Morphological and Phylogenetic Analyses Reveal a New Species of Anthracophyllum (Omphalotaceae, Agaricales) in Zhejiang Province, China

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Abstract

During the investigations of macrofungi resources in Zhejiang Province, China, an interesting wood rot fungus was collected. Based on morphological and molecular phylogenetic studies, it was described as a new species *Anthracophyllum sinense*. *A. sinense* is characterized by its sessile, charcoal black and pleurotoid pileus; sparse, occasionally branches lamellae; oval basidiospores measuring (8–)9–11.2–13(–14) µm × (5–)6–6.6–8(–9) µm, with obvious spore tips; clavate basidia with obvious sterigmata; and non-heteromorphous cystidium. *A. sinense* established a separate lineage that was similar to *A. archeri* and *A. lateritium* in the phylogenetic tree.

Introduction

Anthracophyllum was established by Cesati (1879) based on the material collected from the Peradeniya Royal Botanic Gardens (Pegler and Young 1989) typified by *A. beccarianum* Ces (Cesati 1879; Pegler & Young 1989; Segedin 1994). *Anthracophyllum* is characterized by dark purplish red or black pileus surface with obvious sulcato-striate; sparse lamellae, sometimes intertwined; rudimentary or absent stipe; thin context; ovo-ellipsoid, subglobose spores; heteromorphic or sterile lamella-edge; coralline or diverticulate, hyphoid and usually branched cheilocystidia (Pegler and Young 1989; Segedin 1994).

Subsequently, two new taxa, i.e. *A. dusenii* Henn (1900) and *A. hasselmannii* Rick (1936), were added to *Anthracophyllum*. Pegler and Young (1989) monographed the genus and they recognized and accepted 8 species, and these species can be readily separated into three regions on geography: palaeotropical (*A. nigritum*, *A. melanophyllum*); neotropical and south American (*A. andinum*, *A. berteroi*, *A. discolor*, *A. laterifium*, *A. paxilloides*), and Australasian (*A. archeri*). In the monograph, *A. proximum* was combined to *Marasmiellus rawakensis*. The current name of *A. berteroi* was *Geoglossum berteroi* (Mont.) Colenso (1887) in the Index Fungorum (https://www.indexfungorum.org/, accessed on 24 Feb 2023). Segedin (1994) reported a new species, *A. pallidum*, and combined *Xerotus glaucophyllus* to *A. glaucophyllum*. So far, 11 species of *Anthracophyllum* have been accepted (Samarakoon et al. 2020).

So far, only two species of *Anthracophyllum*, i.e *A. lateritium* (Moncalvo et al. 2002)d *archeri* (Matheny et al. 2007) were studied on molecular phylogeny (Li et al. 2021; Wang et al. 2021).

Materials And Methods

Specimens collection

Fresh samples were collected from Songkengkou in Zhou Village, Jiangshan City, Zhejiang Province, China (E118°37'11", N28°17'46"; altitude 587 meters). Habitat photos were taken and macro characteristics were recorded following the method described by Liao et al. (2018). The macroscopic characteristics were described, and the samples were dried in an electric dryer at 41°C for 24 hours (until the samples were dried), and an appropriate amount of silica gel particles were placed during storage to prevent moisture regain (Yang and Feng 2013; Zhou 2021). The specimens were deposited in the Fungal Herbarium of Jiangxi Agricultural University (HFJAU).

Macroscopic And Microscopic Studies

The macroscopic morphological characteristics mainly come from the on-site records and live photos of basidiomata. Color code complied with Kornerup and Wanscher (1981). The microscopic morphology study is based primarily on the rejuvenation of dry specimen materials in 5% aqueous potassium hydroxide (KOH) aqueous solution and dyeing with 1% ammoniacal Congo red solution. Freehand sections were done using a Nikon SMZ1270 stereomicroscope, following the standard method described in previous studies (Li et al. 2011; Zeng et al. 2012; Hosen et al. 2013; Zeng et al. 2013; Zhou et al. 2022). Microstructures were observed with a Nikon Y-TV55 compound microscope.

The dimensions of the microscopic features are presented in the listed below format: the number of measured basidiospores is given as [n/m/p], which refers to n basidiospores measured from m basidia collected from p places (Yang and Feng 2013; Raghoonundon et al. 2021). Dimensions of basidiospores are given as (a–)b–c–d(– e), where c represents the average, the range b–d represents a minimum of 90% of the measured values (5th to 95th percentile), and extreme values (a and e), whenever present (a < 5th percentile, d > 95th percentile), are in parentheses (Yang and Feng 2013; Raghoonundon et al. 2021). Q is the length/width ratio for the spores, Q_m refers to the average Q of basidiospores ± sample standard deviation (Yang and Feng 2013; Zhou et al. 2022); and other measurements are presented in the same format (Raghoonundon et al. 2021; Zhou et al. 2022).

Dna Extraction, Amplification, And Sequencing

DNA was extracted from the dried specimens (HFJAU12000) with the Hexadecyltrimethy Ammonium Bromide (CTAB) method (Doyle and Doyle 1987; Huang et al. 2000). Two gene regions, the internal transcribed spacer (ITS) region and the largest subunit nuclear ribosomal RNA (LSU), were amplified using the primer pairs ITS1/ITS4, LR0R/LR5 (Vilgalys and Gonzalez 1990). The PCR reaction in this study was 25 μL reaction system (Table 1) (Zhou 2021), the primers and their sequences used for the two target genes are shown in Table 2 (Zhou 2021). PCR amplification was performed using the following thermocycling conditions: pre-denaturation at 94°C for 3 min and denaturation at 94°C for 30 s, annealing at 55°C for 50 s, extend at 72°C for 1 min, the total number of cycles from step denaturation to extension is 39 cycles at 72°C, and a final extension of 10 min at 72°C, finally, keep at 12°C (Hu et al 2012, Yang and Feng 2013). PCR products were detected in 1% agarose gels, and then sent to TSINGKE Biotechnology for both directions sequencing (Zhou et al. 2022). The primers used for sequencing were the same as those for PCR amplification.

Table 1
The reaction system used in this study.

Designation	Dosage (μL)
DNA template	1
Forward primer	1
Reverse primer	1
2 × M5 HiPer plus Taq HiFi PCR mix	12.5
Nuclease-free ddH_2O	9.5

Table 2
Amplification primers information used in this study.

Gene	Primer	Primer sequence (5′-3′)	References
ITS	ITS1	TCCGTAGGTGAACCTGCGG	Gardes and Bruns 1993
	ITS4	TCCTCCGCTTATTGATATGC	White et al. 1990
LSU	LR0R	ACCCGCTGAACTTAAGC	Vilgalys and Hester 1990
	LR5	TTAAAAAGCTCGTAGTTGAAC	Hopple and Vilgalys 1999

Sequence Alignment And Phylogenetic Analyses

Sequences were visualized and edited with BioEdit v7.0.9 (Hall 1999) and submitted online to NCBI website for Nucleotide BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.CGI) to determine which genus the species belongs to (Zhou et al. 2022). According to the BLAST results, all available sequences of and closely related to *Anthracophyllum*s were downloaded from

GenBank, as shown in Table 3. MAFFT version 7 online analysis of data sets was used for all genes (https://mafft.cbrc.jp/alignment/server/) (Katoh and Standley 2013), and Bioedit v7.0.9 (Hall 1999) was used to perform sequence cutting. Finally, in MEGA7 v7.0.26, two genes of each specimen were synthesized into a complete sequence in the order of ITS-LSU and integrated into a FASTA file (Zhou 2021). Then the data sets were analyzed by RAxML version 8 (Stamatakis 2014) for Maximum likelihood (ML) and PhyloSuite v1.2.2 (Zhang et al. 2020) for Bayesian Inference (BI), respectively.

For phylogenetic analysis, the data set was evaluated with Mrmodeltest 2.3 (Nylander 2004), and the results showed that GTR + I + G was the best fitting model for the data set. Statistical support was calculated using 1000 repetitions of nonparametric bootstrapping (Yang and Feng 2013). Bayesian Inference phylogenies were inferred using MrBayes 3.2.6 (Ronquist et al. 2012) under partition model (2 parallel runs, 2000000 generations), in which the initial 25% of sampled data were discarded as burn-in. Read a total of 40002 trees in 2 files (sampling 30002 of them) (Each file contained 20001 trees of which 15001 were sampled) (Ronquist et al. 2012).

Results

Molecular phylogenetic results

The dataset comprised 41 taxa retrieved from GenBank, *Marasmius curreyi* (BRNM 714676), *Marasmius oreades* (PBM 2701), *Moniliophthora perniciosa* (CMR UB 2041) were selected as outgroup (Table 3). Partial nucleotide sequences of ITS (1039 bp), and LSU (879 bp), with 1918 characters, including gaps, were used to determine the phylogenetic placement of the new taxon. The generated ML and Bayesian trees were similar in topology, and the best scoring ML tree is presented in (Fig. 1).

Table 3
Specimens used in molecular phylogenetic study and their GenBank accession numbers.

Species	Voucher/Culture	GenBank accession numbers		Origin	References
		ITS	LSU		
Anthracophyllum archeri	TFB3511_TENN50049	DQ444308	—	Australia	Mata et al. 2007
A. lateritium	TFB4043_TENN50256	DQ444309	—	USA	Mata et al. 2007
A. lateritium	TENN62043[H]	FJ596891	—	USA	Hughes et al. 2009, Unpublished
A. lateritium	AFTOL-ID 973	DQ404387	AY745709	—	Koch et al. 2018
A. sinense	**HFJAU12000**	**ON711250**	**ON711248**	**China**	**This study**
A. sinense	**TBY2021-8-13**	**OL998876**	**—**	**—**	**Unpublished**
C. filamentipes	TENN F-065861 [T]	NR_174048	—	USA	Petersen and Hughes 2021
C. hasanskyensis	TENN-F-060730 [T]	MN897829	—	Russia	Petersen and Hughes 2021
C. polygramma	URM 90015	KY074640	KY088275	Brazil	Unpublished
C. ramealis	TENN F-065145 [E]	NR_174898	—	Belgium	Unpublished
Gymnopanella nothofagi	SGO 163625 [T]	NR_158479	—	Korea	Antonín et al. 2014
Gymnopus brunneiniger Cesar49	Cesar49 [T]	MT232389	MW187070	Mexico	César et al. 2020
G. cremeostipitatus	BRNM: 747547 [T]	NR_152898	NG_060646	Korea	Antonín et al. 2014
G. dryophilus	DUKE 193411	JX536153	—	Sweden	Antonín et al. 2013
Lentinula aciculospora	TENN 56421 [T]	AY016443	—	Costa Rica	Mata et al. 2001
L. boryana	548	OM400526	—	Colombia	Unpublished
L. detonsa	TENN53824	MW508935	—	Costa Rica	Unpublished
L. madagasikarensis	BB06.007 [T]	MW810301	MW810299	Madagascar	Looney et al. 2021
Marasmiellus bicoloripes	CAL1524 [T]	KY807129	KY817233	India	Unpublished
M. boreoorientalis	LE 323323 [T]	MN597452	MN597444	Russia	Unpublished
M. celebanticus	TO HG2281 [T]	JF460781	—	Spain	Perez-De-Gregorio et al. 2011
M. griseobrunneus	CAL 1752 [T]	MK660191	MK660192	India	Sharafudheen and Manimohan 2019
M. istanbulensis	KATO fungi 3596[T]	KX184795	KX184796	Belgrade	Sesli et al. 2017
M. micromphaleoides	TENN F-68165 [T]	KJ416243	KY019645	USA	Petersen and Hughes 2014

Sequences obtained in this study are shown in bold. **T** = holotype, **E** = epitype, **H** = haplotype

Species	Voucher/Culture	GenBank accession numbers		Origin	References
Marasmius curreyi	BRNM 714676	FJ936152	FJ917614	Madagascar	Antonín and Buyck 2006
M. oreades	PBM 2701	DQ490641	—	USA	Matheny et al. 2006
Moniliophthora perniciosa	CMR UB 2041	AY317136	—	Brazil	Arruda et al. 2005
Neonothopanus gardneri	SP:416340	JF344713	JF344714	Brazil	Chew et al. 2015
N. hygrophanus	HMJAU:48223	MW298685	MW250230	Ghana	Hu et al. 2021
N. nambi	ACL251	KJ206982	KJ206956	Malaysia	Chew et al. 2015
Omphalotus flagelliformis	HKAS:76645	KC333363	—	China	Yang and Feng 2013
O. illudens	DMB006 (TENN)	MF773590	—	USA	Unpublished
O. japonicus	CBS 374.51	MH856905	MH868427	Japan	Unpublished
O. nidiformis	CBS 323.49	EU424307	EU365662	Malaysia	Chew et al. 2015
Paramycetinis austrobrevipes	TENN F-50135 T	NR_171220	—	Australia	Petersen and Hughes 2016
P. caulocystidiatus	TENN F-54050 T	NR_171221	—	New Zealand	Petersen and Hughes 2016
Pseudomarasmius efibulatus	TENN-F-056187 T	MK268234	—	New Zealand	Petersen and Hughes 2020
P. glabrocystidiatus	BRNM 718676 T	KF251073	KF251093	Korea	Antonín et al. 2013
Rhodocollybia olivaceogrisea	JLM 2175	KT205399	—	Costa Rica	Mata et al. 2016
R. tenuipes	TENN59546	AY313288	—	Dominican Republic	Unpublished
R. utrorensis	LAH35478 T	MH220536	—	Pakistan	Sattar et al. 2018

Sequences obtained in this study are shown in bold. **T** = holotype, **E** = epitype, **H** = haplotype

The phylogenetic tree demonstrated that the new taxon (*A. sinense* HFJAU12000 and TBY2021-8-13) formed a unique branch with strong bootstrap support (MLB = 100, BPP = 1.00), which is a sister branch to *A. lateritium* (TENN62043 and TFB4043_TENN50256) with a high statistical support (BS = 86%, PP = 0.96). All the species of *Anthracophyllum* clustered in a clade with high support (BS = 100%, PP = 1).

Taxonomy

Anthracophyllum sinense, W.J. Yang, H.Y. Song & D.M. Hu, sp. nov.

MycoBank: 844465

Figure: 2 and 3

Habitat basidiomata grow on dead branches of rotten wood.

Etymology

Latin "*sinense*" means China, referring to the collection from China.

Distribution

Zhejiang Province, China.

Ecology

Clustered or solitary on dead trees under deciduous broad-leaved mixed forest.

Description

Basidiomata gregarious to caespitose, small, pleurotoid. *Pileus* (1.5–)2.0–4.0 cm diam, sessile, flabelliform, or orbicular, convex to applanate, smooth, radially rugose, irregularly radially sulcate, non-viscid, surface brown (8F6), dark brown (6F6) to black (8F1), shell-pink (8A3) to peach (7A4) when young. The edge is nearly wavy, margin often downcurved, complete and smooth. *Lamellae* radiating from a basal point, subventricose, dark brown (6F4), black (8F1), unequal in length, medium width, sparse, with 2–4 through-lamellae and 2–4 lamellulae, edge concolorous, occasionally branches, breakable when dry. *Lamellae* gaps or inner surface with black carbonaceous particles. *Stipe* rudimentary or absent. *Context* thin 0.5–1 mm, brick-red (6E7), consisting of firmly woven and branching hyphae, 2–5 µm diam which can expand to 4–7 µm, hyaline and smooth, blue-green in KOH, and the pigment occurs as patches or only as minute specks of green, scattered through the context; clamp-connexions prominent.

Basidiospores medium to large, [60/1/1] (8–)9–11.2–13(–14) µm × (5–)6–6.6–8(–9) µm, Q = (1.25–)1.38–1.72–2.2(–2.33), Q_m = 1.72 ± 0.31, n = 60, subglobose to broadly ovoid, ellipsoid with a prominent hilar appendix, hyaline, with conspicuous oleaginous contents, with pale brown contents or staining pale brown, amyloid, thin-walled, staining in KOH. *Spore-print* not obtained. *Basidia* (30–)32–38–43(–45) µm × (6–)7–9.2–11(–12) µm, clavate, some with oleaginous contents, tetrasporic. *Sterigmata* (3–)5–6.1–7(–8) µm × 1–1.5(–2) µm. *Lamella-edge* sterile, rarely fertile, scattered to crowded, conspicuous cheilocystidia. *Cheilocysfidia* (23–)26–34.4–46(–51) × (5–)7–8.8–11(–12) µm, hyphoid cylindric to subfusoid, clavate, fusiform, with oleaginous contents, hyaline, thin-walled. *Pleurocystidia* none or scattered and similared to cheilocystidia. Hyphoid pleurocystidia are common, hyaline and thin-walled, and irregularly clavate. *Basidioles* are often abundant, 20–30 µm × 5–8 µm, cylindric, cylindrico-clavate, thin-walled, with an obtusely rounded apex. *Hymenophoral trama* irregular, with woven hyphae hyaline, (3–)4–5 µm diam, slightly inflating to 5–8 µm diam, blue-green in alkaline solution. The clamp-connexions are apparent, and irregular small branches or protrusions can be seen occasionally. *Subhymenial layer* is tightly woven, 9–14 µm broad. *Pileipellis* is a well-developed semierect hypha, forming a prominent Rameales-structrue. *Hyphae* 2–5 µm dime, slightly inflating to 4–7 µm arborization, clavate, irregular, smooth, colorless, with horizontal septum and affluent branches. Hyphae tightly interwoven, thin-walled or with a slightly thickened wall, hyphae terminations inflate subglobose, stain blue-green in KOH solution. *Clamp-connexions* numerous and prominent. Brown pigment soluble in alkaline solution.

Discussion

Morphologically, *A. sinense* conforms to the characteristics of *Anthracophyllum* (Pegler and Young 1989). It has pleurotoid pileus, (1.5–)2.0–4.0 cm diam, sessile; sparse lamellae, occasionally branches; broadly ovoid basidiospores (8–)9–11.2–13(–14) µm × (5–)6–6.6–8(–9) µm, with obvious hilar appendix; the basidium has conspicuous sterigmata. Compared with *A. sinense*, the type species *A. melanophyllum* (≡ *A. beccarianum*) has smaller pileus (1.0–3.0 µm), denser lamellae (9–12 through-lamellae), bigger basidia (35–45 × 8–11 µm) and narrower ovoid-ellipsoid spores (8.5–11×6–7.5 µm) which have refractive contents. It has obvious morphological differences with others of *Anthracophyllum*.

Key to the species of *Anthracophyllum*

1. Pileus flabelliform to semicircular, convex to applanate……2

1. Pileus pyriform, plicato-striate, 0.5–2 cm diam, spores subglobose, broadly ovoid, hilar appendix obvious, 8–14 × 5–9 µm……*A. paxilloides*

2. Pileus surface rugulose, irregularly radially sulcate……3

2. Pileus surface smooth, spores hyaline or with pale brown contents, 35–45 × 8–11 µm……*A. archeri*

3. Pileus over 1 cm diam……4

3. Pileus less than 1 cm diam, spores ovoid to elliptical, 8–14 × 8–10 µm……*A. pallidum*

4. Stipes small……5

4. Stipes rudimentary or absent, sessile……6

5. Spores ovoid-ellipsoid, hilar appendix obvious, spores ovoid-ellipsoid, hilar appendix obvious, 6–8 ×5–7 µm……*A. dusenii*

5. Spores elongate ellipsoid, subglobose, broadly ovoid, hilar appendix not obvious……7

6. Spores ellipsoid to ovoid-ellipsoid……8

6. Spores subglobose, broadly ovoid, hilar appendix obvious, spores subglobose, broadly ovoid, hilar appendix obvious, 8–14 × 5–9 µm……*A. sinense*

7. Spores less than 5 µm long, dark brown, 3 × 1 µm……*A. hasselmannii*

7. Spores over 5 µm long……9

8. Basidia less than 35 µm long, spores oblong ellipsoid, 6.5–9.5 × 3.5–5 µm……*A. nigritum*

8. Basidia over 35 µm long……10

9. Lamellae less than 5 through-lamellae, spores ovo-ellipsoid, 7–10 × 4.5–7 µm……*A. glaucophyllum*

9. Lamellae over 5 through-lamellae……11

10. Spores contents with much refractile granules, spores ovoid-ellipsoid, 8.5–11 × 6–7.5 µm……*A. melanophyllum*

10. Spores with pale reddish brown contents, spores ellipsoid, 8.5–11× 5.5–6.5 µm……*A. discolor*

11. Stipes 0.5–1 mm long, spores elongate ellipsoid, 9.5–15 × 5.5–8 µm……*A. lateritium*

11. Stipes 4–12 mm long, spores subglobose to broadly ovoid, 11.5–16 × 10–13 µm……*A. andinum*

In phylogenetic analyses, our specimens and TBY2021-8-13 (unpublished sequence from GenBank) formed an independent clade with strong support (BS = 100%, PP = 1), and closed to *A. lateritium* (TENN62043 and TFB4043_TENN50256) with high statistical support (BS = 86%, PP = 0.96). *A. lateritium* is characterized by dense lamellae with 9–12 through-lamellae, hyaline

basidiospores, and obvious stipes (0.5–1.0 mm) (Pegler 1987), which differs *A. sinense*. *A. sinense* is also closed to *A. archeri* (AFTOL-ID 973 and TFB3511_TENN50049) in the phylogentic tree (Figure 1). However, *A. archeri* can be easily distinguished from *A. sinense* by its mooth pileus, sparse lamellae with 5–9 through-lamellae, obvious stipes (4.0 mm), and loose Rameales-structure in pileipellis (Segedin 1994; Pegler 1965; Zhou et al. 2022).

Thus far, only *A. lateritium* and *A. archeri* of *Anthracophyllum* have been included in molecular phylogentic studies (Mata et al. 2007, Hughes et al. 2009, Koch et al. 2018). In order to enrich the research diversity of species in *Anthracophyllum*, it is suggested that the taxonomic research of macrofungi should adopt a method combining molecular and morphological analyses to determine the taxonomic status of species and to improve the efficiency and accuracy of the taxonomic study.

The high biodiversity of wood-decaying fungi is one of the important factors for the health of forest ecosystem. *Anthracophyllum* is an essential component of wood-decaying fungi resources and a vital biological resource. Therefore, it has excellent application potential and is worthy of further exploration.

Declarations

Author contribution

Conceptualization, W-J Y, D-M H and H-Y S; Data curation, W-J Y, Q-G C; Formal analysis, W-J Y, F Z, Q-G C, Z- L; Investigation, W-J Y, F Z, Z-H J, Q-G C; Methodology, Y-G, Z-H J, G-J L; Supervision, H-Y S, D-M H, H-Y S, W-J T, Z-F L and M-X; Visualization, W-J Y, F Z; Writing–original draft preparation, W-J Y; Writing–review and editing, W-J Y, H-Y S, D-M H, J Z and J W. All authors have read and agreed to the published version of the manuscript.

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Figures

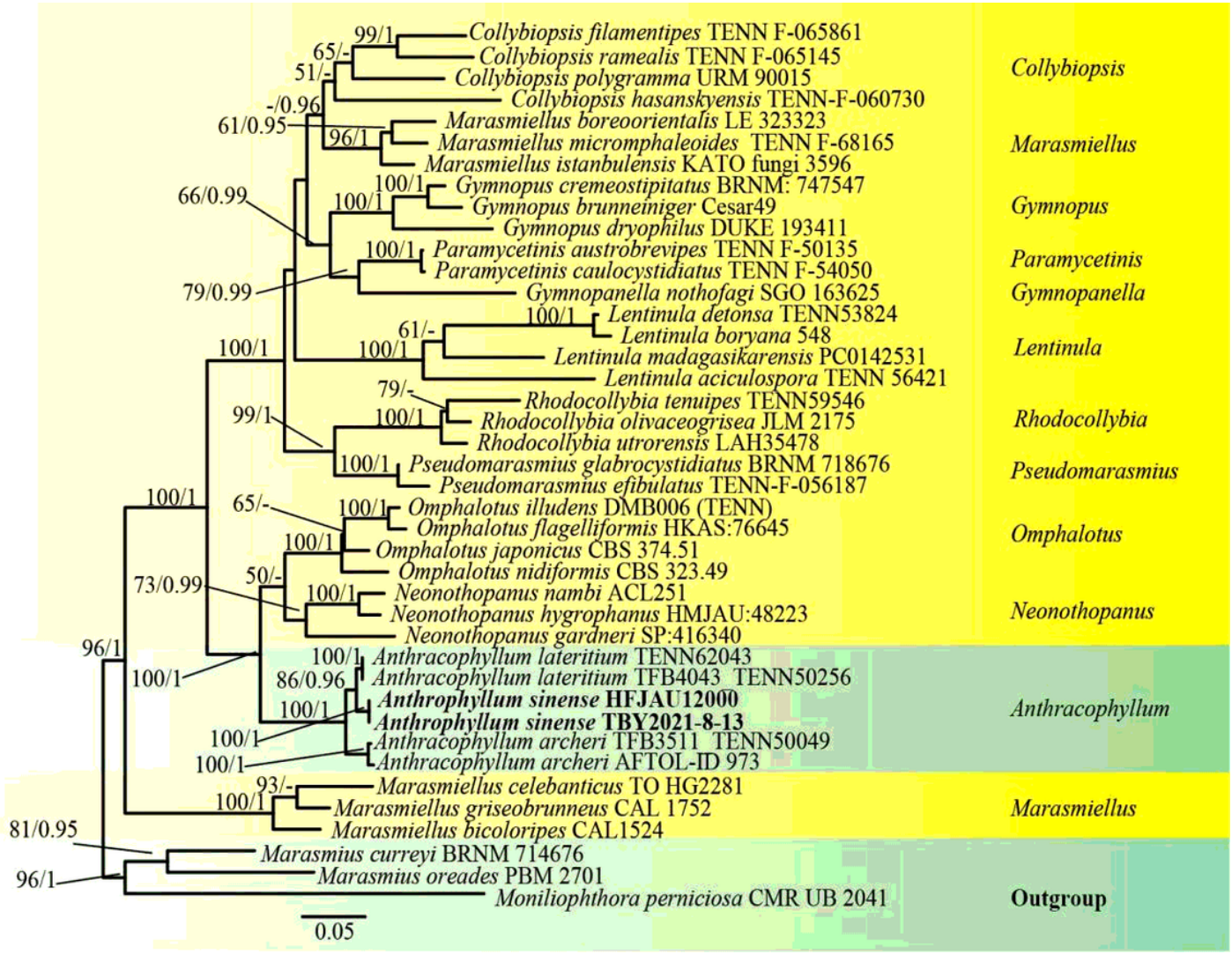

Figure 1

Maximum likelihood phylogenetic tree of *Anthracophyllum* inferred from the combined nuclear dataset (ITS + nrLSU). Maximum Likelihood Bootstrap (MLB, left) ≥ 50% and Bayesian Posterior Probabilities (BPP, right) ≥ 0.95 are shown above supported branches. New species are shown in bold.

Figure 2

Photograph of *Anthracophyllum sinense* sp. nov. (Holotype: HFJAU12000). a: Basidioma Habitat; b: Basidioma; c: Lamella of Basidioma; d: Young Basidioma. Bars = 1 cm. Photos by Wen-Juan Yang.

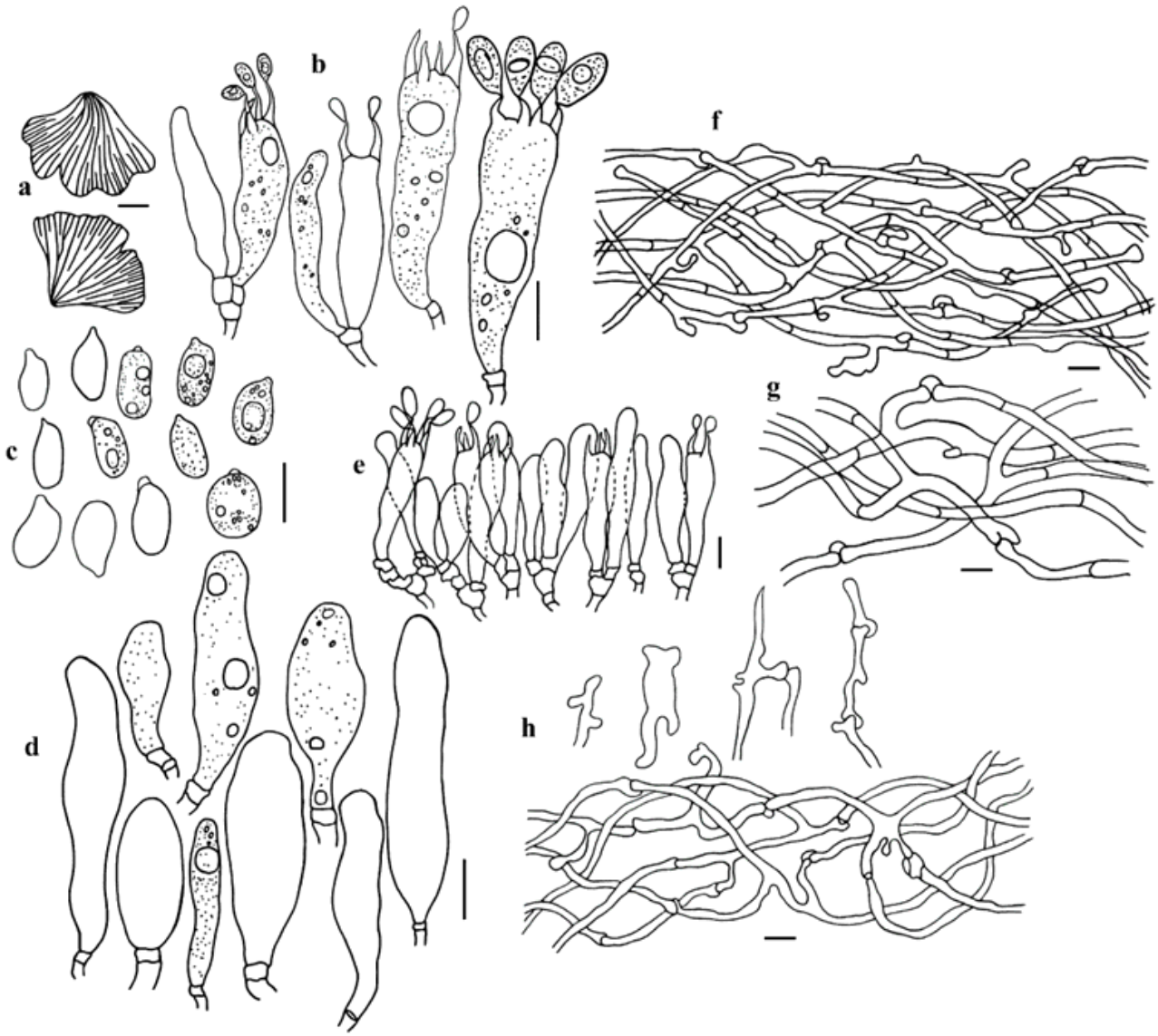

Figure 3

Microscopic features of *Anthracophyllum sinense* sp. nov. a: Basidiomes. b: Basidium. c: Spores. d: Cystidium. e: Hymenophore. f: Hyphoid of context. g: Trama. h: Pileipellis elements. Scale bars: a = 1 cm, b c d = 10 μm, e f g h = 10 μm