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SELECTION OF MARKER REGIONS OF GENES FOR THE MOLECULAR IDENTIFICATION OF HELMINTHS OF THE *ASCARIDOIDEA* SUPERFAMILY FOUND IN COD FISH

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ABSTRACT

In recent years, the consumption of imported fish has increased significantly in Kazakhstan, especially representatives of the cod family, which are characterized by a high level of parasitic invasion. Anisakidosis is a parasitic disease caused by nematodes of the *Anisakidae* family. The main symptoms of this disease are disorders of the gastrointestinal tract and allergic reactions. Humans are random hosts, consuming raw or undercooked fish and seafood. Until recently, it was believed that heat-treated fish was not dangerous, but recent studies show a high degree of allergy to anisakid antigens, which remain active after heat treatment. In addition, existing data indicate differences in the level of allergenicity between different species of nematodes in the family. In this regard, the species identification of anisakids is relevant for taxonomy, monitoring of distribution and minimizing potential risks to humans. Therefore, the purpose of this study is to select marker regions of genes for the identification of nematodes in cod family fish. Of the four types of primers, NC13/NC2 and NC5/NC2 are the most specific and optimal for amplifying the 5.8S and ITS-2 regions of rDNA. The nucleotide sequences obtained by us were identified as nematodes of the species *Anisakis simplex* and *Hysterothylacium aduncum*.

Keywords: nematodes, *Anisakis simplex*, *Hysterothylacium aduncum,* sequencing, fish parasites.

INTRODUCTION

Cod fish such as blue whiting (*Micromesistius*), pollock (*Gadus chalcogrammus*), and haddock (*Melanogrammus aeglefinus*) are important objects of fisheries and aquaculture [1]. Cod family fish are widely consumed due to their high availability, relatively low cost, and valuable dietary properties, including low fat content and rich amino acid composition. However, they are often susceptible to high parasitic infection by various helminths, among which the *Ascaridoidea* superfamily is particularly interesting [2, 3]. The most widespread are the *Anisakidae* and *Raphidascaridae* families [4, 5, 6].

Anisakidosis is a zoonotic disease caused by nematodes of the *Anisakidae* family [7, 8, 9]. The main symptoms of this parasitic disease are gastrointestinal disorders and allergic reactions, including urticaria, angioedema, and anaphylactic shock [10, 11, 12]. Also, the available data indicate differences in the level of allergenicity between individual species of nematodes of the *Anisakidae* family [13, 14, 15].

The *Anisakidae* family includes 12 genera (*Anisakis, Contracaecum, Ophidascaris, Paranisakiopsis, Peritrachelius, Phocascaris, Pseudanisakis, Pseudoterranova, Pulchrascaris, Raillietascaris, Sulcascaris, Terranova*) and more than a hundred species, and the genus *Anisakis* alone includes at least 9 species [16]. Based on morphological and genetic features, the genus *Anisakis* is divided into two groups: *Anisakis* type 1 (including *A. simplex sensu stricto* (ss), *A. pegreffii, A. berlandi, A. typica, A. nascettii and A. ziphidarum*) and *Anisakis* type 2 (including *A. physeteris, A. brevispiculata* and *A. paggiae*) [17, 18].

The life cycle of anisakids includes the change of several hosts: marine mammals, crustaceans and fish, where the larvae go through the stages from L1 to the adult form. A person can accidentally become infected by eating fish with L3 larvae, which leads to their penetration into the mucous membrane of the gastrointestinal tract [19, 20]. Anisakids are cosmopolitan parasites, but the most widespread are in those countries where the consumption of raw fish is a cultural feature [12, 21, 22]. Thus, more than 90% of cases of anisakidosis reported in humans were detected in Japan [23, 24, 25].

Hysterothylacium aduncum, belonging to the family *Raphidascaridae*, parasitizes a large number of fish species and more than 100 species of invertebrates that are intermediate and paratenic hosts, in addition, there have been some reports of their presence in freshwater fish. This nematode species has a circumpolar distribution in the Northern Hemisphere, and is also widespread in many other waters [5, 6, 26]. Since nematodes of the genus *Hysterothylacium* have morphological similarities with anisakids, in many sources they belong to the *Anisakidae* family [27]. In 1996, the first case of noninvasive anisakidosis caused by the larvae of *Hysterothylacium aduncum* was diagnosed. Despite this, there is no precise evidence that this type of nematode has a dangerous effect on humans [28, 29]. Another important factor in the spread of helminths of the *Ascaridoidea* superfamily is an increase in water temperature due to global warming in the world, which led to changes in latitude, oceanographic conditions, as well as water circulation and salinity levels [20].

Molecular methods based on the analysis of specific regions of the genome ensure the accurate identification of the studied species, contributing to the development of the systematics of parasites, monitoring their spread and reducing risks to humans and commercial fish. Thus, the aim of the study is to select marker regions of genes for the identification of nematodes in cod family fish.

MATERIALS AND METHODS

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Sample collection

The parasites were collected from imported fish of the cod family (*Gadus chalcogrammus, Micromesistius, Melanogrammus aeglefinus*) from Astana, caught in the waters of Norway. The anisakides were extracted from the abdominal cavity and muscles of the fish, then washed and cleaned from the cuticle with distillated water. All samples were preserved in 70% ethanol for subsequent molecular and morphological examination. The collection includes several nematode species of the *Ascaridoidea* superfamily.

DNA extraction

For molecular study, genomic DNA was extracted from nematodes by pre-homogenization with lysis buffer and incubation for 2 hours at 56°C, then isolated with the GeneJET Genomic DNA Purification Kit (ThermoFisher, USA, Cat. Nr. K0721) in accordance with the manufacturer's protocol, additionally adding 5 µl of Proteinase K.

PCR analyses

To test and select primers, 10 nematode samples were selected from three fish species: *Gadus chalcogrammus* (n = 3), *Micromesistius* (n = 4), and *Melanogrammus aeglefinus* (n = 3). The PCR analysis was amplified using a set of HS-Taq PCR Biomasters (2×) (Biolabmix, English, Cat. Nr. MH010-1020). The design and parameters for each primer are shown in Table 1. To identify the most specific region of the gene, comparative analyses of sections of ribosomal DNA (rDNA) and mitochondrial DNA (mDNA) were performed. The following regions were used during DNA amplification: 5.8S and 18S. A region of the cytochrome oxidase I (COI) gene was selected for DNA amplification.

Amplicon detection of PCR products was performed using gel electrophoresis using agarose, 1x TBE buffer and ethidium bromide. For the NC13/NC2 primer pair, 1% agarose gel was used, since the amplicons obtained with their help had a size of (475 bp). 0.5% agarose gel was used to separate DNA fragments amplified using NC5/NC2 primers, since the size of the amplicons was 900 bp, for SSU_F_04/SSU_22_R primers (360 bp) and JB3.5/JB4 (450 bp) used 1.8% and 1.2%, respectively.

Sequencing and phylogenetic analysis

The positive amplification products were selected from different hosts for sequencing. The respective amplicons were purified using Exonuclease I (Thermo Fisher Scientific, USA) according to the manufacturer's protocols. Sequencing was performed according to the 3730xl DNA Analyzer 96-Capillary Array (Thermo Fisher Scientific, Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were visually checked using the Bioedit program (version 7.0) and then analysed by BLAST search against the GenBank database (https://www.ncbi.nlm.nih.gov/). Finally, the nucleotide sequences were aligned using the MUSCLE algorithm in the program MEGA11. The tree was rooted by the outgroup *H. aduncum* (PV554186).

RESULTS

During the study, 50 pollock fish samples were analysed, in which 123 larvae of anisakid stage L3 were found, 20 sam-

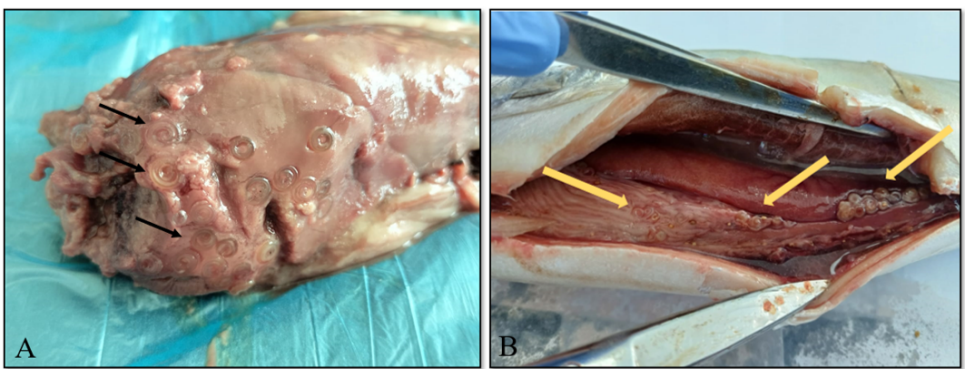

Figure 1. Localisation of anisakides in the abdominal cavity: A – cod infected with anisakides, B – pollock infected with anisakides

Table 1. Primer design and amplification parameters

Primer name	Target gene	Sequence	Parameters	References
NC13/NC2	5.8S	F: 5′-ATCGATGAAGAACGCAGC-3′ R: 5′-TTAGTTTCTTTTCCTCCGCT-3′	95°C 4 min, (94°C 30s, 60°C 30 s, 72°C 45s) 40×, 72°C 7 min	[30]
NC5/NC2	ITS-2	F:5′-GTAGGTGAACCTGCGGAAGGATCATT-3′ R:5′-TTAGTTTCTTTTCCTCCGCT-3′	95°C 4 min, (94°C 30s, 60°C 30 s, 72°C 45s) 40×, 72°C 7 min	[31]
SSU_F_04/ SSU_22_R	18S	F: 3′-GCTTGTCTCAAAGATTAAGCC-5′ R:5′-ATGTGGAGCCGTTTATCAGG-3′	95°C 2 min, (95°C 1 min, 57°C 45 s, 72°C 1 min) 30×, 72°C 10 min	[32]
JB3/JB4,5	CO1	F:5′-TTTTTTGGGCATCCTGAGGTTTAT-3’ R:5′-TAAAGAAAGAACATAATGAAAATG-3’	95°C 5 min, (95°C 50s, 50°C 50 s, 72°C 50s) 35×, 72°C 5 min	[33]

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ples of haddock fish infected with six larvae of anisakid (L3) and 59 whiting fish containing 1178 larvae (L3) of *Anisakis spp*. (Figure 1).

For morphological identification, pre-cleaned larvae were discoloured with 15% lactic acid and microscoped using a light microscope at magnification ×10 (Figure 2). Statistical data on prevalence and invasion intensity are shown in Table 2.

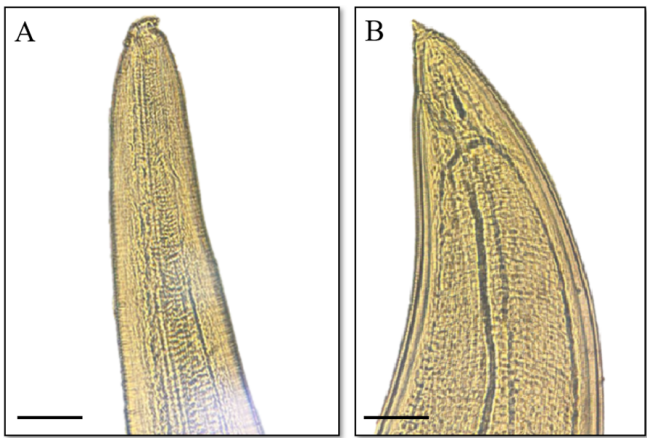

Figure 2. Morphology of larvae of anisakid stage L3 (magnification 10×): A – the front part having a mouth opening B – the back part having a mucron

Anisakid larvae had a characteristic milky color, the length was 15-22 mm, lips with a protruding tooth were located on the front part, and a cone-shaped mucron was located on the back.

To interpret the results of PCR analysis, the gel electrophoresis method was used for the following gene regions: rDNA (5.8S,18S,ITS-2) primers NC13/NC2, SSU_F_04/SSU_22_R and NC5/NC2, respectively, for mDNA, the cytochrome oxidase I (COI) gene region, primer JB3/JB4,5. Figure 3 shows the electrophorogram of the amplification of the primer NC13\NC2.

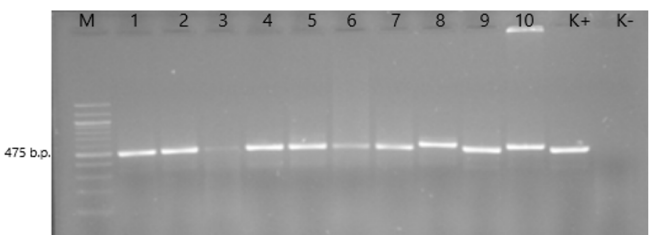

Figure 3. Electropherogram of gDNA PCR products of representative anisakide samples using a universal pair of NC13/NC2 primers: lane (M): DNA marker, lanes: (1-10) – DNA anisakides collected from *Micromesistius* (1-4), *Gadus chalcogrammus* (5-7), *Melanogrammus aeglefinus* (8-10), lane – (K+) – positive control, (K-) – negative control

As can be seen in Figure 3, all samples, with the exception of sample № 3, have clear bright stripes, which indicates high-quality amplification. The amplicons obtained average 475 bp, and small differences in their sizes should be noted, which indicates the species diversity of the samples.

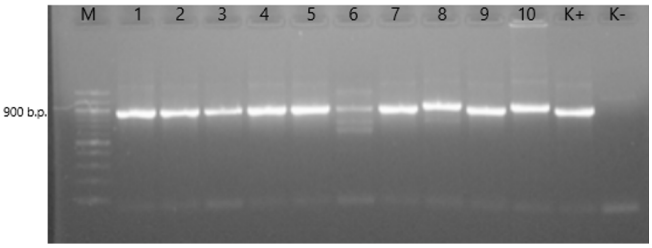

Figure 4. Electropherogram of gDNA PCR products of representative anisakide samples using a universal pair of NC5/NC2 primers: lane (M): DNA marker, lanes: (1-10) – DNA anisakides collected from *Micromesistius* (1-4), *Gadus chalcogrammus* (5-7), *Melanogrammus aeglefinus* (8-10), lane – (K+) – positive control, (K-) – negative control

As shown in Figure 4, all samples have clear bands, but there is some fragmentation. All amplicons are about 900 bp in size.

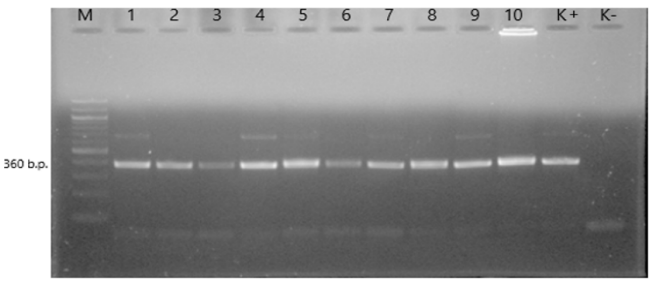

Figure 5. Electropherogram of gDNA PCR products of representative anisakide samples using a universal pair of SSU_F_04/SSU_22_R primers: lane (M): DNA marker, lanes: (1-10) – DNA anisakides collected from *Micromesistius* (1-4), *Gadus chalcogrammus* (5-7), *Melanogrammus aeglefinus* (8-10), lane – (K+) – positive control, (K-) – negative control

As shown in Figure 5, all samples have clear bands, but samples № 1,4,5,7,9 have additional amplicons, which indicates their possible fragmentation, and therefore there is a need to purify PCR products. The resulting amplicons have a size of 360 bp. which corresponds to positive control.

According to Figure 6, this primer has low specificity with nematodes, since only samples № 5 and № 7 have weak amplicons, and a fragmented plume can also be seen in all samples. The accession numbers of the deposited samples are presented in Table 3.

The phylogenetic research study analysed the relationship

Table 2. Prevalence and intensity of *anisakidosis* in marine fish

Host	N infected/N examined	% prevalence	N worms found	Range of intensity	Mean (SD) intensity	*Anisakidae spp.* species identified
Blue whiting	51/59	86	1178	3-151	23 (31)	*Anisakis simplex*
Pollock	31/50	62	123	1-21	4.1 (5.06)	*Anisakis simplex*
Haddock	3/20	15	6	1-3	2 (1)	*Hysterothylacium aduncum*
SD: standard deviation						

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Table 3. Access numbers in the Genebank of nematode samples

№	Isolate name	Helminth species	Genbank (accession number)	
			NC13\NC2	NC5\NC2
1	05-Ma-An-2	*Anisakis simplex*	PV550076	PV596436
2	05-Tc-4	*Anisakis simplex*	PV550173	PV596507
3	05-M-An-2	*Anisakis simplex*	PV550077	PV596463
4	05-M-An-4	*Anisakis simplex*	PV550078	PV596501
5	05-M-An-7	*Anisakis simplex*	PV550097	PV603363
6	05-M-An-8	*Hysterothylacium aduncum*	PV554186	-
7	05-Tc-18	*Anisakis simplex*	-	PV596698

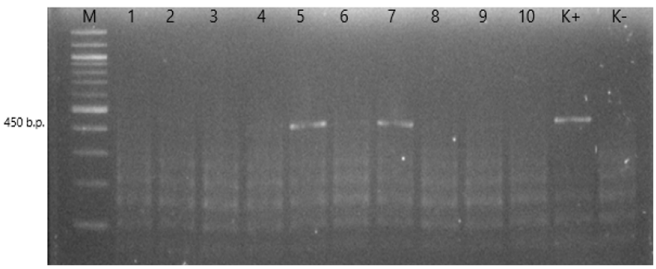

Figure 6. Electropherogram of gDNA PCR products of representative anisakides samples using a universal pair of JB3/JB4,5 primers: lane (M): DNA marker, lanes: (1-10) – DNA anisakides collected from *Micromesistius* (1-4), *Gadus chalcogrammus* (5-7), *Melanogrammus aeglefinus* (8-10), lane – (K+) – positive control, (K-) – negative control

of *Anisakis* species within the family. The tree with the highest log likelihood (Figure 7) is shown. The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura-Nei model [34]. The tree with the highest log likelihood (-2402.41) is shown. This analysis involved 19 nucleotide sequences. There were a total of 1007 positions in the final dataset.

This study incorporated four species of *Anisakis*, as illustrated in Figure 7, where all reference samples were categorized into their respective clades. The species *H. aduncum* (PV554186), included in the analysis, served as an outgroup, enhancing the strength of the phylogenetic tree. It is noteworthy that the isolate 05-M-An-7 of *A. simplex* (PV550097) clustered with other species of *A. simplex* (MT448521, MT448529, PP189864, MT448533), forming a distinct clade. The similarity between isolate 05-M-An-7 *A. simplex* (PV550097) and its most recent ancestor was found to be 37%.

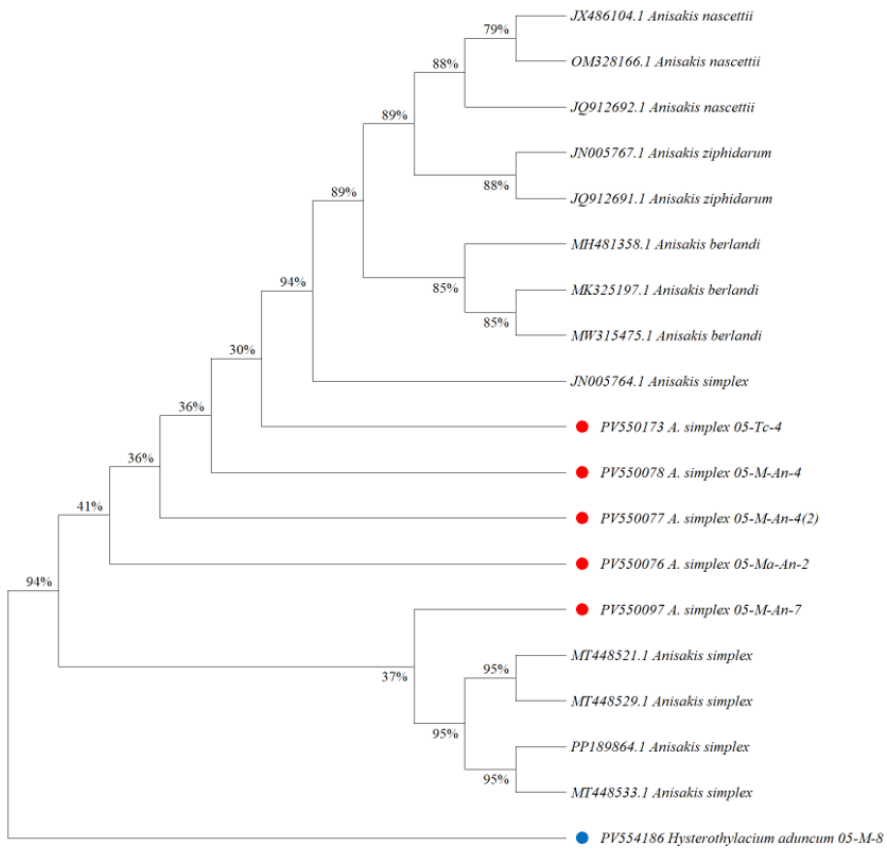

Figure 7. Maximum likelihood (ML) tree for analysing the relationships between *Anisakis* species was constructed from the nucleotide data set. Species name and host species after the accession no.; red dot: roundworms from the present study and blue triangle: outgroup.

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DISCUSSION

The cod family is one of the most consumed fish in Kazakhstan and worldwide. Thus, in 2023 alone, about 819.10 tons of cod family fish were imported to Kazakhstan [35]. According to the latest literature data, this species is often exposed to parasitic infections, which increases the chance of human infection. When infected with anisakides, the severity of the disease and the degree of allergenicity vary from species to species. According to the data presented in Table 2, a high degree of invasion was detected: pollock (*Gadus chalcogrammus*) - 62%, haddock (*Melanogrammus aeglefinus*) - 15% and whiting (*Micromesistius*) - 86%. And the invasion intensity and standard deviation were, respectively: pollock - 4.1 (5.06), haddock - 2 (1) and whiting - 23 (31).

Due to the non-specificity of interspecific morphology, the most accurate identification of nematodes is by molecular methods based on marker regions of genes. In our study, we conducted a comparative analysis of various regions of the rDNA and mDNA genes for the specific molecular identification of nematodes of the *Ascaridoidea* superfamily. The first PCR performed using NC13/NC2 primer pairs aimed at the 5.8S region of rDNA showed that the length of the PCR products is on average 475 bp. The amplicons obtained using this primer come out bright and clear, without any visible fragmentation, and their length varies depending on the type of nematodes. The second PCR using NC5/NC2 primers aimed at the ITS-2 region showed that the length of the PCR products reached about 900 bp. It is worth noting that the amplicons of this primer are bright but have some fragmentation. The SSU_F_04/SSU_22_R primer located in the 18S rDNA region also showed significant fragmentation of amplicons, and the length is 360 bp. This may be due to the presence of non-specific binding sites in the sample. When the JB3/JB4.5 mitochondrial DNA primer was amplified, only weakly luminous amplicons were present, which indicates their non-specificity to the studied samples and the absence of complementary regions in this DNA matrix.

The samples amplified by primer pairs NC13/NC2 and NC5/NC2 were sequenced, and the resulting nucleotide sequences were compared with samples from the GenBank database. The sequencing results showed 98-100% agreement with the samples of the species: *Anisakis simplex* and *H. aduncum*.

The phylogenetic analysis conducted on the ribosomal sequences of the examined samples revealed a significant close relationship among various *Anisakis* species within their taxonomic order, showcasing an identity percentage ranging from 79% to 95%. Notably, the species *H. aduncum*, which was included in this study, was positioned as an outgroup. This finding underscores the importance of exploring intraspecific variability within the *Anisakis* complex, as it sheds light on the complex dynamics of species migration across different hosts. Such insights are crucial for a deeper understanding of the ecological and evolutionary factors influencing these organisms.

CONCLUSION

It can be said that imported fish from Norway has a high degree of infection with nematodes. From this, it can be concluded that such studies are necessary for further monitoring of the parasitofauna of commercial fish species and assessing potential risks to human health.

INFORMATION ON FUNDING

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CONFLICTS OF INTEREST

Authors have no conflict of interest to declare.

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ТРЕСКА БАЛЫҚТАРЫНДА КЕЗДЕСЕТІН ASCARIDOIDEA ТҰҚЫМДАСТАР ҮСТІНІҢ ГЕЛЬМИНТТЕРІН МОЛЕКУЛАЛЫҚ АНЫҚТАУҒА АРНАЛҒАН ГЕНДЕРДІҢ МАРКЕРЛІК АЙМАҚТАРЫН ТАҢДАУ

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ТҮЙІН

Соңғы жылдары Қазақстанда импорттық балықты, әсіресе паразиттік инвазияның жоғары деңгейімен сипатталатын треска тұқымдасының өкілдерін тұтыну айтарлықтай өсті. Бұл балықты тұтыну халық арасында анизакидоздың таралуына әкелген болатын. Анизакидоз – *Anisakidae* тұқымдасының нематодтары тудыратын паразиттік ауру; оның негізгі белгілеріне асқазан-ішек жолдарының бұзылуы және аллергиялық реакциялар жатады. Адам шикі немесе жеткіліксіз түрде пісірілген балық пен теңіз өнімдерін тұтынған кезде ғана кездейсоқ иесі бола алады. Соңғы уақытқа дейін термиялық өңделген балық қауіпті емес деп есептелді, алайда соңғы зерттеулер бойынша термиялық өңдеуден кейін белсенді болып қалатын анизакид антигендеріне аллергияның жоғары деңгейі анықталды. Сонымен қатар, қолда бар дәлелдер сүйене отыра, отбасының нематодтарының әртүрлі түрлері арасындағы аллергенділік деңгейіндегі айырмашылықтарды көрсетеді. Осыған байланысты анизакидті түрлерді сәйкестендіру таксономия, таралу мониторингі және адам үшін ықтимал қауіптерді азайту үшін маңызды. Сондықтан, бұл зерттеудің мақсаты – треска тұқымдас балықтардағы нематодтарды анықтау үшін гендердің маркер аймақтарын таңдау. Зерттеуде пайдаланылған төрт праймерлердің ішінде жоғары телімділік пен оңтайлылықты NC13/NC2 және NC5/NC2 праймерлері көрсетті. Бұл праймерлер 5.8S және ITS-2 рДНҚ аймақтарын амплификациялауға мүмкіндік берді. Біз алған нуклеотидтер тізбегі *Anisakis simplex* және *Hysterothylacium aduncum* түрлерінің нематодтары ретінде анықталды.

Түйін сөздер: нематодтар, *Anisakis simplex*, *Hysterothylacium aduncum*, секвенирлеу, балық паразиттері.

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ВЫБОР МАРКЕРНЫХ УЧАСТКОВ ГЕНОВ ДЛЯ МОЛЕКУЛЯРНОЙ ИДЕНТИФИКАЦИИ ГЕЛЬМИНТОВ НАДСЕМЕЙСТВА *ASCARIDOIDEA*, ОБНАРУЖЕННЫХ У ТРЕСКОВЫХ РЫБ

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АННОТАЦИЯ

В последние годы в Казахстане значительно возросло потребление импортной рыбы, особенно представителей семейства тресковых, которые характеризуются высоким уровнем паразитарной инвазированности. Анизакидоз – паразитарное заболевание, вызываемое нематодами семейства *Anisakidae*. Основными симптомами этого заболевания являются нарушения желудочно-кишечного тракта и аллергические реакции. Человек является случайным хозяином, употребляя в пищу сырую или недостаточно приготовленную рыбу и морепродукты. До недавнего времени считалось, что термически обработанная рыба не опасна, однако последние исследования показывают высокую степень аллергии к антигенам анизакид, которые остаются активными после термической обработки. Кроме того, имеющиеся данные указывают на различия в уровне аллергенности между различными видами нематод семейства. В связи с этим видовая идентификация анизакид актуальна для таксономии, мониторинга распространения и минимизации потенциальных рисков для человека. Поэтому целью данного исследования является подбор маркерных участков генов для идентификации нематод у рыб семейства тресковых. Из четырех видов праймеров наиболее специфичными и оптимальными являются праймеры NC13/NC2 и NC5/NC2, позволяющие амлифицировать области 5.8S и ITS-2 рДНК. Полученные нами нуклеотидные последовательности идентифицированы как нематоды видов *Anisakis simplex* и *Hysterothylacium aduncum*.

Ключевые слова: нематоды, *Anisakis simplex*, *Hysterothylacium aduncum*, секвенирование, паразиты рыб.