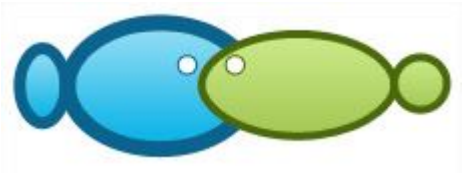

A comparative analysis of Amur carp (*Cyprinus rubrofuscus*) produced from native and cryopreserved sperm using microsatellite loci

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Abstract. The aim of the work was to conduct a comparative analysis of Amur carp (*Cyprinus rubrofuscus*) produced from fresh and defrosted sperm, which was cryopreserved for 24 years, based on the polymorphism of microsatellite loci and to study the heterogeneity of these groups. The genetic structure of carp produced from the defrosted sperm represented a range of amplicons of local stocks. Specific alleles occurred at MFW 6 and MFW 31 loci, which were not observed in the local carp group. This indicates a higher level of polymorphism, which was also confirmed by the higher Shannon biodiversity index at these loci (I = 1.727 in the cryo-carp group vs I = 1.383 in the local carp group). The mean number of alleles per locus (N_a) and the effective number of alleles per locus (N_e) was higher in the cryo-carp group (N_a = 7.25 and N_e = 5.2) compared to the local carp group (N_a = 5.75 and N_e = 3.9). The genetic variability was distributed by 7.7% (F_{st} = 0.077) between the studied carp groups. The microsatellite markers used were found to be highly informative (average PIC = 0.678), and therefore effective for further use in monitoring the changes in the gene pool of cyprinids under the effect of cryopreservation. The obtained results demonstrated the effectiveness of the use of cryopreserved sperm to improve the genetic structure, and also allow assessing the degree of domestication of carp groups. Further analysis of the genetic structure of the offspring and fish sperm can allow developing strategies for preserving the biodiversity of the gene pool of carp of natural and artificial stocks.
Key Words: cryopreservation, defrosted sperm, microsatellites, genetic structure, heterozygosity.

Introduction. The cryopreservation of fish sperm is a promising method for the conservation of gene pool of endangered fish species and valuable objects of aquaculture (Demkina et al 1997; Labbe et al 2001; Golovanova et al 2003; Billard et al 2004; Beh 2004; O'Reilly & Doyle 2007; Martínez-Páramo et al 2009; Ponomareva et al 2009; Cabrita et al 2010; Chew et al 2010; Matishov et al 2012; Shishanova et al 2012; Ananev & Manohina 2013; Asturiano et al 2016; Cherepnin 2016; Martínez-Páramo et al 2017; Ponomareva et al 2017; Kilyakova et al 2019). This method is useful for restoring the heterogeneity of the gene pool of commercial fish stocks, which are in an inhibited condition during domestication (Pronina et al 2010; Osipova et al 2016; Kononenko 2017) or high-quality broodstock of remote fish farms (Yamaner et al 2015; Kilyakova et al 2019), that is quite effective in cases of inbred depressions.

Currently, there is a positive international experience in the use of cryopreserved sperm to restore the population structure of different fish species (Martínez-Páramo et al 2017; Linhart et al 2000; Di Chiacchio et al 2017; Ponomareva et al 2017). In Ukraine, cryocollections of fish sperm are preserved in one of the oldest cryobanks in Europe at the Institute of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine (Kopeika et al 2011) and at the Institute of Fisheries of the National Academy of Agrarian Sciences of Ukraine.

However, despite successful results of the use of cryopreservation, there are still issues about improving the methods of cryopreservation of fish sperm and defrosting it with the preservation of all biological properties, as well as issues about the effect of cryopreserved sperm on the genetic structure of fish populations (Demkina et al 1997;

Bogatyireva & Ponomareva 2009; Ponomareva et al 2009; Asturiano et al 2017; Cherepnin 2016).

There are a number of problems related to the fact that a part of the material intended for cryopreservation cannot be frozen or they are not of good quality after thawing; e.g., spermatozoa lose their ability to fertilize eggs and sometimes offspring is characterized by low viability that indicates low effectiveness of these methods (Zemkov & Akimochkina 2009; Shishanova et al 2012; Cherepnin 2016; Kilyakova et al 2019). The selective mortality of Russian sturgeon (*Acipenser gueldenstaedtii*) embryos associated with the process of sperm cryopreservation was shown to be caused by changes in the genetic structure such as an increase in the number of heterozygotes at the malate dehydrogenase and esterase loci as well as the elimination of alleles with low frequency at the esterase locus (Shishanova et al 2012). Thus, the consequence of the use of cryopreserved sperm may be the selection towards certain isoenzymes and their isoforms as a result of biochemical adaptation under the effect of stress factors (Shishanova et al 2012). Modern science takes significant steps to solve the problem of viability of defrosted material. Creation of a cryocollection requires selection based on genotypes (Golovanova et al 2003; Pronina et al 2010) with several dozens of replicates, which would characterize the genetic polymorphism of valuable biological material, in order to compensate for losses during defrosting and complete reproduction of genetic polymorphism of fish stocks of interest (Golovanova et al 2003; Pronina et al 2010).

A number of works (Demkina et al 1997; Shishanova et al 2012), which studied the effect of cryopreserved sperm on the heterogeneity of fish stocks, were conducted using genetic and biochemical systems (GBS). However, GBSs were later replaced by DNA markers, which have already proven to be convenient and effective tools for studies in the field of population genetic and gene pool management in long-term fish biodiversity conservation programs (Golovanova et al 2003; Abdul-Muneer 2014; O'Reilly & Kozfkay 2014).

The importance of studies on the cryopreserved sperm of different fish species today cannot be underestimated. The extinction of valuable fish species in the wild under the influence of human impact, including possible deterioration of environmental conditions as well as the loss of genetic diversity of aquaculture stocks due to the intensive use of broodstock without regard to their genetic structure have become a driving force for such studies.

For many years, Amur carp (*Cyprinus rubrofuscus*) has been used for industrial hybridization with European carp (*C. carpio*). Due to heterosis effect, the produced hybrids are characterized by higher growth rate, resistance to the most common fish diseases, and their cultivation increases pond productivity by 19-22% (Oleksiyenko et al 2012). However, intensive selection in aquaculture often results in a differentiation from the unique primary genetic structure of "pure forms" of Amur carp; therefore, the cryopreserved sperm can be used as a method of preservation and rational use of their gene pool.

The aim of the work was to conduct a comparative analysis of Amur carp produced from fresh and defrosted sperm based on the polymorphism of microsatellite loci and to study the heterogeneity of these groups.

Material and Method. Two groups of Amur carp were used in the study:

- control group (local carp) included fish produced using fresh sperm of local Amur carp stocks (n = 15), which are descendants of Amur carp brought to the Experimental Fish Farm "Velykyi Lyubin" (Lviv region, Ukraine) from the Russian Far East (Amur River) in 1987 (Savich 1979) and are their eighth generation;

- experimental group (cryo-carp) included carp produced from defrosted sperm (n = 15) at the State Enterprise Experimental Fish Farm "Nyvka" (Kyiv region) (Bezusyi et al 2011). This sperm was obtained from males caught in their native range in the Amur River basin and cryopreserved on June 21-23, 1987 at the thermal fish farm of the Primorsky Hydropower Plant, Russia (Kopeika et al 2011). The cryopreserved sperm was then transported to the cryobank collection of the Institute of Cryobiology and

Cryomedicine of the National Academy of Sciences of Ukraine and stored there (Bezusyi et al 2011; Kolisnyk et al 2014).

Fish for artificial fertilization (three age-7 females and three age-8 males) were brought from the State Enterprise Experimental Fish Farm "Velykyi Lyubin" (Lviv region) to the State Fish Farm of the Institute of Fisheries of the National Academy of Agrarian Sciences of Ukraine "Nyvka" (Kyiv region) in the mid-May 2011. Males and females before fertilization were held separately in 3.2 m^3 plastic tanks for ten days.

Fertilization was conducted using a traditional insemination technique in the third decade of May 2011. Eggs obtained from females were mixed and divided into two parts, which were inseminated either with fresh (a mixture of sperm from three males) or defrosted sperm, respectively. Eggs were incubated in 8 L Weiss jars. The 4-day fry were transported to the Fish Farm "Velykyi Lyubin" and released separately (local and cryo-carp) into 0.64 ha earth ponds at a stocking density of 20,000 ind ha^{-1}. Fish then were cultured according to standard techniques of pond carp aquaculture.

Adult fish were held in 1.75 ha earth ponds at a stocking density of 75 ind ha^{-1}. They were fed with sprouted wheat grains during summer and a mixture of a specialized fish feed Biomar and sprouted wheat grains (50:50) during prespawning period. Age-6+ fish were tagged with individual PIT-tags inserted under the dorsal fin.

The material for the current study was collected in September 2019. Only males were used in experiments. Following exterior parameters were measured: body weight, body length, body depth, body circumference, head length (Oleksiyenko et al 2012). These parameters were measured by a measuring tape to the nearest 1 mm and weighed on electronic balances to the nearest 1 g. Following ratios were calculated based on measurement results: body length to circumference ratio (L:C), body length to depth ration (L:D), and head length to body length ration (H:B). Measured and calculated parameters of two groups of fish were compared using a *t*-test.

Blood was taken from the caudal vein of the measured fish of both groups. Blood was drawn by sterile syringes with heparin solution (25 IU mL^{-1}). Samples were transported at a temperature of 4°C and then stored in a fridge at -20°C. Isolation of total DNA from blood was performed using a commercial DNA-Go kit (BioLabTech LTD). The concentration and quality of the isolated DNA was determined using an Eppendorf biophotometer (Eppendorf, Germany). The genetic structure of two groups of carp was analyzed with the use of four microsatellite (SSR) markers: MFW 6, MFW 15, MFW 23, MFW 31 (Crooijmans et al 1997) (Table 1).

Table 1

Microsatellite markers used in the study for analyzing Amur carp

Locus	*Primer sequence 5'→3'*	*Primer annealing temperature (°C)*
MFW 06	F*: ACCTGATCAATCCCTGGCTC	55
	R**: TTGGGACTTTTAAATCACGTTG	
MFW 15	F: CTCCTGTTTTGTTTTGTGAAA	55
	R: GTTCACAAGGTCATTTCCAGC	
MFW 23	F: GTATAATTGGGAGTTTTAGGG	55
	R: CAGGTTTATCTCCCTTCTAG	
MFW 31	F: CCTTCCTCTGGCCATTCTCAC	55
	R: TACATCGCAGAGAATTCGTAAG	

Note: F* - forward primer sequence; R** - reverse primer sequence.

The polymerase chain reaction was performed using New England Biolabs' Taq 2X Master Mix in a Thermo Scientific™ Arktik™ Thermal Cycler. Amplification was performed under the following conditions: initial DNA denaturation at 95°C for 5 min, following 35 cycles: DNA denaturation at 95°C for 30 sec, primer annealing at 55°C for 45 sec, chain synthesis at 72ºC for 1 min 30 sec; final elongation at 72°C for 10 min.

Electrophoretic separation was performed in a 3% agarose gel with ethidium bromide in 1×TAE buffer. The Quick-load Purple 50 bp DNA Ladder (New England Biolabs) was used as a fragment length marker. The determination of fragment lengths was performed in Totallab v.2.01. Statistical processing was performed in Genalex 6.5.

(Peakall & Smouse 2006, 2012). The polymorphism information content (PIC) was calculated according to the generally accepted formulas for codominant markers (Nagy et al 2012).

The number of different alleles per locus (N_a), effective number alleles per locus (N_e), number of private alleles, observed heterozygosity (H_o), expected heterozygosity (H_e), expected unbiased heterozygosity (uH_e), Shannon's Information Index (I), Hardy–Weinberg probability test and F-Statistics were obtained for each population using GenAlEx version 6.5 (Peakall & Smouse 2006, 2012).

Results. The genetic polymorphism parameters at microsatellite loci, which were determined by a molecular genetic analysis of two groups of Amur carp produced with the use of fresh and cryopreserved sperm, are presented in Table 2 and Figure 1.

Table 2

Genetic variability parameters of the studied groups of Amur carp at four microsatellite loci

Group	*Locus*	*Amplicon size range, bp*	N_a	N_e	H_o	H_e	*Prob*	*PHW*
Group 1 Local carp	MFW 6	150-165	2	1.960	0.000	0.490	0.008	**
	MFW 15	157-296	10	6.095	0.875	0.836	0.862	ns
	MFW 23	95-142	6	5.556	0.600	0.820	0.290	ns
	MFW 31	273-353	5	2.000	0.250	0.500	0.007	**
		Mean	5.750	3.903	0.431	0.661		-
		SE	1.652	1.116	0.192	0.096		-
Group 2 Cryo-carp	MFW 6	124-165	4	2.299	0.100	0.565	0.002	**
	MFW 15	157-286	9	7.692	0.600	0.870	0.203	ns
	MFW 23	95-150	8	6.061	0.500	0.835	0.012	*
	MFW 31	210-345	8	5.128	0.400	0.805	0.079	ns
		Mean	7.250	5.295	0.400	0.769		-
		SE	1.109	1.131	0.108	0.069		-

Notes: N_a - number of alleles per locus; N_e – effective number of alleles per locus; H_o - observed heterozygosity; H_e - expected heterozygosity; Prob-PHW - Hardy–Weinberg probability test: *p < 0.05, ** p < 0.01, n.s. = nonsignificant deviation.

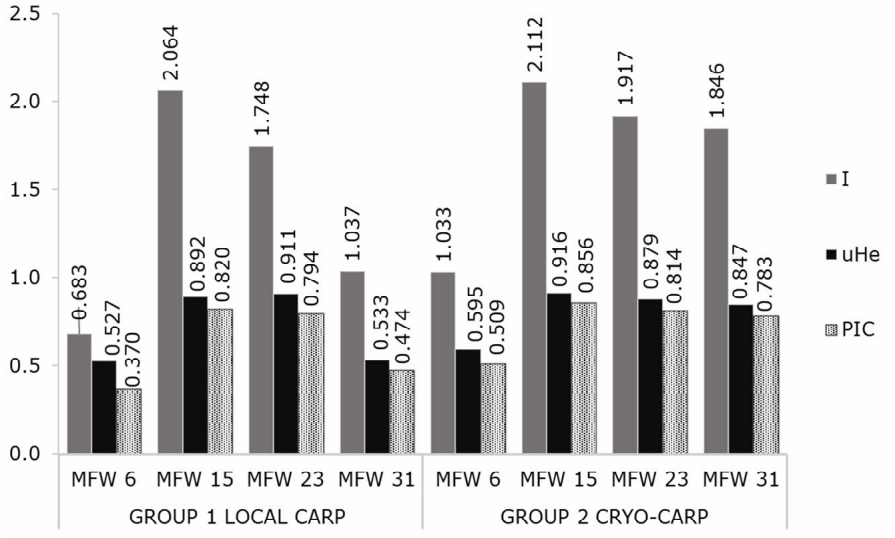

Figure 1. Shannon Index (I), unbiased expected heterozygosity (uH_e), polymorphism information content for two studied groups of Amur carp at four microsatellite loci.

The number of alleles per locus (N_a) varied from two (locus MFW 6) to ten (MFW 15) in the local carp group and from four (MFW 6) to nine (MFW 15) in the cryo-carp group. The average number of alleles per locus and effective number of alleles per locus (N_e) were higher in the cryo-carp group and composed 7.250 (SE 1.109) and 5.295 (SE 1.131),

respectively, compared to the local carp group, where N_a = 5.750 (SE 1.652) and N_e = 3.903 (SE 1.116).

The cryo-carp had a significantly broader range of amplicon sizes at the loci MFW 6 and MFW 31 towards those with a lighter molecular weight of alleles compared to the local carp. The frequencies of the occurrence of specific alleles in the studied groups are presented in Table 3.

Table 3

Frequencies of occurrence of alleles in two groups of carp at four microsatellite loci

MFW 6			*MFW 15*			*MFW 23*			*MFW 31*		
Allele	*Gr 1*	*Gr 2*	*Allele*	*Gr 1*	*Gr 2*	*Allele*	*Gr 1*	*Gr 2*	*Allele*	*Gr 1*	*Gr 2*
124	-	0.050	157	0.313	0.100	95	0.100	0.250	210	-	0.050
137	-	0.100	167	0.188	0.150	106	0.100	0.050	239	-	0.150
150	0.429	0.600	220	-	0.100	112	0.200	0.150	260	-	0.350
165	0.571	0.250	230	0.063	0.100	121	0.200	0.100	265	-	0.150
			236	0.063	0.200	127	0.200	0.150	273	0.063	-
			245	0.063	0.050	135	-	0.050	291	0.688	0.050
			255	0.063	0.100	142	0.200	0.050	318	0.125	0.100
			265	0.063	0.150	150	-	0.200	335	-	0.100
			272	0.063	-				345	0.063	0.050
			280	0.063	-				353	0.063	-
			286	-	0.050						
			296	0.063	-						

Notes: Gr 1 - group of carp produced from fresh sperm; Gr 2 - group of carp produced from cryopreserved sperm.

The Shannon index (I), which depicts the complexity of the population structure based on a quantitative representation of objects within populations and usually varies from 1.5 to 3.5, showed that the cryo-carp group had a more complex genetic structure (1.727) than the local carp group (1.383) (Figure 1).

The average values of the observed (H_o) and expected (H_e) heterozygosity of the two studied groups of carps did not differ significantly for the selected microsatellite loci. However, the unbiased expected heterozygosity (uH_e) showed significant differences between the studied groups of carp at the locus MFW 31 that was also confirmed by the Shannon index for this locus (Figure 1).

The highest observed heterozygosity (H_o) was detected at the loci MFW 15 and MFW 23 indicating significant difference in genetic features at these loci compared to other two studied loci and, therefore, they have the highest differentiating potential for the comparative analysis of the studied groups of Amur carp. This was also confirmed by the polymorphism information content (PIC) (Figure 1). The least informative for both studied groups was the locus MFW 6, while the loci MFW 15 and 23 were characterized by the highest PIC values.

Insignificant deviations from the Hardy–Weinberg distribution was observed at the locus MFW 15 in both groups of Amur carp as well as at the locus MFW 23 for the local carp group and at the locus MFW 31 for the cryo-carp group (Table 2).

The inbreeding coefficient of an individual relative to the total population (F_{is}), inbreeding coefficient of an individual relative to the species (F_{it}), inbreeding coefficient of a population relative to the species (F_{st}) calculated for each analyzed locus are shown in Table 4.

Table 4

F-Statistics and estimates of N_m over all populations for each locus

Parameters	*MFW 6*	*MFW 15*	*MFW 23*	*MFW 31*	*Mean*	*SE*
F_{is}	0.905	0.135	0.335	0.502	0.469	0.163
F_{it}	0.911	0.160	0.356	0.594	0.505	0.162
F_{st}	0.064	0.029	0.031	0.186	0.077	0.037
N_m	3.632	8.503	7.881	1.097	5.278	1.765

Note: N_m - average level of gene flow per generation.

A positive average F_{is} value indicates a 46.9% deficiency of heterozygous genotypes at the studied loci. F_{it} is also positive and averages 0.505 that indicates a 50% deficiency of heterozygotes in the species as a whole. F_{st}, which reflects the degree of differentiation of the studied groups, showed that 92.3% of the genetic variability detected in the studied groups of carp were within the local stock of Amur carp and only 7.7% (F_{st} = 0.077) were distributed between the studied groups. The obtained average value of F_{st} indicates the genetic division of the studied populations. The highest contribution to the interpopulation component of variability was due to the highly polymorphic locus MFW 31 (F_{st} = 0.186).

The Nei unbiased genetic distance between two studied groups was 0.334.

The obtained data of genetic analysis are supported by differences in morphometric parameters (Table 5). According to the analysis of exterior measurements, age-8 males of Amur carp produced from the defrosted sperm were characterized by usually higher parameters than the males of local carp of the same age. However, no significant difference was observed for the body circumference and head length to body length ratio (Table 5).

Table 5

Exterior parameters of age-8 males of Amur carp of different origins (mean±SE)

Parameters	*Genetic origin*	
	local (n = 8)	*cryo (n = 10)*
Body weight, g	2015.0±86.46	2416.5±93.47**
Body length, cm	46.8±0.58	50.7±0.61**
Head length, cm	11.13±0.18	11.85±0.13**
Body depth, cm	12.09±0.26	12.45±0.17*
Body circumference, cm	31.69±0.57	31.4±1.31
L:D ratio	3.88±0.07	4.07±0.04*
L:C ratio	1.48±0.02	1.55±0.01*
H:L ratio	23.76±0.20	23.42±0.36

Note: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Discussion. DNA markers have long been used to study the effect of cryopreservation on the genetic polymorphism of fish offspring produced from cryopreserved sperm, e.g., studies using RAPD markers to assess cryocollections of whitefish (*Stenodus leucichthys*) (Golovanova et al 2003) and studies using microsatellites of brown trout (*Salmo trutta*) produced from cryopreserved sperm (Martínez-Páramo et al 2009). The current study attempted to assess the degree of domestication of Amur carp by confirming changes in their genetic structure because local stocks of this species have a long history of captive breeding and selection, while carp, from which sperm was obtained for cryopreservation, are supposed to be wilder varieties than the domesticated stocks. Microsatellite markers have been found to be useful for cyprinid differentiation and record well the degree of domestication of populations (Tomljanović et al 2013). Studies using microsatellite markers including MFW23 and MFW31 showed that domestic carp being a form of wild carp and having a long history of domestication were characterized by a lower average number of alleles than carp in natural populations (Tomljanović et al 2013). Therefore, the number of alleles per locus may reflect the degree of domestication of carp indicating a change in the genetic structure during long-term selection of carp cultured at fish farms. Tomljanović et al (2013) found that the average value of alleles per locus among five groups of carp caught in natural water bodies was 6.12, while this value for five groups of carp farmed in Croatia was significantly lower and composed 3.86. Similar conclusions were made by Kohlmann et al (2005), who found the highest allelic richness in the group of wild-caught carp (8.221), and the lowest - in farmed carp (4.436).

The values of the average number of alleles per locus for carp of both groups obtained in this study are quite similar to those of carp produced in the study of Tomljanović et al (2013). Thus, fish breeding activities with the groups of Amur carp cultured at the Experimental Fish Farm "Velykyi Lyubin" somewhat narrowed the range of

amplicons but did not lead to a significant loss of genetic diversity in the studied microsatellite loci and, in general, this stock represents the genetic structure of Amur carp of native populations.

Carp breeding works resulted in the elimination of alleles with low molecular weight such as MFW 6 (allele 124, 137 bp) and MFW 31 (alleles 210, 239, 260, 265 bp). It is worth noting that the use of cryopreserved carp sperm in our studies results in an increase in genetic diversity, which can be evidenced by the number of alleles per locus and an increase in the range of amplicon sizes at individual loci.

The PIC index assessed in our work indicates that the microsatellite loci used are informative for the study of polymorphism in the genetic structure of carp. The PIC index for the studied SSR loci was higher than 0.5 that indicates a high level of informativeness for codominant markers. The exceptions were the loci MFW 6 and MFW 31 in the analysis of local groups: they were characterized by a medium level of informativeness as PIC varied between 0.25 and 0.5.

The use of Amur carp males produced from defrosted sperm had a positive effect on the exterior parameters of their offspring. For example, the mean weight and length of cryo-carp exceeded those of local carp by 19.9% and 8.2%, respectively.

Conclusions. The analysis using microsatellite markers showed differences and common features of the genetic structure of Amur carp groups produced from cryopreserved and native sperm. The use of cryopreserved sperm had a positive effect on the variability of microsatellite loci in fish causing an increase in the number of alleles at the studied loci, the range of amplicons compared to the local group, as well as an increase in the biodiversity index. The cryo-carp had specific alleles compared to the local carp of classical selective breeding. The genetic structure of cryo-carp is supposed to be close to that of natural wild populations that indicates a positive effect resulting in an improvement of the genetic potential of brood fish.

The studied loci MFW 15 and MFW 23 proved to be highly informative and therefore can be used for further monitoring work on the analysis of the heterogeneity of carp genetic structure.

The assessment of carp genome diversity using microsatellite markers is one of the methods of restoring genetic structure and increasing its heterogeneity. Further analysis of the genotypes of cryopreserved material and offspring of future generations will allow creating recommendations for the effective use of cryocollections for interspecific hybridization using purebred brood fish (or their genetic material) and monitoring of the obtained offspring.

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