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Research Article

Identification and Molecular Characterization of *Hysterothylacium* (Nematoda: Raphidascarididae) Larvae in Bogue *(Boops boops* L.) from the Aegean Sea, Turkey [1]

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Abstract

Hysterothylacium species are the most prevalent ascaridoid nematode reported from various freshwater, estuarial and marine fish species. However, there are very few studies about molecular identification and characterization of *Hysterothylacium* species in marine fish species from Turkish waters. The aim of the present study was to molecular identification and characterization of Hysterothylacium larvae from *Boops boops* caught off Aegean Sea, Turkey. *Hysterothylacium* larvae were found in 10 of 109 *B. boops.* The prevalence of *Hysterothylacium* spp. larvae was 9.2%. A total of 10 larvae of *Hysterothylacium* spp. were collected from all infected fish. *Hysterothylacium* larvae were genetically identified as *H. aduncum, H. fabri* and *H. reliquens* from Turkish waters by using sequence analyses of rDNA ITS regions in *B. boops* for the first time. In addition, *B. boops* was reported as a new host for *H. reliquens.* Moreover, *H. reliquens* was characterized for the first time by sequencing of the ITS regions from the Turkish waters with the present study.

Keywords: *Hysterothylacium larvae, Boops boops, Molecular identification, rDNA ITS, Aegean Sea, Turkey*

Türkiye'nin Ege Denizi'nden Yakalanan Kupes *(Boops boops)* Balıklarında *Hysterothylacium* Larvalarının İdentifikasyonu ve Moleküler Karakterizasyonu

Öz

Hysterothylacium türleri çeşitli tatlı, acı su ve deniz balığı türlerinden bildirilen en yaygın ascaridoid nematodlardır. Ancak Türkiye sularındaki deniz balıklarında *Hysterothylacium* türlerinin moleküler tanımlanması ve karakterizasyonu konusunda çok az çalışma bulunmaktadır. Bu çalışmada Türkiye'nin Ege Denizi'nden yakalanan *Boops boops*'larda *Hysterothylacium* larvalarının moleküler tanımlanması ve karakterizasyonlarının belirlenmesi amaçlanmıştır. *Hysterothylacium* larvaları 109 *B. boops*'un 10'unda tespit edilmiş olup enfeksiyon oranı %9.2 olarak belirlenmiştir. Tüm enfekte balıklardan toplamda 10 adet *Hysterothylacium* larvası toplanmıştır. Türkiye sularından *B. boops*'larda ilk defa *Hysterothylacium* larvalarından rDNA ITS gen bölgesinin dizi analizleri ile *H. aduncum, H. fabri* ve *H. reliquens* türleri genetik olarak tanımlanmıştır. Ek olarak *B. boops* türü *H. reliquens* için yeni bir konak olarak rapor edilmiştir. Ayrıca, *H. reliquens* ITS gen bölgesinin sekans analizi ile Türkiye sularından ilk kez karakterize edilmiştir.

Anahtar sözcükler: *Hysterothylacium larva, Boops boops, Moleküler tanımlama, rDNA ITS, Ege Denizi, Türkiye*

INTRODUCTION

Hysterothylacium species belonging to the Raphidascarididae family are the most cosmopolitan marine ascaridoid reported as larvae in various fish species [1,2]. At present, there are approximately 70 recognizable *Hysterothylacium* species around the world [3]; however, only two species, *H. aduncum* and *H. fabri*, have been morphologically or

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molecularly described in different fish species from Turkish waters [4-10].

The bogue, *B. boops* is a demersal to semi-pelagic, non-migratory and economically important species of the Mediterranean Sea. The species is prevalent in the Eastern Atlantic, Mediterranean and Black Sea. It is gregarious, and it is found on the shelf or coastal pelagic on different bottoms at a depth range 0-350 m. *B. boops* is omnivorous, feeding mainly on benthic copepods and plants but is also planktonophagous (www.fishbase.org, version 06/2017). *Hysterothylacium* species have been already reported in *B. boops* from different regions of the world [6,11,12].

Morphologic definitions of species in Anisakidae and Raphidascarididae are still complicated and limited. Because of the similarity between the organs morphology and the existence of sibling or cryptic species of ascaridoid larvae, it is often difficult to morphologically identify the *Hysterothylacium* larvae at species level. The absence of many distinctive morphological features and lack of systematic and biological data on *Hysterothylacium* species, are the other important factors limiting the definitive species identification [13-17]. Therefore, genera of *Contracaecum* and *Hysterothylacium* had been often confused in the past [14]. Recently, using PCR and sequencing of ITS (ITS-1 and ITS-2) and 5.8S region have overcome above mentioned problems and these techniques have become absolutely necessary for the accurate or precise identification of ascaridoid nematodes [7,8,15,18-22].

Molecular data on *Hysterothylacium* genus infecting fishes from Turkish waters is still not sufficient. Therefore, with the present study, it has been aimed to specifically determine the existence of different *Hysterothylacium* species in the bogue, *B. boops* (L.) from Turkish waters, and to reveal the genetic characterization of the isolates belonging to determined species.

MATERIAL and METHODS

Fish Collection and Parasitological Examination

Parasitological examinations of *Hysterothylacium* larvae were performed on *B. boops*. A total of fresh and dead 109 samples were purchased from local fishermen between October 2016 and February 2017 caught from the Aegean Sea, Turkey (FAO zone 37.3.1). Fishes were dissected under stereomicroscope (Olympus SZX10 Tokyo, Japan) and examined for the presence of *Hysterothylacium* larvae. Nematodes were recovered from the digestive tract of *B. boops* and then washed in physiological saline and placed in 70% ethanol for molecular investigation. The morphological identification of the collected larvae specimens as *Hysterothylacium* spp. was performed according to the position of the excretory pore, the digestive systems and morphology of the tail [2,23].

PCR Amplification and Sequencing of DNA

All *Hysterothylacium* larvae from *B. boops* were analysed by molecular methods. Genomic DNA (gDNA) was isolated individually by using DNA purification kit (GeneJET Genomic DNA Purification Kit, Thermo Scientific, Waltham, MA, USA) following to the manufacturer's instructions. PCR was conducted to amplify the ITS regions using the NC5/NC2 primers [18] and cycling conditions were modified as follows: at 95°C (5 min), then 30 cycles of at 95°C (1 min), at 55°C (1 min) and at 72°C (1 min) followed by a final extension step at 72°C (5 min). Amplicons were checked on SafeView™-stained 1.5% agarose gel (Applied Biological Materials, Richmond, BC, Canada) and visualized by UV illumination (Quantum CX5, Vilber Lourmat, France). Positive PCR products were purified by using the commercial kit (High Pure PCR Product Purification Kit, Roche, Germany) and sequenced in both directions with using the same primers (NC5/NC2), (Macrogen, Amsterdam, The Netherlands).

Phylogenetic Analyses

Sequences were assembled and edited by Geneious 11.0.2 [24]. Nucleotide sequences were aligned with formerly submitted sequences of *Hysterothylacium* species in GenBank to make species-based identification using the BLASTn algorithm. Genetic distances were analyzed by using the Kimura two-parameter model (Kimura, 1980) with pairwise deletion in Mega 6.0 [25]. The aligned sequences were tested with Mega 6.0 model test to determine the most suitable DNA model according to the correct Akaike's Information Criterion (AIC) to infer the phylogenetic trees [25]. Phylogenetic analysis was conducted by using Maximum-Likelihood (ML) analysis based on Hasegawa-Kishino-Yano (HKY) +G model in PhyML [26] over the South of France Bioinformatics Platform (http://www.atgc-montpellier.fr/phyml/) with 1000 bootstrap replicates [27]. *Raphidascaris acus* (ERURacus) was used as an out group.

RESULTS

Hysterothylacium larvae were isolated from 10 *B. boops* among the total of 109 specimens with a mean prevalence of 9.2%. A total of 10 larvae belonging to *Hysterothylacium* spp. were collected from all infected fish. Each infected fish specimen carried one larva in their digestive tract. gDNAs from all collected larvae were subjected to the PCR analyses. gDNAs from the six larvae showed amplification on agarose gel electrophoresis while the gDNAs from the remaining four larvae were negative in PCR probably due to primer mismatches especially in the 3' end. The six amplified PCR products were sent to DNA sequencing for identification of *Hysterothylacium* species. The BLASTn analysis revealed the presence of three *Hysterothylacium* species. Four larvae were identified as *H. reliquens*. In addition, one was identified as *H. aduncum* and the other

was *H. fabri*. The ITS sequences of *H. reliquens*, *H. aduncum* and *H. fabri* were deposited in GenBank with the accession numbers of MF062506-09, MF062510 and MF062511, respectively.

Phylogenetic tree clearly indicated that all observed *Hysterothylacium* isolates clustered together with same species in monophyletic groups supporting of high bootstrap values over 92% *(Fig. 1)*. *H. aduncum* ERU-H. adun isolate (MF062510) was clustered together with the different geographical isolates of *H. aduncum*. ERU-H. adun isolate from the Aegean Sea, Turkey showed 100% similarity with the isolates of *H. aduncum* reported from Turkey (JX413596-97), Croatia (JQ934882-83), Denmark (JX845135-KU306719), Greenland (KT852549) in GenBank, while it showed 99.2% to 99.5% similarity with some other isolates reported from Eastern Mediterranean Sea, Turkey (KJ748530-31-32). *H. fabri* ERU-H.fab isolate determined as 100% similar with MS003, MS023, MS036 recovered from Italy (KU948632-35-36) and it showed 99.1% similarity with

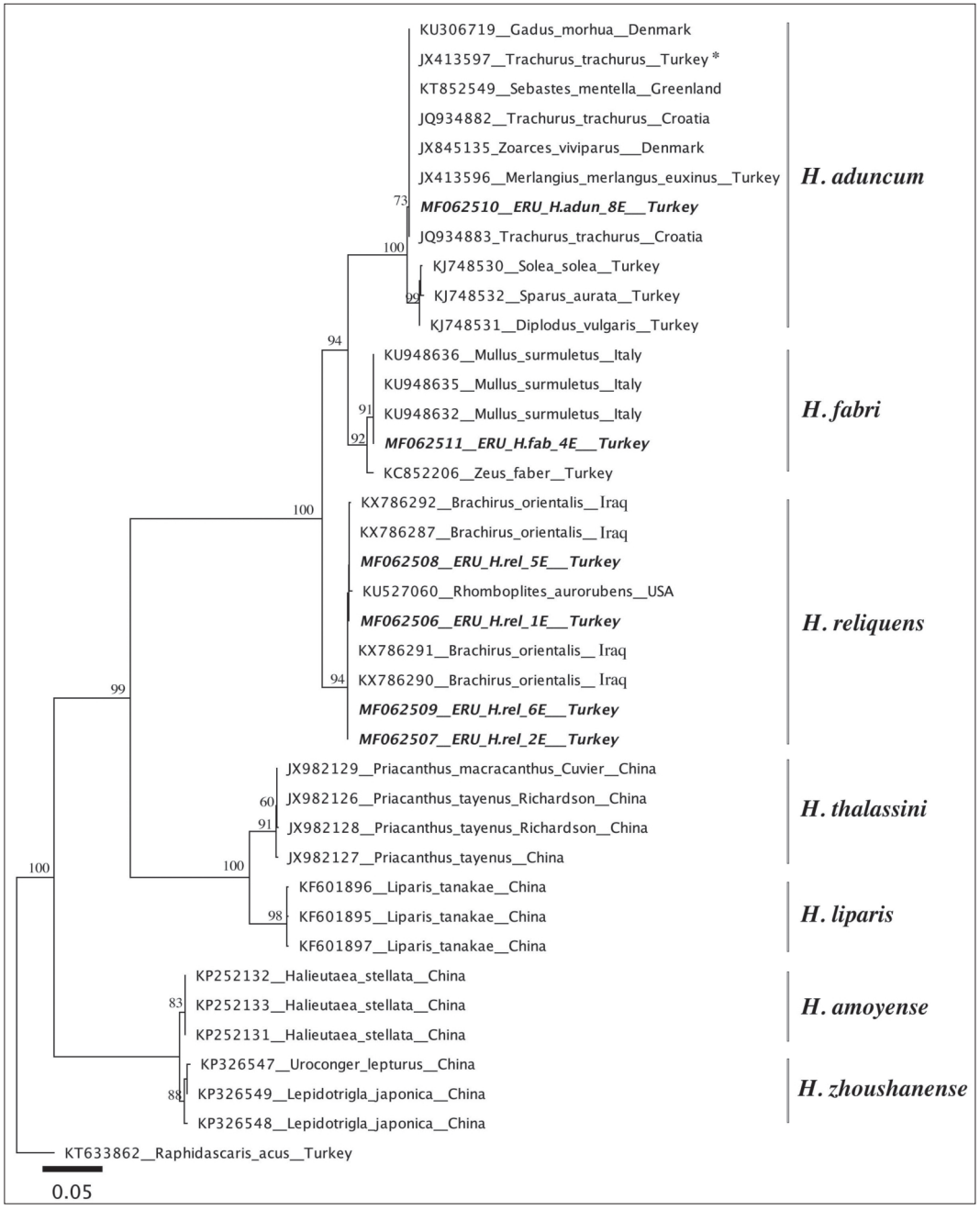

Fig 1. Phylogenetic relationships between *H. aduncum, H. fabri, H. reliquens* and other previously recorded species of *Hysterothylacium* in GenBank as inferred by maximum-likelihood (ML) analysis of ITS regions and 5.8S. *Raphidascaris acus* was used as outgroup taxa. The isolates were given with GenBank accession numbers, hosts and countries. The isolates obtained with the study were shown as bold italic character and other isolates from Turkey were labelled with asterisk. The scale bar represents 0.05% divergence

a recorded isolate (KC852206) from Mediterranean Sea, Turkey. Moreover, the ITS gene sequences of *H. reliquens* ERU-H.rel (MF062506-09) have been obtained for the first time from Turkish waters and no intraspecific nucleotide diversity was observed in the ITS region. ERU-H.rel isolates also showed 99.9% to 100% identity with Arabian Gulf off Basrah, Southern Iraq (KX786287-90-91-92) and Gulf of Mexico, USA (KU527060) isolates. Interspecific genetic differences were determined as 3.8% to 27.2% among the species indicated in *Fig. 1*. Pairwise genetic distance between ERU-H.adun (MF062510) and Sa/Dv/Ss isolates (KJ748530-31-32) from the Eastern Mediterranean Sea, Turkey displayed variation ranged from 0.5% to 0.8%. Similarly, ERU-H.fab isolate (MF062511) indicated 0.9% genetic differences isolate registered in GenBank from Mediterranean Sea, Turkey (KC852206). Pairwise comparison between the *H. reliquens* (ERU-H.rel) and our other species *H. fabri* and *H. aduncum* (ERU-H.fab and ERU-H.adun) displayed 4.3-6.2% interspecific nucleotide differences, respectively.

DISCUSSION

There has been limited knowledge on the *Hysterothylacium* infections in fishes from Turkish waters. To date, only two *Hysterothylacium* species have been morphologically and molecularly reported from different fish species from Turkish waters. *H. aduncum* was found in *Merlangius merlangus euxinus, Trachurus trachurus, Gadus* sp. *Oncorhynchus mykiss, Sparus aurata, Solea solea, Diplodus vulgaris* [4-7,9,10,28-31]. *H. fabri* was identified from *Phycis phycis, Alosa fallax, Coris julis, Trachinus draco, Mullus surmeletus, B. boops, T. mediterraneus, Pagellus acerna, Squalus blainvillei, Symphodus* sp. and *Diplodus annularis* (as *Contracaecum fabri*) [6] and *Zeus faber* [8]. However, there are no molecular studies that confirm the distribution of the *H. reliquens* larvae of *B. boops* in Turkey. In the present study, larvae of *H. reliquens* infecting *B. boops* caught off the Aegean Sea, Turkey were characterized for the first time by sequencing of the rDNA ITS and *H. reliquens* is the first record for the Turkish fish parasite fauna. Moreover, *B. boops* is the new host record for larvae of *H. reliquens*.

In the present study, two previously known *Hysterothylacium* species were characterized by molecular approaches namely *H. aduncum* and *H. fabri*. The obtained sequence from *H. aduncum* (ERU-H.adun) isolate demonstrated 100% identity with previously submitted data in GenBank from (JX413596-JX413597), Croatia (JQ934882-JQ934883), Denmark (JX845135), Greenland (KT852549), Denmark (KU306719). However, ERU-H.adun isolate displayed ITS sequence variation with Sa/Dv/Ss (KJ748530-31-32) isolated from the Eastern Mediterranean Sea, Turkey (range from 0.5 to 0.8%). Similarly, *H. fabri* (ERU-H.fab) isolate showed 100% identity with MS003, MS023, MS036 isolate from Italy (KU948632-35-36), while this isolate indicated 0.9% nucleotide differences with recorded isolate from Mediterranean Sea, Turkey (KC852206). Species belonging to the family of Anisakidae and Raphidascarididae have low host specificity and large intermediate/definitive host populations. This situation can cause spread across a wide area of the world and genetic similarities to be high in these species, while host variability and minimal environmental changes may cause intraspecific genetic differences [9,22,32-34]. *H. reliquens* (ERU-H.rel) isolate showed 99.9% to 100% identity with isolates reported from Arabian Gulf of southern Iraq (KX786287-90-91-92) and Gulf of Mexico, USA (KU527060) isolates. Our isolates were compared with two isolates which only registered in GenBank based on the entire ITS fragment including (ITS-1 and ITS-2) and 5.8S sequences.

The phylogenetic analyses of the ribosomal ITS and 5.8S sequence data set indicated the monophyletic future of the all examined *Hysterothylacium* species with high bootstrap values. Genetic distance analyses also revealed no or low intraspecific genetic distance among the isolates from all examined *Hysterothylacium* species including the isolates obtained in this study. However, Pontaja et al.[17] reported insufficiency of nuclear genomic regions such as rDNA ITS1 and ITS2 in the discrimination of possible interspecific patterns of some *Hysterothylacium* larval types. Our result on *H. reliquens* indicated no intraspecific genetic distance among the obtained isolates with 100% identity to each other. The corresponding isolates also exhibited very low genetic distance to the *H. reliquens* isolates from different countries. This could be attributed to the spreading of the species belonging to the family of Anisakidae and Raphidascarididae across a wide area around the world which might lead high genetic similarities within species. On the other hand, host variability and minimal environmental changes may cause intraspecific genetic differences [9,22,32-34]. In accordance to this inference, phylogenetic tree clearly showed that the *Hysterothylacium* species identified in different fish species *(S. solea*, *S. aurata* and *D. vulgaris)* caught from different waters of Turkey [9] were grouped under different cluster and they exhibited a much more genetic difference to our ERU-H.adun isolate and some other isolates from different countries although they clustered into a monophyletic clade. We also concluded that genetic characterization based nuclear and mitochondrial gene regions with sufficient phylogenetic signal should be conducted to obtain the true identification of the same larval stages of *Hysterothylacium* species from similar geographical areas and to explore the species diversity. In accordance to this inference Shamsi et al.[16] also indicated that, even in the genetic characterization based on the ITS-1 and ITS-2 sequences of the same larval stage, some morphotypes could contain different genotypes.

In conclusion, species of *Hysterothylacium* that are difficult to describe morphologically have been genetically characterized, and three species *(H. aduncum, H. fabri,*

H. reliquens) have been identified in the present study. Findings on *H. reliquens* infecting *B. boops* have been original data contributing the Turkish fish parasite fauna and molecular epidemiology of this parasite. *B. boops* was also characterized as a new host for *H. reliquens* with this study although it has been reported from 25 fish species belonging to eight different orders [22]. Further researches using different host species from various geographical areas are necessary to understand population genetic structure of *Hysterothylacium* species from Turkish waters.

Conflict of Interest

The authors do not have any potential conflicts of interest to declare.

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