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MITOGENOME REPORT

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Complete mitochondrial genome of the bird's nest fungus *Nidula shingbaensis* (Nidulariaceae, Agaricales)

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ABSTRACT

Bird's nest fungi involve six different genera, but only one of these genera (i.e. *Cyathus*) have available mitochondrial genomes (mitogenomes) to date. In this study, we report the first mitogenome in the genus *Nidula* with *Nidula shingbaensis* K. Das & R.L. Zhao 2013 as a representative. The mitogenome is a circular molecule of 65,793 bp with a GC content of 26.2%. There are a total of 43 genes, including 14 typical protein-coding genes, 26 tRNA genes, two rRNA genes, and one free-standing intergenic open reading frame (ORF). Three introns (two in *cox1* and one in *cob*) are present in the mitogenome, with each containing an ORF encoding for a LAGLIDADG endonuclease. Phylogenetic analysis based on mitochondrial amino acid sequences confirms the phylogenetic placement of *N. shingbaensis* in Nidulariaceae in Agaricales. This study serves as a springboard for future investigation on fungal evolution in Nidulariaceae.

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1. Introduction

Bird's nest fungi are charismatic mushrooms resembling tiny egg-filled bird's nests. As they are saprobic, feeding on decomposing substrates, they are often seen growing on mulch, woody chips, fallen logs, leaf litter, or animal dung (Kraisitudomsook and Smith 2024). All Bird's nest fungi are currently classified in the family Nidulariaceae, including six genera, *Cyathus*, *Crucibulum*, *Mycocalia*, *Nidula*, *Nidularia*, and *Retiperidiolia* (Kraisitudomsook et al. 2022). These genera distinguished from each other by differences in morphology and peridiole structure. To date, there is no evidence to suggest that bird's nest fungi are pathogenic to plants or toxic to humans or other animals. On the contrary, some species show significant neurotrophic and neuroprotective activities due to their production of cyathane diterpenoids (Qi et al. 2023).

The genus *Nidula* was originally described in 1902 and currently contains seven accepted species according to statistics in the Fungal Names database (Wang et al. 2023). Different from species in *Cyathus* or *Crucibulum*, the egg-like spore mass (known as peridiole) of *Nidula* does not connect to fruiting bodies (known as peridia) by special cords (known as funiculi) (Brodie 1975). Among *Nidula* species, *Nidula shingbaensis* K. Das & R.L. Zhao 2013 was described as new to science in 2013 (Das and Zhao 2013). It is currently known to be distributed in India and Thailand (Das and Zhao 2013; Kraisitudomsook et al. 2021).

So far, there have been four species in Nidulariaceae with available mitogenomes, and they are all from the genus *Cyathus* (Li et al. 2023). In this study, we report the first mitogenome in *Nidula* with *N. shingbaensis* as a representative. This study will provide a reference for future evolutionary studies among different genera in Nidulariaceae.

2. Materials and methods

2.1. Fungal materials

Specimens used in this study were derived from unknown fallen twigs from Shennongjia Forestry District (N 31.48°, E 110.31°), Hubei, China. A specimen was deposited at the Herbarium of Shanxi University (http://life.sxu.edu.cn/, contact person: Yongjie Zhang, zhangyj2008@sxu.edu.cn) under the voucher number FSXU0224. Both morphology and phylogenetic analysis based on nrDNA ITS sequences identified it as *Nidula shingbaensis* (Figure 1). A culture was obtained, showing identical nrDNA ITS sequence with the fruiting body. The culture was cultivated on potato dextrose agar medium at 28 °C for 15 days, and mycelia were collected and used for DNA extraction.

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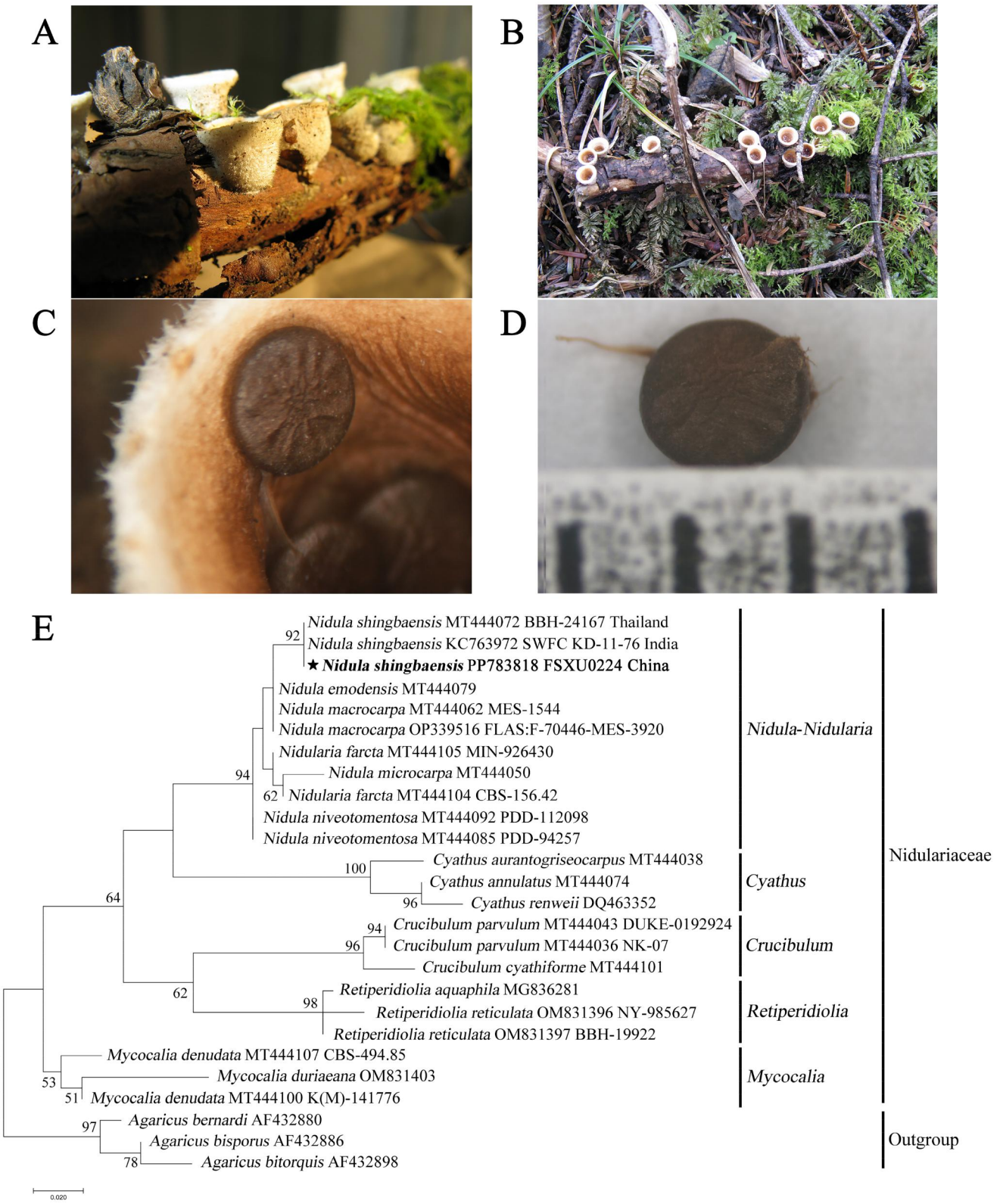

Figure 1. Morphology and phylogenetic identification of the *N. shingbaensis* specimen used in this study. (A, B) Mature basidiocarps. (C, D) Small packets (called peridioles) within nest-shaped fruiting bodies. Photos of the specimen were taken by Yongjie Zhang. (E) Phylogenetic tree based on nrDNA ITS sequences, as implemented with the maximum-likelihood method in MEGA. Bootstrap support values are shown for nodes receiving values larger than 50%. Each fungal taxon is followed by the GenBank accession number of its nrDNA ITS sequence. For species within more than two voucher specimens, the voucher number is also followed. For the focal species *N. shingbaensis*, the country where the specimen was collected is further followed. The short bar at the bottom of the tree is the distance scale (scale bar = 0.020).

2.2. DNA sequencing, mitogenome assembly, and annotation

Genomic DNA was extracted using the cetyl trimethyl ammonium bromide method (Zhang et al. 2010). High-throughput sequencing was performed using an Illumina NovaSeq 6000 sequencing platform in PE150 mode (Novogene, Tianjin, China). Mitogenome sequence was *de novo* assembled using two programs NOVOPlasty (https://github.com/ndierckx/NOVOPlasty) (Dierckxsens et al. 2017) and GetOrganelle (https://github.com/Kinggerm/GetOrganelle) (Jin et al. 2020).

The mitogenome sequence was primarily annotated using MFannot (https://megasun.bch.umontreal.ca/apps/mfannot/) based on the mold mitochondrial genetic code (i.e. Genetic Code 4), but necessary manual corrections were also needed according to previous publications (Zhang et al. 2017; Ren et al. 2021). Open reading frames (ORFs) at intronic and intergenic regions were only considered if they were longer than 300 bp. Introns in protein-coding genes (PCGs) were named according to established nomenclatures (Zhang and Zhang 2019). The circular map of the mitogenome was visualized using OGDRAW (https://chlorobox.mpimp-golm.mpg.de/OGDraw.html) (Greiner et al. 2019). Sequencing depth and coverage plot were drawn according to an online protocol (https://protocols.io/view/generating-sequencing-depth-and-coverage-map-for-o-cswxwffn.html) (Ni et al. 2023). Map of cis-splicing genes was drawn using PMGmap (http://www.1kmpg.cn/pmgmap).

2.3. Phylogenetic position of N. shingbaensis in Agaricales

In order to investigate the phylogenetic position of *N. shingbaensis*, 33 Agaricales species and two Boletales species were chosen as ingroups and outgroups, respectively. Amino acids of the 14 typical PCGs (*atp6*, *atp8*, *atp9*; *cob*, *cox1-3*; *nad1-6*, and *nad4L*) present in fungal mitogenomes were concatenated and used for phylogenetic analysis. Phylogenetic relationships were estimated using both maximum-likelihood (ML) and Bayesian (BI) approaches, with identical settings described in our previous publication (Ren et al. 2021).

3. Results

3.1. Basic features of the N. shingbaensis mitogenome

Both programs NOVOPlasty and GetOrganelle generated an identical circular mitogenome of 65,793 bp for *N. shingbaensis*,

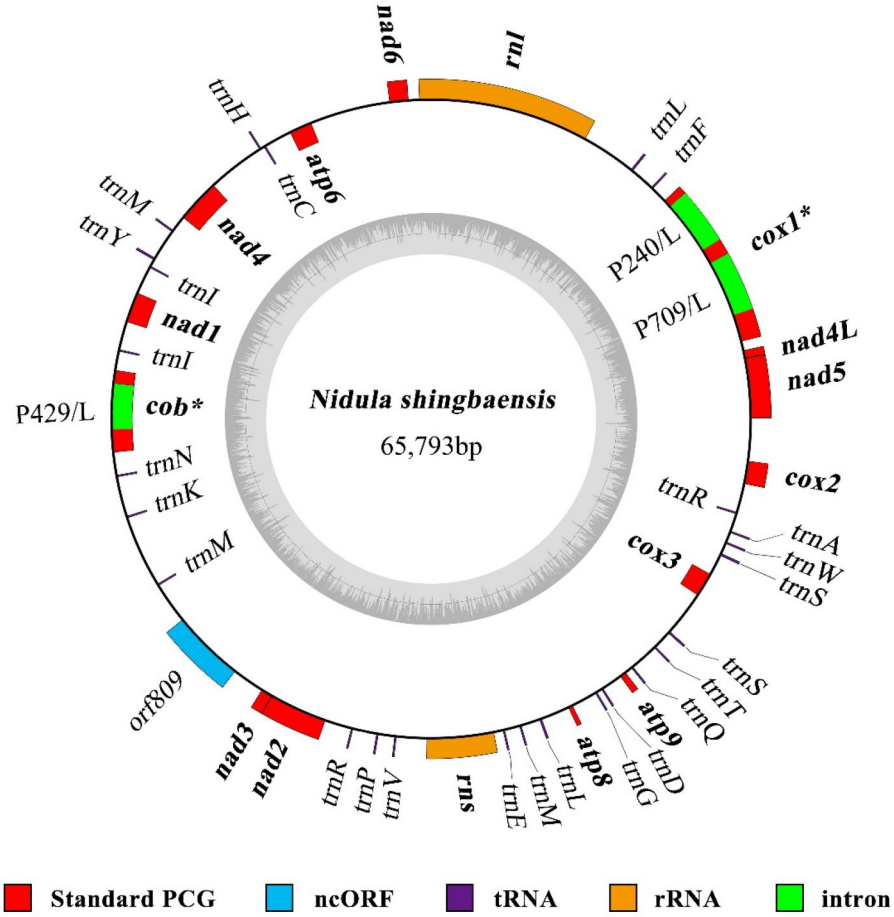

Figure 2. Circular map of the *N. shingbaensis* mitogenome. The outer ring indicates relative positions of different genes, and the inner ring indicates GC contents. Different types of genes/sequences are indicated by different colors as shown at the bottom of the figure. Blocks outside the ring represent forward strand genes, and blocks inside the ring represent reverse strand genes. The 14 standard PCGs and the two rRNA genes commonly found in fungal mitogenomes are shown in bold. Intron-containing genes are followed by an asterisk. For introns, standard intron names (with insertion site information) and functions of intronic ORFs (L, LAGLIDADG endonuclease) are given.

with an average sequencing depth of 1390× (Fig. S1). The mitogenome contains 43 genes, including two rRNA genes (*rnl* and *rns*), 26 tRNA genes, 14 typical PCGs associated with oxidative phosphorylation, and one intergenic ORF (*orf809*) encoding ribosomal protein S3 (Figure 2). The 26 tRNAs encode all 20 canonical amino acids. The 14 typical PCGs encode seven subunits of NADH dehydrogenases (*nad1-6* and *nad4L*), three cytochrome oxidase subunits (*cox1-3*), the cytochrome b (*cob*), and three ATP synthase subunits (*atp6*, *atp8*, and *atp9*). A total of three introns (all belonging to type I) are identified from the mitogenome, including two (named cox1P240 and cox1P709) in *cox1* and one (named cobP429) in *cob* (Fig. S2). Each intron contains an intronic ORF coding for a LAGLIDADG endonuclease. It is noteworthy that intergenic regions (35,146 bp in total length, 53.4%) account for more than half of the mitogenome. No large-fragment duplications, introns, or plasmid-like sequences are present at these intergenic regions.

3.2. Phylogenetic position of N. shingbaensis in Agaricales

Based on the phylogenetic analysis, *N. shingbaensis* clustered with *Cyathus stercoreus* (Schwein.) De Toni 1888, *Cyathus pallidus* Berk. & M.A. Curtis 1869, *Cyathus jiayuguanensis* J. Yu, T.X. Zhou & L.Z. Zhao 2002 and *Cyathus striatus* Willd. 1787, forming the family Nidulariaceae with high support values (99% for ML and 1.00 for BI) (Figure 3). Nidulariaceae further clustered with some other families like Bolbitiaceae and Hymenogastraceae to form the suborder Agaricineae in the order Agaricales. All other Agaricales species clustered according to their known family-level affiliations.

4. Discussion and conclusions

The study represents the first documentation of *N. shingbaensis* in China and the first mitogenome in *Nidula*. The *N.*

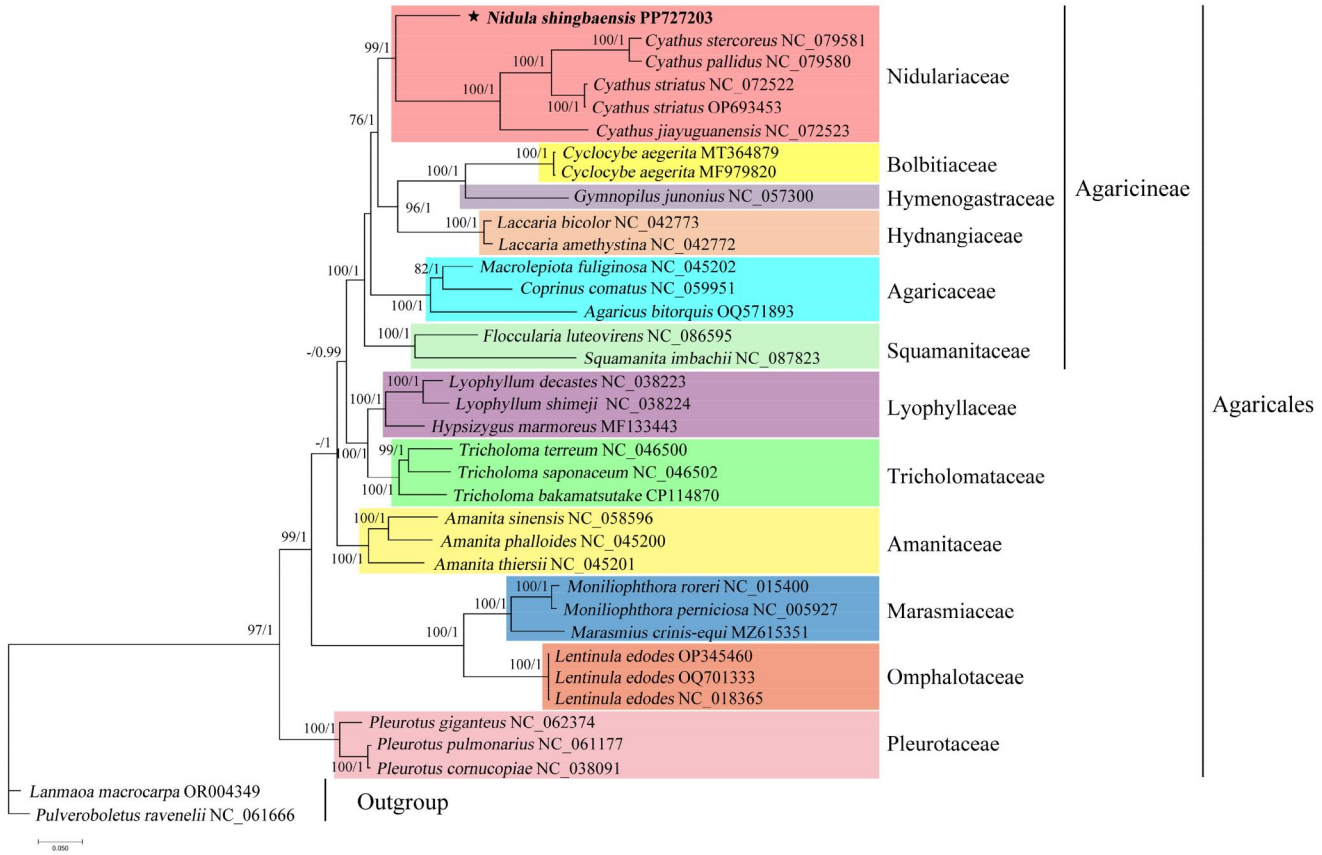

Figure 3. Phylogenetic tree constructed based on 14 core mitochondrial protein-coding genes. The phylogenetic tree shows the evolutionary relationship between *N. shingbaensis* and 33 other Agaricales species, with *Lanmaoa macrocarpa* and *Pulveroboletus ravenelii* as outgroups. This topology is the best topology constructed by Bayesian analysis. Two phylogeny-building methods (BI and ML) generate basically identical topologies. The node support value from ML is before the slash, and the node support value from BI is after the slash. Each fungal family is shown in different colors. The short bar at the bottom of the tree is the distance scale (scale bar = 0.050). Mitochondrial genomes used in the phylogenetic analysis were as follows, along with the corresponding references: *Nidula shingbaensis* PP727203 (this study), *Cyathus stercoreus* NC_079581, *Cyathus pallidus* NC_079580, *Cyathus jiayuguanensis* NC_072523, *Cyathus striatus* NC_072522, *Cyathus striatus* OP693453 (Li et al. 2023), *Cyclocybe aegerita* MT364879 (Liu et al. 2020), *Cyclocybe aegerita* MF979820 (Xu et al. 2017), *Gymnopilus junonius* NC_057300 (Cho et al. 2021), *Laccaria bicolor* NC_042773, *Laccaria amethystina* NC_042772 (Li et al. 2020), *Macrolepiota fuliginosa* NC_045202 (unpublished), *Coprinus comatus* NC_059951 (unpublished), *Agaricus bitorquis* OQ571893 (Zhao et al. 2023), *Floccularia luteovirens* NC_086595 (unpublished), *Squamanita imbachii* NC_087823 (unpublished), *Lyophyllum decastes* NC_038223, *Lyophyllum shimeji* NC_038224 (Li et al. 2019), *Hypsizygus marmoreus* MF133443 (unpublished), *Tricholoma terreum* NC_046500, *Tricholoma saponaceum* NC_046502 (Huang et al. 2021), *Tricholoma bakamatsutake* CP114870 (Ichida et al. 2023), *Amanita sinensis* NC_058596, *Amanita phalloides* NC_045200, *Amanita thiersii* NC_045201 (Li et al. 2023), *Moniliophthora roreri* NC_015400, *Moniliophthora perniciosa* NC_005927, *Marasmius crinis-equi* MZ615351 (Ali et al. 2021), *Lentinula edodes* OP345460 (Kim et al. 2022), *Lentinula edodes* OQ701333 (unpublished), *Lentinula edodes* NC_018365 (unpublished), *Pleurotus giganteus* NC_062374 (Liu et al. 2022), *Pleurotus pulmonarius* NC_061177 (unpublished), *Pleurotus cornucopiae* NC_038091 (Xu et al. 2018), *Lanmaoa macrocarpa* OR004349 (Zheng et al. 2023), and *Pulveroboletus ravenelii* NC_061666 (Cho et al. 2022).

shingbaensis mitogenome at 65,793 bp is obviously smaller than those in *Cyathus*, which ranges from 114,236 bp in *C. pallidus* to 129,263 bp in *C. stercoreus* (Li et al. 2023). Phylogenetic analyses based on mitochondrial amino acid sequences confirm the placement of *N. shingbaensis* in Nidulariaceae (Figure 3). Our results find that Nidulariaceae is sister to a clade of Bolbitiaceae, Hymenogastraceae, and Hydnangiaceae, with moderate to strong support (Figure 3). This is different from previous studies, which showed that Nidulariaceae is sister to Squamanitaceae (Kraisitudomsook et al. 2021; Liu et al. 2021).

For inter-generic relationships of the six genera of bird's nest fungi, there are inconsistencies among different studies (Kraisitudomsook et al. 2021, 2022, 2024). Together with this study, only two genera (*Cyathia* and *Nidula*) have available mitogenomes in Nidulariaceae. Mitogenomes from other genera of bird's nest fungi need to be assembled in the future in order to provide evolutionary insights into inter-generic relationships in Nidulariaceae.

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Ethics statement

The study did not involve humans or animals. In this study, samples can be collected without ethical approval or permission.

Author contributions

Y.J.Z. and S.Z. collected the sample and designed research; Q.Q. and Y.J.Z. performed research and analyzed data; Q.Q., S.Z., and Y.J.Z. wrote the paper. All authors agreed to be accountable for all aspects of the work.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

BioProject, BioSample, and SRA accession numbers related to the sample of this study are PRJNA1104276, SAMN41074236, and SRR28795957, respectively. The mitochondrial genome and the nrDNA ITS sequence that support the findings of this study are openly available in the National Center for Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov, with accession numbers PP727203 and PP783818, respectively.

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