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MOLECULAR CHARACTERIZATION OF LARVAL ANISAKID NEMATODES FROM MARINE FISHES OF MADEIRA BY A PCR-BASED APPROACH, WITH EVIDENCE FOR A NEW SPECIES

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ABSTRACT: One-hundred and fifteen anisakid larvae from 3 different fish hosts, *Aphanopus carbo*, *Scomber japonicus*, and *Trachurus picturatus*, caught in Madeiran waters, were identified by PCR-RFLP. Three distinct species were identified in *A. carbo*, namely *Anisakis simplex sensu stricto*, *Anisakis pegreffii*, and *Anisakis ziphidarum*; 5 in *S. japonicus*, i.e., *A. simplex s.s., A. pegreffii*, *Anisakis physeteris*, *Anisakis typica*, and *A. ziphidarum*; and 3 in *T. picturatus*, i.e., *A. simplex s.s., A. pegreffii*, and *A. typica*. *Anisakis simplex s.s.* was the most frequent species in both *A. carbo* and *S. japonicus* (54% and 23.5%, respectively). *Anisakis pegreffii* and *A. physeteris* occurred with a frequency of 20.6% in *S. japonicus*, whereas in *T. picturatus* the most frequent species was *A. typica* (41.9%), followed by *A. simplex s.s.* (32.3%). Furthermore, *A. carbo* and *S. japonicus* were infected by an apparently undescribed taxon, provisionally named *Anisakis* sp. A. Based on estimations of the genetic distance, this new taxon seems to be more similar to *A. ziphidarum* (0.0335) than to other species of the genus.

Analysis of the genetic structure of both adults and larvae of anisakid species by means of multilocus enzyme electrophoresis provided interesting results at the taxonomic level (Mattiucci et al., 1986; Nascetti et al., 1986). By applying this approach to several species of *Anisakis*, it was concluded that this genus includes more than 6 genetically and morphologically differentiated species, namely *Anisakis simplex sensu stricto, Anisakis typica*, *Anisakis pegreffii*, *Anisakis physeteris*, *Anisakis ziphidarum*, *Anisakis brevispiculata*, and *Anisakis simplex* C (Mattiucci et al., 1997, 2001, 2002; Paggi et al., 1998). Furthermore, molecular techniques, in particular polymerase chain reaction linked to restriction fragment length polymorphism (PCR-RFLP) analysis of the ribosomal DNA (rDNA) internal transcribed spacers (ITS-1 and ITS-2) and the 5.8S rDNA (riboprinting), have been successfully employed for the identification and differentiation of species of *Anisakis* (Matsuura et al., 1992; D'Amelio et al., 2000).

In Madeira, several fish species are frequently infected with anisakids; particularly affected are the black-scabbard fish, *Aphanopus carbo* Lowe, 1839 (Costa et al., 1996) and the epipelagics, chub mackerel, *Scomber japonicus* Houttyn, 1782, and oceanic horse mackerel, *Trachurus picturatus* Bowdich, 1825 (Costa et al., 2003). Both larval types I and II (Berland, 1961) of *Anisakis* occur in mixed infections in these fish hosts. The aim of the present study was to characterize and identify, at the species level, larvae of *Anisakis* collected from these 3 fish species from Madeiran waters. Further information on the geographical and host range of the *Anisakis* species involved is added.

MATERIAL AND METHODS

Larvae of anisakid nematodes were collected alive from the visceral cavities of the black scabbard fish *A. carbo*, chub mackerel *S. japonicus*, and oceanic horse mackerel *T. picturatus* from Madeiran waters (Atlantic Ocean, Portugal) from May 1999 to January 2000. They were either frozen at −80 C or fixed in 70% ethanol, and kept for later molecular identification.

For DNA extraction, the Holmes–Bonner protocol was used. Each specimen was inserted in a sterile 1.5-ml Eppendorf tube, frozen in liquid nitrogen for a few seconds, homogenized with a pestle with 200 μl of Holmes–Bonner buffer (urea 7 M; Tris-HCl 100 mM, pH 8.0; EDTA 10 mM, pH 8.0; NaCl 350 mM; SDS 2%). Purification was done with 100 μl phenol-chloroform, tubes were agitated for 10 min at room temperature, followed by addition of 100 μl TE-CH3COO Na (3 M, pH 5.2) and centrifugation at 14,000 rpm for 10 min. Extraction was performed by 2 additions of 200 μl chloroform-iso-amyl-alcohol (49:1). To the DNA extract, 400 μl (2 volumes {2V}) of pure ethanol was added and the solution was kept at −20 C for 1 hr to allow precipitation of the nucleic acid. The precipitate was centrifuged for 15 min at 14,000 rpm, and the pellet was treated with 70% ethanol; tubes were vacuum-dried for 10 min, and the pellet was resuspended in 100 μl water.

To amplify the rDNA region (spanning the ITS-1, ITS-2, and the 5.8S subunit), 2 universal conserved primers (Bachellerie and Qu, 1993) were used:

Primer A (forward): GTCGAATTCGTAGGTGAACCTGCGGAAGGATCA
Primer B1 (reverse): GCCGGATCCGAATCCTGGTTAGTTTCTTTTCCT.

All PCR reactions were carried out in a final volume of 100 μl, containing 10 mM Tris-HCl, 1.5 mM Mg Cl_2, 200 μM of each dNTP, 25 pmol of each primer, 1.5 U of Taq DNA polymerase (AmpliTaq Gold, Applera, Foster City, California), and 3 μl of purified DNA in a thermocycler (Perkin Elmer -Gene Amp PCR System 2400, Applied Biosystems) under the following conditions: 10 min at 95 C (initial denaturation), 30 cycles of 30 sec at 95 C, 30 sec at 55 C, and 75 sec at 72 C, followed by a final elongation of 7 min at 72 C.

Amplified fragments were directly subjected to RFLP analysis using 3 individual restriction enzymes (*Hinf*I, *Hha*I, and *Taq*I). Two additional restriction enzymes were used when restriction patterns were not clear (*Hae*III, *Hsp*92II). Restriction was performed using 10 μl of PCR products, 0.5 μl of enzyme, 1.5 μl of a specific buffer, and double-distilled H_2O up to a final volume of 15 μl. Digested amplicons were run on 2% agarose gels at 100V (100 volts) at room temperature for 2 hr with ethidium bromide.

PCR products were directly sequenced after purification using the commercial kit Nucleospin Extract (Macherey-Nagel, Duren, Germany). Sequencing was performed by MWG-BIOTECH AG (Ebersberg, Germany) on both strands and manually adjusted by alignment of overlapping regions of opposite strands. The obtained sequences were aligned with previously characterized sequences of *Anisakis* spp., using CLUSTAL W. (1.8) Multiple Sequence Alignments (Thompson et al., 1994).

RESULTS

One-hundred fifteen anisakid larvae from the 3 different fish hosts, *A. carbo*, *S. japonicus*, and *T. picturatus*, were identified

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TABLE I. Restriction fragments obtained with the different enzymes in species of *Anisakis.*

Enzyme	Fragments (in bp)	Species
*Taq*I	330-300-140	*Anisakis ziphidarum*
	330-300-140	*Anisakis* sp. A
	400-320-150	*Anisakis pegreffii*
	430-400-100	*Anisakis simplex s.s.*
	300-280-140	*Anisakis physeteris*
	400-350	*Anisakis typica*
*Hae*III	620-180-90	*A. pegreffii*
	620-180-90	*A. simplex s.s.*
	350-280-180-150	*A. physeteris*
	600-350	*A. ziphidarum*
	600-350	*Anisakis* sp. A
	650-300	*A. typica*
*Hsp*92II	560-340	*A. pegreffii*
	560-340	*A. simplex s.s.*
	750-150	*A. typica*
	700-200	*A. ziphidarum*
	700	*Anisakis* sp. A
*Hinf*I	370-300-250	*A. pegreffii*
	620-250-80	*A. simplex s.s.*
	380-290-270	*A. physeteris*
	620-350	*A. typica*
	370-320-290	*A. ziphidarum*
	620-320	*Anisakis* sp. A
*Hha*I	550-430	*A. pegreffii*
	550-430	*A. simplex s.s.*
	550-430	*A. physeteris*
	320-240-180-160	*A. typica*
	550-430	*A. ziphidarum*
	550-300-80	*Anisakis* sp. A

by PCR- RFLP, on the basis of the diagnostic restriction banding patterns provided by D'Amelio et al. (2000) and recently confirmed by DNA sequencing (Abollo et al., 2003; data not shown). Eighteen of these larvae, analyzed from the black scabbard fish and chub mackerel, produced restriction profiles that did not correspond to any of the patterns so far characterized by PCR-RFLP. Two sibling species of the *Anisakis simplex* complex were identified (*A. simplex s. s.* and *A. pegreffii*) using 2 of the restriction endonucleases (*Hinf*I and *Taq*I). Three additional *Anisakis* species that are not members of the *A. simplex* complex were also identified using the above enzymes according to D'Amelio et al. (2000), i.e., *A. physeteris*, *A. typica*, and *A. ziphidarum*. An undescribed taxon, provisionally named *Anisakis* sp. A, exhibited 2 bands after restriction with the enzyme *Hinf*I (Fig.1; Table I). This pattern did not correspond to any of the species characterized by riboprinting by D'Amelio et al.

TABLE III. Genetic distances between *Anisakis* sp. A, and other species of *Anisakis* (obtained from MEGA-modified). Number of nucleotides in subset 1153, gap sites, and missing information removed in pairwise comparisons.

	Anisakis sp. A
Anisakis simplex C	0.0521
Anisakis simplex s.s.	0.0494
Anisakis pegreffii	0.0521
Anisakis ziphidarum	0.0335
Anisakis physeteris	0.0827
Anisakis typica	0.0798

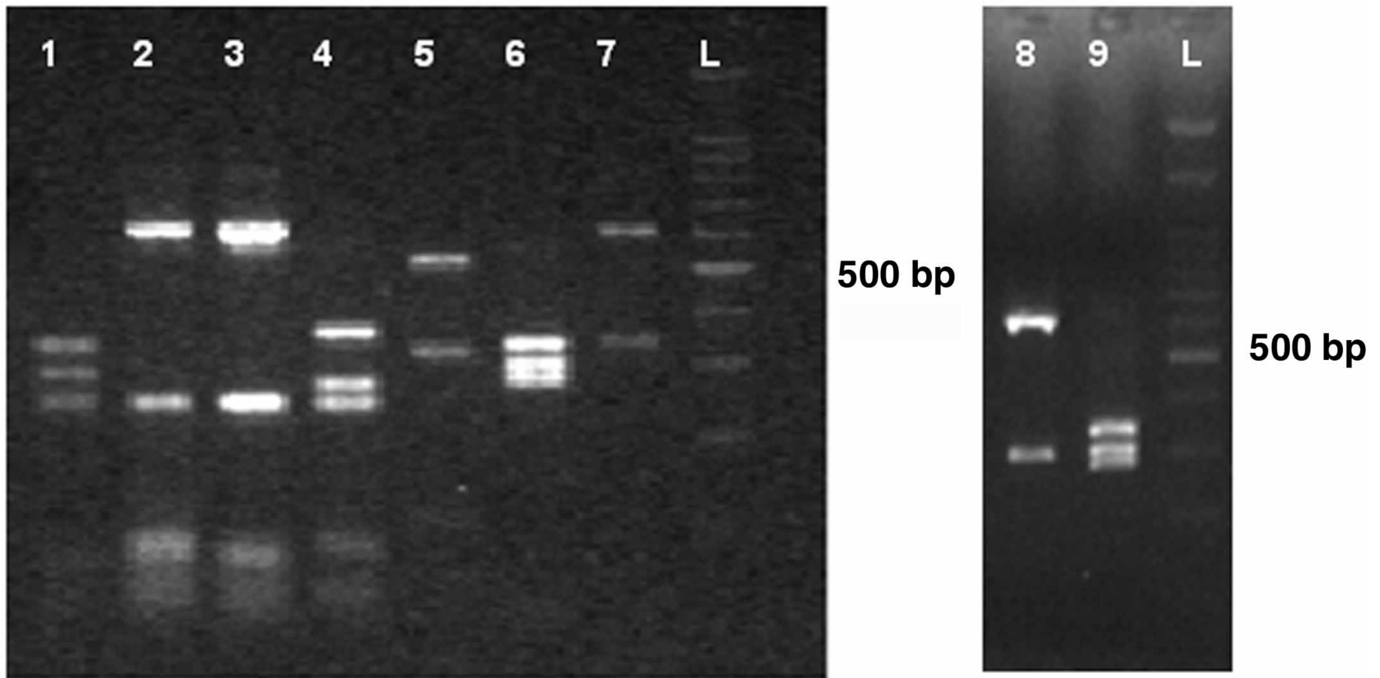
(2000). Restriction with *Taq*I produced 3 fragments in *A. ziphidarum* and *Anisakis* sp. A (bands of 330, 300, and 140 bp), whereas for *A. pegreffii*, *A. simplex s.s.,* and *A. physeteris*, the 3 resulting band patterns were different (bands of 400, 320, and 150 bp; 430, 400, and 100 bp; and 300, 280, and 140 bp, respectively) (Fig. 2). *Anisakis typica* exhibited only 2 fragments, of 400 bp and 350 bp. With *Hha*I, different restriction patterns were obtained for *A. typica* and *Anisakis* sp. A (bands of 320, 240, 180, and 160 bp and 550, 300, and 80 bp, respectively) (Fig. 3). Restriction with *Hae*III yielded 2 identical fragments from *A. ziphidarum* and *Anisakis* sp. A (600 and 350 bp), which differed from the restriction pattern of *A. typica* (bands of 650 and 300 bp). *Anisakis physeteris* showed a unique pattern characterized by 4 bands (350, 280, 180, and 150 bp); the remaining species showed 3 fragments of 620 bp, 180 bp, and 90 bp. With *Hsp*92II, *A. pegreffii* and *A. simplex s.s.* exhibited 2 fragments (560 and 340 bp), *A. typica* exhibited 2 bands (750 and 150 bp), *A. ziphidarum* 2 bands (700–200 bp), whereas *Anisakis* sp. A, exhibited only 1 band, which was longer than 700 bp. The results of the digestion with the different enzymes are summarized in Table I. A potential second undescribed taxon, which was not sequenced, was also found, and identified as *Anisakis* sp. B.

Table II summarizes the results of the molecular identification of the 115 *Anisakis* larvae from the 3 different fish hosts. The Type II larvae were all identified as belonging to *A. physeteris*, whereas the Type I larvae were found to belong to *A. simplex s.s., A. pegreffii*, *A. ziphidarum*, *A. typica*, and the previously undescribed taxon.

Anisakis simplex s.s. was the most frequent species in the black-scabbard fish (54%) and chub mackerel (23.5%). *Anisakis pegreffii* and *A. physeteris* had the same frequency of occurrence in the chub mackerel (20.6%); in the oceanic horse mackerel, the dominant species was *A. typica* (41.9%), followed by *A. simplex s.s.* (32.3%). The new taxon, provisionally indicated

TABLE II. *Anisakis* species identified from *Aphanopus carbo, Scomber japonicus,* and *Trachurus picturatus* by PCR-RFLP.

	Species of *Anisakis*						
Host species	*Anisakis simplex s.s.*	*Anisakis pegreffii*	*Anisakis physeteris*	*Anisakis typica*	*Anisakis ziphidarum*	*Anisakis* sp. A	*Anisakis* sp. B
Aphanopus carbo	26	6	0	0	6	8	2
Trachurus picturatus	10	8	0	13	0	0	0
Scomber japonicus	8	7	7	3	1	6	2



FIGURES 1–3. RFLP patterns obtained by digestion of the ITS region of the rDNA (ITS-1,5,8S gene and the ITS-2) with the restriction enzymes *Hinf*I (**1**), *Taq*I (**2**), and *Hha*I (**3**) shown by the species of the genus *Anisakis*. Lanes: (**1**) *A. pegreffii*; (**2**) *A. simplex s.s.*; (**3**) *A. simplex* C; (**4**) *A. physeteris*; (**5**) *A. schupakovi*; (**6**) *A. ziphidarum*; (**7**) *A. typica*; (from D'Amelio *et al*. 2000); (**8**) *Anisakis* sp. A; (**9**) *A. ziphidarum*; {L,}100-bp ladder.

as *Anisakis* sp. A, was sequenced, aligned, and compared with known sequences for the other *Anisakis* species. The sequence has been deposited in the GenBank (accession number AY260555). Estimation of genetic distances of the unknown and known *Anisakis* species indicated that *Anisakis* sp. A was closer to *A. ziphidarum* than to any of the other species of the genus (Table III).

DISCUSSION

Molecular markers for the identification of nematode species have been successfully developed by several authors (Zhu et al., 1998, 2000; Gasser et al., 1999). For the species within *Anisakis*, in particular, molecular markers were developed based on PCR-RFLP studies on the variable regions of the rDNA, and

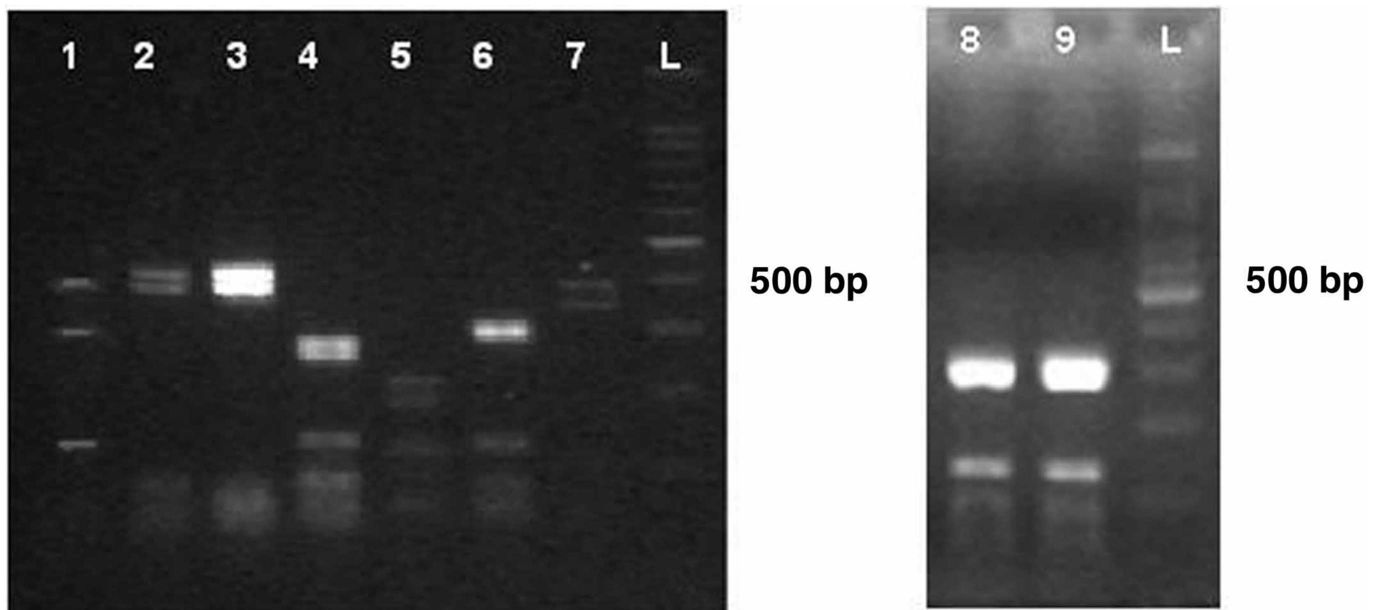


FIGURE 2.

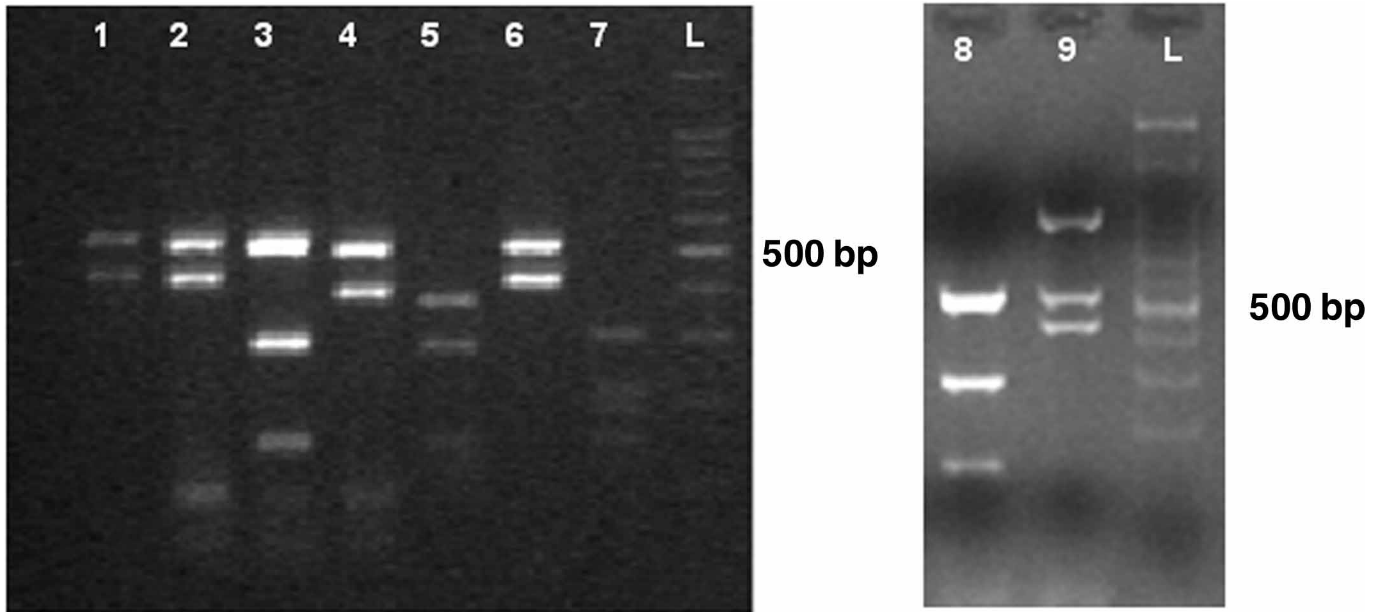


FIGURE 3.

the ITS-1 and ITS-2 regions (D'Amelio et al., 2000). From 5 restriction enzymes used in the present study, *Hinf*I, *Hha*I, *Taq*I, *Hae*III, and *Hsp*92II, 2 provided diagnostic restriction patterns between the species of the *A. simplex* complex, namely *A. simplex s.s.* and *A. pegreffii* (*Hinf*I and *Taq*I). Restriction with *Hinf*I produced 3 fragments, but different profiles in *A. pegreffii* (370 bp, 300 bp, and 250 bp), *A. simplex s.s.* (620 bp, 250 bp, and 80 bp), *A. physeteris* (380 bp, 290 bp, and 270 bp), and *A. ziphidarum* (370 bp, 320 bp, and 290 bp), whereas both *A.typica* and *Anisakis* sp. A exhibited 2 fragments of 620 bp and 350 bp, and 620 bp and 320 bp, respectively. *Hha*I was useful to distinguish between *A. typica* and *Anisakis* sp. A. This enzyme is also helpful to distinguish *A. simplex* C from the other species of the *Anisakis simplex* complex (D'Amelio et al., 2000). *Taq*I was able to distinguish the sibling species (*A. simplex s.s.* and *A. pegreffii*) and the morphologically differentiated species. Similar results were found by D'Amelio et al. (2000). The differences found in restriction patterns for *Anisakis* sp. A (and for *Anisakis* sp. B, which was not sequenced) in the present work, strongly suggested that it belongs to a still unknown *Anisakis* species. Adults of this new species were not available for morphological characterization and molecular comparison. The values of genetic distances obtained for *Anisakis* sp. A seem to indicate that this species is more similar to *A. ziphidarum* than to any other species in the genus (see Table III). However, the value obtained between *Anisakis* sp. A and *A. ziphidarum* (0.0335), suggests that they are not sibling species. The close relationship between *Anisakis* sp. A and *A. ziphidarum* was also confirmed by a preliminary study on their phenetic relationships based on 1,000 bootstrap replicates of a neighbor-joining analysis.

Previous genetic analysis based on allozyme data, performed on larvae in the same fish hosts, confirmed the presence of *A. typica*, *A. simplex s.s., A. pegreffii*, and *A. ziphidarum* in *S. japonicus*, and *A. typica*, *A. simplex s.s.,* and *A. pegreffii* in *T. picturatus* (Mattiucci et al., 2002; Costa et al., 2003). Another 48 larvae, analyzed by multilocus electrophoresis, from *A. carbo*, allowed the identification of *A. simplex s.s., A. pegreffii*, *A. brevispiculata*, *A. ziphidarum*, and *A. physeteris* (see Costa et al., 2003). A further taxon, *Anisakis* sp., was also found. Its correspondence with 1 of the 2 undescribed taxa detected in the present study is still to be confirmed.

Anisakis simplex s.s. is known to have a worldwide distribution, with a mainly benthic or demersal life cycle (Mattiucci et al., 1997). Our data extend its life cycle to the bathypelagic realm. The absence of *A. typica* in the deepwater fish *A. carbo* confirmed that this anisakid species is typical of the epipelagic region (Mattiucci et al., 2002). Its typical definitive hosts, delphinids, are indeed common in the epipelagic region (Jefferson et al., 1993). *Anisakis ziphidarum*, which occurred in both the black-scabbard fish (12.5%) and the chub mackerel (2.9%), can indeed be found in both the pelagic and the deepwater regions; its definitive hosts, *Mesoplodon layardii* and *Ziphius cavirostris* (Paggi et al., 1998), already reported from Madeiran waters (Mathias, 1988), are typical of both regions. Although *A. physeteris* was not identified by molecular methods, its presence in the region was confirmed in a previous study (see Costa et al., 2003). *Anisakis ziphidarum* and the new taxon, *Anisakis* sp. A, seem to be more related to the *A. simplex* complex than to *A. physeteris* and *A. typica*. If this assumption is correct, close correspondence exists between the inferred phylogeny of parasites and that of their hosts (Nascetti et al., 1998; Brooks and Hoberg, 2000; Hoberg and Adams, 2000; Nadler et al., 2000), and the final host for *Anisakis* sp. A could possibly be a member of the Ziphiidae.

The present study emphasizes also the high diversity of *Anisakis* species in the study area, thus suggesting that Madeiran waters may represent a region where the distribution of several *Anisakis* sibling and morphospecies overlaps. However, in contrast with the results described by Abollo et al. (2003), no recombinant genotypes between the restriction patterns of distinct species were detected.

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