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

Öz: Parazitolojik incelemede Ege Denizi’nden mavi mezeit 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’sinin küçük alt birim ribosomal RNA (rrnS) geni ölçülü ve iki yönlü olarak sekanslandı. Anisakis pegreffii’nin rrnS sekansı (MT484284) Akdeniz, Japon, Çin ve Kore sularından Scomber japonicus, Conger myriaster, 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 analizi sağladı. Bu dizi (MT484284), Akdeniz/deki askaridoid nematodların genetik analizinde A. pegreffii’nin yeni rrnS dizisi olarak kullanabilir.

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 definitive 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 HhaI and HinfI 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 HhaI and HinfI 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 coeruleoalba, Mediterranean Sea).
The mitochondrial rrnS gene sequence of adult *A. pegreffii* was obtained from *Stenella coeruleoalba* in the Adriatic and Tyrrenian 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. nascetti* (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 & Yardımcı, 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 Tyrrenian 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.

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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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