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Marine nematode taxonomy in the age of DNA: the present and future of molecular tools to assess their biodiversity

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Summary – Molecular taxonomy is one of the most promising yet challenging fields of biology. Molecular markers such as nuclear and mitochondrial genes are being used in a variety of studies surveying marine nematode taxa. Sequences from more than 600 species have been deposited to date in online databases. These barcode sequences are assigned to 150 nominal species from 104 genera. There are 41 species assigned to Enoplea and 109 species to Chromadorea. Morphology-based surveys are greatly limited by processing speed, while barcoding approaches for nematodes are hampered by difficulties in matching sequence data with morphology-based taxonomy. DNA barcoding is a promising approach because some genes contain variable regions that are useful to discriminate species boundaries, discover cryptic species, quantify biodiversity and analyse phylogeny. We advocate a combination of several approaches in studies of molecular taxonomy, DNA barcoding and conventional taxonomy as a necessary step to enhance the knowledge of biodiversity of marine nematodes.

Keywords – barcoding, marine nematodes, molecular markers, molecular taxonomy.

The phylum Nematoda exhibits high species diversity, as well as high abundances in aquatic (marine or freshwater) and terrestrial environments (Floyd *et al.*, 2002). Nematoda in general are one of the most diverse taxa in the animal kingdom, with estimates ranging from 0.1 to 100 million species (Lambshead, 1993; Coomans, 2002). Only a few thousand of these species have been described, although they represent the most abundant component of the meiofauna in several kinds of ecosystems (Lambshead, 2004; Bhadury *et al.*, 2006a).

Species-level identification of most marine nematodes still relies largely on detailed morphological analysis that requires considerable taxonomic expertise, placing it outside the scope of most routine ecological surveys (De Ley *et al.*, 2005; Bhadury *et al.*, 2006a, b). Nematode identification using morphological characters is not only time-consuming but also problematic, mainly because of the high phenotypic plasticity among populations and the absence of clear diagnostic characters for cryptic species (Avise & Walker, 1999; Derycke *et al.*, 2008; Fonseca *et al.*, 2008).

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The advent of molecular techniques has opened up new possibilities for taxonomic research and has provided an important tool, given that the vast majority of species are not well differentiated morphologically (Godfray, 2002; Seberg *et al.*, 2003; Vogler & Monaghan, 2007). These new techniques improve taxonomic precision and assist in critical examinations of the accuracy afforded by morphological traits that are commonly used in traditional taxonomy (Will & Rubinoff, 2004). Indeed, several studies have already illustrated the advances afforded by the interactive process between morphology and DNA 'barcoding' for systematics (α-taxonomy and phylogeny) (Blaxter, 2004; Hebert *et al.*, 2004; Lee, 2004; Hebert & Gregory, 2005).

DNA barcoding appears to be a promising tool to inventory the biodiversity of free-living marine nematodes (Blaxter *et al.*, 2005; Bhadury *et al.*, 2006a; Meldal *et al.*, 2007; Derycke *et al.*, 2008). DNA barcode sequences have proved useful in identifying clades and evolutionary relationships (De Ley & Blaxter, 2002, 2004; Savolainen *et al.*, 2005).

Large-scale application of molecular data is revolutionising taxonomy, but the validity and relevance of molecular approaches and the concepts on which these techniques are based have been subject to a variety of criticisms (Lipscomb *et al.*, 2003; Wheeler, 2004; Rubinoff *et al.*, 2006; Kohler, 2007; Valdecasas *et al.*, 2008).

This survey presents an overview of molecular approaches for marine nematode taxa, dealing with DNA taxonomy, barcoding, and molecular taxonomy. We also discuss the application of molecular markers that are presently used in studies in molecular taxonomy, and the present number of barcoded sequences available in public databases for marine nematodes. We discuss the prospects for future studies with barcoding of marine nematodes, using multiple approaches such as microarrays, new sequencing technology and metagenomics to elucidate relationships among new taxa.

DNA taxonomy and barcoding for marine nematodes

The history of nematode systematics has been marked by controversy, not only as a result of the development of diverse classification systems but also because relatively few nematologists produce detailed classifications (De Ley & Blaxter, 2002). Nematode identification using morphological characters is often difficult and laborious. To make matters worse, there is no generally accepted species concept by which we can define the unit used, and, furthermore, nematode species can be variable in morphology, and differences between valid species can be obscured by the absence of clear diagnostic differences (De Ley *et al.*, 1999; Derycke *et al.*, 2006, 2007). Although nematodes have been studied for over 100 years, objective criteria for assessing the homology of morphological characters used in phylogenetic reconstructions within the phylum are still lacking (Chilton *et al.*, 2003).

By contrast, the concept of DNA-based taxonomy as proposed by Tautz *et al.* (2003) is essentially based on the barcoding approach as its practical component. The basic procedure of this approach consists of a tissue sample, taken from an individual, from which DNA is extracted. This DNA serves as the reference sample from which one or several gene regions are amplified by PCR and sequenced. The resulting sequence will be an identification tag for the species from which the respective individual was derived (Lipscomb *et al.*, 2003; Mallet & Willmott, 2003; Marshall, 2005). The advocates of taxonomy based mainly on DNA sequences claim that the current practice in morphological taxonomy is not adequate to achieve the aim of a reasonably complete inventory of animal life in a reasonable period of time (Stoeckle, 2003).

Proponents of DNA barcoding have argued that we could use DNA sequences of one or more particular genes to identify nematode species, based on the idea that every species has its own 'diagnostic' sequences, *i.e.*, unique sets of base-pair mutations (Hollingsworth, 1998; Kohler, 2007). The three main aims of DNA barcoding are to: *i*) assign unknown specimens to species; *ii*) enhance the discovery of new species and facilitate identification, particularly in cryptic microscopic and other organisms with complex or inaccessible morphology; and *iii*) increase massively the speed of processing larger data sets (Hebert & Gregory, 2005; Frézal & Leblois, 2008).

DNA barcoding promises rapid, accurate identification of species or molecular operational units by focusing on a short standardised segment of the genome (Hajibabaei *et al.*, 2007). At the gene level it provides, in many animal groups, strong species-level resolution, for example, for birds (Hebert *et al.*, 2004), spiders (Barret & Hebert, 2005), fishes (Ward *et al.*, 2005), and lepidopterans (Janzen *et al.*, 2005). Building upon the idea of the Universal Product Code, found in commercial products as 'barcodes' (Brown, 1997), a few nucleotides may well provide an immediate diagnosis for species (Hebert *et al.*, 2003a; DeSalle *et al.*, 2005; Savolainen *et al.*, 2005).

One of the main disadvantages occurs in sampling shortfalls across taxa that lead to 'barcoding gaps' (Moritz & Cicero, 2004). Many taxa are under-represented, and conclusions based on a restricted data set may be biased (Rubinoff, 2006; Rach *et al.*, 2008).

Recently, several authors have discussed the nature of the taxonomic problems, and potential strategies that could be used to accelerate the pace of the discovery and classification of biodiversity, with a balanced response that would maintain the role of morphology in taxonomy (Mallet & Willmott, 2003; Sites & Marshall, 2003). In fact, barcoding seems prone to failure, except in cases with extremely well developed background knowledge of the taxa sampled and barcoded with an *a priori* understanding of sequence variation among populations and individuals (Wilson, 2004).

Molecular markers for taxonomy of marine nematodes

In an effort to standardise the approach to species identification using molecular techniques, it has been proposed that as many species as possible should be characterised for some genetic markers (Sunnucks, 2000; Blaxter, 2004). However, the main difficulty in molecular taxonomy is to find the ideal gene that discriminates a given species in the animal kingdom.

Several molecular markers have been proposed. The nuclear subunit ribosomal RNA gene is a promising candidate because of its great abundance in the genome and its relatively conserved flanking regions that can provide classifications into molecular taxonomic units (MOTU), as has been shown in meiofauna specimens, including nematodes (Floyd *et al.*, 2002; Blaxter *et al.*, 2005).

There is intense selection in the ribosomal DNA genes because of their vital role in the assembly of proteins in the ribosome. As a consequence, these genes – or at least parts of them – are strongly conserved. Among the ribosomal RNA encoding genes, the small subunit (SSU) rDNA is the most conserved (Holterman *et al.*, 2006).

Nuclear genes

The SSU rDNA gene has proved to be very useful for exploring the phylogenetic relationships within, as well as between, many (though not all) groups of nematodes. The semi-conserved areas in the gene allow the unravelling of the deep phylogenetic relationships within the phylum yet, at the same time, the more variable regions in the gene have enabled investigators to distinguish between families or genera, and, in quite a few cases, even between species (Aleshin *et al.*, 1998; Holterman *et al.*, 2008).

Especially among invertebrates, the SSU rDNA is usually present in several copies that code for SSU rRNA. SSU rDNA sequences are known for a broad range of terrestrial nematode fauna, and are sufficiently variable to permit the differentiation of closely related nematode species (Gasser & Newton, 2000; Fontanilla & Wade, 2008). Consequently, SSU rDNA has received the most attention as a barcoding locus in recent literature (Cook *et al.*, 2005; Bhadury *et al.*, 2006a). The locus has higher phylogenetic information content, with small amounts of polymorphism, and often works well for resolving relationships at different levels of classification (*e.g.*, Félix *et al.*, 2000; Rusin *et al.*, 2003; Foucher *et al.*, 2004).

The LSU rDNA gene has been used for almost 10 years as a source of diagnostic sequences in nematodes, particularly the region that spans the D2 and D3 expansion segments (Thomas *et al.*, 1997; Zheng *et al.*, 2003; Tenente *et al.*, 2004; Subbotin *et al.*, 2007; Fonseca *et al.*, 2008; Kumari *et al.*, 2009). In nematodes, this region covers about 600-1000 bp, fairly close to the 5′ end of the gene. In contrast, the conserved regions alternating with D2 and D3 are highly constant, even across phyla, and provide very robust primer sites. According to De Ley *et al.* (2005), the D2/D3 primer pair has the highest success rate when applying PCR amplification to a phylum-wide selection of nematodes, and, based on our limited testing, it also works well in other phyla of microscopic metazoans. The locus is not known to be subject to significant levels of intraspecific polymorphism, and provides very good separation of cryptic species in some groups (De Ley *et al.*, 1999). Previous studies have included phylogenetic applications of D2 or D3 alone (Litvaitis *et al.*, 2000).

The Internal Transcribed Spacer (ITS) region is another versatile genetic marker located in the repeating array of nuclear 18S and 28S ribosomal DNA genes. The ITS has been used in constructing phylogenetic trees, estimating genetic population structures, evaluating population-level evolutionary processes and determining taxonomic identity (Powers *et al.*, 1997). In marine nematodes, ITS showed highly divergent phylogenetic lineages caused by a common evolutionary process in the *Pellioditis marina* species-complex and the genetic structure of *Halomonhystera disjuncta*. This marker has not been considered a good universal identification tool in marine nematodes for two reasons: *i*) intra-individual variation is fre-

quently observed, which reduces the sequencing signal; and *ii*) a large number of indel events are present within closely related cryptic taxa, rendering alignment between divergent taxa problematic (Derycke *et al.*, 2007, 2008) (Table A1; Fig. 1).

Mitochondrial genes

The mitochondrial gene, cytochrome c oxidase subunit 1 (COI), has been proposed as a candidate locus for a 'universal' diagnostic barcode (Lorenz *et al.*, 2005). COI is widely used for barcoding animals. Current barcoding studies have primarily focused on a single mitochondrial marker as a source of identifying diagnostic barcodes (Rach *et al.*, 2008). Mitochondrial genes such as COI could also provide further information on gene-flow patterns and cryptic-level diversity within marine nematodes. However, amplification of this gene in marine nematodes is extremely difficult and unreliable (Bhadury *et al.*, 2006b).

To date, there are no phylum-wide universal primers for the mitochondrial cytochrome oxidase I gene that work across the Nematoda, and PCR success rates are well below 50% for various taxa within the phylum (De Ley *et al.*, 2005). The reasons for these problems may relate to the emerging evidence that nematode mitochondrial genomes are highly diverse, displaying unusual proper-

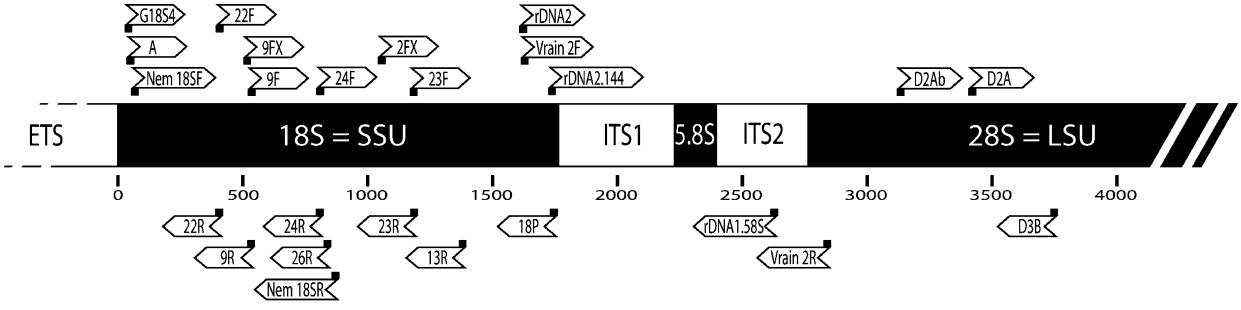

Fig. 1. *Combinations of nuclear primers used in studies of rDNA genes for molecular taxonomy of nematodes.*

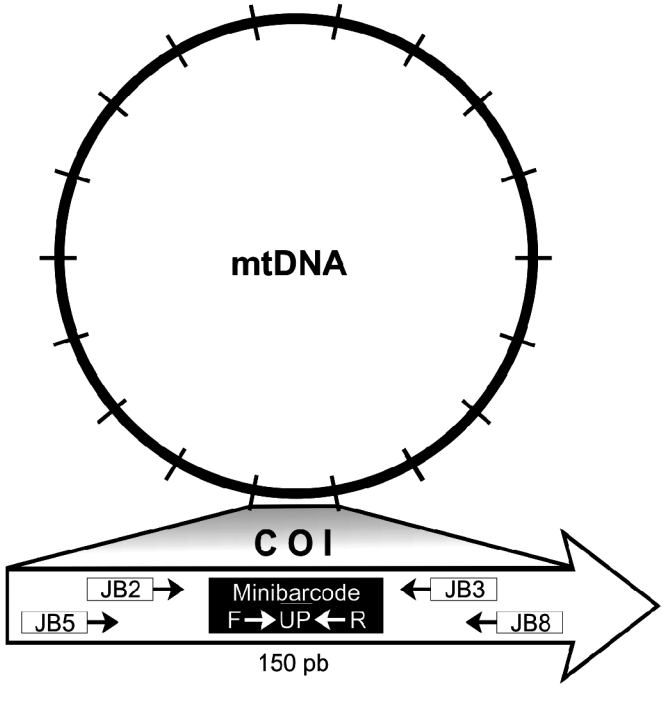

Fig. 2. *Combinations of mitochondrial primers used in studies of molecular taxonomy of nematodes.*

ties such as recombination (Lunt & Hyman, 1997), insertion editing (Vanfleteren & Vierstraete, 1999) and multipartitioning (Armstrong *et al.*, 2000). In addition, mitochondrial genes have higher mutation rates and a fourfold smaller effective size and, consequently, evolve more rapidly than the nuclear genes (Avise, 2000).

Mitochondrial primers are generally used for barcoding studies (Hebert *et al.*, 2003a, b). Derycke *et al.* (2005, 2006, 2007, 2008), using a combination of primers, succeeded in amplifying the COI gene from two nematode species, *P. marina* and *Geomonhystera disjuncta*, and showed cryptic diversity within both taxa (Fig. 2; Table A1). Fonseca *et al.* (2008) also used COI in a survey of integrative taxonomy in two free-living-nematode species complexes. However, it is clear that current primers are not adequate if COI-based DNA barcoding is to work. There is another combination of COI called a minibarcode (short primer for COI segment with 150 bp) that was used to identify the minimum amount of sequence information required for accurate species identification (Meusnier *et al*., 2008). This primer set was tested in marine nematodes and showed high PCR success rates, but the sequences produced showed a lack of phylogenetic signal for discriminating relationships among nematode species (Silva *et al*., unpubl. data) (Fig. 2; Table A1). Thereafter, with the sequencing of new mitochondrial genes, new insights on how to apply other mtDNA can become helpful in studies of molecular taxonomy of marine nematodes.

Available sequenced data of marine nematodes

A total of 600 barcode sequences of marine nematodes have been deposited in the NCBI to date. These barcode sequences were assigned to 150 nominal species from 104 genera. In total, barcoded sequences of 41 species were assigned to Enoplea (Fig. 3) and 109 species to Chromadorea (Fig. 4). Within the latter class, the most sequenced family is Chromadoridae (with 18 species),

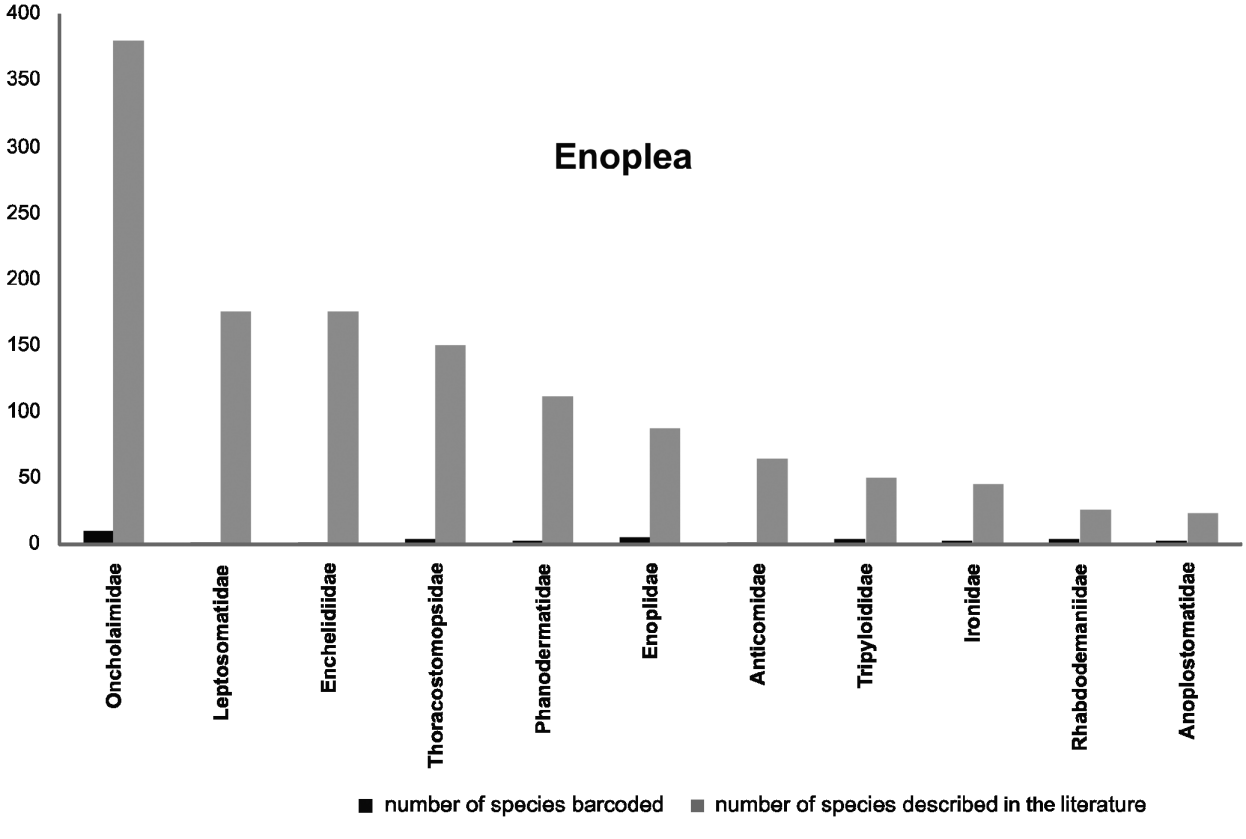

Fig. 3. *Estimated number of nematode species barcoded for SSU/LSU/COI markers in class Enoplea, compared to the number of described nematode species. Source: http://www.nemys.com*

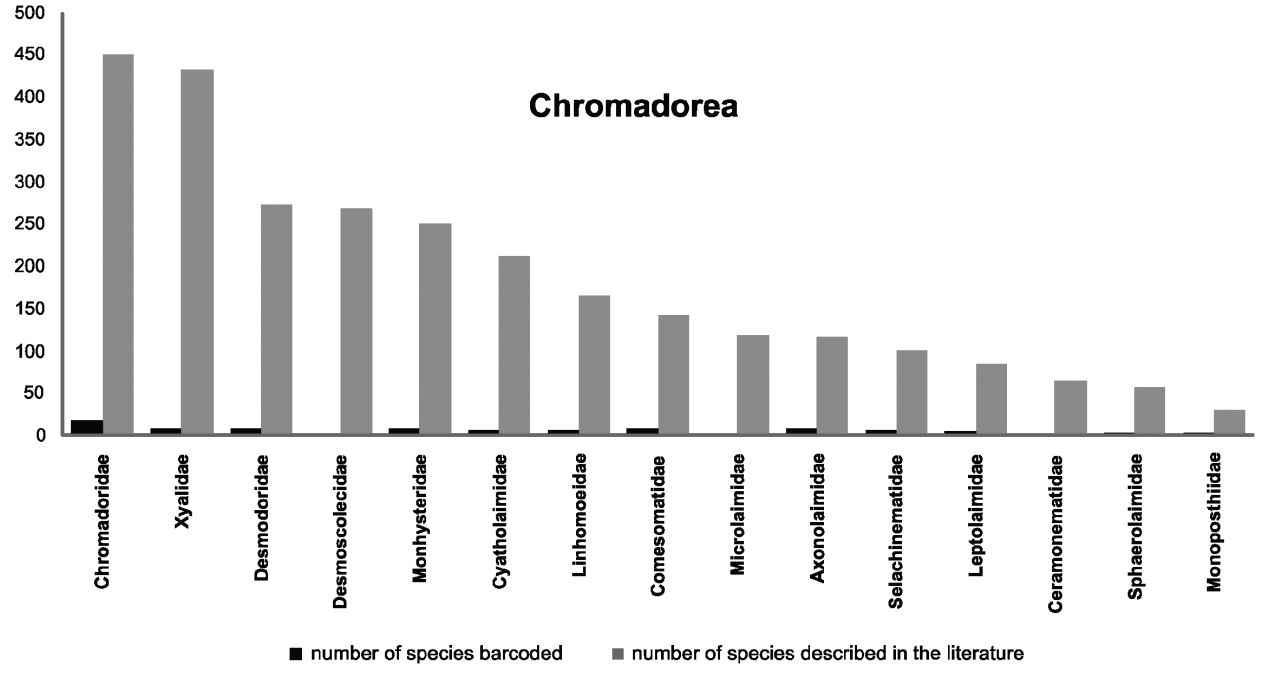

Fig. 4. *Estimated number of nematode species barcoded for SSU/LSU/COI markers in class Chromadorea, compared to the number of described nematode species. Source: http://www.nemys.com*

followed by Desmodoridae, Ethmolaimidae and Xyalidae with nine species each.

According to our analysis, genera of the families Chromadoridae and Oncholaimidae have the largest number of species sequences deposited in GenBank. However, fewer than 20 species are represented, whereas the genus *Oncholaimus* alone has more than 30 valid species described. The number of marine nematode species with barcode sequences in databases is very low compared with the diversity of the phylum. Other nematode groups such as plant parasites are better represented in genetic databases. More sequences are needed in order to increase the number of molecular tags in nematodes. This reinforces the necessity to produce more sequences in studies dealing with free-living taxa in order to increase the number of molecular tags in nematodes. Additional 18S rRNA, COI and 28S sequences from different marine nematode taxa are required in order for the barcoding approach to be more accurate and useful.

Future studies with DNA barcoding in marine nematodes

Two principal elements are proposed in DNA barcoding: *i*) the ability to assign an unknown sample to a known species; and *ii*) the ability to detect previously collected species as distinct. The prospect of assigning an unknown sample to a known one is promising, especially for well known, comprehensively sampled, groups that have been extensively studied by genetic and morphological taxonomy (Meyer & Paulay, 2005).

Nevertheless, it is clear that a comprehensive comparative molecular database is needed against which unknown samples can be compared (Ekrem *et al.*, 2007). The study of marine nematodes has recently focused on molecular tools to describe diversity in ecosystems, and methods of integration of morphological approaches with molecular analyses must be developed in order to cover the diversity of the group.

Several steps must be followed in order to create a DNA-based species identification system. First, we must define a comprehensive barcode sequence library of marine nematodes. Second, we must develop an effective approach for comparing and matching sequences from new specimens to the barcode library (Frézal & Leblois, 2008). Finally, barcoding must be combined with traditional taxonomy in an attempt to integrate several aspects of the biological species concept.

Molecular analysis is making an obvious contribution to taxonomy in helping to discover cryptic species (Lee,

2004). However, it is necessary to know the complete range of diversity of Nematoda in nature in order to understand the possible biological differences between cryptic nematode species, before applying molecular techniques to attempt to solve ecological questions.

Other technologies are available including metagenomics, a method of sequencing DNA from natural samples which thus provides access to a much wider range of genomes to capture the genomic diversity within a natural population (Tringe & Rubin, 2005). The microarray method has been applied in some parasitic nematodes, and is a promising alternative for high-throughput genotype-based diagnostics combining powerful DNA amplification strategies with subsequent hybridisation to develop oligonucleotide probes specific for multiple target sequences, allowing parallel study of the expression of thousands of genes (Schulze & Downward, 2001; Butte, 2002). This technology has been used to generate whole-genome characterisations of aging, wild-type and long-lived individuals of the model organism *Caenorhabditis elegans* (Golden & Melov, 2004).

Conclusions

Marine environments present challenges in assessing the biodiversity of nematodes, challenges that impose limitations using morphology-based species identification and which result in a gross underestimate of the number of species in these habitats; moreover, free-living marine species are poorly represented in public sequence databases. Molecular analyses of a much greater diversity of nematode species are urgently needed to improve the representation of molecular and phylogenetic diversity within this wide-ranging group of organisms.

Further evaluation is needed to select different markers for molecular studies, since a highly conserved gene such as the 18S rRNA may also vary somewhat within some marine nematode populations (Floyd *et al.*, 2005; Bhadury *et al.*, 2008). Hyperdiverse samples of marine nematodes are especially difficult because they may contain very few individuals of each of the many species in the samples, leaving little or no room for assessing whether slightly divergent sequences from similar individuals might represent cryptic species *vs* being attributable to intraspecific sequence variation. Nowadays, the analysis of specimens using the combination of molecular techniques and morphological approach is helping to solve some of these problems or to support existing species complex already described (Fonseca *et al.*, 2008).

Methods for rapid sequence acquisition are already in use at genome sequencing centres and are easily adapted for taxonomic sampling (Blaxter, 2004; Bhadury *et al.*, 2006a, b, 2008). At the same time, morphology-based taxonomic methods should continue to be used in order to develop identification keys for new species of marine nematodes. De Ley and Bert (2002) developed a technique based on video capture editing (VCE), which produces a number of multifocal vouchers of barcoded nematode species. Voucher specimens can be deposited in an open access website database supporting the nematode branch of the tree of life (see: http://nematol.unh.edu). Hence, it is important that studies with model organisms on integrative taxonomy carefully examine the limitations of each strategy in order to choose the best one for future identifications.

Traditional marine nematode taxonomy, based on the analysis of observable morphological characters, may be insufficient if we are to understand fully the species-level biodiversity of this meiobenthic group (Bhadury *et al.*, 2008). An expanding nuclear and mitochondrial sequence database for nematode species will need to be developed to facilitate routine identification of nematodes. The development of high-throughput systems may prove to be more time efficient than traditional microscopy for faunal samples. However, considering a combination of those procedures, it seems that molecular data – based on two or more markers – as used to code the morphological dataset for multivariate analysis, and, ultimately, for pinpointing morphological diagnostic characters, may prove to be very effective (Fonseca *et al.*, 2008), although success in some specific cases is very different from applicability on the scale of ecological surveys. Various novel sequencing technologies are being developed, each aspiring to reduce costs with the aim of producing more sequences of nematode species to be deposited in public databases.

Although integrative taxonomy requires substantial expertise and time, the method is, at present, one of the best ways accurately to delimit species in taxa with unknown biodiversity (Will *et al.*, 2005). Hence, taxonomic revisions are urgently required in the phylum Nematoda in order to understand the group's diversity and to make a compilation of taxonomic descriptions of nematode species.

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Appendix A

Table A1. *Molecular markers used in studies with marine nematodes.*

Primer	Position of amplicons*	Sequence (5′ → 3′)	Source
Nuclear genes			
18S – SSU			
SSUG18S	30-49	GCT TGT CTC AAA GAT TAA GCC	Blaxter *et al.* (1998) Meldal *et al.* (2007) Bhadury *et al.* (2005) Bert *et al.* (2008)
SSUA	39-57	AAAGATTAAGCCATGCATG	Dorris *et al.* (2002)
SSU22R	429-411	GCC TGC TGC CTT CCT TGG A	Blaxter *et al.* (1998) Meldal *et al.* (2007) Bhadury *et al.* (2007) Bert *et al.* (2008)
SSU22F	411-428	TCC AAG GAA GGC AGC AGG C	Blaxter *et al.* (1998)
SSU9FX	530-550	AAG TCT GGT GCC AGC AGC CGC	Meldal *et al.* (2007) Bert *el al* (2008)
SSU9F	573-591	CGCGGTAATTCCAGCTCCA	Blaxter *et al.* (1998)
SSU9R	584-565	AGC TGG AAT TAC CGC GGC TG	Blaxter *et al.* (1998) Bert *et al.* (2008)
SSU24F	868-887	AGR GGT GAA ATY CGT GGA CC	Blaxter *et al.* (1998) Meldal *et al.* (2007)
SSU 24R	885-868	CCCCRRTCCAAGAATTTCACCTC	Meldal *et al.* (2007)
SSU26R	927-907	CATTCTTGGCAAATGCTTTGC	Blaxter *et al.* (1998)
SSU23F	1280-1298	ATT CCG ATA ACG AGC GAG A	Blaxter *et al.* (1998) Bert *el al.* (2008)

Table A1. *(Continued).*

Primer	Position of amplicons*	Sequence (5′ → 3′)	Source
SSU23R	1298-1280	TCT CGC TCG TTA TCG GAA T	Blaxter *et al.* (1998) Bert *et al.* (2008)
SSU13R	1438-1419	GGG CAT CAC AGA CCT GTT A	Blaxter *et al.* (1998)
SSU18P	3′ end	TGA TCC WMC RGC AGG TTC AC	Blaxter *et al.* (1998) Bert *et al.* (2008)
SSU2FX	1108-1129	GGA AGG GCA CCA CCA GGA GTG G	Meldal *et al.* (2007)
SSUDR	1213-1194	CATAAAAGTCTCGCTCGTTA	Dorris *et al.* (2002)
NM18F	345-925	CGCGAATRGCTCATTACAACAGC	Bhadury *et al.* (2008) Bhadury *et al.* (2006) Bhadury *et al*. (2007)
Nem 18SF		CGCGAATRGCTCATTACAACAGC	Floyd *et al.* (2005)
Nem 18SR	998-1015	GGGCGGTATCTGATCGCC	Bhadury *et al.* (2008) Bhadury *et al.* (2006a, b) Floyd *et al.* (2005)
ITS			
RDNA2	2523-2503	TTG ATT ACG TCC CTG CCC TTT	Powers *et al.* (1997)
rDNA1.58S	–	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)
VRAIN 2F	900	CTTTGTACACACCGCCCGTCGCT	Derycke *et al.* (2005) Derycke *et al.* (2008)
VRAIN 2R	900	TTTCACTCGCCGTTACTAAGGGAATC	Derycke *et al.* (2005) Derycke *et al.* (2008)
28S – LSU			
LSU rDNA-D2A	397	TTCGACCCGTCTTGAAACACG	Fonseca *et al.* (2008) De Ley *et al*. (2005) Derycke *et al.* (2008)
LSU rDNA-D3B	397	TCGGAAGGAACCAGCTACTA	Fonseca *et al.* 2008 De Ley *et al.* (2005) Derycke *et al.* (2008)
Mitochondrial DNA			
COI (cytochrome oxidase 1)			
JB2	–	ATGTTTTGATTTTACCWGCWTTYGGTGT	Derycke *et al.* (2005) Derycke *et al.* (2006) Derycke *et al.* (2007)
JB3	426	TTTTTTGGGCATCCTGAGGTTTAT	Derycke *et al.* (2005) Derycke *et al.* (2006) Derycke *et al.* (2007)
JB5	426	TAAAGAAGAACATAATGAAAATG	Derycke *et al.* (2007)
JB5GED	422	AGCACCTAAACTTAAAACATARTGRAARTG	Derycke *et al.* (2007)
JB8	363	CCCCTCTAGTCTWCTATTTCTTAATAC	Derycke *et al.* (2007)
Uncoming genes			
Minibarcode-COI			
Minibar-R1	–	GAAAATCATAATGAAGGCATGAGC	Meusnier *et al.* (2008)
Minibar-F1	–	TCCACTAATCACAARGATATTGGTAC	Meusnier *et al.* (2008)

* Position of amplicon in relation to *Caenorhabditis elegans*.