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Morphological and genetic identification of *Anisakis paggiae* (Nematoda: Anisakidae) in dwarf sperm whale *Kogia sima* from Brazilian waters

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ABSTRACT: Anisakid nematodes have been identified in a wide variety of fish and marine mammal species. In Brazil, *Anisakis physeteris*, *A. insignis*, *A. typica*, *A. nascetti*, and those of the *A. simplex* complex have been reported infecting fishes and cetaceans. In this study, specimens collected from a dwarf sperm whale *Kogia sima* (Owen, 1866) stranded on the northeastern coast of Brazil were identified through morphological and genetic analyses as *A. paggiae*. Anisakids were examined through differential interference contrast light and scanning electron microscopy (SEM). Morphological and morphometric analysis revealed that these specimens belonged to *Anisakis* sp. clade II and more specifically to *A. paggiae*, exhibiting a violin-shaped ventriculus and 3 denticulate caudal plates, which are taxonomic characters considered unique to this species. Genetic analysis based on the mtDNA *cox2* gene confirmed our identification of *A. paggiae*. Phylogenetic trees using both maximum likelihood and neighbor-joining methods revealed a strongly supported monophyletic clade (bootstrap support = 100%) with all available *A. paggiae* sequences. Integrative taxonomic analysis allowed the identification of *A. paggiae* for the first time in Brazilian waters, providing new data about their geographical distribution. Moreover, here we present the first SEM images of this species.

KEY WORDS: Cetacean · Parasitic nematode · Scanning electron microscopy · Light microscopy · mtDNA *cox2* · Brazil

INTRODUCTION

Nematodes of the genus *Anisakis* Dujardin, 1845 are common parasites of marine mammals, the definitive hosts. Humans can become infected by ingesting raw or undercooked fish, the intermediate hosts, thereby acquiring anisakiasis (Mattiucci & Nascetti 2008). In Brazil, anisakid infections reported in the marine cetaceans *Stenella clymene*, *S. longirostris*, *Peponocephala electra*, *Steno bredanensis*, and *Kogia breviceps* (Motta et al. 2008) were identified as *A. typica* (Iñiguez et al. 2011). *Anisakis typica* and *A. physeteris* were previously identified in Brazil by means of genetic markers (D'Amelio et al. 2000, Mattiucci et al. 2002, Mattiucci & Nascetti 2008, Iñiguez et al. 2009, 2011, Borges et al. 2012). Recently, *A. nascettii* was identified and genetically characterized in a Gervais' beaked whale *Mesoplodon europaeus*

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stranded on the northeast coast of Brazil (Di Azevedo et al. 2014). *Anisakis nascettii*, *A. physeteris*, *A. insignis*, *A. typica*, and those of the *A. simplex* complex were therefore the only species identified to date in cetaceans from this country (Luque et al. 2010, Di Azevedo et al. 2014). However, some specimens were reported as *Anisakis* sp. (Carvalho et al. 2010, Luque et al. 2010), suggesting that other species might also be found in the littoral of Brazil.

Anisakis paggiae Mattiucci et al. 2005 was described as a taxon of *Anisakis* sp. clade II in *K. sima* and *K. breviceps* from West Atlantic waters, on the coast of Florida, USA (Mattiucci et al. 2005). *Anisakis paggiae* have also been reported on the coast of Europe (Mattiucci et al. 2007), Caribbean Sea (Colón-Llavina et al. 2009), Gulf of Mexico (Cavallero et al. 2011), coast of Japan (Murata et al. 2011), and in the Irminger Sea, North Atlantic Ocean (Klimpel et al. 2011). Reports of *A. paggiae* in swordfishes from Atlantic tropical–equatorial waters (Garcia et al. 2008, 2011) expanded the geographical distribution of this species to South Atlantic waters (10° S). Recently, *Anisakis* sp. specimens genetically close to *A. paggiae* were identified in a dwarf sperm whale *K. sima* from the Philippine archipelago (Quiazon et al. 2013). In the present study, integrative taxonomy, based on genetic and morphological data, revealed for the first time the presence of *A. paggiae* on the northeast coast of Brazil, providing a new geographical location for this species.

MATERIALS AND METHODS

Samples and morphological analysis

Nematodes were recovered during necropsy of a dwarf sperm whale stranded on Barra das Moitas beach, Amontada municipality in the state of Ceará, northeastern Brazil (03° 01′ 07″ S, 39° 39′ 46″ W), on 2 August 2012. Worms (2 males and 3 females) were collected from stomach contents and conserved in ethanol (70%) until morphological screening. First, all specimens were observed through light microscopy to identify taxonomic characters of genus and species, following Davey (1971) and Mattiucci et al. (2005). Then, 1 male and 3 females had their anterior and posterior regions separated, clarified by Amann's lactophenol, and analyzed with a Zeiss Axiophot microscope, using bright field and differential interference contrast. Mid-section fragments were freeze-dried for molecular studies. One male was reserved for scanning electron microscopy (SEM) following the protocol of Lopes Torres et al. (2013). Two specimens of *Anisakis paggiae* including 1 male (no. 35796a) and 1 female (no. 35796b) were deposited in the Helminthological Collection of Oswaldo Cruz Institute (Coleção Helmintologica do Instituto Oswaldo Cruz-CHIOC), Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro.

DNA extraction and PCR assay

Samples were ground in liquid nitrogen and DNA extraction was conducted using the QIAamp® DNA Mini Kit (Qiagen), as described by Iñiguez et al. (2012). DNA was quantified using a spectrophotometer (Gene Quant II, Pharmacia Biotech). A mitochondrial DNA fragment of 629 bp from cytochrome oxidase gene subunit 2 (mtDNA *cox2*) was amplified using primers and PCR conditions described by Nadler & Hudspeth (2000) and Knoff et al. (2012), respectively. Amplicons were directly sequenced using the Big Dye Terminator v 3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems) in a 3100 Automated DNA Sequencer as recommended by the suppliers.

Sequencing analysis

Sequences were analyzed using the global Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information database) and BioEdit v7.0.4.1 (Department of Microbiology, North Carolina State University, USA). Intra- and inter-specific genetic distances were calculated using MEGA v 6 (Tamura et al. 2013) with the Kimura-2-parameter (K2P) model (Kimura 1980). Maximum likelihood (ML) and neighbor-joining (NJ) phylogenetic trees were inferred using the same software, with the K2P model following the Barcoding CBOL protocol (www.barcodeoflife.ord/content/resources/standards-and-guidelines), and a general time reversible (GTR) model, as selected by the Model Selection tool in MEGA, respectively. Complete deletion and gamma distribution parameters were used. One thousand bootstrap replicates were applied to evaluate the reliability of clusters. *Hysterothylacium aduncum* (GenBank JQ934891) was used as the outgroup. The *cox*2 gene alignment was examined for genetic signatures (i.e. polymorphisms shared only by individuals of a species or a clade), using GeneDoc software v. 2.6.002 (Nicholas et al. 1997). Sequences were translated using the invertebrate mitochondrial code and cytochrome oxidase 2 protein (COII) and also checked for amino acid signatures. The new *A. paggiae* sequence was submitted to GenBank (accession number KF693769).

RESULTS

Anisakid specimens analyzed in this study were morphologically assigned to clade II and were identified as *Anisakis paggiae* according to the taxonomic key of Mattiucci et al. (2005). Measurements were taken and compared to those described in that study (Table 1). Morphological identification was based on 5 adult worms, viz. 3 females and 2 males (1 of which was used for light microscopy and the other for SEM). After light microscopy examination, posterior and anterior portions of 1 male and 1 female were deposited in CHIOC, as mentioned above. Three mid-section fragments were used for molecular analyses.

Morphological data

Light microscopy revealed 3 lips at the anterior end, 2 ventrolateral and 1 dorsal, exhibiting a large

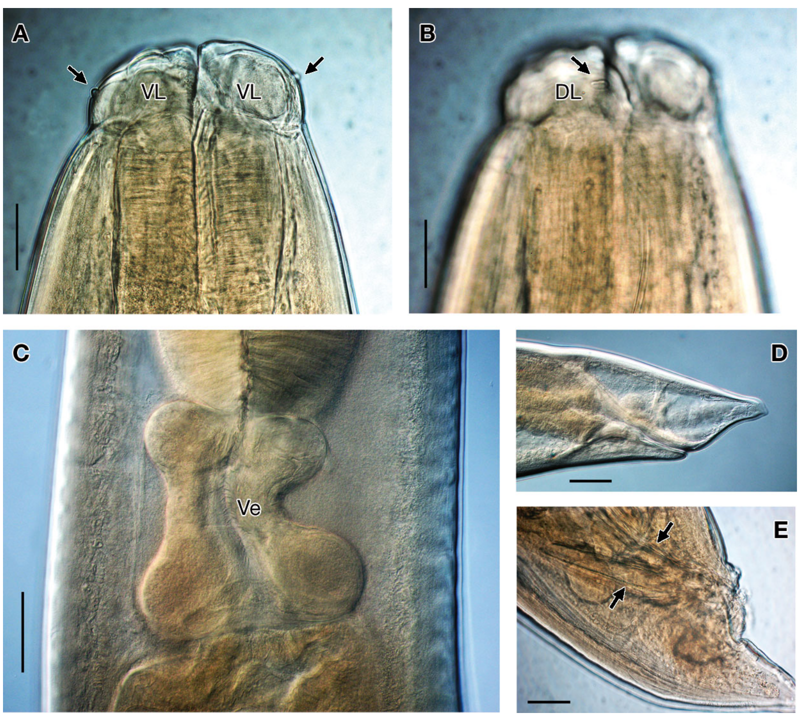


Fig. 1. Differential interference contrast images of *Anisakis paggiae*. (A) Anterior end showing papillae (arrows) in ventrolateral lips (VL). (B) Anterior end, showing large papilla (arrow) in the dorsal lip (DL). (C) Ventriculus (Ve) is short and violin-shaped. (D) Female conical tail with anal opening. (E) Posterior end of male with a pair of spicules (arrows) that are similar in size. Scale bars = (A,B,E) 50 µm, (C,D) 100 µm

Table 1. Morphometric data of *Anisakis paggiae* specimens from the present study and from reference material. The original description of *A. paggiae* was published by Mattiucci et al. (2005). Measurements are in mm; wpl: width of denticulate caudal plates

Source	Body length	Esophagus		Ventriculus		Spicule length		Plectane width		
		Length	Width	Length	Width[a]	Right	Left	wpl1	wpl2	wpl3
Males										
Present study (n = 2)	35, 32	2.3, 2.15	0.39, 0.36	0.4, 0.37	0.24, 0.25	0.18, 0.18	0.19, 0.19	0.049, 0.05	0.036, 0.037	0.04, 0.041
Original description	23–40	2–2.5	0.4–0.45	0.35–0.40	0.24–0.27	0.17–0.21	0.18–0.22	0.049–0.051	0.03–0.04	0.04–0.045
Females										
Present study (n = 3)	28–42	2.5–2.7	0.29–0.32	0.39–0.42	0.28–0.33	–	–	–	–	–
Original description	29–50	2.6–2.8	0.3–0.4	0.41–0.45	0.30–0.35	–	–	–	–	–

[a]Measured at the constriction

papilla (Fig. 1A,B). A short violin-shaped ventriculus could be observed (Fig. 1C, Table 1). The female posterior end exhibited a conical tail with a terminal anus (Fig. 1D), while males had a pair of similar spicules and numerous cloacal papillae (Fig. 1E, Table 1). SEM showed the anterior end with 3 prominent lips ornamented with denticles, amphids, and papillae (Fig. 2A). These papillae were present on the external surface of the lips (Fig. 2A). The internal surface presented cuticular bifid structures with internal denticles organized in plates (Fig. 2A). The dorsal lip presented 2 large papillae, and each ventrolateral lip had a double papilla and an amphid (Fig. 2A–C). At the posterior end, it was possible to observe a tail with a rounded terminal tip, a cuticular surface containing numerous caudal papillae, and 2 cuticular dilatation structures (Fig. 2D). Caudal papillae (nomenclature according to Fagerholm 1989) were as follows: 9 pairs of precloacal papillae were arranged in a single row, 1 pair of single proximal papillae, 1 pair of double paracloacal papillae, and 4 pairs of single postcloacal (distal) papillae (d1, d2, d3, and d4; Fig 2E,F). On the cloacal aperture, there were 3 denticulate caudal plates, wpl1, wpl2, wpl3 (plectanes; Fig. 2F). On the tip of the tail, on the lateral surface of the cuticle, we observed a pair of phasmids (Fig. 2G).

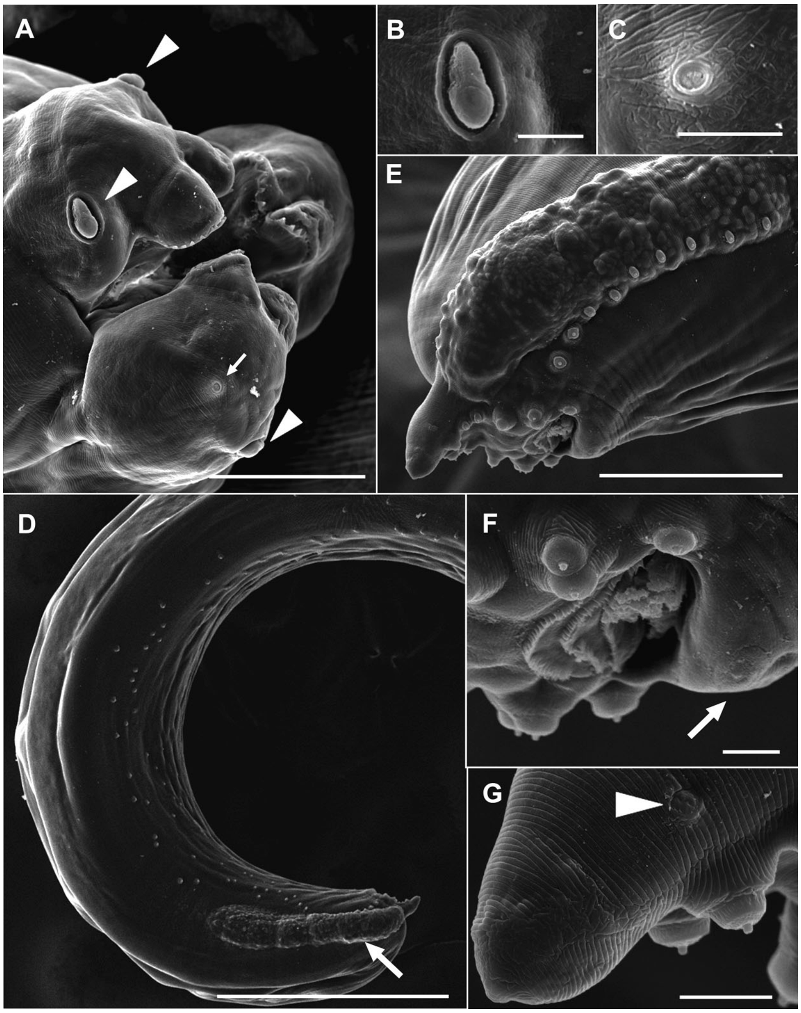


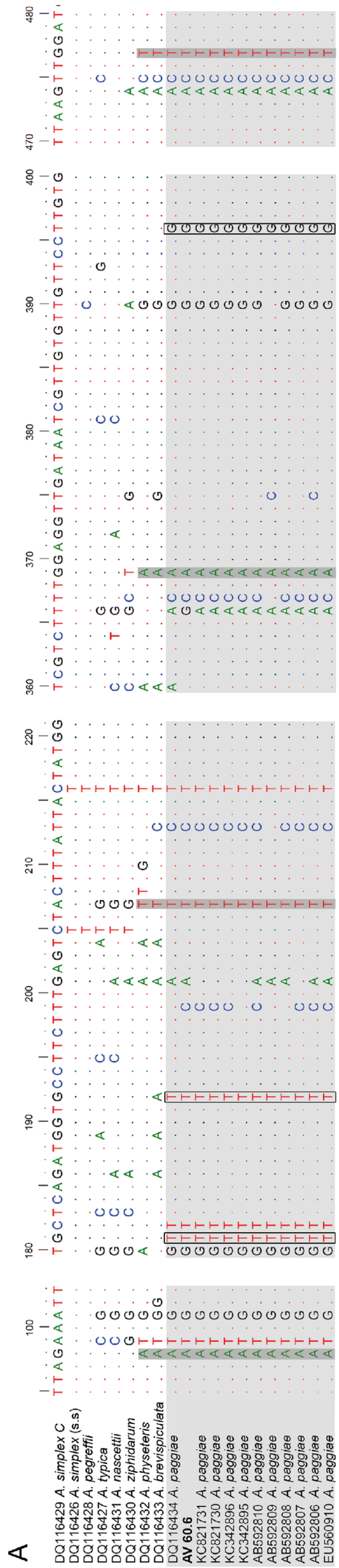
Fig. 2. Scanning electron microscopy of *Anisakis paggiae* male. (A) Anterior end, showing 1 dorsal lip with 2 large papillae (arrowheads) and 2 ventrolateral lips, 1 of which is showing 1 double papilla (arrowhead) and 1 amphid (arrow). (B) Detail of a large papilla. (C) Detail of an amphid. (D) Posterior end, showing numerous proximal papillae and 1 of the lateral cuticular dilatations (arrow). (E) Posterior end, showing 9 pairs of single precloacal papillae, a pair of single proximal papillae, a pair of double paracloacal papillae, and 4 pairs of single postcloacal papillae. (F) Detail of the cloacal region, showing the cuticular median structure (arrow), 3 denticulate caudal plates (plectanes), a pair of single proximal papillae, and a pair of double paracloacal papillae. (G) Rounded tail tip showing the last pair of postcloacal (distal) papillae and a phasmid (arrowhead). Scale bars = (A) 50 µm, (B,C,F,G) 10 µm, (D) 500 µm, (E) 100 µm

Molecular data

The nucleotide sequence of the *cox2* gene confirmed the species identity as *A. paggiae* in sample AV 60.6. Negative results were possibly due to poor quality/quantity of DNA, since specimen conservation was not appropriate for genetic analysis. BLASTn searches revealed 99% identity with *A. paggiae* (GenBank accession number DQ116434). Alignment with all previously characterized sequences of anisakid nematodes (505 bp), using an *A. berlandi* (formerly *A. simplex* sp. C; Mattiucci et al. 2014) sequence (DQ11 6429) as a reference, revealed 4 genetic signatures of clade II (i.e. polymorphisms shared only by individuals of clade II): G98A, A207T, G369A, and G477T, and 3 genetic signatures of *A. paggiae* species: G181T, G192T, and T396G (i.e. polymorphisms shared only by *A. paggiae* sequences; Fig. 3A). Pairwise interspecific analysis with 9 previously characterized *Anisakis* species and the *Anisakis* sp. sequence from this study revealed a high level of genetic identity with *A. paggiae* sequences (K2P = 0.023; SE = 0.004). Intraspecific genetic distances between available *A. paggiae* sequences and the one from this study ranged from K2P = 0.014 (SE = 0.005) to K2P = 0.033 (SE = 0.008). The minimum value of the *A. paggiae* interspecific distance (K2P = 0.123; SE = 0.015) was obtained with *A. ziphidarum.* This value was higher than the maximum value of intraspecific distance of *A. paggiae* (K2P = 0.033; SE = 0.008), indicating the existence of the so-called barcode gap, i.e. a lack of overlap between intra- and interspecific distances (Ratnasingham & Hebert 2007). Consequently, inclusion of the sequence reported here did not affect *A. paggiae* as a taxonomic unit. The generated ML and NJ topologies confirmed the identity *of A. paggiae*. Phylogenetic analysis revealed *Anisakis* sp. from this study clustering with all *A. paggiae* sequences reported in a monophyletic clade with a high bootstrap value (ML = 94 %, NJ = 100 %; Fig. 4). Comparison of the COII protein revealed a specific *A. paggiae* sequence with 168 amino acids from positions 24 to 191 using *A. berlandi* (*A. simplex* C) DQ116429 sequence as a reference (Fig. 3B). The *A. paggiae* COII sequence is characterized by the genetic signatures G28V, N34S, V137I, and V169L, as well as S33N, which is specific to clade II, and A61F, which is specific to *A. paggiae* specimens (Fig. 3B).

DISCUSSION

Phylogenetic analysis based on allozymes and nuclear and mitochondrial DNA divided the genus *Anisakis* into 2 major clades. These clades were also distinguished by larval morphotype (Mattiucci & Nascetti 2008, Mattiucci et al. 2009). The taxonomic key for adult recognition is based mainly on morphological features, including length and shape of both ventriculus and male spicules, as well as the arrangement of male caudal papillae. In this study, we identified *Anisakis* sp. specimens belonging to clade II based on the original description by Mattiucci et al. (2005). The morphologies of the ventricules and male spicules of these specimens were characteristic of species from clade II, viz. *A. physeteris*, *A. brevispiculata*,



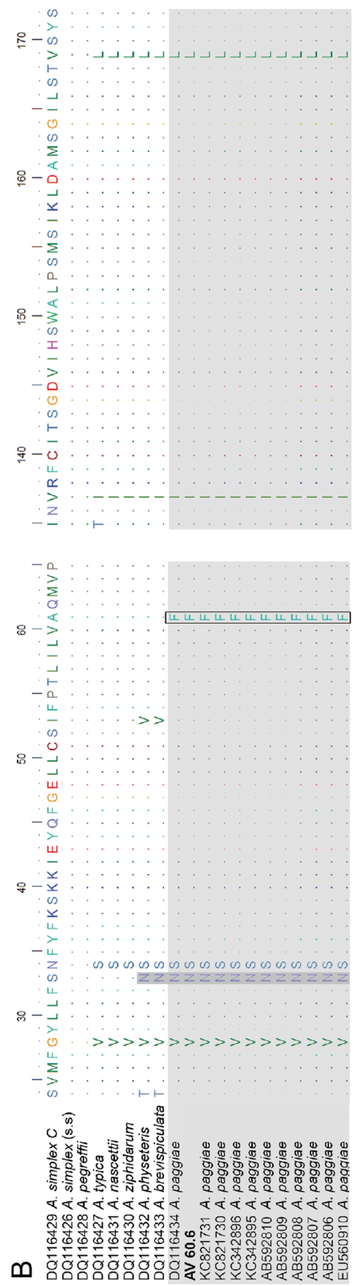


Fig. 3. Alignment of (A) *cox2* gene and (B) COII protein sequences from *Anisakis* spp. and those obtained in this study (AV60.6). GenBank accession numbers and *Anisakis* species names are shown. Points indicate identical position with *A. berlandi* (formerly *A. simplex* sp. C) reference sequence. *A. paggiae* specimens are in light grey. Genetic signatures of clade II and of *A. paggiae* are in dark grey and in boxes, respectively. Only sequence portions with genetic signatures described are shown

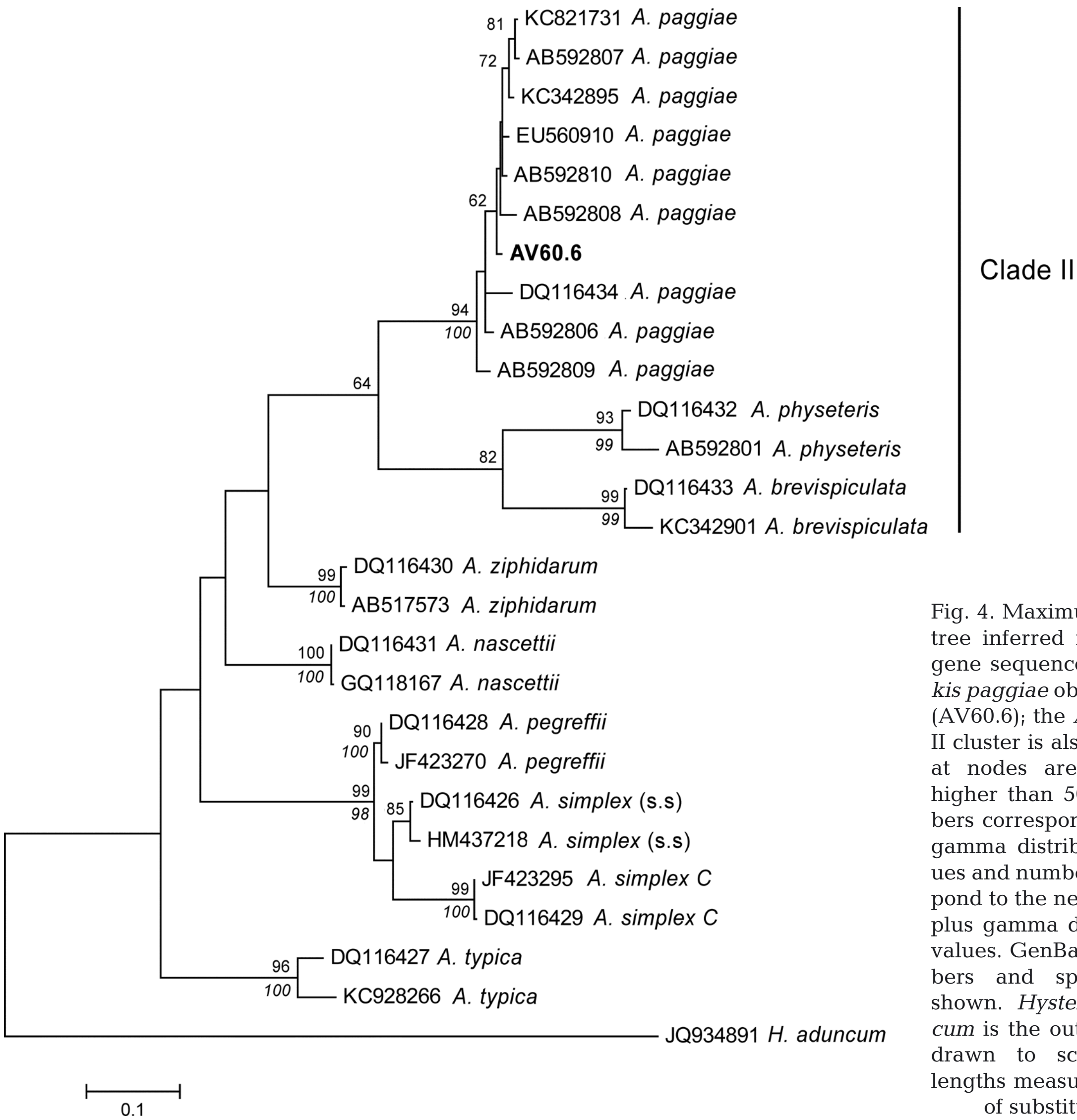


Fig. 4. Maximum likelihood (ML) tree inferred from mtDNA *cox2* gene sequence data from *Anisakis paggiae* obtained in this study (AV60.6); the *Anisakis* spp. clade II cluster is also shown. Numbers at nodes are bootstrap values higher than 50%. Regular numbers correspond to ML GTR plus gamma distribution support values and numbers in *italics* correspond to the neighbor-joining K2P plus gamma distribution support values. GenBank accession numbers and species names are shown. *Hysterothylacium aduncum* is the outgroup. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site

and *A. paggiae.* Spicules and ventricules of males were shorter than 0.35 mm and 0.56 mm, respectively (Table 1), therefore excluding the possibility of specimens belonging to *A. physeteris* and *A. brevispiculata* (Mattiucci et al. 2005). As the spicules were shorter than 0.22 mm and 3 denticulate caudal plates were present, we determined that the species analyzed here was *A. paggiae*.

An adult male *A. paggiae* was analyzed by SEM. Our results showed that the anterior end of this nematode presented denticles that may be related to fixation on host tissue (Motta et al. 2008). The previously described double papillae in the dorsal lip (Mattiucci et al. 2005) did not have an external division, constituting, in fact, a large papilla. A similar structure was observed in *Contracaecum osculatum* by SEM (Fagerholm 1989). Moreover, the originally reported median papilla on the posterior end of males did not have the morphology of a papilla, being a cuticular median structure, as clearly shown by SEM analysis in the present study. Finally, the distribution of papillae in the cloacal region observed by SEM analysis resembled that reported by Mattiucci et al. (2005).

Morphological identification of *A. paggiae* was also confirmed genetically through *cox2* genetic distance comparison, NJ and MP phylogenetic trees, and *in silico* protein and DNA signature analyses. Inter- and intraspecific genetic distances supported our identification of *A. paggiae*. Phylogenetic trees exhibited a monophyletic and strongly supported clade with Brazilian *Anisakis* sp. and all previously character-

Table 2. Hosts and geographical location of *Anisakis paggiae* described to date

Host(s)	Geographical location	Reference
Definitive hosts		
Kogia breviceps and *K. sima*	Florida coast, USA	Mattiucci et al. (2005)
K. breviceps	West Atlantic Ocean (Florida coast)	Valentini et al. (2006)
K. breviceps and *K. sima*	Caribbean Sea	Colón-Llavina et al. (2009)
K. breviceps and *K. sima*	Gulf of Mexico	Cavallero et al. (2011)
K. sima	Atlantic coast of Brazil	Present study
Intermediate hosts		
Merluccius merluccius	Northeastern Atlantic Ocean (Galician coast)	Mattiucci et al. (2005)
	Atlantic coast of Europe	Mattiucci et al. (2007)
Aphanopus carbo	Central Atlantic waters (off Madeira)	Mattiucci et al. (2005)
Xiphias gladius	Atlantic coast of Europe	Mattiucci et al. (2007)
	Atlantic tropical–equatorial waters	Garcia et al. (2008)
	Eastern tropical and Central South Atlantic Ocean	Garcia et al. (2011)
Theragra chalcogramma	Pacific coast of Japan	Quiazon et al. (2009)
Beryx splendens	Pacific coast of Japan	Murata et al. (2011)
Anoplogaster cornuta	Irminger Sea (North Atlantic)	Klimpel et al. (2011)

ized *A. paggiae*. DNA and protein signatures revealed not only polymorphisms specific to *A. paggiae* species, but also to clade II. Our study corroborates the usefulness of the *cox2* genetic marker as a barcode of anisakid species, as previously shown (Valentini et al. 2006, Mattiucci et al. 2009, Knoff et al. 2012, Di Azevedo et al. 2014).

According to the literature (Table 2), the spectrum of definitive hosts of *A. paggiae* is limited to members of the family Kogiidae (Klimpel et al. 2008, 2010), including *Kogia breviceps* and *K. sima.* Regarding the intermediate/paratenic hosts of *A. paggiae*, some fish species have so far been recognized (Table 2). Cephalopods and marine fish, and occasionally crustaceans, are prey items for dwarf sperm whales (Willis & Baird 1998, Culik 2010, Klimpel et al. 2010). Parasite transmission in aquatic ecosystems should be inferred in the context of food webs (Marcogliese 2002). *Xiphias gladius*, *Beryx splenden*, and *Anoplogaster cornuta* were reported as intermediary hosts of *A. paggiae* (Table 2) and are found in Brazilian waters (Froese & Pauly 2014). Therefore, it would be expected that *A. paggiae* could be found in the littoral of this country. Moreover, according to Culik (2010), the geographical distribution of dwarf sperm whales is widespread in tropical and temperate seas, including South Atlantic waters. The abovementioned arguments suggest a possible maintenance of the *A. paggiae* cycle in this region.

Until recently, reports of *A. paggiae* had been limited to boreal waters (Table 2). A record of *A. paggiae* infecting *K. breviceps* along the South African coast, reported by Mattiucci & Nascetti (2006, 2008), is not supported by those studies' referred literature. Therefore, the geographic distribution of *A. paggiae* had been restricted to a range of 15 to 45° N, before it expanded to 10° S with findings in Atlantic tropical–equatorial waters (Garcia et al. 2011). Our data revealed the presence of *A. paggiae* in a new geographical location, i.e. the northeast coast of Brazil, Atlantic coast of South America, suggesting a wider distribution of this species and confirming its austral occurrence.

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