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Genetic features of the formed population of the genus Eisenia worms

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ABSTRACT
It is known that molecular genetic methods can provide researchers with new, more accurate means for differentiating species of earthworms and determining phylogenetic distances among them. Therefore, using neutral molecular markers, ISSR (*Inter-Simple Sequence Repeat*) examined the internal genetic polymorphism of genus *Eisenia* rainworms from the vermiculture array of the Biotechnology Department of the Ukrainian State Chemical Technology University, the parent forms of which were irradiated with an LGN-208B laser for different temporary exposures and cultivated on different organic substrates. Studies have shown that the line of animals has a fairly high level of heterogeneity despite the fact that it was bred from six individuals of the same array. The laser radiation action and the hard substrate during cultivation (bottom deposits) appeared as powerful factors of selective pressure.
Keywords: *genetic variation; laser radiation; molecular markers; alleles.*

1. INTRODUCTION
The intensification of agricultural production several times increased the anthropogenic pressure on regional and local ecosystems, including soil biocenoses [1]. Therefore, the study of the genetic and phenotypic characteristics of animals living in soil-litter interface, particularly genus *Eisenia*, which provide biotransformation and detoxification of the main agricultural and industrial wastes, is relevant [2-5]. These animals are the basis for the formation of various food chains in nature and apart from that provide the formation and fertility of soils [6-11]. Therefore, along with the study and preservation of the genetic diversity of cultural populations, the creation of new populations capable of biotransformation of hard substrates that are not yet used in vermitechnology is particularly important [12]. A new population of the *Eisenia Veneta* worms which capable of processing solid substrates, was formed on the basis of the Biotechnology Department of the Ukrainian State University of Chemical Technology. Parental forms of animals of this population were irradiated with a laser of the type LGN-208 B with a wavelength of 0.63 μm and radiation power of 1 mW at various exposures in time. Therefore, the biological characteristics of these animals should be investigated using molecular genetics methods in order to create breeding biotechnologies. Based on these methods, the potential of living organisms can be widely used. The most important priority in creating local populations is to establish the genetic variability of their individuals.

2. MATERIALS AND METHODS
In ISSR studies, the worms of the following experimental groups were used by primers to isolate DNA from biological material.
1st experiment group: animals that were kept on a soil substrate and were not irradiated;
2nd group: animals that were laser-irradiated for 15 minutes and kept on a soil substrate;
3rd group: the generation of animals irradiated with a laser with an exposure of 15 minutes contained on a soil substrate;
4th group: animals were kept on the substrate bottom deposits and were not subject to laser irradiation;
5th group: animals that were laser-irradiated for 15 minutes and kept on the substrate bottom deposits;
6th group: generation of animals irradiated with a laser with an exposure of 15 minutes contained on the bottom deposits substrate;
7th group: technological type, artificially cultivated in one of the industrial vermiferms.

DNA was extracted from biological material of individuals of the genus *Eisenia* using the standard commercial DNA sorb B kit (Amplisens, Russia) according to the manufacturer's recommendations. Each DNA sample was obtained from one worm. The material for the work was all the tissues of the worms that were stored in ethanol at a temperature of minus 18-20°C. Biological samples were thoroughly ground in a glass homogenizer before the DNA extraction procedure.

The next step after DNA isolation was PCR using the ISSR inter-microsatellite analysis technique. DNA amplification was performed using the ISSR-PCR method in 25 μl of the reaction mixture. The composition of the reaction mixture for conducting ISSR-PCR (using the commercial kit "TermoFisher Scientific" (Fermentas) (Massachusetts, USA) was as follows: the reaction buffer Phusion (2 u/μl) with 7.55 mM MgCl 2-2.5 μl; dNTP mixture – 3.0 μl; 100 pM primer – (0.5-1.0) μl; Tag-polymerase from 2 to 4 units of activity – (0.2-0.4) μl; (0.1-0.2 ng) DNA sample – (1-3) μl; deionized water (in the required amount until the total volume of the mixture is 25 μl). Before PCR, the stock

solution of the extracted DNA was diluted with bidistilled water in a ratio of 1:10. To determine the information content of each primer used in the ISSR experiment, the equivalent of a DNA mixture from representatives of each experimental group was used. Individual typing of individuals for further comparative population-genetic analysis was carried out after the identification of major amplicons of DNA profiles that differed in electrophoregrams. The structure of the primers used for genetic analysis of individuals of various groups of the genus *Eisenia*, and their code designations are shown in the **Table 1**. The primers were synthesized by a firm "Fermentas" (Vilnius, Latvia) according to the indicated nucleotide structure. The temperature regime of amplification with ISSR-primers was determined empirically. Amplification program for S3 primers, UBC 873: 1st cycle: 94°C – 4 min; from 2nd to 31st cycle: 60°C – 2 min; 72°C – 4 min; 94°C – 1 min; 32nd cycle: 60°C – 3 min; 72°C – 7 min. Amplification program for primers S1, S2, S5: 1st cycle: 94°C – 4 min; from 2nd to 31st cycle: 57°C – 2 min; 72°C – 4 min; 94°C – 1 min; 32nd cycle: 57°C – 3 min; 72°C – 7 min. The electrophoretic separation of amplified DNA regions using ISSR technology was carried out in a 2% agarose gel in Tris-borate electrophoresis buffer (TVE: 0.0879 Mtris, 0.089 M boric acid, 0.002 M EDTA pH 8.0) according to the methodological recommendations [13, 14]. After electrophoresis, the gels were stained with a solution of ethidium bromide with a concentration of 0.5 μg / cu. cm for 10 minutes, then the gels were washed (10 times) in distilled water and the gel was left in distilled water for 5 minutes. The gel after staining was placed on a trans-porthole and examined under ultraviolet light of a wavelength of 340 nm, the obtained electrophoregrams were documented by photographing the gels with a Canon digital camera with an orange filter. To more accurately determine the size of the identified amplification products with microsatellite anchored primers in the technology of inter-microsatellite analysis (ISSR-PCR), two molecular weight markers manufactured by ThermoFisher Scientific (USA) were used simultaneously: 1) GeneRuler 100bp Plus DNA Ladder; 2) 1 kbPlus DNA ladder, which allowed a total control over the size of DNA fragments in the range of molecular weights from 100 to 3000 bp. Alleles were identified by determining the "major" amplicons that were clearly reproduced on the electrophoregram after three successive amplification reactions under the most standard identical conditions for PCR and electrophoretic separation and coincided with the sizes on the electrophoregram in the range of molecular weights regarding markers. Amplicon size was determined using the above markers taking into account the average error of ± 5 bp. Amplicons that coincided in size and were detected using different primers were taken into account as the same genetic locus when conducting a total population genetic analysis using five ISSR primers. ISSR profiles were reflected on paper with a millimeter grid on a scale of 1: 2 according to the distance in millimeters between the bands of the molecular weight marker. The source data matrix was entered into the corresponding file of the standard computer program GELSTAT intended for processing polylocus typing data [15]. Statistical processing of numerical data during cluster analysis was carried out in the Statistica 6.0 application package particularly the Past program.

Table 1. Structure and temperature regime of annealing of ISSR primers used in the work.

S1	3'-AGC AGCAGCAGCAGCAGCC-5'	68,42	**57**
S2	3'-AGCAGC AGCAGCAGCAGC G-5'	68,42	**57**
S3	3'-ACC ACC ACCACCACCACC G-5'	68,42	**60**
S5	3'-ATGATG ATGATGATGATG C-5'	36,84	**57**
UBC873	**3'- GAC AGA CAG ACA GAC A-5'**	**50,00**	**60**

3. RESULTS

When analyzing genetic information as an integral component of the earthworm organism, an important element along with identification is the evaluation of frequently repeated DNA regions in ISSR technology. Thus, the expansion of information on genetic variation and species identity of the organism is achieved.

When comparing the nature of the distribution of the calculated frequencies of alleles (amplicons) that are presented in the **Table 2** for representatives of various experience groups, particular attention was paid to the comparative characteristics of representatives of adjacent generations: groups II and III of the experiment – worms to laser irradiation for 15 minutes, and their descendants; groups V and VI are animals that were grown on bottom deposits and were subject to laser irradiation, and their descendants, respectively.

Amplicons 1120 bp in size were found in individuals of group III at the locus (AGC)6G, which were found with a frequency of 0.4 in this sample, but were absent in their parents at all, but the detected difference was not statistically significant (according to the Fisher test). A significant reliable difference was recorded at the locus (ACC)6G with an allele distribution frequency of 1310 bp in size. in individuals of adjacent generations (groups V and VI): this allele was absent in the parental individuals, but its frequency is 0.4 in the offspring of the first generation. Similar patterns were observed in the distribution frequencies of alleles in groups V and VI of the experiment: amplicon frequencies of 1080 bp of the locus (AGC)6G and 1040p. of the locus (ACC) 6G twice as many as the parental forms' properties with a statistically reliable difference.

According to the frequency of distribution of the allele of 1000 bp a statistically possible difference is recorded: in parental forms (experiment group V) this allele is absent, and in descendants of the first generation (group VI) the frequency is 0.6 ($p < 0.5$). Allele size 925 bp was found at the locus (AGC)6G in individuals of group V with a frequency of 0.4 and completely eliminated in their offspring. Note that similar trends were identified for the II and III groups of experience – an allele of 925 bp of size was identified in 20% of the parental forms of group II of the experiment and then that allele was not found at all in the descendants of this group. Unique alleles of 3,000 bp and 595 bp were detected of the locus (ATG)6C with a frequency of 0.2 only in representatives of group IV, which were grown on depleted

substrates (bottom deposits). Also at locus (GACA)4 a unique allele of 250 bp in size was found. only in 20% of individuals of group III. It should be noted that the boundaries of the loci were as follows: (ATG)6C 3000-300 bp; (ACC)6G 1310-500 bp; (AGC)6G 1120-800 bp; (GACA)4 1200-250 bp. A total of 36 DNA fragments were obtained, ranging in size from 250 to 3,000 bp. We performed a cluster analysis of the frequencies of DNA fragments of the studied groups of the *E.veneta* species obtained by ISSR technology with markers S1, S2, S3, S5, UBC 873. On the obtained dendrogram (**Figure 1**) with a high level of confidence, two clusters were identified.

The first cluster included group VI – the generation of animals, which was contained on a substrate of bottom deposits, irradiated with a laser with an exposure of 15 minutes. All other groups of animals were included in the second cluster, including the fourth group – non-irradiated animals that were kept on the substrate of bottom deposits and the fifth group – irradiated parental forms, as a result of further reproduction of which the group VI was obtained. This makes it possible to assert that the genetic features that differentiate this group are the result of the influence of laser radiation on the genetic apparatus of worms of the species *E.veneta*, which appeared in the descendants of irradiated animals in the conditions of their adaptation to a specific substrate (bottom deposits).

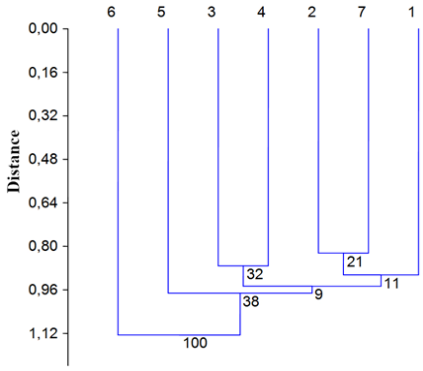

Figure 1. The dendrogram of the distribution of the studied groups of the *E.veneta* species by the frequency of DNA fragments in ISSR technology with markers S1, S2, S3, S5, UBC 873 (tree clustering, pair grouping, Euclidean distance, 1000 iterations).

Table 2. Allelic frequencies and sizes of DNA fragments at the genetic loci of individuals of the species *E.veneta*.

Genetic structure and boundaries of the locus, bp	DNA fragment number	The size of the DNA fragment, bp	Animal groups and allele Frequency						
			I-soil not irradiated	II-soil irradiated, 15 min	III-F1 irradiated, 15 min	IV-bottom deposits not irradiated	V-bottom deposits irradiated, 15 min	VI-F1 irradiated, 15 min	Technological type
S2:(AGC)$_6$G 1120–800	8	1120	0.200	**0.000**[a]	0.400*	0.200	0.200	**0.600***	0.400
	9	1080	0.400	0.200	0.600	0.400	0.200	0.400	0.400
	14	925	0.200	0.200	0.000	0.000	**0.400**	0.000	0.000
S3:(ACC)$_6$G 1310–500	6	1310	0.200	0.000	0.000	0.000	0.000[a]	**0.400***	0.200
	10	1040	0.200	0.000	0.000	0.000	0.000	0.200	0.200
S5: (ATG)$_6$C 3000–300	1	3000	0.000	0.000	0.000	**0.200**	0.000	0.000	0.000
	23	595	0.000	0.000	0.000	**0.200**	0.000	0.000	0.000
UBC873: (GACA)$_4$ 1200–250	11	1000	0.200	0.400	0.400	0.200	**0.000**[a]	**0.600***	0.400
	36	250	0.000	0.000	**0.200**	0.000	0.000	0.000	0.000

At the beginning of the XXI century, the methods of molecular genetics began to be applied to earthworms. The first works were devoted only to individual issues, for example, the relationships between the species *Eisenia fetida* and *Eisenia andrei* [16]. However, the first large work [17], which included facts about a range of European cosmopolitans, showed that earthworms are characterized by very high genetic variability. The authors examined the genetic diversity of the cytochrome oxidase gene fragment in nine cosmopolitan species and found that four of them on the phylogenetic tree are represented by several branches, which were called *genetic linesages*. The number of paired nucleotide substitutions between the lines is approximately 15%, while for most animal species, intraspecific variation rarely exceeds 2%. The existence of such high intraspecific variability was then confirmed for various species [18]. In general, this variability and its causes are not completely understood. It is logical to assume that the detected genetic lines are twin species. In some cases, it probably is. However, the picture is sometimes more complicated. Often, genetic lines that differ by 15% or more in mtDNA do not have noticeable differences with nuclear DNA [19]. It is also possible that differences in nuclear genes exist but there are isolated cases of crossing between lines [20-22]. If this may be the result of an incomplete reproductive barrier between the lines, then the complete absence of differences in nuclear DNA is often explained by introgressions, that is, the replacement of mitochondria of one species by mitochondria of another. MtDNA of earthworms may be characterized by a high nucleotide substitution rate compared to most other animals. It is well known that in animals mtDNA has a higher substitution rate compared to nuclear DNA. The reasons why mtDNA of precisely earthworms should have an increased rate of evolution are not yet known but can probably be explained over time. However, it remains unclear why in some cases there is enormous genetic variation, and in others its absence.

In the abstract base of the NCBI, scientific information on the genetic structure of the species *E.veneta* was not found. However, the results of several scientific works using genetic markers in ISSR and RAPD technologies have proved that the morphological characteristics and structure of the genetic variation of earthworms, in particular, species *E.foetida* and *L.teresttis*, are directly dependent on the substrate environment or biotope environment [22]. When using the ISSR technology in the study of the native population of the species *E.foetida*, 3 to 15 polymorphic loci were detected by ISSR markers in different individuals of the species [23].

4. CONCLUSIONS

The obtained results of a comparative analysis of the frequency distribution of alleles and determination of clusters among groups of experimental animals suggest that the effect of laser irradiation and a depleted cultivation substrate (bottom deposits) turned out to be powerful factors of selective pressure. They led to certain changes in the genetic properties of worms in the *E.veneta* population, namely the loss/appearance of new alleles and significant changes in their frequencies for individual genetic loci in the first generation descendants.

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