Freshwater Ascomycetes: *Minutisphaera* (Dothideomycetes) revisited, including one new species from Japan

By: Huzefa A. Raja, Nicholas H. Oberlies, Mario Figueroa, Kazuaki Tanaka, Kazuyuki Hirayama, Akira Hashimoto, Andrew N. Miller, Steven E. Zelski, Carol A. Shearer

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Abstract:

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Keywords: aquatic | minute fungi | submerged wood | systematics

Article:

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Freshwater Ascomycetes: *Minutisphaera* (Dothideomycetes) revisited, including one new species from Japan

Huzefa A. Raja[1]
Nicholas H. Oberlies
Mario Figueroa
Department of Chemistry and Biochemistry, University of North Carolina at Greensboro, Greensboro, North Carolina 27412

Kazuaki Tanaka
Kazuyuki Hirayama
Akira Hashimoto
Faculty of Agriculture and Life Sciences, Hirosaki University, Bunkyo-cho, Hiroskaki, Aomori 036-8561, Japan

Andrew N. Miller
Illinois Natural History Survey, University of Illinois, Champaign, Illinois 61820

Steven E. Zelski
Carol A. Shearer
Department of Plant Biology, University of Illinois, Urbana, Illinois 61801

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Key words: Aquatic, minute fungi, submerged wood, systematics

Introduction

The freshwater Dothideomycetes is an ecological group of fungi (Shearer et al. 2009) that currently comprise approximately 195 species, which constitute about 32% of the currently known freshwater ascomycetes (Shearer and Raja 2012). Most species of freshwater Dothideomycetes belong in the Pleosporales (Zhang et al. 2012) or Jahnulales (Pang et al. 2002, Campbell et al. 2007, Suetrong et al. 2011), although few taxa have affinities to the Capnodiales and Tubeufiaceae (Shearer et al. 2009).

Based on evaluation of morphological characters and multigene molecular phylogenetic studies, several new families have been assigned recently to the freshwater Dothideomycetes. These families include Aliquandostipitaceae (Inderbitzin et al. 2001), Amniculicolaceae (Zhang et al. 2008, 2009a, b), Lentitheciaceae (Zhang et al. 2009b, c), Lindgomycetaceae (Hirayama et al. 2010), Morosphaeriaceae (Suetrong et al. 2009, Boonmee et al. 2012) and Natipusillaceae (Raja et al. 2012). Although molecular sequence data has provided phylogenetic placements for numerous freshwater Dothideomycetes, a number of Dothideomycetes from freshwater habitats occur as singletons and remain incertae sedis (Shearer et al. 2009).

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[1] Corresponding author. E-mail: haraja@uncg.edu

Minutisphaera Shearer, A.N. Mill. & Ferrer, typified by *M. fimbriatispora* Shearer, A.N. Mill. & Ferrer, is a recently described species from submerged wood in freshwater habitats from USA (Ferrer et al. 2011). It is characterized by small globose to subglobose ascomata with dark brown to black hairs around the ostiole, fissitunicate, oblong to obclavate, eight-spored asci, and one-septate, multiguttulate, hyaline to pale brown ascospores equipped with a gelatinous sheath and numerous filamentous appendages radiating around the spore at the mid-septum. *Minutisphaera* currently is placed in the Dothideomycetes based on morphological as well as nuclear ribosomal sequence data, but its relationship with other taxa within the Dothideomycetes remains unresolved.

During ongoing investigations of freshwater ascomycetes in the USA (Raja et al. 2011b) and Japan (Hirayama et al. 2010), we found interesting taxa from both Aomori (Japan) and North Carolina (USA), which were morphologically similar to *Minutisphaera*. These taxa have dark hair-like structures around the ostiolar region, broadly shaped bitunicate asci, and 1–3-septate, hyaline to brown ascospores with a sheath in material from Japan, and with both a sheath and appendages in material from USA. The ascomata of these taxa, however, appeared more apothecioid than those of the type species of the genus. In addition, collections of *Minutisphaera*-like fungi from USA showed variation in ascospore size. For example, the ascospores of G155-1 were more similar to those reported for *M. fimbriatispora* but the ascospores of G156-1 and G156-2 were comparatively smaller than those of *M. fimbriatispora* (Ferrer et al. 2011). Whether these size variations are intraspecific or result from cryptic speciation and therefore interspecific within *Minutisphaera* is unknown at this time.

The goal of the present study, therefore, was to understand the phylogenetic relationships of *Minutisphaera*-like taxa collected from Japan and North Carolina (USA) with the original collections of *M. fimbriatispora* and within the Dothideomycetes. To address this goal, we undertook a molecular phylogenetic study using partial 18S small subunit nrDNA (SSU) and 28S large subunit nrDNA (LSU). To better understand species boundaries of our collections within *Minutisphaera*, we conducted phylogenetic analyses based on ITS sequences and compared taxa based on morphological characteristics. In addition, as part of ongoing investigations of chemical mycology of freshwater fungi, we screened two strains of *Minutisphaera* from USA (G155-1 and G156-1) for secondary metabolite production because these fungi had not been investigated previously for chemical constituents.

MATERIALS AND METHODS

Morphological studies and fungal isolates.—Methods of morphological observation are described by Tanaka et al. (2009). For ascospore septum position, the decimal system (Shoemaker 1984) was used. Single ascospore cultures were obtained according to Shearer et al. (2004). For the Japanese collections, ascomata formation was induced by placing a small piece of mycelial culture on rice straw agar (RSA; Tanaka and Harada 2003). Colony colors on potato-dextrose agar (PDA; Difco), cornmeal agar (CMA; Difco) and weak oatmeal agar (wOA; 15 g Difco oatmeal agar, 6 g agar, 1 L water; Zhao and Shamoun 2006) were characterized using Rayner (1970). Fungal cultures obtained from Japan were deposited at the Japan Collection of Microorganisms (JCM) and the National Institute of Agrobiological Sciences (MAFF). Cultures from USA were deposited in the Department of Plant Biology Culture Collection at the University of Illinois and Department of Chemistry and Biochemistry Culture Collection at the University of North Carolina at Greensboro (UNCG).

DNA extraction and amplification.— Detailed protocols for DNA extraction and PCR amplification were described by Hirayama et al. (2010). DNA from mycelia was extracted with the ISOPLANT Kit (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's instructions. Partial SSU and LSU and the complete ITS region of nrDNA were amplified with three primer sets, NS1–NS4 (White et al. 1990), LROR–LR7 (Rehner and Samuels 1994) and ITS1/1F–ITS4 (White et al. 1990, Gardes and Bruns 1993). Sequences were assembled with Sequencher 4.9 (Gene Codes Corp.), optimized by eye and manually corrected when necessary.

Taxon sampling and phylogenetic analyses.— Four datasets were assembled for phylogenetic analyses: (i) a SSU dataset that consisted of 80 taxa; (ii) an LSU dataset consisting of mostly the same taxa as in the SSU dataset; (iii) a combined 83 taxa SSU and LSU dataset; and (iv) an ITS dataset with 10 strains of *Minutisphaera* spp. to assess interspecific and intraspecific relationships among members of the genus. For the SSU and LSU datasets, we sampled taxa from the major orders of the Pleosporomycetidae and Dothideomycetidae currently included in the Dothideomycetes (Schoch et al. 2009). Taxa included in the present study were obtained from a study on the molecular phylogeny of freshwater Dothideomycetes (Shearer et al. 2009), as well as other studies on the phylogenetic relationships among dothideomyceteous fungi (Wu et al. 2011, Zhang et al. 2012). We also included members of the Patellariales and other apothecial dothideomycete members such as *Catinella olivacea* (Batsch) Boudier to test whether *Minutisphaera*-like fungi share phylogenetic affinities with the apothecial dothideomycetous fungi. In addition, we included sequences of the *Natipusillaceae* to assess the phylogenetic affinities of *Minutisphaera* spp. with *Natipusilla* spp. because both these freshwater genera possess minute ascomata with bitunicate asci and their ascospores are equipped with gelatinous appendages. Members of the Arthoniomycetes were used as outgroup taxa (Schoch et al. 2009). Alignments were

generated according to Raja et al. (2011a), and subsequently ambiguous regions, gaps and introns were excluded from the final alignment with Gblocks (Castresana 2000, Talavera and Castresana 2007) via the default parameters. We manually deleted a portion of the nucleotides from the 59 and 39 ends due to missing data in most taxa.

Maximum likelihood (ML) analyses were performed on the separate and combined datasets. We used jModeltest (Posada 2008) (with 88 possible evolutionary models) to obtain the best-fit model of nucleotide evolution for each dataset. The Akaike information criterion (AIC) (Posada and Buckley 2004) as implemented in jModeltest selected the TrN+I+G model for the SSU dataset, the TIM3+G model for the LSU dataset, the TrN+I+G model for the combined SSU and LSU dataset, and the TrNef+I model for the ITS dataset. First, we ran separate ML analyses on the individual SSU and LSU datasets using PHYML (Guindon and Gascuel 2003) with 1000 ML bootstrap replicates with a combined nearest neighbor interchange (NNI) and subtree pruning and regrafting (SPR) tree search option in effect. We evaluated bootstrap support (BS) values obtained for the individual SSU and LSU phylogenies for conflict by comparing clades with BS $ 70% (Wiens 1998). Because the topology of the clades obtained in the separate analyses did not show conflicting results, we consequently concatenated the two datasets and performed a ML analysis using PHYML with the same parameters as above with 1000 ML bootstrap replicates to assess clade support (Felsenstein 1985). In addition to the PHYML analysis, we also ran a randomized accelerated maximum likelihood analysis with RAxML 7.0.4 (Stamatakis et al. 2008) on the combined SSU and LSU dataset on the CIPRES Portal 2.0 (Miller et al. 2010) with the default rapid hill-climbing algorithm and GTR model employing 1000 fast bootstrap searches. Clades that received a BS $ 70% were considered significant and robustly supported (Hillis and Bull 1993).

We then ran Bayesian analyses on the combined SSU and LSU dataset and the ITS dataset with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001, 2005) to evaluate clade support by implementing the TrN+I+G model for the combined SSU and LSU dataset and TrNef+I model for the ITS dataset. Constant characters were included, and 10 000 000 generations with trees sampled every 1000 generations were run, resulting in 10 000 total trees. The first 1000 trees that extended beyond the burn-in phase in each analysis were discarded, and the remaining 9000 trees were used to calculate the posterior probability (PP) for each clade. The consensus of the trees was viewed in PAUP 4.0b10 (Swofford 2002). The Bayesian analysis was run twice starting from a different random tree each time to ensure that trees from the same tree space were being sampled. The sequences generated in this study and the alignments used in combined SSU and LSU and ITS phylogenetic analyses were deposited respectively in GenBank (TABLES I, II) and in TreeBASE (www.treebase.org, submission 13647).

Fermentation, extraction and isolation.—Fresh cultures of G155-1a and G156-1 were grown on malt-extract slants, and a piece of agar culture was transferred to a medium containing 2% soy peptone, 2% dextrose and 1% yeast extract (YESD media). After incubation (1 wk) at 22 C with agitation, the cultures were used to inoculate 50 mL rice medium prepared with 25 g rice with 35 mL H_2O in a 250 mL Erlenmeyer flask. This was incubated at 22 C until the cultures showed good growth (approximately 2 wk). To each culture was added 150 mL 1 : 1 MeOH-$CHCl_3$. The mixture was shaken 16 h then filtered, and the solvent was evaporated. Each extract was defatted by stirring vigorously 1 h in a mixture of 25 mL MeOH, 25 mL CH_3CN and 50 mL hexane, then partitioned in a separatory funnel. The bottom layer was collected and evaporated. Each defatted extract (17.25 and 15.84 mg for G155-1a and G156-1 respectively) was purified on semipreparative HPLC over a Phenomenex Gemini-NX C18 (5 mm; 250 3 10 mm; Phenomenex Inc., Torrance, California) column at a 3 mL/min flow with a gradient that initiated with 30 : 70 CH_3CN-0.1% formic acid (aqueous) and increased linearly to 100% CH_3CN over 30 min.

Metabolite identification.— Pure compounds were identified by nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS) analyses. NMR experiments were conducted in $CDCl_3$ with a JEOL ECA-500 (JEOL Ltd., Tokyo, Japan); GC-MS profiles were obtained with a Shimadzu apparatus (QP2010) equipped with a 30.0 m capillary column (ZB-5MS; Phenomenex). Samples were dissolved in $CHCl_3$ and GCMS solution software was used for data processing. The Shimadzu GC-MS Metabolites Spectral Database and NIST 2008 mass spectral library were used to identify pure compounds.

RESULTS

Molecular study.—The original SSU alignment consisted of 3227 nucleotides. After excluding ambiguous regions, introns and nucleotides from the 59 and 39 ends due to missing data in most sequences, the final SSU dataset consisted of 1059 nucleotides. The original LSU dataset consisted of 2566 nucleotides. After ambiguous regions, introns and 59 and 39 ends were delimited and excluded the final LSU dataset consisted of 1227 nucleotides. Because we did not find significant conflicts between the separate SSU and LSU tree topologies based on PHYML BS (data not shown), we concatenated the two genes. The combined SSU and LSU alignment included 2258 nucleotides. PHYML analyses of the combined genes produced a single most likely tree with a log likelihood value of 220151.62 (F_{IG}. 1). All taxa of *Minutisphaera* grouped in a highly supported clade within the Dothideomycetes with 100% PHYML BS and 99% RAxML BS but without significant Bayesian PP value. In the *Minutisphaera* clade there were three distinct clades, labeled A, B and C (F_{IG}. 1). *Minutisphaera fimbriatispora* forms clade A with low support. A strain of *Minutisphaera* sp. (G155-1a) from North Carolina occurred at the base of this clade, which possessed 78% RAxML BS, suggesting that it belongs

TABLE I. Sequences retrieved from GenBank

Species	Voucher information[a]	GenBank accession nos.	
		nucSSU rDNA	nucLSU rDNA
Aliquandostipite khaoyaiensis	CBS 118232	—	GU301796
Aulographina pinorum	CBS 174.90	GU296138	GU301802
Alternaria alternata	CBS 916.96	DQ678031	DQ678082
Alternaria sp. (as *Clathrospora diplospora*)	CBS 174.51	DQ678016	DQ678068
Amniculicola immersa	CBS 123083	GU456295	FJ795498
Amniculicola lignicola	CBS 123094	EF493861	EF493863
Amniculicola parva	CBS 123092	GU296134	FJ795497
Anguillospora longissima	CS869-1D	GU266222	GU266240
Aquaticheirospora lignicola	RK-2006a	AY736377	AY736378
Ascorhombispora aquatica	CAI-1H31	—	EU196548
Asterina phenacis	TH 589	GU586211	GU586217
Asterina weinmanniae	TH 592	GU586212	GU586218
Asterina zanthoxyli	TH 561	GU586213	GU586219
Botryosphaeria dothidea	CBS 115476	DQ677998	DQ678051
Botryosphaeria ribis	CBS 115475	DQ678000	DQ678053
Capnodium coffeae	CBS 147.52	DQ247808	DQ247800
Capnodium salicinum	CBS 131.34	DQ6779977	DQ678050
Catinella olivacea	UAMH 10679	DQ915484	EF622212
Cheirosporium triseriale	HMAS 180703	—	EU413954
Cochliobolus heterostrophus	CBS 134.39	AY544727	AY544645
Cochliobolus sativus	DAOM 216378	DQ677995	DQ678045
Dendryphiella arenaria	CBS 181.85	DQ471022	DQ470971
Dothidea insculpta	CBS 189.58	DQ247810	DQ247802
Dothidea sambuci	DAOM 231303	AY544722	NG_027611
Dothiora cannabinae	CBS 373.71	DQ479933	DQ470984
Elisinoë phaseoli	CBS 165.31	DQ678042	DQ678095
Elisinoë veneta	CBS 164.29	DQ678007	DQ678060
Farlowiella carmichaeliana	CBS 206.36	AY541482	AY541482
Gloniopsis praelonga	CBS 112415	FJ161134	FJ161173
Gloniopsis smilacis	CBS 114601	FJ161135	FJ161174
Guignardia bidwelli	CBS 237.48	DQ678034	DQ678085
Hysteropatella clavispora	CBS 247.34	DQ678006	AY541493
Hysteropatella elliptica	CBS 935.97	EF495114	DQ767657
Jahnula aquatica	R68-1	EF175633	EF175655
Jahnula bipileata	AF220-1	EF175634	EF175656
Jahnula sangamonensis	A402-1B	EF175639	EF175661
Laurera megasperma	AFTOL 2094	GU561841	FJ267702
Lentithecium aquaticum	CBS 123099	FJ795477	FJ795434
Lentithecium arundinaceum	CBS 619.89	DQ813513	DQ813509
Lindgomyces cinctosporae	R56-1	AB522430	AB522431
Lindgomyces ingoldianus	ATCC 200398[T]	AB521719	AB521736
Lindgomyces ingoldianus	JCM16479/NBRC106126	AB521720	JF419899
Lindgomyces rotundatus	JCM 16482/NBRC106127	AB521723	AB521740
Lophiostoma arundinis	CBS 269.34	DQ782383	DQ782384
Lophiostoma crenatum	CBS 629.86	DQ678017	DQ678069
Lophiostoma macrostomum	JCM 13545	AB521731	AB433273
Lophiostoma macrostomum	JCM 13546/MAFF 239447	AB521732	AB433274
Massarina eburnea	HKUCC4054	AF164366	—
Massarina eburnea	CBS 473.64	AF164367	—
Megalohypha aqua-dulces	AF005-2a	GU266228	EF175667
Micropeltis zingiberacicola	IFRDCC 2264	JQ036222	JQ036227
Minutisphaera fimbriatispora	A242-7d	HM196373	HM196366
Minutisphaera fimbriatispora[TYPE]	A242-8a	HM196374	HM196367

TABLE I. Continued

Species	Voucher information[a]	GenBank accession nos. nucSSU rDNA	nucLSU rDNA
Minutisphaera fimbriatispora	A242-8c	HM196375	HM196368
Minutisphaera fimbriatispora	G155-1a[b]	JX474865	JX474859
Minutisphaera sp.	G156-1a	JX474866	JX474860
Minutisphaera japonica	JCM 18561/MAFF 243473	AB733432	AB733438
Minutisphaera japonica	JCM18562/MAFF 243474	AB733433	AB733439
Minutisphaera japonica TYPE	JCM18560/MAFF 243475	AB733434	AB733440
Muyocopron sp.	MFLU (CC) 10-0042	JQ036225	—
Muyocopron sp.	MFLU (CC) 10-0041	JQ036226	JQ036230
Mytilinidon andinense	CBS 123562	FJ161159	FJ161199
Mytilinidon mytilinellum	CBS 303.34	FJ161144	FJ61184
Mycosphaerella fijiensis	OSC 100622	DQ767652	DQ678098
Mycosphaerella graminicola	CBS 292.38	DQ678033	DQ678084
Myriangium duriaei	CBS 260.36	AY016347	DQ678059
Natipusilla decorospora-1a	AF236-1a	HM196376	HM196369
Natipusilla limonensis-1a	AF286-1a	HM196377	HM196370
Natipusilla limonensis	PE3-2a	JX474867	JX474861
Natipusilla limonensis	PE3-2b	JX474870	JX474862
Natipusilla naponensis	AF217-1a	HM196378	HM196371
Natipusilla naponensis	AF217-1b	HM196379	HM196372
Natipusilla bellaspora	PE91-1a	JX474868	JX474863
Natipusilla bellaspora	PE91-1b	JX474869	JX474864
Neomicrothyrium siamense	IFRDCC 2194	JQ036223	JQ036228
Paramicrithyrium chinensis	IFRDCC 2258	JQ036224	JQ036229
Patellaria atrata	CBS 958.97	GU296181	GU301855
Roccellographa cretacea	DUKE 191Bc	DQ883705	DQ883696
Schismatomma decolorans	DUKE 0047570	AY548809	AY548815
		nucSSU rDNA	nucLSU rDNA
Stomiopeltis betulae	CBS 114420	GU214701	GU214701
Tingoldiago graminicola	JCM 16485/NBRC 106131[T]	AB521726	AB521743
Tingoldiago graminicola	JCM 16486/NBRC 106132	AB521728	AB521745
Trypethelium nitidiusculum	AFTOL 2099	GU561842	FJ267701

[a] Source abbreviations: TYPE Type Strains; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CS, and A, Carol Shearer, University of Illinois, Plant Biology Culture Collection; CAI, Lei Cai; RK, Rumpai Kodsueb; TH, T.A. Hoffmann; DAOM, Canadian Collection of Fungi Cultures in Ottawa, Ontario; UMAH, University of Alberta Microfungus Collection and Herbarium; R, Raja H., University of Illinois, Plant Biology Culture Collection; AF, Astrid Ferrer, University of Illinois, Plant Biology Culture Collection; AFTOL, Assembling the Fungal Tree of Life; ATCC, American Type Culture Collection; G, University of North Carolina, Greensboro, Department of Chemistry and Biochemistry Fungal Culture Collection; JCM, Japan Collection of Microorganisms; MAFF, the Ministry of Agriculture, Forestry and Fisheries, Japan; NBRC, National Biological Resources Center, Japan; HKUCC, University of Hong Kong Culture Collection; IFRDCC, International Fungal Research and Development Culture Collection; MFLU(CC), Mae Fah Luang University Culture Collection; OSC, Oregon State University Herbarium, Corvallis, Oregon Genome Databases; PE; Peru freshwater ascomycetes, Department of Plant Biology Culture Collection; DUKE, Duke University Herbarium, Durham, North Carolina.

[b] Newly generated sequences are in boldface.

to *M. fimbriatispora*. Isolates of the newly collected species from Japan formed a well supported clade (B) with 85% PhyML BS, 93% RAxML BS and significant PP; this clade was sister to the *M. fimbriatispora* isolates. Clade C consists of *Minutisphaera* sp. (G156-1a) that occurred on an independent branch sister to *M. fimbriatispora* isolates (A242 and G155) but without significant Bayesian PP and/or PHYML and RAxML BS (FIG. 1). Our molecular results also suggest that *M. japonica* is not phylogenetically related to *Natipusilla* spp. and adds further support to the establishment of Natipusillaceae by Raja et al. (2012) in that all the species currently described in this family formed a monophyletic clade with 100% PHYML and RAxML BS as well as significant BS PP (FIG. 1).

The ITS dataset included 10 strains of *Minutisphaera* spp. and consisted of 687 nucleotides including the primer regions at the 59 and 39 ends.

TABLE II. Newly generated ITS sequences from strains of *Minutisphaera* spp.

Species	Voucher information	Substrate and locality	GenBank accession nos. nucITS rDNA
Minutisphaera fimbriatispora	A242-7c[a]	Submerged wood, IL, USA	JX474871
Minutisphaera fimbriatispora	A242-7d[a]	Submerged wood, IL, USA	JX474872
Minutisphaera fimbriatispora	G155-1a	Submerged wood, NC, USA	JX474873
Minutisphaera fimbriatispora	G155-1b	Submerged wood NC, USA	JX474874
Minutisphaera japonica	JCM 18561/MAFF 243473	Submerged wood, Japan	AB733435
Minutisphaera japonica	JCM 18562/MAFF 243474	Submerged wood, Japan	AB733436
Minutisphaera japonica TYPE	JCM 18560/MAFF 243475	Submerged wood, Japan	AB733437
Minutisphaera sp.	G156-1a	Submerged wood, NC, USA	JX474875
Minutisphaera sp.	G156-2a	Submerged Pinus wood, NC, USA	JX474876
Minutisphaera sp.	G156-2b	Submerged Pinus wood, NC, USA	JX474877

[a] Specimens examined by Ferrer et al. 2011.

After the ends were trimmed and ambiguous regions excluded using Gblocks, the final ITS alignment contained 605 nucleotides. PHYML analyses of the ITS dataset generated a single most likely tree with a log likelihood value of 21334.69 (FIG. 2). *Minutisphaera japonica* (clade B) formed a distinct monophyletic group with 100% PHYML BS, 100% RAxML BS, and $ 95% PP, indicating that it is distinct from the *M. fimbriatispora* clade (A242 7c, 7d and G155-1a, b), which possessed 97% PHYML BS and 99% RAxML BS. Two other strains of *Minutisphaera* in clades C (G156-1) and D (G156-2) may be new taxa in that they are placed in separate groups from both *M. fimbriatispora* and *M. japonica* and each other. However, we have retained them as *Minutisphaera* spp. until additional specimens become available for further investigation.

We calculated the uncorrected p-distances with PAUP* 4.0b10 (Swofford 2002). P-distance calculates the proportion of nucleotide sites that differ between any two sequences. The p-distance can be obtained by dividing the number of nucleotide differences by the total number of nucleotides being compared. In this study as well as a previous study on freshwater Dothideomycetes (Raja et al. 2011b), we used the following criterion to delimit species based on ITS data. To be considered the same species based on ITS data, the taxa being compared should have $ 97% similarity. Among ITS sequences, the average intraspecific variation among different species of *Minutisphaera* was 1.8% whereas the average interspecific difference was 6.7% (data not shown).

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 *M. japonica* as a new and separate taxon within *Minutisphaera*. This placement also is corroborated by morphological data. The taxon from Japan therefore is described and illustrated herein as a new species.

TAXONOMY

When Ferrer et al. (2011) established the genus *Minutisphaera*, they described the ascomata of the type species, *M. fimbriatispora*, as superficial to partly immersed, brown, globose to subglobose, ostiolate, with irregular dark brown hyphae-like structures on the upper part of the ascomata. We observed that ascomata formed on the natural substrate and those formed in axenic culture (*M. japonica*) appear more apothecial at maturity (FIGS. 3–6, 22, 30, 31) than those of the original collections. The ascospores of the fungus from Japan do not possess filamentous appendages around the mid-septum. To include these newly found, additional morphological characters, we emend the genus description of *Minutisphaera* to reflect these observations. The key morphological features distinguishing the *Minutisphaera* species are summarized (TABLE III).

Minutisphaera Shearer, A.N. Mill. & Ferrer, emend

Ascomata on submerged wood, small, globose to subglobose, or apothecioid, erumpent to superficial, brown, with an ostiole and irregularly curved, dark brown to black hyphae-like structures around the ostiole. Peridium thin-walled, composed of *textura angularis* to *globosum*. Pseudoparaphyses septate, with or without enlarged pigmented tips. Asci fissitunicate, eight-spored, ovoid to obclavate, lacking a stalk, rounded at apex. Ascospores 1–2-septate, clavate, multiguttulate, hyaline becoming pale brown, with fusiform gelatinous sheath, with or without numerous filamentous appendages radiating around the mid-septum.

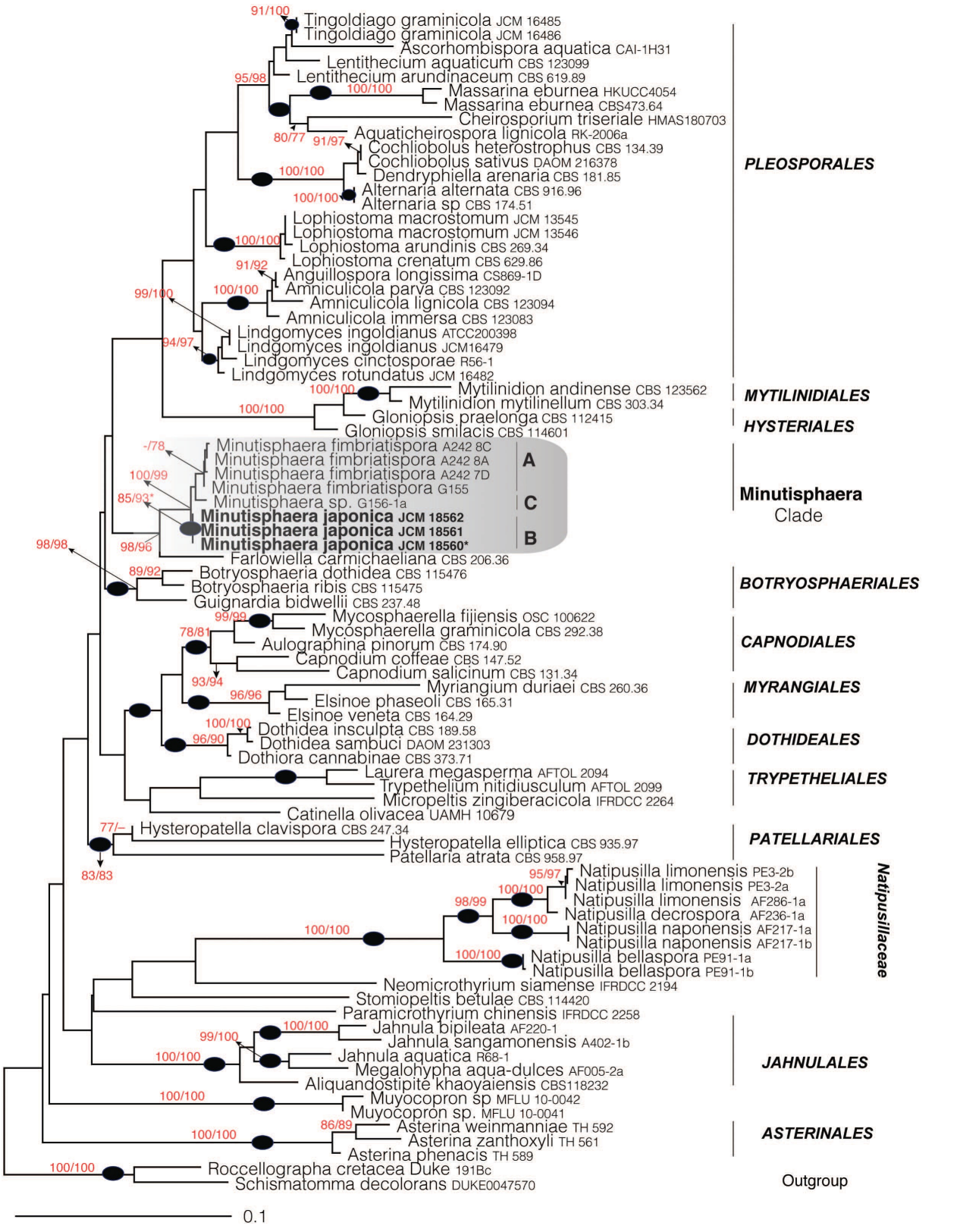


FIG. 1. Phylogram of the most likely tree (2lnL 5 20151.62) from a PHYML analysis of 83 taxa based on combined SSU and LSU nrDNA (2258 bp). Branches with a black oval indicate Bayesian posterior probabilities $ 95%; numbers refer to PhyML/RAxML bootstrap support values $ 70% based on 1000 replicates. An asterisk indicates type specimen. Bar indicates nucleotide substitution per site. Members of Arthoniomycetes were used as outgroup taxa.

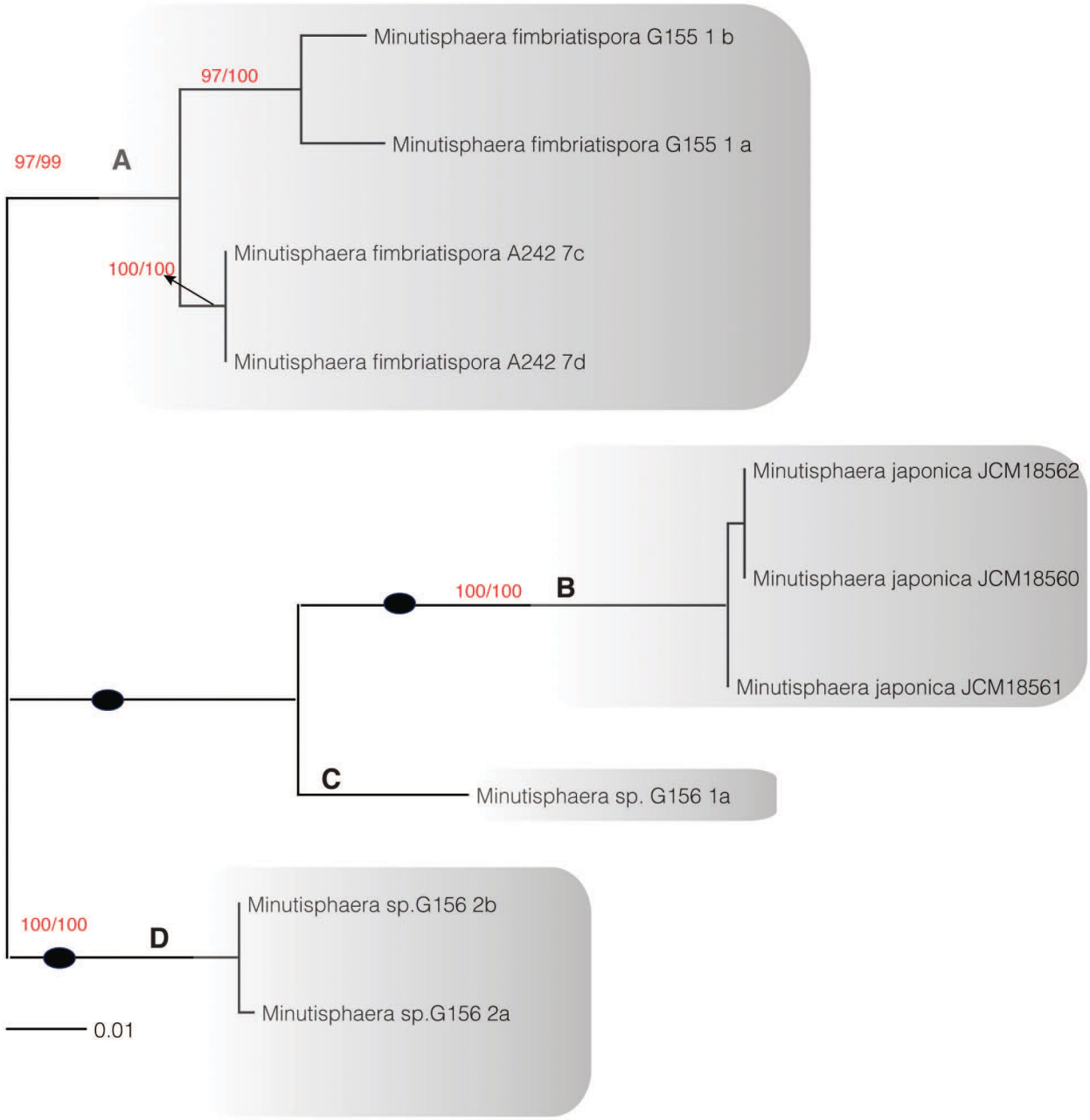


FIG. 2. Phylogram of the most likely tree (2lnL 5 1334.69) from a PHYML analysis of 10 strains of *Minutisphaera* based on ITS nrDNA (605 bp). Support values as in FIG. 1.

Type species: M. fimbriatispora Shearer, A.N. Mill. & Ferrer, Ferrer et al., Mycologia103:415, 2011.

The foregoing description is based on that provided by Ferrer et al. (2011) with these emendments: ascomata sometimes apothecioid; pseudoparaphyses with enlarged tips; ascospores hyaline becoming pale brown, 1–3-septate, with or without gelatinous sheath and appendages radiating around the mid-septum.

Minutisphaera japonica Kaz. Tanaka, Raja, & Shearer sp. nov. FIGS. 3–21.
MycoBank MB801286

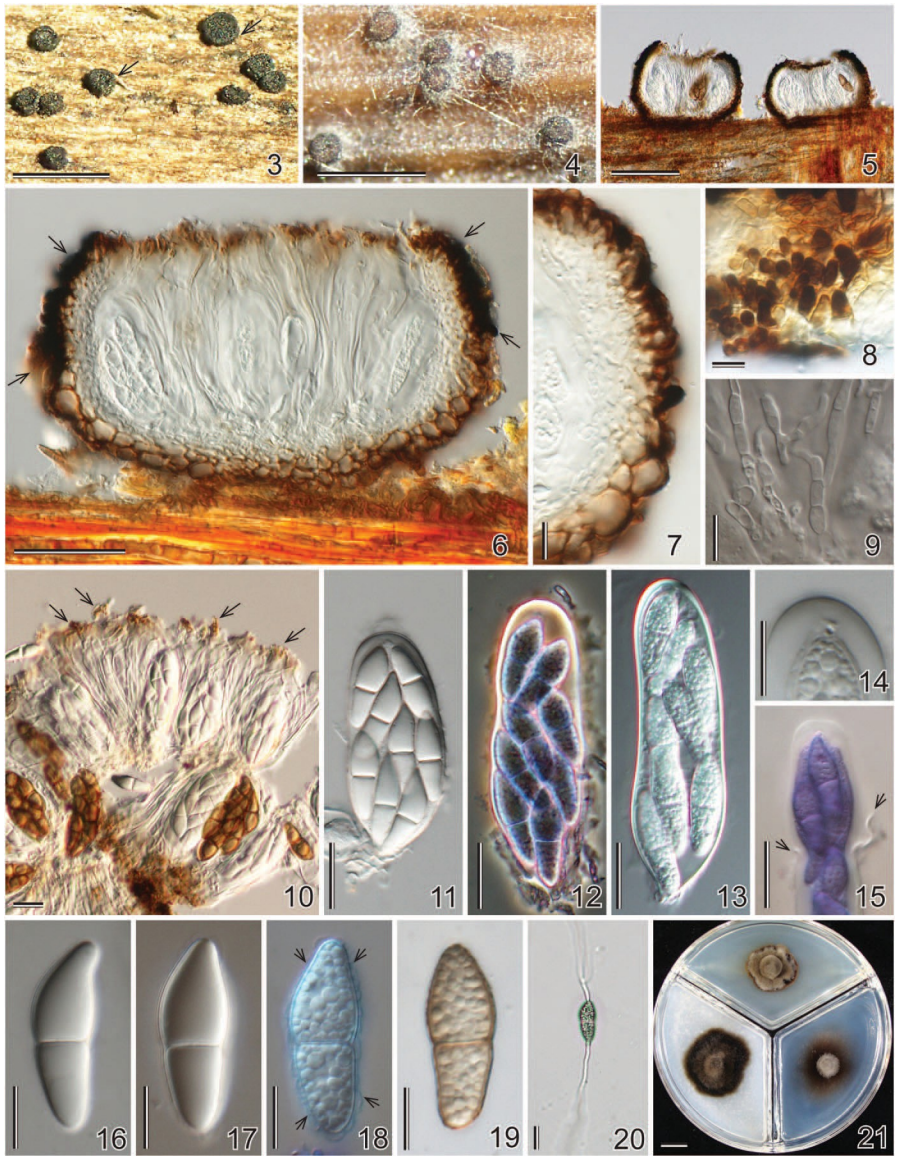
Ascomata on wood 90–130 mm high, 150–300 mm diam, superficial, scattered or in clusters of 2–3, apothecioid at maturity but not hysterithecioid, globose with a flattened top and base, dark brown to dull black, with slightly incurved margin (FIGS. 3–6). Beak absent. Ascomal wall in longitudinal section

TABLE III. Comparison of selected morphological characters among *Minutisphaera* spp.

	Minutisphaera fimbriatispora	*Minutisphaera japonica*	*Minutisphaera* sp. G156-1	*Minutisphaera* sp. G156-2
Habitat	Freshwater	Freshwater	Freshwater	Freshwater
Substrate	Woody debris	Woody debris	Woody debris	Woody debris of *Pinus* sp.
Ascomata	Black, minute; globose to subglobose, ostiolate; perithecoid or apothecioid, collabent	Brown to black, minute; apothecioid at maturity, with flattened base	Black, minute; apothecioid at maturity	Black, minute; apothecioid at maturity
Peridium	Membranous, 2-cell layers wide; with dark hyphae-like apical structures	Membranous, composed of 2 zones, inner zone of rectangular to sub-globose hyaline cells; outer zone with dark hyphae-like apical structures	Membranous; with dark hyphae-like apical structures	Membranous; with dark hyphae-like apical structures
Asci	Numerous, oblong to obclavate, broadly rounded, 8-spored 52–97 × 18–31 mm (A242-8, Type specimen) 58–88 × 18–22 mm (G155)	Obovoid to broadly cylindrical, broadly rounded, 8-spored 55–82.5 × 21.5–32.5 mm	8-spored 48–51 × 17–19 mm	Numerous, broadly clavate, rounded and thickened at the apex, 8-spored 57–70 × 15–23 mm
Ascospores	One-septate, hyaline when young becoming golden brown with age; clavate, with a supramedian septum; upper cell broader, and shorter than tapering basal cell; with gelatinous sheath and numerous filamentous appendages 24–36 × 6–8 mm (A242-8, Type specimen) 22–28 × 6–7 mm (G155)	One-septate, hyaline when young becoming brown with age; broadly fusiform, slightly curved; with a submedian primary septum, upper hemisphere broader than lower hemisphere, slightly constricted at the mid-septum, acute at the apex, rounded at the base; surrounded by an amorphous gelatinous sheath (1–3 mm thick) 25–33 × 9–11 mm	One-septate; with a supramedian septum; upper cell broader, and shorter than tapering basal cell; with gelatinous sheath and numerous filamentous appendages radiating from the mid-septum 20–23 × 5–6 mm	One-septate, hyaline when young becoming, three-septate and golden brown with age; clavate, with a supramedian septum; upper cell broader, and shorter than tapering basal cell; with gelatinous sheath and numerous filamentous appendages radiating from the mid-septum 18–25 × 5–8 mm
References	Ferrer et al. 2011, This study	This study	This study	This study

laterally 15–25 mm wide, of two zones; outer zone 7–15 mm wide of 1–3 layers of polygonal to subglobose brown cells 5–20 × 5–15 mm, with short, thick-walled, dark brown irregularly shaped hairs 7–12 × 2.5–3.5 mm around upper outer wall; inner zone 6–12.5 mm wide, of 2–3 layers of rectangular to subglobose hyaline cells 2–4 × 1.5–2 mm (FIGS. 6–8). Pseudoparaphyses 1.5–3 mm wide, 70–110 mm long, septate, branched; tips of pseudoparaphyses enlarged 3.5–4.5 mm thick and pigmented as in a pseudoepithecium (FIGS. 9, 10).

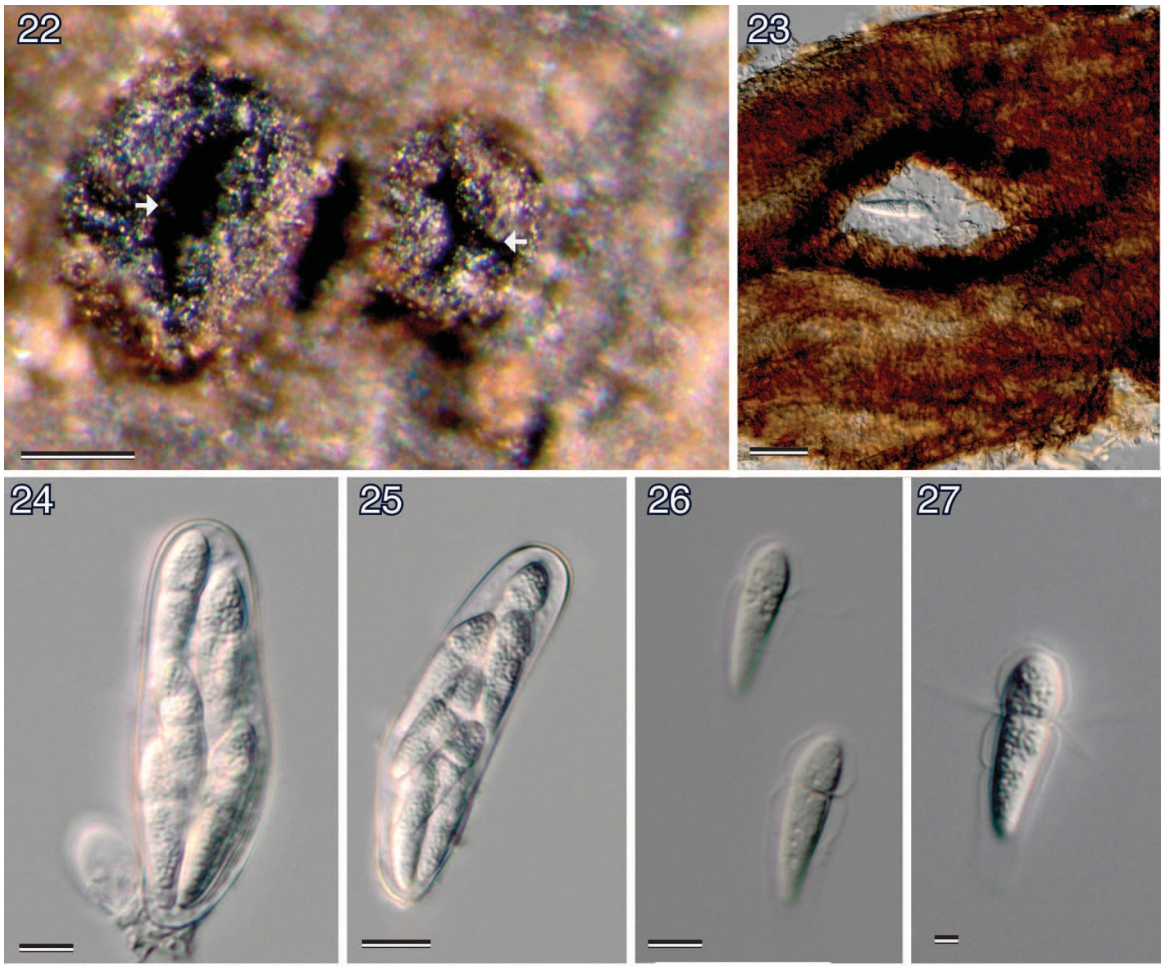
Asci 55–82.5 × 21.5–32.5 mm (av. = 66.8 × 26.5 mm, n = 60), obovoid to broadly cylindrical, fissitunicate, basal, rounded at the apex, with shallow apical chamber, sessile to short-stalked, with eight biseriate to triseriate ascospores (FIGS. 11–15). Ascospores 25–33 × 9–11 mm (average = 29.0 × 9.7 mm, n = 60), L/W 2.5–3.4 (av. = 3.0, n = 60), broadly fusiform, slightly curved, with a median to submedian primary septum (0.50–0.58; av. = 0.54, n = 60), upper hemisphere broader than lower hemi-



FIGS. 3–21. *Minutisphaera japonica*. 3. Apothecioid ascomata of HHUF 30098 on natural substratum (arrows), bar = 500 mm. 4. Ascomata of JCM 18562 in culture, bar = 500 mm. 5, 6. Ascomata in longitudinal section from HHUF 30098, arrows indicate dark hyphae-like structures on ascomatal wall, bars = 100 mm. 7. Ascomatal wall of HHUF 30098, bar = 10 mm. 8. Dark hyphae-like structures on ascomata surface from HHUF 30098, bar = 10 mm. 9. Pseudoparaphyses from JCM 18562, bar = 10 mm. 10. Pseudoparaphyses with enlarged tips (arrows) and asci from HHUF 30096, bar = 20 mm. 11–13. Asci from HHUF 30098 (11), JCM 18562 (12) and JCM 18560, (13) bar = 20 mm. 14. Apex of ascus from JCM 18560 with a shallow apical chamber, bar = 10 mm. 15. Fissitunicate ascus from JCM 18562. Arrows indicate ectoascus, bar = 20 mm. 16–19. Ascospores from HHUF 30096 (16) and HHUF 30098 (17, 19), bars = 10 mm. 18. Arrows indicate gelatinous sheath of ascospore staining with black-blue ink, from JCM 18562, bar = 10 mm. 20. Germinating ascospore of HHUF 30096, bar = 20 mm. 21. Colonies of JCM 18560 on PDA (upper), wOA (left), and CMA (right) after 25 d at 25 C in the dark, bar = 1 cm.

sphere, slightly constricted at the mid-septum, acute at the apex, rounded at the base, hyaline but becoming brown with age, smooth, with small guttules when fresh, surrounded by an amorphous gelatinous sheath (1–3 mm thick) staining with blue-black ink (FIGS. 16–19). Germinating from both ends of ascospores (FIG. 20).

Cultural characters: Colonies on PDA attaining 21–22 mm diam within 25 d at 25 C in the dark, surface velvety in appearance, grayish sepia to smoke gray (Rayner 1970), with an irregular margin. On wOA attaining 25–27 mm diam in the same conditions, fuscous black to dark mouse gray. On CMA attaining 36–39 mm diam in the same conditions, gray



FIGS. 22–27. *Minutisphaera fimbriatispora* (G155-1). 22. Apothecioid ascomata on surface, note how the ostiole opens up to expose the hymenial layer, giving it an apothecioid appearance (arrows), bar = 100 mm. 23. Squash mount of the ascomata showing dark hyphae-like structures around the ostiole, bar = 50 mm. 24. Clavate ascus, bar = 10 mm. 25. Clavate ascus, bar = 20 mm. 26, 27. Ascospores showing gelatinous sheath and filamentous appendages. 26, bar = 10 mm 27. bar = 5 mm.

olivaceous to Hazel (see FIG. 21). On RSA, apothecial ascomata are produced on the surface of rice straw within 2 mo. Asci produced in culture are relatively longer than those formed on natural substratum, but ascospores are almost identical to those found in natural collections: asci (65–)80–105(–128) × (20–)22–27.5(–29) mm (av. 93.5 × 24.9 mm, n = 53); ascospores (24–)28–36(–38) × 9.5–13 mm (av. 31.9 × 11.3 mm, n = 100), L/W 2.4–3.3 (av. 2.8, n = 100), with a submedian primary septum (0.51–0.57[–0.60]; av. 0.54, n = 100). Forcible discharge of ascospores from ascus apex observed.

Anamorph: None observed.

Habitat: On submerged wood in rivers.

Known distribution: Japan.

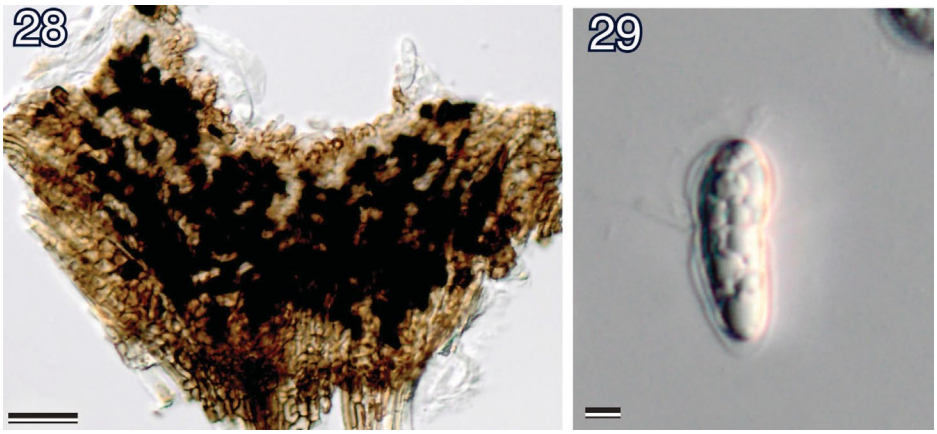
Etymology: "japonica" referring to the country where the new species was collected.

Specimens examined: JAPAN, Aomori, Hirakawa, Aseishi-river, 40.517222N, 140.7675E, on submerged wood, 2 Aug 2003, *K. Tanaka & N. Asama*, KT 1352 (HHUF 30095; single ascospore isolate JCM 18561 = MAFF 243473); Aomori, Nishimeya, Seisyu-trail, Ooshirosawa-stream, 40.547777N, 140.441944E, on submerged wood, 28 Aug 2010, *K. Tanaka*, *K. Hirayama & K. Honda*, KT 2736 (HHUF 30096; single ascospore isolate JCM 18562 = MAFF 243474); *ibid*, KT 2737 (HHUF 30097); *ibid*, KT 2738 (HHUF 30098, HOLOTYPUS designated here; single ascospore isolate JCM 18560 = MAFF 243475).

Comments: The distinctive features of *M. japonica* occur mainly in the ascospores, which are relatively wider (9–11 mm in *M. japonica* vs. up to 8 mm in *M. fimbriatispora*), constricted at the submedian primary septum (vs. supramedian), and without filamentous appendages (TABLE III).

Minutisphaera fimbriatispora Shearer, A.N. Mill. & Ferrer, Mycologia 103:415, 2011 FIGS. 22–27

Anamorph: None observed.



FIGS. 28–29. *Minutisphaera* sp. (G156-1). 28. Squash mount of the ascomata showing dark hyphae-like structures, bar = 20 mm. 29. Ascospores mounted in water with gelatinous sheath and appendages, bar = 5 mm.

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

Known distribution: USA: Illinois, North Carolina, Virginia.

Cultural characters: Colonies on PDA attaining 21 mm diam within 25 d at 25 C in light and dark, surface cotton-like in appearance with some guttation droplets, light gray.

Specimen examined: USA. North Carolina: Piedmont Plateau, Bur-Mil Park, Greensboro, Lake Brandt, 36.170556 N, 79.868333 W, on submerged decorticated wood, 20 Oct 2011, H. Raja, *G155-1*.

Comments: The specimen from North Carolina agrees well with the type description of *M. fimbriatispora* provided by Ferrer et al. (2011). The ascomata of the North Carolina collection (G155-1) were more apothecioid (FIG. 22) than those of the type collection. When the ascomata are young they appear more globose to subglobose, however, at maturity the ostiole tends to widen, and the upper surface of the ascomata becomes more collabent. The aging process subsequently exposes the centrum, giving it an apothecioid appearance.

Results of both molecular phylogenetic analyses (FIGS. 1, 2) as well as morphological examination (FIGS. 22–27) suggest that G155-1a and *M. fimbriatispora* are conspecific.

Chemistry: From the organic extract of *M. fimbriatispora* (G155-1a), two major compounds were isolated and identified as palmitic acid and (*E*)-hexadec-9-en-1-ol (SUPPLEMENTARY FIGS. 1, 2) by comparison of their NMR data with those reported previously (Dictionary of Natural Products, www.chemnetbase.com) and by using the GC-MS Metabolites Spectral Database and NIST 2008 mass spectral library (Babushok et al. 2007).

Minutisphaera sp. G156-1 FIGS. 28, 29

Anamorph: None observed.

Cultural characters: Colonies on PDA attaining 20 mm diam within 25 d at 25 C in light and dark; surface uneven and velvety, light brown to smoke gray with an irregular margin.

Habitat: On submerged wood in a swamp.

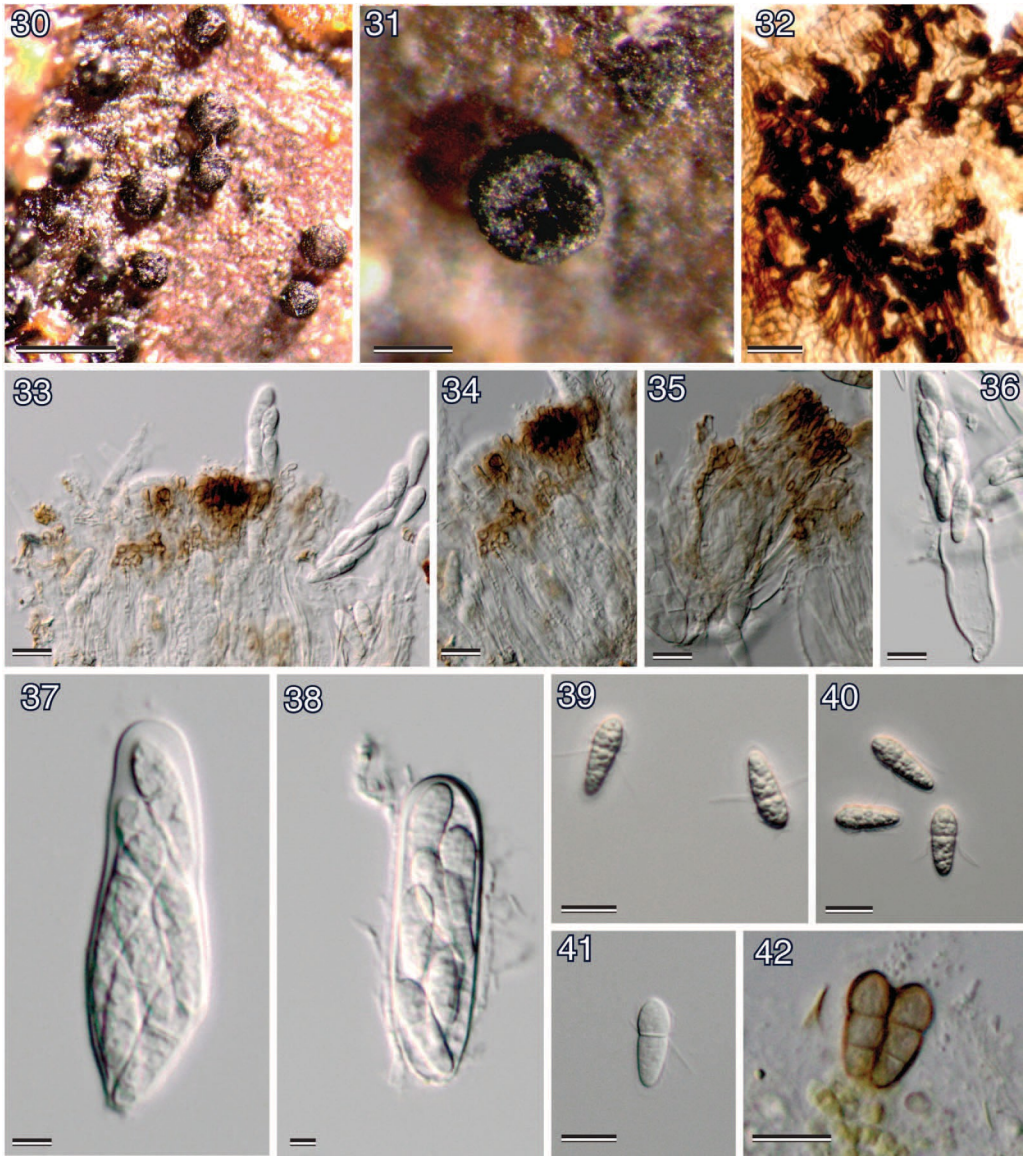
Known distribution: USA. North Carolina.

Specimen examined: USA. North Carolina: Piedmont Plateau, Bur-Mil Park, Greensboro, swampy area behind Lake Brandt, 36.167778 N, 79.868333 W, on submerged decorticated wood, 20 Oct 2011, H. Raja, *G156-1*.

Comments: The ascomata of G156-1 resemble those of *M. fimbriatispora* in having irregular dark brown hyphae-like structures on the upper part of the ascoma wall (FIG. 28). A gelatinous sheath and filamentous medial appendages extend from the ascospores (FIG. 29). Dimensions of the ascospores (20–23 × 5–6 mm) and asci (48–51 × 17–19 mm) were smaller than those of *M. fimbriatispora* (Ferrer et al. 2011) (TABLE III).

Molecular analyses of combined SSU and LSU (FIG. 1) as well as the ITS phylogeny (FIG. 2) separates G156-1a (clade C) from *M. fimbriatispora* (clade A) as well as *M. japonica* (clade B). At present we retain this fungus as *Minutisphaera* sp. because we do not have adequate material for detailed investigation. Further collections as well as molecular phylogenetic analysis of additional axenic culture isolates using ITS and/or perhaps a single-copy protein coding gene such as *MCM7*, which can provide good resolution for species-level relationships among Dothideomycete taxa (Raja et al. 2011a), might shed light on the phylogenetic relationships of this fungus.

Chemistry: From the organic extract of *Minutisphaera* sp. (G156-1), two major compounds were isolated and identified as palmitic acid and



FIGS. 30–42. *Minutisphaera* sp. (G156-2). 30. Ascomata on natural substratum, bar = 200 mm. 31. Single ascoma with collabent surface, bar = 100 mm. 32. Dark hyphae-like structure on ascomata surface, bar = 50 mm. 33. Asci with pseudoparaphyes, bar = 10 mm. 34, 35. Note pseudoparaphyses showing enlarged tips that become brown with age, bars = 10 mm. 36. Fissitunicate ascus, bar = 10 mm. 37. Ascus in glycerin, bar = 10 mm. 38. Ascus in glycerin, bar = 5 mm. 39, 40. Ascospores mounted in water showing compressed gelatinous sheath and filamentous appendages, bars = 20 mm. 41. Ascospore in glycerin with sheath and appendages, bar = 20 mm. 42. Mature, brown three-septate ascospores, bar = 20 mm.

(*E*)-hexadec-9-en-1-ol as indicated for G155-1a (see Supplementary FIGS. 1, 2).

Minutisphaera sp. G156-2 FIGS. 30–42

Ascomata on wood, 160–200 mm high, 157–193 mm diam, black, superficial, scattered or in clusters, globose with a flattened top, which becomes collabent with age, apothecioid at maturity (FIGS. 30, 31). Beak absent. Dark hyphae-like structures present on upper ascoma wall in surface view (FIG. 32). Pseudoparaphyses 50–60 mm long, sparse, interspersed tightly among asci, branched or unbranched; tips of pseudoparaphyses enlarged (1–3 mm) and pigmented brown (FIGS. 33–35). Asci 57–70 × 15–23 mm numerous, broadly clavate, bitunicate, fissitunicate, rounded and thickened at the apex, sessile to short-stalked, with eight biseriate to triseriate ascospores (FIGS. 36–38). Ascospores 18–25 × 5–8 mm (av. = 22 × 7 mm, n = 30), fusiform, multiguttulate, with a supra-median primary septum (0.31–0.41), upper hemisphere broader than lower hemisphere, slightly constricted at mid septum, rounded at the apex, tapering toward the base, hyaline,

and one-septate when young, becoming brown and three-septate with age; surrounded by a gelatinous sheath ca. 1–3 mm wide at the ascospore base; sheath tightly adhered to the sides of the ascospore, with numerous filamentous appendages separating out of the sheath in water and radiating around the ascospore septum (FIGS. 39–42). Ascospores germinating from both apices.

Anamorph: None observed.

Cultural characters: Colonies on PDA attaining 21–25 mm diam within 25 d at 25 C in light and dark, surface undulating and velvety, light brown to light gray.

Habitat: On submerged *Pinus* wood in a lake.

Known distribution: USA. North Carolina.

Specimen examined: USA North Carolina: Piedmont Plateau, Bur-Mil Park, Greensboro, Lake Brandt, 36.170556 N, 79.868333 W, on submerged wood of *Pinus* sp., 27 Mar 2012, H. Raja, *G156-2*.

Comments: Our collection of *Minutisphaera* sp. G156-2 resembles that of *M. fimbriatispora* in that it has black ascomata, the ascomata in surface view possess dark hyphae-like structures, asci are broadly clavate, and ascospores are one-septate (supra-median), multiguttulate, surrounded by a gelatinous sheath and bear numerous filamentous appendages that radiate around the mid-septum. The ascospores (18–25 × 5–8 mm) and asci (57–70 × 15–23 mm) of G156-2 however are smaller compared to those of *M. fimbriatispora* (TABLE III). On morphological grounds, we observed that G156-1 and G156-2 were quite similar in that they had smaller ascospores and asci, but there were minor size differences in their asci and ascospores (TABLE III).

Based on the ITS data, *Minutisphaera* sp. (G156-2) occurs on a separate clade (D) with 100% PHYML and RAxML BS (FIG. 2). The interspecific differences in ITS sequences of the two isolates of G156-2 differed by 4–7% among the ITS strains of *Minutisphaera* spp. included in the analysis. Therefore, it is likely that G156-2 is a distinct species from among the strains included in the analyses. At present, however, we retain G156-2 as *Minutisphaera* sp. until we obtain and examine the morphology of additional collections as well as generate ITS and/or *MCM7* sequences from different populations to examine intraspecific and interspecific relationships among strains of *Minutisphaera* spp. (G 156).

DISCUSSION

Ordinal placement of Minutisphaera *clade within the Dothideomycetes.*—BLAST analysis (Altschul et al. 1990) with other dothideomycete taxa in GenBank (Benson et al. 2012) suggest that the *Minutisphaera* fungi are close to *Farlowiella carmichaeliana*, a member of Hysteriaceae (Boehm et al. 2009a, b) or Pleosporomycetidae genera incertae sedis (Lumbsch and Huhndorf 2010). Based on phylogenetic analyses of combined SSU and LSU data, we could not determine the ordinal position of *Minutisphaera* spp. within the Dothideomycetes (FIG. 1). *Farlowiella carmichaeliana* forms a sister clade with *Minutisphaera* spp. with 98% PHYML BS and 96% RAxML BS (FIG. 1). Ferrer et al. (2011) also recovered high support for a clade including *F. carmichaeliana* and *M. fimbriatispora* in their ML analysis. Species in these genera can be distinguished easily by morphology of the ascomata (hysterothecial in *Farlowiella* vs. perithecioid or apothecioid in *Minutisphaeria*), although they share several characters, such as fissitunicate asci and one-septate ascospores.

Morphological characteristics, such as superficial apothecioid ascomata and pseudoparaphyses with enlarged tips that closely overarch the asci, were observed in *M. japonica* (FIGS. 3–21) as well as in the newly collected material of *M. fimbriatispora* and *Minutisphaera* sp. G156-2 from the USA (NC). These features are characteristic of the family Patellariaceae, Patellariales (Kutorga and Hawksworth 1997, Barr 2001).

To test the hypothesis that species of *Minutisphaera* might have phylogenetic affinities with members of the Patellariaceae within the Dothideomycetes, we included members of the Patellariaceae, such as *Patellaria atrata* (Hedw.) Fr., *Hysteropatella clavispora* (Peck) Höhn and *H. elliptica* (Fr.) Rehm (Boehm et al. 2009a), in the phylogenetic analyses of combined SSU and LSU data. We also included *Catinella olivacea*, a discomycetous fungus that grows on rotting logs. *Catinella olivacea* earlier was placed in the Leotiomycetes based on the morphology, but a more recent study by Greif et al. (2007) suggests that it is closely related to the Dothideomycetes, where it remains incertae sedis at the ordinal and familial rank. Results of the combined 83 taxa, two-gene (SSU + LSU) phylogeny suggested that the *Minutisphaera* clade did not share phylogenetic affinities with either members of Patellariaceae or *C. olivacea* (FIG. 1). This suggests that, thus far, the *Minutisphaera* clade is unique within the Dothideomycetes. Additional studies will be necessary with inclusion of taxa that belong to members of the Patellariaceae, which currently are heavily under represented in GenBank, before a new family can be proposed for this unique freshwater fungal clade.

Comparison of Minutisphaera *spp. to morphologically similar taxa.*—*Minutisphaera japonica* is similar to *Karschia lignyota* (Fr.) Sacc (Hafellner and Grazm 1976); it has flat, stalkless, olive-black ascomata,

cylindrical-clavate, melanized ascospores, asci with thickened apices, pseudoparaphyses that produce a brownish gel, and fruits on damp, rotten wood. *Minutisphaera japonica*, however, differs from *K. lignyota* in having larger asci (55–82.5 × 21.5–32.5 mm vs. 35–45 × 8–11 mm in *K. lignyota*) and ascospores (25–33 × 9–11 mm vs. 10–12 × 3–4 mm in *K. lignyota*). In addition, a gelatinous sheath surrounds ascospores of *M. japonica*, a character not reported for *K. lignyota*. The two taxa also differ in their habitat; *M. japonica* was found in submerged wood in a river in Japan, whereas, *K. lignyota* occurs in a terrestrial habitat.

Minutisphaera japonica is also morphologically similar to *Dactylospora haliotrepha* (Kohlm. & Kohlm.) Hafellner on mangrove (Hafellner 1979) in having an apothecioid ascoma, pseudoparaphyses with an enlarged tip, bitunicate asci, and one-septate brown ascospores. *Dactylospora haliotrepha* was assigned previously to *Buellia haliotrepha* Kohlm. & Kohlm. (Kohlmeyer and Kohlmeyer 1965) but later transferred to a novel genus *Kymadiscus* Kohlm. & Kohlm. The two taxa are quite different, however: *M. japonica* has ascospores that are smooth-walled and surrounded by a gelatinous sheath, whereas those of *D. haliotrepha* have delicate longitudinal septa on the episporium wall and no sheath. *Dactylospora haliotrepha* shares phylogenetic affinities with the subclass Chaetothyriomycetidae of the Eurotiomycetes (Rossman et al. 2010), whereas based on a PHYML analysis of *M. japonica* with taxa in the Chaetothyriomycetidae, *M. japonica* shows no phylogenetic affinities with *D. haliotrepha* (data not shown).

Minutisphaera fimbriatispora should be compared to *Banhegyia setispora* Zeller & Tóth, (Patellariales, Patellariaceae), which originally was described by Naoumoff (1915) for a collection on the bark of *Juniperus communis* L. in the Ural Mountains as *Celidium proximellum* (Nyl.) Karst. var. *uralensis* Naoumoff. Subsequently Zeller and Tóth (1960) collected the same fungus in the Hungarian Bükk Mountains and described it as a novel genus and species. Recent literature on *B. setispora* can be found in Kohlmeyer and Kohlmeyer (1979) and Jones et al. (2009). *Minutisphaera fimbriatispora* and *B. setispora* are morphologically similar in that they have apothecioid ascomata, clavate asci, and one-septate, hyaline to brown ascospores with appendages. They differ, however in that ascospores of *M. fimbriatispora* are surrounded by a gelatinous sheath and bear appendages radiating out of the mid-septum; those of *B. setispora* are polar. Although *B. setispora* is currently placed in the Patellariaceae, no molecular data are currently available to support its inclusion in the family or the order Patellariales.

Chemical analysis of organic extracts of both *M. fimbriatispora* (G155-1a) and *Minutisphaera* sp. (G156-1) revealed the presence of the polyunsaturated palmitic acid and (*E*)-hexadec-9-en-1-ol as the major components of the extracts. Shaw (1966) reviewed the polyunsaturated fatty acid composition of microorganisms, especially fungi, and related this to phylogeny. Stahl and Klug (1996) were able to characterize and differentiate fungi based on their fatty acid profiles. More recently, Spribille et al. (2011) used fatty acid profiles to reveal cryptic species in Mycoblastaceae, a lineage of lichenized Ascomycota. However, our chemical results suggest that the two distinctive species have similar polyunsaturated acids, but further analyses of the secondary metabolic content from additional strains will be required to define the variation in chemical profiles across the *Minutisphaera* clade.

The *Minutisphaera* clade (F_{IG}. 1), which consists entirely of taxa described and reported from freshwater, remains unique within the Dothideomycetes, although morphological characteristics within this clade suggest that they may share putative phylogenetic affinities with members of the Patellariales (such as *Banhegyia setispora* and *Karschia lignyota*) for which sequence data are not currently available in GenBank.

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