Medicine Papers, 2018, 4 (1): 7–14

Detection of Anisakidae larvae (Nematoda) in fish products commercialized in Sicily

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Summary

In this work, a total of 1331 fish samples belonging to 15 species and commercialized in Sicily were examined for the detection of Anisakidae larvae and their morphological and molecular identification. All the fish samples came from FAO zone 37, 27, 41, and 87. The initial inspection of the fish samples was carried out according to the EC Regulation 2075/2005. The larvae detected underwent morphological identification by optical microscopy. A PCR- RFLP of the nuclear ITS region (ITS-1, ITS-2 and 5.8 S subunit) was carried out for the identification of the Anisakidae larvae. While the species belonged to the *Hysterothylacium* spp. were recognized at species level by sequencing of cytochrome oxidase 2 (cox2) region. A total of 3700 larvae were detected during the inspection. A statistically significant number of 370 larvae were subjected to molecular analysis; 330 were identified as *Anisakis pegreffii* Campana-Rouget et Biocca, 1955 (Nematoda Anisakidae), 23 as *Anisakis simplex* (Rudolphi, 1809) sensu strictu, 6 as the recombinant genotype *Anisakis pegreffii*/*simplex* s.s., 1 as *Anisakis physeteris* (Baylis, 1923). The sequencing analysis of the 10 larvae recognized as *Hysterothylacium* spp. verified an identity score of 100% with the *Hysterothylacium fabri* (Rudolphi, 1819) Deardorff et Overstreet, 1980 sequence reported in GeneBank. The results obtained provide interesting data on the presence of parasites belonging to the Anisakidae family in fishery products marketed in Sicily, giving an exhaustive risk analysis for Mediterranean population about raw fish consumption. These findings are an excellent tool for assessing and preventing possible risks due to the consumption of these products.

Key Words

Anisakis; Anisakidae larvae; Molecular analysis; PCR-RFLP; Zoonosis.

Received 16.01.2018; accepted 12.02.2018; printed 28.02.2018

Introduction

Anisakidae is a family of parasitic Nematoda with a cosmopolitan distribution and a life cycle involving invertebrates, fishes, and marine mammals (Mattiucci et al., 2004; Setyobudi et al., 2011; Gaglio et al., 2018). In particular, the parasites of the genus *Anisakis* Dujardin, 1845, are characterised by prominent differences in their genetic structure as well as in ecological traits, such as the geographical distributions, the life cycle, and host preferences (Farjallah et al., 2008; D'Amelio et al., 2010). Anisakidae can act as intermediate, paratenic, or definitive hosts (Zhu et al., 1998). In the intermediate host, the larvae penetrate the intestine and invade the celomatic cavity or the muscle, where they moult in the third stage and become encapsulated (Bernardi, 2009).

Anisakis larvae can be divided as *Anisakis* type I or *Anisakis* type II, on the basis of morphological characteristics (Berland, 1961). *Anisakis pegreffii* is the most detectable Anisakidae species in the Mediterranean Sea, with high prevalence of infestation in different pelagic and demersal fish species (Ferrantelli et al., 2015). Other parasites that can be found in the Mediterranean Sea are *A. physeteris*,

A. brevispiculata Dollfus, 1966, and *A. paggiae* Mattiucci, Nascetti, Dailey, Webb, Barros, Cianchi et Bullini, 2005 (Mattiucci & Nascetti, 2008).

The morphological characteristics of these nematodes are not sufficient for species identification. Therefore, accurate and precise molecular methods were carried out to identify correctly these parasites (Zhu et al., 2000; Abollo et al., 2003; Mattiucci & Nascetti, 2006; Setyobudi et al., 2011).

Based on the cosmopolitan and the heterogeneous distribution of *Anisakis* spp. nematodes, it can be assumed that these parasites could be viewed as reliable markers for the traceability of fish products (Mattiucci et al., 2014; Ferrantelli et al., 2015).

Recently, Italy experienced an increase in the consumption of raw and undercooked fish due to importation of exotic typical products such as sashimi and sushi (Bernardi, 2009).

The Regulation 853/2004 imposes to food operators to freeze fish products consumed raw. This procedure is essential to ensure food safety. In fact, there is a risk of presence of parasites in fish fillets that can lead to zoonotical pathologies if the larvae were to be eaten alive. In particular, *Anisakis* nematodes can induce Anisakiasis, a zoonotic disease caused by the consumption of raw or undercooked fish products. *Anisakis* infection in humans causes significant clinical diseases such as sudden epigastric pain, nausea, vomiting, diarrhea, or allergic reaction (Zhu et al., 1998; Setyobudi et al., 2011; Buchmann et al., 2012). About 20.000 cases of Anisakiasis are reported in the literature; over 90% came from Japan and the rest from the European Union, the United States of America, Canada, New Zealand, Chile, and Egypt (Chai et al., 2005).

The Scientific Panel on biological hazards (BIOHAZ) of the European Food Safety Authority (EFSA) recommended to the member states to carry out coordinated studies, with the purpose to increase knowledge about the zoonotic diseases linked to *Anisakis* (EFSA, 2010).

The present study aimed to estimate the prevalence of infestation of Anisakidae nematodes in the fish products commercialized in Sicily and to estimate the geographical distribution of the Anisakidae infestations.

Material and Methods

A total of 1331 fish samples belonging to 15 species were collected (Table 1). The samples were stored at +4 °C and -20 °C and transported to the laboratories of the National Reference Center for Anisakiasis (C.Re.N.A.) for the preliminary inspection analysis.

Anisakidae parasites research and identification of morphological characters

The fish samples were examined for the presence of Anisakidae nematodes in the stomach, intestine, abdominal cavity, and muscles by visual inspection (Fig. 1). Fish samples were sectioned into a caudocranial sense and opened for the detection of parasites by visual inspection. Parasites were inspectioned by a stereo-microscope (Zeiss CL 1500 ECO; Zeiss, Oberkochen, Germany). A further chloro-peptic digestion of viscera and muscles of all the fish samples was carried out. All the nematode larvae collected were preserved in ethanol 70% for 24 hours and subjected to morphological analysis for the genus identification. The morphological analyses were carried out by optical microscopy (Leica DM 3000; Leica, Wetzlar, Germany) after clarification of parasites with glycerol 80% (Berland, 1961).

Molecular analysis

Subsequently, the larvae were subjected to molecular identification at species level by PCR-based restriction fragment length polymorphism (PCR-RFLP) of the rDNA ITS region, including the internal transcribed spacers ITS-1, ITS-2, and 5.8 S subunit.

The larvae were rehydrated with sterile water, fragmented with a scalpel, and placed in a 2 ml tube with 200 µl of nuclease free water then frozen at –20 °C for 24 h.

DNA extraction

Genomic DNA extraction were conducted with special kits based on the use of silica minicolumns, according to the manufacturer's instructions. The concentration of the extracted DNA was assessed by spectrophotometric method at 260 nm. The solution containing DNA was stored at -20 °C, to avoid re-

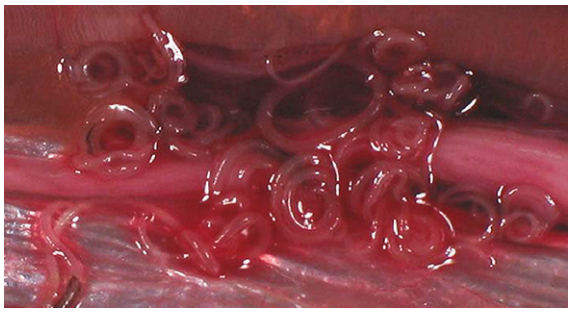

Figure 1. Visual inspection of a fish's viscera for the detection of parasites.

Species	FAO 37	FAO 37.1.3	FAO 37.1.1	FAO 37.2.2	FAO 27	FAO 37.3	FAO 41	FAO 87	Total
Sardinella aurita	31	–	–	–	–	–	–	–	31
Conger conger	–	–	–	8	–	–	–	–	8
Engraulis encrasicolus	434	114	85	–	75	–	–	88	796
Lepidopus caudatus	3	–	2	3	–	–	–	–	8
Loligo vulgaris	–	–	–	10	–	–	–	–	10
Lophius piscatorius	2	–	–	4	–	–	–	–	6
Merluccius merluccius	49	3	13	15	–	–	–	–	80
Sardina pilchardus	106	–	111	32	15	–	–	–	264
Scomber scombrus	11	–	10	2	9	–	–	–	32
Scorpaena scrofa	4	–	–	3	–	1	–	–	8
Todarodes sagittatus	11	4	–	9	–	–	3	–	27
Trachinus draco	4	–	–	2	–	1	–	–	7
Trachurus trachurus	11	–	26	5	–	–	–	–	42
Trigla lyra	–	–	–	5	–	–	–	–	5
Zeus faber	–	–	–	7	–	–	–	–	7
Total	**1331**								

Table 1. Types and number of samples analysed.

peated freezing and thawing which may interfere with the amplification reaction.

DNA amplification

The DNA amplification was carried out by polymerase chain reactions (PCR) of the complete ITS region (ITS-1, ITS-2, and 5.8 S subunit). A PCR master mix containing anhydrous reagents, water, and primers NC5 (5'-GTA GGT GAA CCT GCG GAA GGA TCA TT-3') and NC2 (5'-TTA GTT TCT TTT CCT CCG CT-3') was prepared in reaction tubes, for a final volume of 25 µl.

Subsequently, samples were transferred into a Termal Cycler (2720 Applied Biosystems; Applied Biosystems, Carlsbad, CA, USA) and subjected to the following PCR condition: 95 °C for 10 min; 35 cycles of 30 s at 95 °C, 30 s at 58 °C, and 1.5 min at 72 °C; final polymerisation at 72°C for 15 min. The PCR products were separated by electrophoresis in 1% agarose gel, stained with SYBR safe® (Invitrogen) in Tris-Borate- EDTA buffer, then visualized by UV transilluminator.

The PCR amplification was subjected to restriction fragment length polymorphism (RFLP).

PCR-RFLP analysis

Two different restriction enzymes (HhaI and HinfI) were used for the identification of the *Anisakis*

species. Two different reaction mixes were prepared for each enzyme in a final volume of 20µl containing 3µl of DNA, 13.8µl of distilled water, 1µl of restriction enzyme, 2µl of enzyme buffer and 0.2µl of BSA.

The digestion was performed by incubation at 37 °C over night. The digestion products were electrophoresed in 2% agarose gel (Invitrogen), stained with SYBR safe® and visualized by UV transilluminator.

The size of the fragments were determined by comparison with the molecular weights marker and positive control. The results were interpreted following the keys for the identification of the different species of *Anisakis* described by D'Amelio et al. (2000) and Abollo et al. (2003), implemented by Pontes et al. (2005).

The PCR products were visualized by electrophoresis on 2% agarose gel with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) and visualized by an UV transilluminator.

DNA sequencing

The mitochondrial cox2 region of the larvae examined was also amplified using the primers 210 (5′-CAC CAA CTC TTA AAA TTA TC-3') and 211 (5′-TTT CTA GTT ATA TAG ATT GRT TYA T-3') (20 pmol/µL) with RNAsi and DNAsi-free water, buffer 1x, MgCl2 2 mM, DNTPs 0.2 mM, Taq gold polymerase (6 U), and 10-20 ng of DNA, in a final volume of 50µl. The PCR conditions were as follows: 8 min at 95 °C, 35 cycles of 50s at 95 °C, 1 min at 52 °C, 1 min at 72 °C, and a final extension of 72 °C for 7 min in a Termal Cycler 2720 (Applied Biosystems, Foster City, USA).

The purification of ITS and cox2 gene amplification products was carried out with Illustra GFX PCR DNA and Gel Band Purification kit, following the manufacturer's instructions.

The purified products were sent to Macrogen company (Amsterdam, Holland) for Sanger sequencing. The obtained sequences were compared with previously characterised ITS and cox2 sequences of Raphidascarididae and Anisakidae family present in GenBank™ by the software Basic Local Alignment Search Tool (BLAST).

Results

The results obtained revealed the presence of Anisakidae larvae in 450 fish samples.

In particular, 99 anchovies, *Engraulis encrasicolus* (Linnaeus, 1758), 3 anglers, *Lophius piscatorius* Linnaeus, 1758, 25 hakes, *Merluccius merluccius* Linnaeus, 1758, 12 sardines, *Sardina pilchardus* (Walbaum, 1792), 22 mackerels, *Scomber scombrus* Linnaeus, 1758, 4 red scorpionfishes, *Scorpaena scrofa* Linnaeus, 1758, 6 flying squids, *Todarodes sagittatus* (Lamarck, 1798), 5 greater weevers, *Trachinus draco* Linnaeus, 1758, 35 horse mackerels, *Trachurus trachurus* (Linnaeus, 1758), and 4 John Dory, *Zeus faber* Linnaeus, 1758, were infected by Anisakidae larvae. All the samples of conger, *Conger conger* (Linnaeus, 1758), scabbardfish, *Lepidopus*

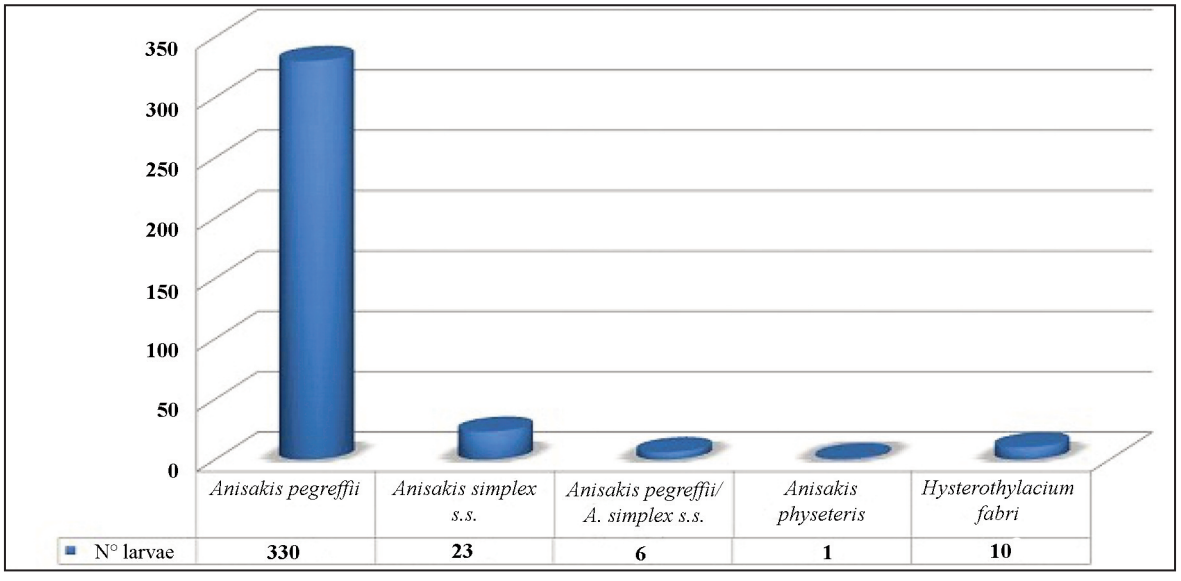

Figure 2. Molecular identification of the Anisakidae larvae.

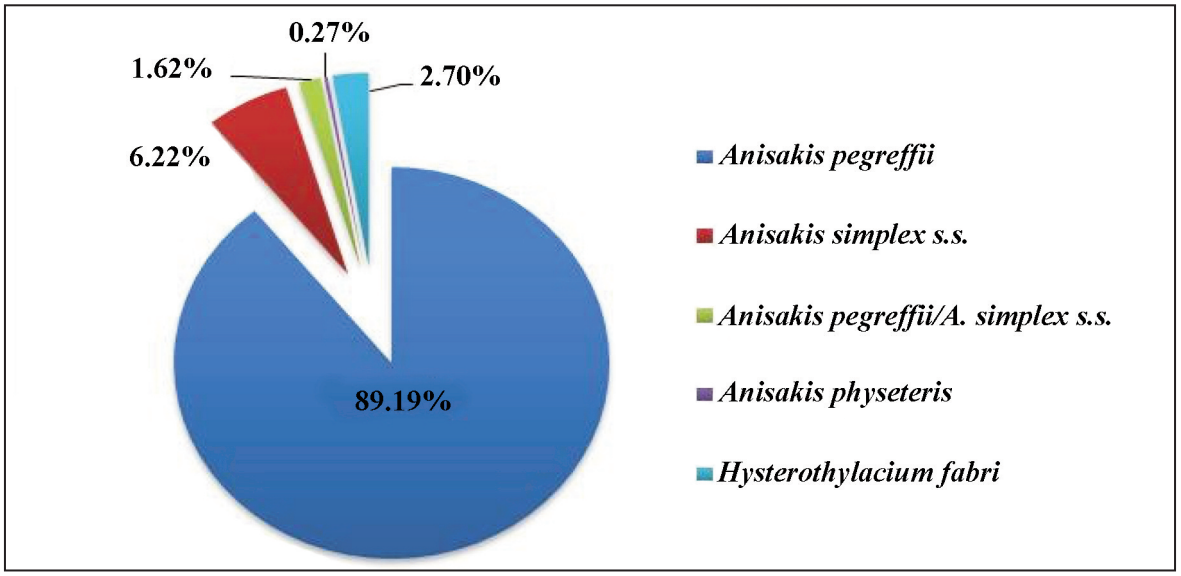

Figure 3. Occurrence of the Anisakidae larvae identified by molecular analysis.

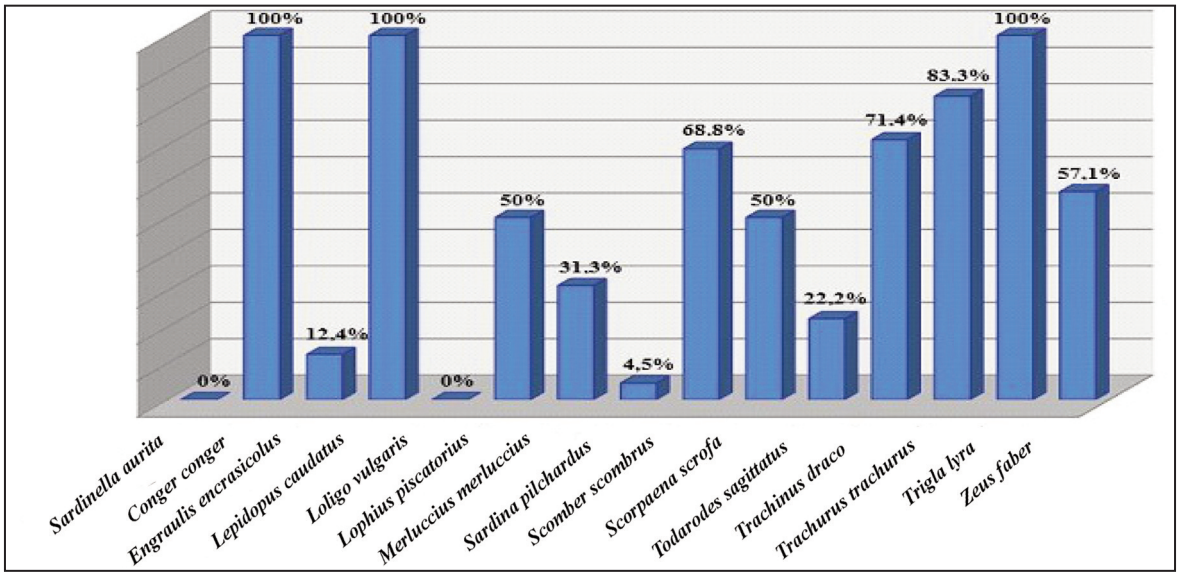

Figure 4. Infestation percentage in the examined species.

caudatus (Euphrasen, 1788), and piper gurnard, *Trigla lyra* Linnaeus, 1758, resulted infected, with a prevalence of 100%. The scabbardfish was the species with the highest mean abundance value (450).

The presence of anisakid nematodes was extensively reported in the horse mackerel, in the anchovy, and in the mackerel. The lower prevalence was found in angler, red scorpionfish, and John Dory.

No Anisakidae larvae were found in sardinella, *Sardinella aurita* Valenciennes, 1847, and common squid, *Loligo vulgaris* Lamarck, 1798. The molecular analysis was carried out on 370 larval samples, of which, 330 belong to *A. pegreffii* (89.2%), 23 to *A. simplex* sensu strictu (6.2%), 6 to the recombinant genotype of *A. pegreffii*/*A. simplex* s.s. (1.6%), 1 to *A. physeteris* (0.3%), and 10 to *Hysterothylacium fabri* (2.7%) (Figs. 2, 3).

The molecular analysis confirmed *A. pegreffii* as the Anisakidae parasite most present in the Mediterranean Sea (FAO zone 37-37.1-37.2-37.3). It was also found in samples of *Engraulis encrasicolus* from the North East Atlantic (FAO zone 27) and in a sample of *Todarodes sagittatus* from the South Atlantic (FAO zone 41).

Furthermore, the presence of *A. simplex* s.s. was found in mackerels and in European anchovies from the North East Atlantic (FAO area 27). Only one sample of salted anchovies from the Mediterranean revealed the presence of *A. simplex* s.s.

The hybrid genotype (*A. pegreffii*/*A. simplex* s.s.), as described by Abollo et al. (2003), was found in samples of *Trachurus trachurus*, *Scomber scombrus*, *Merluccius merluccius*, and *Engraulis encrasicolus* from FAO areas 37.1.3; 37.1.1; 37.2.

Anisakis physeteris was found in only one sample of *Conger conger* from FAO zone 37.2.2. (Sciacca coast). *Hysterothylacium fabri* has been identified in demersal fish samples such as *Conger conger*, *Lophius piscatorius*, *Trigla lyra*, *Trachinus draco*, and *Merluccius merluccius* from FAO areas 37.2 and 37.1. In one case, it has also been identified in one mackerel from the FAO area 27.

The amplification of the mitochondrial cox2 gene (cytochrome oxidase 2) generated a 629-bp sized product. D'Amelio et al. (2010) suggested that this region is highly reliable for species discrimination.

Discussion

One thousand three hundred and thirty-one fish samples were examined for the Anisakidae larvae detection by visual inspection (Table 1).

The percentage of infestation for each species examined is shown in figure 4.

The prevalence values for the species *Sardinella aurita*, *Engraulis encrasicolus*, *Sardina pilchardus*, and *Trachurus trachurus* were lower than what was found in the same species from Northern Sardinia (13.3%, 65.58%, 13.1%, and 100%, respectively) (Piras et al., 2014); otherwise, the *Merluccius merluccius* prevalence found in this work seems to be similar (37.5%).

A lower prevalence of the infestation in the Tyrrhenian Sea has been found for horse mackerel (100%) and anchovies (100%) (Cavallero et al., 2012).

The prevalence values found in this work for anchovies were in contrast to what was found by Cipriani et al. (2018) in South Sicily, giving prevalence values of 0%.

Even the prevalence for mackerel was found lower than the samples collected from the North African coast (Farjallah et al., 2008), giving a prevalence values of 92.3%.

Furthermore, our results confirmed the high prevalence for horse mackerel and scabbardfish reported by Cavallero et al. (2012) in the Tyrrhenian Sea.

Our results for the European hake prevalence were lower than what was reported in literature (Valero et al., 2006; Cipriani et al., 2015) for the Atlantic and the Mediterranean Sea.

The data obtained showed prevalence values much lower than Chaligiannis et al. (2012) in *Merluccius merluccius* and *Scomber scombrus* samples from the Greek coast.

This study confirmed the highest prevalence of *A. pegreffii* in FAO zone 37 (Mediterranean Sea), and the presence of *A. simplex* s.s. and Anisakis pegreffii hybrid forms in the Mediterranean Sea (Costa et al., 2016). In the Mediterranean Sea, the presence of *A. pegreffii*/*A. simplex* s.s. hybrids and *A. simplex* s.s. was mostly detected in FAO sub-areas 37.1.1 and 37.1.3. These two fishing areas are very close to the Strait of Gibraltar, and then to the Atlantic seaboard (Ferrantelli et al., 2015).

A co-infestation between *A. pegreffii* and *A. simplex* s.s. was found only in the horse mackerel samples.

This hybridisation could derive from migratory phenomena from the Atlantic to the Mediterranean Sea (Abollo et al., 2003).

The occurrence of *A. simplex* s.s. was mostly showed in FAO zone 27.

The results provide an exhaustive evaluation on the presence of Anisakidae parasites in fishes caught and commercialized in Sicily in order to have a risk assessment associated with the consumption of these products.

Conclusions

It is possible to confirm a valuable presence of Anisakidae parasites in fish caught and commercialized in the Sicilian territory.

The high prevalence in anchovies found in this work compared to other studies shows the necessity to pay attention to the consumption of raw anchovies products, which represents a major risk for the fish-borne zoonosis anisakiasis in Italy, because of the high consumption of marinated anchovies.

Furthermore, the study confirmed a geographical pattern of distribution of the Anisakidae infestation, showing the highest prevalence of *Anisakis pegreffii* in the Mediterranean Sea.

The results obtained show a satisfactory risk assessment associated with the consumption of fishery products in Southern Italy.

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