Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 31, issue 4, 2021, pp. 187 - 197

TAXONOMY OF ANISAKIDAE INFECTING *MUGIL CEPHALUS* LINNAEUS, 1758 FROM ALGERIAN COAST

Racha BOUBEKEUR[1, 2*], Khaled ABDELOUAHED[2], Haeit ADJMI HAMMOUDI[2], Jean-Paul TRILLES[3], Zouhir RAMDANE[1]

[1]Laboratoire de Zoologie Appliquée et d'Ecophysiologie Animale, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, Algerie
[2]Laboratoire de Parasitologie Mycologie, Hôpital Central de l'Armée, 244 Kouba, Alger, Algerie
[3]Universitée de Montpellier, 34000 Montpellier, France

Abstract: The molecular detection was established for the collected parasitic nematodes (Anisakidae larvae: L3) infecting *Mugil cephalus* Linnaeus, 1758 from the central Algerian coast. From January 2017 to January 2018, 1000 specimens of this fish were sample and studied for their nematode parasites. Our results show very low infection rates (P= 2.5%; MI= 1.2 parasites per infected host) by Anisakidae nematodes (L3). These parasites were collected only in female specimens of *M. cephalus*. Morphological analysis reveals that the collected specimen's nematodes belong to the genus *Anisakis* spp. The 30 collected *Anisakis* larvae (at stage L3) were studied using molecular analysis (PCR targeting the ITS1 ITS2 regions followed by a RFLP and Sanger sequencing targeting the COX2 mitochondrial gene). Sequencing confirms the affiliation of the 30 collected larvae of parasitic nematodes to the species *Anisakis pegreffi* (24 larvae) and *Anisakis simplex sensu stricto* (6 larvae). The results obtained indicate for the first time the presence of parasitic nematodes in *M. cephalus* of the Algerian coast.

Keywords: *Mugil cephalus*, *Anisakis* spp, PCR, RFLP, Algerian coast.

INTRODUCTION

Mugilidae off the Algerian coast are fish of great economic interest. Fishes of this family can be repeatedly attacked by pathogenic parasites impacting subsequently their commercial value. Despite their economic importance, studies of their parasitology are rare. The life cycle of Anisakidae requires at least two hosts of different species to complete their life cycle (Audicana *et al*., 2008). Depending on their stage of development, these nematodes infect invertebrates, fish and mammals (Mattiucci *et al*., 2004; Setyobudi *et al*., 2011; Gaglio *et al*., 2018). They infect intermediate, paratenic or definitive hosts (Zhu *et al*., 1998). In the intermediate host, larvae enter the intestine and invade the abdominal cavity and muscle, where they molt and encapsulate giving the infectious form (Bernardi, 2009). *Anisakis* larvae (L3) may be present in the muscle, which is usually causing therefore a greater risk of contamination. They can infect humans causing Anisakiasis. Humans are an accidental host in which these larvae can cause two types of severe syndromes: digestive and allergic. The classical taxonomy of Anisakidae is based on the morpho-anatomy of specimens: sizes and ratio of the sizes of internal organs, general morphology and morphology of internal organs (excretory and digestive systems, caecum, ventricle, esophagus, sexual organ: spicule), especially in adults (Mattiucci and Nascetti, 2008).

Studies conducted in Algeria are few and focused specially on the taxonomy based on the morph-anatomical and morphometric features of nematodes specimens that infecting teleost marine fishes (Petter and Maillard, 1987; Hassani and Kerfouf, 2014; Ichalal *et al*., 2015; Saadi *et al*., 2019). Parasites of the genus *Anisakis* exhibit differences in their genetic structure, ecological characteristics such as geographic distributions, life cycle and host preferences (Farjallah *et al*., 2008; D'Amelio *et al*., 2010).

Morphological observations of *Anisakis* larvae in type I and II were report for long time (Berland, 1961). For the genus *Anisakis*, the morphological classification of type I or type II larvae is of particular use, according to the criteria given by Shiraki in 1974 (D'Amelio *et al*., 2000). However, many parasitologists claim that the identification of larvae of parasitic nematodes (Anisakidae: *Anisakis*) is not obvious and more difficult especially for the undifferentiated larvae stage. In intermediate hosts, the larvae of parasitic nematodes are not really well differentiating, making, at this stage, the morphological criteria for the development of parasites insufficient to identify the different species. In the Algerian coast, the taxonomic status of the larvae of nematodes infecting Mugilidae is not yet clear (D'Amelio *et al*., 2000). However, the identification of related species can be achieve through the development of molecular markers using PCR-based approaches that have a remarkable sensitivity in detecting variations requiring only small quantities of fresh product or parasitic materials attached to ethanol. Species of the same genus are morphologically similar. The concept of species complex (clade) was developed through these identification techniques. According to phylogenetic analysis, *Anisakis* spp. is separate into two clades. Clade I includes the *Anisakis simplex* complex (*A. simplex* (*s.s*), *A. pegreffii and A. simplex* C *(recently named A. berlandi), A. typica, A. ziphidarum* and *Anisakis* sp. Clade II includes species *A. physeteris, A. brevispiculata and A. paggiae* (Paggi *et al*., 1998; Mattiucci and Nascetti, 2006; Mattiucci and Nascetti, 2007; Mattiucci and Nascetti, 2008; Mattiucci *et al*.,

***Correspondence:** Racha Boubekeur, Laboratoire de Zoologie Appliquée et d'Ecophysiologie Animale, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, Algérie; rachaimane.boubekeur@univ-bejaia.dz*

2009). PCR-RFLP based on transcribed internal spaces of DNA (ADNr) (ITS-1 and ITS-2) provides a useful approach for both distant and closely related identification between Ascaridae as these interspaces have shown high levels for differences in interspecific sequences in the presence of low-level intraspecific variation (Zhu *et al*., 2000). The genetic heterogeneity of nuclear markers is high. The cytochrome oxidase gene 2 (COX2) of DNA_r is known to evolve at a faster rate than nuclear DNA and can potentially provide useful information for phylogenetic reconstruction of related nematode species (Valentini *et al*., 2006).

The aim of this study is to confirm the presence of *Anisakis* larvae in *Mugil cephalus* Linnaeus, 1758 from the central coast of Algeria, and to identify these larvae using morphological and molecular methods.

MATERIALS AND METHODS

Sampling and parasitological study

One thousand specimens of *Mugil cephalus* Linnaeus, 1758 were sample in the fishing port of Algiers (central Algerian coast) from January 2017 to January 2018 (Fig. 1.).

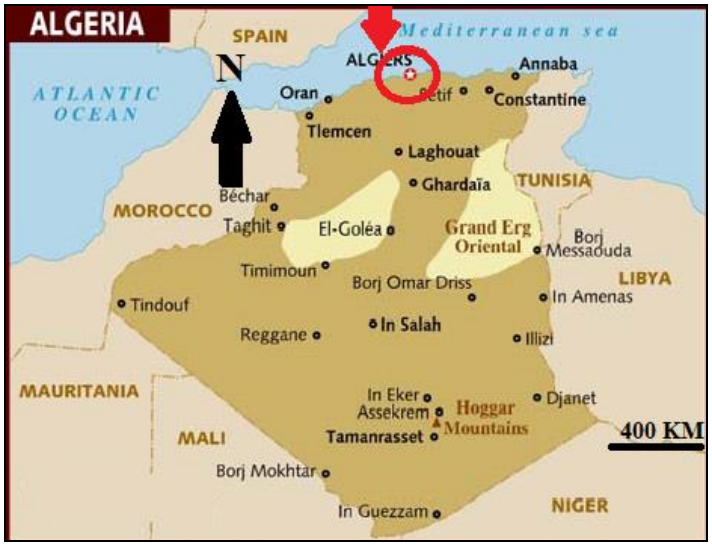


Fig. 1. The sampling area, modified (Wikimedia: orangesmile.com).

After sampling, the specimens were transfered (in a cooler at 4°C) directly to the laboratory for examination. For each specimen, the total length and weight were determined as close as possible (0.1 cm and 0.1 g respectively). The sex of each specimen was determined by macroscopic observation of the gonads. The internal organs (abdominal cavity, intestine, stomach, liver and gonads) of each specimen were examined using a stereo microscope. The fixing site of parasitic nematodes was noted for each infected fish. The collected parasites were stored in 70% alcohol for more detailed identification from their morpho-anatomical characteristics and using identification keys (Petter, 1974; Petter and Maillard, 1987; Petter, 1988; Moravec, 1994; Moravec and Justine, 2017). Morphological analyses were performed using optical microscopy after clarification of parasites with glycerol 80% (Berland, 1961). The parasitological indexes were calculated according to Bush (Buch *et al*., 1997).

Molecular analysis

For more precision in the identification of collected parasitic nematodes we performed a PCR, followed by an RFLP on the ITS1 ITS2 regions of the DNAr, and a Sanger sequencing on the mitochondrial gene Cytochrome Oxydase 2 (COX2). We used the NC5 NC2 (Zhu *et al*., 2000) primer pair targeting the ITS1-5.8, ITS2 regions, as well as the 210 and 211 mitochondrial pair COX2 (Nadler and Hudspeth, 2000).

DNA extraction

The larvae were rehydrated with sterile water fragmented with a scalpel, and placed in a 2 ml tube with 200 µl of water and then frozen at -20°C for 24 hours. The DNA extraction is carried out after recovering the ethanol parasites and frozen in liquid nitrogen using the Qiagen DNA Mini Kit (Qiagen, GmbH, Germany), based on the use of mini silica columns, according to the instructions of the manufacturer. The concentration of the extracted DNA is assess by spectrophotometric method at 260 nm (Zhu *et al*., 2000).

DNA amplification of ITS1- ITS2 region and RFLP

DNA amplification is performed by PCR (polymerase chain reaction) targeting the ITS region (ITS-1, ITS-2, and 5.8 S sub-unit), encoding for a specific DNA fragment of about 1000 bp using the primers NC5 forward (5'-GTA GGT GAA CCT GCG GGA GGAGGA TGA TT-3') and NC2 reverse (5'-TTA GTT TCT TCT CCT CCG CT-3') (Zhu *et al*., 2000). For a final volume of 50 µl, the mix contains 15 µl of tampon (DNA leader), 5 µl of MgCl2, 2 µl of dntp, 1 µl for each primer, and 0.2 µl of taqpolymerase. The PCR

is performed in a MyCyclerTM thermocycler (Bio-Rad, USA), with the following conditions: 10 min at 95°C, then 30 cycles from 1 min to 94°C, 1 min to 57°C and 1 min to 72°C, followed by a final elongation of 5 min to 72°C. PCR products are separate by electrophoresis on a 1.5% agarose gel, and then visualized by a UV lamp.

PCR products are subject to an RFLP PCR, using the restriction enzymes TaqI, HinfI and HhaI (D'Amelio *et al*., 2000; Umehara *et al*., 2006). The mix contains 5 µl of PCR product, 1 µl of restriction enzymes, 2 µl NE buffer, and distilled water up to a final volume of 20 µl. Digestion is at 37°C for 90 min HinfI and HhaI, while digestion with TaqI is carry out at 65°C for 90 minutes. Ten microliters of the digestion product were analyzed by electrophoreses on a 1.5% agarose gel containing ethidium bromide and visualized under the UV lamp.

PCR COX2 and amplified product sequencing

The COX2 mitochondrial region of the larvae examined was also amplified using primers 210 (5'-CAA CTC TTA AAA TTA TC-3') and 211 (5'TTT CTA GTT ATA TAG ATT GRT TYA TYA-3') (Nadler and Hudspeth, 2000). For a final volume of 50 µl, the mix contains 20 µl of buffer (DNA leader), 3 µl of MgCl2, 2 µl of dntp, (20 pmol/l) for each primer, and 0.1 µl of taqpolymerase. The PCR is performed in a MyCyclerTM thermocycler (Bio-Rad, USA), with the following conditions: 10 min at 95°C, then 35 cycles from 5 min to 94°C, 1 min to 52°C and 1 min to 72°C, followed by a final elongation of 7 min to 72°C. PCR products are separate by electrophoresis on a 1.5% agarose gel, and then visualized by a UV lamp. The purification of COX2 gene amplification products was carry out with illustrated GFX PCR DNA and Gel band Purification kit, following the manufacturer's instructions.

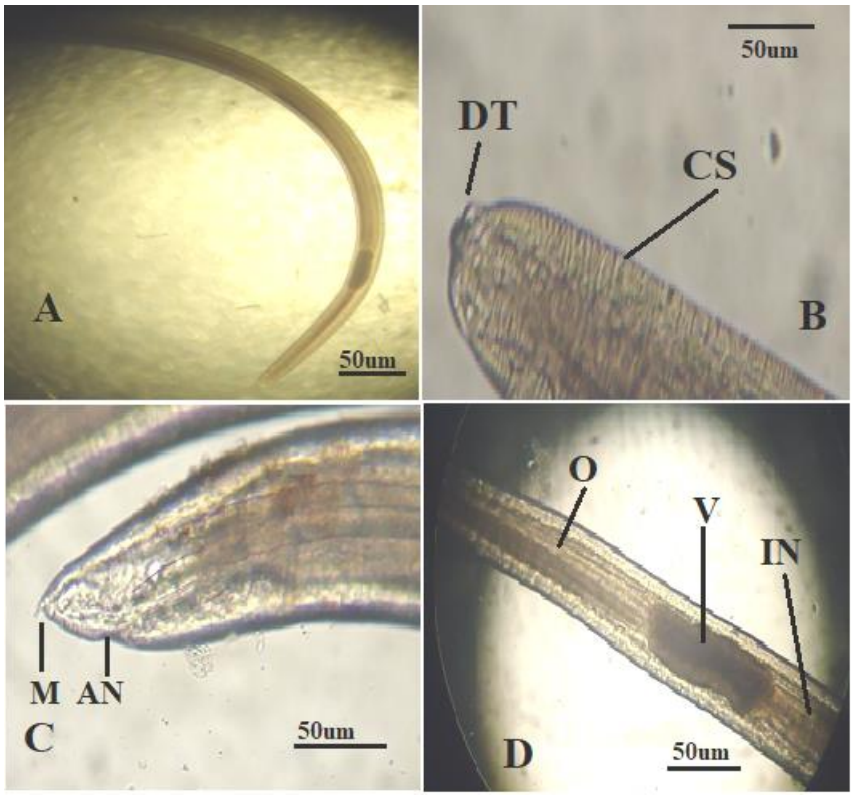
Purified PCR products sent for Sanger sequencing to an outside provider. Each PCR product has sequenced in both directions.

The complementary sequences of the same amplified product were compare to correct any sequencing errors on the Bioedit software (http://www.mbio.ncsu.edu/bioedit/bioedit.htML) and to obtain a "consensus" sequence for each individual analyzed. This consensus sequence was compare to sequences filed in GenBank using the Basic Local Alignment Search Too program (http://blast.ncbi.nlm.nih.gov/Blast.cgi, BLAST (Altschul *et al*., 1990) to identify the species of Anisakidae to which the individual belongs.

RESULTS

Parasitological study

One thousand specimens of *Mugil cephalus* were examined. The weight ranges from 460 to 2540 g and the length from 42 to 63 cm. A good number of both sexes were examined, 510 females and 490 males. Only 25 females were infected (Prevalence= 2,5 %; Mean intensity=1.2 parasites per infected fish). We collected 30 L3 larvae of *Anisakis* spp from tube digestive of adult *M. cephalus* females. The low rate of infection is remarkable especially since males were not infected with parasitic nematodes. The collected L3 larvae are light to yellowish white in color. They are 14 to 30 mm long and have a diameter of 0.5mm (Fig.2 A). The cuticle is streak with large, irregular transverse grooves discontinuous all over the body. Between the cross grooves fine parallel wrinkles are visible at high magnification (Fig.2 B). A mucron is present at the posterior end of the larva (Fig.2 C). The ventral part includes a sharp triangular penetrating tooth and three pre-labial bulges: a back and two sub-ventrals surrounding the oral opening. A white spot at 2 mm in the back of the anterior end corresponds to the esophageal ventricle (Fig.2 D). The internal organs constitute of a complete digestive tract comprising a mouth, an esophagus, an intestine ending in an anus. The esophagus consists of two parts: a muscle part of 1.8 to 2.8 mm located just after the mouth and a fairly elongate glandular part (or ventricle) separated from the intestine by an oblique boundary (Fig.2 D). The larva does not have caecum or esophageal appendage. The excretory organ ends with a pore between the bases of the two labial drafts and is visible in the form of a darker channel (Fig.2 D).



DT: tooth, CS: streaked cuticle, M: mucron, AN: anus, O: esophagus, V: Ventricle, IN: intestine.

Fig. 2. Original photos on the morphology of the collected Larvae of *Anisakis* sp. (Larva L3). A: *Anisakis* sp L3 larva observed at magnification x 400. B: Anterior end of *Anisakis* sp C: Posterior end of *Anisakis* sp. D: Digestive tube of *Anisakis* sp.

Molecular study

Determined 30 larvae of *Anisakis* spp. On the genus level were subject to confirmation of the genus by PCR, determination of the species nematode by an RFLP and sequencing for all nematode.

In first, the Ribosomal fragment ITS1-5.8S-ITS2 was developed on thirty collected larvae, obtaining a fragment of approximately 1,000 Pb (Fig. 3).

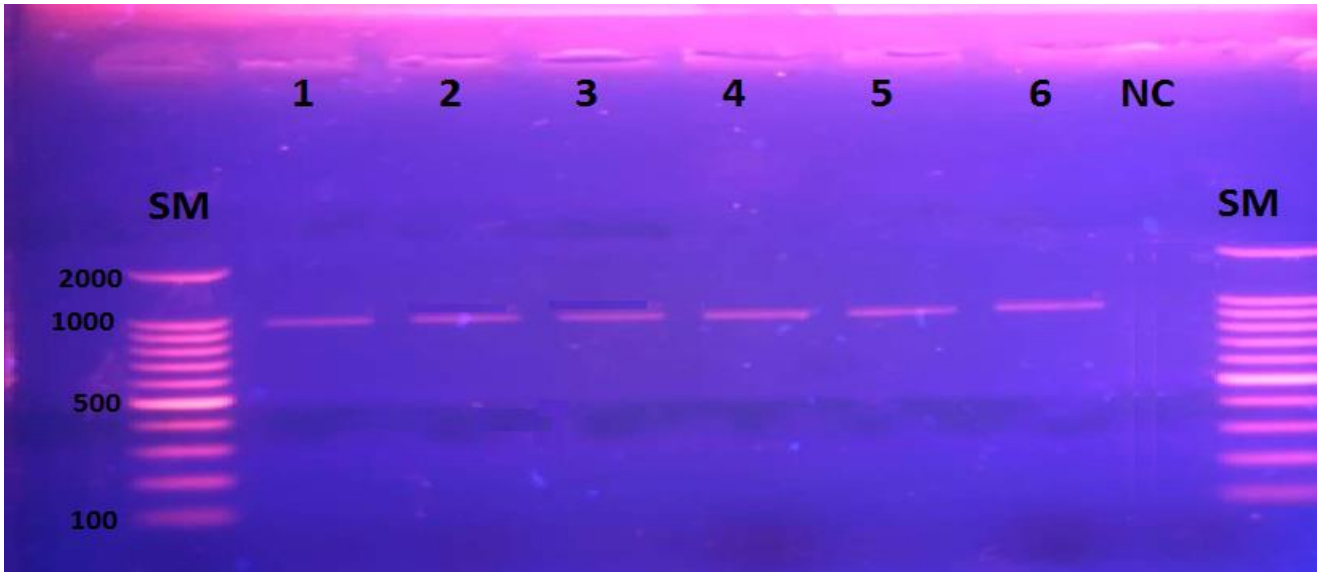


Fig. 3. PCR NC5 NC2 profile (Zhu *et al*., 2000) targeting ITS1 ITS2 regions. SM: size marker. NC: negative control 1-6: DNA extracts fragment -1000PB.

RFLP on ITS1 ITS2 regions

The digestion of PCR products for *Anisakis simplex sensu stricto* larvae with the TaqI presented a pattern of two bands: one of 430 bp, and the other of 400 bp. The HinfI enzyme produced two bands of 620 and 250 bp. HhaI produced two bands (550 and 430 bp). The *A.*

pegreffii larvae presented a pattern of three bands of 370,300 and 250 bp for HinfI enzyme and two of 400,320 for TaqI enzyme. HhaI produced two bands (550 and 430 bp). With the results obtained from these three enzymes, in accordance with the restriction models described by D'Amelio (D'Amelio *et al*., 2000 ; Kijewska *et al*., 2000; Martín-Sánchez *et al*., 2005; Umehara *et al*., 2006) all samples tested yielded the same results, the model of these fragments corresponds to 6 *Anisakis simplex sensu stricto* and 24 *Anisakis pegreffi* (Fig. 4.).

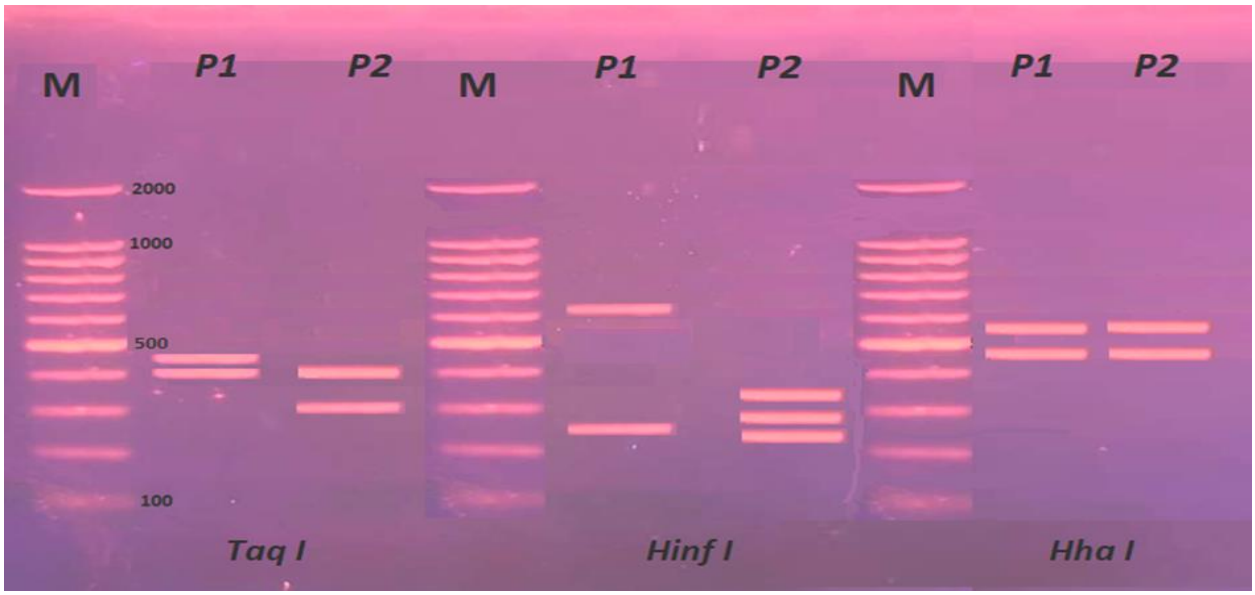


Fig. 4. PCR-RFLP fragment patterns of two *Anisakis* spp. belonging to *Anisakis* Type I (A) after digestion with restriction enzymes. Pattern 1 (P1) - *Anisakis simplex* (*sensu stricto*); Pattern 2 (P2) - *Anisakis pegreffii* M-100 bp marker.

Sequencing on COX2

We also conducted an analysis of COX2 sequencing of the collected larvae. The sequences obtained for the thirty larvae demonstrate the presence of two different species *Anisakis pegreffi* (24 larvae) and *Anisakis simplex sensu stricto* (6 larvae) (Fig. 5).

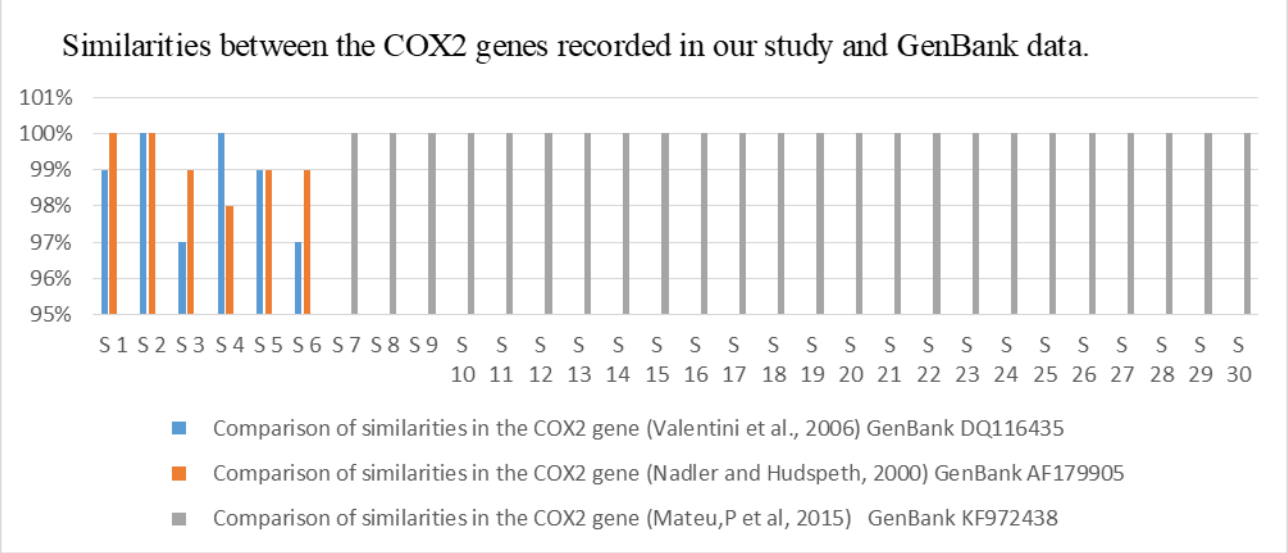


Fig. 5. Similarities between the COX2 genes recorded in our study and GenBank data.

The nucleotide sequences of the COX2 gene of the *Anisakis* genus were collect from public banks of sequences such as the NCBI (National Center for Biotechnology Information). The sequences were standardized by removing redundant sequences using the MAFFT service website. Then the name of the target gene and the identity of the gene and species kept before they were integrate into the bioinformatics analysis pipeline. This sequence database was use for the analytic part of aligning sequences out of sequencing to annotate them. We obtained COX2 sequences from the thirty *Anisakis* larvae; the haplotypes were identifie in an alignment of 600 Pb (data not displayed) (Fig 5). By comparing, the COX2 sequences obtained with the sequences known in GenBank through a BLASTN search. After sequencing the thirty strains we detected 24 *Anisakis pegreffi* similar to the strain GenBank KF972438 (Mateu *et al*., 2015) and 6 *Anisakis simplex sensu stricto*, similar to the two strains GenBank DQ116435 (Valentini *et al*.,

2006) and GenBank AF179905 (Nadler and Hudspeth, 2000). The results obtained are report in (Fig. 6.).

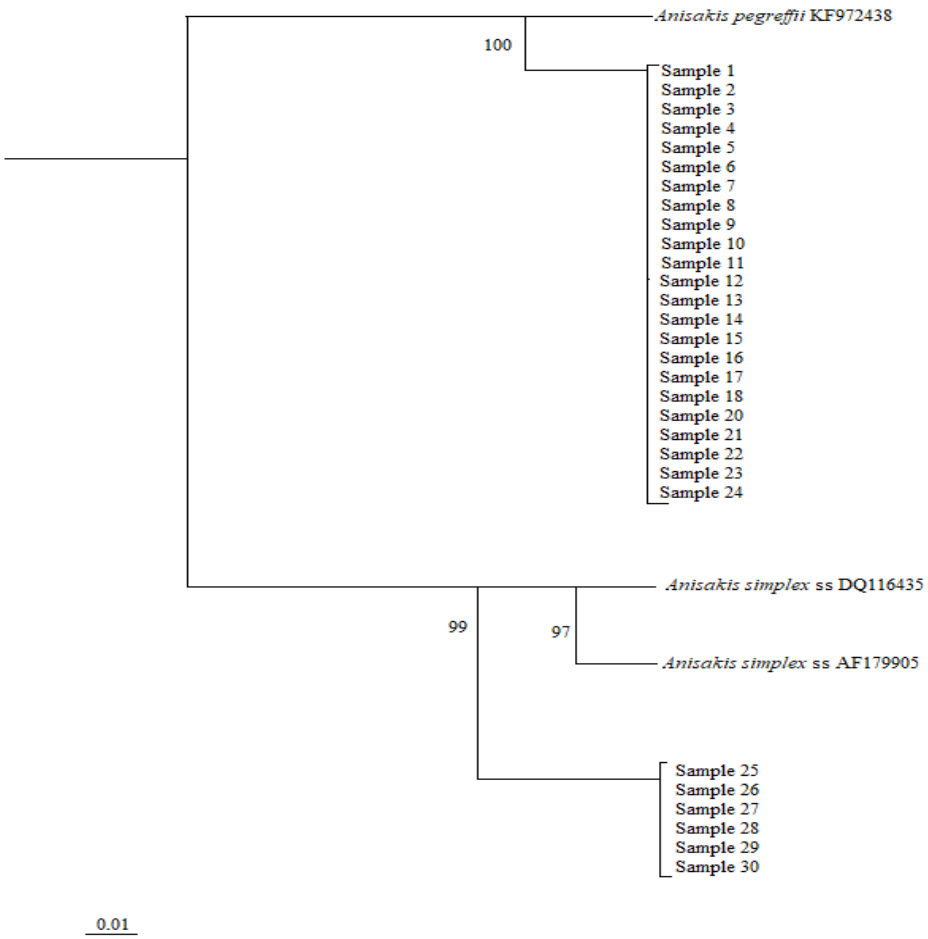


Fig. 6. Phenogram depicting the genetic differences (upon pairwise comparison) among the *Anisakis* our study and different *Anisakis* species from the GenBank for the COX2 genes sequences.

DISCUSSION

In Algeria, *Mugil cephalus* is very popular and its price remains within everyone's reach. Despite the commercial importance of this fish, its parasitofaune was very rarely studied (Ramdane *et al*., 2007a; Ramdane *et al*., 2007b; Ramdane and Trilles, 2008; Ramdane *et al*., 2009). The results obtained indicate for the first time the presence of parasitic Nematodes in *M. cephalus* from Algerian coasts. Molecular analysis identified two species *Anisakis pegreffi* and *Anisakis simplex sensu stricto* by sequencing after confirming the affiliation of the collected L3 larvae of parasitic nematodes at the genus *Anisakis* based on analysis morpho-anatomical. On the basis of observed morphological traits the collected nematodes are identified as type I *Anisakis* larva, this is the only obtained information by morphological examination (Fig. 1). The length of the ventricle and the presence or absence of mucron may distinguish between *Anisakis* Type I and *Anisakis* Type II (Bernardi, 2009). The relationship between the esophagus and the ventricle may be a distinctive morphological trait in differentiating species (Quiazon *et al*., 2008). However, identifications based only on morphological differences can lead to much controversy due to the limitation of valuable taxonomic traits in L3 (Saadi *et al*., 2019).

The measurements recorded on the collected parasite specimens from *Mugil cephalus* show a difference compared to those collected in different fish species from Algerian (Ichalal *et al*., 2015), and Mediterranean coasts (Larizza and Vovlas, 1995). Our specimens are larger compared to those reported in different studies (Table 1). They are characterize by a longer mucron than those found by Ichalal (Ichalal *et al*., 2015), in *Boops boops* and *Trachurus trachurus*, and shorter than those collect by Larizza and Vovlas (Larizza and Vovlas, 1995). The diameter, ventricle, and penetrating tooth also appear to be larger in specimens found in *Mugil cephalus*. Such morphometric differences could be due to certain geographical factors of the environment, knowing that *Mugil cephalus* lives in two different environments (fresh and marine water).

The parasitized specimens of *Mugil cephalus* are adult females larvae (L3) were found in their digestive tract. Several scientific studies have already shown that

Anisakis specimens enter the intestinal wall and are generally encapsulate on visceral organs, and peritoneal mesentery (Smith, 1984). Some factors such as eating behavior of female's specimens or preys disponibility (intermediate hosts) maybe enhance infection by parasitic nematodes. Various studies have already reported factors intrinsic and extrinsic factors that influence the distribution of Anisakidae.

Table 1.

Morphometric data of *Anisakis simplex,* from different studies conducted in the Mediterranean Sea

Host	Tl (mm) D (mm)	Pt (µm)	Ventricle (mm)	Mucron (µm)	O/V	References
Trachurus trachurus	19.37±3.88 0.13±0.02	5.7±1.9	0.28±0.04	9.3±1.56	/	**Ichallal et al., 2015**
Boops boops	17 0.18	4	0.2	10	/	**Ichallal et al., 2015**
Merluccius merhiccius	21.60±3.47 0.41±0.05	/	0.70±0.08	25.69±4.1	/	**Larizza and Vovlas (1995)**
Trachurus trachurus	20.3±3.00 0.43±0.06	/	0.69±0.09	/	/	**Hurst (1984)**
Mugil cephalus	14 ±30 0.5	0.81±0.8 4.8 ±5.5	0.4±0,6	11.5	1.4	**This study**

Tl: Total length; D: Dimeter; Pt: Penetration tooth; O/V: Esophagus/ventricle ratio

It seems that often the larger fish size are more infected by Anisakidae (increasing infection with increasing size) (Bergmann and Motta, 2004). The study on squid infestation (*Illex coindettii*), an intermediate host of *A. pegreffii*, determined the relationship between parasite infection and sex, maturity and host diet (Petric *et al*., 2011). In Spain, a study of (*Trachurus trachurus*) also found that intrinsic factors like sex, diet, immune system, etc. influence the level of Anisakidae infection in fish populations.

A study of wild Pacific salmon showing that fish less than 50 cm in length had lower parasitism intensity (Setyobudi *et al*., 2011), *Contracaecum* sp. infection is higher in male cod than in females (Hemmingsen *et al*., 2000).

In the case of *M. cephalus*, a fish with active displacement between marine water and fresh water, salinity may be a limiting factor to infection with parasitic nematodes. Several studies concerning the resistance of the *Anisakis* sp. different environmental conditions (Augry, 2012) have thus shown that salinity is an effective way to destroy *Anisakis* sp. Salt kill nematodes larvae according to AFSSA (AFSSA 2008) and the CEVPM study (CEVPM 1993).

Species attached to the genus *Anisakis* have different geographical distributions and host spectra. Extrinsic factors including geographic area, climate, ocean currents, water salinity and acidity, depth and seasonality maybe impacting these parasites and their hosts (Bergmann and Motta, 2004). The number of pelagic fish is increasing due to warming coastal waters; allowing parasites of the genus *Anisakis* to expand their paratenic hosts and increasing the probability of infecting their final host.

Farjallah (Farjallah *et al*., 2006) considered season as one of factors influencing the life cycle of fish and thus the level of infection by Anisakidae. In the eastern Mediterranean Sea, in *Phycis blennoides* and *Phycis phycis*, the authors have shown an increase in the prevalence of parasites *A. simplex*, *A. physeteris*, *H. aduncum* and *H. fabri* in spring and summer. At this time of year, the water is warmer, resulting in an increase in the activity of fish, which feed more and have higher growth. As a result, the probability of accumulating parasites is increased (Farjallah *et al*., 2006). In contrast, Smith (Smith, 1984) suggested that the season did not influence the distribution of parasites since the final hosts excrete *Anisakis* eggs throughout the year.

The parasitological examination of the intestines of the *M. cephalus* specimens allowed us to observe the simultaneous presence of small crustaceans and L3 larvae of *Anisakis* sp. The low rate of *M. cephalus* infection with Anisakidae nematodes is probably related to the feeding behavior of *M. cephalus*, as specimens of this species of fish feed mainly on mud and phytoplankton for the majority, but they feed mainly on mud and phytoplankton, but they do may also consume copepods or small crustaceans (FAO, 2009). The study on the stomach contents of *Myctophum punctatum* and *Notoscopelus kroyeri* showed that the consumption of copepods by these two fish increases with their size, thus increasing infection by Anisakidae (Klimpel *et al*., 2008). The study on squid infestation (*Illexcoindettii*), an intermediate host of *A. pegreffii*, highlighted the relationship between parasite infection and sex, maturity and host diet (Petric *et al*., 2011). The authors showed that the prevalence of *A. pegreffii* is high in autumn and low in summer due to the diet that changes with the season but also to the change in age (Petric *et al*., 2011).

Over the past 20 years, our knowledge of the taxonomic position of species of the genus *Anisakis* has improved considerably through the application of other non-morphological techniques: first, iso-enzymatic characterization and subsequent DNA study. In this study, the nuclear DNAr of the ITS1 ITS2 regions is amplify using the NC5/NC2 primers mentioned in previous studies of Zhu (Zhu *et al*., 2000). The Regions of ITS-1 and ITS-2 representing the identified *Anisakis* larvae, the species *Anisakis simplex* is detect by PCR RFLP. The primers NC5/NC2 Zhu (Zhu *et al*., 2000) generally work better than the A/B primers described by D'Amelio (D'Amelio *et al*., 2000), and for this reason, they were use in our study. It has therefore become clear that new genetic markers need to be

developed and implemented (Setyobudi *et al*., 2010). The digestion of PCR products using HhaI produced two fragments (550 and 430 bps), HinfI produced two fragments (620 and 250 bps), and the TaqI produced two bands of 430 and 400 bps. The pattern of these fragments was similar to those previously described by D'Amelio (D'Amelio *et al*., 2000) and Pontes (Pontes *et al*., 2005). The PCR-RFLP of ITS1-5.8S-ITS2 based on the keys provided by D'Amelio (D'Amelio et al., 2000). In addition, subsequently implemented by other authors such as Abollo (Abollo *et al*., 2003), Martin-Sanchez (Martín-Sánchez *et al*., 2005), Marques (Marques *et al*., 2006), Pontes (Pontes *et al*., 2005), Farjallah (Farjallah *et al*., 2008), ect, appears at first glance to be a relatively quick technique to be performed and interpreted.

In our study, sequencing targeting mitochondrial cytochrome oxydase 2 DNA confirmed the identification of the two species *Anisakis pegreffi and Anisakis simplex* (*sensu stricto*) for the thirty larvae collected. We confirm the dominance of *Anisakis pegreffi* in Mediterranean Sea off Algerian coast. Many studies confirm the dominance of *Anisakis* species in Mediterranean Sea (Farjallah *et al*., 2008).

A. simplex is widely distributed throughout the Arctic Circle. It is present in the western and eastern Atlantic Ocean and in the Pacific Ocean (Abe *et al*., 2006; Umehara *et al*., 2006; 2008). The southern limit of this species is for the Northeast Atlantic Ocean around the Strait of Gibraltar area. In the Mediterranean, *Anisakis simplex* has been described in Gadiforms (*Merluccius merluccius* and *Pollachius virens*), perciforms (*Trachurus trachurus*, *Mugil auratus*) and Scombridae (*Scomber scombrus*). In the North Atlantic, the parasite has been described in Clupeiforms (*Clupea harengus*, *Engraulis encrasicolus*, *Sardina pilchardus*), Gadiforms (*Pollachius virens*), Pleuronectiforms (*Max psetta*), Scombridae (*Scomber scombrus*) and Scorpaeniforms (*Scorpaena notata*). Data have been published on the presence of *Anisakis* spp. in fish from the Mediterranean and Atlantic coasts of Morocco (Mattiucci *et al*., 2001; Shukhgalter, 2004; Cisse and Belghyti, 2005; Farjallah *et al*., 2008; Kijewska *et al*., 2009; Abattouy *et al*., 2011).

In Tunisia, *Anisakis simplex* has observed only in common mackerel (*Scomber scombrus*), although other species of *Anisakis* (*A. pegreffii, A. physeteris and A. typica*) have been described in *Merluccius merluccius*, *Lophius* sp., *Thunnus thynnus* and *Xiphias gladius* (Farjallah *et al*., 2008). *Anisakis simplex* was reported in benthic fish from the North Atlantic (Mattiucci *et al*., 2004; 2007). There is a sympatry between *A. simplex* (s.s) and *A. pegreffii* in the waters of the Atlantic Ocean (Marques *et al*., 2006, Mattiucci and Nascetti, 2007) of the Alboran Sea (Mattiucci and Nascetti, 2007) and the Sea of Japan (Umehara *et al*., 2006). *Anisakis simplex* (s.s) and *A. simplex* C are present in intermediate, parathenic and definitive hosts in the eastern Pacific Ocean (Mattiucci *et al*., 1997). Species *A. simplex* has also found off the Korean Peninsula (Bak *et al*., 2014).

In this study, the presence of *Anisakis pegreffi and Anisakis simplex sensu stricto* is report in *Mugil cephalus* for the first time in Algeria. Morphological differentiation of the larval and adult stages of *Anisakis* nematodes is difficult, but the use of molecular methods, such as PCR RFLP from the ITS region of the DNAr, as well as COX2 sequencing, have been shown to be reliable techniques for unequivocal identification and determination of the species. The sequencing is very important for detect exactly the species of nematodes. Thus, we confirm the presence of these two species in Algeria and their repartition in Mediterranean Sea. *Anisakis* infection can be carefully monitor to prevent possible economic losses and potential risk that may affect the health of consumers.

ACKNOWLEDGEMENT

We warmly thank fishermans who helped us in getting samples.

AUTHOR'S CONTRIBUTIONS

Racha Boubekeur: laboratory manipulation, methodology, writing of original draft; Khaled Abdelouahed: supervision; Haeit Adjmi hammoudi: identification of compounds and data processing; Jean-Paul Trilles: data validation, writing and editing; Zouhir Ramdane: proposition of the species, the problematic and data validation, writing and editing.

FUNDING

No source of funding has been provided for this work.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

Abattouy N, Valero A, Benajiba, MH, Lozano, J and Martı´n-Sa´nchez J, *Anisakis simplex* s.1.parasitization in mackerel (*Scomber japonicus*) caught in the North of Morocco – prevalence and analysis of risk factors. International Journal of Food Microbiology, 150: 136–139, 2011.

Abe N, Tominaga K, and Kimata I, Usefulness of PCR-restriction fragment length polymorphism analysis of the internal transcribed spacer region of DNAr for identification of *Anisakis simplex* Complex. Japan Journal Infection Disease, 59: 60-62, 2006.

Abollo E, Paggi L, Pascual S & D'Amelio S, Occurrence of recombinant genotypes of *Anisakis simplex* s.s. and *Anisakis pegreffii* (Nematoda: Anisakidae) in an area of sympatry. Infection Genetics and Evolution, 3: 175–181, 2003.

AFSSA, MIC2007sa0379 opinion of the French Food Safety Agency relating to a risk assessment request for the presence of Anisakids in fishing products and the extension of the exemption from the freezing obligation sanitizing for fish

products whose food is controlled as well as for some species of wild fish, 2008.

Altschul SF, Gish W, Miller W, Myers EW, and Lipman DJ, Basic local alignment search tool. Journal of Molecular Biology, 215: 403–410, 1990.

Audicana MT, and Kennedy MW, *Anisakis simplex* from obscure infectious worm to inducer of immune hypersensitivity. Clinical Microbiology Revue, 21: 360–379, 2008.

Augry S, Current data on anisakids and anisakidosis. Veterinary medicine thesis. Nantes: Oniris - National Veterinary, Food and Food School; University of Nantes 232, 2012.

Bak TJ, Jeon CH, and Kim JH, Occurrence of anisakid nematode larvae in chub mackerel (*Scomber japonicus*) caught off Korea. International Journal of Microbiology, 191: 149 156, 2014.

Bergmann GT, and Motta PJ, Infection by anisakid nematodes Contracaecum spp. in the Mayan cichlid fish Cichlasoma (*Nandopsis*) urophthalmus (Günther 1862). Journal Parasitology, 90:405 407, 2004.

Berland B, Nematode from some Norwegian marine fishes. Sarsia, 2:1–50, 1961.

Bernardi C, Preliminary study on prevalence of larvae of Anisakidae family in European sea bass (*Dicentrarchus labrax*). Food Control, 20:433–434, 2009.

Buch AO, Lafferty KD, Lotz JM & Shostak AW, Parasitology meets ecology on its own terms: Margolis et al. revisited. Journal of Parasitology, 8:575-583, 1997.

Carte du monde, Monde Carte.com, http://www.mondecarte.com/carte/alger-carte .

CEVPM, Study of the frequency of herring net infestation by the *Anisakis simplex* larva, 1993.

Cisse M, and Belghyti D, Helminth parasites of chub mackerel *Scomber japonicus* (Houttuyn, 1782) from the harbour of Mehdia–Kenitra (Atlantic coast of Morocco). Journal of Aquatic Sciences, 20: 63–67, 2005.

D'Amelio S, Busi M, Ingrosso S, Paggi L, and Giuffra E, *Anisakis* sp. In: Liu DY (ed) Molecular detection of foodborne pathogens. CRC Boca Raton, 757–768, 2010.

D'Amelio S, Mathiopoulos KD, Santos CP, Pugachev ON, Webb SC, Picanco M, and Paggi L, Genetic markers in ribosomal DNA for the identification of members of the genus *Anisakis* (Nematoda: Ascaridoidea) defined by polymerase chain reaction-base restriction fragment length polymorphism. International Journal for Parasitology, 30: 223–226, 2000.

FAO, *Mugil cephalus*. In Cultured aquatic species fact sheets. Text by Saleh, M.A. Edited and compiled by Valerio Crespi and Michael. New.CD-ROM (multilingual), 2009.

Farjallah S, Slimane BB, Busi M, Paggi L, Amor N, Blel H, Said K, and D'Amelio S, Occurrence and molecular identification of *Anisakis* spp. from the North African coasts of Mediterranean Sea. Parasitology Research, 102: 371–379, 2008.

Farjallah S, Slimane B, Blel H, Amor N, and Said K, Anisakid parasites of two forkbeards (Phycis blennoides and Phycis phycis) from the eastern Mediterranean coasts in Tunisia. Parasitology Research, 100: 11-17, 2006.

Gaglio G, Battaglia P, Costa A, Cavallaro M, Cammilleri G, Graci S, Buscemi MD, Ferrantelli V, Andaloro F, Marino F, *Anisakis* spp. larvae in three mesopelagic and bathypelagic fishe species of the central Mediterranean Sea. Parasitology International, 67: 23–28, 2018.

Hassani M, and Kerfouf A, Diversity of nematodes from the greater forkbeard Phycis blennoides (Teleostei: Gadidae) in the Western Mediterranean Sea. International Journal Science. Basic and Applied scientific Research, 18: 97-103, 2014.

Hemmingsen W, Halvorsen O, and MacKenzie K, The occurrence of some metazoan parasites of Atlantic cod, Gadus

morhua L., in relation to age and sex of the host in Balsfjord (70 N), North Norway. Polar Biology, 23: 368 372, 2000.

Ichalal K, Ramdane Z, Ider D, Kacher M, Iguerouda M, Trilles JP, Courcot L & Amara R, Nematodes parasitizing *Trachurus trachurus* (L.) and *Boops boops* (L.) from Algeria. Parasitology Research, 114(11): 4059-4068, 2015.

Kijewska A, Burzyn´ ski A & Wenne R, Anisakid parasites of fishes caught on the African shelf. Journal of Parasitology, 95:639–645, 2009.

Kijewska A, Slominska M, Wegrzyn G, and Rokicki J, A PCR RFLP assay for identification of *Anisakis simplex* from different geographical regions. Molecular and Cellular Probes, 14:349–354, 2000.

Klimpel S, Kellermanns E, and Palm H, The role of pelagic swarm fish (Myctophidae: Teleostei) in the oceanic life cycle of *Anisakis* sibling species at the Mid-Atlantic Ridge, Central Atlantic. Parasitology Research, 104(1): 43 53, 2008.

Larizza A, and Vovlas N, Morphological observations on third-stage larvae of *Anisakis simplex* A (Anisakidae: Nematoda) from Adriatic and Ionian waters. Journal of Helminthology, 62(2): 260–264, 1995.

Marques JF, Cabral HN, Busi M. and D'Amelio S, Molecular identification of *Anisakis* species from Pleuronectiformes of the Portuguese coast. Journal of Helminthology, 80, 47–51, 2006.

Martín-Sánchez J, Artacho-Reinoso ME, Díaz-Gavilán M and Valero- López A, Structure of *Anisakis simplex* s.l. population in a region sympatric for *A. pegreffii* and *A. simplex* s.s. Absence of reproductive isolation between both species. Molecular and Biochemical Parasitology, 141: 155–162, 2005.

Mateu P, Nardi V, Fraija-Fernandez N, Mattiucci S, Gil de Sola L, Raga J.A, Fernandez M and Aznar F.J, The role of lantern fish (Myctophidae) in the life-cycle of cetacean parasites from western Mediterranean waters. Deep Sea Research, 95: 115-121, 2015.

Mattiucci S, Paoletti M and Webb SC, *Anisakis nascettii* n. sp. (Nematoda: Anisakidae) from beaked whales of the southern hemisphere: morphological description, genetic relationships between congeners and ecological data." Systematic Parasitology, 74: 199–217, 2009.

Mattiucci S, and Nascetti G, Advances and trends in the molecular systematics of Anisakid nematodes, with implications for their evolutionary ecology and host–parasite co-evolutionary processes. Advances in Parasitology, 66: 47–248, 2008.

Mattiucci S, Abaunza P, Damiano S, Garcia A, Santos MN, Nascetti G, Distribution of *Anisakis* larvae, identified by genetic markers,and their use for stock characterization of demersal and pelagic fish from European waters: an update. Journal of Helminthology, 81: 117–127, 2007.

Mattiucci S, and Nascetti G,"Genetic diversity and infection levels of anisakid nematodes parasitic in fish and marine mammals from Boreal and Austral hemispheres." Veterinary Parasitology, 148: 43–57, 2007.

Mattiucci S and Nascetti G, Molecular systematics, phylogeny and ecology of anisakid nematodes of the genus *Anisakis* Dujardin, 1845: an update. Parasite, 13: 99–113, 2006.

Mattiucci S, Abaunza P, Ramadori L, and Nascetti G, Genetic identification of *Anisakis* larvae in European hake from Atlantic and Mediterranean waters for stock recognition. Journal of Fish Biology, 65: 495-510, 2004.

Mattiucci S, Paggi L, and G Nascetti, Genetic divergence and reproductive isolation between *Anisakis brevispiculata* and *Anisakis physeteris* (Nematoda: Anisakidae). International Journal for Parasitology, 31(1), 9-14, 2001.

Mattiucci S, Nascetti G, Cianchi R, et al, Genetic and ecological data on the *Anisakis simplex* complex with evidence for a new species (Nematoda Ascaridoidea, Anisakidae). Journal of Parasitology, 83: 401-16, 1997.

Moravec F, and Justine JL, Two new species of nematode parasites, Cucullanus epinepheli sp. n. (Cucullanidae) and Procamallanus (Spirocamallanus) *sinespinis* sp. n. (Camallanidae), from marine serranid and haemulid fishes of New Caledonia. Folia Parasitologica, 64: 11, 2017.

Moravec F, Parasitic Nematodes of Freshwater Fishes of Europe. 473 p. Academia and Kluwer Academic Publishers, Prague and Dordrecht, Boston, London, 1994.

Nadler SA & Hudspeth DS, Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: Hypotheses of structural and sequence evolution. Journal of Parasitology, 86: 380-393, 2000.

Petric M, Mladineo I, and Šifner SK, Insight into the short-finned squid Illex coindetii (Cephalopoda: Ommastrephidae) feeding ecology: is there a link between helminth parasites and food composition? Journal of Parasitology, 97(1): 55 62, 2011.

Petter AJ, Larvae of ascarids parasites of fish in the western Mediterranean. Bulltin Museum National History Natural, 10: 347-369, 1988.

Petter AJ and Maillard C, Fish ascarids in the western Mediterranean. Bulletin du Muséum National d'Histoire Naturelle, 4 série, Section A, Zoologie, biologie et écologie animales, 1987.

Petter AJ, Classification test of the Cucullanidae family. Bulltin Museum National. History Natural Paris, 3rd 255 (177): 1469-1490, 1974.

Pontes T, D'Amelio S, Costa G, and Paggi L, Molecular characterization of larval Anisakid nematodes from marine fishes of Madeira by PCR-based approach, with evidence for a new species. Journal of Parasitology, 91:1430–1434, 2005.

Paggi L, Nascetti G, Webb S, Mattiucci S, Cianchi R, and Bullini L, A new species of *Anisakis* Dujardin, 1845 (Nematoda, Anisakidae) from beaked whales (Ziphiidae): allozyme and morphological evidence. Systematic Parasitology, 40: 161-74, 1998.

Quiazon KMA, Yoshinaga T, Ogawa K and Yukami R, Morphological differences between larvae and in vitro-cultured adults of *Anisakis simplex* (sensu stricto) and *Anisakis pegreffii* (Nematode: Anisakidae). Parasitology International, 57 :483–489, 2008.

Ramdane Z, Bensouilah MA and Trilles JP, The Cymothoidae (Crustacea, Isopoda), parasites on marine fishes, from the Algerian fauna. Belgian Journal of Zoology, 137 (1) 67-74, 2007a.

Ramdane Z and Trilles JP, Parasitic copepods (Crustacea: Copepoda) from the Algerian marine fauna. Zootaxa, 1574: 49-68, 2007b.

Ramdane Z and Trilles JP, Cymothoidae and Aegidae (Crustacea, Isopoda) from Algeria. Acta Parasitologica 53(2): 173–178, 2008.

Ramdane Z, Bensouilah MA and Trilles JP, Comparative study of Isopod crustaceans and Copépodes ectoparasites of Algerian and Moroccan marine fish. Cybium, 33(2): 123-131, 2009.

Saadi N, Trilles JP, Amara R and Ramdane Z, Impact of parasitism by nematodes on gonadal anatomy of *Pagellus erythrinus* (L.). Cybium, 43(3): 255-263, 2019.

Setyobudi E, Jeon CH, Lee CH, Seong KB and Kim JH, Occurrence and identification of *Anisakis* spp. (Nematoda: Anisakidae) isolated from chum salmon (*Oncorhynchus keta*) in Korea. Parasitology Research, 108: 585-592, 2011.

Setyobudi E, Soeparno S and Helmiati S, Infection of *Anisakis* sp. Larvae in some marine fishes from the southern coast of Kulon Progo. Yogyakarta.Biodiversitas, (12): 34-37, 2010.

Shukhgalter OA, Parasite fauna of the chub mackerel (Scombridae: Scomber japonicas Houttuyn, 1782) in the Central-Eastern Atlantic Ocean (the Atlantic coast of the North Africa and the

Azores banks). Parasitologiya, 38: 160–170, 2004.

Smith JW, The abundance of *Anisakis simplex* L3 in the body cavity and flesh of marine teleosts. International Journal for Parasitology, 14: 491–495, 1984.

Umehara A, Kawakami Y, Matsui T, Araki J, and Uchida A, Multiplex PCR for the identification of *Anisakis simplex* sensu stricto, *Anisakis pegreffii* and the other anisakid nematodes. Parasitology International, 57:49–53, 2008.

Umehara A, Kawakami Y, Matsui T, Araki J and Uchida A, Molecular identification of *Anisakis simplex* sensu stricto and *Anisakis pegreffii* (Nematoda: Anisakidae) from fish and cetacean in Japanese waters. Parasitology Internationl, 55: 267–271, 2006.

Valentini A, Mattiucci S, Bondanelli P, Webb SC, Mignucci-Giannone AA, Colom-Llavina M.M, and Nascetti G, Genetic relationships among *Anisakis* species (Nematoda, Anisakidae) inferred from mitochondrial cox2 sequences, and comparison with allozyme data. Journal of Parasitology, 92: 156–166, 2006.

Zhu X, D'Amelio S, Paggi L, Gasser RB, (2000) Assessing sequence variation in the internal transcribed spacers of ribosomal DNA within and among members of the *Contracaecum osculatum* complex (Nematoda: Ascaridoidea: Anisakidae) Parasitology Research, 86:677–683, 2000.

Zhu XQ, and Gasser RB, Single-strand conformation polymorphism (SSCP)-based mutation scanning approaches to fingerprint sequence variation in ribosomal DNA of ascaridoid nematodes. Electrophoresis, 19: 1366-1373, 1998.