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Research

Methods

Metabarcoding read abundances of orchid mycorrhizal fungi are correlated to copy numbers estimated using ddPCR

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Summary

- Quantifying the abundances of fungi is key to understanding natural variation in mycorrhizal communities in relation to plant ecophysiology and environmental heterogeneity. High-throughput metabarcoding approaches have transformed our ability to characterize and compare complex mycorrhizal communities. However, it remains unclear how well metabarcoding read counts correlate with actual read abundances in the sample, potentially limiting their use as a proxy for species abundances.
- Here, we use droplet digital PCR (ddPCR) to evaluate the reliability of ITS2 metabarcoding data for quantitative assessments of mycorrhizal communities in the orchid species *Neottia ovata* sampled at multiple sites. We performed specific ddPCR assays for eight families of orchid mycorrhizal fungi and compared the results with read counts obtained from metabarcoding.
- Our results demonstrate a significant correlation between DNA copy numbers measured by ddPCR assays and metabarcoding read counts of major mycorrhizal partners of *N. ovata*, highlighting the usefulness of metabarcoding for quantifying the abundance of orchid mycorrhizal fungi. Yet, the levels of correlation between the two methods and the numbers of false zero values varied across fungal families, which warrants cautious evaluation of the reliability of low-abundance families.
- This study underscores the potential of metabarcoding data for more quantitative analyses of mycorrhizal communities and presents practical workflows for metabarcoding and ddPCR to achieve a more comprehensive understanding of orchid mycorrhizal communities.

Introduction

Mycorrhizal fungi are essential for plant health, soil processes, and ecosystem functioning (Smith & Read, 2008; van der Heijden *et al.*, 2015), and differences in fungal richness and abundance can be expected to have a major impact on plant population dynamics and community composition (Tedersoo *et al.*, 2020). The internal transcribed spacer (ITS) of nuclear DNA (nrDNA) is a widely used DNA barcoding marker to identify fungi in metabarcoding studies using high-throughput sequencing (HTS) techniques, and these techniques have been efficiently implemented to assess the taxonomic diversity of fungal communities in mycorrhizal roots (Lindahl *et al.*, 2013; Nilsson *et al.*, 2019) and other microbial communities (Tedersoo *et al.*, 2022). However, whether the number of reads generated from metabarcoding is quantitative remains controversial (Amend *et al.*, 2010; Lamb *et al.*, 2019; Klunder *et al.*, 2022), and it remains unclear to what extent the number of read counts generated from metabarcoding correlate with the actual number of DNA copies in the original sample due to potential biases arising from each step of metabarcoding (Lamb *et al.*, 2019; Nilsson *et al.*, 2019), including PCR inhibition, unequal amplification, and sequencing errors. Without knowing its quantitative information, metabarcoding remains a tool for assessing species richness and community composition only. Therefore, there is an urgent need to assess the reliability of metabarcoding data for the quantification of mycorrhizal fungal communities by comparing it with other quantitative approaches.

Recently, droplet digital PCR (ddPCR), a technique that has been successfully applied to clinical research, is emerging in ecological research for rapid and accurate quantification of environmental DNA (Capo *et al.*, 2021; Everts *et al.*, 2022). Based on massive

oil-droplet partitioning and separate reactions within each droplet (Hindson *et al.*, 2011), ddPCR yields higher accuracy to measure copy numbers than other digital PCR systems (Nathan *et al.*, 2014; Miotke *et al.*, 2015; Mauvisseau *et al.*, 2019). Therefore, ddPCR has been increasingly applied for biodiversity assessments and biomonitoring, mostly in aquatic ecosystems (Didaskalou *et al.*, 2022). However, the application of this technique has rarely been expanded to terrestrial ecosystems, for example for assessing microbial communities in soil and plant tissues (Holland *et al.*, 2019; Kokkoris *et al.*, 2019). Due to its good tolerances to PCR-inhibiting substances in complex samples (Capo *et al.*, 2021; Mejbel *et al.*, 2021), ddPCR represents a promising approach for quantifying mycorrhizal fungi residing in plant roots. The fungal structures formed in root cells can undergo the physiological process of lysing, and it remains challenging to distinguish intact and degraded fungal structures merely using DNA-based analyses. However, ddPCR shows the potential to tackle this issue using different strategies, for example by assessing fragment size distribution (Fernando *et al.*, 2018; Zhao *et al.*, 2021) or methylation status (Zhao *et al.*, 2023).

Quantitative analyses of fungal abundances may be particularly important for understanding ecological variations in mycorrhizal communities of orchids (McCormick *et al.*, 2018; Jacquemyn & Merckx, 2019). Orchid mycorrhizal communities encompass a wide taxonomic range of Basidiomycota and Ascomycota fungi that exhibit multiple ecological lifestyles (Dearnaley *et al.*, 2012; Wang *et al.*, 2021). Compositional shifts in mycorrhizal communities occur frequently along evolutionary transitions in orchid trophic modes (Wang *et al.*, 2021; Selosse *et al.*, 2022), along ontogenetic stages (Bidartondo & Read, 2008; Waud *et al.*, 2017), and across habitats (Li *et al.*, 2021). Interestingly, instead of abrupt changes in fungal composition, the coexistence of different fungal groups within orchid mycorrhizal communities indicates that compositional shifts occur in a gradual manner (Ogura-Tsujita *et al.*, 2012). Indeed, an increasing number of studies adopting HTS techniques have shown that multiple fungal groups co-occur in the orchid root system with different relative read abundances (Xing *et al.*, 2020; Jacquemyn *et al.*, 2021). However, whether read counts produced by HTS metabarcoding are reliable for quantitative research remains to be validated, hampering a precise understanding of the variation in orchid mycorrhizal communities between species, sites, and life stages.

The objective of this study is to examine whether metabarcoding reads can be used to assess differences in relative abundances of orchid mycorrhizal fungi at the family level. To achieve this goal, we examined the relationship between read accounts generated by metabarcoding and estimates of copy numbers by ddPCR to quantify main groups of orchid mycorrhizal fungi. Here, we focused on the terrestrial orchid species *Neottia ovata* (Kotilínek *et al.*, 2015). Previous studies have shown that the relative composition of its mycorrhizal communities can change between populations found in grasslands and forests, based on read counts (Jacquemyn *et al.*, 2015; Oja *et al.*, 2015; Těšitelová *et al.*, 2015). A more recent study using ITS-based metabarcoding demonstrated that this species can associate with multiple groups of typical orchid mycorrhizal fungi and ectomycorrhizal fungi mainly belonging to eight fungal families, including Serendipitaceae, Ceratobasidiaceae, Inocybaceae, Sebacinaceae, Thelephoraceae, Tulasnellaceae, Russulaceae, and Atheliaceae (Wang *et al.*, 2023). In this study, we designed ddPCR assays to distinguish these main fungal families present in the roots of *N. ovata*. Family-specific copy numbers obtained from ddPCR assays were compared with read counts generated from metabarcoding. Fig. 1 shows a brief workflow of this study.

Materials and Methods

Sample description, DNA extraction, and metabarcoding

The same DNA extracts from the 30 individuals of *Neottia ovata* (L.) Bluff & Fingerh. sequenced in Wang *et al.* (2023) using a MiSeq platform were used to perform the ddPCR assays in this study. In short, root systems were carefully cleaned with running tap water, bleached, and surface sterilized with ethanol. After checking mycorrhizal colonization using light microscopy, five root sections (1 mm thick) per plant were selected for DNA extraction using the CTAB method (Doyle, 1987). The DNA extraction of each sample was suspended in 200 µl Milli-Q water. The concentration and purity of DNA extractions were measured using microfluidic chips with Dropsence 96™ (Trinean, Gentbrugge, Belgium) and subsequently analyzed with DropQuant v.1.5. All DNA extracts showed a similar concentration and purity (Supporting Information Table S1) and were used as templates for PCR amplification. Two sets of primers were used to amplify the fungal nuclear ITS region. The general primer fITS7 (Ihrmark *et al.*, 2012), which was slightly modified from ITS86 (Turenne *et al.*, 1999), was paired with ITS4 (White *et al.*, 1990) to detect a wide range of orchid mycorrhizal fungi. Because amplification of fungi from Tulasnellaceae by general primers is known to be difficult (Waud *et al.*, 2014; Li *et al.*, 2021), the specific primer Tul1F/2F (Wang *et al.*, 2023), which binds *c.* 80 bases upstream of fITS7 in the 5.8S region, was paired with ITS4Tul2 (Oja *et al.*, 2015) to amplify this family. Tul1F/2F was designed based on ITS sequences recorded in the latest global dataset of orchid mycorrhizal fungi (table S1 in Wang *et al.*, 2021).

Since the details of library preparation, sequencing, and bioinformatics have been described in Wang *et al.* (2023), here, we briefly summarize the major procedures to generate the 300-bp paired-end MiSeq dataset of mycorrhizal communities of *N. ovata*. To reduce PCR stochasticity, PCRs were duplicated and merged as one amplicon for each DNA sample and pooled in equal molarity for sequencing using an Illumina MiSeq PE300 platform (BaseClear, Leiden, the Netherlands). After demultiplexing and quality filtering using VSEARCH (Rognes *et al.*, 2016), DNA sequences were clustered into operational taxonomic units (OTUs) based on the commonly used 97% similarity threshold. After taxonomic assignment by Blast searches against the Unite reference database (utax_reference_ dataset_10.05.2021.fasta, https://unite.ut.ee/repository.php), the OTUs annotated as mycorrhizal fungi of *N. ovata* (Jacquemyn *et al.*, 2015; Oja *et al.*, 2015; Těšitelová *et al.*, 2015) were retained for further analyses. Two datasets of read counts of orchid mycorrhizal OTUs produced by the primer pair

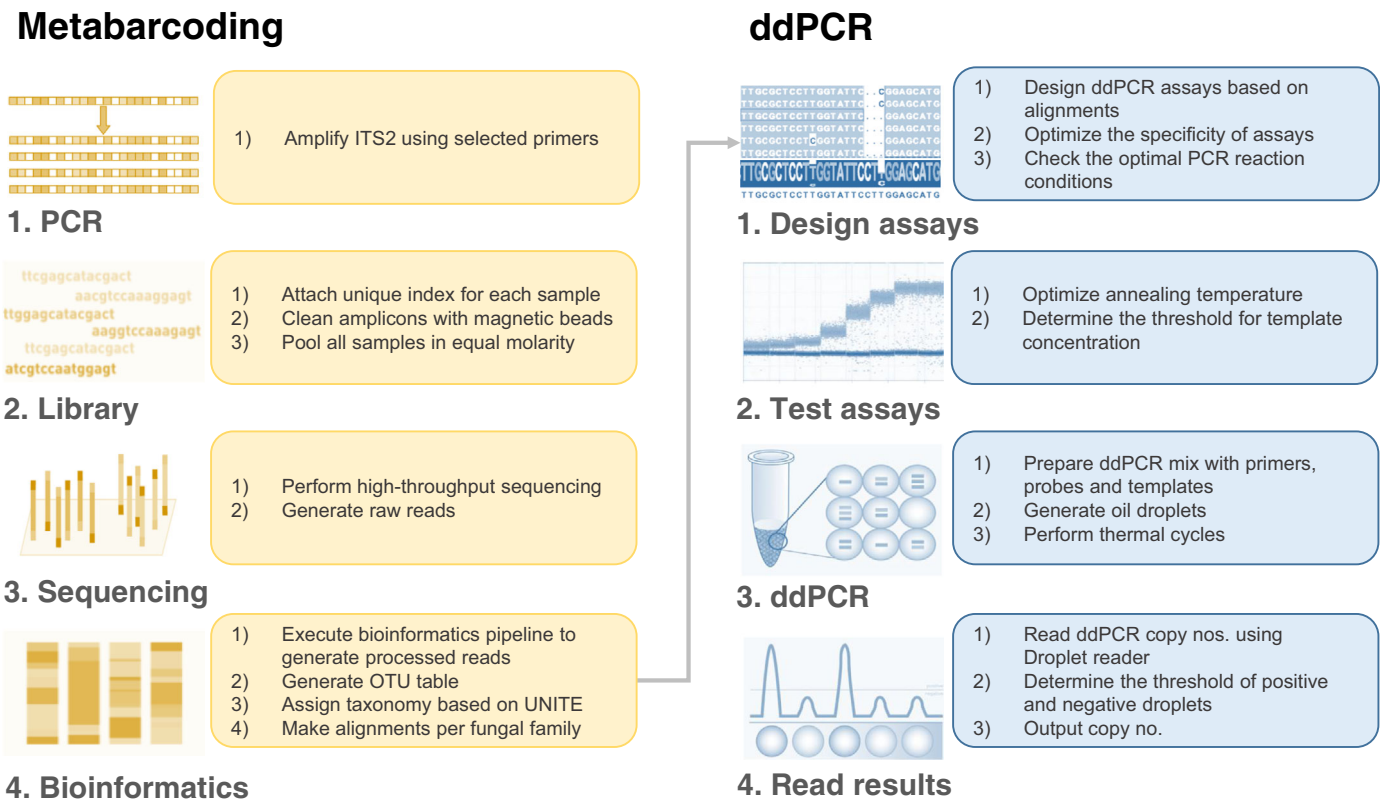

Fig. 1 Workflow of droplet digital PCR (ddPCR) quantification on the basis of ITS2 metabarcoding. In this study, we designed and applied ddPCR assays to target orchid mycorrhizal fungi of the orchid *Neottia ovata* based on metabarcoding data generated by a MiSeq platform. The steps of ddPCR quantification are described in the Materials and Methods section of this study. The procedures for ITS2 metabarcoding are briefly summarized in the Materials and Methods section of this study and described in detail in Wang *et al*. (2023).

fITS7/ITS4 were prepared: one dataset directly retrieved the raw read counts of each OTU within each sample, and the other used the normalized read counts to make sure each sample has the same number of reads. The widely used rarefaction method was adopted for data normalization using the function 'rarefy_even_depth ()' from the R package PHYLOSEQ (McMurdie & Holmes, 2013). The MiSeq data were deposited in the NCBI BioProject database with accession no. PRJNA952620.

Design of ddPCR assays

We designed the ddPCR assay for eight fungal families that were abundant in roots of *N. ovata* (Wang *et al*., 2023), including Serendipitaceae, Ceratobasidiaceae, Inocybaceae, Sebacinaceae, Thelephoraceae, Tulasnellaceae, Russulaceae, and Atheliaceae. The MiSeq dataset of *N. ovata* produced by fITS7/ITS4 (Wang *et al*., 2023) was used as the main resource to design family-specific ddPCR assays, whereas the global dataset of orchid mycorrhizal fungi (Wang *et al*., 2021) was used as supplementary resource to increase the universality of the designed assays for other orchid species. For each fungal family, sequence alignments were built using MAFFT on the GENEIOUS PRIME v.2019.2 software and were screened for relatively conserved regions for primer design. For Tulasnellaceae, which exhibit a highly variable ITS region, the MiSeq sequences of the specific primer pair were also included in the alignment to search for a conserved region in the 5.8S region for primer design. Sequence alignments of each fungal family used for primer design can be freely accessed in the online Figshare database (10.6084/m9.figshare.23904219).

The design of hydrolysis probe assays, PCR protocol optimization, and further check of primer-probe combinations for specificity and efficiency were performed on the basis of the Bio-Rad ddPCR guidelines. For each assay, the annealing temperature, possible secondary structures, and self-/cross-dimers were checked with OligoCal (http://biotools.nubic.northwestern.edu/OligoCalc.html) and Multiple Primer Analyzer (https://www.thermofisher.com/). The specificity of each assay was assessed *in silico* using the NCBI Primer-Blast function (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and double-checked with sequence alignments in GENEIOUS. Alignments of ITS sequences recorded in the global dataset of orchid mycorrhizal fungi (table S1 in Wang *et al*., 2021) were used to ensure that each assay only amplifies the targeted fungal family.

Each hydrolysis probe was attached with a 5′ Reporter Dye (FAM or HEX), a 3′ Quencher (Iowa Black FQ) and an internal quencher (ZEN™) that shortens the distance between dye and quencher. For each assay, double-stranded DNA fragments (gBlocks™) of fungal sequences that cover the product region (Table S2) were used as a positive control. Primers, TaqMan hydrolysis probes, and synthetic gBlocks DNAs were ordered from Integrated DNA Technologies (IDT, https://eu.idtdna.com/).

Optimization of ddPCR assays

Before ddPCR was performed, we tested the optimal reaction conditions of the ddPCR assays using temperature and dilution gradients. Temperature gradients (45–60°C) were created to determine the optimal annealing temperature for each assay with the input of synthetic DNA. Because the MiSeq dataset showed that the read counts varied greatly between fungal families and samples (Wang *et al.*, 2023), we created dilution gradients for each assay to avoid saturation of the ddPCR reaction. Specifically, reactions of an input of 2 µl original DNA templates and 10 times diluted samples were applied for each assay. The copy numbers yielded by 10 times diluted samples were within the detection range of ddPCR, and thus, the 10× dilution factor was suitable for amplifying fungi with relatively high read counts.

Implementation of ddPCR

Each of the 30 DNA templates or 10 times diluted samples were tested in triplicate for each of the nine ddPCR assays, resulting in a total of 810 ddPCR reactions to quantify the number of copies of orchid mycorrhizal fungi. For each droplet-generating unit (one cartridge), we included one negative control using Milli-Q water; for each 96-well ddPCR plate of an assay, we included one positive control using synthetic DNAs (Table S2). The design of the 96-well ddPCR plate can be found in Fig. S1.

Each ddPCR reaction was prepared with 11 µl probe mix (Bio-Rad Supermix; Bio-Rad), 2 µl DNA, 1 µl (10 µM stock) of each forward and reverse primer, 1 µl (10 µM stock) probe, and 6 µl Milli-Q water adding up to a reaction mixture of 22 µl. Subsequently, 20 µl PCR mixture was transferred to a cartridge and mixed with 70 µl of Bio-Rad droplet generation oil to create 10 000–20 000 oil droplets per sample. Detailed procedures for oil-droplet generation can be found in Notes S1. A total of 40 µl of this droplet mixture was then transferred to a 96-well PCR plate and sealed with PCR foil. The target DNA in these plates was finally amplified with a Bio-Rad T100 thermal cycler using the following conditions: 10 min at 95°C, 40 cycles of denaturation for 30 s at 94°C and 1 min at 51°C, then a signal termination of 10 min at 98°C and a hold at 4°C. After amplification, the 96-well plate was transferred to a Bio-Rad QX-200 droplet reader to screen the positive and negative droplets in each well.

Validation of metabarcoding reads using ddPCR

To quantify the number of target copies in ddPCR wells, we used Bio-Rad's QuantaSoft software v.1.7.4. For each sample, triplicates were merged using the merge tool, and the concentrations (copies μl^{-1}) of the positive droplets were estimated using thresholding and Poisson modelling according to the QuantaSoft manual instructions (see details in Notes S1). The original concentrations of the target DNAs were calculated based on the dilution factor and the volume of the ddPCR reaction (Table S3). Raw ddPCR files exported from QuantaSoft can be freely accessed in the online Figshare database (10.6084/m9.figshare.23904219).

The ddPCR copy numbers were compared with the MiSeq read counts at the fungal family level. To investigate the effect of data normalization on MiSeq read abundance, we compared the ddPCR data with the raw and normalized MiSeq dataset of fITS7/ITS4, separately. Within each fungal family, we screened fungal OTUs that could be amplified by ddPCR assays using both a strict and relaxed criterion. A strict criterion does not allow mismatches between one fungal OTU and one assay, whereas a relaxed criterion allows for one or two mismatches. According to the presence and absence of matches between fungal OTUs and ddPCR assays (Table S4), the sum of read counts of fungal OTUs within each fungal family was calculated for each sample. Therefore, a total of 240 data points (rows) were summarized for eight fungal families of 30 DNA samples (Table S5), allowing for comparisons between the ddPCR copy numbers and the corresponding MiSeq read counts. After checking the normality of the data, Spearman's rank correlation coefficient was used to relate ddPCR copy numbers to read counts of the raw and normalized MiSeq dataset, separately.

Results

The specificity and efficiency of ddPCR assays

One primer-probe assay was designed for each fungal family except for Serendipitaceae, for which two assays were designed to detect its high diversity of OTUs in the root communities of *N. ovata* (Figs S2–S9). The specificity of ddPCR assays in amplifying DNAs from the targeting fungal family was confirmed with *in silico* PCRs and manual check of the alignment (Table 1). All ddPCR assays had a wide range of optimal annealing temperatures (45–54°C) according to temperature gradient tests (Table 1).

The designed assays showed a generally high capacity to amplify sequences of each fungal family using raw MiSeq reads (Table 2). For the family Serendipitaceae having the highest read abundance in all orchid samples studied, the ddPCR assays were capable of detecting 93% of the sequencing reads when no or two mismatches were allowed. Regardless of a relaxed or strict calculation, the designed assays could amplify 100%, 77%, and 84% of the reads of Ceratobasidiaceae, Sebacinaceae, and Atheliaceae, respectively. However, for the other families (Inocybaceae, Tulasnellaceae, Thelephoraceae, and Russulaceae), the percentage of MiSeq reads targeted by the designed assay varied depending on the number of mismatches allowed. In addition, among the global dataset of orchid mycorrhizal fungi, several ddPCR assays exhibited high amplification efficiency, detecting > 80% of sequences from Serendipitaceae, Ceratobasidiaceae, and Thelephoraceae (Table 2).

Correlation between ddPCR copy numbers and MiSeq read counts

When using the raw MiSeq dataset, a significant positive correlation was found between read counts and ddPCR copy numbers based on a strict (Spearman's $\rho = 0.74$, $P < 0.0001$) or relaxed calculation (Spearman's $\rho = 0.78$, $P < 0.0001$; Figs 2, S10; Table S6). Similar results were obtained when using normalized

Table 1 Detailed information on newly designed ddPCR assays.

Fungal family	Assay	Name	Sequence	Length (bp)	Average product size (bp)	Optimal T_a (°C)	Specificity of assays	
							In silico PCR	Check alignment
Serendipitaceae	#Ser1	Ser1_F	CRGTGTGATAWGYATCTTCAC	21	175	48–54	Yes	Yes
		Ser1_P	**/56-FAM/**CCTCAAATC**/ZEN/** GGGTGGGACTAC**/3IABkFQ/**	21				
		Ser1_R	TCCTCCGCTTATTGATATGC	20				
	#Ser2	Ser2_F	TCATCGAATCTTTGAACGC	19	160	48–54	Yes	Yes
		Ser2_P	**/56-FAM/**ACCTTGCAC**/ZEN/** CCTTTGGTATTCC**/3IABkFQ/**	20				
		Ser2_R	GGGTACACTCAKGCATTT	18				
Ceratobasidiaceae	#Cera	Cer_F	TTGCGCTCCTTGGTATTC	18	200	45–51	Yes	Yes
		Cer_P	**/56-FAM/**ATACTCAAA**/ZEN/** CAGGCATGCTCC**/3IABkFQ/**	21				
		Cer_R	TATCACGCYGAGTGGAAC	18				
Inocybaceae	#Ino	Ino_F	TTGCGCTCCCTGGTATTC	18	160	45–51	Yes	Yes
		Ino_P	**/5HEX/**ACTCAAACA**/ZEN/** GGCATGCTCCTC**/3IABkFQ/**	21				
		Ino_R1	CACTCCAGRTACCACTAATG	20				
		Ino_R2	CATTTCAGAGGAGCAGGC	18				
		Ino_R3	MATTTCAGGGGAGCMGAC	18				
Sebacinaceae	#Seb	Seb_F	CATCGAATCTTTGAACGCAC	20	200	45–54	Yes	Yes
		Seb_P	**/5HEX/**CCCAAGTCC**/ZEN/** ACCGCTCC**/3IABkFQ/**	17				
		Seb_R	ACTTATCACGCYGTACTATG	20				
Tulasnellaceae	#Tul	Tul_F	CAACGGATCTCTTGGCAT	18	155	45–51	Yes	Yes
		Tul_P	**/56-FAM/**TCGAATCTT**/ZEN/** TGAACGCACCTTG**/3IABkFQ/**	22				
		Tul_R1	GAGATATTCATGACACTCAAC	21				
		Tul_R2	GAGWTGWTCATAATACTCAAC	21				
Thelephoraceae	#The	The_F	CATCGAATCTTTGAACGCAC	20	80	45–51	Yes	Yes
		The_P	**/56-FAM/**AACAGGCAT**/ZEN/** GCCCTTCGGAAT**/3IABkFQ/**	21				
		The_R	TTGAGGTGTTCAYGATACTC	20				
Russulaceae	#Rus	Rus_F	TGATAAGATGTTTCTACGTYTTG	23	160	45–51	Yes	Yes
		Rus_P	**/56-FAM/**TCAAATCGG**/ZEN/** GTGAGACTACCC**/3IABkFQ/**	21				
		Rus_R	TCCTCCGCTTATTGATATGC	20				
Atheliaceae	#Ath	Ath_F	GTCGGCTCCTCTTTAAATG	19	100	45–51	Yes	Yes
		Ath_P	**/5HEX/**ACCACAGCG**/ZEN/** CAACAGGATTATC**/3IABkFQ/**	22				
		Ath_R	CGGTTRGAAGCRGACTTTC	19				

For each assay, the sequences of forward and reverse primers and the probe, the targeting fungal family, the range of optimal annealing temperature (T_a), and the specificity in amplification are displayed. The range of T_a was obtained by temperature gradient tests between 45 and 65°C. The reverse primers attached to R1, R2, or R3 were mixed equally to represent one homologized reverse primer to match with the forward primer. Bold values in each probe sequence represent the position of a 5′ Reporter Dye (FAM or HEX), a 3′ Quencher (Iowa Black FQ), and an internal quencher (ZEN™) that shortens the distance between dye and quencher.

MiSeq reads for comparison (Table S6) based on strict (Spearman's $\rho = 0.74$, $P < 0.0001$) or relaxed calculation (Spearman's $\rho = 0.77$, $P < 0.0001$).

When using the raw MiSeq dataset, correlation analyses within each family (Figs 2, S10; Table S6) showed a significant high correlation between ddPCR and MiSeq data for the fungal families Serendipitaceae (Spearman's $\rho = 0.85$), Inocybaceae ($R = 0.74$ or 0.82), Sebacinaceae (Spearman's $\rho = 0.82$ or 0.84), Thelephoraceae ($R = 0.80$ or 0.83), and Atheliaceae (Spearman's $\rho = 0.90$). However, a relatively low correlation value was found for Ceratobasidiaceae (Spearman's $\rho = 0.51$), Tulasnellaceae (Spearman's $\rho = 0.55$), and Russulaceae (Spearman's $\rho = 0.23$ or 0.39). When using the normalized MiSeq dataset, the correlation values of most families were slightly lower than the raw MiSeq dataset without data normalization (Table S6).

We treated zero values that did not occur simultaneously in the ddPCR and raw MiSeq data set as false zeros. Among the 240 data points obtained for eight fungal families and 30 DNA samples, a total of 54 potential false zeros were found in the raw MiSeq dataset, which is four times higher than the number of possible false zeros produced by ddPCR (Table S5). Most of the corresponding values of these false zeros were found to be relatively small read counts in the MiSeq dataset or small copy numbers in the ddPCR dataset (Table S5). Regarding the distribution of false zeros among

New Phytologist

Table 2 Efficiency of ddPCR assays.

Fungal family	Assay	Raw MiSeq reads	Percentage of MiSeq reads targeted by ddPCR assays (%)	GOM sequences	Percentage of GOM sequences targeted by ddPCR assays (%)
Serendipitaceae	#Ser1 #Ser2	297 235	93	694	95
Ceratobasidiaceae	#Cera	98 384	100	1015	83–100
Inocybaceae	#Ino	42 458	60–93	125	64–91
Sebacinaceae	#Seb	38 371	77	187	37–74
Tulasnellaceae	#Tul	22 238	44–92	1956	10–18
Thelephoraceae	#The	20 453	83–98	255	91
Russulaceae	#Rus	12 112	79–93	411	38–79
Atheliaceae	#Ath	6841	84	13	0–15

For each fungal family, the efficiency of the designed ddPCR assays are represented by the percentage of reads/sequences that can be amplified in the MiSeq dataset of *Neottia ovata* (Wang *et al*., 2023) and in the global database of orchid mycorrhizal fungi (GOM, Wang *et al*., 2021). The total number of raw MiSeq reads presented in all 30 orchid root samples were summed up for each fungal family. For the percent of reads/sequences targeted by ddPCR assays shown by a range, the lower value represents that the reads are exactly targeted by one assay with no mismatches (a strict calculation), while the high value represents that the reads are mapped with one assay with one or two mismatches (a relaxed calculation).

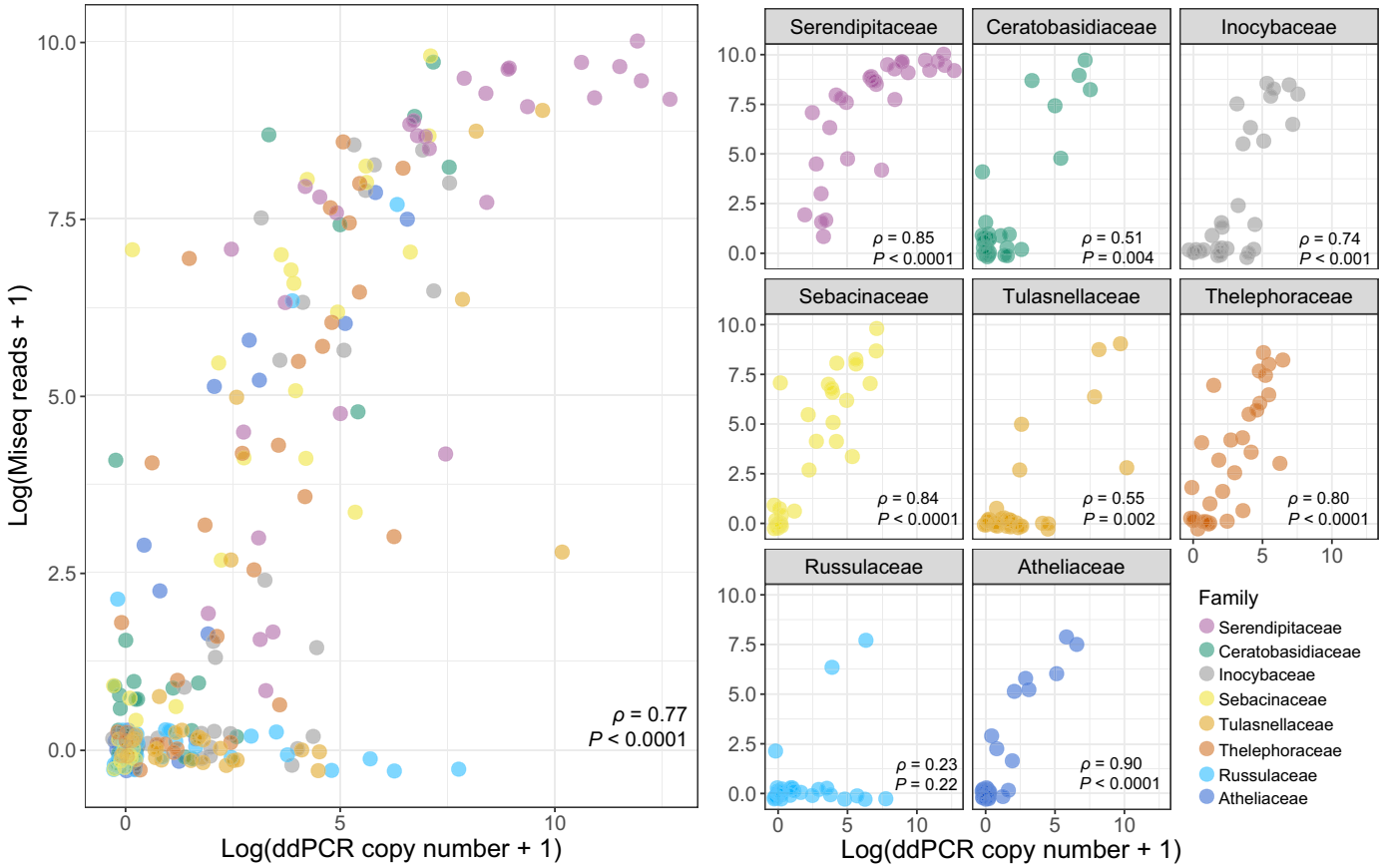

Fig. 2 Correlation between droplet digital PCR (ddPCR) copy numbers and raw MiSeq reads for eight fungal families of orchid mycorrhizal fungi. A strict criterion was adopted that allowed for no mismatches between the two datasets. The left panel shows the correlation for all fungal families and the right panel shows the correlation for each fungal family separately. Spearman's rank correlation coefficient 'ρ' and associated *p*-values are shown. The points in the plot represent each of the eight fungal families detected from a total of 30 root samples of *Neottia ovata*. Points with different colors represent different fungal families. For the purpose of visualization, the values of ddPCR copy number and MiSeq reads were log-transformed. The 'position_jitter ()' function is employed with a value of 0.3 to prevent points from excessively overlapping using the R package GGPLOT2 (Wickham, 2016). Consequently, certain values appear below the zero lines in the plots.

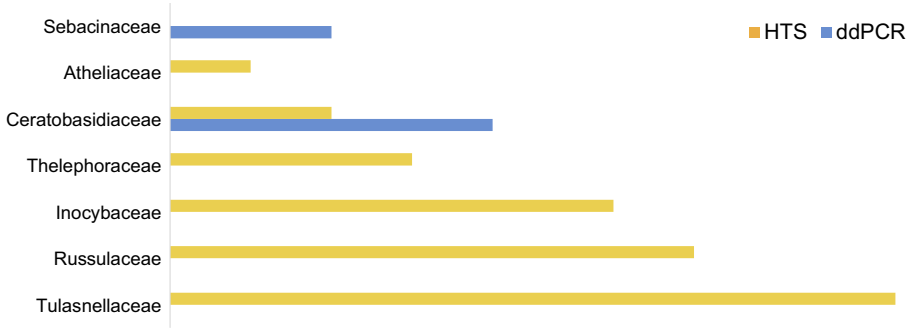

Fig. 3 Comparison of false zeros produced by high-throughput MiSeq sequencing (HTS) and droplet digital PCR (ddPCR). Data points that are positive in one dataset but are zeros in the other are considered false zeros. The raw MiSeq dataset (without data normalization) was used for the calculation.

fungal families (Fig. 3), false zeros produced by ddPCR only occurred in Ceratobasidiaceae (8) and Sebacinaceae (4), while false zeros produced by MiSeq sequencing were found mainly for Tulasnellaceae (18), Russulaceae (13), and Inocybaceae (11).

Discussion

Using newly designed ddPCR assays (Table 1), we investigated whether high-throughput metabarcoding reads can be used to assess the abundances of orchid mycorrhizal fungi of *N. ovata* at the family level. Our results showed that the two data sets were strongly and significantly correlated to each other (Spearman's $\rho = 0.74–0.78$), regardless of whether a normalized (i.e. rarefied) or raw MiSeq dataset was used and whether a strict or relaxed criterion of mismatches with ddPCR assays was adopted (Figs 2, S10; Table S6). However, for some, mostly low-abundant fungal families, the level of correlation between both methods was not very strong (Table S6) and also the number of false zeros occurred primarily in these low-abundance families (Fig. 3; Table S5). These findings indicate that metabarcoding data are suitable for quantitative analyses of mycorrhizal fungi of the orchid species, albeit not for low-abundance fungal families.

Read abundance data from metabarcoding have been increasingly used to describe fungal community composition and to calculate dissimilarity metrics of microbial communities for diversity analyses (Tedersoo *et al.*, 2022; Labouyrie *et al.*, 2023). However, due to potential biases arising from metabarcoding (Lamb *et al.*, 2019; Nilsson *et al.*, 2019), the quantitative information of metabarcoding data is usually approached with caution. For example, raw sequencing reads from metabarcoding were transformed into either a presence-absence metric or normalized metrics of relative abundance data for conducting diversity and community structure analyses (Deagle *et al.*, 2019). Only a few studies have used other techniques to validate the degree to which read counts accurately reflect real abundances (Wood *et al.*, 2019; Wu *et al.*, 2023), leaving the reliability of metabarcoding reads for quantification largely uninvestigated. The present study used ddPCR assays to assess whether metabarcoding reads can be used to quantify diverse mycorrhizal communities that reside in the roots of the terrestrial orchid *N. ovata*. Our results showed a significant positive correlation between read abundances estimated by metabarcoding and copy numbers estimated by ddPCR (Table S6), indicating that the number of reads generated from metabarcoding can be used as a proxy for the relative abundance of fungi for the tested families. These results indicate that sequence reads reliably reflect the biological abundance of fungal groups within the tested samples (Deagle *et al.*, 2019). More importantly, normalized and raw MiSeq data showed similar levels of correlation to ddPCR data (Table S6), indicating that rarefaction commonly used for downstream diversity analyses did not provide more accurate abundance estimations compared with raw read counts. Indeed, rarefaction inevitably converted the low-abundance taxa to zero values (Table S5), causing larger deviations in read abundances from the copy numbers estimated by ddPCR (Table S6). Given the results presented here, the raw reads from metabarcoding approaches could be a favorable choice to achieve more precise interpretation of the mycorrhizal community than data that has undergone normalization.

However, caution should be given to fungal families showing weak correlations between the MiSeq and ddPCR datasets (Table S6), which mostly occurred in families with very low abundances (Table 2). The lack of a consistently high correlation between the two methods could be attributed to several methodological factors. PCR inhibitors are notoriously known to inhibit PCR amplification. Although the same DNA extractions were used for both ddPCR and metabarcoding and the quality of all DNA extracts was similar (Table S1), the level of tolerance to PCR-inhibiting substances can differ between the two methods. With droplet partitioning and individual PCR reactions within each droplet (Hindson *et al.*, 2011), the ddPCR technology has been shown to be highly resistant to PCR inhibitors and allows for reliable detection of low-abundance targets (Taylor *et al.*, 2019). On the contrary, due to its low level of tolerance with PCR inhibitors (Sidstedt *et al.*, 2020), PCR amplifications for amplicon sequencing may contribute to a higher number of false zeros, especially for the low-abundance taxa in Tulasnellaceae, Russulaceae, and Inocybaceae (Fig. 3; Table S5). In addition to potential PCR inhibition, false zeros found in metabarcoding data could be the result from pooling of samples (Manter *et al.*, 2010) and biases and errors caused by sequencing instruments (Stoler & Nekrutenko, 2021). The pooling of mock standardized communities together with tested samples may help to further investigate and even correct for these biases (Tkacz *et al.*, 2018; Tedersoo *et al.*, 2022).

Primer biases could have impacted the detection and quantification of orchid mycorrhizal fungi. While the primer pair fITS7/ITS4 was able to identify a diverse array of orchid mycorrhizal fungi in *N. ovata* (Wang *et al.*, 2023), it remains unclear whether this primer pair provides more accurate assessments of fungal

abundances than other primer pairs, such as the general primer pair ITS3/ITS4OF (White *et al.*, 1990; Taylor & McCormick, 2008) for orchid mycorrhizal fungi and the specific primer pair 5.8S-OF/ITS4Tul for Tulasnellaceae (Taylor & McCormick, 2008; Vogt-Schilb *et al.*, 2020). In addition, the influence of amplicon size on PCR efficiency (Huber *et al.*, 2009) should be taken into consideration. The primer sequences used for ddPCR amplify a smaller amplicon size (< 200 bases) than that used for metabarcoding (on average 300 bases). Although the presented ddPCR assays were designed based on the sequence resources of the MiSeq data, the possibility that ddPCR assays have higher efficiency and fidelity in reaction due to their shorter products cannot be ruled out (Huber *et al.*, 2009).

While metabarcoding and ddPCR data were significantly correlated, the method of choice for quantitative research on microbial communities should be made according to the ultimate objectives of the study. As shown in this and previous studies (Miotke *et al.*, 2015; Capo *et al.*, 2021; Mejbel *et al.*, 2021), the accuracy and sensitivity of ddPCR and its ability to tolerate inhibitors make this molecular method more suitable for quantifying targets from complex samples such as soil, for detecting low-abundance taxa, and for investigating temporal and spatial dynamics, given that a fungal community is well known. Some ddPCR assays provided in this study showed a high efficiency to amplify major groups of orchid mycorrhizal fungi detected from *N. ovata*, as well as sequences recorded in a global dataset (Table 2). Therefore, these assays exhibit the potential to investigate the variation in mycorrhizal communities of *N. ovata* and perhaps of other species and samples comprising a similar subset of fungi. On the contrary, if the identity and diversity of microbial communities are unknown, high-throughput metabarcoding approaches should be chosen at the first place. Metabarcoding approaches are capable of providing a complete picture of microbial community diversity within a sample and thousands of samples can easily be processed simultaneously (Lindahl *et al.*, 2013; Bálint *et al.*, 2016; Tedersoo *et al.*, 2022).

Lastly, it is important to note that both ddPCR copy numbers and amplicon read numbers can be biased in a similar way. The commonly used ITS marker has multiple copies within species (Kiss, 2012; Lofgren *et al.*, 2019) despite its high resolution for fungal identification (Schoch *et al.*, 2012), and thus, ITS-based quantification may not reflect the real abundance or biomass of samples. Future studies are therefore recommended to design and evaluate single-copy markers for absolute quantification. Additionally, future improvements can be achieved by using multiple methods to validate the abundance of fungi obtained from molecular methods with other different estimates of fungal biomass, such as quantifying the rate of fungal colonization in roots and directly measuring fungal biomass perhaps using chemical markers (Wallander *et al.*, 2013; Changey *et al.*, 2022). When methodological challenges in metabarcoding, ddPCR, and other quantitative approaches become sufficiently solved, it can be expected that relative or absolute quantification of fungal abundance will profoundly improve our understanding of plant-fungus interactions and microbial ecology in general.

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Competing interests

None declared.

Author contributions

DW, SIFG, VSFTM and KBT designed the experiment. DW conducted the experiments under the guidance of KBT and VSFTM. DW analyzed data with input from KBT, HJ, SIFG and VSFTM. DW wrote the first draft of the manuscript. All authors commented on and approved the final version of the manuscript.

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Data availability

The data that support the findings of this study are available in the Notes S1; Tables S1–S6; and Figs S1–S10 of this article and are freely accessible from online repositories. Sequence alignments and ddPCR files are openly available in the Figshare repository at doi: 10.6084/m9.figshare.23904219.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Design of ddPCR plate.

Fig. S2 Alignment of Serendipitaceae.

Fig. S3 Alignment of Ceratobasidiaceae.

Fig. S4 Alignment of Inocybaceae.

Fig. S5 Alignment of Sebacinaceae.

Fig. S6 Alignment of Tulasnellaceae.

Fig. S7 Alignment of Thelephoraceae.

Fig. S8 Alignment of Russlaceae.

Fig. S9 Alignment of Tulasnellaceae.

Fig. S10 Correlation between ddPCR copy numbers and raw MiSeq reads allowing two mismatches.

Notes S1 Detailed descriptions of ddPCR procedures.

Table S1 Information of orchid samples and DNA extracts.

Table S2 Information of gBlocks synthetic DNAs.

Table S3 Copy numbers of ddPCR reactions.

Table S4 Raw and normalized MiSeq reads targeted by ddPCR assays or not.

Table S5 Summary of ddPCR copy numbers and MiSeq reads for each fungal family of each DNA sample.

Table S6 Spearman's rank correlation coefficients between copy numbers obtained from ddPCR and read abundances generated from a MiSeq platform.

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