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SPECIES COMPOSITION OF WOLF (*CANIS LUPUS*) HELMINTH FAUNA IN KAZAKHSTAN

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ABSTRACT

The article presents data on the study of the species composition of wolf helminth fauna in the period from 2019 to 2021. A total of 39 wolves (*Canis lupus*) were examined, and their parasite fauna was assessed. The study identified the following types of helminths *Echinococcus granulosus*, *Taenia krabbei*, *Dypilidium spp*., *Mesocestoides spp.*, *Toxascaris leonina*, *Trichinella nativa*, *Dirofilaria repens*. The extensiveness of the invasion wolves by helminths was relatively high in the western part region (96.5%), and towards the north-central part region, this indicator significantly decreased (65.2%). The average number of helminths was high, with some infected animals carrying several types of parasites. Thus, the intensity of invasion by helminths in wolves was 7.6 specimens per infected individual host. The research aimed to study the endoparasitic fauna of wild wolves using complex classical parasitological and molecular genetic methods. Samples of helminths were differentiated by amplification and sequencing using the marker gene cytochrome oxidase *COX1* (*GenBank* accession number: MT877205, MZ505444, MZ669895, MZ724175). There is a need to assess the distribution of helminths in intermediate and final hosts populations and provide appropriate health education to avoid maintaining parasite life cycles and infestations, which will become a public health problem in the future.

Keywords: helminth fauna, molecular, genetic analysis, identification, invasion, parasites.

1 Introduction

Predatory and some other wild mammals play a role in the conservation and spread of invasion in nature. The foci of the most dangerous zooanthroponotic diseases, such as echinococcosis, alveococcosis, opisthorchiasis, diphyllobothriasis and trichinosis, are found in natural communities [1,2]. Of all the wild carnivorous canines in Kazakhstan, the most widespread are the common fox, corsac, wolf and jackal [3, 4, 5].

Determination of wolf helminth fauna in natural biocenosis and agrocenoses is an urgent problem today [3,6]. This is due to the fact that animals play a specific role in the preservation and spread of invasion in nature. In their life cycle, wolf worms go through several stages: an egg, a larva and an adult. In the process of reproduction, most worms leave the host organism, passing into the external environment [7]. Thus, one of the factors contaminating the environment with helminth eggs is the soil, which has properties favorable for maintaining the viability and preserving the invasive origin. This, in turn, determines the possibility of transmission of the invasion to humans [3,7].

European scientists conducted a literature review to compile the final list of parasitic helminths of the wolf *Canis lupus*. Of the 27 studies studied, 72 species of helminths belonging to 40 genera were registered, infecting wolves, of which 93% were identified from the gastrointestinal tract during autopsy. They included 28 species of nematodes, 27 species of cestodes, 16 species of trematodes, and one comb. A meta-analysis of relative prevalence showed that the most common type of helminth is the tapeworm *Taenia hydatigena*, which occurs at a relative frequency of> 30% in any zoogeographic region, and the related tapeworm, *Echinococcus granulosis*, also showed a high meta-prevalence (> 19%). At the same time, the roundworm *Toxascaris leonina* was the dominant helminth species (meta-prevalence 73.9%) in the tundra wolf populations. The analysis of the species composition of helminths found in the wolf populations of European countries is more diverse than the data recorded earlier [8].

Also, in eastern Europe, scientists conducted an epidemiological survey to analyze the real parasitic fauna of Croatian wild wolves. In total, during field studies of the ecology of wolves in 2002–2011. 400 individual fecal samples were collected. Samples with positive *Taniid eggs* were further analyzed by PCR and subsequent sequencing to identify the species. A total of 18 parasite taxa were found here. Most often found in fecal samples of Sarcocystis spp. (19.1%), followed by *Capillaria spp*. (16%), *Ancylostomatids* (13.1%), *Crenosoma vulpis* (4.6%), *Angiostrongylus vasorum* (3.1%), *Toxocara canis* (2.8%), *Hammondia/Neospora spp*. (2.6%), *Cystoisospora ohioensis* (2.1%), *Giardia spp*. (2.1%), *Cystoisospora canis* (1.8%), *Cryptosporidium spp*. (1.8%), *Trichuris vulpis* (1.5%), *Taenia spp*. (1.5%), *Diphyllobothrium latum* (1.5%), *Strongyloides spp*. (0.5%), *Opisthorchis felineus* (0.5%), *Toxascaris leonina* (0.3%), *Mesocestoides litteratus* (0.3%) and *Alaria alata* (0.3%) [9].

There are data on the analysis of 1,041 samples of feces from wolf enclosures of German zoological gardens. Five nematodes (*Ancylostomatidae*, *T. canis*, *T.leonina, T.vulpis, and Capillaria / Eucoleus spp.*), One cestode (*Taeniidae*) and one trematode (*A.alatae*) were found by sedimentation and flotation [10].

In other countries of Europe and North America, the detected parasites and their prevalence are comparable to previous studies of wolf parasites [11].

During the study of the corpses of gray wolves in Turkey by macroscopic and molecular genetic methods, pathogenic parasites such as *T. hydatigena*, *M. litteratus* and *T. britovi* were detected in this animal species for the first time [12].

In the Middle Volga region and the northeast of the European part of Russia, the infestation of wolves was 89–97.8%. Nineteen species of helminths belonging to 3 classes were identified — trematodes (1 species), cestodes (7 species) and nematodes (11 species), including *A. alata*, *D. caninum*, *T. hydatigena*, *T. krabbei*, *T. pisiformis*, *T. polyacantha*, *E. granulosus*, *E. multilocularis*, *C. vulpis*, *T. aerophilus*, *C. plica*, *C. putoria*, *T. nativa*, *U. stenocephala*, *T. leonina*, *T. canis*, *A. caninum*, *S. vulpis*, *M. patens*. The most common helminths in wolves are the trematode *A. alata* (73%), the cestodes *T. hydatigena* and *E. granulosus*, the nematodes *U. stenocephala* and *T. nativa* (68.5%). The listed types of helminths form the basis of wolf helminth fauna in the territory of the Russian Federation [13].

In the article by Fataliev G. G., the complete list of canine helminth fauna in Azerbaijan and the ways of its formation is given. 35 wolves were studied in different ecological zones. This study revealed that 16 species of helminths parasitize wolves, the intensity of invasion was 100%, of the identified 12 species are biohelminths, and 4 species are geohelminths. In this case, the main dominant part was made by such species as *A. alata*, *T. hydatigena*, *T. nativa*, *U. stenocephala*, *T. leonine*, *T. canis* [14].

The revealed composition of helminths in a wolf living in the south-west of the settlement of Kulundy, on the territory of the Russian Federation bordering on Northern Kazakhstan, is represented by 3 types of cestodes, the infection of which was 80%, 2 types of nematodes, the infection rate was 30% and one species of trematodes, the intensity of invasion was 100%. Thus, the total infection of wolves with helminths was 100% [15] (Table 1).

We carried out a literature review of the data and meta-analysis of the prevalence of wolf helminths in Kazakhstan. The table below presents the available literary data on the study of wolves in the country.

The authors of the works presented in the table studied both individual helminths localized in specific organs and the entire organism of the infected host. However, it should be noted that the study of the helminth fauna of animals described in the literature presented was carried out using only classical parasitological methods, such as scatology, macroscopy, and identification of identified samples using identifiers.

As is known, these methods cannot guarantee the accuracy of determining the species composition of the helminth, in contrast to molecular genetic methods, which allow reliably identifying the species of parasites. Since wolves can be infected with a wide range of protozoa and multicellular parasites, which are pathogenic not only for wolves but also for final and many intermediate hosts, including many of which affect the health of free-living, agricultural and domestic animals.

Considering the absence or incompleteness of data in the field of helminthic fauna of one of the main carnivorous carnivores inhabiting the country's territory, we conducted a study of the endoparasitic fauna of wild wolves using molecular genetic research methods.

2 Materials and method

Work with the animals was carried out in a parasitological laboratory of a Veterinary Medicine Faculty and was approved by the Animal Ethics Committee. All procedures complied with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm). Hunters conducted the extraction of wild animals in compliance with all legislative norms. In addition, animals hunted for a limited license under a wildlife management program, and the rules for their use in research comply with the Animal Care and Use program by Guidelines of the American Society of Mammalogists for the use of wild mammals in research and education (http://www.mammalsociety.

Table-1. Species composition of wolf helminthiasis on the territory of the Republic of Kazakhstan

№	Explored area	Type of the studied helminth	Source
1	South Kazakhstan region	*Trichinella spp.*	[16]
		E. granulosus, Taenia spp., D. caninum, M. lineatus, T. canis, T. leonina, Tr. vulpis, M. catulinus, M. moniliformis	[17]
3	Alma-Ata's region	*Trichinella spp.*	[16]
4	West Kazakhstan	*Trichinella spp.*	[18]
5	Kostanay	*Trichinella spp.*	[18]
6	region	*Trichinella spp.*	[18]
		Alaria spp., Taenia spp., Dipilydium spp., Echinococcus spp., Mezocestoda spp., Toxocara spp.	[7]

org/committees/animal-care -and-use # tab3).

Autopsy of wolf carcasses. The fauna of helminths is established by the method of complete and incomplete helminthological dissection [19]. Before opening, carefully examine the skin, paying attention to bumps, growths and bruising. Then the skin is removed and the subcutaneous tissue is examined for the presence of nematodes. The abdominal and thoracic cavities are opened, after which individual organ systems are removed and placed in an appropriate container. After removing the internal organs, the chest and abdominal cavities are examined, scrapings are made, the digestive organs are carefully isolated. The synovial cavities of the joints and individual muscle groups are carefully examined (for trichinosis). After that, they proceed to the opening of particular organs by the method of sequential washing and the compressor method. The collected helminths were fixed in 70% alcohol solution. Coproscopic examinations of animals were carried out by the technique of successive washes, Fülleborn, Kalantaryan, Breza [20].

A method for digesting muscle tissue. The muscle tissue is separated from the skin, gently chopped with a knife or in a meat grinder. Muscle tissue is poured in a 1:10 ratio with artificial gastric juice (8 ml of concentrated hydrochloric acid, 3 g of pepsin per 1 liter of distilled water). The resulting mixture is mixed and placed on a magnetic stirrer for 30 minutes at a temperature of 45°C. Then, the contents are filtered into glass cylinders through a metal filter, the sediment, which contains the parasite larvae, is transferred into a Petri dish.

Methods for coloring helminths. Hematoxylin staining is carried out from 1 to 20 minutes, depending on the size of the object. The colored worms are washed with water and differentiated with 70% acidified alcohol (100 cm3 of alcohol, 15–18 drops of fuming hydrochloric acid), monitoring the process under a microscope. Wash with water, then remove water from sections in one portion of 70% ethanol, two portions of 96% ethanol. During differentiation, the parenchyma brightens to a light pink color, and organs and ducts are clearly visible against its background.

Microscopy with simultaneous photographing of objects in the prepared preparations was carried out using a Canon PS A470 camera, an Olimpus BX-47 microscope at various magnifications. Taxonomic analysis of helminths is established using keys [21, 22, 23, 24, 25].

Isolation of DNA. To isolate DNA from parasites samples, they are homogenized with special pistils in an extraction buffer, 5 µl of Proteinase K is added. Extraction and purification of DNA from the extraction buffer is carried out using the standard phenol-chloroform method, and then the DNA is precipitated by adding 380 µl of isopropanol. Next incubation for 20 minutes at a temperature of –20°C, centrifuged at 12000 rpm for 5 minutes. The resulting DNA pellet was washed with 70% ethanol, dried, and dissolved in 50 µl of 1% TE buffer.

Polymerase chain reaction. DNA is analyzed using the polymerase chain reaction (PCR) with the mitochondrial cytochrome oxidase *COX1* gene. DNA is amplified using a pair of primers, namely forward (JB3_F: TTTTTTGGGCATCCTGAGGTTTAT) and reverse (*JB4.5_R*: TAAAGAAAGAACATAATGAAAATG) [26]. Primers were synthesized by DNA-Sintez LLC, Moscow, Russia. Amplification is carried out in the final reaction mixture in a total vol of 25 µl containing 10 × Taq buffer, 2.5 mM MgCl2, 1 unit. Taq DNA Polymerase, 200 µM dNTP (Thermo Scientific, Carlsbad, CA, USA), 10 pmol of each primer, and 20 ng of sample DNA. PCR is carried out as follows: denaturation at 95°C for 60 seconds, annealing at 50°C for 60 seconds, extension at 72°C for 60 seconds, and final extension for 5 minutes at 72°C. The resulting restriction fragments were subjected to electrophoresis and separated with ethidium bromide with 1.5% agarose gel and using buffer solution 1 × TAE.

Sequencing. According to the manufacturer's specifications, the amplified DNA fragments are sequenced by the Sanger method using the BigDye Terminator Sequencing Kit. Upon completion of sequencing, the reaction products were purified with an acetate-alcohol mixture. Sequencing products were studied using a SeqStudio genetic analyzer (Thermo fisher, USA). The chromatogram analysis and editing were performed using Sequencing Analysis 5.2, Patch 2 (Applied Biosystems).

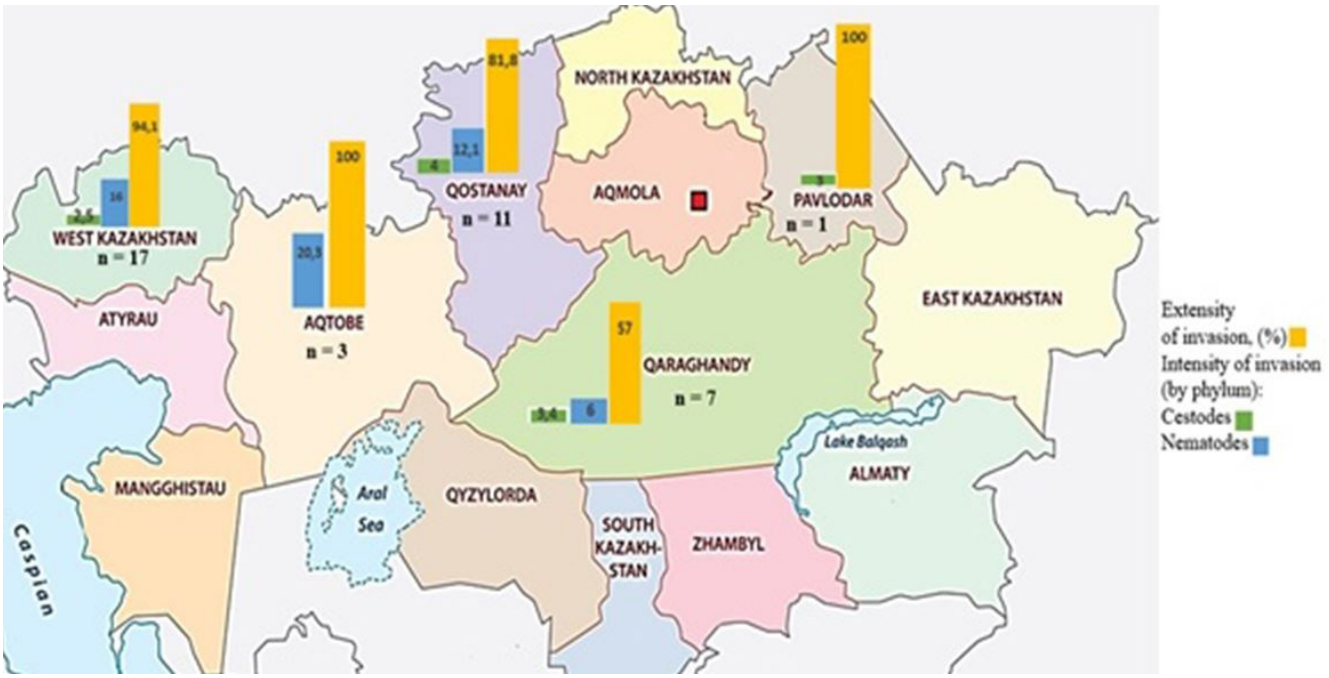
3 Results

For the study, the carcasses of 39 wolves were used, provided by the republican hunting associations by private hunters from various regions of Kazakhstan in the period 2019–2021, after which they were studied in the parasitology laboratory of the Department of Veterinary Medicine.

Figure 1 provides a map showing the area where wolves were caught and the intensity and intensity of infestation by helminths.

The study of the helminth fauna of wolves of the Kostanay region showed the predominant distribution of parasites of the class *Nematoda* and *Cestoda*, including species *Ascaridae*, *Taenia spp*., *Trichinella spp*., *Echinococcus spp*. and *Dirofilaria spp*., extensivity invasion wasa 81. 8%. In the Karaganda region, 7 carcasses of wolves were studied, the helminthiasis infection is 57%, the predominant number of identified parasites belong to the species *Ascaridae*, *Taenia spp*. and *Trichinella spp*.

In samples of the internal organs of a wolf caught in the Pavlodar region, a helminth of the genus *Taenia* was found, the EI, respectively, was 100%. In the delivered and examined sections of the muscle tissue of wolf carcasses of Aktobe region, larvae of *Trichinella spp*. Were found, and the infection was 100%. When examining the internal organs, muscle tissue and diaphragm of wolves of the West Kazakhstan region, it was found that the invasion was 94.1%. Helminths of the genus *Taenia* and Trichinella larvae were widespread, and single specimens of *Mesocestoides spp.*, *Dipylidium spp*. were also found.

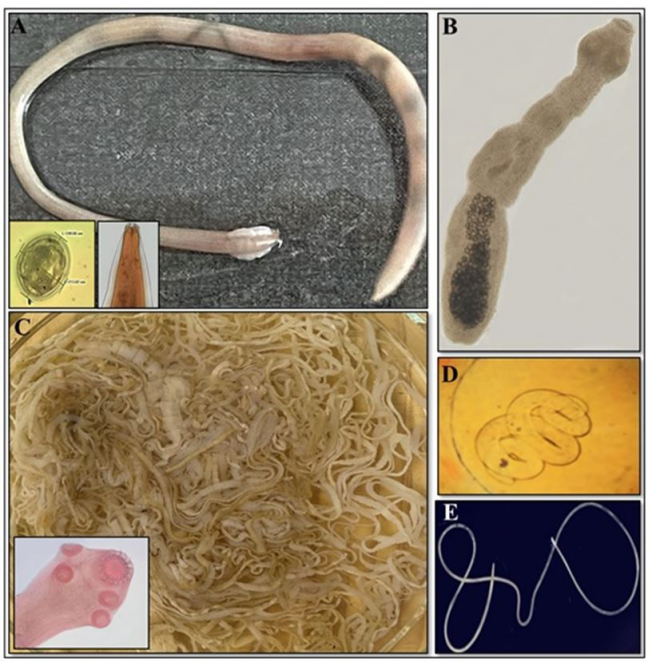


n — number of heads studied

Figure-1. Helminthological indicators of wolves in various areas

Species identification of the found parasites was carried out according to morphological features using determinants. At the same time, special attention was paid to the structural characteristics of the suckers (oral and abdominal), organ systems (digestive, excretory, sexual and nervous), in cestodes, special attention was paid to the structure of scolexes, necks and segments (proglottids) (Figure 2).

In a macroscopic examination, cestodes were identified by morphological features; attention was paid to the presence of 4 suckers on the scolex, the number of segments in the strobilus, and the segments representing a rectangle, the uterus and its lateral branches, as well as spherical eggs.



A –*Toxascaris leonine*; B–*Echinococcus granulosis*; C — *Taenia hydatigena*; D — *Trichinella nativa*; E — *Dirofilaria repens*

Figure-2. Morphological features of the main species of wolf helminths

Echinococcus bubbles were detected by the presence of a transparent liquid in them and the presence of a dense grayish-white connective membrane on the surface.

Roundworms were differentiated by the narrow semi-lanceolate lateral wings located at the head end and the ventricle located between the esophagus and intestines. The isolated roundworm eggs had a rounded appearance, with a thick, smooth shell of a light gray color.

Trichinella was determined as follows: muscle tissue sections were placed in a compressor and examined under slight magnification. Trichinella capsules were egg-shaped, lemon-shaped or round depending on the type of host. Inside them were spirally rolled larvae that were not visible in the calcified capsules.

Dirofilariasis nematodes were recognized by their localization in the host organism and the light yellow color of the filamentous body, tapering towards both ends.

When identifying the cestodes of dipylidiosis, attention was paid to the shape of its proglottides, similar to cucumber seeds or resembling rice grains or sesame seeds.

Differentiation of *Mesocestoides spp.* from other representatives of the cestode class, was carried out on pregnant proglottids, which, as a rule, are smaller than in *Dipylidium caninum* and *Taenia taeniaeformis*, and there were differences in the sizeable amniotic organ present in the segments containing the eggs of this worm, and there was also a hexacanth embryo. At the front end of the worm, there was a scolex with four distinct suckers, on which there was neither a rostellum nor a scolex, which is also a feature of this species.

Helminth samples were differentiated by amplification and sequencing, by the method described above, using a mitochondrial fragment of the marker gene cytochrome oxidase *COX1* (JB3; JB4.5) [26].

Having conducted a comprehensive species identification of helminths using traditional and modern methods, the main species composition of the helminth fauna of wolves in the studied territory, which includes 5 regions of the republic, was determined (Table 2).

Table-2. Helminthofauna of the wolf in the study area

№	The latin name for helminths	Found helminths specimen	Place of localization of helminths	Intermediate host
Cestodes				
1	*Taenia hydatigena*	65	intestines	Cervids, large vertebrates
2	*Mesocestoides spp*	2	small intestine	Ticks, small vertebrates
3	*Dipylidium caninum*	3	small intestine	Fleas, lice
4	*Echinococcus granulosis*	15	intestine	Moose, cervids
Nematodes				
1	*Toxascaris leonine*	10	intestine	Rodents
2	*Dirofilaria repens*	1	heart	Mosquitoes
3	*Trichinella nativa*	185	muscle tissue, diaphragm	Mammalian range

4 Discussion and conclusion

Non-invasive techniques such as macroscopy and coprology are built on morphological features and are often the only option when studying parasitic wildlife. However, the detection and identification of parasites is greatly improved when combined with molecular biology techniques. Studying parasite DNA can provide alternative approaches in diagnosis and are more sensitive and specific if reliable molecular genetic markers are used in tests.

Based on the above, the material obtained after the dissection of the animals was studied in a complex manner, using classical parasitological and molecular genetic methods. These were deposited in the NCBI *GenBank* database (MT877205, MZ505444, MZ669895, MZ724175).

In order to study the evolutionary relationship between different types of identified helminths that have a common ancestor, research was carried out in the field of bioinformatics analysis. For this, a phylogenetic tree was built, showing the evolutionary relationship of taxa (Figure 3).

The evolutionary history was derived using the Neighbor-Joining (NJ) method [27]. An optimal tree with the sum of the lengths of the branches = 132.69336331 is

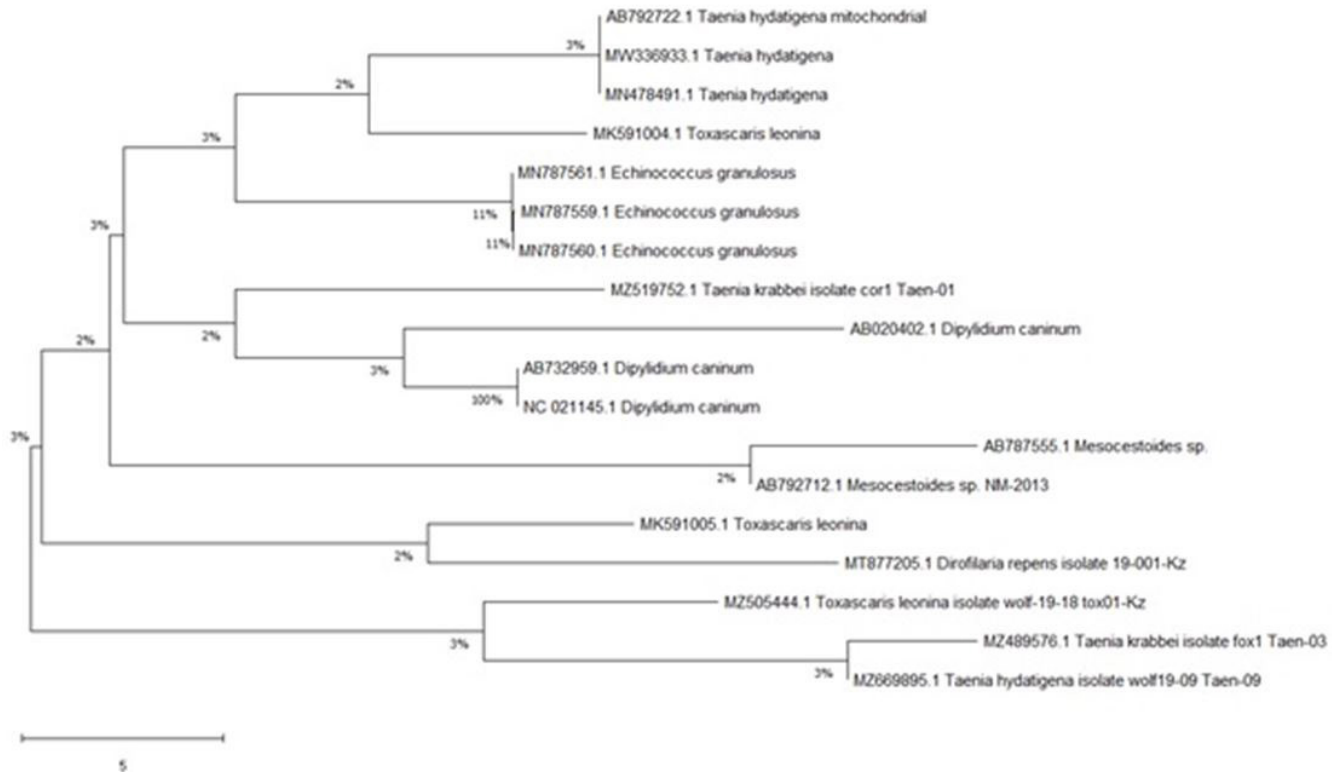


Figure-3. Evolutionary relationships of taxa

shown. The tree is drawn to scale with branch lengths in the same units as the evolutionary distances used to derive the phylogenetic tree. Evolutionary distances were calculated using the maximum complex likelihood method [28] and are expressed in terms of the number of base substitutions per site. This analysis included 18 nucleotide sequences. All ambiguous positions were removed for each pair of sequences (pairwise removal option). There were only 14,297 positions in the final dataset. Evolutionary analysis was carried out in MEGA X [29].

When conducting phylogenetic analysis using methods based on genetic distances (NJ), where the species were first combined into a common tree, and then clusters of closer sequences were sequentially separated, the result is an unrooted tree, with different homology ranging from 2 to 11%.

Thus, the NJ method showed that the *Toxascaris leonine* isolate (MZ505444) formed a well-maintained monophyletic sister clade with the *Taenia hydatigena* branch (MZ669895), which indicates the origin of organisms from a common ancestor. Also, *Dirofilaria repens* (MT877205), formed a clade with nematodes similar in general class because they are connected through one internal knot.

Molecular analysis confirmed the identification of the analyzed samples, which unites related species and their common ancestor, the phenotypic features of which, having been preserved in the descendants, have become standard diagnostic features of this group of taxa.

The analysis of the study of the prevalence showed that the most common type of helminths is the tapeworm of the cestode class *Taenia hydatigena*, which was detected in any zoogeographic region of the country. Also among the nematode class, *Trichinella nativa* was the dominant helminth species in the steppe wolf populations. Ascaris *Toxascaris leonina* has been found in wolf populations in the central and northern parts of the country. During the study of wolf carcasses, a single case of dirofilariasis of the wolf heart caused by the pathogen *Dirofilaria repens* was revealed [30]. However, it should be noted that, during the studies, helminths of the trematode class were not found.

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ҚАЗАҚСТАНДАҒЫ ҚАСҚЫРДЫҢ (CANIS LUPUS)ГЕЛЬМИНТТІК ФАУНАСЫНЫҢ ТҮРКҰРАМЫ

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АБСТРАКТ

Мақалада 2019 жылдан 2021 жылға дейінгі кезеңде қасқырлардың гельминттік фаунасының түрлік құрамын зерттеу деректері келтірілген. Барлығы 39 қасқыр (*Canis lupus*) зерттеліп, олардың паразиттік фаунасына баға берілді. Зерттеу барысында гельминттердің *Echinococcus granulosus*, *Taenia krabbei*, *Dypilidium spp.*, *Mesocestoides spp.*, *Toxascaris leonina*, *Trichinella natativa*, *Dirofilaria repens* түрлері анықталды. Қасқырлардың гельминттермен зақымдануының таралуы облыстың батыс бөлігінде (96,5%) салыстырмалы түрде жоғары болды, ал солтүстік-орталық бөлігінде бұл көрсеткіш айтарлықтай төмендеді (65,2%). Гельминттердің орташа көптігі жоғары болды, кейбір ауру жануарларда паразиттердің бірнеше түрі бар. Осылайша, қасқырлардағы гельминттердің инвазиясының қарқындылығы 7,6 инд. жұқтырған жеке жануарға. Зерттеудің мақсаты кешенді классикалық паразитологиялық және молекулалық-генетикалық әдістерді қолдана отырып, жабайы қасқырлардың эндопаразиттік фаунасын зерттеу болды. Гельминт үлгілері цитохром оксидазасының *COX1* маркерлік генін (*GenBank* қосылу нөмірі: MT877205, MZ505444, MZ669895, MZ724175) пайдаланып амплификация және секвенирлеу арқылы сараланды. Гельминттердің аралық және түпкілікті иесі популяцияларында таралуын бағалау және болашақта қоғамдық денсаулық мәселесіне айналатын паразиттердің үздіксіз айналымын және инвазиясын болдырмау үшін тиісті медициналық білім беру қажет.

Түйін сөздер: гельминт фаунасы, молекулалық, генетикалық талдау, идентификация, инвазия, паразиттер.

ВИДОВОЙ СОСТАВ ГЕЛЬМИНТОФАУНЫ ВОЛКА (CANIS LUPUS) В КАЗАХСТАНЕ

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АБСТРАКТ

В статье представлены данные по изучению видового состава гельминтофауны волков в период с 2019 по 2021 годы. Всего было обследовано 39 волков (*Canis lupus*) и оценена их паразитофауна. В результате исследования выявлены следующие виды гельминтов *Echinococcus granulosus*, *Taenia krabbei*, *Dypilidium spp.*, *Mesocestoides spp.*, *Toxascaris leonina*, *Trichinella nativa*, *Dirofilaria repens*. Экстенсивность инвазии волков гельминтами была относительно высокой в западной части региона (96,5%), а по направлению к северо-центральной части этот показатель значительно снижался (65,2%). Средняя численность гельминтов была высокой, при этом некоторые инфицированные животные были носителями нескольких видов паразитов. Таким образом, интенсивность инвазии гельминтами у волков составила 7,6 экз. на одного зараженного индивидуального хозяина. Целью исследования было изучение эндопаразитарной фауны диких волков комплексными классическими паразитологическими и молекулярно-генетическими методами. Образцы гельминтов дифференцировали путем амплификации и секвенирования с использованием маркерного гена цитохром оксидазы *COX1* (инвентарный номер GenBank: MT877205, MZ505444, MZ669895, MZ724175). Существует необходимость оценки распространения гельминтов в популяциях промежуточных и окончательных хозяев и обеспечение соответствующее санитарное просвещение, во избежание сохранения циркуляции паразитов и инвазии, которые в будущем станут проблемой общественного здравоохранения.

Ключевые слова: гельминтофауна, молекулярный, генетический анализ, идентификация, инвазия, паразиты.