# Anisakid nematodes as possible markers to trace fish products

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## Abstract

In this work a total of 949 fish samples were analysed for the identification of nematode larvae belonging to the Anisakidae family. Biomolecular application for the identification of Anisakidae larvae can be an optimal instrument for the traceability of fish products, described on the Reg. EC 178/2002. Results confirm a correlation between geographical distribution of fishes and presence of specific Anisakid larvae. FAO 37 zone (Mediterranean sea) showed a prevailing distribution of *Anisakis pegreffii* and a minimal presence of *A. simplex s.s.* in hybrid form with *Anisakis pegreffii.* FAO 27 zone showed a prevailing distribution of *A. simplex s.s.* in fish like Brosme (*Brosme brosme*) and infestation prevalence of *Pseudoterranova krabbei* and *P. decipiens s.s.* in *Gadus morhua*. Obtained results validate the hypothesis that molecular biology methods for identifying Anisakidae larvae are effective traceability markers of fish products.

## Introduction

The Regulation (EC) n.178/2002 of 28 January 2002 (European Commission, 2002) defines the term traceability as *the ability to trace and follow the path of a food-producing animals through all stages of production, processing and distribution*. The food sector operators must have systems and procedures that allow the competent authorities to access information on the product in order to guarantee its traceability. Food commercialised in the European Community must therefore be labelled or identified to facilitate their traceability through relevant documentation or information describing geographical origin of the species, as in the case of fish products. Reg. (EC) 2065/2001 (European Commission, 2001) described and listed the fishing areas, following the division implemented by the Food and Agriculture Organization (FAO). Despite these restrictions, consumers can bump into fraudulent suppliers who compromise the veracity of a product. Molecular biology methods, in this case, are a valuable tool in geographical identification of fish stocks. These methods exploit the principles of phylogeography, based on the alignment of DNA sequences obtained from fish and their genetic distance. A precise parassitofauna corresponds to a fish species present in a given area. Co-phylogeny is defined as the set of phylogenetic studies on parasites and their guests. Co-phylogenetic mapping is constructed to provide the best explanation of the phylogeny and to check if parasites have suffered genetic divergence with their guests. Recent studies have shown that phylogeny of the parasites tends to *reflect* that of the infested fish (Desdevises, 2007; Mattiucci *et al*., 2008). Hence, we can assume that parasites could be viewed as reliable markers for the traceability of fish products.

The need to use parasites as markers of fish traceability can be largely met by *Anisakidae* family. In this family of nematodes one can find parasites widely distributed throughout the globe. *Anisakis pegreffii* is the *Anisakidae* most present in the Mediterranean (Mattiucci *et al*., 2004), while Pseudoterranova is most frequent in North-East Atlantic (Desportes and McClelland, 2001). The aim of this study was to verify the correspondence between the parassitofauna of examined fishes and their geographical distribution, through molecular biology methods in order to promote a new methodology in traceability of fish products.

## Materials snd Methods

### Sampling area and methodology

Sampling areas belong to the North-East side of the Atlantic (FAO 27) and to the entire Mediterranean basin (FAO 37). Samplings were carried out from January 2013 to March 2014 within the Monitoring Regional Plan (monitoring plan for the search of Anisakidae larvae in fish products commercialised in Sicily) and as a consequence of research samplings. They were carried out by the veterinary in charge throughout the national territory, preferring products just fished. For each sample species and origin as described on the label (in imported products) and from reports of fishermen were registered (Table 1). Thirteen species of fish were sampled for a total of 949 samples. Samples were stored at 4 and -20°C and analysed by the laboratories of the National Reference Center for Anisakiasis (C.Re.N.A). At first, samples underwent a visual examination for the research of parasites belonging to the family Anisakidae (Figure 1).

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### Anisakidae parasites research and identification of morphological characters

Fish samples were sectioned into a caudo-cranial sense and open for the detection of parasites by visual inspection. Parasites were inspectioned by a stereo-microscope (Zeiss CL 1500 ECO; Zeiss, Oberkochen, Germany). Detected larvae were preserved in 70% ethanol for 24 h and subjected to identification of morphological characters. Genre identification has been carried out by optical microscopy (Leica DM 3000; Leica, Wetzlar, Germany) on parasite samples clarified in glycerol. Morphological characters able enough to discriminate the genre of the larvae and the morfotype of genus *Anisakis* were analysed.

### Anisakidae species object of the study

*Anisakis* and *Pseudoterranova* were the parasites genera considered by this study due to their geographical correlation with the sampling areas (Mattiucci *et al*., 2008). *Anisakis* larvae are divided into morphotype I and II. Morphotype I have parasites more distributed in Mediterranean. *Anisakis pegreffii* is the most detectable Anisakidae species in Mediterranean fish with a prevalence of infestation in scabbard fish (*Lepidopus caudatus*), anchovy (*Engraulis encrasicolus*), horse mackerel (*Trachurus trachurus*), and sardines (*Sardina pilchardus*). *A. simplex s.s.* also belongs to morphotype I and it is a parasite species that infestis in greater measure fish belonging to North-East Atlantic (Portuguese coast, North Sea). *P. decipiens s.s.* and *P. krabbei* are species of *Pseudoterranova* which can be found in North-East Atlantic fish (Costa *et al*., 2013). *P. decipiens s.s.* extends in a range

of distribution including the North-East Atlantic (Scotland, Faroe Islands, Norway, *etc*.) and the Canadian Atlantic (Brattey and Stenson, 1993; Paggi *et al*., 1991). There are simpatry areas with species of the same complex (*Pseudoterranova decipiens* complex) as *P. krabbei* in North-East Atlantic, where it can be found in co-infestation in the same fish. Larval forms of *P. decipiens s.s.* are mostly present in cod of the North Atlantic (Mattiucci *et al*., 1998; Desportes and McClelland, 2001). *P. krabbei* is a parasite found in the North-Eastern side of the Atlantic; its larval form infests Osteichthyes such as the Atlantic cod (*Gadus morhua*) and the black cod (*Pollachius virens*) (Paggi *et al*., 1991).

## Molecular analysis

Larval samples previously preserved in ethanol (70%) were rehydrated with sterile water, fragmented with a scalpel, placed in an *eppendorf* with 200 L of nuclease free water and frozen at –20°C for 24 h.

## DNA extraction

For DNA extraction special kits based on affinity principle pedestals were used (Sigma Aldrich, St. Louis, MO, USA). The concentration of extracted DNA was assessed by spectrophotometric method at 260 nm. The solution containing DNA was stored at -20°C, in order to avoid repeated freezing and thawing which may interfere with the amplification reaction [polymerase chain reaction (PCR)].

## DNA amplification

Polymerase chain reaction was divided into 3 phases. First, preparation of a master mix in reaction tubes with anhydrous reagents, in a 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') mix. NC5 and NC2 are primers that amplify the nuclear rDNA region (ITS1-5.8S-ITS2). Final volume was 25 mL. 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. Amplification products were finally detected by agarose gel elec-

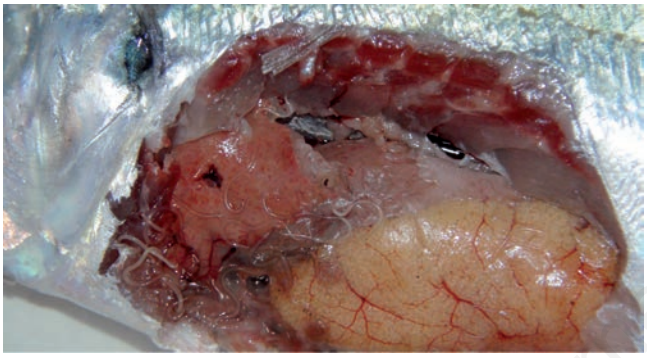

**Figure 1. Visual inspection of *Trachurus trachurus* viscera for the detection of parasites.**

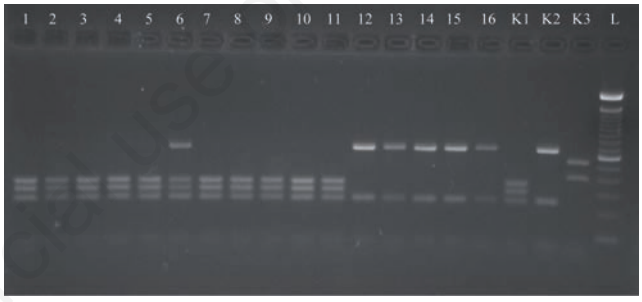


**Figure 2. Restriction pattern with H*inf* I. Lanes 1 to 5 and 7 to 11=*Anisakis pegreffii*; lane 12 to 16=*Anisakis simplex* s.s.; lane 6=*A. pegreffi/A. simplex* s.s.; K1=positive control *A. pegreffii*; K2=positive control *A. simplex* s.s.; K3=positive control *A. physeteris*; L=ladder.**

**Table 1. Analysed samples divided by fish species and fishing area.**

| Type of sample | Scientific name | Fishing area | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | FAO 37.1.3 | FAO 37.1.1 | FAO 37.2.2 | FAO 27 (subarea IXa) | FAO 27 (subarea IV;V) | |
| Anchovy | *Engraulis encrasicolus* | 117 | 107 | 46 | 0 | 0 | 270 |
| Brosme | *Brosme brosme* | 0 | 0 | 0 | 0 | 4 | 4 |
| Tub gurnard | *Chelidonychtis lucernus* | 0 | 0 | 0 | 0 | 6 | 6 |
| Conger | *Conger conger* | 0 | 0 | 2 | 0 | 0 | 2 |
| Hake | *Merluccius merluccius* | 48 | 0 | 8 | 0 | 0 | 56 |
| Atlantic Cod | *Gadus morhua* | 0 | 0 | 0 | 0 | 3 | 3 |
| Scabbard fish | *Lepidopus caudatus* | 2 | 0 | 2 | 0 | 0 | 4 |
| Monkfish | *Lophius piscatorius* | 3 | 0 | 0 | 0 | 0 | 3 |
| Sardine | *Sardina pilchardus* | 178 | 0 | 284 | 0 | 0 | 462 |
| Redfish | *Scorpaena scrofa* | 11 | 0 | 0 | 0 | 0 | 11 |
| Mackerel | *Scomber scombrus* | 10 | 0 | 9 | 8 | 0 | 27 |
| Horse Mackerel | *Trachurus trachurus* | 40 | 10 | 15 | 15 | 0 | 80 |
| Squid | *Todarodes sagittatus* | 17 | 0 | 4 | 0 | 0 | 21 |
| Total of samples | | | | | | | 949 |

trophoresis (1%) and subjected to restriction fragment length polymorphism (RFLP).

### Polymerase chain reaction-restriction fragment length polymorphism

The restriction was performed by the use of two different restriction enzymes (*HhaI* and *HinfI*) with the following sequences: H*haI*:GCG C – CGC G; H*infI*: GANTC – CTNA G. Each sample reached a final volume of 20 µL. Digestion of amplicons was performed by incubation at 37°C O.N. Detection of the digestion products was carried out by electrophoresis in agarose gel (2 %). Restriction fragment length polymorphism data, analysed by electrophoresis, reveal the restriction patterns relating to the different species of *Anisakis*, according to the interpretation key (D'Amelio *et al*., 2000; Pontes *et al*., 2005). Results were interpreted by evaluation of obtained restriction profiles for comparison with the molecular weights marker and positive control (Figure 2). The interpretation of restriction profiles belonging to *A. pegreffi/A. simplex s.s.* hybrid refers to the work of Abollo *et al*. (2003).

### Pseudoterranova DNA sequencing

*Pseudoterranova* identification cannot be executed by PCR-RFLP due to restriction pattern absence, so it was conducted a mitochondrial DNA sequencing. *cox2* mithocondrial region was amplified by the use of primers 210 (5'-CACCAACTCTTAAAATTATC-3') and 211 (5'-TTTTCTAGTTATATAGATTGRTTYAT-3') (20 pmol/µL) with *RNAsi* and *DNAsi*-free water, buffer 1x, $MgCl_2$ 2 mM, DNTPs 0.2 mM, Taq gold polymerase (6 U) and 10-20 ng of DNA, in a final volume of 50 L. The following PCR condition was set: 8 min at 95°C, 35 cycles of 50 s at 95°C, 1 min at 52°C, 1 min at 72°C and a final extension of 72°C for 7 min (Termal Cycler 2720 Applied Biosystems). Polymerase chain reaction products (629 bp fragments), were visualised by electrophoresis on 1.5% agarose gel with Syber Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA). Amplified fragments were purified by GFX Microspin columns and undergo to sequence reaction by Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). The sequence products were purified by G50 columns (GE), denatured and analysed with capillary electrophoresis on automated sequencer 3130 Biotec 69. Obtained sequences were aligned with the most similar sequences available in *GenBank* using the Nucleotide BLAST software (Mattiucci *et al*., 2010; Nadler and Hudspeth, 2000).

## Results

Figure 3 shows the values of infestation prevalence for examined species (expressed as a percentage). Obtained results were normalised by the indication of examined samples for species. Infestation prevalence of the most sampled species (sardines, anchovies, mackerel, nibs) is similar to the one published by other authors (Mattiucci *et al*. 2004). Molecular analysis was carried out on 329 larval samples. For each fish sample a statistically significant number of larvae (≈10%) was examined: 207 belonging to *A. pegreffii,* 64 to *A. simplex s.s.*, 3 to *A. physeteris* and 17 to *A. pegreffii/simplex s.s.* hybrid form, as described by Abollo *et al*. (2003). Sequencing technique placed *Pseudoterranova* larvae as *P. krabbei* (Sequence ID: HM147279) for 36 samples and *P. decipiens s.s.* for 2 (Sequence ID: HM147278.1) (Figure 4). Geographical division showed *A. simplex s.s., P. krabbei, P. decipiens s.s.* belonging to the FAO 27 zone, while *A. pegreffii*, *A. physeteris* and *A. simplex s.s.* species belonging to FAO 37 zone (Figure 5). Obtained results show a species-specific prevalence of host for mackerel and brosme, infested by *A. simplex s.s.* Furthermore, an infestation difference between Atlantic and Mediterranean mackerel has been highlighted: Atlantic mackerel were an infestation by *A. simplex s.s.* larvae, while Mediterranean mackerel showed a prevalence of *A. pegreffii* infestation. *A. simplex s.s.* proved to be present both in

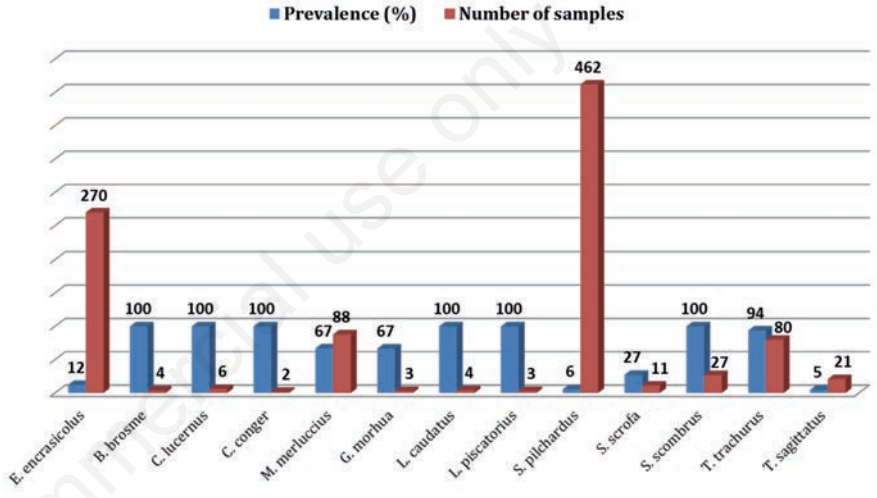


**Figure 3. Infestation prevalence of Anisakidae family larvae according to analysed fish species.**

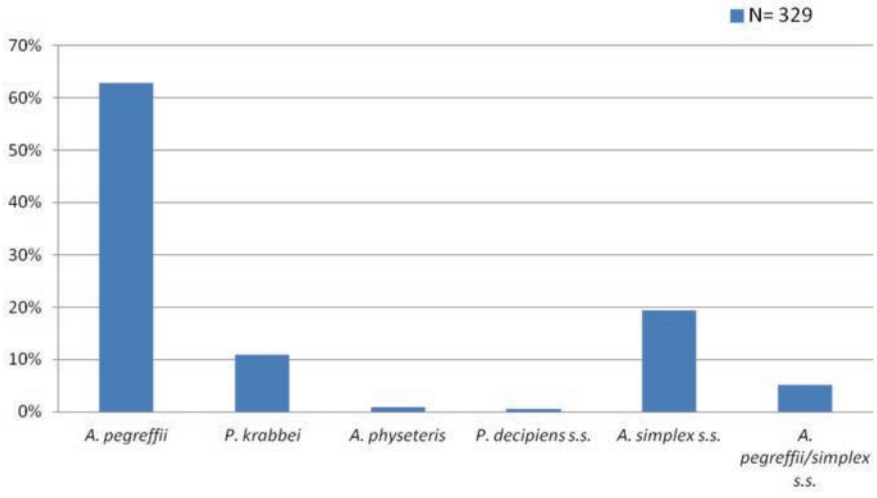


**Figure 4. Prevalece of analysed Anisakidae larve (%).**

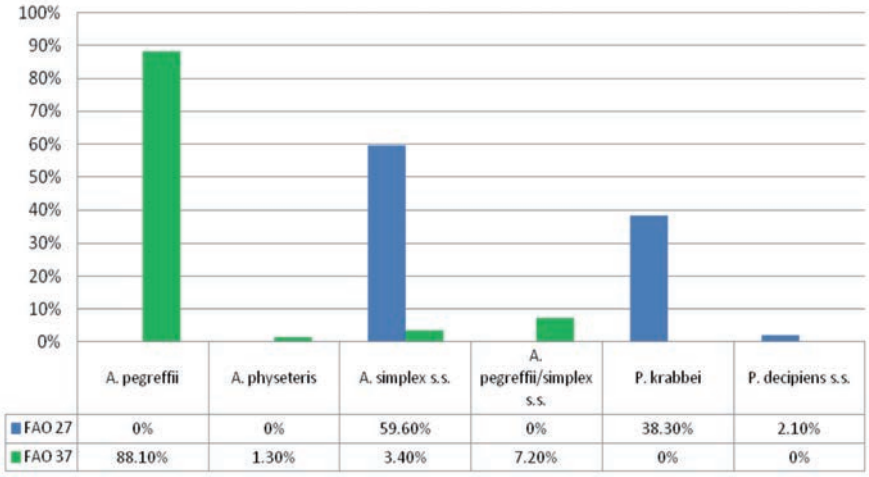


**Figure 5. Anisakidae larvae prevalence according to FAO zone.**

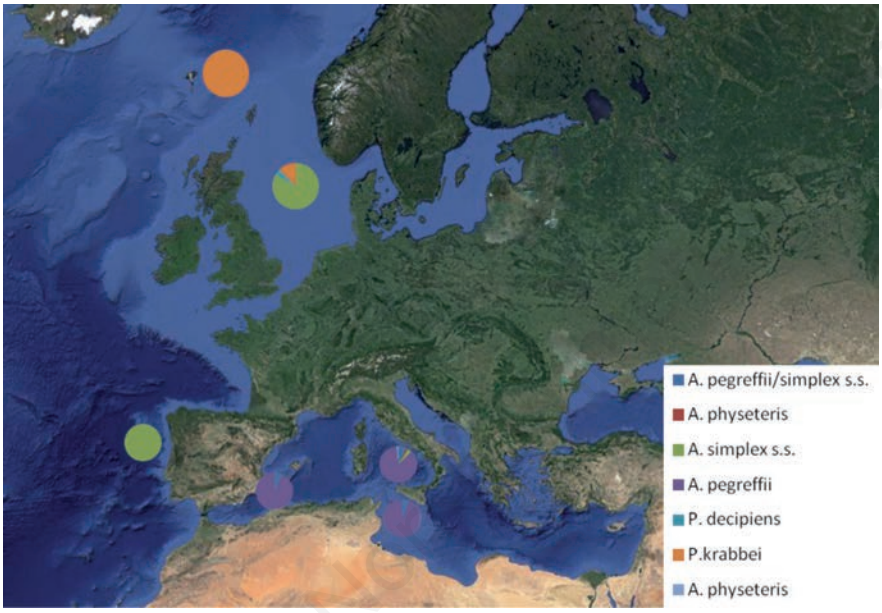


**Figure 6. Infestation prevalence of Anisakidae larvae according to sampling area.**

the North-Eastern Atlantic and in the Mediterranean, where it was found as hybrid form with *A. pegreffii* just as described by Mattiucci *et al*. (2008). Figure 5 evidences a greater presence of *A. simplex s.s.* on FAO 27 zone.

## Discussion

Results showed a marked geographical breakdown of the larvae belonging to the family Anisakidae (Figure 6). This study confirmed the endemism and prevalence of *A. pegreffii* in FAO 37 zone, as demonstrated in previous work (Mattiucci *et al*., 2008), while FAO 27 zone shows the presence of two *Pseudoterranova* species and one of *Anisakis* (*A. simplex s.s.)*. In the Mediterranean area *A. simplex s.s.* mostly comes in the hybrid form with the autochthonous species *A. pegreffii.* This hybridisation could derive from the movement of migratory fish banks (Abollo *et al*., 2003), such as mackerel in the Mediterranean. Obtained results in mackerel (*Scomber scombrus*) prove this hypothesis. In the Mediterranean, the presence of *A. pegreffii/A. simplex s.s.* hybrids and *A. simplex s.s*. was most detected in FAO sub-areas 37.1.1 and 37.1.3 (htpp://www.FAO.org/fishery/area/Area37/en). These 2 fishing areas are very close to the Gibraltar strait and then to the Atlantic seaboard. A confirmation of the distribution specificity of the Anisakidae larvae was also due to the presence of two different *Anisakis* species in *Trachurus trachurus* (horse mackerel). In the Mediterranean horse mackerel (FAO area 37) it was found only the presence of *A. pegreffii*, while in North-East Atlantic horse mackerel only *A. simplex s.s*. larvae were discovered. This study confirmed a co-evolution hypothesis between parasite and its host.

## Conclusions

In conclusion, it is possible to consider Anisakid nematodes as a marker of fish traceability. The application of biomolecular methods for the identification of Anisakidae larvae could be an additional tool for the confirmation of fish product origin and a valid anti-fraud methodology for the protection of EC Reg.178 /2002.

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