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Morphological and molecular diagnosis of *Pseudoterranova decipiens* (*sensu stricto*) (Anisakidae) in imported cod sold in Brazil

Diagnose morfológica e molecular de *Pseudoterranova decipiens* (*sensu stricto*) (Anisakidae) em bacalhau importado vendido no Brasil

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Abstract

An anisakid nematode larva found in cod sold in the state of Minas Gerais, Brazil was studied by light and scanning electron microscopy and by a molecular approach. Mitochondrial cytochrome c-oxidase subunit 2 (mtDNA *cox*-2), 28S rRNA and ITS1, 5.8S and ITS2 regions were amplified using the polymerase chain reaction and sequenced to evaluate the phylogenetic relationships of the larva. The genetic profile confirmed that this larva belongs to the species *Pseudoterranova decipiens* (*sensu stricto*). This is the first molecular and ultrastructural study of *Pseudoterranova decipiens* (*sensu stricto*) in imported cod sold in Brazil. The health implications of these findings are discussed.

Keywords: Anisakiasis, codworm, food safety, public health.

Resumo

Uma larva de Nematoda anisakídeo encontrada em bacalhau comercializado no Estado de Minas Gerais, Brasil foi estudada por microscopias de luz e eletrônica de varredura e por uma abordagem molecular. As regiões da subunidade 2 da citocromo c-oxidase mitocondrial (mtDNA *cox*-2), 28S rRNA e ITS1, 5.8S e ITS2 foram amplificadas usando a reação em cadeia da polimerase e sequenciadas para avaliar as relações filogenéticas da larva. O perfil genético confirmou que esta larva pertence à espécie *Pseudoterranova decipiens* (*sensu stricto*). Esse é o primeiro estudo molecular e ultraestrutural de *Pseudoterranova decipiens* (*sensu stricto*) de bacalhau importado vendido no Brasil. As implicações destes resultados para a saúde são discutidas.

Palavras-chave: Anisakíase, bacalhau, segurança alimentar, saúde pública.

Introduction

Fishes known as cod actually comprise five different species. The term 'cod' originally referred to fishes from the Atlantic and Pacific Oceans that are processed by salting and drying (OLIVEIRA et al., 2012). Among these species, *Gadus morhua* Linnaeus, 1758 and *Gadus macrocephalus* Tilesius, 1810 (Gadidae) are known as Atlantic and Pacific cod that occur, respectively, in the North Atlantic and Pacific Oceans. Brazil imports salted cod mainly from Norway and Portugal, and according to Brazil's Ministry of Fisheries and Aquaculture, 45 million tons were sold in 2011 (BRASIL, 2013). These fish are known to host different helminth parasites, including anisakid nematodes (CHANDRA & KHAN, 1988; HEMMINGSEN & MACKENZIE, 2001; PERDIGUERO-ALONSO et al., 2008; CATALANO et al., 2014; MEHRDANA et al., 2014). In addition to *G. morhua* and *G. macrocephalus*, other species of salted fish are sold erroneously as cod in Brazil, namely saithe *Pollachius virens* (Linnaeus, 1758), ling *Molva molva* (Linnaeus, 1758) and tusk (zarbo) *Brosme brosme* (Ascanius, 1772) (LIMA & SANT'ANA, 2011). Reports of the

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occurrence of anisakid nematodes in cod and other similar salted and dried fish sold in Brazil have begun to appear (PRADO & CAPUANO, 2006).

Due to their zoonotic potential and possibility of inducing allergic reactions (RAMOS, 2011; SUZUKI et al., 2010; NIEUWENHUIZEN & LOPATA, 2013), the occurrence of parasites in fish is considered a quality defect, according to Margeirsson et al. (2007), and as such, they are included in regulatory recommendations for the consumption of salted or dried salted fish in Brazil (BRASIL, 2007). With regard to the harmful effects of these parasites on human health, various authors have produced contradictory results/conclusions about the efficacy of salting to inactivate helminth larvae (RAMOS, 2011). Some authors claim that only live parasites can affect human health (ALONSO et al., 1997; RODRÍGUEZ et al., 2006; AUDICANA & KENNEDY, 2008), while others have demonstrated that even inactivated parasites can cause allergic reactions (FERNÁNDEZ DE CORRES et al., 1996; DEL REY MORENO et al., 2006; VIDACEK et al., 2009).

Cases of human infection resulting from the ingestion of live anisakid larvae have been reported in various geographical regions (MERCADO et al., 2001; YU et al., 2001; CABRERA et al., 2003; NA et al., 2013; QIN et al., 2013; CHOI et al., 2014; RAMANAN et al., 2013). In Brazil, probable human cases are rare (AMATO et al., 2007; CRUZ et al., 2010), although these larvae do occur in different marine fishes in the region (MATTIUCCI et al., 2002; LUQUE et al., 2011; BORGES et al., 2012; FONTENELLE et al., 2013; KNOFF et al., 2013).

Members of the Anisakidae Skrjabin & Karokhin, 1945 in cod include species of the genus *Pseudoterranova* Mozgovoi, 1950, whose larvae reportedly cause human anisakiasis and pseudoterranoviasis through the ingestion of inadequately cooked fish (MATTIUCCI & NASCETTI, 2008; PERDIGUERO-ALONSO et al., 2008; TIMI et al., 2014). Although this genus has been known mainly in terms of the species complex *Pseudoterranova decipiens* (*sensu lato*), it actually includes the following species: *Pseudoterranova decipiens* Krabbe, 1878 (*sensu stricto*); *P. krabbei* Paggi, Mattiucci, Gibson, Berland, Nascetti, Cianchi & Bullini, 2000; *P. bulbosa* Cobb, 1888; *P. azarasi* Yamaguti & Arima, 1942; *P. cattani* George-Nascimento & Urrutia, 2000; *P. kogiae* (Johnston & Mawson, 1939); and *P. ceticola* (Deardorff & Overstreet, 1981) (see MCCLELLAND, 2002; MATTIUCCI & NASCETTI, 2008; ARIZONO et al., 2011; TIMI et al., 2014). These larvae are difficult to differentiate and identify to the level of species based solely on morphology because there are few or no diagnostic characters at this level; hence, the need for species assignation based on genetic analyses. A few recent studies have reported the presence of *Pseudoterranova* larvae in cod based on molecular identification (BUCHMANN & KANIA, 2012; PUFALL et al., 2012). This study aims to identify the species of anisakid larvae occasionally found in salted cod sold as *G. morhua* in the municipality of Viçosa, southeastern Brazil.

Materials and Methods

A single nematode larva removed from three fleshs (1Kg) of salted cod sold in the municipality of Viçosa, state of Minas Gerais, Brazil (20° 45'14" S, 42° 52' 55" W) was washed in distilled water, cut into three pieces and fixed in 70% ethanol. The anterior and posterior regions were cleared in glycerine for examination by light microscopy. These fragments were then washed in 70% alcohol, dehydrated in an ethanol series, critical point dried with CO_2, coated with 20 nm of gold, and examined in a JEOL JSM-6390 scanning electron microscope.

The middle portion of the parasite was prepared for total genomic DNA extraction using the modified phenol/chloroform method developed by Billings et al. (1998). The primers used were 390 (5'-ATCCGTGTTTCAAGACGGG-3') and 391 (5'-AGCGGAGGAAAAGAAACTAA-3') for 28S rRNA gene (NADLER et al. 2005), NC5 (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (5'- TTAGTTTCTTTTCCTCCGCT-3') for ITS1, 5.8S and ITS2 genes (UMEHARA et al., 2006), and 210 (5'-CACCAACTCTTAAAATTATC-3') and 211 (5'-TTTTCTAGTTATATAGATTGRTTTYAT-3') for mitochondrial cytochrome c-oxidase subunit 2 (*cox-2*) (NADLER & HUDSPETH, 1998). PCR was carried out using cycling parameters, as previously described (NADLER & HUDSPETH, 1998; FLOYD et al., 2002; NADLER et al., 2005; UMEHARA et al., 2006). The PCR products were visualized with SYBR green stain (Invitrogen, Eugene, OR, USA) before electrophoresis on 1.5% agarose gel. Amplified PCR products were purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA).

DNA cycle-sequencing reactions were performed using BigDye v.3.1 chemistry (Applied Biosystems, Foster City, CA, USA), while the sequencing reactions were performed in an ABI Prism 3100 genetic analyzer. The sequences were edited using DNASTAR SeqMan (DNASTAR, Inc., Madison, WI, USA) and compared for similarities with sequences from GenBank, using BLAST 2.0 (ALTSCHUL et al., 1990). To examine the phylogenetic relationships, the nucleotide sequences were analyzed using the CLUSTAL W algorithm of the BioEdit Package (THOMPSON et al., 1994; HALL, 1999). The Maximum Likelihood (ML) phylogenetic tree (FELSENSTEIN, 1981) was inferred with MEGA 5.0 software (TAMURA et al., 2011), using the Kimura 2-parameter (K2P) model, and gamma distribution was selected by MEGA 5.0. The Maximum Likelihood tree was resampled by 5,000 bootstrap replicates to evaluate the reliability of the groups. The model of evolution for the Bayesian inference (BI) tree was GTR+γ+I, and default priors were estimated using MrBayes 3.1.2 software (HUELSENBECK & RONQUIST, 2001). After 2,000,000 generations for convergence, 25% of the trees were discarded as burn-in and the rest were used for topology and posterior probability reconstruction.

Results and Discussion

The initial examination using light microscopy revealed that the worm was a third-stage anisakid larva with a boring tooth situated between the ventrolateral lips, the excretory pore at the base of the ventrolateral lips, a long ventriculus but no ventricular appendix, an intestinal caecum and a tail with a mucron. The scanning electron microscopy observations revealed three poorly developed lips, i.e., a dorsal lip bearing a pair of double papillae

and two ventrolateral lips (Figure 1a). The tail bore a terminal mucron (Figure 1b-d). Based on the morphology of the larva, it was identified as a species of *Pseudoterranova* Mozgovoi, 1951.

The subsequent molecular analysis enabled a direct comparison of the ITS1, 5.8S, ITS2 and 28S rDNA and the mitochondrial cytochrome c-oxidase subunit 2 (*cox*-2) sequences with those of *Pseudoterranova decipiens* Krabbe, 1878 (*sensu stricto*) in GenBank (GenBank accession numbers KF806033, KF806034 and KF806035). For *cox*-2 (accession number KF806035), the BLAST results indicated 98% identity with 100% query cover and a maximum score of 399 for *P. decipiens*, and 99% identity with 74% of query cover and a maximum score of 309 for *P. azarasi*. For the 28S gene (accession number KF806033) in relation to *P. decipiens*, there was a 100% identity, 99% query cover and a maximum score of 1382; no match was found for *P. azarasi*. For the sequence KF806034 (ITS1, 5.8S, ITS2 and 28S), the results for *P. decipiens* were as follows: identity = 100%, query cover = 98% and maximum score = 1607, and for *P. azarasi*: identity = 100%, query cover = 95% and maximum score = 1559. While the *cox-2* gene BLAST analysis resulted in a greater similarity to *P. azarasi* than to *P. decipiens*, the maximum score and query cover were greater for *P. decipiens.* For other genes, *P. decipiens* showed the higher values. The higher scores for *P. decipiens* can be explained by a longer query cover for *P. decipiens* than for *P. azarasi*, even though the similarity to *P. azarasi* was closer than to *P. decipiens*. Thus, the BLAST analysis pointed to the species *P. decipiens*. The finding of *P. decipiens* in salted *G. morhua* sold in Brazil is in accordance with a previous report (PEREIRA et al., 2000) and is now confirmed by a molecular approach. The alignment with *Pseudoterranova* species showed eight nucleotide differences between *P. decipiens* (this study) and *P. azarasi* (Figure 2).

Two different phylogenetic trees were generated (ML and BI) and provided similar results. Using the maximum likelihood tree (Figure 3), our sequence (KF806035) was closer to sequences of *P. decipiens* (accession numbers HM147278 and AF179920) than to the sequence of *P. azarasi* (accession number HM147281), despite the low bootstrap value (41%). However, compared to *P. azarasi*, the clade composed of these three *P. decipiens* sequences showed a considerably higher bootstrap value (84%). In the IB tree (Figure 4), the same group of sequences exhibited a reasonable posterior probability (58%) and a higher bootstrap value (89%). In accordance with Timi et al. (2014), our phylogenetic trees strongly

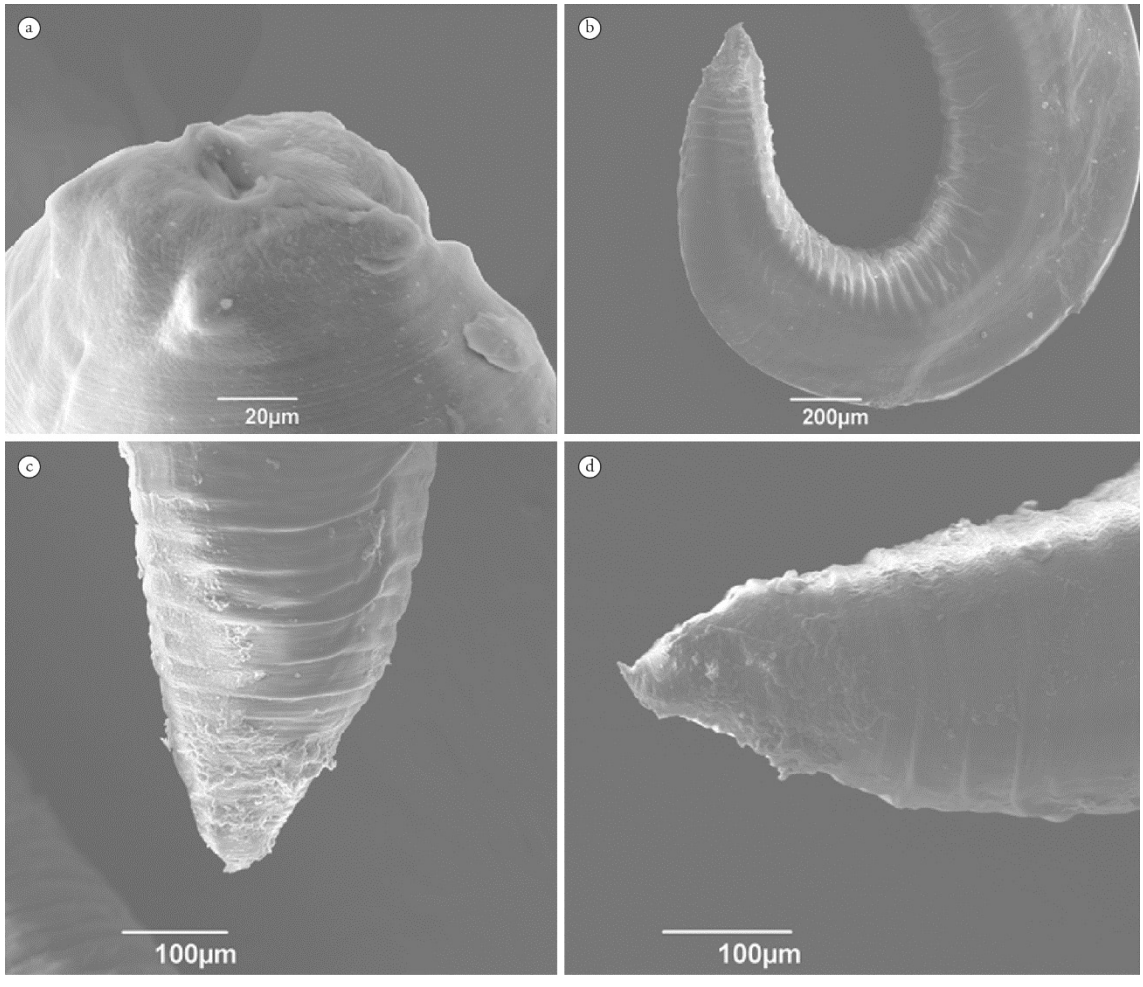

Figure 1. Scanning electron microscopy of L3 larva of *Pseudoterranova decipiens* (*s.s.*): a) anterior region with dorsal lip bearing two double papillae, a boring tooth situated between the two ventro-lateral lips and the oral aperture; b-d) tail with a mucron.

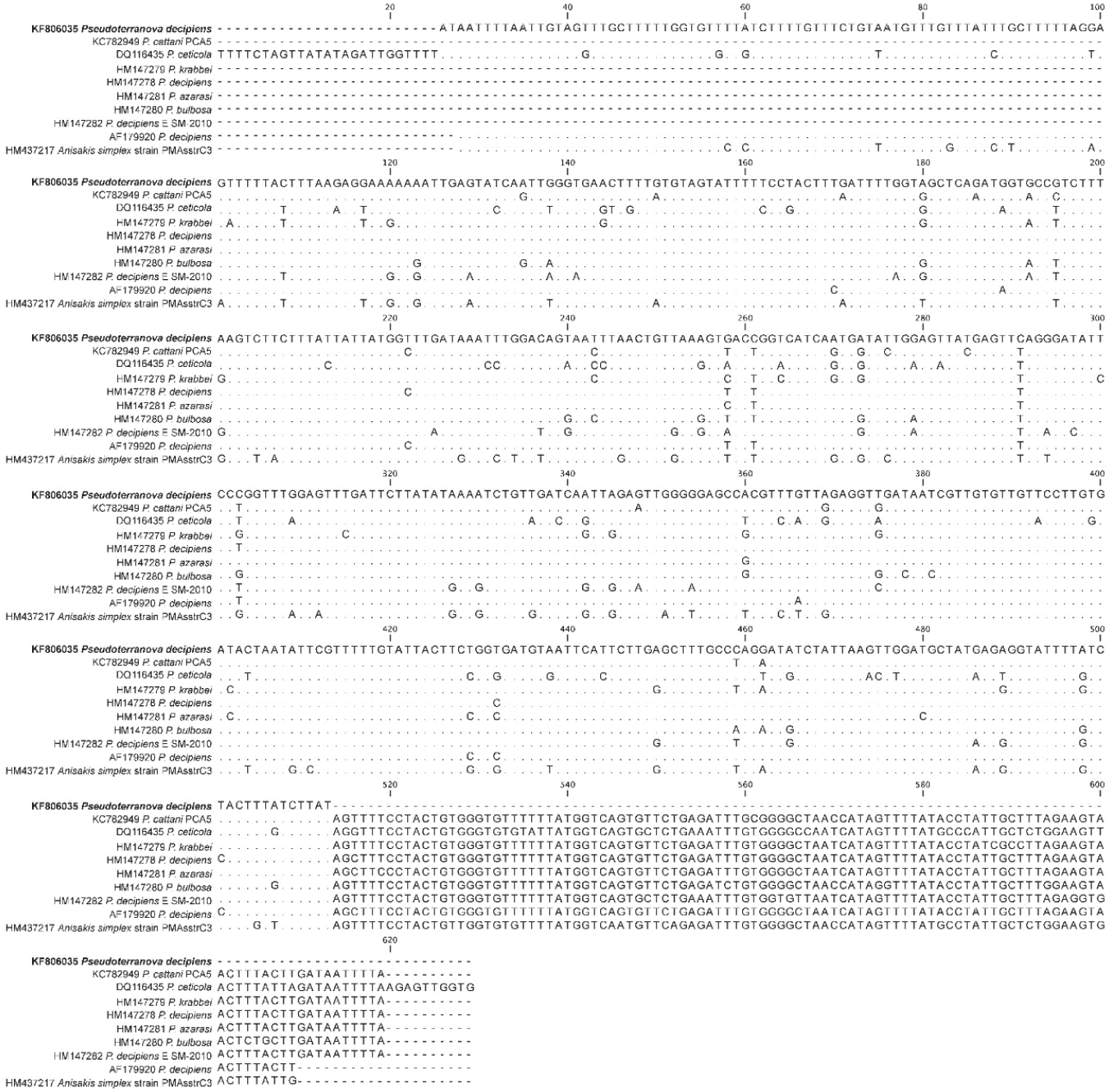

Figure 2. Sequence alignments of *Pseudoterranova* species. The numbers above the sequences represent nucleotide position. The dots indicate equal nucleotides compared with the sequence of *P. decipiens* found herein (in bold). A dash represents a gap.

support *P. decipiens* sp. E (accession number HM 147282-1) as a sister taxon to the remaining species of the *P. decipiens* complex.

Phylogenetic analyses are important, since the species *P. decipiens* and *P. azarasi* are very closely related (MATTIUCCI & NASCETTI, 2008). The identification of *P. decipiens* in commercial food fish such as cod can have both economic and public health impacts, with wild fisheries sustaining considerable losses due to possible recommendations by international or regional regulatory agencies to forbid the consumption of salted and dried fish. As many as 107 parasite species, including both protozoan and metazoan forms, have been identified in *G. morhua* alone (HEMMINGSEN & MACKENZIE, 2001). The finding of codworm *P. decipiens* (*s. s.*) in marketable cod in Brazil therefore rings an alarm bell in terms of a public health risk in this country.

As an emerging zoonosis first diagnosed in the Netherlands in 1955 (VAN THIEL et al., 1960; SAKANARI & MCKERROW, 1989; ISHIKURA, 2003), anisakiasis, the disease caused by the ingestion and establishment of anisakid larvae in the human gastrointestinal tract, has received considerable attention in recent years. There are many reports of its occurrence in marketable cod and other dried, salted

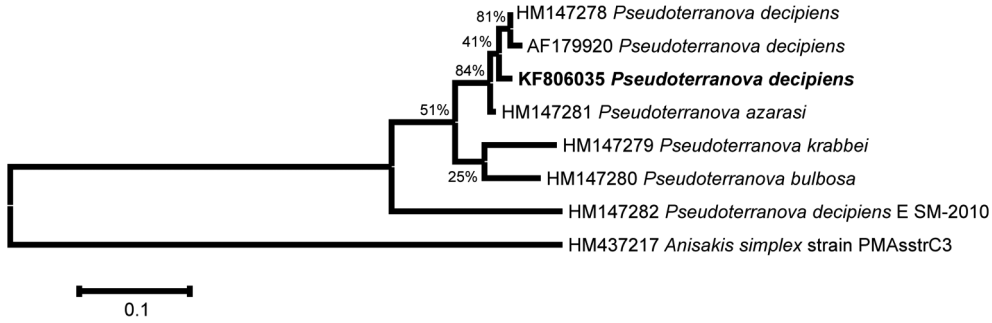

Figure 3. Phylogenetic tree based on the Maximum Likelihood of the partial cytochrome c oxidase subunit II gene, showing the relationship of *Pseudoterranova decipiens* (bold) with others species of *Pseudoterranova*. Numbers (percentage) on the branches indicate 5,000 bootstrap replicates. GenBank accession numbers are shown. Scale bars indicate the nucleotide mutations per site.

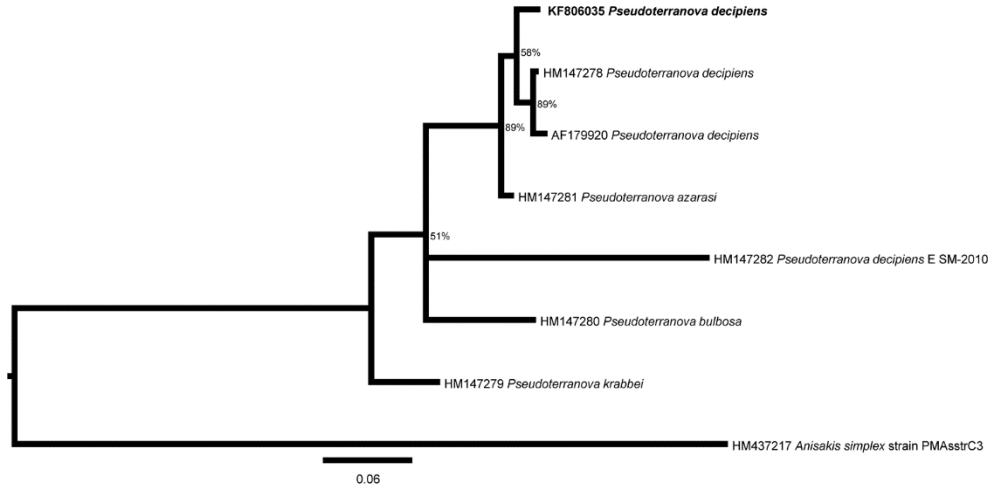

Figure 4. Phylogenetic tree based on the Bayesian inference of the partial cytochrome c oxidase subunit II gene, showing the relationship of *Pseudoterranova decipiens* (bold) with others species of *Pseudoterranova*. Numbers (percentage) on the branches indicate the posterior probability. After 2,000,000 generations a convergence was achieved. GenBank accession numbers are shown. Scale bars indicate the nucleotide mutations per site.

or fresh fish (e.g., TORRES et al., 2007; ARIZONO et al., 2011; MATTIUCCI et al., 2013; NIEUWENHUIZEN & LOPATA, 2013). Anisakiasis, including pseudoterranoviasis, represents an emerging public health problem in Brazil that requires greater attention by physicians and by sanitary and epidemiological services, especially considering the possibility of allergic reactions resulting from the ingestion of these larvae. In a recent recommendation, consultants of Brazil's Ministry of Health included *P. decipiens* on the *Classification List of Biological Risks* as a potential hazard for humans (BRASIL, 2010). This is the first molecular and ultrastructural study of *Pseudoterranova decipiens* (*sensu stricto*) in imported cod sold in Brazil. This report on the identification and occurrence of this parasite in imported cod underscores the need for a rational government program for the risk management of anisakid nematode larvae.

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