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The genus *Neoaquastroma* is widely distributed; a taxonomic novelty, *N. cylindricum* sp. nov. (Parabambusicolaceae, Pleosporales) from Guizhou, China

Samarakoon MC[1,2,3,4], Wanasinghe DN[5,6], Liu JK[2,3], Hyde KD[1,5]* and Promputtha I[4,7]

[1] Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand
[2] School of Life Science and Technology, University of Electronic Science and Technology of China, Chengdu 611731, P.R. China
[3]Guizhou Key Laboratory of Agricultural Biotechnology, Guizhou Academy of Agricultural Sciences, Guiyang 550006, P.R. China
[4] Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
[5] Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, 132, Lanhei Road, Kunming 650201, P.R. China
[6] World Agro Forestry Centre, East and Central Asia, 132, Lanhei Road, Kunming 650201, P.R. China
[7]Center of Excellence in Bioresources for Agriculture, Industry and Medicine, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

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Abstract

Neoaquastroma is a saprobe on dead twigs, characterized by having immersed, globose to sub-globose ascomata, short papillate ostiole, a thin peridium with cells of *textura angularis*, branched, septate pseudoparaphyses, cylindrical clavate, short pedicellate asci with an ocular chamber, overlapping biseriate to triseriate, ellipsoidal to sub-fusiform, hyaline, multi-septate ascospores with a distinct mucilaginous sheath. They have a coelomycetous asexual morph with uniloculate, pycnidial conidiomata, enteroblastic, phialidic, integrated, hyaline, oblong conidiogenous cells, broad-oblong to oval, hyaline, aseptate conidia. Earlier they were only reported from Thailand. In this study, we found another new species to the genus (*Neoaquastroma cylindricum*) from Guizhou, China as the fourth species based on both morpho-phylo evidences. *Neoaquastroma cylindricum* is distinguished from other *Neoaquastroma* species by its cylindric-clavate asci and comparatively small ascospores with a smaller number of transverse septa. Maximum parsimony, maximum likelihood and Bayesian inference analyses of combined LSU–SSU–ITS–*tef1* sequence matrix confirmed its placement within Parabambusicolaceae with a close affinity to *Neoaquastroma krabiense*. The new taxon is compared with other species in *Neoaquastroma* and comprehensive descriptions, illustrations (including DNA based phylogenies) and a synopsis table for relevant species are provided.

Keywords – 1 new species – Dothideomycetes – multi-gene – phylogeny – taxonomy

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Corresponding Author: Kevin D. Hyde – e-mail – kdhyde3@gmail.com

Introduction

Exploration of novel fungal species and placing them in their higher ranks is at a rapid rate during the last few decades mainly due to the advancement of molecular techniques. Those novel discoveries are dominated by phylum Ascomycota Caval.-Sm. with 68 % of the total novel species in 2017 (Willis 2018). Among the ascomycetous classes, Dothideomycetes O.E. Erikss. & Winka is highly diversified and the highest numbers of taxa are accounted (Liu et al. 2017, Wijayawardene et al. 2017, 2018). Pleosporales Luttrell ex M.E. Barr is one of the orders in Dothideomycetes, which is cosmopolitan and hosting a large number of novel species and higher ranks with a high species diversity (Hyde et al. 2016, 2017, 2018, 2019, Crous et al. 2018, 2019, Wanasinghe et al. 2018, Phookamsak et al. 2019).

Parabambusicolaceae Kaz. Tanaka & K. Hiray. (Massarineae; Pleosporales) was introduced by Tanaka et al. (2015) to accommodate the genera *Aquastroma* Kaz. Tanaka & K. Hiray., *Parabambusicola* Kaz. Tanaka & K. Hiray., *Multiseptospora* Phook. & K.D. Hyde and two unnamed *Monodictys* S. Hughes species. The family is characterized by pseudothecioid ascomata with or without stromatic tissues, papillate to apapillate ostioles, clavate to fusiform asci and hyaline or brown phragmospores bearing sexual morph and sporodochial or monodictys-like asexual morph (Ariyawansa et al. 2015, Liu et al. 2015, Tanaka et al. 2015, Li et al. 2016, Wanasinghe et al. 2017a, 2017b, Phukhamsakda et al. 2018). At present, there are six accepted genera in the Parabambusicolaceae *viz*. *Aquastroma*, *Multilocularia* Phook. et al., *Multiseptospora*, *Neoaquastroma* Wanas. et al., *Parabambusicola* and *Pseudomonodictys* Doilom et al. (Wijayawardene et al. 2017, 2018, Phukhamsakda et al. 2018).

Wanasinghe et al. (2017a) introduced the genus *Neoaquastroma* typified by *N. guttulatum* Wanas. et al., which was collected on a dead twig from Thailand. The genus is distinguished from the other related genera by its fully immersed pseudothecia and in well-supported multi-gene based phylogenies (Phukhamsakda et al. 2018). Recently, Phukhamsakda et al. (2018) introduced two new species *viz*. *N. bauhiniae* C. Phukhams. & K.D. Hyde and *N. krabiense* C. Phukhams. & K.D. Hyde from Thailand while increasing the total number of *Neoaquastroma* species up to three. This study provides the morpho-molecular evidence of the new *Neoaquastroma* species, *N. cylindricum* collected from Guizhou, China as the first record outside Thailand.

Materials & Methods

Collection, isolation and morphological studies

Dead twigs of different landscaping plants were collected from Guizhou Academy of Agricultural Sciences (GZAAS) premises, Guiyang, Guizhou, China during May–August 2018. The specimens were placed into paper bags and dried under room temperature for two days. External examinations were made through the stereomicroscope (SteREO Discovery v8) attached with Axio Cam ERc5s while microscopic photography was made by using Nikon ECLIPSE Ni-U compound microscope (Nikon, Tokyo, Japan) attached with Canon EOS 600D camera (Canon Inc., Tokyo, Japan). Measurements were made with the Tarosoft (R) Image Frame Work program and images used for figures were processed with Adobe Photoshop CS6 software (Adobe Systems Inc).

Pure cultures were obtained from single ascospore isolation on potato dextrose agar (PDA) in double-distilled water following Chomnunti et al. (2014) and Samarakoon et al. (2018). Germinated ascospores were transferred on PDA aseptically and incubated at 25–30 °C for 4–6 weeks with frequent observations. The type specimens were deposited in the Mae Fah Luang University Herbarium (MFLU), Chiang Rai, Thailand and the Herbarium of Cryptogams Kunming Institute of Botany Academia Sinica (HKAS), Chinese Academy of Sciences, Kunming, China. The ex-type culture was deposited in the Culture Collection at Mae Fah Luang University (MFLUCC). The new taxon was linked with Facesoffungi and Index Fungorum databases as explained in Jayasiri et al. (2015) and Index Fungorum (http://www.indexfungorum.org).

DNA extraction, PCR amplification and sequencing

DNA was extracted from fresh mycelium by using total DNA extraction kits according to the manufacturer's instructions (Sangon Biotech (Shanghai) Co. Ltd. China). Primers ITS5/ITS4, NS1/NS4 (White et al. 1990), LR0R/LR5 (Vilgalys & Hester 1990), EF1-983F/EF1-2218R (Carbone & Kohn 1999) and Bt2a/Bt2b (Glass & Donaldson 1995) were used to amplify the DNA sequences of the internal transcribed spacers (ITS1-5.8S-ITS2), the partial 18S small subunit rDNA (SSU), the partial 28S large subunit rDNA (LSU), partial translation elongation factor-1α (*tef1*) and partial *β*-tubulin (*tub2*) respectively. The total volume of 25 µl containing 12.5 µl of 2× PCR Master Mix with dye (0.1 U Taq Polymerase/µl, 500 µM dNTP each, 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM MgCl2), 1 µl of each primer, 9.5 µl of double-distilled water and 1µl (100–500 ng) of DNA template.

PCR thermal cycler programs for all gene regions were programmed with an initial denaturation at 94 °C for 3 min and a final extension at 72 °C for 10 min. ITS, LSU and *tub2* gene amplifications were followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 50 seconds and extension at 72 °C for 1 min. The PCR thermal cycles for SSU and *tef1* were followed by 35 cycles of denaturation at 95 °C for 45 seconds, annealing at 56 °C for 50 seconds and extension at 72 °C for 1 min. All the PCR products were immediately subjected to 4 °C and were visualized on 1 % agarose gel stained using Ethidium Bromide with D2000 DNA ladder (Realtimes Biotech, Beijing, China). PCR products were purified according to the company protocols and DNA sequencing was performed using the same primers in an Applied Biosystem 3730 DNA analyzer at Sangon Biotech (Shanghai) Co. Ltd., China.

Phylogenetic analyses

Verified sequences were used for BLAST analyses and related sequences were downloaded from the GenBank (https://www.ncbi.nlm.nih.gov) following recent relevant publications (Wanasinghe et al. 2017a, Phukhamsakda et al. 2018) (Table 1). Individual loci were aligned using FFT-NS-2 Tree-based progressive method, 20PAM/ k=2 Scoring matrix for nucleotide sequences and 1.0 Gap opening penalty settings of MAFFT V.7.036 (http://mafft.cbrc.jp/alignment/server/) (Katoh et al. 2017) and improved when necessary in BioEdit v. 7.0 (Hall 2004) by manual adjustments. Characters were assessed to be unordered and equally weighted. MrModeltest 2.3 was performed for every single gene to estimate the best-fit evolutionary models under the Akaike Information Criterion (AIC) (Nylander 2004) and each resulted GTR+I+G model. Phylogenies were generated using maximum parsimony (MP), maximum-likelihood (ML) and Bayesian inference (BI) analyses using combined LSU–SSU–ITS–*tef1*. All the newly generated sequences were deposited in GenBank for future studies.

MP analysis was carried out using PAUP v.4.0b 10 (Swofford 2002) with the heuristic search option and the number of replications 1,000 each. The tree length (TL), Consistency Indices (CI), Retention Indices (RI), Rescaled Consistency Indices (RC) and Homoplasy Index (HI) were documented. Kishino-Hasegawa tests (KHT) (Kishino & Hasegawa 1989) were performed in order to determine whether trees were significantly different. ML analysis was performed with RAxMLGUI v.1.3 (Silvestro & Michalak 2012) using the ML+rapid bootstrap setting with 1,000 replicates. The Bayesian tree (BI) was generated by using MCMC sampling in MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001, Zhaxybayeva & Gogarten 2002) for 10,000,000 MCMC generations using four chains and partition analysis with 100 sample frequencies which products 100,000 trees. The suitable burn-in phases were determined by traces inspected in Tracer version 1.6 (Rambaut et al. 2014). The first 10,000 (10 % from total) trees were the burn-in phase and were discarded based on tracer analysis. The remaining 90,000 trees were used to calculate the posterior probability in the majority rule consensus tree (when split frequency <0.01). The resulting trees were viewed with FigTree v.1.4.0 (Rambaut & Drummond 2008) and the final layout was done with CorelDRAW Graphics Suite X6. The final alignment and tree were registered in TreeBASE under the submission ID: 25277 (http://www.treebase.org/).

Table 1 Isolates used in this study and GenBank accession numbers.

Taxon	Culture accession number(s)	LSU	SSU	ITS	*tef1*	References
Aquastroma magniostiolata	CBS 139680=MAFF 243824*	AB807510	AB797220	LC014540	AB808486	Tanaka et al. 2015
Aquilomyces patris	CBS 135661*	KP184041	KP184077	KP184002	N/A	Knapp et al. 2015
Aquilomyces rebunensis	CBS 139684*	AB807542	AB797252	AB809630	AB808518	Tanaka et al. 2015
Bambusicola massarinia	MFLUCC 11-0389*	JX442037	JX442041	NR_121548	N/A	Dai et al. 2012
Clypeoloculus akitaensis	CBS 139681*	AB807543	AB797253	AB809631	AB808519	Tanaka et al. 2015
Corynespora cassiicola	CBS 100822*	GU301808	GU296144	N/A	GU349052	Schoch et al. 2009
Corynespora smithii	CABI 5649b	GU323201	N/A	FJ852597	GU349018	Schoch et al. 2009
Falciformispora lignatilis	BCC 21117	GU371826	GU371834	KF432942	GU371819	Schoch et al. 2009
Falciformispora senegalensis	CBS 196.79*	KF015631	KF015636	KF015673	KF015687	Ahmed et al. 2014
Falciformispora tompkinsii	CBS 200.79*	KF015625	KF015639	NR_132041	KF015685	Ahmed et al. 2014
Helicascus elaterascus	HKUCC 7769	AY787934	AF053727	N/A	N/A	Tanaka et al. 2015
Massarina eburnea	CBS 473.64	GU301840	GU296170	N/A	GU349040	Zhang et al. 2009
Monodictys sp.	KH 331=MAFF 243826	AB807553	AB797263	N/A	AB808529	Tanaka et al. 2015
Monodictys sp.	JO 10=MAFF 243825	AB807552	AB797262	N/A	AB808528	Tanaka et al. 2015
Morosphaeria ramunculicola	BCC 18404	GQ925853	GQ925838	N/A	N/A	Suetrong et al. 2009
Morosphaeria velatispora	BCC 17059*	GQ925852	GQ925841	N/A	N/A	Suetrong et al. 2009
Multilocularia bambusae	MFLUCC 11-0180*	KU693438	KU693442	KU693446	N/A	Li et al. 2016
Multiseptospora thailandica	MFLUCC 11-0183*	KP744490	KP753955	KP744447	N/A	Liu et al. 2015
Multiseptospora thailandica	MFLUCC 11-0204	KU693440	KU693444	KU693447	KU705659	Liu et al. 2015
Multiseptospora thailandica	MFLUCC 12-0006	KU693441	KU693445	KU693448	KU705660	Liu et al. 2015
Multiseptospora thysanolaenae	MFLUCC 11-0238*	KU693439	KU693443	N/A	KU705658	Li et al. 2016
Neoaquastroma bauhiniae	MFLUCC 16-0398*	MH023319	MH023315	MH025952	MH028247	Phukhamsakda et al. 2018
Neoaquastroma bauhiniae	MFLUCC 17-2205	MH023320	MH023316	MH025953	MH028248	Phukhamsakda et al. 2018
Neoaquastroma cylindricum	**MFLUCC 19-0489***	**MN473054**	**MN473048**	**MN473060**	**MN481600**	**This study**
Neoaquastroma guttulatum	MFLUCC 14-0917*	KX949740	KX949741	KX949739	KX949742	Wanasinghe et al. 2017a
Neoaquastroma krabiense	MFLUCC 16-0419*	MH023321	MH023317	MH025954	MH028249	Phukhamsakda et al. 2018
Palmiascoma gregariascomum	MFLUCC 11-0175*	KP744495	KP753958	KP744452	N/A	Liu et al. 2015
Parabambusicola bambusina	KH 139=MAFF 243823	AB807537	AB797247	LC014579	AB808512	Tanaka et al. 2015
Parabambusicola bambusina	H 4321=MAFF 239462	AB807536	AB797246	LC014578	AB808511	Tanaka et al. 2015
Parabambusicola bambusina	KT 2637=MAFF 243822	AB807538	AB797248	LC014580	AB808513	Tanaka et al. 2015
Pseudomonodictys tectonae	MFLUCC 12-0552	KT285573	KT285574	N/A	KT285571	Ariyawansa et al. 2015
Stagonospora pseudocaricis	CBS 135132	KF251762	N/A	KF251259	N/A	Quaedvlieg et al. 2013
Trematosphaeria pertusa	CBS 122368*	FJ201990	FJ201991	NR_132040	KF015701	Zhang et al. 2008

Types and authentic strains are indicated with *, newly generated sequences in this study are indicated in bold. "N/A" sequence is unavailable.

Abbreviations: BCC: BIOTEC Culture Collection, Bangkok, Thailand; CABI: Centre for Agriculture and Biosciences International, Egham, UK; CBS: CBS-KNAW Collections, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; HKUCC: The University of Hong Kong Culture Collection; JO: J. Onodera; KH: K. Hirayama; KT: K. Tanaka; MAFF: Ministry of Agriculture, Forestry and Fisheries, Japan; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand.

Results

Topology of phylogenetic analyses

The combined LSU–SSU–ITS–*tef1* sequence matrix comprised of 33 taxa including the newly generated strain. *Corynespora cassiicola* (CBS 100822) and *Corynespora smithii* (CABI 5649b) were selected as outgroup taxa. The concatenated alignment comprised 3586 total characters including gaps (LSU: 1–880 bp, SSU: 881–1919 bp, ITS: 1920–2656 bp, *tef1*: 2657–3586 bp) with 1281 distinct alignment patterns and 23.17 % proportion of gaps and completely undetermined characters, 2538 constant, 270 parsimony uninformative and 778 parsimony informative characters. The MP analysis resulted a single most parsimonious tree (TL= 2706, CI=0.572, RI=0.679, RC=0.388, HI=0.428). The best ML phylogram (Fig. 1) (InL=-17995.951361, α=0.474127, invar=0.473346) resulted with estimated base frequencies A=0.238, C=0.251, G=0.274, T=0.237, substitution rates AC=1.023629, AG=2.050054, AT=1.180510, CG=0.891203, CT=5.263957, GT=1.0000, proportion of variable sites and gamma distribution shape parameter. The tree topologies resulted from ML, MP and BI analyses are similar.

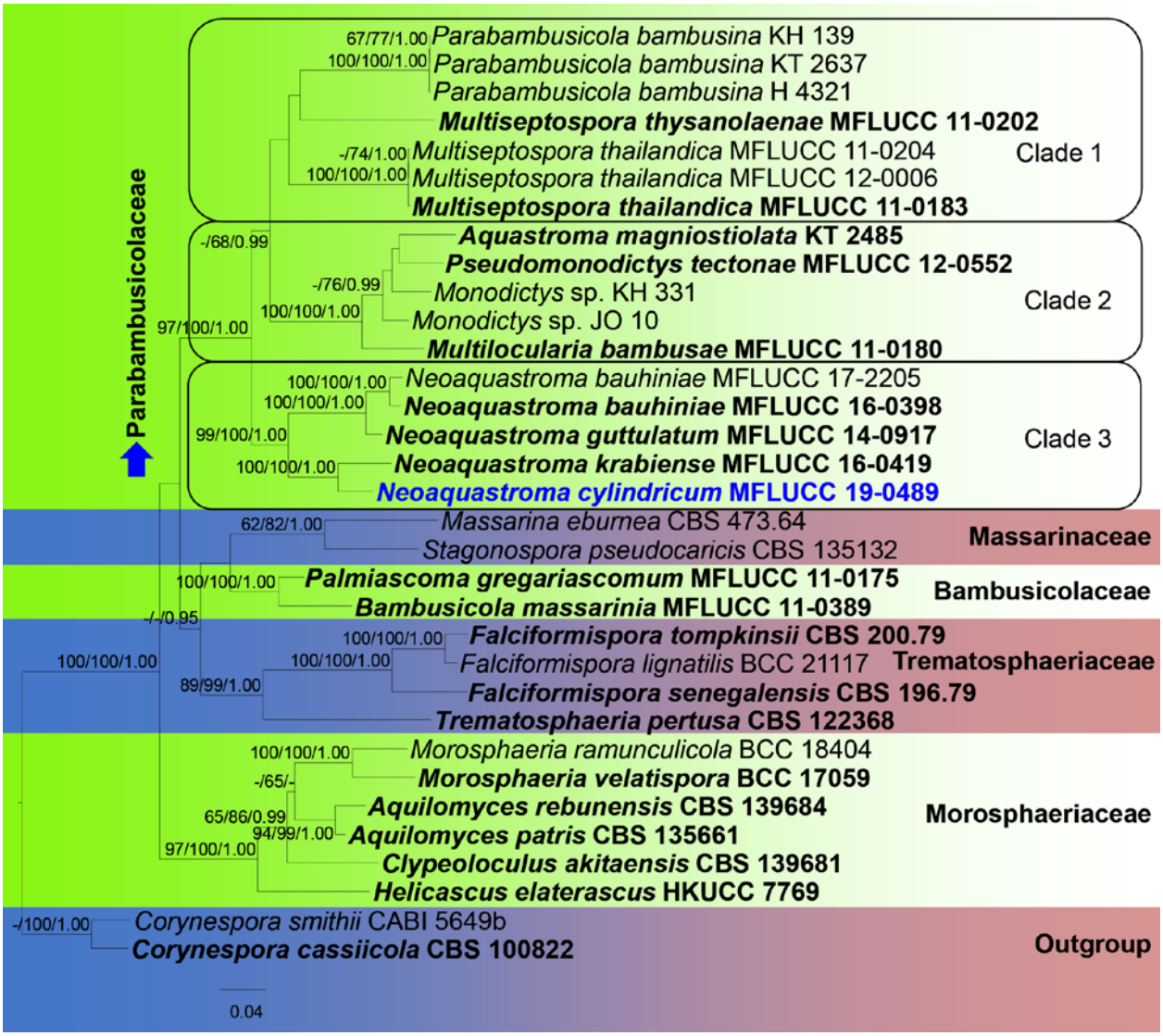

Fig. 1 – Phylogram generated from maximum likelihood (RAxML) based on LSU–SSU–ITS–*tef1* matrix. The tree is rooted in *Corynespora cassiicola* (CBS 100822) and *Corynespora smithii* (CABI 5649b). MP, ML bootstrap supports (≥60 %) and BI posterior probabilities (≥0.95 PP) supports are given above or below the branches respectively. Type strains are in bold and the newly introduced taxon is in blue.

Genera belong to Parabambusicolaceae clustered in three distinct sub-clades as 1, 2 and 3 (Fig. 1). Clade 1 comprises *Parabambusicola bambusina*, the generic type and two species of *Multiseptospora*. Those two species of *Multiseptospora* (*M. thailandica*, *M. thysanolaenae*) are not monophyletic within clade 1. Clade 2 comprises *Aquastroma*, *Multilocularia*, *Pseudomonodictys* and two '*Monodictys*' strains (68 % ML/0.99 PP BI). Clade 3 comprises *Neoaquastroma* species as a monophyletic group basal to clade 1 and 2 with strong statistical support (97 % MP/100 % ML/1.00 PP BI). Our strain MFLUCC 19–0489 clusters together with *N. krabiense* (MFLUCC 16–0419) with high statistical support (100 % MP/100 % ML/1.00 PP BI), but represents a distinct lineage.

In BLASTn searches on NCBI GenBank, the closest matches of our sequences are *Neoaquastroma*. The ITS sequence is 95 % similar to *N. krabiense* (MFLUCC 16–0419) while *tef1* is similar to *N. krabiense* (MFLUCC 16–0419; 97 %), *N. guttulatum* (MFLUCC 14–0917; 96 %) and *N. bauhiniae* (MFLUCC 17–2205; 95 %). However, LSU sequence is 95 % similar to *Monodictys* sp. (KH 331) and 95 % similar to *N. bauhiniae* (MFLUCC 17–2205).

Taxonomy

Neoaquastroma cylindricum Samarak. & K.D. Hyde, sp. nov. Fig. 2

Index Fungorum number: IF556820; Facesoffungi number: FoF06520

Etymology – the epithet "*cylindricum*" referring to cylindric-clavate asci

Holotype – MFLU 19–2110

Saprobic on dead twigs. Sexual morph *Ascomata* 175–220 *μm* high × 150–250 *μm* diam. ($\bar{x}$ = 200 × 220 *μm*, n = 10), immersed in the bark, solitary, scattered or sometimes gregarious, compressed globose, sub-globose, with a flattened base, black to dark brown, smooth, papillate, ostiolate. *Ostiole* 125–135 μm high × 60–75 μm diam. ($\bar{x}$ = 132 × 64 μm, n = 10), centrally located, oblong, filled with hyaline cells. *Peridium* 10–25 μm ($\bar{x}$ = 19 μm, n =10) wide, comprising 2 layers, outer layer pigmented, comprising reddish to dark brown, fused with host tissues, thin-walled cells of *textura angularis*, inner layer composed of hyaline, loosen, cells of *textura angularis*. *Hamathecium* composed of numerous, dense, long, 1.2–2.3 μm ($\bar{x}$ = 1.7 μm, n = 35), filamentous, branched, septate, pseudoparaphyses. *Asci* 80–115 × 14–18 μm ($\bar{x}$ = 91.5 × 15.6 μm, n = 25), 8-spored, bitunicate, fissitunicate, cylindric-clavate, with a short pedicel, apically rounded with broad ocular chamber. *Ascospores* 20–27 × 5–6.5 μm ($\bar{x}$ = 24.5 × 5.7 μm, n = 35), overlapping biseriate, ellipsoidal to sub-fusiform, hyaline, 3–5-transversely septate at maturity, with the primary septum almost median, deeply constricted at the middle septum, constricted at remaining septa, slightly curved, rough-walled, 1–2 prominent guttules in each cell, lacking guttules when over mature, ends remaining cone-shaped, with pointed ends, upper part larger than lower part, surrounded by a 3.7–7.6 μm ($\bar{x}$ = 5.3 μm, n = 10), distinct mucilaginous sheath. Asexual morph: Undetermined.

Culture characteristics – *Colonies on PDA*, reaching 30 mm diam. after three weeks at 25 °C. *Culture* grey, becoming dark-olive brown after three weeks, of dense mycelia, colonies circular, flat, umbonate, raised from the agar in the center, surface rough, dull, covered with aerial mycelium, white mycelium radiating into the agar, pale orange pigment diffusing in the agar from the top view; reverse black, dense, circular, with irregular, fimbriate margin.

Material examined – China, Guizhou, Guiyang, Guizhou Academy of Agricultural Sciences (GZAAS), on dead twigs, 22 July 2018, M.C. Samarakoon, SAMC171 (MFLU 19–2110, holotype; HKAS 102399, isotype) – ex-type living culture MFLUCC 19–0489. Additional sequence: MN481604 (*tub2*).

Discussion

In this study, we introduced *Neoaquastroma cylindricum* isolated from the bark of dead twigs as the first record from China in this genus. The morphological characterization of our new taxon is close affinity to the genus *Neoaquastroma* in having globose to sub-globose ascomata, reddish to

dark brown outer layer and hyaline inner layer peridium, transversely septate ascospores with large guttules and surrounded by a large mucilaginous sheath (Wanasinghe et al. 2017a). However, *N. cylindricum* differs from other *Neoaquastroma* species in having cylindric-clavate asci with comparatively small ascospores (20–27 × 5–6.5 µm) that are having 3–5-transversely septa (Table 2). In addition, *N. bauhiniae* and *N. krabiense* possess a wider cell above central septum of the ascospores which is not noticeable in our species (Phukhamsakda et al. 2018).

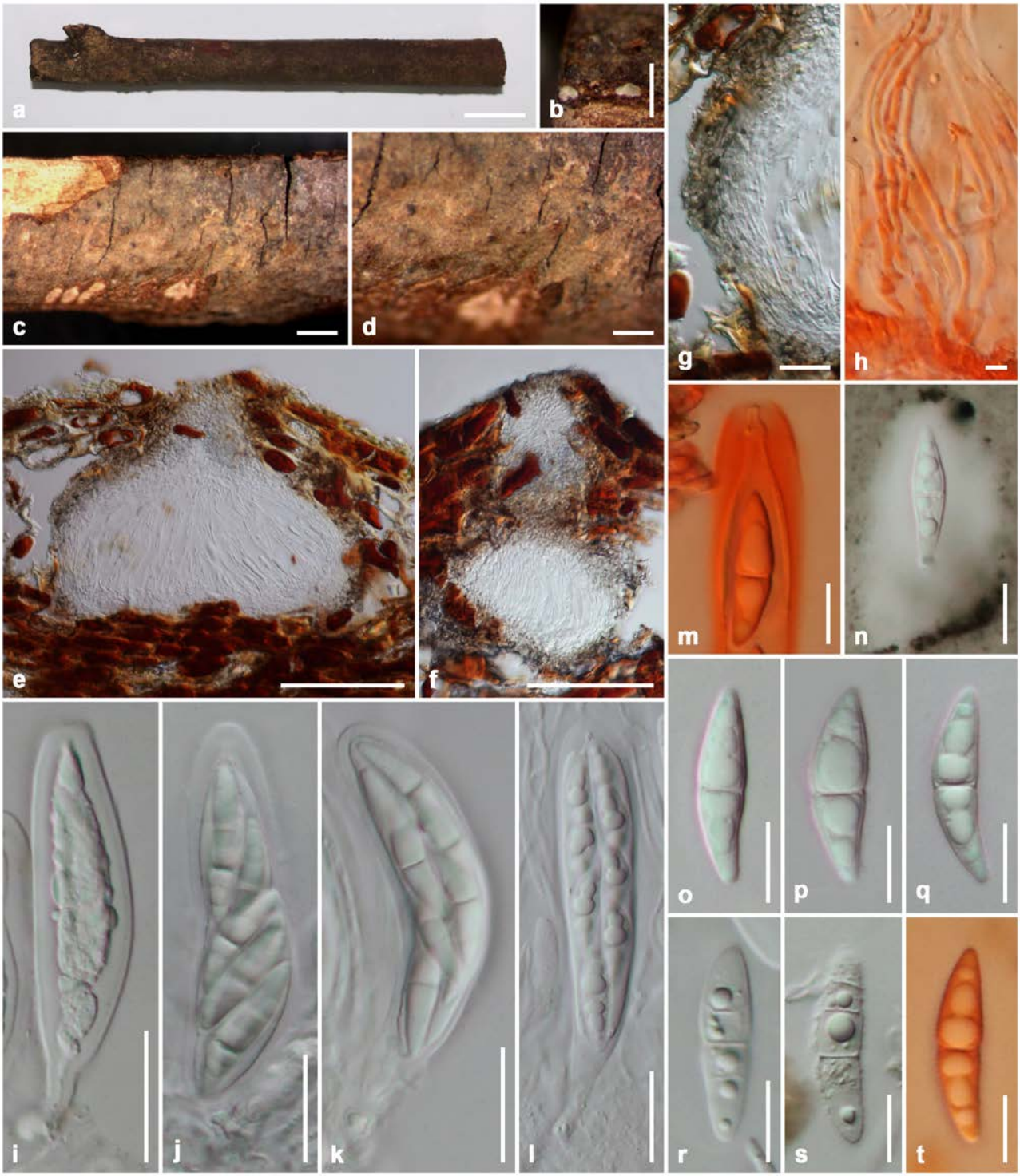

Fig. 2 – *Neoaquastroma cylindricum* (holotype, MFLU 19–2110). a-d Ascomata on the substrate. e, f Vertical section of ascoma. g Peridium. h Paraphyses. i-l Asci. m Ocular chamber (in Congo Red). n-t Ascospores (n in Indian ink, t in Congo Red). Scale bars: a=1 cm, c=1000 µm, b, d=500 µm, g, i-l=20 µm, h, m-t=10 µm.

Phylogenetic trees obtained from MP, ML and BI analyses were similar in overall topology as previous studies (Wanasinghe et al. 2017a, Phukhamsakda et al. 2018). In clade 3, *Neoaquastroma cylindricum* and *N. krabiense* constitute a basal sister-clade to other *Neoaquastroma* species. In a comparison of the ITS (+5.8S) nucleotides of these two strains reveals 49 (8.9 %) nucleotide differences including gaps. Both morph-molecular differentiations justify these two isolates as two distinct taxa (Jeewon & Hyde 2016).

It is worth pointing out that the affinities of the two *Multiseptospora* species are obscure given that there is no reliable support for clade 1 (Fig. 1). This is not a common phenomenon in contrast to those phylogenetic lineages to the remaining taxa in this family. Further taxon sampling with molecular work should be established to resolve the exact classification of *Multiseptospora* species.

Table 2 Synopsis of *Neoaquastroma* species.

Species	Substrate	Country	Ascomata (µm)	Asci (µm)	Ascospores (µm)	Reference
N. bauhiniae	dead twigs, *Bauhinia variegata* (Fabaceae)	Thailand	113–190 × 170–307 semi-immersed to immersed	53–116 × 26–43, oboviod to oblong	37–46 × 9–16 4–7-transversely septate	Phukhamsakda et al. (2018)
N. cylindricum	dead twigs	China	175–220 × 150–250 immersed	80–115 × 14–18 cylindric-clavate	20–27 × 5–6.5 3–5-transversely septate	This study
N. guttulatum	dead twigs	Thailand	240–280 × 210–250 immersed	90–140 × 30–40 clavate	35–50 × 11–15 3–7-transversely septate	Wanasinghe et al. (2017a)
N. krabiense	dead twigs, *Barringtonia acutangula* (Lecythidaceae)	Thailand	404–498 × 290–319 immersed	95–169 × 29–45 oboviod to clavate	50–64 × 9–18 5–8-transversely septate	Phukhamsakda et al. (2018)

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