Article

COI Gene and Algorithmic Analysis for Delimitation of Putative Species in Mexican Marine Nematodes

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Abstract: Nematode biodiversity is mostly unknown; while about 20,000 nematode species have been described, estimates for species diversity range from 0.1 to 100 million. The study of nematode diversity, like that of meiofaunal organisms in general, has been mostly based on morphology-based taxonomy, a time-consuming and costly task that requires well-trained specialists. This work represents the first on the taxonomy of Mexican nematodes to integrate morphological and molecular data. We add seven new morphological records for the Mexican Caribbean: *Anticomid sp1*, *Cylicolaimus sp1*, *Oncholaimus sp2*, *Platycoma sp1*, *Catanema sp1*, Enopliodes spp., and *Metachromadora* spp. We recover 55 COI sequences that represent 20 species. Of the studied sites, Cozumel had 12 species and Cancún had two species (*Rhips sp1* and *Monoposthia mirabilis*) represented by several individuals. All sequences are new for the genetic international databases GenBank and BOLD. Phylogenetic analyses and species delineation methods support the occurrence of the 20 entities and confirm the high resolution of COI sequences in delimiting species. ABGD and mPTP methods disentangled 20 entities, whereas Barcode Index Numbers (BINs) recovered 22 genetic species. DNA taxonomy was demonstrated to be an efficient, fast, and low-cost method to address a taxonomical shortfall of meiofaunal organisms.

Keywords: marine nematodes; mtCOI; DNA taxonomy; meiofauna; Mexican Caribbean

1. Introduction

Meiofaunal nematodes include hyper-diverse, abundant, and worldwide distributed communities of small, free-living marine species that play a key ecological role in benthic energy flow [1-7]. Currently, about 6,500 marine species of meiofaunal nematodes have been formally described [8], but over 100 million species are estimated to exist overall [9,10]. In Mexico, knowledge of the diversity of marine nematodes spans approximately 147 genera and only about 33 species [11-16]. However, the studies are still scarce; the ones mentioned above represent the beginning of the work that remains to be done

The slow advance in the knowledge of the marine nematodes is due to technical difficulties. Marine benthic nematodes are traditionally identified using morphological traits of male genital structures on adults [17,18]; however, adult presence can be very rare in the sediment, and, in most cases, diversity is solely disentangled at a family or genus taxonomic level. Morphology-based taxonomy is a time-consuming task that requires well-trained specialists who are becoming rare [19]. The use of morphological traits, in most cases descriptive and potentially affected by convergent evolution and phenotypic plasticity, could also prevent an accurate quantification of diversity [20-22].

There is an increasing need for methods that can rapidly and cost-effectively estimate nematode diversity in the marine sediments. Molecular tools for taxonomic identification have the potential to overcome the difficulties in nematode species identification, especially when molecular results are

integrated with morphological, ecological, and behavioral assessments [24-29]. Nematodes are genetically well represented in the nuclear markers, especially through the small subunit (18S) and the large ribosomal subunit (rDNA) (28S) [30-33]. However, DNA taxonomy is particularly successful when applied to mitochondrial gene cytochrome oxidase subunit I (COI) sequences [34-38] because of the higher resolution to estimate diversity [37-32, 39].

To disentangle diversity of nematodes, COI sequences should be analyzed using different sophisticated analyses such as Automatic Barcode Gap Discovery (ABGD) [40], Barcode Index Number system (BINs) [41], and Poisson Tree Processes model (PTP) [42]. Integrating different species delineation models should prevent biased conclusions and disclose patterns of diversity and distribution [37, 43, 44, 45].

The main objectives of this study are to (i) improve knowledge of the geographic distribution of meiofaunal nematodes in Mexico, a notoriously poorly investigated area; (ii) add new sequences to the international databases from Mexican nematodes; and, (iii) apply the delimitation models to test COI resolution in marine nematodes.

2. Materials and Methods

Nematode collection and identification

Marine meiofaunal nematodes were collected in 2010 and 2011 from the intertidal zone of different sites along the coast of Quintana Roo state, Mexico (Fig. 1, Table 1). Sediment samples were collected from each site using a Falcon corer (10 × 2 cm; [46]. Individuals were extracted by decantation in the field using two sieves (180 and 63µm mesh) and fixed with DESS solution [47]. In the laboratory, all individuals were separated by morphology, selected, and picked up under a stereo microscope (NIKON SMZ-1). They were put on temporary slides in a drop of MilliQ water covered by a cover slip and photographed by a camera (Canon G11) at different magnifications (10x, 40x, and 100x). Morphological identification was made using available taxonomic keys [47-49].

DNA extraction, PCR amplification and sequencing

Under the microscope, each nematode was picked from the temporary slide using a fine paintbrush and preserved in ethanol. DNA extraction was made by HotSHOT technique [50]. DNA was stored at 4° C and used for genetic amplification. COI amplification was made as follows. PCR reactions were performed in a final volume of 12.5 µl containing 6.25 µl of trehalose 10%, 2 µl of ddH20, 1.25 µl of 10X PCR Buffer, 0.625 µl of MgCl2 50 Mm, 0.125 µl of each primer (10µM), 0.0625 µl of dNTPs (10 mM), and 0.06 µl of Platinum® Taq Polymerase (Invitrogen) [51]. We used two primer sets (LC01490_t1 and HC02198_t1 and LC01490 and HC02198) [52]. For thermocycler conditions, see Table 2. PCR products were visualized on 2% agarose gels stained with ethidium bromide. Amplicons were bidirectionally sequenced with an ABI 3730 capillary sequencer (ABI Carlsbad, CA, USA) using the BigDye© Terminator v.3.1 cycle sequencing kit (Applied Biosystems) at the Canadian Centre for DNA Barcoding (CCDB) following standard protocols for high-volume samples [53].

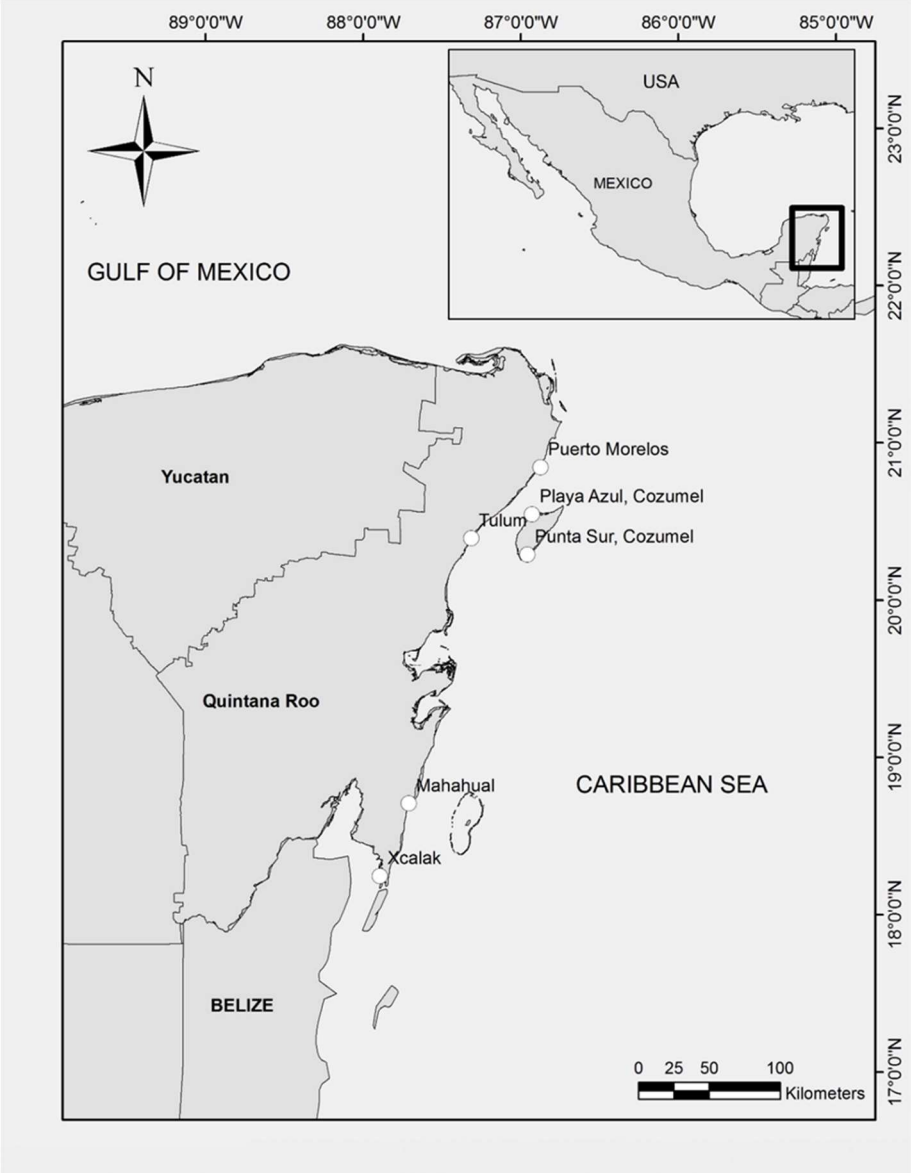

Figure 1. Sampling localities in the Mexican Caribbean.

Table 1. Coordinates (lat/log) of sample sites.

Sites	Lat	Log	Area
Cancun	21.140	-86.677	QRN
Puerto Morelos	20.508	-86.525	QRN
Cozumel (Playa Azul)	20.548	-86.929	QRN
Cozumel (Punta sur)	20.291	-86.959	QRN
Tulum	20.395	-87.315	QRN
Mahahual	18.708	-87.712	QRS
Xcalak	18.2475	-87.8944	QRS

89

Table 2. Primers and termocycling conditions for COI amplification.

Primer name	Sequence (5′-3′)	Reference	Termocycler program
	COI Amplification		
LC01490_t1	TGTAAAACGACGGCCAGTGGTCAACAAATCAT AAAGATATTGG	Folmer et al. 1994 (modified by Floyd et al. 2002)	1 min at 94 ºC, 5 cycles of: 40 s at 94 ºC, 40 s at 45ºC, and 1 min at 72 ºC, followed by 35 cycles of 40 s at 94 ºC, 40 s at 51 ºC and 1 min at 72 ºC, and a final extension of 5 min at 72 ºC.
HC02198_t1	CAGGAAACAGCTATGACTAAACTTCAGGGTGA CCAAAAAATCA		
LC01490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. 1994	
HC02198	TAAACTTCAGGGTGACCAAAAAATCA		

90

Data analysis

Sequences were assembled and edited with the Codon Code Aligner v 3.0.3 software. Clustal W program was used for the sequence alignment using default parameters. Phred score [54] was used to assess the quality of the sequences applying the following categories: no sequences = failed, mean Phred <30 = low quality, 30< mean Phred <40 = medium quality, and mean Phred >40 = high quality. All data are available in the project FMN (Free-Living Marine Nematodes from Quintana Roo, Mexico) on Barcode of Life Data Systems (BOLD, www.boldsystems.org) [55].

Genetic divergence was calculated using Kimura two-parameter (K2P) distance model [56]. Presence of stop codons and indels were verified to discard any contaminants such as NUMTs (nuclear mitochondrial DNA segments) [57,58]. Basic Local Alignment Search Tool (BLAST) [59] and Identification Request, on GenBank and BOLD respectively, were used to identify matches to the sequences generated in this study.

Phylogenetic analysis

A Maximum-Likelihood (ML) tree was constructed in MEGA v6 software using 500 bootstrap replications. The tree was reconstructed with sequences generated in this study and others selected from GenBank according to length, quality, position, and taxonomy (Accession numbers are given in Supplement Table 1). The best-fitting substitution model was the General Time Reversible model with nonuniform evolutionary rates and invariant sites (GTR+G+I) and was chosen with MEGA. COI sequence from Plectus aquatilis was selected as the outgroup because it belongs to a different order of nematodes [60].

Algorithmic analysis

To delimit the putative species and test the resolution of the sequenced COI, we test three methods: (1) Barcode Index Number (BIN) system [40]; (2) Automatic Barcode Gap Discovery (ABGD) [39] with the following parameters: relative gap (X) of 1.1, minimal intraspecific distance (Pmin) of 0.001, maximal intraspecific distance (Pmax) of 0.1, K2P [56] and JC69 (Jukes-Cantor) [61] were selected as distance metrics; and (3) Poisson tree process model (mPTP) selecting single locus species delimitation with p-value 0.001 (http://mptp.h-its.org/#/tree) [41]. thical approval must list the authority that provided approval and the corresponding ethical approval code.

3. Results

3.1. Morphological species

A total of 29 putative morphological species from 102 individuals were identified from the sampled sites in the Mexican Caribbean (Table 3). The Enoplida order was the best represented, followed by Desmodorida, Chromadorida, and Monhysterida and Aerolaimida (Table 4). Among the sampled localities, Cozumel was the site with 12 species; in Cancún, two species were represented by several individuals (Rhips sp1 and Monoposthia mirabilis) (Fig. 2 and 3).

127

Table 3. Morphological species.

ORDER				
Enoplida	**Chromadorida**	**Desmodorida**	**Aerolaimida**	**Monhysterida**
Anticomid sp1	*Actinonema* sp1	*Catanema* sp1	*Odontophora* sp1	Monhysterid sp1
Cylicolaimus sp1	*Rhips* sp1	*Desmodoridae* sp1		Xyala sp1
Enoploides sp1	*Monoposthia mirabilis*	*Desmodoridae* sp2		
Enoploides sp2	*Prochormadorella* sp1	*Epsilonema* sp1		
Enoploides sp3	*Chromadorid* sp1	*Metachromadora* sp1		
Epacanthion sp1	*Chromadorid* sp2	*Metachormadora* sp2		
Halalaimus sp1	*Chromadorid* sp3	*Microlaimidae* sp1		
Metaparoncholaimus sp1		*Spirinia* sp1		
Oncholaimus sp1				
Oncholaimus sp2				
Platycoma sp1				

128

Table 4. Distribution of species identifies by site. 0=Absent 1=Present.

Species \ Sites	Cancún	Cozumel	Puerto Morelos	Tulum	Mahahual	Xcalak
Enoplida						
Anticomid sp1	0	0	1	0	0	0
Cylicolaimus sp1	0	1	0	0	0	0
Enoploides sp1	0	1	0	0	0	0
Enoploides sp2	0	1	0	0	0	0
Enoploides sp3	0	0	0	1	0	0
Halalaimus sp1	0	0	1	1	0	0
Oncholaimus sp2	0	0	0	1	0	0
Platycoma sp1	0	1	1	1	0	0
Chromadorida						
Rhips sp1	1	0	1	0	0	0
Monoposthia mirabilis	1	1	1	0	0	0
Chromadorid sp1	0	0	0	1	1	1
Desmodorida						
Catanema sp1	0	1	0	0	0	0
Desmodoridae sp1	0	1	0	0	1	1
Epsilonema sp1	0	1	1	1	0	0
Metachromadora sp1	0	1	0	0	1	0
Metachormadora sp2	0	0	0	0	1	0
Microlaimidae sp1	0	1	1	0	0	0
Spirinia sp1	0	0	0	0	0	1
Monhysterida						
Monhysterid sp1	0	1	0	0	1	0
Xyala sp1	0	1	0	0	0	0

Figure 2. *Monoposthia mirabilis*. Adult male. (A) Anterior part showing head and esophagus. (B) Posterior part showing tail and spicule.

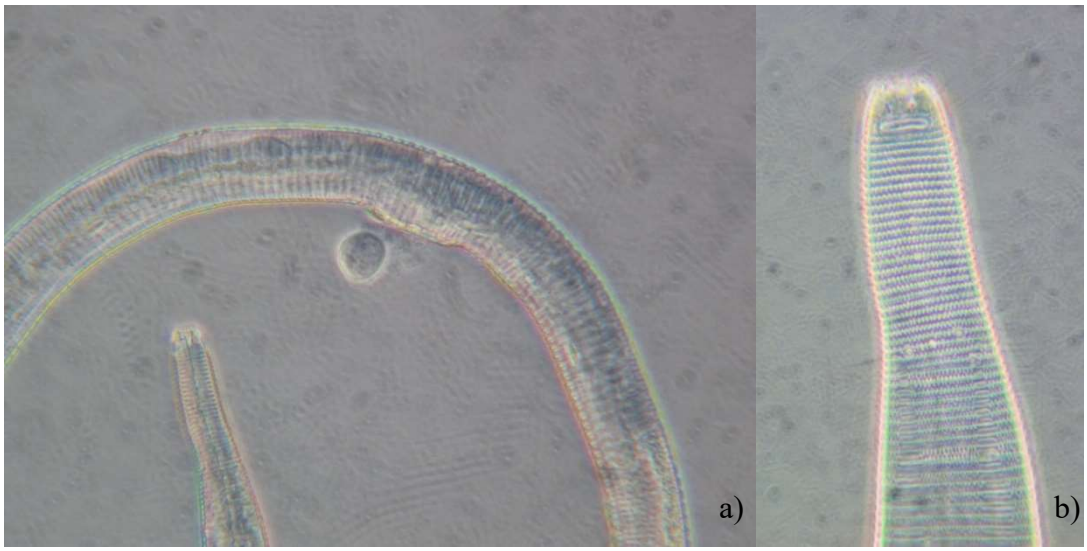

Figure 3. *Rhips* sp1. Adult female. (A) Head with teeth, middle part showing egg. (B) Head showing amphid, ornamented cuticle.

3.2. Amplification and Sequencing

DNA amplification was successful in 67 individuals. Fifty-five sequences were obtained from 20 morphological species; sequences with low quality were discarded (Fig. 4). The genera Actinonema sp1, Epacanthion sp1, Metaparancholaimus sp1, Odontophora sp1, Oncholaimus sp1, Prochormadorella sp1, and some Chromadorids and Desmodorids cannot be amplified (Fig. 4). All sequences are new for the genetic databases GenBank and BOLD. According to Phred score [54], all sequences were of high quality (mean Phred >40). Most of the sequences (91%) had a size greater than 600 bp (651-667 bp), and 9% fell between 502 and 596 bp. There were no stop codons in the alignment. BLAST analysis values were between 71% and 85% with Nematoda. The mean genetic divergence was 0.43% for intraspecific and 26.45% for interspecific, and we observe a clear barcoding gap (Fig. 5).

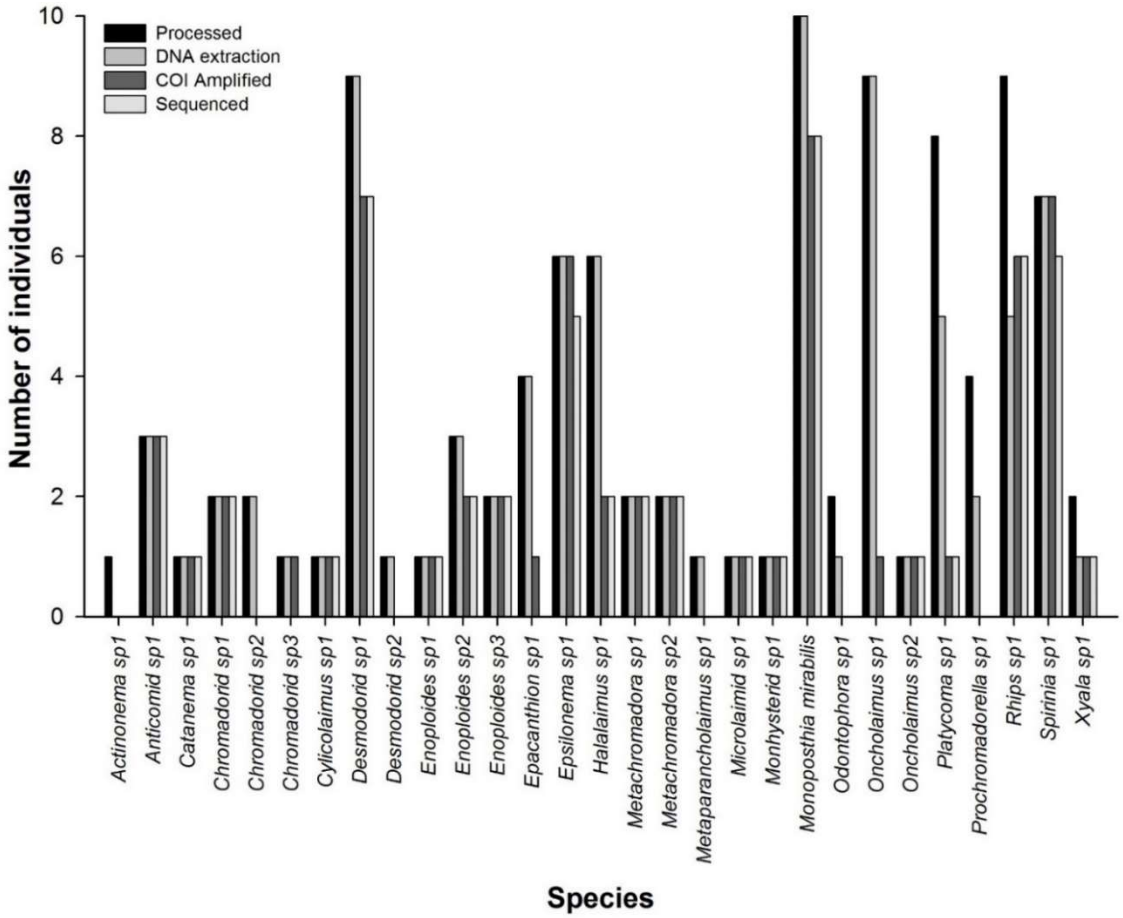

Figure 4. Number of individual free-living marine nematodes that have been successfully processed for DNA extraction, COI amplification, and sequencing.

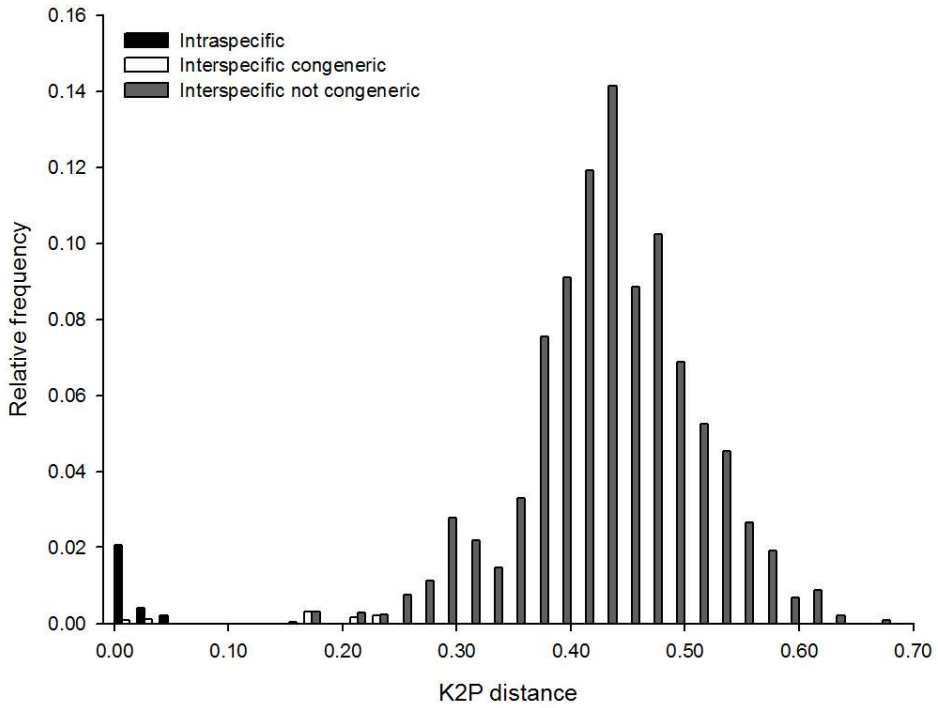

Figure 5. Relative frequencies of K2P distances within species (black), among congeneric species (white), and between species from different genera (grey).

3.3. Phylogenetic analysis

154 Maximum-Likelihood analysis using our 55 COI sequences generated from the present work
155 and an additional 34 sequences from GenBank showed that our sequences grouped in monophyletic
156 clusters represented by the morphological identity. The tree was strongly supported in recent clades
157 by high bootstrap values (>90%) (Fig. 6). The maximum distances values were observed on
158 Epsilonema sp1 clade (4.02%) and Enoploides sp2 (2.24%).

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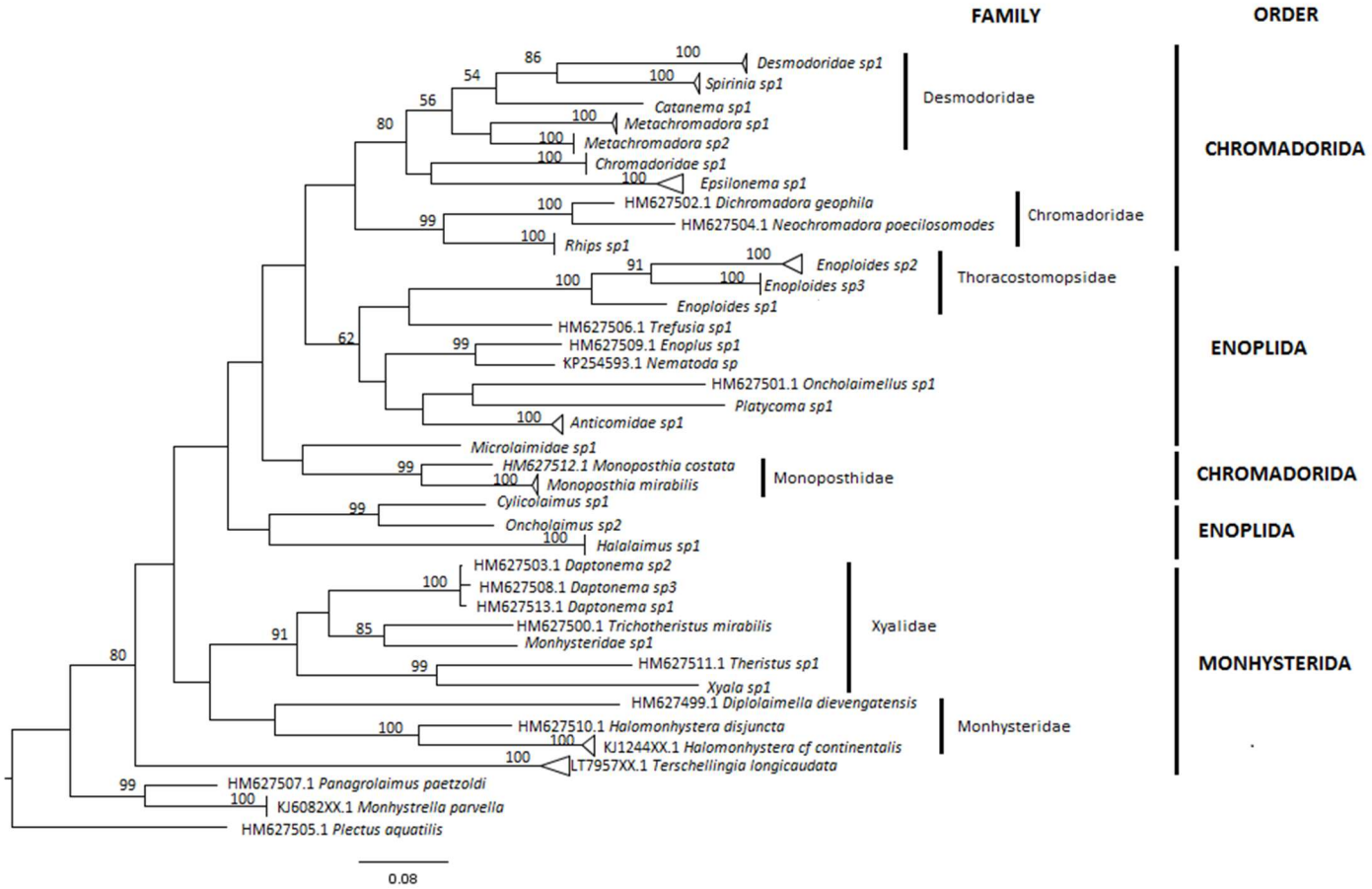

Figure 6. Simplified Maximum-likehood tree reconstructed from COI DNA sequences based on GTR+G+I. Sequences of marine nematodes from the present study are annotated according to their morphological identification. Numbers in parentheses refer to the number of specimens sequenced. Reference sequences obtained from GenBank are indicated with accession numbers. Higher taxon levels are indicated behind the line.

3.4. Delimitation methods

To ascertain the number of entities, the 55 sequences obtained in this work were analyzed using three different methods: ABGD, mPTP, and BINs. ABGD analysis with K2P showed 20 initial and 25 recursive partitions with prior maximal distances (PMD) of 0.0010, 20 initial and 21 recursive partitions with PMDs ranging from 0.0017 to 0.0028, and 20 initial and 20 recursive partitions with PMDs ranging from 0.0046 to 0.0129. With the JC69 model, the same results were observed (see Table 5). The mPTP method based in our phylogenetic tree with p = 0.001 recovered 20 groups for the 20 putative species. BINs split *Enoploides* sp2 and *Epsilonema* sp1: BOLD: AAU8181 (n = 1) and BOLD: AAU8183 (n = 1) for the first species, and BOLD: AAU8184 (n = 4) and BOLD: AAV1242 (n = 1) for the second. A total of 22 BINS were recovered.

Table 5. Entities recovered from the COI sequences with the three different delimitation algorithms.

			ALGORITHMICS																							
	BIN system	**mPTP Model**	**ABGD**																							
p-value		**0.001**																								
Subst.model			**K2P**										**JC69**													
Prior intraspecific divergence (P)			**0.0010**		**0.0017**		**0.0028**		**0.0046**		**0.0077**		**0.129**		**0.0010**		**0.0017**		**0.0028**		**0.0046**		0.0077		0.129	
Partition			I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R
COI-OTUs	22	20	20	25	20	21	20	21	20	20	20	20	20	20	20	25	20	21	20	21	20	20	20	20	20	20

4. Discussion

4.1. Diversity and distribution of meiofaunal marine nematodes in Mexico

This work contributes to improve current knowledge on the diversity and distribution of meiofaunal marine nematodes in Mexico. The faunistic information in Mexico, and in Central America generally, is traditionally scarce, reflecting the low number of taxonomic experts in the area [62]. Until now, a total of seven species has been described from Mexico [11, 63-65]. In the Mexican Caribbean specifically, only small amount of ecological and taxonomic data regard marine nematodes [13-16]. Isla Mujeres and Banco Chinchorro have been the most studied localities, reporting three orders, 17 families, 34 genera, and 51 species, and three orders, 17 families, 49 genera, and 82 species, respectively. The Chromadorida order has been the best represented in both sites [13-16].

This work adds seven new morphological records for the Mexican Caribbean, namely *Anticomid* sp1, *Cylicolaimus* sp1, *Oncholaimus* sp2, *Platycoma* sp1, *Catanema* sp1, *Enopliodes* spp., and *Metachromadora* spp. Individuals of *Desmodorid* sp1, *Chromadorid* sp1, and *Monhysterid* sp1, were not identified at a lower taxonomic level, and it is necessary to collect more specimens to obtain proper identification. The report of taxa belonging to Desmodoridae and Chromadoridae are particularly relevant. These two families are globally represented with high numbers of species (320 and 395, respectively); however, genetic sequences for these families are scarce compared with the number of species described [66,67]. Moreover, species identification within these two families is particularly difficult due to the lack of well-defined diagnostic traits, poor taxonomic keys reference, and absence of updated databases including species lists.

We report for first time meiofaunal nematodes from the Cozumel region, namely *Cylicolaimus* sp1, *Catanema* sp1, *Xyala* sp1, and two species of Enoploides. Interestingly, the molecular analyses allowed to establish that *Platycoma* sp1, *Monoposthia mirabilis*, Desmodoridae sp1, *Epsilonema* sp1, *Metachromadora* sp1, Microlaimidae sp1, and *Monhysterid* sp1 are distributed in Cozumel Island and along to the continental coasts in localities like Cancún, Puerto Morelos, Tulum, Mahahual, and Xcalak.

4.2 DNA Taxonomy

High-quality sequences were obtained by combining existing protocols developed for zooplankton eggs [50] and the protocol by Ivanova et al. [51] for invertebrates. Amplification was 82% successful (55 out of 67 individuals) for the "Folmer region," and 90% of sequences obtained were >600 bp in length. The HotSHOT technique in marine nematodes was successful in 96.55% of the species (28 out of 29 processed).

Importantly, sequences generated in this work are new for the public repository. This work provides the first record of mtCOI for 20 species of the orders Chromadorida, Desmodorida, and Enoplida. We observe low success in the DNA amplification of *Platycom*a sp1, *Oncholaimus* sp1, *Halalaimus* sp1, *Prochromadorella* sp1, *Epacanthion* sp1, and *Odontophora* sp1. This may be explained by the fact that the mitochondrial genome of nematodes is highly diverse and requires design and integration of more specific primers for amplification of genes [68-70].

Phylogenetic analysis supports that each putative species constitutes a monophyletic group and confirms congruence between morphological identification and molecular data. The Desmodoridae clade includes *Catanema* sp1, *Spirinia* sp1, *Metachromadora* sp1, and *Metachromadora* sp2, while that of Thoracostomopsidade includes *Enoplides* sp1, *Enoplides* sp2, and *Enoplides* sp3. *Monoposhia mirabilis* was a sister taxon to *M. costata* from the Monoposthiidae clade. The above showed a congruent topology with the phylogeny of the marine nematodes proposed by Medal and collaborators [71].

4.3. Application of models to disentangle species

Using molecular and species delineation approaches, our results confirm the advantage of disentangling species and estimating marine nematode diversity using the COI gene. Previous

works show that COI resolve better relationship in closely related species (e.g. Desmodorids; [66]). In agreement with Derycke et al. [60], COI is a useful biomarker to disentangle diversity at the species level. Future studies should consider multiple biomarkers to fully solve this issue [62, 32].

Analytical methods ABGD and mPTP recovered a perfect match between the 20 putative species and entities assigned. Both support the presence of all putative species. Though the ABGD method depends on user-selected parameters (distance model and prior limit on intraspecific divergence), we obtain congruent results with both models (K2P and JC69). We corroborated that the value of 0.01 for prior intraspecific divergence (P) has strongest congruence between groups recovered and species defined as proposed by Puillandre et al. [39].

The BINs method showed a 90% match of our putative species. Only *Epsilonema* sp1 and *Enoploides* sp2 were split in four BIN numbers. It is important to consider that this method employs initially single linkage clustering coupled with a 2.2% threshold to establish preliminary OTU boundaries [40]. If the threshold is higher, the method tends to separate a greater number of entities and overestimate diversity. *Epsilonema* sp1 and *Enoploides* sp2 both showed the major intraspecific value >4% (4.02% and 2.24% respectively), but morphologically they are identical. Considering all the criteria, we recognize that all individuals of *Epsilonema* sp1 and *Enoploides* sp2 belong to a single species, as was observed with ABGD and mPTP methods. In BIN numbers, the boundary is retrieved from all taxa available on BOLD database, but, until now, nematodes have been represented with few sequences. For this reason, we should carefully consider the BIN numbers as a tool for disentangling nematode species.

5. Conclusions

Our work contributes to build a more robust database of meiofaunal nematodes, as well as to focus and advance knowledge of meiofauna biodiversity. Barcode sequences from marine nematodes are underrepresented compared with the diversity of the phylum [22]. At present, molecular techniques have opened up new possibilities for taxonomic research in meiofaunal nematodes. Our study combined taxonomy and DNA to delimit the 20 species with robust and integrating parameters. Also, reporting new records from an unexplored region contributes to understanding patterns of diversity and distribution of nematodes worldwide. Although a new set of primers should be designed for some species, our data supports that the COI gene represents a good molecular marker to disentangle diversity. Finally, in the present work, we validate that the intraspecific distance value threshold is 5% as pointed out in previous studies [60, 66]

Author Contributions: "Conceptualization, Arely Martínez-Arce and Francesca Leasi; Data curation, Arely Martínez-Arce, Alberto De Jesús-Navarrete and Francesca Leasi; Formal analysis, Arely Martínez-Arce and Francesca Leasi; Funding acquisition, Arely Martínez-Arce; Investigation, Arely Martínez-Arce, Alberto De Jesús-Navarrete and Francesca Leasi; Methodology, Arely Martínez-Arce; Project administration, Arely Martínez-Arce; Software, Francesca Leasi; Validation, Alberto De Jesús-Navarrete and Francesca Leasi; Writing – review & editing, Arely Martínez-Arce, Alberto De Jesús-Navarrete and Francesca Leasi."

Funding: "This research was funded by the Consejo Nacional de Ciencia y Tecnología (CONACyT) through the Red MEXBOL project no. 271108 and The APC was funded by University of Tennessee at Chattanooga.

Acknowledgments: We thank Jose Angel Cohuo, Mario Yescas, and Blanca Prado for assistance during field work; Jessica Landeros for her help during editing; Ruth Gingold (sweepandmore.com) for proofreading earlier versions of the manuscript; and, finally, the Canadian Center of DNA Barcoding (CCBD) for support during the sequencing process.

Conflicts of Interest: "The authors declare no conflict of interest." "The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results".

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