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MITOGENOME REPORT

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Complete mitochondrial genome sequence and phylogenetic analysis of *Tylopilus brunneirubens* (Boletales, Basidiomycota)

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ABSTRACT

Tylopilus brunneirubens is a common species in southern China. It is known for brown to dark brown pileus, white context turning reddish brown or rust brown when touched and distinct reticulation on the upper stem. However, little is known about its mitochondrial genome and its relationship with other boletes. Our analysis revealed that the mitochondrial genome of this species is a circular DNA molecule that spans 32,389 bp. It contains 15 core protein-coding genes, 24 transfer RNA genes, and two ribosomal RNA genes. The base composition of the mitochondrial genome is as follows: A (37.20%), C (11.32%), G (12.48%), and T (39.00%), with a GC content of 23.80%. Furthermore, a phylogenetic tree based on 24 mitochondrial genomes provided valuable insights into the phylogenetic relationships of *Tylopilus brunneirubens* with other boletes for the first time.

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Introduction

Tylopilus brunneirubens (Corner) Watling and E. Turnbull 1994 is a bolete mushroom species known for its olive-brown to dark brown pileus, white context that turns rust-brown or reddish-brown when touched, distinct half-reticulate stipe, its habitat in tropical to subtropical areas, and its symbiosis with Fagaceae (Li and Yang 2021). The medicinal and edible properties of this species remain unexplored. Basidiocarps of *T. brunneirubens* in their natural habitat are presented in Figure 1. Studying the mitochondrial genome of bolete species can provide valuable insights into their evolutionary history and phylogenetic relationships (Li et al. 2021; Zheng et al. 2023). This study presents the first complete sequence of the mitochondrial genome of *T. brunneirubens*, allowing for an in-depth analysis of its phylogenetic relationships with other species within the family Boletaceae.

Materials

The sample of *T. brunneirubens* was obtained from Jiulingshan National Nature Reserve in Jiangxi Province, China (115°21′09″E, 28°54′43″N) and had been stored at the Cryptogamic Herbarium in the Kunming Institute of Botany, Chinese Academy of Sciences, under the voucher number KUN-HKAS 105257. For further information, please contact Kuan Zhao at key1989@126.com. The identification of the specimen was carried out by the corresponding author. The research conducted on higher fungi adhered to the guidelines established by Jiangxi Science and Technology Normal University and Jiulingshan National Nature Reserve Administration of Jiangxi Province. Field studies were conducted in compliance with local legislation. No specific permission was necessary for the collection as it did not involve any endangered or protected species.

Methods

The basidiocarp tissue was used to extract total DNA using the CTAB method (Doyle and Doyle 1987). The extracted DNA was then sequenced on an Illumina HiSeq 2500 Platform by Sangon Biotech Co., Ltd. (Shanghai, China). The clean reads obtained were assembled by GetOrganelle, utilizing the fungus database (-F fungus_mt) to identify, filter, and assemble the target-associated reads (Jin et al. 2020). The mitochondrial genome was annotated using the MITOS Web Server based on the mitochondrial genetic code 4 (Bernt et al. 2013). The annotated protein-coding genes (PCGs) were refined using the open reading frame (ORF) finder from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). Additionally, the annotated tRNA genes were verified using tRNAscan-SE v1.3.1 (Lowe and Chan 2016). Gene annotation was examined with CPGview (Liu et al. 2023) and intron types (if any) were verified through RNAweasel v5.2.1 (Lang et al. 2007). The gene map

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Figure 1. The basidiocarps of *Tylopilus brunneirubens* collected from Jiangxi Province, China. The grayish red to brownish red or rust brown when bruised and its distinct reticulation on the upper stem are the most distinguished features. Photographed by Kuan Zhao.

was visualized by PMGmap (Zhang et al. 2023, http://www.1kmpg.cn/pmgmap).

A total of 24 mitochondrial genomes were downloaded from NCBI and the Joint Genome Institute (JGI, https://mycocosm.jgi.doe.gov/mycocosm/home) database as indicated by previous studies (Miyauchi et al. 2020; Li et al. 2020, 2021; Shi et al. 2022; Zheng et al. 2023), including 21 from the family Boletaceae and three species from the family Paxillaceae (Boletales) as outgroups. Fifteen core PCGs were extracted and aligned individually using MAFFT v7.037 (Katoh et al. 2019). The alignments were then concatenated to form a matrix by Phyutility v2.6 (Smith and Dunn 2008). The final concatenated matrix was analyzed by MrBayes v3.2.6 and RAxML v8.0.0 for Bayesian inference (BI, Ronquist and Huelsenbeck 2003) and maximum-likelihood (ML, Stamatakis 2006) methods, respectively. BI analyses were conducted under default settings (Site substitution model = Gamma site model (Gamma category = 4; GTR), Chain length of MCMC = 10,000,000, Burn-in = 10%, Model = Yule model) and terminated when the average standard deviation of split frequencies dropped below 0.01. In ML analyses, bootstrap (BS) values were assessed using the ultrafast BS approach under GTR + G model with 1000 replicates.

Results

The mitochondrial genome sequence of *T. brunneirubens* (GenBank accession no. OR619662) spans 32,389 bp and was assembled from 19,780,464 reads, with a mean coverage of ×3677.74 from trimmed sequencing data (Figure S1, supplementary material). The gene map of *T. brunneirubens* is illustrated in Figure 2. The complete mitochondrial genome comprised 15 core PCGs (*atp6*, *atp8*, *atp9*, *cob*, *cox1*, *cox2*, *cox3*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad6*, and *rps3*), 24 transfer RNA genes, and two ribosomal RNA genes. No introns were found in any of the annotated genes. The mitochondrial genome had a base composition of A (37.20%), C (11.32%), G (12.48%), and T (39.00%), with a GC content of 23.80%. The start codon for all 15 PCGs is ATG, and the termination codon for 14 PCGs was TAA, with the exception of nad6, where the stop codon was TAG.

The phylogenetic analysis indicated that the recently sequenced *T. brunneirubens* clustered with *T. plumbeoviolaceoides*, as shown in Figure 3. Additionally, the two *Tylopilus* species also clustered together with the genus *Boletus* sensu stricto, *Hortiboletus* and *Imleria*, which also belong to the subfamily Boletoideae.

Discussion and conclusions

This study presents the first complete mitochondrial genome of *T. brunneirubens*, which is the smallest among all the already sequenced mitochondrial genomes of the family Boletaceae, ranging from 32,883 bp to 48,298 bp (Li et al. 2021; Shi et al. 2022; Zheng et al. 2023). The size of mitochondrial genome of boletes is mainly influenced by the intronic region (Li et al. 2021). In line with this, we found no introns in the *T. brunneirubens* mitochondrial genome. Previously only *T. plumbeoviolaceoides* of the genus *Tylopilus* had been sequenced for mitochondrial genome (Shi et al. 2022). Thus, in our phylogenetic analyses, the two species correspondingly clustered into one clade. However, the two species are different in their morphological characteristics and distribution range. *Tylopilus brunneirubens* has a yellowish brown pileus whereas *T. plumbeoviolaceoides* has a violaceous-brown or purple pileus. In addition, *T. plumbeoviolaceoides* is described from southern

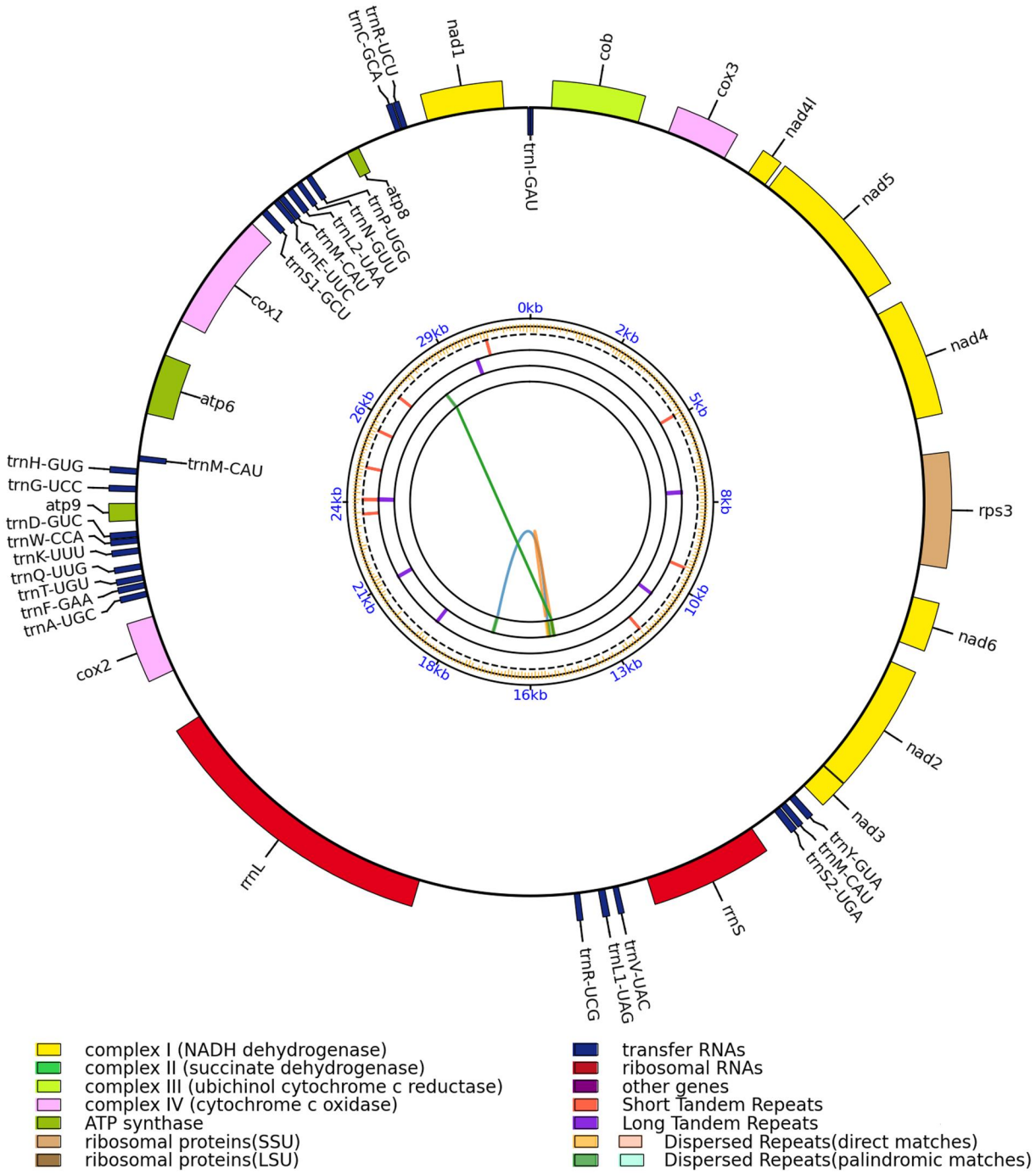

Figure 2. The mitochondrial genome map of *Tylopilus brunneirubens*. Genes shown outside and inside the outer circle are transcribed in counterclockwise and clockwise directions, respectively. The inner circles represent the genome scale, GC content and distributions of short tandem repeats, long tandem repeats, and the dispersed repeats, respectively. The colored parabolas in the center circle represent the dispersed repeats.

China while the newly sequenced *T. brunneirubens* has a wider distribution, which can be found not only in southern China but also in Southeast Asia (Li and Yang 2021). Although the species of the genus *Tylopilus* sensu lato has been split into several genera, such as *Chiua*, *Harrya*, *Sutorius*, and *Zangia*, the genus *Tylopilus* sensu stricto harbors the largest number of species (Wu et al. 2016). In China, more than 30 species of *Tylopilus* sensu stricto has been reported, thus further investigation into the phylogeny of species from the genus *Tylopilus* is required once more mitochondrial genomes are sequenced in the future.

Author contributions

JYH, LT, and YL collected the samples, conducted the analysis, and interpreted the data. JYH drafted the manuscript. KZ conceived and supervised the project, critically reviewed and revised the manuscript, and

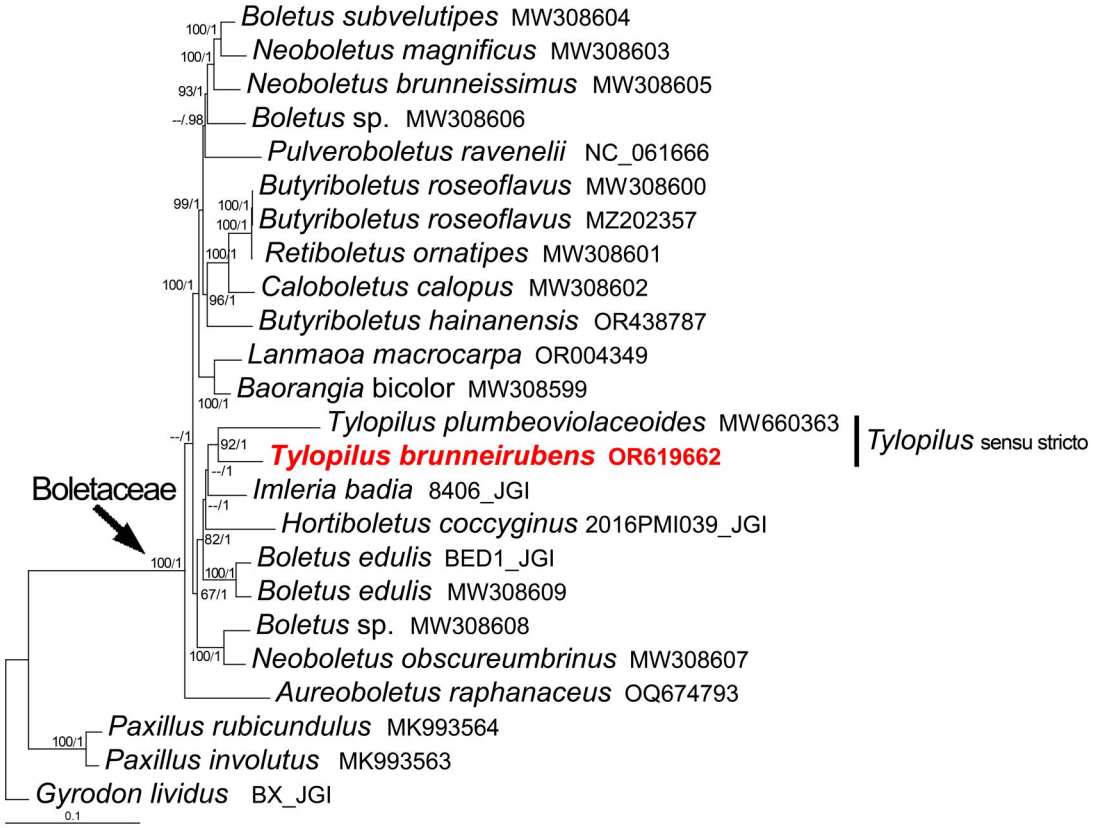

Figure 3. Phylogenetic tree of *Tylopilus brunneirubens* and related taxa based on Bayesian's inference (BI) and maximum-likelihood (ML) analyses of 15 core protein coding genes (*atp6*, *atp8*, *atp9*, *cob*, *cox1*, *cox2*, *cox3*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad6*, and *rps3*). The GenBank accession number from NCBI or the information of voucher specimen from JGI, along with the corresponding references (if any), are provided after the species names. The following sequences were used: *Aureoboletus raphanaceus* OQ674793 (Mu et al. 2024), *Baorangia bicolor* MW308599 (Li et al. 2021), *Boletus edulis* BED1_JGI (unpublished), *Boletus edulis* MW308609 (Li et al. 2021), *Boletus* sp. MW308606 (Li et al. 2021), *Boletus* sp. MW308608 (Li et al. 2021), *Boletus subvelutipes* MW308604 (Li et al. 2021), *Butyriboletus hainanensis* OR438787 (Zeng et al. 2024), *Butyriboletus roseoflavus* MW308600, MZ202357 (Li et al. 2021), *Caloboletus calopus* MW308602 (Li et al. 2021), *Gyrodon lividus* BX_JGI (unpublished), *Hortiboletus coccyginus* 2016PMI039_JGI (unpublished), *Imleria badia* 8406_JGI (unpublished), *Lanmaoa macrocarpa* OR004349 (Zheng et al. 2023), *Neoboletus brunneissimus* MW308605 (Li et al. 2021), *Neoboletus magnificus* MW308603 (Li et al. 2021), *Neoboletus obscureumbrinus* MW308607 (Li et al. 2021), *Paxillus involutus* MK993563 (Li et al. 2021), *Paxillus rubicundulus* MK993564 (Li et al. 2021), *Pulveroboletus ravenelii* NC_061666 (Cho et al. 2022), *Retiboletus ornatipes* MW308601 (Li et al. 2021), and *Tylopilus plumbeoviolaceoides* MW660363 (Li et al. 2021). The newly sequenced mitogenome is marked in red. Numbers near the nodes indicate bootstrap support values (>50%) and posterior probabilities (>0.95). The scale bar refers to 0.1 nucleotide substitutions per character.

approved the final version for publication. All authors discussed and critically revised the results and contributed to the final version of the manuscript.

Ethical approval

No ethical issues were involved in this study. The collection of the mushroom was legal and reasonable. Information of the voucher specimen and who identified it were introduced in the manuscript.

Disclosure statement

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

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Data availability statement

The mitochondrial genome data are available with the accession number of OR619662 in the GenBank of NCBI (https://www.ncbi.nlm.nih.gov/). And the associated BioProject, SRA, and BioSample numbers are PRJNA957944, SRS17371855, and SAMN34274286, respectively.

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