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Genetic Structure of the Antoniny-Zozulenets Intrabreed Type of Ukrainian Leather and Scaly Carps Using Microsatellite Markers

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Abstract

The common carp (Cyprinus carpio) is a dominant aquaculture species in Ukraine with several intra-breed types within two recognized breeds: scaly and leather. Understanding their genetic diversity is crucial for successful breeding programs. This study is focused on a genetic characterization of Antoniny-Zozulenets intrabreed type of scaly and leather carps from the Stara Synyava fish hatchery, which were produced by selective breeding in Ukraine. Microsatellite analysis was conducted using six DNA loci: MFW 02, MFW 06, MFW 09, MFW 15, MFW 23, MFW 31; all of which were polymorphic in both groups. The mean polymorphic information content was 0.903±0.009. The ranges of amplicons for the selected loci were determined. This enabled the characterization of specific genetic features of each breed. Allelic richness ranged from 9.987 to 14.512 for scaly carp and 9 to 13 for leather carp. Notably, loci MFW 02, MFW 09, MFW 23 in the scaly carp group exhibited a wider range and number of alleles compared to leather carp. The Shannon diversity index averaged 2.481±0.060 for scaly carp and 2.348±0.056 for leather carp. The fixation index had negative values, indicating a predominance of heterozygous genotypes (scaly: F=-0.025; leather: F=-0.072). The efficiency of the proposed set of markers was confirmed. These markers could be further used in monitoring genetic diversity and differentiation of carps in Ukraine for breeding and conservation efforts.

Introduction

The common carp (*Cyprinus carpio* L.) is an ancient species domesticated for aquaculture, which is valued for its role as a food source for humans and is widely distributed globally (Balon, 1995; Flajšhans & Hulata, 2006; Kohlmann et al., 2003; Teletchea & Fontaine, 2014). It is the dominant fish species in aquaculture of Ukraine (Hrytsynyak et al., 2022), which currently accounts for nine intra-breed types within two carp breeds (scaly and leather). The leather carp can also be referred to as "framed", which is based on the

translation of locally used nickname. Among Ukrainian carp types, the Antoniny-Zozulenets intra-breed type stands out. It is named after the villages of Antoniny and Velyki Zozulyntsi in Khmelnytskyi Oblast (Ukraine), where the "Antoniny" and "Zozulentsi" fish farms were located respectively (Oborskiy et al., 2022). Being established between the 1930s and 1950s, this intrabreed type was created through selective breeding by reproductive crossing of indigenous outbred carp with leather Galician (Halych) carp (named after the historical region of Galicia (Halychyna), a place in Western Ukraine and southeastern Poland; in some works it is mentioned as Galycian (Hrytsynyak et al., 2022). Antoniny-Zozulenets intra-breed types of Ukrainian leather and Ukrainian scaly carps were officially registered in 2021 (Oborskiy et al., 2022). This intra-breed type showed improved feachures such as fertility, productivity, and winter hardiness. Importantly, it has served as the genetic foundation for other Ukrainian carp types, thus emphasising its crucial role in the Country's selective breeding programs (Oborskiy et al., 2022; Tomilenko et al., 2012).

The valuable genetic stock of original carp forms faces the risk of depletion due to the implementation of active breeding programs as well as changing environmental conditions. For proper monitoring of the carp stocks it is necessary to obtain comprehensive information about its genetic diversity and population structure within and between stocks in various fish hatcheries and farms currently operating in Ukraine. It is needed for sustainable conservation of the selectively bred intra-breed types of carp in this Country.

Molecular methods provide crucial data for evaluating and maintaining genetic diversity to avoid inbreeding of populations both in natural populations and within established broodstocks. It is also helpful while creating broodstocks with desired economically valuable traits. One of the most effective and widely used methods for studying the genetic structure of populations today is microsatellite markers, which allows for the assessment of intraspecific genetic polymorphism, provides the opportunity to assess the differences among populations at the level of allelic variations (Ghelichpour et al., 2013; Napora-Rutkowski et al., 2017). Microsatellite markers have been actively and extensively employed in the past and at present, continuing to date. It allows to study the genetic diversity among various types and varieties of C. carpio (Andarz et al., 2022; Crooijmans et al., 1997; Gu et al., 2012; Kohlmann et al., 2003; Thai et al., 2007). Limited studies have been conducted in Ukraine on carps using microsatellite markers. Previous studies have focused on species such as Galician carp (C. carpio L.), Amur carp (C. rubrofuscus), and silver (Hypophthalmichthys molitrix) and bighead (Hypophthalmichthys nobilis) carps (Grytsyniak et al., 2018; I. Hrytsyniak et al., 2015; Kuts et al., 2021; Yarova et al., 2017). Some Ukrainian carp breeds reared in other countries were also analyzed using microsatellite markers (Napora-Rutkowski et al., 2017), however the information remains limited.

In previous studies of the genetic structure of Antoniny-Zozulenets intra-breed type authors used a panel of protein markers: transferrin (TF), albumin (ALB), esterase (EST, EC 3.1.1.1), malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.1.40), carbonic anhydrase (CA, EC 4.2.1.1), superoxide dismutase (SOD, EC 1.15.1.1), and catalase (CAT, EC 1.11.1.6) (Hrytsyniak et al., 2021; Nagornyuk et al., 2011; Oborskiy et al., 2022; Tarasiuk et al., 2012). This approach allowed for tracking changes in allele

frequencies and heterozygosity levels. The ISSR-PCR method was used to identify markers ((AGC)6C and (AGC)₆G) applicable for genotyping Ukrainian leather and scaly carps of Antoniny-Zozulenets intra-breed type (Nahorniuk et al., 2013). Microsatellite markers have not been employed in the study of Antoniny-Zozulenets intra-breed carp type. Considering the informative nature and advantages of SSR markers in stock analysis, such study would be very useful. We envision that such endeavors can facilitate genetic characterization of each carp breed and type, enable comparative analyses, and aid in identifying the distinctive features of the genetic structure within each structural type. Consequently, this method will enable the monitoring and conservation of unique carp gene pools. The aim of this study is to analyze the genetic structure of Antoniny-Zozulenets intra-breed carp type using microsatellite markers.

Materials and Methods

Sample Collection and DNA Extraction

In 2023, blood samples were collected from a total of 30 carps, comprising 15 leather carps (Figure 1) and 15 scaly carps (Figure 2). These individuals belonged to the Antoniny-Zozulenets intra-breed type's southwestern breeding line, maintained at the Stara Synyava fish hatchery (Khmelnytskyi Oblast, Ukraine). This fish hatchery serves as the sole entity responsible for breeding activities and is part of the State Enterprise "Khmelnytskyi Ribhosp".

Blood samples were obtained from the caudal vein of live fish using sterile syringes containing heparin solution. Total genomic DNA was isolated using DNA-Go kit ("BioLabTech LTD", Ukraine) following the manufacturer's instructions. Isolated DNA was stored at -20°C until futher laboratory analysis.

Microsatellite Amplification and Gel Electrophoresis

The genetic structure of the carp was analyzed using at six microsatellite loci (Crooijmans et al., 1997) (Table 1). These microsatellite loci have been previously used in population genetic studies of different carps in Ukraine, enabling further comparative analysis.

Polymerase chain reaction was conducted using OneTaq[®] Quick-Load[®] 2X Master Mix with Standard Buffer (New England Biolabs) using Thermo Scientific thermocycler (Arktik Thermal Cycler, Finland) under the annealing temperature conditions specified for each primer (Table 1). Amplicons were separated by electrophoresis in 8% polyacrylamide gel (PAGE) in 1×TAE buffer, with specific modifications for fragment separation conditions (4°C, 170 V, 15 mA for 3 hours). Gel plates were stained with ethidium bromide (0.5 μ g/ml gel) in 1×TAE buffer and visualized under UV light on a transilluminator (VILBER LOURMAT ECX-20.M, Germany). Amplicon lengths were determined using TotalLab v.2.01 software (htt://www.totallab.com).





Figure 1. Antoniny-Zozulenets intra-breed type of Ukrainian leather carp.



Figure 2. Antoniny-Zozulenets intra-breed type of Ukrainian scaly carp.

No	Locus	Forward and Reverse Sequence (5'→3')	Annealing temperature (°C)
1	MFW 02	F: CACACCGGGCTACTGCAGAG	55
		R: GTGCAGTGCAGGCAGTTTGC	
2	MFW 06	F:ACCTGATCAATCCCTGGCTC	55
		R:TTGGGACTTTTAAATCACGTTG	
3	MFW 09	F: GATCTGCAAGCATATCTGTCG	55
		R: ATCTGAACCTGCAGCTCCTC	
4	MFW15	F: CTCCTGTTTTGTTTGTGAAA	55
		R: TTCACAAGGTCATTTCCAGC	
5	MFW 23	F:GTATAATTGGGAGTTTTAGGG	55
		R:CAGGTTTATCTCCCTTCTAG	
6	MFW31	F: CCTTCCTCTGGCCATTCTCAC	50
		R:TACATCGCAGAGAATTCGTAAG	

Table 1. Information on microsatellite primers used in PCR

Data Analysis

The software "GenAIEx v.6.5" (Peakall & Smouse, 2012, 2006) was used to determine the following parameters: Observed number of alleles (A), Effective number of alleles (Ae), Number of private alleles, Shannon's Information index Observed (I), heterozygosity (Ho), Expected heterozygosity (He), Expected unbiased heterozygosity (uHe), Inbreeding coefficient (F). Allelic richness (AR) was calculated using FSTAT v.2.9.4 software (Goudet, 1995). The polymorphic information content (PIC) was calculated using CERVUS v.3.0.6 software (Kalinowski et al., 2007). Analysis of molecular variance (AMOVA), Hardy-Weinberg probability test, and Principal coordinate analysis (PCoA) were performed using Genalex v.6.5.

Results

Analysis of selected SSR loci revealed that all of the studied markers were 100% polymorphic in both scaly and leather carps of Antoniny-Zozulenets intra-breed type. The lowest PIC value was recorded for the locus MFW 02 in leather carp (0.842), while the highest was observed for the same locus in scaly carp (0.939). The average PIC value was 0.903±0.009 for scaly carp and 0.879±0.009 for leather carp, resulting in an overall average PIC value of 0.891±0.012, indicative of high polymorphism across all loci, according to the rules for interpreting PIC values (Botstein et al., 1980) (Figure 3).

Specific peculiarities of allelic ranges and frequencies were observed for the studied loci. For the loci MFW 02, MFW 09, and MFW 23 a wider ranges and a greater numbers of alleles were observed in scaly carp

compared to leather carp (Figure 4). Specifically, locus MFW 02 displayed 14 alleles in scaly carp versus 10 alleles in leather carp. At locus MFW 09, the allelic range spanned from 70 to 139 bp in scaly carp, whereas it ranged from 70 to 117 bp in leather carp. For locus MFW 23, scaly carp showcased an allelic range of 89 to 200 bp with 17 allelic variants, whereas leather carp exhibited a narrower range from 133 to 190 bp with 13 alleles.

Private alleles unique to each group were observed at the extremities of the allelic ranges for loci MFW 06, MFW 15, and MFW 31. In locus MFW 06 scaly carp displayed private alleles ranging from 168 to 180 base pairs, encompassing alleles of higher molecular weight. Conversely, leather carp exhibited an extension due to the presence of lighter molecular weight alleles, ranging from 128 to 150 base pairs. A similar scenario was observed at locus MFW 31, with private alleles detected in scaly carp within the range of 355-380 bp, while in leather carp, the range spanned 265-305 bp. Regarding locus MFW 15, private alleles in scaly carp extended from 230 to 250 bp, while in leather carp, heavier private alleles were observed, ranging from 296 to 343 bp.

The number of alleles (A) across all loci ranged from 10 to 17, while the effective number of alleles per locus (Ae) varied from 7.031 to 11.879 (Table 2). The highest allelic richness (Ar) index was recorded for marker MFW 23 (14.512 in scaly carp), whereas the lowest was observed for marker MFW 02 (9.004 in leather carp). On average, scaly carp exhibited a higher Ar value (12.422±0.691) compared to leather carp (11.354±0.564).

Shannon's biodiversity index (I) exhibited noteworthy high levels for locus MFW 23 in both groups

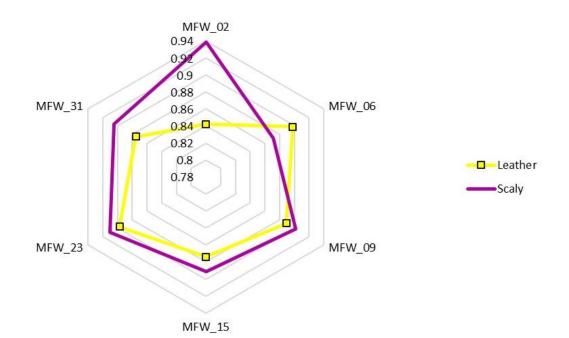


Figure 3. Polymorphic information content of SSR markers in the analysis of Antoniny-Zozulenets intra-breed type of scaly and leather carps.

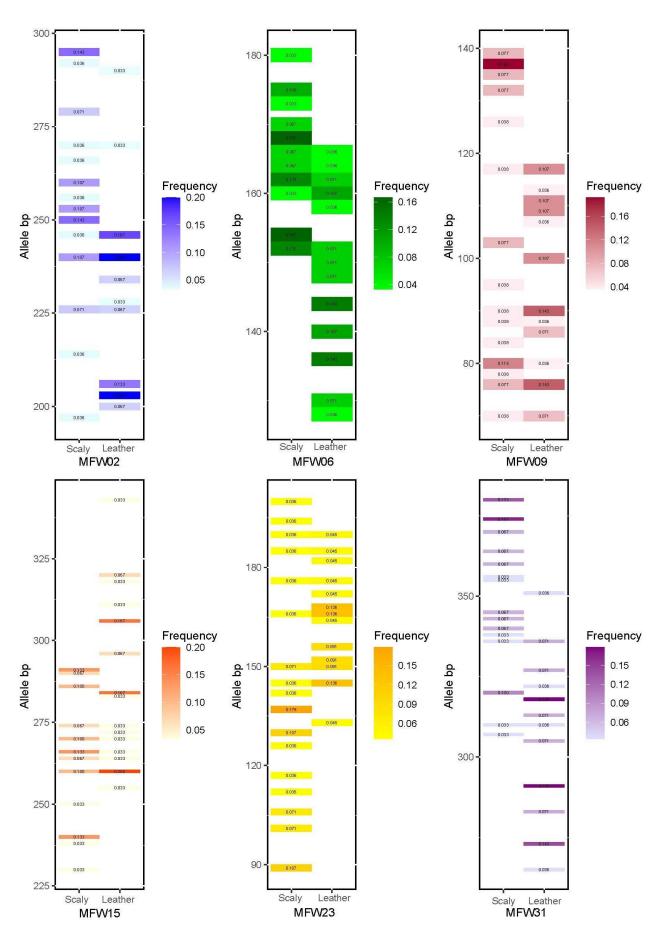


Figure 4. Ranges of allelic variants (bp) for microsatellite loci in scaly and leather carp of Antoniny-Zozulenets intra-breed type

(Table 2). Leather carp displayed an average I value of 2.348±0.056, whereas scaly carp exhibited a slightly higher value of 2.481±0.060.

In the scaly carp group, Ho and uHe were slightly lower at 0.926 ± 0.039 and 0.937 ± 0.005 , respectively, compared to leather carp, which recorded values of Ho= 0.952 ± 0.048 and uHe= 0.923 ± 0.009 . The F value was negative for both groups, with scaly carp at -0.025 and leather carp at -0.072, indicating outbreeding in both groups (Figure 5).

The Hardy-Weinberg test (P<0.05) showed no statistically significant deviations from the equilibrium for all 6 loci in scaly carps. However, deviations were significant for leather carp at loci MFW 02 and MFW 31 (Table 3).

The Fis value suggested a deficit of heterozygous genotypes for locus MFW 09 as indicated by the positive

Fis value of 0.179 (Table 3). Conversely, an excess of heterozygotes was observed for the rest of the microsatellite loci with the inbreeding coefficient ranging from -0.02 to -0.179. The subpopulation Fst indicated a differentiation of 0.042 between the two studied groups. The unbiased genetic distance Nei between the groups was 1.051. Principal coordinate analysis highlighted the differentiation of individuals within the studied groups (Figure 6). The first two components explained 9.36% and 7.97% of the total variations in allelic variants of the studied loci.

Analysis of Molecular Variance demonstrated differentiation between individuals of the two groups, with 8% of genetic variation attributed to variation between groups, while 92% was observed within groups.

Table 2. Genetic Diversity Parameters of Antoniny-Zozulenets Intra-Breed Type of Scaly and Leather Carps

			-			-			
Group	Locus	Ranges, bp	Α	Ae	Ar	I	Но	uHe	F
	MFW 02	197-295	14.000	10.595	12.401	2.484	0.929	0.939	-0.025
	MFW 06	152-180	11.000	8.491	9.987	2.247	1.000	0.913	-0.134
Cash	MFW 09	70-139	15.000	10.903	13.675	2.555	0.769	0.945	0.153
Scaly	MFW 15	230-291	12.000	10.000	10.958	2.378	1.000	0.931	-0.111
	MFW 23	89-200	17.000	11.879	14.512	2.661	0.857	0.950	0.064
	MFW 31	307-380	15.000	11.250	12.997	2.561	1.000	0.943	-0.098
	MFW 02	200-290	10.000	7.031	9.004	2.093	1.000	0.887	-0.166
	MFW 06	128-166	13.000	10.595	11.931	2.453	1.000	0.939	-0.104
Loothor	MFW 09	70-117	12.000	9.800	11.038	2.366	0.714	0.931	0.205
Leather	MFW 15	255-343	15.000	8.654	12.204	2.414	1.000	0.915	-0.131
	MFW 23	133-190	13.000	10.522	13.000	2.453	1.000	0.948	-0.105
	MFW 31	265-351	12.000	8.711	10.944	2.312	1.000	0.918	-0.130

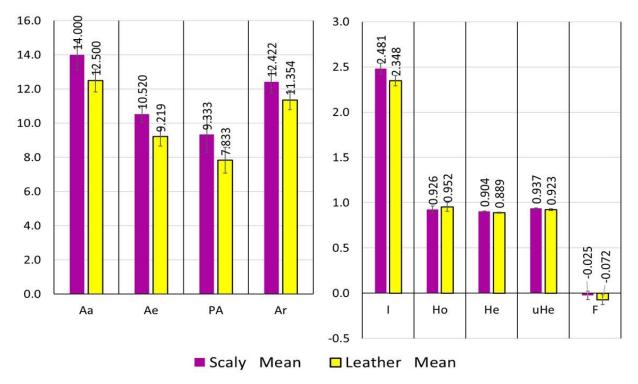
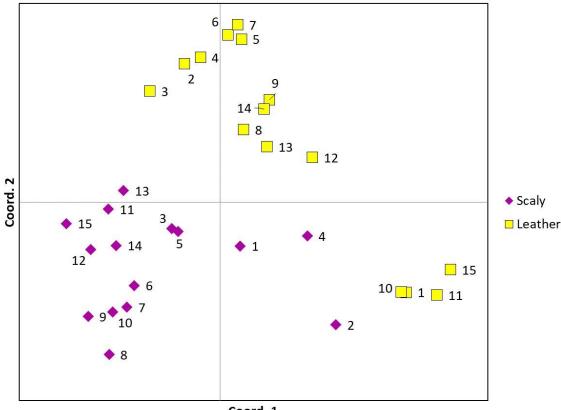


Figure 5. Average values of genetic variability Parameters for the studied microsatellite loci in the Antoniny-Zozulenets intra-breed type of scaly and leather carps.

Table 3. Summary of the Hardy-Weinberg test and F-statistics

Locus	Scaly		Leather		F-statistics		
	Prob-PHW	Signif	Prob-PHW	Signif	Fis	Fit	Fst
MFW 02	0.058	ns	0.031	*	- 0.094	- 0.043	0.046
MFW 06	0.482	ns	0.898	ns	-0.119	- 0.072	0.042
MFW 09	0.214	ns	0.126	ns	0.179	0.208	0.036
MFW 15	0.592	ns	0.326	ns	-0.121	- 0.075	0.041
MFW 23	0.738	ns	0.666	ns	- 0.020	0.017	0.036
MFW 31	0.190	ns	0.042	*	-0.113	- 0.056	0.052
Average					- 0.048	- 0.003	0.042

Notes: Prob-PHW – Hardy-Weinberg probability test; Signif – level of statistical significance; * – P < 0.05; n.s. – non-significant deviation.



Coord. 1

Figure 6. Principal coordinate analysis based on microsatellite analysis of scaly and leather carp.

Discussion

Genetic studies play a crucial role in the management of fish populations in aquaculture because genetic variation and diversity form the foundation of a species' adaptive potential (Teletchea & Fontaine, 2014). Hatchery-based cultivation and selective breeding practices, in the absence of concurrent genetic monitoring, can result in allele loss and reduced heterozygosity. Moreover, a lack of knowledge of the genetic relationships among different fish stocks can adversely affect breeding programs, potentially leading to outbreeding depression from crosses with genetically unrelated or distantly related stocks (McClelland & Naish, 2007). Hulak et al. (2010) highlighted the

importance of describing the genetic structure of individual strains and populations, which is essential for identifying individuals' origins and monitoring undesirable gene introgression during broodstock management. Microsatellites have been widely used to characterize various carp strains in aquaculture facilities globally, not limited to Europe (Hulak et al., 2010; Jewel et al., 2006; Kim et al., 2018; Kohlmann et al., 2005; Napora-Rutkowski et al., 2017; Tomljanović et al., 2013; Tóth et al., 2020). This extensive experience underscores the continued utility of these DNA markers in carp breeding and selection programs. Microsatellite loci have been proven effective in characterizing and differentiating carp populations, including populations from different geographic regions (Kohlmann et al.,

2003). Tóth et al. (2020) summarized numerous studies demonstrating the high discriminatory potential of microsatellite markers: origin of 90% of carp can be identified with a high degree of probability. In this study, we assessed the effectiveness and informativeness of a selected set of microsatellite markers for Antoniny-Zozulenets intra-breed type of scaly and leather carp for the first time. The six DNA markers examined exhibited high PIC with an average value of 0.903. PIC at the same loci were found to be higher in Antoniny-Zozulenets carp compared to Amur carp from the Ukrainian fish hatchery "Velykyi Liubin" (Kuts et al., 2021). Antoniny-Zozulenets carps displayed considerable genetic diversity (I=2.415±0.044), a high number of allele variants per microsatellite locus (A=13.250±0.579), and allelic richness (Ar=11.354±0.564). Specific peculiarities of the genetic structure of two studied groups were elucidated, including the presence of private alleles, enhancing the value of these markers for fish population monitoring. Scaly carp exhibited higher genetic polymorphism indices compared to leather carp as evidenced by parameters such as the number of alleles per locus, count of private alleles, and Shannon's index.

Comparison with Galician carp (Yarova et al., 2017) revealed a higher number of allelic variants in Antoniny-Zozulenets carp at analogous loci (MFW 06, MFW 15, MFW 23, MFW 31). The number of amplicons ranged from 11 to 17 in scaly carp and from 12 to 15 in leather carp, whereas Galician carp exhibited 3 to 6 amplicons. various Ukrainian carp strains Notably, also demonstrate high levels of genetic parameters. Napora-Rutkowski et al. (2017) demonstrated that among various strains of carp cultivated at the Institute of Ichthyobiology and Aquaculture, Polish Academy of Sciences in Golysz (ZIGR Golysz), the Ukrainian strains Up and Ur displayed higher levels of genetic diversity across microsatellite loci. Particularly, the Ukrainian breed Up exhibited the highest average number of alleles per breed (9.27) and private alleles (Apr) among the studied strains.

Microsatellite loci are more sensitive to detecting population bottlenecks and loss of variation due to inbreeding (Kohlmann et al., 2003). In this study, the average Fis was -0.048, indicating a prevalence of heterozygotes over homozygotes in the studied groups. This suggests that the selection activities at fish hatcheries did not lead to negative consequences and the investigated brood fish can be used for further breeding efforts. Deviations from Hardy-Weinberg equilibrium were observed in leather carps at the examined microsatellite loci, while no such deviations were noted in scaly carp. Similar findings were reported in the study by Jewel et al. (2006), where deviations from Hardy-Weinberg equilibrium were more frequent in mirror carps across all three populations, while deviations were observed in only one of the three populations of scaly carp.

The subpopulation Fst indicates weak differentiation between two examined groups, although

noteworthy. Comparable results were observed by Mondol et al. (2006), where the Fst value between scaly and leather carps from Bangladesh was 0.055. In this study, the PCoA plot showed no overlap among individuals from two studied groups with the first component representing 9.36% of total variation. AMOVA analysis showed that variability between groups accounted for 8% of total variation. Hulak et al. (2010) emphasized the significance of inter-population variability, suggesting that it should represent at least 20% of total variability for reliable determination of individual membership to their groups. Therefore, to enhance the discriminatory power of differentiation methods between scaly and leather carps of Antoniny-Zozulenets type, it could be beneficial to utilize SSR markers associated with scale cover patterns developed by Xiao et al. (2015). However, for differentiation of populations from distant regions 4-6 microsatellites should suffice (Kohlmann et al., 2005).

Conclusion

This study provided the details of genetic structure, variability, and intra-population differentiation of scaly and leather carps within Antoniny-Zozulenets intrabreed type. Through the use of SSR markers for carp genotyping, disparities in allele diversity between scaly and leather carps of this intrabreed type were uncovered. These findings emphasise the applicability of the selected set of six markers for genetic profiling of carp populations, facilitating the monitoring of genetic dynamics and the implementation of strategies to enhance breeding efficiency and preserve the genetic integrity of Antoniny-Zozulenets intrabreed type of scaly and leather carps.

Given the demonstrated high differentiating potential of microsatellite markers, as noted in numerous studies, further comprehensive comparative analysis of carps bred in Ukraine is advisable. Such analysis would enable the identification of breedspecific characteristics, monitoring of heterozygosity and genetic diversity under selection pressure, and elucidation of genetic relationships among different carps.

Ethical Statement

All manipulations with European grayling individuals at the fish hatchery for the selection of genetic research material were conducted following the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes (Strasbourg, 1986) ETS No. 123 and UFRC 2014 (Use of Fishes in Research (2014) Guidelines for the use of fishes in research. American Fisheries Society, Bethesda, Maryland. https://fisheries.org/docs/wp/Guidelines-for-Use-of-Fishes.pdf).

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Authors declare there are no financial or any sort of conflicts related to this study.

Author Contribution

Conceptualization: AM, VO; Methodology: AM, OB, TD; Formal Analysis: AM, OB, NB, TD; Investigation: AM, NB, OB, HK, VO; Resources: AM, HK, VO; Visualization: OB; Writing - original draft: AM, OB; Writing-review and editing: all authors. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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