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COMMENTARY

Belowground DNA-based techniques: untangling the network of plant root interactions

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Introduction

Plant roots are a central driver of ecosystem productivity, as plant investments belowground often comprise more than half of total plant biomass (Jackson et al. 1996). Despite this general observation, almost nothing is known about the distribution of roots in ecosystems; generally because roots of different species are morphologically indistinguishable, restricting species identification. This is in strict contrast to plant identification aboveground, which is straightforward after initial taxonomic training. Although in species poor systems containing roots of two species morphological identification has been possible in a few cases (Genney et al. 2002; Janecek et al. 2004; Mommer et al. 2011), disentangling and identifying roots from species-rich systems is impossible. To overcome problems of species identification, pioneering DNA-based techniques have been applied to plant roots (Jackson et al. 1999; Linder et al. 2000) and are now being used in experimental and observational studies of species-rich plant communities (e.g. Mommer et al. 2010; Kesanakurti et al. 2011; Dumbrell et al. 2011). In this paper, we discuss the current state of molecular techniques for plant species identification and quantification from mixed root samples. We focus on crucial aspects in the methodology regarding primer choice, DNA extraction and PCR inhibition, showing the potential caveats and their solutions. Finally we briefly discuss a few questions in the field of root ecology that will be advanced significantly by the appropriate use of these molecular tools.

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Developing methodological tools for root ecology

Untangling species identity and abundance of plant roots has received considerable methodological attention over the last few years. Different approaches making use of biochemical to molecular advances have been followed. The basis of biochemical techniques is the difference in species specific

chemical composition of roots. The use of Near Infrared Reflectance Spectroscopy (NIRS) on milled mixed root samples shows species identity and quantity (Roumet et al. 2006). Alternatively, the differential composition of plant waxes can also be used to differentiate among species, as alkane and alcohol composition are species and tissue specific (Dawson et al. 2000). These biochemical methods provide accurate estimates of species identity and abundance in mixed root samples. However, different environmental conditions change the chemical properties of plant tissues (e.g. atmospheric CO_2 conditions, Soussana et al. 2005 or herbivory, Dawson et al. 2000) which makes the application of these methods more useful in controlled mesocosm experiments with a limited variability in soil chemistry, plant age and plant species richness compared with complex and diverse natural fields.

Molecular methods, based on genomic differences among species do not have these limitations. Jackson et al. (1999) and Linder et al. (2000) pioneered the use of DNA based techniques for indentifying plant roots. They selected many different single root fragments from trees, extracted DNA from each fragment separately and determined species identity using species-specific regions of the DNA (i.e. internal transcribed spacer (ITS)) and compare that to a reference database. Follow up studies used similar approaches to determine species identity using different loci (see references below). Using this qualitative approach to estimate relative species proportion in mixed root samples would require analysis of a very large number of small root parts from one mixed sample. Therefore, Mommer et al. (2008) developed an alternative approach to quantify relative species abundances in mixed root samples via quantitative real-time PCR on species-specific markers. In order to translate the relative abundance to root biomass, relative DNA abundances in species are calibrated against an accurate multi-species reference series.

At the moment, the era of next generation sequencing is in full swing and it is now potentially possible to generate more data from plant roots than can ever be analysed. Thus, it is crucial to focus research and insure a balance between ecological question and methodology. This commentary gives a glimpse of the molecular methodological issues that are topical at the moment.

Primer types: species- specific or universal markers?

Primer choice is a fundamental issue in molecular ecology and thus essential when undertaking molecular analyses of mixed root samples. The choice of primer largely determines which of the two main approaches are used (e.g. quantitative real time-PCR (qPCR) or large scale sequencing). The first option is to use and/or develop species-specific primer sets, which is initially time consuming in terms of finding optimal primers. Generally, ITS (e.g. Haling et al. 2011) or ISSR (Mommer et al. 2008) regions of the DNA of the different study species are amplified, sequenced, and tested for species specificity against reference sequence databases, to identify species-specific primer sets. However, new genomes are increasingly being submitted to sequence repositories (e.g. GenBank, NCBI) and finding species-specific primers will no doubt become easier and quicker. Once developed, species-specific primer sets produce robust and consistent (semi) quantitative species-abundance data, via qPCR (McNickle et al. 2008; Mommer et al. 2010; Haling et al. 2011).

The second option is to use universal DNA-barcode primers targeting plastid genes (Taberlet et al. 2007; Hollingsworth et al. 2009) combined with second generation sequencing techniques (e.g. Roche 454) for data generation. A large scale plant barcoding study concluded that *mat*K and *rbc*L are the most promising markers, based on assessments of recoverability, sequence quality and species discrimination (Hollingsworth et al. 2009). For example, 1000 tropical orchid species could be identified using the *mat*K DNA barcode (Lahaye et al. 2008). *rbc*L was used in a study of root diversity in grasslands (Kesanakurti et al. 2011), but appeared to have insufficient power to discriminate between roots of tree species from Barro Colorado Island, Panama (Jones et al. 2011). Jones et al. (2011), therefore, used *trn*H-*psb*A to discriminate between 33 species from 117 root fragments. However, in recent root studies, an alternative, and often less species specific, barcode (*trn*L) is more frequently used (Brunner et al. 2001; Ridgway et al. 2003; Frank et al. 2010; Dumbrell et al. 2010; Taggart et al. 2011). This variety in the usage of different barcode primers reflects that primer choices will always need to be adjusted to the molecular differentiation found across species within

a particular community or experimental system. At an even finer molecular resolution, primer sets have also been successfully designed to discriminate among roots of different individuals of the same species. For example, Saari et al. (2005) and Lang et al. (2010) have used microsatellite primers to distinguish between individuals of the same tree species.

Species recovery belowground: issues of primer specificity, DNA extraction, PCR inhibition

A problem with DNA barcoding studies on roots is that species recovery belowground appears suboptimal, as several plant species recorded aboveground and rooting in sampled locations, are not detected. Subsequently, studies fail to detect ≈15–30% of aboveground species belowground (Taggart et al. 2011; Kesanakurti et al. 2011) and significantly underestimate biodiversity. Yet reasons for this underestimation of species richness remain poorly investigated, but below we discuss three potential caveats and solutions. A general recommendation beforehand to increase species recovery would be to investigate if the problems are due to the root tissue or are species-specific by analyzing additional aboveground samples, against which belowground sequences can be compared and validated.

A initial problem with species detection may reflect the ability of the barcode region to differentiate between closely related species and/or the specificity of barcode primers. The inability of DNA barcodes to separate closely related plant species is a common problem. Although barcode genes tend to have highly conserved priming regions, many of them (e.g. *trn*L intron) have similar sequences across species within a genus (see Taberlet et al. 2007), leading to the detection of ‘species groups’. Improvements in barcode resolution have proven difficult, for example increasing the number of plastid loci to 3 did not or hardly increased species recovery (Fazekas et al. 2009; Taggart et al. 2011), but as aforementioned primer sets should be intensively tested and adjusted to the experimental system.

A second explanation for the apparent absence of species belowground when already recorded aboveground, can be low quality DNA in root samples. Kesanakurti et al. (2011) showed that 16% of 1531 DNA extracts were of insufficient quality for sequencing, which is a considerable amount. Therefore, knowledge of DNA degradation in roots from natural communities is essential. Riley et al. (2010) showed that DNA yield from dead roots of common pasture plants declined within a few days, even when originally grown in controlled conditions. It is unknown if DNA from some species degrades faster than that of other species, but this could potentially occur. Haling et al. (2011) also investigated the effect on DNA yield of storing root samples, but storage at 4°C for a few days did not decrease the DNA yield in the species tested. However, storage at 20°C did significantly decrease DNA yield (Bainard et al. 2010; Riley et al. 2010).

Other than careful preservation of samples before DNA extraction, another option for increasing DNA yield is to examine new techniques in DNA extraction. For example, work on ancient plant DNA is beginning to provide protocols and commercially available kits specifically designed for degraded and/or old woody root tissues (see Parducci and Petit 2004; Gugerli et al. 2005; Finkeldey et al. 2010). In addition, other methods have been implemented for plant tissue with high polysaccharide levels that may inhibit DNA isolation (Shepherd and McLay 2011). A factor known to decrease DNA extraction is plant age, or more specifically tissue age. DNA yield appeared lower from older plants (Haling et al. 2011) and old woody roots (Finkeldey et al. 2010), as DNA degradation is a controlled cellular process during cell death. In such cases the use of primers that amplify short fragments is recommended (see Sønstebø et al. 2010).

A third limiting and potentially important factor for species identification in roots can be PCR inhibition. Roots and leaves potentially differ in the type of inhibitors that are at work. PCR inhibition in roots often comes from attached humic acids that are present in the soil. Humic acid binds to $MgCl_2$ and inhibits PCR. PCR inhibitors from leaves may come from complex polysaccharides found in the plant tissue. As the more general plant DNA extraction kits are good at removing plant-based inhibitors (e.g. some polysaccharides) but are often not designed for soil, generally more of the soil based inhibitors will remain in final samples. Various methods for removing soil based PCR inhibitors and exist, for example via the addition of proteins that bind to humic acids (Tebbe and Vahjen 1993).

The degree of PCR inhibition can easily be tested by adding an internal DNA standard to the actual DNA extracts. Haling et al. (2011) showed the value of such an approach. If an internal standard (e.g. a known quantity of ground lucerne seeds) was added to the root samples, a strongly significant linear correlation between root biomass and species abundance (based on species-specific quantitative PCR primers) was observed for each of their study species (Haling et al. 2011). By adding this internal standard, they even challenged the most time-consuming part of root research: washing plant roots. Haling et al. (2011) extracted root DNA directly from soil cores and used (qPCR) for further analyses. Interestingly, adding an internal standard to *washed* root samples from any system (experimental or field) will lead to stronger correlations between root biomass and DNA yield via qPCR estimates, resulting in fully rather than semi quantitative data of roots in mixed samples.

Roots: living or dead?

In the grasslands studied by Kesanakurti et al. (2011), 39 species were observed aboveground, but 10 of these were not recovered belowground. Interestingly, also 10 'new' species were found that were not observed in the aboveground samples. This discovery of new species can be explained by sampling error, incomplete aboveground identification or may be due to the fact that species may have been dormant belowground. Another explanation might be that species recovery in DNA extracts of roots may reflect both current and historic species, as it has always been difficult to separate dead roots from living ones. To illustrate this point, Sønstebø et al. (2010) showed that in 15-thousand-year-old permafrost samples up to 47 molecular taxonomic units could be identified. Thus, to disentangle live and dead roots we require methods that screen for biologically active roots. One possibility could be to extract total RNA from samples, and screen for the activity of commonly studied household genes, using species specific qPCR primers.

Exploring bare ground

After these methodological issues raised above have been carefully solved, the way is open for answering key questions in root ecology. One of the long-standing hypotheses that can finally be investigated is if the observed positive relationship between primary production and plant species richness (Hooper et al. 2005) is driven by spatial niche differentiation through differences in rooting distribution (Berendse 1983; Mamolos et al. 1995; Parrish and Bazzaz 1976). Roots of different species may occupy different realized niches. When several species are growing in mixtures the community is expected to explore a broader niche space (i.e. soil volume) and produce more biomass than each of the species separately (Berendse 1983; Fargione and Tilman 2005; Levine and HilleRisLambers 2009). Until recently, no information on the root distributions was available that allowed for testing the spatial niche differentiation hypothesis. The application of molecular markers in a biodiversity experiment showed that root distributions of four grassland species were less different than expected, giving only slight support for niche differentiation (Mommer et al. 2010). However, mixtures produced 40% more biomass belowground than expected as observed more often from grasslands (Tilman et al. 2001; Dimitrakopoulos and Schmid 2004; Reich et al. 2004); and forests (Brassard et al. 2011), suggesting that species-specific below ground interactions are driving the biodiversity-productivity relationship.

Another topic that will benefit from the applying molecular techniques is the horizontal distribution of roots. The question of: if and under which conditions plant roots are territorial? is still inconclusively answered (Schenk et al. 1999). Caldwell and coworkers observed segregated root systems of shrubs and grasses around cold desert shrubs (Caldwell et al. 1991). However, in other environments contrasting results are found. Root distributions of beech (*Fagus sylvatica*) individuals in monoculture stands did not indicate root segregation (Lang et al. 2010). Results of Jones et al. (2011) also suggest that roots in hyperdiverse tropical forests appear to overlap more belowground than do their crowns aboveground, suggesting intense root aggregation. The spatial extent to which belowground plant-plant interactions occur, and how these differ from the aboveground scales occur will be revealed in the coming years, using state of the art DNA techniques.

Root growth is mainly driven by nutrient and water availability, but numerous chemical substances (Bais et

al. 2006) and soil biota (bacteria, fungi, nematodes) (Kardol et al. 2007; Raaijmakers et al. 2009; Bever et al. 2010) are also known to moderate root growth. Since the composition of the rhizosphere community may be driven largely by plant species identity (Bezemer et al. 2010), it is highly likely that interspecific root interactions act partly via these soil organisms. DNA-based investigations of root abundance yield as a 'bonus', information about soil biota, such as arbuscular mycorrhizal (AM) fungi and fungal pathogens that are attached to the plant roots. For example, by using plant DNA barcodes, Dumbrell et al. (2010) showed that the composition and structure of plant-associated AM fungal communities was primarily determined by the local soil environment and not the identity of the host-plant species. However, this is unlikely to be true for the majority of rhizosphere biota, where the species identity of the host-plant is likely to play a major role. Only the application of DNA based techniques allows this to be fully examined in natural systems. The new DNA based methods, currently developed to identify and quantify belowground plant structures in diverse soils will unveil the interactions among plant roots and their soil biota.

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