Abstract

A new species of bird’s nest fungus, *Cyathus tongxinianus*, is proposed based on a combination of morphological and molecular evidence. *Cyathus tongxinianus* is characterized by the cupulate to obconical basidiomata with the smooth outer and inner surface of the peridium, a dimitic hyphal system of peridium walls with generative hyphae having clamp connections, the presence of the fimbriate mouth, brown black to dark grey peridioles, a monomitic hyphal system bearing the generative hyphae with clamp connections, the presence of crystals, and sub-globose, thick-walled basidiospores (8.5–19.5 × 7.5–11). Sequences of ITS and nLSU rRNA markers of the studied samples were generated, and the phylogenetic analyses were performed with maximum likelihood, maximum parsimony and bayesian inference methods on two datasets (ITS+nLSU and ITS). The analyses showed that the new species clustered into the genus *Cyathus* within the family Nidulariaceae based on the ITS+nLSU phylogenetic tree. The phylogenetic analyses based on molecular data from ITS sequences showed that *C. tongxinianus* is sister to *C. annulatus*, and groups with *C. renweii*.

Key words: 1 new species, Molecular phylogeny, Nidulariaceae, Taxonomy, Wood-inhabiting fungi

Introduction

Fungi in the Nidulariaceae, otherwise known as ‘bird’s nest fungi’, are among the least studied groups of Basidiomycota (Kraisitudomsook et al. 2021). Bird’s nest fungi usually grow in decaying wood, on manure, or directly on soil. They have a cosmopolitan distribution and have a rich diversity related to the high diversity of plants growing in boreal, temperate, subtropical, and tropical regions (Brodie 1975, Shinners-Carnelley et al. 2002, Blenis & Chow 2005, Zhao et al. 2008; Kraisitudomsook et al. 2022). Bird’s nest fungi are decomposers that absorb nutrients from dead organic materials (Wicklow et al. 1984), thus, they play an important role in recycling nutrients and decomposing plant debris in natural ecosystems (Kraisitudomsook & Smith 2020).

The genus *Cyathus* 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 their cup-like basidiomata resembling bird nests and lenticular periodioles resembling eggs, five genera viz. *Cyathus*, *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; Kraisitudomsook et al. 2022). Bird’s nest fungi are decomposers that absorb nutrients from dead organic materials (Wicklow et al. 1984), thus, they play an important role in recycling nutrients and decomposing plant debris in natural ecosystems (Kraisitudomsook & Smith 2020).
The comprehensive molecular systematic studies on Cyathus were started in the last two decades (Matheny et al. 2006, Zhao et al. 2007, Kraisitudomsook et al. 2022). An overview of the phylogeny of Agaricales based on a multilocus analysis of a six-gene region supermatrix revealed that the family Nidulariaceae was close to Cystodermataceae, 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 were investigated with ITS and nLSU ribosomal DNA sequences datasets inferred from the neighbor-joining, maximum likelihood, maximum parsimony, and MrBayes analyses, showing that morphological characters and molecular data were incompatible. These include, for example, the peridium plications, variations in peridium hair anatomy, peridiole structure, and fruit-body color. However, 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 and 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. Silva et al. (2016) carried out the phylogenetic reconstruction of Cyathus species based on an alignment of 641 nucleotides of the ITS region, and 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. Investigation considering four loci (ITS, nLSU, TEF1, and RPB2) revealed that 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. Additionally, it was suggested that some bird’s nest fungi species had global geographical distributions, whereas others may have more limited ranges, and that basic morphological characteristics of bird’s nest fungi had likely been lost or gained multiple times (Kraisitudomsook et al. 2021). The phylogenetic research based on five loci (ITS, nLSU, SSU, TEF1, and RPB2) revealed that a new genus Retiperidiolia Kraisit., Choeyklin, Boonprat. & M.E. Sm. (2022: 4) to accommodate this phylogenetically and morphologically unique fungus lineage, in which Cyathus formed a monophyletic lineage, sister to Retiperidiolia (Kraisitudomsook et al. 2022). The phylogenetic analyses based on ITS and nLSU sequences confirmed the monophyly of Cyathus with maximum support, and the infrageneric groups of the most recent classification were unchanged, but the clade striatum was found to be divided into four groups and three subgroups (Cruz et al. 2023). Recently, by using molecular systematics and morphology, one new species of Cyathus from China was introduced (Duan et al. 2022).

During the investigations on bird’s nest fungi in China, we found a species of Cyathus that could not be assigned to any of the described species. The species is here described as C. tongxinianus 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 on 13th September 2000 from Horinger County of Nei Monggol Autonomous Region, China. The 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 macro-morphological descriptions were based on field notes and photos taken in the field and lab. The macro-morphological descriptions follow Brodie (1975). The micro-morphological data were obtained from the dried specimens and observed under a Nikon Eclipse E100 light microscope (Zhao & Wu 2017). The color terms followed Kornerup and Wanscher (1978). Drawings were made with the aid of a fungus plotter (Wu et al. 2022). 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, and 40 ml distilled water) and 5% potassium hydroxide. Spore size data, excluded 5% of the measurements...
from each end of the range, showing them 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 LSU 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.

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<tr>
<th>Species name</th>
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*a* is shown type material, holotype.
Sequencher 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). The sequence alignment was deposited in TreeBase (submission ID 30354). Cystodermella cinnabarina (Alb. and Schwein.) Harmaja (2002: 45) was selected as an outgroup for phylogenetic analyses of combined ITS and nLSU sequences data (Kraisitudomsook et al. 2021). Crucibulum laeve and Nidula niveotomentosa (Henn.) Lloyd (1910: 455) were selected as an outgroup for the phylogenetic analysis of the ITS sequence (Accioly et al. 2018).

Maximum parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI) analyses were applied to the combined three 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 Tree Bisection and Reconnection (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. The best-evolutionary model of each alignment was estimated using jModelTest (Guindon & Gascuel 2003; Posada 2008) under the Akaike information criterion. 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 combined ITS and nLSU sequences (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 ≥70%, a maximum parsimony bootstrap value (BT) of ≥70%, or Bayesian posterior probabilities (BPP) of ≥0.95.

FIGURE 1. Maximum parsimony strict consensus tree illustrating the phylogeny of the new species of Cyathus and related genera in the family Nidulariaceae based on combined ITS and nLSU sequences dataset. Branches are labelled with maximum likelihood bootstrap values ≥70%, parsimony bootstrap values ≥50% and Bayesian posterior probabilities ≥0.95, respectively. Scale bar = 20. The new species is in bold, ✳ corresponds to type species.
Results

Molecular phylogeny

The combined ITS and nLSU dataset (Fig. 1) included sequences from 23 fungal specimens representing 20 species. The dataset had an aligned length of 1535 characters, of which 969 characters are constant, 100 are variable and parsimony-uninformative, and 466 are parsimony-informative. Maximum parsimony analysis yielded one equally parsimonious tree (TL = 1084, CI = 0.7076, HI = 0.2924, RI = 0.8119, RC = 0.5745). The best model for the combined ITS and 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 = 0.009958 (BI), and the effective sample size (ESS) across the two runs is double the average ESS (avg ESS) = 532.5. The phylogeny (Fig. 1) based on the combined ITS and nLSU sequences indicated that the species *Cyathus tongxinianus* was nested within the genus *Cyathus*. 

![Figure 1. Maximum parsimony strict consensus tree illustrating the phylogeny of the new species and related species in *Cyathus* based on ITS sequences. Branches are labelled with maximum likelihood bootstrap values ≥70%, parsimony bootstrap values ≥50%, and Bayesian posterior probabilities ≥0.95, respectively. Scale bar = 20. Type specimen accessions are in bold, * corresponds to type species.]
The ITS dataset alone (Fig. 2) included sequences from 52 fungal specimens representing 44 species. The dataset had an aligned length of 887 characters, of which 347 characters are constant, 223 are variable and parsimony-uninformative, and 317 are parsimony-informative. Maximum parsimony analysis yielded 5000 equally parsimonious trees (TL = 1211, CI = 0.6474, HI = 0.3526, RI = 0.7855, RC = 0.5086). The best model for the ITS dataset estimated and applied in the Bayesian analysis was GTR+I+G (letset 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 = 0.009493 (BI), and the effective sample size (ESS) across the two runs is double the average ESS (avg ESS) = 165.5. The phylogenetic tree (Fig. 2) inferred from ITS sequences revealed that *Cyathus tongxinianus* was sister to *C. annulatus* H.J. Brodie (1970: 749) with supports (86% BS, 81% BT, 0.98% BPP), and nested with *C. renweii* T.X. Zhou and R.L. Zhao (2004: 245) with supports (94% BS, 98% BT, 1.00 BPP).

**Taxonomy**

*Cyathus tongxinianus* Z.Y. Duan & C.L. Zhao, *sp. nov.* Figs. 3, 4, 5, 6
MycoBank no.: MB 848643

**Diagnosis:**—differs from other *Cyathus* species by its exoperidium with cream tomentum, smooth outer and inner surface, brown black peridioles with double-layered cortex, a monomitic hyphal system with clamped generative hyphae and ellipsoid-endage, ovoid-endage basidiospores measuring 8.5–19.5 × 7.5–11 µm.

**Etymology:**—*tongxinianus* (Lat.) in honour of the Chinese mycologist Prof. Tongxin Zhou.

**Holotype:**—CHINA. Nei Monggol Autonomous Region, Hohhot, Horinger County, Nantianmen Forest Farm, E 112°25′, N 40°86′, on fallen branch of angiosperm, 13 September 2000, CLZhao 27588 (SWFC).

**Fruiting body:**—cupulate to obconical, 4–10 mm in high, 3–5 mm in wide at the mouth, 0.1–0.2 mm in wide at the base, without expanding at the top or tapering abruptly at the base; emplacement clearly observed; young fruit bodies clavate, lightly brown (6D4), covered by the cream (5A3) hairs aggregated into tomentum or shaggy clusters; exoperidium slightly brown (6E4), tomentulose, external wall non-striate, covered with cream (4A2), irregular and flexible tufts of the hair; the hairy appearance composed by hyphae with clamp connections, yellowish brown, thick-walled (up to 0.5–2 µm thick), 3–8.5 µm in diameter; endoperidium silvery grey to greyish brown (6E4–7F3), smooth inside; 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, buff to pale brown, slight thick-walled, branched, 2.5–4.5 µm in diameter; skeletal hyphae yellowish to pale brown, thick-walled, 3–5.5 µm in diameter; (2) inner wall layer, hyphal system dimitic, CB–, IKI–, tissues unchanged in KOH; generative hyphae with clamp connections, buff-yellow to pale brown, slight thick-walled, branched, 3.5–5 µm in diameter; skeletal hyphae buff-yellow to pale brown, thick-walled, unbranched, slightly curved, 4–6 µm in diameter; (3) middle layer, pseudoparechymatous, colorless.

**Peridioles:**—angular to irregular, subglobose to broadly ellipsoid, brown black (6F5) to dark grey (8F1), surface irregular, wrinkled, tunica olivaceous buff (4C4), 1.3–2 × 1.2–1.8 mm; cortex double-layered; funicular cord present, funilicus hyphae with clamp connections, thick-walled, branched, yellowish brown, 1.5–4.5 µm in diameter; hyphal system of peridiole monomitic, generative hyphae with clamp connections, colorless, slightly thick-walled, frequently branched, with oil drops inside, 1–5 µm in diameter, CB–, IKI–, tissues unchanged in KOH; crystals present.

**Basidiospores:**—subglobose, ellipsoid-longate, ovoid-longate to broadly ovoid, some irregular, hylane, smooth, thick-walled (wall up to 0.5–2.5 µm thick), CB–, IKI–, with inclusions or oil-like globule, (8–)8.5–19.5(–20.5) × (7–)7.5–11(–11.5) µm, the range of the mean of length × the range of the mean of width = 14.13 × 9.22 µm, Q = 1.5–1.6 (n = 60/2). Basidia not observed.

**Additional specimen examined (paratype):**—CHINA. Nei Monggol Autonomous Region, Hohhot, Horinger County, Nantianmen Forest Farm, E 112°25′, N 40°86′, on fallen branch of angiosperm, 13 September 2000, CLZhao 28521 (SWFC).
FIGURE 3. Basidiomata of *Cyathus tongxinianus* showing in (B) hairs of peridium. Bars: A = 1 cm, B = 1 mm (Holotype: CLZhao 27588). Photoplate by: Zi-Yan Duan.
FIGURE 4. Peridioles of Cyathus tongxinianus showing in (B) a cross-section. Bars: A–B = 1 mm (Holotype: CLZhao 27588). Photoplate by: Zi-Yan Duan.
Discussion

In the present study, Cyathus tongxinianus is described as a new species based on phylogenetic analyses and morphological characteristics. Phylogenetically, the bird’s nest fungi family revealed that Nidulariaceae was resolved as a monophyletic lineage with Squamanitaceae as a potential sister taxon, in which two genera Cyathus and Crucibulum formed independent and well-supported clade individually, and Nidula and Nidularia formed another clade, but each genus is polyphyletic (Kraisitudomsook et al. 2021). Additionally, Kraisitudomsook et al. (2022) 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 formed their own independent clades, and the new species, Cyathus tongxinianus, was nested within the genus Cyathus of the family Nidulariaceae. Based on ITS topology (Fig. 2), C. tongxinianus was sister to C. annulatus and then grouped with C. renweii. However, morphologically C. annulatus differs from C. tongxinianus by having the expanded peridium at the top, the presence of lip of peridium marked by a distinct deep-brown ring, and its wider basidiospores (15–19 × 15.5–17 µm, Brodie 1970); C. renweii differs in its conspicuous striations on the outer and inner surface of the peridium, and larger basidiospores (21–31 × 10.5–13.5 µm, Zhou et al. 2004).
Morphologically, six Cyathus species including *Cyathus batistae*, *C. hortensis* R. Cruz & Baseia (2014: 175), *C. magnomuralis* R. Cruz & Baseia (2014: 177), *C. pedunculatus*, *C. poepiggii* and *C. lignilantanae* R. Cruz & M.P. Martín (2015: 165) are similar to *C. tongxinianus* by having peridioles with double-layered cortex. However, *C. batistae* differs by its expanded mouth of basidiomata, dark brown exoperidium, and smaller basidiospores (9–13 × 5–8 µm) with an apiculus (Silva et al. 2016); *C. hortensis* is distinguished by its basidiomata constricted abruptly at the base and forming a slender stipe, and larger basidiospores (17–34 × 13–20 µm; Cruz & Baseia 2014); *C. magnomuralis* is separated by having the dark brown exoperidium, and larger basidiospores (27–49 × 23–41 µm) with the small apiculus (Cruz & Baseia 2014); *C. pedunculatus* differs by its larger basidiospores (25–34 × 22–29 µm, Silva et al. 2016); *C. poepiggii* is distinguished by its basidiomata with incurved mouths and a slender stipe at the base, and larger basidiospores (30–45 × 18–30 µm, Zhao et al. 2006); and *C. lignilantanae* is separated by having the reddish brown exoperidium, conspicuously striations on the outer and inner walls of the peridium, brownish grey to greyish brown and larger peridioles (2.1–2.3 × 1.8–2 mm; Martin et al. 2015).

Three taxa *Cyathus hookeri* Berk. (1854: 204), *C. olla* (Batsch) Pers. (1801: 237) and *C. stercoreus* (Schwein.) De Toni (1888: 40) are similar to *C. tongxinianus* by having smooth peridium walls. However, *C. hookeri* differs by its basidiomata with short stipe, and single-cortex peridioles (Sharma 2016); *C. olla* is distinguished by its larger basidiomata (10–15 mm height, and 7–10 mm width), greyish exoperidium, pure silver peridioles, and smaller basidiospores (9.8–11.2 × 6.4–8 μm, Dorjey et al. 2013); and *C. stercoreus* differs by having both larger peridioles (2.4–3.1 × 1.8–2.6 μm) and basidiospores (30–41 × 25–31 µm, Gómez & Pérez-Silva 1988).

The family Nidulariaceae is a characteristic group of Agaricomycetes (Basidiomycota), previously studied based on morphological, phylogenetic, and cytological studies in China (Wu et al. 2016; Dai et al. 2021), but its knowledge is far to be complete (Xu et al. 2019, Huang et al. 2020, Ma et al. 2020, Wang et al. 2020, Cai et al. 2023, Yang et al. 2023, Yuan et al. 2023, Zhao et al. 2023). Therefore, the present work contributes to increasing the known fungal diversity of saprotrophs from China and pretends to encourage studies in the Nidulariaceae family.

Acknowledgements

The research was supported by the National Natural Science Foundation of China (Project No. 32170004), the Research Project of Yunnan Key Laboratory of Gastrodia and Fungal Symbiotic Biology (TMKF2023A03), and the High-level Talents Program of Yunnan Province (YNQR-QNRC-2018-111).

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