Techniques for the collection, transportation, and isolation of orchid endophytes from afar: a case study from Madagascar

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**Abstract**

**Background**: Tropical orchids need more study with respect to their mycorrhizal associations. For researchers in distant countries who aspire to study these orchids augmenting their conservation, the great distances involved, coupled with limited funds, pose formidable challenges. These challenges are sometimes exacerbated by political unrest, delays in securing permits, unexpected hardships, and the risk that the biological samples collected (e.g., roots harboring mycorrhizal fungi) will not survive long-distance transport.

**Results**: We describe a protocol for the collection and transport of root samples from Madagascar orchids to labs in the United Kingdom (Kew) and the United States (Illinois) where *Rhizoctonia*-like fungi were subsequently isolated. Three separate trips were made spanning 4 years (2012–2015), with emphasis on the collection of roots from epiphytic, lithophytic, and terrestrial orchids inhabiting the Itremo Massif of the Central Highlands. Collectively, the trips to Madagascar resulted in the isolation of all major groups of *Rhizoctonia*-like fungi (*Ceratobasidium*, *Tulasnella*, *Sebacina*) from all three orchid growth forms (terrestrials, epiphytes and lithophytes). Sampling of terrestrial and epiphytes during the rainy season (January) yielded best results.

**Conclusions**: Our study demonstrates that peloton-forming fungi in root samples can retain viability up to 3 weeks after collection.

**Keywords**: Orchidaceae, Conservation, Epiphytes, *Tulasnella*, *Ceratobasidium*

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**Background**

Orchids have the unparalleled distinction of being the most diverse plant family on earth, but also the most vulnerable to extinction (Swarts and Dixon 2009; Merritt et al. 2014). Part of their vulnerability stems from the family’s susceptibility to acute changes in their environment exacerbated by climate change, as well as their extreme dependence on pollinators and mycorrhizal fungi to complete their life cycles. Of the approximately 25,000 species of orchids worldwide (Dressler 1993; Cribb et al. 2003), about two-thirds are represented by epiphytes and lithophytes (Swarts and Dixon 2009), the majority of which are confined to tropical latitudes in areas prone to deforestation. The remaining one-third consist of terrestrials, many of which occupy cooler temperate zones that are undergoing rapid warming. Collectively, orchids face a conservation crisis of epic proportions, and understanding their biotic and abiotic needs is crucial to their survival.

Mycorrhizal fungi are needed by orchids as a carbon source to initiate germination of their dust-like seeds (Rasmussen 1995). Most of these fungi fall under the category of basidiomycetes in the *Rhizoctonia* complex...
little natural regeneration (Whitman et al. 2011). Many orchid populations that persist from year to year with massive scale has resulted in patches of fragmented the lab is also unclear. Swarts and Dixon (2017) recommended that peloton extraction take place the same day at the germination site, exemplified by recent studies (e.g., Jacquemyn et al. 2014; McCormick et al. 2012; Swarts et al. 2010). Mycorrhizal fungi must also be isolated from living orchid tissues, identified, screened for their ability to germinate seeds in vitro verifying their functionality, and safeguarded in cryopreservation.

During the past 30+ years, much has been published on orchid endophytes recovered from temperate terrestrials (e.g., Currah et al. 1987; Warcup 1981; Zelmer et al. 1996), and more recently the tropical epiphytes (e.g., Pereira et al. 2003; Nontachaiyapoom et al. 2010; Chen et al. 2012; Hoang et al. 2017), but the lithophytes remain in need of more study. The length of time that fungi can remain viable in orchid tissues from field collection to the lab is also unclear. Swarts and Dixon (2017) recommended that peloton extraction take place the same day of root collection, and indeed many studies have adopted this protocol (e.g., Aggarwal et al. 2012; Chutima et al. 2011; Otero et al. 2002; Pereira et al. 2005), as it is generally assumed that fungal pelotons lose their viability shortly after root detachment. Suárez et al. (2006), for example, reported that hyphae in Andean orchid tissues lose viability even after one night of storage in the laboratory regardless of chilling. Nevertheless, samples may be processed 3–4 days after collection if necessary (Swarts and Dixon 2017), or longer in some cases. Zettler et al. 2011, for example, recovered orchid endophytes from root samples that were collected 1-week prior. Similarly, Richardson et al. (1993) had success in isolating a diverse assemblage of Rhizoctonia-like fungi from epiphytes in Costa Rica up to 3 weeks after collection.

In Madagascar, where 90% of the island’s 1000 orchid species are endemic (Tyson 2000), deforestation on a massive scale has resulted in patches of fragmented orchid populations that persist from year to year with little natural regeneration (Whitman et al. 2011). Many orchid species in Madagascar and elsewhere are in dire need of study to keep pace with projected extinction rates this century. For researchers in distant countries who aspire to study these orchids for conservation purposes, the great distances involved, coupled with limited funds, pose formidable challenges. These challenges are often exacerbated by political unrest, delays in securing permits, unexpected hardships, and the risk that the biological samples collected (e.g., roots harboring mycorrhizal fungi) will not survive long-distance transport. As this study has shown, endophytes of orchids persist in root samples at least 3 weeks after they are collected in the field, as Richardson et al. (1993) reported earlier for epiphytes collected in Costa Rica.

In 2012, we were presented with a unique opportunity to collect and study endophytes of rare orchids native to the Itremo Massif Protected Area in the Central Highlands of Madagascar—one of the top five biologically diverse “hotspots” (Tyson 2000). Our primary goal was to isolate, identify, and safeguard Rhizoctonia-like fungi to facilitate orchid conservation in the region (e.g., symbiotic germination). The orchids targeted included endemic species (e.g., Angraecum potensum, Fig. 1), epiphytes (e.g., Bulbophyllum, Polystachya), terrestrials (e.g., Cynorkis flexuosa, Fig. 2), and lithophytes (e.g., Angraecum, Aerangis). One prevailing concern, however, was the great distances between our two labs and the Indian Ocean country (Illinois = 15,300 km, London = 9000 km), namely if orchid endophytes would remain viable in living tissues during lengthy, long-distance transport over rugged terrain and by air.

In this paper, we describe our protocol for the collection and long-distance transport of orchid root samples from Madagascar to labs in the United Kingdom (Kew) and the United States (Illinois) leading to isolation and provisional identification of the endophytes. The goal of this paper is to provide other researchers with a workable framework for recovering Rhizoctonia-like fungi from aged (2–3 week-old) tissue samples acquired from terrestrial, epiphytic, and lithophytic orchids in remote areas.

Methods
Permits
Logistic and taxonomic support for this joint study between Royal Botanic Gardens Kew (Kew) and Illinois College was facilitated by Kew Madagascar Conservation Centre (KMCC) and Parc Botanique et Zoologique de Tsimbazaza (PBZT). During the 5-year project, more than 40 taxa spanning 24 genera were selected for study involving collection and movement of live material (roots, seeds) from Madagascar to labs in the United Kingdom (UK) and the United States (USA). Root samples were shared between the two partners to ensure
that live material could be processed in a timely manner leading to the isolation of *Rhizoctonia*-like fungi from root pelotons. To facilitate the legal collection and international transport of orchid material from Madagascar to the UK (Kew) and USA (Illinois), a CITES permit was obtained which allowed three tubes, each containing seedlings and mature roots per species, to be collected. The rarity of plants in the wild necessitated further restrictions, namely that collections be limited to three each of juvenile and mature plants. Depending on the species and its availability, 1–5 roots per specimen were collected. These were accompanied by a phytosanitary certificate which was secured prior to departure from Madagascar. The CITES permit and phytosanitary certificate were delivered by the Direction Generale des Forets (DGF Nanisana Antananarivo) and Service de la Quarantaine et de l’Inspection Nanisana, respectively. For import of root samples into the USA, an additional permit (PPQ 526) was obtained from the US Department of Agriculture (USDA), as the US Government regards all orchid endophytes in the *Rhizoctonia* complex to be plant pathogens. Samples entering the UK were accompanied by a Phytosanitary Certificate issued in Madagascar, and a UK Letter of Authority.

**Dates and study sites in Madagascar**

Three separate trips to Madagascar took place spanning 4 years (2012–2015), with emphasis on the collection of roots from mature plants and spontaneous seedlings of lithophytes, epiphytes and terrestrials inhabiting the Itremo Massif Protected Area of the Central Highlands. The first two trips (June 2012, April/May 2013) were planned after the rainy season (December to March) in an effort to collect spontaneous seedlings on orchid-rich substrates that may have germinated under the wetter
conditions. The third trip (January 2015) took place during the rainy season to isolate additional fungal strains that may have been inactive during the previous two trips.

**Collection and long-distance transport**
The collection procedure reported by Yokoya et al. (2015) was employed for all three trips to Madagascar, and is summarized below. During the first trip, roots of mature orchids (= those that achieved anthesis or large enough to do so) were primarily targeted for collection to document the location of fungal pelotons in roots, and to isolate fungi in pure culture via hyphal tips. In the two trips that followed, emphasis was placed on collecting roots of seedlings and mature plants alike. Seedlings (= smallest leaf bearing stage, < 2 cm in length) and juveniles (plants > 2 cm in length, no anthesis) were provisionally identified on site by KMCC staff (Rajaovelona and Gardiner 2016) using subtle morphological features (e.g., presence of pseudobulbs) as well as proximity to mature plants on or near the same substrate. The identities of the seedlings were later confirmed by DNA analysis following the procedures described by Yokoya et al. (2015).

To maximize our chances for isolating viable pelotons, younger-appearing roots were collected whenever possible. For epiphytic and lithophytic orchids, these roots appeared translucent to white in color, often with slight greenish pigmentation near the apex (Figs. 1 and 3). For terrestrials, roots that exhibited orange-yellowish patches of color were selected, as well as seedlings in close proximity to mature plants (Fig. 4). After detachment in the field, each root was placed over a small, pre-moistened cotton ball within pre-sterilized glass vial with screw cap (Fig. 5). To permit gas exchange leading up to departure from Madagascar (7–10 days after collection), the caps placed on each vial were not securely tightened. The vials were then placed within a 50 ml capacity centrifuge tube with screw cap (VWR International, LLC, Radnor, PA, USA). 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 so that the root samples would remain as cool as possible (15–25 °C).

For terrestrial orchids, the root collection procedure differed slightly in that soil containing intact root systems (root ball) was also collected. This permitted the roots to remain in a semi-natural (moist) 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. A wet bath towel was then placed
through the zip to facilitate wicking and evaporation to the outside air serving to cool the inside of the bag. Upon arrival at the KMCC base in Antananarivo, 2–7 days after field collection, root samples and root balls were placed into a refrigerator (ca. 6 °C). Approximately 24 h before departure from the country, roots of terrestrial orchids were carefully lifted from soil and rinsed off with UV-irradiated and/or bottled water to remove organic debris to comply with US and UK important regulations. Lateral branch roots were detached and placed over a pre-moistened cotton ball in a pre-sterilized glass vial (8 ml capacity). The screw cap was then tightened firmly and wrapped with a strip of Parafilm “M” (Pechiney Plastic Packaging, Menasha, WI, USA). Caps on glass vials containing roots of lithophytes and epiphytes were also tightened and wrapped with Parafilm “M” at that same time (ca. 24 h 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”. Vials were re-packed into insulated handbags and transported back to labs in the USA and UK as cabin luggage to ensure that samples were maintained at ambient temperature during the duration of each flight.

**Fungal isolation and provisional identification**

Immediately upon arrival into the UK and USA, within 24 and 48 h after departure from Madagascar, respectively, all root samples were placed in refrigeration (4–6 °C) for a period lasting 1–2 weeks. Fungi were isolated from root cortical regions using the method by Zettler et al. (2003), but our procedure differed in that Fungal Isolation Medium (FIM) substituted for Modified Melin-Norkrans’ agar (MMN). Clumps of macerated cortical cells containing pelotons were immersed in FIM containing streptomycin sulfate [(10 ml/l of stock solution = 1 g dissolved in 70 ml); Clements and Ellyard 1979] and incubated at ambient temperature until actively-growing hyphae could be observed under a dissection microscope (typically 1–4 days). Hyphal tips from cortical cells and/or pelotons were then subcultured to Potato Dextrose Agar (PDA, Difco™, Becton, Dickinson and Co., Sparks, MD, USA) using a sterile scalpel. Provisional identification of Rhizoctonia-like fungi to genus level (Ceratobasidium, Sebacina, Tulasnella) reported herein was based on cultural descriptions reported by Currah et al. (1997).

**Results**

**Rhizoctonia-like fungi from Madagascar samples**

Pelotons were observed in mature roots in half of the lithophytes, half of the epiphytes, and both terrestrials (Table 1). Most of the pelotons were observed in the apex of the roots, in the 1–5 cm region from the tip (Table 1). Four of the seven lithophytic Angraecum species harbored pelotons (A. calceolus, A. longicalcar, A. magdalenae, A. rutenbergianum; Table 1). Once plated on agar, all pelotons yielded common conidial fungi (Fusarium sp., Trichoderma sp.), and none resulted in isolates that were assignable to the *Rhizoctonia* complex.

Following the second trip to Madagascar (April–May 2013), root samples returned to Illinois yielded *Rhizoctonia*-like fungi in two of the nine epiphytes (*Aerangis* sp., *Polystachya concreta*), both of which were seedlings (Table 2). Of the 14 terrestrial taxa sampled, roots from six species yielded endophytes assignable to the *Rhizoctonia* complex (*Benthamia rostratum*, *Cynorkis purpurea*, *Eulophia macra*, *Graphorkis concolor*, *Habenaria ambositana*, and *Tylostigma nigrescens*), but none were recovered from the seven lithophytes (Table 2). Collectively, less than half (40%) of the roots of terrestrial orchids spanning all three growth stages (seedlings, juveniles, mature plants) yielded *Rhizoctonia*-like fungi, and 20%
for the epiphytes (Table 2). Seedlings of *C. purpurea* harbored the most diverse assemblage of orchid endophytes with of all three major genera being represented in the samples (*Ceratobasidium, Tulasnella, Sebacina*).

Following the third and final trip to Madagascar coinciding with the rainy season (January 2015), a greater number of *Rhizoctonia*-like fungi were recovered from root samples collected 2–3 weeks prior, including a lithophyte, *Eulophia* sp. (Table 3). Among the six epiphytic taxa sampled, seedlings from *Aerangis* and *Polystachya* yielded *Ceratobasidium* and *Tulasnella* strains, respectively (Table 3). The majority of fungal isolates were acquired from terrestrials, in particular *Cynorkis* species (Table 3).

### Discussion

As to why none of the samples from the first trip yielded *Rhizoctonia*-like fungi is perplexing, but might be attributed, in part, to the time of year of the collecting. For example, sampling during the first trip took place in June, whereas the second took place earlier in the year (April/May) closer to the end of the rainy season. Thus, it is conceivable that the samples collected in June were devoid of *Rhizoctonia*-like fungi because of drier conditions. The two subsequent trips that ensued yielded endophytes assignable to all three major genera of orchid mycorrhizal fungi (*Ceratobasidium, Tulasnella, Sebacina*). For roots of epiphytic and lithophytic orchids, placing detached roots into pre-sterilized vials that remain unsealed possibly served to allow gas exchange (oxygen) for roots and endophytes alike. Moist sterile cotton balls within the vials also may have maintained higher relative humidity levels needed for root and fungus longevity. For terrestrial orchids, keeping roots intact within the moist soil/root ball may have contributed to the high number of *Rhizoctonia*-like fungi acquired from the samples. Placing the vials in an insulated bag during field work, and temporary storage in refrigeration whenever possible, may have also benefited endophyte survival by maintaining cooler temperatures, slowing down metabolic rates in living cells.

Root colonization by endophytic fungi is thought to be influenced by two important factors—the growing season, and the growth stage of the plant (Swarts and Dixon 2017). For temperate terrestrial orchids, Harvais and Raitsakas (1975) and Warcup (1973) found that the most effective fungi for the purposes of seed germination were acquired earlier in the growing season. Huynh et al. (2004) reported that fungi isolated from pelotons in early growth stages were most effective at facilitating seed germination. A prevailing concern we had for collecting smaller roots of seedlings and juvenile stages during the dry season (April/May 2013) was dehydration of the samples given their high surface-to-volume ratio. This potential problem was

### Table 1 Peloton location by root region for orchid samples acquired in the Itremo Massif within the Central Highlands of Madagascar during the first of three trips (June 2012, dry season)

| Growth habit | Orchid species          | n   | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 |
|--------------|-------------------------|-----|----|----|----|----|----|----|----|----|----|----|
| Lithophytic  | *Angraecum calceolus*   | 2/2 | X  |   | X  |   |   |   |   |    |    |    |
|              | *Angraecum longicalcar* | 1/3 |    |   | X  |   |   |   |   |    |    |    |
|              | *Angraecum magdalenae*  | 1/2 |    |   |   |   |   |   |   |    |    |    |
|              | *Angraecum obesum*      | 0/1 |    |   |   |   |   |   |   |    |    |    |
|              | *Angraecum protensum*   | 0/1 |    |   |   |   |   |   |   |    |    |    |
|              | *Angraecum sororium*    | 0/2 |    |   |   |   |   |   |   |    |    |    |
|              | *Angraecum rutenbergianum* | 1/1 | X  | X |    |    |    |    |    |    |    |
|              | *Jumellea ibityana*     | 0/2 |    |   |   |   |   |   |   |    |    |    |
| Epiphytic    | *Bulbophyllum* sp.      | 3/3 | X  | X |    |    |    |    |    |    |    |    |
|              | *Bulbophyllum bicoloratum* | 0/2 |    |   |   |   |   |   |   |    |    |    |
|              | *Jumellea* sp.          | 1/4 | X  |    |    |    |    |    |    |    |    |    |
|              | *Jumellea arborescens*  | 0/2 |    |   |   |   |   |   |   |    |    |    |
|              | *Jumellea intricata*    | 1/2 | X  |    |    |    |    |    |    |    |    |    |
|              | *Polystachya* sp.       | 0/1 |    |   |   |   |   |   |   |    |    |    |
|              | *Polystachya cultriformis* | 1/2 |    |   |   |   |   |   |   |    |    |    |
|              | *Polystachya fusiformis* | 0/1 |    |   |   |   |   |   |   |    |    |    |
| Terrestrial  | *Cynorkis* sp.          | 1/1 | X  |    |    |    |    |    |    |    |    |    |
|              | *Eulophia* sp.          | 1/2 | X  |    |    |    |    |    |    |    |    |    |

Numbers 1–10 reflect the distance (in cm) from the root tip (e.g., 1 terminal end of actively-growing tip, 2 second cm region from root tip), and n the number of roots harboring pelotons/number of roots collected.
apparently avoided by the addition of a moist (sterile) cotton ball placed inside the vial with the sample, as several of *Rhizoctonia*-like isolates were later recovered. Some of these isolates were later tested for their ability to germinate seeds in vitro, with positive results. For example, one of the six strains of *Sebacina*, isolated from a *P. concreta* seedling, was most effective among 14 endophytes tested at inducing rapid in vitro seedling development of *C. purpurea* in symbiotic germination studies that ensued (Rafter et al. 2016). In another experiment, seeds of *H. ambositrana* and *T. nigrescens*, yielded leaf-bearing seedlings in vitro, 49 days after inoculation with *Sebacina* and *Tulasnella* endophytes acquired from seedlings of the same species, respectively (A. Wood, unpub. data).

### Table 2 A summary of the frequency of *Rhizoctonia*-like fungi acquired from roots of orchids inhabiting the Itremo Masif of the Central Highlands of Madagascar during April–May 2013 (dry season)

| Growth habit | Orchid | Site | Sample | Fungus (# strains) |
|--------------|--------|------|--------|-------------------|
| Lithophytic  | *Angraecum coutrixii* | 1, 3 | Seedling | None |
|              | *Angraecum longicalcar* | 2 | Mature | None |
|              | *Angraecum magdalena* | 3 | Seedling | None |
|              | *Angraecum protensum* | 1 | Seedling | None |
|              | *Angraecum rutenbergianum* | 1 | Seedling | None |
|              | *Angraecum sororium* | 3 | Seedling | None |
|              | *Oeceoclades sp.* | 2 | Mature | None |
| Epiphytic    | *Aerangis sp.* | 7 | Seedling | *Ceratobasidium* (3) |
|              | *Aerangus citata* | 5 | Seedling | None |
|              | *Angraecum sp.* | 5 | Seedling | None |
|              | *Angraecum protensum* | 1 | Seedling | None |
|              | *Angraecum rutenbergianum* | 3 | Seedling | None |
|              | *Bulbophyllum sp.* | 3 | Seedling | None |
|              | *Jumellea densifoliata* | 5 | Seedling | None |
|              | *Polystachya concreta* | 1 | Seedling 1 | None |
|              |              |     | Seedling 2 | *Tulasnella* (7), *Sebacina* (6) |
|              | *Polystachya cultiformis* | 7 | Seedling | None |
| Terrestrial  | *Benthamia sp.* | 1 | Mature | None |
|              | *Benthamia glaberrima* | 3 | Mature | None |
|              | *Benthamia rastrum* | 4 | Juvenile | *Tulasnella* (1) |
|              | *Calanthe sp.* | 7 | Mature | None |
|              | *Cynorkis gibbosa* | 7 | Mature | None |
|              | *Cynorkis purpurea* | 7 | Seedling | *Ceratobasidium* (7)
|              |              |     |          | *Tulasnella* (3), *Sebacina* (1) |
|              | *Disa incarnata* | 3 | Mature | None |
|              | *Eulophia macra* | 2 | Mature | *Tulasnella calliospora* (1) |
|              | *Graphorkis concolor* | 7 | Mature | *Ceratobasidium* (1) |
|              | *Habenaria sp.* | 1 | Mature | None |
|              | *Habenaria ambositrana* | 1 | Juvenile | None |
|              |              |     | Seedling | *Tulasnella* (4), *sebacina* (1) |
|              | *Satyrium trinerve* | 4 | Mature | None |
|              | *Tylostigma sp.* | 4 | Mature | None |
|              | *Tylostigma nigrescens* | 4 | Seedling | *Tulasnella* (5) |

Fungal genera listed represent provisional identifications carried out at the time of isolation, based on cultural characteristics described by Currah et al. (1997).

Growth habit reflects the substrate where the individual orchid was actually rooted at the time of collection. Collection sites: 1 exposed rocks, occasional tapia trees, 2 exposed marble outcrop, 3 exposed rocks, sandy stream bed, gnarled small trees, 4 open grassland, moist soil, occasional rocks, 5 reduced forest (canopy ca. 20 m), 6 exposed ridges, montane vegetation, 7 dense shaded forest, downhill stream. With one exception (2), all sites were within 5 km of one another.

Terrestrial seedlings = 3/3, epiphytic seedlings = 2/10, lithophytic seedlings = 0/5
Terrestrial juveniles = 1/2, epiphytic juveniles = NA, lithophytic juveniles = NA
Terrestrial mature = 2/10, epiphytic mature = NA, lithophytic mature = 0/3
Total terrestrial = 6/15 (40%), total epiphytic = 2/10 (20%), total lithophytic = 0/8 (0%)
To what extent mature epiphytic orchids harbor and utilize Rhizoctonia-like fungi in Madagascar needs further study. Due to permit restrictions, only a select few taxa could be collected the third and final year of the study, and the decision was made to target orchid tissues that were most likely to harbor Rhizoctonia-like fungi, namely seedlings. Roots collected from the lone mature epiphyte (Bulbophyllum baronii) were devoid of pelotons, and therefore no endophytes were isolated (Table 3). For future work on the Itremo Massif and the Central Highlands in general, we recommend that roots of mature epiphytes be collected during the rainy season (January). For epiphytic orchids occupying Madagascar’s eastern and northern forests where rainfall is more plentiful throughout the year (> 2000 mm annually; Cribb and Hermans 2009), time of collection may be less critical, as higher moisture levels would be expected to favor fungal activity. To our knowledge, no studies have been reported that document Rhizoctonia-like fungi from Orchidaceae inhabiting the NE portion of the island. Given Madagascar’s considerable biodiversity, securing fungi from both areas and comparing the orchid mycoflora between the two regions seems like a logical next step.

### Table 3 A summary of the frequency of Rhizoctonia-like fungi acquired from roots of orchids inhabiting the Itremo Massif of the Central Highlands of Madagascar during January 2015 (rainy season)

| Growth habit | Orchid | Site | Sample | # Strains and fungus |
|--------------|--------|------|--------|----------------------|
| Lithophytic  | Eulophia plataginea | 1 | Juvenile | None |
|              | Habenaria sp.1 | 1 | Juvenile, mature | None |
| Epiphytic    | Eulophia sp. 1 | 1 | Seedling | Tulasnella (1) |
|              | Jumellea densifolia | 2 | Seedling | None |
|              | Angraecum sp. 1 | 6 | Mature | None |
|              | Aerasnthes sp. 1 | 6 | Juvenile, mature | None |
|              | Bulbophyllum baronii | 2 | Mature | None |
|              | Polystachya sp. 1 | 6 | Seedling | Tulasnella (4) |
|              | Polystachya concreta | 3 | Seedling | None |
|              | Angraecum sp. 2 | 2 | Mature | None |
|              | Benthamia sp. 1 | 3 | Mature | None |
|              | Calanthe sylvatica | 5 | Mature | None |
|              | Cynorkis sp. 1 | 3 | Mature | Ceratobasidium (2) |
|              | Cynorkis sp. 1 | 4 | Mature | Tulasnella, sebacina (2) |
|              | Cynorkis sp. 1 | 7 | Mature | None |
|              | Cynorkis fastigiata | 4 | Mature | Ceratobasidium (1) |
|              | Cynorkis fastigiata | 6 | Mature | Tulasnella (3) |
|              | Cynorkis flexuosa | 1 | Mature | None |
|              | Cynorkis flexuosa | 1 | Seedling | Tulasnella (2) |
|              | Cynorkis gibbosa | 4 | Mature | Tulasnella (4) |
|              | Cynorkis gibbosa | 4 | Juvenile | None |
|              | Cynorkis gibbosa | 7 | Seedling | None |
|              | Cynorkis uniflora | 3 | Seedling | Ceratobasidium (1) |
|              | Cynorkis uniflora | 3 | Mature | None |
|              | Cynorkis uniflora | 7 | Mature | None |
|              | Disa sp. 1 | 4 | Mature | None |
|              | Eulophia sp. 1 | 1 | Seedling | None |

### Table 3 continued

| Growth habit | Orchid | Site | Sample | # Strains and fungus |
|--------------|--------|------|--------|----------------------|
|              | Eulophia plataginea | 1 | Juvenile | None |
|              | Habenaria sp.1 | 1 | Juvenile, mature | None |
| Terrestrial  | Eulophia pliataginea | 1 | Juvenile | None |
|              | Habenaria sp. 1 | 2 | Seedling | Tulasnella (1) |
|              | Habenaria sp. 2 | 4 | Mature | Tulasnella (1) |
|              | Jumellea sp. 1 | 1 | Mature | None |
|              | Polystachya sp. 1 | 5 | Seedling | Tulasnella (1) |
|              | Satyrium sp. 1 | 5 | Seedling | Tulasnella (1) |
|              | Satyrium trinerve | 3 | Mature | None |
|              | Satyrium trinerve | 4 | Seedling | Tulasnella (4) |

Fungal genera listed represent provisional identifications carried out at the time of isolation, based on cultural characteristics described by Currah et al. (1997). Growth habit reflects the substrate where the individual orchid was actually rooted at the time of collection. Collection sites: 1 abandoned mine, rocky grassland, 2 tapia forest, 3 seepage slope, 4 grassland, seepage slope, 5 forest preserve, 6 forest, 7 rocky elevated grassland. Terrestrial seedlings = 6/8, epiphytic seedlings = 2/4, lithophytic seedlings = 0/2. Terrestrial juveniles = 0/3, epiphytic juveniles = 0/2, lithophytic juveniles = NA. Terrestrial mature = 6/18, epiphytic mature = 1/4, lithophytic mature = 1/1. Total terrestrial = 12/29 (41%), total epiphytic = 3/10 (30%), total lithophytic = 1/3 (33%).
As a group, roots of mature lithophytic orchids yielded relatively few *Rhizoctonia*-like fungi with one exception (*Tulasnella* from *Eulophia* sp. 1, Table 3). Mature roots from six lithophytes collected the first year harbored pelotons primarily in the first 4 cm of the root tip (Table 1), yet these pelotons yielded conidial saprophytes (*Fusarium, Trichoderma*), not typical genera in the *Rhizoctonia* complex. As to why this occurred is puzzling and deserves further inquiry. It is conceivable that roots of these lithophytes were subjected to rapid dehydration given their dry placement on sun-exposed rocks with little or no associated (moist) organic debris (Fig. 3). Thus, the pelotons we observed in the tissues may have been formed by *Rhizoctonia*-like fungi during the rainy season, but quickly dried out before the pelotons could be completely digested by the orchid (lysis). Opportunistic saprophytes may then have gained entry as secondary invaders, which may explain why we isolated *Fusarium* in the present study, as well as slow-growing, dark-pigmented endophytes (*Toxocladosporium, Cladophialophora, Lophiostoma*) recovered in samples at Kew (Yokoya et al. 2015). Studies are needed to explore the true nature of the peloton-forming, non-*Rhizoctonia* fungi to determine if these endophytes are potentially harmful and/or benign inhabitants, or if they serve a physiological purpose.

Moisture availability (retention) linked with seasonality may also explain why more *Rhizoctonia*-like fungi were isolated from epiphytes compared to lithophytes. For example, roots of many of the epiphytes we sampled were tightly affixed to crevices of tree bark often in association with lichens and mosses. All three of these substrates would be expected to retain water more effectively than bare rock alone, and could also serve as a carbon source for associated fungi, including the *Rhizoctonia*-like fungi present within the orchid. Members of *Ceratobasidium*, in particular, are known to produce polyphenoloxidases that are involved with lignin breakdown (Rasmussen 1995), and this may explain why roots of some epiphytes, namely *Aerangis*, harbored mostly *Ceratobasidium* (Tables 2 and 3). Thus, the utilization of *Ceratobasidium* strains may afford epiphytes like *Aerangis* with an additional source of organic carbon, and therefore a selective advantage for life in the canopy. For lithophytes, utilization of *Ceratobasidium* at an early (protocorm) stage of development may be critical to life on the rocks in pockets where organic debris and moisture accumulate.

**Conclusions**

Despite the distance, rugged terrain, and length of time between field collection and transport to the laboratory, roots of epiphytic, lithophytic and terrestrial orchids yielded all major groups of fungi in the *Rhizoctonia* complex (*Ceratobasidium, Tulasnella, Sebacina*). These fungi were present in roots of seedlings, juveniles and mature plants, especially terrestrials. Despite their small size, root pieces from seedlings stages of epiphytes (e.g., *P. concreta*) yielded fungi with our method despite being detached in the field weeks prior. In cases where pelot on extraction cannot be accomplished in a timely manner (1–4 days of collection), our study demonstrates that samples retain viability up to 3 weeks after collection.

**Authors’ contributions**

LWZ, KY, JPK and ALS devised the techniques for sample collection and transport. LWZ, LR, KY, JPK, ALS and AEW performed field research in Madagascar, and LR identified Orchidaceae collected. VS headed the team effort. LWZ wrote the manuscript. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

**An explanation of why your manuscript should be published in Botanical Studies**

This manuscript provides details for collection and long-distance transport of root samples from orchids for fungal isolation. The special issue on Orchid Conservation by Botanical Studies is a suitable place for it to be published because the journal has a wide readership for those interested in orchids and their conservation.

**An explanation of any issues relating to journal policies**

Not applicable.

**Availability of data and materials**

The authors confirm that local, national and international guidelines and legislation are followed by acquiring appropriate permissions and licenses for the study.

**Confirmation that all authors have approved the manuscript for submission**

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References

Aggarwal S, Nirmala C, Beri S, Rastogi S, Adholeya A (2012) In vitro symbiotic seed germination and molecular characterization of associated endophytic fungi in a commercially important and endangered Indian orchid, Vanda coerulea Griff. ex Lindl. Eur J Environ Sci 21(1):33042

Chen J, Wang H, Guo SX (2012) Isolation and identification of endophytic and mycorrhizal fungi from seeds and roots of Dendrobinum (Orchidaceae). Mycorrhiza 22:297–307

Chutima R, Dell B, Vessabutr S, Bussaban B, Lumyong S (2011) Endophytic fungi from Pecteilis susannae (L.) Rafin (Orchidaceae), a threatened terrestrial orchid in Thailand. Mycorrhiza 21(3):221–229

Clements MA, Ellyard RK (1979) The symbiotic germination of Australian terrestrial orchids. Am Orchid Soc Bull 48:810–816

Cribb PJ, Hermans J (2009) Field guide to the orchids of Madagascar. Kew Publishing, Royal Botanic Gardens, Kew

Cribb PJ, Kell SP, Dixon KW, Barrett RL (2003) Orchid conservation: a global perspective. (Chapter 1, pp 1–24). Mycorrhizal diversity (Chapter 11, pp 185–203). In: Dixon KW, Kell SP, Barrett RL, Cribb PJ (eds) Orchid conservation. Natural History Publications, Kota Kinabalu, pp 205–226 (ISBN 983-812-078-2)

Curras RS, Sigler L, Hambleton S (1987) New records and new taxa of fungi from the mycorrhizae of terrestrial orchids of Alberta. Can J Bot 65:2473–2482

Curras RS, Zelmer CD, Hambleton S, Richardson KA (1997) Fungi from orchid mycorrhizas. In: Arditto J, Pridgeon AM (eds) Orchid biology: reviews and perspectives, Vol. Springer, Netherlands, pp 117–170

Dressler RL (1993) Phylogeny and classification of the orchid family. Dioscorides Press, Portland

Harvais G, Ratsakasa A (1975) On the physiology of a fungus symbiotic with orchids. Can J Bot 53:144–155

Hoang NH, Kane ME, Radcliffe EN, Zettler LW, Richardson LW (2017) Comparative seed germination and seedling development of the Ghost Orchid, Dendrophylax lindenii (Orchidaceae), and molecular identification of its mycorrhizal fungus from south Florida. Ann Bot 119(3):379–393

Huynh TT, McLean CB, Coates F, Lawrie AC (2004) Effect of developmental stage and peloton morphology on success in isolation of mycorrhizal fungi in Caladenia formosa (Orchidaceae). Aust J Bot 52(2):231–241

Jacomyn J, Hysy T, Bres R, Merckx V, Waud M, Lievens B, Wiegand T (2014) Coexisting orchids species have distinct mycorrhizal communities and display strong spatial segregation. New Phytol 202(2):616–627

McCormick MK, Taylor DL, Juhasova K, Burnett RK, Whigham DF, O’Neill JP (2012) Limitations on orchid recruitment: not a simple picture. Mol Ecol 21(6):511–523

Merritt DJ, Hay FR, Swarts ND, Sommerville KD, Dixon KW (2014) Ex situ conservation and cryopreservation of orchid germplasm. Int J Plant Sci 175:46–58

Moore-Landecker E (1996) Fundamentals of the fungi. Prentice Hall, Upper Saddle River

Nontachayapoom S, Sasirat S, Manooh C (2010) Isolation and identification of Rhizoctonia-like fungi from roots of three orchid genera, Paphiopedilum, Dendrobium, and Cymbidium, collected in Chiang Rai and Chiang Mai provinces of Thailand. Mycorrhiza 20:459–471

Otero JT, Ackerman JD, Bayman P (2002) Diversity and host specificity of endophytic Rhizoctonia-like fungi from tropical orchids. Am J Bot 89(1):1852–1858

Pereira OL, Rollemberg CL, Borges AC, Matsouka K, Kasuya MCM (2003) Epulorhiza-epiphytica sp. nov. isolated from mycorrhizal roots in Brazil. Mycoscience 44:153–155

Pereira OL, Kasuya MCM, Borges AC, de Araújo EF (2005) Morphological and molecular characterization of mycorrhizal fungi isolated from neotropical orchids in Brazil. Can J Bot 83:54–65

Rafter M, Yokoya K, Schofield EJ, Zettler LW, Sarasan V (2016) Non-specific symbiotic germination of Cynorkis purpurea (Thouars) Kraezl., a habitat-specific terrestrial orchid from the Central Highlands of Madagascar. Mycorrhiza 26(6):541–552. https://doi.org/10.1007/s00572-016-0691-6

Rajaevulona L, Gardiner LM (2016) Angraecum longicalcar: saving a critically endangered orchid. Orchid Rev 124(314):95–99

Rasmussen HN (1995) Terrestrial orchids, from seed to mycotrophic plant. Cambridge University Press, Cambridge

Rasmussen HN, Rasmussen FN (2009) Orchid mycorrhiza: implications of a mycophaugous life style. Oikos 118(3):334–345

Richardson KA, Curras RS, Hambleton S (1993) Basidiomycetous endophytes from the roots of neotropical epiphytic Orchidaceae. Lindleyana 8:127–137

Suarez JP, Weiß M, Abele A, Garnica S, Obenwinkler F, Kotsek I (2006) Diverse tulasieloid fungi form mycorrhizas with epiphytic orchids in the Andean cloud forest. Mycol Res 110:1257–1270

Swarts ND, Dixon KW (2009) Terrestrial orchid conservation in the age of extinction. Ann Bot 105:543–556

Swarts ND, Dixon KW (2017) Conservation methods for terrestrial orchids. J Ross Publishing, Plantation

Swarts ND, Sinclair EA, Francis A, Dixon KW (2010) Ecological specialization in mycorrhizal symbiosis leads to rarity in an endangered orchid. Mol Ecol 19(15):3226–3242

Tyson P (2000) The eighth continent: life, death and discovery in the lost world of Madagascar. William Morrow (Harper Collins) Publishers, New York, p 374

Warcup JH (1973) Symbiotic germination of some Australian terrestrial orchids. New Phytol 72:387–392

Warcup JH (1981) The mycorrhizal relationships of Australian orchids. New Phytol 87:371–381

Whitman M, Medler M, Randrianiamandry JJ, Rabakonandrianina E (2011) Conservation of Madagascar’s granite outcrop orchids: the influence of fire and moisture. Lankesteriana Int J Orchidol 11:55–67

Yokoya K, Zettler LW, Kendon JP, Bidartondo M, Stice AL, Skarha S, Corey LL, Knight A, Sarasan V (2015) Preliminary findings on identification of mycorrhizal fungi from diverse orchids in the Central Highlands of Madagascar. Mycorrhiza 25:611–625. https://doi.org/10.1007/s00572-015-0635-6

Zelmer CD, Cuthbertson L, Curras RS (1996) Fungi associated with terrestrial orchid mycorrhizas, seeds and protocorms. Mycoscience 37(4):439–448

Zettler LW, Sharma J, Rasmussen F (2003) Mycorrhizal diversity (Chapter 11, pp 185–203). In: Dixon KW, Kell SP, Barrett RL, Cribb PJ (eds) Orchid conservation and reintroduction of the US federally endangered Hawaiian endemic, Phalanthera holochilus (Hbld.) Krezl. (Orchidaceae). Eur J Environ Sci 1(2):69–70