The Gondwanan connection – Southern temperate *Amanita* lineages and the description of the first sequestrate species from the Americas

Truong, Camille^{1*}; Sánchez-Ramírez, Santiago²; Kuhar, Francisco³; Kaplan, Zacharie¹; Smith, Matthew E.¹

¹University of Florida, Department of Plant Pathology, 2523 Fifield Hall, Gainesville FL 32611, USA

²Department of Ecology and Evolutionary Biology, University of Toronto, 100 Queen's park, Toronto, Ontario, M5S 2C6, Canada

³Centro de Investigación y Extensión Forestal Andino Patagónico (CONICET), Ruta 259, Km 4, Esquel 9200, Chubut, Argentina

*Corresponding author: camille.truong@ufl.edu, phone +1 (352) 2734670

Abstract

Amanita is a diverse and cosmopolitan genus of ectomycorrhizal fungi. Although most species fruit aboveground, some sequestrate taxa have been documented from Australia and the Mediterranean region. We describe *A. nouhrae* sp. nov., a new hypogeous ('truffle-like') species associated with *Nothofagus antarctica* in northern Patagonia. This constitutes the first report of a sequestrate *Amanita* from the Americas. Thick-walled basidiospores ornamented on the interior spore wall ('crassospores') were observed consistently in *A. nouhrae* and its sister epigeous taxon *A. morenoi*. *A. morenoi* is a rarely collected but apparently common species from northern Patagonia that has sometimes

been misidentified as the Australian taxon *A. umbrinella*. Nuclear 18S and 28S ribosomal DNA and mitochondrial 16S and 26S DNA placed these two species in a southern temperate clade within subgenus *Amanita*, together with other South American and Australian species. Based on a dated genus-level phylogeny, we estimate that the southern temperate clade may have originated near the Eocene/Oligocene boundary (ca. 35 Ma $\pm$ 10 Ma). This date suggests a broadly distributed ancestor in the Southern Hemisphere, which probably diversified as a result of continental drift, as well as the initiation of the Antarctic glaciation. By comparison, we show that this clade follows an exceptional biogeographic pattern, within a genus otherwise seemingly dominated by Northern Hemisphere dispersal.

Keywords: ectomycorrhizas, truffles, biogeography, Southern Hemisphere, Agaricales, Nothofagaceae

1. Introduction

Southern Hemisphere biogeography was heavily influenced by the fragmentation of the supercontinent Gondwana during the last 165 million years, causing the isolation and subsequent diversification of the ancestral Gondwanan biota on each separate continent (Raven and Axelrod 1972). The ectomycorrhizal (ECM) plant family *Nothofagaceae* (Fagales) is a prime example of this vicariant scenario, reflected in its current transoceanic distribution in South America, Australia, New Zealand, New Caledonia, and New Guinea (Manos 1997; Sanmartin and Ronquist 2004). Abundant fossil and pollen records place the origin of this plant lineage in the Cretaceous ca. 80 Ma

(million years ago) (Knapp et al. 2005). At the time, Antarctica had a warm-temperate climate and was probably covered by forest until ca. 25 Ma thereby favouring biotic exchanges between what is now South America and Australia. Australia and South America landmasses remained in contact via Antarctica until the final break-up of Gondwana ca. 50–35 Ma (Knapp et al. 2005; Sanmartin and Ronquist 2004). This so called ‘Southern Gondwana’ pattern was first illustrated by Brundin (1966) who demonstrated that phylogeographic patterns of chironomid midges (Diptera) were congruent with the sequential breakup of continents from the Gondwana landmass: African taxa are the earliest diverging lineages followed by a New Zealand–New Caledonia clade and finally a southern South America–Australian–New Guinea clade (Sanmartin and Ronquist 2004).

Despite the wealth of biogeographical data in many plant and animal groups (Sanmartin and Ronquist 2004), there have been fewer studies illustrating similar patterns in fungi. Enhanced diversity in certain ECM fungal lineages (e.g. *Cortinariaceae*) was detected simultaneously in forest soils from South America and Australia (Dickie et al. 2009; Nouhra et al. 2013; Tedersoo et al. 2008), suggesting that this ancient ‘Southern Gondwana’ connection may also occur in fungi (Tedersoo et al. 2014; Tedersoo et al. 2010). Global patterns of ECM fungal diversity also suggest that Austral regions (Australia, New Zealand, and southern South America) share specific fungal lineages, while lacking other lineages commonly found in the Northern Hemisphere (Tedersoo et al. 2010; Truong et al. 2017). These distribution patterns are often consistent with the evolutionary history of their host plants. Due to their long history of isolation, plant lineages native to the Southern Hemisphere (e.g. *Nothofagaceae*) formed specific ECM

associations that are absent in Holarctic (*Pinaceae*, *Betulaceae*, *Fagaceae*) and tropical (*Caesalpiniaceae*, *Dipterocarpaceae*) ECM plant families (Tedersoo et al. 2012). Examples of these ‘Austral’ fungal lineages include the /austropaxillus lineage (*Austropaxillus* and *Gymnopaxillus*) (Skrede et al. 2011), the family *Gallaceaceae* (*Hallingea* and relatives) (Hosaka et al. 2008), and the /descolea lineage (Tedersoo et al. 2010). All of these taxa are known to form ECM associations with trees in the *Nothofagaceae*. The ascomycete genus *Cyttaria* (Peterson et al. 2010) is another example of this ‘host-associated’ biogeographic pattern, this time as an obligate plant pathogens of *Nothofagaceae*. Another scenario is the presence of southern temperate lineages within otherwise cosmopolitan ECM fungal genera, as previously demonstrated in *Amanita* sect. *Caesareae* (Sánchez-Ramírez et al. 2015b), *Inocybe* (Matheny et al. 2009), *Hysterangium* (Hosaka et al. 2008) and *Laccaria* (Wilson et al. 2017).

Sequestrate basidiomycetes (such as false truffles and puffballs) have highly modified fruiting morphologies where basidia mature inside enclosed, belowground (fully hypogeous) or partially emergent (secotioid) basidiomes adapted to passive (non-ballistosporic) spore dispersal (Bougher and Lebel 2001). Several advantages have been suggested to favour the evolution of sequestrate basidiomes, such as protection against drought or cold (Thiers 1984), or enhanced dispersal abilities due to animal mycophagy (Fogel and Trappe 1978; Hosaka et al. 2008; Johnson 1996; Schickmann et al. 2012). With two Mediterranean climatic regions and the presence of numerous mammals as potential dispersal vectors, Australia is a ‘hot spot’ for sequestrate fungi (Bougher and Lebel 2001) including within several families of Agaricomycetes (e.g. *Russulaceae*, *Cortinariaceae*, and *Amanitaceae*). *Amanita* Pers. (*Amanitaceae*, *Agaricales*) is a diverse

and cosmopolitan genus comprising about 500 accepted species and about 1,000 estimated species (Tulloss 2005). The species diversity is relatively low in South America compared to other regions and diversity patterns indicate a disjunction between southern South America and the rest of the continent (Tulloss 2005). The vast majority of species produce typical agaricoid (aboveground) basidiomes, but a few sequestrate taxa have been recorded from Australia and the Mediterranean region. The genera *Torrendia* and *Amarrendia* were initially erected to accommodate these sequestrate taxa. *Torrendia* included taxa with secotioid basidiomes whereas species of *Amarrendia* included the fully sequestrate, hypogeous species. Moncalvo et al. (2002) demonstrated that these taxa were phylogenetically nested within *Amanita* and later Justo et al. (2010) transferred all these sequestrate species to *Amanita*.

Here we describe a new sequestrate *Amanita* species discovered during our mycological explorations in *Nothofagus* forests of Patagonia. We also provide new morphological and molecular data for the sister taxon of *A. nouhrae*, the epigeous species *A. morenoi* Raithelhuber, that was previously known only from the type specimen. We document this mushroom and its unusual globose, ornamented basidiospores and discuss the origin and evolutionary significance of ornamented basidiospores in *A. nouhrae* and *A. morenoi*. Based on multi-gene phylogenies, we elucidate their phylogenetic relationships and track their temporal origins with respect to other *Amanita* species from Australia and southern South America.

2. Materials and Methods

Basidiomes of agaricoid and sequestrate *Amanita* species were collected in *Nothofagus* forest sites in Patagonia (Argentina and Chile) during spring 2015 and 2016 (Southern Hemisphere autumn). Specimens were photographed in the field and fresh tissues were preserved in CTAB buffer for subsequent DNA extraction. Colour codes in descriptions followed the Pantone colour code adopting the CMYK system referring to a Pantone colour bridge (Pantone Inc., New Jersey). Specimens were preserved on a forced-air dryer and stored in plastic bags with silica gel. A stereomicroscope was used to complete measurements of tissues (e.g. peridium, stipe, gleba). Dried material was rehydrated and mounted in water, 3% KOH, Melzer's reagent, and cotton blue to be studied by light microscopy. Spores, basidia, and other microscopic features were measured with ≥ 20 measurements that were averaged to estimate size ranges. Specimens were deposited at the Museo Botánico of Córdoba (CORD), the Museo Nacional de Historia Natural of Santiago (SGO), and the Florida Museum of Natural History (FLAS).

We extracted DNA from representative *Amanita* species collected from South America and Australia using a standard CTAB procedure (Gardes and Bruns 1993). The nuclear 18S and 28S ribosomal DNA and mitochondrial 16S and 26S DNA loci were amplified by PCR following Wolfe et al. (2012b). PCR products were verified by electrophoresis on 1.5 % agarose gel, cleaned with EXO and SAP enzymes (Glenn and Schable 2005), and sequenced with the same primer combinations using the BigDye Terminator v. 3.1 Cycle Sequencing Kit on an ABI3700 DNA sequencer (Applied Biosystems) at University of Florida. Sequences were edited with Sequencher 5.1 and a BLAST search was performed in GenBank to verify their identity. We generated a total of 45 new sequences from 15 specimens and combined them with published datasets,

including all known sequestrate *Amanita* species and as many species as possible from the Southern Hemisphere (Cai et al. 2014; Justo et al. 2010; Sánchez-Ramírez et al. 2015b; Wolfe et al. 2012b). Species of *Pluteus* and *Limacella* were used as outgroup taxa. Species, specimens and Genbank accession numbers are detailed in Supplementary Table S1.

Sequences from each gene were aligned with MUSCLE 3.8.31 (Edgar 2004) using default parameters, then corrected manually and ambiguously aligned regions excluded in Mesquite 3.01. The absence of incongruence among genes was verified before concatenating the data by reconstructing individual gene trees using Maximum Likelihood (ML) with 1000 bootstrap pseudoreplicates and the GTR+G model in RAxML 8.2.8 (Stamatakis 2014) on the CIPRES Portal (Miller et al. 2010). Three partitions (18S, 28S, 16S+26S) were selected with PartitionFinder 1.1.1 (Lanfear et al. 2012) with the BIC criterion. The concatenated dataset was analyzed similarly using ML and Bayesian inference (BI) in MrBayes 3.2.6 (Ronquist et al. 2012) with the GTR+G+I model for 10 million generations, 3 runs, and 4 chains. Convergence of the BI runs was verified in Tracer 1.5 (Rambaut and Drummond 2007) and the majority rule consensus tree was reconstructed by pooling sampled trees after discarding the first 25% as burn-in.

Our molecular dating analysis included an additional set of 327 nuclear large subunit rDNA (28S) sequences deposited in Genbank (Table S2), focusing on species level sampling and records that included geographic location (Sánchez-Ramírez et al. 2017). We used BEAST v2.4.5 (Bouckaert et al. 2014) on a supercomputer mainframe (SciNet, UofT) by choosing a lognormal-distributed clock model, the pure-birth Yule model as a tree prior, and a GTR+G substitution model. A gamma distribution was used

as prior for the uclMean (beta=0.001, alpha=1) and birthRate (beta=0.01, alpha=1) parameters. As calibration points we used two secondary calibrations based on Sánchez-Ramírez et al. (2015b; 2017), calculated from primary fossil data: 1) on the time to the most recent common ancestor (tMRCA) of subgenus *Amanita* (normal distribution with mean=75.0 Ma and SD=1.0 Ma); and 2) on the tMRCA of *Amanita* section *Caesareae* (normal distribution with mean=56.0 Ma and SD=1.0 Ma). We ran two parallel MCMC runs of 100 million generations sampling every 10,000th state. Runs were evaluated for convergence and chain mixing in Tracer v1.6 (Rambaut and Drummond 2007). Then, runs were combined with LogCombiner, removing a 10% burnin, and resampling every 20,000th state. The final tree file included 9000 states, which were processed with TreeAnnotator, estimating node heights using the 'ca' (common ancestor) method (Heled and Bouckaert 2013), and summarized posterior node data to a maximum-clade-credibility (MCC) tree. Only nodes with posterior probabilities ≥ 0.9 were annotated.

3. Results

3.1 Phylogenetic analysis

The final alignment from the four concatenated genes included 2753 bp and 1318 variable characters. The alignment is available in TreeBase (<http://purl.org/phylo/treebase/phyloids/study/TB2:S20736>). The BI majority rule consensus tree (LnL=-29052.09) inferred from the multi-gene dataset is illustrated in Fig. 1. It contains 84 highly supported internodes (posterior probabilities $PP \geq 0.95$). The best-scoring ML tree (LnL=-30259.44) was almost identical to the Bayesian tree, with 71 highly supported internodes (bootstrap values $BS \geq 0.70$). The single-gene tree from the

nuclear large subunit rDNA (28S) was the most complete dataset and had 54 highly supported internodes. Nevertheless, no incongruence was observed among genes and the concatenation of 28S with 18S, 26S and 16S provided additional resolution at important nodes.

As previously reported (Justo et al. 2010; Tulloss et al. 2016; Wolfe et al. 2012b), the genus *Amanita* is subdivided into two highly supported clades corresponding to subgenera *Amanita* and *Lepidella*, while non-ectomycorrhizal species form paraphyletic sister lineages. These species were discovered in the absence of known ECM host plants and are currently considered to be either putative saprobes or root endophytes (Moncalvo et al. 2002; Wolfe et al. 2012a). Redhead et al. (2016) segregated these non-ECM species into the new genus *Saproamanita*. Although the monophyly of ECM *Amanita* species is unequivocal, the generic concept of *Saproamanita* have been contested based on the lack of morphological characters and the fact that this genus may be a paraphyletic grade of species rather than a true monophyletic group (Tulloss et al. 2016). The generic delimitation of *Amanita* is beyond the scope of this paper and we therefore retained all species within the genus *Amanita* until further work clarifies the nomenclature of this group.

All the South American species included in this study belong to subgenus *Amanita*. With the exception of the South American taxon *A. diemii*, all taxa studied formed a highly supported southern temperate clade together with Australian species (Fig. 1). Members of this clade correspond roughly to the ‘*Amarrendia* clade’ described in Justo et al. (2010). This lineage includes both sequestrate (e.g. *A. grandis*, *A. grandispora*, *A. inculta*, *A. pseudoinculta* and *A. oleosa* from Australia, as well as our newly described

species *A. nouhrae* from South America) and epigeous taxa (e.g. *A. umbrinella* and *A. murinoflammeum* from Australia, as well as *A. morenoi* and *A. merxmuelleri* from South America). *Amanita nouhrae* and its sister taxon *A. morenoi* share the presence of ornamented basidiospores (see below) that are absent in their closest known relative *A. merxmuelleri* and other described species from the Southern temperate clade.

3.2 Divergence time estimation

The time-calibrated molecular phylogeny based on our dataset with the addition of 327 28S sequences from Genbank gave an estimated age of the root of the genus *Amanita* (including non-ECM taxa) with a mean of 99.9 Ma [98.0–101.9 HPD] (Fig. 2), fairly close to posterior estimates in other studies (Cai et al. 2014; Ryberg and Matheny 2012). The tMRCA of the southern temperate clade –which includes our newly described species *A. nouhrae*– had a mean of 34.5 Ma [24.3–45.45 HPD], falling near the Eocene/Oligocene boundary (Fig. 2). Within this clade, the South American cluster resulted with a mean tMRCA of 24.4 Ma [13.1–33.2 HPD]. It is worth mentioning that in previous analyses (data not shown), a different calibration prior based on Cai et al. 2014 at the root of the genus *Amanita* (158.5 Ma [116.6–200.7 HPD]) resulted in slightly older ages (ca. 10 Ma), which suggests that our estimates are probably conservative at the upper range of the distribution.

3.3 Taxonomy

Amanita morenoi Raithelhuber, 1986. *Metrodiana* 14: 6 (1986). (Fig. 3)

Type: Argentina, Neuquén, Nahuel Huapi National Park, Lago Espejo, 9 March 1959, coll. I. Gamundi ‘Amanita 75’. BAFC!–Holotype 30.617 (Supplementary Fig. S3)

Macromorphology. Pileus 20–60 mm diam., convex when young, applanate when fully developed, with various shades of beige (P406) to pale grey (P413), cream (P466–468) or brownish (P463–464); margin distinctly crenulate from striations; patches of universal veil irregularly distributed over the disc, almost absent to very abundant, flattened to thickened toward the centre of disc, whitish to beige (P406–407) or greyish (P420–421). Lamellae free to narrowly adnate, 6–8 per cm, white to cream, with approximately one lamellula in between each lamella, margin finely notched when viewed through a hand lens. Stipe 70–120 × 8–10 mm, slightly thicker towards base, cream to beige (P4645, P4655), whitish at apex, distinctly fibrillose, remnants of veil material sparse to very abundant and scaly along stipe, whitish to beige (P406–407) or greyish (P420–421); annulus concolorous with veil remnants (P406–407, P420–421), descending, distinctly striated. Base bulbous, 25 × 18 mm, encircled with cream (P466–467) to beige (P406–407) circumsessile volva. Context trama whitish and smooth, no response with the addition of Melzer’s reagent on any basidiome surfaces; pileus trama slightly darker (P4625, P4635, P4645) on uppermost part where it contacts the pileipellis, 3–6 mm thick above stipe, regularly narrowing towards margins; stipe trama whitish, regularly hollow from base to cap in mature specimens. Odor and taste indistinct.

Microscopic characteristics. Basidiospores globose to subglobose, with an apiculum, hyaline, non-amyloid, (9) 10–12.5 (13) × (8) 9–11.5 (13) μm , Q ratio = 1–1.15, thick-walled (0.8–1 μm), distinctly ornamented on the inner wall (crassospores as described and depicted by (Tulloss and Halling 1997)); ornamentation consisting of small,

evenly distributed hemispherical pits that are covered by an outer layer (ectosporium) appressed to the inner layer, the outer layer eventually collapsing into the cavities; in immature specimens spores are scarce, mostly with thin and smooth cell walls ($<0.8\text{ }\mu\text{m}$), a few ornamented spores visible after careful examination even in young specimens. Basidia clavate-pedicellate, $30\text{--}50 \times 10\text{--}15\text{ }\mu\text{m}$ from base to bottom of sterigmata, typically 4-spored but occasionally 2-spored, thin walled ($<0.5\text{ }\mu\text{m}$), ornamented basidial walls (crassobasidia) not observed; sterigmata short-cylindrical, $3\text{--}6\text{ }\mu\text{m}$ long. Subhymenium trama $30\text{--}50\text{ }\mu\text{m}$ thick, hyaline, consisting of elongated hyphae, tightly interwoven, horizontally distributed; immature specimens with oil droplets abundantly distributed in the hymenial and subhymenial trama and inside basidia; clamp connections infrequent but visible in all trama (hymenium, pileus, stipe).

Habitat and distribution. On soil in *Nothofagus dombeyi* forest in northern Patagonia.

Additional specimens examined. Argentina, Río Negro, Nahuel Huapi National Park, near Lago Escondido, 16 May 2016, coll. M. E. Smith MES1301 (FLAS–F–59751). Chile, Los Ríos, Puyehue National Park, up the road past El Caulle, North of Rio Golgol: 4 May 2016, coll. R. Healy MES1616 (FLAS–F–59753), coll. A. E. Mujic MES1624 (FLAS–F–59754), coll. P. Sandoval MES1588 (FLAS–F–59755); 7 May 2016, coll. B. P. Matheny MES1774 (FLAS–F–59752).

Additional notes. In their description of the type specimen, Tulloss and Halling (1997) depicted basidiospores with regular ‘golf ball’ ornamentation on the inner wall that match perfectly with our own observations of the type specimen (Fig. S3) and our own fresh collections (Fig. 3). These ornamented basidiospores were first reported from

European *Amanita* species by Kotilová-Kubíčková and Pouzar (1988) and were referred to as ‘crassospores’. These crassospores occurred together with ‘normal’ spores (thin and non-ornamented walls) and were observed inconsistently in several *Amanita* species. Kotilová-Kubíčková and Pouzar (1988), and later Tulloss and Halling (1997) therefore postulated that they were anomalous spores resulting from a pathogenic infection or other environmental factors. In our collections of *A. morenoi* and *A. nouhrae*, mature spores were consistently and distinctly ornamented in all studied specimens. Immature specimens with young basidia had few spores in general but crassospores were nonetheless present upon close examination. *Amanita morenoi* and *A. nouhrae* clustered in highly supported sister lineages within our phylogeny (Fig. 1), suggesting that these ornamented spores may be genetically determined and are not due to environmental influences or pathogens. We obtained specimens of *A. morenoi* from several sites on different days, further bolstering the hypothesis that these spores are a normal feature for this species. Although our collections match well with the description of *A. morenoi*, we did not observe the ‘crassobasidia’ described by Tulloss and Halling (1997) in which basidia walls are ornamented in a similar way. We cannot explain this morphological deviation from their observations of the type but morphological studies of additional collections of *A. morenoi* may reveal more about these ‘crassobasidia.’ Tulloss and Halling (1997) also observed crassospores in *A. pseudospreta* from Patagonia, but this species supposedly has a sacate volva and the presence of crassospores was inconsistent within their material. The overall similarities between *A. morenoi* and *A. pseudospreta* and the fact that these two taxa were described from the same region in northern

Patagonia suggest that they could be sister species or perhaps even synonyms. However, careful study of additional material is needed to investigate this hypothesis.

Amanita morenoi was previously known only from the type specimen. In their description of the dry specimen, Tulloss and Halling (1997) depicted a species with a pale brown cap and pyramidal to subconical warts over the disc. They did not have access to fresh material for their descriptions. According to the annotation notes in the holotype packet by Tulloss and Halling: ‘the specimen appears to be somewhat damaged when dried and is not in very good condition. Large thick brown warts that *may have been* truncate or pyramidal decorate the disc.’ We did not observe pyramidal warts over the disc on our fresh material but the thick brown warts fit well with their description. As depicted in Fig. 3, *A. morenoi* specimens with almost identical 18S and 28S rDNA as well as 16S and 26S mitochondrial DNA sequences can be quite variable morphologically, especially in the colour and abundance of veil material left on the pileus and stipe. The overall description of morphology (i.e. striate pileus margin, bulbous base) and crassospores matches well with the type and with the description of Tulloss and Halling (1997). In addition, our collections are from the same area and at the same time of year as the type specimen, in a region where the diversity of *Amanita* species is relatively low. *Amanita morenoi* apparently fruits abundantly during the Austral fall and specimens have been previously misidentified as the Australian taxon *A. umbrinella* (Singer 1954, see also Tulloss in [www. amanitaceae.org](http://www.amanitaceae.org)). In our phylogeny (Fig. 1), *A. umbrinella sensu stricto* (Gilbert 1941) is phylogenetically segregated and clearly distinguished by the thin-walled, smooth basidiospores. Based on these observations, we use the name *A. morenoi* to refer to this taxon.

Amanita nouhrae Truong, Kuhar & M. E. Sm., **sp. nov.**

(Fig. 4 & 5)

MycoBank No.: MB818831

Type: Argentina, Río Negro, Nahuel Huapi National Park, Los Rápidos, 13 May 2016, coll. M. E. Smith MES1921. Holotype–CORD, isotype–FLAS (FLAS–F–59756).

Diagnosis. Basidiome hypogeous, subglobose, with a sterile base almost entirely encircled by the gleba at maturity. Peridium, stipe, and gleba white to cream. Gleba loculate. Basidiospores $12.5\text{--}17.5 \times 14\text{--}20\ \mu\text{m}$, subglobose, hyaline, inamyloid, with an obvious oil droplet, thick spore walls ($1\text{--}2\ \mu\text{m}$) and ornamentation on the inner wall. First sequestrate *Amanita* species recorded from South America. Known only from Nahuel Huapi National Park (northern Patagonia) in association with *Nothofagus antarctica*.

Macromorphology. Basidiome hypogeous, subglobose, 10–16 mm across at maturity, almost entirely enclosed by the universal veil. Peridium white to cream (P465), becoming yellowish (P100) when dry, thickest in the apical part (up to 0.5 mm thick), densely floccose with cream to brownish (P4635, P4645, P4655) universal veil remnants forming patches on upper surface. Gleba hyaline when young, turning white to slightly yellowish (P100) or cream (P468) with age; hymenium loculate, individual locules irregular or angular to circular, up to 0.7 mm across. Sterile base white to cream (P465), with a smooth exterior surface, subglobose when young (up to 1.2 cm diam.), becoming conic and almost entirely encircled by gleba as the basidiome develops. Context trama whitish and smooth, no response with the addition of Melzer's reagent on any basidiome surfaces. Odor and taste indistinct.

Microscopic characteristics. Basidiospores highly variable in size and shape within a basidiome, subglobose to deformed, with an apiculum, hyaline, inamyloid, bearing an oil droplet, 14–20 (22) × (12) 12.5–17.5 (19) µm, Q ratio = 1.05–1.25; mature spores thick-walled (1–2 µm thick) and distinctly ornamented on the inner wall (crassospores), ornamentation consisting of small irregular cavities (0.5 µm diam.) offering a false verrucose appearance that is often obscured by the oil droplet, forming a meshed reticulum of small irregular pits (Moreno et al. (2012) illustrate similar spore ornamentation in their description of *Choiromyces magnusii* (Mattir.) Paol.), inner wall covered by an adpressed outer layer (ectosporium) that eventually collapse into the cavities; in immature specimens, spores rare, with a thin and smooth wall (<1 µm). Basidia clavate-pedicellate, 30–40 x 10–12 µm from base to bottom of sterigmata, 1–3 spored (4-spored basidia not observed), soon collapsing after spore release and therefore challenging to observe in mature basidiomes, ornamented basidia walls (crassobasidia) not observed; sterigmata cylindrical, 7.5–12 µm long. Subhymenial trama consisting of elongated, tightly interwoven hyphae, horizontally distributed, hyaline (lighter than the hymenium), 30–40 µm thick; immature specimens with oil droplets abundantly distributed in the hymenial and subhymenial trama, and inside basidia; peridium composed of large, irregularly polyhedral cells, 20–50 µm long, with thin, yellowish to green-brown walls; clamp connections infrequent but visible in all trama (hymenium, pileus, stipe).

Etymology. This species is named after Dr. Eduardo Nouhra to honour his important and lasting contributions to the study of hypogeous fungi of Patagonia.

Habitat and distribution. In deep soil under *Nothofagus antarctica* in northern Patagonia; associated with areas of animal digging.

Additional specimens examined. Argentina, Río Negro, Nahuel Huapi National Park, Los Rápidos: 8 May 2015, coll. R. Healy MES1126 (FLAS–F–59758); 11 May 2015, coll. L. Fernandez MES1213 (FLAS–F–59759).

Additional notes. At first glance the basidiomes of *A. nouhvae* are similar to an immature button of an epigeous *Amanita*. However, upon closer inspection, the loculate gleba makes it clear that it is a mature, sequestrate basidiome. Thus far this species is known only from a single location in Nahuel Huapi National Park in northern Patagonia. It is possible that this is a rare or narrowly endemic species but it may also be easily overlooked as a button of an epigeous *Amanita* species.

Amanita nouhvae is distinct from any other *Amanita* species due to a combination of the hypogeous, sequestrate habit and the large, thick-walled and ornamented basidiospores. The epigeous sister taxon *A. morenoi* (Fig. 1) has similar basidiospore ornamentation but with a more regularly and evenly distributed pattern on the spore surface. *Amanita nouhvae* differs from the two other known hypogeous species (*A. grandispora* and *A. oleosa*) by its distinct ornamented spores. In addition, spores in both *A. grandispora* and *A. oleosa* are smaller ($13\text{--}17 \times 9.5\text{--}11.5 \mu\text{m}$ and $12.5\text{--}15 \times 9\text{--}11 \mu\text{m}$, respectively) and narrower (Q ratio > 1.30) (Bougher and Lebel 2002). Both species occur in Australia in association with *Eucalyptus* and *A. oleosa* is notably different in having locules in the gleba that are filled with a clear liquid.

4. Discussion

4.1 Southern temperate clade within subgenus *Amanita*

Based on our multi-gene phylogeny, the new species *A. nouhvae* clusters with other epigeous and sequestrate species from Australia and southern South America in a highly supported Southern temperate clade (Fig. 1). With the addition of *A. nouhvae* this clade includes five species of sequestrate Australian taxa; the secotioid species *A. grandis*, *A. inculta*, and *A. pseudoinculta* (with stipitate and partially emergent basidiomes), as well as the hypogeous, fully sequestrate species *A. grandispora* and *A. oleosa* (with astipitate and belowground basidiomes). Four additional epigeous species belong to this clade; *A. merxmuelleri* and *A. morenoi* are from South America whereas *A. umbrinella* and *A. murinoflammeum* are from Australia.

Our time-of-divergence analysis suggests that the tMRCA of the southern temperate is found most likely within the Eocene (Mean=34.5 Ma, Fig. 2). This timeline potentially fits with paleogeographic reconstructions of the final break-up of Gondwana suggesting that complete isolation between Australia and South America occurred around 35 Ma (Knapp et al. 2005; Sanmartin and Ronquist 2004). The initiation of the Antarctic glaciation around that time (DeConto and Pollard 2003) reinforced the termination of biotic exchanges between the two regions. In addition, divergence within the southern temperate clade probably occurred as a result of continental drift and the gradual isolation of both Australia and southern South America from the Antarctic Peninsula in the Eocene (50–35 Ma) and the Oligocene (ca. 30 Ma) respectively, as also documented for other organisms (Sanmartin and Ronquist 2004).

Hosaka et al. (2008) found similar results in one *Hysterangium* lineage (clade II), in which a potential vicariant event was associated with the separation of Australia and

southern South America from a common ancestor distributed across these landmasses. In an alternative scenario, Skrede et al. (2011) found two distinct clades of *Austropaxillus*, one from South America and another from Australasia. They estimated the divergence time of these two clades at ca. 22 Ma, suggesting that dispersal rather than vicariance may have occurred. Wilson et al. (2017) attributed the current worldwide distribution of *Laccaria* to dispersal events from an Australasia ancestor that originated ca. 64 Ma. They estimated the divergence time of two southern South American *Laccaria* species with their Australian sister lineage at 20.49 Ma, but additional sampling from this region may reveal the presence of Southern Gondwanan vicariance from their Australian ancestor. Indeed, long-distance intercontinental dispersal over geological time-scales has been reported rather episodically (Moncalvo and Buchanan 2008; Moyersoen et al. 2003) and the combination of ancient vicariant patterns with more recent dispersal events is a potential scenario in many groups (Sanmartin and Ronquist 2004), including in fungi. For example, a mosaic of vicariant and dispersal events apparently contributed to the historical biogeography of the family Inocybaceae (Matheny et al. 2009). In the case of the *Nothofagaceae*-parasitic genus *Cyttaria*, isolation by vicariance was detected across South American and Australian species in addition to a more recent long distance dispersal event from Australia to New Zealand (Peterson et al. 2010). This pattern matches almost perfectly with the phylogeographic history of the host trees (Knapp et al. 2005). Our incomplete sampling does not comprise many *Amanita* species from New Zealand and only a handful of taxa from Australia. Therefore, we cannot exclude the existence of recent long-distance dispersal events within subgenus *Amanita* or the

Southern temperate clade. Based on our results, however, the ancestor of this southern temperate clade is very likely to be of Southern Gondwanan origin.

Previous biogeographic studies based on dated molecular phylogenies in *Amanita* (and other ECM groups) have shown a pattern where ancient lineages of Paleotropical origins (e.g. Africa or Asia) subsequently disperse through land-bridges, most of them in the Northern Hemisphere (Matheny et al. 2009; Sánchez-Ramírez et al. 2015b). This contrasts with our findings that members of the southern temperate clade are all from Austral regions and share a common ancestor of relatively ancient origin within the genus *Amanita*, potentially due to their long history of isolation. At first glance in our genus-level dated phylogeny (Fig. 2), the lack of sampling and/or diversity in southern South America is noticeable, particularly in comparison with the Australian taxa, which seem to be more commonly associated with Eurasian, African, and even North American lineages. Although more sampling of southern South American species is needed, their estimated diversity seems to be particularly low compared to other regions (Truong et al. 2017). Higher diversity in Australia compared to southern South America could be a result of higher dispersal from the Paleotropics during the Miocene (Sánchez-Ramírez et al. 2015b; Sanmartin and Ronquist 2004). In contrast, South America remained isolated until the Pliocene (ca. 2.5 Ma) when the Panamá Isthmus closed. Moreover, Wilson et al. (2017) estimated that the rate of diversification of Northern Hemisphere *Laccaria* lineages was more than twice that of the Southern Hemisphere. They correlated it to the higher availability of new niches by mean of switching to different ECM host-plant families. Similarly, higher diversification rates in *Amanita* sect. *Caesareae* from temperate regions compared to the tropics was associated with higher number of

ecological niches for ECM fungi, i.e. more complex soil horizons and more diverse ECM hosts (Sánchez-Ramírez et al. 2015a). Niche partitioning such as specificity of host-plant, soil horizons, and/or microclimate, may indeed promote sympatric speciation, as demonstrated in the *A. muscaria* species complex (Geml et al. 2014). In southern South America, the low diversity of *Amanita* species could potentially be explained by limited host availability, as well as reduced dispersal due to isolation and geographic barriers (Tulloss, 2005; Sánchez-Ramírez et al. 2015b).

One notable difference between South American and Australian taxa from the Southern temperate clade is that Australian species have been documented in habitats with *Eucalyptus* or *Acacia* as the primary host trees (Justo et al. 2010, www.amanitaceae.org), whereas southern South American species are associated with *Nothofagaceae*. Sequestrate species are easily overlooked, therefore we suspect that there may be additional Australasian species associated with *Nothofagaceae* that remain unknown or undescribed. In addition, the recent discovery of an *Eucalyptus* fossil in Argentina from the early Eocene (ca. 51.9 Ma) (Gandolfo et al. 2011) falls before our tMRCA estimates of the southern temperate *Amanita* clade. Because of this, we cannot rule out the possibility of a *Eucalyptus*-associated ECM fungal ancestor in southern South America followed by a host switch to *Nothofagus*. Clearly more sampling of Southern Hemisphere *Amanita* species, particularly sequestrate taxa, is needed to resolve broader evolutionary patterns in this group.

4.2 Evolution of the sequestrate habit within *Amanita*

Compelling evidence from molecular studies suggests that sequestrate taxa evolved repeatedly from non-sequestrate ancestors within the Agaricomycetes (Hibbett 2007), e.g. in the Boletales (Binder and Hibbett 2006), the Russulales (Eberhardt and Verbeken 2004), or the Agaricales (Peintner et al. 2001). Once this trait is stable in a lineage, subsequent species radiations seem to occur only into sequestrate species, with no ‘reversed evolution’ into non-sequestrate species (Peintner et al. 2001). As previously reported in Justo et al. (2010), our results corroborate the findings that sequestrate species form polyphyletic lineages within the genus *Amanita* (Fig. 1). Ancestral state reconstruction of the sequestrate habit within our phylogenetic tree (data not shown) indicates that this trait has evolved repeatedly and relatively recently from non-sequestrate ancestors within the genus *Amanita*. For a genus that otherwise includes mostly epigeous species, the southern temperate clade comprises the highest number of sequestrate species to date, with five Australian taxa in addition to our newly described species *A. nouhrae*. Only three additional sequestrate *Amanita* are known from other lineages. The diversity of sequestrate *Amanita* species is still underexplored and our dataset probably underrepresents their true diversity. Nevertheless, based on our current knowledge, the southern temperate clade seems particularly prone to the evolution of the sequestrate habit.

All sequestrate *Amanita* species share similar macro- and micro-morphological features. They can only be distinguished based on a combination of characters, including stipe development (fully hypogeous or not), spore size and shape, the presence of clamp connections, and oil droplets visible in spores and reproductive tissues (Justo et al. 2010). Along with *A. nouhrae*, sequestrate species from the Southern temperate clade all share

abundant clamp connections and oil droplets that are apparently rare or absent in other sequestrate *Amanita* lineages (e.g. *A. arenaria*, *A. torrendii* and '*Amarrendia* sp.' H0909, a phylogenetically unique sequestrate species from Australia that remains undescribed) (Justo et al. 2010). Mature spores in *A. nouhvae* are unusual within *Amanita* because they are ornamented and thick-walled. Interestingly, in South America, both *A. nouhvae* (hypogeous) and *A. morenoi* (epigeous) have similar ornamented basidiospores, while their closest relative *A. merxmuelleri* has spores with thin and smooth walls. These ornamented 'crassospores' were reported inconstantly in other epigeous *Amanita* species and were believed to be an anomalous morph resulting from pathogenic infection (Kotilová-Kubičková and Pouzar 1988; Tulloss and Halling 1997). We found that this character was consistent in all our collections of both *A. morenoi* and *A. nouhvae*, as well as in the type specimen of *A. morenoi*. These two species clustered as highly supported sister lineages within our phylogeny (Fig. 1), suggesting that this character is likely genetically determined. Ornamented spores occur rarely within *Amanita* but are a common feature in many hypogeous fungi regardless of their taxonomic affinity (Trappe et al. 2009). Ornamented, thick-walled spores are believed to be advantageous against the chemical and physical hazard of passing through animal digestive tracts (Fogel and Trappe 1978) or against the harsh climatic conditions in which these sequestrate taxa evolved (Thiers 1984). The examination of further material from various South American *Amanita* species is needed to fully understand the origin and evolution of this character. Nevertheless, the occurrence of a potential epigeous ancestor with ornamented spores may have favoured the evolution of the sequestrate habit in South America. It is notable that immature spores in *A. nouhvae* are similar to the spores described from sequestrate

Australian species. It is difficult to make conclusive assumptions about spore ornamentation because of the paucity of material from most of these sequestrate species. However, it would be interesting to study additional Australian material in the future to see if some of these species may have ornamented spores in very mature specimens.

Amanita nouhvae is found in a cool, moist continental climate in southern South America. In Australia, *A. grandispora* is known from temperate sites in Victoria and Tasmania that are similar to the site where *A. nouhvae* was collected. However, the remaining Australian sequestrate *Amanita* species are typically found in seasonally arid Mediterranean habitats and Justo et al. (2010) suggested that the evolution of sequestrate forms in *Amanita* correlated with aridification processes. Indeed (Sheedy et al. 2016) found an increased rate of sequestration in various Agaricomycetes groups from Australia during a period of aridification after the separation of Australia from Antarctica (ca. 30–15 Ma). Climatic models indicate a similar trend from a moist-warm climate to cooler temperatures and dryer seasons in southern South America after the separation with Antarctica during the Oligocene (ca. 30 Ma), favoring the establishment of steppe and arid vegetation that extended in the entire Patagonian region in the late Miocene (ca. 15 Ma) (Ortiz-Jaureguizar and Cladera 2006). Our data suggest that the divergence of the sequestrate species *A. nouhvae* from an epigeous ancestor is relatively recent (ca. 10-20 Ma, Fig. 2) and corresponds to this period. In addition, *A. nouhvae* is thus far known only from a forest of *Nothofagus antarctica*, a species that typically occurs in the transition with steppe vegetation on the Eastern side of the Andes. The paucity of data from the region does not allow us to make definite conclusions, but it would be interesting to investigate further whether past climatic oscillations toward a dryer climate may have

avored the evolution of sequestrate species in other fungal groups that occur in southern South America, similarly to the pattern observed in Australia.

5. Conclusions

The diversity of ECM fungi in the Southern Hemisphere remains significantly understudied as compared to other regions and future work is likely to reveal many novel taxa (Tedersoo and Smith 2013; Truong et al. 2017). The ‘southern Gondwana’ connection between southern South America and Australia has been demonstrated in various groups (Sanmartin and Ronquist 2004), suggesting a history of biotic exchange that should also be reflected in fungi (Tedersoo et al. 2014; Tedersoo et al. 2010). Here we demonstrated that the new sequestrate species *A. nouhrae* from northern Patagonia is genetically related to sequestrate taxa from Australia. We also estimated the tMRCA of this lineage near the Eocene/Oligocene boundary (ca. 35 Ma $\pm$ 10 Ma), a finding that is congruent with a ‘southern Gondwanan’ origin. Tracing such a relatively ancient connection between southern South America and Australia has been done in a few other ECM fungi so far (e.g. Hosaka et al. 2008; Matheny et al. 2009) and we expect that this pattern is much more common in *Nothofagus*-associated fungi than what has been previously documented.

6. Acknowledgments

This work was supported by the US National Science Foundation grant DEB 1354802 (to MES), an Advanced Postdoc Mobility Fellowship from the Swiss National Science Foundation (to CT), a Friends of the Farlow Fellowship from Harvard University

(to FK), and the UF Institute for Food and Agricultural Sciences IFAS (to MES). Dr. Eduardo Nouhra (University of Cordoba) helped to obtain permits and the Administración de Parques Nacionales of Argentina kindly authorized our collecting expeditions in Parque Nacional Nahuel Huapi and Parque Nacional Lanín under Proyecto 2016/720 (to E. Nouhra). In Tierra del Fuego, Dr. Alicia Moretto provided guidance in obtaining research permits and the Secretaría de Desarrollo Sustentable y Ambiente graciously authorized the collection of specimens under Resolución S.D.S. y A. NRO. 0218/2015 (to CT). The Chilean Corporación Nacional Forestal (Gerencia de Areas Silvestres Protegidas) provided permission to collect fungi in Puyehue National Park under permit No. 014/2014 (to MES). Eduardo Nouhra, Giuliana Furci, and Natalia Fernández offered critical guidance during fieldwork, while Lisandro Fernández, Rosanne Healy, Brandon P. Matheny, Alija E. Mujic, Hugo Pereyra and Pablo Sandoval provided additional collections of fresh specimens. Several members of the research team also graciously provided photographs. Dr. Alfredo Justo and Dr. David Hibbett (Clark University) kindly provided DNA extracts from Australian species of *Amanita*. We thank Susana Pereira and Andrea Romero (BAFC herbarium) for their assistance and curators from the herbaria of CORD, NYBG, and PERTH for generously sending specimens on loan. Laszlo Nagy and two anonymous reviewers provided valuable comments to improve the manuscript before publication.

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Figure 1. Bayesian Inference (BI) majority rule consensus tree from a concatenated dataset of nuclear 18S and 28S ribosomal DNA as well as mitochondrial 16S and 26S DNA. Posterior probabilities (PP) and bootstrap support (BS) from the maximum likelihood (ML) tree are indicated with thick branches (black for $PP \geq 0.95$ and $BS \geq 70$, grey for $PP \geq 0.90$ and $BS \geq 60$, black dashed line for $PP \geq 0.95$ only, grey dashed line for $BS \geq 70$ only). Sequestrate *Amanita* species are in bold. South American species are highlighted in blue and Australian species in green. The genus *Amanita* is subdivided into two highly supported clades corresponding to subgenera *Amanita* and *Lepidella*, while non-ectomycorrhizal species form paraphyletic sister lineages. The new species *A. nouhrae* clusters with other sequestrate species from Australia in a highly supported southern temperate clade (highlighted in grey) that is composed of both sequestrate and epigeous agaricoid taxa.

Figure 2. Time-calibrated maximum-clade-credibility tree of the genus *Amanita*. Only nodes with posterior probability ≥ 0.9 are annotated with horizontal bars representing 95% highest posterior densities (HPD). Individual species are color-coded by the region

where they were found: North America (orange), Eurasia (purple), Africa (red), northern South America (yellow), southern South America (blue), and Australia (green). Names are only shown for Australian and southern South American taxa. Nodes that represent divergence events resulting in an Australian or southern South American lineage are highlighted with a black rhombus. Geological epochs are marked at the bottom. Those abbreviated represent the Paleogene (Pg), the Oligocene (O), the Pliocene (Pl), and the Pleistocene (P), respectively.

Figure 3. *Amanita morenoi*. A. Striated pileus with scaly patches of universal veil; white lamellae with approximately one lamellula in between each lamella (MES1774, Bar = 1 cm). B. Lightly fibrillose stipe, whitish towards the apex, with concolorous striated annulus; bulbous base encircled by circumsessile volva (MES1774, Bar 1 cm). C. Smooth and white context trama, becoming hollow towards the base of the stipe, slightly darker pileipellis evident in cross section (MES1301, Bar 2 cm). D. Globose basidiospores ornamented on the inner wall with small, evenly distributed, hemispherical pits (MES1624, Bar 5 μ m). E. Hymenium showing clavate basidia with short sterigmata and dense subhymenium consisting of tightly interwoven hyphae (MES1624, Bar 10 μ m).

Figure 4. Macroscopic characteristics of *Amanita nouhrae*. A. Holotype collection (MES1921, Bar 1 cm). B. Mature basidiomes showing the thin peridium, loculate gleba, and reduced, conical to subglobose sterile base (MES1921, Bar 5 mm). C. Immature basidiome with whitish peridium and gleba; sterile base distinctive and subglobose

(MES1213, Bar 5 mm). D. Close-up photograph showing the gleba with irregular, angular to circular locules; peridium thin and almost undistinguishable, with universal veil remnants forming patches on upper surface (MES1921, Bar 0.5 mm).

Figure 5. Microscopic characteristics of *Amanita nouhrae*. A. Locule in section showing the hymenial and subhymenial trama (MES1921, Bar 50 μm). B. Hymenium with abundant basidiospores, accumulating from basidia collapsing at maturity; subhymenium dense, consisting of tightly interwoven hyphae (MES1921, Bar 20 μm). C. Single-spored basidia that support thick-walled and distinctly ornamented basidiospores. D. Immature basidium with three young basidiospores (MES1213, Bar=10 μm). E. Hymenial hypha with septum and clamp connection (MES1213, Bar 20 μm). F. Thick-walled and distinctly ornamented mature basidiospores; ornamentation consisting of irregular cavities offering a false verrucose appearance obscured by the pronounced oil droplet (MES1921, Bar 5 μm). G. Immature basidiospores with a thin, smooth wall and distinct oil droplet (MES1213, Bar 5 μm). H. Peridium composed of large, irregularly packed polyhedral cells (MES1921, Bar 20 μm).

Figure 1

Phylogenetic tree of *Amanita* species, showing relationships and time scale (Ma).

Species listed (from top to bottom):

- Amanita* sp.-AUS02 | Australia
- Amanita* sp.-AUS03 | Australia
- Amanita hemibapha* | Australia
- Amanita egregia* | Australia
- Amanita roseolamellata* | Australia
- Amanita* sp. *punctata* | Australia
- Amanita* sp.-H909 | Australia
- Amanita* sp. *myrmeciae* | Australia
- Amanita* sp.-UNSW 03/29 | Australia
- Amanita diemii* | Argentina
- Amanita grandis* | Australia
- Amanita grandispora* | Australia
- Amanita oleosa* | Australia
- Amanita morenoi* | Argentina
- Amanita nouhrai* | Argentina
- Amanita merxmuelleri* | Argentina
- Amanita pseudoinculta* | Australia
- Amanita* sp.-AUS04 | Australia
- Amanita inculta* | Australia
- Amanita murinoflammeum* | Australia
- Amanita umbrinella* | Australia
- Amanita luteofusca* | Australia
- Amanita luteolovelata* | Australia
- Amanita arenaria* | Australia
- Amanita subalbida* | Australia
- Amanita* sp.-10-ZLY-2014 | Australia
- Amanita clelandii* | Australia
- Amanita pyramidifera* | Australia
- Amanita effusa* | Australia
- Amanita conicobulbosa* | Australia
- Amanita ochrophylla* | Australia
- Amanita sublutea* | Australia
- Amanita* sp.-AUS01 | Australia
- Amanita farinacea* | Australia

Time scale (Ma): 100, 75, 50, 25, 0.

Geological periods: Cretaceous, Pg, Eocene, O, Miocene, P, P.

Subgenus labels: subgen. *Amanita*, subgen. *Lepidella*.

Figure 3

Figure 4

Figure 5