Mycorrhiza

Preliminary findings on identification of mycorrhizal fungi from diverse orchids in the Central Highlands of Madagascar, with emphasis on spontaneous seedlings

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	Madagascar. This study will help start to develop a programme for identifying fungal symbionts from this unique biodiversity hotspot which is undergoing rapid ecosystem damage and species loss. The diversity of culturable mycorrhizae, density and their distribution within the Itremo orchid hotspot areas will be discussed.

Preliminary findings on identification of mycorrhizal fungi from diverse orchids in the Central Highlands of Madagascar, with emphasis on spontaneous seedlings

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ABSTRACT

Orchid flora of Madagascar is one of most diverse with nearly 1,000 orchid taxa of which about 90% are endemic to this biodiversity hotspot. The Itremo Massif in the Central Highlands of Madagascar with a Highland Subtropical climate range encompasses montane grassland, igneous and metamorphic rock outcrops, and gallery and tapia forests. Our study focused on identifying culturable mycorrhizae from epiphytic, lithophytic and terrestrial orchid taxa to understand their diversity and density in a spatial matrix that is within the protected areas.

We have collected both juvenile and mature roots from 40 orchid taxa for isolating the orchid mycorrhizal fungi (OMF), to culture, identify and store in liquid nitrogen for future studies. Twelve operational taxonomic units (OTUs), of three known orchid mycorrhizal genera, were recognized by analysis of internal transcribed spacer (ITS) sequences of 86 isolates and, by comparing with GenBank database entries, each OTU was shown to have closely related fungi that were also found as orchid associates. Orchid and fungal diversity were greater in gallery forests and open grasslands which is very significant for future studies and orchid conservation. As far as we know this is the first ever report of detailed identification of mycorrhizae from Madagascar. This study will help start to develop a programme for identifying fungal symbionts from this unique biodiversity hotspot which is undergoing rapid ecosystem damage and species loss. The diversity of culturable mycorrhizae, density and their distribution within the Itremo orchid hotspot areas will be discussed.

INTRODUCTION

About 44% of all vascular plant species are confined to 34 global biodiversity hotspots (Mittermeier et al. 2005). Investing resources to the entire global hotspot could lead to spread the resources too thinly on the ground, making conservation of an entire hotspot area untenable, and therefore conservation approaches must focus on selected areas of maximum diversity and/or endemism (Murray-Smith et al. 2009). Fenu et al (2010) proposed the terms 'micro-hotspots' (*i.e.* endemism-rich areas analogous to biogeographic units) and 'nano-hotspots' (*i.e.* areas less than 3km^2 with an exceptional concentration of endemic taxa). By most estimates, Madagascar is home to 4% of the world's plant and animal species all confined to just 0.4% of the Earth's land surface, earning its reputation as one of the top five biodiversity "hotspots" (Tyson, 2000). About 90% of Madagascar's natural vegetation has been cleared or permanently altered, and the remaining 10% is by no means secure. Roos et al (2004) studied the vascular plants in the Malesian Islands and observed that that species-area relationships of families dependent on species number. They found that island surface area is a predictor for island percent endemism in the study area. Madagascar, world's second largest island has 10,000-12,000 vascular plant species, roughly 1 in 10 (*ca.* 1,000) are orchids, about 90% of which are endemic (Tyson 2000; Moat & Smith 2007).The Itremo Massiff within the Central Highlands is a 'micro-hotspot' home to more than 50 orchid taxa of which majority are endemic and some species are locally endemic (*e.g.* *Angraecum protensum*, *Angraecum magdalane*).

Itremo Massif consists of a plateau of mixed igneous and metamorphic rock at an elevation of 1400-1923m above sea level. The average temperature is in the range of 18-21^{o}C, annual rainfall of 1416mm with a 4-6 month dry season. Savanna is the dominant habitat, with humid gallery forest, remnant tapia (*Uapaca bojeri*) forest and rocky and montane moorland habitats also present. The raising of cattle is commonplace in the region and burning grassland is practiced annually. While some advocate carefully controlled burning as a management strategy for tapia forest in Itremo and elsewhere in the Central Highlands (Alvarado et al. 2013), other habitats can be badly affected (Whitman et al. 2011). We observed evidence of fire damage on two separate rocky ridges and speculate that adjacent man-made grass fires were the cause. In addition to this illegal mining for precious stones is causing habitat degradation in some areas (Vorontsova et al. 2013). Following a proposal in 2008, a 273km^2 area was given Protected Area status in 2012 thanks to Itremo's unique flora and fauna. The area covered by the study represented around 13km^2 both within and adjacent to the protected area (Fig. 1).

In light of the ongoing demise of tropical ecosystems in Madagascar and worldwide, there is an urgent need to document and safeguard the full gamut of life forms in the landscape, and to understand how these biotic agents interact with one another in this age of extinction. Among angiosperms, orchids are particularly vulnerable given their dependency on other organisms – namely mycorrhizal fungi and insect pollinators – to complete their life cycles in nature (Swarts and Dixon 2009). Thus, studying these connections becomes crucial for orchid conservation. While orchids have received considerable study with respect to classification and phylogenetic relationships, other important aspects (*e.g.*, pollination, propagation) have received less attention, and this is especially true of Orchidaceae in Madagascar (Cribb and Hermans, 2009), with a few exceptions (*e.g.*, Nilsson et al. 1992; Whitman et al. 2011). To date, virtually nothing is known about the identity, distribution, and ecology of OMF in Madagascar, nor the physiological roles played by these fungi with native orchids. Madagascar's remote location, coupled with its rugged terrain, continue to pose challenges to specialists seeking to gain access to orchid-rich habitats in search of such knowledge. This is especially true for mycologists faced with the burden of expediting fresh tissue samples harboring viable fungal material (*e.g.*, pelotons) to the lab for further study.

The main objective of the study was to conduct a major study to understand symbiotic relationships of orchids of the Itremo Massif in Madagascar. This study was conducted to achieve the ultimate goal of producing symbiotic propagules for reintroduction by understanding the role of mycorrhizal fungi in seed germination, seedling development, and establishment of plants in the wild. While orchids throughout Madagascar require study, we chose to focus on species inhabiting the Central Highlands – a region encompassing nearly 40% of the island (Cribb and Hermans, 2009) – and spontaneous seedlings in particular. The Itremo Massif has the largest area of exposed quartzitic substrate in Madagascar (du Puy and Moat 1996) with a mixture of ecosystems found nestled within a complex of hills and valleys separated by species-poor grasslands (Cribb and Hermans 2009). Several well-known, showy taxa are found in the region, many of which persist as lithophytes on sun-exposed rocks (*e.g.*, *Angraecum longicalcar*). Others exist as terrestrials of grasslands (*e.g.*, *Benthamia cinnabarina*), moist forests (*e.g.*, *Cynorkis purpurea*), or well-drained soils (*e.g.*, *Habenaria ambositriana*), and a modest number cling to gnarled branches of host trees in open areas as epiphytes (*e.g.*, *Bulbophyllum* sp.).

The orchid seed baiting technique initially developed by Rasmussen and Whigham (1993) remains the most widely used method to identify fungal symbionts that support germination *in situ*. Although this technique has been applied to a large number of species with varied success, namely terrestrials (McKendrick et al. 2000; Batty et al. 2006; Phillips et al. 2011), length of period required for successful baiting is one of its drawbacks in addition to loss of baits (Gale et al. 2010). Given Madagascar's large number of lithophytic taxa, affixing seed baits to exposed rocks also poses a serious practical challenge as we previously discovered. With these limitations, we opted to improve our odds for acquiring germination-phase fungi by targeting spontaneous seedlings on natural substrates, as well as roots from mature phase plants to identify the suite of fungi utilized by these orchids. In this paper, we report the mycorrhizal fungi cultured from spontaneous seedlings and mature orchids in the Central Highlands of Madagascar using morphological and molecular characterization (ITS sequencing). We also provide discussion on the distribution of these isolates in the landscape linked to the specific microhabitats of the orchids. Furthermore, we describe the molecular confirmation of the field identification of spontaneous seedlings collected and propose it as a standard methodology for other collections of a similar nature. Collection and long-distance transportation of fresh orchid material from Madagascar to labs in Europe (Kew) and North America (Illinois) also will be discussed. To our knowledge, this is the first report that documents OMF from orchids of different life forms and diverse ecosystems from Madagascar.

MATERIALS AND METHODS

This joint study was conducted between Royal Botanic Gardens Kew (Kew) and Illionis College with logistic and taxonomic support from Kew Madagascar Conservation Centre (KMCC) and Parc Botanique et Zoologique de Tsimbazaza (PBZT). More than 40 taxa were selected for study within 24 genera. Root samples were shared between two partners and to facilitate the legal collection and international transport of orchid material from Madagascar to the United Kingdom and United States, a CITES permit was obtained which allowed three tubes each containing seedling and mature roots per species to be collected. This was followed by a phytosanitary certificate which was secured prior to departure from the country. For the import of root samples (with soil) and seeds to the US two permits were needed from the United States Department of Agriculture (USDA). The US Government considers all OMF to be plant pathogens due to the ties of some to the *Rhizoctonia* complex. OMF

Study sites

Seven different sites within the Central Highlands were visited during 28 April to 3 May 2013 (Table 1). These sites were all within 50 km of one another with the exception of Analabeby (Fig. 1).

Collection and transportation

The collecting trip was conducted in April/May 2013 to collect spontaneous seedling roots on orchid-rich substrates and mature roots shortly after the rainy season (December to March). Roots of mature orchids and spontaneous seedlings were collected from 40 taxa consisting of nine lithophytic, 15 epiphytic, and 16 terrestrial species, respectively. Spontaneous seedlings were putatively identified on site based on subtle morphological features (*e.g.*, presence of pseudobulbs in *Bulbophyllum* sp.; Fig. 2) as well as proximity to mature plants on or near the same substrate; the identities were later confirmed by DNA analysis as

described later. To maximize our chances for isolating viable pelotons, younger-appearing roots were collected whenever possible. For epiphytic and lithophytic orchids, these roots were those that were translucent to white in color, often with slight greenish pigmentation near the apex (Fig. 2). Upon detachment in the field, each root was placed over a small, pre-moistened cotton ball within pre-sterilized glass vial with screw cap. To permit gas exchange leading up to departure from Madagascar (5-10 days after collection), the caps on each vial were tightened only slightly, then placed within a 50 ml capacity centrifuge tube with screw cap. These tubes were then stored vertically within an insulated handbag for transport from field to shelter. Care was taken to keep the handbag out of direct sunlight at all times so that the root samples would remain as cool as possible (15-25°C).

The root collection procedure for terrestrial orchids differed slightly in that soil containing intact root systems (root ball) was also collected. This permitted the roots to remain in a semi-natural state leading up to departure from Madagascar. A trowel or small shovel was used to gently excavate the soil around individual plants, and to lift the root ball with minimal disturbance to the brittle root systems. Each root ball was then placed into its own separate plastic bag, and the bags were then carefully packed into an insulated handbag for transport. Upon arrival at KMCC base in Antananarivo, Madagascar 2-7 days after field collection, all root samples and root balls were placed into a refrigerator (ca. 6°C). Approximately 24 hrs before departure from the country, roots of terrestrial orchids were lifted from soil and rinsed off with UV-irradiated and/or bottled water to remove soil particles and organic debris. Lateral branch roots, especially those that exhibited orange-yellow patches of coloration, were detached and placed over a pre-moistened cotton ball in a pre-sterilized glass vial. Starch-filled tuberous roots were not retained because previous studies (L. Zettler, unpublished data) have shown that such roots are generally void of pelotons. The screw cap was then tightened firmly and wrapped with a strip of Parafilm "M". Likewise, caps on glass vials containing roots of lithophytes and epiphytes were also tightened and wrapped with Parafilm "M" at that same time (ca. 24 hrs prior to departure by air). All sealed glass vials were then housed in 50 ml plastic (shatter-proof) centrifuge vials which were also firmly tightened and sealed with Parafilm "M". All vials were re-packed into insulated handbags and transported back to labs in the USA and UK as cabin baggage.

Measurement of substrate acidity

Substrate samples were collected at several locations, and consisted of soil and humus in the case of terrestrial habitats, and organic/inorganic debris on the surface and in the cracks of rocks where lithophytic orchids were found. On return to KMCC, the collected samples were analyzed using LaMotte STH Series Combination Soil Testing Outfit (LaMotte, Maryland, USA).

Fungal isolation, initial identification and deposition

Immediately upon arrival at Kew and Illinois 24-48 hrs after departure from Madagascar, all root samples were placed in refrigeration (4-6°C) for a period lasting up to one week, during which time fungal isolations took place. Mycorrhizal fungi were isolated following the method of Zettler et al. (2003). Colonisation of mycorrhiza as pelotons in the cortical region of root sections was scored as percentage of colonization for all three life forms. Clumps of macerated cortical cells containing pelotons were immersed in Fungal Isolation Medium [(FIM); Mitchell 1989] containing streptomycin sulfate (Clements and

Ellyard, 1979) and incubated at 18°C. After 1-4 days, hyphal tips that were observed emerging from cortical cells and/or pelotons under a dissection microscope were subcultured to FIM (Kew) or Potato Dextrose Agar (PDA, Difco™, Becton, Dickinson and Co., Sparks, MD, USA) (Illinois) using a sterile scalpel. OMF were initially distinguished from common molds using previously published descriptions (Zettler et al. 2003). Those that yielded cultural characteristics (*e.g.*, monilioid cells) resembling basidiomycetes in the *Rhizoctonia*-complex (*e.g.*, Tulasnellaceae, Ceratobasidiaceae) were retained for further identification by ribosomal DNA internal transcribed spacer (rDNA ITS) amplification and sequencing. Colony growth rates were recorded during early subculture. To safeguard these strains for the purposes of future work (*e.g.*, symbiotic seed germination) and long-term conservation, fungi isolated in Illinois were deposited into the University of Alberta Microfungus Collection and Herbarium (UAMH), Edmonton, Canada, and Kew for permanent safekeeping.

Molecular identification of fungi by ITS sequencing

To identify each fungal sample, DNA was extracted and sequenced. Genomic DNA was isolated from mycelia using Sigma Extract-N-Amp™ Plant PCR Kit (Sigma Aldrich, St. Louis, Missouri, USA). ITS sequences were amplified using primer combinations ITS1F with ITS4, and ITS1 with ITS4-tul (White et al. 1990; Gardes and Bruns 1993; Taylor and McCormick 2008) and using Sigma Extract-N-Amp™ Plant PCR Kit (Sigma Aldrich, St. Louis, Missouri, USA). The reactions were performed using a programmable thermocycler for 35 cycles of 30 seconds at 94°C, 35 seconds at 53°C, 1 minute at 72°C, with the extension step increased by 5 seconds per cycle. Amplification was verified on 2% agarose gels containing 0.1 mg/ml ethidium bromide in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) or 1×SB (50 mM boric acid, pH 8.5 with sodium hydroxide).

The amplified DNA samples were sequenced by BigDye® (Life Technologies, Carlsbad, California, USA) Sanger sequencing with both forward and reverse primers. The PCR products were cleaned using exonuclease I and shrimp alkaline phosphatase (Affymetrix, Santa Clara, California, USA) to remove residual single-stranded primers and remaining dNTPs before performing the cycle sequencing reactions on a thermocycler for 25 cycles of 10 seconds at 96°C, 5 seconds at 50°C, 4 minutes at 60°C. Reaction products were purified by ethanol precipitation and analyzed using Applied Biosystems 3730xl DNA Analyzer (Life Technologies, Carlsbad, California, USA).

All sequence analyses were performed using Geneious® software package (Biomatters, Auckland, New Zealand). The forward and reverse sequences were checked for accuracy and consensus, and compared to database sequences using BLAST (National Center for Biotechnology Information, Bethesda, Maryland, USA). Sequences that matched *Rhizoctonia*-like fungi were aligned and grouped in to operational taxonomic units (OTUs) based on a conservative similarity threshold of 95%. Representative sequences of each OTU were used to requery the GenBank database using BLAST. Phylogenetic trees were separately constructed for each of the three genera, *Tulasnella*, *Ceratobasidium* and *Sebacina*, together with closely matched sequences from GenBank database. The sequences were aligned with CLUSTALW algorithm, and Neighbor-Joining trees were made using Tamura-Nei genetic distance model.

Molecular confirmation of species identification of orchid seedlings

The DNA of orchid seedlings that yielded *Rhizoctonia*-like fungi was extracted and sequenced in order to confirm their species identification by matching the sequence of the chloroplast DNA region *trnL-F* to that of an identified plant. DNA was extracted from root tissue using Sigma Extract-N-Amp™ Plant PCR Kit (Sigma Aldrich, St. Louis, Missouri, USA) or from desiccated leaf tissue with a modified CTAB (cetyltrimethylammonium bromide) protocol (Doyle and Doyle 1987) followed by chloroform/isoamyl alcohol (24:1) extraction and precipitation in isopropanol. The *trnL-F* sequences were amplified using primer combinations c with d for the *trnL* intron, and e with f for the *trnL-F* intergenic spacer (Taberlet et al. 1991) and using Reddymix™ PCR master mix (Thermo Scientific ABgene, Pittsburgh, Pennsylvania, USA) with additional 150 mM trehalose, 200 µg/ml bovine serum albumin and 0.2% Tween-20 (Samarakoon et al. 2013). The reaction was performed on a programmable thermocycler for 28 cycles of 1 minute at 94°C, 1 minute at 48°C, 1 minute at 72°C, The PCR products were cleaned using QIAquick® columns (Qiagen Inc., East Crawley, UK) and sequenced as described above for fungus ITS sequencing.

RESULTS

Analysis of substrate

Substrate pH was measured on soil collected from three collection sites. At Analabeby (site 1), lithophytic orchids (*Angraecum longicalcar* and *Oeceoclades calcarata*) were on substrates that were noticeably basic, with three separate samples having pH values of 7.8, 8.0 and 8.2, although the soil taken from the surrounding grassland had a pH of 5.8. The rocky outcrops at Ambatoantrano (site 2) had a pH of 5.4, while the marshy, wet soil at Tsinahabeomby (site 4) had a pH of 5.0.

Isolates recovered

Percent of colonisation of fungi in the root cortical regions of 23 orchids is represented in Fig. 3. Most colonization was found in terrestrial taxa followed by lithophytes and epiphytes. Although colonized in smaller percentages epiphytes yielded more mycorrhizae than lithophytes. Of the 40 orchid taxa collected within seven collection sites in the Itremo region of central Madagascar, *Rhizoctonia*-like mycorrhizal fungi were recovered from 12 orchid taxa (Table 2). This determination was based on morphological features matching *Rhizoctonia*-like fungi, and confirmed by sequence data. Most of the *Rhizoctonia*-like mycorrhizal fungi that were isolated were assignable to *Ceratobasidium*, *Sebacina* or *Tulasnella*, (Table 2) based on BLAST searches of their ITS sequences against the GenBank database. Roots of *Cynorkis purpurea*, collected from moist soil adjacent to a clear stream in a shaded forest in Antsirakambiaty, yielded the most diverse assemblage of isolates spanning all three *Rhizoctonia* groups (*Ceratobasidium*, *Tulasnella*, *Sebacina*). The moist dense gallery forest had four orchids with culturable mycorrhizae as had the moist open grassland (Fig. 4 A-B). The open grassland had more *Tulasnella* OTUs than other sites, all

of which were isolated from terrestrial orchids. Site 5 (Small Gallery Forest) had three culturable species of mycorrhizae while the exposed rocky areas had two species with culturable mycorrhizae. The dense moist gallery forest had the most diverse collection of culturable mycorrhizal symbionts compared to any other habitat from the collection of root samples. Site 5 had only *Tulasnella* and *Ceratobasidium* as had the open moist grassland. The epiphytic orchid *Aerangis punctata* had an individual plant that harbored more than one OTU (cer1 and tul7). No other individual plant yielded more than one fungal OTU. In addition, pelotons in our samples also yielded non-*Rhizoctonia* fungi, especially slow-growing dark-pigmented colonies that were assignable to *Toxicocladosporium*, *Cladophialophora* and *Lophiostoma*.

Many isolates fit the typical profile of OMF in pure culture on FIM and/or PDA. In general, these isolates had cream-colored to yellowish orange colonies and usually modest to fast growth rates at 18°C. Colony margins were often entire and submerged slightly with raised aerial mycelia towards the (older) colony center (Fig. 5). Some isolates of *Sebacina* and *Ceratobasidium* yielded noticeable concentric zonation along the surface of the plate (Fig. 5). Upon examination by light microscopy, many of the isolates produced ovoid to barrel-shaped monilioid cells in single or sparsely-branched chains (Fig. 5). In some cases, numerous monilioid cells were evident within one week of subculturing. A few isolates, however, failed to yield monilioid cells, even aged (>2 months) cultures.

ITS sequencing

Analysis of the ITS sequences of 86 *Rhizoctonia*-like fungal isolates revealed the presence of 12 OTUs: four *Ceratobasidium*, one *Sebacina* and seven *Tulasnella* (Table 2). A phylogeny tree was constructed, separately for each genus, which included closely matched examples from the GenBank database (Figs. 6, 7, 8).

All 12 OTUs had orchid-derived fungi among their closest matches from GenBank. In the *Tulasnella* phylogeny tree (Fig. 8), representative sequences from Girlanda et al. (2011) were also included, as the authors described two distinct clades of *Tulasnella* from terrestrial orchids. Similarly to Jacquemyn et al. (2012), all seven *Tulasnella* OTUs in the current study matched with clade A *sensu* Girlanda et al. (2011). The *Ceratobasidium* phylogeny tree (Fig. 7) was also well represented by orchid-derived fungi, although somewhat less than with *Tulasnella*; particularly noticeable was that some branches of the tree also had a number of fungi from Rosaceae and/or Poaceae. In the *Sebacina* phylogeny tree (Fig. 8), fungi found in orchids were clustered on some of the branches while other branches were represented by fungi found in plants mainly of the Ericaceae and Aneuraceae.)

DISCUSSION

Twelve OTUs, of three known orchid mycorrhizal genera, were recognized by analysis of ITS sequences of 86 isolates and, by comparing with GenBank database entries, each OTU was shown to have closely related fungi that were also found as orchid associates. Of the 12 OTUs, two (tul1, tul3) were isolated more than once, from different collections of a species (data not shown), suggesting a certain level of specificity in the association within a background of a diversity of mycorrhizal fungi that

are apparently available to an individual orchid plant. Although this observation could alternatively suggest that the fungi are restricted to specific habitats that coincide with the preferred habitats of their host orchid species, it is very noteworthy that OTU tul3 was isolated from different habitats: from lithophytic *Angraecum protensum* and terrestrial *C. purpurea*. This indicates that at least some of the fungi are able to survive in, and colonize, a range of orchid species in a variety of habitats, but that there is an element of selection by the orchid. Some orchids harbored a surprisingly rich assemblage of different *Rhizoctonia*-like fungi; *Cynorkis purpurea* had a *Ceratobasidium*, *Sebacina* and two OTUs of *Tulasnella*, and is seemingly a 'generalist', with apparently less specificity for particular fungi than other orchid species. With the exception of *C. purpurea* and *Aerangis punctata* (OTUs cer1 and tul7), all other orchids yielded only one type of *Rhizoctonia*-like fungus, again indicating an element of specificity.

The phylogeny tree is based on isolates from root samples, maximum of three plants, which we were allowed to collect by our CITES permit covering all the sites. This stipulation limits our sample size, therefore this does not represent the full genetic diversity of the orchids within the remit of this study. However this preliminary study suggested that a mycorrhizal fungus seems able to associate with different types of orchids and some are specific to only certain orchids. Very notable was that the closest GenBank match of OTU tul7 (from *Aerangis punctata*) was a fungus that was identified in *Aerangis punctata* in Réunion (JF691324 and JF691326, Martos et al. 2012), a neighboring island to Madagascar but some 800 km to the east. OTU cer4, from the terrestrial *C. purpurea*, was found to be closely matched with sequences of fungi from *Pterygodium catholicum* (FJ788724, FJ788725, FJ788812, Waterman et al. 2011), another African terrestrial orchid. It is planned to further investigate such indications of specificity; future collections will focus on roots of a greater number of individual plants and from several different sites for species of high conservation and scientific interests. Fungus-orchid relationships at other stages of the life cycle of selected orchids will be studied by genomic analysis of mycorrhizal fungi in root sections with commonly used genetic markers in addition to ITS.

To our knowledge, this is the first report documenting culturable mycorrhizae from orchids of different life forms from diverse ecosystems from Madagascar, and one of the few from the African region (*e.g.*, Jonsson and Nylund 1979; Mugambi 2001; Bonnardeaux et al. 2007; Waterman et al. 2011; Martos et al. 2012), most of which have used culture-independent molecular methods to detect and identify the fungi. Our success at isolating OMF from remote and inaccessible locations suggests that our method used to collect and transport fresh root samples may have practical merit for other researchers faced with the daunting task of long-distance field work. For the terrestrial orchids in particular, the removal of a root ball (roots with surrounding soil), as opposed to immediate root detachment, followed by transport from the field to Antananarivo days later, may have contributed to the high number of isolates we recovered. For all samples, especially roots of small epiphytic seedlings prone to desiccation, placing fresh tissues over a moist cotton ball, coupled with (cool) incubation in darkness during transport, also may have contributed to the favorable outcome.

These, and numerous other, studies support the hypothesis that tropical orchids, like their temperate counterparts, associate with basidiomycetes in the Chanterellales and Sebacinales with few exceptions (Bidartondo et al. 2004; Selosse et al. 2004). Within this group, the overwhelming majority of isolates fall under the category of '*Rhizoctonia*-like fungi' (Arditti 1992; Otero et al.

2002; Rasmussen 2002; Dearnaley 2007), encompassing teleomorphic genera *Ceratobasidium* (anamorphs = *Ceratorhiza*), *Tulasnella* (anamorphs = *Epulorhiza*), and *Sebacina* (Warcup and Talbot 1980). Tulasnelloid fungi, in particular, have been recovered from orchids with regularity throughout the world (*e.g.*, Nontachaiyapoom et al. 2010; Zettler et al. 2013; Zi et al. 2014), and the orchids of Madagascar appear to fit the same general profile. The Ceratobasidiaceae was also well-represented in Madagascar root samples, paralleling reports mostly from the New World (*e.g.*, Richardson et al. 1993, Zettler and Piskin 2011), and Sebacinaceae fungi were also present among our isolates. Representatives of both Tulasnellaceae and Ceratobasidiaceae were isolated from several orchid species encompassing terrestrial, lithophytic and epiphytic habits, but more epiphytes yielded Ceratobasidiaceae fungi while terrestrials had mostly Tulasnellaceae fungi (Table 2). It is interesting to note that gallery forest and the moist open grassland had the most hits for culturable mycorrhizae, predominantly from terrestrial taxa. The open grassland and adjacent lithophytic habitats are subjected to man-made fire (Whitman et al. 2011). Seedling recruitment and survival are likely to be adversely affected, therefore, conservation of orchids from these areas warrants immediate attention. Gallery forests are also affected, therefore, mapping the orchid diversity and their fungal symbionts need urgent study.

Results of soil/substrate analysis showed that most habitats where orchids were collected in this study showed an acidic pH of 5.0 to 5.8. One exception to this was the lithophytic habitat at Analabeby (site 1) where the substrate pH was consistently basic at 7.8 to 8.2. No *Rhizoctonia*-like fungi were recovered from orchid roots collected from this site. Analabeby (Fig. 1) collection sites features rocky outcrops consisting mainly of marble, whereas the outcrops at the other study areas were of sandstone, granite and quartzite, which explains the difference in pH of the immediate environment of lithophytic plants. It was particularly noticeable that the surfaces of the rocks at Analabeby were devoid of lichen, in contrast to its abundance on the rocks at Ambatoantrano. Lithophytic habitats at other collection sites also had profuse growths of lichen, suggesting that these locations similarly had relatively acidic substrates; mycorrhizae were successfully isolated from lithophytic roots collected at Ambatoantrano (site 2), Ambatoantremo (site 3) and Tsinahabeomby (site 4). In contrast, no mycorrhizal fungi could be isolated from lithophytic roots collected from Analabeby. Epiphytic orchids were also often found in association with lichen; four collections from epiphytic habitats yielded mycorrhizal fungi. These observations could be indicating that mycorrhizal fungi at Analabeby may have a different physiology as a consequence of having adapted to a different environment, and they may therefore have different culture requirements. It may be necessary to adjust the isolation medium composition if mycorrhizae are to be successfully isolated from orchid roots from such environments.

As an alternative to *in situ* seed baiting, we made a conscious effort to collect roots from young orchid plants, including protocorms and very small seedlings. These juvenile plants were identified in the first instance by local botanical specialists based on morphology, habitat and the proximity of mature specimens that could be the likely parents. We set out to bolster our confidence in the identities of the seedlings by seeking a definitive identification using DNA sequence analysis to compensate for the lack of mature morphological features. By matching DNA sequences of the seedlings to those of collections of mature plants and/or GenBank entries, we have been able to confirm the identities of the putatively identified seedlings. As one of our aims is to investigate the specificities in the orchid-fungus relationship, we consider the molecular confirmation of the seedling

identity to be of critical importance, and propose the methodology as standard procedure for our future collections of mycorrhizal collections from juvenile plants in Madagascar, with its diversity of orchid species.

Targeting spontaneous seedlings offers greater flexibility, compared to seed burial (baiting) technique, with positive identification being done within weeks instead of months or years of collecting the material. With the help of fingerprinting techniques as we used here, identifying the seedlings has been made more reliable. As orchid populations continue to decline worldwide, it is conceivable that cross-pollination between unrelated individuals will diminish resulting in lower seed viability. As a result, it may be necessary for future workers to rely more on spontaneous seedlings and less on the standard seed baiting techniques as a means to acquire early germination phase fungi. For critically endangered taxa, use of the seed baiting technique may also be viewed as too wasteful considering that few seed packets typically yield seedlings (protocorms) once retrieved.

CONCLUSIONS AND FUTURE STUDIES

As the first detailed investigation into the identification of culturable orchid mycorrhizae in Madagascar, our findings will form the foundation of our future approach in understanding how the orchid-fungus relationship relates to their native environments. In addition to planned further collections from different locations, different orchid species and at different times of the year, *in vitro* germination tests will be carried out to reveal the specificity/generality of the symbiosis. It is expected that the sum of knowledge will lead to a better conservation strategy that is designed to protect vulnerable orchid taxa by taking into account their need for symbiotic partners. Furthermore this will help to develop a phylogentic analysis of mycorrhizae, their evolution across the land mass of Madagascar and how this affect natural regeneration of orchids in pristine and fragmented ecosystems.

Erratic pollination and low seed set were identified in *Aerangis ellisii* ((Nilsson et al. 1988; Nilsson and Rabakonandrianina 1988) but there may be similarly affected taxa. In the case of *Aerangis ellisii* we have noticed very low or no germination of mature seeds by asymbiotic methods (unpublished data). To develop genetically diverse stocks for species restoration projects is a huge challenge as in vitro asymbiotic germination alone is not an effective tool. If mycorrhizal seed germination can be applied to improve not just seed germination but seedling survival in cases otherwise impossible may be a good step in the right direction. Although data is not available at the moment regarding fungal diversity and density for a specific habitat as part of this study, symbiotic seedlings can be used for reintroduction. Detailed studies to identify the mycobiont populations benefit the resilience of reintroduced orchids in Madagascar and other biodiversity hotspots areas. This will be developed as a major research area to contribute to ecosystem services, conservation and phylogenetic studies.

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Fig. 1. Map depicting the study sites near Itremo in the Central Highlands of Madagascar. Key: Circles = Gallery forest dominated by epiphytic orchids, also terrestrial orchids on river banks; Squares = Rocky montane grassland with lithophytic and terrestrial orchids; Triangles = Montane moorland with terrestrial orchids; T = Terrestrial; L = Lithophyte; E = Epiphyte; L/T = Lithophyte/terrestrial; Cer = *Ceratobasidium*; Seb = *Sebacina*; Tul = *Tulasnella*;. Numbers 2, 3, 4, 5 and 7 denote sites; solid line depicts core protected area. Image © 2014 Google Earth

Fig. 2 a-c. **a**. Spontaneous seedlings of *Angraecum* species affixed to outer bark of a host tree adjacent to a foliose lichen **b**. A small seedling, just past the protocorm stage (denoted by the arrow), was discovered beneath loose bark shown in the palm of a hand (b, top right). **c**. An advanced stage seedling of *Angrecum protensum* displays a young healthy root system with green tipped apex (arrow). Younger roots of spontaneous seedlings were targeted for collection.

Fig. 3. Percent colonisation of cortical cells of root sections by pelotns in lithophytic (first 8 taxa), epiphytic (second group of 7 taxa) and terrestrial orchid roots (last group of 8 taxa). C-*Ceratobasidium*, T- *Tulasnella* and S- *Sebacina* that were isolated from those taxa. Plain bars denote no hits for mycorrhizae.

Fig. 4 a-b. a. Number of orchid species with isolates of different OTUs of mycorrhizal fungi found within five ecosystem types; **b.** Number of OTUs found within the five ecosystem types from three mycorrhizal genera which include *Ceratobasidium*, *Sebaina* and *Tulasnella*.

Fig. 5 a-c. Morphological and cultural characteristics of selected *Rhizoctonia*-like fungi on Potato Dextrose Agar (PDA) >3 months incubation at ambient temperature (22°C) in 9 cm diameter Petri dishes. **a**. *Tulasnella* sp. Isolate 56-9 (UAMH 11781) from roots of *Tylostigma nigrescens* that grew on moist seepage mat in full sun at Tsinahabeomby. **b**. *Ceratorhiza* sp. Isolate 69-13 (UAMH 11789) acquired from roots of *Cynorkis purpurea* in Antsirakambiaty. **c**. *Ceratobasidium* sp. Isolate 71-3 (UAMH 11795) from roots of an *Aerangis* sp., an epiphytic orchid affixed to a host tree in forest at Antsirakambiaty.

Fig. 6. Neighbor-Joining phylogeny tree of aligned sequences of the seven *Tulasnella* OTUs (tul1-tul7) found in this study. Also included in the tree are five closest BLAST matches in GenBank for each OTU, other close matches, and one representative of each of 10 OTUs from clades A and B described by Girlanda et al. (2011) (tulA1-4, tulB1-6). Sequences of clade B *sensu* Girlanda et al. (2011) were used to root the tree. Bootstrap percentages greater than 50%, after 1,000 replicates, are shown.

Fig. 7. Neighbor-Joining phylogeny tree of aligned sequences of the four *Ceratobasidium* OTUs (C1-C4) found in this study. Also included in the tree are five closest BLAST matches in GenBank for each OTU, and other close matches. Sequence of *Sebacina* isolate OUT seb1 was used to root the tree. Bootstrap percentages greater than 50%, after 1,000 replicates, are shown.

Fig. 8. Neighbor-Joining phylogeny tree of aligned sequences of the *Sebacina* OTU seb1 found in this study. Also included in the tree are close BLAST matches in GenBank. Two representative sequences of Sebacinales subgroup A *sensu* Weiss et al. (2004) were used to root the tree. Bootstrap percentages greater than 50%, after 1,000 replicates, are shown.

Figure 1
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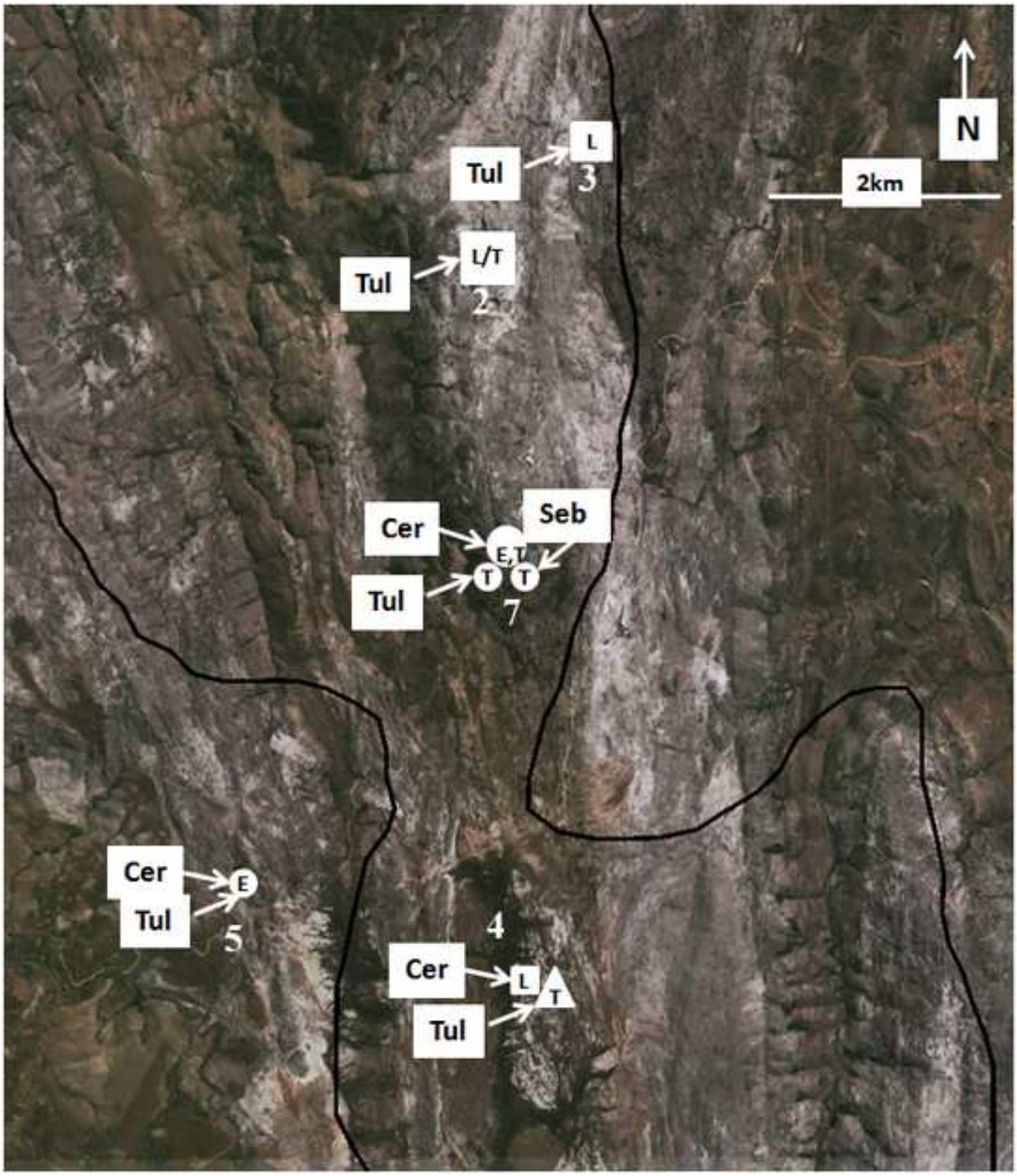

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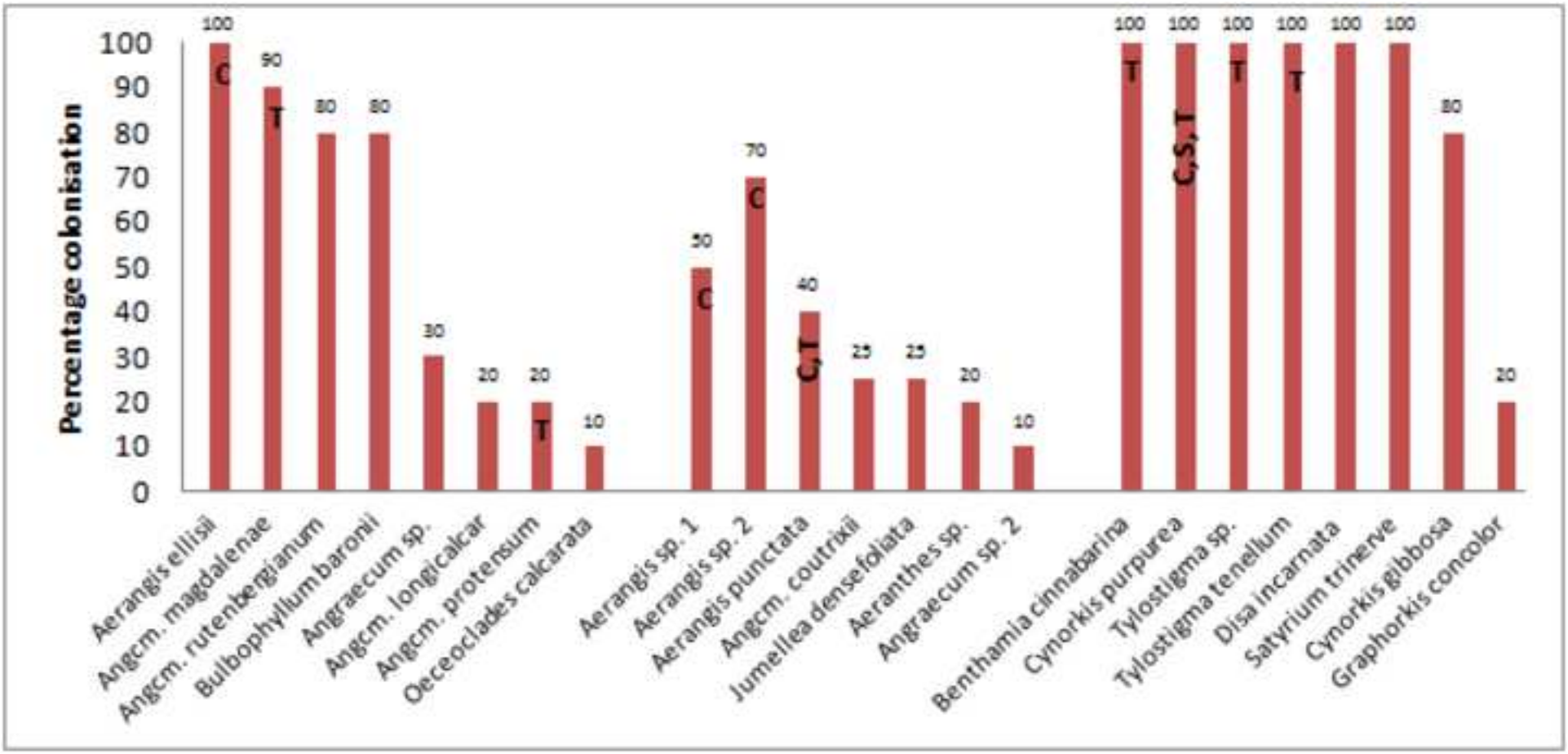

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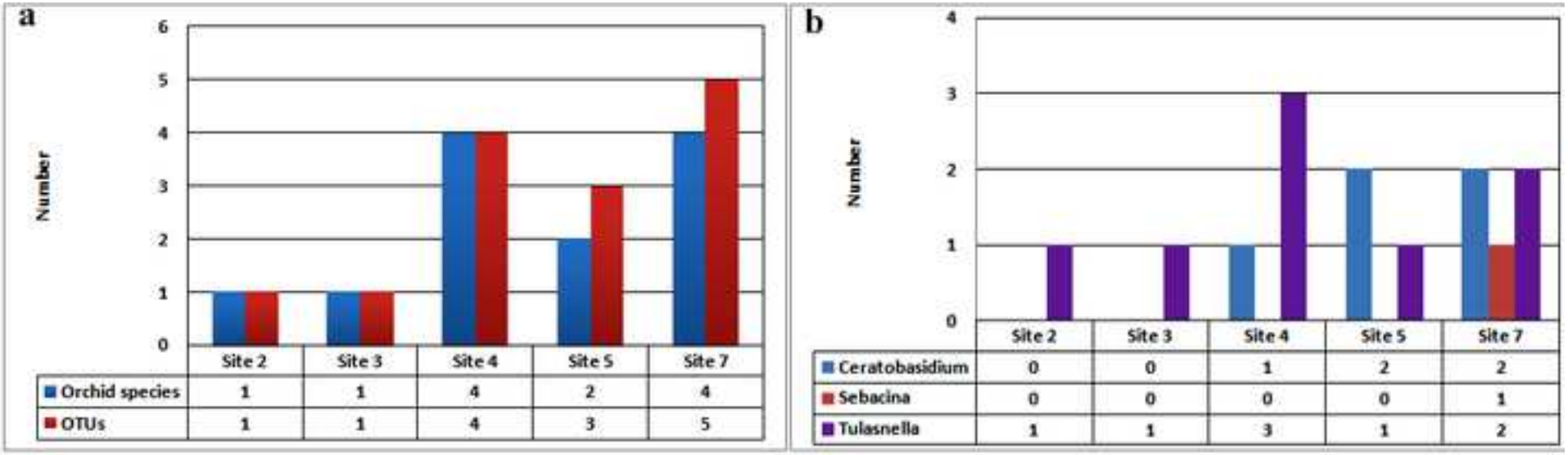

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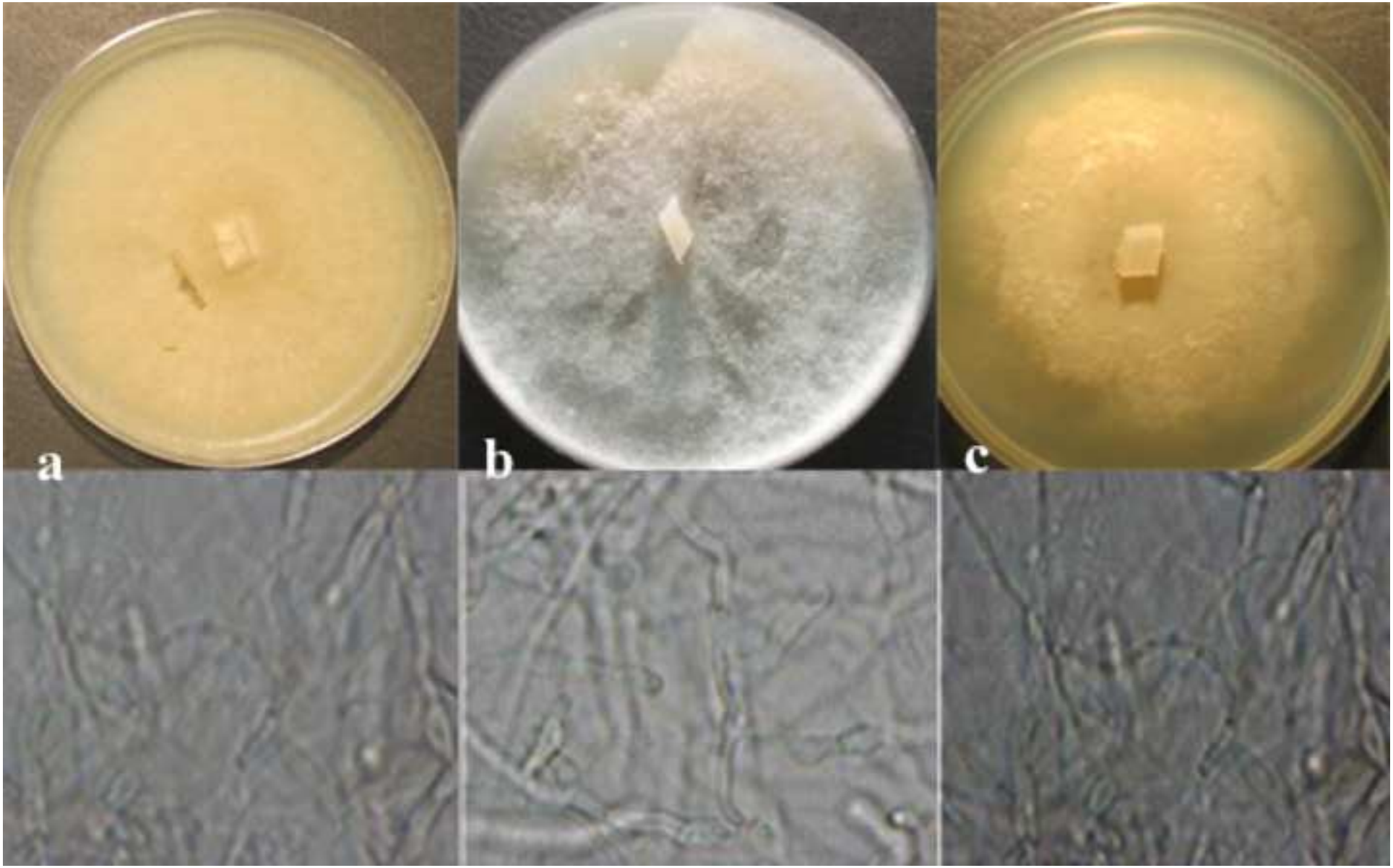

Figure 6

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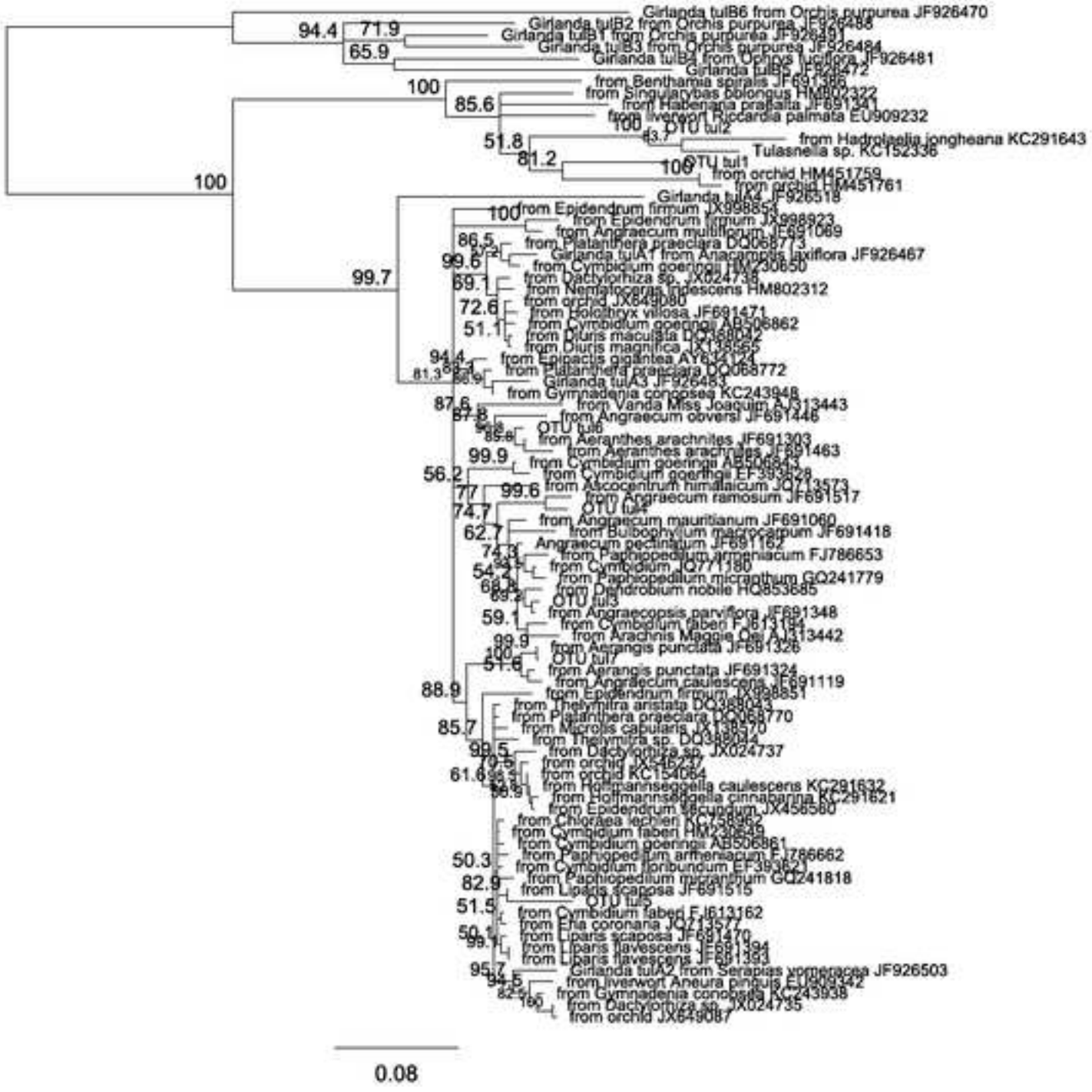

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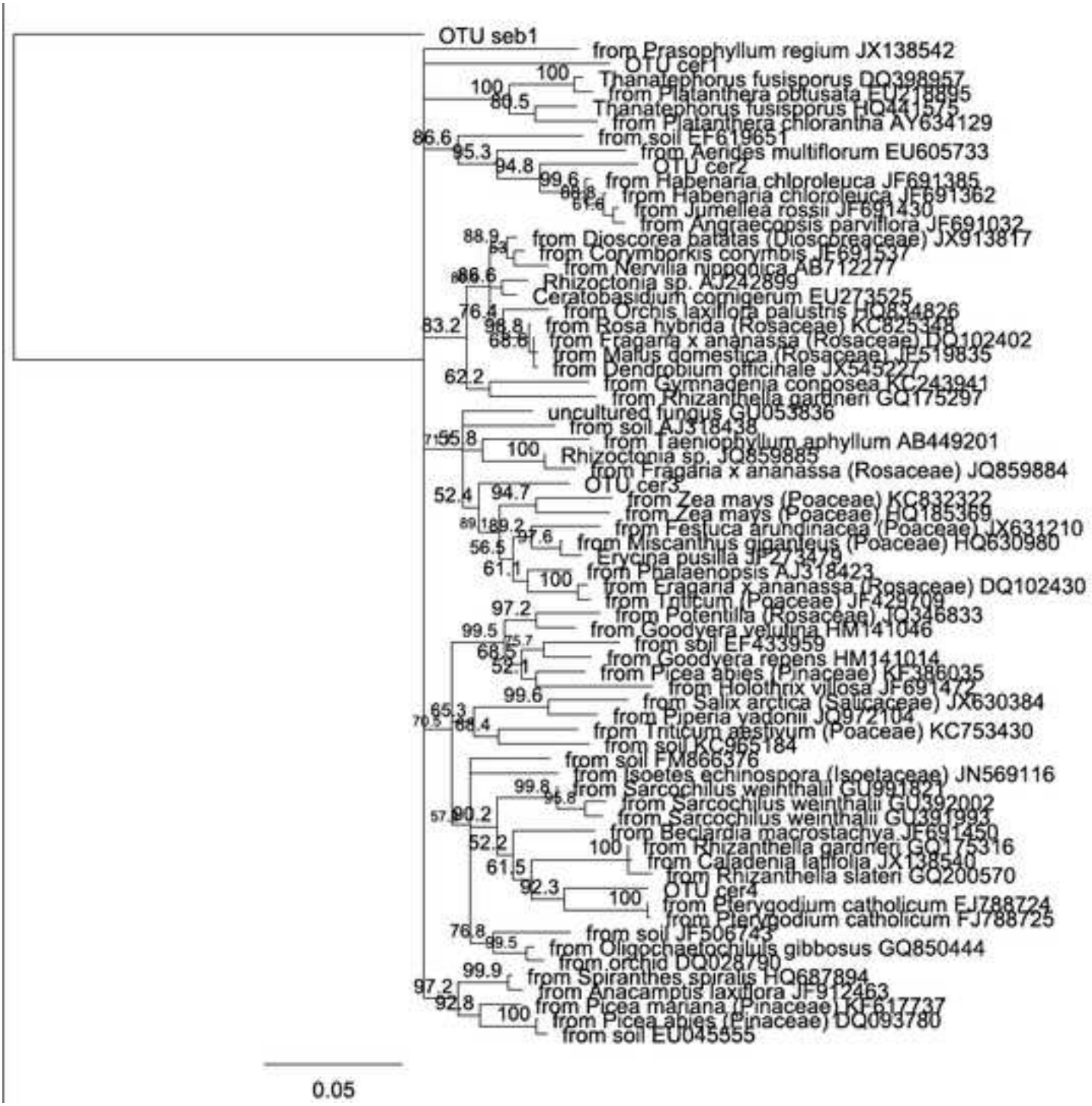

Figure 8
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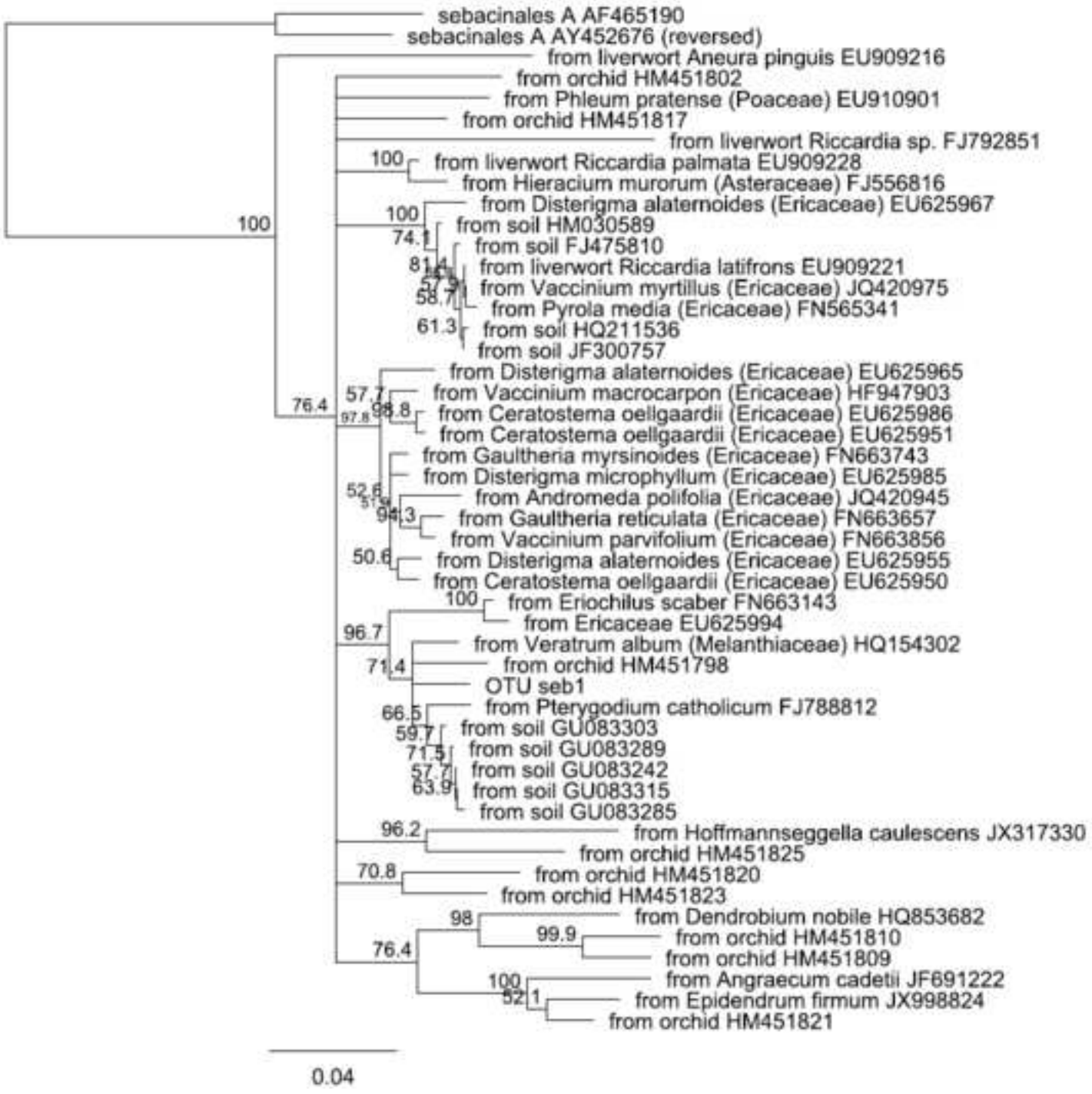

Table 1. General description of the seven orchid collection sites in the Central Highlands of Madagascar.

Site number/name		General description
1	Analabeby	exposed marble outcrop
2	Ambatoantrano	exposed rocks, occasional tapia trees
3	Ambatoantremo	exposed rocks, occasional gnarled small trees, sandy stream bed
4	Tsinahabeomby	open grassland, moist soil, occasional rocks
5	Gallery Forest	reduced forest (canopy ca. 20 m)
6	Soutrihotapaka	exposed ridges, montane vegetation
7	Antsirakambiaty	dense shaded forest, downhill stream

Table 2. Operational taxonomic units (OTUs) of mycorrhizal isolates, and their identities based on ITS sequences.

OTU	Source species	Closest informative matches in GenBank	Identity (%)
Ceratobasidium			
OTU cer1	*Aergs. punctata**, *Aergs*. sp. 1,	HQ687894 uncultured *Ceratobasidium* from *Spiranthes spiralis*, Italy	89.5
OTU cer2	*Aergs. ellisii**	JF691362 uncultured Ceratobasidiaceae from *Habenaria chloroleuca*, Reunion	95.3
OTU cer3	*Aergs.* sp. 2**	HQ630980 *Ceratobasidium* sp. from *Miscanthus giganteus*, USA	94.4
OTU cer4	*C. purpurea**	FJ788724 uncultured Ceratobasidiaceae from *Pterygodium catholicum*, S. Africa	94.8
Sebacina			
OTU seb1	*C. purpurea**	HQ154302 uncultured *Sebacina* from *Veratrum album*, Germany	96.5
Tulasnella			
OTU tul1	*Angcm. magdalenae**	EU909232 uncultured *Tulasnella* from *Riccardia palmata,* Germany	88.1
OTU tul2	*B. cinnabarina* (m)	KC291643 *Tulasnella* sp. from *Hadrolaelia jongheana*, Brazil	97.6
OTU tul3	*Angcm. protensum**, *C. purpurea**	JF691348 uncultured Tulasnellaceae from *Angraecopsis parviflora*, Reunion	99.5
OTU tul4	*C. purpurea**, *T. tenellum* (m)	JF691517 uncultured Tulasnellaceae from *Angcm. ramosum*, Reunion	98.8
OTU tul5	*Tylostigma* sp.(m)	JF691515 uncultured Tulasnellaceae	95.6
OTU tul6	*T. nigrescens*(m)	JF691303 uncultured Tulasnellaceae from *Aeranthes arachnites*, Reunion	98.7
OTU tul7	*Aergs. punctata**	JF691324 uncultured Tulasnellaceae from *Aergs. punctata*, Reunion	99.8

Epiphytic orchids in green; lithophytic orchids in orange; terrestrial orchids in black
Plant host identity confirmed:
(m)- as mature specimen, by morphology
*- to species level by *trnL-F* sequence match
**- to genus level by *trnL-F* sequence match