Received: 31 August 2022 | Accepted: 4 April 2023

DOI: 10.1002/aps3.11523

APPLICATION ARTICLE

Applications in Plant Sciences

A metabarcoding protocol targeting two DNA regions to analyze root-associated fungal communities in ferns and lycophytes

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This article is part of the special issue "Emerging Methods in Botanical DNA/RNA Extraction."

Abstract

Premise: Detailed studies of the fungi associated with lycophytes and ferns provide crucial insights into the early evolution of land plants. However, most investigations to date have assessed fern–fungus interactions based only on visual root inspection. In the present research, we establish and evaluate a metabarcoding protocol to analyze the fungal communities associated with fern and lycophyte roots.

Methods: We used two primer pairs focused on the ITS rRNA region to screen the general fungal communities, and the 18S rRNA to target Glomeromycota fungi (i.e., arbuscular mycorrhizal fungi). To test these approaches, we collected and processed roots from 12 phylogenetically distant fern and lycophyte species.

Results: We found marked compositional differences between the ITS and 18S data sets. While the ITS data set demonstrated the dominance of orders Glomerales (phylum Glomeromycota), Pleosporales, and Helotiales (both in phylum Ascomycota), the 18S data set revealed the greatest diversity of Glomeromycota. Non-metric multidimensional scaling (NMDS) ordination suggested an important geographical effect in sample similarities.

Discussion: The ITS-based approach is a reliable and effective method to analyze the fungal communities associated with fern and lycophyte roots. The 18S approach is more appropriate for studies focused on the detailed screening of arbuscular mycorrhizal fungi.

KEYWORDS

amplicons, DNA sequencing, ferns, ITS, metabarcoding, mycorrhizal fungi, lycophytes, 18S rRNA

Resumen

Premisa: El estudio de los hongos asociados a licofitas y helechos proporciona información crucial sobre la evolución temprana de las plantas terrestres. Sin embargo, hasta el momento, la mayoría de las investigaciones ha evaluado las interacciones helecho–hongo basándose solamente en la observación directa de las raíces. En la presente investigación, establecemos y evaluamos un protocolo de metabarcoding enfocado en dos regiones de ADN para analizar las comunidades fúngicas asociadas a las raíces de helechos y licofitas.

Métodos: Utilizamos dos pares de primer orientados hacia la región ITS ARNr, para la detección de las comunidades fúngicas generales, y la región 18S ARNr, para captar hongos pertenecientes al phylum Glomeromycota (i.e., hongos micorrícicos arbusculares). Para evaluar estos procedimientos, nosotros recolectamos y procesamos raíces de 12 especies de helechos y licofitas distantes desde el punto de vista filogenético.

Resultados: Se observaron claras diferencias de composición entre los sets de datos ITS y 18S: mientras el primero demostró un predominio de los órdenes Glomerales (phylum Glomeromycota), Pleosporales y Helotiales (ambos en phylum Ascomycota), el set 18S reveló la mayor diversidad de hongos micorrizógenos arbusculares. Ninguno de los marcadores moleculares utilizados detectó miembros del phylum Mucoromycota en las muestras. El escalamiento multidimensional no métrico (NMDS) sugirió un papel importante de la región geográfica de origen en la determinación de las similitudes entre muestras.
Discusión: El método basado en la región ITS es consistente, replicable y eficaz para analizar las comunidades fúngicas asociadas con raíces de helechos y licofitos. El enfoque 18S es más apropiado para estudios centrados en la detección de los hongos micorrizógenos arbusculares.

Zusammenfassung
Prämisse: Detaillierte Untersuchungen der mit Lycophyten und Farnen assoziierten Pilze liefern entscheidende Erkenntnisse über die frühe Evolution von Landpflanzen. Die meisten Untersuchungen haben jedoch bisher Farn-Pilz-Interaktionen nur anhand der visuellen Wurzelinspektion untersucht. In der vorliegenden Arbeit wurde ein Metabarcoding-Protokoll etabliert und evaluiert, das auf zwei DNA-Regionen abzielt, um die mit Farn- und Lycophytenwurzeln assoziierten Pilzgemeinschaften zu analysieren.
Methoden: Wir Primerpaare verwendeten, die sich auf die rRNA-Region des ITS konzentrierten, um die allgemeinen Pilzgemeinschaften zu untersuchen, und die 18S rRNA, um Glomeromycota-Pilze (i.e., arbuskuläre Mykorrhizapilze) zu erfassen. Um diese Ansätze zu testen, sammelten und verarbeiteten wir Wurzeln von 12 phylogenetisch entfernten Farn- und Lycophytenarten.
Ergebnisse: Wir fanden Unterschiede zwischen den Datensätzen ITS und 18S. Während die erste die Dominanz der Ordnungen Glomerales (Stamm Glomeromycota), Pleosporales und Helotiales (beide drin Stamm Ascomycota) offenbarte, zeigte die 18S-Datensatz die größte Vielfalt arbuskulärer Mykorrhizapilze. Keiner der Marker konnte Mitglieder des Phylums Mucoromycota in den untersuchten Proben nachweisen. Die Nich-metrische mehrdimensionale Skalierung (NMDS) Ordination deutete auf eine wichtige Rolle der geografischen Herkunftsregion bei der Bestimmung der Ähnlichkeiten der Proben hin.
Diskussion: Der ITS-basierte Ansatz ist konsistent, replizierbar und effektiv, um die gesamten Pilzgemeinschaften zu analysieren, die mit Farn- und Lycophytenwurzeln verbunden sind. Der 18S-Ansatz eignet sich besser für Studien, die sich auf das detaillierte Screening von arbuskulären Mykorrhizapilzen konzentrieren.

Fern and lycophyte studies are fundamental to understanding plant evolution and diversification (Page, 2002; Strullu-Derrien et al., 2018). Paleontological and molecular evidence suggest that fungi from the phyla Glomeromycota and Mucoromycota played an essential role in the colonization of primitive soils by early land plants (Field et al., 2015; Strullu-Derrien et al., 2018; Benucci et al., 2020). Yet, there is little information on the nature, dynamics, and functionality of fern–fungus relationships (Lehnert et al., 2017; Strullu-Derrien et al., 2018). Most studies have followed a morphological approach using visual inspection of the roots to evaluate the fungal colonization status (Kessler et al., 2010a, b; Lehnert et al., 2017). However, accurate identification of fungi can only be achieved by employing molecular tools (Redecker et al., 2013; Öpik et al., 2014).

The rapid development of next-generation sequencing technologies has increased the use of metabarcoding, also known as amplicon sequencing, to identify fungal organisms (Op De Beeck et al., 2014; Öpik et al., 2014; Strullu-Derrien et al., 2018; Semenov, 2021). Metabarcoding analyses allow the simultaneous detection of multiple taxa of a community from a pool of genetic material using taxon-specific primers for DNA amplification and high-throughput sequencing of the barcode marker genes (Semenov, 2021). The main use of amplicon sequencing data is to establish community profiles. However, the taxonomic diversity encountered in each study depends greatly on the specificity of the primers used (Strullu-Derrien et al., 2018; Semenov, 2021).

The internal transcribed spacer or ITS (including the ITS1, 5.8S, and ITS2 regions) in the ribosomal RNA has been chosen as the primary fungal DNA barcode (Schoch et al., 2012). However, despite its efficient detection of members of the phyla Ascomycota and Basidiomycota, DNA amplification targeting the ITS region typically results

in an underestimation of Glomeromycota diversity (Öpik et al., 2013; Op De Beeck et al., 2014; Schlaeppi et al., 2014; Tedersoo et al., 2022). To avoid the poor amplification of arbuscular mycorrhizal fungi (AMF), the small subunit rRNA (SSU rRNA) or 18S region is often employed to analyze these communities (Öpik et al., 2010, 2014; Lekberg et al., 2018).

During the past decade, the molecular analysis of fungal symbioses in ancient plant lineages has received increasing attention (e.g., Field et al., 2015; Benucci et al., 2020; Sandoz et al., 2020; Perez-Lamarque et al., 2022). Bidartondo et al. (2011) found that early-diverging liverwort, hornwort, and fern species are associated with Endogonales fungi (phylum Mucoromycota) rather than Glomeromycota (arbuscular mycorrhizal fungi). Similarly, in their examination of 674 globally collected liverwort species, Rimington et al. (2018) discovered that early-diverging groups were colonized by specific early-diverging fungi (mostly non-Glomeraceae). However, a study analyzing the microbial communities in lycophyte roots in New Zealand revealed that they are simultaneously colonized by dark septate endophytes (DSE), AMF, and Endogonales (Benucci et al., 2020). Molecular data regarding fern–fungal communities are even more scarce than for other ancient plant lineages, and therefore the identity and ecology of fungi in fern roots remain largely unknown (Strullu-Derrien et al., 2018). This might be due to the limited availability of molecular tools (Lehnert et al., 2017; Strullu-Derrien et al., 2018) and the lack of consensus regarding the most efficient protocol to follow. While multiple procedures have been tested and established to analyze fungal communities in angiosperm roots, differences in root structure and chemical composition might render them inefficient in ferns and lycophytes (Pearson, 1969; Vetter, 2018). West et al. (2009), for example, examined the AMF communities linked to eight fern species using the cetyltrimethylammonium bromide (CTAB) DNA extraction protocol and *Glomus*-specific primers. Despite the detection of more than 30 potential fungal ribotypes, they failed to amplify DNA in six of the eight species and the outcome was restricted to one fungal genus. More recently, Sandoz et al. (2020) utilized high-throughput sequencing of the 18S rRNA region to investigate AMF diversity in 71 samples of *Botrychium lunaria* (L.) Sw. (Ophioglossaceae) in the Swiss Alps. They employed the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and primers AMV4.5NF and AMDGR (Sato et al., 2005), identifying five Glomeromycota genera while excluding other fern-colonizing fungi such as DSE. Furthermore, the roots of *Botrychium* are atypically soft for ferns, and this method has not been tested across fern species with different levels of cortex sclerification (Pearson, 1969) and concentration of secondary metabolites in their roots (Vetter, 2018). Perez-Lamarque et al. (2022) assessed the diversity of mycorrhizal fungi in 30 plant species including angiosperms, ferns, and lycophytes on La Réunion island. They processed both the 18S rRNA and the ITS2 regions using the NucleoSpin 96 Plant II kit (Macherey-Nagel, Düren, Germany) and the primer pairs AMADf-AMDGr (Berruti et al., 2017) and ITS86F-ITS4 (White et al., 1990; Turenne et al., 1999). They found that the 18S and ITS2 methods are highly complementary at revealing plant microbiomes, with the 18S region failing to characterize Basidiomycota and Helotiales (Ascomycota) and the ITS2 region missing Mucoromycota fungi. However, carrying out both methods might be impossible due to constraints in time or funding, making it vital to determine which method is most reliable for a particular research question.

In the present study, we aimed to establish and evaluate a metabarcoding protocol targeting two different DNA regions to analyze the composition of the whole fungal and AMF communities associated with the roots of 12 fern and lycophyte species. To achieve this goal, we amplified both ITS and 18S rRNA markers of fungi and used high-throughput sequencing to assess the protocol performance.

METHODS

Plant sampling

We selected two lycophyte and 10 fern species with a wide phylogenetic distribution, representing 12 families: Lycopodiaceae, Selaginellaceae, Aspleniaceae, Blechnaceae, Dryopteridaceae, Gleichenaceae, Lindsaeaceae, Marattiaceae, Ophioglossaceae, Polypodiaceae, Pteridaceae, and Thelypteridaceae. The sampling included three replicates per species for a total of 36 samples ($n = 36$).

We collected the material between April and September 2020 at eight localities in Switzerland and Taiwan (Appendix 1), with the aim of representing both temperate and tropical ecosystems given the influence of geographical and environmental factors in the composition of fungal communities associated with angiosperms (Öpik et al., 2013). We identified the ferns according to Knapp (2011) for Malaysian specimens and Eggenberg et al. (2018) for Swiss specimens.

Approximately 1 g of roots was sampled per specimen, placed in paper bags with silica gel for their rapid desiccation, and stored for up to three months at room temperature before DNA extraction. We implemented and evaluated a metabarcoding protocol to identify root-associated fungi following the general workflow represented in Figure 1.

Molecular analysis

All the replicates in this study were treated in the same way from DNA extraction to bioinformatics.

DNA extraction

We utilized 50 mg of roots per specimen during DNA extraction. Samples were submerged for 2–3 min in liquid

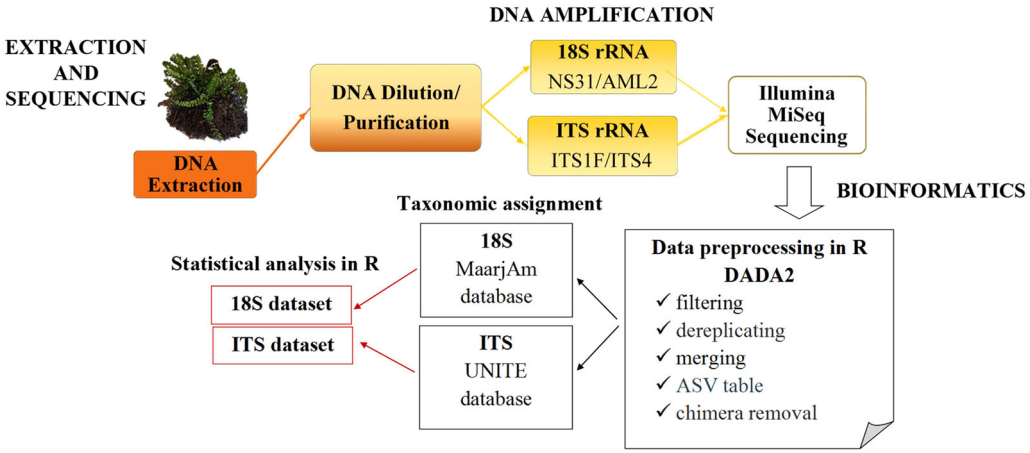

FIGURE 1 General workflow followed during this study resulting in the ITS and 18S data sets.

nitrogen and subjected to three cycles of 3 min/24 Hz in a TissueLyser II (Qiagen), using 5-mm stainless steel beads for mechanical disruption.

We first tested the CTAB method (Doyle and Doyle, 1987), which has been widely used to extract fungal DNA from angiosperms, but it generated low-purity DNA that could not be used in subsequent downstream assays. We also tested the DNeasy Plant Pro Mini Kit (Qiagen), which is recommended by its manufacturer for the extraction of DNA from difficult samples, but again the genetic material recovered was insufficient. We found that the DNeasy Plant Mini Kit was the most successful in extracting fungal DNA from fern roots, yielding the highest quantity of quality DNA for further analyses. We followed the manufacturer's Quick-Start Protocol (Qiagen), except for adaptations in steps 2, 8, and 11. In brief: we added 400 µL of Buffer AP1 to the samples, vortexed them briefly, and left them for 2 min at room temperature. Afterward, we added 4 µL of RNase A, incubated the tubes for 15 min at 65°C in an Eppendorf Thermomixer Compact (Eppendorf, Hamburg, Germany), and inverted them every 5 min. We added 500 µL of Buffer AW2 and centrifuged the mixture for 1 min at 8000 rpm in a Hettich MIKRO 220 R Centrifuge (Hettich, Bäch, Switzerland). After discarding the flow-through, we performed two additional washing steps using the same quantity of Buffer AW2, followed by centrifugation for 2 min at 14,000 rpm. Finally, we added 25 µL of Buffer AE (heated at 65°C) to the spin columns, incubated the tubes for 5 min, and centrifuged for 1 min at 8000 rpm. The resulting material was stored at −20°C.

Dilution and purification

We quantified the DNA concentration of each sample using an Invitrogen Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Basel, Switzerland) following the manufacturer's instructions, and determined the DNA purity using a NanoDrop One (Thermo Fisher Scientific). When the concentration of the studied samples was above 40 ng/µL, we applied a dilution step, because we found that a lower concentration of DNA resulted in purer products and benefited its amplification. We calculated the dilution factor for each sample to obtain a final DNA concentration of 35 ng/µL.

Despite the excellent outcome of the extraction process, we found that a purification step was critical for successful DNA amplification when analyzing fern and lycophyte roots. We utilized the Monarch Genomic DNA Purification Kit (New England Biolabs, Frankfurt am Main, Germany) for DNA purification, adapting the manufacturer's instructions as follows. In brief, we employed 50 µL of each diluted sample and 100 µL of Binding Buffer. The solution was mixed by pipetting, filtered using a purification column, and centrifuged at 14,000 rpm. After discarding the flowthrough, we added 200 µL of Washing Buffer and centrifuged at 14,000 rpm. This washing step had to be repeated twice, after which the filter was placed in a new tube, and 25 µL of heated Elution Buffer was added. Samples were incubated for 2 min and centrifuged for 1 min at 14,000 rpm. We again quantified the resulting DNA using the NanoDrop One and found that this volume of buffer resulted in the greatest DNA yield (different in each sample) with the maximum purity (ratio of absorbance at 260/280 nm ~1.8).

DNA amplification

We developed two PCR protocols: the first to amplify the 18S rRNA region and the second targeting the ITS rRNA region. We tested seven primer pairs during this study (Appendix S1; see Supporting Information with this article), and only two of them were successful for all the fern species as described below. Both forward and reverse primers were synthesized using a tail containing Illumina Nextera consensus sequences (5′-TCGTCGGCAGCGTC-AGATGTGTATAAGAGACAG-Forward_primer-3′, 5′-GTCTCGTGGGCTCGG-AGATGTG

TATAAGAGACAG-Reverse_primer-3′; Illumina, San Diego, California, USA) for a two-step PCR.

18S PCR

Primers NS31 (Simon et al., 1992) and AML2 (Lee et al., 2008) capture the most variable part of the SSU rRNA (18S), offering broad coverage of Glomeromycota taxa (Öpik et al., 2010). We performed a Hot Start DNA amplification using a T100 Thermal Cycler (Bio-Rad Laboratories, Basel, Switzerland) under the following conditions: initial denaturation at 98°C for 2 min; followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 2 min. Reactions were carried out in 25-μL volumes using the Q5 High-Fidelity DNA Polymerase set (New England Biolabs), with each reaction containing 5.0 μL of Reaction Buffer, 5.0 μL of Reaction Enhancer, 0.2 μL of 25 mM dNTP mix, 1.0 μL of each 10 mM primer, 0.25 μL of *Taq* Polymerase, 11.55 μL of ddH_2O, and 1.0 μL of template-purified DNA (>5 ng/μL).

Amplicon size (640–670 bp) was verified by electrophoresis in a 2% agarose gel. We purified the amplified DNA using the same protocol with the Monarch Genomic DNA Purification Kit. Despite many attempts, the DNA amplification of samples CH 1-01 A, CH 1-10 C, and 11E was unsuccessful following the 18S protocol.

ITS PCR

The development of amplicon libraries from the ITS region was very challenging when analyzing multiple phylogenetically distant fern species and targeting their general fungal communities (ITS rRNA region). The variability of the fungal ITS region was evident even using universal primers. Nevertheless, most of the samples (except *Botrychium lunaria* and *Angiopteris lygodiifolia* Rosenst.) were successfully amplified using primer pair ITS86F/ITS4. The PCR conditions developed for these primers are described in Appendix S2.

Finally, the amplification was carried out by EzBiome (Gaithersburg, Maryland, USA) using primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990). In brief, each 25-μL reaction contained 12.5 ng of sample DNA as input, 12.5 μL of 2X KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, Massachusetts, USA), and 5 μL of 1 μM of each primer. The PCR protocol included an initial denaturation step at 95°C for 3 min; followed by 25 cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 30 s); and a final elongation at 72°C for 5 min. The PCR product was cleaned up from the reaction mix using Mag-Bind RxnPure Plus magnetic beads (Omega Bio-tek, Norcross, Georgia, USA), and a second PCR amplification was performed in 25-μL reactions, using the same master mix conditions as described above. Cycling conditions were as follows: denaturation at 95°C for 3 min; followed by eight cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 30 s); and a final 5-min elongation step at 72°C. The resulting fragments were 500–580 bp in length.

Illumina sequencing

The sequencing was carried out by EzBiome. DNA concentration was measured using the QuantiFluor dsDNA System on a Quantus Fluorometer (Promega, Madison, Wisconsin, USA). Before being pooled, libraries were normalized using the Mag-Bind EquiPure Library Normalization Kit (Omega Bio-tek). The pooled libraries were then examined using an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, California, USA) and sequenced (2 × 300 bp paired-end read setting) on the MiSeq system (Illumina).

Bioinformatics

Preprocessing of sequences

All the data generated from amplicon sequencing were subjected to the same quality control pipeline, following the DADA2 ITS Pipeline Workflow 1.8 (https://benjjneb.github.io/dada2/ITS_workflow.html) in R (R Core Team, 2021; v4.1.2), package dada2 (Callahan et al., 2016). Briefly, raw reads were checked for presence of primers and adapters, and the curated reads were filtered by sequence quality with default parameters and a minimum length of 450 bp. Sequences were dereplicated and denoised. Using these data, an amplicon sequence variant (ASV) table was built in which chimeric sequences were identified and removed.

Taxonomic annotation

18S data set

We completed the taxonomic assignment of the 18S ASVs using the MaarjAM database (Öpik et al., 2010), as recommended when targeting AMF and having 18S-based data (Nilsson et al., 2019; Tedersoo et al., 2022). ASVs were considered to be accurately identified at the species level when they had a maximum score of 250 bp, a query coverage >98%, and a similarity >97%; at the genus level, query coverage was ≥95% and similarity ≥90% (Nilsson et al., 2019).

ITS data set

For this data set, the taxonomic assignment used RStudio (RStudio Team, 2020) and the UNITE general FASTA release for Fungi (version 8.3; Abarenkov et al., 2021), which provides rDNA ITS-based identification of Eukaryotes, including curated data from the International Nucleotide Sequence Databases consortium (INSDc).

Statistical analysis

All the statistical analyses were conducted in R (R Core Team, 2021; v4.1.2). The initial data sets, ITS (hereafter referred to as ITS Full) and 18S (18S Full), were filtered to eliminate unidentified sequences at the phylum level. To compare both protocols, we made a subset of ITS Full including solely ASVs identified as part of the phylum Glomeromycota (ITS Glom Full). We also obtained the core microbiome of each data set by filtering out the taxa with a relative abundance lower than 1% in each sample, resulting in six final data sets: ITS Full (whole fungal communities detected by ITS marker), ITS Core (fungal taxa detected by ITS marker with a relative abundance >1%), ITS Glom Full (whole AMF communities detected by ITS marker), ITS Glom Core (AMF taxa detected by ITS marker with a relative abundance >1%), 18S Full (whole AMF communities detected by 18S marker), and 18S Core (AMF taxa detected by 18S marker with a relative abundance >1%).

Rarefaction curves were plotted using the function rarecurve in the vegan package (Oksanen et al., 2022) for ITS Full and 18S Full. We obtained the relative abundance of fungi at phylum level for ITS Full and ITS Core, respectively, making use of the function transform_sample_counts from the phyloseq package. The results were compared using the plot_bar function (ggplot and ggprism packages). To contrast the relative abundance of fungi at genus and species levels in ITS Glom Full and 18S Full, we plotted the results using bar plots generated by Microsoft Excel for Microsoft 365 MSO (Version 2206; Microsoft, Redmond, Washington, USA).

To test the existence of statistical differences among fungal communities, we performed an analysis of similarities (ANOSIM; Clarke, 1993) using the anosim function from the vegan package (Oksanen et al., 2022). We employed the Jaccard index as measure and executed 9999 permutations for the ITS Full and 18S Full data sets, respectively. To calculate and compare compositional similarities among fungal communities within a single fern species and among different fern species, we used EstimateS (version 9.1.0; Colwell, 2009). To visualize the groups' dissimilarities in fungal community composition, we utilized a non-metric multidimensional scaling (NMDS) analysis with the Jaccard index as distance measure using the functions ordinate and plot_ordination in the phyloseq package (McMurdie and Holmes, 2013).

The taxonomic classification and ASV abundance for the ITS Full and 18S Full data sets are provided in Appendices S3 and S4, respectively.

RESULTS

Data quality

We successfully amplified the fungal communities associated with the roots of 12 fern and lycophyte species using a metabarcoding protocol that targeted the 18S rRNA and ITS fungal markers. Samples CH 1-01 A, CH 1-10 C, and 11E did not yield amplicons during amplification of the 18S region and therefore were excluded in the analyses of the corresponding data sets.

We obtained a total of 477,213 and 1,658,998 reads with the ITS and 18S protocols, respectively. The average coverage of fungal reads per sample was 13,256 ± 7693 and 51,844 ± 25,526, respectively. We retained 4409 ASVs in ITS Full, 2172 in 18S Full, and 658 in ITS Glom Full, and for the core microbiomes (>1% relative abundance) 449 ASVs in ITS Core, 391 in ITS Glom Core, and 209 in 18S Core.

Analysis of the rarefaction curves per data set indicated that, regardless of the protocol followed, the number of ASVs recorded reached a saturation level (Figure 2). Unsurprisingly, core microbiomes saturated much earlier than full microbiomes because only common species were included.

Fungal community composition in fern and lycophyte roots

Sequencing analyses revealed the presence of 11 phyla in the examined roots. To illustrate the structure of the fungal communities, we selected three fern (*Angiopteris lygodiifolia*, *Botrychium lunaria*, and *Leptochilus wrightii* (Hook.) X. C. Zhang) and one lycophyte species (*Selaginella delicatula* (Desv. ex Poir.) Alston) with distinctive compositions. The compositional analysis of the fungal communities detected in ITS Full and ITS Core for these four species, and the other eight taxa studied, is shown in Appendices S5 and S6, respectively. The ITS Full and ITS Core sets showed similar outcomes, with all communities dominated by members of the phyla Ascomycota, Basidiomycota, and Glomeromycota, representing more than 85% of the total relative abundances (Figure 3). Interestingly, taxon proportions varied among and within fern species; *A. lygodiifolia* had the most remarkable compositional contrast, with each sample being dominated by a different phylum.

Overall, the ITS approach allowed us to identify 138 fungal species in 115 genera, 68 families, and 24 orders (Appendix S3). The most abundant fungal lineages were those that form mycorrhizae: Glomerales (phylum Glomeromycota), Pleosporales, and Helotiales (both in phylum Ascomycota).

Arbuscular mycorrhizal fungi in fern and lycophyte roots: ITS vs. 18S

To compare the performance of the ITS and 18S methods in detecting AMF, we used the ITS Glom and 18S data sets. The Full and Core microbiome results for both data sets were almost identical (Appendices S7–S10).

Although sequencing analyses revealed the occurrence of well-known Glomeromycota families, we observed marked compositional differences between the ITS and 18S data sets (Figure 4). Both protocols revealed Glomeraceae as the most abundant lineage. However, AMF

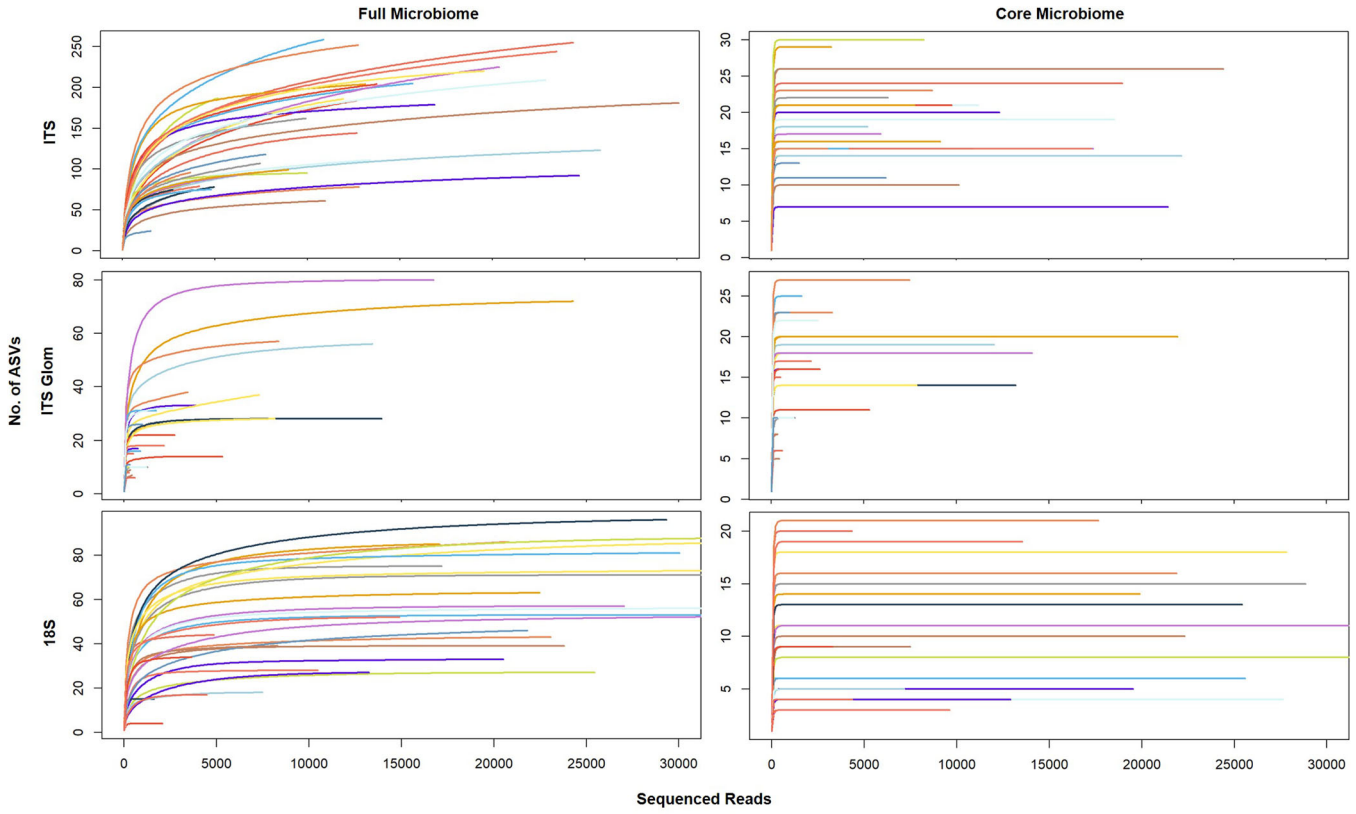

FIGURE 2 Rarefaction curves showing the expected number of species (amplicon sequence variants [ASVs]) as a function of the number of sequenced reads (sample size) for six data sets of whole fungal communities and arbuscular mycorrhizal fungi (AMF) communities associated with 12 fern and lycophyte species. Each colored curve symbolizes a different sample. ITS Full = whole fungal communities detected by ITS marker, ITS Core = fungal taxa detected by ITS marker with a relative abundance >1%, ITS Glom Full = whole AMF communities detected by ITS marker, ITS Glom Core = AMF taxa detected by ITS marker with a relative abundance >1%, 18S Full = whole AMF communities detected by 18S marker, 18S Core = AMF taxa detected by 18S marker with a relative abundance >1%.

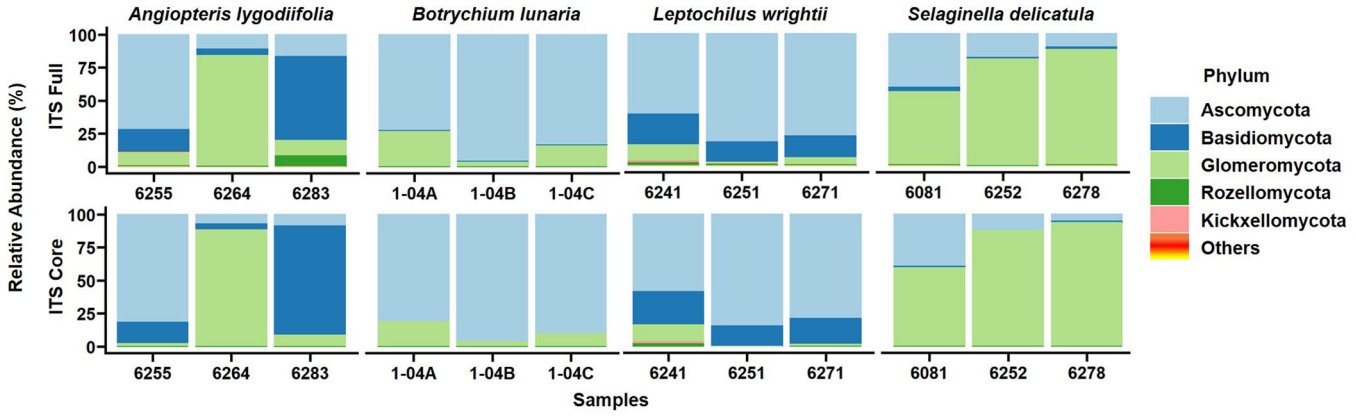

FIGURE 3 The compositional analysis of fungal communities associated with three fern and one lycophyte species at phylum level.

communities identified by ITS also had a predominance of Acaulosporaceae and Gigasporaceae, whereas 18S showed a strong presence of Paraglomeraceae. The latter, together with the families Archaeosporaceae, Claroideoglomeraceae, and Ambisporaceae, were uniquely detected by this marker. Taxon proportions were very variable among and within fern species, with *A. lygodiifolia* (in ITS) and *S. delicatula* (in 18S) displaying the most notable compositional contrasts among samples.

At the genus level, differences were also remarkable; while both protocols detected 42.9% of the fungus genera (Figure 5), 35.7% of them could only be identified using the

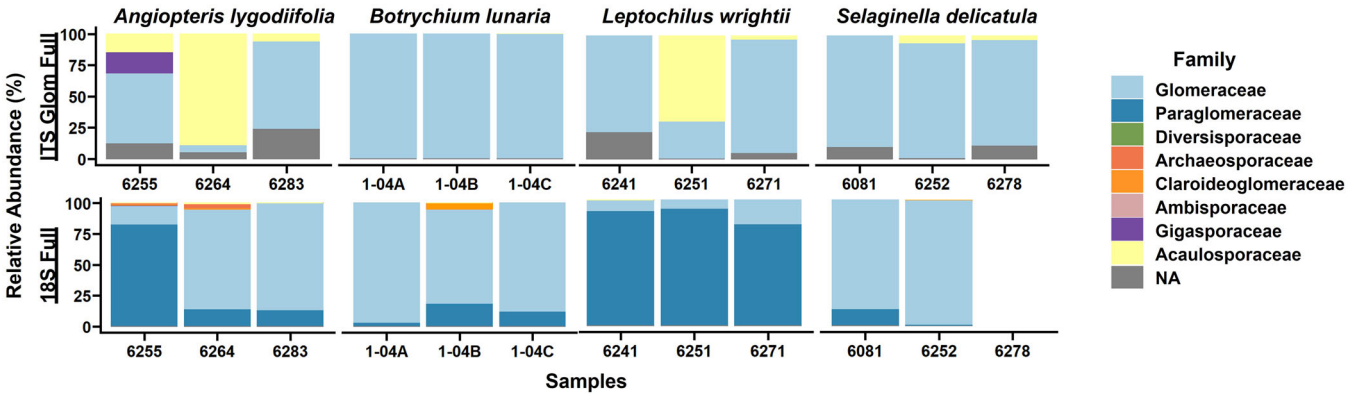

FIGURE 4 Comparison of the ITS Glom Full and 18S Full data sets regarding the compositional analysis of fungal communities associated with three fern and one lycophyte species at family level.

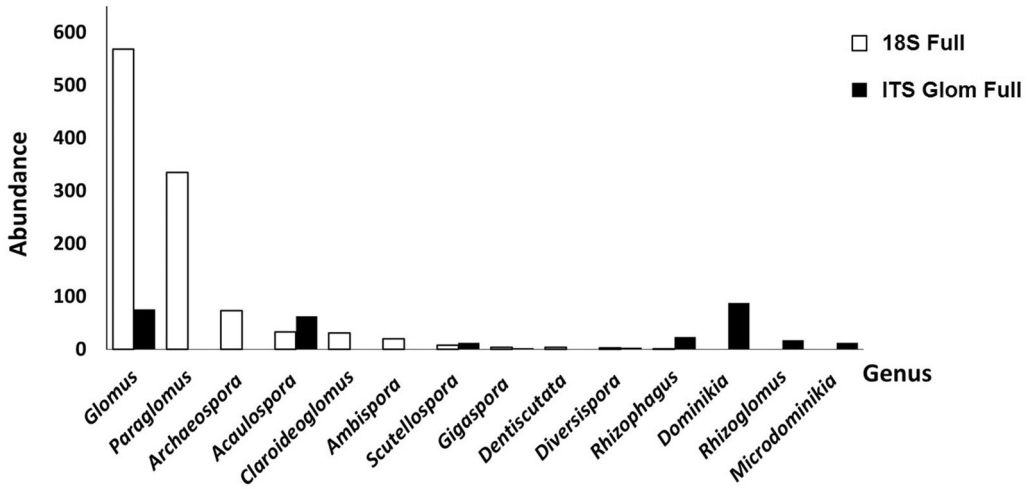

FIGURE 5 Comparison of the ITS Glom Full and 18S Full data sets regarding the abundance of arbuscular mycorrhizal fungi (AMF) genera associated with three fern species (*Angiopteris lygodiifolia*, *Botrychium lunaria*, *Leptochilus wrightii*) and one lycophyte species (*Selaginella delicatula*).

18S approach (*Paraglomus*, *Archaeospora*, *Claroideoglomus*, *Ambispora*, and *Dentiscutata*). In contrast, *Dominikia*, *Microdominikia*, and *Rhizoglomus* were detected only by the ITS protocol.

Despite the limited species-level resolution expected for AMF, we made species-level identifications for 24 fungal species in total, with only three of these shared by the ITS Glom Full and the 18S Full data sets. The most abundant fungal species were *Dominikia aurea* Błaszk., Chwat, G. A. Silva & Oehl and *Ambispora leptoticha* (N. C. Schenck & G. S. Sm.) C. Walker et al.

Fungal community similarity

The ANOSIM results indicated greater dissimilarities of fungal communities among samples of different fern or lycophyte species than among replicates of a single species. These variations of the plant microbiomes were better reflected by ITS ($R = 0.46$, $P < 0.001$) than by 18S ($R = 0.25$, $P = 0.003$) and ITS Glom Full ($R = 0.15$, $P = 0.016$).

Both the NMDS analysis based on Jaccard distance matrices and the ANOSIM analyses showed that the ITS Full ordination provided a clearer picture of community variation (Figure 6). For example, *B. lunaria* and *A. trichomanes* L. subsp. *quadrivalens* D. E. Mey. clusters appear to be well differentiated from the other taxa, indicating unique fungal communities. The geographical factor (Country) showed a greater effect on sample similarities than the corresponding fern species.

DISCUSSION

The recent development of molecular methods for sequencing fungal microbiomes (mycobiomes) in plant roots has led to a fully new understanding of endophytic interactions (Op

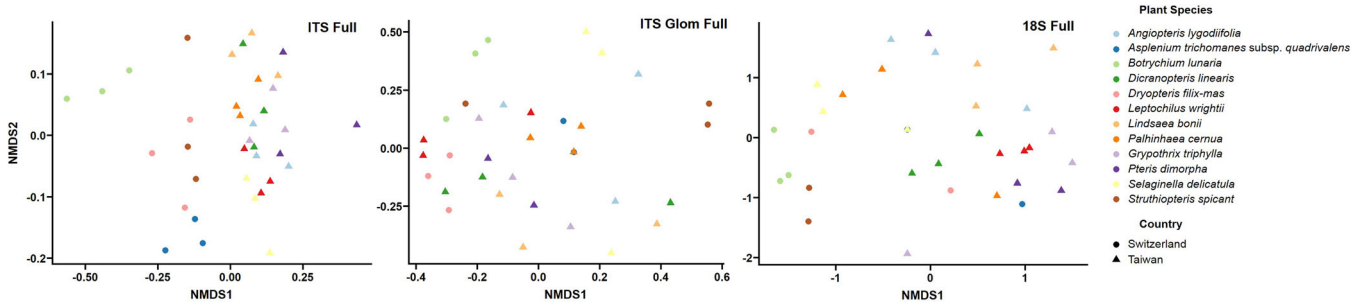

FIGURE 6 Non-metric multidimensional scaling (NMDS) ordination based on a Jaccard distance matrix of the ITS Full, ITS Glom Full, and 18S Full sets. This analysis shows the similarities in fungal community composition among samples based on their country of origin and host plant species.

De Beeck et al., 2014; Nilsson et al., 2019). However, the great majority of these studies have focused on angiosperms (Van der Heijden et al., 2015), leaving other plant clades such as ferns and lycophytes poorly investigated (Wang and Qiu, 2006; Lehnert et al., 2017; Strullu-Derrien et al., 2018). Furthermore, most studies on root mycobiomes have applied existing analytical methods without evaluating their representativeness. In the present study, we used three replicates per species of two lycophyte and 10 fern species broadly spread across the phylogenetic tree to optimize and evaluate an amplicon-based protocol.

Ideally, molecular analyses should be performed immediately after sampling in order to limit DNA degradation; however, working with fresh material is often unfeasible (Lear et al., 2018). Several methods exist to store plant samples for DNA extraction, including cooling, preservation buffers, oven-drying, and silica-drying, with the latter method being used in this study. Silica gel is an inexpensive and reliable material to dehydrate and preserve field-collected roots while keeping them suitable for metabarcoding analyses (Chase and Hills, 1991). Sample preservation methods might affect the quantity and quality of the fungal DNA obtained from plant roots (Bainard et al., 2010; Claassens et al., 2022). Comparing eight preservation methods (including silica-drying) for angiosperm roots, Bainard et al. (2010) found that drying the samples had a negative effect on the recovery of AMF DNA. However, the authors considered the colonization of a single fungal species (*Glomus intraradices*) and stored the samples for 15 weeks. Conversely, Claassens et al. (2022) found a greater number of AMF species when using the oven-drying preservation method. Comparable studies do not yet exist for ferns or lycophytes. In the present study, all of the samples were stored under the same conditions to avoid any unwanted impact on the results.

There are multiple well-established analytical approaches for the quantification of fungi in angiosperm roots (Brundrett, 2009). However, fern roots tend to be more strongly sclerified (Pearson, 1969) and contain high concentrations of polyphenols and terpenoids (Vetter, 2018), which may interfere with PCR (Schrader et al., 2012). The negative effects of inhibitors may be reduced by selecting an appropriate method for nucleic acid extraction and choosing a robust DNA polymerase (Al-Soud and Rådström, 2001), or by diluting the extracted DNA as proposed by Tedersoo et al. (2022). We found that the mechanical disruption of the root tissue using 5-mm steel beads, followed by a nucleic acid extraction with the DNeasy Plant Mini Kit and a purification phase with the Monarch Genomic DNA Purification Kit, yielded sufficient quantities of high-quality DNA. Despite its common usage in angiosperms, in our study the CTAB method generated low-purity DNA that could not be used in further stages. A dilution step increased the chances of successful amplification, although the reduced concentration of the resulting DNA may be inadequate for sequencing company requirements. Therefore, we recommended the incorporation of this step only when the concentration is above 40 ng/μL.

Primer selection is another critical step to obtain high-quality amplicons (Nilsson et al., 2019; Tedersoo et al., 2022). While the ITS rRNA region is the most broadly utilized marker for fungi due to its wide taxonomic coverage (Schoch et al., 2012; Nilsson et al., 2019), it can be highly variable in members of the Glomeromycota and provides insufficient resolution at the species level (Tedersoo et al., 2022). In contrast, despite the common use of the small subunit nuclear rRNA genes (18S region) in studies spanning AMF, this marker provides little information for Ascomycota and Basidiomycota (Nilsson et al., 2019). Mycorrhizal associations in ferns and lycophytes include members of all three above-mentioned phyla (Field et al., 2015; Lehnert et al., 2017; Benucci et al., 2020); therefore, we used the barcoded primer pairs ITS1F/ITS4 and NS31/AML2 to detect the general fungal communities and the AMF communities in fern roots, respectively. According to Manter and Vivanco (2007), the first primer pair yields amplicons with considerable size variability (420–825 bp). Nevertheless, primer barcoding improved the level of resolution (Morgan and Egerton-Warburton, 2017) so that we obtained amplicons of 500–580 bp, and the detected composition for some of the plant taxa coincides with previously published data (Benucci et al., 2020; Sandoz et al., 2020; Perez-Lamarque et al., 2022). The NS31/AML2 pair has been used in previous studies of lycophyte and fern endophytes, providing an accurate estimation of AMF diversity (Öpik et al., 2010; Sandoz et al., 2020;

Perez-Lamarque et al., 2022). As a final point, the selection of a hot start, high-fidelity, thermostable DNA polymerase favored the PCR process, minimizing nonspecific amplification products (Al-Soud and Rådström, 1998; Tedersoo et al., 2022).

Samples CH 1-01 A, CH 1-10 C, and 11E could not be amplified using the 18S rRNA marker but yielded good results when used with the ITS fungal marker. A possible reason for this outcome is the presence of fungi that the 18S-specific primers were unable to amplify (Sandoz et al., 2020; Perez-Lamarque et al., 2022); these samples were composed of >75% Ascomycota and Basidiomycota.

Representativeness of the obtained data

One of the main aims of our study was to assess the sampling completeness depending on the markers and molecular procedures used. Consequently, we calculated accumulation curves of the number of ASVs obtained in relation to the number of reads by sample (Figure 2). When analyzing the full microbiome plots, we encountered great variability in the saturation point of each curve, showing that sampling completeness can be very uneven across samples. In contrast, the core microbiome sampling curves reached saturation after a few hundred reads, which implies that even samples with a low number of reads can reliably be used to study core microbiomes in ferns and lycophytes.

Although this approach minimizes the variation in sequencing depth (number of microbial sequences obtained from each sample) (Risely, 2020; Neu et al., 2021), it might ignore rare taxa that represent a substantial functional portion of many microbial communities (Sogin et al., 2006; Schlaeppi et al., 2014). We conclude that whereas core microbiomes allow a reliable identification of fungal communities associated with the roots of lycophytes and ferns based on a low number of replicates, full microbiomes are less easily captured. Therefore, sampling incompleteness must be taken into account during the analyses.

Characterizing fungal communities in fern and lycophyte roots

The amplification of the ITS region with the primers ITS1F and ITS4 allowed us to characterize the general fungal communities and the AMF communities associated with the studied roots, finding Pleosporales, Heliotales, and Glomerales as dominant lineages. While we were unable to confirm the existence of mutualistic, commensal, or parasitic relationships in our samples based only on the molecular analysis, it is known that Glomerales develop arbuscular mycorrhizae (Brundrett, 2004), whereas Pleosporales and Heliotales include DSE that colonize root tissues (Field et al., 2015; Pressel et al., 2016) and have been reported as being frequently associated with ferns (Lehnert et al., 2017; Perez-Lamarque et al., 2022). The substantial presence of Ascomycota and Basidiomycota in our samples coincides in part with previous reports (Benucci et al., 2020; Perez-Lamarque et al., 2022). However, we did not detect members of Sebacinales or Endogonales with any of the markers, contradicting previous findings for these plant groups (Field et al., 2015; Benucci et al., 2020; Perez-Lamarque et al., 2022). This might be due to their absence in our study plant species or to a limitation of the used primers to detect these endophytic lineages (Rimington et al., 2015). In particular, Endogonales sequences are known to be difficult to amplify using common universal primers, requiring specific primers for their detection (Benucci et al., 2020).

We recorded considerable variation in the mycobiome composition among samples from the same plant species, as previously reported by Perez-Lamarque et al. (2022) for angiosperms, lycophytes, and ferns. In our study, this might be related to a locality effect (Appendix 1), as samples from widely separated localities (e.g., *Angiopteris lygodiifolia*) displayed greater differences in their community composition (similarity index calculated with EstimateS: 0.073 ± 0.040) than samples from the same locality (e.g., *Botrychium lunaria*) (similarity index calculated with EstimateS: 0.133 ± 0.114) (Figure 3). This suggests that particular abiotic (e.g., soil pH, humidity, disturbance) and biotic (e.g., competition, neighboring plants) differences between localities influence the structure and abundance of fungal communities (Brundrett, 2009; Sandoz et al., 2020; Perez-Lamarque et al., 2022). In fact, in addition to the statistically significant dissimilarities between fern species (ANOSIM $R = 0.46$, $P = 1e\text{-}04$), we also found a geographical effect by country when considering the associated general fungal communities (ITS Full) (ANOSIM $R = 0.64$, $P = 1e\text{-}04$), although this was not true of the AMF communities (18S Full, ITS Glom Full) (Figure 6).

Comparing ITS and 18S approaches

The ITS and 18S rRNA markers characterized the studied root microbiomes in different ways (Figure 5). For all of the samples, 18S detected the families Archaeosporaceae, Claroideoglomeraceae, and Ambisporaceae, as well as a dominance of Paraglomeraceae and Glomeraceae. In comparison, ITS found 23% less Glomeraceae and failed to detect the above-mentioned families, but recovered 56% more members of Acaulosporaceae, which was found to predominate in some of the samples (Appendices S5–S10).

Discrepancies in the ability of the two markers to detect AMF communities have been previously reported (Öpik et al., 2014; Lekberg et al., 2018), but the information is very incomplete in the case of ferns and lycophytes. Perez-Lamarque et al. (2022) focused on the complementary capacities of the 18S and ITS protocols to detect the whole fungal community, whereas Chen et al. (2022) only indicated that distinct primer sets captured differences in fungal taxonomic abundance on a single subterranean gametophyte of *Ophioderma pendulum* L. Indeed, the NS31/

AML2 primer set has been reported as highly effective to amplify a wider diversity of AMF taxa than other 18S-specific and ITS-specific pairs (Öpik et al., 2010; Tedersoo et al., 2022). However, fundamental differences in the reference databases used could also have influenced this outcome. When targeting AMF, the UNITE database offers detailed taxonomic annotation centered on the ITS region (Nilsson et al., 2019), whereas the MaarjAM database provides extensive coverage of Glomeromycota sequences based on the 18S rRNA gene (Öpik et al., 2010). Therefore, possible contradictions between databases (e.g., fragments from different genomic regions of the same organism being identified distinctly by each reference set), as well as their limitations, should be considered when interpreting our data (Tedersoo et al., 2022).

In conclusion, detailed studies of the root-associated fungi in lycophytes and ferns can provide important insights into both the early evolution of land plants and their current ecology. Our research offers a starting point for the establishment of consistent protocols to enhance comparability between studies focused on these plant lineages where protocols developed for angiosperms are not effective. Altogether, the 18S approach provides better coverage of the AMF communities associated with the roots of ferns and lycophytes. However, when characterizing the general endophytic communities across different species of these groups, the ITS approach is the best tool. The functional role of non-AMF fungi in ferns and lycophytes is still poorly understood, thus the broader sampling possible with the ITS protocol may uncover crucial relationships that could not be discovered using the 18S protocol. When time or resource constraints prevent the combined use of both the ITS and 18S markers, selecting the appropriate marker will depend on the research questions.

AUTHOR CONTRIBUTIONS

M.K. and P.S. conceived and designed the project. M.K., C.C., and T.G.O. collected plant samples. T.G.O. and S.L. designed and conducted the molecular analyses. T.G.O. completed the bioinformatic and statistical analyses. T.G.O. and M.K. wrote the manuscript. All authors approved the final version of the manuscript.

ACKNOWLEDGMENTS

The authors acknowledge the Swiss National Science Foundation for providing the funds for this study (project 188498).

DATA AVAILABILITY STATEMENT

Sequence data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database with BioProject ID PRJNA876213.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Forward and reverse primer pairs tested during this study.

Appendix S2. PCR conditions using primers ITS86F/ITS4 and the Q5 High-Fidelity DNA Polymerase set (New England Biolabs, Frankfurt am Main, Germany).

Appendix S3. Taxonomic classification and ASV abundance for the ITS Full data set.

Appendix S4. Taxonomic classification and ASV abundance for the 18S Full data set.

Appendix S5. Compositional analysis of whole fungal communities detected by the ITS marker (ITS Full) and associated with 12 fern and lycophyte species. The analysis was performed at phylum level and divided by plant species.

Appendix S6. Compositional analysis of the core fungal communities detected by the ITS marker (ITS Core, relative abundance >1%) and associated with 12 fern and lycophyte species. The analysis was performed at phylum level and divided by plant species.

Appendix S7. Compositional analysis of arbuscular mycorrhizal fungi detected by the ITS marker (ITS Glom Full) and associated with 12 fern and lycophyte species. The analysis was performed at family level and divided by plant species. The absence of columns represents the lack of detection of Glomeromycota families in the sample.

Appendix S8. Compositional analysis of arbuscular mycorrhizal fungi detected by the ITS marker (ITS Glom Core, relative abundance >1%) and associated with 12 fern and lycophyte species. The analysis was performed at family level and divided by plant species. The absence of columns represents the lack of detection of Glomeromycota families in the sample.

Appendix S9. Compositional analysis of arbuscular mycorrhizal fungi detected by the 18S marker (18S Full) and associated with 12 fern and lycophyte species. The analysis was performed at family level and divided by plant species. The absence of columns represents the lack of detection of Glomeromycota families in the sample.

Appendix S10. Compositional analysis of arbuscular mycorrhizal fungi detected by the 18S marker (18S Core, relative abundance >1%) and associated with 12 fern and lycophyte species. The analysis was performed at family level and divided by plant species. The absence of columns represents the lack of detection of Glomeromycota families in the sample.

How to cite this article: Guillen-Otero, T., S.-J. Lee, C.-W. Chen, P. Szoevenyi, and M. Kessler. 2023. A metabarcoding protocol targeting two DNA regions to analyze root-associated fungal communities in ferns and lycophytes. *Applications in Plant Sciences* 11(3): e11523. https://doi.org/10.1002/aps3.11523

APPENDIX 1: Voucher information for the fern and lycophyte species used in this study.

Sample	Family	Species	Authorship	Month	Year	Collection locality	Latitude	Longitude
CH 1-01 A	Dryopteridaceae	*Dryopteris filix-mas*	(L.) Schott	August	2020	Rapperswil-Jona, Switzerland	47°13′44.178″N	8°53′45.440″E
CH 1-01B	Dryopteridaceae	*Dryopteris filix-mas*	(L.) Schott	August	2020	Meilen, Switzerland	47°15′27″N	8°43′37″E
CH 1-01 C	Dryopteridaceae	*Dryopteris filix-mas*	(L.) Schott	June	2020	Zurich, Switzerland	47°21′35″N	8°33′37″E
11E	Blechnaceae	*Struthiopteris spicant*	(L.) Weiss	June	2020	Goms, Switzerland	46°32′41.641″N	8°21′24.418″E
4 C	Blechnaceae	*Struthiopteris spicant*	(L.) Weiss	July	2020	Locarno, Switzerland	46°11′16.990″N	8°48′57.829″E
5B	Blechnaceae	*Struthiopteris spicant*	(L.) Weiss	July	2020	Schwyz, Switzerland	46°59′34.39″N	8°47′31.74″E
Tw 6281	Lycopodiaceae	*Palhinhaea cernua*	(L.) Franco & Vasc.	July	2020	Keelung City, Taiwan	25°03′37.3″N	121°43′27.8″E
Tw 6288	Lycopodiaceae	*Palhinhaea cernua*	(L.) Franco & Vasc.	July	2020	New Taipei City, Taiwan	25°07′50.4″N	121°38′13.8″E
Tw 6286	Lycopodiaceae	*Palhinhaea cernua*	(L.) Franco & Vasc.	July	2020	New Taipei City, Taiwan	25°07′50.4″N	121°38′13.8″E
Tw 6278	Selaginellaceae	*Selaginella delicatula*	(Desv. ex Poir.) Alston	May	2020	New Taipei City, Taiwan	24°46′28.7″N	121°29′45.0″E
Tw 6252	Selaginellaceae	*Selaginella delicatula*	(Desv. ex Poir.) Alston	April	2020	Keelung City, Taiwan	25°08′31.8″N	121°47′02.8″E
Tw 6081	Selaginellaceae	*Selaginella delicatula*	(Desv. ex Poir.) Alston	July	2020	Keelung City, Taiwan	25°08′14.4″N	121°40′15.0″E
Tw 6028	Lindsaeaceae	*Lindsaea bonii*	Christ.	July	2020	Keelung City, Taiwan	25°08′04.8″N	121°37′44.2″E
Tw 6300	Lindsaeaceae	*Lindsaea bonii*	Christ.	June	2020	New Taipei City, Taiwan	25°07′50.4″N	121°38′13.8″E
Tw 6295	Lindsaeaceae	*Lindsaea bonii*	Christ.	June	2020	New Taipei City, Taiwan	25°07′50.4″N	121°38′13.8″E
CH 1-04 A	Ophioglossaceae	*Botrychium lunaria*	(L.) Sw.	June	2020	Leventina, Switzerland	46°28′28″N	8°25′26″E
CH 1-04B	Ophioglossaceae	*Botrychium lunaria*	(L.) Sw.	September	2020	Leventina, Switzerland	46°28′28″N	8°25′26″E
CH 1-04 C	Ophioglossaceae	*Botrychium lunaria*	(L.) Sw.	September	2020	Leventina, Switzerland	46°28′28″N	8°25′26″E
CH 1-10 A	Aspleniaceae	*Asplenium trichomanes* L. subsp. *quadrivalens*	D. E. Mey.	July	2020	Lugano, Switzerland	45°59′30″N	8°47′31″E
CH 1-10B	Aspleniaceae	*Asplenium trichomanes* L. subsp. *quadrivalens*	D. E. Mey.	July	2020	Hombrechtikon, Switzerland	47°15′30″N	8°44′17″E
CH 1-10 C	Aspleniaceae	*Asplenium trichomanes* L. subsp. *quadrivalens*	D. E. Mey.	July	2020	Bellinzona, Switzerland	46°11′36″N	9°00′08″E

Sample	Family	Species	Authorship	Month	Year	Collection locality	Latitude	Longitude
Tw 6264	Marattiaceae	*Angiopteris lygodiifolia*	Rosenst.	June	2020	New Taipei City, Taiwan	25°01′58.4″N	121°36′19.8″E
Tw 6255	Marattiaceae	*Angiopteris lygodiifolia*	Rosenst.	July	2020	Keelung City, Taiwan	25°08′31.8″N	121°47′02.8″E
Tw 6283	Marattiaceae	*Angiopteris lygodiifolia*	Rosenst.	July	2020	Keelung City, Taiwan	25°03′37.3″N	121°43′27.8″E
Tw 6241	Polypodiaceae	*Leptochilus wrightii*	(Hook.) X. C. Zhang	July	2020	Keelung City, Taiwan	25°08′13.8″N	121°45′23.5″E
Tw 6251	Polypodiaceae	*Leptochilus wrightii*	(Hook.) X. C. Zhang	July	2020	Keelung City, Taiwan	25°08′31.8″N	121°47′02.8″E
Tw 6271	Polypodiaceae	*Leptochilus wrightii*	(Hook.) X. C. Zhang	July	2020	Keelung City, Taiwan	25°08′45.6″N	121°46′36.4″E
Tw 6249	Thelypteridaceae	*Grypothrix triphylla*	(Sw.) S. E. Fawc. & A. R. Sm.	July	2020	Keelung City, Taiwan	25°08′31.8″N	121°47′02.8″E
Tw 6294	Thelypteridaceae	*Grypothrix triphylla*	(Sw.) S. E. Fawc. & A. R. Sm.	Abril	2020	New Taipei City, Taiwan	25°07′50.4″N	121°38′13.8″E
Tw 6275	Thelypteridaceae	*Grypothrix triphylla*	(Sw.) S. E. Fawc. & A. R. Sm.	June	2020	Keelung City, Taiwan	25°08′45.6″N	121°46′36.4″E
Tw 6299	Pteridaceae	*Pteris dimorpha* Copel. var. *prolongata*	Y. S. Chao, H. Y. Liu & W. L. Chiou	July	2020	New Taipei City, Taiwan	25°07′50.4″N	121°38′13.8″E
Tw 6020	Pteridaceae	*Pteris dimorpha* Copel. var. *prolongata*	Y. S. Chao, H. Y. Liu & W. L. Chiou	July	2020	Keelung City, Taiwan	25°08′19.5″N	121°45′23.8″E
Tw 6240	Pteridaceae	*Pteris dimorpha* Copel. var. *prolongata*	Y. S. Chao, H. Y. Liu & W. L. Chiou	July	2020	Keelung City, Taiwan	25°04′36.3″N	121°44′14.3″E
Tw 6287	Gleichenaceae	*Dicranopteris linearis*	(Burm. f.) Underw.	July	2020	New Taipei City, Taiwan	25°07′50.4″N	121°38′13.8″E
Tw 6250	Gleichenaceae	*Dicranopteris linearis*	(Burm. f.) Underw.	July	2020	New Taipei City, Taiwan	25°08′31.8″N	121°47′02.8″E
Tw 6268	Gleichenaceae	*Dicranopteris linearis*	(Burm. f.) Underw.	July	2020	Keelung City, Taiwan	25°08′48.7″N	121°45′56.7″E