Uncovering the mycorrhizal community of two *Habenaria* orchids in South Africa

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Abstract

Southern Africa is a biodiversity hotspot for a variety of orchids, including *Habenaria*. However, we know very little about the orchid mycorrhizae that are associated with these orchids. To bridge this gap, we compared the community of orchid mycorrhizae that are associated with two indigenous *Habenaria* species, *H. barbertoni* and *H. epipactidea*, using a high-throughput sequencing platform. We selected these two orchids because their distribution zones overlap in South Africa. Furthermore, *H. barbertoni* is endangered, whereas *H. epipactidea* is not. We hypothesised that the mycorrhizal diversity and composition linked with the roots of these two orchids would overlap, but that some distinct fungal taxa would exist, and these distinct fungi would include unusual taxa. Analyses of the DNA sequence data revealed that the two orchids shared 35 fungal OTUs. Twenty-four and seventeen OTUs were exclusively detected in the roots of *H. barbertoni* and *H. epipactidea*, respectively. Mycorrhizal

fungi from the rust lineage Atractiellales (Atractiellomycetes, Pucciniomycotina) were only detected in the roots of the endangered *H. barbertoni*, which represents the first report of these fungi associated with orchids outside of the Andean rainforest. Our findings increase knowledge of the diversity of mycorrhizae associated with indigenous orchids on the African continent.

Keywords Agaricomycetes, Atractiellomycetes, *Habenaria*, orchid mycorrhizae, Pleosporales

1. Introduction

The Orchidaceae is one of the largest and most diverse families of flowering plants (Givnish et al. 2015; Swarts and Dixon 2009). This diversity is reflected in the ability of the plants to thrive in a wide range of habitats and climatic zones, including tropical, subtropical, and alpine environments (Fay 2010). Orchids have a variety of unusual morphological and physiological characteristics that help them adapt to different ecological environments, such as symbiotic associations with specialised pollinators and mycorrhizal fungi (Byers 2021; Chase et al. 2015; Favre-Godal et al. 2020; Štípková et al. 2020; Tremblay 1992).

Orchid mycorrhizae are fungi that symbiotically associate with orchid roots (Dearnaley 2007; Rasmussen and Rasmussen 2009). These fungi are a fundamental part of the orchid's life cycle because they assist in the germination of seeds and nutrient acquisition throughout the adult orchid's life (Favre-Godal et al. 2020). The vast majority of orchid mycorrhizae are members of the lineage Basidiomycota, with only a few Ascomycota described so far (Dearnaley 2007; Favre-Godal et al. 2020). Orchids were once thought to exclusively associate with fungi from the '*Rhizoctonia*' group (Dearnaley et al. 2012). Newer research has shown that orchid mycorrhizae originate from diverse fungal lineages (Jacquemyn et al. 2015; Kottke et

al. 2008; Kottke et al. 2010; Martos et al. 2009; Martos et al. 2012; McCormick et al. 2018; Oja et al. 2015; Suárez et al. 2006; Suárez et al. 2008; Valadares et al. 2021). However, our knowledge of the mycorrhizal diversity associated with some ubiquitous orchid genera, such as *Habenaria*, is limited.

Habenaria is one of the largest genera of terrestrial orchids with 835 described species (Govaerts et al. 2021). Species from this genus are widely distributed in the tropics and subtropics, extending into temperate areas (Pridgeon et al. 2001). Southern Africa represents one of the major centres of diversity for this genus (Kurzweil and Weber 1992). In South Africa, 29 *Habenaria* species have been identified along the entire eastern coastline (Johnson and Bytebier 2015). Several of these species thrive only in small refugia, such as *H. barbertoni*, *H. bicolor*, *H. humilior*, *H. kraenzliniana* and *H. mossii* while others, such as *H. epipactidea*, *H. arenaria*, *H. clavata*, *H. dregeana* and *H. falcicornis*, are widely distributed (Johnson and Bytebier 2015). Echoing the global trend, several South African *Habenaria* species are becoming rarer and are on the brink of extinction (SANBI Red List of South African Plants 2022, http://redlist.sanbi.org/genus.php?genus=2829)

In this study, we catalogued and compared the diversity of mycorrhizal fungi associated with the roots of two native *Habenaria* species from South Africa, *H. barbertoni* and *H. epipactidea* (Fig. 1A, B). In South Africa, both of these orchid species have overlapping distribution zones, but *H. barbertoni* is of conservation concern, whilst the *H. epipactidea* is not. We hypothesized that (1) the mycorrhizal diversity and composition associated with the roots of these two orchid species would be similar, but that some unique fungal taxa would exist in each species; and (2) the fungi which associate with the endangered *H. barbertoni* will include unusual taxa.

2. Materials and methods

2.1 Collection of orchid samples

In May 2018, three samples each of *Habenaria barbertoni* and *Habenaria epipactidea* were collected from a plot (-25.912111, 28.418389) near Pretoria, South Africa (Fig 1C, D). The two orchid populations in this plot were separated by a linear distance of less than 400 m.

After collection, roots and tubers from each plant were repeatedly rinsed with tap water followed by sterile deionised water. Roots and tuber samples were dried using paper towels and stored at -20 °C until the extraction of DNA.

2.2 Extraction of DNA from orchid roots

All frozen root samples from both orchid species were separately homogenised using liquid nitrogen. Between homogenization of different plant samples, the mortar and pestle were surface sterilised with a 1% (v/v) sodium hypochlorite solution, followed by repeated rinsing with sterile deionized water. Total genomic DNA was extracted from 50 mg of homogenised root tissues using the MoBio Dneasy Powerplant Pro DNA Isolation Kit (Qiagen, Germany) according to the manufacturer's instructions. The concentration of each DNA sample was quantified using a NanoDrop™ 2000 spectrophotometer. Thereafter, all DNA samples were stored at -20 °C until PCR amplification of the internal transcribed spacer (ITS) of mycorrhizal fungi.

2.3 Amplification of fungi from root DNA extracts

For each DNA sample, two sets of PCR reactions were performed targeting the complete ITS region (ITS1 spacer region -5.8S gene -ITS2 spacer region) using orchid mycorrhizae specific primers. The first set of five technical replicates per sample was amplified using the primers ITS1-OF and ITS4-OF (Taylor and McCormick 2008). The second set of five technical

replicates was amplified using the primers ITS1 and ITS4-Tul (Figure S1; Taylor and McCormick 2008; White et al. 1990). The second set of amplifications aimed to capture the diversity of fungi from the Tulasnellaceae (Taylor and McCormick 2008).

PCR reactions of 25 µl included 5 µl of 5 × GoTaq Flexi Buffer (Promega, MI), 2.5 µl of $MgCl_2$ (Promega, MI), 0.1 µl of dNTPs (Promega, MI), 1 µl of BSA (Amresco, OH), 0.125 µl of GoTaq Hot Start Polymerase (Promega, MI), 0.5 µl of each primer, 2 µl (conc. 20 ng/µl) of DNA extracted from roots and the final volume was made up with PCR grade water. The PCR temperature cycling for each primer pair was 96 °C for 2 min, followed by 30 cycles of 94 ˚C for 30 sec, 60 ˚C for 40 sec (ITS1-OF + ITS4-OF) / 54 ˚C for 40 sec (ITS1 + ITS4-Tul), 72˚C for 1 min, and a final elongation for 72 ˚C for 10 min (Taylor and McCormick 2008). Positive amplification of the gene regions was verified using gel electrophoresis.

2.4 Pooling of PCR products

To determine the relative concentrations of the PCR products, the gel images were analysed using the software ImageJ v1.52q (Figure S1; Schneider et al. 2012). To standardize the DNA contribution, the PCR replicates for each DNA sample were pooled into a single unit based on the band intensity. Thereafter, 25 µl of each pooled PCR product was purified twice using Agencourt AMPure XP PCR purification beads (Beckman Coulter Genomics, USA) and visualized using gel electrophoresis (Figure S1).

2.5 Preparation and sequencing of amplicon libraries

Purified PCR products from the pooling step were submitted to Inqaba Biotechnical Industries (Pty) Ltd, South Africa for amplicon library preparation and Illumina MiSeq sequencing. The raw Illumina MiSeq sequencing data were deposited in the NCBI Sequence

Read Archive (https://submit.ncbi.nlm.nih.gov/ subs/sra/) under the accession number PRJNA693177.

2.6 Bioinformatics analyses of high-throughput sequence data

The single-end high-throughput sequencing data were demultiplexed by the sequencing facility. Fungal ITS 1 data were analysed using the bioinformatics workflow provided by Quantitative Insights into Microbial Ecology 2 (QIIME 2) v2020.8 (Bolyen et al. 2019). Filtering, trimming, denoising, and removing singletons and chimaeras were accomplished using the 'q2-dada2' plugin (Callaham et al. 2016). During this step, the filtering settings were set at a Phred quality score of 30 and a sequence length cut-off of 200 bp. All sequences that did not fulfil these criteria were removed from the analyses. The ‘q2-vsearch' plugin (Rognes et al. 2016) was used for *de novo* assembly of the reads with 98 % sequence similarity. Using the UNITE fungal ITS database v8.3 (Abarenkov et al. 2021) as a reference, the ‘qiime feature-classifier' (Rognes et al. 2016) was used to assign taxonomy to the operational taxonomic units (OTUs).

2.7 Mycorrhizal community diversity and composition

The mycorrhizal taxonomic composition of the two orchid species (*H. barbertoni* and *H. epipactidea*) was summarised in a taxonomic tree using the ‘MetacodeR’ package of the R software (Foster et al. 2016; R Core Team 2021). Moreover, a heat map depicting the abundance of taxa between the two orchid species was constructed using the plot heatmap function available through the ‘phyloseq’ package (McMurdie and Holmes 2013) of R software.

To analyse the mycorrhizal diversity of the two orchid species, richness, and Shannon and Simpson diversity indices were calculated for each sample. To analyse mycorrhizal species

richness, the number of taxa per sample was calculated. The effect of the different orchid species on the richness and each diversity index was analysed using a one-way ANOVA followed by a Tukey's Honest Significant Difference (HSD) *post hoc* test to do pairwise comparisons of the means. In all cases, model validity was checked and the 'agricolae' package of the R software was used to analyse mycorrhizal diversity (de Mendiburu 2021; R Core Team 2021).

Differences among the mycorrhizal community composition of *H. barbertoni* and *H. epipactidea* were depicted using a Principal Coordinate Analysis (PCoA). PCoA was conducted on an abundance matrix using Bray-Curtis dissimilarity. To assess whether the two orchid species showed statistically different mycorrhizal community composition, we employed a permutational multivariate analysis of variance (PERMANOVA). The vegan package of the R software (Oksanen et al. 2020; R Core Team 2021) was used to analyse the community composition.

3. Results

3.1 Bioinformatics analyses of high-throughput sequence data

High-throughput sequencing of six root DNA samples resulted in 292,232 raw reads. After quality filtering, 236,419 reads were used for downstream analysis. Assembling of these filtered reads corresponded to 75 fungal OTUs from the phyla Ascomycota (61.3%), Basidiomycota (34.6%), Mortierellomycota (2.6%), and Mucoromycota (1.3%) (Fig. 2, 3).

The fungal OTUs identified in the roots of the two orchid species overlapped but their presence differed amongst orchid species (Fig. 3). For example, fungal OTUs from the orchid mycorrhizal orders Cantharellales and Sebacinales were more abundant in the roots of *H. barbertoni* than in the roots of *H. epipactidea* (Fig. 3). Twenty-four fungal OTUs were unique

to the roots of *H. barbertoni*. Some of these distinct fungal OTUs belonged to the orders Pleosporales, Sebacinales, and Atractiellales (Fig. 3). Sixteen OTUs were unique in *H. epipactidea,* which included taxa from the Pleosporales and Sebacinales (Fig. 3).

A total of 59 fungal OTUs were detected from the roots of *H. barbertoni* (Fig. 3) of which 35 OTUs represented Ascomycota, 17 Basidiomycota while the remaining were from Mortierellomycota and Mucoromycota (Fig. 3). Three fungal orders containing orchid mycorrhizae were detected from the Basidiomycota. These were the Cantharellales (3 OTUs), Sebacinales (1 OTU), and Atractiellales (2 OTUs) (Fig. 3).

Habenaria epipactidea roots harboured a total of 51 fungal OTUs (Fig. 3). Among these, 30 OTUs represented Ascomycota, 18 were from the Basidiomycota, and the rest were from Mortierellomycota and Mucoromycota (Fig. 3). Among the Basidiomycota OTUs, two orders were detected with previously described orchid mycorrhizae. These are the Cantharellales (3 OTUs), and Sebacinales (1 OTU) (Fig. 3).

Apart from orchid mycorrhizal fungi, other fungi were also detected in the orchid roots. These fungi were from the orders Pleosporales, Capnodiales, Chaetothyriales, Hypocreales, Sordariales, Trichosphaeriales, Xylariales, Leucosporidiales, Sporidiobolales, Tremellales, and Mortierellales (Fig. 2, 3). Both orchid species contained a diverse assemblage of fungi from the classes Microbotryomycetes and Tremellomycetes (Fig. 2, 3), including mostly yeast-like unicellular fungi, such as those from the families Leucosporidiaceae, Sporidiobolaceae, Filobasidiaceae, Trimorphomycetaceae, and a few more (Fig. 2, 3).

3.2 Mycorrhizal community diversity and composition

Mycorrhizal community diversity (richness and Shannon and Simpson indexes) did not differ between *H. barbertoni* and *H. epipactidea* ($P > 0.05$; Fig. 4). The PCoA plot showed a

dispersed pattern in the mycorrhizal community composition between the two orchid species (Figure 5). However, the PERMANOVA did not confirm that the orchid species explained the variation in mycorrhizal community composition (F = 6.05, r2 = 0.60, P = 0.1).

4. Discussion

Using high-throughput sequencing, we compared the communities of orchid mycorrhizal fungi associated with the roots of two South African endemic orchid species, *H. barbertoni* and *H. epipactidea*. Analyses of the sequence data revealed a significant number of taxa that were shared between the two orchid species. However, twenty-four fungal OTUs were found exclusively in the roots of *H. barbertoni*, while seventeen were found only in the roots of *H. epipactidea*. The roots of the endangered orchid *H. barbertoni* contained OTUs from the orchid mycorrhizae orders Atractiellales, Cantharellales, and Sebacinales.

A large number of fungal OTUs were found in both orchid species. This was not surprising given that these orchid species are from the same genus, *Habenaria* and have an overlapping distribution in South Africa (Johnson and Bytebier 2015). This was evident at our sampling site where the populations of both orchids were growing close to each other. A similar pattern was also seen in studies comparing mycorrhizal diversity associated with orchids from the same genus (Ercole et al. 2015; Waterman et al. 2011; Xing et al. 2015; Yukawa et al. 2009). However, despite the similarity in the general diversity and community composition, the abundance of many individual shared fungal OTUs differed widely in the two orchid species.

The read abundances of common mycorrhizal OTUs from the Cantharellales differed substantially between the two orchid species. It is, however, well known that read abundances between-species comparisons can be skewed (Amend et al. 2010; Johnson et al. 2021) and

caution must be exercised when assessing hypotheses based on read abundance data (Amend et al. 2010; Anslan et al. 2018; Nilsson et al. 2019). Read counts in our study were consistent between the biological replicates of each orchid species and thus it is unlikely that the variation in abundance of mutually shared fungal OTUs stems from sequencing bias. As a result, we believe the observed variations in abundance are related to plant age and species. Both of these factors influence the structural barriers in plants, influencing the colonization by mycorrhizal fungi (Chomicki et al. 2014; Soudzilovskaia et al. 2020; Teste et al. 2020).

We detected some orchid mycorrhizal fungal OTUs that were unique in one of the orchid species. These included fungal OTUs from the orders Atractiellales and Sebacinales. Among these, two OTUs from the Atractiellales (Atractiellomycetes) were exclusively detected in the roots of *H. barbertoni*. These included *Atractiella rhizophila* and an 'unidentified Atractiellales'. This class is the only lineage of rust fungi (Pucciniomycotina) that form symbiotic associations with orchids (Kottke et al. 2010). To date, Atractiellomycetes were exclusively detected in the roots of epiphytic neotropical orchids from the northern Andean mountain rainforest (Kottke et al. 2010). Finding these fungi associated with a South African orchid species is particularly interesting and further research is needed.

Kottke et al. (2010) suggested that the association between Atractiellomycetes and orchids is an example of an early evolutionary event in the development of orchid mycorrhizae. This group of mycorrhizae originated during the Late Cretaceous, 100-66 mya (Brundrett and Tedersoo 2018). However, classes within Pucciniomycotina, such as the Atractiellomycetes, emerged much earlier, between 211–383 Mya (He et al. 2019; Zhao et al. 2017). As a result, following the mass extinction event during the Cretaceous-Tertiary boundary (65.5 mya), orchids most likely formed symbiotic interactions with Atractiellomycetes and other unique

fungi for adapting to the changing environment (Benton et al. 2021). This and other strategies allowed orchids to radiate rapidly during this period (Ramírez et al. 2007; Zhang et al. 2018).

We detected several OTUs of saprophytic and phytopathogenic fungi from the orders Pleosporales, Capnodiales, Hypocreales, and Xylariales. Pleosporales have been previously described as growth-promoting orchid associated fungi, but their mycorrhizal status has not been confirmed yet (Jacquemyn et al., 2017). In addition, a few OTUs from unknown *Fusarium* species were detected. This genus includes fungi that are pathogenic to several orchid species (Srivastava et al. 2018). However, recent studies have indicated that *Fusarium oxysporum* can also form orchid mycorrhizal associations (Jiang et al. 2019). Similar to those orchid mycorrhizae identified from mycoheterotrophic orchids, some of these saprophytic or pathogenic fungi detected in this study perhaps form symbiotic associations with *Habenaria* (Johnson et al. 2021; Kottke et al. 2010; Martos et al. 2009). Infection trials would be needed to confirm this hypothesis.

We detected an assortment of yeast-like unicellular fungi from the phyla Microbotryomycetes and Tremellomycetes (Basidiomycota). So far, no yeast-like unicellular fungi are known to form symbiotic relationships with plants. However, research revealed that these yeast-like unicellular fungi can increase mycorrhizal colonisation leading to improved nutrient absorption and enhanced stress tolerance in plants (Alonso et al. 2008; Azcón et al. 2013; Botha 2011; Gollner et al. 2006; Mestre and Fontenla 2021; Sampedro et al. 2004; Yurkov et al. 2012). Consequently, these unicellular fungi may be endophytes of the two *Habernaria* species (Scholtysik et al. 2013; Solis et al. 2015) which may perform similar services in the orchid-mycorrhizae symbiosis.

Previously, both Waterman et al. (2011) and Makwela et al. (2022) studied the diversity of mycorrhizae associated with endemic South African orchids. The current study and the one

by Makwela and co-workers were both conducted in the Gauteng Province of South Africa, whereas Waterman and co-workers focussed on the orchid species from the Eastern and Western Cape Provinces. The sampling sites of our study were significantly different from the other study conducted in Gauteng and geographically separated by big urban developments. Nevertheless, comparing the studies from Gauteng revealed both overlapping (Cantharellales and Sebacinales) and unique orchid mycorrhizal fungi. However, the abundance of these overlapping fungal OTUs significantly differed between these studies. For example, Cantharellales were more abundant among *Habernaria* species than in *Brachycorythis conica* subsp. *transvaalensis*, while Sebacinales were present at lower frequencies. It is noteworthy that fungi from the Ceratobasidiaceae were abundant among the orchids from the Cape Provinces but absent from the orchids studied in Gauteng. Based on these studies, we hypothesise that the Ceratobasidiaceae may be widespread in the soil of coastal Southern Africa but are rare inland.

5. Conclusions

We compared the community of mycorrhizal fungi associated with the roots of two indigenous *Habernaria* species from South Africa. Our data showed that the mycorrhizal community associated with these two species was similar, but each orchid also harboured a few unique fungal associates. We also detected mycorrhizal fungi from the Atractiellomycetes that were uniquely associated with the roots of *H. barbertoni.* Previously, these fungi were exclusively reported from orchids living in Peruvian forests. At the same time, we identified several fungi that potentially might fulfil mycorrhizal functions, such as the Pleosporales and *F. oxysporum*. In future, isolations of the fungi identified in this study for use and infection study will be necessary to test their role in the orchids' life history. Overall, the findings of this study add to our understanding of the mycorrhizal diversity associated with indigenous South

African orchids and can ultimately be utilised to help conserve endangered species such as *H. barbertoni*.

Declaration of Competing Interest

The authors state that they have no known competing financial interests or personal connections that may seem to have influenced the work described in this publication.

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488

Figure legends

Figure 1. Above-ground plant with inflorescence, (A) *Habenaria barbertoni* and, (B) *Habenaria epipactidea*. Tuberous root system used in this study for metabarcoding orchid mycorrhizal diversity (C) *Habenaria barbertoni* and, (D) *Habenaria epipactidea.*

Figure 2. Taxonomic composition of fungi associated with *Habenaria barbertoni* and *Habenaria epipactidea* roots. Where available, the heat tree depicts a fungal community structure as a taxonomic hierarchy up to the species level. The size of each node and edge is proportional to the number of OTUs within each taxon, and the colour indicates taxon abundance (sum of reads).

Figure 3. A heat map depicting the fungal OTUs found in the roots of *Habenaria barbertoni* and *Habenaria epipactidea*. The Venn diagram shows the shared and distinct fungal OTUs identified in these two orchids. OTUs detected from both orchid species = black font, *Habenaria barbertoni* only = red font, and *Habenaria epipactidea* only = blue font.

Figure 4. Box plots analysing fungal diversity indices of *Habenaria barbertoni* and *Habenaria epipactidea* roots.

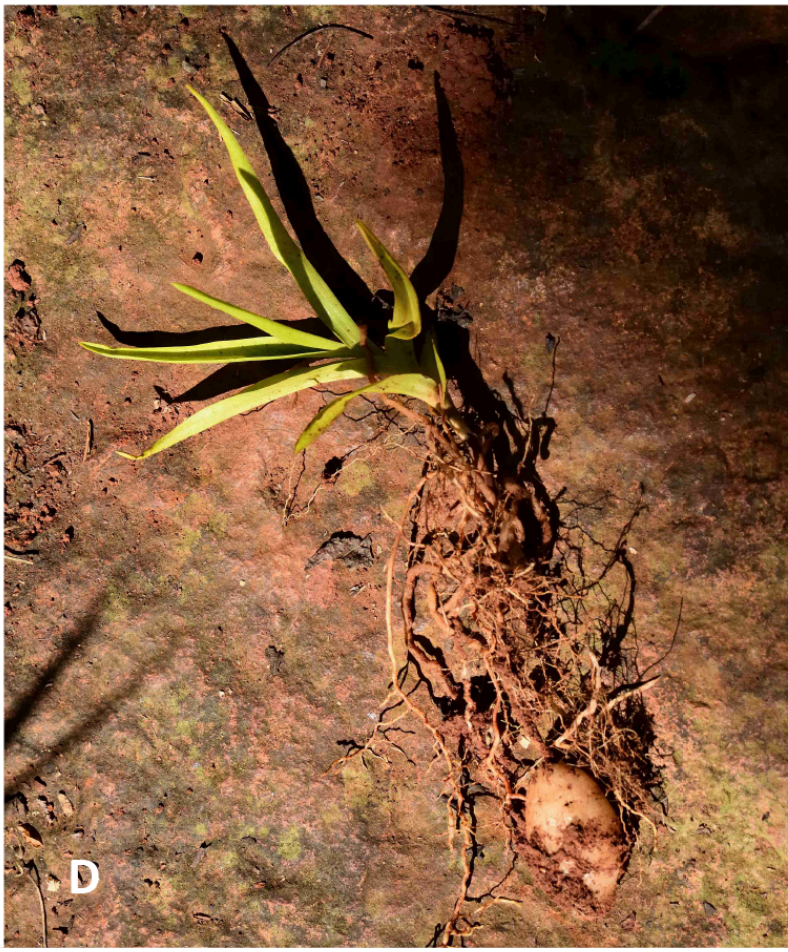
Figure 5. Principal Coordinate Analysis of mycorrhizal community composition associated with the roots of *Habenaria barbertoni* and *Habenaria epipactidea.*

Figure S1. PCR product purification steps used in this study. After merging the replicates for each PCR reaction using either of the primer pairs, the products were purified with Agencourt AMPure XP PCR purification beads. Following that, the relative concentrations of the PCR products were determined using ImageJ v1.52q. To standardise the DNA contribution, the PCR products were pooled into a single unit based on band intensity. All pools were purified in between the steps.


A


B


C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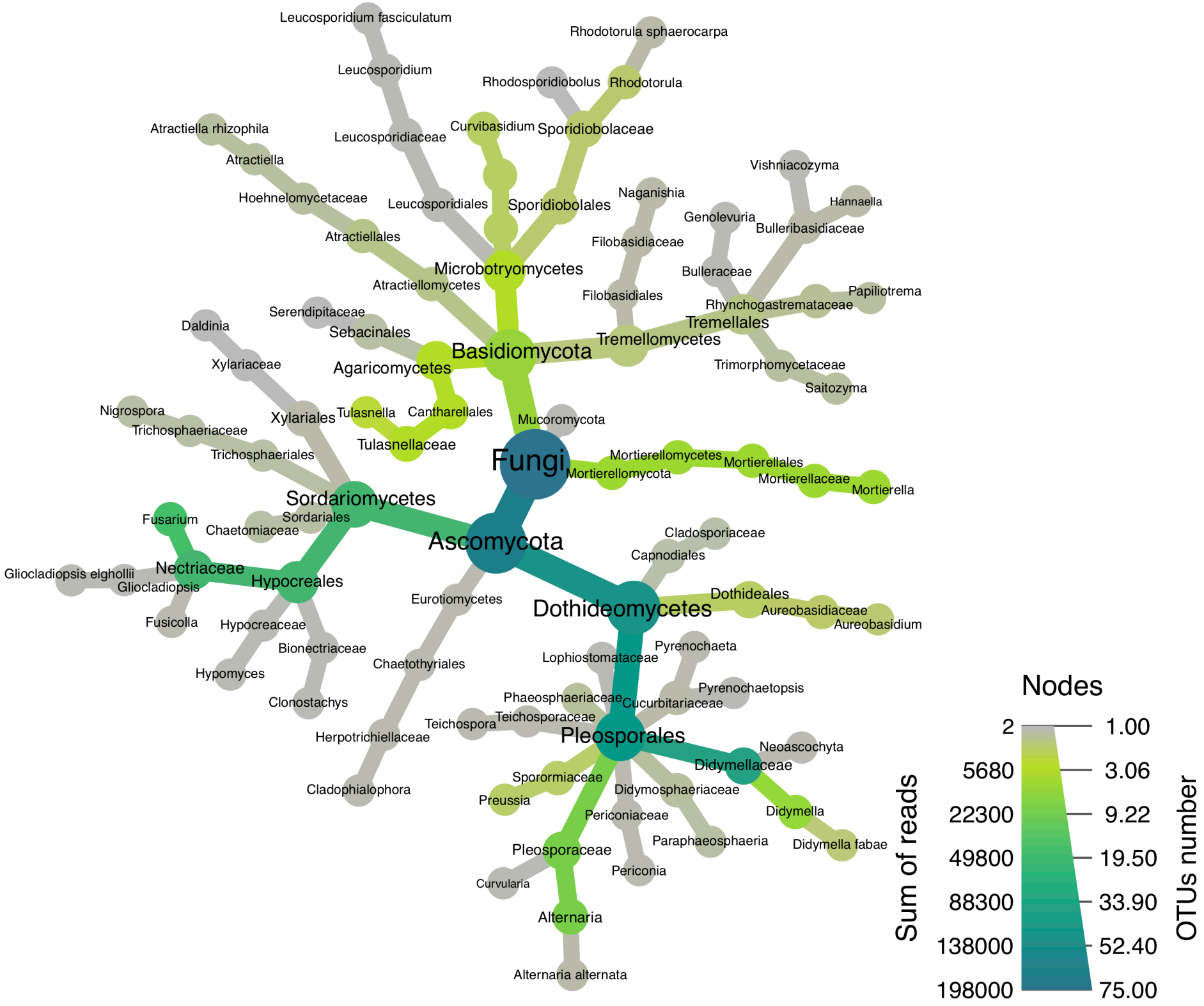
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Rhodotorula sphaerocarpa
Leucosporidium
Rhodosporidiobolus
Rhodotorula
Atractiella rhizophila
Curvibasidium
Sporidiobolaceae
Leucosporidiaceae
Atractiella
Vishniacozyma
Hoehnelomycetaceae
Leucosporidiales
Sporidiobolales
Naganishia
Hannaella
Genolevuria
Bulleribasidiaceae
Atractiellales
Filobasidiaceae
Microbotryomycetes
Bulleraceae
Atractiellomycetes
Papiliotrema
Filobasidiales
Rhynchogastremataceae
Serendipitaceae
Tremellales
Daldinia
Sebacinales
Tremellomycetes
Basidiomycota
Xylariaceae
Agaricomycetes
Trimorphomycetaceae
Saitozyma
Nigrospora
Tulasnella
Cantharellales
Xylariales
Mucoromycota
Trichosphaeriaceae
Tulasnellaceae
Mortierellomycetes
Mortierellales
Trichosphaeriales
Fungi
Mortierellomycota
Mortierellaceae
Mortierella
Sordariomycetes
Fusarium
Sordariales
Chaetomiaceae
Ascomycota
Cladosporiaceae
Capnodiales
Gliocladiopsis elghollii
Nectriaceae
Hypocreales
Gliocladiopsis
Dothideales
Eurotiomycetes
Dothideomycetes
Aureobasidiaceae
Fusicolla
Hypocreaceae
Aureobasidium
Bionectriaceae
Pyrenochaeta
Lophiostomataceae
Chaetothyriales
Hypomyces
Pyrenochaetopsis
Phaeosphaeriaceae
Cucurbitariaceae
Clonostachys
Teichospora
Teichosporaceae
Pleosporales
Herpotrichiellaceae
Neoascochyta
Didymellaceae
Sporormiaceae
Cladophialophora
Didymosphaeriaceae
Preussia
Didymella
Periconiaceae
Paraphaeosphaeria
Didymella fabae
Pleosporaceae
Periconia
Curvularia
Alternaria
Alternaria alternata
Nodes
Sum of reads
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5680
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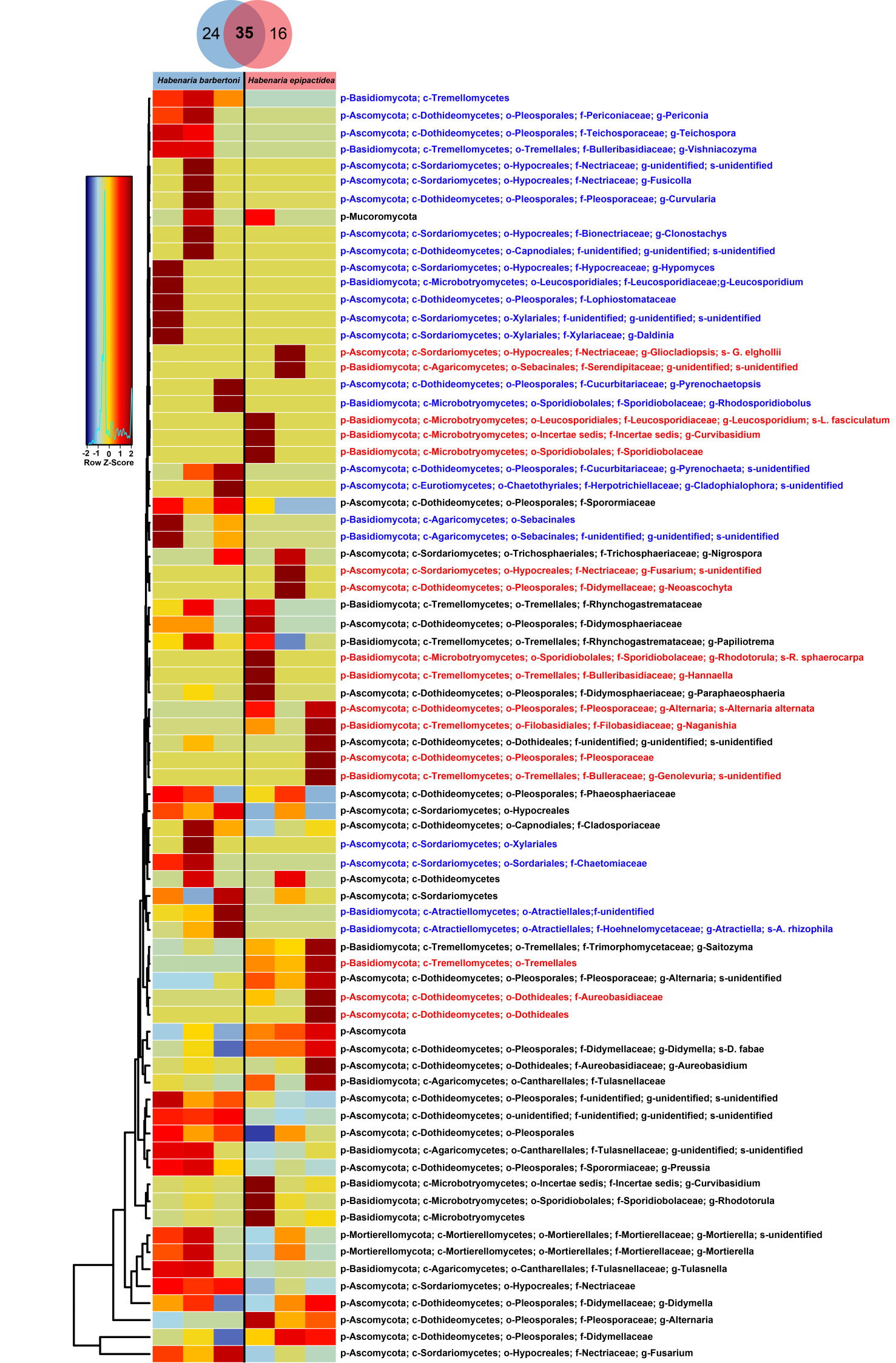
19.50
33.90
52.40
75.00
OTUs number


24
35
16
Habenaria barbertoni
Habenaria epipactidea
-2 -1 0 1 2
Row Z-Score
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p-Ascomycota; c-Dothideomycetes; o-Pleosporales; f-Periconiaceae; g-Periconia
p-Ascomycota; c-Dothideomycetes; o-Pleosporales; f-Teichosporaceae; g-Teichospora
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p-Mucoromycota
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p-Ascomycota
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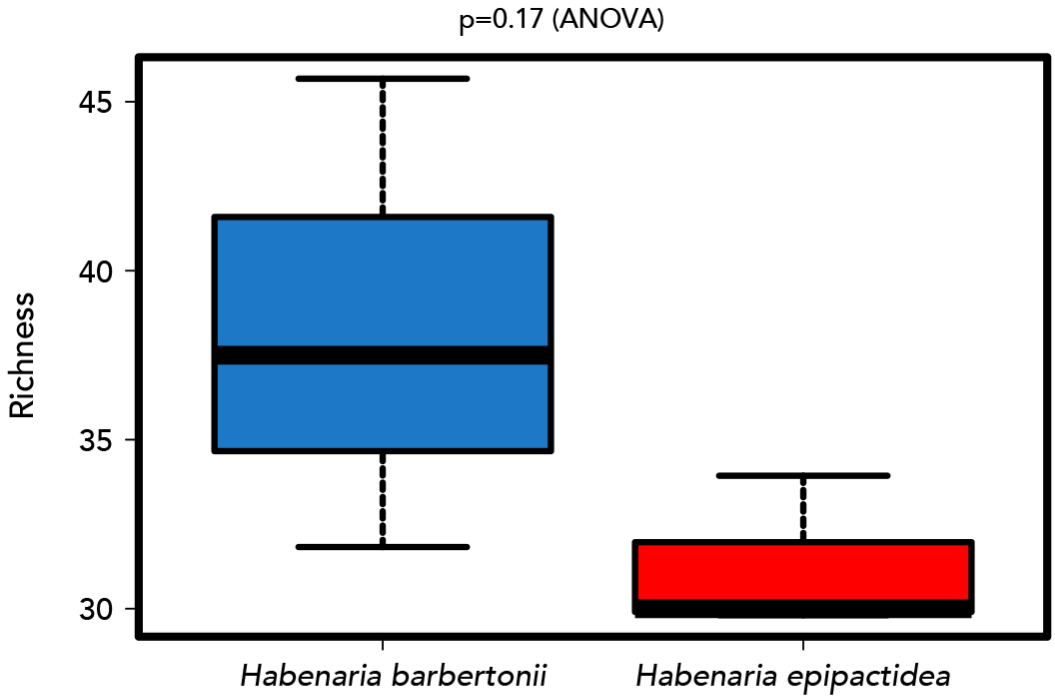
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30
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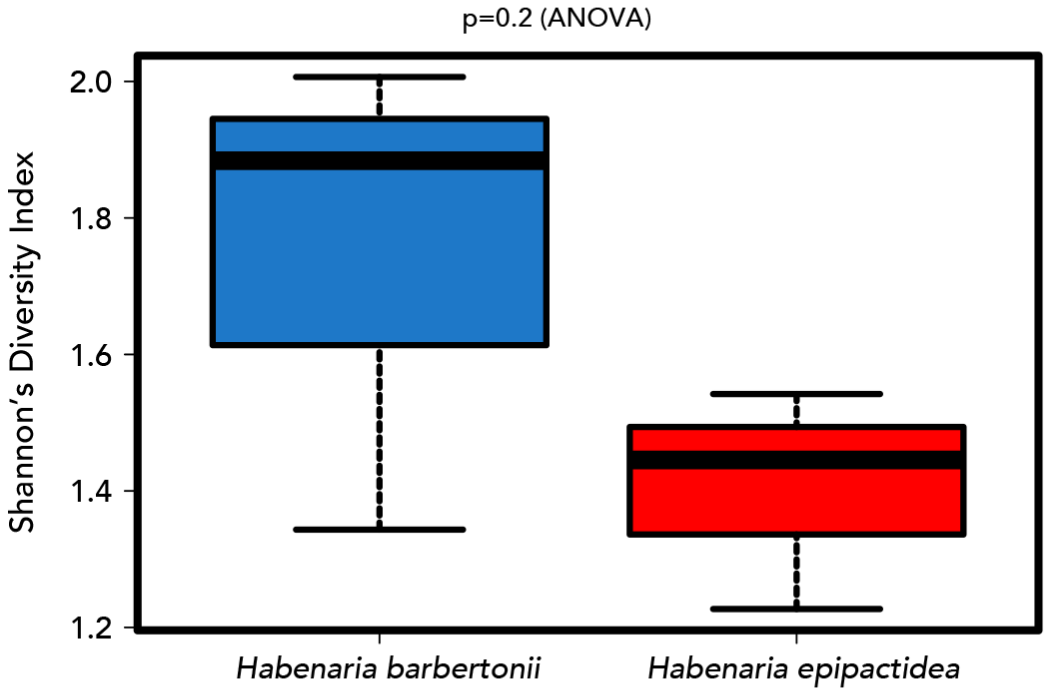
Habenaria epipactidea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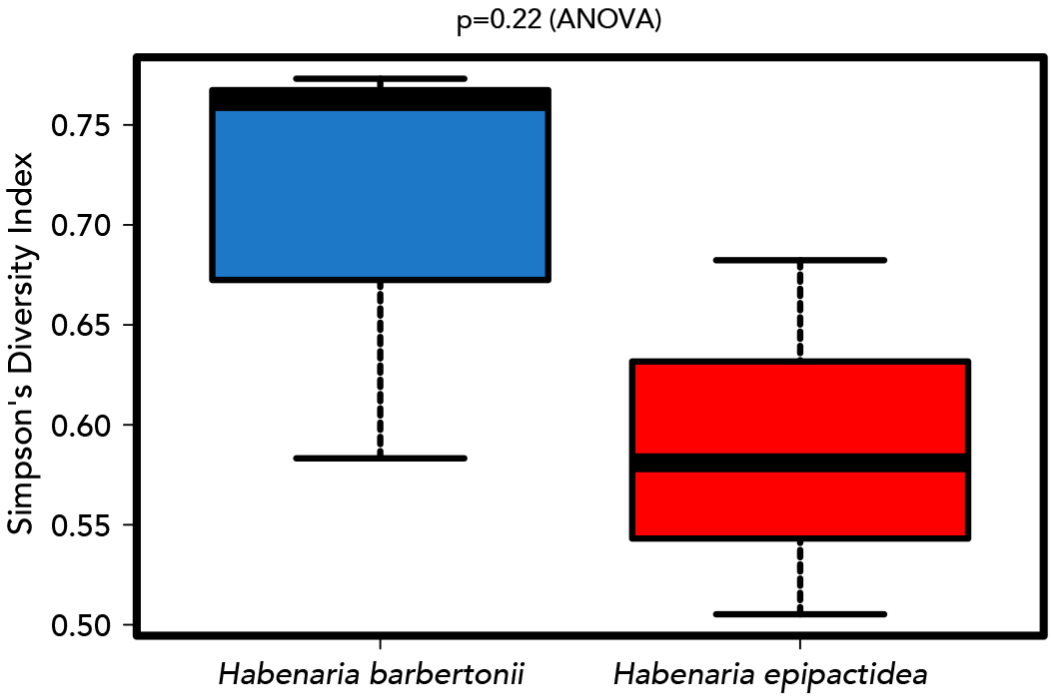
1.8
1.6
1.4
1.2
Habenaria barbertonii
Habenaria epipactidea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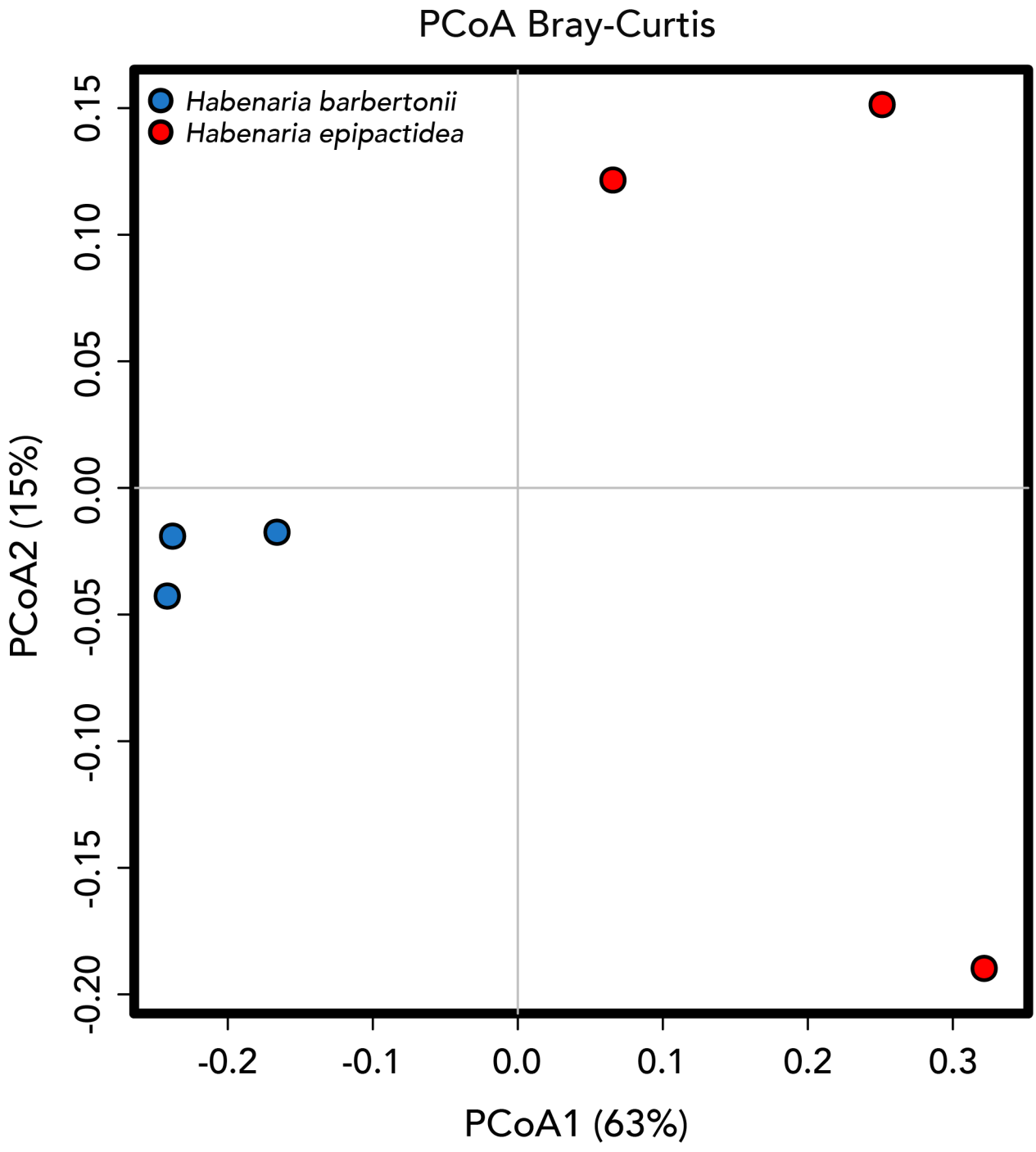
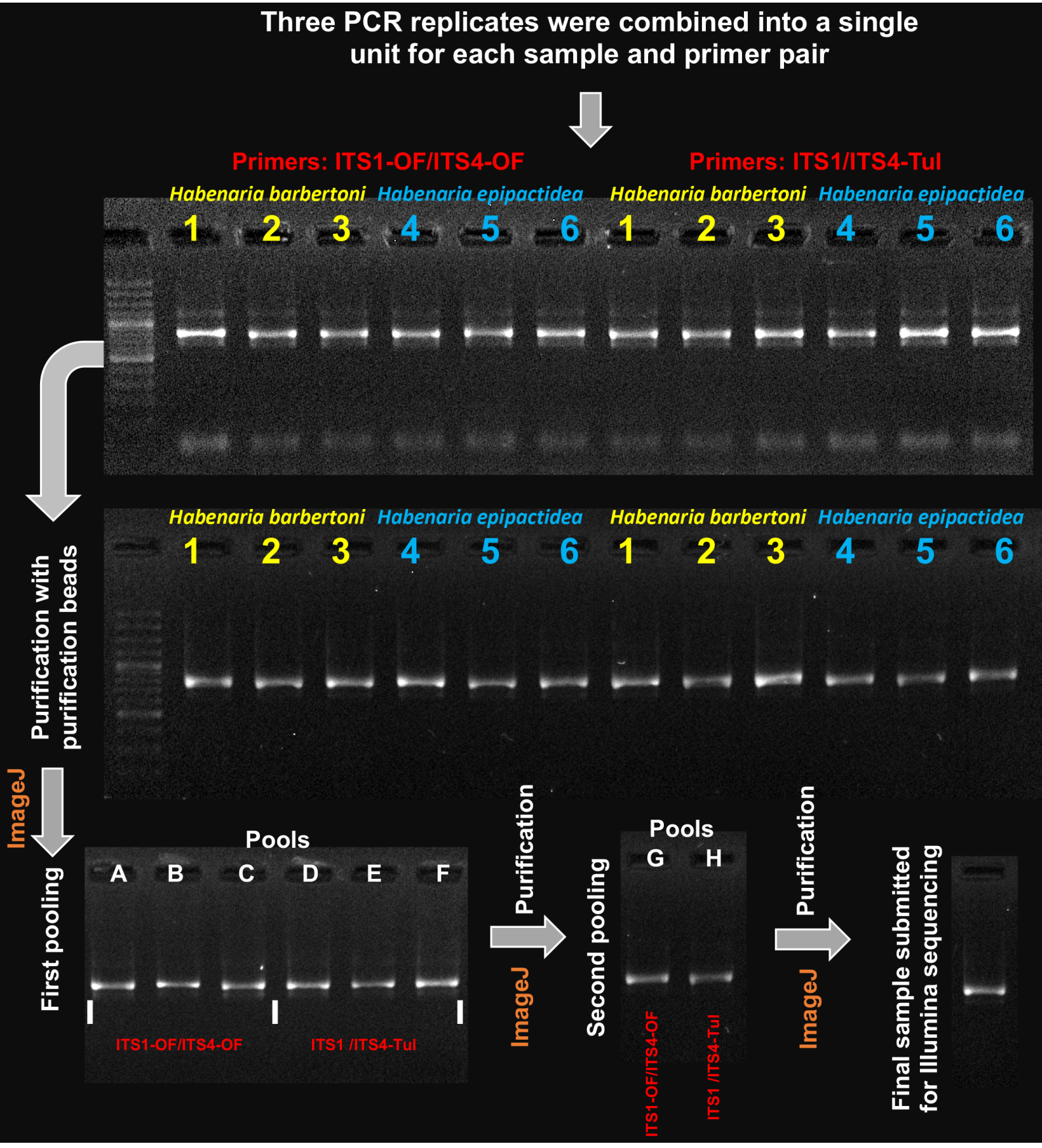
0.60
0.55
0.50
Habenaria barbertonii
Habenaria epipactidea


PCoA Bray-Curtis
Habenaria barbertonii
Habenaria epipactidea
PCoA2 (15%)
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.15
-0.20
-0.2
-0.1
0.0
0.1
0.2
0.3
PCoA1 (63%)


Three PCR replicates were combined into a single unit for each sample and primer pair
Primers: ITS1-OF/ITS4-OF
Primers: ITS1/ITS4-Tul
Habenaria barbertoni
Habenaria epipactidea
1 2 3 4 5 6 1 2 3 4 5 6
Purification with purification beads
ImageJ
First pooling
Pools
A B C D E F
ITS1-OF/ITS4-OF
ITS1 /ITS4-Tul
Purification
ImageJ
Second pooling
Pools
G H
ITS1-OF/ITS4-OF
ITS1 /ITS4-Tul
Purification
ImageJ
Final sample submitted for Illumina sequencing