**An Acad Bras Cienc (2023) 95(1): e20211046 DOI 10.1590/0001-3765202320211046**
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

MICROBIOLOGY

# Morphological and molecular characterization of *Hysterothylacium* spp. parasitizing *Pomatomus saltatrix* and *Pagrus pagrus* of the State of São Paulo, Brazil

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**Abstract:** Raphidascarid nematodes have been the focus of several studies, mainly due to the zoonotic potential of some species, even though the cases are underreported. Due to the difficulty in identifying their larvae, the use of diagnostic techniques involving morphological and molecular analyses has grown in the last 20 years. The present study had as objective the morphological and molecular characterization of the L3 larval types of *Hysterothylacium* collected in *Pomatomus saltatr*ix and *Pagrus pagrus* from the Brazilian coast, close to the municipality of Santos, State of São Paulo. Twenty specimens of *P. saltatrix* were necropsied and *Hysterothylacium* type V (n = 257) and *Hysterothylacium* type X (n = 5) larvae were found. Five specimens of *P. pagrus* were necropsied and all were parasitized by *Hysterothylacium* type V larvae. The analyses showed a genetic proximity relationship between *Hysterothylacium* types V with other *Hysterothylacium* V and with *H. deardorffoverstreetorum*, although this is a *species inquirenda*. Haplotypes for *Hysterothylacium* type X found in the present study formed a monophyletic group with other *Hysterothylacium* X, *H. amoyense,* and *H. zhoushanense*. Through this study, new hosts and localities were registered for *Hysterothylacium* type V and *Hysterothylacium* type X.

**Key words:** ITS, Nematoda, phylogeny, Raphidascaridae, taxonomy.

## INTRODUCTION

Raphidascarid nematodes have been extensively studied in recent years since some species have zoonotic potential (Mattiucci et al. 2013). Fish can harbor the larvae and adults of these nematodes, attracting the interest of researchers to the development of tools (classic or modern) that link conspecific individuals in different stages of development (Cannon 1977, Mattiucci et al. 2002, Borges et al. 2012, Shamsi et al. 2015). Additionally, the understanding of epidemiological, biological, and ecological patterns is only possible after the correct identification of a species, regardless of its stage of development (Kijewska et al. 2002, Mattiucci et al. 2005, Nadler et al. 2005, Zhang et al. 2007).

*Hysterothylacium* Ward & Magath, 1917 includes about 72 valid species being the most abundant and diverse group of ascaridoids parasitic in marine fish, with a worldwide distribution (Moravec & Justine 2015). The identification of these larvae is usually problematic, and different tools for this purpose involving morphological and molecular characterization have been frequently used (Ghadam et al. 2018, Jabbar et al. 2012,

Khammassi et al. 2020, Pantoja et al. 2016, Roca-Geronès et al. 2018, Shamsi et al. 2011, 2013, 2015, 2018, 2020). At least 17 *Hysterothylacium* larval morphotypes have been described, based mainly on characteristics related to the digestive tract and caudal end, in addition to molecular characterization (Cannon 1977, Shamsi et al. 2011).

*Hysterothylacium* larvae have been widely reported in fishes from Brazilian Atlantic Coast, parasitizing more than 35 species of teleosts (Pantoja et al. 2016). However, the use of integrative taxonomy methods (morphology and molecular analyses) to identify these nematodes is limited to a few studies in Brazil (Borges et al. 2012, Knoff et al. 2012, Pantoja et al. 2015, 2016). The few records of parasitism by *Hysterothylacium* spp. in humans can also be related to the lack of a specific diagnosis for the identification of their larvae, making morpho-molecular studies important for actions to control possible parasitic diseases caused by fish consumption (Shamsi et al. 2018, Rahmati et al. 2020).

The anchovy *Pomatomus saltatrix* (Linnaeus, 1766) and the red porgy *Pagrus pagrus* (Linnaeus, 1758) are carnivorous fishes with worldwide distribution in tropical and subtropical waters, commonly found on the Brazilian coast, and are widely consumed and marketed (Froese & Pauly 2019).

In this study, an integrative taxonomy approach was used to evaluate specimens of *Hysterothylacium* found in these two hosts and establish their phylogenetic relationships with other congeners. Therefore, morphological data were combined with DNA sequences from the rDNA region comprising ITS-1, gene 5.8S, and ITS-2.

## MATERIALS AND METHODS

### Collection, processing, and morphological examination

Twenty specimens of bluefish (*P. saltatrix*) and five specimens of red porgy (*P. pagrus*) were purchased frozen and not eviscerated at different commercial points in the municipality of Bauru, State of São Paulo, from February to December 2016. Hosts were originally from the coast of the municipality of Santos, State of São Paulo. The fish were eviscerated through an incision close to the opercula until the cloaca and the stomach, intestine, and mesentery were analyzed. The organs were passed through 75 µm sieves and washed with water and the contents and tissue of the organs were analyzed with a stereomicroscope, for nematodes. Subsequently, the macroscopic examination of fish musculature was performed using a stereomicroscope Bel Photonics STM Pro. The technique of filleting with the removal of sections of the host's musculature and subsequent inspection by transparency using a negatoscope was performed. For the morphological analyses, the larvae were fixed and stored in 70% ethanol.

The anterior and posterior extremities of all nematodes were processed for morphological study, cleared in Amann's Lactophenol, placed on glass slides covered by coverslips, and observed on the Nikon Eclipse E200 microscope equipped with a Moticam 5.0MP image capture system where the photographs were taken and morphometric analyses were performed. The means and ranges measures (in brackets) are given in millimeters.

For scanning electron microscopy (SEM), six specimens were taken from the 70% ethanol, passed in phosphate buffer, and stored in 3% glutaraldehyde for three days. Subsequently, the specimens were post-fixed in 1% osmium tetroxide for 6 hours, dehydrated using a series

of ethanol, and then dried at the critical point through carbon dioxide. The specimens were coated with gold and examined using a ZEISS EVO-MA10 scanning electron microscope with an acceleration voltage of 15 kV.

The SEM images of *Hysterothylacium* type X were not suitable because the specimens were very dehydrated in this preparation, and we decided to use only the images in light microscopy. To avoid repetition of information, we decided to add only the SEM images of *Hysterothylacium* type V, as they are more suitable for visualizing the structures.

## DNA isolation, amplification, and sequencing

The median portion of seven specimens of *Hysterothylacium* from *P. saltatrix* and five specimens from *P. pagrus* were fixed in absolute ethanol. Total genomic DNA was extracted according to the information described in the Wizard commercial genomic DNA purification kit (Promega).

The primers NC5 (forward: 5'-GTAGGTGA ACCTGCGGAAGGATCATT-3') and NC2 (reverse: 5'-TTAGTTTCTTTTCCTCCGCT-3') described by Zhu et al. (1998), were used to amplify the rDNA region comprising ITS-1, 5.8S gene, and ITS-2 (from here and thereafter ITS1-5.8S-ITS2 region). PCR was performed in a 50 µL reaction mixture containing 100-300 ng of template DNA, 1X PCR Gold Buffer, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmol of each primer, and 2.5 U of Ampli Taq Gold™ DNA polymerase (Applied Biosystems, Foster City, CA, USA). PCR cycling conditions were as follows: initial denaturation at 94ºC for 10 min followed by 40 cycles of 94ºC for 45 sec, 55ºC for 45 sec and 72ºC for 45 sec, and a final extension at 72ºC for 7 min. PCR products (10 µL) were enzymatically purified using Exo I (0.6 units) and SAP (0.3 units) enzymes, incubated at 37 ºC for 60 min and 85ºC for 15 min. Finally, the purified product was sequenced following the ABI Prism BigDye™ Terminator v3.1 Cycle Sequencing Kit protocol on an ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA, USA).

## Phylogenetic analysis

The obtained sequences were compared to homologous sequences of *Hysterothylacium* species available in public databases (i.e. NCBI GenBank) (Table I). The software SEQSCAPE 2.5 (Applied Biosystems, Foster City, CA, USA) was used to check variable sites. Sequences from genetically related species that contained the complete ITS region available on GenBank were selected for analysis. Sequences were aligned using Clustal X 2.0 (Thompson et al. 1997) with default parameters. The different haplotypes were detected using the program DnaSP 5.0 (Librado & Rozas 2009) and were compared with sequences available in the NCBI database using the BLAST tool. The software MEGA X (Kumar et al. 2018) was used to estimate the number of nucleotide changes and genetic distances among *Hysterothylacium* taxa, and to determine the optimal model of nucleotide substitution for the dataset based on the Bayesian Information Criterion (BIC) scores.

Phylogenetic analyses were carried out using Bayesian Inference (BI), Neighbor-Joining (NJ), and Maximum Likelihood (ML) methods based on sequences of ITS1-5.8S-ITS2 region. BI analyses were carried out using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). For nodal support estimation based on Bayesian posterior probability the Metropolis-Coupled Markov Chain Monte Carlo process was run for 1 cold and 3 hot chains and 1,000,000 generations, with trees being sampled every 100 generations for a total of 10,000 trees in the initial sample and a burn-in of 25% (i.e. 2,500 trees). The Estimation Sample Sizes (ESS) of the model parameters were checked and the chains are converging.

**Table I. List of nematodes whose sequences were used for analyses and those obtained in the present study. Gene rDNA (ITS1, 5.8S, ITS2).**

| Parasite | GenBank accession no. (gene rDNA) | Host | Locality | Source |
|---|---|---|---|---|
| *Ascaris lumbricoides* | AB571298 | *Homo sapiens* | Japan | Nadler & Hudspeth (2000) |
| *Hysterothylacium* sp. VI | MT635370 | *Platycephalus richardsoni* | Australia | Unpublished |
| *Hysterothylacium* sp. | MT365529 | *Eledone sp.* | Italia | Guardone et al. (2020) |
| *H. rigidum* | HF680324 | *Lophius piscatorius* | Ireland | Unpublished |
| *Hysterothylacium* sp. | MF668813 | *Rachycentron canadum* | USA | Unpublished |
| *H. fortalezae* | KX098563 | *Maurolicus weitzmani* | USA | Andres et al. (2016) |
| *H. auctum* | AF115571 | *Zoarces viviparus* | Baltic Sea | Szostakowska et al. (2001) |
| *H. aduncum* | JX845137 | *Zoarces viviparus* | Denmark | Haarder et al. (2013) |
| *Hysterothylacium* sp. | HM437225 | *Gadus macrocephalus* | South Korea | Unpublished |
| *H. fabri* | KX083575 | *Trigla lyra* | Italy | Costa et al. (2018) |
| *Hysterothylacium* sp. IV | KD203841 | - | China | Shamsi et al. (2013) |
| *Hysterothylacium* sp. IV-A | KP419719 | *Polydactylus sextarius* | China | Zhao et al. (2016) |
| *Hysterothylacium* sp. | HM545895 | *Siganus fuscescens* | China | Unpublished |
| *H. bidentatum* | AY603539 | - | Poland | Unpublished |
| *H. reliquens* | MF062509 | *Boops boops* | Turkey | Şimşek et al. (2018) |
| *Hysterothylacium* sp. | KX083577 | *Lophius piscatorius* | Italy | Costa et al. (2018) |
| *H. thalassini* | JX982129 | *Priacanthus macracanthus* | China | Liu et al. (2013) |
| *H. liparis* | KF601900 | - | China | Guo et al. (2014) |
| *H. sinense* | KX084795 | *Conger myriaster* | China | Unpublished |
| *Hysterothylacium* sp. | AM706344 | *Astroconger myriaster* | China | Zhu et al. (2007) |
| *Hysterothylacium* sp. | MK039148 | *Ephippion guttifer* | Spain | Rodríguez et al. (2019) |
| *Hysterothylacium* sp. | MW699927 | *Epinephelus diacanthus* | Iraq | Unpublished |
| H. zhoushanense | KP326557 | *Lepidotrigla japonica* | China | Zhao et al. (2016) |
| *H. amoyense* | KP252133 | *Halieutaea stellata* | China | Zhao et al. (2016) |
| *H. amoyense* | KY081889 | *Otolithes ruber* | Persian Gulf | Unpublished |
| *Hysterothylacium* sp. | MF668871 | *Scomberomorus maculatus* | USA | Unpublished |
| *Hysterothylacium* sp. | MF668810 | *Coryphaena hippurus* | USA | Unpublished |
| *Hysterothylacium* sp. X | KU594490 | *Sarda sarda* | Brazil | Pantoja et al. (2016) |
| *Hysterothylacium* sp. X | KU594489 | *Priacanthus arenatus* | Brazil | Pantoja et al. (2016) |
| *Hysterothylacium* sp. X | MW817239 | *Pomatomus saltatrix* | Brazil | Present study |
| *Hysterothylacium* sp. X | MW817240 | *Pomatomus saltatrix* | Brazil | Present study |
| *Hysterothylacium* sp. VI | MT635373 | *Platycephalus bassensis* | Australia | Unpublished |
| *Hysterothylacium* sp. VI | MT635372 | *Platycephalus bassensis* | Australia | Unpublished |

**Table I. Continuation.**

| | | | | |
|---|---|---|---|---|
| *Hysterothylacium* sp. VI | MT635371 | *Platycephalus bassensis* | Australia | Unpublished |
| *H. deardorffoverstreetortum* | JF730204 | *Paralichthys isosceles* | Brazil | Knoff et al. (2012) |
| *Hysterothylacium* sp. V | KU594488 | *Zenopsis conchifer* | Brazil | Pantoja et al. (2016) |
| *Hysterothylacium* sp. V | KU594486 | *Paralichthys isoceles* | Brazil | Pantoja et al. (2016) |
| *Hysterothylacium* sp. V | KU594485 | *Merluccius hubbsi* | Brazil | Pantoja et al. (2016) |
| *Hysterothylacium* sp. V | KU594484 | *Menticirrhus americanus* | Brazil | Pantoja et al. (2016) |
| *Hysterothylacium* sp. V | KU594483 | *Lagocephalus laevigatus* | Brazil | Pantoja et al. (2016) |
| *Hysterothylacium* sp. V | KU594482 | *Gymnothorax vicinus* | Brazil | Pantoja et al. (2016) |
| *Hysterothylacium* sp. V | KU594481 | *Caulolatilus chrysops* | Brazil | Pantoja et al. (2016) |
| *Hysterothylacium* sp. V | MW826087 | *Pomatomus saltatrix* | Brazil | Present study |
| *Hysterothylacium* sp. V | MW826088 | *Pomatomus saltatrix* | Brazil | Present study |
| *Hysterothylacium* sp. V | MW826089 | *Pomatomus saltatrix* | Brazil | Present study |
| *Hysterothylacium* sp. V | MW826090 | *Pomatomus saltatrix* | Brazil | Present study |
| *Hysterothylacium* sp. V | MW826091 | *Pomatomus saltatrix* | Brazil | Present study |
| *Hysterothylacium* sp. V | MW826092 | *Pagrus pagrus* | Brazil | Present study |
| *Hysterothylacium* sp. V | MW826093 | *Pagrus pagrus* | Brazil | Present study |
| *Hysterothylacium* sp. V | MW826094 | *Pagrus pagrus* | Brazil | Present study |
| *Hysterothylacium* sp. V | MW826095 | *Pagrus pagrus* | Brazil | Present study |
| *Hysterothylacium* sp. V | MW826096 | *Pagrus pagrus* | Brazil | Present study |
| *Hysterothylacium* sp. V | KU594487 | *Prionotus punctatus* | Brazil | Pantoja et al. (2016) |

NJ and ML analyses were performed in MEGA X. For NJ, and applying the Kimura 2-parameter with a gamma value of 0.5529 to estimate the genetic distances of the matrix (see results section), a total of 1,000 nonparametric bootstrap replicates were performed to assess the reliability of the nodes. Indels were not taken into account (complete deletion option activated). The ML method was also based on the Kimura 2-parameter model. Initial trees for the heuristic search were automatically obtained by applying Neighbor-Join and BioNJ algorithms (implemented in MEGA X) to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with a superior log-likelihood value. All positions containing gaps and missing data were eliminated. As for NJ analysis, the reliability of nodes was estimated using 1,000 nonparametric bootstrap replicates. *Ascaris lumbricoides* Linnaeus, 1758 (accession number AB571298) was used as an outgroup based on previous phylogenetic analyses related to *Anisakis* (Nadler & Hudspeth 2000, Nadler et al. 2005, Pantoja et al. 2015).

## RESULTS

### Morphology

*Hysterothylacium* larval type V sensu Shamsi et al. (2013)

Description (based on 10 specimens): third-stage larvae. Three poorly developed lips, labial papillae not observed; tooth absent (Fig. 2a). Lateral alae present (Fig. 2b). Excretory pore situated immediately posterior nerve ring. Single

small cuticular projection (mucron) present on terminal region of tail (Fig. 2c). Measurements from specimens infecting *P. saltatrix* were: 8.00 (1.00-9.63) length, 0.18 (0.15-0.32) width, 0.48 (0.16-0.65) nerve ring to anterior end, 0.55 (0.20-0.69) excretory pore to anterior end, 0.54 (0.18-1.17) esophagus length, 0.06 (0.02-0.08) ventricule length, 0.58 (0.25-0.69) ventricular appendix length, 0.07 (0.03-0.10) intestinal caecum length and 0.26 (0.16-0.32) tail lenght. The average measures presented in *P. pagrus* were: 9.67 (1.78-11.64) length, 0.26 (0.12-0.40) width, 0.52 (0.34-0.66) nerve ring to anterior ring, 0.58 (0.22-0.75) excretory pore to anterior end, 0.63 (0.22-1.32) esophagus length, 0.09 (0.04-0.13) ventricule length, 0.78 (0.29-1.02) ventricular appendix length, 0.09 (0.05-0.20) intestinal caecum length, 0.33 (0.21-0.45) tail length (Table III).

Host: *Pomatomus saltatrix* and *Pagrus pagrus*.

Location: Santos, State of São Paulo, Brazil.

Prevalence: 75% in *P. saltatrix* and 100% in *P. pagrus*.

Mean abundance: 10.95± 0.56 in *P. saltatrix* and 3.57±0.8 in *P. pagrus*.

Mean intensity: 14.6±0.75 in *P. saltatrix* and 3.57±0.8 in *P. pagrus*.

Site of infection: intestine lumen

*Hysterothylacium* larval type X sensu Shamsi et al. (2013)

Description (based on 5 specimens): third-stage larvae. Three poorly developed lips, unseen labial papillae; tooth absent (Fig. 1a). Excretory pore at level of nerve ring. The average measures presented were: 6.71 (4.01-8.92) length, 0.19 (0.12-0.5) width, 0.35 (0.11-0.46) nerve ring to anterior end, 0.38 (0.16–0.50) excretory pore to anterior end, 0.19 (0.12-0.61) esophagus length, 0.94 (0.53-0.99) ventricule length, 0.70 (0.43-0.81) ventricular appendix length, 0.54 (0.12-0.65) intestinal caecum length and 0.11 (0.5 -0.22) tail length (Table II). Tail presented four delicate spines at the rounded tip (Fig. 1b).

Host: *Pomatomus saltatrix*.

Location: Santos, State of São Paulo, Brazil.

Prevalence: 15%.

Mean abundance: 0.25± 0.05.

Mean intensity: 1.67±0.33.

Site of infection: intestine lumen.

## Molecular and phylogenetic analysis

The BLAST search-related our haplotypes with *Hysterothylacium* sequences available from GenBank. The sequences of ten specimens (PH11, HT06, HT10, HT12, HT13, HT15, PH01, PH06, PH08, PH10) were identical to the *Hysterothylacium* type V species when edited and aligned sequences retrieved from GenBank, while two specimens (H301, H303) had identical sequences to those available for *Hysterothylacium* type X (Supplementary Material - Table SI). The nucleotide sequences obtained in the present study were deposited in GenBank (Accession numbers for 10 specimens identical to *Hysterothylacium* type V: MW826087-MW826096; accession numbers for

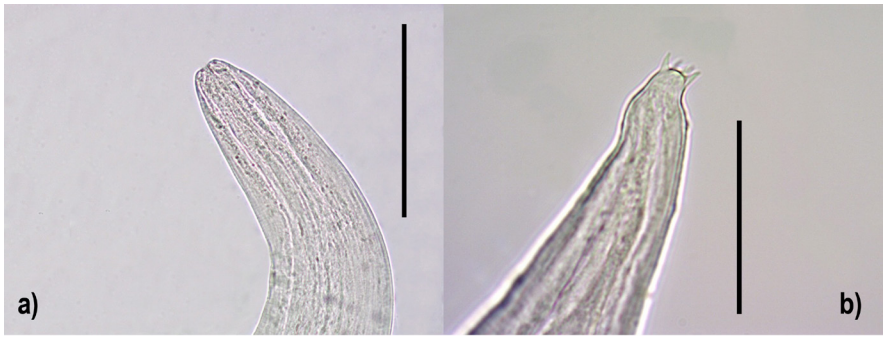


**Figure 1. *Hysterothylacium* type X (third-stage larva). a) Anterior end. b) Posterior end with mucrons. Bars: = 30 µm.**

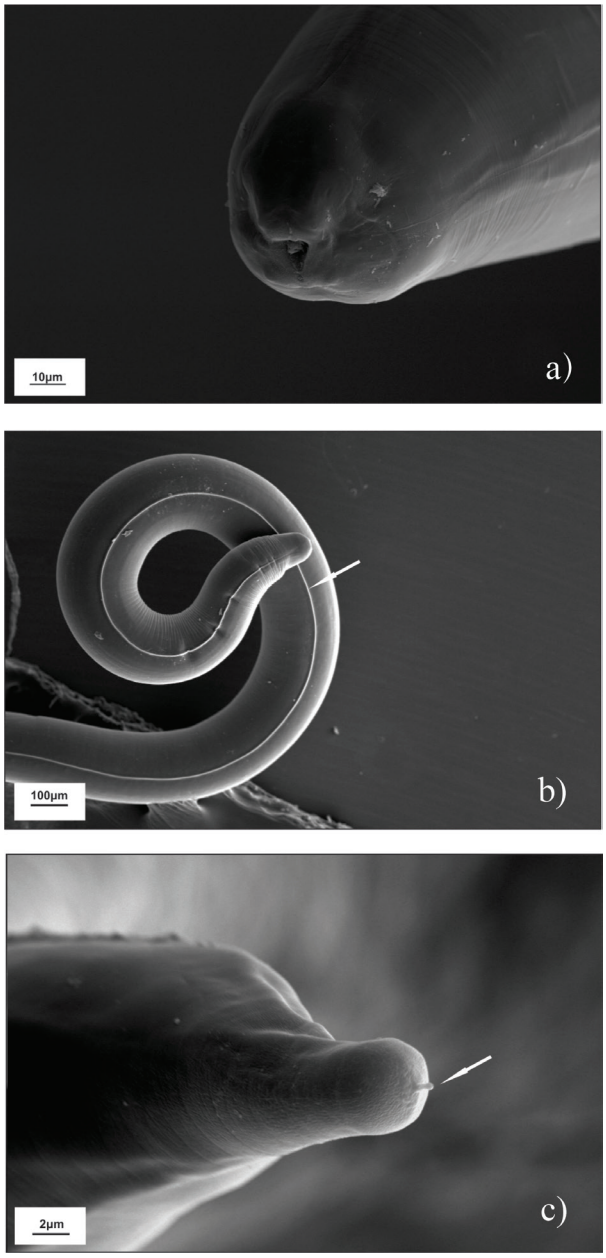


**Figure 2. *Hysterothylacium* type V found in the present study in *Pomatomus saltatrix*. a) cephalic extremity of the body showing three poorly developed lips; b) lateral view showing the lateral ala (arrowed); c) posterior end of the body showing a single prominent terminal spine (mucron) (arrowed).**

two specimens identical to *Hysterothylacium* type X species: MW817239-MW817240).

A 900 bp fragment of the ITS1-5.8S-ITS2 region was amplified for *Hysterothylacium* type V and *Hysterothylacium* type X it was 933 bp. The multiple sequence alignment used had 979 bp using only positions that were unequivocally aligned in all taxa. All samples of *Hysterothylacium* type V were identical. The two samples of *Hysterothylacium* type X were also identical. Among the haplotypes of *Hysterothylacium*. type V and *Hysterothylacium* type X, the difference found was 0.092% about the sequences obtained in this study.

The Kimura 2-parameter with a gamma value of 0.5529 was selected as the optimal model of nucleotide substitution rate using BIC scores. Phylogenetic analyses were based on the alignment of the ITS1-5.8S-ITS2 region sequences together with those of the *Hysterothylacium* genus and other Anisakidae and Raphidascarididae species of the GenBank database (see Accession Numbers on Table I). Many of the analyzed specimens (10) were assigned to *Hysterothylacium* Type V. All of them presented the haplotype which was identical to those described by Knoff et al. (2012) from the host *Paralichthys isosceles* Jordan, 1891, and the *Hysterothylacium* type V haplotypes described by Pantoja et al. (2016) in several species of fish of the Brazilian coast.

The other specimens (2) analyzed were genetically related to *H. amoyense* (Hsü, 1933) and *H. zhoushanense* Li, Liu & Zang, 2012. The haplotypes of these two specimens were identical to the sequences KU594489 and KU594490 of *Hysterothylacium* type X larvae from *Priacanthus arenatus* Cuvier, 1829 and *Sarda sarda* (Bloch, 1793), respectively, captured in Brazil (Pantoja et al. 2016). However, this haplotype showed slight nucleotide differences from the previously described haplotypes of *H. amoyense* and *H. zhoushanense*.

The three phylogenetic reconstructions (Fig. 3) methods used in the present study (i.e. BI, NJ, and ML) showed that haplotypes of *Hysterothylacium* sp. were related to *Hysterothylacium* larvae X, *H. amoyense,* and *H. zhoushanense* formed a

**Table II. Morphometric comparison of larvae L3 of *Hysterothylacium* type X collected in *Pomatomus saltatrix* with larvae L3 of *Hysterothylacium* type X previously reported. All measurements are in mm.**

| | **Present study** | **Pantoja et al. (2016)** | **Shamsi et al. (2013)** | **Jabbar et al. (2012)** |
|---|---|---|---|---|
| Host | *Pomatomus saltatrix* | *Priacanthus arenatus* | *Lutjanus carponotatus* | *Atherinomorus endrachtensis* |
| Length | 6.71 (4.01-8.92) | 6.4 (3.7–8.5) | 2.78 | - |
| Body width | 0.91 (0.5- 0.12) | 0.16 (0.12–0.21) | 0.13 | - |
| Nerve ring | 0.35 (0.11-0.46) | 0.29 (0.21–0.40) | - | 0.23 |
| Esophagus | 0.19 (0.12-0.61) | 0.63 (0.48–0.86) | 0.40 | 0.40–0.60 (0.48) |
| Ventricle | 0.94 (0.53-0.99) | 0.72 (0.40–0.10) | - | - |
| Ventricular appendix | 0.70 (0.43-0.81) | 0.53-0.38 (0.66) | 0.38 | - |
| Intestinal cecum | 0.54 (0.12-0.65) | 0.17 (0.12–0.24) | 0.12 | - |
| Tail | 0.11 (0.5 -0.22) | - | 0.10 | 0.12–0.20 (0.16) |

monophyletic group including the haplotype detected in samples of *Hysterothylacium* type X (01) and *Hysterothylacium* type X (03).

## DISCUSSION

*Hysterothylacium* species are distributed worldwide and often found in fish (Mattiucci & Nascetti 2008, Mattiucci et al. 2014, Shamsi et al. 2016). The fish will be intermediate or final hosts for these parasitic nematodes. The effect of *Hysterothylacium* species on the fish will depend on the site of infection and the parasite abundance, causing tissue erosions and necrosis (Felizardo et al. 2009). Some authors also attribute zoonotic potential to *Hysterothylacium* spp. (Yagi et al. 1996, Moravec 1998). However, such speculations are controversial, mainly due to the few records of human infection by these parasites (Yagi et al. 1996, Fernández-Caldas et al. 1998, Valero et al. 2003, González-Amores et al. 2015). This panorama is also due to the lack of specific diagnostic techniques, and the knowledge regarding the zoonotic larval types responsible for causing human infection remains incomplete (Shamsi et al. 2018).

Since *Hysterothylacium* larvae have problematic identification, morphological and molecular analyses are important for these purposes (Shamsi et al. 2011, 2013, 2015, 2018). In this sense, a single morphotype may exhibit wide morphometric variations (Shamsi et al. 2015). Such variations will depend on the characteristics of the hosts, the intensity of infection, and the parasite ontogeny since its development between the second and fourth larval stages directly influences the morphometric features (Pantoja et al. 2016). Therefore, it is not possible to determine the consistency of morphological characters is consistent for different stages of larval development (Shamsi et al. 2013).

The type V third-stage larvae of *Hysterothylacium* found in this study showed morphological and morphometric similarities with the larvae described by Bicudo et al. (2005), Borges et al. (2012), Knoff et al. (2012), Saad et al. (2012), Jabbar et al. (2012), Shamsi et al. (2013), Pantoja et al. (2016) and Khammassi et al. (2020), collected in several species of marine fish from Brazil and other continents (Shamsi et al. 2013, Khammassi et al. 2020). The morphotype of the present study, even with a shorter body length, presented morphological structures proportionally similar to the specimens found by the cited authors.

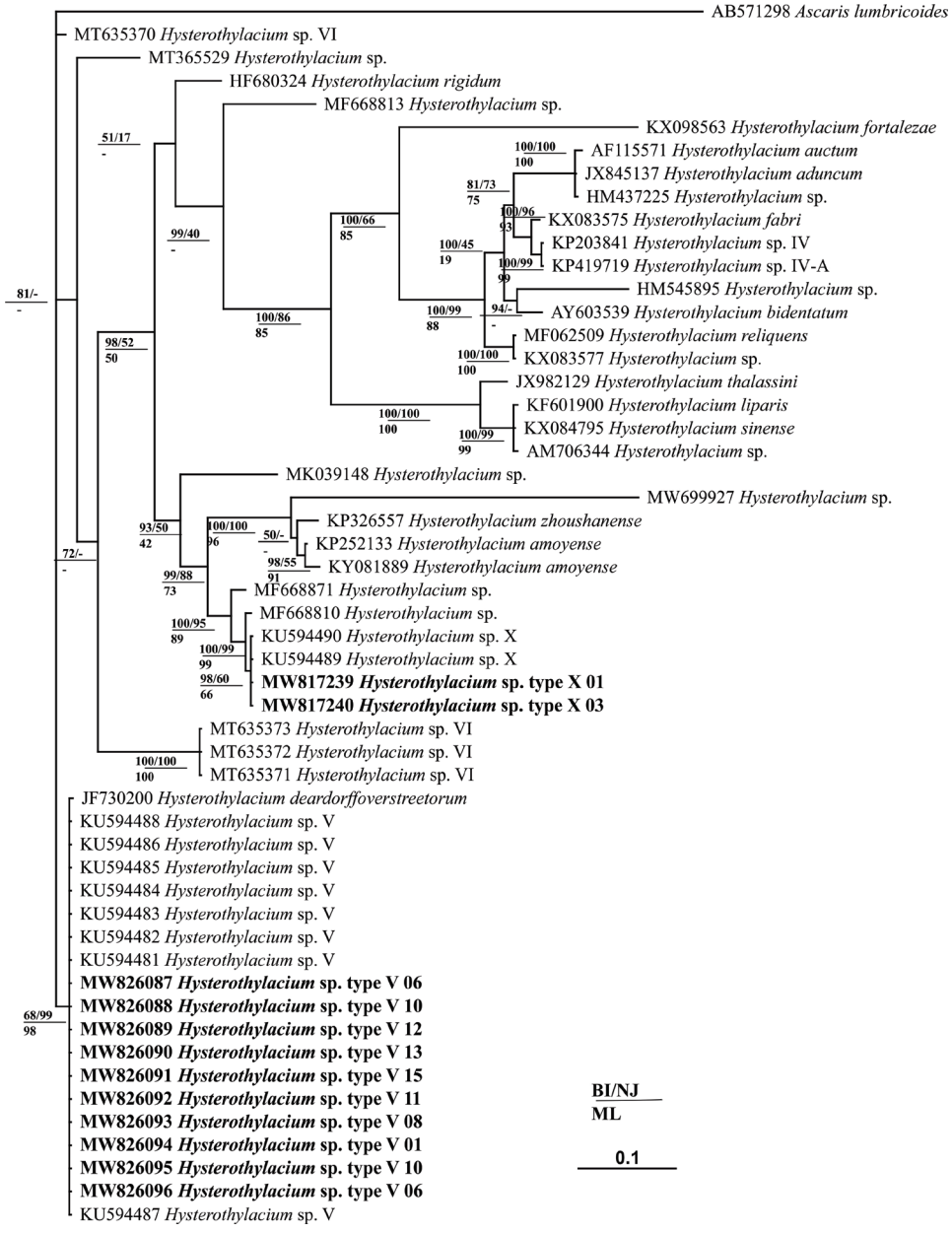


**Figure 3. Phylogenetic tree of the ITS1-5.8S-ITS2 region, evidencing the clustering of *Hysterothylacium* type V with other *Hysterothylacium* species available in Genbank and *Hysterothylacium* type X with *Hysterothylacium* type X species available from Genbank (MEGA 7). Samples from the present study are in bold.**

The larvae identified as *Hysterothylacium* type V in the present study resembled the first description of *Hysterothylacium* type V described by Shamsi et al. (2013). The phylogenetic tree of the ITS1-5.8S-ITS2 region obtained in this study is well-founded and grouped with *Hysterothylacium* type V described by Pantoja et al. (2016) and with *H. deardorffoverstreetorum* described by Knoff et al. (2012). *Hysterothylacium* type VI described by Shamsi et al. (2013) showed some morphological characteristics similar to the *Hysterothylacium* type V specimens of the present study (undeveloped lips, absent flat tooth, blunt tail with a single terminal spine or mucron). In the studies by Shamsi et al. (2013) and Shamsi et al. (2015), *Hysterothylacium* type VI larvae have a surface with tiny spines and intestines with a sinusoidal pattern. The original morphological description of *Hysterothylacium* type V (Shamsi et al. 2013), as well as the

**Table III. Morphometric comparison of larvae L3 of *Hysterothylacium* type V collected parasitizing *Pomatomus saltatrix* and *Pagrus pagrus* with larvae L3 of *Hysterothylacium* type V previously reported. All measurements are in mm.**

| | Present study | Present study | Bicudo et al. (2005) | Saad et al. (2012) | Knoff et al. (2012) | Borges et al. (2012) | Pantoja et al. (2016) | Jabbar et al. (2012) |
|---|---|---|---|---|---|---|---|---|
| Host | *Pomatomus saltatrix* | *Pagrus Pagrus* | *Prionotus punctatus* | *Lophius gastrophysus* | *Paralichthys isosceles* | *Trichiurus lepturus* | *Menticirrhus americanus* | *Lutjanus Carponotatus* (L3) |
| Length | 8.00 (1.00-9.63) | 9.67 (1.78-11.64) | 14.99 (2.97–23.13) | 5.78 (4.44-7.13) | 10.1 (3.62-16.7) | 7.84 (3.42–14.8) | 7.7 (7.5–7.8) | - |
| Body Width | 0.18 (0.5-0.32) | 0.26 (0.12-0.40) | 0.35 (0.08 – 0.56) | 0.20 (0.14-0.26) | 0.25 (0.11-0.40) | 0.24 (0.13–0.4) | 0.26 (0.25–0.27) | - |
| Nerve ring | 0.48 ( 0.16-1.15) | 0.52 (0.34-0.66) | - | - | - | - | 0.29 (0.27–0.31) | 0.22–0.29 (0.25) |
| Esophagus | 0.54 (0.18-1.17) | 0.63 (0.22-1.32) | 0.93 (0.40–1.25) | 0.45 (0.35-0.56) | 0.69 (0.23-1.16) | 0.64 (0.41–0.87) | 0.7 (0.6–0.7) | 0.38–0.46 (0.42) |
| Ventricle | 0.06 (0.02-0.08) | 0.09 (0.04-0.13) | 0.11 (0.04–0.15) | 0.05 (0.04-0.06) | 0.10 (0.05-0.15) | 0.07 (0.04–0.1) | 0.89 (0.87–0.90) | 0.04–0.06 (0.05) |
| Ventricular appendix | 0.58 (0.25-0.69) | 0.78 (0.29-1.02) | 0.58 (0.15–1.16) | 0.34 (0.33-0.35) | 0.86 (0.35-1.37) | 0.59 (0.31–0.84) | 0.83 (0.71–0.88) | 0.20–0.38 (0.31) |
| Intestinal cecum | 0.07 (0.03-0.10) | 0.09 (0.05-0.20) | 0.40 (0.14–0.56) | 0.05 (0.05-0.06) | 0.18 (0.05-0.32) | 0.16 (0.1–0.46) | 0.17 (0.16–0.18) | 0.13–0.20 (0.15) |
| Tail | 0.26 (0.16-0.32) | 0.33 (0.21-0.45) | 0.31 (0.12–0.47) | 0.13 (0.13-0.13) | 0.20 (0.10-0.32) | 0.16 (0.11–0.22) | 0.20 (0.19–0.21) | 0.10–0.19 (0.13) |

*Hysterothylacium* type V of the present study, do not have these characteristics.

Five specimens were assigned to the *Hysterothylacium* type V in *P. saltatrix* and *P. pagrus*, considering the ITS1-5.8S-ITS2 region. These sequences presented the same haplotype, 100% genetically identical to *H. deardorffoverstreetorum* described by Knoff et al. (2012) infecting *P. isosceles* in Brazil. In addition, morphological characters such as anterior extremity with three poorly developed ventrolateral lips, boring tooth absent, lateral alae along the body, excretory pore opening below the nerve ring, and conical tail with mucron are common to *H. deardorffoverstreetorum* and *Hysterothylacium* type V in the present study. However, we will not consider the validity of *H. deardorffoverstreetorum* since the species was described based on third and fourth stage larvae. According to Pantoja et al. (2016), the description of *H. deardorffoverstreetorum* is incomplete and unclear, and the specific diagnosis is flawed because the authors compare their larval stages with adults from other congeners and there is no molecular evidence supporting its validity. Thus, following Pantoja et al. (2016), *H. deardorffoverstreetorum* should be considered as *species inquirenda* and its representatives considered *Hysterothylacium* type V larva.

The third stage larvae of *Hysterothylacium* type X found in this study showed morphological and morphometric similarities with the larvae described by Jabbar et al. (2012), Pantoja et al. (2016), and Shamsi et al. (2013), in fishes collected in Australia and Brazil. Although slightly smaller in size, showed proportionally similar morphological structures.

*Hysterothylacium* larval type X found in the present study was morphologically similar to that described by Shamsi (2013) in Australia, and reported by Pantoja et al. (2016) in Brazil. These specimens showed the absence of cuticular spines throughout the body, absence of a boring tooth, and more than one spine (mucron) on the tail end. The genetic analysis corroborated the morphology, in which the present sequences formed a highly supported assemblage with those from GenBank labeled as *Hysterothylacium* larval type X. The fact that this, and most of the larval types of *Hysterothylacium*, exhibit

low host specificity is commonly observed in parasitic nematode larval forms, with indirect life cycles, and is considered a good strategy for infection of the definitive host (Anderson 2000).

According to Anderson (2000), the definitive hosts for species of *Hysterothylacium* are large pelagic predatory teleosts, and adults of this genus are also described in sharks as shown in Solov'eva & Pozdniakov (1984) and Lakshmi & Sreeramulu (2007). These definitive hosts show long migratory routes through the different oceans, thus transporting their parasites acquired by the trophic chain, as is the case of the raphidascarid and anisakid nematodes, which are then eliminated in feces incorporating in their life cycle several species of intermediate hosts available in marine environments.

The present study provides additional morphological and molecular data on the morphotypes of *Hysterothylacium* third-stage larvae, considering the difficulty of their identification. More in-depth knowledge of these parasites is useful to minimize their threats, therefore, their correct identification and characterization are necessary, since the consumption of sushis and sashimis is growing every day in Brazil. Through this study, new hosts are registered for *Hysterothylacium* type V and *Hysterothylacium* type X in Brazil.

## Acknowledgments

We thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil) (process number: 2016/00513-6) for financial support. Serrano was funded by a FAPESP grant (number: 2017/14332-6) to perform phylogenetic analyses at the University of Santiago de Compostela (ACUIGEN research group) under Manuel Vera supervision.

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# SUPPLEMENTARY MATERIAL

## Table SI

**How to cite**
SERRANO TD, VIEIRA DHMD, PELEGRINI LS, FRAGOSO LV, AGOSTINHO BN, VERA M, PORTO-FORESTI F, DE AZEVEDO RK & ABDALLAH VD. 2023. Morphological and molecular characterization of *Hysterothylacium* spp. parasitizing *Pomatomus saltatrix* and *Pagrus pagrus* of the State of São Paulo, Brazil. An Acad Bras Cienc 95: e20211046. DOI 10.1590/0001-3765202320211046.

*Manuscript received on July 21, 2021;*
*accepted for publication on May 28, 2022*

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