Studies in Fungi 2 (1): 219–234 (2017) www.studiesinfungi.org ISSN 2465-4973
Article

Doi 10.5943/sif/ 2/1/26

Saprobic Dothideomycetes in Thailand: *Muritestudina* gen. et sp. nov. (*Testudinaceae*) a new terrestrial pleosporalean ascomycete, with hyaline and muriform ascospores

Wanasinghe DN[1,2], Jeewon R[3], Tibpromma S[1,2], Jones EBG[4] and Hyde KD[1,2*]

[1]*Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai, 57100, Thailand*
[2]*Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Science, Kunming 650201, Yunnan, People's Republic of China*
[3]*Department of Health Sciences, Faculty of Science, University of Mauritius, Reduit, Mauritius*
[4]*Nantgaredig, 33 B St. Edwards Road, Southsea, Hants, Hampshire PO5 3DH, UK*

Wanasinghe DN, Jeewon R, Tibpromma S, Jones EBG, Hyde KD 2017 – Saprobic Dothideomycetes in Thailand: *Muritestudina* gen. et sp. nov. (*Testudinaceae*) a new terrestrial pleosporalean ascomycete, with hyaline and muriform ascospores. Studies in Fungi 2(1), 219–234, Doi 10.5943/sif/2/1/26

Abstract

The family *Testudinaceae* and its intergeneric classification are poorly understood. This is due to overlap of morphological characteristics in genera and lack of DNA sequence data to infer phylogenetic relationships. The main objective of the present paper is to establish a novel genus, *Muritestudina*, based on distinct morphological characteristics and analyses of combined LSU, SSU, ITS, *rpb2* and *tef1* sequence data. We also fill the gap of our current knowledge on the phylogenetic position of *Testudinaceae*. Based on the morphological characteristics of species representing existing genera of *Testudinaceae*, we herein introduce a new genus, *Muritestudina* with *M. chiangraiensis* as the type species. The new genus is characterized by globose to subglobose, ostiolate ascomata; a peridium of brown to dark-brown cells of *textura angularis*; septate and cellular pseudoparaphyses; cylindric-clavate asci with a distinct pedicel; and hyaline, ellipsoidal and muriform ascospores. The new genus differs from the other genera in *Testudinaceae* in having hyaline, muriform ascospores. Combined analyses of ribosomal and protein coding gene sequence data confirmed that our new taxon belongs in *Testudinaceae* with a close relationship with *Neotestudina rosatii*.

Keywords – Ascomycota – phylogeny – Pleosporales – taxonomy

Introduction

The biology that deals with the classification and nomenclature of Dothideomycetes has undergone considerable change and to-date this largely cosmopolitan class contains 32 orders and 114 families (Liu et al. 2017). Most taxa of Dothideomycetes are plant associated fungi and may be pathogens, endophytes, saprobes, or epiphytes of a wide range of hosts, in terrestrial as well as aquatic habitats (Duong et al. 2006, Kodsueb et al. 2007, Zhang et al. 2008, Hyde et al. 2013, 2014, Wijayawardene et al. 2014, Li et al 2017, Luo et al. 2017). They contribute both positively and negatively to human and economic well-being, by affecting environmental cost and being a risk to agriculture. They also have pharmaceutical and biotechnological significance (Ohm et al. 2012, Stergiopoulos et al. 2012, Torres & Dela Cruz 2015).

Submitted 12 November 2017, Accepted 07 December 2017, Published 29 December 2017
Corresponding Author: Kevin D. Hyde – e-mail – kdhyde3@gmail.com

Pleosporales is the largest order, comprising a quarter of all dothideomycetous species (Kirk et al. 2008). Pleosporales comprises 55 families and has an estimated divergence time (crown age) of ~148–260 MY (Liu et al. 2017). Due to their economic importance and diversity, there has been a great research interest on taxa of this order, which has provided a better taxonomic understanding (Jaklitsch & Voglmayr 2016, Chen et al. 2017, Hashimoto et al. 2017, Wanasinghe et al. 2017a, b, Woudenberg et al. 2017). However, in contrast to the well-resolved families of Pleosporales (*i.e. Camarosporidiellaceae*, *Didymellaceae*, *Didymosphaeriaceae*, *Leptosphaeriaceae*, *Lophiostomataceae*, *Lophiotrematace*ae, *Melanommataceae*, *Nigrogranaceae*, *Phaeosphaeriaceae*, *Pleosporaceae*), many of genera and families are poorly understood (*i.e. Delitschiaceae*, *Halojulellaceae*, *Ligninsphaeriaceae*, *Morosphaeriaceae*, *Salsugineaceae*, *Testudinaceae*, *Wicklowiaceae*, *Zopfiaceae*). Most studies have relied heavily on DNA based sequence analyses based on a limited number of species (*i.e. Testudinaceae*, *Zopfiaceae*). This has resulted in inadequate understanding of the genera and species in these families (Jeewon & Hyde 2007, Zhang et al. 2012). A wider taxon sampling and accurate taxonomic information based on morphological examinations of specimens, coupled with phylogenetic sequence data are needed, to better integrate taxa into appropriate taxonomic ranks in this order.

In this study, we isolated a saprobe from an undetermined, dead, terrestrial substrate in Chiang Rai, Thailand and investigated its phylogenetic relationships as a taxon in *Testudinaceae*. Placement at the family level is inferred based on analyses of combined DNA sequence data and the taxon is introduced as *Muritestudina chiangraiensis* gen. et sp. nov. Morphological similarities and differences, coupled with multi-gene phylogeny of the novel taxon are discussed.

Materials and methods

Isolates and specimens

Fresh fungal material was collected from Chiang Rai Province in Thailand and brought to the laboratory in a Zip-lock plastic bag. Samples were examined with a Motic SMZ 168 Series microscope. Single ascospore isolation was carried out following the method described in Chomnunti et al. (2014). Germinated spores were individually transferred to Potato Dextrose Agar (PDA) plates and grown at 25 °C in the daylight. Isolates including accession numbers of gene sequences are listed in Table 1. Isolates listed as MFLUCC are those maintained in the collection of the Culture Collection of Mae Fah Luang University, Chiang Rai, Thailand. Specimens have been deposited in the Mae Fah Luang University (MFLU) fungarium, Chiang Rai, Thailand. Representative isolates and specimens have been deposited in Thailand Bioresource Research Center, Bangkok, Thailand (TBRC) and National Science and Technology Development Agency, Thailand (BBH). Faces of Fungi and Index Fungorum numbers are provided as outlined in Jayasiri et al. (2015) and Index Fungorum (2017), while new species are justified based on recommendations outlined by Jeewon & Hyde (2016).

Morphological observations

Hand sections of the ascomata, were mounted in distilled water, and the following characteristics were evaluated: ascomata diameter, height, colour and shape; width of peridium; height and diameter of ostioles. Length and width (at the widest point) of asci and ascospores were measured. Images were captured with a Canon 550D digital camera fitted to a Nikon ECLIPSE 80i compound microscope. Measurements were made with the Tarosoft (R) Image Frame Work program and images used for figures processed with Adobe Photoshop CS3 Extended version 10.0 software (Adobe Systems, USA).

DNA extraction, PCR amplifications and sequencing

Mycelia for DNA extraction from each isolate were grown on PDA for 3–4 weeks at 25 °C and total genomic DNA was extracted from approximately 150 ± 50 mg axenic mycelium scraped from the edges of the growing culture. Mycelium was ground to a fine powder with liquid nitrogen

and DNA extracted using the Biospin Fungus Genomic DNA Extraction Kit-BSC14S1 (BioFlux, P.R. China) following the instructions of the manufacturer. DNA to be used as template for PCR were stored at 4 °C for use in regular work and duplicated at -20 °C for long-term storage.

DNA sequence data was obtained from the partial sequences of three ribosomal and two protein coding genes. The genes, primers, references and PCR protocols are summarized in Table 2. Polymerase chain reaction (PCR) was carried out in a volume of 25 µl which contained 12.5 µl of 2 × Power Taq PCR MasterMix (Bioteke Co., China), 1 µl of each primer (10 µM), 1 µl genomic DNA and 9.5 µl deionized water. The amplified PCR fragments were sent to a commercial sequencing provider (BGI, Ltd Shenzhen, P.R. China). The nucleotide sequence data acquired were deposited in GenBank (Table 1). The finalized alignment and tree were deposited in TreeBASE, submission ID: 21850 (http://purl.org/phylo/treebase/phylows/study/TB2:S21850).

Table 1 Genes/loci used in the study with PCR primers, references and protocols.

Locus [a]	Primers	PCR: thermal cycles: [b] (Annealing temp. in bold)	References
ITS	ITS5 ITS4	(95 °C: 30 s, **55** °C:50 s, 72 °C: 90 s) × 35 cycles	White et al. (1990)
LSU	LR0R LR5	(95 °C: 30 s, **55** °C:50 s, 72 °C: 90 s) × 35 cycles	Rehner & Samuels (1994) Vilgalys & Hester (1990)
SSU	NS1 NS4	(95 °C: 30 s, **55** °C:50 s, 72 °C: 90 s) × 35 cycles	White et al. (1990)
rpb2	fRPB2-5f fRPB2-7cR	(94 ˚C: 60 s, **58** ˚C: 60 s, 72 ˚C: 90 s) × 40 cycles	Sung et al. (2007)
tef1	EF1-983F EF1-2218R	(95 °C: 30 s, **55** °C:50 s, 72 °C: 90 s) × 35 cycles	Rehner & Buckley (2005) Liu et al. (1999)

[a] ITS: Part of rDNA 18S (3' end), the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region (ITS2), and part of the 28S rRNA (5' end); LSU: Large subunit (28S); SSU: Small subunit rDNA (18S); *rpb2*: RNA polymerase II second largest subunit; *tef1*: translation elongation factor 1-alpha gene

[b] All the PCR thermal cycles include Initiation step of 95 °C: 5 min, and final elongation step of 72 °C: 10 min and final hold at 4 °C

Molecular phylogenetic analyses

Sequencing and sequence alignment

Sequences generated from different primers of the three genes were analysed with other sequences retrieved from GenBank (Table 2). Sequences with high similarity indices were determined from a BLAST search to find the closest matches with taxa in Pleosporales, and from recently published data (Hashimoto et al. 2017). The multiple alignments of all consensus sequences, as well as the reference sequences were automatically generated with MAFFT v. 7 (http://mafft.cbrc.jp/alignment/ server/index.html; Kuraku et al. 2013, Katoh et al. 2017), and were improved manually when necessary using BioEdit v. 7.0.5.2 (Hall 1999). Ambiguous regions were excluded from the analyses and gaps were treated as missing data.

Phylogenetic analyses

Phylogenetic analyses of both individual and combined aligned data were performed under maximum-likelihood, maximum parsimony and Bayesian criteria. Parsimony analysis was carried with the heuristic search option in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 with the following parameter settings: characters unordered with equal weight, random taxon addition, branch swapping with tree bisection-reconnection (TBR) algorithm, branches collapsing if the maximum branch length was zero. Alignment gaps were treated as missing characters in the analysis of the combined data set, where they occurred in relatively conserved regions. Trees were inferred using the heuristic search option with 1000 random sequence additions, with maxtrees set at 1000. Descriptive tree statistics for parsimony; Tree Length (TL), Consistency Index (CI), Retention Index (RI), Relative Consistency Index (RC) and Homoplasy Index (HI) were calculated

for trees generated under different optimality criteria. The Kishino-Hasegawa tests (Kishino & Hasegawa 1989) were performed in order to determine whether trees were significantly different. Maximum parsimony bootstrap values (MP) equal or greater than 60 % are given above each node (Fig. 1). Other details pertaining to analyses (e.g. consideration of TT ratios, comparison of tree topologies, selection of outgroups etc.) are outlined in Jeewon et al. (2004, 2013).

The evolutionary models for Bayesian analysis and maximum-likelihood were selected independently for each locus using MrModeltest v. 2.3 (Nylander 2004) under the Akaike Information Criterion (AIC) implemented in both PAUP v. 4.0b10. GTR+I+G model is resulted in each locus for Bayesian analysis and maximum-likelihood by AIC in MrModeltest as the best-fit model.

Bayesian analysis was conducted with MrBayes v. 3.1.2 (Huelsenbeck & Ronqvist 2001) to evaluate Bayesian posterior probabilities (BYPP) (Rannala & Yang 1996; Zhaxybayeva & Gogarten 2002) by Markov Chain Monte Carlo sampling (BMCMC). GTR+I+G was used in the command. Six simultaneous Markov chains were run for 2,000,000 generations and trees were sampled every 200th generation. The distribution of log-likelihood scores was examined to determine stationary phase for each search and to decide if extra runs were required to achieve convergence, using the program Tracer 1.5 (Rambaut & Drummond 2007). First 10 % of generated trees were discarded and remaining 90 % of trees were used to calculate posterior probabilities of the majority rule consensus tree. BYPP greater than 0.95 are given above each node (Fig. 1).

Maximum likelihood trees were generated using the RAxML-HPC2 on XSEDE (8.2.8) (Stamatakis et al. 2008, Stamatakis 2014) in the CIPRES Science Gateway platform (Miller et al. 2010) using GTR+I+G model of evolution. Maximum likelihood bootstrap values (ML) equal or greater than 60 % are given above each node (Fig. 1).

Phylograms were visualized with FigTree v1.4.0 program (Rambaut 2012) and reorganized in Microsoft power point (2007) and Adobe Illustrator® CS5 (Version 15.0.0, Adobe®, San Jose, CA).

Table 2 Taxa used in the phylogenetic analyses and their corresponding GenBank numbers. The newly generated sequences are indicated in bold.

Species	Strain no	GenBank Accession no.				
		LSU	**SSU**	**ITS**	***rpb2***	***tef1***
Amniculicola immersa	CBS123083	FJ795498	GU456295	–	GU456358	GU456273
Amniculicola parva	CBS123092	GU301797	GU296134	–	–	GU349065
Angustospora nilensis	MFLU 15-1511	KT944072	KT944071	–	–	–
Anteaglonium abbreviatum	ANM925a	GQ221879	–	–	–	GQ221925
Anteaglonium globosum	ANM925.2	GQ221911	–	–	–	GQ221919
Anteaglonium parvulum	MFLUCC 14-0815	KU922911	KU922912	–	–	KU922919
Anteaglonium parvulum	MFLUCC 14-0817	KU922913	KU922914	–	–	–
Anteaglonium parvulum	MFLUCC 14-0821	KU922915	KU922916	–	–	KU922921
Anteaglonium parvulum	MFLUCC 14-0823	KU922917	KU922918	–	–	KU922922
Anteaglonium thailandicum	MFLUCC 14-0816	KU922909	KU922910	–	–	KU922920
Aquasubmersa japonica	KT2813	LC061586	LC061581	LC061591	LC194420	LC194383
Aquasubmersa japonica	KT2862	LC061587	LC061582	LC061592	LC194421	LC194384
Aquasubmersa japonica	KT2863	LC061588	LC061583	LC061593	LC194422	LC194385
Aquasubmersa mircensis	MFLUCC 11-0401	JX276955	JX276956	JX276954	–	–
Atrocalyx acutispora	KT2436	LC194341	LC194299	LC194475	LC194423	LC194386
Atrocalyx lignicola	CBS122364	LC194342	LC194300	LC194476	LC194424	LC194387
Cryptoclypeus oxysporus	KT2772	LC194345	LC194303	LC194479	LC194427	LC194390

Table 2 Continued.

Species	Strain no	GenBank Accession no.				
		LSU	**SSU**	**ITS**	***rpb2***	***tef1***
Cryptocoryneum brevicondensatum	yone152	LC194349	LC194307	LC096155	LC194431	LC096137
Cryptocoryneum japonicum	KT3300	LC194354	LC194312	LC096160	LC194436	LC096142
Cryptocoryneum longicondensatum	KT2913	LC194360	LC194318	LC096166	LC194442	LC096148
Cryptocoryneum paracondensatum	KT3241	LC194362	LC194320	LC096168	LC194444	LC096150
Cryptocoryneum pseudorilstonei	CBS113641	LC194364	LC194322	LC096170	LC194446	LC096152
Hermatomyces tectonae	MFLUCC 14-1140	KU764695	KU712465	KU144917	KU712486	KU872757
Hermatomyces tectonae	MFLUCC 14-1141	KU764696	KU712466	KU144918	–	KU872758
Hermatomyces tectonae	MFLUCC 14-1142	KU764697	KU712467	KU144919	KU712487	–
Hermatomyces thailandica	MFLUCC 14-1143	KU764692	KU712468	KU144920	KU712488	KU872754
Hermatomyces thailandica	MFLUCC 14-1144	KU764693	KU712469	KU144921	KU712489	KU872755
Hermatomyces thailandica	MFLUCC 14-1145	KU764694	KU712470	KU144922	KU712490	KU872756
Lepidosphaeria nicotiae	CBS 101341	DQ678067	–	–	DQ677963	DQ677910
Lophiostoma arundinis	CBS 621.86	DQ782384	DQ782383	AJ496633	DQ782386	DQ782387
Lophiostoma crenatum	CBS 629.86	DQ678069	DQ678017	–	DQ677965	DQ677912
Lophiotrema bambusae	RP0030	KX672154	KX672159	KX672149	KX672161	KX672162
Lophiotrema eburnoides	KT1424.1	LC001707	LC001706	LC001709	LC194458	LC194403
Lophiotrema fallopiae	KT2748	LC149915	LC149911	LC149913	LC194459	LC194404
Muritestudina chiangraiensis	**MFLUCC 17-2551**	**MG602248**	**MG602249**	**MG602247**	**MG602250**	**MG602251**
Massarina albocarnis	CBS 119345	LC194379	LC194337	LC194503	LC194471	LC194416
Neotestudina rosatii	CBS 690.82	DQ384107	DQ384081			
Polyplosphaeria fusca	KT1616	AB524604	AB524463	AB524789	–	–
Pseudoastrosphaeriella bambusae	MFLUCC 11-0205	KT955475	KT955455	–	KT955414	KT955437
Pseudoastrosphaeriella longicolla	MFLUCC 11-0171	KT955476	–	–	KT955420	KT955438
Pseudoastrosphaeriella thailandensis	MFLUCC 10-0553	KT955477	KT955456	–	KT955411	KT955439
Pseudocryptoclypeus yakushimensis	KT2186	LC194380	LC194338	LC194504	LC194472	LC194417
Pseudolophiotrema elymicola	KT1450	LC194381	LC194339	LC194505	LC194473	LC194418
Pseudotetraploa curviappendiculata	HC4930	AB524608	AB524467	AB524792	–	–
Quadricrura septentrionalis	HC4983	AB524799	–			
Tetraploa sasicola	KT563	AB524631	AB524490	AB524807	–	–
Trematosphaeria wegeliniana	CBS 123124	GU261722	GU261720			
Triplosphaeria maxima	KT870	AB524637	AB524496	AB524812	–	–
Ulospora bilgramii	CBS 101364	DQ678076	DQ678025	–	DQ677974	DQ677921
Verruculina enalia	BCC18402	GU479803	GU479771	–	GU479836	GU479864

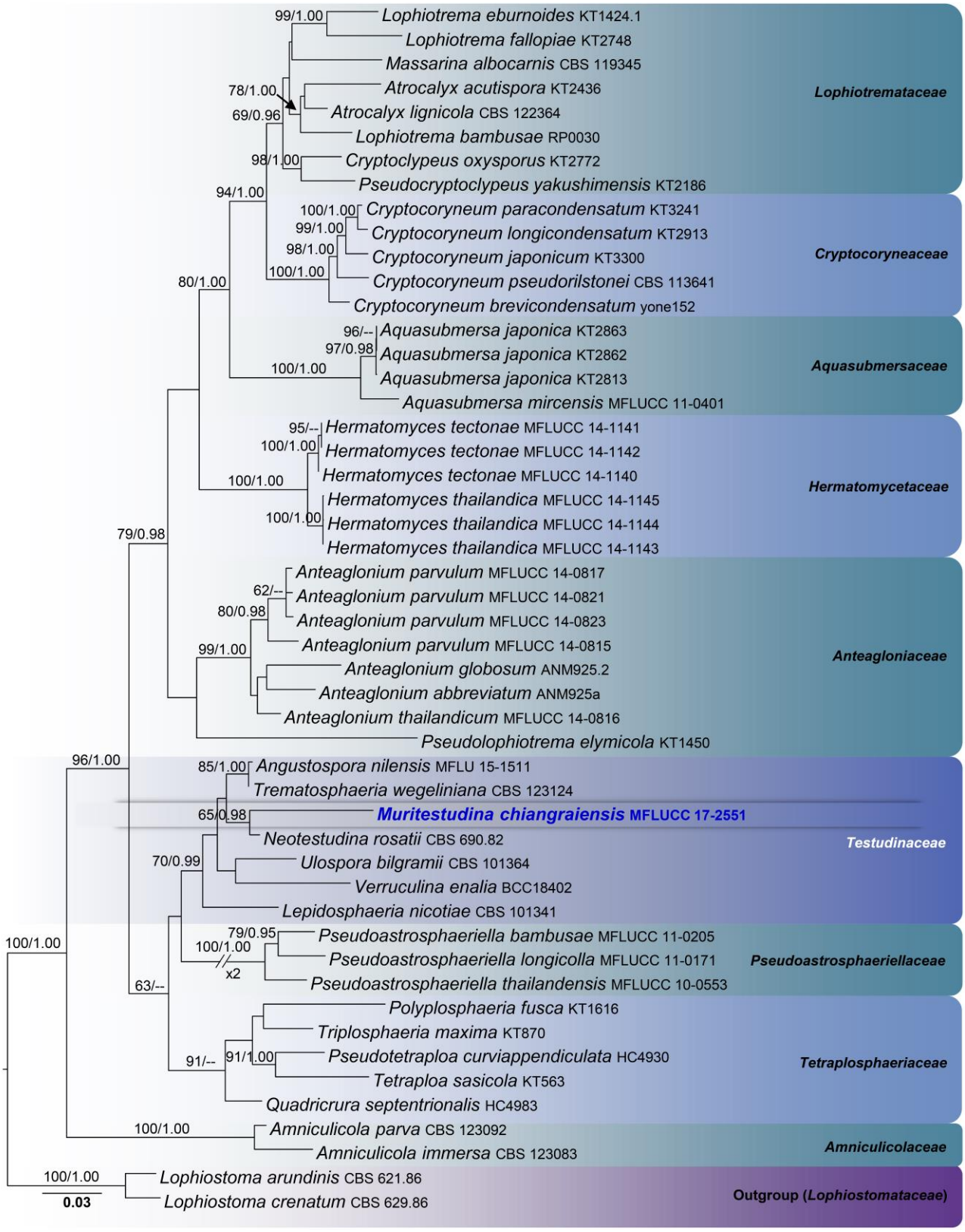

Fig. 1 – RAxML tree based on a combined dataset of partial LSU, SSU, ITS, *rpb2* and *tef1* DNA sequence analyses. Bootstrap support values for ML equal to or greater than 60 %, Bayesian posterior probabilities (PP) equal to or greater than 0.95 are shown as ML/PP above the nodes. The new isolate is in blue. The tree is rooted to *Lophiostoma arundinis* and *L. crenatum* (*Lophiostomataceae*). The scale bar represents the expected number of nucleotide substitutions per site.

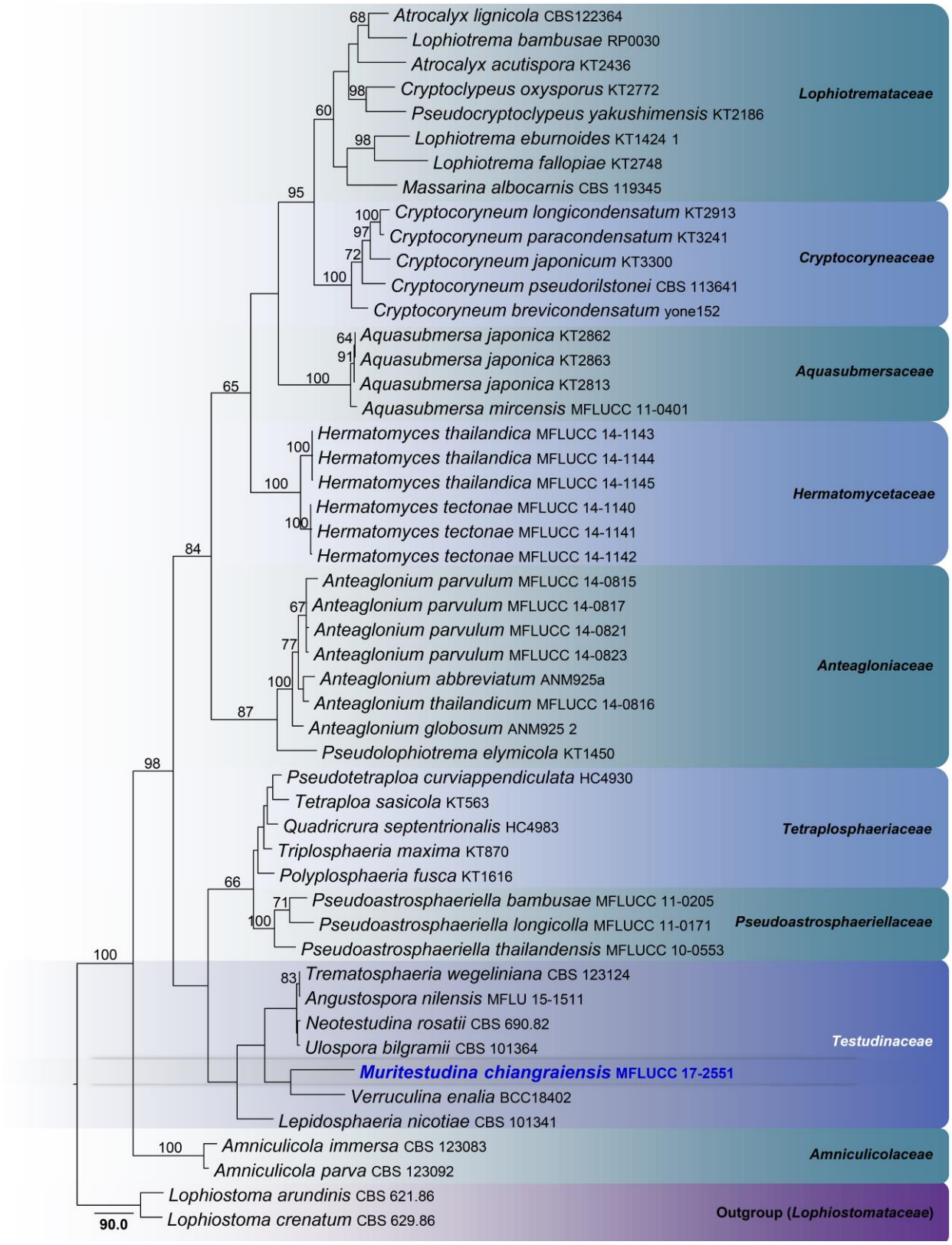

Fig. 2 – The MP phylogram generated from a combined dataset of LSU, SSU, ITS, *rpb2* and *tef1* DNA sequence analyses. Bootstrap support values for MP equal to or greater than 60 % are shown above the nodes. The new isolate is in blue. The tree is rooted to *Lophiostoma arundinis* and *L. crenatum* (*Lophiostomataceae*). The scale bar represents the expected number of nucleotide substitutions per site.

Results

Phylogenetic analyses

The concatenated dataset (LSU, SSU, ITS, *rpb2* and *tef1* loci) consisted of 50 strains with 4527 characters representing nine families (Pleosporales) including the new taxon proposed in this study, and *Lophiostoma arundinis* and *L. crenatum* as out-group taxa. The RAxML analysis of the combined dataset yielded a best scoring tree (Fig. 1) with a final ML optimization likelihood value of -26038.073582. The matrix had 1420 distinct alignment patterns, with 25.01 % of undetermined characters or gaps. Parameters for the GTR + I + G model of the combined LSU, SSU, ITS, *rpb2* and *tef1* were as follows: Estimated base frequencies; A = 0.248965, C = 0.245265, G = 0.270858, T = 0.234913; substitution rates AC = 1.334904, AG = 4.505518, AT = 1.221865, CG = 1.221699, CT = 9.92271, GT = 1.000; proportion of invariable sites I = 0.585813; gamma distribution shape parameter α = 0.497811. The Bayesian analysis resulted in 10001 trees after 2000000 generations. The first 1000 trees, representing the burn-in phase of the analyses, were discarded, while the remaining 9001 trees were used for calculating posterior probabilities in the majority rule consensus tree.

The maximum parsimonious dataset (Fig. 2) consisted of 4527 characters, of which 3310 were constant, 932 parsimony-informative (20.6%) and 285 parsimony-uninformative. The parsimony analysis of the data matrix resulted in 15 equally most parsimonious trees with a length of 4356 steps (CI = 0.422, RI = 0.645, RC = 0.272, HI = 0.578) in the first tree. Tree topologies (generated under ML and Bayesian criteria) from single gene datasets were also compared and the overall tree topology was congruent to those obtained from the combined dataset MP tree (Fig. 2) also recover similar tree topology with respect to the position of the families (except for the *Pseudoastrosphaeriellaceae*). However, there were major differences in statistical support as compared to the RAxML tree (much lower support observed for a number of clades).

The ML tree generated based on sequence analysis of the combined dataset indicates that our new isolate, *Muritestudina chiangraiensis* belongs in *Testudinaceae* and clusters with *Neotestudina rosatii* (CBS 690.82) with 65% ML and 0.98 PP statistical support (Fig. 1). Other species *viz. Angustospora nilensis* (MFLU 15–1511), *Lepidosphaeria nicotiae* (CBS 101341), *Trematosphaeria wegeliniana* (CBS 123124), *Ulospora bilgramii* (CBS 101364) and *Verruculina enalia* (BCC18402) also group in the *Testudinaceae* clade with 70% ML and 0.99 PP statistical support. A notable difference observed herein is the position of our new genus, *Muritestudina*. In our ML analyses, the latter clusters with *Neotestudina rosatii* with moderate support, whereas in the MP tree, it clusters with *Verruculina enalia* in an unsupported subclade.

Taxonomy

Testudinaceae Arx, Persoonia 6 (3): 366 (1971)

Muritestudina Wanasinghe, E.B.G. Jones & K.D. Hyde, gen. nov.

Index Fungorum Number: IF554051; Facesoffungi Number: FoF03866

Etymology – The generic epithet is from the combination of two words Muri and Testudina meaning muriform ascospores in *Testudinaceae*.

Saprobic on unidentified terrestrial dead twigs on the ground. Sexual morph: *Ascomata* scattered, immersed, coriaceous, black, globose to subglobose, ostiolate. Ostiole central, papillate, with an irregular, pore-like opening, plugged by brown, filamentous hyphae, and occasionally lighter in colour. Peridium composed of 4–5-layers with brown to dark-brown, cells of textura angularis fusing and indistinguishable from the host tissues. Hamathecium comprising septate, cellular pseudoparaphyses, situated between and above the asci, embedded in a gelatinous matrix. *Asci* 8-spored, bitunicate, fissitunicate, cylindric-clavate, with a distinct pedicel, apically rounded with an ocular chamber. Ascospores overlapping 2-seriate, hyaline, ellipsoidal, 10–12-transversely

septate, with 2–4-longitudinal septa, muriform, constricted at the septa, guttulate, smooth-walled, surrounded by a thick, wide mucilaginous sheath. Asexual morph: Undetermined.

Type – *Muritestudina chiangraiensis* Wanasinghe, E.B.G. Jones & K.D. Hyde

Muritestudina chiangraiensis Wanasinghe, E.B.G. Jones & K.D. Hyde, sp. nov. Figs 3,4

Index Fungorum Number: IF554052; Facesoffungi Number: FoF03867

Etymology – Named after the locality from where it was collected, Chiang Rai.

Holotype – MFLU 17–2645

Saprobic on unidentified terrestrial dead twigs on the ground. Sexual morph: Ascomata 250–350μm high, 300–400 μm diam. ($\overline{x}$ = 331.1 × 377.2 μm, n = 10), scattered, immersed, coriaceous, black, globose to subglobose, ostiolate. Ostiole central, papillate, with an irregular, pore-like opening, plugged by brown, filamentous hyphae, and occasionally lighter. Peridium 20–30 μm wide, composed of 4–5-layers with brown to dark-brown, cells of textura angularis fusing and indistinguishable from the host tissues. Hamathecium comprising 2–3 μm (n = 30), wide septate, cellular pseudoparaphyses, situated between and above the asci, embedded in a gelatinous matrix. Asci 160–180 × 25–35 μm ($\overline{x}$ = 172.1 × 30.5 μm, n = 35), 8-spored, bitunicate, fissitunicate, cylindrical-clavate, with a distinct pedicel (10–30 μm long; $\overline{x}$ = 21 μm, n=30), apically rounded with an ocular chamber. Ascospores 30–42 × 12–15 μm ($\overline{x}$ = 37.3 × 13.2 μm, n=50), overlapping 2-seriate, hyaline, ellipsoidal, 10–12-transversely septate, with 2–4-longitudinal septa, muriform, constricted at the septa, guttulate, smooth-walled, surrounded by a thick, large mucilaginous sheath. Asexual morph: Undetermined.

Culture characteristics – Colonies on PDA: reaching 3–4 cm diam. after 4 weeks at 25°C, colonies circular, medium dense, flattened, surface slightly rough with edge entire, slightly radiating with concentric rings of mycelium; colony from above, white to creamy, pale brown at the center; from below: honey at the margin, dark brown at the center, producing pale brown pigmentation in agar.

Known distribution – on dead twigs, Thailand.

Material examined – Thailand, Chiang Rai, Mueang Chiang Rai District, Nang Lae, 20.037708N, 99.890775E, on dead twigs of undetermined sp., 25 June 2017, DN Wanasinghe (MFLU 17–2645, holotype (isotype in BBH) – ex-type living culture, MFLUCC 17–2551.

Discussion

The main objective of this paper is to establish a novel genus based on morphological and molecular data, as well as to fill the gap in our current taxonomic knowledge on the phylogenetic position of the *Testudinaceae*. Based on analyses of morphological characters within species representing extant *Testudinaceae* genera, we proposed herein a new genus, *Muritestudina*, with *M. chiangraiensis* as the type species. Despite some superficial similarities and close relatedness between *Muritestudina* and *Neotestudina rosatii* based on our ML analyses, they can be distinguished based on their phenotypes as well as their habitats. *Neotestudina* is a human pathogen which is characterized by clavate asci with an irregular spore arrangement and broadly truncate, brown, ascospores with a smooth wall (Hawksworth 1979). *Muritestudina chiangraiensis* is a terrestrial saprobe which has cylindrical-clavate asci and overlapping biseriate, hyaline, smooth-walled, ellipsoidal, muriform ascospores with large guttules in almost every cell (Fig. 3). Comparison of the LSU nucleotides between our new taxon and *Neotestudina rosatii* (GenBank: DQ384107) used in this analysis reveals 18 (1.8%) differences that sets our taxon apart. On the other hand, our MP analyses depict an unsupported relationship of *Muritestudina* with *Verruculina enalia*. The latter is recorded from marine aquatic habitats (common on mangrove wood) which have cylindrical asci and uniseriate, dark brown, ellipsoidal, ascospores with verrucose to verruculose walls (Hyde et al. 2013, Jones et al. 2015) and hence differs remarkably from our species. A comparison of the 482 ITS (+5.8S) nucleotides with *Verruculina enalia* (GenBank: GQ203796) reveals >50 differences and thus justifies establishment of our new taxon.

The taxonomy of *Testudinaceae* has been primarily based on a few unstable morphological characters and DNA sequence data have been analysed for only a relatively small number of species. Von Arx (1971) introduced *Testudinaceae* to accommodate *Testudina*, *Neotestudina*, *Lepidosphaeria*, *Argynna* and *Pseudophaeotrichum*. The presence of ascomata with a dark peridium, bitunicate asci and dark, 2-celled ascospores (about 10 µm long) has been considered as significant delimiting characters at the familial level. This classification was followed by Hawksworth (1979) as a result of studies with the scanning electron microscopy (SEM), but *Argynna* was excluded from *Testudinaceae* due to its unitunicate asci. Phookamsak et al. (2015) excluded *Lojkania* from *Fenestellaceae* and currently *Testudinaceae* comprises *Angustospora*, *Lepidosphaeria*, *Lojkania*, *Neotestudina*, *Testudina*, *Ulospora*, *Verruculina* as accepted genera (Wijayawardene et al. 2017).

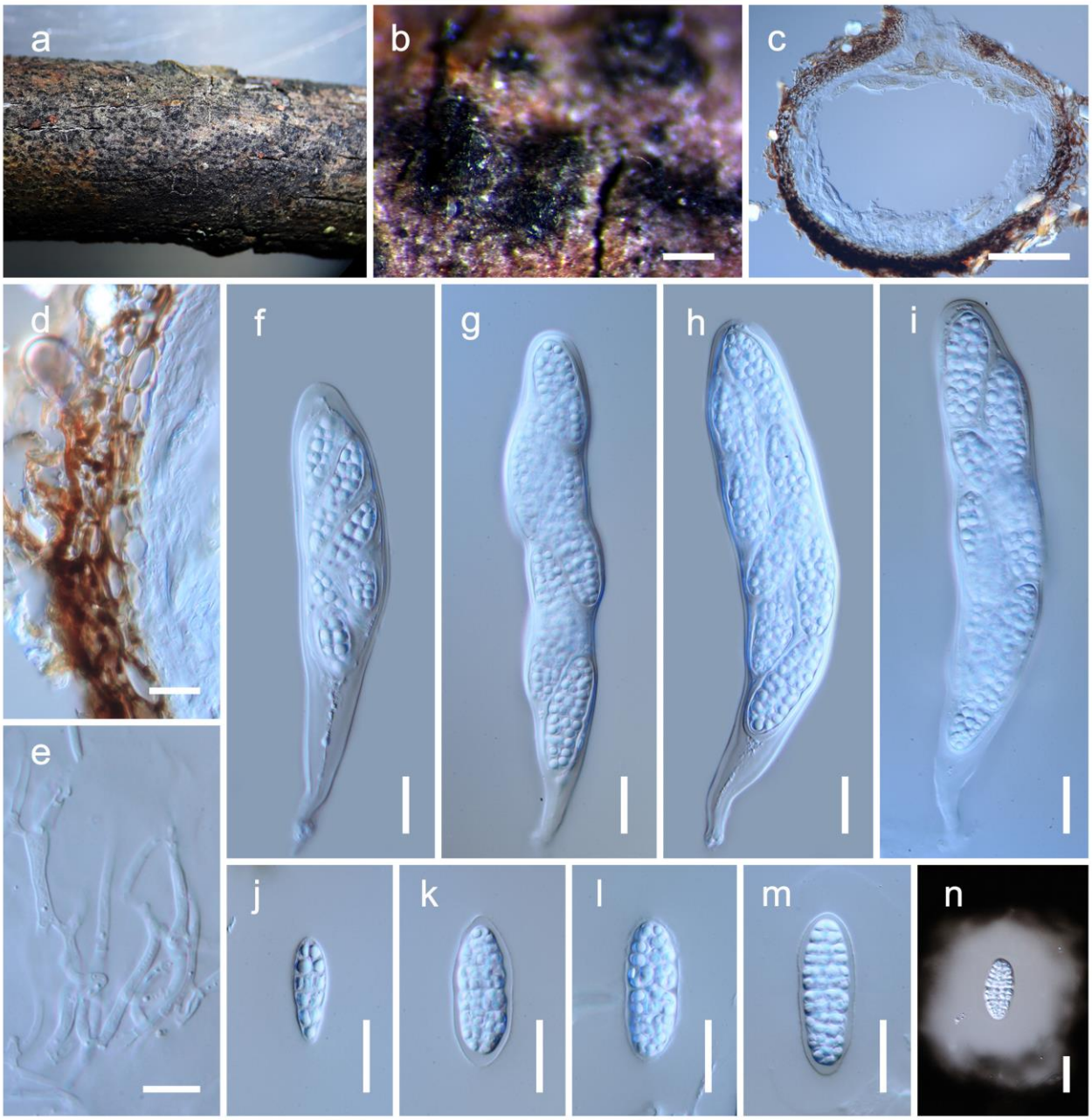

Fig. 3 – *Muritestudina chiangraiensis* (MFLU 17-2645, **holotype**). a, b Appearance of ascomata on host substrate. c Section of ascoma. d Peridium. e Pseudoparaphyses. f-i Asci. j-m Ascospores. n Ascospore stained with Indian Ink. Scale bars: b = 200 µm, c = 100 µm, f–i, j–n = 20 µm, e = 10 µm.

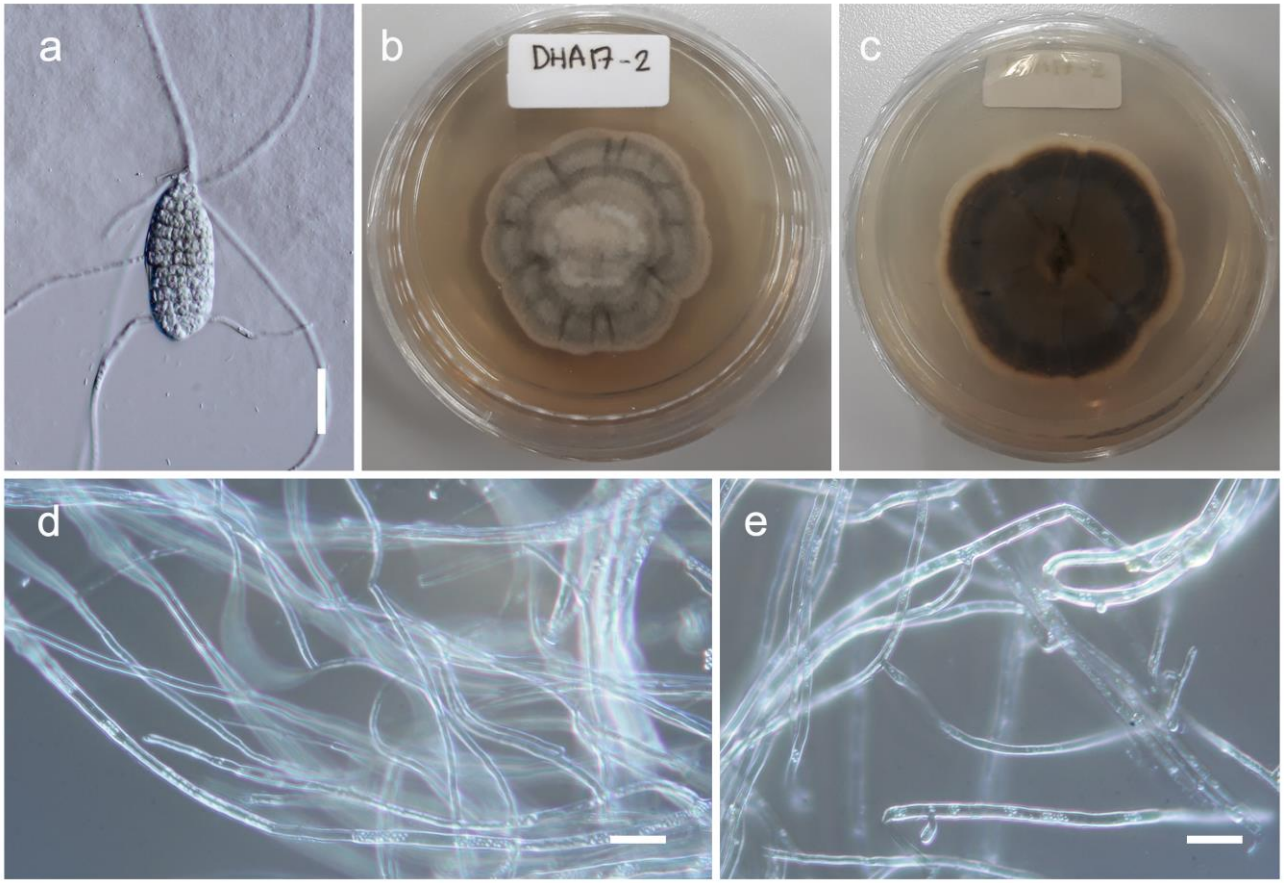

Fig. 4 – *Muritestudina chiangraiensis* (**holotype**). a Germinating ascospore. b, c Culture on PDA (note c reverse). d, e Mycelia. Scale bars: a = 20 µm, d, e = 10 µm.

In an attempt to clarify phylogenetic relationships, we also observe that there is a suite of morphological characters that can delineate species of the *Testudinaceae* from closely related families such as *Pseudoastrosphaeriellaceae* and *Tetraplosphaeriaceae*. Species of *Testudinaceae* are mainly characterized by globose to subglobose ascomata, a peridium comprising cells of *textura angularis*, clavate to cylindrical asci with short pedicels and ellipsoidal, verrucose to verruculose ascospores with mostly one longitudinal septum. *Pseudoastrosphaeriellaceae* are characterized mainly by lenticular or hemisphaerical ascomata, a peridium comprising pseudoparenchymatous cells of *textura angularis*, clavate to cylindric-clavate asci with long pedicels and fusiform to clavate, smooth-walled multi-septate ascospores (Phookamsak et al. 2015). *Tetraplosphaeriaceae* have hemisphaerical ascomata with a flattened base, a peridium comprising polygonal to hyphoid cells, clavate to cylindrical asci with short pedicels and narrowly fusiform to broadly cylindrical, smooth-walled ascospores with 1–3-septa (Hyde et al. 2013). Our ML phylogenies also provide further taxonomic insights to better define the generic boundaries within the *Testudinaceae*. Despite sparse taxon sampling and moderate support for the internal subclades, we believe that the genera should be considered as distinct. Some inconsistencies between the estimated ML and MP trees were observed with respect to the placement of several genera within *Testudinaceae*, but we believe that this may be due to unequal evolutionary rates, different genealogical histories and possibility of insufficient phylogenetic signal of the nuclear and protein genes analysed herein.

From a morphological perspective, the genera of *Testudinaceae* appear to have different morphologies. A summary of the main morphological characteristics that we consider useful at the intergeneric level are summarized in Table 3. For the time being, it seems appropriate to maintain all of the above-mentioned genera in *Testudinaceae*. Interestingly this is the first record of hyaline muriform spores in *Testudinaceae*. Taxa in *Halojulellaceae* and *Shiraiaceae* produce hyaline muriform spores similar to our new isolate, but these families are not closely related to

Table 3 Synopsis of genera in *Testudinaceae*

Genus / Characteristics	***Angustospora***	***Lepidosphaeria***	***Lojkania***	***Neotestudina***	***Testudina***	***Ulospora***	***Verruculina***	***Muritestudina***
Habitat	Aquatic (freshwater)	Aquatic (muddy sand)	Terrestrial (saprotrophic)	Terrestrial (human pathogen)	Terrestrial (saprobic or parasitic)	Terrestrial	Aquatic (common on mangrove wood)	Terrestrial (saprobic)
Ascomata shape	Globose to subglobose	Globose to subglobose	Ovoid to obpyriform	Globose to subglobose	Globose, spherical or tuberous	Subglobose	Ampulliform or depressed ellipsoidal	Globose to subglobose
Ostiole	Present	Present	Present	Present	Present	Absent	Present	Present
Asci shape	Clavate	Clavate	Cylindrical	Clavate	Clavate or spherical	Ovoid to pyriform	Cylindrical	Cylindrical-clavate
Spore arrangement	Overlapping biseriate		Overlapping uniseriate	Irregularly arranged	Uniseriate	Irregularly arranged	Uniseriate	Overlapping biseriate
Spore color	Dark-brown to black	Brown	Pale brown to dark brown, or reddish brown,	Brown	Brown	Brown	Dark brown	Hyaline
Furrows on spores	Lacking furrows	Lacking furrows	Lacking furrows	Lacking furrows	Lacking furrows	three to six deep furrows alternating in each cell of the spore	Lacking furrows	Lacking furrows
Spore surface	Smooth	Minute granulate ornamentation	Smooth	Smooth	Ornamentation a reticulum	Smooth	Verrucose to verruculose	Smooth
Other spore characters	Biconic, polar cells are lighter	Apiculate	Ellipsoidal	Broadly truncate	Ellipsoidal, composed of rounded cells	Ellipsoidal, longitudinal fissures on the ascospores	Ellipsoidal	Ellipsoidal, large guttules in almost each and every cell
Septa in spores	5	1	1	1	1	1	1	Muriform
References	Li et al. 2016	Hawksworth (1979)	Phookamsak et al. (2015)	Hawksworth (1979)	Hawksworth (1979)	Hawksworth (1979)	Hyde et al. (2013)	This study
Number of epithets in Index Fungorum (2017)	1	2	15	4	1	1	1	1

Testudinaceae in DNA based sequence data analyses (Hyde et al. 2013, Liu et al. 2017). We believe that this character as homoplasious and therefore not phylogenetically significant. In addition, *Halojulellaceae* members are recorded from marine habitats and *Shiraiaceae* members only recorded on bamboo, whereas our new isolate was collected from undetermined terrestrial twigs on the ground.

Acknowledgements

Dhanushka Wanasinghe and Saowaluck Tibpromma would like to thank the Molecular Biology Experimental Center at Kunming Institute of Botany for facilities for molecular work. Saowaluck Tibpromma also thanks the Mushroom Research Foundation (MRF), Chiang Rai, Thailand for the financial support of her study. K.D. Hyde would like to thank the National Research Council of Thailand (Mae Fah Luang University) for grants ‘‘Biodiversity, phylogeny and role of fungal endophytes of *Pandanaceae*’’ (Grant No.: 592010200112). R. Jeewon thanks University of Mauritius and Mae Fah Luang University for research support. Ishani D. Goonasekara is thanked for her help during collecting trips.

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