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Contributed Paper

The Genus *Phaeosaccardinula* (Chaetothyriales) from Yunnan, China, Introducing Two New Species

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ABSTRACT

This paper deals with two new species of *Phaeosaccardinula* collected in Yunnan Province of China. *Phaeosaccardinula multiseptata* and *P. dendrocalami* are introduced as new species, based on morphology and molecular data and are compared with related taxa. Descriptions, illustrations and notes are provided for each species. A phylogenetic tree based on analysis of ITS and LSU sequence data show the species to cluster in *Phaeosaccardinula* (*Chaetothyriaceae*) as two distinct clusters. Morphology and analysis of ITS, LSU and EF1a gene data indicate several differences between *P. ficus* and *P. multiseptata* in the first cluster, while the second cluster represents the new species *P. dendrocalami*. Previously, sequence data (ITS and LSU) were only available for *Phaeosaccardinula* species, this paper provides new sequence data for ITS, LSU, EF1a and RPB2 genes.

Key words: Chaetothyriaceae, EF1a, foliar epiphyte, ITS, LSU, phylogeny

1. INTRODUCTION

The *Chaetothyriaceae* was introduced by Hansford in 1946 [1]. The majority of species in this family are superficial on leaves and have no connexion with insects and their secretions [1]. However the ecology of many species is poorly studied and it is unclear whether they are saprotrophic or biotrophic [2]. The mycelia form a dense matt of hyphae, which have the appearance of sooty moulds and are appressed to the host cuticle without penetrating host tissues [3]. The *Chaetothyriaceae* includes 13 genera, with only *Ceramothyrium*, *Phaeosaccardinula* and *Yatesula* lacking setae [4]. *Phaeosaccardinula* has an estimated 14 species [5] which are foliar epiphytes, and have superficial ascomata, with a dark, non-setose pellicle, saccate, bitunicate asci and muriform, hyaline to brownish ascospores [3,6]. More than 40 species have epithets under this genus (Index Fungorum 2013), but many actually belong in *Limacinula* and *Treubiomyces* [7,8]. A new species, *Phaeosaccardinula ficus* Chomnunti & K.D. Hyde, was introduced from northern Thailand by Chomnunti *et al.* [9]. This species with lacks a tessellate scutellum and spores are olivaceous green at the septa and have a

mucilaginous sheath [9].

Phaeosaccardinula javanica (Zimm.) Yamam., *P. longispora* Yamam. and *P. dictyospora* (Petr.) O. Erikss. have been reported from China [7,8,10]. However *P. javanica* is now named *Limacinula javanica* (Zimm.) Höhn. and *P. longispora* Yamam. is *Treubiomyces roseosporus* (Höhn.) D.R. Reynolds [10]. Therefore *P. dictyospora* is the only species in the *Phaeosaccardinula* known from China [10]. The species are listed as *Chaetothyrium javanicum* (Zimm.) Boedijn and *Treubiomyces roseosporus* (= *p. longispora* Yamam.) in Index Fungorum (2013), indicating differences in opinion and need for verification with molecular data. *Chaetothyrium javanicum* has been reported from at least 73 different hosts and surely must represent more than one *Limacinula* species [10].

The purpose of this study is to introduce two new species in the genus *Phaeosaccardinula* and we also add ITS, LSU, EF1a and RPB2 gene sequence data for this poorly known genus.

2. MATERIALS AND METHODS

2.1 Isolates and Morphology

Fungi with sooty mould-like colonization were collected from various living plants in Yunnan. Specimens were taken to the laboratory in an envelope and examined under a microscope (Nikon 80i) for morphological characters. Sections of ascomata were made in a freezing microtome (Leica CM1100). Pure-cultures were obtained by single spore isolation following the methods of Chomnunti *et al*. [11]. After a month, the growing cultures were used for molecular work.

The specimens were air dried and are deposited at the International Fungal Research & Development Centre (IFRD) Herbarium, Kunming, Yunnan, and the cultures are stored in the culture collection of the same institution (IFRDCC) with duplicates in MFLUCC and ICMP.

DNA isolation, amplification and sequencing: Fungal isolates were grown on PDA for 40 days at 26°C in the dark. Genomic DNA was extracted from the growing mycelium using the Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) following the manufacturer's protocol (Hangzhou, P.R. China).

Polymerase chain reaction (PCR) was carried out using known primer pairs LROR and LR5 were used to amplify a region spanning the large subunit rDNA (28S, LSU) [12] and internal transcribed spacers (5.8S, ITS) was amplified by primer pairs ITS5 and ITS4 [13]. The translation elongation factor 1-alpha gene (EF1a) was amplified by using EF1-983F and EF1-2218R primers [14]. The RNA polymerase II second largest subunit (RPB2) was amplified by using RPB2-5F and RPB2-7cR primers [15]. The amplification reaction mixtures were 50 μL which contained 3.0 μL of DNA template, 1.5 μL of each forward and reverse primers, 25 μL of 2 × Easy Taq PCR SuperMix (mixture of *EasyTaq*TM DNA Polymerase, dNTPs, and optimized buffer, Beijing TransGen Biotech Co., Ltd., Chaoyang District, Beijing, PR China) and 19 μL sterilized water. Amplification conditions were set up for initial denaturation of 3 min at 94°C, followed by 38 cycles of 30 s at 94°C, 40 s at 56°C and 60 s at 72°C, and a final extension period of 10 min at 72°C [16]. The PCR products were observed on 1% agarose electrophoresis gels stained with ethidium bromide. Purification and sequencing of PCR products were carried at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (China).

2.2 Phylogenetic Analysis

Blast searches were made to reveal the closest matches in GenBank. All ITS and LSU sequences obtained from GenBank (listed in

Table 1.) [17-22]. The EF1a sequence for *Phaeosaccardinula ficus* was provided by P. Chomnunti (GenBank No. = KF791915). Sequences were aligned and concatenated in BioEdit [23]. Phylogenetic analyses were carried by using RAxMLv. 7.2.7 [24] implemented in RAxMLGui v 1.3 [25] for Maximum likelihood (ML) and MrBayes v. 3.0b4 [26] for Bayesian analyses.

Maximum likelihood (ML) trees searches were done in RAxMLGui v 1.3 [24] and used the 'ML + thorough bootstrap' option. One thousand non parametric bootstrap iterations were run with the general time reversible (GTR) model and a discrete gamma distribution. The resulting replicates were plotted on to the best scoring tree obtained previously. The phylogram with bootstrap values above the branches is presented in Figure 1. by using graphical options available in TreeView [27].

The model of evolution was performed by using MrModeltest v. 3.0b4. Posterior probabilities (PP) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes v. 3.0b4 [28]. Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generation and 10,000 trees were obtained. The first 2,000 trees, representing the burn-in phase of the analyses, were discarded while remaining 8,000 trees used for calculating posterior probabilities in the majority rule consensus tree [29,30]. Bayesian Posterior Probabilities (BYPP) with those equal or greater than 0.80 given below each node (Figure 1).

Table 1. Taxa used in the phylogenetic analysis and their corresponding GenBank numbers.

Species	Culture/voucher	ITS	LSU
Antennariella placitae	CBS 124785	GQ303268	GQ303299
Capnodium coffeae	CBS 147.52	AJ244239	—
Capronia mansonii	—	AF050247	AY004338
Capronia munkii	DAOM 216390	AF050250	EF413604
Capronia semiimmersa	MUCL 40572	AF050259	AF050259
Ceramothyrium carniolicum	CBS 175.95	KC978733	FJ358232
Ceramothyrium thailandicum	MFLU 10–0079	HQ895838	HQ895835
Chaetothyrium brischoficola	MFLU 10–0083	HQ895839	HQ895836
Cladophialophora australiensis	CBS 112793	EU035402	EU035402
Cladophialophora minourae	CBS 556.83	AY251087	FJ358235
Cladophialophora potulentorum	CBS 112222	EU035409	EU035409
Conidioxyphium gardeniarum	CPC 14327	—	GU301807
Cyphellophora laciniata	CBS 190.61	EU035416	FJ358239
Cyphellophora pauciseptata	CBS 284.85	JQ766469	JQ766522
Cyphellophora pauciseptata	CBS 284.85	JQ766470	JQ766523
Cyphellophora suttonii	CBS 449.91	GU225946	—
Cyphellophora vermispora	CBS 227.86	GU225947	JQ766474
Exophiala pisciphila	AFTOL-ID 669	DQ826739	DQ823101
Exophiala salmonis	CBS 157.67	JF747138	AY213702
Leptoxyphium madagascariense	CBS 124766	GQ303277	GQ303308
Microxyphium theae	CBS 202.30	— GU301849	
Phaeosaccardinula dendrocalami	IFRDCC 2649	KF667242	KF667245

Table 1. Continued.

Species	Culture/voucher	ITS	LSU
Phaeosaccardinula dendrocalami	IFRDCC 2663	KF667243	KF667246
Phaeosaccardinula ficus	MFLU 10–0009	HQ895840	HQ895837
Phaeosaccardinula multiseptata	IFRDCC 2639	KF667241	KF667244
Placidiopsis cinerascens	MA 161308	GQ344613	GQ344570
Placidiopsis tenella	L l281352	GQ344616	GQ344575
Placidiopsis tiroliensis	L l281349	GQ344618	GQ344581
Rhinocladiella anceps	CBS 157.54	EU041804	EU041861
Rhinocladiella fasciculata	CBS 132.86	EU041807	EU041864
Trichomerium deniqulatum	MFLU 10–0884	JX313654	JX313660
Trichomerium foliicola	MFLU 10–0054	JX313651	JX313657
Trichomerium foliicola	MFLU 10–0058	JX313653	JX313659
Trichomerium foliicola	MFLU 10–0073	JX313652	JX313658
Trichomerium gloeosporum	MFLU 10–0087	JX313656	JX313662
Veronaea botryosa	CBS 350.65	EU041817	EU041874
Venturia inaequalis	CBS 476.61	EU282478	GU456336

2.3 Individual Data for ITS, LSU, EF1a and RPB2 for Four Strains

Seqman in DNAStar software v5.01 (*www.dnastar.com*) [31] was used to compare the different base pairs of ITS, LSU, EF1a and RPB2 between *P. ficus* and *P. multiseptata* and two strains of *P. dendrocalami*, the details of the difference are listed in Table 2.

Table 2. ITS, LSU, EF1a and RPB2 base pair difference between *P. ficus* and *P. multiseptata* and two strains of *P. dendrocalami*.

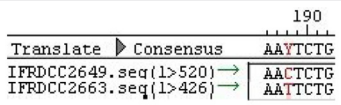
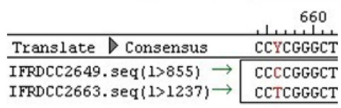
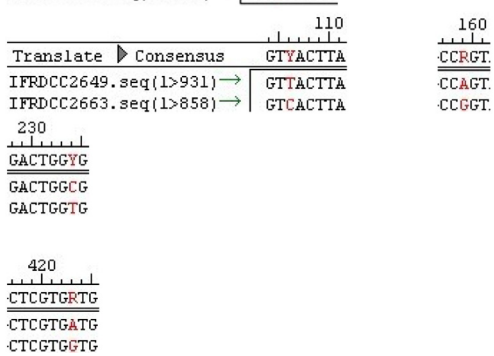
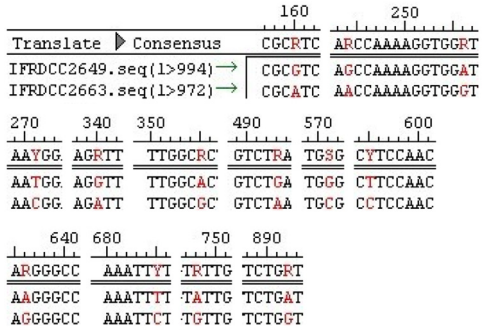
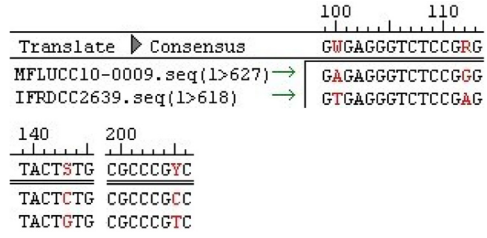
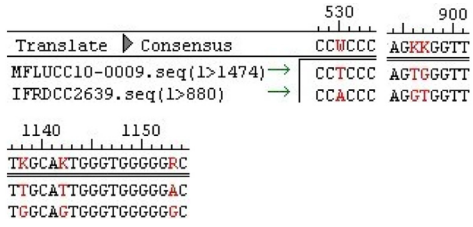
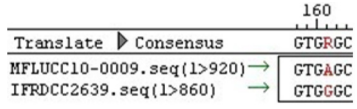
Species name	Gene name	Different bases number	Details of difference
P.dendrocalami (IFRDCC2649 and IFRDCC2663)	ITS	1	
	LSU	1	
	EF1a	4	

Table 2. Continued.

Species name	Gene name	Different bases number	Details of difference
	RPB2	13	
P.ficus (MFLUCC10–0009) and *P.mulitiseptata* (IFRDCC2639)	ITS	4	
	LSU	6	
	EF1a	1	

Note: The different base pairs of the sequence are marked in red. The upper number is the placement of the base pairs. *Phaeosaccardinula ficus* = MFLUCC 10–0009, *P. multiseptata* = IFRDCC 2639, *P. dendrocalami* = IFRDCC 2649 and IFRDCC 2663.

3. RESULTS

Sequence data for ITS, LSU and EF1a genes were obtained for isolates *Phaeosaccardinula multiseptata* (IFRDCC 2639), *P. dendrocalami* (IFRDCC 2649, IFRDCC 2663) and *P. ficus* (MFLUCC 10-0009). RPB2 sequence data were only obtained successfully for isolates of *P. dendrocalami*. Because ITS and LSU sequence data is only available for most members of *Chaetothyriaceae* in GenBank, we use these genes in the phylogenetic analysis. Individual ITS, LSU, EF1a and RPB2 gene sequences are compared manually for the four *Phaeosaccardinula* strains.

3.1 Phylogeny based on combined LSU and ITS gene data set

The combined LSU and ITS data set utilized 37 taxa with *Venturia inaequalis* as the outgroup taxon. The dataset consists with 14.65% missing and gap characters out of a total set of 1,450 characters and the best scoring tree selected with a final ML Optimization Likelihood -10039.136870. Phylogenetic trees obtained from Bayesian and RAxML analyses yielded a best scoring tree with similar overall topology at the genus and family relationships are in agreement with previous work based on a RAxML tree (Figure 1). The phylogenetic hypothesis strongly supports four monophyletic groups, the three new sequences described herein cluster within the *Phaeosaccardinula* (*Chaetothyriaceae)* clade; they are unrelated to species of *Herpotrichiellaceae, Verrucariaceae, Trichomeriaceae* and *Capnodiaceae*. The placement of sequence data from strains *Phaeosaccardinula multiseptata*, *P. dendrocalami* and *P. ficus* in this lineage received 100% bootstrap support and Bayesian posterior probabilities of 1.00, while *Phaeosaccardinula multiseptata* clusters in a subclade with *P. ficus* with 100% bootstrap support and Bayesian posterior probabilities of 1.00. The two strains of *P. dendrocalami* cluster in a subclade with 100% bootstrap support and Bayesian posterior probabilities of 1.00.

Comparisons of indivdual gene data show that *P. multiseptata* and *P. ficus* have four ITS, six LSU and one EF1a base pair difference. The two strains of *P. dendrocalami* have one ITS, one LSU, four EF1a and 13 RPB2 base pairs difference. The further supports *P. multiseptata* being a different species to *P. ficus* while the two strains of *P. dendrocalami* are same species based on molecular evidence.

3.2 Taxonomy

Phaeosaccardinula multiseptata H. Yang & K.D.Hyde, *sp. nov.* (Figure 2)
MycoBank MB 805931
Etymology: from the Latin *multi* and *septate* in reference to the large number of septa found in the ascospores

Foliar epiphytes growing on the upper surface of living leaves forming soot-like coatings. Mycelium superficial, brown to dark brown, hyphae-like dark brown, reticulate to branched, constricted at the septa. Sexual state: Ascomata 156-182 µm diam, 123-135 µm high ($\overline{x}$ = 172 × 124 µm, n = 10) orbicular, with depressed central part, black, scattered on surface of leaving leaves, subglobose to globose, lacking setae, without ostiole in mature ascomata. Peridium 26-31 µm wide ($\overline{x}$ = 28 µm, n = 20), thick-walled, inwardly hyaline to dark brown, comprised of cells of *textura prismatica*, brown towards the outside, comprised of cells of *textura angularis*. Asci 55-69 × 30-40 µm ($\overline{x}$ = 64 × 33 µm, n=20) 4-6-spored, bitunicate, obovoid to oval. Ascospores 38-47 × 13-16 µm ($\overline{x}$ = 45 × 14 µm, n = 30), pale brown to brown, oblong-ellipsoid to reniform, muriform, with obscure 9–12 transversal septa and 7–11 longitudinal septa, surface rough, slightly constricted at the septa. Asexual state: Unknown.

Culture characteristics: Colonies on PDA growing slowly, 3 cm diam after 40 days, surface gray to olivaceous gray, spreading, with obvious folds, velvety, water droplets forming on the surface, some dark mycelium branching at the margin. No asexual state produced on PDA after 60 days.

Material examined: CHINA – Yunnan Province, Jinghong, on living leaf of *Dillenia pentagyna* Roxb., 3 December 2011, Hui Yang, BN04 (IFRD 9041,

holotype), ex-type living culture = IFRDCC 2639 (duplicates in MFLUCC and ICMP). Sequence data: ITS = KF667241, LSU = KF667244, EF1a = KF722789.

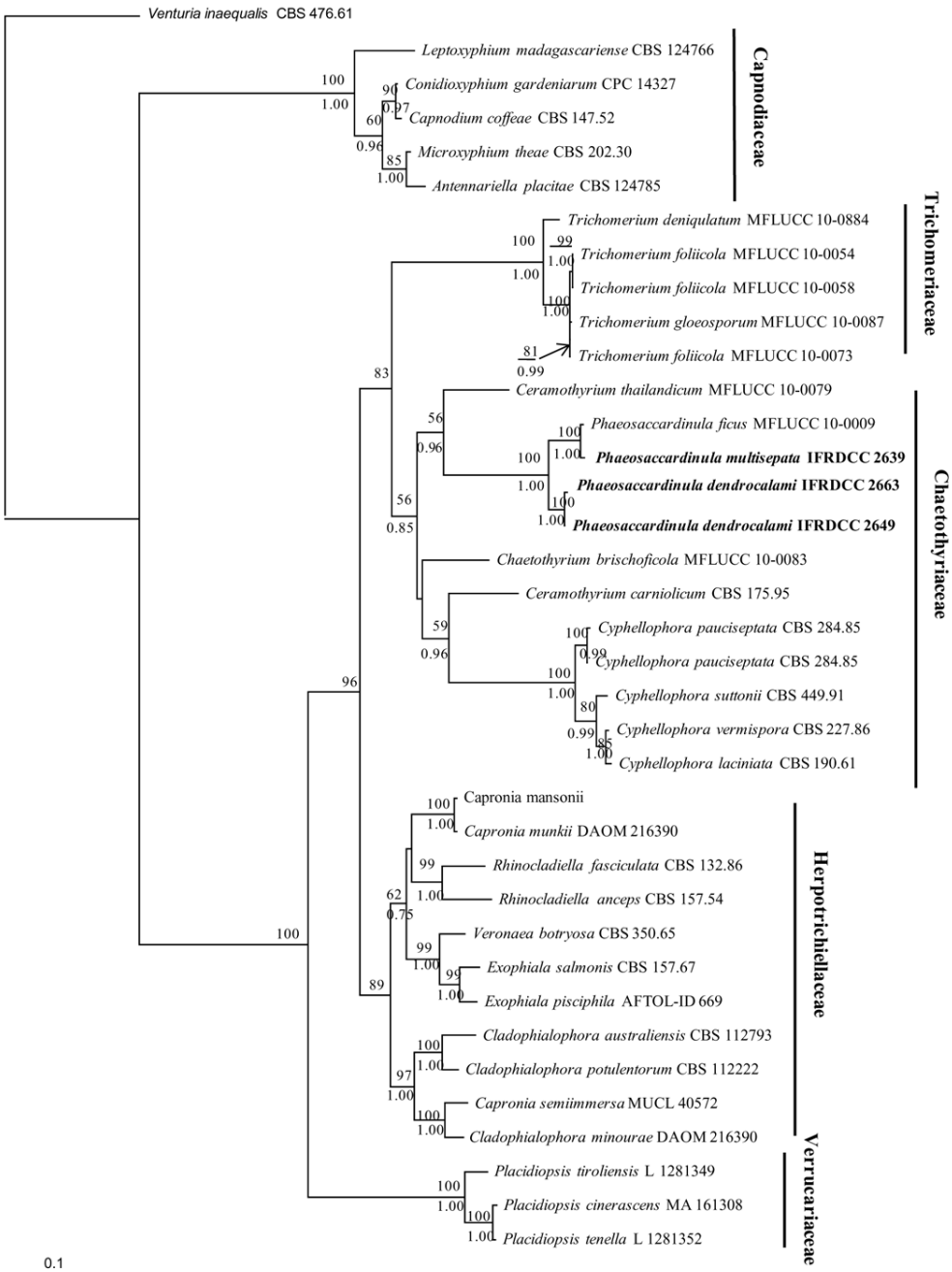


Figure 1. Gene tree constructed using RAxML based on phylogenetic analysis of the nucleotide sequences of combined ITS and 28S nrDNA. Bootstrap support values ≥ 50 % and Bayesian values ≥ 80 % are shown above or below the branch. *Venturia inaequalis* is the outgroup taxon. The original strain numbers are noted after the species names and names of isolates with newly obtained sequences from this study are in bold.

Notes: This species is similar to *Phaeosaccardinula ficus* which was described from *Ficus* in northern Thailand by Chomnunti *et al.* (2012). Our collection differs from *P. ficus* in having smaller ascomata (156-182 × 123-135 µm, $\overline{x}$ = 172 × 124 µm, versus 188-226 × 116-193 µm, $\overline{x}$ = 209 × 174 µm) and asci (55 × 69 × 30–40 µm, $\overline{x}$ = 64 × 33 µm vs 120-185 × 49-64 µm, $\overline{x}$ = 148 × 55 µm), and pale brown ascospores, with a large number of transverse (9-12 vs 7-8) and longitudinal (7-11 vs 6-8) septa. The host *Dillenia pentagyna Roxb* also differs. The ITS sequence data have 4 base pair different, LSU have 6 and EF1a have 1 between our collection and *Phaeosaccardinula ficus*.

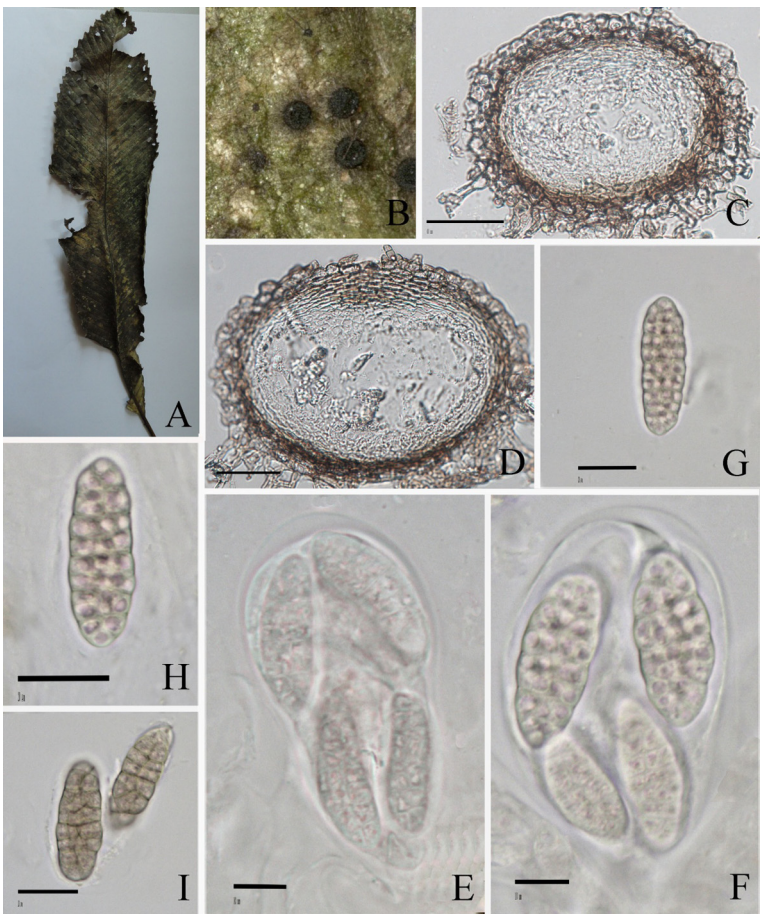


Figure 2. *Phaeosaccardinula multiseptata* (holotype). A. Sooty mold-like appearance on leaf surface. B. Appearance of colony and ascomata on the host surface. C, D. Vertical sections of ascoma. E, F. Asci. G-I. Ascospores. Scale bars: C, D = 40 µm, E, F = 10 µm, G-I = 20 µm.

Phaeosaccardinula dendrocalami H. Yang & K.D. Hyde, *sp. nov.* (Figure 3)
MycoBank MB 805932
Etymology: in reference to its occurrence on *Dendrocalamus*.

Foliar epiphytes growing on the upper surface of living leaves forming a soot-like coating. Mycelium superficial, black, hyphae-like, dark brown to black, reticulate-branched, constricted at the

septa. Sexual state: Ascomata 144-168 µm diam, 105-115 µm high ($\bar{x}$ = 161 × 107 µm, n = 10), black, subglobose to globose, lacking setae, with a central ostiole in mature ascomata. Perdium 32-36 µm wide ($\bar{x}$ = 34 µm, n = 20), thick-walled, inwardly of hyaline of textura prismatica, dark brown to brown towards the outside, comprised 3-4 layers of *textura angularis*. Asci 57-70 × 27-41 µm ($\bar{x}$ = 63 × 32 µm, n = 20) 4–6-spored, bitunicate, oblong-ellipsoid to reniform when young, subglobose to oval when mature, with short pedicel, ocular chamber not visible in mature asci. Ascospores 37-49 × 11-15 µm ($\bar{x}$ = 40 × 13 µm, n = 30), hyaline, oblong-ellipsoid to reniform and fusiform, muriform, with 5–7 transversal septa and 3-5 longitudinal septa, guttulate, constricted at the septum, narrow at the ends. Ascospores germinating on PDA within 12 hours, germ tubes developing from numerous cells of ascospores. Asexual state: Unknown.

Culture characteristics: Colonies on PDA growing slowly, 2 cm diam after 40 days, surface gray to olivaceous green, spreading, velvety, dense floss on the surface, pale brown and smooth at the margin. No asexual state produced on PDA after 60 days.

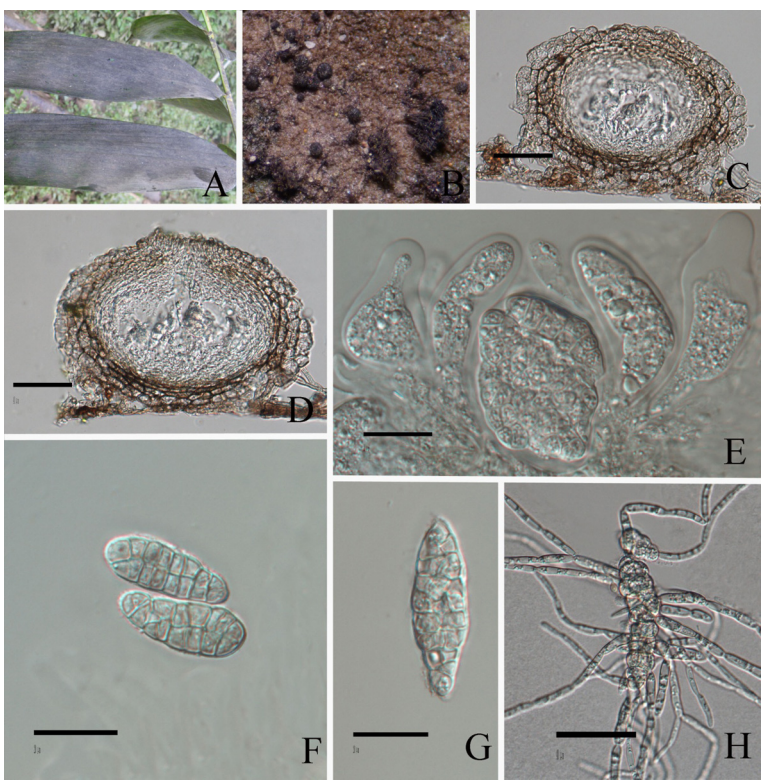


Figure 3. *Phaeosaccardinula dendrocalami* (holotype). A. Sooty mold-like appearance on leaf surfaces. B. Appearance of colony and ascomata on the host surface. C, D. Vertical sections of ascoma. E. Asci. F. G. Ascospores H. Germinating ascospore. Scale bars: C, D = 40 µm, E-G= 20 µm, H = 40 µm.

Material examined: CHINA – Yunnan Province, Lincang, on living leaf of *Dendrocalamus brandisii,* 17 December 2012, Hui Yang, LC11 (IFRD 9042, holotype), ex-type living culture = IFRDCC 2649. Ibid., Ruili, on living leaf of *Schima wallichii (DC.)* Choisy, 15 December 2012, Hui Yang, RL02 (IFRD 409-001), living culture = IFRDCC 2663 (duplicates in MFLUCC and ICMP).

Sequence data: IFRDCC 2649: ITS = KF667242, LSU = KF667245, EF1a = KF722790, RPB2 = KF 753240

IFRDCC 2663: ITS = KF667243, LSU = KF667246, EF1a = KF722791, RPB2 = KF 753241

Notes: *Phaeosaccardinula dendrocalami* was found on *Dendrocalamus brandisii* (bamboo) and is most similar to *P. ficus* and *P. dictyospora* (Petr.) O. Eriksss. *P. ficus* differs from *P. dendrocalami* by its lighter mycelium (brownish and pale brown versus dark brown to black), thinner peridium (20-32 μm, $\overline{x}$ = 27 μm versus 32-36 μm, $\overline{x}$ = 34 μm), larger ascomata (188-226 × 116-193 μm, $\overline{x}$ = 209 × 174 μm versus 144-168 × 105-115 μm, $\overline{x}$ = 161 × 107 μm) and larger asci (120-185 × 49-64 μm, $\overline{x}$ = 148 × 55 μm vs 57-70 × 27–41 μm, $\overline{x}$ = 63 × 32 μm). *P. dictyospora* differs from *P. dendrocalami* in having light brown, larger ascospores (36-61 × 16-24 μm vs 37-49 × 11-15 μm) and a larger number of transversal septa. The ITS and LSU sequence data have only 1 base pair difference, EF1a have 4 and RPB2 have 13 base pairs difference between strains.

4. DISCUSSION

In this study, we illustrate morphology and provide molecular data from three strains of two collections of *Phaeosaccardinula* (*Chaetothyriaceae*) isolated from Yunnan Province, China. Two new species are introduced in *Phaeosaccardinla* and illustrated with photomicrographs and descriptions. ITS LSU, EF1a and RPB2 sequence data for the strains are added to GenBank. *Phaeosaccardinula* species are characterized by ascomata that form beneath a mycelial pellicle on the leaf surface, with ascomata and mycelium that lack setae, bitunicate asci and hyaline to light brown, muriform ascospores [6,10]. The phylogenetic analyses of ITS, LSU combined sequences revealed that the three *Phaeosaccardinula* isolates cluster within *Chaetothyriaceae* in the combined analyses of ITS, LSU sequence data and are remote from *Herpotrichiellaceae, Verrucariaceae, Trichomeriaceae* and *Capnodiaceae*, and other genera, i.e. *Chaetothyrium, Ceramothyrium, Cyphellophora* and *Vonarxia* in *Chaetothyriaceae*. The topology and bootstrap support in the RAxML tree and Bayesian posterior probabilities in Figure 1. indicate that all *Phaeosaccardinula* isolates are the very similar or identical as *P. ficus*. However, both *P. dendrocalami* and *P. multiseptata* differ in morphology and are introduced as new species based on morphological differences. Manual comparison of ITS, LSU EF1a and RPB2 sequence data of *P. multiseptata* and *P. ficus* show that the ITS partial gene has four, LSU six and and EF1a one base pair difference; ITS has 1, LSU has 1, EF1a has 4 and RPB2 has 13 base pairs differences between the two strains (IFRDCC2649 and IFRDCC2663) of *P. dendrocalami*. These strains were isolated from different hosts so 13 different base pairs differences, may indicate they are also distinct species.

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