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Systematics and DNA barcoding of free-living marine nematodes with emphasis on tropical desmodorids using nuclear SSU rDNA and mitochondrial COI sequences

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Summary – The diversity and phylogenetic relationships of the Desmodoridae, a widespread tropical family of free-living marine nematodes, is hitherto poorly known both from molecular and taxonomic points of view. We performed a molecular phylogenetic analysis of marine nematodes to: *i*) disentangle relationships among tropical desmodorid species; and *ii*) compare the performance of the nuclear SSU rDNA and mitochondrial COI nucleotide sequences in 42 and 45 nominal species, respectively, to identify species. We generated 27 new sequences of SSU rDNA belonging to five genera not previously sequenced, and 34 new sequences of COI belonging to six genera and four families not previously sequenced. The SSU rDNA tree confirmed the Enoplida to be a monophyletic sister group to the Chromadorida. The family Comesomatidae is a sister group of the Xyalidae within the Monhysterida. Both DNA markers confirmed the congruence between the morphology- and molecular-based phylogenetic inferences for most of the families. Desmodoridae was a monophyletic group, but the relationships within the family could not be recovered; the subfamilies Desmodorinae and Spiriniinae were not monophyletic meanwhile the monophyly of Stilbonematinae was not fully supported due to a few specimens of questionable identity. COI performed better than SSU rDNA to disentangle relationships among closely related species and suggested the presence of cryptic diversity within Desmodoridae. COI is effective to explore cryptic diversity and barcode species within Nematoda, with a possible threshold of genetic distance of 5% between conspecific and interspecific sequences, but DNA barcoding is limited by the poor knowledge of the diversity and taxonomy of the group and the lack of a good reference database of vouchered COI sequences.

Keywords – 18S rDNA, Chromadorea, Enoplea, molecular, phylogeny, taxonomy.

Nematodes are one of the most diverse groups of metazoans (Zhang, 2011) and are probably the most abundant and ubiquitous (Margulis & Chapman, 2009). They play a key role in the functioning of marine ecosystems and have been extensively studied from a fundamental (*e.g.*, diversity, phylogeny) and applied approach (*e.g.*, biomonitoring). As a result, the phylogenetic relationships within Nematoda have been intensively studied and a robust phylogenetic framework exists for the phylum (Bert *et al.*, 2011). Nevertheless, the taxonomy of many free-living nematodes still remains largely unresolved and debated because of the regular occurrence of parallel evolution (Blaxter *et al.*, 1998; van Megen *et al.*, 2009) and conserved morphology (Holterman *et al.*, 2008).

The first phylogenetic classification of free-living nematodes was established by Lorenzen (1994) using morphological features. It largely still stands for the intrafamilial systematics. Relationships within the phylum have been resolved by using the nuclear small subunit of ribosomal DNA (SSU rDNA) but these studies mostly included parasitic and soil nematodes (*e.g.*, Aleshin *et al.*, 1998; Blaxter *et al.*, 1998). De Ley & Blaxter (2004) in-

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troduced a new classification system based on the combination of morphological and molecular features; importantly, these authors translated the found clades into a Linnean classification. Meldal *et al.* (2007) improved the phylogeny of nematodes by increasing the number of marine taxa in the analysis. The most inclusive phylogenetic study of nematodes currently available contains 1215 sequences of SSU rDNA (van Megen *et al.*, 2009). However, marine taxa remain strongly under-represented (Bert *et al.*, 2011). Moreover, the 46 sequences of marine taxa added by Meldal *et al.* (2007) came from northwest European coastal waters, rendering the representation of marine taxa from tropical regions hitherto scarce. Evidently, information on taxa from tropical areas may reveal novel insights in the systematics and diversity of nematodes.

Against this background, we present a molecular systematic analysis of marine desmodorid nematodes from a tropical coral reef in the Caribbean Sea at Punta Francés, Cuba (Armenteros *et al.*, 2012). The Desmodoridae Filipjev, 1922 is one of the most speciose and diverse groups of marine nematodes (318 species after Hodda, 2011). However, its systematics remains unresolved because of three reasons. First, Desmodoridae comprises many poorly defined taxa (*e.g.*, *Laxonema* Cobb, 1933; *Metadesmodora* Schuurmans Stekhoven, 1942) and only a few recent reviews have been published for speciose groups (*e.g.*, *Desmodora* de Man, 1889 by Verschelde *et al.* (1998) and Stilbonematinae by Tchesunov (2013)). Second, there are conflicting morphological characters (Kampfer *et al.*, 1998). Third, the group is still poorly documented by DNA sequence data, indicating a clear necessity to generate sequence data from taxa that have hitherto been underrepresented.

SSU rDNA is the most widely used DNA marker to study the systematics of nematodes because it is easily amplified across the phylum and it has successfully resolved several taxonomic species-level problems and relationships (Rodrigues Da Silva *et al.*, 2010). Therefore, we used SSU rDNA to explore certain problematic phylogenetic relationships within desmodorid nematodes. In addition, we used the mitochondrial cytochrome oxidase *c* subunit 1 (COI) gene to resolve relationships among closely related and/or cryptic nematode species (Derycke *et al.*, 2005, 2010a) in a DNA barcoding context.

The objectives of this study are hence twofold: *i*) to disentangle phylogenetic relationships among tropical free-living marine desmodorids; and *ii*) to compare the ability of SSU rDNA and COI to identify or differentiate species of marine nematodes.

Materials and methods

Collection and sorting of nematodes

Nematodes were collected in July 2010 in the SW region of the Cuban Archipelago, Punta Francés Reef (21°36′29.68″N, 83°10′34.40″W) (Armenteros *et al.*, 2012). Sediment samples were collected manually with plastic cores (2.6 cm diam. × 8 cm length) from sand flats at 2-3 m depth with scarce vegetation and seagrass meadows of *Thalassia testudinum*.

Samples were sieved over a 45 μm mesh sieve and preserved in DESS solution (Yoder *et al.*, 2006) until further processing. Nematodes were extracted from the samples under a stereomicroscope and again stored in DESS solution.

DNA extraction and sequencing

Specimens were mounted, one by one, on temporary microscope preparations in a drop of distilled water. They were identified and photo-vouchered at magnifications of 100×, 400× and 1000× using an Olympus BX41 light microscope (LM) with interference contrast coupled to a Color View digital camera with the software Olympus cell^D. Identifications were based on published taxonomic descriptions compiled mostly in the NeMys database (available online at http://www.marinespecies.org).

Identified and photographed nematodes were subsequently transferred to individual Eppendorf vials containing worm lysis buffer consisting of 50 mM KCL, 10 Mm Tris, pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP 40 (Tergitol Sigma) and 0.45% Tween 20. Subsequently, they were frozen at −20°C to break the cell walls. Afterwards, 1 μl of proteinase K (10 mg ml^{-1}) was added and samples were incubated during 1 h at 65°C to digest the proteins followed by another 10 min at 95°C to inactivate the proteinase K. Finally, the samples were centrifuged during 1 min at 21 000 *g*. DNA extracts were stored at −80°C.

Fragments of the nuclear small subunit ribosomal DNA (SSU rDNA) and the mitochondrial cytochrome oxidase *c* subunit 1 (COI) genes were PCR amplified. For SSU rDNA, we used the primer set G18S4 (f): 5′-GCT TGT CTC AAA GAT TAA GCC-3′ and 4R (r): 5′-GTA TCT GAT CGC CKT CGA WC-3′ which amplifies a fragment of *ca* 1000 bp. For COI, we used the primer set JB3 (f): 5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′ and JB5 (r): 5′-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG-3′ which amplifies a fragment of 426 bp (Derycke *et al.*, 2010a).

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PCR amplifications were done in 25 μl of reaction volume with the following mix: 1 μl of DNA template, 2.5 μl of 10× PCR buffer, 2.5 μl of Coral Load 10×, 2 μl of $MgCl_2$ (25 mM), 0.5 μl deoxynucleotides (10 mM each), 0.125 μl of primers (25 μM), and 0.125 μl of TopTaq DNA polymerase (5 U μl^{-1}; Qiagen). PCR cycling conditions were: *i*) initial denaturation of 5 min at 95°C; and *ii*) 35 cycles (for COI) or 40 cycles (for SSU rDNA) of (94°C for 30 s, 54°C for 30 s, 72°C for 30 s), followed by a final extension of 10 min at 72°C.

PCR products were loaded on 1.2% agarose gels, run in an electrophoresis chamber and visualised with a BioRad UV system. Amplifications were considered successful when a single band of the expected size was observed in the agarose gel. PCR products were purified using the ExoSAP-IT kit following the instructions of the manufacturer (Affymetrix). Bidirectional sequencing was performed with an ABI 3130xl Genetic Analyzer using the BigDye v1.1 kit; precipitation and purification were done following the instructions of the manufacturer using formamide, ethanol and ethylenediaminetetraacetic acid.

Data analyses and phylogenetic reconstruction

The chromatograms were used for the sequence quality control and sequences were edited by hand when necessary (*i.e.*, gaps, double peaks). Forward and reverse sequences were assembled with the software Chromas Lite v2.01 (available online at www.technelysium.com.au) and a database of all the sequences was built in BioEdit v7.0.5 (Hall, 1999).

The quality of the obtained sequences was individually assured by visual inspection and by a BLAST search in GenBank; all the sequences had high identity to other nematode sequences. In addition, 21 desmodorid SSU rDNA sequences and 27 COI sequences of marine nematodes from GenBank were included in the analyses. Sequences were aligned using ClustalW in the software MEGA 5.10 (Tamura *et al.*, 2011) with the default parameters. The ends of the obtained sequences were trimmed (including primer sequences) and we deleted manually the gaps, ambiguously aligned regions and identical haplotypes. As COI is a protein coding gene, COI sequences were checked for stop-codons using the *Drosophila* mitochondrial DNA as genetic code in the software DnaSP 5.10.1 (Librado & Rozas, 2009).

MEGA 5.10 was used for the selection of the best nucleotide substitution model using the Bayesian Information Criterion. Phylogenetic trees were inferred for SSU rDNA and COI separately using the maximum likelihood (ML) method. Branch support was estimated by bootstrapping over 100 resamplings. Phylogenetic trees were rooted using the midpoint rooting technique as implemented in MEGA 5.10. New sequences have been uploaded to the European Nucleotide Archive; with accession numbers LK54702-LK54728 (for SSU rDNA) and LK054668-LK054701 (for COI). Pairwise *p*-distances between haplotypes were calculated in MEGA 5.10 for SSU rDNA and COI; distances were classified in three exclusive categories: conspecific, interspecific congeneric and interspecific non-congeneric. The definition of these categories was based on the identity of the morphospecies as resulted of the LM microscope observations. We refrained from any nomenclatural change of these morphospecies even when they result in paraphyletic species/genus in the phylogenetic tree.

For the supra-familial classification, we follow De Ley & Blaxter (2004) and Hodda (2011); for the intra-family classification we follow Lorenzen (1994).

Results

SSU rDNA

We obtained 27 new SSU rDNA sequences of which several belong to five genera (*Cheironchus* Cobb, 1917; *Gomphionema* Wieser & Hopper, 1966; *Longicyatholaimus* Micoletzky, 1924; *Steineria* Micoletzky, 1922; and *Viscosia* de Man, 1889) that have not been previously sequenced. The final alignment comprised 51 sequences of 586 bp of which 235 positions were parsimony informative. The best nucleotide substitution model was the Kimura 2-parameter with non-uniform evolutionary rates (K2 + G).

The ML tree recovered several clades of marine nematodes (Fig. 1). Enoplida was recovered as a clade but Chromadorida could not be recovered as a well-supported monophyletic group. The order Monhysterida was recovered as a clade; the exception was *Sphaerolaimus* sp. NN004 which was not placed within the clade. The families Comesomatidae and Xyalidae were placed as sister groups within the order. The families Desmodoridae and Oncholaimidae were also supported as clades. The genera *Longicyatholaimus* and *Cheironchus* were supported as monophyletic taxa.

The Desmodoridae formed a well-supported clade, but each of the three subfamilies in our study (*i.e.*, Desmodorinae, Spiriniinae and Stilbonematinae) were not

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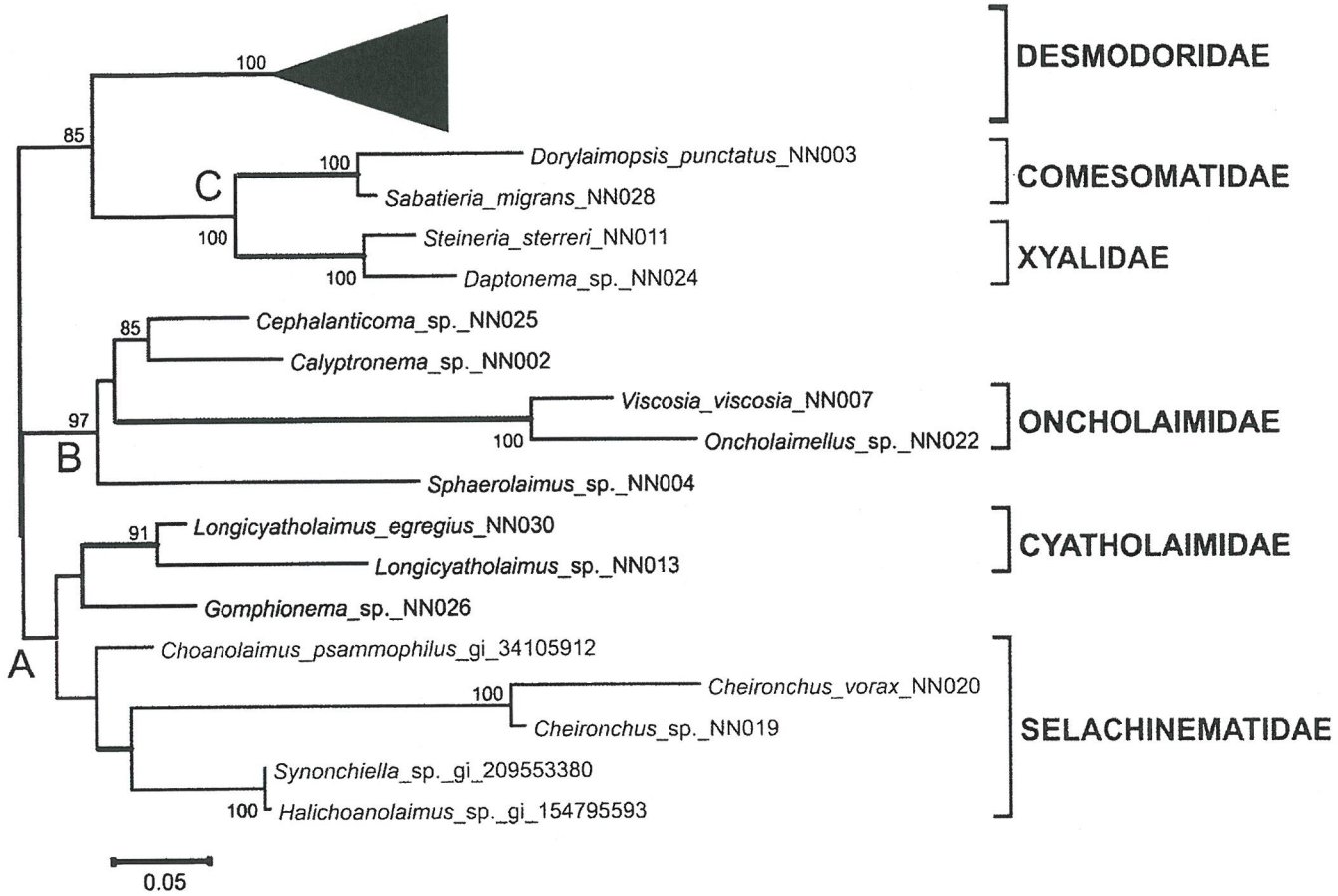

Fig. 1. Maximum likelihood tree of marine nematode species based on 51 DNA sequences of SSU rDNA. The root of the tree is placed based on midpoint rooting technique. Numbers above/below the branches indicate branch support based on 100 bootstrap replicates (values < 75% are not shown). Taxa above the family level are indicated by capital letters on the nodes (A: Chromadorida, B: Enoplida, C: Monhysterida). Families are indicated by square brackets. Sequences obtained in this study are coded as NN.

monophyletic (Fig. 2). Even at the genus level there were several non-monophyletic taxa and/or without bootstrap support, such as *Eubostrichus* Greeff, 1869; *Laxus* Cobb, 1894; *Robbea* Gerlach, 1956; *Spirinia* Gerlach, 1963; and *Stilbonema* Cobb, 1920. A double checking of the identity of these species was made based on photo-vouchers in order to confirm that no misidentified specimens existed in the tree.

Three sequences (*Desmodora_pontica*_AM234628.1, *Spirinia_parasitifera*_AY854217.1 and *Spirinia_parasitifera*_EF527426.1) from GenBank showed abnormally high values of conspecific distances; we did not include them in the descriptive statistics or in the histogram since there was no way to check for possible misidentification. Pairwise *p*-distances overlapped across the three categories: conspecific (mean = 0.022; range = 0.011-0.038), congeneric (mean = 0.054; range = 0.024-0.075) and non-congeneric (mean = 0.12; range = 0.015-0.25). The distribution of the pairwise *p*-distances for SSU rDNA did not show a clear gap between the three categories (*i.e.*, conspecific, congeneric and non-congeneric distances); in fact, non-congeneric distances showed a bimodal distribution (Fig. 3).

COI

COI has been less studied than SSU rDNA in Nematoda, with 2818 sequences belonging to 942 species *vs* 15 415 sequences belonging to 3541 species, respectively (search in GenBank on 16 May 2014). So, with this study,

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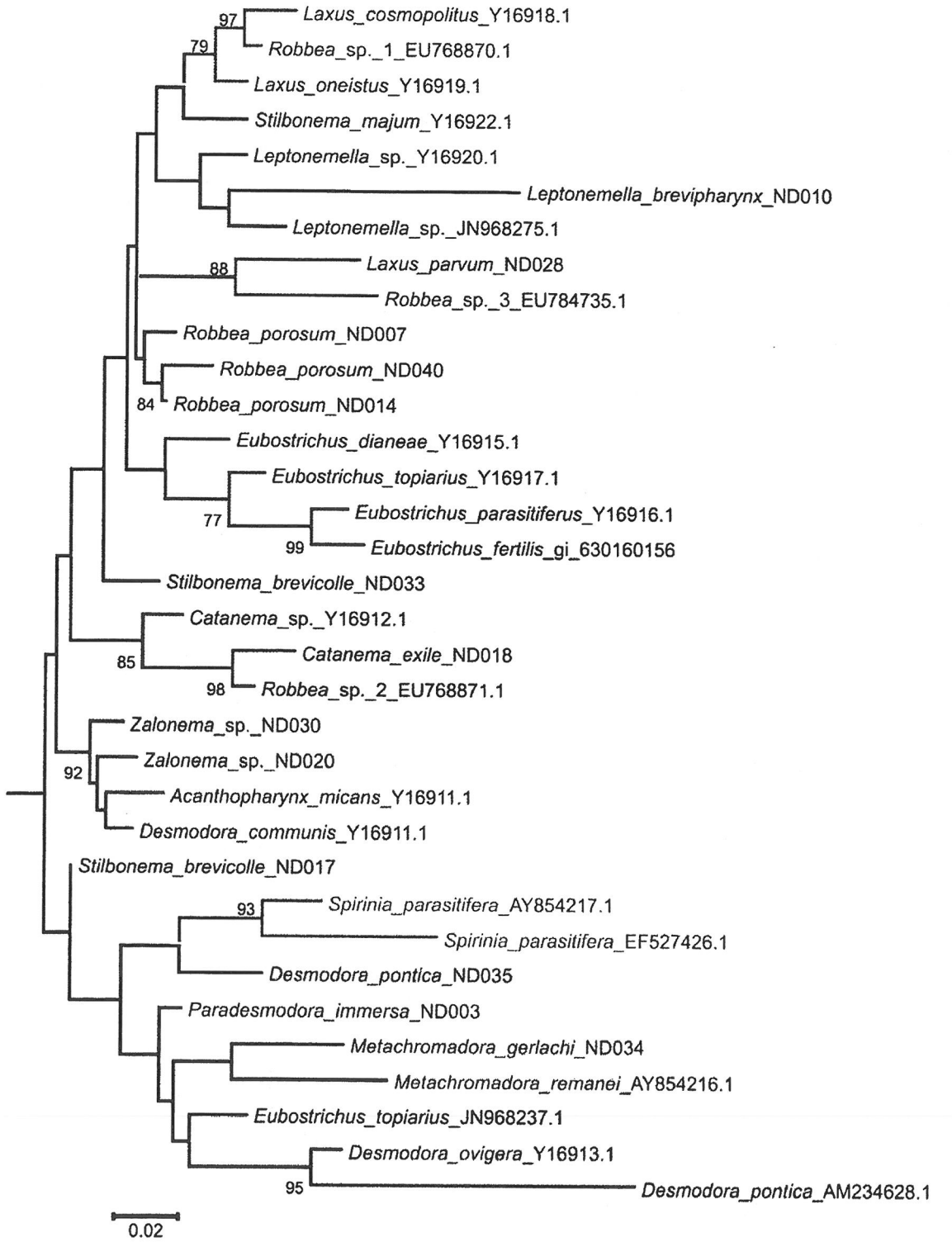

Fig. 2. A section of the maximum likelihood tree (SSU rDNA) in Figure 1 showing only the family Desmodoridae. Numbers above/below the branches indicate branch support based on 100 bootstrap replicates (values < 75% are not shown). Subfamily membership is indicated by colour: Stilbonematinae (red), Desmodorinae (blue) and Spiriniinae (green). Sequences obtained in this study are coded as ND.

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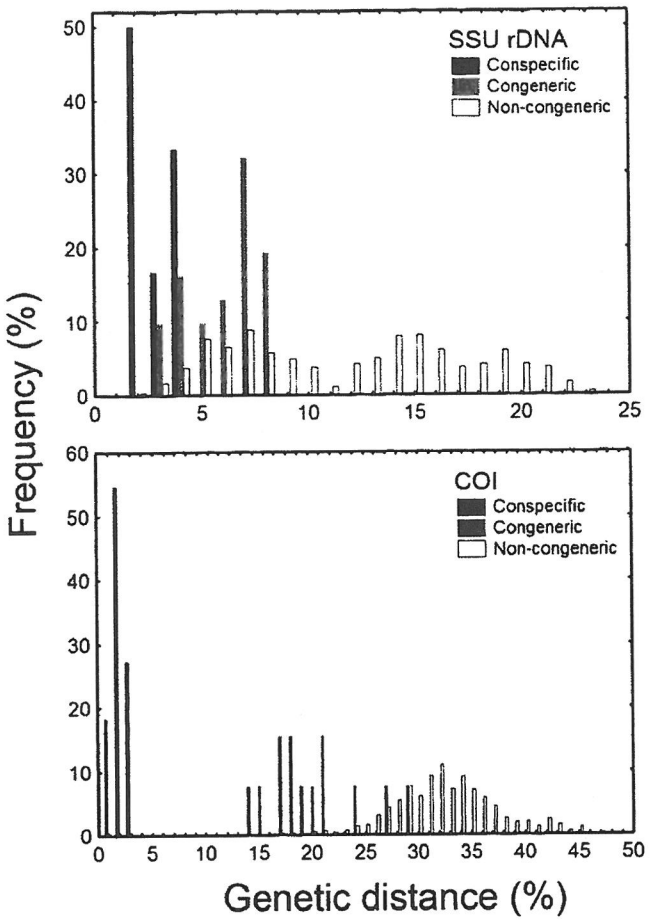

Fig. 3. Relative frequencies of pairwise *p*-distances for SSU rDNA (top) and COI (bottom) genetic markers.

we added 34 new COI sequences to this dataset; including six species of desmodorids not previously sequenced: *Catanema exile*, *Desmodora pontica*, *Paradesmodora immersa*, *Robbea porosum*, *Stilbonema brevicolle* and *Zalonema* sp. The final alignment comprised 61 sequences of 393 bp of which 291 positions were parsimony informative. The nucleotide substitution model that best fitted the data was the General Time Reversible model with non-uniform evolutionary rates and invariant sites (GTR + G + I).

The ML tree based on COI did not provide support for the deep relationships in the phylogenetic backbone of Nematoda (Fig. 4). Four families were recovered as well-supported clades: Xyalidae, Chromadoridae, Cyatholaimidae and Oncholaimidae. Thoracostomopsidae were non monophyletic because *Calyptronema* Marion, 1870, *Enoploides* Ssaweljev, 1912 and *Trileptium* Cobb, 1933 were placed outside the clade. *Dichromadora* Kreis, 1929 (Chromadoridae) was non-monophyletic. In general, for the groups outside of Desmodoridae, there was a good recovery of the relationships between species using the COI marker. The family Desmodoridae could not be recovered as a well-supported clade.

Within Desmodoridae, the three subfamilies (*i.e.*, Desmodorinae, Spiriniinae and Stilbonematinae) appeared non-monophyletic (Fig. 5). Specimens were clustered consistently within the genera *Zalonema* Cobb, 1920 and *Paradesmodora* Schuurmans Stekhoven, 1942. However, *Stilbonema brevicolle* was unresolved because of low bootstrap support. Divergent lineages within morphospecies were found for *Robbea porosum*.

The analysis of conspecific *p*-distances had a very wide range when specimens were classified based solely on morphology (mean = 0.14; range = 0.0028-0.29). However, we detected two lineages which are probably cryptic species (*R. porosum*) and also detected one paraphyletic species (*S. brevicolle*) (Fig. 5). If specimens belonging to these species are removed from the pairwise conspecific group, the values were more consistent (mean = 0.015; range = 0.0028-0.024). The congeneric distances had higher values (mean = 0.19; range = 0.14-0.28) and non-congeneric (mean = 0.32; range = 0.15-0.47). The histogram of the COI pairwise *p*-distances with the removed values showed a clear gap between the conspecific (<2.5% genetic divergence) and the other two groups; the congeneric distances had some overlapping with those of the non-congeneric ones (Fig. 3).

Discussion

The phylogenetic relationships based on SSU rDNA largely agree with those of van Megen *et al.* (2009). The position of Comesomatidae has been uncertain (Holterman *et al.*, 2008), but our results support its membership to the Monhysterida which is in agreement with Lorenzen (1994) and van Megen *et al.* (2009). The monhysterids included in our study support the inclusion of Comesomatidae and Xyalidae in a Monhysterida-dominated clade (5A *sensu* Holterman *et al.*, 2008).

Our results confirm the monophyly of the Desmodoridae (Meldal *et al.*, 2007, 2009). Conversely, the marine desmodorid subfamilies Desmodorinae and Spiriniinae were non-monophyletic in agreement with previous studies (Holterman *et al.*, 2008; van Megen *et al.*, 2009; Armenteros *et al.*, 2014). Consistently with our findings, no defining synapomorphy has been reported for these two subfamilies. Stilbonematinae have been reported to be monophyletic (Kampfer *et al.*, 1998) and indeed most

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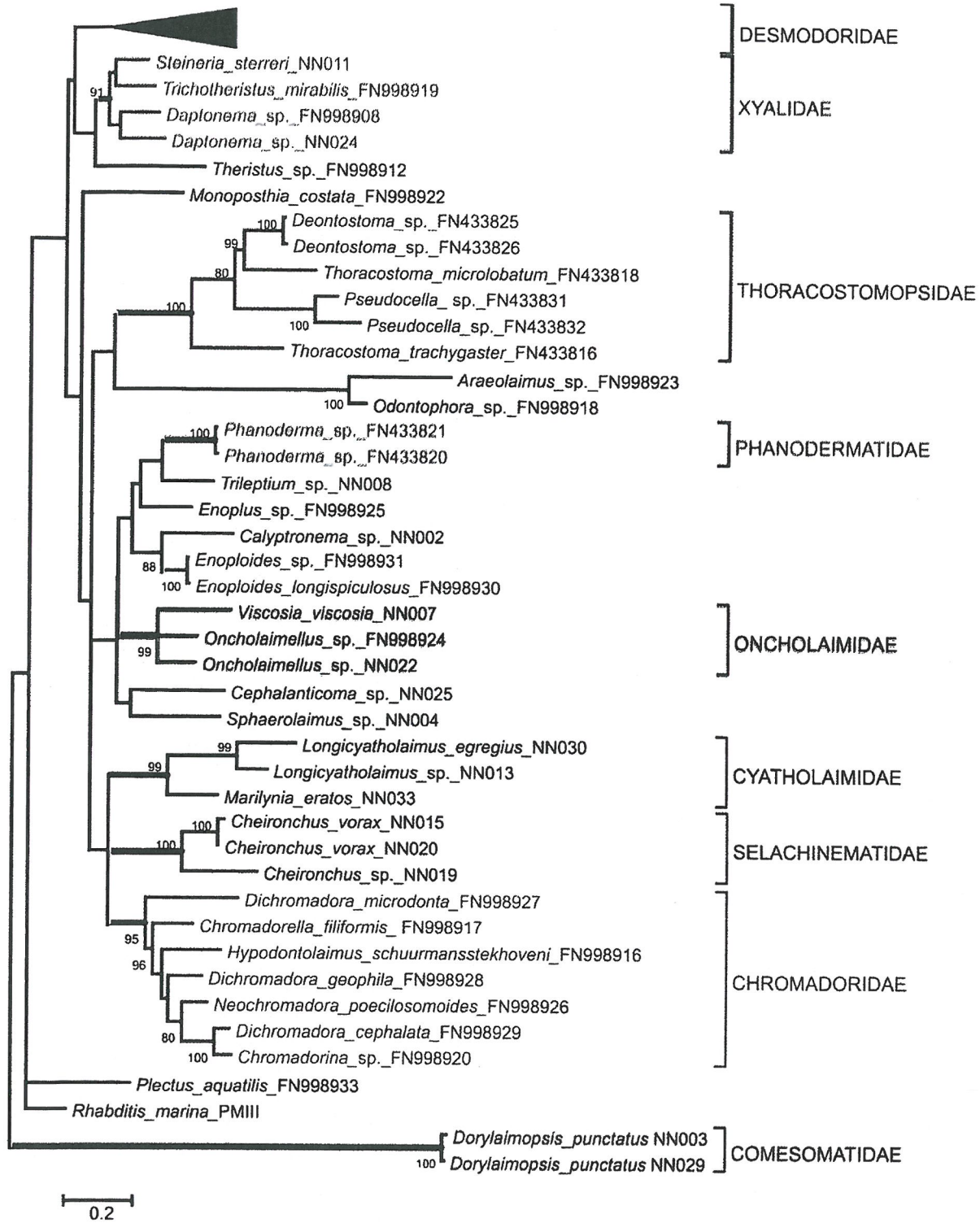

Fig. 4. Maximum likelihood tree of marine nematode species based on 61 DNA sequences of COI. The root of the tree is placed based on midpoint rooting technique. Numbers above/below the branches indicate branch support based on 100 bootstrap replicates (values < 75% are not shown). Families are indicated by square brackets. Sequences obtained in this study are coded as NN.

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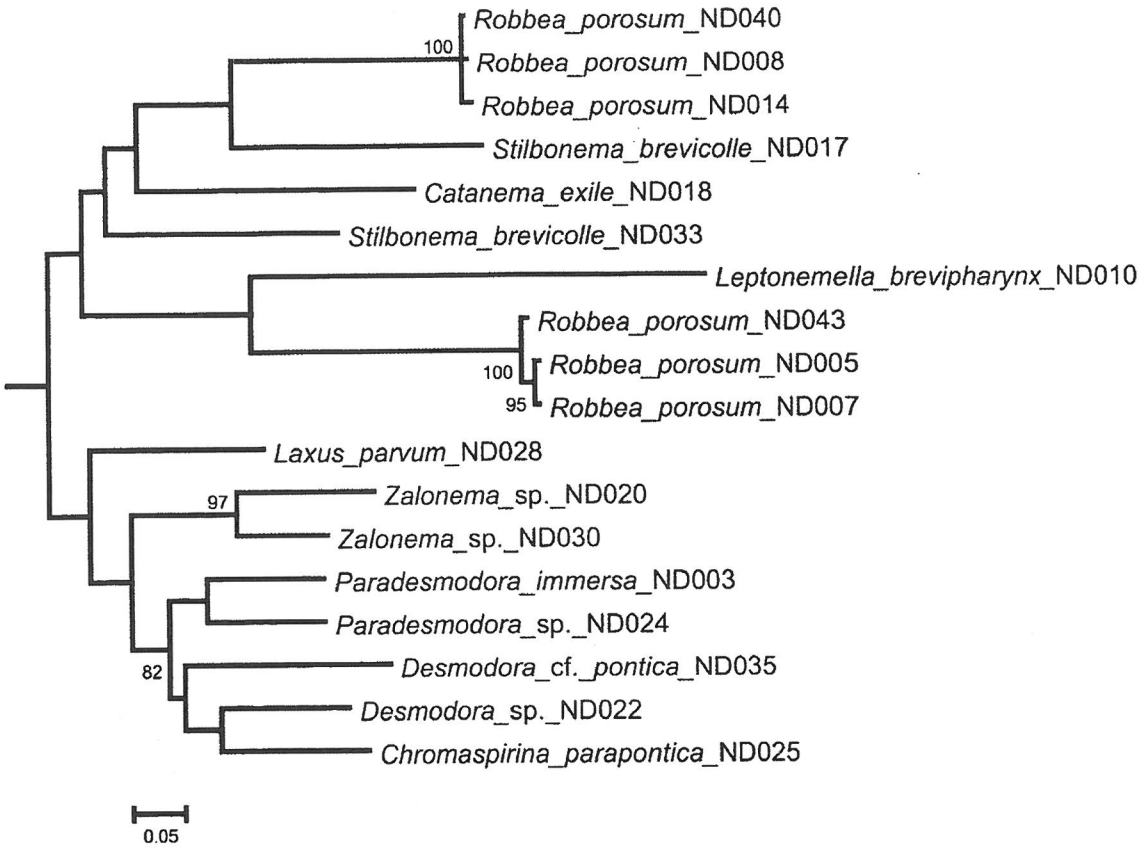

Fig. 5. A section of the maximum likelihood tree (COI) in Figure 4 showing only the family Desmodoridae. Numbers above/below the branches indicate branch support based on 100 bootstrap replicates (values < 75% are not shown). Subfamily membership is indicated by colour: Stilbonematinae (red), Desmodorinae (blue) and Spiriniinae (green). Sequences obtained in this study are coded as ND.

of the stilbonematin species in our analysis clustered together in both trees, thereby supporting this hypothesis. However, there were some specimens placed out of the cluster that we consider questionable because of: *i*) the considerable difficulties to identify some specimens because the coiled habitus; and *ii*) there are some apparently anomalous DNA sequences. Actually, the inclusion of most of the SSU rDNA sequences from GenBank by Armenteros *et al.* (2014) notably weakened the phylogenetic signal of Stilbonematinae and as result they concluded that the subfamily was non-monophyletic. However, Stilbonematinae have been characterised by two synapomorphies: glandular sensory organs (Bauer-Nevelsick *et al.*, 1995) and ectosymbiotic chemoautotrophic bacteria on the cuticle (Polz *et al.*, 1992). Thus, we support the hypothesis of monophyly of Stilbonematinae based on our molecular evidence and consider the existence of possible misidentification of specimens. Finally, future studies to disentangle the systematics within Desmodoridae should include other species and genera.

Several desmodorid SSU rDNA sequences from GenBank were apparently anomalously placed in the tree in the initial performed phylogenetic reconstruction (*e.g.*, *Robbea* sp. JN968260.1 and *Spirinia parasitifera* JN968278.1). The original publications did not provide enough detail to check the species identification of these sequences and therefore were not included in the tree. The vouchering of sequenced nematode specimens with images (photos or videos), as suggested by De Ley *et al.* (2005) is essential for the interpretation and subsequent validation of phylogenetic analyses. For instance, the relatively high value of the *p*-distance (0.20) between specimens NN019 and NN020 of *Cheironchus* spp. suggested that different species may be involved. Further scrutiny of photo-vouchers from several specimens indicated that there were indeed differences in the numbers of

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pre-cloacal supplements (12 *vs* 13) and numbers of turns and size of the amphidial fovea (5.5 *vs* 3.5 turns). We labelled *a posteriori* NN019 as *Cheironchus* sp. and it may be a new species within the genus.

The taxa that were morphologically identified and which had not been previously sequenced (*e.g.*, *Steineria*, *Zalonema*) were placed consistently within clades representing the families. This result supports the high congruence between the morphology- and DNA-based systematics at family-level of marine nematodes (van Megen *et al.*, 2009; Derycke *et al.*, 2010a).

SSU rDNA has been useful to barcode nematode groups with high nucleotide substitution rates (*e.g.*, animal- and plant-parasite species) (Holterman *et al.*, 2006), although it is considerably less informative for discriminating closely related marine nematode taxa (see Bhadury *et al.*, 2006). The use of the I3-M11 partition of the COI gene, as suggested by Derycke *et al.* (2010a), clearly performs much better than SSU rDNA in discriminating species of genera such as *Cheironchus*, *Dichromadora*, *Enoploides* and *Thoracostoma* Marion, 1870. The locus is relatively easy to amplify using JB3-JB5 primers and we obtained a 73% of amplification success without attempting to improve the protocols of Derycke *et al.* (2010a), even if these authors had a higher success: 87.8%.

Our study supports the usefulness of COI for prospecting nematode species and we obtained evidence of cryptic diversity that could be further explored based on the voucher photographs. We found divergent genetic lineages within the morphospecies *R. porosum* and *S. brevicolle* which are flagged as putative cryptic species. For some taxa, *a posteriori* analysis based on photo vouchers revealed morphological divergence (*e.g.*, *Cheironchus*, *Desmodora*, *Paradesmodora*), but for other taxa we could not morphologically differentiate nominal species which were divergent in the tree. Evidence of cryptic diversity has been published for several marine species, namely: *Litoditis marina* (Derycke *et al.*, 2005), *Thoracostoma trachygaster* (Derycke *et al.*, 2010b; Silva de Oliveira *et al.*, 2012), *Halomonhystera disjuncta* (Derycke *et al.*, 2007) and *Terschellingia longicaudata* (Bhadury *et al.*, 2008).

The existence of substantial cryptic diversity in nematodes may be explained by a combination of: *i*) conserved morphology (Holterman *et al.*, 2008); *ii*) fast nucleotide substitution rates both in mitochondrial and nuclear DNA (Blouin *et al.*, 1998; Holterman *et al.*, 2006; Gissi *et al.*, 2008); and *iii*) restricted dispersal and gene flow at large geographical scales (Derycke *et al.*, 2013). The latter point is less relevant in our study given the small area in which the samples were taken (*i.e.*, <10 m distance among cores). However, there is also the possibility of speciation in other regions and secondary contact of the sympatric species.

COI separated clearly conspecific from interspecific sequences (*i.e.*, barcoding gap). All conspecific genetic distances were <3% of genetic divergence, whereas all interspecific distances (within and among genera) were >14%. Therefore, the threshold value of 5% of genetic divergence suggested by Derycke *et al.* (2010a) also holds in our study. COI seems promising for DNA barcoding of nematodes, which is in agreement with previous studies (*e.g.*, Derycke *et al.*, 2010a; Siddall *et al.*, 2012). However, the main limitation for the implementation of COI barcoding for marine nematodes is the poor knowledge of the diversity and taxonomy of the group (Bucklin *et al.*, 2011) and the lack of a good reference database of vouchered COI sequences.

In summary, our main conclusions are: *i*) the monophyly of Enoplida is re-affirmed, with Chromadorida as a sister group; *ii*) the family Comesomatidae is a sister group of Xyalidae within Monhysterida; *iii*) both DNA markers confirm the congruence between morphology- and molecular-based phylogenetic inferences for most of the families included in our study, although non-congruence occurs at intra-familial level (*i.e.*, cryptic species); *iv*) Desmodoridae is a monophyletic group, but the relationships within the family could not be recovered; the subfamilies Desmodorinae and Spiriniinae were non-monophyletic meanwhile the monophyly of Stilbonematinae was not fully supported due to a few specimens of which the identification could be questioned; and *v*) currently, COI is effective to explore cryptic diversity and barcode species within Nematoda, providing a good reference database is available.

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