

A molecular phylogenetic assessment of *Massarina ingoldiana sensu lato*

Kazuyuki Hirayama
Kazuaki Tanaka¹

Faculty of Agriculture and Life Science, Hirosaki
University, 3 Bunkyo-cho, Hirosaki, Aomori 036-8561,
Japan

Huzefa A. Raja

Department of Plant Biology, University of Illinois at
Urbana-Champaign, 265 Morrill Hall, 505 South
Goodwin Avenue, Urbana, Illinois 61801

Andrew N. Miller

Illinois Natural History Survey, University of Illinois at
Urbana-Champaign, Champaign, Illinois 61820

Carol A. Shearer

Department of Plant Biology, University of Illinois at
Urbana-Champaign, 265 Morrill Hall, 505 South
Goodwin Avenue, Urbana, Illinois 61801

Abstract: *Massarina ingoldiana* occurs worldwide on a variety of dead plant substrates in aquatic habitats. This species has been accommodated in *Massarina* or *Lophiostoma* in Pleosporales, Dothideomycetes, but the validity of either of these taxonomic placements has not been confirmed with molecular data. In addition morphological variations occur among different populations of this species causing problems in identification. To evaluate the generic placement and monophyly of *M. ingoldiana* and the taxonomic usefulness of variable morphological features, phylogenetic analyses based on SSU and LSU sequences of ribosomal DNA were conducted for 10 putative strains of this species and its relatives. Phylogenies revealed that *M. ingoldiana sensu lato* is polyphyletic and comprises two distinct lineages within Pleosporales. Neither lineage was congeneric with either *Massarina* or *Lophiostoma*. Based on molecular data and a re-evaluation of morphology, two new genera, *Lindgomyces* and *Tingoldiogo*, are established for the two lineages of *M. ingoldiana sensu lato*. *Lindgomyces* includes four species, *L. ingoldianus* comb. nov. (= *M. ingoldiana sensu stricto*), *L. rotundatus* sp. nov. (= *M. ingoldiana sensu lato*), *L. cinctosporae* sp. nov. and *L. breviappendiculatus* comb. nov. (= *Lophiostoma breviappendiculatum*). A new aquatic family, Lindgomycetaceae, is proposed for *Lindgomyces* and its sister taxon, *Massariosphaeria typhicola*. Isolates of a fungus from submerged *Phragmites*, with ascospores similar

to those of *M. ingoldiana*, occurred in an additional single species lineage distant from that of *M. ingoldiana* (*Lindgomyces*). This fungus is described as *Tingoldiogo graminicola* gen. & sp. nov. The discovery that *Tingoldiogo*, which occurs in a lineage distantly related to *Lindgomyces* but has morphologically similar ascospores and ascospore sheaths, suggests that the elaborate ascospore sheath in *M. ingoldiana* has arisen in two separate lineages as a result of convergent evolution in response to the aquatic environment. The large gelatinous sheath previously was considered one of the most distinctive and stable features for species identification of *M. ingoldiana*.

Key words: Ascomycetes, convergence, evolution, freshwater, Pleosporales, taxonomy

INTRODUCTION

Massarina ingoldiana Shearer & K.D. Hyde is a freshwater ascomycete belonging to Pleosporales, Dothideomycetes (Lumbsch and Huhndorf 2007). This species has been found frequently from partially decomposed woody and herbaceous debris submerged in freshwater habitats in both temperate and tropical regions in eastern and western hemispheres (Shearer and Hyde 1997, Tsui et al. 2000, Shearer 2001, Shearer et al. 2007, Raja et al. 2009). The most distinctive feature of *M. ingoldiana* is the ascospore sheath, which is considered a morphological adaptation to aquatic environments (Shearer and Hyde 1997). In ascospores discharged into water the sheath initially envelops ascospores as a conspicuous shell-like structure and then enlarges bipolarly to a relatively large size (see FIGS. 10–12, Shearer and Hyde 1997). Enlargement of the sheath increases the area of orthogonal projection of the spore thereby increasing the probability of contact with substrate, and the sticky gelatinous sheath increases the ability of a spore to adhere to substrate in moving water.

Pleosporalean ascomycetes with hyaline single septate ascospores that tardily become lightly pigmented and three-septate traditionally have been placed in genus *Massarina*. Liew et al. (2002) however with molecular phylogenetic analyses found that five species of *Massarina*, which possess narrowly fusiform ascospores (*M. armatispora* K.D. Hyde, Vrijmoed, Chinnaraj & E.B.G. Jones, *M. bipolaris* K.D. Hyde, *M. corticola* [Fuckel] L. Holm, *M. fronsisubmersa* K.D. Hyde and *M. rubi* [Fuckel] Sacc.), belong in *Lophiostoma*, a genus morphologically similar to

Massarina. They further suggested that other species with ascospores of similar morphology might have affinity with *Lophiostoma*. Following this suggestion, Hyde et al. (2002) transferred 26 species of *Massarina* to *Lophiostoma*, based solely on the narrowly fusiform ascospores, although "narrowly fusiform" was not clearly defined. *Massarina ingoldiana* was one of the species transferred to *Lophiostoma* by Hyde et al. (2002). Thus far no molecular evidence has been presented to support the validity of these transfers.

A re-assessment of the species monophyly of *M. ingoldiana* also is required. Morphological variations exist among different populations of this species, for example in the thickness of the ascomal wall, the length of the ascomal beak and ascospore features such as pigmentation and septation (Shearer and Hyde 1997). Differences in colony morphology among different isolates under the same culture conditions also occur (Tanaka pers obs). However no attempts have been made to evaluate the monophyly of this species because phenetic differences among different collections have been considered to be intraspecific variations of *M. ingoldiana*.

During our ongoing surveys of freshwater ascomycetes several strains of *M. ingoldiana* and its relatives were obtained from various samples of plant debris in aquatic habitats. The aims of this study were to clarify the taxonomic placement of *M. ingoldiana* at the generic level and to evaluate the monophyly of *M. ingoldiana* based on both morphological comparisons and phylogenetic analyses of partial 18S small subunit (SSU) and 28S large subunit (LSU) of ribosomal DNA. In addition the phylogenetic relationships of *M. ingoldiana* to other Pleosporalean freshwater ascomycetes were evaluated.

MATERIALS AND METHODS

Fungal strains and morphological studies.—Submerged woody and herbaceous debris were collected from a variety of aquatic habitats. Samples were rinsed in distilled or tap water and incubated in moist chambers at room temperature (ca. 25 C) (Shearer 1993). Debris were examined periodically with a dissecting microscope for fruiting bodies of ascomycetes. Ascospores were removed from substrates and crushed in a drop of water on a glass slide and secured with a cover slip to observe ascospores, pseudoparaphyses, asci and ascospores. These structures mounted in water or glycerin were measured at 450× or 1000×. India ink or nigrosin in distilled water was used to detect gelatinous sheaths around ascospores. The ascospore midseptum position was noted with the decimal system (Shoemaker 1984, Raja et al. 2008). To observe details of ascomal anatomy ascospores were boiled in water a few minutes and sectioned with a freezing microtome (HM 400R, MICROM,

Germany) or were embedded in resin and sectioned with a glass knife with an ultramicrotome (Fallah and Shearer 2001). Light microscopy was conducted with an Olympus microscope (BX51) equipped with Nomarski interference differential contrast objectives. Specimens cited in this paper are maintained at the Herbarium of Hirosaki University (HHUF), the University of Illinois Herbarium (ILL) or the Illinois Natural History Survey Herbarium (ILLS).

Single ascospore cultures were obtained according to the methods of Tubaki (1978) or Shearer et al. (2004). Growth rate and colony characteristics were recorded from cultures grown on potato dextrose agar (PDA, Difco) or peptone, yeast extract glucose agar (PYG; peptone 1.25 g, yeast extract 1.25 g, D-glucose 3.00 g; distilled water 1000 mL, agar 18 g) at 20 or 25 C in the dark. Colors were designated according to Kornerup and Wanscher (1978). To validate culture isolations the induction of sexual reproduction was attempted by placing a small piece of mycelial culture on rice straw agar (Tanaka and Harada 2003a). Fungal cultures newly obtained in this study were deposited at the Japan Collection of Microorganisms (JCM), the Ministry of Agriculture, Forestry and Fisheries, Japan (MAFF), and National Biological Resources Center, Japan (NBRC) (TABLE I).

DNA extraction and PCR amplification (Tanaka Lab).—Mycelia were grown in malt extract broth (20 g malt extract, 1000 mL distilled water). DNA from mycelia was extracted with the ISOPLANT Kit (Nippon Gene, Japan) according to manufacturer instructions. Partial SSU and LSU rDNA (ca. 1300 bp of the 5' end) were amplified by the polymerase chain reaction (PCR) with the primer pairs NS1–NS4 (White et al. 1990) and LROR–LR7 (Rehner and Samuels 1994) respectively. Amplifications were conducted in 25 µL PCR mixtures containing 1 µM each primer, 0.125 U TaKaRa Ex Taq (TaKaRa Bio, Otsu, Japan), dNTP mixture (2.5 mM each) and Ex Taq reaction buffer (containing 2 mM Mg²⁺). PCR was carried out as follows: initial denaturation at 94 C for 4 min; 35 cycles of denaturation at 94 C for 1 min; annealing 1 min at 48.8 C for SSU rDNA and 46.2 C for LSU rDNA; an extension at 72 C for 1 min and a final extension at 72 C for 7 min. The size of PCR products were verified with 7.5% polyacrylamide gels stained with ethidium bromide and then sequenced directly at SORGENT Co. Ltd. (Korea).

DNA extraction and PCR amplification (Miller and Shearer labs).—Fungal isolates were grown on cornmeal agar (CMA, Difco) or PYG. For extraction of genomic DNA mycelium axenic cultures was scraped from culture plates with a sterile scalpel and ground to a fine powder in liquid nitrogen with a mortar and pestle. Approximately 400 µL API buffer from the DNAeasy Plant Mini Kit (QIAGEN Inc., Valencia, California) was added to the mycelial powder, and DNA was extracted following manufacturer instructions. Total genomic DNA was observed on a 1% TBE agarose gel stained with ethidium-bromide.

Fragments of SSU and LSU rDNA were amplified by PCR with puReTaq™ Ready-To-Go PCR Beads (Amersham Biosciences Corp., Piscataway, New York) according to

TABLE I. Cultures and GenBank accession numbers of *Massarina ingoldiana* and its related species

Taxa ^a	Source			GenBank number		
	Original number	Culture collection number	Voucher specimen	SSU	LSU	
<i>Lophiostoma macrostomum</i>	KT 635	JCM 13545	HHUF 27290	AB521731	AB433273	
<i>Lophio. macrostomum</i>	KT 709	JCM 13546/MAFF 239447	HHUF 27293	AB521732	AB433274	
<i>Lophio. breviappendiculatum</i> (<i>Lindgomyces breviappendiculatus</i>)	KT 1215	JCM 12702/MAFF 239291	HHUF 28193	AB521733	AB521748	
<i>Lophio. breviappendiculatum</i> (<i>Lindgomyces breviappendiculatus</i>)	KT 1399	JCM 12701/MAFF 239292	HHUF 28194	AB521734	AB521749	
<i>Massarina eburnea</i>	H 3953	JCM 14422	HHUF 26621	AB521718	AB521735	
<i>M. ingoldiana</i> (<i>Lindgomyces ingoldianus</i>)	—	ATCC 200398	ILLS 52289	AB521719	AB521736	
<i>M. ingoldiana</i> (<i>Lindgomyces ingoldianus</i>)	KH 100	JCM 16479/NBRC 106126	HHUF 30006	AB521720	AB521737	
<i>M. ingoldiana</i> (<i>Lindgomyces sp.</i>)	KH 241	JCM 16480/NBRC 106130	HHUF 30007	AB521721	AB521738	
<i>M. ingoldiana</i> (<i>Lindgomyces rotundatus</i>)	KT 966 Type	JCM 16481/MAFF 239473	HHUF 27883	AB521722	AB521739	
<i>M. ingoldiana</i> (<i>Lindgomyces rotundatus</i>)	KT 1096	JCM 16482/NBRC 106127	HHUF 27999	AB521723	AB521740	
<i>M. ingoldiana</i> (<i>Lindgomyces rotundatus</i>)	KT 1107	JCM 16483/NBRC 106128	HHUF 28000	AB521724	AB521741	
<i>M. ingoldiana</i> (<i>Lindgomyces rotundatus</i>)	KH 114	JCM 16484/NBRC 106129	HHUF 30008	AB521725	AB521742	
<i>M. ingoldiana</i> (<i>Tingoldiogo graminicola</i>)	KH 68 Type	JCM 16485/NBRC 106131	HHUF 30009	AB521726	AB521743	
<i>M. ingoldiana</i> (<i>Tingoldiogo graminicola</i>)	KT 891	MAFF 239472	HHUF 27882	AB521727	AB521744	
<i>M. ingoldiana</i> (<i>Tingoldiogo graminicola</i>)	KH 155	JCM 16486/NBRC 106132	HHUF 30010	AB521728	AB521745	
<i>Massarina sp.</i> (<i>Lindgomyces cinctosporae</i>)	R56-1	Raja R56-1	ILL 40791	AB522430	AB522431	
<i>Massariosphaeria typhicola</i>	KT 667	MAFF 239218	HHUF 27779	AB521729	AB521746	
<i>Massariotyphicola</i>	KT 797	MAFF 239219	HHUF 27785	AB521730	AB521747	

^aNames in parentheses indicate proposed taxonomic names.

Huhndorf et al. (2004). Primers NS1 and NS4 for SSU (White et al. 1990) and LROR and LR6 for LSU (Vilgalys and Hester 1990) were used for PCR reactions. PCR products were purified to remove excess primers, dNTP and nonspecific amplification products with the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, California). Purified PCR products were used in 11 μ L sequencing reactions with BigDye[®] Terminators 3.1 (Applied Biosystems, Foster City, California) in combination with SSU primers NS1, NS2, NS3, NS4 (White et al. 1990) and LSU primers LROR, LR3, LR3R, LR6 (Vilgalys and Hester 1999). Sequences were generated on an Applied Biosystems 3730XL high-throughput capillary sequencer at the UIUC Biotec facility.

Taxon selection.—The type species of *Massarina* and *Lophiostoma*, *M. eburnea* (Tul. & C. Tul.) Sacc. and *L. macrostomum* (Tode) Ces. & de Not. respectively, some freshwater relatives of *M. ingoldiana*, such as *Massariosphaeria typhicola* (P. Karst.) Leuchtm., *L. breviappendiculatum* Kaz. Tanaka, Sat. Hatak. & Y. Harada, and one strain of *Massarina* sp., also were sequenced as well as 10 strains of *M. ingoldiana*. New sequences were deposited in GenBank (TABLE I).

Phylogenetic analyses.—To identify species similar to *M. ingoldiana* a BLAST analysis of GenBank was conducted with the sequences of the ex type culture of *M. ingoldiana* obtained from ATCC. SSU and LSU sequences of *M. ingoldiana sensu lato* taxa were aligned along with those of other related species obtained from GenBank. The aligned dataset was subjected to two phylogenetic analyses, maximum parsimony (MP) using a close-neighbor-interchange heuristic search with an initial tree by random addition sequence (100 replicates) and neighbor joining (NJ) based on the Kimura two-parameter model. These analyses were performed with MEGA 4 (Tamura et al. 2007). Characters were weighted equally, and gaps were ignored. The bootstrap values for nodes were computed from 1000 replicates for both MP and NJ analyses. In addition to the analyses of each region analyses based on a combined dataset of SSU and LSU were carried out. Sequences of *Dothidea insculpta* Wallr., outgroup taxon, were used to root trees. Alignments were deposited in TreeBASE (S2516, M4806).

Bayesian analysis employing Markov chain Monte Carlo (MCMC) was performed with MrBayes 3.1.2 (Huelsenbeck et al. 2001, Huelsenbeck and Ronquist 2001) as an additional means of assessing branch support. Modeltest 3.7 (Posada and Crandall 1998) was used to determine the best fit model of evolution for the dataset. The TrN + I + G model was used to run 50 000 000 generations with trees sampled every 1000 generations resulting in 50 000 trees. Two independent analyses were performed with four chains using default settings to ensure that trees were being sampled from the same tree space and that they converged on the same tree. The first 10 000 trees, which extended beyond the burn-in phase in each analysis, were discarded, and the remaining 40 000 trees were used to calculate posterior probabilities.

RESULTS

Analyses of SSU rDNA sequences.—A BLAST analysis of GenBank based on the SSU sequence (ca. 800 bp) of *M. ingoldiana* (ATCC 200398) suggested that this species is close to *Massariosphaeria typhicola* (EF165037) in Pleosporales. In terms of sequence length however these sequences were insufficient for our analyses. Therefore new sequences of SSU and LSU from two strains of *M. typhicola* (MAFF 239218 and 239219) were obtained. An SSU alignment of 45 strains, after excluding insertions of *Delitschia didyma* Auersw. (513–809, 1247–1591) and *Neottiosporina paspali* (G.F. Atk.) B. Sutton & Alcorn (488–842), resulted in a 911 character dataset, of which 147 characters (16.1%) were variable and 106 characters (11.6%) were parsimony informative. The NJ tree generated from this alignment showed that *M. ingoldiana sensu lato* does not constitute a monophyletic clade and 10 strains of *M. ingoldiana* are divided into two lineages (data available in TreeBASE).

Analyses of LSU rDNA sequences.—A 1300 bp segment of LSU sequenced data was obtained from *M. ingoldiana* and its relatives. To analyze these sequences with as many pleosporalean taxa in GenBank as possible ca. 760 characters of the 5' end were used. The data matrix of LSU comprised 78 strains and 779 aligned characters with 246 variable positions (31.6%) and 200 parsimony informative positions (25.7%). A MP analysis of the dataset resulted in 18 equally parsimonious trees with a length of 904 steps (consistency index [CI] = 0.370, retention index [RI] = 0.766). The tree obtained from NJ analysis had a topology similar to that of the MP tree. The MP tree based on the LSU dataset revealed that *M. ingoldiana* is polyphyletic and comprises two distinct lineages (data available at TreeBASE). These two lineages did not cluster with the type species of *Massarina* (*M. eburnea*) or *Lophiostoma* (*L. macrostomum*), genera that previously accommodated *M. ingoldiana*. The ex-type (ATCC200398) and other strains (KT966, 1107, 1096, KH100 and KH114) of *M. ingoldiana*, along with *Massarina* sp. R56-1 and *L. breviappendiculatum*, formed a single clade supported by moderate bootstrap values (82% in MP and 90% in NJ). *Massariosphaeria typhicola* was a sister taxon of this clade. Other strains of *M. ingoldiana* (KH68, 155 and KT891) were separated from the type lineage of *M. ingoldiana* and formed a monophyletic clade with high bootstrap support (99% in MP, 100% in NJ). The MP and NJ analyses indicated that this clade is the sister of *Massaria platani* Ces. (Massariaceae).

Analyses of combined SSU and LSU rDNA sequences.—A combined alignment of the SSU (916 bp) and LSU

(1047 bp) consisting of 54 strains was generated. An insertion of *Ophiosphaerella herpotricha* (Fr.) J. Walker (459–797) in the SSU region was excluded from the alignment. Of 1963 characters 406 (20.7%) were variable, of which 296 (15.1%) were parsimony informative. An MP analysis yielded 29 equally parsimonious trees with a tree length of 1052 steps (CI and RI values respectively of 0.470 and 0.748). A consensus tree was constructed from the 29 MP trees (FIG. 1). Analyses of the combined datasets also generated essentially similar genealogies to the LSU analyses and suggested that *M. ingoldiana sensu lato* was separated into two lineages (clades *Lindgomyces* and *Tingoldiogo*) not belonging to either *Massarina* nor *Lophiostoma*. Clade *Lindgomyces*, which includes the type strain of *M. ingoldiana*, consisted of 10 strains and received relatively high statistical support (MP bootstrap 92%/posterior probability 100%). In clade *Lindgomyces* distinct subclades of *M. ingoldiana* were found. A subclade including the type strain (ATCC200398) and KH100 of *M. ingoldiana* and a subclade with high bootstrap value (95%) comprising four strains of *M. ingoldiana* (KT1096, 1107, 966 and KH114 = *Lindgomyces rotundatus*). *Massarina* sp. R56-1 and *L. breviappendiculatum* clustered together and nested at the basal position of the clade including *M. ingoldiana sensu lato* (MP BS 92%/PP100%). In clade *Tingoldiogo* three strains (KH68, 155 and KT891) of *M. ingoldiana* formed a strongly supported lineage (100%).

TAXONOMY

Phylogenetic analyses based on ribosomal DNA sequences indicated that *M. ingoldiana sensu lato* contains two distinct lineages corresponding to two genera. Placement of these genera was not consistent with that of previously known genera in terms of molecular phylogenetic and morphological aspects. A new genus, *Lindgomyces*, is established for members of clade *Lindgomyces* including the type strain of *M. ingoldiana* and two new species; two new combinations in *Lindgomyces* also are proposed. We further propose a new family, Lindgomycetaceae, based on morphological and molecular phylogenetic differences among members of the *Lindgomyces* clade and other existing families in the Pleosporales. A monotypic genus, *Tingoldiogo*, also is established based on phylogenetic and morphological data.

Lindgomycetaceae K. Hiray., Kaz. Tanaka & Shearer, fam. nov.

MycoBank 515187

Ascomata subglobosa vel globosa, ostiolata. Rostrum breve, centrale. Pseudoparaphyses cellulosa, copiosae,

septatae, ramificantes et anastomosantes. Asci fissitunicati, cylindrici vel clavati, octospori. Ascospores fusiformes vel cylindricae, uniseptatae vel multiseptatae, hyalinae vel brunneae, cum vagina gelatinosa obtectae.

Ascomata subglobose to globose, scattered to crowded, ostiolate. Beak short, central. Ascomal wall of hyaline to pale brown, small, thin-walled cells. Pseudoparaphyses filamentous, numerous, septate, branched, anastomosing. Asci fissitunicate, cylindrical to clavate, rounded at the apex, with an apical chamber, eight-spored. Ascospores fusiform to cylindrical, uni- to multiseptate, hyaline to brown, usually covered with an entire sheath and/or bipolar mucilaginous appendages.

Typus genus: *Lindgomyces* K. Hiray., Kaz. Tanaka & Shearer.

Lindgomyces K. Hiray., Kaz. Tanaka & Shearer, gen. nov.

MycoBank 515188

Ascomata subglobosa vel globosa, erumpentia, ostiolata. Rostrum breve, papillatum, centrale. Pseudoparaphyses cellulosa, copiosae, septatae, ramificantes et anastomosantes. Asci fissitunicati, cylindrici vel clavati, octospori. Ascospores fusiformes vel cylindricae, uniseptatae, hyalinae, cum vagina gelatinosa obtectae.

Ascomata subglobose to globose, scattered to clustered, erumpent, ostiolate. Beak short-papillate, central. Ascomal wall composed of hyaline to pale brown small thin-walled cells. Pseudoparaphyses cellular, numerous, septate, branched, anastomosing. Asci fissitunicate, cylindrical to clavate, rounded at the apex, with an apical chamber, eight-spored. Ascospores fusiform to cylindrical, with a median primary septum, hyaline, usually covered with a sheath or bearing bipolar mucilaginous appendages. Senescent ascospores brown, 3(–5)-septate.

Etymology. An anagram of the last name of Dr T. Ingold in honor of his pioneering studies of freshwater filamentous fungi combined with “myces” L. for fungi.

Species typical. *Lindgomyces ingoldianus* (Shearer & K.D. Hyde) K. Hiray., Kaz. Tanaka & Shearer.

Notes. The general morphology of *Lindgomyces* is superficially similar to that of *M. eburnea*, the type of genus *Massarina* but differs in that the ascomata lack a well developed clypeus and the ascospores are equipped with a prominent gelatinous sheath. *Lindgomyces* is also similar to the type species of *Lophiostoma* (*L. macrostomum*), but the ascomata of *Lindgomyces* lack the slit-like or complex ostiole, thick lophiostomatoid ascomal wall and narrow ascospores of *Lophiostoma* (Hyde et al. 2002).

Lindgomyces ingoldianus (Shearer & K.D. Hyde) K. Hiray., Kaz. Tanaka & Shearer, comb. nov. FIGS. 2–13

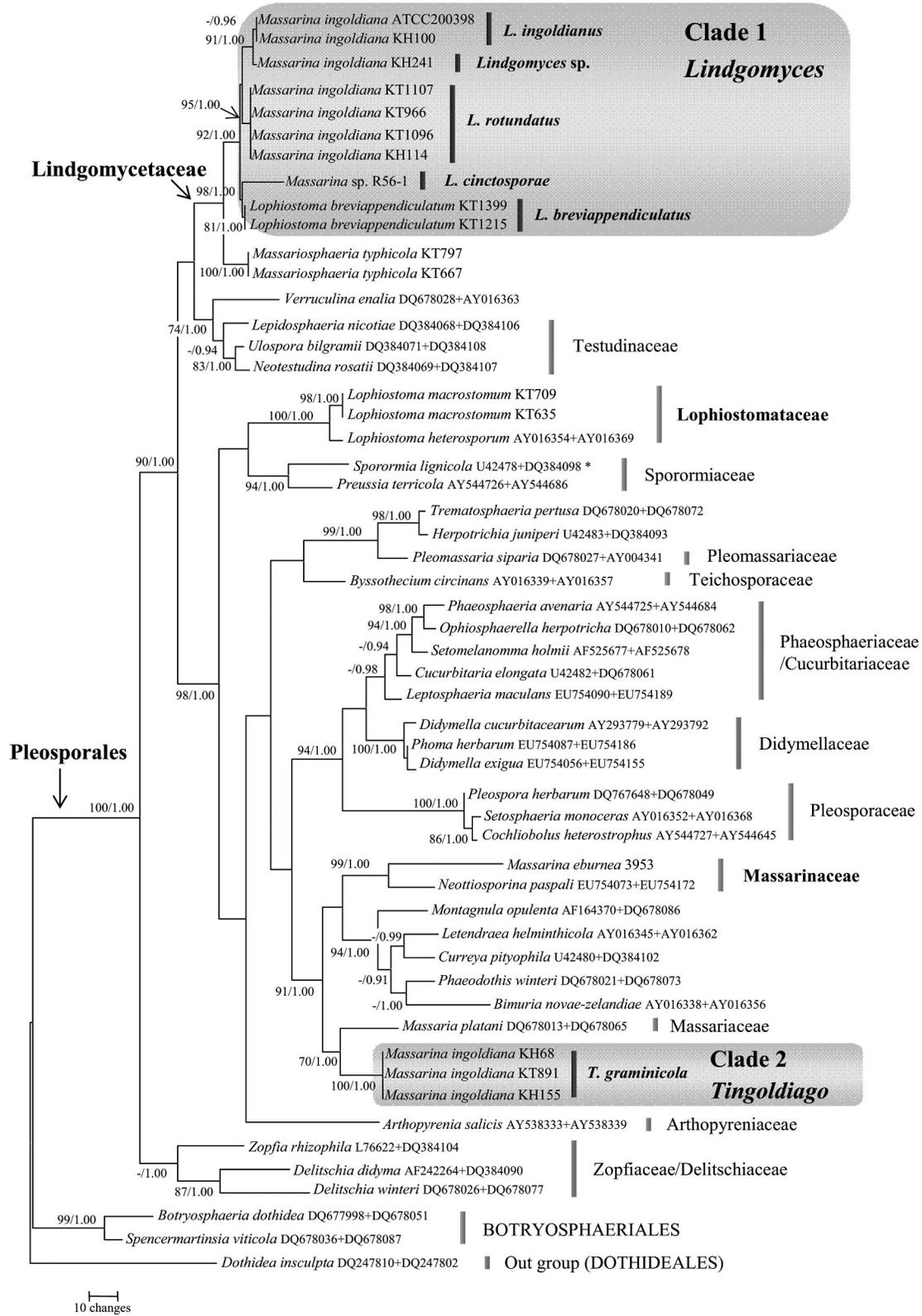
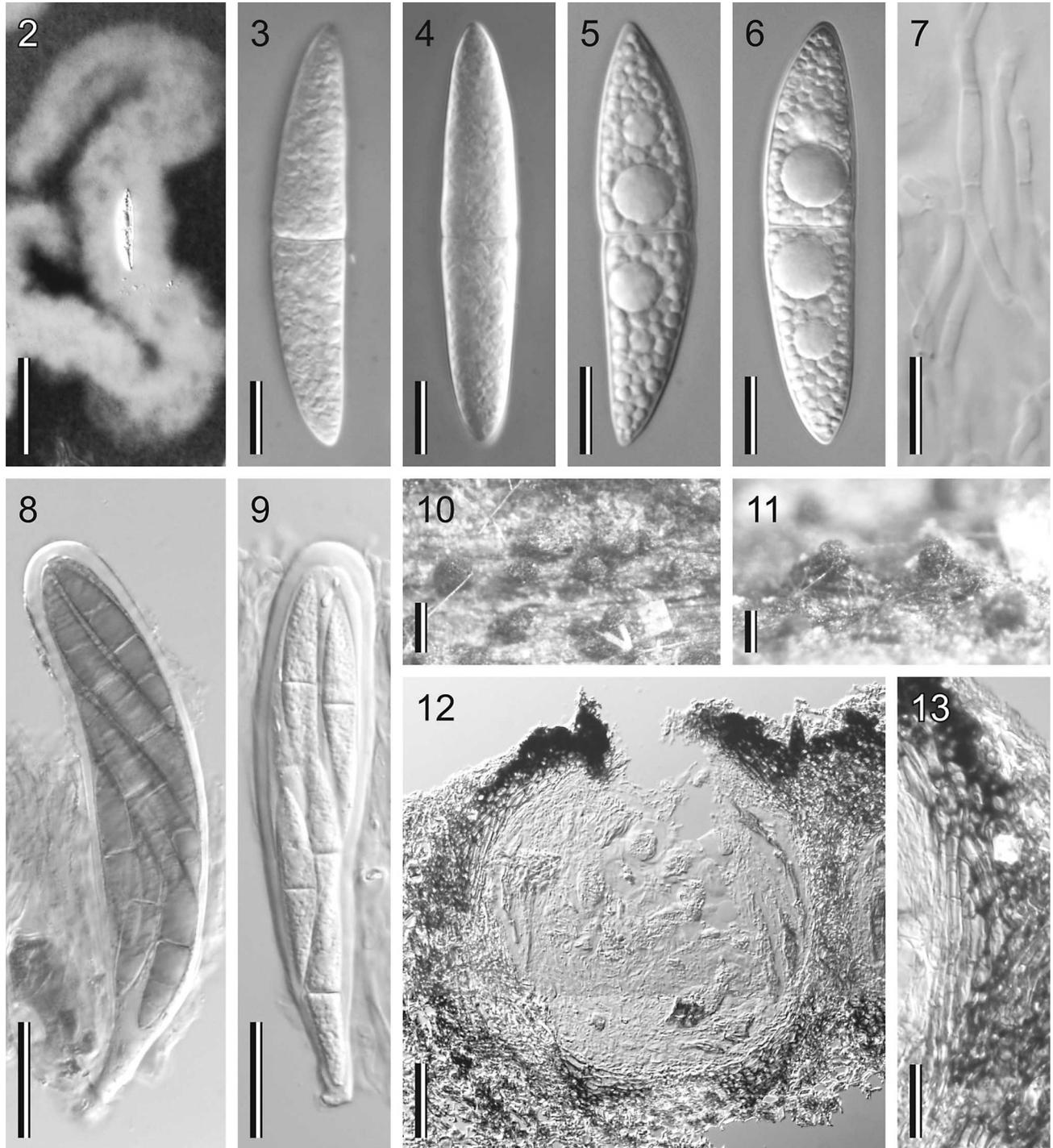


FIG. 1. Consensus tree of the 29 most parsimonious trees based on a combined dataset of SSU (916 bp) and LSU (1047 bp) rDNA. MP bootstrap values greater than 70% and Bayesian posterior probabilities above 0.90 are indicated at the nodes. Tree length = 1052, CI = 0.470, RI = 0.748. *Indicates sequences obtained from two different strains of the same species.



FIGS. 2–13. *Lindgomyces ingoldianus*. 2. Ascospore with a sheath (in India ink). 3–6. Ascospores. 7. Cellular pseudoparaphyses. 8, 9. Asci. 10, 11. Ascomata on host surface. 12. Ascoma in longitudinal section. 13. Ascomal wall. (2, 5 from culture ATCC 200398; 3, 4, 7, 9–13 from ILLS 52289 holotype; 6, 8 from HHUF 30006). Bars: 2, 12 = 50 μm ; 3–7 = 10 μm ; 8, 9 = 30 μm ; 10 = 250 μm ; 11 = 125 μm ; 13 = 20 μm .

Mycobank 515189

= *Massarina ingoldiana* Shearer & K.D. Hyde, Mycologia 89:114, 1997 (basionym).

= *Lophiostoma ingoldianum* (Shearer & K.D. Hyde) Aptroot

& K.D. Hyde, in Hyde, Wong, Aptroot, Fungal Diversity Res. Ser. (Hong Kong) 7:107, 2002.

Ascomata 200–260 μm high, 340–450 μm wide, scattered, immersed to erumpent, subglobose, black,

ostiolate. Ostiole central, rounded. Beak 45–70 μm long, 90–140 μm wide, short-papillate, composed of subglobose brown cells. Ascomal wall 20–35 μm thick, composed of an inner layer of polygonal to subglobose hyaline, thin-walled cells 7–12 \times 2.5–5 μm , and an outer layer of small subglobose, brown cells 2–4 \times 2–4 μm , sometimes poorly developed at the base. Pseudoparaphyses cellular, numerous, 1.5–3 μm wide, hyaline, anastomosing, branched, covered with gelatinous material. Asci (110–)120–167.5(–182.5) \times 25–32.5 μm (\bar{x} = 148.0 \times 27.9 μm , n = 50), fissitunicate, cylindrical to clavate, rounded at the apex, with an apical chamber, with eight overlapping biseriate to triseriate ascospores. Ascospores (47–)50–59 \times 9–11(–12) μm (\bar{x} = 55.3 \times 9.9 μm , n = 50), L/W (4.7–)4.9–6.6 (\bar{x} = 5.6, n = 50), fusiform with acute ends, straight or slightly curved, with the primary septum almost median (0.47–0.54; \bar{x} = 0.50, n = 50), filled with small lipid droplets that have a foamy appearance, slightly constricted at primary septum, with a broad upper cell, hyaline, smooth, becoming three-septate and pale brown with age, surrounded by a gelatinous sheath. Sheath about 1 μm thick at sides, 20–30 μm long at each end. Sheath expands at both ends to form large, sticky appendages up to 240 μm long.

Anamorph. Unknown.

Habitat. On decaying submerged wood in freshwater habitats.

Known distribution. JAPAN, USA.

Specimens examined: USA. WISCONSIN: Adams County, Lemonweir River, 43°46'16"N, 89°53'10"W, on submerged decorticated woody debris, 31 Jul 1992, *CAS and JLC A-39-1* (HOLOTYPE ILLS 52289; culture ATCC 200398). JAPAN. OKINAWA: Iriomote, Oomijya River, 24°23'695"N, 123°51'781"E, on submerged decorticated woody debris, 28 Sep 2007, *KH 100* (HHUF 30006; culture JCM 16479).

Notes. This species is almost identical to *L. rotundatus* (see below) in terms of overall morphology and lignicolous substrates in aquatic habitats but can be distinguished from the latter by relatively larger ascospores with acute ends.

Lindgomyces rotundatus K. Hiray. & Kaz. Tanaka, sp. nov.

FIGS. 14–25

Mycobank 515190

Ascomata 140–210 μm alta, 200–260 μm diam, aggregata, immersa vel erumpentia, subglobosa vel globosa, nigra, cum ostiolo rotundato. Rostrum leviter papillata vel none, centrale, periphysatum, 20–50 \times 60–120 μm . Paries ascomatis 10–20 μm crassus, ex cellularis stratis interna, rectangularata vel subglobosa, brunneis, 5–12 \times 1.5–5 μm , que exostratis diminuta subglobosa, 2–4 \times 2–4 μm composites. Pseudoparaphyses cellulosa, copiosae, 2–4 μm latae, septatae, inter septa 19–22 μm longae, in material gelatinosa. Asci (80–)92.5–150(–152) \times (16–)17.5–27.5 μm ,

fissitunicati, cylindrici vel clavati, apice rotundati, camera vadosae apicali formantes vel none, octosporis. Ascospores (41.5–)43–53 \times 9–12 μm , L/W (3.5–)3.9–5.1(–5.4), cylindricae rotundis, ad septum primo medio constrictae, hyalinae, biguttulatae in cellularibus, membranibus fusiformium gelatinosae.

Ascomata 140–210 μm high, 200–260 μm diam, clustered, immersed to erumpent, subglobose to globose, black, ostiolate. Ostiole central, rounded. Beak absent or short papillate, periphysate, 20–50 \times 60–120 μm wide, composed of subglobose brown cells. Ascomal wall 10–20 μm thick, comprising an inner layer of rectangular to subglobose, pale brown 5–12 \times 1.5–5 μm thin-walled cells, and an outer layer of small subglobose 2–4 \times 2–4 μm brown cells; outer cells interspersed among hosts cells. Pseudoparaphyses cellular, numerous, 2–4 μm wide, septate, with septa at 19–22 μm intervals, hyaline, branched, associated with gelatinous material. Asci (80–)92.5–150(–152) \times (16–)17.5–27.5 μm (\bar{x} = 117.1 \times 23.4 μm , n = 60), fissitunicate, cylindrical to clavate, rounded at the apex, with or without a shallow apical chamber, with eight overlapping biseriate to triseriate ascospores. Ascospores (41.5–)43–53 \times 9–12 μm (\bar{x} = 48.8 \times 10.8 μm , n = 53), L/W (3.5–)3.9–5.1(–5.4) (\bar{x} = 4.5, n = 53), cylindrical with rounded ends, slightly curved, with the primary septum almost median (0.48–0.53; \bar{x} = 0.50, n = 41), constricted at primary septum, with broad upper cell, becoming three-septate late in development, hyaline but becoming brown with age, smooth, with two large guttules in each cell, surrounded by a fusiform gelatinous sheath. Sheath about 4 μm thick at sides, 30–35 μm long at both ends, expanding to form appendages up to 200 μm long.

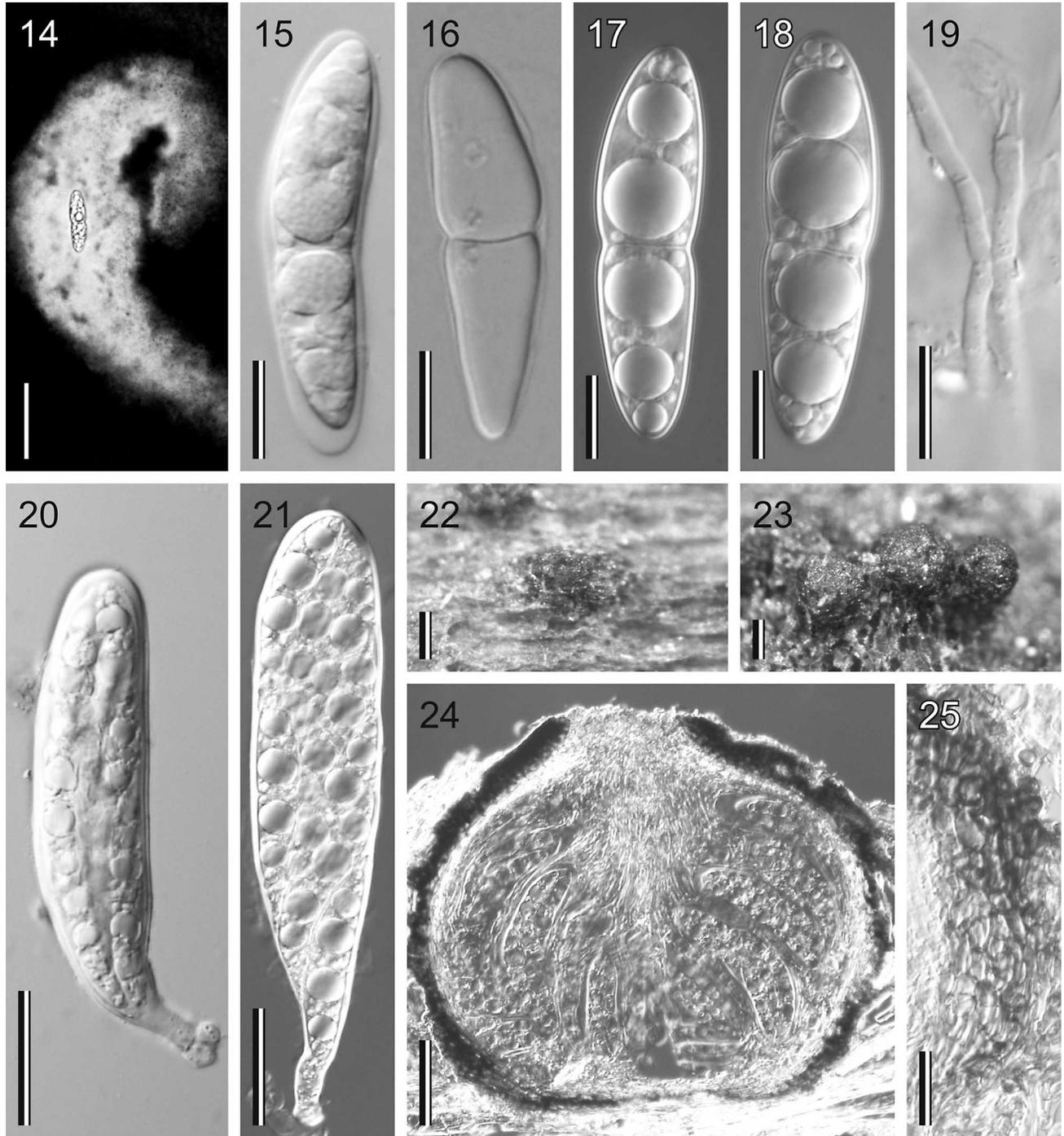
Anamorph. Unknown.

Habitat. On decaying submerged wood in freshwater habitats.

Known distribution. JAPAN, USA.

Etymology. From the Latin, *rotundatus*, in reference to the rounded apices of ascospores.

Specimens examined: JAPAN. AOMORI: Turutamati, Huzimiko, on submerged decorticated *Salix* debris, 8 Jun 2008, *KH 114* (HHUF 30008; culture JCM 16484); Hirosaki, Aoki, Mohei Pond, 40°34'1"N, 140°26'3"E, on submerged woody debris, 7 Dec 2002, *KT 966* (HHUF 27883 HOLOTYPE designated here; culture MAFF 239473); 3 May 2003, *KT 1107* (HHUF 28000; culture JCM 16483); 22 Aug 2003, *KT 1362–1364* (HHUF 29037–29039), 27 Sep 2003, *KT 1479* (HHUF 29041); 25 Oct 2003, *KT 1525–1527* (HHUF 29045–29047); 29 Nov 2003, *KT 1599–1603* (HHUF 29048–29052); Kuroishi, Syounai, Nijinoko, 40°34'4"N, 140°41'3"E, on submerged woody debris, 29 Apr 2003, *KT 1096* (HHUF 27999; culture JCM 16482). USA. WISCONSIN: Adams County, Lemonweir River, 43°46'16"N, 89°53'10"W, on bark of submerged, partly



FIGS. 14–25. *Lindgomyces rotundatus*. 14. Ascospore with a sheath (in India ink). 15–18. Ascospores. 19. Cellular pseudoparaphyses. 20, 21. Asci. 22, 23. Ascomata on host surface. 24. Ascoma in longitudinal section. 25. Ascomal wall. (14 from HHUF 28000; 15, 19, 20, 22–24 from HHUF 27883 holotype; 16, 25 from HHUF 27999; 17, 21 from culture KT966; 18 from culture KT1096.) Bars: 14, 24 = 50 μ m; 15–19 = 10 μ m; 20, 21 = 30 μ m; 22, 23 = 250 μ m; 25 = 20 μ m.

decorticated woody debris, 25 Sep 1993, CAS and JLC A-140-2 (ILLS 52291 PARATYPUS of *Massarina ingoldiana*).

Notes. This species closely resembles *L. ingoldianus* but clearly is separated from it by having slightly smaller ascospores with rounded ends. Analyses based

on LSU and SSU sequences indicate that they are distinct species. A specimen cited in the protolog of *M. ingoldiana* (ILLS 52291) also was re-examined and identified as *L. rotundatus* based on morphological data.

Lindgomyces breviappendiculatus (Kaz. Tanaka, Sat. Hatak. & Y. Harada) K. Hiray. & Kaz. Tanaka, comb. nov.

Mycobank 515191.

= *Lophiostoma breviappendiculatum* Kaz. Tanaka, Sat. Hatak. & Y. Harada, *Mycoscience* 46:88, 2005 (basonym).

Anamorph. Unknown.

Habitat. On submerged wood in rivers.

Known distribution. JAPAN.

Specimens examined: JAPAN. HOKKAIDO: Akkeshi, Ootakita, Satebetsu River, 43°8'13"N, 144°49'3"E, on submerged wood, 3 Jun 2003, *KT 1215* (HHUF 28193; culture MAFF 239291 = JCM 12702); 7 Sep 2003, *KT 1399* (HHUF 28194 HOLOTYPUS; culture MAFF 239292 = JCM 12701).

Notes. For a detailed description and illustration see Tanaka et al. (2005). This species, collected from submerged wood from a river, first was described as *Lophiostoma* based on a generic concept proposed by Hyde et al. (2002), although the most characteristic feature of *Lophiostoma*, the slit-like ostiole of the ascomatal beak, was not found in this species (Tanaka et al. 2005). Certain characteristics of this species, such as lignicolous substrate in freshwater habitat, globose ascomata composed of polygonal cells, clavate asci with fissitunicate dehiscence and fusiform ascospores with a sheath elongated to form bipolar appendages, are in accordance with the concept of *Lindgomyces*.

Lindgomyces cinctosporae Raja, A.N. Mill. & Shearer, sp. nov. FIGS. 26–36

Mycobank 515192

Ascomata in ligno 285–330 µm alta, 374–426 µm diam, subglobose vel conica, nigra, ostiolata, sub clypeo, immersa. Papillae 40–70 × 85–90 µm, centrale, aperiphysatum. Pseudoparaphyses septatae, anastomosantes, 1.5–3 µm diam. Asci 146–198 × 22–35 µm, fissitunicati, clavati, ad apices rotundatos, octospori, brevi pedunculati. Ascosporae 40–58 × 10–18 µm, biseriatis, oblongus-fusiformis, ad apicem rotundum, uniseptatum, ad maturitates trianastomosatae, e vaginae irregulari gelatinosa circumcincta.

Ascomata on wood 285–330 × 374–426 µm, subglobose to conical, black, appearing as raised dome-like structures on substrate, solitary to gregarious, immersed to partly immersed, ostiolate, papillate, clypeate. Beak 40–70 × 85–90 µm, central, conical to cylindrical, aperiphysate. Ascomal wall 45–68 µm wide, in longitudinal section composed of an inner layer of hyaline, elongate, angular, thin-walled cells 5–7 × 3–5 µm, and an outer layer of angular cells occluded with brown amorphous material associated with decomposed wood. Pseudoparaphyses numerous, sheet-like, septate, constricted at the septa, 1.5–3 µm wide, anastomosing. Asci 146–198 × 22–35 µm (\bar{x} = 167 × 29 µm, n = 15), fissitunicate, clavate, broadly rounded at the apex, tapering to a short stipe,

thick-walled, with or without an apical chamber. Ascospores 40–58 × 10–18 µm (\bar{x} = 50 × 14 µm, n = 50), overlapping biseriata, oblong-fusiform, slightly rounded at the apices, hyaline, one-septate when young, with the primary septum almost median (0.46–0.57; \bar{x} = 0.50, n = 50), becoming three-septate and brown with age, with one large and one small guttule in each cell, occasionally filled with numerous small, lipid droplets; upper cell broader than the lower cell; surrounded by a gelatinous sheath ca. 2 µm wide along the side of the ascospores and 3–4 µm long at the ascospore apices, staining in aqueous nigrosin; in water sheath becoming amorphous and spreading to ca. 8–12 µm wide. Colonies on PYG immersed with a dense mat of aerial hyphae; immersed hyphae brown black.

Anamorph. Unknown.

Habitat. lotic, lentic.

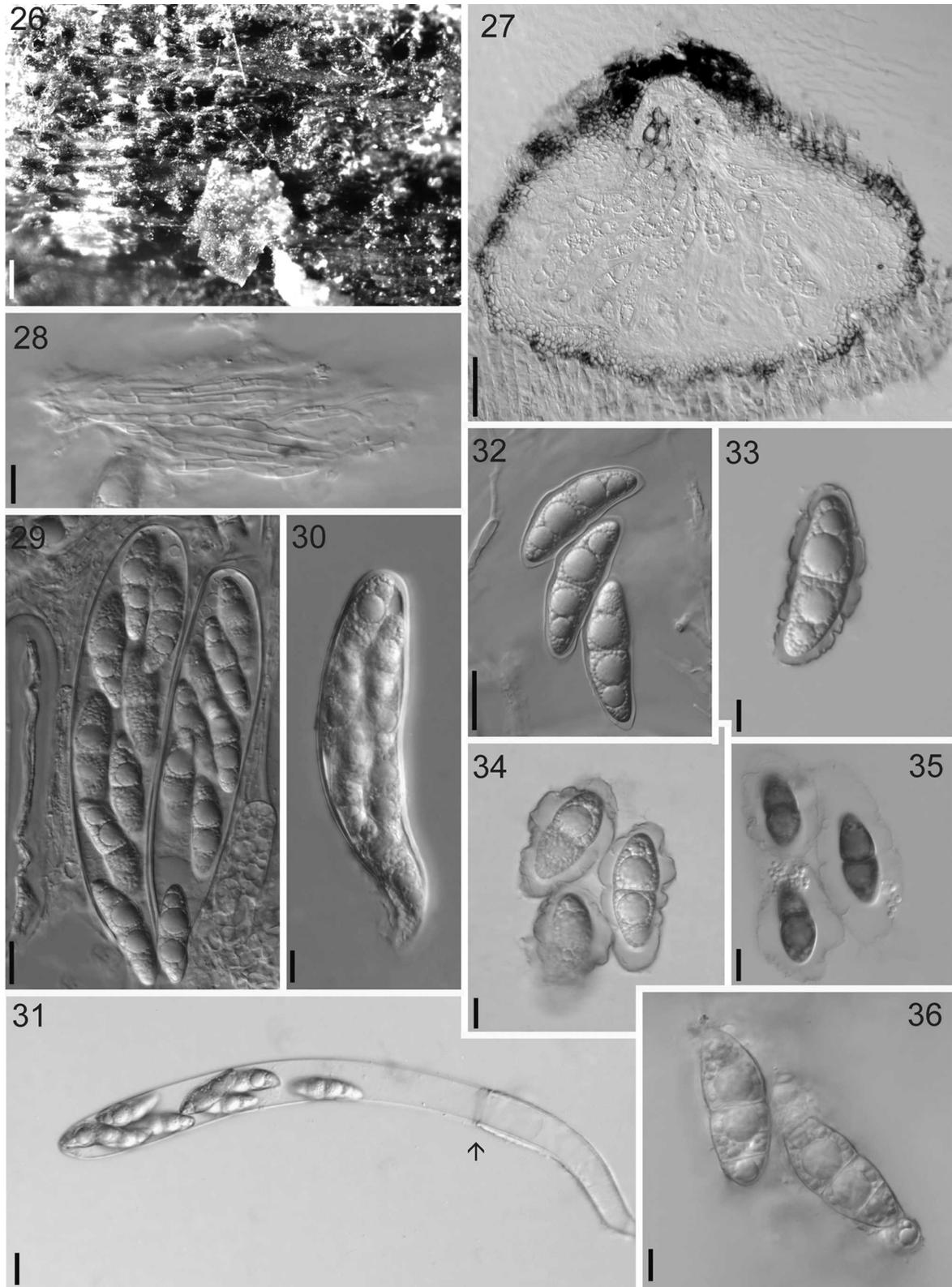
Known distribution. USA.

Etymology. cincto = Latin meaning surround/enclosed referring to the ascospore, which is surrounded by a gelatinous sheath.

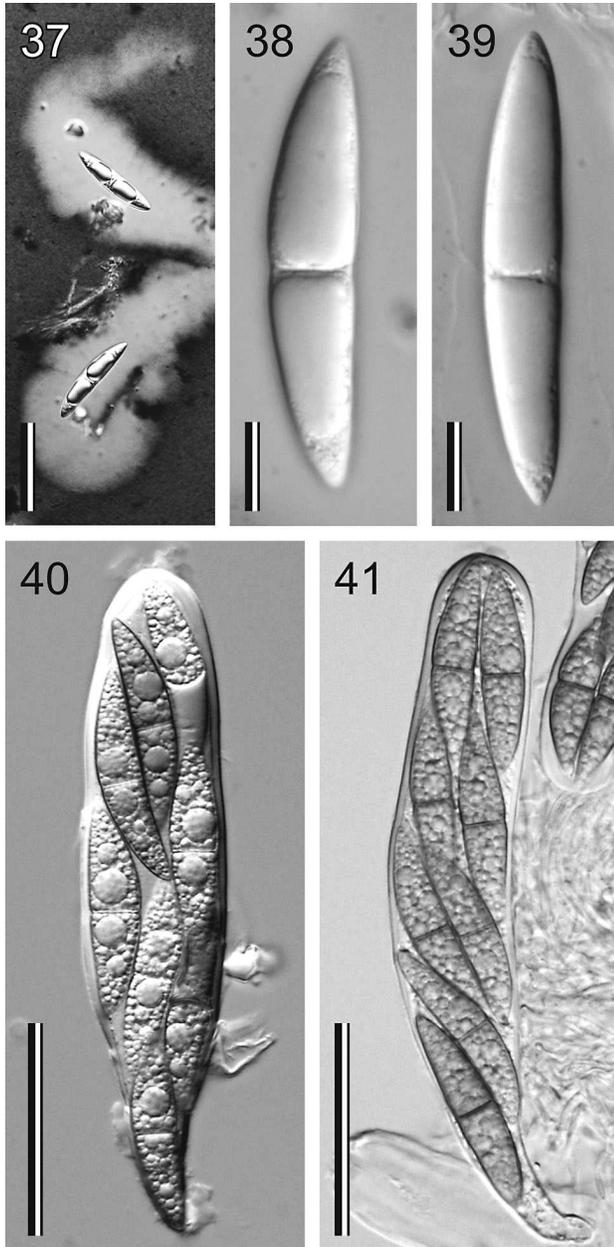
Specimens examined: USA. NORTH CAROLINA, Deep Creek, 35°28'23"N, 83°25'39"W, water 21 C, pH 5, 16 Jul 2000, *Jinx Campbell, A430-1*; NORTH CAROLINA, Cheoah Lake near Little Tennessee River, 35°27'06"N, 83°49'25"W, on submerged wood, water 28 C, pH 4.5, 19 Jul 2000, *Jinx Campbell, A430-3*. TENNESSEE: Great Smoky Mountains National Park, Green Brier Area, 35°45'054"N, 83°06'530"W, water 22 C, pH 5, on submerged wood, 2 Jul 2002, *HAR & Nate Hamburger, R56-1* (ILL 40791 HOLOTYPUS designated here; culture Raja R56-1); Great Smoky Mountains National Park, Forge Creek, 35°33'45"N, 83°50'45"W, water 19 C, 6 Sep 2005, *HAR & ANM, R56-2*; Great Smoky Mountains National Park, Fletcher Creek, on road to Cosby campground, 35°46'52.895"N, 83°13'0.264"W, on submerged wood, pH 5–5.5, 28 Jul 2008, *HAR, ANM, Alberto M. Stchigel & Misericordia Caldach, R56-3*.

Notes. *Lindgomyces cinctosporae* differs from other species of *Lindgomyces* in the relatively wide ascospores and a surrounding ruffled gelatinous sheath. The sheath eventually becomes amorphous and enlarged in water but not nearly to the size of the sheaths of the other *Lindgomyces* species. The ascospores of *L. cinctosporae* are oblong-fusiform and slightly rounded at the apices, whereas those of *L. ingoldianus* are fusiform with acute ends and those of *L. rotundatus* are somewhat cylindrical with broadly rounded apices before fixation.

Thus far *L. cinctosporae* has been reported only from submerged wood collected in Great Smoky Mountains National Park. *Lindgomyces cinctosporae* (isolate R56-1) was tested for the production of extracellular enzymes in vitro and was found positive for cellulase, endoglucanase, beta-glucosidase, xyla-



FIGS. 26–36. *Lindgomyces cinctosporae*. 26. Ascomata on wood. 27. Longitudinal section through the ascoma (note dark clypeus around the ostiole). 28. Cellular pseudoparaphyses. 29, 30. Clavate, thick-walled asci. 31. Fissitunicate ascus with endoascus extending from ectoascus. 32. Ascospores with a gelatinous sheath mounted in water. 33. Ascospores stained with India ink showing gelatinous sheath. 34. Ascospores stained with aqueous nigrosin showing expanding gelatinous sheaths. 35. Ascospore sheath in glycerin. 36. Mature, three-septate ascospores beginning to germinate. Bars: 26 = 1 mm; 27 = 50 μ m; 28, 30, 33–36 = 10 μ m; 29, 31, 32 = 20 μ m.



FIGS. 37–41. *Lindgomyces* sp. KH241. 37. Ascospores with sheath (in India ink). 38, 39. Ascospores. 40, 41. Asci with pigmented ascospores. (37–41 from KH 241.) Bars: 37, 40, 41 = 50 μ m; 38, 39 = 10 μ m.

nase, amylase and laccase and also caused soft rot on balsa wood (Simmonis et al. 2008), suggesting it might contribute to decay of wood in freshwater habitats.

***Lindgomyces* sp. KH 241**

Asci 125–194 \times 20.5–29 μ m (\bar{x} = 149.5 \times 25.2 μ m, n = 30). Ascospores 47–62 \times 8–12 μ m (\bar{x} = 54.8 \times 10.1 μ m, n = 34), L/W 5.0–6.1, with a slightly

FIGS. 37–41

submedian septum (0.51), sometimes three-septate, hyaline to brown.

Anapmorph. Unknown.

Habitat. On submerged wood in intertidal environments.

Known distribution. JAPAN.

Specimen examined: JAPAN. OKINAWA: Iriomote, Geta River, 24°24'N, 123°52'E, on submerged wood, 22 Nov 2008, KH 241 (HHUF 30007; culture JCM 16480).

Notes. All phylogenetic analyses in this study suggest that this fungus is close to *L. ingoldianus*. However we retain this fungus as *Lindgomyces* sp. because of its habitat and ascospore morphology. *L. ingoldianus* usually is found in freshwater habitats and has hyaline, zero-septate (0.50) ascospores when fresh, whereas *Lindgomyces* sp. KH 241 is collected from intertidal regions and has hyaline to brown and 1–3-septate ascospores with a slightly submedian primary septum (0.51).

***Tingoldiagio* K. Hiray. & Kaz. Tanaka, gen. nov.**

Mycobank 515193

Ascomata complanata globosa vel subglobosa, immersa vel erumpentia, ostiolata. Pseudoparaphyses copiosae, septatae. Asci fissitunicati, cylindrici, octospori. Ascospores clavatae, uniseptatae, hyalinae, laeves, cum vagina gelatinosa obtectae.

Ascomata depressed globose to conical, sometimes mammiform, with a flattened base, scattered, immersed to erumpent, with a rounded ostiole. Ascomal wall composed of small hyaline to brown cells. Pseudoparaphyses numerous, septate, immersed in gel matrix. Asci fissitunicate, cylindrical, rounded at the apex, with or without a shallow apical chamber, eight-spored. Ascospores clavate, with a median primary septum, hyaline, smooth, usually with mucilaginous sheath. Senescent ascospores three-septate, pale brown.

Etymology. From the name of Dr C.T. Ingold, in honor of his outstanding contributions to mycology, especially with respect to aquatic filamentous ascomycetes.

Species typical. *Tingoldiagio graminicola* K. Hiray. & Kaz. Tanaka.

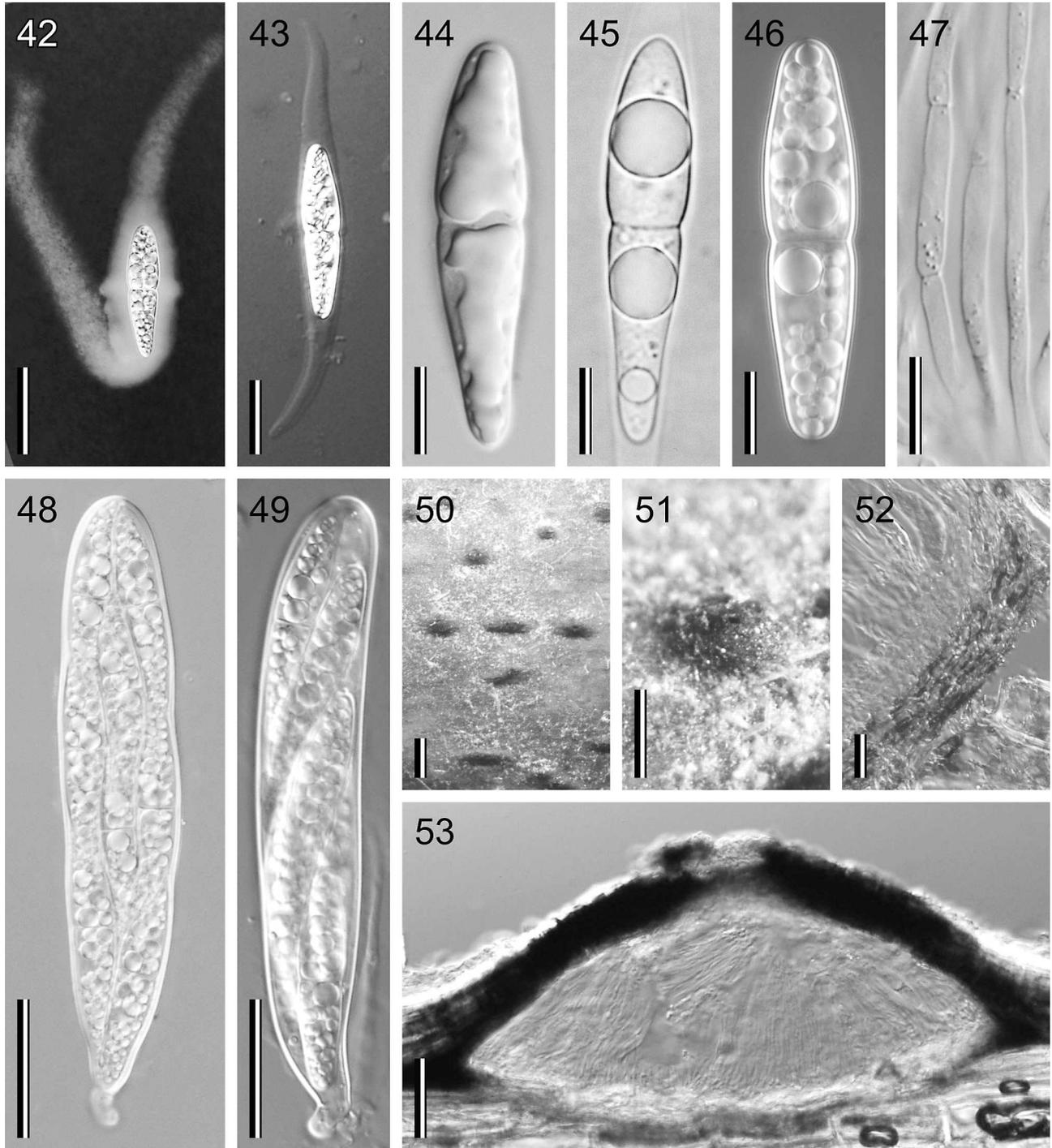
Notes. *Tingoldiagio* resembles *Lindgomyces* with respect to its ascospore and ascospore sheath and aquatic habitat. This genus differs in its graminicolous substrate and depressed globose to mammiform ascomata with a wedge of palisade-like cells at the rim between the base and sides.

***Tingoldiagio graminicola* K. Hiray. & Kaz. Tanaka, sp. nov.**

Mycobank 515194

Ascomata 150–250 μ m alta, 250–450 μ m diam, gregaria,

FIGS. 42–53



FIGS. 42–53. *Tingoldiagio graminicola*. 42, 43. Ascospore with a sheath. 44–46. Ascospores. 47. Cellular pseudoparaphyses. 48, 49. Asci. 50, 51. Ascomata on host surface. 52. Ascomal wall. 53. Ascoma in longitudinal section. (42 from culture KT891; 43, 46, 49 from culture KH68; 44, 47, 48, 50–53 from HHUF 30009 holotype; 45 from HHUF 27882.) Bars: 42, 43, 48, 49, 52 = 30 μm ; 44–47 = 10 μm ; 50 = 500 μm ; 51 = 250 μm ; 53 = 50 μm .

immersa vel erumpentia, depressa globosa vel conica cum deplanato fundi, nigra, cum ostiolo rotundato. Rostrum non. Paries ascomatis 10–50 μm crassus, ex cellulis stratis interna subglobosa hyalina 5–12 \times 2.5–5 μm que exostratis subglobosa parva brunneis 2–3 \times 2–3 μm compositus.

Pseudoparaphyses copiosae, 1.5–4 μm latae, septatae, inter septa 18–20 μm longae, in material gelatinosa. Asci (80–) 87.5–122(–127) \times 18.5–25(–27.5) μm , fissitunicati, cylindrici, apice rotundati, camera vadosae apicali formantes, octosporis. Ascosporae (42–)43.5–53 \times 7.5–11(–12.5) μm ,

L/W (3.6–)4.4–6.3(–6.7), clavatis rotundis, ad septum primo medio constrictae, hyalinae, guttulate, membranifusiformium gelatinosae.

Ascomata 150–250 µm high, 250–450 µm diam, scattered, immersed to erumpent, depressed globose to conical with a flattened base, black, ostiolate. Ostiole central, rounded. Beak absent. Ascomal wall 10–50 µm thick, composed of an inner layer of subglobose, hyaline 5–12 × 2.5–5 µm thin-walled cells, and an outer layer of small subglobose, 2–3 × 2–3 µm brown cells, poorly developed at the base. Pseudoparaphyses numerous, 1.5–4 µm wide, septate with 18–20 µm intervals, hyaline, immersed in gelatinous material. Asci (80–)87.5–122(–127) × 18.5–25(–27.5) µm (\bar{x} = 105.2 × 21.3 µm, n = 62), fissitunicate, cylindrical, rounded at the apex, with or without a shallow apical chamber, with eight overlapping biseriate to triseriate ascospores. Ascospores (42–)43.5–53 × 7.5–11(–12.5) µm (\bar{x} = 47.8 × 9.6 µm, n = 58), L/W (3.6–)4.4–6.3(–6.7) (\bar{x} = 5.1, n = 58), clavate with rounded ends, straight, with the primary septum almost median (0.45–0.53; \bar{x} = 0.49, n = 50), constricted at primary septum, with broad upper cell, narrow basal cell, becoming three-septate late in development, hyaline but becoming brown with age, smooth, guttulate, surrounded by a fusiform gelatinous sheath. Sheath about 1 µm thick at sides, 20–33 µm long at both ends, expanding to form a long appendage up to 180 µm.

Anamorph. Unknown.

Habitat. On stems of *Phragmites* spp. in freshwater habitats.

Known distribution. JAPAN, UK.

Etymology. In reference to the host plant.

Specimens examined: JAPAN. HOKKAIDO: Rishiri, Himenuma, 45°8'11"N, 141°8'53"E, on submerged culms of *Phragmites australis*, 27 Jul 2007, KH 68 (HHUF 30009 HOLOTYPE designated here; culture JCM 16485); 25 Jul 2008, KH 155 (HHUF 30010; culture JCM 16486); AOMORI: Hirosaki, Kadoke, Oowasawa River, 45°34'3"N, 141°31'8"E, on submerged culms of *Phragmites japonica*, 28 Sep 2002, KT 891 (HHUF 27882; culture MAFF 239472). UK. Esthwaite, Blelham Tarn and Windermere, on submerged culms of *Phragmites australis* (IMI 51812, as *Wettsteinina niesslii* E. Müll.)

Notes. The asci and ascospores of *T. graminicola* are similar in size and morphology to those of *L. ingoldianus* and *L. rotundatus*. The morphology and behavior of the ascospore sheath, as observed in a water mount, also are highly similar among the three species. However *T. graminicola* differs from the latter two species in the morphology of the ascomata, the clavate ascospores and its gramineous substrate. Slight differences in colony features also are observed: Colonies of *T. graminicola* (KT891) grow quickly on PDA (63 mm/4 wk in 20 C in the dark) and were clay-

colored (5D5), while colonies of *L. ingoldianus* (ATCC200398) and *L. rotundausa* (KTC966) grew more slowly (15 mm/4 wk) and were greenish-gray (30E2) in both strains.

Material on culms of *Phragmites communis* from the UK identified as *Wettsteinina niesslii* (IMI 51812; Ingold 1955) also was re-examined in this study. Sheath morphology of the fungus could not be clearly observed, probably due to the long-term storage of the herbarium specimen. This fungus however was identified as *T. graminicola* on the basis of the slightly clavate ascospores of 53–60 × 9–12 µm as well as by the illustration of ascospores with a bipolar appendage-like sheath provided by Ingold (1955).

DISCUSSION

Generic placement of M. ingoldiana.—*Massarina ingoldiana* has been reported frequently worldwide since its establishment (Shearer 2001). This species is easily recognized by its most striking feature—ascospores bearing a characteristic gelatinous sheath that enlarges in water (FIG. 2). The taxonomic position of this species however is currently problematic. This fungus originally was described as a species of *Massarina*, based on the pseudothecial ascomata composed of pseudoparenchymatous cells, cellular pseudoparaphyses and one-septate hyaline ascospores (Shearer and Hyde 1997). This classification was accepted by Aptroot (1998), who published ‘‘A world revision of *Massarina*’’, which provides a list of 160 species that had been placed in the genus. Later the generic concept of *Massarina* was changed based on phylogenetic analyses of SSU and ITS sequences of ribosomal DNA by Liew et al. (2002); they suggested that the *Massarina* species with its characteristically narrow fusiform ascospores are close to the morphologically similar genus *Lophiostoma*. Based on ascospore shape, *M. ingoldiana* was transferred to *Lophiostoma* without molecular evidence (Hyde et al. 2002).

Phylogenetic analyses in this study, made on the basis of 10 strains identified as *M. ingoldiana*-like fungi, revealed that the species complex is separated into two distinct lineages within Pleosporales and these clades are not congeneric with either *Massarina* or *Lophiostoma*. Several *Massarina* species, for example *M. phragmiticola* and *M. arundinacea*, both of which like *M. ingoldiana* previously had been placed in *Massarina* and *Lophiostoma*, did not group with *M. eburnea*, the type species of the genus, and they also deviated from the *Lophiostoma* clade (Kodsueb et al. 2007). These results suggest that the current generic concept of *Massarina* and *Lophiostoma* proposed by Hyde et al. (2002) is at least partially insufficient. A

revised circumscription of *Massarina* based on the type species and its related species with molecular phylogenetic evidence would be required to clarify the taxonomic position of other *Massarina* spp. because many species deposited in *Massarina* are based solely on morphological grounds (e.g. Tanaka and Harada 2003b) and are probably polyphyletic. On the other hand the traditional generic concept of *Lophiostoma* (Holm and Holm 1988, Tanaka and Harada 2003a) has characters such as the crest-like beak and slit-like ostiole and is considered to be a natural grouping (see also Tanaka and Hosoya 2008) because the monophyletic clade comprising two strains of the type species (*L. macrostomum*) and *L. heterosporum* (de Not.) M.E. Barr was strongly supported by high statistical values (MP bootstrap 100%/posterior probability 100%; FIG. 1).

In this study two lineages of *M. ingoldiana* were found from phylogenetic analyses based on SSU and LSU sequences; they are clade 1 containing the strain derived from the type specimen of *M. ingoldiana* and clade 2 (FIG. 1). Neither of these lineages possess ascomata with a conspicuous clypeus or cylindrical ascospores surrounded by a firm sheath similar to that of the type species of *Massarina* (*M. eburnea*), and they also lack the crest-like beak and slit-like ostiole at the ascomatal apex that are characteristic features of *Lophiostoma*. Both clades belong to the Pleosporales, but their phylogenetic affinities are distantly related and they can be distinguished by their habitats (i.e. lignicolous in clade 1 and herbaceous in clade 2) and the morphology of ascomata (i.e. nearly globose in clade 1 and lenticular with rim-like side wall in clade 2). Therefore two new genera, *Lindgomyces* and *Tingoldiogo*, are established respectively to accommodate members of clades 1 and 2.

Familial placement of Lindgomyces and Tingoldiogo.—*Lindgomyces*, supported by a high bootstrap and posterior probability (92%/100%; FIG. 1), is a sister clade of *M. typhicola*, and their affinities also received high statistical support (98%/100%, FIG. 1). Although genus *Massariosphaeria* is placed in Lophiostomataceae (Lumbsch and Huhndorf 2007) a molecular phylogenetic study indicates that *Massariosphaeria* is polyphyletic and *M. typhicola* deviates from the type lineage of the genus and its familial placement is obscure (Wang et al. 2007). Phylogenetic analyses based on SSU + LSU sequences (FIG. 1) suggest that *Lindgomyces* (clade 1) might be phylogenetically related to *Verruculina enalia* (Kohlm.) Kohlm. & Volk.-Kohlm. (Didymosphaeriaceae) and three species in Testudinaceae, although no bootstrap support was obtained for these relationships. In addition *Lindgomyces* possesses none of the morphological

features of these families, such as brown and two-celled ascospores (Didymosphaeriaceae, Aptroot 2000) and cleistothecial ascomata and sculptured ascospores (Testudinaceae; von Arx 1971, Hawksworth 1979). Recently a new genus of a *Massarina*-like taxon, *Ammiculicola*, was established (Zhang et al. 2008), and two additional species of this genus as well as *Karstenula rubicunda* (Niessl) M.E. Barr, *Spirosphaera cupreorufescens* Voglmayr, and *Repetophragma ontariense* (Matsush.) W.P. Wu were treated as a monophyletic group characterized by ascomata with a slit-like ostiole, purple staining pigments on substrates and freshwater habitats (Zhang et al. 2009). They further suggested that this assemblage might represent a familial rank. The LSU sequence of *A. lignicola* Y. Zhang & K.D. Hyde, the type species of *Ammiculicola*, was analyzed with the LSU data used for this paper. The MP tree (data available at TreeBASE) revealed that the clade comprising *Lindgomyces* spp. and *M. typhicola* is a sister of the clade comprising the above *Ammiculicola* allied taxa. These relationships however were not supported in our LSU analyses (less than 50% bootstrap value) and were distinct in morphological aspects. The lineage includes genus *Lindgomyces*, and *M. typhicola* is similar to family Massariaceae but can be separated by lacking obvious clypeus at the ascomata. Due to the lack of any existing family in the Pleosporales we proposed a new family, Lindgomycetaceae, to accommodate five *Lindgomyces* species and *M. typhicola*. The nomenclature of *M. typhicola* should be changed and establishment of a new genus might be necessary, but we retain this species in *Massariosphaeria* until further morphological evidence on *Massariosphaeria sensu lato* is obtained.

Tingoldiogo formed a monophyletic clade with *Massaria platani* (Massariaceae, Barr 1979). Their relationship was moderately supported in the combined analyses (70%/100%, FIG. 1) but not in those of SSU and LSU alone (data not shown). Massariaceae is characterized by large ascomata, thick-walled asci and distoseptate ascospores (Barr 1979), but these characteristics were not found in *Tingoldiogo*. The SSU analysis suggested that *Tingoldiogo* is most closely related to *Keissleriella cladophila* (Niessl) Corbaz (Massariaceae, Kodsueb et al. 2007). The relationship between *Tingoldiogo* and *K. cladophila* based on the LSU and the combined dataset could not be analyzed because LSU sequences of this taxon are not available from GenBank. *Tingoldiogo* might have a high degree of affinity with *K. cladophila* but not with Massariaceae, typified by *M. eburnea*, as indicated by the results of our analyses (FIG. 1).

Monophyly of M. ingoldiana sensu lato.—*Massarina ingoldiana*, a common freshwater ascomycete, has

been reported in both temperate and tropical regions throughout latitudes 4–46°N (Shearer 2001). The global distribution of *M. ingoldiana* has been considered to be caused by its high adaptability to various aquatic habitats. Most ascomycetous species (82%) from freshwater habitats are found only either on woody (54%) or on herbaceous (28%) substrates, according to Shearer (2001). Similarly most freshwater ascomycetes (82%) can inhabit only either lotic (34%) or lentic (48%) environments. *Massarina ingoldiana* in contrast has been considered a “generalist species” that can occur on both herbaceous and woody plants in both lotic and lentic habitats (Shearer 2001). In this study putative *M. ingoldiana* was collected on submerged woody plants or the stems of reeds, such as *Phragmites australis* and *P. japonica*, in swamps and streams. Our study however revealed that *M. ingoldiana* as phenotypically defined to date was not a single species.

Considerable morphological variation exists among different populations of *M. ingoldiana* (e.g. ascospore features like dimension, septation, shape and pigmentation) already has been cited in the protolog of this species (Shearer and Hyde 1997). However those features have been interpreted as intraspecific variations and the collections have been treated as a single species primarily based on the sheath morphology of ascospores as the most consistent and distinguishing character. Phylogenetic analyses and morphological re-examination of *M. ingoldiana* in this study proved that ascospore differences have taxonomic significance and that the species *sensu lato* consists of at least three species that belong to two genera. Ascospore characteristics, albeit based on small differences, such as the shape of both ends, were found to be useful criteria in distinguishing species in the *M. ingoldiana* complex. For example *L. ingoldianus*, *L. rotundatus* and *T. graminicola* are characterized respectively by ascospores having acute ends, rounded ends and a slightly clavate form and the range and average of spore measurements differ slightly among all three taxa, although they all have enlarging gelatinous ascospore sheaths.

Convergence and species diversity of M. ingoldiana sensu lato.—The ascospore sheath has been considered the most distinctive and stable feature of *M. ingoldiana*. The sheath is considered to be an adaptation to aquatic habitats because it better enables ascospores to attach to substrates in moving water (Shearer 1993, Goh and Hyde 1996, Jones 2006). A similar expanding sticky sheath has been seen in other bitunicate aquatic ascomycetes, such as *Aliquandostipite crystallinus* Raja, A. Ferrer & Shearer in the Jahnuales (Raja et al. 2005), *Macrospora*

scirpicola (DC.) Fuckel in Pleosporaceae (Ingold 1955, Fallah and Shearer 2001), and *Phaeosphaeria vilasensis* Fallah, Shearer & Leuchtm. in Phaeosphaeriaceae (Fallah et al. 1999). Considering their phylogenetic position in Dothideomycetes, a sheath resembling that of *M. ingoldiana* might not be an uncommon feature and might have evolved repeatedly in aquatic Dothideomycetes.

Of note, the presence of *Tingoldiogo*, an additional lineage distantly related to *Lindgomyces*, initially was considered a putative species of *M. ingoldiana* in this study. It is obvious from our results that an ascospore sheath with remarkable features has arisen as a consequence of convergent evolution in response to aquatic environments. Several examples of convergence are generally known for aquatic fungi (Ingold 1966; Bärlocher 2007, 2009). For instance it is considered that tetradiate, sigmoid, and helical spore morphs found respectively among species in *Lemoniera* (Campbell et al. 2006), *Anguillospora* (Belliveau and Bärlocher 2005) and *Helicosporium* (Tsui and Berbee 2006, Tsui et al. 2006) evolved convergently and therefore are noninformative in terms of defining each genus. However these examples are interspecific phenomena in anamorphic genera. The remarkable convergence found for *M. ingoldiana sensu lato* is probably a rare case because species in *Lindgomyces* and *Tingoldiogo* have been identified phenotypically as species within a single ascomycetous genus, but in fact they are distantly related at familial levels.

We re-examined two specimens on *Scirpus validus* (ILLS 54040) and *Typha latifolia* (ILLS 54041) reported by Fallah and Shearer (2001) as *M. ingoldiana*. Although the species were similar in morphology to *L. ingoldianus* in actuality they might not be conspecific or even congeneric because all structures are considerably smaller than those of *L. ingoldianus* (e.g. 25–36 × 8–11 μm vs. [47]–50–59 × 9–11[–12] μm in the ascospores). The graminicolous nature of these collections might indicate that it is a new species belonging to *Tingoldiogo*. However molecular evidence is needed to confirm this hypothesis. At the same time a fundamental re-assessment of specimens formerly identified as *M. ingoldiana* is necessary to clarify the distribution and species diversity of *M. ingoldiana sensu lato*. The results of our study suggest that “*M. ingoldiana*” instead of being a cosmopolitan and generalist species comprises several species with to some extent distinctly different distributions and substrate preferences.

ACKNOWLEDGMENTS

This work was partially supported by a foundation from the Institute for Fermentation, Osaka, (IFO) to T. Seki.

Financial support from the National Science Foundation and National Institutes of Health (NSF No. DEB 03-16496, 08-44722 and NIH No. R01GM-60600) helped make this research possible. We are grateful for the help of curators at ILLS and IMI who loaned specimens. Any opinions, findings and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the National Science Foundation and National Institutes of Health.

LITERATURE CITED

- Aptroot A. 1998. A world revision of *Massarina* (Ascomycota). *Nova Hedwig* 66:89–162.
- . 2000. A monograph of *Didymosphaeria*. *Stud Mycol* 37:1–160.
- Bärlocher F. 2007. Molecular approaches applied to aquatic hyphomycetes. *Fungal Biol Rev* 21:19–24.
- . 2009. Reproduction and dispersal in aquatic hyphomycetes. *Mycoscience* 50:3–8.
- Barr ME. 1979. On the Massariaceae in North America. *Mycotaxon* 9:17–37.
- Belliveau MJR, Bärlocher F. 2005. Molecular evidence confirms multiple origins of aquatic hyphomycetes. *Mycol Res* 109:1407–1417.
- Campbell J, Shearer CA, Marvanová L. 2006. Evolutionary relationships among aquatic anamorphs and teleomorphs: *Lemonniera*, *Margaritispora* and *Goniopila*. *Mycol Res* 110:1025–1033.
- Fallah PM, Shearer CA. 2001. Freshwater ascomycetes: new or noteworthy species from north temperate lakes in Wisconsin. *Mycologia* 93:566–602.
- , ———, Leuchtmann A. 1999. Freshwater ascomycetes: *Phaeosphaeria vilasensis* (Pleosporales), a new species from a lake in Wisconsin. *Nova Hedwigia* 68:399–404.
- Goh TK, Hyde KD. 1996. Biodiversity of freshwater fungi. *J Ind Microbiol* 17:328–345.
- Hawksworth DL. 1979. Ascospore sculpturing and generic concepts in the Testudinaceae (syn. Zopfiaceae). *Can J Bot* 57:91–99.
- Holm L, Holm K. 1988. Studies in the Lophiostmataceae with emphasis on the Swedish species. *Symb Bot Ups* 28(2):1–50.
- Huelsenbeck JP, Ronquist F. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754–755.
- , ———, Nielsen R, Bollback JP. 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. *Science* 294:2310–2314.
- Huhndorf SM, Miller AN, Fernández FA. 2004. Molecular systematics of the Sordariales: the order and the family Lasiosphaeriaceae redefined. *Mycologia* 96:368–387.
- Hyde KD, Wong WSW, Aptroot A. 2002. Marine and estuarine species of *Lophiostoma* and *Massarina*. In: Hyde KD, ed., *Fungi in marine environments*. *Fungal Divers Res Ser* 7:93–109.
- Ingold CT. 1955. Aquatic ascomycetes: further species from the English lake district. *Trans Br Mycol Soc* 38:157–168.
- . 1966. The tetradiate aquatic fungal spore. *Mycologia* 58:43–56.
- Jones EBG. 2006. Form and function of fungal spore appendages. *Mycoscience* 47:167–183.
- Kodsueb R, Lumyong S, Ho WH, Hyde KD, McKenzie EHC, Jeewon R. 2007. Morphological and molecular characterization of *Aquaticheirospora* and phylogenetics of Massarinaceae (Pleosporales). *Bot J Linn Soc* 155:283–296.
- Kornerup A, Wanscher JH. 1978. *Methuen handbook of colour*. 3rd ed. London: Methuen.
- Liew ECY, Aptroot A, Hyde KD. 2002. An evaluation of the monophyly of *Massarina* based on ribosomal DNA sequences. *Mycologia* 94:803–813.
- Lumbsch HT, Huhndorf SM. 2007. Outline of Ascomycota—2007. *Myconet* 13:1–58.
- Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817–818.
- Raja HA, Carter A, Platt HW, Shearer CA. 2008. Freshwater ascomycetes: *Jahnula apiospora* (Jahnulales, Dothideomycetes), a new species from Prince Edward Island, Canada. *Mycoscience* 49:326–328.
- , Ferrer A, Shearer CA. 2005. *Aliquandostipite crystallinus*, a new ascomycete species from wood submerged in freshwater habitats. *Mycotaxon* 91:207–215.
- , Schmit JP, Shearer CA. 2009. Latitudinal, habitat and substrate distribution patterns of freshwater ascomycetes in the Florida Peninsula. *Biodivers Conserv* 18:419–455.
- Rehner SA, Samuels GJ. 1994. Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. *Mycol Res* 98:625–634.
- Shearer CA. 1993. The freshwater Ascomycetes. *Nova Hedwig* 56:1–33.
- . 2001. The distribution of freshwater filamentous ascomycetes. In: Misra JK, Horn BW, eds. *Trichomyces and other fungal groups*. Enfield: Science Publishing. p 73–98.
- , Descals E, Kohlmeyer B, Kohlmeyer J, Marvanová L, Padgett D, Porter D, Raja HA, Schmit JP, Thornton HA, Voglymayr H. 2007. Fungal biodiversity in aquatic habitats. *Biodivers Conserv* 16:49–67.
- , Hyde KD. 1997. *Massarina ingoldiana*, a new ascomycete from freshwater habitats. *Mycologia* 89:114–119.
- , Langsam DM, Longcore JE. 2004. Fungi in freshwater habitats. In: Mueller GM, Bills GF, Foster MS, eds. *Biodiversity of fungi: inventory and monitoring methods*. San Diego: Elsevier Academic Press. p 513–532.
- Shoemaker RA. 1984. Canadian and some extralimital *Nodulosphaeria* and *Entodesmium* species. *Can J Bot* 62:2730–2753.
- Simonis JL, Raja HA, Shearer CA. 2008. Extracellular enzymes and soft rot decay: Are ascomycetes important degraders in freshwater? *Fungal Divers* 31:135–146.
- Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596–1599.

- Tanaka K, Harada Y. 2003a. Pleosporales in Japan 1. The genus *Lophiostoma*. *Mycoscience* 44:85–96.
- , ———. 2003b. Pleosporales in Japan 3. The genus *Massarina*. *Mycoscience* 44:173–185.
- , Hatakeyama S, Harada Y. 2005. Three new freshwater ascomycetes from rivers in Akkeshi, Hokkaido, northern Japan. *Mycoscience* 46:287–293.
- , Hosoya T. 2008. *Lophiostoma sagittiforme* sp. nov., a new ascomycete (Pleosporales, Dothideomycetes) from Island Yakushima in Japan. *Sydowia* 60:131–145.
- Tsui CKM, Berbee ML. 2006. Phylogenetic relationships and convergence of helicosporous fungi inferred from ribosomal DNA sequences. *Mol Phylogenet Evol* 39:587–597.
- , Hyde KD, Hodgkiss IJ. 2000. Biodiversity of fungi on submerged wood in Hong Kong streams. *Aquat Microb Ecol* 21:289–298.
- , Sivichai S, Berbee ML. 2006. Molecular systematics of *Helicoma*, *Helicomycetes* and *Helicosporium* and their teleomorphs inferred from rDNA sequences. *Mycologia* 98:94–104.
- Tubaki K. 1978. On the Skerman's micromanipulator and microforge (in Japanese). *Trans Mycol Soc Japan* 19:237–239.
- Vilgalys R, Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J Bacteriol* 172:4238–4246.
- von Arx JA. 1971. Testudinaceae, a new family of ascomycetes. *Persoonia* 6:365–369.
- Wang HK, Aptroot A, Crous PW, Hyde KD, Jeewon R. 2007. The polyphyletic nature of Pleosporales: an example from *Massariosphaeria* based on rDNA and RBP2 gene phylogenies. *Mycol Res* 111:1268–1276.
- White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JS, White TJ, eds. *PCR protocol: a guide to methods and applications*. San Diego: Academic Press. p 315–322.
- Zhang Y, Fournier J, Crous PW, Pointing SB, Hyde KD. 2009. Phylogenetic and morphological assessment of two new species of *Amniculicola* and their allies (Pleosporales). *Persoonia* 23:48–54.
- , Jeewon R, Fournier J, Hyde KD. 2008. Multi-gene phylogeny and morphotaxonomy of *Amniculicola lignicola*: a novel freshwater fungus from France and its relationships to the Pleosporales. *Mycol Res* 112:1186–1194.