Genetic and morphological characterisation of a new species of the genus *Hysterothylacium* (Nematoda) from *Paralichthys isosceles* Jordan, 1890 (Pisces: Teleostei) of the Neotropical Region, state of Rio de Janeiro, Brazil

Marcelo Knoff[1], Nilza Nunes Felizardo[1], Alena Mayo Iñiguez[2]/+, Arnaldo Maldonado Jr[3], Eduardo José L Torres[4], Roberto Magalhães Pinto[1], Delir Corrêa Gomes[1]

[1]Laboratório de Helmintos Parasitos de Vertebrados [2]Laboratório de Genética Molecular de Microorganismos [3]Laboratório de Mamíferos Silvestres Reservatórios, Instituto Oswaldo Cruz-Fiocruz, Av. Brasil 4365, 21045-900 Rio de Janeiro, RJ, Brasil [4]Laboratório de Biologia de Helmintos Otto Wucherer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

Taking into account the difficulties of taxonomic identification of larval anisakid nematodes based on morphological characters, genetic analyses were performed, together with those usually applied, in order to identify anisakid larvae found in the flounder Paralichthys isosceles *from the littoral of the state of Rio de Janeiro, Brazil. The analysis of 1,820 larvae revealed a new species, similar to* Hysterothylacium *MD,* Hysterothylacium *2,* Hysterothylacium *KB and* Hysterothylacium *sp regarding the absence of the larval tooth, an excretory pore situated below the nerve ring level, and slender lateral alae. Moreover, the new species differs from* Hysterothylacium fortalezae *and* Hysterothylacium reliquens *with regard to the number and size of spines present on the tail end and from* Hysterothylacium patagonicus *by the absence of interlabia. The maximum parsimony and neighbour joining tree topologies based on the 18S ribosomal DNA gene, complete internal transcribed spacer region and cytochrome oxidase 2 (COII) gene demonstrated that the Brazilian larvae belong to Raphidascarididae and represent a unique genetic entity, confirmed as a new* Hysterothylacium *species. Furthermore, the new species presents COII genetic signatures and shares polymorphisms with Raphidascarididae members. This is the first description of a new anisakid species from Brazil through the integration of morphological and molecular taxonomy data.*

Key words: Raphidascarididae - *Hysterothylacium* - new species - morphology - molecular taxonomy

The study of larval anisakids infecting fishes raises many doubts related to their taxonomic identification and, thus, promotes the proposal of different nomenclatures, thereby increasing difficulties in obtaining their proper specific diagnosis. More than 60 species of the genera *Hysterothylacium* (Raphidascarididae Hartwich, 1954, *sensu* Fagerholm, 1991), which parasitize estuarial, freshwater and marine fishes, have been described around the world (Gopar-Merino et al. 2005). However, *Hysterothylacium* larvae suffered from indefinite taxonomy and are frequently mistaken with the *Contracaecum* genus (Lopes et al. 2011). In South American countries, larvae and adults of *Hysterothylacium* sp. have already been reported from Ecuador, *Hysterothylacium corrugatum* (Deardorff & Overstreet 1981), Argentina, *Hysterothylacium rhamdiae* (Brizzola & Tanzola 1995), *Hysterothylacium patagonense* (Moravec et al. 1997), *Hysterothylacium aduncum* (Incorvaia & Hernández 2006) and from Chile, *Hysterothylacium geshei* (Torres et al. 1998), *Hysterothylacium winteri* (Torres & Soto 2004) and *H. aduncum* (Torres et al. 2010). In Brazil, nematode adults of *Hysterothylacium fortalezae* (Klein 1973) and *Hysterothylacium reliquens* (Norris & Overstreet 1975) were also recovered from teleosteans on the northeastern coast (Guimarães & Cristofaro 1974, Deardorff & Overstreet 1980). However, a number of *Hysterothylacium* sp. larvae have already been reported in 28 teleostean fish species from the littoral of the state of Rio de Janeiro (RJ) (Tavares & Luque 2006). In the same region, *Hysterothylacium* sp. larvae were reported as the most prevalent anisakid nematode in *Pagrus pagrus*, a teleostean fish with significant commercial value (Saad & Luque 2009).

Anisakiasis is considered a zoonosis that can affect humans after the ingestion of raw, poorly cooked or smoked fish meat infected with L_3 larvae. Yagi et al. (1996) reported a case of human infection from Japan due to the ingestion of a fish parasitized with *H. aduncum* (Rudolphi, 1802). Until now, no cases of anisakiasis infection have been reported in Brazil. Overstreet and Meyer (1981) described haemorrhagic lesions and eosinophilia caused by *Hysterothylacium* larvae type MB, recovered from *Paralichthys lethostigma*, in a rhesus monkey experimental animal model. Recently, pathological alterations caused by *Hysterothylacium* larvae were observed in *Paralichthys isosceles* on the southeastern Brazilian coast (Felizardo et al. 2009b). These *Hysterothylacium* larvae were found parasitiz-

Financial support: CNPq, FIOCRUZ
AMI and RMP are CNPq research fellows.
+ Corresponding author: alena@ioc.fiocruz.br
Received 10 May 2011
Accepted 9 August 2011

ing different sites, including musculature and ovaries, with high prevalence. However, morphological analysis, with approaches including epidemiological and zoonotic aspects, revealed the necessity of adopting additional methodologies to permit a more reliable taxonomic identification of these Brazilian larvae.

Genetic and morphological analyses have been utilised in the present investigation, aiming at the proper identification of *Hysterothylacium* larvae parasitizing specimens of *P. isosceles* captured in the littoral of RJ.

MATERIALS AND METHODS

Studied material - From October 2006-March 2008, 1,820 larvae of anisakids of the genus *Hysterothylacium* were recovered from 60 specimens of the flounder *P. isosceles* Jordan, 1890, captured in the littoral of the municipality of Angra dos Reis, RJ (21º15'S 23º23'S, 40º29'W 44º28'W). For morphological analysis, nematodes were fixed, clarified and preserved in accordance with Eiras et al. (2006). The taxonomic identification followed Peter and Maillard (1988), Incorvaia and Díaz de Astarloa (1998), Timi et al. (2001), Bicudo et al. (2005) and Felizardo et al. (2009a). Measurement ranges are in millimetres, with means in parentheses unless otherwise indicated. The nematode larvae reserved for molecular procedures were collected alive and free in the intestine, then were rinsed in a 0.65% NaCl solution, observed under a stereomicroscope and preserved at -20ºC until DNA extraction.

Differential interference contrast (DIC) - Samples were clarified and photographed using an Axiophot Zeiss with micrographic system in a DIC apparatus (Zeiss, Germany).

Scanning electron microscopy - Samples fixed in 70% ethanol were dehydrated in an ethanol series (100º GL), CO_2 critical-point dried, coated in gold and examined and photographed using a scanning electron microscope (JEOL SM-25 SII and Zeiss 962) under an accelerating voltage of 15 kvolts.

Statistical analysis - Pearson's correlation coefficient (*r*) was applied to correlate the total length of the parasites with the biometry of their internal organs to determine the central tendency and dispersion of the measurements in order to ensure that the sample represents the population of larvae (L_3/L_4). A t test with a 5% level of significance was used to determine the correlation coefficient (Serra-Freire 2002).

Deposit of the studied material - Representative specimens were deposited in the Helminthological Collection of the Oswaldo Cruz Institute-Oswaldo Cruz Foundation (CHIOC-Fiocruz).

Genetic analysis – Eleven *Hysterothylacium* larvae were washed with phosphate buffered saline and homogenised in liquid nitrogen for DNA extraction. DNA was extracted individually using a QIAamp® DNA Mini Kit (Qiagen) following the manufacturer's protocol with modifications, as described by Iñiguez et al. (2011). Polymerase chain reaction (PCR) targeting the 18S ribosomal DNA (rDNA) gene, the complete internal transcribed spacer (ITS) region and the cytochrome c oxidase subunit II (*cox*2) gene were performed. The 18S rDNA gene (~1,500 bp) was amplified using the primers SSU-A (forward; 5'-AAAGATTAAGCCATGCATG-3´) and 18P (reverse; 5'-TGATCCWKCYGCAGGTTCAC-3') (Dorris et al. 2002) and the following PCR conditions: 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 250 ng of each primer, 1.5 U Platinum *Taq* Polymerase (Invitrogen) and 25-50 ng of genomic DNA in a volume of 50 µL. The reactions were subjected to an initial cycle of 5 min at 96°C, followed by 40 cycles of 96°C for 30 s, 60°C for 30 s and 72°C for 1 min 50 s in a programmable thermal controller (Mastercycler ep system, Eppendorf). Products were electrophoresed through 1.2% agarose gels and visualised using ethidium bromide staining. The ITS region (~1,000 bp) was amplified using NC5 (forward; 5'-CACCAACTCTTAAAATTATC-3') and NC2 (reverse; 5'- TTTTCTAGTTATATAGATTGRTTTYAT-3') (Zhu et al. 1999) and the PCR and electrophoresis conditions described above. The *cox*2 gene fragment (629 bp) was amplified using 210 (forward; 5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and 211 (reverse; 5'-TTAGTTTCTTTTCCTCCGCT-3') (Nadler & Hudspeth 2000) and 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 300 ng of each primer, 1.5 U Platinum *Taq* Polymerase and 25-50 ng of genomic DNA, in a volume of 50 µL. The reactions were subjected to an initial cycle of 5 min at 96°C, followed by 40 cycles of 96°C for 40 s, 45°C for 40 s and 72°C for 40 s in programmable thermal controller. Both strands of the *Hysterothylacium* PCR products were directly sequenced using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with a 3730 Automated DNA Sequencer (Applied Biosystems, USA).

The sequences were analysed using a global Basic Local Alignment Search Tool search (National Center for Biotechnology Information database) and BioEdit v7.0.4.1 (Department of Microbiology, North Carolina State University, USA). Genetic distance [p-distance, Kimura 2 parameters (K2P)], neighbour joining (NJ) K2P and maximum parsimony (MP) trees were estimated using Molecular Evolutionary Genetics Analysis v. 4.0 software by bootstrap procedures (2,000 replicates) (Tamura et al. 2007). All *Hysterothylacium* sp. sequences of 18S rDNA, ITS and *cox*2 available on the GenBank database were used in the analysis (February 2011). *cox*2 gene sequences were translated using the invertebrate mitochondrial translation code and the cytochrome oxidase 2 (COII) protein thus produced was examined for genetic signatures using GeneDoc software v. 2.6.002 (ps.edu/biomed/genedoc). The new *Hysterothylacium* sp. Brazilian sequences were deposited into the GenBank database with accessions JF718550 and JF730199-JF730214.

RESULTS

Hysterothylacium deardorffoverstreetorum sp. nov. (Figs 1-3)

Description - Description based on 54 third-stage larvae: cuticle with lateral alae extending along the body with a wedge-shaped support, devoid of basal extension. Anterior extremity with a dorsal and two poorly developed ventrolateral lips (Fig. 3A). Cephalic papil-

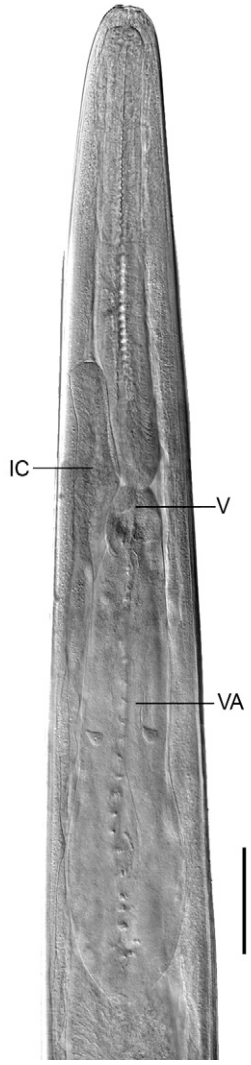

Fig. 1: *Hysterothylacium deardorffoverstreetorum* sp. nov., photomicrograph of anterior portion of a fourth stage larva by differential interference contrast. IC: intestinal cecum; V: ventriculus; VA: ventricular appendix. Bar = 200 µm.

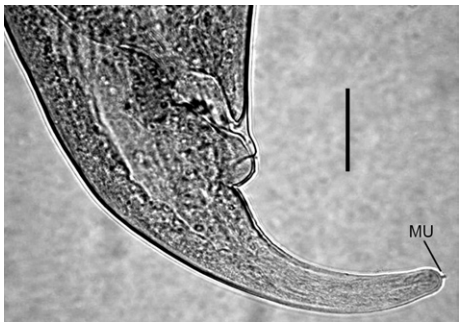

Fig. 2: *Hysterothylacium deardorffoverstreetorum* sp. nov., photomicrograph of posterior end by differential interference contrast. MU: mucron on the posterior end of third stage larva. Bar = 50 µm.

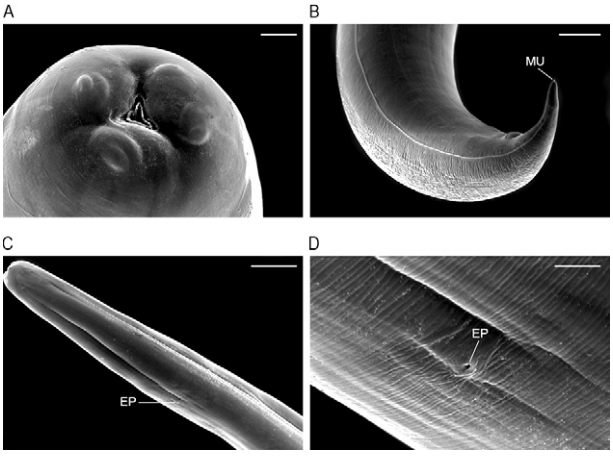

Fig. 3: *Hysterothylacium deardorffoverstreetorum* sp. nov., photomicrographs of third stage larva by scanning electron microscopy. A: anterior end, frontal view; B: mucron (MU); C: anterior portion, local of excretory pore (EP); D: detail of EP. Bars = 10 µm (A, D), 50 µm (B) and 100 µm (C).

lae (Fig. 3A), two pairs in the dorsal lip together with a large papilla and a pair in each ventrolateral lip. Boring tooth absent (Figs 1, 3A). Excretory pore opening below the nerve ring (Fig. 3C, D). Ventriculus nearly spherical (Fig. 1). Ventricular appendix twice as long as the oesophagus (Fig. 1). Intestinal caecum present (Fig. 1). Tail conical, mucron present (Figs 2, 3B). Description based on 50 fourth-stage larvae: morphological characteristics similar to those present in third-stage larvae were observed, except for the more developed lips (Fig. 3A) and the presence of a caudal multispinous process responsible for the cactus-tail format in the former. Additional morphological data and drawings, including anterior portion, posterior end and cross section, are in Felizardo et al. (2009a), as well as the parasitological indexes of prevalence, mean intensity, mean abundance and range of infection. The third-stage and fourth-stage larvae (measurements based on 1,820 specimens): total length 3.62-16.7 (10.1), width 0.11-0.40 (0.25). Oesophagus 0.23-1.16 (0.69) in length, 0.04-0.19 (0.12) in width. Ventriculus 0.05-0.15 (0.10) in length. Ventricular appendix 0.35-1.37 (0.86). Intestinal caecum 0.05-0.32 (0.18). Nerve ring and excretory pore situated 0.12-0.46 (0.29) and 0.25-0.46 (0.36), respectively, from the anterior end. Tail 0.10-0.32 (0.20). Mucron 3-8 µm (5 µm).

Type-host - *P. isosceles* Jordan, 1890.

Sites of infection - Abdominal cavity, abdominal musculature, stomach, stomach mucosa, mesentery, intestine, heart serosa, kidney serosa, liver serosa, ovary, ovary serosa and spleen serosa.

Type-locality - Municipality of Angra dos Reis.

Type data and depository - The holotype and paratypes are deposited in the CHIOC-Fiocruz under the registration 37523a, holotype and 37523b-e, 35771, paratype. The accessions in GenBank are JF718550 and JF730199-JF730214.

Etymology - The Latin name *deardorffoverstreetorum* is given after Drs Thomas L Deardorff and Robin M Overstreet, for their contributions to the knowledge of this group of nematodes.

Remarks - Based on the statistical analysis that determined the external validity of the sample, it can be confirmed that, with 5% probability of error type I, there are significant correlations regarding the following: (i) the body length of the parasite compared with the length of the ventriculus ($r = 0.6904$ in L_4, 0.6184 in L_3) and the intestinal caecum ($r = 0.7207$ in L_4, 0.5415 in L_3), (ii) the oesophagus length compared to the length of the ventriculus ($r = 0.6701$ in L_4 and 0.7462 in L_3), intestinal caecum ($r = 0.6690$ in L_4, 0.6532 in L_3) and ventricular appendix ($r = 0.6089$ in L_4, 0.7792 in L_3), (iii) length of the ventriculus and intestinal caecum ($r = 0.6872$ in L_3 and 0.4949 in L_4). The correlation of the ventriculus length with the ventricular appendix was significant in L_4 ($r = 0.7578$) and accentuated in L_3 ($r = 0.8122$); conversely, the correlation of the body length and the length of the oesophagus was accentuated in L_4 ($r = 0.8614$) and significant in L_3 ($r = 0.6981$). There was an outstanding correlation regarding the body length of the fish and the oesophagus length in the L_4 larvae ($r = 0.4664$), which was unimportant in the case of larval L_3 nematodes ($r = 0.2356$). Also, the correlation of the length of the ventricular appendix with the intestinal caecum was considerable ($r = 0.5068$ in L_4, $r = 0.5759$ in L_3), as well as with the body length of the parasite ($r = 0.5942$ in L_4) and remarkable in L_3 ($r = 0.7223$). The other correlations, although inconsistent ($0.16 < r < 0.30$), are not to be disregarded ($0 < r < 0.15$). Therefore, the results of statistical analysis showed that L_3 and L_4 larvae belong to the same species and that observed variations are related to the vital conditions to which the populations were submitted in the considered environment.

Genetic analysis - Samples of *Hysterothylacium* sp. nov. yielded the expected PCR products of the three genetic regions analysed. The 18S rDNA sequences (1,479 bp) were identical and matched the 18S rDNA *Hysterothylacium* sp. sequences available, with 99% similarity to *Hysterothylacium pelagicum* (U94375) and 98% similarity to *H. reliquens* (U94376) and *H. fortalezae* (U94374). Nematodes studied here revealed low genetic distance values between other Raphidascarididae spp studied so far, including *Raphidascaris acus* (p-distance = 0.01), *Iheringascaris inquies* (p-distance = 0.03) and *Goezia pelagia* (p-distance = 0.06). The MP tree showed that the new species of the genus *Hysterothylacium* and the other anisakid species are in a unique cluster characterising the Raphidascarididae, with a high bootstrap value (Fig. 4). The genus *Hysterothylacium* was not monophyletic, with a clade containing *H. pelagicum*, *R. acus*, *I. inquies* and *G. pelagia* and another clustering *H. reliquens* and *H. fortalezae* (Fig. 4).

The ITS sequences (867 bp) from the new species revealed six ITS haplotypes with a mean genetic distance of 0.003 (p-distance = 0.001-0.007). The MP and NJ trees were inferred based on ITS (758 bp) using 18 Ascaridida ITS sequences, including all available *Hysterothylacium* species: *H. aduncum*, *Hysterothylacium auctum, Hysterothylacium bidentatum*, *Hysterothylacium* (*Contracaecum*) *muraenesoxi* and *Pseudoterranova decipiens* as an outgroup. The MP tree revealed that all *H. deardorffoverstreetorum* sp. nov. grouped in a monophyletic clade with a high bootstrap value (99%) (Fig. 5). Specimens were included in a main cluster (bootstrap = 89%) with *H. muraenesoxi* and *R. acus* species. Another well-supported and large cluster (98%) is formed by *Hysterothylacium* species alone. *Contracaecum* and *Raphidascaris* species (except *R. acus*) are in particular clusters basal to the *Hysterothylacium* groups. The NJ (K2P) tree showed similar topology, with sequences grouped in a strongly supported monophyletic clade (bootstrap = 100%), but the *Raphidascaris* cluster is placed in the largest *Hysterothylacium* group (data not shown).

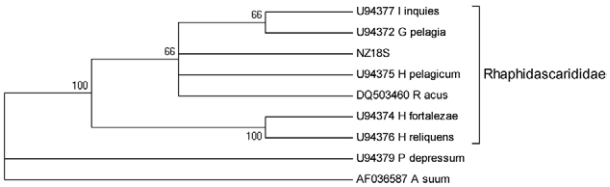

Fig. 4: maximum parsimony tree inferred from 18S ribosomal DNA sequence data from *Hysterothylacium deardorffoverstreetorum* sp. nov. and other Raphidascarididae spp studied. Phylogenetic analyses were conducted in MEGA4. *H. deardorffoverstreetorum* sp. nov. is named as NZ 18S. GenBank accession and the species name are shown. *Ascaris suum* and *Porrocaecum depressum* are outgroups.

The *cox*2 sequences (506 bp) of the new species have a mean genetic distance of 0.03 (0.01 SE) and the K2P distance vs. *H. fortalezae* was K2P = 0.18 (0.01 SE). K2P genetic distances of *H. deardorffoverstreetorum* sp. nov. vs. *H. reliquens* and *H. pelagicum* were higher, with 0.21 (0.01 SE) and 0.23 (0.01 SE), respectively. The tree topologies generated using both MP and NJ methods allocated the new species in a monophyletic clade with a well-supported bootstrap value (99%) and not closely related to other *Hysterothylacium* sp. (Fig. 6) (NJ tree, data not shown). On the MP tree, genus-specific clusters with moderate and strong support were delineated in the genera *Anisakis*, *Ascaris*, *Hysterothylacium* (*H. reliquens* and *H. pelagicum*) and *Toxocara*. The same genus-specific clades, plus a *Contracaecum* clade (bootstrap = 68%), were observed in the NJ analysis (bootstrap > 70%), although the *Hysterothylacium* clade was poorly supported (data not shown). Protein *in silico* analysis of the new species revealed a 168 amino acids COII sequence from positions 51-218 (reference *Ascaris suum*, Genbank X54253) (Fig. 7). The comparison of 20 anisakid COII sequences demonstrated that members of Raphidascarididae contain the combined genetic signatures V69, M77, I80 and N92, except for the highly polymorphic *H. fortalezae* (V69, I77, V80 and S92). The new *Hysterothylacium* specimens share the Raphidascarididae signatures, but also present two particular polymorphisms of isoleucine at positions I133 and I194, constituting genetic signatures (Fig. 7). Other COII genetic signatures, L171 and V232, were also found in *Contracaecum* and *Toxocara* species, respectively.

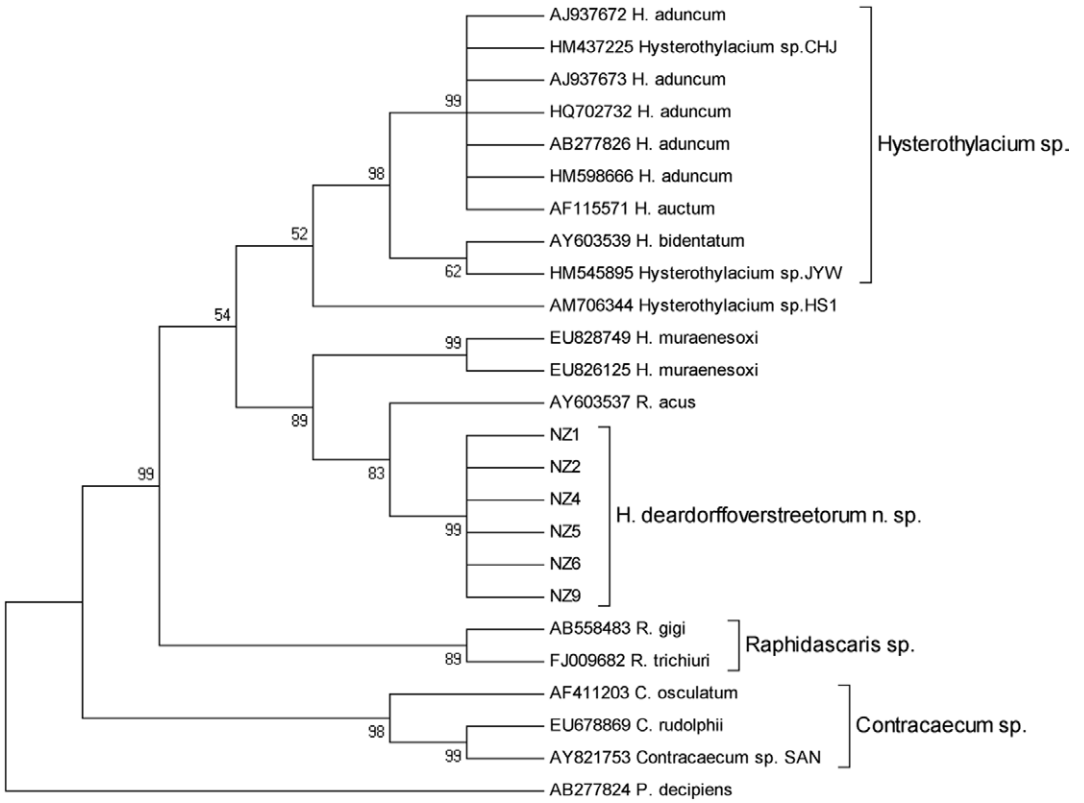

Fig. 5: maximum parsimony tree inferred from internal transcribed spacer (ITS) region sequence data from *Hysterothylacium deardorffoverstreetorum* sp. nov. and other Raphidascarididae spp studied. Phylogenetic analyses were conducted in MEGA4. Only ITS haplotypes from *H. deardorffoverstreetorum* sp. nov. sequences were represented. ITS haplotype sequence from NZ2 was identical to NZ3, NZ7-8 and NZ10-NZ11. GenBank accession and the species name are shown. *Pseudoterranova decipiens* is the outgroup taxa.

DISCUSSION

Larvae of *Hysterothylacium* recovered from *P. isosceles* are here described as a new species, taking into account the defined morphological characteristics of the species. These larvae are similar to *Hysterothylacium* MD of Deardorff and Overstreet (1981), *Hysterothylacium* 2 of Petter and Maillard (1988), *Hysterothylacium* KB of Petter and Sey (1997) and *Hysterothylacium* sp. of Pereira Jr et al. (2004) due to the absence of a larval tooth, an excretory pore located below the nerve ring and the presence of slender lateral alae, together with similarities related to the body size, oesophagus, intestinal caecum, ventricular appendix and rounded tail with mucron. Among the species reported in South America, the third and fourth-stage larvae presently studied and that were recovered from flounders in the Brazilian littoral differ from larvae of *H. fortalezae* (Klein 1973) by the absence of lateral alae in the cephalic region and the presence of six caudal spines and from specimens of *H. reliquens* (Norris & Overstreet 1975), which present numerous spines in the posterior end of the tail (Deardorff & Overstreet 1981). Also, considering the absence of interlabia, they differ from the fourth-stage larvae of *H. patagonense* (Moravec et al. 1997), which infect specimens of *Percichthys trucha* and from the larvae of *H. winteri* (Torres & Soto 2004), in *Eleginops maclovinus*.

The structures that are present in the caudal tip of some species can change in the different stages ($L_3 \rightarrow L_4 \rightarrow$ adults). Thus, the L_3 with mucron and the L_4 with a spiny cluster differ from L_3 and L_4 larvae of *H. fortalezae*, the former presenting six spines and the latter with a variation of 7-12 spines and from larvae of *H. reliquens*, which present the same caudal pattern until they develop into adults (Deardorff & Overstreet 1980, 1981). These ontogenetic modifications have already been reported by Pereira Jr et al. (2004) and Felizardo et al. (2009a).

The above-mentioned morphological characteristics suggest that the larval forms of *Hysterothylacium* MD, *Hysterothylacium* 2, *Hysterothylacium* KB and *Hysterothylacium* sp. should be identified as *H. deardorffoverstreetorum* sp. nov.

The molecular characterisation based on three genetic markers also supports the evidence of a new *Hysterothylacium* species. The 18S rDNA gene of *H. deardorffoverstreetorum* sp. nov. yielded a unique sequence that confirms the taxonomic position on Raphidascarididae. ITS and *cox*2 genetic analyses provided strong evidence that this species is a unique genetic entity, characterising a new anisakid species. The phylogenetic tree topologies were in significant agreement, demonstrating that *H. deardorffoverstreetorum* sp. nov. represents a taxonomic unit genetically distant from other anisakids,

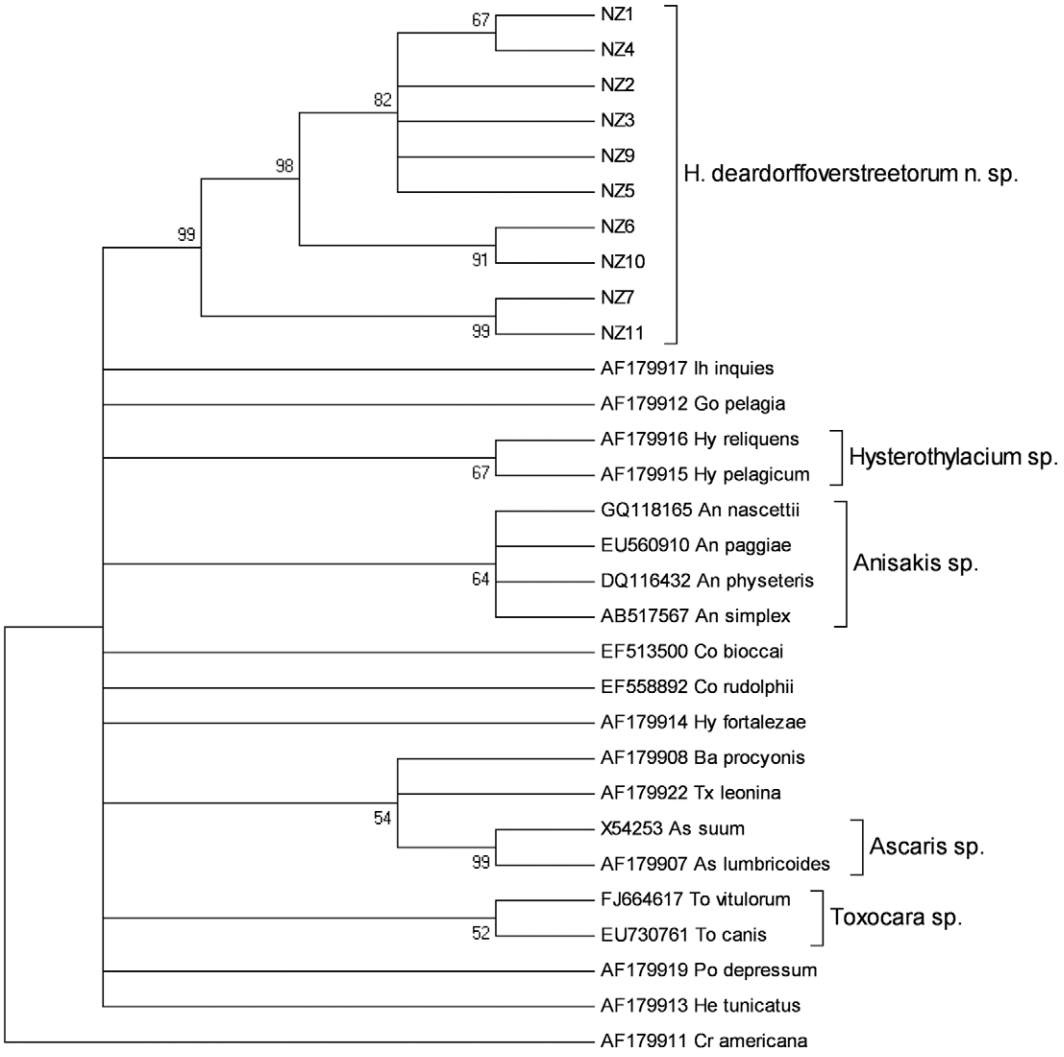

Fig. 6: maximum parsimony tree inferred from cytochrome c oxidase subunit II (*cox*2) gene sequence data from *Hysterothylacium deardorffoverstreetorum* sp. nov. and other Nematode spp studied. Phylogenetic analyses were conducted in MEGA4. *H. deardorffoverstreetorum* sp. nov. sequences are named as NZ1-NZ11. *cox*2 haplotype of NZ8 was identical to NZ3. GenBank accession and the species name are shown. *Cruzia americana* is the outgroup taxa.

even from other *Hysterothylacium* sp. In this regard, both genetic targets permitted the evaluation of all *Hysterothylacium* spp studied (*H. aduncum*, *H. auctum, H. bidentatum*, *H. muraenesoxi, H. reliquens*, *H. fortalezae* and *H. pelagicum*) and therefore confirm that the larvae do not belong to any *Hysterothylacium* sp. genetically described before. Concerning the relationships between congeners, *H. deardorffoverstreetorum* sp. nov., *H. pelagicum* and *R. acus* are in a polytomy on the 18S rDNA tree. Smythe et al. (2006) observed a similar 18S rDNA MP tree topology, with two clades for *Hysterothylacium* spp, one of which contained *H. pelagicum*, *R. acus*, *I. inquies* and *G. pelagia*. Interestingly, using ITS topologies, it was possible to verify two *Hysterothylacium* clusters, one of which grouped specimens of *Hysterothylacium deardorffoverstreetorum* sp. nov., *H. muraenesoxi* and a sequence of *R. acus.* ITS sequences of *H. muraenesoxi* are annotated in the GenBank database as *Contracaecum muraenesoxi*, which requires updating because Li et al. (2008) redescribed and proposed its synonymy. The presence of *R. acus* in the *Hysterothylacium* cluster could indicate a polyphyletic condition of the *Raphidascaris* group, as the well-supported clade of *Raphidascaris* sp. (*Raphidascaris gigi* and *Raphidascaris trichiuri*) is shaped and placed basal to the *Hysterothylacium* groups (MP), or in the largest *Hysterothylacium* clade (NJ). A misclassification of *R. acus* could be also a plausible explanation, as morphological characterisation or molecular analysis of this specimen (AY603537) is not available. The phylogenetic analysis obtained from the mitochondrial DNA (mtDNA) *cox*2 dataset clearly supports *H. deardorffoverstreetorum* sp. nov. as an evolutionarily separate taxon, despite some intraspecific genetic heterogeneity. The use of *cox*2 in this study corroborates the value of this genetic marker as a barcode for molecular taxonomy, allowing the

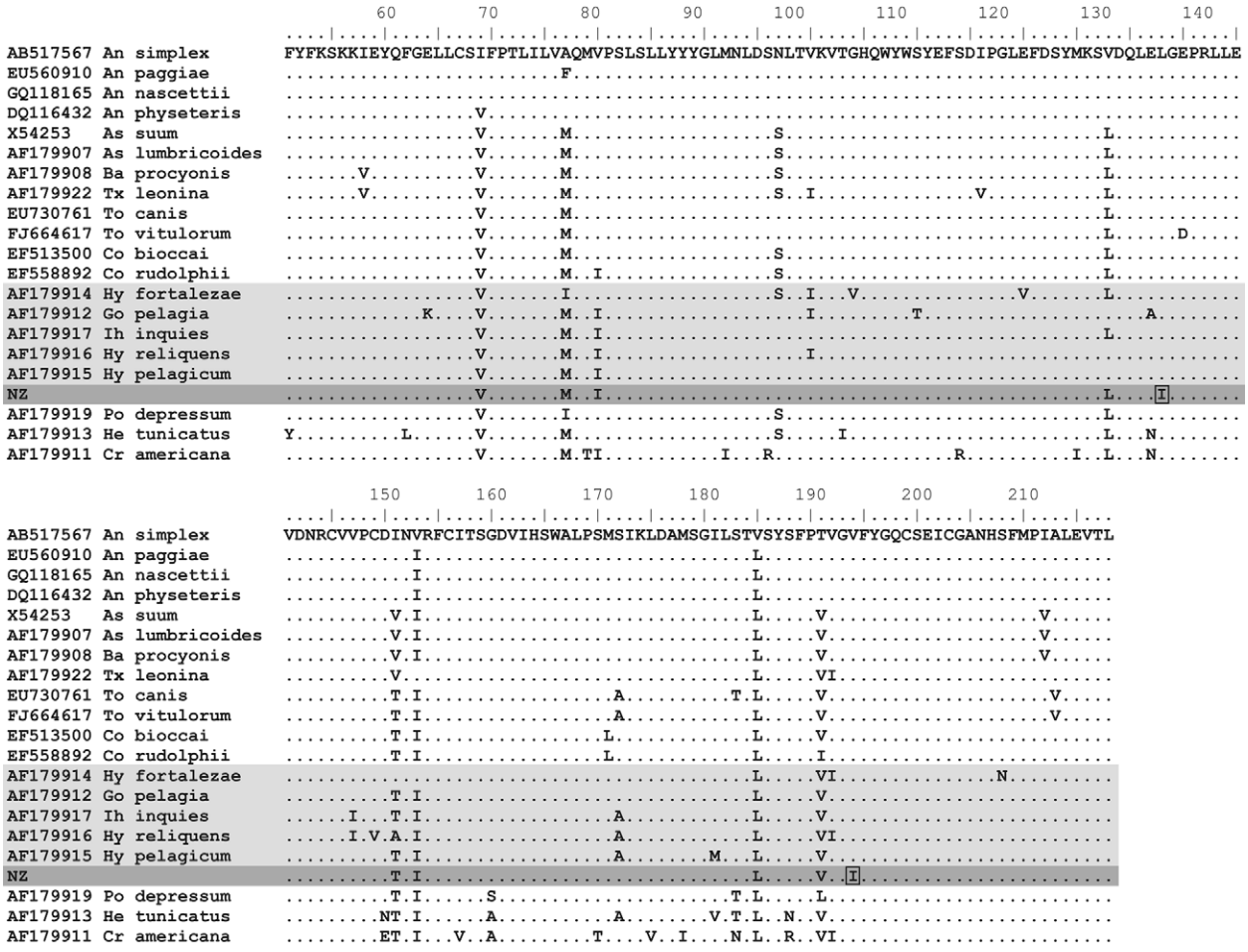

Fig. 7: alignment of amino acids sequence of cytochrome oxidase 2 (COII) from *Hysterothylacium deardorffoverstreetorum* sp. nov. and other Nematode spp studied. Translation code 5: mitochondrial invertebrate. GenBank accession and the name of the species are shown. Points indicate the identity with *Anisakis simplex* reference sequence. Raphidascarididae sequences are in light grey and NZ represents *H. deardorffoverstreetorum* sp. nov. sequence in hard grey. COII genetic signatures of *H. deardorffoverstreetorum* sp. nov. are in box.

identification of presumed new or sibling species as demonstrated and proposed for anisakids nematode research (Valentini et al. 2006, Mattiucci et al. 2009). In addition, *cox*2 gene analyses were in agreement with the observation that *Hysterothylacium* does not represent a monophyletic group. However, different from 18S rDNA topologies, the *Hysterothylacium* genus-specific cluster is formed by *H. reliquens* and *H. pelagicum*. Previous studies also reported these conflicting clades based on rDNA and mtDNA analyses, even when using a combined analysis including morphological traits (Nadler & Hudspeth 2000). In this study, based on the new data from both anisakid specimens and genetic markers (ITS), the unsolved molecular taxonomy of the *Hysterothylacium* group is evident, suggesting a polyphyletic group condition. *In silico* COII protein analysis further supported the taxonomic problem of the *Hysterothylacium* group, as the set of genetic signatures identified the Raphidascarididae species and excluded the *H. fortalezae* member. The presence of Raphidascarididae COII signatures in *H. deardorffoverstreetorum* sp. nov., as well as the detection of particular genetic signatures, provided additional and significant evidence of a new anisakid nematode species.

ACKNOWLEDGEMENTS

To Dr Nicolau Maués da Serra Freire, from National Reference Laboratory of Vectors of Rickettsial diseases/IOC/Fiocruz, for the statistic analysis, Milena Mota and Koko Otsuki, from Laboratory of Molecular Genetics of Microorganisms/IOC-Fiocruz, for their technical assistance, to PDTIS/Fiocruz, for the genomic platform nucleotide sequencing, and Heloisa Nogueira Diniz, from Service Production and Processing of Images/IOC/Fiocruz.

REFERENCES

Bicudo AJA, Tavares LER, Luque JL 2005. Metazoários parasitos da cabrinha *Prionotus punctatus* (Bloch, 1793) (Osteichthyes: Triglidae) do litoral do Estado do Rio de Janeiro, Brasil. *Rev Bras Parasitol Vet 14*: 27-33.

Brizzola SM, Tanzola RD 1995. *Hysterothylacium rhamdiae* sp. n. (Ascaridoidea: Anisakidae) from a neotropical catfish, *Rhamdia sapo* (Pisces: Pimelodidae). *Mem Inst Oswaldo Cruz 90*: 349-352.

Deardorff TL, Overstreet RM 1980. Review of *Hysterothylacium* and *Iheringascaris* (both previously = *Thynnascaris*) (Nematoda: Anisakidae) from the Northern Gulf of Mexico. *Proc Helminthol Soc Wash 93*: 1035-1079.

Deardorff TL, Overstreet RM 1981. Larval *Hysterothylacium* (= *Thynnascaris*) (Nematoda: Anisakidae) from fishes and invertebrates in the Gulf of Mexico. *Proc Helminthol Soc Wash 48*: 113-126.

Dorris M, Viney ME, Blaxter ML 2002. Molecular phylogenetic analysis of the genus *Strongyloides* and related nematodes. *Int J Parasitol 32*: 1507-1517.

Eiras JC, Takemoto RM, Pavanelli JC 2006. *Métodos de estudo e técnica laboratoriais em parasitologia de peixes*, 2nd ed., Eduem, Maringá, 199 pp.

Fagerholm HP 1991. Systematic implications of male caudal morphology in ascaridoid nematode parasites. *Syst Parasitol 19*: 215-228.

Felizardo NN, Knoff M, Pinto RM, Gomes DC 2009a. Larval anisakid nematodes of the flounder, *Paralichthys isosceles* Jordan, 1890 (Pisces: Teleostei) from Brazil. *Neotrop Helminthol 3*: 57-64.

Felizardo NN, Menezes RC, Tortelly R, Knoff M, Pinto RM, Gomes DC 2009b. Larvae of *Hysterothylacium* sp. (Nematoda: Anisakidae) in the sole fish *Paralichthys isosceles* Jordan, 1890 (Pisces: Teleostei) from the littoral of the State of Rio de Janeiro, Brazil. *Vet Parasitol 166*: 175-177.

Gopar-Merino L, Osorio-Sarabia D, Garcia-Prieto L 2005. A new species of *Hysterothylacium* (Nematoda: Anisakidae) parasite of *Ariopsis guatemalensis* (Osteichthyes: Ariidae) from Tres Palos lagoon, Mexico. *J Parasitol 91*: 909-914.

Guimarães JF, Cristofaro R 1974. Contribuição ao estudo da fauna helmintológica de peixes do estado da Bahia. *Atas Soc Biol 17*: 81-85.

Incorvaia IS, Díaz de Astarloa JM 1998. Estudio preliminar de las larvas (Nematoda: Ascaridida) parásito de *Paralichthys orbignyanus* (Valenciennes, 1839) y *Paralichthys patagonicus* (Pisces: Pleuronectiformes) *Bol Chil Parasitol 53*: 38-42.

Incorvaia IS, Hernández DR 2006. Nematodes parásitos como indicadores biológicos de *Macruronus magellanicus*. *INIDEP Inf Tec 61*: 1-24.

Iñiguez AM, Carvalho VL, Motta MR, Pinheiro DC, Vicente AC 2011. Genetic analysis of *Anisakis typica* (Nematoda: Anisakidae) from cetaceans of the northeast coast of Brazil: new data on its definitive hosts. *Vet Parasitol*: *178*: 293-299.

Klein VLM 1973. Helmintos parasitos das espécies *Scomberomorus cavalla* (Cuvier) e *Scomberomorus maculatus* (Mitchill) do litoral cearense. *Contracaecum fortalezae* sp. n. (Nematoda, Ascaridoidea). *Mem Inst Oswaldo Cruz 71*: 199-202.

Li L, Xu Z, Zhang L 2008. Redescription of three species of *Hysterothylacium* (Nematoda: Anisakidae) from marine fishes from the Yellow Sea, China, with the synonymy of *Hysterothylacium muraenesoxin* (Luo, 1999). *Zootaxa 1878*: 55-67.

Lopes LP, Pimpao DM, Takemoto RM, Malta JC, Varella AM 2011. *Hysterothylacium* larvae (Nematoda, Anisakidae) in the freshwater mussel *Diplodon suavidicus* (Lea, 1856) (Mollusca, Unioniformes, Hyriidae) in Aripuana River, Amazon, Brazil. *J Invertebr Pathol 106*: 357-359.

Mattiucci S, Paoletti M, Webb SC 2009. *Anisakis nascettii* n. sp. (Nematoda: Anisakidae) from beaked whales of the southern hemisphere: morphological description, genetic relationships between congeners and ecological data. *Syst Parasitol 74*: 199-217.

Moravec F, Uruwa S, Coria CO 1997. *Hysterothylacium patagonense* n. sp. (Nematoda: Anisakidae) from freshwater fishes in Patagonia, Argentina, with a key to the species of *Hysterothylacium* in American freshwater fishes. *Syst Parasitol 36*: 31-38.

Nadler SA, Hudspeth DSS 2000. Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: hypotheses of structural and sequence evolution. *J Parasitol 86*: 380-393.

Norris DE, Overstreet RM 1975. *Thynnascaris reliquens* sp. n. and *T. habena* (Linton, 1900) (Nematoda: Ascaridoidea) from fishes in the northern Gulf of Mexico and eastern U.S. Seaboard. *J Parasitol 61*: 330-336.

Overstreet RM, Meyer GW 1981. Hemorragic lesion in the stomach of rhesus monkey caused by a piscine ascaridoid nematode. *J Parasitol 67*: 226-235.

Pereira Jr J, Almeida FM, Morais NCM, Vianna RT 2004. *Hysterothylacium* sp. larva (Nematoda: Anisakidae) in *Micropogonias furnieri* (Sciaenidae) from Rio Grande do Sul cost, Brazil. *Atlantica (Rio Grande) 26*: 55-60.

Petter AJ, Maillard C 1988. Larves d'ascarides parasites de poissons en Méditerranée occidentale. *Bull Mus Hist Nat 10*: 347-369.

Petter JA, Sey O 1997. Nematode parasites of marine fishes from Kuwait with a description of *Cucullanus trachinoti* n. sp. from *Trachinotus blochi*. *Zoosystema 19*: 35-59.

Saad CD, Luque JL 2009. Larval Anisakidae in musculature of *Pagrus pagrus* from the State of Rio de Janeiro, Brazil. *Rev Bras Parasitol Vet 18*: 71-73.

Serra-Freire NM 2002. *Planejamento e análise de pesquisas parasitológicas*, EdUFF, Niterói, 195 pp.

Smythe AB, Sanderson MJ, Nadler SA 2006. Nematode small subunit phylogeny correlates with alignment parameters. *Syst Biol 55*: 972-992.

Tamura K, Dudley J, Nei M, Kumar S 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) sofware version 4.0. *Mol Biol Evol 24*: 1596-1599.

Tavares LER, Luque JL 2006. Sistemática, biologia e importância em saúde coletiva de larvas de Anisakidae (Nematoda: Ascaridoidea) parasitas de peixes ósseos marinhos do Estado do Rio de Janeiro, Brasil. In AT Silva-Souza, *Sanidade de organismos aquáticos no Brasil 15*, ABRAPOA, Maringá, 387 pp.

Timi JT, Sardella NH, Navone GT 2001. Parasitic nematodes of *Engraulis anchoita* Hubbs et Marini, 1935 (Pisces, Engraulidae) off the Argentine and Uruguayan coasts, South West Atlantic. *Acta Parasitol 46*: 186-193.

Torres P, Andrade P, Silva R 1998. On a new species of *Hysterothylacium* (Nematoda: Anisakidae) from *Cauque mauleanum* (Pisces: Atherinidae) by brightfield and scanning electron microscopy. *Mem Inst Oswaldo Cruz 93*: 745-752.

Torres P, Quintanilla JC, Rozas M, Miranda P, Ibarra R, San Martin MF, Raddatz B, Wolter M, Villegas A, Canobra C, Hausdorf M, Silva R 2010. Endohelminth parasites from salmonids in intensive culture from southern Chile. *J Parasitol 96*: 669-670.

Torres P, Soto MS 2004. *Hysterothylacium winteri* sp. n. (Nematoda: Anisakidae), a parasite of Chilean rock cod, *Eleginops maclovinus* (Perciformes: Eleginopidae), from south Chile. *Folia Parasitol 51*: 55-60.

Valentini A, Mattiucci S, Bondanelli P, Webb SC, Mignucci-Giannone AA, Colom-Llavina MM, Nascetti G 2006. Genetic relationships among *Anisakis* species (Nematoda: Anisakidae) inferred from mitochondrial *cox*2 sequences and comparison with allozyme data. *J Parasitol 92*: 156-166.

Yagi K, Nagasawa K, Ishikura H, Nagagawa A, Sato N, Kikuchi K, Ishikura K, Ishikura H 1996. Female worm *Hysterothylacium aduncum* excreted from human: a case report. *J Parasitol 45*: 12-23.

Zhu X, Chilton NB, Jacobs DE, Boes J, Gasser RB 1999. Characterisation of Ascaris from human and pig hosts by nuclear ribosomal DNA sequences. *Int J Parasitol 29*: 469-78.