Phylogenomics, biogeography, and trait evolution of the Boletaceae (Boletales, Agaricomycetes, Basidiomycota)

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ABSTRACT

The species-rich porcini mushroom family Boletaceae is a widespread and well-known group of ectomycorrhizal (ECM) mushroom-forming fungi that has eluded intrafamilial phylogenetic resolution despite many attempts using morphological traits and multi-locus molecular datasets. In this study, we present a genome-wide molecular dataset 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 subtropical forests in northwestern Argentina is recovered as an early diverging lineage within the 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 cladogenesis patterns that parallel its fragmentation. Ancestral state reconstruction of morphological traits predicts that the ancestor of the Boletaceae was lamellate with ornamented basidiospores, contrary to the most common “bolete” sporocarp morphology. Transition rates indicate that the lamellate hymenophore and sequestrate sporocarp are reversible traits. Together, this study represents the most comprehensively sampled, data-rich molecular phylogeny of the Boletaceae to date, enabling robust inferences of trait evolution and biogeography in the group.

KEYWORDS:

phylogenomics; Boletaceae; porcini; evolutionary radiation; Gondwana

INTRODUCTION

Evolutionary radiations result from short bursts of relatively rapid diversification and demonstrate the creative power of evolution. Adaptive and non-adaptive radiations may be the most common macroevolutionary patterns, and are fundamental to the origins of biodiversity (Simoes et al. 2016; Futuyma 1998; Schluter and McPeek 2000). Yet, the underlying causes of evolutionary radiations are poorly known for most groups, as most studies have focused on animals and plants (Soulebeau et al. 2015). The other major multicellular eukaryotic group, the Fungi, have largely neglected (Varga et al. 2019).

The porcini mushroom family Boletaceae (Fig. 1) is an example of an evolutionary radiation in Fungi (Bruns et al. 1992). The family is exceptionally diverse (>2000 currently accepted species) and globally distributed, but poorly documented for many regions. Yet, they 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; Sitta and Floriani 2008; Dentinger et al. 2010; Dentinger and Suz 2014). Despite their conspicuous sporocarps (=mushrooms), ecological dominance, and cultural importance, new species of Boletaceae are regularly described from poorly explored habitats around the globe(e.g. Halling et al. 2006, 2023; Fulgenzi et al. 2007, 2008, 2010; Neves et al. 2010; Husbands et al. 2013; Castellano et al. 2016; Chakraborty et al. 2015; Das et al. 2015, 2016; Henkel et al. 2016; Magnago et al. 2017), as well as from wild-collected foods in local markets and even from supermarkets, e.g., (e.g. Das et al. 2015; Dentinger and Suz 2014; Halling et al. 2014). While these new species are increasingly understood in a global phylogenetic context, shedding light on their origin, diversification, and migration in time and space, there has been little progress made towards resolving the backbones in the phylogenies for Boletaceae using legacy loci (Grubisha et al. 2001;

Binder & Hibbett 2006; Drehmel et al. 2008; Dentinger et al. 2010; Nuhn et al. 2016; Wu et al. 2014).

One of the most prominent consequences of this lack of phylogenetic resolution is the recent explosion of new generic names to accommodate newly discovered species, or species that are included in molecular phylogenetic analyses for the first time and that are recovered on long branches with no supported affinity to existing generic names (e.g., Castellano et al. 2016; Henkel et al. 2016; Badou et al. 2022; Halling et al. 2023). Although this is a predictable pattern for evolutionary radiations, it has resulted in a recent proliferation of new genera, many of which are monotypic or require identification of species to recognize, an impractical solution to the problem. Taken together, the current situation is perhaps best described as a quagmire of nomenclatural, taxonomic, ecological, and evolutionary speculation.

Beyond taxonomic concerns, the Boletacaeae presents a unique system to identify the genetic mechanisms that contribute to diversification. Abundant evidence indicates that the Boletaceae underwent an evolutionary radiation 60-100 million years ago (Bruns and Palmer 1989; Binder et al. 2006; Dentinger et al. 2010; Wu et al. 2014, 2016; Sato et al. 2019). This early radiation has been correlated with the convergence of morphological traits, such as lamellate (“gilled”) mushrooms and gasteromycetization (Castellano et al. 2016), which suggests that the development of novel morphological diversity is constrained by relatively few changes in developmental pathways.

Sampling fungi for comparative studies is often a difficult and time-consuming process due to their cryptic somatic lifestyles and the ephemeral, unpredictable sporocarps. This can result in ad hoc taxon sampling that suffers from heavy sampling bias.

Although progress has been made in describing new diversity and construction of molecular phylogenetic datasets, the phylogeny for Boletaceae has been frustratingly difficult to resolve. This is the consequence of molecular phylogenetic datasets that included only one to four loci (Grubisha et al. 2001; Binder & Hibbett 2006; Drehmel et al. 2008; Dentinger et al. 2010; Nuhn et al. 2013; Wu et al. 2014, 2016). While phenomena such as incomplete lineage sorting and hybridization may obscure historical phylogenetic signal, previous datasets for the Boletaceae have consisted of patchy taxonomic and geographic sampling. All these factors' impacts on phylogenetic reconstruction were intensified by the aforementioned rapid radiation event (Bruns et al. 1992; Sato et al. 2017). Without a robust phylogeny, it is impossible to name, classify, reproduce ecological studies, and understand evolutionary history in the Boletaceae. For example, only a few recent studies have included representatives of the exceptionally rich Australian macrofunga (Halling, Fechner, et al. 2015, 2023; Halling, Nuhn, et al. 2012), and the Neotropics and Afrotropics have been conspicuously underrepresented in family-level analyses despite their exceptional species richness (Heinemann 1951; Henkel et al. 2012).

Recent fieldwork has resulted in many new collections from under-sampled regions (B. Dentinger and T. Henkel, unpublished data), and these are now available to include in phylogenetic datasets aiming to achieve the first globally representative sampling of the Boletaceae. Collections-based phylogenomics is also effective for resolving ancient relationships among mushroom-forming fungi (Dentinger et al. 2015, Liimatainen et al. 2021, Tremble et al 2020). No one, however, has yet applied these methods to the Boletaceae. Moreover, whole genome sequencing of mushroom forming fungi provides opportunities to go beyond phylogeny reconstruction. For example, whole genome sequencing can facilitate analyses of the population processes that

generate biodiversity (e.g. Tremble et al. 2022), resulting in a much richer insight into biology than only a few molecular loci can provide on their own.

For this study, we generated the first robust phylogeny of the Boletaceae that utilized a large molecular dataset comprised of 1764 genome-wide loci from 418 taxa across the family collected in many tropical and temperate geographic regions. To obtain sufficiently representative sampling, we targeted fungarium collections including type specimens whenever possible, augmented by recent new collections to fill in geographic and taxonomic gaps. We included samples from regions that have been neglected in the past (e.g., Africa, southern South America, lowland tropical South America, Australia), and wherever possible type species to provide an anchor for a future taxonomic revisions of genera. We also performed the first inclusive biogeographic reconstruction of the Boletaceae and assessment of morphological trait evolution using our highly resolved genomic phylogeny. Overall, we provide unprecedented insight into broad patterns of evolution in this enigmatic fungal group.

MATERIALS AND METHODS

Sampling

Taxon selection focused on obtaining representatives of all currently accepted genera, 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 unrepresented 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 JGI Mycocosm Portal (Grigoriev et al. 2014) for *Boletus coccyginus*,

B. reticuloceps, *Butyriboletus roseoflavus*, *Chiua virens*, *Lanmaoa asiatica*, and *Imleria badia* (Miyauchi et al. 2020, Wu et al. 2022, Kohler et al. 2015). Genomes of *Paxillus involutus*, *Paxillus adelphus*, and *Hydnomerulius pinastri* (Kohler et al. 2015) from JGI were used for outgroups.

DNA extraction and sequencing

Genomic DNA was extracted from 10mg of hymenophore tissue from each specimen. Tissues was homogenized in 2.0 mL screw-cap tubes containing a single 3.0 mm and 8 x 1.5 mm stainless steel beads by bashing them in a BeadBug microtube homogenizer (Sigma-Aldrich, #Z763713) for 120 seconds at a speed setting of 3500 rpm. After physical disruption, DNA was extracted using the Monarch® Genomic DNA Purification Kit (New England Biolabs, Massachusetts; #T3010) with the Monarch® gDNA Tissue Lysis Buffer (#T3011) using double the volume of lysis buffer, 1 hour lysis incubation at 56 C , and 550 µl of wash buffer during each of the wash steps. DNA extract quality was assessed for quality using a NanoDrop 1000 (Thermo Scientific) 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 (Supplementary Table 2). All raw reads and whole genome assemblies are deposited in the Short Read Archive (Bioproject#PRJNA1022813).

Genome assembly and ortholog extraction

Raw sequencing reads were quality-filtered and adapter-trimmed using fastP v0.20.1 (Chen et al. 2018) with default settings. Genome assemblies were produced from quality-filtered reads using SPAdes v3.15.0 (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 v7.397 (Katoh, Rozewicki, and Yamada 2017) with the “L-INS-I” algorithm, and maximum- likelihood gene-trees were inferred using IQ-TREE v2.0.3 (Minh et al. 2020) with automatic model selection in ModelFinder (Kalyaanamoorthy et al. 2017) and ultra- fast bootstrapping (BS, (Hoang et al. 2018)) with 1000 replicates. A summary coalescent species tree was constructed from the resulting gene trees using hybrid-ASTRAL implemented in ASTER (v1.15) (Zhang & 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 gene tree discordance, individual 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 correlations between metrics.

Divergence dating

A timetree 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 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 & Kumar 2000) from two sets of 100 genes: 1) the top 100 genes with well-supported clock-like trees determined using SortaDate

(Smith et al. 2018) and 2) the top 100 genes with the smallest generalized Robinson-Foulds ('gRF') distances to the species tree calculated using the R package 'TreeDist.' The timetree was computed using two sets of calibration constraints. The first included two calibrations: 1) a secondary calibration for the stem age of the Boletaceae from 50-150 mya (Varga et al. 2019, Wu et al. 2022) and 2) a secondary calibration for the stem age of Boletus edulis from 5-13 mya (Tremble et al. 2022). 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). 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. Confidence intervals were computed using the Tao et al. (2020) method. Times were not estimated for outgroup nodes 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 (Ronquist 1994; Ree et al. 2005; Landis et al. 2013). 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 2018), which has unified the most prevalent biogeographic models. Samples were coded in two ways: 1) Paleotropical (consisting of Africa and Tropical Asia), Neotropical (South and Central America), South Temperate (temperate

Australia and New Zealand), or North Temperate (North America, Europe, Northern tem- perate Asia), and 2) by floristic regions of the world, Holarctic including Central America, Neotropical, Chilean-Patagonian, African, Indo-Malesian, Australian, and Novo-Zealandic (Liu et al 2023) (Supplementary Table 3). Central America was combined with the Holarctic region because prior studies have shown that Central American ECM fungi are mostly derived from North American ancestors (Halling 1996). The most likely model was chosen according to AIC and weighted AIC score calculated in BioGeoBEARS.

RESULTS

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 2). 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%. 34 of 418 specimens possessed BUSCO scores less than 20%, and 175 specimens possessed BUSCO scores greater than 90% (Supplementary Table 2). After removing specimens with poor BUSCO recovery, our final dataset included 383 Boletaceae specimens, three outgroup taxa, and 1461 single-copy orthologs.

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

Stem ages for the Boletaceae were estimated at 138-139 mya and 77 mya using the two- and six-calibration sets, respectively. The crown age of the Boletaceae was estimated at 103-105 mya and 63 mya (49-77 mya) using the two- and six-calibration sets, respectively. The diversification of the Boletineae was estimated at 83-87 mya and 58 mya (49-77 mya) using the two- and six-calibration sets, respectively. The origin of *Boletus sensu stricto* (i.e. "porcini) was estimated at 38 mya and 35 mya (29-42 mya), and its diversification was estimated at 29-30 mya and 26 mya (20-34 mya), using the two- and six-calibration sets, respectively.

Ancestral state reconstruction

Ancestral state reconstruction recovered the most likely ancestor of the Boletaceae to be a pileate-stipitate, lamellate mushroom with ornamented spores. The highest transition rates were observed in phenotypic changes from secotioid to gasteroid and from secotioid to pileate-stipitate morphologies, recovering secotioid morphology as being a relatively transient evolutionary state (Table 1). High transition rates were also found to occur from gasteroid to pileate-stipitate morphology, supporting evidence for the reversibility of gasteromycetation.

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 two 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 Gondawana (Fig. 3). Gondwanan separation occurred in two predominant phases: Phase 1, involved the separation of Southern South America, Southern Africa, Australia-Antarctica, and Madagascar-India, which began approximately 180 MYA and was largely completed by 120 MYA (Jokat et al. 2003) and Phase 2, involving the separation of South America and Africa, which was completed 80 MYA (Reguero and Goin 2021). At the split between the Austroboletoidae and Suillelloidae (Fig. 3), we found a putative Phase 1 Gondwanan separation event estimated to have occurred 150-120 MYA, which led to the rapid formation of South Temperate, Neotropical, and Paleotropical lineages. Later, around 90-70 MYA, we found at least five putative Phase 2 separation events, leading to the separation of Paleotropical and Neotropical lineages.

In our four-category paleo-region 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 Phylloboletoidae and Chalciporoideae) was recovered as Paleotropical with high likelihood, as were all subsequent daughter nodes. The coding of the Chalciporoideae and the single *Phylloboletellus* specimen in our dataset likely had a strong influence on ancestral range reconstructions at the deepest nodes. The backbone nodes of the Boletaceae excluding Phylloboletoidae and Chalciporoideae were estimated as Asian in origin, corroborating the four-category analysis, though with less confidence. Migrations between phytogeographic regions were dominated by dispersals between the Indo-Malesian and Holarctic regions (Fig. 3,4).

DISCUSSION

Phylogeny

The fully resolved backbone supports the recognition of eight subfamilies, including two newly defined as Phylloboletelloideae and Suillelloideae(Tremble et al. 2023) with their interrelationships resolved for the first time. The Chalciporoideae was recovered as the earliest diverging group in the Boletaceae, a relationship previously noted by Wu et al. (2014). The enigmatic *P. chloephorus* (Singer & Diglio 1952) is 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 where we recovered *P. chloephorus* (e.g. Wu et al. 2014), but we were not able to include a representative of *P. parasiticus* in this study and cannot corroborate this. Nonetheless, the tree topology has intriguing implications for the role of ecological transitions in Boletaceae diversification. Members of the earliest-diverging Chalciporoideae are ecologically labile can be facultatively ECM or mycoparasitic (Caiafa and Smith 2022) and *P. chloephorus*, the next lineage to branch off, is primarily known from non-ectotrophic forests which suggests a non-ECM ecology (Singer & Diglio 1952). Moreover, *P. parasiticus* is a parasite of *Scleroderma* spp. pseudotruffles (Raidl 1997; Binder and Hibbet, 2006; Nuhn et al. 2013). Altogether, the basal position of these early diverging groups suggests that the ancestor of the Boletaceae was non-ECM, and is in line with a previous study which indicated that the ECM habit emerged with the origin of the six derived subfamilies that subsequently radiated (Sato & Toju 2019). A number of genomic changes coinciding with the emergence of obligate ectomycorrhizality (Wu et al. 2022) further support the view that this shift in nutritional mode has profoundly impacted evolution of the Boletaceae. Our phylogeny also recovered previously undocumented sister relationships between the Zangioideae and Xerocomoideae, and between Austroboletoideae and Suillelloideae. The phylogeny also recovered a new clade composed of the Austroboletoideae, Suillelloideae and Boletoideae.

Taxonomic implications

Many taxonomic changes in the Boletaceae have been proposed in recent years. Primarily these have involved the erection of new genera for phylogenetically unresolved lineages. Many of these new genera are monotypic or composed of only a few species (Castellano et al. 2016; Chakraborty et al. 2015; Henkel et al. 2016; Halling et al. 2023). This burdensome proliferation of new genera has made it difficult or impossible to recognize inclusive groupings without requiring knowledge of the species. On the bright side, the strong nodal support throughout our phylogeny sets the stage for a new, comprehensive and stable generic-level taxonomy which will be addressed in subsequent work when all recently described genera are sampled. Nonetheless, we have delimited higher-level groups into subfamilies following previous authors (Wu et al. 2014), which precipitated the need to formally recognize two new subfamilies (Tremble et al. 2023). The subfamily Boletoideae is also expanded with taxa not previously included.

Biogeography and chronology

Our biogeographic and divergence dating analyses support a Gondwanan origin of the Boletaceae. Subsequent diversification was likely facilitated by vicariance events correlated with continental drift and possible long-distance dispersal. These results support the emerging consensus that numerous lineages of ECM fungi originated in Gondwana or more recently in paleotropical regions (Hosaka et al. 2008, Matheny et al. 2009, Dentinger et al. 2010, Ryberg & Matheny 2011, Kennedy et al. 2012, Sanchez-Ramirez et al. 2015, Han et al. 2018; Hackel et al. 2022; Codjia et al. 2023). Phytogeographic endemism was implied with our biogeographic reconstruction. The strongest migrations occurred recently over the past 50 mya between the Indo-Malesian and Holarctic regions (Figs. 3, 4). We acknowledge the difficulty to determining origins

and dispersal events with certainty in the absence of a fossil record or other corroborating evidence. Nonetheless it is clear from our results and those of other studies 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 & Matheny 2016). Conversely, pure vicariance cannot explain the close phylogenetic relationships seen between distant disjunct taxa. It follows that long-distance dispersals may have occurred, albeit rarely. While long-distance dispersal is demonstrably possible in the Boletaceae (Tremble et al. 2022), and has been documented in diverse ECM lineages (Gelm et al. 2012; Hackel et al. 2022), it is obvious that successful long-distance dispersal is difficult. Most basidiospores do not travel far from the sporocarp (Galante et al. 2011), and the probability of two basidiospores landing in proximity to mate becomes highly unlikely with increasing distance from sporocarps (Peay et al. 2012; Golan and Pringle 2017). Such improbabilities notwithstanding, our biogeographic patterns are consistent with episodic long-distance dispersal of basidiospores or even vegetative mycelium vectored by wind, migrating birds (Elliot et al. 2019), or even rafting vegetation (Thiel et al. 2005).

Our biogeographic reconstructions are consistent with the “Southern Route to Asia” hypothesis (Wilf et al. 2019). This idea proposes that ECM Fagaceae, and their symbiont fungi, originated in Gondwana and drifted on Australia north to Asia. In this scenario the Gondwana ECM habitat tracked climatic niches northward as the Australian plate collided with the Pacific plate. A resulting relictual ECM community remained in an emergent New Guinea and subsequently spread northwest along the montane Australasian archipelago and dispersed onto continental Asia. Many of the inferred dispersal events between Indo-Malesia and other regions, especially the Holarctic, are inferred within the last 20 my (Figs. 3,4), coincident with the collision of

Australia with the Pacific plate in the later Oligocene (Hall 2011). More recent migrations across the Australasian archipelago are likely (Halling et al. 2008), consistent with the many recent dispersal events (Fig. 3).

Biogeographic reconstructions are highly sensitive to taxon sampling and despite our best efforts this dataset is not immune to misleading reconstructions. For example, in both coding schemes the ancestral node of the Chalciporoideae had the highest probability of a North Temperate and North American origin. However, we did not have access to samples of this clade from Asia, Africa or Australia/New Zealand, and this could have had impacts on the reconstruction. Nonetheless we have the most geographically comprehensive sampling for Boletaceae ever compiled, and the first opportunity to examine global-scale biogeographic patterns. Thus, insights into the evolution of the Boletaceae are revealed for the first time, despite mild uncertainty at a minority of nodes.

The origin and evolution of distinctive Boletaceae funga in specific regions 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). Four sampled Chilean species were recovered as ancient lineages of similar age within four of the subfamilies (*Gastroboletus valdivianus* in Xerocomoidae, *Boletus loyita* in Austroboletoidae, *Boletus loyo* in Suillelloidae, *Boletus putidus* in Boletoidae). Moreover, the closest relatives of the Chilean boletes occurred in geographic regions as disjunct as North America, lowland tropical northern South America, and Australia. Furthermore, *B. loyita* and *G. valdivianus* are most closely related to extant Australian taxa and potentially originated prior to final Gondwanan disarticulation (Reguero and Goin 2021). Close relationships between Australian and South American taxa is a

common pattern produced by Gondwanan separation (Feng et al. 2017). Altogether, Chilean boletes likely arose in Gondwana, separated from their Australian sister lineages during Gondwanan disarticulation, and experienced no subsequent speciation for tens of millions of years.

Ancestral range reconstruction recovered an Asian origin of the core genus *Boletus* s. str. (a.k.a ”true porcini”) as previously suggested (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 another study found a similar relationship for the recently described African *Paxilloboletus africanus* (Badou et al. 2022). Furthermore, we estimated the origin of *Boletus* s. str to be 40 mya, which may indicate why the sister lineages to *Boletus* s. str. are endemic to Africa. At 40 mya the Indian subcontinent, which had separated from Africa and Madagascar ~120 mya (Reguero and Goin 2021), was already colliding 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 it is possible that the ancestor of true porcini is also African, and arrived in Asia via the Indian subcontinent with subsequent diversification. However, even more diversity of *Boletus* s. str. is still to be uncovered, including the currently undescribed species from Taiwan, Malaysian Borneo, and the Gulf Coast of the US reported here. Clearly more mycological exploration and specimen collecting are needed to fully document global diversity of *Boletus* s. str. In particular, searches in India and Africa for potential relictual lineages could shed light on the origin of this important charismatic group.

Divergence dating estimated the origin of the Boletaceae to be 138-139 mya, and ancestral range reconstructions suggest it may be even older. Ranking genes with different metrics had little impact on divergence date estimates. However, the two sets

of calibrations gave very different estimates for most nodes. The calibration set that included estimates from Varga et al. (2019) gave younger divergence ages than the calibration set with only two calibrations. In fact, the estimated origin dates of the Boletaceae using the Varga et al. (2019) calibrations were almost half those of the two-calibration set. It is difficult to interpret these divergence dates with any certainty due to a lack of fossil evidence. However, the dates estimated using the Varga et al. (2019) internal calibrations may be viewed with extra skepticism due to extensive topological incongruence of the Varga et al. trees with our topology. The older divergence estimates are corroborated by other studies (e.g., He et al. 2020), and internal dates of this tree largely correspond with other work, such as the ~48 MYA origin of the Stobilomyces group reported by Han et al. (2018). These older divergence estimates also correspond with the origin of ECM Pinaceae in the early Cretaceous (Brundrett & Tedersoo 2018). Therefore, we consider these older estimates be more accurate.

In our ancestral range reconstruction analysis, we found evidence of multiple diversification events that may have been initiated by the breakup of the Gondwanan supercontinent. The putative phase 1 Gondwanan separation event identified within the Boletoidae suggests that the Boletaceae was diverse and widely distributed by at least 120 mya (Jokat et al. 2003), substantially older than the estimated age from our divergence dating analysis but within the 95% confidence interval. Our dates are at best coarse estimates based on secondary calibrations and lack fossil-based calibrations. However, the phylogenetic pattern of vicariance that parallels the breakup of Gondwana is compelling and offers corroborating evidence that our estimated ages are in fact too young. In any case, our divergence estimates suggest that the Boletaceae originated and diversified within the early to late Cretaceous period, when the climate was warmer and wetter than it is now (Hay and Floegal, 2012), when gymnosperms and subsequently

angiosperms diversified (Crisp and Cook, 2011), and when the large supercontinental land masses broke apart (Jokat et al. 2003).

Trait evolution

The longest standing debates of Boletaceae systematics have centred on the evolution of morphological traits and their utility for defining natural generic-level groups. Basidiospore color, ornamentation, and hymenophore arrangement were long emphasized in this debate, on the one hand used as evidence for dividing the Boletaceae into multiple genera (Singer, Pegler & Young, etc.) or treating nearly all Boletaceae as a single genus (Corner 1972). More recently, anatomy of the pileal surface and pigment chemistry have been emphasized (e.g. Binder et al. 2002, Sutara 2014). Yet, despite the tremendous morphological and chemical variability in the Boletaceae, the family is typified by the 'bolete' macro-morphology (i.e. pileate-stipitate mushrooms with tubular hymenophores). In addition, spore ornamentation has been considered to be a derived trait, unifying species into readily recognizable genera such as *Strobilomyces*, *Boletellus*, and *Austroboletus* (Berkeley 1851, Murrill 1909, Corner 1972, Pegler & Young 1981, Wolfe 1980). Contrary to these iconic morphotypes, our ancestral state reconstructions suggest that the ancestor of the Boletaceae was probably a pileate-stipitate mushroom with lamellate hymenophore and ornamented spores. This is almost certainly due in large part to the phylogenetic position of the enigmatic *Phylloboletellus chloephorus* (Singer & Diglio 1952). It is also possible that the obligately ECM habit, characterising most extant Boletaceae, was gained after its origin as evidenced by the habitat of *Phylloboletellus* and the saprotrophic and mycoparasitic abilities of Chalciporoideae (Caiafa et al. 2022). The mycoparasitic *Pseudoboletus parasiticus* and *P. silvaticus* have also been recovered as early-diverging lineages of Boletaceae (Binder & Hibbett 2006, Cortes- Perez et al. 2023) and may have a close affinity with

Phylloboletellus, but we were not able to evaluate their phylogenetic positions in this study. A more thorough investigation of the ecology of early-diverging Boletaceae is needed to test the mycoparasitic hypothesis.

The evolution of gasteroid morphologies has long been thought to be an evolutionary dead-end (Thiers 1984). The transition from pileate-stipitate to gasteroid morphology involves the loss of ballistospory, and regaining this complex mechanism once lost is likely difficult (Hibbett et al. 1997, Hibbett 2004, Sanchez-Garcia et al. 2020). While prior studies have not fully rejected the hypothesis gasteroidization is irreversible (Hibbett 2004; Wilson et al. 2011; Sanchez-Garcia et al. 2020), there is no previous evidence that suggests otherwise. However, our ancestral state reconstructions under two coding schemes strongly supported transition to and from gasteroid forms. This suggests that gain or loss of ballistospory may be a reversible condition. In our analyses, transitions between pileate-stipitate, secotioid, and gasteroid forms indicate that the secotioid condition is intermediate, as suggested by equal rates of transitions to both gasteroid forms and pileate-stipitate forms. Moreover, inferred rates from pileate-stipitate to both secotioid and gasteroid forms was almost zero, suggesting that the secotioid condition is evolutionarily unstable. This result conflicts with previous investigations of secotioid stability, particularly those of *Cortinarius* secotioid taxa, where evidence suggests long term stability (Peinter et al. 2001).

Transitions between hymenophore organization parallels the sporocarp morphologies. The “tubes with crosswalls” condition appears to be an intermediate between lamellate and tubulate forms, with transitions to the latter being 1.3-5.8x greater than in the reverse. However, the greatest transition rate was from lamellate to tubulate forms, indicating that an intermediate morphology may not be necessary or, like the secotioid condition, may be evolutionarily unstable and rarely persists.

Our data provide compelling evidence that lamellate hymenophores, color changes, and gasteromycetization have evolved multiple times in the Boletaceae and are reversible conditions. Based on the phylogenetic distribution of taxa, it is also clear that membranous partial veils have evolved independently many times, although we did not code this explicitly.

CONCLUSION

The Boletaceae underwent a rapid radiation and subsequent long period of phylogenetic instability. These issues had long limited accurate assessment and analysis of trait evolution and conclusive generic-level taxonomic frameworks. Prior molecular phylogenetic studies based on a limited taxon sampling and a few loci generated trees with poor deep-node support and many short branches. This study provides the first statistically well-supported phylogeny for the Boletaceae using 1461 single copy genes from 383 genomes sampled from specimens representing the most taxonomically and geographically comprehensive dataset compiled to date. Our analyses show that the Boletaceae likely arose in Gondwana prior to its breakup and present-day distributions are in part due to vicariance. Episodic long-distance dispersal could not, however, be ruled out for some current distributions. Morphological traits have resulted from of convergent evolution and frequent, reversible recycling of traits that, together with the rapid radiation, has long confounded attempts to achieve a natural classification within the family. This study provides new genomic data and a stable phylogenetic framework that will enable a robust, foundational taxonomy and provide a resource for further analysis of trait evolution.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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LEGENDS

Table 1. Morphological transition rates estimated with Bayestraits.

Figure 1. Selected photographs of Boletaceae collections from two of the most species-rich regions that are 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). Not to scale. All photos used with permission from Bryn Dentinger.

Figure 2: Time-calibrated phylogeny of Boletaceae using 1764 BUSCO genes. Topology is a summary coalescent of individual best ML gene trees using astral-hybrid. Num- bers on branches are quartet probabilities. Branch lengths were converted to time using the top 100 best gene trees estimated using SortaDate in RelTime. 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 pack- age ”ape.” The dashed line represents a constant birth-death rate. The shaded box indicates a period of significant divergent increase from a constant birth-death rate indicative of a rapid radiation.

Figure 3. Biogeographic reconstruction using BioGeoBEARS. Left-hand tree depicts 4-state coding scheme (lig2h2t 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 I and Phase II Gondwanan diversification events, respectively.

Figure 4. Dispersal events and rates inferred from BioGeoBEARS. Top: Map depicting phytogeographic zones that are colored and labeled on the map. Map was ren- dered 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. 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).

Figure 5. Distribution of likelihood frequencies of morphological traits. A-C) Illustrations of coded traits and inferred transition rates. Arrows indicate direction of transition with stroke weights scaled to maximum rate value. D-I) Frequency distributions of state probabilities for six trait codings. Traits and codes are labeled on the x-axis.

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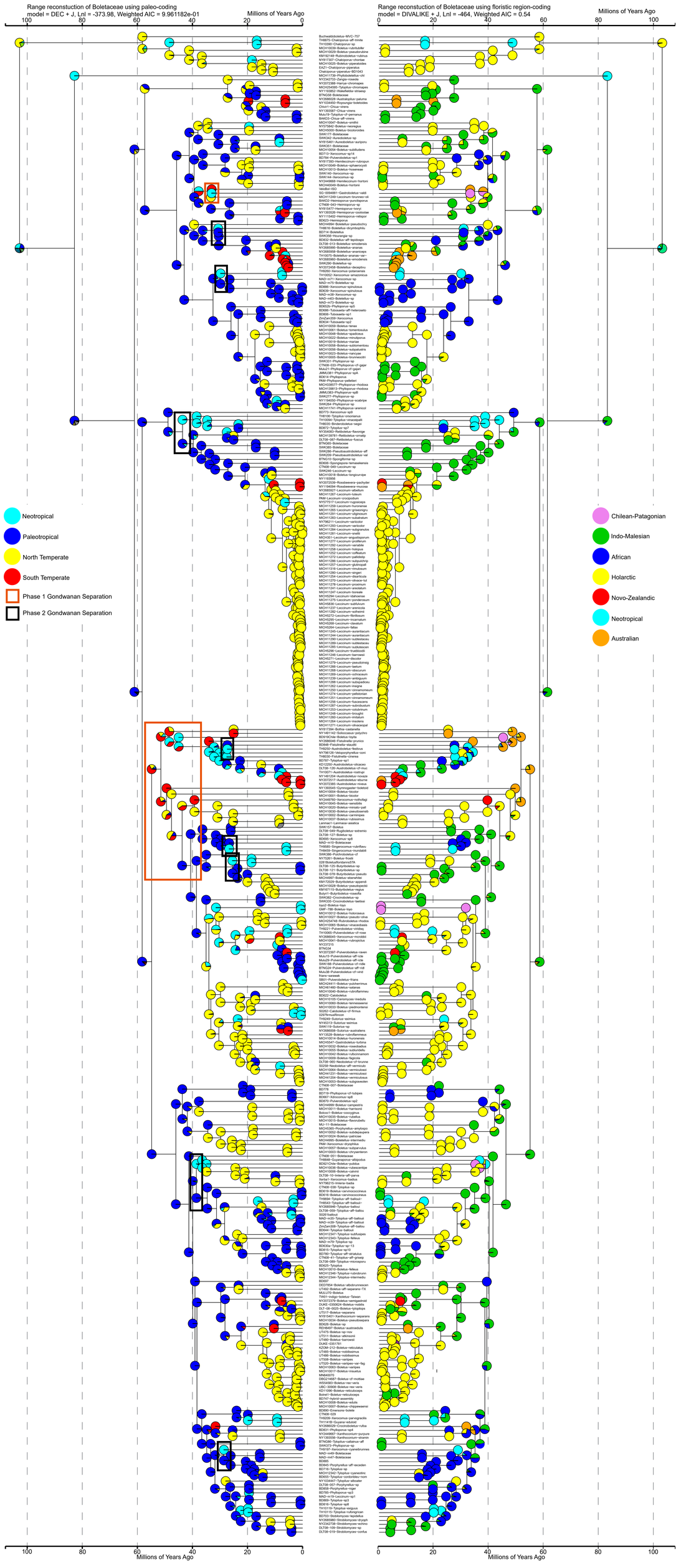
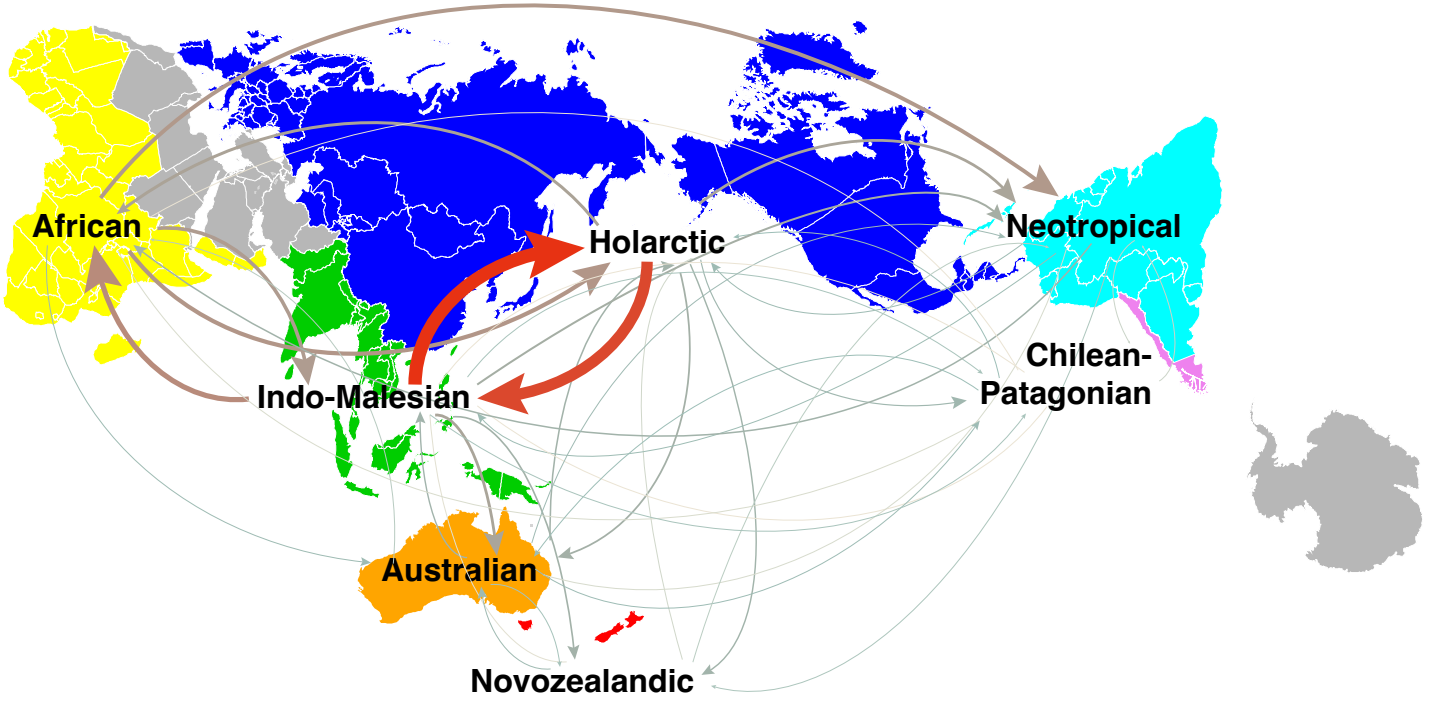
Trait	Transition	Mean rate	Median rate
Ballistospory			
	Yes to No	1.2303	1.0565
	No to Yes	39.8088	35.7478
Spore Ornamentation			
	Smooth to Not Smooth	0.7747	0.7498
	Not Smooth to Smooth	1.741	1.464
Stipe			
	Absent to Present	33.685	27.67
	Present to Absent	0.8415	0.6788
Sporocarp Habit			
	Secoitiod to Pileate-stipitate	51.64	51.47
	Pileate-stipitate to Secoitiod	0.8178	0.7172
	Secoitiod to Gasteroid	53.44	53.90
	Gasteroid to Secoitiod	34.63	31.05
	Pileate-stipitate to Gasteroid	0.8393	0.6749
	Gasteroid to Pileate-stipitate	40.78	36.85
Hymenophore Bruising			
	None to Blue	1.3515	1.2632
	None to Black	0.6921	0.6921
	Blue to None	5.3730	5.3540
	Blue to Black	0.1732	0.1205
	Black to None	3.2329	2.7385
	Black to Blue	1.0294	0.7281
Hymenophore Anatomy			
	Straight to Cross	0.6398	0.6092
	Straight to Lamellete	0.2054	0.1715
	Cross to Straight	3.694	2.960
	Cross to Lamellate	2.4131	1.6755
	Lamellate to Straight	5.483	5.004
	Lamellate to Cross	1.858	1.275

Southeast Asia

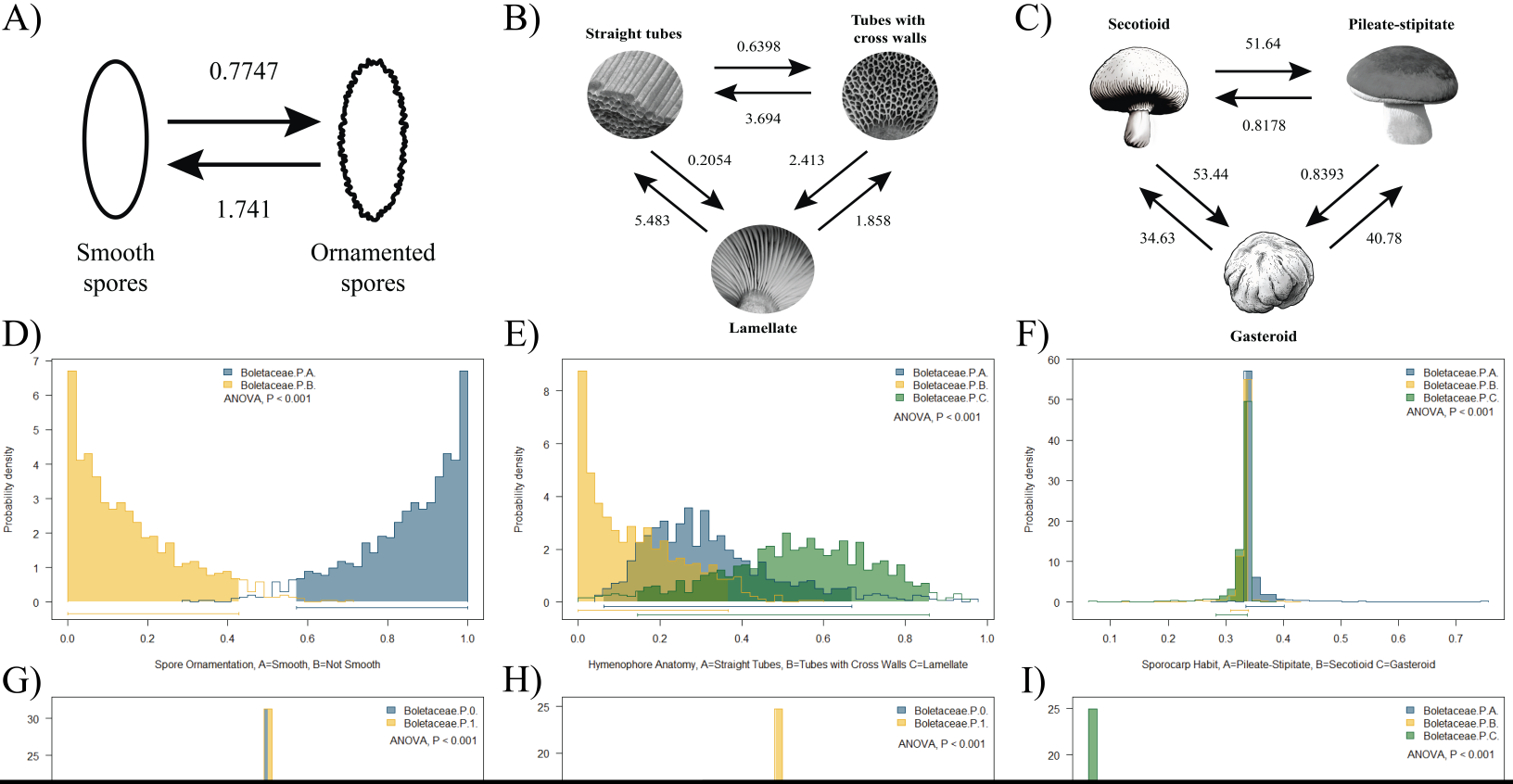
Africa

*1764 BUSCO genes, ≥ 20% complete
*ASTER summary coalescent of best ML gene trees
*branch lengths converted to time in RelTime from 100 top-ranked trees using SortaDate
a
181
11
3
25
50
85
6
22
b
Lineages through time
log(lineages)
rapid diversification
time
BOLETOIDEAE
Boletus s.s.
38 mya (95% CI: 14-90 mya)
30 mya
(95% CI: 11-78 mya)
SUILLELLOIDEAE
55 mya
(95% CI: 26-115 mya)
51 mya
(95% CI: 24-111 mya)
AUSTROBOLETOIDEAE
58 mya
(95% CI: 47-145 mya)
LECCINOIDEAE
61 mya
(95% CI: 31-122 mya)
XEROCOMOIDEAE
83 mya
(95% CI: 47-145 mya)
103 mya
(95% CI: 67-145 mya)
BOLETACEAE
138 mya
(95% CI: 50-150 mya)
57 mya
(95% CI: 28-117 mya)
ZANGIOIDEAE
PHYLLOBOLETELLOIDEAE
CHALCIPOROIDEAE
OUTGROUP
150
100
50
0
Time (millions of years)


Range reconstuction of Boletaceae using paleo-coding
model = DEC + J, Lnl = -373.98, Weighted AIC = 9.961182e-01
Millions of Years Ago
100
80
60
40
20
0
Range reconstuction of Boletaceae using floristic region-coding
model = DIVALIKE + J, Lnl = -464, Weighted AIC = 0.54
Millions of Years Ago
0
20
40
60
80
100
Neotropical
Paleotropical
North Temperate
South Temperate
Phase 1 Gondwanan Separation
Phase 2 Gondwanan Separation
Chilean-Patagonian
Indo-Malesian
African
Holarctic
Novo-Zealandic
Neotropical
Australian



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


A)
0.7747
1.741
Smooth spores
Ornamented spores
B)
Straight tubes
Tubes with cross walls
Lamellate
0.6398
3.694
0.2054
2.413
5.483
1.858
C)
Secotioid
Pileate-stipitate
Gasteroid
51.64
0.8178
53.44
0.8393
34.63
40.78
D)
Boletaceae.P.A.
Boletaceae.P.B.
ANOVA, P < 0.001
Probability density
Spore Ornamentation, A=Smooth, B=Not Smooth
E)
Boletaceae.P.A.
Boletaceae.P.B.
Boletaceae.P.C.
ANOVA, P < 0.001
Probability density
Hymenophore Anatomy, A=Straight Tubes, B=Tubes with Cross Walls C=Lamellate
F)
Boletaceae.P.A.
Boletaceae.P.B.
Boletaceae.P.C.
ANOVA, P < 0.001
Probability density
Sporocarp Habit, A=Pileate-Stipitate, B=Secotioid C=Gasteroid
G)
Boletaceae.P.0.
Boletaceae.P.1.
ANOVA, P < 0.001
H)
Boletaceae.P.0.
Boletaceae.P.1.
ANOVA, P < 0.001
I)
Boletaceae.P.A.
Boletaceae.P.B.
Boletaceae.P.C.
ANOVA, P < 0.001