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Design and evaluation of nematode 18S rDNA primers for PCR and denaturing gradient gel electrophoresis (DGGE) of soil community DNA

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Abstract

Consensus nematode 18S ribosomal DNA primers were designed by aligning available 18S sequences and identifying a variable region flanked by highly conserved regions. These primers were then used to amplify nematode 18S rDNA from whole soil community DNA extracted from a range of European grassland types. Cloning of the PCR amplicons (778 bp) followed by restriction digest analysis (RFLP) resulted in the recovery of 34 unique nematode sequences from the four grasslands studied. Comparison of these data with the limited number of 18S rDNA nematode sequences currently held in on-line databases revealed that all of the sequences could be assigned to known nematode taxa albeit tentatively in some cases. Two of the sequences recovered from the site in the Netherlands (wet, hay-grassland) were recovered in a clade that included a sequence of the genus *Trichodorus* whilst other sequences from this site showed similarity with 18S rDNA sequences of the genus *Prismatolaimus* (five sequences), *Xiphinema* (one sequence) and *Enoplus* (one sequence). Of the remaining sequences, two showed some affinity with *Mylonchulus* (UK, upland peat), four with *Steinernema* (UK) and one sequence with *Mesorhabditis* (Hungary, east European Steppe). Three sequences from the Netherlands and one from Hungary were recovered in a clade that included a sequence of the genus *Pratylenchoides* whilst three further sequences from the Netherlands and two from Hungary were recovered in a clade encompassing the genus *Globodera*. Of the remaining nine sequences, two (NL6, NL62) formed a distinct lineage within the Adenophorea with 90% bootstrap recovery in a paraphyletic clade that included sequences of *Prismatolaimus* and *Trichodorus*. Seven sequences (three from the Netherlands, three from the UK and one from Greece) were left unassigned though the tree topology suggested some relationship (58% bootstrap recovery) with the genus *Cephalobus*. To assess whether primers used to amplify 18S rDNA might be used to fingerprint genetic diversity in nematode communities in soil, the environmental sequence data were used to design a second set of primers carrying a GC-clamp. These primers amplified a 469 bp fragment internal to the region flanked by the primer set used to derive the nematode trees and were used to amplify 18S rDNA for subsequent analysis using denaturing gradient gel electrophoresis (DGGE). DGGE analysis of six major European grassland types revealed considerable genetic diversity between sites. However, the relationships seen with the DGGE data were inconsistent with previous studies where the same soils had been characterized with respect to functional and morphological diversity. To confirm that this second set of primers was amplifying nematode sequences, selected bands on the DGGE gels were extracted, PCR amplified and sequenced. The final alignment was 337 bases. These analyses revealed the presence of sequence signatures from the genera *Paratrichodorus*, *Plectus*, *Steinernema*, *Globodera*, *Cephalobus* and *Pratylenchoides*.

Keywords: Nematodes; 18S rDNA; Denaturing gradient gel electrophoresis; PCR; Grasslands; Soil community DNA; Molecular ecology

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1. Introduction

The application of molecular biology to the analysis of soil microbial community diversity is already well established for bacterial communities (Felske et al., 1998; O'Donnell and Goerres, 1999; Ritchie et al., 2000; McCaig et al., 2001) and is now used increasingly to study soil fungal communities (Kowalchuk et al., 1997; Pennanen et al., 2000; Vainio and Hantula, 2000). Nematodes represent an important part of the soil microfauna that affect directly and indirectly the size, activity and diversity of the soil microflora (Bardgett and Cook, 1998; Ekschmitt et al., 1999; Sonnemann et al., 1999). While much work has been done using large subunit (LSU) 28S (Livaitis et al., 2000) and internal transcribed spacer (ITS) (Szalanski et al., 1997; Zijlstra et al., 1997) regions of the ribosomal RNA gene to determine differences between species and differences at subspecies levels, there are few reports of studies on the use of molecular techniques for studying nematode diversity and community structure in soils. Van der Knaap et al. (1993) used arbitrarily primed PCR, while Floyd et al. (2002) and Foucher and Wilson (2002) used small subunit (SSU) 18S rDNA to characterize isolated nematodes but as yet there are no reports on the use of nematode specific 18S rDNA primers for amplifying directly extracted, soil community DNA. The 18S rRNA gene contains variable regions flanked by conserved regions necessary for reliable primer design and while the ITS regions are divergent between taxa, it can be difficult to align ITS regions between disparate taxa. Although within species variations in ITS, length make these regions suitable for taxonomic analysis (Foucher and Wilson, 2002), they are less effective targets for soil community fingerprinting (Floyd et al., 2002).

Morphological analyses (Bongers and Bongers, 1998; De Goede and Bongers, 1998) have shown nematode diversity to be extensive but unlike bacteria or fungi (O'Donnell et al., 1994) the phylogenetic robustness of such classifications is largely untested. Some studies have suggested that the circumscription of nematode taxa using morphological characters is not consistent with their genetic (McKeand, 1998) and phylogenetic affiliation (Blaxter et al., 1998). There is, therefore, a need, as has occurred with other soil taxa, to develop molecular methods for classifying nematodes and to use these classifications to develop tools for fingerprinting nematode communities in soil.

Molecular analysis of soil community DNA involves the recovery and purification of nucleic acids followed by their PCR amplification using consensus or specific primers (Head et al., 1998). Cloning of the PCR amplicons and their subsequent screening and sequencing provides taxon specific information on the genetic diversity of the target organisms (O'Donnell and Goerres, 1999). A major advantage of the approach is that isolation and culture is unnecessary and, provided primers are available or can be designed, it can be applied to diverse taxa and has the added advantage that bacterial, fungal and nematode communities can be analysed concurrently in the same soil community DNA extract. An alternative to the cloning and sequencing approach is to use one or more of a range of fingerprinting techniques to profile the PCR amplicons (Marsh, 1999; Vainio and Hantula, 2000; Smalla et al., 2001).

Our objective was to establish the potential of molecular ecological approaches in the analysis of soil nematode communities. This has been done by extracting, amplifying, cloning and sequencing soil community DNA followed by phylogenetic analysis to assess the specificity of the proposed nematode consensus primers. The PCR was done using primers designed from SSU 18S rDNA sequences held on-line. The limited data set from which the PCR primers could be designed required that their effectiveness and specificity be evaluated using denaturing gradient gel electrophoresis (DGGE; Muyzer et al., 1993) in a range of European grassland soils (Ekschmitt et al., 1999). Specificity of the primers was determined by excision, amplification and sequencing of bands resolved by DGGE.

2. Materials and methods

2.1. Soils

The six grassland soils used in this investigation lie on a climatic-cross gradient and represent six major natural and semi-natural European grassland types (Table 1). Sites were located in Sweden (Abisko, northern tundra), the United Kingdom (Otterburn, Northumberland, atlantic heath), the Netherlands (Wageningen, wet grassland), Germany (Linden, semi-natural grassland), Hungary (Pusztaszer, east European steppe) and Greece (Mt Vermion, Mediterranean garigue). Full details of the soils and their associated vegetation was provided by Ekschmitt et al. (1999). Although all six soils were used for the DGGE analysis only soils from the UK, Netherlands, Hungary and Greece were used for the cloning and phylogenetic analyses.

2.2. Extraction and purification of soil community DNA

DNA was extracted as described by Macrae et al. (2001) with minor modification. Briefly, soil (1 g) was weighed into a 2 ml silicon sealed screw-top tube containing 0.5 g of glass beads (0.17–0.18 mm diameter, Braun Biotech International). The 0.5 ml of 120 mM extraction buffer (pH 8.0, 30 mM Na_2HPO_4 and 90 mM NaH_2PO_4) and 0.5 ml phenol/chloroform/isoamyl alcohol (25:24:1, v/v) was added to the tube and placed into a Mikro-Dismembrator U (Braun, Biotech, International) for 2 min at 1600 beats min^{-1}. Samples were then centrifuged for 5 min at $12,000 \times g$ and the upper aqueous phase removed and collected in a fresh 2 ml screw-top tube. A further 0.5 ml extraction buffer was added to the original tube and the bead beating and centrifugation steps repeated. Following centrifugation, the aqueous phase was pooled with that

Table 1
Soil properties and vegetation cover

	Sweden	United Kingdom	Netherlands	Germany	Hungary	Greece
Location	Abisko 68°19′N 18°51′E, 450 m ASL[a]	Otterburn, 53°13′N 2°15′W, 295 m ASL	Bennekom 51°59′N 5°40′E, 11 m ASL	Linden 52°32′N 8°41′E, 172 m ASL	Pusztaszer 46°40′N 20°00′E	Mount Vermion 40°20′N 23°10′E, 212 m ASL
Mean annual rainfall (mm)	311	874	790	644	500	352
Soil type	Organic soil (80% OM)	Shallow peat over blue clay	Humic podzol	Stagnofluvic gleysol on loamy-sandy sediments	Chernozem-type sandy soil	Calcareous, 1 m deep
Dominant plants	*Btula nana*, *Cassiope tetragona*, *Empetrum hermaphroditum*, *Vaccinium uliginosum*	*Cynosurus cristatus*, *Anthoxanthum odoratum*, *Holcus lanatus*, *Poa subcaerulea*, *Festuca rubra*, *Deschampsia flexuosa*, *Trifolium repens*	*Holcus lanatus*, *Anthoxanthum odoratum*, *Alopecurus parensis*, *Agrostis capillaris*, *Juncus effucus*, *Phalaris arundinacea*	*Arrhenatheretum elatioris*, *Filipendula ulmaria* sub community	*Cynodontofestucetum pseudovinae*	Degraded pasture land dominated by *Asphodelus aestivus*

[a] ASL, above sea level.

from the first extraction. An equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added and the sample vortexed and centrifuged to remove residual phenol. The upper layer was removed to a fresh tube and a 0.1 volume of 3 M sodium acetate and 0.6 volumes of isopropanol were added. This mix was left at room temperature for 10 min prior to centrifugation at $12,000 \times g$ for 10 min. The supernatant was discarded and the DNA pellet washed in 250 μl 70% (v/v) ethanol. Following centrifugation at $12,000 \times g$ for 5 min, the supernatant was discarded and the sample air-dried at 37 °C for approx. 10 min. Once dry, the DNA was re-suspended in 30 μl H_2O and stored at −20 °C. Prior to PCR amplification, all DNA concentrations were adjusted to 1 μg μl^{-1}.

2.3. PCR amplification of 18S rDNA

Small subunit ribosomal DNA (18S rDNA) was selectively amplified from the soil community, genomic DNA by PCR, using primers designed to anneal to conserved positions within the gene. A nematode consensus primer was designed with reference to an existing but limited data set held on-line at RDP (http://rdp.cme.msu.edu) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Nematode specificity was established by targeting the variable V3 and V5 regions of the 18S rDNA to identify a 'consensus' forward primer, NEMF1 (5′-CGC AAA TTA CCC ACT CTC-3′). The initial specificity of NEMF1 for nematodes was evaluated using the CHECKPROBE routine at RDP and by searching the databases for complementary sequences. A 'universal' 18S rDNA primer that hybridized with highly conserved regions of the eucarya 18S rDNA molecule was used as the reverse primer (5′-AGT CAA ATT AAG CCG CAG-3′; primer S3, Mailwald et al., 1994).

Extracted DNA was amplified by PCR in 30 μl volumes using 9 pM of each primer, 7.5 mM of each dNTP, 45 mM $MgCl_2$ and 0.5 units of *Taq* Polymerase. The reaction conditions were: 94 °C, 1 min (denaturation); 53 °C, 1 min (annealing); 72 °C, 2 min (extension) for 30 cycles using a thermocycler (Hybaid). In addition to the target soil DNA, a 'negative' (without DNA) control sample was included with every PCR amplification run. Following amplification, PCR products were checked for size and specificity by electrophoresis on 1.2% (w/v) agarose (Bio Gene Limited) gels and stained with 3,8-diamino-5-ethyl-6-phenylphenanthridium bromide (ethidium bromide).

Amplified 18S rDNA was cloned using the Invitrogen TOPO TA cloning kit (San Diego, CA) according to the manufacturer's instructions except that all of the recombinant *E. coli* cells (total volume) were plated out. White colonies (*E. coli* containing inserts) were selected, transferred using a sterile cocktail stick to 3 ml LB broth containing ampicillin (100 μg μl^{-1}) and incubated at 37 °C for 16 h. After growing overnight, the transformed *E. coli* cells were harvested by centrifugation and the DNA extracted from the 3 ml culture using Qiagen's QIA prep 8 Miniprep Kit (Boundary Court, Gatwick Road, Crawley, UK) according to the manufacturer's instructions.

Restriction fragment length polymorphism (RFLP) was used to select unique clones for sequence analysis. Extracted DNA (1 μg) was digested at 65 °C for 3 h with the restriction enzyme *Taq1* (Kramel Biotech, Northumberland, UK), electrophoresed on a 2.0% (w/v) agarose gel and stained with ethidium bromide. Gels were visualised at A_{260} using a BioRad Fluor MultiImager (BioRad Laboratories, Hemel Hempstead, UK). Novel clones were identified by eye according to the banding pattern and then sequenced using an ABI 370 automatic sequencer (Perkin Elmer).

2.4. Sequencing and phylogenetic analyses

Sequence fidelity was verified by eye and chimeras checked for using CHECK_CHIMERA at RDP (Maidak et al., 1994). Chimeric sequences and those in which universal internal priming sites for the 18S rDNA gene could not be found were discarded. For the remaining sequences, relatives and representative strains were downloaded from RDP (Maidak et al., 1994) and BLAST (Altschul et al., 1994). Multiple sequence alignments were done with the aid of CLUSTAL X (Jeanmougin et al., 1998) then edited to remove major gaps using Microsoft Word. The final alignment comprised 534 bp. New sequences have been deposited at EMBL, accession numbers AY227210 to AY227243 inclusive and can be retrieved from http://www.ncbi.nlm.nih.gov. TREECON (Van de Peer and de Wachter, 1993) was used to construct phylogenetic trees using the corrected distance of Galtier and Gouy (1995) and neighbour joining (Saitou and Nei, 1987). The stability of the resultant tree topologies was assessed by 100 bootstrap re-samplings of the data (Felsenstein, 1985). Since distance measures represent only one particular model of evolution, the validity of the phylogeny was tested by comparing the topology of the trees derived using the distance algorithm (Galtier and Gouy, 1995) with the most parsimonious tree (a different model of evolution) derived using the DNAPARS routine in the PHYLIP package (http://evolution.genetics.washington.edu/phylip.html; data not shown).

2.5. Denaturing gradient gel electrophoresis

To fingerprint the nematode communities of the six European grassland types, a second primer, internal to the region amplified by the NEMF1 primer was designed. This reverse primer (NEM896r; 5′-TCC AAG AAT TTC ACC TCT MAC G-3′) was used with a NEMF1-GC primer (NEMF1 modified by the addition of a GC-clamp (Muyzer et al., 1993) to the 5′ end of the sequence) to amplify fragments for DGGE analysis. PCR was done as described in Section 2.3. The resultant amplicons were separated using DGGE in a 6% (w/v) acrylamide gel with a 20–40% denaturing urea gradient. Gels were run for 3 h at 200 V then stained with Sybr-Green (1:10,000) for 30 min. Stained gels were visualised and recorded using a Bio-Rad Fluor MultiImager.

3. Results

3.1. RFLP analysis

Based on RFLP analysis (Fig. 1) 34 unique banding patterns in the soils from the UK, the Netherlands, Greece and Hungary (Swedish and German soils were not included) were identified and the 18S rDNA inserts sequenced. These sequences were then compared with those of known nematode sequences held on-line at RDP (http://rdp.cme.msu.edu) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and then used to identify marker sequences for known nematode taxa. Comparison of the 18S rDNA from the environmental clones with those of nematode sequences currently held on-line, revealed that all of the sequences studied showed high similarities to the Nematoda and that of the 34 sequences selected for further study, 25 could be ‘assigned’ to known taxa albeit tentatively in some cases (Fig. 2). Of the remaining nine sequences, seven showed some similarity (58% bootstrap recovery) to those of *Cephalobus* with the remaining two sequences (NL6, NL62) showing some similarity to *Mylonchulus*. Sequences from more type culture isolates are needed to refine these

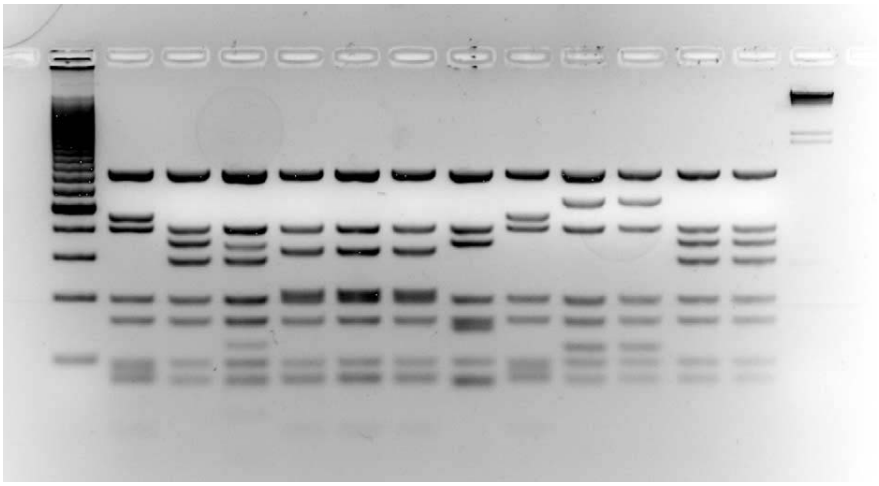

Fig. 1. Typical restriction digest analysis (RFLP) of the PCR amplified nematode 18S rDNA from the UK soil. Soil community DNA was amplified using primer NEMF1 and a consensus eucarya primer as the reverse primer. Amplicons were cloned and digested with *Taq1* then run on 2% (w/v) agarose gels. Amplicons giving unique banding patterns were subsequently sequenced.

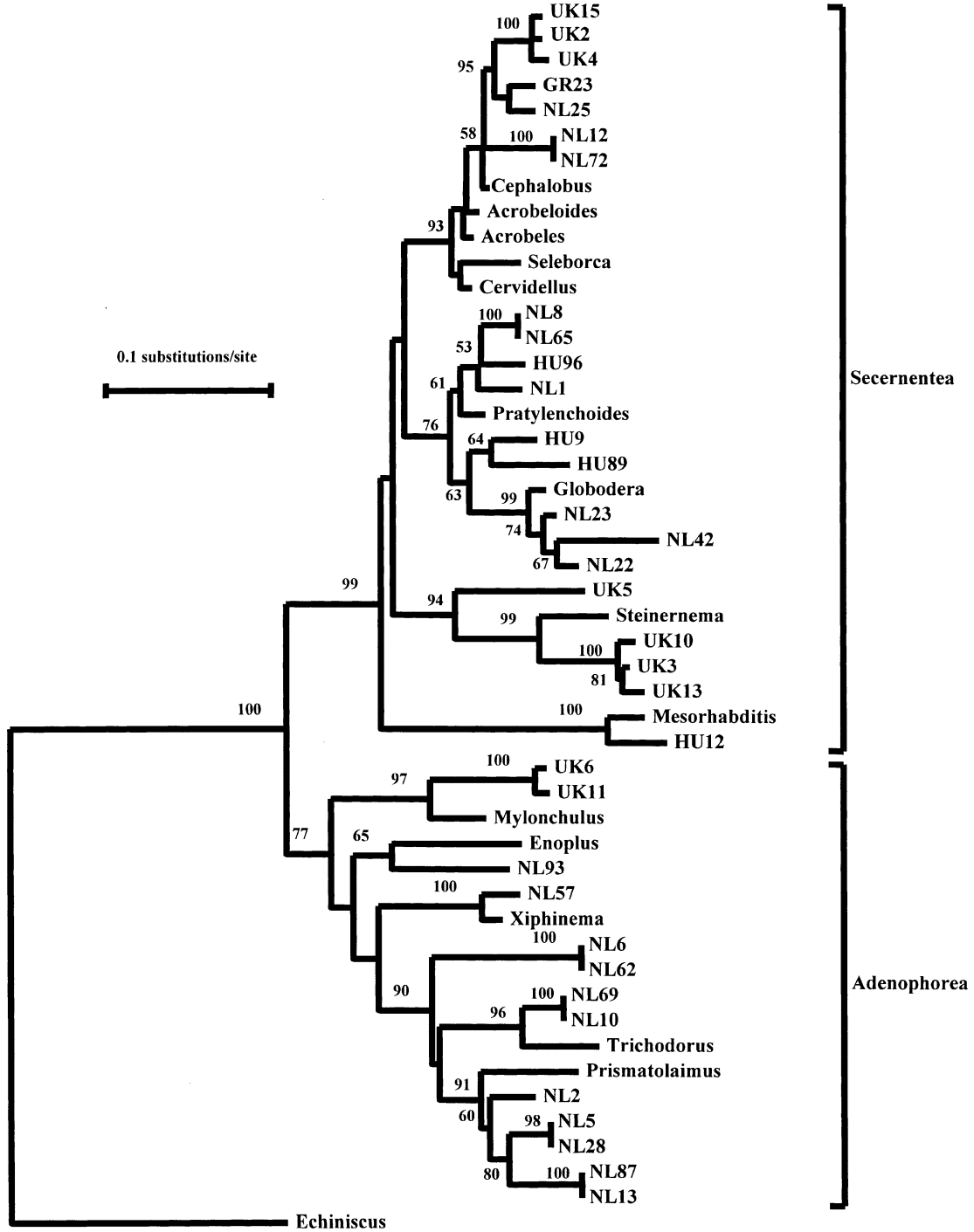

Fig. 2. Phylogenetic analysis showing the relationship between cloned 18S rDNA amplicons and their nearest neighbours held on-line. The tree is based on a final alignment of 534 bases. The distance scale indicates 0.1 substitutions/site. Numbers represent the number of times the clade to the right of the node was recovered in 100 bootstrap re-samplings of the data. *Echiniscus viridissimus* (EMBL Accession No. AF056024, Tardigrada) was used to root the tree. Parsimony analysis of the same data (not shown) supported the overall tree topology.

relationships. Of the 25 sequences assigned to known taxa, 13 were recovered in a polyphyletic clade representing the Adenophorea whilst the remaining 12 sequences were distributed amongst the Secernentea (Fig. 2). To accommodate the different approaches to assess phylogenetic relationships used by different techniques (e.g. distance measures, BLAST comparisons, parsimony) the data were also analysed using parsimony analysis. This uses a different model of evolution to distance measures such as the corrected distance of Galtier and Gouy (1995) and

neighbour joining (Saitou and Nei, 1987) used to derive Fig. 2. Comparison of the tree topology obtained using parsimony analysis (tree not shown) supported the phylogenetic relationships shown in Fig. 2 indicating that the relationships seen in Fig. 2 are largely independent of the model of evolution used to derive the tree.

3.2. DGGE results

Based on DGGE banding patterns these data (Fig. 3) show that the 18S rDNA diversity differs between sites (differences in banding pattern) with certain bands prevalent at each site. Eleven of the dominant bands were cut from the gels (seven from the replicate gel shown in Fig. 3), re-amplified and sequenced. Selected bands are delineated on the gels and identified with a reference number. All of the extracted bands show high sequence similarity to the Nematoda and to each other suggesting that DGGE can be used to fingerprint nematode communities in soils (Fig. 4). Of the 11 bands selected for sequencing, UKex1, NLex3, DEex6 and DEex7 were recovered in clades that included no known nematode sequence. The phylogenetic placement of the remaining seven sequences suggests that they share high sequence similarity with *Paratrichodorus* (DEex8), *Plectus* (SEex9), *Globodera* (NLex4), *Steinernema* (HUex3, HUex4) and *Cephalobus* (HUex5, UKex2).

4. Discussion

Understanding the relationship of below ground diversity and how it affects the functioning and sustainability of soil processes remains one of the major challenges facing soil ecologists. For microbial communities, and in particular bacteria, potential solutions to these problems have focused on the analysis of small subunit rRNA (Head et al., 1998; O'Donnell and Goerres, 1999) and the use of PCR based methods to fingerprint communities (Marsh, 1999; Vainio and Hantula, 2000; Smalla et al., 2001). However, such molecular methods have not been applied routinely to characterize soil nematode communities with much of the published work based predominantly on functional and morphological classifications. Blaxter et al. (1998) in a phylogenetic analysis of 53 small subunit rDNA sequences from a range of nematode taxa have proposed a new evolutionary framework for the phylum Nematoda and

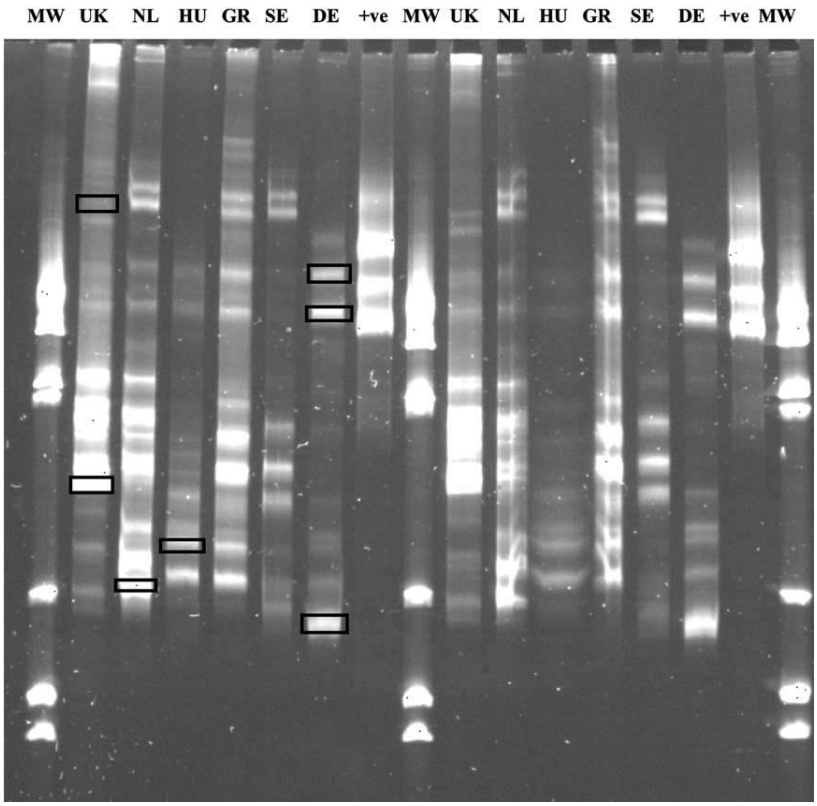

Fig. 3. Denaturing gradient gel of nematode 18S rDNA showing the diversity relationships between European grassland types. The seven boxes mark bands extracted and sequenced to verify specificity of the NEMF1 probe. Sequences UKex1, UKex2, DEex6, DEex7, DEex8, HUex3 and HUex4 (Fig. 4) were recovered from the gel shown. The remaining test sequences NLex3, NLex4, SEex9 and HUex4 were recovered from a separate gel (data not shown). DE, Germany; UK, United Kingdom, GR, Greece, HU, Hungary, NL, Netherlands and SE, Sweden. MW identifies the molecular weight marker used to normalize the gels and +ve shows a positive control included with all analyses.

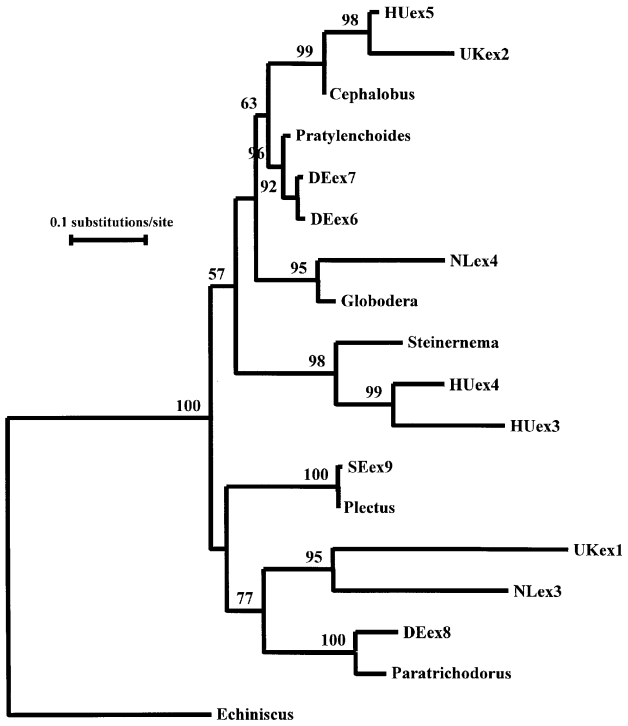

Fig. 4. Phylogenetic analysis showing the relationship between DGGE bands amplified using primer NEMF1 (18S rDNA) and the most similar sequences of known nematodes. The tree is based on an alignment of 337 bases. The distance scale indicates 0.1 substitutions/site. Numbers represent the number of times the clade to the right of the node was recovered in 100 bootstrap re-samplings of the data. *Echiniscus viridissimus* (EMBL Accession No. AF056024, Tardigrada) was used to root the tree.

have made comparisons across functional groupings such as animal-parasitic, plant-parasitic and free-living nematodes. These analyses suggested that convergent morphological evolution in nematodes may be extensive and that the current higher taxa classification of nematodes warrants revision. A similar reappraisal of higher taxa relationships also occurred following the introduction of 16S RNA and rDNA methods to bacterial systematics (Embley et al., 1994). These revisions and the introduction of molecular techniques effectively paved the way for molecular ecology and the analysis of bacterial genetic diversity in natural environments (O'Donnell and Goerres, 1999).

The primers we used were designed using sequences held on-line and subsequently improved using the sequence data generated from the environmental clones. The usefulness of the primers in selectively amplifying nematode 18S rDNA from soil community DNA is seen in Fig. 2 where all of the cloned products lie within the radiation encompassing the Nematoda and show phylogenetic affinities consistent with a nematode origin. Further investigation of the distribution of the cloned nematode sequences and their comparison with the phylogenetic framework for the Nematoda proposed by Blaxter et al. (1998) reveals some interesting results with four of the five clades represented in the clone library (*Prismatolaimus*, *Trichodorus*, *Xiphinema*, *Mylonchulus*, *Enoplus*, *Mesorhabditis*, *Pratylenchoides*, *Globodera*, *Steinernema*). There were no sequences from clade III that are vertebrate and arthropod parasites including taxa from the nematode orders Oxyurida, Spirurida, Rhigonematida and Ascaridida. Whilst one might expect the latter to be present as eggs in animal faeces or present in infected soil arthropods, the relatively small samples used to extract soil community DNA (1 g soil) may have limited their detection and amplification.

Whilst our results show clearly that 18S rDNA signatures can be used to study the genetic diversity of soil nematodes without recourse to their isolation and morphological identification the study as done here cannot be used to infer too much about the genetic diversity of the samples or of the relative abundance of particular taxa. Previous studies using morphological and functional characterisation of nematodes in these soils revealed considerably more nematode diversity within and between these grasslands than is evident here (DeGoede and Bongers, 1998). This in part reflects the fact that we sought specifically to design and evaluate consensus primers for molecular ecological analysis of soil community DNA but may also reflect a lack of congruence between classifications based on morphology and function and those derived using sequence data.

As regards the design of the experiments, it is important to note that RFLP was used to select 'unique' clones prior to sequencing and, therefore, not all of the nematode diversity in each of the grasslands was sampled. RFLP analysis was used to select different digestion patterns prior to sequencing and, therefore, the clones used for phylogenetic analysis all showed a distinctive RFLP banding pattern. RFLP of small subunit rDNA amplicons (amplified ribosomal DNA restriction analysis, Heyndrickx et al., 1996; Moyer et al., 1996) can be used to assess diversity but such analyses usually employ more than one restriction enzyme (Moyer et al., 1994) to achieve the required resolution. Thus, in Fig. 1, some of the clones showing the same banding patterns may in fact originate from different taxa. This is because of limitations in the resolution of the gels and the fact that bands are separated according to size and not sequence. This diversity would not have been sampled here and as such would not be represented on the tree shown in Fig. 2. Although not evaluated, it is also possible, despite the direct amplification of 18SrDNA sequences from four of the five phylogenetic clades described by Blaxter et al. (1998) that the cell lysis procedures failed to lyse particular taxa. This would mean that their 18S rDNA did not form part of the soil community DNA and as such would not be amplified. This is a potential problem for all molecular ecological work and is not unique to nematode ecology. How serious a limitation will only be resolved when such approaches

become more widely applied to studies of soil nematode communities.

Another interesting difference between analysing nematode diversity and that of bacteria using molecular techniques is the possibility that the extent of the diversity recovered is closely linked to the size of the sample making the analysis of larger or more samples critical in establishing taxon diversity. For bacterial communities, this spatial diversity is not so evident (Felske and Akkermans, 1998). In our work only 1 g of soil was extracted for each sample (2 × 1 g for the DGGE analyses) making it possible that sampling rather than lysis or PCR biases (Wang and Wang, 1996; Suzuki and Giovannoni, 1998; O'Donnell and Goerres, 1999) were responsible for the low taxon richness seen in Fig. 2. Effective application of molecular ecological approaches to study nematode diversity will need to address questions of spatial heterogeneity and sample size either by processing larger soil samples (e.g. by pooling DNA from many sub-samples) or possibly by indirect methods where 'soil community DNA' is recovered following extraction of the nematodes from soils; although nematode extraction may introduce sampling biases by selecting for larger and faster swimming taxa and could mean the spatial and temporal separation of nematode characterisation from that of other soil organisms such as bacteria and fungi. Further work is needed to optimise the sampling strategies needed for molecular studies on nematode diversity.

The DGGE analysis (Fig. 3) was used to assess whether the nematode primers (NEMF1-GC and NEM896r) could be used to fingerprint nematode diversity between grasslands. Again the data show the potential of the approach with good resolution of the nematode amplicons in a 20–40% denaturing gradient. Although when used as a fingerprinting method, the caveats about PCR biases (Wang and Wang, 1996; Suzuki and Giovannoni, 1998; O'Donnell and Goerres, 1999) apply equally to DGGE, the technique has been used to provide profiles of the bacterial communities where the dominant bands are seen as representing major components of the community at the time of sampling (Muyzer and Smalla, 1998). Thus, differences in band intensity can be used as a surrogate for relative abundance whilst the number of bands indicates taxon richness with large numbers of bands indicative of diverse, taxon-rich environments (Muyzer et al., 1993; Sievert et al., 1999; Smalla et al., 2001). As seen in Fig. 3, nematode amplifications from the six European grassland types show marked differences in 'diversity' with different amplicons prevalent in different countries. Each of the six grassland types can be differentiated on the basis of their DGGE banding pattern thus providing a means of comparing 'diversity' between sites and of monitoring changes within sites due to changing environmental or management conditions. Whilst these DGGE studies show that molecular ecology techniques can be applied successfully to nematode communities, it was possible, again because of a lack of nematode sequence information, that the primers were not amplifying only nematode 18SrDNA and that the patterns seen with DGGE did not represent nematode sequences. To evaluate this, a number of bands were excised from the gels, amplified and sequenced, then subject to phylogenetic analysis (Fig. 4). Of the bands excised from the gels, all demonstrated high sequence similarity with nematodes showing that the modified primer and the DGGE were targeting and resolving nematode 18S rDNA signal in these soils. Obviously, these studies could be extended to excise all of the bands to ensure that none of the amplicons were from organisms other than nematodes. Such studies, together with more nematode sequences from single isolates, will enhance our understanding of nematode diversity and their phylogenetic affiliations and make possible the routine use of molecular ecology to study nematode communities in soils.

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