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METHODS PAPER

Measurement of belowground diversity of fine roots in subtropical forests based on a quantitative real-time PCR (qPCR) method

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Abstract
Background and aims Direct measurement of fine root diversity is very important to unveil belowground interaction, community dynamics and ecosystem functions in forests, but is limited by the effective method. This study attempted to develop specific primers for a quantitative real-time PCR (qPCR) method to determine diversity of fine roots and test whether this method could be applied in field forests.
Methods We used inter-simple sequence repeat (ISSR) analyses to develop the specific primers and applied the qPCR method to determine the belowground diversity of fine roots in soil samples collected from field tree clusters containing different species.
Results Specific primers were successfully developed to identify six tree species. The relative proportions of each species in known fine-root mixtures predicted by the qPCR method agreed well ($r^2 > 0.86$, $P < 0.001$) with the actual relative proportions of the corresponding species. All aboveground tree species in belowground fine-root samples were detected. But tree species richness and relative proportions of belowground fine roots predicted by the qPCR method differed from aboveground trees within a given cluster.
Conclusions Developing specific primers is the critical step in the qPCR method. The qPCR method can be used to determine belowground diversity of fine roots in forests.

Keywords Belowground diversity · Fine-root mixture · Multiple species forest · Relative proportions · qPCR · Species-specific primers

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Introduction

Belowground plant richness is an important component of plant community (Pärtel et al. 2012). Since fine roots (≤ 2 mm in diameter) of plants (Jones et al. 2011; Lawrence et al. 2012; Jacob et al. 2013) are responsible for water and nutrient uptake to sustain growth and reproduction (Valverde-Barrantes et al. 2014; Mommer et al. 2016), belowground interactions among diverse tree species growing in forests greatly affect the community structure and dynamics (Jacob et al. 2013; Xiang et al. 2015), carbon and nutrient cycling (McNickle et al. 2009; Bardgett et al. 2014; Valverde-Barrantes et al. 2015) and responses of ecosystem functions to global change (Brunner et al. 2001; Jones et al. 2011; Bardgett

and van der Putten 2014). Moreover, fine-root turnover and growth contributes substantially to soil organic matter inputs and productivity in forest ecosystems, respectively (Gill and Jackson 2000; Fukuzawa et al. 2013). Relative proportions of fine roots determine the contributions of a given species to the belowground biomass and production which intimately associate with ecosystem function (de Kroon et al. 2012). Therefore, the species richness and relative proportions of fine roots are critical indicators for understanding the belowground interactions for species assemblages (Schenk 2006; Price et al. 2012) and the mechanisms underlying the relationship between belowground biodiversity and ecosystem function (Jackson et al. 1997; Taggart et al. 2011; Bardgett et al. 2014).

Most previous studies of species diversity in fine roots were conducted in herbaceous plant communities in grasslands (Mommer et al. 2010; Kesanakurti et al. 2011; Hiiesalu et al. 2012, 2014; Lamb et al. 2016). However, the relationship of species diversity between the aboveground and belowground in forests was rarely investigated (Leuschner et al. 2009). In species-rich forests, aboveground tree stems are easily identified by leaf, flower and fruit traits, and the stem numbers, spatial distribution and production can be measured for each species (Rewald et al. 2012; Jones 2015). However, belowground fine root species are difficult to be identified visually according to morphological traits, particularly when fine roots are intermingled or entwined with each other (Mommer et al. 2011; Hiiesalu et al. 2012; Frank et al. 2015). Consequently, belowground interactions and the relationship between belowground biodiversity and functions are still poorly understood in forests containing diverse tree species (Jones 2015).

To identify tree species and to estimate the relative proportions of each species in the fine-root samples collected from the forests, more effective methods are required. Many studies have demonstrated that molecular methods are a reliable approach to distinguish the plant species of fine roots (Mommer et al. 2008; Fisk et al. 2010; Haling et al. 2011; Zeng et al. 2015). Universal primers are used for species identification, and commonly targeted regions include the *trn*L intron (Brunner et al. 2001; Fisk et al. 2010; Taggart et al. 2011; Zeng et al. 2015), the *rbc*L gene (Jones et al. 2011; Kesanakurti et al. 2011) and the *mat*K gene (Lahaye et al. 2008). Unfortunately, these regions often appear to exhibit insufficient variation (Brunner et al. 2001; Saari et al. 2005; Taberlet et al. 2007; Mommer et al. 2011) to allow discrimination of the species in diverse forests, especially some closely related species. Thus, multiple regions must sometimes be applied to discriminate species: Jones et al. (2011) have used the *trn*H-*psb*A and *rbc*L markers, and Taggart et al. (2011) have used three chloroplast (cpDNA) regions (the *trn*L intron, the *trn*T-*trn*L and *trn*L-*trn*F intergenic spacers) to identify grassland species. The universal-primer is a powerful tool to study the tree species richness, but it is required to select a large number of positive clones through screening technology for accurately estimating the relative proportion (Fisk et al. 2010; Zeng et al. 2015). Next-generation sequencing (NGS) technology, such as Illumina and Ion torrent, is another effective molecular method to identify the tree species (Fisk et al. 2010; Hiiesalu et al. 2012). But high cost, time-consuming procedures and special knowledge for data analysis keep this method from taking into application in most laboratories (Grada and Weinbrecht 2013).

Quantitative real-time polymerase chain reaction (qPCR) based on species-specific primers is a rapid and low-cost alternative method used to identify species and to predict species relative proportions in fine roots with high accuracy (McNickle et al. 2008; Mommer et al. 2008, 2011; Haling et al. 2011, 2012). The key to the qPCR method is to find specific primers that possess a sufficiently high resolution power to distinguish tree species in forest communities, though such primers are difficult to identify. In the future, as the number of DNA sequences submitted to the National Center for Biotechnology Information (NCBI) increases, the procedure for developing specific primers will undoubtedly become easier and quicker (Mommer et al. 2011; NCBI Resource Coordinators 2014).

In this study, we developed a qPCR method to identify tree species and to predict the relative proportions of each species in known fine-root mixtures prepared in the laboratory and to distinguish the tree species associated with fine roots collected from the field in Chinese subtropical forests. The objectives of this study were to (1) develop species-specific DNA primers for the qPCR method to identify tree species and predict the relative proportions of each species in known fine-root mixtures containing one to six species; and (2) test whether the qPCR method could be applied to investigate the belowground tree species richness and relative proportions in diverse tree species forests. We have successfully developed six specific primers for dominant tree species in subtropical forests. However, it is unknown whether

the qPCR method with the specific primers could be applied to investigate belowground diversity in field forests. Therefore, tree clusters consisting of different tree species (Leuschner et al. 2009; Jacob et al. 2013; Xiang et al. 2015) were elaborately selected to collect fine root samples for the test of qPCR method.

Materials and methods

Study site description

The fine-root samples were collected from forests located in Dashanchong Forest Farm (28°23′58″-28°24′58″ N, 113°17′46″-113°19′8″E) in Changsha County, Hunan, China. This area of the farm has a typical hilly topography, with an altitude ranging from 55 m to 217.4 m above sea level. This region has a humid mid-subtropical monsoonal climate. According to climate data obtained from 1954 to 2010, the annual minimum and maximum air temperatures are −10.3 °C and 39.8 °C, respectively, with an annual mean of 17.3 °C. The annual average precipitation is 1416.4 mm, and the minimum and maximum annual precipitation is 936.4 mm and 1954.2 mm (Xiang et al. 2015). The soil type is well-drained clay loam red soil developed from slate and shale parent rock, named AllitiUdic Ferrosol, according to Acrisol in the World Reference Base for Soil Resource (IUSS Working Group WRB 2006).

There are four principal forest types in the farm: (1) a monoculture *Cunninghamia lanceolata* plantation; (2) an early successional species-dominated mixed forest stand (dominant species: *Pinus massoniana* and *Lithocarpus glaber*); (3) a deciduous broadleaved specie-dominated mixed forest stand (dominant species: *Choerospondias axillaris*); and (4) a late successional evergreen broadleaved species-dominated forest stand (dominant species: *Lithocarpus glaber* and *Cyclobalanopsis glauca*). Stand characteristics of the four forests were reported in Liu et al. (2014) and Zhu et al. (2016). As succession proceeds, *Liquidambar formosana* is an important pioneer tree species at sites of destroyed natural forests or abandoned clearcut plantations. Thus, we chose six dominant tree species to develop species-specific primers and test whether the qPCR method could be used to predict the relative proportion of each species in known fine-root mixtures manipulated in the laboratory. The six tree species included two evergreen coniferous (*C. lanceolata* and *P. massoniana*), two deciduous broadleaved (*C. axillaris* and *L. formosana*), and two evergreen broadleaved (*L. glaber* and *C. glauca*) species.

Fine-root sampling for each tree species

To develop specific primers and the qPCR method for species identification and relative proportion estimation in the known fine-root mixtures manipulated in the laboratory, we collected fresh leaf and fine-root samples of the six tree species in August 2014. Because it is difficult to develop specific primers that need more leaf and fine-root samples, we also collected some leaf and fine-root samples in November 2014 and April 2015, as supplement when the experiment required. Two trees of each tree species were identified visually according to their morphological traits. Fresh leaf samples of each tree species were collected from the identified trees, placed in centrifuge tubes (10 ml), and stored in liquid nitrogen. For fine-root sampling, we excavated the root starting from the tree stem base and traced the root to the fine roots. The fine roots were dissected, and soil stuck to root was gently removed. The fine roots sampled were placed in centrifuge tubes (10 ml) and then stored in liquid nitrogen. All samples in tubes were transported to the laboratory and stored at −70 °C until analysis. Morphological traits (diameter, specific root length and specific root area) of fine roots for six tree species were presented in Table S1.

Experimental design and tree cluster selection for fine-root sampling

To address the second objective of this study, we applied the qPCR method to identify species and to predict the relative proportion of a species in the field. We selected 4 tree species *P. massoniana*, *C. axillaris*, *C. glauca* and *L. glaber* to form different tree clusters. According to tree clusters method designed by Jacob et al. (2013) and Xiang et al. (2015), the tree clusters contained trees of one, two, three or four different species and represented all 15 possible combinations of the four species along with a species diversity gradient (see Fig. S1). Specifically, there were 4 one-species tree clusters: *P. massoniana* (PM), *C. axillaris* (CA), *C. glauca* (CG) and *L. glaber* (LG); 6 two-species tree clusters: *P. massoniana*-*C. glauca* (PM + CG), *P. massoniana*-*L. glaber* (PM + LG), *C. glauca*-*L. glaber* (CG + LG), *C. glauca*-*C. axillaris* (CG + CA), *P. massoniana*-*C. axillaris* (PM + CA) and *L. glaber*-*C. axillaris* (LG + CA); 4 three-species tree clusters:

P. massoniana-C. axillaris-L. glaber (PM + CA + LG), *P. massoniana-C. glauca-L. glaber* (PM + CG + LG), *L. glaber-C. glauca-C. axillaris* (LG + CG + CA) and *P. massoniana-C. glauca-C. axillaris* (PM + CG + CA); and 1 four-species tree cluster: *L. glaber-P. massoniana-C. glauca-C. axillaris* (LG + PM + CG + CA). Each combination of tree cluster had 4 replicates and there were 60 tree clusters in total. The tree clusters are relatively closed (Fig. S1) and their sizes with average area of about 9.2 m^2 approximately reflect aboveground interactions ranges. Tree species, diameter at breast height (DBH, cm) and tree height (m) were recorded for each tree cluster (Table S2). In August 2015, soil samples were collected at the centre of each cluster by using a steel auger (10 cm in internal diameter) at soil depths of 0–10 cm, 10–20 cm and 20–30 cm (Fig. 1). Soil samples were removed from the auger, placed into plastic bags. A total of 60 tree clusters soil samples were collected and transported to the laboratory for further analysis.

In the laboratory, soil samples from depth 0–10 cm, 10–20 cm and 20–30 cm were pooled for each tree cluster. Fine roots were manually sorted from the soil samples with tweezers. Fine-root samples within 0–30 cm depth from each tree cluster were placed in plastic bags and stored at −70 °C until analysis. Concentrations of soil organic carbon (C) and total nitrogen (N) in tree clusters of different species diversity were presented in Table S3.

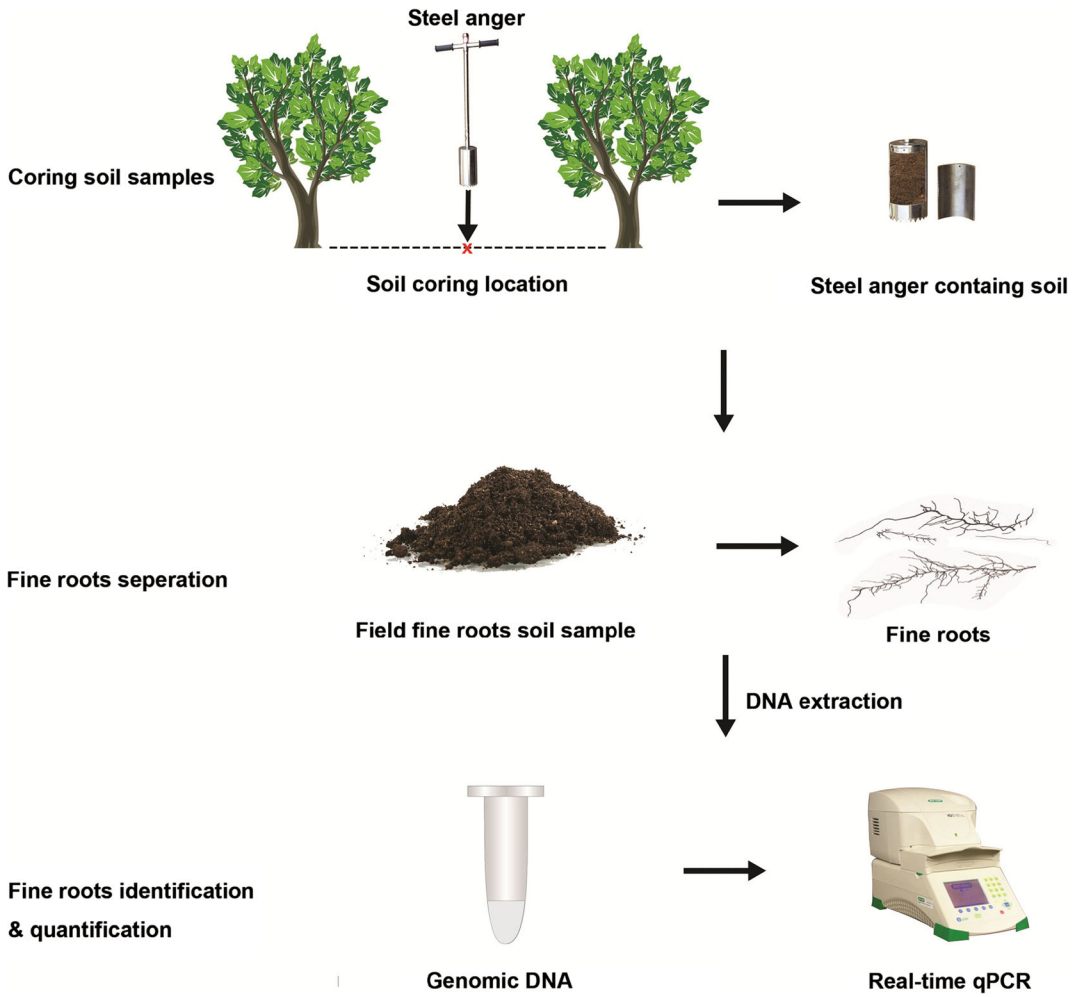

Fig. 1 Flowchart illustrating the determination of tree species and relative proportions in fine-root samples collected from tree-cluster plots containing one to four species established in three subtropical forests. The upper part shows the soil coring locations in the cluster plots. The lower part presents the procedures for the qPCR method to identify tree species and to quantify their relative proportions in the fine-root samples

Genomic DNA isolation

Leaf and fine-root samples were removed from the tubes with tweezers, rinsed with tap water to remove soil particles and dead materials, and then dried on filter paper. Each sample of individual roots was weighed to 0.1 g (fresh weight) and ground for 1 min in a mortar with liquid nitrogen. Each ground sample was collected in a sterile centrifuge tube (1.5 ml). DNA extraction was performed with a Plant DNA Kit (Tiangen Biotech (Beijing) Co., Ltd.), per the manufacturer's protocol. The DNA was then eluted into a sterile centrifuge tube (1.5 ml) in 70 μl of ddH_2O, measured on a NanoDrop-2000 (Thermo Scientific, Wilmington, DE, USA) to determine the DNA concentration, and stored at −20 °C for further study. The values of DNA purification measured by the ratio of absorbance at 260 nm and 280 nm ranged from 1.75 to 1.85, which was considered as standard for pure DNA (Psifidi et al. 2010). Therefore, this slight variation in DNA purification did not significantly affect the qPCR method.

To assess whether the collected individual fine-root samples were accurate, we used primers 5′-CGAA ATCGGTAGACGCTACG-3′ and 5′-GGGG ATAGAGGGACTTGAAC-3′ are from Taberlet et al. (1991) to amplify and sequence the plastid *trn*L(UAA) intron region in the leaf and fine-root samples of each tree species. Then, the plastid *trn*L(UAA) intron region sequences of the root and leaf samples of the same species were aligned with GENEDOC software. If the sequences of the fine roots completely matched those of the leaves, the species of the fine-root sample was accurately identified.

Primer design

To find species-specific DNA fragments to identify and quantify the fine-root species, ISSR analyses were performed using homogeneous templates. Of the 100 ISSR primers (UBC primer set no. 9, Biotechnology Laboratory, University of British Columbia) initially screened, 5 were chose to perform ISSR PCR which could produce highly discernible bands for the six species, including ISSR-811 (5′-GAGA GAGAGAGAGAGAC-3′), ISSR-815 (5′-CTCTCTCTCTCTCTG-3′), ISSR-818 (5′-CACA CACACACACACAG-3′), ISSR-835 (5′-AGAG AGAGAGAGAGAGYC-3′) and ISSR-848 (5′-CACA CACACACACACARG -3′). ISSR PCR reactions were performed on an Eppendorf Mastercycler in a total volume of 20 μl containing 8 μl Quick Taq™ DyeMix (TOYOBO Ltd.), 1 μl (10 μM) ISSR primer and 1.5 μl (20 ng μl^{-1}) of template DNA under the following conditions: 5-min denaturation at 94 °C and 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 52–58 °C, and 2 min extension at 72 °C, followed by a final extension at 72 °C for 5 min. For the six tree species, the PCR resulted in numerous bands on a 2.0% agarose gel in 1 × TAE run 80 V for 1.5 h. A large number of specific PCR products of each species DNA fragment were purified from the gel with a Gel Extraction kit (Tiangen Biotech (Beijing) Co., Ltd.) according to the manufacturer's instructions. The purified DNA fragments were then cloned into the pMD®18-T easy vector (TaKaRa Ltd.), per the manufacturer's directions. Next, the recombinant plasmids were transformed into DH5α *Escherichia coli* competent cells. Finally, positive clones were screened by PCR amplification and sequenced.

From the obtained sequences, primers were designed using Primer 5.0 designer software with melting temperatures (Tm) of 58–62 °C, primer lengths of 18–25 bp, and amplicon lengths of 70–250 bp. To test the specificity of each pair of primers, each pair of primers was run with all the different species on an Eppendorf Mastercycler; the annealing temperature for each species-specific primer set was fixed at 61 °C. The amplification programme was 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 61 °C, and 30 s at 72 °C, with a final elongation step of 5 min at 72 °C. The PCR products were detected via 1.0% agarose gel electrophoresis at 150 V in 25 min to ensure that only the corresponding species yielded specific amplification products and that no primer-dimers were found in any species. Primers sets were chosen for further study based on these results.

Rechecking the specific primers

To ensure the availability and specificity of each primer set, each set of primers was run with all the different species on a Bio-Rad iQ5 real-time PCR detection system using a SYBR Green Kit. Each measurement was performed in triplicate. Reactions were carried out in 20-μl volumes containing 10 μl of SYBR Premix Ex Taq (TaKaRa Ltd.), 0.1 μl (10 μM) each of primer and 1 μl (5 ng μl^{-1}) of template DNA. Reactions were run

using the manufacturer's recommended cycling parameters of 95 °C for 3 min, 40 cycles of 95 °C for 10 s and 61 °C for 30 s, and 95 °C for 1 min, and 55 °C for 1 min and 81 cycles to 55 °C for 10 s. Relying on the melting curve analysis, we verified the availability of each set of primers. qPCR is characterized by the point in time (or PCR cycle) at which the target amplification is first detected, and threshold cycle values (CT-values) are determined as the number of cycles required to amplify the DNA to a detectable level (Wong and Medrano 2005). From the CT-values, the each set of primers was tested again to ensure specificity for the corresponding species and no reaction from the other five species. Nonspecific primers were redesigned.

PCR inhibitors might be presented in the DNA extracted from fine root samples in field environment (Mommer et al. 2008, 2011). Therefore, it is important to monitor the run-to-run qPCR variation for the specific primers. Accordingly, DNA of fine roots for a species collected from a tree was run three times on one qPCR plate (96 wells). Then, DNA of fine roots for same species collected from another tree was run three times on a new qPCR plate. The CT values acquired by the qPCR method within the same species were compared to examine the run-to-run qPCR variation.

Determination of the relative proportion of each species in known fine-root mixtures

After the specific primers were verified by qPCR, we estimated the relative proportions of each different species in the known fine-root mixtures. Given the effect of root diameter on DNA quantification (Fisk et al. 2010), only fresh fine roots (≤ 2 mm in diameter) were chosen and mixed at different ratios to form different known fine-root mixtures. DNA was obtained from these known fine-root mixtures as described above. Each sample was run in triplicate with the above qPCR protocol. The first sample that we mixed contained six different species at a 1:1:1:1:1:1 fresh-weight ratio. This sample was prepared as a reference for the other mixed samples. To reduce the deviation, the 1:1:1:1:1:1 fresh-weight ratio sample was replicated four times. Each species was run a total of 12 times.

To determine the reliability of this method in predicting the relative proportion of each tree species in the known fine-root mixture, we created three types of known fine-root mixtures: a two-species mixture, a three-species mixture and a five-species mixture (Table 1). To ensure the reliability of the quantification, 36 known fine-root mixtures containing two species, 12 known fine-root mixtures containing three species and 6 known fine-root mixtures containing five species were used to estimate the relative proportions of each species. Each species in one mixed test sample was run 3 times. Thus, the CT-values from one mixed test sample were averaged 3 times values for a given species. On one qPCR plate, five samples could be tested simultaneously (5 samples × 3 replicates × 6 species). Given the amount of mixed samples, the quantification results were highly reproducible and provided a reliable basis for the field application.

Determination of species and relative proportion for fine-root samples in the field

We used the qPCR method to determine the species and relative proportion for fine-root samples collected from 60 tree clusters in the field. DNA was extracted from fine roots obtained from each soil sample by using the method described above. Using the six specific primer sets, each sample was run in triplicate through the qPCR protocol by using the aforementioned reaction to identify and quantify the fine-root species.

Data analysis

Data analysis was performed according to Mommer et al. (2008). We compared the species within each sample instead of comparing root samples. Thus, the CT value for each sample (CT sample) of a species was compared with the standard CT-reference value for the corresponding species. Because we used four replicates for 1:1:1:1:1:1 samples and each sample was run in three times, the CT-values of each species in the reference mixed samples were the average of 12 measured values for each species and served as the standard CT-reference values ($CT_{Species,reference}$) for each species.

The CT values for each species in one mixed test sample were averaged 3 times for the species and served as CT-sample values ($CT_{Species,sample}$). The relative proportion (%) for a species was estimated using the procedure proposed by Mommer et al. (2008):

$$\Delta CT_{Species} = CT_{Species,sample\ X} - CT_{Species,reference} \quad (1)$$

Table 1 Tree species combinations in known fine-root mixtures (in each row) prepared in the laboratory to determine species and relative proportions using the qPCR method

Species	*C. lanceolata*	*P. massoniana*	*C. axillaris*	*L. formosana*	*L. glaber*	*C. glauca*
Proportions (%)	0	20	20	20	20	20
	20	0	20	20	20	20
	20	20	0	20	20	20
	20	20	20	0	20	20
	20	20	20	20	0	20
	20	20	20	20	20	0
	0	40	0	60	0	0
	0	60	0	0	0	40
	40	0	0	0	0	60
	60	0	0	0	40	0
	0	0	40	0	60	0
	0	0	60	40	0	0
	0	0	0	40	0	60
	0	0	0	60	40	0
	0	40	0	0	60	0
	40	60	0	0	0	0
	60	0	40	0	0	0
	0	0	60	0	0	40
	60	0	0	0	0	40
	0	0	60	0	40	0
	0	0	40	60	0	0
	0	60	0	40	0	0
	40	0	0	0	60	0
	0	40	0	0	0	60
	0	0	40	0	0	60
	0	0	0	60	0	40
	60	40	0	0	0	0
	0	0	0	40	60	0
	0	60	0	0	40	0
	40	0	60	0	0	0
	0	80	0	20	0	0
	0	20	0	0	0	80
	80	0	0	0	0	20
	20	0	0	0	80	0
	0	0	80	0	20	0
	0	0	20	80	0	0
	0	0	0	80	0	20
	0	0	0	20	80	0
	0	80	0	0	20	0
	80	20	0	0	0	0
	20	0	80	0	0	0
	0	0	20	0	0	80
	10	0	10	0	80	0

Table 1 (continued)

Species	*C. lanceolata*	*P. massoniana*	*C. axillaris*	*L. formosana*	*L. glaber*	*C. glauca*
	80	0	0	0	10	10
	10	10	0	0	0	80
	0	80	0	10	0	10
	0	10	10	80	0	0
	0	0	80	10	10	0
	80	10	10	0	0	0
	10	80	0	0	10	0
	0	10	0	10	80	0
	0	0	0	80	10	10
	0	0	10	10	0	80
	10	0	80	0	0	10

$$\text{Estimated ratios of experimental sample } \left(\mathrm{ER_{Species}}\right) = 2^{-\Delta \mathrm{CTSpecies}} \quad (2)$$

$$\text{Estimated qPCR proportion } (\%) = \mathrm{ER_{Species}}/\mathrm{ER_{Sum}} * 100 \quad (3)$$

Regression analysis was used to compare the estimated proportion by qPCR for each species in the known fine-root mixtures and the actual proportion of fresh-weight fine roots that were mixed in the laboratory. The regression equation, degree of significance and coefficient of determination (r^2) were calculated for each tree species.

The aboveground relative proportion of each species was expressed as percentage of the basal area of the respective species to total of all trees (%). Thus, to test the application of the qPCR method for fine-root samples collected from different tree clusters in the field, the relative proportions of the aboveground for each species were calculated using the following formula:

$$\mathrm{Area_{Species}} = 3.14 * \mathrm{DBH}^2/4 \quad (4)$$

$$\text{Estimated aboveground proportion } (\%) = \mathrm{Area_{Species}}/\mathrm{Area_{Sum}} * 100 \quad (5)$$

The tree species identified and the relative proportions in fine-root samples predicted by the qPCR method were compared with the basal area proportions of aboveground stem of the corresponding species in each tree cluster. The statistical software R 3.2.0 (R Development Core Team 2015) was used to perform all data analysis and to produce figures.

Results

Species-specific primer development

The sequences of the plastid *trn*L(UAA) intron region matched completely between the leaf and fine-root samples of each species. Accordingly, specific DNA fragments were chosen by using ISSR technology. All positive clones were sequenced. The sequences for each tree species were used to perform BLAST searches of the NCBI database, but no corresponding information was found. The sequences of six tree species were submitted to NCBI and the BankIt No. and accession number were assigned to *C. lanceolata* (1955706, KX907334), *P. massoniana* (1959540, KX977402), *C. axillaries* (1959543, KX977403), *L. formosana* (1959536, KX977401), *L. glaber* (1959546, KX977404) and *C. glauca* (1959550, KX977405), respectively. On the basis of these sequences, six pairs of primers were developed (Table 2). Through PCR and qPCR, the specificity of the primers for the corresponding species was verified, and no other species were detected.

When compared the CT-values of the same species collected from different trees, standard errors of CT-values were very low (less than 0.2 for each species). This indicated that the run-to-run qPCR variation and PCR inhibitors have relatively low effects on our qPCR method results.

Table 2 The sets of species-specific primers for the six tree species screened in this study using the qPCR method

Species	Primer set	Forward (5′-3′)	Reverse (5′-3′)
C. lanceolata	ISSR-848	GCAAAAGTGGAATCAACGCTACA	AAGTTTTTCTCTTTCCTTCAGTCCC
P. massoniana	ISSR-835	CTTTGGAGGTTGGGTAAACTCTATT	CCTTTGCTATTGAGAGGTCACAGTT
C. axillaris	ISSR-818	CGCTACCGCAGGTGATGATT	CTGAGACCGAGAGAGAAAGAGAGTG
L. formosana	ISSR-815	TGTGTATTACTCCTATTTCGGTCC	TTGACCAGGGGTTGCTCTTAT
L. glaber	ISSR-811	CATATTGGCTAGGGGAAGAACT	GGCAACAACGAGATGACAGG
C. glauca	ISSR-815	AAAGTCGTTCGTCGCAGGT	TCTCACTCACAAGTCAGAACAAA

Relative-proportions estimates for each tree species in known fine-root mixtures

The qPCR results of the 1:1:1:1:1:1 fresh-weight ratio samples showed that the average species standard CT-reference values ($CT_{Species,reference}$) (± SE) calculated were different for the six species: 28.24 (± 0.28) for *C. lanceolata*, 27.93 (± 0.11) for *P. massoniana*, 26.53 (± 0.07) for *C. axillaris*, 24.08 (± 0.09) for *L. formosana*, 23.54 (± 0.07) for *L. glaber* and 25.19 (± 0.18) for *C. glauca*. The relative proportion of each species in the known fine-root mixtures in 54 test samples was also estimated. In the test samples, the estimated qPCR did not show any signal when the relative proportion of fresh weight for a given species was zero.

Linear regression models between the predicted relative proportion using the qPCR method and the actual proportion of fresh-weight showed significant correlations ($P < 0.001$) for the six tree species (Fig. 2). The regression lines of the six species were very close to the 1:1 lines, with regression slopes ranging from 0.863 for *L. formosana* to 1.034 for *C. lanceolata*. The highest coefficient of determination (r^2) was 0.894 (for *L. glaber*), and the lowest r^2 value was 0.863 (for *L. formosana*) (Fig. 2). However, the predicted proportion explained more than 80% of the variations in the actual proportion.

Species and relative proportion determination for fine-root samples collected in the field

The tree species of aboveground parts in 60 tree clusters were successfully detected using the qPCR method for the corresponding fine-root samples (Fig. 3). In addition, more tree species were found in the fine-root samples collected in several tree clusters than in those collected from the aboveground parts (Fig. 3). For one-species tree clusters, *P. massoniana* DNA was detected in the fine-root samples collected from the CG and LG clusters, and *C. glauca* DNA was detected in the fine-root sample collected from the CA cluster. For two-species tree clusters, *L. formosana* DNA was detected from the fine-root sample collected from the LG + CA cluster, and *C. axillaris* DNA was detected in the fine-root sample collected from in the CG + LG and PM + LG clusters, and *P. massoniana* DNA was detected in the fine-root sample collected from the CG + CA cluster. For three-species tree clusters, *L. formosana* DNA was detected from the fine-root sample collected from the PM + CA + LG cluster.

The relative proportions of fine roots for each tree species predicted by qPCR differed from the basal area proportions of aboveground stem of the corresponding species in a given tree cluster (Fig. 3). For one-species tree clusters, the relative proportions in fine-root samples predicted for *L. glaber* was 21.24%, which differed from that of the aboveground proportion in the LG cluster, whereas *P. massoniana*, which was not included in the aboveground proportion of the cluster, accounted for 78.76% of the fine roots. In the CG cluster, the relative proportion of *C. glauca* in the fine-root sample was 71.14% and that of *P. massoniana* was 28.85%. In the CA cluster, besides *C. axillaris*, the relative proportion of *C. glauca* accounted for 2.5%. For two-species clusters, in the PM + CA cluster, the aboveground proportion of *P. massoniana* was 69.4%, but its belowground proportion was 10.27%. For three-species clusters, the aboveground proportion of *P. massoniana* in the PM + CG + CA cluster was 20.3%, but its belowground proportion reached 48.01%. In the PM + CG + LG cluster, the aboveground proportion of *C. glauca* was 44.4%, but its belowground proportion accounted for 16.5%. In the PM + CA + LG cluster, the aboveground proportion of *L. glaber* was 20.12%, but its belowground proportion was 10.07%. For the four-species cluster, the aboveground proportion of

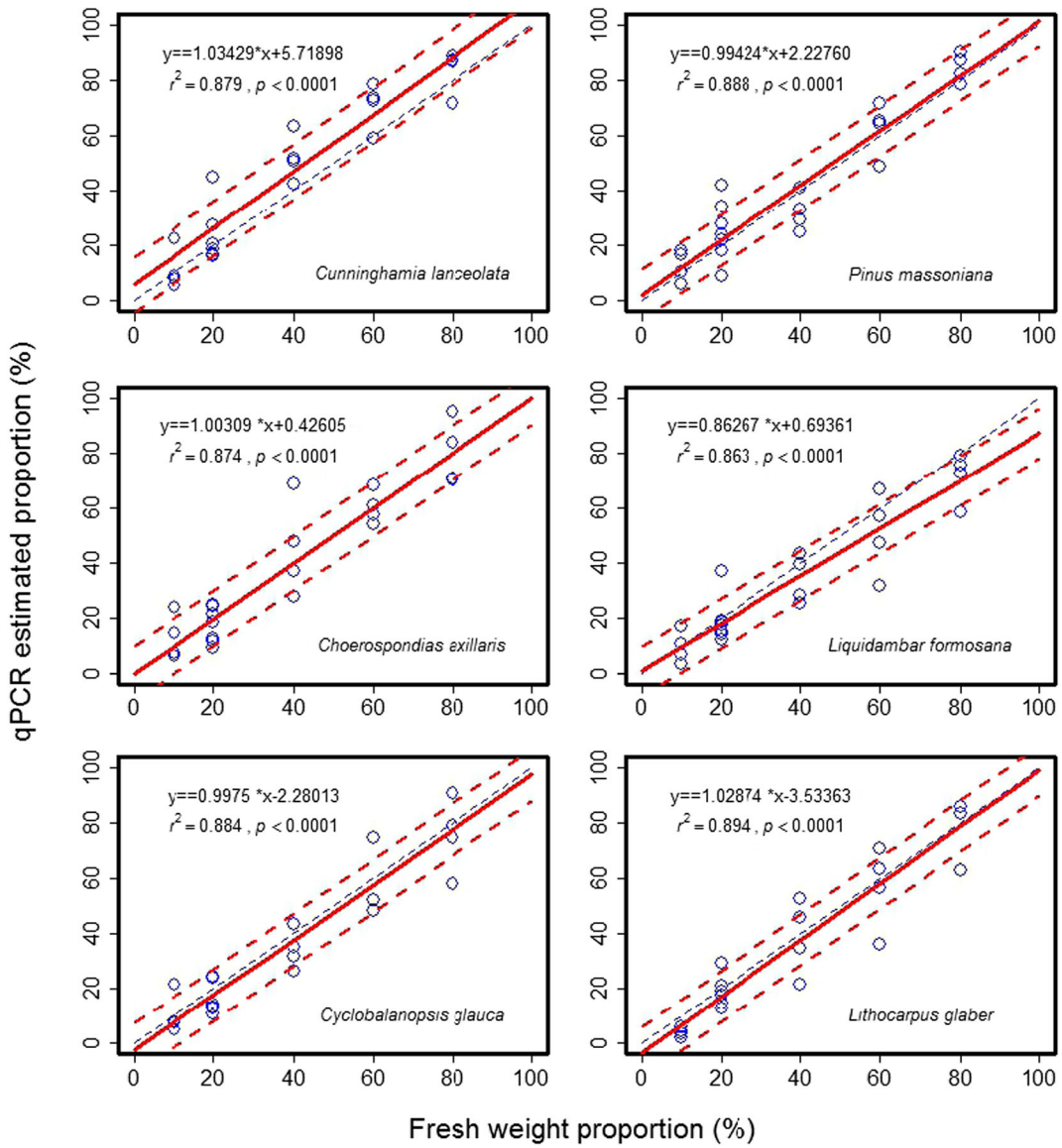

Fig. 2 Comparison of the proportion of a given tree species in known fine-root mixtures predicted by the qPCR method and the actual fresh-weight proportion in known fine-root mixtures prepared in the laboratory with two to six species. Empty blue dots represent the predicted proportions. The solid red line depicts the regression line for predicted versus actual proportions. Dashed red lines represent standard residual lines. The dashed blue line represents the 1:1 regression line

P. massoniana was 27.7%, but its belowground proportion reached 45.25% (Fig. 3).

Discussion

Specific primers development

Developing specific primers is the critical step for the qPCR method to determine the fine root in diverse tree species forests. To design the specific primers, we applied the ISSR method proposed by Mommer et al. (2008). Through ISSR technology, specific primers were successfully designed for six dominant tree species in subtropical forests, laying the foundation for the qPCR method to identify the species of fine roots. After blasted in NCBI, no sequence data were found to be corresponded to the DNA sequence amplifed using our specific primers. This result indicated that this is the first study to develop specific primers for belowground

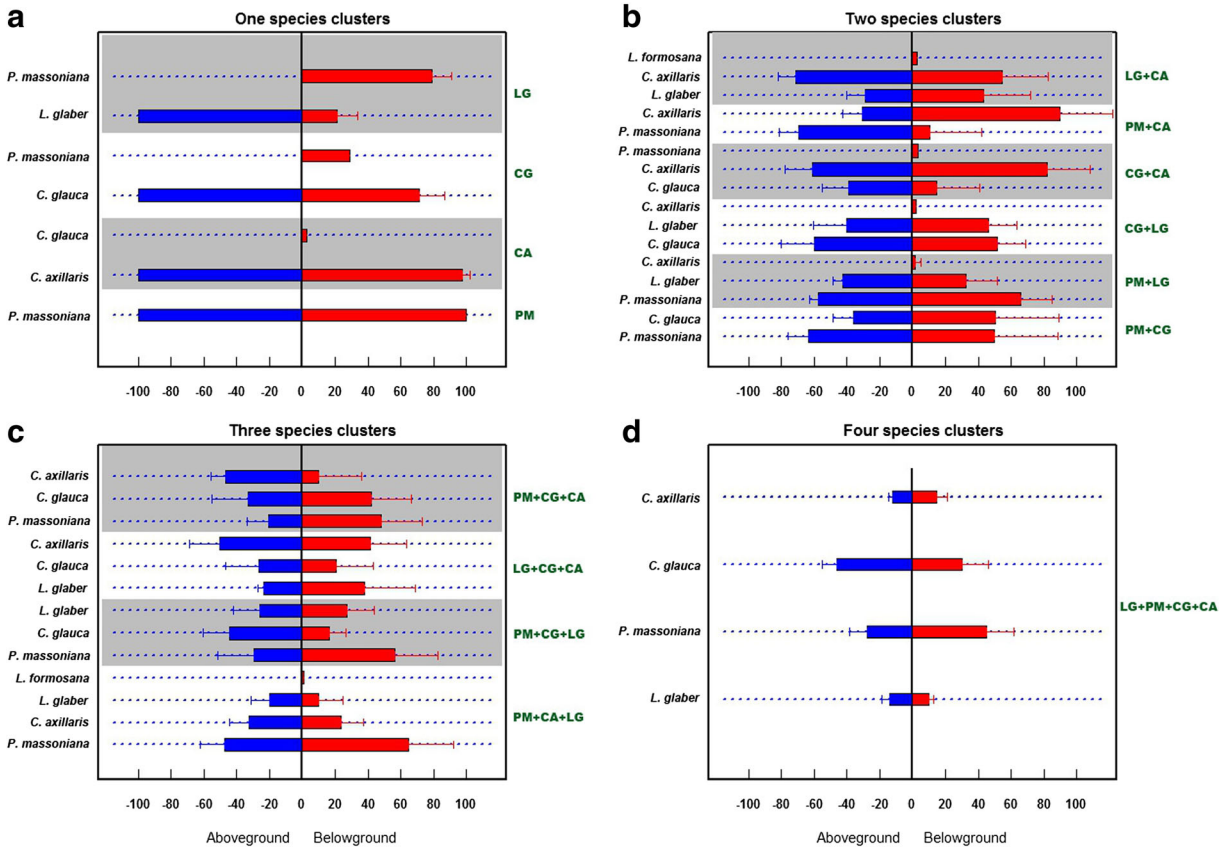

Fig. 3 Tree species and their relative proportions (mean ± SE) in aboveground individual stems (left blue bars) in the tree-cluster plots and the corresponding belowground fine roots (right red bars), as determined by the qPCR method. Tree-cluster plots investigated in the field consisted of one (**a**), two (**b**), three (**c**) and four (**d**) species

research in subtropical forests. Our present work may allow future studies to save the time and cost associated with developing specific primers for the six species.

Measurement of tree species and relative proportions in known fine-root mixtures

The qPCR method could successfully identify all the species in the known fine-root mixtures. In agreement with the result of Mommer et al. (2008), the relative proportion of each tree species predicted by qPCR method was significantly fitted 1:1 line with the actual fresh-weight proportion. This indicates that the qPCR method can be used to estimate the relative proportion of a given species in fine-root samples collected in subtropical forests consisting of many tree species.

The coefficient of determination (r^2) of the regression equation between the predicted and actual proportions varied among the tree species, with the highest values for evergreen broadleaved species and the lowest values for deciduous broadleaved species. However, the r^2 values were larger than 0.86, implying that the variability of the prediction is rather small for the different tree species.

Different morphological traits of fine roots among the six species (Table S1) could influence DNA content (Haling et al. 2011) and relative abundance estimate (Fisk et al. 2010). In this study, the extracted DNA concentrations in fine roots varied with tree species (230 ng.μl^{-1} for *C. lanceolata*, 234 ng.μl^{-1} for *P. massoniana*, 242 ng.μl^{-1} for *C. axillaris*, 246 ng.μl^{-1} for *L. formosana*, 260 ng.μl^{-1} for *L. glaber* and 240 ng.μl^{-1} for *C. glauca*). DNA content variation could be compensated through calculation eqs. (1) and (2), which normalised the estimated ratios of experimental sample with the standard CT-reference values (Pfaffl 2001). Therefore, a large number of replicates for the species standard CT-reference values calculation were extremely important to increase the reliability of the estimate (Mommer et al. 2008).

Measurement of tree species and relative proportions in the field forests

After the test for known fine-root mixtures in the laboratory, the qPCR method was applied to identify tree species in fine-root samples collected from field tree clusters consisting of one to four tree species. All tree species that appeared in the aboveground part of the tree clusters were detected in the fine-root samples. In addition, 8 combinations of tree clusters were found to contain more tree species than the number of corresponding aboveground tree species. There were *P. massoniana* trees around the CG, LG and CG + CA clusters, *C. axillaris* trees around the CG + LG and PM + LG clusters, *C. glauca* trees around the CA clusters, and *L. formosana* trees around the LG + CA and PM + CA + LG clusters (Zhao et al. 2015; unpublished data). The reasons might be that the breadth of belowground roots is much greater than the corresponding aboveground parts (Hawkes and Casper 2002; Schenk and Jackson 2002; Taggart et al. 2011; Hiiesalu et al. 2012), and the fine roots of other species outside the clusters can extend into the sampling region (Kesanakurti et al. 2011).

The relative proportions of tree species in the fine-root samples predicted by the qPCR method differed from the basal area proportions of aboveground stem of the corresponding species, thus indicating that belowground species and distributions are more complex and irregular and that aboveground species diversity does not necessarily mirror the belowground situation. The likely reasons are the unique foraging strategies (de Kroon and Mommer 2006) and imbalanced resource competition (Brisson and Reynolds 1994; de Kroon 2007; Bardgett and van der Putten 2014), which might lead to the high asymmetry between the aboveground and belowground regions. These results indicated that the belowground tree species diversity could not be totally reflected the aboveground parts (Hiiesalu et al. 2012; Frank et al. 2015) and it is important to develop an effective method to identify and quantify the belowground tree species.

In this study, we only developed specific primers for six tree species in subtropical forests. Thus, the limitation is that this method is suitable for the forests containing these six species but could not be applied to the forests with other species beyond these six species. NGS is an effective method to obtain a large number of sequences which could help to find the specific primers more easily (Csencsics et al. 2010). Therefore, with the NGS technology development, the application of the qPCR method will extend to the forests contained a larger number of tree species.

Conclusions

Developing specific primers is the most important step in the qPCR method. In this study, we developed specific primers for six dominant tree species in subtropical forests. Our successful application of the qPCR method based on specific primers in the laboratory and field indicates that the qPCR method is an effective approach to investigate belowground fine-root diversity in subtropical forests. In the field study clearly demonstrates that the aboveground diversity did not mirror the belowground parts. This implies that developing an effective method is critical to determine the relative proportions of fine roots in forests contained diverse species. The NGS technology development can help to find more specific primers easily and the qPCR method has a high potential to unveil the belowground diversity in forests.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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