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FEATURE ARTICLE

Development and evaluation of a DNA-barcoding approach for the rapid identification of nematodes

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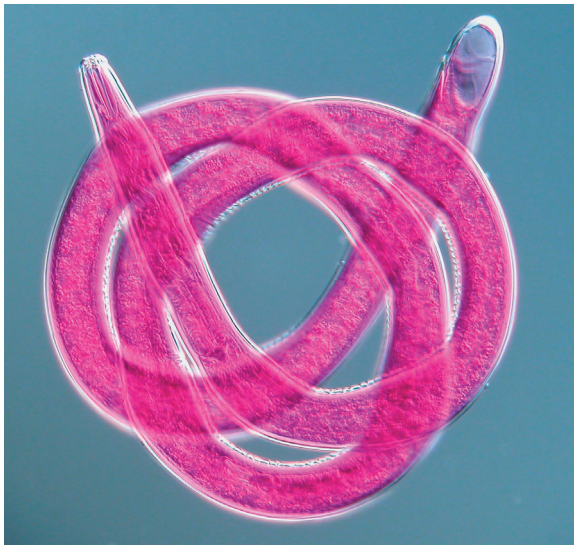
ABSTRACT: Free-living nematodes are abundant in all marine habitats, are highly diverse, and can be useful for monitoring anthropogenic impacts on the environment. Despite such attributes, nematodes are effectively ignored by many marine ecologists because of their time-consuming taxonomy. Nematode diagnostics has traditionally relied on detailed comparison of morphological characters which, given their abundance, is difficult and laborious, meaning that the biodiversity of the group is typically underestimated. Molecular methods such as DNA-barcoding offer potentially efficient alternative approaches to studying the biodiversity of marine nematode communities, allowing these organisms to be more effectively exploited in ecological surveys and environmental assessments. In this study, a number of nuclear and mitochondrial genomic regions were evaluated as potential diagnostic loci for marine nematode species identification. Of these, the 18S ribosomal RNA gene amplified most reliably from a range of taxa, and was therefore evaluated as a DNA barcode. In a comparison of molecular and morphological identifications, over 97% of specimens sequenced were correctly assigned on the basis of a short stretch of 18S rRNA sequence (approximately 345 bp), making this a potentially useful marker for the rapid molecular assignment of unknown nematode species, and evaluation of nematode species richness during ecological surveys or environmental assessments. This study showed that a single marker approach based on amplification and sequencing may prove invaluable in the rapid identification of nematodes during ecological surveys and, indeed, other taxonomically challenging invertebrate taxa.

KEY WORDS: Marine nematodes · Identification · DNA barcoding · 18S rRNA · Ecological survey



Genomic identification based on DNA barcoding was evaluated as a tool to improve identification of nematodes such as *Trissonchulus* sp. (photo). This method makes meiofaunal identification significantly easier and more reliable, particularly for non-experts in taxonomy, as well as in cases where traditional methods are impractical.

Photo: Dr. Tim Ferrero, The Natural History Museum, London, UK

INTRODUCTION

Sound taxonomy underpins almost all biological research, nowhere more so than in ecology. Despite this, there is currently a serious crisis in taxonomic expertise throughout the scientific commu-

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nity (Freckleton & May 1992, Buyck 1999, Hopkins & Frecklenton 2002), resulting in the neglect of many highly diverse groups of organisms. This is particularly the case for many marine invertebrate taxa, especially those from benthic sediments, which contain species-rich communities of metazoans including large numbers of nematodes, polychaetes, crustaceans and molluscs (Grassle & Maciolek 1992, Coull 1999, Lambshead 2004). Global marine nematode species richness may exceed 1 million (Lambshead 2004), only a few thousand of which are described, and these animals are typically the most abundant component of the meiofauna and deep-sea macrofauna (Lambshead 2004). Being so diverse and abundant, marine nematodes are believed to be of great importance ecologically as they play an important role in the decomposition process and recycling of nutrients (Austen 2004) and have proved to be highly sensitive indicators of anthropogenic stress in a range of situations (Lambshead 1986, Austen & McEvoy 1997, Schratzberger et al. 2000). Despite such attributes, the group has seen relatively limited use by marine ecologists and those involved in routine biomonitoring, largely as a result of their relative taxonomic intractability. Specific identification of most marine nematodes relies on detailed morphological analysis (Platt & Warwick 1988) that requires considerable taxonomic expertise, placing it outside the scope of most routine ecological surveys. Also, the overwhelming number of individuals present in a square metre of sediment ($1 \times 10^{5-7}$; Lambshead 2004) impedes attempts to describe nematode communities in detail, even when such expertise is available. In addition to this, the fact that many taxa can also only be reliably identified from adult males has contributed to the relative neglect of nematodes in many infaunal studies (Warwick & Robinson 2000). The use of morphologically-defined operational taxonomic units (or OTUs) on such organisms is also fraught with difficulty (Floyd et al. 2002). Defining discriminatory morphological characters in small, morphologically uniform families that are known to include taxa which are morphologically cryptic, in a manner which can be standardized across a range of investigators, is problematic even to the specialist.

DNA barcoding, based on the analysis of a small segment of the genome, is one potential way of simplifying and speeding up the evaluation and identification of taxa such as nematodes in ecological or biomonitoring studies (Hebert et al. 2003a, Rogers & Lambshead 2004). Genomic regions within an individual can be viewed as genetic 'barcodes' as these regions hold necessary information from their remote or recent evolutionary history. Therefore a DNA barcode in the form of a specific sequence carries both species-specific and phylogenetic information regarding an organism (Blaxter 2004). DNA barcodes can be used in the identification of unknown specimens, to assist the phylogenetic placement of unknown taxa through comparison with known reference sequences, and to enable the definition of molecular operational taxonomic units (MOTUs), whose delineation is not fraught with the difficulties discussed above. Such molecular barcodes are now routine in investigations of prokaryotic diversity (e.g. Cohan 2002), and have also been employed in unicellular eukaryotes, including some planktonic taxa (e.g. Massana et al. 2002, Moreira & López-Garcia 2002). Identifications based on DNA sequences are also increasingly used for metazoans, including soil and parasitic nematodes, (Hebert et al. 2003a, Powers 2004) although to date this type of approach has seen limited application in marine systems.

This study investigated the potential utility of a nucleic acid marker for the rapid identification and evaluation of marine nematode diversity, based on a study of a wide range of taxa from estuarine and shelf waters around southwest Britain. In addition, the reliability of the barcoding approach was tested, by comparing the placement of specimens based on short DNA sequence with that generated by expert morphological identification of the same specimen as part of a broader ecological survey. An approach based on the amplification and sequencing of a short segment of DNA may prove invaluable towards rapid identification of nematodes and other benthic organisms.

MATERIALS AND METHODS

Sediment collection. Sediments were collected subtidally using a van Veen grab from muddy and muddy-sand substrates in SW England from the Tamar estuary (50° 24′ N, 4° 12′ W) at 1 to 5 m depth, from Plymouth Sound at Jennycliff and Plymouth Breakwater (both 50° 20′ N, 4° 08′ W) at 10 and 15 m depth, respectively, from Rame Head (50° 17′ N, 4° 17′ W) at 50 m depth and from Cawsand (50° 19′ N, 4° 11′ W) at 12 m depth. Additionally, sediment samples collected by hand from intertidal mud in the Plym estuary (Saltram) in SW England were also used in this study. All samples were immediately fixed in 1 l storage pots containing 98% molecular grade ethanol (Hayman).

Meiofauna extraction and nematode identification. Each sediment sample was washed twice with tap water on a 63 µm sieve, until the water passing through the sieve became clear, to remove finer sediment components and drive off any alcohol. Extraction then followed Somerfield & Warwick's (1996) flotation method, where the residual sediment and fauna was saturated with Ludox™ (specific gravity 1.15) before being washed into 100 ml beakers. The mixture of

sediment and Ludox was thoroughly stirred and then left for at least 2 h to allow animals to become suspended. The supernatant was poured into a 63 µm sieve to collect the fauna, which was then washed once with distilled water and stored in 98% alcohol. Nematode specimens used for DNA extraction were picked out of the extracted samples using a sterile needle under a stereo microscope (50× magnification) and placed into a cavity block containing approximately 5% glycerol and 10% ethanol. Each specimen was then mounted in glycerol on a separate slide, and a cover slip placed on top and sealed with paraffin wax. Before mounting, slides and cover slips were washed in molecular grade alcohol and dried with tissues. Based on morphological characters, each specimen was identified to genus and species level (wherever possible) under a compound microscope, using pictorial keys for the identification of marine nematodes from North West Europe (Platt & Warwick 1983, 1988). Wherever possible, male specimens were included for taxonomic conformation. All identifications were verified by experienced nematode taxonomists prior to molecular analyses. After identification, cover slips were removed from the slides using a sterile scalpel, and specimens were individually placed in 0.5 ml PCR tubes containing 20 µl of 0.25 M NaOH for DNA extraction. Twenty-six taxa representing the major orders and groups of marine nematodes that dominate SW England benthic environments (Austen 1986, Austen & McEvoy 1997) were morphologically identified prior to molecular analyses (Table 1).

Table 1. List of nematode taxa with family/order position (following Meldal's 2004 classification) and GenBank accession numbers

Taxon	Family/order	Accession number
Daptonema setosum (Bütschli, 1874)	Xyalidae	AM234045
Daptonema sp. Cobb, 1920	Xyalidae	AM234624
Daptonema hirsutum (Vitiello, 1967)	Xyalidae	AM236231
Theristus acer Bastian, 1865	Xyalidae	AM234627
Dorylaimopsis punctata Ditlevsen, 1918	Comesomatidae	AM234047
Sabatieria pulchra (Schneider, 1906)	Comesomatidae	AM234623
Sabatieria celtica Rouville, 1903	Comesomatidae	AM234626
Setosabatieria hilarula (De Man, 1922)	Comesomatidae	AM236043
Metachromadora remanei Gerlach, 1951	Desmodoridae	AM234620
Desmodora pontica Filipjev, 1922	Desmodoridae	AM234628
Spirinia parasitifera (Bastian, 1865)	Desmodoridae	AM236044
Ascolaimus elongatus (Bütschli, 1874)	Axonolaimidae	AM234617
Parodontophora sp. Timm, 1963	Axonolaimidae	AM234630
Axonolaimus helgolandicus Lorenzen, 1971	Axonolaimidae	AM236598
Paralinhomoeus sp. De Man, 1907	Linhomoeidae	AM235216
Terschellingia longicaudata De Man, 1907	Linhomoeidae	AM234716
Cyatholaimus sp. Bastian, 1865	Cyatholaimidae	AM234618
Praeacanthonchus sp. Micoletzky, 1924	Cyatholaimidae	AM234046
Oncholaimus sp. Dujardin, 1845	Oncholaimidae	AM234625
Bathylaimus sp. Cobb, 1894	Tripyloididae	AM234619
Anoplostoma sp. Bütschli, 1874	Anoplostomatidae	AM235215
Halichoanolaimus dolichurus Ssaweljev, 1912	Choniolaimidae	AM234629
Sphaerolaimus hirsutus Bastian, 1865	Sphaerolaimidae	AM234622
Adoncholaimus fuscus (Bastian, 1865)	Enoplidae	AM236232
Adoncholaimus sp. Bastian 1865	Enoplidae	AM236077
Enoploides brunettii Gerlach, 1953	Thoracostomopsidae	AM234621

Reliability of DNA barcodes for ecological surveys. To evaluate the reliability of identifications based on DNA barcodes, sediments from the Tamar and Plym estuaries were fixed in molecular grade ethanol, after which meiofauna were extracted as part of an ecological survey. Forty individuals from each site were randomly selected and fixed on slides for taxonomic identification. After taxonomic identification, unique numbers were assigned to each specimen and these randomised before being subjected to 18S rRNA amplification and sequencing so that further analyses and processing acted as a 'blind test' of the barcoding. These sequences were then included in a phylogenetic analysis and their molecular and morphological placements compared. The identity of individual specimens based on morphological characters, and their unique reference numbers are given in Table 2.

Molecular marker selection for this study. Two nuclear genes, namely 18S rRNA and 28S rRNA (for 28S rRNA primers see De Ley et al. 2005), were tested along with mitochondrial cytochrome C oxidase I (COI) and 16S rRNA genes (for primers see Hebert et al. 2003a, Bhadury 2005) for molecular barcoding evaluation. Further evaluation with the 28S rRNA, 16S rRNA and COI genes was abandoned as a result of unreliable PCR amplification with several representative marine nematode taxa from SW Britain. Therefore, the main focus of this work was to evaluate the potential of 18S rRNA genes for barcoding marine nematodes. In addition, 18S rRNA sequences are generally taxon specific and contain both conserved and variable regions, suitable for primer design and taxonomic distinction, respectively (Blaxter et al. 1998, Floyd et al. 2002). This gene is also present in multiple copies in the nematode genome and, therefore, is a more effective target for amplification than the single copy gene (Floyd et al. 2002).

DNA extraction from a single worm. DNA was extracted using a modification of the method of Floyd et al. (2002). All 0.5 ml PCR tubes were frozen overnight (8 to 9 h) at –20°C, then incubated overnight at 60°C. The tubes were then heated for 3 min at 99°C on a

Table 2. Morphological identifications and corresponding molecular tags for specimens used to test the barcoding concept

Tamar		Plym	
Taxon	Mol. ID	Taxon	Mol. ID
Adoncholaimus fuscus Bastian, 1865	Tamar1	*Praeacanthonchus* sp. Micoletzky, 1924	Plym1
Spirinia parasitifera (Bastian, 1865)	Tamar2	*Anoplostoma* sp. Bütschli, 1874	Plym2
Sabatieria sp. Rouville, 1903	Tamar3	*Paracanthonchus* sp. Micoletzky, 1924	Plym3
Dichromadora sp. Kreis, 1929	Tamar4	*Daptonema setosum* (Bütschli, 1874)	Plym4
Terschellingia longicaudata De Man, 1907	Tamar5	*Metachromadora* sp. Filipjev, 1918	Plym5
Praeacanthonchus sp. Micoletzky, 1924	Tamar6	*Sabatieria pulchra* (Schneider, 1906)	Plym6
Enoploides brunettii Gerlach, 1953	Tamar7	*Terschellingia* sp. De Man, 1888	Plym7
Metachromadora remanei Gerlach, 1951	Tamar8	*Sphaerolaimus hirsutus* Bastian, 1865	Plym8
Sphaerolaimus hirsutus Bastian, 1865	Tamar9	*Theristus* sp. Bastian, 1865	Plym9
Sabatieria celtica Southern, 1914	Tamar10	*Metachromadora* sp. Filipjev, 1918	Plym10
Atrochromadora microlaima (De Mann, 1889)	Tamar11	*Terschellingia* sp. De Man, 1888	Plym11
Terschellingia longicaudata De Man, 1907	Tamar12	*Terschellingia longicaudata* De Man, 1907	Plym12
Terschellingia longicaudata De Man, 1907	Tamar13	*Paralinhomoeus* sp. De Man, 1907	Plym13
Ascolaimus elongatus (Bütschli, 1874)	Tamar14	*Sphaerolaimus hirsutus* Bastian, 1865	Plym14
Terschellingia sp. De Man, 1888	Tamar15	*Sphaerolaimus* sp. Bastian, 1865	Plym15
Viscosia viscosa (Bastian, 1865)	Tamar16	*Axonolaimus helgolandicus* Lorenzen, 1971	Plym16
Terschellingia longicaudata De Man, 1907	Tamar17	*Metachromadora suecica* (Allgén, 1929)	Plym17
Sabatieria celtica Southern, 1914	Tamar18	*Daptonema* sp. Cobb, 1920	Plym18
Setosabatieria hilarula (De Man, 1922)	Tamar19	*Sabatieria* sp. Rouville, 1903	Plym19
Daptonema setosum (Bütschli, 1874)	Tamar20	*Daptonema hirsutum* (Vitiello, 1967)	Plym20
Paralinhomoeus sp. De Man, 1907	Tamar21	*Sabatieria* sp. Rouville, 1903	Plym21
Sabatieria pulchra (Schneider, 1906)	Tamar22	*Sabatieria* sp. Rouville, 1903	Plym22
Terschellingia longicaudata De Man, 1907	Tamar23	*Enoploides* sp. Ssaweljev, 1912	Plym23
Desmodora pontica Filipjev, 1922	Tamar24	*Adoncholaimus* sp. Filipjev, 1918	Plym24
Halichoanolaimus dolichurus Ssaweljev, 1912	Tamar25	*Sphaerolaimus hirsutus* Bastian, 1865	Plym25
Axonolaimus helgolandicus Lorenzen, 1971	Tamar26	*Adoncholaimus* sp. Filipjev, 1918	Plym26
Adoncholaimus sp. Filipjev, 1918	Tamar27	*Enoploides* sp. Ssaweljev, 1912	Plym27
Anoplostoma sp. Bütschli, 1874	Tamar28	*Sphaerolaimus hirsutus* Bastian, 1865	Plym28
Terschellingia longicaudata De Man, 1907	Tamar29	Unidentified Cyatholaimid	Plym29
Theristus acer Bastian, 1865	Tamar30	*Theristus acer* Bastian, 1865	Plym30
Paracanthonchus sp. Micoletzky, 1924	Tamar31	*Metachromadora remanei* Gerlach, 1951	Plym31
Neochromadora sp. Micoletzky, 1924	Tamar32	*Metachromadora remanei* Gerlach, 1951	Plym32
Metachromadora sp. Filipjev, 1918	Tamar33	*Neochromadora* sp. Micoletzky, 1924	Plym33
Cyatholaimus sp. Bastian, 1865	Tamar34	*Sphaerolaimus hirsutus* Bastian, 1865	Plym34
Daptonema normandicum (De Man, 1890)	Tamar35	*Paralinhomoeus* sp. De Man, 1907	Plym35
Daptonema oxycerca (De Man, 1888)	Tamar36	*Sphaerolaimus* sp. Bastian, 1865	Plym36
Terschellingia longicaudata De Man, 1907	Tamar37	*Daptonema hirsutum* (Vitiello, 1967)	Plym37
Metachromadora sp. Filipjev, 1918	Tamar38	*Paralinhomeus* sp. De Man, 1907	Plym38
Praeacanthonchus sp. Micoletzky, 1924	Tamar39	*Terschellingia* sp. De Man, 1888	Plym39
Terschellingia longicaudata De Man, 1907	Tamar40	*Tripyloides* sp. De Mann, 1886	Plym40

heating block and allowed to cool to room temperature before centrifugation for 30 s in a benchtop microcentrifuge (16 000 × *g*). 4 µl of 1 M HCl, 10 µl of 0.5 M Tris-HCl (pH 8.0) and 5 µl of 2% Triton X-100 was added to each tube and the contents mixed briefly and centrifuged for 30 s (16 000 × *g*). Tubes were reheated for 3 min at 99°C and allowed to cool to room temperature. The extract was then used for PCR amplification.

Assembling 18S rRNA sequence database for barcoding evaluation. Two primers, namely MN18F (5′-CGCGAATRGCTCATTACAACAGC-3′) and Nem_18S_R (5′-GGGCGGTATCTGATCGCC-3′) were used to amplify approximately 925 bp of the 18S rRNA gene from 26 marine nematode taxa commonly found in SW England waters. The 18S rRNA sequence database is comprised of 26 taxa representing 4 major orders of the phylum Nematoda, which were tested in this study and subsequently used for identification of unknown nematodes from ecological surveys. To test the molecular barcoding concept in marine nematodes, a small fragment around 100 bp inward from the 5′ end of the 18S rRNA molecule was selected for evaluation. Two primers, MN18F forward and 22R reverse (5′-GCCTGCTGCCTTCCTTGGA-3′), were used to amplify approximately 345 bp PCR fragments from 80 nematodes as part of the survey. The majority of these primers have been used previously in nematode phylogenetics and molecular identification studies (Floyd et al. 2002, 2005, Bhadury 2005).

Routine PCRs were conducted with 5 µl of the extracted DNA, 5 µl 10× buffer with $MgCl_2$, 5 µl of 2 mM deoxyribonucleotide triphosphates (dNTPs), 2 µl of each primer (10 pmol μl^{-1}), 0.5 µl of *Taq* DNA polymerase (5 U μl^{-1}) and water to make a total volume of 50 µl for each sample. For MN18F and Nem_18S_R primers, the thermal cycler parameters were 95°C for 5 min, 37 cycles of 95°C for 1 min, 54°C for 1 min and 72°C for 2 min, and finally one cycle of 2 min at 55°C, 5 min at 72°C followed by a holding temperature of 4°C. For molecular barcoding evaluation, the following

parameters were employed for MN18F and 22R primers: 95°C for 5 min, followed by 37 cycles of 95°C for 30 s, 56°C for 1 min, 72°C for 1 min 30 s and a final extension of 72°C for 5 min and the PCR tubes were cooled at 4°C. In total, 80 ind. from Saltram (Plym estuary) and Saltash (Tamar estuary) were PCR amplified and sequenced as part of an ecological survey towards barcoding evaluation.

Cloning and sequencing of the 18S rRNA gene. PCR fragments from the 26 marine nematode taxa were cloned with pBluescript SK⁻ vector and the pGEM-T Easy vector system (Promega). Plasmid inserts were sequenced in both directions using the T7 and T3 primers for pBluescript SK⁻ and M13F and M13R primers for pGEM-T vector. We sequenced 3 to 4 colonies from each clone to confirm the sequence identity. Sequence traces were checked with Chromas Pro software package (Technelysium) for any ambiguities and/or errors.

Sequence analysis and the reliability of barcode identification. 18S rRNA sequences from 26 taxa generated in this study were aligned in Clustal X (Thompson et al. 1997, Jeanmougin et al. 1998) using the default parameters. Neighbour-joining trees were constructed with Molecular Evolutionary Genetic Analysis (MEGA) v3.0 (Kumar et al. 2004) using gamma-corrected Kimura distance parameters (Blaxter et al. 1998). Additionally, a 345 bp fragment from the 18S rRNA gene representing all the major 26 taxa were subjected to phylogenetic analysis using the parameters mentioned above. For molecular barcoding evaluation, 18S rRNA sequences (345 bp fragment) generated from known nematode taxa in this study, together with selected sequences from the GenBank and EMBL databases [only those whose identification was deemed reliable and published in Meldal (2004) and Cook et al. (2005) were selected; accession numbers AF047888, AY854202, AY854204, AY854209, AY854210, AY854212, AY854224, and AY854225], in addition to 80 sequences from the Tamar and Plym estuaries, were aligned in the Clustal-X program using the default parameters. Neighbour-joining trees were constructed with MEGA v3.0 (Kumar et al. 2004) using gamma-corrected Kimura distance parameters (Blaxter et al. 1998). Bootstrap support values for individual branches were generated using 1000 replicate searches.

RESULTS

18S rRNA sequence from representative marine nematode taxa

Successful amplification and sequencing of the 18S rRNA gene was achieved from 26 nematode taxa from SW England waters, with the majority of taxa possessing unique sequences. A distinct pattern of conserved and variable regions was observed in the 18S rRNA molecule among all these taxa (see Appendix 1, available at www.int-res.com/articles/suppl/m320p001_app.pdf). The partial 5′ end of the 18S rRNA molecule exhibits a mix of conserved and variable regions that were later tested for molecular barcoding. Almost all sequences showed a similarity of 99% and above when compared with the nematode sequences available online in GenBank and EMBL databases. Twenty-six MOTU generated in this study agree with morphological taxon assignment for all the specimens. However, there were discrepancies at the phylogenetic level for some of the taxa based on 18S rRNA sequences (Fig. 1). These discrepancies are beyond the scope of this paper and are not discussed. Amplified taxa along with family/order position and respective GenBank accession number have been detailed in Table 1. Additionally, MOTU patterns generated on a small segment of the 18S rRNA molecule (345 bp fragment) were able to resolve most of the taxa but there were discrepancies for some taxa as mentioned earlier (Fig. 2).

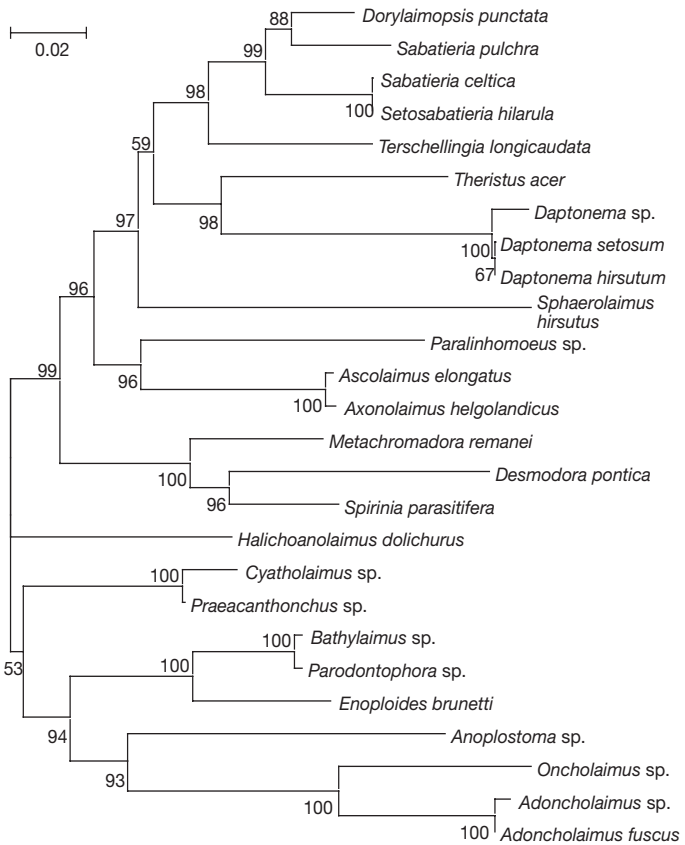

Fig. 1. Phylogenetic resolution (with bootstrap values; 1000 replicates) of representative marine nematode taxa from SW England waters, based on 18S rRNA sequences. Scale bar: 0.02 substitutions per site

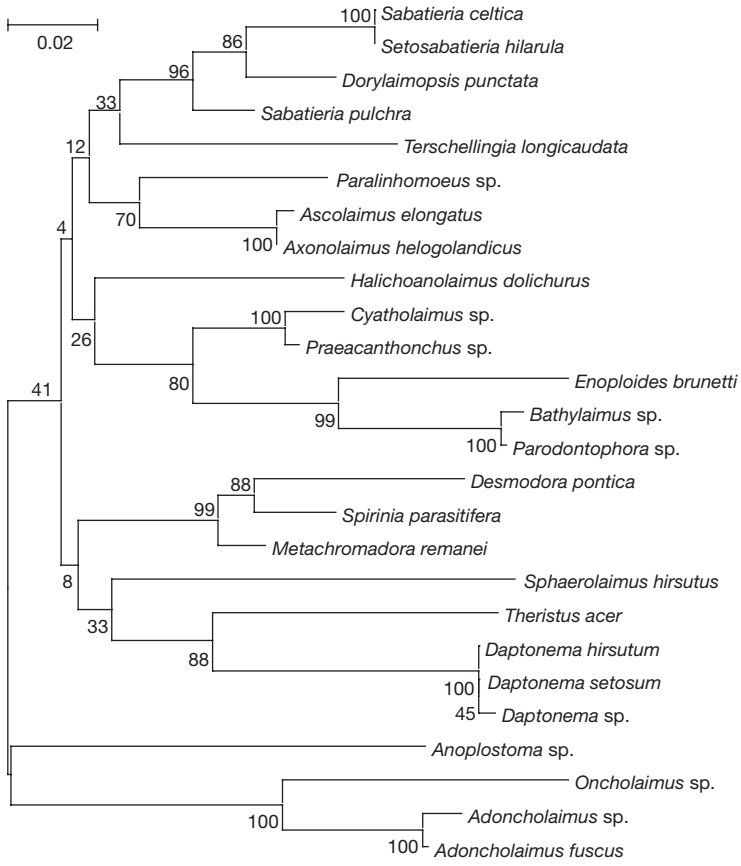

Fig. 2. Phylogenetic resolution (with bootstrap values; 1000 replicates) of representative marine nematode taxa from SW England waters, based on a 345 bp fragment of the 18S rRNA molecule. Scale bar: 0.02 substitutions per site

Molecular barcoding of marine nematodes based on 18S rRNA sequences

Eighty high quality sequences were generated for nematodes from the Tamar and Plym estuaries that had been identified morphologically prior to DNA analyses. The phylogenetic analysis of the 80 sequences along with known marine nematode 18S rRNA sequences (345 bp fragments) showed clear resolution, and the majority of the specimens were resolved to genus and species level in both the trees (Figs. 3 & 4). From the Tamar estuary, only 1 specimen (Tamar 3) was not assignable to species level in the phylogenetic tree. This was placed within the genus *Sabatieria* on the basis of its 18S rRNA sequence, and indeed was identified as *Sabatieria* sp. based on morphological characters prior to molecular analysis. In the Plym estuary, 5 out of 40 specimens were not readily assignable to species level in the tree. Out of these, 2 were assignable to genus level as *Praeacanthonchus* (Plym1), and *Sabatieria* (Plym22), and had been identified as such based on morphological characters prior to molecular analyses. The Plym17 specimen was morphologically identified as *Metachromadora suecica*, and indeed clustered with the *Metachromadora* species included in the tree, despite being relatively divergent, differing by 7 base pairs from *M. remanei*. Plym19 and Plym29 clustered with *Atrochromadora microlaima* and *Dichromadora* sp. in the phylogenetic tree but had been morphologically identified as *Sabatieria* sp. and Cyatholaimid respectively. Sequences generated in this study have been submitted to GenBank and their accession numbers are DQ394725–DQ394804.

DISCUSSION

The main objective of this study was to amplify and sequence the 18S rRNA gene from representative estuarine and marine nematode specimens so as to create DNA sequence profiles which could be used to aid identification of bulk nematode samples. PCR products were recovered from all the individuals and there was no evidence of any complications with the molecular methods. Moreover, the alignment of the sequences and subsequent phylogenetic analysis was straightforward, as indels and polymorphism were uncommon for this gene. 18S rRNA genes were successfully amplified and sequenced from all the taxa tested, and proved to be valuable markers for barcoding studies. Wherever possible, more than one individual from each taxa was sequenced in this study and there was no variation at the intra-specific level between the members of each taxa for 18S rRNA gene. However, the 18S rRNA gene shows high inter-specific variation between taxa as expected. Such patterns have been observed across many metazoan phyla (Abouheif et al. 1998). The mix of conserved and variable regions amongst the 18S rRNA molecule makes it suitable for the design of primers to amplify segments of the gene that are variable amongst different species of nematodes.

Based on PCR amplification and sequencing success rates, the 18S rRNA gene proved to be more consistent as compared to other nuclear and mitochondrial genes. The 18S rRNA gene is generally conserved and has a high rate of amplification success with PCR, and because of this it has received a great deal of attention in recent literature as a barcoding locus (Floyd et al. 2002, Blaxter 2004, Powers 2004). In our study, a region from the 5′ end of the molecule of approximately 345 bp was selected for barcoding studies and evaluation of its potential to assign specimens to genus and

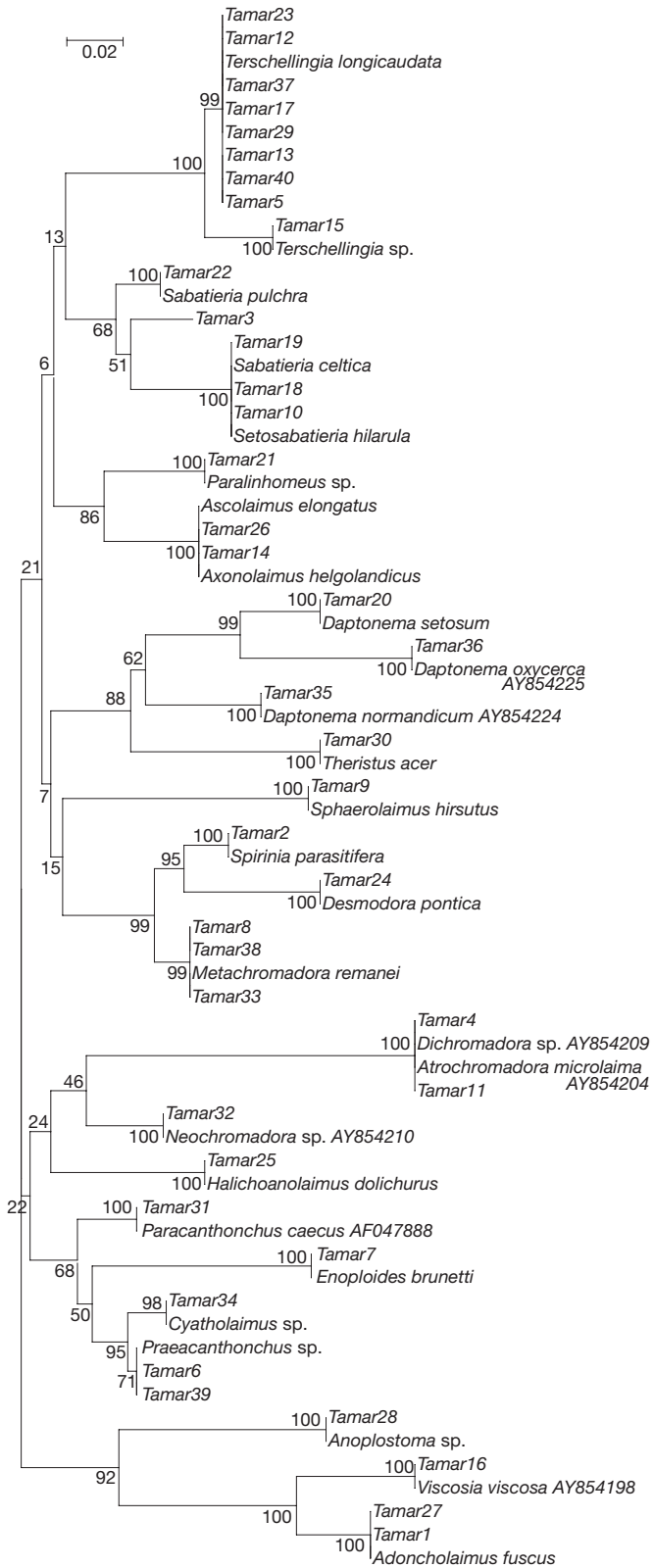

Fig. 3. Neighbour joining tree with bootstrap values (1000 replicates) showing relationship between Tamar estuary nematode 18S rRNA sequences and sequences from known marine nematodes. Scale bar: 0.02 substitutions per site

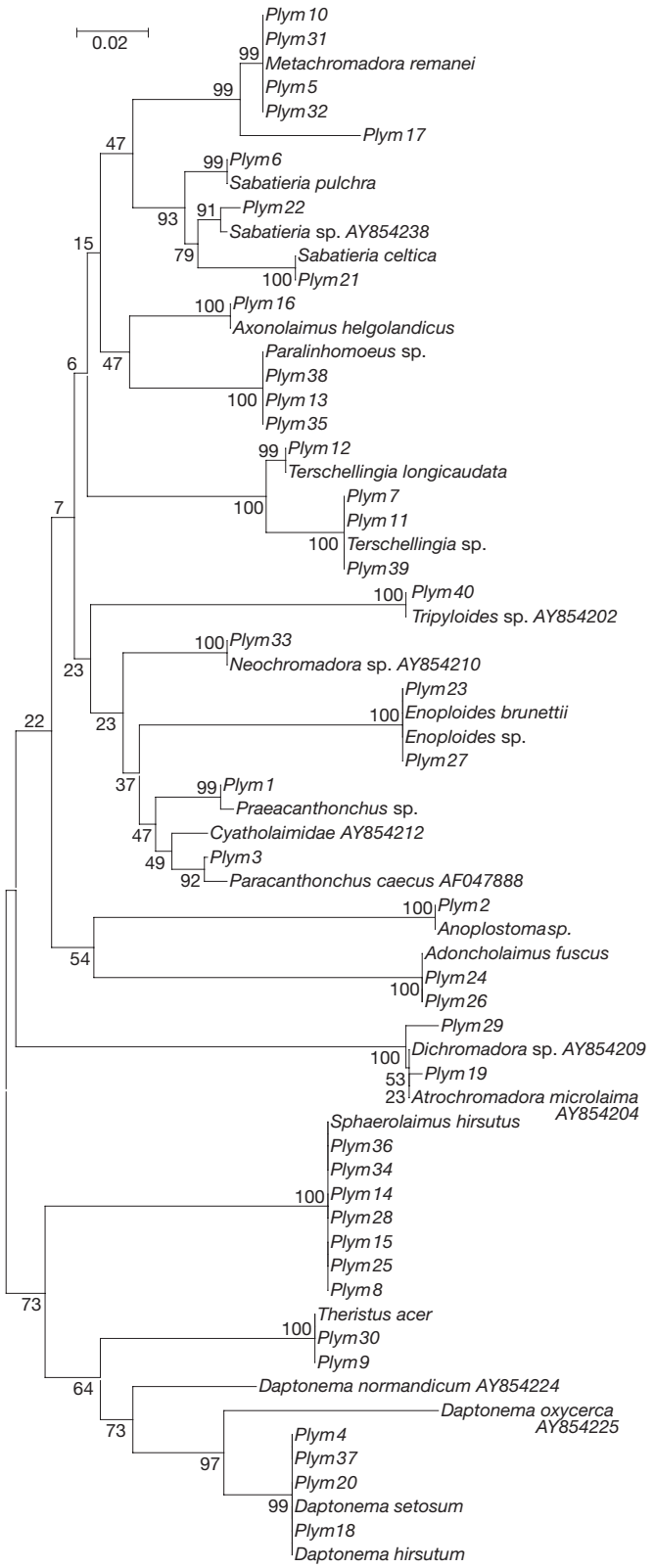

Fig. 4. Neighbour joining tree with bootstrap values (1000 replicates) showing relationship between Plym estuary nematode 18S rRNA sequences and sequences from known marine nematodes. Scale bar: 0.02 substitutions per site

species level. The validity of the technique was evaluated by identifying specimens using traditional taxonomic methods followed by their subsequent randomization, sequencing and inclusion in phylogenetic analysis. MOTU of almost all specimens from the Plym and Tamar estuaries resolved to genus and most of them to species level when compared with representative marine nematode sequences based on phylogenetic analysis. Additionally, the majority of the MOTUs were correctly assigned to genus and species level when compared to nematode sequences held online at GenBank and EMBL.

However there were some exceptions to this (see 'Results; Molecular barcoding of marine nematodes based on 18S rRNA sequences'), most notably for specimens Plym 19 and Plym 29. Misidentification caused as a result of distortion of morphological characters resulting from preservation of nematodes in ethanol or contamination of genomic DNA could have been responsible for the wrong derivation to genus or species level in the tree. Additionally, the possibility of novel cryptic taxa or sequences from previously undescribed species cannot be ruled out. Therefore, amplification and sequencing of other genomic regions for these 2 specimens could provide vital information for subsequent assignment to correct genus and species level.

Based on 18S rRNA amplification and sequencing, 78 MOTU out of 80 specimens were correctly assigned to genus or species level, indicating that the success rate of molecular barcoding using this sequence is close to 98%. At the same time, taxonomic placements of most specimens using molecular data matched those based on morphology, where specimens were identified under the microscope and randomized subsequently to test the barcoding concept. The success rate of the 18S rRNA based DNA barcoding conducted here is consistent with the rate found by Hebert et al. (2003b) for COI across a wide range of Phyla. However, it is slightly lower than that of Hebert et al. (2003a) for nematodes where the success rate was 100% based on COI profiles. However, the number of terrestrial or parasitic nematode species analysed by Hebert et al. (2003b; not published online) was relatively limited (in the context of the broad range of nematode taxa found in the marine environment).

One of the important aspects of the molecular barcoding approach is to carefully consider the cut-off value generated from the bootstrap analysis for accurate genus and species level identification during large-scale ecological surveys. This study is based on a 345 bp 18S rRNA sequence and therefore bootstrap values of 99 and above would ideally correspond to correct genus and species level in a phylogenetic tree. Almost all of the unidentified specimens were correctly assigned to genus and species level when analysed against representative marine nematode taxa and, in the majority of the cases, the bootstrap values were either 99 or 100 (Figs. 3 & 4).

A larger number of 18S rRNA sequences from different marine nematode taxa are required for the barcoding approach to be more accurate and useful. The habitats chosen for this study (mud and muddy sand estuaries) are just one of the habitat types in which a diversity of nematode species are found. An ever-expanding 18S rRNA sequence database will need to be developed to enable and speed up routine identification of nematodes from the full spectrum of marine habitats in which they exist. With the development of high throughput systems and an ever-expanding database of nematode sequences, molecular barcoding approaches may prove to be more time-efficient than traditional microscopy for faunal samples that are, in taxonomic terms, comparatively unknown, or poorly known (e.g. deep-sea samples). Molecular barcoding will be also useful in laboratories where nematode taxonomy expertise does not exist (i.e. throughout most of the world). Routine monitoring will ultimately require the development of mass screening methods such as massively parallel sequencing for speeding up barcoding process (Creer in press). At the same time, traditional taxonomic methods should continue to be used in order to develop keys for new species of marine nematodes so as to generate congruency between the 2 methods (molecular and morphological). This is especially the case for taxa for which amplification of the COI gene is not reliable or for which it does not provide species-level resolution. Traditional approaches may also be enhanced by new methods such as video capture or generation of digital images of nematodes from microscopes (e.g. De Ley & Bert 2002).

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