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Molecular identification of *Anisakis* and *Hysterothylacium* larvae in commercial cephalopods from the Spanish Mediterranean coast

Gabriela Picó-Durán[a,b], Lorena Pulleiro-Potel[a,b], Elvira Abollo[c], Santiago Pascual[d], Pilar Muñoz[a,b,*]

[a] Dpto. Sanidad Animal, Universidad de Murcia, 30100 Murcia, Spain
[b] Campus de Excelencia Internacional Regional "Campus Mare Nostrum", Spain
[c] Centro Tecnológico del Mar, Eduardo Cabello s/n, 36208 Vigo, Spain
[d] Ecobiomar, Instituto de Investigaciones Marinas de Vigo. CSIC, Eduardo Cabello 6, 36208 Vigo, Spain

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ABSTRACT

This study aims to investigate the occurrence of nematode larvae in commercial cephalopods in the Western Mediterranean Sea. A total of 202 animals comprising 123 broadtail shortfin squid (*Illex coindetii*), 34 European squid (*Loligo vulgaris*) and 45 common octopus (*Octopus vulgaris*) were examined using enzymatic digestion. A total of 31 larvae were isolated (prevalence: 14.6%) and identified using molecular analyses which included PCR and sequencing of the ITS (ITS1-5.8S rDNA-ITS2) region. Phylogenetic tree inferred from ITS sequences yielded supported relationships for *Anisakis* (P: 12.2%) and *Hysterothylacium* species (P: 4.1%). All parasites were found parasitizing *I. coindetii* and, as expected, *A. pegreffii* presented the highest prevalence (11.4%). *A. physeteris* was also found with a lower prevalence (1.6%) but confirming the role of the broadtail shortfin squid as paratenic host and, its potential host for anisakidosis transmission. A hybrid larva between *Anisakis simplex* and *A. pegreffi* was also identified. All *Anisakis* larvae were found within the visceral cavity; in contrast most of the *Hysterothylacium* larvae were isolated from the mantle. A significant correlation was found between total nematode prevalence and depth, explained by the presence of larger broadtail shortfin squids inhabiting deeper depths. Therefore, the results obtained in the present study improve the knowledge of the occurrence of *Anisakis* and *Hysterothylacium* species in the *I. coindetii* from the Spanish Mediterranean Sea highlighting the importance of considering *I. coindetii* as a potential hazard for humans if it is consumed raw or not well cooked and the need of further research in other cephalopods.

1. Introduction

Anisakidosis, produced by nematodes of the family Anisakidae or Raphidsacaridae, which are commonly referred to as anisakids, has acquired a high social relevance for causing digestive disorders or initiating hypersensitivity states or allergies (Audicana and Kennedy 2008; Mattiucci et al., 2013). These nematodes comprise a parasitic group widely distributed at geographical level, displaying a complex life cycle in aquatic ecosystem that involves various hosts at different levels in the food-web (Køie, 1993, 2001). Cephalopods and fishes are paratenic hosts for the anisakid larvae, while adult's parasites are found in marine mammals, marine birds or fish. Humans can become part of the cycle as accidental hosts by consuming raw or lightly cooked fish and cephalopods contaminated with third-stage larvae (Audicana and Kennedy, 2008). The genera *Anisakis* and *Pseudoterranova* are described as primarily responsible (Klimpel and Palm, 2011) whereas *Hysterothylacium* and *Contracaecum* are believed to be minor (Valero et al., 2003). According to the scientific opinion of the European Food Safety Authority (EFSA, 2010) protection and prevention are priorities in zoosanitary parasite control of fishery products for human consumption (Brogli and Kapel, 2011). In fact, EFSA (2010) recommended that research should be continued in parasites of public health importance in fishery products, regarding prevalence, intensity, anatomical location, as well as geographical and seasonal distribution.

The present study has investigated the occurrence and taxonomic identification of *Anisakis* and *Hysterothylacium* larvae in three commercially-important cephalopod species in the Spanish Mediterranean coast. Remarkably, cephalopods are of increasing

* Corresponding author at: Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de Murcia, 30100, Murcia, Spain.
E-mail address: pilarmun@um.es (P. Muñoz).

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economic importance which is manifested by the rise in their global landings over recent decades (International Council for the Exploration of the Sea [ICES], 2012). They constitute a large portion of the species fished in the Spanish Mediterranean Sea, with annual landings of approximately 7501 tons between 2004 and 2012 according to the FAO, being one of the main compounding of the Mediterranean diet (Illescas et al., 2007; Osanz Mur, 2001).

2. Materials and methods

2.1. Sample collection

The sampling plan was carried out within the framework of the Mediterranean International Trawl Survey (MEDITS financed by DG MARE and UE members Council Regulation (EC) N° 199/2008) by the Spanish Institute of Oceanography between May and June 2013. Trawlings were conducted in 91 different geographical sectors (Fig. 1 and Supplementary material) which were stratified into three zones based on the differences in seabed topography and water currents according to Lobo et al. (2014). Zone 1 (fishing hauls 1–14) is characterised by a very narrow continental shelf (from a few km to >20 km) and a steep slope to the shelf edge close to the coast. Zone 2 (fishing hauls 15–35) is characterised by a medium width shelf (from a few to >40 km). Zone 3 (fishing hauls 36–91) is characterised by a wide shelf (from a few to >80 km), by the presence of the Columbretes Islands which have been protected as a marine reserve since 1990 and an important current cetaceans passing between the island of Ibiza and the Iberian Peninsula.

Trawlings were conducted with a bottom trawl (model GOC-73) with a 4 m vertical opening and a 20 mm cod end mesh size. Depth was recorded by means of a CTD SBE-37 probe located in the mouth of the gear and ranged between 36.25 and 526 m. Further information on the sampling design and on the characteristics of the gear is available in the MEDITS-Handbook (2012). A total of 202 cephalopods specimens belonging to three different species (123 broadtail shortfin squid (*Illex coindetii*), 34 European squid (*Linaria vulgaris*) and 45 common octopus (*Octopus vulgaris*)) were randomly collected. Each specimen was identified at species level, sexed, weighted at the nearest gram, and the dorsal mantle length (DML) measured from the tip of the mantle to the midpoint between the eyes at the nearest millimeter. Body condition score (K) was calculated following Fulton's index as $K = BW/DML3 \times 100$ (Ricker, 1975) were BW was body weight. Maturity stage was determined according to the three-stage maturity scale described by Sánchez and Obarti (1993) which includes (I) immature (ovary whitish, very small and with no signs of granulation in females; spermatophoric organ transparent or whitish in males), (II) maturing (ovary yellowish with a granular structure; spermatophoric organ with white streaks of sperm) and (III) mature (ovary very large with plenty of eggs; spermatophoric sac with spermatophores). Then, cephalopods were immediately frozen on board and stored at −20 °C. Samples were transferred in frozen conditions to laboratory and stored at −20 °C until examination.

2.2. Parasite isolation and enzymatic digestion

Once thawed, cephalopods were visually examined for the presence of nematodes. The viscera and muscle were digested separately in order to establish whether nematodes were present at the muscular level. The enzymatic digestion method used was based on the Codex Alimentarius Commission (CODEX, 2004) and the EU Regulation (EC) No 2075/2005 (EU, 2016).

Digestion was carried out in a freshly prepared solution of 0.5% (w/v) pepsin (10000 FIP-U/g) and 0.063 M hydrochloric acid in distilled water in a ratio 1:10. The mixture was heated at 37◦C and continuously stirred for 2, 4 or 24 h depending on whether a specimen of *I. coindetii*, *L. vulgaris* or *O. vulgaris* respectively was being digested. Digested tissue was poured through a sieve with a mesh size of 400 μm and flushed carefully with tap water. When the flesh was not dissolved completely, the solution was filtered through a sieve and washed with tap water. Then the remaining flesh was quantitatively replaced in the beaker with a proportional amount of solution and stirred under the same conditions until there were no large pieces left (CODEX, 2004). The presence of nematode larvae was evaluated observing the retained part by sieve on a Petri dish under stereomicroscope. All nematodes found were removed and preserved in ethanol 70% for molecular diagnosis.

2.3. Molecular identification

Genomic DNA purification was performed employing NucleoSpin Tissue Kit (Macherey-Nagel, Easton, PA), according to manufacturer's instructions. DNA quality and quantity was checked in a spectrophotometer Nanodrop® ND-2000 (Thermo Scientific). The entire ITS region (ITS-1, 5.8S rDNA gene and ITS-2) was amplified using the forward primer NC5 (5′-GTA GGT GAA CCT GCG GAA GGA TCA TT-3′) and reverse primer NC2 (5′-TTA GTT TCT TTT CCT CCG CT-3′) (Zhu et al., 2000). The primer pair amplified an approximately 950 bp product. PCR reactions were performed in a total volume of 25 ml containing 1 μl of genomic DNA (100 ng), PCR buffer at 1x concentration, 0.3 μM primers, 0.2 mM nucleotides and 0.625 U Taq DNA polymerase (Roche Applied Science). PCRs were carried out in a GeneAmp PCR System 9700 (Applied Biosystems). The cycling protocol was 10 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min 15 s at 72 °C, followed by 7 min at 72 °C. A negative control (no DNA) was included in all PCR amplifications. The PCR products were separated on a 1% agarose gel in Trisacetate EDTA buffer, stained with Red Safe and scanned in a GelDoc XR documentation system (Bio-Rad Laboratories). PCR products were cleaned for sequencing using ExoSap-It reagent (GE, Healthcare, NJ, USA) for 15 min at 37 °C, followed by inactivation for 15 min at 80 °C. Sequencing was performed in a specialised service (Secugen, Madrid).

Chromatograms were analysed using ChromasPro v.1.41 Technelysium Pty Ltd., All generated sequences were searched for identity using BLAST (Basic Local Alignment Search Tool) through web servers of the National Center for Biotechnology Information (USA). Multiple aligments based on ITS region of nematode parasites were performed using the sequences obtained in this study and others available from GenBank. Aligments were performed using Clustal W (Thompson et al., 1994) included in MEGA6 (Tamura et al., 2013). Maximum parsimony tree was constructed using MEGA6 software and reliability of the inferred tree was tested by 2000 bootstrap replications. The analyses of the ITS1-5.8S-ITS2 region included the sequences obtained in this study (n = 31) and the following sequences deposited in the GenBank: *A. pegreffii* (KJ011495, KF923927), *A. simplex* s.s. (KC663498, KF512906, KF953967, KF953969), *A. physeteris* (JQ912693, JN005754), hybrid beteween *A. simplex* and *A. pegreffii* (KF032056), *Hysterothylacium* sp. (JQ798963), *H. deardorffoverstreetorum* (JF30201) and *H. tetrapteri* (KF601901). The sequence of *Ascaridia columbae* (KF147909) was used as outgroup.

2.4. Statistical analysis

Quantitative parasite descriptors such as prevalence (P), mean intensity (MI), and mean abundance (MA) were calculated according to Bush et al. (1997). Odds ratio and 95% confidence interval were obtained. Normality of the data was tested using a SHAPIRO-test. Pearson's correlation coefficient (r) was applied to determinate the degree of association between prevalence and biological