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Denaturing gradient gel electrophoresis (DGGE) as a tool for identification of marine nematodes

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ABSTRACT: Many phyla of marine invertebrates are difficult to identify using conventional morphological taxonomy. Larvae of a wider set of phyla are also difficult to identify as a result of conservation of morphology between species or because morphological characters are destroyed during sampling and preservation. DNA sequence analysis has the potential for identification of marine organisms to the species level. However, sequence analysis of specimens is time-consuming and impractical when species diversity is very high and densities of individuals huge, as they are in many marine habitats. The effectiveness of the 18S rRNA gene sequences for identification of one species-rich marine group, the Nematoda, is analysed. Following identification of variable regions of the 18S rRNA gene, primers were designed to amplify a small segment of sequences suitable for denaturing gradient gel electrophoresis (DGGE). The effectiveness of DGGE for identifying individual species is analysed. DGGE analysis of natural communities of nematodes detected less than ⅔ of the species present. This fraction of the community probably represents the abundant species in the original samples. It is concluded that DGGE is not a useful tool for analysis of species richness in marine communities as it fails to detect rare species of which there are usually many in the marine benthic environment. However, DGGE may be a useful method for detecting changes in communities that influence the abundance of the most common taxa.

KEY WORDS: Marine nematodes · 18S ribosomal DNA · Denaturing gradient gel electrophoresis

INTRODUCTION

The taxonomic crisis is well recognised throughout the scientific community (e.g. May 1997, Hebert et al. 2003a). It arises from a falling number of taxonomists and a narrow focus of existing expertise that neglects many highly diverse groups of organisms (May 1997, Tautz et al. 2003). This is particularly the case for organisms that live in many marine habitats, especially in the sediments of the seabed. These habitats may represent some of the most species-rich communities of metazoans on the planet and include, in order of numerical importance, nematodes, crustaceans, molluscs and polychaetes (e.g. Grassle & Maciolek 1992). The fact that these groups are studied by relatively few taxonomists is a significant limitation in marine ecology and the assessment of the impacts on biodiversity of global climate change and human activities.

As stated by Tautz et al. (2003), 'Insights into the stability or change of animal and plant guilds require

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species identification on a broad scale.' Such broad-scale species identification for any sample of marine sediment requires a huge effort on the part of several taxonomic experts to sort and identify individual specimens. The time, effort and costs of doing this for routine biomonitoring associated with specific impact assessments, or longer-term environmental studies, are multiplied by the number of samples. However, the problems associated with such studies cannot merely be reduced to practical considerations of scale. Morphological identification of marine species, belonging to the most speciose groups, is problematic through subjectivity of opinion of individual taxonomists, differences in the skills of taxonomists (compare specialist museum taxonomists with non-specialist parataxonomists) and problems of nomenclature and the existence of cryptic species. Synonyms, for example, are thought to over-inflate estimates of species diversity by 20% (May 1997) and sibling species have been commonly found across numerous marine taxa in all habitats (Knowlton 1993). Such problems become particularly intractable when samples from different geographic localities are compared, especially on regional, oceanic or global scales.

The adoption of DNA-based technologies offers one route to increase the speed and cost-effectiveness of species identification for ecological surveys or biomonitoring. This approach is based on the use of DNA sequences as 'barcodes' for species (Floyd et al. 2002, Hebert et al. 2003a). The first step in this process is the identification of regions of the genome that offer sufficient variation to resolve closely related species as well as higher taxa (e.g. Tautz et al. 2003). Such approaches require considerable effort in pilot studies that 'calibrate' sequence information by comparing the genetic distances expected between known species and higher taxa. The efficacy of mitochondrial genes as molecular barcodes for species identification has been explored recently (Hebert et al. 2003a,b). However, mitochondrial DNA can be subject to a number of problems. These include mitochondrial transfer between species, transposing of mitochondrial genes to the nucleus (Tautz et al. 2003), slow rates of mitochondrial evolution leading to low divergences in sequences between species (e.g. Cnidaria; France & Hoover 2002, Hebert et al. 2003b) and differing arrangements of the mitochondrial genome in different taxa that prevent 'universal' primers from amplifying target regions (e.g. nematodes; Okimoto et al. 1991, 1992, Blouin 1998, Keddie et al. 1998, Lavrov & Brown 2001). Nuclear genes may provide an alternative or companion to mitochondrial barcode sequences (e.g. Floyd et al. 2002). However, the most commonly used barcode regions of nuclear DNA—e.g. small subunit (ssu) and large subunit (lsu) rDNA—belong to multigene families, and although these are thought to exhibit concerted evolution there are many cases where intragenomic variation has been detected, especially in the internal transcribed spacer regions (e.g. ITS 1 and ITS 2; Harris & Crandall 2000, Chu et al. 2001).

The second major step in using DNA sequences for species identification is to accelerate the screening process so that it truly becomes a more rapid and cost-effective method than manual identification of specimens. Without such approaches, DNA barcodes will only be an adjunct to traditional methods for species identification from environmental samples. Previously, screening methods have been developed for small numbers of species and vary from the use of species-specific PCRs to PCR product-mobility assays using electrophoresis (for review on identification of marine larvae see Rogers 2001; for terrestrial nematodes, Foucher & Wilson 2002, Foucher et al. 2004).

Free-living marine nematodes typify many of the taxonomic problems associated with poorly studied invertebrate groups. Their identification to species level can be difficult, time-consuming and expensive. Specimens have to be mounted on slides and examined individually using high-power interference microscopy (Warwick et al. 1998). Moreover, only a small fraction of species have been or will be described (Lambshead 1993, Hugot et al. 2001). Therefore, only a few specialists with extensive taxonomic knowledge can work with the group. In addition, identification of juvenile meiofaunal taxa is often only possible by inference based on the presence of adults in the same samples (Litvaitis et al. 1994). The morphological characters used for classification may also be unreliable, and known species, especially those used for biomonitoring, may be complexes of sibling species with different functional responses (Warwick & Robinson 2000).

Free-living nematodes are an important component of the marine ecosystem and are a potentially ideal taxocene for biomonitoring and testing ecological theory. This is because they are a ubiquitous, abundant and hyper-diverse group of organisms in marine, terrestrial and freshwater environments. For ecological studies, work at the species level provides a greater resolution than that at higher taxonomic levels (Cook 2001). Whilst there have been many investigations into the use of functional grouping as a means of classification (Wieser 1952, Romeyn & Bouwman 1983, Jensen 1987, Thistle et al. 1995, Moens & Vincx 1997), recent work has suggested that, until a better understanding of species biology is reached, this approach is of limited use (Cook 2001). Moreover, the functional biology of marine nematode species in the deep sea is often unknown.

The aim of this study was to assess sequence variation in the ribosomal ssu for its power to resolve species

of marine nematodes. In addition, the use of DGGE was assessed as a means of rapidly screening nematode diversity from environmental samples. This is a common approach in studies of microbial ecology (DeLong 1992, Muyzer et al. 1993, Riemann et al. 1999) and has been attempted in terrestrial nematology (Foucher & Wilson 2002). This system has been reported to be fast and reliable, and only requires a modest laboratory set-up in terms of equipment.

MATERIALS AND METHODS

Obtaining and identifying nematodes. Nematodes for assessment of variation in the ssu were collected from mean low water spring (MLWS) tidal height from Southend-on-Sea, UK and Southampton Water at Warsash mud flat (50° 51′ N, 1° 18′ W), Weston shore (50° 53′ N, 1° 23′ W) and Hythe Salt Marsh (50° 52′ N, 1° 23′ W). Nematode samples were extracted *in situ* from 3 pooled core samples (5 cm diameter, to a depth of 2 cm plus any overlying water) by 5 decantations with filtered seawater, through a 45 µm-mesh sieve. All the retained material was washed in the sieve with filtered seawater before being washed with molecular grade 98% ethanol to drive off most of the water. The resultant material was then washed into storage pots with 98% ethanol.

For DGGE analysis of environmental samples, subtidal sediment was collected using a Van Veen grab from the coast off Plymouth, specifically: Saltash (Tamar estuary; 1 to 5 m depth) (50° 24′ N, 4° 12′ W), Plymouth Sound (10 m depth) (50° 20′ N, 4° 08′ W), and off Rame Head (50 m depth) (50° 17′ N, 4° 17′ W). These samples were immediately preserved in 98% ethanol, apart from the Saltash sample which was divided in half and one half preserved in 98% ethanol and the other in 4% formalin in seawater.

In the laboratory, Southend-on-Sea and Southampton Water nematode samples were further extracted by flotation in LUDOX™ 50 (made up to specific gravity of 1.15), after the ethanol was removed from the samples by washing through a 45 µm mesh. In a modification of the LUDOX flotation technique of Platt & Warwick (1983), samples were simply suspended in LUDOX for 24 h before the first extraction and then subsequently resuspended and extracted every 3 h, until all nematodes were extracted. LUDOX was replaced every second day to reduce dilution and consequent reduction in its specific gravity. SW England nematodes were similarly extracted but using a 63 µm mesh with 2 decantations with tap water followed by two 2 h flotations in LUDOX.

Nematode specimens used for morphological identification were picked out of the extracted sample using a binocular microscope, placed into cavity blocks containing anhydrous glycerol and stored in a desiccator until they could be mounted on slides for identification. Using semi-permanent, paraffin-wax ring mounts, nematodes were mounted singly, in anhydrous glycerol, onto clean microscope slides that had been stored in ethanol. It was found that the genomic DNA within specimens degraded in glycerol over a variable amount of time, from days to months, therefore storage time was minimised as far as practicably possible. Nematodes were examined using a high-power interference-contrast binocular microscope and identified to species level using pictorial keys for the identification of marine nematodes from NW Europe (Platt & Warwick 1983, 1988, Warwick et al. 1998). After identification, the coverslips were carefully removed from the microscope slide using a scalpel blade and the identified nematodes were transferred into 0.5 ml PCR tubes with 98% ethanol.

For DGGE analysis of environmental samples from SW England, all nematodes were picked out of the extracted samples using a binocular microscope, transferred in 0.5 ml PCR tubes with 0.25 M NaOH, and directly subjected to DNA extraction and amplification for the ssu (see later subsection) prior to electrophoresis. All nematodes extracted from the formalin-preserved Saltash sample were identified as above.

DNA extraction. A sodium hydroxide DNA extraction method modified from Floyd et al. (2002) was used to extract DNA from individual nematodes. An individual ethanol-preserved worm was placed into 20 µl 0.25 M sodium hydroxide in an 0.5 ml PCR tube, frozen for a minimum of 1 h at –20°C and incubated at 25°C overnight in an agitating incubator. The tube was then heated to 99°C for 3 min and the solution allowed to cool to room temperature before adding 4 µl 1 M hydrochloric acid (HCl), 10 µl 0.25 M Tris-HCl (pH 8.0) and 5 µl 2% Triton X-100 to the sample. This mixture was stirred briefly and then spun in a tabletop centrifuge for a few seconds at 3000 × *g*. It was then heated again to 99°C for 3 min before being allowed to cool to room temperature. The extracted DNA was used as a template for direct PCR amplification or stored at –20°C. In cases where the extraction became solid, an additional 40 µl Tris/HCl buffer were added to redissolve the sample.

DNA amplification and sequencing. The ssu rRNA gene was amplified in 3 sections and contiguous sequences constructed to obtain approximately 1400bp of sequence data for 50 putative species of marine nematode. The primers pairs used were G18S4 and 22R, 22F and 26R, and 24F and 13R (Blaxter et al. 1998; see present Table 1). PCR reactions were performed using the following mix: 50 mM KCl, 10 mM Tris-HCl pH 8.0, 2.75 mM $MgCl_2$, 0.8 mM dNTP mix (Perkin

Elmer), 10 pmol of each primer (Sigma-Genosys), 1 U *Taq* polymerase, 1.0 to 2.0 µl of DNA extract (all reagents Qiagen except where stated). The reaction volume was made up to 10 µl with ultrapure water (Sigma). PCR amplifications were performed on a Perkin Elmer Model 480 or Hybaid PCR express thermocycler under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of 45 s at 94°C, 30 s at 60 to 65°C and 1 min at 72°C (1°C s^{-1} ramping rate), with a final extension phase of 10 min at 72°C and a holding step at 4°C. Products were stored at –20°C prior to sequencing.

PCR products were purified using the Qiagen Qiaquick PCR purfication kit according to the manufacturer's instructions. Electrophoresis of 1µl of PCR product was performed through a 1% agarose gel followed by comparative quantification against a known standard using a Uvidoc 008-XD gel-documentation system and Uvisoft Version 98 gel-quantification software. We used 10 to 20 ng of PCR product as template for cycle-sequencing using the BigDye Terminator kit (Applied Biosystems), according to the manufacturer's instructions. Sequencing products were cleaned using the Dye-Ex Spin Kit (Qiagen) and 0.7 to 1.0 µl of product were analysed using an ABI Prism 377 automated DNA-sequencing system. Sequencing was performed in both directions for confirmation of sequences.

Several other regions of the nematode genome were analysed for ease of amplification and sequencing in several marine nematodes and in *Caenorhabditis elegans*. These included the mitochondrial 16S and 12S rRNA genes, the Cytochrome Oxidase I (COI) and the internal transcribed spacers of the nuclear rRNA multigene family (ITS 1 and ITS 2). The 16S and 12S primers were taken from M. Lange (unpubl.) or designed from genomic data available for *C. elegans* and all other available nematode sequences using Primer 3 (online interface). COI primers were taken from Hebert et al. (2003a). ITS 1 and ITS 2 primers were taken from Powers et al. (1997) and Chilton & Gasser (1999). All primers are detailed in Table 1. For all primer pairs annealing temperatures were varied according to the melting temperatures of the primers.

Sequence analysis of the ssu. Forward and reverse sequences were checked for quality and compared by eye using the Chromas software package (Version 1.45; available at: www.technelysium.com.au/chromas 14x.html). In order to align the sequences and to take into account the secondary structure of the ssu, a profile of already aligned nematode sequences was obtained from the European Ribosomal RNA Database (available at: http://www.psb.ugent.be/rRNA/ssu/list/Eucaryota.html). The new sequences were aligned to this profile using the programme ClustalX (Thompson et al. 1997) using default settings. Further small-scale editing was carried out by hand using the BioEdit software (Hall, online, Version 5.0.9). Substitution rates for the ssu gene were calculated using the HYPHY software package (Kosakovsky Pond et al. 2005; online, Version .96beta, available online at: www.hyphy.org). A sliding windows analysis with a window size of 25 bases and a shift of 5 bases, under local parameter estimation, was run under the Tamura–Nei (Tamura & Nei 1993) model (as selected by ModelTest, Version 3.06; Posada & Crandall 1998). A MrBayes consensus tree was used as the input tree for the analysis. Fig. 1 shows the estimated variation in the substitution rate along the ssu sequence.

Selection of region of ssu for DGGE analysis. DGGE works optimally with sequences of lengths between

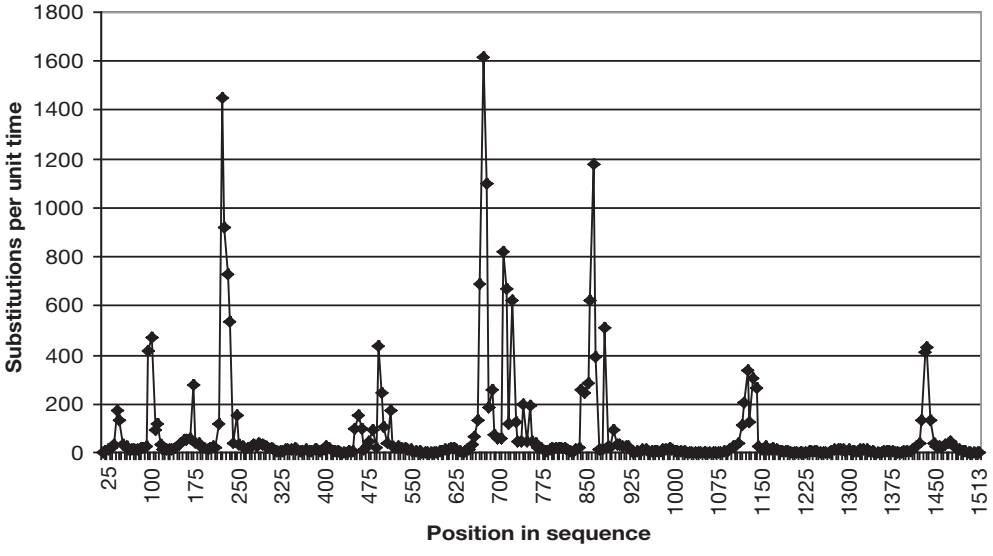

Fig. 1. Substitution rates for 50 marine nematode taxa using sliding-windows analysis

Table 1. Nematode small subunit and other primers used for PCR amplification, including approximate base-pair position in orthologous gene in *Caenorhabditis elegans*

Primer	*C. elegans* position	Sequence	Source
18S			
G18S4	5′ end	GCT TGT CTC AAA GAT TAA GCC	Blaxter et al. (1998)
22R	429–411	GCC TGC TGC CTT CCT TGG A	Blaxter et al. (1998)
22F	411–428	TCC AAG GAA GGC AGC AGG C	Blaxter et al. (1998)
26R	927–907	CAT TCT TGG CAA ATG CTT TCG	Blaxter et al. (1998)
24F	868–887	AGR GGT GAA ATY CGT GGA CC	Blaxter et al. (1998)
13R	1438–1419	GGG CAT CAC AGA CCT GTT A	Blaxter et al. (1998)
26RD	927–907	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCA TTC TTG GCA AAT GCT TTC G	This study
ITS			
rDNA2	2523–2503	TTG ATT ACG TCC CTG CCC TTT	Powers et al. (1997)
rDNA 1.58S	–	ACG AGC CGA GTG ATC CAC CG	Powers et al. (1997)
rDNA 2.144	–	GTA GGT GAA CCT GCA GAT GGA T	Powers et al. (1997)
NC1	3291–3310	ACG TCT GGT TCA GGG TTG TT	Chilton & Gasser (1999)
NC2	3764–3745	TTA GTT TCT TTT CCT CCG CT	Chilton & Gasser (1999)
NC5	2667–2692	GTA GGT GAA CCT GCG GAA GGA TCA TT	Chilton & Gasser (1999)
NC13R	3207–3190	GCT GCG TTC TTC ATC GAT	Chilton & Gasser (1999)
16S			
16SarMOD	10779–10798	CGC CTG TTT ATC AAA AAC AT	M. Lange (unpubl.)
16SbrMOD	11253–11232	CCG GTC TGA ACT CAG ATC ACG T	M. Lange (unpubl.)
12S			
$12SF_1$	1027–1046	GTT CCA GAA TAA TCG GCT AG	Meldal (2004)
$12SF_2$	1311–1326	GAC TCG TGT ATG ATC G	Meldal (2004)
$12SR_1$	1556–1538	CAA CTT ACT CCC CTT TGG G	Meldal (2004)
COI			
LCO1490		GGT CAA CAA ATC ATA AAG ATA TTG G	Hebert et al. (2003a)
HCO2198		TAA ACT TCA GGG TGA CCA AAA AAT CA	Hebert et al. (2003a)

200 to 1000bp in length (Potts 1996). For DGGE analysis, 2 sets of primers were therefore designed by the authors at Plymouth Marine Laboratory and the Natural History Museum respectively. The primers were:

- Primer pr 1: 22F [5′-GCCTGCTGCCTTCCTTGGA-3′]
  26R [5′-CATTCTTGGCAAATGCTTTCG-3′]

- Primer pr 2: G18F [5′-GCTTGTCTCAAAGATTAAGCC-3′]
  22R [5′-GCCTGCTGCCTTCCTTGGA-3′]

These primer sets produced amplicons of approximately 500 and 400 bp respectively. We synthesised 1 of the primers of each pair with the addition of a 39 bp GC clamp at the 5′ end to prevent complete denaturation of DNA molecules during DGGE analysis (Myers et al. 1985; see present Table 1 for 26RD). Primer Set 2 was primarily developed to eliminate co-amplification of fungal DNA that sometimes occurred using Primer Set 1.

Amplification of 18S rDNA for DGGE analysis. Genomic DNA extracted from marine nematodes was used as template for PCR amplification. PCR reactions were carried out in a final volume of 50 µl with the following mix: 5 µl of 10× reaction buffer, 2 µl $MgCl_2$, 0.5 µl dNTPs (0.25 mM), 1 µl bovine serum albumin, 1 µl of each primer, 10 µl of DNA extract and 19.1 µl of ultrapure water. For Primer Set 1, the following cycling parameters were used: 2 min at 96°C, followed by 10 cycles of 1 min at 96°C, 1 min 55°C, 2.5 min at 72°C, then 30 cycles of 1 min at 94°C, 1 min at 55°C, 2.5 min at 72°C followed by 30 min at 72°C and a holding step of 4°C. For Primer Set 2, the programme used was 2 min at 96°C, 35 cycles of 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C and finally 1 cycle of 2 min at 55°C, 5 min at 72°C followed by a holding temperature of 4°C.

Denaturing gradient gel electrophoresis. DGGE was developed using the Ingeny Phor-U DGGE apparatus according to the manufacturers′ instructions. For the

Ingeny Phor-U-based system, a stacking gel was used in addition to the main denaturing gel. Subsequent experiments were performed using Bio-Rad D-Code™ Universal Mutation Detection System (Bio-Rad). Denaturant gradients and electrophoretic parameters are detailed in the following subsection. Gels were stained with SYBR gold nucleic acid stain (Invitrogen), which was found to be more sensitive that ethidium bromide and less expensive than other SYBR dyes.

Resolution and sensitivity of DGGE. To test whether DGGE could separate 18S rDNA amplicons from marine nematodes, genomic DNA from 3 marine nematode taxa, *Sabatieria* sp., *Thalassironus britannicus* and *Enoploides* sp., were amplified individually and as a mixture using the G18FGC forward and 22R reverse primers. The amplification products were loaded in a 25 to 60% denaturing gel and underwent electrophoresis at 60 V for 16 h at 60°C.

To detect the minimum level of DNA that could be resolved in a denaturing gel, genomic DNA extracted from a single *Thalassironus britannicus* individual (total 20 µl) was used as template for PCR amplification using the G18FGC and 22R primers. The templates were added in the following order to carry out PCR amplification: 8, 5, 3, 1, 0.5 and 0.1 µl. The PCR products underwent electrophoresis in a 25 to 60% denaturing gel using previous electrophoresis conditions.

To test the use of DGGE for identification of environmental nematode samples all individual worms from each 'Plymouth' ethanol sample (half of the original grab sample for Saltash) were subjected to total DNA extraction. Small subunit ribosomal DNA (18S rDNA) was selectively amplified using the primers G18FGC and 22R. The resultant amplicons were separated in a 25 to 60% denaturing gradient. Electrophoresis was performed for 16 h at 60 V. Banding patterns were obtained for each environmental site (see Fig. 3). The dominant bands were cut from the gel, reamplified, cloned (using pGEM-T Easy vector system) and sequenced.

RESULTS

Interspecific variation in the ssu

Variation along the ssu showed a pattern of variable and conserved regions as shown by the substitution rate pattern in Fig. 1. This allowed targeting of a relatively short variable stretch (<1000 bp) of the ssu, between Primers 26F and 22R. Genetic distance estimates showed that the 2500 pairwise comparisons between 50 putative species of marine nematodes (Table 2) showed different sequences in all but 6 cases (note that specimens morphologically classed as *Sabatieria punctata* or *Sabatieria* sp. juveniles had slightly different sequences and so were treated separately for this analysis). In these cases, at least 1 of the 2 specimens was a juvenile (juv.) making identification potentially ambiguous (i.e. *Calomicrolaimus* sp. juv. vs. *C. parahonestus*; *S. punctata* vs. *Sabatieria* sp. juv. (2×); *Dichromodora* sp. juv. vs. *Atrochromodora* sp. juv.;

Table 2. Nematodes used in sequence analysis of the ssu

Sequence	GenBank Accession No.
Adoncholaimus fuscus Bastian, 1865	AY854195
Anoplostoma sp. Bütschli, 1874	AY854194
Ascolaimus elongatus (Bütschli, 1874)	AY854231
Atrochromadora microlaima (De Mann, 1889)	AY854204
Axonolaimus helgolandicus Lorenzen, 1971	AY854232
Bathylaimus sp. Cobb, 1894	AY854201
Calomicrolaimus parahonestus (Gerlach, 1950)	AY854218
Calomicrolaimus sp. Lorenzen, 1976	AY854219
Calyptronema maxweberi (De Mann, 1922)	AY854199
Chromadora nudicapitata Bastian, 1865	AY854205
Chromadora sp. Bastian, 1865	AY854206
Chromadorina germanica (Bütschli, 1874)	AY854207
Chromadorita tentabunda (De Man, 1890)	AY854208
Cyartonema elegans Jayasree & Warwick, 1977	AY854203
Cyatholaimid sp.	AY854212
Cyatholaimus/Praeacanthonchus sp.	AY854213
Daptonema hirsutum (Vitiello, 1967)	AY854223
Daptonema normandicum (De Man, 1890)	AY854224
Daptonema oxycerca (De Man, 1888)	AY854225
Daptonema setosum (Bütschli, 1874)	AY854226
Desmodora communis (Bütschli, 1874)	AY854215
Desmolaimus zeelandicus De Man, 1880	AY854229
Dichromadora sp. Kreis, 1929	AY854209
Enoploides brunettii Gerlach, 1953	AY854193
Enoplus communis Bastian, 1865	AY854192
Metachromadora remanei Gerlach, 1951	AY854216
Molgolaimus demani Jensen, 1978	AY854220
Monoposthia costata (Bastian, 1865)	AY854221
Neochromadora poecilosoma (De Man, 1893)	AY854210
Nudora bipapillata Platt, 1973	AY854222
Odontophora rectangula Lorenzen, 1971	AY854233
Oncholaimus sp. Dujardin, 1845	AY854196
Praeacanthonchus punctatus (Bastian, 1865)	AY854214
Sabatieria celtica Southern, 1914	AY854234
Sabatieria punctata 200 (Kreis, 1924)	AY854235
Sabatieria punctata 223 (Kreis, 1924)	AY854236
Sabatieria punctata 343 (Kreis, 1924)	AY854237
Sabatieria sp. 210 Rouville, 1903	AY854238
Sabatieria sp. 335 Rouville, 1903	AY854239
Setosabatieria hilarula (De Man, 1922)	AY854240
Sphaerolaimus gracilis De Man, 1884	AY854227
Sphaerolaimus hirsutus Bastian, 1865	AY854228
Spilophorella paradoxa (De Mann, 1888)	AY854211
Spirinia parasitifera (Bastian, 1865)	AY854217
Syringolaimus striatocaudatus De Mann, 1888	AY854200
Terschellingia longicaudata De Man, 1907	AY854230
Tripyloides sp. De Man, 1886	AY854202
Marine tylenchid	AY854241
Viscosia sp. 251 De Man, 1890	AY854197
Viscosia viscosa (Bastian, 1865)	AY854198

Daptonema hirsutum juv. vs. *D. setosum* juv.; *Setosabatieria hilarula* juv. vs. *Sabatieria celtica* juv.). In 1 case, genetic identity may also reflect a systematic problem, where *D. hirsutum* may be a synonym of *D. setosum*. Only in the comparison of *Setosabatieria hilarula* and *Sabatieria celtica* was an identical sequence found for 2 species that were unlikely to be confused or reflect systematic problems. For all other species (Table 2) a range of genetic distances up to 0.19109 (uncorrected) or 0.22369 (Tamura & Nei corrected distance, Tamura & Nei 1993) were observed. The greatest genetic distance for both measures was observed between the unidentified tylenchid harvested from seaweed and the enoplid nematode *Calyptronema maxweberi*.

The phylogenetic analysis of marine nematodes showed a good resolution of the major nematode orders Enoplida, Chromodorida and Monhysterida. Most species cluster into genera and families described from morphological studies. There were some exceptions, and these are the result of taxonomic problems within the phylum Nematoda. These data are fully explored by Meldal (2004) on the phylogenetics of the phylum Nematoda.

Very limited success was achieved amplifying the mitochondrial COI, 16S and 12S rDNA and nuclear ITS regions for marine nematodes. PCR amplification was unreliable and the use of these regions was abandoned for marine nematodes.

Resolution and sensitivity of DGGE

The results of amplification and separation of DNA from 3 individuals and from a mixed template are shown in Fig. 2. This demonstrated that in this case there is no template bias when the amplicons are separated by DGGE.

The limit of detection of DNA extracted from an individual nematode is shown in Fig. 3. This indicates that the limits of detection are in the region of 0.5 µl of template that represents 2.5% of the DNA extractable from a single nematode. Template concentration is in the range of 0.5 to 3 ng μl^{-1} (n = 10), thus giving a limit of detection for genomic DNA of approximately 250 pg (before PCR and DGGE detection).

PCR/DGGE analysis of nematode samples taken from, Cawsand, Plymouth Breakwater, Rame Head and Saltash (Tamar estuary) is shown in Fig. 4. Approximately 15 bands could be distinguished, representing 15 putative taxa. All the extracted bands (Env 1 to 4) show high sequence similarity to the available nematode sequences held online at GenBank and EMBL. This indicates that the primer/DGGE system is capable of targeting and resolving 18S rDNA of marine nematodes selected from environmental samples. However, an analysis of half the sample from Saltash, using morphological identification, revealed 25 species of nematodes (Table 3). Previous comprehensive surveys based on morphological analysis indicate that at least 40 nema-

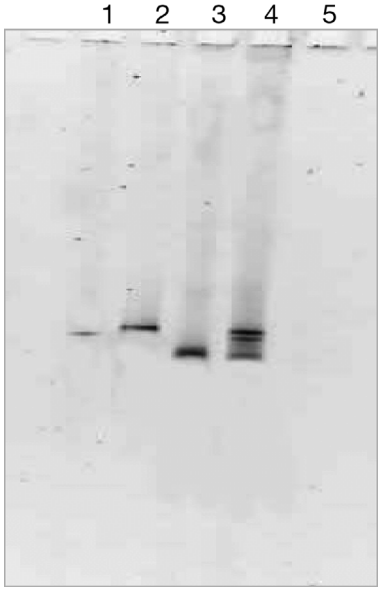

Fig. 2. *Sabatieria* sp. (Lane 1), *Thalassironus britannicus* (Lane 2) and *Enoploides* sp. (Lane 3). PCR-DGGE analysis of 18S rDNA gene in a 25 to 60% denaturing gel. Lane 4: mixed DNA from all 3 nematodes (PCR-amplified); Lane 5: negative control

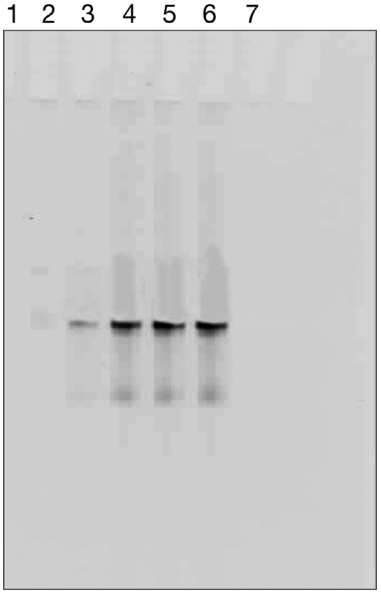

Fig. 3. *Thalassironus britannicus*. DGGE gel showing minimum detectable level of DNA. Lanes 1 to 6: PCR products amplified from 0.1 µl template (Lane 1), 0.5 µl template (Lane 2), 1 µl template (Lane 3), 3 µl template (Lane 4), 5 µl template (Lane 5) and 8 µl template (Lane 6). Lane 7: negative control

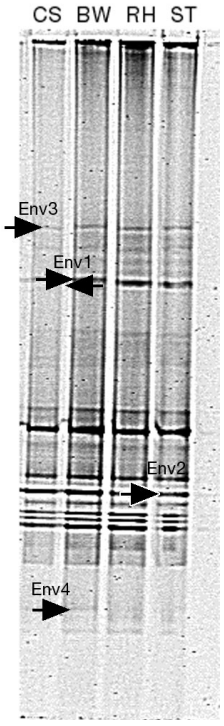

Fig. 4. Banding patterns of marine nematode communities from 4 environmental stations. Lane 1: Cawsand (CS); Lane 2: Breakwater (BW); Lane 3: Rame Head (RH); Lane 4: Saltash (ST). Arrows indicate bands extracted and sequenced

tode species occur in this environment in the Tamar Estuary/Plymouth Sound area (Austen 1986).

A phylogenetic tree was constructed using the sequences from the excised bands and additional marine nematode partial ssu sequences retrieved from GenBank. Prior to phylogenetic analysis, nematode sequences were aligned in Clustal-X using default parameters (Thompson et al. 1997, Jeanmougin et al. 1998). A neighbour-joining tree was constructed with the program MEGA Version 2.1 (Kumar et al. 2001) using gamma-corrected Kimura distance parameters (Blaxter et al. 1998). The phylogenetic placement of the sequences suggests that some of them share high similarity with *Enoplus meridionalis* Steiner, 1921 (similar to Env 3), *Sabatieria celtica* Southern, 1914 (Env 2), *Terschellingia longicaudata* De Man, 1907 (Env 1) and *Thalassironus britannicus* De Man, 1889 (Env 4) (see Fig. 5; EMBL Accession Nos. AJ867815, AJ867816, AJ867817 and AJ867818). Some of these species were specifically identified in the morphological analysis from Saltash or were related to taxa identified in the morphological analysis at the genus or family level.

DISCUSSION

As with other studies on the 18S ssu rDNA region, our study indicated that this gene contains a mix of conserved and unconserved regions in nematodes. This is consistent with the considerable variation in among-site rates of nucleotide substitution that have been observed across many phyla (e.g. Abouheif et al. 1998) and shows that such variation occurs within, as well as among, different phyla. This variation in among-site substitution rates has been observed across different classes of 18S secondary structure (Abouheif et al. 1998). The mix of conserved and unconserved regions amongst the 18S rDNA region makes it suitable for the design of primers to amplify segments of the gene that are variable amongst different species of nematode.

Of the 2500 pairwise comparisons, only 2 species appeared to have the same DNA sequence. These species were both from the family Comesomatidae, and this result may have arisen from homoplasy within the 18S rDNA region for these taxa. Another possible

Table 3. Nematodes identified from Saltash sample by morphological characteristics. Classification to family is according to Meldal (2004). n: number identified

Taxon	Family/order	n
Anoplostoma sp. Bütschli, 1874	Anoplostomatidae	1
Viscosia viscosa Bastian, 1865	Oncholaimidae	3
Microlaimus sp. De Mann, 1880	Microlaimidae	1
Molgolaimus tenuispiculum Ditlevsen, 1921	Microlaimidae	1
Atrochromadora sp. Wieser, 1959	Chromodoridae	1
Chromadora sp. Bastian, 1865	Chromodoridae	1
Ptycholaimellus ponticus (Filipjev, 1922)	Chromodoridae	9
Cyatholaimus sp. Bastian, 1865	Cyatholaimidae	1
Metachromadora remanei Gerlach, 1951	Desmodoridae	1
Metachromadora sp. Filipjev, 1918	Desmodoridae	5
Desmodora pontica Filipjev, 1922	Desmodoridae	2
Sabatieria pulchra (Schneider, 1906)	Comesomatidae	6
Sabatieria celtica Southern, 1914	Comesomatidae	3
Setosabatieria hilarula (De Man, 1922)	Comesomatidae	1
Terschellingia longicaudata De Man, 1907	Linhomoeidae	5
Terschellingia communis De Man, 1888	Linhomoeidae	3
Terschellingia gourbaultae Austen, 1989	Linhomoeidae	1
Sphaerolaimus sp. Bastian, 1865	Sphaerolaimidae	3
Theristus acer Bastian, 1865	Xyalidae	2
Daptonema setosum (Bütschli, 1874)	Xyalidae	8
Daptonema oxycerca (De Man, 1888)	Xyalidae	5
Daptonema normandicum (De Man, 1890)	Xyalidae	9
Daptonema sp. Cobb, 1920	Xyalidae	7
Axonolaimus paraspinosus Stekhoven & Adam, 1931	Axonolaimidae	4
Odontophora sp. Bütschli, 1874	Axonolaimidae	5

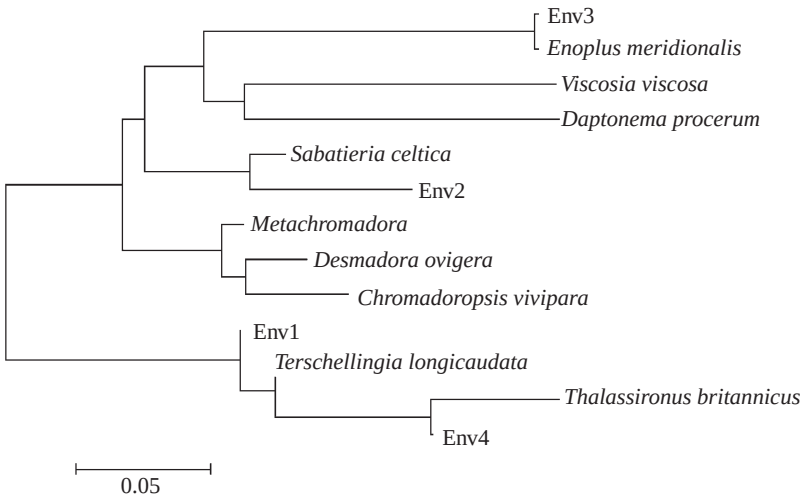

Fig. 5. Phylogenetic tree showing relationship between DGGE bands amplified using G18F and 22R primers (18S rDNA) and almost similar sequences of known nematodes. Scale = 0.05 substitutions/site

explanation is error during DNA-sequencing, but this is unlikely as the 2nd and 3rd amplified segments of the 18S gene overlap, giving a high confidence in sequence data as effectively the same regions were sequenced several times. Taking this in to account, the 18S rDNA gene was able to distinguish 93% of the congeneric species compared and >99% of the total species compared. This is a lower value than the resolution reported for Cytochrome Oxidase I (COI) gene, in which only 1.9% of comparisons between congeneric pairs of species showed a sequence divergence of less than 2% (Hebert et al. 2003b); in this and a previous study (Hebert et al. 2003a), the authors did include nematodes, although these were mainly from the Rhabditina (7 taxa), a suborder of terrestrial free-living and parasitic nematodes, the Spirurina (2 taxa) (Hebert et al. 2003a), or parasitic nematodes from several families (Hebert et al. 2003b, electronic Appendix available at www.pubs.royalsoc.ac.uk). The higher resolution of COI probably reflects the higher mutation rate of mitochondrial genes in most taxa (Avise 1994). However, this may also result in a proportionally greater occurrence of mutations in primer sites for Primers LCO1490 and HCO2198 (Hebert et al. 2003a), with the result that there will be a lower success rate in amplification of a wide range of taxa. It is also known that the arrangement of the mitochondrial genome of at least some nematodes differs from that usually found in metazoans (Okimoto et al. 1991, 1992, Blouin 1998, Keddie et al. 1998, Lavrov & Brown 2001). Together these factors, especially the former, may explain the limited success in amplification of marine nematode material with the COI primers from Hebert et al. (2003a) compared with the 18S rRNA primers. This material originates from a broader range of species and higher taxa than previously examined. Primer-binding problems may be circumvented by more optimisation for COI amplification or by design of nematode-specific COI primers. Ultimately, an approach based on more than 1 marker may be the solution.

One of the critical aspects of methodology using environmental samples is the concentration at which target DNA can be amplified to a level sufficient for detection by DGGE. The technique was only able to detect PCR products at or greater than the nanogram level (with original template quantity of at least 0.25 ng DNA). In marine sediments few nematode species tend to be abundant, with many occurring as only a few or even single individuals in an environmental sample (Heip et al. 1985). This is almost certainly the reason that the DGGE analysis of an environmental sample from Saltash only detected 15 putative taxa, whereas morphological analysis of half of the same sample revealed at least 25 taxa. Whilst there maybe some minor differences in species composition within 2 halves of the same sample, it is unlikely that they will be sufficient to explain the considerable difference in numbers of species detected using molecular versus morphological analysis. Previously, detailed morphological studies of the same locality indicate the presence of at least 40 species, but many of these only occur in relatively low numbers compared to a much higher number of individuals representing a few species like *Terschellingia longicaudata*, *T. communis*, *Sabatieria pulchra*, *Ptycholaimellus ponticus* and others (Austen 1986). This shows a striking similarity with results obtained from DGGE analysis of microbial communities, in that this method only detects the abundant taxa within a sample (Chan et al. 2002, Koizumi et al. 2003). It is also comparable with a recent study on the application of DGGE to elucidate the diversity of nematode communities of soil (Foucher et al. 2004).

Overall, it is apparent that the use of molecular markers for analysis of species richness is not straightforward and is largely determined by the scientific question that is being addressed (i.e. changes in abundance versus changes in species richness). The specificity of primer-binding may mean that many species are missed by sequence-based approaches. Sensitivity and selectiveness of the polymerase chain reaction must also be considered. Different markers obviously have different levels of resolution depending on the

mutation rate of the gene in question within the taxa studied. Determination of what level of genetic divergence to expect between target species inevitably means that a period of cross-calibration of morphologically identified species and molecular data is necessary. This strongly advocates approaches that combine both morphological and genetically based identifications to study the species richness of marine communities. As with all such surveys, care must be taken in the design of a sampling programme, the requirements for which are the same whether a morphological or a molecular study is being undertaken.

To conclude, it seems that the 18S rDNA region is suitable for the identification of the majority of nematode species. For 100% certainty in identification of species, a second region of DNA will be required to ensure identification of each taxon and to confirm identifications made on the basis of 18S rDNA partial sequences. DGGE is only capable of identifying relatively abundant taxa in an environmental sample. This is suitable for identifying major changes in the species composition between samples, but not for a direct assessment of species richness; thus, the technique is useful as a rapid system for community analysis in a similar manner as for microbial ecological studies. Even with such limitations DGGE could be a useful way of detecting changes in communities of marine animals that are small or difficult to identify (i.e. larvae). As such, taxa that are usually beyond the scope of marine biodiversity studies (identified simply to phylum or group level) may be incorporated in analyses in combination with more conventionally identified and analysed material such as macro- or megabenthic organisms.

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