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Molecular identification and population dynamic of *Anisakis pegreffii* (Nematoda: Anisakidae Dujardin, 1845) isolated from the European anchovy (*Engraulis encrasicolus* L.) in the Adriatic Sea

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ABSTRACT

Anchovy *Engraulis encrasicolus* (L.) is a coastal pelagic and euryhaline species that represents the only European species of the family Engraulidae, with a widespread distribution. In Croatia, it is marketed fresh, frozen, salted or marinated and mainly exported to Italy and Spain, however *Anisakis* sp. larval infection is frequently the reason for border rejection. Since it is known that the prevalence and intensity of *Anisakis* infection varies with fish species, fishing area and season, the aim of our study was to identify *Anisakis* sp. parasitizing European anchovy and infer its population dynamic through a 2.5-year period. Larvae were found coiled and encysted on the external wall of intestine (94%) and reproductive organs (6%), rarely in fillets. Prevalence was 76.1% (95% confidence limits 74.51–77.56%), mean abundance 6.59 (bootstrap 95% confidence limits 5.81–7.26) and mean intensity 8.67 (bootstrap 95% confidence limits 7.82–9.35). The partial CO2 mitochondrial DNA sequence of the isolated anisakids confirmed clustering of the anchovy parasite within *A. pegreffii* sister group. Parasite population structure showed plasticity inferred by fishing ground, sampling year and fish gender and size. Compared to anisakid prevalence/abundance in other fish, the European anchovy in the Adriatic Sea represents a moderately high-infected paratenic host, although in the Mediterranean and Atlantic waters, anchovies have shown strikingly lesser values of prevalence. Since this host represents one of the most attractive Mediterranean fisheries products traditionally consumed without thermal preparation that in any case would not disrupt larval antigenicity and prevent human allergies, and given the high prevalence of the anisakid within the host, it is necessary to include anchovy into more firm risk assessment frames in order to develop measures that will support the safe alimentary production and consumption of seafood.

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1. Introduction

Nematode genus *Anisakis* Dujardin, 1845, consists of species inhabiting alimentary tract of aquatic vertebrates, displaying indirect life cycle closely related to the marine food web, where crustacean euphausiids act as intermediate hosts, fish and cephalopods serve as paratenic, and cetaceans and pinnipeds represents the final anisakid hosts. *Anisakis* is generalist and ubiquitous parasite, distributed world-wide from temperate to cold and polar waters, having the only limitation in respect to waters with low salinity, where euphausiids cannot propagate. It has been suggested that 23 cetacean species (dolphins, narvals, whales) and 11 pinnipeds (sea lions) offer

reproductive habitat for this nematode, while hundreds of fish species, acting as paratenic hosts, state in favor of the nematode wide niche distribution (McClelland, 2005).

In humans, the zoonotic disease 'anisakidosis' or 'anisakiasis' represents the clinical situation related to infection by living *Anisakis* larvae (Adams et al., 1997; Oshima, 1987; Vidal-Martinez et al., 1994) when consumed in raw or under-cooked fish (for review see Chai et al., 2005; Dorny et al., 2009; Hochberg and Hamer, 2010). Such gastrointestinal anisakiasis reflected as eosinophilic gastroenteritis is triggered by larval penetration through patient gastrointestinal wall. In severe cases of perpetual cycles of infection, underlined with gastroenteric changes, the process today is considered carcinogenic (Petithory et al., 1990; Yoo et al., 2008). Nematodes are contracted through consumption of thermally unprocessed or lightly processed traditional seafood like sushi and sashimi in Japan, tuna or spard carpaccio, marinated, salted or pickled anchovy in Mediterranean (Rello et al., 2009), smoked or fermented herrings (maatjes)

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in the Netherlands (Levsen and Lunestad, 2010), dry, cured salmon (gravlax) in Norway, raw salmon (lomi lomi) in Hawaii or ceviche in South America (Dias et al., 2011).

Second clinic form of anisakidosis is triggered by perpetual consumption of thermally processed seafood where the whole nematode or its molecular traces induce hypersensitivity in the consumer and wide array of allergic symptoms, from acute urticarial and angioedema, to bronchospasm, fatal respiratory arrest and anaphylaxis (Audicana et al., 2002; Anadón et al., 2010; Daschner and Cuéllar, 2010; Daschner et al., 2012). Rheumatologic and occupational disorders like asthma, gingivostomatitis, conjunctivitis and contact dermatitis, have also been related to allergen exposure in fish-mongers, fishermen or employees in fish-processing industry.

As a response to such pathological spectrum in humans, anisakidosis has been regulated by European Community regulations on food fish and products (Directive 91/493/EC; Decision 93/140/EC), and hygiene rules for the hygiene of foodstuffs (Regulation 853/2004/EC; Regulation, 1276/2011/EC) where fish designated to thermal processing have to be subjected to visual examination, manual extraction of *Anisakis*, and removal from the market if heavily parasitized. In cases of fish intended for marinating, salting and any other treated fish products, if the treatment is insufficient to kill the viable parasite, freezing treatment must consist of lowering the temperature in all parts of the product to at least -20°C for not less than 24 h or -35°C for not less than 15 h. US Food and Drugs Administration imposes similar measures and fish products not for cooking or processing at temperatures $>60^{\circ}\text{C}$ have to be blast frozen at -35°C for >15 h or regularly frozen at -20°C for at least 7 days. In 2010, the European Food and Safety Agency (EFSA, 2010) invoked a scientific board for biological hazards, seeking a scientific opinion on risk assessment for parasites in fisheries products, with *Anisakis* spp. accentuated. The panel inferred three main topics that need further focus in order to understand better this threat: research of anisakid life cycle (geographical and seasonal distribution, abundance, prevalence); research of different farming techniques in respect to incidence of anisakids in farmed fish and research of methods targeting decrease of infectivity and inactivation with technological treatments during fish processing. Given such focus on the larval nematode, *Anisakis* is considered the most significant emerging food-borne zoonosis, although in many Mediterranean countries is still misdiagnosed or underestimated.

Anchovy *Engraulis encrasicolus* (L.) represents the only European species of the family Engraulidae, having a widespread distribution: in eastern Atlantic from the North Sea to the coasts of Morocco and, throughout the Mediterranean, to the Black Sea and Azov Sea (Whitehead, 1986). It is a coastal pelagic and euryhaline species, entering lagoons, lakes or estuaries, whereas in winter migrates in deep waters down to 150 m in Mediterranean forming large shoals (Whitehead, 1986). The migration cycle of this species is related to its age and size, thus smaller fish inhabit the coastal regions while larger (older) anchovies migrate towards the open sea water. It is the most important pelagic fish resource in the Mediterranean Sea (Leonart and Maynou, 2003), being the target species of most purse seine and mid-water pair trawl fishing fleet operating across the basin. This fishery mainly relies on the abundance of juveniles and small individuals with a length of less than 13 cm (Millán, 1999; Rodríguez-Roda, 1977). The anchovies are caught all year around, but mainly within the spring–autumn period (Millán and Villamor, 1992; Sinovčić, 2000). Croatian catch of anchovies in the Adriatic Sea increased four times since 2003 and had reached 13,835 tons in 2009 (CBS, 2010). Anchovies are marketed fresh, frozen, salted or marinated and mainly exported to Italy and Spain, however *Anisakis* infection is frequently the reason for border rejection (EC RASFF, 2010).

Since it is known that the prevalence and intensity of *Anisakis* infection varies with fish species, fishing area and season (Mattiucci

et al., 2002; Tejada et al., 2006; Rello et al., 2009), the aim of our study was to: (i) identify *Anisakis* sp. parasitizing European anchovy from the North and South Croatian fishing area of the Adriatic Sea (GSA 17); (ii) infer population dynamic of the parasite in the European anchovy through a 2.5-year period in given area. Deepening our knowledge of the population dynamic of *Anisakis* sp. in the European anchovy, more firm risk assessment matrices as well as HACCP procedure would be available for this important fisheries product in the Mediterranean.

2. Material and methods

2.1. Sampling

A total of 4600 anchovies (*Engraulis encrasicolus*) of different size categories were sampled bi- to trimonthly between January 2009 and June 2011 (Table 1). Fish were caught from two Croatian fishing areas: North (N = $44^{\circ}00' \text{N}$, $14^{\circ}30' \text{E}$; $44^{\circ}00' \text{N}$, 15°E ; $43^{\circ}30' \text{N}$, $14^{\circ}30' \text{E}$; $43^{\circ}30' \text{N}$, $15^{\circ}00' \text{E}$) and South (S = $43^{\circ}10' \text{N}$, $16^{\circ}05' \text{E}$; $43^{\circ}10' \text{N}$, $16^{\circ}35' \text{E}$; $42^{\circ}50' \text{N}$, $16^{\circ}05' \text{E}$; $42^{\circ}50' \text{N}$, $16^{\circ}35' \text{E}$) Adriatic region (GSA 17) (Fig. 1), iced on board, brought to the laboratory on ice and examined the same day. The temperature of fish upon arrival to the laboratory was $3(\pm 1)^{\circ}\text{C}$ measured by temperature probe. Total fish length (Lt) measured from 9.1 to 21.85 cm (16.54 ± 4.02 cm) and total fish weight was 4.26 to 44.35 g (28.92 ± 14.49 g). Samples were eviscerated and filleted into butterfly fillets. Visceral mass and surface were examined for anisakid larvae under stereomicroscope (Olympus, SZX10). Prior to examination under stereomicroscope with an underneath light source, fillets were compressed between two tick compression glasses in order to observe larvae more easily. Larvae were isolated and morphologically identified as *Anisakis* sp. based on mucron and boring tooth presence and appearance of oesophagus and ventriculus (Olympus, BX51).

Ecological terms describing parasitological dynamic: prevalence (P), mean intensity (I) and mean abundance (A) of infection were used according to Bush et al. (1997).

2.2. Statistical analysis

Prior to statistical analysis, obtained data were tested for normality (Anderson–Darling test) and when necessary log-transformed. Sterne's exact 95% confidence limits were calculated for prevalence, bootstrap 95% confidence limits (number of bootstrap replications = 2,000) for mean abundances, variance to mean ratio as measure of over dispersion and exponent of the negative binomial (k) for the parasite skewness, using Quantitative Parasitology 3.0 software (Reiczigel et al., 2008; Reiczigel and Rózsa, 2005; Rózsa et al., 2000). Since parasites typically exhibit an aggregated (right-skewed)

Table 1

Number of total sampled European anchovy (*Engraulis encrasicolus* L.) in the North (N) and South (S) part of Croatian fishing area of the Adriatic Sea from January 2009 to July 2011.

	2009		2010		2011	
	N	S	N	S	N	S
Jan	80	80	80	80	63	63
Feb	80	80	80	80	63	63
Mar	80	80	80	80	63	63
Apr	80	80	80	80	63	63
May	80	80	80	80	63	63
Jun	80	80	80	80	65	65
Jul	80	80	80	80	–	–
Aug	80	80	80	80	–	–
Sept	80	80	80	80	–	–
Oct	80	80	80	80	–	–
Nov	80	80	80	80	–	–
Dec	80	80	80	80	–	–

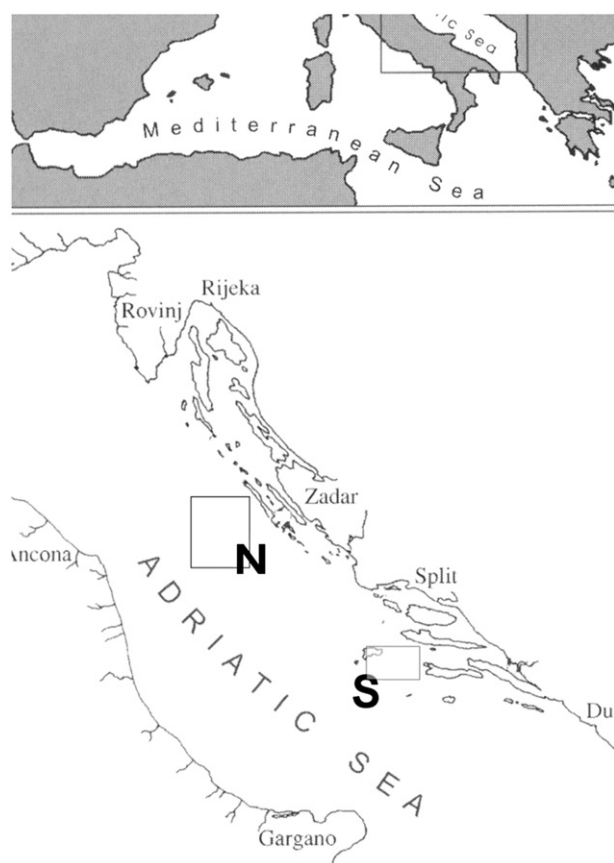


Fig. 1. Geographical area of sampling locations North (N) and South (S) Adriatic Sea are represented by rectangles.

distribution within a host population, the negative binomial model represents the observed data following the maximum-likelihood method (Bliss and Fisher, 1953). Aggregation indices were inferred through variance/mean ratio and discrepancy index D , where $D=0$ and theoretical maximum $D=1$ (Poulin, 1993).

For statistical analysis of differences of abundance between the sexes, size categories, sampling years and geographical area, ANOVA incorporated in statistical package Statistica 7.0 (StatSoft, Inc.) was used. GLM (general linear models) procedure was applied taking into account the fixed effect of all investigated factors. The accepted type I error was 5%. Two-way ANOVA was used to assess the combined effects of sampling years (2009–2011) and geographical areas to abundances, as well as sex and geographic areas. Pairwise comparisons of means for each significant effect were performed using Tukey's test ($p < 0.05$). One-way ANOVA was used to assess the difference in abundances between small and large anchovies. Linear regression model and Pearson's correlation coefficient were used to describe and determine the relation between size categories and prevalence.

2.3. Molecular identification of anisakid larvae

For phylogenetic analysis, genomic DNA was isolated using the QIAGEN DNeasy Blood and Tissue Kit (Qiagen) from eight anisakid larvae isolated from the European anchovy visceral cavity. A ~645 bp fragment of the mitochondrial cytochrome oxidase 2 (CO2) locus was amplified using 0.8 μ M of each of the following specific primers (Petrić et al., 2011): forward primer 5' TCTGACTTCCAATCAGAGG 3' and reverse primer 5' GTCACCTCAAAGCAATGG 3'. The rest of the reaction mix consisted of 2 mM of $MgSO_4$, 220 μ M of dNTP, 22 U/ml of PCR SuperMix High Fidelity DNA polymerase mixture (Invitrogen) and 3 ng/ μ l of template in 50 μ l total reaction volume. The amplification

profile consisted of initial denaturation for 30 sec at 94 °C, 35 cycles of denaturation for 30 sec each at 94 °C, annealing at 52 °C for 30 sec, elongation for 60 sec at 72 °C with final extension of 10 min at 72 °C. PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and sequenced on an ABI 3100 automatic DNA sequencer (Applied Biosystems), using the ABI PRISM BigDye Terminator Cycle Sequencing Kit, in both directions. Sequences were aligned with other anisakid sequences stored in GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html>): *A. pegreffii* DQ116428; *A. simplex* AJ132189; *A. simplex* 1 DQ116426; *A. simplex* C DQ116429; *A. typica* DQ116427; *A. ziphidarum* DQ116430; *A. simplex* SM-2005 DQ116431; *Pseudoterranova ceticola* DQ116435; *A. paggiae* DQ116434; *A. brevispiculata* DQ116433; *A. physeteris* DQ116432, by Clustal X, implemented in the MEGA 5.05 software, using default parameters. The same tool was used to perform neighbor-joining (NJ) analysis, based on p-distance. Reliabilities of phylogenetic relationships were evaluated using non-parametric bootstrap analysis (Felsenstein, 1985) with 2,000 replicates for NJ analysis. Bootstrap values exceeding 70 were considered well supported (Hillis and Bull, 1993). Sequences were added to GenBank and the accession numbers of sequences obtained in this study are following: JQ341905–JQ341912.

3. Results

3.1. Parasite population dynamic

Anisakid larvae were isolated from visceral surface of gastrointestinal tract, with few specimens isolated from muscle fillets (24 in 2009, 53 in 2010, 7 in 2011). Larvae were found coiled and encysted on the external wall of intestine (94%) and reproductive organs (6%). Larval prevalence was 76.1% (95% confidence limits 74.51–77.56%), mean abundance 6.59 (bootstrap 95% confidence limits 5.81–7.26), and mean intensity 8.67 (bootstrap 95% confidence limits 7.82–9.35). Prevalence and mean abundance oscillations in anchovy through the sampling period are depicted in Fig. 2. The smallest infected anchovy measured 9.4 cm and 20.3 cm the largest one. Larvae were aggregated across host populations (left biased), and most anchovies harboured low numbers of parasites (e.g. 1 anisakid in 394 fish) (Fig. 3). The greatest number of parasites ($N=70$) harboured a female of 20 cm and 40.2 g in June 2010 from the South Adriatic region. Variance/mean ratio was 10.355, discrepancy index $D=0.612$. Fitting the negative binomial distribution as a theoretical model to the observed data following the maximum-likelihood method, observed and expected frequencies differ significantly

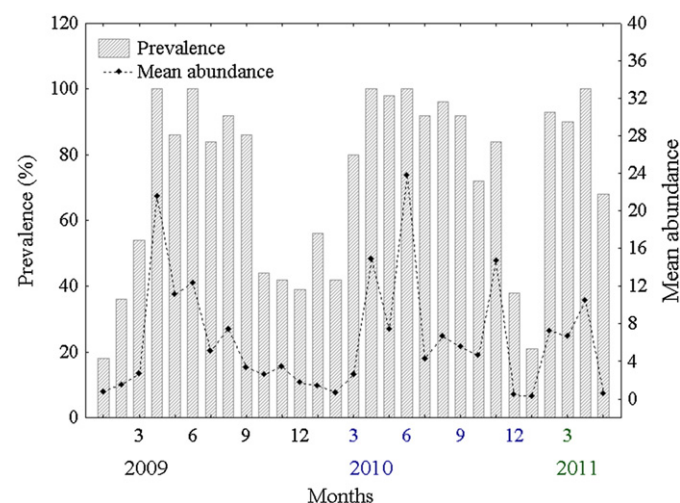


Fig. 2. Oscillation of prevalence (%) and mean abundance of *Anisakis pegreffii* larvae infecting the European anchovy (*Engraulis encrasicolus* L.) in the Croatian fishing area of the Adriatic Sea from 2009 to 2011.

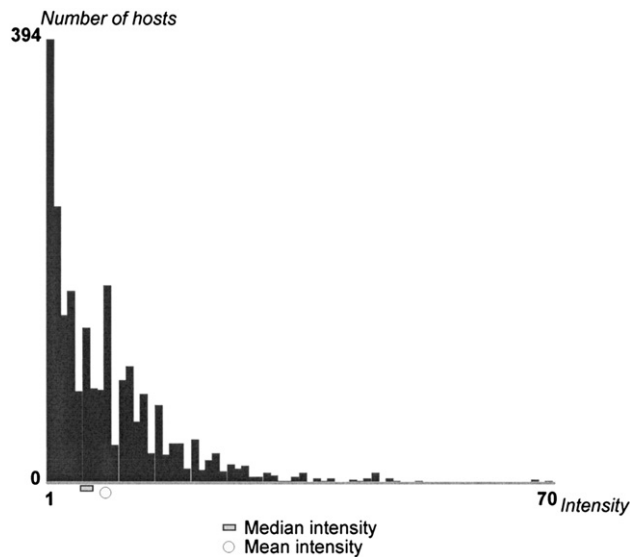


Fig. 3. Intensity of *Anisakis pegreffii* larvae showing left biased aggregation across the European anchovy (*Engraulis encrasicolus* L.) population.

($\chi^2 = 359.99$, $p = 0.05$), with exponent of the negative binomial $k = 0.588$.

Significant differences were found in abundance values through sampling years and habitat. While abundances did not differ in 2009 and 2010, significant difference was observed in 2011, with a decreasing trend. *Post hoc* analysis revealed lower parasite abundance in North compared to South fishing ground (North: prevalence 71.4% with 95% confidence limits 69.08–73.67%; abundance 5.62 with 95% confidence limits 5.31–6.0, mean intensity 7.87, variance/ mean ratio 9.06; South: 81.1% with 95% confidence limits 79.42–82.74%; abundance 7.58 with 95% confidence limits 7.18–8.05, mean intensity 9.34, variance/ mean ratio 10.88). Statistical difference was also noted when comparing abundance values to sex and geographical area: in general males were less parasitized, while female anchovies from South harboured significantly more anisakids compared to North anchovies. No statistical difference was observed between abundances of males in the two fishing grounds.

Higher abundance of anisakids in large anchovies versus small individuals was statistically significant. Linear growth of prevalence with fish total length (TL) and high positive correlation between the parameters were observed ($p = 12.874 \times TL - 124.574$, $R = 0.964$, $p < 0.01$).

3.2. Molecular identification of larvae

The partial CO2 mitochondrial sequence of the isolated anisakid resulted in a 591-bp sequence. Multiple sequence alignment showed that this isolate belongs to *A. pegreffii* (bootstrapping 2,000 generations) and that four haplotypes were present in our sample. Similarly, neighbor-joining analysis generated a consensus tree resulting from 2,000 bootstrap replicates, which clustered anchovy *Anisakis* isolates within *A. pegreffii* clade (Fig. 4).

No ambiguities were present in eight isolates. Estimate of average evolutionary divergence over all *Anisakis* sequences analysed (bootstrap 500), gave the overall mean distance of 0.004 ± 0.001 . There were 11 parsimony informative sites. Within haplotypes isolated in the anchovy, average nucleotide frequencies were: T 45.5%; C 11.8%; A 20.5%; G 22.2%, showing 589 identical base pairs, 2 transitional pairs and none of transversal pairs. Test of the homogeneity of substitution patterns between sequences showed statistically significant (Monte Carlo, 500 replicates) evolutionary pattern of substitution between anchovy larvae 5 and 1 ($p = 0.006$), 2 ($p = 0.020$); larvae 6 and 1 ($p = 0.006$), 2 ($p = 0.020$); and larvae 7 and 5

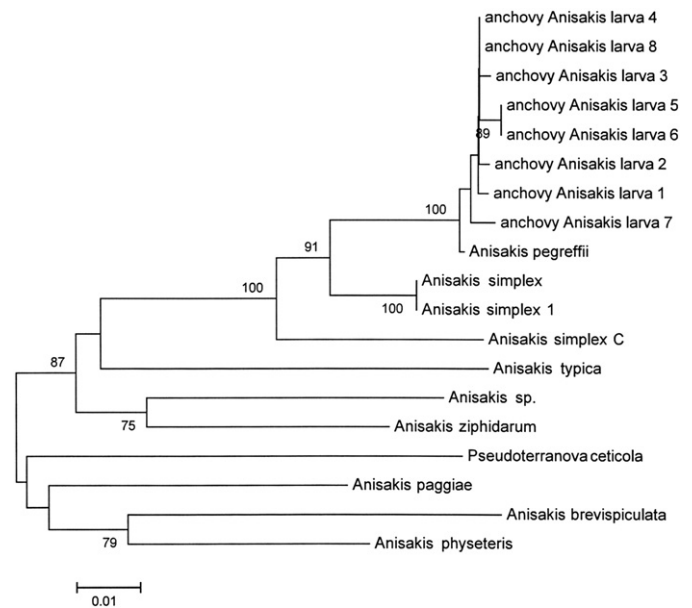


Fig. 4. Unrooted consensus tree generated by neighbor-joining analysis resulting from 2,000 bootstrap replicates. *Anisakis pegreffii* isolated from the European anchovy (*Engraulis encrasicolus* L.) in the Adriatic Sea is represented by 8 isolates clustering with *A. pegreffii* (DQ116428) sister group.

($p = 0.020$), 6 ($p = 0.020$). Maximum likelihood estimate of substitution matrix tested under Tamura–Nei model (Tamura and Nei, 1993) showed that probability of transitional substitution for GA was 10.86; CT 61.39; TC 15.98; AG 11.75.

4. Discussion

Assessing population structure of *Anisakis* sp. in the European anchovy in the Eastern Adriatic Sea, we observed plasticity inferred by fishing ground, sampling year and fish gender. Although previous studies have evaluated *Anisakis* prevalence and abundance in main commercially important species like sea bass (*Dicentrarchus labrax*) (Bernardi et al., 2011), mackerels *Scomber japonicus* (Quiazon et al., 2011; Suzuki et al., 2010), *S. australasicus* (Chou et al., 2011) and *Scomberomorus cavalla* (Dias et al., 2011), Norwegian spring spawning herring (*Clupea harengus*) (Levsen and Lunestad, 2010), anchovy (*E. encrasicolus*) (Rello et al., 2009), as well as few cultured baitfish-fed species—bluefin tuna (*Thunnus thynnus*) (Mladineo et al., 2011) and cobia (*Rachycentron canadum*) (Shih et al., 2010), our study is the first bringing insights in the parasite dynamic in the European anchovy over a 2.5-year period, which inevitably depicts more realistic dynamic that might pass unobserved in 1-year studies.

Parasite prevalence and abundance data show seasonal pattern where peak values have been observed in warmer seasons, congruent for anisakids in other fish hosts. Reasons are related to environmental abiotic and biotic conditions, and authors have suggested that anisakid population is shaped by quantity of shed eggs from migrating population of aquatic mammals in an area, as well as general bloom of plankton in warm season (Chou et al., 2011; Strømnes and Andersen, 2000). Since such factors express fluctuations not only seasonally but over longer temporal periods we argue that parasite surveys extending over 1-year enable more accurate assessment of parasite dynamic in the host population. Similarly, differences in nematode abundances between North and South fishing areas of the Adriatic Sea might be influenced by same conditions. Rello et al. (2009) have demonstrated differences in nematode load between Mediterranean and Atlantic Ocean anchovies, suggesting that the concentration of cetaceans in the Western Mediterranean is the main reason for *Anisakis* increased abundance.

Cetacean population in the Adriatic is approximated to only 220 individuals of the only resident species bottlenose dolphin (*Tursiops truncatus*), however, the knowledge on the present status of dolphins in the Adriatic is rather insufficient (Galov et al., 2011) to draw conclusions in respect to the final host availability.

Although the nematode was present in two thirds of anchovy population, a decreasing trend in abundance was observed in 2011. Data on anchovy stomach contents in the Adriatic could help to postulate whether such phenomenon is related to intermediate or final host availability in the Adriatic. Generally, all life stages of anchovy are zooplanktivorous and the main prey consists of different copepod species (Conway et al., 1998; Betulla Morello and Arneri, 2009; Borme et al., 2009), although data on their parasitisation by *Anisakis* L1 and L2 are missing. Similarly in Algerian waters, Bacha and Amara (2009) have observed that whatever the season, the region or the fish size, anchovy is exclusively zooplanktivorous and copepods were the most present prey, constituting 87% of the prey taken and found in 98% of the anchovy stomachs examined. Up to one third of such prey profile is composed of Euphausiacea, first intermediate host recognised for *Anisakis* today. Although our statistical data unambiguously have shown difference in anchovy abundance between the first two (2009, 2010) and the last sampling year (2011), the reason partially lays in the sampling bias, since the sampling continued till the first half of the 2011, excluding half year of data in the analysis. Moreover, habitat conditions known to affect significantly anchovy population (Basilone et al., 2004) might reflect indirectly to differences in *Anisakis* dynamic as well. Nevertheless, compared to anisakid prevalence/abundance in other fish that range up to 100% (Chou et al., 2011; Mattiucci et al., 2011), the European anchovy in the Adriatic Sea represents a moderately high-infected paratenic host, although in the Mediterranean and Atlantic waters, anchovies have shown strikingly lesser values of prevalence (from 0% to 5.56% and 9.35%, respectively) (de la Torre Molina et al., 2000; Rello et al., 2009; Gutiérrez-Galindo et al., 2010). Previous studies carried in the Adriatic Sea have also evidenced quite different parasite population values (Fioravanti et al., 2003; Losito et al., 2003; Fioravanti et al., 2006) and such underestimation might arise from smaller sample size compared to this study or different environmental conditions.

Significantly higher abundance of anisakids in larger anchovies compared to smaller hosts is attributed to the cumulative effect, when the parasite is acquired through longer life span in larger fish and their continuous re-infestation by feeding. Such observation has been described in many parasitological studies (Bernardi et al., 2011; Petrić et al., 2011; Rello et al., 2009). Interestingly, abundance values were found to differ significantly between fish sexes, with females harbouring more parasites than males. There are biased findings in respect to this observation, since such pattern has not been identified in *Anisakis*-infected spotted mackerel (*S. australasicus*) (Chou et al., 2011) or short-finned squid *Illex coindetii* (Petrić et al., 2011). However, in previous studies the sample comprised approximately 10% of the sample assessed herein, which might contribute to the discrepancy. Moreover, in other host–parasites systems, authors argued that female higher investment in reproduction based on production of larger gametes, makes females more susceptible to parasite infection, finding positive correlation between *Gyrodactilus* spp. and *Raphidascaris acus*, and gonad mass and gonadosomatic index (Šimková et al., 2005).

It is interesting to note that an insignificant number of parasites were isolated from anchovy fillets, contradictory to findings in the wild sea bass (Bernardi et al., 2011), redfish *Sebastes marinus*, cod (*Gadus morhua*) and seithe *Pollachius virens* (Strømnes and Andersen, 1998), fisheries-attractive species in Scotland waters (Petrie et al., 2005) or anchovy from the Atlantic and Mediterranean (Rello et al., 2009). Authors suggested that the distribution patterns between muscle and viscera were governed by the conditions encountered within host tissues, possibly related to the availability of nutrients, like greater

fat reserves in the muscle tissue that induced higher extent of migration towards the muscle. However, *post-mortem* migration is also highly influenced by freshness of the sample, being accelerated with time lapsed from the moment of fish catch (Mladineo, 2001; Suzuki et al., 2010). In our study, anchovies were brought for nematode isolation within couple of hours that did not affect larval *post-mortem* migration. To our knowledge, only Losito et al. (2003) reported a weak prevalence of *Anisakis* larvae in muscle (0.7%) of anchovy from the Adriatic.

The record of *A. pegreffii* represents the first molecular characterisation of the nematode at the species level in the Adriatic Sea anchovy. Its zoogeographical incidence in the Adriatic is not surprising at all, since this is the most frequent anisakid species in the Mediterranean and its adjacent seas. Moreover, Mattiucci et al. (2002, 2008) found the nematode with 100% prevalence in a sample of 116 Adriatic fish, while the short-finned squid *I. coindetii* was suggested as a second paratenic host (Petrić et al., 2011). However, although *A. pegreffii* represents so far the only *Anisakis* species identified in the Adriatic anchovy, novel data indicate that certainly it is not the only one in the Adriatic Sea. Likewise, Blažeković et al. (2012) reported the presence of *A. simplex* and *A. physeteris* isolated from cetacean carcasses of Cuvier's beaked whale (*Ziphius cavirostris*), striped (*Stenella coeruleoalba*) and bottlenose dolphin (*T. truncatus*) found along Croatian coastline, although in much small prevalence (5%), suggesting putative presence of those anisakid species also in fish paratenic host. This sympatry has not been evidenced previously in the Adriatic Sea, but has been observed in Mediterranean (see Mattiucci and Nascetti, 2008 for review). Moreover, Cavallero et al. (2012) have assessed restriction profiles of the nuclear ribosomal ITS region of *Anisakis* spp. larvae recovered from Tyrrhenian Sea fish and have identified *A. pegreffii*, *A. physeteris* and heterozygotes of *A. pegreffii* and *Anisakis simplex* s.s., suggesting wider richness of anisakid species that previously believed in the Adriatic.

Since the European anchovy represents one of the most attractive Mediterranean fisheries product (Solas et al., 2009) traditionally consumed without thermal preparation that has been already related to *Anisakis* zoonosis in men (López Peñas et al., 2000; Foti et al., 2002; AAITO-IFIACI *Anisakis* Consortium, 2011), and given the high prevalence of the anisakid within the host, it is necessary to include anchovy into more firm risk assessment frames in order to develop measures that will support the safe alimentary production or strengthen risk communication to avoid this natural hazard in seafood.

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