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Molecular phylogenetic analyses redefine seven major clades and reveal 22 new generic clades in the fungal family *Boletaceae*

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Abstract Mushrooms in the basidiomycete family *Boletaceae* are ecologically and economically very important. However, due to the morphological complexity and the limited phylogenetic information on the various species and genera of this fungal family, our understanding of its systematics and evolution remains rudimentary. In this study, DNA sequences of four genes (nrLSU, *tef1-α*, *rpb1*, and *rpb2*) were newly obtained from ca. 200 representative specimens of *Boletaceae*. Our phylogenetic analyses revealed seven major clades at the subfamily level, namely *Austroboletoideae*, *Boletoideae*, *Chalciporoideae*, *Leccinoideae*, *Xerocomoideae*, *Zangioideae*, and the *Pulveroboletus* Group. In addition, 59 genus-level clades were identified, of which 22 were uncovered for the first time. These 22 clades were mainly placed in *Boletoideae* and the *Pulveroboletus* Group. The results further indicated that the characters frequently used in the morphology-based taxonomy of *Boletaceae*, such as basidiospore ornamentation, the form of the basidioma, and the stuffed pores each had multiple origins within the family, suggesting that the use of such features for high-level classification of *Boletaceae* should be de-emphasized and combined with other characters.

Keywords *Boletales* · Chemotaxonomy · Convergent evolution · Morphological characters · Multi-gene analyses · New subfamilies

Introduction

Mushrooms in the basidiomycete family *Boletaceae* are mainly characterized by fleshy context and a tubulose, rarely lamellate or loculate hymenophore. They are extraordinarily diverse; about 50 genera and 800 species have been identified in this family (Kirk et al. 2008), but the numbers of species and genera are likely to be higher because large parts of the tropics and the subtropics remain little studied. Many mushrooms in *Boletaceae* have very attractive ornamentations on the pileus, and further ornamentation on the stipe, and/or a diversity of color change in the basidioma when bruised or cut (Smith and Thiers 1971). Some of these boletes have great economic, dietary, and health value. For example, *Boletus edulis* Bull. sensu lato (Porcini) is a gourmet mushroom highly prized in many parts of the world (Feng et al. 2012). According to data from the Bolete Association of Yunnan Province, over 10,000 t of fresh boletes were exported from Yunnan, China to Europe or other regions in 2010. In contrast, some other boletes, such as *B. satanas* Lenz (Devil's mushroom), *B. venenatus* Nagas., *Heimioporus japonicus* (Hongo) E. Horak, and a few species of *Pulveroboletus* Murrill are poisonous, predominantly causing gastrointestinal symptoms of nausea and violent vomiting if eaten raw or fried (Kretz et al. 1991; Matsuura et al. 2007; Sun et al. 2012). Ecologically, most species of *Boletaceae* are important ectomycorrhizal (ECM) fungi in the ecosystem and can form ECM relationships with plants of more than 10 families

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(Thoen and Bâ 1989; den Bakker et al. 2004; Sato et al. 2007; Smith and Pfister 2009; Becerra and Zak 2011; Henkel et al. 2012; Husbands et al. 2013).

The systematics studies of *Boletaceae* have long attracted the attention of mycologists from different parts of the world, with most such studies focused on morphological and/or chemical features (Snell 1941; Smith and Thiers 1971; Corner 1972; Pegler and Young 1981; Singer 1986; Høiland 1987; Watling and Li 1999; Li and Song 2000; Binder and Bresinsky 2002b; Zang 2006; Horak 2011). However, the understanding of morphological evolution in the family remains rudimentary. The morphology-based taxonomy for the family has long been controversial and continues to raise questions. For instance, at higher taxonomic levels, the basidiospore ornamentation in *Boletaceae* was supposed to evolve as a single event and was used to distinguish a group with such ornamentation (subfamily *Strobilomycetoideae* (E.-J. Gilbert) Snell) from groups with smooth basidiospores (subfamily *Boletoideae* and *Xerocomoideae* Singer) (Pegler and Young 1981). However, this treatment was not supported by molecular phylogenetic data (Binder and Hibbett 2007; Nuhn et al. 2013). At lower taxonomic levels, it was suggested that *Xerocomus* Quél. s.l. shared the character of the *Phylloporus*-type hymenophoral trama with the genus *Phylloporus* Quél. (Singer 1986). However, molecular phylogenetic analyses revealed that *Xerocomus* s.l. was polyphyletic. Some species, such as *X. chrysenteron* (Bull.) Quél., *X. badius* (Fr.) E.-J. Gilbert, and *X. parasiticus* (Bull.) Quél., are distinct from *Xerocomus* s.s. and are clustered in different major groups, even though they have the *Phylloporus*-type hymenophoral trama (Binder and Hibbett 2007; Nuhn et al. 2013). Similarly, the phylogenetic relationships among species of *Boletus* L. s.l. or *Tylopilus* P. Karst. s.l. are not completely resolved. Moreover, more and more genera with sequestrate basidiomata were described or proven to be related to *Boletaceae* or *Boletales* E.-J. Gilbert (Yang et al. 2006; Orihara et al. 2010; Halling et al. 2012b; Lebel et al. 2012; Orihara et al. 2012; Trappe et al. 2013). Yet the phylogenetic relationships among these genera in the family scale and the evolution of the sequestrate forms within *Boletaceae* have not been fully clarified, although previous work has shown that sequestrate forms have evolved independently several times from the boletoid ancestors (Binder and Hibbett 2007).

The recent rapid developments of DNA-sequencing techniques and phylogenetic analysis have enabled mycologists to overcome difficulties in fungal taxonomy and systematics, to resolve the phylogeny of fungi, and to elucidate the morphological, ecological, and functional evolution of fungi (Yang 2011). For example, in relation to the boletes, Binder and Hibbett (2007) used a combined matrix of DNA sequences at 5.8S, nrLSU, mtLSU, and *atp6* to reveal six major lineages within *Boletales* at the subordinal level and to estimate the evolution of morphology and ecology of these seven suborders and related inner nodes. Wilson et al. (2012) clarified the diversity and evolution of ectomycorrhizal host associations in the suborder *Sclerodermatineae* Manfr. Binder & Bresinsky. Nuhn et al. (2013) elucidated generic relationships in the suborder *Boletineae* using nrLSU, *tef1*-α, and *rpb1* sequences, and identified 17 clades with four major groups in the family *Boletaceae*, and discussed the morphological characters of each clade. However, the phylogenetic positions of some genera within the *Boletaceae*, such as *Austroboletus* (Corner) Wolfe, *Heimioporus* E. Horak, *Pulveroboletus*, *Sinoboletus* M. Zang, and *Sutorius* Halling et al., and the relationships among many clades have still not been adequately addressed.

Several factors may have contributed to our difficulties in understanding the systematics and phylogenetic relationships among different genera in the *Boletaceae*. First, the convergent evolution of morphological traits (e.g. macromorphological features of basidiomata and micromorphological features of basidiospores) may be common and could mask the extensive biodiversity and increase the phenotypic plasticity in fungi (Binder and Bresinsky 2002a; Hibbett and Binder 2002; Hosaka et al. 2006; Binder and Hibbett 2007; Justo et al. 2010; Wilson et al. 2011). Second, geographic distribution and ectomycorrhizal associations may have significantly influenced speciation rates of higher fungi, making the rates highly variable among lineages and ecological niches (Wang and Qiu 2006; Halling et al. 2008). Third, limited numbers of genera and species were analyzed and less informative genetic markers (nrLSU, mtSSU, etc.) for molecular phylogenetic analyses were used in these previous studies (e.g. Binder and Hibbett 2002, 2007; Matheny et al. 2007; Drehmel et al. 2008; Halling et al. 2012a, b).

To overcome these difficulties and to better understand the phylogeny and morphological evolution within *Boletaceae*, we selected the following four highly informative genetic markers for our molecular analyses: the nuclear ribosomal large subunit (nrLSU), the genes encoding the largest subunit of RNA polymerase II (*rpb1*), the second-largest subunit of RNA polymerase II (*rpb2*), and the translation elongation factor 1α (*tef1-α*). Using samples gathered from many parts of China, together with samples obtained from other parts of the world and sequences available in the GenBank database, our study aims to: 1) establish a phylogenetic framework for *Boletaceae*, and 2) assess the origins and paths of evolution of several key morphological/chemical characters within *Boletaceae*.

Materials and methods

Sample collection

Representative species of the known genera of *Boletaceae*, including as many generic type species as possible, were selected for the study. Voucher specimens made in the last

10 years from China are kept in the Cryptogamic Herbarium (HKAS-KUN) of the Kunming Institute of Botany, Chinese Academy of Sciences. Whenever possible, representative samples from other parts of the world, such as northern/central America (USA, Costa Rica), Europe (Italy, Germany), Australia (New Zealand), and southern Asia (Bangladesh), were also included in our analyses. Over 900 specimens were first screened, which represented 29 known genera, several putative novel lineages, and about 290 species. Duplicate specimens within each species were identified in the initial screening and one to six representatives for each lineage were selected for further analyses. Finally, 192 representative specimens of ca.180 species were included in our further molecular phylogenetic analyses, with the sequence data for 95 species taken mainly from Nuhn et al. (2013) and Halling et al. (2012a, b). In total 39 known genera were included in this study, representing over three-quarters of all known genera in the *Boletaceae*. Information on these samples is detailed in Table S1.

Morphological studies

The macro-morphological characters were taken from detailed field notes and photographs of fresh basidiomata. The color change of the context when exposed, the spore ornamentation, the form of the basidioma, and the presence or absence of stuffed pores were all noted and recorded. Micromorphological features including pileipellis, basidia, basidiospores, pleuro- and cheilocystidia, and stipitipellis were obtained using an Axioskop 40 microscope following the standard method described in previous studies (Li et al. 2011; Zeng et al. 2012, 2013; Hosen et al. 2013). To observe spore ornamentations, small hymenophoral fragments were taken from dried specimens. The fragments were mounted on aluminum stubs with double-sided adhesive tape, coated with gold palladium, and then observed under a Hitachi S4800 or a JEOL JSM-6510 scanning electron microscope (SEM) in accordance with the previous studies by Zeng et al. (2012, 2013) and Hosen et al. (2013).

DNA isolation, PCR, sequencing, and dataset assembly

Genomic DNA was extracted from silica-gel dried or herbarium materials using the CTAB method (Doyle and Doyle 1987). A total of four nuclear loci were sequenced for this study, including three protein-coding gene fragments (*rpb1*, *rpb2*, and *tef1*-α) and one non-protein coding region (nrLSU). The primer pair LROR/LR5 was used to amplify the nrLSU fragments. For the amplifications of *rpb1*, *rpb2*, and *tef1*-α, the commonly used primer pairs RPB1-Af/fRPB1-Cr, bRPB2-6F/bRPB2-7.1R, EF1-F/EF1-R, and EF1-595F/EF1-1160R (Matheny et al. 2002; Mikheyev et al. 2006; http://faculty.washington.edu/benhall/) were used first. However, we were unable to obtain PCR products from many specimens using these primers. Instead, new primers were designed either manually or using Primer 3 (version 0.4.0) (Rozen and Skaletsky 2000) based on sequences available in GenBank and the sequences newly generated in this study. The fragment lengths amplified using these new primers were designed to be around 800–900 bp. The nucleotide sequences and binding sites for these new primers are presented in Table 1 and Fig. 1, respectively. The PCR reaction was conducted on an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) or an Eppendorf Master Cycler (Eppendorf, Netheler-Hinz, Hamburg, Germany) under the following conditions: 94 °C for 4 min, then 35 cycles of 94 °C for 60 s, 53 °C for 60 s, and 72 °C for 80 s, followed by a final extension step of 72 °C for 8 min. The PCR products were purified with a Gel Extraction & PCR Purification Combo Kit (Spin-column) (Bioteke, Beijing, China), and then sequenced on an ABI-3730-XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using the same primers as in the PCR amplification. The products that failed to be sequenced directly were cloned into a PMD18-T vector (Takara, Japan) and then sequenced with primers M13F (5′-GTAAAACGACGGCCAGTGAA-3′) and M13R (5′-CAGGAAACAGCTATGACCAT-3′). The contiguous sequences were assembled with SeqMan implemented in Lasergene v7.1 (DNASTAR Inc., USA).

In this analysis, two datasets of nrLSU were constructed for different types of analysis. For the first dataset (the complete nrLSU dataset), the sequences generated in this study were combined with the representative sequences of *Boletaceae* retrieved from GenBank. This dataset was used to identify the relationships among all of our samples and known related samples in the GenBank (data not shown). The second dataset (the “condensed dataset”) contained only the representative sequences obtained through the selection criteria used for the condensed *rpb*1 dataset (see below). This condensed nrLSU dataset was later concatenated with condensed datasets of *rpb1*, *rpb2*, and *tef1*-α to form the four-gene dataset for final combined analyses. For the *rpb1* sequences, two datasets were used for different purposes. The complete *rpb1* dataset contained all of the sequences obtained in this study and was used to identify lineages below generic rank in our samples, as *rpb1* could provide more parsimony-informative characters for phylogenetic studies of boletes (Dentinger et al. 2010). One to six representatives of each lineage were then chosen to form three condensed datasets, respectively, one for each of the three protein-coding genes *rpb1*, *rpb2*, and *tef1*-α. Each dataset was separately aligned with MAFFT v6.853 using the E-INS-i strategy (Katoh et al. 2002) and was shown in Bioedit v7.0.9 (Hall 1999). The concatenation of the sequences of the four gene markers was completed in Phyutility 2.2 (Smith and Dunn 2008). The fragments of some gene markers of several taxa could not be sequenced and were therefore coded as

Table 1 PCR and sequencing primers for *tef1*-α, *rpb1* and *rpb2*

Primer name	Nucleotide sequence 5′–3′
EF1-B-F1	AGC ATG GGT KCT CGA YAA GCT
EF1-B-F2	CCC AAG TWC ATG GTY ACK GT
EF1-B-R	CG TGR TGC ATT TCY ACG GAA
RPB1-B-F	CGC GTT TTC GRT CGC TTG AT
RPB1-M-F(for *B*[a]. *mirabilis* only)	CT CCC AAA GGT TTC ATC GTC
RPB1-T-F(for *A*[b]. *thibetanus* only)	GTC CTC CCA TCT CGA GGT T
RPB1-B-R	AT ACT YGG GCG RAC RGG RGG
RPB1-M-R(for *B. mirabilis* only)	AC GAA TGG CTT TCA CAG GAC
RPB1-T-R(for *A. thibetanus* only)	CAC GCA GAA TGG CGT TAG TA
RPB2-B-F1	AAG ATY GCY AAG CCT CGT CA
RPB2-B-F2	GAA GGA CAR GCT TGY GGT CT
RPB2-B-R	AAG ATR TTG GCC ATS GTG TCC

Notes: 1) [a] *Boletellus*, [b] *Aureoboletus*; 2) F: Forward primer, R: Reverse primer; 3) "B" in the middle of the primers' name means the primer is designed for all species in family *Boletaceae*, "M" means the primer is only for *Boletellus mirabilis* clade and "T" means the primer is only for *Aureoboletus thibetanus* clade. 4) Binding sites of these primers are shown in Fig. 1

missing data. The intron regions of protein-coding genes were retained in the final analyses, but hypervariable, indel-rich regions were detected and excluded by using Gblocks 0.91b (Castresana 2000) with a less stringent selection of the following settings: minimum number of sequences for a conserved position: 146; minimum number of sequences for a flank position: 146; maximum number of contiguous nonconserved positions: 8; minimum length of a block: 2; allowed gap positions: with half. To calculate the genetic distances (Kimura-2-parameter distance (K2P)) of inter-subfamilies/intra-subfamily and inter-genera/intra-genus, the sequences of exons of three protein-coding genes with the same length were conducted with MEGA 5.05 software (Tamura et al. 2011).

Phylogenetic analyses

The best-fitted substitution model for each gene marker was determined through MrModeltest v2.3 (Nylander 2004) by using Akaike Information Criterion (AIC). GTR+I+G was chosen as the best model for nrLSU, *tef1*-α, and *rpb1* whereas SYM+I+G was selected as the best model for *rpb2*. Phylogenetic analyses using a Maximum Likelihood (ML)

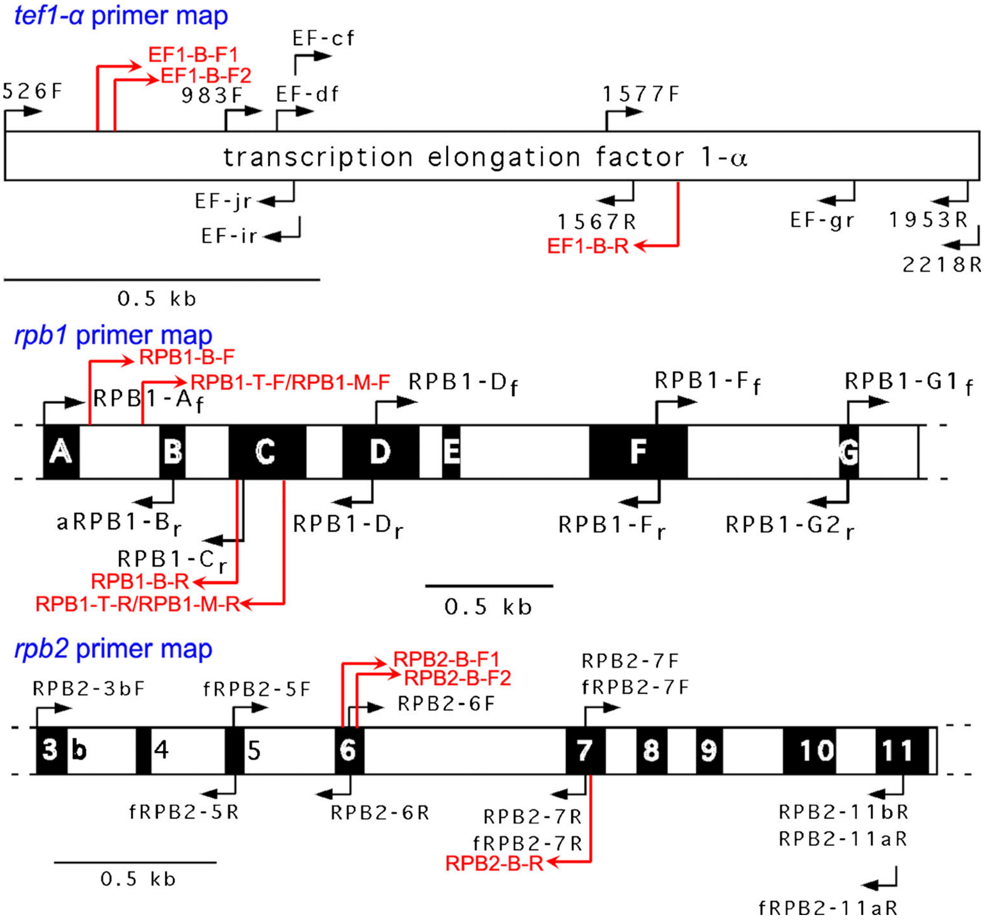


Fig. 1 Binding sites for newly designed primers of *tef1*-α, *rpb1*, and *rpb2* based on http://www.clarku.edu/faculty/dhibbett/Protocols_Folder/Primers/Primers.pdf, shown by the *red arrows* and *letters*

analysis and Bayesian Inference (BI) were subsequently conducted on RAxML v7.2.6 (Stamatakis 2006) and MrBayes 3.2.2 (Ronquist and Huelsenbeck 2003), respectively. For the condensed matrices, single-gene analyses were conducted to assess incongruence among individual genes using the ML method (results not shown). Because no well-supported (BS>70 %, Nuhn et al. 2013) conflict was detected among the topologies of the four genes, their sequences were then concatenated together for further multi-gene analyses. For the four-gene datasets, a partitioned mixed model, with model parameters estimated separately for each gene, was used by defining the sequences of nrLSU, *tef1*-α, *rpb1*, and *rpb2* as four partitions. For the ML analyses, all of the parameters were kept at their default settings, except that the model was set as GTRGAMMAI (Stamatakis 2006), and statistical supports were obtained using nonparametric bootstrapping with 1000 replicates. For the BI analyses, four chains were processed with the generation set as 10 million using the selected model for each gene. The trees were sampled every 100 generations. 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. The stop rule was used when parallel MCMC runs converged (ESS value>200). Finally, 15 million generations were taken to reach the convergence. The trees were summarized and statistical values were obtained using the sump and sumt commands with burn-ins (i.e. the first 25 % of the samples) discarded.

Reconstruction of ancestral character state

To understand the evolution of some key morphological and nutritional characters in *Boletaceae*, most recent common ancestor (MRCA) analyses of three characters, namely basidiospores ornamentation (smooth, ornamented), the form of the basidiomata (stipitate-pileate, gasteroid, secotioid), and the presence or absence of stuffed pores, were performed using BayesMultiState implemented in Bayestraits V1.0 with the MCMC method (i.e. the hyperprior approach) (Pagel et al. 2004). Exponential distribution was selected for the prior and the Rate Dev was set to five or six to ensure acceptance rates of around 20–40 %. Five million iterations were conducted. The probabilities of MRCA states at eight nodes were estimated, including the root node of the family *Boletaceae* and seven major nodes within the *Boletaceae*. Each of the eight nodes supported a group that was resolved as monophyletic with high support, except for the *Pulveroboletus* Group (BS = 55 %, PP < 0.90). The phylogenetic tree used in this analysis was obtained from the ML analysis. Four independent files with the status (coded as 0, 1, 2) of the four characters were constructed for all samples, and probabilities (P=0–1) of each status were represented as arithmetic means.

Results

Phylogenetic analyses

In total 716 sequences generated in this study were included in the four-gene dataset, of which 190, 177, 166, and 183 sequences were obtained for nrLSU, *tef1*-α, *rpb1*, and *rpb2*, respectively. An additional 242 relevant sequences available in GenBank were also retrieved and combined with our own data for the analyses. Those sequences were mainly generated by Nuhn et al. (2013) and Halling et al. (2012a, b) from 95 samples of 95 species of *Boletales* with complete or partial sequences of the four genes mentioned above. The full alignment of the combined data was submitted to the TreeBASE (15253).

The aligned four-gene matrix contained a total of 290 samples and 3,530 aligned bases. Of the 3,530 aligned bases, 2,740 were retained using Gblocks for the final analyses. The ML and BI analyses generated almost identical tree topologies with minimal variation in statistical support values, thus a ML tree was selected for the purposes of display (Fig. 2). In multi-gene analyses, the monophyly of *Boletaceae* was strongly supported by both bootstrap value (BS = 97 %) and posterior probability (PP = 0.99). The family *Paxillaceae* Lotsy was inferred as the closest sister group of *Boletaceae*, again receiving high statistical support (BS = 100 %, PP = 0.99). In total seven major clades could be identified in the family *Boletaceae* (Fig. 2). Two of these seven clades corresponded to the subfamilies *Boletoideae* and *Xerocomoideae* Singer, respectively (Singer 1945, 1947). The remaining five major clades are newly defined in this study. They are named as *Austroboletoideae*, *Leccinoideae*, *Zangioideae*, *Chalciporoideae*, and the *Pulveroboletus* Group. All of these major clades were statistically well supported from all data other than the *Pulveroboletus* Group (BS = 55 %, PP < 0.90). *Chalciporoideae* and *Zangioideae* formed the basal and third-basal groups of *Boletaceae*, respectively, whereas the exact phylogenetic relationships among the remaining five major clades remained unresolved. For these seven subfamily-level major clades, the average sequence divergence (K2P distance) within individual major clades was 0.098±0.024 (average value ± SD) whereas the divergence among major clades was significantly higher, at 0.145±0.02. A dichotomous key of morphological features for these seven major clades is shown in the section of "Taxonomy" below.

Within the seven major clades, 59 clades were found, 25 of which were new. Among the 25 new clades, 22 (the clades numbered but unnamed in Fig. 2) were firstly detected in this study. These new clades were mainly placed in *Boletoideae* and the *Pulveroboletus* Group. Most of the new clades were at the generic level, and a few were probably at the subgeneric level. The average intra-/inter-generic sequence divergences (i.e. the K2P distance) of these lineages were 0.056±0.0249/ 0.104±0.03. Information regarding the systematic positions of these 59 clades is detailed in Table 2. The basidiomata of representative species of 25 new clades are shown in Fig. 3. Eleven of the 39 known genera (*Australopilus* Halling & Fechner, *Aureoboletus* Pouzar, *Chalciporus* Bataille, *Heimioporus*, *Phylloporus*, *Retiboletus* Manfr. Binder & Bresinsky, *Rossbeevera* T. Lebel & Orihara, *Strobilomyces* Berk., *Sutorius*, *Xanthoconium* Singer, and *Zangia* Y.C. Li & Zhu L. Yang) are supported as monophyletic groups by our phylogenetic analyses. However, seven genera (*Austroboletus*, *Boletellus* Murrill, *Boletus*, *Leccinum* Gray, *Porphyrellus* E.-J. Gilbert, *Tylopilus*, and *Xerocomus*) are paraphyletic or polyphyletic. *Austroboletus* is split into two lineages (*Austroboletus* s.s. and species in the *Veloporphyrellus* L.D. Gómez & Singer lineage). *Boletus* s.l. is separated into at least 15 lineages (*Boletus* s.s. corresponding to the porcini mushrooms s.l. in Feng et al. (2012), or the "porcini" clade in Nuhn et al. (2013), and Clades 19, 25, 29, 37, 39–41, 43–46, 48, 49, 51). *Tylopilus* s.l. is clustered in at least 11 lineages (*Tylopilus* s.s., *Australopilus*, *Harrya* Halling et al., *Porphyrellus* s.s., *Sutorius*, and Clades 14, 18, 20, 31, 53, and 54), and *Xerocomus* s.l. harbors six lineages (*Xerocomus* s.s., *Hemileccinum* Šutara, *Xerocomellus* Šutara s.s., and Clades 8, 22, and 27). *Boletellus* s.l. is grouped into three lineages (*Boletellus* s.s., Clade 2, and one part of *Aureoboletus*), and *Leccinum* s.l. includes at least three separate lineages (*Leccinum* s.s., *Rossbeevera*/*Leccinellum* Bresinsky & Manfr. Binder, and Clade 47).

In the subfamily *Austroboletoideae*, *Fistulinella* Henn. formed the basal group, followed by *Veloporphyrellus*, *Mucilopilus* Wolfe, Clade 31, and *Austroboletus*, successively. *Austroboletus* may be the latest divergent group in this subfamily. In *Boletoideae*, *Xerocomellus* s.s. and Clades 27 and 29 were the basal groups, *Afroboletus* Pegler & T.W.K. Young and Clade 18 were located with *Strobilomyces*, and *Xanthoconium* clustered with Clade 25. The relationships of other genera/lineages (*Boletus* s.s., *Porphyrellus*, *Tylopilus*, and Clades 19, 20, 22) were not resolved. In *Chalciporoideae*, *Rubinoboletus* Pilát & Dermek could be regarded as synonymous for *Chalciporus* as suggested by Singer (1986), because *R. rubinus* (W.G. Sm.) Pilát & Dermek, the type species of *Rubinoboletus*, closely clustered with *Chalciporus* (Nuhn et al. 2013). *Buchwaldoboletus* Pilát was the basal lineage of this subfamily. In *Leccinoideae*, *Leccinum*, *Leccinellum*, *Rossbeevera*, and *Octaviania* Vittad. clustered together as a monophyletic clade and *Leccinellum* mixed together with *Rossbeevera. Borofutus* Hosen & Zhu L. Yang was a sister group of gasteroid bolete *Spongiforma* Desjardin et al., consistent with Hosen et al. (2013). *Retiboletus* may be the basal group of this subfamily but this is not statistically well supported. In *Xerocomoideae*, *Heimioporus*, together with *Hemileccinum*/*Corneroboletus* N.K. Zeng & Zhu L. Yang, *Aureoboletus*, and Clade 2, formed a monophyletic clade, in which the latter two were sister groups. *Boletellus* could be close to this clade but this lacks effective support values. Furthermore, the type species of *Sinoboletus* was located within *Aureoboletus*, indicating that *Sinoboletus* was a synonym of *Aureoboletus. Xerocomus*, *Phylloporus*, and Clade 8 were closely related. In *Zangioideae*, two monophyletic clades were retrieved; one was composed of *Zangia* alone and the other of *Australopilus*, *Harrya*, *Royoungia* Castellano et al., and Clades 53 and 54. In addition, *Australopilus* and a species from China (HKAS53379) were located very closely with *Royoungia*. In the *Pulveroboletus* Group, Clades 48 and 49 stayed temporarily at the basal position with low statistical support, and the Clades 45, 46, and 47 formed the fourth, third, and second basal groups, respectively, with moderate to high support values. Another well-supported monophyletic clade contained most members of *Boletus* sect. *Luridi* Fr. and sect. *Calopodes* Fr., genera *Pulveroboletus*, and *Sutorius*, but their relationships are still mostly uncovered. For the genera/lineages (*Bothia* Halling et al., *Gymnogaster* J.W. Cribb, *Pseudoboletus* Šutara, *Solioccasus* Trappe et al., and Clade 51) not belonging to the seven major clades, *Pseudoboletus* formed the second basal group in the *Boletaceae*, congruent with Nuhn et al. (2013), whereas the systematic positions of other genera/lineages remained unclear.

Fig. 2 Phylogenetic relationships among representative specimens of *Boletaceae* inferred from a multigene (nrLSU, *tef1-α*, *rpb1*, and *rpb2*) dataset using the Maximum Likelihood and Bayesian Inference methods (only the ML tree is shown). The bootstrap frequencies (≥50 %) and posterior probabilities (PP ≥ 0.90) are shown at the supported branches. The seven major clades of *Boletaceae* are indicated with solid arrows on the left and the 59 clades are labeled with vertical bars on the right. The superscript "a" showed a new species described in Li et al. (2014, in press) based on molecular data ▶

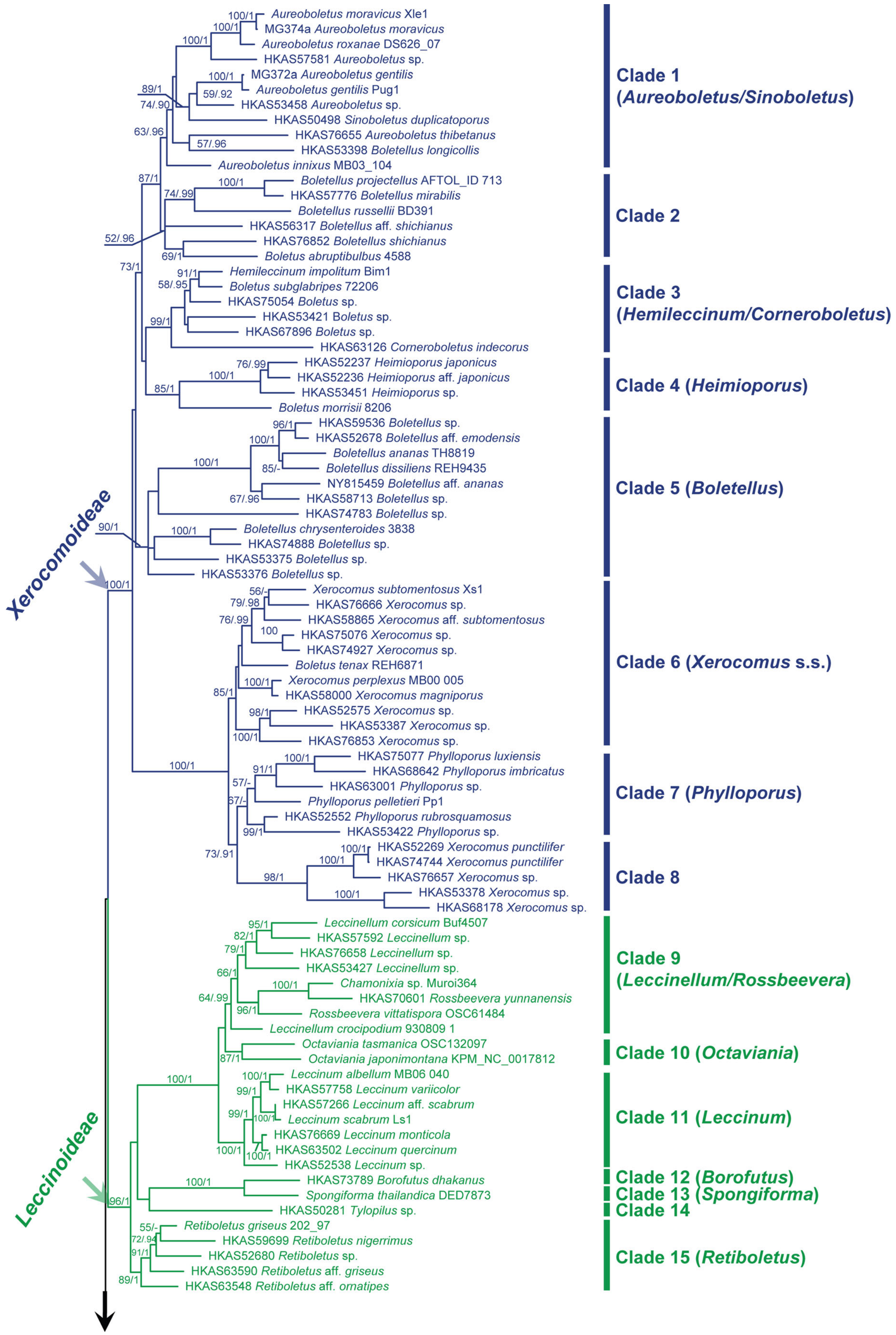
Key morphological characters in *Boletaceae*

Color change when bruised, stuffed pores, and appearance of basidioma

According to field notes (Fig. 4), the color change in the cut or bruised basidioma of boletes was highly diverse and could be partitioned into two major types, bluing and browning-blacking. When the place and speed of color change were considered, varied types could be detected. The stuffed pores occurred in four clades, namely *Boletus* s.s., *Sutorius*, and Clades 25 and 46, and the stipitate-pileate, gasteroid, and secotioid forms of the fruiting body co-existed in this family and evolved multiple times (Fig. 2).

Basidiospores characteristics revealed by scanning electron microscope (SEM)

Eleven major types of basidiospore ornamentations were recognized in the following lineages in the *Boletaceae*: *Strobilomyces*, *Xerocomellus*, *Boletellus*, *Xerocomus*/*Phylloporus*/Clade 8, *Hemileccinum*/*Corneroboletus*,


100/1 Aureoboletus moravicus Xle1
100/1 MG374a Aureoboletus moravicus
100/1 Aureoboletus roxanae DS626_07
HKAS57581 Aureoboletus sp.
100/1 MG372a Aureoboletus gentilis
59/.92 Aureoboletus gentilis Pug1
89/1
74/.90
HKAS53458 Aureoboletus sp.
HKAS50498 Sinoboletus duplicatoporus
63/.96
HKAS76655 Aureoboletus thibetanus
57/.96 HKAS53398 Boletellus longicollis
Aureoboletus innixus MB03_104
Clade 1 (Aureoboletus/Sinoboletus)
87/1
100/1 Boletellus projectellus AFTOL_ID 713
74/.99 HKAS57776 Boletellus mirabilis
Boletellus russellii BD391
HKAS56317 Boletellus aff. shichianus
52/.96
HKAS76852 Boletellus shichianus
69/1 Boletus abruptibulbus 4588
Clade 2
73/1
91/1 Hemileccinum impolitum Bim1
58/.95 Boletus subglabripes 72206
HKAS75054 Boletus sp.
99/1 HKAS53421 Boletus sp.
HKAS67896 Boletus sp.
HKAS63126 Corneroboletus indecorus
Clade 3 (Hemileccinum/Corneroboletus)
76/.99 HKAS52237 Heimioporus japonicus
100/1 HKAS52236 Heimioporus aff. japonicus
85/1 HKAS53451 Heimioporus sp.
Boletus morrisii 8206
Clade 4 (Heimioporus)
96/1 HKAS59536 Boletellus sp.
100/1 HKAS52678 Boletellus aff. emodensis
Boletellus ananas TH8819
100/1
85/- Boletellus dissiliens REH9435
NY815459 Boletellus aff. ananas
67/.96 HKAS58713 Boletellus sp.
HKAS74783 Boletellus sp.
90/1
100/1 Boletellus chrysenteroides 3838
HKAS74888 Boletellus sp.
HKAS53375 Boletellus sp.
HKAS53376 Boletellus sp.
Clade 5 (Boletellus)
Xerocomoideae
100/1
56/- Xerocomus subtomentosus Xs1
79/.98 HKAS76666 Xerocomus sp.
76/.99 HKAS58865 Xerocomus aff. subtomentosus
100 HKAS75076 Xerocomus sp.
HKAS74927 Xerocomus sp.
Boletus tenax REH6871
100/1 Xerocomus perplexus MB00 005
85/1 HKAS58000 Xerocomus magniporus
98/1 HKAS52575 Xerocomus sp.
HKAS53387 Xerocomus sp.
100/1 HKAS76853 Xerocomus sp.
Clade 6 (Xerocomus s.s.)
100/1 HKAS75077 Phylloporus luxiensis
100/1
91/1 HKAS68642 Phylloporus imbricatus
57/- HKAS63001 Phylloporus sp.
67/- Phylloporus pelletieri Pp1
HKAS52552 Phylloporus rubrosquamosus
99/1 HKAS53422 Phylloporus sp.
Clade 7 (Phylloporus)
73/.91
100/1 HKAS52269 Xerocomus punctilifer
100/1 HKAS74744 Xerocomus punctilifer
98/1 HKAS76657 Xerocomus sp.
100/1 HKAS53378 Xerocomus sp.
HKAS68178 Xerocomus sp.
Clade 8
95/1 Leccinellum corsicum Buf4507
82/1 HKAS57592 Leccinellum sp.
79/1 HKAS76658 Leccinellum sp.
66/1 HKAS53427 Leccinellum sp.
100/1 Chamonixia sp. Muroi364
64/.99 HKAS70601 Rossbeevera yunnanensis
96/1 Rossbeevera vittatispora OSC61484
Leccinellum crocipodium 930809 1
Clade 9 (Leccinellum/Rossbeevera)
Octaviania tasmanica OSC132097
87/1 Octaviania japonimontana KPM_NC_0017812
Clade 10 (Octaviania)
100/1
100/1 Leccinum albellum MB06 040
99/1 HKAS57758 Leccinum variicolor
HKAS57266 Leccinum aff. scabrum
99/1
100/1 Leccinum scabrum Ls1
HKAS76669 Leccinum monticola
100/1 HKAS63502 Leccinum quercinum
100/1 HKAS52538 Leccinum sp.
Clade 11 (Leccinum)
100/1 HKAS73789 Borofutus dhakanus
Spongiforma thailandica DED7873
96/1 HKAS50281 Tylopilus sp.
Clade 12 (Borofutus)
Clade 13 (Spongiforma)
Clade 14
Leccinoideae
55/- Retiboletus griseus 202_97
72/.94 HKAS59699 Retiboletus nigerrimus
91/1 HKAS52680 Retiboletus sp.
HKAS63590 Retiboletus aff. griseus
89/1 HKAS63548 Retiboletus aff. ornatipes
Clade 15 (Retiboletus)

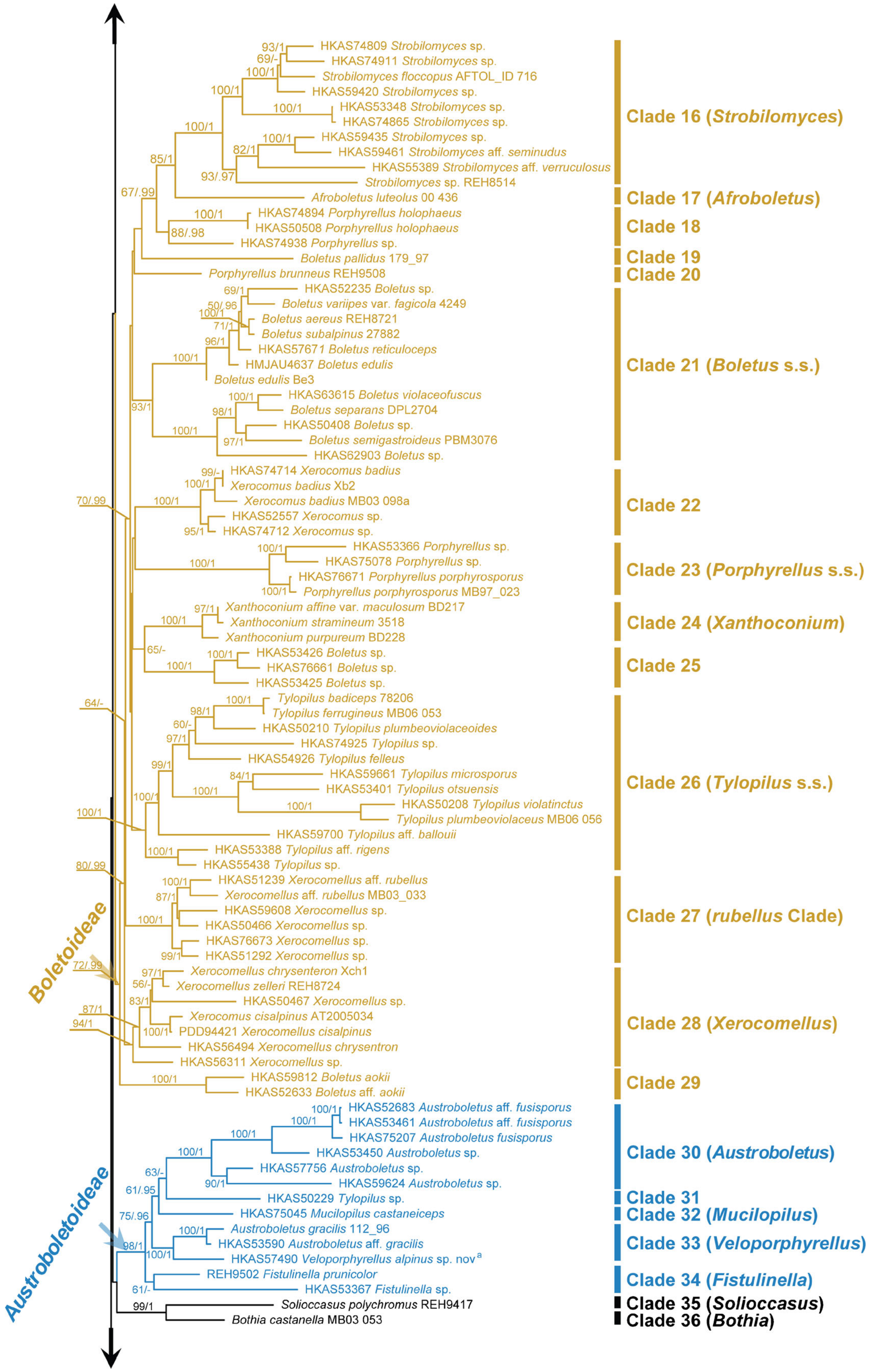


Fig. 2 (continued)

Fig. 2 (continued)

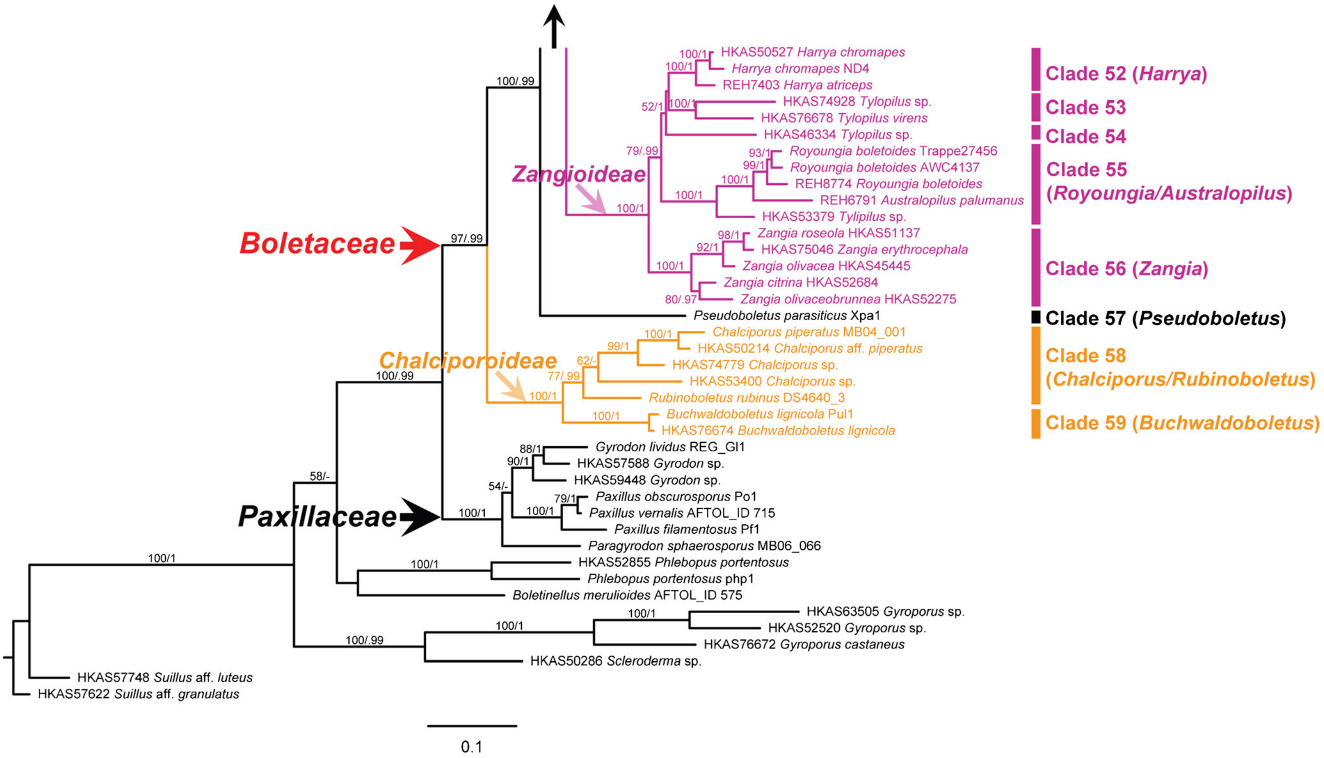


Fig. 2 (continued)

Heimioporus, *Austroboletus*, *Borofutus*, *Spongiforma*, *Afroboletus* (Pegler and Young 1981) and Clade 31, which were shown in Figs. 5 and 6, and described in Table 3. Our phylogenetic analyses indicated that basidiospore ornamentations have originated at least five times in *Boletaceae* and occurred in the subfamilies *Austroboletoideae*, *Xerocomoideae*, *Boletoideae*, and *Leccinoideae*, with predominance in *Xerocomoideae* (Fig. 2). Of these ornamentations, one major type (tiny-shallow pits of Clade 31) and three types (verrucose, and rugulose ornamentation from *Boletellus*, and pinholes from *Hemileccinum*/*Corneroboletus*) reported in *Boletaceae* for the first time (Figs. 5 and 6). The tiny-shallow pitted type of Clade 31 (Fig. 5h), found in the subfamily *Austroboletoideae*, was distinguished by the tiny shallow pits at the apical part of the basidiospores. In the *Boletellus*-type, the verrucose ornamentation, represented by *Boletellus shichianus* of Clade 2, was characterized by distinctive tubercules (with a larger size than the tubercules of *Strobilomyces*) present over the entire surface of the basidiospores (Fig. 5a–3). The rugulose ornamentation, also belonging to Clade 2, had crackled verruca at the basidiospore surface with an incomplete myxosporial layer (Fig. 5a–4). The ornamentations in *Hemileccinum*/*Corneroboletus* (Fig. 5c) were characterized by irregular warts and tiny "pinpricks" on the spore surface (Fig. 6), which suggests a putative synapomorphy of the lineage *Hemileccinum*/*Corneroboletus*.

Of the previously reported basidiospore types, the ornamentations found in the genus *Austroboletus* (Fig. 5i), were very diverse, such as truncate-tuberculate (i-1), reticulate or deep pitted (i-2), meandering-fissured and irregular pitted (i-3), labyrinthoid-ridged (i-4) ornamentations, and irregularly verruculose (i-5), and evolved at least twice independently (Fig. 2). The genus *Strobilomyces* harbored two main types of ornamentations, reticulate or warty-spiny (Fig. 5f). The longitudinally striate ornamentations in *Boletellus* s.l. proved to be polyphyletic and were grouped into three distinct lineages (*Boletellus* s.s. Fig. 5a-1, *Aureoboletus* Fig. 5a-2, and Clade 2). Similarly, *Afroboletus* also owned longitudinal costae but with lower raised ornaments between costae (Pegler and Young1981), which were absent in *Boletellus*. *Xerocomellus* possessed very low fine longitudinal ridges on the basidiospores without the fragmenting myxosporium at maturity (Fig. 5g), unlike that of *Boletellus* (Fig. 5a-1). The lineages *Xerocomus* s.s., *Phylloporus*, and Clade 8 shared the bacillate ornamentation (Fig. 5b) that also could be a synapomorphy of the clade. The species in *Heimioporus* owned a reticulate ornamentation with a persistent myxosporium (Fig. 5d), and *Borofutus* (Fig. 5e) had irregular to regular shallow pits on basidiospores surface, somewhat similar to *Spongiforma*, but lacked the subtruncated distal end with a tiny hole at the apex that is present in *Spongiforma* (Fig. 5j).

Reconstruction of ancestral morphological states

The results from the MRCA analyses of eight specified nodes are shown in Table 4. The MRCA of this family was inferred

Table 2 The known genera and new clades supported by molecular data within the seven major clades of *Boletaceae*

Subfamilies or major clades	Genera or clades	
	Known genera	New clades
Boletoideae	*Afroboletus* Pegler & T.W.K. Young; *Boletus* L. s. s.; *Porphyrellus* E.-J. Gilbert s. s.; *Strobilomyces* Berk.; *Tylopilus* P. Karst. s. s.; *Xerocomellus* Šutara s. s.; *Xanthoconium* Singer	18–20, 22, 25, 27, 29
Austroboletoideae	*Austroboletus* (Corner) Wolfe; *Fistulinella* Henn.; *Mucilopilus* Wolfe; *Veloporphyrellus* L.D. Gómez & Singer	31
Leccinoideae	*Borofutus* Hosen & Zhu L. Yang; *Chamonixia* Rolland[a]; *Leccinum* Gray s. s.; *Leccinellum* Bresinsky & Manfr. Binder; *Octaviania* Vittad.; *Retiboletus* Manfr. Binder & Bresinsky; *Rossbeevera* T. Lebel & Orihara; *Spongiforma* Desjardin et al.	14
Xerocomoideae	*Aureoboletus* Pouzar; *Boletellus* Murrill s. s.; *Corneroboletus* N.K. Zeng & Zhu L. Yang; *Hemileccinum* Šutara; *Heimioporus* E. Horak; *Phylloporus* Quél.; *Sinoboletus* M. Zang *Xerocomus* Quél. s. s.	2, 8
Zangioideae	*Australopilus* Halling & Fechner; *Harrya* Halling et al.; *Zangia* Y.C. Li & Zhu L. Yang	53, 54
Chalciporoideae	*Buchwaldoboletus* Pilát; *Chalciporus* Bataille *Rubinoboletus* Pilát & Dermek	–
Pulveroboletus Group	*Pulveroboletus* Murrill s. s.; *Sutorius* Halling et al.	37, 39–41, 43–49

[a] Based on the results of Lebel et al. 2012 and Orihara et al. 2012

to have smooth basidiospores and open hymenophoral pores, and probably to be stipitate-pileate. For the major clades/subfamilies, particular characters — smooth spores, the stipitate-pileate form, and open hymenophoral pores — were inferred as the ancestral state for all of them, except for the ornamented spore of the MRCA of the subfamily *Xerocomoideae*.

Taxonomy

Of the seven major clades retrieved in the *Boletaceae*, six with high statistical supports are formally erected as subfamilies below, comprising *Boletoideae*, *Xerocomoideae*, and four new subfamilies. The *Pulveroboletus* Group, with low statistical supports, is not erected as a subfamily for the time being.

Boletaceae Chevall., Fl. gén. env. Paris (Paris) 1: 248 (1826) (“ordre”)

Strobilomycetaceae E.-J. Gilbert [as ‘*Strobilomyceteae*’], Les Bolets: 105 (1931)

Ixechinaceae Guzmán, Boln. Soc. mex. Micol. 8: 59 (1974)

Octavianiaceae Locq. ex Pegler & T.W.K. Young [as 'Octavianinaceae'], Trans. Br. mycol. Soc. 72: 379 (1979)

Boletellaceae Jülich, Bibl. Mycol. 85: 357 (1982) [“1981”]

Chamonixiaceae Jülich, Bibl. Mycol. 85: 357 (1982) [“1981”]

Fig. 3 Basidiomata of representative species in new clades of *Boletaceae*. Clade 2 *Boletellus mirabilis* (HKAS57776), Clade 8 *Xerocomus punctilifer* (HKAS52269), Clade 14 *Tylopilus* sp. (HKAS50281), Clade 18 *Porphyrellus holophaeus* (HKAS59407), Clade 22 *Xerocomus badius* (HKAS74714), Clade 25 *Boletus* sp. (HKAS52241), Clade 27 *Xerocomellus* sp. (HKAS68158), Clade 29 *Boletus aokii* (HKAS59812), Clade 31 *Tylopilus* sp. (HKAS50229), Clade 37 *Boletus brunneissimus* (HKAS52660), Clade 39 *Boletus panniformis* (HKAS55444), Clade 40 *Boletus sinicus* (HKAS68620), Clade 41 *Boletus rufo-aureus* (HKAS76849), Clade 43 *Boletus* sp. (HKAS56280), Clade 44 *Boletus* aff. *amygdalinus* (HKAS57262), Clade 45 *Boletus* sp. (HKAS59660), Clade 46 *Boletus* aff. *speciosus* (HKAS59467), Clade 47 *Leccinum extremiorientale* (HKAS63535), Clade 48 *Boletus* sp. (HKAS76850), Clade 49 *Boletus* aff. *carminipes* (HKAS54094), Clade 51 *Boletus* sp. (HKAS75081), Clade 53 *Tylopilus virens* (HKAS74968). Images for Clades 19, 20, and 54 are unavailable, and the last four images show the stuffed pores of four genera (*Boletus* s.s., Clade 25, *Sutorius*, and Clade 46)

Xerocomaceae (Singer) Pegler & Young, Trans. Br. mycol. Soc. 76: 112 (1981)

Typus: *Boletus* L., Sp. pl. 2: 1176 (1753)

Basidioma boletoid, or sequestrate, occasionally phylloporoid. Pileus small to large, scaly, fibrillose, mealy, tomentose, velutinous or glabrous, dry or viscid; margin sometimes projecting; context fleshy, unchanged, bluing, browning, blackening, or reddening when bruised; hymenophore sinuate-adnexed, decurrent, or adnate to depressed, lamellate or tubular or loculate, whitish, pinkish, yellowish to yellow, red, or brown, unchanged or becoming bluing, browning, blackening, or reddening when bruised; when sequestrate, the gleba chambered or tubulose. Stipe (when present) usually fleshy, central or slightly lateral, cylindrical or ventricose-bulbous, solid, smooth, or ornamented with furfuraceous to scabrous squamules, or with reticulate lines. Pileipellis trichodermium, ixotrichodermium, or ixohyphoepithelium, or composed of interwoven hyphae. Spores ovate, subfusiform, or elongate subfusoid, rarely broadly ellipsoid, yellowish brown, brown, or olivaceous brown to pinkish, inamyloid, smooth, or with different types of ornamentation. Basidia ballistosporic or statismosporic, clavate. Cystidia more or less fusoid-ventricose. Hymenophoral trama boletoid or phylloporoid or intermediate. Hyphae without clamp connections. Mostly ectomycorrhizal, occasionally mycoparasitic/saprotrophic.

Austroboletoideae G. Wu & Zhu L. Yang, **subfam. nov.**
MycoBank: MB 805103

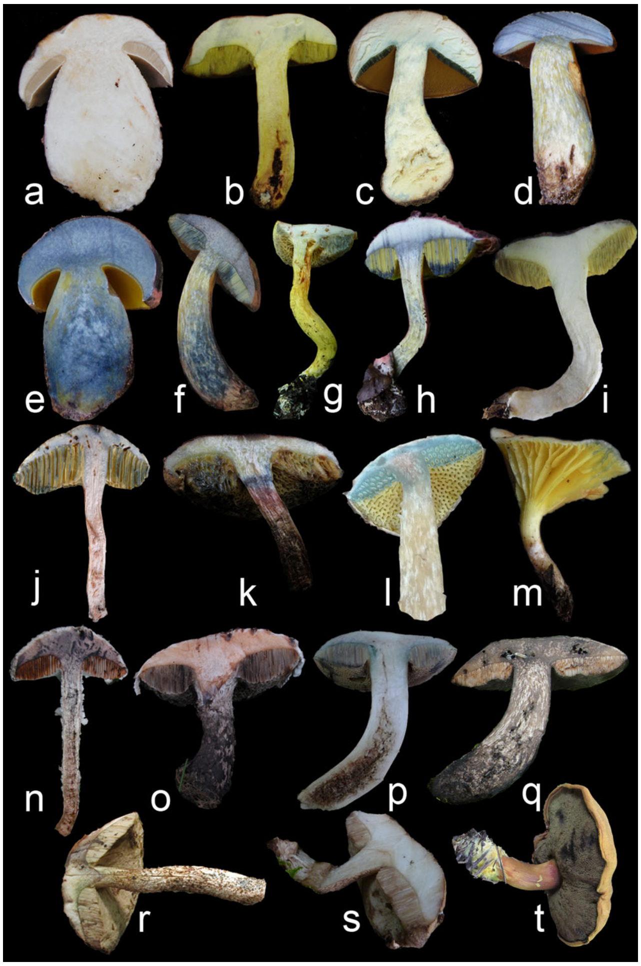


Fig. 4 Types of color change of context in *Boletaceae*. **a**–**f** *Boletus* s.l., **g** *Pulveroboletus*, **h** *Boletellus*, **i**–**l** *Xerocomus* s.l., **m** *Phylloporus*, **n**–**o** *Strobilomyces*, **p**–**q** *Porphyrellus*, **r** *Leccinum*, **s** *Retiboletus*, **t** *Buchwaldoboletus*

Typus: *Austroboletus* (Corner) Wolfe, Bibl. Mycol. 69: 64 (1980) ["1979"]

Basidioma boletoid. Pileus smooth, subtomentose to scurfy, viscid or dry; marginal veil always present; context white, unchanged when bruised; hymenophore tubular, whitish or with pink or purplish tinge, unchanged, rarely browning when bruised. Stipe smooth or ornamented with coarsely reticulations or squalmules; basal mycelium white. Pileipellis ixotrichodermium or trichodermium. Spores fusoid-boletoid, sometimes smooth but usually ornamented with pits, ridges, or tubercules, etc., violaceous gray, yellow-tan, yellow-cinnamon, yellow-gold, yellow-pink, light khaki-brown to rosy brown in KOH, dark yellow-tan, yellow-cinnamon, ochraceous gold to pale rusty in Melzer's, pinkish brown to reddish brown in deposit. Hymenophoral trama boletoid. Ectomycorrhizal.

Exemplar genera: *Austroboletus* (Corner) Wolfe, *Fistulinella* Henn., *Mucilopilus* Wolfe, and *Veloporphyrellus* L.D. Gómez & Singer

Boletoideae

Strobilomycetoideae (E.-J. Gilbert) Snell [as "*Strobilomyceteae*"], Mycologia 33: 422 (1941)

Typus: *Boletus* L., Sp. pl. 2: 1176 (1753)

Basidioma boletoid, or rarely gasteroid or secotioid. Pileus dry, smooth, subtometose, squamose, or floccose; marginal veil absent or present; context white, or yellowish, unchanged or changing to brown, red, black, or blue when cut; hymenophore tubular or rarely glebulous, white, pinkish, sometimes yellowish to yellow, unchanged or changing to brown, red, black, or blue when bruised. Stipe smooth, reticulate, shaggy. Pileipellis generally trichodermium, rarely ixotrichodermium. Spores often fusoid-boletoid, or globose to subglobse, ellipsoid to broadly ellipsoid, usually smooth, or longitudinally striate, or ornamented with *Strobilomyces*-ornamentations, nearly hyaline, bright yellow, yellow-cinnamon to olive-ochraceous, buffy brown in KOH, yellowish, tawny, cinnamon, dull rusty brown to dull grayish yellow in Melzer's, vinaceous, brownish, olive-brown to black in deposit. Hymenophoral trama boletoid or phylloporoid. When gasteroid, basidioma often with a rudimentary stipe; gleba whitish to grayish, bluing when cut; spores with spiny ornamentations. When secotioid, basidoma white, unchanged when cut, and spores smooth. Ectomycorrhizal.

Exemplar genera: *Afroboletus* Pegler & T.W.K. Young, *Boletus* L. s.s., *Heliogaster* Orihara & K. Iwase, *Porphyrellus* E.-J. Gilbert, *Strobilomyces* Berk., *Tylopilus* P. Karst. s.s., *Xerocomellus* Šutara, and *Xanthoconium* Singer.

Chalciporoideae G. Wu & Zhu L. Yang, **subfam. nov**.

MycoBank: MB 805104

Typus: *Chalciporus* Bataille, Bull. Soc. Hist. nat. Doubs 15: 39 (1908)

Basidioma boletoid. Pileus smooth or subtomentose, dry; marginal veil absent or margin incurved; context yellowish to yellow, unchanged or bluing when bruised; hymenophore tubular, red, yellowish to brownish yellow, always suilloid, unchanged or bluing with green tinge when bruised. Stipe smooth. Pileipellis trichodermium. Spores comparatively small (usually not over 10 μm of the length), elongated, or short ellipsoid, smooth, yellowish, pale olivaceous to dingy ochraceous in KOH, rusty brown in Melzer's, olive brown to dull cinnamon in deposit. Hymenophoral trama generally boletoid. Saprotrophic/mycoparasitic.

Exemplar genera: *Buchwaldoboletus* Pilát, and *Chalciporus* Bataille

Leccinoideae G. Wu & Zhu L. Yang, **subfam. nov**.

Octavianiaceae Locq. ex Pegler & T.W.K. Young [as 'Octavianinaceae'], Trans. Br. mycol. Soc. 72: 379 (1979)

Chamonixiaceae Jülich, Bibl. Mycol. 85: 357 (1982) ["1981"]

MycoBank: MB 805105

Typus: *Leccinum* Gray, Nat. Arr. Brit. Pl. (London) 1: 646 (1821)

Basidioma boletoid or gasteroid. Pileus smooth or subtomentose, dry; marginal veil present or absent; context whitish to white or yellow, unchanged or changing to reddish, bluish, grayish, or brownish to blackish when cut; hymenophore tubular or glebulose, whitish, pinkish, yellow, or brown; unchanged or changing to bluish, or brown when cut. Stipe (when present) ornamented with lines, points, dots, squamules, or reticulations. If gasteroid, peridium surface white to grayish white, bluing when bruised, or peridium absent; gleba white when young to brown when mature; Pileipellis trichodermium. Spores usually elongated fusoid-boletoid, or ellipsoid to broadly ellipsoid, smooth or ornamented with pits, spines, rugosities, longitudinal ridges, yellow-brown to dingy ochraceous in KOH, ochraceous-tawny to rusty brown in Melzer's, honey yellow, yellow-brown to cinnamon-brown in deposit. Hymenophoral trama boletoid, or sometimes phylloporoid. Ectomycorrhizal.

Exemplar genera: *Borofutus* Hosen & Zhu L. Yang, *Chamonixia* Rolland, *Leccinellum* Bresinsky & Manfr. Binder, *Leccinum* Gray s. str., *Octaviania* Vittad., *Retiboletus* Manfr. Binder & Bresinsky, *Rossbeevera* T. Lebel & Orihara, and *Spongiforma* Desjardin et al.

Xerocomoideae Singer, Farlowia 2:278 (1945)

Boletellaceae Jülich, Bibl. Mycol. 85: 357 (1982) ["1981"]

Xerocomaceae (Singer) Pegler & Young, Trans. Br. mycol. Soc. 76: 112 (1981)

Typus: *Xerocomus* Quél., in Mougeot & Ferry, Fl. Vosges, Champ.: 477 (1887)

Basidioma boletoid or phylloporoid. Pileus dry or viscid, smooth or subtomentose to tomentose; marginal veil absent or present; context and hymenophore yellowish to yellow, often changing to blue or sometimes reddish or unchanged when bruised; hymenophore tubular or lamellate; Stipe smooth or ornamented with particles or coarse lacerate reticulations. Pileipellis trichodermium or ixotrichodermium. Spores fusoid-boletoid, sometimes ellipsoid, often with reticulate, bacillate, tuberculate or longitudinally striate ornamentations, sometimes with irregularly warts and pinpricks, occasionally smooth, pale ochraceous, pale lemon-yellow, honey brown to dark brown in KOH, pale to dull olive yellowish in Melzer's, mostly olivaceous brown in deposit. Hymenophoral trama usually phylloporoid, sometimes boletoid, or intermediate. Ectomycorrhizal.

Exemplar genera: *Aureoboletus* Pouzar, *Boletellus* Murrill, *Corneroboletus* N.K. Zeng & Zhu L. Yang, *Hemileccinum* Šutara, *Heimioporus* E. Horak, *Phylloporus* Quél., and *Xerocomus* Quél.

Zangioideae G. Wu, Y. C. Li & Zhu L. Yang, **subfam. nov.**

MycoBank: MB 805107

Typus: *Zangia* Y.C. Li & Zhu L. Yang, Fungal Divers. 49: 129 (2011)

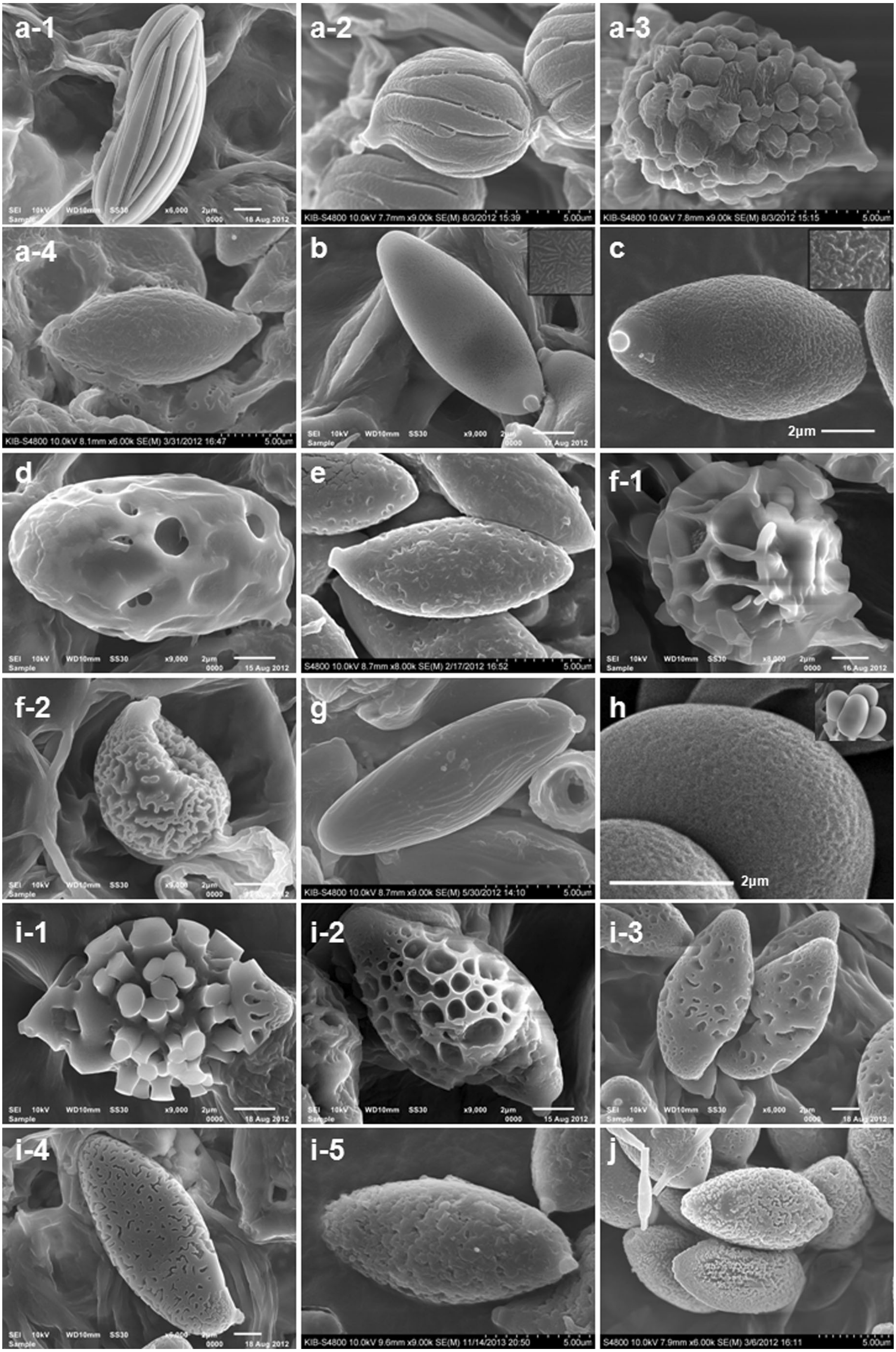
Basidioma boletoid or gasteroid. Pileus dry or viscid, smooth or subtomentose; marginal veil absent; context white or chrome-yellow, mostly unchanged when bruised; hymenophore tubular or glebulose, pinkish brown tinge when mature, mostly unchanged when bruised. Stipe smooth or ornamented with particles, sometimes with reticulations; base and basal mycelium chrome-yellow. If gasteroid, surface of peridium chrome-yellow; gleba brownish to brown, loculate. Pileipellis trichodermium or ixohyphoepithelium. Spores fusoid-boletoid, smooth, nearly hyaline to light olivaceous in KOH, yellowish to tawny occasionally in Melzer's, pinkish- to reddish-brown in deposit. Hymenophoral trama generally boletoid. Ectomycorrhizal.

Exemplar genera: *Australopilus* Halling & Fechner, *Harrya* Halling et al., *Royoungia* Castellano et al., and *Zangia* Y.C. Li & Zhu L. Yang.

Key to the seven subfamilies or major clades

1 Hymenophore suilloid; mycoparasitic/saprotrophic…………………………*Chalciporoideae*
1 Hymenophore non-suilloid; ectomycorrhizal partners of plants………………….…….…...….…….….….…….…2
  2 Hymenophore surface whitish when juvenile…………………………………………….3
  2 Hymenophore surface yellow when juvenile…………………………………....……......…7
3 Base of stipe not bright yellow………………….………4
3 Base of stipe bright yellow…………......…*Zangioideae*
  4 Context of basidioma staining black, brown, red, sometimes blue when bruised……………………...5
  4 Context of basidioma always unchanging when bruised………………………………….……….6
5 Basidiospores smooth; stipe with blackish scales or reticulations (if stipe reticulated, context barely changing when bruised but with retipolides)……….*Leccinoideae*
5 Basidiospores smooth or ornamented; stipe smooth (if so, basidiospores smooth) or with cottony to woolly flocci or reticulations (if so, basidiospores ornamented or context

Fig. 5 Characteristics of spore ornamentations of different clades ▶ revealed by SEM. Elven genera with ornamented spores shown here: **a** *Boletellus*, **b** *Xerocomus*/*Phylloporus*, **c** *Hemileccinum*/*Corneroboletus*, **d** *Heimioporus*, **e** *Borofutus*, **f** *Strobilomyces*, **g** *Xerocomellus*, **h** Clade 31, **i** *Austroboletus*, **j** *Spongiforma*. **a–1** *Boletellus* sp. (HKAS59536), **a–2** *Boletellus longicollis* (HKAS53398), **a–3** *Boletellus shichianus* (HKAS76852), **a–4** *Boletellus* aff. *shichianus* (HKAS56317), **b** *Xerocomus* sp. (HKAS75076), **c** *Corneroboletus indecorus* (HKAS63126), **d** *Heimioporus* aff. *japonicus* (HKAS52236), **e** *Borofutus dhakanus* (HKAS73789), **f–1** *Strobilomyces* sp. (HKAS74809), **f–2** *Strobilomyces* aff. *verruculosus* (HKAS55389), **g** *Xerocomellus cisalpinus* (PDD94421), **h** *Tylopilus* sp. (HKAS50229), **i–1** *Austroboletus* aff. *fusisporus* (HKAS53461), **i–2** *Austroboletus* aff. *mutabilis* (HKAS53450), **i–3** *Austroboletus* sp. (HKAS57756), **i–4** *Austroboletus* sp. (HKAS59624), **i–5** *Austroboletus gracilis* (NY181254), **j** *Spongiforma thailandica* (DED7873)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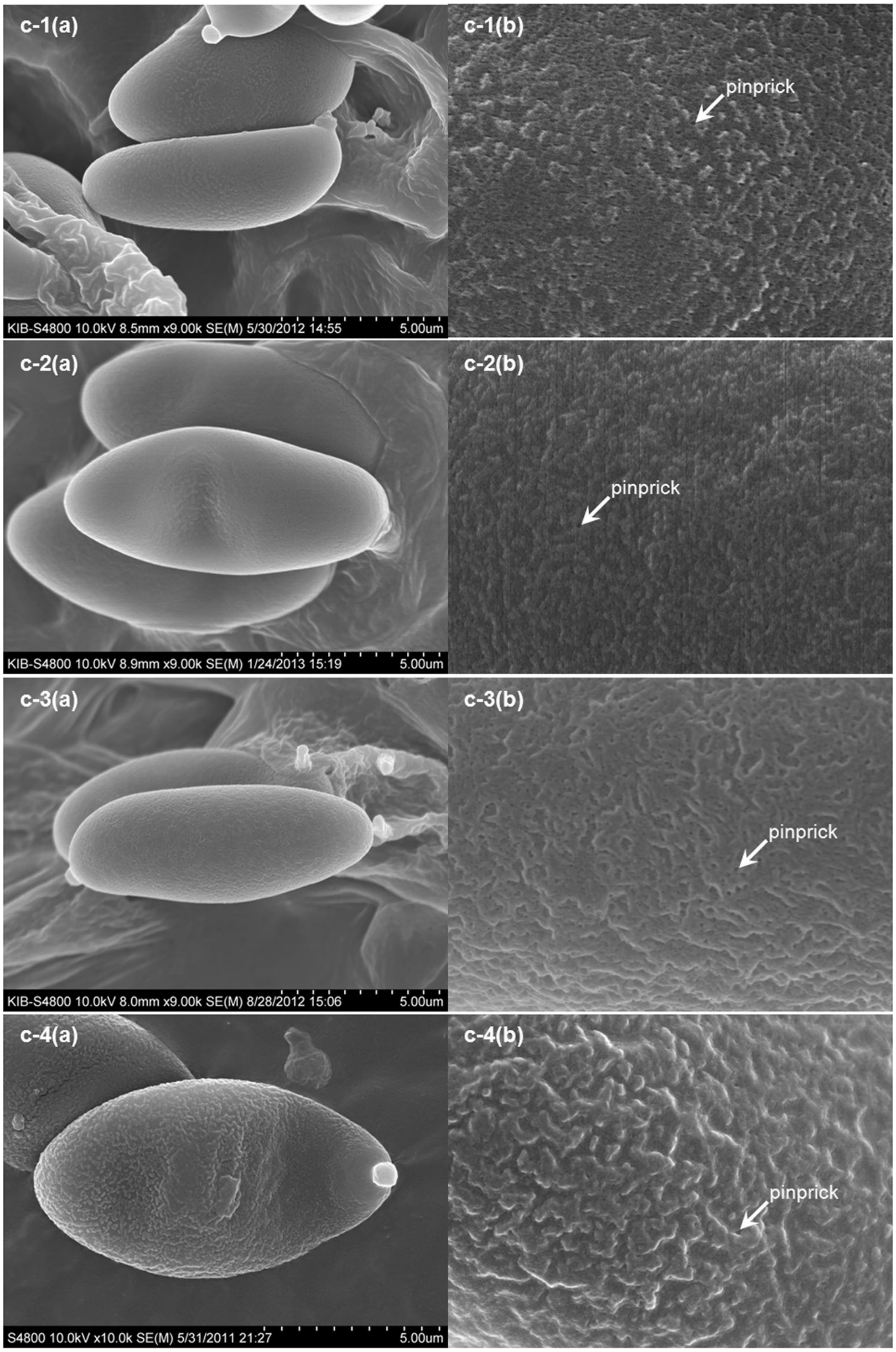

Fig. 6 Characteristics of basidiospore ornamentation of the lineage *Hemileccinum*/*Corneroboletus* revealed by SEM. The images on the left show the general characteristics of the spores, and the images on the right show the close-up features of the spores (the tiny "pinpricks" and irregular warts; the arrows show the "pinpricks"). **c**–**1**: *Boletus* sp. (HKAS67896), **c**–**2**: *Hemileccinum impolitum* (MG373a), **c**–**3**: *Boletus* sp. (HKAS53421), **c**–**4**: *Corneroboletus indecorus* (HAKS63126)

quickly staining when cut)…………………*Boletoideae* (*Afroboletus*, *Porphyrellus* s.s., *Strobilomyces*, and Clades 18–20, 22)

6 Basidiospores smooth; pileus surface dry and with neither marginal veil nor incurved margin……*Boletoideae* (*Boletus* s.s., *Tylopilus* s.s., and *Xanthoconium*)

6 Basidiospores ornamented, sometimes smooth; pileus surface gelatinized or sometimes dry, with marginal veil, or at least with distinctly incurved sterile margin……………………………………*Austroboletoideae*

Table 3 Types of basidiospore ornamentations in the *Boletaceae*

Genera/Lineage	Basidiospore ornamentations[a]
Afroboletus	short ellipsoid spores ornamented with longigudinal costae with lower raised ornaments between costae
Austroboletus	fusoid/elongate-cylindric spores warted to reticulated with broad to narrow ridges and shallow meandering pits (observed in *Austroboletus* s.s.)
	fusoid/elongate-cylindric spores with very narrow-small pits (observed in *A. gracilis* and its allies)
Boletellus	fusoid/elongate-cylindric spores with longitudinal striate ornamentations and retaining the fragmenting myxosporium at maturity (observed in *Boletellus* s.s.)
	ovoid to broadly ellipsoid spores with longitudinal striate ornamentations and retaining the fragmenting myxosporium at maturity (observed in *B. longicollis*/*B. russellii*)
	ovoid to broadly ellipsoid spores with verrucose ornamentations (observed in *B. shichianus*)
	fusoid/elongate-cylindric spores with rugulose ornamentations (observed in *B.* aff. *shichianus*)
Borofutus	fusoid/elongate-cylindric spores ornamented with irregular to regular, conspicuous shallow pits
Heimioporus	ellipsoid spores with reticulate ornamentations (wider mesh)
Heimileccinum/*Corneroboletus*	fusoid/elongate-cylindric spores with irregularly warted and pinholed ornamentations
Spongiforma	fusoid/elongate-cylindric spores with irregularly warted ornamentations and a subtruncated distal end with a tiny hole at the apex
Strobilomyces	subglobose to globose spores with reticulated or echinate or verrucose ornamentations
Xerocomellus	fusoid/elongate-cylindric spores with shallow longitudinal striae and lacking of the fragmenting myxosporium at maturity
Xerocomus/*Phylloporus*	fusoid/elongate-cylindric spores with baccilate ornamentations
Clade 31	fusoid to broadly ellipsoid spores with tiny-shallow pits at the apex

[a] Terms of descriptions of ornamentations mainly based on Pegler and Young (1981), Singer (1986) and original papers of related genera

7 Basidiospores usually smooth (if ornamented, ornamentation longitudinally ridged but indistinctive under a light microscope); hymenophoral pores stuffed or not……..8

7 Basidiospores usually ornamented (if smooth, context unchanging when bruised); hymenophoral pores not stuffed…………………………......…*Xerocomoideae*

  8 Context always staining blue when bruised; hymenophoral pores mostly not stuffed……………9

  8 Context unchanging when bruised; hymenophoral pores stuffed…………......……..*Boletoideae* (Clade 25)

9 Basidioma xerocomoid; pileus always xerocomoid-subtomentose; hymenophoral pores angular; basidiospores smooth or sometimes with indistinctively longitudinal ridges; hymenophoral trama usually phylloporoid-type……………..…*Boletoideae* (*Xerocomellus* s.s., and Clades 27 and 29)

9 Basidioma boletoid; pileus not xerocomoid-subtomentose; hymenophoral pores round; basidiospores smooth; hymenophoral trama usually boletoid-type…………………………..the *Pulveroboletus* Group

Discussion

Redefining the subfamilies and generic lineages of *Boletaceae*

Boletaceae is the most genera- and species-rich family in the suborder *Boletineae* (Binder and Hibbett 2007; Nuhn et al. 2013). Despite the long-term interest and efforts of mycologists, the relationships among the genera within the family have remained largely unresolved. This was mainly due to the lack of a comprehensive specimen sampling and the limited phylogenetic information in morphological characters and in DNA sequences analyzed thus far. Binder and Hibbett (2007) provided a framework for the systematics of the order *Boletales* based on sequences of 5.8S, nrLSU, mtLSU, and

Table 4 Probabilities of ancestral morphological states at eight nodes in the *Boletaceae* estimated using BayesMultiState under MCMC method

Subfamily or Group	Basidiospores		Basidioma form			Hymenophoral pores	
	Smooth	Ornamented	Stipitate-pileate	Gasteroid	Secotioid	Stuffed	Open
Boletaceae	√0.992	√	√0.904	√	√0.078	√1.000	√
Boletoideae	√0.999	√	√0.965	√	√	√1.000	√
Austroboletoideae	√0.959	√	√0.955	—	—	√1.000	—
Leccinoideae	√0.997	√	√0.896	√	√0.064	√1.000	—
Xerocomoideae	√	√0.999	√0.961	—	—	√1.000	—
Zangioideae	√0.999	—	√0.929	√	—0.055	√1.000	—
Chalciporoideae	√0.997	—	√0.924	—	—0.065	√1.000	—
Pulveroboletus Group	√1.000	—	√0.984	—	√	√1.000	√

√=character present, —character absent; probabilities (P=0-1) are represented as arithmetic means. Non-significant probabilities (P<0.05) are not shown *atp*6. Nuhn et al. (2013) elucidated the phylogenetic relationships of the genera in *Boletineae* using nrLSU, *tef1-α*, and *rpb1*. Nevertheless, relationships among many genera/clades in the family *Boletaceae* were still not adequately addressed. As indicated by Dentinger et al. (2010), sequences from the protein-coding gene fragment *rpb1* were phylogenetically more informative than the sequences of nrLSU and *atp*6 combined. Using the *rpb1* sequences, together with sequences from the other three gene markers, nrLSU, *tef1-α*, and *rpb2*, as applied in the AFTOL project (Matheny et al. 2007; Seifert 2009), we constructed a broader and better resolved phylogenetic framework for *Boletaceae*. In total seven subfamily-level major clades (*Austroboletoideae*, *Chalciporoideae*, *Leccinoideae*, *Zangioideae*, *Boletoideae*, *Xerocomoideae*, and the *Pulveroboletus* Group) could be recognized in *Boletaceae*, with the first four subfamilies formally recognized with a taxonomic rank for the first time. The subfamilies *Boletoideae*, *Xerocomoideae*, *Leccinoideae*, and *Chalciporoideae* in this study correspond respectively to the "*Anaxoboletus* Group," "*Hypoboletus* Group," "*Leccinoid* Group," and "*Chalciporus* Group" in Nuhn et al. (2013). Furthermore, 59 clades including 25 putative new genus-level ones were uncovered in the subfamilies. The clades 3, 9, 11, 21, 26, 28, 55, 58, 27, 46 and 51 in our study correspond to the *Hemileccinum* clade, the *Leccinellum* clade, the *Leccinum* clade, the "*Porcini*" clade, the *Tylopilus* clade, the *Xerocomellus* clade, the *Royoungia* clade, the *Chalciporus* clade, the *rubellus* clade, the *regius* clade, and the *bicolor* clade in Nuhn et al. (2013) respectively. Our data further indicated that the *Aureoboletus* clade, the *Strobilomyces* clade, the *Xerocomus* clade, the *badius* clade, the *carminipes* clade, and the *dupainii* clade proposed by Nuhn et al. (2013) should be further split into two (*Aureoboletus* and Clade 2), two (*Strobilomyces* and *Afroboletus*), three (*Xerocomus*, *Phylloporus*, and Clade 8), two (Clades 19 and 22), two (Clades 48 and 49), and four (Clades 37, 39, 40, and 44) separate lineages respectively. The 22 unnamed clades in Fig. 2 were discovered for the first time.

The subfamily *Austroboletoideae* includes *Austroboletus*, *Mucilopilus*, *Veloporphyrellus*, *Fistulinella*, and Clade 31, and shares the characters of conspicuous or inconspicuous marginal veil or projecting, whitish hymenophore, and no color change in the basidioma at injury. *Austroboletus* is divided into two distinct lineages that may be differentiated from each other based on the ornamented stipe (*Austroboletus*) or smooth stipe (species in *Veloporphyrellus*, Li et al. 2014). The SEM results of basidiospores also showed the transition between smooth and ornamented spores in the subfamily (e.g. the transition in Clade 31 shown in Fig. 5h). The reconstruction of the ancestral morphological state inferred that the spore ornamentation was also a derived character in this subfamily (see Table 4).

The subfamily *Boletoideae* circumscribed by Singer (1986) was much broader than that delimited in this study. Singer's *Boletoideae* included almost all of the taxa of currently accepted *Boletaceae* except for *Xerocomus* s.l. and *Strobilomyces* and its allies. Our study indicated that *Boletoideae* should harbor *Afroboletus*, *Boletus* s.s., *Strobilomyces*, *Tylopilus* s.s., *Porphyrellus* s.s., *Xanthoconium*, *Xerocomellus* s.s., and Clades 18–20, 22, 25, 27, 29. *Boletus* s.l. was divided into seven sections by Singer (1986) but has been proven to be polyphyletic (Binder and Bresinsky 2002b; Binder and Hibbett 2007; Dentinger et al. 2010; Feng et al. 2012; Nuhn et al. 2013). The members of sect. *Boletus* are representatives of the present *Boletus* s.s., which is characterized by its whitish hymenophore with stuffed pores when juvenile and unchanged whitish context when bruised (Dentinger et al. 2010; Feng et al. 2012). The sections *Ornatipedes* Singer and *Grisei* (Singer) Singer were erected as the genus *Retiboletus* (Binder and Bresinsky 2002b), species of which shared the compound retipolides. The type species of *B.* sect. *Subpruinosi*, *B. barlae* Fr., was considered to be equal to *B. rubellus* Krombh. (Singer 1947). The latter was transferred to *Xerocomellus* by Šutara (2008) but appears as a species of a new genus (Clade 27) in this subfamily by our analyses. The remaining three sections, *Appendiculati* Konr. & Maubl., *Calopodes*, and *Luridi*, were clustered within the *Pulveroboletus* Group.

Our analyses further suggest that the genus *Porphyrellus* (Singer 1945; Wolfe 1979) should be divided into *Porphyrellus* s.s., Clade 18 represented by *P. holophaeus* (Corner) Yan C. Li & Zhu L. Yang, and Clade 20 represented by *P. brunneus* McNabb. The species of *Porphyrellus* s.s should be restricted to those with a dark basidioma, a typically reddish or purple KOH reaction on the context and without reticulations on the stipe. In contrast, Clade 20 lacks a KOH reaction (McNabb 1967) and Clade 18 is characterized typically by the distinctive reticulations on the stipe and blackish basidioma. In addition, the genus *Xerocomellus* should be subdivided into *Xerocomellus* s.s. and Clade 27 represented by *X.* aff. *rubellus* (Nuhn et al. 2013; see also this study). *Boletus aokii* Hongo and its relatives formed an independent group (Clade 29). Of the remaining two new lineages, Clade 22 is represented by *Xerocomus badius* (Fr.) E.-J. Gilbert, and Clade 25 is morphologically related to *Boletus* s.s. but can be easily distinguished by its yellow stuffed-pore surface and context when juvenile.

The subfamily *Leccinoideae* consists of *Leccinum* s.s., *Leccinellum*, *Borofutus*, *Retiboletus*, *Spongiforma*, and Clade 14. Several gasteroid genera, namely *Rossbeevera*, *Octaviania*, and *Chamonixia*, should also be placed in this subfamily as inferred by Orihara et al. (2012) and Lebel et al. (2012). *Leccinum* s.l. with a pronouncedly scabrous stipe was previously separated into four sections by Singer (1986). Two of the four sections, sect. *Roseoscabra* Singer and sect. *Eximia* Singer, were recently raised to the generic rank, *Harrya* and *Sutorius*, respectively, by Halling et al. (2012a, b). Boletologists have held several contrasting views on section *Luteoscabra* Singer

(Binder and Besl 2000; Bresinsky and Besl 2003; den Bakker and Noordeloos 2005). A new genus *Leccinellum* was erected for this group of organisms based on a nrLSU analysis by Bresinsky and Besl (2003). This view was soon opposed by den Bakker and Noordeloos (2005) due to its supposed paraphyly. Similarly, the results of our study indicated that neither *Leccinum* nor *Leccinellum* were monophyletic without integrating gasteroid *Rossbeevera* and *Octaviania*. Therefore, a possible classification would collapse *Leccinum*, *Leccinellum*, and the three gasteroid genera into a single large genus *Leccinum*, as mentioned in Lebel et al. (2012). *Borofutus* as described recently by Hosen et al. (2013) is the only stipitate-pileate genus with spore ornamentation within the subfamily. According to their results, *Borofutus* clustered closely with the gasteroid *Spongiforma*, another genus with ornamented spores (Desjardin et al. 2009, 2011), which was shown to belong to this subfamily by Nuhn et al. (2013) and this study as well.

The *Pulveroboletus* Group comprises 11 new lineages identified in this study, most of which were arranged in three sections of *Boletus* s.l., namely sects. *Calopodes*, *Appendiculati*, and *Luridi*. The sect. *Calopodes* and sect. *Appendiculati* correspond to Clades 39 and 46, respectively, whereas sect. *Luridi* sensu Singer (1986) is polyphyletic, with its members clustering in at least six lineages (Clades 37, 39–41, 44, and 46). *Boletus bicolor* Peck (1872), an illegitimate homonym of *B. bicolor* Raddi (1806), was previously placed in *B.* sect. *Calopodes* by Zang (2006), but this species and its related species should be arranged in two separate monophyletic groups (Clades 49 and 51). *Boletus sinicus* W.F. Chiu and *Boletus pulverulentus* Opat. with relative allies represented two new independent lineages (Clades 40 and 48). Other lineages in this major clade include *Pulveroboletus*, *Sutorius*, and Clades 43, 45, and 47 including *Leccinum extremiorientale* (Lar. N. Vassiljeva) Singer and its relatives.

Xerocomoideae was proposed by Singer (1945) as a subfamily mainly based on the character of *Phylloporus*-type hymenophoral trama. Three genera, *Xerocomus*, *Phylloporus* and *Tubosaeta* E. Horak, were arranged in the subfamily. This subfamily was subsequently elevated to the family level (*Xerocomaceae*) by Pegler and Young (1981), including five additional genera, namely *Boletellus*, *Gymnopaxillus* E. Horak, *Paxillogaster* E. Horak, *Phylloboletellus* Singer, and *Singeromyces* M.M. Moser. However, the family rank for the group was not supported by molecular data (Binder and Hibbett 2007; Nuhn et al. 2013). Based on our phylogenetic analyses, we locate the boundary of the subfamily *Xerocomoideae* (Fig. 2) differently from previous suggestions to comprise *Xerocomus* s.s., *Phylloporus*, *Hemileccinum*, *Boletellus* s.s., *Heimioporus*, *Aureoboletus*, *Hemileccinum*/*Corneroboletus*, and Clades 2 and 8.

The genus *Xerocomus* circumscribed by Singer (1986) has been proven to be polyphyletic (Binder and Hibbett 2007; Drehmel et al. 2008). Consequently, it was split into five genera by Šutara (2008): *Xerocomus* s.s., *Phylloporus*, *Xerocomellus*, *Hemileccinum*, and *Pseudoboletus*, all of which were supported by molecular data (Nuhn et al. 2013; Zeng et al. 2013; see also this study). However, this classification should be revised. *Xerocomellus* stays in the *Boletoideae* and harbors two distinct lineages (Nuhn et al. 2013; see also this study). *Xerocomus* s.s. of Šutara (2008) should be split into two lineages, namely *Xerocomus* s.s. and Clade 8 represented by *X. punctilifer* W.F. Chiu, both of which owned the bacillate ornamentation on the surface of their basidiospores, but the context of species in Clade 8 changes first to reddish and then to bluish when bruised, which is different from *Xerocomus* s.s.

The genus *Boletellus*, belonging to the redefined subfamily *Xerocomoideae*, was subdivided into eight sections by Singer (1986). Based on our analyses, *Boletellus* s.l. with ornamented spores, olivaceous spore print, and yellow tubular hymenophore, is polyphyletic (*Boletellus* s.s., Clade 2, and one part of *Aureoboletus*). *Boletellus* s.s. may only include the two sections identified by Singer (1986), sect. *Boletellus* and sect. *Chrysenteroidei* Singer, with a length/width ratio of basidiospores over 2. Sharing the gelatinized pileus and marginal veil with *Aureoboletus thibetanus* (Pat.) Hongo & Nagas, *Boletellus longicollis* (Ces.) Pegler & T.W.K. Young, a typical species of section *Ixocephali* Singer, should be transferred to *Aureoboletus*. Section *Mirabiles* Singer represented a distinct genus-level lineage (Clade 2) with *B. russellii* (Frost) E.-J. Gilbert, the type species of sect. *Dictyopodes* Singer, all of which own apparently coarse ridges or reticulations. *Boletellus shichianus* (Teng & L. Ling) Teng, a member of Singer's "Section 8 (unnamed)," together with its allies were also located within Clade 2 that has another type of ornamentation, namely tuberculose or rugulose ornamentation. The *Hemileccinum*/*Corneroboletus* lineage with irregular warts and pinholes on the spore surface contained several genera that may be distinguished by differences in the structure of their pileipellis. *Sinoboletus* M. Zang is a synonym of *Aureoboletus* as they share an ixotrichoderm, a bright yellow hymenohore, and non-color change when bruised. The inclusion of *Tubosaeta*, *Gymnopaxillus*, *Paxillogaster*, *Phylloboletellus*, and *Singeromyces* in *Xerocomoideae* remains to be determined as molecular data are insufficient.

The subfamily *Zangioideae*, the sub-basal group of *Boletaceae* typically characterized by a bright yellow color at the base of stipe, comprises *Australopilus*, *Harrya*, *Royoungia*, *Zangia*, and Clades 53–54, which were separated from *Tylopilus* s.l.

The subfamily *Chalciporoideae*, including *Chalciporus* and *Buchwaldoboletus*, forms the basal group of *Boletaceae*, consistent with the results of Binder and Hibbett (2007), and Nuhn et al. (2013).

Although six of the seven major clades are well supported in our phylogenetic analyses, the relationships among *Austroboletoideae*, *Leccinoideae*, *Xerocomoideae*, *Boletoideae*, and the *Pulveroboletus* Group remain unresolved. This is probably due to two reasons: (i) the limited sampling from regions outside of China; and (ii) the limitation of the gene markers used. Further work with more representative samples and sequences from more gene markers or genomic data may help to resolve such issues.

At the generic level, 25 putative new lineages were uncovered by our study. However, special attention should be paid to the two stipitate-pileate genera, *Boletochaete* Singer and *Tubosaeta*, and the sequestrated genera in family *Boletaceae* during the validation of these new lineages, as they were not included in our molecular analyses.

Morphological complexity and evolution in *Boletaceae*

Exclusive reliance on the morphological features for fungal taxonomy, despite its uses, has created many taxonomic difficulties and confusions. Such problems were often generated due to the convergent evolution and phenotypic plasticity of many of these morphological features. Based on our results, several pairs of characters have displayed evidence for convergent evolution and phenotypic plasticity in *Boletaceae*: smooth versus ornamented basidiospores, changing versus non-changing color of context, stipitate-pileate versus sequestrate (gasteroid and secotioid) appearance, and open versus stuffed hymenophoral pores.

The ornamentation of basidiospores has been used as a key character for recognizing several genera in *Boletaceae*, such as *Strobilomyces*, *Boletellus*, and *Austroboletus*. For example, the patterns of spore ornamentations were used to establish the family *Strobilomycetaceae* [as '*Strobilomyceteae*'] (Gilbert 1931) or the subfamily *Strobilomycetoideae* (Snell 1941) but these groups were later proven to be inappropriate (Binder and Hibbett 2007; Nuhn et al. 2013). Our results indicated that ornamented basidiospores could be found in four distinct subfamilies (*Austroboletoideae*, *Boletoideae*, *Leccnioideae*, and *Xerocomoideae*). The most recent common ancestor of the family was estimated to own smooth basidiospores, and ornamented basidiospores were derived from smooth basidiospores and evolved independently for at least ten times (Fig. 2) in this family.

In the subfamily *Xerocomoideae*, more than half of the genera harbored different types of ornamented basidiospores (Fig. 2). Our analysis indicated that the ornamented basidiospore was the ancestral state of this subfamily (Table 4), and would be a symplesiomorphic character for the lineages within this subfamily, but different types of ornamentation evolved via different paths. For example, longitudinally grooved ornamentation evolved at least twice and occurred in the genera *Boletellus* s.s. and *Aureoboletus* (including *Boletellus longicollis*). In contrast, some other types of basidiospore ornamentation are symplesiomorphic. For instance, species within the clade including the genera *Xerocomus* s.s., *Phylloporus*, and Clade 8 share bacillate ornamentations on their basidiospore surfaces, whereas the basidiospore ornamentation of *Corneroboletus*/*Hemileccinum* represents another different type, with "pinpricks" on the spore surfaces as seen in SEM at 20000× to 50000× magnifications in addition to irregular warts (Fig. 6).

More and more genera with the sequestrate phenotype have been proven to belong to *Boletaceae* or *Boletales* based on analyses of the molecular data (Yang et al. 2006; Desjardin et al. 2009; Orihara et al. 2010, 2012; Halling et al. 2012b; Lebel et al. 2012; Nuhn et al. 2013; Trappe et al. 2013). Based on our data, sequestrate basidioma could be derived from stipitate-pileate form in the *Boletaceae* and can be detected in the subfamilies *Boletoideae*, *Leccinoideae*, and *Zangioideae*, and the *Pulveroboletus* Group, probably as a result of convergent evolution in response to challenge by particular ecological requirements regarding spore dispersal and climate (Reijnders 2000; Binder and Bresinsky 2002a; Hibbett 2007). Thus, our work further suggested that the sequestrate phenotype should be de-emphasized in the taxonomy of the *Boletaceae*. A good example is the transfer of secotioid species (*Gastroboletus subalpinus* Trappe & Thiers and *Secotium areolatum* G. Cunn.) to *Boletus* s.s. by Dentinger et al. (2010) and Nuhn et al. (2013). Similar cases may hold for *Heliogaster columellifer* (Kobayasi) Orihara & K. Iwase versus *Xerocomellus* s.s. (Orihara et al. 2010), or *Borofutus* versus *Spongiforma* (Hosen et al. 2013). These results suggest that attention should be paid to the remaining sequestrate boletes in the future.

The presence of stuffed hymenophoral pores has been proven to be an important characteristic distinguishing *Boletus* s.s. from other groups in *Boletus* s.l. (Dentinger et al. 2010). According to our results, this feature has arisen independently in several different lineages (Clade 25 of *Boletoideae*, *Sutorius* and Clade 46 in the *Pulveroboletus* Group) of *Boletaceae* (Fig. 2). Thus, the character "stuffed pores" should be combined with other characteristics in the taxonomy of *Boletaceae*.

Chemotaxonomy: color change, taste, and chemical reactions

In *Boletales*, the chemotaxonomy of the families *Suillaceae* and *Gomphidiaceae* was presented by Besl and Bresinsky (1997). However, other families have received relatively little attention. In the family *Boletaceae*, some species of the following genera, namely *Austroboletus*, *Boletus*, *Boletellus*, *Chalciporus*, *Chamonixia*, *Leccinum*, *Pulveroboletus*, *Phylloporus*, *Retiboletus*, *Strobilomyces*, and *Xerocomus*, have been analyzed. The chemical compounds responsible for the color changes of the context and the hymenophore, the colors of the basidoma surface and context, and the tastes of the context of the basidioma, etc., were studied and used for taxonomy (Steglich and Esser 1973; Steffan and

Steglich 1984; Høiland 1987; Andary et al. 1992; Hellwig et al. 2002).

According to our analyses, the species and genera in five of the six subfamilies of *Boletaceae* (excluding *Austroboletoideae*), as well as the *Pulveroboletus* Group, may change colors when exposed, such as bluing, reddening, browning, and blackening. Our data indicated that each type of color change has evolved independently several times in *Boletaceae* (Fig. 2). Previous research indicated that the chemical compounds responsible for bluing reactions in boletes were mainly phenols, typically pulvinic acid and its derivatives, derived from the same precursor, L-tyrosine (Høiland 1987; Gill 2003; Nelsen 2010; Zhou and Liu 2010). This suggests that the bluing reactions in different subfamilies of *Boletaceae* are likely to have the same chemical basis. In addition to the bluing reactions, reddening, browning, and blackening are also common in some genera of *Boletaceae*. Only Steglich and Esser (1973) indicated that the red color change of *Strobilomyces floccopus* flesh was caused by the oxidation of L–DOPA, also derived from L-tyrosine. The mechanisms of these reactions need further investigation.

Similarly, the colors of the basidioma surface and context, and the taste of the context, are probably related to specific metabolites. A well-known example is the erection of the genus *Retiboletus* on the basis of the presence of retipolide, a colorless antibiotic (Binder and Bresinsky 2002b). Likewise, *Xerocomus badius*, the only species reported to possess a special pigment, badione A (Steffan and Steglich 1984), formed a unique lineage (Clade 22). Regarding the taste of the context of basidioma, the chemical compounds responsible for the bitterness or pungent are different (Hellwig et al. 2002; Sterner et al. 1987).

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