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ORIGINAL ARTICLE

A new genus *Pseudoaustroboletus* (Boletaceae, Boletales) from Asia as inferred from molecular and morphological data

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Abstract A new genus of Boletaceae, *Pseudoaustroboletus*, is described to accommodate the phylogenetically isolated species *Tylopilus valens* inferred by molecular and morphological data. The lineage consisted of *T. valens* was nested into the Boletaceae clade with high support value using nuclear genes: the large subunit of the nuclear ribosomal RNA (nrLSU), the translation elongation factor 1-alpha (*tef1-α*), and mitochondrial gene: the small subunit (mtSSU) of the ribosomal RNA, based on Maximum Likelihood and Bayesian analyses. Morphologically, *Pseudoaustroboletus* is distinguished from other boletoid lineages by a combination of morphological characters, including the pale pinkish to light pinkish or greyish pink hymenophore, the distinctly reticulated stipe surface, the smooth basidiospores, the brown to dark yellow-brown pigmented cystidia, and the trichodermial brown to dark brown pigmented pileipellis. Geographically, *Pseudoaustroboletus* is currently only known from China, Japan, Malaysia and Singapore under forests dominated by Fagaceae plants. The species and its variety *macrosporus* in this genus are fully documented with taxonomic descriptions and illustrations.

Keywords Boletes · Distribution · Phylogeny · Taxonomy

Introduction

China is one of the hot spots for boletes, and many taxa have been discovered from the region in the past few decades (Chiu 1948; Wen 1985; Zang 1992, 2006; Zang et al. 2006; Li et al. 2011, 2014; Gelardi et al. 2013; Zhao et al. 2014; Zeng et al. 2012). Even so, there are still many species that need to be delimited. Recently, a few collections of boletes from the Provinces of Fujian, Hunan, Hainan, Guangdong and Yunnan were observed to be largely matching the concept of *Boletus albellus* (Massee 1909; non Peck 1888), originally named illegitimately by G. E. Massee from Singapore. The legitimate name, *Boletus valens*, provided by E.J.H. Corner (1972), has been used for this species. It is characterized by the dark or pale fuscous grey to greyish pileus, the pale pinkish to light pinkish or greyish pink hymenophore, the distinctly reticulated stipe surface, the white mycelia on the base of the stipe, the smooth basidiospores, the brown to dark yellow-brown pigmented cystidia, the trichodermial brown to dark brown pigmented pileus covering, and the occasionally yellowish discoloration on the base of the stipe when injured (Corner 1972). Hongo and Nagasawa (1976) regarded it as a member of *Tylopilus* P. Karst. due to the pink to pinkish hymenophore, which was followed by Horak (2011). Meanwhile, Horak (2011) transferred the variety *Boletus valens* var. *macrosporus* Corner to the genus *Tylopilus*. Morphologically, the stipe macro-morphology and spore color are also identical with some species in *Austroboletus* (Corner) Wolfe. Thus, the systematic placement of these taxa remains dubious. With the development and utilization of molecular phylogenetics, some morphologically delimited genera, such as *Boletus* L., *Tylopilus* and *Xerocomus* Que'l., were revealed to be polyphyletic (Kretzer and Bruns 1999; Binder and Besl 2000; Binder and Bresinsky 2002a; Binder and Hibbett 2007; Dentinger et al. 2010; Li et al. 2011; Wu et al. 2014). Several new genera were thus suggested to accommodate clades

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recognized by both morphological and molecular data (Binder and Bresinsky 2002b; Halling et al. 2012a, b; Desjardin et al. 2008, 2009; Šutara 2008; Orihara et al. 2010; Li et al. 2011; Lebel et al. 2012; Hosen et al. 2013; Zeng et al. 2012; Gelardi et al. 2014; Trappe et al. 2013). With careful examination of recent collections of *Tylopilus valens* (Corner) Hongo & Nagas., we found that the combined macro- and micro-morphological features distinguish it from other species in Boletaceae. Our phylogenetic analyses of this species based on the nuclear genes nrLSU and *tef1-α*, and the mitochondrial gene mtSSU, confirm *T. valens* as a unique generic lineage in Boletaceae. We thus propose a new genus, *Pseudoaustroboletus*, based on both morphological data and molecular sequences to accommodate *T. valens* and *Tylopilus valens* var. *macrosporus* (Corner) E. Horak.

Materials and methods

Morphological studies Macroscopic descriptions are based on detailed field notes made on fresh basidiocarps. Microscopic structures were revived in 5 % KOH. Sections of the pileus covering were cut radial-vertically and halfway between the center and margin of the pileus. All microscopic features were drawn by hand. For explanations of spore data, see Yang et al. (2012). Method for scanning electron microscopy (SEM) followed Xiang et al. (2010). Briefly, basidiospores were scraped from the dried hymenophore, pasted onto an SEM stub with double-sided tape, coated with gold-palladium and photographed with an AMRAY 1000B SEM. Specimens examined are deposited in the fungal herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN-HKAS).

Molecular procedures and phylogenetic analyses Protocols for DNA extraction, PCR, sequencing and sequence alignment followed those in Ge and Smith (2013), Xiang et al. (2012), and Zhao et al. (2013). Three DNA fragments were amplified in this study, including nrLSU, *tef1-α* and mtSSU. The primer pair used for amplifying the nrLSU region consisted of LROR and LR5 (Vilgalys and Hester 1990). To amplify the *tef-1α* fragment, the primers EF1-α-F and EF1-α-R were used (Mikheyev et al. 2006). For amplifying mtSSU, we used the primer pair MS1 and MS2 (White et al. 1990).

The phylogenetic analyses were based on two data sets: the single-locus data set for the nrLSU gene and the concatenated multi-locus data set of nrLSU, *tef-1α* and mtSSU. To test for phylogenetic conflict among the three genes, the partition homogeneity (PH) or incongruence length difference (ILD) test was performed with 1,000 randomized replicates, using heuristic searches with simple addition of sequences in PAUP* 4.0b10 (Swofford 2004). The result of the partition homogeneity test showed that the phylogenetic signals present in the different gene fragments were not in conflict ($p=0.89$) and could therefore be combined. In the preliminary analysis, the new genus was found nested in the Boletaceae clade; thus, sequences from samples of its closely related families in Rhizopogonaceae, such as *Rhizopogon nigrescens* Coker & Couch, Suillaceae, such as *Suillus pictus* (Peck) A.H. Sm. & Thiers, and Gomphidiaceae, such as *Gomphidius roseus* Massee, were downloaded from GenBank and included for outgroup rooting in both data sets. The scientific names, collection information and GenBank accession numbers for the sequences used in the combined data set are presented in Table 1.

Both data sets were analyzed using Randomized Accelerated Maximum Likelihood (RAxML) and Bayesian Inference (BI) methods, respectively. All parameters in the ML analysis used the default setting, and statistical support values were obtained using nonparametric bootstrapping with 1,000 replicates. Both data sets were analyzed further with a Bayesian approach (Metropolis-coupled MCMC) using MrBayes 3.1 (Huelsenbeck and Ronquist 2005). The parameter model was selected by the Akaike Information Criterion (AIC) as the best-fit likelihood model with Modeltest 3.7 (Posada and Buckley 2004). The models employed for each of the three partitions were: TrN+I+G for nrLSU, SYM+I+G for *tef-1α* and GTR+G for mtSSU. Posterior probabilities (PP) were determined twice by running one cold and three heated chains in parallel mode, saving trees every 1,000th generation. Runs were terminated once the average standard deviation of split frequencies went below 0.01. Trees were sampled every 1,000 generations. The first 25 % of generations were discarded as burn-in, and Bayesian PP were then calculated from the posterior distribution of the retained Bayesian trees.

Results

Molecular data Twelve sequences, four nrLSU, four *tef-1α* and four mtSSU, were newly generated for this study (Table 1). For the nrLSU data set, sequences representing most of the genera in the Boletaceae were downloaded from GenBank (Castellano et al. 1992; Binder and Fischer 1997; Binder and Besl 2000; Binder and Bresinsky 2002a; Peintner et al. 2003; Binder and Hibbett 2007; Halling et al. 2007, 2008; Desjardin et al. 2008, 2009; Dentinger et al. 2010; Orihara et al. 2010; Li et al. 2011). The final nrLSU data set included 67 nrLSU sequences, and the alignment contained 993 nucleotide sites. The combined nrLSU, *tef-1α* and mtSSU data set consisted of 2,075 nucleotides. In our phylogenetic analyses on both data sets using the ML and BI approaches, very similar estimates of tree topologies were produced. The most significant finding was that the lineage that consisted of *Tylopilus valens* was nested within the Boletaceae clade, and clustered with species in the genera *Chamonixia* and

Table 1 Specimens used in molecular phylogenetic studies and their GenBank accession numbers

Taxon	Voucher	Locality	GenBank accession		
			nrLSU	*tef1-α*	mtSSU
Boletellus ananas	–	–	HQ161853	–	AY615907
Boletinellus merulioides	–	–	AY684153	DQ056287	–
Buchwaldoboletus lignicolla	Pul1	Maindreieck, Germany	JQ326997	JQ327040	–
Chalciporus piperatus	MB 04-001	Rutland, MA, USA	DQ534648	GU187690	AY615908
Chamonixia caespitosa	–	–	AF336245	–	AF213145
Gomphidius roseus	MB95-038	Bavaria, Germany	DQ534669	GU187702	–
Gyrodon lividus	–	–	AF167677	GU187701	–
[*]*Leccinum holopus*	KUN-HKAS 53417	Hunan, central China	HQ326928	HQ326861	HQ326904
[*]*L. manzanitae*	KUN-HKAS 51277	Tibet, SW China	HQ326929	HQ326862	HQ326905
Paxillus vernalis	Pv2	Canada	AY645059	DQ457629	–
Phylloporus rhodoxanthus	–	–	DQ534631	–	M91013
Porphyrellus porphyrosporus	MB 97-023	Bavaria, Germany	DQ534643	GU187734_	–
[*]*Pseudoaustroboletus valens*	KUN-HKAS 59835 (Zeng120)	Hainan, China	KM274871	KM274879	KM274875
[*]*Pseudoaustroboletus valens*	KUN-HKAS 82643 (Lifang507)	Guangdong, China	KM274870	KM274878	KM274874
[*]*Pseudoaustroboletus valens*	KUN-HKAS 53407 (Li1062)	Hunan, China	KM274868	KM274876	KM274872
[*]*Pseudoaustroboletus valens*	KUN-HKAS 52603 (Li915)	Yunnan, China	KM274869	KM274877	KM274873
Rhizopogon nigrescens	MB06-070	Massachusetts, USA	GU187594	GU187744	–
Suillus pictus	–	–	AY612830	AB284698	–
[*]*Tylopilus felleus*[a]	KUN-HKAS 54926	Marburg, Germany	HQ326933	HQ326866	HQ326909
[*]*T. felleus* [b]	KUN-HKAS 55832	Jilin, NE China	HQ326934	HQ326867	HQ326910
[*]*Veloporphyrellus pantoleucus*[a]	F (Gómez 21232–1)	Cartago, Costa Rica	JX984548	–	JX984536
[*]*V. pantoleucus*[b]	F (Gómez 21232-2)	Costa Rica	JX984547	–	JX984535
Xerocomellus chrysenteron	–	–	AF514808	–	M91018
Xerocomus subtomentosus	Xs1	Bavaria, Germany	AF139716	JQ327035	–
[*]*Zangia citrina*	KUN-HKAS 52677	Fujian, SE China	HQ326940	HQ326871	HQ326916
[*]*Z. olivaceobrunnea*	KUN-HKAS 52275	Yunnan, SW China	HQ326947	HQ326875	HQ326923

[*] Sequences obtained in this study and Li et al. (2011, 2014). Others were from GenBank. Superscripts (a, b) are used to relate individual collections of the same taxon to their corresponding sequence data shown in Fig. 2

Leccinum, with high support value on the multi-locus data set in both analyses (Figs. 1 and 2).

Morphological observations In this study, thirteen specimens were examined, representing *P. valens var. valens* and *P. valens* var. *macrosporus*. *Pseudoaustroboletus* is characterized by the pale pinkish to light pinkish or greyish pink hymenophore, the distinctly reticulated stipe surface, the smooth basidiospores, the brown to dark yellow-brown pigmented cystidia, the trichodermial brown to dark brown pigmented pileus covering, and the occasionally yellowish discoloration on the base of the stipe when injured.

Taxonomy

Pseudoaustroboletus Yan C. Li et Zhu L. Yang, gen. nov.

MycoBank: MB 809890

Etymology: Named because of its phenotypic similarity to the genus *Austroboletus*.

Typus generis: *Boletus albellus* Massee (non Peck, 1888), Bull. Misc. Inf., Kew: 206 (1909)≡*Boletus valens* Corner, *Boletus* in Malaysia (Singapore): 161 (1972)

Basidiocarps stipitate-pileate with tubular hymenophore. Pileus hemispherical to applanate, not viscid when wet, with radially arranged filamentous squamules; context white to pallid, unchanged in color when injured. Hymenophore adnate to depressed around apex of stipe, white to pallid when young, and becoming pale pinkish or pinkish to pink when mature, unchanged in color when injured. Spore print pinkish to pink. Stipe pallid to white, reticulate with elongate meshes; context unchanging in color but occasionally with yellowish discoloration on the base of the stipe when bruised; mycelia white. Pileipellis an interwoven trichoderm. Pleurocystidia and cheilocystidia with brown to dark

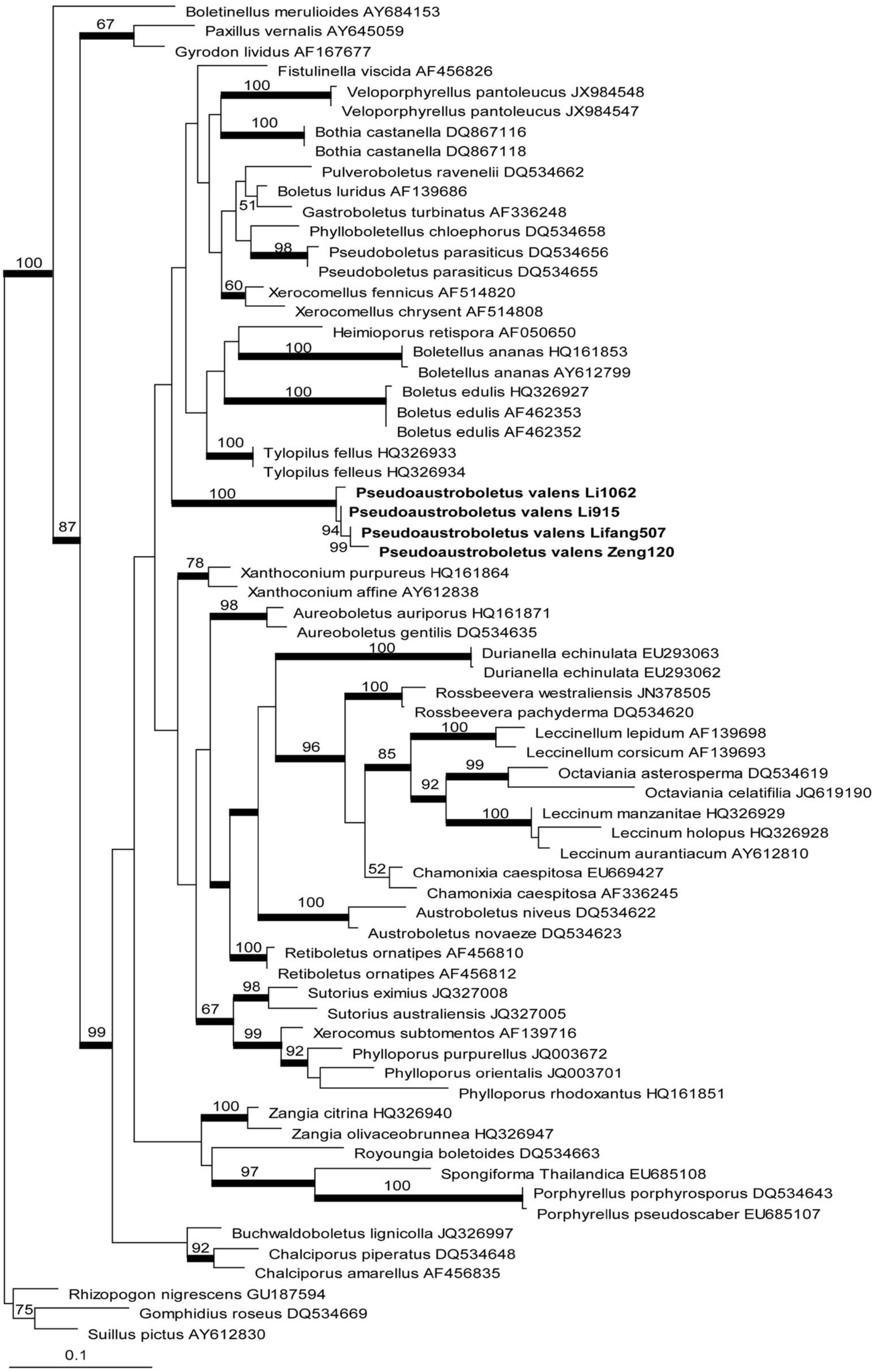

Fig. 1 Cladogram resulting from the nrLSU data set using RAxML. ML BS support values >50 % are indicated above or below the branches. In Bayesian analysis, PP>0.95 are indicated with thick branches. GenBank accession numbers are provided behind the species name

brown vacuolar pigment. Basidiospores smooth, pinkish to light olivaceous to nearly colorless. Clamp connections absent.

Pseudoaustroboletus generally shares the same colored hymenophore, and stipe morphology with some species in *Austroboletus* (Corner) Wolfe. But *Austroboletus* is characterized by the lightly to heavily ornamented spores with pits, warts or reticulations that are significantly different from *Pseudoaustroboletus* (Wolfe 1979; Singer 1986; Fulgenzi et al. 2010).

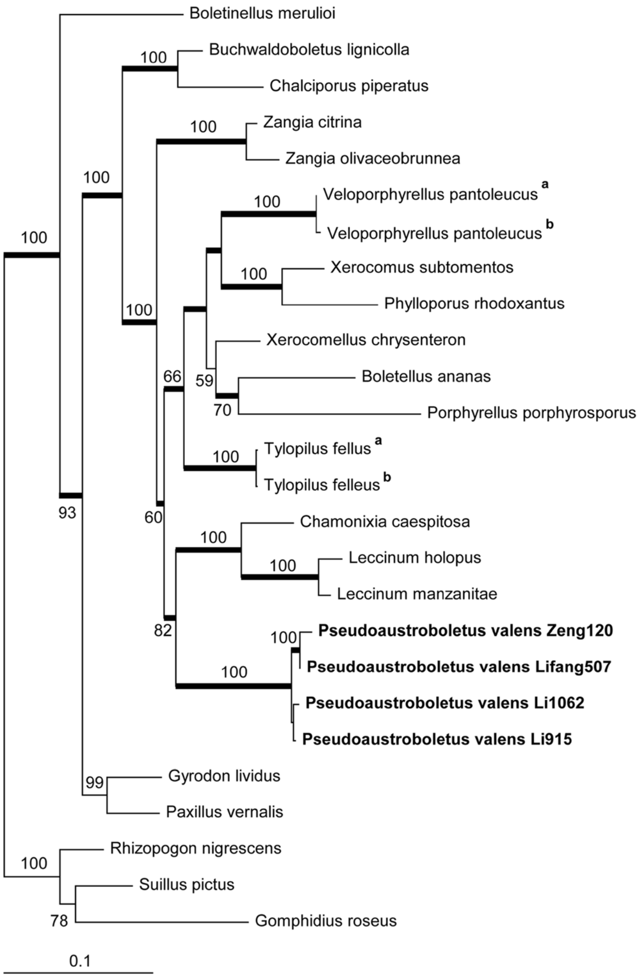

Fig. 2 Cladogram resulting from the combined nuclear (nrLSU and *tef-1α*) and mitochondrial (mtSSU) data set using RAxML. ML BS support values >50 % are indicated above or below the branches. In Bayesian analysis, PP>0.95 are indicated with thick branesch. GenBank accession numbers for the three genes of each species were presented in Table 1

Pseudoaustroboletus valens **(Corner) Yan C. Li et Zhu L. Yang, comb. nov.**

Basionym: *Boletus valens* Corner, *Boletus in Malaysia* (Singapore): (1972) 161 [≡*Boletus albellus* Mass., Kew Bull. (1909) 206 (non Peck, 1888)]

MycoBank: MB 809891

1. *Pseudoaustroboletus valens* (Corner) Yan C. Li et Zhu L. Yang var. *valens* (Figs. 3, 4, and 6, a–f)

Basidiomata 5–8 cm broad, small to medium-sized. Pileus dark grey or pale fuscous grey to greyish white, paler on expansion, subhemispherical or hemispherical to applanate, dry, not viscid when wet; glabrous, with radially arranged filamentous squamules when aged; context in pileus white to pallid, unchanged in color when injured. Hymenophore adnate to depressed around apex of the stipe, pallid to pale pinkish or pinkish to pink, unchanged in color when injured; tubes and pores concolorous, tubes up to 1.5 cm long and pores up to 0.1 cm in diameter, unchanging in color when bruised. Stipe 9–12×1.5–2.5 cm, central, subcylindrical, sometimes enlarge downwards, reticulate with elongate meshes up to 0.5 mm in height, wholly pallid to white or whitish; context in stipe white to whitish unchanging in color when bruised, but some collections occasionally with yellow to yellowish or pinkish stains at the base of stipe on injury; mycelia on the base of stipe white. Taste slightly sour.

Basidia 27–32×8.5–12 μm, clavate, hyaline to light yellowish in KOH, thin-walled, four-spored, occasionally two-spored. Basidiospores [240/12/12] (11.5) 12–15 (16.0)×(4.0) 4.5–5.5 (6.0) μm, [Q=(2.18) 2.33–3.18 (3.50), Q_m=2.69±0.21], boletoid, slightly thick-walled (up to 0.5 μm in thickness), subhyaline to light olivaceous in KOH and yellowish brown in Melzer's reagent, smooth under SEM. Pleurocystidia and cheilocystidia 22–62×6.5–10 μm, fusiform to subfusiform or subfusoid-mucronate to ventricose-lanceolate with a short attenuate apex, thin-walled, brownish to yellowish brown in KOH and brown to dark brown in Melzer's reagent. Caulocystidia forming the meshy ornamentation over surface of stipe, similar to pleurocystidia and cheilocystidia morphologically, some with one septum. Hymenophoral trama bilateral, composed of broad hyaline hyphae 5–11.5 μm in width. Pileus covering (squamules on pileus) composed of more or less vertically to almost radially arranged to loosely interweaved, yellowish brown to brownish or brown, filamentous hyphae 5–10.5 μm in diameter; terminal cells 15–46×6–11 μm, subcylindric. Pileal trama made up of hyphae 3–10.5 μm in diameter, hyaline to yellowish brown in KOH. Clamp connections absent in all tissues.

Habitat: Solitary on the ground in the forest of Fagaceae plants.

Known distribution: Currently known from China, Japan, Malaysia and Singapore.

Specimens examined: China. Hainan Province: Qiongzhong County, Limu Mountain, alt. 750 m, 6 May 2009, Zeng120 (KUN-HKAS 59835). Fujian Province: Zhangping County, Xinqiao Town, Chengkou Village, 3 Sep. 2009, Zeng670 (KUN-HKAS 82641). Yunnan Province: Jinghong City, Dadugang Town, alt. 1400 m, 21 July 2007, Li915 (KUN-HKAS 52603). Hunan Province: Chenzhou City, Yizhang County, Mangshan National Forest Park, alt. 1,800 m, 2 Sep. 2007, Li1062 (KUN-HKAS 53407). Guangdong Province: Fengping County, Heishiding, alt. 200 m, 5 Jun. 2012, Lifang443 (KUN-HKAS 82642); same location, 14 Jun. 2012, Lifang507 (KUN-HKAS 82643), 18 Jul. 2012, Lifang690 (KUN-HKAS 82644) and 15 Oct. 2012, Lifang1081 (KUN-HKAS 77791). Malaysia. Pahang Fraser's Hill, 16 May 1930, Corner (E 98119); Kinabalu, 23 Jan. 1964, Corner RSNB5082A (E 98120). Singapore. Bukit Timah, 16 Aug. 1939, Corner (E 98118); Reservoir Jungle, 4 Sep. 1939, Corner (E 98116).

Fig. 3 Habitat of *Pseudoaustroboletus valens* var. *valens*. **a**–**b.** Li 915 (KUN-HKAS 52603). **c**–**d.** Lifang 507 (KUN-HKAS 82643). **e**–**f**. Lifang 443 (KUN-HKAS 82642)

2.*Pseudoaustroboletus valens* var. *macrosporus* (Corner) Yan C. Li et Zhu L. Yang, comb. nov. (Fig. 5 and 6, g–i)

Basionym: *Boletus valens* var. *macrosporus* Corner, *Boletus in Malaysia* (Singapore): (1972) 162

MycoBank: MB 809892

Basidia 28–35 × 8–10.5 μm, clavate, hyaline in KOH and yellowish in Melzer's reagent, four-spored. Basidiospores (60/3/1) 16–20.5(21) × (5.5)6–6.5(7.5) μm, [Q = (2.46) 2.67–3.33(3.42), Q_m = 3.00 ± 0.16], boletoid, smooth, slightly thick walled (up to 0.5 μm thick), subhyaline to light olivaceous in KOH and yellowish brown in Melzer's reagent, smooth under SEM. Pleurocystidia and cheilocystidia 29–51 × 8–12 μm, subfusoid-mucronate or ventricose-mucronate with a long pedicel, thin-walled, brownish to yellowish brown in KOH and brown to dark brown in Melzer's reagent. Caulocystidia forming the meshy ornamentation over surface of stipe, similar to basidia, pleurocystidia and cheilocystidia morphologically. Hymenophoral trama bilateral, composed of broad hyaline hyphae 4–10 μm in width. Pileus covering composed of loosely interweaved non-viscid, yellowish brown to brownish or brown hyphae 3–7 μm in width; terminal cells 21–56 × 4–9 μm, subcylindrical. Pilea trama hyphae 6–11 μm in width, hyaline to yellowish brown in KOH. Clamp connections absent.

Known distribution: Currently known from Malaysia and Singapore.

Specimen examined: Malaysia. North Borneo, Kinabalu, 23 Jan. 1964, Corner RSNB 5082B (E 98121, isotype).

Discussion

The Chinese collections match well with the descriptions of *Tylopilus valens* provided by Massee (1909), Corner (1972), Hongo and Nagasawa (1976). Studies on collections from Singapore (where the type was collected), Malasia and

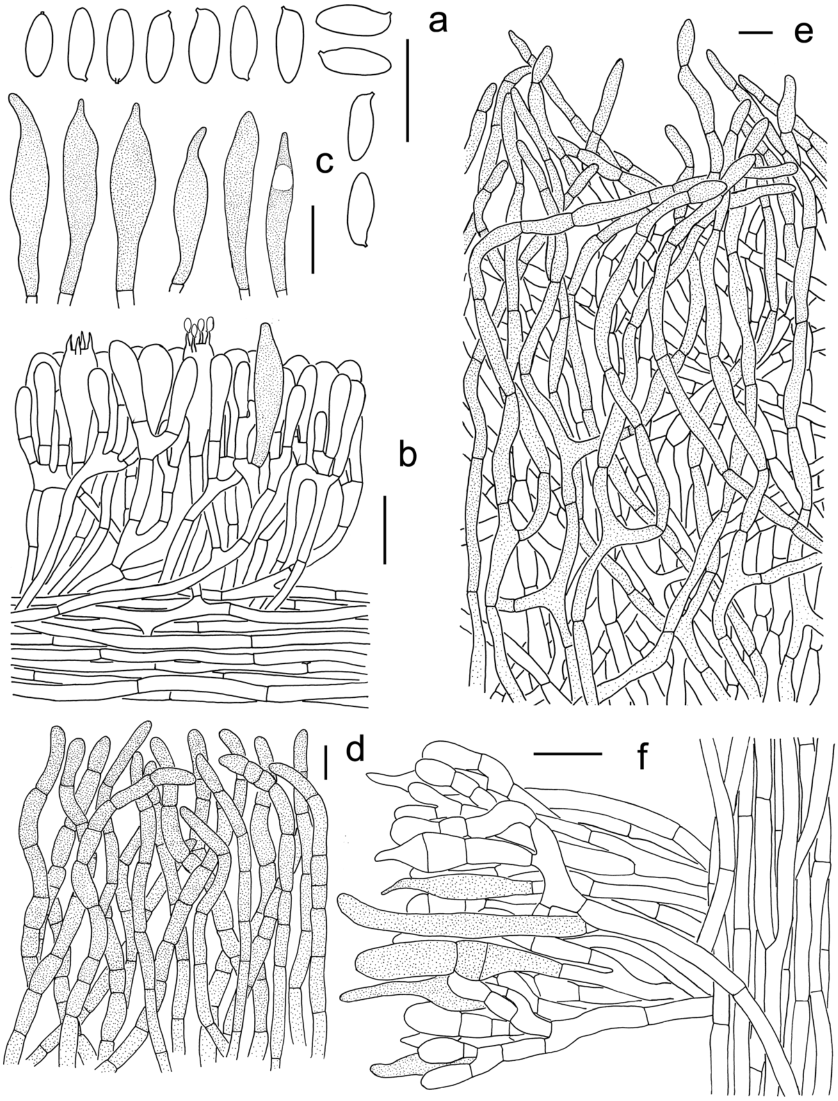

Fig. 4 *Pseudoaustroboletus valens* var. *valens* (**a**–**c**, **f**: from KUN-HKAS 52603, **d**: from KUN-HKAS 53407, **e**: from KUN-HKAS 77791). **a**. Basidiospores. **b**. Basidia and pleurocystidium. **c**. Pleurocystidia and cheilocytidia. **d**. Pileus covering in younger fruitbody. **e**. Pileus covering in mature fruitbody. **f**. Caulocystidia. *Bars*: **a**–**f**= 20 μm

China add more information to the recognition of this species. This taxon is characterized by the dark or pale fuscous grey to greyish pileus, the pale pinkish to light pinkish or greyish pink hymenophore, the distinctly reticulated stipe surface, the smooth basidiospores 12–15×4.5–5.5 μm, the brown to dark yellow-brown pigmented cystidia, and the trichodermial brown to dark brown pigmented pileipellis with hyphae 5–11 μm in diameter. Such combined traits are greatly different from those in the other genera in Boletaceae. Thus, *Pseudoaustroboletus* is proposed to accommodate this species. Geographically, *P. valens* is currently known from southern, southeastern and southwestern China, Japan, Malaysia, Singapore under forests dominated by Fagaceae.

So far, *Pseudoaustroboletus* harbors *P. valens var. valens* and *P. valens* var. *macrosporus*. The var. *macrosporus* is macroscopically identical with the var. *valens* according to Corner's description and our macro-morphological examinations on the isotype. The var. *macrosporus* is mainly distinguished from var. *valens* by its large spore (16–20.5×6–6.5 μm) and relatively narrow hyphae (3–9 μm in width) in the pileus covering. The var. *macrosporus* may represent a separate species. Unfortunately, the authors did not successfully extract DNA from the isotype. Moreover, there are no additional materials after the description of this variety. Thus, further taxonomic treatment of the taxon should wait until more data are available.

In the nrLSU data set, the lineage consisted of *P. valens* was nested within the Boletaceae clade and received high support in ML (100 %) and Bayesian analyses (Fig. 1). But the relationships of this lineage with the other genera in Boletaceae are not well solved. In our multi-locus data set analyses, samples of *P. valens* formed a monophyly group, sistered to the genera *Chamonixia* Rolland and *Leccinum* Gray, with high support value in both analyses (Fig. 2).

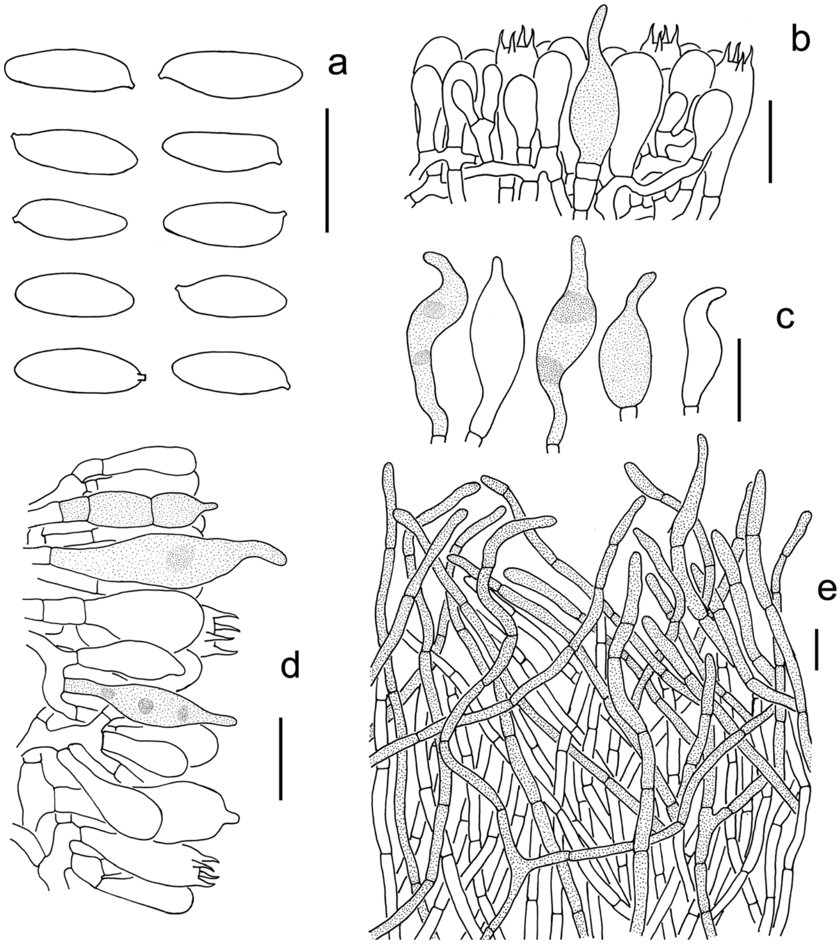

Fig. 5 *Pseudoaustroboletus valens* var. *macrosporus* (Corner RSNB 5082B, E 98121, isotype). **a**. Basidiospores. **b**. Basidia and pleurocystidium. **c**. Pleurocystidia and cheilocytidia. **d**. Caulocystidia. **e**. Pileus covering. Bars: **a**–**e**=20 μm

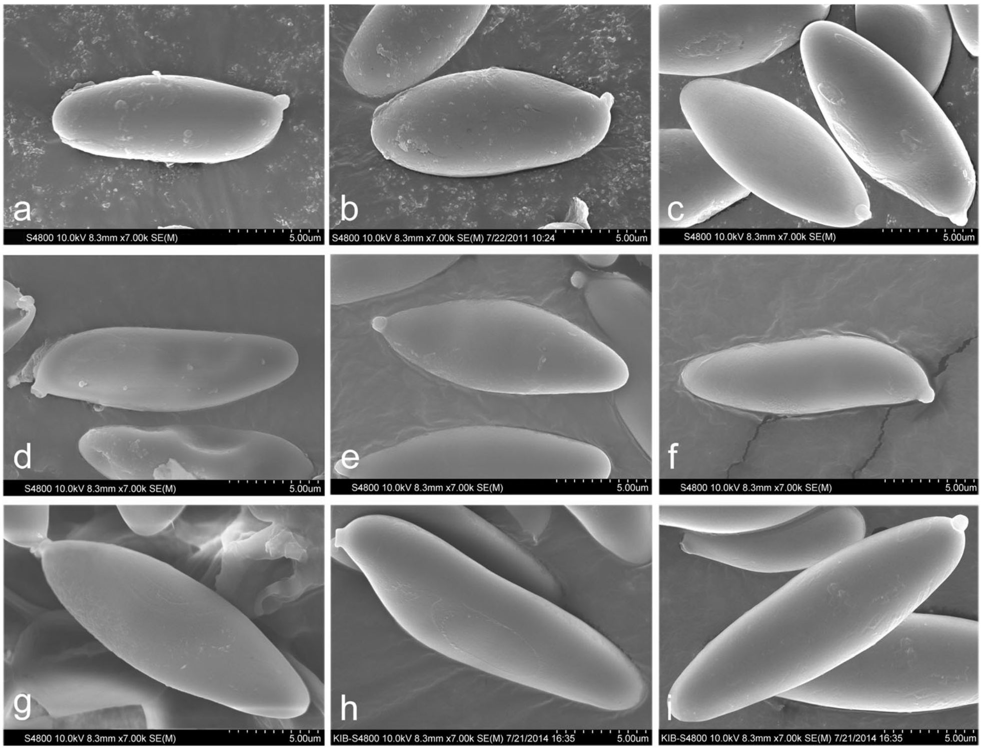

Fig. 6 Basidiopores of *Pseudoaustroboletus* taxa under SEM. **a–c.** *P. valens* var. *valens* (Li915, KUN-HKAS 52603). **d–f.** *P. valens* var. *valens* (Corner RSNB5082A, E 98120). **g–i.** *P. valens* var. *macrosporus* (Corner RSNB 5082B, E 98121, isotype)

However, *Chamonixia* is characterized by the globose whitish basidiomata, the cyanescent discoloration of the peridium when injured, and the longitudinal ridged basidiospores (Smith and Singer 1959; Beaton et al. 1985), while *Leccinum* has a coarsely verrucose stipe, and grayish, brownish or yellowish but never pink to pinkish hymenophores (den Bakker and Noordeloos 2005). Such traits are significantly different from those in *Pseudoaustroboletus*.

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