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Taxonomy and phylogeny of *Sparticola muriformis* sp. nov. on decaying grass

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Abstract

Members of Sporormiaceae are saprobes on plant debris, wood, soil and dung and are sometimes endophytes. In this study, a saprobic species was collected from decaying grass in China. Maximum-parsimony, maximum-likelihood and Bayesian Inference analyses of combined ITS, LSU, SSU, TEF1-α and RPB2 sequence data clarified the phylogenetic affinity in *Sparticola*. The isolate was confirmed as a new species based on morphological and phylogenetic analyses. *Sparticola muriformis* sp. nov. is distinguished from other taxa in Sporormiaceae by having muriform ascospores.

Key words: – Dothideomycetes – muriform – Pleosporales – Sporormiaceae

Introduction

Pleosporales is the largest order in Dothideomycetes comprising a quarter of all Dothideomycetes (Kirk et al. 2008, Zhang et al. 2012, Hyde et al. 2013, Wijayawardene et al. 2014, Jayasiri et al. 2015). Pleosporales, currently comprises 43 families based on multi-gene phylogenetic analyses (Zhang et al. 2016). Various papers have provided backbone trees for various families in this important order (e.g. Phaeosphaeriaceae - Phookamsak et al. 2014, Leptosphaeriaceae - Ariyawansa et al. 2105a, Pleosporaceae - Ariyawansa et al. 2015b, Sporormiaceae - Phukhamsakda et al. 2016). Thus, we have a much better understanding of the group.

Sporormiaceae, a member of Pleosporales comprises mostly saprobic taxa on dung, but has also been recorded on plant debris, soil and wood and occasionally as endophytes (Zhang et al. 2012, Hyde et al. 2013, Kruys 2015, Phukhamsakda et al. 2016). The family contains nine genera including two recently introduced genera, *Sparticola* Phukhams. et al. and *Forliomyces* Phukhams. et al.

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Grass is a major example of standing litter in many temperate and tropical countries (Wong & Hyde 2001) and fungi are important in the decay process (Poon & Hyde 1998, Wong & Hyde 2001, Purahong & Hyde 2011). According to Hawksworth & Rossman (1997) Poaceae is a group of hosts that can be investigated in order to discover new fungal species. We have been surveying the micro-fungi on various members of Poaceae, with the intention of providing a better understanding of their biodiversity, ecology and phylogeny (Poon et al. 1998, Wong et al. 2001). Most taxa in Phaeosphaeriaceae are found on grasses (Phookamsak et al. 2014). Most of our studies to date, however, have been confined to bamboo (Liu et al. 2011, Phookamsak et al. 2015, Dai et al. 2017). In this study, we collected microfungi on dead grass in Yunnan Province, China, and isolated a pleosporalean taxon. Subsequently, with further analyses, we introduce *Sparticola muriformis* sp. nov.

Materials and Methods

Sample collection, morphological studies and isolation

Fresh specimens were collected from different sites in Yunnan Province in China. Collected specimens were processed and examined following the method described in Wanasinghe et al. (2014). Hand-cut sections of the fruiting structures were mounted in water for microscopic studies and photomicrography. The taxon was examined with a Nikon ECLIPSE 80i compound microscope and photographed with a Canon EOS 600D digital camera fitted to the microscope. Measurements of photomicrographs (ascomatal height and width, ostiolar length and width, peridium width, asci length and width, ascospore length and width) were made with the Tarosoft (R) Image Frame Work program and images used for figures processed with Adobe Photoshop CS3 Extended version 10.0 (Adobe Systems, USA).

Single ascospore isolation was carried out following the spore suspension method described in Chomnunti et al. (2014). Germinated spores were individually transferred to potato dextrose agar (PDA) plates and grown at room temperature (10–16 ºC) in the daylight. Colony colour and other characters were observed and measured after a week and again after three weeks. The specimens were deposited in the Mae Fah Luang University (MFLU) Herbarium, Chiang Rai, Thailand and Herbarium of Cryptogams, Kunming Institute of Botany Academia Sinica (HKAS). Living cultures are also deposited at the Culture Collection at Mae Fah Luang University (MFLUCC), Kunming Institute of Botany Culture Collection (KUMCC). Facesoffungi (FoF) and Index Fungorum (IF) numbers were acquired as in Jayasiri et al. (2015) and Index Fungorum (2017).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from fresh fungal mycelium grown on PDA media at 16 ºC for 4 weeks using the Biospin Fungus Genomic DNA Extraction Kit (BioFlux®, Hangzhou, P. R. China) following the instructions of the manufacturer.

The DNA amplification was performed by polymerase chain reaction (PCR) for the partial sequences of five genes, the internal transcribed spacers (ITS1, 5.8S, ITS2), small subunit rDNA (SSU), large subunit (LSU), RNA polymerase II subunit 2 (RPB2) and part of the translation elongation factor 1-alpha (TEF1-α). The ITS gene was amplified using the primers ITS5 and ITS4 (White et al. 1990), the LSU region was amplified using the primer pair LROR and LR5 (Vilgalys & Hester 1990), SSU was amplified using the primers NS1 and NS4 (White et al. 1990). RPB2 was amplified with primers RPB2-5F and RPB2-7cR (Liu et al. 1999) and. TEF1-α gene region was amplified with primers EF1-983F and EF1-2218R (Carbone & Kohn 1999). Polymerase chain reaction (PCR) was carried out following the protocol of Phookamsak et al. (2014). The quality of PCR amplification was confirmed on 1 % agarose gel electrophoresis stained with ethidium bromide. The amplified PCR fragments were sent to a commercial sequencing provider Shanghai Sangon Biological

Engineering Technology & Services Co., Ltd (Shanghai, P.R. China). The nucleotide sequence data acquired were deposited in GenBank (Table 1).

Phylogenetic analyses

Phylogenetic analyses were conducted based upon the combined gene of LSU, SSU, ITS, TEF1-α and RPB2 sequence data. The topologies of the trees obtained from each gene were compared prior to obtain combined gene tree to confirm the correct overall topology of the phylogenetic tree. The combined gene analysis was performed to obtain a well-resolved phylogenetic tree. The reference nucleotide sequences (Table 1) of selected families of Pleosporales were obtained from the GenBank database and recently published data (Ariyawansa et al. 2014, 2015b, Mapperson et al. 2014, Phukhamsakda et al. 2016). The single gene sequences were initially aligned by MAFFT V.7.036 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013), and improved manually where necessary using Bioedit v.7.2 (Hall et al. 1999). The multiple alignments were combined using BioEdit v. 7.0.9.0 (Hall et al. 1999).

Phylogenetic analyses of combined gene trees were performed using Bayesian Inference (BI), maximum likelihood (ML) and maximum parsimony criteria. Maximum parsimony (MP) analysis was carried with the heuristic search option in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2002). Parsimony bootstrap analyses were performed using the full heuristic search option, random stepwise addition, and 1000 replicates, 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 to determine whether the trees inferred under different optimality criteria were meaningfully different.

Evolutionary models for Bayesian Inference and maximum likelihood were selected independently for each locus using MrModeltest v. 3.7 (Posada & Crandall 1998) under the Akaike Information Criterion (AIC) implemented in PAUP v. 4.0b10. ML 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 and Bootstrap support obtained by running 1000 pseudo replicates.

Bayesian Inference (BI) analysis was conducted with MrBayes v. 3.1.2 (Huelsenbeck & Ronqvist 2001) to evaluate Posterior probabilities (BYPP) (Rannala & Yang 1996; Zhaxybayeva & Gogarten 2002) by Markov Chain Monte Carlo sampling (BMCMC). Two parallel runs were conducted, using the default settings, but with the following adjustments: Six simultaneous Markov chains were run for 5,000,000 generations and trees were sampled every 1000th 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 20% of generated trees were discarded and remaining 80% of trees were used to calculate posterior probabilities (PP) of the majority rule consensus tree.

Phylograms were visualized with FigTree v1.4.0 program (Rambaut 2012) and reorganized in Microsoft power point (2016) and Adobe Illustrator CS5 (Version 15.0.0, Adobe, San Jose, CA). The finalized alignment and tree is deposited in TreeBASE, submission ID: 20787 (http://www.treebase.org/).

Table 1. Taxa used in the phylogenetic analyses and their corresponding GenBank numbers (Fig 1). The newly generated sequence is in bold.

Taxon	Culture accession no.	GenBank accession no.				
		LSU	**SSU**	**ITS**	**RPB2**	**TEF1-α**
Amorosia littoralis	NN 6654^{T}	AM292055	AM292056	AM292047	NA	NA
Angustimassarina acerina	MFLUCC 14–0505^{T}	KP888637	KP899123	KP899132	NA	KR075168
Angustimassarina populi	MFLUCC 13–0034^{T}	KP888642	KP899128	KP899137	NA	KR075164
Angustimassarina quercicola	MFLUCC 14–0506^{T}	KP888638	KP899124	KP899133	NA	KR075169
Eremodothis angulata	CBS 610.74^{T}	DQ384105	DQ384067	GQ203757	NA	GU371821
Floricola striata	JK 5603K^{T}	GU479785	GU479751	NA	NA	NA
Floricola striata	JK 5678I	GU301813	GU296149	NA	GU371758	GU479852
Floricola viticola	MFLUCC 15–0039^{T}	KT305993	KT305995	KT305997	NA	NA
Forliomyces uniseptata	MFLUCC 15–0765^{T}	KU721762	KU721767	KU721772	NA	KU727897
Lophiostoma arundinis	AFTOL–ID 1606	DQ782384	DQ782383	NA	DQ782386	DQ782387
Lophiostoma caulium	CBS 623.86	GU301833	GU296163	NA	GU296163	NA
Lophiostoma crenatum	AFTOL–ID 1581	DQ678069	DQ678017	NA	DQ677965	DQ677965
Lophiostoma fuckelii	CBS 101952	DQ399531	FJ795496	NA	FJ795472	NA
Lophiostoma macrostomum	KT508	AB619010	AB618691	NA	NA	LC001751
Lophiostoma compressum	KT 534	JN941379	JN941376	JN942962	JN993492	NA
Massarina corticola	CBS 154.93	FJ795448	FJ795491	NA	FJ795465	NA
Melanomma pulvis–pyrius	CBS 124080^{T}	GU456323	GU456302	NA	GU456350	GU456265
Misturatosphaeria kenyensis	GKM 1195^{T}	GU385194	NA	NA	NA	GU327767
Misturatosphaeria minima	GKM 169N^{T}	GU385165	NA	NA	NA	GU327768
Misturatosphaeria tennesseensis	ANM 911^{T}	GU385207	NA	NA	NA	GU327769
Platystomum scabridisporum	BCC 22835	GQ925844	GQ925831	NA	GU479830	GU479857
Preussia flanaganii	CBS 112.73^{T}	AB470528	NA	AY943061	NA	NA
Preussia funiculata	CBS 659.74^{T}	GU301864	GU296187	NA	GU371799	GU349032
Preussia lignicola	CBS 264.69	GU301872	GU296197	NA	GU371765	GU349027

Table 1. continued. Taxa used in the phylogenetic analyses and their corresponding GenBank numbers (Fig 1). The newly generated sequence is in bold.

Taxon	Culture accession no.	GenBank accession no.				
		LSU	**SSU**	**ITS**	**RPB2**	**TEF1-α**
Preussia minima	CBS 524.50[T]	DQ468046	NA	DQ468026	NA	NA
Preussia sp.	ELV3.2[T]	KF269206	NA	JN418773	NA	NA
Preussia sp.	ELV3.11[T]	KF269205	NA	JN418774	NA	NA
Ramusculicola thailandica	MFLUCC 13–0284[T]	KP888647	KP899131	KP899141	NA	KR075167
Sparticola forlicesenae	MFLUCC 14–0952	KU721764	KU721769	KU721774	NA	NA
Sparticola forlicesenae	MFLUCC 14–1097[T]	KU721763	KU721768	KU721773	NA	NA
Sparticola forlicesenae	MFLUCC 14–0952	KP888647	KP899131	KP899141	NA	KR075167
Sparticola junci	MFLUCC 15–0030[T]	KU721765	KU721770	KU721775	KU727900	KU727898
Sparticola junci	MFLUCC 13–0926	KU721766	KU721771	KU721776	KU727901	KU727899
Sparticola muriformis	**MFLUCC 17–0316**	**KY768862**	**KY768863**	**KY768864**	**KY855380**	**KY768874**
Sparticola triseptata	CBS 614.86[T]	EF165031	EF165036	NA	EF165040	NA
Sporomia lignicola	CBS 363.69[T]	DQ384098	DQ384087	GQ203783	NA	NA
Sporormia fimetaria	UPS:Lundqvist 2302–c	GQ203728	NA	GQ203768	NA	NA
Sporormia fimetaria	UPS:Dissing Gr.81.194[T]	GQ203729	NA	GQ203769	NA	NA
Teichospora rubriostiolata	TR7[T]	NA	NA	KU601590	KU601599	KU601609
Teichospora trabicola	C134[T]	NA	NA	KU601591	KU601600	KU601601
Westerdykella cylindrica	CBS 454.72[T]	AY004343	AY016355	NA	NA	NA
Westerdykella dispersa	CBS 297.56[T]	GQ203753	DQ384085	GQ203797	NA	NA
Westerdykella ornata	CBS 379.55	GU301880	GU296208	AY943045	GU371803	GU349021

[T]Type strain.

NA: not available

ANM: Andrew N. Miller, **ATCC**: American Type Culture Collection, Virginia, USA; **CBS**: Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht,The Netherlands; **GKM**: G.K. Mugambi; **IFRD**: Culture Collection, International Fungal Research and Development Centre, Chinese Academy of Forestry, Kunming, China; **JCM**: The Japan Collection of Microorganisms, Japan; **JK**: J. Kohlmeyer; **KT**: Kazuaki Tanaka, **MFLUCC**: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; **NN**: NovoNordisk culture collection (now Novozymes, Bagsvaerd, Denmark); **SMH**: S.M. Huhndorf; **UPS**: The Museum of Evolution Herbarium, Sweden.

Results

Phylogeny

The combined LSU, SSU, ITS, TEF1-α and RPB2 gene dataset comprises 40 taxa including the new taxon and other taxa from four families in Pleosporales i.e. Sporormiaceae, Lophiostomaceae, Floricolaceae / Teichosporaceae and Amorosiaceae which are available in GenBank. *Melanomma pulvis–pyrius* is selected as the outgroup taxon (Fig. 1). Phylogenetic trees obtained from ML, MP and BI analyses yield trees with similar overall topologies at the species relationships in agreement with previous studies (Schoch et al. 2009, Zhang et al. 2012, de Gruyter et al. 2012, Hyde et al. 2013, Ariyawansa et al. 2014, 2015b, Wijayawardene et al. 2014, Thambugala et al. 2015, Phukhamsakda et al. 2016). In the phylogenetic analyses, our new strain (MFLUCC 17–0316) forms a single lineage separating from other taxa in *Sparticola* (Fig. 1).

RAxML analysis yield a best scoring tree (Fig. 1) with a final ML optimization likelihood value of - 22651.862591. The parameters for the GTR+I+G model of combined LSU, SSU, ITS, TEF and RPB2 were as follows: Estimated base frequencies; A – 0.2458, C – 0.2476, G – 0.2751, T– 0.2315, substitution rates AC – 1.4890, AG – 3.4561, AT – 1.8557, CG – 1.3820, CT – 9.052/ and GT – 1.0000 proportion of invariable sites I – 0.5482 gamma distribution shape parameter 0.6353. The matrix had 1634 distinct alignment patterns, with 42.20 % of undetermined characters or gaps. This maximum parsimony analysis comprised 4330 total characters, of which 3048 were constant, 993 parsimony–informative and 289 parsimony–uninformative. The first tree generated among 1000 equally parsimonious trees is selected (Fig. 1). The Kishino Hasegawa test shows TL – 3656 steps with Consistency Index CI – 0.531, RI – 0.662, RC – 0.352 and HI – 0.469.

Taxonomy

Sparticola muriformis A. Karunarathna & Phookamsak, *sp. nov.*

Index Fungorum number: IF552967, Facesoffungi number: FoF 03188 Fig. 2

Etymology – Name reflects muriform ascospores

Holotype – MFLU 17–0374

Saprobic on grass. **Sexual morph** *Ascomata* 80–134 μm high, 102–112 μm diameter ($\bar{x}$ = 100 × 105 μm, n = 10), semi-immersed and raising host tissue, to erumpent, solitary, scattered, globose to subglobose, brown to dark brown, ostiolate. *Ostiole* 65–70 μm long, 37–41 μm diameter ($\bar{x}$ = 68 × 39 μm, n = 5), central, papillate, composed of 1–2 cell layers, of brown, pseudoparenchymatous cells, filled with hyaline periphyses. *Peridium* 6–9 μm wide, composed of 2–3 layers of lightly pigmented to dark brown, pseudoparenchymatous cells, arranged in a *textura angularis*, cells towards the inside lighter, outer layers fusing and indistinguishable from the host tissues. *Hamathecium* comprising numerous, 1–2 μm wide (n = 15), filamentous, anastomosing, broadly cellular pseudoparaphyses, embedded in a gelatinous matrix. *Asci* 55–83 × 12–18 μm ($\bar{x}$ = 66 × 15 μm, n = 20), 8-spored, bitunicate, fissitunicate, cylindrical to cylindric-clavate, subsessile, thick-walled at the apex, with well-developed ocular chamber, indistinct at maturity. *Ascospores* 18–20 × 5–7 μm ($\bar{x}$ = 19 × 6 μm, n = 40), overlapping 1–2-seriate, phragmosporous to muriform, ellipsoidal, widest at the central cells, hyaline when young, becoming brown at maturity, with 3–5 transverse septa and 1–2 longitudinal septa, constricted at the septa, rounded at both ends, wall rough, verruculose, surrounded by a thick, hyaline, mucilaginous sheath. **Asexual morph** Undetermined.

Culture characteristics − Colonies on PDA reaching 3 cm diameter after 3 weeks at 16–25 ºC, medium dense, flattened to slightly raised, circular, with edge entire, surface smooth, floccose to fluffy; mycelium composed of septate, branched hyphae, colony from above grey to greyish-brown, reverse iron-grey, producing dark brown pigmentation in agar.

Material examined − CHINA, Yunnan Province, Kunming Institute of Botany, Botanical Garden, on leaves of unidentified grass, 28 November 2016, K.V.A. Karunarathna, AKKIB 50 (MFLU 17–0374, **holotype**; HKAS 97367, **isotype**), ex-type living culture, MFLUCC 17–0316, KUMCC 16–0236.

Notes − *Sparticola muriformis* is introduced herein as a novel species based on morphological and phylogenetic supports. *Sparticola muriformis* differs from other *Sparticola* species in having muriform ascospores. While, other *Sparticola* species have phragmosporous ascospores (Phukhamsakda et al. 2016). Phylogenetically, *S. muriformis* forms a separate lineage from other *Sparticola* species with moderate support. (Fig. 1).

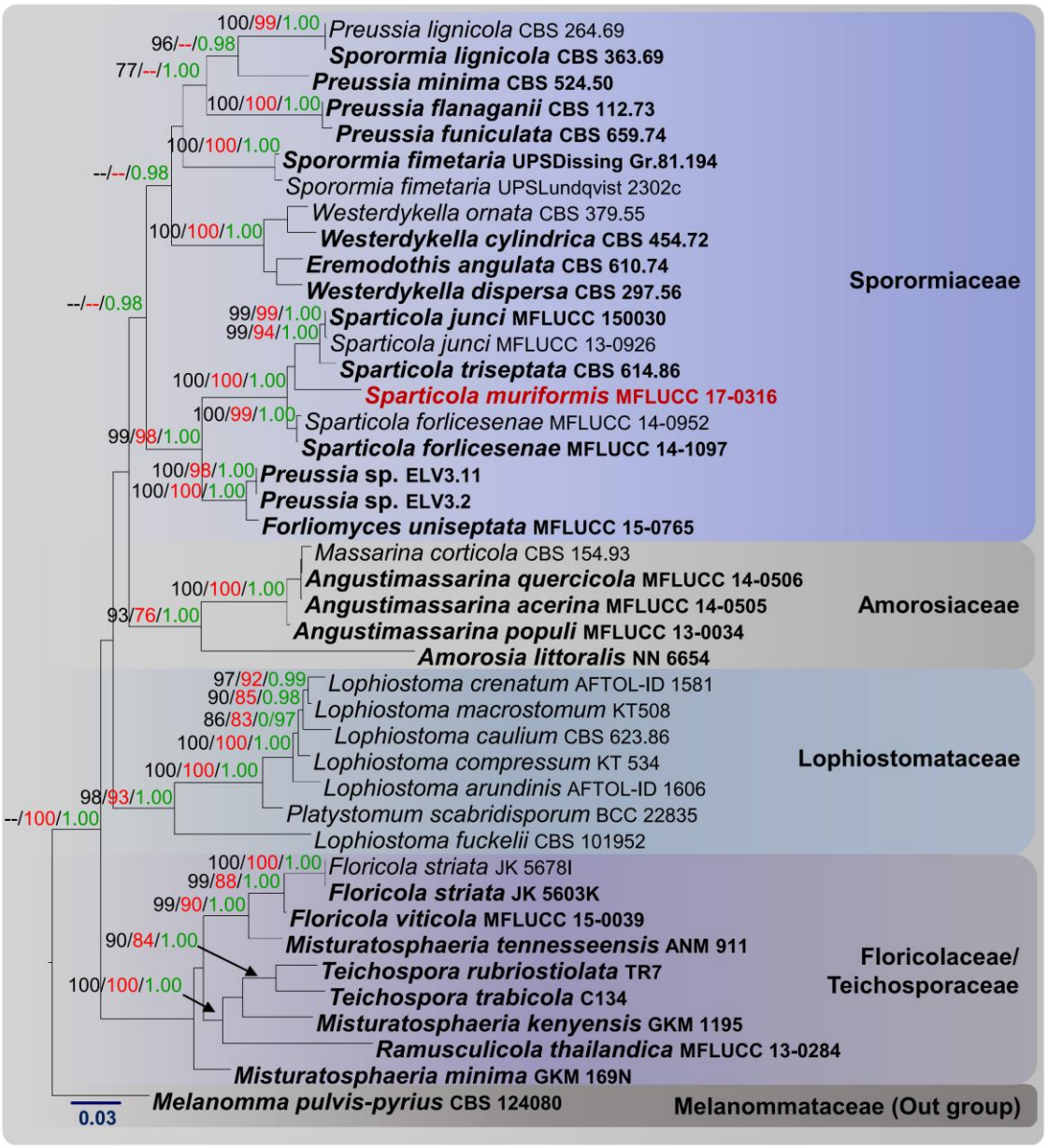

Fig. 1 – RAxML tree based on a combined dataset of LSU, SSU, ITS, TEF1-α and RPB2 partial sequences. Bootstrap support values for maximum parsimony (MP, black) and maximum likelihood (ML, red) higher than 75 % are defined as above the nodes. Bayesian posterior probabilities (PP, green) greater than 0.95 are provided below the nodes. The tree is rooted to *Melanomma pulvis–pyrius* (CBS 124080). Newly generated sequence is indicated in red.

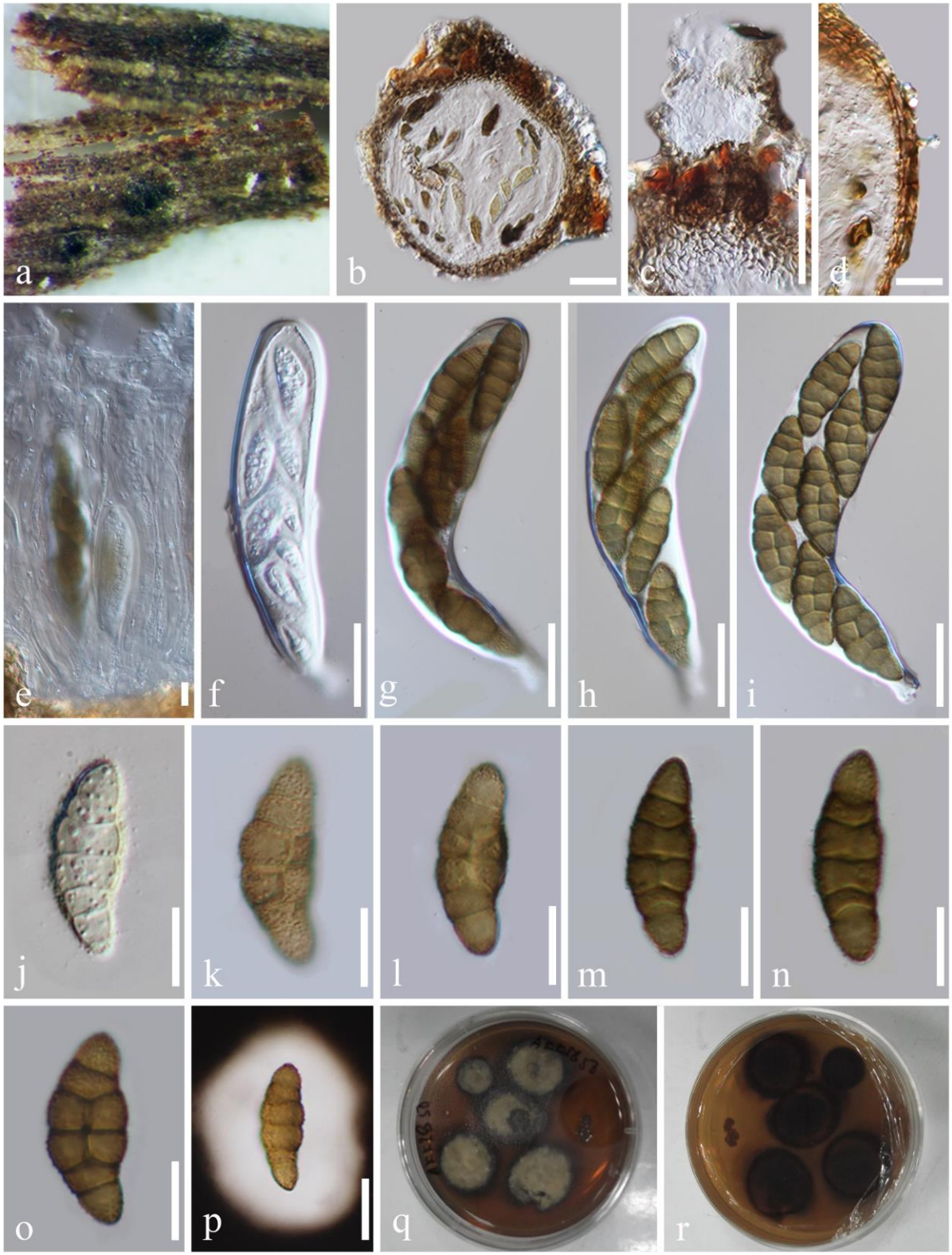

Fig. 2 – ***Sparticola muriformis*** (MFLU 17–0374, **holotype**) **a.** Appearance of ascoma on the host. **b.** Section through an ascoma. **c.** Section through an ostiole. **d.** Section through peridium. **e.** Pseudoparaphyses. **f–i**. Different developing stages of the asci. **j–o**. Ascospores. **p**. Ascospore surrounded by mucilaginous sheath, stained with Indian ink. **q–r.** Culture characteristics (q = from above, r = from below). Scale bars: b–c, f–i = 50 µm, d, e, j–p = 10 µm.

Discussion

Sparticola was introduced by Phukhamsakda et al. (2016) to accommodate saprobic Dothideomycetes occurring on *Spartium* (Fabaceae) and *Tofieldia* (Tofieldiaceae). Three species were first accommodated in this genus, viz. *S. forlicesenae* Wanasinghe et al., the type species, *S. junci* Phukhamsakda et al. and *S. triseptata* (Leuchtm) Phukhamsakda & K.D. Hyde (Phukhamsakda et al. 2016). *Sparticola* differs from other genera in Sporormiaceae in having phragmosporous, yellowish-brown or light brown ascospores, lacking a germ slit at the end of ascospores and those members of *Sparicola* were found in terrestrial habitats (Phukhamsakda et al. 2016). Whereas, other genera in Sporormiaceae produced dark brown, multi-septate ascospores, separating into part spores, with a germ slit at the ends and mostly found as coprophilous fungi (Kruys et al. 2006, Hyde et al. 2013, Phukhamsakda et al. 2016). In this study, we introduce the forth species of *Sparicola* namely *S. muriformis* which was collected on grass from Yunnan, China. The species can be distinguished from other *Sparticola* species due to its ascospores having brown to dark brown and muriform. The morphological comparisons of all taxa in *Sparticola* are shown in Table 2. Based on multi-genes phylogenetic analyses, *S. muriformis* clusters with *S. junci* and *S. triseptata*. It is the first record of *Sparticola* in Asia, while other *Sparticola* were found in Europe.

Table 2 Synopsis of characters of *Sparticola* species discussed in this study.

Character	***S. forlicesenae***	***S. junci***	***S. triseptata***	***S. muriformis***
Ascomata	275–325 × 200–250 µm, immersed to semi-immersed or erumpent, globose to subglobose.	125–200 × 190–230 µm, immersed, globose to subglobose,	260–510 × 360–440 µm, immersed, globose.	80–134 × 102–112 µm, immersed to semi-erumpent, globose to subglobose.
Papilla	80–110 µm × 60–80 µm diam., canal filled with hyaline periphyses	48–76 µm × 87–104 µm diam., canal filled with periphyses	160(–180) µm high, conical, canal filled with periphyses	65–70 µm × 37–41 µm diam., canal filled with hyaline periphyses
Peridium	20–50 µm wide, 7–8 layers	8–12 µm wide, 2–3 layers	24–50 µm wide, 7–8 layers	6–9 µm wide, 2–3 layers
Hamathecium	2–2.5 µm wide, cellular pseudoparaphyses	1.8–2.6 µm wide, cellular pseudoparaphyses	1–2 µm wide	0.8–1.8 µm wide, pseudoparaphyses
Asci	130–150 × 20–30 µm, broadly cylindrical to cylindric-clavate, subsessile	91–160 × 14–23 µm, broadly cylindrical to cylindric-clavate, subsessile	(118–)165–272 × 15–19(–25) µm, narrowly cylindrical, short bulbous pedicel,	55–83 × 12–18 µm, broadly cylindrical to cylindric-clavate, subsessile
Ascospores	30–40 × 10–15 µm, yellowish-brown to brown, curved-fusoid, 5–6(–9) transverse septa, rough-walled	19–29 × 7–12 µm, yellowish, oval to ellipsoid, 1–3 transverse septa, rough-walled	20–36 × 8–12 µm, reddish-brown to brown, ellipsoidal, 3 transverse septa, smooth-walled	18–20 × 5–7 µm, brown to dark brown, ellipsoid, muriform, 5 transverse septa, with 1–2 longitudinal septa, rough-walled.
Host	*Spartium junceum* (Fabaceae)	*Spartium junceum* (Fabaceae)	*Tofieldia calyculatan* (Tofieldiaceae)	Grass litter (Poaceae)
References	Phukhamsakda et al. 2016	Phukhamsakda et al. 2016	Leuchtmann 1987, Phukhamsakda et al. 2016	This study

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