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

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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* for- talezae 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 *Contra- caecum* 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 winteni* (Torres & Soto 2004) and *H. aduncum* (Torres et al. 2010). In Brazil, nematode adults of *Hysterothylacium* for talezae (Klein 1973) and *Hysterothylacium* reliquens (Norris & Overstreet 1975) were also recovered from teleostceans on the northeastern coast (Guimarães & Cristofaro 1974, Deardorff & Over- street 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. larvae. Yagi et al. (1996) reported a case of human infection from Japan due to the ingestion of a fish parasitized with *H. aduncum* (Rudolph, 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-
ing different sites, including musculature and ovaries, with high prevalence. However, morphological analysis, with approaches including epidemiological and zootonic 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 (21°15′S 23°23′S, 40°29′W 44°28′W). For morphological analysis, nematodes were fixed, clarified and preserved in accordance with Eiras et al. (2006). The taxonomic identification followed Peter and Mailard (1988), Incorvaia and Díaz de Asturias (1998), Timi et al. (2001), Bicudo et al. (2005) and Felizardo et al. (2009a). Measurement ranges, with means in parentheses unless otherwise indicated. The nematode larva 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 -20°C 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), CO2 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 kvols.

**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/L). 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 buffer 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 Iglesiz 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 (cox2) gene were performed. The 18S rDNA gene (~1,500 bp) was amplified using the primers SSU-A (forward; 5′-AACAGTAACCATCATCAGT-3′) and 18P (reverse; 5′-TGTATCCWKCYGAGGTTCAC-3′) (Dorris et al. 2002) and the following PCR conditions: 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM MgCl2, 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 NCS (forward; 5′-CACCAACTCTTTAAATTATC-3′) and NC2 (reverse; 5′-TTTCTAGTTATATAGTTGRTTYAT-3′) (Zhu et al. 1999) and the PCR and electrophoresis conditions described above. The cox2 gene fragment (629 bp) was amplified using 210 (forward; 5′-GTAGGTGAACCT -GCGGAAGGATCATT-3′) and 211 (reverse; 5′-TTAGTTTCTTTTCCTCCGCT-3′) (Nadar & Hudspeth 2000) and 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM MgCl2, 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 cox2 available on the GenBank database were used in the analysis (February 2011). cox2 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 accesions 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-
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). Ventricle nearly spherical (Fig. 1). Ventricle 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. Ventricle 0.05-0.15 (0.10) in length. Ventricle 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 $L_1$, $0.6184$ in $L_2$) and the intestinal caecum ($r = 0.7207$ in $L_1$, $0.5415$ in $L_2$),
(ii) the oesophagus length compared to the length of the ventriculus ($r = 0.6701$ in $L_1$ and $0.7462$ in $L_2$),
intestinal caecum ($r = 0.6690$ in $L_1$, $0.6532$ in $L_2$) and ventricular appendix ($r = 0.6089$ in $L_1$, $0.7792$ in $L_2$),
(iii) length of the ventriculus and intestinal caecum ($r = 0.6872$ in $L_1$, and $0.4949$ in $L_2$). The correlation of the ventriculus length with the ventricular appendix was significant in $L_1$ ($r = 0.7578$) and accentuated in $L_2$ ($r = 0.8122$); conversely, the correlation of the body length and the length of the oesophagus was accentuated in $L_1$ ($r = 0.8614$) and significant in $L_2$ ($r = 0.6981$).

There was an outstanding correlation regarding the body length of the fish and the oesophagus length in the $L_1$ larvae ($r = 0.4664$), which was unimportant in the case of larval $L_2$ nematodes ($r = 0.2356$). Also, the correlation of the length of the ventriculus appendix with the intestinal caecum was considerable ($r = 0.5068$ in $L_1$, $r = 0.5759$ in $L_2$), as well as with the body length of the parasite ($r = 0.5942$ in $L_1$) and remarkable in $L_2$ ($r = 0.7223$). 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 $L_1$ and $L_2$ 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 sequence data from MEGA4. The MP and NJ methods, including all available Ascaridida ITS sequences, including all available Hysterothylacium species: H. aduncum, Hysterothylacium auctum, Hysterothylacium bidentatum, Hysterothylacium (Contracaecum) muraenaeoxi 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. muraenaeoxi 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).

The cox2 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 located 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 Raphidascaridae contain the combined genetic signatures V69, M77, 180 and N92, except for the highly polymorphic H. fortalezae (V69, 177, V80 and S92). The new Hysterothylacium specimens share the Raphidascaridae 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.
DISCUSSION

Larvae of Hysterothylacium recovered from P. isoceles 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 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 cox2 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.

The structures that are present in the caudal tip of some species can change in the different stages (L₃ → L₄ → adults). Thus, the L₃ with mucron and the L₄ with a spiny cluster differ from L₃ and L₄ 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).

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.
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. muraenesoxi* 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* spp (e.g. *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) *cox2* dataset clearly supports *H. deardorffoverstreetorum* sp. nov. as an evolutionarily separate taxon, despite some intraspecific genetic heterogeneity. The use of *cox2* in this study corroborates the value of this genetic marker as a barcode for molecular taxonomy, allowing the

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**Fig. 6: maximum parsimony tree inferred from cytochrome c oxidase subunit II (*cox2*) 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. *cox2* haplotype of NZ8 was identical to NZ3. GenBank accession and the species name are shown. *Cruzia americana* is the outgroup taxa.**
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, cox2 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.

**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.

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