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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Genetic Analysis of the Mitochondrial *rrnS* Gene of Zoonotic *Anisakis pegreffii* (Nematoda: Anisakidae) Isolated from *Micromesistius poutassou* (R.) in the Aegean Sea

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Abstract: Ascaridoid nematodes were found in viscera of the blue whiting *Micromesistius poutassou* (Risso, 1826) from the Aegean Sea in the parasitological examination. Collected nematodes were morphologically identified as the third (L3) stage of *Anisakis* type I larvae and then subject to Restriction Fragment Length Polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region to identify the species. Randomly selected larvae were molecularly confirmed as *Anisakis pegreffii* by RFLP analysis. Subsequently, L3 of *A. pegreffii* were amplified and bi-directionally sequenced at the small subunit ribosomal RNA (rrnS) gene. The *rrnS* sequence of *A. pegreffii* (MT484284) had 100% identity with the *rrnS* gene of *A. pegreffii* (JX500050, LC222461, MF140359, MT312511, MT312512) which were found in fish and cetaceans hosts, *Scomber japonicus*, *Conger myriaster*, *Neophocaena asiaeorientalis*, and *Stenella coeruleoalba*, from the Mediterranean Sea, Japanese, Chinese, and Korean waters, respectively. This study provided the genetic analysis of zoonotic *A. pegreffii* from the Turkish marine waters based on the *rrnS* gene for the first time. This sequence (MT484284) can be used as the novel *rrnS* sequence of *A. pegreffii* in the genetic analysis for ascaridoid nematodes in the Mediterranean Sea.

Keywords: *Anisakis pegreffii,* molecular characterization, mtDNA, *rrnS* gene, Turkish coast.

Ege Denizi'ndeki *Micromesistius poutassou*'dan (R.) İzole Edilen Zoonotik *Anisakis pegreffii*'nin (Nematoda: Anisakidae) Mitokondriyal *rrnS* Geninin Genetik Analizi

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Öz: Parazitolojik incelemede Ege Denizi'nden mavi mezgit *Micromesistius poutassou* (Risso, 1826) balığının iç organlarında askaridoid nematodlar bulundu. Toplanan nematodlar morfolojik olarak üçüncü (L3) dönem *Anisakis* tip I larva olarak tanımlandı ve daha sonra türleri tanımlamak için internal transcribed spacer (ITS) bölgesi Restriksiyon Parça Uzunluk Polimorfizm (RFLP) analizlerine tabi tutuldu. Rastgele seçilen larvalar RFLP analizleri ile *Anisakis pegreffii* olarak doğrulandı. Daha sonra *A. pegreffii*'nin L3'ünün küçük alt birim ribozomal RNA (*rrnS*) geni çoğaltıldı ve iki yönlü olarak sekanslandı. *Anisakis pegreffii*'nin *rrnS* sekansı (MT484284) Akdeniz, Japon, Çin ve Kore sularından *Scomber japonicus*, *Congenaer asyager*, *Neophocaena asiaeorientalis* ve *Stenella coeruleoalba* gibi balık ve deniz memelilerinde bulunan *A. pegreffii*'nin *rrnS* geni (JX500050, LC222461, MF140359, MT312511, MT312512) ile %100 benzerliğe sahipti. Bu çalışma ilk kez Türk deniz sularından zoonotik *A. pegreffii*'nin *rrnS* genine dayalı genetik analizini sağladı. Bu dizi (MT484284), Akdeniz'deki askaridoid nematodların genetik analizinde *A. pegreffii*'nin yeni *rrnS* dizisi olarak kullanılabilir.

Anahtar kelimeler: *Anisakis pegreffii*, moleküler karakterizasyon, mtDNA, *rrnS* geni, Türk kıyıları.

INTRODUCTION

Adult nematodes of *Anisakis* Dujardin, 1845 are mainly found in the gastrointestinal canal of marine mammals. Different marine mammals and marine fish or squids serve as definite and intermediate or paratenic hosts. Based on molecular genetic markers such as nuclear and mitochondrial DNA, nine distinct *Anisakis* species have been confirmed worldwide (Mattiucci & Nassetti, 2008; Mattiucci et al., 2018). Among the mitochondrial genes, the cytochrome oxidase I (*cox1*), II (*cox2*), and the small ribosomal subunit of RNA (*rrnS*) have been widely used for the genetic analysis of *Anisakis* species (Mattiucci et al., 2014; Mattiucci et al., 2018; Pekmezci & Onuk, 2020). *Anisakis pegreffii* is zoonotic nematodes, and the dominant species in the Mediterranean Sea, widespread in all the fish species (Mattiucci & Nassetti, 2008; Mattiucci et al., 2018).

Both mitochondrial *cox2* and nuclear ITS data of *Anisakis* species from marine fish were previously reported from Turkish marine waters (Pekmezci et al., 2014). Moreover, genetic analyses of *rrnS* loci of *Hysterothylacium aduncum*, *H. fabri, Contracaecum overstreeti*, and *A. typica* among ascaridoids nematodes of marine fish were molecularly made from the coast of Turkey (Pekmezci, 2019; Pekmezci & Yardimci, 2019; Pekmezci & Onuk, 2020; Simsek et al., 2021). There is no study about the genetic characterization of the mitochondrial *rrnS* gene of *A. pegreffii* from the Turkish marine waters. Therefore, the molecular characterization of the *rrnS* gene of *A. pegreffii* from Aegean Sea is aimed for the first time in the current study.

MATERIALS AND METHODS

Morphological examinations: Nematodes were collected from *Micromesistius poutassou* (R.) in the Aegean Sea coasts of Turkey. Parasites were individually cut into three parts. The anterior and posterior parts were used for morphological identifications. Nematodes were morphologically identified by light microscope according to Berland, (1961) and Petter & Maillard, (1988). Morphologically identified three representative specimens were randomly selected and genetically analysed.

PCR, RFLP analysis and DNA sequencing: The middle parts were extracted for genomic DNA (gDNA) using commercial kits. The internal transcribed spacer (ITS) region (ITS1, 5.8S rDNA, ITS2) of nuclear DNA was amplified using polymerase chain reaction (PCR) with NC5/NC2 primers (Zhu et al., 1998) in a final volume of 50 µl as follows: 2X Hot Start PCR Master Mix, 0.5 µM of each primer, and 10-50 ng gDNA. The PCR reactions were subjected to initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 60 s, 55°C for 60 s, 72°C for 60 s, and a final extension at 72°C for 10 min. All PCR amplicons were electrophoresed on 1% gel and visualized under UV illumination. The ITS region was then digested with *Hha*I and *Hinf*I enzymes using restriction fragment length polymorphism (RFLP) analysis to identify the species (D'Amelio et al., 2000). RFLP patterns were electrophoresed on 1.5% gel and visualized under UV illumination. The small subunit ribosomal RNA (*rrnS*) gene in the mitochondrial DNA of randomly selected three Anisakis species was amplified PCR (D'Amelio et al., 2007). PCR was performed using primers MH3/MH4.5 in a final volume of 50 µl as follows: 2X Hot Start PCR Master Mix, and 0.5 µM of each primer, and 10–50 ng gDNA. The PCR protocols were 95°C for 60 s, 55°C for 60 s, 72°C for 60 s for 30 cycles, and a final extension at 72°C for 10 min. PCR amplicons were electrophoresed using a 1.5% agarose gel. Randomly selected three individuals were purified with a PCR purification kit and sequenced using the MH3/MH4.5 primers with an ABI PRISM 3130xl automatic sequencer using a BigDye Terminator v3.1 Cycle Sequencing kit by Macrogen (Amsterdam, Netherlands).

Genetic analysis: Phred scores of their nucleotide bases were checked, and forward and reverse sequences were assembled and then trimmed to remove MH3/MH4.5 primers in Geneious R11 (Kearse et al., 2012). The assembled sequence was blasted in the GenBank database to examine the nucleotide similarity (Altschul et al., 1990). Obtained the *rrnS* data from GenBank were aligned by ClustalW in MEGA X multiple sequence alignments (Kumar et al., 2018). Pairwise distances were estimated using the K2P model in MEGA X (Kumar et al., 2018).

RESULTS

Ascaridoid nematodes were morphologically identified as the third stage of *Anisakis* type I larvae. Some specimens of *Anisakis* larvae were classified as *A. pegreffii* by RFLP analyses with *Hha*I and *Hinf*I enzymes as previously described by D'Amelio et al., (2000). The amplified *rrnS* gene of *A. pegreffii* was produced ~500 bp in the PCR analyses. After DNA sequencing of *rrnS* gene and trimmed to primers, the 491 bp length products were obtained in the present study. There were no intraspecific nucleotide differences detected within *rrnS* gene of three representatives. Therefore, the *rrnS* sequence of one representative was submitted to GenBank was given the accession number: MT484284. Comparison of the *rrnS* sequence of *A. pegreffii* from the Aegean Sea (MT484284) had 100% sequence similarity with the *rrnS* sequence of different *A. pegreffii* isolates (JX500050; *Stenella coeruleoalba*, Mediterranean Sea), (LC222461; *Scomber*

japonicus, Japan Sea), (JQ900764; human, Italy), (MF140359; *Conger myriaster*, China), (MT312511, MT312512; *Neophocaena asiaeorientalis*, South Korea). The *rrnS* sequence of *A. pegreffii* from the Aegean Sea (MT484284) was most similar to *A. pegreffii* (MT312513, MT312515-MT312518) (99.80%), *A. simplex* (99.59%, JN786323), *A. simplex* x *A. pegreffii* (99.37%, AB831878), *A. berlandi* (*A. simplex* C) (98.98%, JX500049), *A. ziphidarum* (96.54%, JX500053), *A. nascettii* (JX500054; 95.11%), *A. brevispiculata* (JX500056; 93.89%), *A. physeteris* (JX500055; 93.52), *A. paggiae* (JX500057; 93.27%), and *A. typica* (MT395672; 91.11%). Pairwise distance between the *rrnS* sequence of *A. pegreffii* from the Aegean Sea (MT484284) with those for other isolates *rrnS* sequences of *A. pegreffii* (MT312511-MT312518, JX500050) showed divergence levels ranging from 0.00 to 0.41%.

DISCUSSION

Current investigation provides the first molecular data of the *rrnS* gene of *Anisakis pegreffii* from the Turkish marine waters. To date, among ascaridoid nematodes, *Hysterothylacium aduncum, H. fabri*, *A. typica*, and *Contracaecum overstreeti* species from Turkish marine waters have been molecularly characterized based on mitochondrial *rrnS* gene (Pekmezci, 2019; Pekmezci & Yardimci, 2019; Pekmezci & Onuk, 2020; Simsek et al., 2021).

The mitochondrial *rrnS* gene sequence of adult *A. pegreffii* was obtained from *Stenella coeruleoalba* in the Adriatic and Tyrrhenian Sea and firstly recorded in the GenBank under accession number JX500050 by Mattiucci et al., (2014). Here, we report the second record of *A. pegreffii rrnS* sequence (MT484284) from Mediterranean Sea. However, there are eight unpublished data of *A. pegreffii* mitochondrial *rrnS* sequences (MT312511-MT312518) from *Neophocaena asiaeorientalis* in the South Korean waters.

Until now, nine *Anisakis* species have been genetically confirmed different gene loci worldwide (Mattiucci et al., 2018). Among these species, *A. berlandi* (JX500049), *A. pegreffii* (JX500050), *A. simplex* (JX500051), *A. typica* (JX500052; MT395672) *A. ziphidarum* (JX500053), *A. nascettii* (JX500054), *A. physeteris* (JX500055), *A. brevispiculata* (JX500056), *A. paggiae* (JX500057), and *A. simplex* x *A. pegreffii* (AB831878, unpublished data) had genetically characterized for *rrnS* gene, and their sequences were also recorded to GenBank (Mattiucci et al., 2014; Pekmezci & Onuk, 2020). The BLASTn search showed that the *rrnS* sequence of *A. pegreffii* isolate herein exhibits ranged from 91.11% to 99.59% similarity to those of *rrnS* sequences of different *Anisakis* species (Mattiucci et al., 2014; Pekmezci & Onuk, 2020). Therefore, the mitochondrial *rrnS* gene can be used to differentiate among *Anisakis* species. Moreover, the genetic distance between our *rrnS* sequence of *A. pegreffii* (MT484284) and other isolates *rrnS* sequences of *A. pegreffii* (MT312511-MT312518) from South Korean waters was 0.41%. The genetic difference between two isolates belonging to the same species may be related to the isolates being obtained from different geographic areas far from each other.

CONCLUSION

Herein, the novel data of mitochondrial small ribosomal subunit RNA sequence of *A. pegreffii* from Turkish marine waters was achieved for the first time, and this novel *rrnS* data (MT484284) is a second data recorded in GenBank for *A. pegreffii* from the Mediterranean Sea. Furthermore, this novel *rrnS* sequence can be utilized for genetic analysis of ascaridoid nematodes from the Mediterranean Sea.

CONFLICT OF INTEREST

The author declares that they have no competing interests.

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