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Research Article

First Molecular Characterization of *Raphidascaris acus* Bloch, 1779 (Nematoda: Anisakidae) from European eels *(Anguilla anguilla* Linnaeus, 1758) Caught off the Aegean Region Streams, Turkey [1]

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Abstract

In this study, the presence of anisakid nematodes in the European eels *(Anguilla anguilla* L.) was investigated. A total of 30 specimens of eels were caught by local fishermen from the Buyuk Menderes River, Turkey. Nematoda species of *Raphidascaris acus* was found from eels. This is the first record of *R. acus* from *A. anguilla* from Turkish waters. The ribosomal DNA (rDNA) internal transcribed spacer regions (ITS-1and ITS-2) and 5.8S of this parasitic species was amplified and sequenced. Also, all the nematodes were identified as *R. acus* based on nucleotide sequence comparisons. Pairwise comparison between the entire ITS regions and 5.8S of the *R. acus* isolates of *A. anguilla* (GenBank accession number: KT633862) and other *R. acus* isolates from Caspian Sea (KM047505), and Vistula Lagoon, Poland (AY603537) showed differences ranging from 0.0 to 1.9% intraspecific nucleotide differences, respectively. With the present study, *R. acus* infecting *A. anguilla* caught off the Aegean Region streams were characterized for the first time by sequencing of the rDNA ITS regions and 5.8S.

Keywords: *Raphidascaris acus, Anguilla anguilla, ITS gene regions, 5.8S, Aegean Region streams, Molecular characterization*

Ege Bölgesi Akarsularında Yakalanan Avrupa Yılan Balıklarında *(Anguilla anguilla* Linnaeus, 1758) *Raphidascaris acus* Bloch, 1779 (Nematoda: Anisakidae)'un İlk Moleküler Karakterizasyonu

Özet

Bu çalışmada balıkçılar tarafından Büyük Menderes Nehri'nden yakalanan 30 adet Avrupa yılan balığında *(Anguilla anguilla* L.) anisakid nematodların varlığı araştırılmıştır. Yılan balıklarında *Raphidascaris acus* türü nematoda rastlanmıştır. *Raphidascaris acus* Türkiye sularında yakalanan yılan balıklarında ilk kez bildirilmiştir. İdentifiye edilen parazitlerin internal transcribed spacer gen bölgeleri (ITS-1 ve ITS-2) ve 5.8S rDNA'sı amplifiye edilerek sekans analizleri gerçekleştirilmiştir. Nükleotid sekans karşılaştırmaları sonucu bu nematodların *R. acus* olduğu doğrulanmıştır. Yılan balığında tespit edilen *R. acus* izolatının (GenBank erişim numarası: KT633862) ITS gen bölgeleri (ITS-1 ve ITS-2) ve 5.8S bölgesinin ikili hizalamaları sonucunda Hazar Denizi (KM047505) ve Polonya, Vistula Lagünü'nden (AY603537) izole edilmiş *R. acus* izolatları ile arasında sırasıyla %0.0 ve 1.9 oranlarında tür içi nükleotit farklılığı saptanmıştır. Bu çalışmada Ege Bölgesi akarsularında sularında yakalanan yılan balıklarında *(A. anguilla)* saptanan *R. acus*'un ITS gen bölgeleri (ITS-1 ve ITS-2) ve 5.8S rDNA'sı sekanslanarak ilk kez moleküler karakterizasyonu yapılmıştır.

Anahtar sözcükler: *Raphidascaris acus, Anguilla anguilla, ITS gen bölgeleri, 5.8S, Ege Bölgesi akarsuları, Moleküler karakterizasyon*

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INTRODUCTION

Anisakid nematodes of the genus *Raphidascaris* (Railliet & Henry, 1915) are parasites of the digestive tract of a range of marine, brackish and freshwater fishes in world-wide. *Raphidascaris acus* is a cosmopolitan species reported from different host species [1].

Raphidascaris acus has three well developed lips that mouth surrounded. However, the interlabia are rudimentary or absent. Nerve ring is encircling approximately at border of first and second thirds of oesophagus. Excretory pore is located at behind level of nerve ring. The intestinal caecum is absent. *Raphidascaris acus* has various genital papillae. The tail of both sexes is short and conical [2].

Raphidascaris acus has been morphologically identified from Turkish waters [3,4]. However, to date there has not been any study regarding molecular characterization of *R. acus* from fish caught off the Turkish waters. Recently, molecular techniques, using DNA sequencing of the nuclear ribosomal DNA spacers, have been proven to be particularly useful for the accurate identification of ascaridoid nematodes at the species level for eggs, larvae, and adults [5-12]. Nevertheless, before the present study, there had been no reports of characterizing the *R. acus* from the Turkish waters using well-defined internal transcribed spacers (ITS1 and ITS2) and 5.8S region sequence.

Therefore, in the present study, *R. acus* from Aegean Region streams were genetically characterized for the first time by sequencing of ITS regions and 5.8S subunit markers.

MATERIAL and METHODS

Sampling and Parasitological Examination

A total of 30 specimens of *Anguilla anguilla* were caught by local fishermen from the Buyuk Menderes River in Aydın vicinity of Turkey. Fishes were dissected carefully and examined for nematodes in the stomach, intestine, abdominal cavity, and muscles. Nematodes were only found from the intestine and washed in physiological saline. For each nematode, a small piece of the mid-body was cut and stored in 70% ethanol for molecular analyses and anterior-posterior ends of specimens were cleared in lactophenol for morphological studies. The parasites were identified by using the morphology of the labia, the position of the excretory pore, ventricular appendix and the tail [1].

DNA Extraction, PCR Amplification, and Sequencing

Three nematodes (i.e., three individuals were randomly selected among the samples) were subjected to the molecular analysis. Genomic DNA (gDNA) was extracted from the nematodes using the DNA purification kit (Wizard Genomic, Promega) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed to amplify the ITS regions (ITS-1and ITS-2) and 5.8S. PCR reaction (50 µl) was contained 10-50 ng of extracted DNA, 1× TaqBuffer with KCl (Thermo Scientific), 3mM of $MgCl_2$ (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 ITS regions and 5.8S were amplified using the primers NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (5′-TTA GTTTCTTTTCCTCCGCT-3′) [5]. The PCR was performed in an automated thermocycler (Applied Biosystems) and the conditions were modified 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 extension step at 5 min at 72°C. PCR products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide and visualized by UV illumination. Then the positive samples were purified by the commercial kit (High Pure PCR product purification kit, Roche) from the agarose gel. The purified products were commercially sequenced by Macrogen (Netherlands) in both directions, using NC5 and NC2 primers.

Data Analysis and Phylogenetic Tree Construction

The forward and reverse nucleotide sequences were assembled and edited with using Contig Express (Vector Nti® Advance 11.5, Invitrogen, Carlsbad, California, USA). A standard nucleotide Basic Local Alignment Search Tool (BLAST [blastn]) search was conducted [13]. Nucleotide sequences were aligned with the software CLUSTAL W in Mega 6.0 multiple sequence alignments [14]. Genetic distances were calculated using the Kimura two-parameter model with pairwise deletion in Mega 6.0 [15]. Phylogenetic analysis with other known *Raphidascaris* species was conducted using Maximum-Likelihood (ML) analysis in Mega 6.0 [15]. The aligned sequences were tested with Mega 6.0 model test to find the best DNA model to infer the phylogenetic trees [15]. The general-time reversible model (GTR+G) was selected using Akaine Information Criterion (AIC). The evolutionary history was inferred using the ML method based on the GTR+G model for ITS sequences with *Eustrongylides* sp. as an out group. Confidence in the ML trees was determined by analyzing 1.000 bootstrap replicates [16] using the Mega 6.0 program. The sequences of ITS regions and 5.8S of *R. acus* has been deposited in GenBank databases under accession number KT633862.

RESULTS

These species were analyzed in the present paper clearly belongs to the genus *Raphidascaris*. The morphology of our specimens agrees well with the description of *R. acus* [1]. The amplification of the ITS regions and 5.8S was produced a fragment of approximately 1.000 bp from each nematodes. No intraspecific differences were found

in the sequences of *R. acus,* which represented a single genotype. Therefore, only one sequence was submitted to GenBank. The length of ITS sequences of *R. acus* was 866 bp. *Raphidascaris acus* isolates from the Menderes River, Turkey (KT633862) showed 98.1 to 100% identity with various geographical isolates of *R. acus* from the Vistula Lagoon, Poland (AY603537) and Caspian Sea (KM047505) from GenBank, respectively. Pairwise comparison between the present data and other *R. acus* isolates from the Caspian waters (KM047505) and the Poland (AY603537) displayed only 0.0 to 1.9% intraspecific nucleotide differences *(Table 1)*, respectively. Phylogenetic relationships among *R. acus* isolates from *A. anguilla* of the Menderes River, Turkey and the other *Raphidascaris* species isolates as inferred by ML analysis of the ITS sequence are presented in *Fig. 1*.

DISCUSSION

The genus *Raphidascaris* Railliet & Henry, 1915 consists of three subgenera namely *Ichthyascaris* Wu, 1949, *Sprentascaris* Petter & Cassone, 1984, and *Raphidascaris* Railliet & Henry, 1915 [17-19]. The present material is assigned to *Raphidascaris* due to the lips well-developed, excretory pore slightly behind the nerve-ring, posterior directed ventricular appendix, and intestinal caecum absent [1]. *Raphidascaris acus* is found in different freshwater fishes (Esocidae, Salmonidae, Anguillidae, Gadidae) in Europe, Asia and North America [1]. Until now, only one species, *R. acus* has been morphologically identified from Esocidae *(Esox lucius* L.) from Turkish waters [3,4]. To the best of our knowledge, this is the first record of *R. acus* from Anguillidae *(A. anguilla*) from Turkish waters.

Accurate identification of a parasite at any stage of its development has important implications for studying parasite epidemiology and resolving taxonomic problems [20]. Different studies have demonstrated that the ITS regions and 5.8S provide useful genetic markers for the accurate identification of sibling species and morphospecies within ascaridoid species [5,10-12,21].

There are three *Raphidascaris* species [*R. lophii* (Wu, 1949), *R. trichiuri* (Yin & Zhang, 1983), and *R. longispicula* (Li et al., 2012)] belongs to the subgenus *Ichthyascaris* Wu, 1949 [22-24] and two species [*R. acus* (Bloch, 1779) Railliet

Table 1. Pairwise comparison of nucleotide sequence differences (%) in the ITS gene regions and 5.8S among *Raphidascaris acus* (Turkey) isolates and various geographical isolates

Tablo 1. *Raphidascaris acus* izolatı (Türkiye) ile değişik coğrafik bölgelere ait izolatların ITS gen bölgeleri ve 5.8S nükleotit sekans farklılıklarının (%) ikili karşılaştırmaları

Isolate No	GenBank Accession Numbers and Locations	Nucleotide Sequence Differences (%)		
		1	2	3
1	KT633862 Menderes River, Turkey			
2	KM047505 Caspian Sea	0.0		
3	AY603537 Vistula Lagoon, Poland	1.9	1.9	

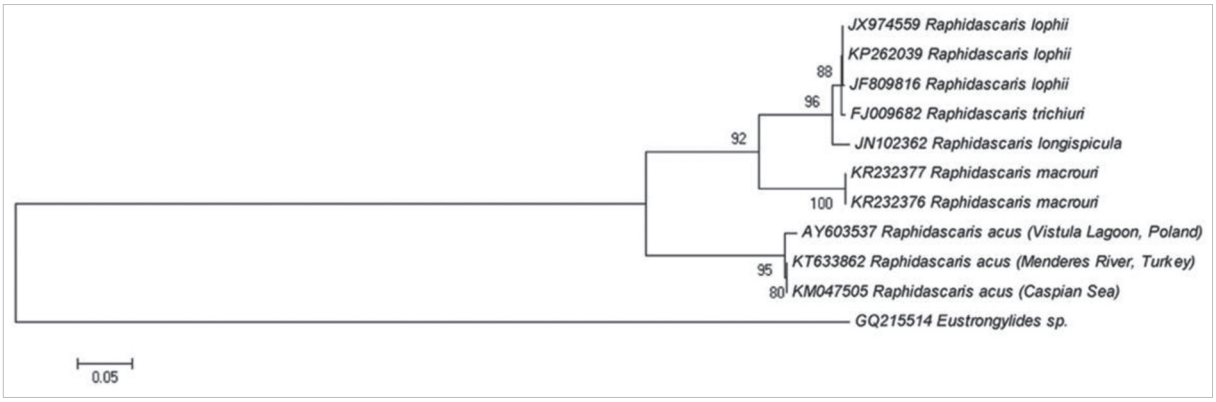

Fig 1. Phylogenetic tree reconstructed using Maximum-Likelihood (ML) analysis of ITS regions and 5.8S. The evolutionary history was inferred using the ML method based on the GTR+G model for ITS sequences with *Eustrongylides* sp. as an out group. The accession numbers of individual sequences determined in the present study are shown in each tree. A scale bar indicates estimated distance. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1.000 replicates) are shown at the internal nodes (>70% only)

Şekil 1. ITS gen bölgeleri ve 5.8S'in filogenetik ağacı Maximum-Likelihood (ML) analiz yöntemi kullanılarak oluşturulmuştur. ITS dizilimlerinin evrimsel sürecine *Eustrongylides* sp.'nin dış grup olarak kullanılması ile GTR+G modelini temel alan ML analiz yöntemi kullanılarak varılmıştır. Bu çalışmada belirlenen sekansların erişim numaraları ağaçta gösterilmiştir. Ölçek çubuğu tahmini uzaklıkları göstermektedir. Bootstrap testinde birbirleri ile ilişkili taksonların filogenetik ağaçtaki tekrar yüzdeleri ağacın iç düğümlerinde gösterilmiştir (>%70)

& Henry, 1915, *R. macrouri* (Pérez-i-García et al., 2015)] belongs to the subgenus *Raphidascaris* Railliet & Henry, 1915 studied of the ITS gene region [10,19].

This study provides the first molecular characterization of *R. acus* from the Turkish waters. *Raphidascaris acus* isolate of Turkey (KT633862) showed 100% identity with isolate of Caspian Sea (KM047505) from GenBank. Nonetheless, the ITS sequence variation between the Turkish and Polish populations was 1.9%. A significant ITS sequence variation between the two populations is evidence on the lacking gene flow between the Turkish waters and Vistula Lagoon, Poland. In this study, bootstrapping of the sequences with ML revealed significant support for one clade containing *R. acus* isolates from the Turkey (KT633862), Caspian waters (KM047505) and the Vistula Lagoon, Poland (AY603537), revealing a close relationship between these isolates *(Fig. 1)*.

As conclusions, the ITS gene sequences of *R. acus* from Turkish waters have been obtained for the first time in the present study, and further researches using more polymorphic genetic markers are required to examine the genetic variability and population genetic structure within *R. acus* from different freshwater and marine fish species and geographical locations in Turkey.

Conflict of Interest

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

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