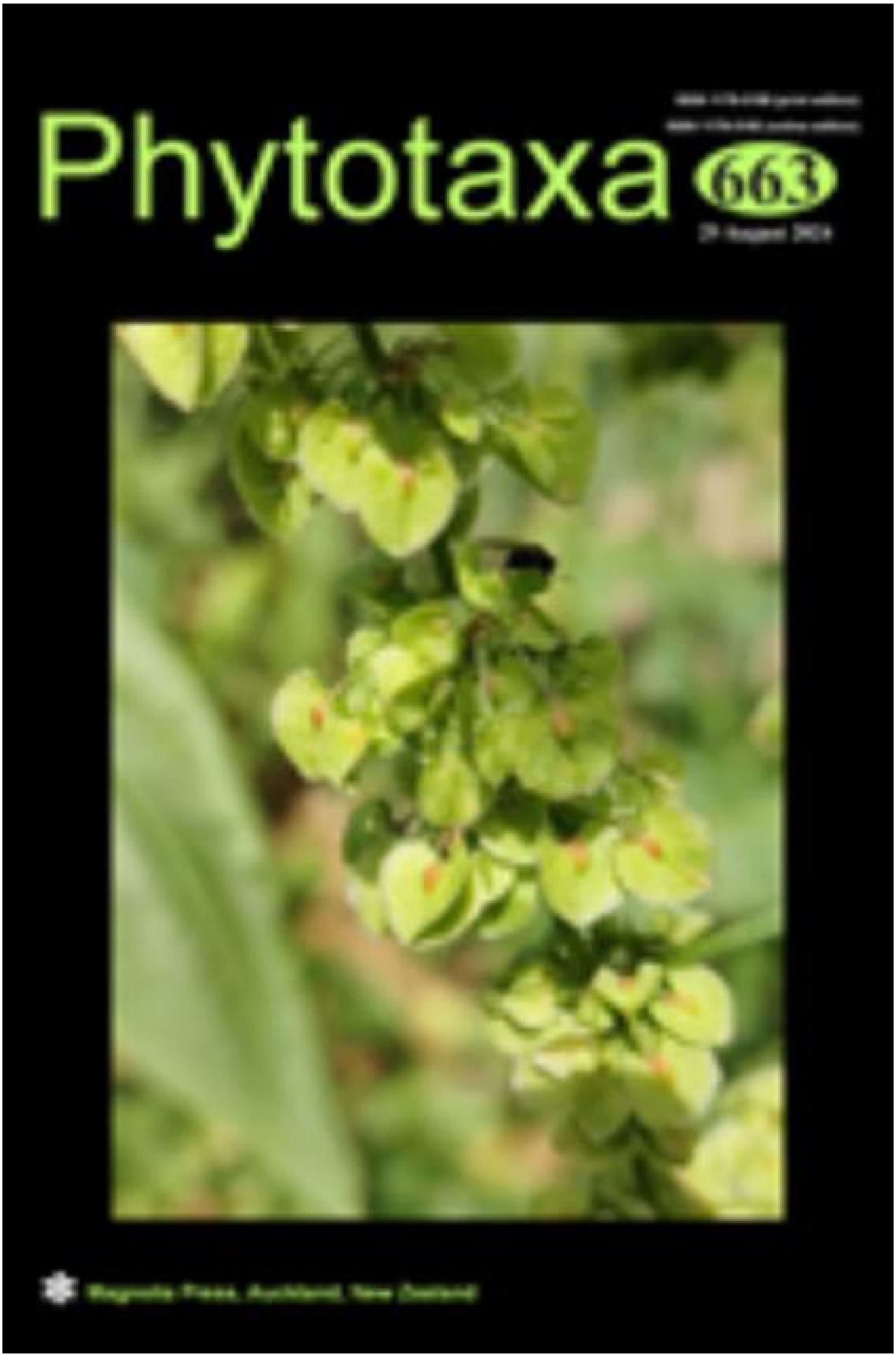

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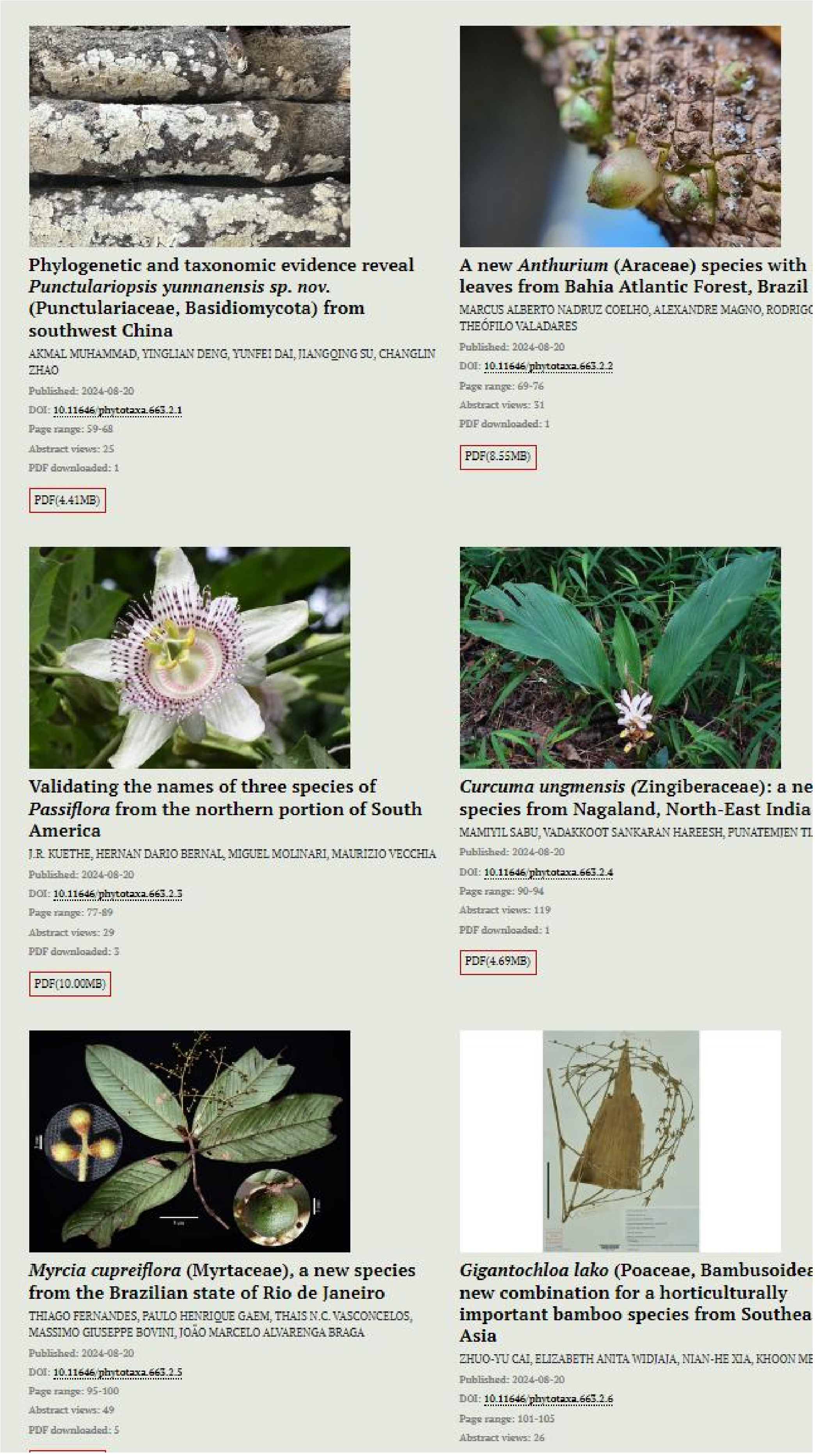

Phylogenetic and taxonomic evidence reveal *Punctulariopsis yunnanensis sp. nov.* (Punctulariaceae, Basidiomycota) from southwest China

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Phylogenetic and taxonomic evidence reveal *Punctulariopsis yunnanensis sp. nov.* (Punctulariaceae, Basidiomycota) from southwest China

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Abstract

A new wood-rotting fungal species, *Punctulariopsis yunnanensis*, is proposed based on a combination of morphology and molecular data. The species is characterized by the resupinate basidiomata with a pinkish buff salmon hymenial surface, a monomitic system, generative hyphae with clamp connections, and subhymenial generative hyphae thick-walled, and the presence of dendrohyphidia, and broadly ellipsoid basidiospores measuring 7.8–12.8 × 6–9.8 µm. Phylogenetic analyses of the combined ITS and nLSU nrDNA gene regions demonstrated that *P. yunnanensis* formed an independent lineage and was sister to *P. subglobispora* with strong statistical support. A full description, illustrations and phylogenetic analysis results of the new species are provided.

Key words: 1 new species, Phylogenetic analyses, Punctulariaceae, Taxonomy, Wood-rotting fungi

Introduction

Corticioid (non-poroid resupinate) basidiomycetes comprise a phylogenetically heterogeneous group of fungi with a relatively simple fruiting body configuration, and corticioid lineages have been found in all major clades of Agaricomycotina (Binder *et al.* 2005, Dai 2011; Ghobad-Nejhad *et al.* 2021; Mao *et al.* 2023) and the resupinate habit may well represent the ancestral form of basidiomycete fruiting bodies (Hibbett & Binder 2002). The taxa of the order Corticiales show considerable ecological and nutritional diversity, including saprophytic, lichenicolous, plant pathogenic, and lichenized lineages inhabiting diverse types of substrata (Lawrey *et al.* 2008; Rungjindamai *et al.* 2008). Punctulariaceae Donk is a small family in Corticiales that accounts for corticioid taxa with simple resupinate basidiomes growing on angiosperm wood (Donk 1964). All species hold dendrohyphidia and have ellipsoid basidiospores negative in Melzer's reagent. The family contains the three genera *Dendrocorticium* M.J. Larsen & Gilb., *Punctularia* Pat., and *Punctulariopsis* Ghob.-Nejh. (Ariyawansa *et al.* 2015).

The genus *Punctulariopsis* (2010: 1529) (Punctulariaceae, Corticiales) was established in 2010, and was typified by *P. subglobispora* (Hallenb. & Hjortstam) Ghob.-Nejh. (2010: 1529) (Ghobad-Nejhad *et al.* 2010). The genus is characterized by basidiocarp resupinate, strictly adnate, hymenium surface reddish-ochraceous, smooth, subgelatinous when fresh, corneous in a dried state, velutinose to pruinose under lens, margin abrupt, loosening, and concolorous with the hymenium. The hyphal system is monomitic, generative hyphae with clamps, yellowish-brown, thin-walled in the hymenial layer, and thickened walled in the basal layer. Subiculum is composed of horizontally arranged, agglutinated hyphae, with a brown tint. Basidia elongate, large clavate, flexuous, with four sterigmata and a basal clamp, walls occasionally thickened. Cystidia absent. Dendrohyphidia are smooth or irregularly encrusted and lightly branched. Basidiospores are broadly ellipsoid, smooth, contents granular, walls moderately thickened (Ghobad-Nejhad *et al.* 2010). Based on the MycoBank database (http://www.MycoBank.org, accessed on May 14, 2024) and the IndexFungorum (http://www.indexfungorum.org, accessed on May 14, 2024), the genus *Punctulariopsis* has four species, as *Punctulariopsis cremeoalbida* (M.J. Larsen & Nakasone) Ghob.-Nejh. (2015: 224), *P. efibulata* (M.J.

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Larsen & Nakasone) Ghob.-Nejh. (2015: 224), *P. obducens* (Hjortstam & Ryvarden) Ghob.-Nejh. (2010: 1529), and *P. subglobispora* (Ghobad-Nejhad *et al.* 2010; Ariyawansa *et al*. 2015). Morphologically, *Punctulariopsis* resembled *Punctularia* but had a thinner basidiocarp, less developed dendrohyphidia, and larger basidia and basidiospores (Ghobad-Nejhad *et al.* 2010). *Punctulariopsis* and *Punctularia* are mainly distributed in tropical and subtropical regions. Both genera have similar habits, but *Punctularia* has a considerably thicker fruiting body with well-developed layers of pigmented hyphae and numerous richly branched dendrohyphidia growing brown, and forming a cathahymenium, smaller basidia, and smaller spores. *Dendrocorticium* has a different fruiting body configuration, and lacks a gelatinous consistency. Unlike *Punctulariopsis*, which has large basidia sparsely hidden within a network of hyphidia, the basidia in *Dendrocorticium* are much smaller and are formed in a distinguishable layer (Ghobad-Nejhad *et al.* 2010).

The nLSU phylogeny showed that the taxa of Punctulariaceae included the three well-supported clades as *Punctulariopsis*, *Dendrocorticium* M.J. Larsen & Gilb., and *Punctularia* (Ghobad-Nejhad *et al.* 2010). All *Vuilleminia* species from Europe and Asia were grouped in the *Vuilleminia* clade together with *Cytidia salicina* and New Zealand *Vuilleminia* (*Australovuilleminia*). *Vuilleminia* (*Punctulariopsis*) *obducens* from Africa and *Vuilleminia* (*Punctulariopsis*) *subglobispora* from South America were nested in *Punctularia* Pat. and *Dendrocorticium* M.J. Larsen & Gilb. in the *Punctularia* clade. The species of these clades were collected on angiosperms and are composed of taxa with clamped hyphae (Ghobad-Nejhad *et al*. 2010). *Vuilleminia subglobispora* and *V*. *obducens* were described in the same year and their phylogenetic analyses of the nLSU and combined datasets were congruent in revealing a close relationship between the two species and their positioning in a well-supported clade (the *Punctularia* clade) together with *Dendrocorticium* and *Punctularia*, while *Vuilleminia* was nested in a different clade (the *Vuilleminia* clade) (Ghobad-Nejhad *et al*. 2010). Ghobad-Nejhad *et al*. (2010) established the genus *Punctulariopsis* to accommodate *P*. *obducens* (Hjortstam & Ryvarden) Ghob.-Nejh., and *P*. *subglobispora* (Hallenb. & Hjortstam) Ghob.-Nejh. (both species were first described in the genus *Vuilleminia*, but shown to represent an independent genus based on morphology and DNA sequence data). Therefore, two *Vuilleminia* species described from Africa and South America are transferred to *Punctulariopsis* by Ghobad-Nejhad *et al*. 2010.

Recently, we have collected a basidiomycetous fungus from Yunnan Province of China, which could not be assigned to any described species in the family Punctulariaceae. In this study, we present morphological and molecular phylogenetic evidence to support the identification of the new species in *Punctulariopsis*.

Materials and methods

Morphological studies.

Macromorphological studies were conducted on field notes, while Petersen (1996) followed the color terms. Micromorphological descriptions were recorded from the dried specimens (Hu *et al.* 2022), and examined under a compound light microscope following Ma *et al*. (2020) and Zhou *et al*. (2023). The following abbreviations were used: KOH = 5% Potassium hydroxide, CB = Cotton Blue, CB- = acyanophilous, IKI = Melzer's reagent, IKI- = both inamyloid and indextrinoid, L = mean spore length (arithmetic average of all spores), W = mean spore width (arithmetic average of all spores), Q = variation in the L/W ratios between the specimens studied, n (a/b) = number of spores (a) measured from given number (b) of specimens. The studied specimens were placed at the herbarium of the Southwest Forestry University (SWFC), Yunnan Province, China.

DNA extraction, amplification, sequencing, *and phylogenetic analysis.*

The EZNA HP Fungal DNA Kit (Omega Biotechnologies Co., Ltd, Kunming) was used to amplify PCR products from dried specimens according to the manufacturer's instructions (Xu *et al*. 2019; Zhang *et al.* 2023).

ITS region was amplified with primer pairs ITS5 and ITS4 (White *et al.* 1990), whereas the nuclear nLSU region was amplified with primer pairs LR0R and LR7 *(http://www.biology.duke.edu/fungi/mycolab/primers.htm).* The PCR conditions for ITS were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles at 94 °C for 40 s, 58 °C for 45 s and 72 °C for 1 min, and a final extension of 72 °C for 10 min. The PCR procedure for nLSU was as follows: initial denaturation at 94 °C for 1 min, followed by 35 cycles at 94 °C for 30 s, 48 °C 1 min, and 72 °C for 1.5 min, and a final extension of 72 °C for 10 min. The PCR products were purified and directly sequenced at Kunming Tsingke Biological Technology Limited Company, Yunnan Province, China. All the newly generated sequences were deposited in GenBank (Table 1).

TABLE 1. Names, sample numbers and corresponding GenBank accession numbers of the taxa used in this study.

Species name	Sample no.	GenBank accession no		References
		ITS	LSU	
Dentocorticium taiwanianum	He4615	MF626362	MF626386	Liu *et al*. 2018
D. taiwanianum	He3777	-	MF626388	Liu *et al*. 2018
D. portoricense	personal: Alden Dirks: ACD0358	ON364085	ON369537	Unpublished
D. portoricense	personal: Alden Dirks: ACD0365	ON364088	ON369540	Unpublished
D. portoricense	He2202	MF626357	MF626381	Liu *et al*. 2018
D. portoricense	He2161	MF626356	MF626380	Liu *et al*. 2018
D. bicolor	He2772	MF626354	MF626378	Liu *et al*. 2018
D. bicolor	He2757	MF626355	MF626379	Liu *et al*. 2018
D. sulphurellum	DLL2011-016	KJ140542	-	Brazee *et al*. 2014
D. sulphurellum	FP11801	JN165018	-	Justo & Hibbett 2011
D. ussuricum	CLZhao 16063	MW582752	-	Unpublished
D. ussuricum	He3278	MF626358	MF626382	Liu *et al*. 2018
D. ussuricum	KUC20121102-32	KJ668553	KJ668406	Unpublished
D. ussuricum	He3322	MF626360	MF626384	Liu *et al*. 2018
Gloeophyllum sepiarium	FP 125002-T	-	AY333806	Ghobad-Nejhad & Ginns 2012
G. abietinum	P254	-	AJ583431	Moreth & Schmidt 2005
Punctularia strigosozonata	AFTOL-ID 1248	DQ398958	-	Unpublished
P. strigosozonata	CBS 345.34	MH855559	MH867064	Vu *et al*. 2019
P. strigosozonata	BHI-F586b	MH558554	-	Haelewaters *et al*. 2018
P. bambusicola	CLZhao 4133	MW559982	MW559984	Guan *et al*. 2021
P. subhepatica	He 5695	MW507075	MW507015	Unpublished
P. subhepatica	He 4436	MW507074	MW507014	Unpublished
Punctulariopsis obducens	MG70	HM046918	-	Ghobad-Nejhad *et al*. 2010
P. cremeoalbida	Burdsall 9616	KR494275	-	Ariyawansa *et al*. 2015
P. efibulata	Burdsall 8824	KR494276	-	Ariyawansa *et al*. 2015
P. efibulata	He 2027	MW507077	-	Ariyawansa *et al*. 2015
P. subglobispora	GB: Hallenberg 12761	NR_119827	-	Ghobad-Nejhad *et al*. 2010
P. subglobispora	FCUG 2535	HM046917	HM046932	Ghobad-Nejhad *et al*. 2010
P. yunnanensis	**CLZhao 21333**	**PP425903**	**PP572972**	**Present Study**
P. yunnanensis	**CLZhao 21393**	**PP425904**	**PP572969**	**Present Study**
P. yunnanensis	**CLZhao 21443**	**PP425905**	**PP572972**	**Present Study**
P. yunnanensis	**CLZhao 21561**	**PP425906**	**PP572970**	**Present Study**
P. yunnanensis	**CLZhao 21683**	**PP425907**	**PP572971**	**Present Study**
P. yunnanensis	**CLZhao 21718**	**PP425908**	**PP572973**	**Present Study**
P. yunnanensis	**CLZhao 29618**	**PP190238**	**PP190235**	**Present Study**
P. yunnanensis	**CLZhao 29620**	**PP190239**	**PP190236**	**Present Study**
P. yunnanensis	**CLZhao 29632**	**PP190240**	**PP190237**	**Present Study**

Sequencher 4.6 (Gene Codes, Ann Arbor, MI, USA) was used to edit the DNA sequences. Sequences were aligned in MAFFT 7 (https://mafft.cbrc.jp/alignment/server/) using the "G-INS-I" strategy and manually adjusted in BioEdit (Hall 1999). Sequences of *Gloeophyllum abietinum* (Bull.) P. Karst. (1882: 80) and *G. sepiarium* (Bull.) P. Karst. (1882: 79) were obtained from GenBank and used as an outgroup to root trees following Ghobad-Nejhad and Duhem (2012) in the ITS+nLSU analysis (Fig. 1). Maximum parsimony analysis was applied to the ITS+nLSU dataset sequences. Approaches to phylogenetic analyses followed Zhao & Wu (2017) and Wang *et al*. (2020), and the tree construction procedure was performed in PAUP* version 4.0b10 (Swofford 2002). All characters were equally weighted and gaps were treated as missing data. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Max-trees were set to 5000, branches of zero length were

collapsed and all parsimonious trees were saved. Clade robustness was assessed using a bootstrap (BT) analysis with 1,000 replicates (Felsenstein 1985). Descriptive tree statistics tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were calculated for each Maximum Parsimonious Tree (MPT) generated. Sequences were also analyzed using Maximum Likelihood (ML) with RAxML-HPC2 on Abe through the Cipres Science Gateway (www.phylo.org; Miller *et al*. 2009). Branch support (BS) for ML analysis was determined using 1000 bootstrap replicates.

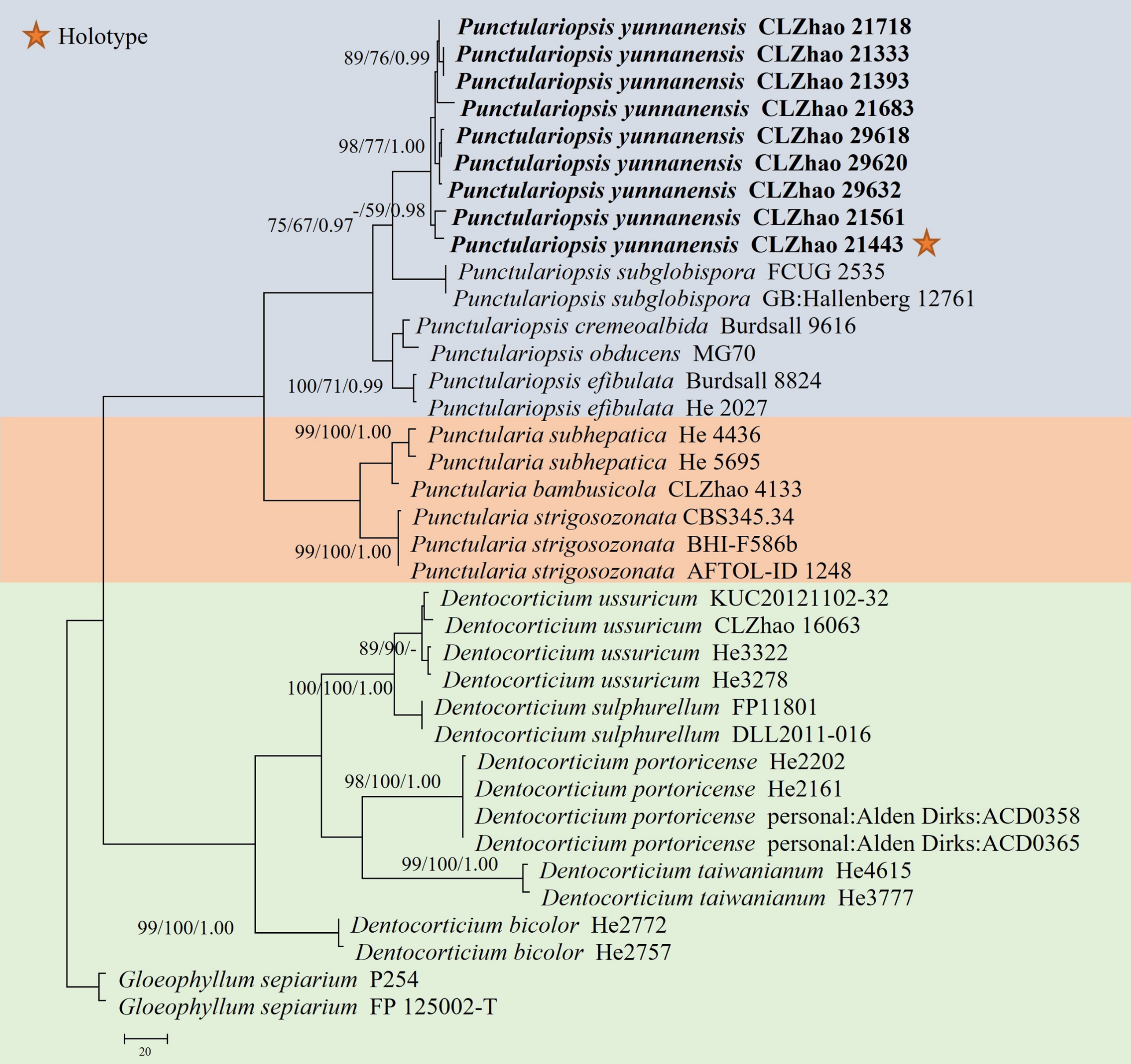

FIGURE 1. Maximum parsimony strict consensus tree illustrating the phylogeny of *Punctulariopsis yunnanensis* and related species in *Punctulariopsis* based on ITS+nLSU sequences. Branches are labeled with maximum likelihood bootstrap equal to or higher than 70%, parsimony bootstrap proportions equal to or higher than 50%, and Bayesian posterior probabilities equal to or more than 0.95 respectively.

MrModeltest 2.3 (Posada & Crandall 1998, Nylander 2004) was used to determine the best-fit evolution model for each dataset for Bayesian inference (BI). BI was calculated in the MrBayes3.1.2 model of DNA substitution and a gamma distribution rate variation across sites (Ronquist & Huelsenbeck 2003). Four Markov chains were run for two runs from random starting trees for 90 thousand generations (Fig. 1), and trees were sampled every 100 generations, while the first one-fourth generations were discarded as burn-in. A majority-rule consensus tree for all the remaining trees was calculated. Branches that received bootstrap support for maximum likelihood (BS), maximum parsimony (BT), and Bayesian posterior probabilities (BPP) greater than or equal to 75 % (BS), 75 % (BT), and 0.95 (BPP) were considered as significantly supported, respectively.

Results

Molecular phylogeny

The ITS+nLSU dataset (Fig. 1) included sequences from 37 fungal samples representing 15 species. The dataset had an aligned length of 1988 characters, of which 1355 characters are constant, 137 are variable and parsimony-uninformative, and 496 are parsimony-informative. The maximum parsimony analysis yielded three equally parsimonious trees (TL = 1041, CI = 0.7646, HI = 0.2354, RI = 0.9135, RC = 0.6985). The best model for the ITS+nLSU dataset is estimated and applied in the Bayesian analysis: GTR+I+G. Bayesian analysis and ML analysis resulted in a similar topology as MP analysis, and the effective sample size (ESS) across the two runs is double the average ESS (avg ESS) = 897.3267.

Phylogenetic analysis (Fig. 1) inferred from the combined ITS+nLSU sequences, was obtained for one species within the genus *Punctulariopsis* and showed that the new species formed a single lineage with a strong support and was sister to the *P. subglobispora*.

Taxonomy

Punctulariopsis yunnanensis A. Muhammad & C.L. Zhao, *sp. nov.* (Figs. 2, 3)
MycoBank no.: MB 853715

Etymology:—*yunnanensis* (Lat.) refers to the type location "Yunnan Province, China".

***Holotype*:**—CHINA. Yunnan Province, Lijiang, Lashihai Nature Reserve, International Wetland Park, Meiquan Village E 100°14′78″, N 26°86′63″, alt. 2437 m, on the fallen branch of the angiosperm, on July 19 2021, *CLZhao 21443* (SWFC).

***Basidiomata*:**—Annual, resupinate, adnate but easily separable, gelatinous when fresh, becoming rigid upon drying, up to 15 cm long, up to 3.5 cm wide, 100–200 µm thick. Hymenial surface smooth, white to cream when fresh, turned cream to pinkish buff to salmon upon drying. Sterile margin narrow, white, up to 1 mm.

***Hyphal structure*:**—Hyphal system monomitic; generative hyphae colorless, thick-walled, with clamp connections, IKI-, CB-; tissues unchanged in KOH. Subiculum almost absent or indistinct, subhymenial generative hyphae colorless, more or less interwoven, thin-walled, rarely branched, 2.6–3.5 µm in diameter, crystals present among generative hyphae.

***Hymenium*:**—Cystidia and cystidioles absent; dendrohyphidia colorless, thin-walled; basidia clavate, flexuous, with four sterigmata and a basal clamp connection, 19.5–46.9 × 5–9.1 µm; basidioles dominant, in shape similar to basidia, but slightly smaller.

***Spores*:**—Basidiospores broadly ellipsoid, thin-walled, smooth, IKI-, CB-, (7.6–)7.8–12.8(–13) × 6.0–9.8 µm, L = 10.11 µm, W = 8 µm, Q = 1.22–1.39 (n = 270/9).

***Additional examined specimens (paratypes)*:**—CHINA. Yunnan Province, Lijiang, Lashihai Nature reserve, International Wetland Park, Meiquan Village, E 100°14′78″, N 26°86′63″, alt. 2437 m, on the fallen branch of angiosperm, 19 July 2021, *CLZhao 21333*, and *CLZhao 21393* (SWFC); Lijiang, Heilongtan Park, Xiangshan, E 100°24′47″, N 26°8944′10″, alt. 2384 m, *CLZhao 21561, CLZhao 21683*, and *CLZhao 21718* (SWFC); Qujing, Qilin District, Cuishan Forest Park, E 103°70′76″, N 25°55′52″, alt. 1875 m, on the fallen branch of angiosperm, 5 November 2022, *CLZhao 26172*, *CLZhao 26282*, and *CLZhao 26691* (SWFC); Qujing, Zhanyi District, Lingjiao Town, Xiajia Village, E 103°67′72″, N 25°88′48″, alt. 1859 m, on the fallen branch of angiosperm, 6 March 2023, *CLZhao 27244* (SWFC); Qujing, Zhanyi District, Dapo Town, Tulo Village, E 103°66′98″, N 25°68′02″, alt. 1859 m, on the fallen branch of *Pyrus xerophila*, 7 March 2023, *CLZhao 27435*, *CLZhao 27441*, *CLZhao 27446*, *CLZhao 27449*, *CLZhao 27452*, and *CLZhao 27484* (SWFC); Zhaotong, Yiliang County, Xiaocaoba Town, Wumengshan National Nature Reserve, E 104°25′66″, N 27°77′10″, alt. 1700 m, on the fallen branch of angiosperm, 13 July 2023, *CLZhao 29618*, *CLZhao 29620*, and *CLZhao 29632* (SWFC), 20 September 2023, *CLZhao 31803*, *CLZhao 31907*, *CLZhao 33481*, *CLZhao 33616*, *CLZhao 33676*, *CLZhao 33695*, *CLZhao 33710*, and *CLZhao 33713* (SWFC).

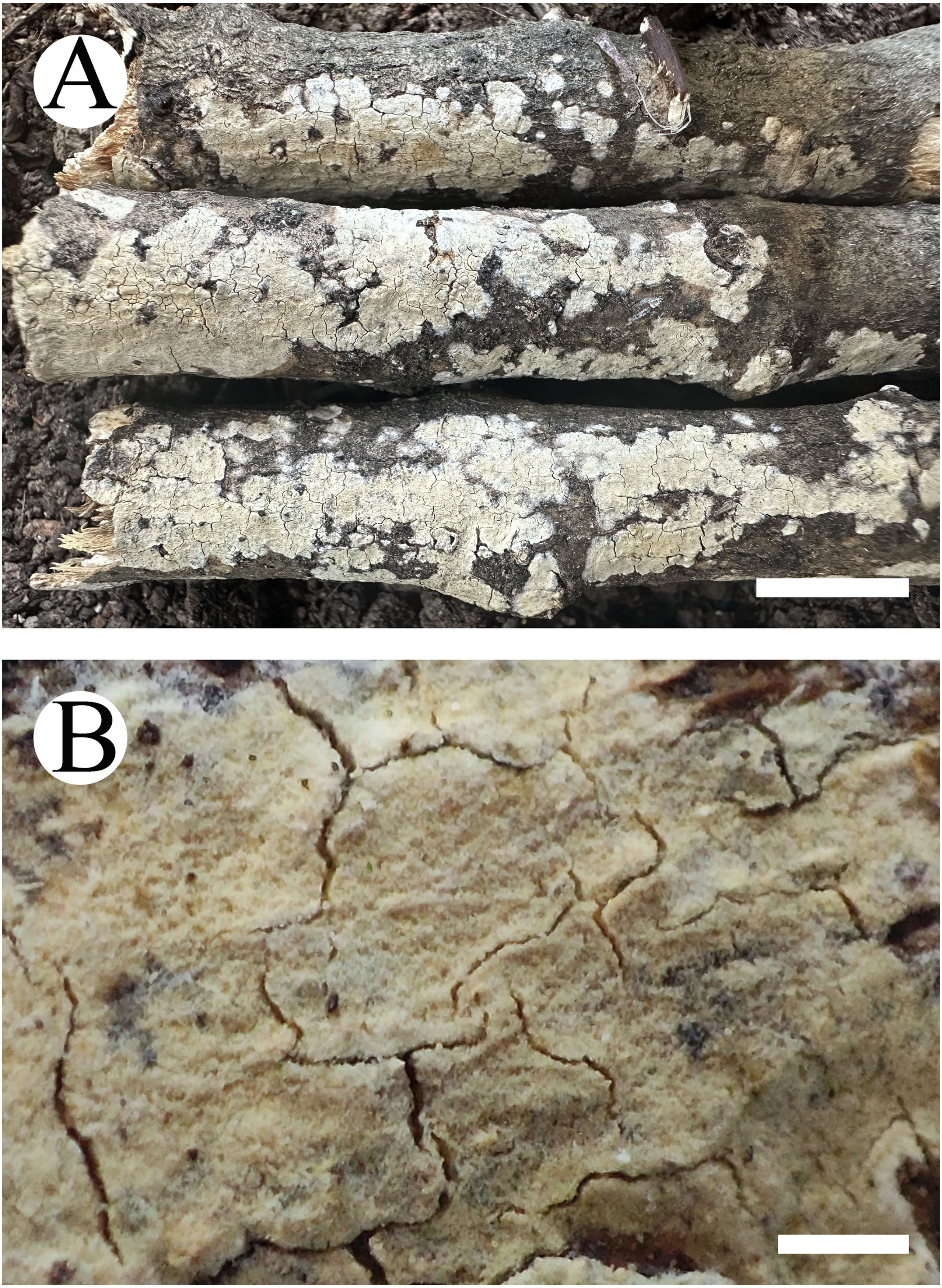

FIGURE 2. Basidiomata of *Punctulariopsis yunnanensis* (holotype). Bars: A = 1 cm, B = 1 mm.

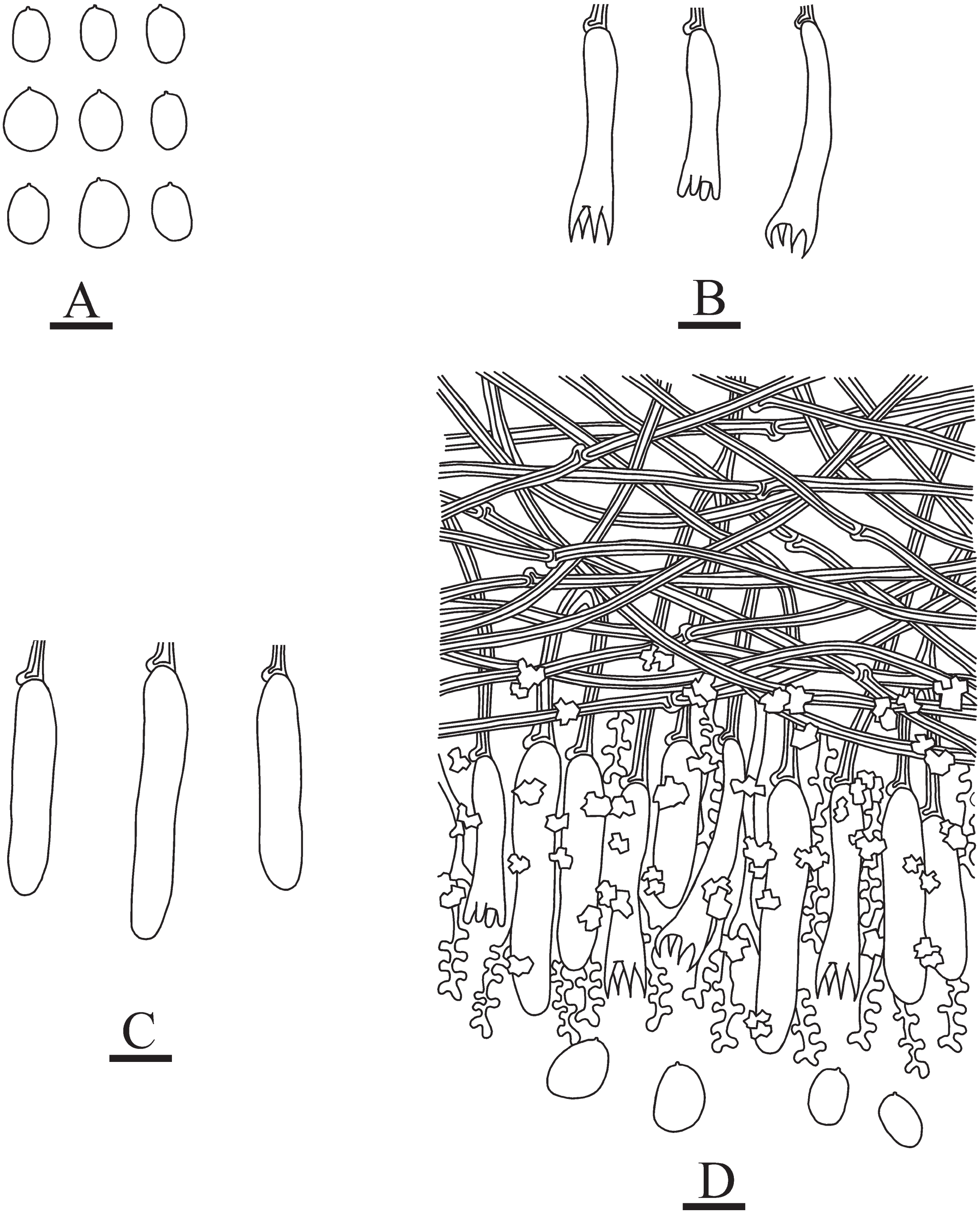

FIGURE 3. Microscopic structures of *Punctulariopsis yunnanensis* (drawn from the holotype). A: basidiospores. B: basidia. C: basidioles. D: a section of hymenium. Bars: A = 5 µm, B–D = 10 µm.

Discussion

In the present study, a new species, *Punctulariopsis yunnanensis*, is described based on phylogenetic analysis and morphological characteristics. Phylogenetically, *P. yunnanensis* is closely related to *P. subglobispora* based on ITS + nLSU nrDNA gene analysis and forms a clade with four previously published (Fig. 1).

Phylogenetically, based on the ITS + nLSU, *Dendrocorticium*, *Punctulariopsis*, and *Punctularia* were grouped in the family Punctulariaceae (Ghobad-Nejhad *et al.* 2010). In the present study, based on the ITS+nLSU data (Fig. 1), the new species was grouped into the genus *Punctulariopsis*. From the phylogram inferred from the ITS+nLSU data (Fig. 1), *P. yunnanensis* was retrieved as a sister to *P. subglobispora.* However, morphologically, *P. subglobispora* differs from *P. yunnanensis* by its pruinose to light ochraceous hymenial surface, subglobose to broadly ellipsoid basidiospores measure 14.5–16 × 9.5–12 µm (Hallenberg & Hjortstam 1996).

During the study on Corticiales taxa, the phylogenetic affinity of two *Corticium* Pers. (1794: 110) species namely *C. cremeoalbidum* (M.J. Larsen & Nakasone) M.J. Larsen (1990: 59) and *C. efibulatum* (M.J. Larsen & Nakasone) M.J. Larsen (1990: 60) were examined for the first time. Phylogenetic analyses of ITS and LSU sequences showed that both *C. cremeoalbidum* and *C. efibulatum* resided well in the genus *Punctulariopsis*, and their morphological characteristics matched the concept of the genus (Ghobad-Nejhad & Duhem 2014). *Punctulariopsis cremeoalbida* and *P. efibulata* were originally described by Larsen and Nakasone (1984) for two corticioid species with effused basidiomes growing on *Vitis* (the former), and on *Vaccinium* (the latter) in North America. The light-colored, thin, adnate basidiomes of both species, their little-branched dendrohyphidia, large and broadly ellipsoid spores with blunt apiculus, and their growth on hardwood match well with the concept of *Punctulariopsis* (Ghobad-Nejhad *et al.* 2010). Both species are apparently known only from their type localities in the USA. With the two species combined in *Punctulariopsis*, the number of species in this genus has grown to four, while *P. efibulata* is currently the only species in the genus lacking clamps on its hyphae (Ariyawansa *et al.* 2015).

Corticioid fungi, a large characteristic group of Basidiomycota, are primarily found in tropical and subtropical regions. To date, only four species of *Punctulariopsis* have been identified, but the diversity of *Punctulariopsis* in China is still not well known. This paper reports a new species, *P. yunnanensis*, which enriches our knowledge of fungal diversity in this area. The discovery of *P. yunnanensis* underscores the need for further fieldwork and molecular analyses to uncover more new taxa and expand our understanding of fungal diversity.

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