Additions to Lindgomyces (Lindgomycetaceae, Pleosporales, Dothideomycetes), Including Two New Species Occurring on Submerged Wood from North Carolina, USA, with Notes on Secondary Metabolite Profiles

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Abstract:

Two new species of freshwater ascomycetes belonging to the genus *Lindgomyces* (Pleosporales, Dothideomycetes) are described and illustrated from submerged wood in North Carolina, USA. *Lindgomyces carolinensis* is characterized by immersed to erumpent ascomata, fissitunicate broadly cylindrical to clavate asci, and fusiform ascospores with acute ends surrounded by a large, fusiform gelatinous sheath. *Lindgomyces cigarospora* morphologically differs from *L. carolinensis* in that its ascospores are fusiform to cylindrical with rounded ends, without a large fusiform gelatinous sheath. These two new species nest in the family Lindgomycetaceae based on analyses of combined SSU and LSU rDNA sequence data. Phylogenetic analyses using ITS sequence data support the establishment of the new taxa as separate species within *Lindgomyces*. In addition to the new species, we report new ITS sequence data for *L. cinctosporus* and *L. griseosporus* from France, and *L. ingoldianus* from North Carolina, USA. We report a video exhibiting fissitunicate ascus dehiscence in *L. carolinensis* showing ascospore discharge and unraveling of the gelatinous sheath in real time. Chemical analysis of the organic extracts of *L. carolinensis* and *L. cigarospora* resulted in two known cyclodepsipeptides, Sch 378161 and Sch 217048. The in situ spatial mapping of these secondary metabolites on fungal cultures indicates the presence of both compounds on the surface of mycelia, as well as being exuded into the surrounding agar.

Keywords: Freshwater fungi | Natural products chemistry | Ribosomal genes | Systematics

Article:

Introduction

Lindgomyces K. Hiray. Kaz. Tanaka & Shearer, typified by *Lindgomyces ingoldianus* (Shearer & K.D. Hyde) K. Hiray., Kaz. Tanaka & Shearer, is a recently established freshwater ascomycete genus belonging to the family Lindgomycetaceae in the Pleosporales, Dothideomycetes (Hirayama et al. 2010). *Lindgomyces* occurs frequently on submerged wood in freshwater habitats mostly in temperate but also in some tropical and subtropical regions of the world (Shearer and Raja 2016). The genus is characterized by globose to subglobose ascomata, fissitunicate, clavate to cylindrical asci that are rounded at the apex, and one-septate, hyaline ascospores with a gelatinous sheath, which sometimes extends to form bipolar mucilaginous appendages (Hirayama et al. 2010). Eleven species have been included in *Lindgomyces*, namely *L. angustiascus* Raja, A.N. Mill. & Shearer, *L. apiculatus* K. Hiray. & Kaz. Tanaka, *L. breviappendiculatus* (Kaz. Tanaka, Sat. Hatak. & Y. Harada) K. Hiray. & Kaz. Tanaka, *L. cinctosporus* Raja, A.N. Mill. & Shearer, *L. griseosporus* Ying Zhang, J. Fourn. & K.D. Hyde, *L. ingoldianus* (Shearer & K.D. Hyde) K. Hiray., Kaz. Tanaka & Shearer, *L. lemonweirensis* Raja, A.N. Mill. & Shearer, *L. madisonensis* Raja & Oberlies, *L. okinawaensis* Tak. Takah. & Kaz. Tanaka, *L. pseudomadisonensis* Tak. Takah. & Kaz. Tanaka, and *L. rotundatus* K. Hiray. & Kaz. Tanaka (Shearer and Hyde 1997; Hirayama et al. 2010; Raja et al. 2011b, 2013a; Zhang et al. 2014; Crous et al. 2015; Hyde et al. 2016; Li et al. 2016).

During our ongoing investigations of freshwater ascomycetes in North Carolina, USA, two additional species on submerged wood were collected. These collections were morphologically similar to *Lindgomyces*, but did not fit the descriptions of any previously described species within the genus. Based on the evaluation of both morphology and phylogenetic analysis of combined partial 18S nrDNA (SSU) and 28S nrDNA (LSU), as well as partial and entire nuclear ribosomal internal transcribed spacer (ITS) regions, two new species of *Lindgomyces* were described and illustrated. In addition, we also report on the secondary metabolite chemistry of the newly collected taxa as part of our continuing studies to characterize the chemical mycology of freshwater ascomycetes (Raja et al. 2013a, b, 2015; El-Elimat et al. 2014a, b, 2017; Paguigan et al. 2016).

Materials and methods

Collection, morphological examination, and fungal isolates

Methods of collection, single-spore isolation, and morphological examination of specimens followed procedures outlined previously (Shearer et al. 2004; Raja and Shearer 2008). Water temperature, pH, latitude, and longitude were recorded in the field. Briefly, morphological characterization of the fungus was done with ascomata found on submerged wood collected from aquatic habitats in North Carolina and incubated in plastic boxes containing moist paper towels at ambient temperatures (ca. 25 °C) under 12/12 h (light/dark) conditions. Measurements of micromorphological characters were made with the Olympus cellSens software. Means and standard deviations (SDs) were calculated for asci and ascospores, based on approximately 25–50 measurements. Ex-type cultures of newly obtained strains were deposited in Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany. Fungal strains were also deposited in the Department of Chemistry and Biochemistry Culture Collection at the

University of North Carolina at Greensboro (UNCG). Voucher specimens of all newly collected taxa are deposited in the Illinois Natural History Survey Fungarium (ILLS).

DNA extraction, PCR amplification, and sequencing

For genomic DNA extractions, a small portion of agar along with mycelia was aseptically cut out from the leading edge of a 2-week-old fungal culture on potato dextrose agar (PDA; Difco) and transferred to a bashing-bead tube with DNA lysis buffer provided by the Zymo Research Fungal/Bacterial DNA Extraction Kit and vortexed vigorously for 10 min. Subsequently, DNA was extracted with procedures outlined in the Zymo Research Fungal/Bacterial DNA MiniPrep. Where possible, the DNA extractions were done in duplicate for each sample. All polymerase chain reaction (PCR) experiments were run using an Applied Biosystems Veriti Thermal Cycler. Partial fragments of 18S, ITS1-5.8S-ITS2, and 28S D1-D2 nuc rRNA genes were PCR-amplified with puReTaq™ Ready-To-Go PCR Beads (GE Biosciences Healthcare, New Jersey), using the following PCR protocol: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, annealing 52 °C for 30 s, and extension 72 °C for 1 min, with a final extension step of 72 °C for 8 min (Schoch et al. 2012). Primers NS1 and NS4 for 18S (White et al. 1990) and LROR and LR6 for 28S (Vilgalys and Hester 1990; Rehner and Samuels 1995) were used for PCR reactions, in addition to 2.5 mL bovine serum albumin (BSA; New England Biolabs, Ipswich, Massachusetts) and/or 2.5 mL dimethyl sulfoxide (Fisher Scientific, Pittsburgh, Pennsylvania). The entire rDNA ITS region was PCR-amplified with primer combinations ITS5 and ITS4 (White et al. 1990; Gardes and Bruns 1993). The PCR products were run on an ethidium bromide-stained 1% agarose gel (Fisher Scientific), along with a 1-kb DNA ladder (Promega) to estimate the size of the amplified band. PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega). Sanger sequencing of the purified PCR products was performed at Eurofins Genomics (http://www.operon.com/default.aspx) with the BigDye Terminator v3.1 Cycle Sequencing Kit. Sequences were generated in both directions with 2 μM sequencing primers: ITS = ITS5, ITS4; 18S = NS1, NS4, NS3, NS4; 28S = LROR, LR3, LR3R, LR6. Sequences were generated on an Applied Biosystems 3730xl high-throughput capillary sequencer. Sequences were assembled with Sequencher 5.3 (Gene Codes Corp.), optimized by eye, and manually corrected when necessary. All newly obtained sequences were subjected to GenBank nucleotide BLAST queries to verify their identities and deposited in GenBank (Table 1).

Table 1

Species used in this study

Species	**Strain**	**GenBank accession numbers**			**References**
		nucSSU rDNA	**nuc ITS**	**nucLSU rDNA**	
Amniculicola immersa	CBS 123083 Type	GU456295	–	FJ795498	Zhang et al. (2009b)
Amniculicola lignicola	CBS 123094 Type	EF493863	–	EF493861	Zhang et al. (2008)
Amniculicola parva	CBS 123092 Type	GU296134	–	FJ795497	Zhang et al. (2008)
Anguillospora longissima	CS869-1D	GU266222	–	GU266240	Shearer et al. (2009)
Bimuria nova-zelandiea	CBS 107.79	AY016338	–	AY016356	Lumbsch and Lindemuth (2001)
Byssothecium circinans	CBS 675.92	AY016339	–	AY016357	Lumbsch and Lindemuth (2001)
Clohesyomyces aquaticus	MFLUCC11-0092	JX276949	–	JX276950	Zhang et al. (2012a)
Cochliobolus heterostrophus	CBS 134.39	AY544727	–	AY544645	Lutzoni et al. (2004)

Species	Strain	GenBank accession numbers			References
		nucSSU rDNA	nuc ITS	nucLSU rDNA	
Cucurbitaria elongata	CBS 171.55	U42482	–	DQ678061	Berbee (1996); Schoch et al. (2006a)
Delitschia didyma	UME 31411	AF242264	–	DQ384090	Winka (2000)
Delitschia winteri	CBS 225.62	DQ678026	–	DQ678077	Schoch et al. (2006b)
Didymella cucurbitacearum	IMI 373225	AY293779	–	AY293792	–
Didymella exigua	CBS 183.55	EU754056	–	EU754155	de Gruyter et al. (2009)
Dothidea insculpta	CBS 189.58	DQ247810	–	DQ247802	Schoch et al. (2006a)
Dothidea sambuci	DAOM 231303	AY544722	–	NG_027611.1	Lutzoni et al. (2004)
Herpotrichia juniperi	CBS 468.64	U42483	–	DQ384093	Berbee (1996)
Hongkongmyces pedis	HKU35	KF314117	JQ435790	KF314111	Tsang et al. (2014)
Lentithecium aquaticum	CBS 123099 Type	FJ795477	–	FJ795434	Zhang et al. (2009a)
Lentithecium fluviatile	CBS 123090	FJ795492	–	FJ795450	Zhang et al. (2009a)
Lepidosphaeria nicotiae	CBS 559.71	DQ384068	–	DQ384106	Kruys et al. (2006)
Letendraea helminthicola	CBS 884.85	AY016345	–	AY016362	Lumbsch and Lindemuth (2001)
Lindgomyces angustiascus	A640-1a Type	JX508280	JX508281	JX508279	Raja et al. (2013a)
Lindgomyces angustiascus	A640-1b	–	JX508282	–	Raja et al. (2013a)
Lindgomyces angustiascus	F60-1	JX508284		JX508283	Raja et al. (2013a)
Lindgomyces angustiascus	G202-1a	JX508287	JX508286	JX508285	Raja et al. (2013a)
Lindgomyces apiculatus	KT 1108 Type	JF419886	JF419892	JF419884	Raja et al. (2011b)
Lindgomyces apiculatus	KT 1144	JF419887	JF419893	JF419885	Raja et al. (2011b)
Lindgomyces breviappendiculatus	KT 1215	AB521733	JF419896	AB521748	Hirayama et al. (2010)
Lindgomyces breviappendiculatus	KT 1399 Type	AB521734	JF419897	AB521749	Hirayama et al. (2010)
Lindgomyces carolinensis	**DSMZ 103499 G618 Type**	**KX655801**	**KX655793**	**KX655800**	This study
Lindgomyces carolinensis	**G618-2**	**KX655803**	–	**KX655802**	This study
Lindgomyces carolinensis	**G833**	–	**KX655797**	–	This study
Lindgomyces carolinensis	**G834**	–	**KX655796**	–	This study
Lindgomyces cigarospora	**DSMZ 103500 G619 Type**	**KX655805**	**KX655794**	**KX655804**	This study
Lindgomyces cigarospora	**G619-2**	**KX655807**	**KX655798**	**KX655806**	This study
Lindgomyces cigarospora	**G619-3**	–	**KX655799**	–	This study
Lindgomyces cinctosporus	R56-1 Type	AB522430	JF419905	AB522431	Hirayama et al. (2010)
Lindgomyces cinctosporus	R56-3	GU266238	–	GU266245	Hirayama et al. (2010)
Lindgomyces cinctosporus	**G825/JF15154**	–	**KX655790**	–	This study
Lindgomyces griseosporus	BJFC200094	–	KC954093	KC769693	Zhang et al. (2014)
Lindgomyces griseosporus	CBS 123100	–	–	JX157876	Zhang et al. (2014)
Lindgomyces griseosporus	**G822/JF15163**	–	**KX655789**	–	This study
Lindgomyces ingoldianus	ATCC 200398 Type	AB521719	JF419898	AB521736	Hirayama et al. (2010)
Lindgomyces ingoldianus	KH 100	AB521720	JF419899	AB521737	Hirayama et al. (2010)
Lindgomyces ingoldianus	**G99-2**	–	**KX655791**	–	Zhang et al. (2009a)
Lindgomyces ingoldianus	**G99-3**	–	**KX655792**	–	This study
Lindgomyces ingoldianus	**G99-5**	–	**KX655795**	–	This study
Lindgomyces sp.	KH 241	AB521721	JF419900	AB521738	Hirayama et al. (2010)
Lindgomyces lemonweirensis	A632-1a Type	JF419890	JF419894	JF419888	Raja et al. (2011b)
Lindgomyces lemonweirensis	A632-1b	JF419891	JF419895	JF419889	Raja et al. (2011b)
Lindgomyces madisonensis	G416-a Type	KT207822	KT207818	KT207820	Crous et al. (2015); Paguigan et al. (2016)

Species	Strain	GenBank accession numbers			References
		nucSSU rDNA	nuc ITS	nucLSU rDNA	
Lindgomyces madisonensis	G416-1b	KT207823	KT207819	KT207821	Crous et al. (2015); Paguigan et al. (2016)
Lindgomyces okinawaensis	KT 3531 Type	LC100019	LC100022	LC100027	Li et al. (2016)
Lindgomyces pseudomadisonensis	KT 2742 Type	LC149912	LC149914	LC149916	Hyde et al. (2016)
Lindgomyces rotundatus	KT 966 Type	AB521722	JF419901	AB521739	Hirayama et al. (2010)
Lindgomyces rotundatus	KT 1096	AB521723	JF419902	AB521740	Hirayama et al. (2010)
Lindgomyces rotundatus	KT 1107	AB521724	JF419903	AB521741	Hirayama et al. (2010)
Lindgomyces rotundatus	KH 114	AB521725	JF419904	AB521742	Hirayama et al. (2010)
Lolia aquatica	MF 644 Type	–	–	HM367732	Abdel-Aziz and Abdel-Wahab (2010)
Lophiostoma macrostomum	KT 635	AB521731	–	AB433273	Hirayama et al. (2010)
Lophiostoma macrostomum	KT 709	AB521732	–	AB433274	Hirayama et al. (2010)
Lophiostoma heterosporum	CBS 644.86	AY016345	–	AY016369	Lumbsch and Lindemuth (2001)
Macrodiplodiopsis desmazieri	CBS 221.37	DQ678013	–	DQ678065	Schoch et al. (2006b)
Massarina eburnea	H3953	AB521718	–	AB521735	Hirayama et al. (2010)
Massariosphaeria typhicola	KT 667	AB521729	–	AB521746	Hirayama et al. (2010)
Massariosphaeria typhicola	KT 797	AB521730	–	AB521747	Hirayama et al. (2010)
Montagnula opulenta	CBS 168.34	AF164370	–	DQ678086	Liew et al. (2000)
Neotestudina rosatii	CBS 690.82	DQ384069	–	DQ384107	Kruys et al. (2006)
Neottiosporina paspali	CBS 331.37	EU754073	–	EU754172	de Gruyter et al. (2009)
Ophiosphaerella herpotricha	CBS 620.86	DQ678010	–	DQ678062	Schoch et al. (2006b)
Phaeosphaeria avenaria	AFTOL-ID 280	AY544725	–	AY544684	Lutzoni et al. (2004)
Phaeodothis winteri	CBS 182.58	DQ678021	–	DQ678073	Schoch et al. (2006b)
Phoma herbarum	CBS 615.75	EU754087	–	EU751486	de Gruyter et al. (2009)
Pleomassaria siparia	CBS 279.74	DQ678027	–	AY004341	Schoch et al. (2006b)
Plenodomus biglobosus	CBS 532.66	EU754090	–	EU754189	de Gruyter et al. (2009)
Pleospora herbarum	CBS 714.68	DQ767648	–	DQ678049	Schoch et al. (2006b)
Preussia terricola	OSC 100098	AY544726	–	AY544686	–
Setomelanomma holmii	CBS 110217	AF525677	–	AF525678	Rossman et al. (2002)
Setosphaeria monoceras	CBS 154.26	AY016352	–	AY016368	Lumbsch and Lindemuth (2001)
Sporormia lignicola	CBS 363.69	U42478	–	DQ384098	Berbee (1996)
Tingoldiago graminicola	KH 68 Type	AB521726	–	AB521743	Hirayama et al. (2010)
Tingoldiago graminicola	KT 891	AB521727	–	AB521744	Hirayama et al. (2010)
Tingoldiago graminicola	KH 155	AB521728	–	AB521745	Hirayama et al. (2010)
Trematosphaeria pertusa	CBS 400. 97	DQ678020	–	DQ678072	
Ulospora bilgramii	CBS 110020	DQ384071	–	DQ384108	Kruys et al. (2006)
Verruculina enalia	CBS 304.66	DQ678028	–	AY016363	Schoch et al. (2006b)
Zopfia rhizophila	CBS 207.26	L76622	–	DQ384104	LoBuglio et al. (1996)

Newly sequenced fungi are in **bold**

AFTOL Assembling the Fungal Tree of Life; *ATCC* American Type Culture Collection; *A* Carol Shearer; *BJFC* Beijing Forestry University culture collection; *CBS* Centraalbureau voor Schimmelcultures; *DAOM* Agriculture and Agri-Food Canada National Mycological Herbarium; *DSMZ* Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures); *F* Florida Freshwater Ascomycetes; *G* University of North Carolina at Greensboro (UNCG), Department of Chemistry and Biochemistry Culture Collection; *IMI* International Mycological Institute; *JF* Jacques Fournier; *KT* Kazuaki Tanaka; *KH* Kazuyuki Hirayama; *MFLUCC* Mae Fah Luang University Culture Collection; *NBRC* National Biological Resource Center, Japan; *OSC* fungal type specimens at OSC, the Oregon State University Herbarium; *R* Raja Freshwater Ascomycetes; *UME* Umeå University, Sweden

Taxon sampling and phylogenetic analyses

Two datasets were assembled for phylogenetic analyses: (i) a combined dataset composed of partial 18S and 28S nrDNA sequences from 76 taxa belonging to 12 families from a total of 28 (Zhang et al. 2012b) to 33 families (Hyde et al. 2013) within the Pleosporales in the Dothideomycetes. We found no clade conflict between the 18S and 28S datasets (data not shown) and, thus, we concatenated the two alignments. Members of the Dothideales were included as outgroup taxa based on previous studies (Hirayama et al. 2010; Raja et al. 2011b, 2013a; Paguigan et al. 2016). Most taxa included in the combined 18S and 28S dataset were obtained from previous molecular phylogenetic studies of freshwater Dothideomycetes, including all previously described *Lindgomyces* spp. (Shearer et al. 2009; Hirayama et al. 2010; Raja et al. 2011b, 2013a; Zhang et al. 2014; Crous et al. 2015). And (ii) a 34-taxa ITS dataset, which consisted of all described species of *Lindgomyces* thus far, including three strains of the two newly isolated *Lindgomyces* spp. (G619, G619-2, G619-3, G618, G833, G834), two strains of *L. apiculatus*, three strains of *L. angustiascus*, two strains of *L. breviappendiculatus*, two strains of *L. cinctosporus*, two strains of *L. griseosporus,* five strains of *L. ingoldianus*, one strain of *Lindgomyces* sp., two strains of *L. lemonweirensis*, two strains of *L. madisonensis*, one strain of *L. okinawaensis*, one strain of *L. pseudomadisonensis*, and four strains of *L. rotundatus*. *Hongkongmyces pedis* Tsang, Chan, Trendell-Smith, Ngan, Ling, Lau, Woo, which is a sister species to *Lindgomyces* (Tsang et al. 2014), was used as an outgroup in the ITS dataset.

Multiple sequence alignments were generated following methods outlined previously (Shearer et al. 2009; Raja et al. 2011a) in MUSCLE (Edgar 2004), which was implemented in SeaView 4.1. The program Gblocks version 0.91b (Castresana 2000) was used via the Gblocks server to remove ambiguous regions such as poorly aligned positions and divergent regions of the DNA alignment using default parameters. Maximum likelihood (ML) analyses were performed on the two datasets with RAxML 7.0.4 (Stamatakis 2006) via the CIPRES Portal 3.3 (Miller et al. 2010). Model selection for ML analyses was accomplished with the program jModelTest 2.1.4 (Darriba et al. 2012) to obtain the best-fit model of nucleotide evolution for each dataset. The Akaike information criterion (AIC) (Posada and Buckley 2004) selected the GTR + I + G model for the 18S-28S dataset and the SYM + G model for the ITS dataset. Using the appropriate models of nucleotide evolution, RAxML analyses were run on the CIPRES server. For both datasets, bootstrap values (BV) (Felsenstein 1980) were obtained with the default rapid hill-climbing algorithm and GTR model employing 1000 fast bootstrap searches using RAxML. Clades that received a BV $\geq$ 70% were considered significant and robustly supported (Hillis and Bull 1993).

Bayesian inference (BI) employing a Markov chain Monte Carlo (MCMC) algorithm were performed on the datasets with MrBayes 3.12 (Huelsenbeck and Ronquist 2001, 2005) using the CIPRES Portal 3.3 as an additional measure of clade support. The selected models based on jModelTest 2.1.4 were implemented and constant characters were included, and four independent chains of Metropolis-coupled MCMC were run for 100 million generations with trees sampled every 1000th generation, resulting in a total of 100,000 trees. Independent BI analysis ensures that the same tree space is being sampled during each analysis and that the trees were not trapped in local optima. To conservatively estimate that the log-likelihood values reached a stable equilibrium, the first 10,000 trees that extended beyond the burn-in were discarded and the

remaining 90,000 trees were used to calculate the posterior probability (PP) in each analysis. Consensus trees were generated and viewed in PAUP 4.0b10 (Swofford 2002). Clades with a PP ≥ 95% were considered significant and strongly supported. The final alignments (SSU and LSU combined datasets and ITS) and the trees obtained were deposited in TreeBase (http://www.treebase.org) under accession number S20137.

Chemical analysis: fermentation and extraction

The fungal fermentations were performed according to procedures described previously (El-Elimat et al. 2014a; Paguigan et al. 2016). Initially, the strains were grown on PDA media for about 14 days. Then, a small agar plug with mycelium was inoculated into liquid YESD media composed of 2% soy peptone, 2% dextrose, and 1% yeast extract, followed by incubation for 14 days at room temperature with shaking at 125 rpm. Once sufficient fungal growth was observed in the seed cultures, they were transferred into 250-mL Erlenmeyer flasks, each containing 30 mL of autoclaved rice medium (10 g rice, deionized H_2O that was twice the volume of rice), and grown at room temperature for a period of 28 days. The solid fermentation cultures were extracted by the addition of 60 mL 1:1 CH_3OH-$CHCl_3$, followed by shaking for 16 h, and filtered under vacuum. To the filtrate, 90 mL of $CHCl_3$ and 150 mL of H_2O were added, stirred for 30 min, and the organic layer was collected and dried in vacuo. The extract was partitioned between 100 mL of 1:1 CH_3OH-CH_3CN and 100 mL of hexane, and the CH_3OH-CH_3CN soluble layer was collected and concentrated in vacuo.

Dereplication of fungal extracts

Dereplication analysis of fungal extracts was implemented according to a method described previously (El-Elimat et al. 2013). The samples were dissolved in CH_3OH-dioxane (1:1) to obtain a final concentration of 2 mg/mL in a total volume of 100 µL. The identity of the compounds was confirmed by matching the retention times, high-resolution electrospray ionization mass spectrometry (HRESIMS), tandem mass spectrometry (MS/MS), and photodiode array detector (PDA) data to those of the standard compounds in our in-house library of 207 fungal metabolites (El-Elimat et al. 2013; González-Medina et al. 2016).

Isolation of compounds from G618

To further confirm the presence of these cyclodepsipeptides, the compounds were isolated from G618 with the rationale that G618 had almost twice as much yield for the extract compared to that of G619. The resulting CH_3OH-CH_3CN extract (90 mg) was adsorbed on Celite 545 and subjected to flash chromatography on a 4 g RediSep Rf Gold Si-gel column, eluting in an increasing gradient of hexane to $CHCl_3$ at a flow rate of 18 mL/min over 68 column volumes and to give 37 fractions, each containing 9 mL. The resulting fractions were then pooled according to their ELSD and UV profiles, which resulted in six combined fractions in total. Fraction 5 (46 mg) was purified by using an Atlantis® T3 Prep Column eluting with a linear gradient from 40 to 60% CH_3CN in H_2O (0.1% formic acid) at a flow rate of 17 mL/min over 30 min to 100% for 15 min to afford compounds **1** (1.3 mg, t_R =19 min) and **2** (2.4 mg, t_R=24 min).

In situ chemical analysis of fungal cultures

Mass spectrometry spatial mapping of the secondary metabolites of fungi in situ (in living culture) can address a series of ecological questions that are lost through a traditional extraction process (Sica et al. 2015, 2016). In order to better understand where the secondary metabolites were located in culture, we analyzed the cultures of all strains of the new species (G618, G833, G834, and G619) via a droplet–liquid microjunction–surface sampling probe (droplet-LMJ-SSP), as reported in detail previously (Sica et al. 2015). Briefly, the droplet-LMJ-SSP was coupled with a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters Corp.) and a Thermo Q Exactive Plus. The HCD fragmentation used a normalized collision energy of 30 eV to obtain MS/MS data. The UPLC separation was performed using an Acquity UPLC BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm) equilibrated at 40 °C and a flow rate set at 0.3 mL/min. The mobile phase consisted of a linear CH_3CN-H_2O (acidified with 0.1% formic acid) gradient starting at 15% CH_3CN to 100% CH_3CN over 8 min. The mobile phase was held for another 1.5 min at 100% CH_3CN before going back to the starting conditions.

Biological evaluations

Compounds **1** and **2** were tested for antimicrobial activity against an array of bacteria and fungi (*Staphylococcus aureus*, *Escherichia coli*, *Mycobacterium smegmatis*, *Candida albicans*, and *Aspergillus niger*) using methods outlined previously (Ayers et al. 2012; Falkinham et al. 2012). The cytotoxicity of the compounds was evaluated against melanoma (MDA-MB-435), breast (MDA-MB-231), and ovarian (OVCAR3) cancer cell lines following procedures reported earlier (Ayers et al. 2011).

Results

Phylogenetic analyses

Nineteen rRNA sequences, 4 (18S), 11 (ITS), and 4 (28S), were newly generated in this study (Table 1). The initial 76-taxa combined 18S-28S alignment consisted of 2876 nucleotides. After excluding ambiguous regions, introns, and nucleotides from the 5′ and 3′ ends due to missing data in many sequences, the final combined 18S-28S alignment consisted of 2294 nucleotides.

RAxML analyses of the combined 18S-28S dataset produced a single most likely tree (Fig. 1). All species of *Lindgomyces* and other genera such as *Clohesyomyces* K.D. Hyde (Hyde 1993; Zhang et al. 2012a), *Massariosphaeria typhicola sensu lato* (Hirayama et al. 2010), *Lolia*(Abdel-Aziz and Abdel-Wahab 2010), and *Hongkongmyces* (Tsang et al. 2014) occurred in a highly supported clade within the family Lindgomycetaceae (Hirayama et al. 2010), with ≥95% PP and 72% RAxML BV. Isolates of the two new species, G618 and G619, formed well-supported clades, with ≥95% PP, 97% RAxML BV for G618 and ≥95% PP, 100% RAxML BV for G619, respectively (Fig. 1).

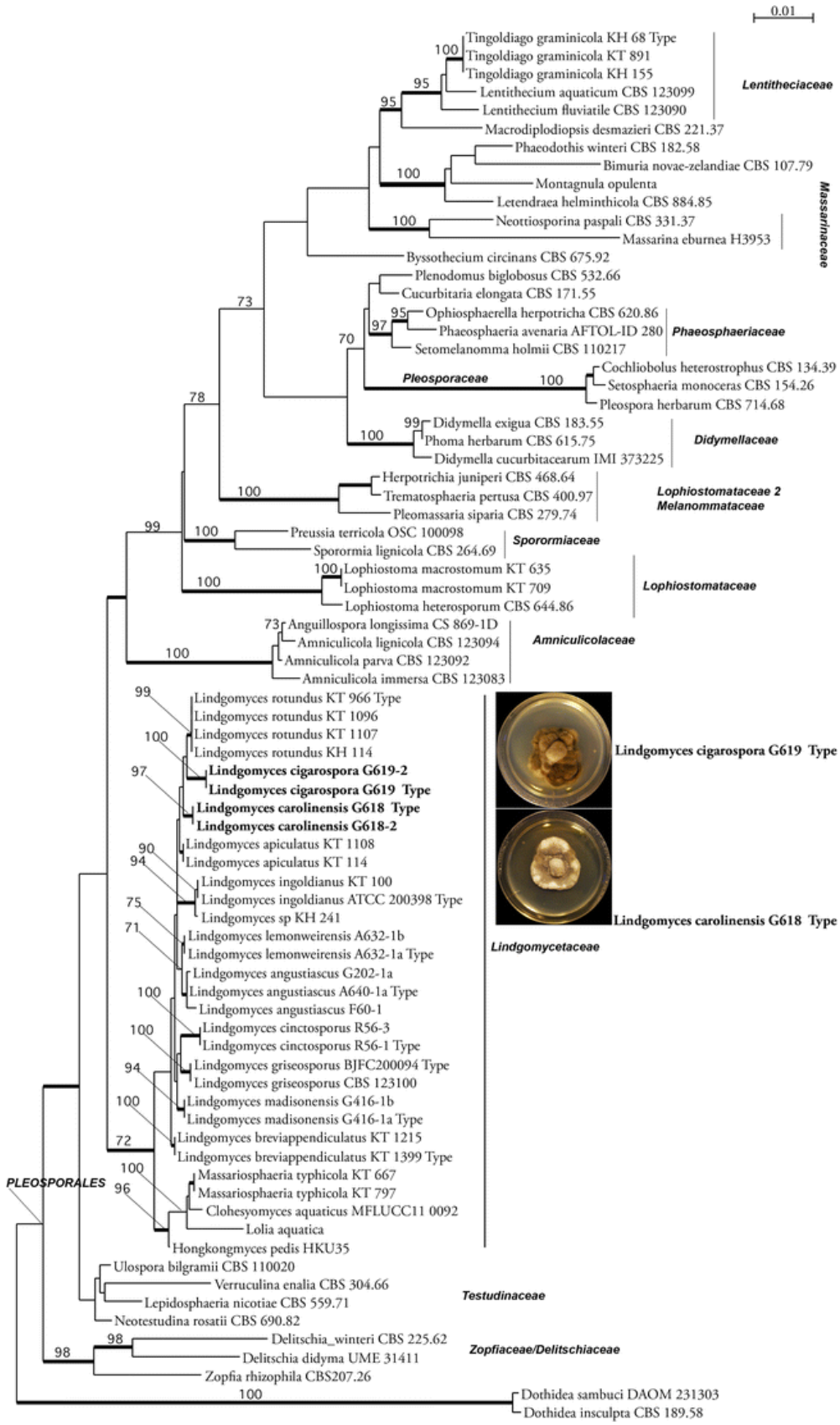

Fig. 1. Phylogram of the most likely tree (−lnL = 10060.22) from an RAxML analysis of 76 taxa based on combined SSU and LSU nrDNA (2294 bp). The *thickened branches* indicate significant Bayesian posterior probabilities ≥95% and the *numbers* refer to RAxML bootstrap support values ≥70% based on 1000 replicates. Members of the Dothideales were used as outgroup taxa. The two new species are shown in **bold**.
The *bar* indicates nucleotide substitutions per site. One-month-old cultures of ex-type strains of *Lindgomyces carolinensis* (G618) and *L. cigarospora* (G619) on potato dextrose agar (Difco) are shown on the right

The initial 34-taxa ITS alignment consisted of 1187 nucleotides, which resulted in a final alignment of 1034 nucleotides when ambiguous regions and portions of 5′ and 3′ ends were discarded from the final analyses. RAxML analyses of the ITS dataset produced a single most likely tree (Fig. 2). In this species-level phylogeny of *Lindgomyces*, strains G618 and G619 showed strong support to indicate that they are new species: G618 was strongly supported in clade 12 along with strains from another collection G833 and G834, with ≥95% PP and 99% RAxML BV, while G619 formed a strongly supported clade 11 with ≥95% PP and 100% RAxML BV.

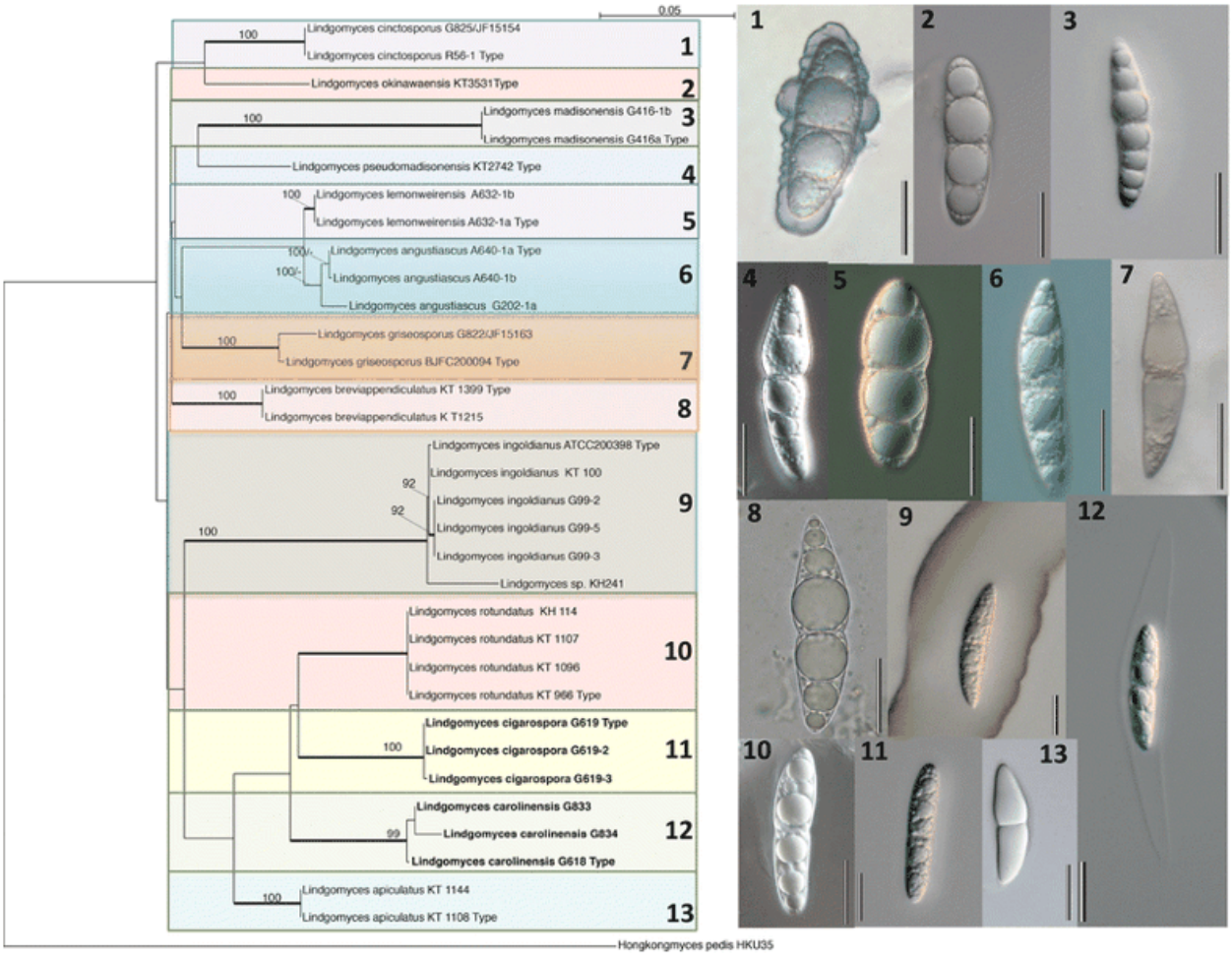

Fig. 2. Phylogram of the most likely tree (−lnL = 5031.41) from an RAxML analysis of 34 taxa based on ITS nrDNA (1017 bp). The *thickened branches* indicate significant Bayesian posterior probabilities ≥95% and the *numbers* refer to RAxML bootstrap support values ≥70% based on 1000 replicates. *Hongkongmyces pedis* was used as outgroup. The two new species are shown in **bold**. The *bar* indicates nucleotide substitutions per site. Morphologically diverse ascospores of 13 species of *Lindgomyces* corresponding to 13 strongly supported clades based on ITS data are shown on the right. Scale bars = 20 µm. Photo credits: clades 2, 4, 5, 8, 10, 13 = Kazuaki Tanaka from holotype, clade 9 = G99-5, clade 3 = G416 from holotype, clade 7 = JF15163, clade 6 = A640-1a, clade 1 = R56-1 from holotype, clades 11 and 12 = G618 and G619 from holotype

Newly sequenced strains G99-2, G99-3, and G99-5 formed a monophyletic clade with the type strains of *L. ingoldianus* (clade 9), with 95% PP and 100% RAxML BV. Strain G822/JF15163

from France formed a monophyletic clade with the type stain of *L. griseosporus*, with ≥95% PP and 100% RAxML BV (clade 7). Finally, strain G825/JF15154 formed a monophyletic clade with the type strain of *L. cinctosporus* (clade 1).

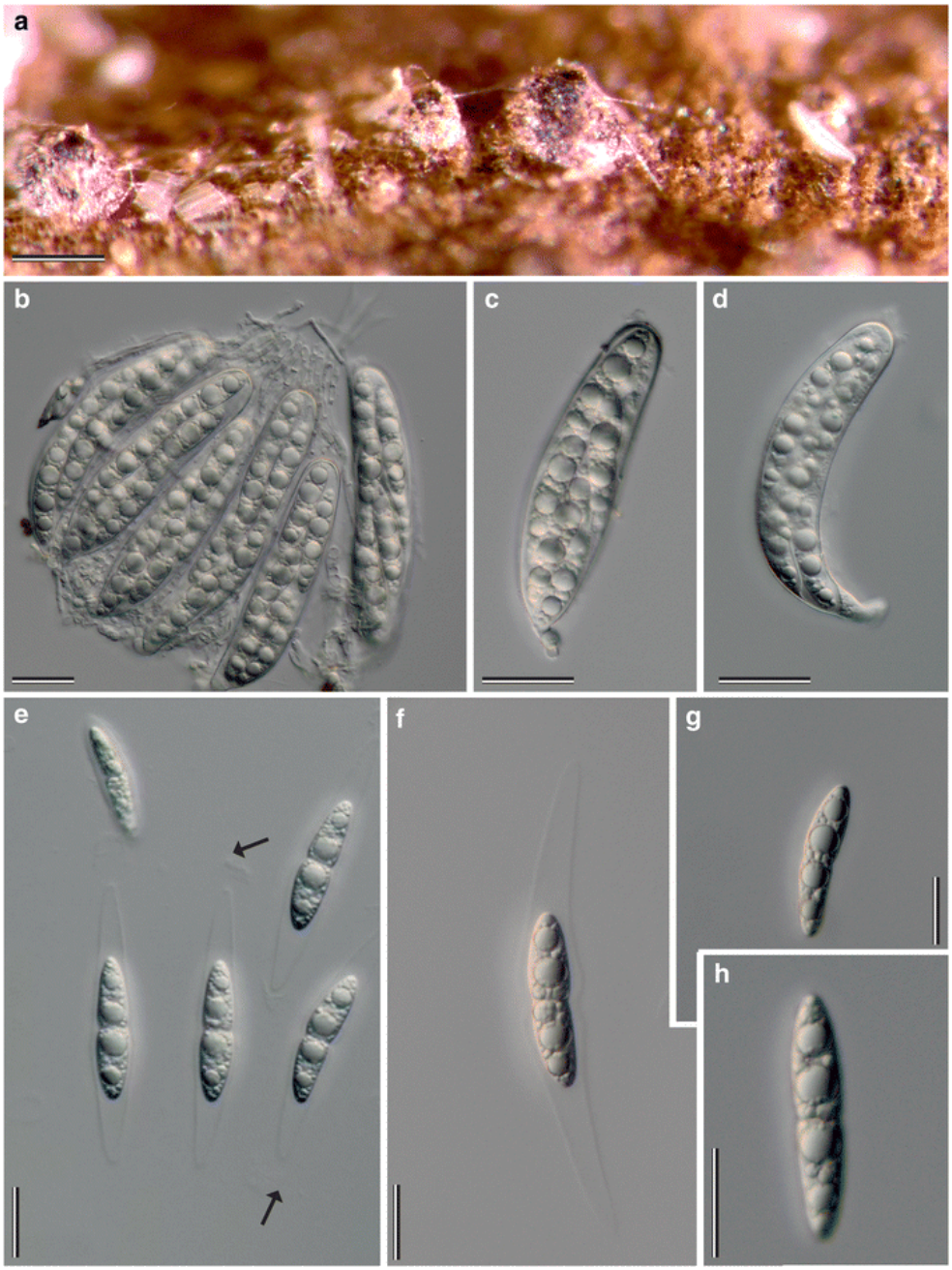

Fig. 3. *Lindgomyces carolinensis* (from HOLOTYPE, ILLS 80483). **a** Ascomata on wood. **b** Asci with interascal pseudoparaphyses. **c**, **d** Ascus. **e**, **f** Ascospores with fusiform gelatinous sheath; note arrows showing gelatinous cap-like structures on either side of the ascospore sheath. **g**, **h** Ascospore without gelatinous sheath in water. Scale bars: **a** = 200 µm; **b**–**h** = 20 µm

The molecular phylogenetic analyses of both the combined SSU and LSU (Fig. 1) as well as the ITS phylogeny (Fig. 2) clearly support the establishment of G618 and G619 as new and separate species within *Lindgomyces*. Moreover, this placement is also corroborated by the examination of morphological characters (Figs. 3 and 4). These collections are, therefore, described and illustrated herein as new species of *Lindgomyces*.

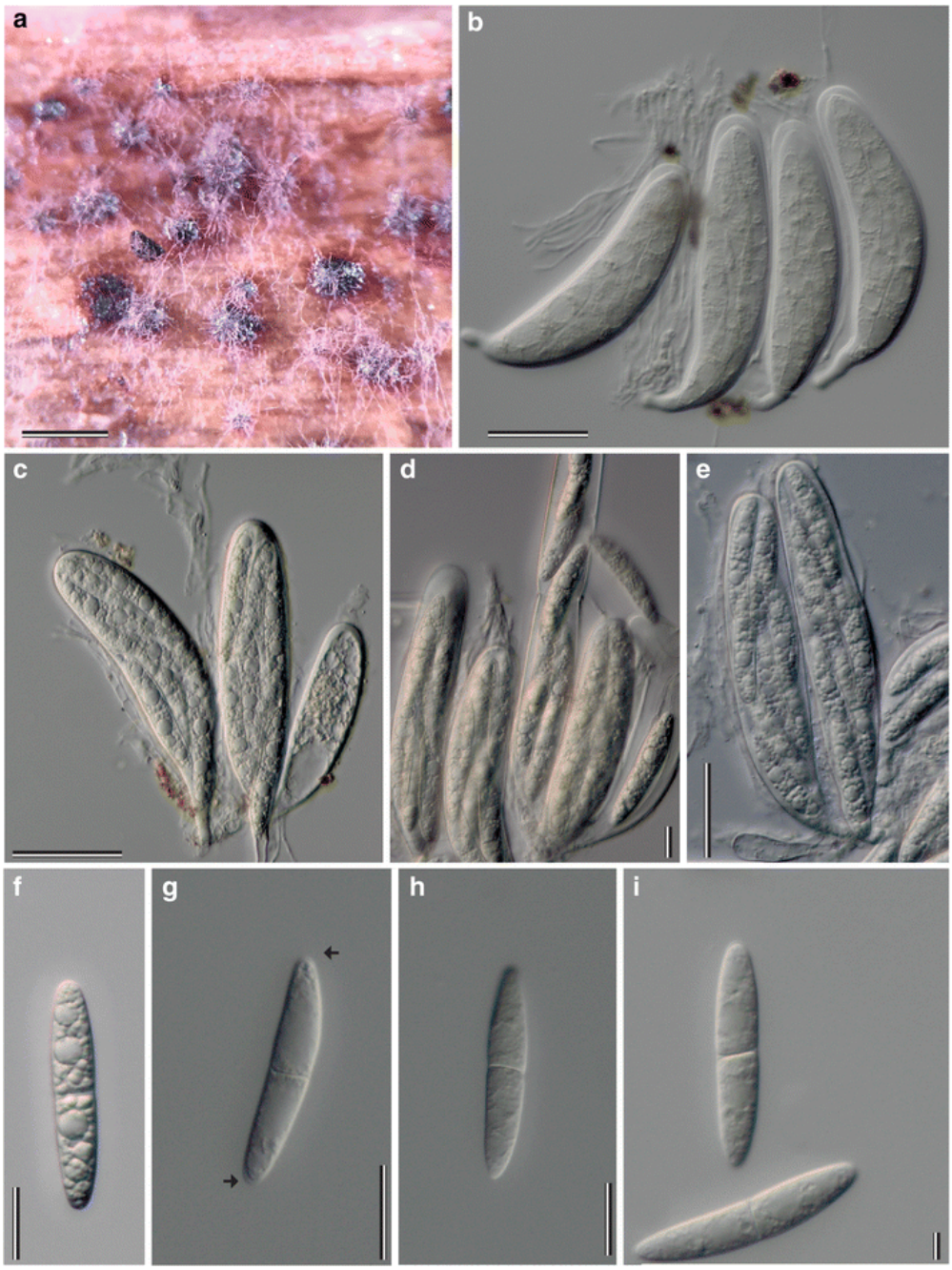

Fig. 4. *Lindgomyces cigarospora* (from HOLOTYPE, ILLS 80482). **a** Ascomata on balsa wood in culture from ex-type culture (DSM 103500, G619; ascomata produced in culture are sterile). **b** Asci with interascal pseudoparaphyses. **c**–**e** Asci; note bitunicate ascus in **d**. **f**–**i** Ascospores; note arrows in **g** showing short, rounded terminal ephemeral appendages on ascospores mounted in glycerin and lactic acid. Scale bars: **a** = 500 µm; **b**, **c**, **e** = 50 µm; **d**, **f**, **g**, **h** = 20 µm; **i** = 5 µm

Taxonomy

Lindgomyces carolinensis Raja, J. Fourn., Paguigan & Oberlies, sp. nov.

Fig. 3

MB 817996

Holotype: **USA**: North Carolina, 35° 9.215′ N, 81° 1.231′ W, Lake Wylie, on submerged wood, 30 August 2014, Huzefa A. Raja and Vincent P. Sica, G618 (ILLS 80483), ex-type living culture DSM 103499, single ascospore isolate from holotype.

Etymology: in reference to the state North Carolina, where the fungus was first collected.

Saprobic on decaying wood. **Sexual morph**: Ascomata 160–170 × 290–330 μm, black, partially immersed becoming superficial to erumpent by sloughing off the wood, scattered, globose to subglobose, ostiolate, short papillate. Peridium composed of two layers, inner layer composed of small, pale brown, isodiametric cells, outer layer of dark brown, pseudoparenchymatic cells, covered with brown amorphous material from the host 20–30 μm wide. Pseudoparaphyses cellular, abundant, ca. 2–3 μm wide, septate, slightly constricted at the septa, anastomosing above the asci. Asci 84–141 × 19–25 μm (mean and SD = 104 ± 12 × 23 ± 2 μm, $n = 50$), broadly cylindrical to clavate, broadly rounded at the apex, cymbiform, fissitunicate, tapering to a short stipe at the base, eight-spored. Ascospores 39–50 × 6–9 (−11) μm (mean and SD = 43 ± 2 × 8 ± 1 μm, $n = 45$), overlapping, biseriate, fusiform with acute ends, straight to slightly curved, broad at the apex, tapering towards the base, one-septate, constricted at the septum; primary septum supra-median to mostly median (0.47–0.53; average 0.5, $n = 50$), hyaline, with two large lipid guttules in each cell, surrounded by a large, fusiform gelatinous sheath 86–125 × 13–15 μm, with gelatinous cap-like structures on either side of the ascospore sheath; sheath evanescent and disappears completely upon contact with water; not visible in glycerin and/or lactic acid.

Additional specimen examined: Hanging Rock State Park, stream draining into Hanging Rock Park Lake, 36° 23.568′ N, 80° 16.162′ W, water 8 °C, pH 4.5, on submerged wood, 3 March 2015, leg. Huzefa A. Raja and Nicholas H. Oberlies, G833 and G834 (ILLS 80484).

Anamorph: None observed.

Known distribution: USA (North Carolina).

Culture characteristics: Colonies on PDA irregular, slow growing, reaching about 25–30 mm in diameter after 21 days at 25 °C, in 12/12 h light/dark. Aerial mycelium pale brown, raised, and fluffy towards the center, with a marginal zone of hyaline to pale brown mycelium. Aerial and submerged hyphae, septate, 3–4 μm wide; colony reverse dark to pale brown.

Notes: *Lindgomyces carolinensis* is unique among the 11 previously described species of *Lindgomyces*, in that the ascospores are equipped with a large fusiform gelatinous sheath,

which enlarges upon contact with water (video, supporting information) and does not stain in India ink or aqueous nigrosin. Once the sheath is fully extended in water, it shows the presence of cap-like structures on the apical apices of the sheath (Fig. 3e). After a few minutes in water, the sheath completely dissolves and becomes invisible (Fig. 3g, h); it is also not visible when the fungal material is fixed in glycerin and lactic acid. *Lindgomyces carolinensis* can be compared to the type species, *L. ingoldianus*, which also has a large sheath; however, the former is different from the latter in that the sheath of *L. carolinensis* does not stain, while that of *L. ingoldianus* stains black in India ink and is long, amorphous, and sticky (Shearer and Hyde 1997; Hirayama et al. 2010). In addition, molecular data (SSU + LSU nrDNA, Fig. 1) and nuclear ribosomal ITS data (Fig. 2) clearly indicate that *L. carolinensis* is a phylogenetically distinct species in comparison to all 11 previously described species currently belonging to *Lindgomyces*.

Chemistry: From the organic extract of the solid fermentation culture of *L. carolinensis* (G618) (132 mg), two known cyclodepsipeptides, Sch 378161 (**1**) and Sch 217048 (**2**) (Fig. 5), were isolated and identified based on comparison of their retention times and HRESIMS and MS/MS fragmentation patterns to standards in our in-house database (El-Elimat et al. 2013). The NMR data for the compounds were also consistent with those reported in the literature (Hegde et al. 1998, 2001; El-Elimat et al. 2017). Additionally, the same compounds were identified in cultures of G618, G833, and G834, all grown on PDA, and analyzed by in situ sampling analysis using a droplet-LMJ-SSP coupled to an UPLC-high-resolution mass spectrometry (HRMS) (Sica et al. 2015) (Fig. 6).

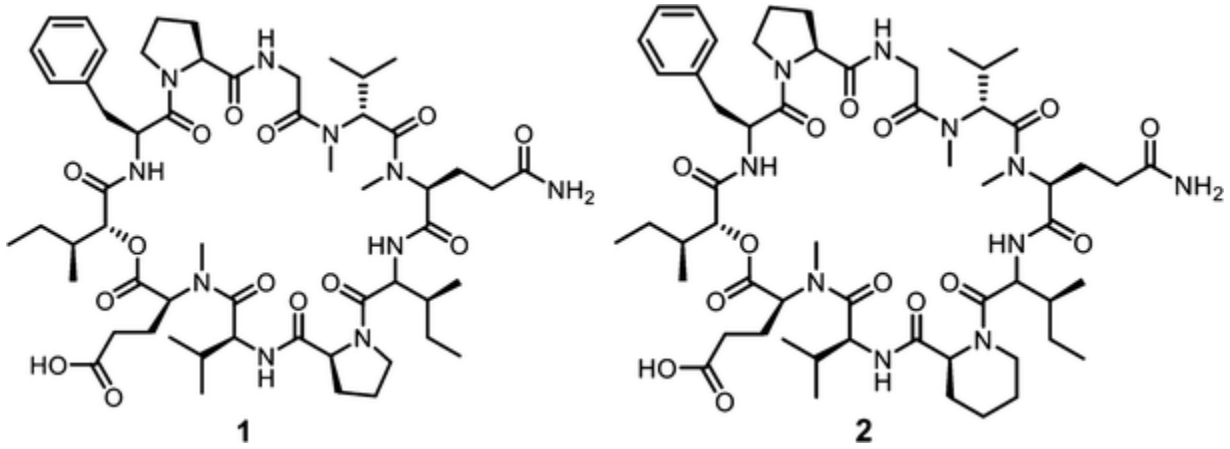

Fig. 5. Structures of cyclodepsipeptides Sch 378161 (**1**) and Sch 217048 (**2**)

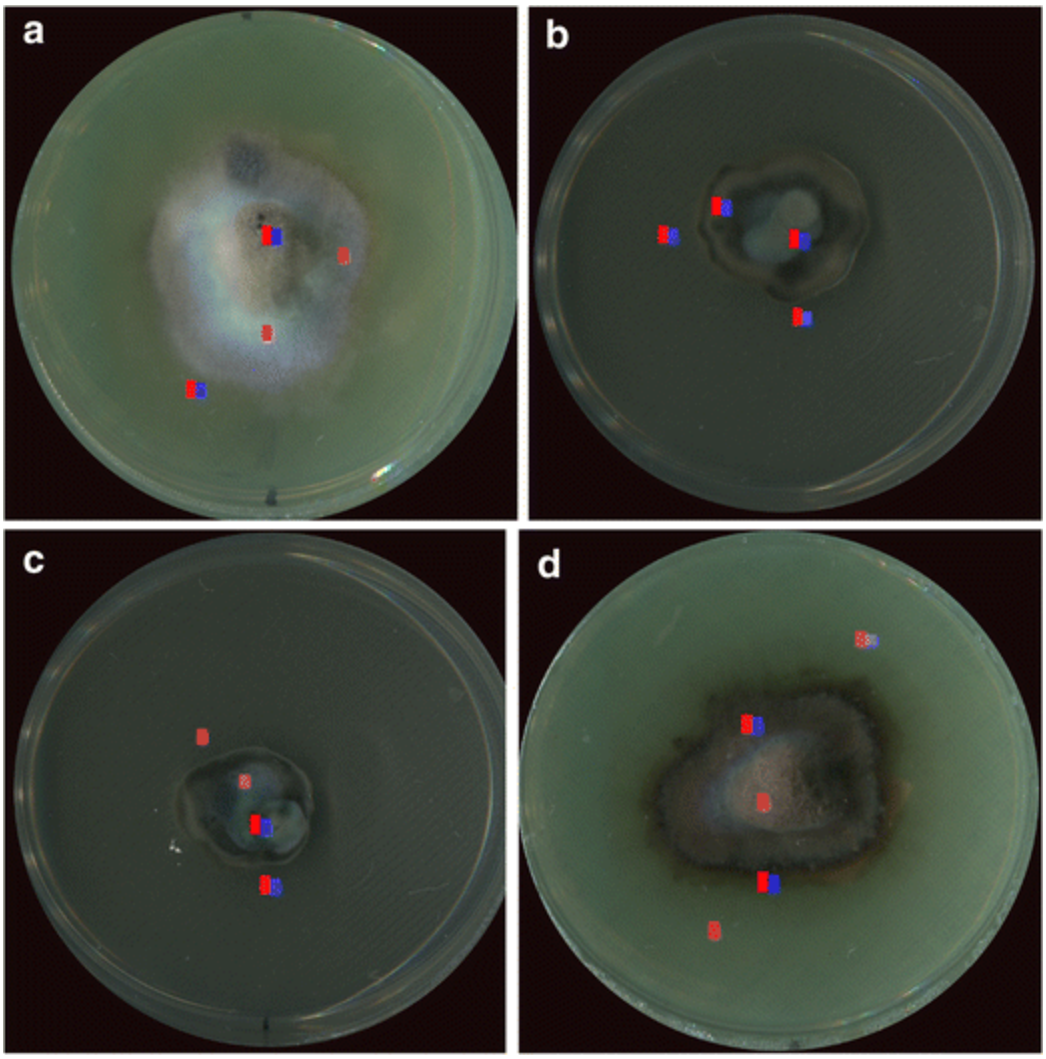

Fig. 6. In situ analysis of the distribution pattern of compounds **1** (*blue*) and **2** (*red*) via a droplet–liquid microjunction–surface sampling probe (droplet-LMJ-SSP). The height of the bars indicates the relative amount of signal detected by high-resolution mass spectrometry (HRMS) within a 5 ppm mass tolerance for the given compounds in cultures of: **a** G618, **b** G833, **c** G834, and **d** G619, all grown on potato dextrose agar (Difco)

Lindgomyces cigarospora Raja, J. Fourn., Paguigan & Oberlies, sp. nov.

Fig. 4

MB 817997

Holotype: **USA**: North Carolina, 35° 9.215′ N, 81° 1.231′ W, Lake Wylie, on submerged wood, 30 August 2014, Huzefa A. Raja and Vincent P. Sica, G619 (ILLS 80482), ex-type living culture DSM 103500, single ascospore isolate from holotype.

Etymology: from Latin *cigaro* = cigar, in reference to the cigar-shaped ascospores.

Saprobic on decaying wood. **Sexual morph**: Ascomata 280–290 × 330–340 µm, black, partially superficial to immersed, gregarious, globose to subglobose, ostiolate, short papillate. Peridium composed of two layers: inner layer composed of small, pale brown, isodiametric cells, *textura angularis* in surface view, outer layer of pigmented, thick-walled, pseudoparenchymatic cells. Pseudoparaphyses cellular, abundant, 1–2 µm wide, septate, slightly constricted at the septa, anastomosing above the asci. Asci 112–162 × 21–32 µm (mean and SD = 131 ± 12 × 26 ± 3 µm, *n* = 25), broadly clavate, thick-walled, and rounded at the apex, with an apical chamber, fissitunicate, tapering to a long stipe at the base, 10–15 µm, eight-spored. Ascospores 44–60 (−70) × 7–10 (−13) µm (mean and SD = 50 ± 4 × 8 ± 1 µm, *n* = 40), overlapping biseriate to triseriate, fusiform to cylindrical with rounded ends, hyaline, one-septate; primary septum supra-median to mostly median (0.46–0.50; average 0.5, *n* = 25), with 2–3 large lipid guttules in each cell, with short, rounded terminal ephemeral appendages, visible when ascospores are mounted in glycerin and/or lactic acid.

Anamorph: None observed.

Known distribution: USA (North Carolina).

Culture characteristics: Colonies on PDA irregular, slow growing, reaching about 25–30 mm in diameter after 21 days at 25 °C, in 12/12 h light/dark. Aerial mycelium dark mouse gray, raised, and fluffy towards the center of the inoculum block. Aerial and submerged mycelium, rough-walled, septate, 2–3 µm wide, floccose, hyaline to pale brown towards the margins; colony reverse dark brown. Submerged to superficial sterile ascomata produced after 28–40 days towards the margins on PDA.

Notes: Morphologically, *L. cigarospora* is comparable with *L. rotundatus*, but the ascospores of *L. cigarospora* are fusiform to cylindrical with rounded ends, equipped with short, rounded terminal, ephemeral appendages, while those of *L. rotundatus* are cylindrical with rounded ends and surrounded by fusiform gelatinous sheath. There is no expanding fusiform gelatinous sheath in *L. cigarospora*, but ascospores of *L. rotundatus* are equipped with a sheath, 4 µm thick at sides, and 30–35 µm long at both ends, expanding to form appendages up to 200 µm long (Hirayama et al. 2010). The morphological data are further corroborated with molecular data, which includes the (SSU + LSU nrDNA, Fig. 1) and nuclear ribosomal ITS data (Fig. 2), clearly indicating that *L. cigarospora* is a phylogenetically distinct species.

Chemistry: Compounds **1** and **2** (Fig. 5) were also detected in the extract of *L. cigarospora*(G619) (87 mg) by dereplication. Similarly, compounds **1** and **2** were also detected in PDA cultures of G619 by in situ sampling analysis using droplet-LMJ-SSP coupled to an UPLC-HRMS system (Sica et al. 2015) (Fig. 6).

Lindgomyces cinctosporus Raja, A.N. Mill. & Shearer [as ‘cinctosporae’], Mycologia 102(3): 738 (2010)

Fig. 7a, d

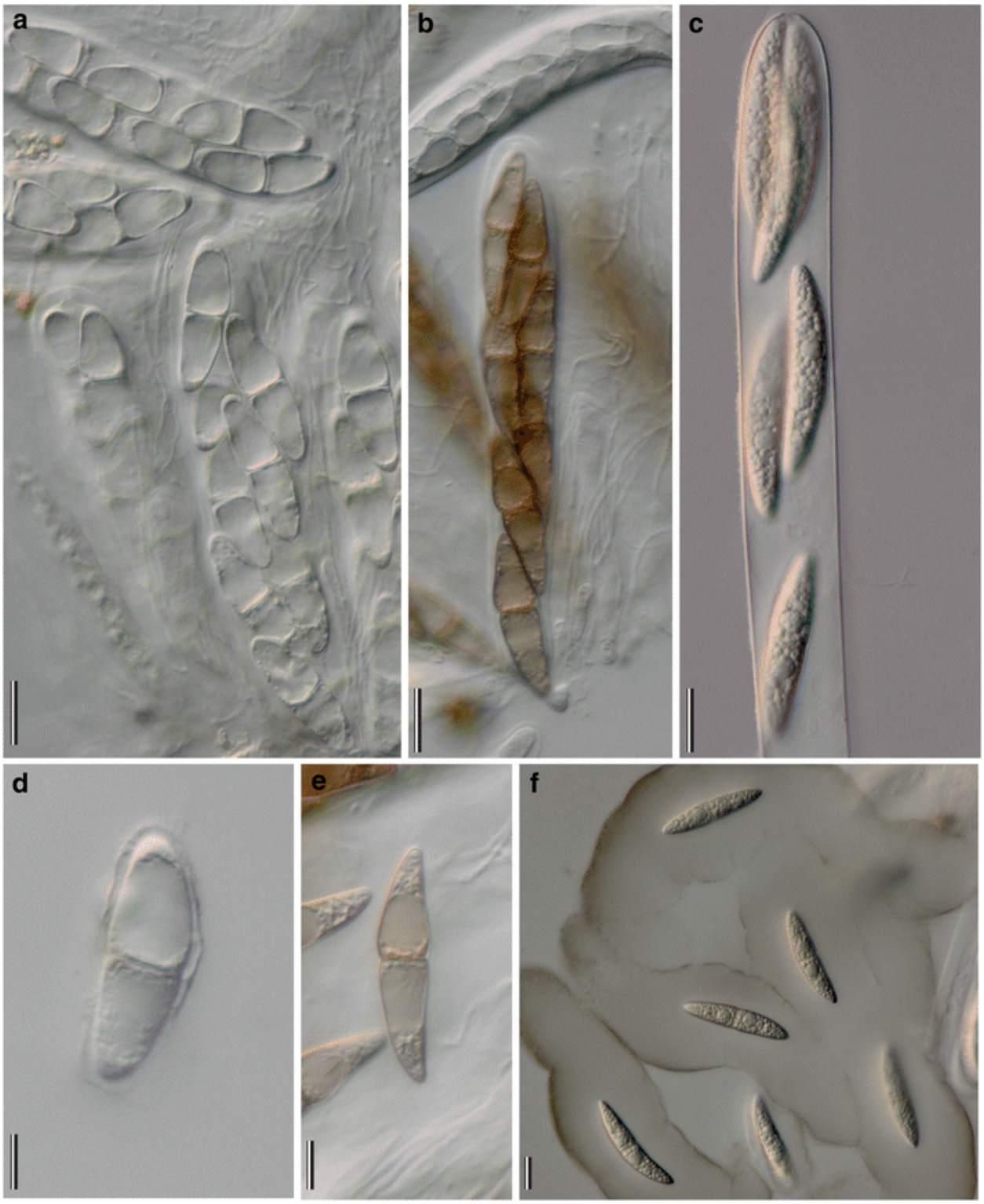

Fig. 7. a, **d** *Lindgomyces cinctosporus* (JF15154/G825). **a** Asci. **d** Ascospore with gelatinous sheath. **b**, **e** *Lindgomyces griseosporus* (JF15163/G822). **b** Asci. **e** Ascospore. **c**, **f** *Lindgomyces ingoldianus* (G99-5). **c** Elongating endoascus in water. **f** Ascospores showing amorphous, sticky gelatinous sheath stained in India ink. Scale bars: **a**–**c**, **f** = 20 μm; **d**, **e** = 10 μm

Habitat: On submerged, dead, corticated or partially decorticated woody debris.

Anamorph: None observed.

Known distribution: USA: North Carolina, France.

Culture characteristics; Colonies on PDA irregular, slow growing, reaching about 25 mm in diameter after 21 days at 25 °C, in 12/12 h light/dark. Aerial mycelium mouse gray towards the center, septate, dark brown to black towards the margin of the colony.

Specimen examined: France: Saône & Loire: Roussillon en Morvan, Gorges de la Canche, stream, 47° 0.000′ N, 4° 0.000′ E, ca. 500 m, on submerged decorticated wood of *Salix* sp., 20 October 2015, Jacques Fournier, JF15154 (ILLS 80647), living culture, G825.

Notes: The specimen from France agrees well with the type description of *L. cinctosporus* provided by Hirayama et al. (2010). The results of both molecular phylogenetic analyses (Fig. 2) as well as morphological examination (Fig. 7a, d) suggest that isolate G825 obtained from specimen JF15154 and *L. cinctosporus* are conspecific. *Lindgomyces cinctosporus* was previously reported from France by Zhang et al. (2014); however, the authors were unable to obtain a pure culture and sequence data from those collections. Our study provides the first phylogenetic verification reporting the occurrence of *L. cinctosporus* from France, as strain G825 forms a strongly supported clade to the type strain of *L. cinctosporus* (R56-1) in the ITS phylogeny, with ≥95% PP and 100% RAxML BV (Fig. 2).

Lindgomyces griseosporus Y. Zhang, J. Fourn. & K.D. Hyde, Mycoscience 55: 44 (2014)

Fig. 7b, e

Anamorph: None observed.

Habitat: On submerged, dead, corticated or partially decorticated woody debris.

Known distribution: France.

Culture characteristics: Colonies on PDA somewhat regular, reaching about 35 mm in diameter after 21 days at 25 °C, in 12/12 h light/dark. Aerial mycelium mouse gray, fluffy, and cotton-like towards the center; dark brown to black towards the margin of the colony.

Specimen examined: France, Saône & Loire: La Grande Verrière, Chateau Bouton, Le Mechet stream, 46° 59.775′ N, 4° 0.000′ E, ca. 315 m, submerged decorticated twig, 22 October 2015, Jacques Fournier, JF15163 (ILLS 80645), living culture G822.

Notes: *Lindgomyces griseosporus* was described by Zhang et al. (2014) and is morphologically characterized by the production of gray, narrowly fusiform ascospores with supramedian primary septum, which lacks a mucilaginous sheath at maturity (Fig. 7b, e). The new isolate, G822 obtained from a specimen collected in France JF15163, is both morphologically and

phylogenetically related to the type sequence of *L. griseosporus*. The isolate from France forms a well-supported clade (≥95% PP and 100% RAxML BV) in the ITS phylogeny with the type strain BFC200094 (CBS 123100) of *L. griseosporus* (Fig. 2).

Lindgomyces ingoldianus (Shearer & K. D. Hyde) K. Hiray., Kaz. Tanaka & Shearer, Mycologia 102: 733 (2010)

Fig. 7c, f

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

Anamorph: None observed.

Habitat: On submerged, dead, corticated or partially decorticated woody debris.

Known distribution: Australia, Brunei Darussalam, France, Japan, USA (FL, ILL, ME, NC, VA, WI).

Culture characteristics: Morphological variation was observed in different strains on the PDA media. Colony morphology of strain G99-2 on PDA was irregular, reaching about 20–355 mm after 21 days at 25 °C, in 12/12 h light/dark. Aerial mycelium was dark gray towards the center and dark brown to black towards the margins. A brown pigment diffused into the surrounding agar.

Strain G99-3 on PDA was irregular, slow growing, reaching about 20 mm in diameter after 21 days at 25 °C, in 12/12 h light/dark. Aerial mycelium mouse gray; dark brown to black towards the margin; no diffusible pigment was observed. Strain G99-5 on PDA reached about 30 mm in diameter after 21 days at 25 °C, in 12/12 h light/dark. Aerial mycelium was mouse gray; mycelium dark brown to black towards the margin of the colony. Dark brown exudates produced on the surface of the mycelium.

Specimens examined: USA: North Carolina, Hagan Stone State Park, freshwater lake, 35° 57.183′ N, 79° 43.566′ W, 232 m, on submerged decorticated wood, 22 March 2012, Huzefa Raja, living culture, G99-2; North Carolina, freshwater stream, 36° 27.888′ N, 80° 1.846′ W, 250 m, water 13 °C, pH 5, on submerged wood, Huzefa Raja & Nicholas Oberlies, 26 April 2013, living culture, G99-3; North Carolina, Lake Brandt at Burmil Park, 36° 10.186′ N, 79° 52.041′ W, 226 m, on submerged decorticated wood, Huzefa Raja, 23 September 2015, living culture, G99-5.

Notes: Three strains of *L. ingoldianus* (G99-2, G99-3, and G99-5) collected from North Carolina form a strongly supported clade (≥95% PP and 100% RAxML BV) with the type strain of *L. ingoldianus* (ATCC200398) in the ITS phylogeny (Fig. 2). Morphologically, the ascospores that were used for the isolation of these strains were equipped with a sticky, amorphous gelatinous sheath, which forms a long gelatinous envelope when stained with India ink in water. This amorphous, sticky, gelatinous sheath is a key morphological feature that separates *L. ingoldianus* from the other species of *Lindgomyces* (Fig. 2).

Discussion

In this study, we describe and illustrate two new species on submerged wood, *L. carolinensis*and *L. cigarospora*, from North Carolina based on morphological and molecular analysis (Figs. 1–4). Hirayama et al. (2010) pointed out that the ascospore shape, especially the shape of ascospore ends, is more significant than ascospore dimensions when distinguishing various species of *Lindgomyces*. The shape of the ascospore ends can also distinguish these two new species. For example, the ascospores of *L. carolinensis* have acute ends, but with a gelatinous sheath, which expands upon contact with water (video, supporting information), while those of *L. cigarospora* have rounded ends and are cylindrical to fusiform in shape, equipped with short, rounded terminal, ephemeral appendages (Figs. 2 and 4). Additional collections of *L. cigarospora* might shed more light on the nature of its ephemeral appendages.

The fissitunicate ascus dehiscence of *L. carolinensis* was visualized under a microscope slide using a video recording (video, supporting information). This is possibly the first time a freshwater Dothideomycetes has been video recorded capturing ascospore discharge and unraveling of the gelatinous sheath in real time. The video shows the extension of the endoascus from the ectoascus and the forcible ejection of the ascospores from the tip of the endoascus. After the ascospores are forcibly ejected and come in contact with the water column, the gelatinous sheath unravels in water. The sheath is fusiform and surrounds the entire ascospore as soon as it touches the water. After the sheath is completely extended in water, it also shows the presence of gelatinous cap-like structures on the apical apices of the sheath. It has been hypothesized that gelatinous sheaths and appendages in both freshwater and marine ascomycetes allow the ascospores to attach to substrates in moving water (Shearer 1993; Fallah et al. 1997; Jones 2006). In *L. carolinensis*, the sheath quickly begins to disintegrate, and in a few minutes, the sheath completely disappears and is not visible when the material is mounted in glycerin and/or lactic acid.

In the past 6 years, since the description of *Lindgomyces* as a separate genus from *Massarina*, based on morphological and molecular data (Hirayama et al. 2010), nine additional new species have been added from investigations of freshwater ascomycetes mainly from temperate geographical locations (Raja et al. 2011b, 2013b; Crous et al. 2015; Liu et al. 2015; Hyde et al. 2016). Except for *L. ingoldianus* (Shearer and Hyde 1997), most species have been collected from France, USA, and Japan, which indicates that *Lindgomyces* spp. prefer temperate geographical locations. All previously described *Lindgomyces* species have been reported from submerged wood (Shearer and Raja 2016), except *L. ingoldianus*, which can occur on herbaceous material (Shearer and Hyde 1997). In terms of habitat, most species have been collected from streams and rivers, while we report herein two collections of *L. ingoldianus* from lakes. Thus, it seems that *L. ingoldianus* might be broadly distributed geographically because it can occur on both woody and herbaceous debris in both lotic (running water) and lentic (standing water) habitats. Thus far, all species of *Lindgomyces* are described and reported from fresh water (Shearer and Raja 2016). Additional collections from different geographical locations, habitats, and substrates will shed light on the ecological preference of *Lindgomyces* spp.

We have been investigating the secondary metabolites from freshwater ascomycetes in North Carolina, primarily due to our interest in this unique ecological niche. Based on our previous studies, this niche has proven to be diverse in both mycological (Raja et al. 2013a, b, 2015; Crous et al. 2015) and chemical discoveries (El-Elimat et al. 2014a, b, 2017; Paguigan et al. 2016). Chemical exploration of the newly described species, *L. carolinensis* and *L. cigarospora*, resulted in the identification of two known cyclodepsipeptides, Sch 378161 (**1**) and Sch 217048 (**2**), and the isolation of both of them were first reported from an unidentified fungal fermentation broth (Hegde et al. 1998, 2001; Chu et al. 2000). Additionally, **2** was recently isolated from an unidentified coprophilous fungus belonging to the Pleosporaceae, along with other closely related cyclodepsipeptides (Isaka et al. 2014). Both compounds have been described to have selective inhibition against human NK_2 receptors (Hegde et al. 1998, 2001), and **2** exhibited antimalarial activity against *Plasmodium falciparum* K1 (Isaka et al. 2014). Compounds **1** and **2** were also tested for antimicrobial and cytotoxicity in this study, but no significant inhibitions were observed.

In situ analysis of the distribution pattern of compounds **1** and **2** on cultures of G619, G618, G833, and G834 grown on PDA not only indicated that these compounds were being produced in the mycelia of these fungi, but also showed that they were being exuded into the surrounding agar (Fig. 6). Fungi often release an array of secondary metabolites into the environment that may influence organisms that interact with them, thus producing a competitive advantage for the producing organism (Rohlfs 2015). Compounds **1** and **2** lacked biological activity (antimicrobial and cytotoxicity) in our studies; thus, their ecological function is currently hard to discern. Investigating the effects of different environmental stimuli on the production of these compounds may help shed some light on their natural functions (Fox and Howlett 2008). Studies are currently ongoing in our laboratory to examine the pure compounds **1** and **2** as well as other *Lindgomyces* strains against a series of diverse biological assays to better predict the biological activity of secondary metabolites from this unique ecological niche.

As to the production of **1** and **2** by G618, G834, G833, and G619, the data demonstrated that these species correlated closely not only phylogenetically, but also in their secondary metabolites profiles. This observation may be useful for classification and/or as potential chemotaxonomic markers for these fungi in the future. Previously, we described and reported other *Lindgomyces* species that produced biosynthetically different classes of secondary metabolites (Raja et al. 2013a; Paguigan et al. 2016). For example, from *L. madisonensis*, compounds with aromatic polyketide structures were identified (Paguigan et al. 2016), while *L. angustiascus* mainly produced the fatty acid 6*E*,9*E*-octadecadienoic acid and the steroid derivative ergosterol peroxide (Raja et al. 2013a), at least in culture. We are currently screening cultures of *L. cinctosporus*, *L. ingoldianus*, and *L. griseosporus* for secondary metabolites to better understand the chemical diversity within this genus of freshwater ascomycetes.

Notes

Acknowledgements: We thank Dr. J.O. Falkinham, III of Virginia Polytechnic and State University for the antimicrobial testing and Dr. J. Burdette of the University of Illinois at Chicago for the cytotoxicity testing. We also thank Drs. V. Kertesz and G.J. Van Berkel of the Mass Spectrometry and Laser Spectroscopy Group, Chemical Sciences Division (Oak Ridge

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