



Morphological characteristics and phylogenetic analyses revealed two additional taxa in *Cyathus* (Agaricales, Basidiomycota)

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Abstract

Two new species of the bird's nest fungus, *Cyathus asiaticus* and *C. prismaticus* are proposed based on a combination of the morphological and molecular evidence. *Cyathus asiaticus* is characterized by having an obconical, campanulate to cupulate basidiomata with the inconspicuously striation on the inner surface of the peridium, smooth mouth, silver gray to black peridioles with funicular cord, a monomitric hyphal system bearing the generative hyphae with clamp connections and broadly ellipsoid, elliptical, subglobose, ovoid, irregular, thick-walled basidiospores with inconspicuous apiculus. *Cyathus prismaticus* is characterised by the infundibuliform, obconical to cupulate basidiomata covered with shaggy or hirsute hairs, presence of the striations on the outer and inner surface of the peridium, peridioles with the funicular, a dimitic hyphal system with generative hyphae having clamp connections, presence of the narrow oblique prisms and subglobose, ovoid, elliptical to broadly ellipsoid, thick-walled basidiospores. Sequences of ITS and nLSU rRNA markers of the studied samples were generated, and the phylogenetic analysis were performed with maximum likelihood, maximum parsimony and Bayesian inference methods on two datasets (ITS+nLSU and ITS). The dataset analysis showed that two new species clustered into the genus *Cyathus*.

Keywords: molecular phylogeny, Nidulariaceae, taxonomy, wood-inhabiting fungi, Yunnan Province

Introduction

In forest ecosystems, wood rotting fungi play essential ecological roles, driving carbon cycling in forest soils, mediating mineral nutrition of plants, and alleviating carbon limitations (Tedersoo *et al.* 2014). The species of *Cyathus* are saprobic, usually growing in decaying wood, on manure or directly on soil are a cosmopolitan group and have a rich diversity related to the high diversity of plants growing in boreal, temperate, subtropical, and tropical regions (Brodie 1975, Shinnars-Carnelley *et al.* 2002, Blenis & Chow 2005, Zhao *et al.* 2008; Kraistudomsook *et al.* 2022).

The genus *Cyathus* Haller (1768: 236) (Nidulariaceae, Nidulariales) was first introduced by Haller (Haller 1768) and later was adopted by Persoon (Persoon 1801), typified by *C. striatus* (Huds.) Willd. (1787: 399). Due to the cup-like basidiomata resembling bird nest and lenticular peridioles resembling eggs, *Cyathus* together with *Crucibulum* Tul. & C. Tul. (1844: 89), *Mycocalia* J.T. Palmer (1961: 58), *Nidula* V.S. White (1902: 271), and *Nidularia* Fr. (1817: 2), are commonly known as bird's nest fungi (Brodie 1975, Gómez & Pérez-Silva 1988, Zhao *et al.* 2008). This genus is characterized by having the deeper or cuped, inverted bell-like basidiomata covered with shaggy or tomentose hairs on the outside; peridium composed of three layers of tissues, inside peridium filled with a number of dark-colored, small, hard lentil-shaped peridioles attached with funicular cords; colorless, thin-walled or thick-walled, smooth basidiospores (Lloyd 1906, Brodie 1974, 1975, Brodie & Sharma 1980, Miller & Miller 1988, Das & Zhao

2012, 2013). Both MycoBank database (<http://www.Mycobank.org>; 9 March 2023) and Index Fungorum (<http://www.indexfungorum.org>; 9 March 2023) register 207 specific and infraspecific names in genus *Cyathus*, but the actual number of species about sixty (Nirajan & Singh, 2021), including 35 species from China (Zhou 2007, Duan *et al.* 2022).

The comprehensive molecular systematics studies on *Cyathus* were rare in the last 20 years (Matheny *et al.* 2006, Zhao *et al.* 2007, Kraistudomsook *et al.* 2022). An overview of the phylogeny on Agaricales based on a multilocus analysis of a six-gene region supermatrix revealed that Nidulariaceae was close to Cystodermateae, in which *Cyathus striatus* and *Crucibulum laeve* (Huds.) Kambly (1936: 167) grouped together within the family Nidulariaceae (Matheny *et al.* 2006). Phylogenetic relationships among *Cyathus* (bird's nest fungi) were investigated with ITS and nLSU ribosomal DNA sequences datasets inferred from the neighbor joining, maximum likelihood, weighted maximum parsimony and MrBayes analyses, in which the morphological characters and the molecular data were incompatible, such as the peridium plications and variations in peridium hair anatomy, peridiole structure and fruit-body color, while the ITS and nLSU datasets supported the recognition of three infrageneric groups herein named the ollum, pallidum and striatum groups (Zhao *et al.* 2007). The phylogeny of *Cyathus* based on ITS and nLSU ribosomal DNA sequences showed that *C. cheliensis* F.L. Tai & C.S. Hung (1948: 161), *C. gansuensis* B. Yang, J. Yu & T.X. Zhou (2002: 313), and *C. megasporus* W. Ren & T.X. Zhou (1992: 25) were respectively accepted as synonyms of *C. limbatus* Tul. & C. Tul. (1844: 78), *C. pygmaeus* Lloyd (1906: 26), and *C. poeppigii* Tul. & C. Tul. (1844: 77) (Zhao *et al.* 2008). On the basis of the morphological and molecular data, Martin *et al.* (2015) studied on the affinities among *Cyathus* species, which showed that this group formed a monophyletic lineage with high support. Phylogenetic reconstruction of *Cyathus* species based on an alignment of 641 nucleotides of the ITS region indicated that three new species as *C. batistae* M.M.B. Barbosa & Baseia (2016: 60), *C. apiculatus* M.M.B. Barbosa & Baseia (2016: 62), and *C. pedunculatus* M.A. Silva, M.M.B. Barbosa & Baseia (2016: 63) were proposed, and discussed the relationships with other species of *Cyathus* (Silva *et al.* 2016). Phylogenetic analysis of the bird's nest fungi investigated with four loci (ITS, nLSU, *TEF1*, and *RPB2*) revealed that the family Nidulariaceae was resolved as a monophyletic lineage with Squamanitaceae as a potential sister branch, and suggested that the species concepts needed to be revisited and refined throughout the family Nidulariaceae and several bird's nest fungi species had global geographical distributions, whereas others may have more limited ranges, and the basic morphological characters of bird's nest fungi had likely been lost or gained multiple times (Kraistudomsook *et al.* 2021). The phylogenetic research using five loci (ITS, nLSU, SSU, *TEF1*, and *RPB2*) revealed that a new genus *Retiperidiolia* Kraisit., Choeyklin, Boonprat. & M.E. Sm. (2022: 4) was accommodated this phylogenetically and morphologically unique bird's nest fungus lineage, in which *Cyathus* formed a monophyletic lineage and then was sister to the genus *Retiperidiolia* (Kraistudomsook *et al.* 2022). Based on molecular systematics and morphology, one new species of *Cyathus* from China was found and recorded (Duan *et al.* 2022).

During the investigations on bird's nest fungi in southern China, we found two species of *Cyathus* that could not be assigned to any of the described species. These species are here described as *C. prismaticus* *sp. nov.* and *C. asiaticus* *sp. nov.* based on the morphological characteristics and molecular phylogenetic analyses.

Materials and methods

Morphological studies

The fresh fruiting bodies of the bird's nest fungi were collected from the Wenshan of Yunnan Province, China. The fresh specimens were dried in an electric food dehydrator at 40 °C, then sealed and stored in an envelope bag and deposited in the herbarium of the Southwest Forestry University (SWFC), Kunming, Yunnan Province, China. The macromorphological descriptions were based on field notes and photos captured in the field and lab. The macromorphological descriptions are based on Brodie (1975). The micromorphological data were obtained from the dried specimens and observed under Nikon Eclipse E100 light microscope following Zhao & Wu (2017). The color terms follow Kornerup & Wanscher (1978). Drawings were made with the aid of a fungus plotter. The measurements and drawings were made from slide preparations stained with Cotton Blue (0.1 mg aniline blue dissolved in 60 g pure lactic acid), Melzer's reagent (3 g potassium iodide, 1 g crystalline iodine, 44 g chloral hydrate, aq. dest. 40 ml) and 5% potassium hydroxide. In presenting spore size data, 5% of the measurements excluded from each end of the range are shown in parentheses. The following abbreviations are used: KOH = 5% potassium hydroxide; CB = cotton blue; CB- = acyanophilous; IKI = Melzer's reagent; IKI- = non-amyloid and non-dextrinoid; 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).

DNA extraction, PCR amplification, sequencing and phylogenetic analyses

The CTAB rapid plant genome extraction kit-DN14 (Aidlab Biotechnologies Co., Ltd, Beijing, China) was used to obtain genomic DNA from the dried fungal specimens according to the manufacturer's instructions (Zhao & Wu 2017). The nuclear ribosomal ITS region was amplified with ITS5 and ITS4 primer pair (White *et al.* 1990). The nuclear nLSU region was amplified with the LR0R and LR7 primer pair (<http://lutzonilab.org/nuclear-ribosomal-dna/>). The PCR procedure for ITS was 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 for 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 sequenced at Kunming Tsingke Biological Technology Limited Company (Yunnan Province, China). All of the newly generated sequences were deposited in NCBI GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) (Table 1).

TABLE 1. List of species, specimens and GenBank accession numbers of sequences used in this study. The new species are in bold.

Species name	Sample no.	Locality	GenBank accession no.		References
			ITS	nLSU	
<i>Crucibulum crucibuliforme</i>	F-C0294427F	USA	—	MW600342	Kraisitudomsook <i>et al.</i> 2021
<i>C. cyathiforme</i>	Mushroom Observer 426306	USA	MW633046	—	Kraisitudomsook <i>et al.</i> 2022
<i>C. laeve</i>	UTC-0025855	USA	MT444042	MT444013	Kraisitudomsook <i>et al.</i> 2021
<i>C. laeve</i>	SWFC 21261	China	DQ463357	—	Zhao <i>et al.</i> 2007
<i>C. parvulum</i>	FLAS-F-66522	USA	—	MW600344	Kraisitudomsook <i>et al.</i> 2021
<i>Cyathus africanus</i>	DAOM 200370 ^a	Tanzania	DQ463347	DQ463330	Zhao <i>et al.</i> 2007
<i>C. albinus</i>	UFRN-Fungos 2239	Brazil	KY176371	—	Accioly <i>et al.</i> 2018
<i>C. amazonicus</i>	URM 80036 ^a	Brazil	KY495280	—	Accioly <i>et al.</i> 2018
<i>C. amazonicus</i>	UFRN-Fungos 2798	Brazil	KY176375	—	Accioly <i>et al.</i> 2018
<i>C. annulatus</i>	MichaelKuo-8200901	USA	MT444076	—	Kraisitudomsook <i>et al.</i> 2021
<i>C. annulatus</i>	DAOM 200366 ^a	Canada	NR119588	NG058740	Zhao <i>et al.</i> 2007
<i>C. apiculatus</i>	UFRN:Fungos 1448	Brazil	KT365516	—	Silva <i>et al.</i> 2016
<i>C. asiaticus</i>	CLZhao 19445^a	China	OP251005	OP251007	Present study
<i>C. asiaticus</i>	CLZhao 19847	China	OP251006	OP251008	Present study
<i>C. aurantogriseocarpus</i>	UFRN:Fungos:2798	Brazil	KX966026	—	Cruz <i>et al.</i> 2017
<i>C. badius</i> Kobayasi	KH:JPN15-1321	Japan	KX906250	—	Cruz <i>et al.</i> 2018
<i>C. batistae</i>	UFRN:Fungos 1449	Brazil	KT365515	—	Silva <i>et al.</i> 2016
<i>C. berkeleyanus</i>	SWFC 20789	China	DQ463355	—	Zhao <i>et al.</i> 2007
<i>C. bulleri</i> H.J.	DAOMC 195062	India	MK020156	—	Vats & Mishra 2021
<i>C. canna</i> Lloyd	CBS 370.80	Costa Rica	MH861275	—	Vu <i>et al.</i> 2019
<i>C. colensoi</i> Berk.	DAOM 200423	India	DQ463344	—	Zhao <i>et al.</i> 2007
<i>C. crassimurus</i>	DAOM 200372 ^a	Hawaii	DQ463350	—	Zhao <i>et al.</i> 2007
<i>C. discoideus</i>	AB 7831	China	KY652080	—	Cruz 2017
<i>C. gansuensis</i>	SWFC 20880 ^a	China	DQ463348	—	Zhao <i>et al.</i> 2007
<i>C. gansuensis</i>	69	China	KC869661	—	Cruz <i>et al.</i> 2018
<i>C. gracilis</i>	AB7873	Japan	KY652081	—	Cruz 2017

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TABLE 1. (Conintued)

Species name	Sample no.	Locality	GenBank accession no.		References
			ITS	nLSU	
<i>C. hookeri</i>	SWFC 20799	China	DQ463346	—	Zhao <i>et al.</i> 2007
<i>C. hortensis</i>	UFRN:Fungos:1819	Brazil	KX906252	—	Cruz <i>et al.</i> 2018
<i>C. ibericus</i>	AH:48137 ^a	Spain	KX858597	—	Crous <i>et al.</i> 2016
<i>C. ibericus</i>	AH:48138	Spain	KX858598	—	Crous <i>et al.</i> 2016
<i>C. intermedius</i>	UFRN:Fungos 1033	Brazil	KT365519	—	Silva <i>et al.</i> 2016
<i>C. jiyuguanensis</i>	SWFC 20846 ^a	China	DQ463341/ NR119585	NG068994	Zhao <i>et al.</i> 2007
<i>C. lignilantanae</i>	MA Fungi 87327	Cape Verde	NR_154827	—	Cruz <i>et al.</i> 2018
<i>C. limbatus</i>	UFRN:Fungos 2238	Brazil	KY176373	—	Accioly <i>et al.</i> 2018
<i>C. magnomuralis</i>	UFRN:Fungos:1817	Brazil	KX906251	—	Cruz <i>et al.</i> 2018
<i>C. minimus</i> Pat.	AB7868	Japan	KY652082	—	Cruz 2017
<i>C. novae-zeelandiae</i>	PDD-76442	New Zealand	MT444096	MT444025	Kraisitudomsook <i>et al.</i> 2021
<i>C. olla</i>	BPI 727227	Canada	DQ463345	DQ463327	Zhao <i>et al.</i> 2007
<i>C. olla</i>	PDD-86833	New Zealand	MT444086	MW600346	Kraisitudomsook <i>et al.</i> 2021
<i>C. pallidus</i>	KKUITN2	Thailand	KU202745	—	Sutthisa & Sanoamuang 2020
<i>C. pallidus</i>	KKUITN3	Thailand	KU202751	—	Sutthisa & Sanoamuang 2020
<i>C. pallidus</i>	NK-01	USA	MT444032	MT444006	Kraisitudomsook <i>et al.</i> 2021
<i>C. parvocinereus</i>	UFRN:Fungos:1814	Brazil	KX906253	—	Cruz <i>et al.</i> 2018
<i>C. pedunculatus</i>	UFRN:Fungos 403	Brazil	KT365518	—	Silva <i>et al.</i> 2016
<i>C. poeppigii</i>	cp-457	Brazil	KT962176	—	Silva <i>et al.</i> 2016
<i>C. prismaticus</i>	CLZhao 2935^a	China	OP251012	OP251011	Present study
<i>C. pyristriatu</i>	MFLUCC:14-0770	Germany	KU865513	—	Richter <i>et al.</i> 2016
<i>C. renweii</i>	SWFC 201406 ^a	China	DQ463352	DQ463333	Zhao <i>et al.</i> 2007
<i>C. setosus</i>	DAOM 200815 ^a	Jamaica	DQ463349	—	Zhao <i>et al.</i> 2007
<i>C. stercoreus</i>	NK-08	USA	MT444037	—	Kraisitudomsook <i>et al.</i> 2021
<i>C. stercoreus</i>	DM4	Canada	KY706156	—	Hay <i>et al.</i> 2019
<i>C. striatus</i>	NK-61	England	MT444056	MT444020	Kraisitudomsook <i>et al.</i> 2021
<i>C. subglobisporus</i>	BBH-14815	USA	MT444063	—	Kraisitudomsook <i>et al.</i> 2021
<i>C. subglobisporu</i>	BBH18348	Thailand	EF613553	—	Zhao <i>et al.</i> 2008
<i>C. triplex</i> Lloyd	SWFC 21077	China	DQ463353	—	Zhao <i>et al.</i> 2007
<i>C. uniperidiolus</i>	AMH:10196	India	MN398297	—	Boonmee <i>et al.</i> 2021
<i>Cystodermella cinnabarina</i>	DBG-20996	USA	—	MW600359	Kraisitudomsook <i>et al.</i> 2021
<i>C. cinnabarina</i>	DBG-29008	USA	—	MW600358	Kraisitudomsook <i>et al.</i> 2021
<i>Nidula candida</i>	UBCF23768	Canada	KC581303	—	Unpublished
<i>N. niveotomentosa</i>	SWFC 3000	China	DQ463358	—	Zhao <i>et al.</i> 2007
<i>N. niveotomentosa</i>	UBC:F19769	Canada	HQ604744	—	Silva <i>et al.</i> 2016

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TABLE 1. (Continued)

Species name	Sample no.	Locality	GenBank accession no.		References
			ITS	nLSU	
<i>Nidularia deformis</i>	CMP	Spain	MW248505	—	Olariaga <i>et al.</i> 2020
<i>N. farcta</i>	CBS-156.42	Sweden	MH856106	MW600352	Kraisitudomsook <i>et al.</i> 2021
<i>N. pulvinata</i>	FH-00822889	USA	MT444039	MT444010	Kraisitudomsook <i>et al.</i> 2021
<i>Retiperidiolia aquaphila</i>	UFRN:Fungos:2944	Brazil	MG836281	MG836282	Kraisitudomsook <i>et al.</i> 2022

^a indicates type material (holotype).

The sequencer 4.6 (GeneCodes, Ann Arbor, MI, USA) was used to assemble and edit the generated sequence reads. Sequences were aligned in MAFFT 7 (<https://mafft.cbrc.jp/alignment/server/>) using the “G-INS-I” strategy. The alignment was adjusted manually using AliView version 1.27 (Larsson 2014). *Cystodermella cinnabarina* (Alb. & Schwein.) Harmaja (2002: 45) was selected as an outgroup for phylogenetic analyses of ITS+nLSU phylogenetic trees (Kraisitudomsook *et al.* 2021). *Crucibulum laeve* and *Nidula niveotomentosa* (Henn.) Lloyd (1910: 455) were selected as outgroups for the phylogenetic analysis of ITS phylogenetic tree (Accioly *et al.* 2018).

Maximum parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI) analyses were applied to the two datasets. Approaches to phylogenetic analyses followed Zhao & Wu (2017). MP analysis was performed in PAUP* version 4.0b10 (Swofford 2002). All of the 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 most-parsimonious trees were saved. Clade robustness was assessed using bootstrap (BT) analysis with 1000 replicates (Felsenstein 1985). Descriptive tree statistics tree length (TL), the consistency index (CI), the retention index (RI), the rescaled consistency index (RC), and the homoplasy index (HI) were calculated for each most-parsimonious tree generated. ML was inferred using RAxML-HPC2 through the Cipres Science Gateway (www.phylo.org) (Miller *et al.* 2009). Branch support (BS) for ML analysis was determined by 1,000 bootstrap replicates and evaluated under the gamma model.

MrModeltest 2.3 (Nylander 2004) was used to determine the best-fit evolution model for the dataset for Bayesian Inference (BI). Bayesian Inference was performed with MrBayes 3.1.2 with a general time reversible (GTR+I+G) 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 500 thousand generations for ITS+nLSU (Fig. 1), or for 600 thousand generations for ITS (Fig. 2) with trees and parameters sampled every 100 generations. The first quarter of all the generations were discarded as burn-ins. A majority rule consensus tree of all remaining trees and posterior probabilities were calculated. Branches were considered significantly supported if they received a maximum likelihood bootstrap value (BS) of $\geq 70\%$, a maximum parsimony bootstrap value (BT) of $\geq 70\%$, or Bayesian posterior probabilities (BPP) of ≥ 0.95 .

Results

Molecular phylogeny

The ITS+nLSU dataset (Fig. 1) included sequences from 28 fungal specimens representing 23 species. The dataset had an aligned length of 2,084 characters, of which 1,494 characters are constant, 153 are variable and parsimony-uninformative, and 437 are parsimony-informative. Maximum parsimony analysis yielded one equally parsimonious trees (TL = 1,192, CI = 0.6971, HI = 0.3029, RI = 0.8157, RC = 0.5687). Best model for the ITS+nLSU dataset estimated and applied in the Bayesian analysis was GTR+I+G (lset nst = 6, rates = invgamma; prset statefreqpr = dirichlet (1,1,1,1)). The bayesian analysis and ML analyses resulted in a similar topology to that of the MP analysis with an average standard deviation of split frequencies = 0.005844 (BI), and the effective sample size (ESS) across the two runs is the double of the average ESS (avg ESS) = 281.

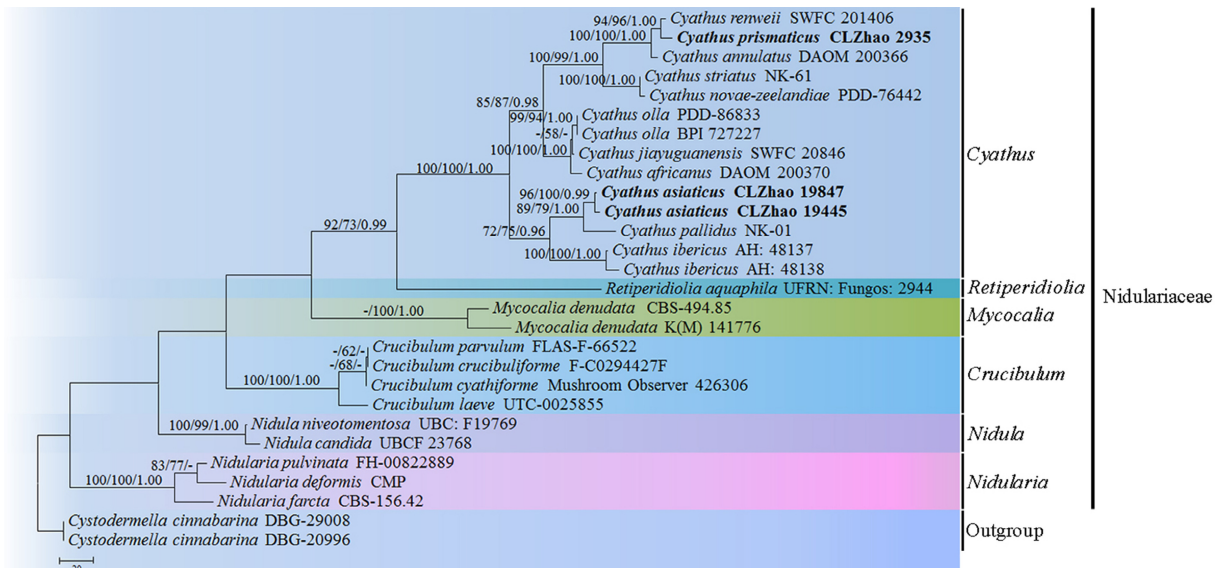


FIGURE 1. Maximum parsimony strict consensus tree illustrating the phylogeny of two new species of *Cyathus* and related genera in the family Nidulariaceae based on ITS+nLSU sequences. Branches are labelled with maximum likelihood bootstrap values $\geq 70\%$, parsimony bootstrap values $\geq 50\%$ and Bayesian posterior probabilities ≥ 0.95 , respectively, Scale bar = 20. The new species are in bold.

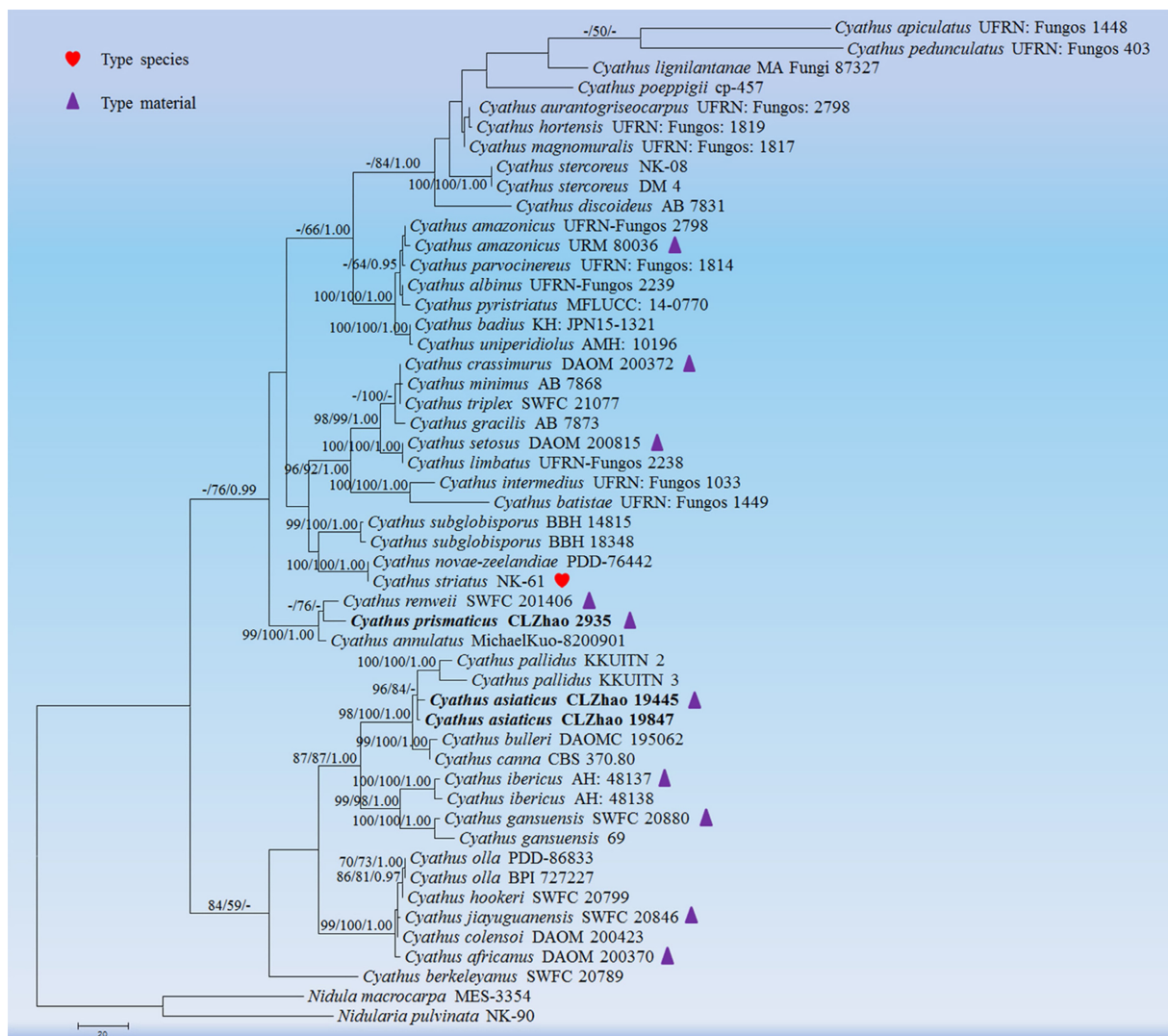


FIGURE 2. Maximum parsimony strict consensus tree illustrating the phylogeny of two new species and related species in *Cyathus* based on ITS sequences. Branches are labelled with maximum likelihood bootstrap values $\geq 70\%$, parsimony bootstrap values $\geq 50\%$ and Bayesian posterior probabilities ≥ 0.95 , respectively. Scale bar = 20. The new species are in bold.

The phylogeny (Fig. 1) based on the combined ITS+nLSU sequences indicated that both species *Cyathus asiaticus* and *C. prismaticus* were nested within the genus *Cyathus*, in which *C. asiaticus* was sister to *C. pallidus* Berk. & M.A. Curtis (1868: 346) with moderate supports (89% BS, 79% BT, 1.00 BPP); *C. prismaticus* closely grouped with *C. renweii* T.X. Zhou & R.L. Zhao (2004: 245) with moderate supports (94% BS, 96% BT, 1.00 BPP).

The ITS-alone dataset (Fig. 2) included sequences from 51 fungal specimens representing 43 species. The dataset had an aligned length of 865 characters, of which 327 characters are constant, 229 are variable and parsimony-uninformative, and 309 are parsimony-informative. Maximum parsimony analysis yielded 5000 equally parsimonious trees (TL = 1,236, CI = 0.6392, HI = 0.3608, RI = 0.7855, RC = 0.5020). Best model for the ITS dataset estimated and applied in the Bayesian analysis was GTR+I+G (lset nst = 6, rates = invgamma; prset statefreqpr = dirichlet (1,1,1,1)). The bayesian analysis and ML analyses resulted in a similar topology to that of the MP analysis with an average standard deviation of split frequencies = 0.009529 (BI), and the effective sample size (ESS) across the two runs is the double of the average ESS (avg ESS) = 122.5.

The phylogenetic tree (Fig. 2) inferred from ITS sequences revealed that *Cyathus asiaticus* was sister to *C. pallidus*, and then grouped with *C. bulleri* H.J. Brodie (1967a: 68) & *C. canna* Lloyd (1906: 27) with high supports (98% BS, 100% BP and 1.00 BPP). Another species *C. prismaticus* was sister to *C. renweii* with lower supports (–BS, 76% BT, –BPP), and then grouped with *C. annulatus* H.J. Brodie (1970: 749) with high supports (99% BS, 100% BT, 1.00 BPP).

Taxonomy

Cyathus asiaticus Z.Y. Duan & C.L. Zhao, *sp. nov.* Figs. 3–6
Mycobank no.: MB 845559

Etymology:—*asiaticus* (Lat.) referring to the provenance (Asian) of the type specimen.

Holotype:—CHINA. Yunnan Province, Wenshan, Pingba Town, Huguangqing Village, E 104°33', N 23°60', on fallen branch of *Pinus yunnanensis* Franch, 11 August 2020, CLZhao 19445 (SWFC).

Fruiting body:—obconical, campanulate to cupulate, 5–10 mm in height, 4–6 mm in width at the upper part, without expanding at the top or tapering abruptly at the base; the base usually attached to the substrate by a conspicuous emplacement, yellowish brown (5E4). *young fruit bodies* clavate, covered by hairs aggregated into tomentum or shaggy clusters, pale yellow (4A3) to yellowish brown (5E4). *exoperidium* yellowish to yellowish brown (5E4), shaggy or hirsute, external wall non-striate, covered with pale yellow (4A3), irregular and flexible tufts of hair; hair hyphae with the clamp connections, yellowish, thick-walled (up to 0.5–2 µm thick), 2.5–11.5 µm in diameter; *endoperidium* concolorous with the exoperidium, non-shiny, smooth to faintly striate, with 0.5–0.7 mm between grooves. *mouth* smooth. *peridium walls* consist of three different layers: (1) outer wall layer: hyphal system dimitic, CB–, IKI–, tissues unchanged in KOH; generative hyphae with clamp connections, yellowish brown, slight thick-walled, frequently branched, 1.5–4.5 µm in diameter; skeletal hyphae yellowish brown, thick-walled, unbranched, 2–4 µm in diameter; (2) inner wall layer: hyphal system dimitic, CB–, IKI–, tissues unchanged in KOH; generative hyphae with clamp connections, yellowish-brown, slight thick-walled, frequently branched, 1.5–4.5 µm in diameter; skeletal hyphae yellowish brown, thick-walled, unbranched, 1–3 µm in diameter; (3) pseudoparenchymatous layer.

Peridioles:—shiny, regular to irregular, elliptical to broadly ellipsoid, silver gray to black, surface irregular, wrinkled, tunica present, slightly thin, dark grey (8F1) to brownish gray (7F2). *cortex* single-layered, 1.5–2.5 × 1.5–2 mm. *funicular cord* present, funiculus generative hyphae with clamp connections, thick-walled, branched, yellowish, 1–4.5 µm in diameter. *hyphal system* monomitic, generative hyphae with clamp connections, colorless, thick-walled, branched, with oil drops inside, 3–7 µm in diameter, CB–, IKI–, tissues unchanged in KOH.

Basidiospores:—3.5–10.5 × 3–5.5 µm, L = 7.0 µm, W = 4.4 µm, Q = 1.6 (n = 90/2), broadly ellipsoid, elliptical, subglobose, ovoid, broadly ovate, some irregular, colorless, smooth, slight thick-walled (up to 0.5–1 µm thick), CB–, IKI–, some basidiospores with the inconspicuous apiculus. Basidia not observed.

Additional specimen examined:—CHINA, Yunnan Province, Wenshan, Pingba Town, Huguangqing Village, E 104°33', N 23°60', on fallen branch of angiosperm, 12 Aug 2020, CLZhao 19847 (SWFC).

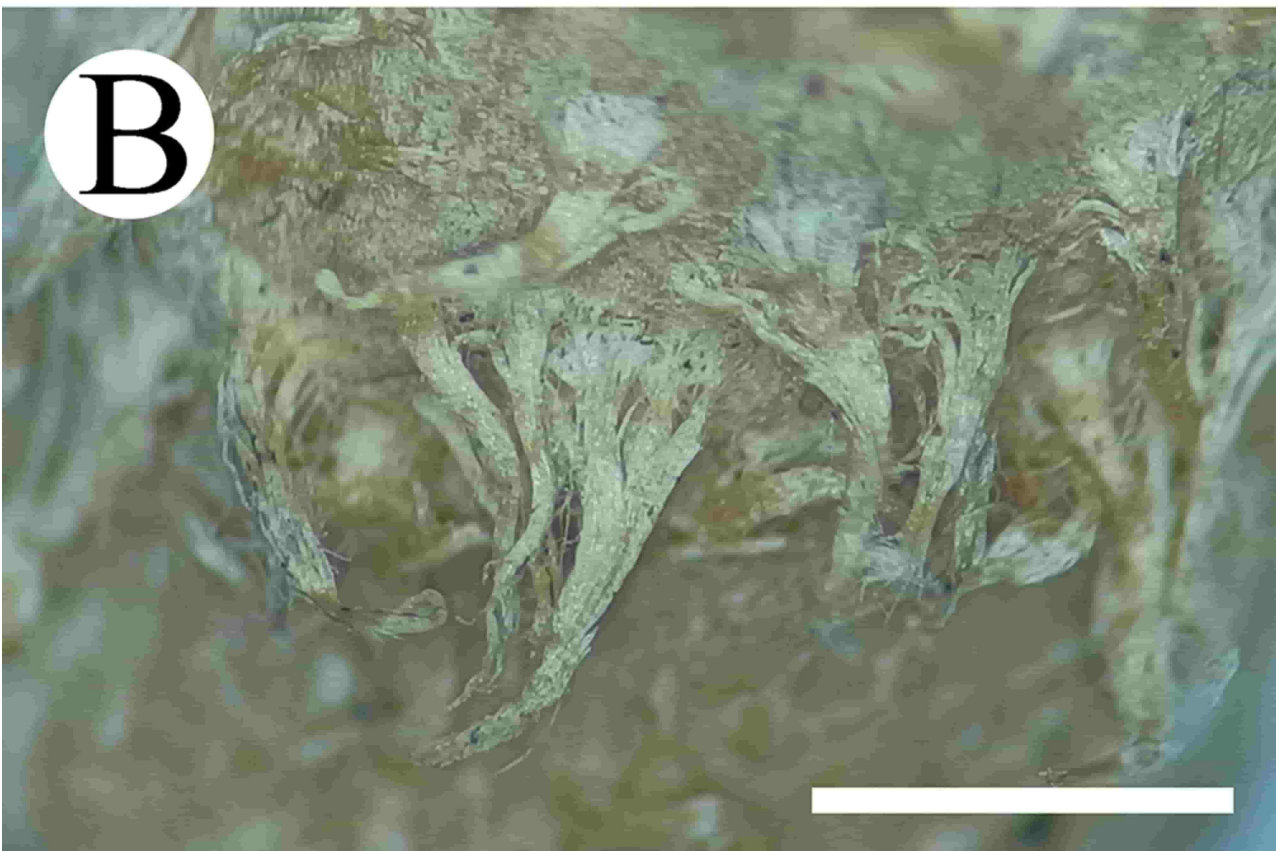
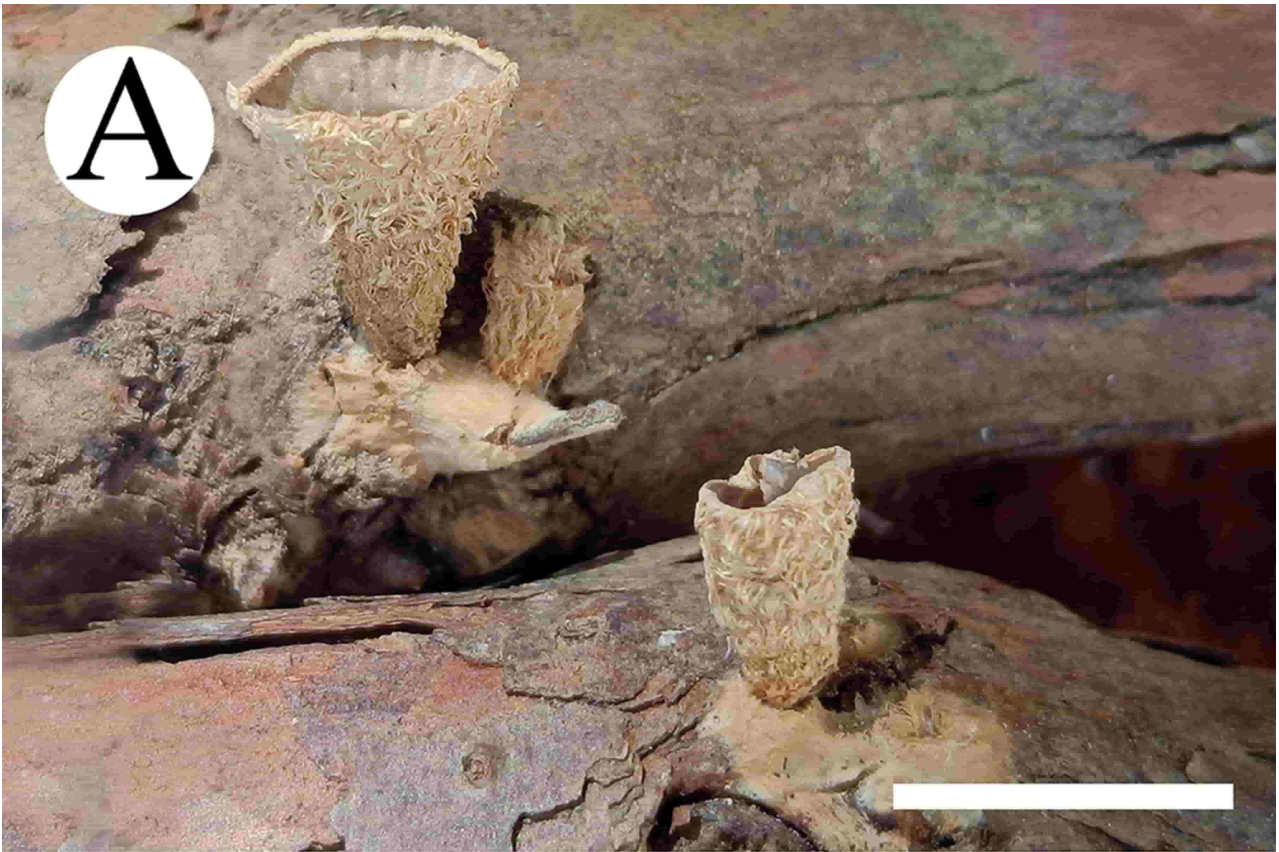
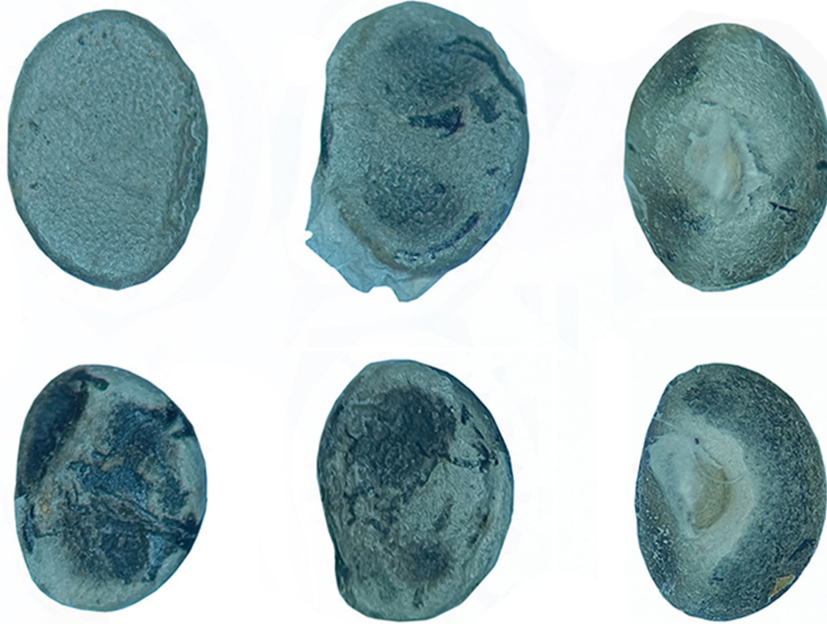


FIGURE 3. Basidiomata of *Cyathus asiaticus*. Bars: A = 1 cm, B = 1 mm (Holotype: CLZhao 19445). Photoplate by: Zi-Yan Duan.

A



B

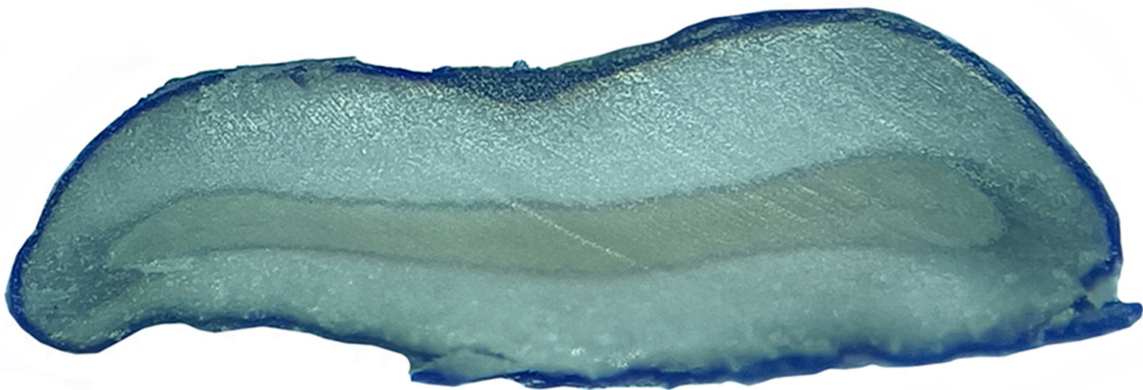


FIGURE 4. Peridiole of *Cyathus asiaticus*. Bars: A–B = 1 mm (Holotype: CLZhao 19445). Photoplate by: Zi-Yan Duan.

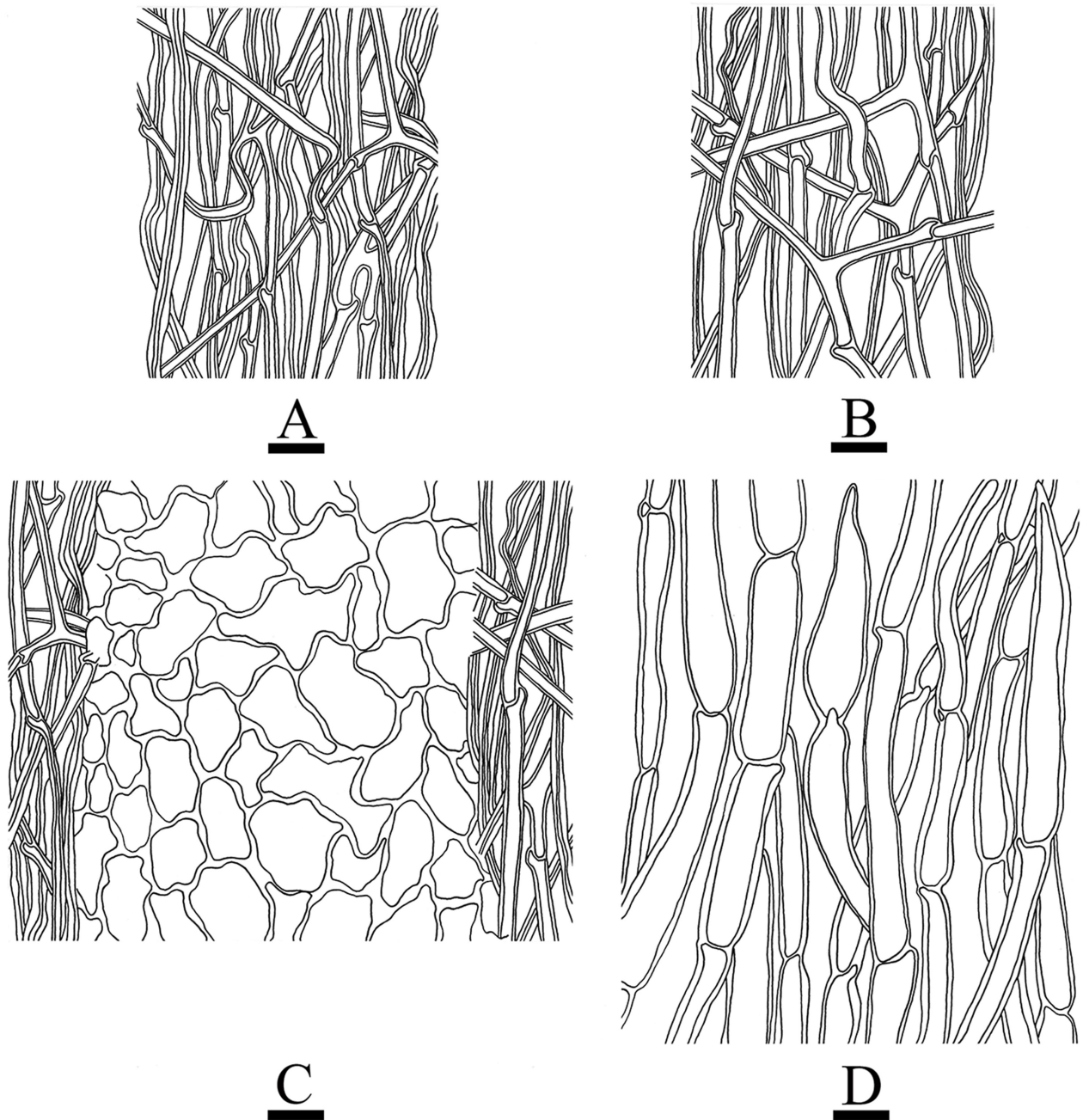


FIGURE 5. Microscopic structures of *Cyathus asiaticus* (drawn from the holotype, CLZhao 19445). A: Outer wall of peridium. B: Inner wall of peridium. C: Three-layered peridium. D: Structure of the hair. Bars: A–D = 10 μm . Drawings by: Zi-Yan Duan.

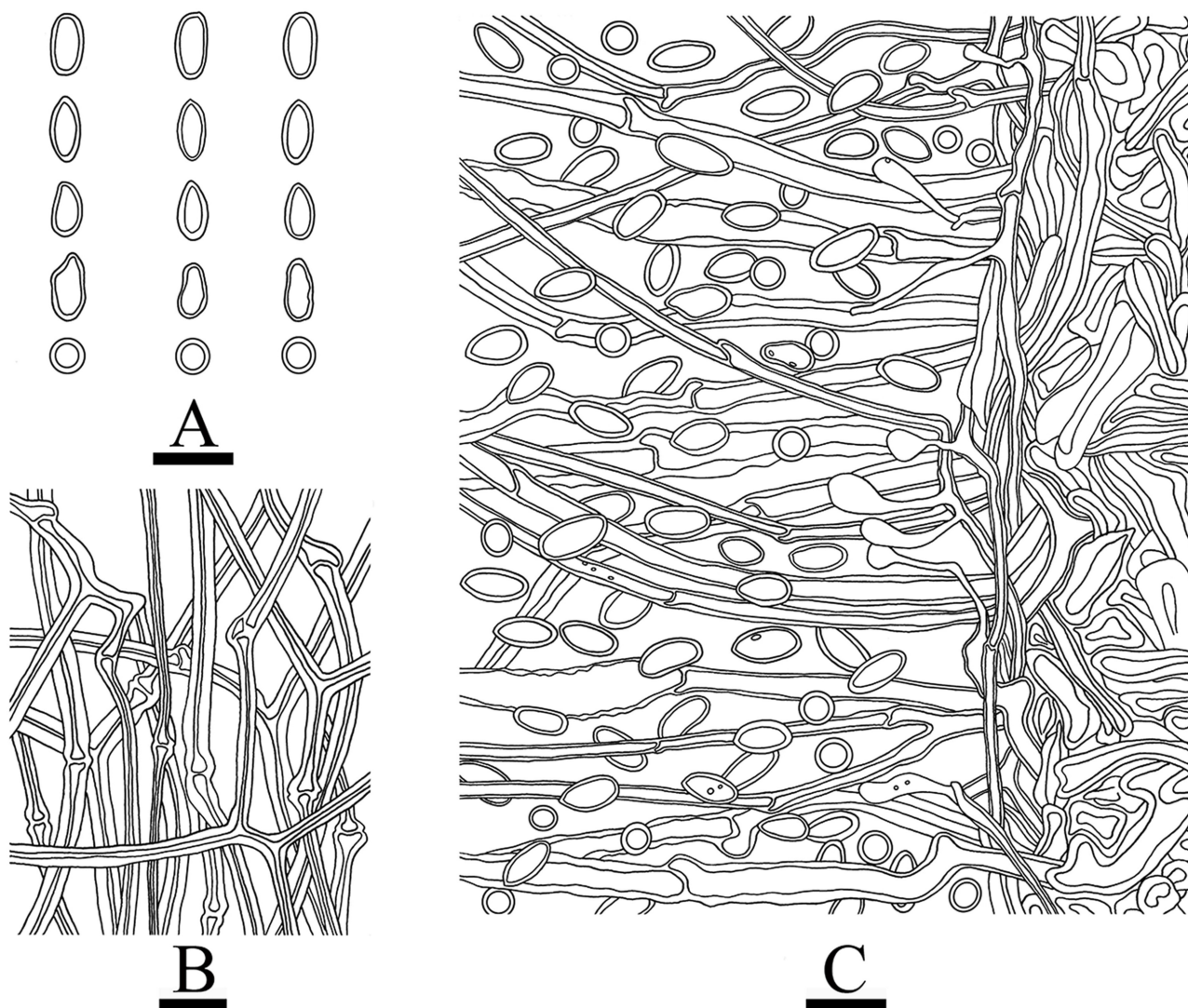


FIGURE 6. Microscopic structures of *Cyathus asiaticus* (drawn from the holotype, CLZhao 19445). A: Basidiospores. B: Generative hyphae of the funicular cord. C: Internal structure of the peridiole. Bars: A–C = 10 μ m. Drawings by: Zi-Yan Duan.

Cyathus prismaticus Z.Y. Duan & C.L. Zhao, *sp. nov.* Figs. 7–10
 MycoBank no.: MB 845560

Etymology:—*prismaticus* (Lat.) referring to the narrow oblique prisms in the peridiole of the specimens.

Holotype:—CHINA. Yunnan Province, Xishan District, Haikou Forest Park, E 102°61', N 24°79', on fallen branch of angiosperm, 16 September 2017, CLZhao 2935 (SWFC).

Fruiting body:—infundibuliform, obconical to cupulate, 7–12 mm in height, 4–6 mm in width at the mouth, non-expanding at the mouth and non-tapering towards the base; presence of the emplacement. *exoperidium* yellowish brown (5E4), shaggy or hirsute, external wall non-striate or only striate at near mouth, covered with pale yellow (4A3), irregular and flexible tufts of hair; hair hyphae with clamp connections, yellowish-brown (5E4), thick-walled (wall up to 0.5–2 μ m thick), 1.5–9 μ m in diameter. *endoperidium* silvery brown to dark brown (5E6–7F4), conspicuously striate with 0.3–0.8 mm between the groves. *mouth* finely fimbriate. *peridium walls* consist of three different layers: (1) outer wall layer: hyphal system dimitic, CB–, IKI–, tissues unchanged in KOH; generative hyphae with clamp connections, brown, slight thick-walled, frequently branched, 1.5–3.5 μ m in diameter; skeletal hyphae colorless to yellowish-brown, thick-walled, unbranched, 1.5–3.5 μ m in diameter; (2) inner wall layer: hyphal system dimitic, CB–, IKI–, tissues unchanged in KOH; generative hyphae with clamp connections, reddish brown, slight thick-walled, frequently branched, 1.5–4.5 μ m in diameter; skeletal hyphae reddish brown, thick-walled, unbranched, 2.5–4 μ m in diameter; (3) pseudoparenchymatous layer.

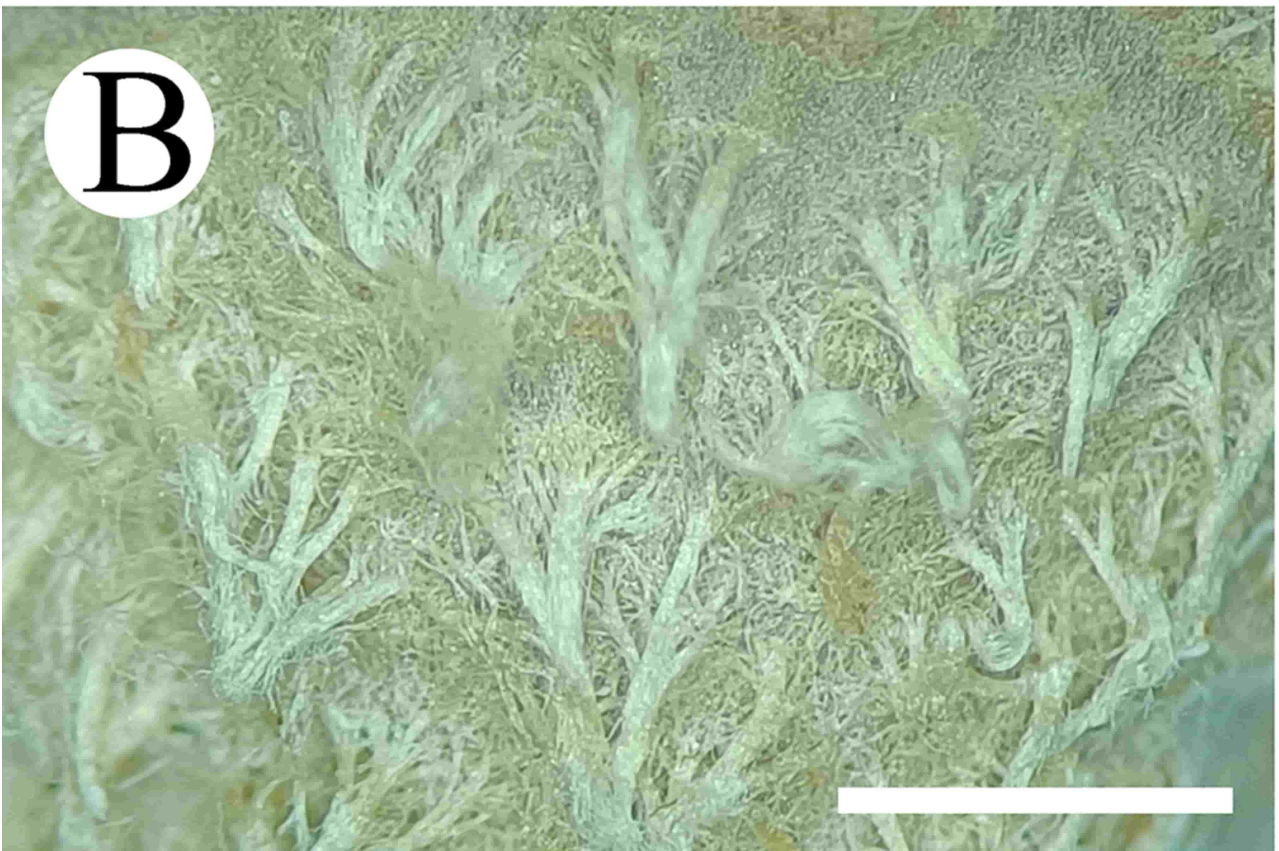
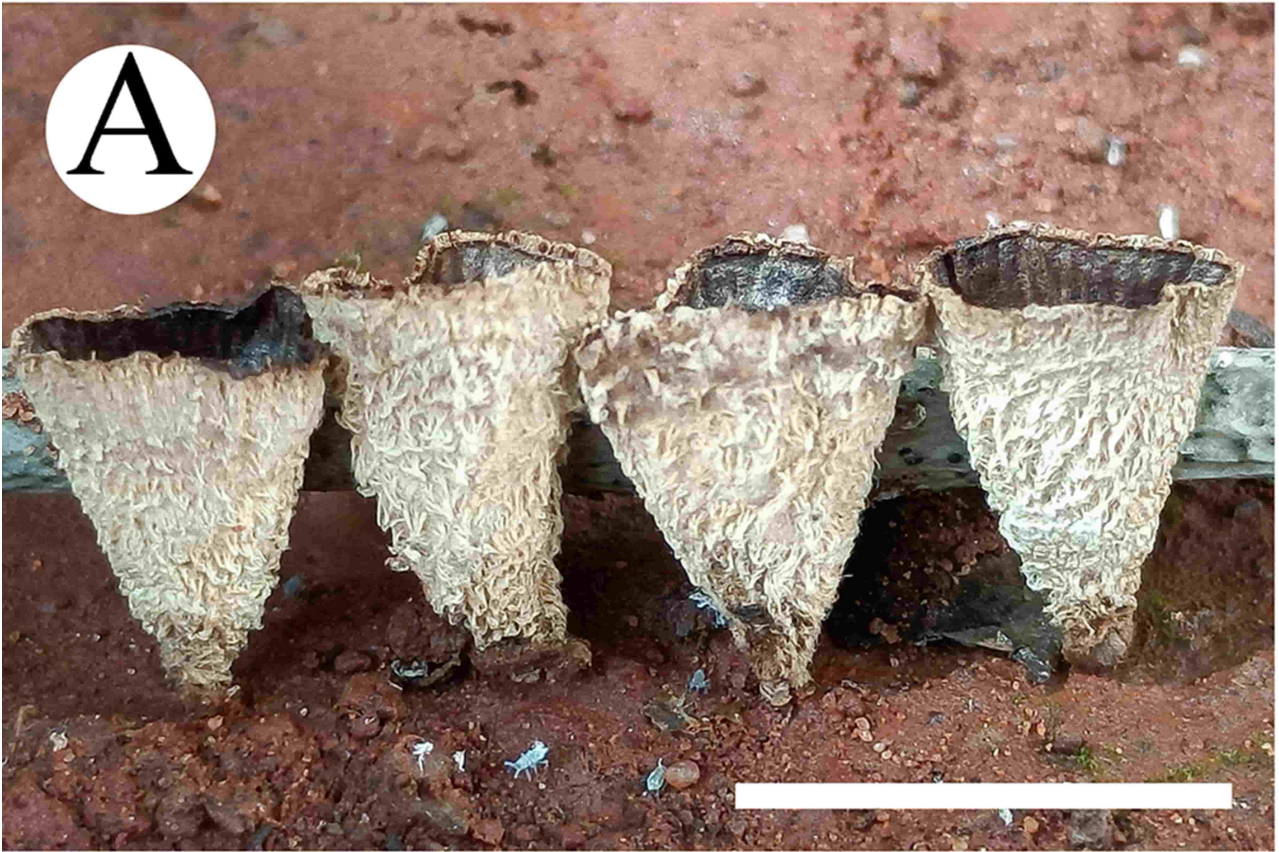
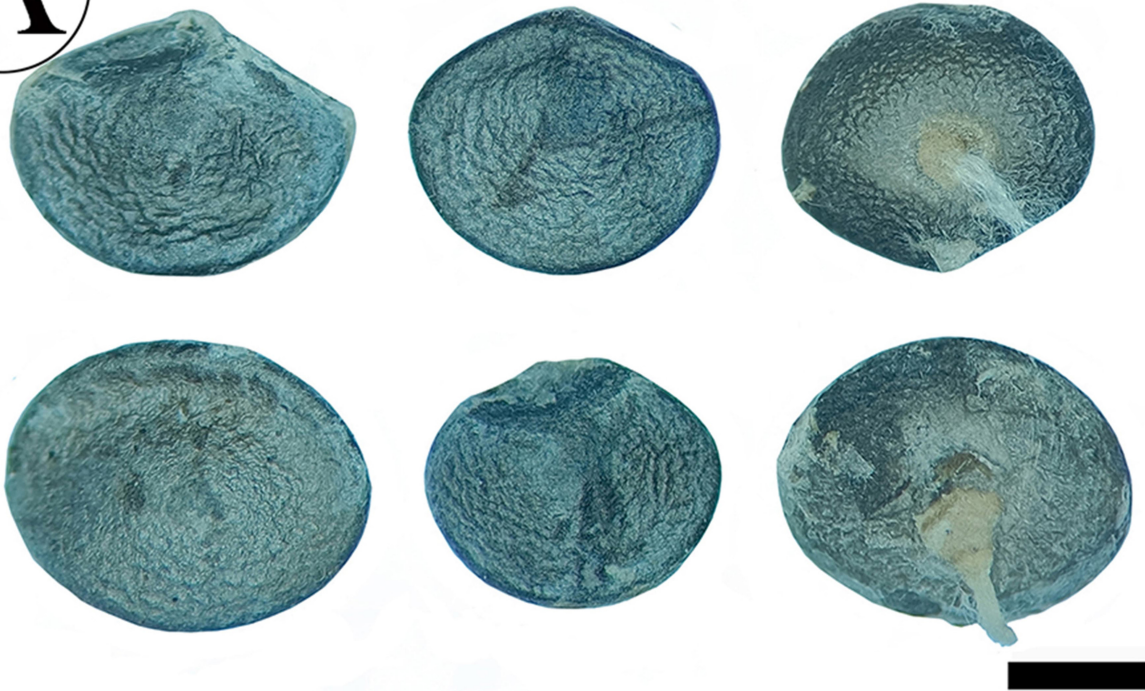


FIGURE 7. Basidiomata of *Cyathus prismaticus*. Bars: A = 1 cm, B = 1 mm (Holotype: CLZhao 2935). Photoplate by: Zi-Yan Duan.

A



B



FIGURE 8. Peridiole of *Cyathus prismaticus*. Bars: A–B = 1 mm (Holotype: CLZhao 2935). Photoplate by: Zi-Yan Duan.

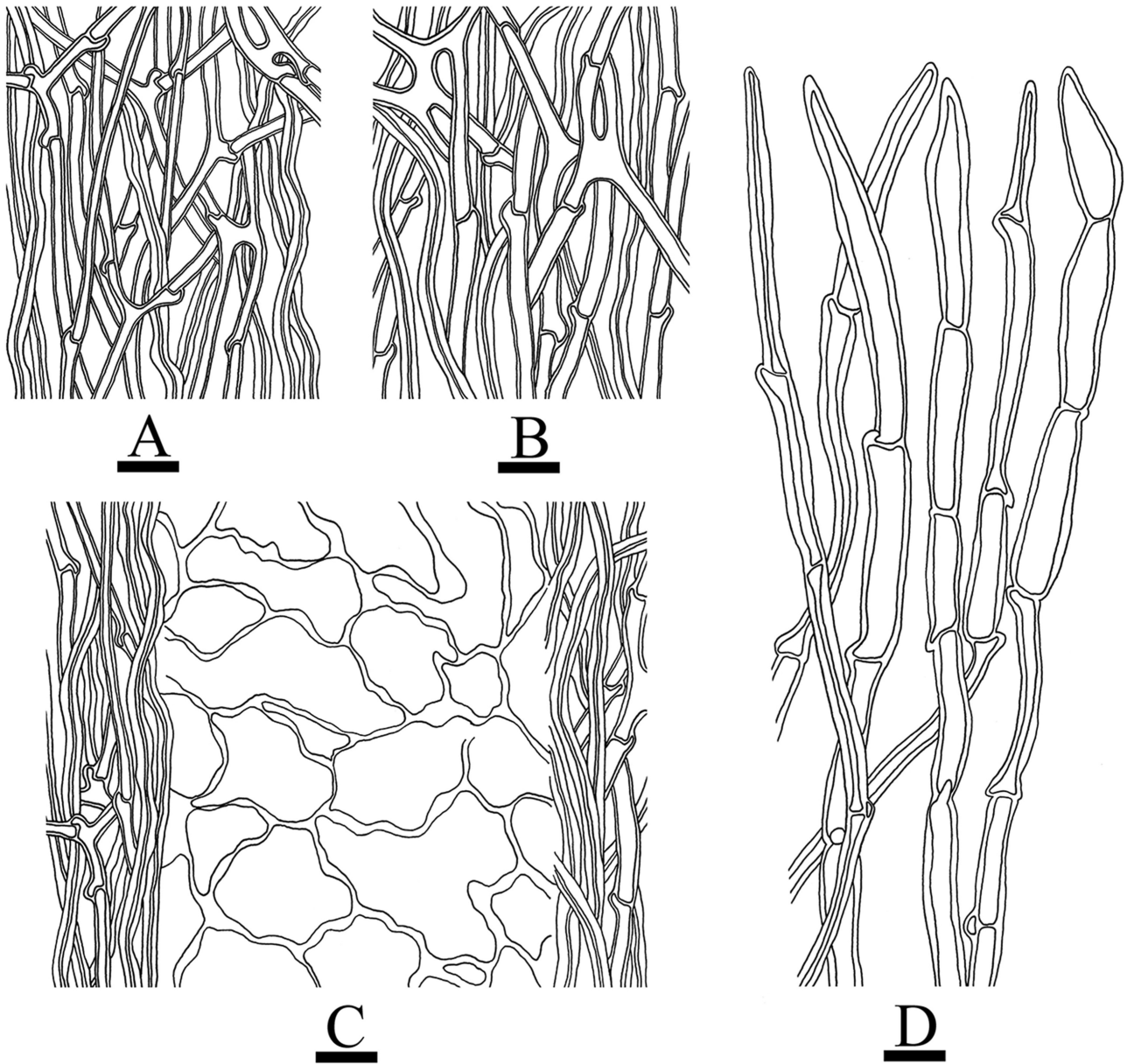


FIGURE 9. Microscopic structures of *Cyathus prismaticus* (drawn from the holotype, CLZhao 2935). A: Outer wall of the peridium. B: Inner wall of the peridium. C: Three-layered peridium. D: Structure of the hair. Bars: A–D = 10 μ m. Drawings by: Zi-Yan Duan.

Peridioles:—shiny, slightly regular, elliptical-triangular, broadly ellipsoid to ovoid, brownish gray (7F2) to dark grey (8F1), surface irregular, wrinkled, tunica thin, brownish. *cortex* single-layered, 2–3 \times 1.5–2.5 mm. *funicular cord* present, funiculus hyphae with clamp connections, thick-walled, branched, pale yellowish, 1–3 μ m in diameter. *hyphal system* dimittic, hyphae CB–, IKI–, tissues unchanged in KOH; generative hyphae with clamp connections, colorless, thin-walled, branched, with oil drops inside, 0.5–5 μ m in diameter; skeletal hyphae colorless, slight thick-walled, unbranched, with oil drops inside, 1–3 μ m in diameter; narrow oblique prisms present.

Basidiospores:—8.5–20(–20.5) \times 7.5–11 μ m, L = 15.68 μ m, W = 8.77 μ m, Q = 1.8 (n = 60/1), subglobose, elliptical to broadly ellipsoid, rarely ovoid, colorless, smooth, thick-walled (up to 0.5–2 μ m thick), CB–, IKI–, with inclusions or oil-like globule, without apiculus. Basidia not observed.

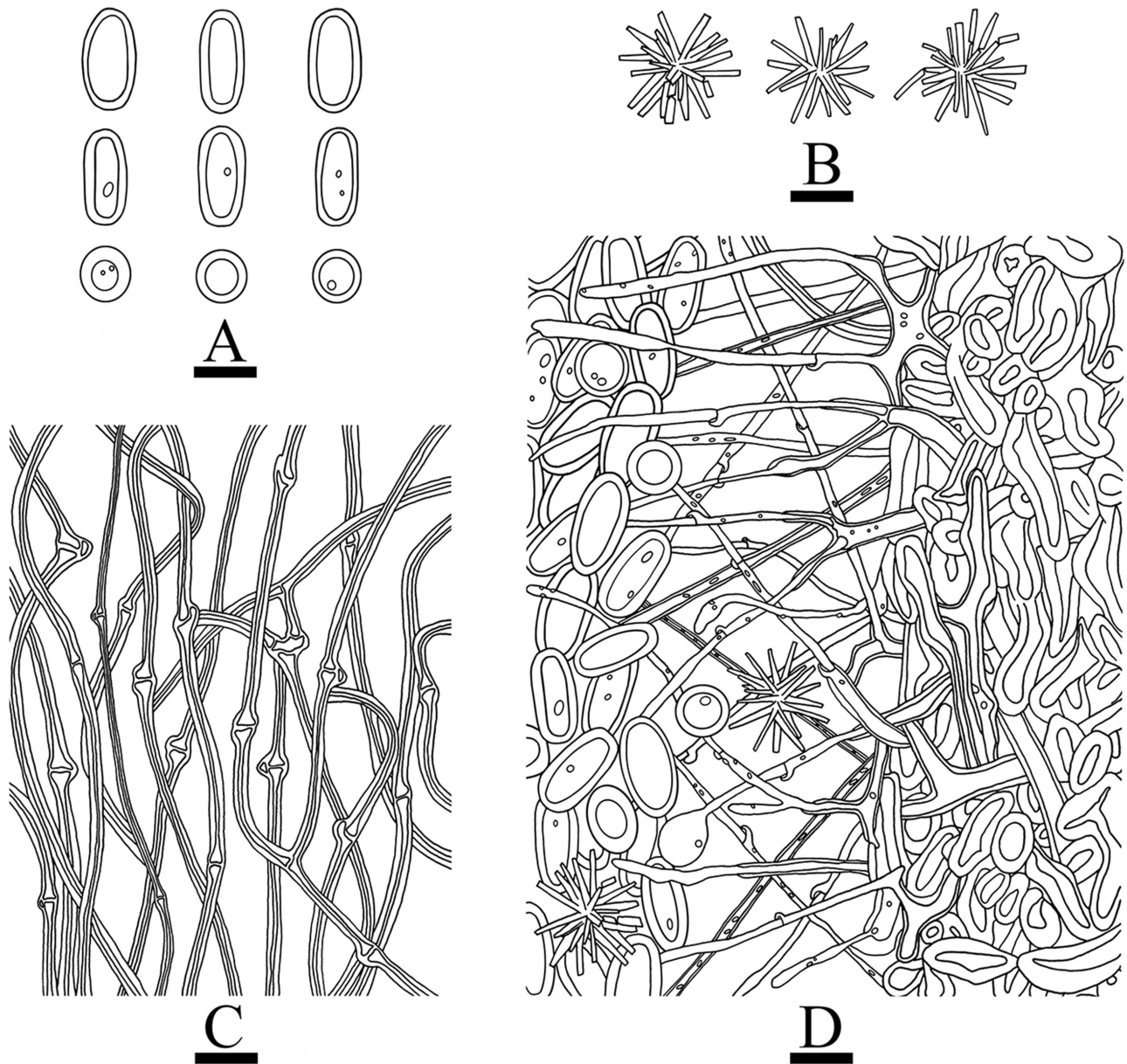


FIGURE 10. Microscopic structures of *Cyathus prismaticus* (drawn from the holotype, CLZhao 2935). A: Basidiospores. B: Narrow oblique prisms. C: Generative hyphae of the funicular cord. D: Internal structure of the peridiole. Bars: A–D = 10 μ m. Drawings by: Zi-Yan Duan.

Discussion

Phylogenetically and taxonomic overview of the bird's nest fungi (Nidulariaceae) revealed that Nidulariaceae was resolved as a monophyletic lineage with Squamanitaceae as a potential sister group, in which two genera *Cyathus* and *Crucibulum* formed an independent and well-supported clade individually, and *Nidula* and *Nidularia* formed a clade together, but each genus is polyphyletic (Kraisitudomsook *et al.* 2021). Additionally, Kraisitudomsook *et al.* (2022) further studied the taxonomy of *Mycocalia* and then proposed a new genus named *Retiperidiolia*. In the present study, based on ITS and nLSU (Fig. 1) datasets, we found that six taxa *Crucibulum*, *Cyathus*, *Mycocalia*, *Nidula*, *Nidularia* and *Retiperidiolia* each formed its own independent clade, and the two new species, *Cyathus asiaticus* and *C. prismaticus*, were nested within *Cyathus* (Nidulariaceae). Based on ITS topology (Fig. 2), *C. asiaticus* was sister to *C. pallidus*, and then grouped with *C. bulleri* and *C. canna*. Another species *C. prismaticus* was sister to *C. renwei*

and then grouped with *C. annulatus*. However, morphologically *C. pallidus* differs from *C. asiaticus* by having the black silver inner wall of peridium, and larger basidiospores ($8\text{--}11 \times 6\text{--}9 \mu\text{m}$, Gómez & Pérez-Silva 1988); *C. bulleri* differs in having the basidiomata abruptly narrowed at base, strongly striate peridium walls, and silvery to dark brown peridioles (Brodie 1967a); *C. canna* is distinct from *C. asiaticus* in having the striate external wall of peridium, brown chocolate to brown rusty peridioles, and wider basidiospores ($6\text{--}8.5 \times 5.5\text{--}6.5 \mu\text{m}$, Cruz 2017). *Cyathus renweii* differs from *C. prismaticus* by having the larger basidiospores ($21\text{--}31 \times 10.5\text{--}13.5 \mu\text{m}$, Zhou *et al.* 2004); *C. annulatus* differs in its expanded peridium at the top, presence of lip of peridium marked by a distinct deep-brown ring, and the wider basidiospores ($15\text{--}19 \times 15.5\text{--}17 \mu\text{m}$, Brodie 1970).

Morphologically, seven *Cyathus* species as *C. africanus* H.J. Brodie (1967b: 1653), *C. apiculatus*, *C. gansuensis*, *C. ibericus* J.C. Zamora & Poveda-Molero (2016: 277), *C. jiayuguanensis* J. Yu, T.X. Zhou & L.Z. Zhao (2002: 314), *C. novae-zeelandiae* Tul. & C. Tul. (1844: 66) and *C. olla* (Batsch) Pers. (1801: 237) are similar to *C. asiaticus* in light of the character by having the single-layered cortex of the peridioles. However, *C. africanus* differs from *C. asiaticus* by its peridium walls with woolly hairs, minutely fimbriate mouth, and wider basidiospores ($8.5\text{--}12 \times 6.5\text{--}8.5 \mu\text{m}$) with distinct apiculus (Brodie 1967b); *C. apiculatus* differs in the basidiomata expanded at the mouth and abruptly tapering to the base, brown to dark brown exoperidium, silvery endoperidium, strongly striate external wall, and larger basidiospores ($22\text{--}37 \times 10\text{--}22 \mu\text{m}$, Silva *et al.* 2016); *C. gansuensis* is distinguished from *C. asiaticus* by its narrow base basidiomata with grayish to dark smoke-gray inner surface, exoperidium with short hairs, and larger basidiospores ($10.5\text{--}13.5 \times 8.5\text{--}10.5 \mu\text{m}$, Yang *et al.* 2002); *C. ibericus* differs from *C. asiaticus* by its whitish to pale brownish grey internal peridium without stripes, and smaller peridioles (0.8–1.2 mm diam, Crous *et al.* 2016); *C. jiayuguanensis* differs from *C. asiaticus* by its short stipe basidiomata with gray-white inner endoperidium and larger basidiospores ($8\text{--}11.5 \times 7\text{--}8.5 \mu\text{m}$, Yang *et al.* 2002); *C. novae-zeelandiae* differs in its dark red exoperidium with stripes, larger peridioles (2.9–3 mm), and larger basidiospores ($9\text{--}15 \times 6\text{--}10 \mu\text{m}$, Gómez & Pérez-Silva 1988); *C. olla* differs from *C. asiaticus* by its larger basidiomata (10–15 mm height, 7–10 mm width), greyish exoperidium, silver to blackish, smooth and shiny inner surface, pure silver peridioles, and larger basidiospores ($9.8\text{--}11.2 \times 6.4\text{--}8 \mu\text{m}$, Dorjey *et al.* 2013).

Four taxa *Cyathus batistae*, *C. colensoi* Berk. (1855: 192), *C. gracilis* H.J. Brodie (1973: 1393) and *C. setosus* H.J. Brodie (1967c: 1) are similar to *C. asiaticus* inferred from the character by having the smooth external wall of peridium and inconspicuously striate inner wall. However, *C. batistae* differs from *C. asiaticus* by its expanded mouth of basidiomata having the stipe, distinctly fimbriate lip of the mouth of peridium with dark brown exoperidium, and double-layered cortex of the peridioles, and smaller basidiospores ($9\text{--}13 \times 5\text{--}8 \mu\text{m}$, Silva *et al.* 2016); *C. colensoi* differs from *C. asiaticus* by its brown peridioles with double cortex, and larger basidiospores ($9\text{--}12 \times 6.5\text{--}9 \mu\text{m}$, Vu *et al.* 2019); *C. gracilis* differs from *C. asiaticus* by having the minutely fimbriate mouth, cortex distinctly double peridioles, and larger basidiospores ($17\text{--}24 \times 10\text{--}14 \mu\text{m}$, Brodie 1973); *C. setosus* is distinguished from *C. asiaticus* by its very dark brown, almost black, larger peridium (7–8 mm height, 8–10 mm width) with the mouth with stiff dark setae, and double-layered peridioles (Brodie 1967c).

Four taxa, *Cyathus lignilantanae* R. Cruz & M.P. Martín (2015: 165), *C. limbatus*, *C. pedunculatus* and *C. poeppigii* are similar to *C. prismaticus* on the basis of the character by having the stripes on the inner and outer walls of peridium. However, *C. lignilantanae* is separated from *C. prismaticus* by having the reddish brown exoperidium, platinum endoperidium, brownish grey to greyish brown peridioles with double-layered cortex (Martín *et al.* 2015); *C. limbatus* differs from *C. prismaticus* by its double-layered peridioles (Góis *et al.* 2021); *C. pedunculatus* is separated from *C. prismaticus* by its the basidiomata base forming a conspicuous pedicel, and smaller peridioles ($1\text{--}1.5 \times 1.5\text{--}2 \text{mm}$) with double-layered cortex, and larger basidiospores ($25\text{--}34 \times 22\text{--}29 \mu\text{m}$, Silva *et al.* 2016); *C. poeppigii* is distinguished from *C. prismaticus* by having narrowly obconical basidiomata with incurved mouths, having a slender stipe at the base, and dark brown to reddish-brown exterior surface of the peridium, double cortex of peridioles, and larger basidiospores ($30\text{--}45 \times 18\text{--}30 \mu\text{m}$, Zhao *et al.* 2006).

Cyathus amazonicus Trierv.-Per. & Baseia (2009: 74), *C. stercoreus* (Schwein.) De Toni (1888: 40) and *C. subglobisporus* R.L. Zhao, Desjardin & K.D. Hyde (2008: 74) resembles *C. prismaticus* based on having subglobose to broadly ellipsoid basidiospores. However, *C. amazonicus* differs from *C. prismaticus* by its dark brown to grayish dark brown exoperidium, gray to brownish gray endoperidium, dark gray peridioles, and wider basidiospores ($14\text{--}19 \times 12\text{--}16 \mu\text{m}$, Trierveiler-Pereira & Baseia 2009); *C. stercoreus* is separated from *C. prismaticus* by its smooth peridium walls, double-layered cortex of the peridioles and larger basidiospores ($30\text{--}41 \times 25\text{--}31 \mu\text{m}$, Gómez & Pérez-Silva 1988); *C. subglobisporus* differs in its greyish brown to pale brown, double-layered cortex of the peridioles and wider basidiospores ($13\text{--}18 \times 12\text{--}16 \mu\text{m}$, Blenis & Chow 2005).

The family Nidulariaceae is a characteristic group of Agaricomycetes (Basidiomycota), which has a number of macro

fungi based on a result of morphological, phylogenetic and cytological studies in China (Wu *et al.* 2020, Dai *et al.* 2021), but the species diversity of macro fungi are still not well known in China, especially in subtropical and tropical areas of this country (Xu *et al.* 2019, Huang *et al.* 2020, Ma *et al.* 2020, Wang *et al.* 2020, Cai *et al.* 2023). The new species, *Cyathus asiaticus* and *C. prismaticus* are from the subtropics, too. Therefore, the present paper enriches the fungal diversity in the Chinese ecosystem, and it is likely that more new taxa will be found after further fieldwork and molecular analyses.

Acknowledgements

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