

## Molecular identification of larvae for Anisakidae family reduced in benthic and pelagic fish

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### ABSTRACTS

The present work, carried out at the Istituto Zooprofilattico Sperimentale della Sicilia “A.Mirri”, Department of Food, Laboratory C.Re.NA (National Reference Center for Anisakiasis), aims to identify species of larvae found in different fish species from the pelagic and benthic environment through molecular analysis (PCR, PCR-RFLP and sequencing) and evaluate the possible ecological relationship between parasites and guests in the two different marine environments.

### KEY WORDS

Anisakidae; PCR-RFLP; benthic; pelagic.

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### INTRODUCTION

In the last ten years, there was an increase in the consumption of raw fish due to the introduction of new eating habits from countries outside Europe (sushi, sashimi, herring, pickled anchovies, etc.), increasing the risk of contracting parasitic diseases, such as Anisakiasis (Audicana et al., 2002). Anisakiasis is a disease caused by nematodes of the genus *Anisakis*, belonging to the Anisakidae family, together with *Pseudoterranova*, genera (Køie et al., 1995; Pozio, 2013). Human infestation occurs by ingestion of third stage *Anisakis* larvae present in raw fish and cephalopods but also undercooked, marinated, pickled, smoked or salted. These parasites are found, at the adult stage, in the abdomen of marine mammals (whales, seals, dolphins), more precisely in the stomach where they are visible to the naked eye. In fish, which are intermediate hosts,

the larval form are normally found in the coelomic cavity where they can be found free or encysted or adherent to the various organs and tissues (Mattiucci & Nascetti, 2008). In the present study larval forms of Anisakidae collected in pelagic, benthic and demersal fishes sampled in Sicily were identified by molecular analysis according to the protocols reported in literature (Cavallero et al., 2015).

### MATERIAL AND METHODS

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 washed, fragmented with a scalpel and frozen at -20 °C for 24 hours. The extraction of DNA was conducted by special kits based on the use of affin-

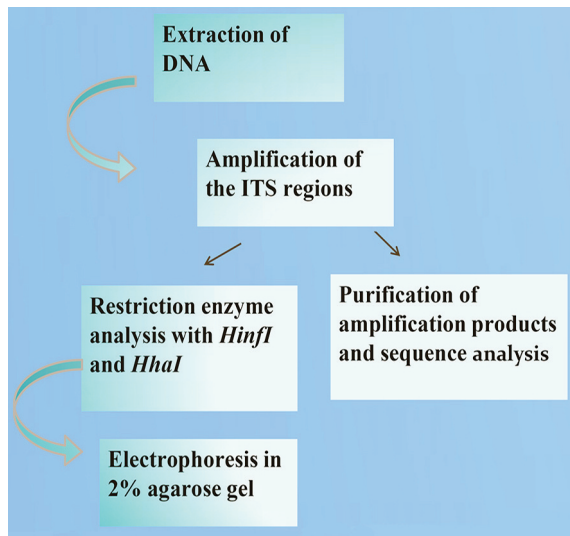


Figure 1. Molecular identification.

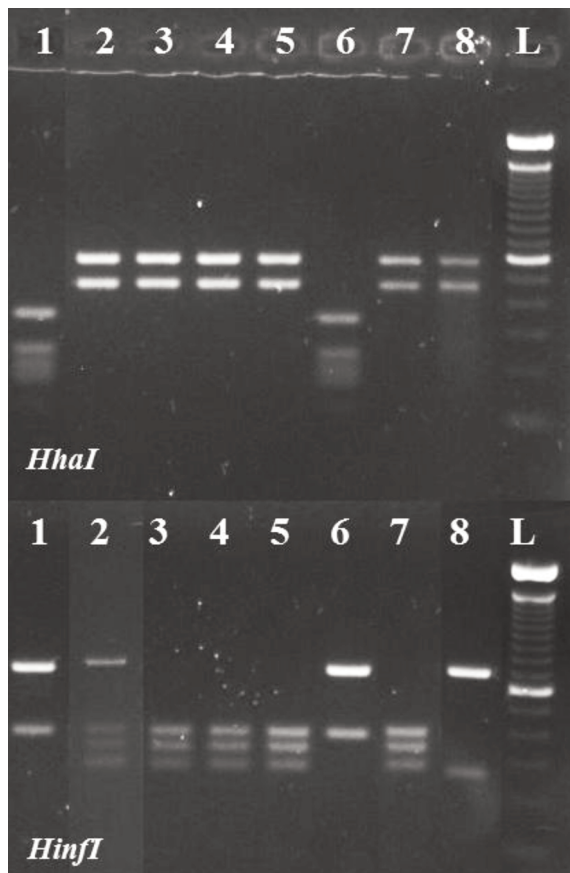


Figure 2. Restriction enzyme analysis with *HinfI* and *HhaI*. Lane 1-6: *Anisakis typica*; lane 2: *Anisakis simplex* s.s./*Anisakis pegreffii* hybrid form; lane 3-4-5-7: *Anisakis pegreffii*; lane 6: *Anisakis simplex* s. s.; L: ladder 100 bp.

ity columns. For the genus *Anisakis* we proceeded to the amplification of the ITS regions (ITS-1, ITS-2 and 5.8 S subunit) of nuclear rDNA by the primers NC5 (5'GTA GGT GAA CCT GCG GAA GGA TCA TT3'), NC2 (5'TTA GTT TCT TTT CCT CCG CT3') (Zhu et al., 1998). The DNA samples have been subjected to restriction enzyme analysis with two restriction enzymes, *HinfI* and *HhaI*, for the identification of *Anisakis* spp. according to genetic key of D'Amelio et al. (2000). The digestion was performed over night at 37°C and the digestion products were electrophoresed in 2% agarose gel (Invitrogen) stained with SYBR safe® and visualized by UV transilluminator (Figs. 1, 2). For the identification of *Hysterothylacium* species a sequence analysis were conducted. Purification of ITS gene amplification products was carried out with Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare) following the manufacturer's instructions. The purified products were sent to Macrogen company (Amsterdam, Holland) for Sanger sequencing (Fig. 3).

## RESULTS AND DISCUSSION

In the seas surrounding Sicily the larvae found in the various species of fish belonged to the *Anisakis* and *Hysterothylacium* genera of the Fischer Anisakidae. The pelagic environment was rich in larvae belonging to the *Anisakis* genus such as *Anisakis pegreffii*, *Anisakis simplex* s.s. as well as the hybrid species *Anisakis pegreffii* / *Anisakis simplex* s.s. In the benthic environment predestined species belonging to the genus *Hysterothylacium* there was also a low presence of larvae of the species *Anisakis pegreffii*. Demersal fish species were found infested both by *Hysterothylacium aduncum* and by *Anisakis pegreffii*. The data obtained indicate a different parasites belonging to the Anisakidae family as well as the fish species of pelagic and benthic marine environments, indicating therefore a difference of infestation in relation to the different habitats (Fig. 4).

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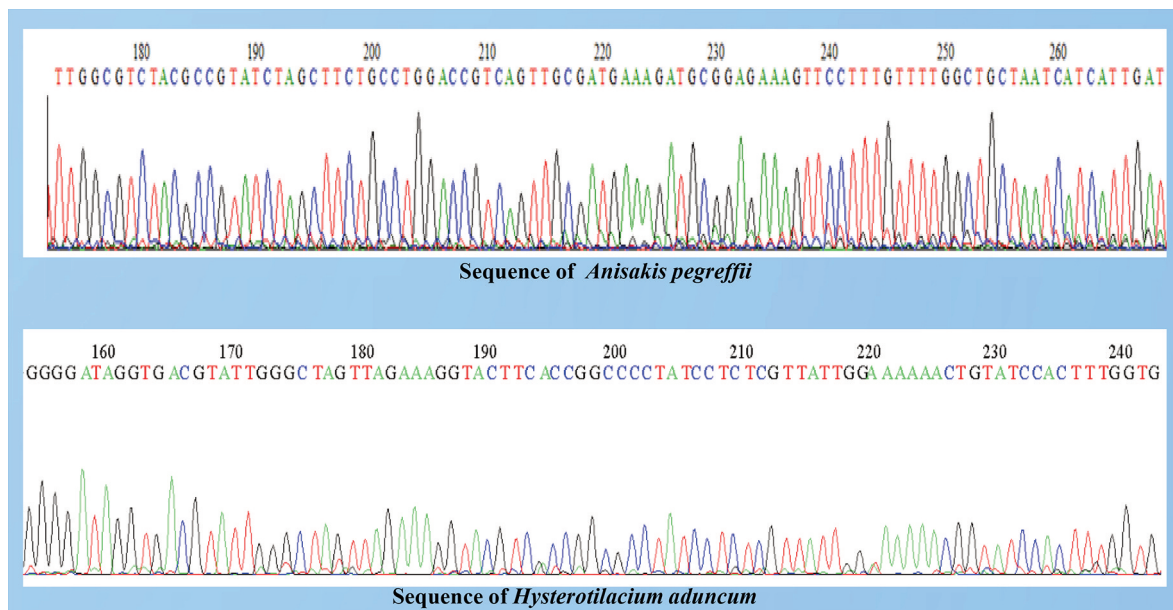


Figure 3. Sanger sequencing.

	Species	n. guests	n. larvae	Larvae species	zone F.A.O.	
Pelagic environment	<i>Sgomber sgombrus</i>	289	259	<i>A. pegreffii</i>	37.12	TP
	<i>Engraulis encrasicolus</i>			<i>A. simplex</i>	37.19	ME
	<i>Sardina pilchardus</i>			<i>A. pegreffii/A. simplex</i>	37.15	SR
					27	Atlantic
Benthic environment	<i>Mullus barbatus</i>	29	8	<i>H. aduncum</i>	37.16	AG
	<i>Scorpaena scrofa</i>			<i>A. pegreffii</i>	37.15	SR
Demersal	<i>Merluccius merluccius</i>	9	604	<i>A. pegreffii</i>	37.12	TP
	<i>Lepidopus caudatus</i>			<i>H. aduncum</i>		
Total		327	871			

Figure 4. Larvae of Anisakidae family found in bentonic and pelagic fish.

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