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. adumcum* 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

Türkiye'nin Karadeniz Kıyılarındaki *Merlangius merlangus euxinus*'u (Linnaeus, 1758) Enfekte Eden *Hysterothylacium aduncum* (Nematoda: Raphidascarididae) Larvasının Küçük Alt Ünite Ribozomal RNA Gen Bölgesine Göre Moleküler Karakterizasyonu

Özet: Türkiye sularında *Hysterothylacium* cinsinin taksonomisi eksik ve belirsizdir. Bu araştırmada Türkiye'nin Karadeniz kıyılarında avlanan *Merlangius merlangus euxinus*'da *H. aduncum* larvaları morfolojik olarak teşhis edildi. *H. aduncum*'un nükleer ribozomal ITS (ITS-1, 5.8S subunit, ITS-2) ve küçük alt ünite mitokondriyal ribozomal RNA (rrnS) gen bölgeleri çoğaltıldı ve sekanslandı. *H. aduncum*'un ITS sekanslarının BLAST analizleri daha önce Türkiye'nin Karadeniz kıyılarından rapor edilen *H. aduncum*'un referans dizisiyle (erişim numarası JX413596) identik olduğunu gösterdi. Türkiye Karadeniz'den *H. aduncum* rrnS geni (MK886768), Akdeniz (MF000685-MF000691) ile Çin sularındaki (MF140344) bulunan *H. aduncum* izolatları ile %97.94-99.56 arasında benzerlik gösterdi. Bununla birlikte, Türkiye Karadeniz'den elde edilen *H. aduncum*'un rrnS dizisi ile Akdeniz (MF000685-MF000691) ve Çin sularındaki (MF140344) diğer *H. aduncum* izolatlarının ikili karşılaştırmasında %0,2 ile %1,7 arasında farklılık gözlendi. Bu çalışma ile ilk kez Karadeniz'den *H. aduncum*'un mitokondriyal rrnS geni moleküler olarak karakterize edildi.

Anahtar kelimeler: *Hysterothylacium aduncum*, moleküler karakterizasyon, mitokondriyal rrnS gen, Karadeniz, Türkiye

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

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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 GGA TCA TT-3′) and NC2 (5′-TTA GTT 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 CGG CTA GAC 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 Mayıs 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

	1	2	3	4	5	6	7	8	9
1-MK886768, Black Sea, Turkey									
2-MF000685, Mediterranean Sea	0.007								
3-MF000686, Mediterranean Sea	0.007	0.004							
4-MF000687, Mediterranean Sea	0.009	0.007	0.007						
5-MF000688, Mediterranean Sea	0.004	0.002	0.002	0.004					
6-MF000689, Mediterranean Sea	0.007	0.004	0.004	0.007	0.002				
7-MF000690, Mediterranean Sea	0.007	0.004	0.004	0.007	0.002	0.004			
8-MF000691, Mediterranean Sea	0.009	0.007	0.007	0.009	0.004	0.007	0.007		
9-MF140344, Chinese waters	0.021	0.017	0.017	0.020	0.015	0.017	0.017	0.015	

Figure 1. Alignment of the rrnS sequences of the *H. aduncum* larvae isolated from Black Sea with respect to the *H.adumcum* 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.

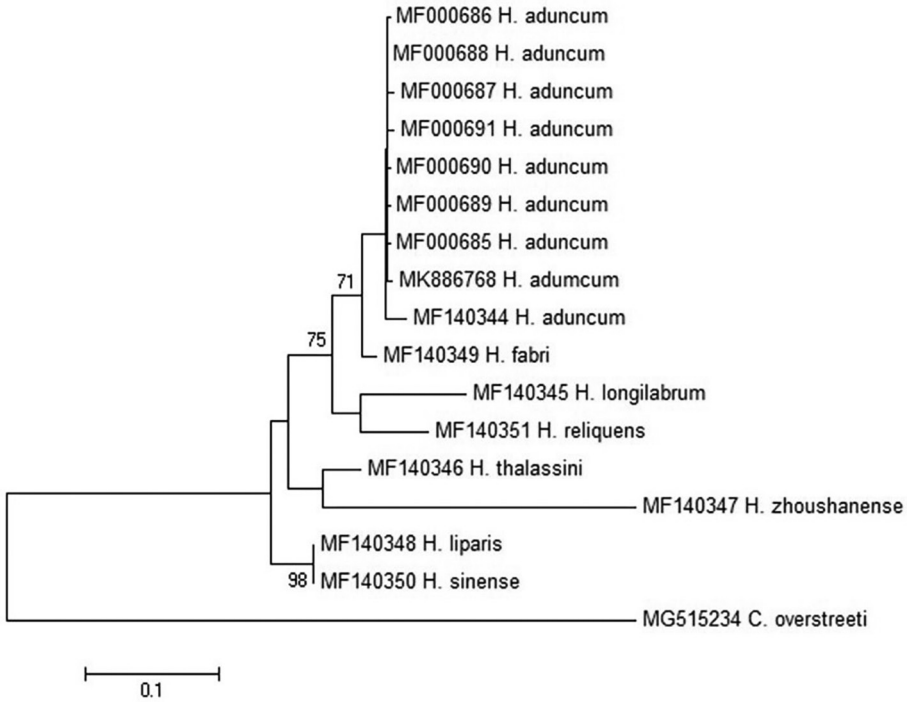

Figure 2. Phylogenetic relationships between *H. aduncum* (MK886768) from the present study and other *H. aduncum* as inferred by maximum likelihood obtained from rrnS gene. The scale bar indicates the distance in substitutions per nucleotide. Bootstrap values were calculated over 100 replicates and percentages ≥70% are shown at the internal nodes. *C. overstreeti* was used as out group

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. adumcum* 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 additional to, geographically distant population of the same *Hysterothylacium* species may be cause intraspecific genetic diffrences.

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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