Morphological and Molecular Diagnosis of Anisakid Nematode Larvae from Cutlassfish (Trichiurus lepturus) off the Coast of Rio de Janeiro, Brazil

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Abstract

Anisakid nematode larvae from Trichiurus lepturus off coast of Rio de Janeiro were studied using light, laser confocal and scanning electron microscopy, in addition to a molecular approach. Mitochondrial cytochrome c-oxidase subunit 2 (mtDNA cox-2), partial 28S (LSU) and internal transcribed spacers (ITS-1, 5.8S, ITS-2) of ribosomal DNA were amplified using the polymerase chain reaction and sequenced to evaluate the phylogenetic relationships between the nematode taxa. The morphological and genetic profiles confirmed that, of the 1,030 larvae collected from the 64 fish examined, 398 were analysed, of which 361 were Hysterobothycium sp. and 37 were Anisakis typica. Larvae of Hysterobothycium sp. were not identified to the species level due to the absence of similar sequences for adult parasites; however, the ITS sequence clustered to the phylogenetic tree with sequences of H. deardorffoverstreetorum, whereas an mtDNA cox-2 and LSU concatenated phylogenetic analysis demonstrated the presence of two clades, both of them under the same name as the larval H. deardorffoverstreetorum. Data on the occurrence of parasites during the winter and summer months were compared using the t-test. The greatest prevalence and intensity of infection were recorded for larval Hysterobothycium, with a prevalence of 51.56% and an intensity of up to 55 parasites per fish. The larval Anisakis exhibit a higher abundance and intensity of infection in the winter months, and those of Hysterobothycium during the summer. However, the t-test indicated no significant differences between the abundance and intensity of infection recorded during the months of collection for either of these larval nematodes. All sequences generated in this study were deposited in GenBank.

Introduction

Anisakid nematodes are parasites with an indirect life cycle, which utilizes hosts at different trophic levels in the food web. Aquatic vertebrates, such as piscivorous fishes, mammals and birds, are definitive hosts and aquatic invertebrates and fishes act as intermediate or paratenic hosts [1,2]. The Anisakidae Skrjabin & Karokhin, 1945 is a major family within the Ascaridoidea as intermediate or paratenic hosts [1,2]. The Anisakidae Skrjabin & Karokhin, 1945 is a major family within the Ascaridoidea as intermediate or paratenic hosts [1,2]. The Anisakidae Skrjabin & Karokhin, 1945 is a major family within the Ascaridoidea as intermediate or paratenic hosts [1,2]. The Anisakidae Skrjabin & Karokhin, 1945 is a major family within the Ascaridoidea as intermediate or paratenic hosts [1,2]. The Anisakidae Skrjabin & Karokhin, 1945 is a major family within the Ascaridoidea as intermediate or paratenic hosts [1,2]. 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Anisakid larvae are usually very difficult to identify to species using morphology due to the lack of differential characters, but when adults are already described and genetically characterized, then such larva can be assigned to a species based on molecules [1,4]. The accurate identification of anisakid species is essential, because there are important pathogens within the group that can cause problems for human and animal health [2,5,6,7]. Molecular tools are therefore valuable for linking anisakid larva to known adults as well as for systematic, evolutionary and ecological studies of these parasites [1,4,5,8,9].

The cutlassfish Trichiurus lepturus L. (Trichiuridae) has a wide distribution, occurring throughout tropical and temperate waters of the world. Previous parasitological surveys on specimens from off the coast of Rio de Janeiro listed the occurrence of anisakid larva identified only to generic level by means of light microscopy [10,11]. In this study, the nematode parasites of T. lepturus from the same region are re-evaluated using light, laser confocal and scanning electron microscopy, and also by the determination of nucleotide sequences from the internal transcribed spacers of ribosomal DNA (ITS-1, 5.8S, ITS-2), the partial 28S (LSU) and mitochondrial cytochrome c-oxidase subunit 2 (mtDNA cox-2).

Results

A total of 1,030 nematode larva were collected from 64 fishes; 398 were analyzed for morphological data and 72 were used for genetic studies. The larvae identified by morphological and molecular approaches as Anisakis typica and Hysterobothycium sp. are characterized below.
Anisakis typica third-stage larva. Thirty seven specimens were collected from the body cavity and mesentery of T. lepturus; their measurements are presented in Table 1. They had the following characteristics: cuticle smooth; lips poorly developed; ventrolateral lips with single and double papilla, dorsal lip with two double papillae; boring tooth present between ventral lips; intestinal caecum absent; ventriculus elongate (Figures 1A–1C). Excretory pore present at the base of ventrolateral lips (Figures 1B, 1D–1E); tail short, round, with mucron (Figure 1F).

Genetic characterization of 22 larva enabled the species determination, with 13 being diagnosed by specific PCR as Anisakis typica (Diesing, 1860); 9 were submitted to PCR for family determination, with 13 being diagnosed by specific PCR as A. typica (positioned at 1D–1E); tail short, round, with mucron (Figure 1F).

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The prevalence of Anisakis typica was 20.31% and the intensity varied from 1 to 10 specimens per fish. Hysterothylacium presented a prevalence of 51.56% and intensity of 1 to 55 per fish. The highest prevalences were found during November and December (Figure 9). Larvae of Hysterothylacium sp. were the most abundant, with mean intensities between 2.5 and 20.5 (Figures 10 and 11). The t-test applied to verify the existence of variation between prevalence, intensity and abundance during winter (August) and summer (December and January) was not significant.

**Discussion**

The larvae of Hysterothylacium sp. are difficult to identify and their similarity with related genera has resulted in taxonomic confusion, with species of Hysterothylacium being identified as Contracaecum or Iheringascaris [12,13,14]. The position of the excretory pore, which has been reported as inconspicuous, is the main difference between larvae of Hysterothylacium (positioned at nerve ring level) and Contracaecum (situated at the base of lips). As mentioned above, the confocal microscopy and SEM were essential to ascertain its location. Likewise, species of Iheringascaris are separated from Hysterothylacium based only in the pattern of annulations of the cuticle [12]. In the present study, the SEM

**Table 1.** Present measurements of Anisakis typica and Hysterothylacium sp.

|                     | Anisakis typica L3 (n = 12) | Hysterothylacium sp. L3 (n = 28) | Hysterothylacium sp. L4 (n = 13) |
|---------------------|-----------------------------|----------------------------------|----------------------------------|
| Body length         | 19.31 (15.34–22.43)         | 7.84 (3.42–14.8)                 | 9.17 (6.55–11.55)                |
| Body width          | 0.45 (0.6–0.35)             | 0.24 (0.13–0.4)                  | 0.27 (0.14–0.35)                 |
| Esophagus           | 1.46 (1.81–1.1)             | 0.64 (0.41–0.87)                 | 0.77 (0.6–0.98)                  |
| Ventriculus         | 0.61 (0.76–0.5)             | 0.07 (0.04–0.1)                  | 0.09 (0.06–0.1)                  |
| Intestinal caecum   | Absent                      | 0.16 (0.1–0.46)                  | 0.28 (0.15–0.4)                  |
| Ventricular appendix| Absent                      | 0.59 (0.31–0.84)                 | 0.67 (0.42–0.94)                 |
| Tail                | 0.12 (0.2–0.08)             | 0.16 (0.11–0.22)                 | 0.16 (0.11–0.25)                 |
| Esophagus/ventriculus| 1.030–0.54                  | 1.070–0.20                      | 1.070–0.14                      |
| Esophagus/caecum    | –                           | 1.04–0.34                       | 1.020–0.57                      |
| Esophagus/ventricular appendix | – | 1.060–1.33 | 1: 0.55–1.00 |

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Figure 1. A–F: *Anisakis typica* larvae: light, CLSM and SEM microscopy. A- Anterior end with boring tooth; B- SEM of lips with papilla, boring tooth and excretory pore; C- Esophagus and ventriculus; D- Position of excretory pore; E- CLSM reconstruction with detail boring tooth and excretory pore; F- SEM of tail with mucron terminal. Abbreviations: e - esophagus; ep - excretory pore; t - tooth; p - papilla; v - ventriculus; m - mucron.

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Figure 2. Alignment of ITS-1 and ITS-2 sequences representing *Anisakis* spp. Dots indicate identity with the first sequence, dashes are inferred insertion-deletion events and * represents our sample.

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micrographs showed the cuticle to lack annulations, as described for *Hysterothylacium* spp., although the phylogenetic analysis showed a close relationship with *Iheringascaris*. In the future, it is possible that species of *Iheringascaris* may be allocated within *Hysterothylacium* [15].

In this study, larvae of *Hysterothylacium* are reported at a high prevalence (51.56%), with an intensity of infection of up to 55 parasites per fish, but could not be identified to species level due to the absence of related adult sequences in the GenBank. Consequently, a specific identification could not be assigned.

Previous genetic analysis of *Anisakis simplex* and *Hysterothylacium aduncum* from *T. lepturus* in Taiwanese waters [16] were described but not formally deposited in the GenBank. However, a comparison with these data showed these species are genetically distinct from the nucleotide sequences obtained in this study.

The similarity among our *Hysterothylacium* sequences for ITS and LSU regions was 100%; on the contrary, our mtDNA *cox-2* sequences exhibited a high genetic heterogeneity. The presence of polymorphism in the mtDNA *cox-2* region has likewise been reported before for other species of nematodes [17]. The K2P distances calculated among the sequences available in GenBank under the name of *H. deardorffovertstreetorum* and the *Hysterothylacium* sequenced here, showed a genetic differentiation ranging from K2P = 0.005 to K2P = 0.08. The present study indicates that the *Hysterothylacium* larvae analyzed were likely to correspond to the larva described as *H. deardorffovertstreetorum*; however, the marked genetic differentiation so far detected at the mtDNA *cox-2* level seems to suggest a possible genetic heterogeneity. This needs to be further investigated by future genetic analysis, likely using other nuclear markers. Indeed, while a comparison with one of the sequences of *H. deardorffovertstreetorum* (accession no. JF730200) resulted in a 100% of similarity for the ITS region, the mtDNA *cox-2* sequences deposited, under the same name, had, at the intraspecific level, a genetic differentiation value with K2P.

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![Figure 3](https://example.com/figure3.png)

**Figure 3.** Maximum likelihood reconstruction between sequences of *Anisakis typica* obtained in this study (*) and sequences of *Anisakis* species from the GenBank, with the tree inferred from the ITS data set. The numbers on the tree branches represent the percentage of bootstrap resampling. *Ascaris lumbricoides* was used as an out group.

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![Figure 4](https://example.com/figure4.png)

**Figure 4.** Maximum likelihood reconstruction between sequences of *Anisakis typica* obtained in this study (*) and sequences of *Anisakis* species from the GenBank, with the tree inferred from mtDNA *cox-2* and LSU data sets. The numbers on the tree branches represent the percentage of bootstrap resampling. *Ascaris lumbricoides* was used as an out group.

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Figure 5. A–H: *Hysterothylicium* sp. larvae: SEM and CLSM microscopy. A- SEM of anterior end with alae and excretory pore; B- Detail of L3 lips with inconspicuous boring tooth and papillae; C- Detail of lips of L4 with dorsal lip showing double papilla; D- CLSM of esophagus; E- CLSM reconstruction with ventriculus, intestinal caecum and esophagus; F- CLSM reconstruction with nerve ring and excretory pore; G- SEM of tail; H- SEM micrograph with a detail of the digitiform tip with terminal mucron. Abbreviations: a - alae; ep - excretory pore; p – papilla; t - tooth; dl - dorsal lip; e - esophagus; ic - intestinal caecum; v - ventriculus; n - nervous ring and m - mucron.
doi:10.1371/journal.pone.0040447.g005
| Genetic region | Species                              | GenBank accession number | Reference                                      |
|----------------|--------------------------------------|--------------------------|------------------------------------------------|
| ITS            | Contracaecum sp.                     | JN005755                 | Unpublished data                               |
|                | Contracaecum muraenesoxi             | EU828749                 | Fang et al. 2009 Exp. Parasitol.               |
|                | Hysterothylacium aduncum             | HQ270433                 | Amor et al. 2011 Parasitol. Res.               |
|                | Hysterothylacium aduncum             | HQ270431                 | Amor et al. 2011 Parasitol. Res.               |
|                | Hysterothylacium aduncum             | JF683734                 | Unpublished data                               |
|                | Hysterothylacium aduncum             | HQ702733                 | Unpublished data                               |
|                | Hysterothylacium aduncum             | AJ937673                 | Zhu et al. 2007 Parasitol. Res.                |
|                | Hysterothylacium aduncum             | HM598666                 | Unpublished data                               |
|                | Hysterothylacium aduncum             | AB277826                 | Umehara et al. Parasitol. Int.                 |
|                | Hysterothylacium auctum              | AF115571                 | Szoekowska et al. 2001 Acta Parasitol.         |
|                | Hysterothylacium bidentatum          | AF103559                 | Unpublished data                               |
|                | Hysterothylacium deardorffoverstreetorum | JF730200            | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz   |
|                | H. deardorffoverstreetorum          | JF730201                 | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz    |
|                | H. deardorffoverstreetorum          | JF730203                 | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz    |
|                | H. deardorffoverstreetorum          | JF730204                 | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz    |
|                | H. deardorffoverstreetorum          | JF730199                 | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz    |
|                | Hysterothylacium fabri               | JQ520158                 | Li et al. 2012 Parasitol. Res.                 |
|                | Hysterothylacium longilabrum         | JQ520159                 | Li et al. 2012 Parasitol. Res.                 |
|                | Anisakis brevispiculata              | AB592793                 | Murata et al. 2011 Parasitol. Int.            |
|                | Anisakis paggiae                     | GU295975                 | Klimpel et al. 2011 Polar Biol.               |
|                | Anisakis physeteris                  | JN968636                 | Kuhn et al. 2011 Plos One                     |
|                | Anisakis paggiae                     | JN968632                 | Kuhn et al. 2011 Plos One                     |
|                | Anisakis simplex                     | JN968904                 | Kuhn et al. 2011 Plos One                     |
|                | Anisakis simplex C                   | JN968654                 | Kuhn et al. 2011 Plos One                     |
|                | Anisakis typica                      | AJ826724                 | Unpublished data                               |
|                | Anisakis typica                      | AB551660                 | Umehara et al. 2010 Int. J. Food microbiol.    |
|                | Anisakis typica                      | EU327686                 | Iniguez et al. 2009 Vet. Parasitol.            |
|                | Ascaris lumbricoides                 | AB571300                 | Arizono et al. 2010 Jpn. J. Infect. Dis.      |
|                | Heterocheilus tunicatus              | AF226592                 | Nadler et al. 2000 Parasitol.                 |
| LSU            | Hysterothylacium pelagicum           | AF226590                 | Nadler et al. 2000 Parasitology               |
|                | Hysterothylacium fortalezae          | U947660                  | Nadler & Hudspeth 1998 Mol. Phylogenet.        |
|                | Hysterothylacium reliquens          | U947662                  | Nadler & Hudspeth 1998 Mol. Phylogenet.        |
|                | Irenhingascaris inquies              | U947663                  | Nadler & Hudspeth 1998 Mol. Phylogenet.        |
|                | Anisakis simplex C                   | AY821754                 | Nadler et al. 2005 J. Parasit.                |
|                | Heterocheilus tunicatus              | AF226592                 | Nadler et al. 2000 Parasitol.                 |
|                | Ascaris lumbricoides                 | AF182298                 | Nadler & Hudspeth 2000 J. Parasitol.          |
| mtDNA cox2     | Hysterothylacium fortalezae          | AF179914                 | Nadler & Hudspeth 1998 Mol. Phylogenet.        |
|                | Hysterothylacium deardorffoverstreetorum | JF730211            | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz   |
|                | H. deardorffoverstreetorum          | JF730213                 | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz    |
|                | H. deardorffoverstreetorum          | JF730205                 | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz    |
|                | H. deardorffoverstreetorum          | JF730208                 | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz    |
|                | H. deardorffoverstreetorum          | JF730207                 | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz    |
|                | H. deardorffoverstreetorum          | JF730206                 | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz    |
|                | H. deardorffoverstreetorum          | JF730209                 | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz    |
|                | H. deardorffoverstreetorum          | JF730212                 | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz    |
|                | H. deardorffoverstreetorum          | JF730210                 | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz    |
|                | H. deardorffoverstreetorum          | JF730214                 | Knoff et al. 2012 Mem. Inst. Oswaldo Cruz    |
|                | Hysterothylacium pelagicum           | AF179915                 | Nadler & Hudspeth 1998 Mol. Phylogenet.        |
|                | Hysterothylacium reliquens          | AF179916                 | Nadler & Hudspeth 1998 Mol. Phylogenet.        |
There are about 60 species of Hysterothylacium which have been formally described based on the morphological features of the adult worm [19,20,21,22]. However, so far, scanty data are available for their molecular analysis. Hysterothylacium sequences determined in this work were not similar to those deposited in the GenBank based on adult characterization. The question remains: could it be a new species, as indicated by the phylogenetic analysis, or a known species based on the morphological features of an adult worm which has not yet been characterized by molecular means? Species descriptions should contain data from as many sources as possible, including morphological information from adult worms, molecular data and phylogenetic analyses, which can be used not only as tools for identifying an isolate specimen but also for understanding its biology and taxonomy.

Hysterothylacium sp. type MB larvae sensu Deardorff and Overstreet [12] were reported from T. lepturus in the Sea of Oman [23], but the authors refrained from naming it. Similarly, unknown anisakid larvae have been reported from fishes using a PCR-based approach as evidence for new species, but the new form was not formally described as adults were not available for morphological characterization and molecular comparison [24]. However, Hysterothylacium deardorffoverstreetorum has recently been proposed based only on morphological features of the larva and a comparison with sequences of the genus deposited in the GenBank, despite their small number [25]. It is possible that, in future when sequences of adults of all or most of the 60 nominal species of Hysterothylacium are deposited in the GenBank, this species will likely sink into synonymy, reinforcing the idea that molecular data should be accompanied by strong morphological evidence based on adult nematodes.

The genotyping of more species will enable GenBank to become a robust tool for identification and phylogenetic analyses. However, at present, the number of sequences of Hysterothylacium deposited in this database represents less than 15% of the valid species. This limitation compromises any phylogenetic results when the objective is to identify a species. For this, it is necessary to characterize a larger number of valid species based on genotypic information and morphological analyses of adult worms in order to enable the genetic identification of Hysterothylacium larvae.

An ITS sequence of the larvae of Contracaecum sp. found in Pagellus bogaraveo in Portuguese waters (accession no. JN005755) also presented 99% similarity with Hysterothylacium sequences from this study. Unfortunately, a formal publication with morphological characterization was not available for comparison.

Within the GenBank, the ITS sequence (accession no. EU828749) identified as Contracaecum muraenesoxi appeared to be very closely related to the sequences determined in this study. Nevertheless, this species was recently synonymized with Hysterothylacium amoyneze [21], explaining its phylogenetic position within the Hysterothylacium and proximity to our sequence. This highlights the fact that taxonomic changes of taxonomic names need, somehow, to be included in the GenBank in order to avoid phylogenetic misinterpretation. Similarly, the phylogenetic analysis showed an LSU sequence of Raphidascaridae sp. (accession no. AY821772) to be closely related to Hysterothylacium sp. from this study, which suggests that the morphological identification of that voucher of R. acus should be revised [15].

In this study, Anisakis typica was identified by molecular data, and our phylogenetic analysis for Anisakis species also indicated three distinct groups of species, agreeing with data from the literature [1].

The prevalence of Anisakis and Hysterothylacium larvae in this study were similar to those previously described in the cutlassfish off the coast of Rio de Janeiro [11]. Significant differences in prevalence were not observed between the winter and summer periods, although a moderate increase in prevalence and abundance was observed at the beginning of summer for Hysterothylacium. The prevalence of Anisakis simplex in fish from Norway, for comparison, was most significant during spring, and the authors have suggested that a small variation in the occurrence of anisakids in tropical waters could be related to the low level of climatic variability typical for tropical weather [26]. The constant presence of definitive hosts along the Brazilian coast may also contribute to the presence of Anisakis and Hysterothylacium during both winter and summer, as observed in this study. Hysterothylacium

Table 2. Cont.

| Genetic region | Species                      | GenBank accession number | Reference                        |
|----------------|------------------------------|--------------------------|----------------------------------|
|                | Iheringascaris inques        | AF179917                 | Nadler & Hudspeth 1996 Mol. Phylogenet. |
|                | Anisakis typica 1            | AB517571                 | Suzuki et al. 2009 Int. J. Food Microbiol. |
|                | Anisakis typica 2            | AB517572                 | Suzuki et al. 2009 Int. J. Food Microbiol. |
|                | Anisakis typica 3            | DQ116427                 | Valentini et al. 2006 J. Parasitol. |
|                | Anisakis nascetti 1          | GQ118169                 | Mattiucci et al. 2009 Syst. Parasitol. |
|                | Anisakis nascetti 2          | GQ118171                 | Mattiucci et al. 2009 Syst. Parasitol. |
|                | Anisakis simplex             | HM488999                 | Setyobudhi et al. 2011 Parasitol. Res. |
|                | Anisakis pegreffii           | JF423263                 | Baldwin et al. 2011 J. Parasitol. |
|                | Anisakis physeteris          | AB592801                 | Murata et al. 2011 Parasitol. Int. |
|                | Heterocheilus tunicatus      | AF179913                 | Nadler et al. 2000 J. Parasitol. |
|                | Ascaris lumbricoidea         | AF179907                 | Nadler & Hudspeth 2000 J. Parasitol. |

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Figure 6. Alignment of mtDNA cox-2 sequences representing *Hysterothyacium* and *Iheringascaris* taxa. Dots indicate identity with the first sequence, dashes are inferred insertion-deletion events and * represents our samples.
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adults have been reported off the Brazilian coast parasitizing the following definitive hosts: 
a Harengula clupeola, Scromberomorus cavalla, S. maculatus, Epinephelus guttatus [27]. These definitive hosts have a
preference for coastal habitats, which may be related to the
prevalence and abundance of Hysterothylacium in T. lepturus.

Adults of Anisakis typica were described from the dolphins Sotalia guianensis and Stenella longirostris off the Brazilian coast. S. guianensis
inhabits coastal waters, whereas S. longirostris prefers oceanic bays
and island regions. A. typica larvae has been reported in Thunnus thynnus and Auxis thazard off Rio de Janeiro [28,29,30,31,32],
indicating that the parasite is common in the area. During
summer, there is an increase in whale-watching along the Rio de Janeiro coast, which is probably related to the seasonal upwelling
in the region responsible for the addition of new elements to the
food webs. At this time these food webs become more complex,
thus promoting anisakid transmission [33,34]. This may explain
the increasing abundance of these parasites in the summer.
Furthermore, the increase in prevalence of anisakids off the coast
during spring and summer could be due to the spawning period of
T. lepturus, whose foraging behaviour increases in order to build
resources for reproduction [35].

This is the first identification of A. typica in T. lepturus in Brazilian
waters with LSU, ITS and mtDNA cox-2 sequences for larvae of
both of A. typica and Hysterothylacium sp. This integrated study has
shown the great need for a linkage between the analysis of
morphological features supplemented by molecular data in order
to enable the accurate identification of anisakid larva and provide
robust taxonomic data.

Materials and Methods

A total of 64 fish were collected off Itaipu beach, Niterói, Rio de Janeiro (22°53′14″S; 43°22′48″O) from August 2010 to January
2011. Prevalence, abundance and mean intensity were calculated
[36]. Data were transformed to attend the assumption of
normality, and t-tests for independent samples were performed
to verify differences between winter and summer months.
Nematodes were cut into three pieces and fixed in 70% ethanol.
The anterior and posterior regions were cleared in glycerine and

Figure 7. Maximum likelihood reconstruction between sequences of Hysterothylacium obtained in this study (*) and sequences of
Hysterothylacium and Iheringascaris spp. from the GenBank, with the tree inferred from mtDNA cox-2 and LSU data sets. The numbers
on the tree branches represent the percentage of bootstrap resampling. Heterocheilus tunicatus was used as an out group.
doi:10.1371/journal.pone.0040447.g007
Figure 8. Maximum likelihood reconstruction between sequences of *Hysterothylacium* sp. larvae obtained in this study (*) and sequences of other anisakid species from the GenBank inferred from the ITS dataset. The numbers on the tree branches represent the percentage of bootstrap resampling. *Heterocheilus tunicatus* was used as an out group.

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Figure 9. Ecological data of *Anisakis typica* and *Hysterothylacium* sp.: prevalence expressed as a percentage.

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mounted as semi-permanent preparations on slides; the middle regions were used for molecular analyses. Specimens were examined using an Olympus CX3 microscope, and measurements were made with the aid of an ocular micrometer are given in micrometres as the mean, followed in parentheses by the range. High resolution confocal images were made using a confocal laser scanning microscope (Zeiss Axiovert 510, META). For scanning electron microscopical observations, some specimens were fixed for 24 hours at 4°C in a solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer containing 3% sucrose at pH 7. The samples were washed in the same buffer and post-fixed overnight in 1% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.2 in the dark. The specimens were dehydrated in an ethanol series, critical point dried with CO2, coated with 60 nm of gold and observed in a Jeol JSM 6390 SEM microscope.

Figure 10. Ecological data of *Anisakis typica* and *Hysterothylacium* sp.: mean abundance (no. of parasites/fish) transformed using the fourth root.
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Figure 11. Ecological data of *Anisakis typica* and *Hysterothylacium* sp.: mean intensity (no. of parasites parasitized fish); the bars represent the standard deviation.
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The middle parts of parasites were prepared for total genomic DNA extraction using a ChargeSwitch gDNA Mini Tissue Kit (Invitegen, Carlsbad, CA, EUA) according to the manufacturer’s instructions. To amplify gene fragments of anisakid nematodes, a set of primers were used: NC5/NC2 [37] for ITS fragments, 2/1F for 25S rDNA and 231/290L [5] for 28S rDNA gene fragments. The primer ITSF/ITSR was used to amplify the ITS region of A. typicum [32]. All PCR reactions were performed in a volume of 50 μl with 20 mM of Tris-HCl at pH 8.4; 50 mM of KCl; 250 μM of each deoxynucleotide triphosphate (dNTPs) and 2 μl of genomic DNA. The concentrations of MgCl2, primers and Taq Gold DNA polymerase (Promega) were 2.5 mM MgCl2, primers and 1 U of Taq; ITSF/ITSR (2.5 μM of each oligonucleotide primer and 1 U of Taq); ITSF/ITSR (2.5 μM of MgCl2, 0.4 μM of each oligonucleotide primer and 1 U of Taq); 211F/210R (0.5 μM of forward and 0.4 μM of reverse oligonucleotides, 2.5 μM of MgCl2 and 1 U of Taq) and for 391/390 (0.4 μM of each oligonucleotide 3 μM of MgCl2 and 1.5 U of Taq). PCR was carried out using a Mastercycler Personal/Eppendorf thermal cycler (Epperdorf, Hamburg, Germany) and cycling parameters as previously described [5,15,32,37].

PCR products were visualized with GELRED (Biotium Inc, Hayward, CA, USA) staining after electrophoresis on 1.5% agarose gels. All amplified PCR products generated were purified with Wizard SV gel and PCR clean up system kit (Promega) following the manufacturer’s instructions and sequenced in both directions using the same primer sets as in the respective PCR assay. DNA cycle-sequencing reactions were performed using BigDye v.3.1 chemistry (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were performed in the ABI Prism 3100 sequence analyzer. Sequences were assembled, edited, in DNASTAR SeqMan (DNASTAR, Inc., Madison, WI), and aligned with Bioedit Sequence Alignment Editor (version 7.0.4.1; http://www.mbio.nscu.edu/BioEdit/bioedit.html). The edited sequences were compared for similarities with sequences from GenBank using BLAST 2.0 (“Basic Local Alignment Search Tool”) (Table 2) [39]. To examine the phylogenetic relationships, the nucleotide sequences were analyzed by CLUSTAL W algorithm of Bioedit Package [39,40]. The sequences of the two mitochondrial genes (mtDNA cox-2 and LSU) were joined using the software Concatenator [41]. Phylogenetic trees were inferred by using the software MEGA 5.0 [42] utilizing the General Time Reversible model (GTR) for ITS sequences and Hasegawa-Kishino-Yano model (HKY) for mtDNA cox-2 and LSU. These models were selected using the program jModelTest [43]. Kimura Two Parameters (K2P) values were calculated by the software MEGA 5.0 [42,44]. Maximum Likelihood method was used to construct trees [45] and were resampled by 100 bootstrap replicates to evaluate the reliability of the groups.

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Author Contributions

Conceived and designed the experiments: CPS JNB HLCS CMN. Performed the experiments: JNB LFGC CPS CMN. Analyzed the data: JNB HLCS CPS CMN. Contributed reagents/materials/analysis tools: CPS CMN. Wrote the paper: JNB HLCS CPS. Fish necropsies and collection of parasites: JNB LF GC CMN. DNA extractions, PCR and sequencing: JNB LFGC HLS CS. Co-supervisor of MsC: CMN. Supervisor of MsC: CPS. Confocal and SEM: CPS LFGC JNB.

References

1. Mattiucci S, Nasce Cetti G (2008) Advances and trends in molecular systematics of anisakid nematodes, with implications for their evolutionary, ecology and host-parasite co-evolutionary processes. In: Rollinson D, Hay SI, editors. Advances in Parasitology. Academic Press, 66: 47–148.
2. Kjelstrup S, Palm HW (2001) Anisakid nematode (Ascaridoidea) life cycles and distribution. Increasing zoontic potential in the time of climate change. Parasitology Research Monographs, 2: 201–222.
3. Anderson RC (2000) Nematode parasites of vertebrates: Their development and transmission. Wallingford: CABI Publishing, 672 p.
4. Mattiucci S, Nascetti G, Gianchi R, Pagli I, Arduino P, et al. (1997) Genetic and ecological data on the Anisakis simplex complex with evidence for a new species (Nematoda, Ascarididea, Anisakidae). Journal of Parasitology, 83: 401–7.
5. Nadler SA, D’Amelio S, Pauletta M, Webb SC (2009) Anisakis simplex (Nematoda: Anisakidae) from the stomach of the Red-Spotted Newt (Notophthalmus viridescens) from Pennsylvania Fishless Ponds. BioOne, 95 (6): 1503–1506.
6. Suzuki J, Murata R, Hosaka M, Araki J (2010) Risk factors for human Hysterothylacium norvegicum infection and association between the geographic origins of marine mammals. Journal of Parasitology, 91 (6): 1413–29.
7. Blouin MS, Yowell CA, Sherry DH (1998) Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. Molecular Biology Evolution, 15: 1719–1727.
8. Nike KC, Li L, Xu Z, Zhang L (2008) Redescription of Anisakidae (Nematoda) parasite of Anisakis simplex. Systematic Parasitology, 74: 199–217.
9. Brizzola SM, Tanzola RD (1995) Hystricholaimus rhamidae sp. n., (Nematoda: Ascarididae) from beaked whales of the southern hemisphere: morphological description, genetic relationships between congeners and ecological data. Systematic Parasitology, 19: 159–163. Review of Anisakidae (Nematoda) parasite of Anisakis simplex. Systematic Parasitology, 22: 121–125.
10. Shih HH (2008) Parasitic helminth fauna of the catfish, Trichurus lepturus, L., and the differentiation of four anisakid nematode third-stage larvae by nuclear ribosomal DNA sequences. Parasitology Research, 93: 189–195.
11. Anisakid Nematode from Trichiurus lepturus (Nematoda: Anisakidae) from the northern Gulf of Mexico. Proceedings of the Biological Society of Washington, 95: 1035–1079.
24. 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. Journal of Parasitology, 91 (6): 1430–1434.

25. Knoff M, Felizardo NN, Ituigmez AM, Maldonado A, Torres EJL, et al. (2012) Genetic and morphological characterisation of a new species of the genus Hysterothylacium (Nematoda) from Paralichthys oviceps Jordan, 1890 (Pisces: Teleostei) of the Neotropical Region, state of Rio de Janeiro, Brazil. Memórias do Instituto Oswaldo Cruz, 107 (2): 196–193.

26. Stromnes E, Andersen K (2000) “Spring rise” of whaleworm (Anisakis simplex; Nematoda, Ascaridoidea) third-stage larvae in some fish species from Norwegian waters. Parasitology Research, 86: 619–624.

27. Luque JL, Aguiar JC, Vieira FM, Gibson DI, Portes Santos C (2011) Checklist of Nematoda associated with fishes of Brazil. Zootaxa, 3082: 1–48.

28. D’Amelio S, Mathiopoulos K, Portes Santos C, Pugachev ON, Webb SC, et al. (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. International Journal for Parasitology, 30: 223–226.

29. Mattiucci S, Paggi L, Nascetti G, Portes Santos C, Costa G, et al. (2002) Genetic markers in the study of Anisakis typhlea (Diesing, 1860): Larval identification and genetic relationships with other species of Anisakis Dujardin, 1845 (Nematoda: Anisakidae). Systematic Parasitology, 51: 159–170.

30. Mello OP, Ramos RMA, Di Benedetto APM (2006) Helminths of the marine tucuxi, Sotalia fluviatilis (Gervais, 1853) (Cetacea: Delphinidae), in northern Rio de Janeiro State, Brazil. Brazilian Archives of Biology and Technology, 49 (1): 145–148.

31. Valenzini A, Mattinucci S, Boudanoff P, Webb SC, Magnacci-Giannone AA, et al. (2006) Genetic relationships among Anisakis species (Nematoda, Anisakidae) inferred from mitochondrial cox2 sequences, and comparison with allozyme data. The Journal of Parasitology, 92 (1): 156–166.

32. Ituigmez AM, Portes Santos C, Vicente ACP (2009) Genetic characterization of Anisakis typhlea and Anisakis physeteris from marine mammals and fish from the Atlantic Ocean of Brazil. Veterinary Parasitology, 165: 356–356.

33. Hassel L, Venturotti A, Magalhaes F, Cuerca S, Siciliano S, et al. (2003) Summer sightings of dwarf minke whales (Balaenoptera acutorostrata) off the eastern coast of Rio de Janeiro State, Brazil. Latin American Journal of Aquatic Mammals, 2: 47–50.

34. Brandini FP (1990) Produção primária e características fotosintéticas do fitoplâncton na Região Sudeste do Brasil. Brazilian Journal of Oceanography, 38: 147–159.

35. Martins AS, Haimovich M (2000) Reproduction of cutlassfish Trichiurus lepturus in the southern Brazil subtropical convergence ecosystem. Scientia Marina, 64 (1): 97–103.

36. Bush AO, Lafferty KD, Lotz JM, Shostak AW (1997) Parasitology meets ecology on its own terms: Margolin, et al. revisited. Journal of parasitology, 83 (4): 573–583.

37. Zhu XQ, Gasser RB, Podolska M, Chilton NB (1998) Characterization of anisakid nematodes with zoonotic potential by nuclear ribosomal DNA sequences. International Journal of Parasitology, 28: 1911–1921.

38. Altshul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology, 215 (3): 403–10.

39. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22 (22): 4673–4680.

40. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium 41: 95–98.

41. Pina-Martins F, Paulo OS (2008) Concatenator: sequence data matrices handling made easy. Molecular Ecology Resources, 8: 1254–1255.

42. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA 5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 28: 2731–2739.

43. Posada D (2008) jModelTest: Phylogenetic model averaging. Molecular Biology and Evolution, 25 (7): 1253–1256.

44. Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution, 16: 111–120.

45. Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. Journal of Molecular Biology and Evolution, 17: 366–376.