Ecology and Genetic Structure of Zoonotic Anisakis spp. from Adriatic Commercial Fish Species

Ivona Mladineo, Vedran Poljak
Institute of Oceanography and Fisheries, Laboratory of Aquaculture, Split, Croatia; Health Ecology Department, Croatian National Institute of Public Health, Zagreb, Croatia

Consumption of raw or thermally inadequately treated fishery products represents a public health risk, with the possibility of propagation of live Anisakis larvae, the causative agent of the zoonotic disease anisakidosis, or anisakiasis. We investigated the population dynamics of Anisakis spp. in commercially important fish—anchovies (Engraulis encrasicolus), sardines (Sardina pilchardus), European hake (Merluccius merlucius), whiting (Merlangius merlangus), chub mackerel (Scomber japonicus), and Atlantic bluefin tuna (Thunnus thynnus)—captured in the main Adriatic Sea fishing ground. We observed a significant difference in the numbers of parasite larvae (1 to 32) in individual hosts and between species, with most fish showing high or very high Anisakis population indices. Phylogenetic analysis confirmed that commercial fish in the Adriatic Sea are parasitized by Anisakis pegreffii (95.95%) and Anisakis simplex sensu stricto (4.05%). The genetic structure of A. pegreffii in demersal, pelagic, and top predator hosts was unstructured, and the highest frequency of haplotype sharing (n = 10) was between demersal and pelagic fish.

Global migration and foodstuff trade, climate change, and novel trends in human eating habits characterized by an elevated demand for raw food are today considered major reasons why reports of food-borne infections, especially zoonotic parasitosis, have increased in the last decade. Therefore, the prevention of and protection against zoonotic parasites in fishery products destined for human consumption have recently become a priority (1). Consequently, health authorities and the fishing industry have become aware of the significance of the nematode genus Anisakis Dujardin, 1845, whose members affect both human health and the commercial value of fish products. Although it is considered one of the most significant emerging food-borne zoonoses, anisakiasis in many countries is still misdiagnosed or underestimated (2).

The genus Anisakis comprises parasites with a complex life cycle (3) and a generalist character that enables it to have a worldwide distribution. Its infective third-stage (L3) larvae are commonly found in the viscera and musculature of many teleost species (4) and migrate postmortem from the abdominal cavity into the fish flesh. Larvae penetrating deeply into the fillets are difficult to detect by visual inspection (5), and a hazard analysis critical control point (HACCP) program must be implemented to reduce the risk (6). Risk management measures for mitigation of anisakidosis have been regulated by the European Community (EC) and the U.S. Food and Drug Administration (7, 8).

Consumption of raw or thermally inadequately treated fishery products represents a public health risk with the possibility of propagation of live Anisakis larvae, the causative agent of the zoonotic disease anisakidosis, or anisakiasis (9, 10). Thermally unprocessed or lightly processed traditional seafood has the highest risk of Anisakis propagation: Japanese sushi and sashimi (9), tuna or spard carpaccio, marinated salted sardines, or pickled anchovies in the Mediterranean (11, 12); smoked or fermented herrings (maatjes) in The Netherlands; (5) dry, cured salmon (gravlax) in Norway; raw salmon (lomi lomi) in Hawaii; or ceviche in South America (13). In humans, larvae penetrate the mucosa of the stomach, small intestine, or colon, causing eosinophilic granuloma formation with severe symptoms of disease (for a review, see references 10, 14, and 15). The other clinical form is triggered by repeated consumption of thermally processed seafood that contains the whole nematode or its molecular traces, inducing hypersensitivity in the consumer and an array of allergic symptoms: acute urticaria and angioedema, bronchospasm, fatal respiratory arrest, and anaphylaxis (16, 17). Rheumatologic diseases and disorders like asthma, gingivostomatitis, conjunctivitis, and contact dermatitis have also been related to occupational exposure to allergens in fishmongers, fishermen, or employees of the fish-processing industry (18).

Although there are many reports on the population dynamics of Anisakis spp. in the Mediterranean (19–22) and the Adriatic Sea (23–25), an updated assessment of infection levels in commercial fish species is important for the implementation of risk analysis. In coastal parts of the Adriatic, traditional home-made, thermally unprocessed fish, mostly pickled, marinated, or salted anchovies and sardines, are particularly well-known ethnic dishes that need to be considered in relation to Anisakis infection in humans, and also because their elevated consumption correlates with the tourist season in the Adriatic. Additionally, Adriatic fish species marketed fresh, frozen, salted, or marinated are also exported to the EC, and Anisakis infection is frequently the reason for rejection at the border (26). Therefore, we investigated the population dynamics of Anisakis spp. in commercially important fish—anchoovies (Engraulis encrasicolus), sardines (Sardina pilchardus), European hake (Merluccius merlucius), whiting (Merlangius merlangus), chub...
mackerel (*Scomber japonicus*), and Atlantic bluefin tuna (*Thunnus thynnus*)—captured in the main Adriatic fishing ground (GSA 17). Molecular identification of isolated larvae was inferred from the mitochondrial DNA (mtDNA) cytochrome oxidase subunit 2 (*cox2*) locus, and a population genetic study was performed to better understand the parasite’s ecological and phylogenetic traits.

**MATERIALS AND METHODS**

**Fish sampling.** The sample consisted of 120 individuals of each commercially important species—anchoovies (*E. encrasicolus*), sardines (*S. pilchardus*), European hake (*M. merluccius*), whiting (*M. merlangus*), and chub mackerel (*S. japonicus*) (*n* = 600)—caught in the wild and 120 bluefin tuna designated for cage culture. The fish (except bluefin tuna) were caught in Croatian fishing area C (43°15′N, 15°0′E; 43°15′N, 16°45′E; 42°10′N, 15°05′E; 42°10′N, 16°45′E), Adriatic region (GSA 17), sampled every month from September 2009 to September 2010. The fish were brought to the laboratory on ice and examined immediately (36 to 48 h postcapture), and the total fork length (cm) and total weight (g) were measured. The temperature of the fish upon arrival at the laboratory was 3°C (±1°C), measured with a temperature probe. The surface of the visceral mass was examined under a stereomicroscope (Olympus; SZX10) for the presence of anisakid larvae. Larvae were isolated and morphologically identified as *Anisakis* spp. based on mucron and boring tooth presence and the appearance of an esophagus and ventriculus (27). The site of parasitization was noted. The abdominal cavity was checked for the presence of nematodes by visual observation, and the pressing-UV method (6) was used to investigate the presence of larval anisakids in the flesh. The prevalence (*P*) (percent), mean intensity (*I*), and mean abundance (*A*) in fish visceral organs and the intensity in fillets (*J*) were calculated according to the method of Bush et al. (28). Wild bluefin tuna (*T. thynnus*) was caught during June 2008 in the central Adriatic waters around the Island of Jabuka (43°5′6″N, 15°28′63″E). The fish (*n* = 120) were transported to the farm, and 30 fish were sampled every 3 months from summer 2008 until spring 2009. The same procedure as for the wild-fish analysis was used for bluefin tuna, except for fillet inspection. Isolated *Anisakis* L3 larvae (*n* = 77) from fish fillets and visceral cavities were morphologically identified as type 1 larvae, washed in phosphate-buffered saline solution, and fixed in ethyl alcohol for molecular identification.

**Statistical analysis.** The data obtained were tested for normality (Anderson–Darling test) and, when necessary, log transformed. Quantitative Parasitology 3.0 software (29) was used to calculate Sterne’s exact 95% confidence limits for prevalence, bootstrap 95% confidence limits (2,000 bootstrap replications) for mean abundances, mean intensity, variance to mean ratio, and the exponent of the negative binomial (*k*). Analysis of variance (ANOVA) (*Statistica* 7.0; StatSoft, Inc.) was used to test the differences in abundance between the sexes and sampling seasons, and pairwise comparisons were performed by the Tukey HSD test (*α* = 0.05). *P* values were corrected using the Bonferroni method of correction. The linear regression model and Pearson’s correlation coefficient were used to determine the relationship between fish length and abundance. Similarities in parasite seasonal abundance were analyzed with PERMANOVA (Primer 6; Primer-E Ltd.) based on the Bray–Curtis similarity index.

**Molecular identification of anisakid larvae.** For phylogenetic analysis, genomic DNA was isolated, amplified, purified, and sequenced at the mitochondrial *cox2* locus (~645 bp), as described previously (30), from anisakid larvae isolated from the fillets and visceral cavities of 6 fish species (*n* = 77). The sequences were aligned with other anisakid sequences stored in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/GenbankSearch.html)—*Anisakis simplex sensu stricto* (DQ116426), *A. pegreffii* (DQ116428), *A. simplex C* (DQ116429), *A. typica* (DQ116427), *A. ziphidiana* (DQ116430), *A. physeteris* (DQ116432), *A. brevispiculata* (DQ116433), *A. poggi* (DQ116434), and *A. nascetti* (DQ116431) (as reported in reference 31)—by Clustal X (32) implemented in MEGA 5.05 software, using default parameters, and further verified by GBLOCKS (http://molevol.cimia.csic.es/castresana/Gblocks.html).

**Genetic diversity and population structure of A. pegreffii.** Molecular diversity was analyzed using DnaSP 5.0 (33) and Arlequin 3.5 (34), and the number of haplotypes (*H*) and polymorphic sites (*S*), haplotype diversity (*h*), nucleotide diversity (*p*), and the average numbers of pairwise nucleotide differences (*k*) were estimated. Pairwise and overall distances among haplotype sequences were calculated in MEGA 5 (35). A substitution model for both genes and the gamma distribution shape parameter for the rate of heterogeneity among sites were determined using Modeltest 3.07 (36), based on the hierarchical likelihood ratio tests (hLRTs). For *cox2* data, the TrN model (37) of evolution with the gamma shape parameter (*G* = 0.01) was selected for the analysis of molecular variances (AMOVA) and phylogenetic analysis. Populations were constructed based on host feeding habits, not fish species, because only a small number of sequences were available for each species. Thus, the pelagic population consisted of nematode sequences from pelagic hosts (*E. encrasicolus, S. japonicus, S. pilchardus*, and *Illex coindeti*—obtained from our previous research [30]), the demersal population consisted of sequences from two demersal hosts (*M. merluccius* and *M. merlangus*), and the top predator population consisted of one cage-farmed host (*T. thynnus*). For evaluation of hypothesized patterns of the spatial genetic structure, a hierarchical AMOVA was used to partition variance components attributable to population variance and to individuals within the populations, where 10,000 permutations were performed to test the significance of pairwise population comparison. Pairwise genetic differentiation between populations was estimated using the fixation index (*Fst*), and statistical significances were tested with 10,000 permutations. Both AMOVA and *Fst* calculations were performed in Arlequin 3.5 (http://cmpg.unibe.ch/software/arlequin35/).

The null hypothesis of population panmixia was also tested in Arlequin 3.5 using an exact test of the differentiation of haplotypes among populations.

Tajima’s *D* and Fu’s *Fs* statistics were calculated to verify the null hypothesis of selectivity neutrality inferred by mtDNA sequences, which would be expected with population expansion.

Mismatch distributions (38) were constructed using Arlequin 3.5. The shapes of the mismatch distributions were used to deduce whether a population had undergone sudden population expansion. The fit between the observed and expected distributions was tested using the Harpending raggedness index (HR1) and sum of squared deviations (SSD) for the estimated stepwise expansion models (39), implemented in Arlequin 3.5. Significance was assessed for the parameters with permutation tests under the null hypothesis that sudden population expansion cannot be rejected. Graphic presentation of genetic diversity and population structure was inferred by activating the R statistics (R.2.15.2) command in Arlequin 3.5.

**Phylogenetic analysis.** Bayesian inference (BI) analysis (40) was performed on the 579-bp sequences using MrBayes v3.1.2 (41) and the Tamura–Nei with gamma distribution (TrN+G) model. Four incrementally heated Markov chains were run for 2,000,000 generations, with sampling every 100 generations, and 5,000 samples were discarded. Default values were used for Markov chain Monte Carlo (MCMC) parameters. A 50% majority rule consensus tree was constructed from the tree output files produced in the Bayesian inference analysis and visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). For tree rooting, *Pseudoterranova decipiens sensu stricto* (AF179920) was used. A median-joining network (42) was used to illustrate *cox2* haplotype distribution among host groups (NETWORK v 4.5.1.6, available at http://www.fluxes-engineering.com/sharenet.htm).

**Nucleotide sequence accession numbers.** The GenBank accession numbers of the sequences obtained in this study are as follows: *T. thynnus*, KC479822 to KC479852; *E. encrasicolus* KC479853 to KC479860; *M. merluccius*, KC479861 to KC479870; *S. pilchardus*, KC479871 to KC479874; *M. merlangus*, KC479875 to KC479883; *S. japonicus*, KC479884 to KC479890.
TABLE 1 Numbers of isolated anisakid larvae (L3) and their parasitisation sites in commercial Adriatic Sea fish

<table>
<thead>
<tr>
<th>Host species</th>
<th>No. of larvae</th>
<th>% parasitation at indicated site</th>
<th>No. of molecularly identified larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L</td>
<td>S</td>
</tr>
<tr>
<td>Anchovy (E. encrasicolus)</td>
<td>827</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sardine (S. pilchardus)</td>
<td>26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hake (M. merluccius)</td>
<td>824</td>
<td>64.53</td>
<td>0</td>
</tr>
<tr>
<td>Whiting (Gadus merlangus)</td>
<td>343</td>
<td>63.72</td>
<td>0</td>
</tr>
<tr>
<td>Chub mackerel (S. japonicus)</td>
<td>1,257</td>
<td>9.07</td>
<td>20.6</td>
</tr>
<tr>
<td>Bluefin tuna (T. thynnus)</td>
<td>202</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Total | 3,479 | 77

TABLE 2 Larval ecological parameters of infection of Anisakis sp. parasites isolated from six fish species over a 1-year period

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Mean host length ± SE (mm)</th>
<th>Mean host wt ± SE (g)</th>
<th>Prevalence (CI) (%)</th>
<th>Mean J (CI), range</th>
<th>Mean A (CI)</th>
<th>Mean J (range)</th>
<th>v/x</th>
<th>k</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. encrasicolus</td>
<td>142.29 ± 7.74</td>
<td>17.73 ± 0.3</td>
<td>81.7 (73.81–87.76)</td>
<td>8.44 (7.26–9.57), 1–23</td>
<td>6.89 (5.83–8.07)</td>
<td>1.25 (1–2)</td>
<td>5.84</td>
<td>0.889</td>
<td>0.507</td>
</tr>
<tr>
<td>S. pilchardus</td>
<td>149.53 ± 12.95</td>
<td>29.8 ± 0.41</td>
<td>3.3 (1.15–8.21)</td>
<td>1.25 (1.18–2.12), 1–5</td>
<td>0.04 (0.01–0.09)</td>
<td>1.37</td>
<td>0.227</td>
<td>0.890</td>
<td></td>
</tr>
<tr>
<td>M. merluccius</td>
<td>342.92 ± 39.71</td>
<td>322.12 ± 6.54</td>
<td>70.8 (62.12–78.41)</td>
<td>9.69 (8.39–11.25), 1–32</td>
<td>6.87 (5.57–8.20)</td>
<td>1.33 (1–2)</td>
<td>7.80</td>
<td>0.566</td>
<td>0.567</td>
</tr>
<tr>
<td>M. merlangus</td>
<td>279.54 ± 18.23</td>
<td>140.3 ± 1.68</td>
<td>65.8 (56.69–73.80)</td>
<td>4.34 (2.32–3.47), 1–13</td>
<td>2.86 (2.32–3.47)</td>
<td>1 (1–1)</td>
<td>3.59</td>
<td>0.790</td>
<td>0.580</td>
</tr>
<tr>
<td>S. japonicus</td>
<td>300.725 ± 8.81</td>
<td>222.63 ± 1.62</td>
<td>100 (96.87–100)</td>
<td>10.48 (9.80–11.28), 1–22</td>
<td>10.48 (9.8–11.28)</td>
<td>2.13 (1–4)</td>
<td>1.60</td>
<td>0.211</td>
<td></td>
</tr>
<tr>
<td>T. thynnus</td>
<td>904.66 ± 99.37</td>
<td>1643 ± 49.1</td>
<td>23.3 (16.59–31.66)</td>
<td>6.5 (5.0–10.00), 1–6</td>
<td>1.78 (1.15–2.67)</td>
<td>10.06</td>
<td>0.091</td>
<td>0.851</td>
<td></td>
</tr>
</tbody>
</table>

a Total mean prevalence with Stern’s exact 95% confidence limits (CI).
b I, intensity.
c A, abundance, with bootstrap 95% CI.
d J, intensity in fillets.
e v/x, variance-to-mean ratio.
f The exponent of the negative binomial (k) showed no statistical difference between observed and expected frequencies at P = 0.05.
g D, discrepancy index (D = 0 to 1).
FIG 1 Graphical representation of genetic diversity and population structures of three *A. pegreffii* populations isolated from hosts from different feeding habitats inferred from the mtDNA *cox2* locus. (A) Frequencies of haplotypes in top predator, demersal, and pelagic fish. (B) Molecular diversity indices: $\theta_w$, average number of nucleotide differences; $\theta_v$, haplotype diversity; $\theta_s$, number of polymorphic sites; $\theta_v$, nucleotide diversity. (C) Haplotype distance matrix inferred from the number of pairwise differences (diff.). (D to F) Observed and expected mismatch distributions with 90 to 99% confidence intervals for *A. pegreffii* populations parasitizing pelagic (D), demersal (E), and top predator (F) fish.
AMOVA, which attributed −0.54% of the genetic variation to variability among populations and 100% variation within populations (see Table S3 in the supplemental material). Over all samples, the nondifferentiation exact P values were not significant (P = 0.83906 ≤ 0.01109), not rejecting the idea that the population of *A. pegreffii* among hosts that feed in three different environmental habitats is panmictic.

**Phylogenetic and network analyses.** The topology of trees built from the cox2 locus using BI (Fig. 2) clustered most of the Adriatic taxa in a younger clade, along with the *A. pegreffii* reference sequence, which in part branched in well-supported groups that formed small, relatively short-branched clades. In the tree’s other segment, polytomy was observed in the lower part within mostly bluefin tuna and a few other fish taxa. No structuring based on habitat use (feeding ground) or fish species was detected. Most of the isolated larvae (*n* = 74; 95.95%) with no ambiguity belonged to *A. pegreffii*, while only three samples (4.05%) clustered with the *A. simplex sensu stricto* isolate. Adriatic isolates of *A. simplex sensu stricto* were isolated from bluefin tuna (*n* = 2) and hake (*n* = 1). The haplotype network showed a star-like phylogeny, with most of the unique haplotypes closely related to the common clade (H6) (Fig. 3), composed of *Anisakis* parasite hosts from all three examined habitats. If *A. pegreffii* underwent expansion, this common central haplotype was probably the ancestral one, from which further stepwise nucleotide substitutions could explain all other unique haplotypes.

**Demographic patterns.** The demographic history of *A. pegreffii* was investigated using mismatch distribution. The goodness-of-fit test showed that no mismatch distributions for sample localities deviated significantly (*P* > 0.05) from predicted values under the sudden-expansion model of Harpending (see Table S4 in the supplemental material). Furthermore, the HRI values were low for all *Anisakis* populations from different habitats, indicating a significant fit between the observed and expected distributions, thereby providing further evidence for population expansion in the past (Fig. 1D, E, and F). The sum of squared deviation also supported a nondeparture from the null hypothesis of population expansion. The 0 values for all cases confirmed the scenario of population expansion; the initial 0 values were always much smaller than the final 0 values (see Table S4 in the supplemental material). Only slight departure from the age expansion parameter (τ) between populations from different habitats suggested that the population expansion may date back to a recent historical period.

The results of Tajima’s D test and Fu’s Fs test are presented in Table S4 in the supplemental material. Tajima’s D values were negative for each population and the populations overall, indicating an excess of rare nucleotide site variants compared to the expectation under a neutral model of evolution, with statistically significant differentiation from the model of neutral evolution. Fu’s Fs test, which is based on the distribution of haplotypes, also showed negative values for each population and the populations overall, indicating an excess of rare haplotypes over what would be expected under neutrality. Following these tests, the hypothesis of neutral evolution was rejected for all populations.

**DISCUSSION**

**Ecological factors of *Anisakis*-fish interaction.** In three commercially important pelagic fish species (anchovies, chub mackerel, and sardines), two demersal fish species (hake and whiting), and one cage-farmed fish species (bluefin tuna) we investigated, previous studies have also identified *Anisakis* spp. in the Adriatic (23, 25, 43, 44). However, no published records exist concerning *Anisakis* infection in sardines and whiting in the east Adriatic, while to our knowledge, *Anisakis* sp. infections in sardines have been recorded only in the eastern Mediterranean (45–47).

We observed a significant difference in the numbers of parasite larvae (1 to 32) in individual hosts and between species, as described previously (23, 25), which underscores the necessity to monitor anisakid infections over a longer time span in order to develop an adequate risk assessment analysis for commercially important fish. Interestingly, the aggregation of the parasite varies with the host species. In anchovies and sardines, it was observed that *Anisakis* spp. have an aggregated, left-biased binominal distribution very common among parasites (48). In contrast, the normal distribution of aggregating larvae was observed in chub mackerel, possibly associated with the limited life span of the parasite and the robustness of the host immune response at a specific length/age (49). We also confirmed the tendency of *Anisakis* larvae toward a particular organ in a specific host (2, 43, 44, 49), which has been related to the availability of nutrients to the parasite (2), although firm evidence has never been provided. We argue that an anatomical relationship between the point of anisakid penetration through the gastrointestinal tract (related to intrinsic host factors, such as gut peristalsis or the quality of its content) and the location of the visceral organ that is close to that point governs the predilection site in the host.

Most commercially important fish species in this study showed high or very high population index values for the parasite, while lower values were observed in farmed bluefin tuna and sardines. Compared to other studies, very similar patterns exist in general, and the variation might be attributed to the initial sampling effort (sample size and period) and/or the sampling ground (ecological and oceanographic characteristics). Similarly, Abattouy et al. (50) concluded that the potential risk factors of *Anisakis* in fish are influenced by the fishing season and fishing grounds and the sex, weight, and length (age) of the fish, for example, previously observed higher values for anchovies (100%) and hake (88%) and lower values for chub mackerel (54%) (43). In the Aegean Sea (47), prevalences in hake (66.6%) and chub mackerel (75%) were similar to our findings, while the prevalences in sardines (5.5%) and anchovies (3.9) were much lower, probably due to the smaller sample size (36 and 77, respectively) and different fishing grounds. Data on the infection of mackerel in the northeast Atlantic Ocean (50) and in reared bluefin tuna (25%) (44, 51) are congruent with this study. We observed oscillations in the seasonal pattern of infection (significant through all sampling seasons, except for spring-autumn) in all examined species except cage-farmed tuna. Temporal variations in the intensity of parasitic infections are common in marine ecosystems due to seasonal changes affecting the physiology of the host (e.g., the intensity of feeding and immune function) (52). The highest level of infection was observed in the warm part of the year, which in the literature has been described as typical for *Anisakis* spp. (50, 53). Likewise, Gutiérrez-Galindo et al. (53) reported the highest intensity in mackerel from the Spanish Mediterranean coast in the summer season. The reasons for seasonal fluctuations in the population dynamics of *Anisakis* spp. are associated with the seasonal fluctuation of biotic and abiotic environmental conditions that indirectly influence the migration of aquatic mammals (final hosts),...
FIG 2 Rooted phylogenetic tree inferred by Bayesian analysis of mtDNA cox2 locus fragments from Anisakis spp., with posterior probabilities shown in different colors (thickest [red] line, 0.9 to 1; thick [orange] line, 0.8 to 0.9; thin [coral] line, 0.7 to 0.8; thinnest [yellow] line, 0.6 to 0.7). A. pegreffii isolated from fish species in the Adriatic Sea is represented by 74 isolates forming a sister clade with A. pegreffii (DQ116428), while 3 isolates (M. merluccius 1, T. thynnus 3, and T. thynnus 4) branched from A. simplex sensu stricto (DQ116426), apart from the A. pegreffii group. (Inset) Magnification of part of the tree with clades composed of A. simplex sensu stricto and A. pegreffii taxa.
the amounts of parasite eggs laid, and zooplankton (intermediate Anisakis hosts) availability (51). Regular seasonal fluctuations of temperature and salinity, the impact of open and deeper waters, and various topographic and hydrographical factors in the fishing area of the eastern Adriatic control the concentrations of chlorophyll a. In turn, phytoplankton has a decisive influence on the distribution and abundance of zooplankton, the first link in the Anisakis life cycle and the main nutritive base for fish. We observed that the host sex had no statistically significant effect on the degree of infection in most studied species, except in hake and whiting, where females were more infected, probably related to the faster growth of females in these demersal species (54). Šimková et al. (55) related the greater infection of the gill monogenean parasites in female fish to the greater investment of females in reproduction, which produces larger gametes than in males. The authors observed a positive correlation between parasite infection and gonad weight and the gonadosomatic index, although this does not fully explain the higher affinity of the parasite for female hosts. In contrast to our study, Mladineo et al. (25) observed a correlation between sex and anisakid infection in a sample of 4,600 anchovies, which highlights the importance of the effect of sample size in parasitic studies.

Positive correlation between the length of the host and the number of larvae was observed in all captured species and was attributed to the cumulative effect of repeated parasite infections, acquired over a longer lifetime for larger (older) fish and constant reinfection through the diet. Bioaccumulation of anisakids that leads to the accumulation of hundreds of parasites in the paratenic host has been described in many studies (21, 23, 30). Mladineo (43) reported a negative correlation for anchovies and a significant positive correlation for hake, while in a more recent work (25), the same author reported a high positive correlation for anchovies. Rello et al. (21) also reported a positive correlation between four groups of anchovies of different lengths and the prevalence of the parasite, and Abattouy et al. (50) recorded a high correlation between parasitic infection and age (length and weight) in S. japonicus from Moroccan coast waters. The only negative correlation in our study was found between the length of the cage-farmed tuna and the abundance of nematodes, indicating that larger individuals were less infected, in contrast to other examined hosts. This pattern has also been described in other parasite species isolated from reared tuna (51), which is very interesting, because the fish in cages are intensively fed the same bait fish they feed upon in the wild, which are significant paratenic hosts for anisakids. This decrease in anisakids (and other parasite species) in tuna has been attributed to the strengthened and stable immunity of properly farmed fish. Similarly, low correlation between the length of mackerel and Anisakis numbers was previously recorded in a group of larger fish (>500 mm), where increasing the length of the hosts decreased parasite intensity. As previously argued, this could be attributed to the immune response of the larger host specimens (49) and limited larval lifetime (2); however, it can have important implications in the risk assessment analysis of categories of large fish. In the case of tuna, this would imply that larger specimens destined for the Japanese sushi and sashimi market are less risky in terms of human anisakidosis than smaller fish, which are usually sold in local markets. Migration of Anisakis sp. larvae in muscle tissue was observed in all sampled host species except tuna and sardines, with an intensity range from 1.00 (whiting) to 2.13 (chub mackerel) larvae. We argue that these numbers might be underestimated, because the visual method we employed, although a reference method of the EC (6), has lower accuracy and sensitivity than molecular methods. Such migrating larvae in fish fillets are the main microbiological (parasitic) hazards in fishery products and thermally untreated dishes made from fish (2), as previously described (21, 49). Because Anisakis prevalence and intensity present the first risk factors for human infection (2), we can conclude that there is a high level of parasite hazard in raw materials originating from our studied species. Moreover, the existence of A. simplex sensu stricto in two Adriatic hosts further aggravates the risk for consumers, as the species has a 12 times greater ability to penetrate into the fillets than A. pegreffii (56). However, such a risk is ultimately negligible, given the very low prevalence and abundance of A. simplex sensu stricto observed in the Adriatic Sea.

**Phylogeny and genetic structure of Anisakis in the Adriatic.** Phylogenetic analysis confirmed that commercial fish in the Adriatic Sea are parasitized by A. pegreffii (95.95%) and A. simplex sensu stricto (4.05%); bluefin tuna and hake), both belonging to the A. simplex complex (57). Previous studies in the Adriatic found only A. pegreffii infection in parthenic hosts (23–25, 30), and our evidence of sympathy of A. simplex in fish is the first recorded for the Adriatic Sea. We argue against the possibility that A. simplex sensu stricto isolated from bluefin is in fact “imported” through tuna feed (North Sea herring) and not an actual Adriatic resident. First, the technical management of the imported bait fish is rigorous; herrings are caught in the North Sea and frozen below −20°C for a period of over 30 days, shipped, and stored deep frozen in order to maintain feed quality. Second, A. simplex sensu stricto was also isolated from hake native to the Adriatic, offering further evidence that the propagation of the species in the Adriatic is achievable. Previously, sympathy of A. simplex sensu stricto and A. pegreffii has been recorded in Mediterranean fish (4), while in the Adriatic, A. simplex sensu stricto was observed, in addition to another anisakid species, A. physeteris, in the final cetacean hosts (58). The authors isolated A. physeteris and A. simplex sensu stricto from Cuvier’s beaked whale (Ziphius cavirostris), spinner dolphin (Stenella coeruleoalba), and bottlenose dolphins (Tursiops truncatus), with an incidence of 5% in sympyty with A. pegreffii (95%).

FIG 3 Haplotype network showing a star-like phylogeny, with most of the unique haplotypes closely related to the common central haplotype (H6). The sizes of the circles match the numbers of sequences belonging to the specific haplotypes. The circle colors represent the three A. pegreffii populations: black, demersal; white, pelagic; gray, top predator. The smallest dark-gray polygonal nodes represent hypothetical haplotypes that were required for the establishment of the existing (sampled) haplotypes.
This strongly supports the presence of anisakid species other than the typical *A. pegreffii* in Adriatic fish, although in a much smaller quantity that makes them almost rare. Cuvier’s beaked whale (*Z. cavirostris*) and spinner dolphin (*S. coeruleoalba*) are highly migratory species whose presence in the open middle Adriatic is occasionally recorded, in contrast to the constantly present bottlenose dolphin (*T. truncatus*) (59), implying dissemination of more than one anisakid species from other seas. Based on a very low incidence of these other anisakid species in Adriatic paratenic hosts, it is still questionable if their colonization and propagation in the Adriatic is successful or is limited to only occasional events.

Analyzing the population genetic structure of *A. pegreffii* in three groups of paratenic hosts divided by their feeding grounds (demersal, pelagic, and top predator), we evidenced the existence of genetically unstructured populations. Mes (60) suggested that parasite ecological characteristics, such as a wide distribution range, the fragmented nature of the habitat, and/or a low expected rate of long-distance dispersal and hermaphroditic reproduction type, contribute to genetic structuring of parasite populations. In contrast, *A. pegreffii* is a generalist, cosmopolitan species with separate sexes, which enables the observed high gene flow and consequent parasite panmixia in the Adriatic. This is further confirmed by negligible *F_\text{ST}* values used to differentiate potential populations. Comparing the haplotype and nucleotide diversity over three anisakid populations, similar values were observed for each of them, further suggesting the existence of a single population that circulates between different levels of the marine food chain in the Adriatic. A combination of high haplotype diversity and low nucleotide diversity is a signature of rapid demographic expansion from a small effective population size (61), which was also supported by Tajima’s D test and the Fu test. The negative value of these tests suggests bias toward rare alleles, typical of recent population expansion and recent colonization events in specific areas. The observed haplotype divergence (*d_{xy}*; 0.2% to 3.5%; average, 0.7%) is mostly in accordance with previous data for anisakid species (4). Mattiucci and Nascetti (4) concluded that such findings confirm that the complex life cycle of these nematodes does not limit gene exchange but enhances it through the high mobility of the different hosts, such as fish and marine mammals.

Three Adriatic haplotypes included anisakids from all feeding grounds (H6, H12, and H16), which can contribute to our knowledge of parasite ecology. The highest frequency of haplotype sharing (*n* = 10) was found between demersal and pelagic fish; whiting and the European hake are demersal, predatory carnivorous species whose main diet consist of small pelagic fish, especially anchovies and sardines (54), supporting our data on shared haplotypes. Thus, both demersal fish species can be considered secondary *Anisakis* paratenic hosts. Haplotypes are also shared between the top predator population and the demersal/pelagic population with similar frequencies (*n* = 4 and 5, respectively). During cage rearing, bluefin tuna is mostly fed small pelagic fish and cephalopods and rarely any demersal species (62). This suggests that part of the tuna infection with *A. pegreffii* originates from its wild juvenile phase prior to rearing (age category, +1 year in 2008). We collected the last reared tuna samples in 2009, which implies that *A. pegreffii* larvae survived in the bluefin for at least 1 year, and probably even longer, enabling a first robust estimate of larval survival within a secondary paratenic host. The second group of *A. pegreffii* haplotypes isolated from the reared bluefin is shared by small pelagic fish, as expected, given the current feeding regime of the bluefin in aquaculture (62).

In conclusion, the risk management measures for *Anisakis* in seafood need to be adapted to each commercial fish species. In this way, all ecological and phylogenetic traits of the host and parasite found in a specific fishing ground will be encompassed, resulting in satisfactory control and mitigation conditions.

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