

Molecular characterisation of Anisakidae larvae from fish in Adriatic Sea

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Abstract In the present study, anisakids from: tuna (*Thunnus thynnus*) fattened in the Croatian farm in middle Adriatic Sea, three different feral fish species caught near tuna farm (*Trachurus trachurus*, *Scomber japonicus* and *Oblada melanura*) and fish marketed in Croatia (*T. trachurus*) were analysed by morphology and molecular methods. Larvae were identified to the species level by PCR restriction fragment length polymorphism and characterised by sequencing of nuclear (internal transcribed spacer) and mitochondrial (cytochrome c oxidase subunit 2) markers. The results revealed diverse Anisakidae community consisting of: *Anisakis pegreffii*, *Anisakis simplex* (s.s.), *Anisakis typica* and *Hysterothylacium aduncum*. This is the first report of *A. typica* in Adriatic Sea, and also the first record of this species in *T. thynnus* as host in Mediterranean Sea. Molecular identification of *H. aduncum* found in co-infection with *Anisakis* larvae type I expands our knowledge of the occurrence of these taxa in the Adriatic Sea. Zoonotic Anisakidae worms found in fish from the Adriatic Sea could represent a risk to acquire parasitic infection/allergies in Croatia.

Introduction

Members of Anisakidae family include parasitic nematodes with complex life cycle. Larval stages can infect many marine and freshwater fish and squid that serve as intermediate/paratenic hosts. Humans can accidentally be infected when consuming raw, undercooked or improperly processed parasitized

fish and squid (Mattiucci et al. 2011). Ingested larvae of the genera *Anisakis*, *Pseudoterranova*, *Contracaecum* and *Hysterothylacium* are a potential human health threat, both as causative agents of zoonosis termed anisakiasis and as food-borne allergens (Hochberg and Hamer 2010). 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 2008). Anisakidae members have a global distribution and their presence in Croatian part of Adriatic Sea was reported previously (Mladineo et al. 2008; Petter and Radujković 1989; Zurak 2010). However, they were not identified by molecular methods that are unavoidable for the accurate species identification. In the present study, Anisakidae species from the fish from the Adriatic Sea were genetically characterised and identified by PCR-RFLP and sequencing of nuclear (ITS, internal transcribed spacer) and mitochondrial (cytochrome c oxidase subunit 2, *cox2*) markers. Determined species are discussed related to the hosts and their geographic origins.

Materials and methods

Sample collection

Parasitological examination of *Anisakis* spp. larvae was performed on different groups of fish samples: tuna farmed in the Middle Adriatic, feral fish close to the tuna farm in the Middle Adriatic (horse mackerel, chub mackerel and saddle bream) and horse mackerels from the fish market (Table 1). Fish were sterile dissected and anisakid larvae were collected, counted, fixed in 75 % ethanol for morphological determination and stored at -80 °C for molecular analysis. Fixed species were stained in Semichon's acetocarmine, dehydrated in graded ethanol series, cleared in methyl salicylate

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Table 1 Sampling data on fish hosts parasitized by Anisakidae larvae

Sample group	Fish host	Sampling date	Locality	Number of fish examined	Number of fish parasitised by Anisakidae larvae	Fish TW (g)	Number of Anisakidae larvae
No. 1	Farmed tuna (<i>Thunnus thynnus</i>)	June 2009 July 2010 November 2010	Farm in the Middle Adriatic N44°01.676' E15°13.173'	n=3	n=1	12×10 ³	226
No. 2	Horse mackerel (<i>Trachurus trachurus</i>)	July/ November 2010	Feral fish close to tuna farm in the Middle Adriatic	n=14	All	305.3±77.0	52.4±3.7
	Chub mackerel (<i>Scomber japonicus</i>)			n=3	All	654.5±50.2	50.3±2.5
	Saddle bream (<i>Oblada melanura</i>)			n=3	All	548.0±43.1	49.3±4.5
No. 3	Horse mackerel (<i>Trachurus trachurus</i>)	February 2011	Fish market 1	n=2	All	189.8±7.3	184.5±38.9
			Fish market 2	n=2	All	166.2±0.2	197.5±20.5

and identified at genus level by light microscopy (Davey 1971; Køie 1993).

Molecular identification of Anisakidae larvae

Anisakidae larvae were identified by analysis of two molecular markers: nuclear (ITS) and mitochondrial (cox2). Total DNA was extracted from a single nematode using the DNA purification kit (DNeasy Blood and Tissue Kit, Qiagen) according to manufacturer's instructions. Polymerase chain reaction (PCR) were performed in the mixture containing 1×PCR buffer, 2.5 mM MgCl₂, 0.4 mM dNTPs, 20 pmol of each primer, 1 U of Taq Polymerase (Applied Biosystems), 50 ng of total DNA and water (Molecular biology reagent, Sigma) to 50 µl. Primers used to amplify partial ITS region and cox2 gene were described previously (Luton et al. 1992; Nadler and Hudspeth 2000). Reaction conditions were as follows: 10 min at 94 °C (initial denaturation), 35 cycles of 30 s at 94 °C (denaturation), 45 s at 56 °C (annealing) and 1 min at 72 °C (extension), with final elongation step of 10 min at 72 °C. ITS products were digested with the restriction endonucleases HhaI and HinfI (Promega), as described previously (D'Amelio et al. 2000; Chaligiannis et al. 2011). Digests were analysed by electrophoresis in 2 % agarose gel. PCR amplicons of ITS region and cox2 partial gene of Anisakidae larvae from various hosts/locations were purified by Gel Extraction Kit (Qiagen) and sequenced commercially (Macrogen, Netherlands). Sequences have been deposited in GenBank under accession numbers JQ934866–JQ934893. Furthermore, they were analysed by GenBank Blast programme and aligned with previously characterised ITS and cox2 sequences of Anisakidae family by ClustalX (Thompson et al. 1997). Only unique sequences were used in phylogenetic trees construction. Phylogenetic trees were inferred using Neighbour-joining method in MEGA programme (Tamura

et al. 2007). The evolutionary distances for NJ tree were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2,000 replicates) is shown next to the branches. Phylogenetic trees were rooted by the included two out-group species, *Ascaris suum* and *Toxocara canis*, as it was described previously by Cavallero et al. (2011).

Results

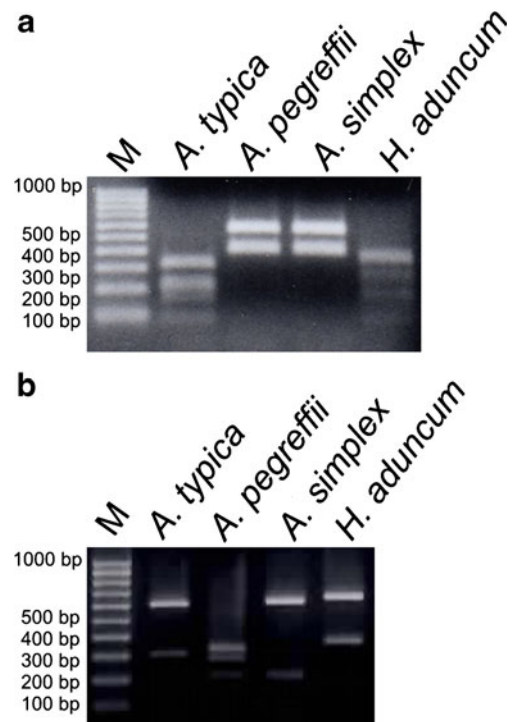
Anisakidae larvae were found in one farmed tuna, all feral fish (horse mackerel, chub mackerel and saddle bream) and all examined horse mackerels from marketed fish (Table 1). Number of Anisakidae larvae was the highest in tuna and marketed horse mackerels. Morphological features based on measurements of 64 Anisakidae larvae identified them as L3 larvae of *Anisakis* Type I (Davey 1971) or *Hysterothylacium* sp. (Køie 1993; Table 2). Identification to the species level was possible only after molecular analysis.

A total of 120 larvae, 20 per fish species collected from different sample groups (Table 1), were subjected to PCR-RFLP analysis of ITS region. Results of restriction digestion of ITS region (approximately 900 bp) with HhaI and HinfI enzymes revealed three different *Anisakis* species: *A. typica* from farmed tuna, *A. pegreffii* from feral and marketed fish and *A. simplex* (s.s.) from marketed fish (Fig. 1a, b). Besides, restriction profile of *Hysterothylacium aduncum* from marketed fish was observed (Fig. 1a, b).

To confirm the results obtained by PCR-RFLP analysis, a sequence analysis of amplified ITS rDNA and mitochondrial cox2 was carried out and NJ phylogenetic trees were constructed. The sequence analyses of partial ITS region (approximately 900 bp) from 18 Anisakidae larvae revealed

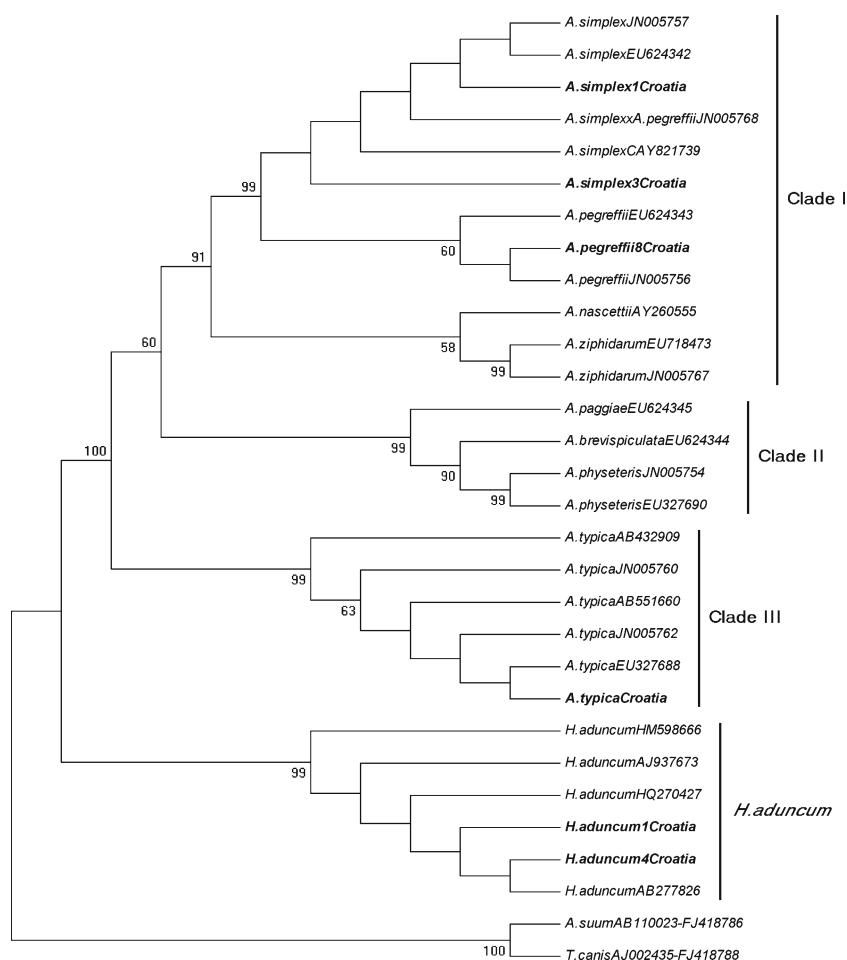
Table 2 Measurements of some morphological characters in *Anisakis* type I larvae and *Hysterothylacium* sp. (larvae were identified to the species level after molecular analyses)

Type of larvae (number measured, fish host)	<i>A. typica</i> (<i>n</i> =15, farmed tuna)		<i>A. pegreffii</i> (<i>n</i> =10, near tuna farm)		<i>A. pegreffii simplex</i> ^a (<i>n</i> =15, market 1)		<i>H. aduncum</i> (<i>n</i> =10, market 1)		<i>A. pegreffii</i> (<i>n</i> =14, market 2)	
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
Body length (mm)	18.58±3.31	13.18–23.02	19.40±3.72	13.53–24.50	7.94±2.26	6.31–13.48	6.88±0.86	5.11–8.08	14.43±2.35	9.75–17.88
Body width (mm)	0.56±0.14	0.40–0.83	0.37±0.07	0.31–0.51	0.24±0.07	0.19–0.44	0.259±0.07	0.20–0.41	0.40±0.06	0.33–0.52
Oesophagus length (mm)	1.31±0.14	1.17–1.61	1.91±0.71	1.33–3.30	1.18±0.22	0.85–1.50	1.15±0.40	0.45–1.75	1.22±0.19	0.74–1.46
Ventriculus length ^b (mm)	0.49±0.07	0.40–0.71	0.63±0.07	0.52–0.73	0.68±0.13	0.50–0.90	0.372±0.06	0.27–0.45	0.51±0.06	0.44–0.63
Body length/body width	33.83±4.33	27.57–41.65	52.82±6.70	43.93–60.62	33.70±5.74	26.85–45.99	27.69±5.96	16.75–34.66	35.87±4.86	27.53–43.19
Body/oesophagus length	12.59±1.81	10.12–14.98	11.28±1.27	10.15–13.21	6.83±1.65	4.25–10.62	6.93±3.44	3.57–15.53	11.72±1.20	10.02–13.75
Body/ventriculus length	32.18±3.65	26.31–35.84	30.63±3.60	26.12–34.94	12.24±5.25	8.04–27.18	18.98±3.93	13.73–24.49	28.44±4.19	21.75–34.78

^a There was no clear morphometric difference between *A. pegreffii* and *A. simplex*^b In the case of *H. aduncum*, ventriculus appendix was measured**Fig. 1** Restriction fragment length polymorphism patterns obtained by digestion of ITS region of Anisakidae larvae with restriction enzymes HhaI (a) and HinfI (b); M molecular marker 1 kb

presence of four different specimens, clustering with similar sequences previously deposited in GenBank (Fig. 2). Sequences of the ITS amplicons were almost identical among the same species. The ITS sequences of *Anisakis* larvae obtained from feral and marketed fish showed 100 % identity and matched the sequence of *A. pegreffii/A. simplex* group. As reported previously (Abollo et al. 2003; Farjallah et al. 2008), sequence divergence of analysed ITS region between *A. pegreffii* and *A. simplex* (s.s.) is only at two polymorphic sites and this difference was used to confirm presence of *A. pegreffii* and *A. simplex* (s.s.) larvae in examined fish. Hybrid-type *A. pegreffii*×*A. simplex* was not confirmed by RFLP-PCR. *A. typica* from farmed tuna had 100 % identity of ITS region with sequences from GenBank. *H. aduncum* from marketed mackerels showed 99.6 to 99.9 % identity of ITS region to the sequences from the GenBank. Another mitochondrial marker, *cox2*, amplified from 10 larvae was used for further analyses of Anisakidae species. Sequence analysis of *cox2* gene (approximately 600 bp) of *A. pegreffii* from feral and marketed fish matched 99.6 to 99.8 % with the sequences of the species *A. pegreffii* deposited in GenBank (Fig. 3). Identity of *cox2* sequence of isolated *A. simplex* (s.s.) species and those from the GenBank was 98.7 to 99.4 %. *Anisakis* species from farmed tuna matched the sequences of *A. typica* previously deposited in GenBank (Valentini et al. 2006) with 99.2 % of similarity. *H. aduncum* from marketed

Fig. 2 Neighbour-joining phylogenetic tree of ITS region sequences obtained in this study (**bold**), reference GenBank sequences and *A. suum* and *T. canis* as outgroup. Values beside nodes correspond to bootstrap percentages (>50 % are shown)



fish showed 91.2 to 92.1 % identity of partial *cox2* sequence to the GenBank sequences of *Hysterothylacium* sp. *Cox2* sequences of *H. aduncum* in fish from Adriatic Sea are the first *cox2* sequence for this species in GenBank.

Discussion

In recent years, high infections with *Anisakis* spp. in the fish from Croatian part of Adriatic Sea were reported (Mladineo et al. 2008; Zurak 2010). However, molecular identification of these parasites to the species level was not made, although species determination is the first parameter for any parasitological survey and epidemiological study (Mattiucci et al. 2011). In the present work, we identified four Anisakidae species in the Croatian part of Adriatic Sea: *A. pegreffii*, *A. simplex* (s.s.), *A. typica* and *H. aduncum*.

Larval identification of examined *Anisakis* spp. was not possible by using certain morphological characters (Table 2). PCR-RFLP analysis of ITS rDNA region amplified with universal primers for ITS regions of different endoparasites (Luton et al. 1992; Krállová-Hromadová et al. 2003) proved to be adequate molecular tool for determination of larger number

of larval specimens of *Anisakis* spp. and *Hysterothylacium* spp. Although for analyses of Anisakidae ITS regions, other primers are usually used (Zhu et al. 1998), the present study confirmed applicability of universal primers pair (Luton et al. 1992). Moreover, ITS amplicons obtained in our study were similar in length with ITS regions that are usually used for Anisakidae molecular analyses (Zhu et al. 1998). Further, sequence analyses of ITS region and *cox2* gene allowed confirmation of distinct *Anisakis* species and also identification of *H. aduncum* species. NJ tree constructed from ITS regions of Anisakidae species provided grouping of *Anisakis* species and also *H. aduncum* into different clades, while those grouping was not clear in NJ tree constructed from the *cox2* sequences. Similar results were published by Cavallero et al. (2011), indicating these two markers as significantly different datasets for Anisakidae species topology.

A. pegreffii is the dominant species of *Anisakis* in Mediterranean Sea, probably due to the occurrence of various dolphin species, such as *Tursiops truncatus*, as the main definitive hosts (Mattiucci and Nascetti 2006). *T. truncatus* is also the only cetacean species that resides in the Croatian part of the Adriatic Sea (Vuković et al. 2010). Due to this fact and previous record of *A. pegreffii* in Adriatic

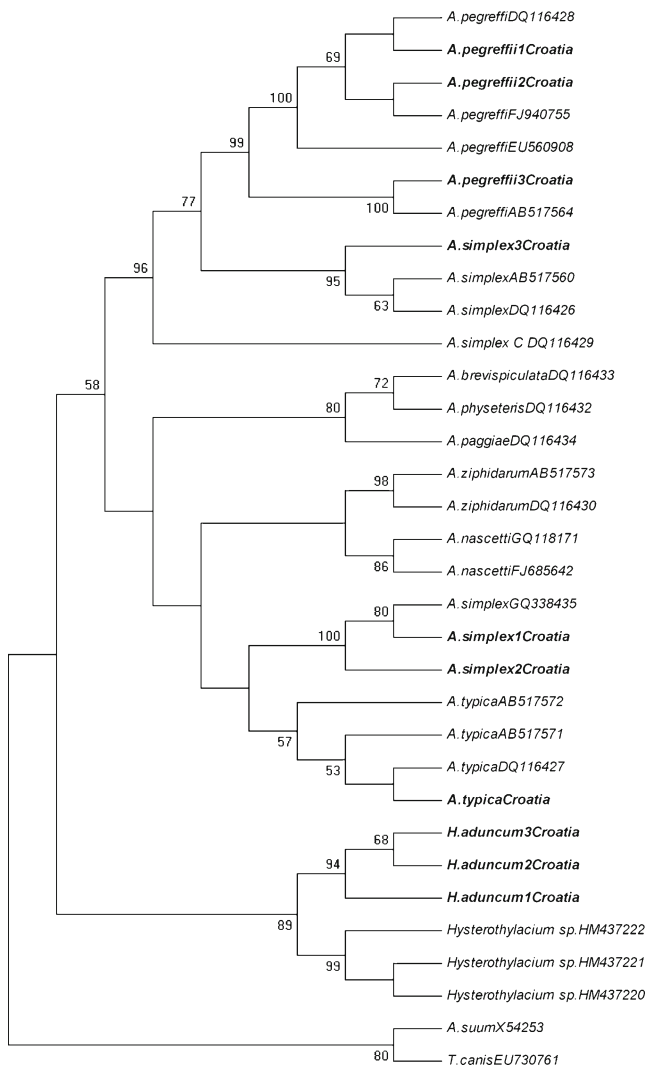


Fig. 3 Neighbour-joining phylogenetic tree of partial cox2 sequences obtained in this study (**bold**), reference GenBank sequences and *A. suum* and *T. canis* as outgroup. Values beside nodes correspond to bootstrap percentages (>50 % are shown)

Sea (Mattiucci and Nascetti 2006), finding of this *Anisakis* species in Croatian part of Adriatic Sea was expected. Horse mackerel (*T. trachurus*), chub mackerel (*S. japonicus*) and saddle bream (*O. melanura*) were the fish intermediate hosts for *A. pegreffii* in this study. These fish species were also previously described as the common intermediate hosts for *A. pegreffii* (Mattiucci and Nascetti 2008).

A. simplex (s.s.) is the dominant in Atlantic and Pacific Oceans, but it is also present in southwestern Mediterranean waters (Farjallah et al. 2008; Mattiucci and Nascetti 2008) and Aegean Sea (Chaligiannis et al. 2011). Although this species was previously described in Croatia by morphological classification (Mladineo et al. 2008; Zurak 2010), our study provide first molecular identification of this species in Adriatic Sea. *A. simplex* (s.s.) was found only in mackerels from marketed fish caught in unknown location in Adriatic

Sea. In studies carried out on fish collected from European markets, morphological identification has shown L3 larvae of *A. simplex* (s.s.) as the most prevalent Anisakidae species, although marketed fish were of different origin (Chaligiannis et al. 2011; Piccolo et al. 1999). Inter-specific hybridization occurring in areas of sympatry of *A. pegreffii* and *A. simplex* (s.s.) species may result in *A. pegreffii/A. simplex* recombinant genotype (Abollo et al. 2003; Chaligiannis et al. 2011; Du et al. 2010; Martín-Sánchez et al. 2005; Umehara et al. 2006). Although in this study *A. pegreffii* and *A. simplex* (s.s.) were found together in the marketed fish, recombinant genotype could not be identified by PCR-RFLP analysis of ITS region.

In our study, *A. typica* was found only in reared Atlantic bluefin tuna, *T. thynnus*. This *Anisakis* species is common in hosts from warmer temperate and tropical waters (Mattiucci and Nascetti 2008). In the Mediterranean Sea, it is distributed in the Eastern Mediterranean Sea off Crete and Cyprus in European hake (Mattiucci et al. 2002) and the North African central Mediterranean coast in European hake, Atlantic mackerel and forkbeard (Farjallah et al. 2008). There are more than 10 known intermediate/paratenic hosts of *A. typica* (Iñiguez et al. 2011), and *T. thynnus* as intermediate host is identified only in Brazil (Mattiucci and Nascetti 2008). Atlantic bluefin tuna displays highly migratory behaviours and trans-oceanic movement (Rooker et al. 2007). Tuna parasite community is remarkably diverse, probably as a result of long distance migration, highly predatory behaviour and longevity (Mladineo et al. 2011; Munday et al. 2003). In Croatia, capture-based tuna aquaculture comprises catch of the small juvenile tuna below 130 cm in fork length and usually 8–15-kg body weight, that through their fattening cycle (1.5–2 years) gain more than 30 kg (Mladineo et al. 2011). Reared tuna are fed by domestic fresh baitfish or imported frozen herrings and sardines. As *A. typica* is found in some fish in Mediterranean Sea (Farjallah et al. 2008; Mattiucci et al. 2002), it is possible that tuna reared in Croatian farm was parasitized by *A. typica* before catching, by feeding with infected fish prey. Absence of *A. typica* in analysed feral fish and fish from the market also indicates possibility that tuna reared in Croatian farm was parasitized by *A. typica* before catching. Mattiucci et al. (2004) suggest that the presence of *A. typica* in eastern Mediterranean Sea could be a consequence of the migration of the definitive/paratenic hosts from the Indian Ocean to Mediterranean waters through the Suez Channel. Farjallah et al. (2008) extended the geographic distribution of *A. typica* to the eastern coast of Tunisia and Libya, and our results show outspread of this anisakid species even northerly in the Adriatic Sea.

H. aduncum is another Anisakidae species found in marketed mackerels from Adriatic Sea. It is a very common fish parasite from North and Baltic Seas, Mediterranean Sea and the North Atlantic and Pacific Oceans (Amor et al. 2011).

This anisakid is also previously described in Adriatic Sea (Klimpel et al. 2007; Petter and Radujković 1989). In this study, *H. aduncum* is detected in marketed mackerel, *T. trachurus*, together with *A. simplex* and *A. pegreffii*. Recent studies also described co-infection of *H. aduncum* with *Anisakis* type I larvae (Chaligiannis et al. 2011; Rello et al. 2009). Rello et al. (2009) showed a higher global parasitization of anchovies (*Engraulis encrasicolus*) by *H. aduncum* than by *Anisakis* spp. in southwestern Europe. Also, *H. aduncum* was the dominant parasite species in *Sprattus sprattus* from North Sea, English Channel, Bay of Biscay and Adriatic Sea (Klimpel et al. 2007). The high levels of infection of this parasite in fish can be explained by the extensive migration of its host species. In the Mediterranean Sea, numerous definitive and intermediate hosts are common in the epipelagic zone, including *T. trachurus* (Amor et al. 2011) which was the only host of *H. aduncum* L3 larvae in our study.

The presence of Anisakidae larvae in examined fish from Adriatic Sea could have public health implications in Croatia. Among the nine species of *Anisakis* so far genetically characterised, the two sibling species, *A. simplex* (s.s.) and *A. pegreffii*, have been identified as agents of human anisakiasis (Mattiucci et al. 2011). *H. aduncum* can also affect human health as causative agent of noninvasive anisakiasis or allergy (Rello et al. 2008). Although well-cooked fish meals are tradition in Croatia, raw domestic fish meat in carpaccio and sushi in some restaurants could represent a risk for human health. 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 conclusion, this study presents first molecular identification of Anisakidae species in fishes from Croatian part of Adriatic Sea. Occurrence of *A. typica* in *T. thynnus* is the first case of this anisakid species in Adriatic Sea and extends its geographical distribution previously limited to the other regions and host species of Mediterranean Sea. The risk of food-borne parasitic infection/allergies after ingestion of fish parasitized with determined *A. pegreffii*, *A. simplex* and *H. aduncum* is a matter of human health concern.

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