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Infection and genetic detection of *Hysterothylacium* spp (Ascaridida: Raphidascaridinae) infesting *Saurida tumbil* (Bloch, 1795) and *Platycephalus indicus* (Linnaeus, 1758) fishes, Iraqi marine water fishes

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Abstract

Hysterothylacium spp Larvae encysted within the mesenteries, peritoneum, and viscera, which is the perfect place to complete their life cycle. More than 4123 larval stages were isolated from 595 *Saurida tumbil* and *Platycephalus indicus* fish. This is a simple fact that these two species are feeding greedily. The prevalence of infection was 95.3- 100 %. Internal transcribed spacers (ITS) gene of nuclear rDNA (rDNA) were amplified by conventional PCR, using the primer sets NC5/NC2. The ITS sequences determined were compared using the algorithm BLASTn with those available in the National Center for Biotechnology Information (NCBI) database. Multiple sequence alignments (MSA) were performed by ClustalW. The result showed that there are 22 distinct taxa within the genus *Hysterothylacium*, followed by 4 common ancestors, with 6 clades grouped. The study showed Sequence heterogeneity in the ITS gene within *Hysterothylacium* spp. of nematode parasite collected from *Saurida tumbil* and *Platycephalus indicus* fish. Moreover, genetic homogeneity between the population of the Arabian Gulf and other regions seemed different through the high levels of gene flow observed somewhat in this qualitative diversity. The most important of which may be the impact of climate.

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Introduction

Saurida tumbil (Bloch, 1795) Greater Lizardfish is widely distributed in the Indo-West Pacific from the east coast of Africa, the Red Sea to eastern Australia, found in shallow coastal sandy or muddy bottoms (1). It feeds on fishes, crustaceans, and squids, whereas the *Platycephalus indicus*, also called the bar-tailed flathead, is found in the Indian and western Pacific oceans. The species has been recorded in the Mediterranean, having invaded as a Lessepsian migrant through the Suez Canal (1). They live on sandy and muddy bottoms of coastal waters, including estuaries, and juveniles have even been taken in freshwater. It is a commercial fish species (1). *Hysterothylacium* species belonging to the Raphidascarididae family are the most widespread fish-parasitizing marine ascaridoids (2,3). The genus *Hysterothylacium* includes about 101 species reported in marine and freshwater (4). Eighteen *Hysterothylacium* larval types have been described worldwide (5-14). A previous essential study of the nematode parasites of marine fishes from Kuwait conducted over 3 years from 1992 to 1995 suggested an apparent convergence of the present eight types - KA-KH - of *Hysterothylacium* spp. (15). The lack of information on the genetic diversity and polymorphisms of the nematode group, which provides a limited morphology, makes them difficult to identify specifically (5).

So, this study aims to provide new information on the diverse structures population and follow molecular

heterogeneity using nuclear ribosomal DNA spacers analysis.

Materials and methods

Ethical approve

The care of experimental animals was consistent with the Republic of Iraq animal welfare laws, guidelines, and policies approved by the University of Basrah (Permit reference number 3/7/13302/2019). All fishes were collected from the fishing area consist of various locations in Iraqi marine waters (29°58 0 33 00 N48°28 0 20 E). Within fishing grounds for human consumption, with required permissions of the Directorate and supported by the Department of Veterinary Microbiology and Parasitology, College of Veterinary Medicine, and Marine Science Center, University of Basrah, Iraq.

Fishing area

The fishing area consists of three various locations, Arabian Gulf (29° 58 0’ 33 00’’ N48°28 ’ 0 20’’ E). This area is unique for fish feeding and spawning. Salinity concentrations in the region range from 40 to 43 ppt, and water temperature from 12.5 to 33.5 °C.

Collection of specimens

595 *Saurida tumbil* (Forster, 1801) and *Platycephalus indicus* (L., 1958) were examined, representing two genera of critical marine fishes. The gill nets fishing was used for collection. Nematodes were washed extensively by physiological saline (pH 7.4) and stored in 70-95% ethanol at -20 °C genomic DNA and PCR amplification. Fishes were identified according to Fish Base (16). Nematodes were identified using morphology (9). Some of the specimens were fixed in 4 % (v/v) hot formaldehyde solution (60°C), preserved in 70 % (v/v) ethanol, and post-fixed in 1 % osmium tetroxide, specimens were then dehydrated by incubating in a graded series of acetone ethanol concentrations (1:1), (1.5 - 0.5) and absolute acetone, 15 min each) for scanning electron microscope study (17). Genomic DNA was extracted from individual larvae by proteinase K treatment and purified using a mini-column (WizardDNA genomic DNA purification Kit, Promega, USA), according to the manufacturer's protocol. The internal transcribed spacers (ITS) of nuclear rDNA (rDNA) were amplified by PCR using the primer sets NC5/NC2 (Forward NC5 (5'-GTA GGT GAA CCT GCG GAA GGA TCA T3’) NC2 Revers (5'-TTA GTT TCT TTT CCT CCG CT-3') (18,19), respectively, under the same conditions as described previously (20). The ITS sequences determined were compared using the algorithm BLASTn with those available in the NCBI database. Phylogenetic relationships and the Evolutionary analysis used the Maximum Likelihood method. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura & Nei model and then selecting the topology with superior log likelihood value. The DNA sequences were aligned for phylogenetic analysis using theClustalW computer program**.** The tree was drawn to scale, with Evolutionary studies conducted in MEGA X version 10.7.1 (21).

Results

Samples were heavily infected with the larval stage (n= 4,123) of two genera of important marine fishes, *Saurida tumbil* (Forster, 1801) and *Platycephalus indicus* (L., 1958) in the Arabian gulf, that showed encapsulated within the mesenteries, peritoneum, and viscera, with a prevalence of infection (91.11 to 100 %) (Table 1) (Figure 1).

Table 1: Fish species, prevalence, the intensity of infection, and total numbers of nematodes in collected fish

Host	Common name	Total larvae.	Intensity	Prevalence %	Infection	No. of fish Exam
Saurida tumbil	Greater lizardfish	3360	12	100	280	280
Platycephalus indicus	Bartail flathead	763	2.65	91.11	287	315

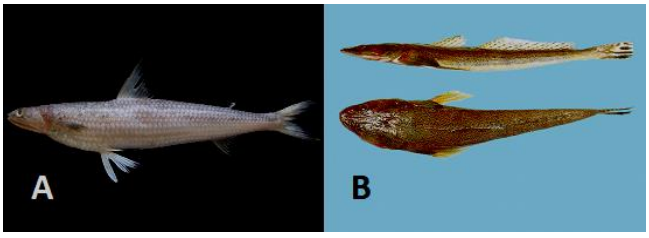

Figure 1: A: *S. tumbil* and B: *P. indicus.*

A detailed examination of the worms collected predominantly showed that they are anisakid larvae. They appeared under a light and scanning electron microscope (SEM) and were cylindrically bodied in a pointy shape at both ends. Lengths measured ranged from 10-20 mm. The front lots of each larva have a boring tooth and four lips that are not significantly distinct. The esophagus was in the anterior part with cylindrical striated muscle. A glandular ventriculus is present in the most larva, and their measurements varied from one sample examined to another based on the species (Figures 2 and 3).

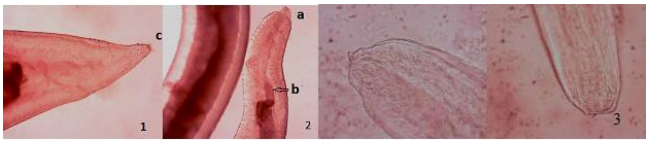

Figure 2: Larvae (L3) of *H. amoyense* viewed under a stereomicroscope; (1) c: posterior end region of the larval stage morphotypes tail. (2) a: cephalic head region shows mouth opening and papillae; b: glandular ventriculus viewed. X=100. (3) Light microscopic *Hysterothylacium* sp. larval. X=400.

Twenty-two representative specimens were subjected to molecular analysis. The sequences of rDNA ITS regions (ITS1-5.8S-ITS2) were submitted to GenBank under the accession numbers (Table 2). Nineteen specimens of *H. amoyense*, query similarity identities percentage was identities 99-100%. Two species of *Hysterothylacium* sp., with query similarity identities percentage of 98%, were recognized, and one species of *Countraceacum muraenesoxi* query similarity identities percentage was 913/917 (99) with Gap 4/917. Unfortunately, since no mature stages were recorded, this cannot be confirmed at the species level. The new larvae cannot be accurately identified at the species level because it is necessary to study the mature adult stage of the parasite, using male and female morphology and characterization of reproductive organs.

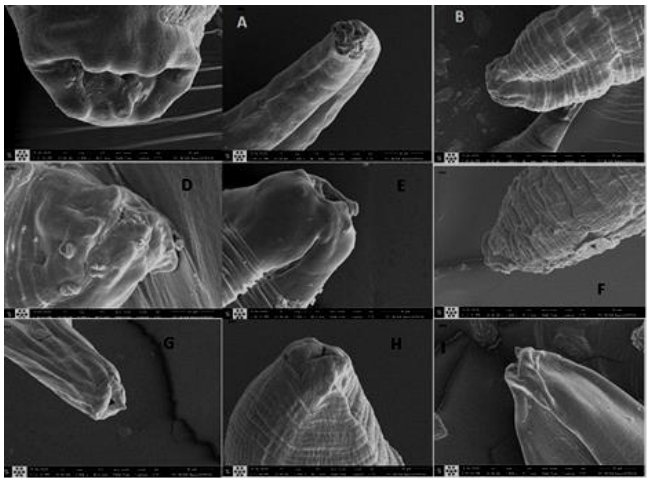

Figure 3: Scanning electron micrographs of *Hysterothylacium* spp) head with dorsal lip and ventral interlabium cephalic extremity with deep cuticular striations on the anterior end.

Table 2: Detailed information of Nematode species, GenBank Accession numbers provided by NCBI for the collected larvae of *P. indicus* and *S. tumbil* of Ascaridoid nematode species with their genetic data including reference source, identical %, GenBank (ITS) reference, and geographical locality

Nematode species	GenBank	Reference	Identical (%)	Gaps (%)	GenBank	Country
H. amoyense	MZ503498	36	912/912(100)	0/912	MT02011.1	China
H. amoyense	MZ509280	37	913/913(100)	0/913	MT020134.1	China
Hysterothylacium sp.	MZ509452	37	809/825(98)	4/825	MT020132.1	China
H. amoyense	MW422786	37	915/916(99)	1/916	MT020132.1	China
H. amoyense	MW423789	37	915/915(100)	0/915	MT02011.1	China
H. amoyense	MW423785	37	896/899(99)	3/899	MT020134.1	China
C. muraenesoxi	MW423788	37	913/917(99)	4/917	MT020132.1	China
H. amoyense.	MW423773	36	917/920(99)	3/920	MT020132.1	China
H. amoyense	MW423786	37	905/920(98)	8/920	MF539813.1	China
H. amoyense	MW423794	2	909/918(99)	4/918	MT020132.1	China
H. amoyense	MW423792	3	864/869(99)	3/869	MF539813.1	China
Hysterothylacium sp.	MW423772	40	599/607(99)	2/607	MK039147.1	North-E Atlantic
H. amoyense	MW404622	37	913/913(100)	0/913	MT020134.1	China
H. amoyense	MW412565	36	830/830(100)	0/830	MF539813.1	China
H. amoyense	MW404617	36	860/860(100)	0/860	MF539813.1	China
H. amoyense	MW404615	37	915/915(100)	0/915	MT020134.1	China
H. amoyense	MW404619	37	915/915(100)	0/915	MT020134.1	China
H.amoyense	MW412836	2	862/870(99)	0/870	MT020134.1	China
H. amoyense	MW412582	2	916/916(100)	0/916	MT020134.1	China
H. amoyense.	MW453086	2	916/916(100)	0/916	MT020134.1	China
H. amoyense.	MW423771	2	915/916(99)	0/916	MT020134.1	China
H. amoyense	MW412588	2	912/912(100)	0/912	MT020134.1	China

The evolutionary relationships of taxa history were inferred using the Neighbor-Joining method (Figure 4). This analysis involved 24 nucleotide sequences. There were 1407 positions in the final datasete analysis.The results showed that there are 22 distinct taxa (taxon) with 4 common ancestors of parasites collection, with grouped 6 clades. With *Hysterothylacium amoyense* with 2 strong nodal support (bootstrap value = 88 %) and 7 sisters taxon.

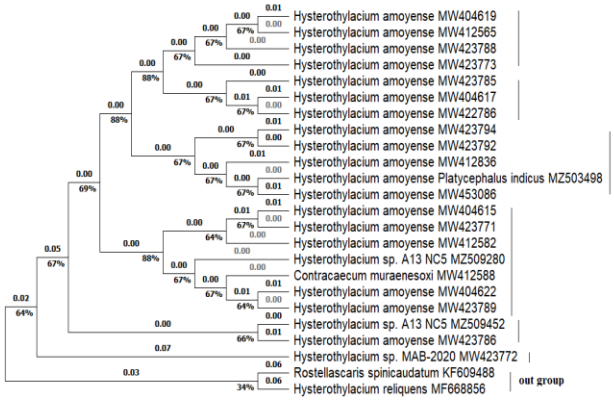

Figure 4: Cladogram Maximum Neighbor-joining (MN) Phylogenetic of ascaridoid nematodes of larvae obtained *(Rostellascaris spinicadatum* and *H.reliquens*) were used as an outgroup.

Multiple alignments show genetic polymorphisms in the Nucleotide of anisakid nematodes within some specimens of *Hysterothylacium* spp. A small group of them show the effect of genetic polymorphisms on morphological appearance in terms of the structure of the parasite cuticle. However, many classifications have kept them within the same species (Figure 2). These genetic variations between individuals, apparently of the same species in the same host, may represent genetic variations at the species level, which can be transferred to new species. It represents the most significant gene diversity in the genus *Hysterothylacium* . which exhibits a very close relationship. Also, this mainly means that there is a considerable diversity of genetic variations at the individual level in the Raphidascarididae family, which may be due to the mutual fertilization of the occupied species belonging to the same family. The result also showed that the resilience of ecosystems of the Arabian Gulf was generally positive because of the high gene flow.

Discussion

The ribosomal RNA encapsulates a wealth of evolutionary information, including genetic variation that can be used to discriminate between organisms at a wide range of taxonomic levels. Uses methods of molecular approaches in the understanding of the biodiversity and systematics of nematode parasites have been utilized for delimiting and identifying anisakid nematodes and also to reveal cryptic species, such as the members of the genus *Anisakis* (22,23). The genetic analysis of larvae in the present study has allowed the specific and genotypic identification and polymorphisms of larvae within single morphotypes based on matching their sequences with the data available in the NCBI.

The targeted DNA sequencing of the ITS region and use of additional genetic loci, such as the ITS-1 region and perhaps mitochondrial gene loci, might support further the specific identification of anisakid nematodes as accurate and efficient for large-scale studies (19,24,25). The analysis revealed four polymorphisms in the ITS region, and the molecular methods showed that each of these larval types was composed of different genotypes. Sequence identity of 100%; nucleotide difference = 0.00%). Thirteen of *Hysterothylacium* spp. were identified based on their molecular characterization showed that those specimens represent different taxon with *Hysterothylacium amoyense* (26,27).

Widely distributed species can show different patterns of genetic structuring influenced by geographic distance, history, and/or natural selection (28). The discrepancy between morphological and molecular identification of the same host larvae species cannot be readily explained. We ensured that the sequence types reported in the present study should correspond to the correct morphotypes. The presence of the same species of individuals possessing heterogeneous genotypes in the same host may cause these genetic differences at the species level, which is recorded by the current study.

According to Froese and Pauly (16), over 200 species of fish are found in Iraqi marine waters, many of which are edible. However, our knowledge of their parasites is poor (29). Although there have been reports of *Hysterothylacium* spp. in Iraqi marine fish, most of these are based on morphology only, providing a limited morphological description that makes specific identification difficult. This information needs to be updated to improve knowledge of the species and the effect of environmental changes on increasing temperatures and climate change. These conditions are essential for the distribution of this type of parasite (30). Higher infections with Raphidascarididae were found than that observed in Bander Abas, Hormozan province, located on the southern coast of Iran on the Arabian Gulf (9). Their study was of 600 fish belonging to five popular species of fish, including *Otolithes ruber, Psettodes erumei, Saurida tumbil, Scomberomorus commerson,* and *Sphyraena jello.* The targeted DNA sequencing of the ITS, ITS-1, and ITS-2 of the rDNA provides genetic markers for the accurate identification of a range of species of Ascaridoids (31-35). It appears that the nematode fauna of the Arabian Gulf shows few similarities with the fauna of the western Pacific Coast and adjacent seas. This also corresponds to the results of the current study,

which shows many similarities with the fauna in the Arabian Gulf (27), in north-east Atlantic waters (30,36), and in China (37).

Conclusion

Larvae from the Arabian Gulf. Valid genetic data deposited in GenBank described here can be used to establish the phylogenetic relationships of *Hysterothylacium* spp. from the Arabian Gulf fish and the rest of the world. Moreover, further research using the same genetic markers is required to examine the genetic variability and population genetic structure within larvae and adults of the *Hysterothylacium* species.

Conflict of interest

The authors declare no conflicts of interest regarding this manuscript's publication and/or funding.

Acknowledgments

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العدوى والكشف الوراثي للطفيليات لأنواع هستيروثلاسيم الاسكارديا التي تصيب اسماك الوحرة وأبوالهيل، أسماك المياه البحرية العراقية

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الخلاصة

تم عزل أكثر من ٤١٢٣ مرحلة يرقات من أنواع هستيروثلاسيم من داخل المساريق، الصفاق، والأحشاء، والذي يعد المكان المثالي لإكمال دورة الحياة من ٥٩٥ سمكة أبو الهيل وسلطان إبراهيم، وهذه حقيقة طبيعية بأن هذين النوعين يتغذيان بشراهة. كان معدل انتشار الإصابة ٩٥,٣-١٠٠٪. تم تضخيم الفواصل الداخلية المنسوخة الجينية للحمض النووي الريبي النووي بواسطة تفاعل البلمرة المتسلسل التقليدي، باستخدام مجموعات التمهيدي وتمت مقارنة تسلسلات الفواصل الداخلية المنسوخة المحددة باستخدام خوارزمية بلاستن مع تلك المتاحة في قاعدة بيانات المركز الوطني لمعلومات التكنولوجيا الحيوية. تم إجراء محاذاة تسلسل متعددة بحيث أظهرت النتيجة أن هناك ٢٢ صنفا متميزا داخل جنس هستيروثلاسيم، يليه ٤ أسلاف مشتركين، مع ٦ مجموعات مجمعة. أظهرت الدراسة عدم تجانس التسلسل في جين الفواصل الداخلية المنسوخة داخل الأنواع العائدة لنفس الجنس من طفيليات الديدان الخيطية التي تم جمعها. كما بدا التجانس الجيني بين تجمعات الطفيليات في منطقة الخليج العربي والمناطق الأخرى مختلفا من خلال المستويات العالية من التدفق الجيني التي لوحظت إلى حد ما في هذا التنوع النوعي. قد يكون أهمها تأثير المناخ.

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