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Molecular profiling of soil animal diversity in natural ecosystems: Incongruence of molecular and morphological results

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ABSTRACT

A major problem facing ecologists is obtaining a complete picture of the highly complex soil community. While DNA-based methods are routinely used to assess prokaryote community structure and diversity in soil, approaches for measuring the total faunal community are not yet available. This is due to difficulties such as designing primers specific to a range of soil animals while excluding other eukaryotes. Instead, scientists use laborious and specialized taxonomic methods for extracting and identifying soil fauna. We examined this problem using DNA sequencing to profile soil animal diversity across two Alaskan ecosystems and compare the results with morphological analyses. Of 5267 sequences, representing 549 operational taxonomic units (OTU), only 18 OTUs were common to both sites. Representatives included 8 phyla, dominated by arthropods and nematodes. This is the most comprehensive molecular analysis of soil fauna to date, and provides a tool to rapidly assess a missing component of soil biodiversity.

1. Introduction

There is growing interest in assessing soil animal biodiversity (Wall et al., 2001; Fitter et al., 2005), largely because soils are recognized as one of the most species rich animal habitats on Earth (Behan-Pelletier and Newton, 1999; Wardle, 2002). For example, up to 89 nematode species were found in <90 cm^3 soil from a tropical forest in Cameroon (Bloemers et al., 1997) and 159 mite species were found in 579 cm^2 soil in a grassland in Kansas (St John et al., 2006a). These soils have many additional invertebrate species that were not identified. Moreover, since soil animals and their interactions with micro-organisms mediate many ecosystem properties and processes, including decomposition, nutrient cycling, carbon sequestration, plant community dynamics, and maintenance of soil structure, an understanding of their diversity is required to maintain ecosystem services from which humans benefit (Wall, 2004). Despite the vast diversity of animals in soil, most biodiversity research has focused on aquatic and aboveground organisms (Behan-Pelletier and Newton, 1999; Wardle, 2002) and little is known about factors that influence patterns of biodiversity in soils at local, regional, or global scales (Ettema and Wardle, 2002). Where soil animal biodiversity has been studied at high resolution (i.e. species level) it has been restricted to a limited number of ecosystems and/or taxonomic groups (Bardgett, 2005). This dearth of information results partly from a lack of methods to rapidly and easily measure soil animal biodiversity at high resolution (Behan-Pelletier and Newton, 1999).

Current methods for characterizing animal biodiversity in soils are based on traditional morphological identification. Thus, they require specialist knowledge for each taxonomic group (e.g. termites, mites, nematodes, rotifers, tardigrades etc.), making identification extremely labor intensive. For example, six person-years were required to identify individuals of one soil animal group (mites) to the species level in a single experiment conducted at one grassland site in Kansas, USA (St John et al., 2006b). In addition, due to the large range in body size and natural history of animals in soils, different extraction methods are needed for different animal groups, precluding the identification of all animal species from individual soil samples. Finally, the challenge of identifying soil animal species morphologically is compounded by the fact that most species are undescribed (Behan-Pelletier and Newton, 1999). Thus, molecular methods that rapidly assess "species"-level diversity of a range of animal taxa from a single soil sample could potentially be an important tool in the study of soil biodiversity.

Molecular techniques for the study of soil biodiversity (typically microbial), such as denaturing gradient gel electrophoresis,

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terminal restriction fragment length polymorphism and phospholipid fatty acid analysis, provide only a broad overview of diversity due to their relatively low level of resolution; in contrast, sequencing provides a detailed measure of biodiversity at a resolution analogous to species level (O'Brien et al., 2005). Today, microbial ecologists often study diversity by extracting bulk DNA from soil, sediment or water, amplifying the bacterial 16S rRNA gene and constructing a sequence-based clone library (Chen and Pachter, 2005; Tringe et al., 2005). However, due primarily to a lack of primers specific to a range of animals, this approach has been limited to relatively small datasets (100–1000 sequences) utilizing fungi (O'Brien et al., 2005), algae and protists (Lawley et al., 2004; Lara et al., 2007), nematodes (Floyd et al., 2002; Griffiths et al., 2006) or mixed microeukaryotes (Fell et al., 2006). Our study reports a dataset of more than 5000 metazoan sequences from two sites, each represented by four replicates.

We developed a primer to amplify the 18S rRNA gene (rDNA) from a broad array of animals and to exclude other eukaryotes (plants, fungi, and protozoa) and bacteria. We used the eukaryotic 18S rDNA gene as a target because it is a homologue of the bacterial 16S rDNA gene, is widely used for phylogenetic study (Blaxter et al., 1998; Griffiths et al., 2006), and there are existing databases (e.g. GenBank) related to this gene (Cole et al., 2003). The 18S rDNA gene has some highly conserved regions used to discern deep phylogenetic relationships at the phylum level, while other regions vary at the species level. Although full-length 18S rDNA sequences have been used extensively in metazoan phylogenetics, the short sequences used in this paper are not expected to reconstruct metazoan phylogeny. Partial 18S rDNA sequences have been used in barcoding (Floyd et al., 2002) and thus can be used to identify operational taxonomic units (OTUs) and can discriminate among even closely related species. These partial 18S rDNA sequences can also be used to identify the closest match in GenBank that can provide some degree of taxonomic information. Some studies have shown a congruence between morphological and molecular analysis in simple systems (Eyualem and Blaxter, 2003). We utilized a molecular approach to assess soil animal diversity in two contrasting arctic ecosystems in Alaska: a boreal forest and arctic tundra. We also identified soil animals morphologically and compared the results.

2. Materials and methods

2.1. Experimental design

The sampling sites were a black spruce (*Picea mariana*) boreal forest near Fairbanks, Alaska (64°46.560′ N; 148°18.267′ W), and an acidic tussock tundra approximately 420 km further north (68°37.246′ N; 149°36.476′ W), which are home of the Bonanza Creek and Toolik Lake Long Term Ecological Research (LTER) sites, respectively. The mean ± SE maximum air temperature between 1990 and 2006 at the forest site was -18.2 ± 1.3 and 16.6 ± 0.3 in January and July, respectively, whereas at the tundra site it was -18.8 ± 1.0 and 15.2 ± 0.5, respectively. Annual precipitation was 342.9 ± 19.1 and 298.3 ± 55.2 mm (mean ± SE) and aboveground net primary productivity was 299.9 ± 15.7 and 140.8 ± 13.3 g m^{-2} at the forest and tundra site, respectively (Knapp and Smith, 2001).

At each site, we established a 900 m transect consisting of four evenly spaced 10 × 10 m plots (i.e. 300 m apart). The location of the transect was selected to be broadly representative of a dominant ecosystem type in the region and relatively pristine, having not experienced major anthropogenic disturbance. Within each transect, plots had similar elevation, aspect, vegetation and soil type. From each plot, we collected 20 soil cores (3.4 cm diameter; 10 cm deep), which were bulked for a single soil sample for each plot.

2.2. Molecular methods

The bulked samples were processed to produce the sieve fractions. Within 24 h of collection, 200 g of fresh soil sample from each of the four plots at each site was hand-mixed and added to 500 ml 95% ethanol which acted as a suspension medium and a preservative. The soil–ethanol suspension for molecular analysis was mixed, left to stand for 2 h, and remixed prior to washing through a series of progressively finer sieves (mesh sizes: 2 mm, 1 mm, 0.5 mm, and 0.1 mm) to separate animals of different body size. The material on the 1 mm, 0.5 mm and 0.1 mm was collected and stored in 95% ethanol, resulting in 12 samples from each of the two sites.

Metazoan specific primers for the 18S rDNA gene were designed from an alignment of 80 species of eukaryote rDNA sequences from GenBank including 69 sequences representative of metazoan phyla and 11 sequences representing non-metazoan phyla. The forward primer 18S11b (5′-GTC AGA GGT TCG AAG GCG-3′) corresponds to positions 1037–1054 of the human sequence (NR_003286 in GenBank) and to a region that is relatively constant among metazoans, but has positions that vary considerably in other eukaryotes. The reverse primer 18S2a (5′-GAT CCT TCC GCA GGT TCA CC-3′) corresponds to positions 1848–1867 of the human sequence. The primers amplify approximately a 830 bp segment.

DNA was extracted from each sample using a modified CTAB extraction procedure (Gawel and Jarret, 1991). Metazoan-specific primers amplified the 18S rDNA (an initial 2 min denaturing step at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min). PCR products were cloned into libraries using Topo TA cloning kits (Invitrogen, Carlsbad, CA) and approximately 200 single pass sequences (519 base pairs after trimming) were obtained from each library using a commercial sequencing facility. The animal-specific primers minimized amplification of plant, protozoa, fungi or bacteria DNA.

Raw sequences were processed with custom software developed in our lab that finds a highly conserved region of the 18S rDNA sequence as an anchor point and then trims upstream and downstream from that point. We optimized the trim points so that only the most accurate part of the sequences were used, and employed a filter that removed poor quality sequences. This produced highly accurate sequences by reducing the length of the read to 519 bp and discarding many poor sequences, approximately 30%. This filtering process resulted in our finding identifiable GenBank matches for 99.9% of the final 5267 sequences.

2.3. Assigning operational taxonomic units (OTUs)

The relationship between molecular based OTUs and morphology-based taxonomic assignments is not well documented. For example, can a group of 18S rDNA sequences that are 95% identical to each other serve as a proxy for a species, a genus or a family? To find out, we analyzed 890 nematode 18S rDNA sequences from GenBank. According to GenBank taxonomy, those sequences represent 18 orders, 139 families, 330 genera and 616 species. Similarly we analyzed 229 mite sequences that represent 7 orders, 61 families, 179 genera and 218 species. We then analyzed both sets of sequences strictly by grouping them into OTUs based on 100%, 99%, 97%, 95%, 90%, 85% and 80% sequence similarity using Sequencher version 4.7 (Gene Codes, Ann Arbor, MI). We compared the number of those sequences that fell into the GenBank assigned taxonomic groupings of species, genus, family and order to the

number of OTUs formed at different sequence similarity thresholds according to Sequencher.

2.4. Identification of OTUs

After each OTU was assigned, a sequence from that OTU was used as a query in a BLAST search. The closest matching sequence contained in GenBank for which there was documented taxonomic information was used as a provisional "identification" of the OTU for subsequent comparison to morphological results. We recognize that GenBank taxonomy is not always accurate but use it here because of the wide taxonomic scope of the project.

2.5. Morphological methods

A 100 g aliquot of fresh soil from each of the four soil plots at each site was used for morphological analysis. For comparison to the molecular approach we extracted soil animals from the sample using sugar flotation, a standard method for small soft-bodied soil invertebrates such as nematodes (Jenkins, 1964). Soil fauna were identified to major taxonomic group (e.g. mites, collembolans, nematodes, etc.) and nematodes were further identified to family. It was not feasible to morphologically identify animals to the species-level due to time limitations.

2.6. Statistical analysis

The frequency of each OTU was tabulated and input for diversity and community structure analysis. Diversity indices including richness, Hurlbert's Probability of Interspecific Encounter (PIE), Dominance and Shannon diversity for 500 sequences from each sample were obtained in EcoSim (Gotelli and Entsminger, 2008) using the rarefaction randomization algorithm included in the program. It randomly resamples different numbers of sequences from the dataset without replacement and determines the number of OTUs in each sample. SigmaStat 3.10 (Systat Software Inc., San Jose, CA) was used for univariate analysis of soil faunal diversity indices. The estimated species richness (S_{Chao1}) was computed by the formula $S_{Chao1} = S_{obs} + {F_1}^2/2F_2$ where S_{obs} is the number of observed OTUs, F_1 and F_2 are the number of OTUs occurring either once or twice using EstimateS 8.0.0 (Colwell, 2006). This analysis is based on randomly resampling different numbers of sequences of 12 samples (four plots and three sieve sizes) at each site. The soil community was analyzed with the non-parametric multivariate analysis procedures of multidimensional scaling (MDS) based on Bray–Curtis community similarity using PRIMER-E statistical software (PRIMER-E Ltd, Plymouth Marine Laboratory, UK). The PRIMER-E software was also used for an analysis of similarity (ANOSIM) that calculated the significance of differences in community structure among plots, sieve sizes and sites. Differences in soil fauna community structure between sample sites were also compared using UniFrac software (Lozupone et al., 2006) using an alignment and neighbor-joining tree calculated with Mega 4.0 software (Kumar et al., 2008).

3. Results

3.1. Assigning operational taxonomic units (OTUs)

Table 1a shows the results of comparing the taxonomy of a dataset of 890 known nematode and 229 known mite sequences from GenBank to the number of OTUs for those sequences as determined by Sequencher using 100%, 99%, 97%, 95%, 85% and 80% identity thresholds. The 99% identity threshold was used as a proxy for "species" in subsequent results. See Section 4.

3.2. Sequences and OTUs

We obtained 5267 sequences from the two Alaskan locations: 2621 from boreal forest and 2646 sequences from the arctic tundra. These represented 262 OTUs from boreal forest and 305 OTUs from tundra using 99% match criteria, which correlates roughly to species level (Table 1a). Only 18 of 549 identified OTUs were found at both locations, yet these 18 OTUs represented 48% of the sequences examined. Approximately 8% of the sequences at each site occurred once (singletons), representing 77% and 71% of the boreal forest and arctic tundra OTUs, respectively.

3.3. Analysis of similarity of soil faunal communities

Primer-E analysis of similarity based on assigned OTUs revealed no significant differences in faunal communities among the four plots at either the boreal forest site ($p = 0.986$) or the tundra site ($p = 0.113$). We bulked 20 core samples at each plot and then processed the bulked samples to produce the sieve fractions. However, we found that sieving did not partition soil fauna among the three sieve sizes at the four plots at each site (boreal forest, $p = 0.638$; tundra, $p = 0.951$). In subsequent diversity and community structure analyses, data from the three sieve sizes were combined for each of the four plots at each site.

Analysis of similarity based on assigned OTUs by Primer-E also showed that soil faunal communities of boreal forest and tundra were significantly different ($p = 0.001$), in agreement with UniFrac analyses, which also found a significant difference ($p = 0.001$).

3.4. Diversity and communities of soil fauna

Indices of richness, Shannon diversity, Dominance and Hurlbert's PIE (Probability of Interspecific Encounter, similar to the Simpson index) all indicated greater diversity in the tundra than in the boreal forest; however, only differences in Hurlbert's PIE and the dominance index were statistically significant (Table 2).

Non-metric MDS analysis shown in Fig. 1 indicated that the similarity of soil faunal communities of the four boreal forest plots is 15%. The similarity of three plots at the tundra site is 15%, while the fourth plot shared only about 10% similarity with the other three plots. The MDS analysis also revealed a 0.01 2-dimensional stress value for the ordination.

3.5. Rarefaction curves

Rarefaction curves did not plateau even with the analysis of >2500 sequences for each site (Fig. 2a), indicating that more OTUs were present than were detected. Rarefaction curves that estimate the number of OTUs using S_{Chao1} (Fig. 2b) showed that the estimated number of OTUs at the boreal forest site was 1320, five times higher than the observed number of 262 OTUs. Similarly, the estimated number of OTUs at the tundra site was 2010, much higher than the 305 OTUs observed at this site. However, these S_{Chao1} values also underestimated total OTU richness since the curves did not reach a plateau (Fig. 2b).

3.6. Community structure based on molecular analyses

Five and six phyla of soil fauna were detected from 2621 boreal forest and 2646 tundra sequences respectively (Table 3). Arthropods were the dominant faunal group in the boreal forest, accounting for 79.9% of the total number of sequences analyzed. Mites accounted for 68.4% of the total number of sequences. Nematodes and annelids accounted for 10.2% and 8.4% of the total number of sequences respectively, while mollusks and tardigrades

Table 1a
890 nematode and 229 mite 18S rDNA sequences from the NCBI database with the numbers of taxonomic levels and the numbers of OTUs grouped by different minimum sequence match levels (%) in Sequencher version 4.7 (Gene Codes, Ann Arbor, MI).

Taxonomic levels	890 GenBank taxonomy of nematode sequences	Number of OTUs grouped by similarity (%)	229 GenBank taxonomy of mite sequences	Number of OTUs grouped by similarity (%)
Sequences	890	712 (100)	229	203 (100)
Species	616	417 (99)	218	148 (99)
Genera	330	313 (97)	179	85 (97)
Family	139	165 (95)	61	50 (95)
Order	18	14 (80)	7	13 (85)

were only 0.1% of soil fauna (Table 3). In the arctic tundra, the dominant soil fauna were arthropods and nematodes, both accounting for 43% (43.0% and 42.8%) of the sequences analyzed, followed by tardigrades (4.8%), gastrotrichs (4.0%), annelids (3.2%) and platyhelminthes (1.0%) (Table 3).

Four and seven nematode orders were detected from 267 boreal forests and 1132 tundra nematode sequences (Table 4). In the boreal forest, Araeolaimida was the most dominant nematode, accounting for 88.8% of soil nematodes, followed by Rhabditida (8.6%), Monhysterida (1.9%) and Enoplida (0.7%) (Table 4). In the arctic tundra, Rhabditida was the dominant nematode group (52.3%), followed by Enoplida (28.8%) and Araeolaimida (11.4%). Four other nematode orders (Mononchida, Chromadorida, Tylenchida and Dorylamida) ranged from 1% to 3% of soil nematodes (Table 4).

3.7. Community structure based on morphological analyses

Four phyla of soil fauna were detected by morphological identification. Nematodes were the most numerically dominant taxa in both boreal forest (60.9%) and tundra (69.8%). Arthropods were the next most numerically abundant group in the boreal forest (19.4%), but only a minor component in the tundra (2.6%). Rotifers were the second major component (26.1%) in the arctic tundra and less dominant (18%) in the boreal forest. Tardigrades contributed 1.3% and 1.5% in the boreal forest and tundra respectively (Table 3).

Eight different nematode orders were identified from 1606 boreal forest and 3214 tundra individuals sorted to order using morphological identification of nematodes (Table 4). The order Tylenchida was the dominant nematode order at both the boreal forest (35.1%) and tundra (47.3%) site, followed by Araeolaimida, 19.5% and 18.9% and Rhabditida, 17.6% and 12.2%, in the boreal forest and tundra respectively. Five other orders were less than 10% of soil nematodes (Table 4).

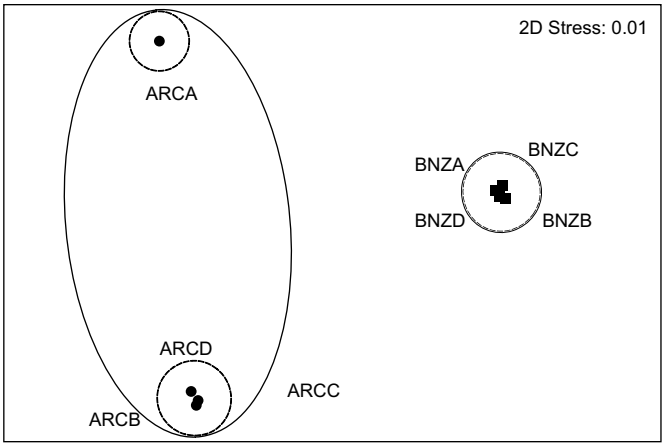

Fig. 1. Non-metric MDS ordination of soil faunal communities using molecular sequencing at four plots within boreal forest and arctic tundra. Symbols in the MDS ordination are: circle, arctic tundra (ARC); square, boreal forest (BNZ). The solid lines represent a similarity level of 10% and the broken lines represent a similarity level of 15%.

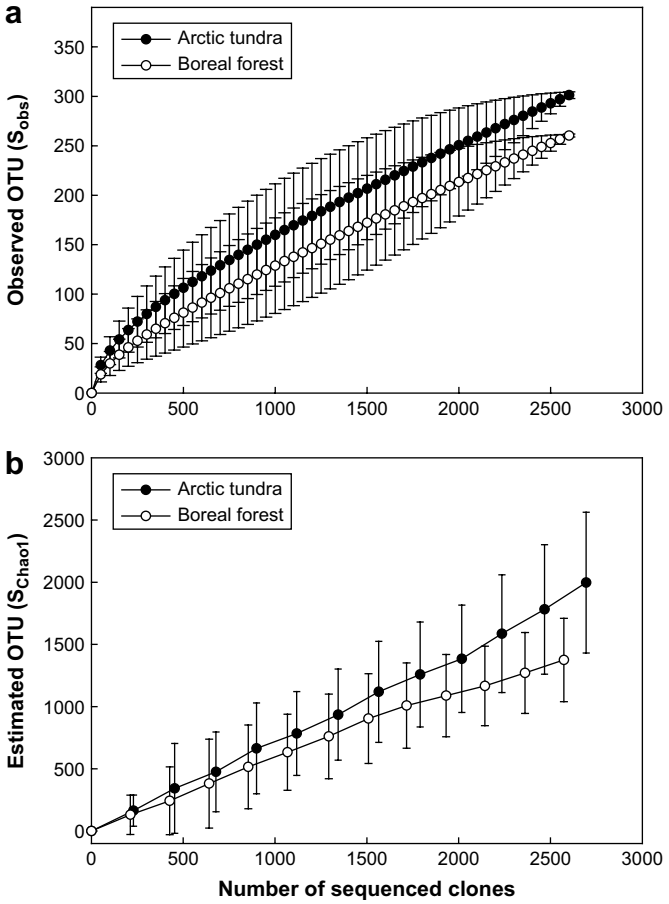

Fig. 2. Rarefaction curves using molecular sequencing of observed (a) and estimated (b) soil faunal OTU richness for boreal forest and the arctic tundra sites. Vertical bars represent the standard deviation. Observed OTU (S_{obs}) obtained from EcoSim by randomization. Each dot on this graph represents the accumulated number of S_{obs} in groups of 50 sequences. Estimated OTU (S_{Chao1}) obtained from EstimateS were based on 12 samples (four plots and three sieve sizes).

4. Discussion

We analyzed a broad cross-section of soil invertebrates using molecular methods with a resolution analogous to the species level. This addresses a missing gap in the assessment of the Earth's biodiversity because: (1) the soil animal community is analyzed from a single sample without taxon-specific extractions; (2) a vast range of well and little known (e.g. undescribed) soil animals are detected; (3) the time required to identify even one phylum (e.g. nematodes) to a resolution analogous to species level is substantially reduced; (4) patterns of soil taxa on various spatial scales can be examined by a standard and unambiguous comparison of OTUs within and between sites; (5) ecosystem level questions about taxa previously addressed only at coarse-scale functional levels (e.g. fungal-, bacterial-, or plant-feeders) can now be tested; and (6) the combination of molecular methods for bacterial, fungal and this method will allow for the first time, a more complete biotic analysis of soils.

4.1. Assignment of OTUs

There is little published information that estimates the percent sequence identity expected for various taxonomic levels. Bacterial studies using 16S rDNA have used values ranging from 95% to 99% sequence identity to define OTUs that may correspond to species (Edwards et al., 2004; Stach and Bull, 2005; Gontang et al., 2007). Another study used a value of 97.5% sequence identity to define OTUs that may correspond to species of mixed micro-eukaryotes (Lawley et al., 2004), while Floyd et al. (2002) suggested 99.5% identity to define OTUs that may correspond to nematode species. In order to estimate the correlation between sequence identity and taxonomic level among soil metazoans, we examined 890 nematode and 229 mite 18S rDNA sequences from species with taxonomies that are well documented in GenBank.

We initially tested 100%, 99%, 97%, 95%, 90%, 85% and 80% criteria for OTUs on a known dataset of 890 documented nematode 18S rDNA sequences in GenBank representing 616 species, 330 genera, 139 family and 18 orders (Table 1a). The 99% criterion of nematode sequences found 417 OTUs, slightly under- representing the number of actual species. The 97% criterion found 313 OTUs which was close to the 330 genera in the GenBank annotations. We chose the 99% criterion as a proxy for species because we conservatively estimate our experimental dataset has an error rate of less than 1% based on expected errors PCR amplification and sequencing. Raw sequences were processed (see methods) to maximize sequence quality. Floyd et al. (2002) used 99.5% based on an analysis of error. We carried out a similar analysis of 229 mite 18S rDNA sequences from GenBank (Table 1a) representing 218 species in 179 genera, 61 family and 7 orders. In this case, the 99% criterion produced slightly fewer OTUs than the number of genera in the sample. Even the 100% criterion produced fewer OTUs than suggested by GenBank taxonomy. Since the mite taxonomy in GenBank is based on morphology, it appears that the 18S rDNA molecule as analyzed here may lack the resolution of detailed morphological analysis. However, we conservatively used the 99% criterion for nematodes, mites and all other taxa analyzed in this study. For both nematodes and mites, the 95% criterion appears to approximate family level relationships and 80–85% approximates order level relationships. The rate of DNA substitution varies among taxa and there is no general link between DNA variation and morphological change (Blaxter, 2004). It appears that at least for mites and nematodes, the most prevalent fauna found in soil, the 99% criterion for assigning OTUs underestimates rather than overestimates species diversity.

The analysis of documented sequences of nematodes and mites from GenBank suggested sequence similarity values that approximate the taxonomic levels of species, genera, family and order (Table 1a). According to those data the 5267 sequences obtained at the two Alaskan sites were predicted to represent 549 species, 250 genera, 180 families and 48–65 orders (Table 1b). Identification of the 549 OTUs using BLAST searches revealed only 118 species, 112 genera, 87 families and 51 orders (Table 1b). The predicted number of OTUs was much higher than the number of BLAST identified OTUs for species, genera and family, but similar for order. This is consistent with the nature of GenBank, where orders are well represented across most phyla, but families, genera and species are progressively less well represented, resulting in 21% predicted species, 45% predicted genera, 48% predicted families to 78–106% predicted orders. These values should increase as GenBank grows.

Table 1b
5267 18S rDNA sequences obtained from two Alaskan sites with the number of OTUs grouped by different minimum sequence match levels (%) and the number of "taxa" identified from GenBank.

Taxonomic levels	Similarity %	Predicted OTUs	GenBank identified OTUs	GenBank identified/ Predicted OTU
Sequences		5267		
"Species"	99	549	118	21%
"Genera"	97	250	112	45%
"Family"	95	180	87	48%
"Order"	85 (80)	65 (48)	51	78–106%

4.2. Biodiversity of OTUs

Rarefaction curves did not reach a plateau for either observed or estimated OTUs at the boreal forest and tundra sites, suggesting that rare soil fauna were not adequately quantified, which further highlights the rich diversity of animals in soils. Our study, and other molecular studies of soil bacterial, fungal and nematode communities, suggest that far more sequence data would be required in order to obtain a plateau in rarefaction curves (Dunbar et al., 1999; McCaig et al., 1999; McGarvey et al., 2004; Tringe et al., 2005). The common factor appears to be the large number of OTUs that are only represented a single time in the data (singletons). In our study, singletons represented 8% of the sequences but accounted for 70–77% of OTUs; other studies of soil nematodes have found 65% of OTUs were represented by singletons (Griffiths et al., 2006), whereas studies of soil fungi (O'Brien et al., 2005) and bacteria (Dunbar et al., 1999) report 66% and 79–87% of OTUs were represented by singletons, respectively. The high number of OTUs represented by singletons in nearly all studies of soil DNA is a common occurrence and suggests that there is a large pool of low abundance organisms of all taxa in soil. In our study, estimated OTUs by S_{Chao1} indicated that soil faunal OTUs could reach 1320 at the boreal forest and 2010 at the tundra, which reflects the enormous faunal diversity found in these soils. Moreover, this value is likely to be an underestimate of soil animal species richness because it was still increasing as more sequences were analyzed.

The large numbers of singletons affect indices commonly used to compare community structure among sites. In our study, the Hurlbert's PIE, which is closely related to Simpson diversity index (Hamilton, 2005), differed significantly between the two sites, while Shannon diversity and species richness indices were not significantly different at the 95% confidence interval (Table 2). Dominant taxa contribute to the Simpson index, while the Shannon index is affected more by rare species such as singletons (Mouillot and Leprêtre, 1999). Our analysis of Dominance (Table 2) confirms that dominance is a major factor differentiating the two sites. This was consistent with the observed soil faunal compositions, in which mites contributed greatly (68.4%) to the soil faunal compositions in the boreal forest (Table 3). Our estimates of soil biodiversity are much higher than other molecular studies of soil eukaryotes when simple rarefaction curves were used and sample sizes were small (e.g. Lawley et al., 2004; Griffiths et al., 2006; Lara et al., 2007) but are consistent with at least one study of soil fungi that used the ACE estimator as well as a larger sample size (O'Brien et al., 2005).

4.3. Community structure of OTUs

Only 18 common OTUs were observed at both the boreal forest and tundra sites, suggesting that environmental factors between

Table 2
Diversity indices (mean ± SE) of soil fauna using molecular sequencing at the boreal forest and tundra sites. Diversity indices are the value obtained by an EcoSim rarefaction calculation at 500 sequences.

Location	Richness (S)	Shannon diversity (H')	Dominance	Hurlbert's PIE
Boreal forest	59.4 ± 5.8 a	2.32 ± 0.13 a	0.43 ± 0.04 a	0.78 ± 0.03 b
Arctic tundra	72.4 ± 13.8 a	2.98 ± 0.31 a	0.20 ± 0.04 b	0.90 ± 0.03 a

Values followed by different letters are significantly different at the $p = 0.05$ level.

Table 3
Taxonomic composition of soil fauna at the boreal forest and tundra sites.

Taxonomic groups	Molecular %		Morphological %	
	Boreal forest	Arctic tundra	Boreal forest	Arctic tundra
Annelida	8.4	3.2	0.0	0.0
Arthropoda	79.7	43.0	19.4	2.6
Ants	0.0	0.0	0.1	0.0
Collembolans	1.1	7.3	0.2	0.3
Diptera	0.0	0.6	0.2	0.4
Mites	68.4	25.7	18.9	1.8
Other arthropoda	10.3	9.4	0.0	0.0
Gastrotricha	0.0	4.0	0.0	0.0
Mollusca	0.1	0.0	0.0	0.0
Nematode	10.2	42.8	60.9	69.8
Platyhelminthes	0.0	1.0	0.0	0.0
Rotifer	0.0	0.0	18.4	26.1
Tardigrade	0.1	4.8	1.3	1.5
Total number counted	2621	2646	2640	4611

Assignments of sequence-based OTUs to the taxonomic groups were estimated by finding the closest match in GenBank to each OTU by BLAST and using the taxonomic listing for that GenBank entry. Values are the percent of the total number of sequences analyzed (molecular) or specimens counted (morphology).

sites, such as soil type, above-ground community structure and climate, may affect the distribution of soil fauna. However, these 18 OTUs, which represented only 3% of the total OTUs identified, accounted for 48% of the sequences, indicating that the most abundant soil animal species were also the most widely distributed. This was also observed in a much smaller molecular study of 438 soil ciliate 18S rDNA sequences from two sites (Lara et al., 2007) where 31 OTUs were identified. Nine OTUs were common to both sites and represented 61% of the sequences. Similar abundance–range relationships have been found in a number of other taxa (Gaston, 1996).

The boreal forest was dominated by arthropods (79.7%) while the tundra was co-dominated by arthropods (43.0%) and nematodes (42.8%) as seen in Table 3. This difference was a major contributing factor to the observed difference between soil faunal community structure at the boreal forest and tundra as evidenced by the MDS analysis in Fig. 1. The site ARCA (Fig. 1) differs from the other three tundra sites because it had four large OTUs containing 319 sequences that were not found at the other plots at the tundra site. This most likely was caused by the patchy distribution of some soil animals that can be caused by microhabitats (Salminen and Sulkava, 1996; Garey and McInnes, 2008). The Primer-E based analysis of similarity also revealed that the differences in the community structure between the boreal forest and tundra sites are significant ($p = 0.001$). However, both MDS and the similarity analyses depend on the OTU assignments made. Therefore Unifrac analysis was carried out as it is phylogenetically based and does not depend on pre-assigned OTUs. Again, the difference in community structure was significantly different between the boreal forest and tundra sites ($p = 0.001$).

About 97% (531 of 549) of OTUs were observed in the boreal forest or the tundra, but not both. The prevalence of OTUs unique to each site suggests that most species are endemic to each site and that cosmopolitan species are rare. Similar evidence for the endemism of eukaryotic organisms was observed in Antarctic soils (Lawley et al., 2004).

4.4. Differences between morphological and molecular analyses of community structure

In a broad sense, the morphological and molecular results agreed in that they both indicate higher diversity in the tundra than in the boreal forest site. However, differences between the two approaches were found at every comparable level of the analyses. This was not unexpected because even with morphological analyses, different methods of extracting organisms from the soil can cause large differences in the perceived community structure. For example, live nematodes are commonly extracted from soil using Baermann funnels, which rely on nematodes actively moving through soil and a tissue barrier into water (Coleman et al., 1999), but this method underestimates less mobile taxa and large taxa. Tullgren funnels are commonly used to extract mites, collembolans, and other microarthropods, but do not efficiently extract inactive organisms or organisms that live in water films within the soil, such as nematodes, tardigrades, and rotifers (Crossley and Blair, 1991; Coleman et al., 1999). As well as varying among taxa, extraction efficiency also differs among species within a taxonomic group. For example, using the sugar flotation method (similar to the variation of this method used in our study), the extraction efficiency of the nematode *Xiphinema index* was almost an order of magnitude lower than that of the nematode *Criconemella xenoplax* (Viglierchio and Schmitt, 1983).

Some of the differences between the molecular and morphological results in our study may result from differences in the methods that were used. For example, the morphological data are based on the abundance of individual animals within a taxonomic group, whereas the molecular data are based on the relative abundance of sequences belonging to that taxonomic group, which may not correspond to the number of animals. The relative abundance of each OTU sequence is related to biomass of the source organisms as well as the DNA content, size and number of the cells within the organism. In addition, 18S rRNA genes are found in clusters of many copies, and the copy number varies enormously among different taxa. The simplest interpretation is that the molecular analysis approximates the biomass of the contributing soil animals (Griffiths et al., 2006). For example, a tiny animal species could appear dominant in terms of the number of individuals counted, but be a minor component in an analysis of DNA sequences. An unidentifiable fragment of an organism might not be counted at all morphologically, yet contribute in terms of DNA sequences. All of these factors, and other factors discussed below, make it difficult to directly compare results from the two methods. However, either method, when used consistently should provide a valid view of the biodiversity and community structure in different ecological systems.

In our study, DNA was extracted directly from soil to avoid bias presented by taxon-specific extraction methods commonly used in morphological studies. Our morphological work on nematodes required the animals to be extracted from the soil prior to analysis, so it is likely that this difference played a major role in the discrepancies we found between the two methods. One soil nematode study extracted whole nematodes from soil using Baermann funnels (Griffiths et al., 2006). The extracted nematodes were analyzed both morphologically and by DNA sequencing of 18S rDNA. Their study found agreement at the order level between the morphological and molecular results after accounting for differences in biovolume among nematodes. For example, in their Fig. 3, Dorylaimida and Mononchida appear to have a large body side and thus are highly represented in their molecular results while Rhabditida and Tylenchida appear to have a small body size and thus are under-represented in their molecular results. We do not find similar results in our data (Table 4). However, their study suggests that the disparity we found between morphological and molecular results could be due in part to the difference in how DNA was extracted directly from the soil compared to how nematodes were extracted for morphological work using sugar flotation in our study.

Our molecular analyses were carried out on DNA extracted from sieved soil. When extracting DNA, we may have obtained DNA not only from the whole animal bodies but also those from the fragments, debris and eggs of soil animals. The sieving was used to

Table 4
Taxonomic composition of nematodes at the boreal forest and tundra sites.

Taxonomic groups	Molecular %		Morphological %	
	Boreal	Tundra	Boreal	Tundra
Chromadorida	0.0	1.9	1.4	2.9
Enoplida	0.7	28.8	2.8	6.9
Tylenchida	0.0	1.7	35.1	47.3
Rhabditida	8.6	52.3	17.6	12.2
Dorylaimida	0.0	1.1	4.6	4.2
Monhysterida	1.9	2.9	0.2	0.8
Mononchida	0.0	0.0	0.7	0.3
Araeolaimida	88.8	11.4	19.5	18.9
Unknown bacterial-feeder	0.0	0.0	9.3	4.2
Unknown omnivore	0.0	0.0	3.7	0.8
Unknown plant parasite	0.0	0.0	4.8	1.8
Unknown predator	0.0	0.0	0.3	0.0
Total number counted	267	1132	1606	3214

Assignments of sequence-based OTUs to orders were estimated by finding the closest match in GenBank to each OTU by BLAST, and using the order listed for that GenBank entry. Values are the percent of the total number of sequences analyzed (molecular) or specimens counted (morphology).

exclude large invertebrates such as adult earthworms and large insects that could have overwhelmed the DNA from smaller soil dwelling animals. The sieving was also designed to at least partially separate the small invertebrates from the soil particles, by excluding particles larger than 2 mm or smaller than 100 μm. It appeared that soil properties varied substantially among sites and with some soils, the 2 mm sieve excluded a large portion of the soil as large clumps and in other soils excluded less. It is likely that some soil animals are difficult to detach from soil clumps and may have been lost in the sieving process. Also, DNA from lysed animals would be lost during the sieving process. Our observations suggest that although sieving did eliminate large whole invertebrates from our samples, it did not effectively partition soil invertebrates by size. In addition, subsequent steps in extracting genomic DNA from the soil, PCR amplifying the 18S rDNA gene, and cloning of the 18S rDNA genes are not without their own potential biases (Waite et al., 2003; Foucher et al., 2004; Griffiths et al., 2006; Donn et al., 2008).

At the broadest view, the molecular analyses found species from seven phyla, while the morphological analysis found species from four phyla (Table 3). Several phyla present in the molecular analyses but absent in the morphological analyses might be difficult to recognize in viewing preserved specimens, particularly gastrotrichs, flatworms, juvenile mollusks and juvenile or fragmented oligochaetes. Rotifers were a large component in the morphological analyses (18.4–26.1%) but were absent in molecular analyses. Examination of one of the metazoan specific primers (18S-11b) revealed several mismatches to all known rotifer 18S rDNA that would explain the discrepancy. Finally, molecular methods found a substantial number of sequences (9.4–10.3%) representing arthropod groups not found in the morphological analysis.

In the molecular analyses, mites were the dominant taxon in the boreal forest (68.4%) while nematodes were dominant in the arctic tundra (42.8%). Together nematodes and mites were represented by 78.6% of the sequences in the boreal forest and 68.5% in the tundra. With morphological methods, nematodes were the dominant taxon in both boreal forest (60.9%) and tundra (69.8%). Mites were the next most dominant in the boreal forest (18.9%) but only a minor component in the tundra (1.8%). Generally, morphological analysis found fewer mites and more nematodes than the molecular analysis. The sugar flotation method is known to be inefficient in recovering mites (Coleman et al., 1999), but the molecular methods could also have been inefficient in extracting and amplifying DNA from nematodes. Our initial methods for extracting DNA from soil using a commercial kit yielded very few nematodes sequences, while the CTAB method resulted in the proportion of nematode sequences reported here. It appears that the method used to extract genomic DNA from the soil can bias the results, particularly with nematodes.

Because of our expertise with nematodes, the most detailed comparison of our morphological and molecular results were with nematodes. The overall diversity of nematode orders was very similar between morphological and molecular analyses. Morphological analysis revealed eight nematode orders, while molecular results found seven of those eight orders (Table 4). The order Mononchida was only a minor component of the morphological analysis but absent in the molecular analysis. Tylenchids were abundant in the morphological analysis but rare in the molecular analysis. Areolaimids appeared much more often in molecular analysis than in the morphological analysis at the boreal forest, but not at the tundra site. We sorted nematode specimens to family in our morphological studies. GenBank contains over a thousand nematode 18S rDNA sequences representing at least 139 families so it seems reasonable that our molecular identification of DNA sequences to family were reasonably accurate. However, at the family level, morphological analyses found nematodes representing 32 families while molecular methods found sequences representing only 15 families (Table 5). We analyzed 1399 nematode sequences in the molecular analysis while we counted 3868 individual nematodes in the morphological analysis. Therefore,

Table 5
Molecular and morphological taxonomic composition of nematodes (family levels) at the boreal forest and tundra sites (see Table 4 for more information).

Taxonomic groups	Molecular %		Morphological %	
	Boreal	Tundra	Boreal	Tundra
Achromadoridae	0.0	1.9	0.0	0.0
Alaimidae	0.0	7.2	0.4	1.0
Anguinidae	0.0	0.0	3.6	2.5
Aphelenchidae	0.0	0.0	2.9	1.3
Aphelenchoididae	0.0	0.0	6.4	2.3
Bastianiidae	0.0	0.0	0.0	0.9
Bunonematidae	0.0	0.0	0.0	0.1
Cephalobidae	0.0	0.0	14.5	4.5
Criconematidae	0.0	0.0	0.1	0.2
Dorylaimidae	0.0	0.0	2.5	2.0
Haliplectidae	0.0	1.9	0.0	0.0
Heteroderidae	0.0	0.0	0.1	0.5
Hoplolaimidae	0.0	0.0	0.0	0.5
Ironidae	0.0	0.1	0.0	0.0
Leptonchidae	0.0	0.0	0.0	0.6
Meloidogynidae	0.0	0.0	4.3	4.3
Monhysteridae	1.9	2.6	0.2	0.8
Mononchidae	0.0	0.0	0.7	0.3
Nordiidae	0.0	0.0	0.0	0.2
Oncholaimidae	0.0	0.1	0.0	0.0
Odontolaimidae	0.0	0.0	1.4	2.9
Paratylenchidae	0.0	0.0	1.3	1.1
Plectidae	88.8	7.2	10.2	14.4
Pratylenchidae	0.0	0.1	1.1	5.2
Prismatolaimidae	0.7	19.3	1.8	4.8
Qudsianematidae	0.0	1.1	1.5	1.4
Rhabdolaimidae	0.0	2.3	0.0	0.0
Teratocephalidae	8.6	52.3	3.1	7.6
Trichodoridae	0.0	0.0	0.6	0.0
Tobrilidae	0.0	0.0	0.0	0.2
Tripylidae	0.0	2.1	0.6	0.9
Tylenchidae	0.0	1.6	24.6	32.0
Tylenchulidae	0.0	0.0	0.0	1.0
Xyalidae	0.0	0.3	0.0	0.0
Unknown bacterial-feeder	0.0	0.0	9.3	4.2
Unknown omnivore	0.0	0.0	3.7	0.8
Unknown plant parasite	0.0	0.0	4.8	1.8
Unknown predator	0.0	0.0	0.3	0.0
Total number counted	267	1132	1606	3214

nematodes were likely under-represented in the sequencing analysis compared to the morphological analysis. With less sampling, it is not unexpected that the molecular analysis found fewer nematode families than did the morphological analysis.

4.5. Challenges and benefits of using molecular methods to assess soil biodiversity

Although different in detail, both molecular and morphological methods indicated higher nematode and lower arthropod relative abundance at the tundra site than at the boreal forest. We conclude that this molecular approach provides a valuable new method for the assessment of biodiversity of animals in soil. The molecular and morphological results from this study are incongruent in most details even though they agree as very broad measures of biodiversity.

From this study, it is clear that the methods can be improved and made more consistent. For example, better primers can be devised that do not exclude particular taxa. Our attempt at fractionating soil fauna by body size with sieving was ineffective, most likely because small soil invertebrates attached to soil particles larger than the size of the animals. Animals smaller than 0.1 mm could have been lost in the sieving process but appear to have been retained on larger soil particles. The sieving process could be simplified and the amount of soil used for the DNA extraction could be increased. Particular attention needs to be paid to standardizing how soil is processed prior to DNA extraction. The DNA extraction method used for this kind of study needs to be tested and refined to minimize extraction bias. Clearly, higher throughput sequencing methods such as pyrosequencing (Ronaghi, 2001; Edwards et al., 2006; Roesch et al., 2007) would provide a clearer view of the biodiversity and community structure in soil samples.

The most important benefits of the molecular approach include: (1) the fine taxonomic detail that molecular based OTUs provide, even with unknown or rare species; (2) the broad taxonomic groups that can be included; (3) the ease with which sites can be compared over both space and time; and (4) the process can be automated and analyzed using bioinformatic approaches. Although we cannot conclude whether molecular or morphological studies better reflect soil animal biodiversity, the molecular approach does provide new information that adds to our understanding of soil animal biodiversity.

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