



Genetic analysis of *Anisakis typica* (Nematoda: Anisakidae) from cetaceans of the northeast coast of Brazil: New data on its definitive hosts

Alena M. Iñiguez^{a,*}, Vitor L. Carvalho^b, Monica R. Alves Motta^c,
Diana C. Sousa Nunes Pinheiro^c, Ana C. Paulo Vicente^a

^a Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro 21045-900, Brazil

^b Associação de Pesquisa e Preservação de Ecossistemas Aquáticos – AQUASIS, Caucaia 61627-010, Ceará, Brazil

^c Programa de Pós Graduação em Ciências Veterinárias da Universidade Estadual do Ceará – PPGCV/UECE, Fortaleza 60740-000, Ceará, Brazil

ARTICLE INFO

Article history:

Received 3 March 2010

Received in revised form

22 December 2010

Accepted 7 January 2011

Keywords:

Anisakid

Anisakis typica

ITS region

Brazilian coast

Cetacean host

ABSTRACT

Anisakids from 5 different species of cetacean, *Kogia breviceps*, *Peponocephala electra*, *Stenella clymene*, *Stenella longirostris* and *Steno bredanensis*, were submitted to genetic analysis. Adults and larvae fixed in ethanol–formalin–acetic acid or in 70% ethanol for periods ranging from 10 months to 10 years were isolated from 9 cetaceans stranded on Ceará coast, Northeast Brazil. The 18S rDNA gene, ITS1, and specific *Anisakis typica* ITS regions were amplified by PCR. 18S rDNA and ITS1 region confirmed *Anisakis* sp. morphological identification but also detected the presence of *Aspergillus* sp. in longer preserved samples. All samples were identified as *A. typica* by ITS species-specific PCR. The study report three new definitive hosts of *A. typica* from the Brazilian Atlantic coast by genetic analysis: *P. electra*, *K. breviceps*, and *S. clymene*.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Nematodes of the family Anisakidae are probably the most prevalent parasites in marine mammals and have been associated with gastric lesions. Transmission is through the consumption of a variety of fish, squid, and crustaceans infected with the anisakid larvae. Accidental ingestion of these larvae by humans can cause the gastrointestinal infection, anisakiasis, and a series of allergic reactions (Couture et al., 2003; Pellegrini et al., 2005; Choi et al., 2009). *Anisakis typica* (Diesing, 1860) was first identified parasitizing *Delphinus delphis* in the Central Atlantic Ocean, but has since been recorded in several delphinid species, and in *Phocoena phocoena* (Phocoenidae), and *Pontoporia blainvillei* (Pontoporidae) (Mattiucci et al.,

2002; Nadler et al., 2005; Valentini et al., 2006; Mattiucci and Nascetti, 2008). There are more than 10 intermediate/paratenic host species characterized by molecular markers including *Thunnus thynnus* and *Auxis thazard* (Scombridae), the latter identified only in Brazil. The definitive and intermediate/paratenic hosts of *A. typica* have an epipelagic distribution in the Atlantic coast off Brazil, Uruguay, and Argentina (D'Amelio et al., 2000; Mattiucci et al., 2002; Pontes et al., 2005), the Atlantic coast off Florida and Caribbean sea (Mattiucci et al., 2005; Colon-Llavina et al., 2009), the Atlantic coast off Portugal and Morocco (Marques et al., 2006), the Somali coast of the Indian Ocean, the Eastern Mediterranean sea off Crete and Cyprus (Mattiucci et al., 2002), and the North African central Mediterranean coast (Farjallah et al., 2008a,b). More recently, *A. typica* infections were confirmed in fish in seas of China, Thailand, Indonesia, Japan and Taiwan, thereby extending its geographical range (Zhu et al., 2007; Chen et al., 2008; Umehara et al., 2010).

* Corresponding author. Tel.: +55 21 38658168; fax: +55 21 22604282.
E-mail address: alena@ioc.ficoruz.br (A.M. Iñiguez).

Table 1

Brazilian anisakid samples used in this study, hosts species and their period of preservation.

Host register	Host species	Host family	Stranding place Beach/municipality	Specimens evaluated	Life stage	Period of preservation (years)
02C1212/70	<i>Steno bredanensis</i>	Delphinidae	Iguape, Aquiraz	10	Adult	10.0
02C1512/79	<i>Peponocephala electra</i>	Delphinidae	Barro Preto, Aquiraz	10	Adult	10.0
02C1512/145	<i>Peponocephala electra</i>	Delphinidae	Icarai, Caucaia	10	Adult	7.0
02C0521/149	<i>Kogia breviceps</i>	Kogiidae	Serviluz, Fortaleza	3	Adult	7.0
02C1152/219	<i>Stenella clymene</i>	Delphinidae	Praia do Diogo, Beberibe	10	L3–L4	3.6
02C1132/221	<i>Stenella longirostris</i>	Delphinidae	Icarai, Caucaia	10	Adult	3.0
02C1131/225	<i>Stenella longirostris</i>	Delphinidae	Praia da Barrinha, Icapui	10	L3–L4	3.0
02C1151/242	<i>Stenella clymene</i>	Delphinidae	Paracuru	10	Adult	2.6
02C1512/273	<i>Peponocephala electra</i>	Delphinidae	Barra do Ceará, Fortaleza	10	Adult	0.1

Until recently, when *Anisakis physeteris* larvae was detected in the fish host, *A. thazard* (Iñiguez et al., 2009), *A. typica* was the only anisakid identified in Brazil by means of genetic markers [multilocus allozyme electrophoresis (MAE), intergenic transcribed spacer (ITS)-, RFLP and mitochondrial cytochrome c-oxidase subunit 2 (mtDNA *cox 2*)] (D'Amelio et al., 2000; Mattiucci et al., 2002; Valentini et al., 2006; Mattiucci and Nascetti, 2007). The study, based on the ITS region, documented the high prevalence of the *A. typica* in *Sotalia guianensis* and *A. thazard* off the Brazilian southeast coast. Recently, we confirmed *A. typica* infection in gastric lesions in cetaceans stranded on the Ceará State coast, in northeast Brazil (Motta et al., 2008). However, there are no records of human anisakiasis in Brazil, possibly by the lack of information about the infection.

In this study anisakid specimens collected from 9 cetaceans belonging to the Delphinidae and Kogiidae families were genetically analyzed. We established a methodology for DNA extraction from fixed specimens, identified all anisakids as *A. typica* and presented new definitive host to this specie.

2. Materials and methods

Nematodes were isolated from 9 hosts comprising 5 cetacean species (Table 1) collected from the Ceará coast, northeast Brazil (Fig. 1.) between 1994 and 2006 (Motta et al., 2008). Nematodes were isolated from the stomach compartments in the gastric lumen and gastric mucosa or the stomach wall mucosa and fixed in either AFA (ethanol–formalin–acetic acid) or 70% ethanol, and preserved for periods ranging from 10 months to 10 years (Table 1). For morphological identification, nematodes were cleared with phenol and identified as *Anisakis* sp. based on morphological characters through microscopy (max $\times 400$) using Davey (1971), and Mattiucci et al. (2005).

In the genetic analysis, three protocols of DNA extraction were applied. In protocol 1, individual parasites were ground in a mortar with liquid nitrogen and DNA was extracted using proteinase K (10 mg/ml), SDS 1%, DTT 1 M treatment (Iñiguez et al., 2002), followed by phenol/chloroform extraction and ethanol precipitation. In protocol 2, DNA extraction was accomplished using a QIAamp® DNA Mini Kit (Qiagen), following the protocol for genomic DNA isolation from formalin-fixed tissues. In protocol 3, individual worms were soaked in PBS for up to 72 h at 4 °C with several buffer changes before being

ground with liquid nitrogen. Subsequently, DNA extraction was conducted using QIAamp® DNA Mini Kit (Qiagen) and the protocol for formalin-fixed tissues, with the following modifications: samples were digested with 200 μ l ATL buffer and proteinase K for 24 h at 56 °C, and DNA was eluted twice in 60 μ l of AE buffer (Leles et al., 2009). For PCR controls, fish and human DNA were extracted from muscle of uninfected fish and from human blood by the DNAzol (Invitrogen) method. DNA was quantified by spectrophotometer (Gene Quant II, Pharmacia Biotech) by absorbance at 260 nm.

PCR was conducted of the 5' end of the 18S rDNA gene (Dorris and Blaxter, 2000) and the ITS1 region, between the 18S rDNA and 5.8S rDNA genes (Zhu et al., 1999) of the nematode genome. *A. typica* specific diagnosis PCR was also used. The reactions were performed on 50 μ l using 10 mM Tris–HCl (pH 8.0) 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 400 ng of each primer, 1.5 U Platinum Taq polymerase (Invitrogen), and 25–50 ng of genomic DNA. The PCR reactions were subjected to an initial cycle of 5 min at 96 °C, followed by 35 cycles at 96 °C for 30 s, 55–60 °C for 30 s, and 72 °C for 60 s in a programmable thermal controller (PTC100 60v, MJ Research, Inc). Products were electrophoresed through 1.5% agarose gels and visualized by UV light after ethidium bromide staining. PCR products were directly sequenced using Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems) in a 3100 Automated DNA Sequencer as recommended by the suppliers. Pairwise/Blast/NCBI, ClustalW (Thompson et al., 1994) and BioEdit v7.0.1 (Hall, 1999) software were used for editing and sequence analysis. To investigate the relationship among *A. typica* from definitive and intermediate/paratenic hosts worldwide, we conducted an analysis of all *A. typica* ITS sequences available on GenBank (12/2010). Identical ITS haplotype sequences from the same host were omitted and identical ITS haplotype from different hosts was duplicated. The dataset comprised 27 ITS sequences (Brazil, 15; China, 3; Indonesia, 3; Philippines, 1; Thailand, 1; Japan, 1; Taiwan, 1; Morocco, 1; Florida/USA, 1). The dataset was submitted to FindModel server (<http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html>) and select Jukes-Cantor (JC) plus gamma as the best fit model. Kimura-2-Parameters (K2P) and Tajima-Nei (TN) models were also applied. Neighbor-Joining (NJ) trees were constructed using 2000 bootstrap replicates, and complete deletion or pairwise deletion parameter, with MEGA v4 (Tamura et al., 2007). Definitive



Fig. 1. Map of Brazil highlighting the geographic localization of Ceará State.

and intermediate/paratenic hosts and the geographic localization were mapped in the tree.

3. Results

All specimens analyzed were identified as *Anisakis* by the presence of three typical lips; a long and muscular esophagus; a long ventricle, sigmoid; unequal spicules; and the presence of various pairs of tail papillae (Davey, 1971; Mattiucci et al., 2005). A *typica* was diagnosed morphologically in 6/9 hosts belonging to four cetacean

species (Table 2). Male specimens were not evaluated from *Kogia breviceps*, compromising the identification at species level.

We successfully amplified DNA only with protocol 3, which included extensive incubations in PBS buffer. PCR results of the 18S rDNA gene yielded 5 positive samples. However, the sequence analysis determined that only one sequence from a nematode specimen with short time of preservation (2.6 years), matched *Anisakis* sp. Four sequences belonged to specimens from 3.6 to 10.0 years of preservation, matched *Aspergillus* sp. 18S rDNA sequences

Table 2Results of morphological and molecular identification and sequences analysis of *Anisakis* from Brazilian waters.

Parasite ID	Life stage	Morphological identification	Molecular target, 18S rDNA	Molecular target, ITS1	Molecular target, <i>A. typica</i> ITS
AQ70	Adult	<i>A. typica</i>	+/C	+/ <i>A. typica</i>	<i>A. typica</i>
AQ79	Adult	<i>A. typica</i>	+/C	+/ <i>A. typica</i> /C	<i>A. typica</i>
AQ145	Adult	<i>A. typica</i>	—	—	<i>A. typica</i>
AQ149	Adult	<i>Anisakis</i> sp.	+/C	+/ <i>A. typica</i> /C	<i>A. typica</i>
AQ219	L3–L4	<i>Anisakis</i> sp.	+/C	—	<i>A. typica</i>
AQ221	Adult	<i>A. typica</i>	—	—	<i>A. typica</i>
AQ225	L3–L4	<i>Anisakis</i> sp.	—	+/ <i>A. typica</i>	<i>A. typica</i>
AQ242	Adult	<i>A. typica</i>	+/ <i>Anisakis</i> sp.	+/ <i>A. typica</i>	<i>A. typica</i>
AQ273	Adult	<i>A. typica</i>	—	+/ <i>A. typica</i>	<i>A. typica</i>
A3 ^a	Adult	<i>Anisakis</i> sp.	<i>Anisakis</i> sp.	<i>A. typica</i>	<i>A. typica</i>

C, contamination with fungus *Aspergillus* sp.; —, negative PCR result.^a *Anisakis* sample used as positive control (Iñiguez et al., 2009).

indicating fungus contamination (Table 2). The ITS1 PCR results showed 6/9 samples (400 bp) corresponding to sequences belonging to *A. typica*. However, two samples (7.0–10.0 years preservation) co-amplified *Aspergillus* sp. ITS1 region (230 bp) (data not shown). *A. typica* specific PCR was effective for the identification of all samples, yielding the expected product of 500 bp. Comparison of the sequences obtained with those available in GenBank databases showed all 9 samples exhibit 98% identity to the *A. typica* reference sequence AY826721. The sequence data reported herein have GenBank Accession Number: FJ161066–FJ161074. Sequence analysis revealed 8 haplotypes among the 9 sequences from this study, all presented 3 deletions at position C178, C183, and T214, and 6 polymorphic sites, C170A, G282C, T346A, G426A, C643T, and T659C.

All genetic analysis conducted showed the same topology tree and similar bootstrap values (data not showed). A NJ tree using JC plus gamma distribution model (Fig. 2), revealed two main clusters. One with a high bootstrap support (88%), comprised only *A. typica* from intermediate/paratenic hosts from Asian coastlines including Indonesia, Thailand, and China. The second cluster (bootstrap 64%), contained most of *A. typica* sequences described, from marine mammals and fish hosts from Brazil, from fish of Asian waters (Indonesia, Japan, Philippines, and Taiwan), a new record of *Merluccius polli* from Morocco and one from the definitive host *Steno bredanensis* of the Florida coast, USA. A short cluster (bootstrap 64%) contained two *A. typica* strains isolated from definitive hosts *S. bredanensis* and *P. electra* from this study.

4. Discussion

The primary methods of helminth parasite preservation in research and museum collections are ethanol or AFA fixation, used to preserve morphological characteristics. Because genomic data of this material is valuable for species identification, taxonomy, phylogeny, as well as disease diagnosis, methods and molecular techniques applied to fixed samples are in an ongoing process of development (Herniou et al., 1998; Li et al., 2000; Dorris et al., 2002; Magalhaes et al., 2008). DNA extraction protocols used on ancient DNA studies has been applied on the recovery of DNA from anisakids specimens fixed on formalin 1% or

ethanol 70% (Valentini et al., 2006; Colon-Llavina et al., 2009). We evaluated two methodologies for DNA extraction of *Anisakis* spp. specimens preserved in either AFA or 70% ethanol. The phenol–chloroform method and the QIAamp® DNA Mini Kit (Qiagen) (protocols 1 and 2) failed to retrieve sufficient DNA to be detected by any of 3 molecular targets used. When we introduced modifications to the protocol of genomic DNA isolation from ethanol- or formalin-fixed tissues, the yields of DNA improved. This may have been a benefit of the initial step of extensive soaking of samples and of the several buffer changes that removed ethanol or formalin and also re-hydrated the sample, as observed by others (Herniou et al., 1998). Moreover, the prolonged proteinase K digestion time enhanced the DNA extraction, as has been previously observed (Li et al., 2000).

Contamination by the fungus *Aspergillus* sp. detected when 18S rDNA and ITS PCR were applied seems to have been related to the time that samples were preserved. *Aspergillus* sp. colonization has been observed in decomposition of human cadavers (Hitosugi et al., 2006). Because the parasites were removed during necropsy from gastric lesions of stranded cetaceans, it is likely that the fungus was co-extracted with nematode. Herniou et al. (1998) pointed out that enteric parasites are surrounded by potential sources of contamination associated with the host: the most recent meal, or microbiological flora and fauna of the digestive system. More positive and valid results were obtained when more specific PCR was applied. Therefore, it was possible to achieve molecular identification of all samples independent of the time that samples were preserved. This suggests that the DNA damage caused by an extended period in ethanol or AFA can be overcome using both an efficient DNA extraction method and suitable PCR primers. The primers used to amplify ITS1 region in this study (NC5F, NC13R, Zhu et al., 1999), which yielded *Aspergillus* sp. amplifications, has been largely applied and useful in nematode genetic analysis. Because their sequences annealing on the conserved 18S rDNA and 5.8S rDNA genes, ITS1 amplifications other than nematode ITS1 could be expected, especially if the sample contains other sources of DNA (ex: fixed samples from necropsy; fecal samples).

A. typica shows remarkable genetic homogeneity even among geographically distant populations (Mattiucci et al., 2002), probably due to the homogenizing effect of genetic

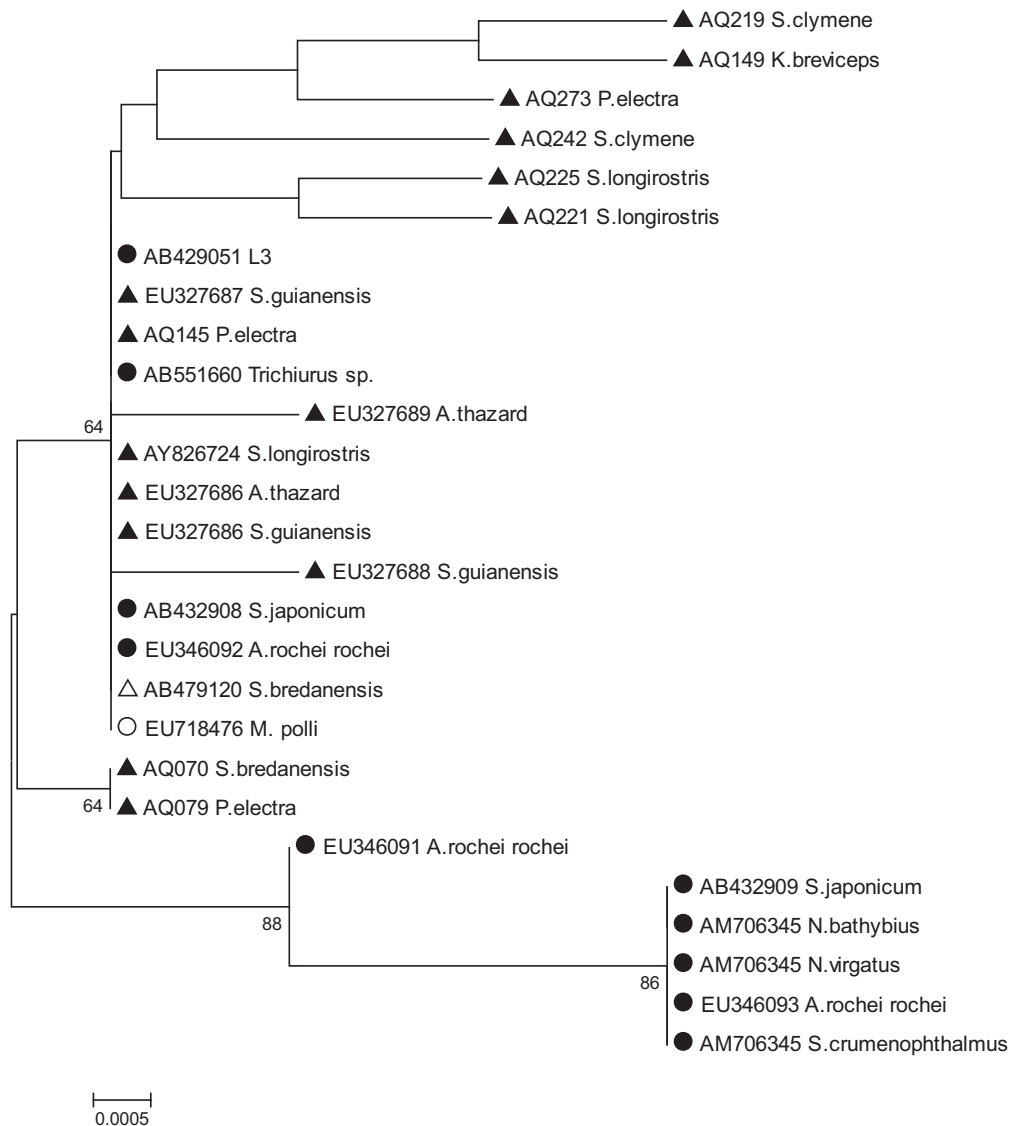


Fig. 2. NJ tree inferred from *Anisakis typica* ITS rDNA sequences obtained in this study and all *A. typica* ITS sequences available on GenBank (12/2010) using JC plus gamma distribution model, and complete deletion parameter (constructed with MEGA v4). The name or GenBank access number of each sample is showed. The definitive and intermediate/paratenic hosts as well as the geographic location are indicated in the tree. Black triangle: Brazilian coast; white triangle: Florida coast; black circle: Asian waters; white circle: Morocco coast. Bootstrap percentages after 2000 simulations >60% are shown at internodes.

flow, enhanced by the high dispersion capacity of intermediate/paratenic and definitive hosts. For the species evaluated in this work, the definitive hosts were primarily oceanic dolphins. We conducted an *A. typica* genetic analysis and mapped hosts and geographical origins. The cluster analysis showed interesting patterns, with a cluster consisting only of intermediate/paratenic hosts from Asian coasts, and another with both intermediate/paratenic and definitive hosts. The main cluster, showed not only a high frequency of the *A. typica*/*S. guianensis* and *A. typica*/*A. thazard* relationship previously reported (Iñiguez et al., 2009), but also revealed the presence of the same *A. typica* ITS haplotype in cetaceans off Brazilian and US coasts and in some intermediate/paratenic hosts from Asian and African coasts. Most cetacean hosts in this study are in sep-

arate groups, and no intermediate/paratenic hosts were identified in the groups. The identification of intermediate/paratenic hosts among this population is crucial to identifying species that conduct the *A. typica* incursion into a novel definitive host species, especially *K. breviceps*. These data may be interpreted to describe the flow of *A. typica* through diverse ecosystems could contribute information on the marine food web structure (Marcogliese, 2002; Lafferty et al., 2006).

We observed *A. typica* parasitizing all cetaceans studied. Therefore we identified 3 previously unreported definitive hosts, by means of genetic markers, *P. electra*, *K. breviceps*, and *Stenella clymene*. These results not only expand the *A. typica* definitive host records to two additional species of Delphinidae (Table 3), but included Kogiidae

Table 3Definitive hosts of *Anisakis typica* detected by genetic analysis.

Cetaceans	Geographic distribution	References
Delphinidae		
<i>Globicephala macrorhynchus</i>	Florida coast, USA	Mattiucci et al. (2005)
<i>Peponocephala electra</i>	Brazilian Atlantic coast	Present study
<i>Sotalia guianensis</i>	Brazilian Atlantic coast	D'Amelio et al. (2000); Valentini et al. (2006); Iñiguez et al. (2009)
<i>Stenella attenuata</i>	Florida coast, Caribbean sea	Valentini et al. (2006)
<i>Stenella clymene</i>	Brazilian Atlantic coast	Present study
<i>Stenella coeruleoalba</i>	East Mediterranean sea	Mattiucci et al. (2002)
<i>Stenella longirostris</i>	Brazilian Atlantic coast	Nadler et al. (2005); Colon-Llavina et al. (2009); Present study
<i>Steno bredanensis</i>	Caribbean sea, Brazilian Atlantic coast	Valentini et al. (2006); Colon-Llavina et al. (2009); Present study
<i>Tursiops truncatus</i>	Florida coast, Caribbean sea	Valentini et al. (2006); Colon-Llavina et al. (2009)
Kogiidae		
<i>Kogia breviceps</i>	Brazilian Atlantic coast	Present study

family. The tanglegram of phylogenies of *Anisakis* spp. and their cetacean hosts has been proposed by Mattiucci and Nascetti (2008). This co-phylogeny analysis revealed individual host–parasite associations suggesting host–parasite speciation events for *Physeter catodon*/*A. physeteris*; *K. breviceps*/*Anisakis brevispiculata*; and *Kogia simus*/*Anisakis paggiae*. The lack of parallelism of the phylogenies of *A. simplex* (s.s.) and *Balenoptera acutorostrata* was detected and believed to be a possible host-switching event association. This explanation could be plausible for the host–parasite association identified in this study, of that *A. typica*/*K. breviceps*. However, more reports are necessary to imply this association on an evolutionary event.

A. typica has been found in co-infections with *Anisakis pegreffii* in specimens of *Merluccius merluccius* from the Sea of Crete; with *A. pegreffii* and *A. simplex* s.s. in *Trachurus picturatus* specimens, and with *A. pegreffii*, *A. simplex* s.s., and *Anisakis ziphidarum* in *Scomber japonicus* specimens from the Atlantic Ocean off Madeira (Mattiucci et al., 2002). The sperm whales *Physeter macrocephalus*, *K. breviceps*, and *K. simus* are the major hosts of *A. physeteris*, *A. brevispiculata*, and *A. paggiae*, respectively (Mattiucci et al., 2001; Mattiucci and Nascetti, 2006). However, based on morphological data, *A. simplex* of clade I has been reported to infect *K. breviceps* in Mexico (Gonzalez Soliz et al., 2006), indicating that other anisakids may be found in this host. *K. breviceps* inhabits deep waters in tropical and warm temperate zones all over the world. It feeds primarily on cephalopods and, less often, on deep-sea fishes and shrimps. The majority of data for this species come from stranding (Jefferson et al., 2008). This report of *A. typica* infecting *K. breviceps* suggests that this host could share dietary items with the other cetaceans evaluated in this study where they co-occur in the Atlantic Ocean. Genetic markers have been shown to be efficient identifiers of *A. typica* definitive hosts. These data provide important information regarding the life-history, behavior, and epizootiological aspects of these parasites (Mattiucci and Nascetti, 2008). The approach used here provides a basis for investigation of the anisakid population structure in cetaceans, as well as their ecology, particularly their life cycles and transmission patterns.

Conflict of interest statement

All authors declare no conflicts of interests.

Acknowledgements

We thank Koko Otsuki (IOC/FIOCRUZ) and Daniela Leles (IOC/ENSP/FIOCRUZ) for their technical assistance and for the PDTIS/FIOCRUZ genomic platform. We thank to the Aquasis team for their work in recovering and performing cetacean necropsies. We are also grateful to Jose Antônio Sena for constructing the Ceará state map. This study was supported by IOC/FIOCRUZ and CNPq.

References

- Chen, Q., Yu, H.Q., Lun, Z.R., Chen, X.G., Song, H.Q., Lin, R.Q., Zhu, X.Q., 2008. Specific PCR assays for the identification of common anisakid nematodes with zoonotic potential. *Parasitol. Res.* 104, 79–84.
- Choi, S.J., Lee, J.C., Kim, M.J., Hur, G.Y., Shin, S.Y., Park, H.S., 2009. The clinical characteristics of *Anisakis* allergy in Korea. *Korean J. Intern. Med.* 24, 160–163.
- Couture, C., Measures, L., Gagnon, J., Desbiens, C., 2003. Human intestinal anisakiosis due to consumption of raw salmon. *Am. J. Surg. Pathol.* 27, 1167–1172.
- Colon-Llavina, M.M., Mignucci-Giannoni, A.A., Mattiucci, S., Paoletti, M., Nascetti, G., Williams Jr., E.H., 2009. Additional records of metazoan parasites from Caribbean marine mammals, including genetically identified anisakid nematodes. *Parasitol. Res.* 105, 1239–1252.
- D'Amelio, S., Mathiopoulou, K.D., Santos, C.P., Pugachev, O.N., Webb, S.C., Picanco, M., Paggi, L., 2000. Genetic markers in ribosomal DNA for the identification of members of the genus *Anisakis* (Nematoda: Ascaridoidea) defined by polymerase-chain-reaction-based restriction fragment length polymorphism. *Int. J. Parasitol.* 30, 223–226.
- Davey, J.T., 1971. A case of *Anisakis* sp. infection in a brown bear from Alaska. *Trans. R. Soc. Trop. Med. Hyg.* 65, 433–434.
- Dorris, M., Blaxter, M., 2000. The small subunit ribosomal RNA sequence of *Strongyloides stercoralis*. *Int. J. Parasitol.* 30, 939–941.
- Dorris, M., Viney, M.E., Blaxter, M.L., 2002. Molecular phylogenetic analysis of the genus *Strongyloides* and related nematodes. *Int. J. Parasitol.* 32, 1507–1517.
- Farjallah, S., Busi, M., Mahjoub, M.O., Slimane, B.B., Paggi, L., Said, K., D'Amelio, S., 2008a. Molecular characterization of larval anisakid nematodes from marine fishes off the Moroccan and Mauritanian coasts. *Parasitol. Int.* 57, 430–436.
- Farjallah, S., Slimane, B.B., Busi, M., Paggi, L., Amor, N., Blel, H., Said, K., D'Amelio, S., 2008b. Occurrence and molecular identification of *Anisakis* spp. from the North African coasts of Mediterranean Sea. *Parasitol. Res.* 102, 371–379.
- Gonzalez Soliz, D., Vidal-Martinez, V.M., Antochiw-Alonso, D.M., Ortega-Argueta, A., 2006. Anisakid nematodes from stranded pygmy sperm whales, *Kogia breviceps* (Kogiidae), in three localities of the Yucatan Peninsula, Mexico. *J. Parasitol.* 92, 1120–1122.
- Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp.* 41, 95–98.
- Herniou, E.A., Pearce, A.C., Littlewood, D.T.J., 1998. Vintage helminths yield valuable molecules. *Parasitol. Today* 14, 289–292.

- Hitosugi, M., Ishii, K., Yaguchi, T., Chigusa, Y., Kurosu, A., Kido, M., Nagai, T., Tokudome, S., 2006. Fungi can be a useful forensic tool. *Legal Med.* 8, 240–242.
- Iñiguez, A.M., Vicente, A.C., Araujo, A., Ferreira, L.F., Reinhard, K.J., 2002. *Enterobius vermicularis*: specific detection by amplification of an internal region of 5S ribosomal RNA intergenic spacer and trans-splicing leader RNA analysis. *E. vermicularis*: specific detection by PCR and SL1 RNA analysis. *Exp. Parasitol.* 102, 218–222.
- Iñiguez, A.M., Santos, C.P., Vicente, A.C.P., 2009. Genetic characterization of *Anisakis typica* and *Anisakis physeteris* from marine mammals and fish from the Atlantic Ocean off Brazil. *Vet. Parasitol.* 165, 350–356.
- Jefferson, T.A., Webber, M.A., Pitman, R.L., 2008. *Marine Mammals of the World: A Comprehensive Guide to their Identification*. Academic Press/Elsevier, 573 pp. (101).
- Lafferty, K.D., Dobson, A.P., Kuris, A.M., 2006. Parasites dominate food web links. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11211–11216.
- Leles, D., Araujo, A., Vicente, A.C., Iñiguez, A.M., 2009. Molecular diagnosis of ascariasis from human feces and description of a new *Ascaris* sp. genotype in Brazil. *Vet. Parasitol.* 163, 167–170.
- Li, J., Liao, X., Yang, H., 2000. Molecular characterization of a parasitic tapeworm (*Ligula*) based on DNA sequences from formalin-fixed specimens. *Biochem. Genet.* 38, 309–322.
- Magalhaes, K.G., Jannotti-Passos, L.K., Caldeira, R.L., Berne, M.E., Muller, G., Carvalho, O.S., Lenzi, H.L., 2008. Isolation and detection of *Fasciola hepatica* DNA in *Lymnaea viatrix* from formalin-fixed and paraffin-embedded tissues through multiplex-PCR. *Vet. Parasitol.* 152, 333–338.
- Marcogliese, D.J., 2002. Food webs and the transmission of parasites to marine fish. *Parasitology* 124 (Suppl.), S83–S99.
- Marques, J.F., Cabral, H.N., Busi, M., D'Amelio, S., 2006. Molecular identification of *Anisakis* species from Pleuronectiformes off the Portuguese coast. *J. Helminthol.* 80, 47–51.
- Mattiucci, S., Paggi, L., Nascetti, G., Abollo, E., Webb, S.C., Pascual, S., Cianchi, R., Bullini, L., 2001. Genetic divergence and reproductive isolation between *Anisakis brevispiculata* and *Anisakis physeteris* (Nematoda: Anisakidae). *Int. J. Parasitol.* 31, 9–14.
- Mattiucci, S., Paggi, L., Nascetti, G., Portes Santos, C., Costa, G., Di Benedetto, A.P., Ramos, R., Argyrou, M., Cianchi, R., Bullini, L., 2002. Genetic markers in the study of *Anisakis typica* (Diesing, 1860): larval identification and genetic relationships with other species of *Anisakis* Dujardin, 1845 (Nematoda: Anisakidae). *Syst. Parasitol.* 51, 159–170.
- Mattiucci, S., Nascetti, G., Dailey, M., Webb, S.C., Barros, N.B., Cianchi, R., Bullini, L., 2005. Evidence for a new species of *Anisakis* Dujardin, 1845: morphological description and genetic relationships between congeners (Nematoda: Anisakidae). *Syst. Parasitol.* 61, 157–171.
- Mattiucci, S., Nascetti, G., 2006. Molecular systematics, phylogeny and ecology of anisakid nematodes of the genus *Anisakis* Dujardin, 1845: an update. *Parasite* 13, 99–113.
- Mattiucci, S., Nascetti, G., 2007. Genetic diversity and infection levels of anisakid nematodes parasitic in fish and marine mammals from Boreal and Austral hemispheres. *Vet. Parasitol.* 148, 43–57.
- Mattiucci, S., Nascetti, G., 2008. Advances and trends in the molecular systematics of anisakid nematodes, with implications for their evolutionary ecology and host–parasite co-evolutionary processes. *Adv. Parasitol.* 66, 47–148.
- Motta, M.R.A., Pinheiro, D.C.S.N., Carvalho, V.L., Viana, D.A., Vicente, A.C.P., Iñiguez, A.M., 2008. Gastric lesions associated with the presence of *Anisakis* spp. Dujardin, 1845 (Nematoda: Anisakidae) in cetaceans stranded on the coast of Ceara, Brazil. *Biota Neotrop.* 8, 4.
- Nadler, S.A., D'Amelio, S., Dailey, M.D., Paggi, L., Siu, S., Sakanari, J.A., 2005. Molecular phylogenetics and diagnosis of *Anisakis*, *Pseudoterranova*, and *Contracaecum* from northern Pacific marine mammals. *J. Parasitol.* 91, 1413–1429.
- Pellegrini, M., Occhini, R., Tordini, G., Vindigni, C., Russo, S., Marzocca, G., 2005. Acute abdomen due to small bowel anisakiasis. *Dig. Liver Dis.* 37, 65–67.
- Pontes, T., D'Amelio, S., Costa, G., Paggi, L., 2005. Molecular characterization of larval anisakid nematodes from marine fishes of Madeira by a PCR-based approach, with evidence for a new species. *J. Parasitol.* 91, 1430–1434.
- Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599.
- Thompson, Julie, D.J., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Umehara, A., Kawakami, Y., Ooi, H.K., Uchida, A., Ohmae, H., Sugiyama, H., 2010. Molecular identification of *Anisakis* type I larvae isolated from hairtail fish off the coasts of Taiwan and Japan. *Int. J. Food Microbiol.* 143, 161–165.
- Valentini, A., Mattiucci, S., Bondanelli, P., Webb, S.C., Mignucci-Giannone, A.A., Colom-Llavina, M.M., Nascetti, G., 2006. Genetic relationships among *Anisakis* species (Nematoda: Anisakidae) inferred from mitochondrial *cox2* sequences, and comparison with allozyme data. *J. Parasitol.* 92, 156–166.
- Zhu, X., Chilton, N.B., Jacobs, D.E., Boes, J., Gasser, R.B., 1999. Characterisation of *Ascaris* from human and pig hosts by nuclear ribosomal DNA sequences. *Int. J. Parasitol.* 29, 469–478.
- Zhu, X.Q., Podolska, M., Liu, J.S., Yu, H.Q., Chen, H.H., Lin, Z.X., Luo, C.B., Song, H.Q., Lin, R.Q., 2007. Identification of anisakid nematodes with zoonotic potential from Europe and China by single-strand conformation polymorphism analysis of nuclear ribosomal DNA. *Parasitol. Res.* 101, 1703–1707.