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INTRASPECIES MOLECULAR DNA POLYMORPHISM AND THREAT OF *Hirudo orientalis*

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ВНУТРИВИДОВОЙ МОЛЕКУЛЯРНЫЙ ПОЛИМОРФИЗМ ДНК И ЗНАЧЕНИЕ *Hirudo orientalis*

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Abstract. Medical leeches play the role of a natural factory for the production of a complex of unique biologically active substances such as hirudin, eglins, bdellins, hementin, bradykinins, which are urgently needed by modern medicine and veterinary medicine. Medical leeches produce a wide range of enzymes such as hyalinodase, destabilase, collagenase, apyrase, elastase and triglyceride. All these substances are biologically active substances of natural origin. It is used to treat a wide range of diseases, such as amenorrhea, osteoarthritis, trauma, and blood stasis syndrome. In modern times, leeches also serve as an important model system for understanding the structure, function, development, regeneration, and repair of the nervous system. The currently seven known species of the *Hirudo* Linnaeus, 1758 genus of the Hirudinidae family are widespread in different regions of the Eurasian contingent. The results of the phylogenetic analysis based on the nucleotide sequences of the oxidase enzyme subunit I (12S r-RNA, COI) allow us to assume that the *Hirudo* genus is monophyletic. In 2005, P. Trontelj and S. Utevsky, while studying the molecular systematics of medical leeches, analyzed 2 samples of medical leeches from Azerbaijan together with others. According to the results of the cluster analysis, two medicinal leech samples of Azerbaijan (*Hirudo sp.* AZ1 and *Hirudo sp.* AZ2) form a separate cluster and are located between *H. medicinalis* and *H. verbana* species. As a result of recent phylogenetic studies, it was determined that those two samples are *H. orientalis* species. The main goal of the conducted research is to study the intraspecies polymorphism of medical leeches collected from different regions of Azerbaijan at the genome level. Isolation of leech chromosomal DNA was performed with the Gene Elute Mammalian Genomic DNA miniprep reagent kit.

Аннотация. Медицинские пиявки играют роль природной фабрики по производству комплекса уникальных биологически активных веществ, таких как гирудин, эглины, бделлины, гементин, брадикинины, которые остро необходимы современной медицине и ветеринарии. Медицинские пиявки производят широкий спектр ферментов, таких как гиалинодаза, дестабилаза, коллагеназа, апираза, эластаза и триглицерид. Все эти вещества являются биологически активными веществами природного происхождения, которые используют для лечения широкого спектра заболеваний, таких как аменорея, остеоартрит, травмы и синдром застоя крови. В наше время пиявки также служат важной модельной

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системой для понимания структуры, функций, развития, регенерации и восстановления нервной системы. Известные в настоящее время семь видов рода *Hirudo* Linnaeus, 1758 семейства Hirudinidae широко распространены в разных районах евразийского контингента. Результаты филогенетического анализа, основанного на нуклеотидных последовательностях субъединицы I фермента оксидазы (12S р-РНК, COI), позволяют предположить, что род *Hirudo* является монофилетическим. В 2005 г. Тронтель П. и Утевский С. при изучении молекулярной систематики медицинских пиявок проанализировали вместе с другими 2 образца медицинских пиявок из Азербайджана. По результатам кластерного анализа две выборки медицинских пиявок Азербайджана (*Hirudo sp.* AZ1 и *Hirudo sp.* AZ2) образуют отдельный кластер и располагаются между видами *H. medicinalis* и *H. verbana*. В результате недавних филогенетических исследований было установлено, что эти два образца относятся к виду *H. orientalis*. Основная цель проведенных исследований — изучение внутривидового полиморфизма медицинских пиявок, собранных из разных регионов Азербайджана, на уровне генома. Выделение хромосомной ДНК пиявки осуществляли с помощью набора реагентов Gene Elute Mammalian Genomic DNA miniprep.

Keywords: Hirudinidae, *Hirudo*, *Hirudo orientalis* intraspecific DNA polymorphism, molecular markers, microsatellite alleles, phylogenetic analysis.

Ключевые слова: Hirudinidae, *Hirudo*, внутривидовой полиморфизм ДНК *Hirudo orientalis*, молекулярные маркеры, микросателлитные аллели, филогенетический анализ.

The species characteristics of medical leeches are so unique that in order to extract the entire complex of biologically active substances from the body of leeches, it is not necessary to spend on their separation, cleaning, packaging, delivery and introduction means [1]. Because when blood is taken from the patient's body, all biologically active substances are scattered by themselves. This allows medical leech to play the role of an ideal tool in solving a wide range of endocrinological problems of a sick person or animal. From an ecological point of view, leeches are rare and endangered species included in the Red Book. It is forbidden to catch medical leech from natural water bodies. In the 21st century, the demand for medical leeches will increase in accordance with the increasing demand for biologically active substances of natural origin in medicine and veterinary medicine. As natural resources of medical leeches decrease, leeches grown in biofactories are used for treatment [2]. The breeding technology of medical leeches is a trade secret not published in the scientific literature. In biofactories, only certified adult leeches are sold, which cannot be studied at different stages of ontogenesis. Therefore, it is important to develop personal biotechnology of reproduction of leeches in laboratory conditions. Medical leeches of the *Hirudo* genus have been used in therapeutic procedures for thousands of years.

Species of medicinal leeches of the Hirudo genus

Today, seven known *Hirudo species* are widespread in different regions of the Eurasian contingent. *Hirudo medicinalis* Linnaeus 1758, *Hirudo verbana* Carena 1820 and *Hirudo orientalis* Utevsky and Trontelj are confirmed by Hildebrandt and Lemke to be of "dubious" taxonomic status because these 3 species mate under laboratory conditions. This data contradicts the data published by Elliott and Kutschera, which states that these leeches are reproductively isolated biospecies, being mutually mating hermaphrodites [3]. 60 years ago, Mann (1962) gave a detailed description of *H. medicinalis*, noting that it is a representative of the class Hirudinea that it is very diverse in color shades, but in Britain the white and gray marking patterns are usually black on the ventral

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surface with green sides and two long red stripes. there is This description refers to the bloodsucker species *H. medicinalis*, the only member of the family Gnathobdellidae (jawed leeches) found in the wild in England and Wales. Sawyer (1986) in his monograph "Biology and behavior of leeches" accepted Mann's opinion that *H. medicinalis* is a "polymorphic taxon", "*H. medicinalis* Linnaeus 1758 (*Hirudo officinalis* Savigny 1822)" collected different color variants of these ringworms. Hirudo specimens with non-pigmented (grey) ventral-ventral sides collected from a small pond in Hungary were proposed to be classified as *H. verbana* Carena, 1820 (*H. officinalis*, Savigny, 1822) taxonomy because they differed from the Linnean type. Five years later, *H. medicinalis* and *H. verbana* were identified as two separate biospecies, and it was noted that both species of leeches live in the freshwater basin and mating does not occur [4]. This result led to the careful description of both species and the determination of the DNA sequence of the genes of the mitochondrial genome [4].

Petrauskina et al. (2009) adult *H. medicinalis* in laboratory conditions of 3 species of medicinal leeches (*H. medicinalis*, *H. verbana* and *H. orientalis*) and *H. verbana*, after keeping the pairs in isolation for a month, within 4 weeks they mated and formed cocoons. But the viability of young leeches is sharply reduced. Female *H. medicinalis* 8 months after birth. and the individuals obtained from the cross of male *H. verbana* were destroyed, that is, the average number of mature individuals was equal to zero. However, most of the individuals obtained from the cross between female *H. medicinalis* and male *H. medicinalis* were destroyed and about 10 individuals survived to adulthood. Thus, the death of the offspring obtained from interspecific crossing was much higher than the death of the offspring obtained from intraspecific crossing. All 3 "leech types" differ in karyotype-chromosome number and there is no evidence of their hybridization in nature, it follows that in the wild *H. medicinalis*, *H. verbana* and *H. orientalis* are reproductively isolated species, differing in morphological and geographical distribution.

The DNA sequence of the cytochrome oxidase c mitochondrial gene subunit I (CO-I) of the variegated variant "var. verbana" of *H. verbana* was determined and deposited in GenBank (no. EF125043). DNA analysis showed that 600 n. c. of CO-I gene. The length of the sequence is identical to the sequence stored in GenBank. It turns out that the leeches used by Hildebrandt and Lemke for histological studies in 2011 are polymorphic varieties of *H. verbana*. Data summarized here according to Utevsky and Trontelj (2005) show that the following 4 well-defined taxa form the species complex: the European medicinal leech *H. medicinalis*, the Mediterranean leech *H. verbana*, the Caucasian leech *H. orientalis* and the African "dragon leech". *H. troctina*.

A new species of medicinal leech, *Hirudo tianjinensis* Liu, was discovered in specimens collected in Tianjin, China. A phylogenetic tree based on the subunit I sequence (COI) of the mitochondrially encoded cytochrome oxidase c gene suggests a sister relationship to *H. nipponia* Whitman, 1886. A key to known species is provided.

Of these, the dry leech belongs to the Haemadipsa genus, and is widely distributed in India, Southeast Asia, and East Asia. *Haemadipsa japonica* Whitman is a dryland species, endemic to Japan, living in the temperate zone between the litter of evergreen and deciduous forests. *H. japonica* feeds on the blood of mammalian hosts such as spotted deer (*Cervus nippon*), wild boar (*Sus scrofa*), Japanese capricorn (*Capricornis crispus*) and humans. In the past, *H. japonica* was distributed only in small forest areas in the mountains. Recently, this leech's range has expanded, possibly due to the growth of the mammal population, and in several prefectures in Japan, leeches have become a serious problem by spreading into human-populated areas.

H. nipponia is a well-known medicinal leech species that has been found in China for many decades. This species is widespread in most regions of China, except Xinjiang and the Tibet

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Autonomous Region (Yang 1996). In ancient folklore, the treatment with medical leeches was widely used in the case of varicose veins and arthrolithiasis [5].

Study of DNA polymorphism among the species of the genus Hirudo with different markers

In 2005, while studying the molecular systematics of medical leeches, P. Trontelj and S. Uvetsky analyzed 2 samples of medical leeches from Azerbaijan together with others. Confusion existed regarding the taxonomic status of the various taxonomic forms. Thus, many different types have been described in the past, but there are currently two generally accepted types. The results of the phylogenetic analysis of the nuclear genome (ITS2+5.8S r-RNA) and two mitochondrial (12S r-RNA, COI) genomes allow us to assume that the *Hirudo* genus is monophyletic. The *Hirudo* genus consists of 3 other forgotten species besides *H. medicinalis* and East Asian *H. nipponia*. They are described either as species or morphologically diverse and are easily identified by color. The type species from Transcaucasia and Iran is *Hirudo sp.* (described as an orientalis variation) is sisterly related. Their relative is *H. verbana* from Southeast Europe and Turkey, which is now propagated in leech farms and used as a "medical leech" [6]. The North African species *Hirudo troctina* is the sister taxon to the West Eurasian species, as a basal split occurred between the *H. nipponia* and West Palearctic clade. The analysis was carried out on a specimen of *Hirudo sp.* (as a variant variety of orientalis) collected from Azerbaijan in 1962 by Shevkunova and Christman.

Genetic sensitivity among the species of *Hirudo* genus was studied based on the areas of mitochondrial (COI and 12S rDNA) and nuclear (ITS1+5.8S+ITS2) genomes of *Hirudo orientalis* in all areas. The sister relationship of *H. orientalis* and *H. medicinalis* was inferred with high posterior probability. *H. orientalis* has a wide and uneven distribution in Central and Middle Eastern parts of Asia with minor genetic differences. The known distribution range occurred in topographically heterogeneous landscapes around the Caspian Sea. Demographic analysis suggests selection of the COI locus under unfavorable respiratory conditions, but an increase in population size cannot be completely ruled out. The trend of genetic variation shows a northward dispersal. The higher haplotype diversity suggests the South Caspian region as a potential historical refuge for these species. Widespread dispersal is thought to have occurred after the Pleistocene glaciations through vertebrate hosts.

The present study presented more haplotypes than those in the 2012 study by Trontelj and Utevsky in *H. orientalis* populations due to their range. In addition, an updated view of the phylogenetic relationships among Hirudo species is described. Significant statistical support for the phylogenetic relationship of *H. orientalis* and *H. medicinalis* was shown, confirming other similarities and affinities of traits (i.e., morphological, chemical composition of saliva, etc.) in the aforementioned species [7].

There is a lack of information on the distribution of *H. orientalis* from some neighboring countries of the Caspian Sea (e.g., Turkmenistan, Armenia and possibly northern Afghanistan). Comprehensive sampling is needed to get a clear picture of the genetic diversity of *H. orientalis* [1].

To date, six known species of *Hirudo* are widespread in different regions of the Eurasian continent. In this study, a new species of medicinal leech, *H. tianjinensis* Liu, 2022. It is described based on specimens collected from Tianjin, China. A phylogenetic tree based on COI suggests a sister relationship to *H. nipponia* Whitman, 1886. A phylogenetic tree showing the results of the phylogenetic analysis of *H. tianjinensis* with other species of Hirudo is given in Figure 1.

Research object and methods

Various individuals of the *H. orientalis* species were collected from reservoirs in the Sheki-Zagatala-Gakh, Lankaran-Astara-Masalli-Jalilabad, Shabran-Guba-Khachmaz regions of Azerbaijan and were starved for a long time in the laboratory so that the blood they sucked from other animals

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was completely used for nutrition and when separating chromosomal DNA, there should be no admixture of foreign DNA.

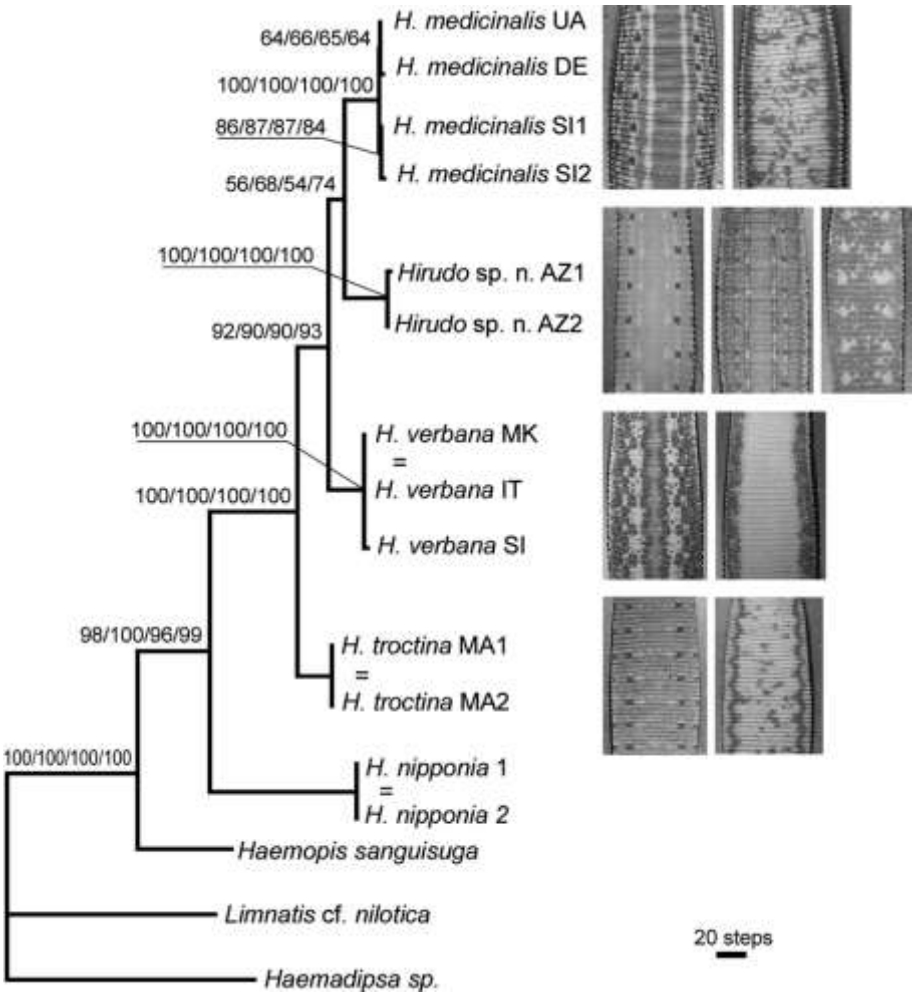

Figure 1. Best phylogenetic tree inferred from a combination of nuclear and mitochondrial DNA sequences of medical leeches. Previously, “H. medicinalis”. Typical, diagnostic dorsal (left) and ventral (right) examples of the midbody region are shown for the species grouped under the name Hirudo sp. n. two versions of the dorsal model are shown. (orientalis color type) [6]

Isolation of chromosomal DNA from different individuals of Hirudo orientalis

Chromosome-DNA isolation of medical leech was performed with the Gene Elute Mammalian Genomic DNA miniprep reagent kit manufactured by Sigma-Aldrich (Steinheim, Germany). A small piece of skin and muscle tissue 2 mm wide was cut from the side of the leech’s body and kept in distilled water for the purpose of dispersing foreign blood cells and removing foreign DNA. The procedure was repeated 3 times, and the samples were placed separately in 2 ml clean, sterile Eppendorf tubes and placed on ice. After the tubes are numbered, 180 µl Lysis T solution is added to the tissue, followed by 20 µl proteinase K, mixed and incubated at 55°C for 2 hours until the tissue is completely dissolved. To get pure DNA from RNA, additional 20 µl of proteinase A is added and 2 min. incubated at room temperature. Then 200 µl of Lyzis C is added, vortexed for 15 seconds and incubated at 70°C for 10 minutes. 500 µl of the column preparation solution is added to the connecting column, centrifuged at 12000 g for 1 minute, the collected solution is discarded in the tube. 200 µl of ethanol (96-100%) alcohol is added to the lysate and mixed in a Vortex for 5-10 seconds. Using wide-tip pipette tips, the homogenized lysate is added to

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the coupling column, centrifuged at 6500-7000g for 1 minute, and the coupling column is placed in a fresh 2 ml tube. On it 500 mkl Column Preparation Solution is added and centrifuged at 12000 g for 1 minute. Then 500 µl diluted Wash Solution is added to it and centrifuged at 6500-7000g for 1 minute. In the second wash, 500 µl diluted Wash Solution is added and centrifuged for 3 minutes at 12,000-16,000 g maximum speed to dry the column. 200µl Elution Solution is added to the middle of the column, incubated at room temperature for 5 minutes and centrifuged at 6500-7000 g. Thus, the chromosomal DNA separated within 1 hour is measured in a spectrophotometer at A260/280 nm wavelength and its quantity and degree of purity are determined [6].

Spectrophotometric determination of DNA purity and concentration

The concentration and degree of purity of DNA extracted from the skin and muscle tissue samples of medical leech were checked by spectrophotometry method. For this purpose, optical densities of DNA samples are measured at wavelengths of 260 and 280 nm in the Epoch Microplate Spectrophotometer (BIOTEK-USA) device with the help of Gen5 software. For properly extracted and suitable PCR DNA samples, the optimal degree of purity is 1.8≥ [A260/A280] =2. The density of the extracted DNA is determined based on the optical density measured at a wavelength of 260 nm.

Primer sequence synthesis

Primer sequences (Table 2) were synthesized, purified and diluted to 100 mM. Then it was prepared as a working solution at a concentration of 10 mM and used during PCR (Table 1).

Table 1

NUCLEOTIDE SEQUENCE OF MICROSATELLITE LOCI PRIMER HM1 NUCLEOTIDE SEQUENCE AND SIZE OF THE AMPLICON TO BE SYNTHESIZED

Primer	*Nucleotide sequence*	*Expected fragment size (n. c.)*	*Ann. temp. (°C)*
Hm1- F	5’CACGACGTTGTAAAACGACTCAGGCGAC ATCCTCTTCATCG -3’	Allele 1-133-139 n. c. and Allele 2-92-104 n. c	50˚C
Hm1–R	5’- ATGGCTACCACTGCGTTGTTG -3’		

Nucleotide sequence of microsatellite loci primer Hm1 nucleotide sequence and size of the amplicon to be synthesized.

Conducting PCR with a specific primer pair

Polymerase chain reaction (PCR) was performed on DNA samples suitable for PCR with specific primers (Table 1) in an Applied Biosystems 2720 Thermal Cycler amplifier. During DNA amplification, the total reaction volume per sample was 25 µl (5 µl sample DNA + 20 µl reaction mixture). After collecting the reaction mixture, the tubes were placed in the PCR apparatus (Applied Biosystems 2720 Thermal Cycler) and the program was compiled in the following sequence. Initially, the reaction starts with denaturation of the DNA strand at 94°C for 3 min. The next 3 stages — denaturation of the DNA strand, hybridization of the primer with the DNA strand and synthesis of the complementary DNA strand by Taq DNA-polymerase, respectively, were performed at 94°C (45 seconds), 50°C (0.5 min), 72°C (45 seconds) occurs in conditions. This is repeated for 35 cycles. Finally, the PCR reaction is completed by completing the synthesis process at a temperature of 72°C for 10 minutes.

Electrophoretic analysis of PCR products

For electrophoretic analysis of PCR products, 1% agarose gel was documented using UVI-pro Gel documentation Systems (Jencons, England).

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Conclusions and discussion

After extracting DNA from 13 samples collected from different regions of the leech with the Gene Elute Mammalian Genomic DNA miniprep reagent kit, the concentrations and purity of the isolated chromosomal DNA were determined using Epoch Microplate Spectrophotometer (BIOTEK-USA) in a volume of 2 µl. The obtained results are given in Table 2.

Table 2

SPECTROPHOTOMETRIC DETERMINATION OF THE AMOUNT AND DEGREE OF PURITY OF CHROMOSOMAL DNA ISOLATED FROM THE SKIN AND MUSCLE TISSUE OF MEDICAL LEECHES

№	*Medicine leach specimens*	*A260 nm*	*A280 nm*	*A260/A280*
1	Control	000	0.01	0.04
2	Sheki-Zakatala	0.854	0.408	2.09
3	Shekii-Zakatala	1.082	0.575	1.88
4	Lankaran -Astara	1.175	0.632	1.85
5	Lankaran -Astara	1.767	0.976	1.81
6	Guba- Khacmaz	1.658	0.914	1.81
7	Guba- Khacmaz	2.145	1.188	1.80
8	Gah	0.695	0.384	1.81
9	Lankaran -Astara	1.403	0.753	1.86
10	Lankaran -Astara	1.898	1.029	1.84
11	Guba- Khacmaz	4.675	2.577	1.81
12	Guba- Khacmaz	2.719	1.513	1.80
13	Guba- Khacmaz	5.981	3.267	1.83
14	Guba- Khacmaz	2.718	1.472	1.84

As can be seen from Table 1, the amount and degree of purity of isolated chromosomal DNA meet the necessary requirements for conducting PCR, and the degradation level of nuclear DNA was checked by conducting electrophoresis in 0.7% agarose gel. The results of electrophoretic analysis of nuclear DNA isolated from the samples are given in Figure 2. As can be seen from the picture, the nuclear DNA separated from the high amount of RNA from the skin and muscle tissue of the leech was not degraded. The isolated nuclear DNA was diluted to 20 ng/µl in sterile deionized water in a sterile box for PCR. At the same time, Hm1 is diluted to 10 mM in F/R primer. Thus, PCR is assembled in the composition given in Table 3 below. The program of PCR with Hm1 primer is repeated at 94°C for 3 minutes, then 94°C (45 seconds), 50°C (30 seconds), 72°C (45 seconds), 35 cycles. At the end, the PCR reaction is completed with the complete completion of the synthesis process of amplicons at 72°C for 10 minutes. After completion of PCR, PCR products are stored at 6-8°C and electrophoretically analyzed in 1.5% agarose gel. The results of electrophoresis are documented by taking a picture of the gel under UV light using the Gel Documentation System. The results are reflected in Figure-3. As can be seen from the picture, 50 n. c. of agarose gel. Lanes below are unreacted excess primers (blue). Mark E. Siddall and colleagues (2007) identified 2 different alleles of the microsatellite locus Hm1 molecular marker and different variations of each allele in the genomic DNA of *H. medicinalis* and *H. verbana* species collected from different European countries.

So, Allele 1 13 3 n. c. length variant was found in samples of *H. medicinalis* species. 136-138-139 n. c. in different individuals of *H. verbana* species. variations were found. Allele 2 variant is 104 n. c. in representatives of *H. medicinalis* species, and 92 n. c. in representatives of *H. verbana* species. variant was discovered. In three samples, amplification of the microsatellite locus with the Hm1 marker on genomic DNA did not occur (samples 9, 12, 13) and the PCR product was not visible on the gel.

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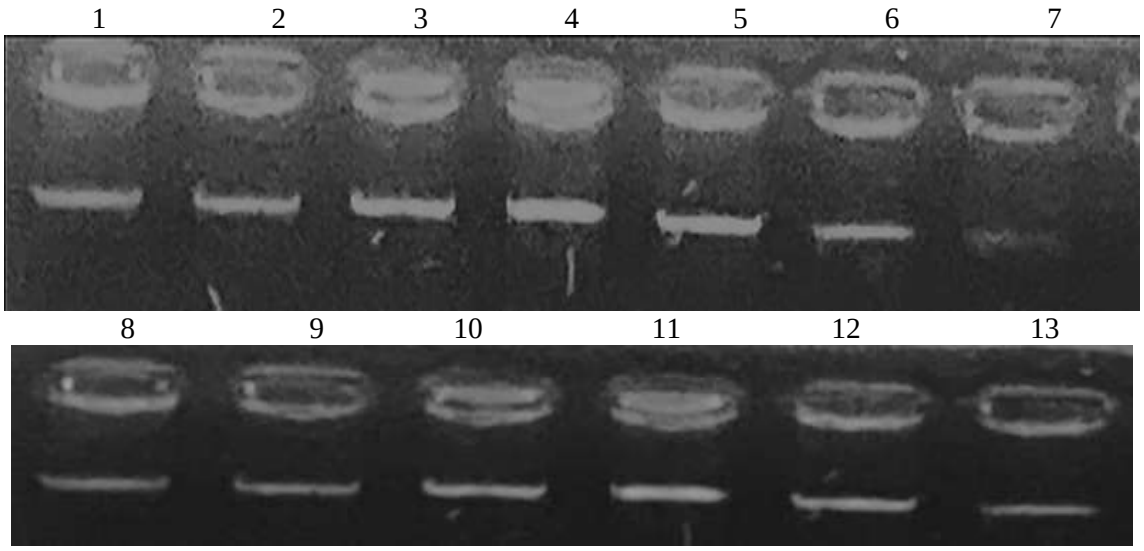

Figure 2. 0.7% agarose gel electrophoretic analysis of chromosomal DNA isolated from medical leeches collected from different areas

Table 3

REAGENTS INCLUDING IN PCR

№	*A PCR reaction composition*		*14 PCR reaction composition*	
1	Matrix DNT	5 mkl	Matrix is added for each sample	5 mkl
2	Reaction buffer - 10^x	2.5 mkl	Reaction buffer - 10^x	35 mkl
3	Primer Hm1-F	2 mkl	Primer Hm1-F	28 mkl
4	Primer Hm1-R	2 mkl	Primer Hm1-R	28 mkl
5	$MgCl_2$ – 25 mM	3 mkl	$MgCl_2$ - 25mM	42 mkl
6	dNTPs – 10 mM	2 mkl	dNTPs-10mM	28 mkl
7	Taq DNAPolymerase-1 mkl / 5 unit	1unit - 0.2 mkl	Taq DNAPolymerase-1mkl / 5 unit	1 unit – 3 mkl
8	Deionzed sterile H_2O	8.3 mkl	Deionzed sterile H_2O	116 mkl
		25mkl		280 mkl

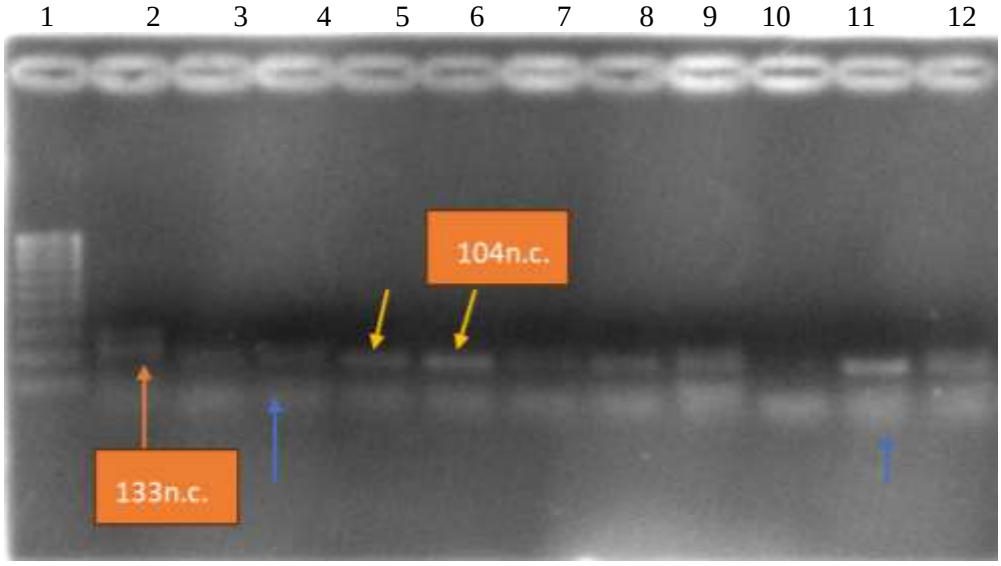

Figure 3. M-DNA marker, each fragment 50 n. c. are different from each other. PCR was performed on the genomic DNA of 13 different H. orientalis leeches with the Hm 1 marker of the microsatellite locus, and the synthesized amplicons or allelic variants were analyzed in 1.5% agarose gel

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According to the obtained results, profiles corresponding to the microsatellite locus of the *H. medicinalis* genome DNA were synthesized on *H. orientalis* genome DNA with Hm1 marker. Based on the results of comparative phylogenetic studies conducted by Peter Trontelj et al. (2005) and Hao Wang et al. (2022) on the nuclear genome of all seven species of the *Hirudo* genus (Aghdam), including samples of leeches collected from Azerbaijan (Aghdam), the leeches from Azerbaijan formed a separate cluster [5, 6, 8]. It is located between *H. medicinalis* and *H. verbana*.

Thus, in the wild, *H. medicinalis*, *H. verbana* and *H. orientalis* are reproductively isolated species, differing in morphological and geographical distribution. Therefore, the synthesis of the corresponding alleles of the microsatellite locus with the Hm1 molecular marker once again indicates that these species are reproductively separated species.

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