

Article

Austroboletus brunneisquamus (Boletaceae, Boletales), a New Ectomycorrhizal Fungus from a Tropical Rainforest, China

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Abstract: *Austroboletus brunneisquamus* (Boletaceae/Boletales), an ectomycorrhizal fungus, is described as a new species from a tropical rainforest in China based on morphological and molecular evidence. It is morphologically characterized by a subtomentose pileal surface when young, which cracks into areolae, having large, pale brown and brown to dark brown scales, a stipe with yellowish brown reticulation, basidiospores measuring (11–)12–14.5(–15) × 6–8(–8.5) μm, with fine cristate to subreticulate ornamentation, and a pileipellis in the form of a cutis. A detailed description, color photographs of fresh basidiomata, and line drawings of microscopic features of the new species are presented.

Keywords: boletes; molecular phylogeny; morphology; new taxon; taxonomy



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1. Introduction

Austroboletus (Corner) Wolfe (Boletaceae, Boletales), typified by *A. dictyotus* (Boedijn) Wolfe, is characterized by a pinkish pore surface at maturity, context usually unchanging in color when injured, a stipe, often with conspicuous reticulation, fusiform or amygdaliform basidiospores with verrucose, warted, irregularly pitted, cristate, subcylindrical, ridged or subreticulate ornamentations, and forming ectomycorrhizae with Fagaceae, Pinaceae and Dipterocarpaceae [1–5]. Species of *Austroboletus* are mostly distributed in tropical regions [6–11]. In China, five taxa of the genus, viz. *A. dictyotus*, *A. fusisporus* (Kawam. ex Imazeki & Hongo) Wolfe, *A. gracilis* (Peck) Wolfe, *A. malaccensis* (Pat. & C.F. Baker) Wolfe and *A. subvirens* (Hongo) Wolfe were reported in previous studies [1,12–15]. Hainan, a tropical area covered mostly by rainforests, is considered as a hotspot of biodiversity, and many new taxa of macrofungi were described from the region in the past [16–25]. With further investigations, more new taxa are expected to be uncovered. During a fieldtrip to the area, we encountered a few collections of *Austroboletus*. Subsequent morphological and molecular phylogenetic analyses confirmed that it is different from all known species of *Austroboletus*, and thus we describe these collections as a new species.

2. Materials and Methods

2.1. Morphological Studies

Collections were described and photographed in the field and deposited in the Fungal Herbarium of Hainan Medical University, Haikou City, Hainan Province of China (FHMU). Color codes are from Kornerup and Wanscher [26]. Sections of the pileipellis were cut radially-perpendicularly and halfway between the center and the margin of the pileus. Sections of

the stipitipellis were taken from the middle part along the longitudinal axis of the stipe. As a mounting medium for microscopic studies, 5% KOH was used. All microscopic structures were drawn freehand from rehydrated material. The number of measured basidiospores is given as $n/m/p$, where n represents the total number of basidiospores measured from m basidiomata of p collections. Dimensions of basidiospores are given as $(a-)b-c(-d)$, where the range $b-c$ represents a minimum of 90% of the measured values (5th to 95th percentile), and extreme values (a and d), whenever present ($a < 5$ th percentile, $d > 95$ th percentile), are in parentheses. Q refers to the length/width ratio of basidiospores; Q_m refers to the average Q of basidiospores and is given with a sample standard deviation [19,20]. Basidiospores from dried specimens were also examined with a FEI Quanta 250 (USA) scanning electron microscope (SEM) [27].

2.2. Molecular Procedures

Total genomic DNA was obtained with the Plant Genomic DNA Kit (KANGWEI Company, China) from materials dried with silica gel according to the manufacturer's instructions. Primer pairs used for amplification were: LR0R/LR5 [28,29] for the nuclear ribosomal large subunit RNA (28S), the nuclear rDNA region encompassing the internal transcribed spacers 1 and 2, along with the 5.8S rDNA (ITS), with ITS5/ITS4 [30], and EF1-2F/EF1-2R [27] for the translation elongation factor 1- α gene (*TEF1*). PCR was performed in a total volume of 25 μ L containing 13 μ L $2 \times$ Taq PCR MasterMix (KANGWEI Company, China), 2 μ L per primer (10 mM), 2 μ L DNA template and 8 μ L nuclease-free water. PCR reactions were performed with 4 min initial denaturation at 95°, followed by 35 cycles of denaturation at 94° for 30 s, annealing at an appropriate temperature (50° for 28S and ITS, 53° for *TEF1*) for 30 s, extension at 72° for 120 s and a final extension at 72° for 7 min. PCR products were checked in 1% (w/v) agarose gels, and positive reactions with a bright single band were purified and directly sequenced using an ABI 3730xl DNA Analyzer (Guangzhou Branch of BGI, China) with the same primers used for PCR amplifications [19,20]. BioEdit [31] was used to compile forward or reverse sequences.

2.3. Dataset Assembly

Five DNA sequences (two of 28S, two of ITS, and one of *TEF1*) from two specimens were newly generated. *Mucilopilus castaneiceps* Hongo was chosen as an outgroup following Wu et al. [1]. To test for phylogenetic conflict among the different genes in the combined dataset, the single-gene phylogenetic trees based on 28S, ITS and *TEF1*, respectively, were analyzed and conducted using the ML method to detect the topologies of genes used. The results of analyses showed that the different gene fragments were not in conflict. Then, the sequences of different genes in the combined dataset were aligned using MUSCLE. The sequences of the different genes were concatenated using Phyutility v. 2.2 for further analyses [19,20] (Table 1).

2.4. Phylogenetic Analyses

The combined nuclear dataset (28S + ITS + *TEF1*) was analyzed with maximum likelihood (ML) and Bayesian Inference (BI). Maximum likelihood tree generation and bootstrap analyses were performed with the program RAxML 7.2.6 running 1000 replicates combined with an ML search. Bayesian analysis with MrBayes 3.1 implementing the Markov Chain Monte Carlo (MCMC) technique and parameters predetermined with MrModeltest 2.3 was performed [19,20]. The best-fit likelihood models for the combined dataset were GTR + I + G, GTR + I + G and SYM + G for 28S, ITS and *TEF1*, respectively. Bayesian analysis of the combined nuclear dataset (28S + ITS + *TEF1*) was repeated for 10 million generations. Trees sampled from the first 25% of generations were discarded as burn-in, and Bayesian posterior probabilities (PP) were then calculated for a majority consensus tree of the retained Bayesian trees [19,20].

Table 1. List of collections used in this study.

Taxon	Voucher	Locality	GenBank Accession No.			References
			28S	ITS	TEF1	
<i>Austroboletus</i> aff. <i>fusisporus</i>	HKAS 53461	Hunan, central China	KF112486	—	KF112214	[32]
<i>Austroboletus</i> aff. <i>rostrupii</i>	G4357	Guyana	KJ786636	—	—	Unpublished
<i>Austroboletus amazonicus</i>	2032 AMV	Colombia	KF714510	KF937309	—	[8]
<i>A. amazonicus</i>	1839 AMV	Colombia	KF714508	KF937307	—	[8]
<i>Austroboletus asper</i>	Perth 06658407	Australia	KP242277	KP242216	—	[4]
<i>A. asper</i>	Perth 8018251	Australia	KP242267	—	—	[4]
<i>A. asper</i>	MEL:2104343	Australia	KP242260	KP242174	—	[4]
<i>A. asper</i>	MEL:2300520	Australia	KP242253	KP242186	—	[4]
<i>A. asper</i>	MEL:2371703	Australia	—	KP242152	—	[4]
<i>Austroboletus austrovirens</i>	BRI:AQ0796003	Australia	KP242228	KP242212	—	[10]
<i>A. austrovirens</i>	BRI:AQ0795791	Australia	KP242225	KP242211	—	[10]
<i>Austroboletus brunneisquamus</i>	N.K. Zeng 4292 (FHMU5875)	Hainan, southern China	MW506828	MZ855494	—	This study
<i>A. brunneisquamus</i>	N.K. Zeng 4294 (FHMU5876)	Hainan, southern China	MW506829	MZ855495	MW512637	This study
<i>Austroboletus dictyotus</i>	HKAS59804	China	JX901138	—	—	[15]
<i>A. dictyotus</i>	HKAS 53450	Hunan, central China	KF112487	—	KF112215	[32]
<i>Austroboletus festivus</i>	FLOR 51599	Brazil	KY888001	KY886202	—	[33]
<i>A. festivus</i>	AMV1941	Colombia	KT724096	—	—	Unpublished
<i>A. festivus</i>	FLOR 51601	Brazil	KY888000	KY886203	—	[33]
<i>A. festivus</i>	AMV1881	Colombia	KT724095	KT724086	—	Unpublished
<i>A. festivus</i>	AMV1800	Colombia	—	KT724085	—	Unpublished
<i>A. fusisporus</i>	HKAS75207	China	JX889720	JX889719	JX889718	[15]
<i>Austroboletus gracilis</i>	112/96	USA	DQ534624	—	KF030425	[34]
<i>A. gracilis</i>	CNV35	—	—	MT345212	—	Unpublished
<i>A. gracilis</i>	NAMA 2017-106	USA	—	MH979242	—	Unpublished
<i>A. gracilis</i>	Mushroom Observer # 310751	Mexico	—	MH167935	—	Unpublished
<i>Austroboletus lacunosus</i>	BRI:AQ0795789	Australia	KP242271	KP242162	—	Unpublished
<i>A. lacunosus</i>	REH9146	Australia	JX889669	—	JX889709	[35]
<i>Austroboletus mucosus</i>	TH6300	Guyana	AY612798	—	—	[36]
<i>Austroboletus mutabilis</i>	BRI:AQ0554121	Australia	KP242241	KP242192	—	Unpublished
<i>A. mutabilis</i>	BRI:AQ0557644	Australia	KP242237	KP242196	—	Unpublished

Table 1. Cont.

Taxon	Voucher	Locality	GenBank Accession No.			References
			28S	ITS	TEF1	
<i>Austroboletus neotropicalis</i>	NY181457	Costa Rica	JQ924334	JQ924301	—	Unpublished
<i>Austroboletus niveus</i>	AD-C 54948	Australia	KP242280	KP242220	—	Unpublished
<i>A. niveus</i>	Perth 6660703	Australia	KP242279	KP242217	—	Unpublished
<i>A. niveus</i>	BRI:AQ0795772	Australia	KP242276	KP242156	—	Unpublished
<i>A. niveus</i>	MEL2053830	Australia	KC552058	KC552016	KC552099	Unpublished
<i>A. niveus</i>	REH9487	Australia	JX889668	—	JX889708	[35]
<i>A. niveus</i>	PDD:105246	New Zealand	—	—	MH594049	Unpublished
<i>Austroboletus novae-zelandiae</i>	TL2578	New Zealand	—	KP191803	—	Unpublished
<i>A. novae-zelandiae</i>	PDD:105097	New Zealand	—	—	MH594051	Unpublished
<i>A. novae-zelandiae</i>	MEL:2370154	Australia	KP242256	KP242175	—	Unpublished
<i>Austroboletus rarus</i>	BRI:AQ0794045	Australia	KP242236	KP242197	—	Unpublished
<i>A. rarus</i>	BRI:AQ0807888	Australia	—	KP242200	—	Unpublished
<i>Austroboletus rionegrensis</i>	INPA 78693	Brazil	—	KY886201	—	[33]
<i>Austroboletus roseialbus</i>	Dodd	Australia	KY872650	KY872653	—	[10]
<i>A. roseialbus</i>	REH10024	Australia	KY872651	KY872652	—	[10]
<i>Austroboletus rostrupii</i>	BRI:AQ0807886	Australia	KP242270	KP242163	—	Unpublished
<i>A. rostrupii</i>	BRI:AQ0796694	Australia	KP242258	KP242179	—	Unpublished
<i>A. rostrupii</i>	TH8189	Guyana	—	JN168683	—	[37]
<i>Austroboletus</i> sp.	HKAS74743	China	KT990527	—	KT990730	[1]
<i>Austroboletus</i> sp.	MEL:2382826	Australia	KP242283	KP242213	—	Unpublished
<i>Austroboletus</i> sp.	BRI:AQ0794271	Australia	KP242259	—	—	Unpublished
<i>Austroboletus</i> sp.	BRI:AQ0794258	Australia	KP242255	KP242182	—	Unpublished
<i>Austroboletus</i> sp.	Perth 8019207	Australia	KP242245	—	—	Unpublished
<i>Austroboletus</i> sp.	BRI:AQ0794222	Australia	KP242234	KP242215	—	Unpublished
<i>Austroboletus</i> sp.	MEL2305143	New Caledonia	KC552060	KC552018	KC552101	Unpublished
<i>Austroboletus</i> sp.	CANB:643163	Australia	—	KP242201	—	Unpublished
<i>Austroboletus</i> sp.	BRI:AQ0794272	Australia	—	KP242159	—	Unpublished
<i>Austroboletus</i> sp.	HKAS 59624	Yunnan, SW China	KF112485	—	KF112217	[32]
<i>Austroboletus</i> sp.	HKAS 57756	Jiangxi, eastern China	KF112383	—	KF112212	[32]
<i>Austroboletus</i> sp.	OR0891	Thailand	—	—	MH614706	[38]
<i>Austroboletus</i> sp.	CM13_006	New Caledonia	—	KY774009	—	[39]
<i>Austroboletus</i> sp.	CY13_008	—	—	KY774008	—	Unpublished
<i>Austroboletus</i> sp.	CYMy36L1	—	—	KY774007	—	Unpublished

Table 1. Cont.

Taxon	Voucher	Locality	GenBank Accession No.			References
			28S	ITS	TEF1	
<i>Austroboletus</i> sp.	LAM 0479	Malaysia	KY091070	—	—	Unpublished
<i>Austroboletus</i> sp.	DD9852	North America	AY612797	—	—	Unpublished
<i>Austroboletus subflavidus</i>	CFMR BZ-3178 BOS-625	Belize	MK601716	—	MK721070	Unpublished; [40]
<i>A. subflavidus</i>	FLAS-F-60635	USA	—	MH016816	—	Unpublished
<i>A. subflavidus</i>	JBSD130771	Dominican Republic	MT580902	MT581525	—	[5]
<i>A. subflavidus</i>	JBSD130772	Dominican Republic	MT580903	MT581526	—	[5]
<i>A. subflavidus</i>	CFMR:DR2859	Dominican Republic	MT580901	MT581523	—	[5]
<i>A. subflavidus</i>	CFMR:DR592	Dominican Republic	—	MT581524	—	[5]
<i>A. subflavidus</i>	CFMR:BZ1824	Belize	—	MT581522	—	[5]
<i>A. subflavidus</i>	CFMR:BOTH-3463	Florida (USA)	MT580900	MT581521	—	[5]
<i>Austroboletus subvirens</i>	OTU1575	Japan	—	MT594990	—	Unpublished
<i>A. subvirens</i>	KPM-NC-0017836	Japan	JN378518	—	JN378458	[41]
<i>A. subvirens</i>	MEL:2382920	Australia	—	KP012789	—	Unpublished
<i>Austroboletus viscidoviridis</i>	REH9993	Australia	—	KY872649	—	[10]
<i>A. viscidoviridis</i>	Perth 7588682	Australia	KP242282	KP242219	—	Unpublished
<i>A. viscidoviridis</i>	BRI:AQ0554020	Australia	KP242243	KP242189	—	Unpublished
<i>Mucilopilus castaneiceps</i>	HKAS50338	China	KT990555	—	KT990755	[1]
<i>M. castaneiceps</i>	HKAS71039	China	KT990547	—	KT990748	[1]

New sequences are shown in bold.

3. Results

3.1. Molecular Data

The combined dataset (28S + ITS + *TEF1*) included 81 taxa with 2225 nucleotide sites, and the alignment was deposited in TreeBASE (S27641). Bayesian analyses resulted in identical topologies to the ML analysis, while statistical supports showed slight differences. Figure 1 is a branch-length phylogram with the support values, and inferred with RAXML. The molecular phylogenetic analyses show that the collections numbered FHMU5875 and FHMU5876 grouped together with a strong statistical support (BS = 99, PP = 1.0), forming an independent lineage within *Austroboletus* (Figure 1).



Figure 1. Phylogram inferred from a combined dataset (28S, ITS and *TEF1*) using RAXML. RAXML likelihood bootstrap (BS $\geq$ 50%) and Bayesian posterior probabilities (PP $\geq$ 0.95) are indicated above or below the branches as RAXML BS/PP.

3.2. Taxonomy

Austroboletus brunneisquamus N.K. Zeng, Chang Xu and S. Jiang, sp. nov. (Figures 2–4).



Figure 2. Basidiomata of *Austroboletus brunneisquamus* ((a) from FHMU5875; (b,c) from FHMU5876, holotype). Photos by Y.G. Fan.

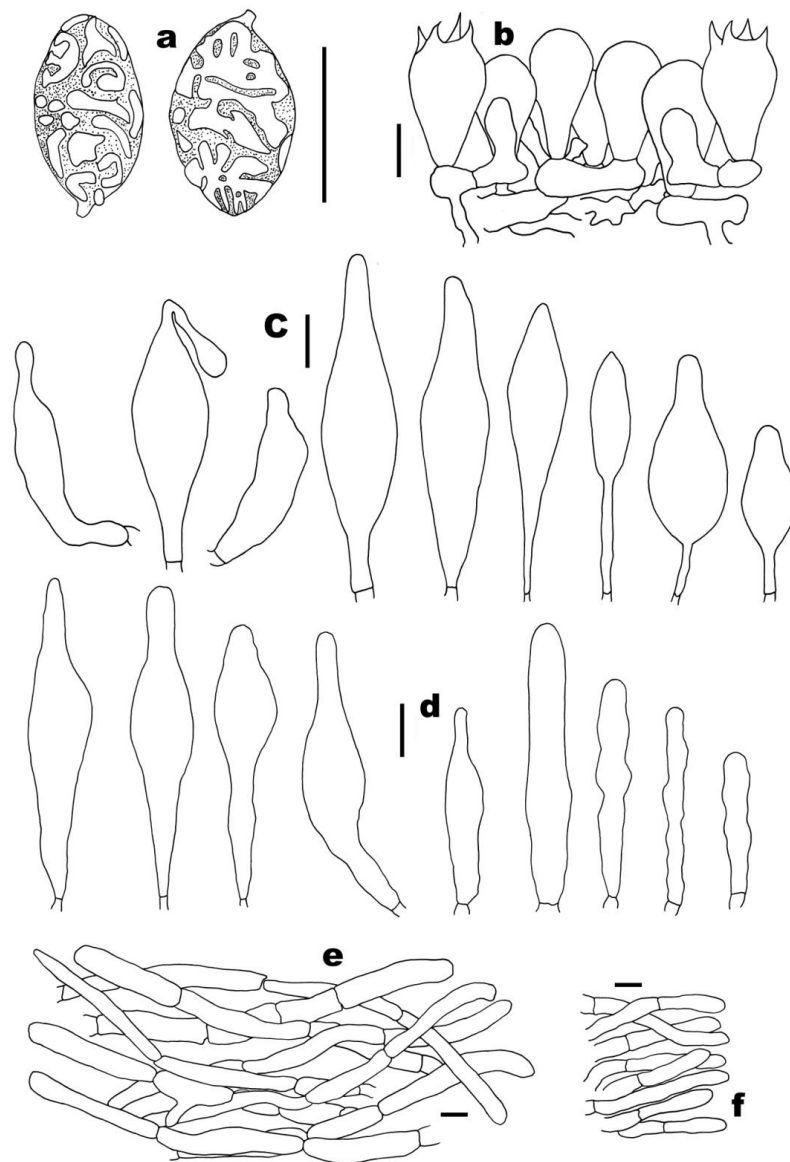


Figure 3. Microscopic features of *Austroboletus brunneisquamus* (FHMU5876, holotype). (a) Basidiospores. (b) Basidia. (c) Cheilocystidia. (d) Pleurocystidia. (e) Pileipellis. (f) Stipitipellis. Bars = 10 μ m. Drawings by C. Xu.

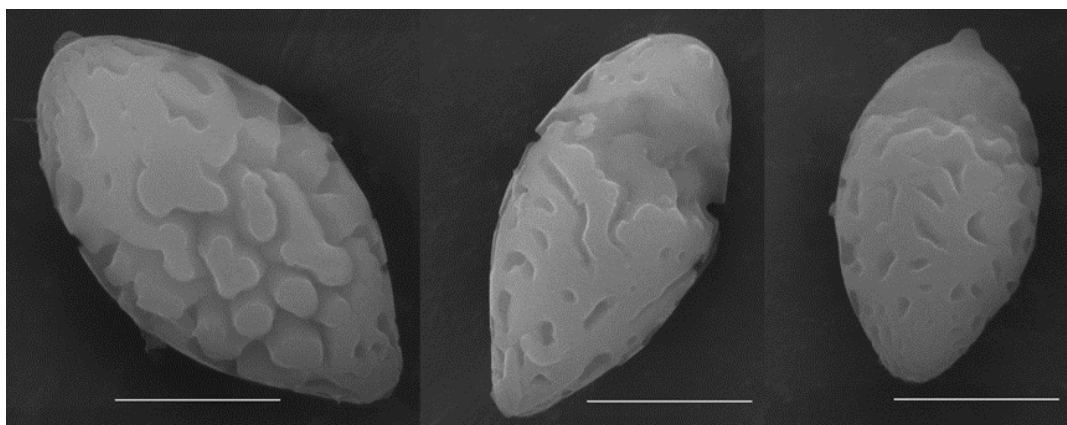


Figure 4. SEM of basidiospores from a dried specimen of *Austroboletus brunneisquamus* (FHMU5876, holotype). Bars = 5 μ m.

Mycobank: MB838577

Etymology—Latin “*brunneisquamus*” refers to the pileus of new species displaying distinctive brown scales.

Diagnosis—Characterized by a subtomentose pileal surface when young, which cracks into areolae, with large, pale brown and brown to dark brown scales, a stipe with yellowish brown reticulation, basidiospores measuring $(11\text{--}12\text{--}14.5\text{--}15) \times 6\text{--}8\text{--}(8.5) \mu\text{m}$, with fine cristate to subreticulate ornamentation, and a cutis in form of pileipellis.

Holotype—CHINA. Hainan Province: Ledong County, Yinggeling of Hainan Tropical Rainforest National Park, $109^{\circ}17'17''$ E, $18^{\circ}51'6''$ N, elev. 650 m, 2 July 2020, N.K. Zeng4294 (FHMU5876). GenBank accession numbers: 28S = MW506829, ITS = MZ855495, TEF1 = MW512637.

Description—*Basidiomata*: small to medium-sized. *Pileus*: 3–6.5 cm in diam, sub-hemispherical when young, then convex to applanate; margin: decurved; surface: dry, subtomentose when young, which cracks into areolae, with large, pale brown (3C7–8) and brown to dark brown (5E7–8) scales; context: 0.5–1.2 cm thick in the center of the pileus, white (1A1), unchanging in color when injured. *Hymenophore*: poroid, depressed around the stipe; pores: subrounded, white (1A1) when young, then pink (8A2), unchanging in color when injured; tubes: 0.6–1.5 cm in length, pinkish (9A2), unchanging in color when injured. *Stipe*: $4.5\text{--}9 \times 0.7\text{--}1.4$ cm, central, subcylindrical, solid; surface: distinctly covered with a yellowish brown (4B5–6) reticulation; context: white (1A1), unchanging in color when injured; basal mycelium: white (1A1). *Odor*: indistinct.

Basidiospores: $[60/3/3] (11\text{--}12\text{--}14.5\text{--}15) \times 6\text{--}8\text{--}(8.5) \mu\text{m}$, $Q = (1.57\text{--})1.60\text{--}2.00\text{--}(2.17)$, $Q_m = 1.80 \pm 0.14$ (including ornamentation), $[60/3/3] (9\text{--}11\text{--}13.5\text{--}14.5) \times 4\text{--}6\text{--}(6.5) \mu\text{m}$, $Q = (1.8\text{--})1.92\text{--}2.78\text{--}(3.25)$, $Q_m = 2.37 \pm 0.29$ (excluding ornamentation), fusiform or amygdaliform, with a fine cristate to subreticulate ornamentation, pale yellowish brown in KOH. *Basidia*: $18\text{--}26 \times 11\text{--}15 \mu\text{m}$, broadly clavate, thin to slightly thick-walled (up to $0.7 \mu\text{m}$), 4-spored, colorless in KOH; sterigmata: 4–6 μm long. *Cheilocystidia*: $27\text{--}54 \times 7\text{--}14 \mu\text{m}$, abundant, fusiform or subfusiform, slightly thick-walled (up to $0.9 \mu\text{m}$), colorless in KOH. *Pleurocystidia*: $27\text{--}62 \times 4\text{--}12 \mu\text{m}$, mostly fusiform, occasionally subclavate or subcylindrical, slightly thick-walled (up to $0.9 \mu\text{m}$), colorless in KOH. *Pileipellis*: a cutis up to 315 μm thick, composed of cylindrical hyphae 6.5–13 μm wide, occasionally branched, slightly thick-walled (up to $0.9 \mu\text{m}$), colorless in KOH; terminal cells: $44\text{--}64 \times 6\text{--}10.5 \mu\text{m}$, subcylindrical, with obtuse apex. *Pileal trama*: made up of hyphae 4–12 μm in diam, slightly thick-walled (up to $0.9 \mu\text{m}$), colorless in KOH. *Stipitipellis*: a trichoderm-like structure, 70–105 μm thick, composed of slightly thick-walled (up to $0.9 \mu\text{m}$) hyphae, 5.5–7.5 μm wide, colorless in KOH; terminal cells: $24.5\text{--}53 \times 6\text{--}6.5 \mu\text{m}$, clavate, subclavate or subcylindrical. *Stipe trama*: composed of parallel hyphae, 4–15 μm wide, cylindrical, slightly thick-walled (up to $0.9 \mu\text{m}$), colorless in KOH. *Clamp connections*: absent in all tissues.

Habitat—solitary on the ground in tropical rainforests dominated by fagaceous trees.

Known distribution—Southern China (Hainan Province).

Additional specimens examined—CHINA. Hainan Province: Ledong County, Yinggeling of Hainan Tropical Rainforest National Park, elev. 650 m, 2 July 2020, N.K.Zeng4291, 4292 (FHMU5874, 5875).

4. Discussion

In the present study, our newly collected specimens were placed into the genus *Austroboletus* with high statistical support (Figure 1), and morphological features of *A. brunneisquamus* are highly compatible with the typical characteristics of the genus.

Phylogenetically, *A. brunneisquamus* is related to *A. appendiculatus* Semwal, D. Chakr., K. Das, Indoliya, D. Chakrabarty and S. Adhikari & Karun. However, *A. appendiculatus*, a species originally described from India [42], has a distinctively larger pileus (7.5–9 cm) with a yellowish orange to grayish yellow surface, a longer stipe (8–12 cm), and large basidiospores measuring $14.2\text{--}16.5 \times 7.3\text{--}9.1 \mu\text{m}$, and it is associated with Dipterocarpaceae trees [42]. Moreover, *A. brunneisquamus* is also morphologically similar to *A. dictyotus*, *A. fusisporus* and *A. subflavidus* (Murrill) Wolfe. However, *A. dictyotus*, originally discovered in Indonesia, has a larger basidioma (pileus up to 11 cm), larger basidiospores measuring $(11\text{--})13\text{--}16 \times (6\text{--})7\text{--}8.5 \mu\text{m}$ with reticulations, and a pileipellis in the form of a trichoderm [1,2]; *A. fusisporus*, originally discovered in Japan, has a smaller pileus with a viscid surface, large basidiospores measuring $13.5\text{--}18.5 \times 8\text{--}11 \mu\text{m}$ with subcylindrical ornamentation, and a trichodermium pileipellis [1,43]; *A. subflavidus* has longer basidiospores measuring $13.1\text{--}19.5 \times 5.5\text{--}8.7 \mu\text{m}$ with $Q_m = 2.26$, a pileipellis in the form of a trichoderm, and a distribution in North America–Central America–northern South America [5]. Molecular evidence provided in this study also indicated that *A. brunneisquamus* is genetically distant from *A. dictyotus* and *A. fusisporus*, respectively, and it is somewhat related to *A. subflavidus*, as these two taxa belong to the same clade (not species level) (Figure 1).

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