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Molecular studies on larvae of *Pseudoterranova* parasite of *Trichiurus lepturus* Linnaeus, 1758 and *Pomatomus saltatrix* (Linnaeus, 1766) off Brazilian waters

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Abstract

Pseudoterranova larvae parasitizing cutlassfish *Trichiurus lepturus* and bluefish *Pomatomus saltatrix* from Southwest Atlantic coast of Brazil were studied in this work by morphological, ultrastructural and molecular approaches. The genetic analysis were performed for the ITS2 intergenic region specific for *Pseudoterranova decipiens*, the partial 28S (LSU) of ribosomal DNA and the mtDNA *cox*-1 region. We obtained results for the 28S region and mtDNA *cox*-1 that was amplified using the polymerase chain reaction and sequenced to evaluate the phylogenetic relationships between sequences of this study and sequences from the GenBank. The morphological profile indicated that all the nine specimens collected from both fish were L3 larvae of *Pseudoterranova* sp. The genetic profile confirmed the generic level but due to the absence of similar sequences for adult parasites on GenBank for the regions amplifyied, it was not possible to identify them to the species level. The sequences obtained presented 89% of similarity with *Pseudoterranova decipiens* (28S sequences) and *Contracaecum osculatum* B (mtDNA *cox*-1). The low similarity allied to the fact that the amplification with the specific primer for *P. decipiens* didn´t occur, lead us to conclude that our sequences don't belong to *P. decipiens* complex.

Keywords

Cutlassfish, blue fish, Anisakidae, Brazil, ultrastructure, larvae

Introduction

The cutlassfish, *Trichiurus lepturus* Linnaeus, 1758 (Actinopterygii, Trichiuridae) and the bluefish, *Pomatomus saltatrix* (Linnaeus, 1766) (Actinopterygii, Pomatomidae) are considered cosmopolitan coastal species and are caught in warm and warm-temperate waters around the world (Froese and Pauly 2011). Cutlassfish has been repeatedly reported in shelf and shelf break demersal trawl surveys off southeast/southern Brazil as one of the most frequent and abundant bony fish species (Martins and Haimovici 1997). Bluefish is a migratory species found in south/southeast coast of Brazil from June to September (Haimovici and Krug 1996).

Previous parasitological surveys on these host species have been performed and, based on morphological characters, reported the presence of anisakid larvae of *Contracaecum* Raillet and Henry, 1912; *Hysteroyhylacium* Ward and Magath, 1917; *Anisakis* Dujardin, 1845 and *Pseudoterranova* Krabe, 1878 from these hosts off Rio de Janeiro (Rêgo *et al*. 1983; Barros and Amato 1992; São Clemente *et al.* 1995; Luque and Chaves 1999; Paraguassú *et al*. 2000; Luque *et al.* 2011; Borges *et al.* 2012).

The adult nematodes of the genus *Pseudoterranova* parasitize marine mammals all over the world (Mattiucci and Nascetti 2008) and the larvae from fish are morphologically difficult to be identified to species level by the absence of diagnostic characters, which only appear in adults. In this paper a morphological and molecular approaches were used to characterize larvae of *Pseudoterranova* from *T. lepturus* and *P. saltarix* of Southwest Atlantic.

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Materials and Methods

A total of 64 cutlassfish were collected off Itaipu beach, Niterói, Rio de Janeiro (22°53′14″S; 43°22′48″W) from August 2010 to January 2011 and 50 bluefish were obtained from markets of Rio de Janeiro from November 2010 to August 2012. Nematodes were measured in total length while in saline medium and subsequently cut into three pieces and fixed in 70% ethanol. The anterior and posterior regions were cleared in glycerine and mounted as semi-permanent slides; the middle regions were used for molecular analysis (Borges *et al*. 2012). Specimens were examined using an Olympus CX3 microscope, and measurements were made with the aid of an ocular micrometer and are given in millimeters as the mean, followed by the range in parentheses. Drawings were made with the aid of a camera lucida. Scanning electron microscopy was carried out with some specimens that were fixed for 24 hours at 4 °C in a solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer containing 3% sucrose at pH 7. The parasites were washed in the same buffer and post-fixed overnight in 1% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.2 in the dark. The samples were dehydrated in an ethanol series, critical point dried with CO_2, coated with gold and observed in a Jeol JSM 6390 SEM microscope.

The middle parts of parasites were prepared for total genomic DNA extraction using the Charge Switch gDNA Mini Tissue Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. PCR assays were conducted using 391 (5'-AGCGGAGGAAAAGAAACTAA-3'), 390 (5'-ATCCGTGTTTCAAGACGGG-3') primers for 28S rDNA gene (LSU) fragments of Anisakidae (Nadler *et al*. 2005) and PD (5′-CGAGTACTTTTTATGGTCGTGAAGT-3′), Universal B(5′-GCCGGATCCGAATCCTGGTTAGTTTCTTTTCCT-3′) primers for the ITS2 intergenic region of *Pseudoterranova decipiens* (Umehara *et al*. 2008) and JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3'), JB4 (5'-TAAAGAAAGAACATAATGAAAATG-3') primers for Cytochrome Oxidase I region of the Mitochondrial DNA (Bowles *et al*. 1992). All PCR reactions were performed in a volume of 50 µl with 20 mM of Tris-HCl at pH 8.4; 50 mM of KCl; 250 µM of each deoxynucleoside triphosphate (dNTPs) and 2 µl of genomic DNA. The concentrations of $MgCl_2$, primers and Taq Gold DNA polymerase (Promega Hot Taq Go Start Madison, WII – USA) were different for each reaction: 391/390 (0,4 µM of each oligonucleotide 3 µM of MgCl2 and 1,5 U of Taq) and PD/Universal B(1,5 µM of MgCl2; 0,5 µM of each oligonucleotide primer; 1 U of Taq). PCR was carried out using a Mastercycler Personal/Eppendorf thermal cycler (Epperdorf, Hamburg, Germany) and cycling parameters followed Nadler *et al*. (2005) and Umehara *et al*. (2008). PCR products were visualized with GELRED (Biotium Inc, Hayward, CA, USA) staining after electrophoresis on 1.5% agarose gels. Amplified PCR products were purified with Wizard SV Gel and PCR Clean up System kit (Promega) following the Manufacturer's instruction and sequenced in both directions using the same primer sets as in the respective PCR assay. DNA cycle-sequencing reactions were performed using BigDye v.3.1 chemistry (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were performed in the ABI Prism 3100 sequence analyzer. Sequences were edited, in DNASTAR SeqMan (DNASTAR, Inc., Madison, WI), and aligned with Bioedit Sequence Alignment Editor (version 7.0.4.1; http://www.mbio.ncsu.edu/BioEdit/bioedit .html). The edited sequences were compared for similarities with sequences from GenBank using BLAST 2.0 – Basic Alignment Search Tool (Altschul *et al*. 1990). To examine the phylogenetic relationships, the nucleotide sequences were aligned by CLUSTAL W algorithm (Thompson *et al*. 1994). Phylogenetic trees and the genetic distances between sequences were inferred with the software MEGA 5.0 (Tamura *et al*. 2011) using Kimura Two Parameters (K2P) for genetic distances (Kimura 1980). Maximum Likelihood method was used to construct trees, which were resampled by 10000 bootstrap replicates to evaluate the reliability of the groups (Felsenstein 1981).

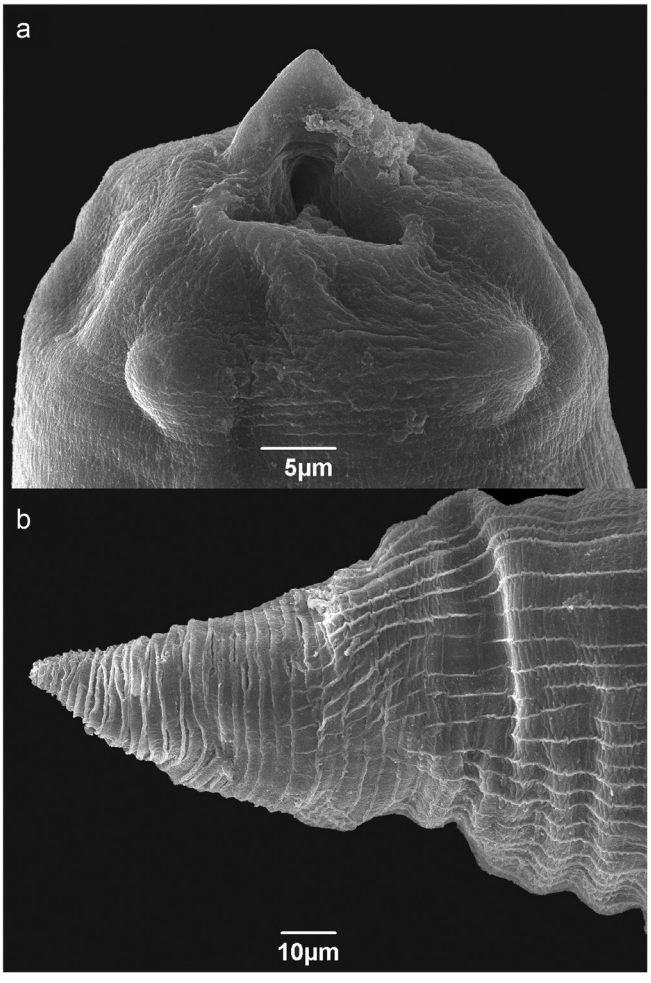

Fig. 1. Scanning electron micrographs of *Pseudoterranova* sp. from *Trichiurus lepturus*: (a) Anterior end with prominent boring tooth; (b) Posterior end with transversal striations and mucron

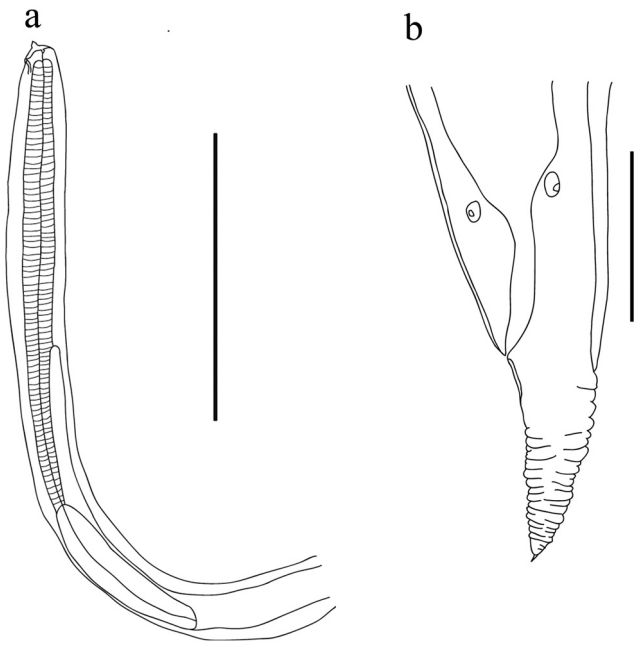

Fig. 2. *Pseudoterranova* sp. from *Pomatomus saltatrix*: (a) Anterior end with boring tooth, oesophagus, long ventriculus and intestinal caecum; (b) Posterior end with transversal striations

Results

A total of 403 anisakid larvae were collected and only 5 from the serosa of the liver of *T. lepturus* belonged to *Pseudoterranova* sp. The prevalence was 1.3% (two fish parasitized) and intensities were two and three specimens per fish. In *P. saltatrix*, a total of 170 anisakid larvae were found and four collected from the serosa of the liver were identified as *Pseudoterranova* sp. The prevalence was 8% (four fish parasitized) and the intensity was one specimen per fish. All 9 specimens of *Pseudoterranova* sp. examined were L3 larva.

Measurements based on five specimens from Trichiurus lepturus. Body 6.19 (4.40–8.10) long by 0.15 (0.10–0.22) wide; three developing lips and prominent boring tooth at anterior end (Fig 1a). Excretory pore on base of lips. Anterior oesophagus 0.7 (0.60–0.78); ventriculus 0.36 (0.30–0.40); ratio esophagus/ ventriculus 1: 0.38–0.63. Intestinal caecum 0.24 (0.17–0.28); ratio esophagus/ caecum 1: 0.28–0.37. Anus 0.12 (0.11–0.15) from posterior end; tip with transversal striations and mucron (Fig 1b).

Measurements based on three specimens from Pomatomus saltatrix. Body 6.3 (5.8–7) × 0.18 (0.19–0.19); three developing lips and boring tooth at anterior end (Fig 2a). Excretory pore on base of lips. Anterior oesophagus 0.79 (0.75–0.82); ventriculus 0.33 (0.30–0.35) (Fig 2a); ratio esophagus/ ventriculus 1: 0.40–0.42. Intestinal caecum 0.40 (0.25–0.55); ratio esophagus/ caecum 1: 0.31–0.67. Tail 0.17 long; tip with transversal striations (Fig 2b).

Out of nine specimens from both fish submitted to genetic PCR reactions conducted with 28S rDNA region primers for Anisakidae (four from each fish), only three specimens (one from *P. saltatrix* and two from *T. lepturus*) were amplified, but the reactions with the specific primer for *Pseudoterranova decipiens* were negative. The sequences obtained for 28S rDNA region presented 100% of similarity with each other and were deposited in GenBank under the accession numbers JQ951961 and KJ174509. The highest similarity level in comparison with the sequences deposited in GenBank for the same genetic region was 89% (*i.e. P. decipiens*); the K2P distances between our sequences and the closest sequences from GenBank were 0.145.

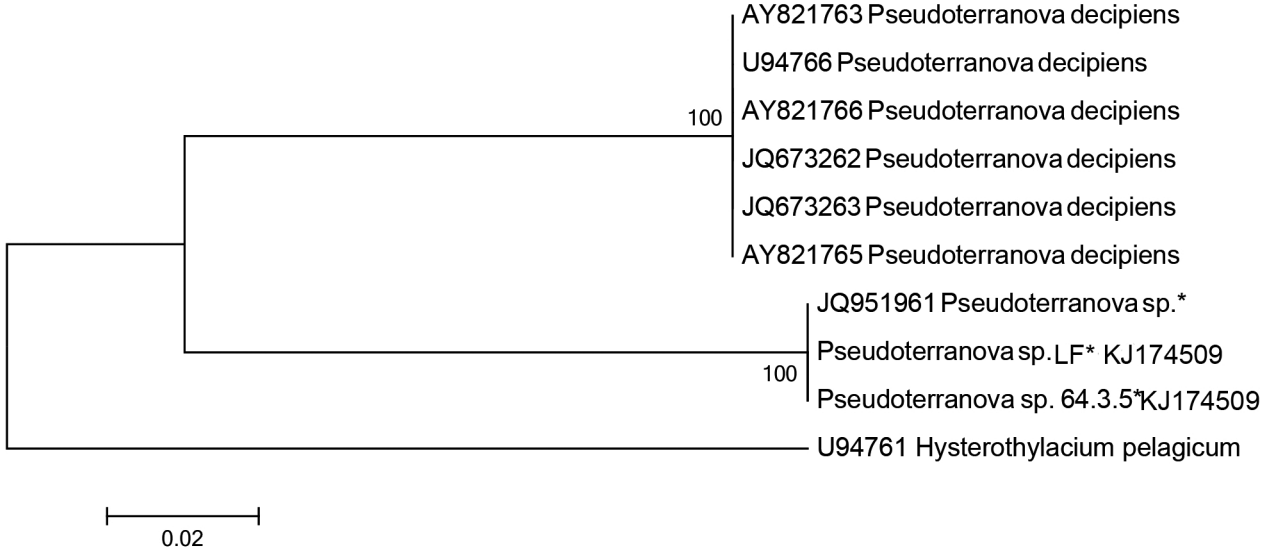

Fig. 3. Phylogenetic analysis of *Pseudoterranova* species obtained in this study (*) and sequences from the GenBank, based on Maximum Likelihood reconstruction of the partial 28S rDNA dataset. *Hysterothylacium pelagicum* was used as outgroup. Bootstrap values are exhibited at the nodes

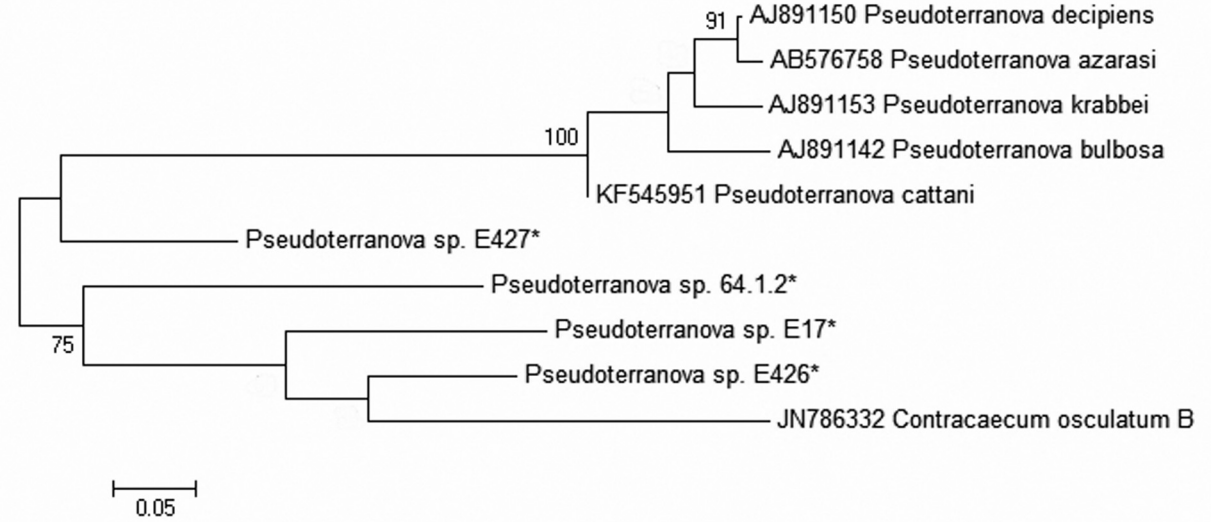

Fig. 4. Phylogenetic analysis of *Pseudoterranova* species obtained in this study (*) and sequences from the GenBank, based on Maximum Likelihood reconstruction of the partial mtDNA *cox*-1 dataset. Bootstrap values are exhibited at the nodes

The phylogenetic tree of *Pseudoterranova* sp. from both fish species confirmed the morphological assignment to the genus, but did not indicate high similarity with 28S rDNA region of the *P. decipiens*, the only species of the genus deposited in GenBank for this region (Fig 3).

Three specimens were also tested for mtDNA- *cox*1 region and the highest similarity level was 89% with *Contracaecum osculatum* B (JN786332). The K2P distances between our sequences and the closest sequence on GenBank ranged from 1.37 to 1.79, however the distances among our sequences varied greatly ranging from 0,145 to 0,187.

The phylogenetic tree of the sequences for the mtDNA *cox*-1 region grouped most of our sequences together with *Contracaecum osculatum* B, with some statistical support. Only one of our sequences remained out of this group and grouped closer to the clade for the other *Pseudoterranova* species (Fig 4).

Discussion

The *Pseudoterranova* sp. larvae were morphologically identified by the presence of long ventriculus and intestinal caecum and the tail with transversal striations and mucron. The phylogenetic tree showed low similarity among the sequences obtained in this study and those of the *P. decipiens* complex deposited in GenBank. According to Mattiucci and Nascetti (2008) species within *P. decipiens* complex presented from 0 to 6.8% of variability for ITS region. However, the highest similarity value found in our analysis was 89% and the lowest K2P distance was 0.145. The LSU region used in this study is more conserved than ITS region and the variability found was higher, therefore our data indicate that the species found belong to another species not yet characterized by LSU region. Our mtDNA *cox*-1 sequences presented great variability among them. Bluoin *et al* (2002) showed that distance between congeners would be as high as 0.16 for mitochondrial genes. The negative amplification of the ITS1, 5.8S, ITS2 intergenic regions (ITS) did not allow a comparison with other *Pseudoterranova* species. The genotyping of more species with the 28S will enable GenBank to become a robust tool for identification and phylogenetic analyses.

In this study only five larvae of *Pseudoterranova* sp. were found parasitizing *T. lepturus* and four in *P. saltatrix* among larvae of *Anisakis typica* and *Hysterothylacium* sp. previously reported (Borges *et al*. 2012). *Balaenoptera borealis* Lesson, 1828 and *Balaenoptera physalus* L., 1758 (Cetacea: Mysticeti) in addition to *Kogia breviceps* (Cetacea: Odontoceti) were reported to be parasitized by *Pseudoterranova* sp. off the Brazilian coast (Santos and Lodi 1998; Pinto *et al*. 2004; Muniz-Pereira *et al*. 2010). A likely explanation for the low prevalence of *Pseudoterranova* sp. in *T. lepturus* and *P. saltatrix* may be the habitat and migration route of definitive hosts along the Brazilian coast. The Mysticeti perform Antarctic breading migrations up to 5ºS only during winter/ spring seasons (Zerbini *et al*. 1997) and *K. breviceps* lives in oceanic waters beyond the edge of the continental shelf (Caldwell and Caldwell 1989). The oceanic habitat of the definitive hosts may probably influence the prevalence of parasitism, reducing the chances of infection in the coastal regions. Similar conclusions have been shown by McClelland *et al*. (2000) that found significant differences comparing infection of larvae of *P. decipiens* in *Gadus morhua* from Canada where the populations of cod inhabiting areas near breeding sites of seals had infection levels significantly higher. This is the first attempt to identify genetically the larvae of *Pseudoterranova* sp. from Brazilian waters.

Conclusions

Our data indicate that the L3 larvae of *Pseudoterranova* sp. found in low prevalences in *P. saltatrix* and *T. lepturus* caught

in the continental shelf off Rio de Janeiro belong to another species not yet characterized by LSU or mtDNA *cox*-1 region.

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