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A revised phylogeny of Boletaceae using whole genome sequences

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ABSTRACT
The porcini mushroom family Boletaceae is a diverse, widespread group of ectomycorrhizal (ECM) mushroom-forming fungi that so far has eluded intrafamilial phylogenetic resolution based on morphology and multilocus data sets. In this study, we present a genome-wide molecular data set of 1764 single-copy gene families from a global sampling of 418 Boletaceae specimens. The resulting phylogenetic analysis has strong statistical support for most branches of the tree, including the first statistically robust backbone. The enigmatic *Phylloboletellus chloephorus* from non-ECM Argentinian subtropical forests was recovered as a new subfamily sister to the core Boletaceae. Time-calibrated branch lengths estimate that the family first arose in the early to mid-Cretaceous and underwent a rapid radiation in the Eocene, possibly when the ECM nutritional mode arose with the emergence and diversification of ECM angiosperms. Biogeographic reconstructions reveal a complex history of vicariance and episodic long-distance dispersal correlated with historical geologic events, including Gondwanan origins and inferred vicariance associated with its disarticulation. Together, this study represents the most comprehensively sampled, data-rich molecular phylogeny of the Boletaceae to date, establishing a foundation for future robust inferences of biogeography in the group.

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INTRODUCTION

The porcini mushroom family Boletaceae is exceptionally diverse and globally distributed, but poorly documented for many regions. Yet, boletoid fungi are prevalent ectomycorrhizal (ECM) mutualists in ecosystems dominated by ECM plants (Peay et al. 2010), and at least eight species are traded globally as wild-collected edible mushrooms (Arora 2008; Dentinger et al. 2010; Dentinger and Suz 2014; Sitta and Floriani 2008). Despite their conspicuous sporocarps, ecological dominance, and cultural importance, new species of Boletaceae are regularly described from around the world (FIG. 1; 2249 currently accepted species; SUPPLEMENTARY TABLE 2) (e.g., Castellano et al. 2016; Chakraborty and Das 2015; Das et al. 2015, 2016; Fulgenzi et al. 2007, 2008, 2010; Halling et al. 2006, 2023; Henkel et al. 2016; Husbands et al. 2013; Magnago et al. 2017; Neves and Halling 2010). Novel taxa have also recently been described from wild-collected foods in markets (e.g., Das et al. 2015; Dentinger and Suz 2014; Halling et al. 2014). New Boletaceae species have been increasingly described in a global phylogenetic context, shedding light on their origin, diversification, and migration. However, over 20 years of molecular phylogenetic studies using legacy loci have made little progress toward resolving the deepest nodes ("backbones") in Boletaceae phylogenies (Grubisha et al. 2001; Binder and Hibbet 2006; Drehmel et al. 2008; Dentinger et al. 2010; Nuhn et al. 2013; Wu et al. 2014).

As a result of this intrafamial phylogenetic uncertainty, there has been a recent explosion of new generic names to accommodate newly discovered species (e.g., Henkel et al. 2016). In addition, new generic names have been erected for species that are included in molecular phylogenetic analyses for the first time and recovered on long branches with no supported affinity to existing named genera (e.g., Badou et al. 2022; Castellano et al. 2016; Halling et al. 2023; Henkel et al. 2016). Few of these studies have followed recommended best practices for naming new genera (Vellinga et al. 2015), namely, failing to establish

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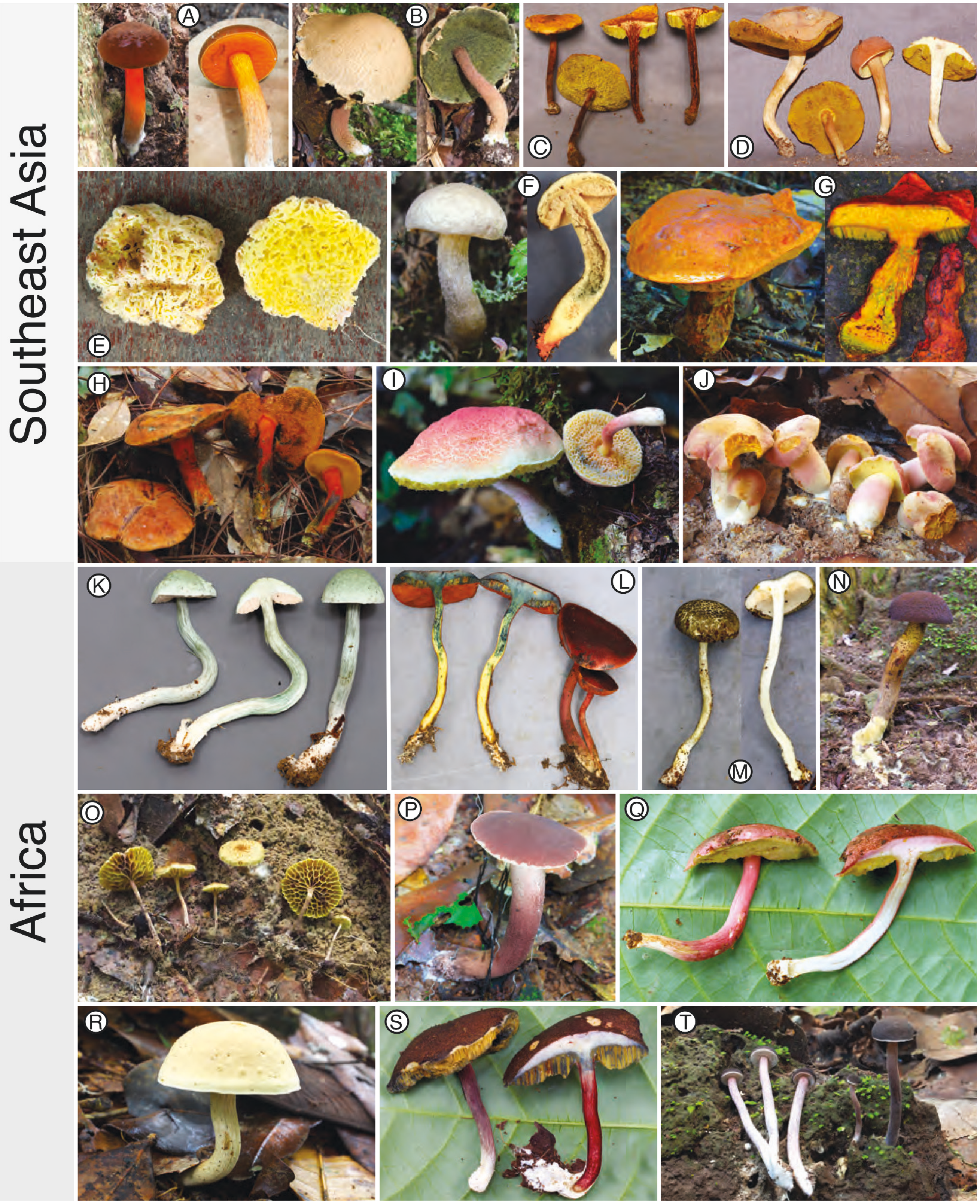


Figure 1. Selected Boletaceae collections from two of the most species-rich regions that were newly sequenced in this study. A. *Boletus cervinococcineus*, Singapore (BD616). B. *Heimioporus punctisporus*, Sarawak (BAKO2). C. Unidentified Boletaceae, Vietnam (CTN-08-0007). D. Unidentified Boletaceae (CTN-08-0029). E. *Spongiforma* sp., Sarawak (BTNG10). F. *Leccinum* sp., Sarawak (SWK246). G. *Crocinoboletus laetissimus*, Sarawak (SWK335). H. unidentified Boletaceae sp., Vietnam (DLT-08-0127). I. *Boletellus* sp., Sarawak (SWK356). J. unidentified Boletaceae, Vietnam (CTN-08-0051). K. *Tylopilus* sp., Cameroon (BD655). L. *Xerocomus* sp. 9, Cameroon (BD773). M. *Fistulinella staudtii*, Cameroon (BD848). N. *Boletellus* sp., Cameroon (BD714). O. *Phylloporus cf. tubipes*, Cameroon (BD719). P. *Tylopilus* sp. 8, Cameroon (BD816). Q. *Xerocomus* sp. 8, Cameroon (BD695). R. *Boletus alliaceus*, Cameroon (BD697). S. *Tubosaeta brunneosetosa*, Cameroon (BD686). T. *Tylopilus* sp., Cameroon (BD716). Images are not to scale.

all genera as monophyletic, insufficient geographic and taxonomic sampling, missing type species, and insufficient statistical support for branches in the tree. Instead, a more inclusive and general application of existing generic names with recognition of new subgenera would enable advancement of biodiversity discovery without an inflation of new generic names, as recommended by Nimis (1998) for lichen taxaonomy. Moreover, many of these new Boletaceae genera are monotypic and require identification to species to be recognized, contradictory to the inclusive nature of taxonomic ranks above the species level and an impractical solution for field identifications. For example, 69 genera (62%) have five or fewer species. Of the 27 genera described in the last 5 years, only one has more than five species (*Erythrophylloporus* = six species), whereas 11 (44%) are monotypic and another 11 (44%) have only two species (SUPPLEMENTARY TABLE 2). Taken together, there remains much taxonomic, nomenclatural, and evolutionary uncertainty in the Boletaceae.

Despite these taxonomic concerns, the Boletacaeae presents a unique system to identify the mechanisms that contribute to rapid diversification in Fungi. The Boletaceae appear to have undergone an early evolutionary radiation between 60 and 100 million years ago (mya) (Bruns and Palmer 1989; Binder and Hibbet 2006; Dentinger et al. 2010; Wu et al. 2014, 2016; Sato et al. 2017; Varga et al. 2019; Sato 2023). This early radiation has been correlated with the convergent evolution of morphological traits, such as the lamellate hymenophore and gasteromycetization (Badou et al. 2022; Castellano et al. 2016; Farid et al. 2018; Smith et al. 2015; Zhang and Li 2018).

Many factors have contributed to difficulties in generating robust phylogenetic reconstructions for the Boletaceae. Although phenomena such as incomplete lineage sorting and hybridization may obscure historical phylogenetic signal, previous data sets for the Boletaceae had patchy taxonomic and geographic sampling. These factors impact accurate phylogenetic reconstruction, possibly exacerbated by the aforementioned rapid radiation event (Bruns et al. 1992; Sato et al. 2017). Without a phylogeny that is based on globally representative taxon sampling and statistically well-supported resolution at all depths of the tree, it is impossible to name, classify, and understand Boletaceae evolution. For example, only a few studies have included representatives of the exceptionally rich Australian boletoid funga (Halling et al. 2012, 2015, 2023). Boletoid fungi from the African and American tropics are rarely represented in family-level analyses despite their exceptional species richness (e.g., Heinemann 1951; Henkel et al. 2012).

Recent field work has resulted in many new collections from undersampled regions (B. Dentinger, T. Henkel, and R. Halling, unpublished data). These specimens are now available to include in phylogenetic data sets in the effort to achieve the first globally representative sampling of the Boletaceae and potentially resolve ancient relationships among mushroom-forming fungi (Dentinger et al. 2016; Liimatainen et al. 2022; Tremble et al. 2020). However, no one has yet applied these methods to the Boletaceae. Moreover, whole genome sequencing of mushroom-forming fungi provides opportunities to go beyond phylogenetic reconstruction. For example, whole genome sequencing can exceed legacy loci in identifying processes in populations that generate biodiversity (e.g., Tremble et al. 2022).

For this study, we generated the first phylogeny of the Boletaceae that utilized a large-scale molecular data set comprising 1764 genome-wide loci from 418 taxa across the family, from both tropical and temperate geographic regions. We included recent new collections from previously undersampled regions, including tropical Africa, southern South America, lowland tropical South America, and Australia. Type species, including type specimens, were sampled to facilitate future taxonomic revisions of genera. Using our highly resolved genome-based phylogeny, we also performed the first inclusive biogeographic reconstruction of the Boletaceae. Overall, we provide new insights into the broad patterns of evolution of this enigmatic fungal group.

MATERIALS AND METHODS

Sampling.—Taxon selection focused on obtaining representatives of all currently accepted genera following Index Fungorum/Species Fungorum, selecting type species whenever possible. Because the current understanding of genera is incomplete and rapidly changing, we could not include representatives of all currently accepted genera that were published during the course of this study. Specimens from geographic regions not represented in prior studies were also included. A total of 418 Boletaceae specimens were gathered from a global distribution using collections made by the authors, those borrowed from four institutions, and donations from citizen scientists (SUPPLEMENTARY TABLE 1). In addition, we utilized genome data publicly available from the Joint Genome Institute (JGI) MycoCosm Portal (Grigoriev et al. 2013) for *Boletus coccyginus, B. reticuloceps, Butyriboletus roseoflavus, Chiua virens, Lanmaoa asiatica*, and *Imleria badia* (Kohler et al. 2015; Miyauchi et al. 2020; Wu et al. 2022). *Paxillus involutus* and *Paxillus adelphus* genomes from JGI were used for outgroups (Kohler et al. 2015).

DNA extraction and sequencing.—Genomic DNA was extracted in one of three ways. (i) Ten milligrams of hymenophore tissue from each specimen was homogenized in 2.0-mL screw-cap tubes containing a single 3.0 mm stainless steel bead and 8 × 1.5 mm ones using a BeadBug microtube homogenizer (Sigma-Aldrich, Saint Louis, Missouri; catalog no. Z763713) for 120 s at a speed setting of 3500 rpm. After physical disruption, DNA was extracted using the Monarch Genomic DNA Purification Kit (New England Biolabs, Ipswich, Massachusetts; catalog no. T3010) with the Monarch gDNA Tissue Lysis Buffer (catalog no. T3011) using double the volume of lysis buffer, 1 h of lysis incubation at 56 C, and 550 μL of wash buffer during each of the wash steps. (ii) An in-house 96-well plate protocol where tissue is physically homogenized, as above, after which 1000 μL of lysis buffer (1% sodium dodecyl sulfate, 10 mM Tris, 10 mM EDTA, 5 mM NaCl, 50 mM dithiothreitol, pH 8.0) is added. To this solution is added 4 μL of RNase A (20 mg/mL), the solution vortexed, and then incubated at 37 C for 10 min. Next, 10 μL of proteinase K (20 mg/mL) is added, the solution vortexed, and then incubated at 56 C overnight on an Eppendorf ThermoMixer (Eppendorf, Hamburg, Germany) with agitation at 400 rpm. After lysis, the tubes are centrifuged at maximum speed (17 000× *g*) to pellet the cellular debris. Then 700 μL of supernatant is removed to a new 1.7-mL microcentrifuge tube with hinged cap to which 162.5 μL 3.0 M potassium acetate (pH 5.5) is added. The solution is mixed briefly and then put on ice for 5 min, followed by a second centrifugation, as above. Avoiding the pellet, the supernatant is removed to a well of a 96-well 10 μM filter plate (Enzymax, Lexington, Kentucky; catalog no. EZ96FTP) set in a 2-mL MASTERBLOCK collection plate (Greiner Bio-One, Monroe, North Carolina; catalog no. 780271). Filtration is achieved through centrifugation at 1500× *g* for 2 min. The flow through is transferred to a new 1.7-mL microcentrifuge tube with hinged cap and centrifuged, as above. Without disturbing the pellet, the supernatant is removed to a new 2.0-mL microcentrifuge tupe with hinged cap and 1000 μL of binding buffer (5 M guanidium hydrochloride, 40% isopropanol) is added and the solution homogenized by pipetting. The binding solution is then transferred to a well of a 96-well long-tip AcroPrep plate (Cytiva Life Sciences, Marlborough, Massachusetts; catalog no. 8133) that was preconditioned by pulling 400 μL Tris-HCl buffer (pH 8.0) through using a vacuum manifold. DNA is bound to the filter by centrifugation at 1500× *g* for 2 min or using a vacuum manifold. The filter is washed twice with 700 μL of wash buffer (20% solution of 80 mM NaCl, 8 mM Tris-HCl, pH 7.5 and 80% ethanol) using centrifugation or vacuum, and then the filter is dried with centrifugation at 1500× *g* for 15 min. Residual ethanol is removed by incubating the filter plate at room temperature for 30 min. To elute the DNA from the filter, 50 μL of elution buffer (0.1× Tris-EDTA buffer, pH 8–9) prewarmed to 60 C is added directly to the filter, incubated for 2 min at room temperature, and eluted into a new 2-mL MASTERBLOCK collection plate with centrifugation at 1500 rpm for 2 min. The elution step is repeated once. (iii) A phenol-chloroform DNA extraction protocol where tissue is physically homogenized, as above, and lysed using the Tissue Lysis buffer from the Monarch Genomic DNA Purification Kit (New England Biolabs; catalog no. T3010S) with double the volume of lysis buffer and a 1-h incubation at 56 C. Then, total lysate was placed in Phase Lock Gel Light tubes (QuantaBio, Beverly, Massachusetts; catalog no. 2302820) along with an equal volume of OmniPur phenol:chloroform:isoamyl alcohol (25:24:1, TE-saturated, pH 8.0) solution (MilliporeSigma; Calbiochem catalog no. D05686) and then mixed by gentle inversion for 15 min using a fixed speed tube rotator. After mixing, tubes were centrifuged at maximum speed (14 000 × *g*) for 10 min, then the aqueous (top) layer was transferred to a new Phase Lock gel tube and the process repeated. DNA precipitation of the aqueous phase was performed by adding 5 M NaCl to a final concentration of 0.3 M and two volumes of room temperature absolute ethanol, inverting the tubes 20× for thorough mixing followed by an overnight incubation at −20 C. DNA was pelleted by centrifugation at 14 000× *g* for 5 min, washed twice with freshly prepared, ice-cold 70% ethanol, air-dried for 15 min at room temperature, and then resuspended in 150 μL of Elution Buffer from the Monarch Genomic DNA kit.

DNA extract quality was assessed for quality using a NanoDrop 1000 (Thermo Scientific, Waltham, Massachusetts) and fragment integrity using agarose gel electrophoresis. Genomic DNAs were sequenced using a combination of paired-end sequencing on the Illumina MiSeq, HiSeq, and Novaseq sequencing platforms (Illumina, San Diego, California) (SUPPLEMENTARY TABLE 2). All raw reads and whole genome assemblies are deposited in the Short Read Archive (Bioproject PRJNA1022813).

Genome assembly, ortholog extraction, and phylogenetic analysis.—Raw sequencing reads were quality-filtered and adapter-trimmed using fastp 0.20.1 (Chen et al. 2018) with default settings. Genome assemblies were produced from quality-filtered reads using SPAdes 3.15.0 (Bankevich et al. 2012) with five k-mer values (k = 77, 85, 99, 111, 127). From each genome, we identified 1764 highly conserved single-copy orthologs using BUSCO with the "basidiomycota odb 10" dataset.

Orthogroups that were present in less than 75% of taxa and taxa with less than 20% ortholog recovery were removed. Retained orthologs were aligned using MAFFT 7.397 (Katoh et al. 2017) with the "L-INS-i" algorithm, and maximum likelihood gene trees were inferred using IQ-TREE 2.0.3 (Minh et al. 2020) with automatic model selection in ModelFinder (Kalyaanamoorthy et al. 2017) and ultrafast bootstrapping (BS; Hoang et al. 2018) with 1000 replicates. A summary coalescent species tree was constructed from the resulting gene trees using ASTRAL-hybrid implemented in ASTER* (1.15) (Zhang and Mirarab 2022). Branch lengths in substitutions/site were estimated under maximum likelihood on the species tree using the "-te" option in IQ-TREE, with a partitioned concatenated alignment of all BUSCO genes used in species tree construction.

Gene tree comparison.—To evaluate discordance, individual gene trees were compared using six metrics calculated in SortaDate (average bootstrap support, clocklike branch lengths, tree length; Smith et al. 2018) and the R package TREEDIST (generalized Robinson-Foulds metrics; Smith 2020, 2022). In addition to data matrix summaries (number of taxa, alignment length), Pearson's correlations were calculated to determine relationships between metrics.

Divergence dating.—A time tree was inferred by applying the RelTime method (Tamura et al. 2012, 2018) conducted in MEGA11 (Stecher et al. 2020; Tamura et al. 2021) to the species tree with maximum likelihood (ML)-estimated branch lengths. To reduce computational burden, time-calibrated branch lengths were calculated using the maximum likelihood (ML) method and the general time reversible substitution model (Nei and Kumar 2000) from two sets of 100 genes: (i) the top 100 genes with well-supported clocklike trees determined using SortaDate (Smith et al. 2018) and (ii) the top 100 genes with the smallest generalized Robinson-Foulds ("gRF") distances to the species tree calculated using the R package TREEDIST. The time tree was computed using two sets of calibration constraints. The first included two calibrations with uniform priors: (i) a secondary calibration for the stem age of the Boletaceae from 50 to 150 my (Varga et al. 2019; Wu et al. 2022) and (ii) a secondary calibration for the stem age of *Boletus edulis* from 5 to 13 my (Tremble et al. 2022). Uniform priors were used because they are less prone to error with secondary calibrations (Schenk 2016). The second set included the former two calibrations plus four of the five internal calibrations using the highly supported core shifts from Varga et al. (2019). All internal calibrations used priors with normal distributions around means, with the minimum and maximum ages of the 95% confidence intervals (CIs) reported by Varga et al. (2019). Because many of the clades in Varga et al. were incongruent with our topology, calibrations were selected using the most inclusive node, except for *Aureoboletus*, which could not be reconciled with our results. The Tao et al. (2020) method was used to set minimum and maximum time boundaries on nodes for which calibration densities were provided, and to compute confidence intervals. Outgroup node ages were not estimated because the RelTime method uses evolutionary rates from the ingroup to calculate divergence times and does not assume that evolutionary rates in the ingroup clade apply to the outgroup.

Ancestral range reconstruction.—Numerous analytical methods for reconstructing historical biogeography exist, accounting for processes such as vicariance, dispersal, and cladogenesis (Landis et al. 2013; Ree et al. 2005; Ronquist 1994). To account for these macroevolutionary processes in our ancestral state reconstruction in the Boletaceae, we utilized BioGeography with Bayesian (and likelihood) Evolutionary Analysis with R scripts ("BioGeoBEARS"; Matzke 2013). Samples were coded in two ways to compare coding by physical geography with coding by phytogeographic regions. We chose to include coding by phytogeographic regions because ectomycorrhizal fungi are likely to track their plant partners due to the obligate nature of their symbiosis. The physical geographic coding scheme was as follows: Paleotropical (consisting of Africa and tropical Asia), Neotropical (South and Central America), South Temperate (Australia and New Zealand), or North Temperate (North America, Europe, northern temperate Asia). The phytogeographic coding scheme used the floristic regions in Liu et al. (2023): Holarctic (including Central America), Neotropical, Chilean-Patagonian, African, Indo-Malesian, Australian, and Novo-Zealandic (SUPPLEMENTARY TABLE 4). Central America was combined with the Holarctic region, as Central American ECM fungi are mostly derived from North American ancestors (Halling 1996). The most likely model was chosen according to the Akaike information criterion (AIC) and weighted AIC score calculated in BioGeoBEARS.

RESULTS

Sampling.—Our samples represent 65/111 (58.5%) currently accepted genera, with many new collections, especially from Cameroon and southeast Asia, that could not be assigned to known genera based on morphology or internal transcribed spacer (ITS) DNA barcode comparisons (SUPPLEMENTARY TABLE 1). Of the 46 genera not represented, 23 (47%) are monotypic, 13 (28%) are ditypic, whereas only two (4%) have more than five species, both of which have only six species (*Erythrophylloporus* and *Hourangia*). Thirty-nine genera have been described since 2000, with 19 of them described in the last 5 years, including six in the year 2023. Many of these are rare or rarely collected and are poorly represented by specimens: of the 46 genera not represented in our data set, 23 have no specimens in MyCoPortal, six are represented by a single specimen, and only six genera are represented by more than 10 specimens.

DNA sequencing, genome assembly, and ortholog extraction.—Whole genome sequencing of 418 specimens resulted in 13 794 532 paired-end reads per specimen on average (SUPPLEMENTARY TABLE 3). On average, genome assemblies possessed an assembly N50 of 12.9 kbp (thousand base pairs), total assembly length of 61.6 Mbp (million base pairs), 53 972 scaffolds, and a BUSCO score of 74.7%. Thirty-four of 418 specimens possessed BUSCO scores less than 20%, and 175 specimens possessed BUSCO scores greater than 90% (SUPPLEMENTARY TABLE 3). After removing specimens with poor BUSCO recovery, our final data set included 384 Boletaceae specimens, three outgroup taxa, and 1461 single-copy orthologs.

Phylogenetic analysis.—The summary coalescent tree resolved most nodes with significant statistical support, including full resolution of the backbone within Boletaceae (FIG. 2). Many of the groups recovered are consistent with previous studies but now with statistical support (Dentinger et al. 2010; Nuhn et al. 2013; Wu et al. 2014). We formally recognize eight subfamilies, including six subfamilies following previous authors (Wu et al. 2014), and two new subfamilies, the Phylloboletelloideae and Suillelloideae (Tremble et al. 2023). Many of the currently accepted genera that are not mono- or oligotypic are polyphyletic. One notable phylogenetic pattern is the polyphyly of the endemic Chilean taxa, all of which were recovered on long branches in separate subfamilies: *Gastroboletus valdivianus* in Xerocomoideae, *Boletus loyita* in Austroboletoideae, *Butyriboletus loyo* in Suillelloideae, and *Boletus putidus* in Boletoideae.

Gene tree comparison.—Average bootstrap support had the highest positive correlation with the number of taxa present (Pearson's coefficient = 0.76), and weak to moderate negative correlations with alignment length (Pearson's coefficient = −0.17), clocklike branch lengths (Pearson's coefficient = −0.13), and total tree length (Pearson's coefficient = −0.20). Generalized Robinson-Foulds distances were weakly to moderately negatively correlated with number of taxa (Pearson's coefficient = −0.33), total length (Pearson's coefficient = −0.30), and clocklike branch lengths (Pearson's coefficient = −0.13), and weakly to moderately positively correlated with alignment length (Pearson's coefficient = 0.29) and average bootstrap support (Pearson's coefficient = 0.17). Clocklike branch lengths and total tree length were weakly positively correlated (Pearson's coefficient = 0.15).

Divergence dating.—Using the two- and six-calibration sets, the following ages were estimated (FIGS. 2, 3): Stem ages for the Boletaceae were estimated at 139 my and 77 my, depending on the calibrations used (TABLE 1). The crown age of the Boletaceae and stem age of Chalciporoideae were estimated at 103–105 my and 63 my (49–77 my). The stem ages of the Phylloboletelloideae were estimated at 83–87 my and 58 my (49–77 my). The radiation of the remaining subfamilies was estimated to have occurred between 61 and 51 mya. The origin of *Boletus* sensu stricto (i.e., "true porcini") was estimated at 38 my and 35 my (29–42 my), and its diversification was estimated at 29–30 mya and 26 mya (20–34 mya).

Ancestral range reconstruction.—Ancestral distribution reconstruction recovered a likely Paleotropical origin of the Boletaceae (DEC+J model chosen with lowest AIC and AICc for both coding sets), with major descendant radiations originating in the Paleotropics (Africa and Asia) and Neotropics (FIG. 3). In addition, we found evidence for multiple diversification events spurred by the separation of Gondwana (FIG. 3). Gondwanan separation occurred in two predominant phases: Phase 1 (southern Gondwanan disarticulation), which involved the separation of southern South America, southern Africa, Australia-Antarctica, and Madagascar-India, beginning approximately 180 mya and largely completed by 120 mya (Jokat et al. 2003), and Phase 2 (western Gondwanan disarticulation), involving the separation of South America and Africa, which was completed 80 mya (Reguero and Goin 2021). At the split between the Austroboletoidae and

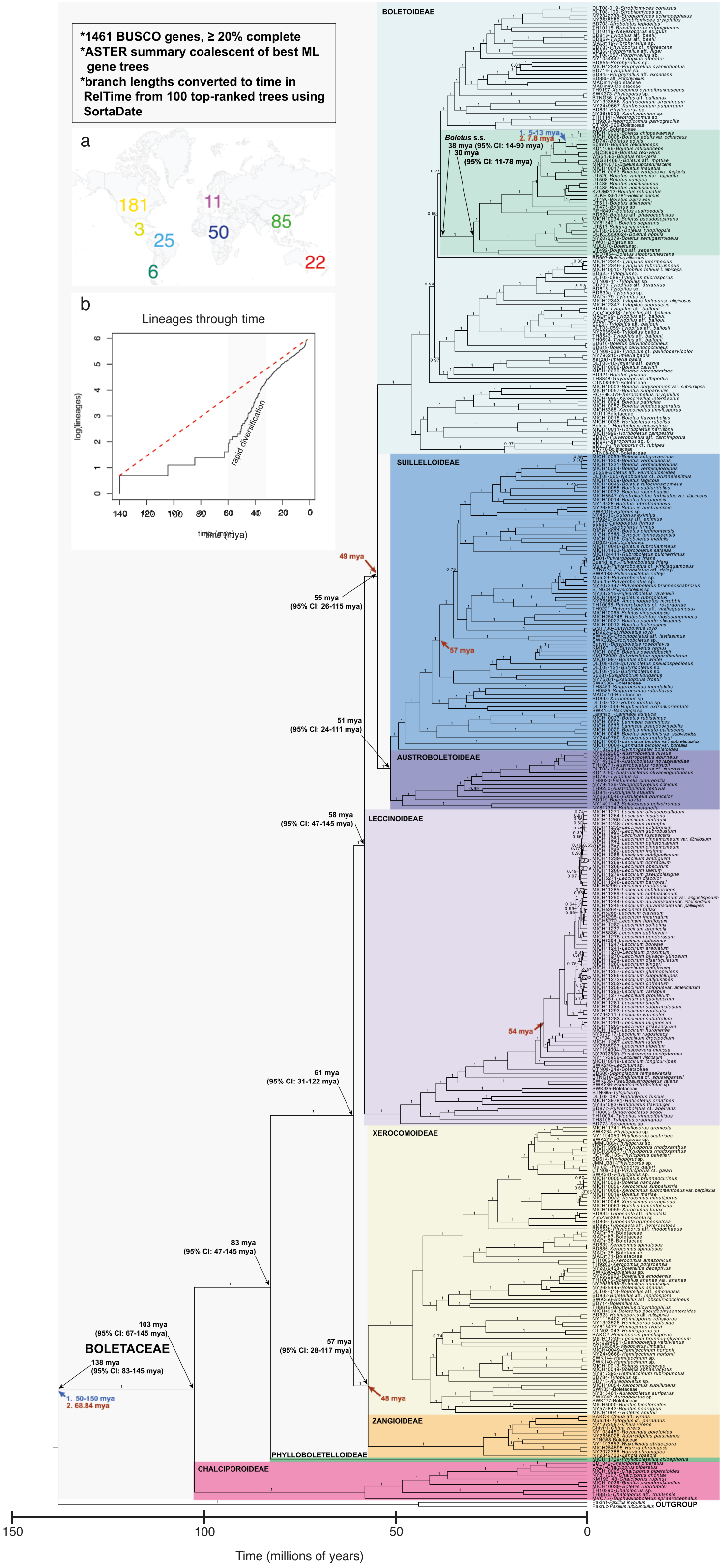


Figure 2. Time-calibrated phylogeny of Boletaceae using 1461 BUSCO genes. Topology is a summary coalescent of individual best ML gene trees using ASTRAL-hybrid. Numbers on branches are quartet probabilities. Branch lengths were converted to time using the top 100 best gene trees estimated using SortaDate in RelTime. Red and blue arrows and numbers indicate node calibrations used for the six-calibration and two-calibration divergence estimations, respectively. Inset a. Map of specimen origins with numbers of specimens from each geographic area. Inset b. Lineages-through-time plot calculated with the "ltt.plot" function in the R package APE. The dashed line represents a constant birth-death rate. The shaded box indicates a period of increased diversification from a constant birth-death rate indicative of a rapid radiation.

Suillelloidae (FIG. 3), we estimated a putative Phase 1 Gondwanan separation to have occurred, which led to rapid formation of South Temperate, Neotropical, and Paleotropical lineages. Later, at least five putative Phase 2 separation events occurred, splitting the Paleotropical and Neotropical lineages. Our divergence time estimation places these events much earlier than the 120 mya and 80 mya reported (estimated at 60 mya for Phase 1—southern Gondwanan separation and 40 mya for Phase 2—western Gondwanan separation in our analyses), which suggests that our divergence time estimation could be consistently underestimating dates.

In our four-category paleoregion coding set, the Boletaceae ancestor was equally likely to be Neotropical or Paleotropical. However, the subsequent node that leads to the rest of the Boletaceae (excluding Phyllobolletoidae and Chalciporoideae) was well supported as Paleotropical, as were all immediate descendent nodes. Our coding of the Chalciporoideae and the single *Phylloboletellus* specimen likely had a strong influence on deep-node ancestral range reconstructions. The backbone nodes of the Boletaceae excluding Phylloboletoidae and Chalciporoideae were estimated as Asian in origin, corroborating the four-category analysis, although with less confidence. Migrations between phytogeographic regions were dominated by dispersals between the Indo-Malesian and Holarctic regions (FIGS. 3, 4).

DISCUSSION

Phylogeny.—The phylogeny supports the recognition of eight subfamilies within the Boletaceae, including the recently defined Phyllobolletoideae and Suillelloideae (Tremble et al. 2023), and their interrelationships were fully resolved for the first time. The Chalciporoideae was recovered as the basal group of the Boletaceae, a relationship previously noted by others (e.g., Wu et al. 2014). The rarely collected *Phyloboletellus chloephorus* (Singer and Digilio 1951) was the next lineage to branch off before the radiation that gave rise to the six additional subfamilies. Previous studies have placed *Pseudoboletus parasiticus* in a position similar to that of *Phylloboletellus chloephorus* in our study (Caiafa and Smith 2022; Nuhn et al. 2013; Sato and Toju 2019; Wu et al. 2014). However, we were unable to include genomic data from a representative of *Pseudoboletus* in our study so cannot assess its putative phylogenetic position.

The tree topology has intriguing implications for the role of ecological transitions in Boletaceae diversification. Members of the Chalciporoideae have not been identified from ECM root tips in situ despite multiple synthesis attempts (Kasuya and Igarashi 1996; Yamada and Katsuya 1995) and are thought to be saprotrophic or mycoparasitic (Caiafa and Smith 2022). As well, *P. chloephorus* may not be ECM given its occurrence in nonectotrophic forests (Singer and Digilio 1951). Moreover, *P. parasiticus* and other *Pseudoboletus* spp. produce sporocarps directly attached to gasteroid *Scleroderma* and *Astraeus* and are assumed to be mycoparasites (Binder and Hibbet 2006; Nuhn et al. 2013; Raidl 1997). Interestingly, Richter and Bruhn (1989) reported ectomycorrhizal synthesis between *Pseudoboletus parasiticus* and red pine (*Pinus resinosa*), leading both Tedersoo et al. (2010) and Sato and Toju (2019) to list *Pseudoboletus* as ectomycorrhizal. However, the report by Richter and Bruhn (1989) is dubious because the specimen they used to produce the ectomycorrhizal synthesis was not attached to a *Scleroderma* sporocarp and could have been misidentified. Altogether, the phylogenetic positions of these groups suggest that a strict ECM ecology is not ancestral for the Boletaceae. This situation corroborates Sato and Toju (2019), who induced that the ECM habit emerged coincident with rapid diversification of the Boletaceae. Genomic changes coinciding with the emergence of an obligate ECM habit further support the view that this nutritional shift has profoundly impacted Boletaceae (Wu et al. 2022).

Taxonomic implications.—Many taxonomic changes in the Boletaceae have been proposed in recent years. In particular, new genera have been erected for phylogenetically unresolved lineages. Many of these new genera are mono- or oligotypic (composed of one or few species) (e.g., Castellano et al. 2016; Chakraborty and Das 2015; Halling et al. 2023; Henkel et al. 2016). These new genera typically lack well-defined, unifying morphological features and are difficult or impossible to recognize without also identifying their species or phylogenetic analysis. Such a lack of unifying features minimizes the information content of these generic names. Nonetheless, the strong nodal support throughout our phylogeny sets the stage for a new, comprehensive and stable generic-level taxonomy. We will address this in subsequent works when all currently accepted genera are sampled.

Biogeography and chronology.—Divergence dating estimated the origin of the Boletaceae to be as old as 139 my and as young as 77 my. Ranking genes with different metrics had little impact on divergence date estimates. However, the two calibrations sets gave very

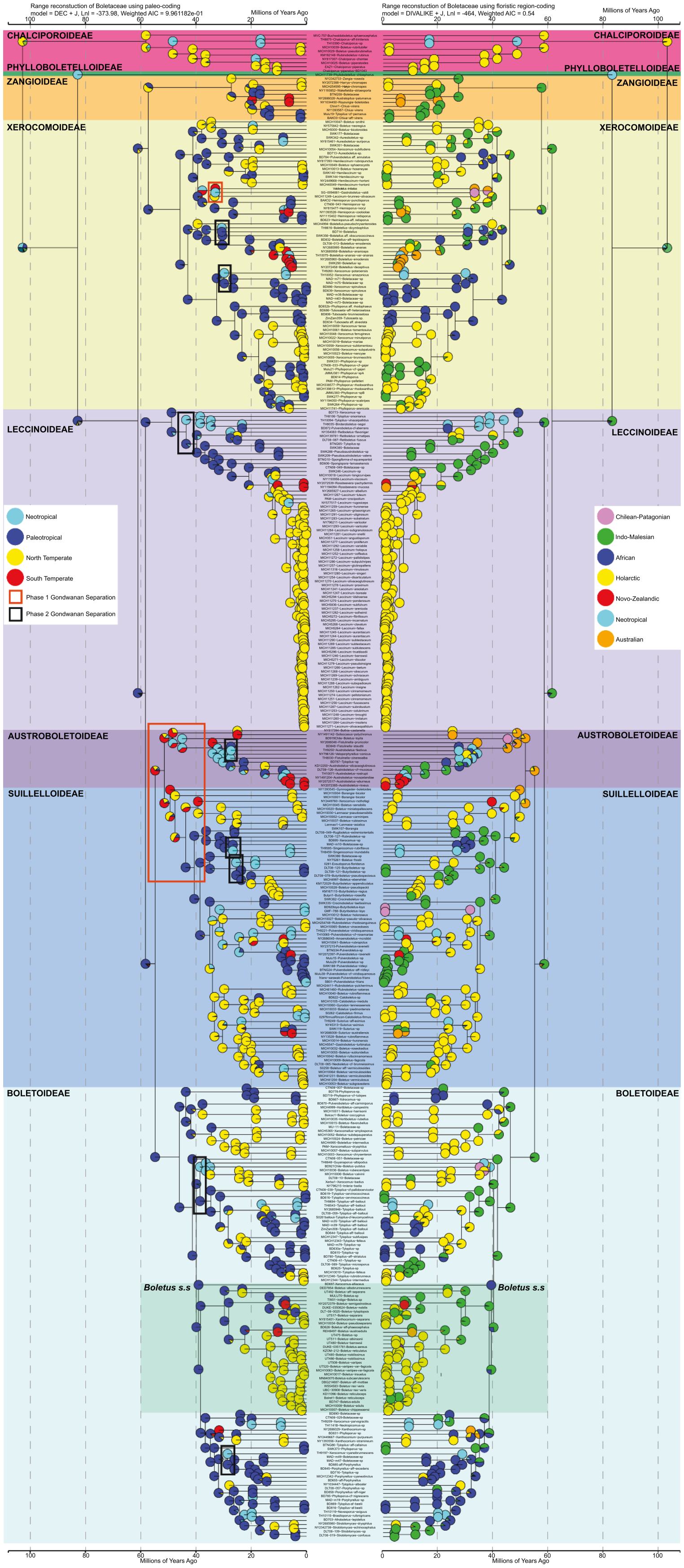
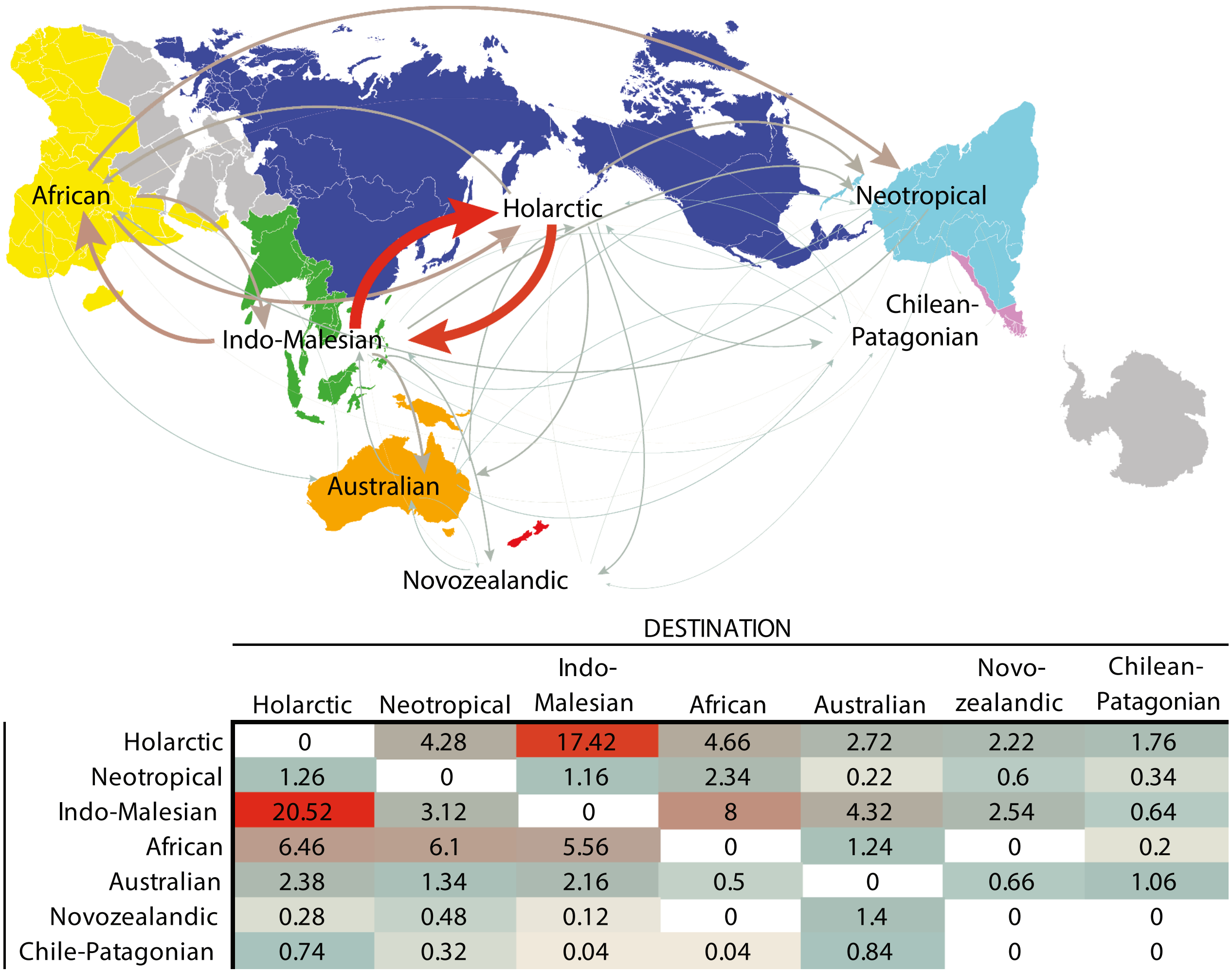


Figure 3. Biogeographic reconstruction using BioGeoBEARS with our two-calibration-point time tree. Left-hand tree depicts 4-state coding scheme (light blue = Neotropical, blue = Paleotropical, yellow = North Temperate, red = South Temperate) and right-hand tree depicts floristic region coding scheme (pink = Chilean-Patagonian, green = Indo-Malesian, blue = African, yellow = Holarctic, red = Novo-Zealandic, light blue = Neotropical, Orange = Australian). Pie charts indicate the proportional likelihood of each state at a node. Red and black boxes indicate Phase 1—southern Gondwanan and Phase 2—western Gondwanan diversification events, respectively.

Table 1. Estimated divergence dates of the Boletaceae using two separate calibration sets and gene ranking approaches.

		Boletaceae			*Boletus* s. str.	
Ranking method	Calibrations	Stem age	Crown age	Crown age	Stem age	Crown age
Generalized Robinison-Foulds distance	2	139.42 (50–150)	105.36 (74.16–144.09)	87.25 (54.58–139.5)	37.92 (16.06–85.72)	28.96 (12.42–67.49)
SortaDate (BS, clocklike, length)	2	138.38 (50–150)	102.77 (67.33–144.83)	82.85 (46.94–144.83)	38.36 (14.37–90.11)	29.70 (11.24–78.46)
SortaDate (BS, clocklike, length)	6	76.73	63.12 (48.90–76.73)	57.53 (48.90–76.73)	35.10 (28.95–41.89)	26.13 (19.90–34.31)



SOURCE \ DESTINATION	Holarctic	Neotropical	Indo-Malesian	African	Australian	Novo-zealandic	Chilean-Patagonian
Holarctic	0	4.28	17.42	4.66	2.72	2.22	1.76
Neotropical	1.26	0	1.16	2.34	0.22	0.6	0.34
Indo-Malesian	20.52	3.12	0	8	4.32	2.54	0.64
African	6.46	6.1	5.56	0	1.24	0	0.2
Australian	2.38	1.34	2.16	0.5	0	0.66	1.06
Novozealandic	0.28	0.48	0.12	0	1.4	0	0
Chile-Patagonian	0.74	0.32	0.04	0.04	0.84	0	0

Figure 4. Dispersal events and rates inferred from BioGeoBEARS using our two-calibration-point time tree. Top: Map depicting global floristic regions (Liu et al. 2023) that correspond with our second ancestral state reconstruction coding region and are colored and labeled on the map. Map was rendered using the "imago" R code (https://github.com/hrbrmstr/imago) to reproduce the AuthaGraph world map projection (http://www.authagraph.com/top/?lang=en). Curved arrows indicate inferred directional dispersal events and are colored by rate values following the table (Bottom). The stroke weight of the arrows has been scaled to percent of the maximum rate value following the values in the table. Bottom: Table of dispersal rates inferred under a DIVAlike+j model in BioGeoBEARS. Rates in the table are mean number of recreated dispersal events between two coding regions. Source regions are at left and destination regions are along the top. Values are colored along a scale from cool to warm (red being maximum).

different estimates for most nodes. For example, the estimated dates of the crown age of the Boletaceae using the Varga et al. (2019) internal calibrations were almost half those of the two-calibration set [63 my (95% CI: 49–77 my) vs. 103 my (95% CI: 67–145 my), respectively]. Although there is some overlap in the confidence intervals, given the lack of fossil evidence, it is difficult to interpret these different estimates. The priors for the internal calibrations were also normally distributed, which may have introduced more error (Schenk 2016). Regardless of the priors used, the dates estimated using the Varga et al. (2019) internal calibrations may be suspect due to extensive topological incongruence of their phylogenetic trees with ours. Our older divergence estimate corroborates the results of He et al. (2020), and our internal dates correspond with other results, such as the ~48 my origin of the *Strobilomyces* group (Han et al. 2018). Our older divergence estimate is also in line with the origin of ECM Pinaceae in the early Cretaceous (Brundrett and Tedersoo 2018). Therefore, we consider the older estimate to be more plausible.

In our ancestral range reconstruction analysis, we found evidence of multiple diversification events that may have been initiated by the breakup of both southern and western Gondwana. The first phase of the Gondwanan separation (southern Gondwana) postulated by Jokat et al. (2003) correlates well with our estimated origin of the Boletaceae; this indicates that the family could have been diverse and widely distributed by 120 mya, substantially older than the estimated age from our divergence dating analysis (103–105 my) using the two-calibration set, albeit within the 95% confidence interval. Still, our dates are at best coarse estimates based on fossil-free secondary calibrations. However, the phylogenetic pattern that parallels the breakup of Gondwana means that a hypothesis of vicariant dispersal cannot be rejected; if that is true, then this may provide corroborating evidence that our estimated ages may, in fact, be too young. For example, in our ancestral range reconstruction, we identified at least eight separate nodes where the immediate descendant nodes are Neotropical and Paleotropical, a pattern that is often a hallmark of western Gondwanan separation (Beaulieu et al. 2013; Regueroand Goin 2021). However, all of these nodes were reconstructed to have occurred in the last 50 my. Unless our divergence estimates are underestimates, this biogeographic pattern would have to have resulted from at least eight independent intercontinental dispersal events or is indicative of descent from common ancestors that once occupied a Holarctic range at high latitudes (e.g., the "boreotropics hypothesis"; Wolfe 1975). Although repeated intercontinental dispersals cannot be entirely ruled out, they would be surprising and unprecedented. Thus, we consider it more likely that our divergence estimates are underestimates, and that these "dispersal events" reflect vicariance due to western Gondwanan separation. However, the alternative hypotheses cannot be rejected with the current dataset, and testing them will require more comprehensive and intentional geographic sampling and, ideally, incorporation of fossils. In any case, our divergence estimates suggest that the Boletaceae originated and diversified within the early to late Cretaceous period. During this time, the global climate was warm and wet (Hay and Floegel 2012), gymnosperms and subsequently angiosperms diversified (Crisp and Cook 2011), and the supercontinents broke apart (Jokat et al. 2003).

Our biogeographic and divergence dating analyses support a Paleotropical origin of the Boletaceae with subsequent diversification during separation of both western and southern Gondwana. Later divergence was likely facilitated by continental drift–based vicariance events and possible long-distance dispersals. Other recent studies have shown that lineages of ECM fungi originated in Gondwana or more recently in Paleotropical regions (Codjia et al. 2023; Dentinger et al. 2010; Hackel et al. 2022; Han et al. 2018; Hosaka et al. 2008; Kennedy et al. 2012; Matheny et al. 2009; Ryberg and Matheny 2011; Sánchez-Ramírez et al. 2015). Endemism paralleling phytogeographic regions was implied with our biogeographic reconstruction. For example, we found that estimated dispersal events were most frequent between the Indo-Malesian and Holarctic regions over the past 50 my. We acknowledge the difficulty of determining origins and dispersal events in the absence of fossils or other corroborating evidence. Nonetheless, our study and others suggest that vicariance may have played a strong role in the distribution of ECM fungal taxa, despite the long-distance dispersal capacity of airborne spores (Matheny et al. 2009; Peay et al. 2010; Peay and Matheny 2016). Conversely, vicariance alone cannot explain the close phylogenetic relationships seen between distantly disjunct taxa. Long-distance dispersals may have occurred, albeit rarely. Although long-distance dispersal is demonstrably possible in the Boletaceae and other ECM lineages, the likelihood of its frequent occurrence is low (Geml et al. 2012; Hackel et al. 2022; Tremble et al. 2022). Most basidiospores do not travel far from the parental sporocarp (Galante et al. 2011), and the probability of two airborne basidiospores landing in close-enough proximity to mate is negatively correlated with increasing distance from sporocarps (Golan and Pringle 2017; Peay et al. 2012). Such improbabilities notwithstanding, our biogeographic patterns are consistent with episodic long-distance dispersal, possibly by aerial dispersal of basidiospores, spores vectored by migrating animals (e.g., Elliott et al. 2019) or somatic mycelia on rafting vegetation (Thiel and Gutow 2005).

Our biogeographic reconstructions are consistent with the "Southern Route to Asia" hypothesis (Wilf et al. 2019). This idea proposes that ECM *Castanopsis* (Fagaceae), and by extension their symbiotic fungi, existed in southern Gondwana during the Eocene and were carried on Australia northward to Asia. In this scenario, the Gondwanan ECM habitat tracked increasingly drier climatic niches on Australia as it moved northward and collided with the Pacific Plate.

A relictual ECM community remained in a newly isolated New Guinea and subsequently spread northwest along the montane Australasian archipelago, followed by dispersal into continental Asia. Many of the dispersal events we found between Indo-Malesia and other regions, especially the Holarctic, are inferred within the last 20 my, coincident with the late-Oligocene contact of Australia with the Pacific Plate (Hall 2011). As suggested by Halling et al. (2012), recent Boletaceae migrations likely occurred across the Australasian archipelago and are corroborated by our inferred recent regional dispersal events. Interestingly, all endemic New Zealand Boletaceae are estimated to be much younger than the separation of New Zealand from Gondwana ~80 mya, strongly suggesting that they arrived via dispersal. Similar patterns have been shown in other Agaricomycetes, such as *Lentinula* and Inocybaceae (Hibbett 2004; Matheny et al. 2009).

Biogeographic reconstructions are highly sensitive to taxon sampling, and our dataset is not immune to equivocal reconstructions. For example, in both coding schemes, the most recent common ancestor of the Chalciporoideae had the highest probabilities of a North Temperate and a North American origin, respectively. However, with no Chalciporoideae samples from Asia, Africa, or Australia/New Zealand in our study, their potential impacts on the reconstruction are unknown. Such sampling gaps notwithstanding, we have the most geographically comprehensive sampling for Boletaceae ever compiled and provide the first opportunity to examine global-scale biogeographic patterns. Insights into the evolution of the Boletaceae are revealed for the first time, despite slight uncertainty at a minority of nodes.

The evolutionary origins of distinctive regional Boletaceae assemblages have long been a mystery (Horak 1977). For example, the endemic Boletaceae of Chile and Argentina have not been included in previous phylogenetic studies, and their morphology-based affinities have been inconclusive (Horak 1977). The recovery of several Chilean species as independent lineages on long branches in four of the subfamilies implies that they have survived in isolation without speciating for millions of years. The closest relatives of these Chilean boletes occurred in geographic regions as disjunct as North America, lowland tropical northern South America, and Australia. *Boletus loyita* and *Gastroboletus valdivianus* were most closely related to extant Australian taxa, suggesting an origin prior to southern Gondwanan disarticulation (phase 1 separation) (Reguero and Goin 2021). Close relationships between southern Gondwanan Australian and southern South American taxa have been documented elsewhere (Feng et al. 2017). It is difficult to determine whether Chilean boletes are relictual species, whose placement on long branches is due to extensive extinction of sister lineages or simply due to no subsequent speciation after southern Gondwana separation. However, ECM genera such as *Lactarius* and *Russula* are also strikingly species-poor in Chile compared with other parts of the world (Nouhra et al. 2019), which may suggest abnormally low ECM net diversification rates in Chile. In all likelihood, Chilean boletes arose in Gondwana, separated from their sister lineages during Gondwanan disarticulation, and either suffered from high levels of extinction or underwent no subsequent speciation for tens of millions of years.

Ancestral range reconstruction recovered an Asian origin of the core, “true porcini” genus *Boletus* s. str., corroborating the results of Feng et al. (2012). However, we cannot entirely rule out an African origin. The Central African endemic *Boletus alliaceus* was recovered here as a sister taxon to *Boletus* s. str., and a similar relationship was found for the recently described *Paxilloboletus africanus* (Badou et al. 2022). Furthermore, we estimated the origin of *Boletus* s. str to be 40 my, which may indicate why the sister lineages to *Boletus* s. str. are endemic to Africa. India separated from Africa and Madagascar ~120 mya (Reguero and Goin 2021) and at 40 mya was already in contact with Asia (Aitchison et al. 2007; Hu et al. 2016). If *B. alliaceus* and *P. africanus* are indeed sister lineages of *Boletus* s. str., then the arrival and subsequent diversification of true porcini in Asia must have been a dispersal event, because the separation of India from mainland Africa (~180–170 mya; Hankel 1994) or Madagascar (83.6–91.6 mya; Storey 1995) occurred long before our estimated age of the *Boletus* s. str. ancestor (~40 mya). Even if a more recent ancestor existed in Madagascar or the Seychelles, the separation of India from these landmasses at ~90 mya (Storey 1995) and ~64 mya (Norton and Sclater 1979), respectively, is still much older than our current age estimates for true porcini. Furthermore, most or all ECM fungi in Madagascar appear to have arrived on the island through dispersal after its separation from Africa (Rivas-Ferreiro et al. 2023), so dispersal is the most plausible mechanism unless ancient Malagasy relict taxa are discovered. In the current study, currently undescribed species of *Boletus* s. str. were recovered from Taiwan, Malaysian Borneo, and the Gulf Coast of the United States, indicating that much more diversity exists in the genus. To sort out the origins and full diversity of *Boletus* s. str., more mycological exploration and whole genome sequencing are needed. In particular, discovery and analysis of true porcini lineages from India and Africa could shed further light on the origin of this charismatic group.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the author(s).

DATA AVAILABILITY STATEMENT

All whole genome sequences are deposited on the National Center for Bioinformation Technology (NCBI) Short Read Archive under the bioproject accession ID Bioproject PRJNA1022813. Genome assemblies are also deposited on the dryad data repository (https://doi.org/10.5061/dryad.dv41ns25n). BUSCO gene alignments, individual gene trees, and our summary coalescent species tree are publicly available on figshare (https://figshare.com/projects/Tremble_et_al_2023_BoletaceaePhylogeny/184027).

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