

## Molecular Detection of *Anisakis pegreffii* in Horse Mackerels (*Trachurus trachurus*) Sold for Human Consumption in Erzurum Province of Turkey

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### Summary

This study was planned to identify six parasites detected in horse mackerels (*Trachurus trachurus*) sold for human consumption in Erzurum province. DNA extraction was performed on six parasites diagnosed as nematode. Species identification was done by PCR and PCR-RFLP analysis of ITS (ITS-1, 5.8S subunit rRNA gene and ITS-2) region of ribosomal DNA (rDNA) and partial sequencing of mitochondrial cytochrome c oxidase subunit 1 (mt-CO1) and mt-CO2 genes. According to the PCR and PCR-RFLP results, all parasites were identified as *Anisakis pegreffii*. Partial sequence of mt-CO1 gene of one randomly selected parasite was corresponding with *A. simplex* and mt-CO2 genes of two parasites were corresponding with *A. pegreffii*. With this study, *A. pegreffii* was molecularly detected for the first time in Turkey.

**Keywords:** *Anisakis pegreffii*, Fish, PCR, PCR-RFLP, Sequencing

## Türkiye'nin Erzurum İlinde İnsan Tüketimi İçin Satılan İstavrit Balıklarında (*Trachurus trachurus*) *Anisakis pegreffii*'nin Moleküler Tanısı

### Özet

Bu çalışma, Erzurum ilinde insan tüketimi için satılan istavrit balıklarında (*Trachurus trachurus*) tespit edilen altı parazitin tür tanısını yapmak amacıyla planlanmıştır. Nematod olarak belirlenen altı parazitten DNA ekstraksiyonu yapılmıştır. Tür tanısı, rDNA'nın ITS bölgelerinin (ITS1, 5.8 alt ünite rRNA ve ITS2) PCR ve PCR-RFLP analizleri ve mitokondrial sitokrom c oksidaz alt ünite 1 (mt-CO1) ve mt-CO2 genlerinin kısmi olarak sekanslanması ile yapılmıştır. PCR ve PCR-RFLP sonuçlarına göre tüm parazitlerin *Anisakis pegreffii* olduğu belirlenmiştir. Rastgele seçilen bir parazitin mt-CO1 geninin kısmi sekans sonucu *A. simplex*, iki parazitin mt-CO2 geninin kısmi sekans sonuçları ise *A. pegreffii* ile uyuşmuştur. Bu çalışma ile *A. pegreffii* Türkiye'de ilk kez moleküller olarak tespit edilmiştir.

**Anahtar sözcükler:** *Anisakis pegreffii*, Balık, PCR, PCR-RFLP, Sekanslama

## INTRODUCTION

The species of the genus *Anisakis* are the parasites of the stomach and intestine of pinnipeds (seal, sea-lion, walrus) and cetaceans (dolphin, narwhal, porpoise, whale). Larvae of *Anisakis* spp. have been reported in the body cavities or tissues of a great variety of marine and anadromous teleosts as well as squids and prawns <sup>1-3</sup>.

Anisakiasis is an important fish-borne zoonosis.

Humans become accidental hosts by eating raw or poorly cooked marine fish or squid harboring larvae. Human anisakiasis occurs worldwide; the majority of cases are reported in Asian countries where consumption of seafood is common. Difficulty in swallowing, stomach pain, abdominal pain, nausea, vomiting, diarrhea, isolated swellings to urticaria and life-threatening anaphylactic shocks are the symptoms of the disease. These symptoms

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can be slight to severe<sup>1,2</sup>.

Recent molecular studies on *Anisakis* revealed the existence of two main clades. One encompassing the species showing the larval stage is indicated as *Anisakis* Type I, and a second sharing the larval morphology as *Anisakis* Type II. The first clade includes the species of *A. simplex* complex (*A. pegreffii*, *A. simplex* sensu stricto and *A. simplex* C), *A. typica*, *A. ziphidarum* and *Anisakis* sp. The second includes the species *A. physeteris*, *A. brevispiculata* and *A. pagie*<sup>4</sup>. These species are genetically characterized by molecular techniques, such as PCR (polymerase chain reaction), PCR-RFLP (restriction fragment length polymorphism), RAPD-PCR (Random Amplified Polymorphic DNA) and DNA sequencing<sup>5-11</sup>.

The aim of this study was to identify nematode species isolated from horse mackerels (*Trachurus trachurus*) sold for human consumption in Erzurum province of Turkey by use of PCR, PCR-RFLP and DNA sequencing techniques.

## MATERIAL and METHODS

### Parasite Materials

Six parasites were detected in horse mackerels (*T. trachurus*) sold for human consumption in Erzurum province of Turkey. The origin of the fishes was determined as Black Sea. Samples were put into 70% ethanol until use.

### DNA Extraction

DNA was isolated from ethanol preserved parasites. Each parasite was cut into small pieces. Prior to DNA extraction, broken material was washed with 5X PBS and then digested for 6 h at 56°C with 250 µl TEN-SDS (50 mM Tris-HCl, pH 8, 5 mM EDTA, 100 mM NaCl, 10% SDS) containing 2 mg/ml Proteinase K. After digestion, 125 µl of 6M NaCl was added and the samples were vortexed for 15 seconds. Then, the samples were centrifuged for 5 minutes at 13.000 g in eppendorf tubes. Finally, the supernatants were transferred to new clean eppendorf tubes, and DNA was extracted using the classical phenol-chloroform and precipitation method. After drying, the DNA was suspended in 50 µl Tris-EDTA buffer (pH 7.6)<sup>12,13</sup>.

### PCR-RFLP Analysis

Universal primers A (5'-gtcgaattcgtaggtgaacctgcggaa ggatca-3') and B (5'-gccggatccgaatcctggtagttctttcct-3') were used for the amplification of rDNA (ITS1, 5.8 subunit rRNA and ITS2)<sup>5,7,14-16</sup>. PCR was carried out in a final volume of 50 µl, containing 23.75 µl DNase, RNase free steril distilled water (Biobasic, Inc), 5 µl 10X PCR buffer, 6 µl 25 mM MgCl<sub>2</sub>, 5 µl 1 mM dNTP mix, 2.5 µl of each primer (50 pmol), 5 µl of template DNA, and 0.25 µl of TaqDNA polymerase (1.25 IU) (MBI, Fermentas). PCR conditions were: 5 min at 95°C (initial denaturation), 35 cycles of 1 min at 95°C, 1 min at 48°C and 1 min at 72°C and finally 5 min at 72°C (final extension).

PCR products were digested with the restriction enzyme *Hinf1* (MBI, Fermentas) by following the manufacturer's instructions. Digested products were separated on agarose gels (3%), stained with ethidium bromide, visualised and photographed on an UV transilluminator (UVP).

### Species-Specific PCR

APE1 (5'-gagcagcagcttaaggcagaggc-3') and B (5'-gccg gatccgaatcctggtagttctttcct-3') primer pair was specific to *A. pegreffii* and used for the partial amplification of 18S-28S rRNA gene<sup>5</sup>. PCR mix and PCR conditions were same as above. PCR products were separated on agarose gels (1.5%), stained with ethidium bromide, visualised and photographed on an UV transilluminator (UVP).

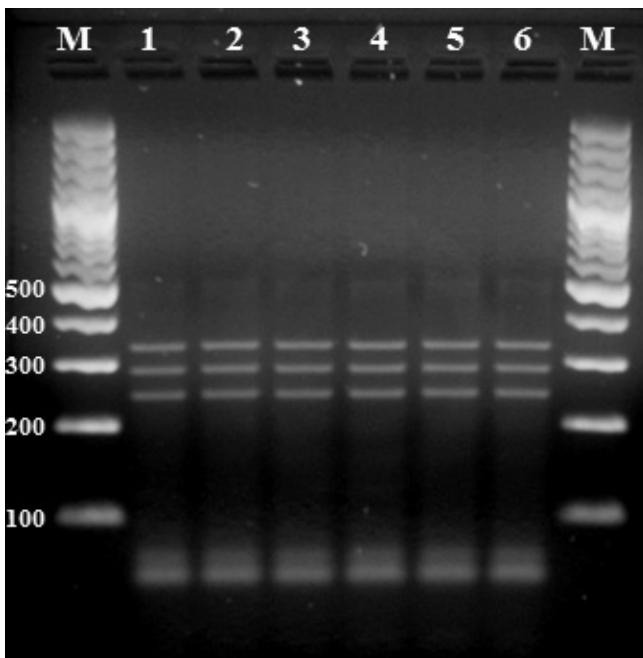
### Sequence Analysis

JB3 (5'-tttttggcatcctgaggttat-3') and JB4.5 (5'-taaa gaaagaacataatgaaaatg-3') primer pair was used to amplify partial mt-CO1, and 211F (5'-tttcttagttatagattgrtyat-3') and 210R (5'-caccaactctaaaatttc-3') primer pair was used to amplify partial mt-CO2 genes of the nematodes<sup>17,18</sup>. PCR was carried out in a final volume of 50 µl, containing 25.75 µl DNase, RNase free steril distilled water (Biobasic, Inc), 5 µl 10X PCR buffer, 5 µl 25 mM MgCl<sub>2</sub>, 4 µl 1 mM dNTP mix, 2.5 µl of each primer (50 pmol), 5 µl of template DNA (100-200 ng), and 0.25 µl of TaqDNA polymerase (1.25 IU) (MBI, Fermentas). PCR conditions were: 5 min at 95°C (initial denaturation), 35 cycles of 1 min at 95°C, 1 min at 50°C and 1 min at 72°C and finally 5 min at 72°C (final extension). PCR products were separated on agarose gels (1.5%), stained with ethidium bromide, visualised and photographed on an UV transilluminator (UVP).

Amplicons of mt-CO1 gene of one parasite and mt-CO2 gene of two parasites were sequenced by a commercial company (Refgen, Ankara, Turkey). Obtained sequences were edited and aligned with CLC main workbench software<sup>19</sup>; then, sequence analysis was undertaken by BLAST algorithms and databases from the National Center for Biotechnology (<http://www.ncbi.nlm.nih.gov>).

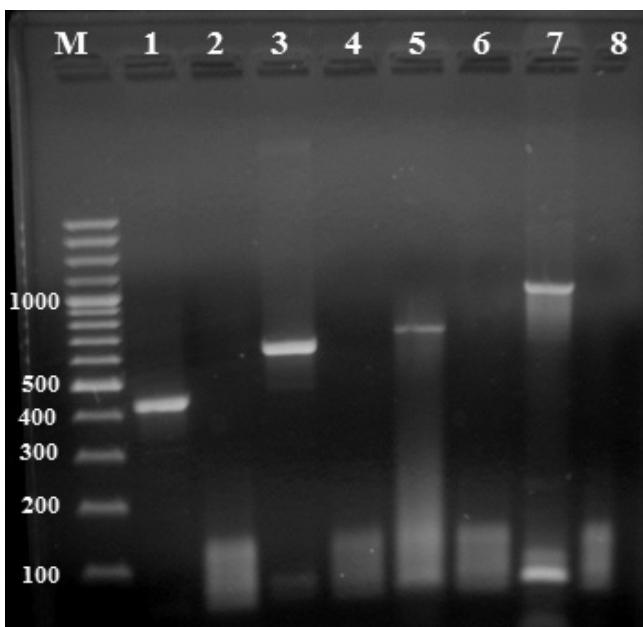
## RESULTS

Digestion of approximately 1000 bp region of rDNA (ITS1, 5.8 subunit rRNA and ITS2) (Fig. 1) with *Hinf1* produced the same banding pattern in all six parasites analyzed. All parasites showed three bands of 250, 300, 370 bp which were specific to *A. pegreffii* (Fig. 2). PCR products of 672 bp were amplified in all of six parasites with primer pair APE1 and B, which is specific for *A. pegreffii* (Fig. 1). Mt-CO1-PCR yielded 446 bp and mt-CO2-PCR yielded 629 bp of amplification products (Fig. 1). When compared to GenBank results, mt-CO1 sequence (GenBank accession no JN102304) was corresponding with *A. simplex* (94-98%), and mt-CO2 results (GenBank accession no JN827308 and JN827309) were corresponding with *A. pegreffii* (97-99%).



**Fig 1.** RFLP analysis of six nematodes using *Hinf1* enzyme M: 100 bp marker, 1-6: Banding patterns of nematode samples obtained from horse mackerels

**Şekil 1.** Altı nematodun *Hinf1* enzimi kullanarak yapılan RFLP analizi M: 100 bc marker, 1-6: İstavrit balıklarından elde edilen nematodların RFLP kalıpları



**Fig 2.** PCR results of six nematodes obtained from horse mackerels, M: 100 bp marker, 1: 446 bp amplicon of mt-CO1 gene, 3: 629 bp amplicon of mt-CO2 gene, 5: 672 bp amplicon of 18S-28S rRNA gene, 7: 1000 bp amplicon of rDNA (ITS1, 5.8 subunit rRNA and ITS2), 2,4,6,8: Negative controls (sterile distilled water)

**Şekil 2.** İstavrit balıklarından elde edilen altı nemetodun PCR sonuçları, M: 100 bc'lik marker, 1: Mt-CO1 geninin 446 bc'lik ürünü, 3: Mt-CO2 geninin 629 bc'lik ürünü, 5: 18S-28S rRNA geninin 672 bc'lik ürünü, 7: rDNA (ITS1, 5.8 altünlü rRNA ve ITS2)'nın 1000 bc'lik ürünü, 2,4,6,8: Negatif kontroller (steril distile su)

## DISCUSSION

Molecular markers have been successfully used for the species level identification of *Anisakis* species. Mattucci et al.<sup>10</sup> reported *A. pegreffii* for the first time in a paraffin embedded granuloma taken from a man with intestinal anisakiasis in Italy. Santoro et al.<sup>20</sup> reported *A. pegreffii* in loggerhead sea turtles (*Caretta caretta*) from Central Mediterranean of southern Italy. Valentini et al.<sup>21</sup> determined the genetic relationship among nine species of *Anisakis* sp. and reported *A. pegreffii* in common dolphin (*Delphinus delphis*) from Spanish coasts of Northeast Atlantic Ocean. In these studies, mt-CO2 gene was used for the detection and phylogenetic analyses of *A. pegreffii*.

Umehara et al.<sup>14</sup> studied on 85 patients with anisakiasis in Hokkaido and Kyushu in Japan. According to this study, 84 out of 85 patients were infected with *A. simplex* s.s., and one was infected with *A. pegreffii*. Same researchers reported these two parasites and a hybrid genotype from fish and cetacean in Japanese waters<sup>7</sup>. Lee et al.<sup>16</sup> studied on 60 larvae obtained from 90 sea fishes and 15 squids. Among 60 isolates, 47 were identified as *A. pegreffii*, 10 as *A. typica*, one as *A. simplex* s.s. and two as hybrid genotypes. Species level identification of parasites was achieved by PCR-RFLP analysis of ITS region.

Quiazon et al.<sup>22</sup> detected *A. simplex* s.s. and *A. pegreffii* in marine fishes in Japanese waters by PCR-RFLP analysis of ITS region and sequencing of mt-CO2 gene. Abe et al.<sup>23</sup> reported *A. simplex* s.s. from a 65-year-old Japanese woman with epigastralgia. Umehara et al.<sup>8</sup> reported *A. typica* in addition to *A. simplex* s.s. and *A. pegreffii* in hairtail fish caught in coasts of Taiwan and Japan. Pontes et al.<sup>15</sup> studied on 150 anisakid larvae in three different species of fish, and they detected *A. simplex* s.s., *A. pegreffii*, *A. zippidaram*, *A. physeteris*, *A. typica* and *Anisakis* sp. in Madeiran waters (Atlantic Ocean, Portugal). Parasites were identified by sequencing and PCR-RFLP analysis of ITS region<sup>15,23</sup>.

In PCR-RFLP analysis, primer pairs A-B and NC2-NC5 were used for the amplification of ITS region, AniF-Ani1R for 5.8 rRNA and JB3-JB4.5 for mt-CO1 genes<sup>7,8,14-16,22-25</sup>. The most used restriction enzymes were *Hinf1*, *Hhal* and *Tag1* for the analysis of both ribosomal and mitochondrial genes<sup>7,8,14-16,22-25</sup>. In the studies in which A and B primer pair was used, RFLP patterns of *Hinf1* provided the discrimination of *A. pegreffii*, *A. simplex* s.s. *A. physeteris*, *A. typica*, *A. zippidaram*, *Anisakis* sp. A and hybrid genotypes of *Anisakis* sp., while the digestion profiles of *Hhal* were same in *A. pegreffii*, *A. simplex* s.s. *A. physeteris* and *A. zippidaram* and different only between *A. typica* and *Anisakis* sp. A. Likewise, digestion patterns of *Tag1* were not sufficient to discriminate *Anisakis* species<sup>7,14-16,22,25</sup>. In this study, we used universal primers A and B for the amplification of ITS region, and *Hinf1* for digestion of ITS products. All six parasites showed three bands of 250,

300, 370 bp which were specific to *A. pegreffii*. Researchers obtained the same banding pattern for *A. pegreffii* in different studies<sup>5,7,14,16,22,25</sup>.

Umeshara et al.<sup>5</sup> and Fang et al.<sup>6</sup> developed species-specific primers from the ITS region of rDNA and used in multiplex PCR for the rapid identification of anisakid nematodes. Umehara et al.<sup>5</sup> achieved to differentiate *A. simplex* s.s., *A. pegreffii* and *A. physeteris*, and Fang et al.<sup>6</sup>, *A. simplex* s.s., *A. pegreffii* from other anisakid nematodes. In these two studies, primer pairs APE1-B and APEF-NC2 were used for the detection of *A. pegreffii*. We used APE1-B primer pair for the detection of *A. pegreffii* and we obtained 672 bp amplification products as obtained by Umehara et al.<sup>5</sup>.

Kim et al.<sup>11</sup> determined the complete mitochondrial genome of *A. simplex* and defined the phylogenetic relationship of *A. simplex* with 13 different nematode species. Noguera et al.<sup>26</sup> studied on 42 Atlantic salmons (*Salmo salar*) in Scotland. They detected Anisakis type I larvae in the morphologic examination. For species level identification, larvae were analyzed by PCR-RFLP of ITS region and sequencing of ITS and mt-CO1 genes. At the end of the study, all larvae were identified as *A. simplex* s.s. Cross et al.<sup>27</sup> detected *A. simplex* s.s. in 37 Atlantic herrings (*Clupea harengus*) caught off the north-west coast of Scotland. They made species level detection by sequencing of large subunit mt-CO1 gene of 161 nematodes.

In sequencing reactions, primer pairs A-B and NC2-NC5 were used for the amplification of ITS region, AnCO1F-AnCO1R for mt-CO1 and 211F-210R for mt-CO2 genes<sup>8,10,15,20-25,27</sup>. In this study, we used primer pairs JB3-JB4.5 and 211F-210R for the amplification and sequencing of mt-CO1 and mt-CO2 genes, respectively. Mt-CO1 result was corresponding with *A. simplex* (94-98%), and mt-CO2 results were corresponding with *A. pegreffii* (97-99%) when compared to GenBank results.

In Turkey, there is limited data on *A. simplex*. This parasite was reported in red mullet (*Mullus surmuletus*) caught near Gökceada, anchovies (*Engraulis encrasicolus*) from Marmara sea, and mackerel (*T. trachurus*), scad (*Trachurus mediterraneus*), whiting (*Merlangius euxinus*) as well as sardines (*Sardina pilchardinus*) caught on Canakkale coast and throughout the Dardanelles Strait of Turkey<sup>28-30</sup>. In these studies, species identification was based on morphological features of parasites. Even though morphology is useful for the diagnosis of *Anisakis* spp., the species of *A. simplex* complex are so far morphologically indistinguishable at both adult and larval stage; consequently, only genetic and molecular methods can be used reliably to identify them at all the developmental stages. Although *A. typica* and *A. ziphidarum* are distinguishable at their male adult stages, this is not possible at their larval stage<sup>4</sup>. In this study, we determined *A. pegreffii* from horse mackerels (*T. trachurus*) by PCR and PCR-RFLP analysis of ITS region

of rDNA and partial sequencing of mt-CO1 and mt-CO2 genes for the first time in Turkey. This study suggests that *Anisakis* species can easily be diagnosed by molecular techniques at species level. We consider that further studies should be carried out in Turkey to detect different *Anisakis* species and understand clearly the transmission ways, epidemiology and control of the disease.

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