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Morphology and phylogeny of *Phaeoseptum mali* sp. nov. (Phaeoseptaceae, Pleosporales) on bark of *Malus halliana*

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Abstract

A novel species, *Phaeoseptum mali* is described based on its distinct morphs and phylogenetic relationships among members of Phaeoseptaceae (Pleosporales). *Phaeoseptum mali* can be distinguished from other species of *Phaeoseptum* by its ascomatal, asci and ascospore characters. A detailed illustration and a description of *P. mali* are provided. Phylogenetic analyses of a combined LSU, ITS, SSU, *tef1*, and *rpb2* sequence dataset confirmed its placement within Phaeoseptaceae and its relationships to *P. aquaticum* and *P. terricola.*

Key words – 1 new species – China – Dicotyledon – Dothideomycetes – Fungus

Introduction

Among the Dothideomycetes (bitunicate ascomycetes), Pleosporales is one of the most diverse orders with many new species recently described incorporating DNA sequence data (Kodsueb et al. 2006, Pinnoi et al. 2007, Zhang et al. 2008, 2009, Wanasinghe et al. 2017a, b, 2018, Wijayawardene et al. 2017, 2018, Hyde et al. 2018). Molecular dating has also been used to clarify evolutionary relationships across these bitunicates (Liu et al. 2017).

The family Phaeoseptaceae was established by Hyde et al. (2018), and contains two accepted genera, *Lignosphaeria* and *Phaeoseptum* (with *P. aquaticum* as the generic type). Putative strains of *Decaisnella formosa* (BCC 25616 and BCC 25617), *Neolophiostoma pigmentatum* (MFLUCC 10–0129), and *Thyridaria macrostomoides* (GKM 1033 and GKM 1159), have also been reported to belong to this family (Ariyawansa et al. 2015, Phukhamsakda et al. 2016, Liu et al. 2017, Hyde et al. 2018). Species of Phaeoseptaceae commonly occur on decaying wood in both terrestrial and aquatic habitats. *Phaeoseptum* was introduced based on collections from a woody substrate submerged in a freshwater ecosystem. According to Zhang et al. (2013), the characteristics of the genus include immersed ascomata, narrowly cellular pseudoparaphyses, cylindrical to broadly clavate asci, and dictyosporous, pale brown ascospores with thickened septa.

In this study, we provide an update on the phylogeny of the Phaeoseptaceae and describe a novel species of *Phaeoseptum* from *Malus halliana* collected in China. The phylogeny is based on a multi-gene dataset.

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Materials & Methods

Sample collection, morphological study, and isolation

Fresh specimens of the bark of *Malus halliana* Koehne were collected from southern part of China, in the botanical garden of Kunming Institute of Botany, Chinese Academy of Science, Kunming, Yunnan, China. The specimens were packed into paper bags and transported to the laboratory. Pure cultures were obtained from single ascospores on malt extract agar (MEA; 62 g/l) in distilled water following the method of Chomnunti et al. (2014). Cultures were incubated at 25 °C for one month under standard light cycles of 12 hrs light/ 12 hrs dark (Liu et al. 2010). The type specimen is deposited in Mae Fah Luang University (MFLU) herbarium. The ex-type living culture is deposited in the Mae Fah Luang Culture Collection (MFLUCC). Faces of fungi number (Jayasiri et al. 2015) and Index Fungorum number (www.indexfungorum.org) are provided. Samples were examined using a Nikon ECLIPSE 80i compound microscope and photographed with a Canon 600D digital camera fitted to the microscope. Measurements were made using Tarosoft (R) Image Frame Work programme and photo-plates were made by using Adobe Photoshop CS6 Extended version 10.0 software (Adobe Systems, United States).

DNA extraction, PCR amplification and sequencing

DNA was extracted from mycelium by using Biospin Fungus Genomic DNA Extraction Kit (BioFlux) (Hangzhou, P.R. China) and gene extraction kit (Bio Basic, Canada). PCR amplification was carried out using primers LROR/LR5 for the nuclear ribosomal large subunit 28S rDNA gene (LSU), NS1/NS4 for the nuclear ribosomal small subunit 18S rDNA gene (SSU) and ITS5/ITS4 for internal transcribed spacer rDNA region and covered 5.8S ribosomal part (ITS1, 5.8S rDNA and ITS2); partial fragments of the translation elongation factor 1-alpha (*tef1*) gene region was amplified using primers EF1-983F and EF1-2218R; and partial RNA polymerase subunit II (*rpb2*) was amplified with primers RPB2-5f and RPB2-7cr (Vilgalys & Hester 1990, White et al. 1990, Carbone & Kohn 1999, Sung et al. 2007). Primer sequences are available at the WASABI database at the AFTOL website (aftol.org). Amplification conditions followed Phukhamsakda et al. (2016). Purified PCR products were sequenced with primers mentioned above by Shanghai Sangon Biological Engineering Technology & Services Co. (Shanghai, P.R. China).

Sequence alignment and phylogenetic analyses

The consensus sequences of the novel species were generated in Geneious (version 7.1.9 created by Biomatters, available from www.geneious.com). Similar sequences were retrieved using BLAST searches in GenBank (www.ncbi.nlm.nih.gov) and also from Ariyawansa et al. (2015), Phukhamsakda et al. (2016), Hyde et al. (2017), and these are listed in the accession table in Appendix 1. Sequences were aligned with MAFTT version 7.220 (Katoh & Standley 2013) online sequence alignment tools (mafft.cbrc.jp/alignment/server), with minimal adjustments of the ambiguous nucleotides by visual examination and manually corrected in AliView programme (Larsson 2014). Leading or trailing gaps exceeding from primer binding site were trimmed from the alignments prior to tree building and alignment gaps were treated as missing data. The concatenation of the multigene alignment was created in MEGA 6 (Tamura et al. 2013).

A maximum likelihood analysis including 1,000 bootstrap replicates was performed at CIPRES using RAxML version 8.2.10 as part of the "RAxML-HPC2 on XSEDE" tool (Stamatakis 2006, Stamatakis et al. 2008, Miller et al. 2010). The general time reversible model (GTR) using proportions of invariable sites were applied with a discrete gamma distribution and four rate classes model were applied for nucleotide substitution. The best scoring tree was selected with a final likelihood value of -29062.646359. Maximum likelihood bootstrap support (MLBS) equal or greater than 70% are given near to each node (Fig. 1).

The majority rule consensus tree (MRC) from the Bayesian-inference analysis based on combined dataset of LSU, SSU, ITS, *tef1*, and *rpb2* sequence data was performed by using MrBayes on XSEDE version 3.2.6 (Ronquist et al. 2012). The model of evolution was inferred

using jModeltest 2.1.7 (Guindon & Gascuel 2003, Darriba et al. 2012). In our analysis, GTR+I+G model was used for each partition. The Bayesian inference posterior probabilities (PP) distribution (Zhaxybayeva & Gogarten 2002) was estimated by Markov Chain Monte Carlo sampling (MCMC). Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 1000th generation, thus 1,000 trees were obtained. The suitable burn-in phases were determined by traces inspected in Tracer version 1.6 (Rambaut et al. 2014). Based on the tracer analysis, the first 200 trees representing 20% of burn-in phase of the analyses were discarded while the remaining trees were used for calculating posterior probabilities in the majority rule consensus tree (critical value for the topological convergence diagnostic set to 0.01).

Phylogenetic trees and data files were visualized in FigTree v. 1.4 (Rambaut & Drummond 2008). The phylogram with bootstrap values and/or posterior probabilities on the branches are presented in Fig. 1 by using graphical options available in Adobe Illustrator CS v. 6. All sequences generated in this study were submitted to GenBank (Appendix 1). The finalized alignment and tree were deposited in TreeBASE, submission ID: 24111 (http://www.treebase.org/). Maximum likelihood bootstrap values equal to or greater than 70% with Bayesian posterior probabilities (PP) equal or greater than 0.90 are presented below or above each node (Fig. 1).

Results

Phylogenetic analyses

The phylogeny based on a combined LSU, ITS, SSU, *tef1*, and *rpb2* sequence data included 38 taxa representing Phaeoseptaceae and closely related families in Pleosporales. The dataset comprised a total of 4693 characters (965 characters for LSU, 617 characters for ITS, 1097 characters for SSU, 926 characters for *tef1*, 1061 characters for *rpb2*). *Aigialus grandis* (BCC 20000) and *Neoastrosphaeriella krabiensis* (MFLUCC 11−0025), from Aigialaceae, were used as the out group. The matrix has 2061 distinct alignment patterns, with 43.3% undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.245073, C = 0.249429, G = 0.276047, T = 0.229452; substitution rates AC = 1.232711, AG = 2.440857, AT = 1.291268, CG = 1.269789, CT = 6.315893, GT = 1.000000; gamma distribution shape parameter α = 0.766883. A best scoring phylogram from maximum likelihood analysis showed similar topologies with the 50% majority rule consensus tree (MRC) from Bayesian-inference analysis. A best scoring RAxML tree resulted with the value of likelihood: -29062.646359 (Fig. 1). Single gene phylogenetic analyses were also performed to compare tree topologies and clade stability with trees generated with the combined gene dataset. Phylogenies from individual genes were congruent with those obtained from the combined dataset (results not shown).

The new strain of *Phaeoseptum* clusters with the two other known species of *Phaeoseptum* (Fig. 1). The species is basal to *P. aquaticum* CBS 123113 and *P. terricola* MFLUCC 10−0102 and all three *Phaeoseptum* species constitute a strongly supported monophyletic clade (100% in MLBT, 1.00 BSPP).

Taxonomy

Phaeoseptum mali Phukhams. & K.D. Hyde, sp. nov. Fig. 2

Index Fungorum number: IF556265; Facesoffungi number: FoF05982

Etymology – the epithet "*mali*" referring to host substrate, *Malus halliana*

Holotype – MFLU 19–0406

Saprobic on dead stems of *Malus halliana* Koehne. Sexual morph *Ascomata* 320–375 × 320–360 μm ($\bar{x}$ = 348 × 327 μm, n = 5), on surface of the host, visible as black spots or having a convex surface, sometime covered with a pseudoclypeus, immersed, solitary, scattered, globose, dark brown hyphae radiating outwards from the perdium wall, coriaceous, black to dark brown, rough-walled, papillate, with an apical ostiole. *Ostiole* central, dark brown to black, papillate, opened pore, ostiolate with periphyses. *Peridium* 5–19(–25 at apex) μm wide diam., multilayer, outer layer

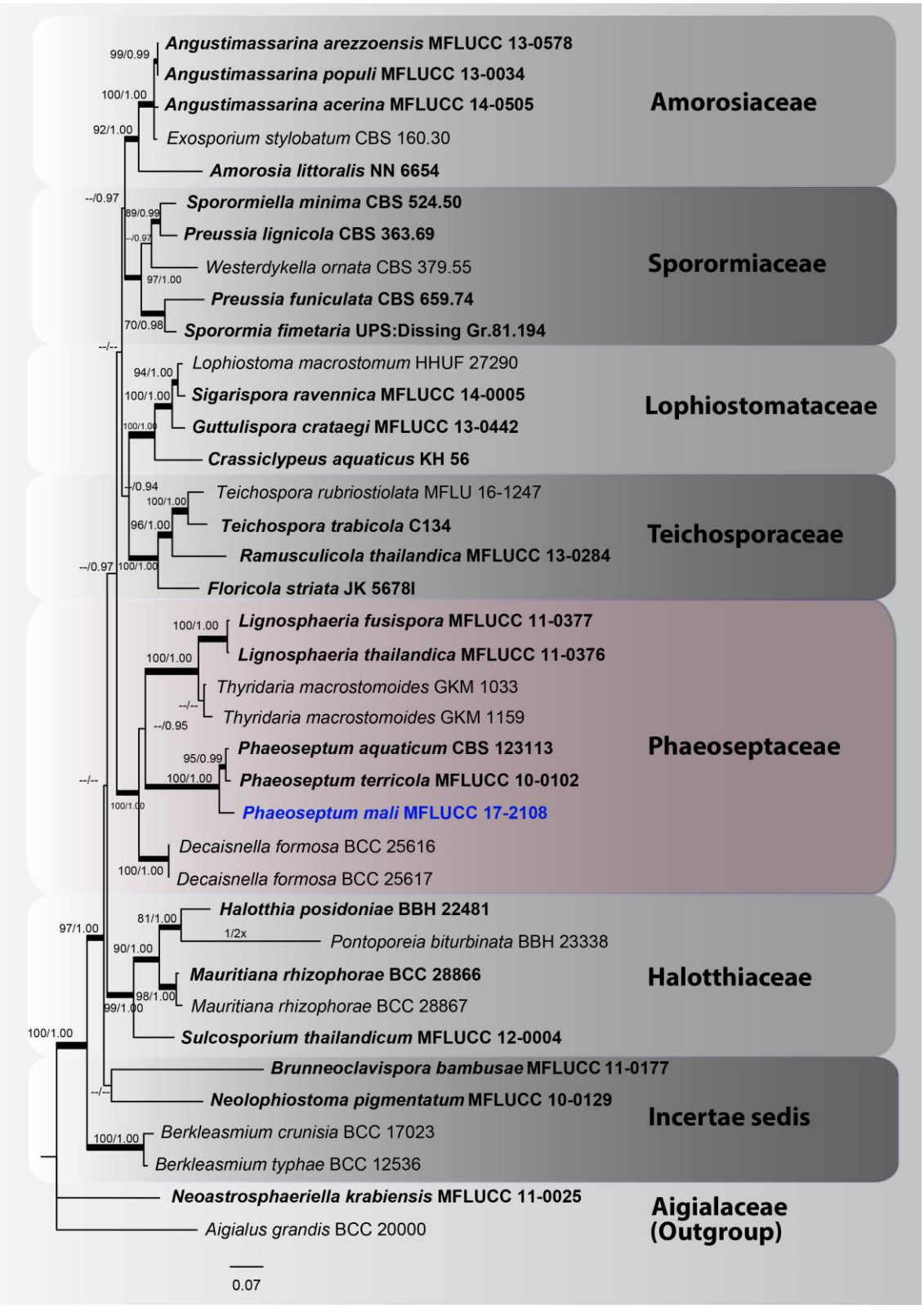

Fig. 1– Phylogram of 50% majority rule consensus tree from the Bayesian-inference analysis based on combined dataset of LSU, ITS, SSU, *tef1*, and *rpb2* sequence data representing Phaeoseptaceae and closely related families in Pleosporales. The tree is rooted with *Aigialus grandis* (BCC 20000) and *Neoastrosphaeriella krabiensis* (MFLUCC 11–0025) in Aigialaceae (Pleosporales). Bootstrap support values for maximum likelihood analysis greater than 70% and clade credibility values greater than 0.90 (the rounding of values to 2 decimal proportions) from Bayesian-inference analysis are labelled below or above the nodes. Ex-type strains are in bold and black, the new isolate is indicated in blue.

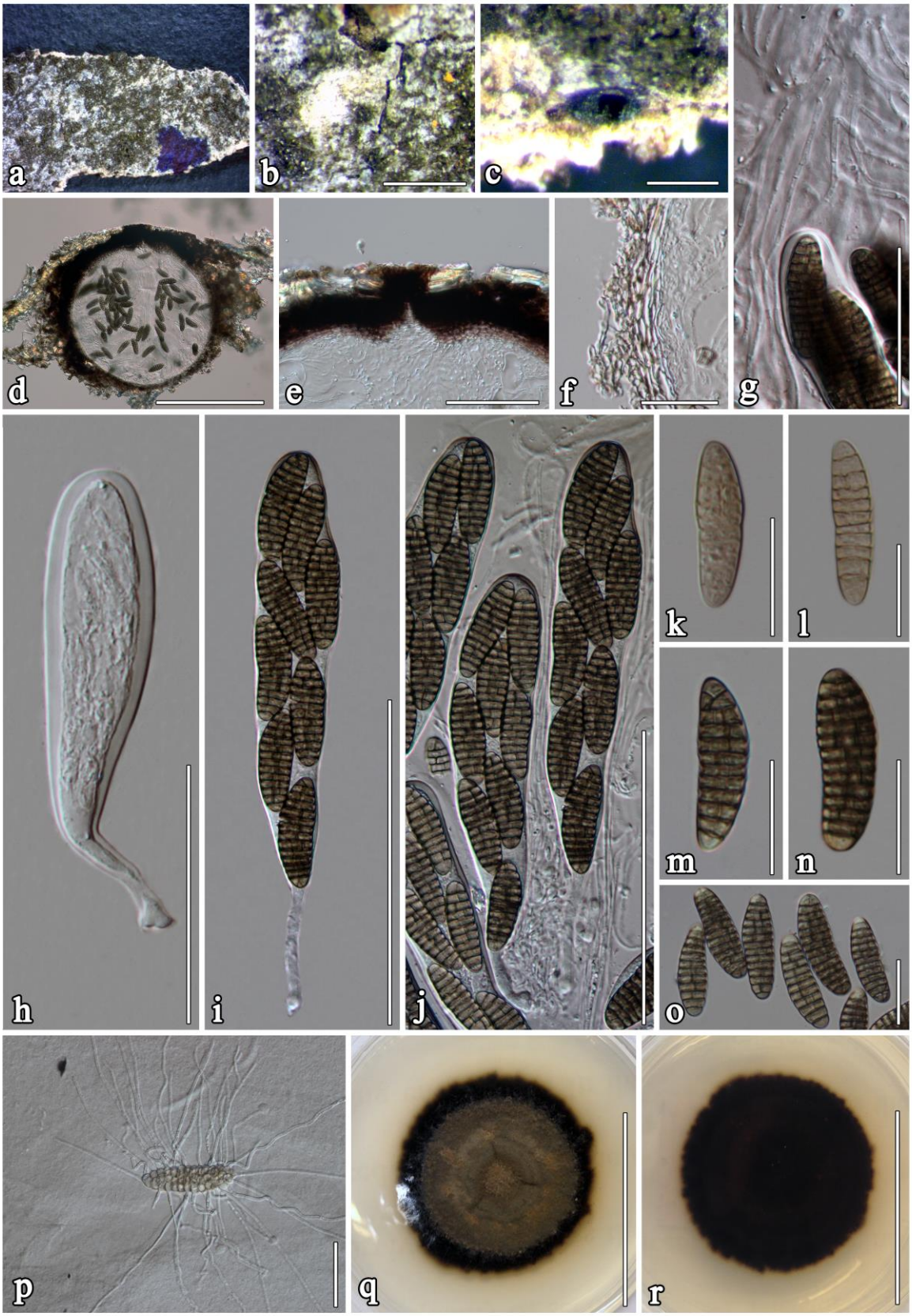

Fig. 2 – *Phaeoseptum mali* (MFLU 19–0406, holotype). a–c Appearance of ascomata on *Malus halliana*. d Vertical section through ascoma. e Ostiole canal. f Section of partial peridium layer. g Trabeculate pseudoparaphyses. h–j Developmental stages of asci. k–o Ascospores. p Germinated ascospore. q, r Culture on MEA. Scale bars: b = 500 µm, c, d = 200 µm, e, g, h = 50 µm, f, k–p = 20 µm, i, j = 100 µm, q, r = 3 cm.

composed of 8–11 layers of dark brown to light brown, somewhat flattened cells of *textura angularis*, the inner layer comprising thin, hyaline cells. *Hamathecium* of dense, 0.9–1.7 μm wide ($\bar{x}$ = 1.4 μm, n = 50), filiform branches, anastomosing above asci, transverse septate, trabeculate pseudoparaphyses. *Asci* 85–190 × 19–32 μm ($\bar{x}$ = 140 × 25 μm, n = 30), 8-spored, bitunicate, fisitunicate, cylindrical-clavate to elongate-clavate, apically rounded, ocular chamber clearly visible when immature. *Ascospores* 27–38 × 8–13 μm ($\bar{x}$ = 33 × 10 μm, n = 70), biseriate, muriform, allantoid, asymmetrical, partial overlapping, broad cylindrical, tapering towards the ends, round at both ends, with 11–14 transverse septa, and 1(–2) longitudinal septum in each cell, initially hyaline, becoming yellowish to brown at maturity, constricted at the median septum, cell above median septum slightly wider than below, rough-walled with verruculose surface, without mucilaginous sheath. Asexual morph Undetermined.

Culture characters – Colonies on MEA reaching 50 mm diam. after four weeks of incubation at 25 °C, from above dark brown radiating outwards, wrinkled folded at the middle, dense, circulate in shape, flattened, umbonate, entire edge, fairly fluffy; reverse black at the middle and dark brown at the edges, orange pigment diffusing into the agar.

Material examined – China, Yunnan Province, Kunming city, in the botanical garden of Kunming Institute of Botany, on decay twigs of *Malus halliana* Koehne (Rosaceae), 21 March 2016, C. Phukhamsakda, CPCN09 (MFLU 19–0406, holotype), isotype in HKAS, ex-type living culture, MFLUCC 17–2108.

Key to the species of *Phaeoseptum*

1. Ascospores 19–25 × 5–7 μm, cylindrical, without a median septum ……..……..*P. terricola*
1. Ascospores with a median septum ……..……2
2. Ascospores 30–38 × 9–12 μm, broadly fusoid with rounded ends ..…….……*P. aquaticum*
2. Ascospores 27–38 × 8–13 μm, broadly cylindrical with rounded ends ……..…...*P. mali*

Discussion

Malus halliana or hall crab apple is considered as a native tree of China and is widely distributed throughout Yunnan Province. Wild and cultivated *M. halliana* commonly occurs in shrubland forests and some basin areas (Zhang et al. 1993, Liu & Tang 2004). It is also a major ornamental tree in Yunnan Province, where it grows in woodland garden with a dappled shade of sunlight (Rhodes & Maxted 2016). At least 20 genera and 40 species of pleosporalean fungi have been recorded from *Malus* spp. (Farr & Rossman 2019, Index Fungorum 2019) but interestingly only three fungi have been recorded on *M. halliana*; these are *Alternaria brassicicola* (Pleosporaceae), *Magnibotryascoma mali* (Teichosporacee) and *Paucispora kunmingense* (Lophiostomataceae) (Gu 2009, Hyde et al. 2017).

Species of Phaeoseptaceae live on woody substrates in various ecosystems. The family was introduced by Hyde et al. (2018), typified by *Phaeoseptum* with the type species *P. aquaticum* Zhang, J. Fourn. & K.D. Hyde. Recent studies on the family have included *Decaisnella* (*D. Formosa*), *Lignosphaeria* (*L. fusispora* and *L. thailandica*), *Neolophiostoma* (*N. pigmentatum*), *Phaeoseptum* (*P. aquaticum* and *P. terricola*), and *Thyridaria* (*T. macrostomoides*((Abdel-Wahab & Jones 2003, Zhang et al. 2013, Ariyawansa et al. 2015, Thambugala et al. 2015, Phukhamsakda et al. 2016, Hyde et al. 2018). Initially, *Phaeoseptum* was reported to belong to the Halotthiaceae based on morphological characters and phylogenetic analysis of LSU sequence data (Zhang et al. 2013). With the discovery of more fungal taxa, the genus was later reported to constitute a monophyletic clade in Phaeoseptaceae (Hyde et al. 2018).

In this study, we introduce *P. mali* isolated from bark of fallen twigs of *Malus halliana*. The species is assigned to *Phaeoseptum* based on its unique morphological features such as globose and immersed ascomata, anastomosing pseudoparaphyses, cylindrical-clavate, pedicellate asci, allantoid and brown muriform ascospores. *Phaeoseptum mali* has ascomata 348 × 327 μm and asci 140 × 25 μm, while *P. terricola* has ascomata 172 × 184 μm and asci 79 × 16 μm which are smaller in size than *P. mali* (Hyde et al. 2018). Based on morphology, *P. mali* is more similar to *P.*

aquaticum, but the latter species has an ascomatal size of 300–400 × 400–600 µm which is larger than *P. mali* (Zhang et al. 2013). In addition, *P. aquaticum* has been reported from submerged material from freshwater habitat while *P. mali* was collected from terrestrial habitats (Zhang et al. 2013). In a BLASTn search on NCBI GenBank, the closest matches to sequences of our isolate (MFLUCC 17–2108) is *P. terricola* strain MFLUCC 10–0102. The ITS sequence is 90% similar (11.6 % nucleotide differences in the ITS regions) while the *rpb2* sequence is 96% similar (4 % nucleotide differences in the *rpb2* regions). Phylogenetic trees obtained from Bayesian analysis and maximum likelihood analysis were similar in overall topology at the family level as previously reported (Ariyawansa et al. 2015, Phukhamsakda et al. 2016, Hyde et al. 2018), except for *Neolophiostoma pigmentatum* (MFLUCC 10–0129) and *Brunneoclavispora bambusae* (MFLUCC 11–0177), which are now classified as *incertae sedis*. Therefore, our new strain is introduced as a new species of *Phaeoseptum* based on guidelines proposed by Jeewon & Hyde (2016).

Phylogeny recovered herein also indicates a different placement of *Neolophiostoma pigmentatum* Boonmee & K.D. Hyde. Based on multigene phylogenetic analyses, *N. pigmentatum* was originally referred to the Halotthiaceae (Ariyawansa et al. 2015, Wijayawardene et al. 2018), while, Hyde et al. (2018) transferred it to Phaeoseptaceae. In our phylogeny, *N. pigmentatum* formed a lineage separated from both Halotthiaceae and Phaeoseptaceae. This topological difference may be because of a highly variable gene region such as ITS which was analysed with increased taxon sampling. Another peculiar phylogenetic finding in this study is the placement of *Thyridaria macrostomoides*, which is similar to *Lignosphaeria* in having phragmosporous, elongate, cylindrical to fusiform and hyaline ascospores. However, the type specimen of *Thyridaria* (*T. broussonetiae* (Sacc.) Traverso) is in a distinct lineage in Thyridariaceae (Jaklitsch & Voglmayr 2016). To clarify the familial placement, the material needs to be re-examined (Abdel-Wahab & Jones 2003, Mugambi & Huhndorf 2009).

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Appendix 1 GenBank accession numbers used in this study. GenBank accessions marked in bold represent new sequences generated in the current study.

Taxon	Culture collection	GenBank accession numbers				
		LSU	SSU	ITS	*tef1*	*rpb2*
Aigialus grandis	BCC 20000	GU479775	GU479739	–	GU479839	GU479814
morosia littoralis	NN 6654	AM292055	AM292056	AM292047	–	–
Angustimassarina acerina	MFLUCC 14–0505	KP888637	NG_063573	NR_138406	KR075168	–
Angustimassarina arezzoensis	MFLUCC 13–0578	KY496722	KY501113	KY496743	KY514392	–
Angustimassarina populi	MFLUCC 13–0034	KP888642	KP899128	KP899137	KR075164	–
Berkleasmium crunisia	BCC 17023	DQ280271	–	DQ280265	–	–
Berkleasmium typhae	BCC 12436	DQ280275	–	DQ28026	–	–
Brunneoclavispora bambusae	MFLUCC 11–0177	KT426562	MG520999	NR_156401	–	–
Crassiclypeus aquaticus	CBS 143639	LC312526	LC312468	LC312497	LC312555	LC312584
Decaisnella formosa	BCC 25617	GQ925847	GQ925834	–	GU479850	GU479824
Decaisnella formosa	BCC 25616	GQ925846	GQ925833	–	GU479851	GU479825
Exosporium stylobatum	CBS 160 30	JQ044447	–	JQ044428	–	–
Floricola striata	JK 5678I	GU301813	GU296149	–	GU479852	GU371758
Guttulispora crataegi	MFLUCC 13–0442	NG_059563	KP899125	NR_154070	KR075161	–
Halotthia posidoniae	BBH 22481	GU479786	GU479752	–	–	–
Lignosphaeria fusispora	MFLUCC 11–0377	KP888646	–	KP899140	–	–
Lignosphaeria thailandica	MFLUCC 11–0376	KP888645	–	KP899139	–	–
Lophiostoma macrostomum	HHUF 27290	AB433273	AB521731	AB433275	LC001752	–
Mauritiana rhizophorae	BCC 28867	GU371825	GU371833	–	GU371818	GU371797
Mauritiana rhizophorae	BCC 28866	GU371824	GU371832	–	GU371817	GU371796
Neoastrosphaeriella krabiensis	MFLUCC 11–0025	JN846729	JN846739	NR_120004	–	–
Neolophiostoma pigmentatum	MFLUCC 10–0129	KT324588	KT324589	KT324587	KT324590	–
Phaeoseptum aquaticum	CBS 123113	JN644072	–	KY940803	–	–
Phaeoseptum mali	**MFLUCC 17–2108**	**MK625197**	**MK625211**	**MK659580**	**MK647990**	**MK647991**
Phaeoseptum terricola	MFLUCC 10–0102	MH105779	MH105780	MH105778	MH105781	MH105782
Pontoporeia biturbinata	BBH 23338	GU479796	GU479763	–	–	GU479837
Preussia lignicola	CBS 363.69	DQ384098	–	GQ203783	–	–
Preussia funiculata	CBS 659 74	GU301864	GU296187	–	GU349032	GU371799
Ramusculicola thailandica	MFLUCC 13–0284	KP888647	KP899131	KP899141	KR075167	–
Sigarispora ravennica	MFLUCC 14–0005	KP698414	KP698415	KP698413	–	–
Sporormia fimetaria	UPS:Dissing Gr.81.194	GQ203729	–	GQ203769	–	–

Appendix 1 Continued.

Taxon	Culture collection	GenBank accession numbers				
		LSU	SSU	ITS	*tef1*	*rpb2*
Sporormiella minima	CBS 524.50	MH868263	–	MH856741	–	–
Sulcosporium thailandica	MFLUCC 12–0004	KT426563	KT426564	MG520958	–	–
Teichospora rubriostiolata	MFLU 16–1247	MG829086	MG829186	MG828974	KU601609	KU601599
Teichospora trabicola	C134	KU601591	–	KU601591	KU601601	KU601600
Thyridaria macrostomoides	GKM 1033	GU385190	–	–	GU327776	–
Thyridaria macrostomoides	GKM 1159	GU385185	–	–	GU327778	–
Westerdykella ornata	CBS 379.55	NG_057861	GU296208	NR_103587	GU349021	GU371803