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Molecular identification of *Anisakis* species from Pleuronectiformes off the Portuguese coast

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Abstract

Anisakid nematodes belonging to the *Anisakis simplex* complex are highly prevalent in several fish species off the coast of Portugal and are an important zoonotic problem in the Iberian Peninsula. Two reproductively isolated sibling species of the *Anisakis simplex* complex were identified from Pleuronectiformes inhabiting the Portuguese coast using restriction fragment length polymorphism (RFLP). Recombinant genotypes corresponding to presumptive *Anisakis simplex* sensu stricto and *Anisakis pegreffii* hybrids were also detected by this technique, as well as the species *Anisakis typica*. Although 25 species of Pleuronectiformes were investigated, *Anisakis* spp. larvae were only found in seven: *Arnoglossus imperialis, Arnoglossus laterna, Lepidorhombus boscii, Citharus linguatula, Platichthys flesus, Dicologlossa cuneata* and *Solea senegalensis*. The occurrence of hybrids in relatively sedentary fishes such as the Pleuronectiformes suggests that the Portuguese coast may constitute an area of hybridization and, therefore, is of particular interest for the study of the process of hybridization and speciation for these anisakids.

Introduction

Recent studies have shown that sibling species (morphologically similar but genetically and ecologically differentiated taxa) are common among endoparasitic helminths. The nematode *Anisakis simplex*, the causal agent of human anisakiasis, has been shown to comprise at least three reproductively isolated sibling species, differing in their genetic structure, life history and geographic distribution – *Anisakis simplex* sensu stricto, *Anisakis pegreffii* and *Anisakis simplex* C (Nascetti *et al.*, 1983, 1986; Mattiucci *et al.*, 1997; D'Amelio *et al.*, 2000). In the Iberian Peninsula, this complex is highly prevalent and has been considered an important zoonotic problem (Abollo *et al.*, 2001) with several cases of human anisakidosis being described (Rosales *et al.*, 1999; de Corres *et al.*, 2001) and more than 150 cases of allergy to this parasite being reported since 1995 (Audicana *et al.*, 2002). *Anisakis simplex* sensu stricto is mainly distributed in the Northern Atlantic and Pacific Oceans, *A. pegreffii* in the Mediterranean Sea and in the Southern hemisphere and *A. simplex* C in the Pacific coast of Canada and in the Southern hemisphere (Mattiucci *et al.*, 1997). Although quite rare in anisakid sibling species, hybrid specimens between *A. pegreffii* and *A. simplex* sensu stricto have been detected by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) (Abollo *et al.*, 2003) and allozyme markers (Mattiucci *et al.*, 2004) and seem to occur more frequently in the areas close to the Gibraltar strait, where the geographic distributions of the two species of parasites overlap. However, no adult hybrids were found in the definitive hosts (*Delphinus delphis*) from the same sympatric area (Mattiucci *et al.*, 2004), thus suggesting a lower hybrid fitness.

The Portuguese coast constitutes a transition area between two zoogeographical areas – the warm-temperate

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and the cool-temperate Atlantic – and is, for a large number of species, including the Pleuronectiformes, the northern or southern limit of their distribution. Along this geographical area 25 species of Pleuronectiformes, belonging to five different families differing in their life history, habitat and diet occur in sympatry. This is expected to be reflected in the parasites infecting fish species as, according to the island biogeographical theory (MacArthur & Wilson, 1967; Kuris *et al.*, 1980), areas of sympatry promote high rates of parasite speciation or colonization by new parasite species and are associated with high parasite diversity. The Portuguese coast has, therefore, a high potential for the study of population interactions and hybrids of *Anisakis* are likely to occur.

Molecular techniques such as multilocus enzyme electrophoretic analysis and restriction fragment length polymorphism (RFLP) are extremely powerful tools for routine identification of *Anisakis* larvae and have proved to be particularly useful when different *Anisakis* species occur sympatrically (e.g. Mattiucci *et al.*, 2002, 2004; Abollo *et al.*, 2003). Using PCR–RFLP analysis of the ribosomal DNA (rDNA) internal transcribed spacers (ITS1 and ITS2), D'Amelio *et al.* (2000) established a molecular key to identify the three sibling species of the *A. simplex* complex, as well as *Anisakis physeteris*, *Anisakis typica*, *Anisakis ziphidarum* and *Anisakis schupakovi*. Using the same technique, Abollo *et al.* (2003) identified, from the Iberian coast, a recombinant genotype corresponding to the presumptive hybrids of *A. simplex* sensu stricto and *A. pegreffii* as this produces a restriction pattern consistent with heterozygote genotypes between alleles diagnostic for the two species.

The aims of the present study were to identify the *Anisakis* spp. larvae infecting the Pleuronectiformes off the Portuguese coast using RFLP genetic analysis and the molecular key devised by D'Amelio *et al.* (2000) and to detect the presence of hybrids and the extension of the hybrid zone.

Materials and methods

The 25 different species of Pleuronectiformes occurring along the Portuguese coast were collected seasonally from January 2003 to December 2004 and investigated for macroparasite infections according to general procedures. Anisakid larvae were found encysted in the mesenteries surrounding the fish gut in only seven species of Pleuronectiformes – *Arnoglossus imperialis, Arnoglossus laterna, Lepidorhombus boscii, Citharus linguatula, Platichthys flesus, Dicologlossa cuneata* and *Solea senegalensis* (Table 1), obtained in five sampling areas along the Portuguese coast (fig. 1). After being removed from the cyst, they were identified as the third larval stage (L3) of *Anisakis* type I (*sensu* Berland, 1961), by the presence of a tooth and a mucron and the absence of both a ventricular appendix and an intestinal caecum, and placed in 70% ethanol to be further subjected to genetic analysis.

DNA was extracted using the Wizard® Genomic DNA purification kit (Promega), following the manufacturers' instructions. Each specimen was placed in 600 μl of a mixture containing EDTA 0.5 M plus nuclei lysis solution and the tissue was crushed by a pestle. Following the addition of 17.5 μl of proteinase K (20 mg ml^{-1}) to each tube, these were incubated for 3 h at 55°C and, after the addition of 3 μl of RNase solution (4 mg ml^{-1}) the tubes were incubated for another 30 min at 37°C. After this period, 200 μl of protein precipitation solution were added, the tubes vortexed vigorously and chilled on ice for 5 min before a series of centrifugations and alcohol precipitations to obtain the DNA pellet. This was air-dried for 20 min, 100 μl of DNA rehydratation solution were added and the DNA incubated overnight at 4°C.

Two conserved primers – NC5 (forward), GTAGGTGAACCTGCGGAAGGATCATT and NC2 (reverse), TTAGTTTCTTCCTCCGCT (Zhu *et al.*, 2000) were used in polymerase chain reaction (PCR) to amplify the rDNA region corresponding to the entire ITS (internal transcribed spacers) – ITS1, 5.8S and ITS2 (1 kb, approximately). PCR amplifications were performed using 0.25 μl of AmpliTaq Gold™ (Promega), 2.5 μl of 10 × PCR buffer II (Promega), 2.5 μl of $MgCl_2$ (Promega), 2 μl of dNTPs (dCTP, dGTP, dATP, dTTP) (Promega), 0.25 μl of each primer and 2 μl of template DNA in a 25 μl final volume of reaction. The PCR was performed in a GeneAmp PCR System 2400 (Applied Biosystems) and the conditions were as follows: 10 min at 95°C, 30 cycles of 30 s at 95°C,

Table 1. Prevalence (%) of *Anisakis* sp. third-stage larvae (L3) in seven species of Pleuronectiformes along the Portuguese coast and species detected by restriction fragment length polymorphism analysis.

Host	Area	Prevalence (%)	*Anisakis* species
Arnoglossus laterna ($n = 78$)	Figueira-da-Foz	13.0	*A. simplex* sensu stricto
Arnoglossus imperialis ($n = 98$)	Figueira-da-Foz	12.2	*A. pegreffii*, hybrid
Lepidorhombus boscii ($n = 361$)	Aveiro ($n = 120$)	15.8	*A. pegreffii*, hybrid
	Figueira-da-Foz ($n = 41$)	4.9	*A. pegreffii*, hybrid
	Setúbal ($n = 200$)	17.5	*A. pegreffii*
Citharus linguatula ($n = 485$)	Aveiro ($n = 78$)	52.5	*A. simplex* s.s., *A. pegreffii*, hybrid
	Figueira-da-Foz ($n = 86$)	61.6	*A. simplex* s.s., *A. pegreffii*, hybrid
	Peniche ($n = 41$)	85.3	*A. pegreffii*
	Setúbal ($n = 120$)	48.3	*A. pegreffii*
Platichthys flesus ($n = 34$)	Setúbal	3.0	*A. typica*
Dicologlossa cuneata ($n = 120$)	Matosinhos	4.0	*A. pegreffii*
Solea senegalensis ($n = 80$)	Figueira-da-Foz	1.3	*A. simplex* s.s.

n, number of hosts examined.

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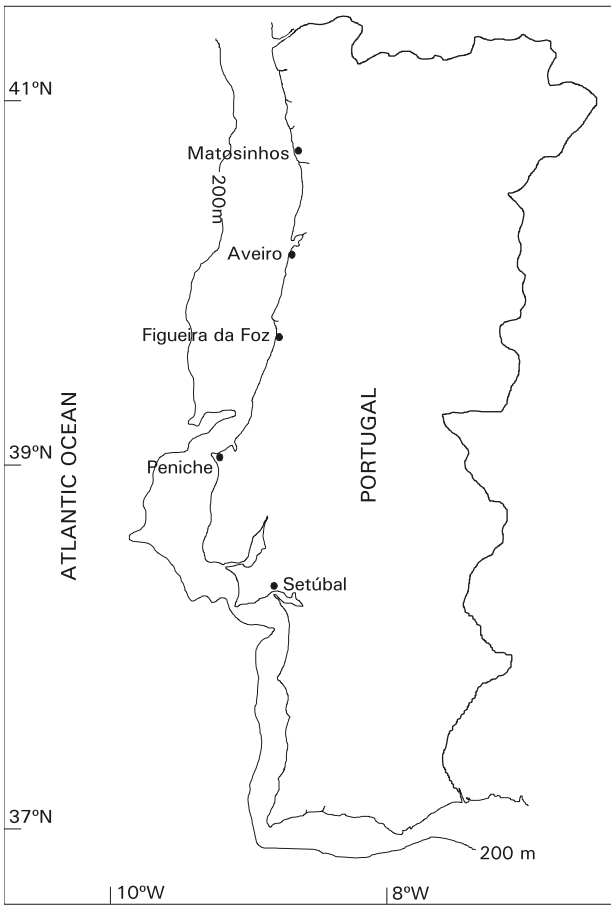

Fig. 1. Location of the sampling areas along the Portuguese coast.

30 s at 52°C and 75 s at 72°C, and a final elongation step of 7 min at 72°C. A negative control, not containing template DNA, was included in the amplifications in order to detect any contamination.

Following PCR, samples were subjected to RFLP analysis using two enzymes, *Hha* I and *Hin*f I whose restriction pattern, according to D'Amelio *et al.* (2000), allows the molecular identification of the *Anisakis simplex* complex species. After digestion, fragments were separated by electrophoresis in a 1.6% agarose gel stained with ethidium bromide (10 mg ml^{-1}) and visualized under UV light.

Whereas *Hha* I produces two fragments of approximately 550 bp and 430 bp in *Anisakis simplex* sensu stricto and *Anisakis pegreffii*, *Hin*f I produces different size fragments for each of these species – three fragments of approximately 620 bp, 250 bp and one shorter than 100 bp in *A. simplex* sensu stricto and three fragments of approximately 370 bp, 300 bp and 250 bp in *A. pegreffii*. According to Abollo *et al.* (2003), a pattern consisting of four fragments of approximately 620 bp, 370 bp, 300 bp and 250 bp corresponds to a recombinant genotype of *A. simplex* sensu stricto and *A. pegreffii*. These two restriction enzymes also allow the identification of *Anisakis typica* by producing four fragments of approximately 320, 240, 180 and 160 bp and two fragments of approximately 620 bp and 350 bp, respectively.

Results

No seasonality was detected in L3 infections of *Anisakis* spp. along the Portuguese coast and, therefore, data from several seasons were pooled for further analyses. The prevalence of L3 infections of *Anisakis* spp. was found to be extremely high in *Citharus linguatula*, exceeding 50% in most locations, whereas in most species it does not infect more than 18% of the fish (table 1).

The restriction patterns produced by *Hha* I and *Hin*f I allowed the identification of the *Anisakis* type I larvae present in the different hosts within each area (fig. 2). Different species of hosts inhabiting the same geographical area showed infections with different parasite species, such as *Arnoglossus imperialis* and *A. laterna*, and different *Anisakis* species were present in the same species of host, as was the case of *Lepidorhombus boscii* and *C. linguatula* from Figueira da Foz (table 1). The recombinant genotype of *Anisakis simplex* sensu stricto and *Anisakis pegreffii* was identified in *A. imperialis*, *L. boscii* and *C. linguatula* and the RFLP pattern corresponding to this recombinant genotype was frequently found (~1:3).

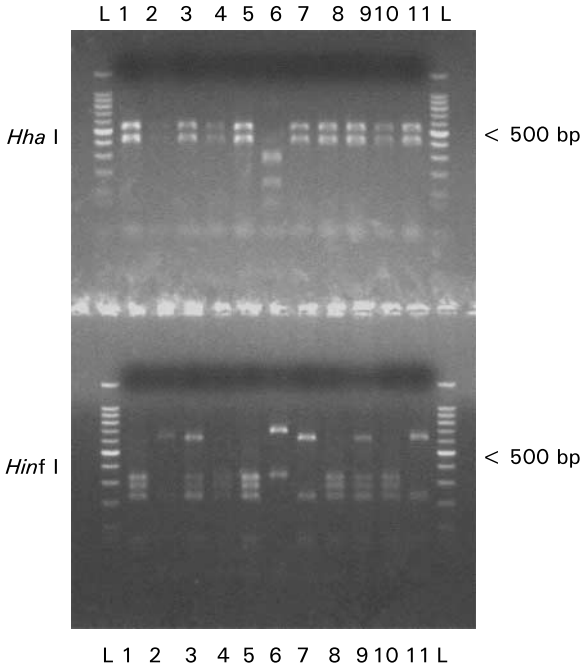

Fig. 2. Restriction fragment length polymorphism pattern produced by *Hha* I (top) and *Hin*f I (bottom). 1, *Anisakis pegreffii* from *Arnoglossus imperialis*; 2, *Anisakis simplex* sensu stricto from *Arnoglossus laterna*; 3, recombinant genotype from *Lepidorhombus boscii*; 4, *A. pegreffii* from *L. boscii*; 5, *A. pegreffii* from *Dicologlossa cuneata*; 6 *Anisakis typica* from *Platichthys flesus;* 7, *A. simplex* s. s. from *Citharus linguatula;* 8, *A. pegreffii* from *C. linguatula*; 9, recombinant genotype from *C. linguatula*; 10, *A. pegreffii* from *C. linguatula*; 11, *A. simplex* s. s. from *Solea senegalensis*; L, 100 bp ladder.

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A species not belonging to the *Anisakis simplex* complex was also found in *Plathichthys flesus* from Setúbal. This was identified as *Anisakis typica* according to its restriction pattern.

Discussion

By providing a useful approach for the specific identification of both distantly and closely related ascaridoid species (Zhu *et al.*, 1999), the PCR–RFLP technique showed that Pleuronectiformes from the Portuguese coast are infected with at least four different taxa of *Anisakis*, those from the *A. simplex* complex being the most prevalent. Some host preferences can be depicted from the present data as these species were found mostly in species of Pleuronectiformes from the families Bothidae (*Arnoglossus laterna, Arnoglossus imperialis*), Scophthalmidae (*Lepidorhombus boscii*) and Citharidae (*Citharus linguatula*), being highly prevalent (48–85%) in *C. linguatula*. This result might be explained by the similar diet of these species, which is mainly composed of crustaceans (personal observation), but may also be a reflection of the phylogenetic relationship between these three families. Although belonging to different clades (Berendzen & Dimmick, 2002), these are the most closely related families of Pleuronectiformes among those considered in the present study.

The presence of *A. simplex* sensu stricto–*A. pegreffii* hybrids on the Portuguese coast has been previously reported by Abollo *et al.* (2003), who found recombinant genotypes in larvae collected from *Micromesistius poutassou* in Peniche. This is, however, a mesopelagic species with seasonal and spawning migrations (Svetovidov, 1984) and, therefore, hybrid infection could have been acquired away from the Portuguese coast. The results obtained in the present study showed that hybrids are common whenever *A. simplex* sensu stricto and *A. pegreffii* occur in sympatry, even in areas hundreds of kilometres away from the Gibraltar strait, where the recombinant genotype was suggested to be more prevalent by Abollo *et al.* (2003). This, associated with the fact that Pleuronectiformes do not migrate great distances, suggests a well established and extensive area of hybridization along the Portuguese coast.

Although, according to Mattiucci *et al.* (2002), the range of *Anisakis typica* extends from 30°S to 35°N in warmer temperate and tropical waters, anisakids found in *Platichthys flesus* inhabiting the central area of Portugal (38°N) were identified as *A. typica.* Moreover, Mattiucci *et al.* (2004) found *A. typica* parasitizing hake off the Mediterranean Sea and the Atlantic coast of Morocco but none were found in the European Atlantic. The present study represents, therefore, an extension of the parasite's geographic range and might be related to the zoogeographic importance of the Portuguese coast which represents the transition between North-eastern Atlantic warm-temperate and cold-temperate regions (Ekman, 1953; Briggs, 1974), being an area of species overlap and thereby promoting host switching.

The infection by different species of *Anisakis* reported here for the Pleuronectiformes might be due to the occurrence in sympatry along the Portuguese waters of several species of cetaceans, such as dolphins and whales, which are definitive hosts for *A. simplex* sensu stricto and *A. pegreffii*. As most examined individuals were infected by a single *Anisakis* sp. larva, no data concerning the existence of multiple species infecting a single host species could be found.

The high prevalence of *Anisakis simplex* sensu lato infection is a matter of concern with regard to public health as anisakiasis is one of the most severe fish-transmitted infections and results from accidental ingestion of third-stage larvae belonging to the family Anisakidae, producing both gastric and intestinal disease (Rosales *et al.*, 1999). To date, global cases of human parasitism caused by *A. simplex* sensu lato constitute approximately 97% of all anisakidosis reported. Although there are difficulties in diagnosis, it has been demonstrated that the L3 of *Anisakis* sp. can pass through the fourth stage in humans, parasitizing the gastrointestinal tract (Rosales *et al.*, 1999; de Corres *et al.*, 2001). This is a common disease in Japan, where thousands of cases of anisakidosis are registered, but is also becoming a concern in Europe, especially in countries with high rates of fish consumption such as Portugal which has one of the highest rates of fish consumption in Europe (56.5 kg *per capita* y^{-1}) (Eurostat, 2001). Although no cases have been reported, which might be due to the habit of consuming fish thoroughly cooked, parasitism by *Anisakis* sp. has been considered for alert notifications in the Rapid Alert System for Food and Feed by the European Food Safety Authority.

The present study showed that the Portuguese coast is an area of high prevalence of *Anisakis simplex* complex parasites, at least in some Pleuronectiforme species, constituting a possible hybridization area for *A. simplex* sensu stricto and *A. pegreffii*. This is therefore an area of particular interest for the study of hybridization and speciation for these anisakids. Species of Pleuronectiformes occurring along the Portuguese coast, by being phylogenetically related but ecologically highly differentiated, might constitute an excellent group of hosts for the study of the hybridization of anisakid species as well as of their interactions. As these parasites are the causal agent of allergic reactions that may include anaphylactic shock (de Corres *et al.*, 2001), more studies concerning the infection dynamics of these *Anisakis* species should be undertaken in order to evaluate the potential of existence of human anisakiasis in Portugal due to the high rate of fish consumption.

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