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A molecular approach to quantify root community composition in a northern hardwood forest — testing effects of root species, relative abundance, and diameter

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Abstract: Research on root community structure is currently limited by the methodologies available. We evaluated a molecular-sequence-based approach to quantify the relative abundance of roots from different plant species in mixed samples. We extracted DNA from mixtures of roots, amplified the *trnL* intron by polymerase change reaction, and identified up to 60 clones from each mixture. We tested the effects of root diameter and species on sequence representation in mixtures. Species were correctly identified in our mixtures. Recovery efficiencies were low for root diameter classes >0.3 mm compared with those <0.3 mm, and species in high abundance in the mixture had relatively low recovery efficiency. American beech (*Fagus grandifolia* Ehrh.) was quantitatively underrepresented compared with yellow birch (*Betula alleghaniensis* Britton) and white ash (*Fraxinus americana* L.). Root identification by sequencing is accurate and can readily be applied to novel systems without new primer development. This technique will be attractive for documenting changes in relative abundances of species, especially as the cost of sequence-based analyses drops. However, the results of such analyses must be considered carefully, as root diameter distribution, abundances, and species can introduce quantifiable biases in the estimation of relative root abundance.

Résumé : La recherche sur la structure des communautés racinaires est limitée par les méthodes disponibles. Les auteurs ont donc évalué l'emploi d'une approche basée sur les séquences moléculaires dans le but de quantifier l'abondance relative des racines de diverses espèces de plantes dans des échantillons mixtes. L'ADN a été extrait des mélanges de racines, puis amplifié par réaction en chaîne par polymérase pour l'intron *trnL*, ce qui a permis d'identifier jusqu'à 60 clones pour chaque mélange. Les effets du diamètre des racines et de l'espèce sur la représentation des séquences dans les mélanges ont été étudiés. Les espèces ont été identifiées correctement dans les mélanges. Le taux de récupération des espèces était faible dans les classes de diamètre des racines > 0,3 mm, comparativement aux classes de diamètre < 0,3 mm; les espèces très abondantes dans le mélange avaient un taux de récupération relativement faible. Le hêtre d'Amérique (*Fagus grandifolia* Ehrh.) était quantitativement sous-représenté comparativement au bouleau jaune (*Betula alleghaniensis* Britton) et au frêne blanc (*Fraxinus americana* L.). L'identification des racines par le séquençage est précise et peut être appliquée directement à de nouveaux systèmes sans avoir à développer de nouvelles amorces. Cette approche sera intéressante pour documenter les changements dans l'abondance relative des espèces, spécialement à mesure que le coût des analyses basées sur le séquençage décroît. Cependant, les résultats de telles analyses doivent être examinés avec précaution étant donné que l'espèce, l'abondance et la distribution diamétrale des racines peuvent introduire des biais quantifiables dans l'estimation de l'abondance relative des racines de différentes espèces.

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Introduction

The relative abundance of roots of different species and their spatial arrangement and temporal pattern are important components of plant community structure. Studies of spatial partitioning, nutrient foraging, and competition among roots have advanced our understanding of plant species interactions (for example, Bliss et al. 2002; Lamb et al. 2009; Meinen et al. 2009). However, the study of root communities in field settings has been limited by the difficulty of

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identifying roots to species. For example, visually distinguishing the fine roots of some hardwood tree species requires examination of secondary growth of higher order roots (Yanai et al. 2008). This approach is not always practical for quantifying species composition in field settings where the finest roots (<0.3 mm), which generally account for most of the root length (Fahey and Hughes 1994; Pregitzer et al. 2002), are most conveniently sampled by methods that do not assure connection to the larger root axes.

Molecular genetic approaches can be used to identify roots but present challenges associated with time, expense, and methods development. Identifying single root fragments using molecular methods based on polymerase chain reaction is straightforward (Bobowski et al. 1999; Linder et al. 2000; Brunner et al. 2001) but can be of limited utility in many situations because of the large numbers of analyses required to estimate the relative abundances of roots of different species in a sample. Extracting community DNA from bulked root samples eliminates some of the effort of extracting multiple fragments per sample. This approach was recently applied using a real-time PCR technique to accurately quantify relative abundance in a root community (Mommer et al. 2008). However, the use of such an approach in new communities is limited by the effort required to develop primer sets specific to each plant species of interest. In contrast, whole-community sequence-based approaches that are now commonly used to describe the structure and composition of soil microbial communities could potentially be applied to root communities without new primer development and are likely to become increasingly economical as the sample throughput possible with these techniques is increasing substantially (Lauber et al. 2009).

We evaluated the potential of a sequence-based approach for quantifying community composition of fine roots, which could easily be applied across different plant communities. We evaluated several possible sources of bias that could affect sequence-based estimates of fine-root community composition in this method. The efficiency of DNA extraction or PCR amplification might differ among species. This could result from differences among species in DNA concentrations, gene composition, tissue chemistry, or root anatomy. Similarly, we may expect root diameter to affect determinations of root relative abundance by species. Tissue chemistry generally varies with root order (Pregitzer et al. 2002; Guo et al. 2004), as older and larger roots, higher in branching order, may be more suberized and have a larger proportion of woody nonliving tissue. To address these possibilities, we tested the effects of species and root diameter on sequence representation and assessed whether these effects influence our ability to accurately estimate root species composition in mixed communities.

Materials and methods

We collected fine roots of American beech (*Fagus grandifolia* Ehrh.), yellow birch (*Betula alleghaniensis* Britton), and white ash (*Fraxinus americana* L.) at the Hubbard Brook Experimental Forest, New Hampshire, USA, in July 2008. The forest is typical of northern hardwoods in species composition, with most stems originating after a partial harvesting around 1918 or after the 1938 hurricane (Likens et al. 1985). We collected roots of known species by tracing root systems from the base of a tree and through the soil to find root systems consisting of first- through fourth-order roots. Roots were cleaned and frozen (–20 °C) until processing in fall 2008. Roots of each species were separated into the following diameter classes: <0.3, 0.3–0.5, and 0.5–1.0 mm. Roots <0.3 mm were first- and second-order roots, and roots >0.3 mm were a mixture of third and fourth order. It was not practical to separate the first- and second-order roots because of the very small size of most first-order roots.

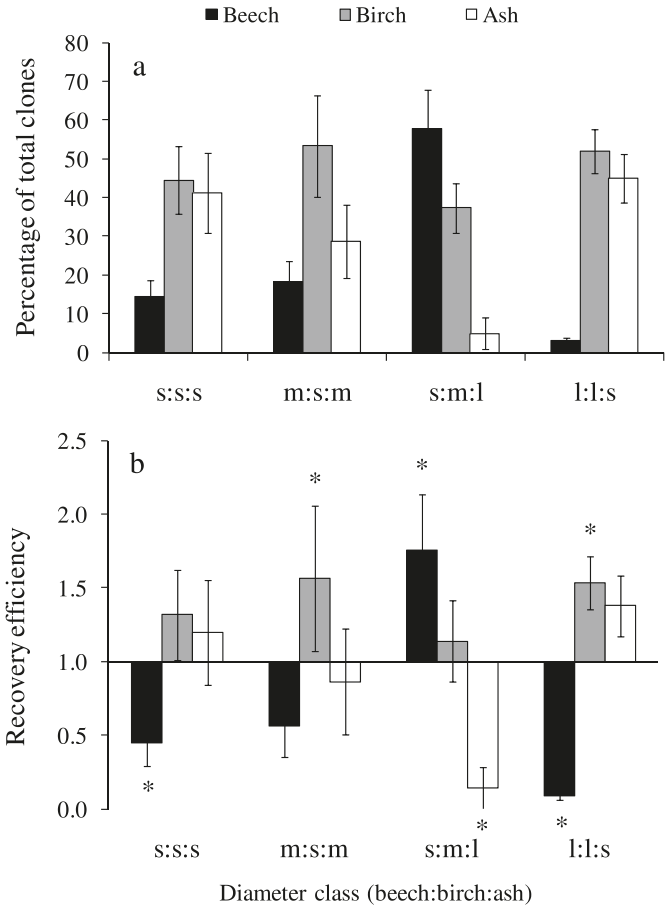
Root material was lyophilized and finely ground in a ball mill. We prepared six different mixtures of various ratios (by mass) of specified diameter classes of the three tree species. Three of these mixtures involved only small-diameter roots but different proportions of the three species. Three additional mixtures involved equal masses of the three species but different diameter classes by species. Each mixture was replicated three times, for a total of 18 samples. Each mixed sample totaled approximately 10 mg.

We extracted DNA from tissue of root systems of each individual species (<0.3 mm diameter class) and from each mixed sample using the MoBio Powerplant DNA isolation kit. Then the plastid *trnL* intron was amplified by PCR using primers C and D (Taberlet et al. 1991), a primer set that has been shown to distinguish a wide variety of tree species (Brunner et al. 2001). We followed the amplification reaction of Brunner et al. (2001), with 3 min of denaturation at 94 °C, 40 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 57 °C, and 2 min of extension at 72 °C, followed by a final extension at 72 °C for 10 min. The template was amplified with similar success from the three individual tree species, and the species identity of the individual root systems that we collected was confirmed by sequencing. We constructed clone libraries using PCR products from each mixed sample using Invitrogen TOPO TA cloning kits following the manufacturer's instructions. Forty to 60 clones per sample were PCR amplified using M13 primers, and PCR products were sequenced at Agencourt Bioscience, Beverly, Massachusetts. Sequences were identified to species by BLAST match (Altschul et al. 1997) to the GenBank nonredundant database.

The results are reported as the proportion of total sequences in a sample contributed by each species. We also calculated recovery efficiency for each species in a sample as the proportion of sequences reported divided by the proportion of mass represented by that species in the sample. Thus, a recovery efficiency <1 means that sequences of that species were underrepresented relative to that mass of that species in the root mixture, and a recovery efficiency >1 means that sequences of that species were overrepresented relative to mass of that species.

We used two-way ANOVAs to test the effects on recovery efficiency (SAS, version 9.1.3, SAS Institute Inc., Cary, North Carolina). The first analysis involved four mixtures of equal masses of the three species but different diameter classes. Each mixture was replicated three times, for a total of 36 observations. The second analysis involved three mixtures of root species in different proportions by mass; each mixture was replicated three times, for a total of 27 observations of recovery efficiency by species. One mixture (equal

Fig. 1. Percentage of total sequences per library (*a*) and recovery efficiency (proportion of total sequences/proportion of total mass) (*b*) by species for different combinations of root diameter classes. s, small (<0.3 mm); m, medium (0.3–0.5 mm); l, large (0.5–1.0 mm). Error bars are standard errors of the mean; $n = 3$ replicate root mixtures. Recovery efficiencies that differ significantly from 1 are indicated by an asterisk ($P < 0.05$).



masses of small roots) was used in both analyses. We tested whether recovery efficiencies differed from 1 using 95% confidence intervals of each species-mass ratio or species-diameter combination.

Results

Each of the three tree species was represented in the clone library of every replicate mixture, indicating that our approach was successful at identifying species of roots in mixed communities. No other species were detected. Amplicon size varied among our species (birch = 444 bp, ash = 548 bp, and beech = 610 bp), but % G+C of the *trnL* intron was very similar (38% G+C in birch, 36% in ash, and 34% in beech).

We studied three diameter classes of roots within our samples of fine roots (all <1 mm). For beech and ash, larger roots (>0.3 mm in diameter) were underrepresented in the clone libraries relative to finer roots (Fig. 1*a*). These mixtures were all 1:1:1 in terms of mass, but recovery efficiency of small-diameter roots of birch exceeded 1 (Fig. 1*b*). Sequences from these small birch roots exceeded 50% of the total when mixed with medium-diameter beech and ash roots (Fig. 1*b*). In contrast, recovery efficiency of large-diameter ash was well below 1 (Fig. 1*b*), such that small-diameter beech accounted for the highest percentage of sequences in the libraries constructed from mixtures of medium-diameter birch and large-diameter ash roots (Fig. 1*a*). For birch, however, larger-diameter roots did not have reduced recovery efficiencies in the clone libraries (Fig. 1*b*, Table 1). This difference in the effect of diameter across the three species is described by a highly significant species-by-diameter interaction (Table 1).

Table 1. Analysis of variance of recovery efficiency in clone libraries.

Source	df	*F*	*P*
Analysis 1			
Diameter class	3	0.01	1.00
Species	2	8.94	0.001
Diameter class × species	6	7.88	<0.0001
Error	24		
Analysis 2			
Mass ratio	2	4.20	0.03
Species	2	30.88	<0.0001
Mass ratio × species	4	3.70	0.02
Error	18		

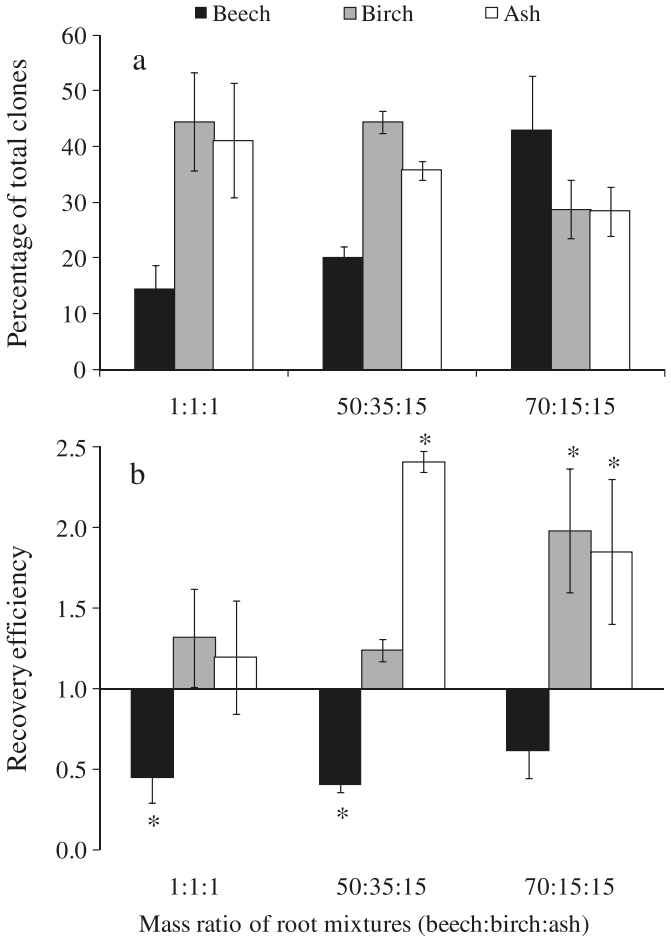
Species effects on recovery efficiency were highly significant (Table 1). Specifically, sequences of beech roots were underrepresented relative to those of birch and ash (Fig. 2*a*), and beech recovery efficiencies were <1 for all mass–ratio mixtures (Fig. 2*b*). Beech accounted for about 15% of total sequences in the 1:1:1 mass–ratio mixture and about 20% of total sequences in the 50:35:15 (beech:birch:ash) mass–ratio mixture, which is less than half of the expected number in both cases. Only in the 70:15:15 (beech:birch:ash) mass–ratio mixture did the percentage of beech sequences exceed that of birch and ash, but beech accounted for 40% of the total rather than the expected 70%. Ash and birch roots had similar representation in the clone libraries when they were present in similar amounts in the mixtures (1:1:1 and 70:15:15) (Fig. 2*a*). Their recovery efficiencies were thus similar in these cases (Fig. 2*b*).

Recovery efficiencies were also sensitive to mass ratios in the root mixture, with species present in smaller amounts having higher recoveries (Table 1). Birch and ash were represented equally at 1:1 mass ratio, but while proportions of clones of the two species followed the order expected at 35:15 mass ratio (Fig. 2*a*), these two species were represented more similarly than expected. The recovery efficiency of ash in this mixture was significantly greater than 1 and substantially exceeded that of birch (Fig. 2*b*).

Discussion

The method we tested involved extracting community DNA from a mixed sample of roots, constructing clone libraries, and quantifying the relative abundance of species based on frequency of sequences in the clone libraries. This sequence-based approach was successful at identifying spe-

Fig. 2. Percentage of total sequences per library (*a*) and recovery efficiency (proportion of total sequences/proportion of total mass) (*b*) by species for mixtures of roots varying in mass ratio (beech:birch:ash). All roots were <0.3 mm in diameter (small-diameter class). Error bars are standard errors of the mean; $n = 3$ replicate root mixtures. Recovery efficiencies that differ significantly from 1 are indicated by an asterisk ($P < 0.05$).



cies of roots in mixed communities, providing an efficient method for estimating relative abundances of roots from different species. Our results show that this type of community analysis should be applied and interpreted carefully because sequence representation can be sensitive to root diameter, species, and possibly also to relative abundance. Given these cautions, the estimates of fine-root relative abundance yielded by this method can be used to test important questions about belowground plant community composition and spatial structure. For example, this approach could be used to describe spatial variation of root deployment by species or differential species responses to resources, competitive interactions, or environmental stresses. The growing ease and economy of sequencing approaches will reduce variation associated with analyzing limited numbers of clones and will make this method increasingly efficient in terms of time, materials, and analysis expense, and highly appropriate for replicated experimental or comparative work. Thus, this sequence-based approach for quantifying root communities in mixed root samples should be an effective method under most circumstances.

Our results emphasize the importance of carefully choosing root diameter classes and show that bias associated with root diameter distributions can be minimized by focusing on the small diameter roots that contribute most of the root length. We found lower sequence representation for roots of larger diameter, even though all of our root classes were <1 mm in diameter. Likely explanations for root diameter effects include differences in gene copy number and tissue chemistry of larger, more structural, and less metabolically active roots. Many studies of fine roots in forest ecosystems sample all roots ≤1 mm diameter or sometimes ≤0.5 mm. However, roots ≤0.3 mm account for most of the fine-root length in this forest type (Fahey and Hughes 1994; Pregitzer et al. 2002), and we found a clear difference in recovery efficiency between <0.3 mm and 0.3–0.5 mm size classes. Therefore, in the forest type we studied, the <0.3 mm size class would probably best represent the species composition of fine-root activity in the ecosystem. This size class corresponds well to roots of low order in our study (first and second), and many tree species have a similar increase in diameter from first to third order roots (Pregitzer et al. 2002). It is possible that the proportion of first and second order roots affects recovery efficiency within our smallest size class, as root order has been shown to predict chemistry and turnover (Guo et al. 2004, 2008). However, root diameter is a more practical classification criterion in field studies using coring methods that disrupt intact root systems.

We found differences among species in the number of sequences detected per unit mass of root material. The most likely potential causes of differential species recovery include effects of root tissue chemistry on DNA extraction efficiency or PCR amplification efficiencies and differences in plastid concentrations or gene copy number. There are reasons to expect that some other sources of bias are not important in our comparisons. Primer regions in each of our species were exact matches with primer sequences, eliminating primer degeneracy as a potential cause of low PCR efficiency (Polz and Cavanaugh 1998). Two other sources of bias, differences in %G+C and amplicon size, can be important in microbial communities that encompass broad taxonomic variation (von Wintzingerode et al. 1997) but appear to result from wider variation in the properties of the target gene than exists among tree species in the *trnL* intron region. Percent G+C was uniformly low in our species, varying among species by only 4%. Amplicon size varied more, with a range almost as great as that reported by Brunner et al. (2001), of 444–672 bp for 30 tree species. However, these sizes fall well between the small and large sizes that have been shown to affect cloning success (Taylor et al. 2007; Huber et al. 2009). Since we tested only three species and found two to be similar, it seems likely that some tree communities will have even fewer differences than we found in recovery efficiency. For more accurate quantification of root community composition, correction factors could be developed for the taxa of interest to account for any differences in recovery efficiencies.

Our test for a bias associated with relative abundance showed that species representing a smaller fraction of the

mass in our root mixtures had disproportionately higher sequence representation. This could be the result of template inhibition, in which replication of the initially more abundant template begins to saturate earlier in the amplification process than that of less abundant templates (Suzuki and Giovannoni 1996). The extreme variation in abundance that we tested (15% vs. 70%) would not be common in more diverse communities. In most cases, the bias associated with relative abundance could be reduced by using fewer amplification cycles (Suzuki and Giovannoni 1996).

In conclusion, the sequence-based approach provides a relatively simple means of identifying the species of roots present in a sample and estimating their relative abundance. Many questions about the relative abundance and spatial distributions of roots, such as the relative response of species to competitive interactions or environmental variables, could be addressed using this approach without further methods development. With the increasing adoption of high-throughput sequencing technologies that do not rely on clone library construction (i.e., Illumina, 454, and ABI Solid sequencing technologies), we expect that the use of this or related DNA approaches will become more widespread, improving the efficiency and feasibility of root research at large scales and across a range of plant community types. We recommend careful attention to the selection of fine-root diameter class in community studies and suggest that further work examining recovery efficiency by species and in relation to abundance can improve the quantitative accuracy of assessing root communities using this approach.

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