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Short Note

The need for standardisation: exemplified by a description of the diversity, community structure and ecological indices of soil nematodes.

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Abstract

Molecular approaches are offering a supplement to, or even the possibility of replacing morphological identification of soil fauna, because of advantages for throughput, coverage and objectivity. We determined ecological indices of nematode community data from four sets of duplicate soil cores, based on morphological identification of nematodes after elutriation from 200g soil and high throughput sequencing (HTS) targeting nematodes both after being elutriated from soils and DNA extracted directly from 10g soil. HTS (at genus and species level) increased the f taxonomic resolution compared to morphology (at family level). DNA extracted from elutriated nematodes identified more nematode taxa than when extracted from soil, due to an enrichment in nematode sequences. Each method also gave a different ecological footprint for the nematode community. Standardisation to previously determined indices based on morphological identification is needed in order to provide more meaningful information about soil quality and for ecological monitoring.

1. Introduction

The study of soil and aquatic micro- and meso-fauna is being transformed by the use of molecular methods (Creer et al., 2010). Not only are the developing molecular methods complementing and even superseding the traditional morphological approaches, they are also developing faster than standard protocols. Philippot et al (2012) highlighted the fact that methodological differences between laboratories, of even the same protocol, are not trivial and hamper comparisons between studies. They urged soil biologists to expand the list of standardised protocols listed by the International Organisation for Standardisation (ISO). This was taken a little further by Römbke et al (2016) who pointed out that when biodiversity data, for example, are being used in a legal context they have to be comparable and lack of standardisation can limit the justification of specific protection measures.

Nematodes are important indicators for soil monitoring (Chen et al., 2010) and there is a large body of existing information based on morphological identification, which has led to well established ecological indices based on nematode traits (Ferris et al., 2001). Morphological identification, though, is often only to the family or trophic group (Porazinska et al., 2009) leaving ecological analyses potentially ambiguous or superficial (Yeates and Bongers, 1999). The level of characterisation of the nematode community is also problematical for DNA based methods, as reliable sequence annotation relies on having curated sequences from vouchered specimens which are not always available. There is a fundamental choice to extract DNA directly from soil or to firstly elutriate nematodes and then extract DNA from those nematodes (here 'elutriation' covers nematode extraction from soil, and 'extraction' refers to DNA). Advantages and disadvantages can be argued for either approach. Elutriating nematodes before extracting DNA will enrich nematodes and diminish other fauna, but takes longer and not all nematodes might be elutriated equally efficiently (Persmark et al., 1992). Directly extracting DNA circumvents issues associated with elutriation and saves time, but relatively small amounts of soil are usually extracted (i.e. <10g rather than the >200g recommended as optimal by Wiesel et al., 2015).

It is important to be able to relate molecular results to the previous body of work using morphological identification, and to have a good understanding of the limitations inherent with each method (Porazinska et al., 2010; Stone et al., 2016; Quist et al., 2016). Currently only the extraction and morphological identification of soil nematodes is covered by an ISO standard (ISO 23611-4). Given the growing interest in biological soil monitoring (Aalders et al., 2009; Turbé et al., 2010; Pulleman et al 2012, Faber et al 2013; Tsiafouli et al 2015; Griffiths et al, 2016), we considered that a reminder of the importance of standardisation for the introduction of the developing molecular methods was timely and relevant. We undertook an initial systematic comparison of nematode community structure and diversity, derived from morphological identification and molecular identification based on DNA extracted either directly from soil or from elutriated nematodes.

2. Materials and methods

From each corner of a square metre grassland plot, we collected two intact soil cores of 5.8cm diameter and 10cm depth (ISO 23611-2) directly adjacent to each other. From one core per corner (n=4) DNA was extracted from a random subsample of 10 g (PowerMax Soil DNA isolation kit (MO BIO Laboratories)) and called 'soil extracted DNA'. The other core per corner (n=4) was used to elutriate the nematodes from 200 g of fresh soil with an Oostenbrink elutriator (ISO 23611-4). Elutriated nematodes were sub-divided and one sample frozen before extracting DNA (Qiagen DNeasy Blood & Tissue Kit), resulting in a so-called "diversity soup" (Yu et al. 2012) and one sample fixed for morphological identification (Yoder et al., 2006). DNA extracts were subjected to DNA metabarcoding (Porazinska et al. 2009; and supplementary details). Nematode relative abundance data (Table 1 and Supplementary tables 1, 2,) were arcsin transformed for principal component analysis (PCA) and one-way ANOVA. Diversity was calculated as Shannon and reciprocal Simpson indices. Functional indices were calculated using the nematode indicator joint analysis (NINJA) programme (Sieriebriennikov et al., 2014).

3. Results

At the family level the DNA based methods revealed more taxa (20) than the morphological analysis (18), while at higher taxonomic resolution the diversity soup method gave more taxa (34 OTU's) than the soil extracted DNA (25 OTU's). Increasing taxonomic resolution significantly increased diversity indices (i.e. Shannon 4.4 versus 6.5) and the diversity soup method revealed greater diversity than the soil extracted DNA (i.e. 1/Simpson 2.0 versus 2.3). From the metabarcoding, 76% of reads from the diversity soup and 7% of reads from soil extracted DNA were nematode sequences. Maturity Index was greatest for the diversity soup community (2.3, 3.4, 2.3 for morphology, diversity soup and soil extracted DNA, respectively), while Basal Index (50, 13, 9) and

Channel Index (33, 15, 4) were both larger for morphology than either DNA method. The communities fell in different quadrants on an enrichment index vs structure index plot (Fig. 1). Principal component analysis revealed a different nematode community composition with each method and by running the analysis to include or exclude rare taxa we could show that patterns are driven by differences in relative abundance of the main taxa rather than the presence / absence of rare taxa.

4. Discussion

The objective of this study was to determine how dependent the metrics for community analysis are on the methods used. Here we show for the first time that different extraction approaches, even an identical high-throughput sequencing approach that targets either DNA of nematodes after being extracted first or directly from extracted DNA, shows not only different taxonomic community composition but most strikingly suggests a different soil quality. We recognise that this is a limited study both in terms of samples analysed and comparatively low sequence depth obtained by 454 pyrosequencing, but the principle was to highlight the crucial need for standardisation in comparing between samples. The pattern of the result would have been the same whether we used 454 pyrosequencing for HTS or another sequencing platform (Luo et al., 2012; Mahe et al., 2015).

The primers (NF-1 and 18Sr2b, Porazinska et al. 2009) give good coverage of soil nematodes and have been widely used, but are not nematode specific and also amplify other eukaryotes. As far as we are aware that there are no universally perfect primers that target all groups of nematodes in the same way, however, primer issues cannot explain differences between the two molecular methods to compare nematode communities. Biases in the extraction/elutriation methods are the only explanation for the observed differences, which implies that we still have only a limited idea how soil nematode communities really look like.

An advantage of the diversity soup method is that most of the other soil eukaryotes are removed by elutriation, thus giving a larger number of reads for nematodes than from the soil extracted DNA. As the technology improves and sequence numbers per sample increase, then the simultaneous study of all soil eukaryotes becomes a practical option (de Groot et al., 2016). The greater taxonomic resolution of the DNA methods cannot be matched by morphology, unless it is a painstakingly detailed study which precludes the throughput necessary in contemporary research (Yang et al., 2014), and could be expected to be more informative about community structure than morphology. That nematode community analyses differed between extraction methods, in aspects of diversity, structure and ecological indices, mirrored results from Quist et al. (2016). Other studies have also noted that different sampling methods give individual community results because of their particular biases, so that there is no 'true' biodiversity dataset (Yang et al., 2014). Despite the diversity soup and morphological methods both starting with the same aqueous solution of nematodes, the profound differences in nematode community structure could be attributed to identification skills and/or PCR biases and were partly explained by the relatively small contribution of Tylenchidae and bacterial-feeding nematodes in the diversity soup, as seen in similar comparisons (Griffiths et al., 2006; Donn et al., 2011, 2012; Darby et al., 2013). The comparison of soil extracted DNA vs. diversity soup might be affected by sample size, as the 10g soil used for direct extraction is much less than the 200g recommended to reliably reveal a soil nematode community (Wiesel et al., 2015). This might explain the lack of larger omnivore and predator nematodes in the soil extracted DNA (such as *Aporcelaimellus*, *Discolaimus*, Dorylaimidae, *Nygolaimus*) (Quist et al., 2017). The calculated functional indices would indicate different soil food web conditions, which is clearly erroneous as we compared the same samples. Therefore method standardisation, including extensive studies using mock communities of known and highly diverse nematode communities, needs to be adopted (as indicated by Darby et al., 2013) in order to be able to compare taxonomic as well as the functional and indicative attributes of soil nematode communities.

5. Conclusion

DNA methods will be increasingly used because of reducing analysis costs, high throughput, greater taxonomic resolution and compatibility with available technical skills. There is a need now to understand the methodological discrepancies (sample size; extraction and PCR biases; primer specificity; read number and taxonomic resolution) identified here and to calibrate the molecular methods to the morphological information. The developing high-throughput molecular methods have to be standardised for ecological and applied indication purposes.

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Data accessibility

The sequence data will be uploaded to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) on acceptance.

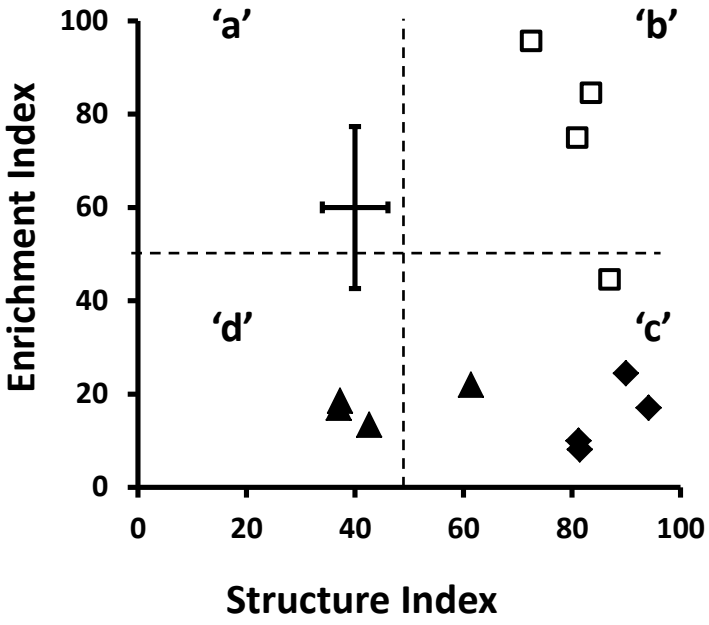

Figure 1. Food web condition of the nematode communities shown by a plot of the Structure and Enrichment indices calculated from: morphological analysis of elutriated nematodes (▲) ; high throughput sequencing of DNA extracted from elutriated nematodes (diversity soup, ◆) and DNA directly extracted from soil (soil extracted DNA, □) amalgamated to allow analysis at the same taxonomic resolution (family level) as the morphological data. n = 4, bar represents the least significant difference ($p<0.05$). Quadrant 'a' represents a disturbed, bacterial energy channel dominated community; 'b' a maturing and balanced community; 'c' a structured, fungal energy channel dominated community, and 'd' a degraded community (Ferris et al., 2001).

Table 1. The percentage distribution of nematode families determined from a morphological examination of elutriated nematodes (morphology); high throughput sequencing of DNA extracted from elutriated nematodes diversity soup) and DNA directly extracted from soil (soil extract). DNA data have been amalgamated to allow analysis at the same taxonomic resolution as the morphological data. The F-statistic (P) was calculated on arcsin transformed data. Detransformed means are presented. Data also presented on the percentage distribution of nematode feeding types. Means followed by a different letter and in bold are significantly different, n = 4.

Nematode family	Method			P
	Diversity Soup	Morphology	Soil Extract	
Alaimidae	**0.16a**	**0.00a**	**1.21b**	**0.002**
Anguinidae	0.04	0.00	0.16	0.244
Aphelenchoididae	**0.79a**	**5.33b**	**1.21a**	**0.007**
Aporcelaimidae	**14.95a**	**7.43a,b**	**0.84b**	**0.045**
Cephalobidae	**23.58a**	**45.37b**	**11.89a**	**0.007**
Diplogasteroidae	0.00	0.00	6.31	0.207
Diphtherophoridae	1.63	0.37	9.31	0.067
Dolichodoridae	0.10	0.18	0.12	0.977
Dorylaimidae	**4.43a**	**0.00b**	**0.28b**	**0.003**
Microlaimidae	**0.72a**	**0.00a**	**10.93b**	**<0.001**
Monhysteridae	**0.12a**	**5.56b**	**0.43a**	**0.012**
Nordidae	0.00	0.12	0.00	0.422
Nygolaimidae	**24.17a**	**0.00b**	**4.55b**	**0.005**
Paratylenchidae	0.04	0.48	0.00	0.516
Plectidae	13.97	13.55	9.54	0.379
Prismatolaimidae	**0.72a**	**0.00a**	**31.42b**	**<0.001**
Qudsianematidae	6.46	1.97	0.45	0.134
Rhabditidae	1.60	2.76	0.92	0.342
Tylenchidae	**0.08a**	**13.28b**	**0.03a**	**<0.001**
Trichodoridae	0.22	0.00	0.24	0.325
Functional groups				
Bacterial Feeders	**41.90a**	**67.67b**	**80.47b**	**0.008**
Fungal Feeders	2.44	5.88	10.56	0.232
Omnivores	**27.23a**	**10.50b**	**2.06b**	**0.025**
Plant Feeders	**0.52a**	**15.31b**	**0.98a**	**0.001**
Predators	**24.17a**	**0.00b**	**4.55b**	**0.005**