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Lindgomyces angustiascus, (Lindgomycetaceae, Pleosporales, Dothideomycetes), a new lignicolous species from freshwater habitats in the USA

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ABSTRACT

In this paper, we describe and illustrate a new species, *Lindgomyces angustiascus*, from submerged wood in lotic and lentic habitats from Florida, North Carolina and Wisconsin, USA. The new species is characterized by black, partially immersed, flattened, globose ascomata; fissitunicate, long, slender, obclavate asci; and one-septate, hyaline, fusiform ascospores with bipolar appendages, each covered with a gelatinous cap. Maximum Likelihood and Bayesian analyses of partial 18S nrDNA and 28S nrDNA, as well as the entire nuclear ribosomal internal transcribed spacer (ITS1, 5.8S and ITS2) region support the placement and establishment of this new species in the Lindgomycetaceae, Pleosporales, Dothideomycetes. Chemical analysis of the organic extract of *L. angustiascus* revealed the presence of 6*E*,9*E*-octadecadienoic acid and ergosterol peroxide as major secondary metabolites.

1. Introduction

Lindgomyces K. Hiray., Kaz. Tanaka & Shearer (Lindgomycetaceae, Pleosporales, Dothideomycetes) is a recently established freshwater ascomycete genus, which currently includes six species, *L. ingoldianus* (Shearer & K.D. Hyde) K. Hiray., Kaz. Tanaka & Shearer (type species), *L. apiculatus* K. Hiray. & Kaz. Tanaka, *L. breviappendiculatus* (Kaz. Tanaka, Sat. Hatak. & Y. Harada) K. Hiray. & Kaz. Tanaka, *L. cinctosporae* Raja, A.N. Mill. & Shearer, *L. lemonweirensis* Raja, A.N. Mill. & Shearer, and *L. rotundatus* K. Hiray. & Kaz. Tanaka (Shearer et al. 2009; Hirayama et al. 2010; Raja et al. 2011a). It is characterized by globose to subglobose ascomata; fissitunicate, cylindrical to clavate asci that are rounded at the apex; and one-septate, hyaline ascospores with a gelatinous sheath, which extends to form bipolar mucilaginous appendages (Hirayama et al. 2010).

As part of our recent investigations of freshwater ascomycetes in North Carolina, we discovered an undescribed species of *Lindgomyces* (G202-1) occurring on submerged wood in a lake from the Piedmont Plateau. The same species

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was previously recorded as an undescribed *Massarina* sp. (F60-1, 2) during a study on the latitudinal, substrate, and habitat distribution patterns of freshwater ascomycetes on submerged wood in Florida (Raja et al. 2009), as well as on submerged wood in a river in Wisconsin (as A640-1). Based on evaluation of morphological characters and combined phylogenetic analysis of partial 18S nrDNA (small subunit-SSU) and 28S nrDNA (large subunit-LSU), as well as the entire nuclear ribosomal internal transcribed spacer (ITS) region, we describe and illustrate a novel species discovered in three different freshwater habitats in the USA. In addition, as part of ongoing investigations of the chemical mycology of freshwater fungi, we screened one of the isolates of the new species (G202) for secondary metabolites production.

2. Materials and methods

2.1. *Sample collection and morphological examination*

Collection methodology and morphological examination of samples followed outlined methods (Shearer et al. 2004; Raja et al. 2009). Specimens were deposited in the University of Illinois Herbarium (ILL). The fungal cultures are maintained at the University of Illinois, Plant Biology Fungal Culture Collection and at the University of North Carolina at Greensboro, Department of Chemistry and Biochemistry Fungal Culture Collection.

2.2. *DNA extraction and PCR amplification*

DNA extraction and PCR amplification of SSU and LSU nrDNA regions were performed following published procedures (Shearer et al. 2009; Hirayama et al. 2010). For amplification of ITS, a combination of ITS1F/ITS1 and ITS4 primers were used (White et al. 1990; Gardes and Bruns 1993) using established thermocycler parameters (Promputtha and Miller 2010).

2.3. *Taxon sampling and phylogenetic analyses*

We compiled two datasets for phylogenetic analyses: (a) a combined SSU and LSU dataset that consisted of 66 taxa, which represented a number of families currently included in the Pleosporales, Dothideomycetes (Schoch et al. 2009; Lumbsch and Huhndorf 2010; Zhang et al. 2012); and (b) an ITS dataset, which consisted of 18 taxa including three strains of the new taxon, two strains of *L. apiculatus*, two strains of *L. breviappendiculatus*, one strain of *L. cinctosporae*, two strains of *L. ingoldianus*, one strain of *Lindgomyces* sp., two strains of *L. lemonweirensis*, and four strains of *L. rotundatus*. *Massariosphaeria typhicola* (P. Karst.) Leuchtm., which is a sister species of *Lindgomyces* (Hirayama et al. 2010; Raja et al. 2011a), was used as the outgroup taxon in the ITS phylogeny, while two taxa in the Dothideales were defined as outgroup for the combined SSU and LSU alignment. Sequences of a number of taxa were obtained from previous studies on Lindgomycetaceae (Shearer et al. 2009; Hirayama et al. 2010; Raja et al. 2011a). In addition, we also included taxa from recently introduced freshwater fungal families in the Pleosporales, such as Amniculicolaceae and Lentitheciaceae (Schoch et al. 2009; Shearer et al. 2009; Zhang et al. 2009a,b). Table 1 lists all taxa used in this study along with their GenBank numbers.

Each of the datasets was aligned initially using the multiple alignment program MUSCLE® (Edgar 2004) set to default parameters as implemented in the program Seaview v. 4.1 (Gouy et al. 2010). Alignments were then optimized by visual examination and corrected manually using MacClade v. 4.08 (Maddison and Maddison 2000). After the datasets were aligned, ambiguous regions, gaps and introns were excluded from the final alignment using the default parameters in the program Gblocks (Castresana 2000; Talavera and Castresana 2007). Nucleotides from the 5′ and 3′ ends were also removed in both the datasets due to missing characters in most taxa.

Maximum Likelihood (ML) analyses were performed on both datasets. JModeltest2 was used (Darriba et al. 2012) (with 1624 possible evolutionary models) based on the implementation of the Akaike Information Criterion (AIC) to obtain the best-fit model of nucleotide evolution. Conflict between the individual SSU and LSU datasets was assessed using the methods outlined in Lutzoni et al. (2004) and Raja et al. (2011b). Based on results of this study as well as previous phylogenetic analyses (Shearer et al. 2009; Hirayama et al. 2010; Raja et al. 2011a), no significant conflicts between separate SSU and LSU trees were found; therefore, the two datasets were concatenated to run the ML analyses. For the combined SSU and LSU dataset, the GTR + I + G model (Rodríguez et al. 1990) was selected. For the ITS dataset, the TIM3ef + G model was selected by AIC in jModeltest2.

For both datasets, ML was performed with 1000 ML bootstrap (BS) replicates with a combined Nearest Neighbor Interchange (NNI) and Subtree Pruning and Regrafting (SPR) tree search option in effect using PHYML (Guindon and Gascuel 2003). Additional ML analyses were performed using RAxML v. 7.0.4 (Stamatakis et al. 2008) on both the combined SSU and LSU dataset as well as on the ITS dataset; these analyses were run on the CIPRES Portal v. 2.0 (Miller et al. 2010) with the default rapid hill-climbing algorithm and GTR model employing 1000 fast BS searches. Clades with a bootstrap value (BSV) $\geq$70% were considered significant and strongly supported (Hillis and Bull 1993).

Bayesian analyses were performed to assess nodal support on the combined SSU and LSU dataset as well as separately on the ITS dataset using MrBayes v 3.12 (Huelsenbeck and Ronquist 2001, 2005) implementing the above models. These analyses were run on the CIPRES Portal v. 2.0 (Miller et al. 2010). Constant characters were included and 100 million generations with trees sampled every 100th generation were run resulting in 100,000 total trees for each of the combined SSU + LSU and ITS datasets. Based on information on prior runs, the first 10,000 trees extended beyond the burn-in phase in each analysis, so these were discarded and the remaining 90,000 trees were used to calculate the Bayesian posterior probability (BPP). The consensus trees were generated in PAUP 4.0b10 (Swofford 2002). The Bayesian analyses were run twice starting from a different random tree each time to ensure that trees from the same tree space were sampled.

Table 1 – Species used in this study.

Species	Voucher info[b]	GenBank accession numbers[a]		
		nucSSU rDNA	nuc ITS	nucLSU rDNA
Amniculicola immersa	CBS 123083	GU456295	–	FJ795498
Amniculicola lignicola	CBS 123094	EF493863	–	EF493861
Amniculicola parva	CBS 123092	GU296134	–	FJ795497
Anguillospora longissima	CS869-1D	GU266222	–	GU266240
Byssothecium circinans	CBS 675.92	AY016339	–	AY016357
Bimuria nova-zelandiea	CBS 107.79	AY016338	–	AY016356
Cochliobolus heterostrophus	CBS 134.39	AY544727	–	AY544645
Cucurbitaria elongata	CBS 171.55	U42482	–	DQ678061
Delitschia didyma	UME 31411	AF242264	–	DQ384090
Delitschia winteri	CBS 225.62	DQ678026	–	DQ678077
Didymella cucurbitacearum	IMI 373225	AY293779	–	AY293792
Didymella exigua	CBS 183.55	EU754056	–	EU754155
Dothidea insculpta	CBS 189.58	DQ247810	–	DQ247802
Dothidea sambuci	DAOM 231303	AY544722	–	NG_027611.1
Herpotrichia juniperi	CBS 468.64	U42483	–	DQ384093
Lentithecium aquaticum	CBS 123099	FJ795477	–	FJ795434
Lentithecium fluviatile	CBS 123090	FJ795492	–	FJ795450
Lepidosphaeria nicotiae	CBS 559.71	DQ384068	–	DQ384068
Leptosphaeria biglobosa	CBS 532.66	EU754090	–	EU754189
Letendraea helminthicola	CBS 884.85	AY016345	–	AY016362
Lindgomyces angustiascus	A640-1a Type	**JX508280**	**JX508281**	**JX508279**
Lindgomyces angustiascus	A640-1b	–	**JX508282**	–
Lindgomyces angustiascus	F60-1	**JX508284**		**JX508283**
Lindgomyces angustiascus	G202-1a	**JX508287**	**JX508286**	**JX508285**
Lindgomyces apiculatus	JCM 13091/MAFF 239601 TYPE	JF419886	JF419892	JF419884
Lindgomyces apiculatus	JCM 13092/MAFF 239602	JF419887	JF419893	JF419885
Lindgomyces breviappendiculatus	JCM 12702/MAFF 239291	AB521734	JF419896	AB521749
Lindgomyces breviappendiculatus	JCM 12701/MAFF 239292 Type	AB521733	JF419897	AB521748
Lindgomyces cinctosporae	R56-1 Type	AB522430	JF419905	AB522431
Lindgomyces cinctosporae	R56-3	GU266238	–	GU266245
Lindgomyces ingoldianus	ATCC 200398 Type	AB521719	JF419898	AB521736
Lindgomyces ingoldianus	JCM 16479/NBRC 106126	AB521720	JF419899	AB521737
Lindgomyces sp.	JCM 16480/NBRC 106130	AB521721	JF419900	AB521738
Lindgomyces lemonweirensis	A632-1a Type	JF419890	JF419894	JF419888
Lindgomyces lemonweirensis	A632-1b	JF419891	JF419895	JF419889
Lindgomyces rotundatus	JCM 16481/MAFF 239473 Type	AB521722	JF419901	AB521739
Lindgomyces rotundatus	JCM 16482/NBRC106127	AB521723	JF419902	AB521740
Lindgomyces rotundatus	JCM 16483/NBRC 106128	AB521724	JF419903	AB521741
Lindgomyces rotundatus	JCM 16484/NBRC 106129	AB521725	JF419904	AB521742
Lolia aquatica	–	–	–	HM367732
Lophiostoma heterosporum	–	AY016345	–	AY016369
Lophiostoma macrostomum	JCM 13545	AB521731	–	AB433273
Lophiostoma macrostomum	JCM 13546/MAFF 239447	AB521732	–	AB433274
Massaria platani	CBS 221.37	DQ678013	–	DQ678065
Massarina eburnea	JCM 14422	AB521718	–	AB521735
Massariosphaeria typhicola	MAFF 239218	AB521729	–	AB521746
Massariosphaeria typhicola	MAFF 239219	AB521730	JF419906	AB521747
Montagnula opulenta	–	AF164370	–	DQ678086
Neotestudina rosatii	CBS 690.82	DQ384069	–	DQ384107
Neottiosporina paspali	CBS 331.37	EU754073	–	EU754172
Ophiosphaerella herpotricha	CBS 620.86	DQ678010	–	DQ678062
Phaeosphaeria avenaria	AFTOL-ID 280	AY544725	–	AY544684
Phaeodothis winteri	CBS 182.58	DQ678021	–	DQ678073
Phoma herbarum	CBS 615.75	EU754087	–	EU751486
Pleospora herbarum	CBS 714.68	DQ767648	–	DQ678049
Pleomassaria siparia	CBS 279.74	DQ678027	–	AY004341
Preussia terricola	DAOM 230091	AY544726	–	AY544686
Setomelanomma holmii	CBS 110217	AF525677	–	AF525678
Setosphaeria monoceras	CBS 154.26	AY016352	–	AY016368
Sporormia lignicola	CBS 264.69	U42478	–	DQ384098
Tingoldiago graminicola	JCM 16485/NBRC 106131 Type	AB521726	–	AB521743
Tingoldiago graminicola	MAFF 239472	AB521727	–	AB521744

(*continued on next page*)

Table 1 – (continued)

Species	Voucher info[b]	GenBank accession numbers[a]		
		nucSSU rDNA	nuc ITS	nucLSU rDNA
Tingoldiago graminicola	JCM 16486/NBRC 106132	AB521728	–	AB521745
Trematosphaeria pertusa	CBS 400. 97	DQ678020	–	DQ678072
Ulospora bilgramii	CBS 110020	DQ384071	–	DQ384108
Verrculina enalia	CBS 304.66	DQ678028	–	AY016363
Zopfia rhizophila	CBS 270.26	L76622	–	DQ384104

a Number in Bold indicates newly obtained sequences in this study.
b CBS, Centraalbureau voor Schimmelcultures; A, Carol Shearer; IMI, International Mycological Institute; UME, Umeå University, Sweden; DAOM, Agriculture and Agri-Food Canada National Mycological Herbarium; JCM, Japan Collection of Microorganisms; MAFF, National Institute of Agrobiological Sciences, Japan; NBRC, National Biological Resource Center, Japan; R, Raja Freshwater Ascomycetes; F, Florida Freshwater Ascomycetes A, Carol Shearer, Ascomycetes; AFTOL, Assembling the Fungal Tree of Life; ATCC, American Type Culture Collection; G, University of North Carolina at Greensboro, (UNCG), Department of Chemistry and Biochemistry Culture Collection.

2.4. *Chemical analysis*

2.4.1. *Fermentation, extraction and isolation*

A fresh culture of G202 was grown on 2% malt extract agar (MEA) (20 g malt extract, 15 g agar, 1000 ml sterile distilled water). After 14–21 d, a piece of agar culture was transferred to a medium containing yeast extract, soy peptone, and dextrose (YESD; 20 g soy peptone, 20 g dextrose, 10 g yeast extract, 1000 ml sterile distilled water). After incubation (7–14 d) at 22 °C with agitation, the culture was used to inoculate 50 ml of a rice medium, prepared using 25 g of rice and 35 ml of H_2O in a 250 ml Erlenmeyer flask. This was incubated at 22 °C until the culture showed good growth (approximately 14 d). To the solid fermentation culture of G202, 150 ml of 1:1 MeOH–$CHCl_3$ was added. The culture was chopped with a spatula and shaken overnight (~16 h) at ~100 rpm at room temperature. The sample was vacuum filtered, and the remaining residues were washed with small volumes of 1:1 MeOH–$CHCl_3$. To the filtrate, 90 ml $CHCl_3$ and 150 ml H_2O were added; the mixture was stirred for 1/2 h and then transferred into a separatory funnel. The bottom layer was drawn off and evaporated to dryness. The dried organic extract was re-constituted in 50 ml of 1:1 MeOH–CH_3CN and 50 ml of hexane. The biphasic solution was then mixed vigorously in a separatory funnel. The MeOH–CH_3CN layer was drawn off and evaporated to dryness under vacuum. The defatted material (127.1 mg) was dissolved in a mixture of $CHCl_3$–MeOH, adsorbed onto Celite 545, and fractionated via a Teledyne ISCO CombiFlash Rf using a 4 g Silica column and a gradient solvent system of hexane–$CHCl_3$–MeOH at an 18 ml/min flow rate and 68.1 column volumes over 18.2 min to afford four fractions. Fraction 2 was eluted with 100% $CHCl_3$ (~13 mg) and was subjected to semi-ipreparative HPLC purification over a Gemini-NX C18 (5 μm; 250 × 10 mm; Phenomenex, Inc., Torrance, CA, USA) column using a mobile phase consisting of CH_3CN–H_2O (acidified with 0.1% formic acid) starting with 90:10 then increasing linearly to 100% CH_3CN within 15 min at a flow rate of 4.7 ml/min to yield five sub-fractions. Sub-fraction 1 yielded compounds **1** (2.97 mg) and **2** (2.49 mg), which eluted at ~10.0 and 18.0 min, respectively.

Identification—The structures of compounds **1** and **2** were elucidated using high-resolution mass spectrometry (HRMS) and Nuclear Magnetic Resonance (NMR) spectroscopy. The HRMS was performed on a Thermo LTQ Orbitrap XL mass spectrometer equipped with an electrospray ionization source. NMR experiments were conducted in $CDCl_3$ with TMS as a reference via a JEOL ECA-500, operating at 500 MHz for ^1H.

3. Results

3.1. *Morphological data*

Examination of specimens based on whole crush mounts of fresh material indicated that the new fungus fits well within the genus *Lindgomyces*. Characteristics of this fungus not observed in any of the six previously described species of *Lindgomyces* include long, apically narrow, obclavate asci and presence of narrow, fusiform ascospores with ephemeral bipolar appendages with gelatinous caps. The morphological data support the establishment of *L. angustiascus* as a novel species within the family Lindgomycetaceae.

3.2. *Molecular data*

The two partial nrDNA regions were concatenated since no significant conflicts were found between the individual SSU and LSU trees. The original combined SSU and LSU alignment comprised 66 taxa and 4747 bp positions. After removal of ambiguous regions with Gblocks and excluding large segments of missing data from the 5′ and 3′ ends, the final alignment included 2312 bp. The base contents were as follows: % GC = 48.07, % A = 26.156, % C = 20.828, % G = 27.979, and % T = 25.037. PHYML analyses of the combined SSU and LSU dataset produced a single most likely tree ($-\ln L$ = 9954.30) (Fig. 1). All species of *Lindgomyces* occurred in a highly supported clade within the family Lindgomycetaceae (Hirayama et al. 2010; Raja et al. 2011a) with ≥95% BPP, 80% PHYML BS, and 80% RAxML BS. Isolates of the new species, *L. angustiascus*, formed a well-supported clade [≥95% BPP, 77% PHYML BS, 96% RAxML BS (Fig. 1)].

The original ITS alignment consisted of 18 taxa and 1197 bp positions. After using Gblocks to remove ambiguous regions from the sequence alignment and excluding the missing data from the 5′ and 3′ ends, the final dataset consisted of 975 bp. The length range of the ITS dataset was 476–975 bp. The base

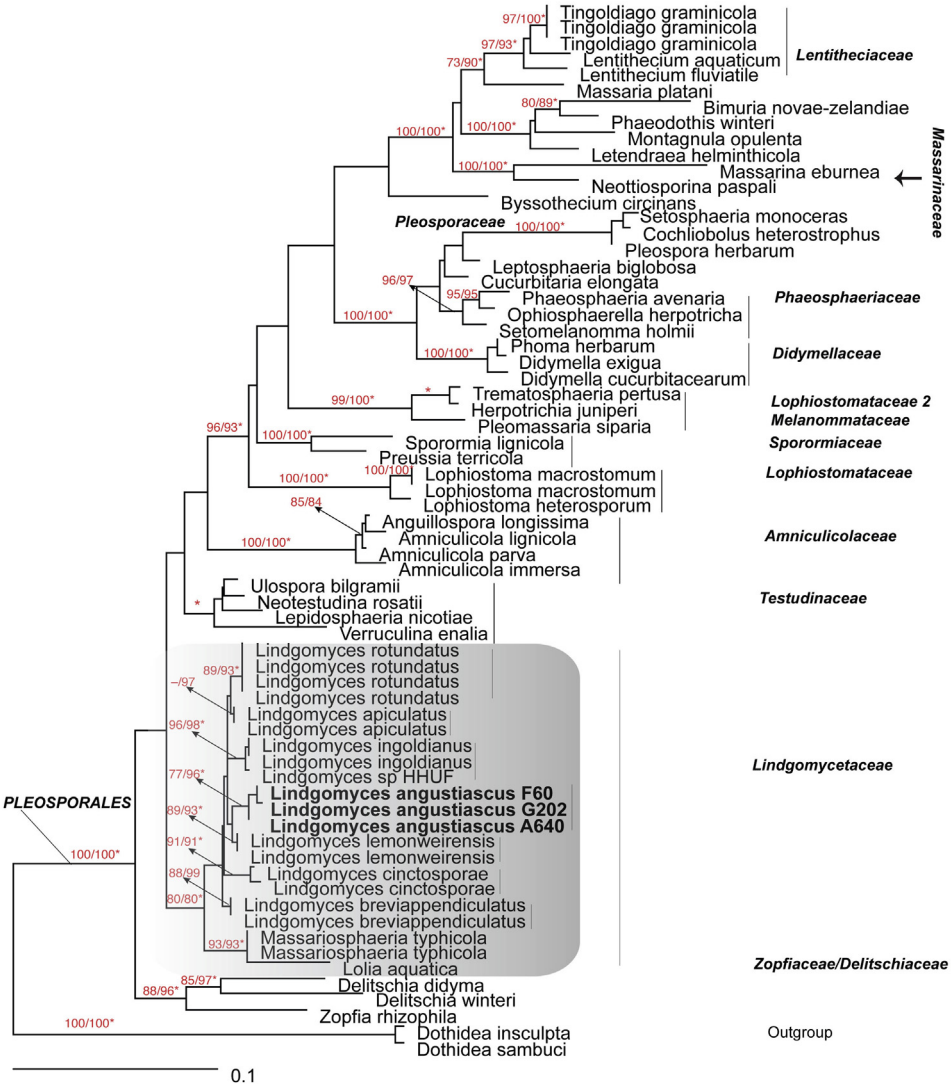

Fig. 1 – Phylogram of the most likely tree (−lnL = 9954.30) from a PHYML analysis of 66 taxa based on combined SSU and LSU nrDNA (2312 bp). Numbers refer to PHYML/RAxML bootstrap support values ≥70% based on 1000 replicates. An astrix indicates significant Bayesian posterior probabilities ≥95%. Members of the Dothideales were used as outgroup taxa. The new species is shown in bold. Classification following Lumbsch and Huhndorf (2010) is shown on the right.

contents were as follows: % GC = 51.802, % A = 22.024, % C = 25.147, % G = 26.654, and % T = 26.174. PHYML analyses of the ITS dataset generated a single most likely tree (−lnL = 3448.21, Fig. 2). Isolates of *L. angustiascus* formed a distinct monophyletic group with ≥95% PP, 99% PHYML BS, and 100% RAxML BS. Both isolates a and b from strain A640-1 grouped together with ≥95% PP, 99% PHYML BS, and 99% RAxML BS. *Lindgomyces angustiascus* occurred as a sister clade to *L. lemonweirensis* with ≥95% PP, 100% PHYML BS, and 100% RAxML BS (Fig. 2). Alignments of both datasets were deposited in TreeBASE (www.treebase.org, submission number (S13461)).

The molecular phylogenetic analyses of both the combined SSU and LSU (Fig. 1) as well as the ITS phylogeny (Fig. 2) added further support to the establishment of *L. angustiascus* within the Lindgomycetaceae. The new species appeared to be a phylogenetically related sister species to *L. lemonweirensis*. This taxon found in Florida, North Carolina, and Wisconsin is therefore described and illustrated herein as a new species of *Lindgomyces*.

3.3. *Chemistry data*

From the organic extract of *L. angustiascus* (G202), two major compounds were isolated and identified as 6*E*,9*E*-octadecadienoic acid (**1**) and ergosterol peroxide (**2**) (Suppl. Fig. 1). The spectral data compared favorably to those reported previously (Dictionary of Natural Products, www.chemnetbase.com) and matched authentic standards.

3.4. *Taxonomy*

Lindgomyces angustiascus Raja, A.N. Mill. & Shearer, sp. nov. Fig. 3

MycoBank no.: MB 801725.

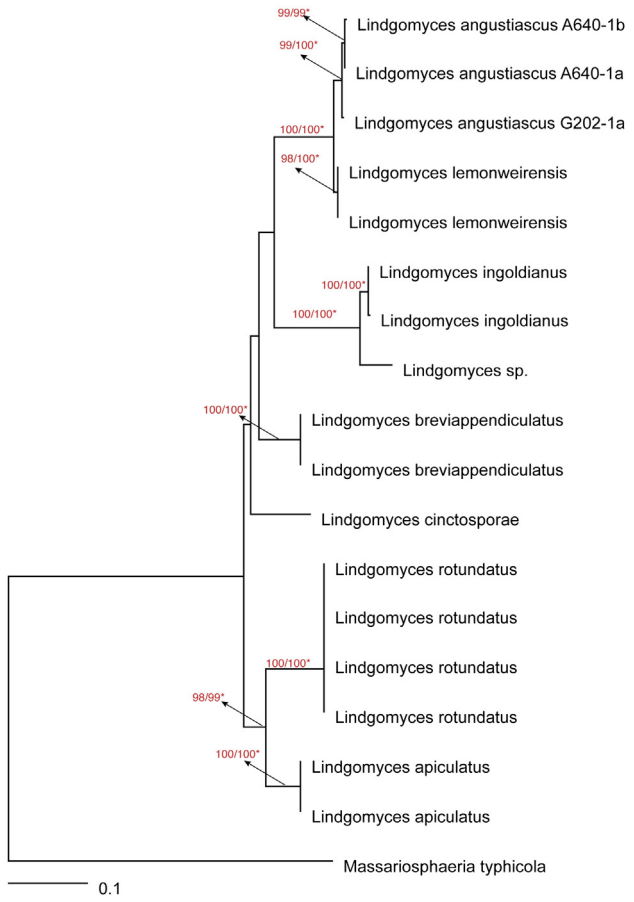

Fig. 2 – Phylogram of the most likely tree ($-\ln L = 3448.21$) from a PHYML analysis of 18 taxa based on ITS nrDNA (975 bp). Support values as in Fig. 1.

Differs from other *Lindgomyces* spp. in characteristic long, slender, obclavate asci with a narrow apex; ascospores newly discharged from the asci possess bipolar apical appendages with gelatinous caps.

Type: USA. WISCONSIN: Lemonweir River at jct. with Rt. HH south of Rt. 82, 43°46′16″N, 89°53′10″W, on submerged decorticated wood, 5 October 2009, Huzefa Raja and Andrew N. Miller, A640-1 (HOLOTYPE designated here, ILL 41201).

Etymology: From L., angustus, "narrow", referring to the narrow ascus apex of this species compared to other species of *Lindgomyces*.

Ascomata on wood 290–395 × 270–280 μm, black, partially immersed, scattered, flattened globose, with a broad flattened base, ostiolate, papillate, clypeate. Papillae 40–50 × 60–90 μm, broad, conical, central; wall of papilla of darkened pseudoparenchymatic cells. Peridium ca. 20–35 μm wide, two-layered; inner layer of hyaline angular cells 10–15 × 3–5 μm, outer layer composed of dark irregularly shaped cells. Pseudoparaphyses numerous, ca. 1–2 μm wide, septate, anastomosing above the asci. Asci 122–203 × 18–28 μm (mean = 158 × 25 μm, $n = 40$), fissitunicate, long, slender, obclavate, narrow and thick-walled at the apex, broader and thinner walled below, slightly curved, eight-spored, overlapping uniseriate at ascus apex, biseriate at ascus base. Ascospores 47–58 × 9–12 μm (mean = 53 × 11 μm, $n = 70$), ellipsoidal to fusiform, tapering at the apices, hyaline, one-septate, multiguttulate, with two to three large guttules in each cell; equipped with bipolar appendage-like structures ca. 2 μm long and initially covered with gelatinous caps; appendages ephemeral in water.

Additional specimens examined: Florida, Apalachicola National Forest, Wood Lake, 30°01′34″N, 84°33′57″W, water 30 °C, pH 8.5, on submerged decorticated wood, July 2004, *Chris Brown* and *Huzefa Raja*, *F60-1*; Apalachicola River at Fort Gadsden Landing, 29°56′25.87″N, 85°0′40.54″W, water 9 °C, pH 6, on submerged decorticated wood, 14 February 2006, *J.L. Crane* and *Huzefa Raja*, *F60-2*; North Carolina, Lake at Hagan Stone Park, 35°57′9″N, 79°44′9″W, on submerged partially decorticated wood, 27 March 2012, *Huzefa Raja*, *Mario Figueroa* and *Daniella Hayes*, *G202-1*.

Culture: Colonies on Potato Dextrose Agar (PDA; Difco, Detroit, MI, USA) growing slowly (20 mm diam in 3 weeks), diffuse, submerged, floccose or cottony, margin even, dark gray at the center, and white toward the periphery.

Anamorph: not observed.

Known distribution: USA (Florida, North Carolina, Wisconsin).

Comments: *Lindgomyces angustiascus* can be distinguished from other species in the genus by the characteristic long, slender, obclavate or cymbiform asci with a narrow apex. Ascospores newly discharged from the asci possess bipolar apical appendages with gelatinous caps (Fig. 3G); however, the appendages are ephemeral and dissolve away quickly in water. Thus far, this species was found occurring only on submerged woody substrates, suggesting that it may prefer lignicolous substrates.

4. Discussion

Using molecular sequence data and morphological evidence, Hirayama et al. (2010) established the family *Lindgomycetaceae* based on *Lind. ingoldianus* (Basionym: *Massarina ingoldiana*), a freshwater member of the Dothideomycetes similar to *M. eburnea* (Tul. & C. Tul.) Sacc., the type species of *Massarina*. The molecular analyses, which included sequences for *M. eburnea*, *Lophiostoma macrostomum* (Tode) Ces. & de Not., and *Lind. ingoldianus*, revealed that these taxa were distantly related in a phylogenetic tree based on a wide range of Dothideomycetes genera. Molecular phylogenetic studies using ribosomal sequence data from different lineages of Dothideomycetes belonging to freshwater, marine and terrestrial bitunicate fungi also showed that Lindgomycetaceae was a unique lineage among the Dothideomycetes and did not share phylogenetic affinities with *Massarina sensu stricto* or Lophiostomataceae (Schoch et al. 2009; Shearer et al. 2009; Raja et al. 2011a). Based on the molecular phylogenetic analysis performed in this study (Fig. 1), it is evident that *L. angustiascus* belongs in the genus *Lindgomyces*, Lindgomycetaceae and is not conspecific with taxa such as *Massarina* or *Lophiostoma*.

Data obtained from both molecular and morphological analyses strongly support the placement of *L. angustiascus* within *Lindgomyces*. The morphological characteristics such as long, slender, obclavate asci and narrow, pointed, fusiform

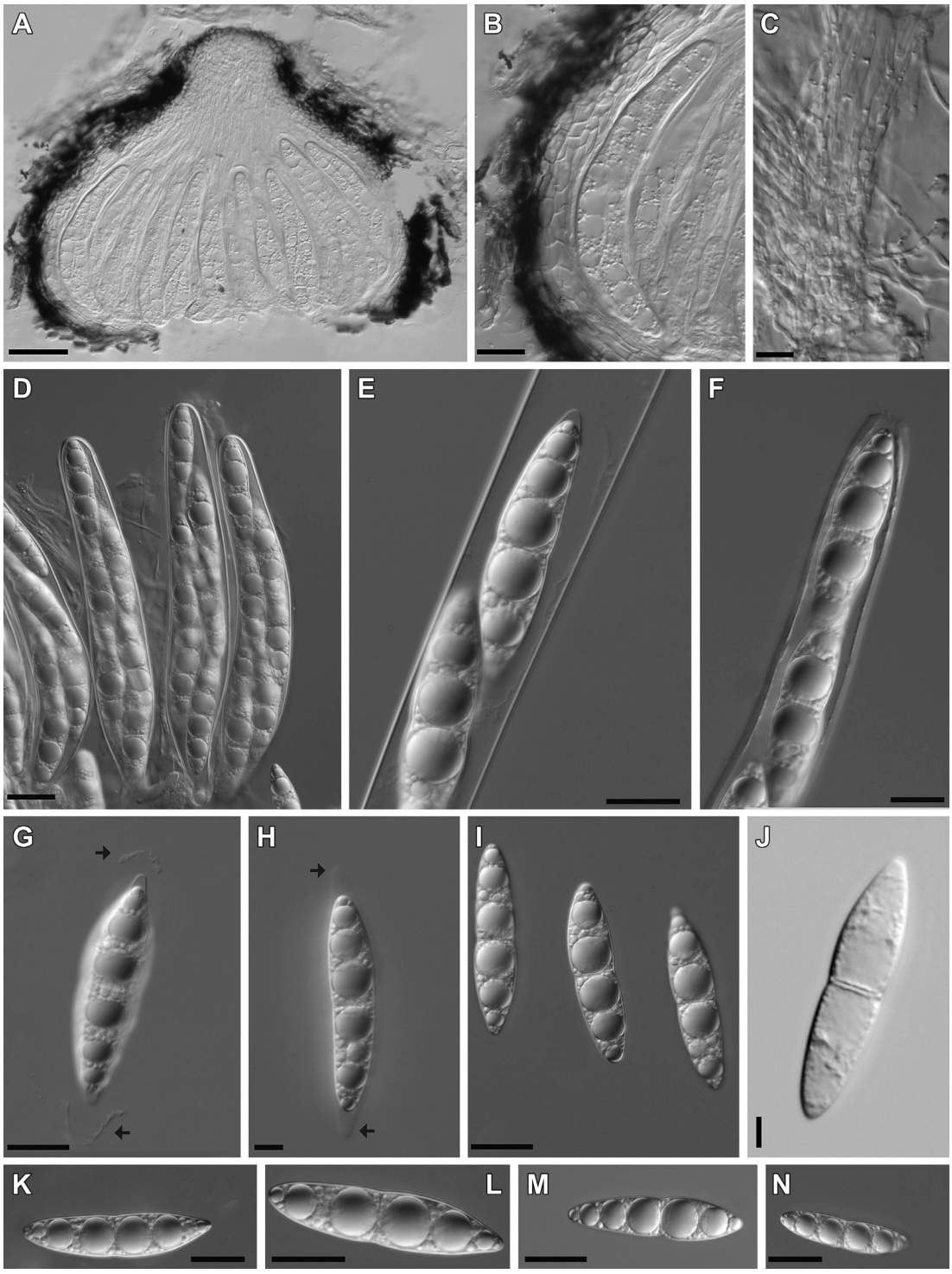

Fig. 3 – *Lindgomyces angustiascus*. A, ascoma in longitudinal section (F60-1); B, ascomal wall (F60-1); C, pseudoparaphyses; D, asci; E, elongating ascus showing ascospores with an apical appendage; F, narrow ascus apex; G, ascospore in water showing apical appendages and gelatinous caps (arrow); H, ascospore showing apical appendages; I, ascospores in water; J, ascospore mounted in glycerin; K–N, ascospores in water. C–N: from the HOLOTYPE. *Bars*: A, 50 μm; B, D–G, I, K–N, 20 μm; C, H, J, 10 μm.

ascospores with ephemeral appendages covered with gelatinous caps in *L. angustiascus* distinguishes this species from all previously recognized species within *Lindgomyces*. Molecular data obtained from combined SSU and LSU sequences and ITS data (Figs. 1 and 2) also supports observations from phenotypic data (Fig. 3).

The ascospores of *Lindgomyces angustiascus* are morphologically similar to those of *L. breviappendiculatus* (Tanaka et al.

2005; Hirayama et al. 2010) and *L. apiculatus* (Raja et al. 2011a) in having bipolar apical appendages. *Lindgomyces angustiascus*, however, differs from the latter two species in having ascospores with similar sized upper and lower cells and not being strongly constricted at the midseptum. In addition, as soon as the ascospores of *L. angustiascus* are released in water, short, gelatinous caps separate from the ascospore apices to release ephemeral, gelatinous appendages (Fig. 3G), a character not observed in other *Lindgomyces* species thus far. A key to species of *Lindgomyces* has been published by Raja et al. (2011a).

The ITS region of the ribosomal operon has been designated as the fungal barcode marker (Schoch et al. 2012). A Blast search (Altschul et al. 1997) of the ITS sequence of isolates of *L. angustiascus* in GenBank (Benson et al. 2012) suggested that the ITS sequence of the new fungus is 97–98% similar to that of *L. lemonweirensis* (JF419894 and JF419895). However, *L. angustiascus* is morphologically distinct from *L. lemonweirensis* in both ascus and ascospore dimensions as well as the morphology of the ascus; the asci of *L. angustiascus* are long, slender and cymbiform with a narrow apex, whereas those of *L. lemonweirensis* are clavate to cymbiform and rounded at the apex (Raja et al. 2011a). In addition, the ascospores of *L. angustiascus* possess bipolar apical appendages with gelatinous caps, while an entire gelatinous sheath surrounds the ascospores of *L. lemonweirensis*. Although the 3% cutoff as a proxy for ITS based species identification works reasonably well in fungi, caution must be used when translating information from sequence data into species names (Nilsson et al. 2008). Previous taxonomic studies using ITS data for species level identification (Harrington and Rizzo 1999) showed sibling species that have recently diverged did not show pronounced differences in ITS sequences, although the taxa under investigation were morphologically distinct.

As part of recent investigations regarding the chemical mycology of freshwater fungi, we are characterizing the chemical constituents of new and unusual ascomycetes collected from various lotic and lentic habitats. A fatty acid, 6*E*,9*E*-octadecadienoic acid (**1**) and ergosterol peroxide (**2**) were the major chemical compounds isolated from strain G202 (Suppl. Fig. 1). Fatty acids have been isolated commonly from filamentous fungi (Stahl and Klug 1996; Jie et al. 1998), and their profiles might be useful in understanding intraspecific variation and chemotaxonomic profiling (Stahl and Klug 1996; Frisvad et al. 2008). Additional natural products chemistry studies on *Lindgomyces* spp. could be conducted to determine if members of Lindgomycetaceae share similar or different fatty acid profiles. Ergosterol peroxide is a steroid derivative that has been isolated from a number of different filamentous fungi including both Ascomycota (Kuo et al. 2003) and Basidiomycota (Krzyczkowski et al. 2009).

It is noteworthy that four out of the seven described species currently placed in *Lindgomyces* including *L. angustiascus* have been described or collected from submerged wood in the Lemonweir River in WI, USA. This new species was collected from three distinct geographical locations along a latitudinal gradient in the USA. Additional collections of submerged woody substrates from lotic and lentic freshwater habitats in different geographical locations will certainly expand the distribution patterns of taxa within the Lindgomycetaceae. Such broad-scale geographical surveys may further add new taxa within this family and expand the suite of morphological and molecular characters that currently encompass this freshwater ascomycete genus.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.myc.2012.12.004.

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