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Molecular identification of Anisakis species (Nematoda: Anisakidae) from marine fishes collected in Turkish waters

Gokmen Zafer Pekmezci[a,*], Ertan Emek Onuk[a], Cenk Soner Bolukbas[b], Banu Yardimci[a], Ali Tumay Gurler[b], Mustafa Acici[b], Sinasi Umur[b]

[a] Department of Aquatic Animal Diseases, Faculty of Veterinary Medicine, Ondokuz Mayis University, Samsun 55139, Turkey
[b] Department of Parasitology, Faculty of Veterinary Medicine, Ondokuz Mayis University, Samsun 55139, Turkey

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ABSTRACT

Anisakid nematodes are important etiological agents for zoonotic human anisakiasis (or anisakidosis). These parasites in the Turkish waters still remain unexplored. This study aims the molecular identification of Anisakis species in Turkey's coast from Black, Aegean and Mediterranean Sea and specifically to screen for zoonotic species in commonly commercialized a total of 1145 fish belonging to 31 different species using both polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) and sequencing of the ribosomal internal transcribed spacer (ITS) regions and the mitochondrial cytochrome *C* oxidase subunit II (cox2) gene. A total of 776 Anisakis type I larvae were isolated in 56/1145 (4.8%) fish of 7 species from Turkish waters. The combining all of our results, e.g., morphology, PCR-RFLP, ITS region, and the cox2 gene, conclusively supported the identification of 3 *Anisakis* spp. taken from marine fish hosts, namely *Anisakis pegreffii*, *Anisakis typica* and Anisakis simplex sensu stricto (s.str.)/*A. pegreffii* hybrid genotype. No Anisakis larvae were isolated from the Black Sea whereas *A. pegreffii*, *A. typica* and *A. simplex* s.str./*A. pegreffii* hybrid genotype was found in the Aegean Sea and *A. pegreffii* was only isolated from the Mediterranean Sea. This study represents the first identification of *A. typica* and *A. simplex* s.str./*A. pegreffii* hybrid genotypes from Turkish waters. Moreover, in the present study first record of the presence of *A. pegreffii* is also reported from Turkish coasts of Aegean and Mediterranean Sea. No zoonotic Anisakis species were found in commonly commercialized 1025 fish belonging to 16 different species from the Black Sea, thus Turkish populations who consume captured fish from the Black Sea may have a less risk of human anisakiasis or allergies. However, the prevalence of larvae were 47.1% and 46% and recognized zoonotic *A. pegreffii* were identified from the Aegean and Mediterranean Sea coast, suggesting a high threat of anisakiasis or allergies for Turkish populations who consume fish originating in these regions.

1. Introduction

Adult nematodes of the genera of most species of the anisakid nematodes *Anisakis* Dujardin, 1845, *Pseudoterranova* Krabbe, 1878 and *Contracaecum* Railliet and Henry, 1913 are parasites of the alimentary tract of aquatic vertebrates. They display indirect life cycles in aquatic

* Corresponding author. Tel.: +90 362 3121919; fax: +90 362 4576922.
E-mail address: zpekmezci@omu.edu.tr (G.Z. Pekmezci).

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ecosystems and involve various hosts at different levels in food webs. Marine mammals (cetaceans and pinnipeds) and fish-eating birds serve as definitive hosts; fish, squids and other invertebrates serve as intermediate or paratenic hosts; and crustaceans serve as first intermediate hosts (Mattiucci and Nascetti, 2006, 2008). Recently, there are nine described species of *Anisakis*, which are further subdivided into two types. Type I consists of *Anisakis simplex sensu stricto* (s.str.), *Anisakis pegreffii*, *Anisakis simplex* C (all three species comprise the *A. simplex* complex), *Anisakis typica*, *Anisakis ziphidarum* and *Anisakis nascettii* whereas type II consists of *Anisakis paggiae*, *Anisakis physeteris* and *Anisakis brevispiculata*. Despite of morphological similarities among some *Anisakis* species, they are found to be genetically different having distinct host preferences, life cycles and zoogeographical distributions (Paggi et al., 1998; Mattiucci and Nascetti, 2006, 2007, 2008; Mattiucci et al., 2009). Moreover, the sequence analyses of nuclear ribosomal DNA spacers have proved the existence of hybrid specimens between *A. pegreffii* and *A. simplex* s.str. (Abollo et al., 2003). *Anisakis* type I or type II larvae can be morphologically identified based on ventriculus length and the presence of a tail spine (or mucron) (Berland, 1961). Species identification based on morphological characters is difficult, especially for larval stages, on the other hand application of genetic markers proved to be an essential tool in their identification (Mattiucci and Nascetti, 2006, 2008). Anisakiasis is an important fish-borne zoonosis caused by larval stages of nematodes of the family Anisakidae (Chai et al., 2005; Hochberg and Hamer, 2010). *A. simplex* s.str., *A. pegreffii* and *A. physeteris* have been shown to cause infection in humans (Mattiucci et al., 2011; Arizono et al., 2012).

Anisakid nematodes are a major public health concern. Anisakiasis is a serious zoonotic disease, and there has been a dramatic increase in its reported prevalence throughout the world in the last two decades (Chai et al., 2005). Despite during the last 20 years the knowledge of these parasites was improved, the European Food Safety Authority (EFSA) recommends that research in this topic should be continued: the collection of systematic data on the complete life cycle, geographical and seasonal distribution, prevalence, intensity, and anatomical location of parasites of public health importance in wild caught fishery products (EFSA, 2010). Although there are various reports on the circulation of these zoonotic parasites in the Mediterranean Sea (Manfredi et al., 2000; Valero et al., 2000; Farjallah et al., 2008b; Angelucci et al., 2011; Meloni et al., 2011; Cavallero et al., 2012; Chaligiannis et al., 2012; Serracca et al., 2013) the amount of information concerning Turkish waters are still poor and requires further investigations. Moreover, a broad knowledge on the distribution of anisakid nematodes is very important in understanding and forecasting possible future infections, thereby serving as pro-active measure in reducing the risk of human anisakidosis and allergies to target consumers locally and internationally (Quiazon et al., 2013).

The present study aims the molecular identification of *Anisakis* species in the Turkey's coast from Black, Aegean and Mediterranean Sea and specifically to screen for zoonotic species in commonly commercialized fish belonging to 31 species using both PCR-RFLP analysis and sequencing of the ITS regions and the *cox2* gene.

2. Materials and methods

2.1. Sampling hosts

A total of 1145 fish belonging to 31 different species of teleosts captured in the sea cages farms (farmed *Oncorhynchus mykiss*, *Dicentrarchus labrax, Sparus aurata*) and fishing-ground of different sites in the coastal waters of Turkey. These sampling sites are Sinop (S) to Trabzon (T) coasts (42°10′ N; 34°55′ E to 41°6′ N; 39°44′ E) in the Black Sea; Gulf of Saros (GS) (40°36′ N; 26°44′ E), İzmir (İ) coast (38°29′ N; 26°46′ E) and Güllük Bays (GB) (37°15′N; 27°34′ E) in the Aegean Sea and Gulf of Antalya (GA) (36°48′ N; 30°42′ E) and İskenderun Bays (İB) (36°46′ N; 36°2′ E) in the Mediterranean Sea between March 2012 and April 2013 (Fig. 1). When sampling for the captured fish species, the most captured fish species for that Sea was considered (For instance *Engraulis encrasicolus* and *Trachurus trachurus* are the most captured fish in the Black Sea). Besides, *Oncorhynchus mykiss*, *Dicentrarchus labrax, Sparus aurata* which are the most cultured three species in the sea cages in Turkey were considered for the cultured fish sampling species. Fish were stored in individual plastic bags in ice boxes and transported to the laboratory and each fish was identified at species.

2.2. Parasitological examination

The fish were necropsied and *Anisakis* larvae were collected from the body cavities. The muscular portion of fishes has been sectioned in less than 5 mm thick fillets and observed within white light to check for larval anisakids under a dissecting microscope (Koinari et al., 2013). *Anisakis* larvae were collected, counted and washed with 0.9% NaCl solution, and preserved in 70% ethanol for further identification. A small piece of the mid-body of each parasite was excised for molecular study, and the rest of the nematode was cleared in lactophenol for morphological examination according to the morphological criteria proposed by Berland (1961) and Peter and Maillard (1988). The larvae morphologically identified as belonging to the genus *Anisakis* were subjected to further molecular characterization by PCR-RFLP to identify the species. Prevalence (*P*), mean intensity (Im), and mean abundance (mA) were calculated by Rózsa et al. (2000) and by using Quantitative Parasitology 3.0 program (Reiczigel and Rózsa, 2005).

2.3. Genomic DNA extraction and PCR-RFLP analysis

Genomic DNA was extracted from individual larvae using the DNA purification kit (Genomic DNA Purification Kit, Thermo Scientific) according to manufacturer's instructions. Polymerase chain reaction (PCR) targeting the ITS regions (ITS-1, 5.8S subunit, ITS-2) and the mitochondrial cytochrome C oxidase subunit II (*cox2*) gene were performed. DNA content was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific) at 260 nm. PCR was carried out in a final volume of 50 μl,

Fig. 1. Map of the study sites. Samples were collected from the Turkey's coast Black Sea, Aegean and Mediterranean Sea.

containing 10–50 ng of extracted DNA, 1× Taq Buffer with KCI (Thermo Scientific), 3 mM of $MgCl_2$ (Thermo Scientific), 0.3 mM dNTPs (Thermo Scientific), 2 pmol of each primer, 2.5 U of Taq DNA Polymerase (Thermo Scientific), and DEPC-treated water. Fragments of ~1000 bp of the ITS region were amplified using the primers NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) (Zhu et al., 1998). The PCR was performed in a Thermo PxE 0.2 thermal cycler (Thermo Scientific) and the conditions were as follows: 15 min at 95 °C, then 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C followed by a final elongation of 5 min at 72 °C. The *cox2* gene fragment (629 bp) was amplified using 210 (5′-CACCAACTCTTAAAATTATC-3′) and 211 (5′-TTTTCTAGTTATATAGATTGRTTTYAT-3′) (Nadler and Hudspeth, 2000) and 1× Taq Buffer with KCI (Thermo Scientific), 2.5 mM $MgCl_2$ (Thermo Scientific), 0.4 mM dNTPs (Thermo Scientific), 2 pmol of each primer, 5 U of Taq DNA Polymerase (Thermo Scientific) and 10-50 ng of genomic DNA, in a volume of 50 μL. Reaction conditions were as follows: 3 min at 94 °C, then 34 cycles of 30 s at 94 °C, 1 min at 51 °C and 90 s at 72 °C followed by a final elongation of 10 min at 72 °C. PCR products were electrophoresed in 1.5% agarose gel (Prona) in a TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) (Thermo Scientific), stained with ethidium bromide (Sigma) and visualized by UV transillumination (DNR, Bio-imaging system). The size of the amplified fragments was estimated by comparisons with the 1000 bp DNA Ladder (Thermo Scientific). The amplified ITS products were analyzed by RFLP technique using the restriction enzymes: *Hha* I and *Hinf* I (Thermo Scientific) that allows the identification of the members of *Anisakis* genus according to the genetic markers described by D'Amelio et al. (2000) and implemented by Pontes et al. (2005). Manufacturer's recommendations were followed with regard to the digestion of ITS PCR products. Finally digested products were electrophoresed in 2% agarose gel (Prona) in a TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) (Thermo Scientific), stained with ethidium bromide (Sigma) and visualized by UV transillumination (DNR, Bio-imaging system). The size of the amplified fragments was estimated by comparisons with the 1000 bp DNA Ladder (Thermo Scientific).

2.4. DNA sequencing

Larvae identified as *A. pegreffii, A. typica* and *A. simplex* s.str./*A. pegreffii* hybrid genotypes using PCR-RFLP analyses were sequenced for further confirmation. Species identities were confirmed by sequencing the ITS regions and *cox2* gene. The ITS region and *cox2* gene amplification products were sent to sequencing company (Iontek Istanbul, Turkey) for purification and sequencing in both directions using NC5-NC2 and 211-210 primers, respectively. Sequencing was carried out directly on purified fragments with ABI PRISIM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA), using the ABI PRISIM® BigDye terminator cycle sequencing ready reaction kit. Sequence quality was assessed using FinchTV v.1.4.0 (Geospiza Inc., Seattle, WA, USA; http://www.geospiza.com).

2.5. Phylogenetic analysis

The obtained sequences were verified by forward and reverse comparisons, assembled and edited with using Contig Express in Vector NTI Advance 11.5 (Invitrogen). The obtained consensus sequences were compared with previously published data for identification by using the Basic Local Alignment Search Tool (BLAST) via GenBank database (Altschul et al., 1990) (http://www.ncbi.nlm.nih.gov/genbank). Sequences were aligned with previously characterized sequences of other known *Anisakis* species, using ClustalW in Mega 5.0 (http://www.megasoftware.net/) multiple sequence alignments (Thompson et al., 1994) and adjusted manually. The electropherograms were analysed with BioEdit (version 7.0.9.0, http://www.mbio.ncsu.edu/bioedit/bioedit.html) (Hall, 1999). Genetic distances were calculated using the Kimura two-parameter model with pairwise deletion in Mega 5.0 (Tamura et al., 2011). The phylogenetic analysis on the sequences data sets obtained at all the *Anisakis* spp. specimens examined was carried out by Maximum Parsimony (MP) by using PAUP* 4.0b10 version (Swofford, 2002). Maximum Parsimony (MP) analysis was performed by using the heuristic search with tree-bisection-reconnection (TBR) branch-swapping algorithm; the reliabilities of the phylogenetic relationships were evaluated using non-parametric bootstrap analysis on 1000 pseudoreplicates (Felsenstein, 1985). Bootstrap values $\geq$70 were considered well supported (Hillis and Bull, 1993). The nucleotide sequences were deposited in GenBank database under the accession numbers: KF032056–KF032067.

3. Result

3.1. Anisakis prevalence

The overall prevalence (%), mean intensity and abundance of *Anisakis* larvae in fish from the Turkish waters were 4.8% (56/1145), 13.6 and 0.6, respectively. No *Anisakis* larvae was isolated from the Black Sea whereas prevalence (%), mean intensity and abundance of *Anisakis* larvae from the Aegean and Mediterranean Sea were 41.7% (33/70), 17.2 and 8.1, and 46% (23/50), 8.5 and 3.5, respectively. For each parasitized fish species the infection indexes are shown in Table 1. An overall of 776 *Anisakis* larvae from both viscera and body cavity, whereas no larvae was isolated from muscle portions, were collected and identified by morphology as L_3 larvae of *Anisakis* type I in fish belonging to 7 species from the Turkish waters. Chub mackerel (*Scomber japonicus*), Atlantic mackerel (*Scomber scombrus*), blue whiting (*Micromesistius poutassou*), European hake (*Merlucius merlucius*), Mediterranean horse mackerel (*Trachurus mediterraneus*) and red mullet (*Mullus barbatus*) were the most highly infected species from the Aegean and Mediterranean Sea. The higher *Anisakis* larvae burden was detected in *S. japonicus* whereas the higher mean intensity and abundance was found in *S. scombrus* from the Aegean Sea. *Anisakis* larvae were not found in 16 fish species from the Black Sea whereas 5 fish species from the both Aegean and Mediterranean Sea (Table 1). Mixed infections were found in the same individual hosts caught along the Aegean coast. In particular the 10% (1/10) of *S. japonicus* were infected by *A. pegreffi* and *A. typica* and the 12.5% (1/8) of *S. scombrus* were infected by *A. pegreffi* and hybrid genotype while 12.5% (1/8) of *M. poutassou* were infected by *A. pegreffi*, *A. typica* and hybrid genotype. In addition, *Hysterothylacium* spp. larvae were found in *E. encrasicolus, M. merlangus euxinus* and *T. trachurus* from the Black Sea.

3.2. Morphological and PCR-RFLP results

All larvae were morphologically identified by light microscopy as *Anisakis* type I larvae. The identification was based on the presence of a long ventriculus with an oblique ventricular-intestinal junction and a rounded tail possessing a mucron. Larvae collected from 56 fish samples (randomly selected three larvae for each sample) were subjected to further molecular characterization by PCR-RFLP to identify the species. All larvae from fish species were PCR positive and results of restriction digestion of ITS region ($\sim$1 kb bp) with *Hinf* I and *Hha* I enzymes revealed three different *Anisakis* species: *A. pegreffii*, *A. typica* and *A. simplex* s.str./*A. pegreffii* hybrid genotypes from the Turkish waters (Fig. 2). Ninety four (94.9%) larvae was detected as *A. pegreffii*, three (3%) larvae hybrid genotype and two (2%) larvae *A. typica* in total of ninety nine larvae from the Aegean Sea whereas all larvae (100%) detected as *A. pegreffii* from the Mediterranean Sea (Table 2). *A. pegreffii* was the dominant species and accounted for 97% of the total number of larvae collected from the Turkish waters. Mixed infections of *A. pegreffii* and *A. typica* were found in *S. japonicus* and *A. pegreffii* and hybrid genotype in both *S. scombrus* and *Zeus faber* from the Aegean Sea. *A. pegreffii*, *A. typica* and hybrid genotypes were rarely found in *M. poutassou* from the Aegean Sea. Furthermore, *A. pegreffii* was found only in *M. merluccius* from the Aegean Sea, whereas *A. pegreffii* was also found in all infected fish samples from the Mediterranean Sea (Table 2).

3.3. Phylogenetic analysis and DNA Sequencing

Sequences analysis of amplified ITS region and *cox2* gene was carried out and MP phylogenetic trees were constructed for the confirmation of PCR-RFLP analysis

Table 1
Fish species from the Black, Aegean and Mediterranean Sea tested for *Anisakis* Type I larvae and their level of infection.

Locality	Fish species	Mean length (range, cm)	Infected/ tested	*P* (%)	Im	mA	Number of collected larvae
Black Sea	*Engraulis encrasicolus*	11.2 (10–19.5)	0/250				
	Trachurus trachurus	13.8 (12.1–18)	0/198				
	Merlangius merlangus euxinus	14.2 (10.5–22)	0/153				
	Mullus barbatus ponticus	12.7 (12–16.9)	0/112				
	Sprattus sprattus	10.2 (6.8–12.5)	0/102				
	Mugil cephalus	18.3 (12–20.3)	0/62				
	Sarda sarda	25.3 (22.4–32)	0/32				
	Alosa fallax	23.8 (20.1–25)	0/30				
	Spicara maena	19.4 (14.8–23)	0/23				
	Belone belone	33.2 (27.2–35)	0/20				
	Pomatomus saltator	25.6 (20–27.9)	0/15				
	Neogobius melanostomus	18.9 (12.7–20)	0/12				
	Platichthys flesus	17 (13.6–22.4)	0/10				
	Psetta maxima	48 (46–50)	0/2				
	Dicentrarchus *labrax* (farmed)	25 (22–28)	0/2				
	Oncorhynchus mykiss (farmed)	42 (40–44)	0/2				
	Total		0/1025	–	–	–	–
Aegean Sea	*Scomber japonicas*	28.1 (19–32.4)	10/10	100	18.4 (8–28)[a]	18.4	184
	Merluccius merluccius	26.2 (20.3–28)	8/10	80	12 (6–20)	9.6	96
	Scomber scombrus	30.3 (22–35.5)	8/8	100	20.2 (10–29)	20.2	162
	Micromesistius poutassou	29.4 (24–33.5)	6/8	75	19 (4–32)	14.2	114
	Boops boops	18 (12–19.5)	0/8				
	Diplodus annularis	14 (11.6–20)	0/7				
	Sarpa salpa	15.6 (13–18)	0/6				
	Dicentrarchus labrax (farmed)	20 (18–26)	0/6				
	Sparus aurata (farmed)	18 (16.5–22)	0/6				
	Zeus faber	31	1/1	100*	13 (1–13)	13	13
	Total		33/70	47.1	17.2	8.1	569
Mediterranean Sea	*Trachurus mediterraneus*	14.5 (11–16.8)	18/30	60	10 (3–30)	6	180
	Mullus barbatus	13.2 (10–14.5)	5/12	41.6	3.4 (3–4)	1.4	17
	Sphyraena sphyraena	33 (30–36)	0/2				
	Pagellus erythrinus	23 (21–25)	0/2				
	Mugil cephalus	26 (22–30)	0/2				
	Liza ramada	28	0/1				
	Umbrina cirrosa	42	0/1				
	Total		23/50	46	8.5	3.9	197
Overall			56/1145	4.8	13.6	0.6	766

mA: Mean abundance of infection.
[a] Im: Mean intensity of infection and its range in parentheses.
* These relative frequencies may be affected by the small sample size.

(Figs. 3 and 4). The amplification of the ITS region produced a fragment of approximately ~1 kb from each larvae. The each ITS PCR products were subjected to direct sequencing giving products 913 bp long. Entire ITS region sequences were determined for 3 larvae of *A. simplex* s.str./*A. pegreffii* hybrid genotypes, 2 larvae of *A. typica* from the Aegean Sea, and 1 larva of *A. pegreffii* from the Mediterranean Sea. Direct sequencing revealed the lengths of ITS-1 and ITS-2 to be 348 and 355 bp for *A. typica*, and 392 and 308 bp for both *A. pegreffii* as well as the proposed hybrid genotype, respectively.

In the present study, sequences of the ITS region was clustered in the same well-supported clade with same species previously sequenced and deposited in GenBank database (Fig. 3). The percent identities among *A. pegreffii* isolates from the Mediterranean Sea (Turkey, KF032066) showed 100% identity with various geographical isolates of *A. pegreffii* from the Korean waters (South Korea, JF683735), Morocco (EU718479), the South China Sea (JX523713), Japan (AB277823, AB196670, EU624343) from GenBank according to Clustal W analyses of ITS region. In the present study, specimens showing the heterozygote restriction pattern were also analyzed by sequencing of the entire ITS region, to confirm the heterozygote status. The hybrid genotype from the Turkish waters (Turkey, KF032056, KF032058 and KF032060) was verified by sequencing, showing the presence of a polymorphism (i.e. C/T) at both positions 280 and 296 in the ITS-1, as reported previously by Abollo et al. (2003), Abe et al. (2005), Umehara et al. (2008), Du et al. (2010), Meloni et al. (2011). Within the present study, all three hybrid genotypes (Turkey, KF032056, KF032058 and KF032060) revealed low genetic variation (genetic distance of 0.001; 1 nucleotide bases difference; alignment positions 148 in ITS-1) compared to the deposited *A. simplex* s.str./*A. pegreffii* isolate from Greece (JF412028) from the GenBank. Also, all hybrid species revealed low genetic variation (0.002; 2; alignment position 369 in ITS-1; alignment positions 714

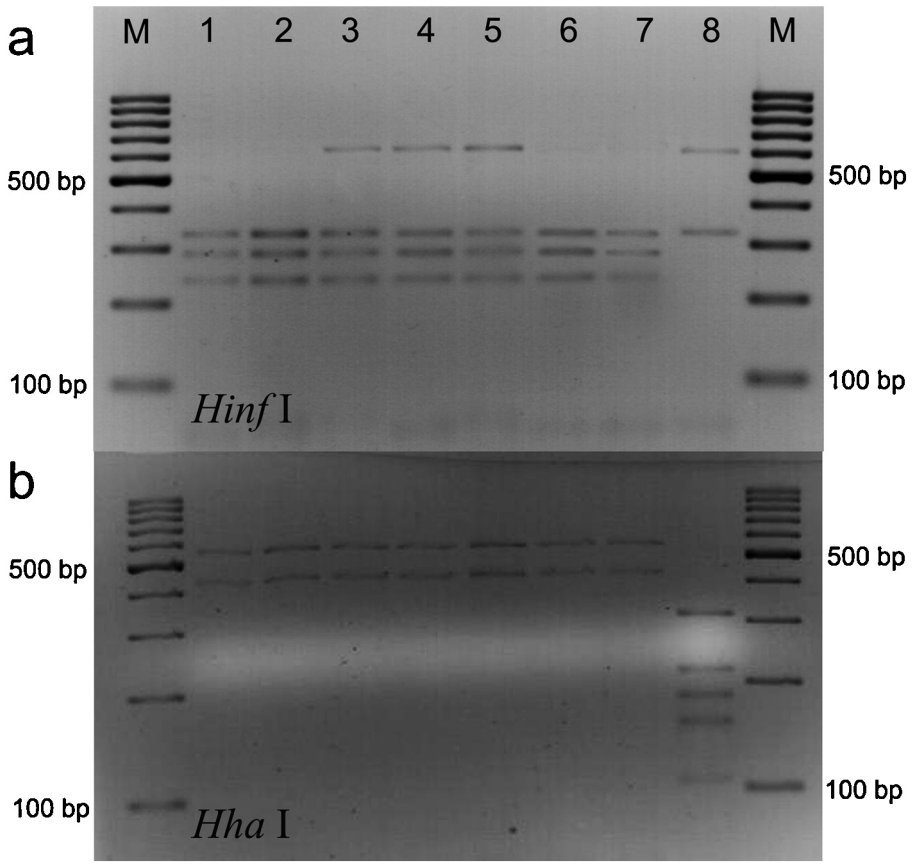

Fig. 2. RFLP patterns obtained by digestion of the ITS region of the rDNA with the restriction enzymes *Hinf* I (a) and *Hha* I (b) shown by the species of the genus *Anisakis*. Lanes: 1–2 *A. pegreffii*, 3–5 hybrid genotype, 6–7 *A. pegreffii*, 8 *A. typica*, M, 100 bp ladder.

in ITS-2) compared to the deposited *A. simplex* s.str./*A. pegreffii* isolate from the Portuguese waters (Portugal, JN005768). However, *A. simplex* s.str./*A. pegreffii* hybrid genotypes (Turkey, KF032056, KF032058 and KF032060), was verified by sequencing, showing the presence of a polymorphism (i.e. C/T) at both positions 280 and 296 in the ITS-1 (Fig. 5). *A. typica* from the Aegean Sea (Turkey, KF032062 and KF032064) had 100% identity with various geographical isolates of *A. typica* isolate from the Portuguese waters (Portugal, JN005760), coasts of Taiwan and Japan (AB432908), the Atlantic Ocean (Brazil, EU327686), the Pasific Ocean (Papua New Guinea, JX648318) from GenBank according to Clustal W analyses of ITS gene. The ITS sequences of *A. typica* in fish from the Aegean Sea are the first ITS sequence for this species in GenBank.

Amplification of the *cox2* gene generated an approximately 630 bp product each larvae from the Turkish waters. The each *cox2* products were subjected to direct sequencing giving products 591 bp long. Within the present study sequences of the *cox2* gene was clustered in the same well-supported clade with same species previously sequenced and deposited in GenBank database (Fig. 4). According to the result of the mtDNA cox2 analysis, larvae with hybrid genotypes were classified as *A. pegreffii* larvae (Fig. 4). The *cox2* sequences of *A. pegreffii* from the Mediterranean Sea (Turkey, KF032067), showed 100% identity with various geographical isolates of *A. pegreffii* from the Adriatic Sea (JQ341912, JQ934889), the Korean waters (South Korea, HQ702744 and JF825530). Sequence analysis of *cox2* gene of *A. typica* from the Aegean Sea (Turkey, KF032063 and KF032065) showed 99.8–100% identity with the Adriatic Sea (JQ934884), the northeastern coast of Brazil (JQ859923), Japan (AB517571), the Pasific Ocean (Papua New Guinea, JX648324). *Cox2* sequences of *A. typica* (Turkey, KF032063 and KF032065) and *A. simplex* s.str./*A. pegreffii* hybrid genotypes (Turkey, KF032057, KF032059 and KF032061) in fish from the Aegean Sea are the first *cox2* sequence for these species in GenBank.

4. Discussion

The morphological study is not useful for the identification at species level the application of a biomolecular method such as PCR-RFLP allows reaching a correct identification of the anisakid larvae (D'Amelio et al., 2000; Serracca et al., 2013). In the present study, RFLP patterns were found similar to previously reported *A. pegreffii*, *A. typica* and *A. simplex* s.str./*A. pegreffii* hybrid genotypes (D'Amelio et al., 2000; Abollo et al., 2003; Abe et al., 2005; Martín-Sánchez et al., 2005; Pontes et al., 2005; Umehara et al., 2006; Farjallah et al., 2008a,b; Du et al., 2010; Suzuki et al., 2010; Cavallero et al., 2012; Smrzlić

Table 2
Anisakis type I larvae identified by PCR-RFLP from fish of Black, Aegean and Mediterranean Sea.

Locality	Fish species	Number of *Anisakis* larvae	PCR-RFLP analysis		
			A. pegreffii	*A. typica*	Hybrid genotype
Black Sea	*Engraulis encrasicolus*				
	Trachurus trachurus				
	Merlangius merlangus euxinus				
	Mullus barbatus ponticus				
	Sprattus sprattus				
	Mugil cephalus				
	Sarda sarda				
	Alosa fallax				
	Spicara maena				
	Belone belone				
	Pomatomus saltator				
	Neogobius melanostomus				
	Platichthys flesus				
	Psetta maxima				
	Dicentrarchus labrax (farmed)				
	Oncorhynchus mykiss (farmed)				
	Total	–	–	–	–
Aegean Sea	*Scomber japonicas*	30	29	1	
	Merluccius merluccius	24	24		
	Scomber scombrus	24	23		1
	Micromesistius poutassou	18	16	1	1
	Boops boops				
	Diplodus annularis				
	Sarpa salpa				
	Dicentrarchus labrax (farmed)				
	Sparus aurata (farmed)				
	Zeus faber	3	2	–	1
	Total	99	94 (94.9%)	2 (2%)	3 (3%)
Mediterranean Sea	*Trachurus mediterraneus*	54	54		
	Mullus barbatus	15	15		
	Sphyraena sphyraena				
	Pagellus erythrinus				
	Mugil cephalus				
	Liza ramada				
	Umbrina cirrosa				
	Total	69	69 (100%)	–	–
Overall		168	163 (97%)	2 (1.1%)	3 (1.7%)

et al., 2012). The ability to identify species of *Anisakis* has important implications for investigating their systematic, population biology and ecology as well as for controlling anisakiasis (Mattiucci and Nascetti, 2006, 2008). It is now recognized that the morphospecies *A. simplex* is not a single species but a complex of three sibling species, *A. simplex* s.str., *A. pegreffii* and *A. simplex* C, differing in their genetic structures, biological cycles and geographical distributions (Nascetti et al., 1986; Mattiucci et al., 1997; Mattiucci and Nascetti, 2006, 2008). These species can also be distinguished by using multilocus enzyme electrophoresis (Nascetti et al., 1986; Mattiucci et al., 1997). Reference individuals initially characterized by allozymes have been used to develop DNA-based approaches for species identification, such as PCR-RFLP (D'Amelio et al., 2000) and direct sequencing of ITS rDNA (Nadler et al., 2005) or mtDNA *cox2* (Valentini et al., 2006). Until now, morphological identification has also been used to identify some third-stage larvae of *Anisakis* species levels in Turkey. *A. simplex*-like larvae have been found in different fish species from the Aegean Sea, but the larvae have been morphologically identified as *A. simplex* by only light microscopy (Akmirza, 2000; Oguz et al., 2000). Therefore, the epidemiology of the *A. simplex* complex, composed of three sibling species, is still unclear in the Turkey's coast.

In the present study, all larvae from fish species were PCR positive and could be further identified as *A. pegreffii*, *A. typica* and *A. simplex* s.str./*A. pegreffii* hybrid genotypes from, the Aegean and Mediterranean coast, Turkish waters (Fig. 2). Therefore this is the first identification of *A. typica* and *A. simplex* s.str./*A. pegreffii* hybrid genotypes from Turkish waters. The present study identified two new fish species as hosts for *A. typica*; *S. japonicus* and *M. poutassou* from the Aegean Sea. Within the present study, anisakid larvae were not found, 1025 examined fish belonging to 16 species, from the Black Sea coast. In contrast, *A. pegreffii* were detected in *T. trachurus*, possible origin of the Black Sea, sold for human consumption in a fish market (Utuk et al., 2012), whereas adult anisakid nematodes were not found in the alimentary canal from stranded as

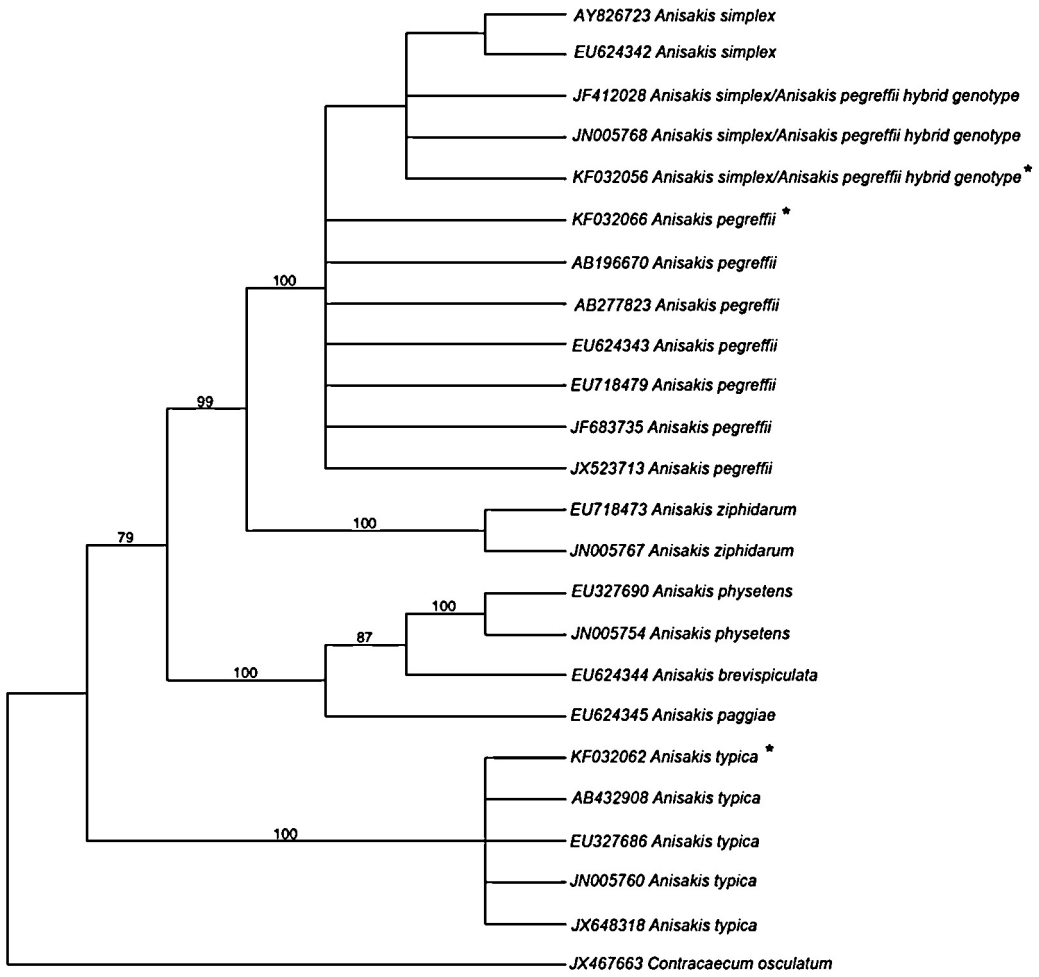

Fig. 3. Phylogenetic relationships between *Anisakis* species from the present study (*) and other *Anisakis* species as inferred by maximum-parsimony (MP) analysis of ITS regions. *Contracaecum osculatum* was used as the out group. The accession numbers of individual sequences determined in the present study are shown in each tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown at the internal nodes (>70% only).

definitive host, *Phocaena phocaena* and *Tursiops truncates*, from the Black Sea (Pekmezci et al., 2013a). However, *A. simplex* reported in the Black Sea as an adult in *P. phocaena* reported by Borcea in a review (Birkun, 2002) and, although it remains listed as a parasite of the Black Sea cetaceans, the record awaits confirmation (Birkun, 2002). The absence of *Anisakis* spp. in the Black Sea might be due to the particular physical and chemical characteristics of this area (Tomczak and Godfrey, 1994). Another Anisakidae species as *Hysterothylacium aduncum* were commonly found different fish species in Black Sea (Ismen and Bingel, 1999; Pekmezci et al., 2013b).

The present results show that *Anisakis* larvae commonly parasitized a great variety of fish in Turkish water, excluding Black Sea, similarly to data collected in a number of surveys carried out in most of the Mediterranean Sea along the European and African coasts (Abollo et al., 2003; Costa et al., 2004; Mattiucci et al., 2007; Farjallah et al., 2008a,b; Mattiucci et al., 2008; Rello et al., 2009; Cavallero et al., 2012; Hermida et al., 2012; Smrzlić et al., 2012; Abattouy et al., 2013; Serracca et al., 2013).

Within the present study, *A. pegreffii* was recorded frequently in the Aegean and Mediterranean Sea coast. In agreement to previously published works carried out in the Aegean and Mediterranean Sea (Farjallah et al., 2008a,b; Mattiucci et al., 2008; Meloni et al., 2011; Cavallero et al., 2012; Chaligiannis et al., 2012; Hermida et al., 2012; Serracca et al., 2013). *A. pegreffi* is the dominant species of *Anisakis* in the Mediterranean Sea, probably due to the occurrence of various dolphin species, such as *T. truncatus*, as the main definitive hosts (Mattiucci and Nascetti, 2006). In the study, chub mackerel (*S. japonicus*), Atlantic mackerel (*S. scombrus*), blue whiting (*M. poutassou*), European hake (*M. merlucius*), Mediterranean horse mackerel (*T. mediterraneus*) and red mullet (*M. barbatus*) were the fish intermediate hosts for *A. pegreffi* in the Aegean and Mediterranean Sea coast.

These results represent the first records of *A. pegreffi* from the Turkey's coast from the Aegean and Mediterranean Sea, and also the first record of this species in these fish species as host in Turkish waters. These fish species were also previously described as the common

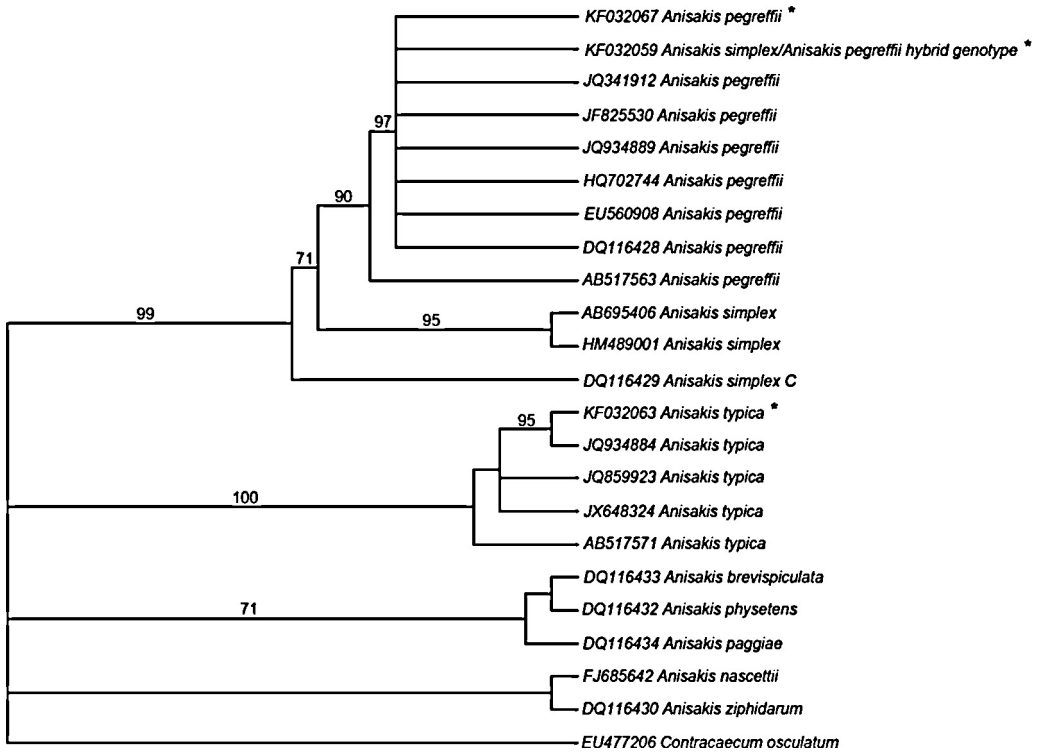

Fig. 4. Phylogenetic relationships between *Anisakis* species from the present study (*) and other *Anisakis* species as inferred by maximum-parsimony (MP) analysis of *cox2* gene. *Contracaecum osculatum* was used as the out group. The accession numbers of individual sequences determined in the present study are shown in each tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown at the internal nodes (>70% only).

intermediate hosts for *A. pegreffi* (Mattiucci and Nascetti, 2008).

A. typica occurred rarely and in mixed infections with *A. pegreffii* in *S. japonicus* and *M. poutassou* from the Turkey's coast from Aegean Sea. To our knowledge, these results represent the first records of *A. typica* in *S. japonicus* and *M. poutassou* from the Aegean Sea, if excluding the results obtained by Mattiucci et al. (2008) in *T. trachurus* from the Aegean Sea. Similarly, *A. typica* was rarely found in mixed infections with *A. pegreffii* in the eastern Aegean and Mediterranean Sea (Mattiucci et al., 2004, 2008; Farjallah et al., 2008b). *A. typica* was detected in definitive hosts and intermediate hosts from tropical and temperate-tropical regions (Mattiucci et al., 2002, 2004). The presence of *A. typica* in the eastern Mediterranean Sea (Aegean 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 (Mattiucci et al., 2004, 2008). *A. typica* has previously been documented from the Atlantic coasts to Mediterranean Sea (Mattiucci et al., 2002, 2004; Farjallah et al., 2008a,b) whereas, this species was not found in different fish species from the Aegean Sea (Chaligiannis et al., 2012).

The existence of a hybrid genotype has been described as the product of inter-specific hybridization occurring in areas of sympatry of the *A. simplex* s.str and *A. pegreffii*, such as the Spanish costs (Abollo et al., 2003; Martín-Sánchez et al., 2005), the Pacific costs of Japan (Umehara et al., 2006), Moroccan and Mauritanian (Farjallah et al., 2008a) and the North African coasts of Mediterranean Sea (Farjallah et al., 2008b), the Yellow Sea of China (Du et al., 2010), the Atlantic and Mediterranean coasts of Morocco (Abattouy et al., 2013), coast of Sardinia (Meloni et al., 2011) and, Tyrrhenian Sea (Cavallero et al., 2012), more recently, Aegean Sea (Chaligiannis et al., 2012). Similarly, previously published study carried out in the Aegean Sea (Chaligiannis et al., 2012) was rarely detected a hybrid genotype in the sympatric area. In the present study, hybrid species was also found for the first time from the Turkish Aegean Sea coast. Within the present study, the sequence analyses of the nuclear rDNA confirmed the presence of two independent polymorphic sites found at positions 280 and 296, corresponding to the sequence differences between *A. simplex* s.str. and *A. pegreffii*, at hybrid genotype in some specimens from the Aegean Sea (Fig. 5). These differences were found at positions 280 and 296 in the ITS-1, and corresponded to the sequence differences between *A. simplex* s.str. and *A. pegreffii* those reported previously (Abollo et al., 2003; Abe et al., 2005; Umehara et al., 2008; Du et al., 2010; Meloni et al., 2011). Therefore, the occurrence of two independent polymorphic sites found to be always heterozygous in the positions 280 and 296 for the same specimen supports that the recombinant genotypes are not really the product of a polymorphism. Although there has been no report of the existence of a hybrid genotype at the adult stage in the Aegean Sea, we found larvae of hybrid in fish. Indeed, the presence of the hybrid genotype in Aegean Sea is thought to be the result of gene exchange between

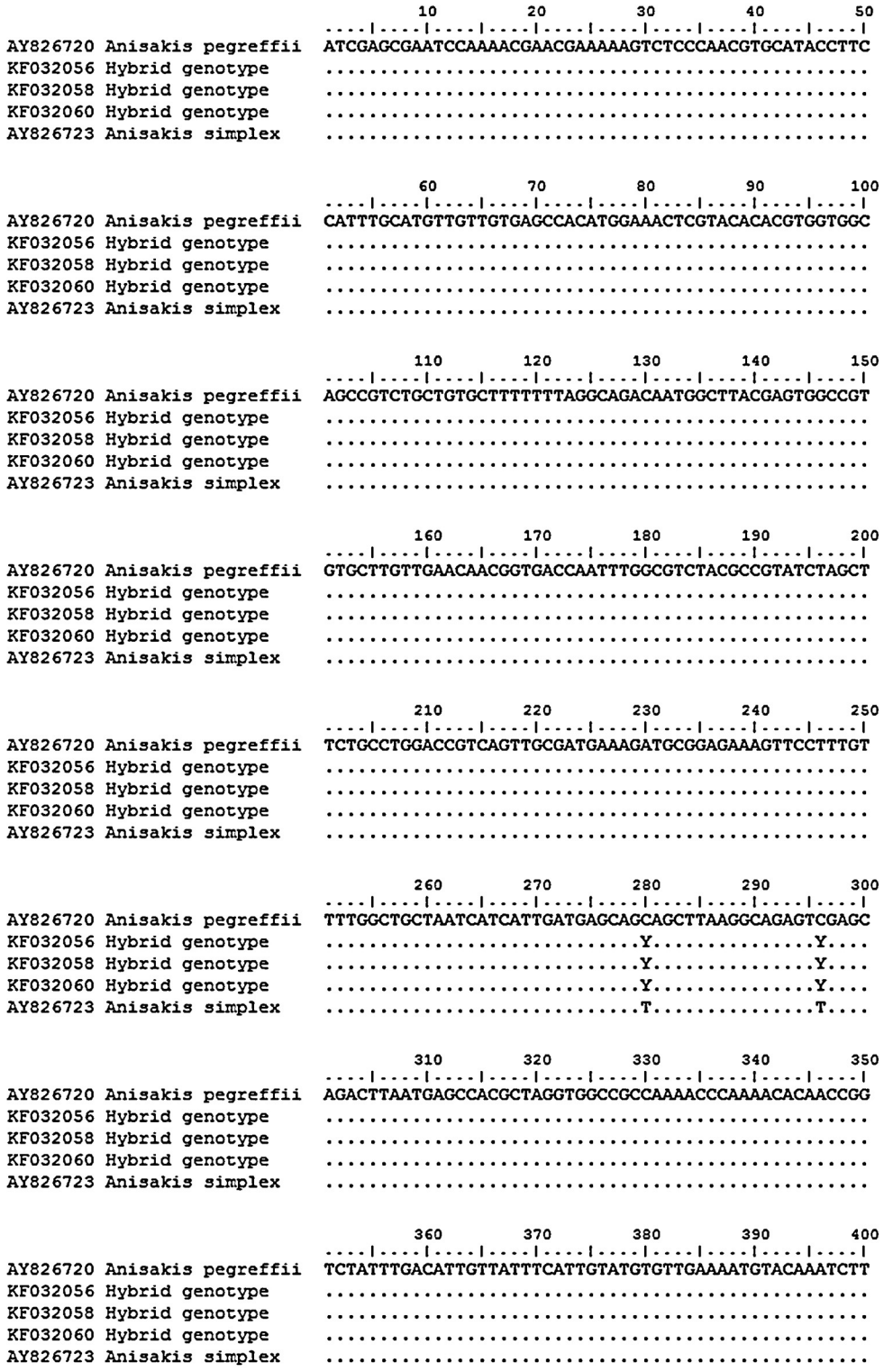

Fig. 5. Alignment of ITS-1 sequences of *Anisakis* taxa. One representative of each unique sequence was included for comparison. Dots indicate identity with the first sequence and dashes are inferred insertion–deletion events. The consensus ITS-1 sequences for the *A. pegreffii* (AY826720), *A. simplex* s.str. (AY826723) and hybrid genotypes (Turkey, KF032056, KF032058 and KF032060) were included in the alignment for comparative purposes. Polymorphic positions are indicated by International Union of Pure and Applied Chemistry (IUPAC) codes: Y = C and/or T.

the sibling species *A. simplex* s.str. and *A. pegreffii* in a sympatric geographic area.

In the present study, at the intraspecific level, a very low level of genetic differentiation at mitochondrial level has been found between the same *Anisakis* species from Turkish waters and the same *Anisakis* species so far sequenced from the various geographical regions; similar values are those reported for conspecific populations of anisakid nematodes (Mattiucci and Nascetti, 2008). Indeed, higher level of differentiation, at the same mitochondrial gene level, are those observed at the interspecific level between *Anisakis* spp. (Mattiucci and Nascetti, 2006). The *cox2* gene revealed to be highly polymorphic in *Anisakis* spp. (Valentini et al., 2006; Mattiucci et al., 2009). Moreover, the small sequence difference of *cox2* marker of the *A. pegreffii* and *A. typica* examined in the present study from those previously reported (Valentini et al., 2006; Quiazon et al., 2009; Suzuki et al., 2010; Setyobudi et al., 2011; Mladineo et al., 2012; Smrzlić et al., 2012; Koinari et al., 2013). The mtDNA is particularly useful for identifying the parental species involved in the production of hybrids, since the vast majority of mitochondrial genomes are inherited uniparentally from the female parent (Feagin, 2000). The analysis of mtDNA sequences showed the existence of a different parental origin for hybrid genotypes (Abollo et al., 2003). Therefore, the parental origin of the hybrid genotypes was probably *A. pegreffii* in this study (Fig. 3).

5. Conclusion

The presence of *A. pegreffi* was the most prevalent species in examined fish from the Aegean and Mediterranean Sea coast could have public health implications in Turkey. *A. simplex* s.str. and *A. pegreffii* have been identified as agents of human anisakiasis (Mattiucci et al., 2011, 2013). Moreover, well-cooked fish parasitized by previously mentioned anisakids may cause allergic processes as these parasites have antigens that act as allergens (Audicana and Kennedy, 2008). In the present study, no *Anisakis* larvae found in the Black Sea. Therefore, Turkish populations who consume captured fish from the Black Sea may have a less risk of human anisakiasis or allergies. Fish are commonly consumed as pan-fried by Turkish population. Although well-cooked fish meals are traditionally consumed in Turkey, raw fish meat in sushi in some restaurants and changes of feeding habits as consequence of the globalization combined with the high prevalence of *A. pegreffii* in marketed fish from the Aegean and Mediterranean Sea could represent a risk to acquire food-borne parasitic infection/allergies also in Turkey.

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