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The molecular phylogeny of aquatic hyphomycetes with affinity to the Leotiomycetes

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ABSTRACT

Aquatic hyphomycetes play a key role in decomposition of submerged organic matter and stream ecosystem functioning. We examined the phylogenetic relationships among various genera of aquatic hyphomycetes belonging to the Leotiomycetes (Ascomycota) using sequences of internal transcribed spacer (ITS) and large subunit (LSU) regions of rDNA generated from 42 pure cultures including 19 ex-types. These new sequence data were analyzed together with additional sequences from 36 aquatic hyphomycetes and 60 related fungi obtained from GenBank. Aquatic hyphomycetes, characterized by their tetraradiate or sigmoid conidia, were scattered in nine supported clades within the Helotiales (Leotiomycetes). *Tricladium*, *Lemonniera*, *Articulospora*, *Anguillospora*, *Varicosporium*, *Filosporella*, and *Flagellospora* are not monophyletic, with species from the same genus distributed among several major clades. The *Gyoerffyella* clade and the *Hymenoscyphus* clade accommodated species from eight and six different genera, respectively. Thirteen aquatic hyphomycete taxa were grouped in the *Leotia-Bulgaria* clade while twelve species clustered within the *Hymenoscyphus* clade along with several amphibious ascomycetes. Species of *Filosporella* and some species from four other aquatic genera were placed in the *Ascocoryne-Hydrocina* clade. It is evident that many aquatic hyphomycetes have relatives of terrestrial origin. Adaptation to colonize the aquatic environment has evolved independently in multiple phylogenetic lineages within the Leotiomycetes.

Introduction

Aquatic hyphomycetes are defined as an ecological group of fungi that inhabit submerged leaf litters, decaying wood (Bärlocher 1992; Suberkropp 1992) and roots of riparian vegetation (Fisher *et al.* 1991; Sati & Belwal 2005), or submerged plants (Kohout *et al.* 2012). Studies of the biodiversity, physiology and ecology of these fungi in recent years resulted in

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better understanding of their critical importance in plant litter decomposition and stream ecosystem functioning (Gessner *et al.* 2007; Krauss *et al.* 2011).

Aquatic hyphomycetes comprise over 300 species; most of them belong to the Ascomycota (Webster 1992; Shearer *et al.* 2007). The characteristic traits of most aquatic hyphomycetes are stauroconidia (e.g. tetraradiate or variously branched) or less frequently scolecoconidia (sigmoid, curved or straight) produced as morphological adaptations to survival and dispersal in aquatic habitats (Webster 1959a; Dix & Webster 1995). The current taxonomic concepts of genera are based on conidial morphology and the mode of conidiogenesis. Most aquatic hyphomycetes are holoanamorphic, however, the direct connections between sexual and asexual states through pure culture (from ascospores to conidial state or, rarely, from conidial state to ascoma) have been established only in about 10 % of all known species (Webster 1992; Marvanová 1997, 2007; Sivichai & Jones 2003).

Within the Ascomycota, according to our present knowledge, aquatic hyphomycetes belong to the subphylum Pezizomycotina and they are distributed among five classes based on relationships to sexual states (Marvanová 2007). Molecular studies (Belliveau & Bärlocher 2005; Baschien *et al.* 2006; Campbell *et al.* 2006, 2009; Vijaykrishna *et al.* 2006; Shearer *et al.* 2009; Seena *et al.* 2010) have confirmed the placement of several aquatic hyphomycetes species in the same major classes: Sordariomycetes (~11 spp.), Dothideomycetes (~10 spp.), Pezizomycetes (1 sp.), Orbiliomycetes (3-5 spp.) and Leotiomycetes (>75 spp., this study). The polyphyly of aquatic hyphomycetes had been already recognized by Webster (1961) who first described the *Nectria lugdunensis* state (Hypocreales) of the aquatic hyphomycete *Heliscus* (Webster 1959b), followed by the description of a *Mollisia* sp. (Helotiales) as the sexual state of *Anguillospora crassa*. Molecular data also revealed the polyphyly of various aquatic hyphomycete genera (Belliveau & Bärlocher 2005; Baschien *et al.* 2006; Campbell *et al.* 2006, 2009). Indeed, based on the morphological studies, the two predominant spore shapes, sigmoid and tetraradiate have evolved multiple times independently in unrelated taxa (Ingold 1966; Webster 1980; Marvanová 2007). The contemporary system based mainly on similarity of conidia and conidiogenesis often does not reflect the phylogenetic relationships among taxa. However, for many genera, we do not yet have enough additional information inferred from phylogenetic studies to replace the taxonomic system based solely on morphology. In particular, sequences from ex-type or authentic cultures are often lacking.

Helotiales is the largest order within the Leotiomycetes which represents a morphologically and ecologically diverse class of Pezizomycotina. It contains an assemblage of fungi that form apothecia with inoperculate, unitunicate asci. Members of Helotiales are saprotrophs in terrestrial and aquatic habitats (including aquatic hyphomycetes), plant pathogens, ectomycorrhizal symbionts or endophytes. Environmental sequencing studies of leaves, roots or rhizosphere often result in a high number of Leotiomycetes sequences (e.g. Kohout *et al.* 2012; Toju *et al.* 2013) of which many are from uncultured fungi. Interestingly, several aquatic hyphomycetes have been reported as plant endophytes that nested in the Helotiales (Sridhar & Bärlocher 1992; Selosse *et al.* 2008). However, the overall systematics of the Helotiales is unstable, and many of the genera are polyphyletic because currently deployed characters are insufficient to delineate taxa (Wang *et al.* 2006a). The whole life cycle is also poorly understood, and many helotialean fungi are known from their sexual state only.

The phylogenetic relationships of the majority of aquatic hyphomycetes and the extent of convergence of morphological conidial characters that delineate the asexual genera are mostly unknown. These gaps in our understanding mostly persist due to the scarcity of sequences of ex-type or authentic cultures. In this study, we analyzed sequence data from the partial large subunit (LSU) and internal transcribed spacer (ITS) regions of ribosomal DNA from 42 pure cultures of aquatic hyphomycetes, including 19 ex-types that were combined with sequence data obtained from GenBank. The objectives of our study were (i) to provide reference (barcoding) sequence data of aquatic hyphomycetes with affinity to the Leotiomycetes using ex-type or authentic cultures from major collections, (ii) to resolve the phylogenetic placement of aquatic hyphomycetes in the Leotiomycetes, and (iii) to examine their evolutionary relationships to helotialean ascomycetes with aquatic and non-aquatic ecology.

Materials and methods

Taxon sampling

We studied 42 isolates of aquatic hyphomycetes including 19 ex-type or ex-neotype cultures from 16 genera and 40 species (Table 1). The molecular data determined from these isolates were compared with sequences of the existing data set of aquatic hyphomycetes in GenBank (36 sequences from 35 species), and sequences of 60 helotialean fungi closely related in ecology and/or taxonomy based on Wang *et al.* (2006a,b; Table 1).

DNA isolation, PCR, and sequencing

Cultures were maintained on 2 % malt extract agar (MEA). Mycelia were harvested directly from MEA plates. Genomic DNA was extracted using the Ultra Clean® Soil DNA Isolation Kit in conjunction with the Vortex Adapter for Vortex-Genie® 2 (MO BIO, Carlsbad, CA, USA) or the FastDNA®SPIN kit for soil in conjunction with the FastPrep® FP120 instrument (Qbiogene, Heidelberg, Germany) according to the manufacturer's instructions. The ITS and the partial LSU regions of rDNA were then amplified by PCR. The ITS region was amplified with primers SR6R (http://www.biology.duke.edu/fungi/mycolab/primers.htm) and LR1 (Vilgalys & Hester 1990), while the primer pair LROR and LR7 (Bunyard *et al.* 1994) was used to amplify a ca. 1400 bp fragment from the LSU region. PCR mixtures contained 10 μl PCR Mastermix M7502 (Promega, Madison, WI, USA), 20 pM of each primer, 40–200 ng of genomic DNA and 8 μl nuclease free water. The PCR was performed with an initial denaturation step for 2 min at 94 °C, followed by 25–35 cycles of denaturation for 1 min at 94 °C, 45 s primer annealing at 46–50 °C (ITS) or 54 °C (LSU) and elongation for 1 min at 72 °C, final extension was for 5 min (10 min for LSU) at 72 °C. The

Table 1 – Sources and GenBank accession numbers of species used in this study. 💧 = Aquatic hyphomycetes,* = type species of aquatic hyphomycete genera. Sequences indicated in bold were generated in this study or from earlier investigations of CB. Strains labelled CCM F-are mostly isolated by L. Marvanová, CB by C. Baschien and VG by V. Gulis. If they were deposited by someone else, the depositor's name is in parentheses.

Species	Strain	Source	GenBank ITS	GenBank LSU
💧 *Alatospora acuminata*	CCM F-37194	Stream foam, CA	AY204590	–
💧 *Alatospora acuminata*	CCM F-02383	Stream foam, GB	AY204587	**KC834018**
💧 *Alatospora constricta*	CCM F-11302, (ex-type, = ATCC 32680)	Stream, angiosperm leaf, USA	**KC834040**	**KC834017**
💧 *Alatospora flagellata*	CCM F-501, ex-type of *Alatospora crassipes*	Stream, *Fagus sylvatica* leaf, CZ	**KC834041**	–
💧 *Alatospora pulchella*	CCM F-502, ex-type	Stream, *Athyrium filix-femina* frond, CZ	**KC834039**	**KC834019**
💧 *Anguillospora crassa*	CCM F-15283	Sessile apothecia on angiosperm twiglet, SK	AY204581	–
💧 *Anguillospora filiformis*	CCM F-20687	Stream foam, CA	AY148104	–
💧 *Anguillospora furtiva*	CB-L16	Stream foam, AT	**KC834038**	–
Arachnopeziza variepilosa	M337	N/A, CA	EU940163	EU940086
💧 *Arbusculina fragmentans*	CCM F-13486, ex-type	Stream foam, SK	**KC834042**	**KC834020**
💧 *Articulospora atra*	CCM F-00684	Stream, *Picea abies* twiglet, CZ	FJ000402	–
💧 *Articulospora tetracladia*	CCM F-12499	Stream foam, CZ	EU998915	EU998915
Ascocalyx abietina	cf870061	Abies sp., CA	U72259	–
Ascocoryne cylichnium	PDD75671	N/A	AY789395	AY789394
Bulgaria inquinans	ZW-Geo52-Clark	N/A	AY789345	AY789344
Cadophora finlandica	IFM50530	N/A	AB190393	AB190423
Cadophora luteo-olivacea	ICMP 18084	*Vitis vinifera*, NZ	HM116747	HM116758
Catenulifera brachyconia	CBS 304.74	*Fagus sylvatica* bark, NL	GU727558	–
Chlorencoelia sp.	ZW-Geo55-Clark	N/A	AY789352	AY789351
Chlorovibrissea sp.	PDD70070	N/A	DQ257353	DQ257352
Ciboria batschiana	CBS 655.78	Acorn, *Quercus robur*, NL	AY526234	–
Cistella spicicola	CBS 731.97	*Diphasiastrum complanatum*, FI	GU727553	–
💧 *Cladochasiella divergens*	CCM F-13489, ex-type, monotypic	Culture contaminant	**KC834043**	–
Cryptosporiopsis rhizophila	CBS110609	*Erica tetralix* root, NL	AY176758	–
Cudonia lutea	wz164	N/A	AF433149	AF433138
💧 *Cudoniella indica*	CBS 430.94 ex-type of *Tricladium indicum*	Submerged *Pinus roxburghii* needles, Kumaun, Himalaya, IN	DQ202513	
Cudoniella sp.	ZW0068	N/A	AY789342	AY789341
Dactylaria dimorphospora	CBS 256.70	Agricultural soil, under potato, NL	U51980	–
💧 *Dimorphospora foliicola*	CBS 221.59, ex-type, monotypic	Stream, *Castanea* leaf, JP	DQ202518	–
💧 *Dwayaangam colodena*	V3.13	*Picea mariana*-needles, CA	AY746351	–
Fabrella tsugae	J. Platt 256	N/A	U92304	AF356694
💧 *Filosporella* cf. *annelidica*	CCM F-11702	Stream foam, GB (E. Descals B 292-1-10)	**KC834044**	–
💧 *Filosporella exilis*	CCM F-13097, ex-type	*Equisetum fluviatile*, BY (VG 98a)	**KC834046**	–
💧 *Filosporella fistucella*	CCM F-13091, ex-type	*Alnus glutinosa submerged roots*, GB (P.J. Fisher 7 DW)	**KC834047**	**KC834021**
💧 *Filosporella versimorpha*	CCM F-11194, ex-type	*Alnus glutinosa* submerged roots, GB (P.J. Fisher WF)	**KC834054**	**KC834022**
💧 *Flagellospora curvula*	CB-M13	Submerged leaf *Cladrastis kentukea*, USA	**KC834045**	**KC834024**
💧 *Flagellospora* sp.1	CCM F-20899	Stream foam, CZ	**KC834050**	**KC834023**
💧 *Flagellospora fusarioides*	CCM F-14583	Submerged leaf, *Crataegus monogyna*, GB	**KC834048**	–
💧 *Flagellospora leucorhynchos*	CCM F-14183	Stream foam, SK	**KC834049**	**KC834025**
💧 *Flagellospora saccata*	CCM F-39994	Stream foam, CA	**KC834053**	–
💧 *Flagellospora* sp. 2	VG 31-4	Submerged leaf, *Rhododendron maximum*, USA	**KC834051**	–
💧 *Fontanospora eccentrica*	CCM F-46394	Stream foam, CA		GQ477305
💧 *Fontanospora fusiramosa*	CCM F-12900	Stream foam, CZ	**KC834052**	GQ477307
💧 *Geniculospora grandis*	UMB-176.01	Stream foam, PT	GQ411354	
Geoglossum glabrum	OSC 60610	N/A	AY789318	AY789317
💧 *Gorgomyces honrubiae*	CCM F-12003, ex-type	Stream foam, ES (A. Roldán AR 9761)	**KC834057**	**KC834028**

Table 1 – (continued)

Species	Strain	Source	GenBank ITS	GenBank LSU
fx1* *Gorgomyces hungaricus*	CCM F-12696, ex-type	Terrestrial, on decaying leaves of *Carpinus betulus*, HU (J. Gönczöl)	**KC834058**	–
🌢 *Gyoerffyella* cf. *craginiformis*	CCM F-09367	*Liriodendron tulipifera*, decaying leaves, terrestrial, NL	**KC834055**	**KC834026**
🌢 *Gyoerffyella entomobryoides*	CBS268.63, ex-type	*Rosa* sp., stem necrosis, NL	**KC834056**	–
fx1 *Gyoerffyella gemellipara*	CCM F-402	*Liriodendron tulipifera*, decaying leaves, terrestrial, NL	**KC834060**	**KC834027**
🌢 *Gyoerffyella rotula*	CCM F-400	Stream foam, SK	**KC834061**	**KC834029**
🌢 *Gyoerffyella tricapillata*	CBS 451.64, ex-isotype	*Rosa* sp. decaying leaf in a pond, GB	**KC834059**	**KC834030**
Helicodendron westerdijkae	ICMP 15521	Aero-aquatic	EF029229	–
Hemiphacidium longisporum	ATCC 26761	*Pinus contorta*, CA	AY645899	–
Heyderia abietis	OSC60392	N/A	AY789290	AY789289
Holwaya mucida	ZW-Geo-138-	N/A	DQ257357	DQ257356
Hyalodendriella betulae	CBS 261.82	*Alnus glutinosa*	EU040232	–
Hyaloscypha vitreola	M39	N/A, FI	EU940231	EU940155
🌢* *Hydrocina chaetocladia*	CCM F-10890, ex-type, monotypic	Submerged alder twigs, GB (J. Webster)	**KC834062**	**KC834031**
Hymenoscyphus scutula	MBH29259	N/A	AY789432	
🌢 *Hymenoscyphus varicosporioides*	FC-2038	Wood, JP	AB481291	AB481292
Hyphodiscus hymeniophilus	MUCL 9042	*Betula* sp., FR	DQ227259	–
Lachnum virgineum	AFTOL49	*Alnus* cones, USA	DQ491485	AY544646
🌢 *Lemonniera aquatica*	CCM F-21799	Stream foam, CZ	–	DQ267627
🌢 *Lemonniera centrosphaera*	CCM F-149, ex-type	Submerged leaf, *Fagus sylvatica*, SK	**KC834063**	**KC834032**
🌢 *Lemonniera cornuta*	CCM F-325	UK, (J. Webster)	–	DQ267629
🌢 *Lemonniera* sp.	CCM F-19299	Stream foam, CZ	–	DQ267633
🌢 *Lemonniera terrestris*	CCM F-11486	Stream foam, SK	–	DQ267634
Leohumicola minima	N086	*Isoëtes echinospora* root, NO	HQ691252	–
Leohumicola verrucosa	CBS 115881	Soil, CA	AY706323	–
Leotia lubrica	ZW-Geo59-Clark	N/A	AY789360	AY789359
🌢 *Loramyces macrosporus*	CBS 235.53 ex-type	Submerged *Equisetum limosum*, UK	–	DQ470957
🌢 *Margaritispora aquatica*	CCM F-11591 monotypic	Submerged *Alnus* leaves, CZ	–	DQ267635
Meria laricis	CBS 298.52	*Larix decidua*, CH	DQ470954	DQ470954
Microglossum olivaceum	FH-DSH97-103	N/A	AY789398	AY789397
🌢 *Miniancora allisoniensis*	CCM F-30487 ex-type, monotypic	Stream foam, CA	**KC834064**	–
Mitrula brevispora	ZW02-012	Aero-aquatic, CN	AY789294	AY789293
Mitrula elegans	ZW-Geo45-Clark	Aero-aquatic, USA	AY789331	AY789330
Mitrula paludosa	MBH50636	aero-aquatic, Europe	AY789424	AY789423
Mollisia "*rhizophila*"	Currah lab1	Aspen roots, CA	JN053274	–
Mollisia cinerea	AFTOL 76	Fallen log, USA	DQ491498	DQ470942
Mollisia dextrinospora	ICMP 18083	*Actinidia deliciosa*, NZ	HM116746	HM116757
Mollisia fusca	CBS 234.71	*Fagus sylvatica*, CH	AY259137	–
Mollisia melaleuca	CBS 589.84	*Picea abies*, DE	AY259136	–
Mollisia minutella	ZK71/08	*Picea abies* needles, CZ	FR837920	–
Mollisia sp.	1.3.s.5.13	*Nothofagus menziesii* leaves, NZ	JN225932	–
🌢 *Mycoarthris corallina*	91A ex-type, monotypic	Stream foam, GB (P.J. Fisher 91A)	AF128440	–
Mycochaetophora sp.	MAFF 239284	*Gentiana scabra*, JP	AB434662	AB469680
🌢* *Mycofalcella calcarata*	CCM F-10289 ex-type	Rotting oak twigs, GB (S. Om-Kalthoum-Khattab HME4405)	**KC834065**	**KC834037**
Neobulgaria pura	CUP 063609	N/A	DQ257366	DQ257365
Neofabraea alba	MM 159	*Malus domestica*, NZ	AY359236	–
Neofabraea malicorticis	DAOM 227085	N/A	AF281386	–
Ombrophila violacea	WZ0024	N/A	AY789366	AY789365
Phialocephala helvetica	D-ZB-40	N/A, CH	AY347413	–
Protoventuria alpina	CBS 140.83	*Arctostaphylos uva-ursi*, CH	EU035444	–
Pyrenopeziza brassicae	CRB	N/A, UK	AJ305236	–
Pyrenopeziza revincta	ARON3150.P	Axenic culture, ascospores, NO	AJ430224	–

(*continued on next page*)

Table 1 – (continued)

Species	Strain	Source	GenBank ITS	GenBank LSU
Rhynchosporium orthosporum	CBS 698.79	*Dactylis glomerata*, CH	AY140669	–
Rhytisma salicinum	BPI1843550	*Salix scouleriana*, USA	AY465516	–
Saccharomyces cerevisiae		N/A	AY247400	J01355
Sclerotinia sclerotiorum	wb197	N/A	AF455526	–
Spathularia flavida	wz214	N/A	AF433152	AF433141
🌢 *Tetrachaetum elegans*	CB-M11, monotypic	Submerged leaf *Cladrastis kentukea*, USA	**KC834066**	–
🌢 *Tetracladium apiense*	CCM F-23199	Stream, plant debris, ES (Gran Canaria)	EU883420	EU883420
🌢 *Tetracladium breve*	CCM F12505	Stream, leaf cf. *Frangula alnus*, PT	EU883431	EU883431
🌢 *Tetracladium furcatum*	CCM F-11883	Stream foam, CZ	EU883432	EU883432
🌢 *Tetracladium marchalianum*	CCM F-26199	Stream foam, CZ	AY204621	AY204612
🌢 *Tetracladium maxilliforme*	CCM F-14286	Stream foam, SK	AF411027	–
🌢 *Tetracladium palmatum*	CCM F-10001	PT (C. Pascoal)	EU883424	EU883424
🌢 *Tetracladium setigerum*	CCM F-10186	Stream foam, CZ	EU883427	EU883427
🌢 *Tricladium alaskense*	VG 69-2, ex-type	Stream, *Carex* sp., Alaska, USA	JQ417290	GQ477338
🌢 *Tricladium angulatum*	CCM F-14186	Stream foam, CZ	AY204611	GQ477311
🌢 *Tricladium attenuatum*	CCM F-06485	CH (J. Rosset)	–	GQ477312
🌢 *Tricladium biappendiculatum*	CCM F-13000	Stream foam, CZ	–	GQ477314
🌢*Tricladium castaneicola*	CCM F-11296	Stream foam, CZ	–	GQ477316
🌢 *Tricladium caudatum*	CCM F-13498	Stream foam, CZ	–	GQ477318
🌢 *Tricladium chaetocladium*	VG 27-1	Stream, *Acer rubrum*, USA	**KC834067**	–
🌢 *Tricladium curvisporum*	CCM F-23387	Stream foam, CA	–	GQ477322
🌢 *Tricladium indicum*	VG 112-1	Foam, USA		GQ477324
🌢 *Tricladium kelleri*	VG 68-1, ex-type	Stream, *Carex* sp., Alaska, USA	JQ417288	GQ477337
🌢 *Tricladium minutum*	CCM F-10203	*Juncus* culms, GB, (E. Descals C181-3-03)	JQ412863	GQ477326
🌢 *Tricladium obesum*	CCM F-14598, ex-type	Stream foam, CZ	**KC834068**	**KC834035**
🌢 *Tricladium patulum*	CCM F-15199	Stream foam, CZ	–	GQ477329
🌢 *Tricladium procerum*	CCM F-16786, ex-type	*Juncus* sp., SK	–	**KC834034**
🌢 *Tricladium splendens*	CCM-F-16599	Stream foam, CZ	AY204635	GQ477333
🌢 *Tricladium terrestre*	CBS 697.73, ex-type	Stream, *Quercus* sp./*Prunus* sp. leaf litter, IE	DQ202519	
🌢 *Varicosporium delicatum*	CCM F-19494	Stream foam, CA	JQ412864	**KC834036**
🌢 *Varicosporium elodeae*	CBS 541.92	Litter, CA	DQ202517	**KC834037**
🌢 *Varicosporium giganteum*	CCM F-10987	Stream foam CA	–	GQ477343
🌢 *Varicosporium scoparium*	CCM F-10303, ex-type	River foam, ES (A. Roldán 9851)	–	GQ477345
🌢 *Varicosporium trimosum*	CCM F-14398	Stream foam,, CZ	–	GQ477346
🌢 *Variocladium giganteum*	CBS 508.71, ex-type	Submerged *Crataegus monogyna* leaf, GB	DQ202520	–
🌢 *Variocladium giganteum*	CCM F-16686	*Juncus* sp., SK	–	GQ477348
Vibrissea albofusca	PDD 75692	N/A, amphibious	AY789384	AY789383
Vibrissea flavovirens	MBH39316	N/A, amphibious	AY789427	–
Vibrissea truncorum	CUP-62562	N/A, amphibious	AY789403	AY789402
🌢 *Ypsilina graminea*	UMB-098.01, monotypic	River foam, PT	GQ411304	–
Zalerion varium	ATCC 169303	Balza wood, river, USA	AF169303	–

quality of PCR amplicons was checked in 1.2 % agarose gels stained with ethidium bromide under UV light using a 100 bp ladder (Promega, Madison/USA). The amplicons were purified using the Ultra Clean PCR Clean-up kit from MO BIO. Primers used for sequencing were SR6R/LR1 for ITS regions and LROR, LR3R, LR3 and LR7 (Vilgalys & Hester 1990) for partial LSU gene. Sequences were generated with an ABI 373 sequencer (Applied Biosystems, Foster City, USA) and analyzed with the sequence analysis software version 3.3 at SMB Dr. Martin Meixner (Berlin, Germany) or University of South Carolina, Engencore (Columbia, SC, USA).

Phylogenetic analyses

All sequences generated were used as queries in the GenBank sequence similarity search tool BLAST [http://

blast.ncbi.nlm.nih.gov/Blast.cgi] with default stringency. The top scoring sequences from the BLAST searches were included in the phylogenetic analyses. Additional sequences from the Leotiomycetes were also added. The full data sets were comprised of 116 ITS and 89 LSU sequences. The combined data set contained 138 taxa of which 64 had both ITS and LSU data concatenated, 55 had only ITS data and 19 had only LSU data (Table 1). *Geoglossum glabrum* and *Saccharomyces cerevisiae* were used as the outgroup taxa.

Phylogenetic relationships were assessed using the ARB software package (Ludwig *et al.* 2004) and MrBayes version 3.2.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003; Ronquist *et al.* 2012). All sequences were aligned using Fast Aligner/ClustalW implemented in ARB V1.03. All alignments were thoroughly examined and manually optimized according to primary and secondary structure information calculated by ARB. Ambiguously aligned nucleotide characters were excluded prior to phylogenetic analyses. The alignment is available on treebase.org under the following link http://purl.org/phylo/treebase/phylows/study/TB2:S14104.

jModeltest 2.1.1 (Darriba *et al.* 2012) was used for the selection of the model of nucleotide substitution that best fits the sequence data employing the Akaike Information Criterion (Akaike 1974). Maximum Likelihood analyses were performed with ARB using RAxML 7.0.3 (Randomized Accelerated Maximum Likelihood, Stamatakis 2006) applying the GTR + I + G model of sequence evolution for the combined data set. Searches were performed with random sequence addition and 100 replicates. Branch support was tested with 1000 replications on bootstrapped data sets. Three independent Bayesian phylogenetic analyses of the combined data sets were performed using the model TIM2ef + G (Posada 2003) revealed by jModeltest for the combined data set. Posterior probabilities for internodes were calculated with the Metropolis-coupled Markov chain Monte Carlo (MCMC) method by running four chains with 26 million generations in each of two runs with trees sampled every 1000 generations. The analyses were ended when the average standard deviation of split frequencies of the two runs was <0.05 (0.0081) and the likelihoods converged to a stable distribution. Additionally, convergence was diagnosed using AWTY (Nylander *et al.* 2008) and Tracer (Rambaut & Drummond 2007). Trees obtained prior to convergence were discarded as 'burn-in' before computing a consensus tree with TreeView version 1.6.6 (Page 1996). Posterior probability support was considered significant with $PP > 0.95$.

For assigning families in Helotiales we used the classifications listed in Myconet (Lumbsch & Huhndorf 2010), Mycobank (http://www.mycobank.org) and The Genera of Hyphomycetes (Seifert *et al.* 2011).

Results

We generated 31 new ITS and 21 new partial LSU sequences in this study. The concatenated data set (ITS and LSU regions) comprised 138 sequences (including 78 sequences of aquatic hyphomycetes and 60 related fungi from other habitats) and 2275 nucleotide positions. After the removal of indels and ambiguous flanking 5′ and 3′ regions, the final data set had 1741 characters.

Maximum Likelihood (RAxML) analyses revealed 1089 distinct alignment patterns and the best tree had a likelihood of $\ln L = -23438.19$, while Bayesian analyses revealed a consensus tree with a likelihood of $\ln L = -23\,433.44$. Both trees recovered the major clades of Leotiomycetes/Helotiales reported by Wang and co-workers (2006a). The comparison of trees inferred with individual data set (ITS or LSU data alone) revealed no significant conflicting clades (data not shown). Fourteen major clades, receiving strong bootstrap support (BS) (>95 %) and posterior probability (PP) (>0.98), were recognized (Fig 1). However, no clade corresponded well to the current circumscriptions of sexual or asexual genera of aquatic fungi that have been established based on conventional morphological characters.

Seventy-eight sequences of aquatic hyphomycetes were placed in nine clades (1–5, 7–8, 11, 14) within the Helotiales (Fig 1). The polyphyly of *Tricladium, Lemonniera, Anguillospora* and *Varicosporium* was confirmed, and newly established for *Articulospora*, *Filosporella* and *Flagellospora* because species from the same genus were placed in several clades.

Clade 1 (100 % BS and 1.0 PP support) contained eight genera of aquatic hyphomycetes including paraphyletic *Gyoerffyella* (five species) and polyphyletic *Varicosporium* (two species), *Fontanospora* (two species), *Articulospora* (*tetracladia*), *Anguillospora* (*filiformis*), *Tricladium* (three species) as well as *Tetrachaetum elegans* and *Cladochasiella divergens*. *Margaritispora aquatica* forms a well supported sister branch to three *Lemonniera* species.

In Clade 2, *Pyrenopeziza brassicae*, *Cadophora*, and *Rhynchosporium* formed a group with the aquatic hyphomycetes *Tricladium alaskense*, *Tricladium kelleri*, *Tricladium curvisporum*, and *Ypsilina graminea*. Clade 3 contained members of the Loramycetaceae/Vibrisseaceae including *Mollisia* s. str. (*M. cinerea*), *Loramyces macrosporus*, and *Variocladium giganteum*, while in Clade 4, *Tricladium procerum* and *Arbusculina fragmentans* were placed along with *Hyaloscypha vitreola* (Hyaloscyphaceae) and *Cadophora finlandica*. Clade 5 contained the monophyletic genus *Tetracladium* and other aquatic hyphomycetes (*Mycoarthris corallina*, *Varicosporium scoparium*) together with *Dactylaria dimorphospora* and *Leohumicola* spp.

Mitrula species and *Ascocalyx abietina* formed an independent Clade 6 with strong support (1.0 PP). Albeit without statistical support, *Tricladium angulatum* was placed adjacent to *Dimorphospora foliicola*, which has a *Hymenoscyphus* teleomorph (Abdullah *et al.* 1981). The *Cudoniella-Hymenoscyphus* clade (Clade 7) also received strong support (100 % BS and 0.98 PP), and this included the aquatic hyphomycetes *Anguillospora crassa*, *Anguillospora furtiva*, *Tricladium obesum*, *Tricladium splendens*, *Tricladium terrestre*, *Tricladium castaneicola*, *Tricladium minutum*, *Tricladium indicum*, *Mycofalcella calcarata*, *Filosporella annelidica*, *Geniculospora grandis*, *V. giganteum*, as well as *Hymenoscyphus varicosporoides*, *Hymenoscyphus scutula*, *Cudoniella* sp. and *Ombrophila violacea*. *Lachnum virgineum* appeared to be the basal taxon in Clade 7. *T. minutum* was placed as a singleton. The *Ascocoryne-Hydrocina* clade (Clade 8) included species of *Ascocoryne*, *Filosporella*, *Varicosporium*, *Articulospora*, *Hydrocina*, *Tricladium* and long branched *Neobulgaria pura*.

The Clade 14 included species of Leotiaceae and Bulgariaceae and twelve aquatic hyphomycete species in four genera.

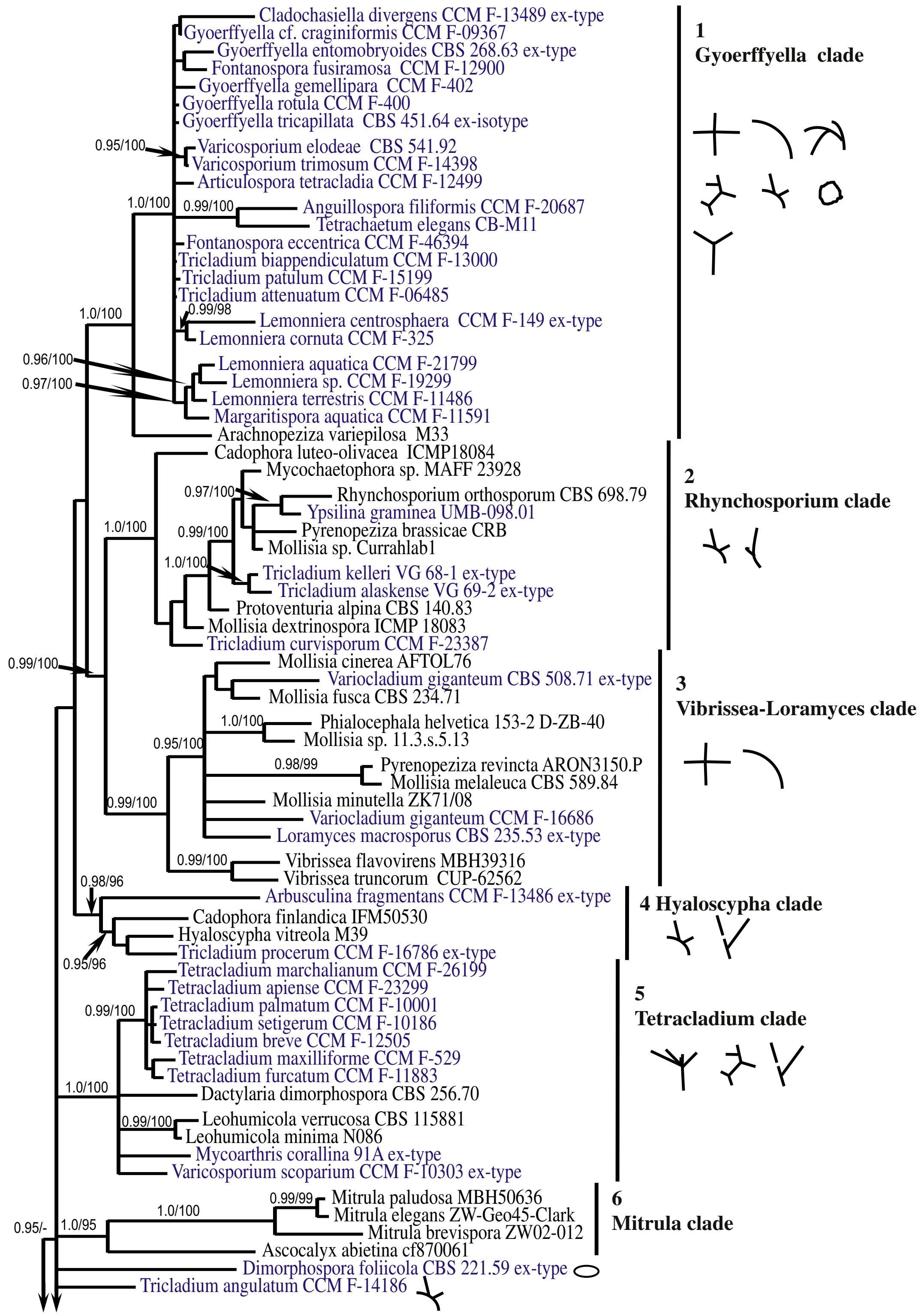

Fig 1 – MrBayes tree obtained from combined ITS and LSU rDNA sequence data. Numbers at the nodes are Bayesian posterior probabilities and ML bootstrap values. Aquatic hyphomycetes are shown in blue. Pictograms indicate major conidial shapes of aquatic hyphomycetes. (Tree Base Nr.: TB2:s14104), curved, includes sigmoid, tricladioid, variously branched (e.g. Varicosporium), *Gyoerffyella*-like, tetraradiate (e.g. *Lemonniera*, *Articulospora*, *Variocladium*, *Geniculospora*, *Alatospora*), straight, dichotomously branched (e.g. *Cladochasiella divergens*), tetrahedral (*Margaritispora aquatica*) *Ypsilina* (single-branched), tetracladioid, arthroconidia, *Dwayaangam colodena*, T-shaped (e.g. *Miniancora allisoniensis*), flail- shaped (e.g. *Gorgomyces*), oval.

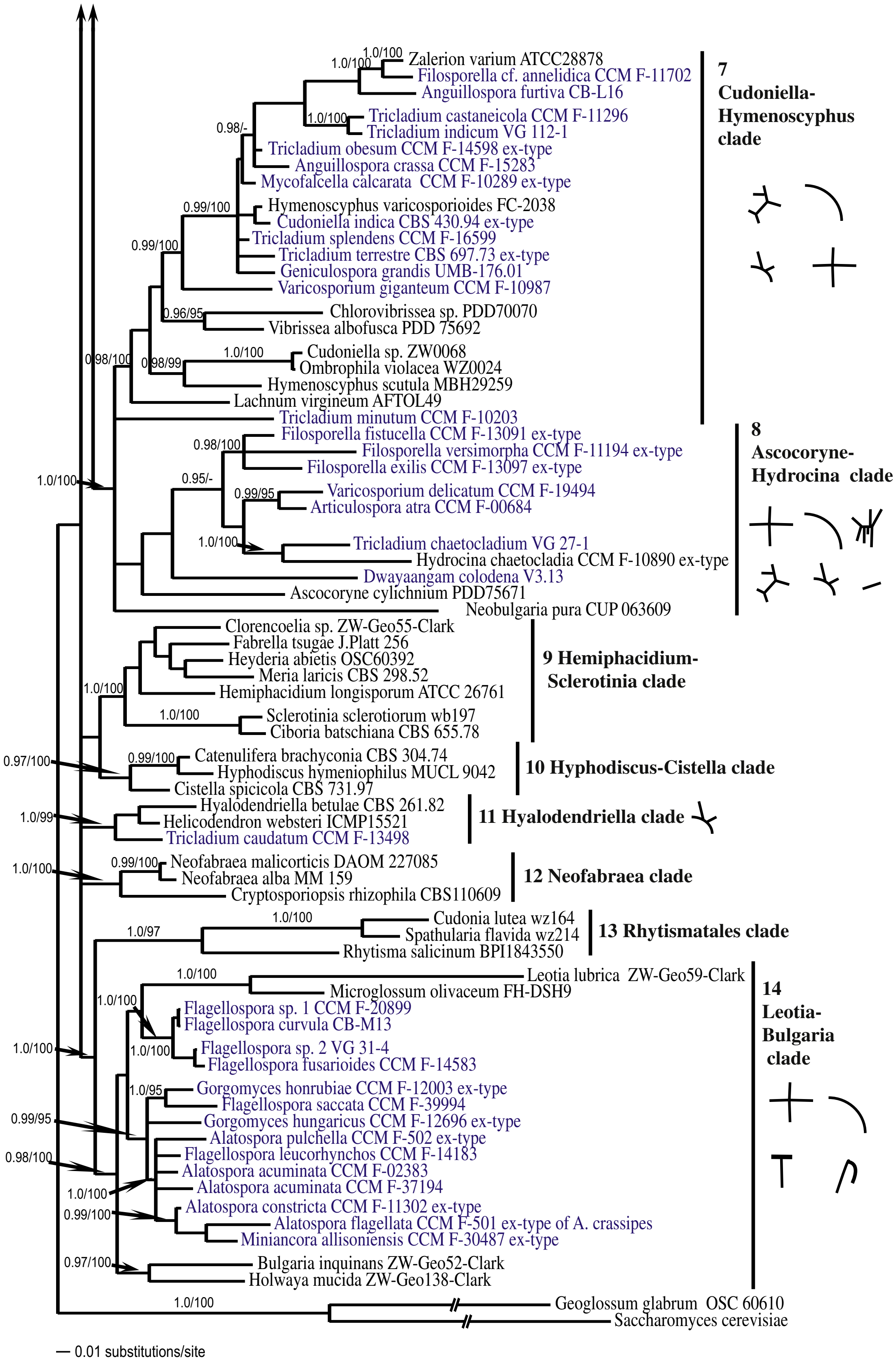

Fig 1 – (continued)

However, none of the four genera is monophyletic. *Alatospora* appeared to be paraphyletic with *Flagellospora leucorhynchos* and *Miniancora allisoniensis* nested within the group. *Gorgomyces honrubiae* did not cluster with *Gorgomyces hungaricus* but showed a sister relationship to *Flagellospora saccata*.

Clades 913 largely corresponded to the clades of *Hemiphacidium, Sclerotinia*, *Dermea*, *Rhytismatales* reported in Wang *et al.* (2006a). These clades did not include aquatic hyphomycetes, with the exception of *Tricladium caudatum* that had affinity (albeit without strong support) to the aero-aquatic fungus *Helicodendron websteri* and *Hyalodendriella betulae* in Clade 11.

Discussion

Molecular phylogeny of aquatic hyphomycetes

We found that at least 75 species of aquatic hyphomycetes belong to the Helotiales and are distributed among nine well to moderately supported clades (Fig 1). We demonstrated that *Articulospora*, *Filosporella*, and *Flagellospora* are polyphyletic, in addition to other polyphyletic genera, *Tricladium*, *Lemonniera*, *Anguillospora*, *Varicosporium* discovered in previous molecular studies (Baschien *et al.* 2006; Campbell *et al.* 2006 2009). The results confirmed that morphological characters, such as conidial shape and conidiogenesis, are not always accurate in defining natural genera. Many (taxa of aquatic hyphomycetes need to be re-defined and delineated, based on molecular studies employing ex-type cultures. Frequent absence of such cultures or even type specimens in most of the larger genera of aquatic hyphomycetes, (e.g. *Anguillospora*, *Articulospora, Flagellospora, Varicosporium, Tricladium*) can be rectified by establishing lecto-, neo- or epitypes.

Aquatic hyphomycetes are distributed throughout the Leotiomycetes (Fig 1). Eight clades, however, contain numerous genera and species and merit further discussion. Clade 1 represents a novel cluster discovered in this study and it contains 22 species from eight aquatic hyphomycete genera. This cluster is a sister group to *Arachnopeziza variepilosa*, which is a saprotrophic discomycete on wood. The prevalent genera are *Gyoerffyella* and *Lemonniera* but neither genus is monophyletic. *Ingoldia craginiformis* (Petersen 1962) was recombined in *Gyoerffyella* by Marvanová (Marvanová *et al.*1967). Later, having seen living material showing only small differences in conidial morphology she synonymized *G. craginiformis* with *Gyoerffyella rotula* (Marvanová 1975). In the present study the terrestrial isolate CCM F-09367 (as *Gyoerffyella* cf. *craginiformis*) appears phylogenetically distant from *G. rotula*.

Lemonniera is polyphyletic because one species (*L. pseudofloscula)* belongs to Pleosporaceae, Dothideomycetes (Campbell *et al.* 2006), even though *Lemonniera* is homogeneous with respect to conidiogenesis and conidial configuration. *Margaritispora aquatica* is very similar to *Lemonniera* in culture characteristics and conidiogenesis but it produces morphologically distinct conidia. However, before *M. aquatica* can be transferred to *Lemonniera*, at least the ex-neotype needs to be examined. Also the type species of *Lemonniera*, *L. aquatica*, has to be neotypified. While most members of Clade 1 are saprotrophs in aquatic environments, *Articulospora tetracladia, Varicosporium elodeae* (Fisher *et al.* 1991) and *Fontanospora fusiramosa* (Marvanová *et al.* 1997) were also reported as facultative endophytes in *Alnus glutinosa* roots growing in aquatic habitats. Furthermore, conidia of *L. aquatica*, *L. terrestris*, *L. cornuta*, *M. aquatica*, *G. gemellipara*, *G. tricapillata*, *V. elodeae* and *T. patulum* were found in the canopy (Bandoni 1981; Mackinnon 1982; Czeczuga & Orłowska 1994; Gönczöl & Révay 2004). *Gyoerffyella. entomobryoides* is described as terrestrial plant pathogen (Boerema & von Arx 1964). Taxonomically, this clade contained mostly asexual genera except for *A. tetracladia*, for which a *Hymenoscyphus* sexual state was described (Abdullah *et al.* 1981) and was later recombined in *Ombrophila* (Baral & Krieglsteiner 1985). However, in our study *Ombrophila violacea* (the type of the genus) was placed in the *Hymenoscyphus-Cudoniella* clade (Clade 7).

Clade 2 forms a strong sister relationship to clade 3 and it accommodates three species of *Tricladium* and *Ypsilina graminea*. Interestingly, all aquatic hyphomycetes in this clade were either reported from arctic streams or are often associated with decaying sedges or grasses (Gulis *et al.* 2012). Some populations of these taxa may have adapted to survive in arctic or subarctic streams that lack trees in the riparian zone, but further studies are required to verify their physiological adaptations. *Ypsilina graminea* was also reported from tree holes in Hungary (Gönczöl & Révay 2003) and India (Karamchand & Sridhar 2008). Common plant pathogens (e.g. *Rhynchosporium orthosporum*, *Pyrenopeziza brassicae*) also belonged to this clade. Apart from aquatic hyphomycetes and plant pathogens, this clade also contained several root associated antarctic dark-septate endophytes (DSE) (Upson *et al.* 2009), as well as root associates *Cadophora* spp. which are asexual states in Dermateaceae (Harrington & McNew 2003).

Clade 3 (*Vibrissea-Loramyces* clade *sensu* Wang *et al.* 2006b) is comprised of Vibrisseaceae, Dermateaceae, and Loramycetaceae. These families include several aquatic teleomorph species such as *Vibrissea flavovirens* with conidial state *Anavirga dendromorpha* (Hamad & Webster 1987), *L. macrosporus* (Ingold & Chapman 1952) and members of *Mollisia*. *Mollisia* is a polyphyletic genus because members are distributed over two clades (2 and 3). *Mollisia* has been reported as sexual state of *Anguillospora crassa* (Webster 1961; in clade 7), *Filosporella* sp. (Webster & Descals 1979), and *Casaresia sphagnorum* (Webster *et al.* 1993). The type species of the genus *Loramyces* is *L. juncicola,* which is linked to *Anguillospora*-like conidial state (Digby & Goos 1987). The sequence of *Variocladium giganteum* (*ex-type*; CBS 508.71) clustered with *Mollisia fusca*, while the other isolate (CCM F-16686) is placed close to *L. macrosporus.* Willoughby & Minshall (1975) observed a microconidial state in their isolate of *V. giganteum*, which they tentatively assigned to *Phialocephala* resembling *P. dimorphospora*. No such microconidial state was described in the protologue of *V. giganteum* by Iqbal (1971) but it was present in the CCM F-16686 isolate. *Phialocephala helvetica,* a cryptic species closely related to *P. fortinii*, appeared in the same clade as both cultures of *Variocladium*. Although most species in Clade 3 are saprotrophs adapted to moist or aquatic conditions, *P. helvetica* is not aquatic and is a dark-septate endophyte (Grünig *et al.* 2008).

The grouping of *Tricladium procerum* with *Hyaloscypha vitreola* in Clade 4 is in agreement with the findings of Campbell *et al.* (2009). Members of the polyphyletic genus *Hyaloscypha* are biotrophic parasites or bryophyte symbionts (Stenroos *et al.* 2010). *T. procerum* was isolated from submerged dead

Juncus stems (Marvanová 1988). *Arbusculina fragmentans* produces fragmenting macroconidia and has a hyaline to pale fuscous phialidic microconidial state. Both aquatic hyphomycetes of this clade are rarely reported from ecological studies.

Clade 5 (*Tetracladium* clade) is comprised of four genera of aquatic hyphomycetes and two *Leohumicola* species. Our analysis confirmed the monophyly of the genus *Tetracladium* (Nikolcheva & Bärlocher 2002; Baschien *et al.* 2006; Letourneau *et al.* 2010). Interestingly, some species of this clade were found associated with roots of terrestrial (Watanabe 1975; *T. setigerum*) and submerged living plants (Kohout *et al.* 2012; *T. furcatum*, *T. setigerum*, *Tricladium* sp., *Leohumicola minima*). Three species of the genus (*T. marchalianum*, *T. maxilliforme*, *T. setigerum*) were also found associated with tree leaves (Czeczuga & Orłowska 1998), and stemflow or in gutters (Gönczöl & Réval 2004). Two *Leohumicola* species are ericoid mycorrhizae-forming fungi (Hambleton *et al.* 2005).

Clade 7 (*Hymenoscyphus-Cudoniella*) is one of the largest groups containing seven genera of aquatic hyphomycetes (e.g. Abdullah *et al.* 1981; Descals *et al.* 1984; Webster *et al.* 1995). Several aquatic hyphomycetes from this clade were reported as root endophytes, e.g. *Tricladium splendens* (Fisher & Petrini 1989) and *A. crassa* (Sati & Belwal 2005). Conidia of *T. splendens* (Karamchand & Sridhar 2008) and *Tricladium castaneicola* (Gönczöl & Révay 2003 2006) were reported from tree holes and from stemflow. *Tricladium* and *Anguillopora* are the two classical, albeit polyphyletic, genera of aquatic hyphomycetes, and their representatives are clustered together. The polyphyly of *Anguillospora* has been demonstrated earlier (Belliveau & Bärlocher 2005; Baschien *et al.* 2006) with species distributed among Dothideomycetes, Orbiliomycetes, and Leotiomycetes. In agreement with the study of Belliveau & Bärlocher (2005), *A. filiformis* was placed in Clade 1 while two other helotialean *Anguillospora* species were placed in Clade 7. All three *Anguillospora* species studied here have thalloblastic percurrent conidiogenous cells and sigmoid conidia.

Tricladium is the largest genus of aquatic hyphomycetes containing 26 species with representatives in Leotiomycetes and Dothideomycetes (Campbell *et al.* 2009; Gulis & Baschien, unpublished). Five *Tricladium* species with dark colonies (*T. castaneicola, T. indicum, T. obesum, T, splendens, T. terrestre*) forming a cluster in the study of Campbell *et al.* (2009, Fig 1) received high support in our Clade 7 as well as in the study of Seena *et al.* (2010). In our study, fifteen *Tricladium* species were distributed in seven clades – an extreme example of polyphyly calling for a taxonomic revision of the genus.

The teleomorph *Cudoniella indica* is grouped with *Hymenoscyphus varicosporioides* which is consistent to data of previous studies (Sivichai & Jones 2003; Campbell *et al.* 2009; Seena *et al.* 2010). Although Sivichai & Jones (2003) suggested that *Hymenoscyphus varicosporoides* and *C. indica* may be conspecific, the low resolution of this clade demonstrated the need to utilize highly discriminatory loci for delineation of genera and species.

Clade 8 (*Ascocoryne sensu* Wang *et al.* 2006a) contained almost exclusively aquatic hyphomycetes with the exception of *Ascocoryne cylichnium* and *Neobulgaria pura*. *Hydrocina chaetocladia* is the sexual state of *Tricladium chaetocladium*, an aquatic hyphomycete (Webster *et al.* 1991). Its position here differs from that published by Wang *et al.* (2005, 2006a,b), who place it near *Mitrula*, but without significant support. Even though three members of *Filosporella* formed a monophyletic group, the genus is polyphyletic because *Filosporella annelidica* nested in Clade 7, while *Filosporella exilis*, *Filosporella fistucella* and *Filosporella versimorpha* in clade 8. Unfortunately, no isolate of the type species of the genus, *Filosporella aquatica*, described from Malaysia (Nawawi 1976) was available for this study. *Filosporella exilis* and *F. versimorpha* produce at least three types of conidia (micro-, macro- and arthroconidia); the latter was isolated from submerged alder roots, as was *F. fistucella* (Marvanová & Fisher 1991; Marvanová *et al.* 1992). The ability to produce different types of conidia might be an adaptation to environmental conditions (e.g. aquatic *vs.* terrestrial) during different stages of life cycle. Apart from *Filosporella,* aquatic hyphomycetes in this clade form branched or tetraradiate conidia. *Dwayaangam colodena*, also isolated from roots, showed affinities to Hyaloscyphaceae (Sokolski *et al.* 2006).

Tricladium caudatum was basal to *Hyalodendriella* (Clade 11; Helotiales incertae sedis, Crous *et al.* 2007) and *Helicodendron websteri* (aero-aquatic fungus), but the placement was poorly supported. The position of *T. caudatum* is uncertain, and it was not clearly resolved in the study of Campbell *et al.* (2009) when it was weakly clustered to *Rhytisma acerinum*.

Clade 14 (*Leotia-Bulgaria* clade *sensu* Wang *et al.* 2006a) is comprised of four aquatic hyphomycete genera. The molecular data appeared to support the separation of *Alatospora* from *Flagellospora* except that *F. leucorhynchos* nested within a cluster of *Alatospora* species. Another species, *Flagellospora saccata*, grouped with *Gorgomyces honrubiae*. While *Alatospora* produces mostly branched conidia (though some isolates tend to produce almost only unbranched conidia), *Flagellospora* has sigmoid to arcuate conidia. Interestingly, the morphology of the phialides in *F. saccata* is different from all other *Flagellospora* species. Two other species of *Flagellospora* are members of the Hypocreales (Ranzoni 1956; Webster 1993). The concepts of *Alatospora* and *Flagellospora* should be revised in light of additional morphological and molecular data (Baschien *et al.*, in preparation).

Ecology and evolution of aquatic hyphomycetes

No clear pattern was evident in the distribution of aquatic hyphomycetes with a particular type of conidial morphology (e.g. tetraradiate or sigmoid) among clades of the Leotiomycetes (Fig 1). Species with tetraradiate or variously branched conidia were found in all nine clades that contained aquatic hyphomycetes. In addition, we found close phylogenetic relationships between aquatic hyphomycete taxa with branched and sigmoid conidia. Moreover, there are species of aquatic hyphomycetes producing both types of conidia in nature as well as in pure culture, e.g. *Alatospora acuminata*, *Pachycladina mutabilis*, *Tricladium indicum*, *Tricladiopsis flagelliformis*. This suggests, that the two shapes may not be genetically fixed at least in some taxa. According to one hypothesis conidia are modified hyphae (Descals 1985; Kendrick 2003). In the case of aquatic hyphomycetes, we can speculate that conidia may have been evolving from simple elongate shapes to more or less branched spores.

In addition to aquatic hyphomycetes, Leotiomycetes also contain aero-aquatic fungi such as *Helicodendron* and fungi that have amphibious lifestyles, e.g. living close by the water or in wet conditions (e.g. *Vibrissea*). The production of different conidial shapes and synanamorphs may also be an adaptation to shifts between aquatic, semi-aquatic and terrestrial habitats.

The molecular data demonstrated that most aquatic hyphomycetes clustered with endophytes, mycorrhizal fungi and saprotrophs in the Helotiales, thus supporting the scenario first suggested by Shearer (1993) that aquatic hyphomycetes evolved from terrestrial plant-associated or litter-associated fungi (Selosse *et al.* 2008). Indeed, conidia of many aquatic hyphomycetes from the genera *Alatospora*, *Anguillospora*, *Flagellospora*, *Gyoerffyella*, *Lemonniera*, *Tetracladium*, *Tricladium* and *Varicosporium* have been reported from the canopy (tree holes, stemflow, throughfall; reviewed in Sridhar 2009). However, as pointed out by Gönczöl & Révay (2006), the group of taxa, whose stauroform or scolecoform conidia are repeatedly observed in rainwater from canopy, stemflow or throughfall (also called 'arboreal aquatic hyphomycetes'), should have a unique, currently poorly understood ecology. Although they resemble aquatic hyphomycetes, the identifications are based on detached conidia only. To our knowledge, a few studies based on pure cultures of fungi from rainwater revealed species that are not found in typical habitats of aquatic hyphomycetes (e.g. Ando & Tubaki 1984a,b). Some species of the genera *Varicosporium*, *Tetracladium*, and *Anguillospora* have been reported as plant endophytes (Nemec 1969; Watanabe 1975; Sati & Belwal 2005). Many plant pathogens, endophytes, root-associated fungi (RAF) and mycorrhizal species belong to the Leotiomycetes (Selosse *et al.* 2008). Indeed, the endophytic lifestyle could possibly facilitate the transition from terrestrial to aquatic habitats. Endophyte and phylloplane fungi are already associated with the substrate when it enters the water (e.g. a leaf during the litter fall), which may have given such fungi a competitive advantage and eventually led to the evolution of spore shapes adapted to aquatic dispersal. Alternatively, it was hypothesized that terrestrial ancestors of the present-day aquatic plants interacted with different groups of ubiquitous RAF, both mycorrhizal and non-mycorrhizal (Kohout *et al.* 2013). The extant free-living aquatic hyphomycetes could have evolved from non-mycorrhizal RAF that once entered aquatic habitats together with their host plants. In fact, many aquatic hyphomycetes are capable of colonizing roots of submerged, riparian or terrestrial plants (Kohout *et al.* 2012 2013).

Conclusions

Seventy-five species of aquatic hyphomycetes and their teleomorphs are associated with the Helotiales, Leotiomycetes. We compiled the largest database of aquatic hyphomycete sequences (75 out of 300 species) and unraveled phylogenetic positions of 29 out of approximately 115 genera of aquatic hyphomycetes. Ribosomal DNA sequence data by themselves are invaluable for the purposes of barcoding and molecular microbial ecology including metagenomics. Many genera of aquatic hyphomycetes are polyphyletic suggesting that conidial adaptations to aquatic dispersal occurred independently in multiple lineages. Many genera and species of aquatic hyphomycetes require typification since type material or ex-type species are often missing. Multilocus sequencing of ex-type strains will be necessary to better resolve phylogenetic relationships.

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