Molecular taxonomy, origins and evolution of freshwater ascomycetes

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Fungi are the most diverse and ecologically important group of eukaryotes with the majority occurring in terrestrial habitats. Even though fewer numbers have been isolated from freshwater habitats, fungi growing on submerged substrates exhibit great diversity, belonging to widely differing lineages. Fungal biodiversity surveys in the tropics have resulted in a marked increase in the numbers of fungi known from aquatic habitats. Furthermore, dominant fungi from aquatic habitats have been isolated only from this milieu. This paper reviews research that has been carried out on tropical lignicolous freshwater ascomycetes over the past decade. It illustrates their diversity and discusses their role in freshwater habitats. This review also questions, why certain ascomycetes are better adapted to freshwater habitats. Their ability to degrade waterlogged wood and superior dispersal/ attachment strategies give freshwater ascomycetes a competitive advantage in freshwater environments over their terrestrial counterparts. Theories regarding the origin of freshwater ascomycetes have largely been based on ecological findings. In this study, phylogenetic analysis is used to establish their evolutionary origins. Phylogenetic analysis of the small subunit ribosomal DNA (18S rDNA) sequences coupled with bayesian relaxed-clock methods are used to date the origin of freshwater fungi and also test their relationships with their terrestrial counterparts. Phylogenies indicate that freshwater ascomycetes have evolved from terrestrial fungi and appear to occur in only three classes. The adaptation to populate freshwater substrates has occurred in several lineages. The earliest possible date, when fungi became adapted to freshwater habitation is estimated at 390 million years ago (MYA).

Key words: ascomycetes, bayesian relaxed-clock, freshwater fungi, lignicolous, molecular dating, phylogenetics.

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Introduction

Lignicolous freshwater ascomycetes inhabit submerged woody material in lentic (lakes, ponds, swamps, pools) and lotic (rivers, streams, creeks, brooks) habitats (Wong *et al*., 1998a; Luo *et al*., 2004), playing an important role in recycling organic matter in the ecosystem. This review comprises of two sections. The first one deals with the biology of these fungi in the tropics, bringing together the large volume of recent data available on this subject and providing facts on their biodiversity and distribution. In the second section, the phylogenetic relationships between freshwater taxa and their terrestrial and marine counterparts are inferred from available rDNA sequences and their possible routes of origins are also explored. Future research needs for these fascinating groups of organisms are mentioned.

Lichens are associations between ascomycetes and algae, which can also occur on river and lake margins subjected to varying water levels (e.g. *Collema dichotomum* and *Verrucaria rheitrophila*). Lichens associated with freshwater environments have been comprehensively reviewed by Hawksworth (2000) and Aptroot and Seaward (2003). These will not be addressed in this paper.

The life cycle of ascomycetes are characterised by sexual (teleomorphic) and asexual (anamorphic) states. In nature, fungi are usually encountered in only one of these states. For a few of these ascomycetes, whose anamorphic states are unknown from nature, teleomorphic/anamorphic connections have been established by nucleic acid similarity and cultural studies (e.g. Huhndorf and Fernández, 2005). Furthermore, based on nucleic acid variation, the anamorphs have been amalgamated with the ascomycete classification, which until recently had been segregated as Fungi Imperfecti (Taylor *et al*., 1999a).

What are freshwater ascomycetes?

Freshwater ascomycetes are defined as ascomycetes which have been recorded in freshwater habitats and which complete part, or the whole of their lifecycle within freshwater environments (Shearer, 1993; Thomas, 1996; Wong *et al*., 1998a). According to this definition, in addition to species that function in water, transient fungi present in water and terrestrial fungi that release spores that are dispersed in water are all regarded as freshwater ascomycetes (Luo *et al*., 2004). Thomas (1996), however, states that the aquatic nature of some substrates is questionable (e.g. emerging parts of a plant), and therefore fungi growing on these substrates cannot be classified as freshwater fungi. Emergent macrophytes are mostly absent in rivers and streams with strong flow rates and high riparian shading (Shearer, 2001). Therefore with the exception of the bases of standing grasses (Van Ryckegem and Verbeken,

2004) and some macrophytes (Luo *et al*., 2004), most plant litter in an aquatic environment is of terrestrial origin. As such, the earlier definitions of freshwater fungi might not be appropriate, as physiological and developmental studies are needed to support the theory that freshwater ascomycetes require water to complete their life cycle (Shearer, 1993).

Fungi that are recorded in freshwater habitats can be indwellers or immigrants. "Indwellers" are fully adapted to aquatic environments and can grow and sporulate in water and are often adapted to dispersal in water, while "immigrants" must continually immigrate from other habitats to maintain their population in water (Park, 1972). Indwellers probably do not have unique characters that define their aquatic existence. Wong *et al*. (1998*a*) points out that there are numerous ascomycete species that commonly occur in freshwater habitats and have not been found in terrestrial habitats, and only these fungi can be confidently categorised as freshwater ascomycetes. Due to the controversies associated with the delimitation of this ecologically distinct group, any fungi that have been isolated from submerged plant substrates will be considered as freshwater ascomycetes and discussed in this paper.

Biodiversity of tropical freshwater ascomycetes

Shearer (1993) listed 288 fungi that had been recorded from freshwater habitats; this number has grown to 511 (Shearer, 2001; Cai *et al*, 2003a). The number increased dramatically during the last ten years because numerous new taxa have been recorded from submerged wood in tropical streams (e.g. Tsui *et al*., 2001a; Vijaykrishna and Hyde, 2006) (see Cai *et al*., 2003b). Only 11 of the 288 fungi (3.82 %) listed by Shearer (1993) were from tropical locations, while 177 of the 511 taxa (34.6 %) have a tropical distribution (Cai *et al*., 2003a). The low ratio of tropical to temperate freshwater ascomycetes had resulted from greater research efforts in temperate regions (Hyde *et al*., 1997). In similar biodiversity studies on fungi on submerged wood in temperate (River Coln: Hyde and Goh, 1999) and tropical streams (Queensland: Hyde and Goh, 1997a,b) it was found that tropical rivers support a greater diversity of fungi (Ho *et al*., 2001; Cai *et al*., 2002). Of the 223 new records or species reported from freshwater habitats since 1993, tropical ascomycetes account for more than 75% of these new records (Cai *et al*., 2003a). The percentage of tropical as compared to temperate taxa is bound to increase as more work is carried out in the tropics (e.g. Tsui *et al*., 2000, Cai *et al*., 2002; Fryar *et al*., 2004, Tsui and Hyde, 2004).

Genera of freshwater ascomycetes

Freshwater lignicolous ascomycetes do not form a monophyletic group, but are an ecological group of organisms. They comprise species from the three classes, Leotiomycetes, Dothideomycetes and Sordariomycetes belonging to the Super class Leotiomyceta, under the subphyla Pezizomycotina, represented by 19 orders (Shearer 2001; Cai *et al*., 2003a) (For classification see, Eriksson *et al*., 2003). The taxonomic position of a large number of these fungi is still uncertain (see Eriksson *et al*., 2003). Representatives of freshwater ascomycetes have been recorded in other habitats (terrestrial or marine). Accordingly, the genera of freshwater lignicolous ascomycetes can be divided in four main groups based on their occurrence. These include 1) genera that are exclusively known from freshwater habitats, 2) genera with both freshwater and terrestrial species, 3) genera with freshwater and marine species and 4) genera that are found in freshwater, marine and terrestrial habitats. The most common genera from freshwater habitats under these categories are listed in Table 1 and each grouping is discussed below. These classifications are probably premature, because recently, several freshwater fungi have been discovered from terrestrial habitats. e.g. *Cataractispora* species have been known only from wood submerged in freshwater (Hyde *et al*., 1999a; Ho *et al*., 2004) however, we have recently collected a species from terrestrial grasses.

Genera exclusively known from freshwater habitats

Most uniquely freshwater genera are confined to the family *Annulatascaceae*, which incorporates genera with some apparently exclusive adaptations to the aquatic environment (Hyde *et al*., 1997, 2000a; Wong *et al*., 1998b; Ho and Hyde, 2000; Tsui *et al*., 2002; Tsui *et al*., 2003; Lee *et al*., 2004). Although most species share a massive apical ring, which possibly facilitates dispersal of spores, the morphology of the ascospores is quite diverse (Ho *et al*., 1999a,b; Wong *et al*., 1999; Lee *et al*., 2004). Species of *Cataractispora* and *Diluviocola* have ascospores with uncoiling thread-like appendages that are believed to aid in dispersal and subsequent attachment in water (Hyde *et al*., 1998, 1999a; Hyde and Goh, 2003). Some species in *Annulatascus* have ascospores with sticky mucilaginous sheaths, and exosporial wall ornamentations, which are thought to aid in the attachment to substrates (Ho *et al*., 1999a,b; Hyde and Wong, 2000a; Hyde and Goh, 2003). In *Fluminicola* ascospores have bifurcate polar appendages (Wong *et al*., 1999), while in *Pseudoproboscispora* ascospores have proboscis-like appendages which uncoil (Wong and Hyde, 1999). Both appendage types are

Table 1. Common freshwater genera with terrestrial, marine/terrestrial or no counterparts (modified from Hyde and Goh, 1998; Tsui *et al*., 2000).

Freshwater only	**Freshwater/ Terrestrial**	**Freshwater/ Marine**	**Freshwater/ Marine/** Terrestrial
Sordariomycetidae			
Aquaticola	*Annulatascus**	*Aniptodera*	*Anthostomella*
Cataractispora	*Ascotaiwania**	*Halosarpheia*	*Phomatospora*
Pseudoproboscispora	*Cercophora*	*Nais*	
Rivulicola	*Ophioceras**	*Savoryella*	
Torrentispora	*Pseudohalonectria**		
Dothideomycetidae			
Jahnula	*Byssosphaeria*	*Quintaria*	*Lophiostoma*
Mamillisphaeria	*Kirschsteiniothelia*		*Massarina*
			Didymella
Leotiomycetidae			
			Vibrissea

probably useful in attachment. The unitunicate genus *Ascosacculus* is characterised by deliquescent asci, with guttulate ascospores and apical appendages and is the only genus within the *Halosphaeriales* found only in freshwater habitats. Other genera of *Halosphaeriales* found in freshwater habitats can also be found in marine and brackish water (Van Ryckegem and Verbeken, 2005).

Jahnula is probably the only common ascomycete genus within the class *Dothideomycetes* found only in freshwater (Table 1). The peridium of large soft-walled cells in *Jahnula* and the dimorphic nature of its ascospores (one ascospore type having bipolar mucilaginous pads) may be important freshwater characteristics (Hawksworth, 1984; Hyde *et al*., 1999b). Another striking feature, are the rhizoid-like structures which enable the ascomata to attach to the substrate while they hang in water which may aid dispersal (Pinruan *et al*., 2002). *Mamillisphaeria* is also known only from freshwater habitats and has dimorphic ascospores, as in *Jahnula*. These characters are thought to be important in dispersal in the freshwater environment (Hyde *et al*., 1996, 1999b; Hyde and Goh, 2003).

There are other genera only known from freshwater habitats, but they have rarely been recorded. *Ascovaginospora* has ascospores with remarkable tetraradiate sheaths (Fallah *et al*., 1997), while in *Ascominuta* ascospores have a swollen sheath (Ranghoo and Hyde, 2000). Both sheath types are probably important in dispersal and subsequent attachment. In *Ascoyunnania*, a recently described ascomycete from submerged bamboo, the hyaline ascospores

germinate within the asci and form dark brown to black, warted secondary spores. These secondary dark spores bear a large hyaline, thin-walled, smooth, non guttulate, ellipsoidal sac-like structure which are remains of the ascospores. The condition where spores directly form secondary spores, without formation of mycelium is known as microcyclic conidiation, where the normal lifecycle of the fungus is bypassed (Cai *et al*., 2005). These spores may help the fungus to survive dry conditions whereas the hyaline ascospores may be adequate for aquatic dispersal.

Genera with both freshwater and terrestrial species

Common freshwater genera within the Sordariomycetes with terrestrial representatives include *Annulatascus*, *Ascotaiwania*, *Cercophora*, *Ophioceras* and *Pseudohalonectria* (Table 1). These genera probably have characteristics that are pre-adapted for dispersal in freshwater. Species of *Pseudohalonectria* were previously only known from freshwater (Hyde, 1995; Shearer, 1989a). However, Hyde *et al*. (1999e) described two new species of *Pseudohalonectria* from terrestrial palms and Promputtha *et al*. (2004) described a new species from leaves of *Magnolia liliifera*. Most *Ophioceras* species are known from freshwater (Tsui *et al*., 2001b), however Fröhlich and Hyde (2000) have identified several species from terrestrial palms.

In most of these genera, it appears that the characters that are important for dispersal in the terrestrial environment may also be important in the aquatic environment. Most species in these genera have the ability to eject their ascospores. Their ascospore appendages or sheaths may be pre-adapted for dispersal in freshwater. Terrestrial and aquatic species of *Phomatospora* have bipolar mucilaginous or hair-like appendages (Fallah and Shearer, 1998; Hyde *et al*., 2000a), species of *Ophioceras* have sigmoid ascospores that probably have mucilage at their tips (Goh and Hyde, 1996) and species of *Saccardoella* have large mucilaginous sheaths (Tsui *et al*, 1998). These spore shapes, and appendages and sheath types, have been shown to be important in the dispersal and subsequent attachment of marine fungi (Hyde and Jones, 1989; Hyde *et al*., 1993).

Bitunicate ascomycetes that occur in freshwater and terrestrial habitats (e.g. *Byssosphaeria* and *Kirschsteiniothelia*) eject ascospores via fissitunicate dehiscence and ascospores are often sheathed (Shearer, 1993). These characteristics appear to be advantageous in both terrestrial and freshwater habitats and are also observed in marine species (Hyde *et al*., 2000b). Some loculoascomycetes have developed elaborate sheaths, which are massive structures to better facilitate attachment (Shearer and Hyde, 1997), or have

uniquely shaped sheaths, e.g. arrow-like (Tsui *et al*., 1999), that arguably aid in attachment to substrata in freshwater.

Genera within the class Leotiomycetes are predominantly terrestrial, however a large number of these species are also found in temperate freshwater habitats. The number of tropical isolations is very low compared to temperate regions. However, the majority of teleomorph connections made for Ingoldian fungi (aquatic hyphomycetes) belong to the class Leotiomycetes (Sivichai and Jones, 2003), and several have been identified from tropical habitats. The conidia of Ingoldian fungi are characterized with branched, sigmoid or helicoid conidia (Gönczöl and Révay, 2004; Sakayaroj *et al*., 2005; Pascoal *et al*., 2005), which help in the attachment of the conidia to substrates.

Genera with freshwater and marine species

The unitunicate genera *Aniptodera*, *Halosarpheia*, *Nais* and *Savoryella* are the most commonly reported ascomycetes that occur in both freshwater and marine habitats (Maria and Sridhar, 2003; Prasannarai and Sridhar, 2003; Tsui and Hyde, 2004) (Table.1). These fungi may have asci that deliquesce early (e.g. *Halosarpheia* species), or are persistent (e.g. *Aniptodera* species) (Shearer, 1989b; Hyde *et al*., 1999d). In some species asci have an apical pore (e.g. *Savoryella* species), but it is not clear if the pore is functional (Jones and Hyde, 1992; Ho *et al*., 1997). A layer of mucilage surrounds the ascospores of *Savoryella* species, which help the ascospores to adhere to the substrate they come in contact with (Read *et al*., 1993). In some *Aniptodera* species and all *Halosarpheia* species, ascospores have bipolar unfurling appendages which aid in the attachment to substrata (Hyde *et al*., 1999d), but other *Aniptodera* species lack appendages. Besides their occurrence in water, there are no distinctive features that can be used to set apart these latter taxa as aquatic fungi.

Genera found in freshwater, marine and terrestrial habitats

The fourth group is genera occurring in freshwater, marine and terrestrial habitats and is best represented by genera within the class Dothideomycetes, *Massarina* and *Lophiostoma* (Hyde and Aptroot, 1997; Aptroot, 1998; Liew *et al*., 2002) (Table 1). Species of *Massarina* are frequently identified in mangrove habitats (Hyde and Borse, 1986; Hyde *et al*., 1990), streams and on terrestrial palms (Hyde and Aptroot, 1997, 1998; Aptroot *et al*., 2000). *Lophiostoma* species are mostly terrestrial (Chesters and Bell, 1970; Holm and Holm, 1988), but some species are also known from aquatic environments

(Shearer, 1993; Hyde and Aptroot, 1998; Liew *et al*., 2002; Hyde *et al*, 2002). There are little to no distinct differences between the species of the above genera that occur in different habitats. Ascomata develop under a thick pseudostroma, asci eject their ascospores during fissitunicate dehiscence, and ascospores generally have a mucilaginous sheath (Aptroot, 1998; Liew *et al*., 2002; Hyde *et al*., 2002), however, aquatic species of *Lophiostoma* have ascospores with elaborate sheaths (*Lophiostoma ingoldianum)* or long appendages (*Lophiostoma frondisubmersum*). These characteristics appear to be advantageous in all the freshwater, marine and terrestrial habitats and may account for the widespread and frequent occurrence of these species.

Species of *Anthostomella*, *Didymella*, *Phomatospora* and *Saccardoella* also occur in all three habitats. *Phomatospora* is a unitunicate genus common in terrestrial habitats (Nograsek, 1990), but has both freshwater and marine species (Fallah and Shearer, 1998; Hyde *et al*., 2000b). Similarly species of the bitunicate genus *Saccardoella* may occur in freshwater, marine and terrestrial habitats (Hyde, 1992; Mathiassen, 1993, Tsui *et al*., 1998). The freshwater and marine taxa of these genera do not have special adaptations, although *Phomatospora* species have ascospores with bipolar mucilaginous pads of hair-like appendages (Fallah and Shearer, 1998; Hyde *et al*., 2000b). *Didymella* and *Saccardoella* species have sheathed ascospores that are similar to those found in terrestrial species of the same genera (Hyde and Aptroot, 1998; Hyde and Wong, 2000b). The genus *Vibrissea* (Class Leotiomycetes), whose species occur in all three habitats, also appear to lack any specific adaptations to the aquatic environment (Hyde *et al*., 1999c).

Distribution of tropical lignicolous freshwater ascomycetes

The biogeography of freshwater ascomycetes has been discussed by Shearer (1993), Hyde *et al*. (1997), Shearer (2001) and Cai *et al*. (2003a). Of the 511 ascomycetes listed by Shearer (2001) and Cai *et al*. (2003a), 177 had a tropical distribution with only 20 also occurring in temperate regions. This low overlap supports the suggestion of Hyde *et al*. (1997) that a distinctive freshwater ascomycete community exists in the tropics. There are numerous taxa that have only been reported from temperate (e.g. *Massarina aquatica*) or tropical regions (e.g. *Aquaticola ellipsoidea*). The conclusion reached for Ingoldian fungi that many species are cosmopolitan with both tropical and temperate distributions seems less well fitting for lignicolous ascomycetes, which appear to have greater temperate or tropical distributions. However, recently many ascomycetes described from tropical freshwater habitats have been discovered in a temperate freshwater habitat (Fallah and Shearer, 2001)

Many freshwater ascomycetes have only been described with limited distributions, but with the availability of more data it is apparent that many species have much wider distributions. *Annulatascus velatisporus* was previously only known from Australia (Hyde, 1992) but is now known to be pan tropical and even occurs in temperate regions (Campbell and Shearer, 2004). These trends indicate that most freshwater ascomycetes have a pan tropical or pan temperate distribution and some may also overlap in warm temperate or subtropical regions.

Physiological studies of freshwater fungi indicate that the optimal growth conditions are identical for both temperate and tropical taxa. The optimum temperature for growth for most tropical freshwater fungi is between 20-25°C, although several isolates exhibited optimal growth rates as low as 15°C or as high as 30°C (Yuen *et al*., 1998). Temperate freshwater fungi also have similar optimum growth rates (Zare-Maivan and Shearer, 1988a,b), showing an optimal temperature of 25°C for most temperate taxa, although they could grow relatively well at temperatures as low as 10°C. This would suggest that there is no such thing as tropical or temperate taxa. Yuen *et al*. (1998) however, concluded that tropical freshwater fungi do not grow well at low temperatures, and so are absent in streams in temperate regions. On the other hand, although temperate species grow best at 25°C, they were not able to grow as rapidly as tropical species and this probably accounts for their absence in tropical streams.

Probably one of the most intriguing age-old problems is how these freshwater species have managed to colonise streams in distance parts of the world. These aquatic fungi can have evolved in one region, so how do they now occur in several continents. Wood-Eggenschwiler and Bärlocher (1985) reviewed the information on the biogeography of the freshwater hyphomycetes and found three basic trends;

1. Cosmopolitan species, although these species may be more common in the tropics or a temperate situation.
2. Species restricted to temperate and cold tropical regions.
3. Species restricted to a very small geographical area.

Wood-Eggenschwiler and Bärlocher (1985) illustrated that aquatic hyphomycetes are not restricted by geographical barriers and that similarities in mycota were found between geographically distant tropical locations, as well as in temperate locations on either side of the equator. Hyde and Goh (2003) illustrated four possible ways by which freshwater ascomycetes appear in different continents. Fungi might have evolved before the split of the continents and then moved with the landmasses, or carried over to different

continents on plant substrates. Fungal spores might have also been dispersed by animals (e.g. birds), or wind.

Origins of freshwater ascomycetes

Overview on the origin of ascomycetes

Ascomycetes are one of the four phyla in the Kingdom Fungi. They are the largest group, containing more than 32,000 species, and have a variety of associations with plants, animals, algae and cyanobacteria. Ascomycetes are distinguished from other fungi, as they form sexual spores within a sac-like structure called the ascus. Ascomycetes are a monophyletic group of fungi, descended from a common ancestral species (Berbee and Taylor, 2001, Keeling *et al*., 2000, Liu *et al*., 1999; Lumbsch, 2000) with Basidiomycetes as sister taxa (Bruns *et al*., 1993; Berbee and Taylor, 1993, 2001). Information concerning the evidence of radiation of major groups of fungi comes from diverse sources.

Evidence on the origin of fungi has been obtained from fossil records (Tiffney and Barghoorn, 1974, Sherwood-Pike and Gray, 1985; Hass *et al*., 1994; Remy *et al*., 1994a,b; Taylor, 1994; Taylor *et al*., 1995, 1992a,b, 1999b, 2004). Based on these records, fungi are presumed to have been present in Late Proterozoic (900-570 million years ago (MYA)). Sherwood-Pike and Gray (1985) reported chains of asexual spores and perforate hyphae from digested rock samples from the Silurian Period (438-408 MYA). Fossil hyphae in association with wood decay, fossil chytrids and *Glomales* (arbuscular mycorrhizae) representatives associated with plants of the Rhynie Chert (Aberdeenshire, Scotland) are reported from the Devonian Period (408-360 MYA) (Hass *et al*., 1994; Remy *et al*., 1994a,b; Taylor *et al*., 1992a, 1995). Fossil "fungi" from the Precambrian era are now considered to be filamentous sheaths of cyanobacteria.

At present, the only oldest definite ascomycete fossils are reported from Rynie Chert dating to the Lower Devonian Period (400 MYA) (Taylor *et al*., 2004). Furthermore, interpretation of earlier fungal fossils is difficult due to lack of diagnostic characters present in modern taxa (Berbee and Taylor, 1993). Due to the lack of interpretational fossil evidence and obscure morphology, the dating of fungal evolution has always been a daunting task for evolutionary biologists and mycologists. These fossil records, however, are crucial calibration points for the molecular dating of fungal radiation (Berbee and Taylor, 2001).

Nonetheless, global environmental changes have left footprints in the DNA of living organisms from which the evolutionary history can be reliably inferred (Brohman and Penny, 2003). With the advent of molecular techniques several investigations have dealt with the origin of various major groups of fungi (e.g. Bruns *et al*., 1993; Berbee and Taylor, 1993, 1995, 2001). Furthermore, most information on the timescale of fungal evolution is derived from analysis of DNA and protein sequence data with molecular clocks (Berbee and Taylor, 2001; Heckmann *et al*., 2001). DNA clock estimates of fungi have proven fruitful, particularly, in dating the evolution of *Glomales* (arbuscular mycorrhizae) (Simon *et al*., 1993, Taylor *et al*., 1995). *Glomales* are ubiquitous root symbionts that have evolved concurrently with land plants and have played crucial roles in the colonization of land by plants, as hypothesised by Pirozynsky and Malloch (1975). This importance of symbiotic association is also suggested by evidence of arbuscular mycorrhizae in the earliest fossil fungi (460 MYA) and in the earliest land plants (Remy *et al*., 1994b; Selosse and LeTacon, 1998; Redecker *et al*., 2000).

Molecular clock estimates based on 18S rDNA have shown that fungi occurred about 800 MYA and the ascomycetes and basidiomycetes diverged about 600 MYA (Berbee and Taylor, 1993). In contradiction, an analysis using multiple protein-coding genes, in fungi and plants, Heckman *et al*. (2001) suggested that fungi originated about 1.5 billion years ago, and the ascomycete-basiodiomycete divergence took place around 1.2 billion years ago. However, analysis based on plastid-encoding genes, suggest that the dates for plants proposed by Heckman *et al*. (2001) may be too early (Sanderson, 2003; Sanderson *et al*., 2004), and this when extrapolated, would also be true for fungi (Lutzoni *et al*., 2004). However there has been no further analysis involving fungi.

Ecological theories: Origin of freshwater ascomycetes

The ‘multiple’ origins of freshwater ascomycetes was first proposed by Shearer (1993). Freshwater ascomycetes could have evolved through several pathways. Accordingly, freshwater ascomycetes could have been present as endophytes, pathogens or saprobes on plants, and have become adapted to aquatic environment when these plants invaded water. Freshwater ascomycetes could have also reached the aquatic environment via tree and shrub litter or from the run-off of rainwater and sediments. As an example, some freshwater ascomycetes (e.g. *Didymella aptrooti*, *Fluminicola bipolaris*) mainly occur on bamboo (Wong *et al*., 1999; Hyde and Wong, 2000a; Cai *et al*., 2003b). In many countries bamboo grow along the banks of rivers and may have provided

a direct pathway for terrestrial ascomycetes to become aquatic species (Cai *et al*., 2003b).

It has also been proposed that freshwater ascomycetes are unique in having unique adaptations, for example their capability to degrade submerged substrates. Most freshwater lignicolous ascomycetes, which have been repeatedly isolated from aquatic habitats, are presumed to be soft rot (a superficial decay type restricted to surface layers of wood) fungi (Yuen *et al*., 2000). These types of decay are thought to be a better adaptation to degrading wood in water-logged conditions (Bucher *et al*., 2004a,b). Furthermore, enhanced mechanisms for dispersal and subsequent attachment in freshwater habitats, as compared to their terrestrial counterparts indicate adaptations to freshwater lifestyles. Various *in vivo* and *in vitro* studies have been carried out to study the ability of tropical freshwater ascomycetes to degrade wood. Yuen *et al*. (1998, 2000) as part of their study on weight loss in wood caused by freshwater fungi, found that the terrestrial fungi they tested caused greater weight losses when incubated on agar than to those incubated in liquid medium. The reverse was true for the freshwater fungi tested. This indicates that freshwater fungi are better adapted to submerged conditions, than terrestrial fungi.

The ability to produce wood degradation enzymes has been studied for both temperate (Abdel-Raheem and Shearer, 2002) and tropical freshwater ascomycetes (Bucher *et al*., 2004a). *In vitro* studies show that tropical freshwater ascomycetes produce cellulases and xylanases causing soft rot cavities and also showed that they are capable of degrading the wood surface when not in water. The study also showed that freshwater fungi could degrade lignin (most refractory component in wood), at least as effectively as some terrestrial ascomycetes which are known to cause white rot decay. It has also been shown that some freshwater fungi are competitive against or may reduce sporulation in other fungi and this may account for the commonness of some fungi in the freshwater milieu (Fryar *et al*., 2001, 2005).

Earlier studies have shown that ascomycete/basidiomycete radiation took place after the invasion of land by plants. Based on this, it is speculated that current day freshwater ascomycetes should have evolved from their terrestrial counterparts. There has been no molecular study that has investigated the origin of freshwater ascomycetes, and evolutionary theories have mainly been based on ecological findings.

Relatives of freshwater ascomycetes

The most likely candidates for the close relatives of the freshwater ascomycetes are among the diverse group of perithecial ascomycetes. The latter are ecologically diverse and include parasites and pathogens of plants and animals, endophytes of grasses and trees, symbionts of arthropods, and decomposers of a wide range of organic substrates (Kumar and Hyde, 2004; Suryanarayanan and Thennarasan, 2004; Zhang *et al*., 2004; Lee *et al*, 2005). There are several terrestrial and marine lineages of fungi within the perithecial ascomycetes that display characters present among the freshwater ascomycetes. Freshwater lignicolous ascomycetes comprise species from the classes Leotiomycetes, Dothideomycetes and Sordariomycetes belonging to the Super class Leotiomyceta, under the subphyla Pezizomycotina, represented by 19 orders (Shearer 2001; Cai *et al*., 2003a). However the majority of the species are restricted to only seven orders, *Eurotiales*, *Halosphaeriales*, *Helotiales*, *Hypocreales*, *Pleosporales*, *Sordariales* and *Xylariales*. The rest of the orders contain less than 10 freshwater taxa (Shearer, 2001).

Molecular clock versus relaxing the clock

The basic approach for estimating molecular dates is the estimation of genetic distance between species, and then applying calibration rates i.e. the number of genetic changes expected per unit time (nucleotide substitution rates), for the conversion of genetic distance into time. Since the proposal of the molecular clock hypothesis by Zuckerkandl and Pauling (1965), which is based on the constancy of evolutionary rate over time, several investigations have dealt with the dating of divergence of organisms. However, there has been constant debate over its validity (Rodriguez-Trelles *et al*., 2001). A number of tests have been developed to test the constancy of evolutionary rate across lineages (Langley and Fitch, 1974; Wu and Li, 1985), and have often rejected the molecular clock (constancy of evolutionary rate over time) in real datasets (see Nei and Kumar, 2000: 188).

Attempts have been made to relax the assumptions of the global molecular clock by allowing variations of the evolutionary rate along the phylogenetic tree (Sanderson, 1997; Rambaut and Brohman, 1998; Thorne *et al*., 1998; Yoder and Yang, 2000; Aris-Brosou and Yang, 2002). Among the newly developed methods, the bayesian relaxed clock molecular clock approach of Thorne *et al*. (1998) is becoming popular. There are several advantages of the bayesian relaxed molecular clock approach. It allows the calculation of divergence time estimates in the presence of rate variation

among lineages. It also allows the incorporation of multiple paleontological constraints as priors (Kishino *et al*., 2001). This method has already been successfully used in the calibration of HIV virus (Korber *et al*., 2000).

Molecular clock in estimating origin of freshwater ascomycetes

Objectives

The primary goal was to test the evolutionary hypothesis that freshwater ascomycetes have evolved from their terrestrial counterparts, based on a phylogenetic approach. For this, 18S rDNA sequences of a number of freshwater ascomycetes are incorporated in a phylogenetic framework containing members of the major groups of ascomycetes and other fungi. Their phylogenetic affinities are also discussed combining recent literature on morphology and phylogeny pertaining to freshwater fungi. These results in turn have been used to investigate the origin of freshwater ascomycetes, attempting to estimate the timescale of their evolution using recently developed molecular tools

Methods

A large number of small subunit ribosomal genes (SSU rDNA) are available for fungi. Sequences were downloaded from GenBank and aligned with terrestrial and marine representative using Clustal X and edited by hand (Chenna *et al*., 2003). Selection of the 18S was mainly due the availability of sequences of a wide range of taxa, and the widespread usage of the gene for molecular clock calibrations. Sequences represented much of the taxonomic and ecological diversity among the three classes where freshwater ascomycetes are sampled. Our final dataset consisted of 94 species representing the major classes of the Kingdom Fungi (Table 2). Apart from representatives from various classes of ascomycetes, members of Chitridiomycota, *Glomales* (Zygomycota) and Basidiomycota were also included in our analyses. Outgroup taxa included 2 members of Metazoa and 1 collar flagellate.

(a) Phylogenetic analyses

Maximum likelihood was performed using the likelihood ratchet methods (Vos, 2003). This method was implemented to search for best trees using the computer programs PAUPRat (Sikes and Lewis, 2001) and Paup* 4b10

Table 2. Sequences of fungal taxa obtained from GenBank, with codes used in the phylogenetic trees.

Name	Code	Accession number	Group/Order
Collar flagellate			
Diaphanoeca grandis	Diaph gra	AF084234	
Basal animal			
Amoebidium parasiticum	Amoeb par	Y19155	Fungi/Metazoa incertae sedis
Ichthyophonus hoferi	Ichth hof	U25637	Fungi/Metazoa incertae sedis
Chytridiomycota			
Chytriomyces hyalinus	Chytr hya	M59758	*Chytridiales*
Chytridium polysiphoniae	Chytr pol	AY032608	*Chytridiales*
Rhizophlyctis rosea	Rhizo ros	AY635829	*Spizellomycetales*
Spizellomyces acuminatus	Spize acu	M59759	*Spizellomycetales*
Neocallimastix frontalis	Neoca fro	M6270	*Neocallimasticales*
Neocallimastix joyonii	Neoca joy	M62705	*Neocallimasticales*
Piromyces communis	Pirom com	M62706	*Neocallimasticales*
Sphaeromonas communis	Sphae com	M62707	*Neocallimasticales*
Zygomycota			
Endogone pisiformis	Endog pis	X58724	*Endogonales*
Gigaspora rosea	Gigas ros	X58726	*Glomales*
Glomus fasciculatum	Glomu fas	Y17640	*Glomales*
Glomus_intraradices	Glomu int	X58725	*Glomales*
Basidiomycota			
Hymenomycetes			
Physalacria maipoensis	Physa mai	AYF42695	*Agaricales*
Pleurotus tuberregium	Pleur tub	AF026595	*Agaricales*
Limnoperdon incarnatum	Limno inc	AF42695	*Aphyllophorales*
Nia vibrissae	Nia vib	AF334754	*Melanogastrales*
Ascomycota			
Dothideomycetes			
Jahnula australiensis	Jahnu aus	AF438182	*Jahnulales*
Jahnula bipolaris	Jahnu bip	AF438181	*Jahnulales*
Jahnula siamensiae	Jahnu sia	AF438180	*Jahnulales*
Patescospora separans	Patec sep	AF438179	*Jahnulales*
Byssothecium circinans	Bysso cir	AY016339	*Pleosporales*
Cochliobolus sativus	Cochl sat	U42479	*Pleosporales*
Herpotrichia diffusa	Herpo dif	U42484	*Pleosporales*
Kirschsteiniothelia elaterascus	Kirsc ela	AF053728	*Pleosporales*
Kirschsteiniothelia maritima	Kirsc mar	AF053726	*Pleosporales*
Leptosphaeria doliolum	Lepto dol	U43457	*Pleosporales*

Table 2 continued. Sequences of fungal taxa obtained from GenBank, with codes used in the phylogenetic trees.

Name	Code	Accession number	Group/Order
Lophiostoma bipolare	Lophi bip	AF164365	*Pleosporales*
Lophiostoma crenatum	Lophi cre	U42485	*Pleosporales*
Massarina australiensis	Massa aus	AF164364	*Pleosporales*
Montagnula opulenta	Monta opu	AF164370	*Pleosporales*
Pleospora herbarum	Pleos her	U43458	*Pleosporales*
Trematosphaeria hydrela	Trema hyd	AF164376	*Pleosporales*
Tubeufia helicoma	Tubeu hel	AF201455	*Pleosporales*
Eurotiomycetes			
Eupenicillium javanicum	Eupen jav	U21298	*Eurotiales*
Hamigera striata	Hamig str	AB003948	*Eurotiales*
Talaromyces flavus	Talar fla	M83262	*Eurotiales*
Leotiomycetes			
Articulospora tetracladia	Artic tet	AY357270	*Helotiales*
Chloroscypha chloromela	Chlor chl	AF203461	*Helotiales*
Dimorphospora foliicola	Dimor fol	AY357274	*Helotiales*
Geniculospora grandis	Genic gra	AY357276	*Helotiales*
Loramyces juncicola	Loram jun	AF203464	*Helotiales*
Phacidium infestans	Phaci inf	AF203466	*Helotiales*
Rhabdocline parkeri	Rhabd par	AF106016	*Heloitiales*
Darkera parca	Darke par	AF203465	Leotiomycetes incertae sedis
Saccharomycetes			
Candida albicans	Candi alb	X53497	*Saccharomycetales*
Debaryomyces hansenii	Debar han	X62649	*Saccharomycetales*
Sordariomycetes			
Camarops microspora	Camar mic	AY083800	*Boliniales*
Chaetomium elatum	Chaet ela	M83257	*Chaetomiales*
Aniptodera juncicola	Anipt jun	U43845	*Halosphaeriales*
Halosarpheia marina	Halos mar	AF352082	*Halosphaeriales*
Halosphaeria appendiculata	Halos app	U46872	*Halosphaeriales*
Lignincola laevis	Ligni lae	U46873	*Halosphaeriales*
Nimbospora effusa	Nimbo eff	U46877	*Halosphaeriales*
Nohea umiumi	Nohea umi	U46878	*Halosphaeriales*
Phaeonectriella lignicola	Phaeo lig	AF050484	*Halosphaeriales*
Chaetopsina fulva	Chaet ful	AB003786	*Hypocreales*
Cordyceps bifusispora	Cordy oph	AF339571	*Hypocreales*
Emericellopsis minima	Emeri min	U44043	*Hypocreales*
Hypocrea lutea	Hypoc lut	D14407	*Hypocreales*
Hypocrea schweinitzii	Hypoc sch	AF164357	*Hypocreales*

Table 2 continued. Sequences of fungal taxa obtained from GenBank, with codes used in the phylogenetic trees.

Name	Code	Accession number	Group/Order
Melanospora zamiae	Melan zam	U78356	*Hypocreales*
Nectria cinnabarina	Nectr cin	U32412	*Hypocreales*
Nectria lugdunensis	Nectr lug	AY204603	*Hypocreales*
Nectria pseudotrichia	Nectr pse	AY342011	*Hypocreales*
Sphaerodothis acrocomiae	Sphae acr	U76340	*Hypocreales*
Microascus cirrosus	Micro cir	M89994	*Miscroascales*
Petriella setifera	Petri set	U43908	*Miscroascales*
Pseduallescheria boydii	Pseud boy	U43915	*Miscroascales*
Ambrosiella sulfurea	Ambro sur	AY497509	*Ophiostomatales*
Endomyces scopilarum	Endom sco	AF267227	*Ophiostomatales*
Ophiostoma quercus	Ophio que	AY497515	*Ophiostomatales*
Ophiostoma stenoceras	Ophio ste	M85054	*Ophiostomatales*
Madurella mycetomatis	Madur myc	AF527811	*Sordariales*
Lasiosphaeria ovina	Lasio ovi	AY083799	*Sordariales*
Sordaria fimicola	Sorda fim	X69851	*Sordariales*
Cainia graminis	Caini gra	AY083801	*Xylariales*
Fasciatispora petrakii	Fasci pet	AY083809	*Xylariales*
Hyponectria buxi	Hypon bux	AF130976	*Xylariales*
Hypoxylon fragiforme	Hypox fra	AB014046	*Xylariales*
Microdochium nivale	Micro niv	AF548077	*Xylariales*
Xylaria carpophila	Xylar car	Z49785	*Xylariales*
Taphrinomycetes			
Taphrina wiesneri	Taphr wie	AY548293	*Taphrinales*
Incertae sedis			
Savoryella elongata	Savor elo	HKUCC	Ascomycetes incertae sedis
Savoryella longispora	Savor lon	HKUCC	Ascomycetes incertae sedis
Ascitendus austriaca	Ascit aus	AF24226	Sordariomycetes incertae sedis
Magnaporthe grisea	Magna gri	AB026819	Sordariomycetes incertae sedis
Ophioceras arcuatisporum	Ophio arc	AF050472	Sordariomycetes incertae sedis
Pseudohalonectria falcata	Pseud fal	AF050477	Sordariomycetes incertae sedis
Pseudohalonectria lignicola	Pseud lig	AF050478	Sordariomycetes incertae sedis
Phomatospora arenaria	Phoma are	CBS 372.92	Xylariales incertae sedis
Phomatospora sp.	Phoma spn	HKUCC	Xylariales incertae sedis

(Swofford, 2000). This method is especially useful for datasets with large number of taxa. A hierarchical maximum likelihood ratio test (LRT) with a tree calculated under Neighbour Joining criterion under the Jukes Cantor model (JC69) (Jukes and Cantor, 1969) was used to select an evolutionary model and estimate all parameters needed for the ML search using the software program MrModelTest 2 (Posada and Crandall, 1998). Gaps were treated as missing data in all analyses.

Bayesian phylogenetic analyses were performed using the program MrBayes 3.04b (Huelsenbeck and Ronquist, 2001) which calculates posterior probability using a MCMC approach with sampling according to the Metropolis-Hastings algorithm (Huelsenbeck and Ronquist, 2001). All of our analyses employed one cold chain and three incrementally heated chains, where the heat of the i^{th} chain is $B = 1/[1 + (i - 1)T]$ and $T = 0.2$. Joint posterior probability distributions were obtained for the phylogeny (including branch lengths) and the parameters of the model of sequence evolution. For the parameters of the model of sequence evolution, the one estimated for ML analyses through MrModeltest (Posada and Crandall, 1998) was used. Three million generations were sampled, with every 100^{th} generation resulting in 30000 trees. Likelihood values stabilised after 10000 to 300000 generations (Fig. 1). To further ensure that we include trees after the chain had reached a stable value, the burnin was fixed at 30000 generations which produced 24000 sampled trees to calculate the posterior probabilities in a consensus tree. The analysis was repeated 3 times starting from random trees to ensure that different tree space were being sampled.

In addition to the bayesian posterior probability, phylogenetic confidence was also assessed using the neighbour joining bootstrap proportions with the model settings as derived through MrModeltest.

(b) Molecular dating

The same original dataset consisting of 94 taxa for the small subunit ribosomal DNA (18S rDNA) was used for dating. As the relaxed molecular clock relies on a topology to infer divergence times, we used the bayesian tree topology (Fig. 1) as previously identified, which conforms to the current views on ascomycete/basidiomycete relationships.

In the bayesian relaxed molecular clock approach (Thorne *et al*., 1998), it is important to use prior constraints on independent calibration points dispersed across the tree in order to reduce potential regional effects (Kishino *et al*., 2001). To provide comparability among studies, we used the calibrations according to Berbee and Taylor (2001) that are comparable with our taxon sampling. Among the four calibration points defining the priors constraints;

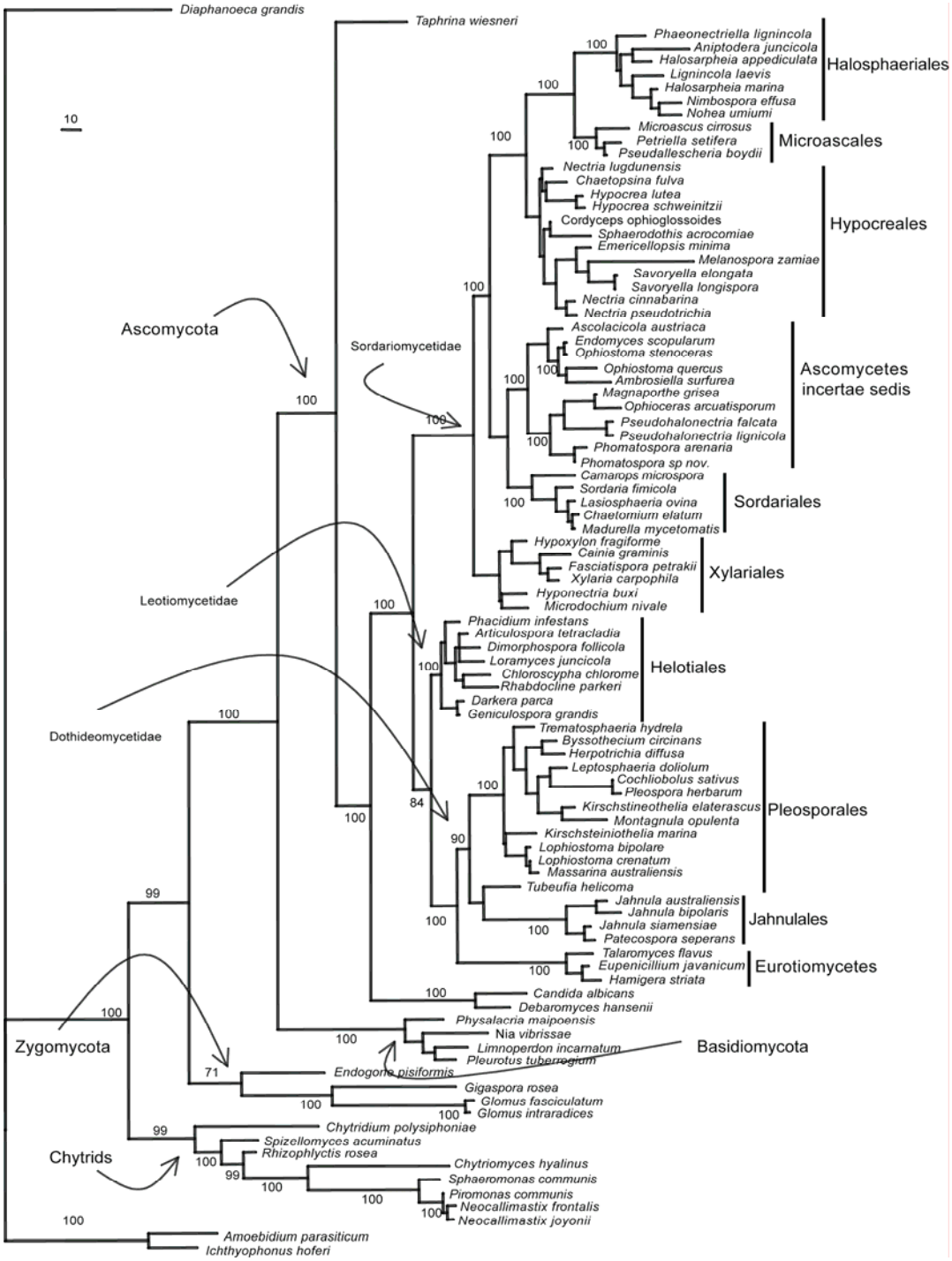

Fig1. Maximum likelihood tree with Bayesian posterior probability (%) at internodes.

three were minimum age constraints and one was a maximum age constraint. In all subsequent bayesian analyses these six prior constraints on calibration were simultaneously used to derive posterior estimates of divergence ages (Kishino *et al*., 2001).

The analysis of divergence time estimation was conducted combining the two different approaches: the likelihood ratio test (LRT: Felsenstein, 1981) and the bayesian approach (Kishino *et al*., 2001) as described in a step-by-step manual by Rutchmann (2004). In brief, the dataset was subject to maximum likelihood analyses using the modules BASEML in the PAML 3.12 program package (Yang, 1997), using the tree shown in Fig. 1. This was done for the estimation of model parameters such as, unequal nucleotide frequencies, transition/transversion rate ratio (parameter: κ) and the rate heterogeneity among sites (Shape parameter: α including a discrete γ model of rates among sites). The bayesian relaxed molecular clock was applied using the software package MULTIDIVTIME. The program ESTBRANCHES was first used to compute branch lengths of the constrained topology. The program MULTIDIVTIME used the variance-covariance matrices produced by ESTBRANCHES to run a MCMC for estimating mean posterior divergence times on nodes with associated standard deviation (SD) and 95% credibility interval. The following settings were used: numstamp (10,000), burnin (100,000), and samplefreq (100). The following priors were used for the expected number of time units between tip and root if there has been no constraint on node times, and 1000 MYA for the minimum possible number of time units between tip and root. Other priors for gamma distribution of the rate at root node and the Brownian motion constant describing the rate variation (i.e. the degree of rate autocorrelation along the descending branches of the tree), were derived from the median branch length for each data set as advised by Thorne *et al*. (1998).

Results and Discussion

Dataset

The final alignment of the 18S rDNA sequences for the 94 taxa was 1641 characters long. The general time reversible model including invariant sites and assuming a discrete gamma distribution (GTR + γ + I) was the best-fit model estimated through MrModeltest 2. The settings for the GTR + γ + I were as follows: R-matrix = (1.1370, 2.9815, 1.4865, 0.7903, 4.9487, 1.0000; base frequencies = (A=0.2471, C=0.2105, G=0.2660; T=0.2764); proportion of invariant sites = 0.3675; and shape of the gamma distribution = 0.6303. This

model of evolution was used in the Maximum likelihood analysis using the likelihood ratchet method. To assess clade stability, in addition to bayesian posterior probability, which is known to show high support for wrong topology, we also assessed clade stability based on a neighbour joining bootstrap proportion. Figure 1 shows the maximum likelihood tree with bayesian posterior probabilities at the internodes.

Phylogeny

Phylogeny shows significant bayesian support for most of the fungal groups and subgroups (Fig. 1). Among the four major clades within fungi, both Ascomycota and Basidiomycota were highly supported, by 100% bayesian posterior probability (BPP), while the neighbour joining bootstrap proportions (NJBP) were 100% and 97 % respectively. Chytrids were moderately supported (Bpp = 99%, NJBP = 83), while, the Zygomycota comprising *Endogone* and the *Glomales* was supported with 100% BPP, but, did not receive clade support for the NJBP. However, the *Glomales* received high support (BPP = 100, NJBP = 98). The major divisions and relationships are similar to those found previously with analyses of fungal genome sequences (e.g. Lutzoni *et al*., 2004).

Test for molecular clock

The molecular clock hypothesis is rejected for the SSU rDNA data set by a likelihood ratio test under the F84 model (Felsenstein, 1984). This reveals that extensive rate variation occurs among different lineages, with fast evolving taxa and slow evolving ones distributed all over the tree. This could also be due to the uneven taxon sampling, with high density of taxa among certain lineages. Thus, the use of a relaxed molecular clock approach designed to accommodate rate variation was preferable for estimating divergence ages with this dataset.

Estimation of divergence time

Times of divergence were estimated for all nodes in the phylogenies as derived through the consensus of 24000 generations of the bayesian analysis, using the 4 calibrations points described in Table 3.

The chronogram obtained on the 18S rDNA data set is presented in Figure 2. Based on this data, the mean posterior age for the basal animal-fungal group, is estimated to be 1150 ± 220 MYA (95% credibility interval: 870-

Table 3. Calibration points for evolutionary analyses.

Divergence points	Paleontological data	Age (Myr)	References
Basal animals – Fungi	Radiation of animals and fungi (minimum age constraint)	965	Doolittle *et al*., 1996
Endogonales - rest of Fungi	Evolution of terrestrial taxa (minimum age constraint)	800	Berbee & Taylor, 2001
Ascomceyetes - basidiomycetes	Identification of fossil clamp connections (minimum age constraint)	600	Berbee & Taylor, 2001
Stomach chitrids - free living chytrids	Stomach chitrids evolution after the evolution of marsupials (maximum age constraint)	150-200	Berbee & Taylor, 2001

1670). This early split is followed by the divergence between the Chytrids and the rest of fungi 1020 ± 140 MYA (820 – 149). Accordingly, the ancestral marine fungi, should have their ancestors, in the Precambrian era (Fig. 2). The evolution of terrestrial fungi, is seen at the divergence between *Endogone* and the rest of fungi at 890 ± 160 MYA (800-1400) and the Ascomycetes-Basidiomycetes divergence at 780 ± 160 MYA (520-1100).

The earliest divergence of freshwater species is seen in the *Jahnulales* at 380 ± 100 MYA (220-660), however most of the freshwater lineages appear to have diverged during the Mesozoic period (66-245 MYA).

The evolution of fungi on land has been one of the most intriguing questions in mycology. Some of the few studies which have attempted to date the evolution of fungi on land, have varied greatly (600 MYA and 1200 MYA) (Berbee and Taylor., 2001; Heckmann *et al*., 2001). The estimated dates in our study based on a 18S rDNA dataset, is at 890 MYA. Our results are much later than the dates estimated by Heckmann *et al*. (2001). Sanderson (2003), also showed that the dates proposed by Heckman *et al*. (2001) to be much earlier, and suggested this might be due to calibration errors and the assumptions of a molecular clock. Our divergence estimates are also in odds with those estimated by Berbee and Taylor (2001), who used the same 18S rDNA gene, yet their dates were around 600 MYA. A possible explanation for the differing conclusions is that the earlier studies were based on the molecular clock hypothesis (Zuckerlandl and Pauling, 1965), which did not hold good for out dataset. It has been suggested that violation of the clock could have drastic effects on date estimation (Brohman and Penny, 2000; Yoder and Yang, 2000).

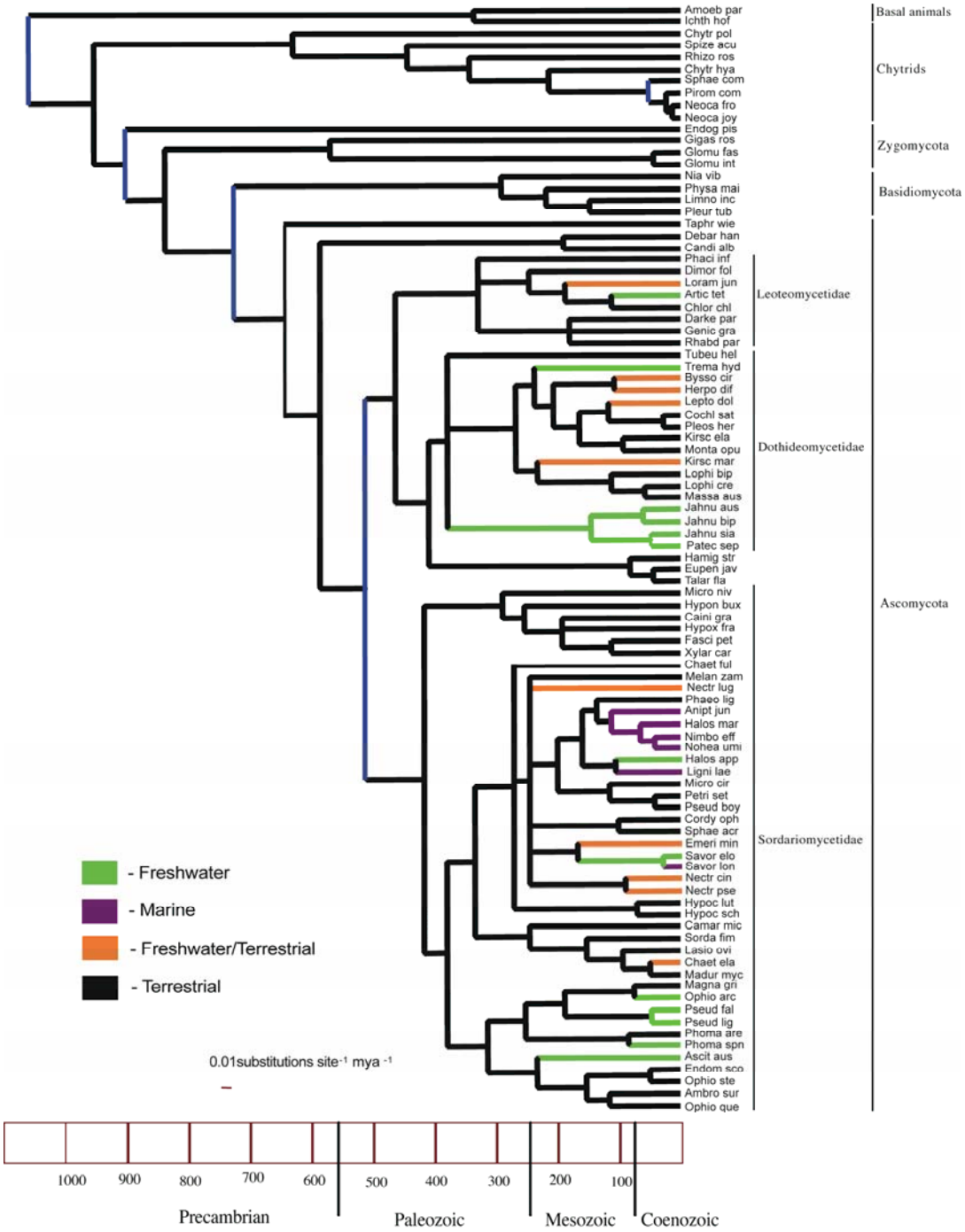

Fig 2. A timescale of fungal evolution. Thick blue lines indicating calibration points used.

The ascomycetes, basidiomycetes and *Glomales*, which diverged after the initial colonization of land, are mainly saprobic on vascular plants. The estimation of their divergence time at 890 MYA is almost two folds older than the most recent dates obtained from the evolution of land plants (483-490 MYA, Sanderson, 2003). With the absence of land plants at 890 MYA, which are the main sources of food for fungi that have colonised land, how did these fungi survive? Berbee and Taylor (2001) suggested that the actual origin of land plants could be much older than their fossil records, or that the fungi were initially associated with the ancestors of the vascular land plants, following their hosts onto land.

The only fossil record which clearly indicates the earliest possible presence of perithecial ascomycetes is at Rhynie Chert deposits dating 400 MYA (Taylor *et al*., 2004). Our dates suggest that the filamentous ascomycetes originated during the Cambrian era (550 MYA) about 150 MY earlier than the fossil record.

Multiple origins of freshwater ascomycetes

The topology of the tree obtained through the maximum likelihood and bayesian analyses accords well with the classification system of fungi derived from other phylogenetic studies (Lutzoni *et al*., 2004) and was assumed to be true for the date estimates. Phylogeny clearly shows that freshwater taxa have evolved independently through several lineages (Fig. 1). Furthermore, it is also evident that freshwater taxa have evolved directly from terrestrial species.

For the convenience of discussing evidence for their origin based on morphological and molecular characters from previously derived phylogenies, freshwater fungi that are present in three classes are discussed into five morphological groups based on characteristics of their asci and ascospores.

Class: Sordariomycetes

Annulatascaceae

The *Annulatascaceae* was introduced by Wong *et al*. (1998b) to accommodate the freshwater genus *Annulatascus* and related genera (Ho and Hyde, 2000). Species in this family have asci with massive apical rings that probably facilitate strong ejection of ascospores in air and under water, and may be adapted for dispersal in freshwater, or in the air when washed to the banks of rivers (Hyde and Goh, 2003). The apical ring comprises of a thick electron dense upper region and an extensively elongated lower region that is

filled with compact electron-dense granules. Only the inner wall layer is responsible for the ring formation, the outer ascus wall layer disintegrates at the apex (Ho and Hyde, 2000). The ascospores in most species are equipped with sheaths or unique appendages (Goh and Hyde, 1996, 1999; Hyde *et al*., 1997; Ho and Hyde, 2000; Ho *et al*., 2004). Appendages in marine fungi are known to be important in the dispersal and attachment of ascospores to substrata (Hyde and Jones, 1989; Hyde *et al*., 2000b). The species can also degrade submerged wood (Yuen *et al*., 1998). It is likely that these three factors are responsible for the success of these fungi in freshwater ecosystems (Hyde *et al*., 1997). The *Annulatascaceae* were originally thought to be unique to freshwater (Wong *et al*., 1998a). However, Fröhlich and Hyde (2000) have discovered two new terrestrial species from palms and two terrestrial species are known from bamboo (Dalisay, 1998) that has taxonomic affinities in this family. There has been no record of any marine genera with morphological similarities to the *Annulatascaceae*.

Annulatascaceae now consists of 14 genera. Recent molecular sequence data have been used to shed light on the phylogeny of *Annulatascaceae*. 28S rDNA sequences have been widely used to analyse the phylogenetic affinities of the *Annulatascaceae* and various views regarding their phylogeny have been reported. Ranghoo *et al*. (1999) analysed partial sequences from the 28S rDNA to determine the taxonomic placement of *Ascotaiwania* and *Ascolacicola* at the familial level. Two *Annulatascus* species and seven other annulatascaceous taxa were also sequenced as part of this study. In the resulting trees these taxa clustered with two sordariaceous species, indicating a closer phylogenetic relationship to the *Sordariaceae* than other pyrenomycete families. The *Sordariales* comprise mostly terrestrial taxa and provide evidence that the *Annulatascaceae* have affinities with terrestrial fungi.

Réblova and Wong (2001) found only *Clohesia corticola* had affinities with the *Sordariales*, while the principal genus *Annulatascus* clustered with *Aquaticola* and *Rhamphoria* representing the *Annulatascaceae*/ *Trichosphaeriaceae* clade. The *Trichosphaeriaceae*, mainly contains terrestrial saprobic species. Recently, Huhndorf *et al*. (2004) found *Annulatascus*, the principle genus of the *Annulatascaceae* shared phylogenetic affinities to the *Ophiostomatales*, which mainly consists of plant pathogens. Due to the lack of bootstrap support, the affinities of *Annulatascaceae* to either one of the two groups (*Trichosphaeriales* or *Ophiostomatales*) could not be confirmed. However, recently, *Annulatascaceae* was found to form a monophyletic clade with members of both the orders with low bootstrap support with the inclusion of additional freshwater taxa (Vijaykrishna *et al*., 2005).

Annulatascaceae was not included in our analysis of the 18S rDNA analysis, because of the absence of data in the GenBank. Phylogenetic studies based on the 28S rDNA show a relationship of *Annultascaceae* to the *Ophiostomatales* (Vijaykrishna *et al*., 2005) indicating a terrestrial origin of the family. Extrapolating the results obtained from our divergence estimates, *Annulatascaceae* members should have evolved between 200 to 300 MYA (Fig. 2).

Terrestrial unitunicate ascomycete genera with aquatic species

Several genera previously known from terrestrial habitats have also been shown to have common freshwater representatives, e.g. *Phomatospora*, *Saccardoella*, *Zopfiella* (Shearer, 1993). Many of the species in these genera occur in terrestrial habitats. Species of *Chaetomium*, *Coniochaeta* and *Nectria* have infrequently been recorded in freshwater (Shearer, 1993). As species in these genera are usually found in terrestrial habitats, the aquatic species are probably not truly aquatic taxa. Species of *Ophioceras* and *Pseudohalonectria* are more frequently identified in freshwater habitats (Chen *et al*., 1995) than in terrestrial habitats (Hyde *et al*., 1999e; Fröhlich and Hyde, 2000).

Phylogenetic ordinal placement of the terrestrial unitunicate genera with aquatic species clearly shows the multiple origins of freshwater ascomycetes. The above species can be seen in three distinct clades within monophyletic pyrenomycetes clade. *Ophioceras*, *Phomatospora* and *Pseudohalonectria* showing a closer relationship to the *Magnaporthaceae*. Whilst *Chaetomium* and *Coniochaeta* are closer to the Sordariales, *Nectria* falls within the Hypocreales, providing further evidence that these aquatic taxa had terrestrial predecessors.

Time estimates of the evolution of terrestrial and freshwater species of *Ophioceras*, *Phomatospora* and *Pseudohalonectria* show a fairly recent evolutionary divergence (approx 40 – 190 MYA).

Halosphaeriales

The *Halosphaeriales* are rather unique amongst freshwater fungi in not having terrestrial representatives, but in being well represented by marine species. The *Halosphaeriaceae* contains two distinct morphological groups: 1) those with appendaged ascospores and early deliquescing asci, and 2) those with persistent asci, often with an apical apparatus (it is not known if this is functional), and mostly with ascospores with polar filamentous unfurling appendages (Hyde *et al*., 1999d, 2000b). The first group is not common in

freshwater and only represented by three species of *Fluviatispora* and one species of *Ayria* (Fryar and Hyde, 2004). Species with early deliquescing asci are rare in freshwater fungi and this character may not be a good adaptation in this milieu. The second group, are however, common in marine and freshwater habitats (Hyde *et al*., 1999d). The possible ability of their asci to eject ascospores, and the presence of ascospores with unfurling filamentous appendages, may be important for dispersal and subsequent attachment in freshwater habitats (Goh and Hyde, 1996).

Spatafora *et al*. (1998) analysed partial sequences from the large and small subunit rDNA from 15 marine representatives of the *Halosphaeriaceae* and integrated this with a data set of homologous sequences from terrestrial ascomycetes. In their analyses, a group of *Halosphaeriales* (e.g. *Aniptodera*, *Ceriosporopsis*, *Corollospora*, *Halosarpheia*, *Nimbospora)* clustered with other terrestrial *Microascales*. Representatives of *Lindra* and *Lulworthia*, which are marine, formed a well-supported clade that was isolated among the perithecial ascomycetes and these genera have recently been accommodated in *Lulworthiales* a new fungal order (Kohlmeyer *et al*., 2000). Spatafora *et al*. (1998) therefore concluded that *Halosphaeriales* and the separate *Lulworthiales* (*Lulworthia/Lindra* clade) were independently derived from terrestrial ancestors. Members of the *Microascales* are characterised by evanescent asci with passively discharged ascospores, which are dispersed by insects. The origin of evanescent asci may have evolved on several occasions being adapted from insect dispersal and dispersal in the marine environment. The Halosphaeriales have been shown to have terrestrial ancestors that have evolved morphologically to adapt to marine environment (Spatafora *et al*., 1998).

In a similar study, partial sequences of the LSU rDNA from representatives of freshwater and marine *Halosphaeriales* clustered into two separate groups (Ranghoo *et al*., 2000). One group contained representatives of *Aniptodera* and *Savoryella*, while the second group contained representatives of the marine *Halosphaeriales* (e.g. *Corollospora*, *Halosphaeriopsis*, *Halosarpheia*, *Nimbospora*). However, the marine and freshwater representatives of the genera *Aniptodera* and *Savoryella* grouped together in their individual genera, rather than separating according to their habitats. Kang *et al*. (2000) analysed the SSU rDNA of four *Halosarpheia* species, *Lignincola laevis* and *Nais inornata* and found that these marine species formed a subclade with taxa from the *Microascales*, indicating a terrestrial origin. Okada *et al*. (1997) also sequenced 19 marine fungi and showed that the *Microascales* and *Halosphaeriales* are phylogenetically related.

Based on SSU and LSU rDNA sequences, Campbell *et al*. (2003), reported that the presence or absence of the unfurling thread like appendages is not a good phylogenetic marker within the aquatic genus *Halosarpheia*. They also found that species occurring in freshwater habitats are phylogenetically distinct from those occurring only in marine habitats. Furthermore, at the morphological level, significant differences can be found between marine and freshwater representatives of the *Halosphaeriales*. Species of *Corollospora*, *Halosphaeriopsis*, *Halosphaeria* and *Nimbospora* are characteristic of the *Halosphaeriales*, having deliquescing asci which lack an apical ring, and ascospores with different kinds of appendages (Jones, 1995). Species of *Aniptodera* and *Savoryella* (marine and freshwater), however, have persistent asci with definite apical rings (Hyde *et al*., 1999d), but in freshwater representatives of *Halosarpheia* the asci deliquesce much earlier than marine representatives of *Halosarpheia* (Campbell *et al*., 2003).

Ranghoo (1998) indicated that marine and freshwater representatives of the *Halosphaeriales* are closely related to each other. Spatafora *et al*. (1998) had already shown that marine *Halosphaeriales* were related to terrestrial *Microascales* and could have originated from them. Accordingly we can infer that freshwater representatives of the *Halosphaeriales* could have terrestrial ancestors.

The data of Spatafora *et al*. (1998), Ranghoo (1998) and Campbell *et al*. (2003) provide support to the hypothesis that the Halosphaeriales are secondary marine ascomycetes, having derived from terrestrial predecessors and do not support the hypothesis of Kohlmeyer and Kohlmeyer (1979a,b), that the *Halosphaeriales* are primary marine fungi. Taking into account the freshwater representatives, the Halosphaeriales are therefore secondary aquatic ascomycetes as a whole. Another intriguing question is: could freshwater habitats be a pathway for certain genera to have become adapted to marine environments? To understand this, a wider species sampling among the Halosphaeriales, can be used to trace ancestral character states.

Class: Dothideomycetes

The Loculoascomycetes have bitunicate asci, which are effective discharge structures (Shearer, 1993). Many terrestrial genera, e.g. *Didymella*, *Lophiostoma*, *Massarina*, contain species that occur in freshwater and marine habitats, while there are a few genera that are restricted to either marine or freshwater habitats, e.g. *Manglicola* and *Jahnula* respectively (Kohlmeyer and Kohlmeyer, 1979 *b*; Hyde and Wong, 1999). The discharge abilities of the asci in these taxa are probably important characters for the survival in aquatic

habitats, as the ascospores need to be ejected through air or possibly in water. Most loculoascomycetes have ascospores with mucilaginous sheaths, and Hyde and Jones (1989) have shown these to be important in dispersal in marine environments. It therefore appears that Loculoascomycete spores may be pre-adapted for dispersal in water and that many genera, e.g. *Leptosphaeria*, *Massarina*, *Trematosphaeria*, have evolved species capable of an aquatic lifestyle.

Comparative analyses based on molecular phylogenetic information have shown the relatedness of marine, freshwater and terrestrial taxa supporting the terrestrial origin of aquatic loculoascomycetes (Spatafora e*t al*., 1995; Liew *et al*., 2000). Marine members of Melanommatales and Dothideales clustered with the terrestrial Dothideales and Pleosporales (Spatafora *et al*., 1995). Liew *et al*. (2000) included two aquatic fungi (i.e. *Massarina australiensis*, *M. bipolaris*) in their analysis of Loculoascomycetes with pseudoparaphyses. These taxa clustered within the groups comprising terrestrial Dothideales and Pleosporales indicating a terrestrial origin.

Nucleotide sequence analyses show that freshwater Dothideomycete taxa have evolved in clearly three distinct lineages. Members of *Jahnula* belonging to the order Jahnulales, form a well-supported clade separate from the other members of the Dothideomycetes. The order Jahnulales was erected to accommodate a very unique group of freshwater ascomycetes, with stalks subtending the ascomata and containing dimorphic ascospores (Pang *et al*., 2002). The Jahnulales show the earliest divergence times for freshwater ascomycetes in our estimates (390MYA). Other freshwater taxa from the class Dothideomycetes are dispersed among terrestrial species. The third group Tubeufiaceae, better represented in aquatic habitats, in their anamorphic form (e.g. *Helicosporium*, *Helicoma*). Therefore, molecular systematics and morphological evidence provide strong evidence that loculoascomycetes have repeatedly gained the ability to inhabit freshwater habitats.

Class: Leotiomycetes

Fungi that produce cup-shaped fruiting structures (apothecial ascomycetes) are commonly known as cup fungi or Discomycetes. Fungi with apotheicial ascomata are found in three classes, viz. Pezizomycetes, Leotiomycetes and Leconaromycetes. Freshwater genera, however, are found only in the Class Leotiomycetes.

Freshwater Discomycetes are not common in tropical freshwater habitats, but are frequently isolated from submerged grasses and twigs in the temperate region (Shearer, 1993). Many discomycete genera found in the aquatic habitats

e.g. *Hymenoscyphus*, *Lophodermium*, *Mollisia*, are better represented in terrestrial habitats. Species of *Apostemidium*, *Niptera* and *Vibrissea* are mostly confined to freshwater environments. The sigmoid or filiform ascospores in species of these genera appear to be adapted for dispersal and attachment in aquatic habitats (Webster and Davey, 1984; Shearer, 1993). *Aquadiscula* and *Loramyces* are genera described to accommodate freshwater species. In *Loramyces* the ascospores are filiform and have a large mucilaginous head and filiform tail and seem to be well adapted for dispersal and subsequent attachment in water (Goh and Hyde, 1996). In *Aquadiscula*, ascospores have a mucilaginous appendage (Shearer and Crane, 1985). The freshwater discomycetes appear to have no other special features, besides the sigmoid and filiform ascospores, adapted to the aquatic habitat. However, the filiform ascospores are also found in terrestrial discomycetes.

A number of Ingoldian fungi have their sexual stages within the Leotiomycetes. These aquatic (Ingoldian) hyphomycetes grow in aquatic habitats and release a large number of conidia, with branched, sigmoid or helicoid conidia, which become trapped in foam (Sakayaroj *et al*., 2005). The foam is however temporary inoculum of conidia, with high viability, which eventually get attached to suitable substrata (Sridhar and Barlocher, 1994; Sakayaroj *et al*., 2005). Therefore, their existence in freshwater habitats in their asexual form is itself an adaptation for their continued occurrence in freshwater.

Conclusion

The major finding of this study is that freshwater fungi, like marine fungi (Spatafora *et al*., 1995), have evolved from terrestrial ancestors. Molecular data show that freshwater ascomycetes have evolved separately through different lineages, which supports the hypothesis proposed by Shearer (1993). Unlike, freshwater hyphomycetes, lignicolous fungi appears appear to have few special adaptations to survive in freshwater habitats. The majority of the characters that appear to be adaptations to freshwater habitats can also be seen in terrestrial fungi (eg. ascospore sheath, and presence of active discharge mechanisms). The lack of exclusive adaptations further indicates, that freshwater ascomycetes share a common ancestor with terrestrial ascomycetes. Morphological and molecular data indicate that the freshwater and marine Halosphaeriales have terrestrial ancestors. One of the major findings of Spatafora *et al*. (1998) is that some of the major lineages of marine fungi were derived from terrestrial predecessors that possessed evanescent asci. Whether the marine representatives of these genera evolved from terrestrial or

freshwater ancestors remains unknown. It would be interesting to select species from these different habitats for molecular studies to investigate the ancestral states of freshwater and marine lineages.

The appearance of freshwater ascomycetes in distant parts of the world has always been intriguing and several possible mechanisms of dispersal have been proposed by Hyde and Goh (2003). Our estimates show the earliest possible origins of freshwater taxa at 390 MYA. This is much earlier than the separation of Pangea into several continents, indicating that the dispersal may have occurred with separation of continents. Evidence which refutes this however, are the freshwater fungal communities on newly formed volcanic islands (e.g. Hawaii), which are also similar to those on older continents (Eldredge and Miller, 1995), indicating that fungi must have arrived in these islands by various dispersal mechanisms (Hyde and Goh, 2003). Studies are needed to understand the biogeography, of commonly found freshwater ascomycetes from different parts of the world. Studies on rDNA sequences have provided valuable insights in the biogeography and evolution of several fungi (Isikhuemhen, 1999; Moncalvo, 2000, 2002). The methods used should be extended to understand the biogeography of widely distributed freshwater fungi, which might provide an answer for the appearance of freshwater fungi in newly formed islands.

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