Molecular Characterization of *Hysterothylacium aduncum* (Nematoda: Raphidascarididae) Larvae Infecting *Merlangius merlangus euxinus* (Linnaeus, 1758) from the Turkish Black Sea Coast Based on Mitochondrial Small Subunit Ribosomal RNA Gene Analysis

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Abstract: The taxonomy of *Hysterothylacium* genus remains incomplete and unclear in Turkish waters. In the present study, *H. aduncum* larvae were morphologically identified from *Merlangius merlangus euxinus* in the Black Sea, Turkey. The nuclear ribosomal internal transcribed spacer region (ITS-1, 5.8S subunit, ITS-2) and the small subunit of the mitochondrial ribosomal RNA (rRNs) gene of *H. aduncum* were amplified and sequenced. The BLAST analysis indicated that obtained ITS sequences were identical to that of the reference sequence of *H. aduncum* (accession no JX413596) recorded previously from the Black Sea, Turkey. The rRNs gene of *H. aduncum* from the Black Sea, Turkey (MK886768) showed 97.94 to 99.56% identity to the isolates of *H. aduncum* from the Mediterranean Sea (MF000685-MF000691) and the Chinese waters (MF140344). Moreover, pairwise comparison between the rRNs sequences of the *H. aduncum* from the Black Sea, Turkey (MK886768) and others *H. aduncum* isolates from the Mediterranean Sea (MF000685-MF000691), the Chinese waters (MF140344) showed differences ranged from 0.2 and 1.7%. Consequently, *H. aduncum* from the Black Sea was characterized for the first time by sequencing of the mitochondrial rRNs gene with the present study.

Key words: *Hysterothylacium aduncum*, molecular characterization, mitochondrial rRNs gene, Black Sea, Turkey

Introduction

*Hysterothylacium* species belonging to family of the Raphidascarididae has a circumpolar distribution in the Northern Hemisphere that mainly found in marine teleost in temperate and cold waters [4] and a few fresh water hosts [16, 21]. To date, there are over 70 recognizable *Hysterothylacium* species with worldwide distribution [17]. However, only three species, *H. aduncum*, *H. fabri* and *H. reliquens* have been molecularly characterized from Turkish waters [11, 18, 20, 25]. These molecular studies have been also proven to be useful for the accurate identification of those *Hysterothylacium* species using DNA sequencing of ribosomal internal transcribed spacer (ITS) regions and the mitochondrial cytochrome c oxidase subunit 1 (cox1) and (cox2) genes [11, 18, 20, 25]. Molecular data on *Hysterothylacium* genus infecting fish from Turkish waters is still not sufficient. Nevertheless, before the present study, there
had been no reports of characterizing the *H. aduncum* from the Black Sea using well-defined mitochondrial rrnS gene sequences. For this reason, in the present study, *H. aduncum* from the Black Sea were genetically characterized for the first time by sequencing of mitochondrial rrnS marker.

**Materials and Methods**

**Parasite Collection and PCR Amplification**

*Hysterothylacium* spp. larvae were collected from *Merlangius merlangus euxinus* in the Black Sea, Turkey between September and November 2018. Larvae were identified using the morphology of the labia, the position of the excretory pore, the intestinal cecum, ventricular appendix and the tail [3, 23]. Three of fourth-stage larvae were randomly selected among the total larvae samples and were subjected to the molecular analysis. Total DNA was extracted from the middle part of larvae using the DNA extraction kit (Thermo Scientific). The nuclear ribosomal ITS region (ITS-1, 5.8S subunit, ITS-2) and the small subunit of the mitochondrial ribosomal RNA (rrnS) gene were targeted for amplifications. ITS regions were amplified using the primers NC5 (5’-GTA GGT GAA CCT GCG GAA GAT CAT TT-3’) and NC2 (5’-TTG TCT TTT CCT CCG CT-3’) [28]. PCR conditions followed the protocol described by Pekmezci et al. [18]. Then, the rrnS gene was amplified using MH3 (5’-TTG TTC CAG AAT AAT CCG GTC CTA TT-3’) and MH4.5 (5’-TCT ACT TTA CTA CAA CTT ACT CC-3’) [6]. PCR conditions were also used according to D’Amelio et al. [6]. PCR products were visualized on 1.5% agarose gel by UV transillumination.

**DNA Sequencing and Phylogenetic Analysis**

Three fourth stage larvae morphologically identified as *H. aduncum* were sequenced using ABI PRISM 310 genetic analyser (Applied Biosystems) for ITS and rrnS genes. The quality of the sequences were checked using Geneious R11 (Biomatters Ltd.) and Vector NTI Advance 11.5 (Invitrogen). Later, sequences were verified by forward and reverse comparisons, assembled, and edited with Contig Express in Vector NTI Advance 11.5 (Invitrogen) and Geneious R11 (Biomatters Ltd.). The obtained consensus sequences were compared with previously published data for identification by using the Basic Local Alignment Search Tool (BLAST) via GenBank database [1]. The rrnS sequences were aligned with others known *H. aduncum* in previous studies [8, 15] using ClustalW in MEGA 7.0 multiple sequence alignments [27] and adjusted manually. Genetic distances were calculated using the Kimura two-parameter model with pairwise deletion in Mega 7.0 [13]. Phylogenetic relationships were inferred using maximum likelihood (ML) with selection of the best model for nucleotide substitution by the Find Best DNA Model test implemented in MEGA 7.0 [13]. The Hasegawa-Kishino-Yano model (HKY + G) was selected using Akaike information criterion (AIC). Bootstrap confidence values were calculated with for 100 repetitions for ML [7]. Bootstrap values ≥ 70 were considered well supported [10]. The nucleotide sequences were deposited in GenBank database under the accession numbers: MK886768 for rrnS gene. Reference specimens and isolated DNA samples are stored at the “Department of Aquatic Animal Diseases, Veterinary Medicine Faculty, Ondokuz Mayis University,” Samsun, Turkey.

**Result**

The amplifications of the rrnS gene and ITS region produced a fragment of approximately 500 bp and 1000 bp from different individuals, respectively, on agarose gels. While the rrnS products were subjected to direct sequencing giving products 480 bp long, ITS products were 900 bp long. No intraspecific nucleotide variability within different individuals was observed in the rrnS gene and ITS region. The *H. aduncum* isolates showed 100% identity to that of the reference sequence of *H. aduncum* (accession from JX413596) recorded previously from the Black Sea, Turkey [18]. The percent identities among *H. aduncum* isolates from Black Sea, Turkey (MK886768) showed 97.94-99.56% identity with various geographical isolates of *H. aduncum* from the Mediterranean Sea (MF000685-MF000691) and the Chinese waters (MF140344) from GenBank according to rrnS gene. Pairwise comparison between the rrnS sequences of the *H. aduncum* isolates from the Black Sea, Turkey (MK886768) and others *H. aduncum* isolates from the Mediterranean Sea (MF000685-MF000691), the Chinese waters (MF140344) showed differences ranged from 0.2 and 1.7% (Table 1). Also, the present rrnS sequence
(MF140344) was aligned with the same gene for *H. aduncum* (MF000685-MF000691 and MF140344) which was previously deposited in GenBank (Fig. 1). Moreover, *H. aduncum* (MK886768) in the Black Sea, Turkey and others *H. aduncum* (EU852345-EU852348 and MF140344) isolates were clustered in the same clade in the ML tree (Fig. 2) inferred from the rrnS sequence analysis.

**Table 1.** Pairwise comparison of nucleotide sequence differences (%) in the rrnS among *H. aduncum* isolates and various geographical isolates

| Pairwise Comparison | (%) |
|--------------------|-----|
| 1-MK886768, Black Sea, Turkey | 0.007 |
| 2-MF000685, Mediterranean Sea | 0.007 |
| 3-MF000686, Mediterranean Sea | 0.007 |
| 4-MF000687, Mediterranean Sea | 0.009 |
| 5-MF000688, Mediterranean Sea | 0.004 |
| 6-MF000689, Mediterranean Sea | 0.007 |
| 7-MF000690, Mediterranean Sea | 0.007 |
| 8-MF000691, Mediterranean Sea | 0.009 |
| 9-MF140344, Chinese waters | 0.021 |

**Figure 1.** Alignment of the rrnS sequences of the *H. aduncum* larvae isolated from Black Sea with respect to the *H. aduncum* have previously been sequenced and deposited in GenBank under the accession numbers. The alignment was performed using BioEdit. Dots indicate identity with the first sequence and dashes are inferred insertion–deletion events.
Discussion

Morphologic identification of larval stage of anisakid and raphidascaridid nematodes is extremely difficult due to the existence of sibling or cryptic species. Therefore, molecular genetic techniques are more reliable for a proper species identification of larval and adult anisakid and raphidascaridid nematodes [12, 28, 18-20, 22, 24-26]. Until now, morphological and molecular data on species of Hysterothylacium genus infecting marine fish from Turkish waters have been still not sufficient. To date, only three Hysterothylacium species have been molecularly reported from different marine fish species from Turkish waters. Hysterothylacium aduncum, H. fabri and H. reliquens species were genetically characterized based on DNA sequencing of ITS regions and the mitochondrial cox1 and cox2 genes from Turkish waters [11, 18, 20, 25]. Moreover, in the present study, larvae of H. aduncum infecting M. merlangus euxinus caught off the Black Sea, Turkey were characterized for the first time by sequencing of the mitochondrial rrnS gene. Phylogenetic analysis revealed that our H. aduncum isolate clustered with others known H. aduncum sequences in a monophyletic clade in rrnS tree (Fig. 1). Furthermore, genetic distance analyses for rrnS gene also revealed a very low intraspecific genetic distance between the obtained H. aduncum isolate (MK886768) and other H. aduncum isolates previously reported (MF000685-MF000691) from the Mediterranean Sea (p distance=0.004 to 0.009) (Table 1). Low intraspecific genetic variability among H. aduncum specimens has also previously been detected in the ITS sequences [2, 9, 12, 14, 18]. Whereas a low level of intraspecific nucleotide difference among the present isolate (MK886768) and others H. aduncum isolates (MF000685-MF000691) from the Mediterranean Sea in the rrnS sequence, H. aduncum only should be considered as a single species. Nevertheless, high intraspecific genetic diversity was detected among our isolate (MK886768) and H. aduncum from the Chinese waters (MF140344) in the rrnS gene (p distance=0.021) (Table 1). In addition to, geographically distant population of the same Hysterothylacium species may be cause intraspecific genetic differences.

Conclusion

In the current study, the mitochondrial rrnS gene sequences of H. aduncum from the Black Sea are determined for the first time. Sequence analysis of the rrnS gene provided a useful approach for the spe-
cific identification of H. aduncum. Moreover, these valid genetic data of H. aduncum (MF140344) can be used to establish the phylogenetic relationships with Hysterothylacium species from the Black Sea and various geographical areas. Further researches using the different genetic markers are required to examine the genetic variability and population’s genetic structure within larvae and adult stage of Hysterothylacium species from the Turkish waters.

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