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Minutisphaera aquaticum sp. nov. increases the known diversity of Minutisphaeraceae

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Abstract

New species of freshwater fungi are constantly being introduced following our studies in Asia. In the present paper, *Minutisphaera aquaticum* sp. nov., is introduced from submerged wood collected in the Mekong River in eastern Thailand, and increases the known diversity of Minutisphaeraceae. *Minutisphaera aquaticum* is characterized by superficial, small globose, dark brown to black ascomata, bitunicate, fissitunicate, obovoid to broadly cylindrical asci and fusiform, hyaline ascospores with a supra-median primary septum and upper cells that are wider than the lower cells. The multigene phylogenetic analysis places the new taxon in a well-supported clade with the species in Minutisphaeraceae (Minutisphaerales). The new species is compared with other *Minutisphaera* species, description and illustration are provided.

Key words – 1 new species – Minutisphaerales – molecular – phylogeny – taxonomy

Introduction

Minutisphaerales, an order of freshwater ascomycetes within the class Dothideomycetes, was recently established by Raja et al. (2015) to accommodate the monotypic genus *Minutisphaera*. Minutisphaerales currently comprises one family, Minutisphaeraceae, with four species and were reported from freshwater habitats in Japan and the USA.

The genus *Minutisphaera* was introduced by Ferrer et al. (2010) with a single species *M. fimbriatispora* in the class Dothideomycetes. Members of this genus are characterized by small, globose to subglobose or apothecioid, erumpent to superficial, brown ascomata, fissitunicate, eight-spored, ovoid to obclavate asci, and 1–2-septate, clavate to broadly fusiform, hyaline to pale brown ascospores, with or without a gelatinous sheath and filamentous appendages. Three additional species, *viz. M. japonica*, *M. fimbriatispora* and *M. aspera*, were later added (Raja et al. 2013, 2015).

In a recent study, lignicolous freshwater fungi were studied along the north-south gradient in the Asian/Australian regions (Hyde et al. 2016). The new taxon, *Minutisphaera aquaticum* is described, illustrated and compared with similar taxa in this article. Phylogenetic analyses of combined LSU, SSU, and ITS sequence data provide evidence for the new species and confirms its placement in *Minutisphaera*.

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Materials & Methods

Collection and examination of specimens

Specimens of submerged decaying wood were collected from Mekong River in Nakhon Phanom province, Thailand. Specimens were brought to the laboratory in plastic bags and incubated in plastic boxes lined with moistened tissue paper at room temperature for one week. Sample examination and morphological studies followed the protocols outlined previously (Luo et al. 2018). Single spore isolations were made on to potato dextrose agar (PDA) and later transferred on to fresh PDA following the method of Chomnunti et al. (2014). Specimens (dry wood with fungal material) are deposited in the herbarium of Mae Fah Luang University (MFLU), Chiang Rai, Thailand. Axenic cultures are deposited in Mae Fah Luang University Culture Collection (MFLUCC). Faces of Fungi and Index Fungorum numbers are registered as outlined in Jayasiri et al. (2015) and Index Fungorum (2018).

DNA extraction, PCR amplification and sequencing

Isolates were grown on PDA medium at 25 °C for one month. Fungal mycelium was scraped off and transferred to a 1.5 ml microcentrifuge tubes using a sterilized lancet for genomic DNA extraction. Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech, China) was used to extract DNA following the manufacturer's instructions. The internal transcribed spacer (ITS), large-subunit rRNA (LSU) and small-subunit ribosomal RNA (SSU) gene regions were amplified using the primer pairs ITS5/ITS4 (Vilgalys & Hester 1990), LROR/LR7 and NS1/ NS4 (White et al. 1990). The amplification was performed in a 25 µl reaction volume containing 9.5 µl ddH_2O, 12.5 µl 2 × Taq PCR Master Mix with blue dye (Sangon Biotech, China), 1 µl of DNA template and 1 µl of each primer (10 µM). The amplification condition for ITS, LSU and SSU consisted of initial denaturation at 94 °C for 3 min; followed by 40 cycles of 45 s at 94 °C, 50 s at 56 °C and 1 min at 72 °C and a final extension period of 10 min at 72 °C. Purification and sequencing of PCR products were carried out using the above-mentioned PCR primers at Sangon Biotech (Shanghai) Co. Ltd. in China.

Phylogenetic analyses

The taxa included in the phylogenetic analyses were selected and obtained from previous studies (Raja et al. 2015, Liu et al. 2017) and GenBank (Table 1). Three gene regions (ITS, LSU and SSU) were used for the combined sequence data analyses. SEQMAN v. 7.0.0 (DNASTAR, Madison, WI) was used to assemble consensus sequences. The sequences were aligned using the online multiple alignment program MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013). The alignments were checked visually and improved using BioEdit.

Maximum likelihood (ML) analysis was performed using RAxML-HPC v.8 (Stamatakis 2006, Stamatakis et al. 2008) on the XSEDE Teragrid of the CIPRES Science Gateway (https://www.phylo.org) (Miller et al. 2010) with rapid bootstrap analysis, followed by 1000 bootstrap replicates. The final tree was selected amongst suboptimal trees from each run by comparing likelihood scores under the GTRGAMMA substitution model.

Maximum parsimony (MP) analyses were performed with PAUP v. 4.0b10 (Swofford 2003) using the heuristic search option with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Clade stability was assessed using a bootstrap (BT) analysis with 1000 replicates, each with 10 replicates of random stepwise addition of taxa (Hillis & Bull 1993).

The program MRMODELTEST2 v. 2.3 (Nylander 2004) was used to infer the appropriate substitution model that would best fit the model of DNA evolution for the combined datasets for Bayesian inference analysis with GTR+G+I substitution model selected. Posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain

Monte Carlo sampling (MCMC) in MRBAYES v. 3.0b4 (Huelsenbeck & Ronquist 2001). Six simultaneous Markov chains were run for 1 million generations, with trees sampled every 100 generations (resulting in 10000 trees). The first 2000 trees, representing the burn-in phase of the analyses were discarded and the remaining 8000 trees were used for calculating posterior probabilities (PP) in the majority rule consensus tree (Larget & Simon 1999).

Phylogenetic trees were represented by FigTree v. 1.4.0 (Rambaut 2012) and edited in Microsoft Office PowerPoint 2016 (Microsoft Inc., United States). Newly generated sequences in this study were deposited in GenBank (Table 1) and the final matrices used for the phylogenetic analyses were submitted to TreeBASE (www.treebase.org; accession number: 25572).

Table 1 GenBank and culture collection accession numbers of species included in the phylogenetic study. The newly generated sequences are shown in bold.

Taxa	**Strain no.**	**GenBank accession numbers**		
		ITS	**LSU**	**SSU**
Aliquandostipite khaoyaiensis	CBS 118232	JN942357	GU301796	AF201453
A. siamensiae	SS 81.02	–	EF175666	EF175645
Asterina cestricola	TH 591	–	GU586215	GU586209
A. fuchsiae	TH 590	–	GU586216	GU586210
A. phenacis	TH 589	–	GU586217	GU586211
A. weinmanniae	TH 592	–	GU586218	GU586212
A. zanthoxyli	TH 561	–	GU586219	GU586213
Asterotexis cucurbitacearum	VIC 24814	–	KP143734	–
A. cucurbitacearum	PMA M 0141224	–	HQ610510	–
Cenococcum geophilum	CG54	KC967410	JN860134	JN860120
Delitschia chaetomioides	SMH 3253.2	–	GU390656	–
D. winteri	CBS 225.62	–	DQ678077	DQ678026
Gloniopsis praelonga	CBS 112415	EU552133	FJ161173	FJ161134
Glonium stellatum	CBS 207.34	–	FJ161179	FJ161140
Hysterium angustatum	CBS 236.34	KX611363	FJ161180	GU397359
Hysterobrevium smilacis	CBS 114601	–	FJ161174	FJ161135
Jahnula aquatica	R 68 -1	JN942354	EF175655	EF175633
Manglicola guatemalensis	BCC 20156	JN819283	FJ743448	FJ743442
M. guatemalensis	BCC 20079	JN819282	FJ743449	FJ743443
Massaria anomia	CBS 591.78	HQ599380	GU301839	GU296169
M. gigantispora	M 26	HQ599399	HQ599397	HQ599447
M. inquinans	M 19	MH875187	HQ599402	HQ599444
Minutisphaera parafimbriatispora	G156-4a	KP309991	KP309996	KP310002
Minutisphaera aquaticum	**MFLUCC 19–0497**	**MN85718**	**MN857176**	**–**
M. parafimbriatispora	G156-4b	KP309992	KP309997	KP310003
M. aspera	G427-1a	KP309989	MH878174	KP309999
M. aspera	G427-1b	KP309990	NG060319	KP310000
M. fimbriatispora	A242-7d	JX474872	HM196366	HM196373
M. fimbriatispora	G155-1a	JX474874	JX474859	JX474865
M. japonica	JCM 18562	AB733436	AB733439	AB733433
M. japonica	JCM 18560b	NR119419	AB733440	AB733434

Table 1 Continued.

Taxa	Strain no.	GenBank accession numbers		
		ITS	LSU	SSU
Minutisphaera sp.	G156-1a	JX474875	–	–
Minutisphaera sp.	G156-2a	JX474876	–	–
Minutisphaera sp.	G156-2b	JX474877	–	–
Myrmaecium rubricosum	CBS 139067	MG708367	KP687881	KP687977
M. rubrum	CBS 109505	MH862829	GU456324	GU456303
Mytilinidion acicola	EB O349	–	GU323209	GU323185
M. andinense	CBS 123562	–	FJ161199	FJ161159
M. mytilinellum	CBS 303.34	HM163570	FJ161184	FJ161144
Oedohysterium insidens	CBS 238.34	–	FJ161182	FJ161142
Psiloglonium araucanum	CBS 112412	–	FJ161172	FJ161133

Results

Phylogenetic analyses

The aligned sequence matrix comprises LSU (1321 bp), SSU (1167) and ITS (486 bp) sequence data for 41 taxa from six orders (Asterinales, Hysteriales, Jahnulales, Minutissphaerales, Mytilindiales and Pleosporales) including two outgroup taxa *Myrmaecium rubricosum* (CBS 139067) and *Myrmaecium rubrum* (CBS 109505). The combined gene analysis comprising 2973 characters after alignment (including gaps), of which 267 were parsimony-informative, 675 were parsimony-uninformative and 2121 characters were constant. The RAxML analysis of the combined dataset yielded the best scoring tree (Fig. 1) with a final ML optimization likelihood value of -14936.216419. The matrix had 975 distinct alignment patterns, with 26.56 % undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.257773, C = 0.221588, G = 0.281573, T = 0.239066; substitution rates AC = 1.252928, AG = 3.119769, AT = 1.187559, CG = 1.297978, CT = 8.312917, GT = 1.000000; gamma distribution shape parameter α = 0.004255. RAxML, MP and Bayesian analyses of the combined dataset resulted in phylogenetic reconstructions with largely similar topologies and the RAxML tree is shown in Fig. 1. Bootstrap support values for RAxML and MP greater than 75% and Bayesian posterior probabilities greater than 0.95 are given at each node (Fig. 1).

In the phylogenetic analyses, all the strains of *Minutisphaera* clustered together with strong support (100 ML/, 100/MP and 1.00 PP). The novel species *Minutisphaera aquaticum* clustered with members of *Minutisphaera*, but in a distinct lineage with good bootstrap support (88 % MLBS/ 75 % MPBS and 1.00 BYPP, Fig. 1).

Taxonomy

Minutisphaera aquaticum D.F. Bao, Z.L. Luo, K.D. Hyde & H.Y. Su, sp. nov. Fig. 2

Index Fungorum number: IF557049; Facesoffungi number: FoF 07084

Etymology – Referring to the aquatic habitat from which the fungus was collected.

Holotype – MFLU 19–2846

Saprobic on decaying wood, submerged in freshwater habitats. Sexual morph: Ascomata 71–90 × 85–98 µm ($\overline{x}$ = 80.5 × 91 µm, n = 5) diam, superficial, scattered, globose, dark brown to black. Ostiole absent. *Peridium* 12–17 µm wide, comprises two layers, outer layers composed of dark brown cells of *textura angularis*, inner layer composed rectangular to subglobose hyaline cells. *Hamathecium* composed of 1.2–2.0 µm wide, cellular pseudoparaphyses, sparse in young ascomata, becoming abundant with age. *Asci* 52–64 × 21.5–27.5 µm ($\overline{x}$ = 58 × 23 µm, n = 20), 8-spored, bitunicate, fissitunicate, obovoid to broadly cylindrical, sessile to short pedicellate, without ocular chamber. *Ascospores* 28–32 × 7.6–8.7 µm ($\overline{x}$ = 30 × 8 µm, n = 30), bi-seriate, hyaline,

fusiform to clavate, straight, curved at both of ends, with a supra-median primary septum, constrict at the septum, upper cell wider and shorter than lower cell, without or with 1-2 large guttules when young, multi-guttulate when mature, smooth-walled, with or without sheath and appendages. Asexual morph: Undetermined.

Material examined – THAILAND, That Phanom, Nakhon Phanom province, on submerged decaying wood in the Mekong River, 13 November 2018, D.F. Bao, B-163 (MFLU 19–2846, holotype), ex-type culture, MFLUCC 19–0497.

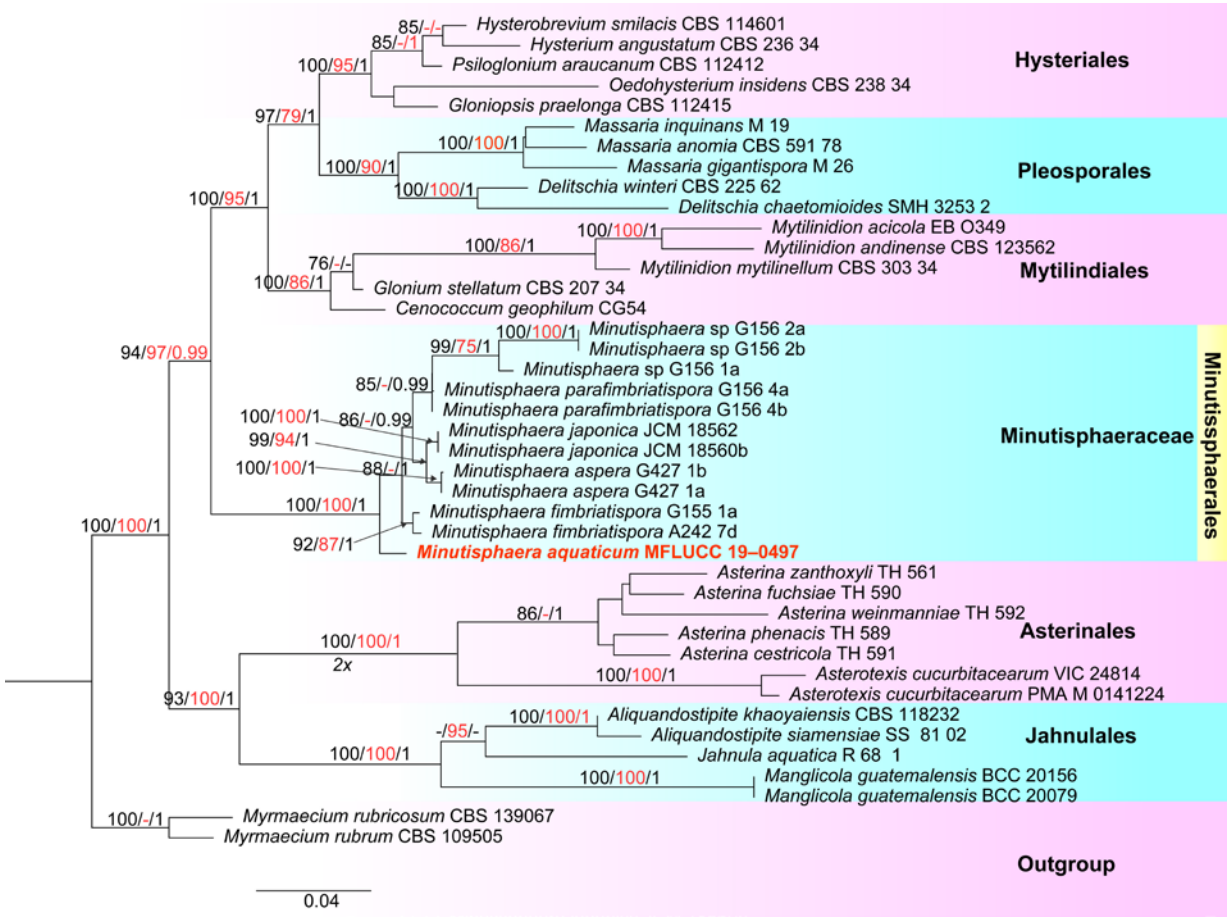

Fig. 1 – Phylogenetic tree based on RAxML analyses of a combined LSU, SSU and ITS dataset. Bootstrap support values for maximum likelihood (ML, black) and maximum parsimony (MP, blue) higher than 75% and Bayesian posterior probabilities (BYPP, red) greater than 0.95 are indicated above the nodes as MPBS / MLBS /PP. The tree is rooted with *Myrmaecium rubricosum* (CBS 139067) and *Myrmaecium rubrum* (CBS 109505). The new isolate is in bold and red.

Discussion

In this study, we introduce a new species, *Minutisphaera aquaticum* with morphological and phylogenetic evidences. *Minutisphaera aquaticum* is most similar to *M. fimbriatispora* and *M. parafimbriatispora* in having superficial, globose ascomata, obovoid to broadly cylindrical, sessile to short pedicellate asci and fusiform ascospores with a supramedian primary septum. *Minutisphaera fimbriatispora* and *M. parafimbriatispora* share similar morphological characters and, Raja et al. (2015) distinguished these two species based on the size of asci and ascospores. *Minutisphaera aquaticum* can be distinguished from these two species by the size of ascomata, asci and ascospores (Table 2). While ascospores of *M. fimbriatispora* and *M. parafimbriatispora* have both sheaths and appendages, ascospores of *M. aquaticum* lacks appendages.

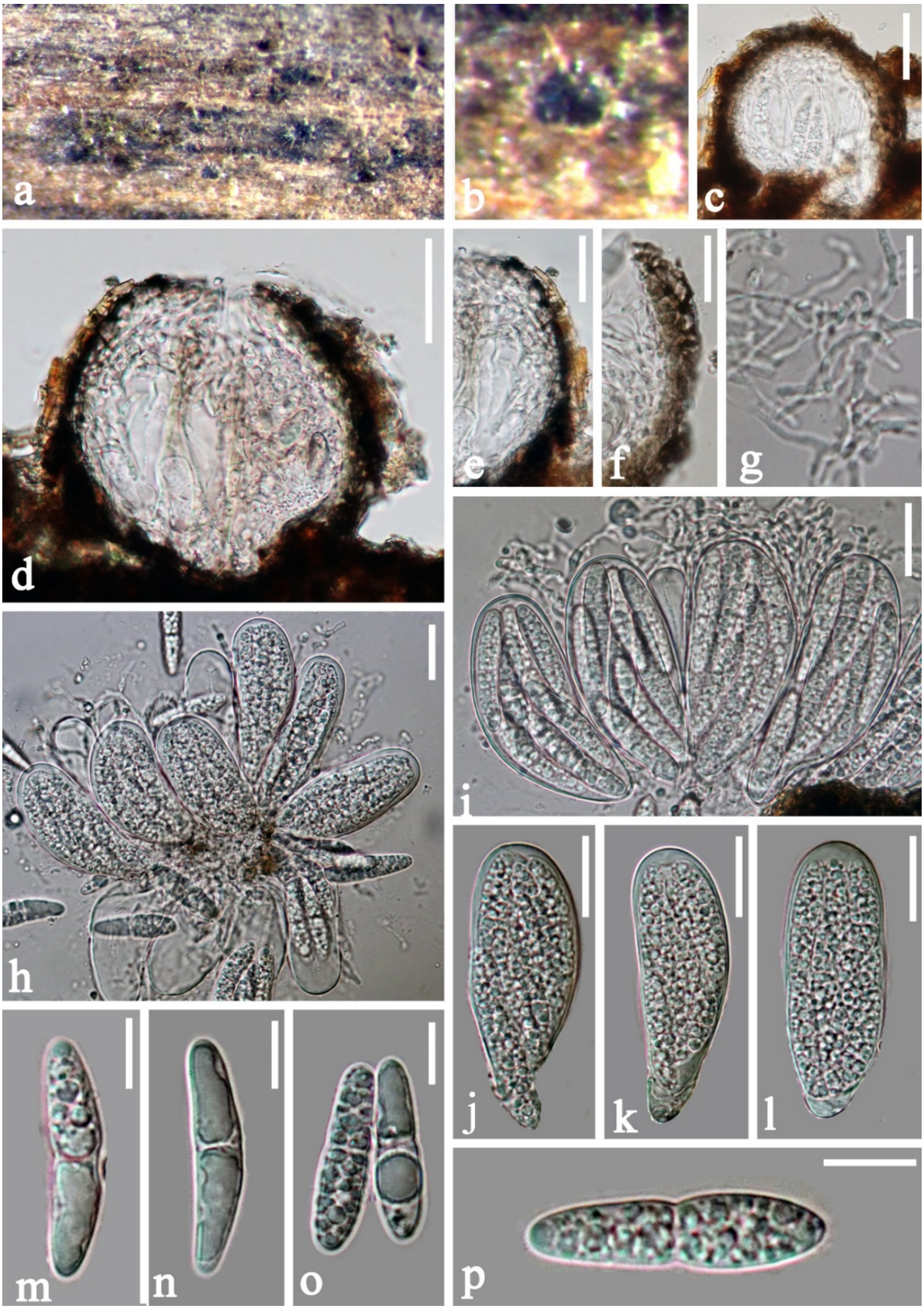

Fig. 2 – *Minutisphaera aquaticum* (MFLU 19–2846, holotype). a, b Ascomata on submerged wood. c, d Sections of ascoma. e, f Peridium. g Pseudoparaphyses. h–l Asci. m–p Ascospores. Scale bars: c, d = 30 μm, e–l = 20 μm, m–p= 10 μm.

Minutisphaera aquaticum can be distinguished from *M. aspera* and *M. japonica* by its ascospores in having hyaline, fusiform to clavate, smooth-walled ascospores with curved ends.

Ascospores of *M. aspera* are broadly fusiform when young, becoming ellipsoidal with age, rough-walled and dark brown when mature. In addition, *Minutisphaera aquaticum* differs from *M. japonica* in having fusiform to clavate, straight ascospores with curved ends. However, ascospores of *M. japonica* are broadly fusiform, slightly curved, acute at the apex and rounded at the base.

In the phylogenetic study by Raja et al. (2015), *Minutisphaera* formed a monophyletic clade in the Dothideomycetes and did not share any relationship with the order of Dothideomycetes. They, therefore, introduced a new order Minutisphaerales to accommodate *Minutisphaera* species. In our phylogenetic study, *Minutisphaera* species formed a distinct clade, sister to Mytilinidiales with strong bootstrap support (100 % MLBS, 95 % MPBS and 1.00 BYPP). This result is similar to the previous studies and supports the placement of Minutisphaerales (Raja et al. 2015).

Lignicolous freshwater fungi have the ability to decay submerged, waterlogged woody debris, (Yuen et al. 1998, Bucher et al. 2004). Members of this family probably decompose lignocellulose in the woody litter, softening the wood, and enhanced nutrient cycling (Wong et al. 1988). Species of *Minutisphaera* are saprobic and have only been reported from submerged wood in freshwater habitats (Ferrer et al. 2011, Raja et al. 2013, 2015). Our study also indicates that *Minutisphaera* species are restricted to freshwater ecosystems and shows the genus to be diverse. Thus, further studies in different regions should also reveal novel species. Morphologically, all the species of this genus have sheaths or having both sheaths and appendages. The formation of sheath and appendages may be related to the habitat (Moss 1990, Jones 1995, Shearer 1993, 2001), where they enhanced the ascospores in attaching to substrates in moving water and aid in dispersal (Shearer 1993, Hyde & Goh 2003, Jones 2006).

Table 2 Size of ascomata, asci and ascospores comparisons of *Minutisphaera* species in this study

Taxa	Ascomata (µm)	Asci (µm)	Ascospores (µm)
Minutisphaera fimbriatispora	110–120 × 120–150	52–97 × 18–31	24–36 × 6–8
M. parafimbriatispora	160–170 ×170–180	48–72 × 15–22	18–25 × 4–7
M. aquaticum	71–90 × 85–98	52–64 × 21.5–27.5	28–32 × 7.6–8.7
M. japonica	90–130 × 150–300	55–82.5 × 21.5–32.5	25–33 × 9–11
M. aspera	235–480 diam	5–80 × 23–42	24–33 × 9–14

Key to species of *Minutisphaera*

1. Ascospores with appendages **2**
1. Ascospores lacking appendages **3**
2. Ascospores18–25 × 4–7 µm ..... ***Minutisphaera parafimbriatispora***
2. Ascospores 24–36 × 6–8 µm .....***Minutisphaera fimbriatispora***
3. Ascospores with verruculose wall and thicker septate when mature .... ***Minutisphaera aspera***
3. Ascospores with smooth wall **4**
4. Ascospores, broadly fusiform, slightly curved, acute at the apex, rounded at the base ....***Minutisphaera japonica***
4. Ascospores fusiform to clavate, straight, smooth walled, with curved ends..... .....***Minutisphaera aquaticum***

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