Letters

How to know the fungi: combining field inventories and DNA-barcoding to document fungal diversity

How many fungi?

The fungi kingdom is among the most diverse eukaryotic lineages on Earth with estimates of several million extant species (O'Brien *et al.*, 2005; Blackwell, 2011; Taylor *et al.*, 2014). Fungi play critical roles in carbon and nutrient cycling of terrestrial and aquatic ecosystems, and they are important pathogens and mutualists (Read & Perez-Moreno, 2003; Taylor *et al.*, 2012; Grossart *et al.*, 2016). More than 80% of plant species form symbioses with fungi and these symbioses have been crucial to the colonization of terrestrial ecosystems (Field *et al.*, 2015a; Selosse *et al.*, 2015). Despite their impacts on primary ecosystem functions, assessments of fungal biodiversity estimate that only *c.* 10% of fungal species have been described (Bass & Richards, 2011; Hibbett *et al.*, 2011).

Traditionally, specimen-based taxonomic studies have been the only way to discover new species. Because most fungi have microscopic life-stages and convergent morphological features (Rivas-Plata & Lumbsch, 2011; Wynns, 2015), many fungal groups remain severely undersampled. DNA-barcoding and high-throughput sequencing methods have provided a new framework for studying fungal biodiversity (Fierer *et al.*, 2012; Schoch *et al.*, 2012; Myrold *et al.*, 2014), and diversity estimates based on environmental sequences have increased exponentially. Although these 'sequence-based classification and identification' methods are a powerful means to rapidly detect hidden diversity, careful interpretation of these data is needed to make accurate inferences (Kõljalg *et al.*, 2013; Lindahl *et al.*, 2013; Nguyen *et al.*, 2015; Hibbett *et al.*, 2016). In particular, many environmental sequences cannot be associated with a known fungal species or lineage. This remains a major challenge to decipher fungal community composition and understand ecological roles of fungi in leaf litter, soil, or inside plants (Yahr *et al.*, 2016). In some cases, these fungi are truly undescribed and their ecological roles are unknown but in other cases they represent described taxa for which no sequence is available (Nagy *et al.*, 2011; Nilsson *et al.*, 2016). DNA barcoding of herbarium specimens and culture collections is extremely valuable to link unidentified sequences to known taxa (e.g. Brock *et al.*, 2009; Nagy *et al.*, 2011; Osmundson *et al.*, 2013; Garnica *et al.*, 2016). DNA sequences have been generated from fungal type specimens > 200 years old (Larsson & Jacobsson, 2004), but in many cases obtaining sequences from historical material is challenging (Dentinger *et al.*, 2010).

Today's threats to biodiversity from habitat loss and climate change are occurring at an unprecedented scale, and it is possible that many species may become extinct before they have been discovered (Costello *et al.*, 2013; Monastersky, 2014). In the need to describe and protect as many species as possible we addressed the following questions: what are the best methods to rapidly document fungal biodiversity? Are traditional, specimen-based approaches still useful?

Exploring the unknown

The Southern Hemisphere harbors many unique fungal lineages that are absent from the Northern Hemisphere (Tedersoo & Smith, 2013; Tedersoo *et al.*, 2014). In southern South America, recent studies based on environmental sequences have detected several previously unknown fungal lineages, thereby demonstrating that fungal diversity is probably much higher than presently known (Nouhra *et al.*, 2013; Geml *et al.*, 2014; Roy *et al.*, 2017).

As part of a project investigating ectomycorrhizal (ECM) fungi of southern South America, our team collected 1430 fungal fruiting bodies during four collecting expeditions, equaling *c.* 50 d with 3–4 collectors per day (Supporting Information Methods S1). We primarily collected ECM fungi in temperate forests dominated by Nothofagaceae but also opportunistically collected nonECM fungi. Vouchered specimens were photographed and dried for future study. Internal transcribed spacer rDNA sequences (ITS, e.g. ITS1-5.8S-ITS2) were obtained from a representative selection of 957 specimens using the Extract-N-Amp Plant kit (Sigma-Aldrich) for rapid DNA extraction and amplification. ITS sequences were clustered into operational taxonomic units (OTUs) at 97–99% similarity against the UNITE 'species hypothesis' dynamic database (Abarenkov *et al.*, 2010a; Kõljalg *et al.*, 2013) using QIIME 1.9.1 (Caporaso *et al.*, 2010). Sequences that did not correspond to an existing 'species hypothesis' in the reference database were subsequently clustered *de novo* at 97% similarity (Methods S1). One representative sequence per OTU was subsequently compared to UNITE+INSD (UNITE and the International Nucleotide Sequence Databases) using MEGABLAST on the PLUTOF workbench (Abarenkov *et al.*, 2010b).

We generated 439 OTUs (Table S1), of which 308 (*c.* 70%) did not match the UNITE dynamic database at 97–99% similarity, and thus did not correspond to any of the 'species hypothesis' currently in UNITE. The efforts of our research group were modest when compared to the high volume and novelty of the data. Although most of these ITS sequences were generated by our research team over a two-year period, they correspond to *c.* 1.5% of the total diversity in UNITE (19 698 representative sequences in v.7.1, August 2016). Comparisons between our dataset and the full

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UNITE database (which also includes singleton sequences) did not alter the number of new OTUs we detected. For comparison, in the one-year period before this study (between August 2015 and August 2016) the global efforts of the scientific community contributed only 360 unique 'species hypotheses' to the UNITE database.

Working with fresh specimens was extremely efficient. The Extract-N-Amp method rapidly generated ITS sequences with a success rate of *c.* 80% (including extraction, amplification and sequencing). The success rate improved to *c.* 90% when partial ITS sequences (e.g. ITS1 + 5.8S only) were included. Success rate of DNA sequencing varies among fungal groups, according to the age of specimens, and based on how they were preserved. Sequencing from type specimens and important historical collections remains the gold standard to link DNA sequences to species names (e.g. Liimatainen *et al.*, 2014; Sánchez-García *et al.*, 2014; Schoch *et al.*, 2014). However, generating sequence data from historical vouchers may be challenging in some groups (25–50% success rate for specimens > 10 years old according to Dentinger *et al.*, 2010). Working with old herbarium specimens is also more time-consuming and sensitive to contamination. It is thus more expensive because it requires more rigorous DNA extraction and purification procedures as well as PCR troubleshooting (Brock *et al.*, 2009; Osmundson *et al.*, 2013). For generating high throughput data, fresh or recently collected specimens are clearly advantageous when compared to preserved herbarium materials.

Morphological examination of specimens combined with BLAST searches helped identify most vouchers to genus level (Table S1). Thirty-two OTUs could only be identified to family (14), order (14), or class (4), mostly because they belong to groups that require extensive systematic revision (e.g. Helotiales). Due to our focus on Nothofagaceae-dominated forests, we collected mostly ECM species (66%), but also many saprobes (31%), and fungi for which the trophic mode is unknown (3%). Agaricales (Basidiomycota) were diverse, abundant, easily sampled and therefore conspicuous in our dataset (Fig. 1c). We also found a large number of Pezizales and Helotiales (Ascomycota), many of which had low similarity to any known sequences. Within Agaricales, *Cortinarius* was the most diverse genus in both species richness and abundance, constituting *c.* 33% of all OTUs (Fig. 1c). The high diversity of *Cortinarius* lineages corroborates previous studies carried out in other southern temperate forests with Nothofagaceae (Tedersoo *et al.*, 2008; Dickie *et al.*, 2009; Nouhra *et al.*, 2013; Fernandez *et al.*, 2015; Horton *et al.*, 2017). In addition, our dataset revealed surprisingly high species diversity in some fungal lineages where only a handful of species have been described from South America (e.g. /austropaxillus, /descolea – Peintner *et al.*, 2001; Skrede *et al.*, 2011). Similar to previous results based on ECM root tips (Nouhra *et al.*, 2013), we detected relatively low diversity in several ECM lineages that are hyperdiverse in other regions of the world (e.g. /amanita, /boletus, /russula-lactarius). We also found several Northern Hemisphere exotic species in South American Nothofagaceae forests, including *Inocybe ochroalba* (MES1236), *Hebeloma mesophaeum* (MES1358) and *Amanita muscaria* (MES1647). All three of these taxa putatively fall into the category of 'introduced species that spread to local hosts' as outlined by Vellinga *et al.* (2009). Evidence of a native South American *Inocybe* species (MES1895) fruiting in pure *Pseudotsuga* plantations suggests that this species may be a potential invasive species in the Northern Hemisphere. We are actively engaged in taxonomic work to compare our collections with described species and formally describe novel taxa (Kneal & Smith, 2015; Trierveiler-Pereira *et al.*, 2015; Kumar *et al.*, 2017).

Approximately one quarter of our OTUs (Fig. 1a, 23–32% according to percent BLAST similarity) matched environmental sequences in UNITE+INSD with no available voucher specimen (Table S1; note that in some cases the closest BLAST match was a GenBank sequence with no corresponding UNITE 'species hypothesis'). In addition, 43 OTUs were < 90% similar to any ITS sequence in UNITE+INSD. It is likely that our assessments may still underestimate the total number of species in some groups

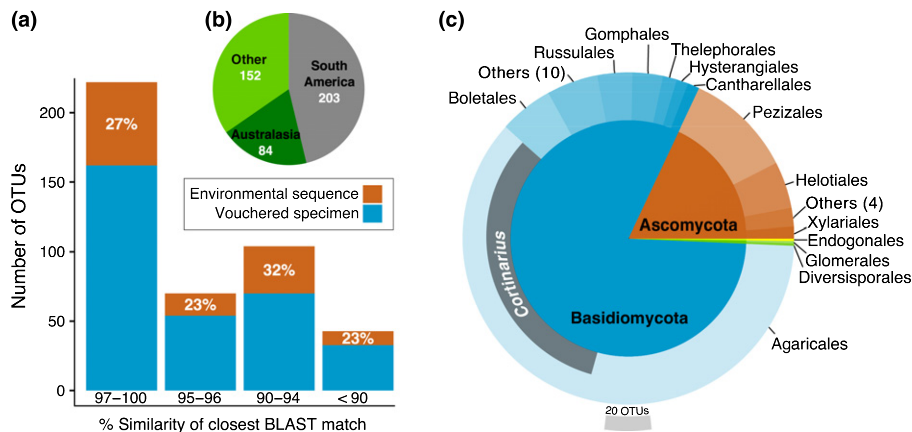

Fig. 1 BLAST sequence similarity of the operational taxonomic units (OTUs) generated by our collections with the UNITE+INSD sequence database (a): OTUs with a closest match to a vouchered specimen are in blue whereas those with a closest match to an environmental sequence are in orange. Geographic distribution of the closest BLAST matches from the UNITE+INSD sequence database (b): most OTUs had a closest BLAST match from South America (grey) or Australasia (dark green), with fewer BLAST matches from other regions (light green); number of corresponding OTUs in white. Phylogenetic affiliations of the OTUs generated in this study depicted by taxonomic order (c): Basidiomycota (blue colors) were highly abundant whereas Ascomycota (orange colors) and Mucoromycota (green and yellow) were less frequent. The species-rich order Agaricales includes many genera but was dominated by the genus *Cortinarius* (grey), which comprised 147 OTUs and represented 33% of the diversity in our dataset.

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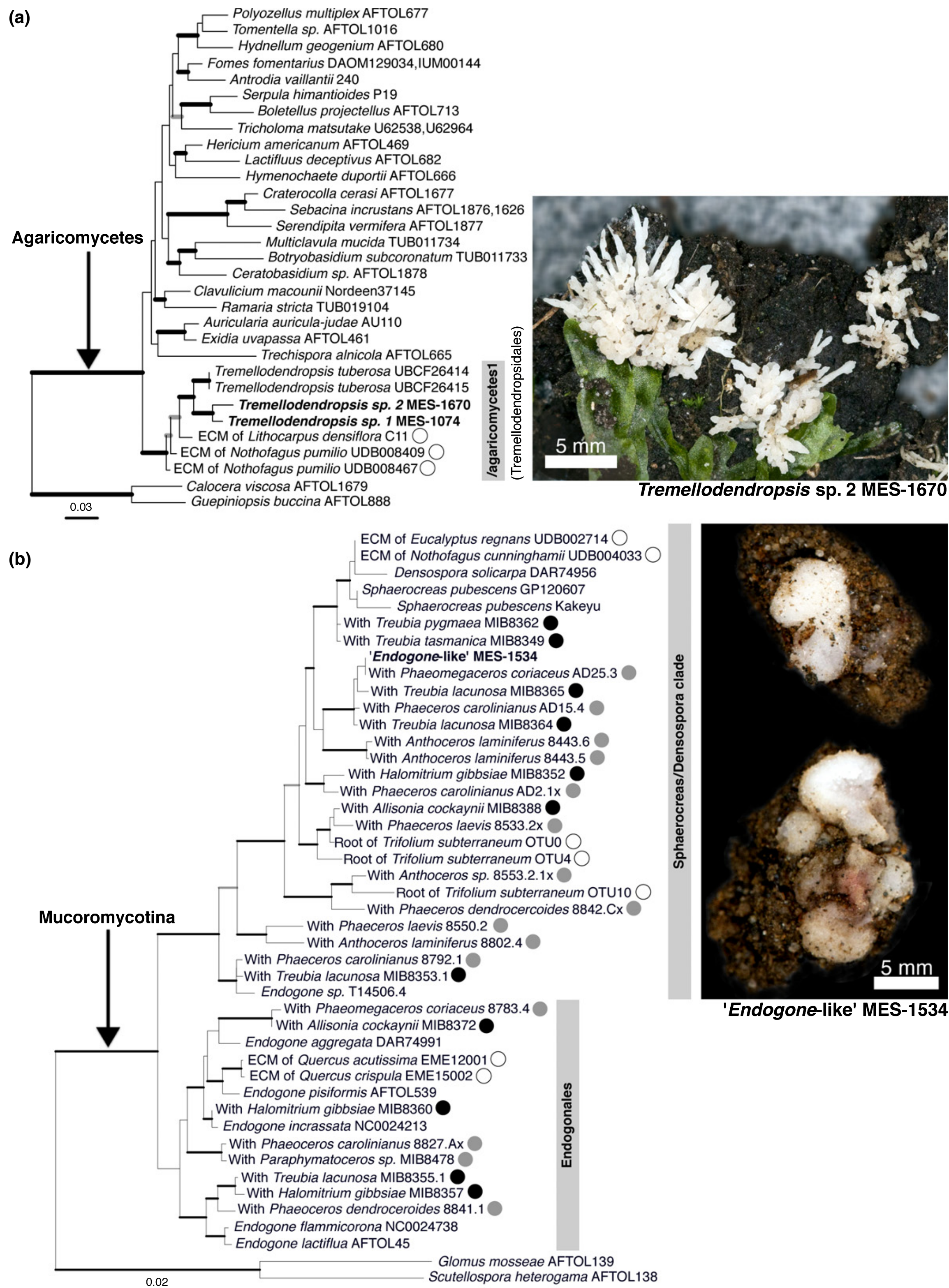

Fig. 2 Maximum likelihood phylogenies with thick bars indicating bootstrap support (BS) ≥ 70 and corresponding posterior probabilities (PP) ≥ 0.95 from Bayesian analysis (in black); BS ≥ 60 and PP ≥ 0.90 (in grey). Phylogenetic placement of novel plant-associated fungi from southern South America based on 18S and 28S rDNA showing (a) the phylogenetic placement of two novel *Tremellodendropsis* species (in bold) in the order Tremellodendropsidales (/agaricomycetes1 in Tedersoo & Smith, 2013); and (b) the phylogenetic placement of an '*Endogone*-like' specimen (in bold) in the *Sphaerocreas-Densospora* clade within the Mucoromycotina. Environmental sequences of fungal symbionts were detected from liverworts (black circles), hornworts (grey circles), ectomycorrhizal or 'arbuscular-like' roots of vascular plants (white circles).

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for which ITS is very similar among divergent species, for example many *Cortinarius* species (Ryberg, 2015; Garnica *et al.*, 2016). Most OTUs (46%) matched sequences originating from South America, but *c.* 20% had a closest match to a sequence from Australasia (Fig. 1b), highlighting both the shortage of sequences from South America and the historical biogeographic connection of these two regions. This biogeographic pattern was particularly striking within some fungal lineages. For example, we found the first evidence of truffle-like species of *Ruhlandiella*, *Amylascus* and *Gymnohydnotrya* (Pezizales) in South America, despite the fact that described members of these genera are so far known only from Australasia (Table S1).

Examples of novel plant-symbiotic fungi

Among the 309 unique OTUs detected, we identified two examples of distinctive plant-associated fungi that illustrate the exciting data generated from our collections. In the first case, we collected two small coralloid fungal specimens whose ITS sequences (KY462416, KY462417) were *c.* 80% similar to each other. The closest ITS BLAST matches for these two OTUs were sequences from ECM root tips of *Nothofagaceae* that corresponded to an anomalous ECM lineage identified by Tedersoo & Smith (2013) as /agaricomycetes1. We subsequently sequenced 18S and 28S rDNA (Methods S1) and found that these specimens are phylogenetically affiliated with *Tremellodendropsis tuberosa* (Fig. 2a). Berbee *et al.* (2016) recently showed that *T. tuberosa* belongs to a unique Agaricomycete lineage in the order Tremellodendropsidales. Our rDNA phylogenies suggest that Tremellodendropsidales includes diverse ECM fungi that associate with a wide range of angiosperms (including *Fagus*, *Eucalyptus*, *Lithocarpus*, and *Nothofagaceae*) across the globe (Figs 2a, S1; Table S2). Together with *T. tuberosa*, our vouchered specimens will provide new insights into the morphology and ecology of this group. They will also allow the description of new species and provide fresh material for the phylogenomic placement of Tremellodendropsidales within the Agaricomycetes.

A second case of unique plant-associated fungi was an *Endogone*-like specimen (KY462475) whose closest ITS BLAST matches are fungal symbionts of nonvascular plants (liverworts and hornworts – Bidartondo *et al.*, 2011; Desiro *et al.*, 2013; Yamamoto *et al.*, 2015) with no corresponding fungal specimens. We generated 18S and 28S rDNA sequences and placed this OTU in the Mucoromycotina (Fig. 2b; Table S2). Our specimen is nested in a large clade composed of multiple lineages, including specimens of *Densospora solicarpa* from Australia and *Sphaerocreas pubescens* from Japan. The *Sphaerocreas-Densospora* clade is sister to the Endogonales, which comprises *Endogone* species and additional sequences of early-diverging plant symbionts. Some members of the Endogonales and the *Sphaerocreas-Densospora* clade are associated with early-diverging plants (Bidartondo *et al.*, 2011; Desiro *et al.*, 2013), whereas others are ectomycorrhizal (Yamamoto *et al.*, 2015, 2016) or 'arbuscular-like' symbionts of vascular plants (Orchard *et al.*, 2016). These fungi have recently been documented from many hosts, habitats, and geographic locations, suggesting that the diversity of species and trophic modes of Mucoromycotina is much higher than previously understood. Because of their inconspicuous habit and the difficulties with culturing and DNA sequencing (Berch & Fortin, 1983; Tedersoo *et al.*, 2016; Yamamoto *et al.*, 2016), these fungi remain poorly represented in sequence databases, culture collections and herbaria, despite the growing evidence that species of Mucoromycotina may have played critical roles in the early colonization of terrestrial habitats (Strullu-Derrien *et al.*, 2014; Field *et al.*, 2015b, 2016; Rimington *et al.*, 2015).

Back to the basics: the power of a collect-and-sequence approach

The examples mentioned earlier are among the most illustrative in our dataset but are by no means the only discoveries. They demonstrate that systematic collecting, documenting, and sequencing from fresh specimens in undersampled regions are efficient and viable methods to capture unknown fungal diversity and provide substantial improvements to public DNA repositories. This approach is particularly relevant to 'fill the gap' of knowledge from geographic regions where comparatively fewer collections exist (e.g. South America – Roy *et al.*, 2017) and this remains an efficient approach to obtain new fungal data at any site.

Although environmental sequencing can rapidly detect diversity and elucidate ecological patterns, these approaches depend on informative sequence databases for fungal identification (Costello *et al.*, 2013). Due to our current lack of knowledge, a large portion of environmental sequences cannot be identified at a meaningful taxonomic level (Nilsson *et al.*, 2016). There is currently a movement to identify and classify fungi known only from sequences (Hibbett *et al.*, 2016). Although we agree that it is critical to compile and validate high-quality environmental sequences, we nonetheless argue that 'traditional' methods should be considered irreplaceable and complementary to 'next-generation' approaches. Unfortunately, in the quest for cutting-edge science it is sometimes the case that 'traditional' methods are viewed negatively by funding agencies. We argue that, along with barcoding herbarium collections, high-throughput collect-and-sequence inventories are highly effective to document fungal diversity and are instrumental for future studies of plant–fungal symbioses. Herbarium vouchers provide much more than just DNA barcodes. Fresh well-documented specimens remain critical to reconstruct robust phylogenies, link sequence data to morphology, and supply ecological data on hosts and substrate associations (Peay, 2014). Specimens can also be used for stable isotopic analyses (Hobbie *et al.*, 2001) and genomic studies (Tedersoo *et al.*, 2016) in a way that environmental samples cannot. Given the increasing threats to biodiversity from habitat loss and climate change, responsibly collecting vouchered specimens with associated data and openly sharing these resources are more necessary today than ever before (Costello *et al.*, 2013; Rocha *et al.*, 2014).

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Author contributions

C.T., A.B.M. and R.H. generated the DNA sequences. C.T. and A.B.M. carried out the analyses. C.T. and M.E.S. wrote the manuscript and constructed the figures and tables. M.E.S. provided expertise at all stages of research. All co-authors (C.T., A.B.M., R.H., F.K., G.F., D.T., T.N., P.A.S-L., N.F., J.M.E., A.M., G.P., D.P., E.N., R.S., M.S-G., P.B.M., and M.E.S.) participated in the field collections.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Maximum likelihood phylogeny of Tremellodendropsidales fungi based on ITS ribosomal DNA.

Table S1 List of operational taxonomic units (OTUs) generated from this study

Table S2 List of species and samples included in the phylogenetic analyses

Methods S1 The full methods for specimen collection, DNA extraction and sequencing, and data analysis.

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