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Advancing nematode barcoding: A primer cocktail for the cytochrome *c* oxidase subunit I gene from vertebrate parasitic nematodes

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Abstract

Although nematodes are one of the most diverse metazoan phyla, species identification through morphology is difficult. Several genetic markers have been used for their identification, but most do not provide species-level resolution in all groups, and those that do lack primer sets effective across the phylum, precluding high-throughput processing. This study describes a cocktail of three novel primer pairs that overcome this limitation by recovering cytochrome *c* oxidase I (COI) barcodes from diverse nematode lineages parasitic on vertebrates, including members of three orders and eight families. Its effectiveness across a broad range of nematodes enables high-throughput processing.

Keywords: barcoding, identification, nematodes, primers

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Introduction

Roundworms (Nematoda) are known to be among the most physiologically and ecologically diverse of metazoan phyla, occupying habitats from the deep sea to deserts, and from the tropics to polar permafrost (Brown *et al.* 1949, 1950; De Ley 2006; Dailey 2009; Asbakk *et al.* 2010; Vanreusel *et al.* 2010). The phylum includes free-living, parasitic, mutualistic, opportunistic and symbiotic taxa (Ott *et al.* 1991; Clarke 2008) and provides a useful model system for the study of human diseases (Fire *et al.* 1998; Barr 2005; Jadiya *et al.* 2011) and a tool for ecosystem surveillance (Sambongi *et al.* 1999; Marcogliese 2005; Ekschmitt & Korthals 2006; Wu *et al.* 2010; Denver *et al.* 2011; Hoess *et al.* 2011; Palm *et al.* 2011). However, nematodes are also a scourge as many species cause disease in crops, livestock and humans (Hodda & Cook 2009; Manguin *et al.* 2010). Despite their importance, the taxonomy of nematodes is poorly studied. Species-level identification has traditionally relied on detailed morphological analysis, a task requiring considerable expertise (Coomans 2000) given the morphological conservatism and small size of nematodes (Creer *et al.* 2010; Powers *et al.* 2011). Aside from being time-consuming, morphology-based identifications are often problematic because of high phenotypic plasticity (Coomans 2002; Nadler 2002), the absence of clear diagnostic characters (Wijova *et al.* 2005; Derycke *et al.* 2008) or their restriction to adults in the numerous groups in which larvae are more often encountered (Anderson 2000). Given these constraints, there is recognition that molecular techniques are critical for taxonomic progress (Godfray 2002; Blaxter 2003). Indeed, there are now online databases, such as NemATOL (http://nematol.unh.edu/), that are dedicated to organizing and storing ecological and molecular data of nematodes.

Several genetic markers have been used for nematode identification, including small and large subunit ribosomal DNA (SSU and LSU respectively), the internal transcribed spacer (ITS) region of ribosomal DNA and cytochrome *c* oxidase subunit I (COI) (Blaxter *et al.* 1998; Floyd *et al.* 2002; Subbotin *et al.* 2008; Elsasser *et al.* 2009; Ferri *et al.* 2009; Siddal *et al.* 2012). The ribosomal DNA small subunit (SSU) was the first marker used, and successfully delineated some nematodes but failed to completely explain previous observations based on morphology (Blaxter *et al.* 1998). As the use of SSU was expanded, it was discovered that the SSU barcode failed to separate many species of nematodes and was better suited for order or family-level discrimination (De Ley *et al.* 2005). The ribosomal DNA large subunit (LSU) was the second marker used in an attempt to develop a nematode phylogenetic classification system, but

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Table 1 Nematode specimens used in this study. Taxa identified using morphology. Classification follows Hodda (2011) unless indicated by *, in those cases classification follows Hodda (2007); ND: No Data

Order	Family	Genus	Number of specimens studied (successfully sequenced)	Host species	Locality	Collection date
Panagrolaimida	Rhabdiasidae	*Rhabdias* sp. 1	1 (1)	*Smilisca baudinii*	Colima: Hwy Colima -Minatitlan	7 July 2008
Panagrolaimida	Rhabdiasidae	*Rhabdias* sp. 2	4 (4)	*Rana* sp.	Nayarit: S of Hyw Barranca del Oro: Barranqueño bridge	25 June 2009
Panagrolaimida	Rhabdiasidae	*Rhabdias* sp. 3	5 (5)	*Rhinella marina*	Colima: Comala	7 July 2008
Panagrolaimida	Rhabdiasidae	*Rhabdias lamothei*	4 (4)	*Leptodeira* sp.	Colima: Hyw 98 Minatitlan -Manzanillo	8 July 2008
Rhabditida	Molienidae*	*Oswaldocruzia* sp.	9 (9)	*Phrynohyas venulosa* *Smilisca baudinii*	Colima: Hyw Colima-Minatitlan Colima: Hyw 98 Minatitlan -Manzanillo	6 July 2008 7 July 2008
Rhabditida	Diaphanocephalidae*	*Kalicephalus* sp.	2 (2)	*Leptodeira* sp. *Imantodes* sp.	Colima: Comala Colima: Ixtlahuacan	27 June 2009 24 June 2009
Spirurida	Heterakidae	*Strongyluris* sp.	1 (1)	*Trimorphodon biscutatus*	Colima: Hwy 98 Minatitlan -Manzanillo	8 July 2008
Spirurida	Pharyngodonidae	*Ozolaimus* sp.	5 (0)	*Ctenosaura* sp.	Colima: Hyw 54 Ixtlahuacan	8 July 2008
Spirurida	Pharyngodonidae	*Parapharyngodon* sp.	4 (4)	*Phrynohyas venulosa*	Colima: Hyw 98 Minatitlan -Manzanillo	8 July 2008
Spirurida	Pharyngodonidae	gen sp. 1	15 (10)	*Sceloporus* sp.	Jalisco: ND	25 July 2009
Spirurida	Pharyngodonidae	gen sp. 2	2 (2)	*Sceloporus formosus*	Veracruz: Hyw Xico Viejo- Matlalapa	28 July 2004
Spirurida	Cosmocercidae	*Aplectana* sp.	18 (17)	*Rana pustulosa* *Bufo* sp. *Leptodeira* sp.	Nayarit: S of Hyw Barranca del Oro: Barranqueño bridge Nayarit: Hyw Uzeta-La Gloria Colima: Comala	24 June 2009
Spirurida	Onchocercidae	*Foleyellides* sp.	11 (11)	*Rana pustulosa* *Rana psilonota*	Nayarit: S of Hyw Barranca del Oro: Barranqueño bridge Jalisco: Zapopan: Barranca del río Santiago	24 June 2009 30 June 2010
Spirurida	Physalopteridae	*Physaloptera* sp.	7 (7)	*Trimorphodon biscutatus*	Michoacan: Hwy 200 between La placita and Maruata	5 July 2008
Spirurida	Physalopteridae	gen sp. 1	5 (5)	*Sceloporus* sp.	Jalisco: ND	25 June 2009
Spirurida	Physalopteridae	gen sp. 2	1 (1)	*Imantodes* sp.	Colima: Hyw Comala -Minatitlán	25 June 2009
Spirurida	Physalopteridae	*Turgida* sp.	1 (1)	*Didelphis virginiana*	Jalisco: Zapopan: Barranca del río Santiago	30 June 2010

requires the amplification of multiple regions to be effective (De Ley *et al.* 2005; Subbotin *et al.* 2008). Similar studies using ITS revealed that a lack of phylum-wide primers combined with difficulties in aligning the extremely variable ITS sequences precluded its use as a universal nematode identification marker amenable to high-throughput platforms (Floyd *et al.* 2002; De Ley *et al.* 2005).

The mitochondrial gene cyctochrome *c* oxidase subunit I (COI) has also been explored as a potential marker on which to base a nematode phylogenetic classification system (Floyd *et al.* 2002; Elsasser *et al.* 2009). In addition to being a mitochondrial gene, COI is translated into an evolutionarily conserved protein and thus has some advantages over SSU, LSU and ITS. However, COI is not immune to the inherent problems associated with nematode barcoding. While the 5′ region of COI has been shown to separate nematodes into proper species (Derycke *et al.* 2010), a phylum-wide primer set has yet to be developed (De Ley *et al.* 2005). In this study, we report the development of a primer cocktail which enables the recovery of COI barcodes from a broad range of nematode parasites of vertebrates in a high-throughput manner and delivers species-level resolution.

Materials and methods

Specimen collection

Ninety-five adult nematodes collected in Mexico from various reptilian, amphibian and mammalian hosts were analysed (Table 1). Each specimen was collected in duplicate (i.e. from the same habitat within the same host), with one stored in 95% ethanol for DNA extraction and the other cleared on a glass slide with undiluted glycerine to enable identification to family, genus or species level using morphological characteristics (Table 1).

Primer design

Cytochrome *c* oxidase subunit I (COI) sequences were obtained from 56 mitochondrial genome sequences from nematodes in GenBank (Table 2) and aligned using online EBI CLUSTALW2 software (Larkin *et al.* 2007). A lepidopteran COI sequence was included in the alignment as a reference for locating the standard primer binding sites (Folmer *et al.* 1994) for COI barcoding (Hebert *et al.* 2003a,b). The forward and reverse primer binding sites were excised from the 56 sequences and

Table 2 Nematode COI sequences used to design cocktail primers

GenBank Accession	Species	GenBank Accession	Species
NC_008231	*Agamermis sp.* BH-2006	AJ556134	*Necator americanus*
FJ483518	*Ancylostoma caninum*	NC_003416	*Necator americanus*
NC_003415	*Ancylostoma duodenale*	GQ888716	*Oesophagostomum dentatum*
GQ398121	*Angiostrongylus cantonensis*	FM161883	*Oesophagostomum quadrispinulatum*
GQ398122	*Angiostrongylus costaricensis*	NC_001861	*Onchocerca volvulus*
NC_007934	*Anisakis simplex*	FN313571	*Radopholus similis*
NC_001327	*Ascaris suum*	NC_008640	*Romanomermis culicivorax*
NC_004298	*Brugia malayi*	NC_008693	*Romanomermis iyengari*
FJ483517	*Bunostomum phlebotomum*	EF175763	*Romanomermis nielseni*
NC_009885	*Caenorhabditis briggsae*	GU138699	*Setaria digitata*
EU407789	*Caenorhabditis briggsae*	NC_005941	*Steinernema carpocapsae*
EU407793	*Caenorhabditis briggsae*	DQ520860	*Strelkovimermis spiculatus*
EU407804	*Caenorhabditis elegans*	NC_008047	*Strelkovimermis spiculatus*
NC_001328	*Caenorhabditis elegans*	AJ558163	*Strongyloides stercoralis*
EU407805	*Caenorhabditis elegans*	GQ888717	*Strongylus vulgaris*
EU407780	*Caenorhabditis sp.*	GQ888718	*Syngamus trachea*
GQ888721	*Chabertia ovina*	GQ888720	*Teladorsagia circumcincta*
HM773029	*Chandlerella quiscali*	DQ520858	*Thaumamermis cosgrovei*
NC_004806	*Cooperia oncophora*	NC_008046	*Thaumamermis cosgrovei*
GQ888712	*Cylicocyclus insignis*	AM411108	*Toxocara canis*
NC_005305	*Dirofilaria immitis*	AM411622	*Toxocara cati*
EU281143	*Enterobius vermicularis*	AM412316	*Toxocara malaysiensis*
NC_010383	*Haemonchus contortus*	FJ664617	*Toxocara vitulorum*
NC_008534	*Heterorhabditis bacteriophora*	GU386314	*Trichinella spiralis*
NC_008828	*Hexamermis agrotis*	NC_002681	*Trichinella spiralis*
GQ888722	*Mecistocirrus digitatus*	GQ888719	*Trichostrongylus axei*
GQ888714	*Metastrongylus pudendotectus*	GQ888711	*Trichostrongylus vitrinus*
GQ888715	*Metastrongylus salmi*	NC_005928	*Xiphinema americanum*

Table 3 Primers used in this study. M13 tails are in lowercase bold

Primer	Sequence (5′→3′)	Reference
NemF1_t1	**tgtaaaacgacggccagt**CRACWGTWAATCAYAARAATATTGG	This study
NemF2_t1	**tgtaaaacgacggccagt**ARAGATCTAATCATAAAGATATYGG	This study
NemF3_t1	**tgtaaaacgacggccagt**ARAGTTCTAATCATAARGATATTGG	This study
NemR1_t1	**caggaaacagctatgact**AAACTTCWGGRTGACCAAAAAATCA	This study
NemR2_t1	**caggaaacagctatgact**AWACYTCWGGRTGMCCAAAAAAYCA	This study
NemR3_t1	**caggaaacagctatgact**AAACCTCWGGATGACCAAAAAATCA	This study
LCO1490_t1	**tgtaaaacgacggccagt**GGTCAACAAATCATAAAGATATTGG	Folmer *et al.* 1994
HCO2198_t1	**caggaaacagctatgac**TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al.* 1994
M13F	TGTAAAACGACGGCCAGT	Messing 1993
M13R	CAGGAAACAGCTATGAC	Messing 1993

phenograms for the two primer binding sites were generated using EBI CLUSTALW2. Both trees revealed three clusters (not shown) and the consensus sequence for each cluster was used to design a primer cocktail consisting of one primer for each cluster (i.e. three forward and three reverse primers). The three primer sequences in each cocktail were tailed with modified M13 sequences (Messing 1993) as described in Ivanova *et al.* (2007). The three forward and three reverse primers were mixed in a 1:1:1 ratio to make the final forward (C_NemF1_t1: NemF1_t1 + NemF2_t1 + NemF3_t1) and reverse (C_NemR1_t1: NemR1_t1 + NemR2_t1 + NemR3_t1) cocktails (Table 3).

DNA extraction, PCR amplification and sequencing

Total DNA was extracted from whole nematodes using standard glass fibre methods (Ivanova *et al.* 2006). After purification, 2 μL of DNA was added to a PCR reaction consisting of 6.25 μL of 10% D-(+)-trehalose dihydrate (Fluka Analytical), 2.00 μL of Hyclone ultra-pure water (Thermo Scientific), 1.25 μL of 10X PlatinumTaq buffer (Invitrogen), 0.625 μL of 50 mM $MgCl_2$ (Invitrogen), 0.125 μL of each primer or primer cocktail, 0.0625 μL of 10 mM dNTP (KAPA Biosystems) and 0.060 μL of 5 U/μL PlatinumTaq DNA Polymerase (Invitrogen) for a total reaction volume of 12.5 μL. Thermal cycling conditions were 94 °C for 1 min, five cycles at 94 °C for 40 s, 45 °C for 40 s, 72 °C for 1 min, followed by 35 cycles at 94 °C for 40 s, 51 °C for 40 s, 72 °C for 1 min and a final extension at 72 °C for 5 min. The resulting amplicons were visualized on a 2% agarose E-gel® 96 precast gel (Invitrogen) and bidirectionally sequenced using M13F and M13R as sequencing primers (Table 3).

Cycle sequencing was performed using a modified BigDye 3.1 Terminator (Applied Biosystems) protocol (Hajibabaei *et al.* 2005). Cycle sequencing conditions were 96 °C for 1 min followed by 35 cycles at 96 °C for 10 s, 55 °C for 5 s, 60 °C for 2.5 min and a final extension at 60 °C for 5 min. Sequencing was performed on an ABI 3730XL capillary sequencer (Applied Biosystems). Traces were assembled and edited using CodonCode v. 3.0.1 (CodonCode Corporation, Dedham, Massachusetts). Trace quality scores were calculated using KB Basecaller (ABI software) and trace statistics were calculated using Sequence Scanner (Applied Biosystems). Sequences have been deposited in BOLD (www.boldsystems.org) under sample ID's MXHEL359–MXHEL453 within the project entitled: Parasitic nematodes from Mexican vertebrates (NEMNP) and in GenBank under accession numbers KC130665 - KC130748.

Since previous studies (Elsasser *et al.* 2009; Derycke *et al.* 2010) reported varying success in barcode recovery with a commonly used primer set (LCO1490 and HCO2198, Folmer *et al.* 1994), we compared the success of sequence recovery with M13-tailed versions of LCO1490

Table 4 PCR success rates of nematode cocktail primers (C_NemF1_t1 + C_NemR1_t1) compared with Folmer primers (LCO1490_t1 + HCO2198_t1)

Primers	Number of PCR positives	Success Rate
C_NemF1_t1 + C_NemR1_t1	85/95	89.5%
LCO1490_t1 + HCO2198_t1	83/95	87.4%

Table 5 Sequencing success rates and trace quality scores of PCR products generated with nematode cocktail primers (C_NemF1_t1 + C_NemR1_t1) or Folmer primers (LCO1490_t1 + HCO2198_t1). Sequencing success rates were calculated by dividing the number of recovered sequences (after editing) by the total number of sequenced samples (i.e. 95)

Primers	Average PHRED Score	Success Rate (661 bp only)	Success Rate (any sequence over 100 bp)
C_NemF1_t1 + C_NemR1_t1	49	88.4%	88.4%
LCO1490_t1 + HCO2198_t1	44	65.2%	75.7%

and HCO2198 (Table 3) and our new cocktail. All PCR reagents were identical between the two primer sets, and the same DNA templates were employed. For each primer set, all 95 nematode samples were sequenced, even if an amplicon was not visible on the E-gel. Sequences were aligned using EBI CLUSTALW2, imported into MEGA5

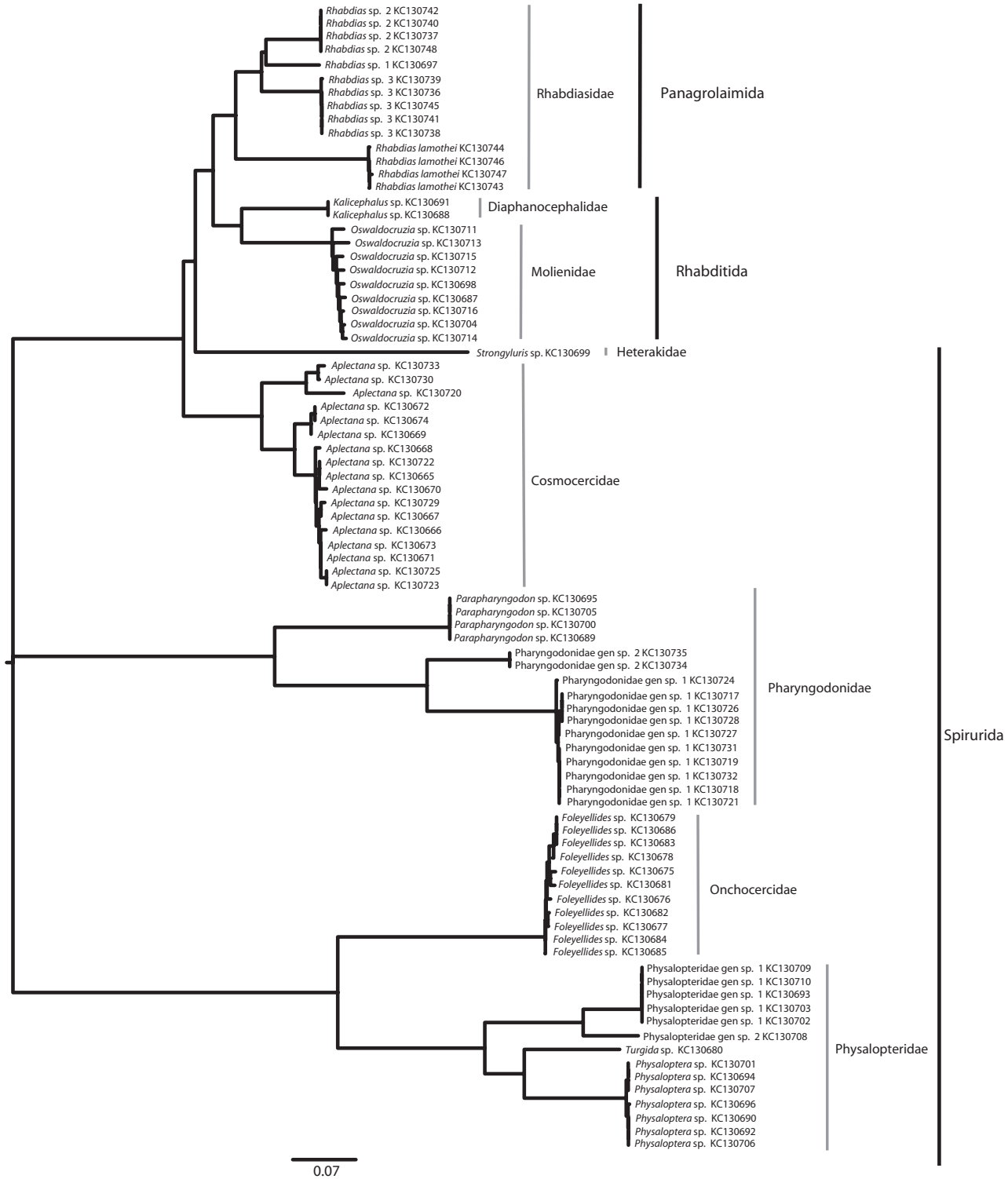

Fig. 1 Neighbour-joining tree of COI barcode sequences generated by the nematode cocktail primers. A divergence of 2% or greater is indicative of a separate operational taxonomic unit. Codes following names of taxa refer to GenBank accession numbers.

(Tamura *et al.* 2011), and a neighbour-joining algorithm (NJ) was used to generate a phenogram.

Results

PCR success rates (Table 4), as measured by the presence or absence of a visible amplicon on the E-gel, were very similar with the Folmer primers (87%) and the new primer cocktail (89%) (Fisher's exact test, $P = 0.8212$). However, there was a marked difference in sequence quality and recovery (Table 5). The traces produced by the primer cocktail ($n = 188$) had a mean PHRED score of 49 (SD = 12), whereas those produced by the Folmer primers ($n = 181$) had a mean PHRED score of 44 (SD = 12) (Student's t-test, $P = 0.0001$). However, any traces with PHRED quality scores between 40 and 50 are usually equally interpretable (personal observation). More importantly, full-length barcodes (661 bp) were recovered from 88% of the specimens with the new primer cocktail, but from just 65% of reactions which employed the Folmer primers (Fisher's exact test. $P = 0.0001$). DNA barcodes were obtained from a total of 84 specimens with 62 yielding full-length sequences with both primer sets, while 12 were only recovered by the cocktail, and another 10 were fully recovered by the cocktail but only partially (~500 bp) by the Folmer primers. Every sequence generated by the cocktail allowed the assignment of its source specimen to an operational taxonomic unit that agreed with its morphological identification (Martínez-Salazar 2008; Velarde-Aguilar, personal observation) (Fig. 1). Individuals from all genera were successfully barcoded except *Ozolaimus* (Table 1); its failure may reflect poor DNA preservation since we observed that ethanol partially evaporated from the vial that kept these specimens, and sequences were successfully recovered from members of closely related genera.

Discussion

The Nematoda may be the most species-rich phylum of animals, with approximately 27 000 described species (Hugot *et al.* 2001; Hodda 2011), but taxonomic knowledge must progress significantly to validate this hypothesis. The ribosomal DNA small subunit (SSU) (Blaxter *et al.* 1998), large subunit (LSU) (Subbotin *et al.* 2008) and internal transcribed spacer (ITS) region (Floyd *et al.* 2002; De Ley *et al.* 2005) have all been used as a tool for species discrimination, but the lack of phylum-wide primers or their failure to delineate closely allied species in certain nematode groups limit their utility in the analysis of nematode diversity (De Ley *et al.* 2005). The COI gene has also been explored as a potential marker for species identification (Floyd *et al.* 2002; Elsasser *et al.* 2009) because of its effectiveness in other major animal phyla (e.g. Campagna *et al.* 2010; Clare *et al.* 2011; Kumar *et al.* 2012; Weigt *et al.* 2012). The barcode region of COI has delivered species-level resolution in certain nematode lineages (Derycke *et al.* 2010), but sequence recovery has proven difficult (De Ley *et al.* 2005). The primer cocktail developed in this study appears to overcome this difficulty as it recovered full-length barcode sequences from nematodes belonging to three orders and eight families (Fig. 1), while 25% of the PCR products from Folmer primers contained co-amplified sequences, perhaps reflecting poor binding with the target COI gene. Moreover, the sequences recovered from our cocktail were able to differentiate congeneric species, such as the four species of *Rhabdias*, each from a different host and showing consistent morphological differences as detected by Martínez-Salazar (2008) (Fig. 1; Table 1). Although we examined various taxa of nematodes parasitic of vertebrates, further testing is required to validate the effectiveness of our primer set across the phylum. We examined representatives from three of the six currently recognized nematode orders parasitic on vertebrates (Hodda 2011), all representatives of the Class Chromadorea. However, it is possible that our primer cocktail is effective across a large diversity of nematodes because the primers were designed based on members of the Class Dorylaimea and other orders of Chromadorea of medical and veterinary importance. An obvious next step will involve testing barcode recovery from representatives of other orders of parasitic and free-living nematodes.

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S.W.J.P. wrote the initial manuscript. M.G.V.A., V.L.R. and P.D.N.H. edited and contributed to the manuscript. M.G.V.A. and V.L.R. collected and identified all nematode specimens. S.W.J.P. designed nematode primers and performed all molecular laboratory work and sequence editing/interpretation. M.G.V.A. and V.L.R. analysed and interpreted genetic-distance results.

Data Accessibility

DNA sequences: GenBank accessions KC130665 - KC130748 and BOLD (www.boldsystems.org) sample ID's MXHEL 359-MXHEL453.

A spreadsheet with sampling and taxonomic details for each individual, GenBank accession numbers for its DNA sequences, and its BOLD entry uploaded as online supplementary material.

DNA sequence alignment used to design primers, final DNA sequence alignment and phylogenetic data: BOLD (www.boldsystems.org) project 'Parasitic nematodes from Mexican vertebrates' (NEMNP).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Specimen collection and taxonomic details. BOLD process ID's and GenBank accession numbers are listed for each specimen.