DISTRIBUTION AND GENETIC FEATURES OF THE CAUSATIVE AGENT OF *ALARIA ALATA* IN WILD CARNIVORES OF THE STEPPE AND FOREST-STEPPE ZONES OF KAZAKHSTAN

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ABSTRACT

This article presents data on the study of the *Alaria spp.* pathogen isolated from wild carnivores (corsacs, foxes, wolves) caught in the territory of Karaganda, Kostanay, Akmola, and East Kazakhstan regions in the period from 2019 to 2022. As a result of the autopsy of wild animals, a collection of *Alaria spp.* was collected, the intensity of invasion varied from 1 to 1489 samples/animal, and the prevalence of invasion ranged from 10 to 36.11%. The primary taxonomic affiliation of the pathogen was carried out using determinants. Genetic identification was carried out by amplification of a section of the *internal transcribed spacer 2* (*ITS2*) marker gene. The resulting 557 bp amplicons. sequenced using the Sanger method. The nucleotide sequences were deposited in the international GenBank database under accession numbers: from the fox - OM630451, ON248128, ON248130, from the corsac - OM630460, ON248043, ON248044, ON248045, from the wolf - ON358106. Bioinformatics analysis to build a phylogeny showed that the samples of *Alaria alata* studied by us were combined into a separate cluster by identity, which ranged from 90-98%.

Keywords: wild carnivores, *Alaria alata*, intensity, extensiveness, *ITS2*, genetic identification.

1 INTRODUCTION

Alaria alata is a parasitic trematode from the family *Diplostomatidae* of the genus *Alaria*. The genus *Alaria* includes the following species - *A. mustelae, A. intermedia, A. marcianae, A. arisaemoides, A. canis (syn. A. americana), A. taxideae*, which are found in North and South America (Möhl et al. 2009), as well as the species *A. alata* common in Europe [1].

The life cycle of the genus *Alaria* is complex and includes definitive, intermediate, and paratenic hosts (Figure 1). The definitive hosts are predators, including foxes, wolves, raccoons, lynxes, martens, badgers, dogs, and cats [2, 3]. They become infected by eating frogs and tadpoles containing mesocercari.

Carnivores infected with *Alaria spp.* excrete parasite eggs into the external environment with feces, which, under optimal temperature conditions (21-27°C), mature within 11-12 days until the formation of miracidia. The miracidia released from the eggs penetrate the freshwater mollusks *Planorbis planorbis* and *Planorbis vortex*. In shellfish, at a temperature of 22-24°C for 37-45 days, and at a temperature of 18-19°C for 77 days, miracidia turn into cercariae. The cercariae that have left the body of mollusks are actively introduced into additional hosts - tadpoles and frogs, where they develop to the stage of metacercariae. Animals that are the definitive hosts become infected by eating frogs, tadpoles, and reservoir hosts infested with alaria metacercariae. The full development cycle of *Alaria spp*. from egg to sexually mature stage (under favorable temperature conditions) lasts 92-114 days. Representatives of the *Canidae* family were considered the definitive hosts, but other predators, such as the wolf, fox, and corsac (*Felidae*), as well as representatives of mustelids, have now been found [1].

Parathenic hosts such as wild boars, mice, rats, martens, ferrets and pigs, as well as wild birds and some species of snakes and lizards, can also participate in the life cycle of this parasite. Like the definitive hosts, they can become infected

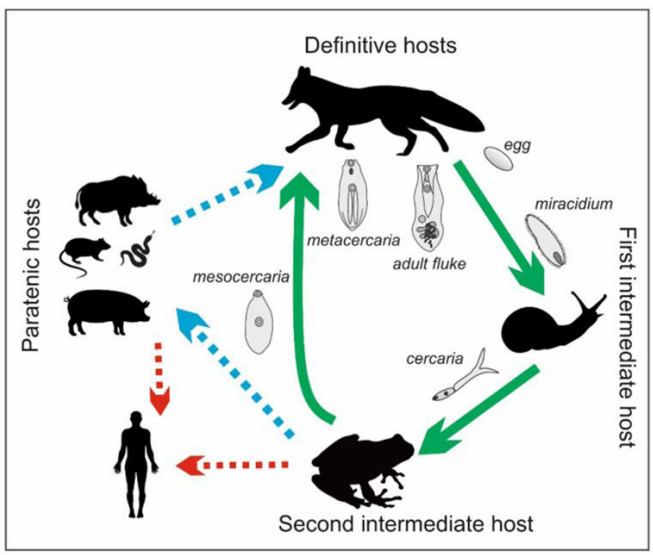


Figure 1. Life cycle of *A. alata* (by J. Karamon).

by eating intermediate mesocercariae (tadpoles and frogs) or other paratenic hosts [4]. Within these hosts, the mesocercariae do not reach the adult trematode stage; however, they can survive for months in the connective tissue between the muscles or in the adipose tissue, which is a kind of reservoir for this fluke for definitive hosts or other paratenic hosts.

The migration of mesocercariae from one paratenic host to another does not reduce the infectivity of the parasite [1]. In addition, humans can become paratenic hosts by consuming mesocercariae, which are present in raw or undercooked game, pork, frog legs, or snails [5]. A. alata infections in humans cause alariosis.

Epizootiological aspects of the trematode *Alaria spp.* on the territory of Kazakhstan have not been studied, there is only partial data obtained from a comprehensive assessment of the parasite fauna of different animal species. Thus, there are reports of detection of Alaria alata species in dogs of Zhambyl, Pavlodar, Akmola and North Kazakhstan regions of Kazakhstan [6, 7, 8].

Molecular genetic studies to identify *Alaria alata* are carried out using traditional marker regions of the genome. Thus, Riehn K. et al. based on complete small subunit ribosomal RNA gene (ssrDNA) and partial (D1–D3) large subunit ribosomal RNA gene (lsrDNA) were used to estimate the phylogeny of *A. alata.* For the selection of a suitable primer set in the 445 to 747 bp region of the *A. alata* genome [9]. Kästner C. et al. in their research for the genetic identification of *Alaria spp*. in wild carnivores, the 18S rDNA region was used; the length of fragments of trematode PCR products was about 750 bp. [10]. This points to the available tools for genetic identification of the trematode *Alaria spp.*

It should be noted that the study of the genetics of Alaria *spp.* a pathogen circulating in Kazakhstan has not been carried out. Taking into account all the above gaps in the study of the trematode *Alaria alata*, we set a goal to study the prevalence and genetic characteristics of *Alaria alata* in wild carnivores of the steppe and forest-steppe territory of the Republic of Kazakhstan.

2 MATERIALS AND METHOD

Sample collection

Alaria isolated from wild carnivores (corsacs, foxes, wolves) caught in the territory of Karaganda, Kostanay, Akmola and East Kazakhstan regions in the period from 2019 to 2022 were taken as research material. Work with the animals was carried out in a parasitological laboratory of a Veterinary Medicine Faculty and was approved by the Animal Ethics Committee (No. 1 dated July 24, 2019). 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). Parasitological sampling methods have been applied, such as the serial drain method and the simple centrifugation method.

Statistical analysis

In the present study, three main indicators of the abundance of parasites were used: the extensiveness of invasion, intensity, and abundance index, which, in fact, are indicators of host infestation. Differences in the prevalence of infections among regions were estimated by a Chi-square test. All statistical analyses were performed using Statistix 10 (Analytical Software).The differences were considered statistically significant when $p < 0.05$.

DNA extraction method

To isolate DNA, one part of an adult worm was homogenized in an Eppendorf centrifuge tube by the standard phenol-chloroform method with proteinase K followed by ethanol precipitation [11]. The amount and purity of the isolated DNA were determined by measuring the absorbance at 260 and 280 nm using a NanoDrop 2000 instrument (Thermo Scientific, USA). DNA was dissolved in ddH2O and stored at –20°C.

PCR and sequencing

The studied helminths were differentiated by amplification using the ribosomal internal transcribed spacer *ITS2* (F: GAACATCGACATCTTGAACG, R: GGAACGACCTGAACACCA) [12]. For amplification, a mixture was prepared in a total volume of 25 µl containing 25 ng of DNA, 1U of DNA polymerase (Thermo Scientific, USA), 0.2 mM of each dNTP, 1x PCR buffer, 2.5 mM MgCl2, 10 pmol of each primer. The PCR program was performed on a SimpliAmp cycler (Thermo Fisher Scientific, USA).

Amplified DNA fragments were sequenced using the Sanger method using the BigDye terminator sequencing kit according to the calculation for a total volume of 25 µl for each sample - dH2O - 18 µl, 5x buffer - 5 µl, BigDye - 0.5 µl, primer - 0 .5 µl, PCR product – 1 µl. The primer sequences used were the same as for PCR. To ensure sequencing accuracy, the amplified fragments were sequenced with two primers: forward and reverse. The sequencing products were studied on an ABI 3130XL genetic analyzer (Applied Biosystems, USA). Chromatogram analysis and editing were performed using Sequencing Analysis 5.2, Patch 2 (Applied Biosystems, USA).

The multiple alignments of obtained sequences were performed using the ClustalW algorithm in MEGA (v.11) software [13]. Alignments were exported as a NEXUS extension and used as input for the DnaSP6 program [14]. The phylogenetic analysis was constructed according to the neighbor-joining (NJ) method using MEGA (v.11) software.

3 RESULTS

The work was carried out based on the Scientific Research Platform of Agricultural Biotechnology and the Laboratory of Parasitology of the Department of Veterinary Medicine of the S. Seifullin Kazakh Agrotechnical University.

Pathogen *Alaria spp*. was isolated from foxes (*Vulpes vulpes*), corsacs *(Vulpes corsac*) and wolves (*Canis lupus*) (*Linnaeus*) caught in various regions of Kazakhstan. Figure 2 shows the trematodes found during the autopsy.

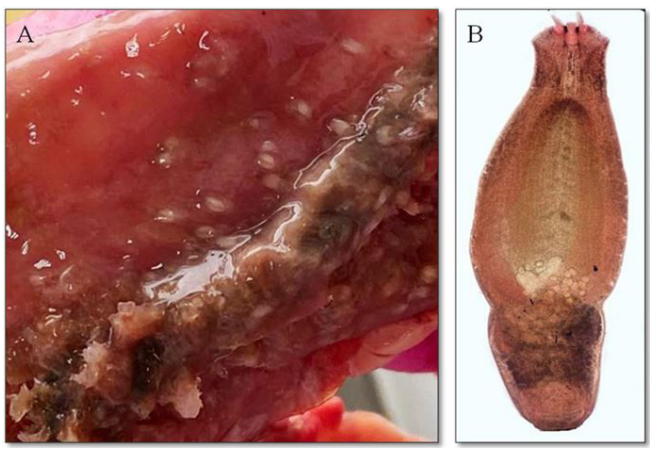


Figure 2 - *Alaria spp.:* A) localization in the intestine; B) mature miracidia

During the parasitological autopsy of animals, trematodes of the genus *Alaria spp.* were found mainly in the anterior small intestine in almost all infected animals (figure 1A). The body of *Alaria spp.* in the adult stage is 3 to 6 mm long and 1 to 2 mm wide, and it is divided into two sections. The front part of this fluke has a wing-like shape and ends in an additional clinging Brandes organ. It contains four clavate cells, which despite their glandular appearance do not have ducts (figure 1B) [15].

According to the results of a comprehensive study of the distribution of *Alaria spp*. on the territory of Kazakhstan, the

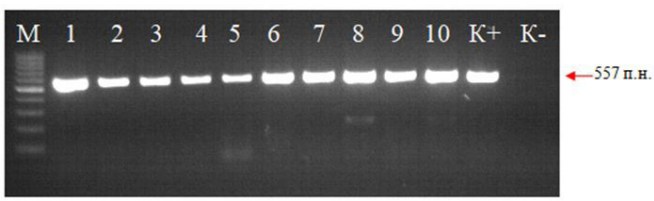
following data were obtained: in the Karaganda region, 31% of the studied foxes (8 out of 26 animals) were infected, and in the Akmola region, 36% of foxes (13 out of 36 animals) were carriers of trematodes, and in the Kostanay region, the percentage of infected foxes was 40% (2 animals out of 5). In the East Kazakhstan region, 10 wolves were studied, of which 10% were carriers of the trematode *Alaria spp.*. The results of the extensiveness and intensity of the invasion of various animal species on the territory of Kazakhstan are presented in Table 1.

Table 1 - Extensiveness and intensity of invasion

Place of capture	Host	EI, %	II
Karagandy region	*Vulpes vulpes*	30,76	13,625
Kostanay region	*Vulpes vulpes*	40	11
Akmola region	*Vulpes vulpes*	36,11	148,61
	Canis lupus	10	1
East Kazakhstan region	*Vulpes corsac*	14,28	10

As can be seen from Table 1, the maximum rate of invasion extensiveness was 36-40% in foxes in the Kostanai and Akmola regions. Due to a small sample of material, the smallest number of applicants is among corsacs in the East Kazakhstan region and wolves in Akmola region. Invasion intensity as well as turnover according to a fair assessment of the material.

For the genetic identification of isolated trematodes *Alaria spp.,* work was carried out to optimize the parameters of the PCR protocol with a specific primer for the amplification of the *ITS2* gene region. The reaction mixture was prepared in a volume of 25 µl for each sample: DNA polymerase 1U - 0.5 µl, 2 mM dNTP - 3 µl, 1-x PCR buffer - 3 µl, 2.5 mM MgCl2 - 3 µl, 10 pmol of each primer - 1 µl. The standard program was used with optimization of the annealing temperature of 57°C. Detection of the result was performed on a 1.5% agarose gel prepared in 1×TAE buffer and EtBr. The result of the electropherogram is shown in Figure 3.



M, DNA marker (100-1000 bp); 1-4 - DNA samples of *Alaria spp.* isolated from corsacs; 5-9 - DNA samples of *Alaria spp.* isolated from foxes; 10 DNA samples of *Alaria spp.* isolated from the wolf; K+, positive control; K- - negative control

Figure 3 – The results of the electropherogram of PCR products *Alaria spp.*

Figure 3 shows the results of PCR analysis with DNA samples from *Alaria spp.* and specific primers. The length of the obtained amplification products was 557 bp, which corresponds to the literature data. There is no specific band in the negative control, which excludes contamination during PCR.

Helminth samples were differentiated by amplification and sequencing as described above using the ribosomal fragment of the marker gene of the internal transcribing spacer *ITS2*.

Having carried out a complex species identification of helminths using traditional and modern methods, the main species of helminth was identified as *Alaria alata*.

Alignment of the *ITS2* rDNA sequences obtained from all analyzed mesocercariae showed 100% sequence identity, which indicates the absence of variability for this genetic marker (figure 4). Sequences confirming the identification of the species, already deposited in GenBank under accession numbers from the fox - OM630451, ON248128, ON248130, corsac - OM630460, ON248043, ON248044, ON248045, wolf - ON358106.

In the *ITS2* locus, among 10 sequences showing from 96.03% to 100% identity, there are differences from each other from one to two single nucleotide variants.

For sequencing, 3 samples were taken from each animal. The deposition was subjected to 1 sample from each animal from different regions of the country. A total of 8 samples were deposited: 3 samples from the corsac, 3 samples from the fox and 1 sample from the wolf. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model [16] which is presented on figure 5. The tree with the highest log likelihood (-15167.30) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with a superior log likelihood value. This analysis involved 17 nucleotide sequences. There were a total of 8431 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [17].

A monophyletic clade was developed that united related species according to their common ancestor, the phenotypic features of which, preserved in the descendants, became the common diagnostic features of this group. The bootstrap method analyzed the statistical assessment of the resulting tree's reliability. The number of Bootstrap Replications = 300.

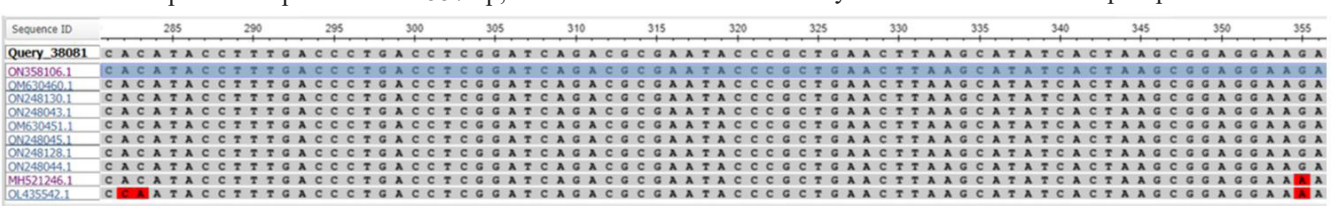


Figure 4 - Alignment of 10 different sequences *ITS2* of *Alaria spp.* isolates collected from wild carnivores (fox, wolf, corsac).

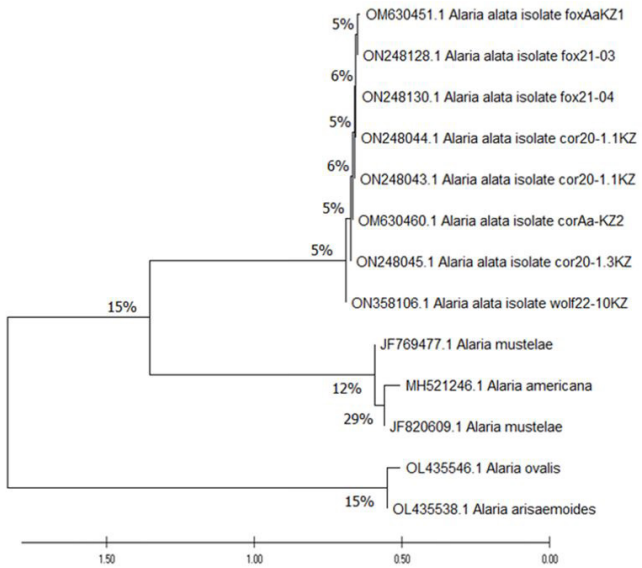


Figure 5 - A neighbor-joining haplotype tree of *Alaria alata*

As can be seen from the phylogenetic analysis, the samples of *Alaria alata* studied by us were combined into a separate cluster by identity. As with other *Alaria* species, the number of substitutions in pairwise sequence comparisons is higher, with genetic distances ranging from 5 to 15%.

4 DISCUSSION AND CONCLUSION

In Kazakhstan, a wild carnivore is one of the most popular types of hunting and can cause a risk of human infection with different parasites. Due to a lack of knowledge of prevalence data of *A. alata* in wolves, foxes, and corsacs, this study aimed to examine the occurrence of *A. alata* in wild carnivores in Kazakhstan territory over a significant period of time.

In the article, for the first time, molecular genetic identification of *A. alata* was carried out, since there is no research data in domestic and foreign publications. Domestic publications provide data on the conduct of a complete and incomplete helminthological autopsy according to Skryabin, where the percentage of obtained trematodes is mainly given, but there are no specific figures on the intensity and extensiveness of the invasion of the spread of alariasis in the territory of Kazakhstan. An autopsy of wild carnivores with alariasis reveals peritonitis, catarrhal, sometimes hemorrhagic, and inflammation of the mucous membrane of the stomach and intestines. Therefore, alariasis can infect domestic animals and people, which can affect their health.

In this regard, there is a need to study the spread of alariasis, as well as to conduct genetic identification to establish the characteristics of the pathogen *Alaria alata* in wild carnivores.

Our study showed a significant infection of wild carnivores with alariasis in the studied areas of the country. The intensity of invasion varied from 1 to 148.61 specimens per animal. Data on the extent of invasion were from 10 to 40% for infected animals.

The molecular analyses of the *ITS2* gene, initially done to confirm the morphological identification of the parasite, demonstrated no genetic variability between different isolates, collected from different wild carnivores.

The identifications of ten different sequences based on the *ITS2* gene (figure 4) analysis introduce a new area of investigation of *A. alata* as no intraspecific genetic differences had been previously reported in Asian *A. alata* isolates.

The phylogenetic analysis produced a monophyletic clade (figure 5) with low bootstrap values which suggest a lack of evolutionary differentiation among the *A. alaria* sampled. Alternatively, this may indicate a lack of informative sites given the relatively short DNA sequences collected. Further marker development and sequencing would be required to differentiate between these possibilities. Bioinformatics analysis for the study of phylogeny clearly showed that the samples we studied belong to the same clade. In particular, you can pay attention to a significant difference in divergence with other species of this family.

5 FUNDING

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РАСПРОСТРАНЕНИЕ И ГЕНЕТИЧЕСКИЕ ОСОБЕННОСТИ ВОЗБУДИТЕЛЯ *ALARIA ALATA* ДИКИХ ПЛОТОЯДНЫХ ЖИВОТНЫХ СТЕПНОЙ И ЛЕСОСТЕПНОЙ ЗОНЫ КАЗАХСТАНА

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АБСТРАКТ

В данной статье представлены данные по изучению возбудителя A*laria spp.*, выделенного от диких плотоядных животных (корсаки, лисы, волки), отловленных на территории Карагандинской, Костанайской, Акмолинской и Восточно-Казахстанской областей в период с 2019 по 2022 годы. В результате проведенного вскрытия диких животных была собрана коллекция алярий, интенсивность инвазии варьировала от 1 до 1489 экз./животное, экстенсивность инвазии составляла от 10 до 36,11%. Первичная таксономическая принадлежность возбудителя проводилась с помощью определителей. Генетическая идентификация была проведена путем амплификации участка маркерного гена *internal transcribed spacer 2 (ITS2)*. Полученные ампликоны длиной 557 п.н. секвенировали по методу Сэнгера. Нуклеотидные последовательности были депонированы в международной базе данных GenBank под инвентарными номерами: от лисы – OM630451, ON248128, ON248130, корсака – OM630460, ON248043, ON248044, ON248045, волка – ON358106. Биоинформатический анализ для построения филогении показал, что изученные нами образцы *Alaria alata* были объединены в отдельный кластер по идентичности, который составил от 90-98%.

Ключевые слова: дикие плотоядные, *Alaria alata,* интенсивность, экстенсивность, *ITS2*, генетическая идентификация.

ҚАЗАҚСТАННЫҢ ДАЛАЛЫҚ ЖӘНЕ ОРМАНДЫ ДАЛАЛЫҚ ЗОНАЛАРЫН МЕКЕНДЕЙТІН ЖАБАЙЫ ЖЫРТҚЫШТАРДАҒЫ *ALARIA ALATA* ҚОЗДЫРҒЫШЫНЫҢ ТАРАЛУЫ ЖӘНЕ ГЕНЕТИКАЛЫҚ ЕРЕКШЕЛІКТЕРІ

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ТҮЙІН

Бұл мақалада 2019-2022 жылдар аралығында Қарағанды, Қостанай, Ақмола және Шығыс Қазақстан облыстарының аумағында ауланған жабайы жыртқыштардан (қорсақ, түлкі, қасқыр) оқшауланған *Alaria spp*. қоздырғышының зерттеу бойынша мәліметтер көрсетілген. Жабайы жануарларды тексеру нәтижесінде алариялардың коллекциясы жиналып, инвазия қарқындылығы 1-ден 1489 инд/жануарға дейін, ал инвазия экстенсивтілігі 10-нан 36,11% дейін ауытқығаны байқалды. Қоздырғыштың бастапқы таксономиялық сәйкестігі анықтағыштарды қолдану арқылы жүзеге асырылды. Генетикалық идентификация *internal transcribed spacer 2 (ITS2)* маркер генінің бір бөлігін амплификациялау арқылы жүзеге асырылды. Амплификация нәтижесінде алынған 557 ж.н. ампликондарды Сэнгера әдісі арқылы секвенирледі. Нуклеотидтер тізбегі халықаралық GenBank дерекқорына келесі номерлермен тіркелді: түлкіден оқшауланған қоздырғыштар - OM630451, ON248128, ON248130, қарсақтан оқшауланған қоздырғыштар - OM630460, ON248043, ON248044, ON248045, қасқырдан оқшауланған қоздырғыш - ON358106. Филогения құру үшін биоинформатикалық талдау нәтижелері зерттелген *Alaria alata* үлгілері 90-98% аралығындағы сәйкестілік бойынша жеке кластерге біріктірілгенін көрсетті.

Кілтті сөздер: жабайы жыртқыштар, *Alaria alata,* инвазия қарқындылығы, инвазия экстенсивтілігі, *ITS2*, генетикалық идентификация.