Chandralata Raghukumar *Editor*

Biology of
Marine Fungi

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Biology of Marine Fungi

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Preface

The late Dr. Stephen Thomas Moss of Portsmouth Polytechnic, UK, edited the book "Biology of Marine Fungi" in the year 1986. The present book, appearing nearly 25 years later, is dedicated to the memory of Dr. Moss, the eminent mycologist who left us prematurely. His expertise covered marine, as well as the trichomycetous fungi and electron microscopy of fungi. None of the authors who have contributed in the present book were authors in the 1986 book, indicating that a new crop of marine mycologists have appeared on the scene, bringing new ideas, techniques and approaches, and having been groomed and guided by the earlier generation in their quest to understand the biology of marine fungi.

There have been quite a few books on marine fungi. Johnson and Sparrow's pioneering monograph in 1961, the book on higher marine fungi by Kohlmeyer and Kohlmeyer in 1979 and in recent times, reviews on diverse topics in marine mycology published as "Fungi in marine environment" by Kevin Hyde in 2002 as a special issue of the journal *Fungal Diversity* are examples. Several books published as proceedings of the marine mycology symposia containing original research papers have appeared regularly. The present book is aimed to present marine fungal biology in its broad perspective, with several reviews and extensive literature coverage that will help the students and teachers working in this field.

With increasing realization of the importance of oceans in our daily lives, the diversity of marine microbes, flora and fauna has attracted the attention of the scientific community. Several exciting papers on marine fungi have appeared in the last two decades. Thus, fungi have been reported from extreme marine environments, such as hydrothermal vents, deep-sea trenches, cold methane seeps, deep-sea subsurface, hypersaline, anoxic and suboxic waters. Culture-dependent as well as culture-independent approaches are helping in assessing marine fungal diversity. Several new phylotypes have been reported based on these techniques, which may contribute to the hidden diversity of fungi on this planet. This should pave way for developing methods to culture the uncultured fungi.

Unraveling the functional diversity of fungi using metagenomics approach is slowly gathering momentum, the first report being that of fungi in coral holobiont.

This approach emphasizes the ecological role of fungi present in any niche. With increasing availability of molecular tools, the metagenomics of fungi in various marine environments will become a popular approach in future.

In this book, I have tried to include reviews on some of the recent advances made in different topics. The role of unicellular thraustochytrids, although not strictly fungi, but eukaryotic osmoheterotrophs, in marine food web is discussed by Bongiorni in the first chapter. The oomycetous pathogens of marine fisheries and shell fisheries with their descriptions and symptoms are illustrated by Hattai. The chapter on fungal endosymbionts of algae highlights their diversity and metabolites. The diversity and ecology of planktonic fungi in the world's oceans and their significance in ocean carbon and nutrient cycling is reviewed by Wang. Fungi associated with sponges and corals and their secondary metabolites have enthused interest of pharma companies. In light of this, one chapter discusses the ecology of fungi in corals and their role in coral ecosystem. Dead Sea with its extremely high salt content is an extremophilic environment. A chapter is devoted to fungi in this environment. Another chapter discusses diversity and adaptation of fungi growing in hypersaline environment of salterns. Sumathi et al. have reviewed molecular diversity of fungi in oxygen-deficient environment from world oceans. Nagano and Nagahama discuss the cultured and uncultured fungal diversity in deep-sea sediments. This chapter highlights the presence of novel fungal lineages, their adaptations and ecological role in extreme environments. Pang et al. discuss fine tuning of identification of marine ascomycetes by classical morphological taxonomy using several new criteria. Sridhar et al. provide new data on diversity of marine fungi from beaches of Portugal. A chapter is devoted to describe marine genera of Xylariaceae of the phylum Ascomycota. Sarma has compiled information on diversity of marine fungi occurring on the mangrove plant *Rhizophora* in 18 different sites world over. Finally, two chapters describe biotechnological potentials of marine fungi in various fields of industrial enzymes, secondary metabolites and bioremediation.

The untapped potential of oceanic resources is being increasingly realized. These resources are in the field of bioactive molecules, enzymes, nutraceuticals, antibiotics, energy, aquaculture, source of gene pool and biodiversity. Most papers contributed in this book have tried to address the current global understanding of the biology, role and biotechnological potential of fungi in the oceans.

Dr. Steve Moss: An Appreciation
of an Outstanding Mycologist and Friend

Dr. Steve Moss (General Secretary and President Elect) and Professor Tony Whalley (Honorary Treasurer) preparing for the AGM of the British Mycological Society in 2000

Stephen (Steve) Thomas Moss was born in Buxton, Derbyshire, but was very much a Norfolk man. In his youth, he was an outstanding swimmer at county level, a fact which he kept modestly to himself, and this is not surprising because those who knew Steve will remember his gentle and unassuming nature. I first met Steve in Florida, USA, at the International Mycological Congress (IMC2) in 1977 and it was clear then that he was a very talented individual but was also warm and friendly. In future years, we became close friends through the British Mycological Society and working together in Malaysia, China, and Thailand. Steve was an outstanding communicator with endless patience as a teacher and at the same time inspirational. Many will remember his operation of a scanning electron microscope at the 100th anniversary exhibition of the British Mycological Society

in London in September 1996. He captivated school children, members of the public, and members of parliament over the 3-day event and did a wonderful job in bringing mycology alive. His ability to enthuse his audience was exceptional and his appearance on the popular TV show, Big Breakfast, was educational, entertaining, and memorable.

As a mycologist, he was exceptionally talented with strong international recognition for his research on thraustochytrids and marine fungi. He did not achieve this easy way having studied part-time for his M.Sc. at Birkbeck College, University of London, funding his studies as a part-time lecturer or demonstrator at a range of technical colleges in the London area. Later these teaching experiences in the technical colleges provided him with the foundation to become a teacher and communicator par excellence. At Birkbeck, he was introduced to fungi by one of the best-known mycologists at that time, Terence Ingold, and this is when his interest and love of fungi started. On completion of his M.Sc. he moved to the University of Reading to undertake his PhD under the guidance of Michael Dick. It was here that he became a proficient electron microscopist. This was followed by an NSF-funded Research Associateship with Robert Lichtwardt at the University of Kansas (USA) which resulted in many publications on the Trichomycetes and established him as a world authority. He then returned to the UK taking up a position at the University of Portsmouth where he formed a very productive partnership with Gareth Jones working mainly on marine ascomycetes and biodeterioration of submerged woods. Electron microscopes played an important part of his studies and many of his publications demonstrate his considerable skill in producing unrivaled images of appendages of trichospores and attachment mechanisms of marine ascomycete spores. He could easily have succeeded as an electron microscopist.

Perhaps it was the electron microscope which was responsible for the detailed and meticulous way Steve tackled his academic career and through his many contributions to the British Mycological Society. He was a devoted and outstanding general secretary and many council members and officers remarked on the emails sent in the early hours! I was particularly fortunate to have the support of Steve during my presidential year and when he assumed office as president he was alive with ideas and an exciting vision for the future of the society. Sadly much of this was lost with his untimely and tragic death at such an early age.

We worked together in Malaysia, China, and Thailand which Jan could sometimes join and I have many fond memories of these trips. He ignored a cobra in the Gombak Reserve in Malaysia to collect a *Xylaria* specimen I had requested. He spent many late nights sorting out problems with the electron microscopes at the University of Malaya and training staff and students. Steve even sorted out the plumbing in the university guest house in Wuhan, China, when we were there running a fungal workshop. It was perhaps his encouragement and interaction with students both in the UK and in the abroad which stands as a testimony to Steve. He was an inspirational teacher and always found time to help to resolve problems. It was hardly surprising that his lectures and workshops were highly sought after. He passionately and expertly promoted mycology with the general public,

academics, politicians, and the media at large. He was also a dedicated family man and when time permitted would take his dinghy out on the Solent with his daughter Becky as crew. His wife Jan strongly supported him in his research and later BMS activities and visits with them to the Jolly Roger near their home in Gosport were highly recommended.

Two measures which indicate the respect and love for Steve were demonstrated by the presence of an exceptional number of mourners at his funeral with former students and colleagues traveling from far corners of the world and the frequent references to Steve when I am with our friends and former students in Thailand, Malaysia, and China. Steve's memory lives on!

Tony Whalley

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Chapter 1
Thraustochytrids, a Neglected Component of Organic Matter Decomposition and Food Webs in Marine Sediments

Lucia Bongiorni

Contents

Abstract Decomposition of organic matter in marine sediments is a critical step influencing oxygen and carbon fluxes. In addition to heterotrophic bacteria and fungi, osmoheterotrophic protists may contribute to this process, but the extent of their role as decomposers is still unknown. Among saprophytic protists, the thraustochytrids have been isolated from different habitats and substrates. Recently, they have been reported to be particularly abundant in marine sediments characterized by the presence of recalcitrant organic matter such as seagrass and mangrove detritus where they can reach biomass comparable to those of other protists and bacteria. In addition, their capacity to produce a wide spectrum of enzymes suggests a substantial role of thraustochytrids in sedimentary organic decomposition. Moreover, thraustochytrids may represent a food source for several benthic microorganisms and animals and may be involved in the upgrading of nutrient-poor organic detritus. This chapter presents an overview on studies of thraustochytrids in benthic ecosystems and discusses future prospectives and possible methods to quantify their role in benthic food webs.

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1.1 Introduction

Marine sediments are the largest habitat on our planet in terms of areal coverage. These habitats are greatly dependent upon the supply of organic material falling from the water column, most of which enters and accumulates as polymeric organic compounds. The balance between organic matter degradation and preservation in these habitats has immense consequences for the global carbon and oxygen cycles (Berner 1989; Hedges and Keil 1995). Part of this organic matter is labile, i.e., immediately degraded, while another part (such as humic and fulvic acids, structural carbohydrates, etc.) is recalcitrant to microbial decomposition and accumulate easily in marine sediments (Mayer et al. 1995; Middelburg et al. 1999). The enzymatic hydrolysis of high molecular weight and recalcitrant organic polymers is usually the rate-limiting step in mineralization in natural environments (Sigee 2005). While prokaryotes have been for a long time considered the main players of organic matter decomposition (Sime-Ngando and Colombet 2009; Weinbauer and Rassoulzadegan 2004), it is known that apart from fungi, several protists (e.g., heterotrophic nanoflagellates and photosynthetic dinoflagellates) can also make important and unique contributions in this process (Mohapatra and Fukami 2004; Stoecker and Gustafson 2003).

Among saprophytic marine protists, the thraustochytrids (stramenopilan fungi) have been found to be abundant in coastal sediments (Bongiorni and Dini 2002; Bongiorni et al. 2005a, b; Santangelo et al. 2000). Thraustochytrids, together with heterotrophic protozoa, are highly adapted to solid substrate pervasion and are involved in the breakdown, scavenging, and mineralization of highly refractory organic matter through extracellular digestion (Fig. 1.1, Bongiorni and Dini 2002; Bongiorni et al. 2005a, b; Raghukumar 2002; Santangelo et al. 2000). Due to these peculiar characteristics, thraustochytrids likely represent an important component of the food webs in benthic environments characterized by high levels of organic matter such as seagrass beds and mangrove areas (Raghukumar and Raghukumar 1999; Raghukumar et al. 1994). However, till now, knowledge on the quantitative relevance of thraustochytrids within the benthic microbial loop is poor especially
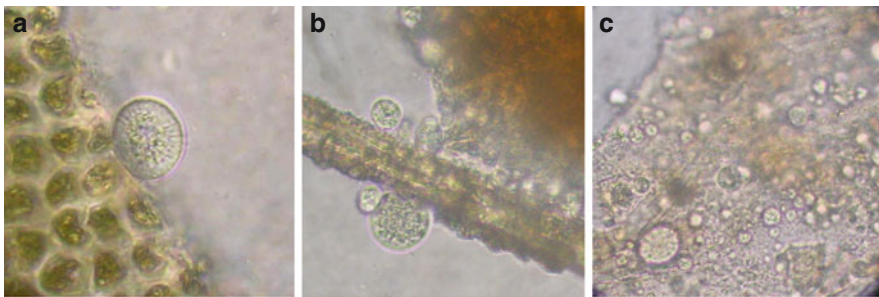
Fig. 1.1 Thraustochytrid cells colonizing dead algae (**a**, **b**) and crustacean exoskeleton (**c**) substrates

due to the lack of appropriate methodologies to measure their abundance, biomass and production in marine sediments. This chapter summarizes the state of the art regarding the study of thraustochytrids in marine sediments and discusses possible research developments with focus on novel methods and approaches that might provide new insights into their ecological role.

1.2 Methodologies for Detecting and Counting Thraustochytrids in Sediments

Knowledge of abundance and distribution of microorganisms are indispensable for understanding their ecological roles. It is therefore evident that the development of methods for accurate determination of their numbers and biomass is a crucial step. Although thraustochytrids abundance has been investigated in the pelagic realm of several geographic locations (see Raghukumar 2002, 2008 and literature cited), studies on sediments have been for a long time hampered, like for other protists, by the lack of adequate counting methodologies. This is especially due to difficulties in separating cells from sediments. Numbers of benthic thraustochytrids were estimated for the first time using the Most Probable Number (MPN) technique modified by Gaertner (1968). The modification consisted in using pine pollen to bait fresh sediment samples. This method, later applied on Mediterranean Sea shores' samples by Bongiorni and Dini (2002) resulted to be efficient in detecting relative abundance and seasonal fluctuation of local thraustochytrid populations. However, this technique presents several limitations. Direct counts might result in underestimating densities as not all thraustochytrid species can grow on pine pollen (Raghukumar 2002). Moreover, labour intensive analysis of fresh material and cultures imposes the examination of small number of replicated samples, thus limiting the extent of *in situ* experimental designs (Santangelo et al. 2000). In the last decades, counting methodologies have been largely improved by staining bacteria and protists with fluorochromes and observing them under the epifluorescence microscope (Sherr et al. 1993). The acriflavine direct detection method (AfDD) elaborated by Raghukumar and Schaumann (1993) specifically for thraustochytrids has allowed enormous advances in the study of these organisms. Acriflavine is a fluorochrome that stains the nucleic DNA and the cell wall of thraustochytrids (made of sulphated polysaccharides) making them fluorescing yellow/green and orange/red, respectively. The uniqueness of the method consists in the fact that autotrophic cells can be discriminated by chlorophyll-autofluorescence, and most other heterotrophic protists can be recognized by their shape or by the lack of a red-fluorescencing cell wall. However, the problem in using fluorochrome for detection of benthic microorganisms has always been the background fluorescence due to sediments. This problem has been partially solved by dividing cells from sediments on the base of their densities mainly by centrifuging samples in a nonlinear silica-gel gradient (Alongi 1993; Epstein 1995, 1997a, b;
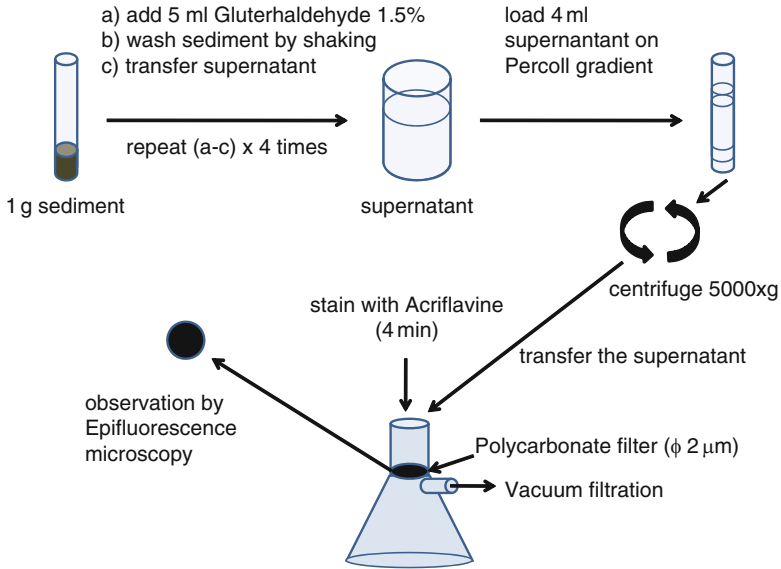
Fig. 1.2 Main steps of the method to enumerate thraustochytrids in sediments as described in Santangelo et al. (2000). Sediment samples are fixed and washed for four times by shaking with 1.5% glutaraldehyde-seawater solution. From each washing step, the supernatant is collected (total ca. 20 ml). Successively an aliquot (4 ml) of the supernatant is loaded on a Percoll gradient. The supernatant and the gradient are centrifuged, concentrated on a polycarbonate 2 μm pore-membrane and stained with acriflavine before observation under an epifluorescence microscope

Starink et al. 1994; Tao and Taghon 1997). A similar method was successfully applied by Santangelo et al. (2000) to simultaneously enumerate the thraustochytrid and ciliate components of benthic protistan community (Fig. 1.2). This protocol combined with the one developed by Raghukumar and Schaumann (1993) for thraustochytrids and that by Epstein (1995) for nano, micro- and meiobenthos consists in separating cells from sediments as described before and then staining them in sequence with acriflavine for thraustochytrids, diamino-2-phenylindole hydrochloride (DAPI) and fluorescein isothiocyanate (FITC) for ciliates (Santangelo et al. 2000). The same method was also used for counting on same samples different component of protistan community (thraustochytrids, ciliates and heterotrophic nanoflagellates, Bongiorni et al. 2005a). However, even the acriflavine method might underestimate the true abundances of thraustochytrids as small cells have a tiny wall and most thraustochytrid zoospores lack cell walls. Because of such problems, the developments of new methods able to specifically and precisely detect thraustochytrid cells are becoming essential (Takao et al. 2007).

Molecular techniques allow direct investigation of the community structure, diversity and phylogeny of microorganisms in almost any environment and quantification of the individual types of microorganisms or entire microbial communities. These techniques can be applied in conjunction with microscopic and biochemical methods to study diversity and functioning of bacterial and fungal communities.

The use of fluorescently labelled oligonucleotide probes complementary to unique target sites on the rRNA (Fluorescent *In Situ* Hybridization – FISH) allows microscopic examination of individual cells in complex microbial assemblages (Theron and Cloete 2000). These techniques have been widely used for analysing population composition of natural bacterial communities (DeLong et al. 1989; Giovannoni et al. 1988; Glöckner et al. 1996; Ishii et al. 2004; Llobet-Brossa et al. 1998). Recently, Takao et al. (2007) established a FISH protocol, and its efficiency, targeting thraustochytrid phylogenetic group (TPG). Another molecular method employing the use of real-time quantitative PCR (qPCR) assay has been successfully applied to detect QPX, a thraustochytrid which parasitized hard clams (Liu et al. 2009; Lyons et al. 2006). This technique is highly sensitive for measuring copy numbers of target nucleic acid sequences. It has been used to detect and quantify prokaryotes as well as eukaryotes, with the power of identification at genus and species levels, from a large number of habitats (Galluzzi et al. 2004; Skovhus et al. 2004; Takai and Horikoshi 2000). Although the detection of thraustochytrids through molecular techniques seems to be promising, further improvements are needed before they will become routine tools for monitoring thraustochytrid abundances in natural environments. Next developments will have to include the design of primers specific for this group of organisms, their validation on a wide representative of thraustochytrid strains and their optimization for application in different types of natural samples, including sediments and mud.

1.3   Roles of Thraustochytrids in Benthic Food Webs

The roles played by fungi and thraustochytrids in benthic food webs have so far received little attention (Raghukumar 2002). Thraustochytrids densities in marine sediments and benthic detritus are the highest reported in literature for that group (Table 1.1) exceeding values measured in planktonic environments (typically of few hundred cells per ml of water). In benthic habitats, protists are known to be sensitive to environmental conditions and generally display a significant numerical response to organic enrichment (Danovaro 2000; Manini et al. 2003). Organic matter represents an important food source for thraustochytrids as suggested by the positive correlation found between thraustochytrid densities and amounts of total sediment organic matter in Mediterranean beaches and intertidal areas (Bongiorni and Dini 2002; Bongiorni et al. 2004; Santangelo et al. 2000). Although thraustochytrids might often represent a negligible fraction of microbial abundance and a minor fraction of the total microbial biomass (Bongiorni and Dini 2002; Bongiorni et al. 2005b), they can reach extremely high densities in the presence of large amounts of organic matter recalcitrant to enzymatic decomposition. Thraustochytrids are found to be particularly abundant in marine sediments underlying seagrass (*Posidonia oceanica*) beds and mangrove detritus (Table 1.1, Bongiorni et al. 2005a; Wong et al. 2005). Moreover, they were reported in high densities in polluted harbour and in sediments characterized by high protein and lipid contents

Table 1.1 Average thraustochytrid abundance in sediments and organic detritus of different benthic habitats

Area	Habitat type	Abundance	Method	References
North Sea (Germany)	Fladen Ground	18–73 cells ml^{-1a}	Modified MPN	Gaertner and Raghukumar (1980) Raghukumar and Gaertner (1980)
Southern-North Sea	–	10–18.5 cells ml^{-1a}	Modified MPN	Gaertner (1967)
NE-Atlantic Ocean (Africa)	Upwelling region	<0.001–22 cells ml^{-1a}	Modified MPN	Gaertner (1982)
W- Atlantic Ocean	–	<0.001–65.7 cells ml^{-1a}	Modified MPN	Gaertner (1982)
NW-Mediterranean Sea (Italy)	Surf zone sediments	44 ± 40 cells ml^{-1}	Modified MPN	Bongiorni and Dini (2002)
NW-Mediterranean Sea (Italy)	Subtidal sediments	61 ± 53 cells ml^{-1}	Modified MPN	Bongiorni and Dini (2002)
NW-Mediterranean Sea (Italy)	Subtidal sediments	42.4 ± 35.2 cells ml^{-1}	Percoll + AfDD	Santangelo et al. (2000)
S-Mediterranean Sea (Italy)	Fish farm, sediments	3.35 ± 0.88 × 10^3 cells g^{-1}	Percoll + AfDD	Bongiorni et al. (2005a)
S-Mediterranean Sea (Italy)	Subtidal sediments	0.96 ± 0.36 × 10^3 cells g^{-1}	Percoll + AfDD	Bongiorni et al. (2005a)
S-Mediterranean Sea (Italy)	Fish farm (*Posidonia* bed)	13.45 ± 3.87 × 10^3 cells g^{-1}	Percoll + AfDD	Bongiorni et al. (2005a)
S-Mediterranean Sea (Italy)	Sediments (*Posidonia* bed)	1.47 ± 0.76 × 10^3 cells g^{-1}	Percoll + AfDD	Bongiorni et al. (2005a)
E-Mediterranean Sea (Cyprus)	Fish farm, sediments	17.49 ± 5.71 × 10^3 cells g^{-1}	Percoll + AfDD	Bongiorni et al. (unpublished)
E-Mediterranean Sea (Cyprus)	Subtidal sediments	11.66 ± 1.18 × 10^3 cells g^{-1}	Percoll + AfDD	Bongiorni et al. (unpublished)
E-Mediterranean Sea (Cyprus)	Fish farm (*Posidonia* bed)	15.03 ± 4.91 × 10^3 cells g^{-1}	Percoll + AfDD	Bongiorni et al. (unpublished)
E-Mediterranean Sea (Cyprus)	Sediments (*Posidonia* bed)	7.79 ± 0.78 × 10^3 cells g^{-1}	Percoll + AfDD	Bongiorni et al. (unpublished)
Central Adriatic Sea (Italy)	Surf zone sediments	6.71 ± 1.04 × 10^3 cells g^{-1}	Percoll + AfDD	Bongiorni et al. (2005b)
Central Adriatic Sea (Italy)	Port, subtidal sand-mud	7.34 ± 1.91 × 10^3 cells g^{-1}	Percoll + AfDD	Bongiorni et al. (2005b)
N-Adriatic Sea (Goro lagoon, Italy)	Mussel farm, mud	34.59 ± 2.71 × 10^3 cells g^{-1}	Percoll + AfDD	Bongiorni et al. (unpublished)
Arabian Sea (India)	Anoxic sediments	64–2622 CFU g^{-1b}	Agar plating	Sumathi and Raghukumar (2009)
China Sea (Hong Kong)	Leaves detritus (Mangrove area)	4.8–560 × 10^3 CFU g^{-1a}	Agar plating	Wong et al. (2005)
China Sea (Hong Kong)	Sediments (Mangrove area)	0.1–1.6 × 10^3 CFU g^{-1a}	Agar plating	Wong et al. (2005)

[a]Density range
[b]Total fungal abundance (thraustochytrids were the next most abundant fungi after *Aspergillus* spp. and *Humicola* sp.)

due to fish-farm biodeposition (Table 1.1, Bongiorni et al. 2005a, b, Bongiorni et al. unpublished). In *P. oceanica* beds characterized by the presence of a highly refractory organic matter composition, dominated by structural carbohydrates (up to 70% of the biopolymeric C fraction), thraustochytrids largely dominated over heterotrophic nanoflagellates biomass (0.124 vs. 0.044 µg C g^{-1}), reaching up to 50% of protist total abundance (as sum of thraustochytrids, nanoflagellates and ciliates, Bongiorni et al. 2005a). Experiments carried on pattern of microbial colonization of dead mangrove leaves and detritus showed that thraustochytrid density and biomass increase in an advanced state of the decomposition process (Fell and Findlay 1988; Raghukumar et al. 1994, 1995). In decaying brown algae, thraustochytrid biomass peaked only after 21 days of the experiment almost equalling bacterial biomass at that time (0.07 vs. 1.1% detrital carbon, Sathe-Pathak et al. 1993). A similar phenomenon was also observed in decaying kelp (Miller and Jones 1983). All these results point to a key role of thraustochytrids in the degradation of the most refractory portion of sedimentary organic matter.

Production of enzymes has been reported sporadically for thraustochytrids (Bahnweg 1979a, b; Bremer and Talbot 1995; Raghukumar and Raghukumar 1999; Raghukumar et al. 1994; Sharma et al. 1994). Enzymes such as cellulase, amylase and xylanase were observed to be produced by several thraustochytrids strains (Bremer and Talbot 1995; Nagano et al. 2011; Raghukumar et al. 1994; Sharma et al. 1994). A later study on the enzymatic profiles of 11 benthic and epiphytic thraustochytrid strains showed that these organisms can produce a wide pool of enzymes involved in the hydrolysis of all classes of organic compounds (Bongiorni et al. 2005b). Benthic thraustochytrids exhibited a good production of lipase, a selection of protease and a poor presence of enzymes degrading carbohydrates. The dominance of enzymatic activities such as alkaline phosphatase and aminopeptidase suggested that thraustochytrids might be implicated in sediment N and P cycling. Moreover, 8 out of 11 strains produced high amount of N-acetyl-β-glucosaminidase, a chitin degrading enzyme (Bongiorni et al. 2005b), thus confirming previous observation (Heiland and Ulken 1989). Chitin occurs commonly as an exo- and endoskeletal material in many marine organisms, and represents a dominant fraction of particulate carbon and nitrogen in the marine sediments (Gooday 1990; Place 1996). However, more studies are needed to determine and quantify the importance of thraustochytrids in the degradation of these refractory polymers. It is also worth of note that a rough estimate of thraustochytrid contribution to the total extracellular enzymatic activities in two types of sediments showed that these organisms may contribute up to 4% of the total β-D-glucosidase, 1% of aminopeptidase and 1.4% of alkaline phosphatase activities (Bongiorni et al. 2005b). However, these data could be underestimates since they were based only on culturable thraustochytrids. If it is postulated that a certain portion of thraustochytrids could be either unculturable or not culturable with the available nutrient media, their contribution to total enzymatic activities in sediments would become even more consistent.

Thraustochytrids are potentially involved in different processes along benthic food webs. Estimates of thraustochytrids biomass in organic-rich sediments suggest

that thraustochytrids could likely represent a significant food source in the microbial loop (Bongiorni et al. 2004, 2005a; Fenchel 1980; Raghukumar and Balasubramanian 1991; Santangelo et al. 2000). Different studies observed ciliates and amoebae feeding on thraustochytrid cells (Raghukumar and Balasubramanian 1991; Ulken 1981) and the association found between thraustochytrids and ciliates abundance in Mediterranean sediments seems to support this hypothesis (Bongiorni et al. 2004). Probably, another important role of these stramenopilan fungi is to supply essential nutrients to detritivores. Thraustochytrids represent a source of valuable vitamins, amino acids (e.g., lysine and methionine), sterols and polyunsaturated fatty acids (PUFAs) that cannot be de novo synthesized by detritus-feeding animals (Arts et al. 2001; Huang et al. 2001; Phillips 1984; Unagul et al. 2005; Zhukova and Kharlamenko 1999). Thraustochytrids might be also implicated, as other heterotrophic protists, in the improvement of the biochemical constituents of poor quality detritus for subsequent use by higher trophic levels, a phenomenon dubbed "trophic upgrading" (Tang and Taal 2005). Several detritivores ingest detritus as a way to feed upon living microorganisms attached to the particles. It has not been clearly demonstrated whether thraustochytrids by colonizing organic particles and detritus can function as trophic upgraders in detrital food webs. Although the role of thraustochytrids in all these energetic pathways remains to be studied in detail, the little clues available suggest that thraustochytrids may represent important energy links within the benthic food webs.

1.4 Conclusions and Future Perspectives in the Study of Thraustochytrids in Marine Sediments

The high thraustochytrid abundances and biomass constantly found in marine sediments suggest their prominent role in benthic habitats. Benthic thraustochytrids produce a wide spectrum of enzymatic activities which are likely to be important in the degradation of the recalcitrant portion of organic matter and in sediment biogeochemical cycles. Moreover, thraustochytrids may be implicated in the transformation of poor nutritive detritus into a more palatable food for detritivores. However, studies on such mechanisms are still poor and more benthic habitats need to be explored in regard to the occurrence of thraustochytrids. The deep-sea is largely unexplored and contains unique benthic features such as canyons, seamounts, deep-water coral reefs, hydrothermal vents and fluid seepages that support unique microbial and faunal communities. Thraustochytrids have been detected or isolated in deep-waters and only occasionally in deep-sea sediments including hydrothermal vents (Damare and Raghukumar 2008; Edgecomb et al. 2002; Raghukumar and Raghukumar 1999; Raghukumar et al. 2001). Ability of these organisms to produce active proteases at high pressure has been documented, thus suggesting a contribution to decomposition processes in deep-sea conditions (Raghukumar and Raghukumar 1999). Thraustochytrids have been also reported to

inhabit anoxic sediments (Sumathi and Raghukumar 2009) but no studies have been attempted to understand their role in these habitats.

The lack of information on thraustochytrids (and in particular in marine sediments) is mainly due to methodological difficulties in quantifying their abundance, biomass and production. Development of new techniques for assessing thraustochytrid abundance and improvements of existing ones has been described. A special effort should be also specifically directed towards the development of techniques for measurements of thraustochytrid biomass, productivity and role in different sedimentary food webs. There are presently no biochemical methods to measure the biomass of thraustochytrids, as a distinctive signature compound such as ergosterol for eumycota fungi has not been found yet. Thraustochytrid biomass is usually estimated by measuring cell biovolume under epifluorescent microscopy and then multiplying the value obtained by the conversion factor 310 fg C μm^{-3} (Kimura et al. 1999). However, since this factor was obtained by coastal planktonic thraustochytrids, in order to have more realistic measurements of biomass in sediment, more C/N measurements from different thraustochytrid strains isolated from representative benthic habitats are urgently needed. Fatty acid signatures (22:5ω6 and 22:5ω3 fatty acids) have been used as alternative method to measure thraustochytrid biomass in mangrove detritus (Fell and Findlay 1988). Thraustochytrid-derived fatty acids, 14:0, 16:0 22:6ω3 and 22:5ω6, were also used to track thraustochytrid diet of krill in controlled conditions (Alonzo et al. 2005). The use of stable isotope (^{13}C)-labelled substrates in combination with biomarker analysis represents a new approach in microbial ecology enabling direct identification of microbes involved in specific processes and also allows for the incorporation of microorganism into food web studies (Boschker and Middelburg 2002). Another recent technique, the stable-isotope probing (SIP), offers a powerful tool for identifying microorganisms that are actively involved in specific metabolic processes. Following this technique ^{13}C-DNA, produced during the growth of metabolically distinct microbial groups on a ^{13}C-enriched carbon source, can be resolved from ^{12}C-DNA by density-gradient centrifugation. DNA isolated from the target group of microorganisms can be characterized taxonomically and functionally by gene probing and sequence analysis (Radajewski et al. 2000). It is likely that the use of multidisciplinary approaches such as combining molecular, biochemical and microscopic tools with isotopic tracers' experiments may provide considerable insights into the dynamics of thraustochytrids in benthic environments.

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Chapter 2
Diseases of Fish and Shellfish Caused by Marine Fungi

Kishio Hatai

Contents

Abstract Fungal diseases are problematic in cultured fish and shellfish, their seeds, and sometimes wild marine animals. In this chapter fungal diseases found in marine animals, especially in Japan, are described. Pathogens in the fungal diseases are divided into two groups. One of them is marine Oomycetes, which cause fungal diseases in marine shellfish and abalones. The diseases caused by the fungi of this group and the fungal characteristics are introduced. The pathogens include members of the genera *Lagenidium, Haliphthoros, Halocrusticida, Halioticida, Atkinsiella*, and *Pythium*. On the other hand, some fungal diseases caused by mitosporic fungi are also known in marine fish and shellfish. The diseases caused

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by these fungi and the fungal characteristics are described. The pathogens include members of the genera *Fusarium, Ochroconis, Exophiala, Scytalidium, Plectosporium*, and *Acremonium*.

2.1 Introduction

Many diseases of fish and shellfish have been observed in studies at monitoring sites in the oceans around the world. The impact of these diseases on population sizes in marine ecosystems in general is poorly understood. Marine fishes and prawns are very popular as seafood especially in Japan, because the Japanese like to eat them raw as "sushi" or "sashimi." Therefore, the culture of fish and shellfish and their seed production are important industries in the sea around Japan. The yield from these industries is gradually increasing along with demand. However, the industry is facing serious problems with infectious diseases. These diseases include fungal infections. No strategies are currently available for effectively controlling fungal diseases with antifungal substances. Therefore, some of these diseases cause high mortality rates, which results in significant economic losses.

In this chapter some of the most economically important diseases caused by species of Oomycetes and mitosporic fungi, found mainly in marine fish and shellfish in Japan, are described. Procedures for identification of these pathogens are also included. Accurate identification of pathogens is necessary in studies designed to improve production rates.

2.2 Fungal Diseases of Shellfish Caused by Oomycetes

Five fungal genera have been reported in Japan as pathogenic Oomycetes of marine shellfish including abalone. For classification of the fungi, an observation on the mode of zoospore production is essential and important. All fungi of the marine Oomycetes were isolated from the lesions using PYGS agar (peptone, 1.25 g; yeast extract, 1.25 g; glucose, 3.00 g; agar, 12–15 g; sea water, 1,000 mL). For inhibition of the most bacterial growth, an addition of ampicillin and streptomycin sulfate in the medium is required. After fungal colonies develop on the agar plates, each one is transferred onto fresh PYGS agar to make a pure culture. The fungi are maintained at 20–25°C and subcultured on PYGS agar approximately at monthly intervals. For morphological observations, the isolates were inoculated into PYGS broth and incubated at 25°C for 3–5 days. Small colonies in PYGS broth are rinsed twice with sterilized artificial seawater and transferred into Petri dishes containing 25 mL of sterilized artificial seawater, and then incubated at 25°C to induce zoospore production. The fungi of the five genera are illustrated in Fig. 2.1.
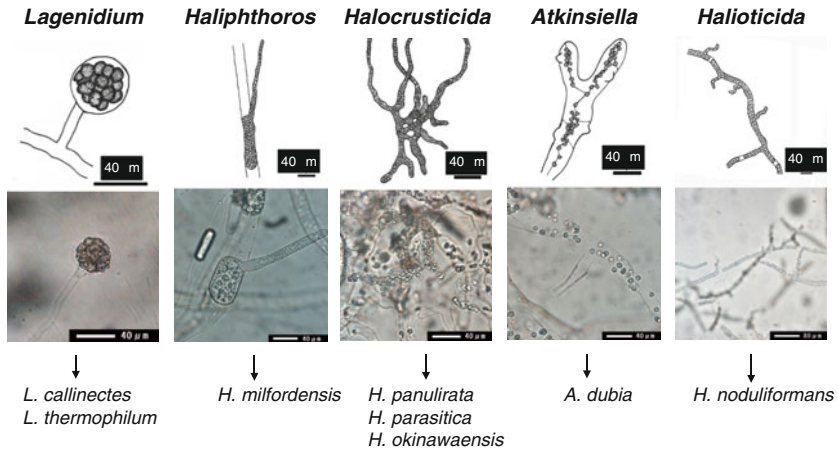
Fig. 2.1 Mode of zoospore production in the fungi of the five genera reported as pathogenic Oomycetes of marine shellfish including abalone in Japan

2.2.1   Lagenidium *Infection*

Species of *Lagenidium* have been found on various hosts from both freshwater and marine habitats (Sparrow 1960, 1973a). As parasites of animals, *L. callinectes* has been described from ova of the blue crab, *Callinectes sapidus* (Couch 1942; Rogers-Talbert 1948; Bland and Amerson 1973) and ova of the barnacle, *Chelonibia patula* (Johnson and Bonner 1960); *L. chthamalophilum* has been isolated from ova of the barnacle, *Chthamalus fragilis* (Johnson 1958), while *L. giganteum* has been reported in mosquito larvae, *Daphne* and copepods (Couch 1935). *Lagenidium callinectes* has been reported from certain marine algae (Fuller et al. 1964). In addition, unidentified species of *Lagenidium* were isolated from cultivated crustaceans, e.g., white shrimp, *Penaeus setiferus* (Lightner and Fontaine 1973), the Dungeness crab, *Cancer magister* (Armstrong et al. 1976) and the American lobster, *Homarus americanus* (Nilson et al. 1976).

A new fungus, *Lagenidium scyllae*, was isolated from ova and larvae of the mangrove crab, *Scylla serrata*, in Philippine (Bian et al. 1979). This fungus was very similar to *L. callinectes*. However, there were some differences between the two. Discharge tubes of *L. scyllae* were longer than those of *L. callinectes*. In the vesicle of *L. callinectes*, a gelatinous envelope is obvious and protoplasmic material never fills more than half of the inside vesicle (Couch 1942), whereas, in that of this fungus, the gelatinous envelope was not seen and the protoplasmic material nearly fills in the whole inside vesicle. According to Couch (1942) and Bland and Amerson (1973), zoospores of *L. callinectes* were discharged by the bursting of vesicle, which was rather persistent for several hours after the spores had emerged. In *L. scyllae*, however, all the spores were simultaneously discharged by rapid deliquescence of the vesicle or the spores were liberated one by one through the

opening of vesicle. Moreover, the collapsed vesicles were never persistent. It seemed to be related to the thin and non-gelatinous characters of the vesicle. The mangrove crab is widely distributed in the tropical Pacific Ocean and the Indian Ocean, while the blue crab is in the Atlantic Coast of North America. In the relation to the distributions of the hosts, there is no overlap between that of *L. callinectes* and that of *L. scyllae*. As a result, it was reported as a new species.

Lagenidium callinectes was isolated from the eggs and zoea of the marine crab, *Portunus pelagicus* for the first time in Japan (Nakamura and Hatai 1995a). Masses of protoplasm flowed into the tip of discharge tubes, where vesicles appeared. Each protoplasmic mass was connected in a chain with a protoplasmic thread. The volume of the vesicles increased with the continuous entry of protoplasmic masses, division into initial zoospores, and active movement of zoospores. The way of zoospore liberation varied: sometimes they were released simultaneously by rupture of the vesicle, sometimes singly through a hole in the vesicle wall. When zoospores were discharged singly, vesicles usually persisted for a few minutes (Fig. 2.2).
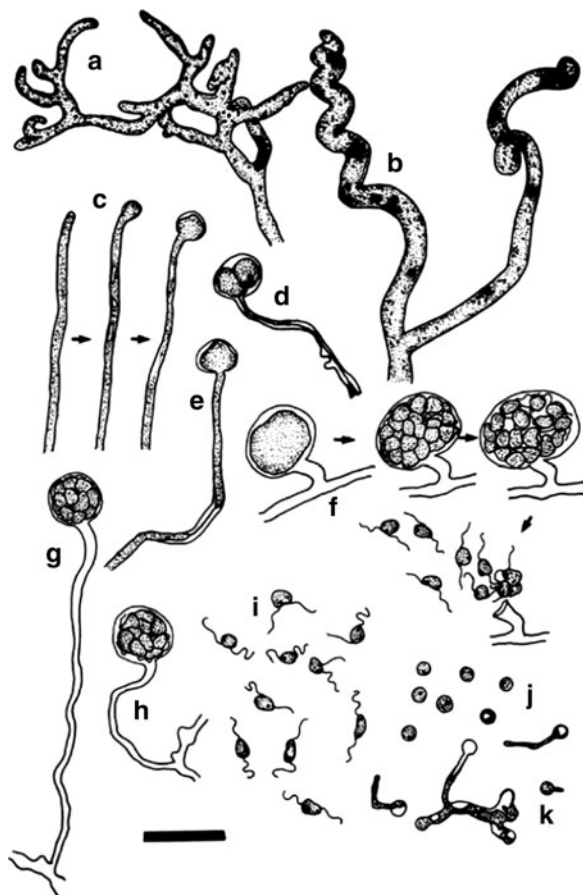
Fig. 2.2 Morphological characteristics of *L. callinectes* isolated from an egg of *P. pelagicus*. Scale = 50 μm. (**a**) Irregularly branched hyphae with numerous shiny rod granules; (**b**) Coiled hyphae in PYGS broth; (**c**) Vesicle formation; (**d**, **e**) Protoplasmic masses flow into the vesicle with a protoplasmic thread; (**f**) Division into initial zoospores and zoospores liberation; (**g**, **h**) Mature vesicles; (**i**) Zoospores; (**j**) Encysted zoospores; (**k**) Germination (Nakamura and Hatai 1995a)

Lagenidium callinectes was also isolated from larvae of mangrove crab, *Scylla serrata*, in Bali, Indonesia (Hatai et al. 2000).

A fungal infection occurred in the eggs and larvae of mangrove crab, *Scylla serrata*, affecting the seed production in Bali, Indonesia. The fungus isolated in August 1993 was a new species in the genus *Lagenidium*, and named *L. thermophilum*, because of its rapid and thermotolerant growth and unique discharge process. Masses of protoplasm occupied nearly all of the vesicles and divided into individual zoospores with two flagellae. The envelopes of the vesicles were not apparent. Zoospore liberation occurred after the vesicles separated from the discharge tubes, namely the vesicles left the discharge tubes before the zoospores were released (Nakamura et al. 1995). The manner of zoospore discharge varied: either zoospores were all discharged simultaneously when the vesicles burst, or they were released in ones or twos through opening in the vesicles. Generally, the former was observed among the bigger vesicle and the latter among the smaller ones. Collapsed vesicles were not persistent. The isolate grew at 15–45°C with an optimum at 30–40°C. This species differed from *L. callinectes* in its salt requirements. As *L. callinectes* grew on media containing seawater or 1–2.5% (w/w) NaCl, it seems to be a marine fungus. However, as *L. thermophilum* also grew on media without seawater, it is obvious that it is not exclusively marine.

L. thermophilum was also found in eggs and larvae of black tiger shrimp, *Penaeus monodon*, at a hatchery in August 2000, Thailand (Muraosa et al. 2006). The characteristics feature of asexual reproduction of the fungus was that zoospores swam away in seawater after the vesicle separated from the discharge tube (Fig. 2.3). This was the first report of *L. thermophilum* infection in black tiger shrimp in Thailand.
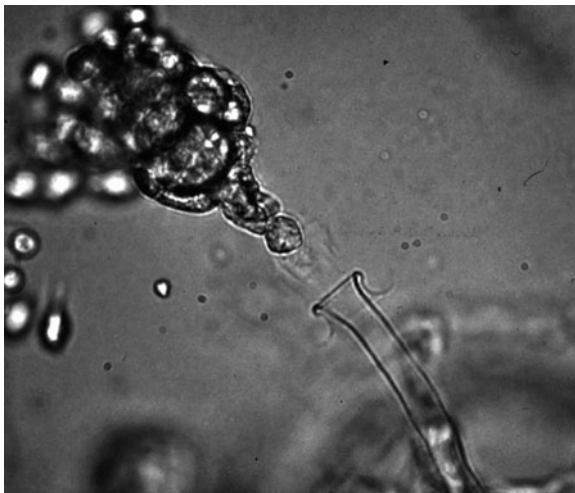
Fig. 2.3 Zoospores swim away in seawater after the vesicle separated from the discharge tube

2.2.2 Haliphthoros *Infection*

The genus *Haliphthoros* was a monotypic genus erected by Vishniac (1958) as the type of the family Haliphthraceae (Saprolegniales). *Haliphthoros milfordensis*, the type species of the genus, has been reported as an endoparasite of eggs of the oyster drill, *Urosalpinx cinerea* (Vishniac 1958). Since then it has been isolated from juveniles of the American lobster, *Homarus americanus* (Fisher et al. 1975), adults of the white shrimp, *Penaeus setiferus* (Tharp and Bland 1977), and a few marine algae (Fuller et al. 1964).

A new species, *H. philippinensis*, was isolated from larvae of the jumbo tiger prawn, *Penaeus monodon* in Philippines (Hatai et al. 1980). The hyphae were stout, branched, irregular, non-septate, developing within the bodies of larvae of the prawn, and it was holocarpic. In pure cultures, the hyphae were homotrichous, at first somewhat uniform, sometimes highly vacuolated, 10–37.5 μm in diameter, becoming fragmentary by means of cytoplasmic constriction with age (Fig. 2.4). Fragments with a dense cytoplasm were variable in size and shape, globose, elongate or tubular, often with protuberances, up to 190 × 100 μm, not disarticulated, connected in bead-like chains, functioning as sporangia and developing discharge tubes which were straight, wavy or coiled, up to 620 × 7.5–12.5 μm. Zoospores were polyplanetic. Encysted spores were spherical, 5–7.5(−12.5) μm in diameter, producing a delicate germ tube. Germ tube was simple, sometimes once branched and up to 250 μm in length. Sexual reproduction was not observed. The fungus showed a close resemblance to *H. milfordensis*, but it differed from the latter in a number of features as described below.

When the fragment with protuberance on the medium was transferred into sea water, the protuberance might again constrict and transform into another sporangium, or might extend and serve as a part of the discharge tube. The sporangia of

Fig. 2.4 Fragment (*arrow*) formation of genus *Haliphthoros*

this fungus are very variable in shape and often with protuberances: globose, elongate, tubular, or irregular-shaped. These are distinctive for the fungus. In this fungus, discharge of zoospores is also peculiar and diverse from that of *Haliphthoros* reported previously. The zoospores were released not only through the orifice of discharge tube but also through the opening of the sporangium. According to Vishniac (1958) and Fuller et al. (1964), zoospores of *H. milfordensis* were monoplanetic and monomorphic. This fungus, however, has polyplanetic and polymorphic zoospores including primary and secondary types. *H. philippinensis* has a wide range of temperature requirement for its growth. Possibly owing to its tropical habitation, it could be tolerant even up to 36°C. This feature was different from that of *H. milfordensis* which could not grow at 35°C (Vishniac 1958).

Haliphthoros milfoldensis infection was found in abalone, *Haliotis sieboldii*, temporarily held in aquaria with circulating sea water adjusted to 15°C by a cooling system in Japan (Hatai 1982). The typical external symptom of diseased abalones was flat or tubercle-like swelling formed on mantle, epipode and dorsal surface on foot (Fig. 2.5). The mycelium was always observed in the lesions. Zoospores of a fungus, which was isolated from the lesion, formed within the fragment were liberated through the orifice of discharge tube. Encysted spores were spherical, usually 7 μm in diameter. The fungus grew at a temperature range of 4.9–26.5°C, with optimum of 11.9–24.2°C.

In June 1994, fungal diseases occurred in the eggs and zoeae of crab, *Portunus pelagicus*, in Japan. Fragments of the isolated fungus were clearly constructed of concentrated masses of protoplasm in the hyphae, tuberculate, saccate or irregular, and quite variable in size and shape. They changed into zoosporangia-producing discharge tubes. Many vacuoles appeared in the zoosporangia and the extending
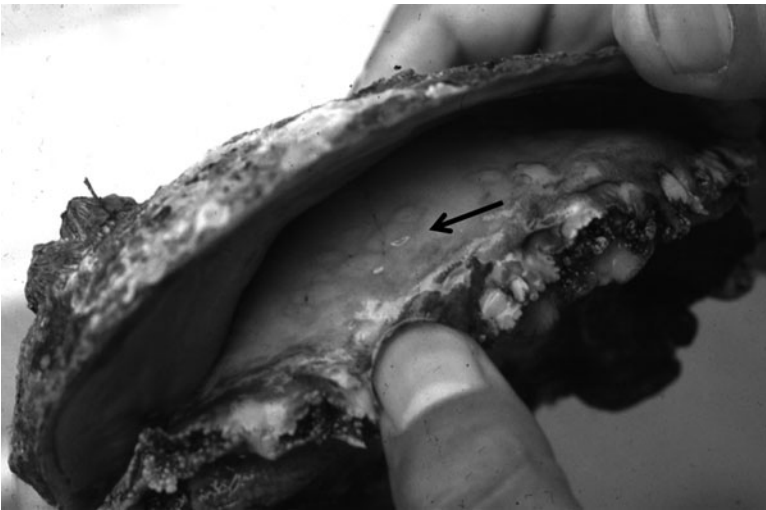
Fig. 2.5 The typical external symptom of diseased abalones was flat or tubercle-like swelling (*arrow*) formed on mantle

discharge tubes, and were also observed in the active mycelia. It was identified as *Haliphthoros milfordens* (Nakamura and Hatai 1995a).

In July 1997, *Haliphthoros milfordens* infection occurred in the eggs and zoeae of the mangrove crab, *Scylla serrata*, in Bali, Indonesia. The mortality rate reached almost 100% in the larvae (Hatai et al. 2000). The colonies on PYGS agar were whitish and reached a diameter of 20–25 mm after 5 days at 25°C. Hyphae in PYGS broth were stout, aseptate, branched with numerous shiny spherical granules, and sometimes concentrated masses of protoplasm were observed in the hyphae. In artificial seawater, fungal fragments were clearly observed to be concentrated masses of protoplasm in the hyphae. They changed into zoosporangia-producing discharge tubes. One discharge tube was usually formed on the lateral side of each zoosporangium. Division of the protoplasm started in the sporangia and continued in the discharge tubes just before zoospore liberation (Fig. 2.6).

In March 2001, *Haliphthoros milfordens* was isolated from larvae of the black tiger prawn, *Penaeus monodon* in Nha Trang, Vietnam (Chukanhom et al. 2003). This was the first report of disease in the black tiger prawn in Vietnam.
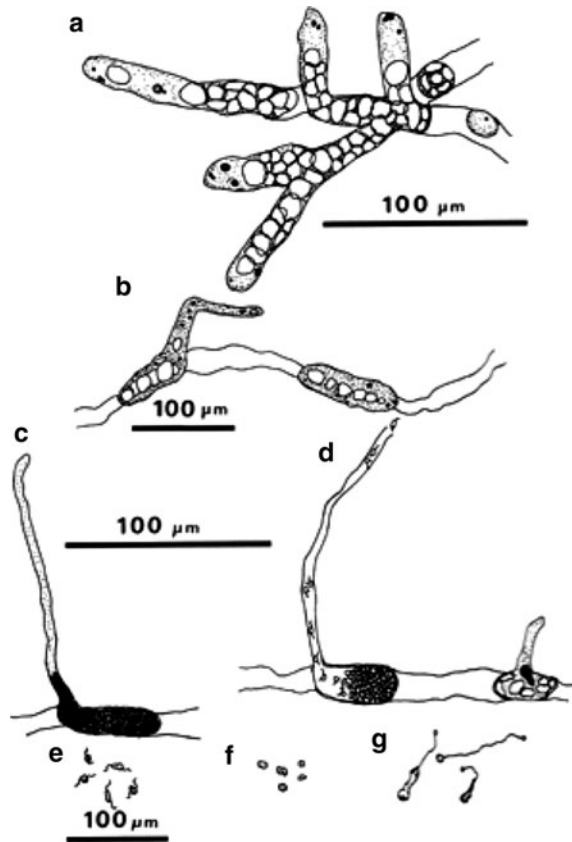
Fig. 2.6 Morphological characteristics of *Haliphthoros milfordensis* isolated from a zoea of *S. serrata*. (**a**) Hyphae in PYGS broth; (**b**) Fragments. Discharge tube formation on the left fragment; (**c**) Zoospore formation; (**d**) Zoospore liberation; (**e**) Zoospores; (**f**) Encysted zoospores; (**g**) Germination (Hatai et al. 2000)
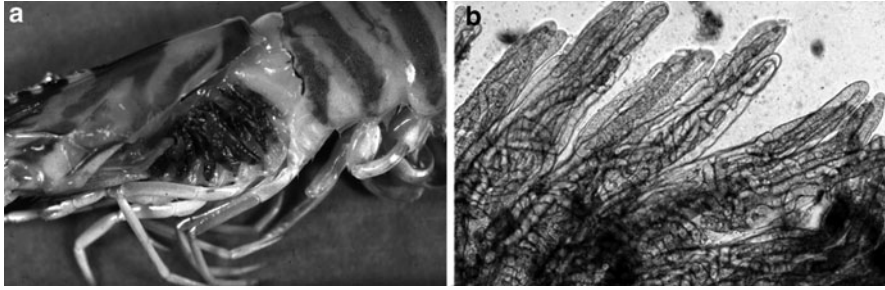
Fig. 2.7 *H. milfordensis* infection in juvenile kuruma prawns, *Penaeus japonicus*. (**a**) Showing *black* gills. (**b**) Hyphae found in gills. Hyphae are only growing within gills

H. milfordensis was also isolated from gill lesions of juvenile kuruma prawns, *Penaeus japonicus*, with black gill disease (Fig. 2.7) at a private farm in August 1989, Japan (Hatai et al. 1992). *H. milfordensis* has been known as a pathogen of various marine organisms. Especially, the fungus has been well known to be an important fungal pathogen of eggs and larvae of crustaceans. However, this was the first case of *H. milfordensis* infection in juvenile kuruma prawn. This condition has previously been known in kuruma prawn as the typical clinical sign of *Fusarium solani* infection.

2.2.3 Halocrusticida *Infection*

A new genus *Halocrusticida* gen. nov. (Lagenidiales, Haliphthoraceae) was proposed for the six species formerly reported as the fungi in the genus *Atkinsiella* except *A. dubia* (Nakamura and Hatai 1995b). These six species of *Atkinsiella* (Table 2.1) were reported from various aquatic animals (Martin 1977; Bian and Egusa 1980; Nakamura and Hatai 1994, 1995a; Kitancharoen et al. 1994; Kitancharoen and Hatai 1995). A key to the species of *Halocrusticida* is described in Table 2.2. Mycelia contained granular clusters without oil droplets and vacuoles on *A. dubia*, but many vacuoles and numerous shiny granules were found on the others. Central protoplasmic masses supported by several protoplasmic threads in the process of zoospore production were observed on *A. dubia*, but not on the others. The most apparent difference between *A. dubia* and the other six species of *Atkinsiella* was the behavior of zoospores in the first motile stage. Zoospores encysted within zoosporangia and discharge tubes following the first motile stage in *A. dubia*, while zoospores in the first motile stage were released from zoosporangia in the other six species.

The definition of the genus *Halocrusticida* is as follows. Thallus is endobiotic, holocarpic, stout, and branched. Zoosporangia are the same in size and shape as thalli. Discharge tubes develop one to several per sporangium. Zoospores in the first motile stage emerge from the zoosporangia. Zoospores are monoplanetic or

Table 2.1 Six species reported previously as *Atkinsiella*[a]

Species	References	Host	Locality
A. entomophaga	Martin (1977)	Insect eggs	USA
A. hamanaensis	Bian and Egusa (1980)	Eggs and larvae of *Scylla serrata*	Japan
A. parasitica	Nakamura and Hatai (1994)	Rotifer (*Brachionus plicatilis*)	Japan
A. awabi	Kitancharoen et al. (1994)	Abalone (*Haliotis sieboldii*)	Japan
A. okinawaensis	Nakamura and Hatai (1995a)	Zoea of the crab (*Portunus pelagicus*)	Japan
A. panulirata	Kitancharoen and Hatai (1995)	Philozoma of spiny lobster (*Panulirus japonicus*)	Japan

[a]Nakamura and Hatai (1995b)

Table 2.2 Key to species of *Halocrusticida*

1 Colonies filamentous, less than two tubes produced from each sporangium . . . .*H. awabi*
1 Colonies lobed, bulbous. 2
  2 Encysted spores more than 9 μm, parasitic on insect eggs . . . .*H. entomophaga*
  2 Encysted spores less than 9 μm, parasitic on crustaceans 3
3 Branched discharge tubes present 4
3 Branched discharge tubes absent 5
  4 Zoospores generally formed two or more deep in the discharge tubes. . . . .*H. okinawaensis*
  4 Zoospores generally formed in a single row in the discharge tubes. . . . .*H. parasitica*
5 Pigmentation from gray to light brown, optimum temperature for growth 30–32°C . . . .*H. hamanaensis*
5 No pigmentation, optimum temperature for growth 25°C. . . . . *H. panulirata*

diplanetic, isokont, laterally biflagellate. Germinating zoospore has a slender germ tube. Sexual reproduction is absent. It is parasitic on aquatic animals, especially marine crustaceans.

Halocrusticida hamanaensis was originally reported as *Atkinsiella hamanaensis* (Bian and Egusa 1980). The fungus was isolated from ova of mangrove crab, *Scylla serrata* in Japan. The swollen hyphal tips up to 150 μm in diameter contained dense cytoplasm. Each sporangium was formed through the formation of septa and several lateral or terminal discharge tubes. The discharge tubes were straight or wavy, measuring 40–1,150 × 5–15 μm. Zoospores measured 6.3 (5–10) × 4.5 (3.8–5) μm in size, were pyriform or slipper-shaped, with two lateral flagella. The encysted spores were 5 (4.5–7.5) μm in diameter, spherical, subglobose, or angular. The encysted spore in sterile sea water developed a hair-like filament, 10–270 μm in length.

Halocrusticida awabi was originally reported as *Atkinsiella awabi* (Kitancharoen et al. 1994). The fungus was isolated from diseased abalone, *Haliotis sieboldii* in Japan. It showed external signs of infection of tubercle-like swelling on the mantle and melanized lesions on the peduncle. The hyphae were stout, irregular, branched, 16–140 μm diameter. Sporangia were formed through the formation of septa and lateral or terminal discharge tubes which were wavy or coiled. Zoospores

were pyriform, biflagellate, and diplanetic. The encysted spore generally developed a hair-like filament with globular enlarged tip in PYGS broth. Direct germination without filament formation also occurred occasionally. The fungus was exclusively marine and grew best in shrimp extract medium at 25°C.

Halocrusticida parasitica was originally reported as *Atkinsiella parasitica* (Nakamura and Hatai 1994). In May 1992 the rotifer, *Brachionus plicatilis* did not increase in number when it was bred in a concrete tank as food supply for seed production of crustaceans and fishes. Because protozoa were observed microscopically on the surface of rotifers, a bath treatment with 25 ppm formalin was first conducted to solve the problem in the tank. However, no increase in the number of rotifers in the tank was found following the treatment. Further detailed microscopical observation revealed thick, non-septate hyphae measuring about 10 μm diameter in the eggs and bodies of many rotifers examined. Discharge tubes were extended outside the bodies (Fig. 2.8), and zoospores with lateral biflagella were released into the seawater through the tubes. Vesicles were not formed at the tip of discharge tubes (Nakamura and Hatai 1994; Nakamura et al. 1994a). The fungus isolated from the rotifer was characterized by producing monoplanetic, lateral biflagellate zoospores, and infrequently branched discharge tubes. The zoosporogenesis of the species is shown in Fig. 2.9.
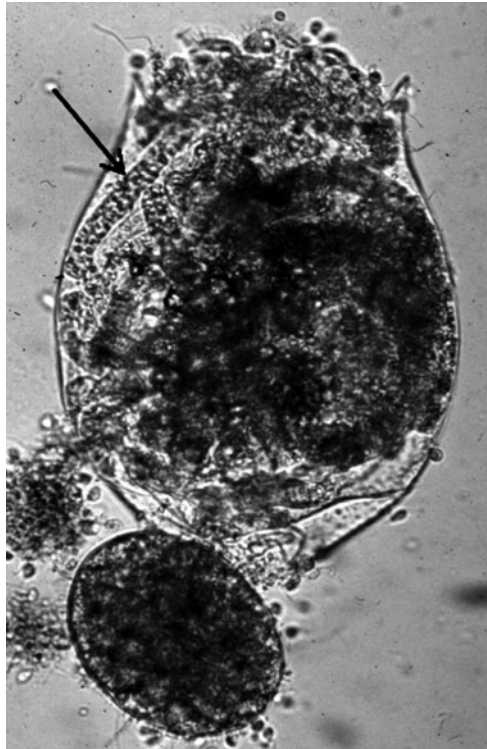
Fig. 2.8 Hyphae in bodies of a rotifer (*arrow*)

Fig. 2.9 Zoosporogenesis of
Halocrusticida (*Atkinsiella*)
parasitica. Scale bar =
50 μm. (**a**) Numerous large
vacuoles appeared at an early
stage of zoosporogenesis, and
later discharge tubes
developed. (**b**) Zoospore
formation in a zoosporangium
and a discharge tube at
the final stage of
zoosporogenesis. (**c**–**f**)
One to three discharge
tubes formed from
a zoosporangium. (**g**, **h**)
Branched discharge tubes

Halocrusticida panulirata was originally reported as *Atkinsiella panulirata* (Kitancharoen and Hatai 1995). This species was isolated from philozoma of the diseased spiny lobster, *Panulirus japonicus* in Japan. The fungus exhibited slow growth, occasionally submerged, with a creamy white, raised moist colony. Hyphae were stout, arranged in radiating pattern, irregularly branched, 10–22 μm diameter, occasionally separated by cross walls into subthalli. Thalli occasionally consisted of swollen features. Sporangia formed from the subthalli had one to three or partly coiled discharge tubes at the terminal or subterminal area. Zoospores were pyriform or reniform, biflagellate, isokont, and diplanetic. Encysted spores germinated as a hair-like filament with a globular enlarged tip in sterilized synthetic seawater, and directly as stout initial hyphae in PYGS broth. Gemmae spontaneously occurred in 3-day-old culture in PYGS broth at 25°C (Fig. 2.10). They were characterized by saccate-lobed-chained, thick-walled dense cytoplasmic and non-vacuolate features, width of 179–270 μm and various lengths up to 18 mm. Gemmae not only developed new thalli on PYGS agar or in PYGS broth, but also in sterilized synthetic seawater.
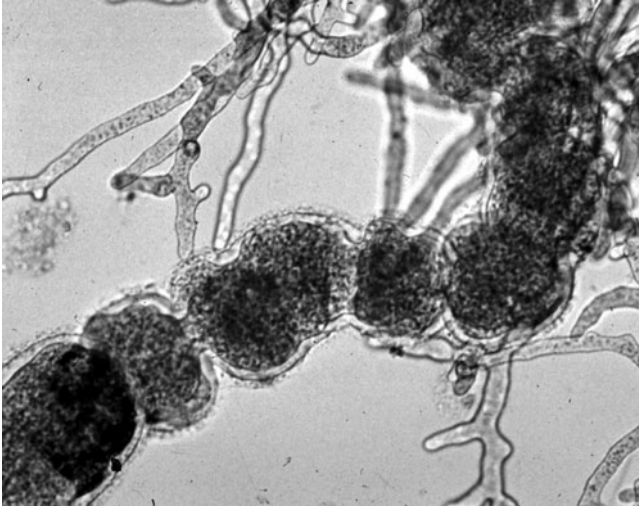
Fig. 2.10 Gemmae (*arrow*) spontaneously occurred in *Halocrusticida panulirata* culture in PYGS broth at 25°C

Halocrusticida okinawaensis was originally reported as *Atkinsiella okina-waensis* (Nakamura and Hatai 1995a). The new fungus was isolated from infected eggs and zoeae of the marine crab, *Portunus pelagicus*. Hyphae were stout, non-septate at first, irregularly branched with numerous shiny rod granules, 10–38 μm width. In seawater, hyphae were divided into subthalli with septa. Gemmae were present with thick walls, 22–190 μm in diameter. Zoosporangia were the same size and shapes as subthalli and gemmae. Discharge tubes were produced laterally or terminally from the sporangia, usually coiled or wavy. Each sporangium extended one to several discharge tubes. In the discharge tubes, zoospores were produced in more than two rows. The discharge tubes were 6–10 μm diameter and 40–510 μm length. Zoospores were laterally biflagellate, diplanetic, 4.7 × 6.3 μm on average. Germination was observed about 3 h after spores had encysted, with a hair-like filament measuring 5–190 μm length (Fig. 2.11).

2.2.4 Halioticida *Infection*

Halioticida infection was reported from abalone, *Haliotis* spp. in Japan (Muraosa et al. 2009). The genus was classified in Peronosporomycetes (formerly Oomycetes) as a new genus. The class Peronosporomycetes contains species that are pathogens of many commercially important plants, fish, and crustaceans (Kamoun 2003). Among the marine invertebrates, infections resulting from some members of the Peronosporomycetes cause problematic diseases, especially in the seed production of marine crustaceans such as shrimp and crabs. On the other hand,

Fig. 2.11 Morphological
characteristics of
Halocrusticida okinawaensis
isolated from a zoea of
P. pelagicus. Scale = 50 μm.
(**a**) Hyphae in PYGS broth;
(**b**) A zoosporangium with
three discharge tubes
(*arrows*); (**c**) Zoospores
released from the orifices of
two discharge tubes. Another
zoosporangium with one
discharge tube is on the right;
(**d**) Zoospores; (**e**) Encysted
zoospores; (**f**) Secondary
zoospores released from
cysts; (**g**) Germination
(Nakamura and Hatai 1995a)
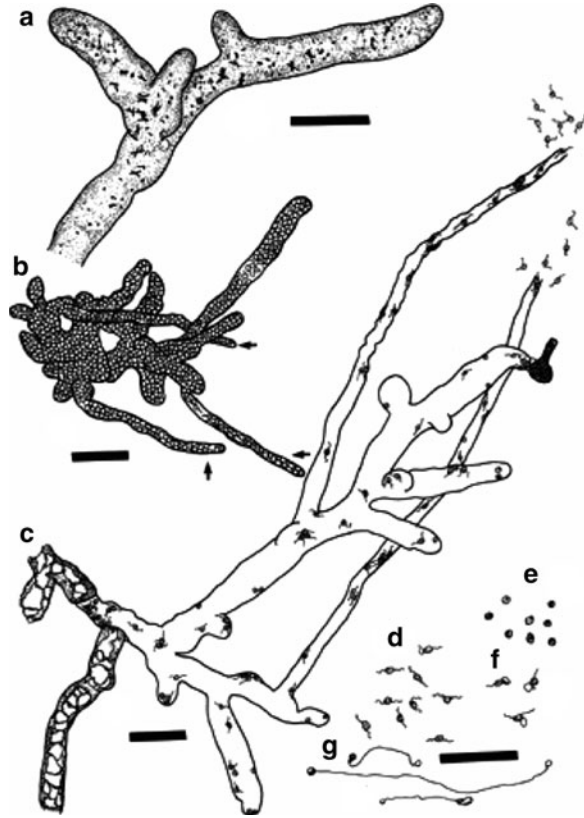
Haliphthoros milfordens (Hatai 1982), *Halocrusticida awabi* (Kitancharoen et al. 1994), and *Atkinsiella dubia* (Nakamura and Hatai 1995b) have been reported as causative agents of such diseases in abalone, *Haliotis sieboldii*. Recently, a new fungus belonging to the Peronosporomycetes was isolated from white nodules found on the mantle of three species of abalone, *Haliotis midae* imported from the Republic of South Africa, *Haliotis rufescens* imported from the Republic of Chile and the United Mexican State, and *Haliotis sieboldii* collected in Japan. They were stocked for sale in the same tank and died from the infection. The daily mortality of stocked abalone was about 1%. The clinical sign of a moribund abalone was the presence of white nodules on the mantle (Fig. 2.12). In the lesions of the white nodules, thick and aseptate hyphae were present. The fungus was isolated from moribund abalones using PYGS agar. The manner of zoospore formation in the fungus isolated from the lesion was totally different from that of the genera *Halocrusticida* and *Atkinsiella*, but similar to that of the genus *Haliphthoros*. However, the isolate differed from the genus *Haliphthoros* as follows. In artificial seawater, the fragments were formed by constricting protoplasm in the hyphae such as in the genus *Haliphthoros*, but the protoplasm constriction was weaker, and fragments were longer, with smaller space between them, than those of
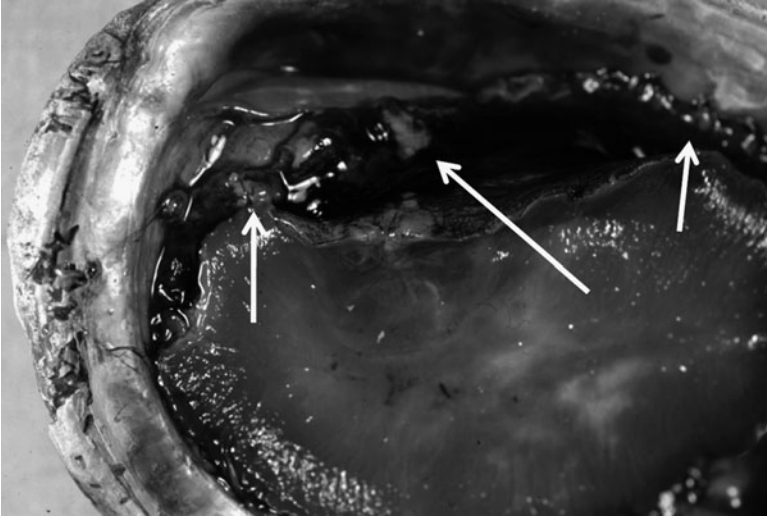
Fig. 2.12 Clinical sign of a moribund abalone. Note the presence of white nodules on the mantle (*arrows*)
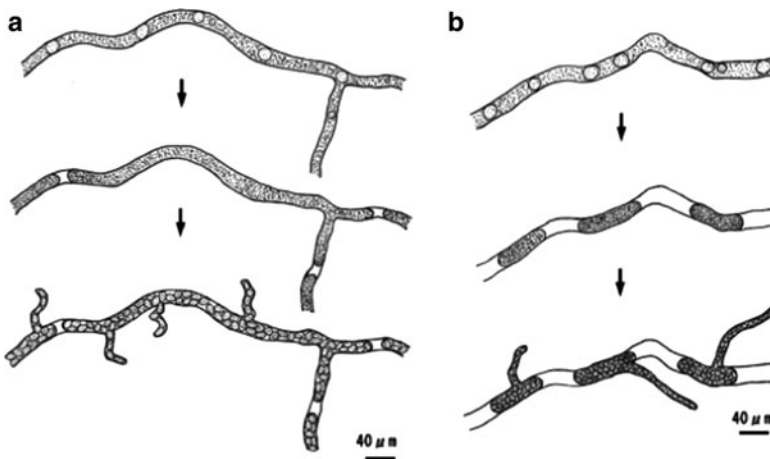
Fig. 2.13 Differences in zoospore formation between *Halioticida noduliformans* (**a**) and *Haliphthoros milfordensis* (**b**). (**a**) Fragments are longer, and spaces between adjacent fragments are smaller than those of *H. milfordensis*. One to several discharge tubes are formed. (**b**) Fragments with only one tube are shorter, and space between fragments are larger than those of *H. noduliformans*

Haliphthoros (Fig. 2.13). The species under the genus *Haliphthoros* form only one discharge tube from a zoosporangium (Vishniac 1958; Hatai et al. 1980, 1992, 2000; Nakamura and Hatai 1995a; Chukanhom et al. 2003), but the fungus from abalone has one or more discharge tubes formed from each zoosporangium.

The size of zoospores was 7.0–8.5 × 9.5–12.5 μm (width × length). From the results mentioned above, the isolate was recognized to have unique morphological characteristics in the family Haliphthoraceae.

Four isolates from white nodules and nine peronosporomycete species isolated from various marine invertebrate animals were used for analysis on the D1/D2 region of LSU rDNA. In the phylogenic tree based on LSU rDNA, the isolate was not classified into the subclass Peronosporomycetidae, Saprolegniomycetidae, or Rhipidiomycetidae, but as a new clade with the genera *Haliphthoros* and *Halocrusticida* (Fig. 2.14). Within this new clade, the four isolates from abalone, *Haliphthoros* spp. and *Halocrusticida* spp. were grouped in the respective independent subclade. *Atkinsiella dubia* and *Lagenidium* spp. were included in Saprolegniomycetidae and Peronosporomycetidae, respectively. The phylogenetic analysis also supported that the four isolates were classified into a new genus and species belonging to the family Haliphthoraceae based on their morphological characteristics. As a result, it named *Halioticida noduliformans* as new genus and species (Muraosa et al. 2009).

Dick (2001) proposed a new taxonomic system for Peronosporomycetes, in which Peronosporomycetes were subdivided into three subclasses: Peronosporomycetidae, Rhipidomycetidae, and Saprolegniomycetidae. Under this taxonomic system, the genera *Haliphthoros*, *Halocrusticida*, and *Atkinsiella* were classified in Haliphthoraceae – Salilagenidiales – Saprolegniomycetidae, and the genus *Salilagenidium*, which was named as a new genus by Dick (2001) for marine species of the genus *Lagenidium*, was classified in Salilagenidiaceae – Salilagenidiales – Saprolegniomycetidae. Molecular phylogenetic analysis by Muraosa et al. (2009) showed that only *Atkinsiella dubia* was included in the subclass Saprolegniomycetidae, but the genera *Haliphthoros*, *Halocrusticida*, and *Halioticida* were not included within the three subclasses proposed by Dick (2001). Furthermore, the genus *Lagenidium* (Salilagenidium) was included in the subclass Peronosporomycetidae in the analysis of Muraosa et al. (2009). Cook et al. (2001) also suggested that the genera *Atkinsiella* and *Lagenidium* (*Salilagenidium*) were classified into the subclass Saprolegniomycetidae and Peronosporomycetidae, respectively, and the genera *Haliphthoros* and *Halocrusticida* were not included in the three subclasses, according to their molecular phylogenetic analysis using the mitochondrially encoded cytochrome *c* oxidase subunit 2 (*cox*2) gene. Thus, the taxonomic position of genera *Haliphthoros*, *Halocrusticida*, *Atkinsiella*, and *Lagenidium* is still confusing.

In December 2006, a *Halioticida* infection was found in wild mantis shrimp, *Oratosquilla oratoria* in Tokyo Bay, Japan (Atami et al. 2009). Fungi were found in the gills of mantis shrimp (Fig. 2.15), isolated from lesions using PYGS agar, and identified by morphological observation and molecular analysis. The fungus formed fragments in the hyphae and several discharge tubes developed from each fragment. Zoospores were formed within the fragments and released into the seawater through the tops of discharge tubes. Based on the characteristics of zoospore production mode, the fungus was classified into the genus *Halioticida*. It was compared by molecular analysis of the D1/D2 region of the large subunit
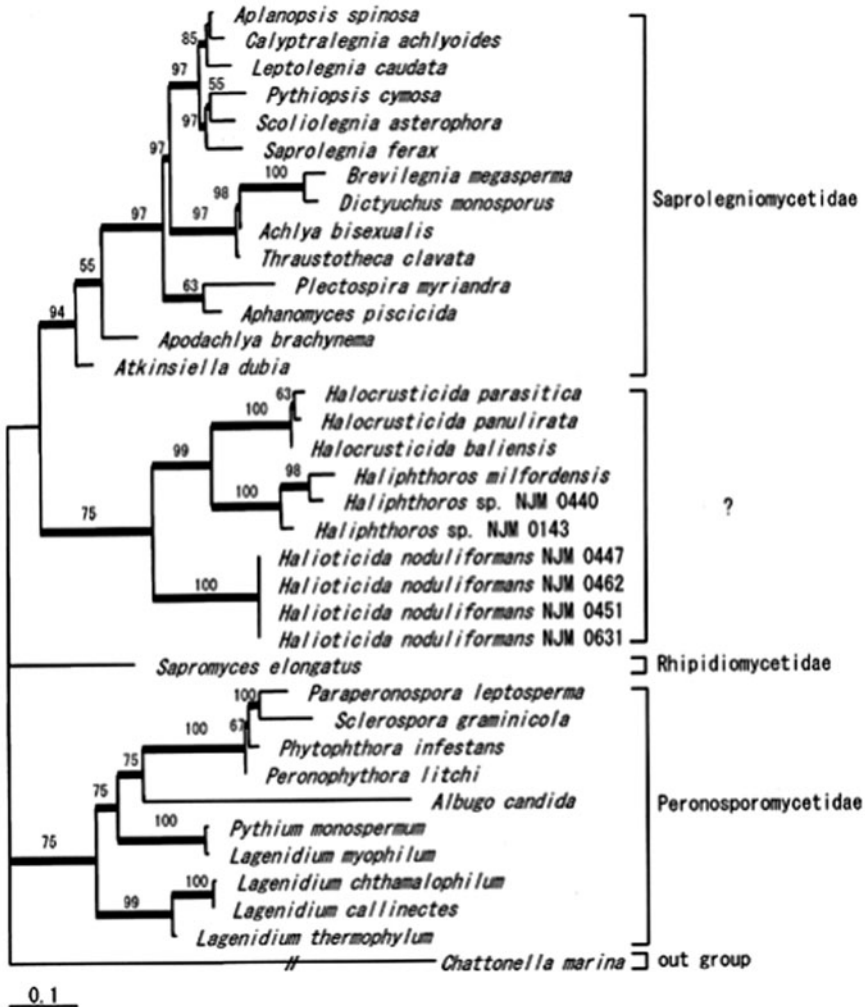
Fig. 2.14 Maximum-likelihood tree based on the D1/D2 region of LSU rDNA. Numbers on branches show bootstrap values (1,000 replicates, above 50% are indicated)

ribosomal RNA gene with *Halioticida noduliformans* isolated from abalone, *Haliotis* spp. (Muraosa et al. 2009). As a result, the sequences of the fungus isolated from mantis shrimp showed 99–100% homology at the D1/D2 domain of the large subunit ribosomal RNA gene sequence with *Halioticida noduliformans*. Histopathological observation indicated that the fungus grew in an aerobic environment, because the hyphae were found mainly in the gills and base of gills. The fungus grew well at 15–25°C, with optimal temperature of 20°C, which corresponds with *H. noduliformans* (Muraosa et al. 2009). The fungus could not
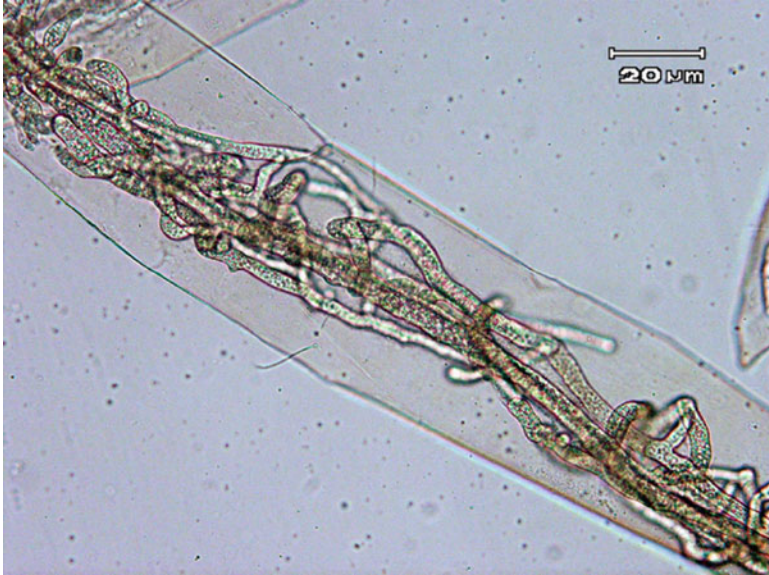
Fig. 2.15 Fungus found in the gills of mantis shrimp

grow on PYG agar or PYG agar with NaCl and KCl, but grew on PYGS agar. This suggested that it was an obligate marine fungus.

2.2.5   Atkinsiella *Infection*

Atkinsiella dubia was originally isolated from eggs of pea crab, *Pinnotheres pisum* in England (Atkins 1954), and assigned to the genus *Plectospira*. Atkins observed the same species on the eggs of *Gonoplax rhomboids* and succeeded in experimentally infecting the eggs of some species of crustaceans. The morphology of the fungal parasite on the eggs of crab was studied at that time. Later, Vishniac (1958) established a new family, Haliphthoraceae (Saprolegniales), for holocarpic biflagellate filamentous fungi, including *Haliphthoros milfordensis* and Atkins' fungus, which was renamed *A. dubia*, although she did not actually observe *A. dubia*. Its morphology and development in pure culture were followed by Fuller et al. (1964) and Sparrow (1973b) from marine algae and the eggs of various crabs, respectively.

Dick (2001) classified *Atkinsiella dubia* and *Haliphthoros* spp. into Saprolegniomycetidae, but at present the genus *Haliphthoros* is classified into different clade, *Haliphthoros/Halocrusticida* clade (Sekimoto et al. 2007), or unknown group (Muraosa et al. 2009), because they constructed different clades from phylogenetic analysis.

During the survey of the fungi belonging to Lagenidiales on marine animals without clinical signs, an interesting fungus was isolated from the mantle of abalone, *Haliotis sieboldii*, in Chiba Prefecture, Japan. The same fungus was also obtained from the gills of swimming crab, *Portunus trituberculatus* in Chiba Prefecture. The fungus was characterized by crystalline, tuberculate and moist colonies, dimorphic and diplanetic zoospores, and zoospores which remained in the zoosporangia during the first motile stage (Fig. 2.16), and identified as *Atkinsiella dubia*, new to Japan (Nakamura and Hatai 1995b).
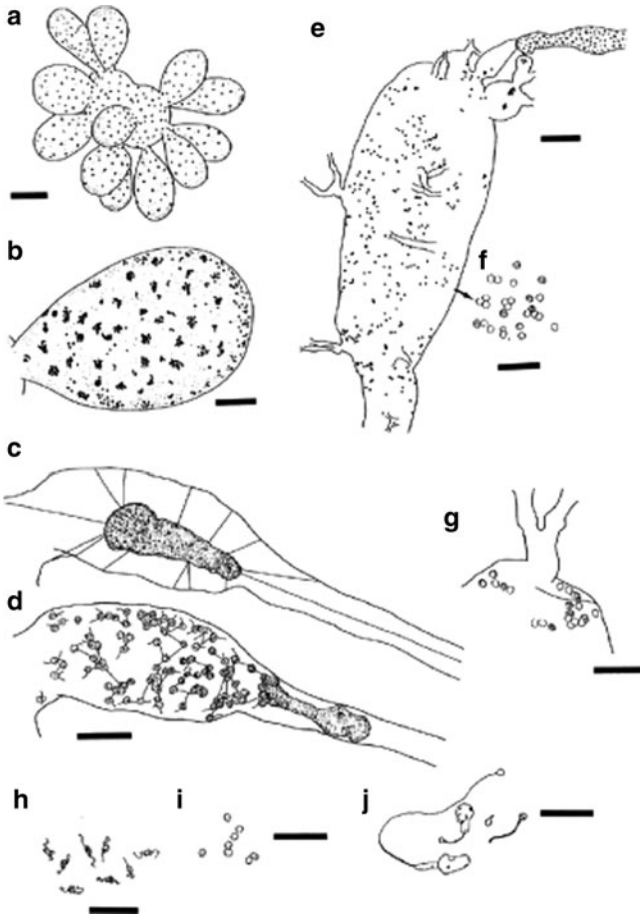
Fig. 2.16 *Atkinsiella dubia*. (**a**, **b**) Mycelium with granular clusters. (**c**) A protoplasmic mass supported by several protoplasmic thread. (**d**) Loose net-works of zoospores. These differentiated into free individual zoospores in the first motile stage. (**e**) A zoosporangium with branched discharge tubes. (**f**) Empty encysted zoospores, and encysted zoospores with protoplasm from which zoospores in the second motile stage will emerge. (**g**) A branched discharge tube with flared openings. (**h**) Zoospores in the second motile stage. (**i**) Encysted zoospores after the second motile stage. (**j**) Germination. *Scales*: (**a**, **e**) 150 μm; (**b**) 70 μm; (**c**, **d**, **g**–**j**) 50 μm; (**f**) = 40 μm

Roza and Hatai ([1999](#)) reported that heavy mortalities reaching 100% among larvae of the Japanese mitten crab, *Eriocheir japonicus*, occurred in Yamaguchi Prefecture, Japan. Under the microscope, infected zoeal larvae were filled with numerous aseptate hyphae. The infected fungus was inoculated on PYGS agar and incubated at 25°C for 7–10 days. Colonies on PYGS agar were attaining a diameter of about 25 mm in 15 days, crystalline, tuberculate, and moist; moderately heaped at the center. Mycelia in the broth were aseptate, radially branched, stout, swollen up to 150 μm in diameter, with clusters of shiny spherical granules, without oil droplets and vacuoles. Granular clusters were evenly distributed inside mycelia, generally consisting of several granules. Mycelia in seawater developing narrow branches (discharge tubes) were followed by zoospore production. Gemmae were present. Zoospores in the first motile stage were produced after 30 h at 25°C. Protoplasmic masses due to gathering of granular clusters on zoosporogenesis were supported at the center of zoosporangia by several protoplasmic threads; differentiated into loose networks of zoospores, then into free individual zoospores in the first motile stage. Zoosporangia were the same in size and shape as the mycelia, with several discharge tubes extending from each zoosporangium. Zoospores in the first motile stage were swimming dully and encysting within zoosporangia and discharge tubes, and biflagellate, subglobose to globose, 3–6 μm in size. Zoospores in the second motile stage were released one by one from encysted zoospores within zoosporangia and discharge tubes, swimming freely for a long time; laterally biflagellate, pyriform, slipper-shaped, isokont, 2–7 μm. Zoospores were dimorphic and diplanetic. Encysted spores were globose to subglobose, 3–7 μm in the first motile stage and 3.5–6 μm in the second motile stage. Discharge tubes were unbranched or occasionally branched, straight or tapering with flared openings, rarely with a central swelling, 4–9 μm in width, 5–16 μm in length. Germination was observed at 6–8 h after spores with slender germ tube were transferred to broth. This fungus was identified as *A. dubia*. This was the first report of mass mortality in crustaceans due to *A. dubia* infection. The optimum growth temperature was at 25°C, and grew only on PYG agar containing 2.5% NaCl and PYGS agar.

2.2.6 Pythium *Infection*

This infection was first reported as *Lagenidium myophilum* infection from marine shrimp (Hatai and Lawhavinit [1988](#)). Later, Muraosa et al. ([2009](#)) made clear that the fungus was included into the genus *Pythium* by phylogenic tree. *Pythium myophilum* (*Lagenidium myophilum*) infection occurred in the abdominal muscles and swimmerets of adult northern shrimp, *Pandalus borealis*, cultured at the Japan Seafarming Association (JASFA). Pure cultures of *P. myophilum* were consistently isolated from the partly blackened abdominal muscle (Fig. [2.17](#)) and the inside of the swimmerets of the adult northern shrimps. Growth of the fungus on PYGS agar was observed at 2 days after incubation. Microscopical observation of the blackened areas of the lesions showed them to be filled with hyphae and the pathogenic

Fig. 2.17 *Pythium myophilum* isolated from the partly *blackened* abdominal muscle (*arrow*)

Fig. 2.18 A juvenile coonstripe shrimp infected with *Pythium myophilum*. The lesions look *whitish* (*arrows*)

fungus to grow only in the tissue of shrimp. The optimum temperature for growth of this fungus was 25°C , but it also grew at the low temperature of 5°C. It would thus be able to infect northern shrimps living in cold seawater; the temperature of the Japan Sea was approximately at 5°C. In pure culture, the hyphae were somewhat uniform with a diameter of 7–10 μm and generally vacuolated. Vesicle formed at the end of discharge tube were measuring 86–240 × 7–10 μm in diameter. Zoospores were 12.9 × 9.6 μm, globose, reniform, pyriform or elongate, monoplanetic and laterally biflagellate. Encysted zoospores were spherical, 5.5–12.0 μm in diameter. Sexual reproduction was not observed.

In 1991, a fungal infection occurred in the larvae of coonstripe shrimps, *Pandalus hypsinotus*, artificially produced at Hokkaido in Japan. Mortality was 100%. In 1993, the infection also occurred in juvenile coonstripe shrimps (Fig. 2.18), which had been reared in tanks after seed production. Mortality was about 70%

(Nakamura et al. 1994a, b). The pathogenic fungi isolated from the lesions were same as those caused by *Pythium myophilum* reported by Hatai and Lawhavinit (1988). *P. myophilum* is pathogenic toward adult northern shrimp, larval and juvenile coonstripe shrimps and Hokkai shrimp, *Pandalus kessleri* (Hatai, unpublished). *P. myophilum* infections have only been in Japan, and these shrimps of the genus *Pandalus* are known to live only in the deep areas of the sea off the coast of Japan. It was interesting that these hosts seemed to be highly sensitive to *P. myophilum*.

2.3 Diseases of Fish and Shellfish Caused by Mitosporic Fungi

2.3.1 Fusarium *Infection*

Some species in the genus *Fusarium* such as *Fusarium solani*, *F. moniliforme*, and *F. oxysporum* have been isolated from kuruma prawn, *Penaeus japonicus*, with black gill in Japan. Among these species, *F. solani* subsequently was reported as an important pathogen.

F. solani was originally proposed as a genus *Fusarium* (Wollenweber 1913). Later the section included five species, ten varieties, and four forms (Wollenweber and Reinking 1935). Snyder and Hansen (1941) combined three species (*F. solani*, *F. martti*, and *F. coeruleum*) into *F. solani*. This taxonomy, however, was not approved by Gerlach and Nirenberg (1982). Booth (1971) and Gerlach and Nirenberg (1982), included four and six species in the section *Martiella*, respectively, from conidiogenesis and shapes of conidia which are major criterions for the classification. The main species, *F. solani*, in the genus *Fusarium* has been later reported in the literatures as *formae specials* (f. sp.), mating population (MP 1–MP VII), or anamorph of *Nectria haematococca* due to its polytypic appearances (Matuo and Snyder 1973). Because of its pathogenic importance, studies on the biological specification of *F. solani* species has also been developed (O'Donnell 2000). Previous studies on the taxonomy of this complex fungal species have contributed valuable information on the limits of the specification and evolutionary relationships within species, *F. solani* (Matuo and Snyder 1973; Hawthorne et al. 1992; Suga et al. 2000; O'Donnell and Gray 1995; O'Donnell 2000). However, *F. solani* isolated from aquatic animals including marine crustaceans and fishes have never been studied in detail in previous reports.

Black gill disease of pond-cultured kuruma prawn, *Penaeus japonicus*, was first reported in Japan (Egusa and Ueda 1972). They demonstrated that a fungus belonged to the genus *Fusarium* was the causative agent and gave the fungus a temporary designation, BG-Fusarium. Since their report, the disease has often broken out among pond cultured kuruma prawn in various districts. Hatai et al. (1978) investigated a taxonomical position of the BG-Fusarium isolated from gill lesions of kuruma prawn with black gill disease (Fig. 2.19). The fungus produced micro- and macro-conidia on conidiophores and chlamydospores. As a result, the

Fig. 2.19 Kuruma prawn infected with *Fusarium solani*. Note gills showing *black color*

BG-Fusarium was identified as *Fusarium solani* according to Booth (1971, 1977) from the characteristics of the fungus on Potato Sucrose Agar. They demonstrated that the pathogenic fungus could be isolated from wet sand in ponds with fungal infection, but not from it without fungal infection, and was capable of surviving for long time in wet sand. Khoa et al. (2005) also reported *Fusarium solani* infection of kuruma prawn (Fig. 2.20). They demonstrated that the fungus showed pathogenicity to kuruma prawn by intramuscular injection. Phylogenetic analyses based on the sequences of its internal transcribed spacer region, including 5.8 S ribosomal DNA and a partial 28 S ribosomal DNA region, showed that all strains tested were monophyletic. And the strains isolated from the diseased kuruma prawn and the phytopathogenic *Fusarium solani* were clearly distinguished by the morphological and phylogenetical characteristics (Khoa et al. 2005).

Fusarium moniliforme was also isolated from gill lesions of kuruma prawn with black gill disease at a private farm in Japan (Rhoobunjongde et al. 1991). The colonies of the fungus cultured on upper surface of potato dextrose agar (PDA) were floccose, creamy white, undersurface a lavender to violet, but did not grow on mycobiotic agar containing cycloheximide. Fungal hyphae were hyaline and 2.5–6.0 μm in diameter. Conidiogenous cells with long monophialides were abundantly formed laterally on aerial mycelium or on sympodially branched conidiophores, and were hyaline, subulate 2.0–4.0 μm in diameter. Macroconidia were present, but only rarely and their appearance varied from slightly sickle- to cigar-shaped, three to four septa, rarely five septa and 26.0–50.0 μm in length. Microconidia were abundant and variable on shape and size from ovoid to elliptical, zero to one septa, rarely two septa, 6.0–20.0 μm in length, and were produced in chains mostly from a simple conidiophores (Fig. 2.21) and false heads on PDA and KCl medium but especially with longer chains on the KCl medium. Chlamydospore was absent. This was the first case of *F. moliniforme* infection in crustacean.
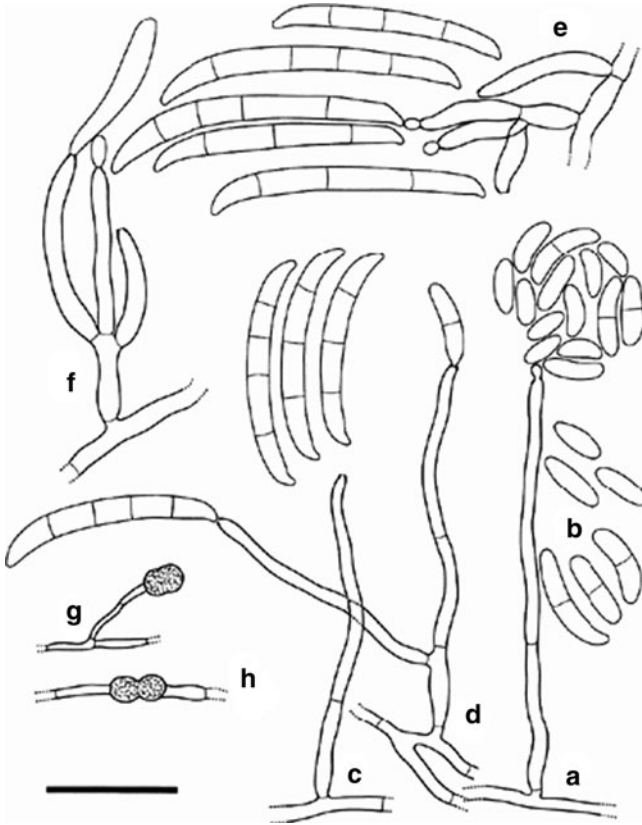
Fig. 2.20 Microscopic morphology of *Fusarium solani* isolated from an infected *Penaeus japonicus*. Scale bar = 25 μm. (**a**) Aerial conidiophores is long and unbranched, slightly narrow toward the apex, monophialidic, producing abundant zero to one-septate conidia that cohere in a false head. (**b**) Oval or ellipsoid one-cell conidia and subcylindric or slightly curved two-cells conodia. (**c**) Unbranched aerial conidiophores bear three to four-septate conidia and are slightly curved with a short and blunt apical cell and slightly notched basal cell. (**d**) A lateral branched aerial conidiophores producing one to four-septate conidia. (**e**) Irregularly and verticillately branched sporodochial conidiophores, bearing monophialides and producing three and four-septate conidia. Conidia extend from the basal part and curve to the apex. The dorsal side is more curved than ventral side, and there is a blunt apical cell and slightly notched basal cell. (**f**) A sporodochial conidiophores verticillately forming monophialides in the early stage of sporulation. (**g**) Terminal chlamydospore from conidiophores is smooth-walled, globose. (**h**) Intercalary chlamydospores in the hyphae are smooth-walled, globose, and in a pair

Khoa and Hatai (2005) reported *Fusarium oxysporum* infection in cultured kuruma prawn *Penaeus japonicus* in Japan. The infection was the first case in kuruma prawn. The infected prawn showed black gills, but the other apparently looked healthy. Fungal hyphae with septa and canoe-shaped conidia were clearly observed in wet-mount preparations of the prawns with black gills. Colony of the *F. oxysporum* grew well on PDA at 25°C. Mycelia were delicate, felt-like, and

Fig. 2.21 Microconidia are produced in chains mostly from simple conidiophores
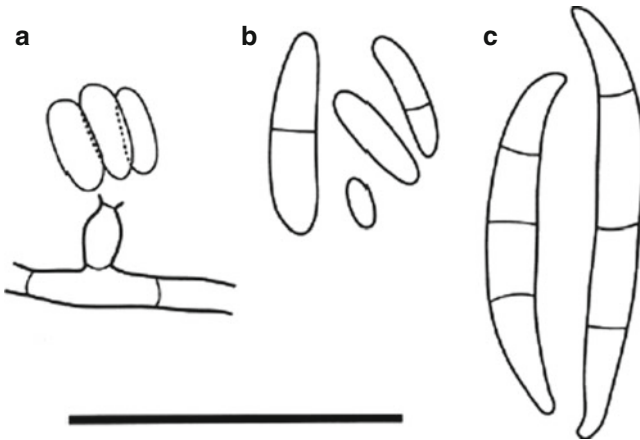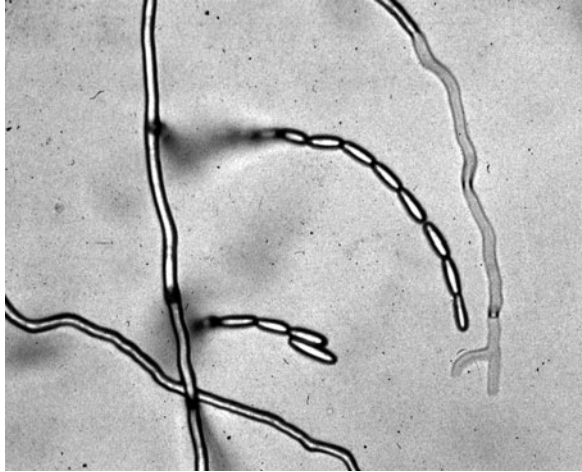
Fig. 2.22 Morphology of *Fusarium oxysporum* . Scale bar $= 30$ μm. (**a**) Short aerial conidiophore, unbranched, monophialidic conidiophore, producing one-cell conidia in false head. (**b**) Aerial conidia with one to two cells. (**c**) Three-septate sporodochial conidia, tapering toward both ends with a pointed apical cell and a slightly hooked basal cell

funiculous flat appressed. Pigment on PDA was white at first, gradually changed to pale beige in the center of the agar plate, and purple or dark violet in the aged cultures. Chlamydospores were not observed. Arial conidiophores were usually single, unbranched and mostly short, 5–15 μm in length, and produced one cell or two cells conidia in false head at 4-day culture (Fig. 2.22). Aerial conidia were usually oval, cylindrical or ellipsoid, straight or slightly curved. One-cell conidia were $5 \pm 2.5 \times 2.5 \pm 0.5$ μm, and two cells conidia were $8.5 \pm 3.5 \times 0.7$ μm. Sporodochial conidiophores were occasionally observed on SNA cultures (Nirenberg 1990), and monophialidic, irregularly or verticillately branched, and

produced three to five septate conidia, predominantly three septate conidia. Sporodochial conidia were usually curved, equally tapering toward both ends with a pointed apical cell and a slightly hooked basal cell. Three septate conidia were $27 \pm 3.7 \times 2.7 \pm 0.3$ μm. The prawns artificially injected with *F. oxysporum* showed typical black gills, and the clinical sign was similar to that of prawn naturally infected with fungus.

F. oxysporum infection was also found in cultured red sea bream, *Pagrus major*, in Japan (Hatai et al. 1986). In almost all cases, no external signs were observed, but kidneys of the fish were remarkably swollen and discolored. The other organs, however, appeared to be normal. The fungus was isolated by inoculating a piece of kidney on Sabouraud dextrose agar (SA agar) at 25°C, and a pure culture was obtained. The fungus was identified as *Fusarium oxysporum* as described by Booth (1971).

In Vietnam a new *Fusarium* infection occurred in black tiger shrimp, *Penaeus monodon* (Khoa et al. 2004). Infected shrimps showed typical signs of black gill disease and mortalities about a month prior to harvest. The isolated fungus was identified as *F. incarnatum* from the detailed morphological and molecular phylogenic analyses. The fungus showed the pathogenicity to kuruma prawn by experimental infection. Optimal temperature for the fungus ranged from 20 to 30°C. The fungus grew drastically at 35°C, but did not at 5 and 40°C.

2.3.2   Ochroconis Infection

The fungal infection in fishes caused by *Ochroconis humicola* was first reported from the kidney of coho salmon, *Oncorhynchus kisutch* (Ross and Yasutake 1973). Later, the infection was reported from rainbow trout, *Salmo gairdneri* (Ajello et al. 1977), Atlantic salmon, *Salmo salar* (Schaumann and Priebe 1994).

In Japan, *Ochroconis humicola* infection has been found in marine cultured fish. First description was from devil stinger, *Inimicus japonicas* (Wada et al. 1995). The diseased fish were about 1.4 g in body weight, and had some ulcers on the body surface. The fish examined showed little appetite, but no mortality was recorded. The center of the lesion was necrotic and sloughed, leaving trunk muscles exposed in a crater-shaped cavity surrounded by an erosion periphery. Direct microscopical examination of the exposed trunk muscles revealed numerous septate fungal hyphae. Fungal colonies were slow growing, slightly domed, velvety to floccose, and pale brown in color. Hyphae were septate, pale brown in color, and 1–2 μm in width. Conidia were usually sparse, $1.8–2.2 \times 7.0–10.0$ μm, two-celled, pale brown in color and cylindrical with rounded ends. The reproductive mode of the conidia was sympodial. The fungus was identified as *Ochroconis humicola* according to de Hoog and von Arx (1973) and Howard (1983). Later, *O. humicola* infection was found in marine-cultured fish, red sea bream, *Pagrus major*, and marbled rockfish, *Sebasticus marmoratus* (Wada et al. 2005). The average body weight of the fish examined was 1.2 g for red sea bream and 1.0 g for marble
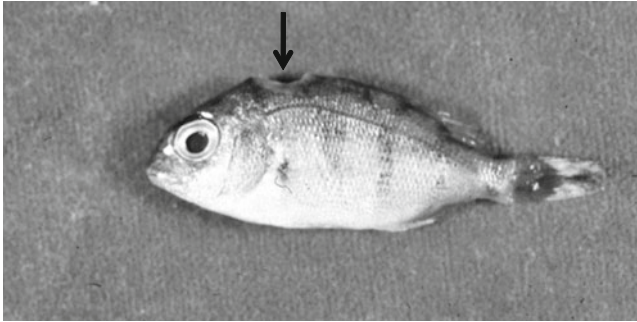
Fig. 2.23 Ochroconis infection in red sea bream. Severe ulceration (*arrow*) formed around the base of the dorsal fins

rockfish. Both cases showed apparent lesions on the body surfaces. In the red sea bream, severe ulceration was found around the base of the dorsal fin (Fig. 2.23), while erosive and/or ulcerative lesions mainly appeared at the mouth regions in the marbled rockfish.

In April 2004, a fungal infection occurred in cultured young striped jack, *Pseudocaranx dentex* at a fish farm in Japan (Munchan et al. 2006). The water temperature in this month was 17–18.5°C. The examined 0-year-old fish were 6–10 cm in body length and 5–10 g in body weight. Moribund fish with fungal infection showed disease sign such as distended abdomen kidney. Numerous brownish hyphae were found in squash preparation of the kidney under microscopy. The cumulative mortality of the disease reached 25% (62,000 out of 250,000 fish) for 1 month after the disease was recognized. Histopathology showed that fungal hyphae were found in the musculature, spleen (Fig. 2.24) and kidney. The granulomas consisted of massive fungal elements and outer layers surrounded by epitheloid cells. No bacteria or parasites were found in the examined tissues. Munchan et al. (2009a) compared the histopathology of young striped jack experimentally infected with dematiaceous fungus *O. humicola* with that of spontaneously infected fish. Moribund and freshly dead fish from both groups showed similar histopathology, and appeared to have been killed due to hyphae penetrating the visceral organs. Fish that survived the infection appeared to be able to suppress the fungal growth by well-established inflammatory reaction involving mycotic granulomas and granulation tissues. The results suggested that two types of *O. humicola* infection occur in young striped jack: an acute type infection, which is characterized by penetrating hyphae that cause direct tissue destruction and a chronic type infection, which is characterized by severe inflammatory reaction that causes function disorders of the affected organs. All fungi isolated from diseased fish were identified as the same fungus. Colony of the isolate showed dark brown to black color when observed from the reverse side of the plate and no visible exudates diffused into the medium, and it was flat, very slow-growing on PDA plate. Colony radii on PDA incubated at 25°C reached 30.1 mm after 4 weeks. Hyphae were septate, 2–3 μm in diameter, pale brown in color, and aerial hyphae
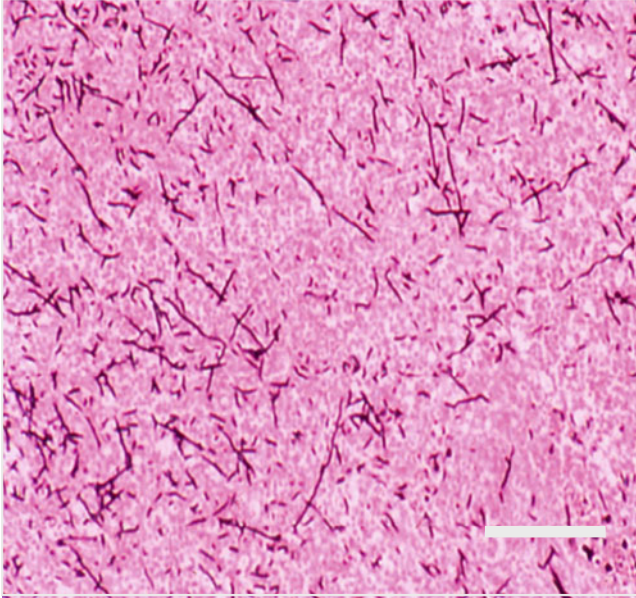
Fig. 2.24 Histopathological finding of spleen in diseased fish. Note many fungal hyphae in the spleen. Grocott-HE stain, Bar = 100 μm
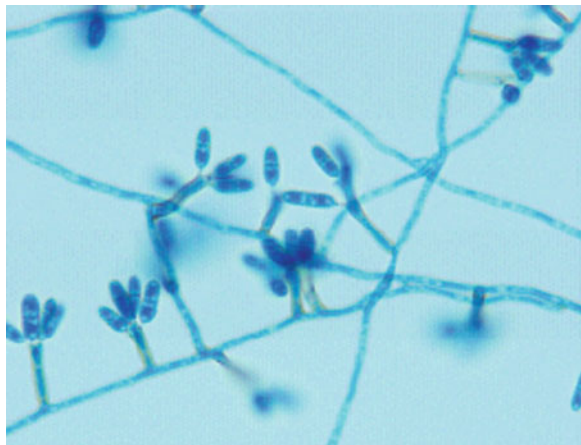
Fig. 2.25 Conidia of *Ochroconis humicola*: two-celled, cylindrical to oblong, constricted at the septum

were sparse. Conidiophores were predominantly cylindrical, average 2.5 × 7.5 μm had denticles at each tip. Conidia were holoblastic, two-celled, cylindrical to oblong with rounded ends, average 2.5–4.5 × 5.5–12.5 μm, smooth-walled, and pale brown in color (Fig. 2.25). The isolate was as *Ochroconis humicola* from these characteristics. The isolate grew at 10–30°C, but not at 35°C. The isolate could grow up to 9% NaCl indicating that *O. humicola* could grow in an environment with

a wide range of salinity. Itraconazole (for oral administration), with an MIC (MFC) range of 0.06–0.13 (0.0625–0.125) μg/mL was chosen for in vivo treatment. In vivo treatment with itraconazole of striped jack experimentally infected with *O. humicola* was conducted for 50 days. No fish died, but gray to white nodules were found in the visceral membrane, kidney, liver, and spleen in the fish. Granulomatous inflammatory reactions were histopathologically found in all fish injected with conidia of *O. humicola*. Clinical signs and histopathological findings indicated that itraconazole showed no efficacy for curing the fish infected with *O. humicola* (Munchan et al. 2009b).

2.3.3   Exophiala *Infection*

Fungal infection caused by the genus *Exophiala*, known as black yeast, has been reported in several species of fish. The first report was by Carmichael (1966) who described a systemic infection of cutthroat trout, *Salmo clarki*, and lake salmon, *Salvelinus namaycush*. The causative agent was initially named a *Phialophora*-like fungus but later classified as *Exophiala salmonis*. Fijan (1969) reported a systemic mycosis in channel catfish, *Ictalurus punctatus*, later the fungus was identified as *E. pisciphilus* (McGinnis and Ajello 1974). Later, *E. salmonis* infection was reported from Atlantic salmon, *Salmo salar* (Richard et al. 1978; Otis and Wolke 1985). On the other hand, *E. pisciphila* infection was reported from smooth dogfish, *Mustelus canis* (Gaskins and Cheung 1986), Atlantic salmon (Langdon and McDonald 1987). *E. psychrophila* infection was also reported from Atlantic salmon (Pedersen and Langvad 1989).

In Japan, *Exophiala* infection occurred in cultured striped jack, *Pseudocaranx dentex*, in 2005 (Munchan et al. 2009c). One hundred out of 35,000 fish died per day and mortalities continued for 1 month. Diseased fish showed swelling of the abdomen and kidney distension. Microscopic examination of the kidney of diseased fish revealed numerous septate hyphae, pale brown in color, in squash preparations. Histology revealed abundant fungal hyphae and conidia in gill, heart, and kidney. Fungal hyphae were accompanied by cell necrosis and in influx of inflammatory, mainly mononuclear cells. The fungus isolated from the diseased fish had septate hyphae, pale brown in color and 1.8–3.0 μm in diameter. The colony morphology of the fungus after 1 week of incubation on PDA at 25°C was initially a black yeast form. It then became woolly and velvety and olive brown in color but black on the reverse side after 4-week incubation. Conidiogenous cells were conspicuous annellides (Fig. 2.26), short or cylindrical or fusiform in shape. Conidia were one-celled, ellipsoidal with smooth walls, accumulated in balls at the apices of annellides that tended to slide down, 1.5–2.0 μm in width and 3.0–5.0 μm in length. The fungus was classified into the genus *Exophila* based on its morphology and as *Exophiala xenobiotica* based on sequences of the ITS1-5.8S-ITS2 regions of rDNA. This is the first record of this fungus in a marine fish.

Fig. 2.26 Conidiogenous
cells: conspicuous annellated
zones, short or cylindrical
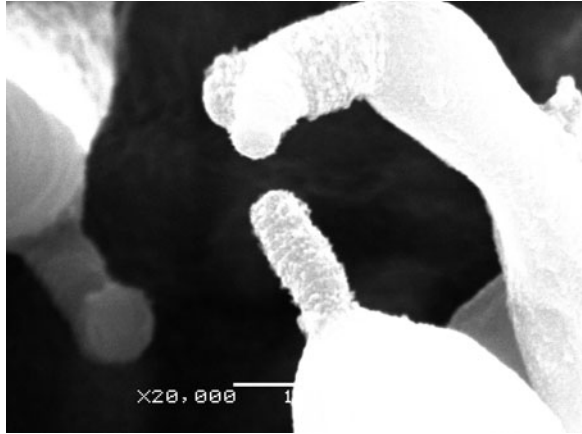
Fig. 2.27 The lesion was only limited in the skin, which is involving ulcerative skin lesion (*arrow*) in the fish

On the other hand, different *Exophiala* infection occurred in Japanese flounder, *Paralichthys olivaceus* in Japan (Kurata et al. 2008). The lesion was only limited in the skin, which is involving ulcerative skin lesions in the fish (Fig. 2.27). The water temperature during the period was approximately 17–21°C. A dematiaceous fungus was only isolated from the fish skin with ulcerative and erosion. The fungal colonies were dark brown to olive black in color. The fungus produced conidia (2.0–3.0 × 2.7–5.0 μm) of an elliptic or obovoid shape and with no or one septum. Conidia were formed as a cluster on the tip of conidiogenous cells. Annellations on the tip of conidiogenous cells were observed under scanning electron microscopy, but were inconspicuous under light microscopy. The fungus grew well at 25°C, but no growth was observed at 37°C. The fungus was identified as an *Exophiala* species, with different morphological, biological and molecular characteristics from three previously described pathogenic *Exophiala* species. The fungus had

a high similarity of 99.6% with *Capronia coronate* from the phylogenetic tree of *Exophiala* spp. based on the sequence of the D1D2 domain of large subunit ribosomal DNA (LSU rDNA). Histology showed that fungal hyphae extended laterally in the dermis, and were absent from the epidermis and musculature of the skin lesions and kidneys of the diseased fish. An inflammatory response with granuloma occurred in the dermis involving accumulations of epitheloid cells around the hyphae. The granulomas were surrounded by lymphocyte-like cells. Epidermal degeneration was observed above the inflamed dermis, suggesting that the inflammatory response caused epidermal damage. Experimental infection reproduced hyphal extension and infiltration of inflammatory cells in the dermis of the flounder, confirming the pathogenicity of the fungus.

2.3.4 Scytalidium *Infection*

Iwatsu et al. (1990) reported first *Scytalidium* infection in striped jack, *Pseudocaranx dentex* with systemic mycosis in Japan. The external clinical signs were blackish patches and ulcers formed on the surface, especially at the basement of dorsal fin, at the tip of snout, and the anal area (Fig. 2.28). No apparent clinical signs were found in the internal organs. Numerous pale brown, septate hyphae, and arthroconidia were found in the lesions of the surface and various internal organs by direct microscopical examination. The fish was reared in sea water with a temperature of about 18°C. The mortality was about 6% of the original population. A fungus was isolated from the lesions of the surface and the internal organs. Experimental infection using striped jack showed that the fungus was a causal agent of the mycosis. The fungus was isolated on PYGS agar. The colonies were dark green and conidia showing dark green were abundantly produced. Mycelium immersed or superficial, composed of straight or sinuous, sometimes curled, smooth, cylindrical, hyaline to mid-brown, branched, rather thick-walled, septate. Stromata were absent. Conidiophores were micronematous, mononematous, straight or flexuous, hyaline to pale brown and branched or unbranched, smooth-walled. Conidiogenous cells were undifferentiated, fragmenting and forming

Fig. 2.28 *Scytalidium* infection in striped jack, *Pseudocaranx dentex* with systemic mycosis. The external clinical signs were *blackish patches* and ulcers formed on the surface, especially at the basement of dorsal fin, at the tip of snout, and the anal area

Fig. 2.29 Arthroconidia of
Scytalidium infestans formed
in extended chains
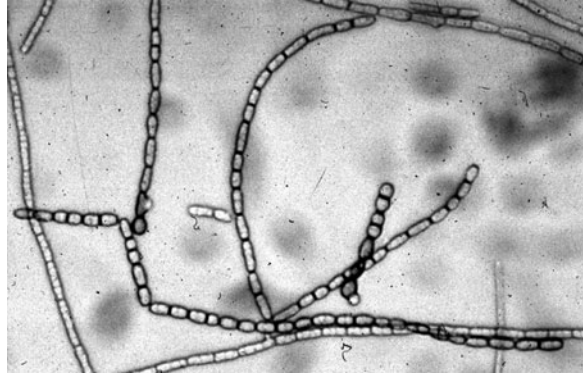
arthroconidia. Arthroconidia of one-type, formed in extended chains (Fig. 2.29), hyaline to mid-brown, dry, simple, rather thick-walled, smooth or verrucose, oblong, dolioform or broadly ellipsoidal, truncate at both ends, 0-1(-3) septate, not easily detached. Chlamydospores were absent. It did not grow at 37°C. As a result, the fungus was a new species of the genus, and named *S. infestans*.

The genus *Scytalidium* was originally erected by Pesante (1957), based on *S. lignicola*. The fungus was characterized by possession of arthroconidia of two types: hyaline, thin-walled, cylindrical ones formed by fragmentation of undifferentiated hyphae, and brown, thick-walled, broadly ellipsoid ones borne in an intercalary fashion. The generic concept was expanded when Sigler and Carmichael (1974) described *S. acidophilum*, a species possessing only dematiaceous arthroconidia. They considered that the genus was characterized by dematiaceous intercalary or terminal arthroconidia formed by fragmentation of undifferentiated hyphae and that the presence of hyaline arthroconidia was not essential for the genus delimitation.

Futhermore, *Scytalidium infestans* infection was first found in red sea bream, *Pagrus major* (Hanjavanit et al. 2004) in Japan. Ulcerative lesions were observed from head to dorsal part of the body surface of red sea bream. Histopathologically, numerous, frequently septate fungal hyphae were observed in the lesions. A fungus was isolated in pure culture from each lesion using PYGS agar. Colonies were dark green in color, and arthroconidia formed in extended chains. It was identified as *S. infestans* according to Iwatsu et al. (1990).

2.4 Infection in Mantis Shrimp by Mitosporic Fungi

The mantis shrimp, *Oratosquilla oratoria*, is an economically important and delicious culinary crustacean species. One of the famous Japanese, sushi dishes, is made from the meat of mantis shrimp. This shrimp is living in mud in the coastal areas of Japan and is the most dominant species. Fungal infection of mantis shrimp

has never been reported in Japan, but it has been known that many mantis shrimp died and the production decreased from 1991. Moribund mantis shrimp were sampled and examined. As a result, it was made clear that the mortality was caused by fungal infection (Duc et al. 2009). They had fungal infection in the gills. Gills of almost mantis shrimp with naturally fungal infection showed brown discoloration (Fig. 2.30). Some gills disappeared due to the fungal infection. Numerous conidia and hyphae inside the gill lamella were observed under microscope (Fig. 2.31). The results of histological examination showed that fungal elements were present in the gills. Fungal hyphae were encapsulated in base of gills.
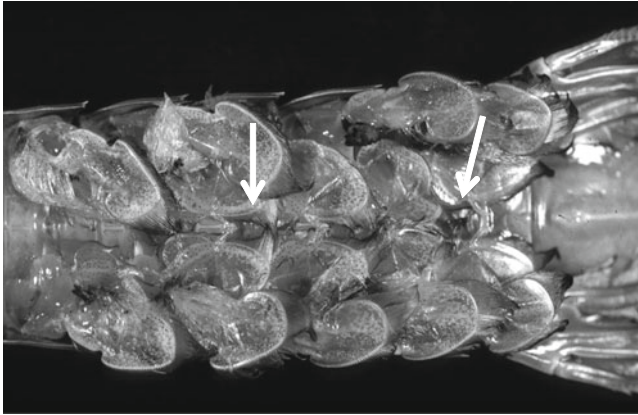
Fig. 2.30   Gills of most mantis shrimp with naturally fungal infection showed *brown discoloration* (*arrows*)
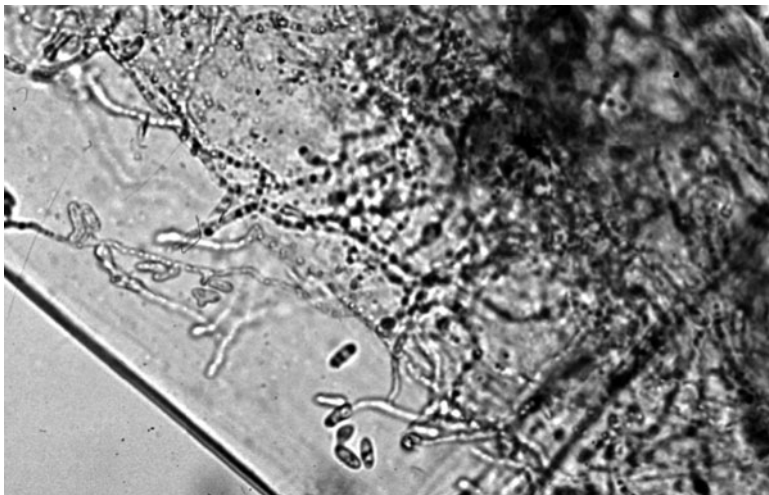
Fig. 2.31   Numerous conidia and hyphae inside the gill lamella observed under microscope

Infected gills were washed three times in sterile physiological saline (0.85% NaCl) and inoculated on PYGS agar. Ampicillin and streptomycin sulfate were added to the medium to inhibit bacterial growth. Plates were incubated at 25°C for 2–4 days. The single spore culture method was applied to obtain pure culture (Ho and Ko 1997). As a result, two kinds of fungi were isolated from the lesions. They were easy to recognize in culture by their growth. The morphological and physiological characteristics, and DNA analysis and sequencing were examined. One of them showed slow growth, and was identified as a new species, *Plectosporium oratosquillae*. The other one exhibited fast growth, and was identified to *Acremonium* sp. (a member of the *Emericellopsis* marine clade) from phylogenetic analysis of ITS and β-tubulin sequences. And it was also a new species, but teleomorph development in culture failed. It was the first report on fungal infection in mantis shrimp (Duc et al. 2009).

Duc and Hatai (2009) carried out experiments to determine pathogenicity of anamorphic fungi *Plectosporium oratosquillae* and *Acremonium* sp., which were isolated from gills of marine shrimp *Oratosquilla oratoria* caught in Japan. Cumulative mortality of the mantis shrimp injected with a high dose (5.0×10^6 conidia/mL) and a low dose (5.0×10^4 conidia/mL) of *P. oratosquillae* reached 100% and 60% at day 25, respectively. Cumulative mortality of the shrimp injected with the high dose and the low dose of the *Acremonium* sp. reached 100% and 80% at day 25, respectively. The gill lesions in the shrimp experimentally infected with the fungi showed many brown spots in the gill filaments, which were similar to the clinical sign of mantis shrimp naturally infected with the fungi. Histopathologically, the hyphae and conidia were found in the gill filaments and heart, and the hyphae were encapsulated by hemocytes in the gill filaments and the base of gills. The result confirmed that these two anamorphic fungi were pathogenic to mantis shrimp.

Duc et al. (2010a) demonstrated the pathogenicity of both the fungi isolated from mantis shrimp to kuruma prawn *Penaeus japonicus* by intramuscular injection of conidial suspensions. These fungi caused mortality in the injected kuruma prawn. Especially cumulative mortality in kuruma prawn injected with 0.1 mL of a conidial suspension with 5×10^6 conidial/mL of *Acremonium* sp. reached 100%. The results indicated that the both fungi were also pathogenic to kuruma prawn. The prawn is important cultured crustacean in Japan, and lives in the same environmental conditions.

Acremonium sp. isolated from diseased mantis shrimp was susceptible *in vitro* to three kinds of antifungal agents: voriconazole, amphotericin B, and terinafine hydrochloride (Duc et al. 2010b). They selected voriconazole to treat kuruma prawn, which had been intramuscularly injected with 0.1 mL of 5.0×10^4 conidia/mL of *Acremonium* sp. Voriconazole was administered orally at doses of six and 2 mg/kg body weight per 7 consecutive days, or intramuscularly injected at doses of 4 and 2 mg/kg body weight per day for 3 consecutive days. Both treatments were started 6 h after injection of the conidial suspension. They demonstrated that voriconazole was an efficient antifungal agent against *Acremonium* sp. from the gross features, mortality, and histopathological observations.

2.5 Future Research

The culture of fish and shellfish and their seed production are important industries in Japan. Diseases caused by fungi result in significant economic losses. Thorough descriptions of many important diseases of fish and shellfish and procedures for identification have been presented in this chapter. It is hoped that these data will be helpful for future research in Japan and in other parts of the world desiring to increase production of commercial fisheries. Some of these obligate host–parasite associations offer excellent tools for research on disease development at cellular and molecular levels. The defense reactions of animals that escape infection will be worth investigating.

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Chapter 3
Fungal Endosymbionts of Seaweeds

T.S. Suryanarayanan

Contents

Abstract Seaweeds are being studied for their role in supporting coastal marine life and nutrient cycling and for their bioactive metabolites. For a more complete understanding of seaweed communities, it is essential to obtain information about their interactions with various other components of their ecosystem. While interactions of seaweeds with herbivores such as fish and mesograzers and surface colonizers such as bacteria and microalgae are known, their interactions with marine and marine-derived fungi are little understood. This chapter highlights the need for investigations on the little-known ecological group of fungi, viz. the fungal endosymbionts, that have intimate associations with seaweeds.

3.1 Introduction

Seaweeds are constituents of highly productive ecosystems and play a major role in nutrient cycling and stabilizing the habitat (Mann 1973). While a lot of information has accumulated on the roles played by fungi in structuring and maintaining

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terrestrial ecosystems like forests (Rayner 1998), their role in orchestrating an important and integral part of the marine ecosystem consisting of seaweeds is not clear. In this context, there is a need to study the biology of fungal endosymbionts of seaweeds with the ultimate aim of aiding in the maintenance and utilization of seaweeds.

3.2   Endomycobiota of Seaweeds

Fungi form parasitic, saprobic or asymptomatic associations with seaweeds. Most of the studies on fungal associations with seaweeds are concerned with the first two ecological groups of fungi (Kohlmeyer 1968; Raghukumar 2006, 2008; Kohlmeyer and Volkmann-Kohlmeyer 2003; Ramaiah 2006; Solis et al. 2010). There are only a few studies on the asymptomatic fungal endosymbionts of seaweeds (Raghukumar et al. 1992; Jones et al. 2008; Zuccaro et al. 2008; Loque et al. 2010; Suryanarayanan et al. 2010b); these studies are based on cultural and molecular methods and have shown that Ascomycetes and anamorphic fungi are the predominant endosymbionts of seaweeds (Fries 1979; Zuccaro et al. 2003, 2008; Tsuda et al. 2004; Schulz et al. 2008; Harvey and Goff 2010). Our study on the endosymbionts of several brown, red and green algae of southern India also showed that anamorphic fungi are dominant in all these algae (Suryanarayanan et al. 2010b). Molecular analysis using LSU rRNA PCR-DGGE showed that the brown alga *Fucus serratus* harbours fungi belonging to the Halosphaeriaceae, Lulworthiaceae, Hypocreales and Dothidiomycetes (Zuccaro et al. 2008). It appears that certain anamorphs of Ascomycetes such as *Sigmoidea marina* could even grow systemically inside algal thalli (Zuccaro et al. 2008). With reference to the species diversity of the endomycobiota harboured by seaweeds, the red and brown algae support a higher species diversity than the green algae (Suryanarayanan et al. 2010b, Table 3.1). The short life cycle of some of the green algal species and the characteristically slow growth of the endosymbionts could together be responsible for the green algae harbouring a low diversity of these fungi (Zuccaro and Mitchell 2005). Although the common endophyte genera such as *Phyllosticta*, *Colletotrichum*, *Phoma* and *Pestalotiopsis* observed among terrestrial plants are absent from the seaweeds, the pattern of distribution of endosymbionts is similar to that of the endophyte assemblages in terrestrial plants with a few dominant fungal species of low host specificity able to colonize taxonomically disparate seaweeds (Suryanarayanan et al. 2010b). Marine-derived fungi are more commonly isolated as endosymbionts of seaweeds than true marine fungi; it is not clear what factors determine the diversity and distribution of this ecological group of organisms. Harvey and Goff (2010) state that differences among algal host species are less important than geographic isolation in determining genetic covariation of their fungal endosymbionts. Environmental factors such as salinity gradient may select fungal species that colonize seaweeds (Mohamed and Martiny 2010). Another factor that can determine the composition of the endosymbiont assemblage is the antifungal metabolites produced by seaweeds to deter fungal colonization

Table 3.1 Fungal symbionts of some seaweeds

S. No	Fungus	Host
1.	*Acremoniella* sp.	Brown alga: *Padina tetrastromatica*[a]
2.	*Acremonium* sp., *Acroconidiella* sp., *Asteromyces cruciatus, Botrytis cinerea, Dendryphiella salina, Emericellopsis minima, Epicoccum* sp., *Geomyces* sp., *Gliocladium* sp., *Humicola fuscoatra, Lindra obtusa, Microascus* sp., *Mycosphaerella* sp., *Nodulisporium* sp., *Periconia* sp., *Scopulariopsis* sp., *Sigmodocea marina, Verticillium cinnabarinum*	Brown alga: *Fucus serratus*[b]
3.	*Alternaria* spp.	Green alga: Ulva linza[c]
		Brown algae: *Stoechospermum marginatum*[a], *Sargassum* sp.[a], *Turbinaria conoides*[a], *Fucus serratus*[b]
		Red alga: *Gelidiella acerosa*[a]
4.	*Aphanocladium* sp.	Red alga: *Gracilaria* sp.[a]
5.	*Arthrinium* sp.	Brown algae: *Fucus* sp.[c], *Fucus serratus*[b]
6.	*Aspergillus* spp.	Green algae: *Caulerpa racemosa*[a], *C. scalpelliformis*[a], *C. sertularioides*[a], *Halimeda macroloba*[a], *Ulva fasciata*[a], *U. lactuca*[a]
		Brown algae: *Dictyota dichotoma*[a], *Lobophora variegata*, *Padina tetrastromatica*[a], *P. gymnospora*[a], *Stoechospermum marginatum*[a], *Sargassum ilicifolium*[a], *Sargassum* sp.[a], *Sargassum wightii*, *Turbinaria* sp.1[a], *T. conoides*[a], *T. decurrens*[a], *Fucus serratus*[b]
		Red algae: *Portieria hornemanii*[a], *Gelidiella acerosa*[a], *Gracilaria crassa*[a], *G. edulis*[a], *Grateloupia lithophila*[a], *Halymenia* spp.[a]
7.	*Aureobasidium pullulans*	Green alga: *Caulerpa racemosa*[a]
8.	*Chaetomium* spp.	Green algae: *Caulerpa racemosa*[a], *C. sertularioides*[a], *H. macroloba*[a], *Ulva fasciata*[a], *U. lactuca*[a]
		Brown algae: *Lobophora variegata*[a], *Padina tetrastromatica*[a], *P. gymnospora*, *Stoechospermum marginatum*, *Turbinaria conoides*[a], *T. decurrens*[a], *Ceramium* sp.[c], *Turbinaria* sp.1[a], *Fucus serratus*[b]
9.	*Cladosporium* spp.	Green algae: *Caulerpa racemosa*[a], *C. sertularioides*[a], *U. lactuca*[a]

(continued)

Table 3.1 (continued)

S. No	Fungus	Host
		Brown algae: *Padina gymnospora*[a], *Sargassum wightii* [a], *Turbinaria* sp.1[a], *T. conoides*[a], *Fucus serratus*[b]
		Red algae: *Portieria hornemanii*[a], *Grateloupia lithophila*[a], *Halymenia* spp.[a]
10.	*Colletotrichum* sp.	Brown algae: *Sargassum* sp.[a], *Turbinaria* sp.1[a]
11.	*Coniothyrium* sp.	Brown alga: *Fucus serratus*[b]
		Red alga: *Corallina elongata*[c]
12.	*Corollospora* sp.	Brown algae: *Sargassum* sp.[c], *Fucus serratus*[b]
13.	*Curvularia* spp.	Green algae: *Caulerpa scalpelliformis*[a], *Ulva fasciata*[a]
		Brown algae: *Turbinaria* sp.1[a], *T. decurrens*[a]
14.	*Drechslera* spp.	Brown algae: *Sargassum* sp.[a], *Turbinaria* sp.1[a], *T. decurrens*[a]
		Red alga: *Halymenia* spp.[a]
15.	*Emericella nidulans*	Brown algae: *Lobophora variegata*[a], *Stoechospermum marginatum*[a], *Turbinaria* sp.1[a], *T. conoides*[a], *T. decurrens*[a], *Sargassum wightii*[a]
		Red alga: *Halymenia* sp.2[a]
16.	*Fusarium* spp.	Green algae: *Caulerpa racemosa*[a], *C. sertularioides*[a]
		Brown algae: *Turbinaria* sp.1[a], *Fucus serratus*[b]
17.	*Geniculosporium* sp.	Red alga: Polysiphonia sp.[c]
18.	*Haloguignardia* sp.	Brown algae: *Cystoseira* sp.[d], *Halidrys* sp.[d]
19.	*Helicosporium* sp.	Brown alga: *Sargassum* sp.[a]
20.	*Lindra thalassiae*	Brown alga: *Sargassum cinereum*[e]
21.	*Memnoniella* sp.	Red alga: *Portieria hornemanii*[a]
22.	*Monilia* sp.	Red alga: *Gracilaria* sp.[a]
23.	*Monodictys* sp.	Brown algae: *Padina tetrastromatica*[a], *Turbinaria* sp.1[a]
24.	*Mucor* sp.	Green alga: *Caulerpa racemosa*[a]
		Brown alga: *Fucus serratus*[b]
25.	*Myrothecium* sp.	Green alga: *Caulerpa sertularioides*[a]
26.	*Nigrospora* sp.	Green alga: *Ulva lactuca*[a]
		Brown algae: *Lobophora variegata*[a], *Stoechospermum marginatum*[a], *Sargassum* sp.[a], *S. wightii*[a], *Turbinaria decurrens*[a]

Table 3.1 (continued)

S. No	Fungus	Host
27.	*Oidiodendron* sp.	Brown algae: *Sargassum wightii*[a], *Fucus serratus*[b]
28.	*Paecilomyces* spp.	Green algae: *Caulerpa scalpelliformis*[a], *Halimeda macroloba*[a], *Ulva fasciata*[a]
		Brown algae: *Padina tetrastromatica*[a], *Sargassum wightii*[a], *Turbinaria* sp.1[a], *Fucus serratus*[b]
		Red algae: *Gracilaria edulis*[a], *Gracilaria* sp.[a], *Halymenia* sp.1[a]
29.	*Penicillium* spp.	Green algae: *Caulerpa racemosa*[a], *C. scalpelliformis*[a], *C. sertularioides*[a], *H. macroloba*[a], *U. lactuca*[a]
		Brown algae: *Padina tetrastromatica*[a], *P. gymnospora*[a], *Sargassum* sp.[a], *S. wightii*[a], *Fucus serratus*[b], *F. vesiculosus*[c]
		Red algae: *Portieria hornemanii*[a], *Gelidiella acerosa*[a], *Halymenia* sp.2[a]
30.	*Phaeotrichoconis* sp.	Brown alga: *Turbinaria* sp.1[a]
31.	*Phialophora* sp.	Green alga: *Caulerpa sertularioides*[a]
		Brown algae: *Turbinaria conoides*[a], *T. decurrens*[a]
32.	*Phoma* sp.	Green alga: *Caulerpa racemosa*[a]
		Brown algae: *Padina gymnospora*[a], *Turbinaria conoides*[a], *Fucus* sp.[c], *F. serratus*[b], *F. vesiculosus*[c]
		Red algae: *Gelidiella acerosa*[a], *Halymenia* sp.1[a]
33.	*Phomopsis* sp.	Brown alga: *Fucus serratus*[b]
		Red alga: *Portieria hornemanii*[a]
34.	*Pyrenochaeta* sp.	Green alga: *Caulerpa racemosa*[a]
35.	*Taeniolella* sp.	Brown alga: *Sargassum* sp.[a]
36.	*Tolypocladium inflatum*	Brown alga: *Fucus vesiculosus*[c]
37.	*Trichoderma* sp.	Green alga: *Halimeda macroloba*[a]
		Brown algae: *Dictyota dichotoma*[a], *Padina gymnospora*[a], *Fucus serratus*[b]
		Red alga: *Gracilaria crassa*[a]
38.	*Trimmatostroma* sp.	Red alga: *Portieria hornemanii*[a]
39.	*Varicosporium* sp.	Brown algae: *Sargassum* sp.[a], *Turbinaria* sp.1[a]

[a]Suryanarayanan et al. (2010b)
[b]Zuccaro et al. (2008)
[c]Schulz et al. (2008)
[d]Harvey and Goff (2010)
[e]Raghukumar et al. (1992)

(Kubanek et al. 2003; König et al. 2006; Lam et al. 2008). Adaptations by a few fungal species to tolerate or detoxify these compounds along with the constant availability of host tissue for colonization in seaweed beds can lead to the evolution of generalist endosymbionts capable of colonizing taxonomically unrelated seaweeds. Such adaptations can possibly explain the constant presence of certain marine-derived fungal genera such as *Aspergillus*, *Cladosporium* and *Penicillium* as endosymbionts in different seaweeds (König et al. 2006; Zuccaro et al. 2008). For instance, Suryanarayanan et al. (2010b) isolated three species of *Cladosporium* from *Caulerpa racemosa*, *C. sertularioides*, *Ulva lactuca*, *Padina gymnospora*, *Sargassum wightii*, *Turbinaria conides*, *Turbinaria* sp.1, *Portieria hornemanii*, *Grateloupia lithophila* and *Halymenia* spp. Zuccaro et al. (2008) isolated several *Cladosporium* sp. from *Fucus serratus*. *Aspergillus terreus* was most frequently isolated as an endosymbiont from *Caulerpa scalpelliformis*, *Helimeda macroloba*, *Ulva lactuca*, *Ulva fasciata*, *Lobophora variegata*, *Padina gymnospora*, *Stoechospermum marginatum*, *Sargassum ilicifolium*, *Portieria hornemanni* and *Gracilaria edulis* (Suryanarayanan et al. 2010b). Marine-derived fungi such as *Aspergillus* spp., apart from dominating the endosymbiont assemblage of seaweeds (Suryanarayanan et al. 2010b), also dominate the fungal assemblages of marine invertebrates of different geographical locations such as the North Sea, the Mediterranean, the Caribbean and the great Barrier Reef (Höller et al. 2000) attesting to their adaptation to occupy such a niche as the inner tissues of seaweeds or marine animals. Such a widespread occurrence of marine-derived fungi may be indicative of their passive migration from terrestrial habitats (Alva et al. 2002). However, since these fungi are better adapted to marine environments than their terrestrial conspecifics (Zuccaro et al. 2004; König et al. 2006) and survive in seaweeds which produce antifungal metabolites (Kubanek et al. 2003; Lam et al. 2008), it is likely that they are not casual residents of the seas but have coevolved with the seaweeds (Zuccaro et al. 2004; Suryanarayanan et al. 2010b). Seaweeds are known to produce secondary metabolites that deter microbial colonization; these metabolites determine the abundance and composition of the microbial community associated with the seaweeds as they select those microbes that can tolerate or detoxify the chemicals (Nylund et al. 2010). The observation that the spores of *Acremonium fuci* associated with *F. serratus* can germinate only in the presence of tissues of the alga and not in seawater alone points to host specificity among seaweed endosymbionts and adds credence to the view that marine-derived fungi are not mere migrants from land habitats (Zuccaro et al. 2004). Furthermore, the widespread occurrence of marine-derived fungi as endosymbionts as opposed to the few obligate marine fungi such as *Ascochyta salicorniae* (König et al. 2006), *Lindra* sp., *Lulworthia* sp. and *Corollospora* sp. (Zuccaro et al. 2008) suggests that the endosymbiotic marine-derived fungi constitute a special community. It is pertinent to mention here that genetic variations exist in a single morphological species of seaweed fungal endosymbiont (Harvey 2002). Molecular studies to discern variations in genes and gene expressions between fungal conspecifics of marine and terrestrial habitats are needed to substantiate this hypothesis.

3.3 Fungal Endosymbionts of Seaweeds as Drug Source

Although a simple procedure of inoculating surface-sterilized segments of algal thalli on suitable growth media yields several endosymbiotic fungi, the nature of association of these fungi with their algal hosts is not clear. As has been reported for endophytes of terrestrial plants (Ganley et al. 2004; Suryanarayanan and Murali 2006), the endosymbiont assemblage of an alga can consist of different ecological groups of fungi such as latent pathogens, true endosymbionts or casual residents. Irrespective of the nature of association that these fungi have with their seaweeds, their capacity to produce secondary metabolites is considerable (Bugni and Ireland 2004; Ebel 2010). They produce novel metabolites showing various bioactivities which are of pharmaceutical importance (Saleem et al. 2007). The extraordinary synthetic ability of these fungi and the fact that natural products are superior to combinatorial synthesis of drugs due to greater number of chiral centres, enhanced steric complexity and capacity to bind to targets (Feher and Schmidt 2003; Koehn and Carter 2005) have renewed the search for novel drugs from marine fungi (Jones et al. 2008; Suryanarayanan et al. 2010a).

Endosymbionts of seaweeds produce, among other bioactive metabolites, strong antioxidants (Suryanarayanan et al. 2010b). An *Epicoccum* sp. associated with *Fucus vesiculosus* produces epicoccone, a novel antioxidant (Abdel-Lateff et al. 2003a). *Wardomyces anomalus*, an endosymbiont of green algae produces a novel xanthone with antioxidant and radical scavenging properties (Abdel-Lateff et al. 2003b). Synthesis of antioxidants may be a strategy among endosymbionts to counter the defence reactions of their algal hosts (Dring 2005). Many fungi isolated from green, red and brown algae also produce antialgal, antifungal and antiinsect metabolites (Suryanarayanan et al. 2010b) which may help in deterring colonization of algal thalli by other microbes thereby reducing competition and in warding off herbivores.

Although marine-derived fungi are marine isolates of terrestrial species, they are prolific producers of novel metabolites of unprecedented structures (Bugni and Ireland 2004; Oh et al. 2006; Meyer et al. 2010; Kjer et al. 2010; Gao et al. 2011) which are not produced by their terrestrial conspecifics (Osterhage et al. 2000). These metabolites exhibit anticancer, antibacterial, antiplasmodial, anti-inflammatory, nematicidal, antiviral and antiangiogenic activities (Bhadury et al. 2006; Newman and Hill 2006; Bhatnagar and Kim 2010; Ohkawa et al. 2010). Marine-derived fungi associated with marine algae have been reported to produce novel metabolites including aromatic polyketides (Pontius et al. 2008), alkaloids (Tsuda et al. 2004), sesquiterpenes (Bugni and Ireland 2004) and terpenes (Klemke et al. 2004; König et al. 2006; Lösgen et al. 2007). These fungi are thus an important source of pharmacologically active metabolites or potential lead compounds for drug synthesis (Jensen and Fenical 2002; Blunt et al. 2008, 2010) (Table 3.2). Production of bioactive metabolites by fungi depends largely on culture conditions. Manipulating physical factors such as pH, salinity and aeration (Bode et al. 2002) and altering growth medium composition or adding certain inhibitors and precursors to the medium (Llorens et al. 2004) increase or induce the synthesis of bioactive

Table 3.2 Some bioactive metabolites isolated from fungal endosymbionts of seaweeds

Fungus	Host	Secondary metabolite(s)	Activity	References
Aspergillus versicolor	Penicillus capitatus	Sesquiterpenoid nitrobenzoyl esters	Antitumour	Belofsky et al. (1998)
Unidentified fungus	Ceralodictyon spongiosum	Anthracycline-derived pentacyclic metabolite	–	Shigemori et al. (1999)
Acremonium sp.	Cladostephus spongiosus	Hydroquinone	Antioxidant	Abdel-Lateff et al. (2002)
Drechslera dematioidea	Liagora viscida	10 new sesquiterpenoids	Antimalarial	Osterhage et al. (2002)
Aspergillus terreus	Marine alga	Chiral dipyrrolobenzoquinone derivatives	UV-A protection	Lee et al. (2003)
Penicillium citrinum	Actinotrichia fragilis	Alkaloid	Anticancer	Tsuda et al. (2004)
Apiospora montagnei	Polosiphonia violacea	Diterpene	Anticancer	Klemke et al. (2004)
Penicillium sp.	Dictyosphaeria versluyii	Decalactones	–	Bugni et al. (2004)
Unidentified fungus	Gracillaria verrucosa	Cyclopentenone derivatives	–	Li et al. (2004)
Myrothecium sp.	Enteromorpha compressa	Cyclopentenone derivatives	Tyrosinase inhibition	Li et al. (2005)
Geniculosporium sp	Polysiphonia sp.	11 new tricyclic sesquiterpenes	Antimicrobial	Krohn et al. (2005)
Fusarium sp.	Codium fragile	Cyclic tetrapeptide	Anticancer	Ebel (2006)
Emericella nidulans var. acristata	Green alga	Prenylated Polyketides	–	Kralj et al. (2006)
Chaetomium globosum	Polysiphonia urceolata	Benzaldehyde derivative	Antitumour and antioxidant	Wang et al. (2006)
Botrytis sp.	Enteromorpha compressa	Cyclopentenone derivatives	–	Li et al. (2007)
Aspergillus niger	Colpomenia sinuosa	Diels–Alder adduct of a steroid and maleimide	–	Zhang et al. (2007a)
Aspergillus niger	Colpomenia sinuosa	Naphthoquinoneimine	Antifungal	Zhang et al. (2007b)
Aspergillus niger	Colpomenia sinuosa	Novel sphingolipids	Antifungal	Zhang et al. (2007c)
Aspergillus flavus	Enteromorpha tubulosa	2-Pyrone derivatives	Induction of cAMP production	Lin et al. (2008)

Beauveria felina	*Caulerpa* sp.	Cyclodepsipeptides	Antimycobacterial, Antibacterial, Antifungal and Cytotoxic	de Vita-Marques et al. (2008)
Pseudoanguillospora sp.	*Polyides rotundus*	New isochromans	Antimicrobial	Kock et al. (2009)
Penicillium chrysogenum	*Laurencia* sp.	Polyketide and glycerol derivatives	Antifungal	Gao et al. (2011)
Cadophora malorum.	*Enteromorpha* sp.	New hydroxylated sclerosporin derivatives	Inhibition of fat deposition in cells	Almeida et al. (2010)
Curvularia sp.	*Gracilaria folifera*	New macrolides	Antimicrobial	Dai et al. (2010)
Chaetomium globosum	*Ulva pertusa*	7 new cytochalasan derivatives	–	Cui et al. (2010)
Aspergillus oryzae	*Heterosiphonia japonica*	2 new indoloditerpenes derivatives	Antibiotic	Qiao et al. (2010)
Exophiala oligosperma	*Laurencia similis*	Phenoxynaphthalene	–	Li et al. (2011)
Coniothyrium cereale	*Enteromorpha* sp.	New phenalenone derivatives	Antibiotic and inhibitor of human leukocyte elastase	Elsebai et al. (2011)

| Control | 0.17 M | 0.6 M | 1.2 M |
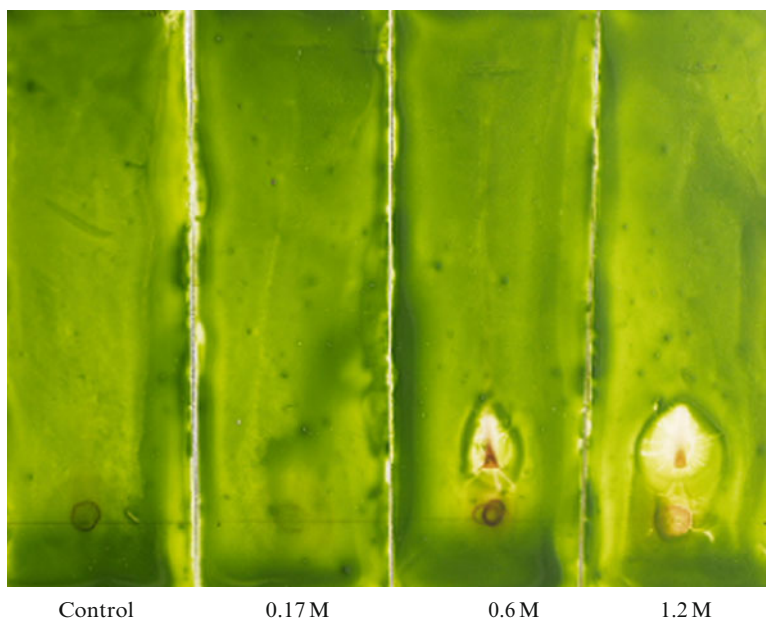
Fig. 3.1 Autobiogram showing the production of antialgal substance(s) by *Aspergillus terreus* endosymbiont of *Ulva lactuca* in NaCl-amended medium

metabolites by the fungi. For example, Miao et al. (2006) reported that the antibiotic activity of a marine-derived fungus increased with salinity of the growth medium. Extending the argument that marine-derived fungi are not recent chance immigrants from terrestrial habitats, we studied the secondary metabolite production in *Aspergillus terreus* isolated from the alga *Ulva lactuca* as influenced by sodium chloride in the growth medium. The fungus produces antialgal substance(s) only when grown in a medium with 3.5 or 7% NaCl and not in the absence of the salt (Fig. 3.1) (Suryanarayanan and Venkatachalam, unpublished). This suggested that cultivation of marine fungi in NaCl medium can result in the production of metabolites not synthesized or only synthesized in undetectable levels in normal growth medium (Bugni and Ireland 2004). Similarly, co-cultivation of marine-derived fungi with microbes of marine habitats activates the silent gene clusters of the fungi enabling them to synthesize novel metabolites (Brakhage and Schroeckh 2011). A marine-derived *Emericella* sp. synthesizes emericellamides when co-cultured with the marine actinomycete *Salinispora arenicola* due to enhanced expression of emericellamide gene cluster (Oh et al. 2007). *Pestalotia* sp., another marine-derived fungus, synthesizes pesatlone, an antibiotic effective against methicillin-resistant *Staphylococcus aureus* when cultured with a marine bacterium (Cueto et al. 2001). The synthetic ability of the fungi can also be enhanced by the presence of small molecule elicitors in the growth medium as they specifically effect the transcription of secondary metabolite gene clusters (Pettit 2010). For instance, Christian et al. (2005) reported that the marine-derived

fungus *Phomopsis asperagi* produces novel chaetoglobosins when the growth medium is amended with the sponge metabolite jasplakinolide. The addition of small molecule elicitors to the fermentation medium specifically influences the synthesis of bioactive molecules by fungi (Pettit 2010). Thus, the synthetic ability of seaweed endosymbionts can be enhanced by altering the culture conditions. In this context, Kjer et al. (2010) have recently described methods for isolating marine-derived endophytic fungi and extracting bioactive metabolites from them.

3.4 Lessons from Fungal Endophytes

We have very limited information on the diversity and nature of association of fungal endosymbionts of seaweeds. Culture methods should be complemented by molecular methods to obtain a more complete picture of the diversity of the endosymbionts as has been done for endophyte of terrestrial plants (Arnold and Lutzoni 2007; Unterseher and Schnittler 2010). Although direct comparisons of seaweeds with terrestrial plants may not be ideal due to differences in their tissue construction, environment and propagule density and survival (Schiel and Foster 2006), the more intense studies conducted on endophytes of terrestrial plants provide us with blueprints for future lines of investigations on seaweed endosymbionts. For instance, endophyte infections render plants more tolerant to abiotic stress (Rodriguez et al. 2008). Endophytes elaborate metabolites that make their hosts more resistant to biotic stress such as infection by pathogens (Arnold et al. 2003) or damage by herbivores (Wagner and Lewis 2000). The strong antioxidants produced by endophytes also play a role in protecting the plant hosts from stresses (White and Torres 2010). Since many endosymbionts of seaweeds produce antimicrobial metabolites and strong antioxidants and since some marine algae such as *Ulva* lack structural and chemical defences (Moksnes et al. 2008), it would be worthwhile determining the role of endosymbionts in stress tolerance and survival of seaweeds. Endophyte infections can also negatively affect a terrestrial plant host by increasing its susceptibility to drought (Arnold and Engelbrecht 2007) and reducing its photosynthetic efficiency (Pinto et al. 2000). There is no information regarding the cost and benefit of endosymbiont association with seaweeds. Some fungal pathogens survive as symptomless endophytes in tissues of terrestrial plants and induce disease under suitable environmental conditions or when the host's defence is weakened (Suryanarayanan and Murali 2006; A'lvarez-Loayza et al. 2011). If pathogenic fungi can exist as latent pathogens in seaweeds, they can be harnessed to control undesirable and invasive seaweeds which are detrimental to marine ecosystems (Anderson 2007). Plant endophytes are known to produce numerous bioactive compounds including those elaborated by their hosts (Stierle et al. 1993; Weber 2009). Though many seaweeds produce antibacterial, antiviral and cytotoxic compounds (Kamenarska et al. 2009), there is no information on how their synthetic ability is affected by the presence of fungal endosymbionts. If a fungal endosymbiont produces the same bioactive metabolite of industrial

importance as its algal host, extraction and purification of the chemical will be easier since marine-derived fungi can be cultured easily. Genotypically similar endophytes of land plants vary in their secondary metabolite profile, indicating that screening of a fungal species once will not capture the entire metabolic spectrum of the endophyte (Seymour et al. 2004). This may be true of the fungal endosymbionts of seaweeds as well since we observed variation in the biological activities of the metabolites of *Aspergillus terreus* isolated from different seaweeds (Suryanarayanan et al. 2010b). Another important aspect of seaweed–endosymbiont association is the diversity and distribution of these fungi. Information on land plant–endophyte diversity provide us with a background with which this question can be tackled. Firstly, both molecular and culture methods should be employed to assess the endosymbiont diversity since they complement each other to provide a more complete data as has been demonstrated for terrestrial plants and endophyte association (Arnold et al. 2007). Furthermore, different organs or tissues of seaweeds with complex structures will have to be screened since different tissues of a single plant species can harbour different endophyte species (Kumaresan et al. 2002). It is also necessary to screen seaweeds during different seasons as the endophyte assemblage of plants is altered by seasons (Suryanarayanan and Thennarasan 2004). Foliar endophytes persist in fallen leaves and initiate tissue degradation by functioning as pioneer leaf litter fungi (Sun et al. 2011). In this context, the role of fungal endosymbionts in the degradation of old and dead seaweed tissues has to be addressed.

3.5 Future Directions

Considering the economic importance of seaweeds and the significant roles they play in maintaining the coastal marine ecosystem, their biology, including their interactions with other organisms need to be investigated in detail. Among the various types of organisms that are known to be associated with seaweeds, the endosymbiotic fungi warrant special attention since their biosynthetic potential is unique and many of them synthesize novel metabolites of unprecedented molecular architecture. The bioactivities of such metabolites indicate that they have pharmaceutical and agricultural applications. Studying the diversity and distribution of seaweed endosymbionts and using modern methods of genome-guided search for discovering their bioactive metabolites will help in harnessing the biotechnological potential of these fungi.

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Chapter 4
Diversity and Biogeochemical Function of Planktonic Fungi in the Ocean

Guangyi Wang, Xin Wang, Xianhua Liu, and Qian Li

Contents

Abstract Microbial communities play critical biogeochemical roles in the functioning of marine ecosystems. Recent advances in molecular methods and environmental genomics have greatly advanced our understanding of microbial prokaryotes and their diversity and functional ecology in the world's oceans. Large populations of heterotrophic eukaryotes are well documented in the oceans and yet, their diversity and function remain relatively unknown. Particularly, large populations of planktonic fungi have long been known to exist in coastal and oceanic waters but the diversity and ecology of planktonic fungi remain one of the most under-studied microbial topics. Recent studies have revealed novel diversity and interesting ecological functions of planktonic fungi and suggest that they are a potentially important component in marine microbial food web. This chapter

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will review the diversity and ecology of planktonic fungi in the world's oceans and discuss their significance in ocean carbon and nutrient cycling.

4.1 Introduction

Fungi have long been known to exist in marine environments. Obligate marine fungi are distinct from terrestrial and freshwater fungi in their taxonomy and morphology as well as their adaptations to an aquatic habitat (Kohlmeyer and Kohlmeyer 1979; Moss 1986; Jones and Mitchell 1996; Meyers 1996; Jones 2000). They are more precisely defined as fungi that can complete their entire life cycles within the sea, i.e., those that can grow and sporulate exclusively in a marine or estuarine habitat (Kohlmeyer and Volkmann-Kohlmeyer 2003). Facultative marine fungi refer those originating from freshwater or terrestrial environment that are also capable of growth and sporulation in the sea (Kohlmeyer and Kohlmeyer 1979). In addition, fungi from marine environments are often referred to as "marine-derived fungi," which include obligate and facultative marine fungi. In this chapter, the term "marine fungi" mostly refers to both obligate and facultative marine fungi unless otherwise specified. Marine fungi primarily include the *Ascomycetes*, the *Basidiomycetes*, and the *Chytridiomycetes* (Kohlmeyer and Kohlmeyer 1979; Kohlmeyer and Volkmann-Kohlmeyer 1991; Fell and Newell 1998; Hyde and Sarma 2000; Kohlmeyer et al. 2004). The *Oomycetes* and *Labyrinthulomycetes*, formerly known as pseudofungi or fungal-like organisms, were originally classified as fungi and have now been reclassified under the new Kingdom of Straminipila (Wang and Johnson 2009). Nevertheless, they are still commonly studied by marine mycologists and mycoplankton ecologists (Fell and Newell 1998; Raghukumar 2002; Kis-Papo 2005; Hibbett et al. 2007). Some marine fungi may occur in freshwater or terrestrial habitats. For example, both *Savoryella lignicola* and *Lignincola leavis* have been found in both marine and fresh habitats (Jones 2000). So far, there are about 467 described obligate or facultative higher marine fungi from 244 genera largely derived from marine substrata using cultivation-based methods (Kohlmeyer and Volkmann-Kohlmeyer 1991, 2003; Hyde and Sarma 2000; Rand 2000; Vrijmoed 2000; Kohlmeyer et al. 2004; Kis-Papo 2005; Pang and Mitchell 2005; Wang et al. 2008a, b). They are divided into a majority of *Ascomycota* (97%), a few *Basidiomycota* (~2%), and anomorphic fungi (<1%). The largest order of marine ascomycetes is the *Halosphaeriales*, with some 45 genera and 119 species. It is estimated that approximately 1.5 million fungal species exist, but only 70,000 species (less than 5%) have been described (Hawksworth 1991). Of these 70,000 species, 468 are marine fungi (0.67%). This suggests that, based on this ratio, there are a total of 10,029 species of marine fungi (Kis-Papo 2005). Hence, the vast majority of marine fungi remain to be discovered and described, particularly planktonic forms in the water column or symbiotic ones in marine animals. This chapter provides a review of the diversity and functional ecology of fungi in marine environments with a focus on water column.

4.2 Marine Fungal Taxonomy

Fungi have historically been identified and classified using morphological characteristics, such as sexual structure. However, fungi, especially microscopic forms, often have few useful morphological features and show pronounced morphological variability (Brasier 1997; Petersen and Hughes 1999; Höller et al. 2000; Burnett 2003). Many fungi are anamorphic without sexual production, yet possess a surprisingly high level of genetic variation (Harrington and Rizzo 1999; Talhinhas et al. 2002). In addition, an increasing number of morphologically indistinguishable (cryptic) species have recently been described by Grunig et al. (2007). Accordingly, the use of morphology for fungal identification and classification purposes can be severely biased. In the past decade, the ability to identify species at the molecular level has changed our understanding of the species concept for different groups of fungi (Hibbett et al. 2007). Particularly, molecular phylogenetic approaches avoid subjectivity in determining the limits of a species by relying on the concordance of more than one gene genealogy and have been approved as well suited for teleomorphic and anamorphic fungi (Taylor et al. 2000).

Ribosomal DNA (rDNA) sequence has been proven to be one of the most useful techniques in aiding fungal identification and resolving taxonomic positions at different levels (Hillis and Dixon 1991). The ribosomal gene cluster comprises three regions coding for the 5.8 S, 18 S, and 28 S rRNA. Some of the rDNA fragments are useful for delineation of higher taxonomic ranks, such as classes and phyla (5.8 rRNA gene, parts of the Large Subunit rRNA (LSU) 1 and 2 regions and Small Subunit rRNA (SSU) genes) (Bruns et al. 1991; Sugiyama 1994), and others are used for separation of genera and species (e.g., Internally Transcribed Spacer (ITS) 1 and 2 regions, Domain 2 of the LSU) (White et al. 1990; Bruns et al. 1991; Hillis and Dixon 1991; Sugiyama 1994). Some of these regions have successfully been used to study fungal communities in marine environments (Pang and Mitchell 2005; Gao et al. 2008, 2010; Li and Wang 2009).

In six decades of marine mycology research, identification of fungi derived from marine environments had largely used morphology-based approaches (Hyde 2002; Li and Wang 2009; Wang and Johnson 2009), which have significantly contributed to the development of marine mycology. However, the majority of fungi derived from marine environments (e.g., sponges) are mitosporic; about one-third of these fungi cannot be identified using the morphology-based approach (Morrison-Gardiner 2002). Furthermore, these approaches are well known to detect only a small fraction of marine fungal communities in natural environments. Hence, identification of marine fungi from natural environments is at best difficult, and in most cases not possible (Gao et al. 2008). Recently, a combination of the morphology-based approach with the molecular method has identified an unprecedented diversity of fungi from marine environments (e.g., sponges) (Li and Wang 2009; Wang and Johnson 2009). In particular, molecular methods have identified a novel diversity of unculturable fungal communities in marine habitats (Pang and Mitchell 2005; Gao et al. 2008, 2010).

4.3   Fungi in the Water Column

Planktonic fungi (also called mycoplankton) include filamentous free-living fungi and yeasts, and those associated with planktonic particles or phytoplankton (Wang and Johnson 2009; Gao et al. 2010). Fungi have long been known for their important role in processing detrital organic matter from terrestrial plants (Carlile et al. 2001). Although they occur in different marine ecosystems and have been postulated to play a major role in the detrital food web (Fell and Newell 1998; Raghukumar 2005; Gulis et al. 2006; Gessner et al. 2007), their role in decomposing marine organic matter and in marine biogeochemical cycles is still poorly understood in comparison with those in terrestrial environments (Fell and Newell 1998; Clipson et al. 2006; Gutierrez et al. 2010). Particularly, in coastal environments the productivity often exceeds the consumption of herbivores and a large fraction of primary organic matter become available to consumers as detritus (Newell 1982). In such ecosystems, productivity depends on the mineralization of wood, leaves, and other detrital plant materials. Fungi play an essential part in this process in terrestrial and freshwater systems (Hyde et al. 1998; Gulis et al. 2006). Hence, the ecological function of fungi in a water column can be significant, but has been largely ignored. To that end, this section is dedicated to the fungal communities (culturable and unculturable) and ecology of fungal populations in the water columns of the world's oceans.

4.3.1   Abundance and Existence of Living Fungi in Seawater

A typical milliliter of seawater contains over 10^3 fungal cells (or propagules) (Gao et al. 2008). Although marine yeasts are ubiquitous in seawater (Vogel et al. 2007; Kutty and Philp 2008; Chen et al. 2009), direct detection of living fungal mycelia using the Calcofluor White stain method has occurred only recently (Fig. 4.1). The recent discovery of the active forms of filamentous fungi in seawater provides an unequivocal piece of evidence for their potential significance in the marine detrital food web. In freshwater ecosystems, fungal biomass can account for as much as 18–23% of the total mass of detritus (Gessner and Chauvet 1994; Methvin and Suberkropp 2003; Gessner et al. 2007). Fungal biomass associated with leaf detritus reaches annual maximum values after leaf litter enters a stream and becomes colonized (Suberkropp 1991). Maximum fungal biomass associated with decaying leaves typically is one to two orders of magnitude greater than bacterial biomass found on the same leaves and, therefore, microbial biomass occurring in leaves is also dominated by fungi in freshwater ecosystems (Gulis et al. 2006). Recently, fungal mycelia, ranging from 1 to 3 μm in diameter and from 10 to >200 μm in length were observed as individual filaments or aggregates in the coastal upwelling ecosystem off central Chile (Gutierrez et al. 2010) and the Hawaiian coastal waters (Want et al. unpublished data) using the fluorescent
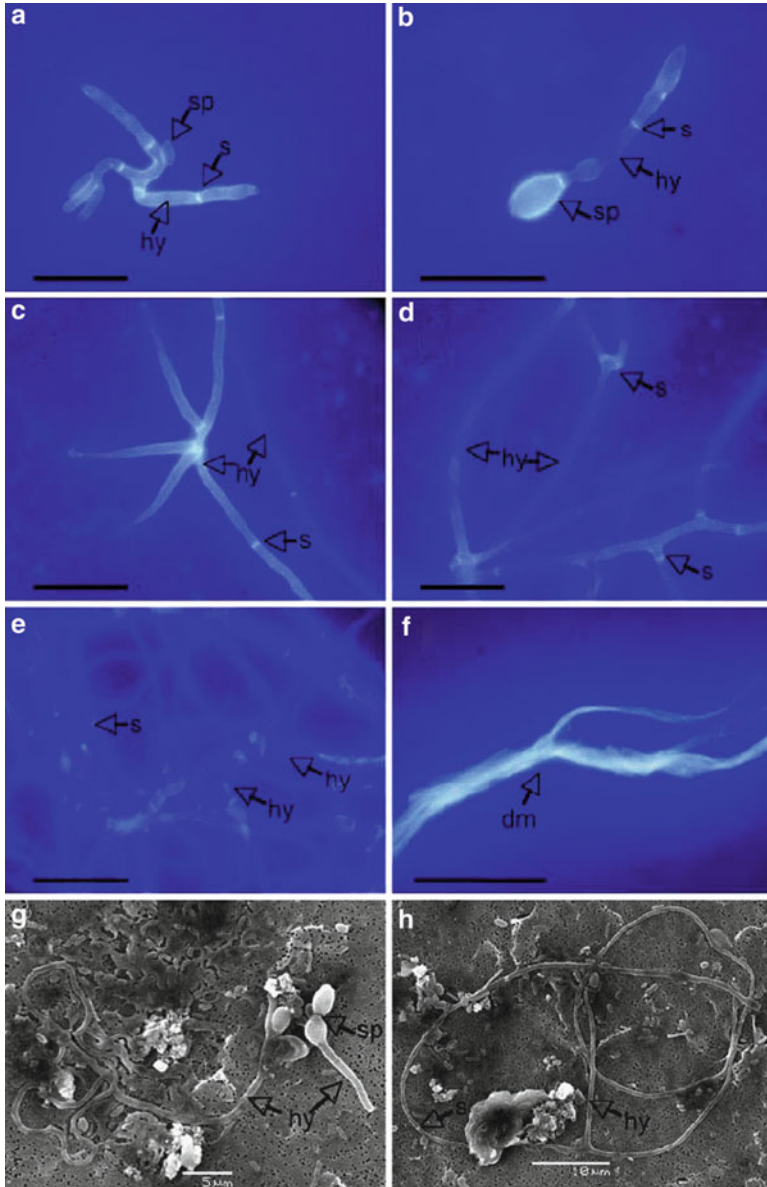
Fig. 4.1 Fungal mycelia detected by epifluorescence (**a–e**) and scanning electron microscopy (**g**, **h**) in the water column of the coastal upwelling ecosystem off central Chile during austral spring of 2008 (October) and summer of 2009 (early March). Fungal structures were clearly distinguished from detrital material (**f**). Scale bars correspond to 10 μm. *sp* spores; *hy* hyphae; *s* hyphal septa; *dm* detrital material (Gutierrez et al. 2011)

staining method. These filaments or aggregates can reach up to 20 μm in diameter and over 50 μm long, similar to fungal mycelia detected in deep-sea sediments in the Indian Basin (Damare and Raghukumar 2008) and water-stable aggregates associated with mycorrhizal fungi in soils (Tisdall and Oades 1982). Because organic aggregates in seawater represent a major growth habitat for planktonic microbial communities, the combined metabolic activities of fungi and prokaryotic microbes can promote a highly efficient mineralization of particulate organic matter to dissolved organic matter in seawater (Kiorboe and Jackson 2001; Gutierrez et al. 2010). Furthermore, fungal biomass displays spatial and temporal variations in the coastal marine ecosystems (Fig. 4.2). Fungal biomass ranged from 0.03 to 0.12 μg C/l of seawater in the top 15 m with a decreased trend toward bottom water to values between 0.006 and 0.01 μg C/l of seawater in the coastal upwelling ecosystem off central Chile during the winter (Gutierrez et al. 2010). During the summer, fungal biomass in the coastal water can reach ~6 μg C/l of seawater at the depth of 7 m and range from 0.19 to 0.44 μg C/l of seawater below 30 m (Fig. 4.2). In addition, the vertical profile of fungal biomass concurred with those of chlorophyll *a* and physical parameters of the water columns (e.g., temperature and oxygen) (Fig. 4.2c, d). Hence, like heterotrophic microplankton, fungi are active in the water column and respond to primary production activity and organic matter availability (Gutierrez et al. 2010). Further investigation of the biological activities of planktonic fungal communities could significantly improve our understanding of ecological function of marine microbial communities in the ocean carbon cycling and decipher the potentially important role of fungi in the current model of marine microbial food web.

4.4   Diversity of Culturable Fungi in Seawater

Because of the unique physio-chemical environments in seawater, planktonic fungi can be quite distinct from those occurring in the marine substrata. Several marine fungi, including *Antennospora quadricornuata*, *Arenariomyces* spp., *Corollospora* spp., and *Torpedospora radiate*, are commonly found in coastal and oceanic waters (Jones and Hyde 1990; Jones 2000). However, some fungi, such as *Lignincola laevis* and *Periconia prolifica*, occur in both seawater and substrata (i.e., mangroves). Because of their adaption to the oceanic environments, planktonic fungi (e.g., *Halosphaeriales*) can grow under submerged conditions and usually have asci that deliquesce early and release their ascospores passively (Fazzani and Gareth Jones 1977). They even produce ascospores with elaborate appendages, which enhance their floatation, impaction and attachment to planktonic particles (Jones 2000).

Compared with fungi in other marine habitats, the fungal populations in seawater are poorly investigated. As of now, over 186 fungal species have been isolated from seawater and are members of the *Asomycota*, *Basidiomycota*, and *Zygomycota* phyla (Kutty and Philp 2008; Roth et al. 1964; Steele 1967; Zhang et al. 1989). The first extensive investigation of fungal populations in seawater occurred in the
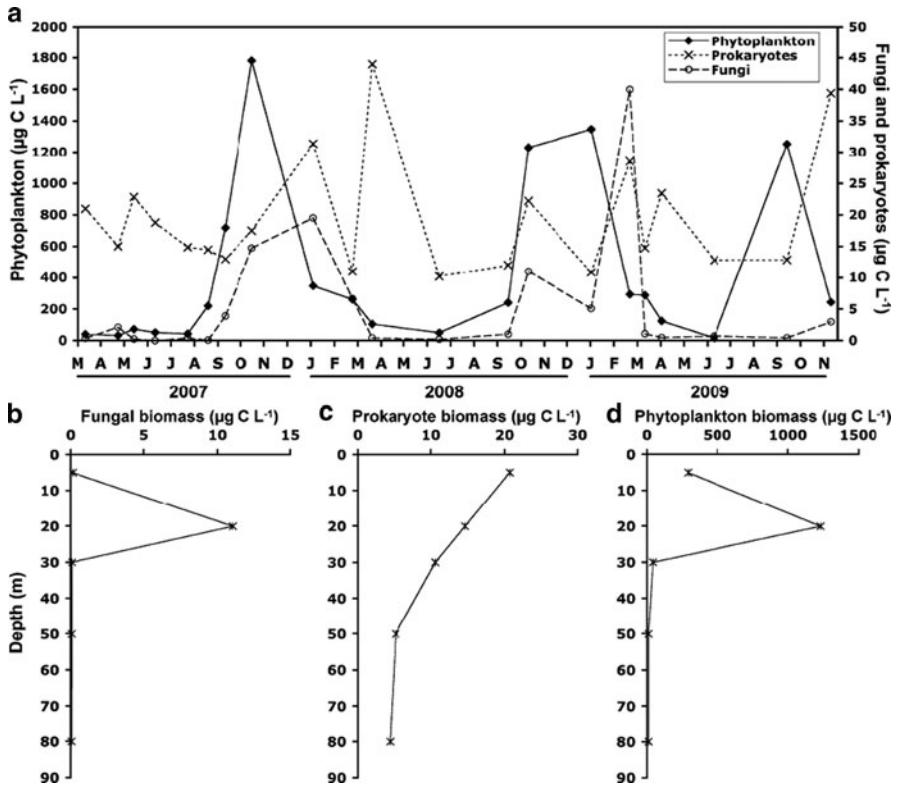
Fig. 4.2 Temporal variation of phytoplankton, fungal and bacterial biomass in surface waters (**a**) and vertical biomass profiles of fungi (**b**), prokaryote (**c**) and phytoplankton (**d**) during austral spring (October) of 2008 at station 18. The coefficient of variation of duplicate samples was less than 20% for fungal biomass (Gutierrez et al. 2011)

northwestern subtropical Atlantic Ocean using cultivation methods (Roth et al. 1964). All eulittoral water samples contained relatively high fungal populations with a minimum of ten different fungal species, ranging from 187 to 296 colonies per liter. Water samples obtained more distant from land and in deep waters displayed a more than tenfold decrease in average fungal population density and about a 50% reduction in the number of species. A similar trend was observed with progression into abyssal regions. Fungi were found in 80% of the 227 water samples collected from offshore stations (i.e., at least 3 miles from land), with most of these yielding less than five viable propagules per liter and giving rise to three or fewer fungal species. The deepest sample, which yielded *Deuteromycetes* (also called the Fungi Imperfecti because they have no sexual reproduction), was collected at the depth of 4,450 m. Three genera, *Cladosporium* spp., *Alternaria* spp., and *Nigrospora* spp., were the dominant fungi in eulittoral and oceanic waters. Both *Aureobasidium pullulans* and *Dendryphiella arenaria* were commonly present in

seawater samples collected in this region. In another study of fungi in the waters of the Hawaiian Islands and elsewhere in the central Pacific (Steele 1967), all sample stations yielded fungal colonies. Of the 59 water samples, 44 of them yielded 50 colonies or less and the remainder gave count of over 50. The average number of fungal isolates from these samples ranged from 0.06 to 3.94 isolates per milliliter with the average of 0.14 per sample. The number of species ranged from 1 to 17 per sample. A total of 126 species representing 59 genera were isolated from these samples. Generally, coastal waters yielded a higher number of isolates than either the surf or oceanic waters; oceanic waters gave a very low average number of isolates. Furthermore, *Aureobasidium pullulans* and *Rhodotorula* spp. were commonly found in oceanic waters and also among those predominant in all isolations from water. In the South China Sea, water samples were collected from 24 stations between 6°02′03″–11°00′11″N and 112°24′55″E at the surface and at depths of 25 m and 100 m. Culturable fungi derived from the membrane filtration method contained mainly marine yeasts, but very few filamentous forms (Vrijmoed et al. 1993). The numbers of colony forming units (CFUs) varied among the stations for both filamentous and yeast forms. The fungal counts (CFUs per 100 ml seawater) decreased with increasing depth with average values of 196, 123, and 104 CFUs per 100 ml seawater in water samples collected at the surface and at the depths of 25 and 100 m, respectively. Among the cultural fungi, yeasts were the dominant populations at each individual depth and present at almost all three depths of all the stations and only water samples collected at two stations at 100 m did not yield yeast forms. The yeast counts decreased with increasing depth with the average counts of 196 CFUs per 100 ml seawater collected from the surface and 104 CFUs per 100 ml seawater collected at 100 m. On the other hand, the filamentous forms were much lower in numbers and were identified at both the surface and at 25 m collected from 11 stations (45.8%), and at 100 m from 9 stations (37.5%). Filamentous forms of these cultural fungi were dominated by *Aspergillus* spp. and *Cladosporium* spp. with many unidentified mycelial and non-sporulating state. Furthermore, fungi were also identified in the water samples collected from the ocean area of the Northwest Antarctic Peninsula (Zhang et al. 1989). Finally, a temporal study of fungal population dynamics in the Lagos lagoon of Nigeria (north of the Gulf of Guinea, Atlantic Ocean) revealed the seasonal variations of fungal populations (Akpata and Ekundayo 1983). The increased fungal populations were observed in the rainy season (March–October) when salinity was low and the concentration of organic matter was high. The low fungal populations in the lagoon occurred in the dry season (November–February) when salinity was generally high and the concentration of organic matter was low. All the isolated fungi had terrestrial origins with *Aspergillus* spp. and *Penicillium* spp. as the dominant fungal populations. Fungal populations in the lagoon were negatively correlated with water turbidity. Existing studies revealed higher yeast populations in the seawater samples than those of filamentous forms of fungi in the seawater samples (Vrijmoed et al. 1993; Kutty and Philp 2008). Generally, populations of planktonic fungi decrease with increased distance from land and increased depths, with certain species dominating in heavily contaminated seawaters. As such, some of the

planktonic fungi could merely be contaminants from terrestrial sources, surviving passively in the water column. Nevertheless, several lines of evidence suggest the existence of indigenous marine fungi in the water columns of the world's oceans (Gao et al. 2008).

Fungal growth in seawater is influenced by various physio-chemical parameters. Temperature, organic matter content, and total ionic concentration influence the population of *Saprolegniacea* in tropical waters (Ritchie 1959). *Zygomycetes* (formerly called *Phycomycetes*) were only observed in estuarine waters of low salinity (<0.5%) (Johnson and Sparrow 1961). The diversity of culturable marine fungi is likely controlled by a wide range of biological, chemical, and physical factors (Jones 2000). Because the cultured fungi only represent a small fraction (less than 5%) of all fungal communities, the intrinsic relationship between physio-chemical environmental factors and planktonic fungi is largely unknown.

4.4.1 Diversity of Uncultured Fungi in Water Column

Molecular methods have been commonly used to reveal or survey uncultured microbial communities. These methods combine PCR-amplified ribosomal RNA gene (or spacer regions) sequences with screening strategies (e.g., DGGE) for quick assessment of diversity and identification of molecular phylotypes in environmental samples (Pang and Mitchell 2005). These methods were originally developed for the assessment of bacterial communities and then adapted for the analysis of fungal communities. Indeed, the recent development and application of molecular methods have significantly advanced our understanding of the diversity and ecology of fungal communities in terrestrial habitats (e.g., soils). Application of molecular approaches enables unculturable fungal communities to be directly detected from environmental samples. The most commonly used molecular methods include, but are not limited to, rRNA clone library construction, terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), amplified rDNA restriction analysis (ARDRA), and amplified ribosomal intergenic spacer analysis (ARISA) (Gao et al. 2008). These methods have been mainly used to analyze fungal communities associated with marine substrata and yielded novel fungal diversity (Pang and Mitchell 2005). Application of these methods for the analysis of fungal communities in seawater is rare.

The first molecular analysis of fungal communities in water columns was carried out for water samples collected from the Hawaiian coast (Gao et al. 2010). In that analysis, DGGE, in conjunction with clone library construction, revealed interesting spatial diversity and novel phylotypes of planktonic fungi. Mycoplankton communities displayed distinct lateral and vertical variations in their diversity and composition. Nearshore waters had greater diversity and species richness than those of oceanic water samples (Fig. 4.3). However, no difference in the diversity of planktonic fungal communities was observed among
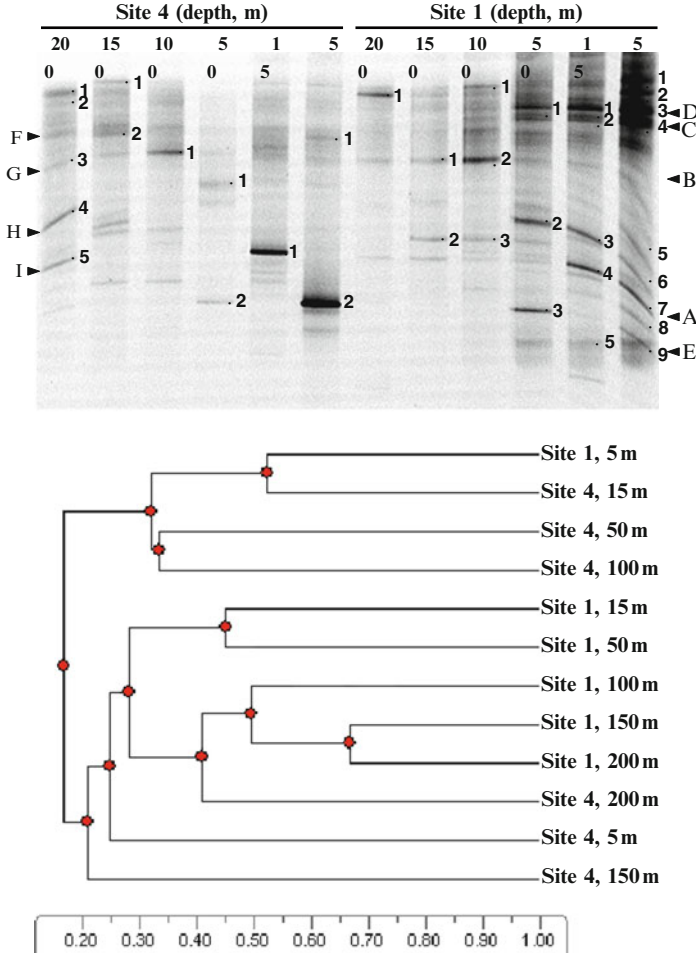
Fig. 4.3 DGGE analysis of planktonic fungi derived from the coastal waters of the island of Oahu (**a**). DGGE bands cloned are side-labeled with dot and number. UPMA tree based on matrix of distance calculated from the difference in DGGE band patterns among water samples from sites 1 (coastal) and 4 (open ocean) (**b**) (Gao et al. 2010)

nearshore and open ocean stations for water samples from the depths below 150 m. Interestingly, DGGE analysis of water samples from the coastal zone off central Chile revealed the similar trend of lateral variations with higher number of fungal phylotypes at surface of coastal stations than in offshore areas (Gutierrez et al. 2010) (Fig. 4.4). Of the 46 identified fungal phylotypes in the Hawaiian coastal waters, 42 are members of *Basidiomycota* and the rest belong to *Ascopmycota* (Fig. 4.5). All the ascomycete phylotypes are members of the class *Dothideomycetes*. In particular, one of the ascomycete phylotypes was
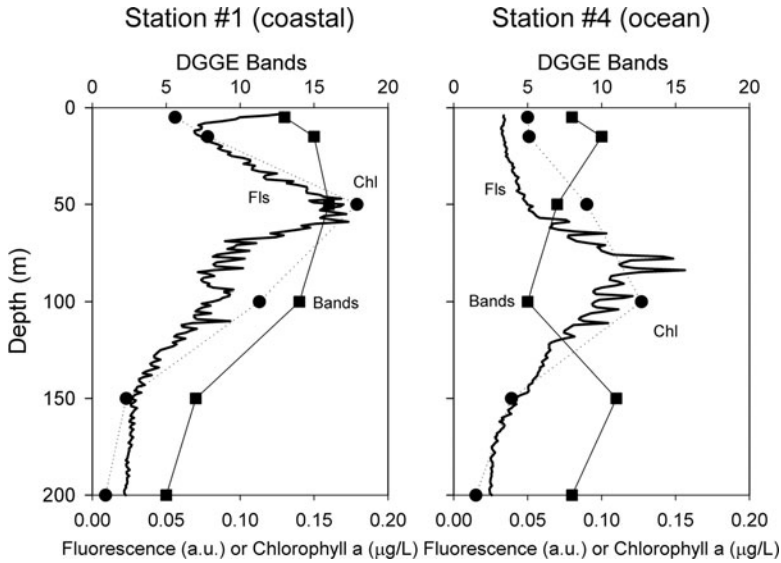
Fig. 4.4 Vertical profile of DGGE band numbers, fluorescence, and chlorophyll-*a* of Hawaiian waters (Gao et al. 2010)

closely matched to the marine yeast *Aureobasidium pullulans*, a complex commonly found in marine environments. The majority (93%) of basidiomycete phylotypes were not affiliated with any member of the described fungal taxa and are likely to be new fungal phylotypes (Fig. 4.5). Furthermore, the results of this study suggest that high primary production likely supports high population of fungal communities and plays a more dominant role in determining mycoplankton diversity in deep-water samples than in shallow areas (Figs. 4.2 and 4.4). Biological, geochemical, and chemical factors, which govern the diversity and composition of mycoplankton in the nearshore waters, may differ from those in the open ocean (Fig. 4.4). Because of the dynamic nature of ocean ecosystems, the relationship between planktonic fungi and their physio-chemical environments can be very complicated. Overall, the rich information on mycoplankton derived from molecular analysis confirms the significance and necessity for the further exploration of marine mycoplankton in the world's oceans. At the same time, these molecular studies clearly indicate that the development of fungal primers with a satisfactory degree of success in discriminating against other marine organisms while maintaining a broad range of compatibility with planktonic fungal communities can significantly benefit the study of unculturable fungi in water column. Novel fungal phylotypes have been detected from the deep-sea waters (3,000 and 4,000 m) with yeasts as the predominant forms (Bass et al. 2007). However, molecular surveys indicate that fungi are relatively rare in the deep-sea habitats (Bass et al. 2007; Raghukumar et al. 2010).
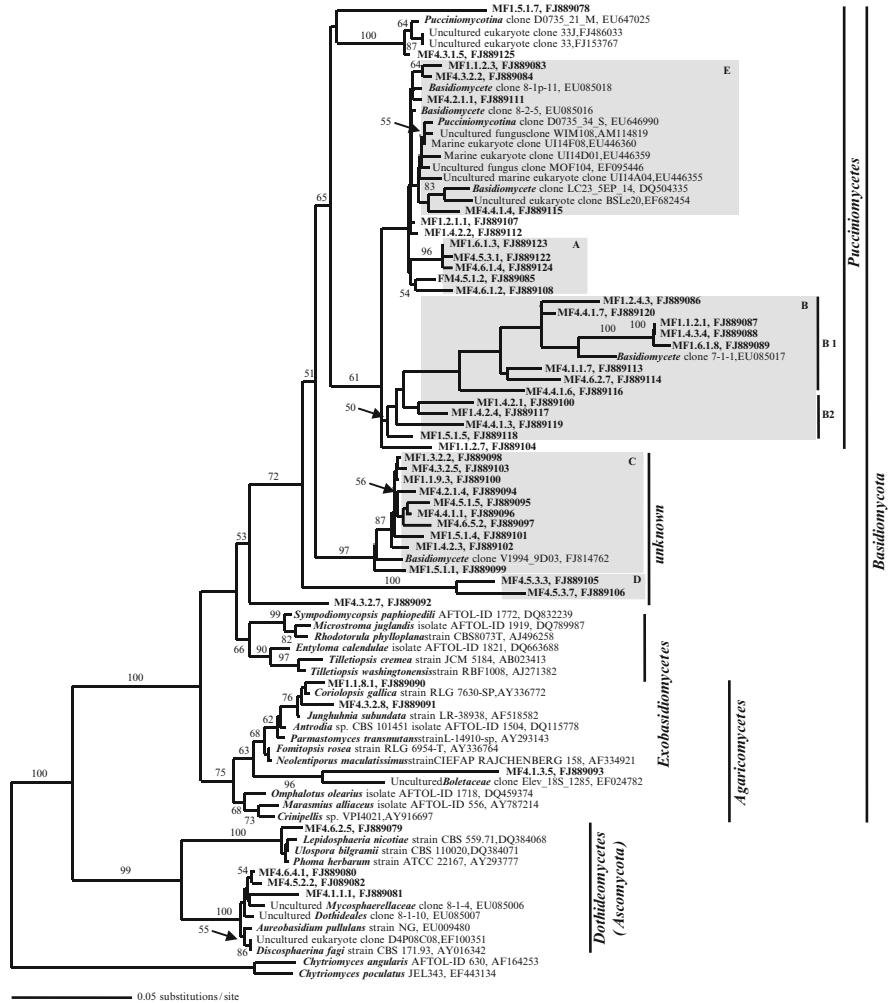
Fig. 4.5 Neighbor-joining tree based on fungal 18 S rRNA gene sequences derived from Hawaiian waters (Gao et al. 2010)

4.5 Biogeochemical Functions of Planktonic Fungi

The ecological importance of fungi in the marine ecosystems is often underestimated or ignored completely, and yet they represent a diverse range of saprobes, pathogens, and symbionts that form an integral part of coastal systems (Newell 1994; Hyde et al. 1998). Of their many ecological functions, marine fungi are best known to be major decomposers of woody and herbaceous substrates in coastal marine ecosystems (Hyde et al. 1998). However, these functions are largely

derived from marine substratum studies or in vitro data and remain to be proven in the water column (Fell and Newell 1998; Raghukumar 2004).

4.5.1 Nutrient Cycling

Fungal distribution in marine ecosystems is largely dependent on the quality and availability of organic carbon (Clipson et al. 2006). Fungi are well known to secrete a battery of hydrolytic enzymes with particular capability to degrade and recycle organic matter, including recalcitrant materials that are less suitable for mineralization by bacteria and other saprotrophic groups. It should be recognized that bacteria and fungi can occupy different ecological niches in detritus (Newell 1994; Raghukumar 2005). Bacteria live on the surface of particulate detritus and have high substrate affinity with the capability to utilize even low concentrations of nutrients and to multiply quickly (Raghukumar 2005). Furthermore, small detrital particles of high surface to volume ratios tend to support more bacteria than fungi. Although filamentous fungi grow more slowly when compared to bacteria, they indeed have the ability to penetrate relatively persistent particulate detritus much more efficiently than the latter by virtue of their filamentous habit and apical growth (Raghukumar 2005). Fungi, including filamentous *Oomycetes*, are particularly adept at utilizing large lignocellulose-predominated substrates of high C:N ratio. The unicellular yeasts are considered to occupy a similar niche as bacteria and live on the surface of particles, or even in the presence of high concentrations of dissolved organic matter (DOM) (Raghukumar 2005). Fungal biomass was significantly greater than bacteria on leaves and wood, confirming the predominance of fungal biomass in larger sized organic matter in freshwater detrital ecosystems (Findlay et al. 2002).

Fungi have generally been considered unimportant in open water detrital processes. Several lines of evidence indicate that thraustochytrids fungi play a major role in detrital dynamics of water columns (Raghukumar 2002; also, refer the corresponding chapter on thraustochytrids). Open ocean waters are mostly low in available organic matter with low fungal biomass. Nevertheless, open ocean waters can have relatively high levels of organic matter when algal blooms occur. Little effort has been made to study fungal biomass of open ocean waters under the algal bloom conditions. On the other hand, coastal seawaters contain much higher biomass availability and complexity, particularly in the coastal areas with highly productive habitats such as salt marshes and mangroves. Application of the fungal sterol, ergosterol, in the quantification of fungal biomass associated with decaying plant liter and the technique for measuring in situ instantaneous growth rates of fungi from rates of [^{14}C]acetate incorporation into ergosterol have yielded rich information on the assessment of the magnitude of fungal contributions to the cycling of carbon and the flow energy in freshwater ecosystems (Gulis et al. 2006). These studies indicated that fungi are a significant decomposer of particulate detritus and play a key role in detrital food webs of freshwater ecosystems. It would

not be surprising to find that fungi play a similar ecological role in the marine detrital food webs using these approaches. Indeed, direct detection in conjunction with microscopic counting indicates that fungal biomass in surface waters of the upwelling ecosystems off central-southern Chile is comparable to that of prokaryotes (Bacteria plus Archea) during active upwelling period (Gutierrez et al. 2011). In general, fungi can convert plant carbon into fungal biomass and also into CO_2 as a result of their respiratory activities (Gessner et al. 2007). Additionally, fungal decomposition of plant liter and feeding activities of detritivore consumers (invertebrates) can facilitate in the export of plant carbon as either fine particulate organic matter (FPOM) or dissolved organic matter (DOM) (Gulis et al. 2006). The role of fungi in the biogeochemical cycle of nitrogen has been grossly underestimated and their biomass can account for virtually all of the nitrogen present in decaying standing plant biomass in salt marshes (Newell 1996). Overall, studies of planktonic fungi in nutrient recycling are rare and would be an interesting research topic for future study.

4.5.2 Marine Microbial Food Webs

Large populations of fungal cells exist in coastal seawaters. Several lines of evidence support that planktonic fungi play an important role within the microbial food web in the coastal oceans (Gao et al. 2008). First, fungi have been reported as parasites of large marine phytoplankton (e.g., diatoms and dinoflagellates) (Johnson and Sparrow 1961; Drebes 1974; Raghukumar 1986; Elbrachter and Schnepf 1998; Ramaiah 2006; Wang and Johnson 2009). Some of these parasitic fungi can infect one and/or multiple marine phytoplankton species, up to 75% of the total natural marine phytoplankton populations (Tillmann et al. 1999). The fast-growing fungal parasites can consume the major part of the host phytoplankton within the water column, imposing a large impact on trophic web structure by directly contributing to phytoplankton lysis or death (Walsh 1983). Therefore, fungal parasites can be successful competitors against zooplankton in controlling energy flow and food web dynamics (Tillmann et al. 1999). Second, infection by planktonic fungal parasites can be important in regulating the dynamics of algal blooms. For example, parasitic *oomycetes* and/or chytrids have been observed to infect *Pseudo-nitzschia multiseries* and *P. pungens* cells in Cardigan Bay of Eastern Prince Edward Island (PEI), Canada (Bates et al. 1998; Elbrachter and Schnepf 1998). Lastly, zoospores of chytrid fungi and fungal-like *oomycetes* contain large amount of polyunsaturated fatty acids (PUFAS), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA), with a high cholesterol concentration (Raghukumar et al. 1994; Raghukumar 2002; Kagami et al. 2007). Spores of these fungi (2–3 μm) are well within the preferred range of food particle size and can be grazed efficiently by zooplankton (Geller and Müller 1981; Kagami et al. 2007). Evidence accumulating from some investigations supports the idea that these spores are food of good quality for zooplankton (Müller-Navarra et al. 2000). During an epidemic, the

density of these spores can be on the same order of magnitude or even more than the density of edible phytoplankton cells (i.e., several thousands per milliliter) in freshwater ecosystems (Kagami et al. 2007). Hence, planktonic fungi may enhance the growth and reproduction of some zooplankton, especially when large inedible phytoplankton species dominate the marine phytoplankton community (Kagami et al. 2007).

4.6 Conclusion

Evidence accumulating from culturable and molecular analyses clearly indicates that large populations of fungi with novel diversity exist in the water columns of the world's oceans. These planktonic fungi display interesting temporal and spatial (lateral and vertical) variations. Higher diversity and a greater abundance of fungi are found in the surface and coastal waters than in open-ocean and deep water samples. The diversity and abundance of planktonic fungi positively correlate with the primary production and availability of organic matter. The physio-chemical parameters of seawater (e.g., temperature and salinity) also impact the distribution of planktonic fungal communities. Several lines of evidence also support the idea that planktonic fungi may play significant role in microbial food webs through parasitism of marine phytoplankton. Given the significant contribution of fungi in nutrient (e.g., carbon and nitrogen) cycling of freshwater ecosystems, fungi may play an important role in ocean nutrient cycles, particularly in the detritus marine ecosystems (e.g., coastal waters). Overall, our understanding of the diversity and functional aspects of planktonic fungi is rather limited. Further investigation of this important, but largely understudied, eukaryotic microbial group will surely contribute to the global efforts to understand the role of microbes in ocean carbon cycling as well as global climate changes.

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Chapter 5
Fungi and Their Role in Corals and Coral Reef Ecosystems

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Contents

Abstract Fungi in coral reefs exist as endoliths, endobionts, saprotrophs and as pathogens. Although algal and fungal endoliths in corals were described way back in 1973, their role in microboring, carbonate alteration, discoloration, density banding, symbiotic or parasitic association was postulated almost 25 years later. Fungi, as pathogens in corals, have become a much discussed topic in the last 10 years. It is either due to the availability of better tools for investigations or greater awareness among the research communities. Fungi which are exclusive as endoliths (endemic) in corals or ubiquitous forms seem to play a role in coral reef system. Fungi associated with sponges and their role in production or induction of

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secondary metabolites in their host is of primary interest to various pharmaceutical industries and funding agencies. Fungal enzymes in degradation of coral mucus, and plant detritus hold great promise in biotechnological applications. Unravelling fungal diversity in corals and associated reef organisms using culture and culture-independent approaches is a subject gaining attention from research community world over.

5.1 The Coral Reef Ecosystem

Coral reefs, oases in the blue deserts of the ocean, are home to colourful, hard and soft corals, sponges, a diverse population of fishes, holothurians, calciferous algae and other myriad communities (Connell 1978). They support tourism, food production and coastal protection from natural hazards. Their diverse life forms have vast biotechnological potentials. Corals fall in two categories: (1) the hard reef-building or hermatypic corals and (2) the soft or ahermatypic corals. The reef-building scleractinian corals (hard corals) possess calcium carbonate skeletons and are responsible for building reefs. They harbour symbiotic dinoflagellates *Symbiodinium*, also called zooxanthellae. The soft corals lack the calcium carbonate skeletons and lack symbiotic zooxanthellae.

Corals offer a diverse and organically rich environment for microorganisms. These are mucus layer on the coral, coral polyps, gastroderm cavity, gastrovascular spaces, organic matrix of the skeleton and calcium carbonate skeleton (Fig. 5.1). Corals with their microbial community of viruses, bacteria, archaea, cyanobacteria, fungi and endolithic algae form complex holobionts in the tropical nutrient-limited waters (Bourne et al. 2009). The symbiotic algae are a major source of crucial nutrients for scleractinian corals, while the microbes form mutualistic and
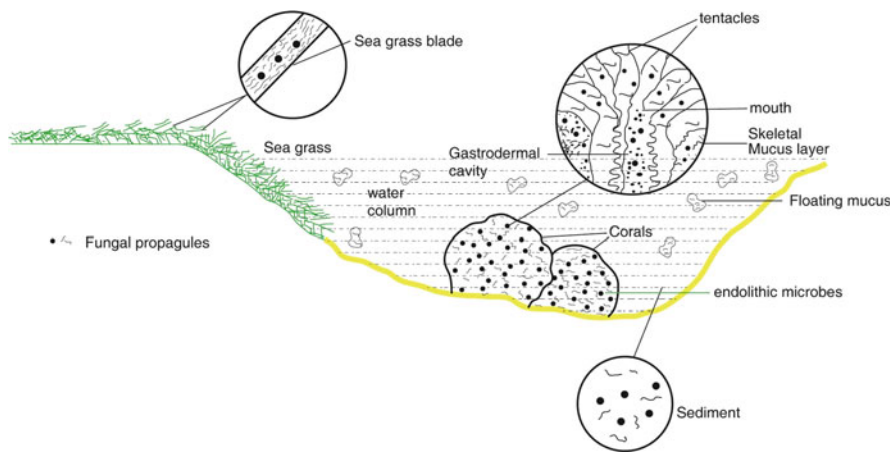
Fig. 5.1 Schematic diagram showing fungal distribution in corals and reef ecosystem in a coral reef lagoon

occasionally parasitic associations. Bacterial diversity associated with different coral species (Bourne and Munn 2005; Kvenefors et al. 2010), mucus (Ritchie 2006), coral reef sediments and water column (Van Duyl and Gast 2001) has been studied in detail. Its diversity in corals has been shown to differ spatially (Olson et al. 2009; Littman et al. 2009). Bacterial diversity has been studied using culture-dependent (Sabdono and Radjasa 2008) and culture-independent approaches (Rohwer et al. 2001; Cooney et al. 2002; Vega Thurber et al. 2009). A varied bacterial diversity in healthy, bleached (Bourne et al. 2008) and diseased corals (Sekar et al. 2008; Sunagawa et al. 2009), and as a response to various pollutants (Kuntz et al. 2005; Smith et al. 2008), has been reported. It is hypothesized that environmental stressors that affect the host physiology will have an impact on microbial community (Ainsworth et al. 2010). Archaeal diversity associated within a single coral species (Wegley et al. 2007), in three species of scleractinian corals from geographically different locations (Siboni et al. 2008; Lins-de-Barros et al. 2010), in healthy and stressed corals (Vega Thurber et al. 2009), in surface microlayer of corals (Kellogg 2004) has been documented in detail by sequencing the 16S rRNA gene. Diversity of cyanobacteria in healthy and diseased corals is also known to a certain extent (Frias-Lopez et al. 2003). After the initial study of fungal diversity in different species of corals (Kendrick et al. 1982) there are increasing reports of fungi in corals, both by culture-dependent and culture-independent methods (Ravindran et al. 2001; Wegley et al. 2007). We attempt to review the fungal diversity in corals and coral reefs and discuss their possible ecological role in this ecosystem.

5.2 Fungal Diversity in Coral Reef Ecosystems

5.2.1 Autochthonous (Indigenous or Native) Coral Fungi

The generally accepted broad ecological definition of marine fungi is that the obligate marine fungi grow and sporulate exclusively in a marine or estuarine habitat, and those from freshwater or terrestrial milieus that are able to grow and also sporulate in the marine environment are termed facultative marine fungi (Kohlmeyer and Kohlmeyer 1979). The best method to determine if a fungus is truly marine or not is to collect pieces of substrate from marine environment and inspect them for the presence of fungal hyphae or fruiting structures (Hyde and Pointing 2000). Based on this method, obligate marine fungi native to corals belonging to the class Ascomycetes were reported from corals (Kohlmeyer and Volkmann-Kohlmeyer 1992). Most of these fungi were reported from coral reefs off the coast of Belize (Central America). Among these, the genus *Koralionastes* with five species was placed in a new family Koralionastetaceae (Kohlmeyer and Volkmann-Kohlmeyer 1987). These five species were found attached to coral slabs

in shallow water and were associated with crustaceous sponges. *Koralionastes angustus* and *K. giganteus* are reported from Belize, *K. ellipticus* and *K. ovalis* from Belize and Australia and *K. violaceus* from Australia and Fiji (Kohlmeyer and Volkmann-Kohlmeyer 1990). However, these fungi were not cultured and these authors suggested that they obtain their nutrition through close association with encrusting sponges (see Kohlmeyer and Volkmann-Kohlmeyer 2003). It is worth pursuing whether these fungi show any host specificity. There is a need for systematic survey and also to develop special culturing methods and media for obtaining such fungi in cultures.

5.2.2   *Allochthonous (Non-native) Coral Fungi*

In contrast to native fungi, several marine-derived terrestrial fungi find home in corals. The organic matrix of the carbonate skeletons is utilized by fungi to grow inside corals as endolithic saprotrophs. Kendrick et al. (1982) isolated saprotrophic fungi from several live hard corals from the Caribbean and the South Pacific using common mycological culture media (Table 5.1). These were identified to be common terrestrial fungi. The bioeroding and boring nature of two of these fungi, *Aspergillus versicolor* and *Penicillium stoloniferum*, was demonstrated by inoculating them in the coral *Siderastrea siderea*. The corals were incubated in sea water for 28 days and fungal boring was demonstrated by resin-cast method. In this technique, the dehydrated coral specimens are infiltrated with resin. After heat-induced polymerization, the carbonate is removed with HCl and the resin-cast borings are studied by scanning electron microscopy. Using the same technique, the presence of endolithic black septate fungi in skeletons of hard corals was demonstrated (Le Campion-Alsumard (1979), Le Campion-Alsumard et al. (1995a), Bentis et al. (2000) and Golubic et al. (2005). These fungi were neither cultured nor identified. *Aspergillus*-like conidiophores of endolithic fungi were observed in the scleractinian coral *Porites lobata* from Mayotte Island, Mozambique channel in the Indian Ocean (Priess et al. 2000) and in the same coral species on the island Moorea in the French Polynesia (Golubic et al. 2005). Bak and Laane (1987) observed a dark mycelial fungus with perithecia, probably belonging to Ascomycetes in black bands of *Porites* species in the eastern part of the Indonesian Archipelago. Partial dissolution of such black bands with EDTA (ethylenediamine tetracetic acid) revealed the presence of branched filaments. Sections of five species of massive corals from the Andaman Islands in the Bay of Bengal revealed the presence of a septate dark brown mycelial fungus on the subsurface of the dead patches (Raghukumar and Raghukumar 1991). The fungus formed a distinct dense brown to black zone of 0.5–1.5 cm width immediately below the surface layer of the corals. The fungus was isolated after partial dissolution of the coral skeleton with EDTA and a pure culture was obtained. It was identified to be a *Scolecobasidium* sp. It was isolated from several coral species showing necrotic patches in the Lakshadweep Island in the Arabian Sea as well (personal observation). Ravindran et al. (2001) also isolated

Table 5.1 Bioeroding saprotrophic fungi associated with various coral species (modified from Kendrick et al. 1982)

Host species	Location	Fungal species
Acropora sp.	Lizard islands Great Barrier Reef (GBR), Australia	*Aspergillus versicolor, Hormonema dematioides, Pithomyces chartarum*, sterile hyaline mycelium with swellings
	Heron islands, GBR	Sterile dark mycelium with swellings
A. hyacinthus	Heron islands, GBR	*A. versicolor, Humicola alopallonella*
A. palmata	Barbados, West Indies	*Aspergillus sydowii, Penicillium expansum, P. stoloniferum*
A. palifera	Lizard islands GBR	Sterile dark mycelium with swellings
	Heron islands, GBR	*Bipolaris rostrata*
Diploastrea heliopora	Lizard islands, GBR	*Aspergillus restrictus, Asteromella sp., Phialophora bubaki*
	Rarotonga, Cook islands	*Hormonema dematioide*
	Heron islands, GBR	Sterile hyaline mycelium with swellings
Diploria labyrinthiformis	Barbados, West Indies	*P. stoloniferum*
Goniastrea retiformis	Lizard islands, GBR	*A. versicolor, Phialophora bubaki*
	Rarotonga, Cook islands	*Hormonema dematioides*
G. australensis	Heron islands, GBR	*Paecilomyces lilacinus*, sterile hyaline mycelium with swellings
Meandrina meandrites	Barbados, West Indies	*Acremonium* sp.
Montastrea annularis	Barbados, West Indies	*A. sydowii, Cladosporium sphaerospermum*
M. cavernosa	Barbados, West Indies	*Paecilomyces godlewski*
Porites sp.	Rarotonga, Cook islands	*Cladosporium sphaerospermum, Wallemia ichthyophaga*, sterile dark mycelium with swellings
P. australensis	Lizard islands, GBR	*A. versicolor, Phialophora bubaki*
	Rarotonga, Cook islands	*Hormonema dematioides*
	Heron islands, GBR	*Penicillium restrictum*
P. porites	Barbados, West Indies	*A. sydowii, Penicillium avellaneum*
Stylophora pistillata	Rarotonga, Cook islands	*Hormonema dematioides*

common terrestrial fungi from healthy, pink-line syndrome-affected, bleached and dead (devoid of polyps) patch of *Porites lutea* from the Lakshadweep Island in the Arabian Sea (Table 5.2). The presence of two of these fungi in the coral skeleton was confirmed using polyclonal immunofluorescence probes. *Conidia*-like structures were observed within the coral skeleton by the authors. Besides isolation, they also observed fungal filaments directly in coral skeleton down to a depth of 3–7 cm in the coral sections of ~1 cm thickness. Fungal biomass constituted up to a 0.05% of the weight of the corals. Several ascomycetous fungi were isolated and obtained in pure culture from healthy and diseased branched coral, *Acropora formosa* (Yarden et al. 2007). These fungi were identified using internal transcribed spacer (ITS) sequences along with morphological features (Table 5.2). In both the

Table 5.2 Fungi in corals

Host	Location	Fungal species	State of the host/location in the host	References
Montastrea annularis	Venezuelan Reefs	Non-septate branched hyphae, possibly a lower marine fungus	Diseased showing black band, hyphae/epidermis, gastrodermis and mesoglea	Ramos-Flores 1983
Porites spp.	Indonesian Archipelago	Dark haphae with perithecia, probably belonging to Halosphaeriaceae (Ascomycota)	Healthy corals with black bands in coral skeleton	Bak and Laane 1987
Montipora tuberculosa, Porites lutea, Goniopora sp.	Andaman islands, Bay of Bengal, Indian Ocean	*Scolecobasidium* sp.	Necrotic patches in the corals/below the subsurface of corals	Raghukumar and Raghukumar 1991
Porites lobata	Moorea islands, French Polynesia	Unidentified septate mycelia	In the pore spaces emptied by the polyps	Le Campion-Alsumard et al. (1995a, b)
Porites lutea and *P. lobata*	Mayotte islands, Mozambique Channel and Moorea islands, French Polynesia	*Aspergillus*-like fungus	Within the coral skeletons attacking endolithic algae	Priess et al. 2000
Porites lutea	Lakshadweep islands, Arabian Sea	Dark, orange and hyaline non-sporulating mycelial forms, *Acremonium* sp., *Fusarium* sp., *Aspergillus* sp., *Cladosporium* sp., Mycelial yeasts, *Labyrinthula* sp., *Chaetomium* sp., *Aureobasidium* sp.	Skeleton of healthy and pink-line syndrome- affected corals and from dead patches	Ravindran et al. 2001
Acropora formosa	Wistari and Heron Reefs, GBR	*Phoma* sp., *Alternaria* sp.	Healthy coral	Yarden et al. 2007
		Phoma sp., *Humicola fuscoatra, Cladosporium* sp., *Penicillium citrinum,*	With brown band	
		Phoma sp., *Fusarium* sp., *Aureobasidium pullulans, Alternaria* sp.	Skeletal eroding	
		Phoma sp., *Cladosporium* sp.	Brown band and skeletal eroding	

Acopora sp., *A. secale*, *Porites* sp.	Lakshadweep islands, Arabian Sea	*Corallochytrium limacisporum*	Coral mucus	Raghukumar 1987
Coral, fresh coral mucus, floating and attached mucus detritus	Lakshadweep islands, Arabian Sea	*Corallochytrium limacisporum*, *Thraustochytrium motivum*, *Labyrinthuloides minuta*, *L. yorkensis*, *Ulkenia visurgensis*		Raghukumar and Balasubramanian 1991
Fungia granulosa	Gulf of Eilat, Israel	Thraustochytrid strain Fng1	Surface mucus	Harel et al. 2008
Various coral species	GBR, Australia	*Alternaria*, *Aspergillus* , *Beauveria* spp., *Dreschlera* spp., *Humicola* spp., *Monilia* spp., *Oididendron* spp., *Penicillium* spp., *Phialophora* spp., *Phoma* spp., *Torulomyces* spp., *Pestlotiopsis*-like sp., *Absidia* spp., unidentified fungus	Surface-sterilized segments	Morrison-Gardiner 2002

above instances (Ravindran et al. 2001; Yarden et al. 2007), alterations in fungal prevalence correlated with coral health. Report on fungi isolated from Australian Great Barrier Reef corals indicated the presence of several fungi, almost all of them being common terrestrial ones (Morrison-Gardiner 2002). There were 54 distinct fungal taxa indentified in addition to the presence of several non-sporulating fungi. Majority of the isolates were mitosporic fungi. Corals from near shore locations showed increased presence of fungi compared to that from offshore locations (Table 5.2). These results indicated that fungi, although terrestrial, are apparently an important component of corals.

5.2.3   Fungi in Other Reef Organisms

After the report of aspergillosis disease in the sea fan (soft coral) *Gorgonia ventalina* (Smith et al. 1996) caused by *Aspergillus sydowii*, a sudden spate of interest in mycoflora of this species is observed worldwide. Toledo-Hernández et al. (2007) isolated 15 species of terrestrial fungi from *Gorgonia ventalina* collected from Puerto Rico. These authors compared the methods of isolating fungi using two strategies: plating of tissue fragments of different sizes versus plating of homogenized tissue. The results showed that homogenization of small tissue fragments yielded better estimate of the fungal diversity. The fungi isolated were identified using ITS gene sequences and all of these were terrestrial fungi (Table 5.3). In a further study, Toledo-Hernández et al. (2008) compared fungal diversity in healthy and aspergillosis-affected *Gorgonia ventalina* collected from 15 reefs around Puerto Rico. These were identified by sequencing the nuclear ribosomal ITS region and by morphology. Thirty terrestrial fungal species belonging to 15 genera were isolated from 203 sea fan colonies (Table 5.3). Healthy sea fans harboured a greater fungal diversity than that of diseased ones. In another study, Koh et al. (2000) isolated 16 fungal genera and 51 species, including 2 yeasts, from 10 species of gorgonian corals in Singapore by culturing. Marine sponges collected from coral reefs of Palau and Bunaken Island, Indonesia harboured several filamentous fungi (Namikoshi et al. 2002). About 83% and 98% of the sponges collected from Palau and Bunaken Island, respectively, harboured fungi. Unfortunately, the authors have not provided the diversity of fungal species obtained in this study. These results indicate that fungi are residential flora within gorgonians too.

Seagrasses are major primary producers in coral reef ecosystems (Bakus et al. 1994), playing an important role in the food web. Seagrasses are angiosperms, capable of growing and flowering in submerged seawater of lagoons and intertidal regions. They form one of the major biological zones of coral reefs, responsible for nutrient recycling and creating sedimentary environment (Macintyre et al. 1987). *Thalassia hemprichii* is one of the dominant seagrasses in the Lakshadweep Islands

Table 5.3 Fungi associated with sea fan and sponges

Host	Location	Fungal genera	State of the host/location in the host	References
Several sponges (n = 10)	Fringing reef south of Singapore	*Acremonium strictum, A. aculeatus, A. cervinus, A. nutans, A. pulverulentus, A. flavus, A. ochraceus, A. ornatus, A. terricola, Cladosporium musae, C. sphaerospermum, Fusarium sp., Gliomastix cerealis, G. luzulae, G. murorum, Microascus triganosporus, Oidodendron griseum, Penicillium brevi-compactum, P. camemberti, P. canescens, P. citrinum, P. decumbens, P. frequentans, P. implicatum, P. janthinellum, P. lanoso-, P. lilacinum, P. notatum, P. oxalicum, Phoma-like, Scolecobasidium humicola, Stibella sp., Trichoderma pseudokoningii, T. hamatum, T. harzianum, T. koningii, T. longibrachiatum, Tritirachium sp., Verticillium sp., black yeast, white yeast, sterile isolates*	Healthy sponges	Koh et al. (2000)
Several sponges (n = 10)	Fringing reef south of Singapore	*Acremonium butryi, A. furcatum, A. strictum, A. aculeatus, A. foetidus var. pallidus, A. kangawensis, A. nutans, A. ficuum, A. flavus, A. ornatus, A. terricola, A. wentii, Chaetophoma sp., Cladosporium musae, C. sphaerospermum, Fusarium sp., Gliomastix cerealis, G. luzulae,*	Unhealthy sponges	Koh et al. (2000)

Table 5.3 (continued)

Host	Location	Fungal genera	State of the host/location in the host	References
		G. murorum, Hymenula sp., *Oidodendron griseum, Penicillium brevi-compactum, P. camemberti, P. canescens, P. citrinum, P. frequentans, P. implicatum, P. janthinellum, P. lilacinum, P. steckii, Scolecobasidium humicola, Sporotrichum* sp., *Trichoderma pseduokoningii, T. hamatum, T. harzianum, T. longibrachiatum, Tritirachium* sp., *Verticillium* sp., *Virgaria* sp., black yeast, white yeast, sterile isolates		
Several sponges	Coral reefs of Palau and Bunaken islands, Indonesia	Filamentous fungi (not identified)	Sponge tissue and fluid	Namikoshi et al. (2002)
Sea fan *Gorgonia ventalina*	San Juan, Puerto Rico	[a]*Aspergillus flavus/oryzae* (4,6), *A. ustus* (1,1), *A. niger* (0,1), *A. unguis* (1,2), *A. sydowii* (1,0), Xylariales (1,0), *Stachybortys chartarum* (1,0), *Cladosporium sphaerocarpum* (0,2), *Rhodotorula nympheae* (0,1), *Xylaria hypoxylon* (0,1), *Gloeotinia tremulenta* (1,0), *Penicillium citrinum* (0,3), *P. coffeae* (0,1), *P. citreonigrum* (1,1), *P. steckii* (0,1)	Healthy tissue	Toledo-Hernández et al. (2007)
Gorgonia ventalina	Puerto Rico[a]	[b]*Aspergillus aculeatus* (2,1,0), *A. flavus/ oryzae* (14,5,9), *A. melleus* (1,0,0), *A. niger* (3,0,2), *A. niger/foetidus/ awamori* (0,1,0), *A. ochraceus* (0,0,1),	Healthy tissue from healthy *G. ventalina*, healthy tissue from diseased sea fan and diseased tissue from diseased colony	Toledo-Hernández et al. 2008

A. sydowii (10,0,0), *A. tamarii* (2,0,1), *A. terreus* (3,2,1), *A. unguis* (1,0,0), *A. ustus* (4,1,0), *A. versicolor* (2,0,1), *Candida* sp. (2,0,0), *Chalaropsis* sp. (0,0,1), *Cladosporium* sp. (0,0,1), *C. cladosporioides* (3,3,1), *C. sphaerosperma* (0,1,0), *Davidiella tassiana* (2,0,0), *Gloetinia temulenta* (4,1,2), Helotiaceae (0,1,0), *Hypocrea lixii* (0,0,1), *Nectria* sp. *Bionectria* (0,0,1), *N. haematococca* (0,1,0), *Penicillium chrysogenum* (2,0,0), *P. citreonigrum* (0,1,0), *P. commune* (0,1,0), *P. minioluteum* (1,0,1), *P. citrinum* (23,1,4), *Pichia guillermondi* (1,0,0), *Rhodotorula nymphaeae* (1,0,0), *Stachybotrys chartarum* (1,0,0), *S. chlorohalonata* (0,1,0), *Trichoderma harzianum* (3,1,0), *Tritirachium* sp. (1,0,4), *Xylaria hypoxylon* (0,0,1), unknown (6,5,13)

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[a] Numbers within parenthesis indicate observed frequency per colony by plating tissue fragments and homogenized tissue respectively
[b] Numbers within parenthesis indicate number of isolates obtained by plating healthy tissue from healthy *G. ventalina* colony, healthy tissue from a diseased colony and diseased tissue from diseased colony respectively

Fig. 5.2 (**a**) Seagrass beds
exposed during low tide in
Kavaratti island of the
Lakshadweep Archipelago.
(**b**) Sea grass beds and
detritus on the same location

in the Arabian Sea (Jagtap 1998) and consequently it contributes enormously to
detrital material in the intertidal and shallow lagoons of these islands (Fig. 5.2).
Detrital material of this seagrass harboured nearly 3% of fungal biomass and 0.23%
thraustochytrid biomass (Sathe and Raghukumar 1991) playing an important role in
biogeochemical cycling of nutrients.

5.2.4   Straminopilan Fungi in Corals

Labyrinthulomycetes, comprising labyrinthulids, aplanochytrids and thrausto-
chytrids, belong to the Kingdom Stramenopila (Dick 2001). Despite being not
considered as true fungi, mycologists consider them as straminopilan fungi and
include them in marine mycology (Raghukumar 2002). Therefore, they are
included in this review. The presence of these truly aquatic "fungi" in corals and
coral reef ecosystem is very sparsely recorded. Fresh coral mucus, floating
and attached mucus detritus proved to be rich sources of these protists. Raghukumar
and Balasubramanian (1991) isolated *Thraustochytrium motivum, Labyrinthuloides
minuta, L. yorkensis* and *Ulkenia visurgensis* from these sources in coral reefs of the
Lakshadweep Islands in the Arabian Sea. Mucus detritus attached to corals yielded
1.9×10^6 thraustochytrid g^{-1} mucus, whereas fresh mucus collected from corals
yielded 20 thraustochytrid ml^{-1} mucus. Harel et al. (2008) reported the presence of

a new unidentified thraustochytrid Fng1 in the coral mucus and surface of the hermatypic coral *Fungia granulosa* and *Favia* sp. from the Gulf of Eilat. A mention must be made here about the presence of a novel marine protist, *Corallochytrium limacisporum* in the coral lagoon (Raghukumar 1987). This single-cell marine protist, a choanozoan belonging to the supergroup Opisthokonta, forms a sister clade with fungi (Cavalier-Smith and Chao 2003). Sumathi et al. (2006) demonstrated the presence of fungal signatures, namely, ergosterol and gene sequence of alpha aminoadipate reductase (α-AAR), a key enzyme involved in lysine synthesis in fungi in *C. limacisporum*. Several isolates of this protist were obtained from coral mucus from a coral lagoon in the Lakshadweep group of islands of the Arabian Sea (Raghukumar and Balasubramanian 1991). The presence of *C. limacisporum* in three corals, *Acropora* sp., *A. secale* and *Porites* sp. was confirmed using an immunofluorescence probe (Raghukumar and Balasubramanian 1991). Such studies on the yet unknown marine eukaryotic diversity in coral reef ecosystems may be a key to understanding the origin of fungi.

5.2.5 Culture-Independent Approaches for Assessment of Diversity

Few studies have focussed on the diversity of coral-inhabiting fungi using culture-independent approach. Metagenomics or community genomics approach describes the taxonomic components, their relative abundance and metabolic potentials. The microbial metagenome of *Porites astreoides* collected from Bocas del Toro, Panama showed fungi to be the dominant community, contributing 38% to the genome of the coral (Wegley et al. 2007). These belonged to Ascomycota, Basidiomycota and Chytridiomycota divisions. The majority of coral-associated fungal sequences (93%) were most similar to Ascomycota, comprising Sordariomycetes, Euratiomycetes, Saccharomycetes and Schizosaccharomycetes classes. In another instance, sequences showing similarity with aquatic chytrids were reported from corals (Vega Thurber et al. 2009). This is the first report of a chytrid being reported from corals. However, the corals in this study were maintained under different stress conditions in the laboratory microcosm experiments. Among these, *Harpochytrium*, the species closely related to chytrid sequences, is a zoosporic fungus found as an epibiont in algae (Sparrow 1960).

5.3 The Role of Fungi in Corals

5.3.1 Fungi as Carbonate Borers

Phototrophic algae and heterotrophic fungi are the two most commonly occurring endoliths in calcium carbonate structures of corals. Very often, cyanobacterial

filaments and conchocelis stages of red algae are also seen as endoliths (Bentis et al. 2000). Both the groups grow in the skeletal structure and are detected in early stages of coral growth (Le Campion-Alsumard et al. 1995a, b) producing tunnels of different diameters. These authors (Le Campion-Alsumard et al. 1995a; Golubic et al. 2005) further demonstrated that fungal hyphae are also part of the endolithic community and they parasitize algal filaments by producing haustoria. In the process they release a dark tannin-like substance that stains the hyphae, algal filaments and the coral skeleton. These result in black bands in corals called density-banding (Priess et al. 2000). Coral's defence response to fungal boring is by covering the perforations produced by fungal hyphae with dense fine-grained carbonate over the borehole. This results in a difference between skeletal carbonate and the repair carbonate, which is fine-grained aragonite, a unstable form of carbonate. Thus boring fungi play an important role in biomineralization of coral skeleton. These benign endolithic fungi can become opportunistic pathogens when the equilibrium is disturbed by environmental stressors. At present we do not know what effects these penetrating fungal hyphae have on corals, their mechanism of penetration and the role their enzymes play in this process.

5.3.2 Fungi as Pathogens in Reef Ecosystems

Besides natural disasters and climate warming, diseases have contributed to coral decline worldwide (Harvell et al. 2002). Disease outbreaks result in significant loss in coral cover and also cause a shift of the coral dominant community towards an algal-dominant ecosystem (Bourne et al. 2009). Characterization and establishing a disease must prove Koch's postulates (Andrews and Goff 1985). Several fungal associations with diseased corals were reported, such as a lower marine fungus associated with black line disease of the star corals, *Montastrea annularis* (Ramos-Flores 1983), endolithic fungi in hard corals *Porites lobata* (Le Campion-Alsumard et al. 1995a) and a *Scolecobasidium* sp. associated with scleractinian corals, *Porites lutea, Goniopora* sp. and *Montipora tuberculosa* (Raghukumar and Raghukumar 1991). About 600 km of the coral reefs was affected along the coasts from Tanzania to Kenya and fungal association has been reported in the affected corals (McClanahan et al. 2002). However, these fungi were not proven to be the actual pathogens.

The only well-characterized fungal disease is that of aspergillosis of gorgonians (sea fans) caused by *Aspergillus sydowii* (Smith et al. 1996). The sea fans *Gorgonia ventalina* and *G. flabellum* affected by the disease show lesions and necrotic patches. The disease is wide spread in the Caribbean (Nagelkerken et al. 1997a). Both tissue and skeleton disappear in the diseased sea fans. The necrotic tissue along the edges of the lesions is either lighter or dark purple in colour, in contrast to the coloration of the healthy tissue (Nagelkerken et al. 1997a). Smith et al. (1996) showed that disease could be established in healthy colonies by inoculating them with pure cultures of *A. sydowii* or by grafting infected tissue onto the healthy

tissue. Recent report (Rypien and Baker 2009) shows that the spread of the disease can also be through a vector. In this case a snail, *Cyphoma gibbosum*, was demonstrated to transmit the pathogen by feeding on the gorgonians. Aspergillosis was highly prevalent in the Caribbean in 1995 where >90% of gorgonians were affected (Nagelkerken et al. 1997a). Temperature plays crucial role in the virulence of the fungus *A. Sydowii* to the sea fan coral *Gorgonia ventalina*. The fungus grows at an optimum temperature of 30°C suggesting that increased water temperature during the summer is likely to promote its pathogenicity (Alker et al. 2001). African dust has been shown to be the source of *A. sydowii* in Caribbean gorgonian corals (Shinn et al. 2000). The transport of dust during Saharan dust storms from Africa to the Atlantic and Caribbean coral reef communities carries nutrients, including iron (Hayes et al. 2001), organic pollutants (Garrison et al. 2006) and pathogenic organisms (Shinn et al. 2000). The presence of spores of *A. sydowii* in the African dust was shown as a proof of it being the primary source of the coral pathogen (Weir-Brush et al. 2004). However, a systematic study involving specific culture techniques to study the fungal flora of the dust collected from Africa and the Cape Verde Islands in the Atlantic could not detect *A. sydowii*, suggesting that African dust may not be a viable source of this coral pathogen (Rypien 2008). Genetic structure of global samples of *A. sydowii*, including isolates from diseased corals, diseased humans and environmental sources, revealed that disease-causing isolates are not genetically distinct from environmental isolates (Rypien et al. 2008). These authors further suggested that no specific virulence factor in the pathogen was responsible and that any isolate of *A. sydowii* could cause aspergillosis. Environmental stressors and host immune system may play a vital role in causing the disease. Absence of *A. sydowii* in diseased sea fans collected from 13 reefs in Puerto Rico and its presence in healthy samples by culture-dependent approach suggested that *A. sydowii* was not the pathogen in these samples (Toledo-Hernández et al. 2008). However, reports of *Aspergillus* species causing disease in another species of the sea fan, *Annella* sp., in Southeast Asia after the 2004 tsunami (Phongpaichit et al. 2006) and in a marine sponge *Spongia obscura* (Ein-Gill et al. 2009) keeps the *Aspergillus*-as-pathogen debate alive.

As the fungus can be cultured and the host is easy to maintain in laboratory, aspergillosis disease of the sea fan *Gorgonia ventalina* is taken as a model system to understand host–pathogen interactions in context with environmental stressors. Climate warming is one of the environmental drivers of diseases. Effects of these have been studied on the aspergillosis disease as well. Increased temperature (31.5°C), caused 176% increase in the activity of host-derived antifungal compounds, but also increased the pathogen's growth rate, providing an opportunity to the pathogen to establish itself before the host could defend itself (Ward et al. 2007). Increased temperature caused loss in zooxanthellae abundance indicating that zooxanthellae were not responsible for high level of anti-fungal activity. With increasing global warming it is predicted that aspergillosis will continue to play havoc in gorgonian sea fan communities. Immune response of the host against the pathogen included production of anti-fungal lipid metabolites (Kim et al. 2000) and melanin deposition as a physical barrier to prevent fungal

expansion (Petes et al. 2003), production of peroxidases that show anti-fungal activity (Mydlarz and Harvell 2007) and production of exochitinase against the fungal pathogen *A. sydowii* (Douglas et al. 2007).

Fungi are the dominant pathogens of algae (Kohlmeyer and Kohlmeyer 1979). Several fungal pathogens of green, brown and red algae (Table 5.4) were reported from the Lakshadweep Islands in the Arabian Sea (see references in Raghukumar 2006). Most of these pathogens belonged to Chytridiomycota, comprising aquatic fungi that are pathogens of algae and phytoplankton. Littler and Littler (1998) described infection of crustose coralline algae by an unidentified fungus in American Samoa. The pathogen occurred in shallow reef habitat but was not restricted to calm waters. The spreading dense black fungal bands caused death of coralline algae. Sparse information available on fungal pathogens of algae from coral reefs indicates that this field is open for survey with modern molecular tools. Their occurrence in coral reef fauna, either as pathogens or saprotrophs would play a role in population dynamics and nutrient recycling, respectively.

Table 5.4 Fungi as algal pathogens in coral reef ecosystem

Locality	Host algae/class	Fungi/class	References
Lakshadweep islands Arabian Sea	*Cladophora repens, C. fascicularis, Rhizoclonium* sp./ Chlorophyta	*Coenomyces* sp./ Chytridiomycota	Raghukumar 1986
Lakshadweep islands, Arabian Sea	*Cladophora expansa, C. fascicularis, C. frescatii, Rhizoclonium* sp./ Chlorophyta	*Olpidium rostriferum*/ Chytridiomycota	Raghukumar 1986
Lakshadweep islands, Arabian Sea	*Cladophora frescatii, C. gracilaris*/ Chlorophyta	*Sirolpidium bryopsidis*/ Chytridiomycota	Raghukumar 1986
Lakshadweep islands, Arabian Sea	*Cladophora* sp., *C. frescatii, C. repens, Rhizoclonium* sp./ Chlorophyta	*Labyrinthula* spp./ Labyrinthulomycota	See Raghukumar 2006
Lakshadweep islands, Arabian Sea	*Sargassum* sp./ Phaeophyta	*Lindra thalassiae*/ Ascomycota	See Raghukumar 2006
Lakshadweep islands, Arabian Sea	*Centroceras clavulatum*/ Rhodophyta	*Chytridium polysiphoniae*/ Chytridiomycota	See Raghukumar 2006
Lakshadweep islands, Arabian Sea	*Thalassia hemprichii* Seagrass	*Acremonium, Chaetomium, Graphium, Humicola, Penicillium* spp., *Labyrinthuloides minuta, Thraustochytrium motivum*	Sathe and Raghukumar (1991)
American Samoa	Crustose coralline alga/ Chlorophyta	An undescribed fungal Pathogen	Littler and Littler (1998)

5.3.3 Secondary Metabolites from Coral Reef Fungi

Coral reefs are an important source of pharmaceutical, nutraceutical, enzymes, cosmetics, pesticides and other novel commercial products. Coral reefs contain diversity of sessile animals, such as corals, sponges, tunicates, molluscs, bryozoan and echinoderms. These sessile animals have developed unique chemical defence mechanisms against predation, biofouling, diseases, environmental perturbations and other stressors. These chemicals are either synthesized by the organisms themselves or their endobiontic microorganisms. If these valuable compounds are to be obtained from reef organisms, a system for sustainable management of harvesting these potentially valuable invertebrates has to be in place. On the other hand, endobiontic microorganisms such as bacteria, fungi and actinomycetes, which produce these compounds or induce the hosts to produce, can be cultured on a large scale for extraction and characterization of bioactive molecules. Several novel compounds with potential medical applications have been derived from fungi associated with various reef organisms (Table 5.5). A number of antimicrobial compounds have been reported from coral reef associated fungi (Namikoshi et al. 2000; Bugni and Ireland 2004).

Fungal presence in corals of different health may, to a limited extent, give insight into the type of fungal succession and their r- or K-strategies. Fungi with r-strategy may grow and reproduce very fast and utilize most of the available organic matter. Whereas, K-strategists would grow slowly, may produce a lot of antimicrobial compounds. Such ecological information is important in drug discovery and screening programmes.

Table 5.5 Secondary metabolites from coral reef organisms

Host/locality	Fungus	Compound	Activity	References
Unidentified soft coral	Unidentified fungus	Spiroxins A-E	DNA-cleaving antitumor antibiotic	McDonald et al. (1999)
Coral reef at Yap	*Paecilomyces* sp.	Paecilospirone	Curing effect on deformed fungal mycelia	Namikoshi et al. (2000)
Mangrove wood from coral reef in Pohnpei	*Phomopsis* sp.	Phomopsidin	Inhibitory activity in microtubule assembly assay using purified porcine brain microtubule protein	Namikoshi et al. (2000)
Coral reef at Pohnpei	*Penicillium* sp. *Fusarium* sp. *Colletotrichum* sp.	Griseofulvin Fusarielin Deoxyfusapyrone	Antifungal activity	Namikoshi et al. (2000)
Coral reef at Yap	*Acremonium* sp.	Fusapyrone	Antifungal activity	Namikoshi et al. (2000)
Sponge tissue, Okinawa	Unidentified strain # 97F95	Verrucarin	Unidentified strain # 97F95	Namikoshi et al. (2000)
Coral reef at Pohnpei	Unidentified strain # 95F137	Verrucarin L acetate	Unidentified strain # 97F95	Namikoshi et al. (2000)

5.3.4 Enzymes of Fungi from Coral Reefs

No information on extracellular enzymes of coral-associated fungi or bioeroding endolithic fungi is available. Coral mucus contains sugars, fatty acids and proteins (Meikle et al. 1988). Thraustochytrid protists displayed very good proteolytic enzyme activity at alkaline pH (Colaço et al. 2006; Raghukumar et al. 2008b). Therefore, thruastochytrids present in coral mucus might play a role in their degradation.

Little research has been aimed at understanding the fungal degradation of polysaccharides such as carrageenans, agar, alginate and laminarin of algae inhabiting coral reefs and their role in nutrient cycling. However, some information on degradative enzymes produced by fungi associated with seagrasses in coral reef lagoon in the Arabian Sea is known (see Raghukumar et al. 2008a). A basidiomycetous fungus NIOCC #312 isolated from decaying *Thalassia hemprichii* leaves from one of the islands in the Lakshadweep in the Arabian Sea displayed lignin-degrading activity (Raghukumar et al. 2008a). It mineralized about 24% of U-ring^{14}C-labelled lignin to $^{14}CO_2$ within 24 days when grown in seawater medium. The lignin-degrading enzymes, lignin peroxidase, manganese peroxidase and laccase were produced when the fungus was grown in seawater medium. It decolorized several synthetic dyes, bleach plant effluent (paper mill effluent) and molasses spent wash (molasses-based distillery effluent) effectively within 6 days. The fungus showed growth, lignin mineralization and decolourization of coloured industrial pollutants in sea water medium (Raghukumar 2008). Several seagrass species occurring in shallow coral reef lagoons, at different stages of decomposition, may harbour fungi with an array of lignocellulose degrading enzymes. These resources are still untapped. These fungi may prove to be a good source of salt-tolerant enzymes as the salinity in lagoons is 25–33 psu (practical salinity units).

5.3.5 Other Possible Roles

A recent metagenomic approach in coral holobiont revealed the presence of fungal genes involved in carbon and nitrogen metabolism, suggesting their role in conversion of nitrate and nitrite to ammonia, enabling fixed nitrogen to cycle within the coral holobiont (Wegley et al. 2007). Besides, genes encoding cell wall synthesis, chitin synthase and glutamine and nitrogen metabolism were also found during these studies on the coral *Porites astreoides*. To understand their role in nutrition, such studies need to be pursued further using radiolabelled substrates. Domart-Coulon et al. (2004) presented experimental evidence of interactions between coral cells and fungal resident, a *Cryptococcus* sp. strain F19-3-1 in *Pocillopora damicornis* coral skeleton. Coral cells established in primary culture were shown to obtain short-term extension of survival in the presence of the fungal strain F19-3-1,

indicating that this fungus produced a cryoprotective effect which selectively enhanced the survival of skeletogenic cell types.

The photosynthetic dinoflagellates are rich in intracellular solute of dimethyl-sulfoniopropionate (DMSP). When DMSP is released from zooxanthellae, it is broken down to dimethyl sulphide (DMS) mostly by bacteria. DMS oxidation products are cloud condensation nuclei, causing cloud cover over the oceans (Sievert et al. 2007). Recently, the coral pathogen *A. sydowii* has been demonstrated to catabolically produce DMS from DMSP. It contained dddP gene, encoding the enzyme that releases DMS from DMSP (Kirkwood et al. 2010). The ability to catabolise DMSP may give such fungi selective access to an abundant substrate present in the ecosystem. The role of coral-associated fungi in DMS production needs to be assessed.

Corals in the tropical oceans protect their tissues from damaging doses of ultraviolet radiation. This protection is considered to be offered by the symbiotic zooxanthellae living in their tissue through mycosporine-like amino acids (MAA). Some members of MAAs are found in fungi (Dunlap and Shick 1998). It will be worthwhile to study how much of MAAs are contributed by the coral-associated fungi in corals and what role they play in absorbing and dissipating UV energy.

5.4   Future Directions

Studies on microbial diversity, especially bacteria and the symbiotic dinoflagellate of coral holobiont, have received a lot of attention recently. Although the presence of fungi in this holobiont has been recognized, not enough information on their diversity and role is known. Cultivation-based methods for fungi have limitations in obtaining slow-growing true marine fungi, which means that there is an urgent need to develop methods to cultivate the uncultured. Modifications of microencapsulation method used for microbial cultivation (Zengler et al. 2002) should be considered for culturing true marine fungi from corals. Low light intensities, oxygen concentrations, pH, carbon and nitrogen sources should be standardised for obtaining fungi from corals. For in situ analysis of residential mycota in corals, culture-independent approach needs to be used for a large-scale quantification on spatial and temporal scale. Methods such as fluorescence in situ hybridization (FISH) or CARD-FISH (catalysed reported deposition) or double-labelling of oligonucleotide probes for FISH (DOPE-FISH), used for the identification, quantification and characterization of phylogenetically distinct population of prokaryotes (Amann and Fuchs 2008; Stoecker et al. 2010), need to be developed for studying fungal diversity in corals. Such probes are being used in the detection of phylloplane mycoflora (Inácio et al. 2010), freshwater fungi in streams (Baschien et al. 2008) and environmental samples (Mitchell and Zuccaro 2006). A protocol for FISH developed by Sterflinger et al. (1998) for detecting hyphomycetes in stone monuments can be easily applied for detecting specific slow-growing fungi in corals. However, these authors encountered a problem of substrate autofluorescence

or other associated fungi. To overcome this problem, they recommend the use of digoxigenin (DIG)-labelled probes which can be detected colorimetrically by antibodies specific for digoxigenin, which are coupled with alkaline phosphatase. After addition of the enzyme substrate (4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate), the intracellular blue-purple precipitate is detected by light microscopy (Schröder et al. 2000). Thus, colorimetric *in situ* hybridization (CISH) is a promising method for the detection of specific fungi in healthy or diseased corals.

Filamentous fungi isolated from corals by several researchers may be mostly allochthonous, as coral skeletons are perforated and may harbour dormant propagules of terrestrial fungi. However, in cases where actively growing mycelia were observed in healthy corals, there is a possibility that they are autochthonous forms. Whether these coral-inhabiting fungi differ physiologically from their terrestrial counterparts is a much-debated topic (Kohlmeyer and Volkmann-Kohlmeyer 2003). It needs to be assessed whether they play any active role in coral holobiont as symbionts, or pathogens, or saprotrophs. The role of *A. sydowii*, a ubiquitous terrestrial fungus causing aspergillosis in sea fan, was cited as an example in this controversy about proving the role of marine-derived fungi. However, recent studies have caused serious concern about it being the sole pathogen (Toledo-Hernández et al. 2008), and is considered being an opportunistic pathogen (Rypien et al. 2008). Such stringent studies are required to be undertaken for proving the presence of marine-derived fungi in corals.

Assuming that some of the fungi may be symbiotic, at what stage the corals start harbouring fungi still is a total black box. In one instance it was demonstrated that endosymbiotic yeasts in the three species of the sponge *Chondrilla* were directly propagated by vertical transmission in the female (Maldonado et al. 2005). These authors reported that a large number of yeast cells (ca. 4.4 cells per 10 μm^2) were transmitted from the soma through the oocytes to the fertilized eggs. Is it the case with yeasts associated with some of the coral species at least? Using immunofluorescence probe, the presence of the thraustochytrid *Ulkenia visurgensis* in gut of hydrozoa was demonstrated (Raghukumar 1988). Do the coral polyps harbour yeasts or thraustochytrids within the coelenteron as shown in the case of hydrozoa?

Do fungi provide nutrition in the event of bleaching, when the zooxanthellae are expelled? Several workers have reported fungal presence in bleached and diseased corals (Ravindran et al 2001; Yarden et al. 2007). Straminopilan fungi found on the surface and in the mucus of corals may provide nutrition in the form of polyunsaturated fatty acids helping corals to survive during bleaching event (Harel et al. 2008).

Do the physiological changes occurring within the host coral in response to environmental stressors alter the endobiontic fungal populations or turn some of these benign fungi into opportunistic pathogens? Fungi associated with corals in healthy, bleached, stressed and diseased corals, succession of fungi at different stages of coral growth, in different species of corals, spatial and temporal variations are some of the issues which need to be addressed using polyphasic approaches of culture-dependent and culture-independent techniques, metagenomics and other modern molecular tools available.

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Chapter 6
Fungal Life in the Dead Sea

Aharon Oren and Nina Gunde-Cimerman

Contents

Abstract The waters of the Dead Sea currently contain about 348 g/l salts (2 M Mg^{2+}, 0.5 M Ca^{2+}, 1.5 M Na^+, 0.2 M K^+, 6.5 M Cl^-, 0.1 M Br^-). The pH is about 6.0. After rainy winters the surface waters become diluted, triggering development of microbial blooms. The 1980 and 1992 blooms were dominated

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by the unicellular green alga *Dunaliella* and red Archaea. At least 70 species (in 26 genera) of Oomycota (Chromista), Mucoromycotina, Ascomycota, and Basidiomycota (Fungi) were isolated from near-shore localities and offshore stations, including from deep waters. *Aspergillus* and *Eurotium* were most often recovered. *Aspergillus terreus*, *A. sydowii*, *A. versicolor*, *Eurotium herbariorum*, *Penicillium westlingii*, *Cladosporium cladosporioides*, *C. sphaerospermum*, *C. ramnotellum*, and *C. halotolerans* probably form the stable core of the community. The species *Gymnascella marismortui* may be endemic. Mycelia of Dead Sea isolates of *A. versicolor* and *Chaetomium globosum* remained viable for up to 8 weeks in Dead Sea water; mycelia of other species survived for many weeks in 50% Dead Sea water. Many isolates showed a very high tolerance to magnesium salts. There is no direct proof that fungi contribute to the heterotrophic activity in the Dead Sea, but fungi may be present at least locally and temporarily, and their enzymatic activities such as amylase, protease, and cellulase may play a role in the lake's ecosystem.

6.1   Introduction: The Dead Sea Ecosystem

The Dead Sea (Fig. 6.1) is one of the most saline bodies of water on Earth. Currently its waters contain about 348 g/l total dissolved salts. The ionic composition is dominated by divalent cations (2 M Mg^{2+}, 0.5 M Ca^{2+}) rather than monovalent cations (1.5 M Na^+, 0.2 M K^+). Cl^- (6.5 M) and Br^- (0.1 M) are the main anions, and the water has a pH of about 6.0. The maximum depth is about 310 m, and the lake is aerobic down to the bottom. Surface water temperatures vary from about 18°C in winter to 35°C in summer. The lake is saturated with respect to sodium ions, and massive amounts of NaCl precipitate to the bottom as halite crystals. The result is a decrease in sodium content with an increase in the relative concentrations of divalent cations (Table 6.1). In summer, a seasonal thermocline develops and floodwaters that enter occasionally in winter may cause the formation of a less saline upper water layer. Still, in the past decade complete mixing occurred at least once every year (a holomictic regime) (Oren 2003a).

Currently the lake's waters are too hostile for life of even the best salt-adapted microorganisms. No algae are found in the water column, and only a small community of halophilic Archaea (family *Halobacteriaceae*) survives (Bodaker et al. 2010). However, occasionally the winter floods from the surrounding area and water inflow from the Jordan River can be so massive that a diluted upper water layer is formed that remains for several years (a meromictic regime). When this happened following the exceptionally rainy winters of 1979–1980 and 1991–1992, dense microbial blooms developed in the diluted epilimnion, consisting of the unicellular green alga *Dunaliella* as primary producer and red halophilic Archaea

Fig. 6.1 Map of the Dead
Sea, indicating the sites
mentioned in the text. The
sampling stations are
indicated by *open squares*
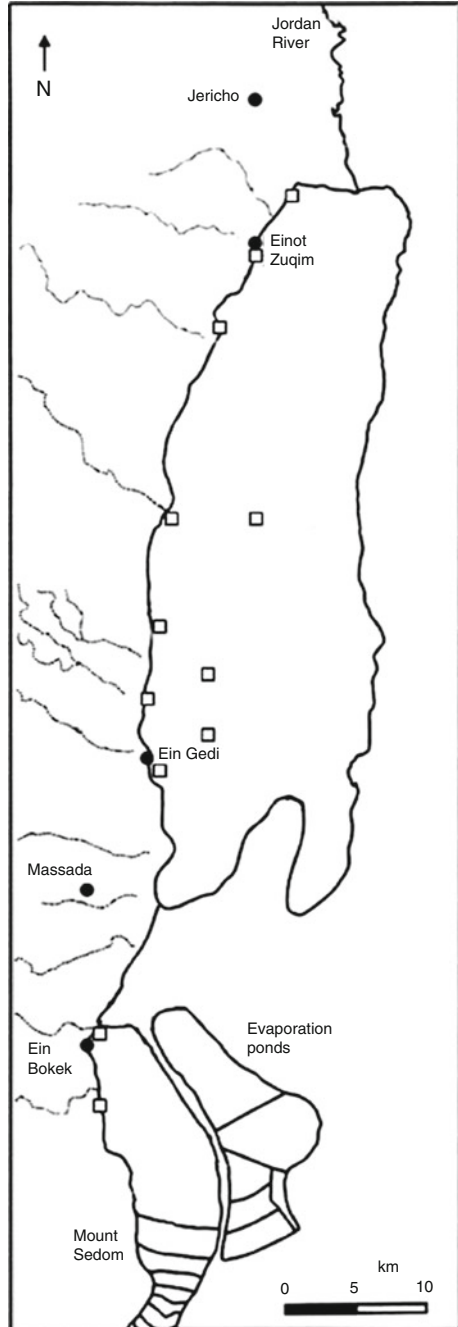
Table 6.1 Dead Sea ionic composition and salt concentrations

Year	1959–1960		1977	1996	2007
	Upper water mass	Lower water mass	Average	–	–
Na^+ (M)	1.57	1.84	1.73	1.59	1.54
K^+ (M)	0.17	0.19	0.18	0.20	0.21
Mg^{2+} (M)	1.49	1.75	1.81	1.89	1.98
Ca^{2+} (M)	0.41	0.43	0.43	0.44	0.47
Cl^- (M)	5.56	6.18	6.34	6.34	6.48
Br^- (M)	0.06	0.07	0.07	0.07	0.08
SO_4^{2-} (M)	NR	NR	0.005	0.005	0.005
$(Na^++K^+)/(Mg^{2+}+Ca^{2+})$[a]	0.92	0.93	0.85	0.77	0.71
Total dissolved salts (g/l)	298	335	339	339	347

Data were derived from Beyth (1980), and unpublished data (I. Gavrieli, the Geological Survey of Israel, personal communication)
NR not reported
[a]Molar ratio

of the family *Halobacteriaceae* (Oren 1988, 1993, 1997, 1999, 2000, 2003b; Oren and Gurevich 1995; Oren et al. 1995, 2005).

Fungi are not a prominent component of the Dead Sea ecosystem: direct microscopic examination of the waters, including during periods of microbial blooms, never showed fungal mycelia, and metagenomic approaches, applied to samples collected both during the 1992 microbial bloom and during a recent period of very sparse life in the lake, did not provide evidence for fungal presence (Bodaker et al. 2010; Rhodes et al. 2010). Still, there is no a priori reason why the lake could not support growth of fungi. A relatively low pH generally favors fungi (Buchalo et al. 1998, 1999), and the water activity of undiluted Dead Sea water of about 0.66 is still above the a_w of 0.61 considered as the lower limit for fungal life (Brown 1990; Kushner 1978). Moreover, halophilic and highly halotolerant fungi are known: some species of *Aspergillus* and *Penicillium* grow above 25% NaCl (Tresner and Hayes 1971). Fungal species isolated from the salterns such as the extremely halotolerant black yeast *Hortaea werneckii* can grow from 0 to 32% NaCl (Gunde-Cimerman et al. 2000), while the halophilic *Wallemia ichthyophaga* cannot grow without the presence of at least 10% NaCl in the medium (Zalar et al. 2005a). Besides, recent evidence indicates that fungi are much more tolerant to very high concentrations of Mg^{2+} ions, particularly abundant in the waters of the Dead Sea, than previously expected. In particular, different species of the genera *Cladosporium* and *Wallemia*, both isolated from the Dead Sea waters, excel in this property (Sonjak et al. 2010). Therefore, research efforts were dedicated to survey the Dead Sea for fungi and to characterize the properties of those recovered from the lake. A monograph has been devoted to the description of the Dead Sea fungi and their properties (Nevo et al. 2003). This chapter reviews the result of these investigations and some not yet published investigations.

6.2   The Fungi of the Dead Sea

6.2.1   Approaches for the Isolation of Dead Sea Fungi

Most studies on Dead Sea fungi were performed with samples collected in the period 1995–2003, when the lake was holomictic. Different approaches were used: mixing of water samples with molten agar and incubation of the solidified agar in Petri dishes, filtration of water samples through 0.45-μm-pore-sized Millipore filters and incubation of the filters on agar plates, and also enrichment cultures in which Dead Sea water samples were added to portions of liquid media.

Media used were generally conventional media employed in mycological studies, such as glucose-yeast extract, malt extract agar, or Czapek agar, and these were prepared with different concentrations of Dead Sea water (10, 20, 40, or 50%, by volume) or different NaCl concentrations (17–32% NaCl). Media without added salt were used as well. To prevent overgrowth of prokaryotic microorganisms, different antibiotics were used: streptomycin and chloramphenicol or tetracycline, at a concentration of 100 μg/ml each. Cultures were incubated at 20, 26, and 37°C (Buchalo et al. 1999, 2000b; Butinar et al. 2005a; Kis-Papo et al. 2001, 2003c).

6.2.2   Fungal Diversity in the Dead Sea Water Column

The first fungal isolates were retrieved from Dead Sea surface water sampled in the vicinity of the freshwater springs of Einot Zuqim, and these included *Ulocladium chlamydosporum*, *Penicillium westlingii*, and *Gymnascella marismortui* (Buchalo et al. 1999, 2000b). Further studies of the spatial and temporal diversity in the fungal community of the lake were based on sampling of different near-shore localities and at a number of stations offshore, including collection of deep water samples down to the bottom (304 m) in the center of the lake (Fig. 6.1; Table 6.2) (Buchalo et al. 1999, 2000b; Butinar et al. 2005b; Kis-Papo et al. 2001, 2003a, c, d; Molitoris et al. 2000; Wasser et al. 2003).

The annotated list compiled by Wasser et al. (2003) encompasses 70 species of filamentous fungi, belonging to 26 genera, 10 orders, and 3 divisions. Ascomycota dominate with 66 species (only 12 of which have known teleomorphs); Oomycota and Mucoromycotina are represented with three and one species, respectively. Nearly half of the species are melanin-containing micromycetes. The Eurotiales are well represented with 33 species, including *Aspergillus* (19 species including *Emericella*) and *Penicillium* (13 species). Other genera frequently encountered were *Chaetomium* (Sordariales; 5 species) and *Cladosporium* (Davidiellaceae, Capnodiales; 5 species). These features can be considered as characteristic for mycobiota of a highly stressful environment. The list compiled by Wasser et al. (2003) was further enlarged by additional isolates obtained by Gunde-Cimerman et al. (unpublished data), and is shown in Tables 6.2 and 6.3.

Table 6.2 Systematic diversity of the Dead Sea microfungi (adapted from Wasser et al. 2003)

Division	Order or class	Number of taxa	
		Genera	Species
Oomycota	Pythiales	1	1
Mucoromycotina	Mucorales	2	3
Ascomycota	Capnodiales	1	13
	Dothideales	1	1
	Eurotiales	4	35
	Hypocreales	6	10
	Onygenales	2	2
	Ophiostomatales	1	1
	Pleosporales	5	12
	Sordariales	2	6
Saccharomycetes	Saccharomycetales	2	3
Basidiomycota	Russulales	1	1
	Sporidiobolales	1	1
	Tremellales	2	2
	Polyporales	2	2
	Wallemiales	1	1
Total		34	94

Many of the fungi recovered from Dead Sea water samples are non-halophilic, non-xerophilic, terrestrial species, and many species were also reported from soils in the surrounding lakes (Sect. 6.2.5). The genera most often recovered were related species with sexual stages in *Eurotium* and asexual ones in *Aspergillus*. *Eurotium* and its associated *Aspergillus* anamorph are known as xerophilic, adapted to matric stress, and is also often encountered in hypersaline waters (Tresner and Hayes 1971; Pitt and Hocking 1997). Thus, six species of the genus (*E. amstelodami*, *E. repens*, *E. herbariorum*, *E. rubrum*, *E. chevalieri*, and a potential new species "*E. halotolerans*") were repeatedly isolated from environments >17% salt, including coastal saltern ponds in Spain, Portugal, France, Slovenia, Israel, Namibia, and the Dominican Republic. *E. amstelodami* was also isolated from an industrial evaporation pond at the southern end of the lake (Butinar et al. 2005b, Gunde-Cimerman et al. 2005, 2009). However, *E. herbariorum* and *E. rubrum* grew poorly on agar media prepared with 50% Dead Sea water (Kis-Papo et al. 2003a). *Ulocladium chlamydosporum* is also poorly adapted to salinity of Dead Sea (Molitoris et al. 2000). *A. versicolor*, *E. herbariorum*, and *P. westlingii* were most abundant in winter; *A. sydowii*, *Cladosporium cladosporioides*, and *C. sphaerospermum* were found in comparable numbers the year round. Hypersaline waters of salterns on different continents most frequently harbor *A. niger*, *E. amstelodami*, and *P. chrysogenum* and to a lesser extent *A. sydowii*. *E. herbariorum*, and *A. versicolor* were isolated only sporadically, and they were considered only as temporal inhabitants, not adapted to long-term survival (Butinar et al. 2011). The diversity of *Cladosporium* species isolated appears to be higher than previously reported (Wasser et al. 2003). Besides *C. cladosporioides* and

Table 6.3 Fungal species reported from Dead Sea water

Group/class	Order	Genus	Species[a]
Oomycota	Pythiales	*Pythium*	*Pythium* sp.
Mucoromycotina	Mucorales	*Absidia*	*A. corymbifera* (Cohn) Sacc. et Trotter
			A. glauca Hagem, 1908
	Mucorales	*Circinella*	*C. rigida* G. Sm.
Ascomycota	Hypocreales	*Acremonium*	*A. implicatum* (J.C. Gilman et E.V. Abbott (W. Gams)
			A. persicinum (Nicot) W. Gams
			A. rutilum W. Gams
	Pleosporales	*Alternaria*	*A. alternata* (Fr.) Kessler
			A. pluriseptata (R. Karst., Har. et Peck) Jorst.
			Alternaria sp.
	Eurotiales	*Aspergillus*	*A. caespitosus* Raper et Thom
			A. carneus (Tiegh.) Blochwitz
			A. flavus Link
			A. fumigatus Fresen.
			A. niger Van Tiegh.
			A. penicilloides Speg.
			A. phoenicis (Corda) Thom de Currie (*A. niger* (nom. cons.), or *A. tubingensis*)
			A. ochraceus G.Wilh.
			A. proliferans G.Sm.
			A. sclerotiorum G.A. Huber
			A. sydowii Bainier et Sartory (Thom et Church)
			A. terreus Thom
			A. tubingensis Mosseray
			A. ustus (Bainier) Thom et Church
			A. versicolor (Vuill.) Tirab.
			A. wentii Wehmer
	Dothideales	*Aureobasidium*	*A. pullulans* (de Bary) Arnaud
	Saccharomycetales	*Candida*	*C. glabrata* (H.W. Anderson) S.A. Mey. et Yarrow
			C. parapsilosis (Ashford) Langeron & Talice)
	Sordariales	*Chaetomium*	*C. aureum* Chivers
			C. flavigenum van Warmelo
			C. funicola Cooke
			C. globosum Kunze
			C. nigricolor L.M. Ames
	Onygenales	*Chrysosporium*	*Chrysosporium* sp.
	Capnodiales	*Cladosporium*	*C. cladosporioides* (Fresen.) G.A. de Vries
			C. aff. *cladosporioides*
			C. halotolerans Zalar, de Hoog & Gunde-Cim.
			C. herbarum (Pers.) Link

Table 6.3 (continued)

Group/class	Order	Genus	Species[a]
			C. aff. *herbarum* (Pers.) Link
			C. aff. *inversicolor* Bensch, Crous & U. Braun
			C. macrocarpum Preuss
			C. oxysporum Berk. et M.A. Curtis
			C. ramotenellum K. Schub., Zalar, Crous & U. Braun 2007
			C. sphaerospermum Penz.
			C. aff. *sphaerospermum.*
			C. tenellum K. Schub., Zalar, Crous & U. Braun 2007
		Cladosporium sp.	
	Pleosporales	*Curvularia*	*C. protuberata* R.R. Nelson et Hodges
	Eurotiales	*Emericella*	*E. nidulans* (Eidam) Vuill.
			E. purpurea Samson et Mouchacca
			E. neopurpurea (a new species under description)
	Hypocreales	*Engyodontium*	*E. album* (Limber) de Hoog
	Eurotiales	*Eurotium*	*E. amstelodami* L. Mangin
			E. herbariorum (F.H. Wigg.) Link
			E. rubrum W. Bremer
	Onygenales	*Gymnascella*	*G. marismortui* Buchalo et al.
	Hypocreales	*Isaria*	*I. farinosa* (Holmsk.) Fr.
	Saccharomycetales	*Meyerozyma*	*M. guillermondii* (Wick.) Kurtzman & M. Suzuki
	Hypocreales	*Myrothecium*	*M. roridum* Tode
	Eurotiales	*Penicillium*	*P. brevicompactum* Dierckx
			P. chrysogenum Thom
			P. citrinum Thom
			P. commune Thom
			P. corylophilum Dierckx
			P. crustosum Thom
			P. fellutanum Biourge
			P. glabrum (Wehmer) Westling
			P. implicatum Biourge
			P. restrictum J.C. Gilman et E.V. Abbott
			P. steckii K.M. Zalessky
			P. variabile Sopp
			P. westlingii K.M. Zalessky
	Pleosporales	*Phoma*	*P. leveillei* Boerema et G.J. Bollen
	Ophiostomatales	*Sporothrix*	*S. guttuliformis* de Hoog
	Hypocreales	*Stachybotrys*	*S. chartarum* (Ehrenb.) S. Hughes
	Pleosporales	*Stemphylium*	*Stemphylium herbarum* E.G. Simmons
	Sordariales	*Thielavia*	*T. terricola* (J.C. Gilman et E.V. Abbott) C.W. Emmons
	Hypocreales	*Trichoderma*	*T. koningii* Oudem.
			T. aff. *atroviridae.*
			Trichoderma sp.

(continued)

Table 6.3 (continued)

Group/class	Order	Genus	Species[a]
	Pleosporales	*Ulocladium*	*U. alternariae* (Cooke) E.G. Simmons
			U. atrum Preuss
			U. chlamydosporum Mouchacca
			U. oudemansii E.G. Simmons
			U. tuberculatum E.G. Simmons
Ulocladium sp.			
Basidiomycota	Polyporales	*Bjerkandera*	*Bjerkandera* sp.
	Tremelalles	*Cryptococcus*	*C. albidus* var. *kuetzingii* (Fell & Phaff) Fonseca, Scorzetti & Fell
	Sporidiobolales	*Rhodotorula*	*R. laryngis* Reiersöl
	Russulales	*Stereum*	*S. hirsutum* (Willd.) Pers.
	Polyporales	*Trametetes*	*T. versicolor* (L.) Lloyd
	Tremellales	*Trichosporon*	*T. mucoides* E. Guého et M.T. Sm.
	Wallemiales	*Wallemia*	*W. sebi* (Fr.) Arx

Data were derived from Buchalo et al. (1998, 1999, 2000a, b), Butinar et al. (2005a, b), Kis-Papo et al. (2001, 2003c), Molitoris et al. (2000), and Wasser et al. (2003, unpublished results)

[a] Lists of synonyms can be found in the Index Fungorum (http://www.indexfungorum.org)

C. sphaerospermum, *C. halotolerans* and *C. ramnotellum* also represent the core fungal community (Zalar et al. 2007).

Fungal diversity increased near the outlets of less saline springs near the shore. Some of the Dead Sea fungi could therefore have entered the lake with river or spring water or from the air. Species richness was highest in winter, and varied greatly between sampling localities. This implies an unstable and variable community with very few constant species. More than 40% of the diversity was isolated >0.5 km offshore. Most species recovered were isolated during one season only and from a limited number of sampling sites. No single species had a temporal or spatial frequency of occurrence of >75%.

Only a few species were consistently isolated from the water column, and these may constitute the core of the Dead Sea mycobiota. These are *Aspergillus sydowii*, *A. versicolor*, *Eurotium herbariorum*, *Cladosporium cladosporoides*, and *C. sphaerospermum*. Moreover, at least for some of these species the spores and mycelia survived prolonged suspension in Dead Sea water (Sect. 6.3.1). Surprisingly, halotolerant black yeasts, the dominant representatives of fungi in hypersaline waters of salterns worldwide, were not isolated from the lake (Butinar et al. 2005c; Gunde-Cimerman et al. 2000), but only from the surface of halophytic plants growing on the shores (Gunde-Cimerman et al., unpublished data).

6.2.3 Gymnascella marismortui: *An Endemic Dead Sea Fungus?*

One of the Ascomycota recovered from Dead Sea surface water near the Einot Zuqim springs in January–November 1995 was a new species of the genus *Gymnascella*, now described as a new species, *Gymnascella marismortui*

(Buchalo et al. 1998, 1999, 2000a). At the time seven isolates were obtained, but to our knowledge it was not found in later studies. It is an obligate halophile that grows optimally between 0.5- and 2 M NaCl, or in media containing 10–30% by volume of Dead Sea water, and some growth was even obtained in 50% Dead Sea water media (Molitoris et al. 2000). High sucrose concentrations instead of salt supported only a slow growth. Mycelia retained their viability for 4 weeks in undiluted Dead Sea water and 12 weeks in Dead Sea water diluted to 80% (Kis-Papo et al. 2003a). Also its spores survived prolonged suspension in Dead Sea water better than the spores of other species (Kis-Papo et al. 2003d) (see also Sect. 6.3.1).

This species might thus be well adapted to life in the Dead Sea. Thus far it was not reported from other sites, and until further notice it may be considered endemic to the lake (Kis-Papo et al. 2003d). Further studies should ascertain the temporal and spatial distribution of *G. marismortui* to decide whether it is a true inhabitant of the Dead Sea, or whether its habitat is restricted to the areas of lowered salinity where freshwater from the Einot Zuqim springs mixes with the brines, and/or other areas with a reduced salinity due to freshwater influx.

G. marismortui displays several enzymatic activities of interest: urease (optimal production at 27°C at 17.5% salt), cellulase (best at lower temperatures), and amylase, but in contrast to other Dead Sea fungi tested (Sect. 6.3.2) it did not display any dye decolorization activity (Molitoris et al. 2000).

6.2.4   Yeasts in the Dead Sea

In an unpublished M.Sc. thesis, Kritzman (1973) reported the isolation of an osmophilic yeast from the Dead Sea that grew in a medium with 15% glucose and 12% salt. Unfortunately no cultures have been preserved. It should be noted that in the early 1970s the lake was stably meromictic, and the conditions were less extreme than today (Table 6.1).

The list of 70 Dead Sea fungi given by Wasser et al. (2003) included a single yeast species (*Candida glabrata*). Butinar et al. (2005a) reported the isolation of a *Candida glabrata*-like isolate, *Candida parapsilosis*, *Rhodotorula laryngis*, and *Trichosporon mucoides*. In addition, *Cryptococcus albidus* and *Meyerozyma guillermondii* were isolated (Gunde-Cimerman, unpublished results). *T. mucoides* was found also in the salterns of Eilat.

6.2.5   Comparison of the Fungal Community of the Dead
Sea Waters with that of the Surrounding Soils

Of the 70 species reported from Dead Sea water (Wasser et al. 2003), 46 were previously isolated from the terrestrial shore, including the commonly found *Eurotium herbariorum*, *Aspergillus versicolor*, and *Cladosporium cladosporoides*.

The fungal diversity of soils in the Dead Sea area has been investigated by a number of groups (Guiraud et al. 1995; Steiman et al. 1995, 1997; Grishkan et al. 2003, 2004; Volz and Wasser 1995). Grishkan et al. (2003) found 78 micromycete species from 40 genera in soils from western shore of the lake. The majority contained melanin, and most isolates did not show special halophilic or thermophilic properties. The same was even true for the mycoflora of the saline Arubotaim Cave, a Holocene karst cave within the halite mountain of Mount Sedom (Grishkan et al. 2004). Soils from the Dead Sea shore and from oases in the area do not have a highly unusual fungal flora (Steiman et al. 1994, 1995, 1997). Many species encountered have a worldwide distribution. A number of new fungal species isolated from the area were described: *Aspergillus homomorphus*, *A. pseudo-heteromorphus* (Steiman et al. 1994), *Exserohilum sodomii* (Guiraud et al. 1997), and *Bipolaris israeli* (Steiman et al. 1996).

6.3 Special Adaptations and Properties of Dead Sea Fungi

6.3.1 Survival of Fungal Spores in Dead Sea Water

The procedures used to isolate fungi from the Dead Sea (Sect. 6.2.1) do not discriminate between vegetative mycelia and spores, and therefore do not provide information whether the fungi may actually be growing in the Dead Sea water or may have entered the lake as resistant conidiospores, ascospores, basidiospores, or chlamydospores, depending on the species, and had survived for prolonged periods in the lake as spores. Therefore, the survival of spores and vegetative mycelia was examined after suspension in the undiluted and diluted Dead Sea water (80%, 50%, 10%, by volume), comparing the result with the survival of strains of the same species or closely related ones isolated from the Dead Sea terrestrial shore and from a control Mediterranean environment (Kis-Papo et al. 2003a, d). Mycelia were grown in media containing 10% Dead Sea water, checked microscopically for absence of spores, and then transferred serially to increasing concentrations of the Dead Sea water (30–50–80%) to prevent severe osmotic shock. Spores were added to Dead Sea water or its dilutions to a density of 200/ml. After incubation periods of up to 12 weeks the number of viable spores and the presence of viable mycelium were tested by plating on suitable media (for *Gymnascella* and *Eurotium* supplemented with 30% Dead Sea water).

Mycelia of *Aspergillus versicolor* and *Chaetomium globosum* remained viable for up to 8 weeks in undiluted Dead Sea water; four isolates (*A. versicolor*, *Eurotium herbariorum*, *Gymnascella marismortui*, and *C. globosum*) retained their viability for 12 weeks in water diluted to 80%. In water diluted to 50% of its original salinity, mycelia of all species survived for many weeks.

Ascospores of *G. marismortui* survived very well, with 70% viable spores in undiluted Dead Sea water and nearly 100% in 80% Dead Sea water at the

termination of the experiment. Ascospores of an *Emericella nidulans* isolated from a non-saline environment proved less resistant than conidia: after 14 days, 40% of the conidia were still viable, as compared to 5% of the ascospores. This is contrary to the common finding that ascospores have improved stress resistance compared with conidia; the tolerance of sexual spores is one of the reasons why they are more frequently encountered in stressful environments (Jin and Nevo 2003). Spores of *Aspergillus sydowii* and *Eurotium herbariorum* (a xerophilic genus that can grow in 50% Dead Sea water) survived for 3 months in undiluted and diluted water; however, spores of these species obtained from the shore of the lake proved less tolerant (10–20% survival only). Spores isolated from a site with low salt concentrations survived for less than 2 months (Kis-Papo et al. 2003a, d).

When spores and mycelium of six different species of the genus *Eurotium*, most frequently isolated from salterns, were tested for viability in hypersaline waters, at the highest salinity (30% NaCl) spore viability of most species decreased drastically within the first week and then with a slower pace. Viability of *E. herbariorum* spores remained unchanged after the initial decrease of 65%, while only up to 15% of the spores of the other species remained viable. The highest adaptive ability of survival in hypersaline water was shown for *E. rubrum* spores, since almost half of them survived. Surprisingly, mycelia of all tested species survived 10 weeks of incubation even at the highest tested salinities. Mycelia of *E. amstelodami*, *E. herbariorum*, *E. repens*, and *E. chevalieri* remained viable throughout the test period of 12 weeks (Butinar et al. 2005b).

6.3.2 Enzyme Production and Dye Decoloration by Dead Sea Fungi

Many fungi possess exoenzymes and display interesting metabolic properties such as decolorization of dyes and degradation of other toxic chemicals. Therefore, selected fungal isolates from the Dead Sea were tested for different enzymatic activities: amylase, caseinase, urease, and cellulase (Buchalo et al. 1999; Molitoris et al. 2000). *P. westlingii* and *U. chlamydosporum* were the best urease producers, whereas *G. marismortui* was the best cellulase producer. Enzyme production generally decreased with increasing temperature and salinity, but in some cases (*G. marismortui* amylase, cellulase, urease, *U. chlamydosporum* amylase, and caseinase) optimal production was observed at intermediate salinities and temperatures.

Dye decolorization was tested as a model system to evaluate the potential of Dead Sea fungi to degrade pollutants. Dyes of different chemical groups were included in the tests: anthraquinones (remazol brilliant blue R, disperse blue), heterocyclic compounds (fluorescein, methylene blue, bromophenol blue), azo dyes (methyl orange, methyl red, reactive orange), and diazo dyes (congo red, sudan black B, reactive black). The anthraquinone and the monoazo dye methyl red

were most rapidly decolorized, while the diazo dyes were only poorly degraded. Among the mitosporic taxa, *U. chlamydosporum* was the best dye degrader, an unnamed *Acremonium* species was the weakest. Among the ascomycetes *E. nidulans* and *A. phoenicis* were the best decolorizers (Buchalo et al. 1999; Molitoris et al. 2000).

6.3.3 Genetic Diversity of Dead Sea Fungi and Comparison with Related Isolates from Other Environments

To further examine whether fungi isolated from the Dead Sea differ from strains of the same species found in less salt-stressed ecosystems, the genetic diversity was investigated using fluorescent amplified fragment length polymorphism (AFLP) markers as a high-resolution fingerprinting tool (Kis-Papo et al. 2003b). Three species were included in these studies: the cosmopolitan *Aspergillus versicolor*, the halophilic *Eurotium amstelodami*, and *E. herbariorum*. *A. versicolor* showed the highest level of gene diversity with 78% of polymorphic loci from the 605 bands scored. *E. herbariorum* showed the lowest percentage of polymorphic loci (31%), and all isolates including those from the lake shore clustered in one group. *E. amstelodami* had two significant genotypes with no apparent correlation to distribution. In both *E. amstelodami* and *A. versicolor* the water column population was significantly differentiated from populations from the Jordan River and a non-saline environment in the north of Israel (Nahal Keziv), but not from the populations of the Dead Sea shore. For the halophilic *E. amstelodami*, the freshwater of the Jordan River is no less stressful than the saline water of the Dead Sea, and accordingly low polymorphism levels were found in the Jordan River samples. In the three species the shore isolates were not differentiated from strains from Dead Sea surface water, which may be explained by migration between the Dead Sea and the shore populations.

Genetic diversity showed an increase from the mild north to the stressful Dead Sea area culminating in the sea surface, but dropped to very low genetic diversity in the water column collected from 30 to 300 m depth, where only a few adapted ecotypes survive. Clearly the highly stressful Dead Sea environment lowers the diversity of its fungi with only a few adapted ecotypes surviving. In addition, the percentage sexually reproducing species increases with stress, but drops dramatically at the highest stress levels, which select for few well-adapted but homozygous genotypes (Kis-Papo et al. 2003b, e).

Further genetic studies were made of the EhHOG gene of a Dead Sea isolate of *Eurotium herbariorum*, encoding a key component of a signal transduction cascade that plays an essential role in the osmoregulatory pathway. When EhHOG was expressed in a *S. cerevisiae hog1Δ* mutant, the growth and aberrant morphology of the *hog1Δ* mutant were restored under conditions of high osmotic stress. Moreover, intracellular glycerol content in the transformant increased to a higher level than

that in the mutant during salt-stress conditions. Such genes from Dead Sea fungi may be a promising resource to improve salt tolerance of plants. Preliminary results showed that indeed the EhHOG gene may provide increased stress tolerance in transgenic *Arabidopsis* (Jin et al. 2005).

6.4 How Important Are Fungi in the Dead Sea Ecosystem?

6.4.1 Occurrence of Fungi on Wood Submerged in Dead Sea Water

A promising place to look for development of fungal mycelia is submerged wood and wooden structures. Thus, *Cladosporium* sp. was found on submerged pine wood in Great Salt Lake at a salt concentration of 290–360 g/l salt (Cronin and Post 1977). Zalar et al. (2005b) reported that the black yeasts *Hortaea werneckii* and *Trimmatostroma salinum* degrade wood immersed in hypersaline waters of the salterns. Isolates of both species showed xylanolytic and lignolytic activity under hypersaline and non-saline conditions; *T. salinum* displayed cellulolytic activity as well. These results suggested an active lignicolous saprobic role of halophilic fungi in hypersaline water environments.

The finding of fungal mycelia on near-shore wooden structures submerged in the Dead Sea was mentioned in several publications (Buchalo et al. 1998, 1999, 2000a, b; Kis-Papo et al. 2001, 2003b; Molitoris et al. 2000). However, nowhere were the findings documented in-depth. Kis-Papo et al. (2003b) stated that "live mycelium of nine fungal species, including *Eurotium amstelodami*, *E. herbariorum*, and *E. rubrum*, were found growing on driftwood submerged near the aquatic shore," and cited papers by Kis-Papo et al. (2003c) and Buchalo (2003), but these sources do not provide any additional information. Buchalo et al. (2000b) described an experiment in which 1–2 cm long pieces of wood on which fungal hyphae were observed under a binocular microscope were placed in Petri dishes on filter paper wetted with sterile Dead Sea water and incubated at 28°C for 30–40 days, the filter paper being regularly wetted with sterile distilled water. However, the results of this experiment were not reported.

6.4.2 Isolation of Vegetative Fungi in the Dead Sea by Baiting

Baiting experiments to isolate fungi, using media containing starch and cellulose were set up in the summer of 2000 at the lake surface at an offshore station and again in the spring of 2003 in near-shore areas. No fungal mycelia were detected after 6 weeks of incubation (Kis-Papo et al. 2003c). Possibly the baits selected were not appropriate, since most halotolerant and halophilic fungi display strong

proteolytic and lipolytic activities and less cellulose and starch degrading ones (Gunde-Cimerman, unpublished results).

6.4.3   Final Comments: Are Fungi Important in the Dead Sea Ecosystem?

A great variety of fungal species were isolated from the lake, and a stable core of species was detected in different seasons and depths, including species such as *A. sydowii*, *A. versicolor*, *E. herbariorum*, *P. westlingii*, *C. cladosporioides*, and *C. sphaerospermum*. These species were also found in deep samples in summer, when the thermocline is expected to act as a barrier preventing migration of spores or mycelium from the surface to the deeper water. New fungi may be expected to enter the lake continuously as spores by rain, runoff, and wind, but due to the harsh conditions there is little chance for intruding fungal species originating from a non-saline environment to survive for prolonged times in Dead Sea brines.

Proof of vegetative growth of mycelia in the Dead Sea is still missing, and growth experiments also showed that even the best-adapted species do not grow in undiluted Dead Sea water. We do not know whether indeed fungi did grow in the upper water layers of the Dead Sea at the time of the blooms of *Dunaliella* and red halophilic Archaea that developed in 1980 and in 1992 following massive rain floods diluting the upper meters of the water column by 15% and 28%, respectively. However, based on the information reviewed above, it appears that a potential for fungal growth in the Dead Sea does exist. Development of mycelium may be possible only during rare episodes in which the salinity of the upper water layers becomes reduced as a result of massive freshwater inflow. In addition, areas near the mouth of the Jordan River and freshwater springs around the lake may be dilute havens where fungi may get the chance to germinate, grow, develop mycelia, and mature conidiophores in the diluted brines and complete their life cycle.

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Chapter 7
The Mycobiota of the Salterns

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Contents

Abstract Solar salterns are constructed as shallow multi-pond systems for the production of halite through evaporation of seawater. The main feature of salterns is the discontinuous salinity gradient that provides a range of well-defined habitats with increasing salinities, from moderate to hypersaline. These present one of the most extreme environments, because of the low levels of biologically available water and the toxic concentrations of ions. Up to the year 2000, hypersaline environments were considered to be populated almost exclusively by prokaryotic

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microorganisms till fungi were reported to be active inhabitants of solar salterns. Since then, numerous fungal species have been described in hypersaline waters around the world. The mycobiota of salterns is represented by different species of the genus *Cladosporium* and the related meristematic melanized black yeasts, of non-melanized yeasts, of the filamentous genera *Penicillium* and *Aspergillus* and their teleomorphic forms (*Eurotium* and *Emericella*), and of the basidiomycetous genus *Wallemia*. Among these, two species became new model organisms for studying the mechanisms of extreme salt tolerance: the extremely halotolerant ascomycetous black yeast *Hortaea werneckii* and the obligate halophilic basidiomycete *Wallemia ichthyophaga*.

7.1 Introduction

7.1.1 The Saltern Ecosystem

Thalassohaline hypersaline environments are generally considered to be those originating by evaporation of sea water and with halite (NaCl) concentrations of greater than 10% (m/w) (Oren 2002). These provide some of the most extreme habitats in the World. They are common all around the globe, and include, for example, marine ponds and salt marshes that are subjected to evaporation, salt or soda lakes, and sea-salt and man-made salterns (Trüper and Galinski 1986). Solar salterns, the focus of this chapter, are composed of multiple shallow ponds that are located in tropical, subtropical, and temperate parts of the World. In these ponds, the NaCl is gradually concentrated as the seawater evaporates.

Salt production in salterns usually begins with seawater as the initial source of brine, which is evaporated through a series of ponds, to the final pond where the NaCl and other salts precipitate out of the saturated brine. The bittern that remains after this crystallization of the halite is rich in magnesium chloride and provides a special ecological habitat within the salterns. Variable water activities (a_w) because of the increasing concentrations of NaCl, as well as the low oxygen concentrations and high light intensity, present life-limiting parameters in salterns (Brock 1979).

The Sečovlje solar salterns (Fig. 7.1a) were established in the ninth century as a man-made system of ponds, and they are located in the northern Adriatic Sea, in the Gulf of Trieste in Slovenia. The sub-Mediterranean climate and unstable weather conditions allow the production of salt only during the summer, when climate is arid. The strong local winds enhance evaporation of water and keep the temperatures of the water in brines moderate (18–32°C). When the water reaches a suitable concentration of salt, it is directed over the wooden barriers to the next of the 16 evaporative ponds separated by canals. These are the only salterns along the eastern Adriatic coast where salt is produced according to the traditional procedures, with the daily gathering of the saturated brine on the cultivated microbial mat covering the salterns, known as the petola. The role of the petola is dual; it prevents the mixing of the crystallized halite with the mud at the bottom of
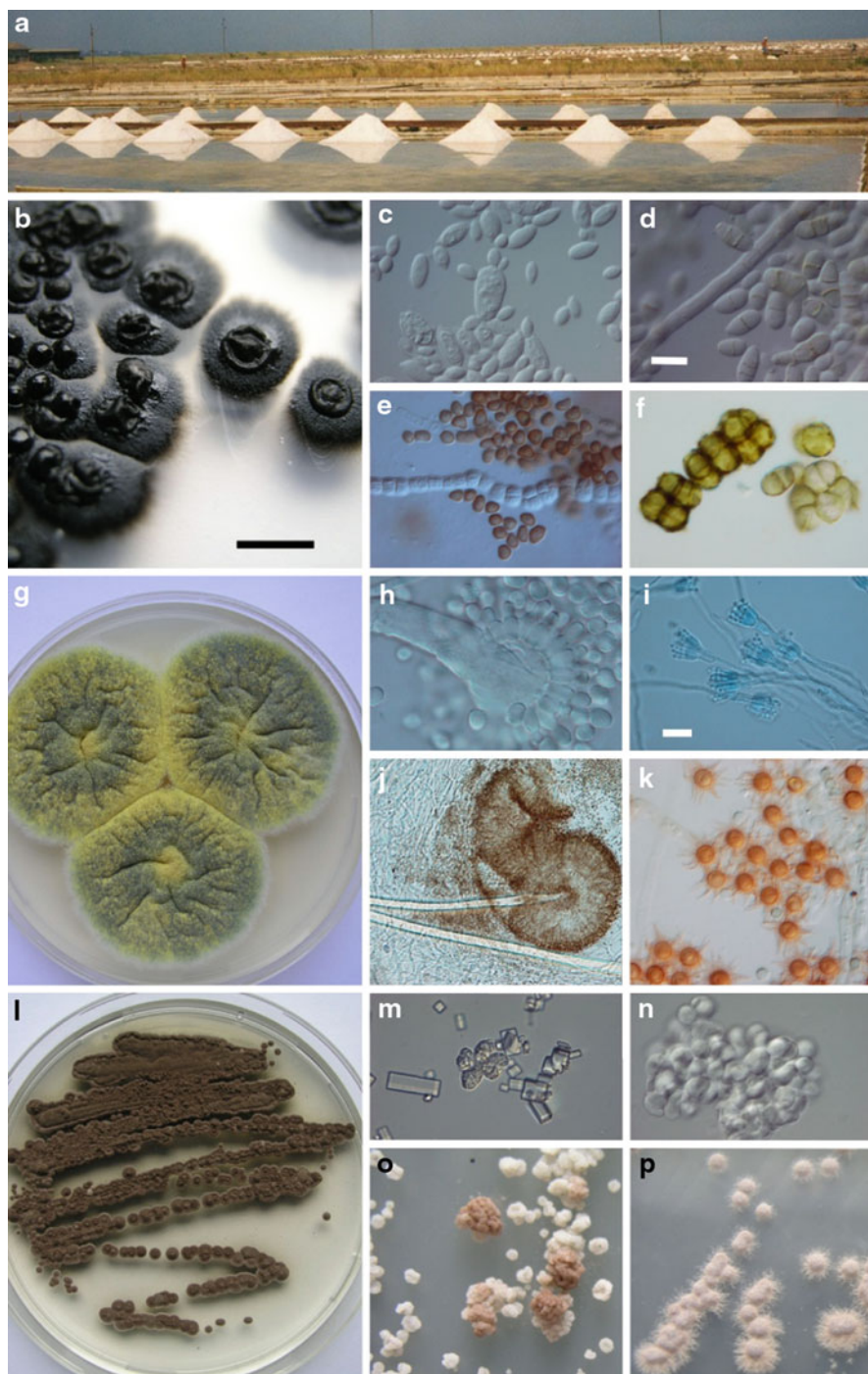
Fig. 7.1 Representatives of the saltern mycobiota. (**a**) salterns Sečovlje; (**b**) *Hortaea werneckii* colonies; (**c**) *Aureobasidium pullulans* budding cells; (**d**) *Hortaea werneckii* budding cells;

the ponds, and it prevents the incorporation of undesired ions of iron and manganese into the halite crystals (Pahor and Poberaj 1963).

The solar salterns Sečovlje with their successive evaporation ponds, the salt-pan mud, and the wooden fences provide a relatively simple ecosystem that is popular for studies of halotolerant and halophilic microorganisms.

These hypersaline waters in salterns were at first believed to be populated almost exclusively by archaea, bacteria, and the eukaryotic alga *Dunaliella salina* (Rodriguez-Valera et al. 1981; Javor 1989; Oren 2005). However, the high diversity of eukaryotic microorganisms in hypersaline waters became evident soon after fungi were first reported as active inhabitants of solar salterns (Gunde-Cimerman et al. 2000). Numerous halotolerant and halophilic fungi were initially isolated from the Sečovlje solar salterns (Gunde-Cimerman et al. 2000), and later also from salterns around the World: La Trinidad in the Ebro River Delta and Santa Pola on the Mediterranean coast of Spain, Camargue in France, and the salterns on the Atlantic coast in Portugal, and in Namibia, the Dominican Republic and Puerto Rico (Butinar et al. 2005a, b, c; Cantrell et al. 2006).

Numerous halotolerant and extremely halotolerant fungi (Zalar et al. 1999a, b, c; Gunde-Cimerman et al. 2000; Butinar et al. 2005a, b, c; Zalar et al. 2005b, 2007, 2008a, b; Butinar et al. 2011) and a few halophilic representatives (Zalar et al. 2005a) have been isolated from these hypersaline waters.

The xerophilic fungi that can grow at low a_w were previously known only from domestic environments, as they can contaminate food preserved through the reduction of the biologically available water, by means of drying, freezing, or solute addition (Pitt and Hocking 1977, 1997). It was assumed that these food-borne fungi that can grow at low a_w reflect a general xerophilic phenotype, and that they would therefore not populate natural hypersaline environments.

7.1.2 The Definition of Halophily in Fungi

Over the years, several definitions for the description of the diverse abilities of fungi to adapt to a wide range of salt concentrations have been proposed. For these fungi that prefer reduced a_w, the most commonly used adjectives were xerophilic and osmophilic, as these fungi can grow at a_w below 0.85; this corresponds to 17% NaCl or 50% glucose added to a growth medium (Gunde-Cimerman et al. 2005). However, these fungi are not only able to grow at low a_w; some also show preferences

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Fig. 7.1 (continued) (**e**) *Phaeotheca triangularis* conidia and hyphae; (**f**) *Trimmatostroma salinum* meristematic clumps; (**g**) *Eurotium amstelodami* in culture; (**h**) *Eurotium chevalieri* anamorph; (**i**) *Penicillium crustosum* conidiophores; (**j**) *Aspergillus niger* conidiophores; (**k**) *Emericella stella-maris* ascospores; (**l**) *Wallemia muriae* in culture; (**m**, **n**) *W. ichthyophaga meristematic clumps;* (**o**) *W. ichthyophaga* colonies; (**p**) *W. sebi* colonies. Scalebar indicated in (**b**) is 10 mm, and also applies for (**o**, **p**); scalebar on (**d**) indicates 10 μm, and is also valid for (**c–h**, **k**, **n**); scalebar on (**i**) indicates 20 μm and is valid for (**i**, **j**, **m**)

for certain chemical natures of the solutes that lower the a_w (de Hoog et al. 2005; Gunde-Cimerman and Plemenitaš 2006). Hence these osmotolerant/osmophilic fungi can live in environments that are rich in sugar, whereas those that are halotolerant/halophilic can live in environments that are rich in salt. In contrast to obligate halophilic archaea and bacteria, in the fungal kingdom, no evidence of obligate requirements for salt was reported until only recently. When fungi were found to constitute active communities in the hypersaline water of solar salterns, they were considered halophilic if they were regularly isolated from water at 17–32% NaCl, primarily on saline selective media, and if they were able to grow *in vitro* on 17% NaCl (Gunde-Cimerman et al. 2000). These fungi were then shown to sustain a range of different salt concentrations, right across the whole salinity range. Later, a few fungal species that show superior growth on media with NaCl as the controlling solute and necessarily require lowered a_w for growth were first reported (Zalar et al. 2005a); hence the term halophilic fungi was challenged again. Thus, halotolerant and extreme halotolerant are now the terms used to describe fungi that can grow across a range of different salt concentrations, even from fresh water to NaCl saturation (Gunde-Cimerman and Plemenitaš 2006), and the term halophily remains reserved for those that require salt for growth.

7.2 The Mycobiota of the Salterns

7.2.1 Methods for the Isolation of the Fungi from the Salterns

To avoid selection of certain mycobiota that are favored by a particular method, four different methods of isolation have often been used in the studies of fungal diversity in hypersaline brines: (a) filtration of the saline and placing the membrane filter on different selective media; (b) agar baits in dialysis tubing and glass tubes that were left for 5 months in crystallization ponds and then placed on media; (c) enrichment of saline with glucose and yeast extract; and (d) spreading of biofilms on selective media (Gunde-Cimerman et al. 2000).

The selective media have low a_w (at least 0.89, and lower) because of the supplements of either salt or sugar. In the salt-based selective media, 10–32% NaCl is added to malt-extract yeast agar media, and for the sugar-selective media, these are supplemented with 50–70% glucose, or with 18% glycerol (Gunde-Cimerman et al. 2000; Butinar et al. 2005a). The enumeration of the colony-forming units (CFU) per 100 ml hypersaline water can be performed with a general-purpose medium (dichloran rose bengal chloramphenicol [DRBC]; a_w 1.0) (King et al. 1979) and a medium for the detection of moderate xerophiles (Hocking and Pitt 1980). Bacterial overgrowth is prevented by adding different antibiotics to all of the selective and enumeration media, like chloramphenicol (100 mg l^{-1}). The cultures are incubated for 1–10 weeks at 25°C, and examined every few days (Gunde-Cimerman et al. 2000).

7.2.2 Diversity of the Saltern Mycobiota

After a decade of research into the fungal diversity in salterns, together with new taxa, a number of fungal genera with high diversities of halotolerant and halophilic species have been described. The melanized fungi isolated are represented by meristematic black yeast-like hypomycetes of the following species: *Hortaea werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum*, *Aureobasidium pullulans*, and the phylogenetically related genus *Cladosporium* (Gunde-Cimerman et al. 2000).

A number of nonmelanized yeast species have been described for the different salterns and salt lakes worldwide. The most abundant among these are *Pichia guilliermondii*, *Debaryomyces hansenii*, and *Candida parapsilosis*, to name but a few (Butinar et al. 2005a). Among the filamentous fungi that appear with the highest frequencies there are different species of the genera *Aspergillus* and *Penicillium* (Butinar et al. 2011). Representatives of the newly described basidiomycetous class Wallemiomycetes cover the order *Wallemiales* with the genus *Wallemia*, and these were recently isolated from hypersaline waters of man-made salterns on different continents in Europe, Asia, Africa, and North America. Interestingly, the genus *Wallemia* represents one of the most xerophilic and halophilic fungal taxa known to date (Zalar et al. 2005a). Morphology and growth features of some of these fungi are shown in Fig. 7.1b–p. Their geographical distribution and salinity range for growth are listed in Table 7.1.

7.2.3 The Black Yeasts

Black yeasts are a group of dark pigmented (melanized) polymorphic hyphomycetes that besides filamentous have also the ability of yeast-like growth. Their slowly expanding melanized colonies have been detected in different extreme environments, such as on rock surfaces in arid, semi-arid, hot, and cold desserts that were previously considered to lack eukaryotic extremophiles (Gorbushina et al. 1996; Sterflinger and Krumbein 1997; Sterflinger et al. 1999).

These black yeasts are characterized by thick, melanized cell walls, with slow, often meristematic, growth and proliferation with endoconidiation. A similar morphology, which is regarded as an "extremophilic ecotype," was observed also with fungi isolated from the hypersaline waters of salterns (Gunde-Cimerman et al. 2000). Because of their morphological plasticity and polymorphic anamorphic stages, their certain identification can only be correctly performed by complementing morphological criteria with molecular methods and physiological tests of carbon and nitrogen metabolism without or with high NaCl concentrations. The morphological types of black yeasts isolated from the Sečovlje salterns were speculated to be different species, as they showed different growth patterns in the presence of salt. This was confirmed by additional molecular data that were obtained by sequencing the internal

Table 7.1 The most frequent fungal species isolated from hypersaline water of salterns across the globe

Group/class	Order	Species	Salinity range (NaCl) (%)	Geographical distribution											
				Slovenia	Spain		France	Portugal	Namibia	Dominican Republic		Puerto Rico	Israel	Bosnia and Herzegovina	USA (Utah)
				Sečovlje	La Trinidad	Santa Pola	Camargue	Samouco	Skeleton coast	Salterns	Lake Enriquillio	Cabo Rojo	Eilat	Ston	Great Salt Lake
Ascomycota	Capnodiales	*Hortaea werneckii*	0–32	L X	X	X	X	X	X		X	X			
		Phaeotheca triangularis	0–26	L X	X	X	X	X	X		X	X			
	Helotiales	*Trimmatostroma salinum*	0–24	L X											
	Dothideales	*Aureobasidium pullulans*	0–18	L X	X	X	X	X	X		X	X			
	Capnodiales	*Cladosporium cladosporioides*	0–17[a]	S X			X				X	X		X	
		Cladosporium herbarum	nd												
		Cladosporium oxysporum	nd							X	X	X	X		
		Cladosporium sphaerospermum	0–20	S X						X		X			
		Cladosporium halotolerans	0–20	S X		X			X		X			X	
		Cladosporium dominicanum	0–20	S						X	X				
		Cladosporium velox	0–20	S X											
		Cladosporium psychrotolerans	0–17	S X						X					
		Cladosporium spinulosum	0–17	S X											
		Cladosporium salinae	0–17	S X		X									
		Cladosporium fusiforme	0–17	S X											
		Cladosporium ramotenellum	nd	X											
		Cladosporium subinflatum	nd	X											
		Cladosporium tenuissimum	nd	X					X						
		Cladosporium tenellum	nd										X		
		Cladosporium herbaroides	nd	X									X		
		Cladosporium macrocarpum	nd	X											
		Cladosporium subtilissimum	nd	X											
		Cladosporium bruhnei	nd	X											
	Saccharomycetales	*Candida parapsilosis*	0–17[a]	S X				X							
		Debaryomyces hansenii	0–17[a]	S					X						X
		Metschnikowia bicuspidata	nd												X
		Pichia guilliermondii	0–17[a]	S X			X						X		
		Yarrowia lipolytica	nd												

(continued)

Table 7.1 (continued)

Group/class	Order	Species	Salinity range (NaCl) (%)		Slovenia Sečovlje	Spain La Trinidad	Spain Santa Pola	France Camargue	Portugal Samouco	Namibia Skeleton coast	Dominican Republic Salterns	Dominican Republic Lake Enriquillio	Puerto Rico Cabo Rojo	Israel Eilat	Bosnia and Herzegovina Ston	USA (Utah) Great Salt Lake
	Eurotiales	*Eurotium amstelodami*	0–27.5	S	X	X	X			X	X	X		X		X
		Eurotium chevalieri	0–22.5	S	X	X	X									
		Eurotium herbariorum	0–25	S	X	X								X		
		Eurotium rubrum	0–20	S	X											
		Eurotium repens	0–25	S	X	X										
		Eurotium halotolerans	0–27.5	S												
		Emericella filifera	0–17	S												
		Emericella stella-maris	0–17	S												
		Aspergillus niger	0–17[a]	S	X	X	X	X			X		X			
		Aspergillus flocculosus	0–17[a]	S	X						X	X				
		Aspergillus caesiellus	0–17[a]	S	X											
		Aspergillus ochraceus	0–17[a]	S	X	X	X						X			
		Aspergillus flavus	0–17[a]	S	X	X							X			X
		Aspergillus roseoglobulosus	0–17[a]	S							X					
		Aspergillus tubingensis	0–17[a]	S	X								X	X		
		Aspergillus melleus	0–17[a]	S									X			
		Aspergillus sclerotium	0–17[a]	S												
		Aspergillus versicolor	0–17[a]	S										X		
		Aspergillus sydowii	0–17[a]	S						X	X			X		
		Aspergillus wentii	0–17[a]	S	X	X					X					
		Aspergillus flavipes	0–17[a]	S									X			
		Aspergillus terreus	0–17[a]	S												
		Aspergillus candidus	0–17[a]	S	X	X							X			
		Aspergillus penicillioides	0–17[a]	S	X								X			
		Aspergillus restrictus	0–17[a]	S	X											
		Aspergillus proliferans	0–17[a]	S	X											
		Aspergillus nidulans	0–17[a]	S									X			
		Aspergillus fumigates	0–17[a]	S	X											
		Penicillium chrysogenum	0–17[a]	S	X	X	X							X		

		Species	Range		Ref1	Ref2	Ref3	Ref4	Ref5	Ref6
		Penicillium brevicompactum	0–17[a]	S						
		Penicillium citrinum	0–17[a]	S	X			X	X	X
		Penicillium oxalicum	0–17[a]	S			X			X
		Penicillium steckii	0–17[a]	S	X					
		Penicillium sizovae	0–17[a]	S	X					
		Penicillium westlingii	0–17[a]	S	X					
		Penicillium freii	0–17[a]	S	X					
		Penicillium cyclopium	0–17[a]	S	X					
		Penicillium solitum	0–17[a]	S	X					
		Penicillium albocoremium	0–17[a]	S	X					
		Penicillium crustosum	0–17[a]	S	X					
Basidiomycota	Wallemiales	*Wallemia sebi*	0–27	L	X	X		X		
		Wallemia muriae	0–25	L	X	X			X	
		Wallemia ichthyophaga	9–32	L	X					X
	Tremellales	*Trichosporon mucoides*	nd							
	Sporidiobolales	*Rhodosporidium babjevae*	0–13[a]	S	X	X				
		Rhodosporidium sphaerocarpum	0–17[a]	S	X	X				

Data were adopted from Gunde-Cimerman et al. (2000); Butinar et al. 2005a, b, c, 2011; Diaz-Munoz and Montalvo-Rodriguez 2005; Zalar et al. 2005a, 2007, 2008a; Cantrell et al. 2006; Schubert et al. 2007, and unpublished data (Gostinčar, personal communication)

nd no data; *X* the presence of the species in the salterns; *L* salinity range determined in the liquid media; *S* salinity range determined in solid media

[a] Maximum tolerated NaCl concentration not determined

transcribed spacer (ITS) rDNA and by restriction fragment length polymorphism (RFLP) of small-subunit (SSU) and ITS rDNA. The representatives of the black yeasts identified include: *H. werneckii* (Zalar et al. 1999c) and *P. triangularis* (Zalar et al. 1999a; c), both comprise the order *Capnodiales*; *A. pullulans* (Zalar et al. 1999c), from the order *Dothideales*; and *T. salinum*, a newly described species from the order *Helotiales* (Zalar et al. 1999b).

Initially, these black yeasts were isolated from the Adriatic salterns, but they were also later identified in hypersaline waters of six salterns on three continents. These were La Trinidad in the Ebro River Delta and Santa Pola on the Mediterranean coast of Spain, Camargue in France, the Atlantic coast in Portugal, and in Namibia and the Dominican Republic. Exceptionally, *T. salinum* was detected only in the Adriatic salterns (Zalar et al. 1999b). *H. werneckii* represents 70–80% of all of the isolates, followed in abundance by *T. salinum* and *P. triangularis*, while *A. pullulans* appears mainly at lower salinities (Butinar et al. 2005b). The extremely halotolerant nature of the black yeasts was first noted when they were isolated from the saline media in the highest numbers according to two peaks, one at the beginning of the sampling season (May), and the other and during the crystallization (August) (Gunde-Cimerman et al. 2000); this was later confirmed by *in vitro* studies.

Hortaea werneckii has also been named as *Cladosporium werneckii*, *Exophiala werneckii*, *Dematium werneckii*, *Pullularia werneckii*, *Aureobasidium werneckii*, *A. mansonii*, *Sarcinomyces crustaceous*, and *Pheoannellomyces werneckii* in the past, but has been because of special conidiogenesis described in a new genus (Nishimura and Miyaji 1983). It was long known as the primary etiological agent of a human surface skin problem called tinea nigra, a superficial fungal infection of the human hand that is frequent in warmer areas of the world. The ecology of *H. werneckii* was linked to the presence of salt, as it was isolated from seawater (Iwatsu and Udagawa 1988), marine fish (Todaro et al. 1983), salted freshwater fish (Mok et al. 1981), and beach soil (de Hoog and Guého 2010). Its ecological niche has been suggested to be intermittently drying salty pools (de Hoog and Gerrits van den Ende 1992) or highly saline water of the crystallization ponds in the solar salterns (Gunde-Cimerman et al. 2000). Among all of the melanized fungi, *H. werneckii* is dominant in the hypersaline waters of the Adriatic salterns and of all of the other salterns that have environmental salinities above 20% (Gunde-Cimerman et al. 2000; Butinar et al. 2005b; Diaz-Munoz and Montalvo-Rodriguez 2005; Cantrell et al. 2006). *H. werneckii* is most abundant (1,400 CFU l^{-1}) at 23% salinity, and remains present with low CFU also after the end of the salt production season. *H. werneckii* is the only black yeast that can grow across the whole range of NaCl concentrations, from 0% to NaCl saturation, with a broad optimum from 6 to 14% NaCl (Butinar et al. 2005b), and it is thus considered to be the most halotolerant fungus so far described.

Trimmatostroma salinum is the only black yeast-like fungus from hypersaline waters that occurs only in the Adriatic salterns, and almost exclusively in one pond, where it was discovered growing on the wooden fence immersed in hypersaline

water (Zalar et al. 1999b, 2005b). *T. salinum* peaks at 25% salinity (700 CFU l^{-1}) and it can appear within broad environmental salinities, as 8–25% NaCl (Butinar et al. 2005b). The broad salinity range for in-vitro growth is from 0 to 24% NaCl, with the optimal values at 2–8% NaCl.

Phaeotheca triangularis was detected in all of the ponds of the Adriatic salterns throughout the year, as well as in crystallizer ponds of other salterns that have been sampled (Butinar et al. 2005b). It shows high adaptability to saline conditions, as it appeared most abundantly (100–340 CFU l^{-1}) in the range of 18–25% NaCl; at lower salinities, its CFUs decrease to less than 50 CFU l^{-1}. In contrast to *H. werneckii*, *P. triangularis* cannot grow *in vitro* on 32% salt, with 24% (or at times also 26%) NaCl reported as its maximum. Its optimal concentration is much narrower, as 6–12% NaCl. *P. triangularis* has been most often isolated from storage ponds with relatively constant salinity. It has also been considered to be an oligotroph, as it can grow on nutritionally poor agar media, and it has been most frequently isolated from low-nutrient storage ponds (Butinar et al. 2005b). *P. triangularis* can form biofilms on solid and liquid saline media, and it frequently appears in sampled microbial biofilms (Gunde-Cimerman et al. 2000; Butinar et al. 2005b). *P. triangularis* is thus an extremely halotolerant species with a narrow ecological amplitude (Gunde-Cimerman et al. 2000).

Aureobasidium pullulans is a cosmopolitan species that can be found in different environments that have fluctuating water activities, such as the phyllosphere (Andrews et al. 1994), foods, feedstuff, bathrooms (Samson et al. 2002), polluted water (Vadkertiova and Slavikova 1995), and others. It has also been found in osmotically stressed environments, such as on rocks and monuments (Urzi et al. 1999), on surface sediments and detritus in salt marshes (Torzilli et al. 1985), and in the hypersaline waters of the Adriatic salterns (Gunde-Cimerman et al. 2000; Butinar et al. 2005b). According to multilocus molecular analysis, the strains of *A. pullulans* from the hypersaline water of the Sečovlje salterns have segregated mostly to the globally ubiquitous variety (var.) *pullulans* (Zalar et al. 2008b). In all of the ponds of the Adriatic salterns and of other sampled salterns, *A. pullulans* occurs with low CFU (up to 50 CFU l^{-1}), and generally at environmental salinities below 8% NaCl. The counts are the highest (800 CFU l^{-1}) at 5% salinity, before or after the salt production season. *A. pullulans* is regarded as a halotolerant species, rather than halophilic, as it can tolerate up to 18% NaCl in vitro and grows optimally on medium without NaCl (Butinar et al. 2005b).

The sampling and inspection of wooden boards that support the walls of the crystallization ponds that are immersed in the hypersaline waters of the active solar salterns of Sečovlje have demonstrated active lignicolous saprobic roles of the halophilic fungi in the hypersaline water. Melanized hyphae in the black-stained parts of the wood from these walls were recognized as belonging to *H. werneckii* and *T. salinum*. These show xylanolytic and lignolytic activities under hypersaline and nonsaline conditions; and *T. salinum* alone shows cellulolytic activity. This

suggests a complementary role of these two black yeasts for the invasion of wood in hypersaline environments (Zalar et al. 2005b).

7.2.4 The Genus *Cladosporium*

The cosmopolitan genus *Cladosporium* is currently in revision process (Crous et al. 2007; Bensch et al. 2010), but is accommodating over 772 different species names (Dugan et al. 2004). It was phylogenetically placed into the order Capnodiales, after the discovery of a teleomorph stage *Davidiella* (Braun et al. 2003). A few of these species, namely *C. herbarum, C. cladosporioides, C. sphaerospermum, C. tenuissimum*, and *C. oxysporum*, have often been isolated from habitats characterized by low a_w, such as sugary and salty foods (Samson et al. 2002), Egyptian salt marshes, the rhizosphere of halophytic plants, and the phylloplane of Mediterranean plants (Abdel-Hafez et al. 1978). The minimal a_w for growth are 0.82, 0.85, and 0.86 for *C. sphaerospermum, C. herbarum*, and *C. cladosporioides*, respectively. In the hypersaline waters of the Sečovlje solar salterns, *Cladosporium* strains are among the most frequently found and are the most abundant of the melanized fungi (Gunde-Cimerman et al. 2000; Butinar et al. 2005b). They have the broadest occurrence among all of the melanized fungi throughout the year in the ponds of the Adriatic salterns, as well as in other hypersaline environments such as Cabo Rojo solar salterns in Puerto Rico (Cantrell et al. 2006). Interestingly, they occur with the highest CFU (1,000–3,600 CFU l^{-1}) between 15 and 25% NaCl, and as the NaCl concentrations increase, they seem to be gradually replaced by different species of black yeasts. As these were mainly obtained from sugar-based media, they are considered as xerotolerant or xerophilic. First, *C. herbarum, C. cladosporioides, C. sphaerospermum*, and *C. oxysporum* were identified (Butinar et al. 2005b; Cantrell et al. 2006). Then with the different taxonomic methods that were performed later, *C. ramotenellum, C. tenellum, C. subinflatum*, and *C. herbaroides* were also shown to be part of the hypersaline mycobiota (Schubert et al. 2007).

Strains at that time identified as *C. sphaerospermum*, which was later recognized as a species complex of cosmopolitan as well as specialized air-borne species, have been reported from a wide range of habitats, including osmotically non-stressed niches, and they show pronounced osmotolerant behavior as they can grow at very low a_w (0.82) (Hocking et al. 1994).

In a wide ecological and phylogenetical study of isolates from the hypersaline waters from Mediterranean salterns, from different coastal areas along the Atlantic Ocean, and from the Red Sea, the Dead Sea, and the salt Lake Enriquillio (Dominican Republic), seven more species of the *Cladosporium* genus were newly described for these hypersaline water: *C. halotolerans, C. dominicanum, C. velox, C. psychrotolerans, C. spinulosum, C. salinae*, and *C. fusiforme*. *C. psychrotolerans* and *C. spinulosum* are currently known only from hypersaline water. Interestingly, a pathogen on the human skin, *Cladosporium langeronii* (= *Hormodendrum langeronii*), is halotolerant, although it has not yet been recorded from hypersaline environments. The maximum NaCl concentration in the growth media for the

development of colonies of these various halotolerant *Cladosporium* representatives has been reported to be 17–20% (Zalar et al. 2007).

7.2.5 The Nonmelanized Yeasts

Only a few reports on the existence of the osmophilic yeasts in natural hypersaline waters have appeared in the literature, although these yeasts have been recognized for their tolerance to high concentrations of sugars or salt. A few species, such as *Torulopsis famata, Rhodotorula rubra, Pichia etchelsii, C. parapsilosis*, and *D. hansenii*, are known to be able to grow above 10–15% NaCl (Samson et al. 2002). Most strains of *Metschnikowia bicuspidata* var. *bicuspidata* have been found to be associated with diseased brine shrimps (*Artemia salina*) from salt lakes and ponds with 10% NaCl, and they appear to require 2% NaCl to grow in vitro (Blackwell 2001). Lahav et al. (2002) described two yeasts, *Pichia guilliermondii* and *Rhodotorula mucilaginosa,* that can survive the extremely high salinity (between 3% and saturation) and pH (2.0–10.0) fluctuations.

Until 2005, there was no evidence that natural hypersaline brines contain nonmelanized yeast populations. Then, yeast diversity was reported for hypersaline waters of eight different salterns and three salt lakes worldwide. These were the Dead Sea, Enriquillo Lake in the Dominican Republic, and the Great Salt Lake in Utah, USA (Butinar et al. 2005a). This diversity included *P. guilliermondii, D. hansenii, Yarrowia lipolytica, M. bicuspidata, C. parapsilosis, Rhodosporidium sphaerocarpum, Rhodosporidium babjevae*, and *Trichosporon mucoides*. In bittern (water rich in magnesium chloride) from the La Trinidad salterns (Spain), two new species were discovered and provisionally named as *Candida atmosphaerica*-like and *Pichia philogaea*-like. Among the isolates obtained, *P. guilliermondii, D. hansenii, Y. lipolytica*, and *C. parapsilosis* were already known contaminants of low a_w food, whereas *R. sphaerocarpum, R. babjevae*, and *T. mucoides* were identified for the first time in hypersaline habitats. Moreover, the ascomycetous yeast *M. bicuspidata*, which is known to be a parasite of the brine shrimp, was isolated as a free-living form from Great Salt Lake brine. The frequency of its occurrence was low, as the counts only occasionally reached several hundred cells per liter across all of sampled salterns (on average, between 0 and 300 CFU l^{-1}). The most frequently occurring species in the Adriatic salterns were identified as *P. guilliermondii* (up to 270 CFU l^{-1}) and *C. parapsilosis*. They have also been sporadically isolated in other salterns (Butinar et al. 2005a).

In contrast to the melanized fungi that appear in the highest numbers during the crystallization in the solar salterns (Gunde-Cimerman et al. 2000; Butinar et al. 2005b), nonmelanized yeasts were isolated primarily outside the salt production season (Adriatic salterns) or in waters with NaCl concentrations below 20% (Butinar et al. 2005a). In addition, these yeasts were never isolated on 32% NaCl medium, but mainly on medium with 10% NaCl, and to a lesser extent on media with 17–25% NaCl (Butinar et al. 2005a).

7.2.6   The Order Eurotiales

The group of filamentous fungi that have been isolated from different salterns around the World is mainly represented by the order *Eurotiales*, or more precisely, by the teleomorphic genera *Eurotium* and *Emericella* and the anamorphic *Aspergillus,* and *Penicillium.* Tolerance for high salt concentrations has for a long time been recognized for many species of the ubiquitous food-borne genera *Aspergillus* and *Penicillium* (Tresner and Hayes 1971), but only recently have their biodiversity, together with their teleomophic forms, been investigated in the hypersaline waters of salterns from different geographical locations (Cantrell et al. 2006; Butinar et al. 2011).

The mycotoxin-producing genus *Eurotium* was for a long time known to comprise species that are contaminants of foods and feedstuffs that are preserved by high concentrations of sugar or NaCl. They can grow at a_w as low as 0.7, and are therefore considered xerophilic (Pitt and Hockering 1997). Occasionally, different *Eurotium* species have been isolated from natural habitats with low a_w, including saline soils and waters (Abdel-Hafez et al. 1978) and the Dead Sea (Kis-Papo et al. 2001). In a study of the mycodiversity of hypersaline water of different salterns from Europe, Asia, Africa, and North America, six different *Eurotium* species were identified, namely *E. amstelodami, E. chevalieri, E. herbariorum, E. rubrum, E. repens*, and *Eurotium* sp. The last one is probably a new species, and it has tentatively been named "*E. halotolerans.*" Most (74%) of the 208 *Eurotium* spp. isolates obtained belonged to *E. amstelodami*, followed by *E. repens* and *E. herbariorum* (10% of the isolates). These three species are probably part of the indigenous mycobiota of salterns, while other *Eurotium* species are believed to be only temporal inhabitants or occasional air-borne contaminants of low-salinity brines. During the salt production season, two pronounced peaks of occurrence appear: first, at the 10–15% NaCl range, with counts up to 5,000 CFU l^{-1}, and then in the 18–25% NaCl range, with the highest counts (up to 30,000 CFU l^{-1}); outside the season of salt production, the counts for all of the *Eurotium* species remain below 100 CFU l^{-1}. The salinity growth ranges determined in vitro are broad, ranging from 0 up to 27.5% NaCl, and the spores and mycelia of these species can survive long-term exposure to solutions of up to 30% NaCl, with growth stimulated up to 10% NaCl for *E. rubrum*, *E. chevalieri*, and *E. amstelodami* and up to 12.5% NaCl for "*E. halotolerans,*" *E. repens*, and *E. herbariorum. E. amstelodami* and "*E. halotolerans*" have the broadest salinity range, growing up to 27.5% NaCl, followed by *E. repens* and *E. herbariorum* (up to 25%), and *E. chevalieri* (22.5%) and *E. rubrum* up to 20% (Butinar et al. 2005c). Some of the *Erotium* species (e.g., *E. amstelodami* and *E. rubrum*) prefer media with sugar to media with NaCl, indicating that they have a xerophilic, rather than a halophilic, character (Wheeler and Hocking 1988).

The representatives of genus *Emericella*, which are recognizable by hülle cells in the chleistothecial walls and ornamented ascospore, have frequently been isolated from dry substrata in hot and arid areas worldwide. These appear to be well adapted to dry and warm climates (Samson and Mouchacca 1974) and low a_w (Zalar et al. 2008a). The soil representative *E. nidulans* was isolated also from desert saline soil

(Samson and Mouchacca 1974), while from the hypersaline water of the Sečovlje salterns in Slovenia, two newly described halotolerant species, *E. filifera* and *E. stella-maris*, were reported. The ascospores of *E. filifera* form long appendages that emerge radially from narrow stellate crests, and the ascospores of *E. stella-maris* have star-shaped equatorial crests (Zalar et al. 2008a). *E. striata* was obtained from Lake Enriquillo in Dominican Republic (Butinar et al. 2011).

The genus *Aspergillus* inhabits hypersaline waters that cover the greatest global diversity. *A. niger* and *A. caesiellus* contribute to the stable fungal communities in natural hypersaline waters, while *A. ochraceus*, *A. flavus*, *A. roseoglobulosus*, and *A. tubingensis* are primarily or exclusively present in hypersaline localities at higher environmental temperatures. *A. melleus*, *A. sclerotiorum*, and holomorphic species *Petromyces alliaceus* have been recognized within these taxonomic groups, although they have appeared only locally. *A. versicolor* and *A. sydowii* are both common in marine environments and in dry foods, and these have also been identified as part of the fungal communities in the hypersaline environments. *A. wentii*, *A. flavipes*, *A. terreus*, and particularly *A. candidus* have been repeatedly isolated from Adriatic salterns, whereas *A. penicillioides*, *A. proliferans*, and *A. restrictus* have been found only sporadically at salinities below 10% NaCl. *A. fumigates* is common in arid environments (deserts) at high temperatures, and has been found consistently in solar salterns, although it is also most abundant at salinities below 10% NaCl (Butinar et al. 2011).

Although many species in the subgenus *Penicillium* grow well in salted foods, only five species have been recognized as part of the indigenous fungal communities here. *P. chrysogenum* is a common, widely distributed species, which appears regularly in saline lakes and salterns worldwide, while *P. brevicompactum* is particularly common in Adriatic salterns. The other three species, two of which are most probably new, are seen consistently at low counts or at low salinities, or appear in hypersaline waters only sporadically. A series of soil-borne *Penicillium* species has also been identified as part of the hypersaline mycobiota, namely *P. citrinum*, *P. oxalicum*, and *P. steckii*. Although *P. sizovae* and *P. westlingii* are also common, their counts decrease with increased salinity and they are therefore considered as temporal inhabitants. Four Penicillia isolates identified as *P. nordicum* were isolated from salt used for human consumption and from the salting of foods, at counts of 5 CFU g^{-1} salt crystals (Butinar et al. 2011).

To conclude, in hypersaline environments, the pan-global stable mycobiota of the order *Eurotiales* are represented by *A. niger*, *E. amstelodami*, and *P. chrysogenum*, and possibly also by *A. sydowii*, *A. candidus*, and *E. herbariorum*, which are also quite abundant, although more locally distributed (Butinar et al. 2011).

7.2.7 The Genus Wallemia

Fungi from the genus *Wallemia* are xerophilic food- and air-borne fungi (Samson et al. 2002) that have also been isolated from soil (Domsch et al. 1990), sea salt (DasSarma et al. 2010; Butinar et al. 2011), and hypersaline water (Zalar et al. 2005a).

Until recently (Zalar et al. 2005a), a single cosmopolitan species *Wallemia sebi* was recognized in the genus which was known as the causative agent of allergological problems arising in farmer's lung disease (Lappalainen et al. 1998; Roussel et al. 2004). Its unresolved phylogenetic position was worked out when a large group of strains were collected globally from environments with low a_w (e.g., foods, hypersaline waters from different part of the World) and the sequence data of their different genes were analyzed (Zalar et al. 2005a; Matheny et al. 2006). On the basis of their unique morphology, xerotolerance, and isolated phylogenetic position, a new basidiomycetous class of Wallemiomycetes and the order *Wallemiales* were proposed for the genus *Wallemia*.

The genus *Wallemia* is an early diverging lineage of Basidiomycota and it segregates into at least three species that have been identified so far: *W. ichthyophaga, W. sebi*, and *W. muriae*. These species have been isolated consistently, although with low counts (maximally, 40 CFU l^{-1}), from hypersaline waters of salterns in Slovenia, Spain, the Dominican Republic, Israel, and Namibia. This is particularly extraordinary, because basidiomycetous fungi rarely show xerophilic or halophilic characteristics, as they generally appear outstandingly intolerant to NaCl (Tresner and Hayes 1971). Also within the Ascomycota there are only a few known fungi, such as *Basipetospora halophila*, *Polypaecilum pisce* (Wheeler et al. 1988), and *H. werneckii* (Gunde-Cimerman and Plemenitaš 2006), which are stimulated by NaCl, although with no obligate requirements.

Interestingly, tests on xerotolerance of the *Wallemia* spp. have shown that it represents one of the most xerophilic fungal taxa (Zalar et al. 2005a). The two halophilic *Wallemia* species, *W. muriae* and *W. ichthyophaga,* necessarily require media with low a_w, whereas *W. sebi* grows also on media without additional solutes. The narrow a_w ranges for growth of *W. muriae* and *W. ichthyophaga* are 0.984–0.805 and 0.959–0.771, respectively. Moreover, *W. ichthyophaga* shows preference for certain solutes for the lowered a_w, as it shows poor growth that is characterized by prolonged growth phases and smaller colonies on media with high concentrations of glucose, compared with media with high concentrations of salt (Zalar et al. 2005a; Kralj Kunčič et al. 2010). As it can thrive in media with NaCl necessarily above 1.7 M and up to saturation (5.3 M NaCl), this makes it the most halophilic fungi known to date. *W. sebi* can grow over a wider range of a_w (0.997–0.690) in glucose/fructose media (Pitt and Hocking 1977), but in media with NaCl as the major solute, its lowest a_w for growth has been reported to be 0.80 (Pitt and Hocking 1977; Zalar et al. 2005a), corresponding to 4.5 M NaCl.

7.3 Special Adaptations and Properties of the Fungi in the Salterns

The responses of eukaryotic cells to low a_w and high concentrations of toxic ions involves complex alterations in gene expression that lead to metabolic changes and subsequent adaptation to the new conditions (Yale and Bohnert 2001; Petrovič et al. 2002; Vaupotič and Plemenitaš 2007). The mechanisms of salt tolerance in fungi have

been mostly studied in salt-sensitive *Saccharomyces cerevisiae* (Blomberg and Adler 1992; Blomberg 2000; Hohmann 2002), the halotolerant yeast *D. hansenii* (Larsson and Gustafsson 1987; Andre et al. 1988; Larsson et al. 1990; Larsson and Gustafsson 1993; Prista et al. 1997; Almagro et al. 2000) and the extremely halotolerant black yeast *H. werneckii* (Turk et al. 2004, 2007a; Kogej et al. 2005, 2006a, b, 2007; Plemenitaš et al. 2008). The last species has been proposed as a model organism for eukaryotic halophily studies. Physiological and molecular adaptations of the true halophilic representative known so far, *W. ichthyophaga*, are only at an early stage.

The pathway for the sensing of osmolarity changes is of vital importance for the survival of the cell. In *S. cerevisiae*, this pathway is known as the high-osmolarity glycerol (HOG) signaling pathway, which is also part of one of the best understood mitogen-activated protein kinase (MAPK) cascades (Hohmann 2002). The existence of a similar signaling pathway has been demonstrated and extensively studied in *H. werneckii* (Turk and Plemenitaš 2002; Plemenitaš et al. 2003; Lenassi and Plemenitaš 2005, 2007; Plemenitaš et al. 2008; Fettich et al. 2011). An important level of adaptation to hypersaline environment is the need to balance the osmotic pressure of the medium by accumulating and/or synthesizing compatible organic solutes (Oren 1999) and maintaining low salt concentrations within the cytoplasm. Additional adaptations at the level of the plasma membrane composition (Petrovič et al. 1999; Turk et al. 2004; Gostinčar et al. 2008, 2009) and the cell wall structure (Kralj Kunčič et al. 2010), which represent the first line of defense against environmental stress (Mager and Siderius 2002), are also required to prevent damage of the cells in such environments.

Numerous morphological adaptations that are reflected in the extremophilic ecotype characterized by meristematic growth, pigmentation, and changes in colony morphology are believed to have important roles for successful growth under extremely saline conditions (Kogej et al. 2006a; Kralj Kunčič et al. 2010). The morphology of black yeasts uniquely depends on the environment, as isodiametrical cells are typical in water and hyphal growth is shown only on solid media. This optimization of the volume-to-surface ratio, together with meristematic growth, thick melanized cell walls, extracellular polysaccharides, and adhesion and propagation with endoconidiation, is interpreted as a response to stress factors such as low a_w, low nutrition, and high temperatures (Sterflinger et al. 1999). A study of the morphological adaptations to moderate and high NaCl concentrations of *Wallemia* spp. using different microscopy approaches revealed an impact of high concentrations of NaCl on the cell morphology of the *Wallemia* spp. (Kralj Kunčič et al. 2010). Hyphal compartments of *W. sebi* and *W. muriae* are thicker and shorter, and their mycelial pellets are larger at high salinity. The phylogenetically distinct *W. ichthyophaga* differs from the other two *Wallemia* spp. at the level of its morphology also, as it forms sarcina-like multicellular clumps that are composed of compactly packed spherical cells. The size of the cells does not respond to increased salinity, whereas these multicellular clumps become significantly large. Clustered growth allows the sheltering of the cells in the interior and minimizes the number of cells that are directly in contact with the hostile environment. Therefore, the ability to grow meristematically or in the form of multicellular clumps is

believed to greatly enhance their survival in stressful environments (Wollenzien et al. 1995; Palkova and Vachova 2006).

The presence of extracellular polysaccharides is part of the protection against desiccation in rock-inhabiting fungi (Selbmann et al. 2005), and it might have a protective function at high sanities, as a pronounced extracellular polysaccharide layer has been observed in all the three *Wallemia* spp. (Kralj Kunčič et al. 2010) and for *T. salinum* (Kogej et al. 2006a). Furthermore, an increase in the thickness of the multilayered cell wall occurs at higher salinities in all the three *Wallemia* spp., although it is especially pronounced in *W. ichthyophaga*. The extremely thick cell wall of *W. ichthyophaga* is however an exception in the fungal responses to extremely saline conditions known to date (Kralj Kunčič et al. 2010).

A common strategy of osmoadaptation in response to increased salinity in the environment in most eukaryotic microorganisms, as well as in halotolerant and halophilic fungi, is based on the synthesis and cytoplasmic accumulation of a mixture of polyols as compatible solutes. Among these, glycerol is the most significant, and it has been measured in the highest amounts in the extremely halotolerant black yeast *H. werneckii* (Petrovič et al. 2002; Kogej et al. 2007) as well as in the halophilic *W. ichthyophaga* (our unpublished data). Besides glycerol, *H. werneckii* accumulates erythritol, arabitol, and mannitol (Kogej et al. 2007), and interestingly, mycosporine-glutaminol-glucoside also, as complementary compatible solutes (Kogej et al. 2006b). *H. werneckii* and *A. pullulans* keep their intracellular concentrations of sodium and potassium cations low in environments with high concentrations of salt, and they are therefore considered as Na^+-excluders (Kogej et al. 2005). So far, only the halotolerant yeasts *D. hansenii* (Prista et al. 1997) and *P. guilliermondii* (Lahav et al. 2002) have been shown to maintain relatively high internal concentrations of sodium when coping with salt stress, together with the production and intracellular retention of compatible solutes, particularly glycerol (Prista et al. 1997; Lahav et al. 2002).

Glycerol can easily pass through lipid bilayers because of its small molecular mass. Therefore, eukaryotic cells using glycerol as a compatible solute either accumulate lost glycerol using energetically costly transport systems or change their membrane structure by an increased sterol content or reduced membrane fluidity (Oren 1999). Interestingly, in the black yeasts *H. werneckii* and *P. triangularis*, the total sterol content remains mainly unchanged with increased salinity (Turk et al. 2004), while the plasma membrane is significantly more fluid over a wide range of salinities, in comparison with the membranes of salt-sensitive and halotolerant fungi (Turk et al. 2004, 2007a). Higher plasma-membrane fluidity results from an increase in the unsaturated fatty acid content and length, because of the salt stress (Turk et al. 2004, 2007a). One of the mechanisms that allows the precise regulation of membrane fluidity in *H. werneckii* and *A. pullulans* is the change in the expression of fatty-acid-modifying enzymes, such as desaturases and elongase (Gostinčar et al. 2008, 2009). Similar to halophilic/halotolerant black yeasts, the membrane fluidity in the osmotolerant *D. hansenii* is not significantly affected at high levels of NaCl, although the sterol-to-phospholipid ratio and the

fatty acid unsaturation increase (Turk et al. 2007b). As high salt tolerance correlates well with higher membrane fluidity, this is of crucial importance for tolerance to salt stress.

Hortaea werneckii can grow at very high salinities, which require large amounts of intracellular glycerol, while at the same time it maintains a very fluid membrane and a constant sterol content. Instead of modifying its membrane properties *H. werneckii* uses its melanized cell wall to reduce glycerol leakage from cells at the optimal salinities (Kogej et al. 2007). The outer part of the melanized cell wall has a continuous layer of melanin granules that minimizes glycerol loss from the cells, as this layer creates a mechanical permeability barrier for glycerol by reducing the size of the pores in the cell wall (Jacobson and Ikeda 2005). At higher salinities, melanization is diminished, and glycerol retention is less effective; therefore, the growth rates and biomass yields of *H. werneckii* are reduced (Kogej et al. 2007).

7.4   The Ecological Types of Fungi in the Salterns

Fungal species can be classified into three ecological groups according to their abilities to inhabit extreme environments. First, there are the mesophiles, which predominantly inhabit environments without extreme conditions. The second group is represented by the generalists, which tolerate a variety of moderately stressful conditions, but not the extreme ones, and they have their growth optimum under moderate conditions. As a result of their limited competition with mesophiles and their inability to survive the most extreme conditions, they are often predominantly found under moderately stressful conditions. In hypersaline environments, *A. pullulans* is a good example of a generalist, as it can tolerate up to 18% NaCl, but not in saturated NaCl solution, and it grows optimally without NaCl. The third group comprises the specialists, which are extremely halo tolerant or even halophilic, and their growth optima are shifted toward extreme conditions. They predominantly inhabit extreme habitats, as they cannot compete with species in moderate environments, or are simply not able to survive moderate conditions. Examples of the specialists among the hypersaline mycobiota are *H. werneckii*, which grows under extreme (up to saturation) salt conditions as well as without salt, and *W. ichthyophaga*, which necessarily requires 10% NaCl and also grows in saturated NaCl solution. Therefore, adaptive extremophiles with a broad ecological amplitude (e.g., *H. werneckii*) and obligate extremophiles with a narrow ecological amplitude (e.g., *W. ichthyophaga*) have been distinguished. Both *H. werneckii* and *W. ichthyophaga* are found in hypersaline environments, and they are characterized by growth optima at extreme salinity. This narrow-amplitude strategy is more of an exception among the fungal kingdom, because *W. ichthyophaga* is so far the only representative of halophilic fungi with an obligate salt requirement (Gostinčar et al. 2010).

7.5   The Importance of the Saltern Mycobiota

Over the last few years, fungi thriving under conditions that are extreme from an anthropocentric point of view, and which thus live at the so-called ecological periphery, have deserved and received increasing scientific attention. Some halotolerant and halophilic fungi have possible important biotechnological applications. These will arise from studies of their basic characteristics and adaptation mechanisms, at the level of their secondary metabolites, cell membranes, intracellular and extracellular enzymes, genetic transfer systems and intracellular osmolytes, and especially of their compatible solutes, which have a wide range of applications because of their ability to stabilize proteins and nucleic acids (Arakawa and Timasheff 1985; Kurz 2008). The salt-tolerant black yeast *A. pullulans* produces exopolymer pullulan that has a broad spectrum of use in the food and pharmaceutical industry (Leathers 2003; Singh and Saini 2008), and the extremely halotolerant black yeasts *T. salinum* and *H. werneckii* have been shown to produce extracellular hydrolytic enzymes that are active at high salt concentrations and that could therefore have important roles in different industries (Zalar et al. 2005b). *H. werneckii* also produces antibiotic compounds that remain to be commercially exploited (Brauers et al. 2001). Indeed, many halophilic and halotolerant fungi synthesize specific bioactive metabolites under stress conditions, and particularly at increased salt concentrations, when higher hemolytic activities have been seen (Sepčić et al. 2011).

Progressive salinization represents a serious agricultural problem worldwide, as 10 million hectares of arable land is lost in this way annually. Genetic manipulations of crops that have increased salt tolerance have still not yielded satisfactory results. The use of halophilic and halotolerant fungal genetic sources might provide the desired improvement in the breeding of such crops in the future. The halotolerant *H. werneckii* has been shown to be a promising source of salt-tolerant transgenes for agriculture. In yeast, as well as in plants, Hal2 is a sodium- and lithium-sensitive $3'$-phosphoadenosine-$5'$-phosphatase, which is an important determinant for halotolerance (Gläser et al. 1993). Thus, overexpression of novel isoenzymes or of Hal2-like proteins from *H. werneckii* can remarkably increase the halotolerance of *S. cerevisiae* (Vaupotič et al. 2007).

Microorganisms and their metabolites can affect the salt production in the evaporating ponds of salterns, as they can physically affect the evaporation process and as their by-products can chemically modify or bind with the dissolved ions (Javor 2002). Moreover, the biological systems in the salterns can also "contaminate" the salt that is used for food preservation. It has been known for a long time that haloarchaea can be introduced into food via salt and can spoil heavily salted proteinaceous products (Norton and Grant 1988; Grant 2004). Recently, different fungi have been isolated from salt used for human consumption and for food salting (Butinar et al. 2011).

As many fungi from salt and hypersaline water produce the same mycotoxins as those found on the salted meat products, the salt is most probably the contamination source for some toxinogenic fungi (Andersen 1995; Larsen et al. 2001; Butinar

et al. 2011). Precautionary measures should therefore be considered seriously, such as heat treatment of salt prior to adding it to the meat products, to kill the fungal conidia (Butinar et al. 2011).

7.6 Conclusion

The taxonomic and physiological characterization of halotolerant/halophilic fungi isolated from different salterns and hypersaline lakes on three continents have shown a surprising diversity of fungi. The reports of existance of fungi along with other complex microbial communities in the salterns has improved our understanding of interlinked microbial and chemical processes. As evaporation and mineral precipitation are intimately linked to microbial communities and their products, microbial activity, including fungal, can affect both the quality and quantity of salt. The mycobiota can thus be "imported" in natural hypersaline environments through air, but it can also be "exported" as living cells bounded to salt crystals, and in this way contaminating our foods and homes.

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Chapter 8
Morphological Evaluation of Peridial Wall, Ascus and Ascospore Characteristics in the Delineation of Genera with Unfurling Ascospore Appendages (Halosphaeriaceae)

Ka-Lai Pang, Wai-Lun Chiang, and Jen-Sheng Jheng

Contents

Abstract In the Halosphaeriaceae, taxa with unfurling ascospore appendages and related species constitute 61 species (in 21 genera). Recent phylogenetic analyses of the rRNA genes have advanced our knowledge on the relationships between genera in the family, especially the group with unfurling ascospore appendages. However, many new genera resulting from these studies lack distinctive morphological characteristics from closely related taxa. In this chapter, peridial wall layers of the ascomata and morphology of asci and ascospores are re-examined to determine if these structures offer useful information for the delineation of genera. In particular, shape parameters (aspect ratio, convexity, elongation, shape factor, sphericity, area, perimeter, diameter max, diameter mean and diameter min) of ascospores were calculated to determine if these parameters can provide extra characters for the delineation of taxa. Results suggest that peridial wall structure alone is insufficient to separate genera in the Halosphaeriaceae. Shape parameters of ascospores can provide additional characters but more taxa are required to test their efficacy. Ascus shape and length of stalk are further characters that should be calculated for

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taxonomical consideration. Morphology of the ascomatal wall and shape of asci and ascospores in genera with unfurling ascospore appendages in the Halosphaeriaceae are partially concordant with their phylogeny, suggesting a more thorough examination of these characters for the delineation of taxa in the family.

8.1 Introduction

In the book "Biology of Marine Fungi," edited by the late Steve Moss, Jones et al. (1986) evaluated the criteria that could be used to delineate genera in the Halosphaeriaceae and Farrant (1986) studied the morphology of two genera with unfurling ascospore appendages, *Aniptodera* and *Halosarpheia*, based on ultrastructural observations. This chapter re-evaluates the morphology of a larger number of genera with unfurling ascospore appendages. Jones et al. (2009) documented a total of 530 marine fungi, with 142 species (in 56 genera) belonging to the Halosphaeriaceae. Genera with unfurling ascospore appendages include *Aniptodera*, *Ascosacculus*, *Cucullosporella*, *Halosarpheia*, *Magnisphaera*, *Moana*, *Nais*, *Natantispora*, *Oceanitis*, *Ophiodeira*, *Panorbis*, *Phaeonectriella*, *Saagaromyces*, *Tirispora*, *Trichomaris* and *Tunicatispora*. The genera *Anisostagma*, *Iwilsoniella*, *Lignincola*, *Neptunella* and *Thalassogena*, although without ascospore appendages, are morphologically related, in terms of morphology of asci and ascospores (Pang et al. 2003a). A total of 61 species are included in these 21 genera.

For the last decades, sequence analysis of the nuclear ribosomal RNA genes has advanced our knowledge on the phylogenetic relationships between genera in the Halosphaeriaceae, especially the group with unfurling ascospore appendages and resulted in a number of new genera. *Halosarpheia* was split into a number of genera, including: *Ascosacculus* (*A. aquatic*, *A. heteroguttulata*), *Magnisphaera* (*M. spartinae*), *Natantispora* (*N. lotica*, *N. retorquens*), *Oceanitis* (*O. cincinnatula*, *O. unicaudata*, *O. viscidula*), *Panorbis* (*P. viscosus*), *Saagaromyces* (*S. abonnis*, *S. ratnagiriensis*) (Campbell et al. 2003, Pang et al. 2003a, b, Dupont et al. 2009). Unfurling ascospore appendages were inferred to be an unimportant character in the delineation of genera but can be used to delimit species (Pang et al. 2003a). *Nais glitra*, although without appendages, formed a monophyletic group with *Halosarpheia abonnis* and *H. ratnagiriensis* and consequently, *Saagaromyces* was established for these three species (Pang et al. 2003a). After these revisions, *Halosarpheia sensu stricto* currently includes *H. fibrosa*, *H. trullifera* and *H. unicellularis,* while further collection and investigation are required to clarify the taxonomical position of the remaining *Halosarpheia* species. Morphologically, many of the newly established genera are well circumscribed. *Magnisphaera* differs from *Halosarpheia* in having multi-septate ascospores with a rough ascospore wall (Jones 1962, Campbell et al. 2003). Ascospores of *Oceanitis* species are elongate-fusiform, many times septate which differ from all other *Halosarpheia* species (Dupont et al. 2009). *Saagaromyces* is characterized by large ascomata, large asci with a long stalk and large ellipsoidal ascospores (Pang et al. 2003a). On the other

hand, *Ascosacculus*, *Natantispora* and *Panorbis* are morphologically very similar to species assigned to *H. sensu stricto*. Is sequence analysis the only way to separate these genera in the Halosphaeriaceae? Have we carefully examined all the morphological traits for the delineation of the above genera?

The morphological characters defining the Halosphaeriaceae include perithecial ascomata, clavate asci which are mostly deliquescing, presence of catenophyses, ellipsoidal/fusiform, mostly hyaline ascospores with appendages (Spatafora et al. 1998). Taxonomic reviews of the family have been undertaken by many authors, including Kohlmeyer (1972), Jones et al. (1986), Jones (1995) and Pang (2002). Sakayaroj et al. (2011) recently constructed a phylogeny of the Halosphaeriaceae, using all available sequences of the small subunit (SSU) and large subunit (LSU) ribosomal RNA and RNA polymerase II subunit (RPB2) genes, to investigate the phylogenetic relationship between genera. Terminal clades (*H. sensu stricto*, *Oceanitis*, *Remispora* and *Saagaromyces*) were well supported by bootstrap and posterior probability, but internal nodes (intergeneric relationship) were poorly supported. Genera could be separated on the basis of ascospore appendage morphology and ontogeny, while other characters were inferred to be taxonomically unimportant at the generic level, including appendage ontogeny, presence of catenophyses/periphyses and deliquescent/persistent nature of asci. Genera with unfurling ascospore appendages were polyphyletic.

Traditional ascomatal characters used in the classification of marine fungi included colour, shape, texture, neck length and size. In *Ceriosporopsis* and *Lulworthia*, colour of the ascomata was dependent on degree of maturation, and size and shape were affected by the wood type (Meyers 1957, Johnson 1958). Cavaliere and Johnson (1966) discovered that in *Lulworthia grandispora*, ascomata were membranous when grown on birch wood and balsa wood, while they were carbonaceous when grown on Whatman #1 filter paper. Nakagiri and Tubaki (1986) examined the ascomatal wall structure of *Corollospora* species and concluded a basic two-layered peridium, but they considered that this character is not taxonomically significant at the generic level.

Most taxa in the Halosphaeriaceae have deliquescing asci, in particular, oceanic species, which limit its use as a systematic character. Other taxa retain the apical structures (thickening, pore) of asci after their transition from the terrestrial habitats to the sea (Pang 2002). *Aniptodera* is one of the genera that are characterized by asci with an apical thickening, pore, retraction of plasmalemma and thick-walled ascospores (Shearer and Miller 1977). Insufficient attention has been placed on the importance of shape of asci and the length of ascus stalk in the taxonomy of the family. For instance, *Halosarpheia kandeliae* is unique in *Halosarpheia* in having an exceptionally long stalk (Abdel-Wahab et al. 1999). Phylogenetically, it is unrelated to *Halosarpheia fibrosa*, the type species of the genus (Sakayaroj et al. 2011), suggesting that the length of ascus stalk can be an important delineating character at the genus level.

Ascospore shape, colour and wall thickness have been used in the separation of taxa in the Halosphaeriaceae. *Carbosphaerella*, selected *Corollospora*, *Nereiospora* and *Phaeonectriella* are genera with coloured ascospores, but they

were not monophyletic (Sakayaroj et al. 2011). *Phaeonectriella* differs from *Aniptodera* only in the dark-coloured ascospores while other characters are extremely similar (Eaton and Jones 1970). Concerning wall thickness, only selected species of *Aniptodera* possess thick ascospore walls. As a result, these characters offer little resolution in the delineation of genera. In this chapter, peridial wall layer of ascomata and the morphology of asci and ascospores are re-examined to determine if these structures offer useful information for the delineation of genera in the Halosphaeriaceae, especially those with unfurling ascospore appendages.

8.2 Material and Methods

Drift, attached and trapped wood were collected from mangrove forests and rocky shores around Taiwan. Wood samples were placed in zip-lock plastic bags and transported to the laboratory at the National Taiwan Ocean University, Keelung, Taiwan (R.O.C.). Wood pieces were incubated in sealed plastic trays lined with moist tissue paper and observed periodically for sporulating structures.

Morphology of ascomata immersed, erumpent or exposed on wood was observed under an Olympus SZ61 stereomicroscope (Tokyo, Japan) with photographs taken on an Olympus DP20 Microscope Camera (Tokyo, Japan). For sections of fruiting bodies, wood pieces with ascomata were cut out from a larger piece of collected wood and fixed by immersion in FAA solution (5% formaldehyde and 5% glacial acetic acid in 50% ethanol) overnight at 4°C. The fixed samples were rinsed three times in 50% ethanol. The samples were then dehydrated in a graduated t-butanol/ethanol/water series (10/40/50, 20/50/30, 35/50/15, 55/45/0, 75/25/0, 100/0/0, 100/0/0, in percentage), and infiltrated gradually and embedded in paraffin (Paraplast, Leica). Paraffin sections (7–10 μm) were cut on a FRM-200P rotary microtome (Tokyo, Japan), floated on 42°C water bath to relax compression and mounted on microscope slides. Dried sections were deparaffinized and rehydrated through a graded series of ethanol. The sections were then stained with 1% safranin O in 50% ethanol (10 s) and 0.5% Orange G in 95% ethanol (30 s). After washing and dehydration, each stained section was permanently mounted with a cover slip and Permount (Fisher, USA). Specimens were observed on an Olympus BX51 microscope (Tokyo, Japan) and light micrographs taken.

For asci and ascospores, ascomata were cut open with a razor under the stereomicroscope. Centrum material was removed with a fine forceps/needle and mounted in a drop of sterile sea water on a slide. Morphology of asci and ascospores was observed on the compound microscope and photographed. For the quantification of ascospore shape, ascospores were first outlined and shape parameters determined by ANALYSIS v3.2 LS image analysis software (Olympus Soft Imaging Solutions GmbH, Germany). The shape parameters included aspect ratio, convexity, elongation, shape factor, sphericity, area, perimeter, diameter max, diameter mean, diameter min and their definitions are given in Table 8.1. These data
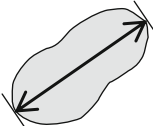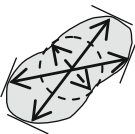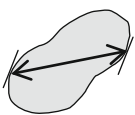
Table 8.1 Calculation methods of shape parameters of ascospores

Max (b/a)	Aspect Ratio: The maximum ratio of width and height of a bounding rectangle for the particle
0.6 0.8 1	Convexity: The fraction of the particle's area and the area of its convex hull
1 5 20	Elongation: The elongation of the particle can be considered as lack of roundness. It results from the sphericity
0.5 0.8 1	Shape Factor: The shape factor provides information about the "roundness" of the particle. For a spherical particle the shape factor is 1, for all other particles it is smaller than 1
1 0.8 0.6	Sphericity: Describes the sphericity or "roundness" of the particle by using central moments
	Area: The area of a particle is (number of pixels of the particles) times (calibration factors in X and Y direction)
	Perimeter: The sum of the pixel distance along the closed outer boundary

(continued)

Table 8.1 (continued)

	Diameter Max: To evaluate the maximum diameter of a particle, the diameter of different evaluation axes will be determined. To evaluation axis is varied in "1" steps and the maximum diameter of each angle is determined
	Diameter Mean: The arithmetic mean of all diameters of particle (for angles in the range 0° through 179° with step width 1°
	Diameter Min: The minimum diameter of a particle (for angles in range 0° through 179° with step width 1°)

were then entered into PASW Statistics 18 (SPSS Inc., Chicago, IL, USA) for a cluster analysis. In brief, the clusters are group of species with similar characteristics. To form the clusters, the procedure began with each species in a separate group. It then combined the two species that presented the closest shape parameters and formed a new group. After recomputing the distance (squared euclidean) between the groups, the two groups then closest together were combined. This procedure was iterated until only one group remained.

8.3 Results and Discussion

8.3.1 Peridial Wall Layer

Peridial wall structure of taxa with unfurling ascospore appendages and related species in the Halosphaeriaceae, based on paraffin sections, is shown in Fig. 8.1. Most species have a one-layered peridium, while in *Aniptodera chesapeakensis*, *A. salsuginosa*, *H. fibrosa*, *P. viscosus*, *Saagaromyces* spp., it is two layered. Cells are mainly elongate or forming *textura angularis*, with or without large lumina. However, the peridial wall of *Aniptodera longispora* and the outer wall layer of *H. fibrosa* are made up of *textura globulosa*, and in *Natantispora retorquens*, the wall is of flattened, rectangular cells.
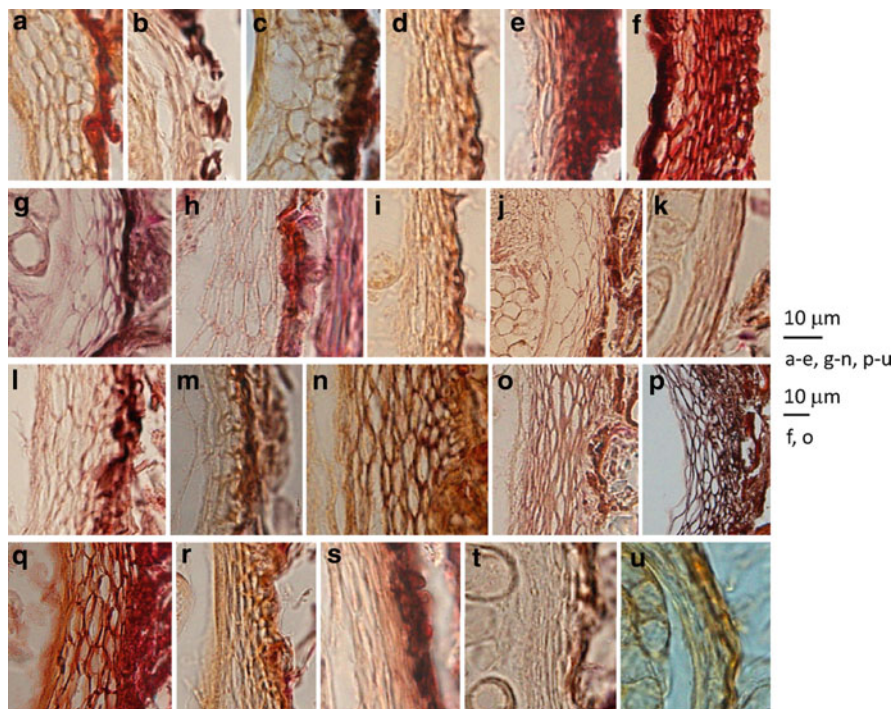
Fig. 8.1 Peridial wall layer. (**a**) *Aniptodera chesapeakensis*, (**b**) *A. lignatilis*, (**c**) *A. longispora*, (**d**) *A. salsuginosa*, (**e**) *Cucullosporella mangrovei*, (**f**) *Halosarpheia fibrosa*, (**g**) *H. marina*, (**h**) *Lignincola laevis*, (**i**) *L. tropica*, (**j**) *Moana turbinulata*, (**k**) *Natantispora retorquens*, (**l**) *Neptunella longirostris*, (**m**) *Oceanitis cincinnatula*, (**n**) *Panorbis viscosus*, (**o**) *Saagaromyces abonnis*, (**p**) *S. ratnagiriensis*, (**q**) *S. glitra*, (**r**) *S. abonnis* c.f., (**s**) *Thalassogena sphaerica*, (**t**) *Tirispora mandoviana*, (**u**) *T. unicaudata*

In *Aniptodera*, *A. longispora*, *A. lignatilis* and *A. salsuginosa* have a different wall layer pattern, when compared with *A. chesapeakensis* (an isolate with ascospore appendages). Sakayaroj et al. (2011) showed that *A. lignatilis* and *A. longispora* did not form a monophyletic group with *A. chesapeakensis* in a phylogenetic analysis of three genes. However, it is unknown if the isolate of *A. chesapeakensis* in the GenBank was with unfurling appendages. *Aniptodera longispora* grouped with *Cucullosporella mangrovei*, while *A. lignatilis* was in a group comprising *Ascosacculus*, *A. chesapeakensis*, *Nais inornata*, *Okeanomyces* and *Thalespora* (Sakayaroj et al. 2011). Peridial wall of *C. mangrovei* is composed of flattened, polyhedral cells (Fig. 8.1e), which is different from *A. longispora* (Fig. 8.1c). In *Halosarpheia*, wall of *Halosarpheia marina* (Fig. 8.1g) differs from that of *H. fibrosa* (Fig. 8.1f) in having one layer of cells of *textura angularis* with large lumina. Similarly, the peridial wall of *Lignincola laevis* (Fig. 8.1h) and *L. tropica* (Fig. 8.1i) is made up of elongate, polyhedral cells, but those cells of *L. laevis* are with large lumina. Sakayaroj et al. (2011)

also showed that *L. tropica* grouped with *H. sensu stricto* and *H. marina* with *P. viscosus*. Peridial wall of *L. tropica* and *H. marina* is also different from that of *H. sensu stricto* and *P. viscosus* (Fig. 8.1n), respectively.

Saagaromyces is a monophyletic genus (Sakayaroj et al. 2011), and *S. abonnis*, *S. glitra* and *S. ratnagiriensis* possess a similar peridial wall structure, i.e., two layers with the outer layer of cells of *textura angularis* and the inner layer of elongate, polyhedral cells. A variant of *S. abonnis* with smaller ascospores from mangroves of Taiwan has a one-layered peridium of flattened, polyhedral cells (Fig. 8.1r). Both *Tirispora mandoviana* and *T. unicaudata* have a one-layered peridium of flattened, polyhedral cells, but their monophyly requires a molecular study since they differ significantly in morphology. Ascospores of *T. mandoviana* are relatively thin walled and constricted at the septum, compared with *T. unicaudata* (Fig. 8.2u, v).

These results collectively suggest that peridial wall structure alone is insufficient to separate genera in the Halosphaeriaceae. However, the sections illustrated in this study provided only a two-dimensional view of the cells of the peridium, while cells
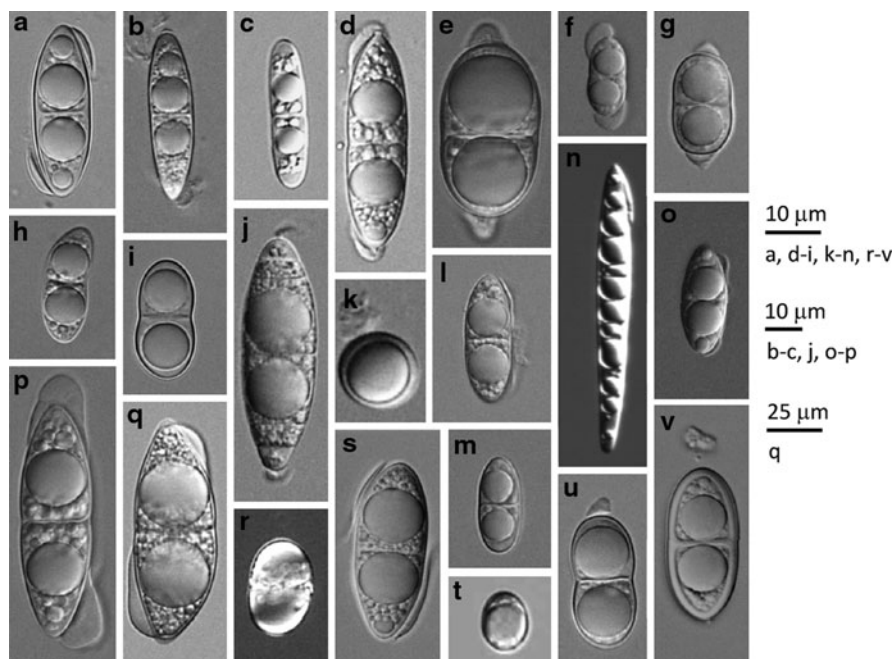

Fig. 8.2 Ascospores. (**a**) *Aniptodera chesapeakensis*, (**b**) *A. lignatilis*, (**c**) *A. longispora*, (**d**) *Cucullosporella mangrovei*, (**e**) *Halosarpheia fibrosa*, (**f**) *H. kandeliae*, (**g**) *H. marina*, (**h**) *Lignincola laevis*, (**i**) *L. tropica*, (**j**) *Magnisphaera spartinae*, (**k**) *Moana turbinulata*, (**l**) *Natantispora retorquens*, (**m**) *Neptunella longirostris*, (**n**) *Oceanitis cincinnatula*, (**o**) *Panorbis viscosus*, (**p**) *Saagaromyces abonnis*, (**q**) *S. ratnagiriensis*, (**r**) *S. glitra*, (**s**) *S. abonnis* c.f., (**t**) *Thalassogena sphaerica*, (**u**) *Tirispora mandoviana*, (**v**) *T. unicaudata*

of the peridium are three-dimensional. Sectioning from different planes is necessary to accurately reflect the shape of the cells.

8.3.2 Ascospores

Ascospores of taxa with unfurling appendages are ellipsoidal/fusiform, uni-septate and possess large oil globules (Fig. 8.2). The hamate ascospore appendages are evidently different from one another in terms of the length and width prior to unravelling, and whether the appendages readily uncoil in sea water, but there has been little attempt to interpret these characteristics to differentiate these closely related taxa. Nakagiri and Ito (1994) investigated the effect of salinity on the unfurling of ascospore appendages in *A. salsuginosa* with appendages unfurling more readily at low salinities. Very likely, these characteristics are not of evolutionary significance, since this group of fungi acquired the unfurling appendages through convergent evolution (Pang et al. 2003a). However, the length of the hamate appendages of *N. retorquens* always exceeds the mid-septum of the ascospores, an essential characteristic for the identification of this species (Shearer and Crane 1980).

Some ascospores are with pointed apices (e.g., *A. lignatilis*, *Saagaromyces* spp.) while others are with rounded ends (e.g., *H. marina*, *T. mandoviana*). Moreover, spores of *L. tropica* and *T. mandoviana* are constricted at the septum and others are not (e.g., *H. fibrosa*, *P. viscosus*). Spore shape of species within one genus is often different, e.g., (1) *A. chesapeakensis*, *A. lignatilis* and *A. longispora* (Fig. 8.2a–c), (2) *L. laevis* and *L. tropica* (Fig. 8.2h, i), (3) *H. fibrosa*, *H. kandeliae* and *H. marina* (Fig. 8.2e–g) and (4) *T. mandoviana* and *T. unicaudata* (Fig. 8.2u,v). All these species were shown to be polyphyletic (Sakayaroj et al. 2011), with the exception of *Tirispora*, since no sequence is available for *T. mandoviana*.

In order to determine if ascospore morphology can be used as a systematic character, shape parameters (aspect ratio, convexity, elongation, shape factor, sphericity, area, perimeter, diameter max, diameter mean, diameter min; Table 8.1) of the ascospores of taxa with unfurling ascospore appendages and related taxa were calculated (Table 8.2). A dendrogram from the cluster analysis is shown in Fig. 8.3. Two of the groups are concordant with the phylogenetic tree presented by Sakayaroj et al. (2011), including the group comprising *S. abonnis*, *S. glitra* and *S. ratnagiriensis* and *A. lignatilis* and *C. mangrovei*. However, other groups are inconsistent and the reason can be one of the followings: (1) ascospore shape alone cannot differentiate genera and it requires a number of morphological characters, or (2) many sequences of genera with unfurling ascospore appendages in the GenBank are early sequences of short length and a second sequence is required to confirm their identity. Nevertheless, the statistics used in this study offer an objective and accurate method to assess shape of ascospores and provide extra characters to delineate these taxa. More taxa from the Halosphaeriaceae should be included in the calculation to have a more thorough analysis of the ascospore shape.

Table 8.2 Shape parameters of ascospores of genera with unfurling appendages and related taxa ($n = 8$)

Species	Aspect ratio	Convexity	Elongation	Shape factor	Sphericity	Area (μm^2)	Perimeter (μm)	Diameter max (μm)	Diameter mean (μm)	Diameter min (μm)
Aniptodera chesapeakensis	2.38	0.99	2.37	0.75	0.18	190.02	56.37	24.40	20.62	10.29
Aniptodera lignatilis	4.08	0.97	4.17	0.51	0.06	540.57	114.99	53.62	47.53	14.07
Aniptodera longispora	3.66	0.98	3.75	0.57	0.07	520.52	107.23	48.41	42.98	14.18
Cucullosporella mangrovei	3.48	0.97	3.41	0.58	0.09	776.07	130.38	60.43	51.83	18.12
Halosarpheia fibrosa	1.95	0.98	1.96	0.84	0.26	415.57	78.95	31.73	27.51	16.35
Halosarpheia marina	1.75	0.98	1.74	0.87	0.33	163.48	48.46	18.65	16.46	10.98
Lignincola laevis	3.12	0.95	3.23	0.59	0.10	124.02	51.41	23.13	20.06	7.89
Lignincola tropica	1.80	0.98	1.81	0.86	0.31	181.32	51.51	20.09	17.57	11.26
Moana turbinulata	1.08	0.98	1.08	0.99	0.87	148.51	43.39	14.58	13.93	13.24
Natantispora retorquens	3.15	0.99	3.14	0.62	0.10	162.02	57.49	26.13	22.45	8.57
Neptunella longirostris	2.01	0.98	2.04	0.82	0.25	81.59	35.39	14.47	12.43	7.37
Saagaromyces abonnis	2.52	0.98	2.54	0.71	0.17	576.64	101.40	43.61	37.17	17.83
Saagaromyces abonnis c.f.	2.18	0.98	2.19	0.77	0.21	295.23	69.27	29.63	24.97	13.43
Saagaromyces glirra	1.63	0.98	1.66	0.89	0.37	921.48	113.89	43.53	38.26	26.73
Saagaromyces ratnagiriensis	2.41	0.98	2.40	0.74	0.18	852.55	120.57	51.79	43.96	22.05
Thalassogena sphaerica	1.09	0.99	1.09	0.99	0.87	70.69	30.01	10.03	9.60	9.09
Tirispora mandoviana	1.86	0.98	1.86	0.85	0.29	162.11	48.91	19.06	16.80	10.60
Tirispora unicaudata	3.06	0.97	3.13	0.64	0.10	186.28	60.37	26.58	23.27	9.19
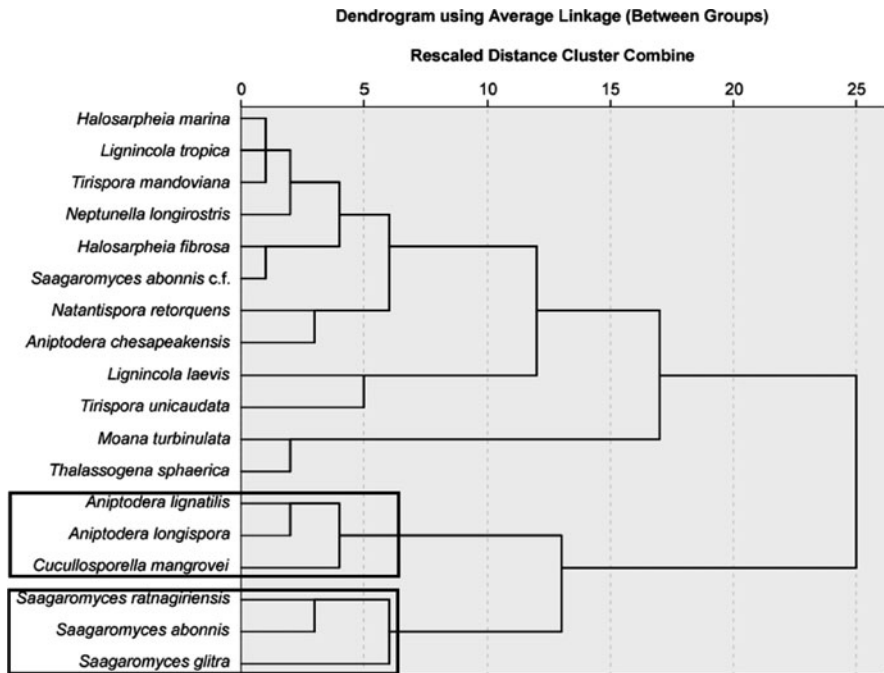
Fig. 8.3 A dendrogram from a cluster analysis of the shape parameters

8.3.3   Asci

Asci of selected taxa in the Halosphaeriaceae with unfurling ascospore appendages and related species are predominantly clavate (Fig. 8.4). Some of them have a flattened apex (e.g., *L. tropica*, *T. unicaudata*), while others have retraction of plasmalemma (e.g., *S. abonnis*, *Thalassogena sphaerica*) and a long stalk (e.g., *Saagaromyces* spp.). *Moana turbinulata* is unique in the complex in having subglobose asci with a long stalk (Fig. 8.4i). A long stalk is also a characteristic feature of *H. kandeliae*. Phylogenetically, *H. kandeliae* was unrelated to *H. sensu stricto* but clustered with *Saagaromyces* spp. but they were not monophyletic (Sakayaroj et al. 2011). A new genus should be introduced to accommodate *H. kandeliae*, but a second sequence is required to confirm its phylogenetic placement. Pang (2002) suggested the use of length/width ratio to describe the shape of the ascus to determine if it is a useful character to differentiate taxa in the Halosphaeriaceae. Shape parameters of asci should also be calculated, however, ascus wall for some of the taxa is very thin and the arrangement of ascospores inside it affects its shape. An average of many asci would give more accurate parameters, but asci of many taxa in the Halosphaeriaceae are deliquescent before maturity, especially the oceanic taxa.

10 µm
a, f-k, p-r

10 µm
b-e, l, o

25 µm
m-n
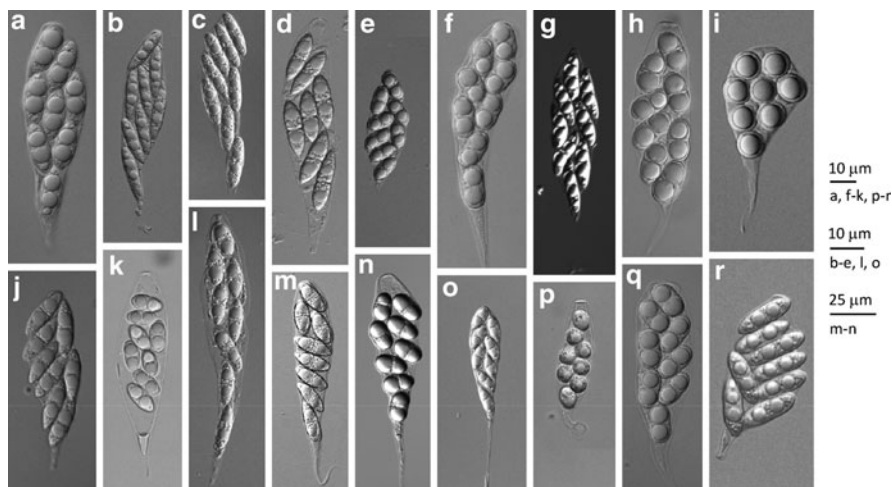
Fig. 8.4 Asci. (**a**) *Aniptodera chesapeakensis*, (**b**) *A. lignatilis*, (**c**) *A. longispora*, (**d**) *Cucullosporella mangrovei*, (**e**) *Halosarpheia fibrosa*, (**f**) *H. marina*, (**g**) *Lignincola laevis*, (**h**) *L. tropica*, (**i**) *Moana turbinulata*, (**j**) *Natantispora retorquens*, (**k**) *Neptunella longirostris*, (**l**) *Saagaromyces abonnis*, (**m**) *S. ratnagiriensis*, (**n**) *S. glitra*, (**o**) *S. abonnis* c.f., (**p**) *Thalassogena sphaerica*, (**q**) *Tirispora mandoviana*, (**r**) *T. unicaudata*

8.4 Concluding Remarks

Spatafora et al. (1998), based on phylogenetic analysis of the rRNA genes, suggested the terrestrial origin of the Halosphaeriaceae. Marine fungi probably have rapidly radiated and evolved independently a wide range of ascospore appendage morphology to increase entrapment and contact area for attachment to substrata for germination and growth (Jones 2006). About one third of taxa in the Halosphaeriaceae possess unfurling ascospore appendages and there are two possibilities: (1) it is an intermediate type of ascospore appendage between no appendage and complex appendages, or (2) it is an efficient type of appendage for attachment to substrates and is an inherited character or gained through horizontal gene transfer. Whether all these taxa are able to interbreed is also a possibility that requires further research. Due to the advancement of molecular techniques and phylogenetic analysis, many genera in the family, such as *Natantispora* and *Panorbis* were defined based on sequence data and these genera lack distinctive morphological traits from other allied taxa. In this study, characters of the ascomatal wall structure and shape of the asci and ascospores in genera with unfurling ascospore appendages in the Halosphaeriaceae are partially concordant with their phylogeny, suggesting a more thorough examination of these characters for the delineation of taxa in the family.

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Chapter 9
Cultured and Uncultured Fungal Diversity in Deep-Sea Environments

Takahiko Nagahama and Yuriko Nagano

Contents

Abstract The importance of fungi found in deep-sea extreme environments is becoming increasingly recognized. In this chapter, current scientific findings on the fungal diversity in several deep-sea environments by conventional culture and culture-independent methods are reviewed and discussed, primarily focused on culture-independent approaches. Fungal species detected by conventional culture methods mostly belonged to Ascomycota and Basidiomycota phyla. Culture-independent approaches have revealed the presence of highly novel fungal phylotypes, including new taxonomic groups placed in deep branches within the phylum Chytridiomycota and unknown ancient fungal groups. Future attempts to culture these unknown fungal

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groups may provide key insights into the early evolution of fungi and their ecological and physiological significance in deep-sea environments.

9.1   Introduction

Deep sea is recognized as an extreme environment, characterized by the absence of sunlight irradiation, predominantly low temperatures (occasionally extremely high, up to 400°C near hydrothermal vents) and high hydrostatic pressure (up to 110 MPa). Despite these extreme conditions, it is now well known that life is abundant in deep-sea environments.

Whereas marine fungi in shallow water, such as coastal and offshore areas (especially mangrove forests), have been well studied with respect to ecological roles and taxonomic positions, a knowledge of "marine fungi" in deep-sea regions of marine environments is limited. In general, it has been believed that extraordinary (occasionally large) creatures inhabit deep sea. However, fungal strains cultured from deep-sea sources have yet to illustrate any remarkable difference (in terms of taxanomic novelty and morphological characteristics) from terrestrial fungi. However, the possibility of unique fungi inhabiting the floor of deep sea remains. This theory may be answered through the application of culture-independent molecular techniques for fungal communities. In this chapter, recent progress on the biodiversity of cultured and uncultured fungi in deep-sea environment will be reviewed.

9.2   Sampling

Great depth and elevated hydrostatic pressure make deep sea a difficult environment to access. However, continuing improvements in technology have made it possible to collect samples from the bottom of the deepest ocean, at depths of 10,000 m.

There are two basic approaches to reach deep sea. One is using a human occupied vehicle (HOV), a deep-sea submarine which can carry people inside to the ocean depths. These types of research vessels have some advantages, such as allowing scientists to make direct observations of deep sea. At present, there are few HOVs which can dive to great depths. The Japanese HOV Shinkai 6500, for example, is capable of diving at depths of up to 6,500 m. An alternative method is to use a remotely operated vehicle (ROV), which is capable of diving at depths greater than 10,000 m and can collect samples from some of the ocean's deepest environments. Equipment loaded on both HOV and ROVs usually include a variety of equipment, such as cameras (television, still photograph, video) and manipulators that enable the placement of objects on the seafloor and the collection of various samples. Both methods are limited in the length of time that can be spent

in deep sea to observe and collect samples, because of the time taken to reach the ocean depths.

The basic sampling methods for investigating fungal communities in deep-sea environments do not differ fundamentally from those used for shallow marine environments. However, collecting samples from deep sea involves higher costs and more complicated equipment. For example, all equipment is required to be resistant to high pressure. For fungal diversity research, sediments are the most popular and investigated areas for deep-sea environment sampling, as fungi are normally most prominently found in sediment. However, water columns, deep-sea habitants, sunken woods, and other materials are also investigated. For water sampling, some known samplers are the Nansen bottle, the Niskin sampler, and the van Dorn sampler. Several types of samplers are used to collect deep-sea sediments, such as a grab sampler, box corer, gravity corer, and piston corer. A grab sampler is useful in collecting soft, sandy, or silt sediments from the surface of the deep-sea floor. Common grab samplers used are the Smith-McIntyre and van Veen grab samplers, which have an effective sampling area of 0.1 m^2 or smaller. A box corer is used to collect the very top surface layers and the sediment underneath, and is available in various sizes. A large box corer has a sampling area of 0.25 m^2. Box corers are used to collect mud and silt but not sand. A gravity corer and piston corer are used to collect samples as a tube of sediment. These types of samplers can collect long deep-sea sediment cores and are useful for investigating fungal diversity in various depths from the seafloor. Gravity corers generally collect sediment layers >2 m in length. Piston corers are similar in appearance to gravity corers but can penetrate much deeper into the seafloor and collect much longer and older core sediment. Currently, the most advanced drilling capabilities in the world are found on the deep-sea drilling vessel, CHIKYU, which is able to bring up sediment core from 7,000 m below the seafloor. One major concern of using the above types of samplers for investigating deep-sea fungal communities is contamination from other environments, such as surface and shallow environments. In order to overcome this problem, sterile sediment samplers (Ikemoto and Kyo 1993) have been developed. These samplers can collect deep-sea sediment into sterile bottles, bringing up samples to the surface without contamination. This method is often applied in fungal diversity studies which use molecular-based methods, because even a minor contamination of samples can cause a major problem in the accuracy of results. Some disadvantages of this method are that the sterile sediment samplers are limited in collecting samples from the surface layer of sediments and can only take a small portion (up to 50 ml) of samples.

9.3 Isolation of Deep-Sea Fungi

The isolation procedure for deep-sea fungi varies depending on the type, volume, and shape of collected samples. Water samples are mostly filtered through membranes and are then used for isolation due to the low number of fungal cells

in the limited volume of water samples. Solid sources, such as sunken wood or deep-sea animals, are normally applied to agar plate media or liquid media after being broken into small pieces or homogenized. Sediment samples are normally directly plated onto medium or mixed with medium agar before they get cold and solid. Mixing with an agar method can separate aggregated sediment particles and usually isolate more diverse fungi than a direct plating method. Samples can first be diluted with sterile sea water, if they contain high numbers of fungi. Particle plating techniques (Bills and Polishook 1994) are also used by some researchers (Jebaraj et al. 2010). In this method, particles that pass through 200-μm mesh but are retained on the 100-μm mesh are spread plated. When the sampling volume is too low or the content contains low numbers of fungi, the sample can be enriched with liquid media. However, it should be noted that enrichment often changes the proportion of the fungal population and also leads to a disappearance of the minority species or slower growing species.

Culture media often used for the isolation of fungi from deep-sea environments are not so different from media used for terrestrial fungi. Several media are used to investigate culturable fungal communities in deep-sea environments (Damare et al. 2006; Jebaraj et al. 2010; Burgaud et al. 2009; Le Calvez et al. 2009), and most investigators use organic media, mainly consisting of malt extract, yeast extract, peptone, glucose, and potato starch. Media are usually prepared in artificial sea water with the addition of antibiotics, such as streptomycin, penicillin, and chloramphenicol to inhibit bacterial growth. Media are also occasionally used at 1/5 strength of normal condition, in order to simulate the low nutrient conditions found in deep sea. Damare et al. (2006) compared several different media, namely, malt extract agar, malt extract broth, corn meal agar, Sabouraud's dextrose agar, Czapek Dox agar, and Czapek Dox broth for isolation of fungi from deep-sea sediments. They concluded that malt extract agar and malt extract broth were generally suitable for the isolation and growth of deep-sea fungi. It is important to note that using fresh samples dramatically increases the number of culturable fungi, compared to using old samples which may have been kept at $-20°C$ or $-80°C$ after sampling. Media are normally incubated between 4 and 30°C, although occasionally incubated at higher degrees, such as 45°C for samples from hydrothermal vents, where the temperature can reach very high, up to 400°C. However, to date thermophilic fungi have not yet been isolated from deep-sea environments. Culturing periods can range from 2 weeks to several months. However, a long culturing time is necessary for isolating deep-sea fungi, as some fungi grow extremely slow. It is also recommended to employ a medium that can suppress the growth of fast growing fungi, along with other fungal media for isolating slow growing deep-sea fungi. As one of the most characteristic conditions of deep sea is extremely high pressure (up to 110 MPa), pressure incubation (up to 30 MPa) has been performed in some studies (Lorenz and Molitoris 1997; Raghukumar and Raghukumar 1998; Damare et al. 2006). In these studies, some fungi showed tolerance to pressure but piezophilic fungi have not yet been reported.

The methods described earlier are what have been done in previously reported studies. However, since our knowledge on the ecological role of deep-sea fungi is

very limited and many niches have not yet been explored, various new isolation media and conditions are being experimented with. Culturing with anaerobic conditions, high-pressure conditions, or in situ cultivating methods may yield interesting results in the near future.

9.4 Detecting Deep-Sea Fungal Diversity by Molecular Methods

With recent technology advances, molecular-based methods are more often used for studying fungal diversity in deep-sea environments. The advantage of using molecular methods is mainly the requirement of only a small amount of samples and the ability to detect difficult-to-culture species, including rare species. DNA-based approaches have confirmed extensive diversity of fungal communities and revealed previously undetected diversity in deep-sea environments (Bass et al. 2007; Lai et al. 2007; Le Calvez et al. 2009; Nagano et al. 2010; Nagahama et al. 2011). Typical procedures for detecting fungal diversity are (1) extracting DNA from samples, (2) amplifying fungal DNA by PCR using pan-fungal primers, (3) sequencing of PCR products, (4) identifying detected species by comparing previously published data and assigning unknown taxa to a phylogenetic clade. DNA extraction from environmental samples is normally performed with various readymade kits after homogenization or treated with six cycles of freeze-thawing in liquid nitrogen/boiling. The most popular targeted genes for fungal diversity studies in deep-sea environments are SSU rRNA and ITS regions, including 5.8 S rRNA. Targeting SSU rRNA is suitable for phylogenetic study, especially for determining the phylogenetic position of unknown or highly novel fungal taxa, which are often detected from deep-sea environments. Targeting ITS regions appears to be a sensitive means of detecting fungal DNA. However, this region is usually too short and variable for complicated phylogenetic study. It is strongly noted that detectable fungal diversity varies strongly depending on the primers used to amplify DNA (Nagano et al. 2010; Jebaraj et al. 2010). It is considered that a strong bias by the selection of PCR primers can limit detected fungal diversity (Amend et al. 2010). Therefore, it is recommended to use multiprimer sets for obtaining better resolution of true fungal diversity in deep-sea environments.

9.5 Comparison Between Culture-Independent Studies of Deep-Sea Fungi

The taxonomic distribution of fungal clones from deep-sea resources was compared according to recent studies using culture-independent approaches (Table 9.1). Of these, nine 18 S rDNA libraries were constructed and investigated in the following

Table 9.1 Appearances of fungal taxa as environmental clones in various deep-sea environments

	18S rDNA									ITS			
Author	Bass et al. (2007)	López-García et al. (2007)	Le Calvez et al. (2009)	Sauvadet et al. (2010)	Singh et al. (2010)	Eloe et al. (2011)	Jebaraj et al. (2010)	Quaiser et al. (2011)	Nagahama et al. (2011)	Lai et al. (2007)	Nagano et al. (2010)	Singh et al. (2010)	Burgaud et al. (unpublished)
Source	Various (including hydrothermal)	Hydrothermal	Hydrothermal	Water and hydrothermal	Sediment	Water	Sediment and water	Water and sediment	Sediment (cold seep)	Sediment	Sediment (including cold seep)	Sediment	Water
Depth	<4,000 m	750–900 m	1,700m, 2,630 m	500–3,000 m	3,992–5,377 m	6,000 m	200 m	1,000–1,260 m	850–1,200 m	350–3,011 m	1,174–10,131 m	3,992–5,377 m	1,750–3,750 m
Target of primers used	Fungi	Eukaryota	Fungi	Eukaryota	Fungi	Eukaryota	Both	Eukaryota	Fungi	Fungi	Fungi	Fungi	Fungi
Ascomycota													
Dothideomycetes	O	O			O	O	O		O	O		O	O
Eurotiomycetes	O	O			O	O	O	O	O	O		O	
Leotiomycetes									O		O		
Saccharomycetes	O			O	O				O	O	O	O	O
Sordariomycetes			O	O		O	O		O	O	O	O	O
Basidiomycota													
Agaricomycetes	O		O	O					O				
Cystobasidiomycetes													O
Entorhizomycetes							O		O				
Exobasidiomycetes	O	O	O	O		O	O		O	O		O	O
Microbotryomycetes	O				O	O			O			O	O
Tremellomycetes	O		O	O		O				O	O	O	O
Ustilaginomycetes						O	O						
Wallemiomycetes				O	O								
Chytridiomycota													
Chytridiomycetes	O		O	O									
Other basal lineage													
LKM11 clade	O										O		
KD14 clade	O							O	O				

O: Taxa presented by phylotypes

studies: Bass et al. (2007), López-García et al. (2007), Le Calvez et al. (2009), Jebaraj et al. (2010), Sauvadet et al. (2010), Singh et al. (2011), Eloe et al. (2011), Quaiser et al. (2011), and Nagahama et al. (2011). Four ITS libraries were constructed and investigated in the following studies: Lai et al. (2007), Singh et al. (2010), Nagano et al. (2010), and Burgaud et al. (unpublished, only in database)., In total, 16 taxa, comprising 14 fungal classes and two uncultured clone groups, have been recognized from the deep-sea resources by culture-independent methods to date.

Ten of 16 taxa were common between 18 S rDNA and ITS libraries, and five taxa, namely, Agaricomycetes, Entorrhizomycetes, Wallemiomycetes, Chytridiomycetes, and KD14 clade were only obtained from the 18 S rDNA library. Cystobasidiomycetes were only obtained from the ITS library in one study. These differences may be caused by primer biases or inhabitation to specific substrates. Geographical limitation may represent the absence of fungal taxa. However, conclusion based on such limited information is difficult.

The taxonomic distribution of deep-sea fungal clones detected by culture-independent methods will now be discussed in more detail.

9.5.1   Ascomycota

Eurotiomycetes and Saccharomycetes were the most frequently detected fungal taxa from deep-sea environments by culture-independent methods, followed by Dothideomycetes and Sordariomycetes (Table 9.1). Some operational taxonomic units (OTUs) of Leotiomycetes have occurred in limited areas (Table 9.1). The majority of OTUs belonging to Eurotiomyces were composed of members of the *Aspergillus*/*Penicillium* group, which are known to be globally distributed fungal taxa. It is suggested that these taxa were also ubiquitous in marine environments (including in deep sea), as well as terrestrial environments. It may appear doubtful that these fungi are indigenous to deep sea. However, evidence of physiological adaption of the *Aspergillus* species to deep-sea environments has been reported (Raghukumar and Raghukumar 1998; Raghukumar et al. 2004; Damare et al. 2006; Damare and Raghukumar 2008). Saccharomycetes are a class of ascomycetous yeasts which often have been isolated from oceanic regions by culturing approaches (Fell 1976). In deep-sea environment, OTUs of genera *Candida*, *Debaryomyces*, *Kodamaea*, *Metschnikowia*, *Pichia,* and their relatives have been detected. Especially, OTUs of *Metschnikowia* with very long evolutionary distances from other ascomycetes have appeared as hitherto uncultured taxa from some deep-sea environments (KD10; Bass et al. 2007; Nagano et al. 2010). Recently, closely related clones were also culture-independently detected as Daphnia parasites in lake environments (Wolinska et al. 2009). Therefore, it may be possible that the *Metschnikowia*-like yeasts in deep-sea environment would also be associated with deep-sea meiobenthos, such as small crustacean. Dothideomycetes are a class of bitunicate ascomycetes, having the order Pleosporales with many marine species.

Dothideomycetes are also reported from deep-sea environment; however, they were not the marine pleosporales but *Aureobasidium*, *Cladosporium,* or *Hortaea*. These frequent species have common characters of adaptation or resistance to/against low temperature and high osmotic pressure, which may be essential keys to survive under deep-sea conditions. Moreover, this may indicate that these fungi originated from terrestrial regions. Also, *Phoma* clones have often appeared in deep-sea studies (Lai et al. 2007; Singh et al. 2011). *Phoma* is known to associate not only with land plants but also with marine plants, such as seaweed and seagrass, as well as with marine invertebrates, such as sponge (Kohlmeyer and Volkmann-Kohlmeyer 1991). The Sordariomycetes were also detected in many studies. However, their phylotypes were few and unique to the area. From oxygen-depleted regions, a number of OTUs close to *Fusarium oxysporum*, which is known as a denitrifying fungus, have been reported (Jebaraj et al. 2010).

9.5.2 Basidiomycota

The most ubiquitous class within the phylum of Basidiomycota detected in deep-sea environments was Exobasidiomycetes (Table 9.1). The majority of the clones belonging to this class are related to the genus *Malassezia*. The genus of *Malassezia* is well known as the causative agents of skin diseases in mammals, along with marine mammals, such as seals or sea lions (Guillot et al. 1998; Nakagaki et al. 2000; Pollock et al. 2000). Whereas *Malassezia* phylotypes present ubiquitous occurrences in deep-sea environments by molecular methods, the isolates have not been cultured from seawater and sediments. It may be considered unsuccessful, because they would possess an anaerobic or hydrothermal-specific metabolism (López-García et al. 2007). In addition, because molecular detection of *Malassezia* sp. from terrestrial soil nematodes has been reported (Renker et al. 2003), the species may be living in small marine invertebrates, such as nematodes or polychaetes, which also inhabit deep-sea sediments. These may have different growth requirements from skin-inhabiting *Malassezia* spp. Moreover, this fungal group has been reported to occupy the majority of eukaryotic diversity found in deep-sea subsurface sediments (Edgcomb et al. 2011). Other classes of Ustilaginomycotina, Ustilaginomycetes, and Entorrhizomycetes were also noted. However, distribution and phylotype was limited. The ustilaginomycetous anamorphic yeasts *Pseudozyma* were detected as uncultured clones and were cultured from deep-sea bivalves (Konishi et al. 2010). The xerophilic characteristics of phylotypes of Wallemiomycetes from deep-sea environments may be a focus for discovery (Sauvadet et al. 2010; Singh et al. 2011). The parasitic smut fungus genus *Entorrhiza*, the taxon which indicated an early divergence in Basidiomycota, is also detected in deep sea. However, clones were separated by long evolutionary distances from known sequences. Two classes of the Pucciniomycotina, Cystobasidiomycetes, and Microbotryomycetes were found with the typical marine yeast orders, Sporidiobolales and Erythrobasidiales. Rare occurrences of Erythrobasidiales

in deep-sea phylotypes were consistent with culturing studies (Nagahama et al. 2001). Cryptococcus-related phylotypes were a major component of Tremellomycetes, some of which were psychrotolerant and were reported from polar or alpine regions (Connell et al. 2008; Turchetti et al. 2008). It may be noted that clones of *Cryptococcus curvatus* were the major component of eukaryotic clones from deep-sea cold seep regions (Takishita et al. 2006). In marine environment, agaricomycetous fungi are scarce and mostly reported from mangrove woods (Jones et al. 2009). Unexpectedly, clones of Agaricomycetes have been detected from deep-sea environments (Bass et al. 2007; Le Calvez et al. 2009; Sauvadet et al. 2010; Nagahama et al. 2011). Because the phylotypes have high similarity (99–98%) with published sequences from the terrestrial Agaricomycetes, it is possible that these may just be contaminants from plant materials by terrestrial runoff. However, distribution was limited in the hydrothermal and cold-seep areas, which may indicate that the agaricomycetous fungi could survive in chemosynthetic communities.

9.5.3 *Chytridiomycota and Other Basal Fungal Lineages*

The chytrids (Phyla Chytridiomycota and Blastocladiomycota) were believed to retain ancient forms before the colonization of land by fungi. However, the majority of chytrids had not been found in marine but freshwater environments. Although reasons why they were lost are unknown, they may still remain at the bottom of deep sea. Interestingly, the culture-independent analyses have unveiled the presence of chytrids in deep-sea environments, especially environments associated with hydrothermal vents communities. The deep-sea chytridian phylotypes were almost considered novel, because their sequences differed remarkably from published species (98–93%). No phylotype of the Phylum Blastocladiomycota was found in the deep-sea chytridian clones, but all were found within the Chytridiomycetes. Some phylotypes related to the chytrids and Zygomycota with lower affinities (<90%) on the blast search belonged to the clone group known as the LKM11 clade or the KD14 clade. The LKM11 clade is an extensive clone group (except *Rozella* spp.) recognized as a sister group of the Kingdom Fungi (Lara et al. 2010). Members of the LKM11 clade have appeared from aquatic (mostly freshwater) environments, such as lake (Šlapeta et al. 2005; Lefranc et al. 2005; Takishita et al. 2007a; Lepère et al. 2006, 2007, 2008; Mangot et al. 2009), water springs (Luo et al. 2005), artificial water system (van Hannen et al. 1999; Laurin et al. 2008), and marine environment (Savin et al. 2004; Takishita et al. 2007b). Some authors consider members of the LKM11 clade to participate in anaerobic ecosystem (van Hannen et al. 1999; Takishita et al. 2005, 2007b). Also in deep-sea environments, the LKM11 clones are likely to appear only from anaerobic sediments. Interestingly, it is suggested that the LKM11 clade involved *Rozella* (Lara et al. 2010), placing the earliest divergent within fungi (James et al. 2006; Bruns 2006). Likewise, the KD14 clade (Bass et al. 2007) consists of clones associated with the

Table 9.2 Appearance and distribution of the KD14 clade

Area	Source	Depth	Clones	References
Gulf of California	Anaerobic bacterial mats	1,575	1	Bass et al. (2007)
Sagami Bay, Japan	Methane cold seep sediment	1,178	1	Takishita et al. (2007a,b)
		1,174	1	Nagano et al. (2010)
		850–1,200	>70	Nagahama et al. (2011)
Kings Bay, Svalbard, Arctic	Deep-sea sediment	20	2	Tian et al. (2008)
Sea of Marmara	Deep-sea sediment	1,260	4	Quaiser et al. (2011)

anaerobic environment. These clones have been detected (Table 9.2) only from sediments of anaerobic bacterial mat (Bass et al. 2007), methane cold seep (Takishita et al. 2007a, b; Nagano et al. 2010), Svalbard in Arctic (Tian et al. 2008), and the Sea of Marmara (Quaiser et al. 2011). Clones of this clade have not yet been detected from hydrothermal vents, in spite of occupying the majority of fungal libraries of methane cold seep (Nagahama et al. 2011). These fungi are not likely to participate with the chemosynthetic ecosystem but just anaerobic sediments in low temperature. This clade is a more coherent clone group than LKM11 and is phylogenetically isolated from known lineages.

9.6   Comparison Between Cultured and Uncultured Fungal Diversity in Deep-Sea Environments

Fungal species isolated from deep-sea environments by conventional culture methods mostly belonged to the Phylum Ascomycota and limited yeast species of Basidiomycota (Table 9.3). The majority of ascomycetous clones in deep sea were closely related to terrestrial species on SSU phylogeny and these have been cultured by conventional isolation methods as well (Burgaud et al. 2009; Singh et al. 2010).

There are no culture isolation reports of Zygomycota and Chytridiomycota from deep-sea environments to date (Table 9.3). Since Zygomycota have not been detected by culture-independent methods either, Zygomycota appears to be very rare or nonexistent in deep-sea environments. Chytridiomycota have been detected as one of the major fungal components in several deep-sea environments, such as hydrothermal vents and methane cold seep but only by culture-independent methods. Le Calvez et al. (2009) revealed the ancient evolutionary lineage within the phylum of Chytridiomycota in deep-sea hydrothermal ecosystems. Nagahama et al. (2011) also reported that deep-branching basal fungi occupied a major portion of fungal clones detected in deep-sea methane cold-seep sediments. The majority of these Chytridiomycota and basal fungi reported from deep-sea environments is highly novel. These novel fungi may have unique ecological and physiological features, so it may be extremely difficult to culture these unknown phylotypes.

Table 9.3 Appearance of fungal taxa as cultured strains in deep-sea environments

	Filamentous fungi and yeasts					Yeasts		
Author	Damare et al. (2006)	Le Calvez et al. (2009)	Burgaud et al. (2009)	Jebaraj et al. (2010)	Singh et al. (2010)	Nagahama et al. (2001)	Gadanho et al. (2005)	Burgaud et al. (2009)
Source	Sediment	Hydrothermal	Hydrothermal	Sediment and water	Sediment	Sediment	Hydrothermal	Hydrothermal
Depth	4,800–5,400 m	1,700 m, 2,630 m	700–3,700 m	<200 m	4,000–5,500 m	1,000–11,000 m	800–2,400 m	700–2,700 m
Isolation method	1–30 atm., various temp.	1 atm., 25°C	1 atm., various temp.	1 atm., room temp.	1–30 atm., various temp.	1 atm., various temp.	1 atm., various temp.	1 atm., various temp.
Ascomycota								
Dothideomycetes	O	O	O	O	O			O
Eurotiomycetes	O	O	O	O	O			
Leotiomycetes			O				O	
Saccharomycetes	O[a]					O		
Sordariomycetes	O	O	O	O	O		O	
Basidiomycota								
Agaricomycetes								
Cystobasidiomycetes	O[a]			O	O	O	O	
Entorrhizomycetes								
Exobasidiomycetes			O	O	O			
Microbotryomycetes	O[a]				O	O	O	O
Tremellomycetes					O		O	O
Ustilaginomycetes					O			
Wallemiomycetes				O				
Chytridiomycota								
Chytridiomycetes								
Other basal lineage								
LKM11 clade								
KD14 clade								

O: Taxa presented by phylotypes
O[a]: Taxa expected from morphology

Similar to other environmental studies, culture-dependent approaches and culture-independent approaches often provide different results on the fungal diversity. Culture-independent approaches have been able to detect diverse fungi, including many novel fungal phylotypes, which may be difficult to culture. Le Calvez et al. (2009) reported the striking difference on their results investigating deep-sea fungal diversity between culture-dependent and culture-independent methods. They isolated only Ascomycota spp. by culture-dependent methods. However, none of Ascomycota spp. were detected by culture-independent methods but Basidiomycota spp. and Chytridiomycota spp. were detected. This study suggested that the abundance of Ascomycota in the deep-sea environmental samples was extremely low, despite the frequent isolation of ascomycetous fungi from various samples investigated. Cultivation resulted in the amplification of rare organisms. Many factors can be responsible for these different results, making it very difficult to judge which methods are more accurately depicting the true fungal diversity in deep-sea environments. The absence of non-Dikarya fungi as chytrids by culture-dependent analyses can be caused as a result of culturing deep-sea fungi using an agar medium with antibiotics. Most chytrids cannot be isolated by direct plating. In addition, chloramphenicol, an antibiotic widely used in fungal isolation, is known to prevent growth of some of the chytrids (Gleason and Marano 2011). In general, culture-independent methods are thought to be able to detect wider ranges of fungi and reflect investigated environments more directly. However, culture-independent methods are also easily biased by many processes, such as PCR primer selection and DNA extraction methods. Thus, it is recommended to combine both conventional culture methods and culture-independent methods when investigating fungal diversity. Also, in order to investigate the true abundance of deep-sea fungi, it is necessary to combine with other methods, such as direct microscopic observation with appropriate staining, FISH, measuring the ergosterol (uniquely present in cell membranes of higher fungi), metagenomic approaches, and new powerful tools expected to be developed in the future.

9.7 Future Direction

Although recent studies on deep-sea fungi have been more extensively researched, our knowledge and understanding of deep-sea fungi is still limited. Unlike deep-sea bacteria, fungal taxa, which are physiologically adapted to low temperatures, high hydropressure, and play important roles in the ecosystem, have not yet been discovered. Culture-independent analyses revealed the presence of hitherto unknown fungal lineages in deep sea, which were difficult to grow via conventional culturing methods. Such uncultured taxa may be fungi which have effectively adapted to deep-sea environments. Also, these unknown fungal groups may provide key insights into the early evolution of fungi and their ecological and physiological significance in deep-sea environments. Deep-sea exploration of uncultured fungi should continue to be investigated thoroughly.

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Chapter 10
Molecular Diversity of Fungi from Marine Oxygen-Deficient Environments (ODEs)

Cathrine Sumathi Jebaraj, Dominik Forster, Frank Kauff, and Thorsten Stoeck

Contents

Abstract Molecular diversity surveys of marine fungi have demonstrated that the species richness known to date is just the tip of the iceberg and that there is a large extent of unknown fungal diversity in marine habitats. Reports of novel fungal lineages at higher taxonomic levels are documented from a large number of marine habitats, including the various marine oxygen-deficient environments (ODEs). In the past few years, a strong focus of eukaryote diversity research has been on a variety of ODEs, as these environments are considered to harbor a large number of organisms, which are highly divergent to known diversity and could provide insights into the early eukaryotic evolution. ODEs that have been targeted so far include shallow water sediments, hydrothermal vent systems, deep-sea basins, intertidal habitats, and fjords. Most, if not all, molecular diversity studies in marine ODEs have shown, that contrary to previous assumptions, fungi contribute significantly to the micro-eukaryotic community in such habitats. In this chapter, we have reanalyzed the environmental fungal sequences obtained from the molecular diversity survey in 14 different sites to obtain a comprehensive picture of fungal diversity in these marine habitats. The phylogenetic analysis of the fungal environmental

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sequences from various ODEs have grouped these sequences into seven distinct clades (Clade 1–7) clustering with well-known fungal taxa. Apart from this, four environmental clades (EnvClade A, B, C, and D) with exclusive environmental sequences were also identified. This has provided information on the positioning of the environmental sequences at different taxonomic levels within the major fungal phylums. The taxonomic distribution of these environmental fungal sequences into clusters and clades has also shown that they are not restricted by geographical boundaries. The distribution pattern together with the reports on the respiratory abilities of fungi under reduced oxygen conditions shows that they are highly adaptive and may have a huge ecological role in these oxygen deficient habitats.

10.1 Marine Fungal Diversity: Historical Background

Fungi thrive in a number of habitats and display specific adaptations to these habitats in their structure and biochemistry (Maheshwari 2005). Some of these habitats colonized by fungi include the outer stratosphere (Wainwright et al. 2003; News Letter ISRO 2009), extremely cold habitats in polar regions (Robinson 2001), deep sea sediments (Raghukumar et al. 2004; Edgcomb et al. 2011), hypersaline brine with saturated salt concentrations (Edgcomb et al. 2009), extremely dry soil crusts (States and Christensen 2001), and hot springs with temperatures ranging up to 62°C (Maheshwari et al. 2000).

The presence of fungi in the marine environment was initially studied in 1936 (Sparrow 1936), followed by Barghoorn and Linder (1944). Since then, mycologists have tried to estimate the extent of fungal diversity and to understand their ecological role in the various niches of the marine ecosystem (Kohlmeyer and Kohlmeyer 1979; Gessner 1980; Raghukumar et al. 1992; Sathe-Pathak et al. 1993; Prasannari and Sridhar 2001; Raghukumar et al. 2004; Damare et al. 2006). These studies used traditional methods involving isolation and cultivation of fungal cultures. Estimates have shown that using such strategies only about 5% of the total fungal diversity is documented so far from the marine environment (Kis-Papo 2005). These diversity estimates are expected to be incomplete and down-numbered, because many of the fungal cultures show an inability to grow and sporulate under laboratory conditions (Jones and Hyde 2002). Advancements in the field of molecular ecology have revolutionized the study of fungal diversity. Molecular biological techniques, particularly the analysis of nucleotide sequences and phylogenetic approaches, have greatly influenced fungal systematics (Guarro et al. 1999). Molecular tools, such as Random Amplified Polymorphic DNA (RAPD) and Terminal Restriction Fragment Length polymorphism (T-RFLP) analysis of the amplified internal-transcribed spacer (ITS) region or the small subunit of ribosomal RNA (SSU rRNA) gene region, are widely known. The nuclear large subunit region of ribosomal RNA gene (LSU rRNA) is also used to identify the fungal cultures isolated from marine ecosystems (Roberts et al. 1996; Buchan et al. 2002), and direct PCR amplification from various environmental samples also targets these

loci. Studies based on the culture-independent approach have proved to be a powerful tool and have showed that the diversity of marine fungi is more extensive than documented previously (Pang and Mitchell 2005).

A strong focus of molecular diversity research has been on micro-eukaryotes from a variety of marine environments, such as shallow water anoxic sediments (Dawson and Pace 2002; Stoeck et al. 2003), hydrothermal vent systems (Edgcomb et al. 2002; López-García et al. 2003, 2007), oxygen-depleted deep basins (Stoeck et al. 2003, 2006), anoxic fjords (Behnke et al. 2006; Stoeck et al. 2007b; Zuendorf et al. 2006), saline meromictic lakes (Takishita et al. 2007a), deep sea habitats (Takishita et al. 2005, 2007b), hypersaline systems (Alexander et al. 2009), a few oxygen-depleted freshwater systems (Dawson and Pace 2002; Luo et al. 2005; Slapeta et al. 2005), and terrestrial habitats (Schadt et al. 2003). These studies, using eukaryote specific primers, have revealed a much larger diversity in all micro-eukaryotic supergroups, including alveolates, stramenopiles, amoebozoa, excavates, discicristates, plants and ophisthokonts (Dawson and Pace 2002; Stoeck and Epstein 2003; Stoeck et al. 2003; Takishita et al. 2005; Luo et al. 2005; Behnke et al. 2006; Alexander et al. 2009). Most, if not all, environmental diversity studies conducted in oxygen-deficient systems have shown that fungal sequences are a significant component of the micro-eukaryotic community. This indicates, that molecular diversity surveys hold a great promise in the discovery of novel fungi. In order to fully exploit the potential of these taxonomically and economically novel fungi, it is inevitable to obtain cultures. Hence, an integrated approach combining molecular, cultivation-independent studies with classical isolation and cultivation techniques offers a greater chance of understanding the fungal diversity and their ecological significance in the diverse niches they occupy (Jebaraj et al. 2010).

10.2 Ecological Significance of Fungi in ODEs

A combination of high productivity and limited mixing or circulation with poorly oxygenated waters leads to the development of mid-water ODEs in the world oceans (Wyrtki 1962). These are regions where the dynamic steady state between oxygen supply and consumption is altered and the oxygen concentration is <0.2 ml l^{-1}. Such permanent anoxic zones are seen in the eastern Pacific Ocean, Indian Ocean, and in West Africa, where the fixed N is transformed in the water column to dissolved N gas through denitrification (Kamykowski and Zentara 1990). Denitrification is a major sink for fixed N and is therefore crucial to budget the loss of oceanic nitrogen through this microbial-mediated process in the ODEs.

Fungi were one of the taxonomic groups that were thought to play only a minor role in ecosystem processes of anoxic environments (Dighton 2003), but molecular analysis has shown that they occupy a greater portion in the microbial diversity of the ODEs. Studies under laboratory conditions have shown that many members of kingdom *Fungi* have physiological adaptations which allow them to thrive in environments with varying oxygen concentrations (Zhou et al. 2002). Generally,

fungi are major decomposers in the marine environment and can actively degrade woody and herbaceous substances with the help of the lingo-cellulolytic, extracellular enzymes produced by them (Newell 1996; Hyde et al. 1998). Fungi play a key role in the decomposition and biochemical transformation of organic matter in the marine habitat because fungal enzymes can efficiently breakdown recalcitrant compounds like phenolics and tannins. They also play an important role in the C and N cycle and aid in the release of the inorganic nutrients back into the ecosystem (Dighton 2007). During this process, the delicate equilibrium of the oxygen consumption during the degradation of the organic matter and oxygen replenishment is maintained. A shifted balance due to increased organic load leads to oxygen depletion, and the change in oxygen concentration alters the microbial community structure (Goregues et al. 2005). In conditions where low oxygen condition prolongs, the diversity of the communities is known to reduce to a few forms which are able to tolerate anaerobic growth conditions (Levin 2003). But surprisingly, the molecular diversity from the oxygen-depleted habitats has shown that there is a wider and richer diversity than previously known (Epstein and López-García 2007).

Many fungal taxa were recently shown to possess metabolic adaptations to utilize nitrate and/or nitrite as an alternative for oxygen (Kurakov et al. 2008). Involvement of fungi in such anaerobic dissimilatory processes was largely unknown, and initial studies that reported growth of fungi in anoxic environments suggested that they required supplementation of certain inorganic salts, such as nitrate, selenite, or ferric iron in the culture media (Gunner and Alexander 1964). Bollag and Tung (1972) were the pioneers to show N_2O production by fungi when growing under anaerobic conditions. They also demonstrated growth of fungi under these conditions when supplemented with nitrate or nitrite and suggested, that nitrate respiration may be a possibility in fungi. Screening fungal cultures from terrestrial origin for their denitrification activity revealed, that indeed several different taxa were involved in this process (Shoun et al. 1992; Usuda et al. 1995). For the first time, Shoun and his group (1992) could confirm that dissimilatory nitrate reduction or microbial denitrification was also possible in fungal cultures, with fungal cultures such as *Fusarium oxysporum*, *Cylindrocarpon tonkinense,* and *Aspergillus oryzae* releasing N_2O under oxygen-depleted conditions. This was a major breakthrough, as denitrification was previously thought to be an alternative form of respiration exclusively within bacterial and archaean communities (Knowles 1982). A recent study has also demonstrated that fungi isolated from anoxic marine waters of the Arabian Sea could contribute to the denitrification process (Jebaraj and Raghukumar 2009).

Denitrification activity has been shown in terrestrial fungi belonging to *Ascomycota*, *Basidiomycota*, and *Zygomycota* (Kurakov et al. 2008). This shows that the fungal denitrification activity is prevalent among the major groups, but the molecular basis on how the members of Kingdom *Fungi* acquired the ability is still unknown. Perhaps, fungi may have a pivotal role in the nitrogen cycle in marine ODEs, but the contribution of fungi to anaerobic denitrification has not yet been quantified.

10.3   What Novel Fungal Diversity Emerges from Molecular Diversity Surveys in Marine ODEs?

In order to identify hotspots of fungal diversity and to assess the degree and distribution of fungal diversity in ODEs, we have analyzed eukaryote SSU rRNA gene meta-data from 12 environmental molecular diversity surveys, targeting 14 different sampling sites (Table 10.1). In the phylogenetic analysis of the fungal tree of life that included fungal environmental sequences ($n = 385$) from various ODEs and representative sequences of described species from all major phylogenetic fungal lineages (Hibbett et al. 2007), we identified seven major clades (clades 1–7) and four environmental clades (EnvClade A, B, C, and D) (Fig. 10.1). Clades 1–7 (Figs. 10.2–10.7) encompassed numerous environmental fungal sequences grouping within known fungal taxa, and EnvClade A, B, C, and D (Figs. 10.8a, b and 10.9a, b) contain environmental fungal sequences that are unrelated to previously described taxa.

Kingdom *Fungi* is one of the major taxonomic group where new phyla, orders, genera, and species are being added frequently with novel ecological and physiological adaptations. It includes subkingdom *Dikarya*, consisting of phylum, Ascomycota and Basidiomycota, phylum Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Microsporidia, Glomeromycota, and the basal groups (Hibbett et al. 2007). The reanalysis of the environmental fungal sequences obtained from various ODEs used in this study grouped them together with the sequences of well-described fungal taxa. The sequences clustered within members of phylum *Basidiomycota* (Clades 1, 2 and EnvClade A), *Ascomycota* (clades 3–5), *Chytridiomycota* (Clade 6 and EnvClade B, C, D), and some belonged to undescribed basal lineages (Clade 7) of kingdom *Fungi* (Fig. 10.1).

Environmental sequences of phylum *Basidiomycota* analyzed from the various ODEs grouped within subphyla *Ustilagomycotina* and *Pucciniomycotina*, and are

Table 10.1 List of oxygen-deficient environments (ODEs) analyzed

| S. No.	Region	Geographical location	References
1.	Cariaco, anoxic basin	Equatorial Atlantic	Stoeck et al. (2003)
2	Lucky Strike, vent region	Mid Atlantic Ridge	Le Calvez et al. (2009)
3	Elsa, Vent region	Mid Atlantic Ridge	Le Calvez et al. (2009)
4	Menez Gwen vent region	Mid Atlantic Ridge	Le Calvez et al. (2009)
5	Greenland, tidal flat	North Atlantic	Stoeck et al. (2007a)
6	Guaymas, vent region	North Pacific	Edgcomb et al. (2002)
7	Mariager fjord	Baltic Sea	Zuendorf et al. (2006)
8	Japan, Tagiri vent site	North Pacific	Takishita et al. (2005)
9	Lost City, vent region	Mid Atlantic Ridge	López-García et al. (2007)
10	Framvaren Fjord	North Sea	Behnke et al. (2006)
11	L'Atalante saline basin	Mediterranean	Alexander et al. (2009)
12	Sippewisset salt marsh	North Atlantic	Stoeck and Epstein (2003)
13	California, sediment	North Pacific	Dawson and Pace (2002)
14	Gotland anoxic basin	Baltic Sea	Stock et al. (2009)
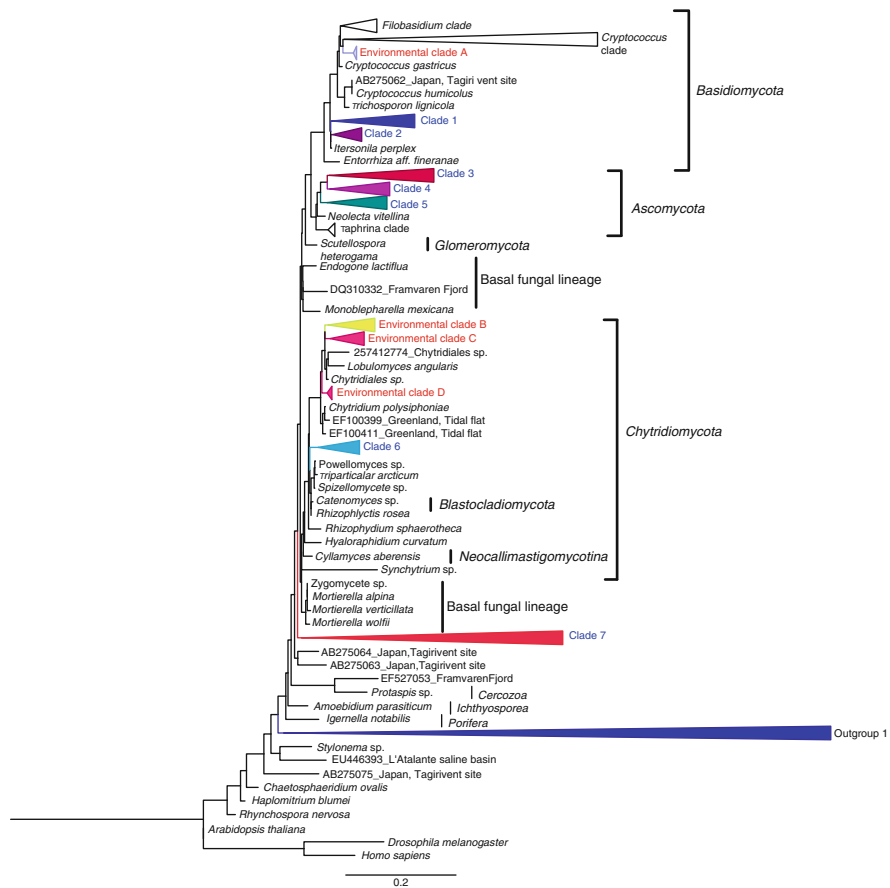
Fig. 10.1 Phylogenetic tree of the SSU rRNA sequences of Kingdom *Fungi* showing the position of environmental sequences obtained from various ODEs used for analysis. The tree shows the positioning of clades 1–7 and env clades A–D which will be explained in subsequent figures. The sequences are represented by their NCBI accession number, followed by the regional location from where each sequence was isolated

represented as clade 1 (Fig. 10.2). Clade 1 has a cluster of environmental fungal sequences obtained from two anoxic vent regions and three major ODEs of the world oceans. This cluster has been recognized as the "hydrothermal and/or anaerobic fungal group" of a similar study that surveyed the eukarotic diversity in an anoxic vent ecosystem (López-García et al. 2007). The composition of this cluster is predominated by environmental sequences, and the only named species is *Malassezia* spp. belonging to *Exobasidiomycetes* of *Ustilagomycotina*. Though *Malassezia* spp. are known to be human pathogens, environmental fungal sequences closely grouping with this genus have been identified from various habitats, such as the deep-sea regions (Lai et al. 2007), anoxic basins (Dawson and Pace 2002; Alexander et al. 2009; Jebaraj et al. 2010), and hydrothermal vent
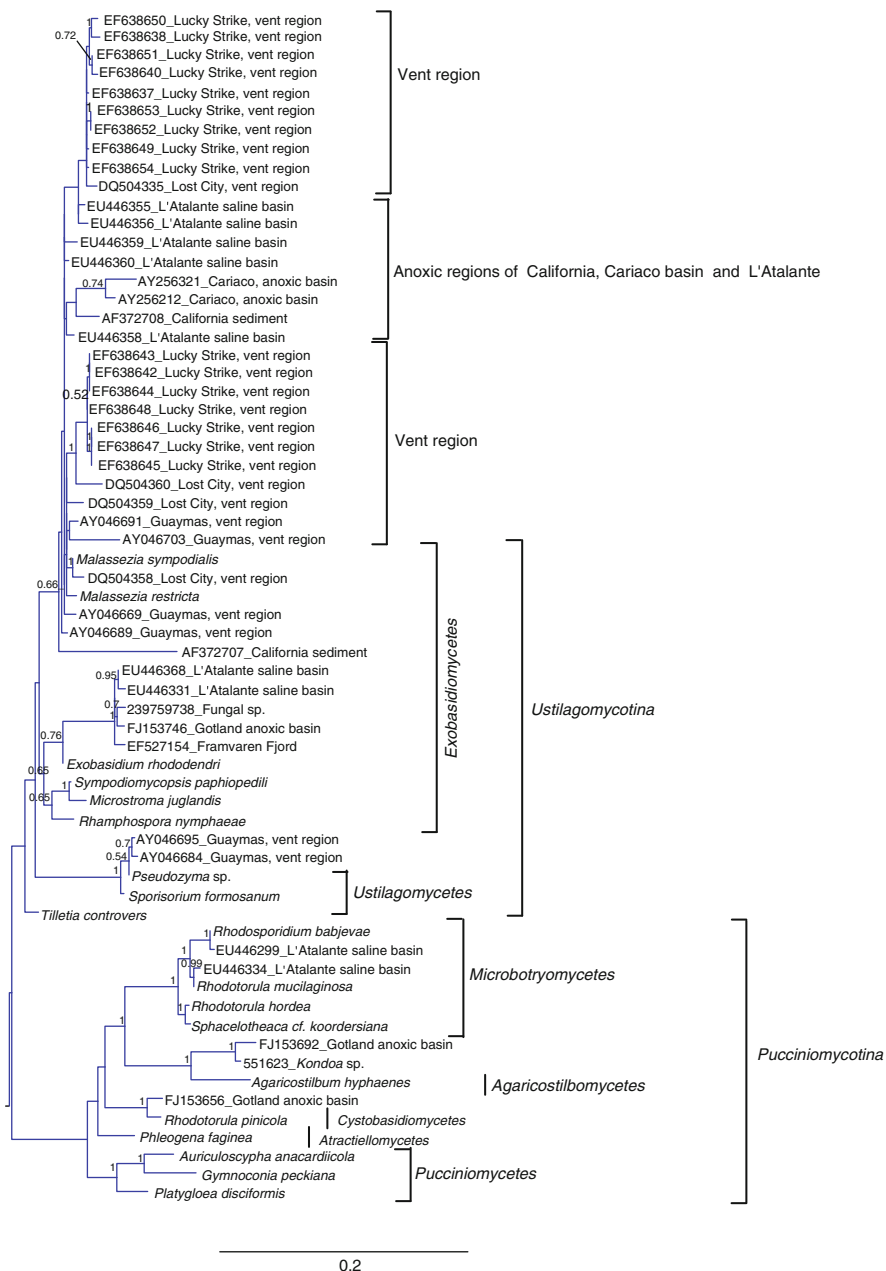
Fig. 10.2 Clade 1: Phylogenetic tree of the SSU rRNA sequences of phylum *Basidiomycota, Ustilagomycetes,* and *Pucciniomycetes* showing the position of environmental sequences obtained from various ODEs used for analysis. The sequences are represented by their NCBI accession number, followed by the regional location from where each sequence was isolated

Fig. 10.3 Clade 2:
Phylogenetic tree of the SSU
rRNA sequences of
phylum *Basidiomycota,*
Agaricomycetes showing the
position of environmental
sequences obtained from
various ODEs used for
analysis. The sequences are
represented by their NCBI
accession number, followed
by the regional location from
where each sequence was
isolated
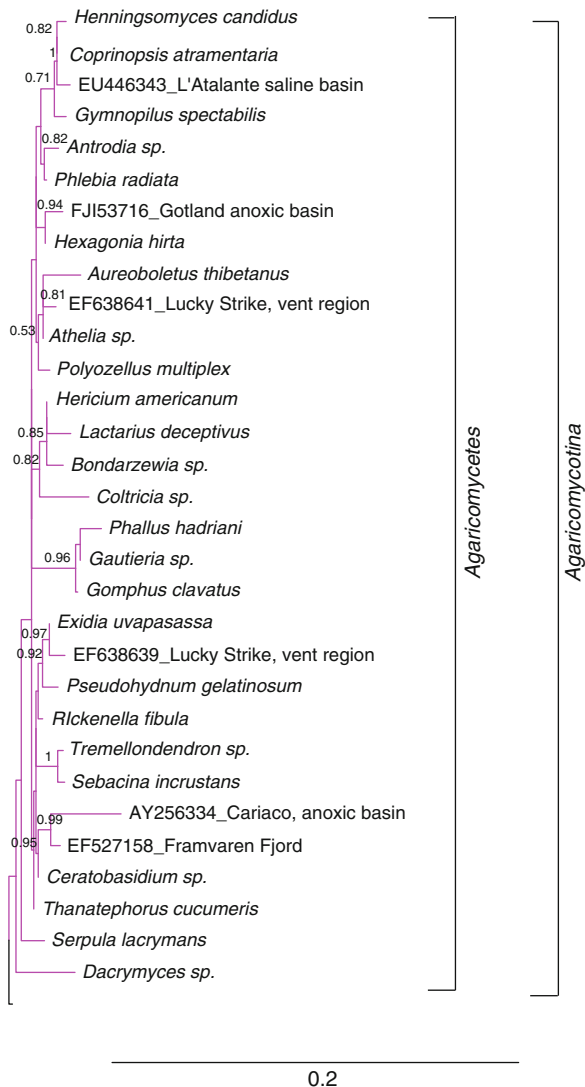
regions (López-García et al. 2007). The life style of *Malassezia* does not give us any
insights into the ecological significance of the fungal environmental sequences
obtained from these regions. This also shows that the molecular approach using
SSU rRNA as a marker to study the microbial richness and diversity has its
shortcomings. A combined approach using both the cultivation based and molecu-
lar studies could help in resolving these issues. Nevertheless, this analysis has
identified a promising target within the "hydrothermal and/or anaerobic fungal
group" for the isolation of novel fungal lineages which have not much similarity
to known fungal taxa (Jebaraj et al. 2010). The environmental sequences obtained
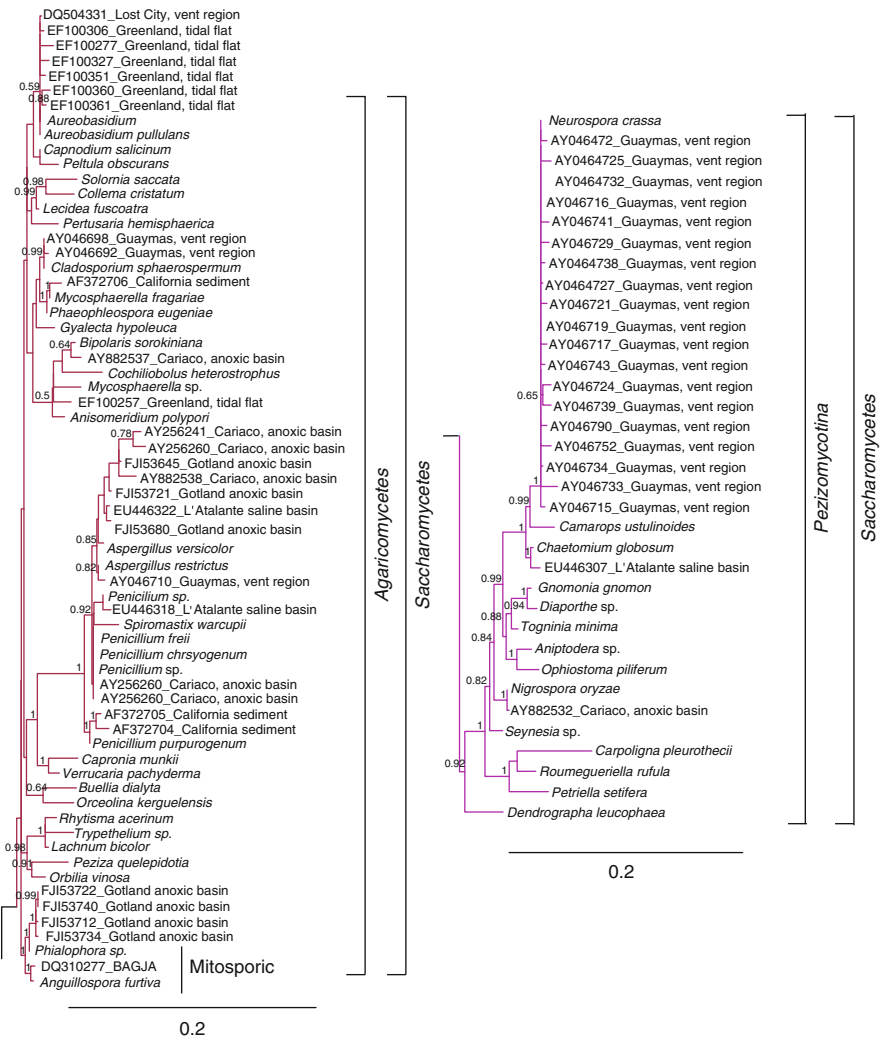
Fig. 10.4 (**a**) Clade 3 and (**b**) Clade 4: Phylogenetic tree of the SSU rRNA sequences of phylum *Ascomycota, Saccharomycetes, Pezizomycotina* showing the position of environmental sequences obtained from various ODEs used for analysis. The sequences are represented by their NCBI accession number, followed by the regional location from where each sequence was isolated

from the various ODEs also show that the microbes may have different physiological adaptations to thrive in extreme conditions, such as high sulfide conditions (Framvaren fjord), elevated temperatures (Mid-Atlantic ridge hydrothermal field), and high salt conditions (L'Atalante basin). Clade 1 (Fig. 10.2) also has another environmental cluster that groups with high divergence to *Exobasidium* sp. (Jebaraj et al. 2010) within *Ustilagomycotina*. This shows that phylum Ustilagomycotina
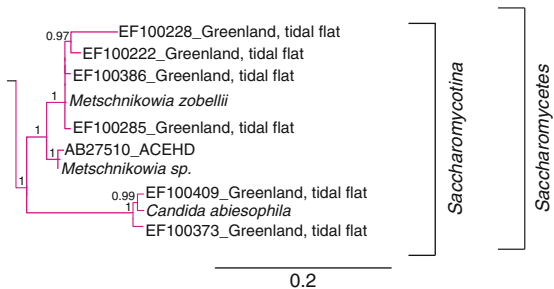
Fig. 10.5 Clade 5: Phylogenetic tree of the SSU rRNA sequences of phylum *Ascomycota, Saccharomycetes, Saccharomycotina* showing the position of environmental sequences obtained from various ODEs used for analysis. The sequences are represented by their NCBI accession number, followed by the regional location from where each sequence was isolated

Fig. 10.6 Clade 6: Phylogenetic tree of the SSU rRNA sequences of phylum *Chytridiomycota, Chytridiomycetes* showing the position of environmental sequences obtained from various ODEs used for analysis. The sequences are represented by their NCBI accession number, followed by the regional location from where each sequence was isolated
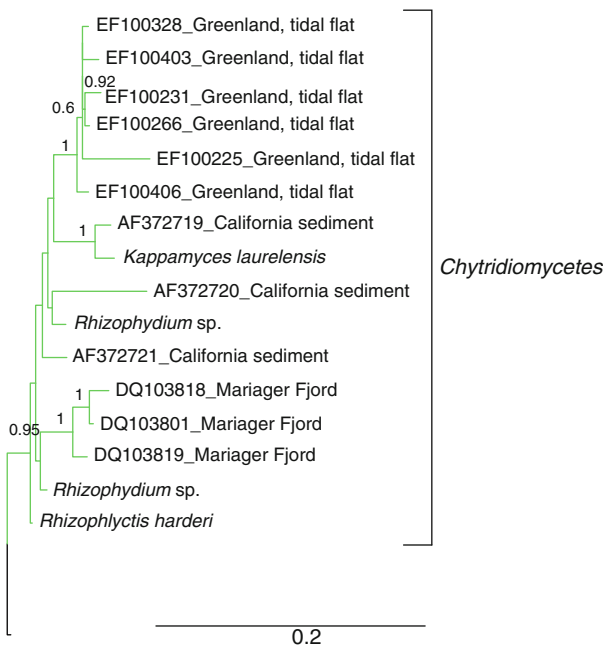
could hold a number of unknown taxa, which could be identified using group specific studies and targeted isolation of cultivable forms belonging to this group.

Clade 2 (Fig. 10.3) includes environmental sequences that group with described fungal sequences belonging to the *Agaricomycetes* of the subphylum *Agaricomycotina*. This clade has representative sequences from anoxic basins of Cariaco, L'Atalante, Gotland basins, and from the Lucky Strike vent site. *Agaricomycetes* include members of *Fungi* which have well-developed fruiting bodies, like mushrooms and wood-rot fungi. The distribution of this group is
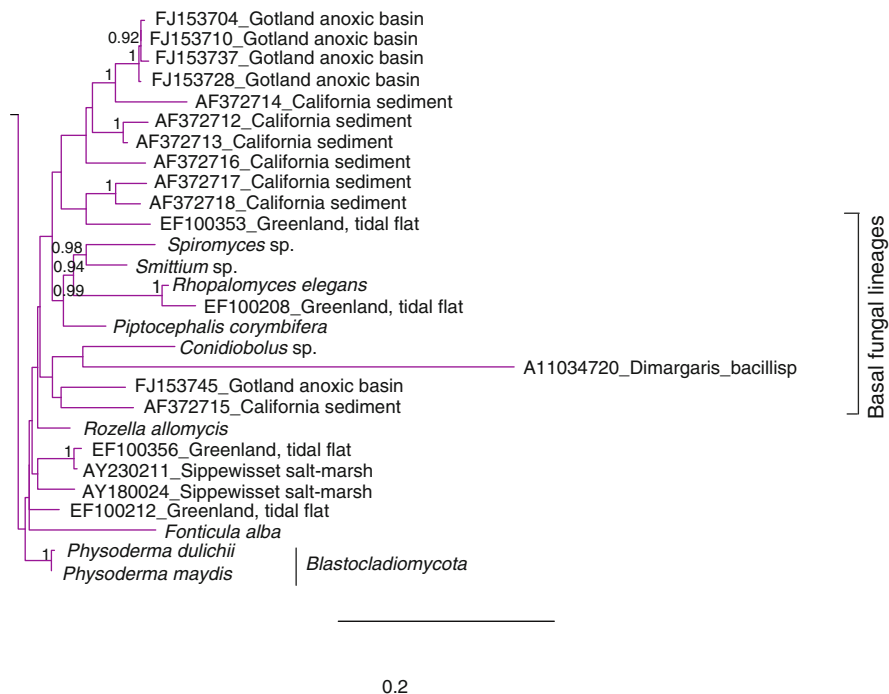
Fig. 10.7 Clade 7: Phylogenetic tree of the SSU rRNA sequences of the basal fungal lineage showing the position of environmental OTUs obtained from various ODEs used for analysis. The sequences are represented by their NCBI accession number, followed by the regional location from where each sequence was isolated

generally restricted to terrestrial habitats. Only a few of them are present in fresh water habitats, but they have rarely been isolated from the marine realm. Some species which are present in the marine environment are known only from their anamorphic stage, and the spores are released passively to the marine conditions (Jones and Choeyklin 2008). The presence of environmental sequences belonging to this group from various anoxic and vent regions shows that *Agaricomycetes* may have a broader distribution than what is documented so far. An alternative interpretation may be that the genetic signatures obtained in environmental studies come from "ancient" extracellular material or from resting stages of the fungi, which are inactive in marine waters. However, some studies amplified SSU rRNA genes from environmental RNA (cDNA) as template, which is indicative of actively growing and metabolically active cells (Stoeck et al. 2007b).

The EnvClade A (Fig. 10.8a) includes sequences exclusively from the Mid-Atlantic vent region and the Norwegian Framvaren Fjord, grouping within the *Tremellomycetes* (*Agaricomycotina*), some of which are closely related to *Cryptococcus* sp. (Fig. 10.1), also belonging to phylum *Basidiomycota*. These groups are generally yeast-like forms and are known to be widespread in the marine

Fig. 10.8 (**a**) env clade A, (**b**) env clade B: Phylogenetic tree of the SSU rRNA sequences of environmental sequences obtained from the various ODEs used for analysis. The sequences are represented by their NCBI accession number, followed by the regional location from where each sequence was isolated

Fig. 10.9 (**a**) env clade C, (**b**) env clade D: Phylogenetic tree of the SSU rRNA sequences of environmental sequences obtained from the various ODEs used for analysis. The sequences are represented by their NCBI accession number, followed by the regional location from where each sequence was isolated
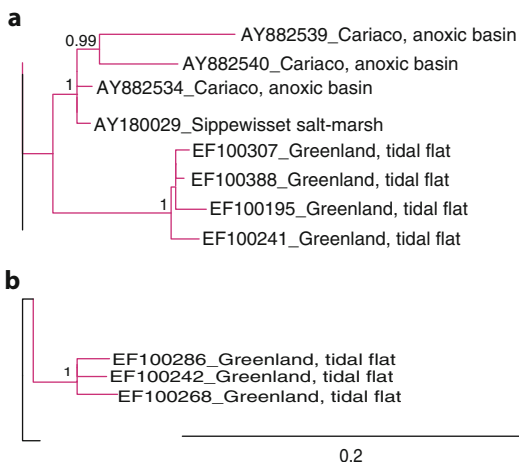
environment as pathogens of marine mammals, such as dolphins and whales (Miller et al. 2002). Other members of this clade, such as *Cystofilobasidium* sp., are also common in the marine environment (Fell et al. 2001). Other than the occurrence of these groups in marine environment, their ecological role is not well studied. The tremellomycete yeasts are rich in carotenoid pigments and are known to break down sugars by fermentative mode. Detailed studies on these groups of organisms can give us more insights into their biochemistry and physiological adaptations to marine ODEs.

Environmental sequences analyzed from the different ODEs are grouped in the classes *Pezizomycotina* (clade 3 and 4) and *Saccharomycotina* (clade 5) of the phylum *Ascomycota*, subphylum *Saccharomycetes* (Figs. 10.4 and 10.5). Environmental fungal sequences belonging to nine different ODEs are grouped within the *Ascomycota*, and these sequences are relatively closely related to cultured organisms. *Pezizomycotina* contains most of the well-known fungal species which are ubiquitous in nature. Clade 3 (Fig. 10.4a) has representative sequences belonging to oxygen-depleted regions of Greenland, intertidal sediments in California, the Cariaco, Gotland, and L'Atalante basins as well as the Framvaren fjord. Environmental sequences from the Mid-Atlantic ridge and the Guaymas vent regions were also included. Clade 4 (Fig. 10.4b) branches within the *Pezizomycotina* and includes a large number of representatives from the Guaymas vent region in the Gulf of California, Mexico. These sequences group with *Neurospora crassa*, a fungal model organism that is spread across geographical boundaries. *Neurospora* spp. are found in high numbers in predominantly tropical and subtropical terrestrial regions, such as Haiti and the Ivory Coast (Turner et al. 2001). A large number of fungal species belonging to the *Pezizomycotina* occurs in marine environments as filamentous forms, and their secondary metabolites are well studied. Clade 5 (Fig. 10.5) includes environmental sequences obtained from anoxic regions of Greenland and Japanese methane seeps. These sequences are related to known species of the *Saccharomycotina*. Our phylogenetic analyses demonstrate that none

of the clusters are specific to a single anoxic habitat type. Instead, the sequences of each clade include genetic signatures from a variety of different marine ODE sites, all of which are united by oxygen deficiency, but have distinct environmental settings (like high pressure, high salinity, high or low temperature). This indicates the highly versatile potential of fungi to adapt to a variety of extreme environmental conditions.

Phylum *Chytridiomycota* was only recently described as a monophyletic phylum (Hibbett et al. 2007). Members of this phylum generally are associated with soil and freshwater habitats. Some have been reported from marine environments, generally as parasites on marine algae (Kerwin et al. 1992) and phytoplanktons (Kagami et al. 2007). In contrast to other fungal phyla, many extremophiles have been described within the *Chytridiomycetes* (Zack and Wildman 2004). They can thrive in extreme environments such as the periglacial soils at high altitudes, in the permafrost, acidic environments, mineral rich, hot hydrothermal vent regions (Gleason et al. 2010), and in ODEs (Lockhart et al. 2006). In our analyses, Clade 6 (Fig. 10.6) and Env Clades B, C, D (Figs. 10.8b and 10.9a, b) group within the *Chytridiomycota*. Where the Clade 6, and the environmental clades (Env Clade) C and D include sequences from a variety of different marine ODEs (Figs. 10.6 and 10.9a, b), Env Clade B includes exclusively hydrothermal vent sites (Fig. 10.8b). Fungal sequences from anoxic sediments in California branch in Clade 6 and group with *Kappamyces laurelensis*, a genus that has been described only few years back (Letcher and Powell 2005). Environmental fungal sequences obtained from Mariager fjord in Denmark (Baltic Sea) group with *Rhizophydium* spp., well-characterized marine parasites of phytoplanktons (Kagami et al. 2007). Interestingly, sequences obtained from intertidal anoxic sediments in western Greenland appear to be very divergent to any described fungal species and their phylogenetic position is strongly supported. The members of this cluster and the sequences from the three Env Clades B, C, and D have high bootstrap support, and most likely they represent new genera or even higher taxonomic categories. Members of phylum *Chytridiomycota* are being extensively studied for their phylogenetic affiliations as new members are being continuously added. Members of *Neocallimastigomycetes* are the only known obligate anaerobes, which have recently been upgraded to phylum level (Hibbett et al. 2007). Members of this group thrive under anaerobic conditions by a fermentative mode of respiration and can only survive in the anaerobic gut of rumens. Molecular analysis has shown that these groups are also present in natural anaerobic landfill areas (Lockhart et al. 2006).

There is a considerable number of fungal lineages, which cannot readily be assigned to any of these phyla and are therefore classified as "basal fungal lineages." The "true" phylogenetic position of many such lineages is discussed controversially. It remains to be seen whether further (environmental) sequencing efforts will strengthen the phylogenetic placement of these unassigned basal fungal lineages. In our study, environmental sequences from oxygen-depleted regions of Greenland, Gotland regions, intertidal sediments in California and Massachusetts grouped within the basal fungal group, clade 7 (Fig. 10.7). Interestingly, most of these sequences are from coastal regions (intertidal soft sediments), identifying such habitats as prime targets for the discovery of further basal fungal lineages.

The resolution of this clade needs to be clarified and further molecular diversity surveys with specific primer sets targeting basal fungal lineages might reveal more diversity within this group, and will possibly provide more sequences that help to elucidate the phylogenetic position of these taxa.

Our analyses show that, in general, the distribution of specific fungal lineages is not limited by geographical boundaries, and fungal sequences could be isolated from marine ODEs across the world oceans. This raises the question, whether one of the most discussed dogmas in microbial ecology, namely, "everything is everywhere, but the environment selects" (Baas Becking 1934) holds true for the fungi.

10.4 Methodological Considerations of Molecular Diversity Surveys and Future Directions

The number of sequences that could be assigned to *Chytridiomycota* or to basal fungal lineages is decisively lower than the number of sequences belonging to *Ascomycota* or *Basidiomycota*. This observation is likely ascribed to the following two reasons: (a) Chytridiomycota and basal fungi are indeed rare in natural (marine) habitats or have a restricted distribution in specific habitats compared to *Ascomycota* and *Basidiomycota*, for example, the basal fungal lineages are confined to anoxic intertidal soft-bottom habitats (Fig. 10.8). (b) Alternatively, methodological issues, like primer bias, could account for the low number of specific fungal taxon groups. If the latter is the case, the application of fungal-specific primer sets for the amplification of taxonomic marker genes could overcome this obstacle. Fungal-specific PCR primers generally target the ITS, SSU rRNA, or the LSU rRNA regions. Although these primer sets were designed to specifically amplify members of the fungal community from the environmental samples, cross-kingdom amplification is observed, which reduces the diversity estimates from the region of interest (Pang and Mitchell 2005). The SSU region has been the region of interest for a large number of studies because of the strong public database libraries that contain representative sequences representing all the major fungal groups. Studies targeting this region were able to amplify representatives of *Ascomycota, Basidiomycota, Chytridiomycota* and *Zygomycota* (Bass et al. 2007; Schadt et al. 2003; Malosso et al. 2006; Jebaraj et al. 2010), and also some members of the basal fungal lineages (Dawson and Pace 2002; Stoeck et al. 2007a) in various extreme habitats, such as the deep sea, frozen environments, and ODEs. Some studies suggest that the SSU rRNA gene is not the best choice for fungal diversity analysis, because of its low resolving power and because it may not always be sufficient to identify fungal species and strains (Hugenholtz and Pace 1996). A more powerful alternative might be the faster evolving ITS regions I and II which separate the ribosomal subunit genes from each other (O'Brien et al. 2005). Diversity estimates based on the ITS region may also have some disadvantages, as in some instances members belonging only to phylum *Ascomycota* and *Basidiomycota* were amplified

(Lai et al. 2007). These discrepancies may arise because not all of the fungal lineages are equally represented (Bruns 2001). The LSU rRNA region has been the most preferred region to study the distribution of some specific groups of *Fungi*, such as the mycorrhizal fungi (van Tuinen et al. 1998; Zuccaro et al. 2003). The target specificity of the primers targeting the LSU rRNA region is high, with the ability to preferentially amplify the target DNA from the fungal biomass, which is present in association with the plant material. Environmental studies using the LSU rRNA region as the target are very limited, in comparison with studies that use SSU rRNA as the marker gene. However novel fungal sequences were retrieved from the tundra soil based on LSU rRNA studies (Schadt et al. 2003). Taxon-specific studies using separate primers to target each of the fungal phyla have revealed a large diversity of *Fungi* from freshwater habitats (Nikolcheva and Bärlocher 2004). Hence, future environmental diversity studies could benefit from targeting primers specifically designed to amplify selected fungal phyla, and also following a multi-PCR approach, where all the major regions of taxonomic interest, such as the ITS, SSU rRNA, and LSU rRNA, could be amplified from the environmental DNA. This may be a versatile tool to amplify the fungal DNA across the various taxonomic communities, as the different primer sets can complement or compensate the strengths and disadvantages of each other.

The phylogenetic analyses of fungal sequences conducted in this study, which were based on publicly available SSU rRNA genes obtained from environmental diversity surveys, have revealed the extent of unknown diversity of *Fungi* in a variety of oxygen-deficient marine habitats on high taxonomic levels. The results show that *Fungi* are indeed much more diverse and abundant in anoxic habitats than previously assumed. Furthermore, most fungal lineages are widely distributed in different habitats with distinct environmental settings, indicating the versatility of *Fungi* and their capabilities to adapt to a variety of environmental conditions. This makes *Fungi* one of the most successful eukaryotic kingdoms, and with only a few exceptions (e.g. *Chytridiomycota* lineages that exhibit a more restricted distribution), they are able to colonize a large variety of natural habitats. Further studies, which specifically target fungal genes in environmental samples combined with culturable diversity studies, are needed for a deeper understanding of their distribution and their ecology in the marine ODEs.

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Chapter 11
Assemblage and Diversity of Fungi on Wood and Seaweed Litter of Seven Northwest Portuguese Beaches

K.R. Sridhar, K.S. Karamchand, C. Pascoal, and F. Cássio

Contents

Abstract Three hundred and fifty woody litter and one hundred and forty seaweed litter sampled from seven beaches of Northwest Portugal were assessed for the filamentous fungal assemblage and diversity. The woody litter was screened for fungi up to 42 months using damp chamber incubation. They consisted of 36 taxa (ascomycetes, 21; basidiomycetes, 3; anamorphic taxa, 12) comprising 10 core group taxa ($\geq 10\%$) (ascomycetes, 8; basidiomycete, 1; anamorphic taxa, 1). The total fungal isolates ranged between 150 and 243, while the number of fungal taxa per wood ranged between 3 and 4.9. The seaweed litter was screened up to four months in damp chamber incubation. They encompassed 29 taxa (ascomycetes, 16; basidiomycetes, 2; anamorphic taxa, 11) comprising 15 core group taxa (ascomycetes, 9; basidiomycete, 1; anamorphic taxa, 5). Total fungal isolates ranged between 56 and 120, while the number of fungal taxa per seaweed segment ranged

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between 4.8 and 6.3. Fifteen taxa of ascomycetes, two of basidiomycetes, and four anamorphic taxa were common to wood and seaweed litter. On both the substrates, two arenicolous fungi *Arenariomyces trifurcates* and *Corollospora maritima* were the predominant fungi (72.6–85.9%). The species abundance curves showed higher frequency of occurrence of fungal taxa in seaweed than woody litter. Our study revealed rich assemblage and diversity of marine fungi on wood and seaweed litter of Northwest Portugal beaches. The fungal composition and diversity of this survey have been compared with earlier investigations on marine fungi of Portugal coast.

11.1 Introduction

The fascinating ecological group of marine fungi has worldwide geographic distribution on a variety of substrata in different habitats. Filamentous marine fungi involve in decomposition and nutrient turnover of organic matter in marine ecosystem (Fell and Master 1980). They have been extensively surveyed in temperate regions (e.g., Kohlmeyer and Kohlmeyer 1979; Koch and Petersen 1996; Petersen and Koch 1997; Shearer et al. 2007) as well as tropical mangroves (e.g., Kohlmeyer 1984; Volkmann-Kohlmeyer and Kohlmeyer 1993; Jones and Alias 1997; Schmit and Shearer 2003, 2004; Sarma and Hyde 2001; Maria and Sridhar 2002). However, survey of marine fungi has been confined to localized areas especially in Southeast Asia, Europe, and North America (Kohlmeyer 1983; Shearer et al. 2007). Therefore, extensive survey is warranted in wide geographical areas to understand the pattern of fungal distribution more precisely. The global occurrence of marine fungi based on morphological and molecular phylogeny is 530 taxa in 321 genera (Ascomycota, 424 taxa in 251 genera; anamorphic fungi, 94 taxa in 61 genera; Basidiomycota, 12 taxa in 9 genera) (Jones et al. 2009). The decadal taxonomic studies on marine fungi (1840–2009) were highest during 1990–1999 (156 taxa) followed by 1980–1989 (135 taxa) and 1960–1969 (75 taxa). The described taxa ranged between 1 and 43 in rest of the 14 decades.

The main areas surveyed in European region include continental shelf from the Canaries and Azores to Greenland and North-West Russia including Mediterranean shelf and Baltic Seas (Landy and Jones 2006). The checklist revealed occurrence of 338 taxa of marine fungi in the European marine waters (Landy and Jones 2006). In contrast, 830 km long Portuguese coast is relatively less explored for marine fungal resources. Studies on marine fungi of Portugal coast was initiated with description of new marine basidiomycete taxon (*Nia globospora*) on the baits of *Spartina maritima* in Mira River estuary by Barata et al. (1997). Subsequently, she reported the occurrence of 20 marine fungi (ascomycetes, 15; anamorphic taxa, 5) associated with the culms of salt marsh halophyte *S. maritima* in three estuaries (Tagus, Sado, and Mira rivers) (Barata 2002). In another study, sterilized stems of *S. maritima* were baited up to 13 months in the estuary of River Mira to explore marine fungi and recorded 26 taxa (ascomycetes, 17; anamorphic taxa, 7; basidiomycetes, 2) (Barata 2006). The woody litter (*Arundo donax*) and herbaceous stems (*Phragmites*

sp. and *S. maritima*) immersed in two beaches (Rainha and Guincho) were incubated up to 10 months in laboratory and recorded 35 fungal taxa (ascomycetes, 27; anamorphic taxa, 6; unidentified taxa, 2) (Figueira and Barata 2007). Autoclaved wood pieces (*Fagus sylvatica* and *Pinus pinater*) were baited in two coastal locations (Cascais and Sesimbra) up to 12 months by Azevedo et al. (2010) yielded 26 fungal taxa (ascomycetes, 13; anamorphic taxa, 12; basidiomycete, 1) on laboratory incubation up to 12 months. All these studies were confined to the mid- and southwest coast of Portugal and the marine fungi of Northwest coast are not explored. Furthermore, although Portuguese coasts are endowed with rich seaweed flora (Pereira et al. 2006; Araújo et al. 2009), there seems to be no information on their association with fungi or detritus decomposing fungi. Hence, the present Chapter aims at understanding the assemblage and diversity of higher fungi on woody litter and seaweed litter of seven beaches of Northwest Portugal. Based on the present study and published reports, marine fungi on three substrata (woody litter, seaweed litter and culms of *Spartina*) of Portuguese coast are compared.

11.2 Materials and Methods

11.2.1 Study Sites and Samples

The coast of Portugal has rocky regions separated by white sandy beaches, which are often large in size and mainly used for recreation purpose. Seven beaches along the northwest coast (Moledo, Afife, Carreço, Foz do Neiva, Apúlia, Costa Nova, and Mira) were randomly selected for wood and seaweed litter collection (Fig. 11.1, Table 11.1).

About 60–70 woody litter (~1.5–2 cm diam.) preferably embedded in sand or entangled in rocky crevices from each beach were collected in clean polythene bags. They were rinsed in filter sterilized seawater in the laboratory to eliminate sediments and debris. Each wood was cut into 15 cm length and 50 wood samples per beach were incubated separately on the wet sand bed soaked in filter sterilized seawater (salinity, 34 ppt) in airtight polythene bags (23 ± 2°C) at 12-h photoperiod. They were screened once a month up to 2 years for the growth of anamorphs and teleomorphs. Moisture of sand bed was maintained by addition of sterile distilled water after each observation. From each beach, 20–30 detached seaweed thalli were sampled. They were cleaned in the laboratory, cut into segments (1–1.5 × 6 cm), and 20 pieces per beach (composite of 5–6 seaweed taxa) were incubated on sterile sand bed and observed as described below.

The fungi grown on wood and seaweed samples were mounted in seawater for morphological examination and later they were stained with cotton blue in lactophenol. Fungi isolated were identified based on monographs and primary literature (e.g. Ellis 1971, 1976; Kohlmeyer and Kohlmeyer 1979; Ellis and Ellis 1987; Hyde and Jones 1988; Kohlmeyer and Volkmann-Kohlmeyer 1991; Hyde et al. 2000; Jones et al. 2009).

Fig. 11.1 Map showing the
sampling sites of woody and
seaweed litter along the
Northwest Portugal coast

Table 11.1 Sampling sites of wood and seaweed litter along the Portugal coast

Beach	Location	Characteristics
Moledo	41°51′N 8°51′W	Sandy beach near Caminha Town (iodine rich)
Afife	41°46′N 8°52′W	Sandy beach near Viana do Castelo Town
Carreço	41°45′N 8°52′W	Sandy and rocky beach near Viana do Castelo Town (protected sand dunes)
Foz do Neiva	41°37′N 8°48′W	Sandy beach with adjacent endemic vegetation
Apúlia	41°29′N 8°46′W	Sandy beach (protected sand dunes)
Costa Nova	40°37′N 8°45′W	Sandy beach (fine sand grains)
Mira	40°27′N 8°48′W	Sandy beach (protected sand dunes)

11.2.2 Data Analysis

The frequency of occurrence (%) and relative abundance (%) of each fungal taxon
on wood and seaweed samples were determined:

$$\text{Frequency of occurrence } (\%) = [(\text{Number of wood pieces colonized})/$$
$$(\text{Totalwood pieces examined})] \times 100,$$

Relative abundance $(\%) = [(\text{Frequency of occurrence of fungal taxon})/$
$(\text{Total of frequency of occurrence fungal taxa})] \times 100.$

The Simpson's (D') and Shannon's (H') diversities (Magurran 1988) and Pielou's evenness (J') (Pielou 1975) of fungal taxa on wood and seaweed samples were calculated:

$$D' = 1/\Sigma(p_i)^2,$$

$$H' = -\Sigma(p_i \ln p_i),$$

where p_i is the proportions of individual that taxon i contributes to the total number of taxa.

$$J' = (H'/H'_{\text{max}}),$$

where H'_{max} is the maximum value of diversity for the number of taxa present.

To compare the richness of fungi based on number of isolations and number of wood and seaweed samples assessed, the expected number of taxa was calculated by rarefaction indices (Ludwig and Reynolds 1988). The expected number of taxa, $E_{(t)}$, in a random sample of n isolations taken from a total population of N isolations was estimated:

$$E_{(t)} = \sum_{i=1}^{s} \left\{ 1 - \left[\binom{N - n_i}{n} \Big/ \binom{N}{n} \right] \right\},$$

where n_i is the number of fungal isolations of the ith taxon.

Sorensen's similarity coefficient (C_s) (%) was calculated based on Mueller-Dombois (1981):

$$C_s(\%) = [2c/(a + b)] \times 100,$$

where a is total number of taxa in beach 1, b is total number of taxa in beach 2, and c is number of taxa common to beach 1 and 2.

11.3 Results

11.3.1 Fungi on Woody Litter

Assessment of 50 wood samples per beach yielded isolations ranging between 150 (3 isolations/wood) (Apúlia) and 243 (4.9 isolations/wood) (Mira) (Table 11.2). Total taxa per locations ranged from 9 (Foz do Neiva, Moledo and Apúlia) to

Table 11.2 Fungal assemblage species richness and diversity on woody litter collected from seven beaches of Northwest Portugal

Beach	Assemblage[a]				Species richness[b]		Anamorph (A)	Teleomorph (T)	A/T ratio	Diversity		
	Sc	Sco	Fi	Fpt	Total taxa	$E_{(t140)}$				Simpson	Shannon	Pielou's Evenness
Moledo	50	50	235	4.7	9	48	4	5	0.80	0.842	2.787	0.897
Afife	50	50	240	4.8	15	47	3	12	0.25	0.884	3.450	0.883
Carreço	50	46	171	3.4	10	49	2	8	0.25	0.856	3.020	0.909
Foz do Neiva	50	50	194	3.9	9	49	1	8	0.13	0.821	2.698	0.851
Apúlia	50	48	150	3.0	9	45	1	8	0.13	0.856	2.953	0.931
Costa Nova	50	49	206	4.1	12	49	4	8	0.50	0.860	3.110	0.868
Mira	50	47	243	4.9	10	49	1	9	0.11	0.861	2.373	0.895

[a]Assemblage: Sc, Number of wood samples collected; Sco, Number of wood samples colonized; Fi, Total fungal isolations on wood; Fpt, Number of fungal taxa per wood

[b]Species richness: $E_{(t140)}$, Expected number of fungal taxa out of 140 random isolations)

15 (Afife); however, the range of expected number of taxa out of 140 random isolations in seven beaches was narrow (45–49 taxa). The range of teleomorphs was higher than anamorphic taxa per beach (5–12 vs. 1–4) with A/T ratio ranging from 0.11 (Mira) to 0.80 (Moledo). The Simpson and Shannon diversities were highest in Afife (0.884 and 3.450), while they were least in Foz do Neiva (0.821) and Mira (2.373), respectively. The Pielou's evenness was highest in Apúlia (0.931) and least in Foz do Neiva (0.851).

Three hundred and fifty woody litter showed 36 taxa (ascomycetes, 21; basidiomycetes, 3; anamorphic taxa, 12). Eight ascomycetes and one each of basidiomycete and anamorphic taxon belonged to core group (≥10%) (ascomycetes: *Arenariomyces parvulus, A. trifurcates, Chaetomium cochlioides, Corollospora angusta, C. maritima, C. pulchella, Dryosphaera navigans, Halosphaeriopsis* sp.; basidiomycete: *Nia vibrissa*; anamorphic taxon: *Bactrodesmium linderi*) (Table 11.3). Among them, *Arenariomyces trifurcatus, B. linderi,* and *Carollospora maritima* were the most frequent taxa (42.1–82.1%) with high relative abundance (10.1–19.7%). Five taxa out of 21 ascomycetes have been reported from the Portugal and up to 11 typical marine ascomycetes are new records to Portugal coast. Interestingly, among three taxa of *Chaetomium* on woody litter (*C. cochlioides, C. gobosum,* and *C. reflexum*), *C. cochlioides* belonged to core group (16.2%). Out of three basidiomycetes, an unknown taxon possessing sigmoid basidiospores was recorded from Costa Nova. *N. vibrissa* and *N. globospora* have been reported from Portugal. Typical marine anamorphic taxa, *B. linderi* and *Astreomyces cruciatus* are new record to Portugal coast. Up to 10 anamorphic taxa seems to be terrestrial fungi and unlike marine fungi their frequency of occurrence was very low (0.9–1.8%). Sorensen's similarity of fungi between 21 beach pairs was least between Afife and Costa Nova (14.8%) and highest between Foz do Neiva and Mira (73.7%) (Table 11.4). Only seven beach pairs showed similarity 50% and above, while it was ranged between 14.8 and 44.4% in rest of the pairs.

11.3.2 Fungi on Seaweed Litter

Assessment of 20 seaweed samples per beach yielded isolations ranging between 56 (2.8 isolations/wood) (Costa Nova) and 125 (6.3 isolations/wood) (Carreço) (Table 11.5). Total taxa per location ranged from 4 (Costa Nova) to 13 (Apúlia), however, the expected number of taxa out of 45 random isolations in all beaches was uniform (19 taxa). The range of teleomorphs was higher than anamorphic taxa per beach (3–8 vs. 1–6) with A/T ratio ranging from 0.33 (Costa Nova) to 0.86 (Apúlia). The Simpson diversity was highest in Apúlia (0.902) and the Shannon diversity in Moledo (3.923), while they were least in Costa Nova (0.705 and 1.876). The Pielou's evenness was highest in Foz do Neiva (0.956) and least in Moledo (0.924).

Table 11.3 Occurrence of fungal taxa on woody litter sampled from seven beaches of Northwest Portugal (out of 50 samples in each beach)

Taxon	Occurrence in beaches							FO (%)	RA (%)	[a]Report
	Mo	Af	Ca	FN	Ap	CN	Mi			
Ascomycetes										
[b]*Corollospora maritima* Werderm.	49	45	45	45	20	39	35	82.1	19.7	2, 3, 5
[b]*Arenariomyces trifurcates* Höhnk	42	50	40	38	35	31	39	80.9	19.4	3
[b,c]*Dryosphaera navigans* Jørg. Koch & E.B.G. Jones	–	15	–	40	–	–	35	26.5	6.4	–
[b,c]*Corollospora pulchella* Kohlm., I. Schmidt & N.B. Nair	–	–	09	35	–	–	27	20.9	5.0	–
[b]*Halosphaeriopsis* sp.	–	29	15	04	15	–	03	19.1	4.6	–
[b,c]*Arenariomyces parvulus* Jørg. Koch	–	05	10	09	15	–	19	17.1	4.1	–
[b]*Chaetomium cochlioides* Palliser	24	10	–	–	21	–	–	16.2	3.9	–
[b,c]*Corollospora angusta* Nakagiri & Tokura	–	–	35	–	–	05	–	11.8	2.8	–
[b]*Nereiospora* sp.	–	20	11	–	–	–	–	9.1	2.2	–
[b,c]*Carbosphaerella leptosphaerioides* I. Schmidt	–	–	–	05	–	25	–	8.8	2.1	–
[c]*Lignincola laevis* Höhnk	30	–	–	–	–	–	–	8.8	2.1	2, 4
Chaetomium globosum Kunze	–	15	–	10	–	–	–	7.4	1.8	–
[c]*Dryosphaera tropicalis* Kohlm. & Volkm.-Kohlm.	–	05	–	–	–	–	20	7.4	1.8	–
Phaeosphaeria orae-maris (Linder) Khashn. & Shearer	–	–	17	–	–	–	–	5.0	1.2	3, 5
[b,c]*Verruculina enalia* (Kohlm.) Kohlm. & Volkm.-Kohlm.	–	11	–	–	–	–	04	4.4	1.1	–
Lulworthia fucicola G.K. Sutherl.	–	–	–	–	–	11	–	3.2	0.8	3, 4
[b,c]*Dactylospora haliotrepha* (Kohlm. & E. Kohlm.) Hafellner	–	–	–	–	06	–	03	2.7	0.7	–
[b,c]*Lignincola tropica* Kohlm.	–	–	–	–	09	–	–	2.7	0.7	–
[c]*Leptosphaeria orae-maris* Linder	–	–	–	–	–	06	–	1.8	0.4	–
Chaetomium reflexum Skolko & J.W. Groves	–	05	–	–		–	–	1.5	0.4	–
[b,c]*Hydronectria tethys* Kohlm. & E. Kohlm.	–	–	–	–	05	–	–	1.5	0.4	–
Basidiomycetes										
[b]*Nia vibrissa* R.T. Moore & Meyers	42	–	–	–	–	24	–	19.4	4.7	2, 5
[b]*Nia globospora* Barata & Basilio	–	05	–	–	–	–	–	1.5	0.4	1, 2
[c]Unknown sp. (sigmoid spores)	–	–	–	–	–	06	–	0.3	0.1	–
Anamorphic taxa										
[b,c]*Bactrodesmium linderi* (J.L. Crane & Shearer) M.E. Palm & E.L. Stewart	38	–	–	05	25	45	35	42.1	10.1	–
[b]*Aspergillus flavus* Link	–	20	–	–	–	–	–	5.9	1.4	–
[b,c]*Astreomyces cruciatus* Moreau & R. Moreau	–	–	16	–	–	–	–	4.7	1.1	–
	–	06	–	–	–		–	1.8	0.4	–

(continued)

Table 11.3 (continued)

Taxon	Occurrence in beaches							FO (%)	RA (%)	[a]Report
	Mo	Af	Ca	FN	Ap	CN	Mi			
Camposporium pellucidum (Grove) S. Hughes										
Curvularia brachyspora Boedijn	–	–	–	–	–	06	–	1.8	0.4	–
[b]*Aspergillus puniceus* Kwon-Chung & Fennell	–	–	05	–	–	–	–	1.5	0.4	–
Curvularia crepinii (Westend.) Boedijn	05	–	–	–	–	–	–	1.5	0.4	–
Dortomyces stemonitis (Pers.) F.J. Morton & G. Sm.	–	05	–	–	–	–	–	1.5	0.4	–
Drechslera fugax (Wallr.) Shoemaker	04	–	–	–	–		–	1.2	0.4	–
Tetracoccosporium paxianum Szabó	–	–	–	–	–	05	–	1.5	0.4	–
Wardomyces anomalus F.T. Brooks & Hansf.		–	–	–	–	04	–	1.2	0.4	–
Diplocladiella scalaroides G. Arnaud	03	–	–	–	–	–	–	0.9	0.1	–

FO%, Percent frequency of occurrence; RA%, Percent relative abundance; arranged in decreasing order

Beaches: *Mo* Moledo; *Af* Afife; *Ca* Carreço; *FN* Foz do Neiva; *Ap* Apúlia; *CN* Costa Nova; *Mi* Mira

[a]Reported from Portugal: 1, Dead culms of *Spartina maritima*: Barata et al. 1997; 2, Baited sterilized young stem of *S. maritima*: Barata 2006; 3, Stems of *Phragmites* sp. and *Spartina maritima*: Figueira and Barata 2007; 4, Beach woody litter: Figueira and Barata 2007; 5, Baited wood pieces of *Fagus sylvatica* and *Pinus pinaster*:Azevedo et al. 2010

[b]Also found in seaweed litter

[c]New to Portugal marine habitats

A total of 140 seaweed samples consist of 29 taxa (ascomycetes, 16; basidiomycetes, 2; anamorphic taxa, 11). Nine ascomycetes, one basidiomycete, and five anamorphic taxa belonged to core group ($\geq$10%) (ascomycetes: *A. trifurcatus, C. cochlioides, C. angusta, C. maritima, C. pulchella, Halosphaeriopsis* sp., *Dactylospora haliotrepha, D. navigans,* and *Verruculina enalia*; basidiomycete: *N. vibrissa*; anamorphic taxa: *Aspergillus flavus, A. puniceus, A. oxysporum, A. tamari,* and *B. linderi*) (Table 11.6). Among core group taxa, *A. trifurcatus, Aspergillus tamari, B. linderi,* and *C. maritima* were the most frequent taxa (40.7–85.9%) with relative abundance ranging from 6.2 to 16.3%. Three taxa out of 16 ascomycetes have been reported from the Portugal and up to 12 typical marine ascomycetes are new records. Among ascomycetes, *C. cochlioides* belonged to core group (13.3%). Among basidiomycetes, *N. vibrissa* and *N. globospora* have been reported from Portugal coast. Out of 11 anamorphic taxa, *Periconia prolifica* is known from Portugal, while three taxa *A. cruciatus, B. linderi,* and *Dendryphiella arenariae* are new records. Up to seven anamorphic taxa seems to be typical terrestrial fungi, and four of them were more frequent (14.8–40.7%). Soresen's similarity of fungi between 21 pairs of beaches was least between Costa Nova and Mira (26.7%) and highest between Afife and Carreço and Foz do Neiva and Mira (60%) (Table 11.4). Only five pairs of beaches showed similarity up to 50% and above, while it was ranged between 26.7 and 47.6% in rest of the beach pairs.

Table 11.4 Sorensen's similarity coefficient (%) of fungal taxa on woody litter and seaweed litter (in parenthesis) from seven beaches of Northwest Portugal

	Afife	Carreço	Foz do Neiva	Apúlia	Costa Nova	Mira
Moledo	25 (52.6)	21.1 (31.6)	33.3 (55.6)	44.4 (36.4)	38.1 (46.2)	31.6 (30)
	Afife	40 (60)	50 (31.6)	41.7 (34.8)	14.8 (28.6)	56 (28.6)
		Carreço	52.6 (42.1)	42.1 (43.5)	27.3 (28.6)	50 (47.6)
			Foz do Neiva	55.6 (45.5)	38.1 (46.2)	73.7 (60)
				Apúlia	28.6 (35.3)	63.2 (50)
					Costa Nova	27.3 (26.7)

11.3.3 Fungal Composition in Wood and Seaweed Litter

Altogether wood and seaweed litter yielded 44 taxa (ascomycetes, 22; basidiomycetes, 3; anamorphic taxa, 19). Woody litter represented by 81% of total fungi, while seaweed litter consists of 70% (Tables 11.3 and 11.6). Twenty one taxa were common to both substrates (ascomycetes, 15; basidiomycetes 2; anamorphic taxa, 4). Teleomorphs of wood and seaweed litter were more similar than anamorphic taxa. Anamorph/teleomorph ratio (0.61 vs. 0.50), Simpson diversity (0.923 vs. 0.900), Shannon diversity (4.116 vs. 3.994), and Pielou's equitability (0.867 vs. 0.773) were higher in seaweed than woody litter.

Seven teleomorphs were confined to woody litter (*Chaetomium globosum, C. reflexum, Dryosphaera tropicalis, Leptosphaeria orae-maris, Lulworthia fucicola, Phaeosphaeria orae-maris,* and unknown basidiomycete), while only one ascomycete was restricted to seaweed litter (*Zopfiella latipes*) (Table 11.3). Among the anamorphic taxa, eight were confined to woody litter (*Camposporium pellucidum, Curvularia brachyspora, C. crepinii*, *Diplocladiella scalaroides, Dortomyces stemonitis, Drechslera fugax, Tetracoccosporium paxianum*, and *Wardomyces anomalus*), while seven were confined to seaweed litter (*Aspergillus niger, A. tamari, D. arenariae, Fusarium oxysporum, P. prolifica, Sporidesmium afrormosiae,* and *Trichoderma harzianum*) (Table 11.6).

Eight core group teleomorphs were common to both substrates (*A. trifurcatus, C. cochlioides, C. angusta, C. maritima, C. pulchella, Halosphaeriopsis* sp., *D. navigans,* and *N. vibrissa*) (Tables 11.3 and 11.6). Only one core group anamorphic taxon was common to wood and seaweed (*B. linderi*). Interestingly, on both substrates two arenicolous fungi *Arenariomyces trifurcates* and *Corollorpora maritima* were dominated in all beaches (72.6–85.9%).

Beach-wise comparison of fungal assemblage, richness, and diversity between wood and seaweed litter drastically varied (Tables 11.2 and 11.5). Although overall occurrence of fungi was higher in woody litter, on plotting frequency of occurrence against species sequence, the frequency of occurrence of fungi in seaweed litter was higher than woody litter (Fig. 11.2). The overall rarefaction curve of woody litter resembles that of all beaches except for the beach Apúlia, so also the curve of seaweed litter except for the beach Costa Nova (Fig. 11.3). The range of expected number of taxa, $E_{(t)}$, out of 140 random isolations of woody litter per beach was

Table 11.5 Fungal assemblage species richness and diversity on seaweed litter collected from seven beaches of Northwest Portugal

Beach	Assemblage[a]				Species richness[b]		Anamorph (A)	Teleomorph (T)	A/T ratio	Diversity		
	Sc	Sco	Fi	Fpt	Total taxa	$E_{(t45)}$				Simpson	Shannon	Pielou's Evenness
Moledo	20	20	104	5.2	9	19	3	6	0.50	0.856	3.928	0.924
Afife	20	20	95	4.8	10	19	4	6	0.67	0.880	3.173	0.955
Carreço	20	17	125	6.3	10	19	4	6	0.67	0.878	3.115	0.950
Foz do Neiva	20	20	110	5.5	9	19	3	6	0.50	0.868	3.030	0.956
Apúlia	20	20	121	6.1	13	19	6	7	0.86	0.902	3.505	0.947
Costa Nova	20	19	56	2.8	4	19	1	3	0.33	0.705	1.876	0.938
Mira	20	19	120	6.0	11	19	3	8	0.38	0.888	3.208	0.948

[a]Assemblage: Sc, Number of seaweed samples collected; Sco, Number of seaweed samples colonized; Fi, Total fungal isolations on seaweed samples; Fpt, Number of fungal taxa per seaweed sample

[b]Species richness: $E_{(t45)}$, Expected number of fungal taxa out of 45 random isolations)

Table 11.6 Occurrence of fungal taxa on seaweed litter sampled from seven beaches of North-west Portugal (out of 50 samples in each beach)

Taxon	Occurrence in beaches							FO (%)	RA (%)	[a]Report
	Mo	Af	Ca	FN	Ap	CN	Mi			
Ascomycetes										
[b]*Arenariomyces trifurcatus* Höhnk	15	14	9	20	15	29	14	85.9	16.3	3
[b]*Corollospora maritima* Werderm.	19	15	17	10	05	16	16	72.6	13.8	2, 3, 5
[b, c]*Verruculina enalia* (Kohlm.) Kohlm. & Volkm.-Kohlm.	–	–	–	10	15	–	11	26.7	5.1	–
[b]*Halosphaeriopsis* sp.	–	10	05	–	14	–	–	21.5	4.1	–
[b, c]*Corollospora pulchella* Kohlm., I. Schmidt & N. B. Nair	–	–	04	19	–	–	05	20.7	3.9	–
[b, c]*Dryosphaera navigans* Jørg. Koch & E.B.G. Jones	–	–	–	10	–	–	15	18.5	3.5	–
[b]*Chaetomium cochlioides* Palliser	14	04	–	–	–	–	–	13.3	2.5	–
[b, c]*Corollospora angusta* Nakagiri & Tokura	–	–	17	–	–	–	–	12.6	2.4	–
[b, c]*Dactylospora haliotrepha* (Kohlm. & E. Kohlm.) Hafellner	–	–	–	–	09	–	05	10.4	1.2	–
[b, c]*Hydronectria tethys* Kohlm. & E. Kohlm.	–	–	–	–	10	–	–	7.4	1.4	–
[b, c]*Lignincola tropica* Kohlm.	–	–	–	–	10	–	–	7.4	1.4	–
[b, c]*Arenariomyces parvulus* Jørg. Koch	–	05	04	–	–	–	–	6.7	1.3	–
[b, c]*Carbosphaerella leptosphaerioides* I. Schmidt	–	–	–	–	–	09	–	6.7	1.3	–
[c]*Zopfiella latipes* (N. Lundq.) Malloch & Cain	04	–	–	05	–	–	–	6.7	1.3	–
[b]*Nereiospora* sp.	–	05	–	–	–	–	–	3.7	0.7	–
[b]*Lignincola laevis* Höhnk	03	–	–	–	–	–	–	2.2	0.4	2, 4
Basidiomycetes										
[b]*Nia vibrissa* R.T. Moore & Meyers	16	–	–	–	–	–	19	25.9	4.9	2, 5
[b]*Nia globospora* Barata & Basilio	–	–	–	–	–	–	05	3.7	0.7	1, 2
Anamorphic taxa										
[b, c]*Bactrodesmium linderi* (J.L. Crane & Shearer) M.E. Palm & E.L. Stewart	20	–	–	15	09	15	–	43.7	8.3	–
Aspergillus tamari Kita	09	10	15	–	05	–	16	40.7	7.7	–
[b]*Aspergillus flavus* Link	–	–	15	16	04	–	09	32.6	6.2	–
[b]*Aspergillus puniceus* Kwon-Chung & Fennell	–	15	10	–	–	–	–	18.5	3.5	–
Fusarium oxysporum E.F. Sm. & Swingle	04	11	–	05	–	–	–	14.8	2.8	–
[b, c]*Astreomyces cruciatus* Moreau & R. Moreau	–	–	10	–	–	–	–	7.4	1.4	–
Aspergillus niger Tiegh.	–	05	–	–	–	–	–	3.7	0.7	–
[c]*Dendryphiella arenariae* Nicot	–	–	–	–	05	–	–	3.7	0.7	–
Periconia prolifica Anastasiou	–	–	–	–	–	–	04	3.0	0.6	5
Sporidesmium afrormosiae M.B. Ellis	–	–	–	–	03	–	–	2.2	0.4	–
Trichoderma harzianum Rifai	–	–	–	–	03	–	–	2.2	0.4	–

FO%, Percent frequency of occurrence; RA%, Percent relative abundance; arranged in decreasing order

Beaches: *Mo* Moledo; *Af* Afife; *Ca* Carreço; *FN* Foz do Neiva; *Ap* Apúlia; *CN* Costa Nova; *Mi* Mira

[a]Reported from Portugal: 1, Dead culms of *Spartina maritima*: Barata et al. 1997; 2, Baited sterilized young stem of *S. maritima*: Barata 2006; 3, Stems of *Phragmites* sp. and *Spartina maritima*: Figueira and Barata 2007; 4, Beach woody litter: Figueira and Barata 2007; 5, Baited wood pieces of *Fagus sylvatica* and *Pinus pinaster*: Azevedo et al. 2010

[b]Also found in woody litter

[c]New to Portugal marine habitats

narrow (45–49), but on pooling isolates of all beaches the $E_{(t)}$ raised to 117 taxa (Table 11.2 and Fig. 11.3). Similarly, the $E_{(t)}$ out of 45 random isolations of seaweed litter was 19 which raised to 40 on pooling isolates of all beaches (Table 11.5 and Fig. 11.3). The $E_{(t)}$ out of 700 random isolations of pooled data was 308 in woody litter, while it was 140 in seaweed litter. On comparison of Soresen's similarity of fungi between wood and seaweed litter, it was least (wood) or near to least (seaweed) between Afife and Costa Nova (14.8 and 28.6%), while it was highest between Foz do Neiva and Mira (73.7 and 60%) (Table 11.4). The similarity of fungi on seaweed litter of Afife vs. Carreço was also highest (60%)

11.4 Discussion

Driftwood in marine habitats has been considered as the most important ecological niche for marine fungi (Hyde et al. 2000). Although marine fungi of European region are relatively well known (Kohlmeyer 1983; Landy and Jones 2006; Shearer et al. 2007), studies on marine fungi of Portugal coast are scanty. Available literature deals with marine fungi mainly on salt marsh halophyte (*Spartima maritima*) in estuaries of Portugal (Barata et al. 1997; Barata 2002, 2006). Stems of monocots (*Phragmites* sp. and *S. maritima*) collected from two beaches were studied for marine fungi by Figueira and Barata (2007). Two studies dealt with marine fungi associated with naturally accumulated woody litter (*A. donax*) (Figueira and Barata 2007) and baited woody litter (*F. sylvatica* and *P. pinater*)
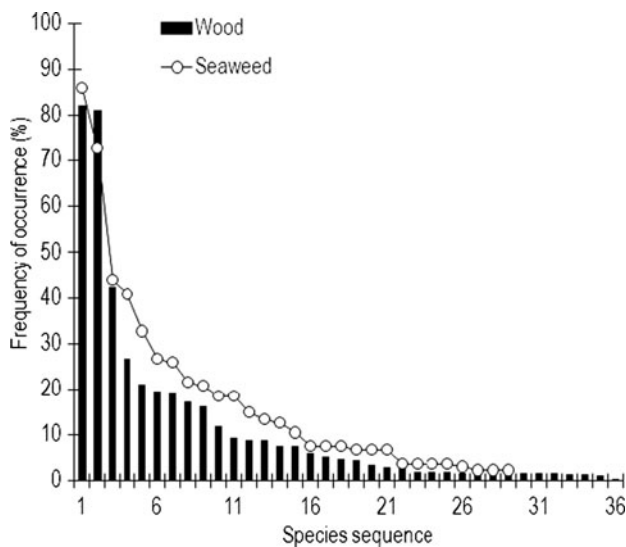
Fig. 11.2 Species abundance curves of fungi recorded on woody and seaweed litter (Fungal taxa on each substrate were arranged in descending order)
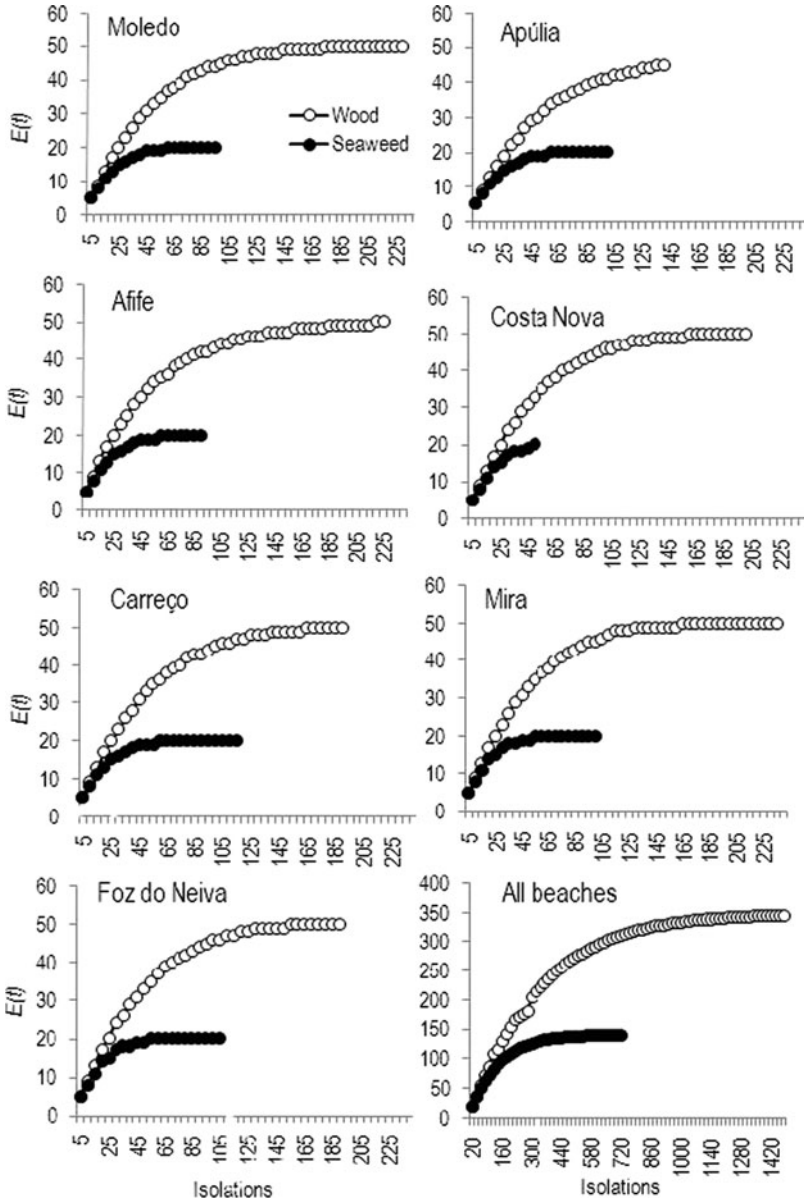
Fig. 11.3 Comparison of rarefaction curve of fungi on woody and seaweed litter of seven beaches of Northwest Portugal coast (number of isolations vs. expected number of taxa)

(Azevedo et al. 2010). Although intertidal regions of Portugal coast are endowed with a variety of seaweeds (e.g., Pereira et al. 2006; Araújo et al. 2009) there seems to be no studies on their association or decomposition by fungi.

11.4.1 Fungi on Woody Litter

Incubation of woody litter sampled from seven beaches of Northwest Portugal up to 24 months in our study yielded 24 typical marine fungi (teleomorphs, 22; anamorphic taxa, 2) (Table 11.3). Among them, only seven teleomorphs are known from the Portugal coast, but none of anamorphic taxa are common to earlier studies. Overall 14 marine fungi (teleomorphs, 12; anamorphic taxa, 2) are new records to Portugal coast.

Our study also yielded many arenicolous fungi on woody litter. Interestingly, the top-ranked two taxa belonged to arenicolous group (*Areneriomyces trufurcatus* and *Corollospora maritima*) with cosmopolitan distribution (Kohlmeyer 1983; Kohlmeyer and Volkmann-Kohlmeyer 1993; Nakagiri and Tokura 1994). Fruit bodies of arenicolous fungi usually develop on wood or animal substrates on long-term incubation (e.g., Ananda et al. 1998; Prasannarai and Sridhar 2003; Ananda and Sridhar 2004). The dominance of arenicolous or nonarenicolous fungi on woody litter may support or suppress other fungi (Tan and Leong 1992; Tan et al. 1995; Jones 2000). For example, *Aigialus parvus* and *V. enalia* suppressed *Lignincola laevis* in co-culture, while *L. laevis* enhances formation of ascomata in association with *V. enalia*. Similarly, *C. maritima* and *V. enalia* have been considered as "aggressive" taxa (Miller et al. 1985; Tan et al. 1995). As diverse fungal flora was seen in our study, the dominant arenicolous fungi might have positive rather than negative interaction with co-occurring fungi.

Short-term incubation (2–4 months) of woody litter yields primarily terrestrial fungi, but long-term incubation (6–12 months) leads to dominance of marine fungi (e.g., Ravikumar et al. 2009). The present study revealed some commonly occurring terrestrial fungi on woody litter (e.g., Ascomycetes: *Chaetomium* spp.; Anamorphic taxa: *Aspergillus* spp., *Camposporium* sp., *Curvularia* spp., *Diplocladiella* sp., *Dortomyces* sp., *Drechslera* sp., *Tetracoccosporium* sp., and *Wardomyces* sp.). However, except for *A. flavus, C. cochlioides,* and *C. globosum* (5.9–16.2%), the rest were found in low frequency (0.9–1.8%). Contribution toward decomposition of organic matter by so-called terrestrial fungi in marine habitats cannot be ruled out (Sridhar et al. 2010). It is interesting to note that seawater, sea foam, and beach sand in Saudi Arabia yielded many terrestrial fungi along with typical marine fungi (Bokhary et al. 1992). Similarly, about 36 taxa of terrestrial fungi were isolated from the hypersaline salterns in Puerto Rico (Cantrell et al. 2006). Among them, *A. flavus, A. niger,* and *C. globosum* were common to our study. Fungal taxa such as *A. niger* and *C. globosum* were also inhabitants of hypersaline waters in temperate and tropical habitats (see Cantrell et al. 2006). Cantrell et al. (2006) classified *A. flavus* as moderately halotolerant taxon, while *A. niger* and *C. globosum* as highly halotolerant taxa. Diverse taxa of *Aspergillus* and *Penicillium* have been reported from the marine sediments from different region (e.g., Sponga et al. 1999; Takami 1999; Damare et al. 2006; Ramesh et al. 2006; Jebraj and Raghukumar 2009; Das et al. 2009).

The data on the mean number of fungi per sample in a substrate help comparing different hosts or regions. The mean number of fungi per wood sample in the present study was ranged between 3 and 4.9. It is higher than mangrove woody litter in Indian ocean (1–1.7), Hong Kong, Macau (1.2–1.7), Southeast Asia (0.9–2.6) (see Maria and Sridhar 2003), Red Sea mangroves of Egypt (1.1–1.3) (Abdel-Wahab 2005), Mediterranean Sea (1–2.8) (Abdel-Aziz 2010), Bahamas Islands (0.9–1.9) (Jones and Abdel-Wahab 2005), and partially resembles the reports on mangrove wood in Malaysia (3.3) and west coast of India (1.5–4.6) (Maria and Sridhar 2004). However, the mean fungi per wood in our study is higher than the report on woody litter (3–4.9 vs. 0.96) (Figueira and Barata 2007) and immersed wood segments (1.11–1.72) (Azevedo et al. 2010) from Portugal. But mean fungi per wood partially resemble the reports on stems of *S. maritima* (1.2–3.7) from Portugal coast (Barata 2006).

11.4.2   Fungi on Seaweed Litter

Incubation of seaweed litter collected from seven beaches of Northwest Portugal up to 4 months in our study yielded 21 typical marine fungi (teleomorphs, 17; anamorphic taxa, 4 taxa) (Table 11.6). Among them, five teleomorphs and an anamorphic taxon are known from the Portugal coast (Barata et al. 1997; Barata 2006; Figueira and Barata 2007; Azevedo et al. 2010). Overall 13 marine fungi (teleomorphs, 10; anamorphic taxa, 3) are new records to the Portugal coast. The mean number of fungi per seaweed litter ranged between 2.8 and 6.3, which is equivalent or higher than woody litter (Tables 11.2 and 11.5) and other substrata assessed in marine and mangrove habitats (see Sect. 11.4.1). The mean fungi per seaweed litter is higher than woody litter and immersed wood segments (0.96–1.72) (Figueira and Barata 2007; Azevedo et al. 2010) and partially resembles with that of stems of *S. maritima* (1.2–3.7) (Barata 2006).

A variety of arenicolous fungi were inhabitants of seaweed litter in our study and top-ranked two species belonged to cosmopolitan arenicolous fungi (*A. trifurcatus* and *C. maritima*). Unlike woody litter, in seaweed detritus in our study the arenicolous fungi grew within 2–4 months of incubation. Beyond this period, they cannot be handled as they become more amorphous. Seaweed detritus were host for many saprophytic marine fungi including arenicolous fungi (see Zuccaro and Mitchell 2005). Molecular studies on healthy thalli of brown seaweed *Fucus serratus* from temperate region yielded several arenicolous fungi along with nonarenicolous fungi (Zuccaro and Mitchell 2005). As seen in woody litter, besides typical marine fungi, a variety of terrestrial fungi were inhabitants of seaweed detritus in our study (*Aspergillus* spp., *Chaetomium* sp., *Fusarium* sp., *Sporidesmium* sp., and *Trichoderma* sp.). Some of these fungi were also endophytic in seaweeds of the southeast coast of India (Suryanarayanan et al. 2010). Molecular studies of live and dead thalli of *Fucus serratus* and other seaweeds (of Antarctica) also yielded several terrestrial fungi and arenicolous fungi (Zuccaro and Mitchell 2008; Loque et al. 2009).

11.5 Conclusions

Our study revealed rich fungal population because of assessment of mixture of wood and seaweed litter from many beaches of Northwest Portugal. Most of the earlier studies were confined to assess fungal assemblage mainly from the saltmarsh plant species *S. maritima* (Barata et al. 1997; Barata 2002, 2006; Figueira and Barata 2007) except for two studies on beach woody litter (Figueira and Barata 2007) and immersed wood segments (Azevedo et al. 2010). As seen in the present study, the frequency of occurrence or percentage occurrence of some fungi were very high on immersed wood (e.g., *Cirrenalia macrocephala*, 48.6%; *Lulworthia* sp., 88.9%) (Azevedo et al. 2010) and monocot stems (*Dictysporium pelagicum*, 66.7%; *Halosarpheia retorquens*, 70%; *Lulworthia* sp., 88.6%; *Phialophorophoma litoralis*, 59.2%; *Phoma* sp., 56.7%; *Sphaerulina oraemaris*, 57.5%) (Barata 2002, 2006) in the Portugal coast. The basidiomycete, *N. globospora* isolated from *S. maritima* of Mira river estuary (Barata et al. 1997), was also reported to colonize on the baited stem of *S. maritima* in Mira saltmarsh (Barata 2002, 2006). In our study, this fungus was an inhabitant of seaweed litter of Mira beach and the woody litter of the beach Afife. None of the fungi in our study was comparable to fungi found on *S. maritima* segments submerged in two salt marshes (Barata 2006). This may be due to assessment of young and senescent stems of *S. maritima* or possibly due to substrate specificity. There seems to be considerable difference in fungal population on organic matter between Northwest and Southwest Portugal as these coasts are influenced by the major currents Canary and Azores, respectively. Our study provided a baseline data on the fungal composition and diversity on wood and seaweed litter of Northwest Portugal. Future explorations may consider sampling and assessment of organic matter from Northwest and Southwest locations simultaneously to provide a clear picture on the fungal assemblage and diversity to strengthen the mycogeography of marine fungi.

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Chapter 12
Xylariaceae on the Fringe

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Contents

Abstract The Xylariaceae is one of the best-known pyrenomycete families (Ascomycota) and is distributed throughout the world. The majority are wood inhabitants and are prevalent in tropical and subtropical regions. *Halorosellinia oceanicum* is the most widely distributed in mangroves and can be regarded as truly manglicolous being frequently recorded as the dominant member of the family in such environments in S.E. Asia. In Malaysian mangroves, members of the Xylariaceae have been found to be numerically important with up to 9% present in one mangrove ecosystem. A further twelve xylariaceous genera are reported as occurring as their teleomorphs in mangrove forest and their immediate surroundings including *Anthostomella*, *Astrocystis*, *Biscogniauxia*, *Camillea*, *Daldinia*, *Fasciatispora*, *Hypoxylon*, *Kretzschmaria*, *Nemania*, *Nipicola*, *Rosellinia* and *Xylaria*. Furthermore, the presence of species from a number of these taxa, especially species of *Anthostomella* and *Xylaria*, are regularly isolated as endophytes from a variety of mangrove plant species. Mangrove Xylariaceae are also well known for their ability to produce novel and often bioactive metabolites.

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12.1 Introduction

What constitutes a marine fungus is open to debate but a generally accepted definition of marine fungi is that provided by Kohlmeyer and Kohlmeyer (1979) "obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat; facultative marine fungi are those from fresh water or terrestrial milieus able to grow (and possibly sporulate) in the marine environment". Jones et al. (2009) adopted a broad interpretation of what we consider to be marine and included species saprotrophic on decaying culms of maritime grasses such as *Spartina* species, *Juncus roemerianus*, *Phragmites communis*, mangrove fungi, and especially those on the palm, *Nypa fruticans*. We review the recorded Xylariaceae, including endophytes, although many of these belong to *Xylaria* Hill ex Schrank and are only identified to generic level. Also included are descriptions of *Halorosellinia oceanica* (Schatz) Whalley, E.B.G. Jones, K.D. Hyde and Laessoe *Camillea selangorensis* M.A. Whalley, Whalley and E.B.G. Jones and *C. malaysianensis* M.A. Whalley. *Halorosellinia oceanica* because it is the most widely distributed and is similar to, and may be confused with *Nemania maritima* Y.-M. Ju and J.D. Rogers, which occurs in Taiwan, Malaysia and appears to be common in Hong Kong (Ju and Rogers 2002; Jones et al. 2009), *C. selangorensis*, which is only the second species of *Camillea* to be recorded in the old world and which may prove to occur more extensively on *Ficus* bordering mangroves in S.E. Asia and elsewhere and *C. malaysianensis* because of possible confusion with the widely distributed *C. tinctor* (Berk.) Læssøe, J.D. Rogers and Whalley (Whalley et al. 1999). The Xylariaceae is one of the best known and widely distributed families of the Ascomycota. Ju and Rogers (1996) recognized 38 and Whalley (1996) 40 genera but with subsequent additions, the number is now 65 or more depending on individual opinion (Lumbsch and Huhndorf 2007). Although the genera *Hypocopra* (Fr.) J. Kickx fil. *Poronia* Willd., *Podosordaria* Ellis & Holw. and *Wawelia* Namyslowski occur on dung and a number of *Xylaria* species occur on leaves, seeds or are associated with insects, the family is mainly one of wood inhabitants. The vast majority of these wood inhabitants are considered to be saprobes but several species are well-known phytopathogens. *Rosellinia necatrix* Prill. causes a world-wide serious white root rot of many commercial plants, *Entoleuca mammata* (Wahlenberg:Fr.) J.D. Rogers & Y.-M. Ju is a major pathogen of aspen trees in North America and species of *Biscogniauxia* Kuntze induce canker formation in trees, which are water-stressed (Edwards et al. 2003; Nugent et al. 2004). Currently there is considerable interest in the Xylariaceae as they are now found to be almost ubiquitous endophytic inhabitants with a high presence in tropical plants (Whalley 1996). They are also known to produce an impressive array of novel natural compounds (Whalley and Edwards 1995; Stadler and Hellwig 2005). It is this ability to produce a wide range of chemical structures coupled with their common endophytic occurrence, which has stimulated considerable research into the family.

12.1.1   The Xylariaceae and the Marine Environment

Not many members of the Xylariaceae are recognized by researchers in the family as belonging to the marine environment, but there are scattered references to species of endophytic *Xylaria* isolates from mangroves (Lin et al. 2001; Okane et al. 2001; Liu et al. 2006; Xu et al. 2008) and "marine-derived" isolates (Rukachaisirkul et al. 2009), in relation to their ability to produce novel metabolites. *Hypoxylon croceum* J.H. Miller isolated from driftwood in a mangrove in Florida, USA, was found to produce a new sordarin derivative, hypoxysordarin and a novel hypoxylactone in addition to the previously known sordarin with both hypoxysordarin and sordarin exhibiting antifungal properties (Daferner et al. 1999). It appears that *H. croceum* is a frequent inhabitant of mangroves occurring in the Bahamas and East Coast USA (Stadler pers. comm.). Furthermore, Jones et al. (2008) provide a recent review of the potential of marine fungi for the discovery of new chemicals whilst Pan et al. (2008) reviewed the bioactive compounds from fungi in the South China Sea, which included compounds from *Xylaria* species.

There are representatives of twelve xylariaceous genera occurring as teleomorphs, *Anthostomella* Sacc., *Astrocystis* Berk. & Broome, *Biscogniauxia* Kuntze, *Camillea* Fr. *Daldinia* Ces. & De Not., *Fasciatispora* K.D. Hyde, *Halorosellinia* Whalley, E.B.G. Jones, K.D Hyde & Læssøe, *Hypoxylon* Bull., *Kretschmaria* Fr., *Nemania* S.F. Gray *Nipicola* K.D. Hyde, *Rosellinia* De Not. and *Xylaria* Hill ex Schrank which have to date been found in mangroves or in vegetation immediately adjacent to mangroves (Hyde 1992; Whalley et al. 1996, 1999, 2000; Smith and Hyde 2001; Jones and Abdel-Wahib 2005; Jones et al. 2009). It is mainly through the studies of E.B. Gareth Jones and Kevin D. Hyde, their co-workers and students that a substantial number of these taxa have been described from the marine environment. There are at least four species of *Anthostomella* recorded from *Nypa fruticans* or growing on *Spartina* or *Juncus* (Lu and Hyde 2000; Jones et al. 2009) and there are considerably more species occurring on mangrove wood from the Bahamas (Abdel-Wahab and Jones unpublished data). Species of *Anthostomella* together with *H. oceanica* and possibly *N. maritima* Y.-M. Ju & J.D. Rogers are members of the Xylariaceae which are widely distributed in the marine environment. *Nipicola* also occurs on the intertidal palm, *N. fruticans* and is clearly very closely related to *Anthostomella* but lacks an iodine-positive subapical apparatus and has black ascospores in addition to other microscopical differences (Hyde 1992; Jones et al. 2009). We are not convinced that the two species of *Nipicola, N. carbospora* K.D. Hyde and *N. selangorensis* K. D. Hyde can be logically separated from *Anthostomella*. Smith and Hyde (2001) in describing *Astrocystis nypae* and *A. selangorensis* from Malaysia maintained the separation of the genus from *Rosellinia*. We agree with Smith and Hyde (2001) in keeping separate status for *Astrocystis* and although we recognize that these genera are closely related, we do not follow Ju and Rogers (1990) in their merger of the genera and consider in particular the *Acanthodochium* anamorph of *Asterocystis* to provide strong evidence for their separation (Whalley 1996; Smith and Hyde 2001).

Two species of *Fasciatispora*, *F. nypae* K.D. Hyde and *F. lignicolla* Alias, E.B.G. Jones & Kuthub. are reported as marine inhabitants, although other members of the genus are non-marine in their distribution (Jones et al. 2009). Species of *Biscogniauxia*, *Daldinia*, *Hypoxylon*, *Kretschmaria*, *Rosellinia* and *Xylaria* were not included by Jones et al. (2009) in their classification of marine fungi and their inclusion here is open to debate. Certainly a wide range of these taxa were recorded for Kuala Selangor Nature Park in woodland immediately adjacent to the mangrove (Whalley et al. 1999) and several species of *Hypoxylon* have been found in a mangrove environment in Thailand (Whalley et al. 1994). It is noteworthy that representatives of these genera are reported to occur commonly as endophytes, especially in tropical plants (Whalley 1996).

   Halorosellina oceanica was originally described from Florida, USA, by Schatz (1988) as *Hypoxylon oceanicum* Schatz for an ascomycete fungus growing on *Rhizophora* mangle in a Floridian mangrove, although later considered to be a member of the Xylariaceae, it was not accepted as a *Hypoxylon* species and the genus *Halorosellinia* was, therefore, erected to accommodate it (Whalley et al. 2000). *Halorosellina oceanica* is a widespread mangrove inhabitant originally recorded as a *Rosellinia* sp from Sierra Leone by Aleem (1980). It has since been reported from the Seychelles (Hyde and Jones 1988; Jones and Hyde 1990), Brunei (Hyde 1988), N. Sumatra (Hyde 1989a), Thailand (Hyde 1989b; Hyde et al. 1990), Malaysia (Jones and Tan 1987; Jones and Kuthubutheen 1989; Alias et al. 1995), Philippines (Jones et al. 1988), Singapore (Tan et al. 1989), South Africa (Steinke and Jones 1993), Taiwan (Ju 1990), Hong Kong (Vrijmoed et al. 1994) and Australia (Hyde 1990). It has recently been reported as one of the most frequently collected marine fungi in Malaysian mangroves with an occurrence of 6.2% of all species recorded (Alias et al. 2010).

   Camillea selangorensis, described from the Kuala Selangor Nature Park, Selangor, Malaysia, was only the second species of the genus to be recorded from outside of the Americas and West Africa. It has also been recorded from the edge of a mangrove in Phuket Island, Thailand (Whalley et al. 1999). The Kuala Selangor Nature Park was also the type locality for a second species of *Camillea*, *C. malaysianensis* M.A. Whalley (Whalley et al. 1999). Kuala Selangor Nature Park contains a wide range of habitats including mangrove forest, secondary forest, estuary, mud flats and a brackish water lake system. From the late 1980s, the water table in the park has been dropping and mangrove species are declining and being replaced by more dry-adapted species. The two species of *Rhizophora*, four species of *Bruguiera*, three species of *Avicennia* and two species of *Sonneratia*, which occur in the mangrove, have decreasing numbers of representatives in the secondary forest. As these taxa decreased, the presence of *Ficus microcarpa* L. has increased and this is the host for *C. selangorensis*. The increase in number of specimens of *C. selangorensis* from surveys in 1993 and 1997 may well be the result of the increase in the numbers of the *Ficus*. Whalley et al. (2002) recorded two species of *Biscogniauxia*, two species of *Camillea*, Daldinia *eschscholzii* (Ehrenb.:Fr.) Rehm, fourteen species of *Hypoxylon*, including one considered likely to be previously undescribed, three species of *Nemania*, a small-spored

Kretschmaria deusta (Hoffm.:Fr.) P.M.D. Martin, two species of *Rosellinia* and ten species of *Xylaria*. In 1993, *H. haematostroma* Mont. and *H. subgilvum* Berk. & Broome were the most frequently recorded species but by 1997, *C. selangorensis*, *H. lenormandii* Berk. & M.A. Curtis apud Berk. and *X. cubensis* (Mont.) Fr. had become more dominant, which is probably a result of the drier conditions. *Halorosellinia oceanica* was confined to the true mangrove forest. It is probable that one of the *Nemania* species found will, with re-examination, prove to be *N. maritima*. In a preliminary study of Xylariaceae in mangrove forest or bordering mangroves in Malaysia and Thailand, Whalley et al. (1994) recorded *Halorosellinia oceanica* (as *Hypoxylon oceanicum*), *Hypoxylon bovei* var. *microspora* Speg.var.*microspora* J.H. Miller, *H. subgilvum* and *H. lenormandii* (as *H. nectriodeum* Sacc. & Trott.). Thus H. *lenormandii* and *H. subgilvum* would appear to be regular inhabitants of the mangrove fringe in S.E. Asia.

12.1.2   *The Xylariaceae as Endophytes of Mangrove Plants*

In their examination of endophytes from *Bruguiera gymnorrhiza* in the Shira River Basin, Iriomote Is. (Okane et al. 2001) reported on xylariaceaeous fungi and Pang et al. (2008) isolated an endophytic *Xylaria* species from *Kandelia candel* (Rhizophoraceae) in Mai Po Nature Reserve, Hong Kong. Although members of the Xylariaceae are common endophytic inhabitants of tropical plants (Whalley 1996; Rodrigues and Petrini 1997), they do not appear to be common or universally present in mangrove plants unlike the situation in other tropical plants. In our studies on Thai plants and their endophytes (Mekkamol 1998; Ruchikackorn 2005; Chareprasert et al. 2006) and those of Lumyong et al. (2004) and in Hong Kong by Photita et al. (2001), the Xylariaceae are indeed common and well represented. Promputtha et al. (2007) in their study of endophytic fungi from *Magnolia liliifera* recorded a range of species of *Xylaria* and other members of the Xylariaceae and indicated that *Xylaria* species from leaves were endophytic, although teleomorphic material for these species was not present even in the immediate vicinity. We also believe that many of the leaf-inhabiting *Xylaria* species are endophytic and under suitable environmental conditions develop their teleomorph on fallen leaves. *Xylaria aristata* Mont. and *X. amphithele* F.S.M. Gonzalez & J.D. Rogers are species found on leaves in Thai forests but their occurrence is rare (Thienhirun and Whalley 2004). We suggest that this is a direct reflection of the nutritional status of the leaf and its immediate environment, which either allows or prevents continued development of the fungus. It is also significant that these and other leaf-inhabiting teleomorphic *Xylaria* species are small and fragile and usually, if found, only contain a few perithecia or more frequently are immature (Thienhirun 1997). The seed-inhabiting species of *Xylaria* are different and in our view are a result of the seed or fruit acting as a "bait" once the fruit or seed is in the litter/soil layer (Rogers 1979a, b; Whalley 1996). Certainly *X. carpophila* Pers ex Fr., which occurs on fallen beech mast in temperate countries, there is clear evidence to show that this is the case. It is also

noteworthy that *Xylaria magnolia* var. *microspora* J.D. Rogers, Ju and Whalley, which is common on fallen fruits of *Magnolia* in Northern Thailand (Rogers and Ju 2002), was not isolated in the Hong Kong study. This perhaps provides additional support that the fruits act as baits and that *X. magnolia* var. *microspora* is not endophytic. Interestingly Sakayaroj et al. (2010), when reporting on endophytic assemblages from the tropical seagrass *Enhalus acoroides* in Thailand, failed to isolate *Xylaria* or other xylariaceaous fungi and noted that this was in agreement with studies on seagrasses in Bermuda, USA, Hong Kong, the Philippines and Puerto Rico. Kumaresan and Suryanarayanan (2001) investigated seven dominant mangrove species of an estuarine forest in south India and did not isolate any *Xylaria* species or other xylariaceaous taxa. They reported that many endophytes were common to more than one host and that the endophyte assemblage of each mangrove species was dominated by different endophytic species. In an earlier study of endophytes of halophytic plants from the same mangrove, no Xylariaceae were isolated with species of *Colletotrichum*, *Phomopsis*, *Phyllosticta* and *Sporormiella* being ubiquitous (Suryanarayanan and Kumaresan 2001).

In extensive surveys of three mangrove sites in Thailand, selected for their differences in dominant mangrove tree species and different environmental parameters, it was found that the presence of endophytic xylariaceous taxa also differed (Chareprasert et al. 2010). Thus *Daldinia eschscholtzii* was isolated from *Avicennia alba* Bl., *Rhizophora apiculata* Bl. and *R. mucronata* Poir. from the Chanthaburi site and from *R. apiculata*, *R. muccronata*, *Xylocarpus granatum* Koen. and *X. moluccensis* Roem from the Ranong mangrove. The percentage colonization frequency ranged from 0.67–3.33% at the Chanthaburi site to 6.00–7.33% at Ranong. *Daldinia eschscholtzii* is a common endophyte in Thailand and S.E. Asia and has been isolated from a wide range of plant species. It proved to be a dominant isolate from teak trees in Chiang Mai Province, Northern Thailand (Mekkamol 1998) and was a frequent isolate from teak in the vicinity of Bangkok (Chareprasert et al. 2006). Endophytic species of *Xylaria* were generally less widespread, with only *Xylaria* sp. 1 occurring on more than a single mangrove species, being isolated from nine out of thirteen mangrove species investigated. The remaining *Xylaria* species were restricted to one or two hosts (Chareprasert et al. 2010). The species of Xylariaceae recorded, although regularly present, were always in the minority, with *Phyllosticta* as the dominant genus in all mangrove plants from the Chanthaburi site and also in two plant species from Prachuap Khiri Khan. The dominant fungi in almost all mangrove species at the Ranong location were different for each host plant (Chareprasert et al. 2010), which is in agreement with the findings of studies on Indian mangrove endophytes where the dominant endophytes were also different for each host plant in the mangrove community (Kumaresan and Suryanarayanan 2001). In common with other reports on endophytic fungi as a source of bioactive compounds (e.g. Huang et al. 2007), many of the culture extracts exhibited a range of antibacterial activities, with *Xylaria* sp. 1, which was isolated from *A. ilicifolius* leaves, showing strong inhibition of both Gram-positive and Gram-negative bacteria. Additionally, crude extracts of many of the endophytic fungal isolates exhibited anticancer activity by the MTT assay

with *Xylaria* species 1, 3 and 5 exhibiting cytotoxicity against the Acute T cell leukaemia Jurkat cell line and *D. eschscholzii* proving to be strongly inhibitory to the colorectal adenocarcinoma SW620 cell line.

In their recent review of marine fungi from Malaysian mangroves, Alias et al. (2010) reported several Xylariaceae as common species of fungi occurring in this environment at between 5 and 9% occurrences. In addition to *H. oceanica*, other Xylariaceae included *N. carbospora* and *F. selangorensis*, *Fasciatispora lignicola* and *F. nypae*, *N. maritima* and *A. nypae*, *A. nypensis* and *A. nypicola*. *Nipicola carbospora* was reported to be cosmopolitan. Thus, from our own observations and those referred to here, members of the Xylariaceae are regular inhabitants of mangroves and their fringes.

12.2 Taxonomic Aspects

Halorosellinia oceanica (Schatz) Whalley, E.B.G. Jones, K.D Hyde & Læssøe. Mycological Research 104: 370 (2000).

Pseudostromata seated on decorticated wood, occasionally embedded at the base, pulvinate to hemispherical, 0.4–0.8 mm diam., single, in clusters of up to 30 uniperitheciate pseudostromata, linear to suborbicular, surface leathery in fresh material, when young covered with a whitish hyphal layer bearing the anamorph (Ju 1990), at maturity black, generally with conspicuous ascomatal projections. In section pseudostromata comprising host cells filled with light-brown fungal cells in the form of *textura globulosa* or amorphous black fungal material. *Ascomata* immersed in pseudostroma, subglobose to hemispherical, soft to leathery, black, *ostioles* papillate. *Peridium* 25–35 μm wide, comprising tissue of *textura porrecta*, fusing at the outside with the pseudostromata. *Paraphyses* 2–2.5 μm wide at the base, abundant, persistent, remotely septate. *Asci* eight-spored, 177–219 μm, spore-bearing part (112)135–140 μm, stipe 37–79 μm, cylindrical, unitunicate, subapical apparatus dark blue in Melzer's iodine reagent, tapering with a distinct apical rim, (4.7–)5.6–6.6 × 4.2–4.7 μm. *Ascospores* uniseriate to obliquely uniseriate or partially biseriate at the upper end of the ascus, dark grey-olive to opaque brown, more or less inequilaterally ellipsoid, ventral side varying in degree of convex curvature, upper end broadly rounded, lower end slightly pointed, one-celled throughout ascospore development, (17.9−)18.7−26(−28) × 7.5−13(−13.5) μm, biguttulate, wall smooth and relatively thick, without appendages or loosening perispore, germination slit usually clearly seen on the ventral side, straight, conspicuous, ½ to total length of spore (Fig. 12.1a–f).

Ascospores germinated freely to form strongly growing white to whitish grey colonies similar to those produced by other xylariaceous taxa such as *Xylaria*, *Kretzschmaria*, *Nemania* but no anamorphs detected. According to Ju (1990), there is an anamorphic form with strongly geniculate conidiophores, which is typical for a number of xylariaceous genera.
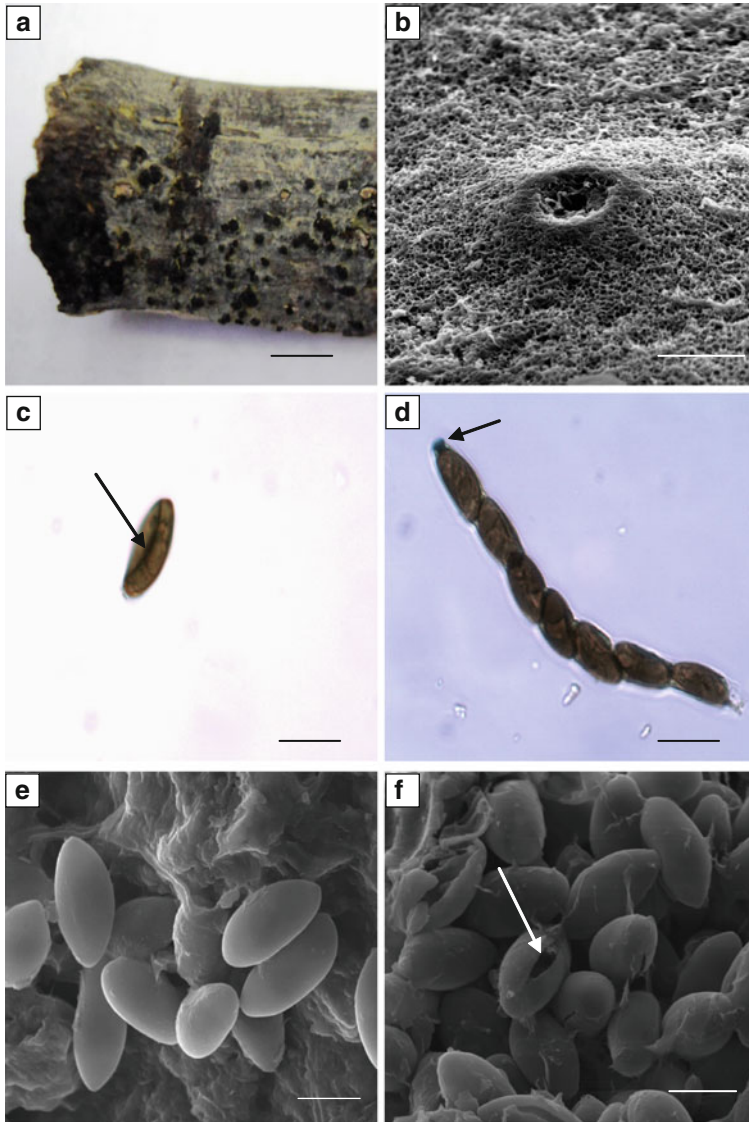
Fig.12.1 *Halorosellinia oceanica*. (**a**) Stromata. (**b**) Ostiolum. (**c**) Ascospore with germ slit (*arrow*). (**d**) Ascus with apical apparatus (*arrow*). (**e**) Ascospores. (**f**) Ascospores with germ slit (*arrow*). Bar markers: (**a**) 1 cm; (**b**) 100 μm; (**c–f**) 15 μm

Camillea selangorensis M.A. Whalley, Whalley & E.B.G. Jones. Sydowia 48:246 (1996).

Stromata erumpent through bark, circular to orbicular, 7–16 × 6–10 mm or occasionally elongated, 11–29 × 6–10 mm c.2–3 mm thick, with applanate to convex apex, surrounded by a slightly raised rim, black. *Ostioles* finely papillate

becoming punctate with age. *Perithecia* packed in a brownish-black, brittle, entostroma, basally seated. *Asci* cylindrical, 106–146 × 3.5–5.8 μm, sp. p. 85–103 μm, stipe 17–53 μm. *Apical* apparatus prominent, dark blue in Melzer's iodine reagent, more or less rhomboid, 2.5–3.8 μm tall and 3.5–5.8 μm wide. *Paraphyses* long, tread-like, unbranched, 152–182 μm long and 7.5 μm at their widest point, persistent. *Ascospores* obliquely uniseriate, hyaline or dilute yellow, one-celled, ellipsoid inequilateral. Minutely warted by light microscopy, strongly verrucose by scanning electron microscopy, 10.0–13.8 × 3.8–6.3 μm, germ slit absent. Anamorph not known (Fig. 12.2a–d).

Camillea malaysianensis M.A. Whalley Kew Bulletin 54: 716 (1999).

Stromata erumpent through bark, orbicular to elongate, 4–5 × 2–3 cm, c. 1.5–2 mm thick, with applanate to convex apex, black. *Ostioles* papillate. *Perithecia* basally seated in black, brittle entostroma, 1 mm high and 0.2–0.4 mm in diam. *Asci* cylindrical, 8-spored, 129–179 × 7.5–11.25 μm, spp. 88–126 μm, stipe 41–53 μm. Apical apparatus prominent, dark blue in Melzer's iodine reagent, more or less rhomboid, 5 μm tall and 3.75 μm wide. Ascospores obliquely uniseriate, hyaline or dilute yellow, one-celled, ellipsoidal, smooth by light microscopy, distinct poroid ornamentation by scanning electron microscopy, 8.75–15 × 5–6.25 μm, germ slit absent. Anamorph unknown.
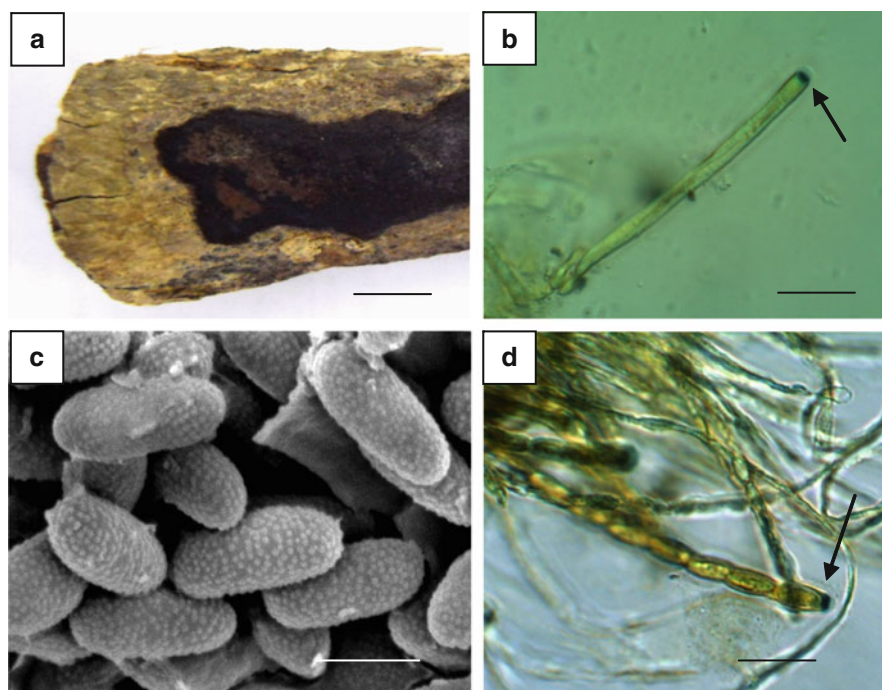

Fig.12.2 *Camillea selangorensis*. (**a**) Stromata. (**b**) Immature ascus with apical apparatus (*arrow*). (**c**) Ascospores. (**d**) Ascus with apical apparatus (*arrow*). Bar markers: (**a**) 2 cm; (**b**, **d**) 20 μm; (**c**) 10 μm

Camillea malaysianensis is superficially similar to *C. tinctor* (Berk.) Læssøe, J.D. Rogers & Whalley but differs in its considerably smaller ascospores and also does not produce the yellow staining of the wood which is characteristic for that species and which usually appears to be present in collections from Malaysia and Thailand. Unlike *C. malaysiensis*, which so far has only been recorded from the type locality, *C. tinctor* is widespread occurring in the Americas, West Africa, Thailand, Malaysia, Singapore and Papua New Guinea (Læssøe et al. 1989).

12.3   Conclusions

Halorosellinia oceanica is a manglicolous member of the Xylariaceae, which appears to be restricted to this habitat. Other taxa which might also be included here are several species of *Anthostomella*, *Nemania maritima* and *Nipicola carbospora* and *N. lignicola*. *Camillea selangorensis* and *C. malaysianensis* have to date only been reported from forest immediately adjacent to mangroves. *Hypoxylon subgilvum* and *H. lenormandii* are regular inhabitants in the mangrove fringe in S.E. Asia. Species of *Xylaria* and *D. eschscholzii* occur as endophytes in a variety of mangrove species. Many endophytic isolates from mangroves, including Xylariaceae, produce a range of novel compounds and many exhibit antimicrobial and anticancer activities.

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Chapter 13
Diversity and Distribution of Marine Fungi on *Rhizophora* spp. in Mangroves

Vemuri Venkateswara Sarma

Contents

Abstract *Rhizophora* spp. occurring in mangrove habitats are excellent hosts for marine fungi. The morphological adaptations of this host plant provide suitable niche for the marine fungi. This review deals with diversity and ecology of marine fungi occurring on *Rhizophora* spp. Two hundred and one fungal species have been recorded on *Rhizophora* plant species consisting of more than ten fungal species specific to this host. In mangroves, no other genus other than *Rhizophora* accommodates as many as 201 marine fungi. *Rhizophora* plant species are highly suitable hosts as they show a variety of niches with vertical zonation, specificity, preferential colonization and succession. Thus this host could be considered as one of the important hosts to investigate marine fungi. The current review provides updated

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information based on the available literature with checklists on diversity and distribution of fungi on *Rhizophora* spp.

13.1   Introduction

Mangroves are the important "land builders" in tropical and subtropical coastal regions. They are considered "interface" habitats of upland terrestrial and coastal estuarine ecosystems (Lugo and Snedaker 1974). They reduce tidal currents, cause extensive deposition of mud and silt, and provide surfaces for attachment of marine organisms (Odum 1971). Their importance lies in generation of leaf and other detritus, which supply nutrients for primary production and thus major energy source for fisheries (Heald and Odum 1970). Mangroves with their rich load of organic detritus support a large number of animal communities and are breeding and nursery grounds for shell fisheries (Jones and Alias 1997). Marine Fungi play an important role in the production of organic detritus in mangroves (Kohlmeyer and Kohlmeyer 1979). Their importance in nutrient regeneration cycles as decomposers of dead and decaying organic matter in marine ecosystems has been well documented (Fell and Master 1980; Raghukumar et al. 1994; Hyde et al. 1998).

Marine fungi are not restricted to any particular taxonomic group but are rather an ecologically and physiologically defined group. They comprise an estimated 1,500 species, excluding those that form lichens (Hyde et al. 1998), although 444 formerly described higher marine fungi have been reported (Hyde et al. 2000). Recently Jones et al. (2009) reported 530 marine fungi.

13.1.1   Substrata for Marine Fungi

Marine fungi grow on diverse substrata including wood, sediments, algae, dead corals, calcareous tubes of mollusks, decaying leaves, seedlings, prop roots and pneumatophores of mangroves, seagrasses and living animals, and in the guts of crustaceans (Kohlmeyer and Kohlmeyer 1979; Hyde 1996). Wood in the open ocean (drift wood) and decaying/decomposing substrata in mangrove habitats are the two predominant substrata, which support marine fungi. Mangrove plants are categorized as "typical mangrove plants" and "mangrove associated plants." There are around 30 "typical mangrove plants" and more or less equal number of "mangrove associated plants" that occur in mangroves forests around the world.

13.1.2   Marine Fungi on Mangroves

Kohlmeyer and Kohlmeyer (1979) listed 42 fungi from mangroves. After a lapse of 24 years roughly around 600 fungi have been reported from mangroves, which

include marine, estuarine and terrestrial fungi (Schmidt and Shearer 2003). Terrestrial fungi and lichens occupy the aerial parts of mangrove plants, whereas the marine fungi occur at lower parts such as trunks and roots, which are permanently or intermittently inundated with water (Kohlmeyer 1969; Kohlmeyer and Kohlmeyer 1979). There will be an interface and an overlap of marine and terrestrial fungi occurring at the high tide mark (Kohlmeyer 1969; Kohlmeyer and Kohlmeyer 1979). The interest of mycologists, however, has been more intense on marine fungi occurring in mangroves with special reference to hard substrata such as wood, roots and prop roots.

13.1.3 Marine Fungi on Rhizophora

Among the "typical mangrove plants" species belonging to genera *Aegiceras*, *Avicennia*, *Bruguiera*, *Ceriops*, *Excoecaria*, *Kandelia*, *Lumnitzera*, *Nypa*, *Rhizophora*, *Sonneratia* and *Xylocarpus* are the most prominent. Within these species *Avicennia* and *Rhizophora* are not only found most commonly but are also abundant throughout the world. Between these two genera several interesting fungi are known from *Rhizophora* spp. Further, the species belonging to this genus have been excellent experimental hosts for several biodiversity and ecological investigations including nutrient recycling, frequency of occurrence, vertical zonation, substrate and seasonal occurrence. Several studies have been conducted on biodiversity and ecology of fungi on *Rhizophora*. Hence it is worthwhile to review the diversity and geographical distribution of fungi reported from *Rhizophora* spp. worldwide.

13.2 Diversity

Kuthubutheen (1981) investigated the phylloplane fungi of *Rhizophora* spp. and reported: *Cephalosporium*, *Cladosporium oxysporum*, *Codinaea simplex*, *Corynespora*, *Fusarium*, *Pestalotiopsis guepini*, *Pestalotiopsis versicolor*, *Zygosporium masoni*. Similarly, in an earlier investigation on the leaves of *Rhizophora*, Fell et al. (1975) found that the majority of the genera were ubiquitous saprophytes (e.g., *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Curvularia*, *Cylindrocephalum*, *Drechslera*, *Fusarium*, *Myrothecium*, *Nigrospora*, *Penicillium*, *Pestalotia*, *Phyllosticta*, *Trichoderma* and *Verticilium*).

   More than 201 lignicolous filamentous fungi belonging to 139 genera are known from *Rhizophora* spp. (Table 13.1). Thus, approximately one-third of total number of marine fungi are recorded on *Rhizophora* spp. These comprise 126 species of ascomycetes in 85 genera; 5 species of basidiomycetes in 3 genera; 70 species of anamorphic fungi in 51 genera of which 10 species in 9 genera are coelomycetes; 60 species in 42 genera are hyphomycetes. A total of 51 bitunicate ascomycetes and

Table 13.1 Lignincolous filamentous marine fungi recorded on *Rhizophora* spp. from Neotropics, Indian Coasts, South East Asia and other sites

S. No.	Name of the species	Neotropics (N & S America)[1]	Belize, Fiji, Hawai[2]	Mandai, Siingapore[3]	Ranong, Thailand[4]	Kuala Selangor, Malaysia[5]	Morib, Malaysia[6]	Irimoto Island, Japan[7]	North Sumatra[8]	Queensland, Austrlaia[9]	Pichavaram, TN, India[10]
1	*Acrocordiopsis patilii* Borse & K.D. Hyde	–	–	–	+	–	–	–	–	–	–
2	*Aigialus grandis* Kohlm. & S. Schatz	–	+	+	+	–	+	+	+	+	+
3	*A. mangrovei* Borse	–	+	+	+	–	–	+	+	–	+
4	*A. parvus* S. Schatz & Kohlm.	–	–	+	+	+	–	–	–	–	+
5	*A. striatispora* K.D. Hyde	–	+	–	+	–	–	–	–	–	–
6	*Aigialus* sp.	–	+	–	–	–	–	–	–	–	–
7	*Aniptodera chesapeakensis* Shearer & M.A. Mill.	–	+	+	+	–	–	–	+	–	+
8	*A. longispora* K.D. Hyde	–	+	–	+	–	–	–	–	–	–
9	*A. haispora* Vrijmoed, K.D. Hyde & E.B.G. Jones	–	–	–	–	–	–	+	–	+	–
10	*A. mangrovei* K.D. Hyde	–	–	–	+	+	–	–	–	–	–
11	*Aniptodera* sp.	–	–	–	–	+	–	–	–	–	–
12	*Antennospora quadricornuta* (Cribb & J.W. Cribb) T.W. Johnson	+	+	–	+	+	+	–	–	–	+
13	*Anthostomella* sp.	–	–	–	–	–	–	–	–	–	–
14	*Ascocratera manglicola* Kohlm.	–	+	–	+	–	+	–	–	–	+
15	*Ascocratera* cf. *manglicola* Kohlm.	–	–	–	+	–	–	–	–	–	–
16	*Astrosphaeriella striatispora* (K.D. Hyde) K.D. Hyde ≡*Trematosphaeria striatispora* K.D. Hyde	–	–	–	–	–	–	–	–	–	–
17	*Bathyascus grandisporus* K.D. Hyde & E.B.G. Jones	–	–	–	+	–	–	–	–	–	–
18	*Bathyascus mangrovei* Ravikumar & Vittal	–	–	–	–	–	–	–	–	–	+
19	*Bathyascus* sp.	–	–	–	+	–	–	–	–	–	–
20	*Belizeana tuberculata* Kohlm. & Volkm.–Kohlm.	–	+	–	+	–	–	–	–	–	+
21	*Biatriospora marina* K.D Hyde & Borse	–	+	–	–	–	–	–	–	–	–
22	*Byssothecium obiones* (P. Crouan & H. Crouan) M.E. Barr	–	–	–	–	–	–	–	–	–	–

	Species										
23	*Capillataspora corticola* K.D. Hyde	−	−	−	−	−	−	−	−	−	−
24	*Caryosporella rhizophorae* Kohlm.	−	+	−	+	−	−	+	−	+	+
25	*Coccostroma* sp.	−	−	−	−	−	−	−	−	−	+
26	*Corollospora pulchella* Kohlm., I. Schmidt & N.B. Nair	−	−	−	−	−	−	−	−	−	−
27	*Coronopapilla mangrovei* (K.D. Hyde) Kohlm. & Volkm.–Kohlm.	−	+	−	−	−	−	−	−	−	−
28	*Cryptosphaeria mangrovei* K.D. Hyde	−	−	−	+	−	−	−	−	−	−
29	*Cucullosporella mangrovei* K.D. Hyde & E.B.G. Jones	−	−	−	+	−	−	+	−	+	−
30	*Dactylospora haliotrepha* (Kohlm . & E. Kohlm.) Hafellner	−	+	+	+	+	+	+	+	+	+
31	[a]*Diatrypasimilis australiensis* J. Zhou & Kohlm	−	−	−	−	−	−	−	−	−	−
32	[b]*Didymella avicenniae* Patil & Borse	−	−	−	−	−	−	−	−	−	+
33	*Durella* sp.	−	−	−	−	−	−	−	−	−	−
34	*Etheirophora bleapharospora* (Kohlm. & E. Kohlm.) Kohlm. & Volkm.–Kohlm.	+	+	−	−	−	−	−	−	+	−
35	[b]*Eutypa bathurstensis* K.D. Hyde & Rappaz	−	−	−	−	−	−	−	−	−	−
36	*Gnomonia* sp.-like	−	−	−	−	−	+	−	−	−	+
37	*Haiyanga salina* (Meyers) K.L. Pang & E. B. G. Jones, Yusoff & S. T. Moss ≡*Antennospora salina* (Meyers) Yusoff, E.B.G. Jones & S.T. Moss,	−	−	−	−	−	−	−	−	−	−
38	*Haligena* sp.	−	+	+	−	−	−	−	−	−	−
39	*Halomassarina thalassiae* (Kohlm. & Volkm.–Kohlm.) Suetrong, Sakayaroj, E.B.G. Jones, Kohlm., Volkm.–Kohlm. & C.L. Schoch, ≡*M. thalassiae* Kohlm. & Volkm.–Kohlm.	−	+	+	+	+	+	−	−	+	+
40	*Halorosellinia oceanica* Whalley, E.B.G. Jones, K.D. Hyde & Laessøe	−	+	+	+	+	+	−	+	−	+
41	*Halosarpheia fibrosa* Kohlm. & E. Kohlm.	+	−	−	−	−	+	+	−	+	−
42	*H. marina* (Cribb & J.W. Cribb) Kohlm.	−	−	+	+	+	+	−	+	+	+

Table 13.1 (continued)

S. No.	Name of the species	Neotropics (N & S America)[1]	Belize, Fiji, Hawai[2]	Mandai, Siingapore[3]	Ranong, Thailand[4]	Kuala Selangor, Malaysia[5]	Morib, Malaysia[6]	Irimoto Island, Japan[7]	North Sumatra[8]	Queensland, Austrlaia[9]	Pichavaram, TN, India[10]
43	*H. minuta* Leong	–	–	+	+	–	–	–	–	–	–
44	*Halosarpheia* sp.	–	+	–	–	–	–	–	–	–	+
45	*Hapsidascus hadrus* Kohlm. & Volkm.–Kohlm.	–	+	–	–	–	–	–	–	–	–
46	*Heleococcum japonense* Tubaki	–	–	–	–	–	–	–	–	–	+
47	*Helicascus kanaloanus* Kohlm.	+	–	–	–	–	–	–	–	–	–
48	*Hypocrea* sp.	–	–	–	–	–	–	–	–	–	–
49	*Hypophloeda rhizospora* K.D. Hyde & E.B.G. Jones	–	+	–	+	–	+	–	–	–	–
50	*Hypxylon hypomiltum* Montagne	–	–	–	+	–	–	–	–	–	–
51	*Hypoxylon* sp.	–	–	–	–	–	+	–	–	–	–
52	[c]*Hysterium* sp.[b]	–	–	–	–	–	–	–	–	–	–
53	*Kallichroma tethys* (Kohlm. & E. Kohlm.) Kohlm. & Volkm.–Kohlm.	+	+	–	+	+	+	–	–	–	–
54	*Kirschsteiniothelia maritima* -like	–	–	–	–	–	–	–	–	–	–
55	[b]*Julella avicenniae* (Borse) K.D. Hyde	–	+	–	–	–	+	–	–	–	–
56	*Leptosphaeria australiensis* (Cribb & J.W. Cribb) G.C. Hughes	+	+	–	+	–	+	–	–	+	+
57	[b]*Leptosphaeria* cf. *avicenniae* Kohlm. & E. Kohlm.	–	–	–	–	–	–	–	–	–	–
58	[b]*Leptosphaeria*.cf. *peruviana* Speg	–	–	–	–	–	–	–	–	–	–
59	[b]*Leptosphaeria salvinii* Catt.	–	–	–	–	–	–	–	–	–	–
60	*Leptosphaeria* sp.	–	–	–	–	–	–	–	+	–	–
61	*Lignincola laevis* Höhnk	+	+	+	+	+	+	–	+	+	+
62	*L. tropica* Kohlm.	+	+	–	–	–	–	–	–	–	–
63	*Limacospora sundica* (Jørg. Koch & E.B.G. Jones) Jørg. ≡*Ceriosporopsis sundica* Jørg Koch & E.B.G. Jones	–	–	–	–	–	–	–	–	–	–

#	Species									
64	*Lineolata rhizophorae* (Kohlm.& E. Kohlm.) Kohlm.& Volkm.–Kohlm	+	+	–	+	–	–	+	+	+
65	*Lophiostoma armatisporum* (K.D. Hyde, Vrijmoed, Chinnaraj & E.B.G. Jones) Liew, Aptroot & K.D. Hyde, *Massarina armatispora* K.D. Hyde, Vrijmoed, Chinnaraj & E.B.G. Jones.	–	–	–	–	–	–	–	–	–
66[d]	[d]*Lophiostoma. rhizophorae* (Poonyth, K.D. Hyde, Aptroot & Peerally) Aptroot & K.D. Hyde	–	–	–	–	–	–	–	–	–
67	*Lulworthia grandispora* Meyers	–	+	+	+	+	–	–	–	+
68	*Lulworthia* sp.	+	+	+	+	+	+	+	+	+
69	*Manglicola guatemalensis* Kohlm. & E. Kohlm.	+	–	–	–	–	–	–	–	–
70	*Marinosphaera mangrovei* K.D. Hyde	–	+	–	+	+	+	+	+	–
71	*Massarina lacertensis* Kohlm. & Volkm.–Kohlm.	–	–	–	–	–	–	–	+	–
72	*Massarina* sp.	–	–	–	–	–	–	–	–	+
73	*Massariosphaeria typhicola* (P. Karst.) Barr	–	–	–	–	–	–	–	–	–
74	*Melaspilea mangrovei* Vrijmoed, K.D. Hyde & E.B.G. Jones	–	–	–	+	–	–	–	–	–
75	*Morosphaeria ramunculicola* (K.D. Hyde) Suetrong, Sakayaroj, E.B.G. Jones & C.L. Schoch ≡*Massarina ramunculicola* K.D. Hyde	–	+	+	+	+	+	–	–	–
76	*Morosphaeria velataspora* (K.D. Hyde & Borse) Suetrong, Sakayaroj, E.B.G. Jones & C.L. Schoch ≡*Massarina velataspora* K.D. Hyde & Borse	–	+	–	+	–	–	–	–	+
77	[b]*Mycosphaerella pneumatophorae* Kohlm.	–	+	+	+	–	–	–	–	–
78	*Nais inornata* Kohlm.	–	+	+	+	–	–	–	–	–
79	*Nais* sp.	–	–	–	–	–	–	+	–	+

(continued)

Table 13.1 (continued)

S. No.	Name of the species	Neotropics (N & S America)[1]	Belize, Fiji, Hawai[2]	Mandai, Singapore[3]	Ranong, Thailand[4]	Kuala Selangor, Malaysia[5]	Morib, Malaysia[6]	Irimoto Island, Japan[7]	North Sumatra[8]	Queensland, Australia[9]	Pichavaram, TN, India[10]
80	*Natantispora retorquens* (Shearer & J.L. Crane) J. Campb., J.L. Anderson & Shearer ≡*Halosarpheia retorquens* Shearer & J.L. Crane	–	–	+	–	–	–	–	–	–	–
81	*Neptunella longirostris* (Cribb & J.W. Cribb) K.L. Pang & E.B.G. Jones ≡*Lignincola longirostris* (Cribb & J.W. Cribb) Kohlm	–	–	–	+	+	–	–	–	–	+
82	*Okeanomyces cucullatus* (Kohlm.) K.L. Pang & E.B.G. Jones ≡*Halosphaeria cucullata* (Kohlm.) Kohlm	–	+	–	–	–	+	–	–	–	+
83	*Ophiodeira monosemeia* Kohlm. & Volkm.–Kohlm.-like	–	+	–	+	–	–	–	–	–	–
84	*Oceanitis cincinnatula* (Shearer & J.L. Crane) J. Dupont & E.B.G. Jones ≡*Halosarpheia cincinnatula* Shearer & J.L. Crane	–	–	+	+	–	–	–	–	–	+
85	*O. viscidula* (Kohlm. & E. Kohlm.) J. Dupont & E. B.G. Jones ≡*Haligena viscidula* Kohlm. & E. Kohlm., Nova Hedw. **9**: 92, 1965	+	–	–	–	–	–	–	–	–	–
86	*Panorbis viscosus* (I. Schmidt) J. Campb., J.L. Anderson & Shearer ≡*Halosarpheia viscosa* (I. Schmidt) Shearer & J.L. Crane	–	–	+	+	–	–	–	–	–	–
87	*Paraliomyces lentifer* Kohlm.	+	–	–	–	–	–	–	–	–	–
88	*Patellaria atra* (Hedw.) Fr., ≡*Lecanidion atratum* (Hedw.) Endl.	–	–	–	–	–	–	–	–	–	–
89	*Passeriniella savoryellopsis* K.D. Hyde & Mouzouras	–	+	–	+	–	–	–	–	–	–

#	Species														
90	*Passereniella mangrovei* G.L. Maria & K.R. Sridhar	–	–	–	–	–	–	–	–	–	–	–	–	–	–
91	*Payosphaeria minuta* W.F. Leong	–	+	–	+	–	–	–	–	–	–	–	–	–	+
92	*Payosphaeria* sp.	+	–	–	–	–	–	–	–	–	–	–	–	–	–
93	*Pedumispora rhizophorae* K.D. Hyde & E.B.G. Jones	–	–	–	–	–	–	–	–	–	–	–	–	–	–
94	*Phaeosphaeria neomaritima* (R.V. Gessner & Kohlm.) Shoemaker & C.E. Babc	+	–	–	–	–	–	–	–	–	–	–	–	–	–
95	*Phaeosphaeria oraemaris* (Linder) Khashn. & Shearer	–	+	–	+	–	–	–	–	–	–	–	–	–	–
96	*Polystigmina* sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	–
97	*Pyrenographa xylographoides* Aptroot	–	–	–	–	–	+	–	–	–	–	–	–	–	–
98	*Quintaria lignatilis* (Kohlm.) Kohlm. & Volkm.–Kohlm.	–	+	+	+	+	+	+	+	–	+	–	–	+	+
99	*Rhizophila marina* K.D. Hyde & E.B.G. Jones	–	+	–	+	–	+	+	+	+	+	+	–	+	+
100	*Rimora mangrovei* (Kohlm. & Vittal) Kohlm., Volkm–Kohlm., Suetrong, Sakayaroj & E.B.G. Jones ≡*Lophiostoma mangrovei* Kohlm. & Vital	–	+	–	+	+	–	–	+	–	+	–	–	+	+
101	*Saccardoella mangrovei* K.D. Hyde	–	–	–	+	+	–	–	–	–	–	–	–	–	–
102	*S. rhizophorae* K.D. Hyde	+	–	–	+	–	–	+	+	–	–	–	–	–	+
103	*Saagaromyces abonnis* (Kohlm.) K.L. Pang & E.B.G. Jones ≡*Halosarpheia abonnis* Kohlm.	+	–	–	+	–	–	+	+	+	–	–	–	–	–
104	*S. glitra* (J.L. Crane & Shearer) K.L. Pang & E.B.G. Jones, ≡*Nais glitra* J.L. Crane & Shearer	–	–	–	+	–	+	–	+	–	–	–	–	–	+
105	*S. ratnagiriensis* (S.D. Patil & Borse) K.L. Pang & E.B.G. Jones ≡*Halosarpheia ratnagiriensis* S.D. Patil & Borse	–	–	–	+	–	–	+	+	–	–	–	–	–	–
106	*Savoryella lignicola* E.B.G. Jones & R.A. Eaton	–	–	–	–	–	+	–	+	–	–	–	–	–	–

(continued)

Table 13.1 (continued)

S. No.	Name of the species	Neotropics (N & S America)[1]	Belize, Fiji, Hawai[2]	Mandai, Singapore[3]	Ranong, Thailand[4]	Kuala Selangor, Malaysia[5]	Morib, Malaysia[6]	Irimoto Island, Japan[7]	North Sumatra[8]	Queensland, Austrlaia[9]	Pichavaram, TN, India[10]
107	S. longispora E.B.G. Jones & K.D. Hyde	–	–	–	+	–	–	–	–	–	–
108	S. paucispora (Cribb & J.W. Cribb) Jörg. Koch	–	–	+	–	–	–	–	–	–	–
109	Splanchnonema britzelmayriana-like	–	–	–	–	–	–	–	–	–	–
110	Splanchnonema sp.	–	–	–	–	–	–	–	–	–	–
111	Sporormiella grandispora (Speg.) Ahmed & Cain	–	–	–	–	–	–	–	–	–	+
112	S. minima (Auersw.)S.I. Ahmed & Cain	–	–	–	–	–	–	–	–	–	+
113	Swampomyces armeniacus Kohlm. & Volkm.–Kohlm.	–	+	–	–	–	–	–	–	–	+
114	S.wampomyces cf. armeniacus Kohlm. & Volkm.–Kohlm.	–	–	–	+	–	–	–	–	–	+
115	S. triseptatus K.D. Hyde & Nakagiri	–	+	–	+	–	–	+	–	–	–
116	Thalassogena sphaerica Kohlm. & Volkm.–Kohlm.	–	+	–	+	–	–	–	–	–	–
117	Torpedospora radiata Meyers	+	+	–	–	–	+	–	–	–	–
118	Trematosphaeria mangrovei Kohlm.	+	–	–	–	–	–	–	–	–	–
119	Trematosphaeria sp.	–	–	–	–	–	–	–	–	–	+
120	Tirispora unicaudata E.B.G. Jones & Vrijmoed, ≡Aniptodera indica Ananda & Sridhar,	–	–	–	–	–	–	–	–	–	–
121	Tirispora mandoviana V.V. Sarma & K.D. Hyde	–	–	–	–	–	–	–	–	–	–
122	Tirispora sp.	–	–	–	–	–	–	–	–	–	–
123	°Tubeufia setosa Sivan. & W.H. Hsieh	–	–	–	–	–	–	–	+	–	–
124	Verruculina enalia (Kohlm.) Kohlm. & Volkm.–Kohlm.	+	+	+	+	+	+	+	+	–	+
125	Zignoella sp.	–	–	–	–	–	–	–	–	–	–
126	Zopfiella sp.	–	–	–	–	–	–	–	–	–	–

Basidiomycetes

#	Species											
127	*Halocyphina villosa* Kohlm.	+	+	+	+	+	+	+	–	+	+	+
128	*Halocyphina* sp.	–	+	+	–	–	+	–	–	–	–	–
129	°*Calathella mangrovei* E.B.G. Jones & Agerer	–	–	–	–	–	–	–	–	–	–	–
130	*Calathella* sp.	–	–	–	–	–	+	–	–	–	–	–
131	*Nia vibrissa* R.T. Moore & Meyers	+	+	+	–	–	–	–	–	–	–	–
132	*Acremonium* sp.	–	–	–	–	–	–	–	–	–	–	–
133	*Alternaria* sp.	–	–	–	–	–	–	–	–	–	–	–
134	*Alveophoma* sp.	–	–	–	–	–	–	–	–	–	–	–
135	*Anguillospora marina* Nakagiri & Tubaki	–	–	–	–	–	–	–	–	–	–	–
136	*Arthrobotrys oligospora* Fresen.	–	–	–	–	–	–	–	–	–	–	–
137	*Bactrodesmium linderii* (Crane & Shearer) M. E. Palm & E.L. Stewart	–	–	–	–	–	–	–	–	–	–	–
138	*Bactrodesmium* sp.*/*	–	–	–	–	–	+	–	–	–	–	–
139	*Brachysporiella gayana* Batista	–	–	–	–	–	–	–	–	–	–	–
140	*Cirrenalia basiminuta* Raghuk. & Zainal	–	–	–	–	–	–	–	–	–	–	+
141	*Cirrenalia macrocephala* (Kohlm.) Meyers & Mooore	+	–	+	+	–	–	–	–	–	–	+
142	*Cirrenalia pseudomacrocephala* Kohlm.	+	–	+	+	+	+	+	–	+	–	–
143	*Clavatospora bulbosa* (Anast.) Nakagiri & Tubaki	–	+	+	+	+	+	–	–	–	–	–
144	*Cumulospora marina* I. Schmidt	–	–	–	–	–	–	–	–	–	–	–
145	*Cytospora rhizophorae* Kohlm. & E. Kohlm.	+	–	+	–	–	–	–	–	–	+	–
146	*Cytospora* sp.	–	–	–	–	–	–	–	–	–	–	–
147	*Dactylella* sp.	–	–	–	–	–	–	–	–	–	–	–
148	*Delortia palmicola* Pat.	–	–	–	–	–	–	–	–	–	–	–
149	*Dendryphiella arenaria* Nicot	–	–	–	–	+	–	–	–	–	–	–
150	*Dendryphiella salina* (G.K. Sutherl.) G.J.F. Pugh & Nicot	+	–	+	–	–	–	–	+	–	–	–
151	*Dictyosporium elegans* Corda	–	–	–	–	–	+	–	–	–	–	–

(continued)

Table 13.1 (continued)

S. No.	Name of the species	Neotropics (N & S America)[1]	Belize, Fiji, Hawai[2]	Mandai, Siingapore[3]	Ranong, Thailand[4]	Kuala Selangor, Malaysia[5]	Morib, Malaysia[6]	Irimoto Island, Japan[7]	North Sumatra[8]	Queensland, Austrlaia[9]	Pichavaram, TN, India[10]
152	*Dictyosporium pelagicum* (Linder) G.C. Hughes	+	–	–	–	–	+	–	–	–	–
153	*Diplocladiela scalaroides* G. Arnaud ex M.B. Ellis	–	–	–	–	–	–	–	–	–	–
154	*Ellisembia vaga* (C.G. & T.F.L.) Subram.	–	–	–	–	–	–	–	–	–	–
155	*Endophragmia alternata* Tubaki & Saito	–	–	–	–	–	–	–	–	–	–
156	*Epicoccum purpurascens* Ehrenb.:Schlecht.	–	–	–	–	–	–	–	–	–	+
157	*Hansfordia* sp.	–	–	–	–	–	–	–	–	–	–
158	*Helicoma muelleri* Corda	–	–	–	–	–	–	–	–	–	–
159	*Helicomyces roseus* Link.	–	–	–	–	–	–	–	–	–	–
160	*Helicoon* sp.	–	–	–	+	–	–	–	–	–	–
161	*Helenospora varia* (Anastasiou) E.B.G. Jones ≡*Zalerion varium* Anastasiou	+	–	–	–	+	–	–	–	–	+
162	*Hydea pygmea* (Kohlm) K.L. Pang & E.B.G. Jones ≡*Cirrenalia pygmea* Kohlm.	+	+	+	+	–	+	–	+	–	+
163	*Leptosphaeria salvinii* Cattaneo (anamorph, nakatea state)	–	–	–	–	–	–	–	–	–	–
164	*Matsusporium tropicale* (Kohlm.) E.B.G. Jones & K.L. Pang ≡*Cirrenalia tropicalis* Kohlm.	+	–	–	+	+	–	+	+	–	–
165	*Menispora cilliata* Corda	–	–	–	–	–	–	–	–	–	–
166	*Monodictys pelagica* Johnson & E.B.G. Jones	–	–	–	–	–	–	–	–	–	+
167	*M. putredinis* (Wallr.) S. Hughes	–	–	–	–	–	–	–	–	–	–
168	*Monodictys* sp.	–	–	–	–	–	–	–	–	–	–
169	*Mycoenterolobium platysporum* Goos	–	–	–	–	–	–	+	–	–	–
170	*Paramassariothea* sp.	–	–	–	–	–	–	–	–	–	+
171	*Phaeoisaria clematidis* (Fuckel) S. Hughes	–	–	–	–	–	–	–	–	–	–
172	*Periconia prolifica* Anastasiou	+	+	–	+	+	+	–	+	+	+
173	*Phragmospathula phoenicis* Subram. & Nair	–	–	–	–	–	–	+	–	–	–

No.	Species	1	2	3	4	5	6	7	8	9	10
174	*Phragmospathula* sp.	–	–	–	–	–	–	–	–	–	–
175	*Phialophorophoma* cf. *litoralis*	–	–	–	–	–	–	–	–	–	+
176	*Phoma suadae* Jaap	–	–	–	–	+	–	–	–	–	–
177	*Phoma* sp.	+	–	+	–	+	+	+	+	+	+
178	*Phomopsis mangrovei* K.D. Hyde	–	–	–	–	+	+	–	–	–	–
179	*Phomopsis* sp.	–	–	–	–	–	–	–	–	–	–
180	*Rhabdospora avicenniae* Kohlm. & E. Kohlm.	–	–	–	–	+	–	+	–	–	–
181	*Robillarda rhizophorae* Kohlm	+	–	–	–	–	–	–	–	–	–
182	*Sporidesmium* sp.1	–	–	–	–	–	–	–	–	–	–
183	*Sporidesmium* sp. 2	–	–	–	–	–	–	–	–	–	–
184	*Sporoschisma* sp.	–	–	–	–	–	–	–	–	–	–
185	*Stachybotrys mangiferae* Misra & Srivastava	–	–	–	+	–	–	+	–	–	–
186	*Stachylidium bicolor* Link.	–	–	–	–	–	–	–	–	–	–
187	*Stagonospora* sp.	–	–	–	–	–	–	–	–	–	–
188	*Taeniolella stricta* (Corda) S. Hughes	–	–	–	–	–	–	–	–	–	–
189	*Tetracrium* sp.	–	–	–	–	–	–	–	–	–	–
190	*Trichocladium achrasporum* (Meyers & R.T. Moore) Dixon	+	+	–	+	+	+	+	+	+	+
191	*T. alopallonellum* (Meyerss & R.T. Moore) Kohlm. & Volkm.–Kohlm.	+	+	–	+	+	+	+	+	+	+
192	*T. constrictum* I. Schmidt	–	–	–	–	–	–	–	–	–	–
193	*T. melhae* E.B.G. Jones, Abdel–Wahab & Vrijmoed	–	–	–	–	–	–	–	–	–	–
194	*Trichocladium opacum*-like	–	–	–	–	–	–	–	–	–	–
195	*Trichocladium* sp.	–	–	–	–	–	–	–	–	–	–
196	*Trimmatostroma* sp.	+	–	–	–	–	–	–	–	–	–
197	*Varicosporina ramulosa* Meyers & Kohlm.	+	–	–	–	–	–	–	–	–	–
198	*Verticillium* sp.	–	–	–	–	–	–	–	–	–	–
199[f]	*Xylomyces rhizophorae* Kohlm. & Volkm.–Kohlm.	–	–	–	–	–	–	–	–	–	–
200	*Xylomyces* sp.	–	–	–	–	+	+	+	+	–	–
201	*Zalerion maritimum* (Linder) Anast.	–	–	–	–	–	–	–	–	–	–

Table 13.1 (continued)

S. No.	Name of the species	Krishna delta, AP, India[11]	Godavari delta, AP, India[12]	A & N Islands, India[13]	North Malabar, Kerala, India[14]	Nethravati, Karnataka, India[15]	Udyavara, Karnataka, India[16]	Mauritius[17]	Kampong Kapok, Brunei[18]	Total no. of mangrove sites in which a fungus was recorded[19]
1	*Acrocordiopsis patilii* Borse & K.D. Hyde	–	–	+	–	–	–	–	+	3
2	*Aigialus grandis* Kohlm. & S. Schatz	+	+	+	+	–	–	–	+	13
3	*A. mangrovei* Borse	–	–	+	–	+	+	+	–	8
4	*A. parvus* S. Schatz & Kohlm.	–	+	+	–	–	–	–	+	7
5	*A. striatispora* K.D. Hyde	–	–	–	–	–	–	–	–	1
6	*Aigialus* sp.	–	–	–	–	–	–	–	–	1
7	*Aniptodera chesapeakensis* Shearer & M.A. Mill.	+	+	+	+	+	+	+	+	11
8	*A. longispora* K.D. Hyde	–	–	–	–	–	–	–	+	5
9	*A. haispora* Vrijmoed, K.D. Hyde & E.B.G. Jones	–	+	–	+	–	–	–	–	2
10	*A. mangrovei* K.D. Hyde	+	+	+	–	+	+	–	+	8
11	*Aniptodera* sp.	–	–	–	–	–	+	+	+	4
12	*Antennospora quadricornuta* (Cribb & J.W. Cribb) T.W. Johnson	–	–	+	–	+	+	–	+	10
13	*Anthostomella* sp.	–	–	–	–	–	–	–	+	1
14	*Ascocratera manglicola* Kohlm.	+	–	+	–	–	–	–	–	6
15	*Ascocratera* cf. *manglicola* Kohlm.	–	–	–	–	–	–	–	–	1
16	*Astrosphaeriella striatispora* (K.D. Hyde) K.D. Hyde ≡*Trematosphaeria striatispora* K.D. Hyde	–	–	+	–	–	–	–	+	2
17	*Bathyascus grandisporus* K.D. Hyde & E.B. G. Jones	–	–	+	–	–	–	–	+	3
18	*Bathyascus mangrovei* Ravikumar & Vittal	–	–	–	–	–	–	–	–	1
19	*Bathyascus* sp.	–	–	–	–	–	–	–	–	1
20	*Belizeana tuberculata* Kohlm. & Volkm.–Kohlm.	–	–	+	–	–	–	–	+	5
21	*Biatriospora marina* K.D. Hyde & Borse	–	–	+	+	–	–	–	–	3

22	*Byssothecium obiones* (P. Crouan & H. Crouan) M.E. Barr	+	–	–	–	–	–	–	–	1
23	*Capillataspora corticola* K.D. Hyde	–	–	–	–	–	+	+	–	1
24	*Caryosporella rhizophorae* Kohlm.	–	+	+	+	+	+	+	+	9
25	*Coccostroma* sp.	–	–	–	–	–	–	–	–	1
26	*Corollospora pulchella* Kohlm., I. Schmidt & N.B. Nair	+	–	–	–	–	–	–	–	1
27	*Coronopapilla mangrovei* (K.D. Hyde) Kohlm. & Volkm.–Kohlm.	–	+	+	+	+	–	+	–	4
28	*Cryptosphaeria mangrovei* K.D. Hyde	+	–	–	–	–	–	–	+	3
29	*Cucullosporella mangrovei* K.D. Hyde & E.B.G. Jones	–	–	+	–	+	+	+	–	4
30	*Dactylospora haliotrepha* (Kohlm. & E. Kohlm.) Hafellner	+	+	+	+	+	+	+	+	16
31[a]	*Diatrypasimilis australiensis* J. Zhou & Kohlm	–	–	–	–	–	–	–	–	1
32	[b]*Didymella avicenniae* Patil & Borse	–	–	–	–	–	–	–	–	1
33	*Durella* sp.	–	–	+	+	–	+	–	–	2
34	*Etheirophora bleapharospora* (Kohlm. & E. Kohlm.) Kohlm. & Volkm.–Kohlm.	–	+	–	–	+	–	+	+	5
35	[b]*Eutypa bathurstensis* K.D. Hyde & Rappaz	–	–	+	+	–	–	–	–	1
36	*Gnomonia* sp.-like	+	–	–	–	–	–	–	–	2
37	*Haiyanga salina* (Meyers) K.L. Pang & E. B. G. Jones, Yusoff & S. T.Moss ≡*Antennospora salina* (Meyers) Yusoff, E.B.G. Jones & S.T. Moss,	–	–	–	–	–	–	–	–	1
38	*Haligena* sp.	–	–	–	–	–	–	–	–	1
39	*Halomassarina thalassiae* (Kohlm. & Volkm.–Kohlm.) Suetrong, Sakayaroj, E.B.G. Jones, Kohlm., Volkm.–Kohlm. & C.L. Schoch, ≡*M. thalassiae* Kohlm. & Volkm.–Kohlm.	+	+	+	+	–	–	+	+	9
40	*Halorosellinia oceanica* Whalley, E.B.G. Jones, K.D. Hyde & Laessøe	+	+	+	+	+	+	+	+	15

(continued)

Table 13.1 (continued)

S. No.	Name of the species	Krishna delta, AP, India[11]	Godavari delta, AP, India[12]	A & N Islands, India[13]	North Malabar, Kerala, India[14]	Nethravati, Karnataka, India[15]	Udyavara, Karnataka, India[16]	Mauritius[17]	Kampong Kapok, Brunei[18]	Total no. of mangrove sites in which a fungus was recorded[19]
41	*Halosarpheia fibrosa* Kohlm. & E. Kohlm.	–	–	–	–	–	–	+	+	6
42	*H. marina* (Cribb & J.W. Cribb) Kohlm.	+	–	–	+	–	–	+	+	11
43	*H. minuta* Leong	–	–	+	–	–	–	–	+	4
44	*Halosarpheia* sp.	+	–	–	–	–	–	+	–	4
45	*Hapsidascus hadrus* Kohlm. & Volkm.–Kohlm.	–	–	–	–	–	–	–	–	1
46	*Heleococcum japonense* Tubaki	+	–	–	–	–	–	–	–	2
47	*Helicascus kanaloanus* Kohlm.	–	–	+	–	–	–	–	–	2
48	*Hypocrea* sp.	+	–	–	–	–	–	–	–	1
49	*Hypophloeda rhizospora* K.D. Hyde & E.B.G. Jones	–	–	–	–	–	–	–	+	4
50	*Hypoxylon hypomiltum* Montagne	–	–	–	–	–	–	–	–	1
51	*Hypoxylon* sp.	+	+	–	–	+	+	–	–	5
52	[c]*Hysterium* sp.[b]	+	+	–	–	–	–	–	–	2
53	*Kallichroma tethys* (Kohlm. & E. Kohlm.) Kohlm. & Volkm.–Kohlm.	+	+	+	–	+	+	+	+	12
54	*Kirschsteiniothelia maritima* -like	–	+	–	–	–	–	–	–	1
55	[b]*Julella avicenniae* (Borse) K.D. Hyde	–	–	–	–	–	–	–	–	1
56	*Leptosphaeria australiensis* (Cribb & J.W. Cribb) G.C. Hughes	+	+	+	–	+	+	+	+	13
57	[b]*Leptosphaeria* cf. *avicenniae* Kohlm. & E. Kohlm.	–	–	–	–	–	–	–	+	1
58	[b]*Leptosphaeria.*cf. *peruviana* Speg	+	+	–	–	–	+	–	–	3
59	[b]*Leptosphaeria salvinii* Catt.	–	–	–	–	+	–	–	–	1
60	*Leptosphaeria* sp.	+	–	–	–	–	–	+	+	4
61	*Lignincola laevis* Höhnk	–	–	+	–	+	+	+	+	13
62	*L. tropica* Kohlm.	–	+	–	–	–	–	–	+	3
63	*Limacospora sundica* (Jørg. Koch & E.B.G. Jones) Jørg.	–	–	–	–	+	+	–	–	2

	Species								
	≡*Ceriosporopsis sundica* Jørg Koch & E.B.G. Jones								
64	*Lineolata rhizophorae* (Kohlm.& E. Kohlm.) Kohlm.& Volkm.–Kohlm	+	–	–	–	+	–	+	9
65	*Lophiostoma armatisporum* (K.D. Hyde, Vrijmoed, Chinnaraj & E.B.G. Jones) Liew, Aptroot & K.D. Hyde,	–	–	–	–	+	+	–	1
	Massarina armatispora K.D. Hyde, Vrijmoed, Chinnaraj & E.B.G. Jones.								
66[d]	≡*Lophiostoma. rhizophorae* (Poonyth, K.D. Hyde, Aptroot & Peerally) Aptroot & K.D. Hyde	–	–	–	–	–	–	–	1
67	*Lulworthia grandispora* Meyers	+	+	+	+	+	+	+	11
68	*Lulworthia sp.*	+	+	+	+	+	+	+	16
69	*Manglicola guatemalensis* Kohlm. & E. Kohlm.	–	–	–	–	–	–	+	2
70	*Marinosphaera mangrovei* K.D. Hyde	–	+	–	–	+	+	+	8
71	*Massarina lacertensis* Kohlm. & Volkm.–Kohlm.	–	–	–	–	–	+	–	2
72	*Massarina sp.*	+	–	–	–	–	+	–	3
73	*Massariosphaeria typhicola* (P. Karst.) Barr	+	–	–	–	–	–	–	2
74	*Melaspilea mangrovei* Vrijmoed, K.D. Hyde & E.B.G. Jones	–	–	–	–	–	–	–	1
75	*Morosphaeria ramunculicola* (K.D. Hyde) Suetrong, Sakayaroj, E.B.G. Jones & C.L. Schoch	–	–	–	–	–	–	–	3
	≡*Massarina ramunculicola* K.D. Hyde								
76	*Morosphaeria velataspora* (K.D. Hyde & Borse) Suetrong, Sakayaroj, E.B.G. Jones & C.L. Schoch	+	+	–	+	+	+	+	9
	≡*Massarina velataspora* K.D. Hyde & Borse								

(continued)

Table 13.1 (continued)

S. No.	Name of the species	Krishna delta, AP, India[11]	Godavari delta, AP, India[12]	A & N Islands, India[13]	North Malabar, Kerala, India[14]	Nethravati, Karnataka, India[15]	Udyavara, Karnataka, India[16]	Mauritius[17]	Kampong Kapok, Brunei[18]	Total no. of mangrove sites in which a fungus was recorded[19]
77	[b]*Mycosphaerella pneumatophorae* Kohlm.	–	–	–	–	–	–	–	–	2
78	*Nais inornata* Kohlm.	–	–	–	–	–	–	–	–	2
79	*Nais* sp.	–	–	+	–	–	+	–	–	3
80	*Natantispora retorquens* (Shearer & J.L. Crane) J. Campb., J.L. Anderson & Shearer ≡*Halosarpheia retorquens* Shearer & J.L. Crane	–	–	–	–	–	–	–	–	1
81	*Neptunella longirostris* (Cribb & J.W. Cribb) K.L. Pang & E.B.G. Jones ≡*Lignincola longirostris* (Cribb & J.W. Cribb) Kohlm	+	–	–	–	–	–	–	–	4
82	*Okeanomyces cucullatus* (Kohlm.) K.L. Pang & E.B.G. Jones ≡*Halosphaeria cucullata* (Kohlm.) Kohlm	–	–	–	–	–	–	–	–	3
83	*Ophiodeira monosemeia* Kohlm. & Volkm.–Kohlm.–like	–	–	–	–	–	–	–	+	3
84	*Oceanitis cincinnatula* (Shearer & J.L. Crane) J. Dupont & E.B.G. Jones ≡*Halosarpheia cincinnatula* Shearer & J.L. Crane	–	–	–	–	+	+	–	–	4
85	*O. viscidula* (Kohlm. & E. Kohlm.) J. Dupont & E. B.G. Jones ≡*Haligena viscidula* Kohlm. & E. Kohlm., Nova Hedw. **9**: 92, 1965	–	–	–	–	–	–	–	–	1
86	*Panorbis viscosus* (I. Schmidt) J. Campb., J.L. Anderson & Shearer ≡*Halosarpheia viscosa* (I. Schmidt) Shearer & J.L. Crane	–	–	–	–	–	–	+	+	4
87	*Paraliomyces lentifer* Kohlm.	–	–	–	–	–	–	–	–	1

88	*Patellaria atra* (Hedw.) Fr., ≡*Lecanidion atratum* (Hedw.) Endl.	+	–	–	–	–	+	3
89	*Passeriniella savoryellopsis* K.D. Hyde & Mouzouras	–	–	–	–	+	–	4
90	*Passereniella mangrovei* G.L. Maria & K.R. Sridhar	–	–	+	+	–	–	2
91	*Payosphaeria minuta* W.F. Leong	–	–	–	–	–	–	1
92	*Payosphaeria* sp.	–	–	–	–	–	–	1
93	*Pedumispora rhizophorae* K.D. Hyde & E.B.G. Jones	+	–	–	–	–	–	2
94	*Phaeosphaeria neomaritima* (R.V. Gessner & Kohlm.) Shoemaker & C.E. Babc	–	–	–	–	–	–	1
95	*Phaeosphaeria oraemaris* (Linder) Khashn. & Shearer	–	–	–	–	–	–	1
96	*Polystigmina* sp.	–	–	–	–	–	+	1
97	*Pyrenographa xylographoides* Aptroot	–	–	–	–	–	–	1
98	*Quintaria lignatilis* (Kohlm.) Kohlm. & Volkm.–Kohlm.	+	+	–	–	–	+	11
99	*Rhizophila marina* K.D. Hyde & E.B.G. Jones	+	+	+	–	+	+	10
100	*Rimora mangrovei* (Kohlm. & Vittal) Kohlm.Volkm–Kohlm., Suetrong, Sakayaroj & E.B.G. Jones ≡*Lophiostoma mangrovei* Kohlm. & Vital	+	+	+	+	–	–	9
101	*Saccardoella mangrovei* K.D. Hyde	–	–	–	–	–	–	1
102	*S. rhizophorae* K.D. Hyde	+	–	–	–	–	–	5
103	*Saagaromyces abonnis* (Kohlm.) K.L. Pang & E.B.G. Jones ≡*Halosarpheia abonnis* Kohlm.	+	+	–	–	+	–	8
104	*S. glitra* (J.L. Crane & Shearer) K.L. Pang & E.B.G. Jones, ≡*Nais glitra* J.L. Crane & Shearer	–	+	–	–	–	+	5

Table 13.1 (continued)

S. No.	Name of the species	Krishna delta, AP, India[11]	Godavari delta, AP, India[12]	A & N Islands, India[13]	North Malabar, Kerala, India[14]	Nethravati, Karnataka, India[15]	Udyavara, Karnataka, India[16]	Mauritius[17]	Kampong Kapok, Brunei[18]	Total no. of mangrove sites in which a fungus was recorded[19]
105	*S. ratnagiriensis* (S.D. Patil & Borse) K.L. Pang & E.B.G. Jones ≡*Halosarpheia ratnagiriensis* S.D. Patil & Borse	+	+	+	–	+	+	–	+	8
106	*Savoryella lignicola* E.B.G. Jones & R.A. Eaton	+	–	+	+	+	+	+	+	9
107	*S. longispora* E.B.G. Jones & K.D. Hyde	–	–	–	–	+	+	–	–	3
108	*S. paucispora* (Cribb & J.W. Cribb) Jørg. Koch	–	–	+	+	+	+	–	–	5
109	*Splanchnonema britzelmayriana*-like	–	+	–	–	–	–	–	–	1
110	*Splanchnonema* sp.	–	–	–	–	–	–	–	+	1
111	*Sporormiella grandispora* (Speg.) Ahmed & Cain	–	–	–	–	–	–	–	–	1
112	*S. minima* (Auersw.)S.I. Ahmed & Cain	–	–	–	–	–	–	–	–	1
113	*Swampomyces armeniacus* Kohlm. & Volkm.–Kohlm.	–	–	+	–	–	–	+	–	4
114	*S.wampomyces* cf. *armeniacus* Kohlm. & Volkm.–Kohlm.	–	–	–	–	–	–	–	+	3
115	*S. triseptatus* K.D. Hyde & Nakagiri	–	–	–	–	–	–	–	–	2
116	*Thalassogena sphaerica* Kohlm. & Volkm.–Kohlm.	–	–	–	–	–	–	+	+	4
117	*Torpedospora radiata* Meyers	–	–	–	–	–	–	–	+	4
118	*Trematosphaeria mangrovei* Kohlm.	–	–	–	–	–	–	–	–	1
119	*Trematosphaeria* sp.	–	–	–	–	–	–	–	+	2
120	*Tirispora unicaudata* E.B.G. Jones & Vrijmoed, ≡*Aniptodera indica* Ananda & Sridhar,	–	–	–	–	+	+	–	–	2
121	*Tirispora mandoviana* V.V. Sarma & K.D. Hyde	–	–	–	–	+	+	–	–	3

No.	Species									Total
122	*Tirispora* sp.	–	–	–	–	+	+	–	–	2
123	ᵃ*Tubeufia setosa* Sivan. & W.H. Hsieh	–	+	–	–	–	–	–	–	1
124	*Verruculina enalia* (Kohlm.) Kohlm. & Volkm.–Kohlm.	+	+	+	+	+	+	+	+	16
125	*Zignoella* sp.	–	–	–	–	+	–	–	–	1
126	*Zopfiella* sp.	–	–	–	–	–	+	–	–	1
127	*Halocyphina villosa* Kohlm.	+	+	+	+	+	+	+	+	15
128	*Halocyphina* sp.	–	–	–	–	–	–	–	–	1
129	ᵃ*Calathella mangrovei* E.B.G. Jones & Agerer	–	–	–	–	–	–	–	–	1
130	*Calathella* sp.	–	–	–	–	–	–	–	–	1
131	*Nia vibrissa* R.T. Moore & Meyers	–	–	–	–	–	–	–	+	2
132	*Acremonium* sp.	–	–	–	–	+	+	+	–	2
133	*Alternaria* sp.	–	–	–	–	+	+	+	–	2
134	*Alveophoma* sp.	–	+	–	–	–	–	–	–	1
135	*Anguillospora marina* Nakagiri & Tubaki	–	–	–	–	+	+	+	–	2
136	*Arthrobotrys oligospora* Fresen.	–	–	–	–	+	+	+	–	2
137	*Bactrodesmium linderii* (Crane & Shearer) M.E. Palm & E.L. Stewart	+	+	–	–	+	+	+	+	5
138	*Bactrodesmium* sp.*l*	–	–	–	–	–	–	+	–	1
139	*Brachysporiella gayana* Batista	–	–	–	–	+	+	+	–	2
140	*Cirrenalia basiminuta* Raghuk. & Zainal	–	+	–	–	–	–	+	–	3
141	*Cirrenalia macrocephala* (Kohlm.) Meyers & Mooore	+	–	+	–	+	+	+	–	4
142	*Cirrenalia pseudomacrocephala* Kohlm.	–	–	–	–	–	–	–	+	7
143	*Clavatospora bulbosa* (Anast.) Nakagiri & Tubaki	–	–	–	–	+	+	+	–	4
144	*Cumulospora marina* I. Schmidt	–	–	–	–	+	+	+	–	2
145	*Cytospora rhizophorae* Kohlm. & E. Kohlm.	+	+	+	+	–	–	–	–	5
146	*Cytospora* sp.	–	–	–	–	–	–	–	+	1

(continued)

Table 13.1 (continued)

S. No.	Name of the species	Krishna delta, AP, India[11]	Godavari delta, AP, India[12]	A & N Islands, India[13]	North Malabar, Kerala, India[14]	Nethravati, Karnataka, India[15]	Udyavara, Karnataka, India[16]	Mauritius[17]	Kampong Kapok, Brunei[18]	Total no. of mangrove sites in which a fungus was recorded[19]
147	*Dactylella* sp.	–	–	–	–	–	–	–	–	1
148	*Delortia palmicola* Pat.	–	–	–	–	+	+	–	–	2
149	*Dendryphiella arenaria* Nicot	–	–	–	–	–	–	–	–	1
150	*Dendryphiella salina* (G.K. Sutherl.) G.J.F. Pugh & Nicot	–	–	–	–	–	–	–	–	1
151	*Dictyosporium elegans* Corda	–	–	–	–	–	–	–	–	1
152	*Dictyosporium pelagicum* (Linder) G.C. Hughes	–	–	–	–	–	–	–	+	3
153	*Diplocladiela scalaroides* G. Arnaud ex M.B. Ellis	–	–	–	–	–	+	–	–	1
154	*Ellisembia vaga* (C.G. & T.F.L.) Subram.	+	+	–	–	–	–	–	–	2
155	*Endophragmia alternata* Tubaki & Saito	–	–	–	–	+	+	–	–	2
156	*Epicoccum purpurascens* Ehrenb.:Schlecht.	–	+	–	–	–	–	–	–	2
157	*Hansfordia* sp.	–	–	–	–	+	+	–	–	2
158	*Helicoma muelleri* Corda	–	–	–	–	–	+	–	–	1
159	*Helicomyces roseus* Link.	–	–	–	–	+	+	–	–	2
160	*Helicoon* sp.	–	–	–	–	–	–	–	–	1
161	*Helenospora varia* (Anastasiou) E.B.G. Jones ≡ *Zalerion varium* Anastasiou	+	+	+	+	+	+	–	–	9
162	*Hydea pygmea* (Kohlm) K.L. Pang & E.B.G. Jones . ≡ *Cirrenalia pygmea* Kohlm.	+	+	+	+	+	+	+	+	15
163	*Leptosphaeria salvinii* Cattaneo (anamorph, nakatea state)	–	–	–	–	–	+	–	–	1
164	*Matsusporium tropicale* (Kohlm.) E.B.G. Jones & K.L. Pang ≡ *Cirrenalia tropicalis* Kohlm.	–	+	–	–	+	+	+	+	10
165	*Menispora cilliata* Corda	–	–	–	–	+	+	–	–	2

166	*Monodictys pelagica* Johnson & E.B.G. Jones	–	–	–	–	–	+	–	3
167	*M. putredinis* (Wallr.) S. Hughes	–	–	–	–	–	+	–	2
168	*Monodictys* sp.	+	–	–	–	–	–	–	1
169	*Mycoenterolobium platysporum* Goos	–	–	–	–	–	–	–	1
170	*Paramassariothea* sp.	–	–	–	–	–	–	–	1
171	*Phaeoisaria clematidis* (Fuckel) S. Hughes	–	–	–	–	+	+	–	2
172	*Periconia prolifica* Anastasiou	+	+	+	+	–	+	+	15
173	*Phragmospathula phoenicis* Subram. & Nair	–	–	–	–	–	–	–	1
174	*Phragmospathula* sp.	–	+	–	+	–	+	–	2
175	*Phialophorophoma* cf. *litoralis*	–	–	–	–	–	–	–	2
176	*Phoma suadae* Jaap	–	–	–	–	–	–	–	1
177	*Phoma* sp.	+	+	+	+	–	+	+	14
178	*Phomopsis mangrovei* K.D. Hyde	+	+	+	+	–	–	–	3
179	*Phomopsis* sp.	+	+	–	–	–	+	–	4
180	*Rhabdospora avicenniae* Kohlm. & E. Kohlm.	–	–	–	–	–	–	–	1
181	*Robillarda rhizophorae* Kohlm	–	–	–	–	–	–	–	1
182	*Sporidesmium* sp.1	–	–	–	–	–	+	–	1
183	*Sporidesmium* sp. 2	–	–	–	–	–	+	–	1
184	*Sporoschisma* sp.	–	–	–	–	+	–	–	1
185	*Stachybotrys mangiferae* Misra & Srivastava	–	–	–	–	–	–	–	1
186	*Stachylidium bicolor* Link.	–	–	–	–	+	+	–	2
187	*Stagonospora* sp.	–	–	–	–	–	–	–	1
188	*Taeniolella stricta* (Corda) S. Hughes	–	–	–	–	+	+	–	2
189	*Tetracrium* sp.	–	–	–	–	+	+	–	2
190	*Trichocladium achrasporum* (Meyers & R.T. Moore) Dixon	+	+	+	+	+	+	+	13
191	*T. alopallonellum* (Meyerss & R.T. Moore) Kohlm. & Volkm.–Kohlm.	+	+	–	–	–	+	–	12
192	*T. constrictum* I. Schmidt	–	–	–	+	–	–	–	1

(continued)

Table 13.1 (continued)

S. No.	Name of the species	Krishna delta, AP, India[11]	Godavari delta, AP, India[12]	A & N Islands, India[13]	North Malabar, Kerala, India[14]	Nethravati, Karnataka, India[15]	Udyavara, Karnataka, India[16]	Mauritius[17]	Kampong Kapok, Brunei[18]	Total no. of mangrove sites in which a fungus was recorded[19]
193	*T. melhae* E.B.G. Jones, Abdel–Wahab & Vrijmoed	–	–	–	–	+	+	–	–	2
194	*Trichocladium opacum*-like	–	–	–	–	–	–	–	+	2
195	*Trichocladium* sp.	+	–	–	–	–	+	–	+	2
196	*Trimmatostroma sp.*	+	+	–	–	–	–	–	–	3
197	*Varicosporina ramulosa* Meyers & Kohlm.	–	–	–	–	–	–	–	–	1
198	*Verticillium* sp.	–	–	–	–	+	+	–	–	2
199[f]	*Xylomyces rhizophorae* Kohlm. & Volkm.–Kohlm.	–	–	–	–	–	–	–	–	1
200	*Xylomyces* sp.	–	–	–	–	+	+	–	+	3
201	*Zalerion maritimum* (Linder) Anast.	–	–	–	–	+	+	–	–	2

The fungal species occurring on aerial woody parts, rhizosphere, rhizoplane, phylloplane, phyllosphere and endophytic fungi *are not included* in the above list as the table was intended mainly for marine fungi colonizing dead and degrading samples of *Rhizophora* spp.

[a]Chalkley et al. (2010)

[b]Doubtful of the occurrence of the fungus on this host

[c]Doubtful whether it is truly marine fungus

[d]Hyde et al. (2002)

[e]From Chorao mangroves, Mandovi River, Goa, India included in CD–ROM on mangrove fungi, National Institute of Oceanography, Goa 403004, India

[f]Kohlmeyer and Volkmann-Kohlmeyer 1998

[g]No. of sites in which was fungus recorded: 1. Mostly from Neotropics (Kohlmeyer and Kohlmeyer 1979); 2. Belize, Fiji, Hawaii Islands, South America (B. Volkmann-Kohlmeyer and Kohlmeyer 1993; Kohlmeyer et al. 1995); 3. Mandai mangrove, Singapore (Leong et al. 1991); 4. Ranong mangrove, Thailand (Hyde et al. 1990); 5. Kuala Selangor & Kampong Sementa, Malaysia (Jones and Kuthubutheen 1989); 6. Morib, Malaysia (Alias and Jones 2000); 7. Irimoto island, Japan (Nakagiri 1993); 8. North Sumatra (Hyde 1989); 9. Queensland, Australia (Kohlmeyer and Volkmann-Kohlmeyer 1991); 10. Pichavaram, Tamil Nadu state, East coast of India (Ravikumar and Vittal 1996; Sridhar 2009); 11. Krishna delta, Andhra Pradesh state, east coast of India (Sarma and Vittal 1998–1999); 12. Godavari delta, Andhra Pradesh state, east coast of India (Sarma and Vittal 1998–1999); 13. Andaman and Nicobar Islands, East coast of India (Chinnaraj 1993); 14. Kerala state, West coast of India (Nambiar and Raveendran 2009); 15 Nethravati mangroves, Mangalore, Karnataka state, South West coast of India (Sridhar and Maria 2006); 16. Udyara, Karnataka state, South west coast of India (Maria and Sridhar 2003); 17. Mauritius (Poonyth et al. 1999); 18. Kampong Kapok, Brunei (Hyde 1990a, b; Hyde 1991); Kampong Danau (Hyde 1988); 19. No. of sites in which was fungus recorded

75 unitunicate ascomycetes have been reported so far from *Rhizophora* spp. The order Halosphaeriales alone consist 37 species, while 38 species belong to other unitunicate ascomycetes. More than 50 species possess active spore dispersal mechanism, either by having a pore in the ascus or by fissitunicate mechanism of dispersal.

On *Rhizophora* spp., most of the marine fungi belong to ascomycetes and sizeable portion to anamorphic fungi (35%) with only a few members belonging to basidiomycetes. Three marine basidiomycetes occur on *Rhizophora* spp. and all are tiny in size and belong to the family Lachnellaceae of the order Agaricales. All have reduced basidiomes, possibly as an adaptation to an aquatic environment where large fruit bodies would not survive (Jones 1988). Hibbett (2007) considers the minute forms of cyphelloid basidiomycetes to be related to selection of spore production from minimal substrates (Jones et al. 2009).

A close examination of the classification of the 85 marine fungal genera of Ascomycota occurring on *Rhizophora* spp. shows that though they belong to 6 of the 10 classes under the division Pezizomycotina of the Phylum Ascomycota, only two classes have large representation and they are Dothideomycetes (36 genera) and Sordariomycetes (42 genera). Whereas in the class Dothideomycetes, the order Pleosporales is represented by as many as 25 genera in the Sordariomycetes, the order Halosphaeriales is represented by 20 marine fungal genera. Three genera could not be assigned to any class and were kept incertae sedis under unitunicate ascomycetes. Including this, totally 85 marine fungal genera belonging to ascomycota has been recorded on *Rhizophora* spp.

A comparison between *Avicennia* spp. and *Rhizophora* spp., the two predominant mangrove tree species, shows that the following fungi are specific to *Avicennia*: *Adomia avicenniae*, *Camarosporium roumeguerii*, *Bathyascus avicenniae*, *Cryptovalsa* sp., *Didymella avicenniae*, *Eutypa bathurstensis*, *Eutypella naqsii*, *Julella avicenniae*, *Leptosphaeria avicenniae*, *Mycosphaerella pneumatophorae*, *Rhabdospora avicenniae*, *Zopfiella latipes*, *Zopfiella marina*. The following species have been found to be specific on *Rhizophora* spp: *Caryosporella rhizophorae*, *Cryptosporella mangrovei*, *Etheirophora blepharospora*, *Hypophloeda rhizospora*, *Lineolata rhizophorae*, *Lophiostoma rhizophorae*, *Pedumispora rhizophorae*, *Rhizophila marina*, *Sacardoella rhizophorae*, *Trematosphaeria mangrovei*, *Cytospora rhizophorae*, *Phomopsis mangrovei*, *Robillarda rhizophorae*, *Xylomyces rhizophorae*.

Many new genera and new species have been described after Hyde et al. (2000) in addition to transfers and/or creating new genera and new species. For an update on these new fungi/names, the following publications have been referred (Campbell et al. 2003; Pang et al. 2003; Jones et al. 2009; Suetrong et al. 2009; Abdel-Wahab et al. 2010) which are included in Table 13.1.

13.3 Geographical Distribution

Prior to 1980s, most of the studies on marine fungi in mangroves, particularly with reference to *Rhizophora* spp. was conducted in Neotropics covering mangrove formations in the coastal regions sandwiching North and South America including the coast of Florida, USA, Caribbean Islands, Belize and other surrounding sites (West Atlantic Ocean and Pacific Ocean regions). The host species studied were *Rhizophora mangle*, *R. racemosa* and *R. stylosa* (Kohlmeyer and Kohlmeyer 1979). A few scattered studies were also conducted from West African region particularly Sierra Leone (Aleem 1980). Around 42 mangrove fungi were reported by Kohlmeyer and Kohlmeyer (1979) in their treatise on higher marine fungi, of which 33 were on *Rhizophora* spp.

Subsequent studies of marine fungi in mangroves in the following two decades were from South East Asia, South Asia, Far East, Seychelles, Mauritius and Australia. Among these, South East Asia including Brunei, Malaysia, Thailand, Philippines and Singapore, has been thoroughly sampled. A large number of marine fungi have been recorded on mangroves from this region (Table 13.1). Many mangrove formations are yet to be explored including Mahanadi mangroves and Sunderbans on the east coast of India, Indonesian mangroves and the African and South American continents.

Fungi occurring on *Rhizophora* spp. from eighteen sites are listed in Table 13.1. These sites may be divided into three regions, viz., (a) Neotropics covering the mangrove formations in between North and South America and Belize, Fiji and Hawaii, (b) South East Asia covering Singapore, Thailand, Malaysia and Brunei, (c) Indian Ocean coasts particularly South and East coast of India and (d) Miscellaneous containing Japan, Mauritius, Australia and North Sumatra. From the available data, it could be seen (Table 13.1) that 60 marine fungi have been recorded on *Rhizophora* spp. from Neotropics and Belize and Hawaii islands belonging to 44 ascomycetes, 2 basidiomycetes and 14 anamorphic fungi. The following fungi were not recorded in the other 3 regions of the 4 mentioned above and they are *Aigialus* sp., *Haligena* sp., *Halosarpheia* sp., *Hapsidascus hadrus*, *Helicascus kanaloanus*, *Oceanitis viscidula*, *Paraliomyces lentifer*, *Trematosphaeria mangrovei*, *Dendryphiella salina*, *Robillarda rhizophorae* and *Varicosporina ramulosa*. Though these fungi have not been recorded on *Rhizophora* spp., there is a possibility of their presence on other hosts.

Trichocladium is represented by 6 species; *Aigialus*, *Aniptodera* and *Leptosphaeria* are represented by 5 species each, while *Halosarpheia*, *Saagaromyces*, *Savoryella*, *Tirispora* and *Cirrenalia* are represented by 3 species each. Most of the marine fungal genera recorded on the host *Rhizophora* spp. are monospecific or have two species. From India a total of 142 marine fungal species (comprising 89 Ascomycota, 1 Basidiomycota and 21 anamorphic fungi) have been recorded. Out of these, 94 marine fungi comprising 40 Ascomycota, 8 Basidiomycota 1 and 30 Anamorphic fungi have been recorded from west coast of India. While 48 fungi (40 Ascomycetes and 8 Anamorphic fungi) were not recorded from the west coast, around 47 marine fungi (16 ascomycetes, 1 basidiomycete and 30 anamorphic fungi)

recorded from west coast of India have not been recorded from east coast of India. Between the coasts, 47 marine fungi (33 Ascomycetes, 1 basidiomycete and 13 anamorphic fungi) are common. Jaccard's Index expressed in percentage showed 33% similarity between the two coasts of India. This shows that there is considerably less commonality between the two coasts and more fungi are unique in the each coast. The reason for variation in fungal diversity could be attributed to the temperature and salinity differences between the two coasts. On the east coast the salinity is normally less and temperatures are high and vice versa are true in the case of west coast (Vinayachandran and Kurian 2008). Of the fungi recorded from east coast of India on *Rhizophora* spp., 77 % are ascomycetes, whereas only 52% of ascomycetes are found in west coast of India. Similarly, the anamorphic fungi recorded on *Rhizophora* spp. from east coast of India is only 22%, whereas it is 46% from west coast of India. However, the total number of marine fungi recorded on *Rhizophora* from both the coasts did not vary much (95 and 94%, respectively).

From the South East Asian region, 103 marine fungi have been reported to occur on *Rhizophora* spp. (76 ascomycetes, 3 basidiomycetes and 24 anamorphic fungi). Though the number of marine fungi recorded on *Rhizophora* spp. from this region is low when compared to the Indian coastline, South East Asia could well be the richest in terms of marine fungal diversity in mangroves. For example, Alias et al. (2010) have reported more than 139 marine fungi covering all mangrove plants from Malaysia with many waiting for formal identification. Similarly, 154 fully identified marine fungi have been reported from Thailand by Jones et al. (2006) that include hosts *Nypa fruticans* and *Rhizophora* spp.

A comparison between the marine fungi reported from the South East Asia and Indian coasts showed that 40 marine fungi reported from the South East Asia are not found in the Indian coasts, while 73 marine fungi found in the Indian coasts are not reported from the S.E. Asia. In between these two regions, 63 fungi have been found to be commonly occurring on *Rhizophora* spp. While 55 fungi are common to the South East Asian region and the East coast of India, 44 marine fungi have been found to be common on *Rhizophora* spp. occurring in the South East Asia and the west coast of India. The reason that relatively more number of marine fungi are common between the east coast of India and the South East Asia could be due to the geographical proximity where the Bay of Bengal waters extend and mix into waters of the Indian Ocean in the South East Asia.

Most of the marine fungi reported from the Mauritius have been reported from the Indian mangroves. Same is the case with North Sumatra and Australia. In Japan, with exception of three or four species, all other marine fungi reported are the commonly occurring on *Rhizophora* spp. reported from all other regions.

13.3.1   Cosmopolitan Fungi

Out of 201 marine fungi recorded on *Rhizophora* spp. from 18 different mangrove formation/sites throughout the world, only 30 species have been recorded in more

than 9 out of the 18 sites (Table 13.1). *Dactylospora haliotrepha*, *Lulworthia* sp. and *Verruculina enalia* from 16 sites, *Halorosellinia oceanica*, *Halocyphina villosa*, *Hydea pygmea* and *Periconia prolifica* were reported from 15 sites; *Phoma* sp. 14 sites; *A. grandis*, *Leptosphaeria australiensis*, *Lignincola laevis* and *Trichocladium achrasporum* in 13 sites; *Kallichroma tethys* and *Trichocladium alopalonellum* in 12 sites; *Aniptodera chesapeakensis*, *Halosarpheia marina*, *Lulworthia grandispora*, *Quintaria lignatilis* in 11 sites; *Antennosopora quadricornuta*, *Rhizophila marina* and *Matsusporium tropicale* 10 sites; *Caryosporella rhizophorae*, *Halomassarina thalassiae*, *Lineolata rhizophorae*, *Morosphaeria velatospora*, *Rimora mangrovei*, *Savoryella lignicola*, *Helenospora varia* in 9 sites; *Aigialus mangrove*, *Aniptodera mangrovei*, *Halomassarina thalassiae*, *Marinosphaera mangrovei*, *Saagaromyces abonnis* and *Saagaromyces ratnagiriensis* in 8 sites. These fungi could be characterized as having cosmopolitan occurrence and hence could be described as having "high ecological amplitude."

13.3.2 Marine Fungi with Restricted Distribution on *Rhizophora* spp.

Of the 201 marine fungi reported on *Rhizophora* spp. from 18 mangroves sites listed (Table 13.1), seventy-one marine fungal species occurred only in any one particular mangrove sites; forty-four in any two mangrove sites; twenty-four in any three mangroves sites and 17 in any four mangroves sites. Though fungi with restricted distribution add to the diversity of the species to a particular site, it also shows the vulnerability of the species to extinction due to their endemic occurrence and selection pressure. Though the exact reason for their restricted distribution is not known, it could be hypothesized that they are of recent origin in geological timescale and speciation, and hence have not reached other mangrove sites.

13.4 Adaptations

On driftwood not many groups of ascomycetes are found but are mostly dominated by Halosphaeriales. It is different in the case of mangroves where marine fungi belonging to several groups of fungi could be recorded. The reasons could be (a) mangroves have regular freshwater in-surge from rivers which not only reduces salinity but also brings propagules of various groups of fungi, (b) intermittent exposure and inundation by sea water during high tides, (c) special adaptations of the mangrove plants, (d) fixed plant parts of some of the mangroves that provide decaying substrata for colonization by marine fungi, (e) less or no turbulence in mangroves when compared to high seas or beaches. The above factors might have provided a natural selection to develop a unique marine mycota in mangroves.

While Halosphaeriales prefer lower parts of the mangroves where inundation takes place, the loculoascomycetes (Pleosporales, Dothideomycetes) prefer the intertidal parts. This clearly shows that mangroves in general, and *Rhizophora* spp. in particular, are influenced by tidal amplitude where the sea water inundation promote fungi belonging to Halosphaeriales and other unitunicate ascomycetes, wheras the intertidal region and the exposure of the plant parts promote the loculoascomycetes with fissitunicate mechanism (Hyde 1988, 1990a; Hyde et al. 1993; Alias and Jones 2000; Sarma and Vittal 2002). Many representatives of Halosphaeriales and other unitunicates having an apical ring at the apex of the asci and bitunicates having fissitunicate mechanism are suited for an active dispersal of the spores in the mangrove environment. Though the fungi have adaptations to withstand exposure to sunlight, desiccation and dryness, they also have features of typical aquatic mode of life, where the spores of many of the marine ascomycete genera, also have appendages or mucilaginous sheaths for attachment to the surfaces. While the appendages is found more in the genera of Halosphaeriales, the mucilaginous sheaths are found more in the order Pleosporales.

Fungi that occur above the water mark have different strategies. The apothecoid fungi viz., *Lecanidion atratum* and *Dactylospora haliotrepha* that occur superficially produce superficial ascomata and have a gelatinous substance in their fruit bodies, particularly the latter fungus. In these two fungi, as they are exposed to sunlight and heat, they form melanized fruit bodies to escape from the solar radiation. Another fungus in this category is *Hysterium* sp. which produces hysterothecia and produces superficial fruit bodies. Further, many of the fungi growing above tidal mark mainly belong to mitosporic fungi, e.g., *Epicoccum purpurascens*, *Trimmatostroma* sp. (Vittal and Sarma 2006).

The status of a few fungi occurring on this host is doubtful and a few others are actually terrestrial fungi but occur occasionally in the intertidal region. These are highlighted in Table 13.1 and they require further studies to establish whether they are marine fungi or "salt-tolerant fungi." Typical mangrove plants in general accumulate salt through their roots and excrete it out through salt glands on the leaves. That would mean the aerial parts have considerable amount of salt concentrations. Hence the fungi growing on the aerial parts of the mangrove plants have to cope up with this excreted salt. From this angle, it could be said that the fungi occurring on aerial parts of the mangroves are "salt-tolerant fungi."

Plant parts in mangroves are exposed intermittently to sea water for different periods of time or upper parts mostly exposed to sun light and radiation without inundation by water. This kind of a condition has allowed more number of loculoascomycetes with active spore dispersal mechanism to thrive in mangroves. Most of these loculoascomycetes are not found on driftwood samples. But these submerged parts are also exposed for short periods or longer periods of time if the tidal range is small. Hence the marine fungi (Halosphaeriales and other Unitunicate ascomycetes) are exposed to sun light, radiation and desiccation, even if it is for shorter periods of time. Due to these reasons the Halosphaeriales commonly recorded from mangroves are not the ones found on driftwood samples. Similarly, typical Halosphaerialean fungi with elaborate appendages, bristles and sheaths are not found in mangroves or

have low percentage occurrence in mangroves. The main factor that influences mycota seems to be the continuous connection of the drift wood samples with the sea water and their non-exposure even for shorter periods of time, which distinguishes "driftwood-marine fungi" from "mangrove wood-marine fungi."

Within mangroves, once again, each plant has its own special adaptations. For example, *Rhizophora* spp. have prop roots that branch out from the main trunk and are fixed into the marshy mangrove habitat and characteristic viviparous seedlings, whereas *Avicennia* spp. possess negatively geotropic pneumatophores and a large trunk with aerial branches which are not subjected to inundation. Hence in the case of *Avicennia* spp. the dead and fallen woody litter on the mangrove floor is the available substratum for marine fungi to colonize. Rarely the roots of *Avicennia* spp. on the banks of the canals and tributaries are exposed and are available for fungal colonization. These factors contribute to the difference in marine mycota occurring on *Avicennia* spp. or other mangrove species. However, there could be an overlap of marine fungi between *Rhizophora* spp. and *Avicennia* spp., indicating their wide host range and high ecological amplitude.

13.5   Conclusions

Rhizophora spp. are important hosts for marine fungi. Accommodating more than 201 fungi is a unique feature of this host genus. No other genus supports so many fungi. *Nypa fruticans* is another host which accommodates more than 100 marine fungi. This is followed by *Avicennia* spp. However, the advantage of *Rhizophora* spp. is that in addition to being a host for rich fungal diversity, it is also one of the ideal plants for ecological experimentation including vertical zonation, frequency of occurrence, substrate preferences and various other ecological observations.

We need to study the relationship between the chemistry/biochemistry of the host and the number of fungi growing on *Rhizophora* spp. and compare fungal diversity and the chemistry of prop roots, wood and leaves to substantiate the hypothesis that "the chemistry of the host plays a major role in supporting the diversity of fungi on a particular host." Further, there are more than 10 fungi that are specific to *Rhizophora* spp. and around 40 fungi are considered as "core group fungi" (most frequent) that thrive on this host. In future, it would be worthwhile to direct studies on these fungi, at molecular level, and also trace the biochemical pathways, to observe which particular carbohydrate or other compounds they prefer to thrive on this host. It is recommended that marine mycologists provide data on marine fungi occurring on each mangrove host separately in future so that maintaining a database will be easy.

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Chapter 14
Biotechnology of Marine Fungi

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Contents

Abstract Filamentous fungi are the most widely used eukaryotes in industrial and pharmaceutical applications. Their biotechnological uses include the production of enzymes, vitamins, polysaccharides, pigments, lipids and others. Marine fungi are a still relatively unexplored group in biotechnology. Taxonomic and habitat diversity form the basis for exploration of marine fungal biotechnology. This review covers what is known of the potential applications of obligate and marine-derived fungi obtained from coastal to the oceanic and shallow water to the deep-sea habitats. Recent studies indicate that marine fungi are potential candidates for novel enzymes, bioremediation, biosurfactants, polysaccharides, polyunsaturated fatty acids and secondary metabolites. Future studies that focus on culturing rare and novel marine fungi, combined with knowledge of their physiology and biochemistry will provide a firm basis for marine mycotechnology.

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14.1   Introduction

Freshwater and terrestrial bacteria and fungi have played a major role in biotechnology by virtue of their biochemical diversity and adaptations to a variety of environmental conditions (Bennett 1998). In comparison, marine microorganisms, particularly marine fungi are yet to find a prominent place in biotechnology. The oceans are home to a vast biodiversity (Ray 1988; Norse 1993). Most of marine microbial diversity has not been discovered and characterized, both taxonomically, as well as biochemically. It is logical, therefore, to conclude that marine microorganisms hold vast promise for improved and novel biotechnologies. Marine bacteria have been major targets for biotechnology research. On the contrary, marine fungi have attracted attention from biotechnologists only in recent years. This review focuses on what is known of marine fungal biotechnology so far, their potentials and future directions.

14.2   Diversity and Habitats of Fungi in the Marine Environment

Taxonomic and phylogenetic diversity of organisms, as well as their occurrence and adaptation to various habitats often provide clues to their potential applications in biotechnology (Raghukumar 2008a). Marine fungi may fall under 'true fungi' of the Kingdom Fungi or the 'straminipilan fungi' of the Kingdom Straminipila. Taxonomic diversity of marine fungi can be assessed by direct observations of sporulating structures, culturing and metagenomics. Culturable and taxonomically characterized marine fungi belong either to obligate or facultative (marine-derived) fungi (Kohlmeyer and Kohlmeyer 1979). The former are not found in freshwater and terrestrial environments, and grow and reproduce exclusively in the sea. Most of these are morphologically distinct. Some, such as the Halosphaeriales and Lulworthiales also constitute taxonomically distinct orders among the Ascomycetes (Jones et al. 2009). Current evidence points out that these are terrestrial fungi that have colonized marine habitats and have become adapted to those conditions (Spatafora et al. 1998). About 530 species of obligate marine fungi are known so far, most of these occurring on decomposing lignocellulosic material in the coastal environment, although a few have been described from decaying wood in the deep sea (Jones et al. 2009). The very fact that these are obligately marine, points out to their novelty compared to terrestrial fungi and makes their study attractive for biotechnological applications. In contrast to the obligate forms, 'facultative' or marine-derived fungi belong to terrestrial species. These have been isolated from a wide variety of marine habitats and it is strongly believed that these terrestrial species have adapted to marine conditions and probably have special biochemical properties (Damare et al. 2006a, b). The straminipilan, marine fungi, the Labyrinthulomycetes were once considered 'true' fungi, but are now known to

belong to the Kingdom Stramenopila (Raghukumar 2002). Metagenomic studies of marine habitats have revealed a large diversity of hitherto uncultured fungi (Edgcomb et al. 2002; Burgaud et al. 2009; Singh et al. 2011). Most of these do not appear to correspond to known fungal taxa and are of much interest to marine biotechnologists.

Salinity is the most defining feature of the oceanic environment. Mechanisms of salinity tolerance in fungi have been discussed by Jennings (1986) and Blomberg and Adler (1992). Physiological and biochemical adaptation of fungi to salinity can be of relevance to biotechnology. The ability to withstand salinity has been addressed from the point of view of biotechnology in the yeast *Saccharomyces cerevisiae*. Overexpression of the *HAL1* gene in yeast has a positive effect on salt tolerance by maintaining a high internal K^+ concentration and decreasing intracellular Na^+ during salt stress. Gisbert et al. (2000) have successfully introduced *HAL1* gene from this yeast into tomato. Higher level of salt tolerance was recorded in the progeny of two different transgenic plants bearing four copies or one copy of the *HAL1* gene. This has the scope for designing salt-tolerant crops.

In terms of nutrients in the marine environment, lignocellulosic substrates from coastal environments have been known for a long time to harbour a distinct diversity of obligate marine fungi, the lignicolous fungi (Kohlmeyer and Kohlmeyer 1979; Sridhar 2005). Among these, the manglicolous fungi that grow on decaying woody material in mangroves comprise an important group. Mangroves are detritus-based ecosystems and substantial fungal populations are involved in detritus processing (Raghukumar 2004). Mangrove fungi are the second largest group among the marine fungi.

Marine endophytic fungi that live inside the living tissues of seagrasses and macroalgae constitute an interesting ecological group of fungi (Raghukumar et al. 1992; Sathe-Pathak et al. 1993; Thirunavukkarasu et al. 2011).

Coral reefs provide an excellent habitat for fungi. Fungi grow both on the surface and interior of scleractinian corals. They are found both within the polyps and calcium carbonate skeleton of such corals. In the latter case, they exist as endoliths by penetrating the calcium carbonate structures. Coral reef fungi are also found as endobionts, being associated with living organisms such as coral polyps, sponges and holothurians, or as saprotrophs in coral mucus, plant detritus, sediments and water column. They are not only responsible for occasional coral diseases but may also have a mutualistic role in healthy corals (Le Campion-Alsumard et al. 1995). The report of aspergillosis disease in the sea fan *Gorgonia* (Smith et al. 1996) has kindled a worldwide interest in mycoflora of soft corals. Fungi have been isolated from the gorgonian soft coral, *Gorgonia ventalina* collected from Puerto Rico (Toledo-Hernández et al. 2007). Koh et al. (2000) isolated 16 fungal genera and 51 species, including 2 yeasts from 10 species of gorgonian corals in Singapore by culture-dependent technique. The microbial metagenome of *Porites astreoides* collected from Bocal del Toro, Panama, showed fungi to be the dominant community, contributing 38% to the genome of the coral (Wegley et al. 2007).

The deep sea is an extreme environment, characterized by temperatures around 2°C and high hydrostatic pressures. Detailed studies on deep-sea sediments of

Central Indian Basin suggest that fungi are an important component therein (Damare et al. 2006a; Damare and Raghukumar 2008; Singh et al. 2010). Most of the fungi isolated from these sediments belong to terrestrial species. Burgaud et al. (2009) have successfully cultured fungi from various benthic organisms in deep-sea hydrothermal vents. Physiological studies showed that these were adapted to deep-sea conditions. Several of these appear to be undescribed forms. Such fungi are important tools for biotechnology research. Raghukumar et al. (2008) isolated fungi and thraustochytrids for the first time from a shallow water hydrothermal vent site at the D. João de Castro Seamount (DJCS) located in the North Atlantic, between the Azorean islands São Miguel and Terceira lying on the Terceira Rift. Thrausto-chytrids isolated from this habitat had a high tolerance to heavy metals and secreted protease even under metal stress. Bass et al. (2007) have reported the dominance of yeasts in the deep sea. Singh et al. (2011) applied a metagenomics approach to detect fungi from the deep-sea sediments of the Central Indian Basin, using a multiple primer approach that included 18 S rDNA and ITS primers. The sea is also a window for 'virtual time travel', making it possible to culture fungi from a past of different environmental conditions. For example, Raghukumar et al. (2004) cultured fungi that had been presumably buried for nearly 420,000 years at about 365 cm below the sea floor at 5,000 m depth in the Central Indian Ocean. Such fungal 'dinosaurs' and 'wooly mammoths' might have their own uses.

Salt pans are an attractive source for extremophilic microorganisms. The high salinity in hypersaline environments exerts an osmotic effect and also adversely affects protein structure and enzyme function (Cooke and Whipps 1993). No obligate marine fungi have been detected so far in salt pans. However, other fungi, such as black yeasts, *Hortaea werneckii*, *Phaeotheca triangularis*, *Aureobasidium pullulans* and *Trimatostroma salinum* have been isolated from salt pans of 15–30% salinity (Zalar et al. 1999a, b; Gunde-Cimerman et al. 2000). Out of these, the black yeast and *Phaeotheca triangularis* appear to be obligate halophiles, while the rest are facultative halotolerant.

Yet another extreme environment for marine fungi are the anoxic habitats. Fungi are primarily aerobic heterotrophs and were thought to play a negligent role in the ecosystem processes of anoxic environments (Dighton 2003). However, many fungi were recently shown to possess metabolic adaptations to utilize nitrate and (or) nitrite as an alternative for oxygen (Shoun et al. 1992). Jebaraj and Raghukumar (2009) have shown that fungi isolated from anoxic marine waters of the Arabian Sea are capable of growth under oxygen-deficient conditions while performing anaerobic denitrification, a characteristic that is bound to interest biotechnologists.

14.3 Enzymes from Marine Fungi

Fungi produce a variety of extracellular enzymes, such as proteases, laccase, amylases, xylanases and cellulases that have found applications in all fields of life, including food, beverages, detergents and medicines (Table 14.1). Enzymes

Table 14.1 Enzymes from marine fungi with potential applications

Enzyme	Source of isolation	Classification of compound	Substrate	References
Laccase	Basidiomycetous fungi	Copper containing protein	Phenolic compounds	Raghukumar et al. (1999)
Manganese peroxidase	Basidiomycetous fungi	Heam protein	Phenolic compounds	Raghukumar et al. (1999)
Lignin peroxidase	Basidiomycetous fungi	Heam protein	Phenolic compounds	Raghukumar et al. (1999)
Alkaline Protease	*Aspergillus ustus*	Serine protease	Azocasein	Damare et al. (2006a)
Cellulase	Lignicolous mangrove fungi	–	Cellulose	Eriksson et al. (1990)
L-Glutaminase	*Beauveria bassiana*	–	L-Glutamine	Keerthi et al. (1999)
Xylanase	*Aspergillus niger*	Glycoside hydrolase	Paper pulp	Raghukumar et al. (2004)
Gelatinase	*Halosphaeria mediosetigera*	Metalloproteinase	Corn meal	Pisano et al. (1964)
Amylase	*Aspergillus flaviceps*	Glycoside hydrolase	Starch	Frolova et al. (2001)

obtained from marine fungi are likely to differ from those produced by terrestrial fungi because of their differences in taxonomic diversity and environmental adaptations (Abe et al. 2001, 2006; Damare 2007).

Most research on enzymes from marine fungi has focused on lignocellulases obtained from fungi in the coastal environment. Fungal degradation of lignin has attracted the attention of researchers because lignin-degrading enzymes have applications in using lignocellulose as a renewable resource for the production of paper products, feeds, chemicals and fuels (Kuhad et al. 1997). Fungi degrade lignin by production of the extracellular enzymes, lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. These enzymes degrade or modify not only lignin, but also several aromatic, recalcitrant environmental pollutants such as those that occur in crude oil wastes, textile effluents and organochloride agrochemicals which are a cause of serious environmental pollution (Mtui and Nakamura 2004; Kiiskinen et al. 2004). Marine mangrove fungi have proven to be an important source of lignocellulose degrading enzymes (Raghukumar et al. 1994; Grant et al. 1996; Pointing et al. 1998; Pointing and Hyde 2001). Pointing and Hyde (2001) suggested that most marine fungal strains capable of decay activity are likely to be soft-rot fungi, with relatively few capable of white-rot decay. Raghukumar et al. (1994) demonstrated the presence of laccase, xylanase and cellulase activities in several obligate marine as well as marine-derived fungi isolated from mangroves. Luo et al. (2005) have reported cellulolytic, xylanolytic and ligninolytic enzymes from fungi obtained from tropical and sub-tropical mangroves. Most of the fungi were ascomycetes, except for one basidiomycete, *Calathella mangrovei* and a mitosporic fungus, *Cirrenalia tropicalis*. A marine-derived fungus, designated NIOCC #312 produced all the three lignin degradative enzymes, namely LiP, MnP and laccase to varying extents when grown in sea water medium containing sugarcane bagasse fibres, pine and poplar wood shavings as carbon and nitrogen source (Raghukumar

et al. 1999). Yet another marine-derived, white-rot fungus isolated from decaying mangrove wood, designated NIOCC #2a produced enhanced levels of laccase in the presence of several phenolics and lignin-derivatives (D'Souza-Ticlo et al. 2006). The enzyme showed optimum activity at 70°C, with half-life for 90 min at 70°C. It was active in the presence of 1 mmol NaCl and was not inhibited by Pb, Fe, Ni, Li, Co and Cd at 1 mmol. Verma et al. (2010) reported laccase production in marine-derived asco- and basidiomycetes, and demonstrated decolorization and detoxification of raw textile mill effluents by these fungi.

Deep-sea fungi are likely to produce interesting enzymes. Thus, a deep-sea yeast isolated from the Japan Trench at a depth of 4,500–6,500 m, showed the presence of an endopolygalacturonase, active at 0–10°C with no loss in activity up to 100 MPa at 24°C (Abe et al. 2006). High level of superoxide dismutase activity was detected in a *Cryptococcus* strain isolated from deep-sea sediments (Abe et al. 2001). A deep-sea isolate of *Aspergillus ustus*, cultured from sediments of the Central Indian Basin from the depths of 5,100 m, produced a low-temperature active protease (Damare et al. 2006b). The enzyme had a broad pH range of 6–10, with an optimum at pH 9. The optimum temperature for protease activity was 45°C and approximately 10% of the activity was retained at 2°C. Its activity remained unaffected in the presence of 0.5 M NaCl, equivalent to seawater salinity of 29 psu (practical salinity units). Daniel et al. (2006) proposed that it might be possible to perform a reaction requiring high temperature at lower temperature under elevated hydrostatic pressure. If so, enzymes of marine microorganisms adapted to such conditions would be ideal candidates.

Enzymes degrading polysaccharides are of great interest (Schaumann and Weide 1990; Pointing et al. 1998). To increase the yield of desired products, glucanases are used for destroying the cellular walls of plants and microorganisms. Using these enzymes, the structure and biological role of glucans can be established. Highly purified b-1, 3-glucanase has been successfully employed for the enzyme transformation of laminaran into a biologically active glucan, translam (Zvyagintseva et al. 1995).

The Labyrinthulomycetes, by virtue of their ubiquitous and abundant occurrence in the oceans are considered to play an important role in mineralization processes and are, therefore, potential sources of useful enzymes (Raghukumar 2008b). Although the presence of several degradative enzymes have been described in these organisms, such studies have been sporadic and of preliminary nature (Sharma et al. 1994; Bongiorni et al. 2005; Raghukumar et al. 2010; Nagano et al. 2011). Salt-dependent alkaline lipase production in two *Thraustochytrium* isolates was reported recently (Kanchana et al. 2011). Enzymes from these fungi deserve more attention.

14.4   Bioremediation Using Marine Fungi

Terrestrial and freshwater microorganisms, particularly bacteria, are now extensively used to treat and remedy a number of polluted environments (Brar et al. 2006). Marine microorganisms in general, and fungi in particular, have not been studied adequately for such applications. A good example of the potentials of

marine-derived fungi for application in bioremediation of industrial pollutants is that of a ligninolytic marine-derived, mangrove fungus, designated NIOCC #2a (Verma et al. 2010, 2011). This fungus has been shown to decolorize and detoxify a wide variety of textile mill and molasses-based raw industrial effluents accompanied by reduction in COD and phenolics.

14.4.1   Hydrocarbon Degradation

The marine environment in many parts of the world suffers from chronic oil pollution (GESAMP 2007). Catastrophic oil pollutions such as the BP disaster in the Gulf of Mexico have often resulted in tremendous environmental damage. Marine fungi have not been adequately studied as candidates for bioremediation of marine oil spills. Ahearn and Meyers (1972) were amongst the first to discuss hydrocarbon degradation by marine fungi. Some of the most important and ecologically damaging components of oil pollution are the polycyclic aromatic hydrocarbons (PAHs). Ligninolytic fungi are interesting from this point of view, because they can oxidize PAHs by producing a non-specific enzymatic extracellular complex that is normally used for lignin depolymerization. These lignin-degrading enzymes are comprised of lignin-peroxidase (LiP), MnP and laccase (Hamman 2004; Peng et al. 2008). White-rot fungi possess powerful extracellular lignin-degrading enzymatic systems that can degrade a broad variety of different pollutants and have been extensively studied for this purpose (Junghanns et al. 2005; Sette et al. 2008). A novel PAH metabolic pathway described in fungi involves hydroxylation by cytochrome P-450 monooxygenase through a sequence of reactions that is similar to the reactions that are involved in mammalian metabolism (Capotorti et al. 2004). This pathway is shared by many non-ligninolytic fungi that could effectively degrade PAHs (Krivobok et al. 1998; Ravelet et al. 2000). The use of marine fungi in the bioremediation of polluted marine environments may be achieved because of their tolerance to saline conditions. Passarini et al. (2011) have recently reported that a marine *Aspergillus sclerotiorum* CBMAI 849 (isolated from cnidarians) was able to reduce 99.7% pyrene and 76.6% benzo[a] pyrene after 8 and 16 days, respectively. They also reported substantial amounts of benzo[a]pyrene (>50.0%) depletion by a *Mucor racemosus* CBMAI 847. They attributed these reductions showed by *A. sclerotiorum* CBMAI 849 and *M. racemosus* CBMAI 847 to their ability to metabolize pyrene to the corresponding pyrenylsulfate and benzo[a]pyrene to benzo[a]pyrenylsulfate, suggesting that the mechanism of hydroxylation is mediated by a cytochrome P-450 monooxygenase, followed by conjugation with sulfate ions.

   Cooney et al. (1993) have reported obligately marine fungi growing in artificial sea water with single hydrocarbon as their sole source of carbon and energy. They observed that the unsaturated compound 1, 1,4-tetradecadiene and the methyl-branched compound pristane were used by several fungi, while none of the fungi used aromatic hydrocarbons as their sole source of carbon. The isolates reported by

them were *Corollospora lacera*, *C. maritima* and *Lulworthia* sp. They found that four of the five fungi examined form microbodies when the fungi were grown on hydrocarbons as sole C-source but not when they were grown on glucose.

Asphaltenes constitute the most recalcitrant components of oil. Certain parts of the world's beaches, such as those in Goa, India, are polluted by tarballs formed from asphaltenes on a regular, annual basis. Organisms that are capable of degrading these are of tremendous biotechnological interest. Raikar et al. (2001) reported extensive degradation of tarballs by thraustochytrids isolated from coastal waters and sediments of Goa coast. They found that there was a 71% reduction of tarball contents by the thraustochytrids inoculated to tarball-enriched sediment after a month's incubation. Up to 30% of tarballs added to peptone broth were degraded in 7 days. This showed for the first time that thraustochytrids play a definite role in tarball degradation in sediments.

14.4.2 *Heavy Metals*

Various toxic heavy metals are released into the environment through natural as well as anthropogenic sources (Shukla and Singhal 1984). These cause serious environmental and health problems, because of their bioaccumulation in the food web. Heavy metals have been reported from the sea sediments (Karageorgis et al. 2005), resulting from river run off and anthropogenic activities. Conventional physicochemical treatment technologies (Dermont et al. 2008) become less effective and more expensive when metal concentrations are in the higher range (1–100 ppm). Many biological sources including marine algae, fungi, yeast and bacteria remove toxic metals from the environment through adsorption as well as their metabolic activity (Davis et al. 2003; Malik 2004; Kadukova and Vircikova 2005).

The potential of living and dead fungal biomass has been recognized for the removal of heavy metals through absorption (Bishnoi and Garima 2005). The latter seems to be a preferred alternative due to the absence of toxicity limitations, absence of requirements of growth media and nutrients in the treatment and also due to the fact that the biosorbed metals can be recovered and the biomass reused. There are not many reports available on the use of marine fungi for biosorption of metals in bioremediation application. Babich and Stotzky (1983) reported that the growth of some marine fungi exposed to nickel was less depressed in the presence of magnesium compared to their growth in the presence of nickel alone. Hicks and Newell (1984) found that exposing *Phaeospharia typharum*, a salt marsh fungus, to mercury at metal concentration of 0.74 mg L^{-1} resulted in no significant change in growth as compared to the cultures grown in the absence of mercury. Taboski et al. (2005) discovered the effect of lead and cadmium on the growth of two species of marine fungi, *Corollospora lacera* and *Monodictys pelagic*. A marine fungus *Aspergillus candidus* has been shown to grow successfully in the presence of arsenic (25 and 50 mg L^{-1}). The fungus decreased the amounts of the metal by means of bioaccumulation. Raghukumar et al. (2008) have shown the capability of

thraustochytrids from shallow water hydrothermal vents to withstand high levels of heavy metals, as described in Sect. 14.2. A metal-tolerant yeast *Lodderomyces elongisporus* was isolated from metal-contaminated site and found to show considerable tolerance towards various heavy metals (Rehman et al. 2008). Production of the antioxidant enzyme, superoxide dismutase was found to increase as a defensive mechanism against high concentration of $CuSO_4$ in *Cryptococcus* sp., isolated from deep-sea sediments of the Japan Trench (Abe et al. 2001). Marine fungi deserve to be studied in greater detail for applications concerning heavy metal removal.

14.5 Biosurfactants and Polysaccharides from Marine Fungi

Biosurfactants were used as hydrocarbon dissolution agents during the late 1960s, but their applications have been greatly extended recently as an improved alternative to chemical surfactants (carboxylates, sulphonates and sulphate acid esters), especially in food, pharmaceutical and oil industry, because of their diversity, environmentally friendly nature, possibility of large-scale production, selectivity, performance under extreme conditions and potential applications in environmental protection (Karanth et al. 1999; Banat et al. 2000; Rahman et al. 2002).

A lipid–carbohydrate–protein complex from tropical marine yeast *Yarrowia lipolytica* was extracted from cell wall (Zinjarde and Pant 2002). Recently, a biosurfactant (novel glycolipid) producing yeast was isolated from *Calyptogena soyoae* (deep-sea cold-seep clam) in the deep sea (Konishi et al. 2010). This yeast showed abundant production of glycolipid biosurfactants at around 30 g L^{-1} for 96 h. The isolation of novel forms of such biosurfactants may prove promising for degradation of complex hydrocarbons contaminating marine environment.

An endosymbiotic isolate of *Aspergillus ustus* (MSF3) from the marine sponge *Fasciospongia cavernosa*, collected from the peninsular coast of India, was reported to produce high amounts of biosurfactants (Kiran et al. 2009). The biosurfactant produced by MSF3 was partially characterized as glycoprotein. The partially purified biosurfactant showed a broad spectrum of antimicrobial activity. It was also suggested that the biosurfactant could be used for the microbially enhanced oil recovery process. Sun et al. (2009) have reported 3 different polysaccharides PS1-1, PS1-2 and PS2-1 from a marine fungus *Penicillium* sp. F23-2. All three polysaccharides primarily consisted of mannose with variable amounts of glucose, whereas their glucoronic acid contents, molecular weights and glycosidic linkage patterns were different. All the three polysaccharides showed good antioxidant properties, especially scavenging abilities on superoxide radicals and hydroxyl radicals.

14.5.1 Production of Omega-3 Polyunsaturated Fatty Acids

The omega-3 polyunsaturated fatty acid (ω-3 PUFA), docosahexaenoic acid (DHA; 22:6ω-3) is important in human health and aquaculture (Horrocks and Yeo 1999).

While fish oil has been the commercial source of DHA for many years, microbial DHA from thraustochytrids has now captured a large market in the world, both as an adult and infant nutritional supplement and as ingredients in aquaculture feeds (Fan and Chen 2007; Raghukumar 2008b). Martek Biosciences, USA, are the major commercial manufacturers of DHA from *Schizochytrium*. Commercial processes have been improved and optimized to yield high biomass in excess of 100 g dry weight of the organism per litre culture, of which at least 50% constitute lipids, and a further 25% of this as DHA. There is a tremendous international interest in obtaining better strains, improved processes and also the molecular biology of the fatty acid synthesis pathways of thraustochytrids to enable the development of recombinant methods to produce DHA (Lippmeier et al. 2009). Thraustochytrids have now become very important organisms for marine biotechnology, in addition to their usefulness for DHA production. Thus, carotenoid and enzyme production of these organisms, besides synthesis of various essential fatty acids other than DHA are attracting attention.

14.6   Secondary Metabolites from Marine Fungi

Microorganisms have historically been a rich source of leads for pharmaceutical development, particularly for antibiotics. Over 20,000 microbial metabolites have been described, most of which were isolated from the terrestrial environment. The trend of tapering off in drug discovery from terrestrial sources has led to exploration of marine organisms for the purpose. Marine fungi have become an important component of this search.

As early as 1968, a marine isolate of the fungus *Cephalosporium acremonium* obtained from the sea near a sewage outfall of the coast of Sardina was reported to produce a number of antibiotic substances (Godzeski et al. 1968). A penicillinase-sensitive antibiotic substance named 'Antibiotic N', active against gram-negative bacteria, was isolated from this source. Szaniszlo et al. (1968) reported production of capsular polysaccharide by marine filamentous fungi. In recent years, marine fungi have proven to be a rich and promising source of novel anticancer, antibacterial, antiplasmodial, anti-inflammatory and antiviral agents and many new metabolites have been isolated from them (Kobayashi and Ishibashi 1993; Davidson 1995; Liberra and Lindequist 1995; Daferner et al. 2002; Bhadury et al. 2006; Ebel 2006). Bhadury et al. (2006) have reviewed novel antibacterial, antiviral, antiprotozoal compounds isolated from marine-derived fungi and their future commercial exploitation as drugs, using metabolic engineering and post-genomics approaches.

Some of the secondary metabolites produced by marine fungi and their characteristics are listed in Table 14.2. Amongst the fungi from different marine environments, marine mangrove fungi have proved to be most significant source of new bioactive compounds (Lin et al. 2001).

More than 700 unique molecular structures had been discovered from marine fungi by the end of 2008. These are divisible into most of the major classes of natural products, mirroring the situation for cultured terrestrial-derived fungi.

Ebel (2006) has provided strong evidence that 'marine-derived' fungi, although taxonomically belonging to terrestrial fungi, are an interesting ecological group that produce unique compounds. In his review, he reported 240 new compounds described from marine-derived fungi described between 2002 and 2004. A variety of secondary metabolites not found in terrestrial fungi possibly act as a chemical defence, enabling marine-derived fungi to survive competition with native microorganisms (Fenical and Jensen 1993; Gallo et al. 2004). It is believed that the use of seawater for isolation and growth of marine-derived terrestrial species enhances recovery of fungi that yield secondary metabolites, as compared to the use of media prepared in distilled water. Compounds such as the neomangicols, which are structurally unprecedented halogenated sesterterpenes that possess cytotoxic and antibacterial properties and various metabolites that exhibit cytotoxicity, anti-inflammatory and antifungal activities, as well as inhibitors of viral topoisomerase and protein tyrosine phosphatase enzymes have been cited as examples (Alvi et al. 1998; Toske et al. 1998; Schlingmann et al. 1998; Hwang et al. 1999; Belofsky et al. 2000; Rowley et al. 2003). One possible example is *Emericella variecolor* (anamorph: *Aspergillus variecolor*). Strains of this fungus have been the source of a variety of natural products, mainly sesterterpenes with unusual polycyclic skeletons, for example, astellatol (Sadler and Simpson 1989) or variecolin (Hensens et al. 1991), and prenylated xanthones (Chexal et al. 1974; Kawahara et al. 1988). Brauers et al. (2000) found a remarkable diversity of secondary metabolites in the preliminary HPLC screening produced by a strain of *E. variecolor* isolated from the sponge *Haliclona valliculata*. Detailed chemical analysis revealed evariquinone (1, 2, 3- trihydroxy-6-methyl-8-methoxyanthraquinone), which is a new anthraquinone, besides the known 1,2,3,8-tetrahydroxy-6-methylan-thraquinone (7-hydroxyemodin). Other new compounds were prenylxanthone and isoemericellin, accompanied by the biosynthetically related known metabolite shamixanthone. Furthermore, the C-glycosidic depside stromemycin was identified and patented for its metalloproteinase-inhibiting activity (Hopmann et al. 2001).

Coral reefs are a rich source of fungi that produce secondary metabolites. These may be produced as a result of association with other biotic components present in the reefs. Namikoshi et al. (2000) have reported two new compounds, paeci-lospirone and phomopsidin, and seven known compounds, chaetoglobosin A, griseofulvin, fusarielin A, fusapyrone, deoxyfusapyrone, verrucarins J and L ace-tate from marine-derived fungi collected in tropical and sub-tropical coral reef environments. An anticancer compound, Sorbicillactone A has been isolated from a strain of *Penicillium chrysogenum* cultured from the Mediterranean sponge *Ircinia fasciculate*. This compound exhibited a highly selective activity against the murine leukemic lymphoblast cell line L5178y, and has antiviral and neuro-protective properties. Owing to its excellent antileukemic properties, this com-pound has also qualified for human trials (Bringmann et al. 2007; Bhatnagar and Kim 2010).

Table 14.2 Secondary metabolites produced by marine fungi

Secondary metabolite	Source of isolation and habitat	Type of compound	Activity	References
Fusidic acid	*Stilbella aciculosa*	Steroid	Antibacterial	Kuznetsova et al. (2001)
Aspergillitine	*Aspergillus versicolor* (isolated from the sponge *Xestospongia exigua*)	Chromone-derivative	Antibacterial	Lin et al. (2003)
Guisinol	*Emericella unguis* (obtained from a mollusc)	Depside	Antibacterial	Nielsen et al. (1999)
Penicillic acid	*Aspergillus* sp. (Coral reef of South china Sea)	–	Mycotoxin, potential carcinogen and mutagen	Hou-jin et al. (2010)
Varixanthone	*Emericella variecolor* (sponge derived)	–	Antibacterial	Malstrom et al. (2002)
7-deacetoxyyanuthone A	*Penicillium* sp.	Polyoxygenatedfarnesyl cyclohexenones	In vitro activity against methicillin and multidrug-resistant *S. aureus*	Li et al. (2003)
Microsphaeropsin	*Microsphaeropsis* sp. (derived from the sponge *Myxilla incrustans*)	Eremophilane derivative	Antifungal	Holler (1999)
Penicitides A	*Penicillium chrysogenum* (endophytic fungus from a marine red algal species of the genus *Laurencia*)	Polyketide derivatives	Inhibits pathogenic fungus *A. brassicae*, human hepatocellular liver carcinoma cell line	Gao et al. (2011)
Penicimonoterpene	*Penicillium chrysogenum* (endophytic fungus from a marine red algal species of the genus *Laurencia*)	Monoterpene derivative	Inhibits pathogenic fungus *A. brassicae*	Gao et al. (2011)
Zopfiellamides A and B	*Zopfiella latipes*	Pyrrolidinone derivative	Inhibits *Nematospora coryli* and *Saccharomyces cerevisiae*	Daferner et al. (2002)
Seragikinone A	Unidentified marine fungus (derived from the Rhodophyte *Ceratodictyon spongiosum*)	Anthracycline-related pentacyclic compound	Weak antifungal activity against *C. albicans*	Shigemori et al. (1999)
	Keissleriella sp. YS4108	–		Liu et al. (2002)

3,6,8-trihydroxy-3-[3,5-dimethyl-2-oxo-3(E)-heptenyl]-2,3-dihydronaphthalen-1(4H)-one		Inhibits *C. albicans*, *T. rubrum* and *A. niger*	
Enniatin B	*Fusarium* sp.	Exhibits antibiotic activity against *S. aureus* and vancomycin-resistant enterococci	Jiang et al. (2002)
Cyclodepsipeptide			

Metabolites exploited in pharmaceutical and agricultural industries are widespread among endophytic fungi (Petrini et al. 1992). These fungi produce an extraordinary diversity of metabolites some of which have therapeutic value as novel antibiotics or anticancer chemicals (Gunatilaka 2006; Suryanarayanan et al. 2009; Weber 2009). Thirunavukkarasu et al. (2011) have recently reported production of L-asparaginase from a *Fusarium* sp. isolated from the thallus of *Sargassum wightii* and a sterile mycelial form isolated from the thallus of *Chaetomorpha* sp. showed maximum activity of the enzyme.

With more access to the deeper parts of oceans, the information about the fungi from deep sea is increasing rapidly and the forms obtained would prove very good source of novel biomolecules.

Most of the herbicides in use today are chemical moieties. Decreasing chemical heterogeneity of herbicides targeting fewer mechanisms of action is increasing the prevalence of herbicide resistance (Lein et al. 2004). Motti et al. (2007) screened extracts of 449 marine-derived fungi for inhibition of pyruvate phosphate dikinase (PPDK), which hinders growth in C4 plants. This enzyme occurs primarily in plants, but not reported so far from vertebrate or invertebrate animals, with the exception of protozoan, *Giardia*, potentially minimizing the risk of PPDK inhibitors exhibiting adverse toxicological effects. They isolated unguinol, a known compound and found it to inhibit PPDK via a novel mechanism of action which also translates to an herbicidal effect on whole plants.

14.7   Future Directions

Marine mycotechnology has already revealed much promise. Interesting enzymes, novel metabolic properties and secondary metabolites have been discovered from obligate and marine-derived fungi from a variety of marine habitats, ranging from coastal to oceanic and shallow water to the deep sea. There is scope to examine them for many other useful compounds, such as extracellular polysaccharides. The premise that genetic diversity based on taxonomy and adaptations to environmental conditions is the source of novel applications needs to be examined more thoroughly in future for marine fungi. Further studies on the biology of marine fungi may reveal interesting physiological and biochemical characteristics that will be useful in novel biotechnology applications. Culturing of hitherto uncultured marine fungi and more detailed studies on the physiology and biochemistry of rare and interesting ones will truly lay a foundation for the marine fungal technology.

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Chapter 15
Degradation of Phthalate Esters by *Fusarium* sp. DMT-5-3 and *Trichosporon* sp. DMI-5-1 Isolated from Mangrove Sediments

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Abstract Phthalate esters (PAEs) are important industrial compounds mainly used as plasticizers to increase flexibility and softness of plastic products. PAEs are of major concern because of their widespread use, ubiquity in the environment, and endocrine-disrupting toxicity.

In this study, two fungal strains, *Fusarium* sp. DMT-5-3 and *Trichosporon* sp. DMI-5-1 which had the capability to degrade dimethyl phthalate esters (DMPEs), were isolated from mangrove sediments in the Futian Nature Reserve of Shenzhen, China, by enrichment culture technique. These fungi were identified on the basis of spore morphology and molecular typing using 18S rDNA sequence. Comparative investigations on the biodegradation of three isomers of DMPEs, namely dimethyl phthalate (DMP), dimethyl isophthalate (DMI), and dimethyl terephthalate (DMT), were carried out with these two fungi. It was found that both fungi could not completely mineralize DMPEs but transform them to the respective monomethyl phthalate or phthalate acid. Biochemical degradation pathways for different DMPE isomers by both fungi were different. Both fungi could transform DMT to monomethyl terephthalate (MMT) and further to terephthalic acid (TA) by stepwise hydrolysis of two ester bonds. However, they could only carry out one-step ester hydrolysis to transform DMI to monomethyl isophthalate (MMI). Further metabolism of MMI did not proceed. Only *Trichosporon* sp. was able to transform DMP to monomethyl phthalate (MMP) but not *Fusarium* sp. The optimal pH for DMI and DMT degradation by *Fusarium* sp. was 6.0 and 4.5, respectively, whereas for *Trichosporon* sp., the optimal pH for the degradation of all the three DMPE isomers was at 6.0. These results suggest that the fungal esterases responsible for hydrolysis of the two ester bonds of PAEs are highly substrate specific.

15.1 What Do We Know About Phthalate Esters and Their Microbial Degradation?

15.1.1 World-Wide Consumption

Phthalate esters (PAEs), or phthalates, are industrially synthetic compounds principally functioning as plasticizers to provide flexibility, workability, and distensibility for consumer products such as building materials, home furnishings, clothing, and packing of food and medicine (Staples et al. 1997a; Cartwright et al. 2000; Wang et al. 2004; Xu et al. 2005a, b). These are also used as additives in the manufacture of paints, adhesives, cardboard, lubricants, and fragrances (Gu et al. 2005). *Ortho*-PAEs alone account for 80% of plasticizers in the world (Baikova et al. 1999; Wang et al. 2003). As plasticizers, PAEs comprise as much as 30% of the polymer matrices in plastic products (Wang et al. 2003). The consumption of PAEs reaches millions of tonnes world-wide every year (Harris and Sumpter 2001). Dimethyl phthalate esters (DMPEs), with the simplest structures amongst PAEs, have three isomers, namely dimethyl phthalate (*ortho*-DMP; *ortho*-arrangement of the two carboxyl groups), dimethyl isophthalate (DMI; *meta*-), and dimethyl terephthalate (DMT; *para*-).

DMP is used as a plasticizer in cellulose ester-based plastics including cellulose acetate and cellulose butyrate (Staples et al. 1997a). DMI is mainly used to synthesize sodium dimethyl isophthalate-5-sulfonate to improve the dyeing properties of polyesters (Gu et al. 2005). DMT is generally applied in the production of polyesters, such as polyethylene terephthalate (PET) and polytrimethylene terephthalate (PTT).

The phthalate plasticizers do not bind covalently to the plastic resin and thus are able to migrate into the environment during manufacture, usage, and disposal (Bauer and Herrmann 1997; Staples et al. 1997a). Due to the large world-wide consumption of PAEs, they have been widely detected in both aquatic and terrestrial environments, including agricultural soils (Xu et al. 2008), river water and sediments (Tan 1995; Long et al. 1998), marine water and sediments (Peterson and Freeman 1982; Xie et al. 2005), sewage sludge and effluents (Fromme et al. 2002), and landfill leachates (Paxéus 2000; Jonsson et al. 2003).

15.1.1.1 Toxicity

Toxicity of PAEs has attracted increasing attention due to their widespread use and ubiquitous occurrence in the environment. Known as endocrine-disrupting chemicals, PAEs may affect the normal function of the reproductive system and development of humans and animals at very low concentrations (Jobling et al. 1995; Colón et al. 2000; Gu et al. 2005; Xu et al. 2005b). Some PAEs, such as di-2-ethylhexylphthalate (DEHP) and di-isononyl phthalate (DINP), are suspected to be mutagens and carcinogens (David and Gans 2003; Chang et al. 2004; Xu et al. 2006). In addition, several low molecular weight PAEs (with alkyl side chains of less than six carbon atoms) have been demonstrated to have acute or chronic toxicity on aquatic organisms (microorganisms, algae, aquatic invertebrates, and fish) at concentrations below their water solubility (Staples et al. 1997b). As a result, six PAEs, including DMP, have been designated as priority pollutants by the United States Environmental Protection Agency due to their endocrine-disrupting activity (Gu et al. 2005).

15.1.2 Bacterial Degradation

Microbial degradation is suggested to be the principal mechanism for removal of PAEs in the environment (Staples et al. 1997a). The biochemical pathways and molecular mechanism of degradation of PAEs by bacteria have been well documented. A large number of bacterial species capable of degrading PAEs were isolated from activated sludge, mangroves, soils, rivers, and the marine environment (Chatterjee and Dutta 2003; Chang et al. 2004; Li et al. 2005a, b; Nalli et al. 2005; Xu et al. 2005b; Wang and Gu 2006a, b; Fang et al. 2007). It was demonstrated in some studies that construction of a bacterial consortia was more efficient to completely mineralize PAEs than single species (Vega and Bastide 2003; Wang et al. 2003; Li and Gu 2007). Degradation of PAEs by bacteria is

initiated by esterases which catalyze stepwise hydrolysis of both carboxylic ester linkages to form phthalate monoester and then phthalic acid (PA) (Gu et al. 2005; Li et al. 2005b; Li and Gu 2006). Two distinct pathways have been elucidated for the transformation of PA to protocatechuate by Gram-positive and Gram-negative bacteria, respectively (Chang and Zylstra 1998; Stingley et al. 2004). Protocatechuate is further metabolized by *ortho*-cleavage pathway or *meta*-cleavage pathway (Keyser et al. 1976; Eaton and Ribbons 1982; Williams et al. 1992; Harwood and Parales 1996; Eaton 2001). Some functional operons involved in the metabolism of PA have been identified and characterized in *Arthrobacter keyseri* 12B, *Mycobacterium vanbaalenii* PYR-1, *Burkholderia cepacia* DBO1, *Pseudomonas putida* NMH102-2, and other bacterial strains (Chang and Zylstra 1998; Eaton 2001; Stingley et al. 2004).

15.1.3 Fungal Degradation

On the other hand, degradation of PAEs by fungi has received surprisingly little attention. The potential role of fungi in degradation of a wide range of recalcitrant compounds, including polycyclic aromatic hydrocarbons (PAHs), benzene-toluene-ethylbenzene-xylenes (BTEX), chlorophenols, polychlorinated biphenyl, munitions waste, and pesticides has been demonstrated (Tortella et al. 2005). The limited reports for fungal degradation of PAEs are mainly grouped into two categories: degradation of PAEs by pure fungal cultures (Table 15.1) (Sivamurthy et al. 1991; Ganji et al. 1995; Pradeepkumar and Karegoudar 2000; Lee et al. 2004; Hwang et al. 2008) and by purified fungal enzymes (Table 15.2) (Kim et al. 2002, 2003, 2005; Kim and Lee 2005; Ahn et al. 2006). The role of fungi on the environmental fate of PAEs remains largely unknown.

Most reports for degradation of PAEs by pure fungal cultures focused on two functional groups of filamentous fungi, namely the ligninolytic fungi (also commonly referred as the white-rot fungi) and non-ligninolytic fungi, respectively.

Many ligninolytic fungi have shown the ability to metabolize PAEs. The fungus *Sclerotium rolfsii* was able to transform DMT to terephthalic acid (TA) through the formation of monomethyl phthalate (MMT) as the intermediate. However, TA was the dead-end product and further metabolism was not observed. Hwang et al. (2008) investigated the degradation of three PAEs (DMP, diethyl phthalate (DEP), and butyl benzyl phthalate (BBP)) by several white-rot fungi, including *Irpex lacteus*, *Merullius tremellosus*, *Polyporus brumalis*, *Trametes versicolor*, *Schizophyllum commune*, *Formitella fraxinea*, *Pleurotus ostreatus*, and *T. versicolor* MrP1, MrP13, and MnP26 (transformant of the Mn-repressed peroxidase gene (*MrP*) and Mn-dependent peroxidase gene (*MnP*) of *T. versicolor*). All these fungi were able to degrade the three PAEs and *P. ostreatus* showed the highest degradation rates among the tested fungi. Degradation of BBP by *P. ostreatus* showed that fungal mycelium was much more efficient to degrade the substrate than the supernatant of the liquid culture, indicating that the initial metabolism of BBP by

Table 15.1 Degradation of PAEs by fungal cultures

Fungal species	Substrates[a]	Degradation	References
Ligninolytic fungi			
Sclerotium rolfsii	DMT	Incomplete	Sivamurthy et al. (1991)
Irpex lacteus	DMP, DEP, BBP	NM	Hwang et al. (2008)
Merullius tremellosus	DMP, DEP, BBP	NM	Hwang et al. (2008)
Polyporus brumalis	DMP, DEP, BBP	NM	Hwang et al. (2008)
Trametes versicolor	DMP, DEP, BBP	NM	Hwang et al. (2008)
Schizophyllum commune	DMP, DEP, BBP	NM	Hwang et al. (2008)
Formitella fraxinea	DMP, DEP, BBP	NM	Hwang et al. (2008)
Pleurotus ostreatus	DMP, DEP, BBP	NM	Hwang et al. (2008)
Phanerochaete chrysosporium	DBP	NM	Lee et al. (2004)
Trametes versicolor	DBP	NM	Lee et al. (2004)
Daldinia concentrica	DBP	Incomplete	Lee et al. (2004)
Polyporus brumalis	DBP	Incomplete	Lee et al. (2007)
Non-ligninolytic fungi			
Aspergillus oryzae	DEHP	NM	Chai et al. (2008)
Aspergillus sydowii	DEHP	NM	Chai et al. (2008)
Aspergillus ustus	DEHP	NM	Chai et al. (2008)
Fusarium graminearum	DEHP	NM	Chai et al. (2008)
Fusarium morniforme	DEHP	NM	Chai et al. (2008)
Fusarium sporotrichioides	DEHP	Incomplete	Chai et al. (2008)
Penicillium citrinum	DEHP	NM	Chai et al. (2008)
Penicillium expansum	DEHP	NM	Chai et al. (2008)
Penicillium frequentans	DEHP	NM	Chai et al. (2008)
Curvularia lunata	DEHP	NM	Chai et al. (2008)
Trichoderma viride	DEHP	NM	Chai et al. (2008)
Rhizopus stolonifer	DEHP	NM	Chai et al. (2008)
Aspergillus niger	DMP	Complete	Pradeepkumar and Karegoudar (2000)
Aspergillus niger	DMT	Complete	Ganji et al. (1995)
Yeasts			
Rhodotorula rubra	DOP, DOTP	Complete	Gartshore et al. (2003)
Saccharomyces cerevisiae	DEP, DBP, BBP	NM	Begum et al. (2003)

NM not mentioned

[a]Abbreviations are explained in the text

P. ostreatus may be catalyzed by mycelium-associated enzymes or intracellular enzymes rather than extracellular enzymes. Lee et al. (2004) reported that *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Daldinia concentrica* were able to completely degrade dibutyl phthalates (DBP) with the formation of a large number of products, including *o*-phenyl acetic acid, isobenzofuranone, *O*-anisic acid, phenylethyl alcohol, di-butyl-4-mehtoxy phenol and 1,2-benzenedicarboxylic acid, diisooctyl ester. Another study on the degradation of DBP by *Polyporus brumalis* recorded that DBP was metabolized to phthalic acid anhydride (PAA) through trans-esterification and de-esterification pathways with DEP and mono-butyl phthalate

Table 15.2 Enzymatic degradation of several PAEs by two purified fungal enzymes, *Fusarium oxysporum* f. sp. *pisi* cutinase and *Candida cylindracea* esterase

PAEs	Enzymes	Degradation rate	Degradation products	Toxic effects of degradation products	References
Di-2-ethylhexyl-phthalate (DEHP)	Cutinase	Fast	1,3-Isobenzofurandione (IBF)	Non-toxic	Kim et al. (2003)
	Esterase	Slow	1,3-Isobenzofurandione (IBF), unidentified compound (X)	Toxic hazard, causing oxidative stress and damage to protein synthesis	
Dipropyl phthalate (DPrP)	Cutinase	Fast	1,3-Isobenzofurandione (IBF)	Non-toxic	Kim et al. (2005)
	Esterase	Slow	1,3-Isobenzofurandione (IBF), propyl methyl phthalate (PrMP）	Toxic hazard, causing oxidative stress and damage to protein synthesis	
Dipentyl phthalate (DPeP)	Cutinase	Fast	1,3-Isobenzofurandione (IBF)	Non-toxic	Ahn et al. (2006)
	Esterase	Slow	1,3-Isobenzofurandione (IBF) , pentyl methyl phthalate (PeMP)	Toxic hazard, causing oxidative stress and damage to protein synthesis	
Butyl Benzyl phthalate (BBP)	Cutinase	Fast	1,3-Isobenzofurandione (IBF)	Non-toxic	Kim et al. (2002)
	Esterase	Slow	1,3-Isobenzofurandione (IBF), butyl methyl phthalate (BMP)	Toxic hazard, causing oxidative stress and damage to protein synthesis	
Dibutyl phthalate (DBP)	Cutinase	Fast	1,3-Isobenzofurandione (IBF)	Non-toxic	Kim and Lee (2005)
	Esterase	Slow	1,3-Isobenzofurandione (IBF), butyl methyl phthalate (BMP)	Toxic hazard, causing oxidative stress and damage to protein synthesis	

(MBuP) as intermediates, respectively (Lee et al. 2007). At the same time, some derivates of PAA were also detected as by-products in trace amounts, including α-hydroxyphenylacetic acid, benzyl alcohol, and *o*-hydroxyphenylacetic acid.

Degradation of PAEs by non-ligninolytic fungi has also been documented. Chai et al. (2008) reported the degradation of DEHP by 14 non-ligninolytic fungi, including *Aspergillus oryzae*, *A. sydowii*, *A. ustus*, *Fusarium graminearum*, *F. morniforme*, *F. sporotrichioides*, *Penicillium citrinum*, *P. expansum*, *P. frequentans*, *Curvularia lunata*, *Trichoderma viride*, and *Rhizopus stolonifer* species. All these fungal strains were able to metabolize DEHP. *Fusarium* species demonstrated the highest degradation efficiencies among the tested fungi. Degradation products of DEHP by *F. sporotrichioides* were identified as *o*-phthalic acid, 2-ethylhexanol, and 2-ethylhexanoic acid. In addition, degradation of DMPE isomers was observed in *Aspergillus niger*. Pradeepkumar and Karegoudar (2000) reported that *A. niger* was capable of completely metabolizing DMP through MMP, PA, and protocatechuate. Protocatechuate was further oxidized via *ortho*-cleavage pathway. Another study investigated the degradation of DMT by *A. niger* (Ganji et al. 1995). DMT was completely metabolized by the fungus with the formation of MMT, TA, and protocatechuate as the intermediates. The aromatic metabolite, protocatechuate, was further metabolized by *A. niger* via *meta*-cleavage pathway in this report, totally different from the study of DMP degradation reported by Pradeepkumar and Karegoudar (2000), in which protocatechuate was degraded by the *ortho*-cleavage pathway.

Compared to filamentous fungi, degradation of PAEs by yeasts has been largely overlooked; only limited information is available. The yeast *Rhodotorula rubra* could completely mineralize bis-2-ethylhexyl adipate (B(EH)A), di-octyl phthalate (DOP), and dioctyl terephthalate (DOTP) while partially transformed dipropylene glycol dibenzoate (D(PG)DB) and diethylene glycol dibenzoate (D(EG)DB) (Gartshore et al. 2003). Incomplete degradation of two dibenzoate plasticizers, D(PG)DB and D(EG)DB, resulted in the production of two toxic metabolites, dipropylene glycol monobenzoate and diethylene glycol monobenzoate, respectively. By contrast, no metabolites were observed and the toxicity of media did not increase during the degradation of B(EH)A, DOP, and DOTP by *R. rubra*. It should be noted that glucose was required as a co-substrate to degrade PAEs by this yeast because PAEs could not support substantial growth of the yeast when serving as the sole source of carbon and energy. In addition, *Saccharomyces cerevisiae* also displayed the capability to degrade DEP, DBP, and BBP (Begum et al. 2003), following an efficiency order of DBP > BBP > DEP.

Other studies focused on the degradation of PAEs using purified fungal enzymes. A Korean research group undertook comparative investigations on enzymatic degradation of PAEs using two purified fungal enzymes, *Fusarium oxysporum* f. sp. *pisi* cutinase and *Candida cylindracea* esterase (Kim et al. 2002, 2003, 2005; Kim and Lee 2005; Ahn et al. 2006). They compared the degradation efficiency of these two enzymes on several PAEs, including DBP, BBP, DEHP, di-*n*-propyl phthalate (DPrP), and di-*n*-pentyl phthalate (DPeP), and obtained similar results. Degradation of PAEs by fungal cutinase had some advantages

over yeast esterase: (1) fungal cutinase showed high stability in ester-hydrolytic activity and was able to degrade PAEs much faster than the yeast esterase, (2) enzymatic degradation products of PAEs by fungal cutinase, mainly 1,3-isobenzo-furandione (IBF) were non-toxic, whereas the hydrolysis of PAEs (such as BBP and DBP, DPrP, DPeP, and DEHP) by yeast esterase produced toxic compounds, including IBF, butyl methyl phthalate (BMP), propyl methyl phthalate (PrMP), pentyl methyl phthalate (PeMP), and an unidentified compound (X) which could lead to oxidative stress and damage to protein synthesis in bacteria.

15.1.4 Bacterial Degradation of PAEs in Mangrove Sediments

Mangrove ecosystems, commonly found in the inter-tidal estuarine zone of tropical and subtropical regions, are preferential sinks of man-made pollutants from fresh waters as well as tidal waters (Tam 2006). Mangrove microorganisms may play an important role in the degradation of recalcitrant pollutants; examples of bacteria isolated from mangrove sediments capable of completely mineralizing PAEs are *Pseudomonas fluorescens* B-1, *Pasturella multocida* Sa, and *Sphingomonas paucimobilis* Sy (Li et al. 2005b; Xu et al. 2005b, 2006; Li and Gu 2006). Others such as *Rhodococcus ruber* Sa exhibited only partial degradation (Li et al. 2005a). Li and Gu (2007) also reported complete degradation of DMI by the cooperation between two mangrove sediment bacteria *Klebsiella oxytoca* Sc and *Methylobacterium mesophilicum* Sr.

15.1.5 Investigation on Fungal Degradation of PAEs in Mangrove Sediments

Very few reports are available on the degradation of PAEs by mangrove fungi to date. An investigation was undertaken recently to explore if these fungi could play a similar role as bacteria (Luo 2010). The site of investigation was a mangrove wetland, the Futian National Nature Reserve (22°32′N, 114°05′E) with an area of 304 ha located at the border of Shenzhen, one of the cities with top economic growth in China. The Reserve is suffering from increasing pollution burden derived from the rapid urbanization and industrialization of the city which is mainly related to the discharge of domestic sewage, livestock, and industrial wastewater (Tam et al. 1995; Tam 2006). High concentrations of PAHs and heavy metals were found in the sediments (Zhang et al. 2004) and elevated levels of bisphenol A (BPA) were also recorded in the tidal waters (Li et al. 2009). These findings indicate that the sediments of this Reserve were sinks of pollutants from all possible sources, including PAH-related chemicals, such as PAEs. Therefore, this site was selected for the investigation with the aims to (a) isolate PAE – degrading fungi from

sediments of this Reserve, (b) to assess the degradation ability of isolated fungi, and (c) to compare the degradation pathways of different isomers of the PAEs by the isolated fungi (Luo et al. 2009; Luo 2010; Luo et al., 2011).

15.2 Isolation and Screening of Dimethyl-Phthalate Ester (DMPE): Degrading Fungi from Mangrove Sediments

15.2.1 *Enrichment Cultures and Isolation of Fungi*

DMPE – degrading fungi were isolated from mangrove sediments by an enrichment culture technique. Enrichment cultures were initiated by inoculating ca. 5.0 g wet mangrove sediments into 100 ml of mineral salt medium (MSM) with 100 mg l^{-1} of either one of DMPE isomers (DMP, DMI, and DMT) as the sole source of carbon and energy in 250 ml Erlenmeyer flasks. The MSM was modified from Hartmans et al. (1989) supplemented with vitamin solution (Kao and Michayluk Vitamin Solution, Sigma-Aldrich, Germany) and antibiotics (penicillin G (sodium salt) 0.5 g l^{-1}, and streptomycin sulfate 0.5 g l^{-1}) (Luo et al. 2009). The initial pH of the culture medium was adjusted to 5.5 $\pm$ 0.1. The flasks inoculated with mangrove sediments were incubated at 25°C on a rotary shaker at 150 rpm in the dark. After 1 week of incubation, the fungal mycelia/yeast biomass were observed and 5 ml of aliquot cultures were extracted and transferred to a new 250 ml Erlenmeyer flask containing 100 ml fresh culture medium identical to the initial one. DMPE – degrading cultures were obtained through six serial enrichment transfers.

The final enrichment culture was diluted 100 times with MSM and then plated on MSM agar plates. Single colonies were picked up and transferred to fresh MSM plates as pure cultures. A total of 30 fungi were isolated from the enrichment cultures, including 12 DMP-degrading fungi, 6 DMI-degrading fungi, and 10 DMT-degrading fungi. The fungal isolates were maintained on MSM slants and stored at 25°C. MSM agar plates and slants were similar to enrichment cultures but supplemented with 1.2% of agar.

15.2.2 *Characterization of the Selected Fungal Isolates*

The fungal isolates were identified on the basis of microscopic spore morphology. Most of the DMP and DMI-degrading isolates, with three exceptions could not be identified because they were non-sporulating cultures. The three isolates, DMP-3-1, DMI-5-1, and DMI-5-2 were identified as *Aureobasidium pollulans* c.f., *Trichosporon* sp., and *Penicillium* sp., respectively. Ten DMT-degrading isolates belong to two genera, eight of which were *Fusarium* spp. and the remaining two were *Aureobasidium* spp.

For selected strains used for detailed investigation of DMPE degradation (DMT-5-3 and DMI-5-1), 18S rDNA gene sequencing was further employed to confirm their taxonomic position determined by examination with an optical microscope (Luo 2010, Luo et al. 2011). The results are illustrated in Figs. 15.1–15.4.

15.2.3 Preliminary Investigation of DMPE Degradation Capability of Selected DMP-/DMI-/DMT-Degrading Fungi

The DMPE degradation capability of the 30 fungal isolates was indirectly tested through another growth assay in MSM amended with each DMPE as sole source of carbon and energy as described previously. Fungal isolates which showed a fast and good growth in the liquid medium after 1 week incubation were then subjected to further DMPE-degrading screening. Three such isolates were selected which were later identified to be *Fusarium* sp. DMT-5-3, *Trichosporon* sp. DMI-5-1, and *Penicillium* sp. DMI-5-2. As shown in Fig. 15.5, *Trichosporon* sp. and *Fusarium* sp. showed much higher DMPE degradation ability compared with *Penicillium* sp. 80% of 84 mg l^{-1} of DMI was metabolized by *Trichosporon* sp. over the 8-day of
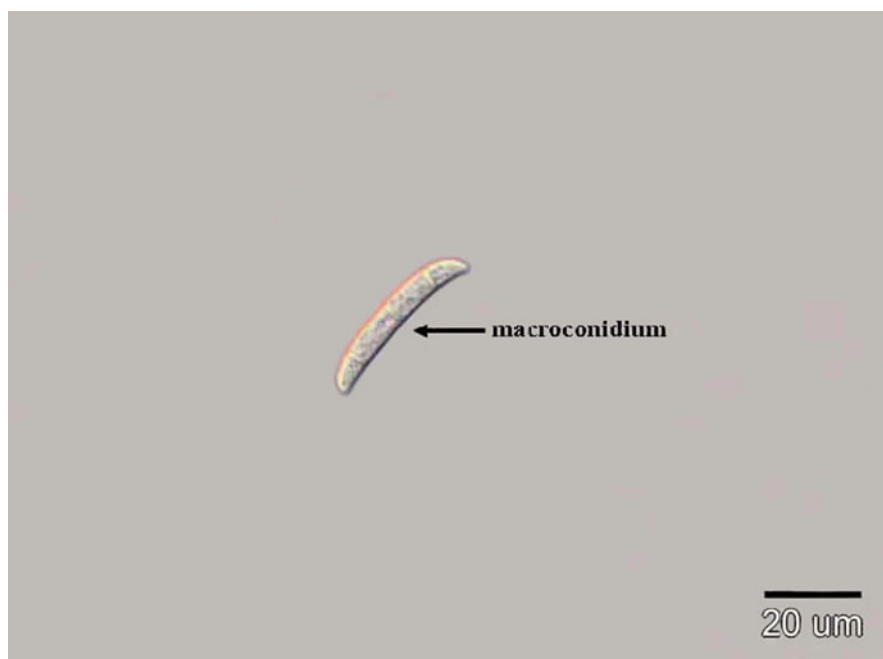
Fig. 15.1 Optical micrograph showing macroconidium of *Fusarium* sp. DMT-5-3 grown on corn meal agar
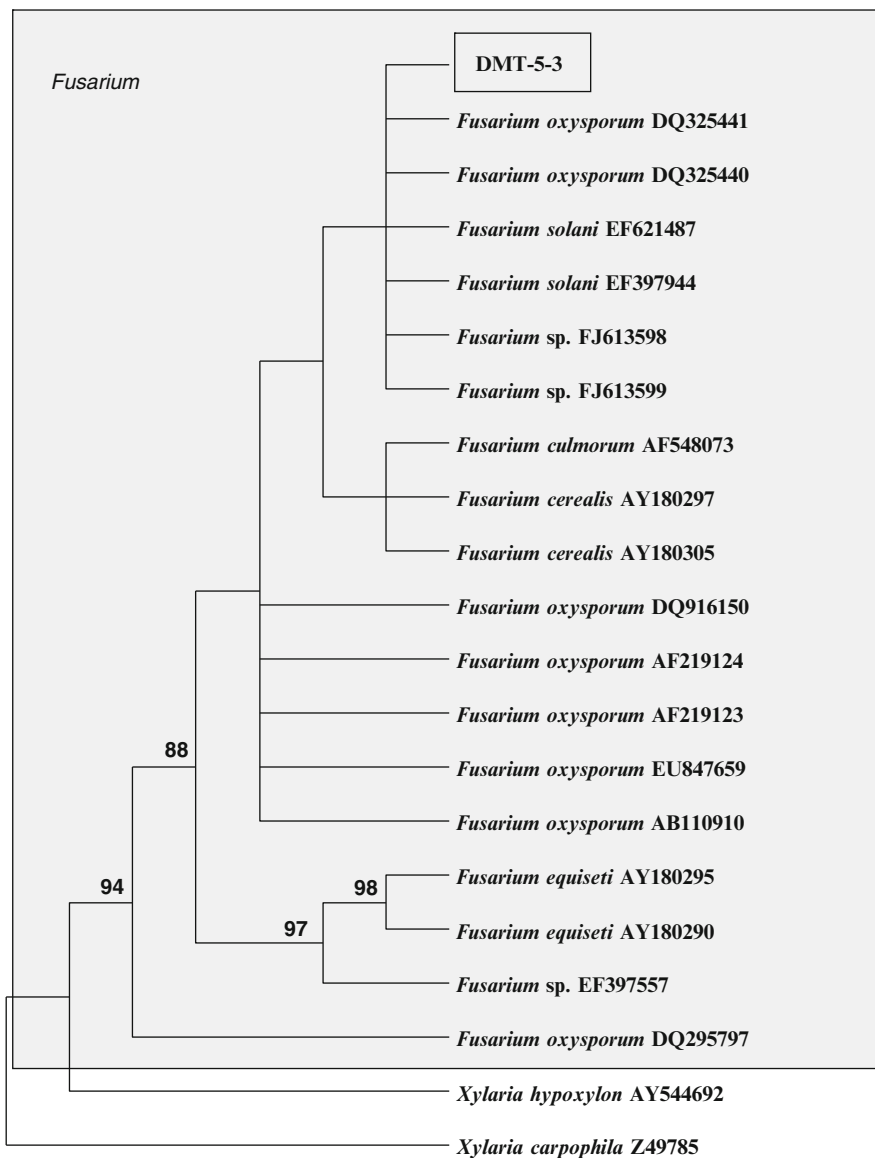
Fig. 15.2 The phylogenetic 18S rDNA-based tree showing the relationship between the fungal strain DMT-5-3 and selected members of genus *Fusarium*

incubation (Fig. 15.5a). By contrast, only 15% of 80 mg l^{-1} of DMI was degraded by *Penicillium* sp. after 8 days (Fig. 15.5b). *Fusarium* sp. was able to completely degrade 22 mg l^{-1} of DMT after 4 days of incubation (Fig. 15.5c). Therefore, *Trichosporon* sp. and *Fusarium* sp. were chosen for further investigation.
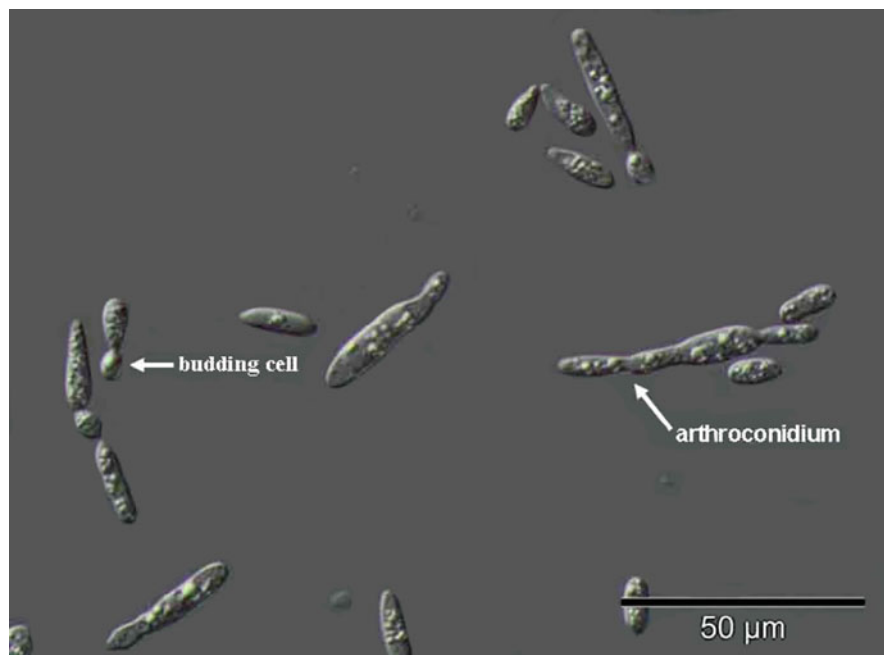
Fig. 15.3 Optical micrograph of *Trichosporon* sp. DMI-5-1 grown in potato dextrose broth. (With kind permission from Springer Science: Luo et al. 2011)

15.3 Degradation of DMPEs by *Fusarium* sp. DMT-5-3

15.3.1 Degradation of the DMPE Isomers

The culture medium for degradation experiments was prepared by dissolving either one of three DMPE isomers (DMP, DMI, and DMT) or one of the probable DMPE-degradation intermediates (monomethyl isophthlate (MMI), MMT, isophthalic acid (IA), and TA) as the sole source of carbon and energy in MSM at a concentration of ca. 30 mg l^{-1}. The medium was sterilized by passing through a 0.2-μm membrane filter (Toyo Roshi Kaisha, Japan). Active inocula were prepared by subculturing the fungus DMT-5-3 onto a MSM agar plate supplemented with 50 mg l^{-1} of the three DMPE isomers (DMP, DMI, and DMT). Six agar plugs (6 mm in diameter) with active fungal mycelia were aseptically inoculated into 125 ml Erlenmeyer flasks with 50 ml of culture medium. The control was composed of culture medium without inoculum (for detecting abiotic degradation). Triplicate flasks were set up for each treatment. The flasks were incubated on an orbital shaker at 150 rpm and 25°C. At scheduled times, three flasks for each treatment were extracted as follows: the fungal mycelia were harvested for cellular
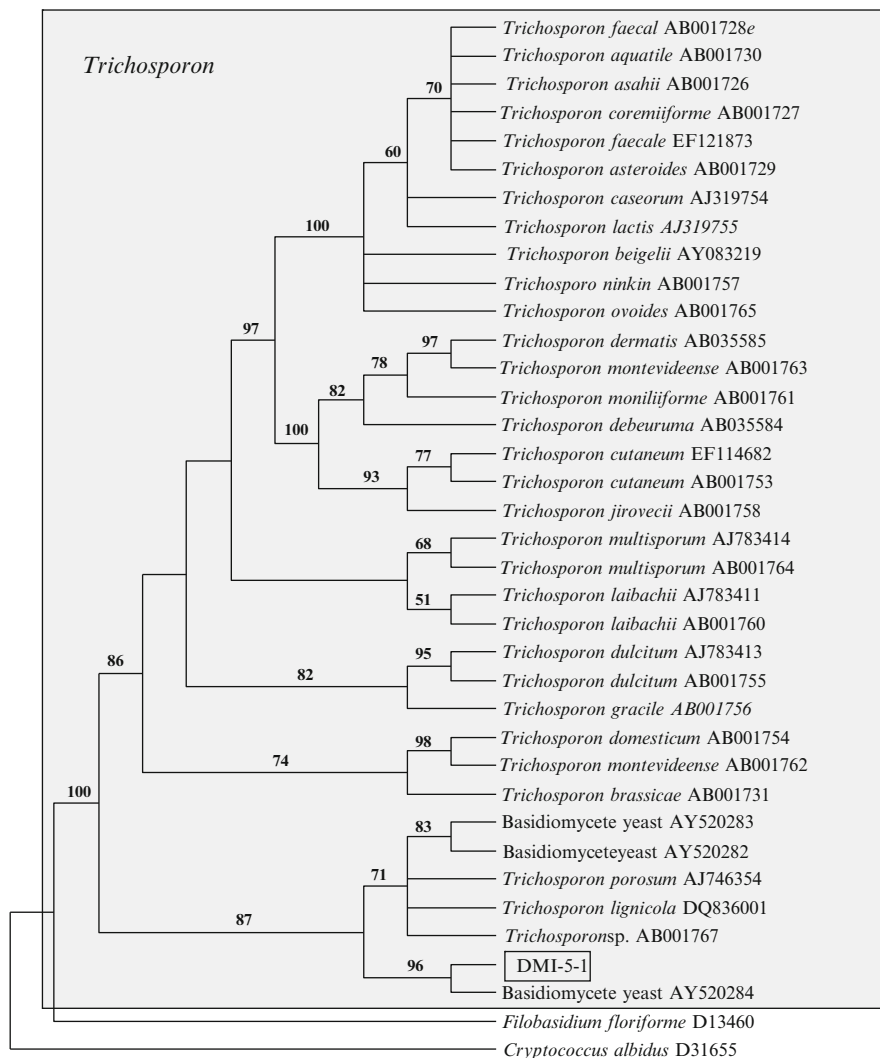
Fig. 15.4 The phylogenetic 18S rDNA-based tree showing the relationship between the yeast strain DMI-5-1 and selected members of genus *Trichosporon* (with kind permission from Springer Science: Luo et al. 2011)

protein measurement by a method adapted from Philips and Gordon (1989); and an aliquot of 2 ml of the cell-free supernatant was filtered and stored frozen ($-20°C$) for HPLC analysis adapted from Wang and Gu (2006a). The results are illustrated in Fig. 15.6. *Fusarium* sp. was able to degrade DMI and DMT, but not DMP (Luo et al. 2009).

Fig. 15.5 (**a**) Degradation of
DMI by *Trichosporon* sp.
(**b**) Degradation of DMI by
Penicillium sp.
(**c**) Degradation of DMT by
Fusarium sp. Curves include
DMI (*filled square*), MMT
(*filled triangle*), and control
(*open diamond*). *Error bars*
show standard deviations
amongst the triplicate
samples
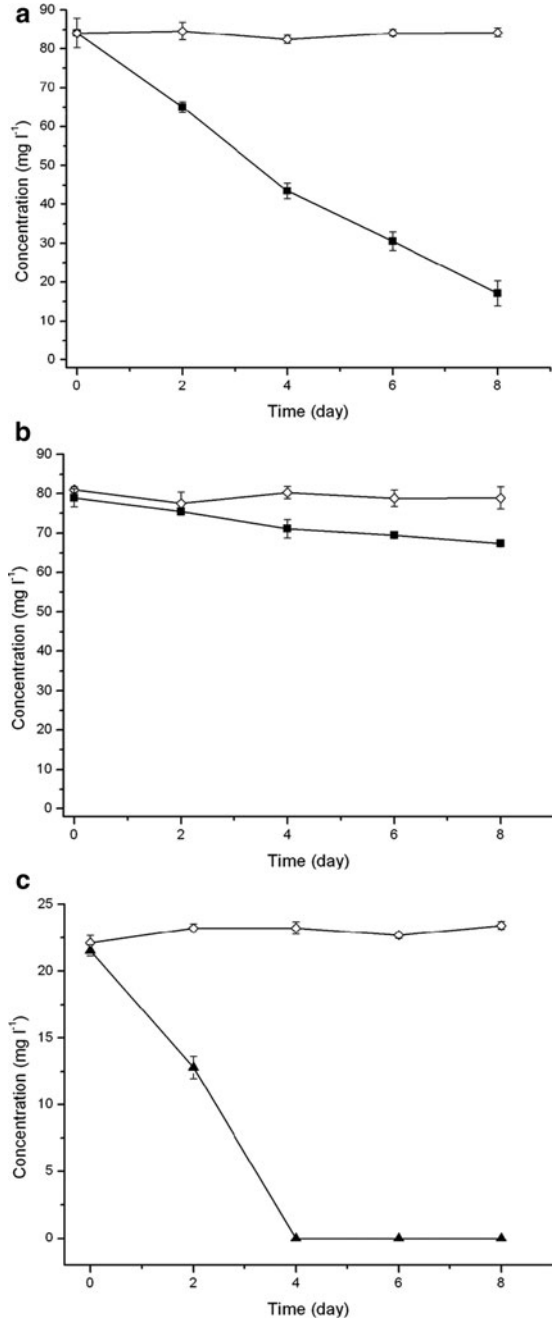
15.3.2   Degradation of DMP

The concentration of DMP remained unchanged over the 24-day incubation period, and none of the expected intermediates (MMP and PA) appeared in the culture medium (data not shown), indicating that *Fusarium* sp. was not able to metabolize DMP (Fig. 15.7a).

15.3.3   Degradation of DMI

As shown in Fig. 15.6a, 24 mg l^{-1} of DMI completely disappeared in 24 days. MMI appeared and accumulated to a peak level of 28 mg l^{-1} after 24 days. IA was not observed in the culture medium over the 24-day incubation period, indicating that further metabolism of MMI did not proceed. The degradation of DMI was accompanied by an increase in cellular protein concentrations, indicating the growth of the fungus during the degradation process. In order to confirm the proposed degradation pathway, degradation of the two suspected intermediates (MMI and IA) was also assessed using this fungus. The results show that neither MMI nor IA was metabolized (data not shown). All these results suggest that *Fusarium* sp. could only transform DMI to MMI through one step of ester hydrolysis, but lacked the ability to hydrolyze the second carboxylic ester-linked methyl group from MMI (Fig. 15.7b).

15.3.4   Degradation of DMT and MMT

Similarly, the fungus was capable of transforming DMT. As shown in Fig. 15.6b, DMT was quickly metabolized by this fungus from 20 mg l^{-1} to 0.7 mg l^{-1} in 12 days. Meanwhile, the intermediate MMT gradually accumulated to the maximum level of 20.3 mg l^{-1} in 8 days, after which it remained constant. Another intermediate, TA, was also detected in the culture medium from day 4 and its concentration remained at 5.3 mg l^{-1} after 24 days. Cellular protein concentrations increased with the depletion of DMT, suggesting that DMT was utilized by this fungus as the sole source of carbon and energy. At the same time, MMT and TA were also used as the initial substrates for the fungus to confirm the degradation pathway.

MMT, with an initial concentration of 29.5 mg l^{-1} was degraded to 24.1 mg l^{-1} over 24 days of incubation. Simultaneously, TA was observed as a degradation intermediate in the culture and accumulated to 5.3 mg l^{-1} after 24 days (Fig. 15.6c). However, further degradation of TA was not observed (data not shown). Based on these results, it can be concluded that *Fusarium* sp. was capable of transforming
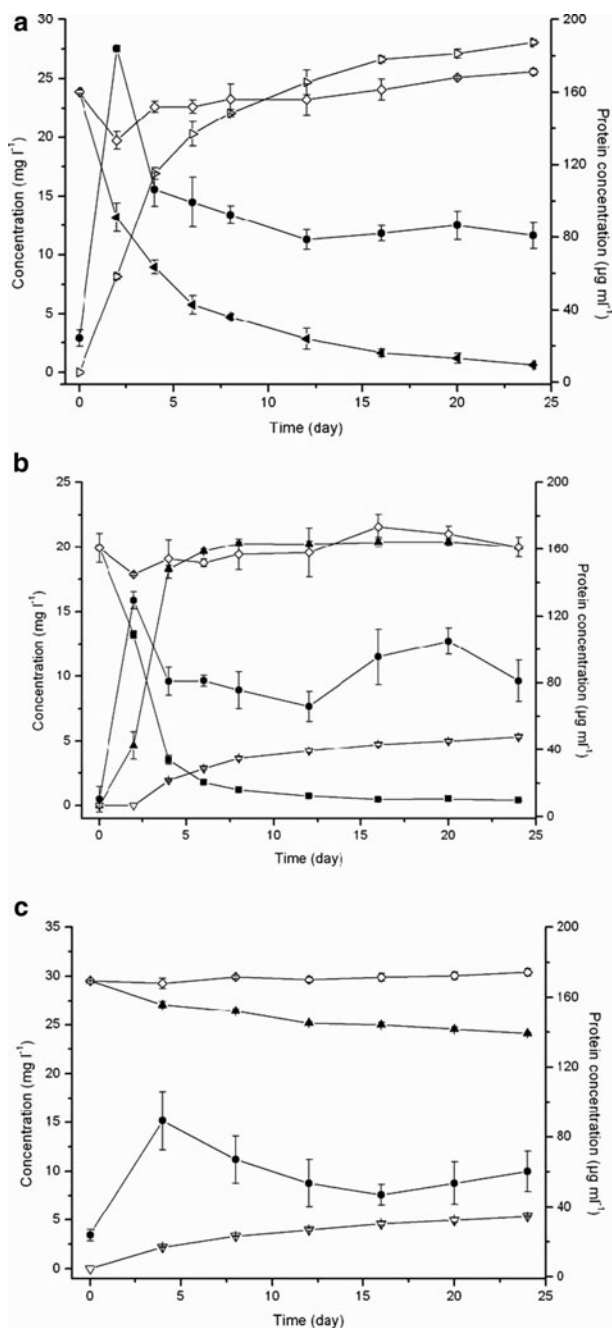
Fig. 15.6 Degradation of DMPEs and suspected intermediates by *Fusarium* sp. over 24 days: (**a**) DMI, (**b**) DMT, and (**c**) MMT. Curves include DMI (*left pointed filled triangle*), MMI (*right pointed open triangle*), DMT (*filled square*), MMT (*filled triangle*), TA (*open inverted triangle*), protein (*filled circle*), and control (*open diamond*). *Error bars* show standard deviations among the triplicate samples (reprinted from Marine Pollution Bulletin with permission from Elsevier: Luo et al. 2009)
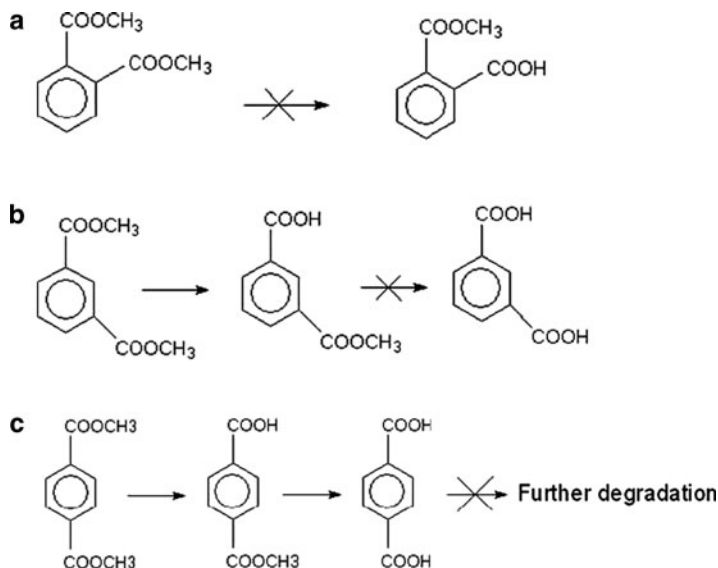
Fig. 15.7 Proposed biochemical degradation pathways for dimethyl phthalate esters (DMPEs) by *Fusarium* sp. (**a**) dimethyl phthalate (DMP), (**b**) dimethyl isophthalate (DMI), and (**c**) dimethyl terephthalate (DMT) (reprinted from Marine Pollution Bulletin with permission from Elsevier Luo et al. 2009)

DMT to MMT and then to TA by stepwise hydrolysis of two carboxylic ester bonds (Fig. 15.7c).

15.3.5   Effect of pH on the Degradation of DMPEs

Preparation of the degradation experiment was similar to the procedures described in Sect. 15.3.1. Initial pH of culture medium was adjusted to four levels (4.5, 5.0, 5.5, and 6.0) using appropriate buffers to investigate the effect on the degradation of DMPEs by the test fungus. At days 4 and 10, an aliquot of 2 ml of cell-free supernatant was extracted from the culture medium, filtered, and stored frozen ($-20°$C) for HPLC analysis. The removal percentages of DMPEs after 4 and 10 days of incubation were calculated. The differences of removal percentages of each isomer of DMPEs among four pH levels and between two incubation days were compared by two-way ANOVA (SigmaStat 3.0). The differences of removal percentages of each DMPE isomers under different pH levels on the same incubation time were compared by Tukey Test (SigmaStat 3.0).

As shown in Table 15.3, both pH levels and incubation time were able to significantly influence the degradation of DMPEs ($p < 0.05$). The interaction between pH and incubation time was not significant for DMI degradation ($p = 0.435$) but was statistically significant for DMT degradation ($p < 0.05$).

Table 15.3 A comparison of removal percentages of DMPEs by *Fusarium* sp. DMT-5-3 under different pH levels after two incubation days using two-way ANOVA

Source of variation	Degrees of freedom	Sum of square	Mean square	F	P
DMI degradation					
pH	3	1294.725	431.575	56.762	<0.001
Incubation days	1	2012.458	2012.458	164.683	<0.001
pH × incubation days	3	21.920	7.307	0.961	0.435
Residual	16	121.653	7.603		
Total	23	3450.756	150.033		
DMT degradation					
pH	3	973.883	324.628	119.719	<0.001
Incubation days	1	907.847	907.847	334.804	<0.001
pH × incubation days	3	545.533	181.844	67.062	<0.001
Residual	14	37.962	2.712		
Total	21	2404.823	114.515		

The significant interactive effect between pH and incubation time on DMT degradation was probably related to the ability of the fungus to almost completely degrade DMT at all pH levels after 10 days of incubation, indicating that the incubation time was too long and the influence of pH on DMT degradation cannot be demonstrated clearly (Fig. 15.8b).

However, comparison of removal percentages of each DMPE isomer under different pH levels on the same incubation day by Tukey Test demonstrated that the optimal pH for DMI degradation by *Fusarium* sp. was 6.0, while that for DMT degradation was 4.5 (Fig. 15.8a, b).

15.4 Degradation of DMPEs by *Trichosporon* sp. DMI-5-1

15.4.1 Degradation of DMPE Isomers

The procedures for this experiment were as described for *Fusarium* sp. in Sect. 15.3.1. The results are illustrated in Fig. 15.9. *Trichosporon* sp. was not able to completely mineralize DMPEs but transform them to respective phthalate monoester or PA (Luo et al. 2011).

15.4.2 Degradation of DMP

As shown in Fig. 15.9a, in 24 days, 21.5 mg l^{-1} of DMP was reduced to 6.7 mg l^{-1}, MMP was detected as an intermediate in the culture medium and accumulated to 17.4 mg l^{-1}. Another suspected intermediate, PA, was not observed in the culture medium, indicating that further metabolism of MMI did not proceed. Degradation

Fig. 15.8 Effect of pH levels on the degradation of DMPEs by *Fusarium* sp. after 4 days and 10 days of incubation: (**a**) DMI degradation and (**b**) DMT degradation. *Bars* with different letters are significantly different at $p < 0.05$ (Tukey Test). *Error bars* show standard deviations among the triplicate samples (reprinted from Marine Pollution Bulletin with permission from Elsevier: Luo et al. 2009)
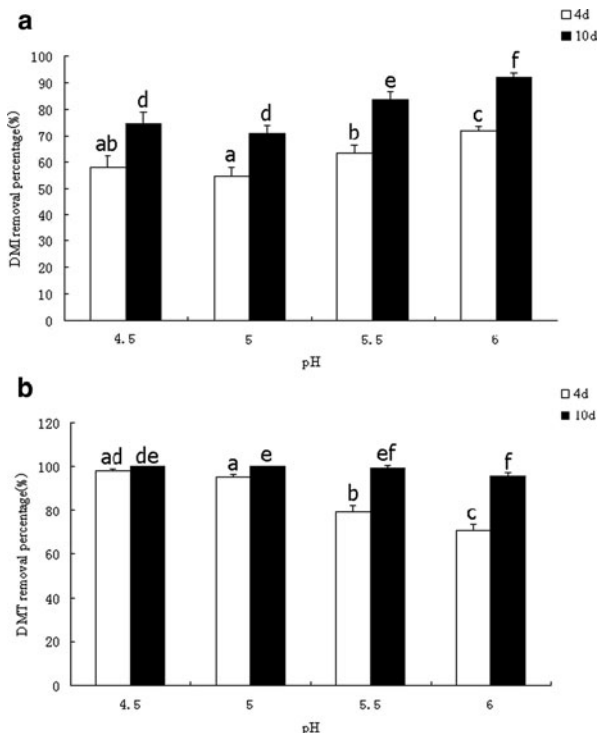
of DMP was accompanied by an increase in cellular protein concentrations, indicating the growth of the yeast during the degradation process. In order to confirm the degradation pathway, degradation of two suspected intermediates (MMP and PA) was carried out and neither MMP nor PA was degraded (data not shown). These results collectively suggest that *Trichosporon* sp. could only carried out one-step ester hydrolysis to transform DMP to MMP, but lacked the ability to remove the second carboxylic ester-linked methyl group of DMP (Fig. 15.10a).

15.4.3 Degradation of DMI

DMI at 20.2 mg l^{-1} completely disappeared after 16 days of incubation (Fig. 15.9b). The intermediate MMI gradually accumulated to peak level of 27.4 mg l^{-1} at the same time and remained constant afterwards. No expected intermediate IA appeared in the medium over the 24-day incubation period, suggesting that MMI was the last end product of DMI degradation. Cellular protein concentration increased with the depletion of DMI, indicating that DMI was utilized by this yeast as the sole carbon source. No MMI or IA was utilized when they were used as the initial substrates (data not shown). Based on these results,

Fig. 15.9 Degradation of dimethyl phthalate esters (DMPEs) and suspected intermediates by *Trichosporon* sp. (**a**) dimethyl phthalate (DMP), (**b**) dimethyl isophthalate (DMI), (**c**) dimethyl terephthalate (DMT), and (**d**) monomethyl terephthalate (MMT). *Curves* include dimethyl phthalate (DMP) (*filled square*), monomethyl phthalate (MMP) (*filled triangle*), dimethyl isophthalate (DMI) (*filled inverted triangle*), monomethyl isophthalate (MMI) (*left pointed filled triangle*), dimethyl terephthalate (DMT) (*right pointed filled triangle*), monomethyl terephthalate (MMT) (*open triangle*), terephthalic acid (TA) (*open inverted triangle*), protein (*filled circle*), and control (*open diamond*). *Error bars* show standard deviations among the triplicate samples (with kind permission from Springer Science: Luo et al. 2011)
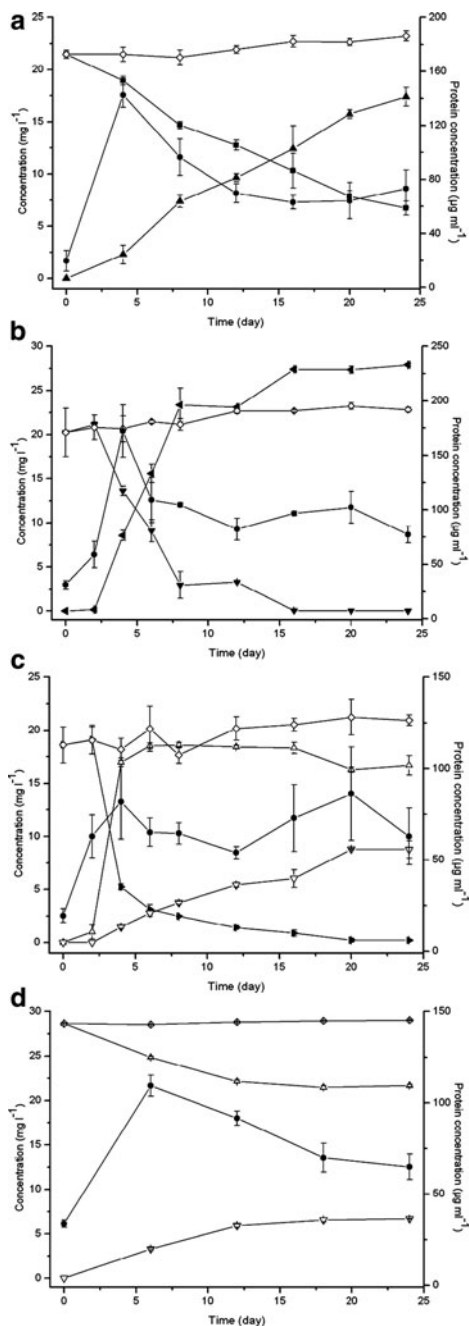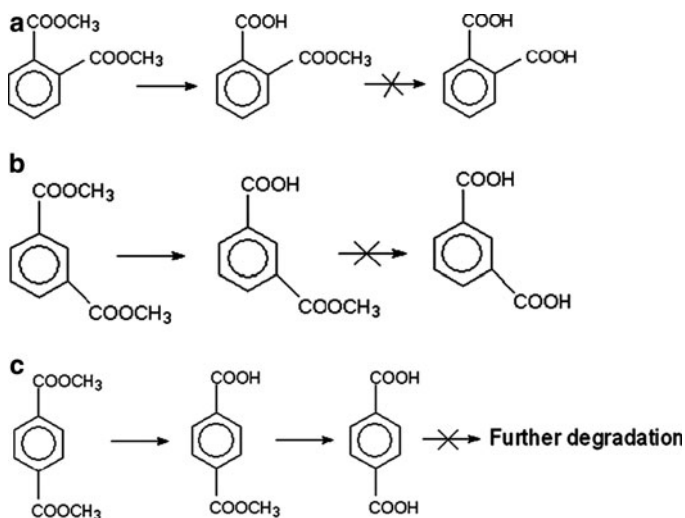
Fig. 15.10 Proposed biochemical degradation pathways for dimethyl phthalate esters (DMPEs) by *Trichosporon* sp. (**a**) dimethyl phthalate (DMP), (**b**) dimethyl isophthalate (DMI), and (**c**) dimethyl terephthalate (DMT) (with kind permission from Springer Science: Luo et al. 2011)

Trichosporon sp. also carried out one-step ester hydrolysis of DMI to MMI and could not further degrade MMI, which was similar with DMP degradation (Fig. 15.10b).

15.4.4   Degradation of DMT

DMT was gradually metabolized from 18.6 mg l^{-1} to 0.2 mg l^{-1} within 20 days of incubation (Fig. 15.9c). At the same time, MMT was observed as an intermediate and quickly accumulated to a maximum level of 18.5 mg l^{-1} in 6 days, after which it remained constant. TA was also detected as another intermediate in the culture medium from day 4 and gradually accumulated to 8.8 mg l^{-1} over the 24-day experimental period. Cellular protein concentration of the fungus increased with the degradation of DMT, suggesting the increase of yeast biomass during the DMT degradation. Confirmation experiment using MMT and TA as the initial substrates showed that the yeast was able to degrade MMT, but not TA (data not shown).

15.4.5   Degradation of MMT

MMT was degraded from 28.6 mg l^{-1} to 21.6 mg l^{-1} over the 24-day incubation period, and expected intermediate TA accumulated simultaneously in the culture

Table 15.4 A comparison of removal percentages of DMPEs by *Trichosporon* sp. DMI-5-1 under different pH levels after 2 incubation days using two-way ANOVA

Source of variation	Degrees of freedom	Sum of square	Mean square	F	P
DMP degradation					
pH	3	413.131	137.710	16.886	<0.001
Incubation days	1	3513.925	3513.925	430.888	<0.001
pH × incubation days	3	25.848	8.616	1.057	0.395
Residual	16	130.481	8.155		
Total	23	4083.386	177.539		
DMI degradation					
pH	3	15596.098	5198.699	265.519	<0.001
Incubation days	1	4138.700	4138.700	211.380	<0.001
pH × incubation days	3	483.352	161.117	8.229	0.002
Residual	15	293.691	19.579		
Total	22	21986.723	999.396		
DMT degradation					
pH	3	16746.105	5582.035	291.384	<0.001
Incubation days	1	625.324	625.324	32.642	<0.001
pH × incubation days	3	147.577	49.192	2.568	0.093
Residual	15	287.355	19.157		
Total	22	18450.535	838.661		

medium and finally reached to 6.7 mg l^{-1} after 24 days (Fig. 15.9d). Further metabolism of TA did not proceed by this yeast (data not shown). These results indicate that *Trichosporon* sp. carried out stepwise ester hydrolysis to transform DMT to TA through MMT (Fig. 15.10c).

15.4.6 Effect of pH on the Degradation of DMPEs

The procedures for this experiment were as described for *Fusarium* sp. and the results are illustrated in Table 15.4 and Fig. 15.11. As shown in Table 15.4, both pH levels and incubation time were able to influence the degradation of DPMEs significantly ($p < 0.05$). The interaction between pH and incubation time was not significant for degradation of DMP ($p = 0.395$) and DMT ($p = 0.093$), but was statistically significant for degradation of DMI ($p < 0.05$). The latter was probably related to the ability of the fungus to almost completely remove DMI at three pH levels (5, 5.5, and 6.0) after 10 days of incubation, indicating that the incubation time was too long and the influence of pH on DMI degradation could not be demonstrated clearly under the experimental conditions (Fig. 15.11b). Moreover, removal percentages of each isomer of DMPE under four pH levels at the same incubation day were compared by Tukey Test and the results indicated that the optimal pH for degradation of all the three isomers was at 6.0 (Fig. 15.11).
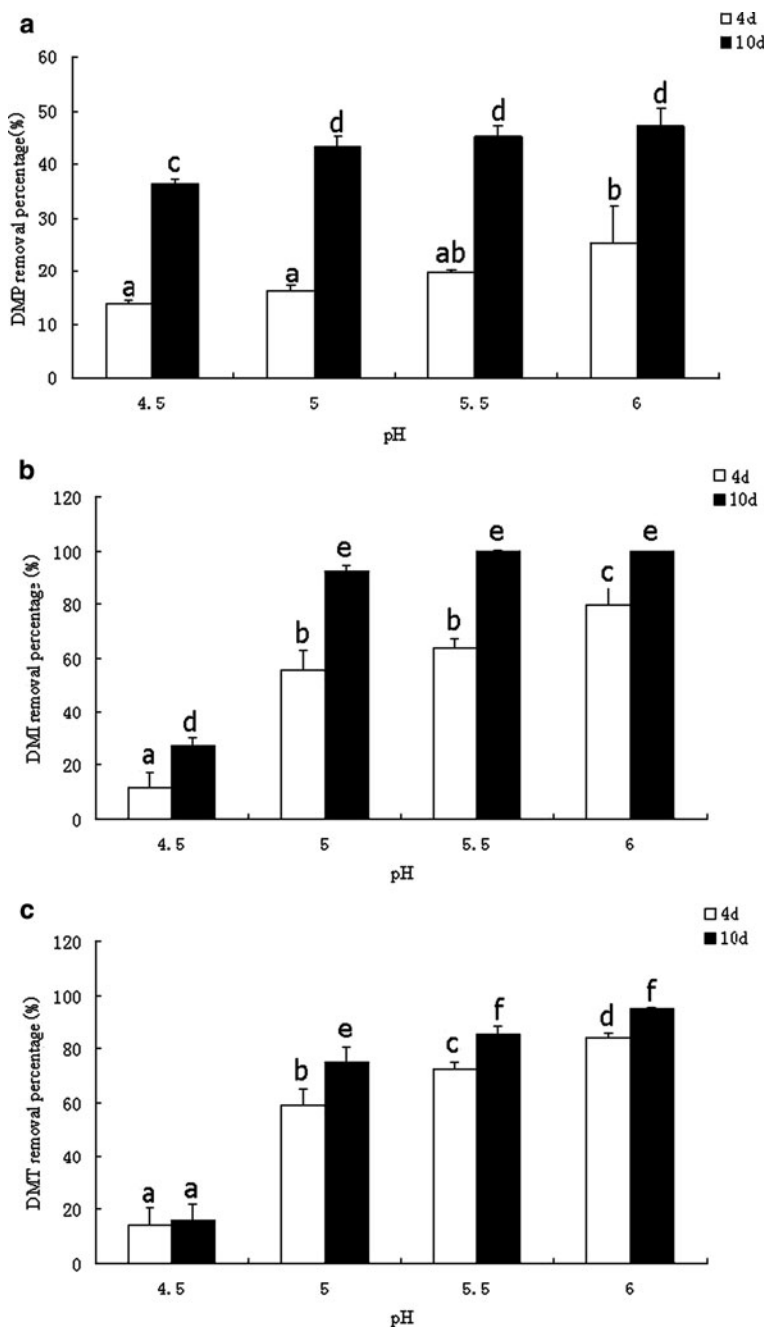
Fig. 15.11 Effect of pH levels on the degradation of DMPEs by *Trichosporon* sp. after 4 and 10 days of incubation: (**a**) DMP degradation, (**b**) DMI degradation, and (**c**) DMT degradation. *Bars* with different letters are significantly different at $p < 0.05$ (Tukey Test). *Error bars* show standard deviations among the triplicate samples

15.5   Discussion

The ability to partially degrade DMPEs by two mangrove sediment fungi, namely *Fusarium* sp. DMT-5-3 and *Trichosporon* sp. DMI-5-1 is demonstrated in this investigation. *Fusarium* is a large genus which is widely distributed in soil and on plants. *Fusarium* species have the significant ability to degrade a wide range of recalcitrant compounds, such as cellulose, xylan, pectin (Kang and Buchenauer 2000), herbicides (Bordjiba et al. 2001), 2,4,6-trinitrotoluene (TNT) (Weber et al. 2002), and PAHs (benzo(a)pyrene, pyrene, phenanthrene, anthracene, and benz(a) anthracene) (Verdin et al. 2004; Li et al. 2005c; Chulalaksananukul et al. 2006; Wu et al. 2010a, b). In addition, Chai et al. (2008) recently reported that three strains of *Fusarium* were able to degrade DEHP. *Trichosporon* is a widely distributed basidiomycetous yeast in the environment (Middelhoven et al. 2004; Kaszycki et al. 2006). *Trichosporon* species have the potential to metabolize a wide variety of organic compounds, including benzene compounds (phenol, benzoic acid, phenylacetic acid, cinnamic acid, and their derivatives as well as some other monoaromatic compounds) (Middelhoven et al. 1992; Middelhoven 1993; Sampaio 1999), cyclopentanol (Iwaki et al. 2004), biarylic compounds (biphenyl, dibenzofuran, and diphenyl ether) (Sietmann et al. 2002), and PAHs (phenanthrene) (MacGillivray and Shiaris 1993). As the structure characteristics of DMPEs were similar to the above benzene compounds, it is not surprising to isolate *Fusarium* and *Trichosporon* species capable of degrading DMPEs from mangrove sediment in this study.

Previous studies suggest that microbial degradation of PAEs is initiated by stepwise hydrolysis on both carboxylic ester bonds to form PA through phthalate monoester (Sivamurthy et al. 1991; Ganji et al. 1995; Pradeepkumar and Karegoudar 2000; Gu et al. 2005; Li et al. 2005b; Li and Gu 2006). *Fusarium* sp. as well as *Trichosporon* sp. isolated from the mangrove sediments in this investigation transformed three isomers of DMPEs to respective phthalate monoester or PA, which show a good agreement with previous reports.

A large number of bacteria such as *Pasturella multocida*, *Sphingomonas paucimobilis*, *Variovorax paradoxus*, and *Burkholderia cepacia* exhibited the capability to completely mineralize PAEs without leaving any by-products under the laboratory conditions (Li et al. 2005b; Li and Gu 2006; Wang and Gu 2006a, b; Wang et al. 2008). Complete degradation of PAEs was also observed in filamentous fungus *Aspergillus niger* and yeast *Rhodotorula rubra*. These observations indicate that PAEs can be completely removed from the environment by a number of microbial species acting on their own. On the other hand, the present study shows another possibility for the fate of PAEs under laboratory conditions, i.e., incomplete degradation of PAEs with the formation of phthalate monoester and PA as the end products. Similar findings were also observed in bacterial species *Rhodococcus ruber* and *Sphingonmonas yanoikuyae*, and fungal species *Sclerotium rolfsii* (Sivamurthy et al. 1991; Li et al. 2005a; Wang and Gu 2006a). In addition, there are reports describing how a bacterial consortia could completely mineralize PAEs,

in which *Arthrobacter* sp. and *Klebsiella oxytoca* were only able to partially transform phthalate diesters to monoesters, and further metabolism of phthalate monoesters required the cooperation of *Sphingomonas paucimobilis* and *Methylobacterium mesophilicum* (Vega and Bastide 2003; Li and Gu 2007). These observations and our results suggest that utilization of reconstituted consortia, including both fungi and bacteria, may be more efficient to completely mineralize PAEs than single species of either group.

A series of enzymes were involved in the metabolism of DMPEs, including esterase, phthalate dioxygenase and protocatechuate dioxygenase (Ganji et al. 1995; Chang and Zylstra 1998; Stingley et al. 2004). Partial transformation of three isomers of DMPEs to respective phthalate monoesters or PAs by both test fungi in this study indicate that these two mangrove sediment fungi were only able to produce esterase but not other DMPE metabolism-related enzymes. Esterases are reported to show high regio- and stereo-specificity (Bornscheuer 2002). Previous reports for degradation of DMPEs by bacterial consortia had demonstrated that two different species were involved in hydrolysis of two identical ester bonds of DMPEs, suggesting that the esterases responsible of hydrolysis of the first and second ester linkages of DMPEs are different (Vega and Bastide 2003; Li and Gu 2007). It was demonstrated with *Micrococcus* sp. YGJ1 that there were two distinct esterases involved in the metabolism of dialkyl phthalates (DAPs), including DAP esterase and monoalkyl phthalate (MAP) esterase (Akita et al. 2001; Maruyama et al. 2005). DAP esterase was responsible for the hydrolysis of DAP to MAP, which was further transformed to PA by the action of MAP esterase. An esterase isolated from *Rhodococcus erythropolis* was reported to be only capable of hydrolyzing DMP and DMI, but not DMT (Kurane 1997). All these observations suggest that esterases are highly substrate specific and there may be different esterases responsible for hydrolysis of different isomers of DMPEs and monomethyl phthalate esters (MMPEs). The present study strongly supports this notion (Table 15.5). Partial transformation of DMI to MMI by both the test fungi in this study indicated that they lacked the capability to produce MMI esterase to further metabolize MMI to IA. Both test fungi were able to transform DMT to TA via MMT, indicating that they were able to produce MMT esterase. However, both the test fungi were not able to transform MMP and MMI to the respective PA, indicating that the MMT esterase produced by the test fungi lacked the ability to hydrolyze the second carboxylic ester-linked methyl group from MMP and MMI. Therefore, the difference in hydrolysis ability of test fungi for different isomers of DMPEs and MMPEs in this study demonstrated high substrate specificity of fungal esterases.

Table 15.5 Hydrolysis ability of test fungi on different isomers of DMPEs and MMPEs

Fungal strain	DMP	DMI	DMT	MMP	MMI	MMT
Fusarium sp. DMT-5-3	No	Yes	Yes	ND	No	Yes
Trichosporon sp. DMI-5-1	Yes	Yes	Yes	No	No	Yes

ND not determined

15.6 Conclusion and Future Studies

This is the first detailed study of degradation of DMPEs by mangrove sediment fungi. Two mangrove sediment fungi, *Fusarium* sp. DMT-5-3 and *Trichosporon* sp. DMI-5-1, could not completely mineralize DMPEs but transform them to respective MMP or PA. The biochemical degradation pathways for different DMPE isomers for both the fungi were different, indicating that the fungal esterases involved in the cleavage of two carboxylic ester linkages of DMPEs are highly substrate specific. Complete mineralization of DMPEs requires the cooperation of other microorganisms.

This study demonstrates the potential of mangrove sediment fungi in the removal of PAEs in the environment. However, this is just a case study. The degradation mechanisms of PAEs in fungal system as well as the role of mangrove sediment fungi in the fate of PAEs in the aquatic environment remain largely unknown. There are still some aspects worthy of further investigation: (1) diversity of PAE-degrading fungi in the mangrove environments, (2) identification and characterization of the key enzymes and genes involved in PAE degradation from mangrove sediment fungi, and (3) degradation of PAEs using a co-culture system of PAEs degrading fungi and bacteria isolated from mangrove sediments to explore the possible synergistic degradation in the natural environment.

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