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Surveying soil faunal communities using a direct molecular approach

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ABSTRACT

Soil faunal communities are often phylogenetically diverse and the accurate assessment of the taxonomic structure of these communities is both time-consuming and requires a high level of taxonomic expertise. Here we describe a DNA sequence-based methodology for characterizing soil micro- and mesofaunal communities that is similar to the molecular approaches commonly used to survey soil microbial diversity. The technique involves the direct extraction of faunal DNA from soil, PCR amplification of the extracted DNA with metazoan-specific primers, followed by the construction of clone libraries and direct sequencing of individual PCR products. We used this technique to characterize micro- and mesofaunal community composition from six individual soils representing two land-use types. The technique captured the more abundant faunal groups in the soils (nematodes, Collembola, Acari, tardigrades, enchytraeids) and provided sufficient taxonomic resolution to describe the overall structure of the communities. We compared the results obtained using this molecular approach to results obtained using a traditional, microscopy-based approach and found that the results were broadly similar. However, since biases are inherent in both methods it remains unclear which method provides a more accurate assessment of soil faunal community composition. Although this molecular approach has some distinct disadvantages over the more widely-used direct extraction methods, one advantage is that the taxonomic identification it can provide will be more accurate and consistent across research groups, facilitating effective comparisons of mesofaunal surveys.

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1. Introduction

Fauna are an integral component of the soil biota and faunal communities are typically phylogenetically diverse. The most abundant micro- and mesofauna in soil include nematodes, microarthropods (Collembola, Acari), enchytraeids, and to a lesser extent, tardigrades, rotifers, and proturans, as well as immature stages of many larger species of soil fauna. Typically, the composition of nematode and mesofaunal communities is determined by extracting the fauna from soil using a combination of approaches such as modified-Tullgren and Baermann funnels, flotation, filtration, and direct sieving followed by identification and quantification of the organisms by microscopy (Edwards, 1991; Coleman et al., 1999, 2004). In general, there are three key limitations associated with the use of these standard approaches for faunal community analysis. First, extraction efficiencies can be fairly low and, perhaps more importantly, extraction efficiencies are known to vary for different

faunal taxa and between different soil types (see André et al. (2002) and references therein). Second, the identification of soil micro- and mesofauna can be a difficult and laborious task, often requiring a high level of taxonomic expertise (André et al., 2001). This is particularly true for the nematodes where identification can be challenging and morphological-based classification is not necessarily consistent with phylogenetic classification (Blaxter et al., 1998; Waite et al., 2003). Third, given that no one method extracts all faunal groups, it is often necessary to use multiple extraction techniques to fully survey and characterize the faunal community in a given soil sample (Petersen and Luxton, 1982; Coleman et al., 1999; André et al., 2002; Coleman et al., 2004).

Molecular techniques for community analysis are now widely used in soil microbiology, and molecular approaches have greatly expanded our knowledge of soil microbes. Amongst these, sequence-based approaches whereby DNA is extracted directly from soil, specific genes are amplified, and the amplified products are cloned and sequenced can provide a wealth of information on the composition and diversity of soil microbial communities. While similar techniques have been applied to specific faunal taxa (Waite

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et al., 2003; Hogg and Hebert, 2004; Griffiths et al., 2006; Read et al., 2006), we know of no previously published study that has used sequence-based approaches to survey soil faunal community composition by extracting DNA from soil and sequencing a specific gene for community-level analyses. There are a number of possible advantages to this approach. First, the taxonomic identification of members of the soil faunal community can be determined more readily. Second, the collection of sequence data allows overall community composition and the taxonomic identity of specific fauna from a given soil to be directly compared between research groups in a consistent manner. Third, if the molecular approaches used to assess bacterial and fungal community composition can be applied to soil micro- and mesofaunal communities, the same DNA sample could (in theory) be used for an integrated assessment of soil faunal and microbial diversity without having to compare results obtained using a very different set of techniques. Molecular techniques of community analysis are definitely no panacea, but they may provide distinct advantages that complement the more traditional methods used to analyze soil faunal communities.

Here we show that we can extract faunal DNA directly from soil and use a sequence-based approach to characterize and compare the faunal communities. We tested the technique using soils collected from pasture and hardwood forest plots in South Carolina, USA. Our objectives were to demonstrate the utility of the approach, compare the molecular approach with more traditional methods for analyzing faunal communities, and highlight some of the pitfalls and limitations associated with the method.

2. Materials and methods

2.1. Soil collection

Soil samples were collected from six locations, across two replicated land-use types (grazed pasture and hardwood forest) within the Calhoun Experimental Forest, which is administered by the USDA Forest Service and located in the Piedmont region of northwestern South Carolina (34.5°N, 82°W). Additional details on the sampling locations and the soils in this landscape can be found in Lauber et al. (2008). Individual 100 m² plots were established across the two land-use types (three plots per land-use type) and each plot was sampled in June 2007 with two replicate cores (15 cm diameter, 7.5 cm deep) collected from randomly-chosen locations within each plot. Immediately after collection, the two cores were halved and one of each half composited, sieved to 4 mm to remove roots and stones, and thoroughly homogenized. For the molecular analyses a 20 g sub-sample of the soil from these plot samples was placed on dry ice in the field and then immediately returned to the laboratory and stored at –80 °C for later DNA extraction. Another sub-sample (~20 g of dry weight soil equivalent) of this soil was added to Baermann funnels for the wet extraction of nematodes. The remaining halves (~500–1000 g dry weight soil equivalent), which were not sieved, were placed within 24 h of collection into modified-Tullgren funnels for the dry extraction of mesofauna.

2.2. Metazoan community analysis using a sequence-based approach

DNA was isolated from the six collected soil samples using the MoBio Power Soil DNA Extraction kit (MoBio Laboratories, Carlsbad, CA) with a modified protocol. The 20 g soil sample was ground in a mortar with pestle in liquid nitrogen and then a 1 g sub-sample of soil was placed in a bead tube for extraction. Bead tubes were heated to 65 °C for 10 min, then shaken horizontally for 2 min at maximum speed with the MoBio vortex adapter. The remaining steps were performed as directed by the manufacturer. DNA was

extracted from three replicate 1 g soil sub-samples per plot and the replicate DNA samples from each plot were pooled together prior to PCR amplification.

The metazoan small-subunit 18S rRNA genes were amplified using a metazoan-specific forward primer 18S11b (5' GTCAGAG GTTCGAAGGCG 3') (Hamilton, 2003) and the universal eukaryotic 18S2A reverse primer (5' GATCCTCCGCAGGTTACC 3') (Nishida and Sugiyama, 1993). The 18S11b forward primer was designed to specifically amplify metazoan DNA when paired with the 18S2A primer. The forward primer was designed *in silico* to be specific for metazoa using a database of 69 metazoan and 11 non-metazoan non-animal sequences obtained from the SILVA rRNA database (Pruesse et al., 2007). This alignment was used to identify regions of the 18S gene where the metazoan sequences were divergent from the non-metazoan sequences by several bases yet conserved across the diversity of metazoa. The 18S11b and 18S2A primer set was tested *in vitro* by PCR amplification of mammalian, nematode, fungal, and algal genomic DNA at various annealing temperatures. The annealing temperature at which non-metazoan DNA failed to amplify was found to be 55 °C. The primer set excludes rotifers, but includes most major faunal taxa found in soils and marine sediments (Hamilton, 2003). More details on the 18S11b primer design and testing can be found in Hamilton (2003).

The PCR reaction contained 1X PCR Buffer (Invitrogen, Carlsbad, CA), 1.5 mM MgCl₂, 0.2 μM of each primer, 200 μM dNTPs, 0.5U Taq DNA polymerase (Invitrogen, Carlsbad, CA) and 1.0 μL template DNA. Amplification was accomplished by initial denaturation at 94 °C for 3 min followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s with a final extension at 72 °C for 10 min. Each DNA sample was amplified in quadruplicate and the amplicons were pooled by plot and run on a 1.5% agarose gel. The pooled amplicons were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and cloned into the pCR4-TOPO-TA cloning vector, followed by transformation into TOP10 chemically competent cells using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA). Transformations were plated onto Luria-agar plates containing 50 μg mL⁻¹ ampicillin and incubated overnight at 37 °C. Sixty-four individual colonies from each plot were picked and amplified directly using M13 forward and M13 reverse primers using standard protocols. M13 PCR products (18S genes) were submitted to Agencourt Biosciences (Beverly, MA) for single-pass sequencing using the 18S11b primer.

2.3. Analysis of sequence data

Sequences of insufficient size (<400 bp) were removed from the analysis and the remaining sequences were compared to a database of 500 metazoan sequences downloaded from the Silva database (Pruesse et al., 2007) (<http://www.arb-silva.de/>). This database was comprised of all nematode, Acari, Collembola, and enchytraeid sequences available from the SILVA database, as well as other selected sequences from the Arthropoda, Annelida, Rotifera, Tardigrada, and Turbellaria. Sequences obtained from the soil clone libraries were compared to the sequences in the database using the BLAST algorithm (Altschul et al., 1997) and only those sequences from our libraries that were a close match to those in the database (e-values < 5.00e–30 and a percent identity > 80%) were considered for further analysis. Approximately 80% of the sequences obtained from the soil clone libraries were deemed to be close matches to known metazoa and suitable for analysis of the faunal communities. The 20% of the sequences not included in subsequent analyses were either probable chimeras or close matches to fungi. Representative sequences from this study have been deposited in the GenBank nr database and have accession numbers FJ610759 through FJ611226.

2.4. Faunal community analysis by direct extraction

Mesofaunal extractions via modified-Tullgren funnels were conducted until the soil was dry to the touch (7–10 days). Using stereo-microscopes, we sorted mesofauna in the resulting samples to order and mites were sorted to major group (Oribatida, Mesostigmata and Prostigmata). A sub-sample of individuals from each group was dried (65 °C) to estimate biomass per individual and, subsequently, total biomass per group. Nematode extractions via Baermann funnels were conducted for 24 h. Nematodes were not sorted into specific groups and, after visual inspection for fauna other than nematodes, were placed into tin cups, dried (65 °C), and weighed, allowing us to determine total nematode biomass. Soils used in both the modified-Tullgren and Baermann funnel extractions were removed after the assays, dried (105 °C), and weighed, allowing us to estimate the relative abundances of the faunal groups in units of biomass per gram soil.

3. Results and discussion

Clone libraries from the three pasture plots (Gr1, Gr2, and Gr3) were dominated by nematode sequences (Table 1, Fig. 1A) with nematodes representing 75–92% of the metazoan sequences in those libraries. In comparison, the libraries from the hardwood forest plots (Ha1, Ha2, Ha3) had relatively more Acari sequences (accounting for 30–85% of the metazoan sequences). Other mesofaunal groups, including Collembola, tardigrades, enchytraeids, and arachnids were less abundant, accounting for less than 15% of the sequences from the six individual libraries (Table 1, Fig. 1).

If we compare the results obtained using the molecular approach to results from the standard approach whereby nematodes and mesofauna were directly extracted from soil and then identified by microscopy, we find some general similarities but also some important differences (Fig. 1). With both methods, the nematodes and Acari were found to be the most abundant fauna in all plots, with oribatids the dominant group of Acari. However, the estimated relative abundances of nematodes across the 6 soils were different with the two techniques and poorly correlated ($r^2 = 0.02$, $P > 0.4$). Likewise, if we compare the ratios of Acari to nematodes across the six soils, we also find that the two techniques give very different results ($r^2 = 0.16$, $P > 0.3$). The differences between the two methods are clearly evident when comparing the relative abundances of the dominant groups as determined in samples Gr1 and Ha3 (Fig. 1). With the molecular method we could identify a few sequences belonging to tardigrades, yet no tardigrades were identified using the direct extraction method. Tardigrades may have been wet-extracted, but we did not observe them under the stereo-microscope. Enchytraeids, in contrast, were identified by direct extraction in samples Ha1 and Ha2 (with relative abundances of 3% and 1.2%, respectively) and these same soils were also the only two soils from which enchytraeid sequences were identified (Table 1).

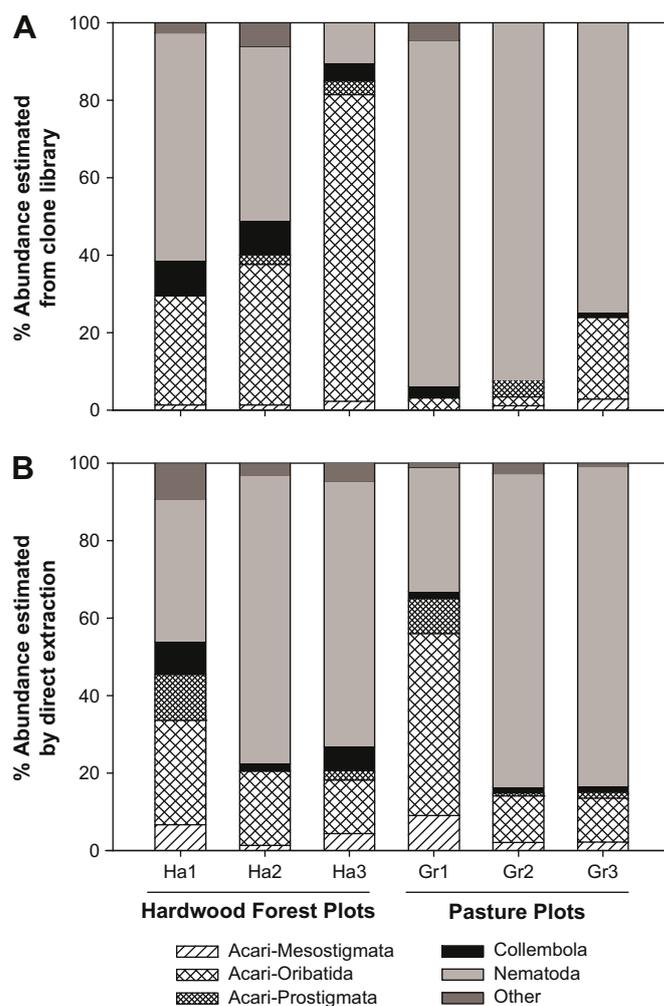


Fig. 1. Relative abundances of the dominant metazoan groups, as estimated from the clone libraries (1A) and by direct extraction (1B). More detailed information on the clone library results are presented in Table 1. The 'other' metazoa in Fig. 1B are primarily Symphyla, Diplura, Enchytraeidae, and Protura.

A direct comparison of the two approaches is difficult and it is not surprising that the two approaches do not necessarily yield identical results (see Fig. 1). The molecular approach is based on the relative abundance of the 18S rRNA genes as represented in each library which may not be directly correlated with relative biomass levels as determined by the direct extraction procedures owing to differences in the ratios of 18S rRNA gene copy numbers to biomass across taxa. The amount of soil from which the DNA was extracted (three 1 g replicates from a 20 g soil sub-sample) was smaller than

Table 1

Relative abundances of various metazoan taxa as determined by the representation of sequences in the clone libraries. N = the number of sequences included in each library that were of sufficient quality to permit taxonomic assignment. Data are presented as percentages of the total number of sequences in each library that were a close match to the particular taxonomic group. The 'other' category represents sequences with close matches to unidentified arachnids. 'Ha' soils are from the deciduous forest plots and 'Gr' soils are from the pasture plots.

Soil	N	Collembola (%)	Acari-Oribatida (%)	Acari-Prostigmata (%)	Acari-Mesostigmata (%)	Nematoda-Chromadorea (%)	Nematoda-Enoplea (%)	Tardigrada (%)	Enchytraeidae (%)	Other (%)
Ha1	78	9.0	28.2	0	1.3	19.2	39.7	0	1.3	1.3
Ha2	80	8.8	36.3	2.5	1.3	17.5	27.5	1.3	1.3	3.6
Ha3	86	4.7	79.1	3.5	2.3	3.5	7.0	0	0	0
Gr1	65	3.1	3.1	0	0	6.2	83.1	3.1	0	1.4
Gr2	88	0	2.3	4.5	1.1	14.8	77.3	0	0	0
Gr3	71	1.4	21.1	0	2.8	28.2	46.5	0	0	0

the amount of soil processed during the direct extraction procedure (~500–1000 g for the modified-Tullgren extractions and ~20 g for Baermann extractions). This difference in soil volumes may partially account for the different results obtained using the two approaches as there could be significant small-scale heterogeneity in faunal communities that may be apparent even after a careful homogenization of the soils. There may also be biases associated with our molecular approach that lead to the preferential amplification or preferential extraction of 18S rRNA genes from some taxa over others. However, it is important to recognize that the direct extraction techniques are also prone to their own set of biases given that the techniques can favor the extraction of some taxa over others and extraction efficiencies for individual taxa are not likely to be constant across different soil types (Petersen and Luxton, 1982; André et al., 2002). Because both methods have their own set of biases and potential sources of error, the lack of direct congruence between the two methods does not mean that the molecular method is less suitable than the direct method for faunal community analyses or vice versa.

One of the difficulties with using sequence-based approaches for community analysis is that, unless clone libraries are large, the abundance of very rare taxa will be difficult to assess because the dominant taxa will represent the bulk of the sequences in a library. For this study, we sequenced only 65–88 sequences per soil sample, a number of sequences likely to be sufficient for describing the general structure of the soil mesofaunal communities, but an insufficient number of sequences if our goal was to describe the full extent of mesofaunal diversity in each soil sample. However, the sequence-based method was able to identify sequences from taxa that were missed with the direct extraction approach. Perhaps more importantly, one limitation of the molecular approach is that the clone libraries only provide estimates of the proportional abundances of the taxonomic group in a given community, not actual abundances, and this may be an important limitation depending on the research question of interest.

One of the most important advantages of the sequence-based approach is that the data are readily comparable between studies conducted by different researchers working with different soils given that taxonomic identification is less prone to 'technician error' in taxonomic identification. The primer set described here targets 18S rRNA genes and provides a broad characterization of soil faunal communities, but other genes could be targeted if more detailed phylogenetic information on individual taxa was desired. Although it is debatable whether the molecular approach is faster than the more standard approaches for analyzing micro- and mesofaunal communities, the identification of most faunal taxa by microscopy is not trivial nor is it rapid, especially considering that there are often few experts with adequate training in the morphological identification and classification of specific taxonomic groups (André et al., 2001). The molecular approach may be particularly useful given that many studies in soil ecology already involve DNA extraction for soil microbial community analyses and the DNA could subsequently be used for corresponding faunal community analyses. As sequence-based approaches become more commonly used by soil ecologists, we expect that the molecular approach described here (or variants thereof) will likely become even more valuable as a tool for the study of soil fauna.

In conclusion, we have shown that a molecular approach can be used to survey soil faunal communities in much the same way that

sequence-based techniques are currently used to survey soil microorganisms. The molecular technique was able to capture differences in faunal communities from two different land-use types at a broad taxonomic resolution, in a manner comparable to traditional, direct extraction techniques. We are not proposing that the sequence-based approach described here should replace more commonly-used methods of faunal analysis as no single method provides an unbiased characterization of faunal communities; nor is any one method optimal for all research questions. Rather, we have demonstrated that a molecular approach can complement more traditional methods used to survey faunal communities, providing an expanding toolbox by which soil ecologists and taxonomists may explore faunal communities.

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