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RESEARCH ARTICLE

First Molecular Characterization of *Hysterothylacium Fabri* Larvae (Nematoda: Raphidascarididae) in the Mediterranean Sea Based on the Small Subunit Ribosomal RNA Gene Sequence

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ABSTRACT

Hysterothylacium fabri is commonly reported in various Mediterranean fish. However, there is no data on the mitochondrial ribosomal RNA (*rrnS*) gene sequence of *H. fabri* larvae in the Mediterranean Sea waters. Therefore, we aimed to reveal molecular characterization of *H. fabri* based on the *rrnS* gene sequence in the current study. Firstly, the fourth stage of *H. fabri* larvae were identified at parasitological examination from *Mullus barbatus* in the Mediterranean Sea. Morphologically identified *H. fabri* larvae herein were also supported by the sequencing of the nuclear ribosomal ITS gene regions. In the next step, the *rrnS* gene of these larvae was molecularly analysed. Our *H. fabri* isolate (MK886659) showed 96.42% identity with *H. fabri* reported from China (MF140349) based on the *rrnS* gene. The nucleotide difference based on the *rrnS* gene between Chinese isolate (MF140349) and our *H. fabri* isolate (MK886659) was determined as 3.2%. Consequently, for the first time, the mitochondrial *rrnS* gene sequencing of *H. fabri* from the Mediterranean waters were performed in the current study.
Keywords: *Hysterothylacium fabri*, Mediterranean Sea, Mitochondrial *rrnS* gene, Molecular characterization

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Akdeniz'deki *Hysterothylacium Fabri* (Nematoda: Raphidascarididae) Larvasının İlk Kez Küçük Alt Ünite Ribozomal RNA Gen Bölgesinin Sekansına Göre Moleküler Karakterizasyonu

ÖZ

Hysterothylacium fabri sıklıkla farklı türdeki Akdeniz balıklarında bildirilmektedir. Ancak, Akdeniz sularındaki *H. fabri* larvasının mitokondriyal ribozomal RNA (*rrnS*) gen bölgesinin sekansı üzerine herhangi bir veri bulunmamaktadır. Bu yüzden, bu çalışmada, *H. fabri*'nin *rrnS* gen bölgesinin sekansına göre moleküler karakterizasyonunu ortaya koymayı amaçladık. İlk olarak, Akdeniz'deki *Mullus barbatus*'tan dördüncü dönem *H. fabri* larvaları parazitolojik muayenede teşhis edildi. Burada morfolojik olarak teşhis edilen *H. fabri*, nükleer ribozomal ITS gen bölgesinin DNA dizi analizi ile de desteklendi. Bir sonraki aşamada ise bu larvaların *rrnS* geni moleküler olarak analiz edildi. *RrnS* gen bölgesine göre, *H. fabri* izolatımız (MK886659) Çin'den rapor edilen *H. fabri* (MF140349) ile %96,42 oranında benzerlik gösterdi. Çin (MF140349) izolatı ile *H. fabri* (MK886659) izolatımız arasındaki nükleotid farklılığı ise *rrnS* genine göre %3,2 olarak belirlendi. Sonuç olarak bu araştırma ile ilk kez Akdeniz sularından *H. fabri*'nin mitokondriyal *rrnS* sekanslaması gerçekleştirildi.
Anahtar sözcükler: Akdeniz, *Hysterothylacium fabri*, Mitokondriyal *rrnS* gen, Moleküler karakterizasyon

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INTRODUCTION

Hysterothylacium species are marine ascaridoids and over 70 recognizable species have been described in the worldwide (Moravec and Justine 2015). *Hysterothylacium* species were reported as larvae and adults in various marine teleost from the Mediterranean Sea (Barcala et al. 2018; Costa et al. 2018; Gazzonis et al. 2017; Keskin et al. 2015; Pekmezci et al. 2014; Petter and Maillard 1988; Roca-Gerones et al. 2018; Simsek et al. 2018; Szostakowska et al. 2001; Tedesco et al. 2018). Until now, five *Hysterothylacium* larval morphotypes (III, IV, V, VIII, IX) and *H. reliquens, H. fabri, H. aduncum,* and *H. girgenti* have been genetically identified using ribosomal and mitochondrial DNA markers in the Mediterranean waters (Barcala et al. 2018; Costa et al. 2018; Keskin et al. 2015; Pekmezci et al. 2014; Roca-Gerones et al. 2018; Simsek et al. 2018; Tedesco et al. 2018; Khammassi et al. 2020). There has been limited knowledge on the *H. reliquens*, *H. fabri,* and *H. aduncum* in marine teleost from Turkish Mediterranean waters (Keskin et al. 2015; Pekmezci et al. 2014; Simsek et al. 2018). Although there are molecular studies for the certain identification of marine ascaridoid species, the taxonomy of the genus *Hysterothylacium* in Mediterranean waters remains incomplete and unresolved (Costa et al. 2018). Moreover, there is no study on the *rrnS* gene sequence of molecular characterisation of *H. fabri* larvae in Mediterranean waters. Therefore, for the first time, we performed the molecular characterization of *rrnS* mitochondrial gene of *H. fabri* from the Mediterranean waters.

MATERIALS AND METHODS

Fish Sampling, Parasite Collection, and Morphological Examination

For this study, ethics committee approval was not needed because no handling of live marine teleost specimens was involved. A total of forty-five fresh and dead *Mullus barbatus* (L.) caught from the Turkish Mediterranean coast were purchased from local fishermen between March 2018 to May 2018 and examined under a stereomicroscope for the presence of *Hysterothylacium* spp. larvae in the digestive tract and visceral cavity. All nematodes were mechanically removed, washed in saline, and stored in ethanol solution. A middle portion of nematodes was used for genetic identification and other portions were individually cleared with lactophenol for using morphological identification. The nematodes were identified using the location of the excretory pore, the digestive systems, and the posterior ends (Petter and Maillard 1988; Tedesco et al. 2018). A subsample of five specimens of the fourth stage of *H. fabri* larvae was randomly selected and subjected to molecular analysis using a small middle portion of the nematodes.

DNA Extractions, PCR Amplifications, DNA Sequencing, and Phylogenetic Analysis

Genomic DNA was extracted from individual nematodes using the DNA extraction kit (GeneJET Genomic DNA Purification Kit, Thermo Scientific, Waltham, MA, USA) following to the manufacturer's instructions. The PCR amplification targeting the nuclear ribosomal ITS gene and the mitochondrial ribosomal RNA (*rrnS*) gene were performed with the primers NC5/NC2 and MH3/MH4.5 primer pairs, respectively (D'Amelio et al. 2007; Zhu et al. 1998). For ITS gene, PCR mixture was prepared according to previously described by Pekmezci et al. (2014). PCR was carried out in a final volume of 50 µl, containing 10–50 ng of extracted DNA, 1× Taq Buffer with KCl (Thermo Scientific), 3 mM of MgCl2 (Thermo Scientific), 0.3 mM dNTPs (Thermo Scientific), 2 pmol of each primer, 2.5 U of Taq DNA Polymerase (Thermo Scientific), and DEPC-treated water. The PCR conditions were as follows: 15 min at 95 °C, then 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C followed by a final elongation of 5 min at 72 °C. For *rrnS* gene, PCR mixture was prepared according to previously described by D'Amelio et al. (2007) and PCR reaction (50 µl) was contained 20–40 ng of extracted DNA, 10 mM Tris-HCl (pH 8. 3), 50 mM KCl (Thermo Scientific), 3 mM MgCl2 (Thermo Scientific), 1 mM of dNTPs (Thermo Scientific), 50 pM of each primer, 0. 5 ml of Taq DNA Polymerase (Thermo Scientific). The PCR conditions were as follows: 10 min at 95 °C, then 35 cycles of 30 sec at 95 °C, 30 sec at 55 °C and 30 sec at 72 °C followed by a final elongation of 7 min at 72 C.

Positive PCR products were purified and sent to Macrogen company for DNA sequencing. The raw sequence data were analysed using Geneious R11 (Kearse et al. 2012). The contigs were compared with all *Hysterothylacium* species sequences using the BLASTn algorithm via GenBank (Altschul et al. 1997). Pairwise distances using a Kimura 2-parameter and best of the nucleotide substitution model were calculated in MEGA 7.0 (Kumar et al. 2016). The TN93+G+I model for *rrnS* gene sequences was selected using Akaine Information Criterion. The *rrnS* datasets were used for maximum likelihood phylogenetic tree by bootstrap of 1000 replicates (Felsenstein 1985). The newly generated *rrnS* sequence was deposited in the GenBank under the accession number: MK886659.

RESULTS

The prevalence of the *Hysterothylacium* larvae in the Mediterranean Sea was detected as 10% (4/40) in *M. barbatus*. The morphology of selected specimens agrees well with the description of the fourth stage of *H. fabri* larvae (Figure 1).

Figure 1: Microphotographs showing main morphological features of *H. fabri* larvae from the Mediterranean Sea: (A) anterior end (scale: 200 µm), (B) posterior end (scale: 50 µm).

The final length of the contig nucleotide sequences of the *rrnS* and ITS gene regions were 490 bp and 955 bp, respectively. No intraspecific nucleotide differences were detected in the sequence analyses of partial *rrnS* and ITS region. Our *H. fabri* isolates displayed 98.90-100% identity to that of the ITS sequences of *H. fabri* (accession from JX974558, MH211474-94, MF539787-89, JQ520158, KU948632-37, KC852206) recorded previously from the Mediterranean Sea, South Korean waters, and Chinese waters (Chen et al. 2018; Pekmezci et al. 2014; Tedesco et al. 2018; Zhang et al. 2018). Moreover, our *H. fabri* isolates (MK886659) showed 96.42% identity with *H. fabri* from China (MF140349) based on *rrnS* gene. The nucleotide difference based on *rrnS* gene between the Chinese isolate (MF140349) and our *H. fabri* isolate (MK886659) was determined as 3.2%. In phylogenetic analysis, our Mediterranean waters isolate of *H. fabri* (MK886659) and China isolate of *H. fabri* (MF140349) were also clustered together in the ML tree (Figure 2) according to *rrnS* dataset.

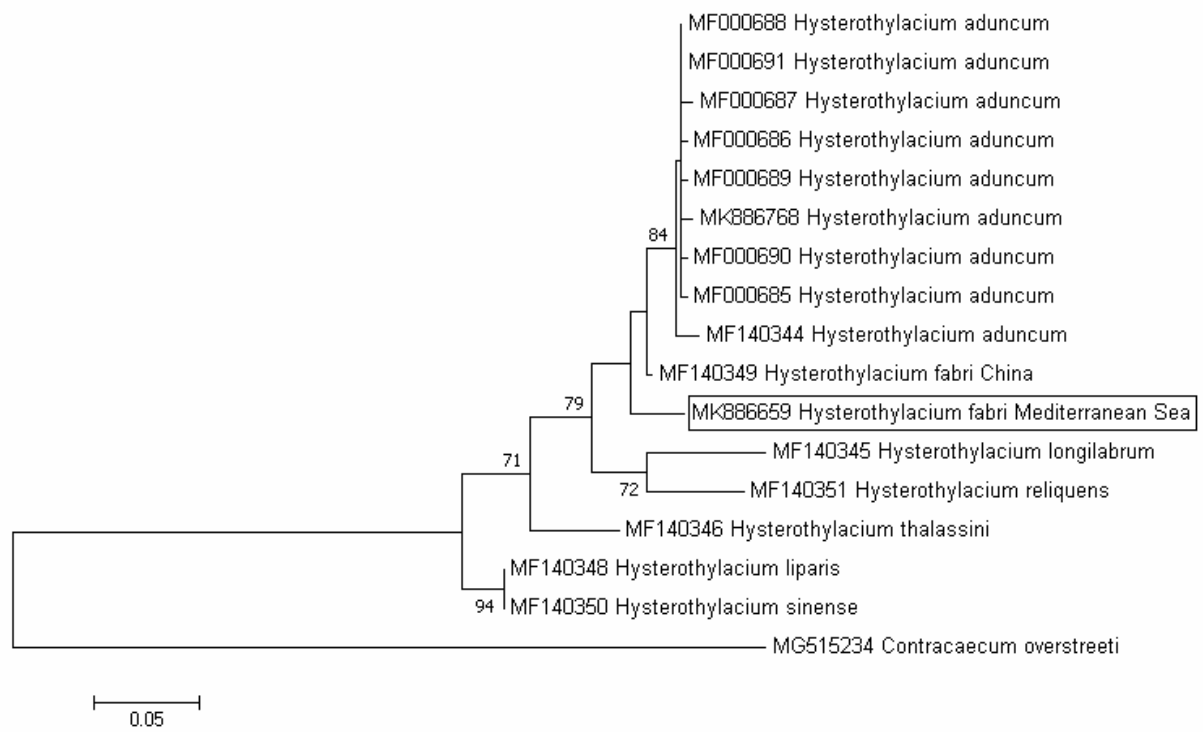

Figure 2: Phylogenetic relationships between our *H. fabri* (MK886659) isolate and other *Hysterothylacium* species as inferred by maximum likelihood obtained from *rrnS* gene. The scale bar indicates the distance in substitutions per nucleotide. Bootstrap values were calculated over 1,000 replicates and percentages ≥ 70% are shown at the internal nodes. *Contracaecum overstreeti* was used as out group.

DISCUSSION

The paper on morphological identification of *Hysterothylacium* larvae published by Petter and Maillard (Petter and Maillard 1988) described the third (L3) and fourth (L4) stage of *H. aduncum* larvae, L3 and L4 of *H. fabri* larvae, L4 of *H. arnoglossi* larvae and L3 and L4 of *Hysterothylacium* sp. I and II larvae from the Mediterranean Sea. Recently, L3 and L4 stage and adult of *H. fabri* were also morphologically identified from the Mediterranean Sea (Tedesco et al. 2018). In the present study, our specimens were morphologically identified as L4 of *H. fabri* according to the identification key (Petter and Maillard 1988; Tedesco et al. 2018). Moreover, the morphological identification of *H. fabri* reported herein was also supported by molecular analyses. Our ITS regions sequence of L4 of *H. fabri* isolate displayed 98.90-100% identity (97% query cover) with the ITS sequences of *H. fabri* (JX974558, MH211474-94, MF539787-89, JQ520158, KU948632-37, KC852206) from the Mediterranean Sea, South Korean waters, and Chinese waters (Chen et al. 2018; Pekmezci et al. 2014; Tedesco et al. 2018; Zhang et al. 2018). Therefore, previously morphologically classified L4 of *H. fabri* larvae were also genetically confirmed as *H. fabri* based on the ITS regions. Herein, for the first time, we also determined the *rrnS* mitochondrial sequences of *H. fabri* from the Mediterranean waters. However, there was one sequence for the *rrnS* gene of *H. fabri* (MF140349) recorded previously from China deposited in GenBank for comparison (Li et al. 2018). Interestingly, the %3.2 genetic distance was observed among *rrnS* sequences of *H. fabri* comparable to those reported (Li et al. 2018) and the present study. Nucleotide identity of the ribosomal and mitochondrial DNA higher than 96% for Anisakidae and Raphidascarididae are considered to be the same species (da Fonseca et al. 2016). Moreover, other known *H. aduncum* species exhibited low level of intraspecific nucleotide differences (p distance=0.004 to 0.009) between the Black Sea (MK886768) and the Mediterranean Sea isolates (MF000685-MF000691) for *rrnS* gene, whereas; high intraspecific genetic diversity (p distance=0.021) in the same species was also detected between Black Sea (MK886768) and China (MF140344) isolates (Pekmezci 2019). Therefore, the *rrnS* mitochondrial nucleotide differences between the China and our Mediterranean isolates of *H. fabri* should be considered as intraspecific nucleotide variation. Additionally, there may be intraspecific nucleotide differences between the remote communities of the same *Hysterothylacium* species from different geographic area. Moreover, several authors accept *H. fabri* collected from marine fish in the Mediterranean and Chinese waters to be a complex comprising at least two or three sibling species (Guo et al. 2014; Martin-Sanchez et al. 2003).

In the present study, high nucleotide variability (3.2 % nucleotide difference) was detected in the *rrnS* sequences of Mediterranean (MK886659) and the China (MF140349) isolates of *H. fabri*. Thus, we also considered *H. fabri* as sibling species according to *rrnS* gene.

In conclusion, for the first time, we determined the mitochondrial *rrnS* data of *H. fabri* from the Mediterranean Sea in the present study. These molecular results provided species-specific genetic identification of *H. fabri* from the Mediterranean waters based on *rrnS* gene. More studies are needed to investigate the nucleotide variability by using various gene markers both larvae and adults of *Hysterothylacium* species from the Mediterranean waters.

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Ethics Committee Information: This study is not subject to the permission of HADYEK in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Conflict of Interest: The authors declared that there is no conflict of interest.

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