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MICROSATELLITE-BASED GENETIC VARIABILITY AND DIFFERENTIATION OF HATCHERY AND FERAL COMMON CARP *CYPRINUS CARPIO* L. (CYPRINIDAE, CYPRINIFORMES) POPULATIONS IN CROATIA

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Abstract - Common carp production has an important status in Croatian aquaculture. In addition, the sport fishing of common carp in open waters is very popular, but it is often based on stocking from fish farms. Using fifteen microsatellites, 243 individuals from 5 hatchery and 5 feral populations have been analyzed. A total number of 148 alleles were recorded. However, the mean number of alleles per locus was remarkably low. Pairwise F_{ST} values (0.026-0.130) were significant (P<0.01), demonstrating differentiation among populations. The Markov chain method test showed that all the populations deviated from HWE (P <0.05). After sequential Bonferroni correction only the Vrana lake was in HWE in all the loci but MFW20. The factors that may result in genetic divergence and significant reduction of the observed heterozygosity are discussed. AMOVA results for 10 populations indicate that the percentage of the variation among populations was 6.26%, which is lower than the variation within populations (91.04%).

Key words: *Cyprinus carpio*, common carp, microsatellites, population genetics, Croatia

INTRODUCTION

The Common carp (*Cyprinus carpio* L.) is an economically important species to the aquaculture of Croatia. Common carp culture in Croatia developed considerably at the turn of 19th to 20th century. The first carp stocks were introduced from Germany and the Austro-Hungarian Empire (nowadays Czech Republic and Hungary). Decades of efforts in selection resulted in the well-known Našice and Poljana strains, kept in the live gene bank in Szarvas (Treer and Kolak 1994; Gorda et al. 1995). The hybrids of Našice and Israeli Dor-70 strains happen to be the most successful crossbreeds in Israel (Wohlfarth and Moav 1990). However, according to the discussion at the Budapest conference, Flajšhans and Gall (1995) presumed that the Našice strain had disappeared from its original fish farm due to uncontrolled crossbreeding.

Nowadays, fish farmers try to certify their stocks through the Croatian Chamber of Commerce as genuine Croatian product, which is also a quality stamp for the fish (Treer et al. 1996). But this poses potential risks associated with the loss of genetic variation and an increase in inbreeding (Schonhuth et al., 2003). Therefore, the information on genetic diversity of these hatchery stocks is ur-

Fig. 1. Sampling locations for common carp (*Cyprinus carpio* L.) in Croatia
1 Draganići, 2 Končanica, 3 Poljana, 4 Grudnjak, 5 Našice, 6 Sava, 7 Kupa, 8 Drava, 9 Danube, 10 Vrana lake

gently required in order to sustain the quality of the broodstock.

Sport fishing of common carp in open waters is often based on stocking from fish farms. Hence, the hybridization of feral populations becomes an increasing problem (Memiş and Kohlmann 2006).

Microsatellites are highly variable genetic markers that are inherited codominantly in a Mendelian pattern. In comparison to other molecular markers, microsatellite markers are sensitive and promising in population genetics studies, especially those populations that are closely related (O'Connel and Wright 1997). As genetic variability of feral and hatchery stocks of common carp has been successfully investigated in Chinese feral and hatchery varieties (Zhou et al. 2004), microsatellites were considered to be a good molecular marker system in our investigation, too.

The aim of our study was to explore genetic variability within and among five hatcheries and five feral populations from different Croatian regions using microsatellite DNA, a hyper-variable molecular marker.

MATERIALS AND METHODS

Fin clip samples of 243 individuals of *C. carpio* were collected in 2005. Details of sampling localities, sample codes and sample sizes are presented in Fig. 1 and Table 1. Fish were obtained from the following fish farms: Našice, Grudnjak, Končanica, Poljana and Draganići in the Croatian part of the Pannonian

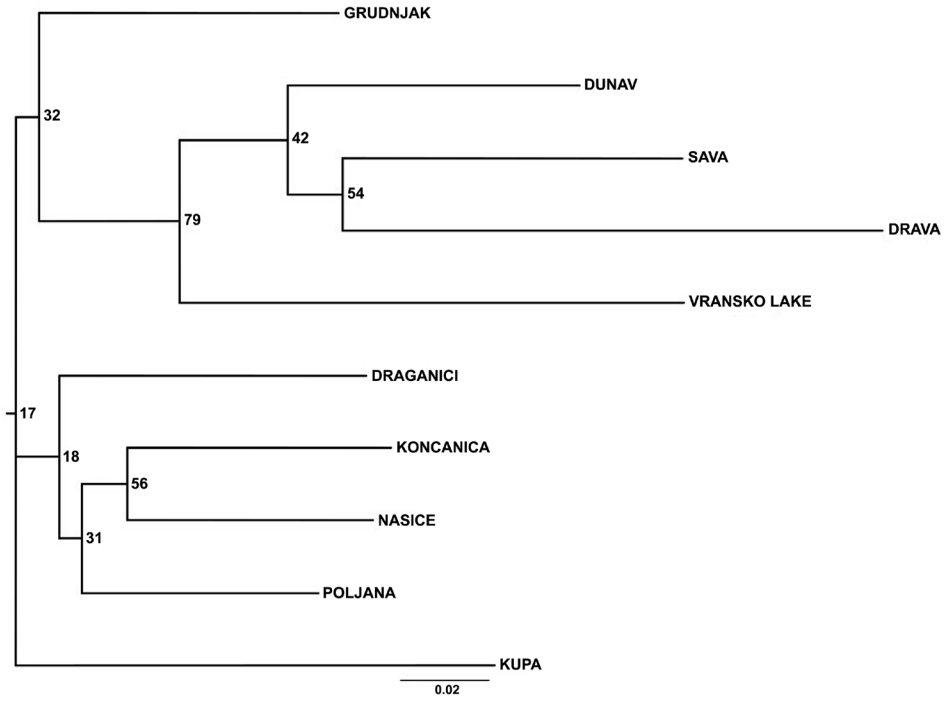

Fig. 2. UPGMA dendogram based on D_A distance for 10 populations of common carp using 15 microsatellite loci. The abbreviation of each population is shown in Table 3.

Plain. Semi-intensive common carp culture is characteristic for all of these farms. Feral common carps were caught from some of the largest Croatian rivers, the Sava, Danube, Drava and Kupa, and also from Lake Vrana. The attribute "feral" does not necessarily mean "native", moreover, it stands for self-reproducing populations in natural waters (rivers and lakes).

Total DNA was extracted from individual caudal-fin samples using DNAeasy Tissue Kit (QIAGEN). Some extractions gave low DNA yields so the final elution was brought down to 100 μl. Microsatellite variation was examined at fifteen microsatellite loci (MFW1, MFW4, MFW7, MFW9, MFW12, MFW31, MFW16, MFW20, MFW23, MFW29, MFW3, MFW13, MFW17, MFW26, MFW28) with primer sequences described in Crooijmans et al. (1997). The temperature profile of the PCR was 95°C for 15 min of an initial denaturing cycle followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s, an extension cycle at 72°C for 1 min followed by final extension step at 72°C for 10 min. One primer of each pair was fluorochrome labeled to enable the determination of allele sizes by ABI 3730 Genetic Analyzer and the ABI GeneScan software.

The genetic diversity of each population has been estimated as the mean number of alleles per locus (A), observed (Ho) and expected heterozygosities (He) following Nei (1978) using GENETIX 4.05.2 (Belkhir et al. 2004). Inbreeding coefficients (F_{IS}) for each population and pairwise F_{ST} values among all the pairs of populations were calculated and tested by permutations using the same software. Allelic richness based on 11 individuals per locus and population was calculated using FSTAT (Goudet 2001). Each population was tested for departure from Hardy-Weinberg equilibrium expectations using the Markov-chain method with 10,000 dememorization steps, 500 batches and 10,000 subsequent iterations. Linkage disequilibrium was tested across all the pairs of loci using GENEPOP 4.0 (Raymond and Rousset 1995). Levels of significance for this test were adjusted by sequential Bonferroni correction (Rice 1989).

To estimate and test the significance of genetic differentiation among feral populations and hatcheries, as well as among all the populations, we performed a hierarchical Analysis of Molecular Variance (AMOVA) implemented in ARLEQUIN 3.0 (Excoffier et al. 2005). The estimated components of molecular variances were tested against zero using 10000 permutations.

Genetic distances among populations (Cavalli-Sforza and Edwards 1967) were calculated using the PHYLIP program package, version 3.67 (Felsenstein 1993). An UPGMA dendrogram was reconstructed based on the genetic distance matrix using the same program. The reliability of the dendrogram was estimated by bootstrapping (1000 replicates) and implemented by PHYLIP program package. The dendrogram was visualized in TreeView, version 1.6.6 (Page 1996).

RESULTS

Variation in number of alleles as well as allele size ranges are shown in Table 2. A total of 148 alleles were recorded over all the loci. The number of alleles per locus varied from 6 (MFW12 and MFW17) to 20 (MFW20).

The average number of alleles for all the populations varied from 2.2 (Našice) to 7.4 (Danube). Variability levels across populations estimated as observed heterozygosities ranged from 0.369 to 0.612, and estimated as expected heterozygosities ranged from 0.654 to 0.736 (Table 3).

The Vrana Lake had the highest observed heterozygosity of all the populations while Kupa had the lowest. The Markov chain method test showed that all the populations deviated from HWE (P <0.05). After sequential Bonferroni correction, only Lake Vrana was in HWE in all the loci but MFW20, and only MFW9 and MFW29 loci were in HWE in all the populations (Table 4).

F_{IS} value varied from 0.2146 (Vrana lake) to 0.4014 (Grudnjak). The number of private alleles in all ten populations was relatively low (one in hatchery populations; three in feral populations) (data not shown).

AMOVA results for 10 populations indicate that the percentage of variation among the populations was 5.62%, which is lower than the variation within the populations (61.26%) (Table 5). Genetic differentiation among 10 populations, as well as among the feral and hatchery populations was both significant.

The pairwise F_{ST} values among all the populations are shown in Table 6. The largest F_{ST} genetic distance (0.424) was measured between Draganići and Drava, indicating that these two populations diverged the most, while two geographically very close hatchery populations, Končanica and Poljana, had the smallest genetic distance (0.136).

The UPGMA dendogram generated from a matrix of Nei's genetic distances among the populations (Fig. 2) revealed three cluster groups among the ten populations. In the first cluster the Grudnjak population was separated from the others. Four feral common carp populations were in the second cluster, and hatchery populations in the third cluster, together with the feral Kupa population. Furthermore, the dendrogram showed that genetic divergences among the studied hatchery populations of common carp were relatively small. Consequently, with the exception of the Grudnjak population, all the studied populations might belong to the same group.

DISCUSSION

A precise estimate of population structure and genetic distances from microsatellite data depends on the sample size, number of loci, number and size range of alleles (Ruzzante 1998). Scoring six loci in Atlantic cod (*Gadus morhua*), Ruzzante (1998) examined the effect of sample size on seven genetic distance measures and two structure metrics. He concluded that 50-100 individuals are needed for a good estimation. The sample size in the present study was obviously lower than the one recommended. Therefore, these results should be taken with caution.

Table 1. Details of samples, population abbreviations, population names, locations, population types and sample size of common carp (*Cyprinus carpio* L.) in Croatia

Pop. abbreviation	Pop. name	Location of sampling	Pop. type	N
D	Draganići	Fish farm "Draganići"	Hatchery	37
K	Končanica	Fish farm "Končanica"	Hatchery	37
N	Našice	Fish farm "Našička Brežnica"	Hatchery	37
G	Grudnjak	Fish farm "Grudnjak"	Hatchery	37
P	Poljana	Fish farm "Poljana"	Hatchery	22
S	Sava	Sava River	Feral	11
Du	Dunav	Danube River	Feral	22
Dr	Drava	Drava River	Feral	16
Ku	Kupa	Kupa River	Feral	13
V	Vrana	Vrana lake	Feral	11

Pop.=populations, *N*=sample size.

Table 2. Characteristics of microsatellite common carp markers

Marker	Allele size range (bp)	No. of alleles
MFW01	167-221	7
MFW03	144-182	12
MFW04	130-170	7
MFW07	189-279	14
MFW09	86-144	10
MFW12	86-178	6
MFW13	172-200	9
MFW16	121-173	14
MFW17	242-278	6
MFW20	232-256	20
MFW23	122-144	13
MFW26	122-148	8
MFW28	284-304	7
MFW29	147-183	7
MFW31	282-302	8

Table 3. Genetic variations at all the loci in ten populations

Population	H_o	H_e	Average no. of alleles	F_{IS}
G	0.4468	0.7324	4.9	0.4014
D	0.4955	0.6826	3.6	0.2864
K	0.4865	0.7015	5.1	0.3188
N	0.4757	0.6829	2.2	0.3158
P	0.5000	0.6968	3.5	0.3036
Du	0.4909	0.7364	7.4	0.3539
S	0.4970	0.7237	5.6	0.3556
Dr	0.4833	0.6658	5.8	0.3035
Ku	0.3692	0.6544	5.4	0.4676
V	0.6121	0.7364	6.4	0.2146

On the other hand, by using fifteen highly polymorphic loci the right resolution could be achieved in order to differentiate the common carp populations studied. Instead, genetic variability might have been underestimated because of the rare alleles and genotypes that were absent from the samples due to the suboptimal sample size. Such missing alleles and genotypes might also be the reason for significant

Table 4. Deviations from Hardy-Weinberg proportion for 10 populations in 15 loci. Values in italics indicate the loci that significantly deviate from HW ($P < 0.05$)

	MFW1	MFW9	MFW31	MFW12	MFW4	MFW7	MFW20	MFW23	MFW29	MFW16	MFW13	MFW17	MFW26	MFW28	MFW3
G	0,00	1,00	0,00	1,00	0,00	0,00	0,00	0,00	1,00	0,02	0,00	0,00	0,00	0,00	0,00
D	0,00	1,00	1,00	1,00	0,00	1,00	0,00	0,00	1,00	1,00	0,00	0,00	0,00	0,00	0,00
K	0,65	1,00	0,00	1,00	1,00	0,00	0,00	0,00	1,00	1,00	0,00	0,00	0,00	0,00	0,00
N	0,00	1,00	0,23	0,00	1,00	0,05	0,00	0,00	0,08	0,00	0,00	0,00	0,00	1,00	0,00
P	1,00	1,00	0,89	1,00	1,00	1,00	1,00	0,00	1,00	0,90	0,00	0,83	0,80	0,00	1,00
Du	1,00	1,00	1,00	0,80	1,00	0,00	0,93	0,00	0,44	1,00	0,00	0,00	0,00	0,00	0,00
S	1,00	1,00	1,00	1,00	1,00	1,00	0,00	0,53	1,00	1,00	1,00	0,03	0,15	0,12	0,00
Dr	1,00	1,00	1,00	1,00	1,00	1,00	0,00	1,00	1,00	0,00	0,42	0,00	1,00	0,00	1,00
Ku	0,00	1,00	0,00	1,00	1,00	0,00	0,00	1,00	1,00	1,00	1,00	0,00	0,03	1,00	0,00
V	1,00	1,00	1,00	1,00	1,00	0,35	0,00	1,00	1,00	1,00	1,00	1,00	0,44	0,18	1,00

Table 5. AMOVA analysis results for 10 populations of common carp based on 15 loci

Source of variation	*d.f.*	Sum of squares	Variance components	Percentage of variation	P
Among groups	1	53.754	0.15799	2.68	0.0107
Among populations	8	184.872	0.33115	5.62	0.0000
Among individuals within populations	233	1677.409	1.79403	30.44	0.0000
Within populations	243	877.500	3.61111	61.26	0.0000
Total	485	2793.535	5.89429		

Table 6. F_{ST} values among all pairs of the populations of common carp. All the values are significant (p<0.01)

	D	K	N	P	Du	S	Dr	Ku	V
G	0.0718	0.0639	0.0640	0.0398	0.0728	0.0608	0.1058	0.0630	0.0477
D		0.0772	0.0533	0.0562	0.0905	0.1013	0.1287	0.0567	0.0749
K			0.0516	0.0435	0.0867	0.0729	0.0944	0.0700	0.0637
N				0.0319	0.0972	0.0825	0.1308	0.0583	0.0612
P					0.0784	0.0661	0.1110	0.0592	0.0617
Du						0.0264	0.0456	0.0840	0.0291
S							0.0462	0.0779	0.0271
Dr								0.1090	0.0571
Ku									0.0621

deviations from the Hardy-Weinberg equilibrium observed in all the populations.

A significant deviation from Hardy-Weinberg equilibrium found in all the loci could also be explained either by a sample bias or by the presence of null alleles in all populations. In the presence of null alleles, the heterozygotes possessing a null allele would be erroneously recorded as homozygotes for the variant allele and this would lead to a deficiency of heterozygosity (Table 4).

In terms of observed heterozygosity, our results (0.3692-0.6121) are quite similar to those found in hatchery populations from Indonesia (Aliah and Taniguchi 1999) and from Hungary (Lehoczky at al. 2005). The mean number of alleles found in five hatchery stocks (3.86) was lower than the number found for five feral stocks (6.12). This can be considered as a reflection of the probable limited diversity within the hatcheries in comparison to the feral stocks. Since most of the carp farms are small-to-medium sized, a relatively low number of spawners is to

be assumed. Furthermore, the preservation of genetic variability within the existing stocks is important because this variability defines the adaptive potential of the species in hatchery management. Genetic changes in hatcheries occur due to an unavoidable process of aquatic animals being breed in captivity for generations. It entails genetic changes caused by either selection, reduction of effective population size (number of broodstocks contributing to a succeeding generation, N_e), inbreeding or combinations of them all (Doyle, 1983).

In comparison to natural stocks, lower genetic variability in hatchery stocks has been reported for many species, e.g., turbot, *Scophthalmus maximus* (Coughlan et al. 1998), common carp (Kohlmann et al. 2005), Japanese flounder, *Paralichthys olivaceus* (Sekino et al. 2002), Atlantic salmon, *Salmo salar* (Skaala et al. 2004), Kuruma prawn, *Marsupenaeus japonicus* (Luan et al. 2006). The findings of the present study are similar to the earlier observations. This is mainly due to a small founder population and an ultimately small effective population size (N_e), (Falconer and Mackay 1996).

Hence, we suggest that the reduction of allelic diversity in hatchery stocks might be the result of founder events or occasional bottleneck effects during the breeding process. Among hatchery stocks, the highest heterozygosity is found in Poljana (0.5), which could be the result of refreshing from new fish stocks (Božić, 2009). Our results support the prediction of Kirpitchnikov (1999) about the genetic similarity of stocks originating from the same geographic region. The largest distance between Drava and Draganići is likely the result of the geographical separation. In addition, the large distance between Grudnjak and the other nine populations is the result of the geographical separation of the stocks and the effects of different selective breeding. The Končanica stock is genetically very close to Našice. These two fish farms have been managed by the same organization for many years, so the same spawning material has probably been used in both farms (Treer et al., 2000). The results of this study indicate that in Croatia common carp stocks that are well defined no longer exist. On the other hand, it is possible that uncontrolled mixing has made some stocks disappear, as probably happened with the stock of Končanica.

The introduction of hatchery carp is a big issue for feral populations in open waters (Memiş and Kohlmann 2006). This is evident in Kupa population, which by genetic distance belongs to the hatchery populations.

This study is the first genetic study to deal with Croatian common carp based on microsatellite DNA markers. It has demonstrated that microsatellite markers are a powerful tool in monitoring the genetic condition of different strains of common carp in Croatia. A higher genetic variability of the feral carp populations in comparison to the hatchery stocks has been found. This is particularly important in light of the global threat to feral carp populations. In Croatia, five hatchery stocks of common carp have important status in aquaculture, so our results on the genetic variability within/among them and the relationship among them can provide a new background of knowledge in population conservation and breeding programs.

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