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Research Article

Characterization of *Hysterothylacium persicum* (Nematoda: Raphidascarididae) in *Scomberomorus commerson* from the Persian Gulf using morphological and molecular approaches

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Abstract

Narrow-barred Spanish mackerel (*Scomberomorus commerson*) is one of the most important commercial fish species in the Persian Gulf and Oman Sea. Parasites of the genus *Hysterothylacium* (Ascaridoidea: Anisakidae) are common ascaridoid nematodes of fish-eating birds and sea mammals. The aim of this study is to identify morphological and molecular characteristics of the *Hysterothylacium* on *S. commerson* from April 2012 to December 2013. The morphological identification of *Hysterothylacium* specimens (n=10) was performed based on characteristic features, measurements and comparison with other publications. Larvae were counted, fixed in 70% ethanol and cleared in lactophenol for 48 h for identification. The molecular species description was based on a polymerase chain reaction (PCR) of sequences comprising ITS-1 and ITS-2. After the blast of nucleotide sequences, with the other recognized sequencing recorded in the GenBank, the most similarity was observed to *Hysterothylacium persicum*. Based on the morphological analysis and sequencing, the Hysterothylacium specimen was described as *H. persicum*. It is the first morphological and molecular identification of *H. persicum* in *S. commerson* from Iran. A combination of morphological description with a molecular technique seems to be the best practice for identifying anisakid larvae.

Introduction

The Scombridae fish family contains 15 genera and about 50 species of epipelagic and generally migratory marine fish. It includes species of high commercial interest, such as mackerels, bonitos and tunas. Scombridae were distributed widely in the Persian Gulf and Oman Sea. *Scomberomorus commerson* (Teleostei, Perciformes, Scombridae) is a mackerel of the Scombridae family. This fish is a large and fast pelagic predator which is found in vast numbers in the tropical and sub-tropical waters (Kaymaram *et al.,* 2010). *Scomberomorus commerson* is one of the most important and commercial species in the Persian Gulf and Oman Sea, which has many important roles in the foods of humans in the South of Iran.

Parasites play a crucial role in the biology and physiology of fish (Shamsi and Barton, 2023). Adult anisakid nematodes of the genus *Hysterothylacium* are common parasites in the digestive tract of fishes in marine, brackish, and freshwater environments. The genus Hysterothylacium includes more than 90 species, which are distributed in different continents (Pekmezci and Umur, 2015). Different species of *Hysterothylacium* have been reported from a wide variety of freshwater and saltwater fishes (Suthar and Shamsi, 2021). The occurrence of *Hysterothylacium* sp. larvae was reported from Black sole fish, Brachirus orientalis (Bagherpour *et al*., 2011), greater lizardfish, (Saurida tumbil), Japanese thread fin bream (*Nemipterus japonicus*) (Dadar *et al.,* 2016), Pick handle barracuda (*Sphyraena jello*) (Taheri Mirghaed *et al.,* 2016) and *Platycephalus indicus* (Azodi *et al.,* 2019) in Iran.

Fish-eating birds and marine mammals act as a final host, adult parasites live in the stomach or small intestines of these hosts. Cephalopods may act as a first intermediate host (Roumbedakis *et al*., 2018) and various species of fish act as a second intermediate host. Adult worms, also, live in the intestines of marine mammals, dolphins whales and seals and the stomach of ducks, geese, swans, and a large number of aquatic birds. In humans, ingestion of raw or semi-raw fish, lightly salted or smoked fish leads to a severe disease known as anisakidosis (Mostafa *et al.,* 2023). Symptoms of this disease depend on the location of the larval in organs. Third stage larvae in humans are likely to pierce the wall of the stomach or small intestine and symptoms such as vomiting, abdominal pain, fever, bloody diarrhea, eosinophilia, and emaciation were observed. In this disease, serologic tests particularly the enzyme-linked immunosorbent assay (ELISA) and Western blot are very useful for clinical evaluation (Shamsi and Barton, 2023). No human anisakiasis has been yet reported from Iran, this issue could be attributed to the cuisine habit of fish in studied areas as well as other parts of the country.

The genus *Hysterothylacium* includes 89 accepted species, one taxon inquirendum, two nomina dubia, and ten unaccepted species (Hossen and Shamsi, 2019), but GBIF (2021) enlisted 97 species in this genus. Since morphological analysis of *Hysterothylacium* species is difficult and subjective, the developed molecular methods can be helpful and

useful. In the last decade, molecular biology techniques such as PCR and random amplified polymorphic DNA analysis have been used for the identification of *Hysterothylacium* species (AlGabbani *et al*., 2021). PCR assays were used to differentiate among species of anisakid nematodes using the first and second internal transcribed spacers (ITS-1 and ITS-2) of ribosomal DNA (Simsek *et al.*, 2018). It is thought that the combination of morphological and molecular methods can be the best diagnostic strategy to identify the *Hysterothylacium* species. This study presents the identification of Hysterothylacium species on *S. commerson* using both morphological and molecular methods.

Materials and methods

One hundred *S. commerson* were caught from the Persian Gulf in Bandar Abbas in Hormozgan Province, south of Iran from April 2012 to December 2013. The fish were transported to the central laboratory of Shahrekord University for further analysis. The mean length and weight of fish were 40±19 cm 5.0±0.2 kg, respectively. Then, the skin, abdominal cavity, stomach, sub-serous tissues, the contents of the stomach, intestine, livers, spleens, and gonads were inspected for parasites through naked eyes and under the standard investigation (Moravec, 2004; Buchmann, 2007).

The nematode larvae were primarily identified based on morphological characteristics (Shamsi *et al.*, 2013; Dadar *et al.*, 2016). Larvae were counted, fixed in 70% ethanol and cleared in lactophenol for 48 h for identification. Each parasite was photographed by a digital camera under a light microscope. Measurements were made under a microscope by means of eyepiece micrometer and the drawings were made. Photographs and drawings images of parasites were used for morphological analysis. Then, a middle piece of each nematode was fixed and stored in 70% ethanol for molecular analyses (Dadar *et al.*, 2016).

DNA was extracted using a DNA isolation kit (MBST, Iran) according to the manufacturer's instructions. Two pairs of primers were designed based on the sequence comprising ITS-1 and ITS-2 of *Hysterothylacium* sp. (Accession no: JN005755.1). Primer forward: (5’-GTAGGTGAACCTGCGGAAGGATC-3’) was derived from nucleotides 1 to 23 of a sequence of 18S rRNA gene of *Hysterothylacium* spp. (Accession no. JN005755.1). Primer reverse (5’-TTAAATTCAGCGGGTAATCACG-3’) was derived from nucleotide 903 to 924 of the sequence of the 28S rRNA gene of *Hysterothylacium* spp. (Accession no: JN005755.1).

The PCR was performed in a total reaction volume of 100 µL containing: 10 µL of 10X PCR buffer, 3 µL $MgCl_2$ (50 mM), 2 µL of dNTP (10 mM each), 0.5 µL Taq DNA polymerase (5 U, Fermentas), 2 µL of each primer (20 µM), 74.5 µL dH_2O and 8µL of template DNA.

The reaction was repeated for 39 cycles under the following conditions: 5 min at 94°C (1 cycle), 45" at 94°C, 45" at 53°C, 45" at 72°C (37 cycles) and finally, PCR was completed with the additional extension step at 72°C for 10 min.

Distilled water was used as a negative control in each PCR reaction. PCR products were separated on 1.5% agarose gel in 0.5× Tris–borate–ethylene diaminetetraacetic acid (EDTA) buffer and stained with Ethidium Bromide and visualized by UV.

The PCR products were purified using a PCR purification kit (MBST, Iran) following the manufacturer's instructions. Purified fragments were sequenced from both sites of each PCR product using a method based on Sanger *et al.* (1977). Sequencing was carried out using the same primers as used for PCR amplification, by Kowsar Company. Sequences were aligned using the computer program Clustal and then adjusted manually. The consensus data of all sequences were evaluated by the NCBI Basic Local Alignment Search Tool (Blast) to confirm matching rRNA gene. The ClustalW multiple alignment software was used to align the nearly complete rRNA genes of Iranian *Hysterothylacium* isolates with the different sequences present in the NCBI database. MEGA 6.0 was used to construct a phylogenetic tree using the Maximum Likelihood (ML) approach (Tamura *et al.*, 2013). The tree with the highest log likelihood (-1719.1878) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 21 nucleotide sequences.

Results

Hysterothylacium larval were identified in 4 out of 100 fish samples (Fig. 1). The range of infection to *Hysterothylacium* was between 1-3 parasites. These larvae are characterized by excretory pores at the level of the nerve ring. The ventriculus had a nearly oval shape, the intestinal caecum was extended anteriorly and ventricular appendix was projected posteriorly. Four rectal glands were present. The conical tail was tipped with the arranged spines in a circle. Average length and width of body 7 mm (6–8) and 0.10 mm (0.07-0.13), respectively; lacking boring tooth; excretory pore opening just below nerve ring; nerve ring located from the 0.24mm (0.19-0.26) from the anterior end; muscular esophagus was 0.54 mm (0.44 – 0.61) long followed by a glandular ventriculus 0.05 mm long (0.04-0.06) and 0.03 mm (0.02–0.04) width; intestinal caecum 0.08 mm long (0.07-0.12); anus 0.12 mm (0.11-0.14) from the posterior end and tail conically shaped with a single spine. The survey revealed larval type consistent with *Hysterothylacium* type C in *S. commerson* (Fig. 2).

In this study, the amplification of nucleotide sequences of ITS-1 and ITS-2 were obtained from the individual *Hysterothylacium* parasite. After PCR assay, DNAs extracted gave the expected 889 bp PCR fragment, which is specific for Hysterothylacium parasite. After purification and sequencing of the PCR product, the nucleotide sequences revealed 889 bp lengths that were compared with

other *Hysterothylacium* sequences available in public databases (i.e. NCBI GenBank), By a BLAST search. Results showed that they are the most similar to *H. persicum* . The sequence of *H. persicum* isolated from *S. commerson* in the present study was deposited in Gen Bank under ID number 2736622. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-1719.1878) is shown (Fig. 3).

Figure 1: Macroscopic *Hysterothylacium* larvae isolated from the intestine of *Scomberomorus commerson*.

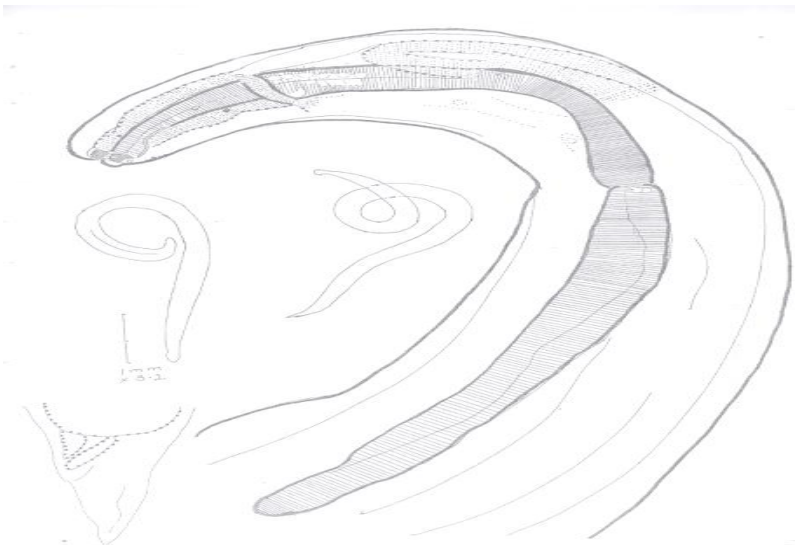

Figure 2: Drawing a picture of *Hysterothylacium* larvae type C from *Scomberomorus commerson*.

Initial tree (s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were

eliminated. There was a total of 766 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

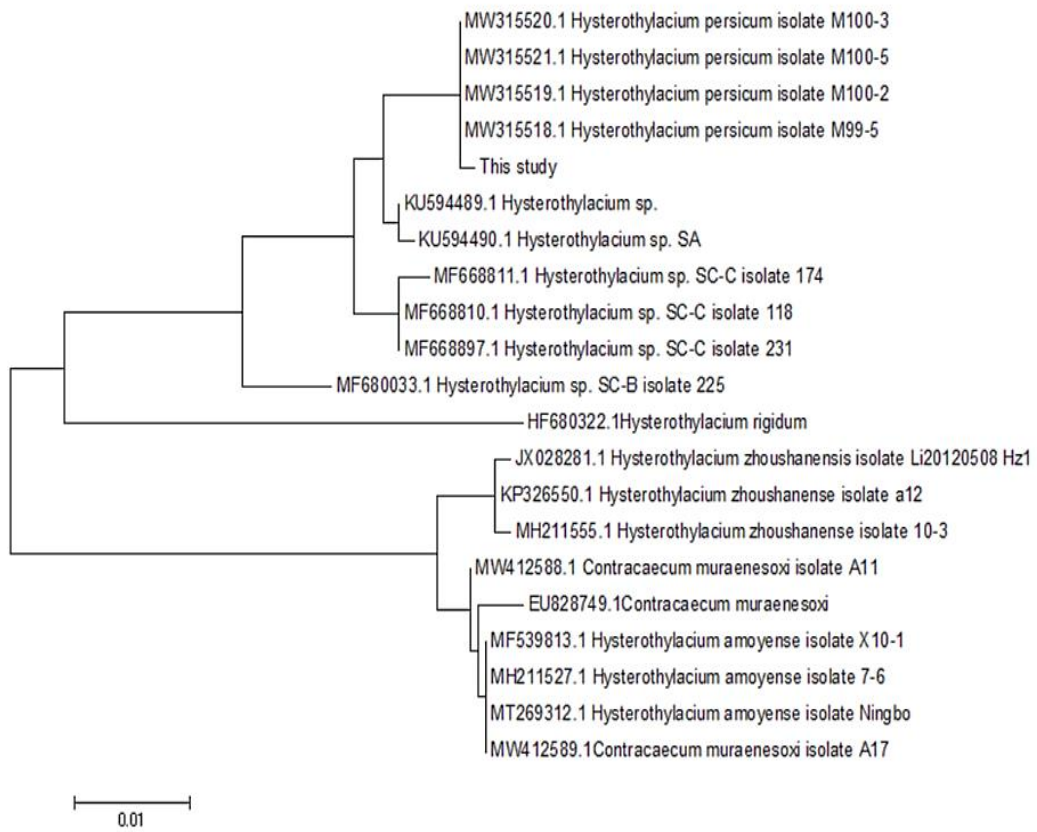

Figure 3: Molecular phylogenetic analysis by maximum likelihood method.

Discussion

The genus Hysterothylacium has numerous species, the taxonomic status of many being unclear. Hence, species identification and distinguishing between some of them are not feasible (AlGabbani *et al.*, 2021). Therefore, an identification of these species using improved approaches such as scanning electron microscopy and molecular techniques is essential. In Iran, only one systematic study of this group has been conducted (Shamsi *et al.*, 2016).

One problem facing researchers when studying species of *Hysterothylacium* in Iran is that most of these species were poorly described. The present study supported the distinction among species of *Hysterothylacium* based on morphological data and was useful in confirming the taxonomic status of individual species. In the present study, using morphological as well as molecular methods, the systematic and taxonomic status of one species of *Hysterothylacium* in Iran has been reexamined.

In this study, from 100 pieces of *S. Commerson*, 4 pieces were infected with *H. persicum*. The range of contamination to *Hysterothylacium* was between 1-3 parasites, which were identified by morphological and molecular methods.

There have been other studies on *Hysterothylacium* species in the Persian Gulf, such as the investigation by Taheri Mirghaed *et al.* (2016) which found a prevalence of 12% and a mean intensity of 4.8 in *S. jello*. Dadar *et al.* (2016) also collected anisakid larvae from two species of fish in the Persian Gulf, which were similar to *Hysterothylacium* type C. These studies highlight the presence of *Hysterothylacium* species in the Persian Gulf and the need for further research on their taxonomy and identification.

It is important to highlight the significance of phylogenetic analysis in this study, which confirmed the presence of *H. persicum* in the Persian Gulf and its similarity to other *H. persicum* in other regions. This analysis can provide insights into the evolutionary relationships and divergence of different species and can aid in the development of a comprehensive taxonomy for this group of nematodes. In our survey, molecular identification of *Hysterothylacium* was carried out by previously designed specific primers using PCR techniques. After the blast of nucleotide sequences, with the other recognized sequencing recorded in the Gene Bank, most similarity was observed to *H. persicum*. *H. persicum* was first described in the Persian Gulf by Shamsi *et al.* (2016). In parallel study, the phylogenetic analyses of the current study showed that the highest similarity to *H. persicum* isolated from selected edible fish from the Persian Gulf (Shahmsi *et al.*, 2016) and those previously described from Australasian (Shamsi *et al.,* 2016) and Iraqi waters (Ghadam *et al.,* 2018; Bannai, 2018). The result of the current study agrees with the suggested of Mattiucci and Nascetti (2008) and Zhao *et al.* (2017) identified the existence of "sibling species" within the ascaridoids, morphologically very similar but genetically different in some specimens. Molecular identification using PCR techniques and specific primers can be a useful tool in identifying *Hysterothylacium* species. The blast of nucleotide sequences with those recorded in the Gene Bank can provide insights into the similarity of different species. For example, investigations in the Gene Bank revealed that species of the same genus of *Hysterothylacium* in studying gene locus were similar to 99%. However, further extensive studies are needed to provide more informative and useful data.

In conclusion, the present study provides the first report of *H. persicum* from *S. Commerson* in Iran using both morphological and molecular methods. The combination of these techniques can aid in identifying *Hysterothylacium* species and confirming their taxonomic status. Considering the limited number of samples, it is evident that conducting molecular studies on a larger number of nematodes and investigating seasonal parasite contamination is essential in future studies. Further research on the taxonomy and identification of *Hysterothylacium* species is necessary, as many species remain poorly described and their taxonomic status unclear. The use of improved approaches such as scanning electron microscopy and molecular techniques can aid in species identification and differentiation. Studies such as the present one, which utilized both

morphological and molecular methods, can be useful in confirming the taxonomic status of individual species.

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Conflicts of interest

The authors declare that they do not have any conflict of interest.

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