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PRESENCE OF ANISAKIS PEGREFFII IN FARMED SEA BASS (*DICENTRARCHUS LABRAX* L.) COMMERCIALIZED IN SOUTHERN ITALY: A FIRST REPORT

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Highlights

- 151 farmed European sea bass samples were examined for *Anisakis* larvae detection
- Two nematode larvae were found in the viscera of only one sample (prevalence 0.7%)
- The larvae were confirmed as *Anisakis pegreffii* by PCR-RFLP and sequence analysis
- The larvae detected belonged to a sample from farms of Greece (FAO 37.3)
- This work give a first report on the presence of *Anisakis* in farmed European sea bass

ABSTRACT

We examined 151 European sea bass (*Dicentrarchus labrax* L.) samples from farms and fish markets of Sicily (Southern Italy) for Anisakidae larvae detection. All the samples were examined by visual inspection and modified chloro-peptic digestion. Two nematode larvae were found in the viscera of only one European sea bass sample from a farm located in Greece (FAO 37.3), giving a total prevalence of infestation of 0.7%. No other parasites were found after chloro-peptic digestion of the samples. The larvae were morphologically ascribed, at genus level, to morphotypes I and molecularly identified as *Anisakis pegreffii*. To the best if our knowledge, this is the first report on the presence of anisakid parasites in farmed European sea bass of Mediterranean Sea. Our findings suggest that the risk of exposure to Anisakidae nematodes in farmed European sea bass remains very low. However, further data on Mediterranean farms are needed to have a detailed risk analysis.

***Keywords**: Anisakis spp.;* Aquaculture; Farmed fish; Visual inspection; PCR-RFLP.

1. INTRODUCTION

Anisakis and other nematodes belonging to the Anisakidae family are parasites with a worldwide distribution and a complex life cycle which involves several marine organisms at different trophic levels (Costa et al., 2016; Ferrantelli et al., 2015; Mattiucci and Nascetti, 2006). Anisakids parasites are of interest for health because of their high zoonotic capability. These nematodes are known to cause clinical manifestations called Anisakiasis or Anisakidosis in humans after the consumption of raw or undercooked parasitized fish. The clinical manifestations of Anisakiasis and Anisakidosis include epigastric pain, nausea, vomiting, diarrhoea and urticaria (Mattiucci et al, 2013).

In addition, several authors confirmed the capability of certain *Anisakis simplex* complex proteins to induce acute allergic reactions in humans (Audicana and Kennedy, 2008). Gastroallergic reactions

associated with *Anisakis pegreffii* infection, were reported in Italy (Mattiucci et al, 2013). In response to these findings, the European Food Safety Authority (EFSA) published a Scientific Opinion on risk assessment of parasites in fishery products (EFSA, 2010). The EFSA scientific opinion declare that the risk of infection with anisakids is negligible only for farmed Atlantic salmon reared in floating cages or onshore tanks and fed on compound feedstuffs. In this regard, most of the studies reported the absence of anisakid nematodes in farmed Atlantic and Pacific salmon (Angot and Brasseur, 1993; Lunestad, 2003). Farmed fish have not generally been regarded as infected with anisakid larvae. Apart from farmed Atlantic salmon, sufficient monitoring data are not available for any other farmed fish therefore the EFSA encourage epidemiological studies on the fish species reared in European territory. At present, few studies have focused on the prevalence of anisakid nematodes in farmed fish of Mediterranean Sea such as European Sea Bass (*Dicentrarchus labrax* L.) and Gilthead Sea Bream (*Sparus aurata* L.) (Peñalver et al., 2010). Of these, no information on infection with larval anisakids are available. However, the experimental susceptibility of Gilthead Sea Bream (*Sparus aurata* L.) and European bass (*Dicentrarchus labrax* L.) towards *Anisakis* has been demonstrated (Macrì et al, 2012). European Sea Bass is one of the principal fish farmed in the Mediterranean. Although seabass are farmed in seawater ponds and lagoons, the bulk of production comes from sea cage farming.

The probability of infection of the wild juvenile fish used as basis for this culture method is still unknown. In Mediterranean, the majority of the wild fish belonged to Italian and Greek coasts are infected with larvae belonging to the *A. pegreffii* species (Abollo et al, 2003; Chaligiannis et al, 2012; Ferrantelli et al., 2015). Given this, the present work aimed to evaluate the presence of anisakid nematodes in farmed European sea bass produced and commercialized in Southern Italy to obtain a detailed risk assessment on the presence of zoonotic anisakid larvae in farmed fish of Mediterranean.

2. MATERIALS AND METHODS

2.1 Sampling plan and sample collection

A total of 83 European sea bass from 2 farms located in Licata (40 samples) and Pachino (43 samples), (Sicily, Southern Italy) were sampled during the period of January 2015 to August 2016. The farm sited in Licata (37°05'N 13°56'E) adopt offshore floating cages and compound feed supply as a rearing technique. The farm sited in Pachino (36°42'N 15°07'E) adopt an indoor rearing technique for hatchery and open sea floating cages for fattening. The European sea bass of this farm are fed exclusively with fishmeal. Further, 68 farmed European sea bass samples were collected from 13 fish markets of north-west Sicily. Based on what was stated on the label, all the 68 farmed fish samples came from farms located in Greece (FAO 37.3). All the fish samples described above were intact. The samples were stored at + 4 °C and transferred to the laboratories of the Centro di Referenza Nazionale per le Anisakiasi for the morphometric and inspection analysis.

2.2 Inspection of the samples and morphological analysis of the larvae

The fish samples were initially submitted to morphometric analysis by the calculation of maximum standard length and Fulton's condition index - K. A parasitological exam was performed on fish samples examining accurately the coelomic cavity and muscle by visual and stereoscopic inspection. For visual and stereoscopic inspection, the fish were dissected by making an incision along the ventral line in a caudocranial direction. Subsequently, a chloro-peptic digestion of viscera and muscles was carried out according to the protocol reported by Cammilleri et al.(2016). All the nematode larvae collected were washed in saline solution, fixed in 70 % ethanol and cleared with glycerol for morphological identification by light microscopy Leica DM 2000 (Wetzlar, Germany), following the taxonomic keys (Berland, 1961).

2.3 Molecular analysis

All the larvae were submitted to molecular identification at species level by PCR- based restriction fragment length polymorphism (PCR-RFLP) analysis of the rDNA comprising the internal transcribed spacers ITS (ITS-1, 5.8S gene, and ITS-2) region.

Furthermore, a PCR amplification and sequencing of the ITS region was carried out. The larvae were washed, fragmented with a scalpel and frozen at -20 °C for 24 hours. Genomic DNA extraction were conducted by ~~special~~ kits based on the use of affinity columns (Omega Bio-tek Inc., Norcross, USA), according to the manufacturer's instructions.

The nuclear rDNA containing ITS region was amplified using NC5 (5'-GTAGGTGAACCT GCGGAAGGATCATT-3') and NC2 (5'-TTAGTTTCTTTTCCTCCGCT- 3') primers. The PCR reactions were carried out as follows: 2 mM $MgCl_2$, 0,2 mM of each dNTP, 20 pmol/µl of each primer, buffer AmpliTaq Gold 1X, 3.0 U AmpliTaq Gold DNA Polymerase (AB) and 20-25 ng of genomic DNA, in a final volume of 50 µl. The PCR was performed according to the following conditions: 10 min at 95 °C, 35 cycles of 30 sec at 95 °C, 30 sec at 58 °C and 75 sec at 72 °C, followed by a final elongation of 15 min at 72 °C on a Thermal Cycler 2720 (Applied Biosystems). The PCR products were separated by electrophoresis in 1% agarose gel, stained with SYBR safe® (Invitrogen, Carlsbad, USA) in Tris- Borate- EDTA buffer and visualised by UV transilluminator (GelDoc Imaging System, Euroclone). The PCR amplification produced a ~1 Kb fragment that was subjected to RFLP analysis using two restriction enzymes HhaI and HinfI for the identification of *Anisakis* species (D'Amelio et al., 2000). All restriction reactions were carried out in a final volume of 20 µl containing 3 µl of DNA, 13.8 µl of distilled water, 1 µl of restriction enzyme, 2 µl of enzyme buffer and 0.2 µl of BSA. The digestion was performed on a Thermal Cycler 2720 (Applied Biosystems) over night at 37 °C. The digestion products were electrophoresed in 2 % agarose gel (Invitrogen, Carlsbad, USA) stained with SYBR safe® and visualized by UV transilluminator. The size of fragments was determined by comparison with the molecular weights marker and the results were

interpreted following the keys for the identification of anisakid species described by D'Amelio et al. (2000). The ITS gene amplification products were also purified with Illustra GFX PCR DNA (GE Healthcare, Boston, Massachusetts) and Gel Band Purification kit (GE Healthcare) following the manufacturer's instructions for sequencing analysis. The purified products were sent to Macrogen company (Amsterdam, Holland) for Sanger sequencing.

2.4 Data analysis

Infestation parameters such as prevalence and mean intensity were examined using the software Quantitative Parasitology 3.0. The raw sequence data were manually analyzed for quality assessment and compared with previously characterized ITS sequences of Anisakidae published for identification by using the Basic Local Alignment Search Tool (BLAST) via GenBank.

2.6 Submission of sequence data to databases

Nucleotide sequence data reported in this paper are available in the GenBank™ databases under the accession number: MH197040 (*Anisakis pegreffii* ITS region).

3. RESULTS

All the European sea bass samples were of commercial size with a mean weight and standard deviation (SD) of 402.78 ± 49.72. The weight, maximum standard length, Fulton's condition index and infestation parameters of the fish samples examined are shown in Table 2. A Fulton's condition - K index above 2 was found in 12 samples from Sicilian and Greek farms. Five samples came from Greek farms showed a K index below 1. Two nematode larvae were found in an European sea bass sample from farms of Greece after visual inspection, giving a prevalence of infestation of 0.7%, a mean abundance of 0.01 and a mean intensity of 1. The larvae were located under the visceral serosa of intestine. No additional larvae were found after chloro-peptic digestion. No co-infestation with

other parasites was found. The weight of the affected fish was lower than the mean weight, detecting a K index of 0.83. The morphological analysis allowed to identify parasites as L3 *Anisakis* larvae belonging to the morphotype I (*sensu* Berland 1961). The amplification of ITS region produced a single band of ≈1000 bp for all the specimens. The PCR- RFLP analysis of ITS region, obtained with H*inf*I restriction enzyme produced one pattern with three strong bands at 370, 300 and 250 bp corresponding to *Anisakis pegreffii*. The digestion with H*ha*I produced one pattern with two bands (550-430 bp) (Figure 2). The PCR-RFLP results were confirmed by sequence analysis. The alignment of rDNA ITS sequences with the sequences deposited in GenBank confirmed the results obtained by PCR-RFLP analysis. The alignment with BLAST showed a 99% of identity and 100% query cover with sequence of *A. pegreffii* from Mediterranean Sea (KY426257.1).

4. DISCUSSION

To our knowledge this is the first report on the presence of *Anisakis* larvae in farmed European sea bass. Although digestion method is considered a valid method to recover larvae from viscera and muscle tissue, no additional parasites were found. Our findings seemingly contrast previous studies that confirmed the absence of *Anisakis* nematodes in 259 farmed European sea bass from Southeast Spain (Peñalver et al., 2010). No *Anisakis* larvae were found even in 1040 European sea bass samples from North Italy (Menconi et al., 2017) farms but a *Hysterothylacium fabri* larva was detected in only one sample, suggesting the possibility for European sea bass in open-net cages to feed on live food in addition to the feed offered. Unfortunately, we were unable to identify the farming practice used for the import European sea bass sample showing the presence of *Anisakis* parasites.

Based on what is reported on the National Aquaculture Sector Overview of the Food and Agriculture Organization of the United Nations (FAO), Greek aquaculture is dominated by the farming of marine finfish in offshore cages, specifically of gilthead sea bream and European sea bass (FAO, 2005).

Therefore, it is possible that this that this sample infected by *Anisakis* was reared in offshore open-net cages. This farming practices could allow the exposure of fish to the crustacean intermediate host (Peñalver et al., 2010). Furthermore, the European sea bass sample infected by *Anisakis* showed a low Fulton's K index (0.83). Given that the Fulton's K condition index is considered a good indicator of the general welfare of the fish (Lambert and Dutil, 1997), we assume particular stressed condition that lead the fish to feed on infected crustaceans and small fish entering the cage.

Most of the studies on the presence of anisakid parasites in farmed fish have focused on salmonids. Several authors have demonstrated the absence of anisakids in farmed Atlantic salmon (*Salmo salar*) (Angot and Brasseur, 1993; Lunestad, 2003). Marty (2008) reported the presence of an anisakid larva in a farmed Atlantic salmon, although molecular identification was not carried out. The presence of these parasites was verified even in runts of farmed Atlantic salmon (Mo et al., 2013). Based on the prevalence values reported in literature (Bernardi et al., 2011), the relative risk of anisakids in European sea bass is estimated to be 136 times greater in wild forms than in farmed forms.

At present, there are few data on the prevalence of anisakid parasites in farmed European sea bass (Menconi et al., 2017; Mladineo et al., 2010; Peñalver et al., 2010). This could be because these fish are not usually consumed raw and consequently are not considered a potential risk in terms of zoonosis. Nevertheless, several studies that point out the allergic potential of *Anisakis* spp. in clinical reports and the presence of thermally stable allergens in processed fish products (Fæste et al., 2015; Audicana and Kennedy, 2008) encourage to increase the monitoring data on farmed fish of Mediterranean, in order to clarify the effects of different farming practices on the prevalence of Anisakidae nematodes and fulfil the EFSA Panel on Biological Hazards' requests (EFSA, 2010). In conclusion, we showed for the first time the presence of Anisakidae nematodes in farmed European sea bass. Therefore, our findings should be considered in future risk analyses on the fish species farmed in Mediterranean. However, due to the low prevalence found in this work, the risk of exposure to anisakid parasites in farmed fish remain very low.

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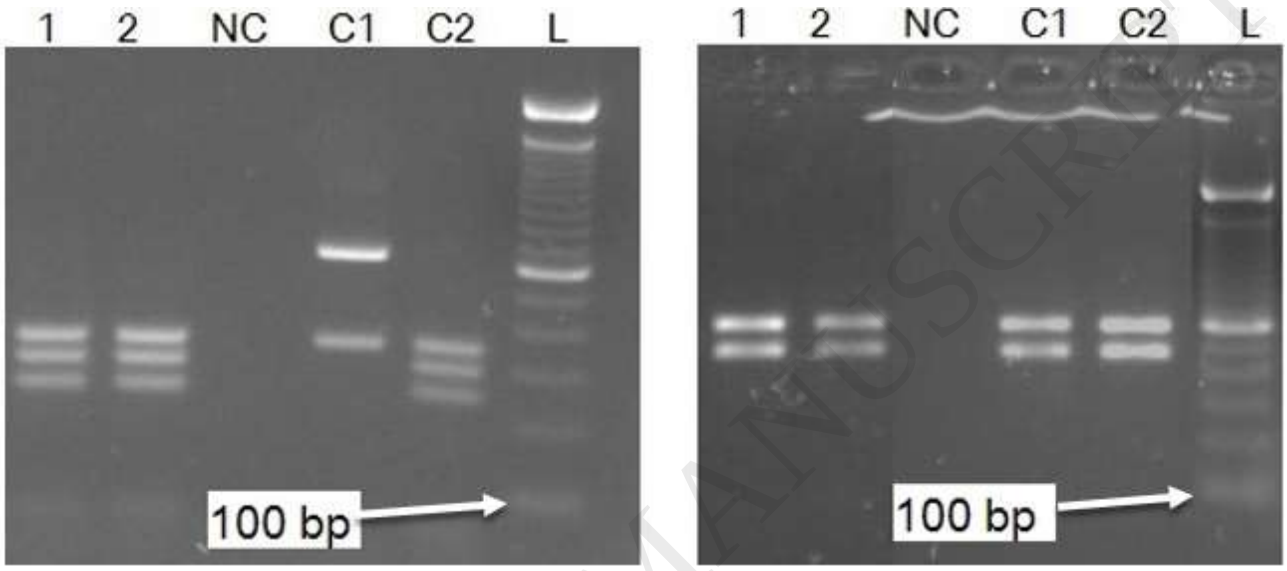
Figure captions

Figure 2. RFLP pattern of PCR products of Anisakis pegreffii from infected sea bass after digestion with H*inf*I and H*ha*I (lanes 1-2: *Anisakis pegreffii*; lane NC: Negative control; lane C1: positive control *Anisakis typica*; lane C2: positive control *Anisakis pegreffii*; lane L: 100 bp DNA size marker.



Table

Table 2. Morphometric and infestation data of the European sea bass samples analysed.

	N	MSL (mean ± SD)	Weight (mean ± SD)	Fulton's K index (mean ± SD)	Fulton's K index (min-max)	Anisakidae larvae	Prevalence (%)
Pachino	43	29.3 ± 2.09	406.3 ± 53.10	1.6 ± 0.38	1.21-2.27	0	-
Licata	40	29.5 ± 1.77	405.9 ± 39.27	1.5 ± 0.25	1.29-2.09	0	-
Greece (FAO 37.3)	68	29.4 ± 2.32	398.6 ± 60.97	1.6 ± 0.45	0.76-2.45	2	1.5
Total	151	29.4 ± 2.01	402.7 ± 49.72	1.6 ± 0.36	0.76-2.45	2	0.7%