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ORIGINAL ARTICLE

Molecular phylogeny of *Caloboletus* (Boletaceae) and a new species in East Asia

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Abstract *Caloboletus* has recently been erected to accommodate *Boletus calopus* and its allies. Our phylogenetic analyses based on four gene markers (ITS, nrLSU, *tef1*-α and *rpb1*) recognized six distinct species within the genus. Morphologically, the genus is characterized by the combination of the yellow hymenophore, smooth spores that are olive-brown in deposit, an interwoven pileipellis, a bluish color-change and a distinctly bitter taste of the context. A new species was described and a key to the six species of *Caloboletus* is provided. Members of *Caloboletus* are mainly distributed in subtropical and temperate zones in the Holarctic and are often associated with plants of Pinaceae and Fagaceae.

Keywords Boletes · New species · Phylogeny · Taxonomy

Introduction

Boletus L. is one of a few generic names of mushrooms proposed by Linnaeus (1753), and has been widely studied by mycologists from all over the world (Fries 1836; Singer 1947, 1986; Smith and Thiers 1971; Corner 1972; Pegler and Young 1981; Zang 1983; Høiland 1987; Watling and Li 1999; Li and Song 2000; Binder and Bresinsky 2002; Horak 2005, 2011; Dentinger et al. 2010). It was divided into several sections, mainly based on morphological characters such as the colors of the hymenophore, the color-change of the context when exposed to air and the taste of the basidioma (Fries 1836; Elrod and Blanchard 1939; Singer 1947, 1986; Smith and Thiers 1971; Zang 1983, 1999, 2006). The most widely used infrageneric treatment within *Boletus* was proposed by Singer (1986). He divided *Boletus* into seven sections, namely, *Boletus*, *Appendiculati* Konr. and Maubl., *Calopodes* Fr., *Grisei* Singer ex Singer, *Luridi* Fr., *Ornatipedes* Singer and *Subpruinosi* Fr. ex Singer (Singer 1986). Boletes with yellow pores, a bluish color-change and a bitter taste were placed in the sect. *Calopodes* (Fries 1836; Peck 1896; Elrod and Blanchard 1939; Singer 1947, 1986; Dick 1960; Hongo 1960; Hills and Vassiliades 1999; Zang 1999, 2006; Šutara and Špinar 2006; Ortiz-Santana et al. 2007; Lukić 2009; Assyov 2012; Takahashi et al. 2013) or a subsection in *B*. sect. *Boletus* (Smith and Thiers 1971; Thiers 1975; Zang 1983).

As shown in an increasing number of studies, the genus *Boletus* is not monophyletic (Binder and Hibbett 2006; Drehmel et al. 2008; Nuhn et al. 2013, Wu et al. 2014), and the taxonomic statuses and phylogenetic positions of the sections are largely unsolved. Memebers of the sect. *Ornatipedes*, along with *B. griseus* in sect. *Grisei*, were transferred to the genus *Retiboletus* (Binder and Bresinsky 2002). Dentinger et al. (2010) and Feng et al. (2012) have recommended restricting the genus *Boletus* sensu stricto (s. str.) to those species once retained in *B*. sect. *Boletus*. Recently, *Butyriboletus* D. Arora and J.L. Frank and *Caloboletus* Vizzini were erected to accommodate boletes once usually placed in sect. *Appendiculati* and sect. *Calopodes*, respectively (Arora and Frank 2014; Vizzini 2014). *Caloboletus* mainly corresponds to the *dupainii* clade in Nuhn et al. (2013) and the clade 39 of *Pulveroboletus* Group in Wu et al. (2014).

Molecular techniques have already strongly contributed to modern concepts in the fungal taxonomy (Taylor et al. 2000; Weiβ 2010; Hibbett et al. 2011; Yang 2011). Combined with molecular data and morphological characters, a batch of new

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genera of boletes were erected (Halling et al. 2007, 2012a, b; Desjardin et al. 2008, 2009; Li et al. 2011; Zeng et al. 2012; Hosen et al. 2013). These techniques have also helped to recognize some new lineages in the family Boletaceae (Nuhn et al. 2013; Wu et al. 2014).

This study addressed the following objectives: (1) to compare the morphological features between *Caloboletus* and related taxa; (2) to elucidate species diversity and their phylogenetic relationships within the genus *Caloboletus*; (3) to describe a new species from China.

Materials and methods

Morphological studies

Macroscopic descriptions and detailed notes were made based on field notes and images of basidiomata. Color codes follow Kornerup and Wanscher (1981). For microscopic observation, the dried materials were sectioned and mounted in 5 % KOH solution. Sections of the pileipellis were cut anticlinally, both at the center and the margin of the pileus. Sections of the stipe were taken from the central part along the longitudinal axis of the stipe. Pileipellis, basidia, basidiospores, pleurocystidia and cheilocystidia, and stipitipellis were obtained using an Axioskop 40 microscope following the standard method described in previous studies (Li et al. 2009, 2011; Zeng et al. 2012, 2013; Hosen et al. 2013). All microscopic features were drawn by hand. In the descriptions of basidiospores, the abbreviation [*n*/*m*/*p*] means *n* basidiospores measured from *m* basidiomata of *p* collections; *Q* is used to mean "length/width ratio" of a spore in side view; *Q* means average *Q* of all basidiospores±sample standard deviation. To observe the surfaces of spores, small hymenophoral fragments were taken from dried specimens, and the fragments were mounted on aluminum stubs with double-sided adhesive tape, coated with gold palladium, and then observed under a Hitachi S4800 scanning electron microscope (SEM). Specimens examined are deposited in the Cryptogamic Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (HKAS), the National Botanical Garden of Belgium (BR), the Herbarium of the Royal Botanic Gardens at Kew (K), the Herbarium of New York Botanical Garden (NY) and the Herbario Musaei Nationalis Pragae (PRM). The descriptions of species appear in alphabetical order by species epithet. The generic name *Boletus* is abbreviated as "*B.*" and *Butyriboletus* as "*Bu*", while "*Caloboletus*" is abbreviated as "*C.*"

DNA extraction, PCR and sequencing

Total DNA was extracted from basidioma dried by silica gel or from herbarium specimens using the CTAB method (Doyle and Doyle 1987). The primer pair used for amplifying the ITS and nrLSU region was ITS5/ITS4 (White et al. 1990) and LROR/LR5 (Vilgalys and Hester 1990), respectively. EF1-595 F and EF1-1160R (Rehner 2001), RPB1-Af and RPB1-Cr (Dentinger et al. 2010) were used to amplify *tef1-α* and *rpb1*, respectively. However, only a part of samples can be successfully amplified using these primer sets. Thus, two primer pairs ITS5/ITS2, ITS3/ITS4 were used to amplify the ITS region (White et al. 1990), another two primer pairs LROR/LR3, LR3R/LR5 (Vilgalys and Hester 1990) were used to amplify the nrLSU region. To amplify *tef1-α* and *rpb1*, several pairs of primers designed by Wu et al. (2014) were used even though these primer pairs also failed to amplify from some specimens with poor quality. Thus, two primer pairs were newly designed, EF1-AM-R (GAGGCTGGTATCTCCAAGG) and EF1-AM-R (AACAAGCTGCTCGTGGTG) for *tef1-α*, and RPB1-AM-F (CTCTTGCCTGTGACGCAG) and RPB1-AM-R (CCTCTTTGGGATCGTCAG) for *rpb1*. These additional primer pairs allowed us to amplify most of the remaining sequences for the present study.

PCR reactions were conducted on an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) or an Eppendorf Master Cycler (Eppendorf, Netheler-Hinz, Hamburg, Germany) The PCR programs were as follows: pre-denaturation at 94 °C for 3 min, then followed by 35 cycles of denaturation at 94 °C for 40s, annealing at 50 °C (for ITS), 53 °C (for nrLSU), 55 °C (for tef1-*α* and *rpb1*) for 40s, elongation at 72 °C for 90s, a final elongation at 72 °C for 8 min was included after the cycles. PCR products were purified with a Gel Extraction and PCR Purification Combo Kit (Spin-column) (Bioteke, Beijing, China). The purified products, cloned when necessary, were then sequenced on an ABI-3730-XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using the same primers as in the original PCR amplifications, yet the cloned products were sequenced using M13F and M13R (Messing 1983).

Phylogenetic analyses

75 sequences, including 56 newly generated in this study and 19 retrieved from the GenBank, were used in the phylogenetic analyses. DNA sequences of four loci (ITS, nrLSU, *tef1-α* and *rpb1*) were independently aligned with MAFFT v6.8 (Katoh et al. 2005) and manually optimized in BioEdit v7.0.9 (Hall 1999). The data sets were then analyzed using RAxML v7.2.6 (Stamatakis 2006) and MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) for Maximum Likelihood (ML) and Bayesian Inference (BI), respectively.

To assess incongruence among individual genes before they are concatenated together, single-gene analyses were conducted using the ML method to detect the topologies of the three genes (BS>70 %, Nuhn et al. 2013). Due to no significant incongruence detected, the resulting four alignments (ITS, nrLSU, *tef1-α* and *rpb1*) were then concatenated using Phyutility (Smith and Dunn 2008). Unavailable sequences of the loci of a few species were treated as missing data in the phylogenetic analyses. Final concatenated alignment has been deposited in TreeBASE (http://purl.org/phylo/treebase; submission ID 15618).

For both BI and ML analyses of the combined data set, the substitution model suitable for the four gene partitions of the combined data set were individually determined using the Akaike Information Criterion (AIC) complemented in MrModeltest v2.3 (Nylander 2004). GTR+I+G was chosen as the best model for all the data sets. For ML analysis, all parameters were kept default except for the model choosing as GTRGAMMAI (Stamatakis 2006), and the statistic supports were calculated using nonparametric bootstrapping with 1 000 replicates. Bayesian analyses with four chains were conducted by setting generations to three million for the combined data set, and runs were terminated once the average standard deviation of split frequencies went below 0.01 (Huelsenbeck and Ronquist 2005). Other parameters were kept at their default settings. The chain convergence was determined using Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/) to ensure sufficiently large ESS values. Trees were summarized and posterior probabilities (PPs) were calculated by using sump and sumt command complemented in MrBayes by discarding the first 25 % generations as burn-ins.

According to recent studies on the phylogeny of Boletaceae (Nuhn et al. 2013; Wu et al. 2014), *B. amygdalinus* Thiers, *B. sinicus* W.F. Chiu and *Bu. appendiculatus* (Schaeff.) D. Arora and J.L. Frank, which are close to *Caloboletus*, were selected as outgroups. The scientific names, collection information and GenBank accession numbers for the sequences used in the combined data set are presented in Table 1.

Results

Morphologic observations

42 specimens were examined, including 33 collections of *Caloboletus*, four collections of *Butyriboletus* and five collections of *Boletus* s. str. *Caloboletus* is characterized by its yellow hymenophore, a bluish color-change when bruised, an interwoven pileipellis, smooth spores and a bitter taste. These combinations of characters make *Caloboletus* distinct from the other existing genera in Boletaceae, whereas, *Boletus* s. str. has a mild taste, a trichodermatous pileipellis and an unchanged context when exposed to air or injured. *Butyriboletus* strongly resembles *Caloboletus* as they both have the yellow tubes, colored reticulum on the stipes and bluish color change. However, the former tastes mild, not bitter.

Molecular data

As no well-supported conflict (BS>70 %, Nuhn et al. 2013) was detected among the topologies of the four genes, their sequences were then concatenated together for further multigene analyses. The combined data set included nine species and the alignment contained 2713 nucleotide sites (including gaps), consisting of 622, 783, 567 and 741 sites (including gaps) for ITS, nrLSU, *tef1-α* and *rpb1*, respectively.

Sequences generated from 17 collections of *Caloboletus* formed an independent clade with high support value (BS=100 %, PP=1.0). This clade was further grouped into two major subclades, one including *C. firmus* and *C. yunnanensis*, and the other including *C. calopus*, *C. inedulis*, *C. panniformis* and *C. radicans*. Within the cluster of *C. panniformis*, the six samples were grouped into two statistically supported smaller lineages, one lineage associated with *Abies* and/or *Picea* in subalpine forests, the other with subtropical *Pinus yunnanensis* (Fig. 1).

ML and Bayesian analyses produced very similar estimates of tree topologies, and, thus, only the tree inferred from the ML analysis is shown (Fig. 1).

Taxonomy

Caloboletus Vizzini. Index Fungorum 146:1, 2014.

Basidioma stipitate-pileate with tubular hymenophore. Pileus hemispherical, convex or applanate; context pallid to yellowish, blues quickly when exposed. Hymenophore and tubes yellow to pallid yellow, turning bluish to blue quickly when injured, then back to original color slowly. Stipe central, smooth or covered with colored reticulum, especially in the upper parts. Pileipellis composed of interwoven filamentous hyphae. Hymenophoral trama boletoid. Basidiospores smooth, typically boletoid, subfusiform, ellipsoid to somewhat oblong, slightly thick-walled (Fig. 2), subhyaline in KOH and yellowish brown in Melzer's reagent. Pleurocystidia and cheilocystidia lageniform, thin-walled. Clamp connections absent. Taste bitter.

Distribution: Mainly distributed in subtropical and temperate zones in the Holarctic. Associated with members of Pinaceae and Fagaceae.

Type of the genus: ***Caloboletus calopus*** (Pers.) Vizzini.

1 *Caloboletus calopus* (Pers.) Vizzini, Index Fungorum 146:1, 2014.

Basionym: *Boletus calopus* Pers., Synopsis methodica fungorum:513, 1801.

Boletus subtomentosus subsp. *calopus* (Pers.) Pers., Mycologia Europaea 2:139, 1825.

Dictyopus calopus (Pers.) Quél., Enchiridion Fungorum in Europa media et praesertim in Gallia Vigentium:160, 1886.

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

Species	Isolate/voucher/strain	Collector/ identifier	Location	Genbank accessions			
				ITS	nrLSU	*tef1*-α	*rpb1*
Caloboletus calopus	BR50201590638-05	De Kesel A.	Montenau, Belgium	KJ605655	KJ184554	KJ184566	KJ184560
Caloboletus calopus	Bc1*	N. Arnold	Bavaria, Germany	DQ679806	AF456833	JQ327019	–
Caloboletus firmus	MB 06-060	M. Binder	Chestnut Ridge Park, NY, USA	–	KF030278	KF030408	KF030368
Caloboletus firmus	NY00796115	R.E. Halling	Cayo, Belize	KJ605656	KJ605678	KJ619464	KJ619473
Caloboletus inedulis	MB06-044	M. Binder, E. Both	Erie Co., NY, USA	–	JQ327013	JQ327020	KF030362
Caloboletus inedulis	HKAS80478	Z.W. Ge	Florida, USA	KJ605657	KJ605671	KJ619465	KJ619474
Caloboletus panniformis	HKAS55444	B. Feng	Deqin, Yunnan, China	KJ605658	KF112334	KF112165	KF112506
Caloboletus panniformis	HKAS56164	Y.C. Li	Lijiang, Yunnan, China	KJ605667	KJ605674	KJ619466	KJ619475
Caloboletus panniformis	HKAS57410	B. Feng	Shangri-la, Yunnan, China	KJ605659	KJ184555	KJ184567	KJ184561
Caloboletus panniformis	HKAS63560	G. Wu	Lijiang, Yunnan, China	–	KJ605675	KJ619468	KJ619476
Caloboletus panniformis	HKAS77506	K. Zhao	Lijiang, Yunnan, China	KJ605660	KJ605660	KJ619469	KJ619477
Caloboletus panniformis	HKAS77530	K. Zhao	Dali, Yunnan, China	KJ605661	KJ605670	KJ619470	KJ619478
Caloboletus radicans	HKAS80856	J. Xu	INRA Centre de Nancy, France	KJ605662	KJ184557	KJ184569	KJ184563
Caloboletus yunnanensis	HKAS69214 (holotype)	Y.J. Hao	Baoshan, Yunnan, China	KJ605663	KJ184556	KJ184568	KJ184562
Caloboletus yunnanensis	HKAS58694	Q. Cai	Dali, Yunnan, China	KJ605664	KJ605672	KJ619470	KJ619479
Caloboletus yunnanensis	HKAS63040	L.P. Tang	Baoshan, Yunnan, China	–	KJ605676	KJ619471	KJ619480
Caloboletus yunnanensis	HKAS74864	G. Wu	Nujiang, Yunnan, China	KJ605665	KF112415	KF112200	KF112508
Boletus amygdalinus	112605ba*	B. Neil	Mendocino Co., CA, USA	FJ235148	JQ326996	JQ327024	KF030360
Boletus sinicus	HKAS56304	Y.C. Li	Shangri-la, Yunnan, China	KJ605666	KJ605673	KJ619483	KJ619482
Butyriboletus appendiculatus	BR50200892955-50	Vanholen B.	Zoniënwoud, Belgium	KJ605668	KJ605677	KJ619472	KJ619481

Accessions numbers in boldface indicate newly generated sequences. An asterisk (*) at the isolate number emphasizes that the sequences were represented by two strains for *Caloboletus calopus* and *Boletus amygdalinus*

Tubiporus calopus (Pers.) Maire, Publicacions del Instituto Botánico Barcelona 3 (4):46, 1937.

Materials examined: BELGIUM, Montenau, Omgeving Klooster, 02 Oct 2011, De Ceuster RSN, (BR50201706674-40); Vencimont, 24 Aug. 2005, De Kesel A. 4087 (BR 50201590638-05).

Notes: *Tubiporus* P. Karst. (1881) and *Dictyopus* Quél. (1886) were typified by *B. luridus* (Schaeff.) P. Karst. and *B. edulis* Bull., respectively. *Boletus luridus* is the type species of *Boletus* sect. *Luridi*.

2 ***Caloboletus firmus*** (Frost) Vizzini, Index Fungorum 146:1, 2014.

Basionym: *Boletus firmus* Frost, Bull. Buffalo Soc. Nat. Sci. 2:103, 1874.

Material examined: BELIZE, Cayo District, Mountain Pine Ridge, Macal River near Guacamaya Bridge, 04 Oct 2003, R.E. Halling 8520 (NY 00796115).

Notes: Recognizable by its red to red orange pore surface, *B. firmus* once was regarded as a synonym of *B. luridus* (Murrill 1909). However, subsequent studies uncovered that *B. firmus* and *B. luridus* were distinctive species, and both were placed in sect. *Luridi* and *B. luridus* was the type (Dick 1960; Singer 1986). Recent studies indicated that *B. firmus* is related to *B. calopus* and its allies (Nuhn et al. 2013; Wu et al. 2014).

3 ***Caloboletus inedulis*** (Murrill) Vizzini, Index Fungorum 146:1, 2014.

Basionym: *Ceriomyces inedulis* Murrill, Mycologia 30:523, 1938.

Materials examined: USA, Florida, Gainesville, University of Florida, under an oak, 16 Jun 2012, Z.W. Ge 3226 (HKAS 80478); New York, Bronx Co. Bronx, the New York Botanical Garden, N end of Azalea Way, 01 Jul 2008, R.E. Halling 9010 (NY 1034406).

Notes: *Ceriomyces* Murrill was erected in 1909 and typified by *Ceriomyces crassus* Murrill. Now this genus is a synonym of *Boletus* (Fr.) Singer (Singer 1945) and can be discounted as a mixture of several modern genera (Halling 2012a).

4 ***Caloboletus panniformis*** (Taneyama and Har. Takah.) Vizzini, Index Fungorum 146:1, 2014.

Basionym: *Boletus panniformis* Taneyama and Har. Takah., Mycoscience 54:459, 2013.

Materials examined: CHINA, Gansu Province, Diebu County, in a forest of *Abies*, alt. 3000 m, 12 Aug 2012, X.T. Zhu 687 (HKAS 76536); Guizhou Province, Weining County,

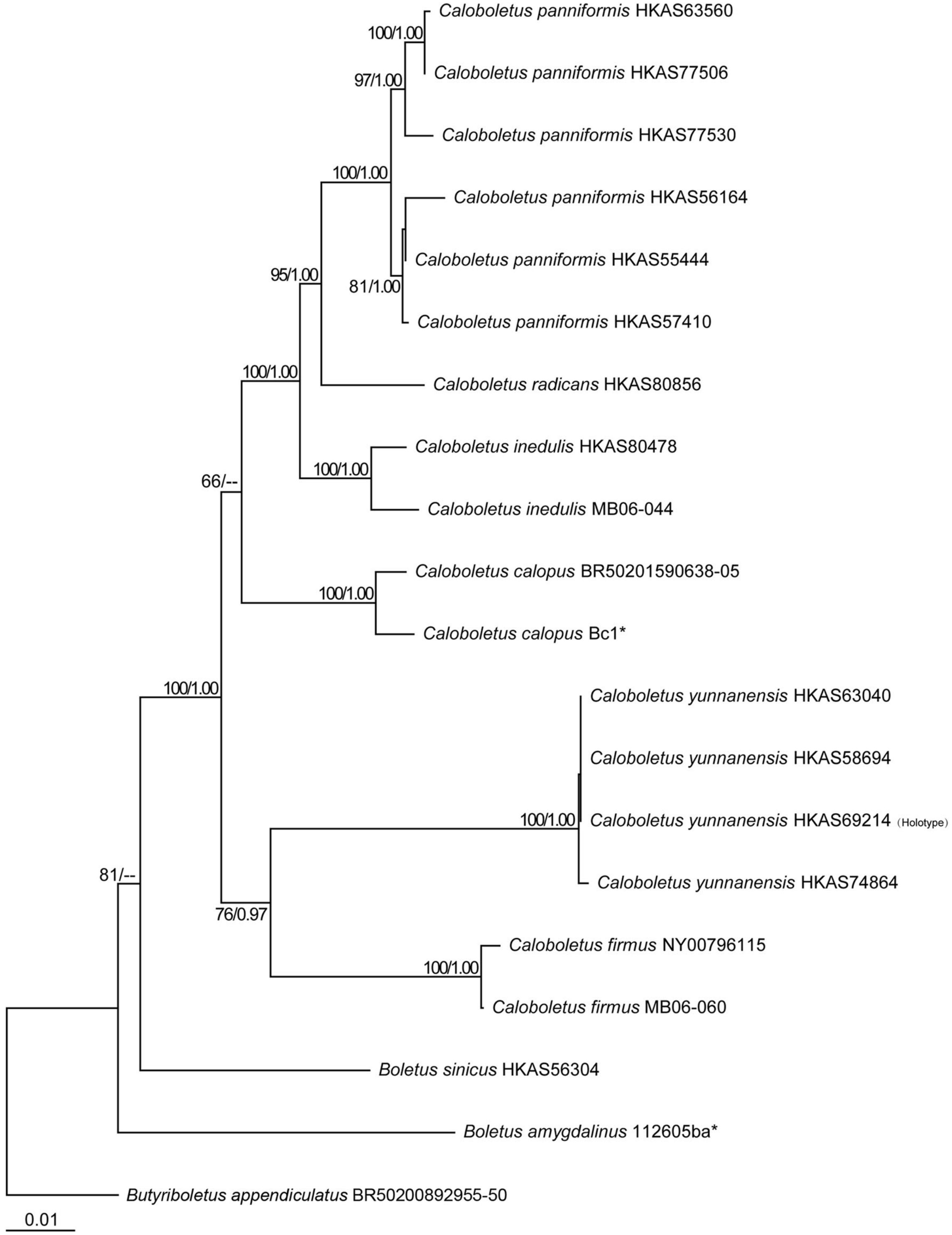

Fig. 1 Maximum-Likelihood phylogenetic tree generated from combined analysis based on DNA sequences of four molecular markers (ITS, nrLSU, *tef1*-α and *rpb1*). Support values >50 % BS for ML and PPs > 0.95 from BI are indicated along nodes

24 Jun 1993, X.L. Wu 4190 (HKAS 29249, as *B. calopus* by Zang, 2006); Sichuan Province, Kangding County, in a mixed forest, alt. 3700 m, 30 Aug 1984, M.S. Yuan 870 (HKAS 15556); the same place, in a mixed forest of *Abies*, *Betula* and *Quercus semecarpifolia*, alt. 3300 m, 9 Aug 1996, M.S. Yuan 2644 (HKAS 31098, as *B. calopus* by Zang, 2006); Xizang Autonomous Prefecture, Mangkang County, 16 Jul 2009, B. Feng 462 (HKAS 57191); Yunnan Province, Binchuan County, in a mixed forest dominated by *Pinus yunnanensis*, alt. 2280 m, 20 Sep 2012, K. Zhao 180 (HKAS 77530); Deqin

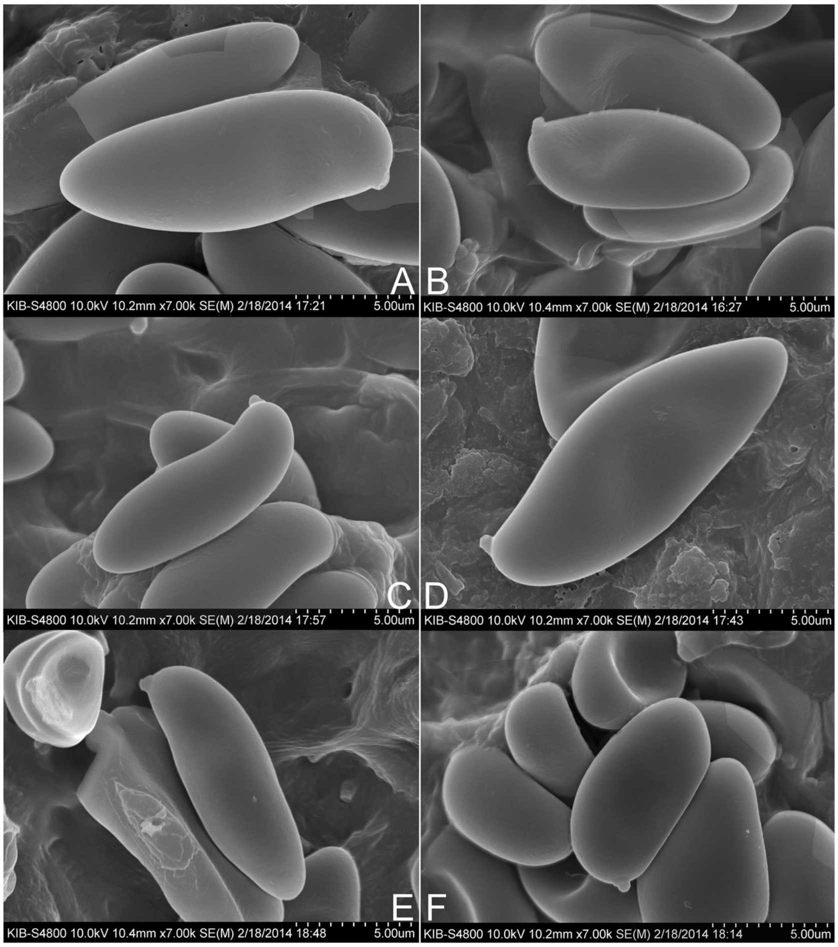

Fig. 2 Spores of *Caloboletus* under SEM. **a**. *C. calopus* (BR 50201590638-05). **b**. *C. firmus* (NY 00796115). **c**. *C. inedulis* (NY 1034406). **d**. *C. panniformis* (HKAS 57410). **e**. *C. radicans* (HKAS 80856). **f**. *C. yunnanensis* (HKAS 69214, holotype)

County, in a forest dominated by *Abies* and *Picea*, alt. 3600 m, 19 Aug. 2008, B. Feng 333 (HKAS 55444); the same place, in a forest dominated by *Abies*, alt. 3500 m, 3 Aug 2013, K. Zhao 296 (HKAS 80721); Kunming City, Xishan, 29 Jul 1994, L.Y. Yang 94048 (HKAS 29341, as *B. calopus* by Zang, 2006); Shangri-la County, in a mixed forest dominated by *Abies* and *Rhododendron*, alt. 4100 m, 08 Aug 2009, B. Feng 617 (HKAS 57346); the same place, in a forest of *Picea*, 24 Aug 2009, B. Feng 654 (HKAS 57383); the same place, in a forest of *Picea likiangensis*, alt. 3750 m, 25 Aug 2009, B. Feng 681 (HKAS 57410); the same place and same time, G. Wu 137 (HKAS 57669); Simao County, alt. 1680 m, 24 Jun 2000, M. Zang 12491 (HKAS 36338, as *B. calopus* by Zang, 2006); Yulong County, in a forest of *Abies*, 20 Jul 2008, Y.C. Li 1310 (HKAS 56164); the same place, in a mixed forest dominated by *Abies* and *Quercus*, alt. 2490 m, 12 Jul 2010, Y.J. Hao 32 (HKAS 68004); the same place, under *Pinus yunnanensis*, alt. 2600 m, 20 Aug 2010, G. Wu 329 (HKAS 63560); the same place, under *Pinus yunnanensis*, alt. 2500 m, 16 Sep 2012, K. Zhao 153 (HKAS 77506).

Notes: *Caloboletus panniformis* was recently described from Japan. Our data indicated that this taxon is a common species widely distributed in China.

5 ***Caloboletus radicans*** (Pers.) Vizzini, Index Fungorum 146:1, 2014.

Basionym: *Boletus radicans* Pers., Synopsis methodica fungorum:507, 1801.

Versipellis radicans (Pers.) Quél., Enchiridion Fungorum in Europa media et praesertim in Gallia Vigentium:158, 1886.

Materials examined: BELGIUM, Meise, Nationale Plantentuin, in a forest of *Abies*, alt. 3000 m, 11 Aug 2008, O. van de Kerckhove 1053 (BR 50201669036-37); FRANCE, Institut National de la Recherche Agronomique (INRA) Centre de Nancy, in a forest of oak, alt. 40 m, 26 Aug 2013, J Xu 2 (HKAS 80856).

Notes: *Versipellis* Quél. (1886) was typified by *Boletus edulis*, which is the type species of *Boletus* s. str.

6 ***Caloboletus yunnanensis*** Kuan Zhao and Zhu L. Yang, **sp. nov**.

MycoBank: MB 809303

Figure 3a–b, 4a–f.

Etymology: Named after Yunnan, where the type was collected.

Holotype: CHINA, Yunnan Province, Baoshan City, Longyang District, in a forest of *Pinus yunnanensis*, 1,860 m, 11 Aug 2010, Y.J. Hao 230 (HKAS 69214).

Fig. 3 *Caloboletus yunnanensis* in its natural habitat (holotype) **a**. Mature basidiomata. **b**. Bluish color changes when injured (image taken immediately after sectioning). Bars: a–b=1 cm

Basidiomata (Fig. 3) 5–10 cm in diameter, hemispherical to convex, margin inrolled slightly; surface dry, covered with fibrillose to tomentose squamules, ochreous (4D7–8) to pallid yellow (5C5–6) when mature; context 1.5–2.5 cm thick, pink (11A2–3) to reddish (12A2–3), the lower part becoming light blue quickly when injured, then back to original color slowly. Hymenophore adnate around stipe, yellowish brown (3A6–7) to pale yellow (4B7–8) when mature, changing into blue quickly when injured, then back to original color slowly; pores angular, 0.35–0.5 mm board, densely arranged; tubes 5–8 mm in length, becoming blue quickly when bruised. Stipe 3–6×1–1.8 cm, subcylindrical, tapering upwards, yellowish grey (5B2–3) to grey (5B1) to grayish (1B1) but becoming purplish (8B3–4) upwards, apical part yellowish (2A4–5) to yellow (3A5–6), smooth, unchanging in color when injured; mycelia at the base of the stipe white; context red- brown, becoming bluish slowly when injured, then back to original color slowly. Odor not distinctive. Taste is bitter.

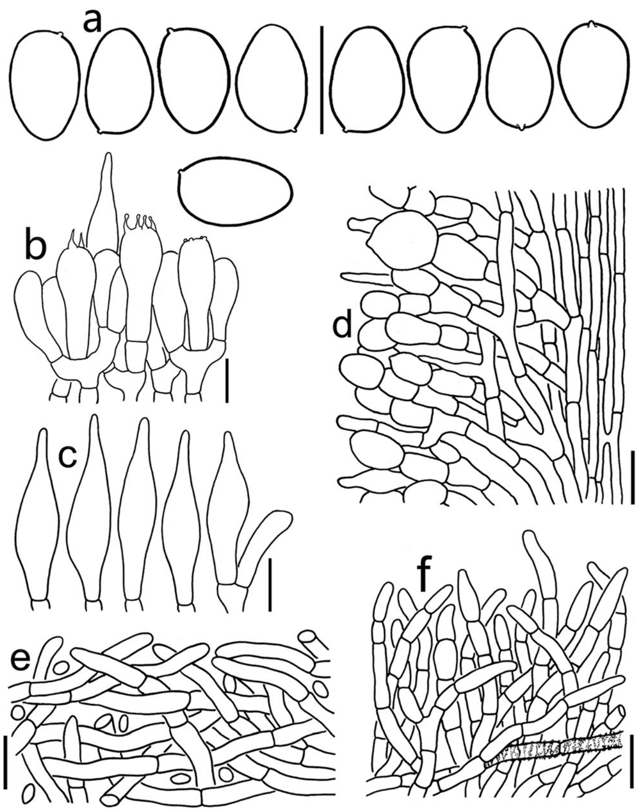

Fig. 4 Microscopic features of *Caloboletus yunnanensis* (holotype). **a**. Basidiospores. **b**. Basidia and pleurocystidium. **c**. Cheilocystidia. **d**. Stiptipellis. **e**. Interwoven pileipellis from the margin of the surface. **f**. Trichodermatous pileipellis from the center of the surface. Bars: a–c=10 μm; d–f=20 μm

Basidiospores (Fig. 4a) [120/5/4] (7) 8.5–9 (10.5)×(5) 6.5–7 (8) μm, [*Q*=(1.67) 1.77–1.82 (2.13), ***Q***=1.81±0.02], ovoid-ellipsoid, subhyaline in KOH and yellowish brown in Melzer's reagent. Basidia (Fig. 4b) 22–30×8.5–12 μm, clavate, hyaline in KOH and yellowish in Melzer's reagent, four-spored, sometimes two-spored. Hymenophoral trama boletoid. Cheilocystidia (Fig. 4c) smooth and thin-walled, hyaline in KOH, narrowly lageniform to lageniform, 27–42×6–9 μm, inamyloid; pleurocystidia similar with cheilocystidia in shape and size, but rarely seen. Pileipellis (Fig. 4e–f) interwoven at the margin but irregular, densely trichodermatous in the center of the surface, hyphae 4–7 μm in diameter, smooth, thin-walled, hyaline, sometimes with brown extracellular pigments; pileal trama composed of interwoven hyphae 3.5–5 μm in width. Stipitipellis (Fig. 4d) composed of ellipsoid, subglobose to broadly clavate inflated cells in short chains; stipe trama composed of vertically arranged hyphae. Clamp connections absent in all tissues.

Habitat and distribution: Solitary to scattered under *Pinus yunnanensis* or in mixed forests dominated by *P. yunnanensis*. Currently only known from Yunnan Province, southwestern China.

Additional specimens examined: CHINA, Yunnan Province, Baoshan City, in a mixed forest dominated by *P. yunnanensis*, alt 1450 m, 9 Aug 2009, L.P. Tang1220 (HKAS 63040); Dali City, Nanjian County, in a coniferous forest, alt. 2000 m, 28 Jul 2009, Q. Cai 27 (HKAS 58694); Nujiang City, Lushui County, Chongren Village, in a mixed forest dominated by *P. yunnanensis*, alt. 1800 m, 7 Aug 2011, G. Wu 550 (HKAS 74864).

Notes: *Caloboletus yunnanensis* is characterized by its basidioma with a smooth stipe and unique spores with an inconspicuous suprahilar depression, and a symbiotic relationship with *Pinus yunnanensis*. This species is somewhat similar to *C. inedulis*, as they both have a yellow brown pileus and relatively slender stipe. However, the spores of *C. inedulis* are typically boletoid, with a conspicuous suprahilar depression and there are reddish reticula on its stipe.

For the convenience of identification of the species, a key is given below.

Key to the species in *Caloboletus*

1 Stipe smooth; spores boletoid, with obvious suprahilar depression..2
1 Stipe with red or yellow reticula; spores ovoid-ellipsoid, with an inconspicuous suprahilar depression .... *C. yunnanensis*
2 Pileus surface smooth or tomentose; in coniferous or broad-leaved forests; in Europe or America 3
2 Pileus surface felty-tomentose to velvety; in coniferous forest, such as *Abies* spp., *Picea* spp. and *Pinus yunnanensis*; in East Asia .... *C. panniformis*
3 Stipe with whitish or reddish reticula, without rooting 4
3 Stipe with yellowish reticula, often rooting ... *C. radicans*
4 Hyphae of the context amyloid 5
4 Hyphae of the context inamyloid .... *C. inedulis*
5 Pore surface yellow to pallid yellow; spores larger (9–19×5–6 μm); in Europe .... *C. calopus*
5 Pore surface red to red orange; spores smaller (9–15×3.5–5 μm); in North and Central America .... *C. firmus*

Discussion

Our phylogenetic analyses based on four gene markers (ITS, nrLSU, *tef1-α* and *rpb1*) recognized six distinct species within the genus *Caloboletus*. Morphologically, the genus is characterized by the combination of the yellow hymenophore, smooth spores which are olive-brown in deposit, an interwoven pileipellis, a bluish color-change and a distinctly bitter taste of the context.

The bitter taste is an important feature to recognize *Caloboletus*. The compounds responsible for the bile-like bitterness of *Caloboletus* are calopins and cyclocalopins (Hellwig et al. 2002). Vizzini (2014) listed 11 species in the genus *Caloboletus*. The systematic positions of five of the species have been verified in our study. In addition, we have added a new species, *C. yunnanensis*, in the genus. Among the six remaining taxa, *B. peckii* Frost should not be placed in *Caloboletus*. Although *B. peckii* tastes bitter, its context turns blue when exposed and then becomes light red (Smith and Theirs 1971; Bessette et al. 2000). Furthermore, recent phylogenetic analyses indicated that this species didn't nest within the clade comprising *B. calopus* and its allies (Nuhn et al. 2013; Wu et al. 2014).

Additionally, a species originally described from western America, i.e., *B. frustosus* Snell and Dick (≡*B. calopus* var. *frustosus* (Snell and Dick) Miller and Watling) may be a member of *Caloboletus*. However, due to the lack of well dried specimens for molecular phylogenetic analyses, its systematic positions can only be settled in the near future.

Geographically, members of *Caloboletus* are mainly distributed in subtropical and temperate zones in the Holarctic and often associated with plants of Pinaceae and Fagaceae. It is interesting to note that species of *Caloboletus* show distinct geographical distribution patterns. *Caloboletus calopus* and *C. radicans*, have only been found in Europe so far, while *C. firmus* and *C. inedulis* are restricted in North and Central America. *Caloboletus panniformis* and *C. yunnanensis* have only been found in East Asia (Taneyama and TakaHashi 2013; the present work). It is noteworthy that *C. panniformis* not only grows in highland forests dominated by *Abies* and/or *Picea*, but also can be found under *Pinus yunnanensis*. Interestingly, populations with different hosts were nested into two subgroups within the *C. panniformis* clade (Fig. 1), suggesting that the host specificity may contribute to their ongoing divergence. The intraspecific variation between the two sublineages is morphologically mainly reflected in the size of the spores. Spores of populations associated with *Pinus yunnanensis* ([80/4/4] 11–13 (15)×3.5–4.5 (5.0) μm) are smaller than those with *Abies* and/or *Picea* in highland forests ([100/5/5] (12) 13–16 (19)×(4) 5–6 (6.5) μm; see also Taneyama and TakaHashi 2013). Based on the description provided by Das (2013), the Indian "*B. rubripes*" should be *C. panniformis*.

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