

## Detection of Anisakidae larvae in fish products commercialized in Sicily

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### ABSTRACTS

In this work a total of 1331 fish samples belonging to 15 species (*Engraulis encrasicolus*, *Alaccia aurita*, *Loligo vulgaris*, *Trigla lyra*, *Conger conger*, *Merluccius merluccius*, *Zeus faber*, *Lophius piscatorius*, *Sardina pilchardus*, *Lepidopus caudatus*, *Scorpaena scrofa*, *Scomber scombrus*, *Trachurus trachurus*, *Todarodes sagittatus*, *Trachinus draco*) from Sicilian commercialized in Sicily (FAO 37, FAO 37.1.3, FAO 37.1.1, FAO 37.2.2, FAO 37.3, FAO27, FAO 41, FAO87), were examined for the detection of Anisakidae larvae and molecular identification. The fish samples were examined for the research of nematodes by visual inspection and digestion method according to the EC Regulation 2075/2005. Detected larvae were subjected to morphological identification through the optical microscopy. Subsequently, the DNA was extracted and the molecular identification of the larvae was conducted by RFLP-PCR of the nuclear ITS region (ITS-1, ITS-2 and 5.8 S subunit). The polymerase chain reactions targeting the cytochrome c oxidase subunit II (cox2) was performed and subjected to sequence reaction. A number of 370 larvae have been identified at species level, of which 330 belong to the *Anisakis pegreffii*, 23 to the *Anisakis simplex* sensu strictu, 6 to the recombinant genotype *Anisakis pegreffii/simplex* s. s., 1 to *Anisakis physeteris* and 10 to *Hysterothylacium fabri*. The data obtained provide interesting reflections on the presence of parasites belonging to the Anisakidae family in fishery products marketed in the Sicilian territory. These findings are an excellent tool for assessing and preventing possible risks due to the consumption of these products.

### KEY WORDS

*Anisakis*; nematodes; larvae; PCR.

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### INTRODUCTION

In 2002, the Parliament of the European Union have developed a revision on the Community legislation about the production, marketing and food control, aimed to increasing the level of safety of food products. The EC Regulation 2002/178 laying

down the general principles and requirements of food law and constituting the European Food Safety Authority (EFSA). Subsequently, a group of regulations, commonly called “hygiene package” were established from 1 January 2006. These regulations have redefined the existing EU provisions on hygiene of production and food marketing and have

set new rules on the food control for Competent Authorities and food sector operators. In recent years in Italy, we have seen an increasing consumption of raw fish due by importation of exotic typical products such as sashimi and sushi, and the increased consumption of different Italy culinary preparations such as marinated anchovies or carpaccio.

The Regulation 853/2004 imposes the food sector operators to freeze fish products eaten raw, this procedure ensuring food security. In fact there is a risk of parasites presence in fish fillets that can lead to zoonotical pathologies if larvae are eaten alive. The BIOHAZ panel of the European Food Safety Authority (EFSA recommended member states to carry out coordinated studies aimed to increase knowledge about the zoonotic diseases linked to Anisakis (Scientific Opinion on risk assessment of parasites in fishery products and EFSA Panel on Biological Hazards (BIOHAZ)). The study aims to estimate the prevalence of infestation of Anisakidae nematodes in the fish products commercialized and bred in Sicily and estimate the geographical and seasonal distribution of Anisakidae infestation

**MATERIAL AND METHODS**

The fish samples were examined for the research of nematodes in the stomach, intestine, abdominal cavity and muscle by visual inspection and digestion method. Detected larvae are subjected to identification at genus level, through the optical microscopy (Leica DM 2000), according to morphological characters (Berland, 1961). Subsequently, the larvae were subjected to molecular analysis. Genomic DNA extraction were conducted by special kits based on the use of affinity columns, according to the manufacturer’s instructions. Polymerase chain reactions targeting the complete ITS region ITS-1, 5.8S, ITS-2 and the cytochrome c oxidase subunit II (cox2) were performed. Purification of ITS and cox 2 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 sequences were compared with previously characterised ITS and cox2 sequences of Raphidascarididae family published for identification by using the Basic Local Alignment



Figure 1. Anisakidae larvae infestation.

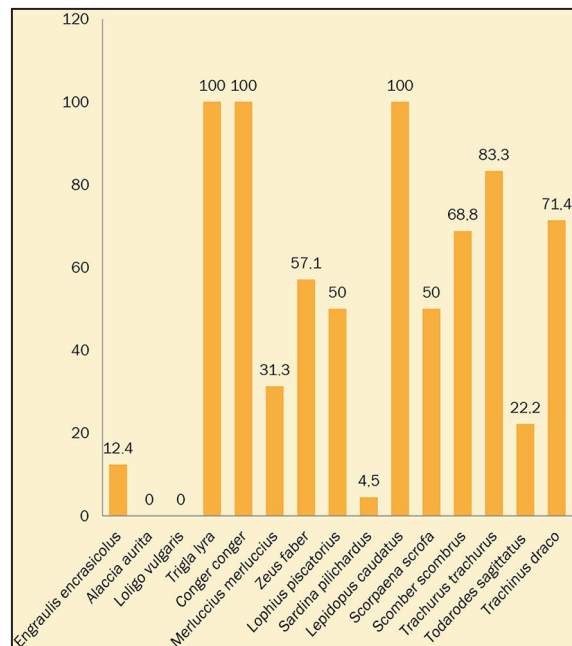


Figure 2. Infestation prevalence in the examined species.

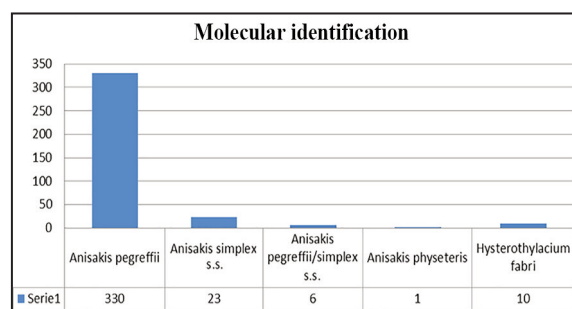


Figure 3. Molecular identification of Anisakidae larvae.

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
<i>Alaccia aurita</i>	31								31
<i>Engraulis encrasicolus</i>	434	114	85		75			88	796
<i>Loligo vulgaris</i>				10					10
<i>Trigla lyra</i>				5					5
<i>Conger conger</i>				8					8
<i>Merluccius merluccius</i>	49	3	13	15					80
<i>Zeus faber</i>				7					7
<i>Lophius piscatorius</i>	2			4					6
<i>Sardina pilichardus</i>	106		111	32	15				264
<i>Lepidopus caudatus</i>	3		2	3					8
<i>Scorpaena scrofa</i>	4			3		1			8
<i>Scomber scombrus</i>	11		10	2	9				32
<i>Trachurus trachurus</i>	11		26	5					42
<i>Todarodes sagittatus</i>	11	4		9			3		27
<i>Trachinus draco</i>	4			2		1			7
<b>Totale</b>	<b>1331</b>								

Table 1. Type and number of samples analysed.

Search Tool (BLAST) via GenBank™. Sequence Alignment was conducted using ClustalW 2.1 and MEGA 6.05 software. Evolutionary relationship among the *Hysterothylacium* haplotypes was analysed using Neighbor Joining (NJ) trees (Tamura et al., 2007). The evolutionary distances for NJ tree were computed using the Maximum Composite Likelihood method (Tamura et al., 2004).

## RESULTS AND DISCUSSION

One thousand three hundred and thirty-one fish samples were examined for Anisakidae larvae detection by visual inspection (Table 1) The prevalence of infestation for each species examined were calculated (Fig. 1, 2). The collected larvae were examined for genre identification by optical microscopy, while the molecular identification was performed by RFLP-PCR (Fig. 3). The results ob-

tained revealed the presence of Anisakidae larvae in 450 fish samples. The comparison of the results obtained with Chaligianis have showed the lower values in the samples of *Merluccius merluccius* and *Scomber scombrus*, indicating a higher rate of infestation in fish species from the Greek coast. Molecular analysis confirm *Anisakis pegreffii* as the Anisakidae parasite most present in Mediterranean Sea (FAO zone 37-37.1-37.2-37.3) and also found in samples of *Engraulis encrasicolus* of from the North East Atlantic (FAO area 27) and in a sample of *Todarodes sagittatus* from the South Atlantic (FAO zone 41). Furthermore, the presence of *Anisakis simplex sensu strictu* has generally been identified in mackerel or in European anchovy from North-East Atlantic (FAO area 27) and only in one case in salted anchovies samples derived from the Mediterranean (FAO zone 37). The hybrid genotype (*Anisakis pegreffii*/*Anisakis simplex* s.s.) was found in samples from probable areas of sympathy (FAO 37.1.3; 37.1.1; 37.2) between the

*Anisakis pegreffii* and the *Anisakis simplex* s. s., in samples of *Trachurus trachurus*, *Scomber scombrus*, *Merluccius merluccius* and *Engraulis encrasicolus*. The species *Anisakis physeteris* was found in a single sample of *Conger conger* caught in FAO 37.2. The *Hysterothylacium fabri* has been identified in demersal fish samples such as *Conger conger*, *Lophius piscatorius*, *Trigla lyra*, *Trachinus draco*, *Merluccius merluccius* from FAO 37.2 and FAO 37.1 and in one case in one mackerel from the FAO area 27.

The results provide an exhaustive evaluation on the presence of Anisakidae parasites in fish caught and commercialized in Sicily in order to have a risk assessment associated with the consumption of these products (see also D'Amelio et al., 2000; 2010; Evans et al., 2001; Abollo et al., 2003; Bernardi, 2009; EFSA, 2010; Buchmann & Kania, 2012; Chaligiannis et al., 2012).

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