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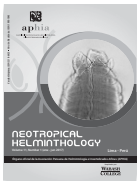

ORIGINAL ARTICLE / ARTÍCULO ORIGINAL

MOLECULAR IDENTIFICATION AND MORPHOLOGICAL CHARACTERIZATION OF *ANISAKIS* SPP. L3 LARVAE (NEMATODA: ANISAKIDAE) IN *SCOMBER COLIAS* GMELIN, 1789 (PERCIFORMES: SCOMBRIDAE) FROM NORTHERN ARGENTINA

IDENTIFICACIÓN MOLECULAR Y CARACTERIZACIÓN MORFOLÓGICA DE LARVAS L3 DE *ANISAKIS* SPP. (NEMATODA: ANISAKIDAE) EN *SCOMBER COLIAS* GMELIN, 1789 (PERCIFORMES: SCOMBRIDAE) DEL NORTE DE ARGENTINA

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ABSTRACT

We performed the molecular identification of nematode third-stage larvae parasites of *Scomber colias* Gmelin, 1789 collected on the northern coast of Argentina, and described the morphological and morphometric features of these parasites. Internal organs and muscles of 31 *S. colias* specimens were analyzed. The larvae genetic identification was performed by PCR-RFLP (*Polymerase Chain Reaction - Restriction Fragment Length Polymorphism)* using amplification of nuclear DNA fragment (ITS1, ITS2 and 5.8S) and cleavage with specific restriction enzymes (*Hinf*I, *Hha*I) for identification. For morphological and morphometric analysis, the specimens were observed in trinocular microscopy and in scanning electron microscopy. About 369 nematodes were collected in 68% of the analyzed fishes. All parasites were in the internal organs of the host. No larval forms were detected in the muscles. The molecular characterization produced four different patterns: *Anisakis pegreffii* Campana-Rouget & Biocca, 1955; *Anisakis typica* (Diesing, 1860); a hybrid of *Anisakis* spp; and other larvae that showed no molecular patterns for *Anisakis* (Karl Rudolphi, 1809). Morphological-morphometric differences between *A. pegreffii* and *A. typica* larvae were observed. Except the larval tooth, all structures of *A. pegreffii* had much higher dimensions than those found in *A. typica*. In addition, the posterior end of *A. pegreffii* tapered more gradually and a mucron accompanied this tapering. In *A. typica* the posterior end is more robust and the mucron is very tapered. This is the first contribution to molecular identification with morphological characterization of *Anisakis* spp. in *S. colias* from Argentina.

Keywords: chub mackerel – fish parasites – genetic identification – polymerase chain reaction – South America – zoonotic potential

RESUMEN

Se realizó la identificación molecular de larvas de nemátodos en el tercer estadío del *Scomber colias* Gmelin, 1789 recogidos en la costa norte de Argentina, y la caracterización morfológica y morfométrica de estos parásitos. Los órganos internos y los músculos de 31 muestras de *S. colias* fueron analizadas. La identificación genética de larvas se realizó por PCR-RFLP *(Polymerase Chain Reaction - Restriction Fragment Length Polymorphism)* mediante la amplificación de un fragmento de ADN nuclear (ITS1, ITS2 y 5,8S) y el escote con enzimas de restricción específicas (*Hinf*I, *Hha*I) para la identificación. Para el análisis morfológico y morfométrico, los especímenes se observaron en el microscopio trinocular y en microscopía electrónica de barrido. Alrededor de 369 nematodos fueron recolectados en el 68% de los peces analizados. Todos los parásitos se encontraron en los órganos internos del huésped, y no se detectó formas larvarias en los músculos. La identificación molecular produjo cuatro diferentes patrones *Anisakis pegreffii* Campana-Rouget & Biocca, 1955; *Anisakis typica* (Diesing, 1860); un híbrido de *Anisakis* spp; y otras larvas que no muestran patrones moleculares correspondientes a *Anisakis*. Se observaron diferencias morfológicas y morfométricas entre las larvas de *A. pegreffii* y *A. typica*. Excepto el diente larvario, todas las estructuras de *A. pegreffii* tenían dimensiones mucho mayores que los encontrados en *A. typica*. Además, el extremo posterior de *A. pegreffii* se estrecha más lentamente y el mucrón acompaña esta conicidad. En *A. typica* el extremo posterior es más robusto y el mucrón es muy afilado. Esta es la primera contribución a la identificación molecular con la caracterización morfológica de *Anisakis* spp. en *S. colias* de Argentina.

Palabras clave: caballa – parásitos de los peces – identificación genética – reacción en cadena de la polimerasa – América del Sur – potencial zoonótico

INTRODUCTION

Fish meat is the most required source of animal protein worldwide and possesses a significant market value (Sidonio *et al.*, 2012), due to it being one of the most important food animals for human consumption, with great nutritional emphasis relative to the quantity and quality of its protein, its high digestibility, vitamins and minerals presence and, especially, by being a source of essential fatty acids like omega-3, which comprises the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Sartori & Amancio, 2012).With the increased consumption of raw fish in America, typical of oriental and the Andes region cuisine, diseases previously unreported in humans have started to emerge. The most common among these diseases is anisakiasis, transmitted through consumption of raw or undercooked fish. In South America, Smith (1999) considers the species of the genus *Anisakis*, *Contracaecum* and *Pseudoterranova* as mainly responsible for these infections. About 20000 cases of anisakiasis have been registered worldwide, with over 90% of them in Japan (Chai *et al.*, 2005). Anisakiasis in humans can occur through ingestion of raw fish meat or that insufficiently treated by heat, salt or smoke, containing the third stage larvae. In this case man acts as an accidental host and the larvae do not complete their development. These parasites may penetrate the digestive tract of humans and invade others organs causing a number of pathological effects (Lymbery & Cheah, 2007).

However, it is possible that the ingested larvae do not attach to the gastrointestinal mucosa and are eliminated by vomit or faeces, which would represent a case considered as asymptomatic (Acha & Szifres, 2003). Thus, according to Germano & Germano (1998), this may be a reason for the lack of reported cases of human anisakiasis in many Latin American countries, where there is a progressive increase in the raw fish consumption, raising the possibilities of anisakiasis becoming an emerging zoonosis in the future.

The pathology caused by accidental ingestion by eating raw or undercooked fish infested with *Anisakis* spp. larvae causes allergic disorders, mainly characterized by acute hives generalized and swelling (angioedema) able to result in

anaphylactic shock (López-Serrano *et al.*, 2000). Symptoms occur usually between four and 72 h after ingestion of the parasitized fish. The acquisition of only fresh fish and their rapid evisceration can prevent larvae migration to the host muscle, considered one of the ways to prevent anisakiasis (Ventura *et al.*, 2008). However, ingestion of dead parasites in fishes after cooking or those preserved may also cause an allergic reaction (Audicana *et al.*, 2002).

The larval stages of *Anisakis* species in general do not have host-parasite specificity, and can be found in a wide variety of teleost fishes and also pelagic, benthic-pelagic and benthic crustaceans (Busch *et al.*, 2012). By using intermediate hosts of different trophic levels and habitats within the marine food chains, the *Anisakis* larvae become abundant in virtually all bathymetric levels (Kuhn *et al.*, 2013).

The natural presence of larvae in fish muscle is characteristic of some anisakid species (as *A. simplex* and *P. decipiens*), with recognized zoonotic importance, but the presence of other species of anisakid larvae in somatic musculature may be a consequence of *post-mortem* migration or during the freezing process (Lymbery & Cheah, 2007).

For any parasitological research and epidemiological study to be carried out with the parasites, the identification of the exact species that occurs in a given geographical area is the first parameter to be considered (Mattiucci *et al.*, 2011). Before the use of molecular techniques for diagnosis, anisakid larvae identification had been difficult because the species morphology is virtually indistinguishable among congeners, often leading to erroneous determinations (Klimpel & Palm, 2011; Kuhn *et al.*, 2013). The importance of such information also relies on the costs that the presence of anisakids implies for the fishery industry, reducing both the quality and the market value of the products (Timi *et al.*, 2014).

The application of methods such as PCR-RFLP (*Polymerase Chain Reaction - Restriction Fragment Length Polymorphism*) to taxonomic studies of *Anisakis* spp. have revealed nine different species with different preferences for hosts and zoogeographic regions (Klimpel *et al.*, 2004, 2008; Valentini *et al.*, 2006; Mattiucci & Nascetti, 2008). The determination of anisakids based on genetic molecular markers provides unambiguous identification tools for these helminths with zoonotic potential and is thus an essential requirement for proper epidemiological research (Mattiucci & Nascetti, 2008).

Scomber colias Gmelin, 1789 (Scombridae), commonly known as chub mackerel is an important commercial fish species with a wide distribution, covering the South and North Atlantic and the Mediterranean Sea (Collette & Nauen, 1983). This species is a relevant diet component of large pelagic fishes such as sharks, as well as marine mammals (like dolphins and whales) (Lockwood, 1988). This species was included in the IUCN Red List of Threatened Species in 2011 and listed as Least Concern, although there are indications of regional declines in some populations (IUCN, 2011).

Based on the foregoing, the present study aimed to perform molecular identification of *Anisakis* spp. L3 larvae parasitizing *S. colias* collected on the northern coast of Argentina, as well as characterize morphological and morphometric traits of these nematode species.

MATERIALS AND METHODS

Samples collection

The 31 samples of *S. colias* from the northern coast of Argentina were acquired at CEASA (Municipal Market) of the city of Bauru, SP, from March to June 2014. The fish were frozen and uneviscerated. The specimens were taken to the Laboratório de Ictioparasitologia of the Universidade do Sagrado Coração, where they were examined for parasites.

The fish were eviscerated through an incision near to the cloaca. The internal organs of the abdominal cavity were examined. These organs were passed through 75µm sieves and washed with water. The organ walls and contents were analyzed with a stereomicroscope looking for anisakid nematodes. Musculature was examined through filleting technique and inspection by transparency using a negatoscope.

The nematodes found were measured for total length and then sectioned into three parts: the anterior and posterior regions were separated for taxonomic study, fixed and preserved in ethanol 80% and subsequently clarified with Amann's Lactophenol following the methodology of Eiras *et al.* (2006) and the middle region was used for molecular study and preserved in ethanol 100%.

Molecular and morphological analysis

The total genomic DNA of larvae collected was extracted and purified according to the information described in the commercial Wizard genomic DNA purification kit (Promega). The larvae genetic identification were made by PCR-RFLP through amplification of nuclear DNA fragment (internal transcribed spacer - ITS1, ITS2 - and ribosomal subunit 5.8S) and subsequent cleavage with specific enzymes for the identification of *Anisakis* species (D'amelio *et al.,* 2000).

The PCR amplification reaction was conducted using the primers *forward* A (5′ GTCGAATTCGTAGGTGAACCTGCGGAAGG ATCA 3′) and *reverse* B (5′ GCCGGA TCCGAATCCTGGTTAGTTTCTTTTCCTCCG CT 3′) with a total volume of 20 µl containing 200 µM of each dNTP (dATP, dTTP, dGTP and dCTP), $MgCl_2$ 2.0 mM, buffer *Taq* 1X (20 mM Tris-HCl, pH 8.4 and 50 mM KCl), 0.5 units (U) of *Taq polymerase* (Invitrogen), 0.4 µM of each *primer* and 20-50 ng of genomic DNA. Amplification cycles followed an initial denaturation program at 94°C for 5 min followed by 35 cycles of denaturation at 94ºC for 30s, hybridization at 55°C for 30s and extension at 72°C for 30s with a final extension of 10 minutes at 72ºC (D'amelio *et al.,* 2000).

Two RFLP reactions were performed, one using the restriction enzyme *Hinf*I and another using the enzyme *Hha*I. Both had a final volume of 15 µl containing 7 µl of the PCR products, enzyme buffer 1X (100mM Tris-HCl, pH 7.9, 500mM NaCl, 100mM $MgCl_2$ and 10mM DTT), BSA 0.1mg/ml and 5 enzyme units (10U/ul) (Promega), and incubated for 2 hours at 37ºC and for 20 minutes at 65ºC.

The PCR-RFLP products were applied to agarose gel electrophoresis 1.5% stained with Nancy-520 (Sigma-Aldrich), using the molecular weight marker ladder 1Kb (Invitrogen), visualized under UV light and captured with a digital camera (OLYMPUS, CAMEDIA, C-5060 5.1 Megapixel). Individuals were identified as certain species within the genus *Anisakis* when presenting electrophoretic band sizes corresponding to the species-specific pattern cleavage described by D'amelio *et al.* (2000) to one or both RFLPs *Hinf*I and *Hha*I.

For the morphological analysis of individuals with scanning electron microscopy (SEM), some specimens were dehydrated through a graded series of decreasing ethanol, from 70% to 50%, and finally in 30% ethanol. The samples were dried in hexamethyl disilazane, coated with gold and examined in a FEI Quanta 200 scanning electron microscope at the Centro de Microscopia Eletrônica of Instituto de Biociências, UNESP, Botucatu, SP. A trinocular microscope was used for morphometric analysis (Nikon E200). Specimen measurements were obtained by a computerized image analysis system (Motic, Moticam 5.0MP). Measurements are given in micrometers (µm) and presented with the mean followed by the maximum and minimum values in parentheses.

RESULTS

Among the 31 specimens of *S. colias* analyzed, 21 (67.7%) were infected by at least one species of Nematoda. A total of 369 nematode specimens were collected (about 12 parasites per fish examined). The parasites were found in host stomachs, intestines and gonads. There were no parasites in the muscles of the analyzed fish. The fish showed a mean standard length of 27.94 ± 1.86cm and mean weight of 390.59 ± 61.30g.

Genetic identification of L3 type larvae (third stage larvae) by PCR-RFLP using the two *Hinf*I and *Hha*I restriction enzymes produced four different patterns: 222 individuals were identified as *Anisakis pegreffii* Campana-Rouget & Biocca, 1955, 66 as *Anisakis typica* (Diesing, 1860), 9 as a possible hybrid of *Anisakis* spp; and 72 other larvae did not amplify or showed no molecular patterns for the genus *Anisakis* (Table 1).

Table 1. Species identification based on PCR-RFLP patterns (bp = base pairs).

N. of individuals	Band Size - RFLP (bp)		Molecular identity
	HinfI	HhaI	
222	370, 300, 250	550, 430	*A. pegreffii*
66	620, 350	320, 240, 180, 160	*A. typica*
9	620, 370, 300, 250	550, 430	hybrid genotype
72	variable band size or no amplification	variable band size or no amplification	not identificated

Individuals identified as *A. pegreffii* showed 370, 300 and 250 bp (base pairs) after the restriction enzyme *Hinf*I, 550 and 430 bp after the enzyme *Hha*I (Figures 1A and 1B). The bands corresponding to *A. typica* were 620 and 350 bp after the *Hinf*I cleavage and 320, 240, 180 and 160 bp by the *Hha*I enzyme (Figures 1A and 1B). The hybrid presents bands of both species, *A. pegreffii* and *A. typica,* for RFLP with the enzyme *Hinf*I with bands 620, 370, 300 and 250 bp (Figure 2), while for RFLP analysis with the enzyme *Hha*I bands were 550 and 430 base pairs (Sample 18, Figures 2A and 2B). Individuals that were not identified showed a band pattern not corresponding to any *Anisakis* species for this marker (D'amelio *et al.,* 2000) or showed no amplification in the gel (Figures 1 and 2).

Table 2. Measurements of *Anisakis* spp. third stage larvae samples, *Scomber colias* parasites, collected in Argentina (*Anisakis pegreffii*: n = 10; *Anisakis typica*: n = 7) (TL = total length, MW = maximum width, SD = standard deviation).

	Anisakis pegreffii		*Anisakis typica*	
	Mean ± SD	min-max	Mean ± SD	min-max
Parasite (TL)	19725.7 ± 2067.3	15509-22999.2	10243.3 ± 1211.4	9272.3-11951.2
Parasite (MW)	468.3 ± 95.9	332.1-590.5	244.9 ± 6.7	210.8-251.6
Larval tooth (TTL)	14.8 ± 3.5	10.5-21.5	16.2 ± 2.3	12.9-18.5
Esophagus (ETL)	2498.6 ± 927.4	1659.6-4541.3	1632.4 ± 173	1297.3-1789.6
Esophagus (EMW)	199 ± 48.5	132.4-282.6	113.5 ± 17.4	77.7-134.2
Ventricle (VTL)	1260.6 ± 606.3	606.9-2169.5	120.1 ± 5.8	114.9-129.9
Ventricle (VMW)	269.8 ± 66.7	180.6-403.5	90.5 ± 3.4	85.4-94.4
Gut (GTL)	15836.0 ± 2524.8	12425.6-19909.5	8594.1 ± 1077	7715-10110.8
Gut (GMW)	323.9 ± 62	233-434.5	173.2 ± 9.6	165-186.7
Nervous ring (RTL)	113 ± 29.2	58.6-140	39.4 ± 5.4	34-44.7
Nervous ring (RMW)	116.6 ± 13.8	95.4-142.9	83.5 ± 3.9	79.6-87.4
Mucron (MTL)	34.2 ±7.8	23.2-49.2	15.1 ± 3.8	10.0-19.0
ETL/VTL	2.2 ± 0.6	1.4-2.8	13.2 ± 1.7	11.0-15.0
TL/ETL	8.7 ± 2.9	4.7-12.7	6.8 ± 0.7	5.7-7.3
TL/VTL	19.4 ± 9.3	9.1-33.3	85.8 ± 12.2	78.7-104

After the molecular analysis, some morphological and morphometric differences between the larvae of *A. pegreffii* and *A. typica* were observed. Except the larval tooth length, all other *A. pegreffii* structures presented much larger dimensions than those found in *A. typica* (Table 2; Figure 3A and 3C). Another more obvious difference is the relationship between measurements of esophagus and ventricle lengths and the parasite total length. *Anisakis typica* larvae showed higher values of ETL/VTL and TL/VTL than those found in *A. pegreffii*, since the *A. typica* esophagus length can be on average 13 times larger than the ventricle, while in *A. pegreffii* we noted that the esophagus length is twice as large as the ventricle (Table 2). Regarding the external morphology, at the anterior end of *A. pegreffii* it can be observed that the larval tooth is much less pronounced than in *A. typica* and the posterior end of *A. pegreffii* was more gently tapered slowly and the mucron accompanies this thinning while in *A. typica* the posterior end is more robust (or less tapered) and the mucron is well tapered (Figures 3 A-D).

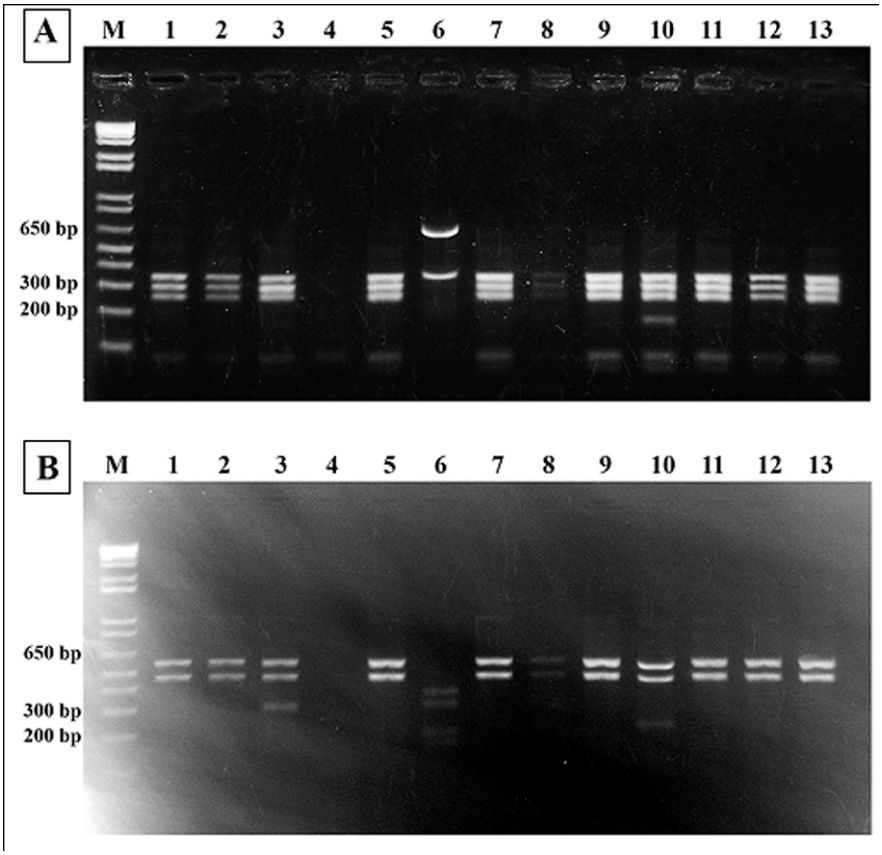

Figure 1. Genetic identification of *A. pegreffii* (Lanes 1, 2, 3, 5, 7, 9-13) and *A. typica* (Lane 6) individuals through PCR-RFLP using the restriction enzymes *Hinf*I (A) and *Hha*I (B). M: molecular weight marker 1kb; bp: base pairs.

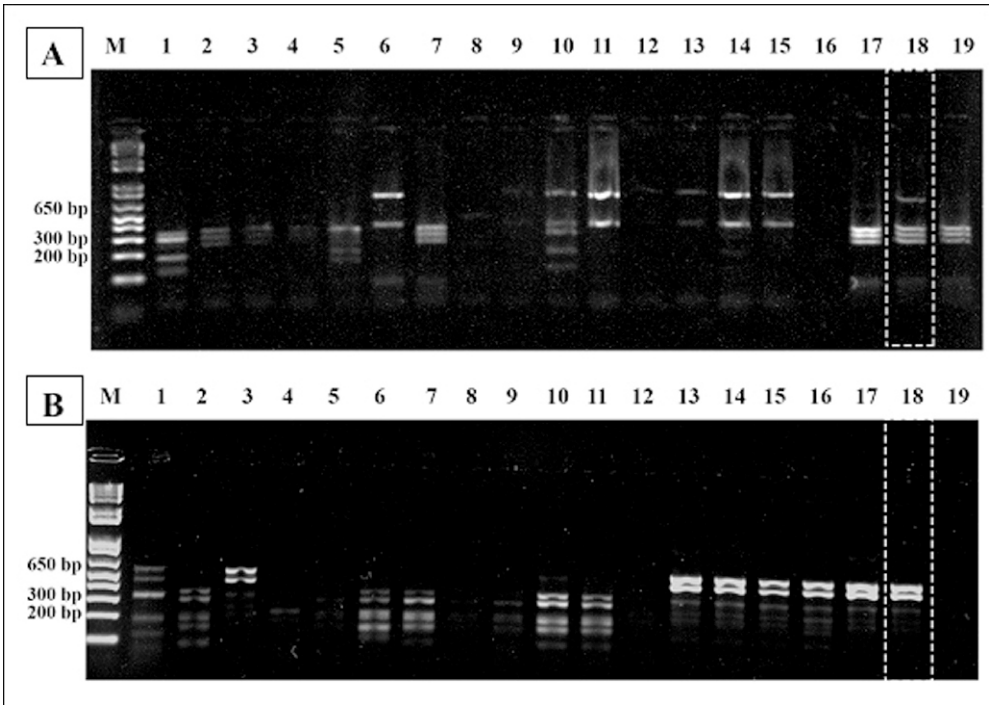

Figure 2. Genetic identification of a hybrid individual (Lane 18, highlighted by white dashes) through PCR-RFLP using the restriction enzymes *Hinf*I (A) and *Hha*I (B). M: molecular weight marker 1kb; bp: base pairs.

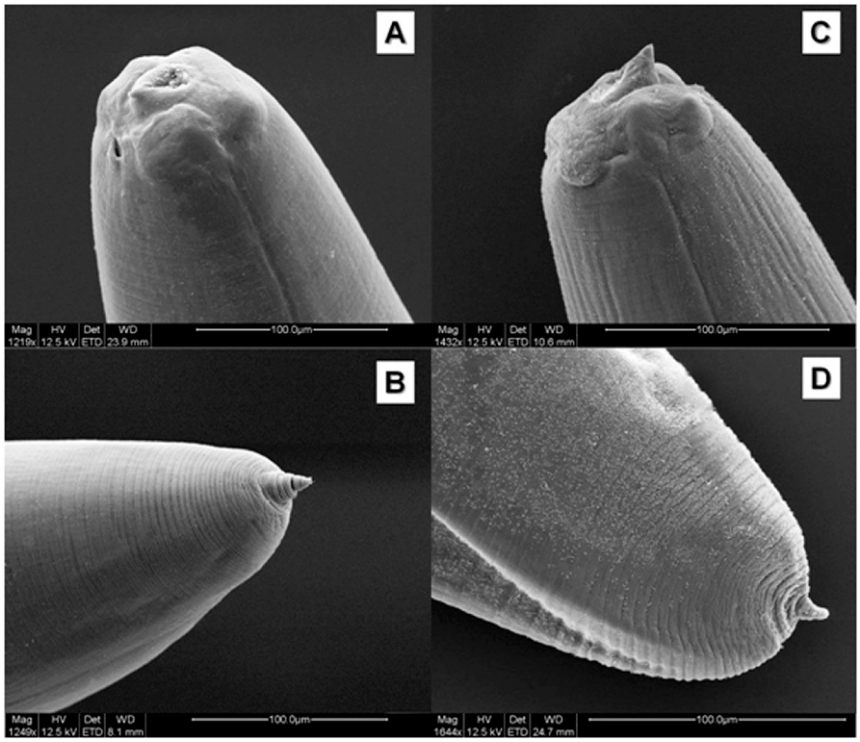

Figure 3. Morphology of third-stage larvae of *Anisakis* spp. parasites of *Scomber colias.* Scanning electron microscopy (SEM). A) *Anisakis pegreffii*, anterior; B) *Anisakis pegreffii*, posterior region highlighting the mucron; C) *Anisakis typica*, anterior; D) *Anisakis typica*, posterior region with mucron.

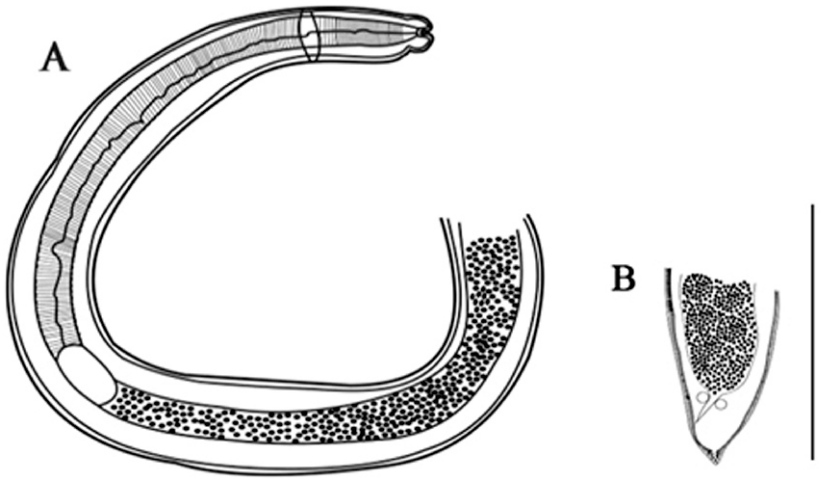

Figure 4. Internal morphology of *Anisakis typica* parasite of *Scomber colias*. A) anterior region, highlighting the esophagus, ventricle and nervous ring; B) posterior region. Scale: 10 mm.

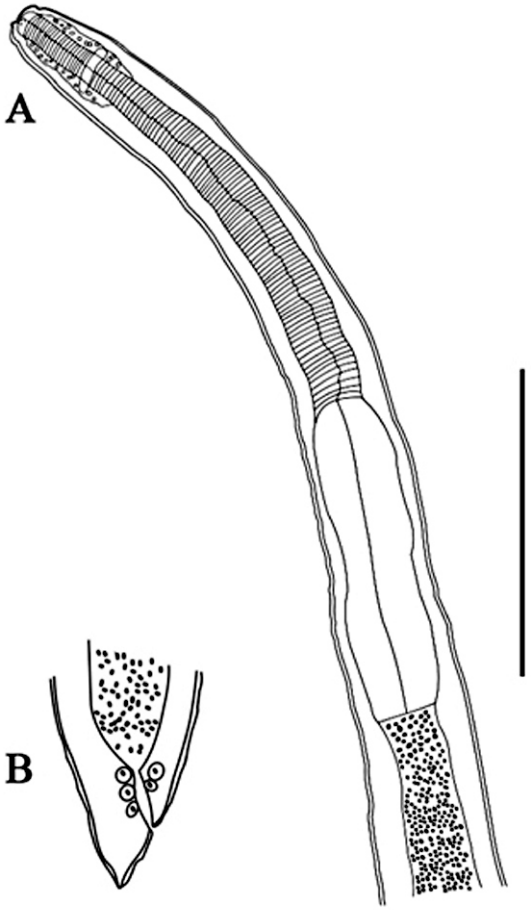

Figure 5. Internal morphology of *Anisakis pegreffii* parasite of *Scomber colias*. A) anterior region, highlighting the esophagus, ventricle and nervous ring; B) posterior region. Scale: 10 mm.

DISCUSSION

The present work is the first contribution to the molecular identification with morphological and morphometric characterization of *Anisakis* spp. in *S. colias* captured on the north coast of Argentina. All larvae found were located in the internal organs of the hosts, and no larvae were detected in muscles, although there are such records in the literature (Cremonte & Sardella, 1997; Costa *et al*., 2011; Molina-Fernandez *et al.,* 2015).

It can be affirmed that the risk of anisakiasis is real and must always be taken into consideration, despite the few official records of this zoonotic disease in Argentina (Menghi *et al.,* 2011), possibly as a consequence of its symptoms which can be easily confused with various diseases. However, as was observed in this work, the presence of *Anisakis* mainly in the mesentery and viscera can limit its zoonotic potential (Mattos *et al.,* 2014). Nevertheless, it is worthwhile to consider that some specimens of *S. colias* analyzed showed high intensity of parasitism by anisakids (up to 82 parasites/fish).

The lack of morphological differences among the larval stages of anisakids can occur due to factors such as similar selection pressures causing the conservation of morphology; consequently, some morphological characteristics have little or no taxonomic value due to evolutionary co-adaptation of these endoparasites in their stable habitat, represented by their definitive hosts. In other words, populations of parasites isolated in their host diverged genetically but preserved morphological characteristics, making it essential to use molecular tools for the correct diagnosis of these species (Mattiucci & Nascetti, 2008).

However, Murata *et al.* (2011) conducted a study of morphological and molecular characterization of *Anisakis* larvae and concluded that these larvae are differentiated not only by genetic analysis, but also by morphological characteristics present in the L3, which corroborates the results obtained in this present study, since the SEM assisted in the description of the external morphology with viewing important taxonomic structures for this parasite group. This technique has helped in the study of the external morphology of many nematodes and therefore been considered a very important tool in taxonomic studies (Eisenback, 1985).

Anisakis pegreffii was the parasite with highest incidence in this work, and this fact has been observed in other studies with the same host as well as in other marine fish species such as *Engraulis encrasicolus* (Linnaeus, 1758)*, Micromesistius poutassou* (Risso, 1827) and *Trachurus trachurus* (Linnaeus, 1758) (Costa *et al.*, 2011; Mladineo *et al.*, 2012; Piras *et al.*, 2014). Among the nine *Anisakis* species described and genetically characterized to date, two, *A. simplex s. str*. and *A. pegreffii*, have been identified as major agents of human anisakiasis, with very significant reports in countries like Italy and Japan (Umehara *et al.*, 2007; Mattiucci *et al.*, 2011).

Anisakis typica utilizes several species of aquatic mammals of the Delphinidae, Phocoenidae and Pontoporidae families as final hosts. This species is distributed in temperate and tropical waters, and their populations, even from remote locations, apparently have low intraspecific genetic homogeneity (Mattiucci *et al.*, 2002), and this pattern can also be observed in populations *of A. pegreffii*, according to Mattiucci *et al.* (1997). Also according to these same authors, this fact would indicate high levels of gene flow in these nematodes, which can be explained by the high vagility of intermediate and definitive hosts involved in their life cycles.

The hybridism among species belonging to the genus *Anisakis* has already been reported in other studies (Abollo *et al.*, 2003; Kuhn *et al.*, 2013). It is possible that hybridization in *Anisakis* allows an adaptation to particular environmental conditions or may be a consequence of the presence of incomplete barriers, enabling meetings among species, but it could be a reflection of the radiation within the genus *Anisakis*.

The four species belonging to the genus *Scomber* are commonly infected by anisakids, especially by members of the genus *Anisakis*. *Scomber australasicus* has the highest variability of species recorded (*A. pegreffii, A. simplex* s.s*., A. typica*, *A. paggiae*, *A. physeteris*, *A. brevispiculata* and a recombinant genotype), followed by *S. japonicus* (*A. pegreffii*, *A. simplex* s.s., *A. typica*, *A.*

physeteris, *A. ziphidarum* and a recombinant genotype), by *S. colias* (*A. pegreffii*, *A. physeteris*, *A. nascettii* and *A. typica*), and *S. scombrus* (*A. pegreffii*, *A. simplex* s.s. and *A. physeteris*) (Abollo *et al.*, 2001; Pontes *et al.*, 2005; Costa *et al.*, 2011; Bak *et al.*, 2014; Chen & Shih, 2015).

The results obtained in this study may assist health authorities and veterinarians to better control the parasite occurrence, from the fish production phase to their marketing, thus decreasing the morbidity and mortality rates in captivity, with improvement in the fish quality for the consumer, and prophylactically, to prevent the spread of zoonoses transmitted by fish.

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