Abstract: An understanding of factors influencing the distribution of plant roots is intimately linked to our understanding of basic ecosystem functions such as nutrient flux and productivity. However, it is not usually possible to measure root distributions because it is difficult to identify the roots of different species when they are grown in mixture. This is because the roots of most species are not visually distinguishable. We designed a simple, PCR-based method for the identification of roots in mesocosm experiments, which we have applied to 10 co-occurring grassland species. Species-specific primers based on ITS sequences from GenBank were evaluated in PCR assays using either homogeneous or heterogeneous DNA templates, as well as DNA extracted from mixed-root samples from multiple combinations of species. The species-specific primers reported here produced accurate identifications, free from both false negatives and false positives, in 100% of our assays. We also evaluated the sensitivity of our system and demonstrated detection of species when they comprised as little as 0.05 ng of target DNA mixed in a total of 2.5 ng of multi-species template DNA. Our PCR-based method for root identification in mesocosms is more cost effective, and simpler to apply than previously described methods.

Key words: root identification, grasslands, species-specific primers, root foraging.

Introduction

Factors that affect the productivity and nutrient flux within ecosystems have great potential to affect ecosystem structure and function. Most plant species have the ability to nonrandomly place their roots in regions of high soil fertility (Drew 1975; Crick and Grime 1987; Campbell et al. 1991). This ability leads to higher nutrient capture, and thus greater productivity of individual plants, than if roots were randomly arranged in the soil. An understanding of these basic patterns of root distribution should be intimately linked to our understanding of ecosystems. However, almost nothing is known about how important these root responses are in natural systems (Hodge 2004; Kembel and Cahill 2005). A major limitation of our ability to link patterns of small scale root distributions to larger scale ecosystem processes has been a lack of robust methods for identifying roots of co-occurring species (Bobowski et al. 1999; Linder et al. 2000; Brunner et al. 2001; Ridgway et al. 2003; Moore and Field 2005).

Essentially all of the evidence concerning root placement in soil comes from mesocosm experiments. Mesocosms are useful tools for addressing questions about root distributions, because in a field setting it is impossible to know the distribution of plant roots before the start of an experiment. Furthermore, it has been necessary for studies concerning root distributions to focus on individual plants grown in isolation (e.g., Campbell et al. 1991; Lamb et al. 2004), or on single species grown in monoculture (e.g., Casper and Cahill 1996,
1998; Franzen et al. 2001). This is because the fine roots of different species are not visually distinguishable and species-specific differences cannot typically be measured when more than one species co-occur. However, the results of single species mesocosm experiments are of limited relevance to natural systems, where plants rarely occur in the absence of interspecific competition (Robinson et al. 1999; Franzen et al. 2001; Bliss et al. 2002; O’Brien et al. 2005; Rajaniemi 2007).

As a response to these technical challenges, several methods have been developed that use DNA-based methods to identify individual root fragments, one at a time (Bobowski et al. 1999; Linder et al. 2000; Brunner et al. 2001; Ridgway et al. 2003). These methods are all based on either DNA sequencing or RFLP keys, which work best on homogeneous single-species samples. While such methods represent a significant technological advance, it should be clear that even a modest sized mesocosm experiment would contain an impractically large number of root fragments, each of which would have to be isolated and analyzed individually. Moore and Field (2005) recognized this fact and took root identification one step further. They showed that samples containing mixtures of up to four species could be distinguished with their RFLP keys. However, this method is not easily scalable, in part because RFLP patterns have an increasing chance to blend together and overlap as more species co-occur in a sample.

Each of these previously described root identification methods begins with PCR, and proceeds to use various downstream reactions to characterize the PCR products and assign taxonomic identity to each specimen. It would be much more cost and time effective if PCR were the first and last step in the process. Furthermore, since questions about root distributions are almost always confined to mesocosms, the high taxonomic resolution of more expensive techniques, such as DNA sequencing or RFLP keys, are not required. Our goal was to develop a new method for root identification which was (i) applicable to multi-species samples of any number of species, and; (ii) more cost and time effective than previously described methods. In this paper we report a set of species specific PCR primers designed for this purpose. We show that these patterns are robust and species specific, and can lead to species identifications using simple agarose gel electrophoresis.

**Materials and methods**

**Species and tissue collection**

We selected 10 naturally co-occurring grassland species to develop this method. Species selection was influenced by our primary goal of addressing central questions in plant ecology. We included species from a wide range of natural abundances (common to rare), members of all grassland functional groups (shrubs, grasses, forbs) and species which have been extensively studied in previous mesocosm studies (e.g., *Achillea millefolium* L. and *Poa pratensis* L.) (Table 1). Leaf tissue and seed were field collected from spatially separated individuals by haphazardly sampling along an 11 km transect at the University of Alberta Kinsella Ranch (53°00.950’N, 111°32.403’W) every 2 weeks during the summer of 2005. Leaf tissue from each species was always

<table>
<thead>
<tr>
<th>Species (family)</th>
<th>Species specific primer sequence</th>
<th>GenBank accession No.</th>
<th>Herbarium accession No.</th>
<th>PCR product size (bp)</th>
<th>Multiplex grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rosa arkansana</em> var. <em>arkansana</em> Porter (Rosaceae)</td>
<td>TCA CGC CGG TGT TCA GTA</td>
<td>AJ631862.1</td>
<td>116082</td>
<td>C</td>
<td>186</td>
</tr>
<tr>
<td><em>Festuca hallii</em> (Vasey) Piper (Poaceae)</td>
<td>CGG ATG CAC TGC GTT TAG T</td>
<td>AF532952.1</td>
<td>116074</td>
<td>D</td>
<td>558</td>
</tr>
<tr>
<td><em>Achillea millefolium</em> L. (Asteraceae)</td>
<td>CGT CAA TGA CAC ATT CAC CAG</td>
<td>AF532952.1</td>
<td>116074</td>
<td>E</td>
<td>497</td>
</tr>
<tr>
<td><em>Poa pratensis</em> L. (Poaceae)</td>
<td>GGT CcT TAA GGc CAT CAC</td>
<td>AF171183.1</td>
<td>116074</td>
<td>B</td>
<td>588</td>
</tr>
<tr>
<td><em>Koeleria macrantha</em> (Ledeb.) J.A. Schultes (Poaceae)</td>
<td>GGG TCT TTA GAG GCC ATC G</td>
<td>Z96910.1</td>
<td>116074</td>
<td>C</td>
<td>552</td>
</tr>
<tr>
<td><em>Artemisia ludoviciana</em> Nutt. (Asteraceae)</td>
<td>AAA GCG TCG AAA GGA TCA AA</td>
<td>AF171183.1</td>
<td>116074</td>
<td>E</td>
<td>497</td>
</tr>
<tr>
<td><em>Thermopsis rhombifolia</em> (Nutt. Ex Pursh) Nutt. ex Richardson (Fabaceae)</td>
<td>GGG AcG CAc TAG ACA ATC T</td>
<td>AF007468.1</td>
<td>116074</td>
<td>A</td>
<td>208</td>
</tr>
<tr>
<td><em>Astragalus agrestis</em> Douglas ex G. Don (Fabaceae)</td>
<td>GCA TGC ACA TGA TAG ATC T</td>
<td>L107591.1, L107581.1</td>
<td>116074</td>
<td>A</td>
<td>486</td>
</tr>
<tr>
<td><em>Campanula rotundifolia</em> L. (Campanulaceae)</td>
<td>GAC AAG GAA GGG GTC AAA TG</td>
<td>DQ304651.1</td>
<td>116074</td>
<td>D</td>
<td>136</td>
</tr>
<tr>
<td><em>Bromus inermis</em> L. Leyss. (Poaceae)</td>
<td>CAA CAC AAG AGA TGA CCA GCA</td>
<td>AY367915.1</td>
<td>116074</td>
<td>A</td>
<td>224</td>
</tr>
</tbody>
</table>
sampled from flowering individuals to guard against misidentification. Leaf tissue was immediately dried using silica gel and stored at –20 °C (Chase and Hills 1991). Seeds were stored at 4 °C. Reference specimens were collected at the same time from the field site and deposited in the University of Alberta Vascular Plant Herbarium (ALTA) (Table 1).

**Species specific primer design**

We selected the internal transcribed spacer (ITS) region of ribosomal DNA as the site for primer design. This was done for three reasons: (i) the ITS region has been successfully applied to the problem of root identification (Linder et al. 2000; Moore and Field 2005); (ii) sequence variability in the ITS region has been shown to be among the most useful tools for phylogenetic inference (White et al. 1990; Baldwin et al. 1995; Alvarez and Wendel 2003); (iii) the ITS region is one of the most widely available DNA sequences in GenBank. Any available DNA sequence could conceivably be used to design species-specific primers and, in all cases, specificity must be demonstrated empirically.

Based on the general topology of the ITS region (Baldwin et al. 1995; Alvarez and Wendel 2003), and the fact that we did not require high taxonomic resolution for mesocosm experiments, we reasoned that we did not need to design two unique primers for each species. Instead, we designed a single species-specific primer that could be paired with the universal ITS5m primer (Saar et al. 2001) in all PCR reactions. By doing this, we reduced the amount of unique sequence necessary for positive species identification to about 20 bp. ITS sequence data for each species was downloaded from GenBank (Table 1). Primers were designed using the Primer3 program (Rozen and Skaletsky 2001). The PCR product size was designed to be approximately 500 bp for half of our focal species, and 200 bp for the other half (Table 1). This was done to add additional landmarks for the visualization and quantification of PCR products, and to potentially permit multiplexing of PCR reactions. The assignment of species to each PCR product size class was based on the best location for a primer, as predicted by Primer3.

**DNA extractions**

All DNA extractions were done using a powerful method for DNA extraction specifically designed for roots and described by Brunner et al. (2001).

**Specificity using homogeneous templates**

To test the species specificity of each primer, we conducted PCR with genomic DNA of two individuals of each of the 10 species, in a full factorial design of each combination of a unique primer and genomic DNA from an individual species. Reactions were performed in a total volume of 15 µL containing 1X PCR buffer (New England Biolabs, Toronto, Ont.), 0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP (Invitrogen, Burlington, Ont.), 0.25U of Taq polymerase (New England Biolabs) and 2.5 ng of template DNA. Reaction temperature conditions were as follows: 1 min initial denaturation at 94 °C, and 25 cycles of 30 s denaturation at 94 °C, 30 s annealing, 30 s extension at 72 °C, followed by a final extension of 5 min at 72 °C. The optimal annealing temperature differed among primers (Table 1). Seven µL of each reaction was run on a 1% (w/v) agarose gel and visualized using ethidium bromide to confirm successful amplification.

Primers that failed to be species specific were redesigned as necessary. If re-designed primers were also nonspecific, we used locked nucleic acid (LNA, Exiqon, Woburn, Mass.) modifications to increase primer melting temperature and thus, primer specificity (Letertre et al. 2003).

**Multiplex PCR**

To minimize the number of PCR reactions, combinations of primers were tested in multiplex PCR reactions until five multiplex mixtures of two primers each (plus the universal primer) were identified (Table 1). To compensate for potential differences in efficiency related to target sequence length, we added 0.2 µmol/L of the species specific primer that corresponded to the ∼200 bp target sequence, and 0.4 µmol/L of all other primers in all multiplex PCR reactions. Although further multiplexing could potentially reduce the cost of the assay, we did not attempt to optimize our method for a larger number of primers in a single reaction. Increasing the number of primer pairs in a PCR reaction increases the potential for undesirable interactions among components of the reactions. Moreover, because PCR efficiency is related to the length of amplified product, the large range of product sizes that would be required for electrophoretic resolution of bands from 10 multiplexed primers could differentially affect the sensitivity of detection for individual species. This is especially true in our case since different products competed not only for typical components of the reaction but also for access to the ITS5m primer.

**Assay of DNA extracted from a mixture of roots**

We grew monocultures of 8–10 focal species in a greenhouse. Root tissue was collected and dried in silica gel (Chase and Hills 1991). Eight different combinations of four randomly selected species were made by mixing equal biomass of root tissue of four species (see supplementary data2, Table S1). Combinations of species were limited to only 8 of our 10 focal species, because the field-collected seed from *Rosa arkansana* Porter and *Astragalus agrestis* Douglas ex G. Don did not germinate. The DNA was extracted from each root mixture, and the species present were identified using five multiplex PCR reactions per sample as described above.

**Assays of heterogeneous templates**

We mixed genomic DNA samples that had been extracted from individual species (rather than mixing the tissue and then extracting the DNA). This was done so that we could include DNA from *R. arkansana* and *A. agrestis* in our validation of our method. 16 different mixtures of genomic

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2 Supplementary data for this article are available on the journal Web site (http://botany.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 3735. For more information on obtaining material refer to: http://cisti-icist.nrc-cnrc.gc.ca/cms/unpub_e.html.
DNA were made that included five mixtures each of 4, 6, and 8 randomly selected focal species, and one mixture of all 10 species (see supplementary data2, Table S2). DNA stocks of each species were diluted to a concentration of 10 ng/μL and mixtures were created by adding equal volumes of the DNA from each species to a constant final volume.

Assays of sensitivity
To test the limits of detection, we performed PCR reactions on a dilution series of three samples containing two selected species each. In these reactions, the DNA of one species was not diluted and remained common, while the second target species was increasingly diluted making it increasingly rare. The abundances of the target species in these reactions were: 10%, 8%, 6%, 4%, 2%, 1%, and 0.5% of 2.5 ng total DNA, and the common species made up the remainder of the DNA in these samples. The species were randomly selected and included Campanula rotundifolia L. (136 bp), Festuca hallii (Vasey) Piper (558 bp), and Bromus inermis Leyss. (224 bp) as target species, and A. millefolium as the common species in all three cases.

Results
Specificity using homogeneous templates
After reaction conditions were optimized, all primers produced species-specific PCR products in a full factorial design of each combination of a unique primer and genomic DNA from an individual species. In the case of P. pratensis, species specificity was achieved only after introducing LNA modifications to the primer (Table 1). It was also necessary to introduce LNA modifications into the Thermopsis rhombifolia (Nutt. Ex Pursh) Nutt. ex Richardson primer for use in multiplex group B (Table 1). This was not necessary to achieve species specificity for the T. rhombifolia primer, but only to ensure proper annealing at the increased annealing temperature required for the P. pratensis primer in a multiplex reaction.

Assays of DNA extracted from a mixture of roots
In the multispecies root mixtures, we always detected all species known to be present in a mixture (true positives), and never detected anything known to be absent (false positives) (see supplementary data2, Table S1). This was true regardless of the combination of species in a mixture.

Assays of heterogeneous DNA templates
Similarly, in the multi-species DNA mixtures we never observed any false positives or false negatives (see supplementary data2, Table S2). That is, our DNA based species identification method worked perfectly in 100% of our assays. The accuracy of the method was not influenced by the number, identity, or even the abundance of species in an unknown sample.

Assays of sensitivity
When 2.5 ng of mixed DNA from more than one species was used as a template in our assays, we were able to detect the presence of as little as 0.05 ng (2%) of DNA from within this mixture. To put this measurement of sensitivity in perspective, our standard DNA extraction procedure yields an average of 3000 ng of DNA from a 5 mg root sample, and thus, 0.05 ng is theoretically the amount of DNA that could be extracted from 83 ng of root tissue. However, owing to practical limitations on isolating roots from soil, the actual amount of root mass that can be detected in practice is expected to be somewhat higher.

Discussion
Our objective was to demonstrate that identification of soil-grown roots from a mixture of species is possible using PCR and simple gel electrophoresis. The species-specific primers we report here provided reproducible positive species identifications in 100% of our validation assays. As with any application of PCR, the method is extremely sensitive. We were able to detect extremely low absolute amounts of template DNA using PCR. This PCR-based method is more cost and time effective than previous methods designed for root identification because it relies exclusively on PCR and does not require any downstream reactions. Furthermore, a PCR-based method is more robust than previous methods because it can be applied directly to mixed species samples of any number.

Because our technique is PCR-based, it may be more easily adapted to certain applications than previously described (e.g., RFLP) methods. For example, future modifications of our method may allow for quantification of relative species abundance, using quantitative real-time PCR with fluorescent probes or dyes such as SYBR green (Mommert et al. 2008). Resolution and throughput of species detection could also be increased using multiple fluorescent dyes in automated DNA sequencers. Neither of these applications are possible using previously described root identification methods.

We have shown that, when present, very small amounts of template DNA present in a multi-species sample can be detected using PCR. However, in practice, the soil type and thoroughness with which the roots are washed prior to DNA extraction can influence the yield and quality of extracted DNA, and may influence sensitivity (Brunner et al. 2001; G. McNickle, unpublished data, 2007). Thus, instead of attempting to validate every possible set of extraction and growing conditions, we have shown that the basic behaviour of our species-specific primers in PCR reactions was robust. Researchers should calibrate the sensitivity of the method on actual roots for their particular set of growing conditions prior to application of the method.

The method we have presented here should not be confused with DNA barcoding. Although DNA barcoding methods also seek to make species identifications, the overarching goal of DNA barcoding initiatives is the development of methods for species identification that are universal across all taxonomic groups (Hebert et al. 2002; Savolainen et al. 2005). Our method stands in contrast: we were interested in distinguishing between a small set of specific, ecologically interesting species in mesocosms. Because the identities of all potential members of a mesocosm experiment can be known from the outset of the experiment, we did not require the universally high taxonomic resolution of standard DNA barcoding methods. Instead, we developed...
a simple, cost-effective technique with sufficient resolution for the identification of each species within a mesocosm. We have shown only that our method works consistently in mixed species samples from mesocosms in which only those 10 species occur. Application to other systems where any additional species outside our 10 focal species might occur (e.g., the field, or in pots with additional species) would require empirical testing of the species specificity of our primers against the DNA of those additional species.

Our understanding of the factors influencing root distributions has been overwhelmingly dependent on results from mesocosm experiments with single plants grown in isolation (Robinson et al. 1999; Franzen et al. 2001; Bliss et al. 2002; O’Brien et al. 2005; Rajaniemi 2007). Despite a proliferation of types of experiments in the literature there has been very little success in drawing connections between conclusions based on single plant-pot experiments and patterns of plant communities in the field. This is partially because there is little data available on the interaction between interspecific competition and root foraging ability. With a robust method for identification of roots to the level of species, previous single plant experiments can now be replicated to include multi-species competition treatments which more closely approximate the competitive environment a plant experiences in the natural world.

Acknowledgments

We thank the Natural Sciences and Engineering Research Council of Canada for Discovery Grants to J.F.C. and M.K.D., and Canadian graduate scholarship and post-graduate scholarship awards to G.G.M. The Alberta Conservation Association is thanked by G.G.M. and J.F.C. for an integrated, cost-effective technique with sufficient resolution for the identification of each species within a mesocosm. We have shown only that our method works consistently in mixed species samples from mesocosms in which only those 10 species occur. Application to other systems where any additional species outside our 10 focal species might occur (e.g., the field, or in pots with additional species) would require empirical testing of the species specificity of our primers against the DNA of those additional species.

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We thank the Natural Sciences and Engineering Research Council of Canada for Discovery Grants to J.F.C. and M.K.D., and Canadian graduate scholarship and post-graduate scholarship awards to G.G.M. The Alberta Conservation Association is thanked by G.G.M. and J.F.C. for a biodiversity grant. The Alberta Ingenuity Fund is thanked by G.G.M. for a graduate student scholarship. We thank Mary DePauw and Corey Davis for their advice throughout this project.

References


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