DNA and RNA metabarcoding reveal distinct seed-borne mycobiota

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Research Article

Keywords: microbiome, high-throughput sequencing, viable community, metabolic activity, relic DNA, phytosanitary risk

Posted Date: February 17th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-8095745/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Environmental Microbiome on July 3rd, 2026. See the published version at https://doi.org/10.1186/s40793-026-00923-6.

Abstract

Background

Tree seeds harbor diverse fungal communities, including both pathogens and mutualists, that can influence plant health. These communities comprise living, metabolically active organisms as well as dormant or dead cells. Because only active fungi interact with their hosts, distinguishing active from inactive taxa is crucial, especially for environmental and phytosanitary monitoring. Traditional culturing methods capture living fungi but account for only a small fraction of the total fungal diversity. Currently, these methods are increasingly replaced by high-throughput DNA metabarcoding, which detects a broader range of taxa. However, DNA persists after cell death and occurs in dormant cells, preventing distinction between active and inactive fungi. In contrast, RNA metabarcoding detects metabolically active organisms and may better reflect living fungal communities than the other two methods, though its use in assessing plant-associated fungi remains underexplored. We used culturing, DNA-, and RNA-based metabarcoding to compare fungal communities associated with seeds of three key European tree species (*Fagus sylvatica*, *Abies alba*, *Pinus sylvestris*).

Results

DNA and RNA metabarcoding detected largely distinct, non-overlapping fungal communities, with differences primarily driven by rare active taxa in the RNA dataset. Several cultured genera—likely representing abundant and metabolically active taxa—were shared between both metabarcoding approaches.

Conclusions

These results highlight the complementary nature of the three methods for characterising seed-associated fungi. Combining culturing, DNA- and RNA-based metabarcoding may provide the most comprehensive assessment of fungal diversity, while RNA metabarcoding alone offers a promising opportunity to identify the active members of fungal communities for improved environmental and phytosanitary monitoring.

Background

Seeds are essential for plant reproduction and dispersal and act as selective microbial habitats. Packed with oils, starches, and sugars, their resources are protected by seed coats and chemical defences [1], allowing access only to specialized microorganisms [2]. Molecular studies show that tree seeds host diverse fungi [3], including plant pathogens [4–6], and fungi that protect plants against biotic and abiotic stresses [7]. However, few of these fungi may be metabolically active, while the remainder are dead or

dormant. Differentiating these fractions is key to understanding the roles of seed-associated fungi in their hosts and their impact on tree health.

Plant-associated fungi have been traditionally studied by growing them from plant tissues on nutrient media and identifying the isolates based on morphology and/or with molecular techniques [8]. This approach has been widely used to explore fungal biodiversity and ecology [9–11] and remains essential for obtaining living cultures, describing new species and conducting phenotyping assays (e.g., host range, virulence, optimal growth temperatures). For studying new host-pathogen associations, living cultures are also necessary to fulfil Koch's postulates. However, culturing captures only a subset of fungal diversity—mainly abundant fungi—excluding taxa that are difficult or impossible to isolate [3], but which may affect tree health [12]. Moreover, culturing is time-consuming and costly [13], limiting its usefulness for large-scale applications such as environmental monitoring or phytosanitary screening.

Recent advances in high-throughput sequencing (HTS) have enabled the rapid and sensitive analysis of many samples simultaneously. Amplicon-based HTS (i.e., metabarcoding) has become widely used for profiling fungal communities from various substrates, including plant tissues, to address questions related to their diversity and biosecurity [14, 15]. This approach typically targets the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS) region—a universal fungal barcode [16]. Sequencing platforms such as Illumina or PacBio are then used to characterise fungi from DNA extracted directly from bulk, host or environmental samples. However, DNA metabarcoding cannot distinguish living, metabolically active from dead or dormant organisms [14]. For example, Carini et al. [17] showed that over 40% of ITS sequences in soil belong to dead organisms, inflating diversity and skewing community profiles. This raises concerns when using DNA metabarcoding to study fungal responses to environmental change or in biosecurity [17, 18], where only viable organisms pose a biosecurity threat that requires mitigation measures. Additionally, distinguishing dead from living organisms is essential for evaluating the effectiveness of phytosanitary treatments. Unlike DNA, RNA exists only in metabolically active cells and some viruses, and degrades rapidly after cell death [19, 20]. In fungi, the rDNA operon is transcribed into a precursor ribosomal RNA (rRNA) that includes the ITS region. Targeting this precursor allows identification of fungi actively transcribing rRNA [21], resulting in a more accurate characterisation of the living community. While the potential of using RNA for improved biological monitoring has been recognised in environmental fields such as water research [22], the approach remains largely underexplored, including for detecting active fungi in plant samples.

Studies using DNA and RNA metabarcoding revealed different total and active microbial communities in temperate soil, wood [23, 24], ballast water [18, 25] and ocean [26]. However, other studies found no differences. For example, 92% of fungal taxa in *Picea abies* needles overlapped between DNA and RNA datasets [27], and similar patterns were observed in Arctic soil [28]. The differences between total and active fungal communities may depend on environmental factors such as resource availability and temperature, which influence fungal metabolism [29, 30] and DNA/RNA turnover [31]. Methodological biases may also contribute to the observed discrepancies, highlighting the need to assess how well

these two approaches reflect fungal assemblages—especially when distinguishing active fungi from dead or dormant ones is critical for ecological inference and biosecurity.

In this study, we characterised fungal communities associated with commercially traded seeds of three ecologically and economically important European tree species—common beech (*Fagus sylvatica* L.), silver fir (*Abies alba* Mill.), and Scots pine (*Pinus sylvestris* L.)—using traditional culturing and DNA- and RNA-based metabarcoding. We assessed incidence-based and abundance-weighted alpha diversity (i.e., OTU richness and Inverse Simpson index, respectively) and beta diversity (i.e., Sørensen and Morisita-Horn dissimilarities, respectively) across tree species and molecular methods. We also compared taxonomic and lifestyle composition of fungal communities assessed with DNA and RNA metabarcoding within each tree species and compared fungal communities detected by each metabarcoding method with those from culturing. We hypothesised that DNA metabarcoding would reveal greater diversity than RNA metabarcoding, as RNA-based communities represent the active subset of the DNA-detected communities. Moreover, we hypothesised that fungi obtained by culturing would more closely resemble RNA-based communities than DNA-based ones, because culturing primarily recovers metabolically active fungi at the time of isolation.

Methods

Study material

A total of 16 seed lots (i.e., batches of seeds from specific locations and tree species; *F. sylvatica* (n = 6), *A. alba* (n = 5), *P. sylvestris* (n = 5)) were obtained in winter 2019 from European commercial seed suppliers. Seeds were stored at 4°C until fungal assessment using culturing, DNA- and RNA-metabarcoding in spring 2020.

Fungal assessment by culturing

Seeds were surface sterilized by subsequent immersion in 70% ethanol (1 min), 1% sodium hypochlorite (5 min), 70% ethanol (30 sec), and sterile water (30 sec) [32], then dried in a laminar flow hood. For fungal assessment, 90 seeds per seed lot were crushed with a sterile pestle and placed on nutrient media containing streptomycin (0.05 mg/mL). To capture a broad range of fungi, three media with varying nutrient content were used—water agar (WA 15 g/L; VWR Chemicals, Solon, Ohio, USA), malt extract agar (MEA; ME 30g/L; Duchefa Biochemie, Haarlem, the Netherlands & WA 15 g/L; VWR Chemicals) and potato dextrose agar (PDA 39g/L; Merck KGaA, Darmstadt, Germany). Thirty seeds were plated per tree species x medium combination. Plates were checked every five days for 30 days. Emerging colonies were transferred to PDA (39 g/L; Merck KGaA) to obtain pure cultures. Isolates were grouped based on macromorphological traits (i.e., colour, texture, form, margin).

One isolate per morphotype was selected for DNA extraction. Mycelia were scraped from agar, freeze-dried overnight, and DNA was extracted using the E.Z.N.A.® SP Plant DNA Kit (Omega Bio-Tek, Norcross, Georgia, USA) following the manufacturer's instructions. DNA concentrations were measured using a DS-

11 UV-Vis Spectrophotometer (DeNovix, Wilmington, Delaware, USA) and diluted to 5 ng/µl. DNA extracts with concentrations below 5 ng/µl were not diluted. The ITS region was amplified in 25 µl reactions, containing 2 µl DNA, 8.5 µl nuclease-free H_2O, 12.5 µl DreamTaqPCR Master Mix (2X), and 1 µl each of ITS1 (F: TCCGTAGGTGAACCTGCGG) and ITS4 (R: TCCTCCGCTTATTGATATGC) primers [33]. PCRs were run on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) under the following conditions: 95°C for 2 min; 35 cycles of 95°C for 1 min; 55°C for 45 sec; 72°C for 1.5 min; final extension at 72°C for 5 min. Amplification success was confirmed by gel electrophoresis. PCR products were purified and Sanger-sequenced by Macrogen-Europe (Amsterdam, the Netherlands) using the same primers as in PCRs.

Forward and reverse sequences were trimmed and assembled into consensus sequences using CLC Main Workbench 26 (Qiagen, Aarhus, Denmark). Study sequences were compared against the National Centre for Biotechnology Information (NCBI) core nucleotide (nt) database using MEGABLAST [34, 35], excluding sequences annotated as uncultured or environmental samples, to ensure reliable taxonomic assignment.

Fungal assessment by metabarcoding

Fungi were also assessed from 30 seeds per seed lot using DNA and RNA metabarcoding. Seeds were surface sterilised as for culturing, then ground in liquid nitrogen using RNase *AWAY*™ Surface Decontaminant-treated mortars and pestles (Thermo Fisher Scientific, Waltham, USA). DNA was extracted using the E.Z.N.A.® SP Plant DNA Kit (Omega Bio-Tek) following the manufacturer's instructions. DNA quantity was determined with a Qubit dsDNA BR Assay kit on a Qubit 3.0 Fluorometer (Thermo Fisher Scientific). Extracts were sent to Novogene (Cambridge, UK) for PCR, library preparation and sequencing. The ITS2 region was amplified using primers ITS3 (F: GCATCGATGAAGAACGCAGC) and ITS4 (R: TCCTCCGCTTATTGATATFC) [33], and libraries were sequenced on an Illumina MiSeq (2 x 250 bp).

RNA was extracted using the E.Z.N.A.® Plant RNA kit (Omega Bio-Tek) following the manufacturer`s protocol for difficult samples, from the same ground seed pool used for DNA extractions. Multiple extractions per sample were pooled to obtain sufficient RNA for complementary DNA (i.e., cDNA) synthesis. After pooling, RNA quantity was measured using the Qubit™ RNA Broad Range (BR) Assay Kit with Qubit 3.0 Fluorometer (Thermo Fisher Scientific), and quality was assessed using the Agilent RNA 6000 Pico Kit and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, California, USA). DNA contamination was removed using TURBO DNase (Invitrogen™, Thermo Fisher Scientific) with a 60 min incubation (4 U/reaction), confirmed by 35-cycle ITS PCR (ITS1/ITS4 primers; [33]) as described above and the absence of bands in 1.2% TAE agarose gel electrophoresis. RNA was cleaned and concentrated with the RNeasy MinElute Cleanup Kit (QIAGEN, Hilden, Germany), and cDNA synthesised using the SuperScript™ IV First-Strand System (Invitrogen™, Thermo Fisher Scientific) with random hexamer primers and RNase H treatment. The resulting cDNA was purified with AMPure XP beads (1:4 bead:sample ratio) and stored at − 80°C. Samples were sent to BMKGENE (Münster, Germany) for PCR, library preparation and Illumina NovaSeq 6000 sequencing (2 x 250 bp) of the ITS2 region using ITS3 and ITS4 primers [33].

Samples were demultiplexed by the sequencing facility, yielding separate forward and reverse FASTQ files for each sample and dataset (i.e., DNA and RNA). Bioinformatic processing was performed using a custom QIIME2-based pipeline [36]. Adapters and primers were removed with Cutadapt (i.e., error rate 0.1; [37]). Quality filtering, denoising, read merging and chimera removal were conducted using DADA2 [38]. Amplicon sequence variants of both DNA and RNA datasets were clustered into OTUs at 97% similarity using VSEARCH [39], and taxonomic assignment of the merged dataset was performed with a Naïve Bayes classifier [40] in QIIME2 against the UNITE v8.2 database [41]. Taxonomic assignments at species level were not considered, because species identification based on the ITS2 region is often unreliable, particularly for certain fungal groups [42].

Fungal lifestyle assignments

Fungal lifestyles were assigned to OTUs based on genus-level matches using the *FungalTraits* database [43], referencing the “primary_lifestyle” column. The "plant pathogen" category was retained as is, while the following classification adjustments were made:

1) "ectomycorrhizal", "arbuscular_mycorrhizal" = "mycorrhiza",

2) "foliar_endophyte", "root_endophyte" = "endophyte",

3) "soil_saprotroph", "dung_saprotroph", "litter_saprotroph", "nectar/tap_saprotroph","wood_saprotroph”, “pollen_saprotroph", "unspecified_saprotroph" = "saprotroph",

4) "mycoparasite","animal_parasite","epiphyte","lichen_parasite", "algal_parasite”, "sooty_mold", "lichenized" = "other".

Statistical analyses

All analyses were performed using R Statistical software [44].

To account for differences in sequencing depth (i.e., number of reads per sample ranged from 7,939 to 1,179,119 with a mean ± se of 48,663 ± 6,590) and retain all the samples, the dataset was rarefied to a minimum number of reads per sample (i.e., 7,939 reads in a DNA sample of *P. sylvestris*; *Rarefy* function, GUniFrac package; [45]) prior to alpha and beta diversity analyses [46]. Alpha and beta diversity analyses were conducted using incidence-based and abundance weighted metrices. While incidence-based diversity measures capture overall diversity patterns by giving equal weight to all taxa regardless of abundance, abundance-weighted measures emphasize patterns driven by dominant taxa by assigning greater weight to abundant taxa.

Alpha diversity was assessed using OTU richness (i.e., incidence-based metric) and Inverse Simpson Index (i.e., abundance weighted metric; [47], calculated using the *hill_taxa* function from the package hillR [48, 49]). Alpha diversity metrics (i.e., response variables) were compared between explanatory variables—the methods (i.e., DNA and RNA metabarcoding) and tree species (i.e., *F. sylvatica*, *A. alba* and

P. sylvestris). To account for overdispersion, OTU richness was analysed using negative binomial models (i.e., *glm.nb* function, MASS package; [50]). Inverse Simpson Index was modelled with a gamma distribution generalized linear model (GLM) using log link (i.e., *glm* function, stats package; [50]) due to its non-integer nature. Models with and without the interaction term were compared based on Akaike information criterion (AIC). Models without the interaction term were retained (i.e., lower AIC) and further evaluated for factor significance (i.e., *Anova* function, car package; [51]). Estimated marginal means for significant factors and pairwise comparisons were computed with the function *emmeans* from the emmeans package [52].

Beta diversity was assessed using Sørensen dissimilarity (i.e., incidence-based metric), and Morisita-Horn dissimilarity (i.e., abundance-weighted metric). Pairwise distances between samples were calculated using the *vegdist* function from the vegan package [53]. Overall effects of method and tree species (i.e., explanatory variables) on fungal community composition (i.e., Sørensen and Morisita-Horn dissimilarity; response variables) were tested using Permutational multivariate ANOVA (i.e., PERMANOVA; adonis function, vegan package; [53]), without interaction term to match alpha diversity models. Differences in fungal community composition between the methods and tree species were visualized using non-metric multidimensional scaling (i.e., NMDS; *metaMDS* function, vegan package, [53]).

As PERMANOVA revealed significant differences in fungal communities among tree species (see Results), taxonomic composition was assessed separately for each tree species and method, using a non-rarefied dataset to preserve full abundance information across samples [54]. We focused on reads assigned to fungal genera, enabling inference of ecological roles and host associations. First, the number of reads associated with each distinct fungal genus was calculated for every method and tree species. The genera were then ranked by read count, and the ten most abundant genera per method and tree species were selected for further analysis. All remaining reads were grouped as either “Other” (i.e., reads assigned to genera outside the top ten) or “Unassigned” (i.e., reads not assigned to any genus). Relative abundances of the top ten genera, and genera classified as “Other” or “Unassigned” were plotted across the methods and tree species. In addition, indicator species analysis (i.e., *multipatt* function, indicspecies package, [55]) was performed across all genera, irrespective of their read count, to identify those strongly associated with either the DNA or RNA dataset for each tree species.

Variation in fungal lifestyles was evaluated using all non-rarefied reads assigned to a specific lifestyle category or classified as “Unassigned”. For each method and tree species, the relative abundance of reads associated with each lifestyle were calculated and plotted.

Finally, we identified fungal genera shared between culturing and each metabarcoding dataset, as well as those unique to each dataset. This was first done for all tree species together to assess the overall trend, and for each tree species separately, to assess the differences across tree species.

Results

Of the 3,167,108 non-singleton reads assigned to 1,797 OTUs in the merged dataset comprising DNA and RNA reads, approximately 50% were fungal (i.e., 1,557,227 reads and 942 OTUs). Rarefaction curves indicated that the sequencing depth was sufficient to capture all fungal OTUs across all samples (Supplementary Fig. 1).

More than 60% of the fungal reads originated from *A. alba* samples, while the remaining reads were similarly distributed between the other two species (Table 1). Moreover, the variation in the number of reads per sample was smaller for *A. alba* than for the other two species (Supplementary Fig. 1). Overall, a similar number of reads was obtained from both sequencing methods, although this varied among tree species. Read counts were comparable between methods in *F. sylvatica* and *A. alba*, whereas the *P. sylvestris* DNA dataset was almost twice the size of the RNA dataset (Table 1). *Pinus sylvestris* samples contained twice as many OTUs as *A. alba*, with *F. sylvatica* falling between the two (Table 1). The total number of OTUs in all samples of each of the three tree species was three to four times higher in the RNA dataset than in the DNA dataset (Table 1).

The rarefied dataset that was used for the alpha and beta diversity analyses consisted of 254,048 non-singleton fungal reads equally distributed across all samples, as all samples were rarefied to the same number of reads (i.e., 7,939 reads). Overall, these reads were assigned to 872 OTUs (Table 1). Although 70 OTUs (i.e., around 7%) were lost during rarefaction, the number of OTUs in the RNA dataset remained three to four times higher than that in the DNA dataset (Table 1).

Table 1

Fungal read and OTU counts across three tree species and DNA and RNA metabarcoding datasets. Number of fungal reads and OTUs in samples belonging to the three tree species (i.e., *F. sylvatica*, *A. alba*, *P. sylvestris*; numbers of seed lots are indicated in brackets) analysed by DNA and RNA metabarcoding are indicated. Numbers are shown for the DNA and RNA datasets and for the combined dataset (i.e., all data) considering all fungal reads and rarefied fungal reads (in the brackets).

	Reads			**OTUs**		
Tree species	DNA	RNA	All data	DNA	RNA	All data
F. sylvatica (n = 6)	105,396	177,420	282,816	108	361	417
	(47,634)	(47,634)	(95,268)	(102)	(342)	(392)
A. alba (n = 5)	444,834	522,881	967,715	93	232	277
	(39,695)	(39,695)	(79,390)	(72)	(173)	(202)
P. sylvestris (n = 5)	212,820	93,876	306,696	152	519	628
	(39,695)	(39,695)	(79,390)	(139)	(512)	(610)
Total	763,050	794,177	1,557,227	247	801	942
	(127,024)	(127,024)	(254,048)	(215)	(512)	(872)

Alpha diversity

RNA metabarcoding revealed approximately twice as many OTUs per sample compared to DNA metabarcoding (Fig. 1A; $\chi^2 = 25.95$, df = 1, $p < 0.001$). This pattern persisted for abundance-weighted alpha diversity (i.e., Inverse Simpson Index; $\chi^2 = 17.90$, df = 1, $p < 0.001$), although the mean values of the Inverse Simpson Index were roughly ten times lower than those of OTU richness (Supplementary Fig. 2A).

Overall, OTU richness per sample differed significantly between tree species (Fig. 1B; $\chi^2 = 8.18$, df = 1, $p < 0.05$), with the lowest richness observed in *A. alba* samples and the highest in *P. sylvestris* samples. A similar pattern was observed for the abundance-weighted diversity ($\chi^2 = 15.12$, df = 2, $p < 0.001$) which was around ten times lower than OTU richness—*F. sylvatica* and *P. sylvestris* exhibited more than twice the diversity of *A. alba* (Supplementary Fig. 2B).

Beta diversity

When all OTUs were considered, regardless of their abundance, DNA and RNA metabarcoding revealed significantly different fungal communities associated with the same seeds (Fig. 2A; F = 11.49, $R^2 = 0.25$, $p < 0.001$). Of the total 872 OTUs identified in the rarefied dataset, only 100 (~ 11%) were shared between the datasets, while around 13% and 75% were unique for the DNA and RNA dataset, respectively (Fig. 2B). The proportion of OTUs shared between the two metabarcoding methods further differed across tree species: approximately 7% of OTUs were shared in *P. sylvestris*, compared to 13% in *F. sylvatica* and 21% *in A. alba* (Supplementary Fig. 3A–C). Comparable results were obtained using the non-rarefied dataset (Supplementary Fig. 4A-E). Significant differences in the composition of dominant fungal communities—assessed by Morisita-Horn dissimilarity, which gives greater weight to OTUs with higher read counts—were also observed between DNA and RNA datasets (Supplementary Fig. 5A; F = 1.97, $R^2 = 0.05$, $p < 0.05$). However, as indicated by R^2 values, the relative importance of the method in explaining the differences in fungal community composition decreased from 25% to 5% when an abundance-weighted beta diversity measure was used suggesting that the differences between methods were largely driven by rare OTUs.

Fungal communities also differed among tree species, both when analysing the entire community (Fig. 2C-D; F = 2.84, $R^2 = 0.13$, $p < 0.001$) and when focussing on dominant taxa (Supplementary Fig. 5B; F = 1.75, $R^2 = 0.28$, $p < 0.001$). The proportion of variation explained by tree species increased from 13% to nearly 28% when switching from incidence-based to abundance-weighted metrics, respectively, as indicated by R^2 values, suggesting that taxa with higher read counts were more host-specific than rare taxa.

Taxonomic compositional differences between methods and tree species

Out of a total of 1,557,227 fungal reads and 942 fungal OTUs in the non-rarefied dataset, the majority of reads and OTUs were assigned at the class, order, and family levels. At the genus level, around 72% of

reads and 64% of OTUs were classified to 338 distinct genera (Supplementary Table 1).

The proportion of fungal reads and OTUs assigned across taxonomic levels was broadly consistent between DNA and RNA datasets (Supplementary Table 1). The vast majority of reads and OTUs were assigned at high taxonomic levels (i.e., class, order, family) in both datasets. Approximately 74% of reads and 68% of OTUs in the DNA dataset, and 71% of reads and 65% of OTUs in the RNA dataset, were assigned to 110 and 305 unique genera, respectively. Seventy-seven genera (~ 23%) appeared in both datasets, while 33 (~ 10%) and 228 (~ 67%) were unique for DNA and RNA dataset, respectively.

Approximately 85% of reads were assigned to genera in both DNA and RNA datasets of *F. sylvatica*, compared to 73% in *A. alba* and 61% in *P. sylvestris* (Fig. 3). The proportion of reads classified as "Other" (i.e., reads assigned to genera outside the top ten most abundant genera per method and tree species) was around only 3% in the *A. alba* DNA and RNA, and *P. sylvestris* DNA datasets. Higher proportion of reads classified as "Other" was found in the *P. sylvestris* RNA dataset (~ 14%) and, especially, in both *F. sylvatica* datasets (i.e., ~ 16% in the DNA and ~ 26% in the RNA dataset; Fig. 3).

Taxonomic composition of dominant genera (i.e., the top ten most abundant genera per method and tree species) varied across tree species and sequencing methods (Fig. 3). The only genus consistently identified among the top ten genera across all methods and tree species was *Aspergillus*. Among the top ten genera identified for each method and tree species, five were shared between the DNA and RNA datasets in *F. sylvatica* (i.e., *Alternaria*, *Apiognomonia*, *Aspergillus*, *Diaporthe*, *Gibberella*), and in *A. alba* (i.e., *Aspergillus*, *Diplodia*, *Hormonema*, *Lambertella*, *Penicillium*), and two in *P. sylvestris* (i.e., *Aspergillus*, *Hormonema*). Those shared genera accounted for a high proportion of reads in each dataset (i.e., *F. sylvatica*: 53% in DNA and 32% in RNA; *A. alba*: 60% in DNA and 55% in RNA; *P. sylvestris*: 44% in DNA and 40% in RNA).

Indicator species analysis revealed significant, dataset-specific genus associations with either DNA or RNA datasets (Supplementary Table 2). Across tree species, the genera *Mycocentrospora*, *Paraleptosphaeria*, and *Phoma* were associated with the DNA datasets, whereas *Fusarium*, *Kazachstania*, and *Vishniacozyma* were associated with the RNA datasets, although with varying relative abundances. In *F. sylvatica*, four genera were associated with the DNA and 17 with the RNA dataset. Among these, several indicator genera ranked among the top ten genera per method in *F. sylvatica—Paraleptosphaeria* was an indicator of the DNA, whereas *Vishniacozyma* and *Lophodermium* were representative of the RNA dataset. In *A. alba*, nine genera were indicators of the DNA dataset and ten of the RNA dataset. Among the top ten genera per method in *A. alba*, *Caloscypha* was strongly linked to DNA, while *Fusarium*, *Lophodermium* and *Neocatenulostroma* emerged as indicators of the RNA dataset. For *P. sylvestris*, 12 genera were associated with the DNA dataset and nine with the RNA dataset. Within the top ten genera per method in *P. sylvestris*, *Caloscypha*, *Fibulochlamys*, *Podospora*, and *Lambertella* were DNA-associated, whereas *Colletotrichum*, *Kazachstania*, and *Vishniacozyma* were strongly associated with the RNA dataset. Additional genus-level associations were observed but fell outside the top ten most abundant genera per method and species (Supplementary Table 2).

Lifestyle compositional differences between methods and tree species

Of the total reads and OTUs in the non-rarified dataset (i.e., 1,557,227 reads and 942 OTUs), a primary lifestyle could be assigned to 1,128,634 reads (74%) representing 596 OTUs (64%). Among these, most fungal reads and OTUs belonged to saprotrophs (72% of reads, 59% of OTUs), followed by plant pathogens (25% of reads, 24% of OTUs), while mycorrhizal fungi, endophytes, and taxa categorized as "Other" each accounted for only a small fraction of the reads and OTUs (Supplementary Table 3).

The proportion of fungal reads and OTUs assigned to a lifestyle was largely consistent across DNA and RNA datasets—around 74% and 71% out of 763,050 and 794,177 reads and 68% and 64% out of 247 and 801 OTUs in DNA and RNA dataset could be assigned to a lifestyle. Moreover, the proportions of reads and OTUs assigned to each lifestyle were largely similar between the metabarcoding datasets (Supplementary Table 3).

Approximately 85%, 75%, and 65% of reads from *F. sylvatica*, *A. alba*, and *P. sylvestris*, respectively, were classified into fungal lifestyles, with varying proportions between the two datasets (Fig. 4). In *F. sylvatica*, plant pathogens accounted for 57% of DNA reads and 39% of RNA reads, while saprotrophs were represented with 29% of DNA reads and 38% of RNA reads. Saprotrophs were the most abundant guild in *A. alba* (60%) and *P. sylvestris* (52%) in both datasets. Plant pathogens constituted 15% and 12% of DNA and RNA reads in *A. alba* samples and 7% and 9% in *P. sylvestris* DNA and RNA dataset, respectively. In *F. sylvatica* and *A. alba*, mycorrhizal fungi (i.e., 740 and 392 reads) and endophytes (i.e., 217 and 233 reads) were exclusively detected in the RNA dataset. Unlike *F. sylvatica* and *A. alba*, *P. sylvestris* contained endophytes in both DNA and RNA datasets at similar read counts (i.e., 85 and 51, respectively). Mycorrhizal fungi in *P. sylvestris* were detected in both datasets but with only three reads in the DNA compared to 201 reads in the RNA dataset.

Comparison of metabarcoding datasets with traditional culturing data

A total of 1,553 fungal isolates were obtained across all seed samples and those were assigned to 203 morphotypes. Out of 203 representative isolates (i.e., one representative isolate per morphotype) which were selected for sequencing, 145 yielded good-quality sequences. Of those, 121 sequences were assigned to 31 distinct genera, compared to 110 and 305 genera identified through DNA and RNA metabarcoding, respectively. Of the 31 genera identified through culturing, ten (~ 32%) were found exclusively in the culturing datasets. In contrast, 17 (~ 55%) of the cultured genera were also detected in both DNA and RNA datasets and two additional cultured genera were detected in each of the metabarcoding datasets. Although the overlap between culturing and metabarcoding was broadly similar—with most cultured genera appearing in both metabarcoding datasets—the RNA dataset contained a higher number of unique genera, whereas the DNA dataset comprised of fewer unique taxa (Fig. 5).

Most of the isolates originated from *F. sylvatica* seeds (i.e., 1,300 isolates representing 145 distinct morphotypes). Out of the 114 representative isolates which yielded good quality sequences 91

representative isolates could be assigned to one of 24 fungal genera (i.e., corresponding to 976 isolates and 91 morphotypes). Almost half of the cultured fungal genera (i.e., eleven) were detected by both DNA and RNA metabarcoding approaches, while an additional two (i.e., *Oliveonia*, *Phacidium*) and three genera (i.e., *Cladosporium*, *Chaetomium*, *Clonostachys*) were exclusive to the DNA or RNA datasets, respectively. Notably, eight genera were identified solely through culturing (i.e., *Discosia*, *Epicoccum*, *Xylaria*, *Didymosphaeria*, *Plagiostoma*, *Biscogniauxia*, *Radulidium*, *Boeremia*; Table 2., Supplementary Fig. 6).

From *A. alba* seeds, 231 isolates representing 44 morphotypes were obtained from seeds. Good quality sequences were obtained from 19 representative isolates, covering 97 isolates belonging to 19 morphotypes. All representative sequences could be assigned to one of the eight genera, five of which were detected in both DNA and RNA datasets (i.e., *Penicillium*, *Trichoderma, Fusarium, Aspergillus, Talaromyces*), while three were exclusive to the culturing dataset (i.e., *Sydowia*, *Mucor*, *Akanthomyces*; Table 2., Supplementary Fig. 6).

From *P. sylvestris* seeds, 22 isolates representing 14 morphotypes were obtained. Good quality sequences were obtained from 12 representative isolates (morphotypes), covering 20 isolates. All but one representative isolate was assigned to one of seven genera. Three genera were shared between DNA and RNA datasets (i.e., *Penicillium*, *Fusarium*, *Aspergillus*), two were shared with RNA dataset (i.e., *Coniochaeta*, *Pseudopithomyces*) and two were detected only via culturing (i.e., *Boeremia*, *Sydowia*; Table 2., Supplementary Fig. 6).

Table 2

Fungal genera cultured from three tree species and their detection in DNA/RNA metabarcoding datasets Fungal genera detected in the culturing data of the three tree species (i.e., *F. sylvatica*, *A.alba*, *P. sylvestris)* are listed. Number of isolates belonging to each genus and number of morphotypes those isolates belong to is indicated, as well as the information about if the genera were detected in either of the two metabarcoding datasets (i.e., DNA, RNA).

Genus	Number of isolates	Number of morphotypes	Present in DNA dataset	Present in RNA dataset
Fagus sylvatica				
Alternaria	318	13	x	x
Apiognomonia	244	18	x	x
Fusarium	125	13	x	x
Discosia	57	5		
Diaporthe	42	4	x	x
Epicoccum	33	5		
Cladosporium	25	4		x
Penicillium	24	5	x	x
Aureobasidium	14	3	x	x
Ceratobasidium	10	1	x	x
Oliveonia	10	1	x	
Clonostachys	9	4		x
Trichothecium	9	1	x	x
Seimatosporium	8	2	x	x
Xylaria	8	2		
Didymosphaeria	6	1		
Muriphaeosphaeria	6	1	x	x
Chaetomium	5	1		x
Biscogniauxia	5	1		
Radulidium	5	1		
Neosetophoma	4	2	x	x
Plagiostoma	3	1		
Boeremia	3	1		

Genus	Number of isolates	Number of morphotypes	Present in DNA dataset	Present in RNA dataset
Phacidium	3	1	x	
unidentified	324	54		
Abies alba				
Penicillium	28	7	x	x
Trichoderma	27	2	x	x
Sydowia	25	2		
Fusarium	7	2	x	x
Aspergillus	6	3	x	x
Mucor	2	1		
Talaromyces	1	1	x	x
Akanthomyces	1	1		
unidentified	134	25		
Pinus sylvestris				
Penicillium	5	5	x	x
Fusarium	5	1	x	x
Boeremia	4	2		
Coniochaeta	3	1		x
Aspergillus	1	1	x	x
Sydowia	1	1		
unidentified	3	3		

Discussion

Traditional culturing methods reveal limited fungal diversity, are resource-intensive, and have largely been replaced by the high-throughput metabarcoding approaches in studies where living cultures are not required. Metabarcoding studies are predominantly DNA-based, which does not allow differentiation between metabolically active and inactive taxa, including dormant or dead organisms. In contrast, RNA metabarcoding targets living, metabolically active organisms, potentially providing a more ecologically relevant snapshot of fungal communities compared to DNA metabarcoding. RNA metabarcoding has shown potential for studying active environmental microbiota [18, 22, 25, 56]. However, the method has rarely been applied in studies of plant-associated microbiota. Given its high potential for ecological and

phytosanitary monitoring, it is crucial to evaluate the RNA metabarcoding method against other commonly applied methods for studying plant-associated fungi before it can be widely implemented.

In this study, we compared incidence-based and abundance-weighted seed-borne fungal diversity and community composition between DNA and RNA metabarcoding methods and tree species. Additionally, we assessed the overlap between fungal genera identified through culturing and those revealed by each metabarcoding method. Our results show that the two metabarcoding approaches capture distinct and largely non-overlapping fungal communities, with the observed differences primarily driven by rare OTUs in the RNA dataset. However, both approaches recovered similar cultured genera, which likely represent abundant and metabolically active taxa.

Fungal diversity and community composition across metabarcoding and culturing datasets

Contrary to our hypothesis, RNA metabarcoding revealed a higher number of fungal OTUs than DNA metabarcoding, including many unique OTUs, resulting in highly divergent community profiles. This unexpected outcome likely results from the high abundance of taxa represented by dead or dormant forms [57], as well as the large diversity of metabolically active rare or low-abundance taxa in dormant tree seeds. In complex or competitive environments—such as dormant seeds—metabolic activity often originates from low abundance taxa [58], as demonstrated by rare bacterial taxa showing high metabolic activity in atmospheric [56, 59] and oceanic environments [60]. While DNA metabarcoding typically captures highly abundant taxa, RNA metabarcoding targets metabolically active fungi, leading to discrepancies in community profiles, especially when incidence-based measures are used. However, although abundance-weighted alpha diversity was also lower in the DNA than in the RNA dataset, the divergence between DNA- and RNA-based community profiles decreased when abundance-weighted metrics were applied. Among the most abundant genera identified per tree species and method, about half were shared between the DNA and RNA datasets in two out of three tree species. This indicates that many dominant fungi are generally metabolically active, and that discrepancies between methods largely reflect differences among rare active taxa.

Methodological factors likely contributed to the observed differences in fungal communities between the DNA and RNA datasets. A first bias may have been introduced during nucleic acid extraction, as DNA and RNA were obtained from subsamples of one seed lot and using different extraction kits. Previous studies have demonstrated that the choice of extraction kit can significantly influence microbial community composition [61], a bias that could be mitigated by using co-extraction kits that allow simultaneous isolation of both DNA and RNA [62]. In addition, subsampling procedures differed between the extractions. While a single DNA extraction was performed per sample, RNA was extracted in duplicates and pooled to obtain sufficient input for reverse transcription. This variation in the amount of starting material may have led to higher alpha diversity in the RNA than DNA dataset and shifted community composition—an effect previously shown for seed mycobiota of *P. sylvestris* [63]. Nonetheless, our study accounted for differences in sampling effort through rarefaction and the use of abundance-weighted diversity metrics [64, 65], helping to mitigate potential confounding effects.

Furthermore, the reverse transcription step required to synthesize cDNA from RNA introduces biases such as uneven abundances of correctly transcribed molecules. The reverse transcription can also introduce artefacts due to incorrect primer binding or unpredictable reverse transcriptase behaviour [66]. These factors likely contributed to discrepancies between fungal community profiles derived from DNA and RNA. Additional bias may occur during PCR amplification of the ITS region originating from both DNA and cDNA, and library preparation, including primer-template mismatches and selective amplification of certain taxa [67]. Finally, DNA and RNA libraries were sequenced on separate runs, which may have introduced further variation, although, this effect is likely less pronounced than the other previously mentioned methodological sources of bias [61].

We found no evidence supporting our hypothesis that cultured fungi would more closely resemble the RNA-derived community than the DNA-derived one. Approximately 50% of cultured genera were detected by both metabarcoding approaches, while an additional 6% was uniquely recovered by either DNA- or RNA-based metabarcoding. These results suggest that both metabolic activity and abundance influence the success of isolation of the fungus. If culturing primarily captured metabolically active taxa, a stronger overlap with the RNA dataset—representing rare but active fungi—would have been expected. Instead, our culturing approach likely favoured abundant and competitive fungi [8]. Alternative strategies such as dilution-to-extinction culturing [68] or use of different media [69] could help recover a greater diversity of rare yet cultivable taxa and potentially increase overlap with RNA-based profiles. As expected, culturing recovered substantially lower diversity than metabarcoding, reflecting the well-known difficulty of growing many fungal taxa under standard laboratory conditions [8]. Moreover, taxa detected only by culturing may reflect stochastic recovery or primer bias between methods. As isolates are critical for species description, functional studies, and phenotypic assays, continued improvement of culture-dependent techniques remains essential. Developing more sensitive and high-throughput culturing approaches will be key to bridging molecular and culture-based views on fungal diversity.

Taxonomic and functional fungal community composition across metabarcoding datasets and tree species

Tree species identity was a major driver of the community composition of dominant fungi, explaining approximately 30% of the overall observed variation. The high host specificity of seed-borne fungi observed in our study aligns with findings from both woody and non-woody plants [70, 71]. Although, several dominant fungal genera were associated with already known hosts, not all of them were previously known to occur in seeds. For example, the genus *Apiognomonia* was the most abundant in *F. sylvatica* seed, likely corresponding to *A. errabunda*, a common endophyte of *F. sylvatica* known to cause anthracnose under wet spring conditions or following insect damage [72], and was also identified in the culturing dataset. *Abies alba* seeds were dominated by OTUs assigned to the genus *Lambertella* which has been previously found in *A. koreana* needles [73]. *Pinus sylvestris* seeds were dominated by the genus *Hormonema*, likely corresponding to *Sydowia polyspora* (i.e., old name is *H. dematioides*), a well-known endophyte and opportunistic pathogen in conifers and pre-emergent seed pathogen in *P. ponderosa* [6, 74], which was also identified in the culturing dataset.

Some fungal genera observed in seeds of specific tree species were found to be strongly associated with one of the metabarcoding datasets. For example, the genus *Paraleptosphaeria* was strongly associated with the DNA dataset of *F. sylvatica*. This genus is known to be saprobic, fungicolous, or pathogenic, and has been found in grasslands and on stems and leaves of various plants. However, *Fagus* has not been listed among confirmed hosts in recent studies [75, 76]. The genus *Caloscypha*, likely represented by the species *C. fulgens*—a well-known conifer seed pathogen [77] was strongly associated with the *A. alba* and *P. sylvestris* DNA dataset. The genus *Lophodermium* showed strong association with the RNA data of *F. sylvatica* and *A. alba* and was represented by multiple OTUs identified to several species known as needle endophytes or pathogens of conifers [78]. Association of *Lophodermium* with *F. sylvatica* may stem from environmental or laboratory contamination. The genus *Neocatenulostroma* was strongly associated with the RNA dataset of *A. alba* and could correspond to *N. abietis*, species which was isolated from various substrates and was found as a saprobe or endophyte in pine needles, but also as a pathogen on a wide range of conifer hosts [79]. In *P. sylvestris*, *Colletotrichum* was strongly associated with the RNA dataset. Some *Colletotrichum* species are known pathogens of pines, although their occurrence in conifers is less frequent than in broadleaf hosts [80]. These results highlight the value of combining DNA and RNA-based metabarcoding to uncover both inactive and active fungal associations, some of which may have implication for tree health.

Similar to findings by Mittelstrass et al. [3] most fungal reads in our study were assigned to saprotrophs and plant pathogens, which appeared in similar proportions across DNA and RNA metabarcoding datasets of all three host species. This suggests that these functional groups are not only abundant but also metabolically active in dormant tree seeds. Although dormant seeds may not seem like suitable habitats—given that saprotrophs rely on dead organic matter and plant pathogens require a susceptible host—research suggests that many potentially saprotrophic or pathogenic taxa can persist in asymptomatic plant tissues as endophytes (i.e., asymptomatic, commensal or weakly mutualistic inhabitants; [43]) and shift to saprotrophic or pathogenic lifestyle under favourable conditions [81]. Mycorrhizal fungi and endophytes were detected almost exclusively in RNA datasets, likely due to their low abundance, which may have prevented their detection by DNA metabarcoding. The sporadic occurrence across seed lots and low abundance of mycorrhizal fungi in seeds supports the notion that their seed-transmission is uncommon in trees as previously suggested [82]. However, the detection of taxa belonging to mycorrhizal fungi in seeds of various non-woody plant species [70] highlights the need to further investigate mycorrhizal transmission from seeds to seedlings. Low numbers of endophytic taxa are likely associated with their underrepresentation in databases such as the FungalTraits [43], underscoring the need for more targeted research into their diversity and function.

Conclusions

Our results show that DNA and RNA metabarcoding provide distinct but complementary insights into fungal communities and have valuable potential for high-throughput ecological and biosecurity monitoring. DNA metabarcoding has already been widely adopted for assessing biodiversity of different organisms in both environmental and host-associated samples. Its ability to detect living and non-living

taxa provides a comprehensive perspective on total fungal community associated with the studied system. In contrast, RNA-based metabarcoding is still less commonly applied, despite its focus on metabolically active organisms which may enable real-time insights into ecosystem dynamics and functional responses to environmental change. Moreover, RNA metabarcoding might be particularly promising for biosecurity application where rapid detection and identification of living pests and pathogens is critical for decision making and timely intervention.

In our study, RNA metabarcoding revealed a higher diversity, primarily of rare taxa, compared to DNA metabarcoding, making it particularly effective for detecting low-abundance pathogens, at least when dominant taxa are inactive or dormant (e.g., ensuring absence of pathogens in treated samples). However, its inability to detect metabolically inactive yet viable organisms represents a limitation as dormant pathogens may escape detection and later become active. This could be tackled with applying viability PCR (vPCR) using propidium or ethidium monoazide which enables selective suppression of DNA from membrane-compromised cells, allowing DNA-based assays to target only intact, viable fungi and thus reduce false positives from relic DNA [83, 84]. Nevertheless, vPCR also has important limitations. Its effectiveness can vary depending on cell wall structure and environmental matrix composition, and incomplete dye penetration or binding may lead to false negatives. Moreover, its applicability to mixed environmental samples such as those that need to be ground prior to DNA/RNA extraction, remains uncertain. If technical obstacles are overcome, performing DNA metabarcoding with and without vPCR pretreatment could help differentiate living from dead organisms. Combining this with RNA-based methods can further help identify the metabolically active members of the community, leading to more accurate ecological interpretations and risk assessments.

Looking ahead, advances in sequencing technologies and bioinformatics are expected to increase the sensitivity and resolution of metabarcoding methods, enabling more precise taxonomic identification and functional profiling. In the face of growing environmental pressures and accelerating species movement through trade, the combined use of DNA and RNA metabarcoding holds strong potential for ecosystem management and biosecurity, by revealing total and active communities. While culturing remains essential for studying fungal diversity, pathogenicity and functional traits, traditional approaches recover only a subset of the taxa detected by metabarcoding, typically the dominant and readily cultivable species. Future efforts should thus focus on developing more sensitive, high-throughput culturing techniques capable of capturing a broader spectrum of fungal diversity. Ultimately, integrating all three approaches—DNA- and RNA-based metabarcoding together with advanced culturing—offers the most comprehensive framework for studying fungal diversity and strengthening biosecurity, by capturing both active and inactive taxa as well as culturable and unculturable species.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data Availability

Raw metabarcoding sequence reads have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB96213.

ITS barcode sequences generated from cultured specimens have been deposited in GenBank under accession numbers PX736467–PX736489, PX740711–PX740835, and PX745229–PX745242. All ITS sequences used in this study, together with their corresponding accession numbers, are provided in Supplementary Table 4.

Competing Interests

The authors declare that they have no competing interests.

Funding

This work was funded by the Swiss National Science Foundation SNSF (Grant: P500PB_211040) and by the Swiss Federal Office for the Environment (FOEN) (Finanzhilfevertrag betreffend wissenschaftlich-technischen Tätigkeiten im Bereich Waldschutz).

René Eschen was supported by CABI. CABI is an international intergovernmental organisation, and we gratefully acknowledge the core financial support from our member countries (and lead agencies) including the UK (Foreign, Commonwealth & Development Office), China (Chinese Ministry of Agriculture and Rural Affairs), Australia (Australian Centre for International Agricultural Research), Canada (Agriculture and Agri-Food Canada), Netherlands (Directorate-General for International Cooperation) and Switzerland (Swiss Agency for Development and Cooperation). See https://www.cabi.org/about-cabi/who-we-work-with/key-donors/ for full details.

Authors' contributions

IF conceived and designed the study with input from RE, SP, and MC. Data collection was carried out by IF, PS, and KS. PS supervised all laboratory activities related to RNA metabarcoding. IF performed the data analysis and prepared the initial manuscript draft with input from MC. All co-authors reviewed the draft and contributed to the preparation of the final version of the manuscript

Acknowledgments

We thank Beatrice Tolio, Delnia Sepahvand, and Diana Marčiulynienė for their valuable assistance in the laboratory. We thank Beat Ruffner for his guidance with processing ITS sequences from cultured fungal isolates.

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Figures

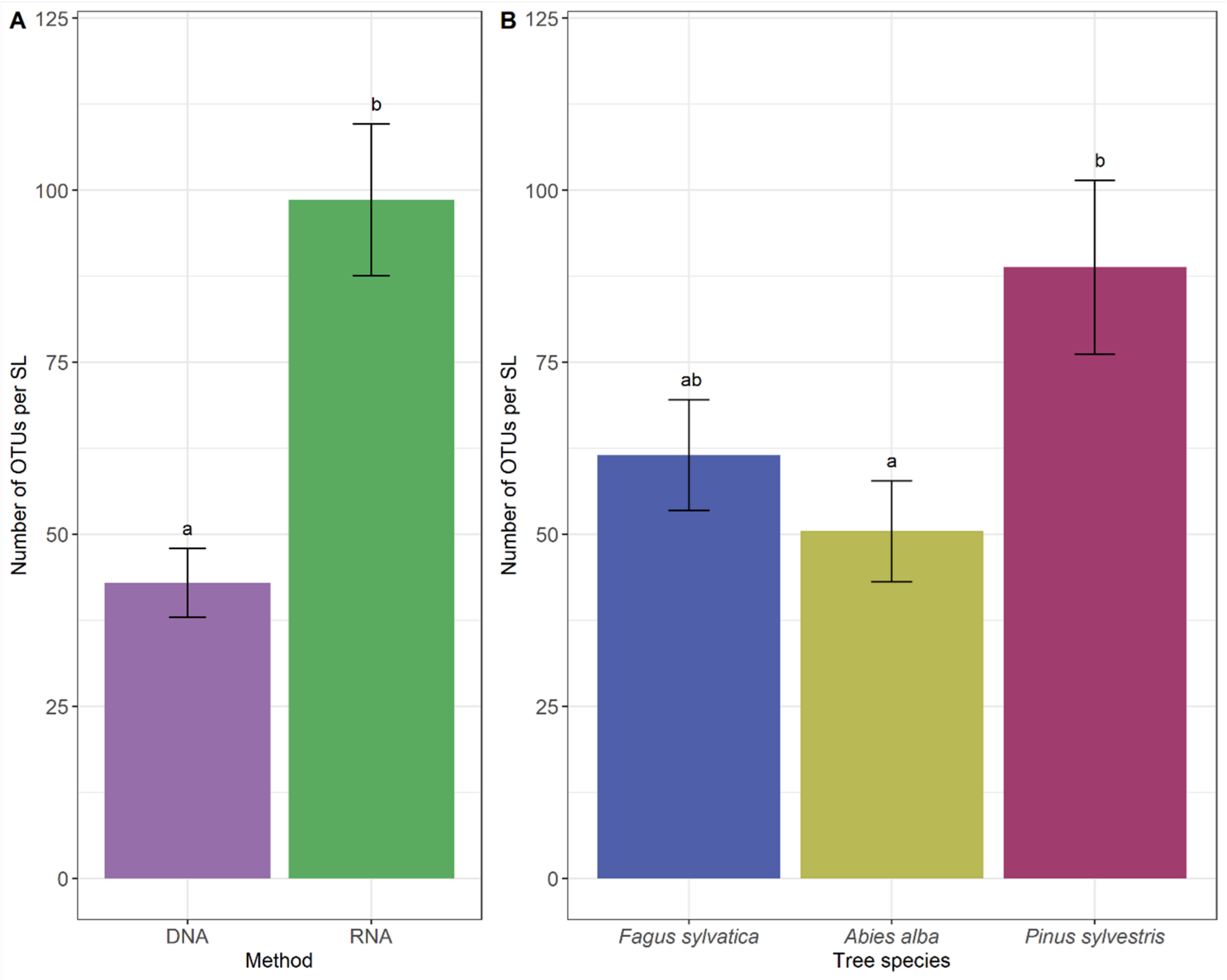

Figure 1

OTU richness per seed lot across metabarcoding methods and tree species. OTU richness (i.e., number of observed OTUs) per seed lot comparing (A) different methods and (B) tree species is shown. Bars represent estimated means ± standard errors (SE). The model was run on the rarefied dataset. Different letters above the bars indicate statistically significant differences ($p < 0.05$).

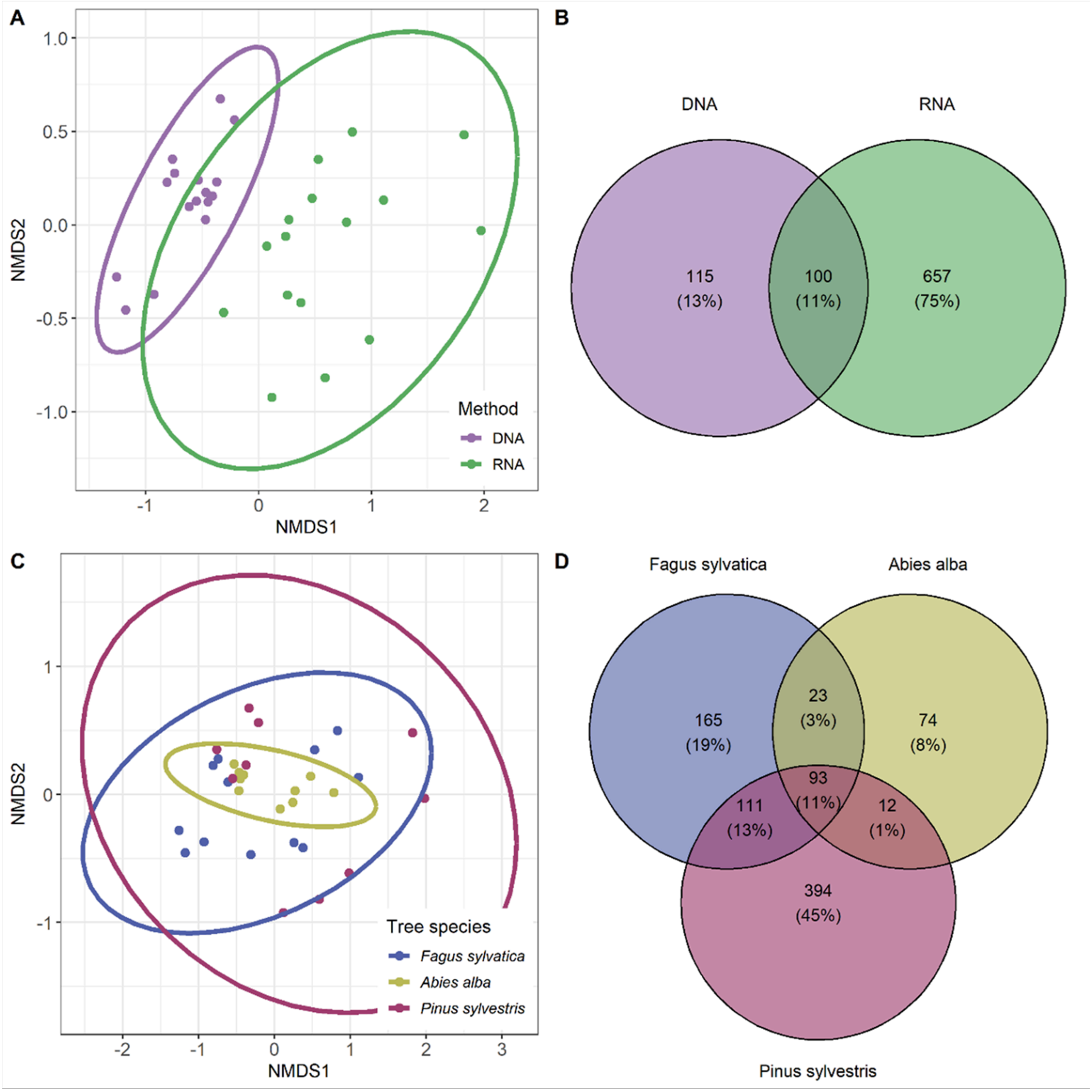

Figure 2

Differences in fungal community composition across metabarcoding methods and tree species. Differences in fungal community composition between samples assessed with DNA and RNA metabarcoding (A, B) and samples belonging to different tree species (C, D) are shown. Non-metric multidimensional scaling ordination plots (A, C) are based on Sørensen distances and represent incidence-based community patterns. Stress value is 0.12. Points represent individual samples, coloured by method (A) or tree species (C). Ellipses represent 95% confidence intervals. Venn diagrams (B, D) are

based on incidence data and illustrate the overlap in fungal operational taxonomic units between the two methods (B) or across three tree species (D), highlighting both shared and unique OTUs. All visualisations are based on the rarefied dataset.

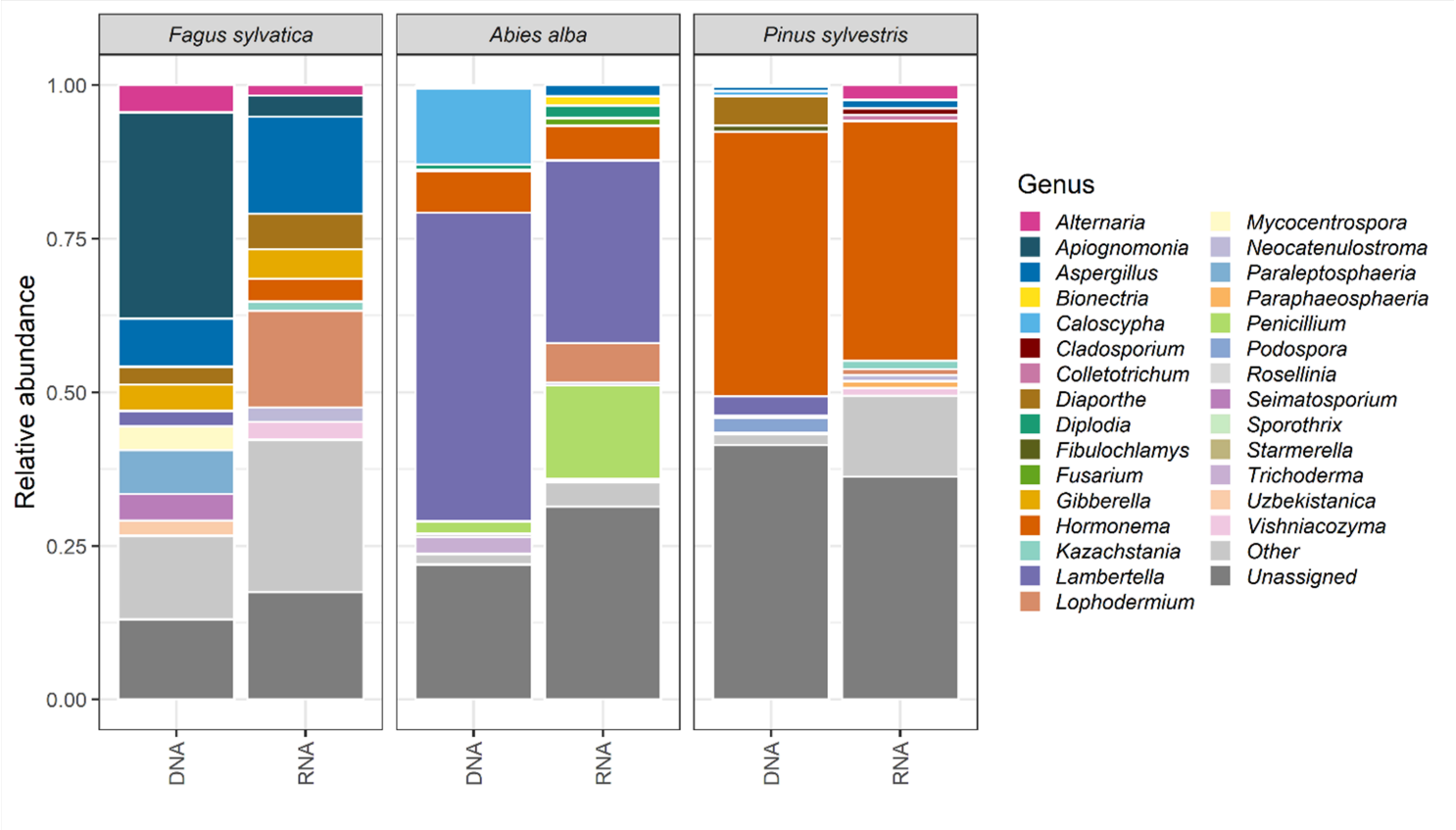

Figure 3

Dominant fungal genera in seeds of three tree species from DNA and RNA metabarcoding. Taxonomic profiles of dominant fungal communities in seeds of *Fagus sylvatica*, *Abies alba*, and *Pinus sylvestris*, based on DNA and RNA metabarcoding are shown. Bars show the relative abundance of the ten most abundant fungal genera per tree species and metabarcoding method. Reads assigned to genera outside the top ten are grouped under “Other,” while those not classified at genus level are labelled “Unassigned.” Calculations are based on non-rarefied dataset.

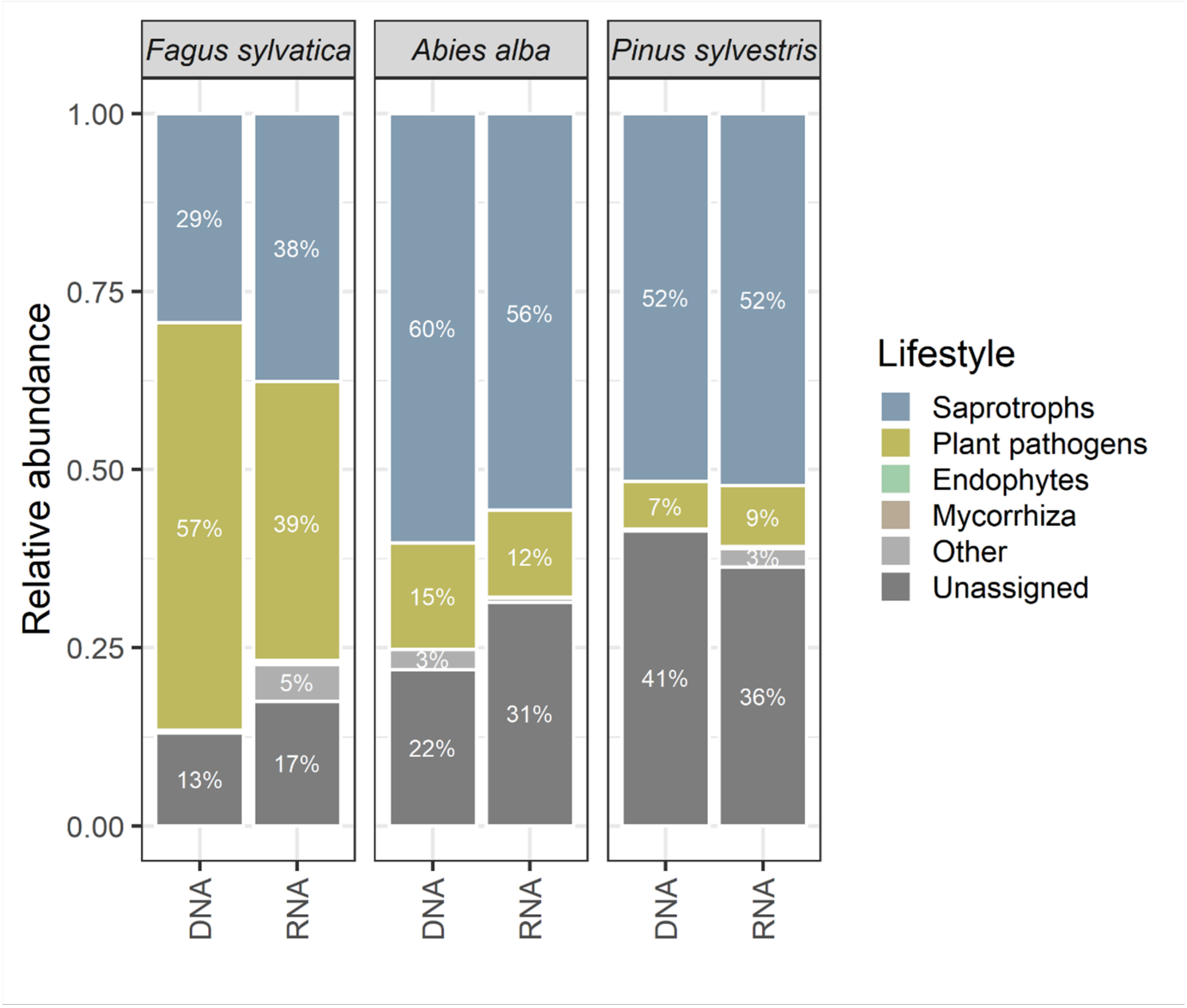

Figure 4

Fungal lifestyle composition in seeds of three tree species from DNA and RNA metabarcoding. Lifestyle profiles of fungal communities in seeds of *Fagus sylvatica*, *Abies alba*, and *Pinus sylvestris*, based on DNA and RNA metabarcoding are shown. Fungal lifestyles were assigned using FungalTraits database. Bars show the relative abundance of different fungal lifestyles per tree species and metabarcoding method. Percentages are not shown for lifestyles representing less than 1% of reads per method and tree species. Calculations are based on non-rarefied dataset.

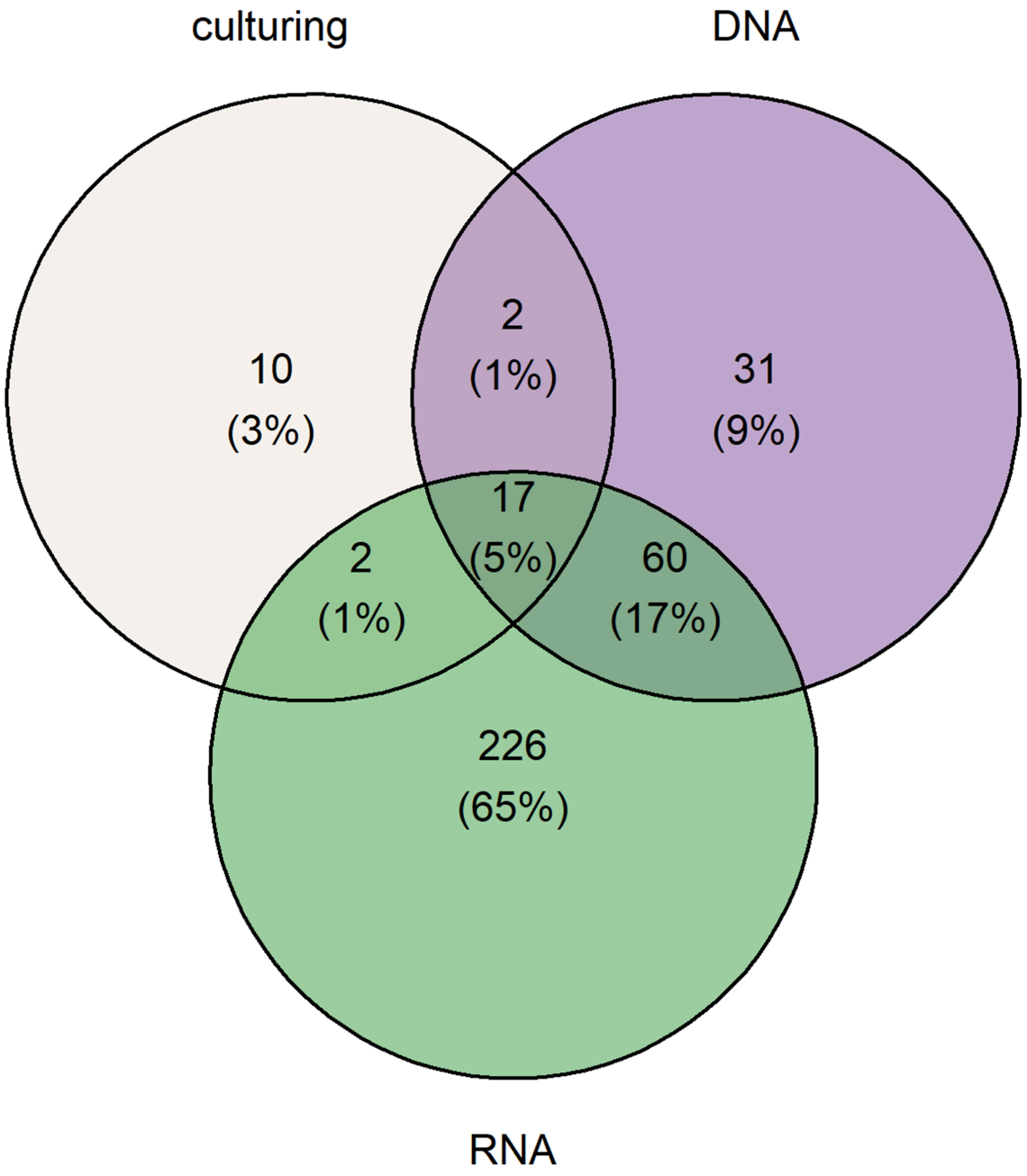

Figure 5

Overlap of fungal genera detected by culturing and DNA/RNA metabarcoding methods. Venn diagram illustrates the overlap in fungal genera detected by culturing and two metabarcoding methods (i.e., DNA and RNA), highlighting shared and unique OTUs. The visualization is based on the non-rarefied dataset.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Franicetal.2025SupplementaryMaterial.docx