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Comparative Analysis of Mitochondrial COI and 18S rRNA Genes in the Identification of Marine Nematodes

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Abstract. Free-living nematodes are the most abundant metazoan organisms in marine sediments. They are important in many ecological processes and are used as bio-indicators. Yet, they remain the least described taxon, because morphological diagnostic features are difficult to observe due to their small body size. DNA barcoding may overcome the problems associated with morphology and may lead to a quicker identification of marine nematodes. In this study, the amplification and sequencing success of both gene fragments were compared in a wide range of marine nematodes. 73 species belonging to 56 genera were sampled from Paulina Polder (the Netherlands) and Belgium North Sea. Our results demonstrate that 18S is easily amplified in marine nematodes compared to COI (57% vs 43% amplification success). Sequencing success was higher for 18S rRNA genes than for COI (61% vs 39%) genes. Neighbor joining analysis using the K2P-model showed that both genetic markers cluster into well-defined clades congruent with known taxonomic families and orders that have been delineated based on morphology. Pairwise genetic distance for the 18S sequences showed that ≈74% of intraspecific comparisons showed a genetic divergence ≤3% while about 77% of interspecific comparisons were above 3%. For COI sequences, 98% of intraspecific comparisons showed a genetic divergence of ≤8% and >8% was observed for about 94% of all interspecific comparisons. This study establishes the fact that although, the 18S rDNA may prove invaluable in the identification of marine nematodes, COI gene may be a better choice. However this gene is hampered by low amplification and sequencing rates.

Keywords: DNA Barcoding . COI . 18rDNA . Molecular Identification. Marine nematodes.

INTRODUCTION

Nematodes have been reported to be the most abundant metazoans on the surface of the earth with about 10^8 individuals distributed within a square meter (Decraemer & Hunt, 2006). In marine sediments alone, about 10^5 -10^7 free-living nematodes have been reported to exist in every square meter (Lambshead, 2004). Yet, the number of described nematode species is estimated at only 27,500 species (Hallan, 2007) out of the over one million species predicted by Lambshead (2004). It is a well-known fact that marine nematodes play an important role in many ecosystems. Their roles in bioturbation, nutrient decomposition and nutrient mineralization as well as in food web

interactions have been well reported (Moens and Vincx, 1997; Lawton *et al.*, 1998). Their sensitivity to changes in the environment caused by pollution has been exploited in their use as bio-indicators in the environment (Austen and McEvoy, 1997; Boyd *et al.*, 2000). Despite their important roles, discriminating species remains a difficult task that requires great taxonomic expertise, leaving a greater part of knowledge on nematode species diversity in a state of enigma. According to Hammond (1992), it has been estimated that since only few taxonomists can identify more than 0.01% of the estimated 10-15million nematode species, about 15,000 taxonomists will be required to identify life if our reliance on morphological diagnosis is to be sustained.

While identification based on morphological diagnosis is possible given the availability of an in-depth taxonomic skill and high throughput equipment, it is often saddled by certain limitations. First, there is a high chance of species misidentification due to both phenotypic plasticity as well as genetic variability (Hebert *et al.*,2003a). Second, certain life stages or sex may be needed before a complete identification is possible in some cases; hence in the absence of such a criterion, identification is either impossible or based on guess work (Knowlton, 1993; Jarman and Elliott, 2000). Third, the identification of cryptic species i.e. two or more distinct species that were classified as the same due to morphological similarity (Bickford *et al.*, 2007).

DNA Barcoding, has over the years since its discovery in 2003 continued to unfold the indecipherable taxonomic bottlenecks associated with identification of nematodes in marine sediments as well as in those associated with plants and other large metazoans (Floyd *et al.*, 2002; De Ley *et al*., 2005; Bhadury *et al*., 2006a, b;Holterman *et al.*, 2008; Derycke *et al.*, 2010a). Although various segments of the genome have been used as molecular identification markers in taxonomic and ecological studies, cytochrome *c* oxidase subunit I gene (*cox*-1 = COI) of the mitochondrial DNA (mtDNA) was proposed as the standard barcode for identifying many animals (Hebert *et al*., 2003a). This is chiefly due to two reasons; the availability of robust universal primers that can amplify the COI gene in a wide range of animals (Folmer *et al.*, 1994; Zhang and Hewitt, 1997). It has been shown that COI can provide species identification with more than 90% resolution (Hajibabaei *et al.*, 2006a; Hajibabaei *et al.*, 2006b; Hajibabaei *et al.*, 2007; Meusnier *et al.*, 2008). Hence, effort towards expanding the standard sequence reference database of COI continues to increase (Marshall, 2005; Silva *et al.*, 2010). Barcoding studies in nematodes have traditionally used a fragment of the ribosomal 18S gene, however, its resolution is may not be sufficient for species-level identification especially when closely related species are involved (Derycke *et al.*, 2010b). It is against this background that this study was conducted to compare the reliability and performance of the two barcode genes – 18S and COI in species identification for marine nematodes through the use of sequence alignment, calculation of the number of differences between species and constructing phylogenetic trees.

MATERIALS AND METHODS

Taxon Sampling

Sediments were collected from six stations in Paulina Polder which is located in the polyhaline zone of The Westerschelde estuary (The Netherlands) during low tide in September, 2011. The stations included: Canal Sediment (51° 20.913‟

N, 3° 43.354‟E), Before Experimental Plot Sediment (51° 21.0171‟ N, 3° 43.496‟E), After Experimental Plot Sediment (51° 20.984‟ N, 3° 43.484‟E), Silt Sediment (51° 21.149‟N, 3° 43,911‟E), Biofilm-based Sediment (51° 21.068‟ N, 3° 43.854‟E) and Sea Grass Plot Sediment (51° 20.998‟ N, 3° 43.897‟E). Three replicates of the top 5 cm were collected for all sampled stations. All samples were immediately fixed in a compound of 20% DMSO (Dimethyl sulfoxide), 0.25 M disodium EDTA (Ethylenediaminetetraacetic acid) and saturated with NaCl (Sodium chloride) known as DESS (Yoder *et al,*. 2006). Samples from the subtidal zone of the Belgium North Sea collected from a previous study, was further added to increase the chances of obtaining more species for this study.

Nematode Extraction and Sample Preservation

Each sediment sample was washed twice with tap water after removing DESS using a doubly-arranged set of sieves (1mm and 38µm respectively) until the water passing through the set of sieves became clear. The content of the 1mm sieve was properly rinsed until only fractions larger than 1mm in width were left. Ludox™ (specific gravity 1.18) was used to saturate the washed sample in the 38 µm sieve and left for 3 minutes. The Ludox-added sample was then distributed into tubes and centrifuged after been calibrated with a weight balance. Centrifugation was maintained at 3000 rpm for 12 minutes. Supernatants from all the tubes were poured into the 38µm sieve, then into a clean labeled empty sample pot. The residues were twice subjected to the same procedure each time pouring the supernatant into the sample pot but after the third centrifugation, all collected supernatants were poured into the 38µm sieve and rinsed with tap water to get rid of the ludox. The content in the 38µm sieve was then immersed in DESS and poured into the rinsed labelled sample pot.

Morphological Identification and Vouchering of Specimens

Adult nematodes, especially males were picked-out from each sample pot one after the other under a dissecting microscope, rinsed off with distilled water and each individual specimen was mounted on a temporary slide and identified under a LEICA DMR research microscope. Identification, were possible was made to species level based on morphological dissimilarities and morphometric measurements using Nematotheek (A Collection of Publications on Marine Nematodes Descriptions) and identification guides (Warwick & Platt: Part I, II and III). Diagnostic features of the specimen were digitally captured using LEICA Application Suit. The vouchered specimen was then transferred into an Eppendorf tube containing 20 µL worm lysis buffer (50 Mm KCl, 10 mTris Ph 8.3, 2.5 mM MgCl2, 0.45% NP40, 0.45% Tween 20) and stored at -20°C.

DNA Extraction and Amplification of the COI and 18S regions

To extract genomic DNA of each specimen, proteinase K (1µl of 10g/ml) was added into each Eppendorf tube containing a single nematode in worm lysis buffer. This was followed by incubation at 65°C for one hour and the proteinase K was denatured at 95°C for 10minutes. Finally, the extracted DNA was centrifuged for one minute at 20°C and stored at 4°C. PCR-Mix was prepared for each primer set separately in total volumes of 25 µL containing 15.13 µL distilled water, 2.5 µL of 10x PCR buffer, 2.5 µL dye, 2 µL MgCl2, 0.5 µL dNTP, 0.25 µL of each primer

(25 nM), 0.125µL TopTaq polymerase (Qiagen) and 2 µL DNA. Three test samples were randomly chosen each time to test the amplification success of the primers based on the eight different primer sets used and PCR cycling conditions optimization (Table 1). To amplify CO1, JB3-JB5 primer set was chosen based on its amplification success under pre-set optimized condition of the PCR cycler. The conditions were: initial denaturation of 5 minutes at 94°C, 35 cycles of (94°C for 30s; 50°C for 30s; 72°C for 30s) and a final extension of 10 minutes at 72°C. To amplify 18S, G18S4-4R primer set was used under optimized PCR cycler conditions: initial denaturation of 5 minutes at 94°C, 45 cycles of (94°C for 30s; 54°C for 30s; 72°C for 1 min) and a final extension of 10 minutes at 72°C. PCR products were loaded on 1% agarose gels containing 0.003% ethidium bromide and visualized using BioDoct-ItTM Imaging System (UVP). Each gel contained one lane with 4 µL of DNA Mass Ladder (Invitrogen), one lane which contained 4µL of positive control (PCR product containing 2µL DNA of *Litoditis marina*), one lane which contained 4 µL of negative control (PCR product without DNA) while all other lanes contained 4µL PCR products of the specimens under scrutiny. Amplifications were considered successful when a band of the expected size was observed on agarose gel and when aspecific bands were absent. Primers used for amplification and sequencing are denoted by double asterisks while those used for only for sequencing are denoted by a single asterisk. Those without asterisks were only used to test amplification success.

Sequencing

Five (5) µL of PCR products which gave reliable bands and void of aspecific bands were added into 96-well plates containing 5 µL of primers: G18S4 and 4R were used as forward and reverse primers respectively to sequence the 18S gene while JB3 and JB5 were used to sequence the CO1 gene (Table 1). Sequencing plates were then sent to Macrogen for sequencing. To investigate the biological origin of the sequences as being nematode sequences, all sequences were first subjected to a Basic Local Alignment Search Tool search using BLASTn for 18S sequences and BLASTx for the CO1 sequences. The reliability of sequences was then checked using BioEdit sequence alignment editor (Hall, 1999) and EditSeq v. 7.1. The obtained chromatogram for each sequence was used as a yardstick in the selection of usable and non-usable sequences. Chromatograms with clearly defined nucleotide peaks and high fluorescent signals were considered usable while those with multiple peaks and low signals were considered non-usable. Forward and reverse sequences of COI and 18S were edited, assembled and merged into consensus sequences using SeqMan ProTM software (Lasergene v 8.0.3, DNASTAR®). All COI sequences were translated using the invertebrate mtDNA genetic code in SeaView v. 4 (Gouy *et al.*, 2010). 18S sequences were not translated since the 18S region is non-coding. Prior to alignment, sequences were manually trimmed to remove all ambiguous characters such as gaps and unresolved base calling errors.

Table 1: List of Primer sequences used in this study

Primer	Sequence	Reference
JB3 (F)**	5‟ TTT TTT GGG CCT GAG GTT TAT 3‟	Bowles *et al.,* 1992
JB5 (R)**	5‟ AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG 3‟	Bowles *et al.,* 1992
JB2 (F)	5‟ ATG TTT TGA TTT TAC CWG CWT TYG GTG T 3‟	Derycke *et al.,* 2007
JB5GED (R)	5‟ AGC ACC TAA ACT TAA AAC ATA RTG RAA RTG 3‟	Derycke *et al.,* 2007
G18S4**	5‟ GCT TGT CTC AAA GAT TAA GCC 3‟	Blaxter *et al.* 1998
4R**	5‟ GTA TCT GAT CGC CKT CGA WC 3‟	Blaxter *et al.* 1998
F04 (F)	5‟ AGAGGT GAAATTCTTGGATC 3‟	Blaxter *et al.* 1998
22R (R)	5‟ GCC TGC TGC CTT CCT TGG A 3‟	Blaxter *et al.* 1998
9R*	5‟ AGC TGG AAT TAC CGC CGC TG 3‟	Blaxter *et al.* 1998
G10 (F)	5‟TCW ACW AAT CAT AAA GAT ATT GG 3‟	LCOmod, *S. Derycke*
G11 (R)	5‟ ACT TCS GGR TGA CCA AAA ATCA 3‟	HCOmod, *S.Derycke*
18P (R)	5‟ TGA TCC WMC RGC AGG TTC AC 3‟	Blaxter *et al.* 1998

Data Analysis

Amplification and Sequencing success

Amplification success of each primer-set was calculated by dividing the number of successfully generated amplicons (those which gave reliable bands) by the total number of specimens ran over agarose gel for the specific primer-set. Sequencing success for all primers used to sequence the genes under study were calculated by dividing the sum of successfully sequenced reactions of the forward and reverse primers by the total sum specimens sequenced. To obtain sequencing success for each fragment, both forward and reverse percentages for individual primer-set were added. For intra- and inter-species-level analysis, nucleotide-sequence divergences were computed using the Kimura‟s 2 Parameter (K2P) substitution model in MEGA v.5.0. Intra- and interspecific genetic distances were calculated using all COI and 18S sequences.

Sequence Alignment and Phylogenetic analysis

The CO1 and 18S rRNA sequences were separately aligned using Muscle (Edgar, 2004) as implemented in SeaView v. 4. Subsequently, both ends in all sequences containing the forward and reverse primers were trimmed off. While it is true that this process may

lead to the loss of some phylogenetically informative segments of the sequences, it also eradicates the inclusion of questionable characters that may lead to wrong phylogenetic inference (Floyd *et al.,* 2002). The aligned sequences were subsequently used to calculate pairwise distances using the K2P-model in MEGA v. 5. (Tamura *et al.,* 2011). Finally, neighbor joining (NJ) trees for both genes under study were constructed in MEGA v. 5 using the K2P-model because it is the most widely used model for DNA barcoding and in the inference of barcoding gaps (Hebert *et* al., 2003a). Node support was evaluated with 1000 bootstrap pseudoreplicates.

RESULTS

Taxon Sampling

A total of 170 specimens were screened and vouchered yielding 73 species belonging to 56 genera of marine taxa. About 33 bidirectional sequences were obtained for COI analysis from this study. In addition 60 COI sequences from previous studies were added to broaden the scope of phylogenetic analysis. Fifty-seven (57) bidirectional sequences were obtained for the 18S rRNA gene.

Amplification and Sequencing success of 18S rRNA and COI

The G18S4-4R primer set outperformed JB3-JB5 primer set in terms of number of specimens 18S gene amplified. Among the 170 specimens tested, 57% of the specimens were successfully amplified by G18S4-4R, although aspecific bands were produced in most lanes in the agarose gels due to primer dimers. JB3-JB5 had 43% amplification of the COI region without the production of aspecific products. Aspecific bands were produced when G18S4-4R primer set was used to amplify the 18S region in *Paroxystomina micoletzkyi, Parodontophora cobbi, Viscosia abyssorum, Theristus acer, Eleutherolaimus, Ptycholaimellus pandispiculatus, Odontophora* sp3*, Chromadorina,* sp1 *Antomicron* sp1 and *Chromadorita* sp1. However, distinct bands were produced when JB3-JB5 primer pairs were used to amplify the COI region of the same species of nematode. Sequencing success was calculated for the four separate primers used in the study: JB3(F), JB5(R), G18S4(F) and 4R(R). JB5 outperformed JB3 (46.22% vs 42.62%) in terms of sequencing success of the COI-amplified specimens. JB3 also produced more ambiguous chromatograms than the reverse primer. On the contrary, for the sequencing of 18S, G18S4 outperformed 4R (76.70% vs 68.46%) of the amplified specimens. Ambiguous chromatograms were more in 4R sequences than in sequences obtain using G18S4. Sequencing success of the COI fragment was clearly higher than the 18S- amplified genes (61% vs 39%).

Assembled COI sequences were subsequently compared with sequences in GenBank database to check if they originated from the phylum nematoda. Most of the hits reported hereafter had coverage of 99 or 100% and maximum identity higher than 85%. About 65.59% of all COI sequences blasted in GenBank matched with the exact nematode species sequences in the database while 34.41% of the sequence did not show resemblance to the exact nematode sequences. However, some of the sequences that did not match still showed that they are of nematode origin (Table 2) as revealed by the Taxonomy Section of BLASTx. The sequences of *Ascolaimus* sp1, *Bolboliamus* sp1, *Microlaimus* sp1, *Sabatiera* sp1 and *Enoplolaimus attenautes* showed similarity with organisms from four different phylums: *Chattonella marina* (100%): *Onchrophyta, Cryptosporidum*

parva (99%): Apicomplexa, *Colwellia psychrerythraea* (100%): Proteobacteria-gamma, *Megischus bicolor* (100): Arthropoda and *Thelyphonidae* sp (88%): Arthropoda respectively.

Molecular Phylogenetic Analysis of COI sequences

Nucleotide insertions were observed in the COI sequence alignment of *Daptonema setosum*_JB5, *Praeacanthonchus* sp1, *P. punctulatus*, *Praeacanthonchus* sp_sofsam and *Sphaerolaimus penicillus* while some nucleotide single deletions were observed in the nineteenth position in *Microlaimus sp1*, *M. punctulatus*, *Microlaimus* sp1_P and *Bolbolaimus sp.* There appeared to be a pattern of occurrence among species of *Praeacanthonchus*: Insertions with about nine amino acid residues were observed. In the case Sphaerolaimus-derived sequences, only *Sphaerolaimus penicillus* showed a seven-amino acid long insertion on the 84th position, no insertion was observed in the others. Based on the COI sequences, eighty sequences used in constructing the NJ tree showed congruence with those of known morphologically-derived families and orders of marine nematodes. However there were discrepancies at the phylogenetic level for species positioned at the basal "pseudoclade" of the NJ tree (Figure 1). *Theristus acer*_HL, *Daptonema setosum*_JB5 and *Sphaerolaimus hirsutus*_NX1 formed the "pseudoclade" since there was no basis for the clustering of their sequence. Another group of species also showed incongruent pattern of phylogenetic positioning within the tree: *Microlaimus* sp1_P, *M. punctulatus*_KM, *Metachromadora remanei*, *Theristus* sp1_TZ, *Theristus* sp1, *Araeolaimus* sp1, *Enoplus* sp1, *Bathylaimus australis* and *Sphaerolaimus penicillus*. This incongruence likely confirms the BLASTx similarity search as shown in Table 2 since the sequences of all these nine species are of a different origin. Although discrepancies exist in the position of a few species within the NJ tree, most of the branches formed eight well supported clades with bootstrap supports ranging from 93% to 100%. Low bootstrap support was however observed in the clades of Enoploidea (40%), Chromadorina (56%) and Monhysteridae. Pairwise genetic distance using K2P substitution model for the COI sequences showed that about 98% of intraspecific comparisons showed a genetic divergence ≤8% while about 94% of interspecific comparisons were above 8%.

Molecular Phylogenetic Analysis of 18S sequences

The multiple alignment of all 18S sequences showed that nucleotide insertions were noticed in *Monoposthia mirabilis*_NQ, *Bathylaimus* australis, *Bathylaimus* denticaudatus and *Anoplostoma viviparum.* Deletions were observed in the 20th position in *Monoposthia mirabilis*_NQ, on the 179th, 82nd and 67th positions in *Bathylaimus* australis, *Bathylaimus denticaudatus* and *Anoplostoma viviparum* respectively. No deletion was observed in all sequences except in *Anoplostoma viviparum*. Based on the aligned 18S sequences, 57 sequences used in constructing the NJ tree showed congruence with those of known morphologically-derived families and orders of marine nematodes. However there were discrepancies at the phylogenetic level for species positioned at the basal "pseudoclade" of the NJ tree (Figure 2). *Monoposthia mirabilis*_NQ, *Microlaimus honestus*_Y3 and *Hypodontolaimus inaequalis*_H3 formed the "pseudoclade" given the fact there was

no bases for their clustering. Another group of species also showed incongruent pattern of phylogenetic positioning within the tree: *Sphaerolaimus hirstus*_LK, *Metadasynemoides* sp1, *Ethmolaimus pratensis, Anoplostoma viviparum, Stylotheristus* sp1 and *Tubolaimoides tenuicaudatus*. These incongruence likely confirm the BLASTn similarity search since the sequences of all these nine species are of a different origin (Table 4). Although discrepancies exist in the position of a few species within the NJ tree, most of the branches formed nine well supported clades with 100% bootstrap support. A bootstrap support was however observed in the Xyalidae clade (90%). Pairwise genetic distance using the K2P substitution model for the 18S sequences showed that ≈74% of intraspecific comparisons showed a genetic divergence ≤3% while about 77% of interspecific comparisons were above 3%.

DISCUSSION

DNA barcoding is increasingly becoming important in the identification of marine nematodes just like their plant-parasitic nematode counterparts. Its application in environmental metagenetics has been largely exploited (Porazinska *et al.*,2009a; Porazinska *et al.*,2009b; Creer *et al.,* 2010; Porazinska *et al.*,2010; Bik *et al.,* 2011). The nuclear ribosomal 18S rRNA and mitochondrial COI genes have been employed in the search for a universal barcode in marine nematodes (Bhadury *et al.,* 2006a; Martinez-Arce and Rochas-Olivares 2009; Derycke *et al.,* 2010a). Nonetheless, arguments exist on which of these genetic markers are wholly representative. This study was undertaken to compare the reliability and performance of both genes in the identification of marine nematode species.

Polymerase chain reaction products were recovered from most of the specimens whose 18S rRNA were amplified than in those specimens whose COI genes were amplified. However the difference in amplification was surprisingly almost similar. There were no complications with the molecular methods; hence the higher amplification percentage of 18S rRNA could not have happened by chance. In fact higher values than 57% were expected since rRNA genes have been reported to be easily amplified by universal primers due to their highly conserved flanking regions (Blok and Power, 2009; Meyer *et al.,* 2010). Furthermore, their repetitive arrangement within the genome provides excessive amounts of template DNA for polymerase chain reactions (Floyd *et al*., 2002; Bhadury *et al.,* 2008). Surprisingly, only a little more than half of the specimens were amplified in this study. This may have resulted from various possibilities. One of such possibilities may be a likely inadequacy in the amplification of the 18S rDNA of the particular organism, in this case marine nematodes. Although other 18S-designed primers including 18P, G10-G11 and F04-22R (Blaxter, 1998) were tested at the preliminary amplification stage, they hardly gave any bands and this makes G18S4-4R a better choice. Sequencing similarity analysis using GenBank database for comparing 18S-derived sequences allowed identification of the individual nematode sequences as closely related to sequences derived from named taxa in the database. All 18S derived-sequences appeared to be

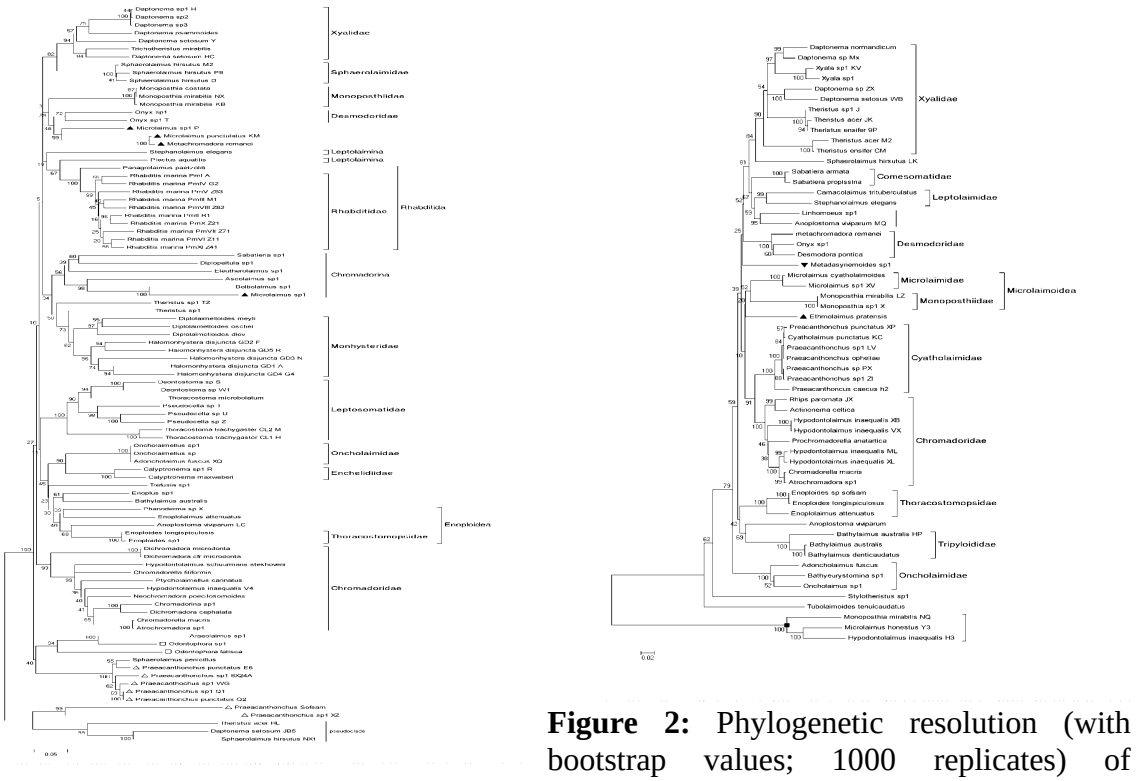

Figure 2: Phylogenetic resolution (with bootstrap values; 1000 replicates) of successfully sequenced specimens, based on nuclear ribosomal 18S sequences.

of a nematode origin hence gave some degree of reliability on the source of sequences used.

A phylogenetic analysis based on the 18S sequences discriminated groups into morphologically correct families and in some cases orders in this study. While popular opinion holds it that the discriminatory power of the 18S is weak in the delimitation of nematode species (Fitch *et al.*, 1995; Silva *et al.*, 2010), the phylogenetic analyses in this study contradict such findings to a slight degree. Distinct monophelytic groups were recovered from the phylogenetic tree comprising species showing low divergence. Inner nodes in the tree were well supported with bootstrap values of either 99 or 100. Other works have shown that the semi-conserved or conserved areas in the 18S gene allow the unraveling of deep phylogenetic relationships within the phylum (Aguinaldo *et al.* 1997; Blaxter, 1998;
Bhadury *et al.*, 2006a and Meldal *et al.*, 2007). However, there were some exceptions (Figure 2). Three species: *Microlaimus honestus*_Y3, *Monosposthia* sp_NQ and *Hypodontolaimus inaequalis*_H3 were wrongly assigned and formed a "pseudo-clade" at the base of the NJ tree. Such a placement whether haphazardly assigned within or anywhere else on the tree may reduce its reliability of the tree topology (Bhadury *et al.*, 2007). This may perhaps be due to misidentifications caused as a result of distortion of morphological characters resulting from preservation in DESS or wrong judgments during morphological identification. After all, BLASTn search showed that *Microlaimus honestus*_Y3 and *Hypodontolaimus*

Figure 1: Phylogenetic resolution (with bootstrap values; 1000 replicates) of successfully sequenced specimens, based on COI sequences.

*inaequalis*_H3 share a high

resemblance to *Calomicrolaimus* sp and *Neochromadora.* Since the certainty in the morphological status is debatable, verifications may be required by using the initial vouchers/pictures taken of these specimens.

Bacterial contamination of genomic DNA could also possibly have been another reason for the wrong derivation. Therefore, amplification and sequencing of other genomic regions for these three specimens could provide vital information for subsequent assignment to correct genus or species as the case may be. Aside from the amplification and sequencing success, the 18S rDNA genetic distance matrix for the sequences tested, indicated that divergence values within species were ≤0.03. Although no standard threshold values exist for intra- and inter-specific variation among species of marine nematodes. A repetition of this study using a higher number of 18S rDNA sequences may confirm or in effect, disregard this value. In any case, the need to have a threshold in the delineation of species may be needful as this may ease species diagnosis (Hebert *et al.,* 2003b). Although the use of threshold value is highly debatable, it should be noted that these values may likely depend on the metrics used and number of congeneric taxa employed in a given study (Jansen and Vepsalainen, 2009).

Unlike the 18S rDNA, COI gene proved difficult to sequence (94.15% vs 55.20%). However some success of sequencing was achieved even though it was low. Low amplification and sequencing rates of COI fragments have been reported for marine nematodes (De Ley *et al.,* 2005; Bhadury *et al.,* 2006b; Derycke *et al.,* 2010b). The high number of variable regions and indels at the primer sites in COI-generated sequences may likely impair amplification (Creer *et al.,* 2010). Development of plausible primers that could successfully amplify and sequence the COI fragment to high degree is therefore needful. However, the obtained COI-derived sequence was able to largely discriminate almost all morphological species. *Microlaimus* sp1, *Theristus* sp1 and *Theristus* sp1_TZ and *Sphaerolaimus penicillus* had random positions within the phylogenetic tree and at the base of the tree is a pseudoclade consisting *Sphaerolaimus hirsutus, Daptonema setosum*_JB5 and *Theristus acer*_HL. The non-clustering they show with their relative species or genera on the phylogenetic tree may be due to a contamination caused by bacteria such as *Wolbachia;* a bacteria which has been found to cause extreme COI divergence in infected organisms. Although this phenomenon of extreme divergence in the COI has been reported in insects (Gerth *et al.,* 2011; Smith *et al.,* 2012; Xiao *et al.,* 2012) and filariids in which they occur at an appreciable frequency (Bordenstein *et al.,* 2003). No report exists in marine nematodes yet. However, this has been reported in a large group of invertebrates and vertebrates (Avise *et al.,* 1994; Thomaz *et al.,* 1996; Walker *et al.,*1997; Waters and Burridge 1999; Lindell *et al.,* 2008). Since large-scale barcoding projects rely largely on mitochondrial COI sequences, a screening of marine nematofauna for possible occurrence of *Wolbachia*-COI interference may give more insights to the interpretations of phylogenetic inferences. Introspection of the use of DNA-Barcoding reveals some inherent shortcomings of this promising identification tool.

Another possibility for the non-clustering of the species may be due to the occurrence of nuclear mitochondrial DNA, abbreviated as NUMT (Song *et al.,* 2008). NUMTs

are described as any transfer or transposition of cytoplasmic mitochondrial DNA sequence into the nuclear genome of a eukaryote organism (Lopez *et al.,* 1994). They are also referred to as pseudogenes. Although these NUMT„s remain transcriptionally inactive, reports have shown that they can be co-amplified with orthologous mitochondrial DNA, hence increase the likelihood of a misled amplification output based on the use of a universal or conserved primers (Bensasson *et al*., 2001; Song *et al*., 2008). NUMTs have been reported in a broad range of organisms (more than 82 eukaryotes according to Bensasson *et al*., 2001) ranging from nematodes (Gibson *et al*., 2007) to mouse and human beings (Richly & Leister 2004). The latter study showed that 99% of the mitochondrial sequences were transferred to the different parts of the nuclei in mouse and humans. Although there is no elaborate published description as well as characterization of NUMTs in marine nematodes yet. Regardless of the possible occurrence of NUMTs, a strict quality control of the COI sequences is needful. However, the COI genetic distance matrix for the sequences tested, indicated that divergence values within species were ≤0.08 K2P genetic distance. Although no standard threshold values in the COI exist for intra- and inter-specific variation among species of marine nematodes. Derycke *et al.* (2010a) suggested that an intraspecific divergence of 0.05 K2P distance will discriminate species within marine taxa. This discordance in values does not make any of the results incredible. In fact, it has been reported in a study by Jansen *et al.* (2009) that demarcating threshold for delimiting species may vary with metrics used and also the number of congeneric taxa. The success of the barcoding approach also depends on the distribution of genetic distances between conspecific individuals and heterospecific individuals given that failures in barcode clustering are proportional to the overlap between both distributions. It has been shown that lineages diversify more quickly within species than between species (Pons *et al.,* 2006). This is due to the fact that diversification within species is driven by mutation at a rate higher than speciation within lineages. Hence, the branch length between species tends to be much deeper than between conspecific individuals leading to a gap in the distribution of the pairwise distance between conspecific individuals and between species that has been referred to the barcoding gap (Meyer *et al.,* 2010).

In a nutshell, assessing the biodiversity of nematodes in marine sediments still poses a big challenge to many taxonomists due to the inherent limitations of the widely used morphology-based approach for identification. The implication of this deterrent is the under-estimation of species richness within marine environments. Although there is a trade-off between using 18S genes that are easy to amplify, but which by their nature are highly conserved and underestimate the true number of species, and using COI genes that give a better description of the number of species, but which are more difficult to amplify. The evaluation of the performances of these two genetic markers linked directly to their taxonomic placement unveils a novel approach for marine nematode identification instead of the use of operational taxonomic units (Porazinska *et al.,*2009a; Porazinska *et al.,*2009b).

This study has established the fact that DNA barcoding using 18S rDNA and COI can be used to identify

marine nematode communities. The performance of 18S rDNA outperforms COI in terms of amplification and sequencing success. This has made the 18S rDNA a typical example of a gene employed in environmental metagenetics for biodiversity studies and analysis on eukaryotes, specifically nematodes (Porazinska *et al.*,2009a; Porazinska *et al.*,2009b; Creer *et al.,* 2010; Porazinska *et al.,*2010; Bik *et al.* 2011). However, COI showed higher resolution in the identification of species but the generation of high profile primers for the amplification and sequencing of its fragment may improve future results.

Therefore given the shortcomings of working with systems of identification separately, an integrative taxonomy in which a combination of molecular approach with vouchering of important structures, morphometric measurements as well as making judgments based on valid identification keys may solve the recurrent ambiguous identification challenges within some taxa in the marine nematode communities.

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