

Occurrence and molecular identification of *Anisakis* spp. from the North African coasts of Mediterranean Sea

Sarra Farjallah · Badreddine Ben Slimane ·
Marina Busi · Lia Paggi · Nabil Amor · Hager Blel ·
Khaled Said · Stefano D'Amelio

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Abstract Larval forms of the genus *Anisakis* were reported infecting several fish species from the North African coasts of central Mediterranean Sea. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis was used to investigate the occurrence of larval forms of different *Anisakis* species in teleost fishes and squid from North African coasts of the Mediterranean Sea and to establish the geographical and host range of these parasites in this area. A total of 282 *Anisakis* larvae were identified by PCR-RFLP from 13 teleost fish species and one cephalopod species captured at different sites off the Algerian, Tunisian and Libyan coasts. The type I larvae were found with a frequency of 93.62% and were identified as belonging to the following species: *Anisakis simplex* s. str., *Anisakis pegreffii*, *A. simplex* s.str./*A. pegreffii* hybrids and *Anisakis typica*. The type II larvae were found to belong to *Anisakis physeteris*, with the frequency of 6.38%. The record of *A. simplex* s.str./*A. pegreffii* hybrids, previously recorded from the Spanish and Portuguese Atlantic coasts and the Alboran Sea, extends their geographic distribution to the Tunisian coasts. The occurrence of *A. simplex* s.str. and hybrids away from their known area of distribution may predict the successful use of *Anisakis* larvae for tagging *Scomber scombrus* fish stocks for

fisheries management purposes. Moreover, the results reported provide valuable information regarding the diversity of *Anisakis* species in the study area, indicating that several *Anisakis* sibling and morphospecies coexist in the North African coasts of the Mediterranean Sea.

Introduction

The life cycles of Anisakid nematodes involve an important and diverse array of marine hosts, with a certain degree of specificity in their geographic distribution and their host preference in major areas of the marine ecosystem (Mattiucci et al. 1997). Larvae of these nematodes are, also, a major problem for commercial fishing industries (Abollo et al. 2001) and are of potential human health hazard, both as potential causative agents of anisakiasis and as food-borne allergens (Daschner and Pascual 2005). By using multilocus enzyme electrophoresis, the analysis of the genetic structure of both adults and larvae of *Anisakis* species has provided interesting results at the taxonomic level (Mattiucci et al. 1986, 1997; Nascetti et al. 1986). At present, it has been shown that *Anisakis* larvae type I (sensu Berland, 1961) comprises the larval stages of six distinct species, namely, *Anisakis simplex sensu stricto*, *Anisakis pegreffii*, *A. simplex* C, *Anisakis typica*, *Anisakis ziphidarum* and *Anisakis* sp. A, whilst in *Anisakis* type II (sensu Berland, 1961), three species are so far included, *Anisakis physeteris*, *Anisakis brevispiculata* and *Anisakis paggiae* (Mattiucci et al. 1998, 2001, 2002, 2005; Paggi et al. 1998; Pontes et al. 2005), which have different genetic structures, life cycles and geographical distributions (Martin-Sanchez et al. 2005). The genetic differentiation among the *Anisakis* species was also detected in the region spanning the ITS1, the 5.8S and the ITS2 of the nuclear ribosomal DNA,

S. Farjallah (✉) · B. B. Slimane · N. Amor · H. Blel · K. Said
Unité de Recherche: Génétique, Biodiversité et Valorisation des
Bioressources UR/09-30,
Institut Supérieur de Biotechnologie de Monastir,
Monastir 5000, Tunisia
e-mail: sarra_farj@yahoo.fr

M. Busi · L. Paggi · S. D'Amelio
Parasitology Section, Department of Sciences of Public Health,
University of Rome,
“La Sapienza”, P.le Aldo Moro 5, Box 6 Roma 62,
00185 Rome, Italy

providing useful markers based on polymerase chain reaction–restriction fragment length polymorphisms (PCR-RFLPs) for their identification (D’Amelio et al. 2000; Abollo et al. 2003; Pontes et al. 2005; Marques et al. 2006). Recently, the PCR-RFLP and sequence analyses of nuclear ribosomal DNA spacers have proven the existence of hybrid specimens between *A. pegreffii* and *A. simplex* s.str. as results of an interspecific hybridization between the two species of the *A. simplex* complex (Abollo et al. 2003). The genus *Anisakis* was previously reported infecting some fish species from the North African coasts of central Mediterranean Sea (Farjallah et al. 2006), although there are no studies that document distribution of the *Anisakis* larvae at species level in this area. The aim of the present work was to investigate, using RFLP genetic analysis and the molecular keys devised by D’Amelio et al. (2000) implemented by Pontes et al. (2005), the occurrence of larval forms of *Anisakis* spp. in teleost fishes and squid caught in several localities of Algerian, Tunisian and Libyan coasts and to establish the geographical and host range of these parasites in the area.

Materials and methods

Hosts and parasites

A total of 282 larvae of anisakid nematodes were collected from 13 teleost fish and one cephalopod species captured at different sites off the Algerian, Tunisian and Libyan coasts (North African central Mediterranean coasts) from October 2006 to January 2007 (Table 1, Fig. 1). The nematodes were repeatedly washed in 0.9% saline solution, fixed in 75% ethanol and morphologically identified at genus level by light microscopy.

DNA extraction and PCR amplification

Total DNA was extracted using the DNA purification kit wizard genomic (Promega) according to the manufacturer’s instructions. DNA was eluted in 100 µl of elution buffer (10 mM Tris, 1 mM EDTA) and kept at –20°C until use. PCR was conducted using 5 µl of the extracted DNA as a

Table 1 Collecting data and taxa of *Anisakis* identified by PCR-RFLP from the sampling localities

Locality	Host species	N_h	N_p	<i>Anisakis simplex</i> s.str.	<i>Anisakis</i> <i>pegreffii</i>	<i>Hybrid</i> <i>genotype</i>	<i>Anisakis</i> <i>physeteris</i>	<i>Anisakis</i> <i>typica</i>
Tunisia								
Tabarka	<i>Merluccius merluccius</i>	10	15		9		6	
Bizerte	<i>Scomber scombrus</i>	20	27	2	20	1	3	1
	<i>Merluccius merluccius</i>	11	14		10		2	2
Tunis	<i>Scomber scombrus</i>	8	22	5	16	1		
Nabeul	<i>Trichiurus lepturus</i>	1	9		9			
Kelibia	<i>Scomber japonicus</i>	13	5		5			
Sousse	<i>Lophius piscatorius</i>	2	9		9			
	<i>Micromesistius poutassou</i>	12	11		11			
Monastir	<i>Todarodes sagittatus</i>	17	13		13			
	<i>Muraena helena</i>	22	14		14			
	<i>Scomber scombrus</i>	31	9	2	7			
	<i>Phycis phycis</i>	15	9		3		2	4
	<i>Phycis blennoides</i>	23	8		4		4	
Mahdia	<i>Thunnus thynnus</i>	8	2		2			
	<i>Echiichthys vipera</i>	2	3		3			
	<i>Trachinus draco</i>	1	2		2			
	<i>Xiphias gladius</i>	3	2		2			
Sfax	<i>Scomber scombrus</i>	10	13	2	11			
	<i>Merluccius merluccius</i>	7	10		10			
Gabes	<i>Scomber scombrus</i>	10	16	1	14			1
Ras Ejdair	<i>Merluccius merluccius</i>	13	14		13			1
Algeria								
El Marsa	<i>Merluccius merluccius</i>	15	7		7			
Cap Rosa	<i>Merluccius merluccius</i>	15	7	1	5		1	
Libye								
Tripoli	<i>Merluccius merluccius</i>	16	41		24			17
Total		285	282	13	223	2	18	26

N_h Number of host examined, N_p number of parasites tested

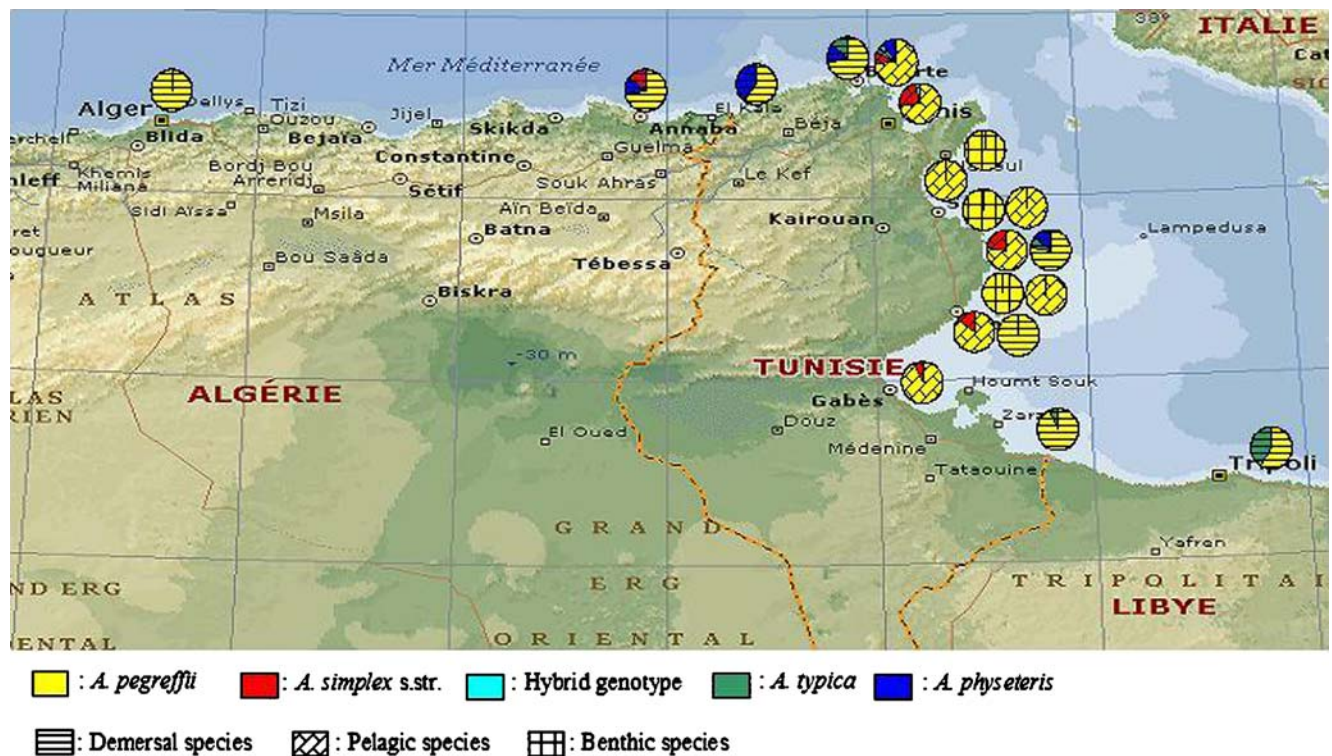


Fig. 1 Distribution patterns of *Anisakis* species larvae, identified by rDNA genetic markers in different localities, represented by the different habitats of the fishes parasitised

template (20–40 ng) and 50 µl reaction mixture containing 2.5 U AmpliTaq Gold (Applied Biosystems), 10 mM Tris-HCl (pH=8.3), 50 mM KCl (Applied Biosystems), 3 mM MgCl₂ (Promega), 1 mM of dNTPs (dCTP, dGTP, dATP, dTTP; Promega) and 0.05 µM of each primer. The entire ITS (ITS1, 5.8S rDNA gene and ITS2) was amplified using the primers NC5 (forward: 5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (reverse: 5'-TTAGTTTCTTCCTCCGCT-3'; Zhu et al. 1998). The PCR was performed in a GeneAmp PCR System 2400 (Applied Biosystems), and the conditions were as follows: 10 min at 95°C, then 30 cycles of 30 s at 95°C, 30 s at 55°C and 75 s at 72°C followed by a final elongation of 7 min at 72°C. A negative control (no DNA) was included in all PCR amplifications. Reactions were carried out using a Perkin-Elmer Gene Amp PCR System 2400. Five millilitres of the amplification products were visualised on 1% ethidium-bromide-stained agarose gels to check the quality of amplification.

Digestion of PCR products with restriction enzyme

The restriction enzymes, *Hinf*I and *Hha*I, were used in RFLP analysis of rDNA for the identification of the members of *Anisakis* genus according to the genetic markers defined by D'Amelio et al. (2000) implemented by Pontes et al. (2005). Restrictions were performed using 10 µl of PCR products, 3 µl of distilled water, 0.5 µl of restriction enzyme, 1.5 µl of a specific buffer and 0.2 µl bovine serum albumin up to a

final volume of 15.2 µl. The digestion was performed for 90 min at 37°C, and the digestion products were evaluated on 2% ethidium-bromide-stained agarose gels.

DNA sequencing and analysis

The PCR products of ribosomal DNA were purified using the commercial kit NucleoSpin Extract (Macherey-Nagel) according to the manufacturer's instructions. Sequencing was performed by MWG-BIOTECH AG (Ebersberg, Germany). Sequences were aligned with previously characterised sequences of *Anisakis* spp. using ClustalW (Thompson et al. 1994) and adjusted manually. The electropherograms were analysed using Chromas 2.13.

Results

PCR-RFLP and sequence analysis of ribosomal DNA

Two hundred eighty-two anisakid larvae from the 13 different fishes and one squid species were identified by PCR-RFLP on the basis of the diagnostic restriction banding patterns (D'Amelio et al. 2000; Pontes et al. 2005). The amplification of the rDNA region (spanning the ITS-1, ITS-2 and the 5.8S subunit) produced a fragment of approximately 1,000 bp. Digestion of the amplified ITS of

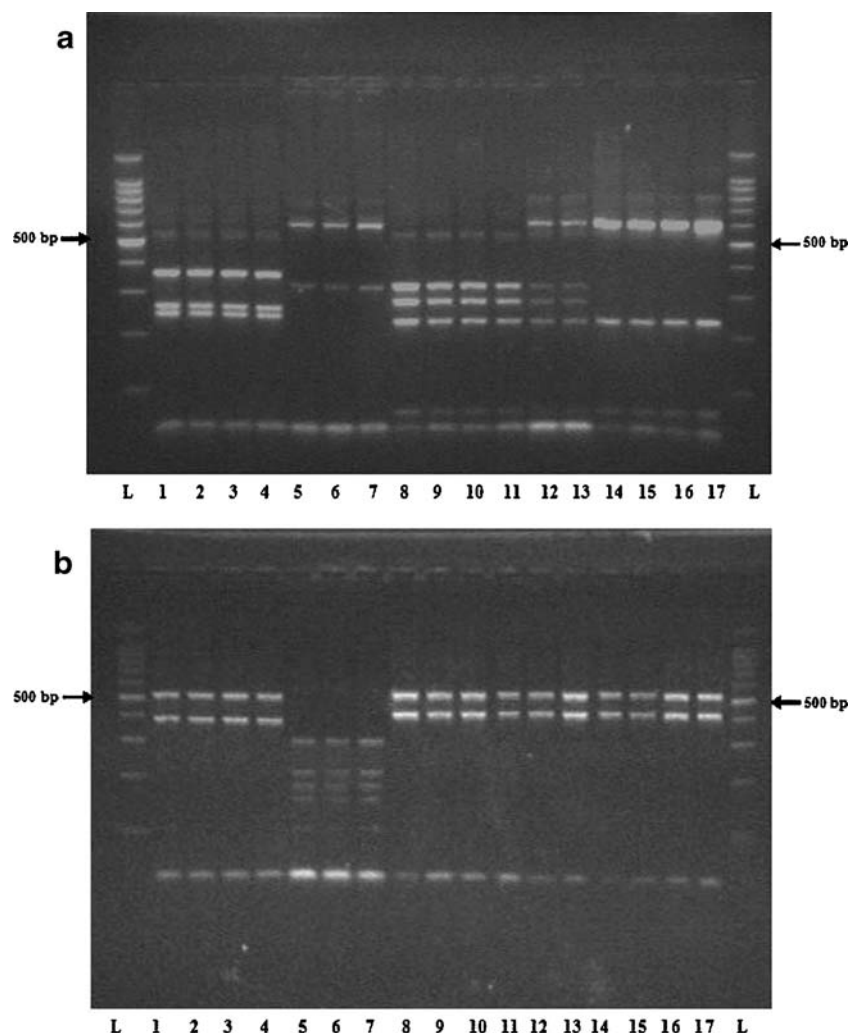
larval nematodes with *Hinf*I produced three different patterns corresponding to two sibling species of the *A. simplex* complex (*A. simplex* s.str. and *A. pegreffii*) and a hybrid genotype between the two species, whilst digestion with *Hha*I produced the same pattern for all of them (Fig. 2). Two additional *Anisakis* species that are not members of the *A. simplex* complex were also identified using the above enzymes, *A. physeteris* and *A. typica* (Fig. 2). Digestion with *Hinf*I produced two strong bands and a weak band (620, 250 and 80 bp, respectively) in *A. simplex* s.str., whilst *A. pegreffii* showed three bands of 370, 300 and 250 bp. Furthermore, the hybrid genotype showed four fragments corresponding to all the bands observed in *A. simplex* s.str. and in *A. pegreffii* (Fig. 2a). On the other hand, digestion with *Hha*I produced two fragments of 550 and 430 bp in *A. simplex* sensu lato and recombinant genotypes (Fig. 2b). Using *Hinf*I, *A. typica* exhibited two fragments (610 and 350 bp), whilst *A. physeteris* exhibited three bands (380–290–270 bp). The restriction with *Hha*I produced four fragments in

A. typica (bands of 320–240–180–160 bp) and two fragments in *A. physeteris* (bands of 540–420 bp; Fig. 2). The ITS region of homozygote and heterozygote larval individuals, belonging to *A. simplex* complex, was further studied by DNA sequencing. The sequence analyses revealed the presence of two polymorphic sites found at positions 244 and 260, corresponding to the sequence differences between *A. simplex* s.str. and *A. pegreffii*, at heterozygote status in some specimens of parasites, as reported previously by Abollo et al. (2003; Fig. 3).

Geographical distribution and host range of *Anisakis* spp. in the sampling area

The type I larvae were all identified as belonging to *A. simplex* s.str., *A. pegreffii*, *A. typica* and the recombinant genotype, whereas the type II larvae were found to belong to *A. physeteris*, with the frequency of 93.62 and 6.38%, respectively (Table 1). Seventy-nine percent of the larval forms examined were identified as *A. pegreffii*, 9.21% as

Fig. 2 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 (a), *Hha*I (b) shown by the species of the genus *Anisakis*. Lanes: 1–4 *A. physeteris*; 5–7 *A. typica*; 8–11 *A. pegreffii*; 12–13 recombinant genotype; 14–17 *A. simplex* s.str.; L 100-bp DNA ladder



<i>A. pegreffii</i>	ACC TTC CAT TTG CAT GTT GTT GTG AGC CAC ATG GAA ACT CGT ACA CAC --- --G TGG TGG [60]
Hybrid	 [60]
<i>A. simplex</i> s.str.	 [60]
Hybrid	 [60]
<i>A. pegreffii</i>	 [60]
AY826723	 [60]
AY826720	 [60]
AY826721	... G... ..G... ..A... ..T... [60]
AY826724	... GC... ..G... ..C... ..GT TTG TG...A [60]
AY821749	 [60]
AY821740	 [60]
AY826722	 [60]
<i>A. pegreffii</i>	CAG CCG TCT GCT GTG CTT TTT TTA GGC AGA CAA TGG CTT ACG AGT GGC C-G TGT -GC TTG [120]
Hybrid	 [120]
<i>A. simplex</i> s.str.	 [120]
Hybrid	 [120]
<i>A. pegreffii</i>	 [120]
AY826723	 [120]
AY826720	 [120]
AY826721	A... -G T... ..T... ..C... [120]
AY826724	T... ..G... ..CG ..G... ..T... ..C... [120]
AY821749	 [120]
AY821740	 [120]
AY826722	 [120]
<i>A. pegreffii</i>	TTG AAC AAC GGT GAC CAA TTT GGC -GT CTA CGC CGT ATC TAG CTT CTG CCT GGA CCG TCA [180]
Hybrid	 [180]
<i>A. simplex</i> s.str.	 [180]
Hybrid	 [180]
<i>A. pegreffii</i>	 [180]
AY826723	 [180]
AY826720	 [180]
AY826721	 [180]
AY826724	T... ..C... ..G... [180]
AY821749	 [180]
AY821740	 [180]
AY826722	 [180]
<i>A. pegreffii</i>	GTT GCG ATG AAA GAT GCG GAG AAA GTT CCT TTG TTT TGG CTG CTA ATC ATC ATT GAT GAG [240]
Hybrid	 [240]
<i>A. simplex</i> s.str.	 [240]
Hybrid	 [240]
<i>A. pegreffii</i>	 [240]
AY826723	 [240]
AY826720	 [240]
AY826721	..A... ..C... ..A... ..CG...G... [240]
AY826724	..A... ..G... ..C-T... [240]
AY821749	 [240]
AY821740	 [240]
AY826722	 [240]
<i>A. pegreffii</i>	CAG CAG CTT AAG GCA GAG TCG AGC AGA CTT AAT GAG CCA CGC T-- AGG TGG CCG CCA AAA [300]
Hybrid	... Y... ..Y... .. [300]
<i>A. simplex</i> s.str.	... T... ..T... .. [300]
Hybrid	... Y... ..Y... .. [300]
<i>A. pegreffii</i>	 [300]
AY826723	... T... ..T... .. [300]
AY826720	 [300]
AY826721	... --C... ..G... ..T... ..CT...G... [300]
AY826724	... --G... ..T... .. [300]
AY821749	... T... ..T... .. [300]
AY821740	 [300]
AY826722	T... .. [300]
<i>A. pegreffii</i>	CCC AAA ACA CAA CCG GTC TAT TTG ACA TTG TTA TTT CAT TGT ATG TGT TGA A-- AAT GTA [360]
Hybrid	 [360]
<i>A. simplex</i> s.str.	 [360]
Hybrid	 [360]
<i>A. pegreffii</i>	 [360]
AY826723	 [360]
AY826720	 [360]
AY826721	A... ..C AG. ATG C... TGA CA...AC GTT... [360]
AY826724	C.A... TTG ..CA... ..GA... TGA ..A... T-- T... [360]
AY821749	 [360]
AY821740	 [360]
AY826722	 [360]

Fig. 3 Alignment of ITS-1/5.8S/ITS-2 sequences of *Anisakis* taxa. One representative of each unique sequence was included for comparison. Dots indicate identity with the first sequence, and dashes are inferred insertion-deletion events; standard IUB ambiguity codes are used, i.e. Y=C/T, R=A/G. The consensus ITS-1/5.8S/

ITS-2 sequences for the *A. pegreffii* (Accession number: AY826720), *A. simplex* s.str. (AY826723), *A. simplex* C (AY826722), *A. typica* (AY826724), *A. physeteris* (AY826721) and *Anisakis* sp. (AY821749, AY821740) were included in the alignment for comparative purposes

Fig. 3 (continued)

Fig. 3 (continued)

parasitising the demersal fish species, as *Merluccius merluccius* (92%; Table 1).

Different studies have demonstrated that the ITS region (ITS1, the 5.8S and the ITS2) of nuclear rDNA provide useful genetic markers for the accurate identification of sibling species and morphospecies within Anisakid species (Zhu et al. 1998, 2000, 2001, 2002; D'Amelio et al. 2000; Abollo et al. 2003; Pontes et al. 2005). In the present study, the PCR-RFLP technique allowed the identification of different biological species of *Anisakis*, varying in their relative proportions, throughout the sampling area. In the Mediterranean Sea, both in the western and eastern basins, all the fishes examined were infected by the most widespread species present in the Mediterranean Sea,

A. pegreffii. The high abundances recorded for this species from the Mediterranean Sea can be explained by the occurrence of various dolphin species, such as the bottlenose dolphin *Tursiops truncatus* (Montagu, 1821), one of the main definitive hosts of *A. pegreffii* (Mattiucci et al. 2004). Interestingly, we also report the distribution of *A. simplex* s.str. in *Scomber scombrus* Linnaeus, 1758 from different area of sampling. *A. simplex* s.str. is known to have a worldwide distribution mainly with a benthic or demersal life cycle (Mattiucci et al. 1997; Abollo et al. 2001). This species was previously documented from the European Mediterranean Sea in some pelagic fishes, such as *S. scombrus* L., *Thunnus thynnus* (Linnaeus, 1758) and *Trachurus trachurus* (Linnaeus, 1758; Nascetti et al. 1986; Paggi et al. 1998; Mattiucci et al. 1997, 1998, 2001).

In this study, the sequence analyses of the ribosomal DNA has confirmed the presence of two independent polymorphic sites found at positions 244 and 260, corresponding to the sequence differences between *A. simplex* s.str. and *A. pegreffii*, at heterozygote status in some specimens. These results represent the first records of hybrids from the Mediterranean Sea, if excluding the results obtained by Abollo et al. (2003) in the Alboran Sea. A relatively high number of recombinant individuals was previously detected in Gibraltar strait waters, thus, suggesting that this can represent an hybrid zone for the two sibling species within the *A. simplex* complex (Abollo et al. 2003; Marques et al. 2006).

The size of the fragments obtained for *A. typica* after digestion with *Hinf*I and for *A. physeteris* after digestion with *Hha*I is slightly different than those reported in Pontes et al. (2005), probably due to a better resolution of gels in the present study. Regarding *A. typica*, the occurrence of this species in *Phycis phycis* (Linnaeus, 1766) represents the record of new intermediate/paratenic hosts, whilst its occurrence in *S. scombrus* and *M. merluccius* (Linnaeus, 1758) was already described by Mattiucci et al. (2002, 2004).

Most of the cetaceans reported in literature as definitive hosts of *A. typica* belong to the family Delphinidae, with the exceptional record of the phocoenid *Phocoena phocoena* (Linnaeus, 1758) and the pontoporiid *Pontoporia blainvillei* (Linnaeus, 1758; Mattiucci et al. 2002). Its typical definitive hosts (delphinids) are common in the epipelagic zone (Pontes et al. 2005), which explain the occurrence of this species in various pelagic fish species as suitable intermediate and paratenic host, such as *M. merluccius*, *T. thynnus*, *Auxis thazard* (Lacepède, 1800), *Trachurus picturatus* (Bowdich, 1825) and *S. scombrus*. The present findings extend the geographical range of this species to include the North African central Mediterranean coast, thus, confirming the presence of this species in the central-eastern part of Mediterranean as reported by Mattiucci et al. (2004). These authors claimed that the presence of *A. typica* in eastern Mediterranean Sea could be a consequence of the migration

of the paratenic or definitive hosts from the Indian Ocean to Mediterranean waters through the Suez Channel. Our findings extend the geographic distribution of *A. typica* to the eastern coasts of Tunisia and to Libya, whilst no occurrence of this species has been recorded in the westernmost collecting sites. Several larvae of *A. physeteris* were also recovered during this study, and its occurrence from the Mediterranean Sea in different fish hosts was already known (Nascetti et al. 1986; Orecchia et al. 1989; Mattiucci et al. 2001, 2004; Farjallah et al. 2006; Valero et al. 2006). Its main definitive host is the sperm whale, *Physeter catodon* Linnaeus, 1758. Pre-adults (L4) of *A. physeteris* have been recorded also in the beaked whale, *Ziphius cavirostris* Cuvier, (1823) from the Mediterranean Sea, suggesting a similar feeding behaviour shared by the sperm whale and the beaked whale in the Mediterranean Sea (Mattiucci et al. 2001). At larval stages, *A. physeteris* is typical of epipelagic paratenic hosts of the Mediterranean and Atlantic Ocean (Costa et al. 2004).

From a geographical perspective, the results obtained improve the picture of the occurrence of *Anisakis* species in the Mediterranean coasts of North Africa. Mattiucci et al. (2004) found *A. pegreffii* and *A. physeteris* in hake from western Mediterranean Sea and six species of *Anisakis* from the Atlantic coast of Morocco. The record of *A. simplex* s.str./*A. pegreffii* hybrids extends their geographic distribution to the Tunisian coasts. Furthermore, the finding of recombinant genotypes and of *A. simplex* s.str., in *S. scombrus* from the North African coasts of the Mediterranean Sea, is in accordance with the migratory mode of life for this fish characterised by important movement from Atlantic to Mediterranean (Punzón et al. 2004; Villamor et al. 2004; Dransfeld et al. 2005). The use of some endoparasite species and their infection levels as biological tags in fish stock discrimination is well documented and recurrently deeply reviewed (Beverly-Burton et al. 1977; George-Nascimento 1996; MacKenzie 2002; Mattiucci et al. 2002, 2004, 2005). The parasite fauna of an adult fish reflects not only the environmental conditions and the geographical region where it lives but also its feeding behaviour (Mattiucci and Nascetti 2006). The occurrence of *A. simplex* s.str. and of recombinant genotypes away from their area of distribution may predict the successful use of species of *Anisakis* larvae, identified by PCR-RFLP, for tagging *S. scombrus* fish stocks for fisheries management purposes, given the assumption that the parasite data are related to differences in the life history of individual fish hosts and considering fish stock in a spatial scale. Moreover, the results reported provide valuable information regarding the diversity of *Anisakis* species in the study area, thus, suggesting that the North African central Mediterranean coasts correspond to an area where the distribution of several *Anisakis* sibling and morphospecies overlaps.

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