

Infection levels and diversity of anisakid nematodes in blackspot seabream, *Pagellus bogaraveo*, from Portuguese waters

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Abstract The blackspot seabream, *Pagellus bogaraveo*, is a sparid fish of great economic importance in the northeast Atlantic. The main aim of this work was to assess the infection levels and diversity of anisakid nematodes parasitizing *P. bogaraveo* from Portuguese waters. The anisakid larvae were identified by polymerase chain reaction-restriction fragment length polymorphism analysis and ten different patterns were observed, four of which were not previously reported in the literature. Moreover, several species were detected for the first time in this host: *Anisakis simplex* × *Anisakis pegreffii* hybrids, *Anisakis ziphidarum*, *Anisakis typica*, *Anisakis physeteris*, as well as three undescribed anisakids *Anisakis* sp. PB-2009, *Anisakis* sp. PB-2010, and *Contracaecum* sp. PB-2010. The ITS1-5.8S-ITS2 region was sequenced and analyzed phylogenetically, revealing that our anisakids were distributed by the two

distinct clades reported previously, corresponding to the two recognized larval morphotypes. Moreover, a group of organisms, including our specimens from Madeira and the previously reported *Anisakis* sp. HC-2005, cluster together and seem to belong to clade I. A certain degree of intraspecific diversity was also detected. Samples from mainland waters had the highest infection levels and were dominated by *A. pegreffii*. Madeira had the highest diversity overall, dominated by *Anisakis* sp. PB-2010. Fish from the Azores had the lowest infection levels, and the species with the highest relative abundance was *A. physeteris*. The anisakid nematode communities were relatively similar in mainland waters but very distinct in both the Azores and Madeira islands, suggesting the existence of at least three different stocks of *P. bogaraveo* in the northeast Atlantic.

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Introduction

The blackspot seabream, *Pagellus bogaraveo* (Brünnich, 1768), is a greatly appreciated fish with high commercial value. It is a sparid fish whose geographical distribution encompasses the Northeast Atlantic and the Mediterranean, occurring at depths of up to 800 m (Spedicato et al. 2002). It is a protandrous hermaphroditic species, with most individuals maturing first as males and later changing sex to females (Micale et al. 2002). This feature seems to increase the species' vulnerability to fishing pressure, as evidenced by the collapse of the Cantabrian Sea *P. bogaraveo* stock (Erzini et al. 2006). Little is known about its population structure in the northeast Atlantic, although several studies seem to point to low genetic variability (Stockley et al. 2005; Lemos et al. 2006). Most of the fishery catches of this species are from Portugal and Spain, and recently, this fish

has been introduced into aquaculture (Basurco et al. 2011). Recent shifts in gastronomic habits, especially the consumption of raw fish, have contributed to an increased awareness of fish parasites as a health risk. Anisakid larvae, especially *Anisakis* spp., can infect humans and cause anisakiosis, a condition that includes a number of mild to severe gastrointestinal problems, as well as acute allergic reactions (Audicana et al. 2002; Falcão et al. 2008; Hochberg and Hamer 2010). Recently, *A. simplex* extracts have been included in the standard sets of allergens for the evaluation of food allergies (Audicana and Kennedy 2008).

Parasites have been widely used as biological tags to study fish stocks, and anisakid nematodes are especially suited for this purpose because once acquired they tend to remain in the host fish for long periods of time (MacKenzie and Abaunza 1998). However, as fish are generally intermediate or paratenic hosts of these parasites (with marine mammals as definitive hosts), only larvae occur in fish, exhibiting little morphological differences, which hinders their identification. Some formerly recognized species have been shown to be complexes of cryptic species, e.g., *A. simplex sensu lato* (s.l.), with genetic but not morphological differentiation (Mattiucci and Nascetti 2008). Molecular markers, in particular, the rDNA region spanning the internal transcribed spacers ITS1 and ITS2, and the 5.8S subunit are widely used in taxonomic studies of nematodes and have been an invaluable tool to discriminate among several closely related anisakid species (Mattiucci and Nascetti 2008).

In the northeast Atlantic, several species of the genus *Anisakis* have been reported in teleosts. At high latitudes, *A. simplex* s.s. is often the only species found in these hosts (e.g., Klimpel et al. 2007, 2008). Recently, Klimpel et al. (2011) have also detected *Anisakis paggiae* in a deep-sea fish at latitudes of 52°–58°N. In general, *A. simplex* s.s. becomes less abundant towards the south. *Anisakis pegreffii* is the most common anisakid in the Mediterranean Sea (e.g., Abollo et al. 2003; Mattiucci et al. 2004; Martín-Sánchez et al. 2005) and has been detected in Atlantic areas with Mediterranean influence. In sympatric areas of *A. simplex* s.s. and *A. pegreffii*, hybrid genotypes have been detected, namely in the Cantabrian Sea, Portuguese mainland coast, Gibraltar area, and the Azores (Abollo et al. 2003; Martín-Sánchez et al. 2005; Marques et al. 2006; Sequeira et al. 2010). In Portuguese waters, several other *Anisakis* species have also been detected, including *Anisakis typica*, *Anisakis physeteris*, and *Anisakis ziphidarum*, the latter only in islands' waters (Marques et al. 2006; Sequeira et al. 2010). Anisakid diversity seems to increase towards lower latitudes, and, around Madeira, several species occur, including *A. simplex* s.s., *A. pegreffii*, *A. ziphidarum*, *A. typica*, *A. physeteris*, *Anisakis brevispiculata*, and *Anisakis nascetti* (Costa et al. 2004; Pontes et al. 2005; Sequeira et al. 2010). The distribution of anisakid species is undoubtedly related

to the presence of their definitive hosts and conditioned by the local availability of suitable intermediate and paratenic hosts (Klimpel et al. 2010).

The main aim of this work was to determine the infection levels of *P. bogaraveo* by anisakid nematodes, as well as to assess their diversity at different locations in Portuguese waters by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and direct sequencing of the internal transcribed spacer (ITS) region.

Material and methods

Fish sampling and parasitological analysis

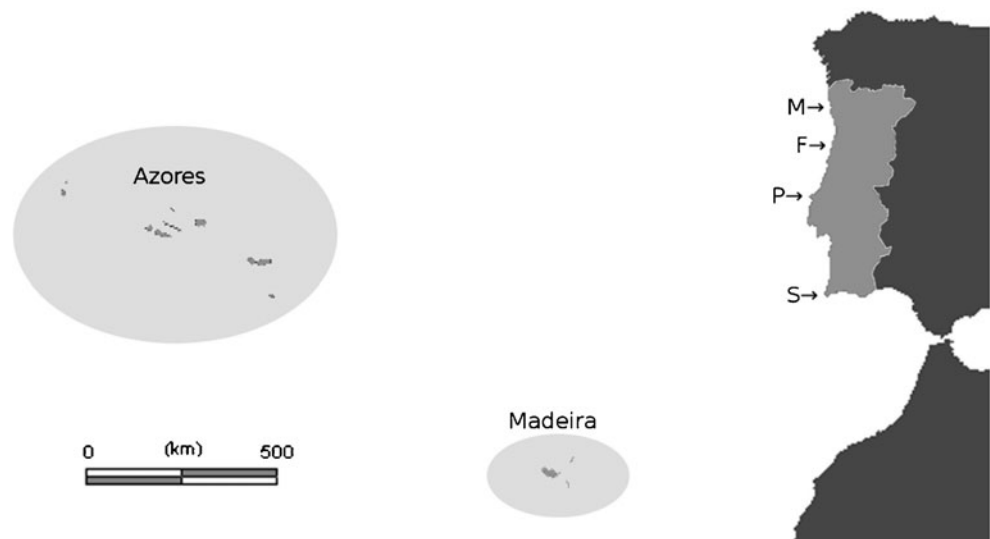
A total of 269 specimens of *P. bogaraveo* from Portugal Exclusive Economic Zone, namely Matosinhos (42), Figueira da Foz (52), Peniche (42), Sagres (30), Madeira (36), and the Azores (67) (see Fig. 1) were acquired from commercial catches. The fish were transported in ice, placed in individual plastic bags, and frozen until further observation. After defrosting, specimens were measured, dissected, and examined for the presence of anisakid larvae in the body cavity, viscera, and mesenteries, under a stereomicroscope. All anisakid larvae detected were removed, counted, washed in physiological saline 0.9% (p/v) and preserved in 70% (v/v) ethanol. For each location, infection levels of anisakid larvae (prevalence, intensity, and abundance) were determined following Bush et al. (1997).

DNA extraction, PCR-RFLP, and sequencing

DNA was extracted by crushing and grinding the anisakid larva with a sterile pestle in an Eppendorf tube containing 20 µl of buffer (50 mM Tris-HCl, pH 8.0, with 10 mM EDTA) and using the QIAamp DNA MiniKit (QIAGEN, Germany) following the protocol for DNA extraction from tissues. The DNA was eluted with 20–30 µl of water.

Polymerase chain reaction (PCR) amplification of the ITS region was performed using the NC5 forward (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 reverse (5'-TTAGTTTCTTCCTCCGCT-3') oligonucleotide primers (Zhu et al. 1998). PCRs were carried out in a MyCycler™ thermal cycler (Bio-Rad laboratories Inc., Hercules, CA, USA) using 0.5 U of Taq polymerase and 1× PCR reaction buffer supplemented with 1 mM MgCl₂ (GE Healthcare, Buckinghamshire, UK), 200 mM deoxynucleoside triphosphates, 1 mM of each primer, 16% (p/v) of bovine serum albumin and 50 to 250 ng of DNA, with the following program—94°C for 10 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 70s, and a final extension of 72°C for 7 min. Agarose gel electrophoresis

Fig. 1 Sampling locations in Portuguese waters (northeast Atlantic); *M*, Matosinhos; *F*, Figueira da Foz; *P*, Peniche; *S*, Sagres; Madeira; and the Azores islands



was performed with standard protocols using $1\times$ TAE buffer (Sambrook and Russell 2001).

At least 30 larvae were randomly selected among the total sample from each location for the molecular identification in order to detect, with a 95% confidence level, all the species present with a relative abundance $\geq 10\%$ (Simon and Schill 1984). After PCR, about 200 ng of the ITS amplicon (~ 1 kb) was digested with the restriction enzyme FastDigest® *Hinf*I (Fermentas, York, UK), according to manufacturer instructions. When further discrimination was necessary, the endonucleases FastDigest® *Hha*I and/or *Taq*I were also used. The RFLP patterns were evaluated by electrophoresis using 2–2.5% ethidium-bromide-stained agarose gels.

The DNA fragments were isolated from agarose gels using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). Purified PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* DH5 α competent cells following the instructions of the manufacturer. White colonies were grown overnight at 37°C, in liquid LB medium supplemented with 100 $\mu\text{g ml}^{-1}$ of ampicillin, with shaking. Plasmid DNA was isolated from *E. coli* cultures using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Saint Louis, MO, USA), and the insert was sequenced at STAB Vida (Lisbon, Portugal). Novel sequences associated with this study were deposited in GenBank (Accession numbers: JN005754 to JN005769).

Phylogenetic analysis

The ITS1-5.8S-ITS2 sequences from the anisakid species isolated in this study were aligned with a series of *Anisakis* spp. reference species and *Pseudoterranova decipiens*, using the ClustalW (Larkin et al. 2007) plug-in

present in the Geneious Pro package (Drummond et al. 2011). This alignment was used to build a neighbor-joining (NJ) phylogenetic tree (Saitou and Nei 1987) using *P. decipiens* as the outgroup. This tree was computed using the MEGA5 software (Tamura et al. 2011), considering the Kimura-2 parameter model (Kimura 1980) with a rate variation among sites modeled with a gamma distribution (as selected by the Model Selection tool built in MEGA). For the bootstrap analysis, 1,000 pseudo-replicates were used (Felsenstein 1985).

Statistical analysis

Statistical analyses were carried out using IBM® SPSS® Statistics software. The nonparametric Mann–Whitney *U* test was used to perform a pairwise comparison of fish length and anisakid abundance between locations (differences were considered significant at $p < 0.05$). The relation between fish length and anisakid abundance was analyzed by Spearman rank correlation. A pairwise analysis of the relative abundance of the identified anisakid species was carried out between locations using Chi-square test (differences were considered significant at $p < 0.05$).

Results

Infection levels

Most anisakid larvae were found encapsulated in flat coils in the fish body cavity, mesenteries, and on the outside of viscera, sometimes forming large clusters. Some larvae were free in the body cavity and, occasionally, inside visceral organs.

Fish length and infection levels are presented in Table 1. Fish length varied significantly among locations, with fish from Madeira and Sagres being the smallest and the ones from Peniche being the largest. Most of the sampled fish were infected, with prevalence reaching 100% in Peniche and Matosinhos. The fish from Azores were the only exception with a prevalence of 36%. Abundance of infection varied significantly among locations, showing very low values in the Azores, low values in Sagres, and high values in Peniche. A significant positive correlation was found between anisakid abundance and fish length ($r=0.273$, $n=268$, $p=0.000$).

Identification and geographical distribution of anisakid species

Two hundred five anisakid larvae isolated from *P. bogaraveo* (at least 30 randomly selected from the total sample from each location, see section “Material and methods”) were identified by PCR-RFLP pattern analysis. Ten different patterns were observed (Table 2). Five patterns were previously described (D'Amelio et al. 2000), corresponding to the species *A. simplex* s.s., *A. pegreffii*, *A. ziphidarum*, *A. physeteris*, and *A. typica* (Table 2 and Electronic supplementary material Fig. S1: lanes 1, 2, 4, 5, 6, respectively). One pattern consisting of the combined patterns of both *A. simplex* s.s. and *A. pegreffii* was found, corresponding to the recombinant genotype first described by Abollo et al. (2003) (Table 2 and Electronic supplementary material Fig. S1: lane 3). Four novel patterns were also detected. For most of the Madeira specimens, the 869-bp PCR fragment was not cut by *Hinf*I, and since this pattern is typical for both the species, *A. paggiae* and *A. brevispiculata* (Quiazon et al. 2009; Murata et al. 2011; Cavallero et al. 2011), the fragment was subjected to digestion with *Hha*I and *Taq*I (Table 2 and Electronic supplementary material Fig. S1: lane 7). However, the patterns obtained did not correspond to either *A. paggiae* (Cavallero et al. 2011; Murata et al. 2011) or *A. brevispiculata* (Sequeira et al. 2010; Murata et al. 2011;

Cavallero et al. 2011). Subsequently, the PCR fragment obtained for *Anisakis* sp. PB-2010 was cloned and sequenced (JN005761), and it was found to have 99% similarity to *Anisakis* sp. HC-2005 in *Hoplostethus cadenati* from the African shelf (Kijewska et al. 2009). In two specimens from Madeira, *Anisakis* sp. PB-2009, a different pattern was observed (Table 2), and their sequences (JN005758 and JN005759) had 94% similarity to the above-mentioned *Anisakis* sp. HC-2005 as well as *Anisakis* sp. PB-2010. Two other unknown patterns (Table 2 and Electronic supplementary material Fig. S1, lanes 8 and 9) were also found and the respective fragments sequenced: One of the sequences (JN005769) showed 98% similarity to *Contracaecum* sp. 2 SX-2008 (Fang et al. 2010), a sequence that in fact is more similar to several *Hysterothylacium* spp. than to any *Contracaecum* spp., and therefore our specimen was identified as *Hysterothylacium* sp. PB-2010, whereas the other (JN005755) has 88% similarity to *Contracaecum muraenesoxi* (Luo 1999; Fang et al. 2010) and was identified as *Contracaecum* sp. PB-2010.

The number of anisakid nematodes from each species, their relative abundances, and statistical significance of differences among locations is presented in Table 3. The relative abundances of anisakid species in each sampling location are depicted in Fig. 2.

In the samples from mainland waters, the relative abundance of larvae belonging to the *A. simplex* complex was very high (between 89% in Sagres and 100% in Figueira da Foz and Matosinhos), whereas in the Azores it was considerably lower (55%), reaching only 12% in Madeira. *A. pegreffii* was the most abundant species in all the mainland samples, whereas in the Azores *A. physeteris* presented the highest relative abundance (42%), and in Madeira *Anisakis* sp. PB-2010 was the dominant species with a relative abundance of 56%. This species, as well as the closely related *Anisakis* sp. PB-2009, only occurred in Madeira, and the larvae were mostly encysted in the stomach wall (69%).

Anisakid species richness decreased with latitude, with the highest diversity in Madeira (eight species) and lowest in

Table 1 Number of fish examined (*N*), fish total length, anisakid infection levels (prevalence, intensity, and abundance) in each sampling location and significance of differences between locations (similar letters indicate no statistically significant differences at $p<0.05$)

Location	<i>N</i>	Fish total length (cm)			Prevalence (%)	Intensity		Abundance		
		Mean±SD (min–max)	Median			Mean±SD (min–max)	Median	Mean±SD	Median	
Matosinhos	42	32.1±1.7 (27.1–35.0)	32.4	b	100	12.6±11.9 (1–48)	8.5	12.6±11.9	8.5	d
Figueira da Foz	52	32.7±1.5 (28.8–37.6)	32.7	b	92	10.5±11.9 (1–49)	6	9.7±11.8	6	cd
Peniche	42	37.4±3.1 (33.2–48.2)	36.6	c	100	43.4±82.9 (1–449)	18	43.4±82.9	18	e
Sagres	30	30.0±0.7 (28.2–31.1)	30.1	a	73	9.1±15.9 (1–76)	4.5	6.7±14.2	2	b
Madeira	36	30.2±1.7 (27.6–34.2)	30.1	a	83	14.9±26.3 (1–125)	5.5	12.4±24.6	5	bc
Azores	67	33.5±4.1 (27.0–43.3)	32.5	b	36	2.67±2.62 (1–10)	2.6	1.0±2.0	0	a

Table 2 ITS region PCR product and PCR-RFLP fragment sizes obtained for the anisakids identified in this study

Species	PCR product (bp)	Fragment sizes (bp)		
		<i>Hinf</i> I	<i>Hha</i> I	<i>Taq</i> I
<i>A. simplex</i> s.s.	951	615, 232, 67, 37	530, 421	424, 378, 65, 54, 30
<i>A. pegreffii</i>	951	331, 284, 232, 67, 37	530, 421	378, 293, 131, 65, 54, 30
<i>A. simplex</i> s.s. × <i>A. pegreffii</i>	951	615, 331, 284, 232, 67, 37	530, 421	424, 378, 293, 131, 65, 54, 30
<i>A. ziphidarum</i>	929	332, (287, 273), 37	516, 413	(285, 282), 128, 65, 54, 30
<i>A. typica</i>	954	594, 326, 34	308, 212, 180, 153, 101	380, 338, 65, 54, 28
<i>Anisakis</i> sp. PB-2009	896	578, 284, 34	526, 370	293, 171, 125, 91, 65, 54, 40, 28, 21, 8
<i>Anisakis</i> sp. PB-2010	869	869	372, 211, 172, 114	391, 171, 65, 54, 40, 30, 21, 8
<i>A. physeteris</i>	898	360, 260, 241, 37	511, 387	272, 242, 133, 65, 54, 30, 12
<i>Hysterothylacium</i> sp. PB-2010	1,033	685, 349	307, 271, 184, 150, 74, 48	324, 298, 147, 118, 65, 54, 28
<i>Contracaecum</i> sp. PB-2010	953	422, 339, 192	349, 306, 150, (77, 71)	306, 142, (112, 107), 65, 54, 46, 45, 41, 30, 5

Fragments that appear as a single band are indicated in brackets. Fragments in italics might not be visible in the gel

Matosinhos and Figueira da Foz, where only *A. simplex* s.s., *A. pegreffii*, and hybrid genotypes between these two species were found.

Phylogenetic analysis

Phylogenetic analyses were performed to assess the relative positioning of the anisakid species identified in this study. A NJ algorithm was used to compute a phylogenetic tree of all isolates and a number of reference species, using *P. decipiens* as the outgroup. The resulting tree (Fig. 3) revealed the two distinct clades previously identified by a number of different authors (e.g., Mattiucci and Nascetti 2008). Moreover, we were able to identify a group of specimens from Madeira (*Anisakis* sp. PB-2009 and *Anisakis* sp. PB-2010)

that cluster together with a species temporarily described as *Anisakis* sp. HC-2005. This group of organisms (Fig. 3, dashed line) seem to belong to clade I, with a high bootstrap (100%) support. In order to validate these phylogenetic results, a maximum-likelihood tree was also constructed (data not shown). This tree fully supported the topology of the NJ one (Fig. 3), both in the clade separation and in the relationship between clade I and the undescribed species.

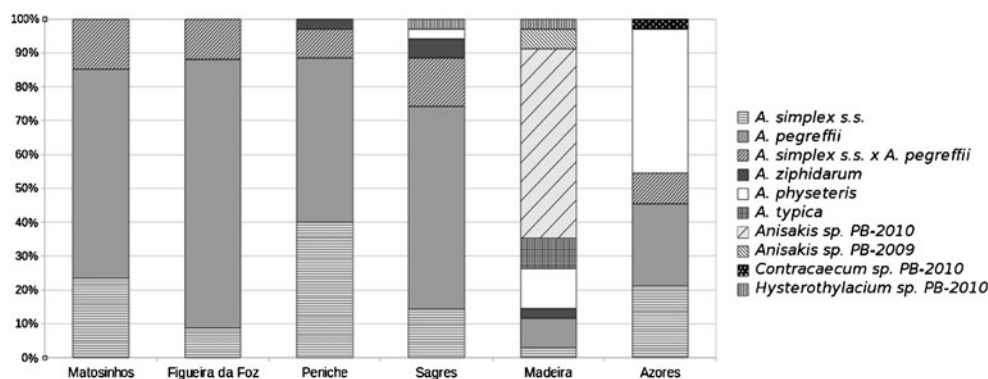
Most species showed some degree of intraspecific variability, as can be seen in the estimates of evolutionary divergence and their standard errors (Table S1 of the Electronic supplementary material). Of the four *A. simplex* s.s. sequenced, JN005764 and EU624342 were identical, whereas JN005757 differ from JN005764 and EU624342 0.002 base substitutions per site. On the other hand, within

Table 3 Number and relative abundance (percent) of anisakid species detected in the different locations and significance of differences between locations

Anisakid species	Matosinhos (34)			Figueira da Foz (34)			Peniche (35)			Sagres (35)			Madeira (34)			Azores (33)		
	No.	%		No.	%		No.	%		No.	%		No.	%		No.	%	
<i>A. simplex</i> s.l.	34	100	c	34	100	c	34	97	c	31	89	c	4	12	a	18	55	b
<i>A. simplex</i> s.s.	8	23	ab	3	9	a	14	40	b	5	14	a	1	3	a	7	22	ab
<i>A. pegreffii</i>	21	62	bc	27	79	c	17	48	ab	21	60	bc	3	9	a	8	24	a
<i>A. simplex</i> × <i>A. pegreffii</i>	5	15	–	4	12	–	3	9	–	5	14	–	0	0	–	3	9	–
<i>A. ziphidarum</i>	0	0	–	0	0	–	1	3	–	2	6	–	1	3	–	0	0	–
<i>A. typica</i>	0	0	–	0	0	–	0	0	–	0	0	–	3	9	–	0	0	–
<i>Anisakis</i> sp.	0	0	a	0	0	a	0	0	a	0	0	a	21	61	b	0	0	a
<i>Anisakis</i> sp. PB-2009	0	0	–	0	0	–	0	0	–	0	0	–	2	6	–	0	0	–
<i>Anisakis</i> sp. PB-2010	0	0	a	0	0	a	0	0	a	0	0	a	19	55	b	0	0	a
<i>A. physeteris</i>	0	0	a	0	0	a	0	0	a	1	3	a	4	12	a	14	42	b
<i>Hysterothylacium</i> sp. PB-2010	0	0	–	0	0	–	0	0	–	1	3	–	1	3	–	0	0	–
<i>Contracaecum</i> sp. PB-2010	0	0	–	0	0	–	0	0	–	0	0	–	0	0	–	1	3	–

Similar letters indicate no statistically significant differences at $p < 0.05$

Fig. 2 Relative abundances of anisakid species in each sampling location

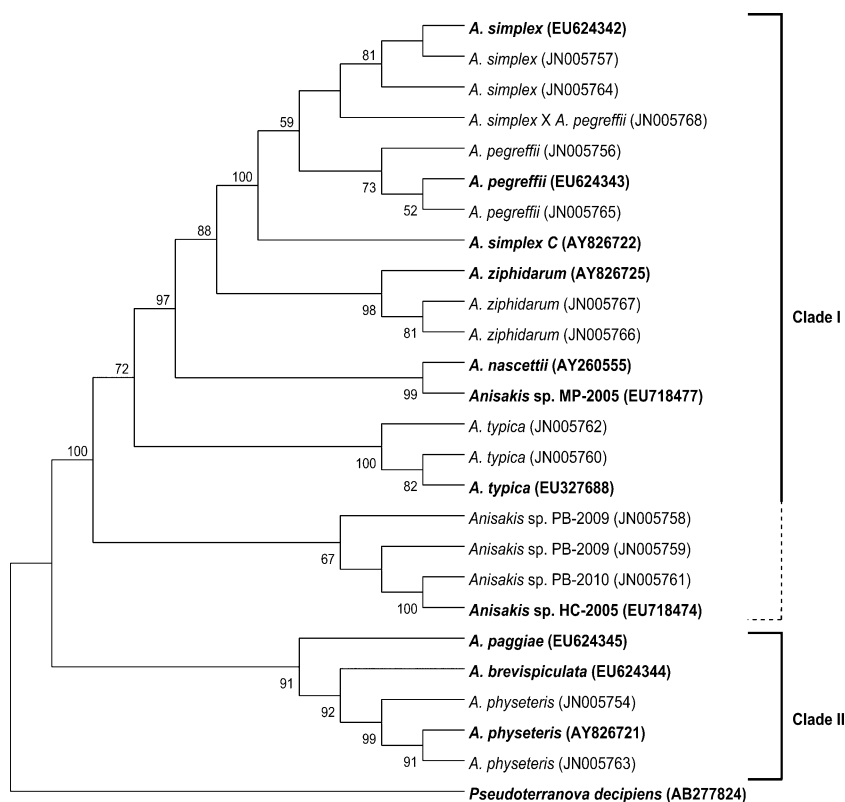


the species *A. pegreffii*, the distance ranged between 0.001 and 0.006. The two consistent differences that have been reported between *A. simplex* s.s. and *A. pegreffii* in positions 280 and 296 of the ITS-1 regions, T in *A. simplex* s.s. and C in *A. pegreffii* (Abollo et al. 2003; Ceballos-Mendiola et al. 2010; Du et al. 2010), were also detected in our sequences. However, a hybrid sequence (JN005768), which had a C at position 280 but a T at position 296 of the ITS-1 region, is reported here for the first time. The two *A. ziphidarum* sequences from Sagres have a 0.005 phylogenetic distance. *A. physeteris* sequences showed a maximum difference between two individuals of 0.008 base substitutions per site. In contrast, *A. typica* were only separated by a maximum of 0.002, whereas *Anisakis* sp. PB-2009 0.003.

Discussion

In this study, a wide diversity of anisakids was detected in *P. bogaraveo* from Portuguese waters. Previously, only *A. simplex* s.s., *A. pegreffii*, and *Hysterothylacium* spp. had been detected in this host (Oliveira Rodrigues et al. 1975; Costa et al. 2004; Mladineo 2006). Consequently, *A. simplex* s.s. × *A. pegreffii*, *A. ziphidarum*, *A. typica*, *Anisakis* sp. PB-2009, *Anisakis* sp. PB-2010, *A. physeteris*, and *Contraecum* sp. are reported for the first time in *P. bogaraveo*. This diversity could be related to the wide habitat range and feeding habits of this host, which feeds on numerous benthic, benthopelagic, and pelagic species, including smaller fish, crustaceans, molluscs, and numerous other

Fig. 3 Neighbour-joining phylogenetic tree of ITS1-5.8S-ITS2 sequences obtained in this study, reference GenBank sequences, and *P. decipiens* as the outgroup. Values beside nodes correspond to bootstrap percentages, and only those above 50% are represented



invertebrates (Morato et al. 2001), thus being exposed to various possible intermediate and paratenic hosts of anisakid nematodes.

Very high prevalences of *Anisakis* spp. infection were detected in mainland Portuguese waters, as reported by other authors (Cruz et al. 2009; Sequeira et al. 2010) and in agreement with findings from Galicia (Abollo et al. 2001; Mattiucci et al. 2004). Characteristic upwelling phenomena occur all along the western Portuguese coast promoting high primary production, feeding a wealth of organisms including the intermediate hosts of anisakid larvae. Anisakid abundance levels were particularly high in Peniche and lower in Sagres, which can be explained by the differences in fish size, since anisakid abundance was found to be positively correlated with fish length. This correlation has been detected in numerous fish species, e.g., *Trachurus trachurus* and *Micromesistius poutassou* (Cruz et al. 2005, 2007) and is rather common for parasite species that have long life spans in the host, since the fish tend to accumulate these parasites as they feed on infected prey.

Almost all of the identified anisakid larvae from mainland waters belonged to the *A. simplex* complex. This coincides with previous studies undertaken in this region (Abollo et al. 2003; Marques et al. 2006; Mattiucci et al. 2008; Sequeira et al. 2010), and it is related to the presence of several suitable definitive hosts, namely, several species of Delphinidae (Santos-Reis and Mathias 1996). However, whereas Mattiucci et al. (2008) found in *T. trachurus* an increasing occurrence of *A. pegreffii* from North to South, and the opposite tendency regarding *A. simplex* s.s., this was not observed in our study in which *A. pegreffii* had a higher relative abundance in all mainland samples.

Hybrid genotypes *A. simplex* s.s. × *A. pegreffii* were detected with relative abundances (9–15%), similar to those found by Abollo et al. (2003) off the mainland Portuguese coast and in the Cantabrian Sea, although lower than those found in both the Atlantic and Mediterranean southern coasts of Spain (Abollo et al. 2003; Martín-Sánchez et al. 2005). At the moment, it is not clear whether *A. simplex* s.s. and *A. pegreffii* are in the process of speciation, or if they have been geographically separated in the past, but have secondarily come into contact and, in the absence of barriers, behave as a single species when in sympatry, as suggested by Martín-Sánchez et al. (2005). Furthermore, the occurrence of hybrids in very distant geographical regions, such as China (Du et al. 2010), suggests that this may be a worldwide occurrence whenever *A. simplex* s.s. and *A. pegreffii* occur in sympatry.

Besides *A. simplex* s.l., only three other species were detected in mainland waters: *A. ziphidarum* (Peniche and Sagres), *A. physeteris*, and *Hysterothylacium* sp. (Sagres). *A. ziphidarum* had not been previously detected in the

Portuguese mainland waters. This species has beaked whales of the family Ziphiidae as definitive hosts (Mattiucci and Nascetti 2008), and its presence may be explained by the regular occurrence in these waters of Cuvier's beaked whale, *Ziphius cavirostris* (Santos-Reis and Mathias 1996). *A. physeteris* had previously been detected by Sequeira et al. (2010) in Peniche, also in very low numbers, most probably as a result of the rare occurrence of its definitive host *Physeter macrocephalus* (Mattiucci et al. 2001; Santos-Reis and Mathias 1996). *Hysterothylacium* sp. has often been detected in Portuguese waters, both in the mainland (Cruz et al. 2005, 2007) and in Madeira (Costa et al. 2004), where it was also found in the present study.

The diversity of anisakid nematodes isolated from Madeira fish was remarkable (eight species). Previously, both Pontes et al. (2005) and Sequeira et al. (2010) have detected high anisakid species richness (seven species) in fish from Madeira. Studies conducted at similar latitudes (Moroccan and Mauritanian Atlantic coasts) also reported high diversity of anisakid species (Farjallah et al. 2008; Kijewska et al. 2009). The region that includes Madeira, the Canary Islands, and the North African Atlantic coast is located at the confluence of different biogeographic areas, namely the North Atlantic, the Central Atlantic, and the Mediterranean, a factor which greatly contributes to an increased biodiversity. Numerous species of whales and dolphins occur in this region, including the definitive hosts for several anisakid species (Santos-Reis and Mathias 1996; Mattiucci and Nascetti 2008). Madeiran waters seem in fact to represent a region where the distribution of several anisakid species overlaps, as suggested by Pontes et al. (2005), including more typically northern species such as *A. simplex* s.s. and tropical/warmer temperate ones such as *A. typica* (Mattiucci and Nascetti 2008).

The Madeiran anisakid nematode community was dominated by an as yet-undescribed species, *Anisakis* sp. PB-2010. *Anisakis* sp. PB-2010 is most probably the same species as *Anisakis* sp. HC-2005 (Kijewska et al. 2009) and closely related to *Anisakis* sp. PB-2009. This would suggest the existence of several as yet undescribed species in this region, including *Anisakis* sp. B from *Aphanopus carbo* and *Scomber japonicus* (Pontes et al. 2005), *Anisakis* sp. HD, and *Anisakis* sp. type II HD (Sequeira et al. 2010) and *Anisakis* sp. in *Xiphias gladius* from Cape Verde (Garcia et al. 2011).

The Azores sample was markedly different from both Madeira and the mainland. Prevalence and abundance of infection were much lower than in all other samples, including the ones with similar and smaller fish length. Anisakid diversity was also much lower in the Azores than in Madeira, although many marine mammals also occur in this region (Santos-Reis and Mathias 1996). This may be related to different feeding habits of *P. bogaraveo* in this oceanic

region. Morato et al. (2001) reported that the main components of the blackspot seabream's diet in Azorean waters are pelagic invertebrates, mainly salps (Thaliaceae), which are not known to harbor any nematode parasites. The dominant species of anisakid in the Azores was *A. physeteris*, with a relative abundance significantly different from all other samples. This was also the dominant species in *Helicolenus dactylopterus* in this region (Sequeira et al. 2010), in sharp contrast to Madeira and the mainland. The occurrence of this parasite in large numbers in the Azores can be due to the presence of a large and stable population of its main definitive host, the sperm whale *P. macrocephalus* (Mattiucci et al. 2001; Pinela et al. 2009).

At the intraspecific level, we found a certain degree of variability, in accordance with findings by other authors (Ceballos-Mendiola et al. 2010; Mattiucci and Nascetti 2007; Palm et al. 2008). Differences were found among individual nematodes both from the same location, as well as from different locations. Indeed, Mattiucci and Nascetti (2008) refer that most of the genetic diversity in *Anisakis* spp. occurs within, rather than among, populations. High intraspecific variability is a feature that can be explained by the environmental heterogeneity experienced by anisakid nematodes with heteroxenous life cycles that involve both ectothermic and endothermic hosts (Mattiucci and Nascetti 2007). At the same time, high rates of gene flow promoted by the high dispersal capacity of their paratenic and especially, definitive hosts, maintain a worldwide homogeneity among populations (Mattiucci and Nascetti 2008).

Conclusions

In conclusion, the diversity of anisakid species in *P. bogaraveo* from Portuguese waters is high. Ten PCR-RFLP patterns were observed, four of which were novel. Seven anisakid taxa were detected in this host for the first time. The phylogenetic analysis (ITS region) revealed that our anisakids are distributed by the two distinct clades reported previously and that a group of organisms, including undescribed specimens from Madeira and a species temporarily described as *Anisakis* sp. HC-2005, cluster together and seem to belong to clade I.

The anisakid nematode communities were relatively similar in all locations in mainland waters but very distinct in both the Azores and Madeira islands, possibly due to differences in the local distribution of both intermediate and definitive hosts. These results suggest the existence of at least three different stocks of *P. bogaraveo* in the northeast Atlantic, but further studies are necessary to confirm this hypothesis.

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