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Version: Accepted Version

Article:
Magkourilou, E., Moeskjaer, S., Hipperson, H. et al. (2026) A novel ITS primer minimises plant DNA co-amplification and expands fungal coverage. Fungal Biology, 130 (8). 101875. ISSN: 1878-6146

https://doi.org/10.1016/j.funbio.2026.101875

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A novel ITS primer minimises plant DNA co-amplification and expands fungal coverage

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Keywords: fungi; Illumina; ITS; metabarcoding; mycorrhizae; PacBio; primers; sequencing

Abstract

Fungal metabarcoding is a vital tool for understanding plant-microbiome dynamics and assessing how natural and anthropogenic drivers influence the diversity of plant-associated fungal communities across environments. Commonly used fungal ITS primers have inherent biases, often co-amplifying off-target plant host DNA while underrepresenting ecologically important groups (such as the mycorrhizal-containing phyla Glomeromycota and Mucoromycota). Here, we conducted both *in silico* and experimental comparisons of commonly used fungal primer combinations against a newly designed primer developed to reduce plant host binding and provide a more balanced fungal taxonomic coverage. Primer performance was evaluated across diverse habitat types, using plant root tissue and soil samples. Metrics for species richness and community diversity varied significantly depending on the primer pair used, with performance influenced by both habitat and sample type. Despite this variability, our novel primer demonstrated the most consistent, reliable, and balanced performance. This makes it a suitable option for capturing a wide portion of the

fungal community (including mycorrhizal fungi) without having to employ multiple primer pairs.

Introduction

Despite major technological advances in recent decades and growing efforts to describe global biodiversity, only an estimated 3-to-8% of extant fungal species have been formally described (Blackwell 2011; Hawksworth and Lücking 2017). Fungi are already recognised as among the most ubiquitous life forms on Earth, yet many regions, including those likely to contain the greatest diversity, remain understudied (Hyde et al., 2018). Appreciation of fungal diversity has expanded considerably in recent years, driven largely by advances in molecular sequencing techniques and the development of a wide range of primers targeting different fungal groups (Tedersoo et al., 2020).

However, the sequencing-based approaches that have driven such advances are inherently subject to multiple methodological biases related to the choice of marker region, primer pair, PCR conditions and sequencing platform used (Paloi et al., 2021; 2023). Most foundational investigations into the molecular ecology of fungi, particularly within the phyla Basidiomycota and Ascomycota, have relied on primers targeting the internal transcribed spacer (ITS) region (Schoch et al., 2012), widely regarded as the standard fungal DNA barcode. The ITS region comprises subregions ITS1 and ITS2 separated by the 5.8S ribosomal RNA (rRNA) gene, and it is relatively variable due to frequent insertion and deletion events (Nagy et al., 2012). This variability makes it well-suited for differentiating between closely related taxa, but less appropriate for assembling phylogenies (Tedersoo et al., 2020; Furneaux et al., 2021; Kolaříková et al., 2021). When selecting between the subregions for metabarcoding, ITS1 often provides better species-level discrimination but suffers from length variability that can induce PCR bias. On the other hand, although ITS2 offers more uniform fragment sizes and conserved primer binding sites, it often exhibits lower resolution in certain fungal groups. In any case, despite neither region completely capturing all taxa on its own, both subregions tend to capture overall community patterns similarly (Bazzicalupo et al. 2013; Blaalid et al. 2013).

An ideal fungal primer should amplify DNA from all fungal groups evenly without amplifying non-target organisms such as plants, other non-fungal eukaryotes, bacteria, or archaea (Martin and Rygiewicz, 2005). However, in practice, many commonly used primers exhibit biases. This is exemplified by mycorrhizal fungi (e.g., Lumini et al., 2010), a group of fungi that form symbiotic relationships with the roots (or rhizoids) of approximately 82% of land plant species (Brundrett and Tedersoo, 2018). Mycorrhizal associations play important ecological roles in terrestrial ecosystems, influencing plant nutrition, productivity, and community dynamics (van der Heijden et al., 2008). The term 'mycorrhiza' covers several distinct functional groups, including arbuscular mycorrhizal (AM) fungi, ectomycorrhizal (EcM) fungi, ericoid mycorrhizal (ERM) fungi, orchid mycorrhizal (OM) fungi, as well as the more recently delineated Mucoromycotina 'fine root endophytes' (MFRE; Orchard et al., 2017; Howard et al., 2025). MFRE can form nutritionally mutualistic associations with a wide variety of host plants and are now widely recognised as being mycorrhizal, akin to AM fungi (Field & Pressel, 2018; Hoysted et al., 2019, 2023; Prout et al., 2024; Howard et al., 2025). One reason MFRE remain relatively understudied is the challenge of distinguishing them morphologically from other root endophytes or in 'cryptic' colonisations where characteristic plant-fungal interfaces are not present (Field et al., 2015). Although high-resolution microscopy can, in some cases, differentiate MFRE from AM fungi (Albornoz et al., 2020), molecular approaches are increasingly used to reliably characterise fungal diversity in environmental samples.

Traditionally, mycorrhizal fungi were thought to span four fungal phyla: Ascomycota, Basidiomycota, Mucoromycota and Glomeromycota (Naranjo-Ortiz and Gabaldón, 2019). However, *sensu* Spatafora et al. (2016), Glomeromycota, encompassing all known AM fungi, are sometimes treated as a sub-phylum within Mucoromycota referred to as Glomeromycotina. Molecular evidence further supports that Glomeromycotina comprises all AM fungi and is phylogenetically distinct from Mucoromycotina, the other major Mucoromycotan lineage, which includes the MFRE (Orchard et al., 2017), most notably in the orders Densosporales and Endogonales (Desirò et al., 2017; Tedersoo et al, 2024; Cole et al., 2025). Although MFRE and AM fungi are therefore often separated at the subphylum level (Mucoromycotina vs. Glomeromycotina), here we follow the phylum-level distinction (Mucoromycota vs

Glomeromycota) used in major reference databases such as UNITE (Abarenkov et al., 2024) and EUKARYOME (Tedersoo et al., 2024).

Despite their ecological importance, the diversity of mycorrhizal fungi remains substantially underestimated. For example, recent syntheses suggest that there could be as many as 55,500 species of EcM, the majority of which are currently classified as 'dark' taxa, known primarily from environmental DNA sequences, while only around 300 species have been formally described (van Galen et al., 2025). Similar knowledge gaps exist for other mycorrhizal fungal groups. For example, global estimates suggest that more than 8,000 AM fungal species may exist, although only 200–300 have been formally described, while approximately 600 MFRE species are hypothesised and only one species, *Planticonsortium tenue*, has been formally described to date (Walker et al., 2018). Despite research on MFRE being limited due to them lacking in comprehensive global diversity assessments, they are increasingly becoming of broader research interest (Lutz et al., 2025).

The underrepresentation of several mycorrhizal taxa at least partly relates to molecular and phylogenetic differences between fungal groups (e.g., Ascomycota and Basidiomycota or Glomeromycota and Mucoromycota) and the primers used to capture fungal diversity. For example, several ITS-targeting primers have been shown to underrepresent certain Glomeromycota taxa (Lumini et al., 2010), favour certain fungal families (Kohout et al., 2014), or co-amplify plant DNA (Alguacil et al., 2011). Moreover, maybe unsurprisingly considering their aforementioned phylogenetic distinctness, even primers designed specifically for AM fungi may fail to detect MFRE (Orchard et al., 2017). Indeed, although some studies have attempted to simultaneously characterise AM fungi and MFRE communities (e.g., Albornoz et al., 2021; 2022; Mansfield et al., 2023), subsequent analyses have confirmed that the primers used may bias against detection of MFRE (Seeliger et al., 2024).

In Glomeromycota, the ITS region is highly variable, and studies often instead target the immediately adjacent 18S Small Subunit (SSU) rRNA, which is more conserved (Chemidlin Prévost-Bouré et al., 2011; Błaszkowski et al., 2021; Furneaux et al., 2021; Kolaříková et al., 2021). Although targeting the 18S is better suited for phylogenetic reconstruction, it comes at the cost of lower species-level resolution (Kolaříková et al., 2021). These trade-offs are particularly important when assessing

closely related species or when employing short-read sequencing technologies such as Illumina, which typically generate read lengths of 300–600 base pairs. On the other hand, long-read sequencing, such as third-generation Pacific Biosciences (PacBio) platforms, utilises the parallel processing of high-throughput sequencing and can simultaneously target the 18S, ITS, and/or 28S regions to gain a greater degree of taxonomic and phylogenetic resolution (Hugerth and Andersson, 2017; Castaño et al., 2020; Kolaříková et al., 2021). However, despite these advantages, PacBio sequencing remains comparatively more expensive than short-read platforms and offers a lower data yield per run (Jeon et al., 2023).

Understanding the strengths and limitations of specific sequencing technologies and of the primers used is crucial for accurately interpreting the resulting data and applying appropriate caveats. Additionally, minimising the amplification of non-target organisms is essential to reduce sequencing costs, maximise useful data yield (e.g., targeting fungal vs plant sequences), and improve the detection of rarer taxa. Recently developed primers targeting the 18S rRNA region have improved detection of MFRE relative to AM fungi (Seeliger et al., 2024). However, the use of broadly inclusive fungal primers (rather than AM- or MFRE-specific primers) has recently been highlighted as particularly valuable, as comprehensive datasets allow for a better understanding of how mycorrhizal fungi associate with the wider fungal microbiome, including plant pathogenic fungi (Lutz et al., 2023). That said, there has been no comprehensive assessment of commonly used fungal primers with respect to their ability to capture a wide diversity of fungal groups, including ecologically important yet underrepresented in reference databases groups such as AM fungi and MFRE, while minimising amplification of non-target DNA.

In this investigation, we designed a general fungal primer targeting the ITS1 subregion that would allow a less biased assessment of fungal diversity (e.g., amplify Glomeromycota and Mucoromycota as effectively as Ascomycota and Basidiomycota) without amplifying plant DNA. To compare the performance of our novel candidate primer against commonly used ITS primers, we first performed *in silico* testing to assess the specificity of the primers for fungal DNA and their coverage across major fungal phyla (Table 1). Following the *in silico* testing, we validated the results *in vitro* using complex environmental samples from a range of

158 diverse habitats to maximise the robustness of our findings. Our pipeline combined
159 short-read Illumina and long-read PacBio sequencing approaches to improve
160 phylogenetic resolution within the poorly-characterised Mucoromycota lineage and
161 more accurately assess primer performance in complex environmental samples. We
162 aimed to determine whether there are primer biases in terms of 1) target (i.e., fungal)
163 vs non-target (e.g., plant) groups; and 2) taxonomic richness and composition,
164 focusing on overall fungal diversity as well as specifically for the exemplar under-
165 represented Glomeromycota (AM fungi) and Mucoromycota (including MFRE) phyla.

166 **Table 1. Primers as used in the current study.**

Primer name	Code	Sequence (5'-3')	Binding site	Reference
ITS1F	FZ	CTTGGTCATTTAGAGGAAGTAA	18S	Gardes and Bruns, 1993
1624F	FY	CCTTTGTACACACCGCCCGTCG	18S	Hadziavdic et al., 2014
ITS86R	RA	TTCAAAGATTCGATGATTCAC	5.8S	Vancov and Keen, 2009**; original Turenne et al., 1999*
fITS7R and fITS7oR mix	RB***	CAAARATTYGATGATTCAC*	5.8S	Mix as used in Lekberg et al. 2018*; original Ihrmark et al., 2012*, but slightly modified by Kohout et al., 2014*
gITS7R	RB***	CAAAGAYTCGATGAYTCAC*	5.8S	Ihrmark et al. 2012*
fITS9R	RC	TCRCNNTTYGCTGCGTTC*	5.8S	Ihrmark et al. 2012*

58A2R	RD	CTGCGTTCTTCATCGAT	5.8S	Martin and Rygiewicz, 2005
5.8SFun24R	RE	CCAAGAGATCCRTTGYTRAAA	5.8S	Novel primer
5.8S_FunR	RF	AG**T**WGATCCRTTGYYRAAAGTT****	5.8S	Taylor et al. 2016*

167 * Report/use the forward primer targeting the ITS2 subregion instead of the ITS1
168 ** As noted by Op De Beeck et al. (2014), Vancoc and Keen (2009) contain a typo in the
169 ITS86R, as it was not an exact reverse complement of ITS86F originally developed by
170 Turenne et al. (1999)
171 *** Same code as in the experimental analysis; added to the PCR mix together
172 **** This is the reverse complement of the 5.8S_Fun primer used in Taylor et al. (2016), with
173 the addition of one extra T (marked in bold) near the 5′ end

Materials and Methods

Primer selection and *in silico* analysis

First, we performed *in silico* testing of 5.8S and 18S primers, which are commonly used in fungal diversity studies (Table 1; Figure 1), to assess their specificity for fungal DNA and their theoretical coverage of fungal phyla and therefore potential reduced bias. This assessment included a novel candidate primer (5.8SFun24R) to investigate whether it improved fungal coverage whilst minimising co-amplification of plant DNA. The novel candidate primer was designed by aligning 5.8S sequences from diverse fungal groups with non-target eukaryotic taxa. Following the *in silico* screening procedures described below, candidate regions were selected based on high sequence conservation within Fungi and intentional 3′ mismatches against non-target groups to prevent non-specific amplification.

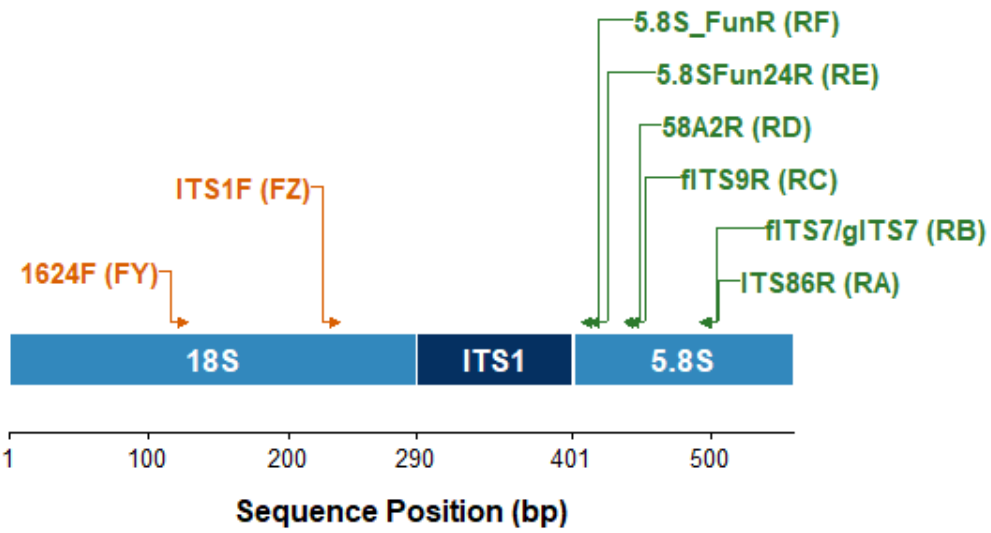


Figure 1. Location of the primers used in this study along the 18S/ITS1/5.8S rRNA gene region. Positions are shown against the sequence of FR750032.1 *Funneliformis mosseae*, which contains a partial 18S rRNA gene.

Primer matching was performed *in silico* against EUKARYOME ('General-EUK-long v2.0'; Tedersoo et al., 2024), a curated eukaryotic reference database comprising the combined 18S (SSU) - ITS1 - 5.8S - ITS2 - 28S (LSU) regions of multiple fungal, plant and animal taxa. All sequences with a minimum length of 50 bases were scanned for primer binding sites using *cutadapt* (v4.5; Martin, 2011) on the

University of Sheffield High Performance Computing cluster. For each primer (Table 1), matching was performed, allowing 0, 1, or 2 mismatches. Reverse-complement matching was enabled to account for both strand orientations, and only sequences containing a valid primer match were retained.

The number of sequences successfully captured for each primer-mismatch combination for each database was recorded and compared against the total number of database sequences for each phylum in order to determine the percentage of sequences successfully captured (i.e., capture efficiency). Subsequently, the information for the major fungal phyla, as well as an average of other phyla (including those belonging to plants and animals), was filtered into a clean table.

Field sampling

We tested the primer pairs' (Table 1) performance on complex environmental samples comprising three replicate root and soil samples from four contrasting habitats; 1: Agricultural field (Spen Farm, University of Leeds; 53.861348, -1.344814), 2: Woodland (Froggatt Woods; 53.312241, -1.619469), 3: Heathland (Longshaw Estate; 53.317010, -1.616952), and 4: Calcareous Grassland (Bakewell; 53.206262, -1.655300). The dominant plant taxa were recorded at each location. *Triticum aestivum* alone was present in the agricultural field; the woodland understory was dominated by *Trifolium*, *Rubus*, *Pteridium*, *Sorbus*, *Plantago*, and *Dactylis*. *Calluna*, *Festuca*, *Juncus*, and *Holcus sp.* dominated the heathland. *Veronica*, *Trifolium*, *Plantago*, *Holcus*, *Juncus*, *Festuca*, *Ranunculus*, and *Cirsium sp.* dominated the grassland. All samples were taken from between 0 and 20cm soil depth, sealed in plastic bags for transport to the lab, frozen overnight, and freeze-dried for storage before DNA extraction. Root samples were homogenised using a TissueLyser II (QIAGEN, Germany) prior to DNA extraction.

DNA extraction

DNA extractions were carried out using the PowerSoil® DNA Isolation kit (MO BIO Laboratories, QIAGEN, Venlo, Netherlands) for soil samples and the DNeasy® Plant Mini Kit (QIAGEN, Venlo, Netherlands) for root samples, following the

manufacturer's instructions. Extracted DNA was stored at -20°C until further processing.

Sequencing

A dual-sequencing approach was undertaken. Illumina sequencing targeting mostly the ITS region was used to obtain high-depth, quantitative profiles of fungal community composition. PacBio long-read sequencing was employed to simultaneously capture parts of the 18S, ITS1 and 5.8S regions, enabling improved taxonomic resolution and phylogenetic placement of fungal taxa. Both sequencing approaches were performed on the same DNA samples, but using different sets of primers as described in detail below. Ultimately, as our main aim was to compare results from different ITS1 targeting primers for ecological study, the data presented throughout this paper stem from the short-read Illumina sequences. However, as detailed below, PacBio long-read sequences were leveraged to improve taxonomic assignment.

PacBio sequencing

PCR amplification, library construction and sequencing

DNA was diluted 1:50 before being amplified in a two-step PCR to generate a pool of indexed amplicons for all 24 samples. For the first round of PCR, 1µl of each diluted sample was mixed with 10µl of Q5® High-Fidelity DNA Polymerase (Q5® Hot Start High-Fidelity 2X Master Mix, Massachusetts, USA), 7µl of water, and 1 µl each of the customised (tailed with an overhang adapter) forward and reverse primers (10µM; Sigma Aldrich, Merck KGaA, Darmstadt, Germany) 3NDf[GGCAAGTCTGGTGCCAG (Cavalier-Smith *et al.*, 2009)] and 5.8SFun24R [TTATYARCAAYGGATCTCTTGG] spanning a region of ~1,500 bp (from a highly conserved region of the 18S rRNA gene to a conserved sequence located in the 5.8S rRNA gene) in a reaction volume of 20 µl and the following cycling conditions: 95°C for 3 min, 30 cycles of 95°C for 30s, 59°C for 30s and 72°C for 30 s with a final 2 min extension at 72°C. PCR products were bead-cleaned using 0.6x AMPure XP Beads (Beckman Coulter Inc., CA, USA). The second round of PCR used 8µl of bead-cleaned PCR1 product with 10µl of Q5® High-Fidelity DNA Polymerase (Q5® Hot Start High-Fidelity 2X Master Mix, Massachusetts, USA), 1µl of water, and 0.5 µl each of forward and reverse indexed primers (10µM) that incorporate the overhang adapter from PCR1 to

allow sample multiplexing for sequencing. The total reaction volume was 20 µl and the following cycling conditions were used: 95°C for 3 min, 10 cycles of 98°C for 15s, 65°C for 30s and 72°C for 90s with a final 7 min extension at 72°C. PCR2 products were quantified using a Qubit broad range assay (Thermofisher Scientific, Waltham, MA, USA), pooled in equimolar amounts and further purified and concentrated using AMPure XP Beads (Beckman Coulter Inc., CA, USA).

PacBio library preparation was carried out using the SMRTbell® Express Template Prep Kit 2.0, and Single Molecule Real Time (SMRT) circular consensus sequencing (CCS) was performed on the PacBio Sequel I using Sequencing Primer v4 and Sequel I Binding Kit 3.0 (Pacific Bioscience, CA, USA). Sequence reads containing at least five passes and with a minimum predicted accuracy of 0.9 were generated using CCS v6.0.0 in SMRT Link v10.1.0 and retained for further analysis.

Bioinformatic analysis

Filtering and demultiplexing of the CCS reads output from PacBio were carried out using cutadapt (Martin, 2011) and FASTX-Toolkit (Hannon, 2010). The PacBio CCS bam file was converted to fastq format using BBMap v38.45 (Bushnell, 2019).

Cutadapt *v3.2* was used to remove the primer sequences, allowing the default 10% error rate and retaining only trimmed sequences. Fastq barcode splitter from the FASTX-Toolkit package v0.0.13 was used to demultiplex the sequences into sample-specific files based on the primer indexes used, allowing the default value of 1 mismatch.

Subsequent analysis was carried out in R v4.0.3 (R Core Team, 2022), following the workflow described by Dirks and Jackson (2020), in turn largely based on using DADA2 (Callahan et al., 2016) to assign reads to Amplicon Sequence Variants (ASVs). More specifically, samples were filtered to a minimum quality of 2, a minimum length of 100bp and a maximum ‘expected error’ of 2, before denoising and chimaera removal, following which 1,915 ASV sequences were exported as a FASTA file. *ITXs* (Bengtsson-Palme *et al.*, 2013) was then used to extract the ITS1 and SSU regions of the reads. BLAST (Altschul et al., 1990) nucleotide database searches were run using standalone blast on the University of Sheffield High-Performance Computing Cluster against the National Centre for Biotechnology Information (NCBI) nt database (Sayers et al., 2020) for 713 sequences with a

detected ITS1 region, excluding blast hits to ‘uncultured’ or ‘environmental’ accessions.

Turning PacBio reads into a project-specific taxonomic reference database

Step 1: Taxonomic assignment using BLASTn

Ultimately, the full-read portion of the sequences that passed the ITSx and BLAST prefiltering were assigned taxonomy using BLASTn (Camacho et al., 2009) as part of Taxonomizr (Sherrill-Mix, 2025) and employing a Lowest Common Ancestor (LCA) method against the EUKARYOME reference database (‘General-EUK-long v2.0’; Tedersoo et al., 2024). More specifically, BLAST alignments for each ASV were pre-filtered at a 97% identity threshold, and only those with a bitscore within 2% of the maximum score for each specific ASV were retained. Taxonomy was determined using a strict LCA approach. To enforce hierarchical integrity, if a consensus could not be reached at a given taxonomic level, all subsequent lower levels were automatically designated as ‘unassigned’.

Step 2: Phylogenetic inference using IQ-tree and epa-ng

To further improve taxonomic assignment, the 18S regions of the PacBio long-read sequences were extracted to be used for phylogenetic inference.

Sequence Preparation and Reference Backbone Construction

The 18S regions of the PacBio long-read ASVs were isolated and dereplicated. To provide an evolutionary framework, a curated reference backbone was constructed using sequences from the EUKARYOME database (‘General-EUK-long v2.0’; Tedersoo et al., 20240). The database was dereplicated at 99% identity and subsampled to maximise taxonomic diversity, including representative sequences of all fungal phyla that contained more than 100 sequences in EUKARYOME (e.g., Chytridiomycota, Ascomycota, and Basidiomycota), alongside Nuclearia sequences to serve as an outgroup root (Supplementary Table 1). To maximise the phylogenetic placement of fungal groups used as examples in this study (i.e., Glomeromycota and Mucoromycota phyla), a deeper representative sampling was performed. For Glomeromycota and the Mucoromycotan orders Endogonales and Densosporales—which are believed to harbour most MFRE taxa (Desirò et al., 2017; Tedersoo et al., 2024; Cole et al., 2025)—sequences were selected to ensure representation across all classified families. The 18S regions for both the query ASVs and the reference

backbone were explicitly extracted using Barrnap (Seeman, 2013) to ensure homologous comparisons.

Alignment and Phylogenetic Inference

Alignment was performed using a two-step approach in MAFFT (Katoh and Standley, 2013): the reference backbone was first aligned with high accuracy using the L-INS-I iterative refinement method, after which the query ASVs were added to the stable backbone alignment. The resulting alignments were trimmed using ClipKIT (Steenwyk et al., 2020) to remove phylogenetically uninformative regions. A maximum-likelihood constraint tree of the reference backbone was generated using IQ-TREE2 (Minh et al., 2020), utilising ModelFinder to select the optimal evolutionary model and 1000 ultrafast bootstraps for branch support. A final maximum-likelihood tree incorporating all sequences was then constructed, topologically constrained by the reference backbone.

Taxonomic Assignment via Phylogenetic Placement

Query ASVs were phylogenetically placed onto the reference backbone tree using EPA-ng (Barbera et al., 2019). Taxonomy was subsequently assigned based on these placements using Gappa (Czech, Barbara and Stamatakis, 2020). To ensure rigorous and conservative taxonomic assignments, results were filtered to retain only placements with high confidence (accumulated Likelihood Weight Ratio > 0.8) and low phylogenetic uncertainty (Expected Distance between Placement Locations < 0.064, representing roughly one average branch length of the reference tree). Placements failing these thresholds were designated as ‘unassigned’.

Step 3: Taxonomic integration

To establish a final, high-resolution taxonomic assignment for each long-read ASV, classifications derived from BLAST against the EUKARYOME database and phylogenetic placement via EPA-ng were integrated using a lineage-aware, cascading logic. BLAST classifications served as the foundational taxonomy. For ASVs subjected to phylogenetic placement, the EPA-ng-derived taxonomy was evaluated from kingdom to species level. An EPA-ng assignment was used to fill ‘unassigned’ gaps in the BLAST taxonomy only if all higher-level taxonomic levels (e.g., kingdom, phylum) were strictly identical between the two methods to ensure that deeper phylogenetic assignments were not appended to conflicting higher-level

lineages.

Illumina

PCR amplification, library construction and sequencing

Samples were amplified using 12 combinations of primers designed to amplify partial fungal 18S, full ITS1 and partial 5.8S rRNA gene sequences (Figure 1, Table **1**). Amplification was performed in a two-step process, using target-specific primers modified to include Illumina overhang adapter nucleotide sequences in the first-round PCR, followed by the ligation of indices in a secondary PCR to allow for multiplexing.

First-round PCR conditions were optimised by initially testing DNA template dilutions (1:1, 1:10, and 1:100) to balance DNA concentration against potential PCR inhibitors; a 1:10 dilution was found to be most effective and was utilised for all subsequent reactions. Additionally, three Taq polymerase enzymes were screened for efficiency using three randomly selected root and three soil DNA samples: Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, USA), KAPA HiFi DNA Polymerase (KAPA Biosystems, Wilmington, USA), and MyTaq™ DNA Polymerase (Bioline, Little Clacton, UK). Q5® High-Fidelity DNA Polymerase was selected for all downstream experiments as it demonstrated the highest efficiency and produced the strongest bands. To determine the optimal annealing temperature, all primer combinations were tested on a gradient thermal cycler with a temperature range from 50°C to 60°C across a 96-well plate. All primer combinations performed well across this range, only failing at the extreme temperatures, and an annealing temperature of 55°C was selected for all subsequent reactions.

First-round PCRs were performed in triplicate in 20 µl reaction volumes containing 1 µl of DNA template (1:10 dilution), 0.2 µl Q5® High-Fidelity DNA Polymerase, 4 µl Q5® High-Fidelity Buffer (5x), 0.4 µl dNTP mix (10 mM each), 0.3 µl Bovine Serum Albumin (BSA; 20 mg/ml), and 1 µl of each primer (10 µM). Primers fITS7R, fITS7oR and gITS7R (i.e., RB) were added together. Thermal cycling was conducted on a 96-well thermal cycler with the following parameters: an initial denaturation step at 95°C for 3 min; followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 2 min. Negative controls were included on all plates to monitor for contamination.

Following amplification, the triplicate PCR products from each sample were pooled to create a 24 µl total sample volume. The pooled samples were purified using AMPure XP PCR Purification beads (Beckman Coulter Ltd, High Wycombe, UK) according to the manufacturer's instructions. Briefly, 20 µl of AMPure XP beads were added to each sample and mixed by pipetting. Following a 5 min incubation at room temperature, the 96-well plate was placed on an Invitrogen™ Magnetic Stand-96 (Thermo Fisher Scientific, Waltham, USA) until the solution cleared, after which the supernatant was carefully removed and discarded. The beads were washed twice by adding 200 µl of 80% ethanol to each well, removing the majority of the ethanol and allowing the beads to air-dry for 10 min before removing the plate from the magnetic stand and resuspending the beads in 52.5 µl of 10 mM Tris (pH 8.5) by pipetting. After a 2 min room-temperature incubation, the plate was returned to the magnetic stand until the solution cleared. The supernatant containing the purified PCR product was then carefully transferred to a clean tube and frozen.

The purified first-round PCR product was subsequently used in a short-cycle secondary PCR to attach a unique combination of Nextera XT i5 and i7 indices (Illumina, Inc, San Diego, USA) to each sample. Each 50 µl reaction contained 5 µl of purified PCR product, 5 µl of Nextera i5 index, 5 µl of Nextera i7 index, 0.5 µl of Q5® High-Fidelity DNA Polymerase, 10 µl Q5® High-Fidelity Buffer (5x), and 1.5 µl dNTP mix (10 mM each). Thermal cycling conditions consisted of an initial denaturation at 95°C for 3 min; followed by 8 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. Secondary PCR products were run on an agarose gel and imaged using SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, USA) to verify successful amplification.

The resulting Nextera XT amplicons were purified using AMPure XP PCR Purification beads as described above, with minor modifications to concentrate the sample, using 45 µl of PCR product to produce 25 µl of cleaned product. Final PCR products were quantified using QuantiFluor® dsDNA Dye quantification assays (Promega, Chilworth, UK). A working solution was prepared by creating a 400-fold dilution of QuantiFluor® dsDNA Dye (Promega Corp., Madison, USA) in TE buffer. Next, 2 µl of each sample was mixed with 200 µl of the QuantiFluor working solution in a black 96-well microplate. Fluorescence was measured on a FLUOstar® OPTIMA

spectrofluorimeter (BMG LABTECH, Ortenberg, Germany) with excitation at 485 nm and emission at 545 nm. Absolute DNA concentrations were calculated using a standard curve generated from a serial dilution of known DNA standards. Finally, the amplicons were pooled in equimolar concentrations to form a single multiplexed library. Sequencing was conducted across two lanes of a HiSeq 2500 system using 250-bp paired-end (250PE-RR) reads at The Earlham Institute (Norwich Research Park, Norwich, UK). Raw Illumina Sequences were uploaded to the National Center for Biotechnology Information (accession no. PRJNA1483938).

Bioinformatic analyses

One grassland soil sample for primer pair 'FERY' failed at sequencing, so it was not included in the analysis. Demultiplexed sequences with primer sequences removed using Cutadapt version 4.5 and -O 3 (Martin, 2011) were processed using the DADA2 pipeline (Callahan et al., 2016) on the High-Performance Computing Cluster at the University of Sheffield. In brief, within each primer pair dataset, the relevant primers were identified and subsequently removed using *cutadapt* (Martin, 2011). Subsequently, low-quality reads and chimaeras, and singletons were removed from the library using *filterAndTrim*. Next, the sequence error rates were assessed using *learnErrors*, and dereplication was performed using *derepFastq* functions, where forward and reverse reads were subsequently merged to form ASVs. Unmerged forward reads were also retained to rescue ASVs that lacked sufficient overlap so as not to introduce any bias in the comparison between primer pairs due to the variable nature of the ITS1 subregion. To prevent redundancy and double-counting, a per-sample exclusivity filter was applied: unmerged forward ASVs were only kept if a successfully merged counterpart was not present in the same sample. Finally, chimeric sequences were removed de novo from both the merged and rescued unmerged ASV datasets using the consensus method.

Taxonomic assignment

Taxonomy of short-read Illumina sequences was assigned in two steps. First, taxonomy was assigned against the project-specific reference database containing our long-read PacBio sequences. Taxonomy was also assigned against EUKARYOME. For each of these two steps, taxonomic classifications from the DADA2 and BLASTn pipelines were integrated to maximise resolution. More

specifically, taxonomy was assigned using *assignTaxonomy* of *DADA2* (Callahan et al., 2016) against the EUKARYOME reference database ('DADA2-EUK-long v2.0'; Tedersoo et al., 2024). For assignments against the project-specific long-read PacBio database, a minimum bootstrap confidence was set as 80%, whereas for assignments against EUKARYOME, it was set as 60%.

ASVs were also assigned taxonomy using BLASTn (Camacho et al., 2009) as part of Taxonomizr (Sherrill-Mix, 2025) and employing an LCA method against the EUKARYOME reference database ('General-EUK-long v2.0'; Tedersoo et al., 2024). BLAST alignments were filtered as described above (see section Step 1: Taxonomic assignment using BLASTn) except that a 99% identity threshold, as well as a minimum 80% length coverage, was used when the project-specific long-read PacBio database was used. A minimum 50% length coverage was introduced for assignment against the EUKARYOME database.

Within each step (i.e., taxonomy assigned against our project-specific long-read PacBio database or against EUKARYOME), DADA2 assignments were prioritised, but if the lowest taxonomic assignment from DADA2 matched the BLASTn classification at that same level, subsequent lower-level annotations from BLASTn were appended to the taxonomy.

Ultimately, to maximise classification resolution and ensure taxonomic accuracy, ASV assignments from the project-specific PacBio long-read database and the broader EUKARYOME database were integrated using a level-by-level consensus algorithm. The taxonomy stemming from the project-specific long-read database was established as the primary reference; however, rules were incorporated to handle conflicting assignments and capture broader eukaryotic diversity. First, if an ASV was classified within kingdom Fungi using the project-specific database, but the same ASV was classified as a non-fungal kingdom using EUKARYOME, the EUKARYOME lineage completely superseded the project-specific assignment. Additionally, if both databases agreed on the Fungi kingdom, but EUKARYOME identified a fungal phylum explicitly absent from the project-specific reference database, the EUKARYOME lineage was prioritised to preserve novel taxonomic diversity.

Statistical analyses

Statistical analysis and data visualisation were carried out in R (R Core Team, 2025) using RStudio (Posit team, 2025). Briefly, for each primer set, the ASV table, taxonomy table and metadata were imported to create a phyloseq object using the R package `phyloseq` (this unfiltered, full dataset is used under 'All taxa' in the Results; McMurdie and Holmes, 2013). The phyloseq objects were filtered to create a version where singletons were removed (cf. Brown et al., 2015), and only fungal ASVs classified to at least the phylum level were retained (i.e., this filtered dataset is used in 'All fungal taxa with a classified phylum' in the Results).

Forward primer comparison (FY: 1624F vs FZ: ITS1F)

The first comparison looked at the difference between forward primers FY (1624F) and FZ (ITS1F). To evaluate the differential performance of these primers in recovering specific taxonomic groups, read and ASV counts were aggregated at the phylum level for each biological sample. Taxonomic assignments were categorised into 11 target groups: Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, Mortierellomycota, Mucoromycota and Rozellomycota, referring to the main fungal phyla, 'Other fungal' (fungal ASVs not belonging to the target phyla), 'Unknown fungal' (fungal ASVs assigned lacking phylum-level resolution), 'Other non-fungal' (non-fungal ASVs that had a phylum assignment, the majority of which were plant) and 'Unknown non-fungal' (non-fungal ASVs lacking phylum-level resolution).

Generalised linear mixed models (GLMMs) were constructed using the `glmmTMB` package (McGillycuddy et al., 2025) to assess the interactive effect of reverse primer and taxonomic group on the recovery of reads and ASVs. Because the response variables were overdispersed count data, models were fit using a negative binomial error distribution (nbinom2 parameterisation). To account for the non-independence of data originating from the same physical sample, biological sample identity was included as a random intercept in all models. Prior testing confirmed that habitat and sample type did not significantly interact with the primer, and they were therefore excluded as fixed effects. Model assumptions, including dispersion and zero-inflation, were verified by assessing simulated residuals using the `DHARMa` package (Hartig, 2025). Type III Analysis of Variance (ANOVA) with sum-to-zero contrasts was used to test for the global significance of fixed effects. Following the GLMMs,

estimated marginal means (EMMs) for each primer and taxonomic group combination were extracted on the response scale using the `emmeans` package (Lenth and Piaskowski, 2026). Pairwise comparisons between primers within each taxonomic group were conducted, and compact letter displays were generated using the `multcomp` package (Hothorn, Bretz and Westfall, 2008) to denote statistically significant differences ($p < 0.05$).

Alpha diversity

Next, for each phyloseq object, metrics, including total read abundance, observed ASVs, and alpha diversity measures, Shannon diversity, and Simpson diversity, were calculated for each sample using the `vegan` package (Oksanen et al., 2026). To assess the effects of primer selection, habitat type, and sample type on these metrics, GLMMs were again constructed using the `glmmTMB` package. To account for the hierarchical structure of the experimental design, sampling replicate was included as a random intercept in all models. Fixed effects included the two-way interactions between primer, habitat, and sample type. For overdispersed count data (Observed ASVs and Total Reads), models were fitted using a negative binomial distribution (nbinom2). Shannon diversity, which represents continuous, unbounded data, was modelled using a Gaussian distribution. Simpson diversity, which is strictly bounded between 0 and 1, was modelled using a Beta distribution with a logit link function; a dispersion formula (~ primer + habitat) was sometimes included to stabilise model residuals. Model assumptions and residual fit were validated using simulated residuals via the `DHARMa` package (Hartig, 2025). The global significance of fixed effects and their interactions was first evaluated using a Type III ANOVA via the `car` package (Fox and Weisberg, 2019). The extraction of EMMs and subsequent post-hoc comparisons were dictated by these results and were performed using the `emmeans` package (Lenth and Piaskowski, 2026). Specifically, if a significant interaction between the primer and environmental context (habitat and sample type) was detected, EMMs were extracted, and pairwise comparisons were conducted within each specific environment. Conversely, in the absence of significant interactions, EMMs were extracted for the main effect of the primer, marginalised across all environmental factors. All pairwise comparisons were performed on the response scale, with p-values adjusted using the Benjamini-Hochberg False Discovery Rate (FDR) method (Benjamini & Hochberg, 1995).

Plotting of the data reflected the statistical modelling (i.e., only faceted by sample type and/or habitat if the interaction with primer was significant). Compact letter displays denoting statistical significance ($P < 0.05$) were generated using the `multcomp` package (Hothorn, Bretz and Westfall, 2008). All plots were generated using `ggplot2` (Wickham, 2016).

Beta biodiversity

Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity and a Permutational Multivariate Analysis of Variance (PERMANOVA) implemented in the `pairwiseAdonis` package (Martinez Arbizu, 2020) was used to assess the impact of primer selection on fungal community structure. The phyloseq object was pruned to remove empty taxa and samples with zero total abundance to prevent artefacts in distance calculations. A Bray-Curtis dissimilarity matrix for each primer was produced to account for both the presence/absence and the relative abundance of fungal taxa. Community clustering was visualised via PCoA using the `ggplot2` package, where the *stat_ellipse ()* function was used to calculate 95% confidence ellipses for each habitat using a multivariate t-distribution to highlight group-level differences. A PERMANOVA using the *adonis2 ()* function was performed to quantify the drivers of fungal community variation. The model was structured to evaluate the marginal effects of habitat and sample type, as well as their interaction. Statistical significance was assessed using 999 permutations with the by = "margin" setting to weight contributions by variance. Homogeneity of variances across groups was tested using the *betadisper ()* function from the `vegan` package (Oksanen et al., 2026). p-values were adjusted for multiple comparisons where applicable, and a significance threshold of 0.05 was used.

Relative abundance

Raw ASV counts were converted to relative abundances (0–100%) and aggregated at the phylum level for each sample. Fungal sequences lacking assignment at the phylum level were grouped as ‘Unknown fungal’, while fungal sequences of minor fungal phyla were categorised as ‘Other fungal’, and those assigned to non-fungi as ‘Other non-fungal’ if assigned at the kingdom level, or ‘Unknown non-fungal’ (if unassigned at both kingdom and phylum levels). Differences in community composition across primers, habitats, and sample types were assessed using a

PERMANOVA (Bray-Curtis dissimilarity, 999 permutations), which indicated significant interactive effects. To account for these interactions, relative abundances are faceted by habitat and sample type, illustrating how technical primer bias shifts across different ecological contexts.

Differential Abundance Analysis using ANCOM-BC2

To evaluate the differential recovery of taxonomic groups by the primers, ASV counts were aggregated at the phylum level. Following aggregation, the phylum-level count matrices and corresponding metadata were merged into a single, unified phyloseq object. A global pseudocount of 1 was uniformly applied to the merged count matrix to handle zero-count values and stabilise downstream logarithmic calculations.

Differential abundance analysis was conducted using Analysis of Compositions of Microbiomes with Bias Correction 2 (ANCOM-BC2; Lin and Peddada, 2024). The generalised linear mixed model in ANCOM-BC2 was configured to assess the fixed effect of using each primer, with the standard 'RE' primer designated as the baseline reference for all comparisons. To account for the non-independence of samples processed by multiple primers, the unique sample identity was included in the model as a random intercept.

The raw natural log fold changes and standard errors estimated by ANCOM-BC2 were mathematically converted to Log2 fold changes and Log2 standard errors to facilitate interpretation. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg FDR method (Benjamini & Hochberg, 1995). Finally, phyla demonstrating statistically significant differential abundance (adjusted $p < 0.05$) compared to the RE reference primer were extracted, mapped back to their respective kingdoms, and visualised as a forest plot displaying the bias-corrected Log2 fold changes and standard errors.

Results

In silico analysis

Between the two forward primers, FZ (ITS1F) generally showed an equal or higher theoretical coverage for all fungal groups; however, it also captured a very high proportion of non-target Viridiplantae sequences (Table 2). On the other hand, FY

(1624F) showed an acceptable capture (>60%) of most fungal groups and virtually no coverage of Viridiplantae sequences (Table 2).

For the reverse primes, we found that with 0 mismatches, only RB (fITS7R/fITS7oR/gITS7R) and our novel candidate primer RE (5.8SFun24R) primers had a good (>80%) theoretical coverage for Glomeromycota and Mucoromycota, with RC (fITS9R), RD (58A2R) and RF (5.8S_FunR) severely underperforming (i.e., <25% theoretical capture) for one or both phyla (Table 2). However, as expected, sequence capture efficiency increased when allowing for 1 or 2 mismatches. Specifically, the average capture of all fungi across all primers increased from around 60% with 0 mismatches to 87% with 1 mismatch and 95% with 2 mismatches. The most dramatic shift was observed for RF, which went from only 0% average coverage at 0 mismatches to 80% at 1 mismatch, and reached 95% at 2 mismatches. Across all fungal phyla, RC and RE have the highest capture efficiency with 0 mismatches at ~75%; however, when allowing for more mismatches, RD and RC improved significantly to reach ~93% and 98% for 1 and 2 mismatches, respectively. However, when more mismatches were allowed, most primers also showed high capture efficiency for plant phyla. Specifically, RC captured 67% of plant sequences with 0 mismatches, increasing to 95% and 97% with 1- or 2-mismatches. RD also captured the majority of plant sequences when mismatches were allowed (e.g., 93% and 97% for 1 and 2 mismatches, respectively). In contrast, RE and RF either did not match plant sequences at all or only matched 48% of sequences with 2 mismatches (RE).

Our novel candidate primer, RE, was the most balanced, as with only 1 mismatch, it successfully captured 100% of Glomeromycotan and 98% of Mucoromycotan sequences whilst only capturing 1% of plant sequences. At 2 mismatches, RF is predicted to be the optimal primer, as it would capture 95% of fungal sequences and almost no plant sequences, but this is at the expense of low matching at 0 or 1 mismatches to either general fungi or our exemplar taxa.

636 **Table 2. *In silico* evaluation of primers.** Percentage of sequences in the EUKARYOME database captured using *cutadapt* (v4.5;
637 Martin, 2011), allowing for 0, 1 or 2 mismatches. For the main fungal phyla, the total number of sequences in EUKARYOME is
638 shown in parentheses. For 'other fungal phyla' as well as for the plant kingdom Viridiplantae, the global average sequence capture
639 efficiency is shown. Numbers have been rounded to the nearest whole number. Darker red indicates a high capture efficiency, and
640 lighter yellow indicates low capture efficiency. * In the experimental analysis, all RB primers were added to the PCR mix together.

Taxonomic Group	FZ: ITS1F			FY: 1624F		
	0	1	2	0	1	2
Ascomycota (18906)	93	97	98	46	91	98
Basidiomycota (16890)	94	99	100	88	95	98
Chytridiomycota (5885)	95	99	100	62	96	100
Glomeromycota (4542)	95	100	100	95	100	100
Mortierellomycota (2728)	95	100	100	90	96	100
Mucoromycota (1846)	94	100	100	39	66	69
Rozellomycota (7082)	87	98	99	51	71	80
Other fungal phyla (avg)	94	99	99	72	94	98
All fungi (avg)	93	99	99	67	91	95
Viridiplantae (avg)	92	96	97	1	1	1

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Taxonomic Group	RA: ITS86F			RB: fITS7*			RB: fITS7o*			RB: gITS7*			RC: fITS9			RD: 58A2F			RE: 5.8SFun24R			RF: 5.8S-FunF		
	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
Ascomycota (18906)	72	93	97	80	94	97	75	95	99	85	95	98	92	98	99	91	98	99	88	98	99	1	92	98
Basidiomycota (16890)	91	98	98	92	98	98	93	98	99	93	98	99	92	97	98	92	98	99	90	97	98	0	91	97
Chytridiomycota (5885)	44	79	96	44	79	96	72	95	97	46	80	96	84	98	99	20	91	99	6	70	96	0	85	99
Glomeromycota (4542)	51	91	100	54	92	100	85	99	100	55	92	100	23	96	100	25	97	100	95	100	100	0	19	97

Mortierellomycota (2728)	90	95	98	91	95	98	92	98	99	91	98	99	95	99	100	95	99	99	94	99	100	0	95	99
Mucoromycota (1846)	63	92	97	63	92	97	72	97	98	80	95	98	75	96	100	76	96	100	82	98	100	0	95	100
Rozellomycota (7082)	6	23	47	10	39	62	10	29	62	20	52	75	26	73	90	27	88	95	45	74	82	1	53	77
Other fungal phyla (avg)	18	41	63	19	42	65	34	57	91	24	60	85	48	63	97	28	61	95	57	80	86	0	76	87
All fungi (avg)	62	81	89	65	83	91	70	86	94	69	87	95	75	92	98	67	93	98	74	91	96	0	80	95
Viridiplantae (avg)	16	48	80	13	47	79	19	53	88	17	75	91	67	95	97	20	93	97	1	2	48	1	1	1

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Forward primer comparison (FY vs FZ)

Overall, primer pairs containing the FY (1624F) forward primer produced a modelled-derived average of 10% more ASVs for all fungal phyla than primer pairs containing the FZ (ITS1F) forward primer (ranging from a 10% decrease in Glomeromycotan ASVs to a 132% increase in 'Unknown fungal' ASVs; Supplementary Figure 1). This increase was statistically significant for 'Rozellomycota', 'Unknown fungal', 'Other non-fungal' and 'Unknown non-fungal' phyla, even though, in all cases except for 'Rozellomycota' and 'Other non-fungal' phyla, the FZ primers produced considerably more reads than the FY primer (on average, FZ primers produced 43% more fungal reads than FY primers). Although FZ yielded more raw reads, FY-based primer pairs recovered equal or greater numbers of unique ASVs. Moreover, as already shown, FY provided strong theoretical coverage of target fungal groups while virtually eliminating non-target Viridiplantae contamination; thus, the FY forward primer was selected for all downstream comparative analyses.

Reverse primer comparisons

Reads vs ASVs

The relationship between sequencing depth (total reads) and total ASV richness varied slightly among primers, with RC and RD capturing more overall ASV richness as sequencing depth increased (Supplementary Figure 2A). In contrast, the slopes of RA, RB, and RF plateaued sooner, yielding the lowest total ASV richness regardless of total sequencing depth. Our novel candidate primer, RE, demonstrated an intermediate total yield. When restricting the analysis to fungal reads and fungal ASVs (Supplementary Figure 2B), RD and RC again predicted the highest fungal ASV richness; however, the performance gap between the other primer pairs was smaller. RA and RF captured the lowest fungal ASV richness across most of the observed sequencing depths. Finally, evaluating the proportion of fungal reads against the proportion of fungal ASVs (Supplementary Figure 2C) revealed distinct trade-offs in primer specificity. Notably, RC demonstrated the poorest fungal specificity; even when fungal sequences comprised a high percentage of its total reads (e.g., >80%), its resulting fungal ASV percentage remained low (below 50%), indicating that FYRC amplifies a considerable amount of non-fungal diversity. In contrast, RB, RE, and RF exhibited strong fungal specificity, as for these primer pairs, a high proportion of

fungal reads translated into a high proportion of fungal ASVs (reaching ~60% to over 75% at the upper limits). RA maintained a more stable slope with a moderate proportion of fungal ASVs across the observed range of read percentages.

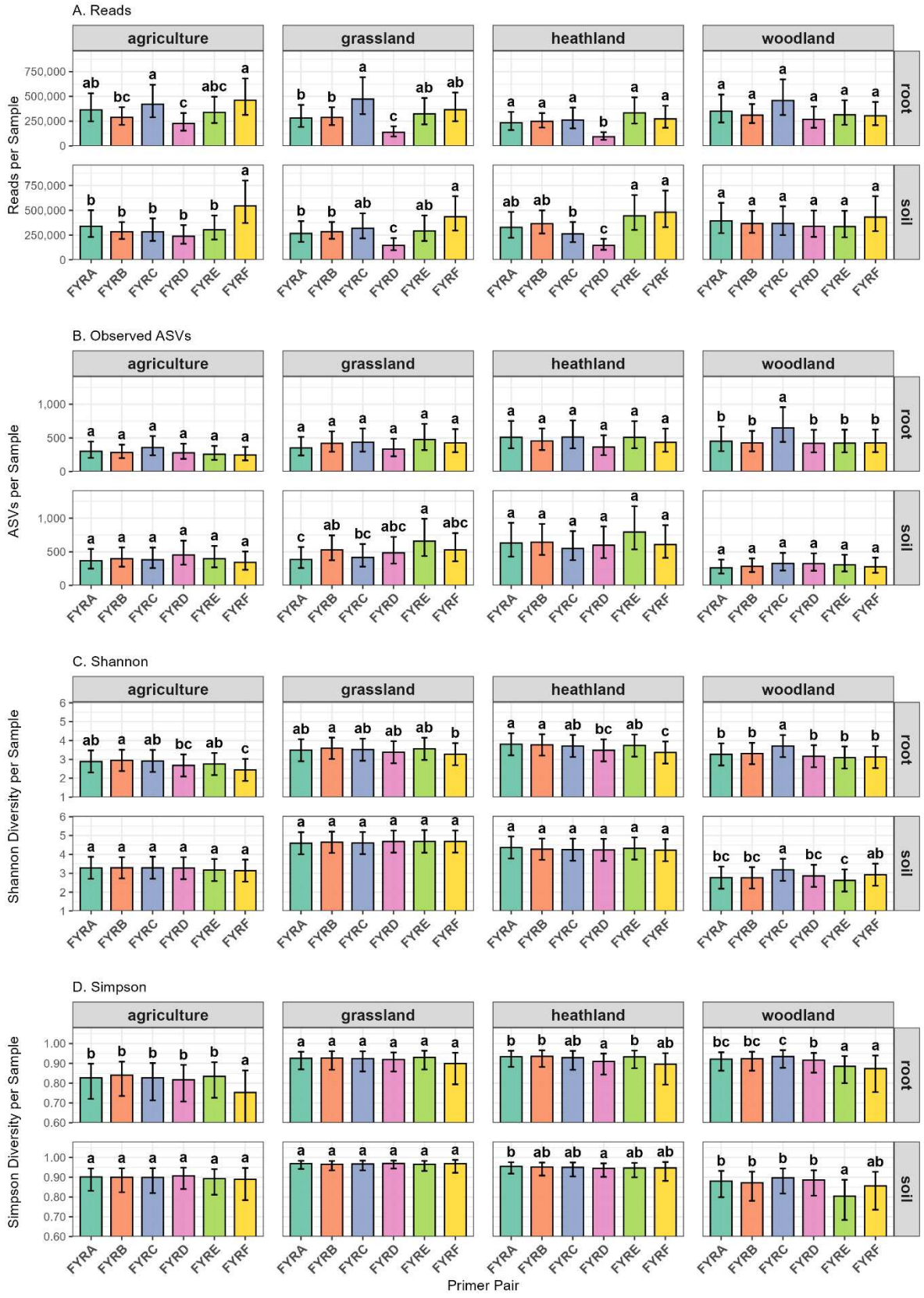


Figure 2. Impact of primer selection on total read abundance and alpha diversity metrics across different habitats and sample types using fungal

sequences that have been assigned to at least the phylum level only. Bar plots display the estimated marginal means derived from generalised linear mixed-effects models for (A) Total reads per sample, (B) Observed ASV richness, (C) Shannon diversity, and (D) Simpson diversity. Error bars represent the asymmetric 95% confidence intervals (lower and upper confidence limits) extracted from the models. Different letters placed above the confidence intervals denote statistically significant differences ($P < 0.05$) between primers within a specific facet, determined via post-hoc pairwise comparisons of the estimated marginal means using a False Discovery Rate adjustment.

Alpha Biodiversity

All taxa

When using the full dataset, the RC and RF primers produced, on average, approximately 100,000 more sequence reads per sample (Supplementary Figure 3A). The number of ASVs produced by each primer was influenced by the habitat and the type of sample analysed (ranging from 356 ASVs per agricultural root sample with primer RF to 6,741 ASVs per heathland soil sample with RC). In general, RC and RD primers produced the most unique ASVs (2,729 and 1,754 ASVs on average per sample, respectively; Supplementary Figure 3B). In terms of Shannon (Supplementary Figure 3C) and Simpson (Supplementary Figure 3D) diversity measures, RC and RD showed the highest values, particularly in the soil samples.

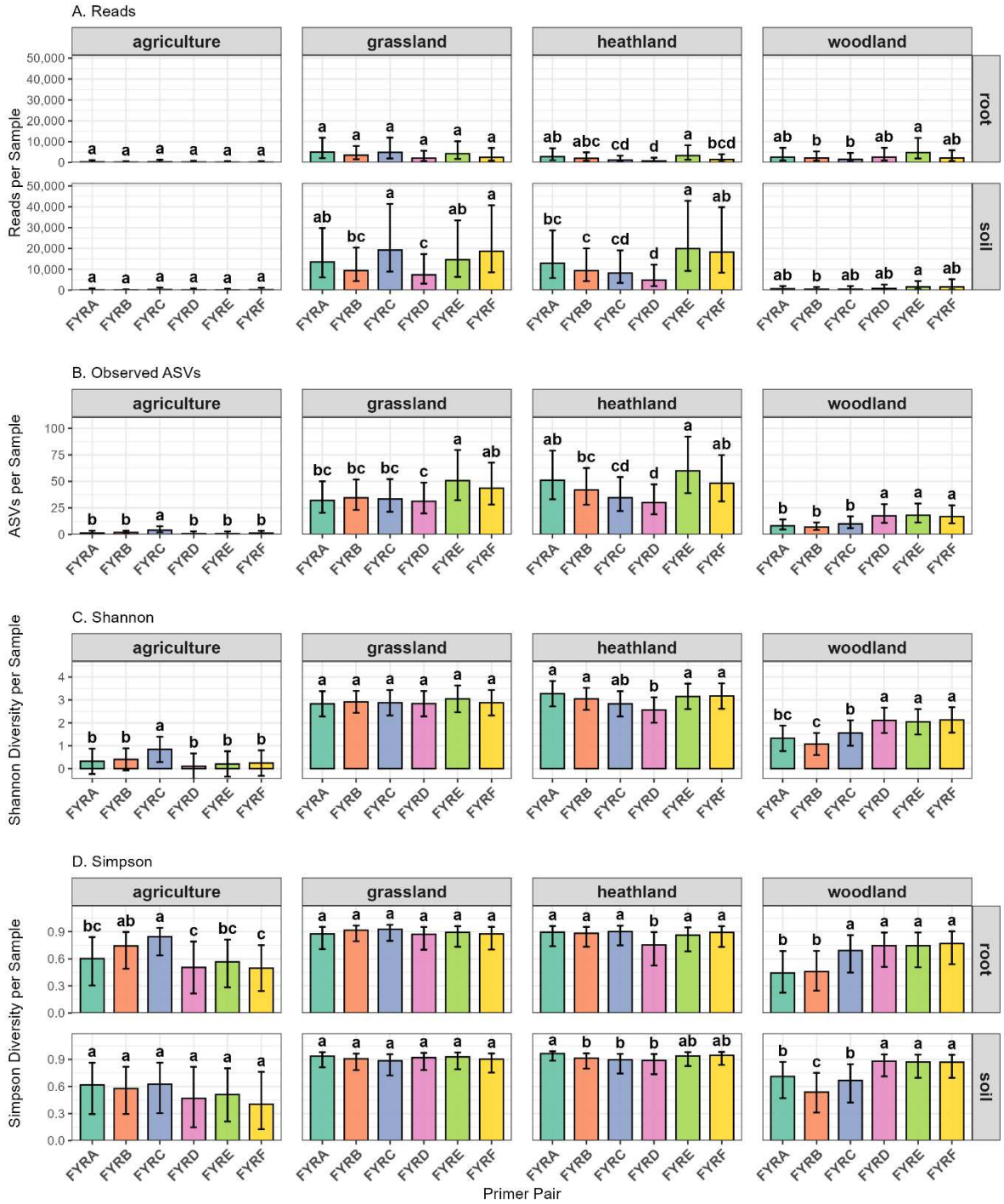


Figure 3. Impact of primer selection on total read abundance and alpha diversity metrics across different habitats and sample types using Glomeromycotan sequences only. Bar plots display the estimated marginal means derived from generalised linear mixed-effects models for (A) Total reads per sample, (B) Observed ASV richness, (C) Shannon diversity, and (D) Simpson diversity. Error bars represent the asymmetric 95% confidence intervals (lower and upper confidence limits) extracted from the models. Different letters placed above the

confidence intervals denote statistically significant differences ($P < 0.05$) between primers within a specific facet, determined via post-hoc pairwise comparisons of the estimated marginal means using a False Discovery Rate adjustment.

All fungal taxa with a classified phylum

When the dataset was filtered to only keep fungal ASVs that had been assigned to at least a phylum level, the picture changed slightly. Although there was still a trend for the reads produced by RC and RF to be higher in some cases (most notably for grassland and woodland root samples for RC and agricultural samples for RF), overall, these were far more in line with the other primers (Figure 2A). In woodland roots, RC produced more ASVs than all other primers, but RC underperformed relative to RE in grassland soil (Figure 2B). Alpha diversity measures (Figure 2C and D) were largely similar across primers, with the one noticeable difference being in woodland roots, where RC again showed higher Shannon diversity than all other primers.

Glomeromycota and Mucoromycota

When focusing on Glomeromycota (i.e., AM fungi), reads varied significantly between habitat and sample type, but RD generally underperformed (2,421 reads on average per sample for RD versus 6,495 for RF; Figure 3A). Most richness was captured in the grassland and the heathland; the two habitats where RE performed better than most primers (51 and 70 Glomeromycotan ASVs on average per sample, respectively, for grassland and heathland samples; Figure 3B). Shannon diversity varied based on primer and habitat combinations (Figure 3C), whereas for Simpson diversity, the sample type was also an influential factor (Figure 3D). Agricultural samples showed very low richness and diversity overall, but RC generally outperformed the other primers in these samples.

When focusing on Mucoromycota, reads also varied significantly between habitats and sample types, but RD again generally underperformed (409 reads on average per sample for RD versus 1,995 for RF; Figure 4A). Most richness was captured in the heathland, where again RD produced the lowest number of ASVs (12 Mucoromycotan ASVs with RD versus 19 with RE; Figure 4B). Shannon and Simpson diversity varied based on primer and habitat combinations, with differences between primers more pronounced in agriculture and heathland (Figure 4C).

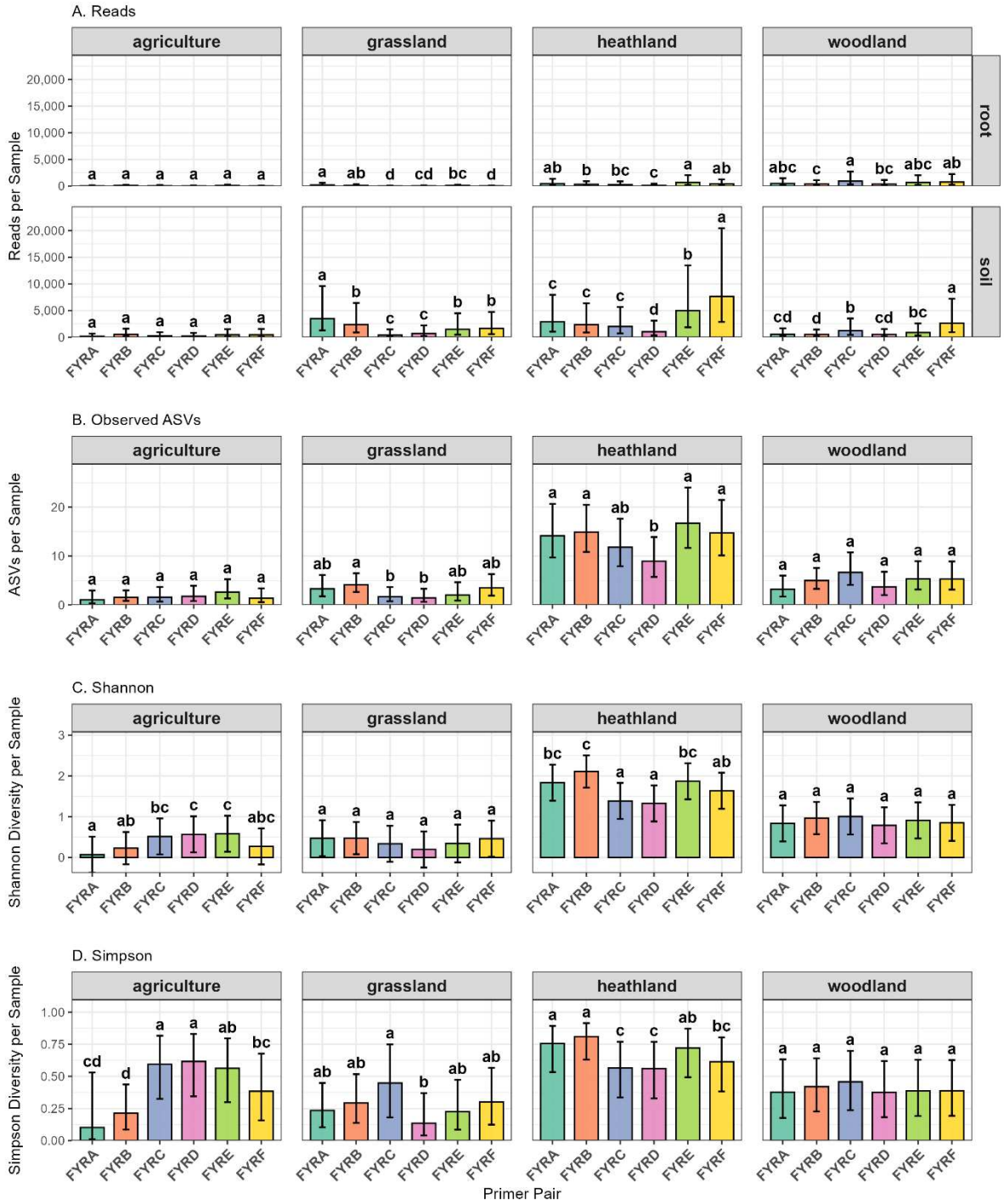


Figure 4. Impact of primer selection on total read abundance and alpha diversity metrics across different habitats and sample types using Mucoromycotan sequences only. Bar plots display the estimated marginal means derived from generalised linear mixed-effects models for (A) Total reads per sample, (B) Observed ASV richness, (C) Shannon diversity, and (D) Simpson diversity. Error bars represent the asymmetric 95% confidence intervals (lower and upper confidence limits) extracted from the models. Different letters placed above the

confidence intervals denote statistically significant differences ($P < 0.05$) between primers within a specific facet, determined via post-hoc pairwise comparisons of the estimated marginal means using a False Discovery Rate adjustment.

Relative abundance of phyla

All taxa

The relative abundance of phyla varied between primers, but this was also further influenced by habitat and sample type (Figure 5). In general, RD and RC showed the highest relative abundance of non-target taxa (i.e., Unknown non-fungal or Other non-fungal; averaging at around 19% and 13%, respectively, across all samples), with RE and RB showing the lowest values of non-target taxa (both averaging at around 4%; Figure 5).

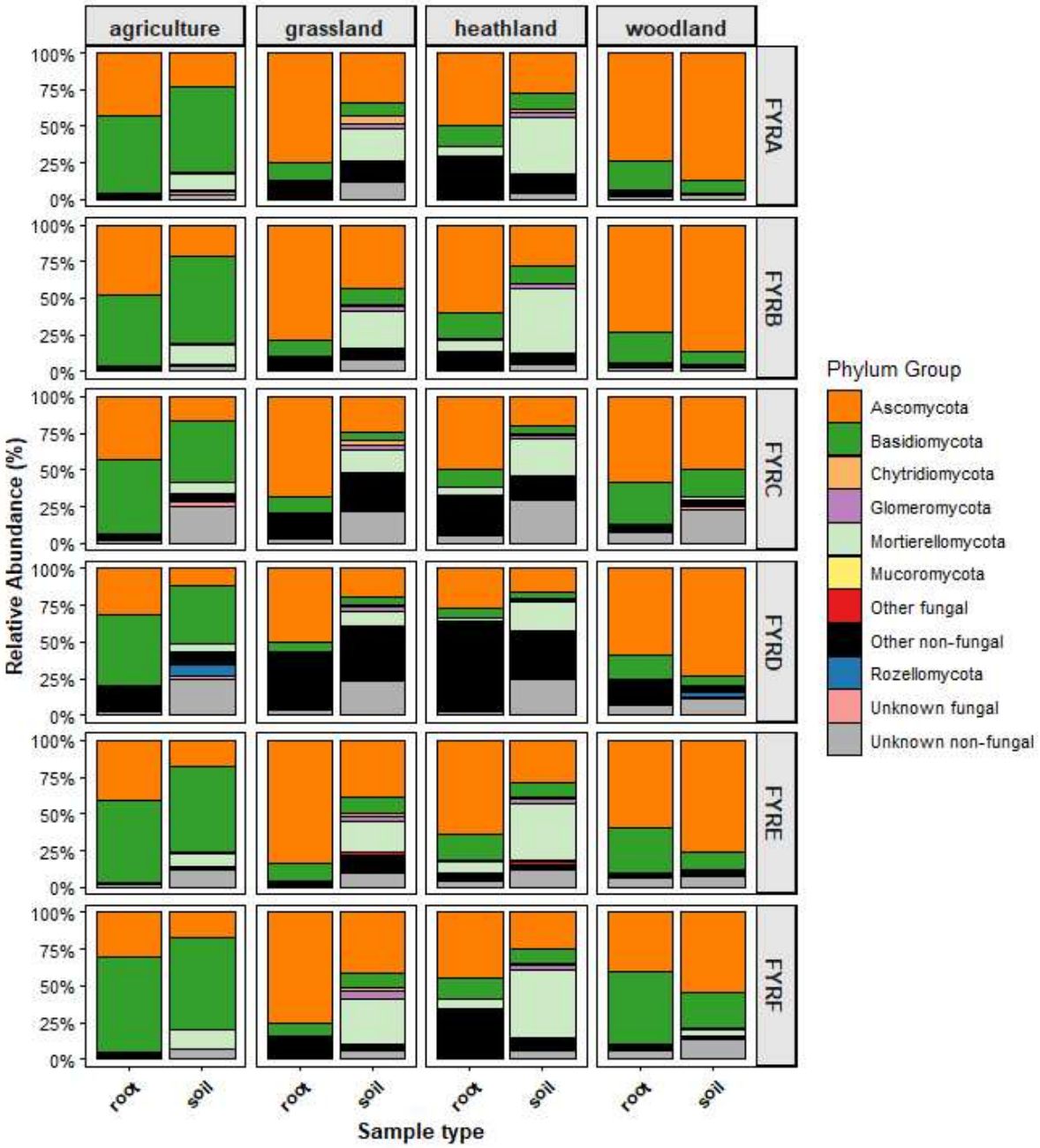


Figure 5. Relative abundance of phylum groups across primers, habitats, and sample types. Raw ASV counts were converted to relative abundances (0–100%) and aggregated at the phylum level for each sample. Fungal sequences lacking assignment at the phylum level are grouped as ‘Unknown fungal’, while fungal sequences of rare fungal phyla are categorised as ‘Other fungal’. Non-fungal sequences are presented as ‘Other non-fungal’ unless they lack a specific phylum-level assignment, in which case they are presented as ‘Unknown non-fungal’.

When evaluating the differential abundance of non-target (i.e., non-fungal) taxa, the novel candidate primer RE performed noticeably better than primers RC and RD

(Figure 6). Specifically, RC overamplified 18 non-target phyla relative to RE, while only two phyla showed the opposite trend. Similarly, RD overamplified 21 non-target phyla compared to RE. Although seven phyla were more abundant with RE than RD, these taxa exhibited a much lower average log-fold change (LFC) in magnitude (-1.7 for RE-favoured phyla vs. 3.83 for RD-favoured phyla). While the remaining pairwise comparisons (RA, RB, and RF) yielded fewer extreme differences, RE consistently proved superior at minimising non-target amplification overall (average LFCs: RA vs. RE, -2.6 vs. 2.8; RB vs. RE, -2.4 vs. 3.22; RF vs. RE, -3.0 vs. 4.3).

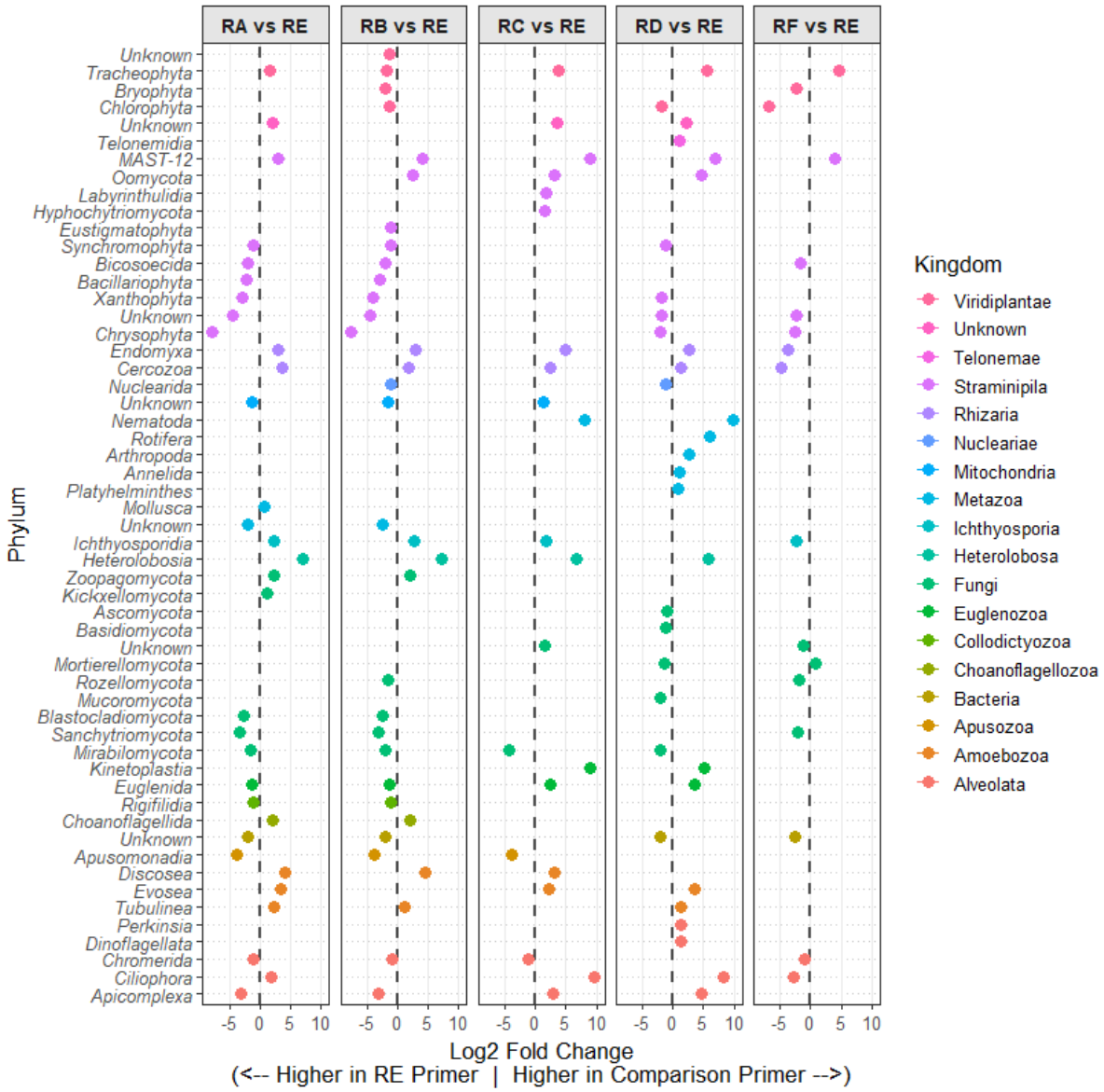


Figure 6. Differential taxonomic capture of non-fungal phyla for the different primer tests relative to the RE novel candidate primer. Forest plot displaying the bias-corrected Log_2 Fold Change (LFC) in phylum-level relative abundances for each

783 evaluated primer compared to RE. Differential abundance was calculated using
784 ANCOM-BC2, controlling for sampling fraction bias, and utilising sample replicate as
785 a random effect. Only phyla exhibiting statistically significant differential abundance
786 (Benjamini-Hochberg adjusted $p < 0.05$) are shown. Points represent the LFC
787 estimate, and error bars indicate the standard error of the LFC. Colours denote the
788 Kingdom-level classification of each phylum.

789 *All fungal phyla with a classified phylum*

790 The relative abundance of fungal phyla again varied between primers, but this was
791 also further influenced by habitat and sample type (Figure 7). The highest
792 Glomeromycota relative abundance was 6.7%, recorded by RD in grassland soil.
793 The highest Mucoromycotan relative abundance was 1.4%, recorded by RF in
794 heathland soil. In general, RA showed the highest average relative abundance of
795 Glomeromycota (1.69%) and RF for Mucoromycota (0.50%; Figure 7). In general,
796 primers agreed that the relative abundance of Mortierellomycota and
797 Glomeromycota was higher in grassland and heathland soils, that grassland,
798 heathland and woodland habitats (especially the roots) were dominated by
799 Ascomycota and that in agriculture, Basidiomycota dominated. Rozellomycota were
800 relatively more abundant in agricultural soils, with primer RD, whereas primers RA
801 and RC suggested a higher relative abundance of Chytridiomycota in grassland soils
802 than other primers.

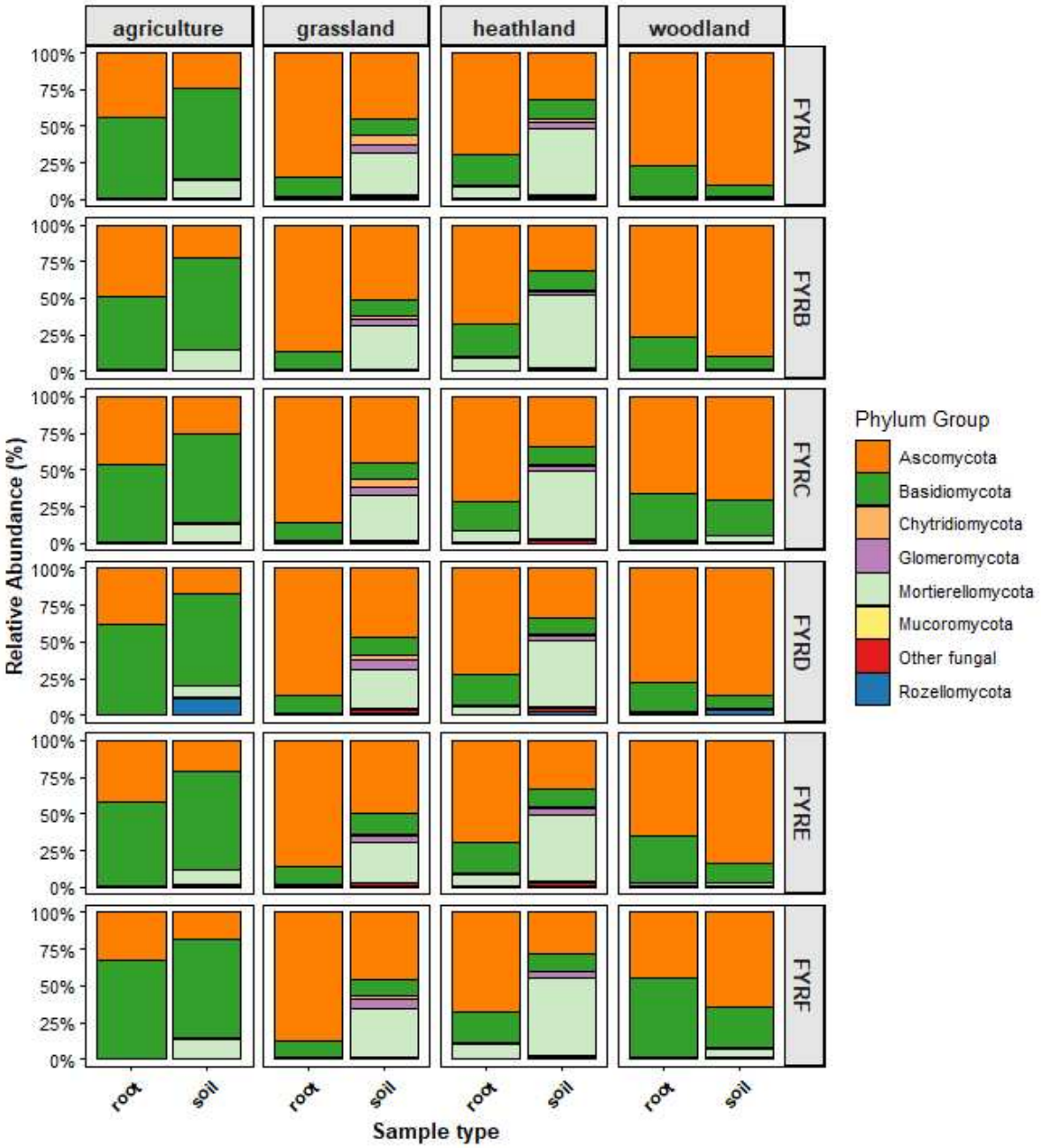


Figure 7. Relative abundance of fungal phylum groups across primers, habitats, and sample types. Raw ASV counts were converted to relative abundances (0–100%) and aggregated at the phylum level for each sample. Fungal sequences of rare fungal phyla are categorised as 'Other fungal'.

When evaluating the differential abundance of fungal taxa, most differences appeared between RD and RE, where five fungal phyla (including Mucoromycota) were statistically more abundant with RE (Figure 8). No statistically significant differences were observed for Glomeromycota between any of the pairwise comparisons.

Figure 8. Differential taxonomic capture of fungal phyla for the different primers tested relative to the RE novel candidate primer. Forest plot displaying the bias-corrected Log_2 Fold Change (LFC) in phylum-level relative abundances for each evaluated primer compared to RE. Differential abundance was calculated using ANCOM-BC2, controlling for sampling fraction bias, and utilising sample replicate as a random effect. Points represent the LFC estimate, and error bars indicate the standard error of the LFC. Phyla exhibiting statistically significant differential abundance (Benjamini-Hochberg adjusted $p < 0.05$) are shown in red points.

Mucoromycotan and Glomeromycotan orders only

The relative abundance of Mucoromycota and Glomeromycota orders varied between primers, but as for comparisons between phyla, this was also further influenced by habitat and sample type (Figure 9). For example, the Mucoromycotan order Densosporales, which likely contains many MFRE taxa (Desirò et al., 2017; Tedersoo et al, 2024; Cole et al., 2025), was relatively more abundant in heathland root samples, especially evidenced with primers RC and RF (Figure 9). Primers generally agreed in determining the relative abundance of woodland samples, even though, again, the FC primer showed a slightly higher relative abundance of Densosporales in the roots. Contrary to all other primers, RC suggested almost no Mucorales in grassland roots. Overall, RB was an outlier in that it detected no substantial amounts of Paraglomerales. In general, the characterisation of the relative abundance of agricultural samples between the different primers was the least consistent, possibly influenced by the low levels of ASVs detected in these samples.

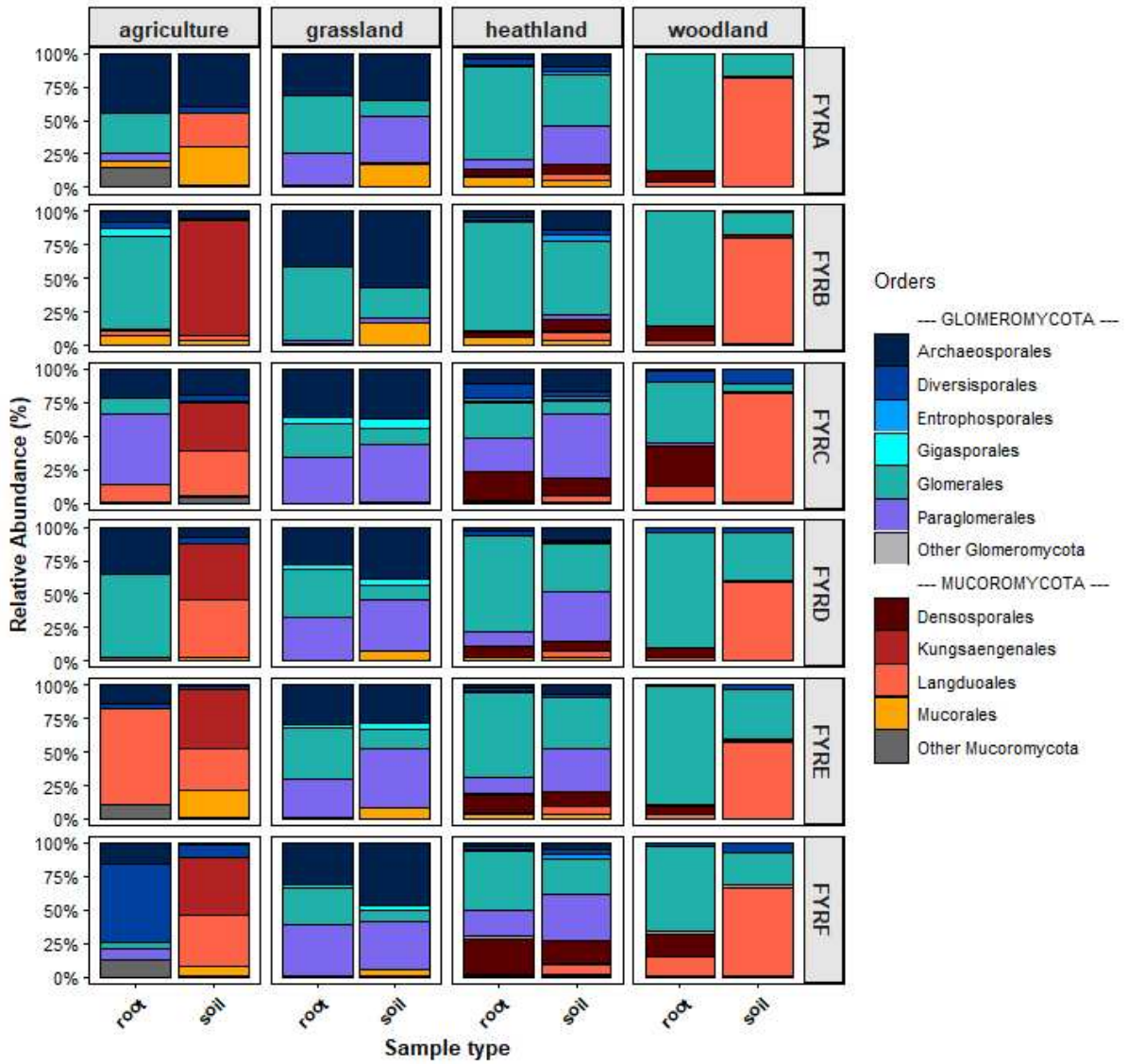


Figure 9. Relative abundance of Glomeromycotan and Mucoromycotan orders across primers, habitats, and sample types. Raw ASV counts were converted to relative abundances (0–100%) and aggregated at the order level. Data are presented as mean relative abundances across biological replicates. The 10 most abundant orders across the dataset are shown individually and organised by their respective phyla (Glomeromycota and Mucoromycota). Sequences from remaining less abundant taxa are categorised as 'Other Glomeromycota' or 'Other Mucoromycota'.

When evaluating the differential abundance of Glomeromycotan and Mucoromycotan taxa, Mucorales were more abundant with the RE primer relative to RC, and the 'Unknown' order and the AM fungal orders Paraglomerales and Gigasporales were more abundant with RE relative to RB (Figure 10).

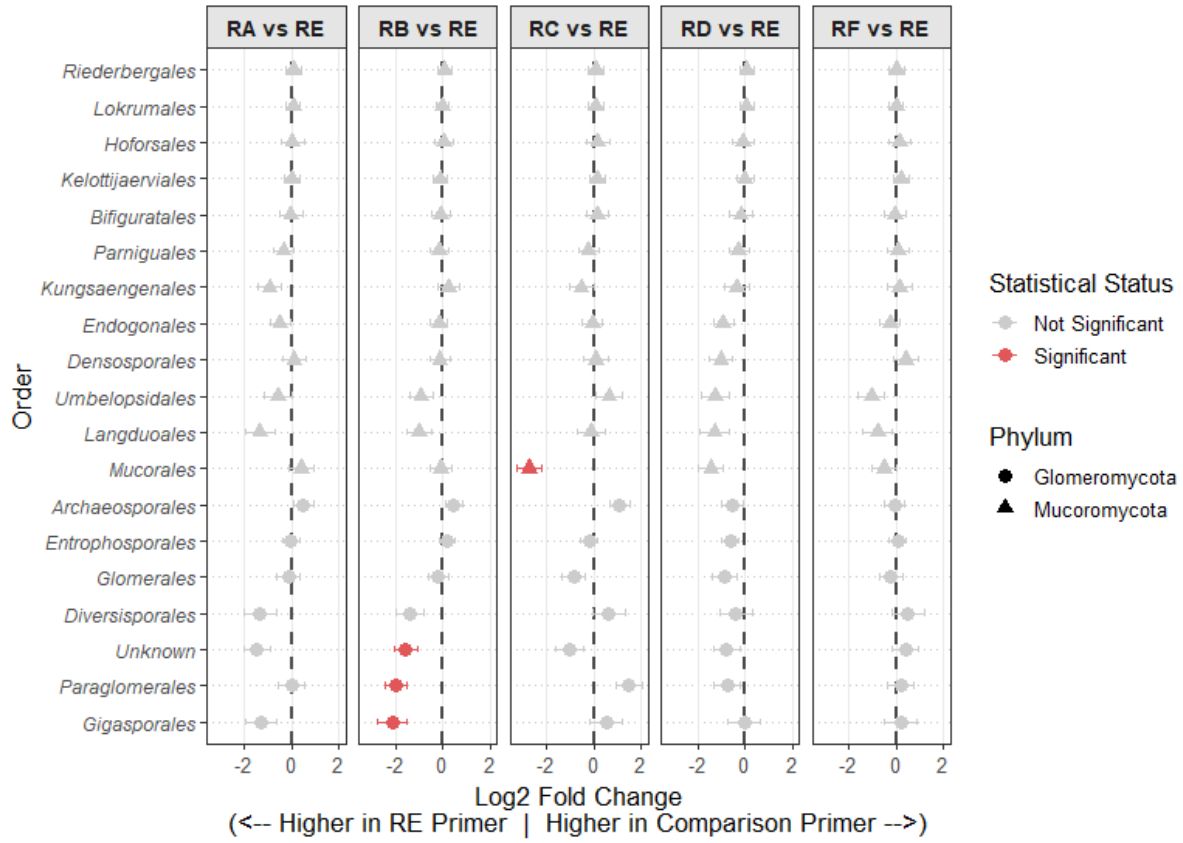


Figure 10. Differential taxonomic capture of Glomeromycotan and Mucoromycotan orders for the different primers tested relative to the RE novel candidate primer. Forest plot displaying the bias-corrected Log_2 Fold Change (LFC) in order-level relative abundances for each evaluated primer compared to RE. Differential abundance was calculated using ANCOM-BC2, controlling for sampling fraction bias, and utilising sample replicate as a random effect. Points represent the LFC estimate, and error bars indicate the standard error of the LFC. Orders exhibiting statistically significant differential abundance (Benjamini-Hochberg adjusted $p < 0.05$) are shown in red points.

Discussion

Here, we compared five ITS primers that are commonly used in fungal metabarcoding studies with a novel candidate primer to evaluate their performance in amplifying fungal DNA from environmental samples. The selected ITS primers have been used in a variety of fungal community studies (e.g., Lekberg et al. 2018; Tedersoo et al., 2016), whilst our novel candidate primer RE (5.8SFun24R) has so far already been used in one published study within our group (Durant et al., 2025).

867 Our primary aim was to assess the specificity of these primers for fungal rather than
868 plant DNA and to evaluate their ability to amplify all fungal groups in a relatively
869 unbiased manner. To test coverage of relatively poorly represented fungal groups,
870 we assessed the Glomeromycota and Mucoromycota phyla containing key
871 mycorrhizal taxa where amplification of plant DNA can prove particularly problematic.
872 To improve taxonomic resolution of our database, we followed a gold-standard
873 approach where we utilised PacBio long-read sequencing to achieve a phylogenetic
874 placement of our reads within an expanded reference framework.

875 Both *in silico* (Table 2) and experimental analyses (Supplementary Figure 2) confirmed
876 that the primers tested varied substantially in the proportion of plant DNA they
877 amplified. Notably, our novel candidate primer RE showed lower specificity for plant
878 DNA and greater specificity for fungal targets than the other primer sets tested
879 (Supplementary Figure 3). However, because the sequencing depth in our study was
880 exceptionally deep (mean = 382,631; s.d = 129,273 reads across all primer pair
881 combinations with the forward primer FY: 1624F), fungal diversity was largely
882 recovered even by those primers that co-amplified substantial amounts of plant DNA
883 (Figure 2A). Still, fungal reads did not always translate into fungal ASVs, and in fact,
884 our novel candidate primer RE overall recovered the greatest fungal diversity despite
885 not showing the highest number of fungal reads (Figure 2A & B). That said, patterns
886 of fungal ASV richness differed among habitats and sample types. In root samples,
887 where relatively lower fungal diversity may be expected, fungal ASV richness was
888 largely similar among primers except in woodland, where primer RC (fITS9R)
889 recovered more ASVs than all primers (Figure 2B). However, in grassland soil, primer
890 RE recovered more ASVs than RA and RC (Figure 2B). Differences were also evident
891 in the recovery of our exemplar groups. For instance, for Glomeromycotan ASVs
892 recovered from heathland samples, primer RD (58A2R) performed worse than all
893 primers except for RC, while primers RA and RB (fITS7R/fITS7oR/gITS7R) and RC
894 recovered fewer Glomeromycota ASVs in woodland compared to RD, RE, and RF
895 (5.8S_FunR; Figure 3B). Together with RF, our novel candidate primer RE also
896 performed better in recovering Glomeromycotan ASVs from grassland soils (Figure
897 3B).

All ITS primers used in this study captured major fungal phyla, including Glomeromycota, which contains exclusively AM fungi, and Mucoromycota, which includes plant symbiotic MFRE. Previous studies using 18S and ITS primers have suggested that MFRE might tolerate, or even benefit from, disturbed conditions relative to some AM fungi, as MFRE diversity has been observed to be greater in agricultural sites when compared to AM fungal diversity in the same sites (Albornoz et al., 2023; Seelinger et al., 2024). However, our results showed the opposite pattern, with Glomeromycota exhibiting greater diversity than Mucoromycota in our agricultural field samples. In fact, all primers recovered very low Mucoromycotan ASV richness in the agricultural plot (ranging from 0 to 6 ASVs), contrasting with previous work using 18S Mucoromycotina-specific primers on a wheat field trial in Northumberland, UK, where richness ranged from around 25 to 50 ASVs (Seelinger et al., 2024).

This discrepancy could reflect genuine ecological differences between the agricultural sites' samples or differences in agricultural management practices. For example, even though MFRE are likely less discriminatory than AM fungi in their soil nutrient exploration (Košnar, Šmilauer and Šmilauerová, 2025), they have been shown to respond strongly to N-addition (Seelinger et al., 2024; Košnar et al., 2026) as well as to background phosphorus availability (Košnar et al., 2026). Differences in sampling time could have also influenced the abundance and detectability of mycorrhizal (AM and MFRE) taxa. Numerous studies have shown that AM fungal communities display pronounced temporal dynamics, with both abundance and community composition varying between years, across seasons and plant developmental stages (Bennet et al., 2013; Cotton et al., 2015). For example, in our study, despite agricultural samples containing more Glomeromycotan than Mucoromycotan ASVs, reads showed the opposite trend even though the overall mean read count across all primers for both groups was really low in agricultural samples (~ 92 reads for Glomeromycota and 211 for Mucoromycota). Another factor to consider is the application of agrochemicals such as pesticides and fungicides, which may further shape fungal community composition in agricultural soils (Zhang et al., 2024) and reduce the function of beneficial plant symbionts (Edlinger et al., 2022). Fungicides, either introduced into the soil through direct application or indirectly as emerging contaminants, have been shown to alter fungal diversity in

soils and impair nutrient transfer between AM fungi and plants (Durant et al., 2025; Köninger et al., 2026). Together, these factors suggest that relatively small differences in agricultural management (including fertiliser inputs and agrochemical use), or sampling timing, may lead to substantial variation in the relative abundance of both AM and MFRE fungal groups. Finally, it is important to note that biological replication in this study was relatively low (3 samples from root and soil, respectively, from each of the four sampled habitats) and that higher replication might have revealed smaller differences. Moreover, we only sampled one representative area for each habitat type, and a more widespread and comprehensive selection of habitats would have increased our ability to generalise from our results.

Alternatively, differences between previous studies using 18S primers and this comparison between general ITS primers could reflect the limited ability of the latter to capture Mucoromycotan diversity, similar to primer bias documented previously in fungal metabarcoding studies, including those targeting AM fungi (Kohout et al., 2014). That said, ITS primers are clearly capable of detecting substantial Mucoromycotan diversity under certain conditions, as demonstrated in our heathland samples, where up to 45 ASVs were detected, suggesting that Mucoromycota are more present in heathland soils and/or that the types present in heathland soils are best captured by the primers tested. In any case, the relatively low prevalence of Mucoromycotan sequences in our study suggests that using a dedicated primer set may be preferable if focusing on this group alone. That said, despite the limitations discussed, the use of broad target ITS primers may still be merited when simultaneous detection of multiple fungal groups is required.

In summary, despite the exceptionally high sequencing depth employed in our study, potentially minimising detection differences between primers, the novel candidate primer RE yielded similar or superior results to the other primers tested (Table 3). Still, as suggested by previous studies (e.g., Tedersoo et al., 2015), no primer will capture the fungal diversity with absolute accuracy, and the taxa of most interest for the research question, as well as the importance of habitat and sample type, need to be considered. In studies with lower sequencing depth, primer choice is likely to have a stronger influence on the detection of fungal diversity. In particular, primers

that co-amplify large amounts of plant DNA may reduce the recovery of low-abundance yet ecologically important taxa such as AM fungi and MFRE.

Table 3. Summary of comparison measures. Primers are ranked from 1 to 6 based on their relative performance to each other. Rank position '1' is assigned to the best-performing primer for that category. Differences are not necessarily always statistically significant. *In silico* comparisons refer to results with 1 mismatch.

	RA: ITS86R	*RB: fITS7R/fITS7oR/gITS7R*	*RC: fITS9R*	*RD: 58A2R*	*RE: 5.8SFun24R*	*RF: 5.8SFunR*
In silico fungal capture (%)	81	87	92	93	91	80
Rank	**5**	**4**	**2**	**1**	**3**	**6**
In silico Glomeromycota capture (%)	91	99	96	97	100	19
Rank	**5**	**2**	**4**	**3**	**1**	**6**
In silico Mucoromycota capture (%)	92	97	96	96	98	95
Rank	**5**	**2**	**3**	**3**	**1**	**4**
In silico plant capture (%)	48	75	95	93	2	1
Rank	**3**	**4**	**6**	**5**	**2**	**1**
Fungal reads (mean ×10³ per sample)	321	304	356	201	340	417
Rank	**4**	**5**	**2**	**6**	**3**	**1**
Fungal reads (mean % per sample)	88	93	72	61	91	87
Rank	**3**	**1**	**5**	**6**	**2**	**4**
Total fungal ASVs (mean per sample)	419	436	472	424	490	426
Rank	**6**	**3**	**2**	**5**	**1**	**4**
Fungal ASVs (mean % per sample)	59	61	25	31	49	56
Rank	**2**	**1**	**6**	**5**	**4**	**3**
Glomeromycotan ASVs (mean % per sample)	3.08	2.74	0.97	1.34	2.81	3.44
Rank	**2**	**4**	**6**	**5**	**3**	**1**
Mucoromycotan ASVs (mean % per sample)	0.69	0.85	0.24	0.25	0.63	0.77
Rank	**3**	**1**	**6**	**5**	**4**	**2**
Relative abundance of non-fungal phyla (mean % per sample)	11	7	27	39	9	13
Rank	**3**	**1**	**5**	**6**	**2**	**4**

Uniquely detected fungal families (Supplementary Figure 5)	7	15	5	4	7	16
Rank	**3**	**2**	**4**	**5**	**3**	**1**
Total rank score	**44**	**30**	**51**	**55**	**29**	**37**

It is also important to note that studies of MFRE remain limited by taxonomic uncertainty and the lack of well-curated reference databases linking molecular sequences with morphological descriptions. To address this limitation and to accurately compare the selected primers using a gold-standard approach, in our study, we coupled short-read Illumina with long-read PacBio sequencing. That said, we acknowledge that this is not typically feasible, and we stress the importance of expanding reference databases to improve the resolution and ecological interpretation of currently underrepresented taxa such as MFRE in future metabarcoding studies. This is necessary in order to limit identification bias, whereby the quality and abundance of reference sequences of taxa influence the probability of them being detected (Tedersoo et al., 2015). We finally note that even though the incorporation of the PacBio long-reads in this study was important to compare the tested primers as accurately as possible, ultimately, this did not drastically increase the assignment rate of our Illumina short-reads ASVs (Supplementary Figure 4).

Acknowledgments

This work was supported by the Natural Environment Research Council UK Newton Critical Zone Observatory Fund [NE/N00745X/1 and NE/S009132/1 to T.J.D., A.C., S.M.], the Royal Society through the Faraday Discovery Fellowships Fund, provided by DSIT – [FDF/S2/251044 to K.J.F., E.M.] and a European Research Council Consolidator Grant 'MYCOREV' [865225 to K.J.F., T.J.D., S.M., E.M.]

Author contributions

T.J.D. and K.J.F. designed the research and collected the environmental samples; S.M., H.H., A.C., R.S. and A.E. each performed aspects of the sample processing; E.M. led the data analysis, interpretation of results and writing of the manuscript. For

the purpose of open access, the author has applied a Creative Commons Attribution (CC BY) licence to any Author Accepted Manuscript version arising.

Data availability

The raw Illumina and PacBio Sequences have been uploaded to the National Center for Biotechnology Information (accession no. PRJNA1543541).

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