Variation in the functional traits of fine roots is linked to phylogenetics in the common tree species of Chinese subtropical forests

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Cong Liu · Wenhua Xiang (ORCID) **· Limei Zou · Pifeng Lei · Yelin Zeng ·
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Abstract

Aims The phylogenetic variations of fine root traits, which are related to plant growth and development as well as to physiological and ecological processes, are not fully understood. This study aimed to: (1) examine how tree species and sampling methodology affect the anatomical, morphological and nutrient traits of fine roots; and (2) determine whether phylogenetic signals affect fine root trait relationships and influence comparison of root traits between the branch order-based and diameter-based cut-off sampling categories.

Methods Fine root samples of 16 subtropical forest tree species were obtained and their anatomical, morphological and nutrient traits were studied. The phylogenetic signals of trait variations were calculated to determine trait relationships.

Results Tree species and sampling methodology significantly affected fine root traits ($p < 0.05$). Mean root diameters, root tissue density (RTD) and carbon-to-nitrogen ratio were the lowest in the first-order category and highest in the ≤ 2 mm category. The reverse pattern was found for specific root length, specific root area and nitrogen concentration. Morphological traits showed significant phylogenetic signals; however, nutrient traits did not reflect phylogenetic conservatism. Phylogenetic factors influenced correlations between traits for the first-order root economics spectrum. Root traits were multidimensional and RTD was loaded on a novel phylogenetic principal component analysis.

Conclusions Functional traits of fine roots are multidimensional for subtropical tree species and closely linked to phylogeny. Morphological traits of first order roots showed a much stronger phylogenetic signal than those of roots ≤ 2 mm (traditionally defined fine roots). The findings improve understanding of root trait strategies in response to environmental changes.

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Introduction

The anatomical, morphological and physiological traits of plants have evolved to adapt to the environments in which they grow (Chandler and Bartels 2008). Trait-based approaches have increasingly been used to investigate how evolutionary and environmental changes affect resource-use strategies and habitat preferences among plant species. However, most previous studies have focused on aboveground plant traits (Reich et al. 2003; Bardgett et al. 2014). Fine roots are the most active and plastic organ with which trees obtain soil nutrients and water (Eissenstat and Yanai 2002; Pregitzer 2002), and play a key role in carbon (C) budgets and biogeochemical cycles in forest ecosystems (Jackson et al. 1997; Norby and Jackson 2000; Clemmensen et al. 2013). The functional traits of fine roots exhibit plasticity in trade-offs between resource acquisition and conservation in response to environmental variations (Comas et al. 2012). Therefore, elucidation of the anatomical, morphological and physiological traits of fine roots within an architectural system are critical for understanding plant response to variability in the availability of soil resources and driving ecosystem processes (Reich 2014; Kong et al. 2016).

Fine roots are usually defined arbitrarily based on diameter (i.e., ≤ 2 mm) (Wells and Eissenstat 2001; Pregitzer et al. 2002). However, this definition ignores the functional roles of fine roots relative to their various orders or comparative positions within the root system because, depending on root order, fine root traits and physiological processes vary dramatically and in a non-linear fashion (McCormack et al. 2015). Over the past two decades, many studies have discussed fine root definitions and classification methods, which can be generally categorized into three types: (i) classification based on diameter, i.e. lower than a specific threshold (e.g. ≤ 0.5 mm, ≤ 1 mm or ≤ 2 mm) (Liu et al. 2010a; Valverde-Barrantes et al. 2013); (ii) segmenting fine roots based on root order system (Guo et al. 2004, 2008b; Kong et al. 2014) and (iii) the classification of functional modules (i.e., absorption or transportation) (McCormack et al. 2015). Each type has its own advantages and disadvantages. The diameter-based cut-off approach can be effective within species. For example, a recent research paper summarized an analytic modeling method with which to determine a precise specific diameter threshold (0.3 mm and 1 mm) for elucidating fine root function in *Fagus sylvatica* (Montagnoli et al. 2018).

However, the diameter-based approach is not a suitable method for the comparison of traits among species (Valenzuela-Estrada et al. 2008; McCormack et al. 2012; Kong et al. 2014). Functional classifications are generally intended to mirror the order-based system, because this style of approach is more widely appropriate in field campaigns on fine root traits across species (McCormack et al. 2015). Numerous studies have classified first- to third-order roots as the absorptive functional group (Comas et al. 2002; Holdaway et al. 2011; Liao et al. 2014) while the four- and fifth orders were responsible for transportation because of significant secondary development (Guo et al. 2008b). It is important to note that the relationships between fine root traits, and their functions, are generally affected by fine root definitions and their corresponding sampling categories (Freschet and Roumet 2017). We should consider various fine root sampling criteria and take various relevant factors, including sites and vegetation types, into account in order to design suitable methodology; this would enhance the comparability of fine root functional traits (McCormack et al. 2015; Freschet and Roumet 2017).

Numerous studies have emphasized significant differences in many fine root traits within the root branching hierarchy. Smaller diameter or lower order (most distal or first- to third orders) roots exhibit high specific root lengths (SRL), nitrogen (N) concentrations and respiration rates, but low C storage, carbon-to-nitrogen (C/N) ratios and shorter life-spans (Eissenstat et al. 2000; Pregitzer et al. 2002; Guo et al. 2004; Hishi and Takeda 2005). In contrast, higher orders (typically including four- and fifth orders; Guo et al. 2008a, b; McCormack et al. 2015) are longer lived; however, mycorrhizal colonization is reduced or even disappears. Root anatomy is closely related to physiological activities (Hishi 2007; Guo et al. 2008b; McKenzie and Peterson 2015). Different tissue developmental features can be observed on the cross-cut structure of primary and secondary roots (Peterson et al. 1999; Guo et al. 2008b). Primary roots, including cortical tissue and primary vascular tissue, are susceptible to mycorrhizal infection and are essential for the entry of water and nutrients into transport tissue (Peterson et al. 1999; Wells and Eissenstat 2003). The secondary growth of roots is a complex process involving activities of vascular/cork cambium and formation of secondary xylem/phloem and cork cells (Peterson et al. 1999). Secondary roots have low absorption capacity, strong transport capacity and resistance to external stress, and

are mainly responsible for carbon and energy storage (Wells and Eissenstat 2003; Hishi 2007).

Cross-species analyses of the functional traits of plants have unraveled trade-offs associated with life-history acclimatization and resource economy strategies. For example, the leaf economics spectrum (LES) is used to describe a universal pattern of ecological strategies by which to acquire and retain multiple limiting resources (Wright et al. 2004). In general, investment in "inexpensive" leaves promotes fast growth but a short lifespan, while long-lived leaves are costly and offer low return. For example, plants with leaf traits of high specific leaf area, low tissue density and high N and phosphorus (P) concentrations generally support a high photosynthetic efficiency (Wright et al. 2004; Osnas et al. 2013; Collins et al. 2016). Furthermore, plants are complex organisms that must synchronize between aboveground and belowground resource capture strategies. It is assumed that fine roots support an analogous economics spectrum that supports aboveground leaves, with a close correlation between morphological, physiological and nutrient traits, thus highlighting a trade-off between resource acquisition and conservation (Wahl and Ryser 2000; Lavorel et al. 2007; Roumet et al. 2016). For example, for fine roots that are thinner in diameter, lower in root tissue density (RTD) and higher in SRL, branching intensity and nutrient concentrations generally enable rapid resource acquisitive strategies with shorter root life-spans (Roumet et al. 2006; Holdaway et al. 2011; McCormack et al. 2012; Collins et al. 2016). At the opposite end of the spectrum, a thicker root diameter and higher root C/N are indicative of resource conservation (Prieto et al. 2015; Fort et al. 2016; Weemstra et al. 2016). However, sufficient evidence related to the "root economics spectrum" (RES) among woody species has yet to be obtained to substantiate this assumption (Comas and Eissenstat 2009; Hobbie et al. 2010; Chen et al. 2013).

Leaf traits are only weakly phylogenetically structured and evolutionarily labile (Baraloto et al. 2012; Flores et al. 2014), while root traits appear to have a higher capacity for phylogenetic conservatism (Milla and Reich 2011; Gu et al. 2014; Kong et al. 2014). Studies have shown that the morphological traits of absorptive roots exhibit greater variability than nutrient traits among species, and stronger phylogenetic signals than the morphological traits of leaves (Kong et al. 2014; Valverde-Barrantes et al. 2015). Thus, we predict

that phylogenetic effects may contribute to the higher similarity of fine root traits among closely related species than those that are distantly related. Comparative analyses that exploit phylogenetic relationships among species across an independent root branching architecture have been used to quantify variations and trade-offs in fine root functional traits (Valverde-Barrantes et al. 2013; Kong et al. 2014; Roumet et al. 2016). Compared with LES syndromes, phylogenetic relationships have far more rarely been reported to be a factor by which to explain RES. Recently, limited data that relate to the wide variation in root traits among existing species have been published, thus reflecting evolutionary patterns (Comas et al. 2012). Because root trait variations among species are extremely high, the incorporation of phylogenetic affiliations and natural selection provides a reference for forecasting how belowground processes respond to global change.

The subtropical region of China is a typical ecological transitional zone that is sensitive to global change. Native forests are composed of both ancient and modern plant species that belong to diverse phylogenetic lineages. However, few studies have examined differences in the functional traits of fine roots among species in this subtropical zone, especially as it pertains to the different fine root sampling categories. Variations in the functional traits of fine roots among tree species in the subtropics and tropics are higher compared with those in the temperate zone, and different sampling methodologies capture different functional root characteristics (Chen et al. 2013; Kong et al. 2014; Gu et al. 2014). Thus, the application of phylogeny and the comparison of fine root sampling categories across species can advance our understanding of coevolutionary mechanisms related to the physiological and functional traits of plants in this region.

In this study, we compared the functional traits of fine roots among 16 subtropical tree species according to the branch order-based classification, and the diameter-based cut-off method (i.e., fine roots of the first-order; first-three-orders; ≤ 1 mm and ≤ 2 mm in diameter). The objectives of this study were to: (1) examine how tree species and sampling methodology affect the anatomical, morphological and nutrient traits of fine roots; (2) investigate the relationships between anatomical traits, morphological traits and nutrient concentrations across tree species; and (3) determine whether phylogenetic signals affect fine root trait relationships and influence comparison of root traits between the branch order-based and diameter-based cut-off sampling categories.

Materials and methods

Study site and species selection

This study was conducted in the Dashanchong Forest Park (latitude 28°23′58″–28°24′58″ N, longitude 113°17′46″–113°19′08″ E), Changsha County, Hunan Province, China. This park belongs to a typical low hilly area with an altitude ranging from 55 to 217 m above sea level (MASL). The climate is characterized by a mid-subtropical monsoon climate. The mean annual air temperature is 17.3 °C, with a maximum monthly mean temperature of 30.0 °C in July and a minimum monthly mean temperature of 5.5 °C in January. Annual precipitation ranges from 936.4 mm to 1954.2 mm, and mean annual precipitation is 1416.4 mm, mainly occurring from April to August. The soil type is well-drained clay loam red soil developed from slate and shale parent rock, classified as Alliti-Udic Ferrosol according to Chinese Soil Taxonomy and Acrisol according to the World Reference Base for Soil Resource (IUSS Working Group WRB 2006; Liu et al. 2014; Xiang et al. 2015).

Fine root samples were obtained from a *Cunninghamia lanceolata* monoculture plantation established in 1966 and from regenerated secondary forests after firewood collection was outlawed in the late 1950s. We selected 16 dominant tree species with contrasting phylogeny and root architecture that covered different taxonomic (angiosperms versus gymnosperms) and foliage types (evergreen versus deciduous) (Table S1). The species included two coniferous gymnosperms, namely, *C. lanceolata* (Taxodiaceae) and *Pinus massoniana* (Pinaceae), five deciduous broadleaved angiosperms, namely, *Liquidanbar formosana* (Hamamelidaceae), *Choerospondias axillaris* (Anacardiaceae), *Liriodendron chinense*, *Michelia alba* (Magnoliaceae) and *Sassafras tzumu* (Lauraceae), and nine evergreen broad-leaved angiosperms, namely, *Cyclobalanopsis glauca*, *Lithocarpus glaber* (Fagaceae), *Manglietia insignis*, *Manglietia chingii*, *Michelia maudiae*, *Michelia chapensis*, *Michelia figo*, *Parakmeria lotungensis* (Magnoliaceae) and *Cinnamomum camphora* (Lauraceae).

Root excavation and preliminary processing

Fine root sampling was conducted in July 2013 during the highest period of aboveground and belowground tree growth (Liu et al. 2014). For each species, samples were obtained from four trees (one individual from each of the four selected trees) by manual excavation within a 2 m distance of the tree stem (trunk) and at a soil depth of 0–20 cm. Intact roots, including more than five branch orders, were collected by cutting after soil had been removed. The roots were handled with great care to avoid breakage. The collected root samples were divided into two subsamples: one was placed in a plastic bag with moistened paper, labeled and instantly placed on ice before being transported to the laboratory within a timeframe of two hours, then frozen for subsequent dissection and morphological analysis. The other subsample was washed lightly in deionized (DI) water, followed by immediate fixation in a formalin-aceto-alcohol (FAA) solution (90 ml 50% ethanol, 5 ml 100% glacial acetic acid, 5 ml 37% methanol) for anatomical analysis.

Root dissection

Before dissection, root segments were placed in DI water (1 °C) to prevent drying. Soil particles clinging to the samples were carefully removed with forceps and the roots were rinsed clean with DI water, according to protocols described by other studies (Pregitzer et al. 2002; Guo et al. 2004; Erktan et al. 2018). Cleaned root segments were then dissected into different orders according to the procedure described by Pregitzer et al. (2002) and Guo et al. (2004, 2008b), with the most distal roots regarded as the first-order (Fitter et al. 1991). Given that a certain proportion of the higher-order root diameter was greater than 2 mm, we included only the first five orders, and only live fine roots from trees were analyzed in this study. This was because the diameter of first-order roots in some Lauraceae and Magnoliaceae species may be thicker than 0.5 mm (Valenzuela-Estrada et al. 2008; McCormack et al. 2012; Kong et al. 2014). Consequently, we selected four sampling categories: fine roots of the first-order; first-three-orders; ≤ 1 mm and ≤ 2 mm in diameter.

Root morphology and nutrient concentration measurements

After dissection, fine root segments of each subsample were placed in a transparent tray (20 cm × 15 cm) filled with DI water to facilitate root spreading. The segments were then scanned by order in grayscale at 400 dpi with a

1 mm filter using an automatic threshold method (Expression 10000XL, Seiko Epson Corporation, Nagano, Japan). The scanned images of each segment allowed us to measure root morphological traits using WinRHIZO Pro 2009 software (Régent Instruments Inc., Quebec, Canada). Morphological traits included mean root diameter (mm), total root length (mm^{-2} of soil), total root surface area (m^2 m^{-2} of soil) and total root volume (cm^3 m^{-2}). After morphological analysis, the dry biomass of roots was determined by drying at 70 °C until a constant weight was obtained (0.0001 g). Image analysis and mass were used to calculate SRL (mg^{-1}), specific root area (SRA) (m^2 kg^{-1}) and RTD (g cm^{-3}).

Each fine root segment was then cut into smaller pieces and finely ground with a ball grinder (Mixer Mill MM 400, Retsch GmbH, Germany) for chemical analysis. Total C and N concentrations were determined using a dry combustion method with a macro elemental analyzer (Vario Max CN, Elementar Analysensysteme GmbH, Hanau, Germany).

Anatomical root assessments

The complete root systems of another subsample, fixed in FAA solution, were dissected into individual orders. For each species, sixteen segments were randomly selected for each order (from first to fifth). Cross sections of fine root material were obtained by using the paraffin sectioning method. After gradient alcohol dehydration (alcohol concentrations of 70%, 85%, 95% and 100%), safranin-fast green staining, wax-impregnation (using xylene as a drying solvent at 45 °C in an oven for 20 h), and paraffin embedding, tissues were cut into thin sections, and dewaxed to achieve transparency. Finally, 10 μm thick sections were prepared for analysis. For each root segment, three cross-sections were selected and photographed under a compound microscope (BX51; Olympus Corporation, Tokyo, Japan) to measure cortex thickness and stele diameter to the nearest 1 μm (Guo et al. 2008b).

Phylogenetic reconstruction

A molecular phylogeny map was generated for the 16 tree species in this study. For each species, we conducted a National Center for Biotechnology Information (NCBI; Bethesda, Maryland, USA) search of three sequence regions: *rbcL*, *matK*, and *trnH-psbA*, which have commonly been used in published phylogenies (Pei et al. 2011; Swenson et al. 2012). A DNA supermatrix was established following the methods described by Kress et al. (2009) and was then analyzed through RA × ML via the CIPRES supercomputer cluster to construct a phylogenetic tree using the maximum likelihood (ML) method (Stamatakis 2006). The divergence times were estimated using divergences in DNA sequence data and the molecular clock was implemented in r8s version 1.70 (Sanderson 2003) using the nonparametric rate smoothing (NPRS) approach to obtain a chronogram tree (Fig. 1 and Fig. S2).

Data analysis

Two-way analysis of variance (ANOVA) was used to examine the effects of tree species, sampling methodology and their interaction with the functional traits of fine roots. The percentage of variance (V.e, in %) of tree species, sampling categories, or their interactions, were indicated by an increase in the value of multiple R^2 due to the addition of these terms to the model. Trait variables were log-transformed prior to statistical analysis when the data were heteroscedastic and had non-normal distribution of residual error. Pairwise comparisons were evaluated using Tukey's honest significant difference (HSD) test ($p < 0.05$). We also used Pearson's correlation coefficients (PCC) and the phylogenetically independent contrasts (PIC) method to test correlations between root traits across the five root orders.

We tested the hypothesis of high phylogenetic "conservatism" in root traits by conducting K statistics, which is a method used to measure the intensity of phylogenetic signals associated with functional traits and examine the relationships between functional traits and phylogeny for all tree species (Blomberg et al. 2003). A K statistic greater than 1 implied that the functional traits are stronger than the phylogenetic signal traits, derived using a model of Brownian motion. This indicated that the functional traits exhibited a phylogenetic signal. Otherwise, the functional traits were less affected by phylogenetic relationships than the Brownian motion model, indicating that no phylogenetic signals were involved. When measuring functional traits with phylogenetic signals, the species in the phylogenetic tree are usually randomly permuted 999 times to calculate the K statistic. If the observed value was greater than the K statistic of the zero model at a level of $\alpha = 0.05$, this implied that the functional trait had a
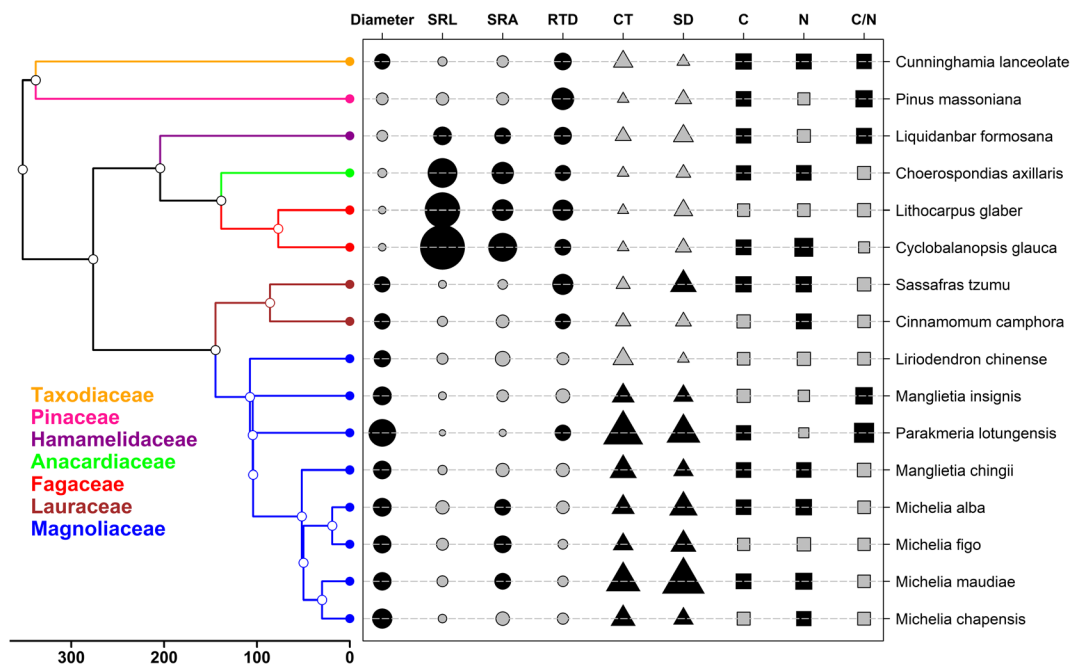
Fig. 1 Relationship between phylogenetic traits and morphological traits (circular symbol) [mean diameter, specific root length (SRL), specific root area (SRA), and root tissue density (RTD)], anatomical traits (triangle symbol) [cortex thickness (CT) and stele diameter (SD)], and nutrient concentrations (square symbol) [root C, N concentrations and root C/N ratio] of the first-order roots of 16 tree species. Symbol size indicates the proportion (%) of functional trait values for each species, with smaller symbols closer to the mean value; black symbols represent values above the mean, and gray symbols represent values below the mean. The chronogram tree with divergence times estimated for speciation events was generated using the r8s test in conjunction with the nonparametric rate smoothing (NPRS) approach (version 1.7; Sanderson 2003); open circles represent ancestral nodes; filled circles represent sampled species. Numbers indicate millions of years

phylogenetic signal; conversely, the trait had weak or no phylogenetic signals (Blomberg et al. 2003).

To determine how multiple morphological and anatomical root traits aligned with root nutrients, we selected first-order roots for our analysis. This was because first-order roots are comparable to leaves in terms of resource acquisition by virtue of their rapid turnover rate and high physiological activity (Guo et al. 2008a; Wang et al. 2018). We used standard major axis (SMA) regression to evaluate the relationships between the traits of first-order roots. SMA is more appropriate in situations where both variables are subject to error and the two variables have different units of measurement (Friedman et al. 2013). Following this, we also used the PIC to calculate correlation coefficients to test the relationships between first-order root morphology, anatomy variation and nutrient traits without phylogenetic influence. Finally, phylogenetic principal component analysis (pPCA) was conducted to determine the major axes of functional trait variations in the first-order roots (Revell 2009).

Statistical analysis was conducted using R programming language (version 3.4.3, The R Foundation for Statistical Computing; www.r-project.org) using package *picante* (Kembel et al. 2010), *ape* (Paradis et al. 2004), *smatr* (Warton et al. 2012), *phytools* (Revell 2012) and *vegan* (Dixon 2003).

Results

Effect of tree species and sampling categories on the functional traits of fine roots

Tree species, and sampling categories, caused significant effects upon the anatomical, morphological and nutrient traits of fine roots ($p < 0.05$) (Table 1), except for the effect of tree species upon stele diameter. In terms of the relative percentage of variance (V.e %), tree species had a greater effect on functional traits than sampling categories. The interactive effects of tree

species, and the sampling categories, were significantly associated with morphological traits but anatomical and nutrient traits (Table 1).

The roots of the gymnosperms (i.e., *C. lanceolata* and *P. lotungensis*) and Magnoliaceae (i.e., *M. insignis*, *M. chingii*, *M. maudiae*, *M. chapensis*, *M. figo* and *P. lotungensis*) were thick with thick cortices, and had a short SRL and a low RTD; the root diameter of Anacardiaceae and Fagaceae (i.e., *C. glauca* and *L. glaber*) were thinner (Fig. S3). The range in variation of the first-order root diameter showed a three-fold difference between the thinnest diameter (*L. glaber*: 0.23 ± 0.01 mm) and the thickest (*P. lotungensis*: 0.84 ±

0.02 mm). The SRL showed more than ten-fold difference between the longest (*C. glauca*: 150.61 ± 17.95 mg^{-1}) and the shortest (*P. lotungensis*: 11.40 ± 0.50 mg^{-1}) (Fig. S3).

For the sampling categories, anatomical traits (cortex thickness and stele diameter), mean root diameters and RTD were the lowest in the first-order category and the highest in the ≤2 mm category; the opposite pattern was observed for SRL and SRA (Table 2; Fig. S1). Differences in root diameter, SRL and SRA were significant among sampling categories, and anatomical and nutrient traits were significantly different among the sampling categories ($p < 0.0001$). For all species, root C/N was highest in the ≤2 mm root sampling category (Fig. S3).

Table 1 Two-way analysis of variance (ANOVA) for the effects of tree species and sampling categories on morphological [root diameter (mm), specific root length (SRL, m g^{-1}), specific root surface area (SRA, m^2 kg^{-1}) and root tissue density (RTD, g cm^{-3})], anatomical [cortex thickness (μm) and stele diameter (μm)] and nutrient [root C, N concentrations (%) and C/N ratio] traits. d.f., degrees of freedom; V.e %, the percentage of variance explained

Parameters	Items	Factor	d.f.	F-value	p value	V.e %
Morphology	Root diameter	Species	15	53.95	< 0.0001***	60.77
		Category	3	131.52	< 0.0001***	29.63
		Species×Category	45	2.84	< 0.0001***	9.60
	SRL	Species	15	73.73	< 0.0001***	74.70
		Category	3	68.44	< 0.0001***	13.87
		Species×Category	45	3.76	< 0.0001***	11.44
	SRA	Species	15	27.03	< 0.0001***	56.30
		Category	3	73.69	< 0.0001***	30.70
		Species×Category	45	2.08	< 0.0001***	13.00
	RTD	Species	15	40.35	< 0.0001***	80.13
		Category	3	27.01	0.0131*	10.73
		Species×Category	45	1.54	< 0.0001***	9.15
Anatomy	Cortex thickness	Species	15	7.52	< 0.0001***	49.41
		Category	3	29.15	< 0.0001***	38.31
		Species×Category	45	0.62	0.9748	12.28
	Stele diameter	Species	15	0.95	0.5108	11.38
		Category	3	28.72	< 0.0001***	69.00
		Species×Category	45	0.54	0.9935	19.62
Nutrients	Root C	Species	15	43.95	< 0.0001***	88.42
		Category	3	15.30	< 0.0001***	6.16
		Species×Category	45	0.90	0.6618	5.42
	Root N	Species	15	17.35	< 0.0001***	76.75
		Category	3	15.08	< 0.0001***	13.34
		Species×Category	45	0.75	0.8885	9.90
	Root C/N	Species	15	10.62	< 0.0001***	62.21
		Category	3	19.74	< 0.0001***	23.13
		Species×Category	45	0.83	0.7714	14.66

*, ** and *** indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively

Table 2 Differences in anatomical, morphological and nutrient traits of fine roots among four sampling categories of the 16 tree species investigated

Items	First-order				First-three-orders				≤ 1 mm				≤ 2 mm			
	M	Min	Max	SE	M	Min	Max	SE	M	Min	Max	SE	M	Min	Max	SE
Diameter	0.48a	0.21	1.00	0.01	0.58b	0.21	1.56	0.01	0.58b	0.21	1.00	0.01	0.78c	0.21	1.99	0.01
SRL	51.96a	8.33	270.24	3.25	33.25b	1.69	270.24	1.40	29.07b	2.37	270.24	1.18	23.44c	0.41	270.24	0.97
SRA	62.68a	22.79	215.52	2.02	46.06b	5.24	215.52	1.03	41.41c	5.24	215.52	0.92	36.29d	2.14	287.74	0.87
RTD	0.16a	0.06	0.32	0.00	0.20b	0.05	1.32	0.00	0.23c	0.04	1.32	0.01	0.23c	0.01	1.67	0.00
CT	378.64a	166.65	984.88	23.55	474.20a	166.65	1162.28	16.02	570.99b	166.65	1596.46	19.89	680.28c	166.65	1853.00	22.97
SD	107.93a	41.75	235.82	6.67	156.68a	41.75	488.65	6.02	240.18b	41.75	898.33	12.96	297.27c	41.75	951.11	14.11
RC	46.48a	40.88	50.08	0.36	47.35ab	40.88	53.10	0.21	47.89bc	40.88	55.37	0.18	48.05c	40.88	55.37	0.16
RN	3.46a	2.54	4.54	0.06	3.28ab	2.33	5.39	0.04	3.17bc	1.74	5.39	0.04	3.08c	1.54	5.39	0.04
C/N	13.64a	10.69	18.59	0.26	14.72ab	9.24	20.61	0.19	15.55c	9.24	30.18	0.22	16.22c	9.24	32.18	0.23

SRL specific fine root length (m g^{-1}), SRA specific fine root surface area (m^2 kg^{-1}), RTD fine root tissue density (g cm^{-3}), CT cortex thickness (μm), SD stele diameter (μm), RC root carbon concentration (%), RN root nitrogen concentration (%), C/N root carbon and nitrogen concentration ratio, M mean, Min minimum, Max maximum, SE standard error. Distinct letters indicate significant differences between different sampling categories ($p < 0.05$)

Differences in fine root traits between first-order and those of the other sampling categories were more significant for modern taxa (Anacardiaceae and Fagaceae) than ancient taxa (gymnosperms and Magnoliaceae). Ancient taxa showed significant differences in morphological traits between the ≤1 mm and ≤2 mm sampling categories, while modern taxa (Anacardiaceae and Fagaceae) showed only slight differences in all fine root traits when compared between the ≤1 mm and ≤2 mm sampling categories (Fig. S3).

Relationship between anatomical, morphological and nutrient traits in fine roots

Anatomical traits (cortex thickness and stele diameter) were significantly and positively correlated to root diameter ($r = 0.78$ and 0.61, respectively, both at $p < 0.0001$), but were negatively correlated to SRL and SRA ($r = -0.61$ and -0.67, respectively, $p < 0.0001$ for cortex thickness; $r = -0.52$ and -0.67, $p < 0.0001$ for stele diameter) (Fig. S4a). Anatomical traits (cortex thickness and stele diameter) were significantly and negatively correlated to N concentrations ($r = -0.45$ and -0.56, respectively, $p < 0.0001$), but exhibited a positive correlation to root C/N ($r = 0.46$ and 0.63, respectively, $p < 0.0001$) (Fig. S4a).

For first-order roots, the root N concentration was negatively correlated to diameter and RTD but positively and significantly correlated to SRL and SRA (SMA $R^2 = 0.38$, $p < 0.05$; Fig. 2a). Root C/N was significantly and positively correlated to root diameter and negatively correlated to SRA (SMA $R^2 = 0.29$ and 0.47; $p < 0.05$; Fig. 2b).

In general, traits associated with resource acquisition (i.e., SRL, SRA and root N concentration) exhibited significant and positive relationships ($r = 0.50$–0.90, all at $p < 0.0001$) with each other. Fine root traits associated with resource conservation (i.e., root diameter and C/N) were significantly and negatively correlated to SRL and SRA ($r = -0.63$ and -0.60, respectively, $p < 0.0001$ for root di-

Fig. 2 Relationships between N concentration (**a**), root C/N ratio ▶ (**b**) and morphological traits, the anatomical traits of first-order roots. Data show mean ± standard error ($n = 16$). Dotted lines indicate standard major axis (SMA) regression lines. Coefficients of determination (R^2) are shown for both SMA regressions (SMA R^2) and phylogenetic independent contrast correlation values (PIC R^2). Morphological traits include SRL (specific fine root length), SRA (specific fine root surface area) and RTD (fine root tissue density). Anatomical traits are represented by CT (cortex thickness) and SD (stele diameter). SMA is the slope for the standardized major axis
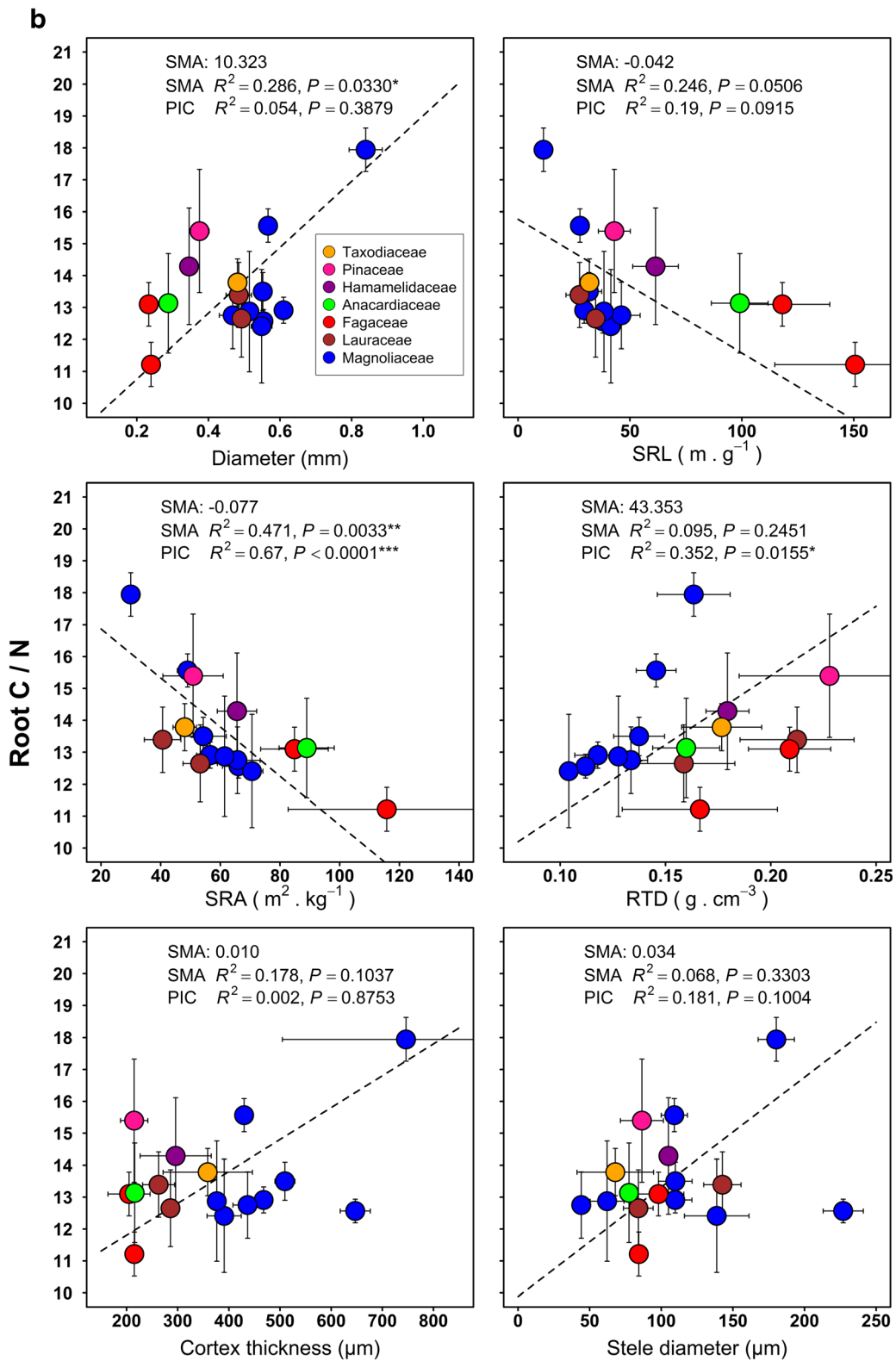
a
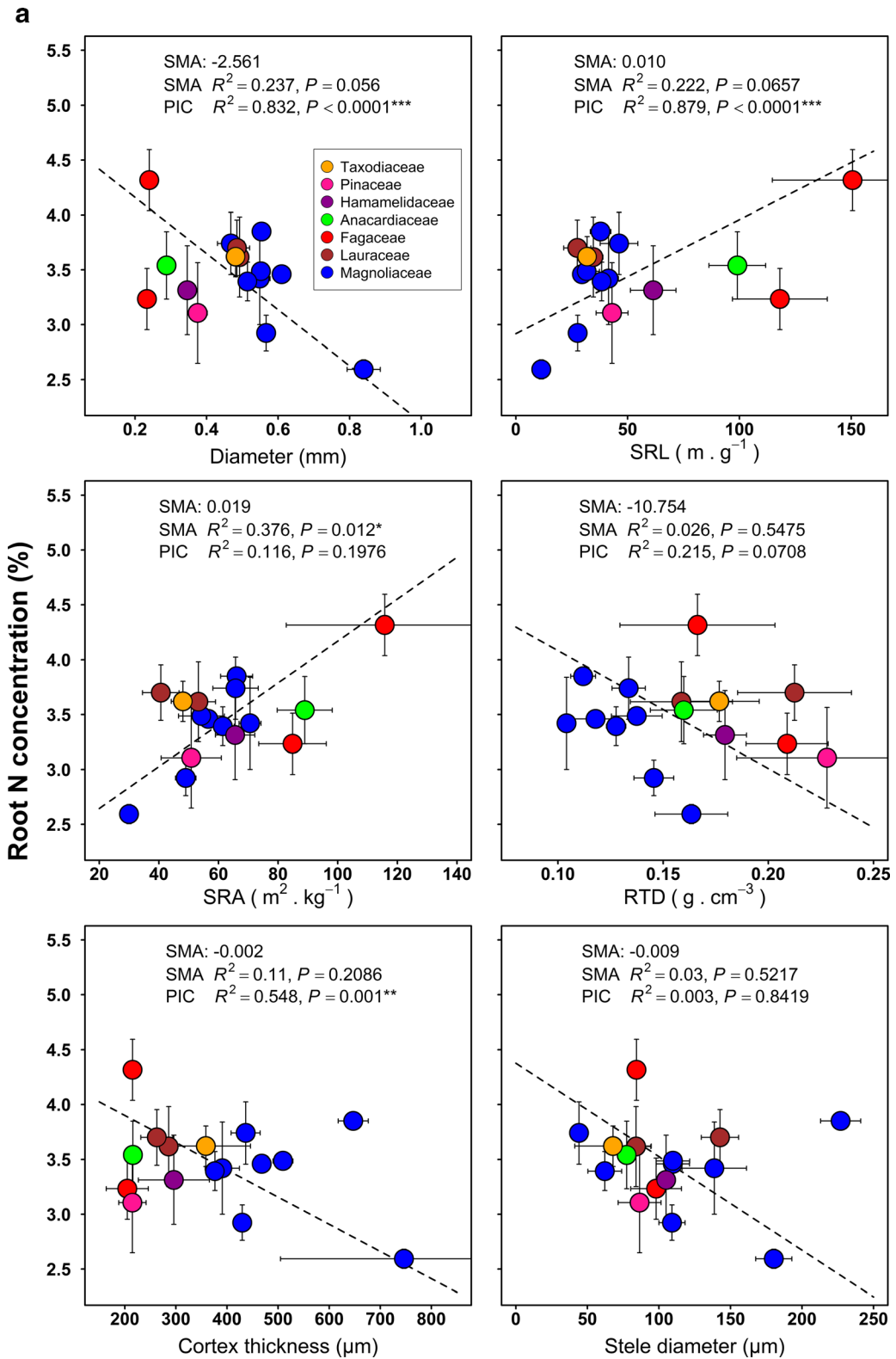
Fig. 2 (continued)

ameter; $r = -0.48$ and -0.66, respectively, $p < 0.0001$ for root C/N) (Fig. S4a). Phylogenetically independent contrasts showed that correlations between SRL, SRA and root N concentration increased after the phylogenetic influence was removed ($r = 0.87$ and 0.88, $p < 0.0001$; Fig. S4b).

Phylogenetic structure of fine root traits

When data from the 16 tree species were pooled together, root diameter, SRL and RTD exhibited phylogenetic signals according to Blomberg's K values for the first-order and ≤ 1 mm sampling categories (Table 3). Root diameter ($K = 1.05$), SRL ($K = 1.57$) and RTD ($K = 1.10$) of first-order roots yielded strong phylogenetic signals; SRA ($K = 0.86$) and cortex thickness ($K = 0.76$) showed moderate phylogenetic conservatism ($p < 0.05$). For nutrient concentrations, only root C/N ratio showed a significant phylogenetic signal (first to third-orders: $K = 0.69$, ≤ 1 mm: $K = 0.75$, ≤ 2 mm: $K = 0.67$, all at $p < 0.05$).

For all sampling categories, morphological and anatomical traits had more obvious phylogenetic structure than nutrient concentrations. First-order root functional traits showed stronger phylogenetic structure among species compared with the other sampling categories (Fig. 1; Fig. S2).

Furthermore, PICs were conducted for first-order roots to exclude the impact of universal phylogenetic signals; this yielded variable results. The correlations between root N concentrations and diameter, SRL and cortex thickness were significant after the phylogenetic influence was

eliminated (PIC $R^2 = 0.55$–0.88, $p < 0.001$; Fig. 2a). The relationship between root C/N and diameter became insignificant (PIC $R^2 = 0.05$, $p > 0.05$), while the correlation between root C/N with SRA and RTD were stronger (PIC $R^2 = 0.67$ and 0.35, respectively, both at $p < 0.05$) after removing the phylogenetic effect (Fig. 2b).

Results from pPCA of all functional traits of first-order roots showed that the first two axes of the pPCA explained 79.8% of the total variation (Fig. 3). The first pPCA axis accounted for 54.7%, and, at the negative extremum, we found traits representing resource acquisition strategy (SRL, SRA and root N concentration). Traits representing resource conservation strategy (diameter, stele diameter and root C/N) were found at the opposite positive extremum. The second pPCA axis accounted for an additional 25.1% of variation and was heavily loaded on RTD and cortex thickness (Table 4).

Discussion

Variation in the functional traits of fine roots among tree species and sampling categories

Our study showed that tree species significantly impacted upon the functional traits of fine roots and could explain greater than 50% of the observed variation in morphological and nutrient root traits. These results are consistent with previous studies that identified coexisting species in subtropical forests with very different root morphology (Chen

Table 3 Phylogenetic signals from Blomberg's K statistic with p values of root morphological [root diameter (mm), specific fine root length (SRL, m g^{-1}), specific fine root surface area (SRA, m^2 kg^{-1}) and fine root tissue density (RTD, g cm^{-3})], anatomical [cortex thickness (μm) and stele diameter (μm)] and nutrient [root C, N concentrations (%) and C/N ratio] traits for the different sampling categories

Root orders	First-order		First-three-orders		≤ 1 mm		≤ 2 mm	
Traits	K	P	K	P	K	P	K	P
Root diameter	**1.05**	**0.001**	0.62	**0.021**	**1.32**	**0.001**	0.47	0.163
SRL	**1.57**	**0.001**	**1.30**	**0.001**	**1.30**	**0.001**	**1.19**	**0.001**
SRA	0.86	**0.003**	0.68	**0.006**	0.69	**0.024**	0.55	0.074
RTD	**1.10**	**0.002**	**1.44**	**0.001**	**1.85**	**0.001**	**1.40**	**0.001**
Cortex thickness	0.76	**0.017**	0.78	**0.011**	0.57	0.052	0.40	0.243
Stele diameter	0.20	0.916	0.23	0.814	0.43	0.190	0.19	0.897
Root C	0.23	0.908	0.29	0.808	0.26	0.816	0.32	0.635
Root N	0.37	0.353	0.41	0.210	0.52	0.090	0.50	0.066
Root C/N	0.54	0.117	0.69	**0.035**	0.75	**0.018**	0.67	**0.021**

The bold numbers indicate that the effects are significant

Fig. 3 Ordination of first-order fine root morphological traits (green), anatomical traits (orange) and nutrient concentrations (red) and the projection in multiple factor dimensions for 16 tree species (see Table S1 for corresponding species abbreviations). Axis values represent the first two phylogenetic principal component analysis (pPCA) axes, and the amount of variation explained by each axis. SRL, specific fine root length (m g^{-1}); SRA, specific fine root surface area (m^2 kg^{-1}); RTD, fine root tissue density (g cm^{-3}); CT, cortex thickness (μm); SD, stele diameter (μm)
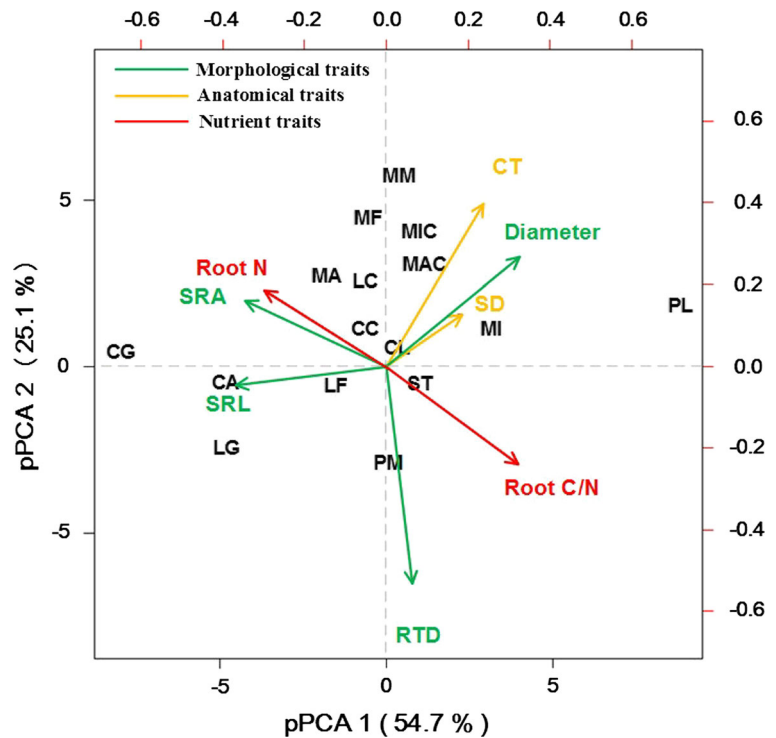
et al. 2013; McCormack et al. 2015); these authors also found that the range of root trait variation in coexisting species was much greater in subtropical regions than in temperate regions. Many fossil records (Doyle and Hickey 1976; Hickey and Doyle 1977) and current paleoclimatic models (Beerling and Woodward 2001) have indicated that the common ancestor of extant angiosperms (e.g. Magnoliaceae) originated in the tropics and subtropics humid zone (Baylis 1975; Feild et al. 2009). In ever-wet habitats, there is less selective pressure imposed on first-order roots, e.g. desiccation stress, which would be a prerequisite for the existence of a large number of thick root species (Chen et al. 2013).

Our analyses showed that, as branch order increased, root diameter, RTD, cortex thickness and stele diameter increased significantly, whereas SRL and SRA

decreased (Table 2). This pattern of variation is in agreement with that observed in previous studies (Pregitzer et al. 2002; Guo et al. 2008b; Liu et al. 2010b; Picon-Cochard et al. 2012; Dong et al. 2015). Anatomical traits in the presence or absence of secondary xylem have been considered to represent a key partition of root function (Esau 1965; Hishi 2007; Valenzuela-Estrada et al. 2008). First-order roots mainly function as a resource uptake mechanism and are often thin, with a high proportion of cortical material and a low stele/root diameter ratio. An increase in the proportion of stele diameter in the other root orders indicated that higher order roots have superior axial transportation capability (Guo et al. 2008b). Our findings showing that first-order roots had the highest mean root N concentrations, and the lowest root C/N, are generally consistent with

Table 4 Loading scores of functional traits for the first-order roots of 16 tree species based on phylogenetic principal component analysis (pPCA) along two explanatory axes

Variables	Diameter (mm)	SRL (m g^{-1})	SRA (m^2 kg^{-1})	RTD (g cm^{-3})	Cortex thickness (μm)	Stele diameter (μm)	Root N (%)	Root C/N	Explained variance (%)
pPC 1	**0.84**	**−0.95**	**−0.90**	0.16	**0.62**	**0.49**	**−0.77**	**0.84**	54.74
pPC 2	0.47	−0.08	0.28	**−0.93**	**0.70**	0.22	0.33	−0.42	25.06

SRL specific fine root length, *SRA* specific fine root surface area, *RTD* fine root tissue density. Strong loadings (absolute value ≥0.50) are shown in bold

previous reports on temperate tree species (Pregitzer et al. 1997, 2002; Guo et al. 2008b; Hishi and Takeda 2005; Li et al. 2010). Root chemistry changes with an increase in order; this has been suggested to be associated with anatomical trait differences among branch orders (Pregitzer et al. 2002; Guo et al. 2004, 2008a). Given the high metabolic and cortical cell proportions, first-order roots generally have high N concentrations to support their physiological activities (Pregitzer et al. 2002; Alexander et al. 2004; Guo et al. 2004). In contrast, fourth- and fifth-order roots mostly consist of secondary vascular tissues and exhibit low physiological activities under low N requirements (Pregitzer et al. 2002; Guo et al. 2004).

Diameter-based cut-off sampling classifications (e.g. ≤ 2 mm) include a set number of root orders (generally between two and five) with different structures and functions (Chen et al. 2013). In our present study, the fine root sampling categories for Fagaceae species, based on the ≤2 mm cut-off approach, included five orders with a mean diameter lower than 0.5 mm, while roots sampled from Magnoliaceae species in the first-three-order category had a mean diameter greater than 0.5 mm. As expected, sampling category effects were driven by differences among phylogeny. Some Magnoliaceae roots at ≤1 mm had a lower diameter than the first-three-orders, while that of Fagaceae was significantly higher. In general, our results provide evidence for a profound difference in trait values between first-order root samples and other sampling categories (the first-three-orders, ≤ 1 mm and ≤2 mm). To extend our knowledge of root ecology, the selection of the first-order sampling category might be suitable for functional comparison across species. However, a greater number of species, which are widely distributed across study sites, should be included in future studies. In addition, trait-based approaches can be summarized and formalized based on comparative analysis of the root traits across a large number of species to enable improved understanding of root trait variation and adaptation to their environment (Laliberté 2017; Erktan et al. 2018).

Does the "root economics spectrum" exist?

We found that fine root nutrient concentrations were significantly correlated with the morphological traits of 16 subtropical species. This observation, which indicates the existence of a belowground trade-off between resource acquisition and conservation, remained similar when phylogenetic relatedness was also considered (Fig. 2), thus

supporting the existence of RES. This indicated that, at one end of the spectrum, the species exhibited resource conservation traits (i.e. high root diameter, cortex thickness, stele diameter and root C/N values). At the other end of the spectrum, the species exhibited resource-acquired traits (i.e. high SRL, SRA and root N concentration values) (Fig. 3).

The identification of contrasting economic strategies of fine roots among different tree species may provide novel insights into the RES (Chen et al. 2013; McCormack et al. 2015). Recent studies have not been able to identify a significant relationship between the chemical and morphological traits of fine roots across species, thus suggesting that the morphological and chemical traits of fine roots are independent of each other (Chen et al. 2013; Valverde-Barrantes et al. 2015). Across the five orders of roots, we found that N concentrations were negatively correlated with both root diameter and cortex thickness (Fig. S4). This may be due to the fact that the relative cortex proportion decreases as root diameter increases, and because the amount of N stored in the cortex also decreases accordingly. When focusing on first-order roots, N concentrations were not significantly correlated with root diameter, SRL or cortex thickness. These results are consistent with those of a recent study that examined root traits in over 300 plant species across broad climate gradients (Ma et al. 2018). However, our correlations were significant after phylogenetic influences had been eliminated (Fig. 2), thus supporting the fact that some covariations between N concentrations and morphological traits may be overshadowed by evolutionary constraints (Ma et al. 2018). The ancient thick-roots species (Magnoliaceae) exhibit conservative strategies to allocate more carbon to the mycorrhizae for resource acquisition, while species with thin roots have a larger SRL and higher exploitative capacities (Hodge 2004), greater rates of nutrient uptake (Eissenstat and Yanai 1997; Comas et al. 2012) and higher N concentrations (Comas et al. 2012; Jia et al. 2013). The evolutionary conversion from ancient plant taxa with thicker roots to more modern irradiated plant species with thinner and higher RTD roots may be advantageous in terms of more efficient soil exploration and the ability to better penetrate the soil matrix. Even a slight evolutionary change in RTD of first-order roots may dramatically alter the length of soil explored per unit of C input (Ma et al. 2018) to enable higher nutrient and water absorption capacities (Fan and Jiang 2010; Beyer et al. 2013).

Roots are multidimensional organs that carry out composite functions (Kramer-Walter et al. 2016). Contrary to

the exclusive acquisition-conservation axis of leaves, the multidimensional framework is more applicable when interpreting variations of interspecific root traits (Kembel et al. 2008; Kramer-Walter et al. 2016; Roumet et al. 2016). Multiple root traits are subject to two major variability dimensions: variation in RTD and variation in root diameter and SRL (Kramer-Walter et al. 2016). In contrast, root diameter, SRL and RTD are correlated along one dimension, while a second orthogonal dimension is depicted by root branching intensity (Kong et al. 2014). Our pPCA results showed the existence of two leading dimensions: the first dimension included root diameter, SRL, SRA, stele diameter and a chemical feature, while the second dimension included RTD and cortex thickness. Moreover, the 16 tree species used in this study, which were from different families, were segregated along multidimensional axes (Table 4; Fig. 3).

Fine roots play a key role in water and nutrient uptake from the soil matrix which exhibits high spatial and temporal heterogeneity in resources. Compared with leaves, plants have fewer spatial constraints in which to construct roots (Kramer-Walter et al. 2016); this gives rise to more possibilities from which to generate differing trait combinations to adapt to a wider range of environmental conditions (Laughlin 2014). For example, RTD can be explained partly by the respective proportions of lignified stele and parenchymatous cortical tissue, which is also related to the availability of mycorrhizal habitats (Hummel et al. 2007; Kong et al. 2017; Valverde-Barrantes et al. 2017). Roots are capable of adjusting their absorption rate via mycotrophy without necessarily adjusting RTD or its diameter (Comas et al. 2012; Kong et al. 2017). These strategies provide more ways for nutrient acquisition and reduced constraints on trade-offs in root morphology traits (Valverde-Barrantes et al. 2015; Weemstra et al. 2016). The potential existence of multiple resource-uptake strategies could explain the lower integration of RTD and cortex thickness to the primary axis of acquisition-conservation and indicates that species disperse along the major axis of belowground variation, resulting in high functional coordination among species (de la Riva et al. 2018).

Modification of root traits with the evolution of tree species

Evolutionary constraints have been frequently used to explain variations in fine root traits (Comas and Eissenstat 2009; Valverde-Barrantes et al. 2015). The third purpose of this study was to associate the functional traits of fine roots

with phylogeny to explore the significance of evolutionary patterns. An evolutionary pattern across various woody taxa has been reported previously, and some traits (such as diameter, SRL and RTD) are structured phylogenetically in low order roots (Comas and Eissenstat 2009; Kong et al. 2014; Valverde-Barrantes et al. 2015). In plants, a larger diameter, or a smaller SRL, in first-order roots occurs during the initial stages of phylogeny compared with that in the advanced evolutionary stages (Comas and Eissenstat 2009; Chen et al. 2013). We found that there were potential effects of evolutionary shifts in root functional traits (Fig. 1) and that the phylogenetic structure was most explicit in first-order roots (Fig. S2). This is consistent with previous studies that found that early-divergent phylogenetic species (i.e., primitive angiosperms such as Magnoliaceae and all gymnosperms) generally had shorter, thicker roots and lower SRL (Pregitzer et al. 2002; Comas et al. 2012; Chen et al. 2013).

As expected, the morphological traits of roots exhibited a higher phylogenetic structure in our study, implying that inherent evolutionary adaptability between major phylogenetic clades result in differences in root morphology (Valverde-Barrantes et al. 2017). As evolution progresses, water and nutrients became more limited, and tree species in subsequent evolution stages (such as *Quercus*, *Aralia* and *Fagus*) adjust their functional traits to acquire these limited resources (Espeleta and Donovan 2002; Meier and Leuschner 2008). To maximize water absorption, thin roots with reduced cell cortices experience less resistance to water conduction (Eissenstat and Achor 1999), and a higher SRL tends to gain greater hydraulic conductivity (Solari et al. 2006; Hernández et al. 2009).

Our results showed that stele diameter yielded no significant phylogenetic signals (Table 3). This was different from some previous studies in which a high degree of evolutionary conservatism for stele diameter was observed (Gu et al. 2014; Kong et al. 2014). This implies that stele diameter is markedly affected by environmental factors. We also found that stele diameter had no significant effect on root nutrient traits (Fig. 2) and that, with species evolution, the reduced range of stele diameter was lower than that of cortex thickness (Fig. 1). For absorptive roots, the root stele is completely surrounded by a cortex which can act as an "isolation belt" to provide a cushion against environmental stress (Alexander et al. 2004). Therefore, the trend in the evolutionary strategy of roots of different tree species is to put more effort into reducing cortex thickness

compared to stele diameter during species evolution along the chronosequence (Gu et al. 2014). For example, the size ratio of stele diameter to cortex thickness is greater for species that diverged later than for those that diverged earlier (Kong et al. 2014). Moreover, we found that the fine roots of *C. glauca* and *L. glaber* had thinner root cortex thicknesses and higher stele/root diameter ratios than other species. This might imply that the roots of some Fagaceae species (e.g. *C. glauca* and *L. glaber*) are weaker in terms of their uptake ability but stronger in other service functions such as anchorage, transport and storage capacities.

Recent studies have suggested that the patterns that emerged in the evolution of root traits for angiosperms are likely to be related to global climate change from the Cretaceous to the late Tertiary period (~120–10 Million Years Before Present [MYBP]) (Fletcher et al. 2008; Comas et al. 2012). To adapt to a decline in atmospheric CO_2 and temperature, coupled with an increase in drought during this period (Savin 1977; Retallack 2001), the fine roots of modern angiosperm lineages reduced xylem vessel size and reinforced lignification for greater water utilization efficiency. Allometric relationships between the root cortex and stele size could therefore contribute to the adaptation of plants to drying conditions (Kong et al. 2017). To facilitate subsistence and expansion in drier and colder environments, root systems exhibit increased SRL and RTD and decreased diameter to alleviate construction costs and improve uptake efficiency (Feild et al. 2009; Comas et al. 2012).

Conclusions

Results from this study indicate that the use of appropriate sampling categories, while taking into account phylogeny, is essential when investigating the functional traits of fine roots in subtropical tree species. Although we determined the existence of RES for resource acquisition and conservative traits, even when the phylogenetic structure was included, a single RES cannot capture mechanisms associated with belowground trade-offs. A multidimensional system offers a comprehensive way to better understand RES and root ecological strategies in response to environmental change. The high variation in root functional traits, as it pertains to the subtropical coexistence of tree species,

appears to arise from both phylogeny and adaptation under complex water and nutrient supply conditions. Moreover, the constant adjustment of root functional traits may represent a crucial evolutionary strategy for terrestrial plant species in adapting to selective pressures associated with global climate change. The elucidation of variable patterns in the functional traits of fine roots, as based on plant evolution, may be a promising strategy for understanding tree root adaptability to global environmental change from a plant economic perspective. Nevertheless, more tree species should be sampled to reinforce these conclusions in the future studies. Meanwhile, our study can be used as a supplement to the root functional traits dataset in subtropical area and provide broader observations for a more complete understanding of tree species.

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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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