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Use of ISSR markers for genotyping an experimental group of largemouth bass *Micropterus salmoides* (LACEPEDE, 1802), reared in ponds of Polissia of Ukraine

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The aim of this study was to investigate the genetic structure of the experimental group of largemouth bass reared in the ponds of Polissia of Ukraine using ISSR markers. To accomplish these tasks, ISSR genotyping of the genetic structure of largemouth bass was performed using four fragments of trinucleotide loci. The genetic structure of the experimental group of largemouth bass of the pond fish farm "Nyvka" was characterised using 4 primers B – $(GAG)_6C$; C – $(AGC)_6G$; D – $(ACC)_6G$ and E – $(AGC)_6C$. Fins fragments were used for the study. In the course of the work, the optimal conditions for ISSR-PCR analysis were selected. The study revealed a number of factors that affect the efficiency of these markers: DNA concentration, number of amplification cycles. For 4 markers, 80 alleles with a molecular weight of 160–1320 bp were identified. The ranges of amplicons for the selected markers were determined: marker B – from 150 to 1186 bp; marker C – from 640 to 200 bp; D – from 1320 to 225 bp; and E – within 630–160 bp. The most polymorphic marker is marker B – 26 alleles, the least polymorphic marker is marker E – 15 alleles. In the studied experimental group of largemouth bass, the effective number of alleles varied from 10.2 for marker E to 12.2 for marker C, D. The indicators of genetic variability were determined by calculating allelic frequencies, the maximum level of available heterozygosity is 0.918 for marker C, D, and the lowest for marker E is up to 0.902. A method has been proposed that makes it possible to analyse the genetic structure of the experimental group of largemouth bass using these primers and to implement genetic information at different stages of the selection process.

Key words: ISSR-PCR, genetic structure, largemouth bass, DNA–markers, genotype, amplicons, genetic polymorphism, molecular genetic marker.

Problem statement and analysis of recent research. Largemouth bass *(Micropterus salmoides)* is the largest representative of the family Centrarchidae, originating from North American waters. At present, this species is present in pond aquaculture in more than 50 countries of the world, on all continents except Antarctica and Australia [1, 2, 3]. As an aquaculture object, largemouth bass is one of the most valuable freshwater species grown in fish farms [4, 5, 6, 7]. The limited supply of stock for sport fishing and commercial fish markets helps to keep the price of largemouth bass high. The market demand for largemouth bass, primarily as one of the world's most popular recreational and sport fisheries, exists for all sizes, from fingerlings to trophy fish [7, 8, 9, 10].

Largemouth bass was first brought to the territory of modern Ukraine at the end of the 19th century (1889) from Germany to the ponds of Prince Horchakov in the village of Korostyshevo, Kyiv province, where successful attempts were made to breed them for sport fishing [11]. In October 1949,

the Ukrainian Institute of Pond, Lake and River Fisheries (now the Institute of Fisheries of the National Academy of Agrarian Sciences of Ukraine) successfully transported 1840 yearling trout by plane to the fish farm "Pushcha-Vodytsia" in Kyiv region [11]. In Ukraine, from the first half of the 1950s to the first half of the 1960s, a study was conducted on the introduction of brown trout into the hydroecosystem of the Shatsk Lakes [12, 13]. Subsequently, the number of largemouth bass in the Shatsk Lakes decreased significantly [14, 15]. during the reclamation fishery in 2018, which was carried out in Luka Bay of Lake Svityaz, among other fish species, individuals of largemouth bass (Micropterus salmoides Lac.) were caught [16] and transferred to the Institute of Fisheries of the NAAS of Ukraine (Kyiv) for further study [17]. The reasons that did not contribute to the adaptation of the brown trout to the conditions of water bodies in Ukraine include: competition from local predators, inconsistency of temperature conditions of water bodies with those optimal for this species, inbreeding due to a small number of breeders [18].

One of the methods that allows, to a certain extent, to analyse the genetic structure, assess the genetic diversity of populations, the degree of their inbreeding and genetic distances between populations is the inter simple sequence repeat (ISSR) analysis of DNA polymorphism. This approach allows amplification of DNA fragments located between two closely spaced sequences that are considered unique. As a result, a significant number of species-specific PCR products are obtained, represented by discrete bands on the electrophoregram. Considering that the ISSR method has high reproducibility, it is successfully used in international [19, 20] and domestic practice [21, 22]. The popularity of these methods is primarily due to the ability to adequately assess both inter- and intra-population variability of the studied animals, identify species, populations, lines, and in some cases, for individual genotyping [23].

For polylocus genotyping of different fish species, ISSR markers are used, the advantages of which include high polymorphism, simplicity of equipment for analysis and data processing. The possibility of using the ISSR-PCR method to assess the genetic structure of carp species from the fishery of Ukraine was previously shown on the example of carp species from the fishery of Ukraine [21, 24, 25], but the performance of intermicrosatellite markers has not been characterised. This method is useful for determining the genetic profile of different fish species, determining the level of heterozygosity of breeding stocks and conducting phylogenetic studies [26, 27]. The study of the genetic structure of the experimental group of largemouth bass reared in Ukraine using intermicrosatellite analysis of DNA polymorphism has not been carried out before.

The aim of the research. The purpose of this work was to study the genetic structure of an experimental group of largemouth bass raised in ponds of Polissia of Ukraine using ISSR markers.

Material and methods of research. Biological material of an experimental group of largemouth bass (n-30) from the ponds of The State enterprise «Experimental Fish Farm «Nyvka»» was studied. Fragments of fins preserved with 96% ethyl alcohol were used as samples for the study. Total DNA was allocated with the commercial Quick – DNA kit. MiniPrep Kit ("BioLabTech LTD", Ukraine).

ISSR genotyping of the genetic structure of largemouth bass was made with four fragments of trinucleotide loci, which were previously used for population genetic studies of different fish species in Ukraine (Table 1) [28, 29, 30, 31, 32].

Table 1 – **ISSR primers for analyzing the genetic structure of largemouth bass used in the study**

Primer	Annealing temperature of primers (°C)
$(GAG)_6C$	58
$(AGC)_6G$	55
$(ACC)_6G$	58
$(AGC)_6C$	60

Polymerase chain reaction (PCR) was made with a Thermo scientific thermocycler (Arktik Thermal Cycler, Finland) amplifier using the ThermoScientific DreamTaq Green PCR Master Mix kit (2X). Amplicons were separated by electrophoresis in a 2% agarose gel. To study amplicons, the molecular mass marker Gene Ruler 1kb DNA Ladder (Thermo Scientific) was chosen.

Amplification products were visualized with ethidium bromide dye (0,5 μg/ml gel) on a transilluminator with ultraviolet radiation. Processing of the obtained data was made with generally accepted computer programs POPGENE 1.31 [33] and a special macro GenAlE×6.5 for MS-Excel [34].

Research results and discussion. The genetic structure of the experimental group of largemouth bass from the ponds of The State enterprise «Experimental Fish Farm «Nyvka»» was analyzed using four oligonucleotides: B – $(GAG)_6C$; C – $(AGC)_6G$; D – $(ACC)_6G$ and E – $(AGC)_6C$.

The indicators that characterize the genetic polymorphism of largemouth bass according to selected ISSR markers were calculated (Table 2). As a result of the analysis of polymorphism at loci, 80 alleles with a length of 160 bp – 1320 bp were identified. The amplicon ranges for the selected

markers were determined: marker B – from 150 bp to 1186 bp; marker C – from 640 bp to 200 bp; D from 1320 bp to 225 bp; and E – within 630 bp – 160 bp. The most polymorphic is marker B – 26 alleles; the least polymorphic is marker E – 15 alleles. The obtained results showed that the markers have a high degree of polymorphism (Figure 1).

In the studied group of largemouth bass, using primer B, a total of 26 alleles were identified with a size in the range of 1186 bp – 150 bp. Allelic variants 1186 bp, 1050 bp, 925 bp, 850 bp, 817 bp, 754 bp, 660 bp, 630 bp, 580 bp, 510 bp were observed with the same percentage – 7,7%; 1000 bp, 780 bp, 600 bp were 11,5%. In the largemouth bass group, the largest proportion form amplicon variants with a length of 415 bp and 300 bp – 27%.

In the group of largemouth bass using primer C, a total of 16 alleles were detected, the size of which was in the range of 640 – 200 bp. Allelic variants 640 bp, 530 bp, 450 bp, 395 bp, 378 bp, 280 bp, 200 bp were found with the same percentage – 12.5%; 389 bp, 322 bp, was 31.2%. The 432 bp allelic variant formed the largest proportion in the study group – 43.8%.

When using primer D, a total of 24 alleles with the size in the range of 1320 bp – 225 bp were detected. Variants of amplicons with length 560 bp and 320 bp formed the largest portion of the study group – 20,8%. Other amplicons were found with almost the same percentage.

A total of 15 alleles were detected with primer E, the size of which ranged from 630 to 160 bp. The largest proportion in the study group was the 280 bp allelic variant – 47.7%. The allelic variants of 600 bp and 400 bp were found with the same percentage – 33.3%. The 420 bp and 340 bp amplicon variants accounted for 26.7%, and the 325 bp and 300 bp amplicons for 20%. Other amplicons were found with almost the same percentage.

As a result of the genetic analysis, it was found that three amplicons were found to be specific for primer C: 385 bp, 322 bp were detected in 31.2 % and 432 bp were detected in 43.8 % of largemouth bass, and three amplicons were found to be specific for primer E: 600 bp, 400 bp – 33.%, 280 bp – 47.7 %. As a result of the study, two primers were identified that can be used for genotyping of largemouth bass. The identified amplicons with high frequency demonstrate the peculiarities of the genetic structure of the studied group of largemouth bass.

The average value of alleles per locus is 20. The largest number of alleles was observed for primers $(AGC)_6G$, $(ACC)_6G$ and amounted to 12,2. The least polymorphic in terms of the number of amplicons per locus is the primer $(AGC)_6C$.

For the four primers studied, the effective number of alleles varied from 10,2 to 12,2. Thus, from the point of view of the number of effective alleles per locus, all the studied markers are highly polymorphic (Table 2).

Table 2 – **Indicators of genetic polymorphism by ISSR markers of largemouth bass**

Primer	Amplicon length (bp)	Number of alleles	n_e	H_0	H_e	F_{is}
$(GAG)_6C$	150-1186	26	10,4	0,785	0,904	0,132
$(AGC)_6G$	200-737	15	12,2	0,765	0,918	0,167
$(ACC)_6G$	225-1320	24	12,2	0,833	0,918	0,093
$(AGC)_6C$	160 - 630	15	10,2	0,754	0,902	0,164
Average (Na)		20	11,3	0,784	0,789	0,332

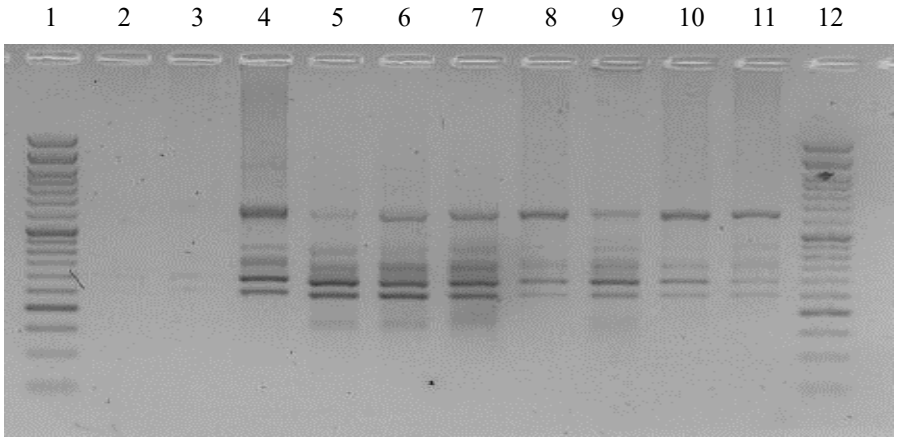

Fig. 1. **Electrophoretic spectrum of amplicons in largemouth bass (ISSR-PCR), was obtained using primer E - lanes № 2–11; 1–12 molecular weight marker 1kb DNA Ladder.**

Actual heterozygosity for these primers was calculated. The lowest value of actual heterozygosity (H_0) was observed for the primer $(AGC)_6C$ and was 0,754, and the highest heterozygosity of 0,833 was recorded for the primer $(ACC)_6G$. The average value of actual heterozygosity was 0,784.

The highest value of expected heterozygosity (H_e) was observed for primers $(AGC)_6G$, $(ACC)_6G$ and amounted to 0,918, the lowest value of expected heterozygosity was for primer $(CTC)_6C$ – 0,842. The average value of expected heterozygosity for four primers was 0,789. A sufficiently high value of average heterozygosity indicates a high level of genetic variability.

For each locus, the heterozygosity index was used to calculate the fixation index ^{Fis} (inbreeding coefficient of individuals relative to the population), which can be used to identify and assess the predominance of heterozygotes in the population. For four primers, the predominance of heterozygotes was observed, as the inbreeding coefficient ranged from 0.093 to 0.167. The average fixation index is 0.332, which also indicates the predominance of heterozygotes over homozygotes in the studied population.

Based on selected markers, 80 alleles with a length of 160 bp – 1320 bp were obtained. The amplicon ranges based on the used markers were determined: marker B from 150 bp to 1186 bp; marker C from 640 bp to 200 bp; D from 1320 bp to 225 bp; and E within 630 bp – 160 bp. The most polymorphic was marker B – 26 alleles, the least polymorphic was marker E – 15 alleles. The analyzed markers are informative for the analysis of the genetic structure of largemouth bass, because molecular markers are considered useful for studies of genetic polymorphism in cases where the average heterozygosity value is in the range of 0,3–0,8 [35, 36, 37]. Analysis of the genetic structure of largemouth bass using ISSR markers has established that they all have a wide range of alleles and a fairly high level of genetic diversity, which is confirmed by studies of other authors with different fish species [27, 38, 39, 40].

During the ISSR analysis of the genetic structure of largemouth bass, it was established that three amplicons were identified with primer C as specific: 385 bp, 322 bp were present in 31,2 % and 432 bp were present in 43,8% of largemouth bass individuals; using primer E, three amplicons were determined to be specific: 600 bp, 400 bp – 33,0%, 280 bp – 47,7%. As a result of research, two primers, that can be used for largemouth bass genotyping, have been identified. Certain amplicons with high frequency demonstrate features of the genetic structure of the studied group of largemouth bass.

Conclusions. The use of molecular genetic markers (ISSR-PCR) to study the genetic structure of largemouth bass is currently the most informative and justified method from the point of view of achieving the goal. The data on the genetic structure of the experimental group of largemouth bass were obtained by means of intermicrosatellite analysis of DNA polymorphism. The results of research, obtained during the study, showed that the selected markers have a high degree of polymorphism and can be used for population genetic analysis of this species. Taking into account the insufficient number of studies on largemouth bass in Ukraine, we consider it reasonable to continue work in this direction.

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Використання ISSR-маркерів для генотипування експериментальної групи великоротого окуня *Micropterus salmoides* (*LACEPEDE, 1802*), вирощеного у ставках Полісся України

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Метою цієї роботи було дослідити генетичну структуру експериментальної групи великоротого окуня, вирощеного у ставках Полісся України, за використання ISSR-маркерів. Для виконання поставлених завдань проведено ISSR-генотипування генетичної структури великоротого окуня із застосуванням чотирьох фрагментів тринуклеотидних локусів. Охарактеризовано генетичну структуру експериментальної групи великоротого окуня ставового рибного господарства ДПДГ «Нивка» *з* використанням 4-х праймерів: B – $(GAG)_6C$; C – $(AGC)_6G$; D – $(ACC)_6G$ та E – $(AGC)_6C$. Для досліджень використовували фрагменти плавців. У ході роботи було підібрано оптимальні умови проведення ISSR-PCR аналізу. Дослідження дали змогу виявити ряд факторів, що мають вплив на ефективність цих маркерів: концентрація ДНК, кількість циклів ампліфікації. За 4-ма маркерами виявлено 80 алелів з молекулярною масою 160 п.н – 1320 п.н. Визначено діапазони ампліконів за обраними маркерами: маркером B – від 150 до 1186 п.н.; за маркером C – від 640 до 200 п.н.; D – від 1320 до 225 п.н.; та E – в межах 630 – 160 п.н. Найбільш поліморфним є маркер B – 26 алелів, найменш поліморфними є маркер E –15 алелів. У дослідженій експериментальній групі великоротого окуня ефективне число алелів варіювало від 10,2 (маркер E) до 12,2 (маркери C, D). Визначено показники генетичної мінливості за розрахунками алельних частот. Максимальний рівень наявної гетерозиготності становить для маркерів C, D – 0,918, найменший для маркера E – до 0,902. Запропоновано метод, який дає можливість за використання зазначених праймерів провести аналіз генетичної структури експериментальної групи великоротого окуня і реалізувати генетичну інформацію на різних стадіях селекційного процесу.

Ключові слова: ISSR-PCR, генетична структура, великоротий окунь, ДНК-маркери, генотип, амплікони, генетичний поліморфізм, молекулярно-генетичний маркер.

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