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RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

Molecular investigation of nematodes isolated from three economical fish species taken from Çanakkale (Türkiye) fish market

Çanakkale (Türkiye) balık pazarından alınan üç ekonomik balık türünden izole edilen nematodların moleküler incelenmesi

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Abstract: This study was based on the molecular identification of endoparasites sampled from three economically valuable fish species caught from the waters of the Canakkale (Türkiye). Subjected samples in the study were from chub mackerel (*Scomber japonicus* Houttuyn, 1782), anchovy (*Engraulis encrasicolus* Linnaeus, 1758), and bogue (*Boops boops* Linnaeus, 1758) without gender discrimination. The nematode parasites obtained from the samples were sent to molecular diagnostic laboratories in alcohol and the results were interpreted. As a result of the study, *Anisakis typica* (Diesing, 1860) Baylis, 1920 and *Anisakis pegreffii* Campana-Rouget and Biocca, 1955 were isolated from the chub mackerel fish and *Anisakis pegreffii* species were also isolated from anchovy and bogue. This parasite was the most isolated nematode parasite among all nematodes.

Keywords: *Anisakis pegreffii*, *Anisakis typica*, phylogenetic tree, molecular, Çanakkale, Aegean Sea

Öz: Bu çalışma, Çanakkale (Türkiye) sularından yakalanan ekonomik açıdan değerli üç balık türünden örneklenen endoparazitlerin moleküler tanımlanmasına dayanmaktadır. Çalışmada cinsiyet ayrımı yapılmaksızın kolyoz (*Scomber japonicus* Houttuyn, 1782), hamsi (*Engraulis encrasicolus* Linnaeus, 1758) ve kupes (*Boops boops* Linnaeus, 1758) balıklarından örnekler alınmıştır. Örneklerden elde edilen nematod parazitler alkol içerisinde moleküler tanı laboratuvarlarına gönderilmiş ve sonuçlar yorumlanmıştır. Çalışma sonucunda kolyoz balıklarından *Anisakis typica* (Diesing, 1860) Baylis, 1920 ve *Anisakis pegreffii* Campana-Rouget ve Biocca, 1955, hamsi ve kupes balıklarından ise *Anisakis pegreffii* türleri izole edilmiştir. Bu parazitin tüm nematodlar arasında en fazla izole edilen nematod parazit olduğu görülmüştür.

Anahtar kelimeler: *Anisakis pegreffii*, *Anisakis typica*, filogenetik ağaç, moleküler, Çanakkale, Ege Denizi

INTRODUCTION

Seafood, which is one of the most valuable food sources, especially fish, contains all the amino acids required for the protection and development of tissues in the human body, being rich in unsaturated fatty acids (omega 9, omega 6, and omega 3) and being easily digestible, vitamins and minerals necessary for humans. It is one of the most valuable food sources that can meet this need with its rich content, high cholesterol, and blood pressure, cardiovascular diseases, diabetes for adults, and protection of the human body against some types of cancer (Ozkan and Koca, 2006; Turan et al., 2006).

Fish production has shown a rapid increase in both aquaculture and hunting due to the increase in demand in recent years. In addition to the increased production amount with the increase in the need, it also brought the existence of serious disease factors. While the diseases observed in the aquaculture area can be easily eliminated, the diseases cannot be prevented in the hunting area. Because the organisms that cause these diseases are widely spread in nature. Some commercially important fish species, which are not yet produced, are under the threat of disease factors that can be found free and need a living thing to cling to at another stage of their lives. So much so that disease agents with this complex life cycle can also use humans and other mammals as a destination. In this context, anisakids, which are included in the life cycle of fish, have been declared by the European Food Safety Authority (2010) as the "biological hazard" of the highest importance in seafood products (Biohaz, 2023). Various negative factors reduce the quality and nutritional value of fish meat. Among these factors, various diseases caused by viruses and bacteria as well as parasites were reported previously (Ozkan and Koca, 2006). Although fish parasites rarely cause problems in the natural environment, it is known that they reduce the nutritional value of fish in aquaculture, negatively affect their growth, reproduction, and nutrition, cause serious disease outbreaks, and thus adversely affect the fishing industry (Quiazon, 2015).

The genera *Anisakis* (Dujardin, 1845), *Pseudoterranova*, and *Hysterothylacium* are nematode parasites of the family Anisakidea (Ángeles-Hernández et al., 2020). *Anisakis simplex* (Rudolphi, 1809) Dujardin, 1845*, Anisakis pegreffii,* and *Pseudoterranova decipiens,* (Krabbe, 1878) Gibson, 1983 are of main public health interest. Anisakiasis is a zoonotic disease caused by the consumption of raw or undercooked seafood containing larval nematodes of the *Anisakis*, *Pseudoterranova*, and *Contracaecum* genera in the Anisakidae family. People catch this infection by consuming some seafood raw or undercooked. Consumption of fish after being caught, frozen without being cleaned, and stored for a certain period, caused an increase in anisakiasis events. On the other hand, if the fish are cleaned as soon as they are caught, the risk of infection decreases when the parasites in the digestive system are removed before they pass into the muscles (Sarımehmetoğlu and Doğanay, 1999; Hochberg and Hamer, 2010; FAO/WHO, 2012; Ludovisi et al., 2017). Anisakiasis is clinically acute or chronic in the human gastrointestinal tract. In addition, allergies occur in individuals sensitive to this parasite. Seafood has a very important place in microorganisms transmitted to humans by food. It is necessary to know the disease factors transmitted by seafood, which has become an important source of human food, and to fight effectively. Although parasites are very common, because seafood is consumed and cooked due to Turkish cultural habits, infections due to such parasites are very rare. Due to the increase in consumption of popular and traditional foods specific to Europe and the Far East in Türkiye in recent years, society needs to be informed about these issues (Kurşun and Erol, 2008; Oktener, 2004).

Species belonging to the genus *Contracaecum*, and especially species belonging to the genera *Anisakis* and *Pseudoterranova*, have been associated with anisakiasis/anisakidosis, both in terms of stomach problems and as fish-borne pathogens producing allergic reactions. Although other nematodes such as *Hysterothylacium* species of the Rhaphidascarididae family are not thought to be pathogenic, data have been reported in studies to cause allergic reactions in humans. Since the *Hysterothylacium* parasite seriously inhibits the growth rate and health of the fish it uses as a host, it can cause death by lowering the immune systems of the fish. A group of these parasitic creatures called zoonosis includes individuals belonging to the Anisakidae family (Audícana et al., 2002; Mattiucci et al., 2017; Li et al., 2016).

Anisakid nematode larvae are the most isolated parasites from aquatic organisms. Nematode parasites are creatures with a complex life cycle. They use different intermediate hosts throughout their lives. Parasitic nematodes use different intermediate hosts throughout their lives - fish and invertebrates. Their final hosts are usually humans or other mammals. These creatures, which pass to humans in larval three and larval four stages, are called zoonotic parasites. It is transmitted to humans by consuming raw or undercooked seafood. Species identification is difficult when nematodes are in larval form. The naming of species may not go beyond genus size in microscopic examinations in terms of morphology. Molecular diagnostic techniques are used for definitive diagnosis. Sometimes, even molecular diagnostic methods may not provide an easy and accurate diagnosis for the identification of the living thing. While some subspecies will cause the living thing to diverge with a single gene, it may create the idea that it shows the gene sequence of the same parasite (Balbuena et al., 2000; Chaia et. al., 2005).

Humans are infected with two species of *Anisakis*, *A. simplex* stricto, and *A. pegreffii*. It has also been confirmed by molecular techniques that these two species cause human infections. Improving taxonomic definitions by using molecular diagnostic methods as well as traditional methods for specific identification will help to understand the biological, ecological, and epidemiological aspects of nematodes by shedding light on their life cycles and geographical distributions.

MATERIALS AND METHODS

Fish were sampled monthly from the Canakkale Fish Market for 12 months between September 2019 and 2020. In the research, a total of 1337 fish, and nematode parasites were collected.

As a result of the observations made, generally, free-moving nematode parasite individuals were found buried on the internal organ surfaces of the fish. All parasites in each fish were carefully collected and taken into 99.9% absolute ethanol for microscopic examinations and molecular determinations.

Molecular diagnosis of nematodes isolated from fish species carried out with manufacturer procedure (Pekmezci, 2021; Mattiuci et al., 2011). EurX GeneMATRIX Tissue & Bacterial DNA isolation kit (Poland) used for DNA isolation from fish species. Spectrophotometric measurement was performed on Thermo Scientific Nanodrop 2000 (USA) device to control the amount and purity of DNA obtained after DNA isolation. Targeted genes for species identification in molecular diagnosis of nematodes, primers of the COII region (211F: 5'-TTT TCT AGT TAT ATA GAT TGR TTY AT-3')- (210R: 5'- CAC CAA CTC TTA AAA TTA TC-3') (PCR optimization was done a company) was replicated in a kyratec thermocycler device. In the study, the PCR mix was adjusted to be 35 µl (Table 1). The following PCR cycle was then performed (Table 2).

Table 1. PCR mixes for the COII gene region

Compound	Stock Concentration	Reaction Concentration
PCR Buffer	10X	1X
MgCl2	25mM	1,5 mM
dNTP mix	20 mM	0,2 mM
F. Primer	10 µM	0,3 µM
R. Primer	10 µM	0,3 µM
Taq DNA Polymerase	5U/ µl	2 U
DNA template	3 µl	
Makeup to 35 µl with PCR-grade water.		

Table 2. The following PCR cycle was then performed

Stage	Definition	Temperature	Duration	
1	initial denaturation	95°C	5 min.	40 cycle
2	denaturation	95°C	45 sec.	
3	annealing	54°C	45 sec.	
4	extension	72°C	60 sec.	
5	final extension	72°C	5 min.	
	stop	4°C	∞	

The amplification products obtained by PCR were run on a 1.5% agarose gel prepared with 1x TAE buffer at a 100-volt current for 90 minutes by electrophoresis. PCR products showing single bands in the gel were purified according to the procedures for sequencing using the MAGBIO "HighPrep™ PCR Clean-up System" (AC-60005) purification kit. Sequence analysis was performed at the Macrogen Netherlands laboratory with the ABI 3730XL Sanger sequencing device (Applied Biosystems, Foster City, CA). The nematode species were detected using the NCBI blast program.

The results of the sequence analysis of the COII gene region were used in MolBio Tools program, which can perform online restriction analysis, and in silico restriction, cuts were performed with HaeIII, HhaI, and HinfI enzymes. The nematode *A. pegreffii* with the code NC_034329.1 retrieved from the NCBI genome database was used as a reference.

RESULTS

This study was carried out to determine the nematode species isolated from some fish with high economic value taken from the Canakkale (Türkiye) fish market of the isolated 137 parasites. The figures showed that the most common parasitism was in chub mackerel, and it was the slightest common fish species with 2 parasites in the bogue included in the study. The COII gene region was used for the molecular characterization of nematodes isolated from fish samples. After PCR amplification of the COII gene region, DNA sequence analysis was performed with the ABI 3730XL Sanger-sequencing device (Applied Biosystems, Foster City, CA). The obtained COII gene sequences were analyzed using BLAST tool on NCBI database and the isolated nematode samples were identified. As a result of the sequence analysis, nematodes (99.5%-100%) isolated from anchovy determined as *A. pegreffii*, those isolated from Bogue (99.50-99.83%) determined as *A. pegreffii* and the nematodes isolated from chub mackerel were defined as *A. pegreffii and A. typica* (99.67%-100%). The COII gene sequences of identified nematodes were submitted on the GenBank platform and accession numbers for sequences were attained and accession numbers were shown in Figure 1.

The determining phylogenetic relationship of identified nematodes, all the COII sequences were aligned using MEGA 11 software and ClustalW v1.6 alignment tool with default parameters. The Neighbour-joining tree was constructed by using the Bootstrap method with 1000 replicates. A bootstrap value of above %60 was given in branches (Figure 1).

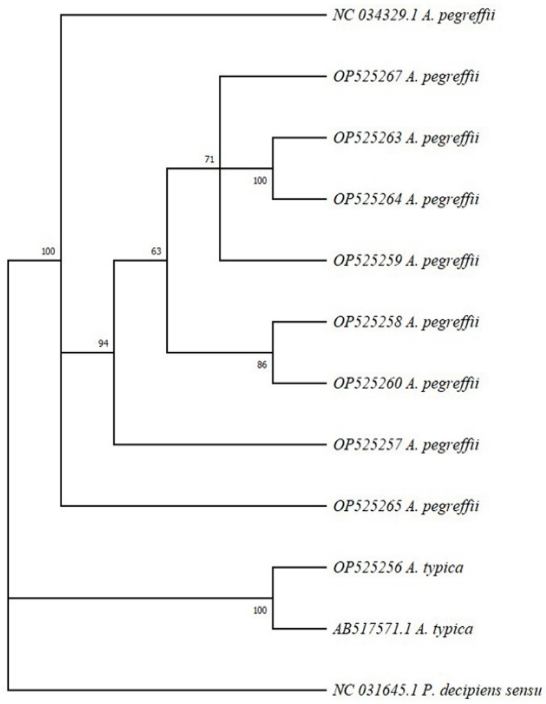

Figure 1. Phylogenetic tree for nematodes

All *A. pegreffii* species showed two restriction profiles because of *in-silico* restriction cuts. While 11 *A. pegreffii* in the first profile did not have cleavage sites for HaeIII and HhaI enzymes, it was found to have two cleavage sites for HhaI enzyme (302-205-88).

It was determined that the nematode parasite *A. pegreffii* from Bogue 3 similarly did not have a cleavage site for the enzyme HaeIII and HhaI but had a single cleavage site for the enzyme HinfI (305-301) (Table 3). *A. pegreffii* nematode with the code NC_034329.1 obtained from the NCBI genome database was used as a reference and it was seen that it had the same restriction profile as 11 species as a result of *in-silico* analysis. As a result of in-silico restriction analysis of the COII gene region of the nematode parasite from Spanish mackerel 1, it was seen that there was no cut-off region for the HhaI enzyme, but it had two cut-off regions for HaeIII and a single cut-off region for HinfI.

As a result of DNA sequence analysis, the difference of *A. pegreffii* nematode from Bogue 3 from other nematode species is due to the difference in a single nucleotide, 5'-AANTC-3', while it should be 5'-G^ANTC-3' in the sequence corresponding to the restriction cut site for the HinfI enzyme. is thought to be possible.

The nematodes that are the subject of the study; It was isolated from the gastrointestinal systems of fish, body cavities with gonads and pyloric caeca, and the liver. No parasite formation was observed on edible meat during the study.

Table 3. Restriction profile of detected nematodes

Rest Profile	Host	Identified Species	Seq Length (bp)	HaeIII	HhaI	HinfI
P-1	Anchovy 3	*A. pegreffii*	595	-	-	302-205-88
	Chub Mackerel 3	*A. pegreffii*	600	-	-	306-206-88
	Bogue 1	*A. pegreffii*	604	-	-	304-212-88
	NC_034329.1	*A. pegreffii -Ref*	697	-	-	333-249-88-27
P-2	Bogue 3	*A. pegreffii*	606	-	-	305-301
P-3	Chub Mackerel 1	*A. typica*	576	359-135-82	-	306-270
	KF356652.1	*A.typica-Ref*	600	356-162-82	-	303-209-88

DISCUSSION

Considering the zoonotic characteristics of Anisakid species, food safety and public health need to determine the presence and occurrence of Anisakid nematode larvae, especially in different fish species used for human consumption. In addition, the European Food Safety Authority (EFSA, 2010) recommends that it is important for public health to regularly collect data on the microhabitats, infection rates, densities, seasonal and geographical distributions, and life cycles of parasites in fish caught from the natural environment. The data of the species is due diligence in the fish caught from the Northern Aegean Sea coasts of our country and includes basic data of the species.

The study was carried out in the Canakkale region of the North Aegean Sea. In the study, 137 nematode larvae were collected. From the isolated parasites, 30 were selected by visual separation under the microscope and the molecular diagnosis was made. The remaining parasites were identified by the traditional method. A molecular examination of these larvae revealed that most of them were *A. pegreffii*, and one isolate was *A. typica*. The parasites found were living freely in the internal organs and body cavities of the fish. While it was observed that the most infected species was chub mackerel, two nematode isolations were observed from the bogue examined in the study. Obtained nematodes were identified by molecular analysis. The molecular technique used is sequencing by sequence analysis. In a study conducted in the Aegean Sea, it was determined that the nematodes obtained were also individuals belonging to the Anisakidae family. The PCR-RLFP technique was used in the study, and it was defined as *A. pegreffii* and *A. pegreffii* hybridized with *A. simplex* (Chaligiannisa et al., 2012).

Klimpe et al. (2011) investigated the deep-water life cycle of *Anisakis paggiae,* in the Irminder sea and their distribution among Kogiid Whales. In the study, they stated that the whale is a typical final host and stated that the *A. paggiae* species was transported to other seas by migratory whales. In addition, nematodes were isolated from the stomach, pyloric caeca, intestine, gonads, and body cavity of the creature, and their identification was made using PCR amplification and sequencing of ITS-1, 5.8S, and ITS-2. In our study, nematodes were collected from the specified regions, and sequencing with the sequencing method was used to identify the samples.

DNA, ITS-1 analysis in anisakid nematodes isolated from the muscles of Codfish in a study from the Norwegian Seas: amplification, NC5 (forward) 5 GTA GGT GAA CCT GCG GAA GGA TCA TT 3 and NC13R (reverse) 5 GCT GCG TTC TTC ATC was constructed using GAT 3 primers. As a result of the study, *Anisakis simplex*, *Pseudoterranova decipiens*, *Pseudoterranova krabbei*, and their hybrid formations were identified. In the sampling areas named FAO ILA1 and ILA2, the prevalence of infection with Anisakidae nematodes was high, 88% in FAO IIa1 and 75% in FAO IIa2, but the parasite fauna composition was different. While the prevalence of infection with FAO IIa1' *Pseudoterranova* sp. was 14% lower, this rate was found to be 39% in FAO IIa2. A reverse trend was observed for the higher prevalence of *Anisakis* infection in FAO IIa1 (88%) than in FAO IIa2 (55%). The intensity of infection with Anisakidae parasites differed in both areas: *Pseudoterranova* spp. Up to 8 parasites per infected fish (abundance 0.44) in FAO IIa1 and up to 53 parasites per fish (abundance 4.34) in FAO IIa2; *Anisakis* spp. They stated that there are up to 30 parasites per fish (abundance 4.46) and up to 25 parasites per fish (abundance 2.32), respectively (Nadolna-Altyn et al., 2022).

Bannai and Jori (2022) conducted a study on the infection of anisakid nematodes, which they isolated from two marine fish species they caught from the northwest of the Arabian Gulf. The study contains various information about the structure of the anisakid population. The nematode parasites isolated from the peritoneum and viscera of *Epinephelus diacanthus* and *E. coioides* fish were subjected to molecular analysis by amplifying the internally transcribed ITS and ITS-1 spacers of nuclear rDNA (rDNA) by PCR using NC13R DNA products, NC5/NC2 and SS1/ primer sets. As a result of evolutionary analyzes based on percent identity in the GenBank database in MEGA X., nematodes isolated from anisakid nematodes, especially *Hysterothylacium* spp. showed that it belonged to nine different taxa. They noted that the presence of individuals of the same species in a host might cause these genetic variations at the species level. We also saw in our study that most nematodes isolated from four different fish individuals

belonged to the *A. pegreffii* species, and one of them belonged to the *Anisakis typica* species. This showed us how wide a distribution network the individuals belonging to the anisakidae family are and that they can form an advanced population of very different fish individuals.

Liu et al. (2022) aimed to summarize the prevalence of anisakid infection in fish in their study in eastern China. They conducted a systematic review and meta-analysis using five bibliographic databases (PubMed, CNKI, ScienceDirect, WanFang, and VIP Chinese Journal Databases). They reported that anisakid nematodes are common in a wide variety of fish species, and the prevalence of anisakid nematodes in fish in China is 45.5%. They found that fresh fish had the highest prevalence rate (58.1%). The highest prevalence rate was observed in East China (55.3%), and fish from the East China Sea showed the highest prevalence of anisakid nematodes (76.8%). In these studies, they stated that molecular techniques were used as well as traditional methods for species determination. In our study, the presence of nematode parasites embedded in the visceral surfaces of the fish bought fresh from the fish market or freely circulating in the body cavity were detected in some fish species, especially from the Canakkale fish market and which can be bought and consumed by people from all income levels. It was observed that the annual nematode rate was 4.26%. It has been determined that the most nematode fish in the Canakkale Strait are chub mackerel. The highest prevalence was calculated in March with 47 (10.68%).

To minimize the negative effects of parasite species, it is necessary to consider issues such as which type of parasite is sheltered in which fish, the seasonal prevalence of parasites, and the effects of length and weight factors on housing. It is more important to investigate parasites in fish with high economic value and used for feeding purposes. Because when enough information about parasites is obtained, it will be easier to eliminate the environments that create them. Thus, because of combating very dangerous ones, the desired efficiency will be achieved. At the same time, increasing the studies on fish parasites will enable the introduction of new species to the parasite fauna of our country and the reaffirmation of existing species.

CONCLUSION

The most fundamental issue with fish and one of the most economically significant issues with aquaculture products is "parasitic diseases" which are secondary disease agents. When parasites are transmitted to fish, they have a negative impact on the health of the fish and cause issues with commercial marketing. Due to their zoonotic characteristics, they not only cause illnesses and economic losses in fish but also major health issues in humans.

As a result, in the data obtained from the study, it was determined that *A. pergeffii* is the most common anisakid nematode in the Canakkale region. The incidence of this species, which can cause infection in humans if not consumed carefully, in economic species has shown that fish should be cleaned by complying with hygienic conditions, and consumption as raw or undercooked is the most important factor in transmission to humans. Since fish is not consumed raw in Türkiye, no parasitic diseases related to seafood have been observed in humans.

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AUTHOR CONTRIBUTION

All authors took part in designing the research, collecting, and writing the manuscript. All authors took part in a part of the article.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

This study numbered HADYEK-2019-1900050960 was evaluated at the meeting of the Animal Experiments Local Ethics Committee dated 04.04.2019 and numbered 2019/03, and it was decided that ethics committee (HADYEK) permission was not required for this research.

DATA AVAILABILITY

The data supporting the conclusions of this paper are available in the main paper.

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