Retiboletus Huanggangensis, A New Species of Retiboletus From China

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Abstract

A new species of the genus *Retiboletus*, *Retiboletus huanggangensis*, collected from Huanggang mountain, Wuyishan Nature Reserve, Yanshan County, Shangrao City, Jiangxi Province, China, is described based on morphological and phylogenetic analysis, nuclear ribosomal large subunit (nrLSU), the translation elongation factor1-α gene (*TEF1-α*) and the RNA polymerase II second largest subunit gene (*RPB2*) were used for phylogenetic analysis. *Retiboletus huanggangensis* is morphologically characterized by its white to grayish black to black pileus, white to pale yellow to black stipe, white to pale yellowish white hymenophore, white to grayish white or pale yellow context, prominently and coarsely reticulate over the upper 3/4 or overall, changing orange yellow to rusty yellow when its pileus injured. For phylogenetic analysis, the specimens from Huanggang mountain, grouped together with *Retiboletus fuscus*, but they have some morphological differences, *Retiboletus fuscus* has slightly narrower basidia, and pileipellis much narrower. Descriptions and hand drawings of the new species and comparisons with similar species are shown below.

Introduction

The genus *Retiboletus* Manfr. Binder & Bresinsky was established by Binder and Bresinsky (Binder and Bresinsky 2002), typified by a species from North America, *R. ornatipes* (Peck) Binder & Bresinsky. Before 2002, some species of *Retiboletus* were mistaken for *Boletus* spp., e.g. *R. fuscus* originally was described as *Boletus griseus var. fuscus* (Hongo 1974), *R. kauffmanii* (Lohwag) N.K. Zeng & Zhu L. Yang was regarded as *Boletus kauffmanii* (Lohwag 1937; Horak 1987), *R. flavoniger* was identified as *Boletus flavoniger* Halling, G.M. Muell & L.D. Gómez (1999). But DNA phylogenetic analyses and chemotaxonomical characters prompted Binder and Bresinsky (2002) to classified *Retiboletus* as a novel genus of Boletaceae. According to the results of chemotaxonomical characters (Hellwig 1999), the phylogenetic analysis of LSU sequence was proposed by Binder and Bresinsky, which proved Hellwig's findings.

*Retiboletus* is characterized by the following characters: basidiomata small to medium-sized, hymenophore pallid, grayish white or pall yellow, unchanging or yellowish brown or orange-brown when injured; stipe reticulate; context white, pall yellow or bright yellow, unchanging or changing light yellow to orange-brown; clamp connections absent in all tissues; spore greenish brown to yellow-brown in KOH, basidiospores smooth, ellipsoid to subsub fusoid to sub fusoid (Binder and Bresinsky 2002; Zeng et al. 2016; Liu et al. 2020).

So far, 16 species of *Retiboletus* have been described, of which 9 species have been reported from China (Binder and Bresinsky 2002; Wu et al. 2016; Zeng et al. 2016; Zeng et al. 2018; Liu et al. 2020).

Recently, we investigated the diversity of Macrofungi in Wuyishan Nature Reserve, Jiangxi Province, and collected several specimens of Boletaceae. Through morphological and phylogenetic analysis, we found a new species of *Retiboletus*.

Materials And Methods

Specimens collection

The specimens were collected on the ground of coniferous and broad-leaved mixed forest in Wuyishan Nature Reserve, on July 31 and August 23, 2021.
Firstly, clean up the sundries around the specimen, put a scale and label on the edge of the specimen, take photos, and finally dig out the specimen with a shovel and put it in the sampling box.

**DNA extraction, amplification, and sequencing**

The whole genome DNA was extracted from dried specimens (HFJAU10002, HFJAU10003, HFJAU10004) by optimized CTAB method (Doyle 1987; Huang et al. 2000). The primer pairs LR0R–LR5 and TEF1-983F–TEF1-1567R were used to amplify the large ribosomal subunit sequence region (Vilgalys and Hester 1990; James et al. 2006) and the translation elongation factor 1-α region (Wu et al. 2014), respectively. The RNA polymerase II second largest subunit gene (*RPB2*) was amplified following the method described by Réblová et al. (2011). PCR products were detected in 1% agarose gels, and then sent to TSINGKE Biological Technology for sequencing.

**Macroscopic and microscopic studies**

Macroscopic features were mainly made from the field records and photographs of the basidiomata, color codes followed Kornerup & Wanscher (1981). Micromorphological descriptions are based on dried materials rehydrated in 5% KOH and stained in ammoniacal Congo red. Freehand sections were done on Nikon SMZ1270 following the standard method described in previous studies (Li et al. 2011; Zeng et al. 2012; Hosen et al. 2013; Zeng et al. 2013). Microstructures were observed by Nikon Y-TV55.

The number of measured basidiospores is given as n/m/p, which means that the measurements were made on n basidiospores from m basidiomata of p collections. The sizes of basidiospores are given using the notation (a)b–c(d), where the range b–c represents a minimum of 90% of the measured values, and extreme values (a and d) are given in parentheses. Q represents the ratio of length/width of the spores. \(Q_m\) refers to the average Q of basidiospores \(\pm\) sample standard deviation.

**Phylogenetic analysis**

Visualization and editing of the sequences were done with BioEdit v7.0.9 (Hall 1999), and submitted to NCBI online website for Nucleotide BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine which genus the species belongs to. Based on BLASTn results, all available sequences of this genus used in recent studies and closely related to *Retiboletus* were downloaded from GenBank, shown in Table 1.

Sequence datasets were aligned on the online website MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/Katoh and Standley 2013), misaligned sequences and gaps were sent to PhyloSuite (Zhang et al. 2020), and reduced by Gblocks (Gerard and Jose 2007). The data was then analyzed using RAxML version 8 (Stamatakis 2014) and MrBayes v3.2 (Ronquist et al. 2012) for Maximum Likelihood (ML) and Bayesian Inference (BI), respectively.

For ML analyses, based on the evaluation of nrLSU dataset, using GTRGAMMA1 as evolutive model (Stamatakis 2006), choosing the rapid bootstrap analysis with the supports were calculated, using nonparametric bootstrapping with 1000 replicates (Joseph 1985).

For BI analyses, substitution models of partition in the datasets were determined using the Akaike Information Criterion (AIC) implemented in PartitionFinder 2 (Lanfear et al. 2016). For the combined dataset GTR+I+G, SYM+G and SYM+G were chosen as best-fit likelihood models for LSU, *TEF1*-α and *RPB2* partitions, respectively. Two
MCMC runs with four chains each were run for 4,000,000 generations and sampled every 1000 generations. At the end of the runs, the average deviation of split frequencies was 0.005509; Other parameters were kept at their default settings; Trees were summarized and posterior probabilities (PPs) were calculated after discarding the first 25% generations as burn-in.

Branches that received bootstrap support for Maximum Likelihood (ML) and Bayesian posterior probabilities (BPP) greater than or equal to 50% (BS) and 0.95 (PP), respectively, were considered credible.

All phylogenetic trees were visualized using FigTree v1.4.4.

**Result**

**Molecular phylogenetic results**

Eight sequences (3 of nrLSU, 2 of *TEF1-α*, 3 of *RPB2*) from 3 collections were newly generated.

The Maximum likelihood and Bayesian phylograms have no conflict in topology, so only the ML trees with both BS and PP values are shown (Fig.1). The nrLSU, *TEF1-α* and *RPB2* datasets consisted of 48 sequences (including 45 from GenBank and 3 new generated), 40 sequences (including 38 from GenBank and 2 new generated), 24 sequences (including 21 from GenBank and 3 new generated), respectively. The combined nuclear dataset (nrLSU+ *TEF1-α*+*RPB2*) contains 112 sequences with 2182 nucleotide sites after reducing gaps by Gblocks.

In the three-gene phylogenetic analysis, *R. huanggangensis* was sister to *R. fuscus* with bootstrap support (BS = 73%) (Fig. 1) but was independented from the collection from southwest China. And it formed a large clades with *R. ater, R. griseus, R. nigerrimus, R. nigrogriseus, R. pseudogriseus, R. sinogriseus and R. zhangfeii* with higher statistical support (BS = 67%, PP = 1).

**Table 1.** Specimens used in molecular phylogenetic study and their GenBank accession numbers.
| Species              | Voucher     | Locality      | Accession     | Reference       |
|---------------------|-------------|---------------|---------------|-----------------|
| **Leccinum duriusculum** | HKAS101160  | Austria       | MZ675541     | Meng et al. 2021 |
| **L. parascabrum**   | HKAS:99903T| China         | MW413911     | Meng et al. 2021 |
| **L. versipelle**    | HKAS97997   | China         | MZ675545     | Meng et al. 2021 |
| **L. versipelle**    | HKAS99380   | China         | MZ675546     | Meng et al. 2021 |
| **Retiboletus ater** | Li1215T     | SW China      | MT010611     | Liu et al. 2020  |
| **R. ater**          | Li1224      | SW China      | MT010612     | Liu et al. 2020  |
| **R. brevibasidiatus** | OR0570  | Thailand      | MT085476     | Chuankid Boontiya et al. 2021 |
| **R. brunneolus**    | LC_LJW23    | SW China      | MT010615     | Liu et al. 2020  |
| **R. brunneolus**    | HKAS 52680  | SE China      | KF112424     | Wu et al. 2016   |
| **R. flavoniger**    | RH7247T     | Costa Rica    | AF456828     | Binder and Bresinsky 2002 |
| **R. flavoniger**    | RH7189      | Costa Rica    | AF456829     | Binder and Bresinsky 2002 |
| **R. fuscus**        | FHMU1403    | Yunnan, SW China | MH367473  | Zeng et al. 2018   |
| **R. fuscus**        | HKAS63624   | China         | KT990635     | Wu et al. 2016   |
| **R. fuscus**        | OR0738      | Thailand      | MT085472     | Chuankid Boontiya et al. 2021 |
| **R. fuscus**        | OR0231      | China         | MG212600     | Vadthanarat et al. 2018 |
| **R. fuscus**        | HKAS74756   | SW China      | KT990636     | Wu et al. 2016   |
| **R. fuscus**        | HKAS:63590  | USA           | KF112417     | Wu et al. 2016   |
| **R. griseus**       | MB03-079    | USA           | KT824030     | Vadthanarat et al. 2016 |
| Species         | Voucher      | Locality       | Accession       | Reference                  |
|-----------------|--------------|----------------|-----------------|----------------------------|
|                 |              |                | nrLSU | TEF1-α | RPB2 |                  |
| *R. griseus*    | snBoth       | USA            | KF030308 | KF030414 | — | Binder and Bresinsky 2002 |
|                 | Halling10162 | USA            | MT010608 | MT010618 | — | Liu et al. 2020 |
| *R. huanggangensis* | HFJAU10002 | China          | OL744444 | OL963527 | OL963530 | This study |
| *R. huanggangensis* | HFJAU10003T | China          | OL744445 | OL963526 | OL963528 | This study |
| *R. huanggangensis* | HFJAU10004 | China          | OL744446 | — | OL963529 | This study |
| *R. kauffmanii* | HKAS68590    | Yunnan, SW China | KP739283 | — | — | Zeng et al. 2016 |
| *R. kauffmanii* | HKAS 63584E  | Yunnan, SW China | KT990634 | KT990828 | KT990465 | Wu et al. 2016 |
| *R. kauffmanii* | OR0278       | Yunnan, SW China | — | MG212601 | MG212643 | Vadthanarat et al. 2018 |
| *R. kauffmanii* | HKAS63584    | SW China       | KP739282 | KP739301 | KP739299 | Zeng et al. 2016 |
| *R. nigerrimus* | Tyni1        | Japan          | AF456832 | — | — | Binder and Bresinsky 2002 |
| *R. nigrogriseus* | BC0179     | Thailand       | — | MT085474 | MT085478 | Chuankid Boontiya et al. 2021 |
| *R. nigrogriseus* | OR049       | Thailand       | — | KT824033 | KT824000 | Vadthanarat et al. 2016 |
| *R. nigrogriseus* | FHMU2045    | Southern Chin  | MH367475 | MH367487 | — | Zeng et al. 2018 |
| *R. nigrogriseus* | FHMU2800T   | Southern Chin  | MH367476 | MH367488 | — | Zeng et al. 2018 |
| *R. ornatipes*  | 201/97T*     | USA            | AF456815 | — | — | Binder and Bresinsky 2002 |
| *R. ornatipes*  | Halling10163 | USA            | MT010617 | MT010626 | — | Liu et al. 2020 |
| *R. ornatipes*  | MBsn         | USA            | — | MT219516 | MT219515 | Chuankid Boontiya et al. 2021 |
| Species            | Voucher | Locality          | nrLSU   | TEF1-α | RPB2 | Reference                  |
|--------------------|---------|-------------------|---------|--------|------|----------------------------|
| R. ornatipes       | 93/97   | USA               | AF456825| —      | —    | Binder and Bresinsky 2002  |
| R. pseudogriseus   | Zeng647 | Southern Chin     | MT010613| MT010623| —    | Liu et al. 2020           |
| R. pseudogriseus   | FHMU375 | Southern Chin     | MH367477| MH367489| —    | Zeng et al. 2018          |
| R. pseudogriseus   | FHMU2205| Fujian, SE China  | MH367478| MH367490| —    | Zeng et al. 2018          |
| R. pseudogriseus   | HKAS 83950^T| SE China    | KP739285| —      | —    | Zeng et al. 2016          |
| R. retipes         | 96/97   | USA               | AF456830| —      | —    | Binder and Bresinsky 2002  |
| R. retipes         | 22/97   | USA               | AF456831| —      | —    | Binder and Bresinsky 2002  |
| R. retipes         | 116/96  | USA               | AF456823| —      | —    | Binder and Bresinsky 2002  |
| R. sinensis        | HKAS83957| SE China       | KP739291| KP739303| —    | Zeng et al. 2016          |
| R. sinensis        | HKAS59832| SE China       | KT990633| KT990827| KT990464| Wu et al. 2016             |
| R. sinensis        | HKAS83955| SE China       | KP739289| KP739302| —    | Zeng et al. 2016          |
| R. sinogriseus     | LJ258   | NE China          | MT010610| MT010620| —    | Liu et al. 2020           |
| R. sinogriseus     | LJ260^T | NE China          | MT010609| MT010619| —    | Liu et al. 2020           |
| R. aff. kauffmanii | HY56    | NE China          | MT010616| —      | —    | Liu et al. 2020           |
| R. vinaceipes      | CFMR:DR-1035^T| Dominican Republic| MN250180| —      | —    | Ortiz-Santana et al. 2007  |
| R. vinaceipes      | CFMR:BZ-2386| Belize         | MN250190| —      | —    | Ortiz-Santana et al. 2007  |
| R. zhangfeii       | HKAS59699| SE China          | JQ928627| JQ928582| JQ928603| Wu et al. 2016             |
Species | Voucher | Locality | Accession | Reference
---|---|---|---|---
*R. zhangfeii* | HKAS 83963 | Fujian, SE China | KP739297 | Zeng et al. 2016
*R. zhangfeii* | HKAS 83961 | Fujian, SE China | KP739295, KP739305 | Zeng et al. 2016
*R. zhangfeii* | HKAS53418 | China | KT990630, KT990824, KT990462 | Wu et al. 2016
*Tylopilus sp.* | 204/97 | USA | AF456813 | Binder and Bresinsky 2002

Sequences obtained in this study are shown in bold. SW = southwestern; NE = northeastern; SE = southeastern.

T= holotype, E= epitype, T* = type species

**Taxonomy**

*Retiboletus huanggangensis* F. Zhou, Y. Gao, HJ. Hu, HY.Song & DM. Hu, sp. nov.

MycoBank No: 842302

Figures. 2 and 3

**Etymology:** "huanggangensis" referring to the collection site of this species (Huanggang Mountain).

**Type:** China. Jiangxi Province: Shangrao City, Yanshan County, Wuyishan Nature Reserve, Huanggang mountain, alt. 1600 m, August 23, 2021, Fan Zhou, WYS236 (holotype: HFJAU10003).

**Description**

Basidiomata small to medium-sized. *Pileus* 18.4–72.2 mm diam, hemispherical to applanate, surface dry, densely subtomentose, pale yellowish white (3A1) to grayish black (5E4) at the margin and black (30F1) towards center; context 4–15 mm in thickness in the center of the pileus, pale yellow (4A2), changing orange yellow (2B6) to rusty yellow (5B7) when injured. *Hymenophore* always adnate around the stipe; pores angular, tubes up to 11 mm long, white (29A2) when young and yellowish white (1A2) in age, becoming brownish yellow (30B4) when injured. *Stipe* 13–30 × 26–150 mm, central, clavate to flexuous, solid; surface dry, prominently and coarsely reticulate over the upper 3/4 or overall, pale yellow (2A2) in the upper part and black (1F3) downwards when young, white (3A1) on upper part and lower part black (3F4) in age, changed to pale yellow (5A2) when injured; basal mycelium white. Odor indistinct.

*Basidia* 30–36 × 10.5–13 μm, thin-walled, clavate, four-spored; sterigmata 2–5 μm long. *Basidiospores* [60/3/3] (7.0–)10–13.5 × 3.5–5.5 μm, Q = (1.75–)2.00–3.00(–3.33), Q_m = 2.40 ± 0.34, amygdaloid to subfusiform to ellipsoid, grayish yellow to pale yellow to yellowish brown in KOH, smooth. *Hymenophoral trama* boletoid. *Cheilo- and Pleurocystidia* 27–46 × 8–10.5 μm, abundant, ventricose to fusiform or ellipsoid, thin-walled, hyaline or with golden yellow contents, without encrustations. *Pileipellis* a trichoderm about 130–220 μm thick.
Composed of intertwined hyphae, brown to grayish brown in KOH, thin-walled hyphae 5–12 µm diam; terminal cells 22–74 × 4–12 µm, clavate or subterete, with obtuse apex. *Pilea trama* composed of thin-walled hyphae 3–11 µm diam. *Stipitipellis* hymeniform about 30–80 µm thick, composed of smooth, hyaline or grayish brown hyphae, narrowly or broadly clavate, ventricose or subfusiform; terminal cells 20–45 × 5–12.5 µm and occasionally with clavate, four-spored basidia 22–42 × 9–12 µm. *Stipe trama* composed of cylindrical, slightly thick-walled (up to 0.5 µm) parallel or interlaced hyphae 5–11 µm diam. *Clamp connections* absent in all tissues.

**Habitat:** Solitary or group on the ground in forests of Pinaceae and Fagaceae.

**Distribution:** Jiangxi Province, China.

**Additional specimens examined:** China. Jiangxi Province: Shangrao City, Yanshan County, Wuyishan Nature Reserve, Huanggangshan, alt. 1300 m, 31 July 2021 WYS083 and alt. 1650 m August 23, 2021, WYS277 (HFJAU10002; HFJAU10004).

**Discussion**

*Retiboletus huanggangensis* is characterized by the white to grayish black to black pileus, white to pale yellow to black stipe, white to pale yellowish white hymenophore, white to grayish white context. It generally shares the same colored pileus and hymenophore with *R. nigerrimus* (R. Heim) Manfr. Binder & Bresinsky and *R. ater* Hai Y. Liu, Yan C. Li & Tolgor Bau. However, *R. ater* can be easily differentiated by its context unchanging when injured (Liu et al. 2020). *Retiboletus huanggangensis* has slightly longer basidiospores than *R. ater* (8–10.5 × 3–4.5 µm), and slightly wider basidia (26–38 × 6–10 µm). *Retiboletus nigerrimus* differs from *Retiboletus huanggangensis* by having a distinctive blue tinge on the pileus, a lemon yellow pileal context and longer basidiospores (11.5–14.5 × 3.6–4.6 µm) (Heim 1963).

In the phylogenetic analysis (Fig. 1), the materials of *R. huanggangensis* from Jiang xi, China clustered with Japanese and southwestern China collections named “*R. fuscus*” (Hongo 1974). And formed a large clades with *R. ater*, *R. griseus*, *R. nigerrimus*, *R. nigrogriseus*, *R. pseudogriseus*, *R. sinogriseus* and *R. zhangfeii*. However, *R. fuscus* has narrower basidia (21–30 × 7–8 µm), and pileipellis much narrower (100–120 µm) (Zeng et al. 2016). *R. griseus* has grayish Pileus, stipe with yellow tinge, and known from North/Central America (Zeng et al. 2016). *R. nigrogriseus* has shorter stipe (4.5–6 × 1–2.5 cm) and basidia (26–31 × 6–9 µm), context white, staining brownish to fuliginous when injured (Zeng et al. 2018). *R. pseudogriseus* has wider pileus (5.5–9 cm), context white, changing brown when injured, has shorter stipe (6–7 × 1–1.5 cm) and slightly narrower basidia (23–29 × 8–10 µm), and pileipellis thick than *R. huanggangensis* (Zeng et al. 2016). *R. sinogriseus* has grayish-brown to brown pileus, always cracked into small squamules on grayish to whitish background, has shorter and narrower stipe (6–8 × 1.1–1.5 cm), and pileipellis thinner than *R. huanggangensis* (Liu et al. 2020). *R. zhangfeii* has wider pileus (5–10 cm), dark purple when young, context gray-white, changing brown to blackish brown when injured (Zeng et al. 2016).

**Declarations**

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**Figures**

**Figure 1**

Maximum likelihood phylogenetic tree of *Retiboletus* inferred from the combined nuclear dataset (nrLSU+*TEF1*-α*+RPB2*). Bootstrap frequencies ≥ 50% and posterior probabilities ≥ 0.95 are shown above supported branches. Newly sequenced collections are boldfaced in black. Species vouchers are provided after the species name.
Figure 2

Habitat of the new *Retiboletus* species. A-B: HFJAU10004. C: HFJAU10002. D: HFJAU10003. Bars = 2 cm. Photos by Fan Zhou.
Figure 3

Microscopic features of *R. huanggangensis* (HFJAU10003). a: Basidiospores. b: Basidia. c: Cheilo- and Pleurocystidia. d: Pileipellis. e: Stipitipellis. Bars = 10 μm. Drawings by Fan Zhou.