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Research Report

Digital PCR enables direct root biomass quantification and species profiling in soil samples

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Abstract

Roots support plant growth and resilience and are a major route for carbon sequestration. Thus, the study of roots in agricultural and natural systems is essential to develop strategies to mitigate and adjust to climate change. Methods to quantify root biomass in mono- and mixed crop systems are therefore in high demand. A promising approach is to exploit the correlation between root biomass and nuclear DNA. The use of qPCR for the quantitative analysis of root samples has been reported. Here, we show how digital PCR can be used to quantify root DNA from soil samples harboring single species or species mixtures. This molecular method has several advantages over more time-consuming methods, including enhanced sensitivity and absolute quantification of target DNA, increased accuracy and reliability, and the ability to quantify roots directly from soil in different species mixtures. We developed a DNA-based digital droplet PCR (ddPCR) method for root species profiling and biomass quantification directly from soil samples under semi-field conditions. Our findings suggest that implementing this ddPCR method can substantially simplify and improve root quantification of specific species, even in crop mixtures. This method offers a more time- and labor-efficient alternative to traditional techniques (e.g. root separation or C^{13} labeling). The complement of primer–probe sets presented here can be continuously expanded to include additional plant species, thus broadening the scope of this DNA-based ddPCR method.

Introduction

Root studies are essential for understanding plant survival, growth, and reproduction, and are becoming increasingly important in breeding efforts for a climate-resilient future (Smit et al. 2000). Root biomass determination is often associated with great uncertainty and sampling of large (typically $> 10 \times 10 \times 10$ cm) soil samples followed by extraction of roots with over 30–90 min of processing per sample (Herrero et al. 2014; Han et al. 2021). Accurate profiling at the species level is essential for studying root dynamics, ecosystem services, and weed-competitive traits (Mommer et al. 2008, 2011; Barneze et al. 2024). Furthermore, in a changing climate, it is essential to understand the role of roots in nutrient uptake, soil water utilization, and carbon cycling (Smit et al. 2000). Biological carbon sequestration in soil through plants shows tremendous promise for reducing human "carbon footprint" (Lal 2004).

Root biomass is typically determined by extraction, drying, and weighing, sometimes with image analysis, root separation, or species assignment (Gregory et al. 2009). Core-break, rhizotron, wax layer, monolith, and root washing/scanning methods are time-consuming and may underestimate root biomass by over a third, especially for fine roots, according to X-ray imaging and 13C tracer studies (Manske and Vlek 2002; Pierret et al. 2016).

Quantitative real-time PCR (qPCR) was applied to identify species diversity of roots in mixed samples (Mommer et al. 2008; Randall et al. 2014; Zeng et al. 2015). Riley and coworkers (2010) suggested specific qPCR probes for quantifying DNA from soil. The approach improved the efficiency of root studies by enabling direct quantification of species-specific DNA (Haling et al. 2011). Initial studies successfully used qPCR to quantify roots in greenhouse experiments. Recently, this method has also been applied in natural ecosystems (Izquierdo et al. 2019).

Here we report the use of ddPCR as a precise and efficient DNA-based approach for quantifying root biomass with botanical resolution, overcoming qPCR limitations such as sensitivity to PCR inhibitors and primer efficiency variability (Taylor et al. 2017). By partitioning the PCR into thousands of nanoliter droplets, each acting as an independent microreactor, ddPCR provides a digital output for absolute quantification using Poisson statistics. We hypothesized that ddPCR on the specific Internal Transcribed Spacer (ITS2) ribosomal barcode (Yao et al. 2010; Haling et al. 2011) DNA can enable DNA quantification from individual plant species. By measuring barcode DNA in complex soil samples, ddPCR allows for identifying specific species proportions and facilitates the estimation of total DNA content, and from this, the root biomass.

Results

We have devised a strategy (Fig. 1) based on the development of species-specific primer–probe sets (Fig. 1 A, Supplementary Fig. S1), the establishment of standard curves for the DNA/biomass correlation (Fig. 1 B-), and a workflow to prepare and process a DNA sample from a representative soil sample (Fig. 1 C-D). Soil sampling per se is outside our system boundaries, but we have

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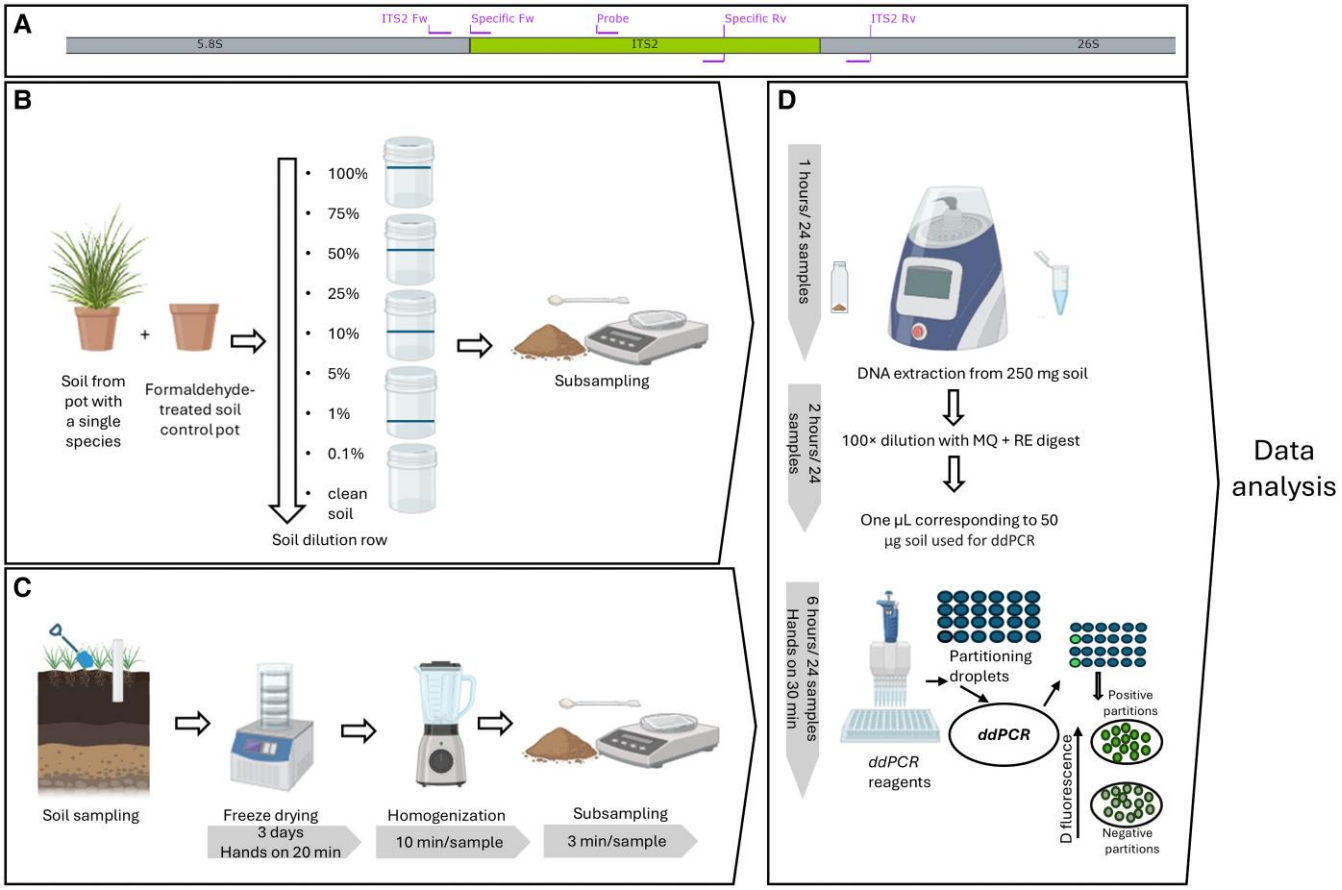

Figure 1. Outline of workflows and key concepts. **A)** Schematic representation of the ITS2 region with indication of universal primers, species-specific primers, and probe. **B)** Workflow to create a dilution series of roots in the soil matrix for the purpose of making a standard curve. **C)** Sample homogenization and subsampling to create small but representative subsamples. **D)** Soil DNA extraction and ddPCR analyses (receiving input from workflows **B** and **C)**.

worked on the assumption that most applications will involve samples >100 g to represent the heterogeneous distribution of roots. The samples are homogenized and subsampled (Fig. 1 C, 15-min hands-on pr sample). DNA is extracted from the subsample and quantified by ddPCR (Fig. 1 C, 9-min hands-on pr sample).

Root biomass measurement

Root biomass and density in the semi-field pot experiment were determined using conventional separation (Table 1). The measurements provided a reference for standard curves.

ddPCR primer-probes

Primer–probe sets for each of the 10 species were tested for efficiency and specificity. Positive controls were DNA purified from leaves and roots in a soil matrix. MilliQ (MQ) water was used as a negative control. Efficiency was defined by the clear separation of positive and negative groupings. Specificity was tested with mixtures of DNA from leaves of grasses, legumes, and forbs, respectively (Table 1). Nine of ten probes showed high specificity and similar signal amplitude between target DNA samples from leaves and soil, respectively (Fig. 2 A1–9).

The perennial ryegrass (*Lolium perenne*) specific primer–probe set reacted to pooled DNA from non-target grasses (Fig. 2 A10). Testing against individual grasses (Fig. 2 A11) showed that the signal originates from hybrid ryegrass (*Lolium hybridum*). This hybrid of perennial ryegrass and Italian ryegrass (*Lolium multiflorum*) likely exhibits cross-reactivity due to both parental ITS2 alleles. Reexamination of the amplicon sequencing chromatograms confirmed that alleles identical to perennial ryegrass are present in hybrid ryegrass (Supplementary Fig. S2). The below-threshold cloud associated with the *hybrid ryegrass* is likely derived from the Italian ryegrass ITS2 allele, differing from the probe's target sequence by only one nucleotide.

Standard curves

Standard curves were generated on the assumption that diagonal soil core subsamples are representative of a pot, enabling correlation between ddPCR- and separation-based quantification of root biomass. The standard curve data can be visualized as side-by-side 1D plots. A representative example with perennial ryegrass shows a soil dilution series containing perennial ryegrass roots, ranging from 100% to 0.1% (Fig. 2 A12–14), the remaining 1D plots for standard curves are in Supplementary Fig. S3. The negative control is MQ water. Negative and positive droplets remain consistent across dilutions, with positive droplet counts decreasing proportionally and reproducibly. The count of positive and negative droplets was converted to template molecule number, i.e. absolute quantification of the target species DNA in the sample, by Poisson statistics through the QX manager software. Template molecule number was correlated with biomass observed by weighing to produce standard curves (Fig. 2B). Eight of

Table 1. Plant species selected for probe development and additional species used to test probe specificity

Family	Species	Common name	Variety	Root biomass (g/L)
Poaceae	*Lolium perenne*	Perennial ryegrass	Arsenal	18
	Lolium hybridum	Hybrid ryegrass	AberEcho	17.5
	Dactylis glomerata	Cocksfoot	Donata	2.1
	Festuca arundinacea	Tall fescue	Swaj	16.9
	Phleum pratense	Timothy	Presto	18.9
	Festuca pratensis	Meadow fescue	Liherold	12.8
Fabaceae	*Trifolium pratense*	Red clover	Larus, Semperina, Taifun	6.8
	Trifolium repens	White clover	(X unknown), Munida, AberCrest	3.5
	Lotus corniculatus	Bird's-foot trefoil	Leo	1.8
	Medicago sativa	Lucerne (Alfalfa)	SW Nexus	0.93
	Trifolium hybridum	Alsike clover	Aurora	3.3
	Medicago lupulina	Black medick	Virgo	2.6
	Ornithopus sativus	Serradella	*NA*	1.6
	Trifolium incarnatum	Crimson clover	Heusers Ostsaat	1.7
Forbs	*Plantago lanceolata*	Plantain	*NA*	7
	Carum carvi	Carum	Rekord	1.4
	Achillea millefolium	Common yarrow	*NA*	1.4
	Cichorium intybus	Chicory	Spadona	4.8

The root biomass in the pot experiment was determined by conventional separation and formed the basis for standard curve development.

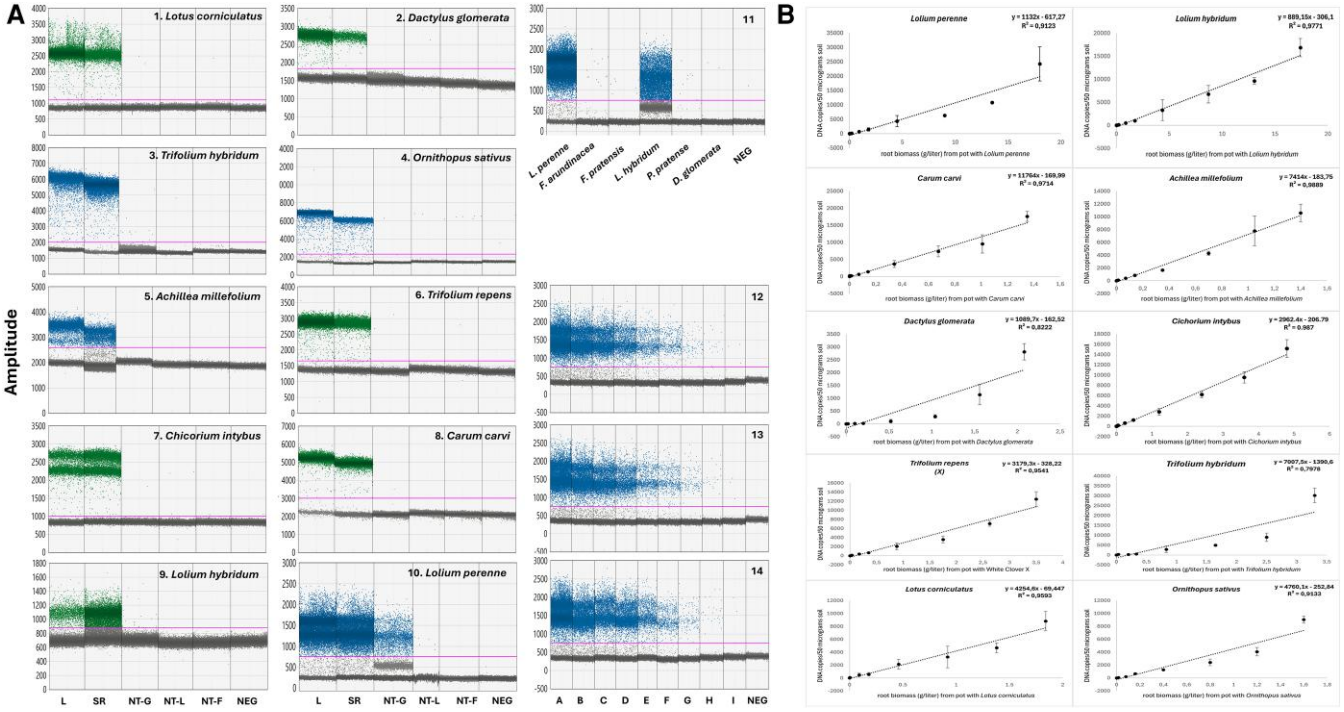

Figure 2. Validating probes and generating standard curves. **A)** ddPCR 1D plots showing (1–10) indicated species-specific probe design signal with samples: L: target species leaf-derived DNA, SR: target species soil matrix root-derived DNA, NT-G: non-target grasses, NT-L: non-target legumes, NT-F: non-target forbes and NEG: water negative control. The pink lines indicate thresholds for calling positive droplets, and colored points indicate the signal amplitude of individual droplets showing a FAM (blue) or HEX (green) signal. Gray droplets are counted as negative. Plot 11 is a test of the *L. perenne* probe against individual non-target grasses performed to identify the source of signal from NT-G in plot 10. Plots 12–14 illustrate the triplicate repetitions of the soil matrix dilution row used for the *L. perenne* standard curve. The percentage of soil from a pot with *L. perenne* is A: 100%, B: 75%, C: 50%, D: 25%, E: 10%, F: 5%, G: 1%, H: 0.1%, and I: 0% (soil negative control) NEG: water negative control. **B)** standard curves for the correlation between probe signal and target species root biomass. The trendline equation and coefficient of determination (r^2) are given in the top right corner of the diagrams.

ten standard curves had R^2 values >0,90 and six were >0,95 across 3 orders of magnitude soil dilutions.

Root biomass in species mixtures

The feasibility of ddPCR for species-specific root biomass quantification was tested using single-species and four-species mixed soil samples. DNA purified from single-species root samples was measured alone and in a combination containing DNA from four species in the proportions perennial ryegrass (10%), birdsfoot trefoil (*Lotus corniculatus*) (20%), white clover (*Trifolium repens*)(X) (30%), and common yarrow (*Achillea millefolium* (40%). The species-specific template count in the mixture was compared to the count in the original sample, which is expected to return the same percentage that the sample constitutes of the mixture. Two species

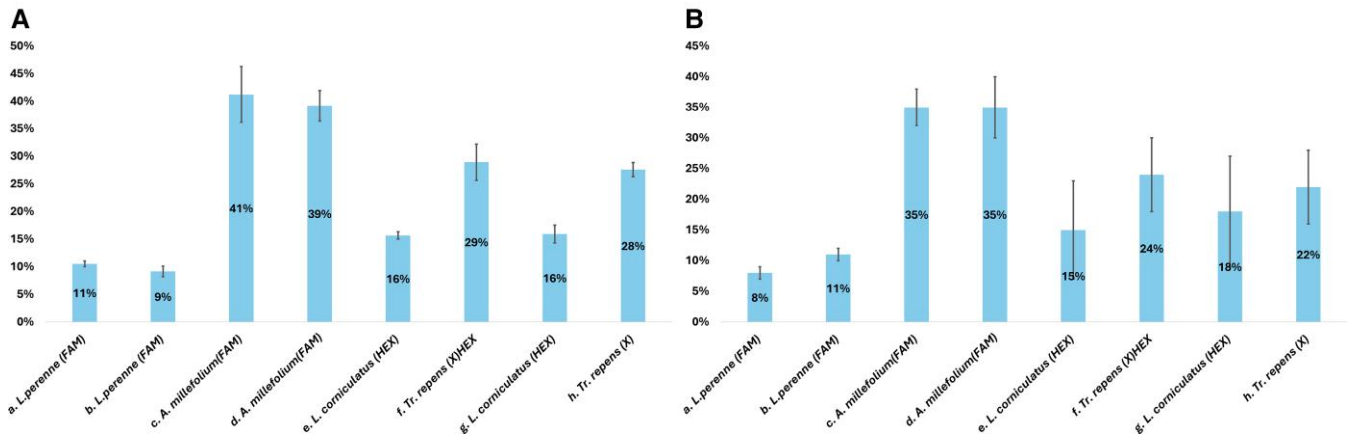

Figure 3. The histograms show the measured relative amounts of the target species *L. perenne* (expected 10%), *L. corniculatus* (expected 20%), *Tr. repens* (expected 30%), and *A. millefolium* (expected 40%) in complex mixtures. The measurements were performed in dual-plex reactions using one FAM-labeled and one HEX-labeled probe. Each target species was detected in the presence of 1 of 2 different companion probes (A + B and A + C). The probe pairs were a. *L. perenne* (FAM) (*L. perenne* FAM + *L. corniculatus* HEX), b. *L. perenne* (FAM) (*L.perenne* FAM + *Tr.repens* (X) HEX), c. *A. millefolium* (FAM) (*A. millefolium* (FAM) + *L.corniculatus* HEX), d. *A. millefolium* (FAM) (*A. millefolium* (FAM) + *Tr. repens* (X) HEX), e. L. corniculatus (HEX) (*L. perenne* FAM + *L. corniculatus* HEX), f. *Tr. repens* (HEX) (*L. perenne* FAM + *Tr. repens* (X) HEX), g. *L. corniculatus* (HEX) (*A. millefolium* (FAM) + *L. corniculatus* HEX), and h. *Tr. repens* (HEX) (*A. millefolium* (FAM) + *Tr.repens* (X) HEX). Panel **A** displays the results of a complex mixture of DNA purified from leaves and combined in the liquid phase, while panel **B** shows the same mixture proportions combined as soil with roots from which the DNA was subsequently extracted.

were quantified at the time using differentially labeled probes. Panel A of Fig. 3 presents the results of species-specific probe identification and quantification in these mixed DNA samples.

The perennial ryegrass probe showed expected biomass proportions of 11% when paired with the birdsfoot trefoil probe and 9% when paired with the white clover (X) probe. The common yarrow probe exhibited biomass proportions of 41% when paired with birdsfoot trefoil and 39% when paired with White clover (*X*). The birdsfoot trefoil probe showed proportions of 16% when paired with both perennial ryegrass and common yarrow, while the white clover probe demonstrated proportions of 29% and 28% when paired with perennial ryegrass and common yarrow, respectively.

Next, the experiment was performed with mixtures of soil with roots. Soil samples from individually grown pot plant species were processed as mentioned earlier, and soil was mixed in the proportions, perennial ryegrass (10%), birdsfoot trefoil (20%), white clover (X) (30%), and common yarrow (40%) for a total of 10 g. DNA was extracted from 250 mg of mixed soil. Figure 3, panel B shows the results of the mixed soil experiment. The perennial ryegrass probe approximated the expected percentage when paired with both primer–probe sets for (i) birdsfoot trefoil and (ii) *white clover* (*X*), 8% and 11%, respectively. The common yarrow probe paired with (iii) birdsfoot trefoil or (iv) *white clover* (X), showed 35% for both combinations. The *birdsfoot trefoil* probe when paired with (v) perennial ryegrass or (vi) common yarrow showed 15% and 18% respectively. *White clover* paired with (vii) *perennial ryegrass* or (viii) common yarrow showed 24% and 22% respectively.

Overall, the two experiments gave similar results, although with higher standard deviations for the samples mixed in the soil.

Discussion

This study aimed to develop a DNA-based method for root biomass determination and species profiling from soil. While qPCR uses amplification curves, ddPCR relies on endpoint measurements of an ultra-partitioned PCR. PCR efficiency is less critical as long as each partition is clearly positive or negative (Quan et al. 2018). We hypothesized that this would make ddPCR suitable for soil DNA samples. Nuclear DNA correlates with root biomass, and barcode sequences enable rapid amplification and sequencing. The ITS2 ribosomal region was chosen for species-specific primer–probe sets in ddPCR (Timpano et al. 2020). ITS2, a ribosomal RNA region between 5.8S and 28S rRNA genes, is widely used in genetic studies for species identification in seed plants (Qin et al. 2017). Eukaryotes contain tens to thousands rDNA copies, and this variation can influence DNA–biomass ratios across species, necessitating the experimental determination of these ratios. While concerted evolution typically homogenizes rDNA copies, the complexities of studying rDNA in hybrid organisms arise due to challenges related to gene similarity and recombination (Karlep et al. 2013). In a ddPCR context, we propose that a standard curve is used to relate a given primer–probe pair directly to the root biomass. Knowledge of rRNA gene copy number and ratio of ITS2 variants detected is therefore not a prerequisite. In total, 10 different species were included in the study. Thorough cross-check analyses with DNA samples from 18 perennial species, including the 10 we present probes, confirmed that it is possible to make specific probes amplifying the desired ITS2 target. One exception was hybrid ryegrass, which was detected by the perennial ryegrass probe. However, this is expected since hybrid ryegrass is a hybrid of perennial ryegrass and Italian ryegrass.

ddPCR signals from soil dilutions with known root densities correlated with biomass, demonstrating DNA extraction and detection reproducibility across concentrations spanning four orders of magnitude. The dependency of the DNA–biomass correlation on factors such as plant age and season (Haling et al. 2011) or root type (e.g. tap root vs fibrous roots, primary vs secondary roots) are issues that need further investigation, but they will apply to any DNA-based method. Finally, we used the method to analyze soil samples with roots from up to four different species. The study validated the approach as a method for root biomass determination and species profiling in a soil sample.

Our study demonstrates ddPCR as a powerful tool for biomass determination and species profiling in complex soil samples. The

high level of qualitative and quantitative accuracy observed in the current application on roots aligns with already established applications in, for example, pathogen detection, diagnostics, rare mutation detection, and gene copy number variation analysis, where the technology has already gained traction (Hou et al. 2023).

The ddPCR method requires an initial investment in the development and validation of primer–probe sets and corresponding standard curves. Here, probes were designed for 10 species, which can be applied in future research, and expanding the probe library over time will help fulfill the potential of the approach. However, primer–probe sets must be re-validated within the specific context of their application. Since probes are typically 18 to 30 base pairs long and highly sensitive to single-nucleotide polymorphisms, genetic variants within a species may go undetected, while non-target species not included in the original control group might be detected. Nonetheless, our study demonstrates that, in a well-defined and tested setup, primer–probe sets can achieve high specificity.

So far, specific probes have been successfully developed for all ten species using the ITS2 ribosomal barcode region. It remains to be seen whether this region has limitations for broader species application. The most well-known alternative is the ITS1 region (Letsiou et al. 2024). Barcode regions enable rapid sequencing and simplify specificity predictions, but primer–probe sets are not limited to these regions. With more plant genomes available, machine learning may enhance probe design through genomic comparisons.

To our knowledge, this technique surpasses existing methods for root biomass determination and species profiling in soil samples. Once established, it is user-friendly, generates large datasets, and supports multiplex species identification. This holds promising potential for selection and breeding programs aimed at increasing carbon allocation to root systems without compromising above-ground yield (Heinemann et al. 2023), even in mixed cropping systems like intercropping or grasslands. Additionally, the ongoing effort to develop improved tools for measuring, reporting, and validating soil carbon changes (Smith et al. 2020) could benefit from ddPCR's ability to quantify root carbon inputs across different cropping systems.

Materials and methods

Plant materials

Seeds of grasses, legumes, and forbs were sourced from DSV, Holstebro, Denmark.

ITS2 amplification and sequencing

Genomic DNA was extracted from leaves of the plants as described (Møller et al. 2003).

PCR was carried out using universal primers targeting the ITS2 region for grasses and modified primers for legumes and forbs obtained from (Timpano et al. 2020; Table 2) using Herculase II

Table 2. Sequences and key parameters of universal primers used for amplifying ITS2 sequences and of primers and probes for droplet digital PCR assays for grass–legume–forb species

Species	Variety	Target	Primer and probe sequences	Threshold	Amplicon length
Poaceae Grass family	Universal	ITS2(C)-Fw	5′-ATGCGATAC**C**TGGTGTGAAT-3′	N/A	375 bp
		ITS2(T)-Rv	5′-CCGCTTATT**T**ATATGCTTAAA-3′		
Fabaceae and herbaceous plant species	Universal	ITS2(T)-Fw	5′-ATGCGATAC**T**TGGTGTGAAT-3"	N/A	375 bp
		ITS2(G)-Rv	5′-CCGCTTATT**G**ATATGCTTAAA-3′		
Lolium perenne (Perennial ryegrass)	Arsenal	Fw	5′-GCTCCCACCCAACTAACT-3′	750	247bp
		Rv	5′-CTTAAATTCAGCGGGTAGTC-3′		
		Probe	6FAM -AGTGCCTC**T**GGCGCGTAG-BHQ1		
Lolium hybridum (Hybrid ryegrass)	Aber Echo	Fw	5′-GCTCCCACCCAACTAACT-3′	880	247bp
		Rv	5′-CTTAAATTCAGCGGGTAGTC-3′		
		Probe	HEX -TAAACGCAGTGCCTCCGG- BHQ1		
Dactylis glomerata (Cocksfoot)	Donata	Fw	5′-ATACTAAACGCAGTGCATCC-3′	1730	121bp
		Rv	5′-TTAAATTCAGCGGGTGGT-3′		
		Probe	HEX-ACATGATTGCCTCAATAGACCCTAGTAAC-BHQ1		
Trifolium repens (White clover)	X (unknown)	Fw	5′-ATAGGTGGTGGCTGTGTTAC-3′	1450	155bp
		Rv	5′-CCGCTTATTGATATGCTTAAA-3′		
		Probe	HEX-TGTGATGCTCTATTGAATTTAGGCCT-BHQ1		
Trifolium hybridum (Alsike clover)		Fw	5′-GTATTGTGCAGGGTGAATGT-3′	2000	187bp
		Rv	5′-TCTCATCACGAGCGTTTAG-3′		
		Probe	6FAM-TTACGCACGAGACCAAGTCATG-BHQ1		
Lotus corniculatus (Bird's-foot trefoil)		Fw	5′-AAATGCTTCGTGCTATGC-3′	1100	151bp
		Rv	5′-TGATAAGGTTACCCATGATTG-3′		
		Probe	HEX-ATGGTAGGGTATGCCATGATGG-BHQ1		
Ornithopus sativus (Serradella)		Fw	5′-CGATGCCATTAGGTTGAG-3′	2300	159bp
		Rv	5′-GGAAAACACATACCATGGAC-3′		
		Probe	6FAM-ATGAT**AC**TTGGGGCGAATGTTG-BHQ1		
Cichorium intybus (Chicory)		Fw	5′-CAACCTGCCTTCCTTATTG-3′	1000	174bp
		Rv	5′-ACACATTGGGGTCTTCATC-3′		
		Probe	HEX-TCGTGCGTCGTGAGCTGT-BHQ1		
Carum carvi Carum		Fw	5′-ACAAAAGTGAGTCTCCGATG-3′	2300	176bp
		Rv	5′-GCTTAAACTCAGCGGGTAG-3′		
		Probe	HEX-AACTTCTTGTCTTGTCACGTGAATCC-BHQ1		
Achillea millefolium (Common yarrow)		Fw	5′-GAACCATCGAGTTTTTGAAC-3′	2590	182bp
		Rv	5′-AACCTAGTTCGTGTGTCCAT-3′		
		Probe	6FAM-AAATATCTGTTGGGGGCGGATA-BHQ1		

The fluorophore labeling of the probes is indicated, as well as the threshold fluorescence amplitude used to call positive droplets.

(Agilent Technologies) according to the manufacturer's instructions and with 6% DMSO. Each 25-μL reaction included 5–10 ng plant genomic DNA. The PCR program was 35 rounds of a two-step cycle with annealing/elongation for 45 s at 53 °C or 55 °C for primer sets 1 and 2, respectively. The program had a final elongation step of 90 s at 72 °C.

Amplicons were purified using NucleoSpin Gel and PCR clean-up kit and were sequenced by Macrogen Europe, Amsterdam, the Netherlands, using the same primers as for the PCR.

Probes and primers

The ITS2 rRNA sequencing results were cross-checked with the NCBI GenBank database. All sequences matched the expected species with sequence identity ranging from 93% to 99%. The ITS2 sequences were aligned using the CLC Main workbench 23 (Qiagen), and probe positions were chosen by visual inspection of the alignment for unique sequence motifs. Species-specific hydrolysis probes with a predicted Tm of 63 °C were designed at the unique sites using the CLC workbench primer properties tool with the default settings. Probes were synthesized with either fluorescein (FAM) or hexachlorofluorescein (HEX) fluorophores and black hole quencher (BHQ) quencher, and amplification primers were designed to flank the probe as outlined in Fig. 1A using the Primer3plus program, aiming for an annealing temperature of 55 °C. All oligos were synthesized by Merck. The primer–probe sets are listed in Table 2 and illustrated graphically in Supplementary Fig. S1.

Root-soil samples

From May 3 to June 30, 2022, a semi-field pot experiment was established at Research Center Flakkebjerg, (55°19′26.6″N 11°23′23.9″E). Water was supplied through dripping tubes. Pots measured a diameter of 25 cm, height of 23 cm, and volume of 11 liters. The soil mixture consisted of 40% screened loam, 40% unfertilized peat moss, 10% sand, and 10% organic soil amendments. Pots were supplemented with 10 g fertilizer (Multicote 6 NPK 18–6 to 12 + micronutrients, Haifa group).

All 18 species, of which red and white clover, were represented by 3 varieties each were sown. For grasses, 30 seeds were sowed per pot. For legumes and forbs, 50 seeds were sown per pot.

Samples were collected after 60–70 days of growth. Above-ground parts were isolated using a scalpel. Soil samples for DNA extraction were collected from the root zone using a metal soil core cylinder (inner diameter, 6 cm; height, 23 cm) and immediately transferred to −20 °C. The remaining soil in the pots was collected, and the roots were thoroughly rinsed with tap water to remove soil particles. The roots were dried in a Jouan laboratory oven at 62°C for 2–3 days. The dried roots were weighed on an analytical balance. The soil cores were lyophilized (CoolSafe by LaboGene).

DNA-free soil was prepared by soaking the soil in 8% formaldehyde overnight. Formaldehyde was removed by two washes with MQ water each followed by centrifugation at 7000 g for 5 min and finally drying in a fume hood.

DNA extraction

The soil cores were processed in their entirety. Large roots were isolated from the samples and ground into powder using a Tube Mill (IKA). The ground roots were thoroughly mixed into the soil. After thorough mixing, samples were transferred to 20-ml containers, which were filled half with soil, and 10–12 glass beads were added to enhance homogenization in a SPEX 2010 Geno/Grinder set to 1500 strokes/min with the rate dial at 500. Each sample was processed twice, each for 150 s.

Soil DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Germany), with the protocol modification that samples were shaken in a FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Santa Ana, CA, USA) at a speed of 5.5 m/sec for 30 s across two cycles, with a 300-second pause between cycles, instead of the suggested vortexing.

Random distribution of template molecules among the droplets, was facilitated by *Pst*I restriction enzyme digestion of gDNA prior to ddPCR. *Pst*I does not digest the ITS2 sequence in any of the studied species. A preliminary dilution experiment indicated that a 100× dilution of the digested DNA was appropriate for both DNA from leaf and soil extractions. The digestion consisted of 17.5-μL undiluted DNA sample (10–100 ng/μL) in a total volume of 20 μL of cutsmart buffer with 0.5-μL *Pst*I enzyme. This digestion was carried out for 2-hrs at 37 °C.

ddPCR

Primer–probe sets were combined with 2× Supermix for Probes (no dUTP) (Bio-Rad Technologies). One microliter sample of DNA was used per reaction, which was processed to generate nanoliter droplets using the QX200 Automated Droplet Generator (Bio-Rad) and Automated Droplet Generation Oil for Probes (Bio-Rad).

PCR was performed in a C1000 Touch Thermal Cycler (Bio-Rad). The program was based on the manufacturer's recommendations with modifications according to primer Tm and amplicon length. The steps were 10 min, 95 °C; 39 cycles each consisting of 30-s denaturation at 94 °C, annealing for 1 min at 55 °C, and extension for 30 s at 72 °C. Each transition maintained a ramp rate of 2 °C per second for optimal primer/probe coordination. The cycling steps were followed by a final incubation for 10 min at 98 °C.

Standard curves

Standard curves were generated to correlate droplet counts to biomass. Core samples were used directly as the highest root density sample, whereas the remaining points were generated by diluting soil from the core samples with control soil. Dilutions were 75%, 50%, 25%, 10%, 5%, 1%, and 0.1% sample soil (Fig. 1).

Reactions were analyzed on a QX200 droplet reader, and data was collected and processed with QX Manager 1.2 Standard Edition software (Bio-Rad Technologies). Fluorescence thresholds for calling negative vs. positive droplets were established by visual identification of groupings.

Sequence data

All sequence data is presented in Supplementary Fig. S1.

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Author contributions

N.S. performed the research, analyzed the data, and wrote the paper. C.K.M.A. designed the research, analyzed the data, and wrote the paper. P.L.G. designed the research and wrote the paper. J.R. designed the research and wrote the paper. U.J. designed the research. H.B.P designed the research, supervised and wrote the paper.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Figure S1. Alignment of the ITS2 sequences isolated in the current study.

Supplementary Figure S2. Amplicon sequencing chromatogram aligned to the perennial ryegrass probe sequence.

Supplementary Figure S3. 1D amplitude plots of ddPCR assays conducted on soil samples containing DNA from ten plant species across a dilution series.

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Conflict of interest statement. We have no conflicts of interests or disclosures.

Data availability

The data related to this manuscript is presented in the manuscript and supplementary data. The corresponding author is available for further inquiries.

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