Original Research

Journal of Advanced Veterinary Research
(2024) Volume 14, Issue 1, 85-90

Molecular and morphological characterization of *Anisakis simplex* in frozen and smoked herring and mackerel fish species in Egypt

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ARTICLE INFO

Recieved: 04 October 2023

Accepted: 30 November 2023

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Keywords:

Anisakis simplex
Herring fish
ITS rDNA
Mackerel
Sequencing.

ABSTRACT

Food-borne zoonoses are of serious public health concern, with a high risk on human health in both developed and developing countries. Thus, this study aimed to determine the prevalence of zoonotic *Anisakis* larvae in smoked imported herring fish (*Clupea harengus*) and frozen mackerel (*Scomber scomberus*) from Assiut and Menoufia governorates, respectively in Egypt. All herring specimens were heavily infected with *Anisakis* larvae, while 9 out of 15 mackerel fish were infected. A total of 434 *Anisakis* larvae were collected from stomach, abdominal cavity, liver, between skin, muscles and gonads of 45 herring fish. In frozen mackerel, number of detected larvae was 78. Sequencing and phylogenetic analysis of internal transcribed spacer rDNA (ITS-rDNA) ribosomal DNA confirmed that the *Anisakis* larvae belonged to *Anisakis simplex* species. Furthermore, by employing light and scanning electron microscope, the morphological characters of *Anisakis* larvae were also determined. These findings highlight the importance of detecting health hazards in seafood products for helping in the management and prevention of anisakiasis.

Introduction

Food-borne zoonoses are serious public health concerns, posing high risk on human health in both developed and developing countries (Shamsi, 2019). According to the World Health Organization (WHO), food-borne diseases caused 420,000 deaths in 2010 (WHO, 2015). Moreover, WHO reported that 56 million of human parasitic infections resulted from ingestion of infected raw or undercooked fish products (WHO, 2012). Among the commonest human infections is anisakiasis, a serious zoonosis that causes gastrointestinal symptoms and may be associated with severe hypersensitivity reactions (Audicana and Kennedy, 2008). Anisakiasis is caused by nematode third stage larvae (L3) of the genus *Anisakis* (Fang *et al*., 2011; Martínez-Rojas *et al*., 2021), which was considered by the European Food Safety Authority as the main biological hazard in marine products (EFSA, 2010). To date, the first two species of the genus *Anisakis* being responsible for the most reported cases of human infection are *Anisakis simplex*, and *Anisakis pegreffii* (Martin-carrillo *et al*., 2022).

Anisakis is found globally and has a complex life cycle. Cetaceans and other marine mammals serve as definitive hosts, whereas crustaceans (Euphausiacea) serve as first intermediate hosts (Akbar and Ghosh, 2005). The latter may be consumed by the second intermediate, i.e., the paratenic hosts, which can be either fish or cephalopods. *Anisakis* larvae parasitize in the mesenteries, viscera, and muscles of some marine fishes as the paratenic hosts (Ivanović *et al*., 2017). The larvae may also reach the peritoneal cavity and other organs, as well as the gastrointestinal mucosa of infected people, where they may cause abscesses or eosinophilic granulomas (Martin-carrillo *et al*., 2022). Their main clinical symptoms in the digestive tract include issues that suddenly occur, as colic, nausea, vomiting and diarrhea, or allergic (Martin-carrillo *et al*., 2022).

Until now, the treatment of anisakiasis includes the endoscopic removal of the worm from the gastrointestinal tract using surgical resection (Martin-carrillo *et al*., 2022). Nevertheless, the best preventive strategy against anisakiasis is to avoid consuming raw or insufficiently thermally treated marine fish (Ziarati *et al*., 2022). *Anisakis* has been detected in more than 200 fish and 25 cephalopods worldwide (Klimpel *et al*., 2004). Numerous studies have reported the presence of *Anisakis* larvae in a wide range of fish species in the Mediterranean Sea as well as in the Atlantic Ocean, including mackerel, frigate mackerel, European hake, Atlantic chub mackerel, European anchovy, and herrings (Mladineo *et al*., 2012; Abou-Rahma *et al*., 2016; Eissa *et al*., 2018; Mostafa *et al*., 2020; El Meghanawy *et al*., 2021; Martin-carrillo *et al*., 2022).

In Egypt, herring is among the top imported fish species due to its low price. Additionally, smoked herring fish is one of the most popular traditional foods among Egyptians, particularly during the Easter and spring breaks (Ghanem *et al*., 2019). Smoked herring fish is stored and transported in wooden boxes at room temperature until delivered to markets. According to General Authority for Fish Resources Development (GAFRD), Egypt mainly imports herring fish from Norway and the Netherlands (GAFRD, 2016). Herring fish is considered a frequent host of *Anisakis simplex* in various stocks of Atlantic herring including the Norwegian and the Dutch ones (Levsen and lunestad, 2010; Guardone *et al*., 2019). *A. simplex* sensu stricto is the only *Anisakis* species detected in The Norwegian herring fish (Levsen and lunestad, 2010). Likewise, the first human anisakiasis infection due to the consumption of salted herring has been described in the Netherlands in 1960 (Guardone *et al*., 2019).

Similarly, mackerel (*Scomber scomberus*) is one of the highly con-

ISSN: 2090-6277/2090-6269/

sumed fish species in Egypt (El-Dengawy *et al*., 2017). Egypt is considered one of the top ten countries that consume mackerel (El-Dengawy *et al*., 2017). Therefore, examination and inspection of these types of fish species for foodborne pathogens are of a major concern for management and avoidance of these infections (Costa *et al*., 2016).

Unfortunately, conventional techniques depending on the morphological features of *Anisakis* larvae are not enough to identify the larvae to the species level. Hence, it is crucial to employ molecular techniques for accurate and reliable characterization of these zoonotic worms, even if the morphological characteristics are unclear. So far, recent PCR-based assays such as polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP), mitochondrial cytochrome oxidase subunit 1 gene (COI) sequencing, sequencing of ribosomal DNA internal transcribed spacer (ITS rDNA), and real-time PCR have been precisely utilized worldwide to identify these species (Martin-Carrillo *et al*., 2022; Najjari *et al*., 2022; Thabit and Abdallah, 2022).

Due to the scarcity of studies concerning the occurrence and DNA profile of *Anisakis* larvae in Egypt, the present study aimed to combine morphological and molecular identifications based on the sequencing of DNA ITS-1 region of anisakid worms, to identify the species of their larvae in imported smoked herring (*Clupea harengus*) and frozen mackerel (*Scomber scomberus*).

Materials and methods

Fish sample collection and processing

A total number of 45 imported smoked herring fish *Clupea harengus* (local name: renga) were collected from 3 local markets at Assiut governorate in the South of Egypt in September 2021. In addition, 15 frozen mackerel (Scomber scombrus) were purchased from 2 different local markets at Menoufia governorate in Egypt around the period of September 2022. Each sample was carried in a plastic bag, to be stored in the deep freezer (-20˚C) until examination. Fish were allowed to thaw, then their weights and body lengths were recorded. Fishes were autopsied separately in Petri dish according to Kabata (1992). This study has been approved by the Ethical committee of the Faculty of Veterinary medicine, Assiut University, Assiut, Egypt, according to the OIE standards for use of animals in research under the number 06/2023/0063. After dissection, the abdominal cavity of every specimen was examined. Skin, muscles, body cavity, internal organs and gonads were visually examined for the presence of larvae. To examine further for parasites, the compression technique and the digestion method were used according to Jackson *et al*. (1981) and Manfredi *et al*. (2000). The number of parasites and the attachment site were noted for each fish. Macroscopic and microscopic examination of different organs were carried out for detection of any visible nematode larval parasites.

Morphological examination of isolated larvae using light microscope

The collected larvae were cleaned by washing several times with isotonic saline solution. The relaxed larvae were fixed in bottles containing a mixture of 70% alcohol and 5% glycerin. For microscopic examination, the larvae were mounted on slides with few drops of lactophenol and covered by a cover slip. Identification of the encountered larvae was done according to the keys of the nematode parasites of vertebrates (Chitwood, 1963).

Quantitative assessment of larval occurrence in fish was done by calculating the prevalence (P) = number of infected fish/numbers of examined fish, mean intensity (MI) = total number of isolated larvae per fish species/number of infected fish belonging to this species and mean abundance (MA) = total number of isolated larvae per fish species/number of examined fish belonging to this species (Bush *et al*. 1997).

Scanning electron microscopic (SEM) examination of isolated larvae

For scanning electron microscopy, the freshly recovered larvae were washed in physiological saline solution, then fixed in 5% gluteraldehyde for 24 h. The specimens were washed three times in phosphate buffer and post fixed in sodium tetroxide for 1.5 hours. Specimens were dehydrated in ethanol series (90%, 95%, absolute ethanol) and then mounted in the special holders, coated with gold- palladiums and examined using scanning electron microscope. The worms were examined by a JEOL, JSM-5400 LV SEM (JEOL Ltd.) operated at 15 kV, in the Electron Microscopy Unit, Assiut University

Molecular analysis of Anisakid larvae

DNA extraction

In the laboratory of molecular biology of Zoology Department in the Faculty of Science of Menoufia university (Egypt), total genomic DNA was individually extracted from 20 Anisakid larvae, which were harvested from smoked imported herring fish and frozen mackerel. DNA extraction was carried out from samples preserved in 70% ethanol, after being blot dry in a clean tissue paper, using Chelex® resin (Sigma-Aldrich, Germany). DNA extraction protocol was previously described by Estoup *et al*. (1996) with slight modifications. Briefly, each Anisakid larva was placed in a 1.5 mL sterile tube containing 300 µL of Chelex resin (10%) combined with 3 µL of proteinase K (600 U mL−1). The tubes were incubated at 65ºC for 90 minutes to complete disruption of cell membrane and release of DNA. Then, the tubes were incubated in 100ºC for 20 minutes to inactivate Proteinase K. DNA was stored in the fridge at 4ºC for subsequent analysis. Other DNA aliquots were placed at - 20ºC for long time storage.

Polymerase chain reaction (PCR)

The rDNA region including the internal transcribed spacer 1 (ITS-1), 5.8S rRNA gene, the ITS-2 was amplified utilizing universal primers FW-NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and RV-NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) (Zhu *et al*. 1999). A PCR reaction mixture (25 µL) was prepared containing 50 ng DNA, 12.5 µL 2X MyTaq™ Red Mix (Bioline), 0.4 µM of each primer. PCR assays were performed in the thermal cycler TC-512 (Techne, UK) using the following program: an initial denaturation at 95 ºC for 10 min, followed by 35 cycles of denaturation at 95ºC for 30 sec, annealing at 55ºC for 30 sec, extension at 72ºC for and a final extension at 72°C for 7 min to complete the extension of fragments. PCR runs included negative controls. After the reaction, the amplicons were electrophoresed through 1% agarose gel electrophoresis stained by 0.5 µg µL−1 ethidium bromide, and visualized using a UV transilluminator (Biometra, Germany). PCR products were sent for sequencing in Macrogen Inc. (South Korea), using the Sanger sequencing method. The generated ITS-1 sequences were submitted in GenBank database using BanKit tool.

Molecular analysis

The obtained sequences were corrected and edited manually using Chromas lite 2.1.1 software. To identify the Anisakid species, homologies of the nucleotide sequences, obtained by sequencing their ITS-1 region, were compared in GenBank database with BLAST tool (http://blast.ncbi.nlm.nih.org). Identification of local similarities' regions with GenBank references was carried out. Identity > 98% and alignment value E= 0 were the cut-off values used for identification at the species level. The resulting nucleotide sequences obtained in the current study were aligned with other 10 reference sequences representing different species of genus *Anisakis*. These ITS-rDNA reference sequences represent five different *Anisakis* species that were previously detected in frozen and smoked fish-

es. The retrieved reference sequences belonged to *A. simplex*, *A. pegreffii*, *A. typica*, *A. nascettii*, and A. physeteris. Another ITS-1 reference was also retrieved from the GenBank database, representing the Anisakid nematode Contraceacum sp. as an outgroup. Alignment was employed using the Clustal W algorithm integrated in MEGA software (v 11, Tamura *et al.* 2021). Then, a phylogenetic tree was constructed with MEGA 11 software using the Neighbor-Joining method. Kimura -2 parameter substitution model was used to estimate the genetic variations among all sequences. One thousand replications were employed as bootstraps to calculate the nodal support values and thus evaluate the reliability of the phylogenetic tree clades.

Results

Prevalence of Anisakis larvae and site of infection

Based on gross examination of *Clupea harengus* fish, all specimens collected from Assiut city were found infected with *Anisakis*. Heavy infection by the larvae were found in the stomach or abdominal cavity, either free or encapsulated on the external surfaces of the host digestive tract and other visceral organs. Also, the larvae were found between skin and muscles, liver, and gonads (Fig. 1).

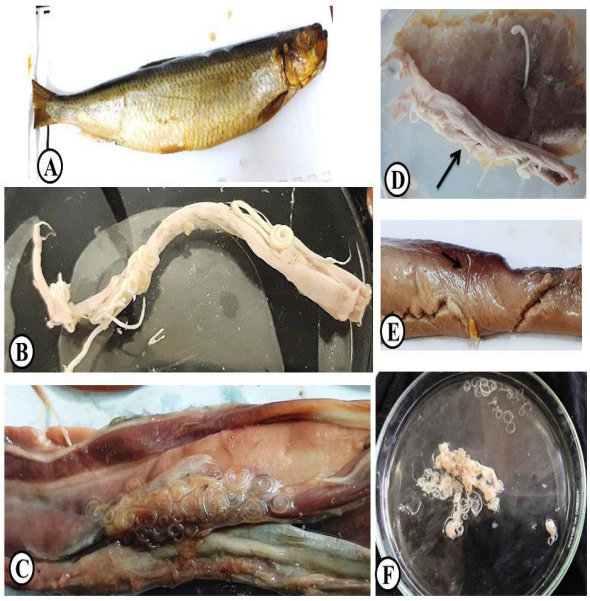

Fig. 1. Infected *Clupea harengus* with the third stage of larvae of *Anisakis simplex*. (A) A photograph of *Clupea harengus*. (B-F) Gross examination of different organs of naturally infected *Clupea harengus* with *Anisakis simplex*. (B) Intestine of *Clupea harengus* infected with numerous third stage larvae of *Anisakis*. (C) Abundance of *Anisakis* larvae encapsulated in mesenteries. (D) Heavy infestation by *Anisakis* larvae attached between muscles & skin (arrow), note anterior part of larvae firmly attached to the muscle. (E) *Anisakis* larva penetrate female gonad. (F) Anisakid larvae recovered from infected fish.

The prevalence of infection was 100 %, and the mean intensity and the mean abundance were 9.64 and 9.64 respectively. The larval burden ranged from 4 to 156 larvae per fish.

In frozen mackerel (Scomber scombrus) collected from Menoufia, 9 out of 15 fishes (60 %) were found infected with *Anisakis* larvae, which were mainly existed in liver, intestine, muscles, and body cavity. The prevalence of larvae in each fish ranged from 5 to 10 larvae (Fig. 2). The lowest overall *A. simplex* prevalence was recorded in the examined mackerel. The prevalence of *Anisakis* larvae at different groups of standard lengths and weights of the fish are presented in Table 1.

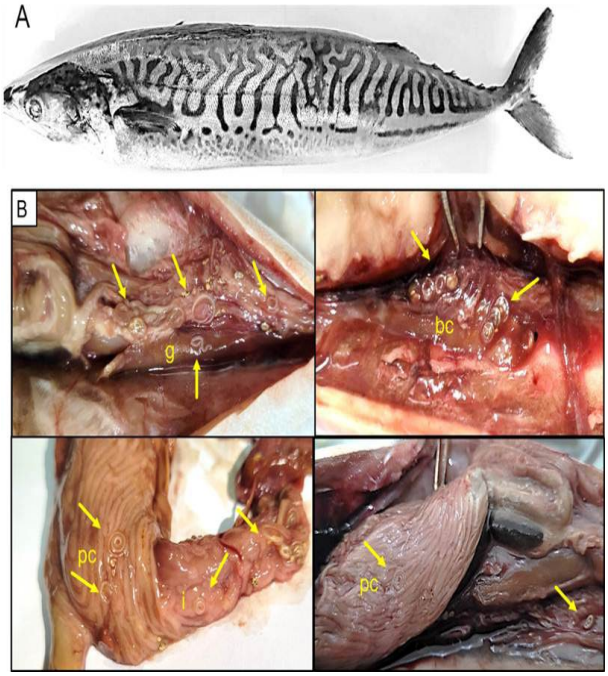

Fig. 2. Infected frozen mackerel (Scomber scombrus) with *Anisakis simplex* larvae. A: An adult mackerel fish. B: Encapsulated *Anisakis simplex* larvae in gonads (g), body cavity (bc), intestine (i), and pyloric caeca of mackerel.

Morphology of the isolated larvae

Combining both light and scanning electron microscopy to reveal morphological details of the third stage larvae isolated from herring fish was employed. The larvae had brownish, elongated body, thickest posteriorly, tapering gradually towards anterior end, the body length was 18±7 mm. Mouth was triangular anteroventrally with a ventral boring tooth, located ventral to mouth and projecting, besides three small lips surrounding the mouth (Fig. 3A and Fig. 4B). Three large lips were present at the anterior end: two large ventrolateral and one dorsal. The dorsal one was somewhat shorter. The lips were somewhat wider than the body (Fig. 4A). Excretory pore situated just behind nerve ring, or open ventrally at anterior end and located between bases of subventral lips (Fig. 3A and Fig. 4A). The esophagus was cylindrical, the ventriculus was short and the intestinal caecum ran anteriorly at nearly half or more of the muscular esophagus (Fig. 3C). The cuticle was characterized by fine transverse striations (Fig. 3C and Fig. 4C) and irregularly wrinkled near tail. The tail was short, bluntly rounded and had a small mucron (Fig. 3 D and Fig. 4 D).

Table 1. Prevalence of third stage *Anisakis* larvae in herring fish and mackerel. Species and number of fish collected, number of larvae detected, site of infection and BLAST results of the examined larvae.

			Fish						BLAST results	
Common name	Scientific name	Collection place	No. of examined fish	No. of infected fish	No. of larvae detected	Fish length (cm)	Fish weight (g)	Sites of infection	Species detected	Identity (%)
Herring fish	*Clupea harengus*	Assiut	45	45	434	30.04±0.21	319.8±5.98	Intestine, muscles, gonads, body cavity, skin and liver	*Anisakis simplex*	99.5
Mackerel	*Scomber scombrus*	Menoufia	15	9	78	31.0±2.0	52.0±10.0	Intestine, pyloric caeca, body cavity, and gonades	*Anisakis simplex*	98.7

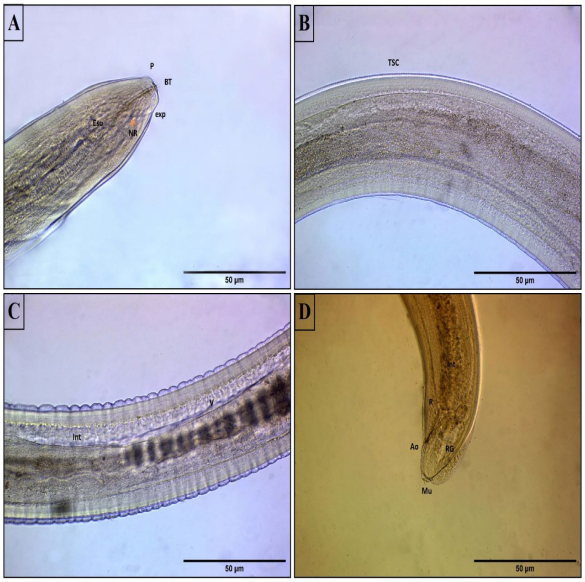

Fig. 3. Photomicrographs of third-stage larvae of the *Anisakis simplex* larvae showing: (A) Anterior end of *Anisakis simplex*, papillae (P), boring tooth (BT), e eosophagus, nr nerve ring, excretory pore (exp), (B) Transverse striations of the cuticle (TSC), (C)Ventriculus region (v) & intestine, (D) Posterior end showing rectal region (RG) mucron (MU) anal opening (AO).

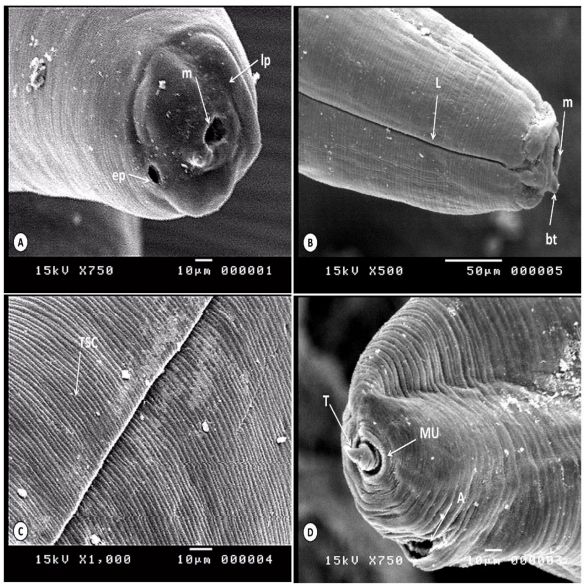

Fig. 4. Photomicrographs of scan electron microscopy of *Anisakis simplex* larvae showing: (A) Anterior part details; mouth (m), labia papillae (lp), excretory pore (ep). (B) Lateral line (l), note boring tooth (bt). (C) Transverse striations of the cuticle (TSC). (D) Posterior end showing tapered process (T), mucron (MU) and anus opening (A).

Molecular and phylogenetic identification of Anisakid larvae

Total DNA was successfully extracted from 20 Anisakid larvae. PCR amplification utilizing the universal primers of rDNA region representing ITS-1, 5.8S, the ITS-2 resulted in ~ 1000 bp long amplicons. The total length of the targeted rDNA region after trimming was between 604 - 700 bp. Only one specific band at the expected size was detected in the gel photo for all samples, which indicates the absence of nonspecific amplifications and primer dimers. PCR assay resulted in good quality sequences with separated needle-shaped peaks that indicate the absence of contamination by other, exogenous DNA, than that of the anisakid DNA. After comparison of the obtained sequences with GenBank reference sequences using nucleotide BLAST (nBLAST), the identified species for all samples was *A. simplex*, with 98.7- 99.5% identity. The sequences obtained in this study were found in given GenBank accession numbers OQ420303-OQ420304. Furthermore, the phylogenetic tree exhibited four main clades, each clade represented only one *Anisakis* species. All bootstrap values were above 75%. The sequences obtained in this study clustered on the same clade with their references for the species detected by BLAST methodology, which confirmed the species assignment. Specifically, all analyzed larvae were phylogenetically belonged to *Anisakis simplex* (Fig. 5 and Fig. 6).

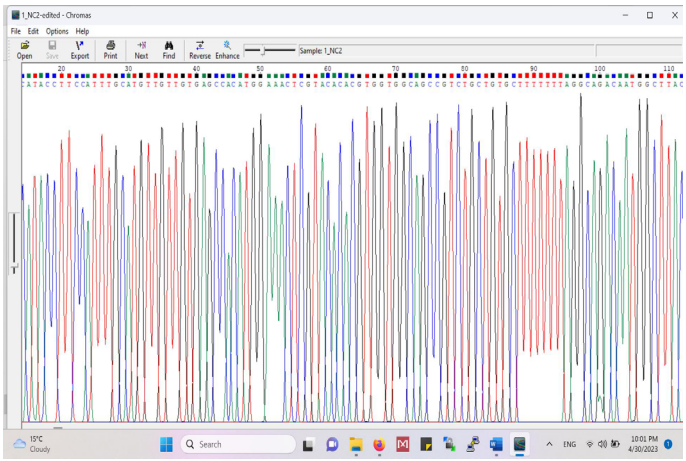

Fig. 5. Obtained *Anisakis simplex* ITS- 1 sequence chromatogram with separated needle-shaped peaks.

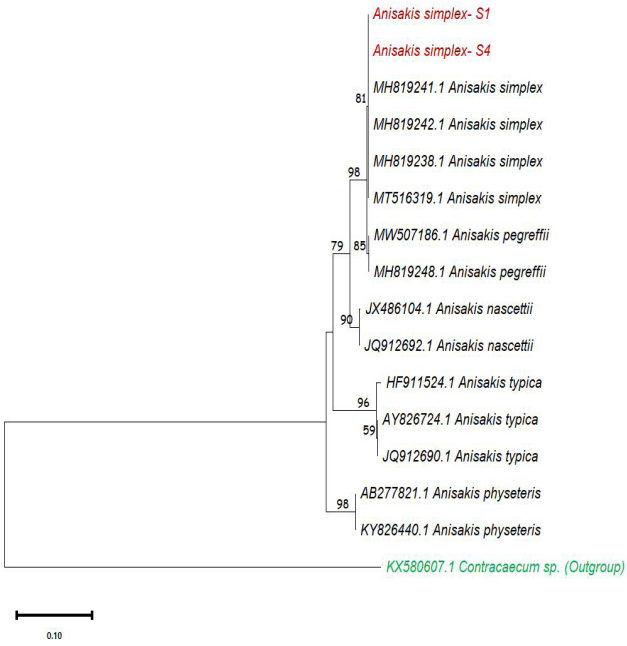

Fig. 6. Phylogenetic tree was constructed using neighbor-joining method and kimura -2 parameter substitution model and based on ITS-1 sequences obtained from *Anisakis* larvae. GenBank references were identified by their accession numbers. Bootstrap values were indicated on clades.

Discussion

Anisakiasis is a zoonotic disease produced by species of the family *Anisakidae* (Lymbery and Walters, 2014). The Anisakid genera includes *Anisakis*, *Pseudoterranova*, and *Contracaecum* (Aibinu *et al.*, 2019). Mostly, *Anisakis* larvae remain encapsulated in or on the visceral organs. However, some larvae may show a potential public health risk when they migrate from viscera to the fish flesh (Levsen *et al.*, 2018). As a result, humans may be infected when consuming insufficiently cooked marine fishes or shellfish products containing 3rd stage larvae of different anisakid species (Audicana and Kennedy, 2008). To date, nine species of *Anisakis* have been confirmed using genetic and/or biochemical methods, i.e. *A. simplex* sensu stricto, *A. pegreffii*, *A. berlandi*, *A. ziphidarum*, *A. nascettii*, *A. paggiae*, *A. physeteris*, *A. brevispiculata* and *A. typica* (Mattiucci *et al.*, 2017; 2018). Two species of the genus *Anisakis* were reported as the causative agent of infections in humans, i.e. *A. simplex*, sensu stricto (s.s), and *A. pegreffii* (Mattiucci and Nascetti, 2008). Thus, proper identification of host fishes of *Anisakis* species is very important to both human health

and fish disease diagnosis.

The first case of anisakid disease was described in 1876 by Leuckhart (Leuckart 1876); the disease was however only widely recognized in the 1960s, when epidemics of anisakidosis occurred in the Netherlands with the consumption of lightly salted herring (Van Thiel *et al*., 1960; Van Thiel, 1962). In that case, the larvae were identified as *A. simplex* 3rd-stage larvae. Since then, many cases of this zoonotic infection have been described in other countries such as Japan where consumption of raw fish is customary (Suzuki *et al*., 2010; Arizono *et al*., 2012). Over 20,000 cases of anisakiasis had been reported worldwide prior to 2010 (EFSA Panel on Biological Hazards (EFSA, 2010), with the highest prevalence (over 90%) from Japan (Baird *et al*., 2014).

Similarly, the present study revealed that all the collected nematode larvae were identified as *A. simplex*, which were characterized in smoked *C. harengus* and frozen *S. scomberus* by combining both molecular and morphological procedures. During the past decades, juveniles of different anisakid species were previously described in Egypt; from the Red Sea fishes (Abdou, 2005; Abdou and Dronen, 2007; Arafa *et al*., 2009; Morsy *et al*., 2015; Abou-Rahma *et al*., 2016) and from the Mediterranean Sea at Alexandria city (Nada and Abd El-Ghany, 2011; Abou Zaid *et al*., 2018). They were also reported from other countries on the Red Sea, mainly in Yemen (Al-Zubaidy, 2010; Al-Zubaidy *et al*., 2012) and Kingdom Saudi Arabia (KSA) (Hassan *et al*., 2013; Ibrahim *et al*., 2018). Many studies were conducted on *Anisakidae* parasitizing Mediterranean fishes with high commercial value, as in Greece (Papoutsoglou, 1975; Chaligiannis *et al*., 2012), Spain (Valero *et al*., 2000; Valero *et al*., 2006; Rello *et al*., 2008; Gutiérrez-Galindo *et al*., 2010), Italy (Larizza and Vovlas, 1995), and North African central Mediterranean coasts (Farjallah *et al*., 2008).

Morphological identification of *Anisakis* spp. to species level is difficult and sometimes impossible, especially for the larval stage occurring in fish. Larvae represents the infective stage to humans and other mammalian hosts. Morphological assessments are mainly based on the shape and length of the ventriculus and the presence/absence of a caudal spine (mucron). The former morphological characters, the presence/ absence of a boring tooth in the anterior body of the nematode, and the relative distance from the boring tooth to the excretory pore, are also used to distinguish among *Anisakis* spp. developmental stages. These criteria can also be utilized to differentiate the other frequently occurring nematode species present in fish, i.e. Pseudoterranova spp., Contracaecum spp. and Hysterothylacium spp. (Berland, 1989; Levsen & Berland, 2012; Mattiucci *et al*., 2018). The foremost problems in the identification of anisakid larvae in fishes is that L3 larvae cannot be easily differentiated morphologically, especially between *A. simplex* (s.s.) (Rudolphi 1809) and A. pegreffi Campana-Rouget et Biocca 1955 (Quiazon *et al*., 2008). Our morphological analyses support and agree with previous reports (Rocka 2004; Nada and Abd El-Ghany, 2011; Borges *et al*. 2012) as well as in concordance with the previously characterized *Anisakis* spp. These morphological characters of larval nematodes were like that obtained by Shih *et al*. (2010) and Nada and Abd El-Ghany (2011).

Due to lack of precise identification of nematodes larvae depending on morphological characteristics, especially in case of absence of these external characters, it was crucial to depend on reliable molecular techniques. Although, many studies confirmed that mtDNA COX 2 gene is highly polymorphic in *Anisakis* sp., sequencing the ITS-1 and ITS-2 nuclear ribosomal DNA coupled with phylogenetic analysis also furnishes robust protocol for *Anisakis* genetic composition identification (Aibinu *et al*., 2019). Various molecular tools have been employed for species-specific identification of different *Anisakis* species. Sequencing of mtDNA COII gene revealed the presence of A. pegreffi, and *A. physeteris* in fish of the Southeastern Pacific Ocean (Aco Alburqueque *et al*., 2020) and anisakid L3 in Chilean hakes (Muñoz-Caro *et al*., 2022). Additionally, another study employed PCR–RFLP analysis of the total ITS rDNA region (ITS1, 5.8S and ITS2) and detected the incidence of A. pegreffi, *A. simplex*, and *A. physeteris* in Central-Western Mediterranean Sea (Costa *et al*. 2016). The current study utilized the universal primers NC2, and NC 5 described by Zhu *et al*. (1999) for the amplification and sequencing of the entire ITS rDNA region and its phylogenetic analysis for precisely identification the *Anisakis* larvae. Our results allowed the detection of *A. simplex* in herring fish collected from local markets in Egypt. Similarly, A more recent Egyptian studies confirmed the incidence of *A. simplex* in herring (El Meghanawy *et al*., 2021) and frozen mackerel (Arafa *et al*., 2019) in Egypt by molecular analysis of ITS region.

In the current study, *A. simplex* larvae were discovered either free, i.e., in the abdominal cavity and the intestinal lumen, or encapsulated in a tightly coiled spiral form in the mesenteries, gonads, liver and between skin and muscles. However, Setyobudi *et al*. (2019) noticed that *Anisakis* larvae were mostly found in the digestive tract (47.2%) and body cavities (46.0%) and only very few of *Anisakis* larvae were found in the liver (3.5%), gonads (1.5%) and muscle (1.8%) of the Indian mackerel (Rastrelliger spp.). Consequently, Tejada *et al*. (2015) suggested that the removal of belly flaps diminished the risk of consumers' infection and sensitization as prevalence and intensity were reduced to 75.6 and 5.9%, respectively.

It is worth mentioning that most of the examined herring fish in Egypt are consumed as ready to eat smoked product, which poses more risk for humans, yet this depends on the efficiency of smoking process in eliminating the anisakid larvae. Higher prevalence (98–100%) was previously reported in various sizes of Norwegian spring spawning herring (*Clupea harengus*), which live in the northeast Atlantic water (Levsen and Lunestad, 2010). In a previous study conducted by Levsen *et al*. (2005), 15–60% of *Clupea harengus* has been shown to harbor *A. simplex*.

Regarding the prevalence of *A. simplex* larvae, our results revealed its existence in herring fish more than in mackerel. Likewise, versatile previous research reported the presence of *A. simplex* larvae in Mediterranean mackerel with lower overall prevalence than in expected (Levsen *et al*., 2018). One reason for this difference may be related to the feeding behavior of the two fish species. Herring are filter feeders that consume plankton and other tiny organisms. So, they may accumulate *Anisakis* larvae in their gut if they feed on infected plankton. On the other hand, mackerel is an opportunistic feeder that mainly consume small fish and crustaceans. Additionally, mackerel tends to migrate to deeper, colder waters where *Anisakis* larvae are less prevalent. Further explanation for that is related to fish size and length, as it has been reported before (Morozinska-Gogol, 2019). In this regard, Podolska (2009) reported that parasites were found only in herring more than 21 cm in length. In addition, 1-2 years old herring are free of nematodes and parasites that are accumulated in oldest fish.

Anisakids are known to survive and to be resistant to different treatment conditions such as freezing, microwaving, heating, salting, as well use of anthelmintic drugs and condiments (Brutti *et al*., 2010; Tejada *et al*., 2015). Storage, after-harvest handling, and fish preparation are the focus of preventive measures. Migration of larvae into the muscle might be prevented by immediate evisceration of fish after being caught (Chen *et al*., 2018).

Although cooking or freezing kills *Anisakis* worms, this method does not eliminate their allergenic reactions. However, the recommended and common method to kill the larvae in fish before consumption is the application of high or low temperatures (Chen *et al*., 2014). The US Food and Drug Administration (FDA) recommended that fish be cooked to a temperature of at least 63–74°C before consumption (Beldsoe and Oria, 2001). Larvae of *Anisakis* species have been observed to be alive if frozen for a short period at −20°C. The FDA has recommended that fish be kept frozen at −20 °C for at least 168 h or blast-frozen at −35°C for at least 15 h (Beldsoe and Oria, 2001).

Conclusion

Employing both molecular sequencing of ITS-rDNA and morphological techniques revealed the prevalence of *Anisakis simplex* in smoked herring fish and frozen mackerel in Egypt. A high prevalence of *A. simplex* larvae was detected in herring fish more than in mackerel. The obtained results provide valuable information on the presence of *Anisakis* in Egypt, suggesting more surveillance studies to be conducted on other fish species to protect humans and minimize the public health risks by such parasites.

Acknowledgments

The authors acknowledge various markets in the governorates of Assiut and Menoufia, For helping with sample collection.

Conflict of interest

The authors declare no conflict of interests.

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