Historical Biogeography and Diversification of Truffles in the Tuberaceae and Their Newly Identified Southern Hemisphere Sister Lineage

Gregory Bonito14, Matthew E. Smith14, Michael Nowak13, Rosanne A. Healy2, Gonzalo Guevara3, Efren Cázares4, Akihiko Kinoshita11, Eduardo R. Nouhra5, Laura S. Domínguez5, Leho Tedersoo6, Claude Murat7, Yun Wang9, Baldomero Arroyo Moreno8, Donald H. Pfister10, Kazuhide Nara11, Alessandra Zambonelli12, James M. Trappe4, Rytas Vilgalys1

1 Department of Biology, Duke University, Durham, North Carolina, United States of America, 2 University of Minnesota, Department of Plant Biology, St. Paul, Minnesota, United States of America, 3 Instituto Tecnológico de Ciudad Victoria, Tamaulipas, México, 4 Department of Forest Ecosystems and Society, Oregon State University, Corvallis, Oregon, United States of America, 5 Instituto Multidisciplinario de Biología Vegetal, Córdoba, Argentina, 6 Institute of Ecology and Earth Sciences and the Natural History Museum of Tartu University, Tartu, Estonia, 7 Institute National de la Recherche Agronomique et Nancy University, Champenoux, France, 8 New Zealand Institute for Plant & Food Research Ltd, Christchurch, New Zealand, 9 Department of Plant Biology, University of Córdoba, Córdoba, Spain, 10 Farlow Herbarium, Harvard University, Cambridge, Massachusetts, United States of America, 11 Department of Natural Environmental Studies, Graduate School of Frontier Science, The University of Tokyo, Chiba, Japan, 12 Dipartimento di Scienze Agrarie, Università di Bologna, Bologna, Italy, 13 Institute of Systematic Botany, University of Zürich, Zürich, Switzerland, 14 Department of Plant Pathology, University of Florida, Gainesville, Florida, United States of America

Abstract
Truffles have evolved from epigeous (aboveground) ancestors in nearly every major lineage of fleshy fungi. Because accelerated rates of morphological evolution accompany the transition to the truffle form, closely related epigeous ancestors remain unknown for most truffle lineages. This is the case for the quintessential truffle genus Tuber, which includes species with socio-economic importance and esteemed culinary attributes. Ecologically, Tuber spp. form obligate mycorrhizal symbioses with diverse species of plant hosts including pines, oaks, poplars, orchids, and commercially important trees such as hazelnut and pecan. Unfortunately, limited geographic sampling and inconclusive phylogenetic relationships have obscured our understanding of their origin, biogeography, and diversification. To address this problem, we present a global sampling of Tuberaceae based on DNA sequence data from four loci for phylogenetic inference and molecular dating. Our well-resolved Tuberaceae phylogeny shows high levels of regional and continental endemism. We also identify a previously unknown epigeous member of the Tuberaceae – the South American cup-fungus Nothojafnea thaxteri (E.K. Cash) Gamundi. Phylogenetic resolution was further improved through the inclusion of a previously unrecognized Southern hemisphere sister group of the Tuberaceae. This morphologically diverse assemblage of species includes truffle-like species (e.g. Gymnohydnotrya spp.) and non-truffle forms that are endemic to Australia and South America. Southern hemisphere taxa appear to have diverged more recently than the Northern hemisphere lineages. Our analysis of the Tuberaceae suggests that Tuber evolved from an epigeous ancestor. Molecular dating estimates Tuberaceae divergence in the late Jurassic (~156 million years ago), with subsequent radiations in the Cretaceous and Paleogene. Intra-continental diversification, limited long-distance dispersal, and ecological adaptations help to explain patterns of truffle evolution and biodiversity.

Citation: Bonito G, Smith ME, Nowak M, Healy RA, Guevara G, et al. (2013) Historical Biogeography and Diversification of Truffles in the Tuberaceae and Their Newly Identified Southern Hemisphere Sister Lineage. PLoS ONE 8(1): e52765. doi:10.1371/journal.pone.0052765

Editor: Jason E. Stajich, University of California Riverside, United States of America

Received September 8, 2011; Accepted November 22, 2012; Published January 2, 2013

Copyright: © 2013 Bonito et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This research was supported through NSF award #0641297, REVSYS: Phylogenetic and Revisory Systematics of North American Truffles (Tuber). Support was also provided by the Friends of the Farlow, which enabled the senior author to conduct research at the Farlow Herbarium. M.E.S. participated via a postdoctoral fellowship from Harvard University Herbaria. D.H.P. received funding from the David Rockefeller Center for Latin American Studies at Harvard University that supported field work in collaboration with M.E.S. Collections and microscopy were enabled by a grant from the Iowa Science Foundation to R.H. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Dr. Yun Wang works at the New Zealand Institute for Plant & Food Research Ltd. The authors confirm that there are no relevant declarations relating to the employment, consultancy, patents, products in development or marketed products. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials. The remaining authors have declared no competing interests.

* E-mail: gregory.bonito@duke.edu

Introduction

Truffles are fungi that produce fruiting bodies with spores sequestered in a spherical mass, belowground or at the soil surface [1]. Many groups of truffles produce volatile aromatics. Although truffles have evolved in nearly every major group of fleshy fungi and over 100 times independently within the Ascomycota, Basidiomycota, and Mucoromycotina [2], the majority of transitions to a truffle form occur in ectomycorrhizal (EcM) fungal lineages [1]. This pattern suggests that the symbiotic association with plants may be an important driver in the evolution of truffle
The hypogeous fruiting habit of the truffle offers several selective advantages. Truffles are characterized by a low surface area-to-volume ratio, therefore a large number of spores are produced in a small packet of tissue. Furthermore, while epigeous fruiting bodies are directly exposed to weather, truffles are buffered against moisture and temperature fluctuations that might otherwise damage or inhibit development of spores. Truffle-forming fungi have evolved novel mechanisms for spore dispersal via small animals that are correlated with the loss of active spore discharge. Many different animal species are attracted by the odors produced by truffle species [1] and truffle spores have been found in the fecal deposits of rodents, marsupials, reptiles and gastropods, suggesting that these animals are important dispersal agents of truffle spores [12,13]. Indeed, truffle fruitbodies usually have durable, thick-walled spores that can withstand and possibly benefit from the passage through the digestive tract of animals [14]. The convergent evolution of these traits across a diversity of truffle lineages suggests that the transition from epigeous to hypogeous fruiting is driven by strong selection for traits that promote animal dispersal. Spore deposition via animal mycophagy may be a more targeted dispersal mechanism than wind or water dispersal [15], because animals that consume truffles are also likely to deposit their nutrient-rich and spore-laden fecal pellets near the roots of suitable host trees. Similarly, truffle consumption by highly dispersive animals may promote fungal colonization of new or distant habitats [16].

Loss of forcible spore discharge and adaptation to the hypogeous habit is often followed by extreme morphological changes, as seen in many different truffle lineages [1]. These morphological enigmas obscure taxonomic relationships between truffles and their epigeous relatives [1,4]. Because morphological changes found in truffles appear to evolve rapidly, this form is likely due to the loss of function of a single or small set of genes that program the epigeous life history [17,18]. Species in the Tuberaceae have undergone extensive morphological modifications compared to epigeous relatives within the Pezizales. For instance, species in the Tuberaceae typically have spherical or irregularly shaped asci and eight or often fewer ascospores per ascus. In contrast, epigeous species of Pezizales routinely have cylindrical asci with 8 spores per ascus (Fig. 1).

Previous phylogenetic studies of Tuberaceae have resolved two monophyletic Northern hemisphere clades, Tuber and Choanomyces, and a Southern hemisphere clade that includes Dingliya, Rudellomyces, and Labyrinthomyces [4,7]. The sister group of the Tuberaceae remains unresolved [4]. The related Helvellaceae, previously regarded as the sister clade of the Tuberaceae (albeit without statistical support), are comprised of species producing either aboveground “elfin saddle” or sessile cup-shaped fruitbodies (e.g. Helvella – see Fig. 2a), or those with a truffle form (e.g. Balsamia) [4].

Dating the origin and diversification of fungi can be a contentious science, but methods for molecular dating are improving [19]. Because nucleotide substitution rates often differ between fungal lineages, a penalized likelihood method and fossil calibrations were used by Padovan et al. to estimate divergence dates within the Ascomycota based on a Bayesian phylogeny of 18S SSU rDNA [5]. They estimated the split of Tuber from other Pezizales occurred around 529 million years ago (Mya). In a more recent study focusing on the historical biogeography of Tuber, a molecular clock approach (with secondary calibration) was used to estimate the divergence times of major Tuber clades based on phylogenies inferred from multiple loci (18S rRNA, 5.8S-ITS2 rRNA, and β-tubulin) [20]. Their results indicate that Tuber began to diverge during the Triassic or Jurassic between 271-140 Mya. However, these studies were limited by regional sampling and phylogenetic uncertainty, which may confound divergence time estimations.

Here, we estimate the phylogeny of a global sample of Tuberaceae employing both Maximum Likelihood (ML) and Bayesian inference methods based on DNA sequences of four genetic loci: ITS rRNA (ITS), 28S large subunit rRNA (LSU), elongation factor 1-α (EF1α), and RNA polymerase subunit II (RPB2). The main aims of this study were to: 1) estimate the phylogeny and divergence times for major clades of the Tuberaceae; 2) examine their major biogeographic patterns; 3) map characters to the phylogeny and reconstruct important ancestral morphological and ecological character states; and 4) determine their relationships to Southern hemisphere taxa of hitherto unknown phylogenetic affinities. We also used the expanded data set to test monophyly of the genus Tuber.

Because long-distance dispersal is often limited in fungi with hypogeous fruitbodies [21] we predicted that the biogeographic patterns of the Tuberaceae would fit a vicariance mode of distribution. Specifically, we hypothesized that 1) Tuber and Tuberaceae are monophyletic lineages composed strictly of truffle taxa; 2) most species and some lineages have restricted distributions at the continental scale, with major disjuncts between Northern and Southern hemisphere Tuberaceae; 3) spore ornamentation is a variable/plastic character that may vary between and (to a lesser extent) within Tuber clades; 4) divergence times of major clades within the Tuberaceae would track angiosperm radiations; and 5) inclusion of Southern hemisphere taxa would improve understanding of biogeographic patterns in the Tuberaceae.

Materials and Methods

Taxon Sampling

This global sampling of Tuberaceae integrated data from research programs in Europe [20], Asia [22], North America [6,23] Central America, and South America, as well as extensive sampling of both public and private herbaria (Table S1). As outgroups, we used taxa belonging to the hypogeous genus Balsamia and epigeous...
genus *Helvella* [4] belonging to the *Helvellaceae* because these have been presumed to be the closest living relatives of the *Tuberaceae*. Our sampling includes representatives of *Tuber* previously analyzed in single locus (ITS) analyses [6] that comprised of 123 ITS phylotypes and represented approximately 70% of the accepted species, as well as 37 undescribed *Tuberaceae* species. We also included representatives from all the major *Tuberaceae* clades known from Japan and presented by Kinoshita et al. (2011).

*Paradoxa* is a rare genus comprised of two species and is known only from the Northern Hemisphere (China and Italy). Although specimens of *Paradoxa* could not be obtained during the course of this study, specimens morphologically resembling *Paradoxa* were included [22]. We exclude taxa represented by only a single locus, with the exceptions of *Underwoodia columnaris* (which we included only to test the monophyly with Southern hemisphere taxa originally described as *Underwoodia*) and *Tuber sinuosatum* and *Tuber cf. excavatum* (which are the only known representatives of the *aestivum* and *excavatum* clades in Asia). Throughout this paper we adopt the use of rankless clade names following that of Moncalvo et al. [24], where the clade name is written in lowercase non-italicized letters and preceded with the symbol “/”.

**Molecular Data**

Standard and touchdown polymerase chain reaction (PCR) protocols and fungal-specific primer sets (Table S2) were used to amplify and sequence four gene regions: the internal transcribed spacer ribosomal RNA gene (ITS), the 28S large subunit ribosomal RNA gene (LSU), elongation factor ($EF_1\alpha$), and the second largest subunit of RNA polymerase II (RPB2). $EF_1\alpha$ and RPB2 could not be amplified for many *Tuber* species. To address this problem we designed a new set of primers with enhanced specificity for the *Tuberaceae*: $EF_1\alpha$ *Tuber*_f ($5'\text{ AGC GTG AGC GTG GTA TCA C} 3'$ – forward), $EF_1\alpha$ *Tuber*_r ($5'\text{ GAG ACG TTC TTG ACG TTG AAG} 3'$ – reverse), and RPB2 *Tuber*_f ($5'\text{ Y AAY CTG ACY TTR GCY GTY AA} 3'$) paired with the reverse primer RPB2 _Tuber*_r ($5'\text{ CR GTT TCC TGY TCA ATC TGA} 3'$). Sequences produced for this study have been deposited in GenBank under the accession numbers JQ925626-

---

Figure 1. Hypothesized evolution of a truffle lineage. In this scenario the habitat of an epigeous species with 8-spored, uniseriate asci becomes more arid (A). Selection for reduced water loss results in an enclosed truffle form that has hymenium-lined chambers and asci that are shorter and more clavate in form (B). The ability to forcibly discharge spores is lost and selection for other means of spore dispersal intensifies, leading to spore dispersal through animal mycophagy. Continued selection results in a truffle species that fruits belowground and has a solid gleba stuffed with spherical asci packed with irregular numbers of spores (C). doi:10.1371/journal.pone.0052765.g001
Figure 2. Morphological diversity and characters of truffles and their relatives. A. An “Elfin-saddle” cup-fungus Helvella lacunosa Fr. Asci line the outside of the fertile cap, which is borne upon a stipe composed of vegetative tissue; B. the “earth-tooth” fungus Underwoodia singer Gamundi & E. Horak. A layer of fertile tissue lines the outside of the tooth-shaped cap. C. Gymnohydnotrya sp. collected under Nothofagus pumilio (Poepp & Endl.) Krasser in Argentina and similar to sequences from Nothofagus mycorrhizas. Fertile asci line both the inside and the outside the Multigene Tuberaceae Phylogeny.
fruiting body. D. *Choriomyces alveolatus* (Harkn.) Trappe, a *Pinaceae* associate from western North America. E. A knobby-shaped representative of the/*puberulum* lineage, a clade of small, whitish truffles. F. *Tuber canaliculatum* Gilkey has a peridium covered in minute warts and its ascii contain one or two reticulate spores. G. Flask-shaped ascus of the spiny-spored *Tuber lyonii* Butters with a stem at the point of attachment. H. Representative of the/*japonicum* lineage. I. Swollen beaded hyphae from the outer peridium of species belonging to the/gibbousum lineage. J. Large pyramidal warts cover the outer surface of *Tuber aestivum*. K. The spores of *Choriomyces meandriformis* Vittad. are ornamented with unusual pitted tubules. L. Species in the/*excavatum* lineage have a thick outer peridium and a partially enclosed internal cavity. M. Species in the/*maculatum* lineage have ellipsoid, alveolate-reticulate ascospores. N. The spores of *Tuber sp.*13 of the/melanosphorium clade are particularly spiny. O. The spores of *Tuber spinoreticulatum* Uecker & Burds have spines that are irregularly connected by ridges that form a partial reticulation. Scale bars: A, B, C, D, E, F, H, J, L = 1 cm; G, I, K, M, N, O = 10 μm. doi:10.1371/journal.pone.0052765.g002

Phylogenetic Reconstruction

Sequence alignments were initially performed in MUSCLE [25] individually for each locus. Alignments were visually inspected and ambiguous regions were excluded in Mesquite 2.5 [26]. Best-fit nucleotide substitution models were chosen through the Akaike information criterion, penalizing more complex models by one likelihood unit per additional free parameter, and ML phylogenetic trees for individual loci were estimated under these models in PAUP* [27]. Conflict among the four loci was assessed through strong incongruence of nodes based on 1000 ML bootstrap replicates (>70%) and posterior probabilities (≥99%) of credible Bayesian trees. Because no strongly supported nodes were in conflict, the data sets were combined into a single matrix with four partitions. We conducted maximum likelihood (ML) and Bayesian inference (BI) analyses on individual and combined data sets. The ITS, LSU, EF1α, and RPB2 partitions included 274, 746, 813, and 733 characters, respectively, for a combined data matrix of 2568 characters. The number of included taxa were 99 (ITS), 96 (LSU), 80 (EF1α), and 67 (RPB2). Maximum likelihood analyses on the concatenated data were conducted with RAxML applying a GTRGAMMAI substitution model with parameters unlinked. ML bootstrap replicates (1000) were computed in RAxML under a GTRMIXI model, which infers an initial tree using the GTRCAT model, and then optimizes the tree topology using a GTRGAMMAI model. For Bayesian phylogenetic estimations, independent analyses were conducted with MrBayes [28]. Partitions were unlinked under either HKY+G+I (ITS and RPB2) or GTR+G+I (EF1α and LSU) nucleotide substitution models. Parallel runs with four chains were allowed to run 50,000,000 generations, sampling every 500 generations. Trees were sampled after the same likelihood plateau was reached between runs. MrBayes and RAxML analyses were computed through the CIPRES web portal (www.phylo.org).

In an attempt to better resolve the *Tuberaceae* a second round of analysis was performed with a more conservative alignment having fewer taxa and with/gymnohydnottya as an outgroup. In these analyses introns within EF1α were excluded and amino acid positions were coded to compare alternative partition assignments. This more conserved alignment was also used in divergence time estimation analyses (below). Best-fit nucleotide substitution models were determined with PartitionFinder [29] under the Bayesian information criterion, which favors simpler models compared to the Akaike information criterion. The partitions included 40, 154, 77, 728, 635, and 733 characters for ITS1, 5.8 S, ITS2, LSU, EF1α and RPB2, respectively, for a combined data matrix of 2374 characters. Phylogenetic inferences were also conducted on a matrix consisting of 8 unlinked partitions: 1) SYM+I+G for 3.8S and RPB2 position 1; 2) JC for RPB position 2; 3) K80+I+G for RPB position 3; 4) F81+I+G for EF1α position 1; 5) JC1+G for EF1α position 2; 6) GTR+G for EF1α position 3; 7) SYM+G for ITS1 and ITS2; 8) K80+I+G for LSU. Parallel runs with four chains were allowed to run 20,000,000 generations, sampling every 1000 generations.

Divergence Time Estimation

Molecular divergence time analyses were performed with the BEAST v1.7.2 software package [30] based on an alignment containing the four gene regions (ITS, LSU, EF1α, and RPB2) for a subset of the samples (one unique specimen per species – see above). Temporal calibration of divergence time analyses was achieved by fixing the absolute rate of molecular evolution for LSU locus (6.5×10^{-10} substitutions per site per million years) [31]. The evolutionary rates of the ITS, EF1α and RPB2 regions were estimated relative to the fixed LSU rate using a relaxed clock model with an uncorrelated exponential prior distribution with a mean of 1.0×10^{-3} substitutions per site per million years assigned to the mean rate of each region. It is important to emphasize that this prior is on the mean of the rate of each locus, and rate heterogeneity is modeled at each of these loci by an exponential distribution to avoid over constraining the rate and rate variation at these loci. Because all known *Tubaria* species are presumed to be ectomycorrhizal, we assume that the most recent common ancestor (MRCA) of these species was also ectomycorrhizal. Conservatively, we applied a maximum age constraint to the age to the MRCA of the *Tubaria* based on recent age estimates of the *Pinaceae* [32] (i.e. <250 million years ago), the oldest known lineage of obligate EcM hosts. A standard uniform prior, meaning an equal probability (i.e. flat distribution) between 0 and 250 Ma was applied to this node (node 2 in Fig. 5).

The sequence data were partitioned by gene region, with the exception of the ITS region, which was divided into three unique partitions: ITS1, 5.8S, and ITS2. The clock models and substitution models of the resulting six partitions were unlinked in BEAST analyses. The substitution models for the partitions were either HKY (RPB2) or GTR+G+I (LSU, ITS1, 5.8S, ITS2, EF1α) substitution models. We used gene partitions rather than codon positions because when data partitions become small the ability to estimate parameters for the substitution model or clock models suffers. The birth/death speciation model was employed, and a fully resolved starting tree was provided for each analysis. Three independent and identical BEAST analyses were each run for 30 million generations, sampling parameters and trees every 1000 generations. Parameters from the resulting 30 thousand generations for each of the three runs were examined for convergence, stationarity, and suitable effective sample sizes in the program Tracer v1.5 [33]. Based on this, a burn-in of 3000 trees was removed from each run, leaving 27,000 trees from each run, which were combined (81,000 trees) and used to generate a maximum clade credibility tree annotated with various parameter summary statistics using the program TreeAnnotator v1.7.2.
Ancestral Character State Reconstructions

We reconstructed ancestral character states for ECM host plants of Tuber by phylogeny using the maximum likelihood model Markov k-state 1 parameter model in Mesquite [26]. Hosts were coded either as gymnosperms, angiosperms, or both (Table S1), considering a global database of Tuber ITS sequences that included host information were considered [6]. Ancestral state reconstructions were also carried out on fruitbody type (epigeous vs. hypogeous) in the Tuberinae and gymnolohydrorya using an asymmetrical 2-parameter Markov-K model. In this model, parameter values for the transition from an epigeous to hypogeous fruiting body were relatively high (≥10^-1) compared to the transition from hypogeous to epigeous fruiting habit, reflecting the reality that in nature forcible spore discharge is more easily lost than acquirad [21]. The program RASP [34] was used to statistically assess patterns of vicariance and dispersal across the genus using a distribution of equally probable Bayes trees and coding species by their geographical origins. Although Tuber aestivum, T. excavatum, T. puberulum, T. oligoperum and T. rufum have been reported from Northern Africa [20], collections from Africa were not available for study, and consequently, we did not include this biogeographic region in our analyses.

Results

Phylogenetic Analyses

Individual loci (Fig. 3) and combined molecular data (Fig. 4) confirm that the Tuberinae is monophyletic as are both of the Northern hemisphere genera Tuber and Choiromyces. In contrast, genera of Southern hemisphere Tuberaceae were not resolved as monophyletic and are in need of taxonomic revision. Phylogenies of Tuber based on individual loci reconstructed the same major clades (Fig. 3). However, the LSU phylogeny does not resolve the/ puberulum lineage or place T. magnatum within the/aestivum lineage. Because there was no strongly supported conflict between single gene phylogenies, we combined the data sets to improve phylogenetic resolution. Eleven major clades can be recognized within Tuber based on the concatenated dataset (Figs. 4 & 5). The/ rufum,/melanosporum,/puberulum,/maculatum, and/macro- sporum clades are distributed across the entire Northern hemisphere (Europe, Asia, North America, Central America and Northern Africa), yet are characterized by a high degree of species- level endemism. On the other hand, several Tuber clades are endemic to particular continents: gennadii and/multimaculatum to Europe,/japonicum to Asia, and/gibbosum to North America. The/aestivum and/excavatum groups are distributed across Europe and Asia. Economically important Tuber species are interspersed within six of the eleven major clades in Europe, Asia, and North America (Fig. 4, Table S3).

A number of noteworthy discoveries came from our inclusion of Southern hemisphere taxa from South America (7) and Australia (21). First, we sequenced two novel Tuber species from multiple root samples collected in Argentina, indicating that Tuber is not strictly a Northern hemisphere genus. These sequences were placed in the/puberulum lineage and were derived from ectomycorrhizas sampled in natural stands of Nothojafnea spp. and Salix humboltiana Willd., both native to South America [35]. These findings support the anomalous report of a native Argentinean Tuber species, T. australis Spec. [36]. Second, we demonstrate that the epigeous South American cup-fungus, Nothojafnea thaxteri (Cash) Gamundi, represents an early diverging lineage within the Tuberinae and is closely related to the Australian truffle genera Reddellomyces, Labyrinthomyces, and Dingleya (Fig. 4). Our findings show that N. thaxteri is the closest known extant epigeous relative of the genus Tuber. This is the first report of an epigeous (non-truffle) species in the Tuberinae sensu stricto. Third, we identified a previously unrecognized Southern hemisphere clade, which is supported as the sister group to the Tuberinae. This clade (/ gymnolohydrorya) is known from South America and Australia and contains taxa that form either epigeous (Underwoodia pro parte) or hypogeous (Gymnohydnotrya) fruitbodies. Our phylogenetic treatment of Nothojafnea and gymnolohydrorya constitutes the first evidence that these taxa are related to Tuberinae.

Estimated Divergence Times

Median date estimates for the origin of the Tuberinae, based on a maximum age constraint of <250 Mya, are in the late Jurassic (Fig. 5 - node 2) at 156 million years ago (Mya). We estimated that Tuber diverged from other genera in the early Cretaceous (156 Mya) (Fig. 5 - node 4), and by the end of the Cretaceous (65 Mya) most of its extant subgeneric lineages were present. However, major radiations within these lineages occurred during the Paleogene (Fig. 5). The divergence time estimates and confidence intervals are summarized for Tuber clades in Table 1. Estimates for the mean ages of the MRCA of Tuber clades (Fig. 5) are:/multimaculatum (121 Mya)/aestivum (101 Mya)/rufum (86 Mya)/melanos- porum (79 Mya)/puberulum (65 Mya)/japonicum (46 Mya)/ excavatum (43 Mya)/maculatum (67 Mya)/macrosorum (43 Mya)/gennadii (48 Mya)/and gibbosum (27 Mya). Our age estimates for MRCA of Tuberinae and its newly recognized sister lineage (/gymnohydnotrya – Fig. 5 - node 1) was 160 Mya, which corresponds to the late Jurassic. The estimated divergence of the/ labyrinthomyces lineage (Fig. 5 - node C) at 43 Mya is relatively recent compared to Tuber.

Evolutionary rates of the ITS1, 5.8S, ITS2, EF1a and RP2B regions were calculated relative to fixed LSU rates using a relaxed clock model with uncorrelated exponential prior distributions (see methods). Our mean posterior rate estimates in substitutions per site per million years for the specific partitions are as follows: ITS1 = 1.72E-3; 5.8S = 3.02E-4; ITS2 = 2.07E-3; EF1a = 4.01E-4; RP2B = 3.78E-4.

Ancestral Character State Reconstructions

Extant species in a number of Tuber clades can associate with angiosperms, Pinaceae, and parasitic orchid monocots (e.g. T. aestivum Vitad.) [6]. Character state reconstructions indicate the most recent common ancestor to the Tuberinae was likely an ectomycorrhizal symbiont of angiosperms. There appear to be multiple independent shifts to Pinaceae hosts, particularly at the nodes of the/gibbosum,/melanosporum, and/puberulum clades. Divergence date estimations (reported above) place the MRCA of both the Tuberinae and/gymnohydnotrya lineage in the early age of angiosperms, yet it is possible that these ancestral species were mycorrhizal with Pinaceae, or lived as endophytes, pathogens or saprotrophs.

Spore ornamentation is one of the most important characters for truffle taxonomy. Consequently, we were interested in reconstructing the evolution of spore characters in the genus Tuber. Spores of Tuber species are either ornamented with an alveolate-reticulate pattern (e.g. honeycomb design – Fig. 2 M), spines (Fig. 2 N), or spino-reticulation (e.g. spines connected by ridges Fig. 2 O). Based on our analyses, alveolate-reticulate ornamentation is the plesiomorphic (ancestral) condition for Tuber. At least two independent transitions from alveolate-reticulate to spiny ornamentation have occurred: one in the ancestor of the/ melanosporum/–/rufum lineage, and another in the ancestor of T. panniferum Tul. These are depicted by an asterisk (*) above the nodes of these clades in figure 4. Several apparent reversals from
Figure 3. Phylogenetic reconstructions of *Tuber* based on maximum likelihood analysis of four individual loci: internal transcribed spacer region (ITS), 28 s large subunit rDNA (LSU), elongation factor 1-alpha (EF1a), and RNA polymerase II (RPB2). Models and
likelihood scores for each locus are: ITS = Sym +G+I (-3960.627); LSU = GTR +G+I (-8732.114); EF1a = GTR +G+I (7374.012); RPB2 = K80 (5880.021). Clade names and node labels are consistent with each other and with figures 4 and 5. Taxa in the Helvellaceae were excluded from the ITS analysis because of the alignment challenges imposed by sequence divergence.

doi:10.1371/journal.pone.0052765.g003

spiny to alveolate-reticulated spores have also occurred (e.g. T. laotongense, T. pseudexcavatum).

Fungal fruiting bodies can have diverse forms. The epigean Nothojafnea cf. thaxteri specimen occurs within the Tuberaceae, a family historically considered to contain strictly hypogeous taxa. Moreover, the/gymnomyctera and/labyrinthomyces lineages include both epigean and hypogeous species. Accordingly, ancestral state reconstructions of fruitbody habit in the Tuberaceae were conducted to determine how many transitions to a belowground fruiting habit occurred in this lineage. Unweighted parsimony and single parameter likelihood models indicated that the ancestor to Tuberaceae was hypogeous and that a single transition to an epigeous fruiting form occurred in the ancestor of Nothojafnea. However, as mentioned previously, the ability to regain forcible spore discharge is considered highly unlikely in fungi and we know of no unequivocal cases where this has been previously shown. Using a 2-parameter model we found that models with forward to reverse (epigean → hypogeous) transition ratios of 10:1 or greater reconstruct the ancestor of Tuber as most likely epigeous. Similarly, the ancestor of the/labyrinthomyces lineage and/ labyrinthomyces-choiromyces lineages are reconstructed as epigeous.

Inter-continental dispersal is evident in most major Tuber clades. Our results indicate that vicariance alone cannot explain the modern distribution of extant Tuberaceae (Fig. 6). Although there is still uncertainty concerning the origin of Tuber, putative geographical origins of the most common ancestors for most clades can be resolved. North America appears to be the ancestral area of the/ gibbosum./maculatum./rufum, and/melanosporum clades, whereas Europe is likely the ancestral area of the/aestivum./ excavatum, and/gennadii clades. Asia is ancestral area for the/japonicum lineage.

Discussion

Multiple Independent Evolutionary Transitions to the Truffle form in the Tuberaceae

Truffles are derived from aboveground fruiting ancestors, however, historically the family Tuberaceae has been regarded to be composed of strictly hypogeous species [4,7]. We show here for the first time that the Argentinean cup-fungus Nothojafnea thaxteri (Cash) Gamundi is the earliest diverging member in the Southern hemisphere/labyrinthomyces lineage, and the only known epigean species that can be placed within the Tuberaceae. We infer from asymmetric 2-parameter ancestral state reconstructions that at least three transitions to belowground fruiting have occurred within the Tuberaceae: one leading to the rest of the/labyrinthomyces lineage, a second transition leading to the genus Choiromyces, and a third transition leading to Tuber. Multiple independent transitions to a truffle fruiting habit are also evident in/ gymnomyctera, the sister lineage of the Tuberaceae. These truffles appear to be derived from an epigeous “earth-tooth” fungus, with affinities to Underwoodia sinesis Gamundi & E. Horak (Fig. 2B).

Historical Biogeography of Tuber

Our data provide high statistical support for the monophyly of Tuber and Tuberaceae, in agreement with our initial hypothesis and previous studies [6,20,22,23]. We estimate that Tuber began diverging in the early Cretaceous, around 142 Mya (Fig. 5 - node 4), which would coincide with the emergence of Eudicots and near complete tectonic breakup of Pangea [37]. This date is also within the range estimates of 140–271 Mya calculated by Jeandroz et al. [20] using molecular clock approaches of ribosomal and beta-tubulin genes. Overall, the divergence date estimates for commonly recognized Tuber clades by Jeandroz et al. [20] were younger for the shallower nodes and older for deeper nodes compared with our divergence date estimates. These discrepancies are likely due to differences in taxon sampling, phylogenetic resolution, and methods for dating divergence times. In particular, Jeandroz et al. [20] assumed a linearized tree approach and a single fixed calibration point meaning that topological and branch length uncertainty are not accounted for in their divergence time estimates. In contrast, Bayesian methods developed and used here are better able to deal with this uncertainty [30]. Further, we have included a more thorough phylogenetic sampling within the family Tuberaceae, the genus Tuber, and within each of the major Tuber clades leading to a more complete and resolved model for the phylogenetic structure of this family.

Our synthesis confirmed relationships among major Tuber clades that were detected in previous studies [6,20,22,23] but we also uncovered new biogeographical patterns such as the occurrence of Tuber in South America. Global diversity of Tuber species is high, and this may be partly due to a high level of regional endemism [6,22,38]. For instance, Kinoshita et al. [22] recently reported more than 20 undescribed Tuber species in Japan, including members of the/japonicum lineage. The addition of phylogenetically dispersed representatives of these species provided insights on the historical biogeography of Tuber, but the relationship between the/japonicum and/gennadii lineages to the rest of Tuber was still not fully resolved with this dataset.

While we must reject a strict vicariance model for explaining the biogeography of Tuber, particularly for the/puberulum group that is the most widely distributed clade, most species and many Tuber clades appear to have restricted distributions at the continental scale in accordance with our hypothesis. For instance, the/ gibbosum./japonicum./gennadii, and/multimaculatum clades appear to be restricted to single continents. Consistent with a vicariance model of diversification, Europe and Asia (which have had greater geographic connectivity) share more Tuber lineages (but not species) with each other (e.g./excavatum and/aestivum) than they do with North America. However, some lineages (i.e./ rufum./melanosporum, and/macrosporum) are distributed across Europe, Asia, and North America, indicating past dispersal (or migration) between the continents, putatively via the Beringia Land Bridge (between North America and Asia) and the Thulean North Atlantic Land Bridge (between Europe and North America). Major disjuncts were observed between the (almost entirely) Northern hemisphere Tuber and Southern hemisphere/ labyrinthomyces and/gymnomyctera lineages. Our date estimates of the divergence of these lineages (156–160 Mya) correspond well with the early splitting of Gondwana and Laurasia, except in the case of/choiromyces. Our data indicates that/choiromyces diverged from/labyrinthomyces 94 Mya, well after the split of Gondwana and Laurasia, thus dispersal must be invoked to explain this biogeographic distribution.

We estimate that the most recent common ancestor of the/japonicum clade radiated around 46 Mya (Fig. 5 - node D) in Asia. Species in the/japonicum group are light in color and have
Figure 4. Maximum likelihood (ML) phylogenetic reconstruction of the Tuberaceae phylogeny based on ITS, 28S rDNA, EF1α, and RPB2 gene regions. Thickened branches represent ML bootstrap support >70 and posterior probabilities of 100. ML bootstrap values above nodes.
are based on 1000 replicates. Posterior probabilities are presented below nodes. Thickened branches without numbers received maximum ML and Bayesian support values. Reconstructed ancestral host plant associations (based on maximum likelihood) are represented at internal nodes by circles; black for ancestors in symbiotic association with angiosperms, white for ancestors in symbiotic association with Pinaceae, and gray for ancestors in symbiotic association with angiosperms and Pinaceae. Nodes supported by transitions in spore ornamentation from alveolate-reticulate to spiny are shown with an asterisk *. Economically important species are denoted by the symbol $ after their name and geographic origin. The phylogeny is rooted with taxa from the Helvellaceae including species of epigeous Helvella and hypogeous Balsamia. Major lineages of Tuber and Tuberaceae are indicated to the right of the tree. The Tuberaceae form a monophyletic group, which is resolved as a sister group to a previously unrecognized Southern hemisphere lineage (gymnohydnotrya). Type specimens are denoted by the superscripts: $ holotype, $ isotype, $ paratype. doi:10.1371/journal.pone.0052765.g004

Figure 5. Bayesian Divergence Time Estimates for Truffles. The maximum clade credibility chronogram estimated in BEAST is shown with nodes placed at the median age. Node bars (grey) represent the node age 95% highest posterior density (HPD) for nodes receiving at least 50% Bayesian posterior probability. The median age is provided for labeled nodes (A–P) that are discussed in the text and node age parameters are presented in Table 1. doi:10.1371/journal.pone.0052765.g005
irregularly alveolate-reticulated spores that are pale yellow at maturity [22]. Also, species in this clade tend to have only one or two spores per ascus, fewer than most other *Tuber* species.

The *gennadii* lineage is another early diverging clade within *Tuber*. This group appears restricted to Europe. There has been much confusion regarding the taxonomy of the species within this clade. Originally described as *Terfezia gennadii* by Chatin in 1896, Patouillard transferred this species to *Tuber* in 1903. More recently Alvarez et al. (1992) placed this species into the monotypic genus *Loculotuber* because of its distinct morphology of chambers (locules) lined with fertile asci. However, our data place it as a distinct clade within the genus *Tuber*. Alvarado et al. [39] have identified two species in this clade (*T. gennadii* and *T. lacunosum*) and we estimate that their most recent common ancestor radiated in Europe around 48 Mya (Fig. 5 - node E) in association with angiosperm hosts.

*Tuber multimaculatum* Parlade, Trappe & I.F. Alvarez is the sole representative in the *multimaculatum* lineage (Fig. 5 - node L), and is only known from a few collections [11]. Possibly due to its long branch on the phylogeny, its exact placement within the genus *Tuber* differs depending on which gene is used to reconstruct the phylogeny. *Tuber multimaculatum* was estimated to have shared a common ancestor with other *Tuber* species 121 Mya (Fig. 5 - node L). Distinctive features of *T. multimaculatum* include 1-spored or 2-spored asci that have notable apical thickenings in the ascus walls, as well as large ellipsoid ascospores that have finely meshed alveolate reticulations.

The *macrosporum* lineage is characterized by the presence of small warts on the outside surface of the peridium and typically 2- or 3-spored asci containing relatively large alveolate-reticulate spores. We show for the first time that this group occurs in Asia, Europe, and North America. Some species in this group are associated with angiosperm hosts, but others are associated with species of *Pinaceae*. We estimate that the most recent common ancestor of this clade radiated in Europe around 43 Mya (Fig. 5 - node F) but the geographical origin and ancestral host group were poorly resolved.

The *gibbosum* lineage is composed of four species of light-colored truffles that are characterized by beaded hyphae (Fig. 2I)
that emerge from their peridia [23]. The/gibbosum lineage is unique in that species in this clade appear to associate exclusively with Pinaceae hosts, particularly with Pseudotsuga but also with Pinus [23,40]. This lineage is restricted to western North America and our molecular dating results indicate that the most recent common ancestor of this clade radiated in the Western North America around 27 Mya (Fig. 5 - node G) in association with Pinaceae. Estimated dates for the radiation of species with the/gibbosum lineage correspond closely with the estimated age of the Pseudotsuga radiation in western North America (~22 Mya) [41]. We hypothesize that the transition to a conifer host may have facilitated species diversification within this Tuber lineage.

The/maculatum lineage is composed of light-colored truffle species that have a smooth to cracked outer peridium and elliptical alveolate-reticulate ascospores [42]. The majority of species in this lineage are undescribed, but they appear to be associated with angiosperm hosts and are mainly distributed in North America and Europe [6]. We estimate that the most recent common ancestor of this clade radiated in North America around 67 Mya (Fig. 5 - node H) in association with angiosperm hosts. Jeandroz et al. [20] calculated a similar divergence date (65 Mya) at this node based on molecular clock analysis of 5.8S and ITS2 rDNA.

The/puberulum lineage is one of the most diverse in Tuber. Species in this clade produce light-colored truffles that have a smooth to cracked peridium and globose to subglobose ascospores with alveolate-reticulation. The multigene phylogeny (Fig. 4), phylogenetic trees based on individual loci (Fig. 5), and previous published studies [6,20] recover the/puberulum clade, but bootstrap support values are low. Species in this clade are distributed across Europe, Asia, North America, South America, and northern Africa and they are found in association with Pinaceae, angiosperms, or both. The two South American species included in this study were recovered from ectomycorrhizas (Nouhra et al., unpublished) and were placed on a long branch in the phylogram (we were only able to amplify ITS and LSU from these root tips, despite multiple attempts to amplify other loci).

Although ectomycorrhizae of the European species T. melanosporum have been formed on Nothofagus in a greenhouse [43], this is the first evidence of a Tuber species from natural stands of Nothofagus. Many Tuber species in the/puberulum clade are known to associate with Salix spp. [6], but we are not able to determine at this time whether these Tuber species tracked the migration of Salix to South America, or whether these Tuber species were present in South America prior to the immigration of Salix (e.g. associated with Nothofagus). We estimate that the most recent common ancestor of the/puberulum clade diverged 65 Mya (Fig. 5 - node I) but their geographical origin and ancestral host group were poorly resolved. Our results indicate that species in the/puberulum lineage are well adapted for long-distance dispersal compared to other Tuber species. For instance, they are the only group of Tuber naturally represented in the Southern hemisphere (e.g. Argentina), but dispersal by ship on roots of seedlings of European and North American mycorrhizal host trees is likely for the species reported for New Zealand [44]. Jeandroz et al. [20] calculated the divergence date of this node (Fig. 4 - node I) at 33 Mya based on molecular clock analysis of 5.8S and ITS2 rDNA.

Species in the/excavatum lineage are characterized by a basal cavity, a thick and hard peridium, and coarsely reticulated ascospores. They are symbionts of angiosperms and are distributed in both Europe and Asia. We estimate that the most recent common ancestor of this clade radiated in Europe or Asia around 43 Mya (Fig. 5 - node J) in association with angiosperms. This clade also appears to contain many cryptic species sharing similar morphology [6].

The/aestivum lineage is also distributed across Europe and Asia. Species in the group occur mostly in association with angiosperms, although T. aestivum may also associate with some Pinaceae hosts. This clade is characterized by the highest level of morphological diversity of the genus and appears to have been among the first Tuber clades to diversify. We estimate its most recent common ancestor radiated in Europe around 101 Mya (Fig. 5 - node K) in association with angiosperms. Jeandroz et al. [20] calculated the divergence date of this node similarly at 70 Mya based on molecular clock analysis of 5.8S and ITS2 rDNA.

The/melanosporum lineage is distributed across Europe, Asia, and North America. Most species in this clade are characterized by large peridial warts and darkly pigmented ascospores ornamented with spines that sometimes connect to form a reticulum. We estimate that the most recent common ancestor of this clade radiated in association with Pinaceae in North America around 79 Mya (Fig. 5 - node M) followed by subsequent dispersal events to Asia and Europe. Jeandroz et al. [20] calculated the divergence date of this node at 76 Mya based on molecular clock analysis of 5.8S and ITS2 rDNA.

The/rufum lineage is well supported as the sister group to the/melanosporum lineage and is also distributed across Europe, Asia, and North America. Species in the/rufum lineage are primarily found with angiosperm hosts [6] and are characterized by a smooth to minutely warted peridium with light-colored ascospores ornamented with spines. In a few species, such as in T. sporeretulatum, the spines may connect to form a partial reticulum (Fig. 20). The most recent common ancestor of this clade was estimated to have radiated in North America around 86 Mya (Fig. 5 - node N) in association with angiosperm hosts. This lineage later dispersed to Asia and Europe. Jeandroz et al. [20] calculated the divergence date of this node at 70 Mya based on molecular clock analysis of 5.8S and ITS2 rDNA.

**Tuberaceae Radiated with Angiosperm Host Plants during the Cretaceous and Paleogene**

The Tuberaceae are presumed to share an ancient ectomycorrhizal ancestor because this is the nutritional mode for all of the extant species [4]. Alternatively, the ectomycorrhizal habit may have been acquired independently in the Tuberaceae, gymnohydnortrya clade, and Hebelia clade, but this seems unlikely given that no saprotrophic fungi are documented for any of these three lineages. Northern hemisphere Tuberaceae species are associated with a wide diversity of host plants including both monocot and dicot angiosperms and Pinaceae, but their ancestral ectomycorrhizal hosts are unknown. In accordance with our hypothesis, ancestral state reconstructions recover the ancestor of Tuber (and Tuberaceae) as the most likely ectomycorrhizal with angiosperm hosts (Fig. 4 - nodes 4, 2). The hypothesis of an ancient symbiotic association between Tuberaceae and angiosperm host plants is also supported by the fact that extant members of the/labyrinthomyces and the/gymnohydnortrya lineages are exclusively associated with angiosperms (they occur in the Southern hemisphere where Pinaceae do not naturally occur). Species of the Northern hemisphere genus Choiromyces may associate with either angiosperms or Pinaceae [45,46,47]. It appears that multiple independent ecological transitions from angiosperm to Pinaceae hosts have also occurred in individual Tuber species (e.g. T. canaliculatum Gilkey, T. indicum Cooke & Massie, T. borichii Vittad.) and for entire clades (e.g./gibbosum). We find it interesting that many clades of Tuber have species susceptible to orchid parasitism [6,48], which raises many questions pertaining to plant-fungus interactions.
Our molecular dating results place the Tuberaceae origin at the end of the Jurassic period (156 Mya), during the early radiation of angiosperm Eudicots [49], and are thus congruent with our hypothesis that the Tuberaceae initially co-radiated with angiosperm hosts. Although most of the major Tuber clades had evolved by the end of the Cretaceous, the origin of most species-rich lineages occurred during the mid-Paleogene (30–54 Mya), a time when angiosperms, Pinaceae, and other plant-associated fungi were all experiencing major evolutionary radiations [50]. These dates generally correspond to estimated radiations in major ectomycorrhizal host plant lineages including the Fagaceae, Betulaceae, Salicaceae and Juglandaceae [51,52,53,54]. We posit that the diversification of ectomycorrhizal angiosperm hosts during this period may have driven the diversification within Tuber and possibly other ectomycorrhizal lineages [55].

Other studies have shown that many other fungal groups were undergoing radiations during the Cretaceous period. For instance, Matheny et al. [56] used a relaxed molecular clock multi-locus approach to study the historical biogeography and diversification of a family of ectomycorrhizal basidiomycetes, the Inocybaceae. Their analyses indicate that the major clades within this family diverged during the Cretaceous (143 Mya) in association with angiosperms. The genera Amanita and Hygrophorus also likely have Cretaceous crown group origins [55]. O’Donnell et al. [57] studied the historical biogeography of the true morels (Morchella), a saprotrophic and biotrophic ascomycete lineage that is related to the Tuberaceae, and they used a multi-locus strict molecular clock approach. They found that Morchella diverged from its closest relatives in the early Cretaceous (126.6 Mya) and exhibited high continental endemism and provincialism. In another study, Sung et al. [58] examined fungal-animal symbionts in the Hypocreales using a Bayesian relaxed molecular clock approach and a fossil calibrating point. Their results indicated that the major families within the Hypocreales all diverged during the Cretaceous. Thus, many fungal groups appear to have undergone radiations during this geological period.

Other Lineages in the Tuberaceae

Choiromyces is a monophyletic genus broadly distributed in the Northern hemisphere. However, it appears more closely related to the Southern/labyrinthomycoses lineage than to Tuber (Fig. 4 - node 3). The hypogeous fruitbodies of Choiromyces are subglobose or irregular in form (Fig. 2D) and are characterized by a solid gleba having a hymenium with paraphyses and clavate ascis usually bearing eight-spores [39]. These ascospores have distinct ornamentation of either pits or pitted tubes (Fig. 2K).

The Southern hemisphere Tuberaceae also form a monophyletic group, the/labyrinthomycoses lineage. Taxa include both truffle and the cup-shaped forms. The cup fungus Nothojafnea thaxteri has 8-spored, cylindrical asci with uniserate spores whereas the truffle genera Dingleya, Labyrinthomycoses, and Reddellomyces are morphologically diverse. They can have between one to eight ascospores, their asci can be uniseriate, cylindrical, or saccate, and they have widely diverging peridial morphologies [60,61,62]. However, the generic boundaries between these truffle genera are not particularly clear since morphological characters are not consistent with the phylogeny (Fig. 4). We estimated relatively short divergence times between taxa in the/labyrinthomycoses lineage, which may explain some of the taxonomic problems with this group.

Enigmatic Taxa – Nothojafnea, Gymnohydnotrya, and Underwoodia

As we initially hypothesized, the inclusion of Southern hemisphere taxa contributed greatly to a better understanding of the Tuberaceae, to the identification of its sister lineage, and provided novel data concerning their evolution and biogeography. Nothojafnea, Gymnohydnotrya, and Underwoodia are three genera of enigmatic Pezizales whose phylogenetic positions are poorly known. Our phylogeny shows for the first time that Nothojafnea and Gymnohydnotrya are affiliated with the Tuberaceae. We also confirmed that the genus Underwoodia is polyphyletic. The North American type species, U. columariss, is allied with Helvella and Balansia in the Helvellaceae, whereas the two Southern hemisphere species, U. singeri (South America) and U. bautoni (Australia) are allied with Gymnohydnotrya. Together these Southern hemisphere species form a previously unrecognized sister group to the Tuberaceae/gymnohydnotrya, which will be formally described and named in a separate paper. Moreover, U. singeri and U. bautoni are not sister species and it is likely that Gymnohydnotrya truffles have evolved multiple times within the Southern hemisphere “Underwoodia”.

Nothojafnea is one of the enigmatic ectomycorrhizal genera whose taxonomic placement has long remained a mystery [2]. Only two species are described in the genus, N. thaxteri and N. australiana from South America, and N. thaxteri (E.K. Cash) Gamundi from Argentina and Chile [63,64]. Based on the ornamented spores and prominent apothecial hairs, the genus was described in the family Pyronemataceae [64]. There is strong support for the placement of N. thaxteri in the/labyrinthomycoses lineage and sister to the Australian truffle genera Dingleya, Labyrinthomycoses, and Reddellomyces (Fig. 5 - node C). Both species of Nothojafnea are considered ectomycorrhizal symbionts since they fruit directly on soil beneath ectomycorrhizal plants. The holotype species, N. cryptotricha, is found with Myrtaceae genera including Eucalyptus and Melaleuca [64,65] whereas N. thaxteri has only been found with Nothofagus [66]. Warcup [65] provided further verification of the symbiotic ecology of Nothojafnea when he synthesized ectomycorrhizas of N. cryptotricha in pot cultures with Melaleuca uncinata R. Br. etc.

As with Nothojafnea, the taxonomy and ecology of the genus Gymnohydnotrya is poorly known [2]. Gymnohydnotrya originally accommodated three Australian truffle species, G. australiana B.C. Zhang & Minter, G. echinulata (G.W. Beaton) B.C. Zhang & Minter, and G. ellipsospora (J.W. Cribb) B.C. Zhang & Minter [67]. The genus is characterized by light colored ascomata with an externally facing hymenium (exothecium), a basal hyphal tuft and no peridium. Microscopically, Gymnohydnotrya species tend to have 8-spored asci and hyaline, ornamented ascospores [67]. Gymnohydnotrya species are considered ectomycorrhizal because they fruit in soil and leaf litter beneath Eucalyptus and other Australian ectomycorrhizal plants [2,67,68]. Here an isolate from healthy root tips of Nothofagus obliqua was strongly supported as a member of the/gymnohydnotrya lineage, providing the first direct evidence for its ectomycorrhizal lifestyle. Although there are currently no species of Gymnohydnotrya described from South America, Roland Thaxter collected a Chilean truffle in 1906 that fits morphologically in the genus Gymnohydnotrya (Smith & Pfister, unpublished data). Thaxter’s specimen may correspond to the ectomycorrhizal symbiont sequenced from Nothofagus obliqua roots in Argentina or may point to further undescribed diversity in the/gymnohydnotrya lineage. Zhang and Minter [67] suggested that Gymnohydnotrya belonged within the Helvellaceae but also suggested possible affinities with Hydnotrya (Discinaceae). Our analysis indicates that Gymnohydnotrya species actually belong to the previously unknown Southern hemisphere lineage (gymnohydnotrya). We estimate the initial divergence of the Southern hemisphere Tuberaceae (labyrinthomycoses) at 43 Mya and/gymnohydnotrya at 72 Mya, with radiations during the Paleogene.
This would coincide with the radiation of the Southern hemisphere genus *Nothofagus* (40–55 Mya) and the fragmentation of South America, Australia, and Antarctica (30–50 Mya) [69].

**Summary**

In this study we reassessed the biogeography and origin of the *Tuberaceae* and their relatives using multiple loci and a global sampling of taxa. Multiple independent transitions from an aboveground to a belowground truffle fruiting body form have occurred in the *Tuberaceae* and in its newly recognized sister lineage/gymnohydrotrya. Our data indicate that the *Tuberaceae* most likely radiated from a common angiosperm-associated ectomycorrhizal ancestor in the late Jurassic. Subsequent radiations of major clades within the family have occurred on different continents during the Cretaceous and Paleogene, periods when many ectomycorrhizal angiosperm groups were also radiating. Several long-distance and intercontinental dispersal events have since occurred in several of the major clades within the *Tuberaceae*, including/puberulum and/or choiromyces. We hypothesize that, in some cases, dispersal events of ancestral truffle species may have been correlated with host plant migration (e.g. with the migration of *Salix* into the Southern hemisphere), but that in other cases host switching may have facilitated intercontinental diversification through founder events. Finally, we have identified an epigeous species belonging to the *Tuberaceae* (*Nothojafnea cf. thaxteri*), providing the first evidence that the *Tuberaceae* is not composed strictly of truffle fungi.

**References**

1. Trappe J, Molina R, Luoma D, Cázares E, Pihl D, et al. (2009) Diversity, Ecology, and Conservation of Truffle Fungi in Forests of the Pacific Northwest. In: United States Department of Agriculture FS, Pacific Northwest Research Station., editor. PNW-GTR-772: Portland, OR: USA pp. 194
2. Tedersoo L, May TW, Smith ME (2010) Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. Mycorrhiza 20: 217–263.
3. Millo A, Murat C, Bonfante P (2006) Truffles: much more than a prized and local fungal delicacy. Fems Microbiology Letters 260: 1–8.
4. Laruse T, Hansen K (2007) Truffle trouble: what happened to the Tuberellas?. Mycological Research 111: 1073–1099.
5. Padovan AGB, Sanson GFO,Brunstein A,Briones MRS (2005) Fungi evolution revisited: Application of the penalized likelihood method to a Bayesian fungal phylogeny provides a new perspective on phylogenetic relationships and divergence dates of ascomycota groups. Journal of Molecular Evolution 60: 726–735.
6. Bonito GM, Gryganskyi AP, Trappe JM, Vilgalys R (2010) A global meta-analysis of *Tuber* ITS rDNA sequences: species diversity, host associations and long-distance dispersal. Molecular Ecology 19: 4994–5008.
7. O’Donnell K, Cigelnik E, Weber NS, Trappe JM (1997) Phylogenetic relationships among ascomycetes truffles and the true and false morel inferred from 18S and 28S ribosomal DNA sequence analysis. Mycologia 89: 46–65.
8. Trappe JM (1979) The orders, families, and genera of hypogeous Ascomycotina (truffles and their relatives). Mycologia 9: 297–340.
9. Trail F (2007) Fungal canons: explosive spore discharge in the Ascomycota. FEMS Microbiology Letters 276: 12–18.
10. Singer R, Harris B (1987) Mushrooms and Truffles: Botany, Cultivation, and Utilization: Lubrecht & Cramer Ltd. 389 p.
11. Alvarez IF, Parlade J, Trappe JM (1992) *Loculotuber gennadii* gen. et comb. nov. in its newly recognized sister Tuberaceae and their relatives using multiple loci and a global sampling of taxa. *Tuberaceae*. Mycological Research 103: 203–208.
12. Berbee ML, Taylor JW (2010) Dating the molecular clock in fungi - how close are we? Fungal Biology 24: 1–16.
13. Jeanrous S, Murat C, Wang YJ, Bonfante P, Le Tacon F (2008) Molecular phylogeny and historical biogeography of the genus *Tuber*, the ‘true truffles’. Journal of Biogeography 35: 815–829.
14. Castellano MA, Trappe JM, Maser Z, Maser C (1989) Key to spores of the genera of hypogeous fungi of north temperate forest with special reference to animal mycophagy. Eurika, California Mad River Press. 186 p.
15. Frank JL, Angliss S, Carrington EM, Taylor DS, Vitarte B, et al. (2009) Rodent dispersal of fungal spores promotes seedling establishment away from mycorrhizal networks on *Quercus garryana*. Botany-Botanique 87: 821–829.
16. Cázares E, Trappe JM (1994) Spore dispersal of ectomycorrhizal fungi on a glacier forefront by mammal mycophagy. Mycologia 86: 547–540.
17. Bums TD, Fogel R, White TJ, Palmer JD (1989) Accelerated evolution of a false-truffle from a mushroom ancestor. Nature 339: 140–142.
18. Martin MP, Hogberg N, Linnell J (1999) *Mummtites mucoides*, a hypogeous relative of *Rhizina mucoides*. Mycological Research 103: 203–208.
19. Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference using posterior probabilities. Bioinformatics 19: 1572–1574.
20. Lanfear R, Calcott B, Ho SYW, Guindon S (2012) PartitionFinder: Combined Selection of Partitioning Schemes and Substitution Models for Phylogenetic Analyses. Molecular Biology and Evolution 29: 1695–1701.

**Supporting Information**

**Table S1** Collection information and GenBank accession information for taxa sampled. (XLS)

**Table S2** Primers used in this study. (XLS)

**Table S3** Economically important *Tuber* species determined by 2009 market prices (USD) in the USA. (XLS)

**Acknowledgments**

Many thanks to the North American Truffling Society and other collectors who provided the material used in this research. Particularly, Joey Spatafora and Richard Halse of the Oregon State University Department of Botany Herbarium for extensive herbarium services for accessions of the collections cited here plus many additional collections. Connie Robertson for curating truffle collections at the Duke Herbarium. The National Fungus Collections and Herbarium of the Università di Bologna generously lent specimens for study. We thank Paul Manos, David Swoford, Michael Castellano, Hiroshi Sasaki, Pei-Gui Liu, Yongjin Wang, Juan Chen, François Le Tacon, Terri Porter, Jason Jackson, Andrée Gryganskyi and Michelle Hersh for providing specimens, sequences, photos or valuable discussion for the preparation of this manuscript. Anthony Bonito graciously prepared scientific illustrations.

**Author Contributions**

Conceived and designed the experiments: GB JT MS RV MN. Performed the experiments: GB MS MN RH. Analyzed the data: GB MS MN. Contributed reagents/materials/analysis tools: RH GG EC AK EN LD MS RV MN. Wrote the paper: GB MS MN LT RV JT LD EN DP KN.

**Contact**

Juan Chen, Francois Le Tacon, Terri Porter, Jason Jackson, Andrii Gryganskyi for providing specimens, sequences, photos or valuable discussion for the preparation of this manuscript. Anthony Bonito graciously prepared scientific illustrations.
30. Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7: -.
31. Otalora MAG, Martinez I, Aragon G, Molina MC (2010) Phyllogeography and divergence date estimates of a lichen species complex with a disjunct distribution between the western United States and Mexico. American Journal of Botany 97: 216–223.
32. Clarke JT, Warnecky RCM, Donoghue PCJ (2011) Establishing a time-scale for plant evolution. New Phytologist 192: 266–301.
33. Rambaut A, Drummond AJ (2007) Tracer v1.4. Available: http://beast.bio.ed.ac.uk, editor.
34. Yan Y, Harris AJ, He X (2011) RASP (Reconstruct Ancestral State in Phylogenies). Available: http://mnhscueducn/soft/blog/RASP.
35. Newsholme C (1992) Willows: The genus Salix: Batsford Ltd. 1-224 p.
36. Spegazzini (1887) Anales Soc. Ci. Argent. 10.
37. Smith SA, Beaulieu JM, Donoghue MJ (2010) An uncorrelated relaxed-clock model reveals a recent origin of flowering plants. Proceedings of the National Academy of Sciences of the United States of America 107: 5897–5902.
38. Marjanovic Z, Grebene T, Markovic M, Glisic A, Milenkovic M (2010) Ecological specificities and molecular diversity of truffles (genus Tuber) originating from mid-west of the Balkan Peninsula. Sydowia 62: 67–87.
39. Alvarado P, Moreno G, Manjón JL (2012) Comparison between Tuber genadini and T. oligoporus lineages reveals the existence of the new species T. cistophilum (Tuberaeaceae, Pezizales). Mycologia 104: 894–910.
40. Smith ME, Doshan GW, Premier AK, Rizzo DM (2009) Are true multithrost fungi the exception or the rule? Dominant ectomycorrhizal fungi on Pinus sabiniana differ from those on co-occurring Quercus species. New Phytologist 182: 295–299.
41. Wei XX, Yang ZY, Li Y, Wang XQ (2010) Molecular phylogeny and biogeography of Pseudotubera (Pinaceae): Insights into the floristic relationship between Taiwan and its adjacent areas. Molecular Phylogenetics and Evolution 55: 776–785.
42. Guevara G, Bottino G, Trappe J, Cazares E, Williams G, et al. (in press) New North American truffles (Tuber spp.) and their ectomycorrhizal associations Mycologia.
43. F. Palffier N, Brunel N, Santelices R (2007) Synthesis and establishment of Tuber sambucorum and T. cistophilum on two Nothofagus species in Chile. Mycorrhiza 17: 627–632.
44. Bulman SR, Visnovsky SR, Hall IR, Guerin-Laguerre A, Wang Y (2010) Molecular and morphological identification of tuber-producing Tuber species in New Zealand. Mycological Progress 9: 205–214.
45. Comandini O, Cunto M, Rinaldi AC (2006) An overview of Otto’s ectomycorrhizal fungi. Mycorrhiza 16: 381–395.
46. Kotorega E, Matuszak M (2008) Hypogeous fungi of Lithuania: a preliminary checklist. Acta Mycologica 43: 133–138.
47. Weden C, Sonny L, Burman R, Backlund A (2009) The Edible Truffle Glomus versiformis and Its use in Sweden. Acta Botanica Yunnanica Supplement XVI: 94–96.
48. Bidartondo MI, Burghardi B, Grubauer G, Bruno TD, Read DJ (2004) Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. Proceedings of the Royal Society of London Series B-Biological Sciences 271: 1709–1806.
49. Bell CD, Solnit DE, Solnit PS (2010) The age and diversification of the angiosperms re-revised. American Journal of Botany 97: 1296–1303.
50. Matheny PB, Aime MC, Bougher NL, Buyck B, Desjardins DE, et al. (2009) Out of the Palaeotropics? Historical biogeography and diversification of the cosmopolitan ectomycorrhizal mushroom family Inocybaceae. Journal of Biogeography 36: 577–592.
51. Mindell RA, Stockey RA, Beard G (2007) Castanopsis spinosa gen. et sp nov (Fagaceae): Castanoid fruits from the Eocene of Vancouver Island, Canada. American Journal of Botany 94: 351–361.
52. Ramirez JL, Cevallos-Ferriz SRS (2000) Leaves of Salicaceae (Salix and Populus) from Oligocene sediments near Tepexi de Rodriguez, Puebla, Mexico. International Journal of Plant Sciences 161: 521–534.
53. Manos PS, Solnit PS, Solnit DE, Manchester SR, Obi SH, et al. (2007) Phylogeny of extant and fossil Juglandaceae inferred from the integration of molecular and morphological data sets. Systematic Biology 56: 412–430.
54. Sims HJ, Herendeen PS, Lupia R, Christopher RA, Crane PR (1999) Fossil flowers with Normapolles pollen from the Upper Cretaceous of southeastern North America. Review of Palaeobotany and Palynology 106: 131–151.
55. Ryberg M, Matheny PB (2012) Asynchronous origins of ectomycorrhizal clades of Agaricales. Proceedings of the Royal Society B-Biological Sciences 279: 2003–2011.
56. Matheny PB, Aime MC, Bougher NL, Buyck B, Desjardins DE, et al. (2009) Out of the Palaeotropics? Historical biogeography and diversification of the cosmopolitan ectomycorrhizal mushroom family Inocybaceae. Journal of Biogeography 36: 577–592.
57. O’Donnell K, Rooney AP, Mills GL, Kuo M, Weber NS, et al. (2011) Phylogeny and historical biogeography of true morels (Morchella) reveals an early Cretaceous origin and high continental endemism and provincialism in the Holartic. Fungal Genetics and Biology 49: 362–385.
58. Sung GH, Poinar GO, Spatafora JW (2008) The oldest fossil evidence of animal parasitism by fungi supports a Cretaceous diversification of fungal-arthropod symbiosis. Molecular Phylogenetics and Evolution 49: 495–502.
59. Moreno G, Alvarado P, Manjón JL, (2011) Phylogenetic affiliation of Chonoryces magnusii and C. sambucorum (Tuberaceae, Acomyces) from Spain. Mycological Progress DOI 10.1007/s11557–011–0762–1.
60. Beaton G, Weste G (1977) New Zealand truffles (Tubera spp.) and their ectomycorrhizal associations Mycologia.
61. Beaton G, Malajczuk N (1986) A New Species and a Variety of Labyrinthomyces in Western-Australia. Transactions of the British Mycological Society 86: 503–506.
62. Trappe JM, Claridge AW, Claridge DL, Liddle I (2008) Desert truffles of the Australian aridbelt: Ecology, ethnomycology, and taxonomy. Economic Botany 62: 497–506.
63. Gamundi JI (1971) Algunos discomycetes de Chile. Bol. Soc. Argent. Bot. 13: 295–304.
64. Gamundi JI (1971) Algunos discomycetes de Chile. Bol. Soc. Argent. Bot. 13: 295–304.
65. Warcup JH (1990) Occurrence of ectomycorrhizal and saprophytic discomycetes after a Wild fire in a eucalypt forest. Mycological Research 94: 1065–1069.
66. Cook LG, Crisp MD (2005) Not so ancient: the extant crown group of Nothofagus and Its Use in Sweden. Acta Botanica Yunnanica Supplement XVI: 94–96.
67. Warcup JH (1990) Occurrence of ectomycorrhizal and saprophytic discomycetes after a Wild fire in a eucalypt forest. Mycological Research 94: 1065–1069.
68. Gamundi JI (1971) Algunos discomycetes de Chile. Bol. Soc. Argent. Bot. 13: 295–304.
69. Gamundi JI (1971) Algunos discomycetes de Chile. Bol. Soc. Argent. Bot. 13: 295–304.
70. Warcup JH (1990) Occurrence of ectomycorrhizal and saprophytic discomycetes after a Wild fire in a eucalypt forest. Mycological Research 94: 1065–1069.