

# Spatial patterns of plant diversity below-ground as revealed by DNA barcoding

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## Abstract

Our understanding of the spatial organization of root diversity in plant communities and of the mechanisms of community assembly has been limited by our ability to identify plants based on root tissue, especially in diverse communities. Here, we test the effectiveness of the plastid gene *rbcL*, a core plant DNA barcoding marker, for investigating spatial patterns of root diversity, and relate observed patterns to above-ground community structure. We collected 3800 root fragments from four randomly positioned, 1-m-deep soil profiles (two vertical transects per plot), located in an old-field community in southern Ontario, Canada, and extracted and sequenced DNA from 1531 subsampled fragments. We identified species by comparing sequences with a DNA barcode reference library developed previously for the local flora. Nearly 85% of sampled root fragments were successfully sequenced and identified as belonging to 29 plant species or species groups. Root abundance and species richness varied in horizontal space and were negatively correlated with soil depth. The relative abundance of taxa below-ground was correlated with their frequency above-ground ( $r = 0.73$ ,  $P = 0.0001$ ), but several species detected in root tissue were not observed in above-ground quadrats. Multivariate analyses indicated that diversity was highly structured below-ground, and associated with depth, root morphology, soil chemistry and soil texture, whereas little structure was evident above-ground. Furthermore, analyses of species co-occurrence indicates strong species segregation overall but random co-occurrence among confamilials. Our results provide insights into the role of environmental filtering and competitive interactions in the organization of plant diversity below-ground, and also demonstrate the utility of barcoding for the identification of plant roots.

**Keywords:** barcoding, checkerboard sampling, competitive interactions, roots, species co-occurrence, species diversity

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## Introduction

The spatial and temporal organization of biological diversity within communities has long interested ecologists

because of its relation to assembly processes (Cody & Diamond 1975), community dynamics and ecosystem function (Tilman *et al.* 1997; Webb *et al.* 2002; Silvertown 2004; Hooper *et al.* 2005). The magnitude and distribution of species diversity is the product of two nonmutually exclusive mechanisms: environmental filtering (Cody & Diamond 1975; Weiher *et al.* 1998; Weiher & Keddy 1999) and interspecific competition for limiting resources (Tilman 1982, 1988). In the first case,

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the distribution of species within communities reflects spatial heterogeneity in the environment, coupled with differences in the establishment and persistence of species because of niche differentiation (Weiher & Keddy 1999; Cornwell *et al.* 2006). Species tend to sort according to the scale and grain of the abiotic environment, and therefore tend to coexist with ecologically and phylogenetically similar species compared with the regional species pool (Losos 2008). In the second case, community structure reflects the past history of interspecific interactions (e.g. competition; Tilman 1988). These interactions cause exclusion of similar species and coexistence of dissimilar species relative to the regional pool (MacArthur & Levins 1967; Abrams 1983; Tofts & Silvertown 2000).

The analysis of plant communities has played a central role in the study of community structure, community assembly and coexistence. Researchers have examined patterns of species coexistence, ecomorphological dispersion of coexisting species (Armbruster *et al.* 1994; Weiher *et al.* 1998), phylogenetic relations of co-occurring species (Webb *et al.* 2002; Cadotte *et al.* 2009), and factors governing the distribution and magnitude of diversity, such as extrinsic disturbance (e.g. fire, soil erosion; Reice 1994) and interspecific interactions (Hartnett & Wilson 1999; Reynolds *et al.* 2003). In most cases, these factors are related to patterns of species or trait diversity observed in the vegetative communities above-ground. Attention to community structure below-ground, consisting of roots, seeds and rhizomes, and its relation to above-ground patterns is poorly understood (Casper & Jackson 1997). This is surprising given that the primary organs of nutrient acquisition exist below-ground (Jackson *et al.* 2008), and that strong abiotic gradients in water, nutrients, and soil texture exist in vertical and horizontal space. Some studies (Wilson 1988; Casper & Jackson 1997) suggest that below-ground interactions may, in fact, be more important than above-ground interactions for organizing diversity.

The general lack of information on root community structure reflects the practical difficulties in identifying roots to the level of species, quantifying diversity and mapping root distributions (Mommer *et al.* 2008). Various methods have been used in the past, with mixed success. Researchers have attempted to map roots through detailed excavation of soil profiles (Kummerow *et al.* 1977; Brisson & Reynolds 1994), but roots are often entangled and difficult to trace back to individual plants. Identification of roots based on morphology may be possible to a limited extent (Cutler *et al.* 1987) but is generally not possible at a community scale, given similarities in root architecture, colour and structure among species. As a result, biologists have tended

to focus on the relation between above-ground plant diversity and abundance, and below-ground properties, such as root biomass and density (Hook *et al.* 1994; Mou *et al.* 1995). More recently, ecologists have used chemical composition as a species marker when diversity is limited (Caldwell *et al.* 1996; Roumet *et al.* 2006). Similarly, the extent of root systems has been inferred using radioactive tracers or stable isotopes to determine from where resources are being drawn (Caldwell & Eissenstat 1987; Mamolos *et al.* 1995).

DNA-based methods offer a potentially powerful set of tools for exploring fine-scale patterns of species diversity below-ground through the analysis of roots. They allow taxa to be identified by comparing DNA sequences (Linder *et al.* 2000) or restriction-fragment lengths (Bobowski *et al.* 1999; Ridgway *et al.* 2003) against reference databases. The efficacy of DNA-based methods, including DNA barcoding, in the study of root organization in plant communities has not been evaluated in detail. Jackson *et al.* (1999) and Linder *et al.* (2000) used variability at the nuclear ribosomal internal transcribed spacer region, ITS-1, for studying root distributions, although in these studies relatively few species were investigated. More recently, Brunner *et al.* (2001) mapped fine roots of a community of 30 tree species using restriction-fragments derived from a plastid intron, and Taggart *et al.* (2010) used the size of labelled amplification products for three noncoding plastid loci to identify single and mixed root samples in a grassland.

The various DNA-based approaches have different strengths and limitations. Restriction-fragment analyses are simpler and may offer a cost-effective method in some situations. Plant DNA barcoding as currently implemented (e.g., CBOL Plant Working Group, 2009) formalizes the use of standardized sequence-based markers from the plastid genome, whose homology is clear-cut. The homology of fragment length-based markers is generally inherently harder to determine, especially for distantly related taxa (Olmstead & Palmer 1994), and the nuclear ribosomal ITS sequences used in some studies (Linder *et al.* 2000) may be problematic in some plant groups, due to concerns about paralogy, pseudogenes, contamination and other sequencing artefacts (e.g. Alvarez & Wendel 2003; Feliner & Rossello 2007). For animals, barcode researchers have reported high rates of species discrimination using a portion of the mitochondrial region cytochrome *c* oxidase 1 (COI or *cox1*) (Hebert *et al.* 2003). In plants, two plastid genes, *rbcL* and *matK*, have been identified as suitable core barcode markers and endorsed by the international plant barcoding community (CBOL Plant Working Group 2009), although it is also recognized that rates of species resolution are generally lower than in animals

(CBOL Plant Working Group 2009; Fazekas *et al.* 2009). Despite the promise of DNA-based approaches, DNA barcoding has yet to be applied to an analysis of spatial variation in root diversity in plant communities.

Here, we use plant DNA barcoding to identify species in the root flora of an old-field in southern Ontario, Canada, and to quantify the diversity and spatial organization of species in relation to each other and to abiotic gradients in the soil. We address the following specific questions: (i) How effective is the plant plastid barcode *rbcL* for discriminating species based on root fragments? (ii) How diverse is the local root community and is there a relation between frequencies of species below- vs. above-ground? (iii) Is there heterogeneity in the taxonomic diversity of roots in vertical and horizontal space? and (iv) To what extent does under-ground community structure reflect interactions among species or with the abiotic environment?

## Materials and methods

### Sampling design

We sampled plant diversity above- and below-ground within a 30 × 30 m plot in an old-field at the Koffler Scientific Reserve (44° 03' N, 79° 29' W), a University of Toronto field site near Newmarket, Ontario (hereafter KSR). Within this area, we randomly located four 5 × 5 m plots (Fig. 1a) and subdivided them into 25, 1 m × 1 m quadrats. We documented the above-ground flora in each plot by recording the presence/absence of all seed-plant species in each quadrat monthly, from June to September (plots were checked more frequently to ensure no short-lived plants were missed). Plants were identified to species, when possible, based on morphological keys; some individuals lacking reproductive parts could only be identified to genus. The relative

frequency of each taxon was estimated as the proportion of the quadrats occupied, to the nearest 1%.

To sample root diversity, we exposed a 1 m (height) × 1 m (width) soil profile in the centre of each sampling plot (Fig. 1b). The vertical face of each profile faced one of the four cardinal directions (randomly assigned) and was accessed by a soil pit dug from one side of the sampling plot. We subdivided two 1-m-tall vertical transects (columns), 80 cm apart, into 5 × 5 × 5 cm soil cubes (20 contiguous cubes per column, 40 per sampling plot). A total of 160 soil cubes were sampled across all four plots and eight columns. We collected all root fragments from each soil cube (a total of 3800 fragments), placed them in envelopes and then in sealable plastic bags with silica gel to dry them rapidly at room temperature for storage prior to DNA extraction.

### Soil nutrient and texture analysis

In each plot, we sampled a third vertical soil column for soil nutrient and texture analysis, located between the two root sampling columns. Soil samples were collected from five depths: 0–10; 20–30; 40–50; 60–70 and 80–90 cm. For each sample, we estimated total nitrogen (% dry), phosphorus (mg/L soil dry), potassium (mg/L soil dry), magnesium (mg/L soil dry) and pH at the University of Guelph, Lab Services, Soil and Nutrient Laboratory. We used a soil texture analysis kit (LaMotte Co.) to estimate the proportions of sand, silt and clay in each soil sample.

### DNA barcoding of root fragments

We identified root fragments using the core DNA barcode *rbcL* (CBOL Plant Working Group 2009) at the Canadian Centre for DNA Barcoding (CCDB), University of Guelph, Canada. We focused on this DNA region as it has a high rate of recoverability using currently available standard primers: *rbcLaF* and *rbcLajf634R* (Fazekas *et al.* 2008; CBOL Plant Working Group 2009) and we therefore expected it to provide a satisfactory level of species identification in the context of a local flora without adding additional loci (Fazekas *et al.* 2008). We did not attempt the other officially sanctioned barcoding region, *matK* (CBOL Plant Working Group 2009), because future workers may wish to use environmental samples of pooled under-ground material, for which multilocus species assignment may be impractical (in pooled samples, there may be no straightforward way of deciding which sets of loci comprise those from any single species). A main goal of our study was therefore to see how well a single locus can perform in identifying plants to species in this context.

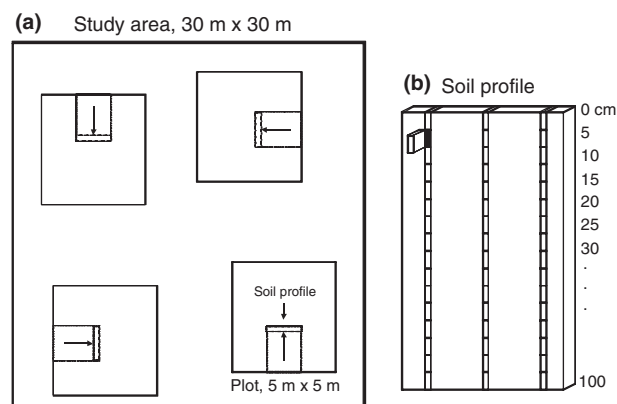


Fig. 1 Sampling design: (a) overview; (b) vertical section of soil profile.

We extracted DNA from root fragments  $\geq 2$  cm in length using a semi-automated, membrane-based protocol (Ivanova *et al.* 2008). We focussed on this size range based on a preliminary experiment to determine the minimum root length necessary for routine recovery of adequate DNA. We extracted DNA from fine root fragments of 1, 2, 3 and 4 cm in length from five test species, *Erigeron* spp., *Plantago lanceolata*, *Poa pratense*, *Populus tremuloides* and *Vitis riparia*, and verified that samples at least 2 cm in length amplified best for the *rbcL* primers used here, probably because some shorter fragments were dead or degraded. From the 3800 root fragments collected, we randomly sampled up to 10 fragments, each at least 2 cm in length per soil cube for a total of 1531 fragments.

We amplified DNA with a modified protocol from the Canadian Centre for DNA Barcoding (<http://www.ccdb.ca/pa/ge/research/protocols/amplification>). In each reaction, we included 6.25  $\mu\text{L}$  of 10% trehalose, 2  $\mu\text{L}$  of dd H<sub>2</sub>O, 1.25  $\mu\text{L}$  of 10 $\times$  buffer (Sigma-Aldrich), 0.625  $\mu\text{L}$  of 50 mM MgCl<sub>2</sub>, 0.188  $\mu\text{L}$  of 10  $\mu\text{M}$  of each primer (Sigma-Aldrich), 0.0625  $\mu\text{L}$  of 10 mM dNTPs (Sigma-Aldrich), 0.09  $\mu\text{L}$  of Taq polymerase (5 U/ $\mu\text{L}$ ; Sigma-Aldrich) and 2  $\mu\text{L}$  of DNA template ( $\sim 20$  ng/ $\mu\text{L}$ ). All PCR reagents except primers and Taq polymerase were added in volumes specified in the protocol. We found that increasing the volume of *rbcL* primers and Taq polymerase 1.5 times (0.188 and 0.09  $\mu\text{L}$ , respectively) the amounts in the original protocol facilitated amplification of root DNA. Conditions for amplification of *rbcL* include initial denaturation at 95 °C for 1 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 40 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Concentrations of the amplified product were determined for a subset of samples using a Nanodrop spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA), and ranged from 100 to 120 ng/ $\mu\text{L}$ . We performed sequencing reactions on both strands. In each sequencing reaction, we added 0.25  $\mu\text{L}$  of dye terminator mix v3.1, 1.9  $\mu\text{L}$  of 5 $\times$  sequencing buffer, 5  $\mu\text{L}$  of 10% trehalose, 1  $\mu\text{L}$  of 10 mM primer, 0.9  $\mu\text{L}$  of dd H<sub>2</sub>O and 0.5–1.2  $\mu\text{L}$  of the unpurified PCR reaction. Sequencing reactions were performed in a thermocycler under the following conditions: initial denaturation at 96 °C for 2 min, followed by 30 cycles of denaturation at 96 °C for 30 s, annealing at 55 °C for 15 s and extension at 60 °C for 4 min. (<http://www.ccdb.ca/pa/ge/research/protocols/sequencing>). Sephadex-purified cycle sequencing products were run on an ABI 3730XL sequencer (Applied Biosystems). We edited DNA sequences using Sequencher (Gene Codes Corp., Ann Arbor, MI) for base-calling and contig preparation. Bidirectional contigs were produced for all but 11 samples.

We identified each root fragment by comparing its *rbcL* sequence to a reference database of sequences for >400 vouchered species collected previously at KSR and identified using morphological characters (K. S. Burgess, unpublished data, [http://www.boldsystems.org/views/idrequest\\_plants.php](http://www.boldsystems.org/views/idrequest_plants.php)). We searched the database with each root sequence using the stand-alone BLAST algorithm (Altschul *et al.* 1990) (Version 2.2.22). When the query sequence had a bit score of >1000 and 99% identity with a single sequence in our reference library, it was assigned that species name as the best match. Poorer matches were treated as unresolved given the current information. We identified all unresolved taxa to family by searching the GenBank database.

### Analysis

We examined variation in number of root fragments and species richness per soil cube using ANOVA as implemented in JMP (v8.0, SAS Institute Inc. 2009) with depth, plot and column nested within plot (random effect) as sources of variation. We compared means using Tukey's HSD. Variation in all soil nutrient and texture variables was tested with respect to plot and depth. We also compared their relative frequency of each species in the eight soil columns to the relative frequency in the 100 above-ground quadrats using a Pearson correlation coefficient.

We examined patterns of species co-occurrence within soil cubes using EcoSim v7.72 (Gotelli & Entsminger 2004). We compared these patterns with a null expectation using 50 000 Monte Carlo randomizations of the data, in which species frequency and cube species diversity were held constant. Data for all taxa that were present in at least 5% of soil cubes were included ( $N = 10$  taxa). We measured species co-occurrence using two different indices: (i) Stone & Roberts (1990) *c*-score: the *c*-score measures the average number of 'checkerboard units (CU)' between all possible pairs of species that occur at least once in the matrix. The number of CU for each species pair is calculated as:  $\text{CU} = (r_i - S)(r_j - S)$ , where  $S$  is the number of sites containing both species; and  $r_i$  and  $r_j$  are the row totals for species  $i$  and  $j$ ; and (ii) the number of checkerboard species pairs; this index follows directly from Diamond's (1975) assembly rules analysis. For this index, EcoSim scans the rows of the matrix and tabulates the number of species pairs that never co-occur in any site. Any *c*-score or checkerboard number that is higher than the random expectation reflects a high degree of segregation of species, whereas a low score or number reflects a high degree of species co-occurrence. We conducted co-occurrence analysis on root fragments collected from all soil cubes sampled. To further understand the role of spatial

variation in the environment and phylogeny in relation to species co-occurrence, we repeated the analysis on shallow (<20 cm) and deep (>20 cm) soil cubes separately, and also individually for two plant families (Poaceae, Asteraceae).

We used multivariate ordination methods implemented in CANOCO 4.5 (ter Braak & Smilauer 2002) to test for spatial community structure below- and above-ground. For root diversity, we used the species presence/absence data with soil cube as the experimental unit ( $N = 160$ ). For above-ground structure, we used the presence/absence of species within the 100 quadrats. A detrended correspondence analysis (DCA; ter Braak 1986) was first used to identify the length of the ordination axis (i.e. the extent of variation in species scores) and determine the need for either a linear or unimodal ordination model. The length of the above-ground gradient (3.04 SD) and below-ground gradient (5.35 SD) justified the use of a canonical correspondence analysis (CCA) in both cases to characterize variation among experimental units using species data and a suite of environmental variables. The CCA of above-ground diversity was based on 100 above-ground quadrats comprising 39 taxa and eight environmental variables [nitrogen (N); phosphorus (P); potassium (K); magnesium (Mg); pH; sand; silt; clay] and one covariable (habitat type: forest or old-field based on proximity to adjacent forest). The CCA of below-ground diversity was based on 160 below-ground soil cubes and 29 taxa (identified using DNA barcoding) constrained by nine environmental variables (those listed above, plus the depth of sample) and two covariables (column position and habitat type). We controlled for non-independence among soil cubes within columns by including column as a covariate in the CCA and transforming variables with a generalized least squares (PGLS) transformation.

We used a discriminant function analysis (DFA; Ramsey & Schafer 1997) to separate clusters initially identified in the CCA (i.e. below-ground community) with respect to six functional traits [life form (grass, sedge, forb, shrub, tree), life cycle (perennial, annual and biennial), root morphology (fibrous, tap), growth strategy (r vs. k strategy) seed type (direct seed banker, direct on site, windborne on site), shade tolerance (shade tolerant, shade intolerant)]. We scored all species for these traits using a database reported in the FOIBIS database (Flora of Ontario Integrated Botanical Information Service; <http://www.uoguelph.ca/foibis/>). Using the CCA clusters as a priori groups and the six traits, the DFA tests whether the groups can be discriminated and indicates the relative contribution of traits in separating the groups. The analysis was repeated with each trait separately to detect the effects of collinearity.

## Results

### Root fragment abundance

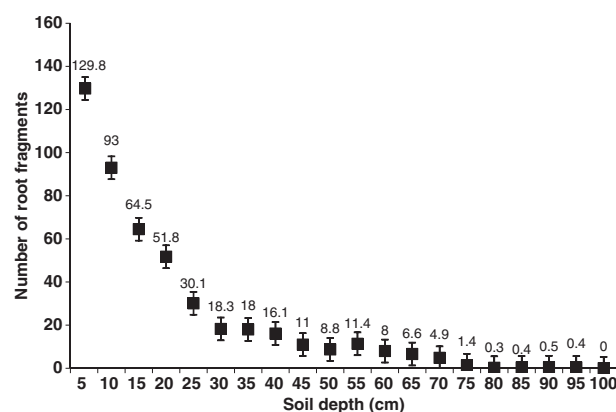
In total, we collected 3800 root fragments from 160 soil cubes from eight vertical columns in four sampling plots. The mean number of fragments per soil cube differed among columns within plots but not among plots (Table 1a). In addition, mean fragment number declined significantly with depth (Fig. 2). Mean fragment number dropped sharply from 130 at 5 cm to 18.3 at 30 cm and then more gradually to 0.0 at 100 cm. Mean values at 45 cm depth or greater were different

**Table 1** Analysis of variance of number of fragments and number of plant species per 5 cm<sup>3</sup> soil cube

Source of variation	d.f.	Sum of squares	F-ratio	P
(a) Number of fragments				
Depth	19	186 614.50	92.92	<0.0001*
Plot	3	3561.05	1.78	0.2900
Column (plot)	4	2667.55	6.31	0.0002*
Plot × depth	57	17 205.45	2.86	<0.0001*
Error	76	8033.45		
(b) Number of species				
Depth	19	628.32	26.67	<0.0001*
Plot	3	17.12	1.13	0.4383
Column (plot)	4	20.28	4.09	0.0047*
Plot × depth	57	67.51	0.96	0.5683
Error	76	94.22		

\* Indicates significant value.

Cubes were sampled from two vertical columns in each of four sampling plots in an old-field community in Ontario, Canada.



**Fig. 2** Mean number ( $\pm$ SE) of root fragments present as a function of depth in the soil column. Each mean is the result of observations from eight vertical soil columns, distributed among four sampling plots in an old-field community in Ontario, Canada.

from cubes at or above 25 cm. Values at depths of 5 through 25 cm were significantly different from each other, except between 15 and 20 cm (Fig. 2).

### Root taxonomic diversity

We amplified *rbcL* in 96% of 1531 samples and obtained high-quality contigs for 1285 root fragments and unidirectional sequences for 11 fragments (total 84.6% sequencing success); a success rate that was relatively constant with soil depth (Fig. 3). We submitted these 1296 sequences to GenBank (Accession nos: HQ623676-HQ624971). The remaining 16% of samples produced chromatograms of lower quality, making base-calling unreliable.

Using the stand-alone BLAST algorithm and an *rbcL* reference database for KSR, we identified 26 taxa from 11 families among the root fragments (Table S1, Supporting information). All root sequences that have a BLAST score value less than 1000 and percent similarity less than 99% were designated as unresolved taxa. We found three such taxa, which were then identified to family (two to Pinaceae, one to Fabaceae) using a search of GenBank. In total, we identified 29 taxa. Seventeen taxa (58.6%) were identified to species; nine taxa were identified to genus and three to family. Of all the 29 taxa identified, more than 55% ( $N = 16$ ) belong to two families: Asteraceae and Poaceae. The remaining 13 taxa comprised 10 genera from 10 families. The genera *Agrostis*, *Cirsium*, *Hieracium*, *Lolium*, *Poa*, *Prunus*, *Solidago* and *Symphyotrichum* contain multiple species at KSR, but these could not be distinguished by their roots using the *rbcL* barcode. Only one species was identified for each of the remaining genera.

The taxa identified ranged in abundance from 1 fragment (*Clinopodium vulgare*, *Hieracium* spp., *Leucanthemum*

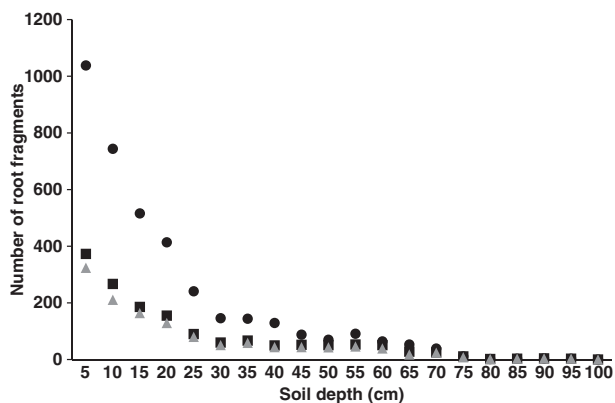


Fig. 3 Number of root fragments observed (circle), sampled (square) and successfully sequenced (triangle) as a function of depth in the soil column. Each value is the sum of samples in eight vertical soil columns.

*vulgare*, *Lolium* spp., *Medicago lupulina*, *Prunus* spp., *Quercus macrocarpa*) to 365 fragments (*Solidago* spp.). Root fragment abundance was related to root architecture. Species with fibrous roots were more abundant in the soil samples than those with tap roots ( $F_{1,24} = 3.8$ ,  $P = 0.06$ ;  $\bar{x}_{\text{fibrous}} = 79.07$  fragments,  $\bar{x}_{\text{taproot}} = 9.5$  fragments).

The relative frequency of taxa in root samples was positively correlated with their relative frequency in quadrats above-ground ( $r = 0.73$ ;  $N = 29$ ,  $P < 0.0001$ ; Fig. 4). Nineteen taxa were detected in both above- and below-ground samples although not always in the same proportions (Fig. 4). Some taxa, such as *Poa* spp., *Solidago* spp. and *Symphyotrichum* spp. were frequent both below- and above-ground, whereas *Hieracium* spp., *Populus deltoides* and *Vitis riparia* were infrequent above- and below-ground. However, asymmetries in frequency were common. Six species, *Carex aurea*, *Convolvulus arvensis*, *Erigeron philadelphicus*, *Leucanthemum vulgare*, *Rudbeckia hirta* and *Vicia cracca* were more common above-ground than below; whereas species, such as *Populus deltoides*, *Medicago lupulina* and *Vitis riparia* were more frequent below-ground. Ten taxa (*Clinopodium vulgare*, *Lolium* spp., *Phalaris arundinacea*, *Prenanthes altissima*, *Prunus* spp., *Quercus macrocarpa*, *Tragapogon dubius* and the three unresolved taxa) were only found in the root samples but not in the above-ground vegetation of the sampling plots.

### Spatial organization of root diversity

The mean number of species that we detected ranged from 0 to 11 per cube. Mean species richness per cube differed among vertical columns within plots but not between plots (Table 1b). It also decreased significantly with soil depth. In contrast to the patterns for root fragment abundance, the decline in species richness occurred in a stepwise fashion with significant reductions at 15–20 and 60–65 cm (Fig. 5). Based on a Tukey's means comparison, there are no significant differences among cubes within the 5–15 cm range, from 20 to 60 cm and from 80 to 100 cm (Fig. 5).

Root distribution profiles for 10 taxa present in at least eight (5%) of the 160 soil cubes illustrate the taxonomic variation in the maximum depth and abundance of roots through the soil column (Fig. 6). Two of the most abundant taxonomic groups, *Poa* spp. and *Solidago* spp. were present in all columns at 5–15 cm depth. These species declined with increasing depth and occurred up to 75 and 80 cm depth. Other taxa, such as *Bromus inermis* and *Symphyotrichum* spp. also declined markedly with depth but had only moderate abundance. In contrast, species such as *Elymus repens* and *Phalaris arundinacea*, although present to 65–75 cm depth, have relatively uniform low frequencies through

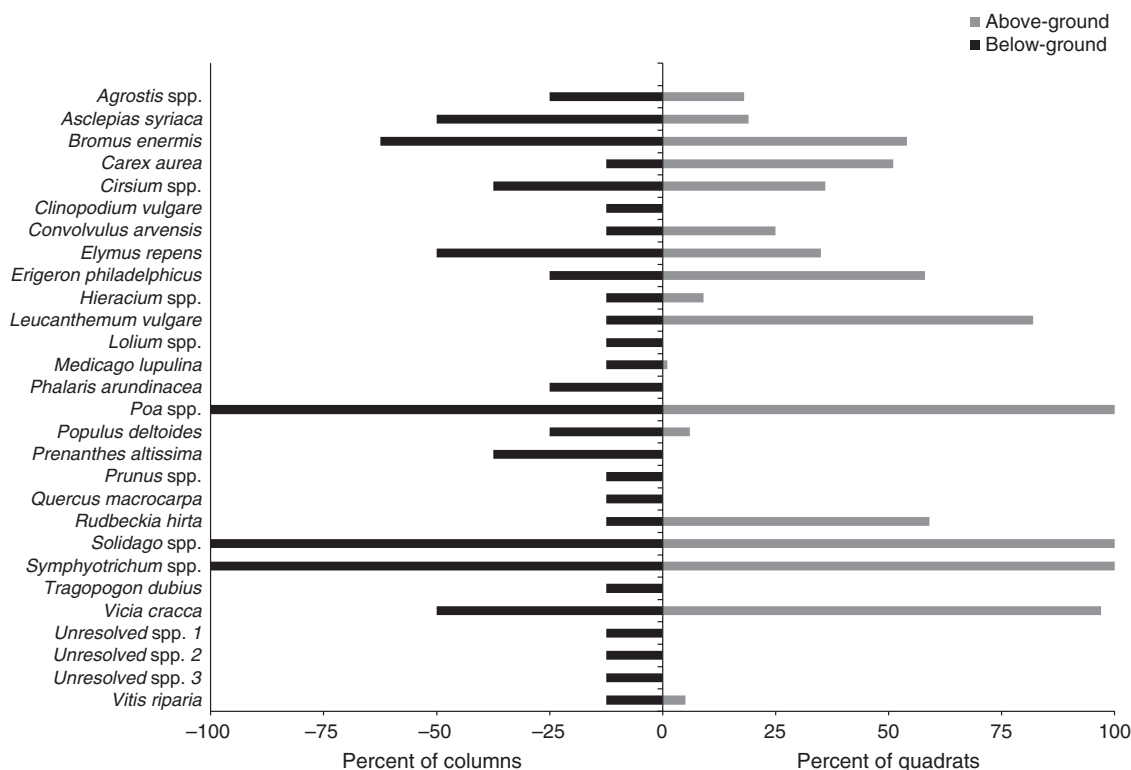


Fig. 4 Relative abundance of 28 taxa above-ground and below-ground. Percentages were calculated from eight soil columns below-ground and from 100 quadrats above-ground. All *Symphyotrichum* taxa in roots samples were collapsed into a single category to match the taxonomic designation above-ground.

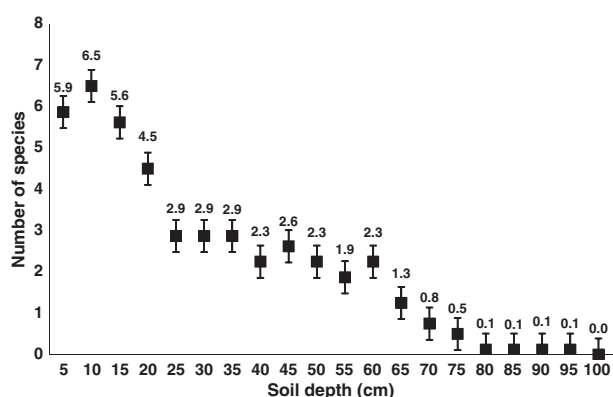


Fig. 5 Mean ( $\pm$ SE) number of genetically distinct taxa detected as a function of depth in the soil column. Each mean is the result of observations from eight vertical soil columns.

the soil depth. *Prenanthes altissima* had a consistently shallow distribution (Fig. 6).

*Soil texture and nutrient characteristics*

Based on ANOVA results, there were no significant differences among plots for any of the nutrient variables. Nitrogen, phosphorous and potassium concentrations

decreased significantly ( $P < 0.05$ ) with increasing soil depth (Table 2, Fig. S1, Supporting information). Magnesium levels were higher in the top 20 cm and bottom 60–100 cm than in the middle depths. Sand and clay content varied significantly among plots and soil depths. On average, plots closest to the forest (B and C) had higher sand content and lower clay content than plots A and D. For all plots, sand content decreased and clay content increased with depth.

*Multivariate analysis of community structure*

In the analysis of species co-occurrence in soil cubes, the *c*-score and checkerboard indices were both significantly larger than expected under a random model (*c* value = 332.51,  $P = 0.012$ ; checkerboard index = 6,  $P = 0.00$ ), indicating strong species segregation. Within the top 20 cm of soil, the checkerboard index was still significantly positive (checkerboard index = 7,  $P = 0.0013$ ), whereas the *c*-score was not significant (*c* value = 30.75,  $P = 0.27$ ). Below 20 cm, both indices had significantly larger values (*c* value = 153.77,  $P = 0.046$ ; checkerboard index = 12,  $P = 0.006$ ) than the null model. Analysis of coexistence among members of Asteraceae and Poaceae, separately, showed that both

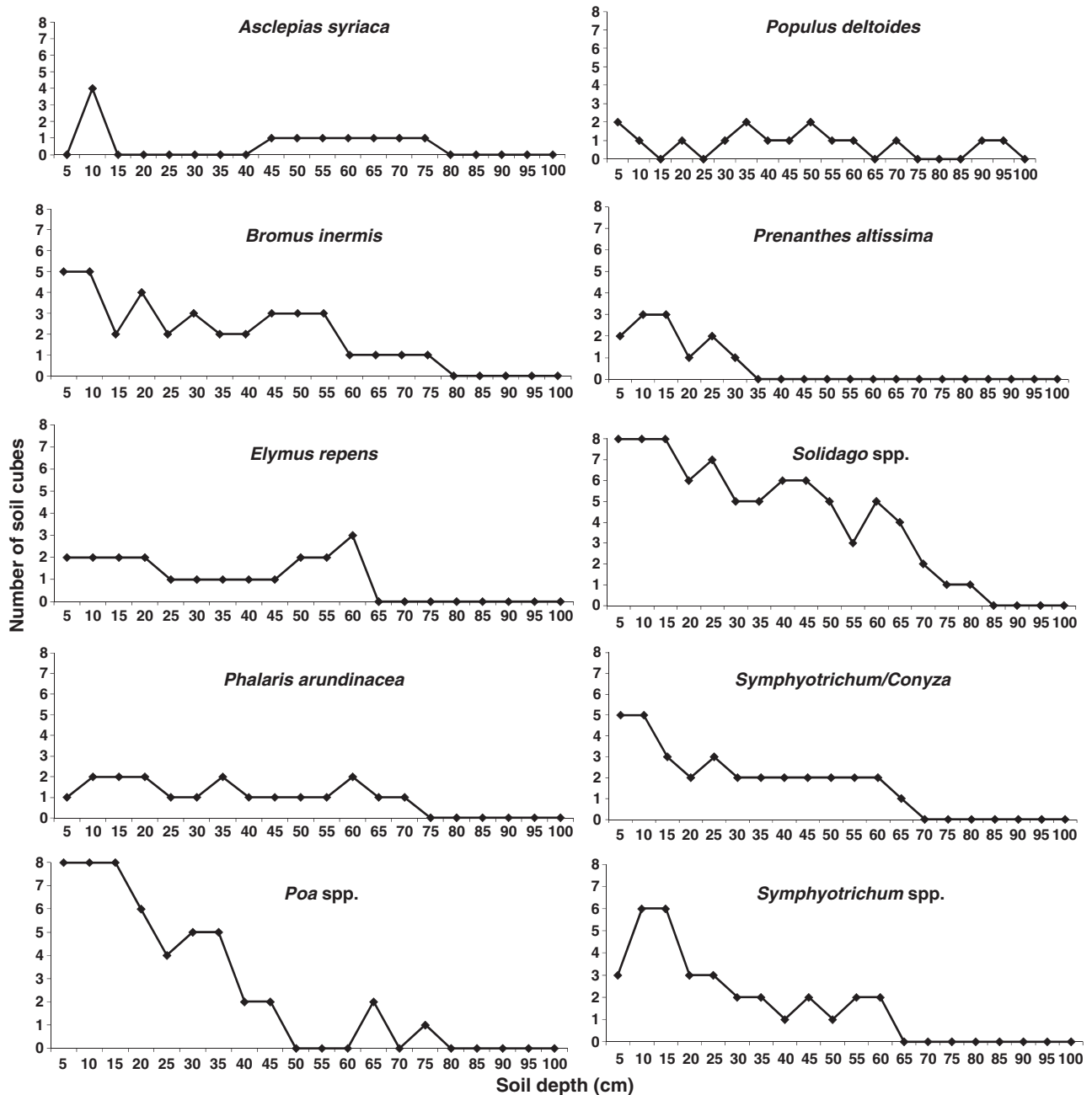


Fig. 6 Root profiles of 10 taxa identified by DNA barcoding for taxa occupying at least eight cubes. *Symphyotrichum* and *Conyza* have identical score and similarity index.

indices of coexistence were not significantly different from random expectations ( $c$  value = 89.9,  $P = 0.158$ ; checkerboard index = 43,  $P = 0.239$ ), suggesting no strong segregation among members of these two families.

The ordination of above-ground variation in diversity revealed no apparent spatial structure. Most variation was explained by the first two axes; however, low eigenvalues (Table 3) and intermixed quadrats from different plots (Fig. 7) indicate a lack

of structure. Although correlations between species scores and environment were moderate (Table 3), they are not biologically meaningful because of the small degree of variation represented on the first four axes.

In contrast to the results for above-ground diversity, below-ground diversity exhibited considerable community structure. The CCA resulted in ordinations with clusters of experimental units (based on species counts) associated with depth, position relative to adjacent forest, dominant species and minerals (Fig. 8). The first



**Table 2** Summary of analysis of variance for eight soil nutrient, pH and texture variables

Soil variable	Plot		Depth	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Total nitrogen (N)	1.86	0.190	65.34	<0.0001
Phosphorus (P)	0.62	0.615	39.88	<0.0001
Potassium (K)	1.04	0.410	15.14	0.0001
Magnesium (Mg)	0.65	0.600	5.83	0.0076
pH	2.29	0.130	6.01	0.0068
Sand (%)	4.46	0.025	23.68	<0.0001
Silt (%)	1.88	0.187	9.82	0.0009
Clay (%)	5.51	0.013	23.74	<0.0001

For all variables the effects of plot and soil depth had 3 and 4 d.f., respectively (error d.f. = 12).

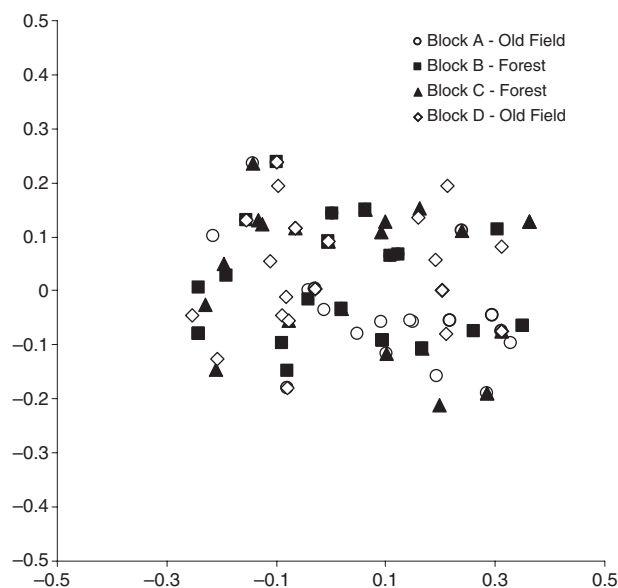
**Table 3** Summary of multivariate ordinations (canonical correspondence analysis) of above-ground and below-ground plant diversity in an old-field community in Ontario, Canada

Summary variables	Sampling	Axis			
		1	2	3	4
Eigenvalues	Above-ground	0.058	0.025	0.015	0.011
	Below-ground	0.351	0.174	0.013	0.090
Species/environment correlations	Above-ground	0.518	0.515	0.510	0.515
	Below-ground	0.821	0.572	0.557	0.501
Cumulative % variance of species data explained	Above-ground	40.1	60.2	71.1	79.3
	Below-ground	71.9	88.1	89.1	92.2

The majority of variation is explained by the first and second axes (shaded). Low eigenvalues indicate no apparent structure above-ground.

CCA axis explained 71.9% of the variation in the species composition data along a gradient of considerable length (Table 3). This axis had significant ( $P < 0.01$ ) canonical correlations with depth of sample, that is, strong separation of shallow (5–35 cm) experimental units from deeper ones, and potassium concentration (Table 4). The second CCA axis explained an additional 16.2% of the variation in the data along a shorter, yet considerable gradient (Table 3). This axis is significantly ( $P < 0.01$ ) correlated with N, P, Mg, pH and soil texture variables, sand, silt and clay (Table 4). Experimental units from plots that are closest to the forest are most clearly separated in shallow soils but less so in deep soils (Fig. 8).

The DFA was able to distinguish the multivariate clusters from the CCA based on a suite of plant life-

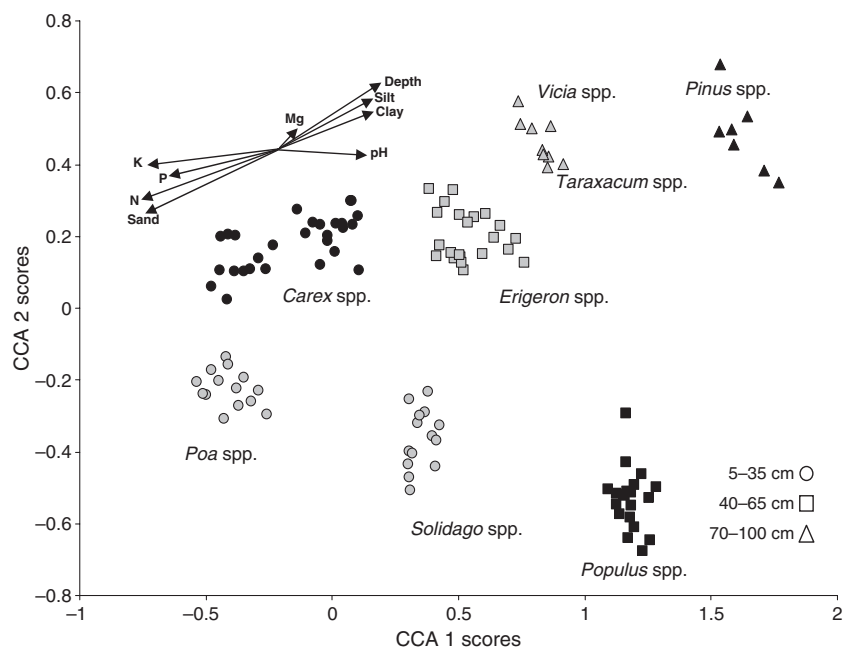


**Fig. 7** Canonical correspondence analysis of 100 above-ground vegetation quadrats from four sampling plots (block). Plots are located in an old field and are classified according to their proximity to an adjacent forest. Quadrat scores are plotted with respect to two CCA axes, which reflect relationships to eight environmental variables (N, P, K, Mg, Sand, Silt, Clay and pH) and one covariable (habitat type: soil plots near forest and away from it).

history traits. There were significant differences among groups ( $P < 0.01$ ), confirming the strong community structure below-ground. There were significant differences ( $P = 0.026$ ) based on the first two canonical functions. The first discriminant function accounted for 69% of the differences between groups (variability in the discriminant scores). The second function accounted for an additional 1% of the between-group variance. Root architecture (fibrous vs. tap) was significantly correlated with the species score in the CCA ( $P = 0.003$ ;  $r = 0.987$ ). The other traits (life form, lifecycle,  $r/k$  strategist, seed type, shade tolerance) were not significantly ( $P > 0.05$ ) correlated with the CCA species scores whether analysed together or separately. Eighty-seven percent of the groups (representing 29 taxa) were correctly classified using the DFA.

## Discussion

The DNA barcoding locus *rbcL* was effective at characterizing the taxonomic diversity of roots in an old-field plant community at the Koffler Scientific Reserve, Canada. We amplified 96% of samples (amplification failures may be due to senescence, but there were no differences in amplification success by depth), and successfully sequenced nearly 85%. This sequencing



**Fig. 8** Canonical correspondence analysis of root samples from 160 cubes representing 29 taxa identified using DNA barcoding. Cube scores are presented in relation to two CCA axes and nine environmental variables (N, P, K, Mg, sand, silt, clay, pH and soil depth) and one covariable (habitat type: soil plots near forest and away from it in old field). CCA1 axis has significant ( $P < 0.01$ ) canonical correlations with soil depth and potassium, and CCA2 axis has significant ( $P < 0.01$ ) canonical correlations with N, P, Mg, pH, sand, silt and clay.

**Table 4** Correlations and canonical coefficients for environmental variables used in canonical correspondence analysis of 160 cubes and 29 taxa (identified using DNA barcoding)

Variable	Interset correlation		Canonical coefficient		<i>t</i> -Value	
	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2
Nitrogen (N)	0.7892	<b>-0.1124</b>	-0.3121	<b>-2.8523</b>	-1.3975	-5.5487
Phosphorus (P)	0.7462	<b>-0.0251</b>	0.0173	<b>1.6165</b>	0.1148	4.8731
Potassium (K)	<b>-0.6821</b>	-0.1075	<b>0.8921</b>	0.1452	4.3812	0.3312
Magnesium (Mg)	-0.1528	<b>0.0109</b>	0.1986	<b>0.7332</b>	1.5347	2.6412
pH	-0.6318	-0.0376	0.3315	0.6823	1.7745	1.7176
Sand	0.6411	<b>0.0734</b>	5.1571	<b>-39.519</b>	0.7511	-2.7188
Silt	-0.5615	<b>-0.0109</b>	2.3387	<b>-19.5792</b>	0.7026	-2.7734
Clay	-0.6345	<b>-0.1319</b>	2.8851	<b>-24.3628</b>	0.7429	-2.9337
Depth of sample	<b>0.7316</b>	-0.1022	<b>-0.7631</b>	-0.3272	-4.8412	-0.9617

Bold values indicate significant correlations.

success rate was lower than rates reported for *rbcl* in a previous study conducted on plant diversity of southern Ontario (100%, Fazekas *et al.* 2008). Lower sequencing success may reflect, in part, lower quantity of DNA yield per extraction in root tissue compared with leaves and the heterogeneity in tissue quality that results from selecting root fragments randomly (within the >2 cm length criterion). Of the 29 unique sequences that we obtained, 17 (58.6%) were identified to species, 9 were

identified to genus and 3 to family. This old-field community is dominated by a small number of plant families (10 and 6 species in Asteraceae and Poaceae, respectively). Several genera at the site (e.g., *Solidago*, *Symphotrichum*, *Poa*) are represented by multiple species (based on above-ground morphology) that were not distinguishable genetically using *rbcl* (Fazekas *et al.* 2008). This may be owing to a variety of different reasons, including incomplete lineage sorting,

hybridization, imperfectly understood species boundaries and taxonomy, and a simple lack of informative variation between species (Fazekas *et al.* 2009).

Within our sampling area there were differences between the patterns of diversity observed below-ground vs. above-ground. Overall 29 species or species groups were detected below-ground using sequence barcodes, whereas 39 species or species groups were observed in the quadrats above-ground. The relative abundances of the taxa detected below-ground were significantly correlated with their abundance above-ground, suggesting that the ranking of biomass above-ground for any species is indicative of allocation to biomass below-ground. Ten of 29 taxa detected below-ground were not observed in the above-ground plots. These species were relatively uncommon overall and thus may have been missed because of sampling error in time or space, or because of incomplete identification based on above-ground morphology (e.g., because informative reproductive characters were not obtained, as was the case for members of *Symphyotrichum* during the census period). Alternatively, individuals of some species may enter a state of root quiescence or dormancy, in which case no above-ground growth may be initiated in some years (Lesica & Steele 1994). Lastly, some species may appear below-ground but not above-ground because their radius of root extension exceeded the perimeter of the above-ground canopy. This was probably the case for *Prunus*, which occurred in the vicinity but not in the above-ground plots.

One of the most striking results of this study was the strong spatial structure in root diversity compared with the near absence of structure in the above-ground quadrats. To our knowledge, this contrast has not been described before. In the CCA analysis, soil cubes grouped into several tight clusters based on root taxonomic diversity and environment. These clusters corresponded to cubes dominated by different plant taxa and soil depth, nutrient content and texture. In contrast, there was no detectable clustering of quadrats from the above-ground survey. The contrasting spatial patterns of diversity may reflect, in part, the differences in size of the sampling units above-ground (1 × 1 m quadrats) and below-ground (5 × 5 × 5 cm cubes). The structure of root diversity in this sampling design may be affected by sampling error or the presence of environmental heterogeneity on different spatial scales. However, sampling error among soil cubes would not produce the strong association between root diversity and soil environment that we observed. Further, much of the variation in diversity and environment in the soil occurred among columns and plots, which exists on a scale that is comparable to the above-ground sampling scale. Therefore, the strong community structure of

roots is not likely to be a result of sampling at a smaller scale. Given that biomass above- and below-ground has similar access to the species pool, it is more likely that the community structure below-ground reflects different environmental gradients and the greater potential for interactions among taxa in the soil.

Species diversity in roots may be structured if there is strong environmental heterogeneity in the soil profile and species are being filtered according to differences in environmental niches (Fitter 1994; Weiher *et al.* 1998). Our soil analysis confirms that nutrient concentrations and texture vary with depth such that individuals that persist near the soil surface may have higher N, P, K and lower Mg requirements. Niche partitioning, at least with respect to depth, may also occur as a result of differences in root architecture. Plants with fibrous roots may be more likely to persist in shallow soil, whereas those with tap roots and secondary root thickening may be more likely (or less constrained) to exploit deeper zones (Le Roux *et al.* 1995; Casper & Jackson 1997). Alternatively, community structure may reflect the results of differential competitive interactions among species. That is, the range of environmental heterogeneity is suitable for establishment by the general pool of old-field species but different competitive abilities in different parts of the site affect the species that can coexist. At this stage, we have no direct evidence for assessing the relative importance of these two factors.

Further insights into the causes of root community structure come from the analysis of species co-occurrence. Overall, values of the checkerboard and *c*-index were strongly positive compared with a null model of random association, indicating that species are generally found in isolation (i.e. are spatially segregated). Unexpectedly, the patterns of coexistence shift to random (and nearly negative) when analysed for the two dominant plant families (Poaceae, Asteraceae) separately. Such values result when species are more likely to co-occur than to be present individually. Such associations among confamilial species but not species from different families suggest a role for environmental filtering. If confamilials are more similar to each other than to species in other families, one would expect competition to result in clearer segregation (i.e. positive values of coexistence) among them. Instead, we see more coexistence among confamilials than among the whole sample. The most logical interpretation is that confamilial species are found in close proximity because they either share common root attributes or share common physiological tolerances and thus establish in similar environments. More in-depth community phylogenetic analyses (Kembel & Hubbell 2006; Kress *et al.* 2009) were not performed here because of the dominance of

two large families, but may be feasible at other, richer sites.

Several studies have identified competition as an important factor in the organization of plant diversity below-ground (Fowler 1986; Wilson 1988; De Kroon *et al.* 2003), and such interactions may account for some of the patterns in community structure that we observed. Although this cannot completely explain the spatial proximity of congeners as revealed in the coexistence analysis, it may reinforce some of the structure initially formed through environmental filtering. This is consistent with the observation that some of the most abundant taxa have fibrous root systems, a root system that is able to rapidly proliferate in resource-rich volumes of soil, depleting the resources before competing plants do (Grime 1979; Casper & Jackson 1997). This root architecture may allow these taxa to proliferate in top layers of soil, where N, P and K are more abundant, before other species do. Previous studies have also demonstrated that species with fibrous root systems have a disproportionate competitive advantage over species less able to proliferate in nutrient-rich areas of soil (Fransen *et al.* 2001). Another below-ground trait that may provide a competitive advantage is the ability to occupy soil space. Species whose roots can occupy more space than others are likely to be more successful in competition (Casper & Jackson 1997).

Our study has two results with broad ecological implications. First, this is the first attempt to apply DNA barcoding to describe the patterns of below-ground community structure in a complex community. This molecular tool was effective in terms of successfully amplifying root fragments. Species identification was successful in that only three taxa could not be resolved from the local reference database. This success rate will probably vary in future applications depending on the nature of the taxonomic diversity in a given region. Some sequences were identified to genus, not species. This is partly because of the incomplete nature of the reference database but also because of the taxonomically complex nature of several of the important species groups in this particular flora and upper limits on species resolution possible using plastid-based DNA barcoding markers (Fazekas *et al.* 2008). Nevertheless, identification of species or species groups at this level still represents a powerful advance in the ecological analysis of roots and below-ground processes. Other DNA-based approaches, such as restriction-fragment based methods (Bobowski *et al.* 1999; Brunner *et al.* 2001; Ridgway *et al.* 2003) have been used successfully to identify roots. However, sequence-based approaches are likely to be increasingly used in future applications, and the development of DNA barcoding references will provide a powerful tool-box for ecologists interested in

studying below-ground floras. Next-generation sequencing technologies, including 454 technology (Roche Group), may reduce the time and cost of sequencing enormously and may also facilitate single-locus environmental PCR-style studies (De Koning *et al.* 2008).

An additional noteworthy result is that we detected an asymmetry in patterns of diversity below-ground vs. above-ground in the same community. Community structure was more evident below-ground compared with the same horizontal area above-ground. The mechanisms driving this difference are unclear; however, previous studies have demonstrated that species below-ground segregate their root systems in response to competition and allelopathy (Schenk *et al.* 1999). Future work using DNA barcoding in other communities and along successional gradients should help advance our understanding of the environments and community processes operating among roots in the soil. This has the potential to provide fine-scale insights into the development of plant communities underground, and the mechanisms by which diversity arises and is organized over time.

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## Supporting information

Additional supporting information may be found in the online version of this article.

**Table S1** Summary of species or species groups and their root attributes that were identified in an old field using the *rbcL* DNA barcode.

**Fig. S1** Mean ( $\pm$ SE) concentration of total nitrogen, phosphorus, potassium, and magnesium and pH as function of depth in four soil plots at Koffler Scientific Reserve (KSR), Ontario, Canada.

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