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Genetic Diversity and Differentiation of Common Carp (*Cyprinus carpio* L.) in the southern part of Caspian Sea by using microsatellite markers

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Abstract

Eight microsatellite loci were used for analysing eight populations of common carp (*Cyprinus carpio* Linnaeus 1758) in Iranian waters of the Caspian Sea. Mean expected heterozygosity within populations ranged from 0.21 to 0.92, and the mean number of effective alleles per population ranged from 1.26 to 12.25. Of the analysed loci, 9 out of 64 possible tests were found to deviate significantly ($P<0.05$) from the Hardy–Weinberg equilibrium. A low level of population differentiation (F_{ST}) was observed among populations; however, significant differentiation was evident between some populations ($p<0.05$). An analysis of the distribution of genetic variation indicated within population variation is very high (90.0%), while among populations within groups and among groups is low (3.0% and 1.0% respectively). Phylogenetic neighbor-joining tree analysis showed that the south western populations are closely related to the south middle populations, and the Tajan River population is not closely related to any other carp populations. Thus, the microsatellite markers used in the present study demonstrated significant importance in monitoring the genetic diversity of the common carp. In addition, our results proved the efficiency of the conservation programme and highlighted the need for better control of genetic variability in the common carp populations in the Caspian Sea.

Introduction

Common carp (*Cyprinus carpio* Linnaeus 1758) belongs to the family Cyprinidae, the largest family among freshwater teleosts (Balon 1995). Common carp has a long history of domestication and numerous strains and breeds have been developed from its ancestor, the wild common carp, *C. carpio*, both in Europe and Asia. The natural distribution range of wild carp in Eurasia is currently divided into disjunct western (Caspian, Aral and Black Sea basins) and eastern (East and South-East Asia) areas, which were supposedly isolated during multiple Pleistocene glaciations (Balon 1995).

In recent decades, common carp selection was aimed at developing high-productive breeds, as well as breeds resistant to different diseases and acclimatised to new habitats. Based on differences in morphological and ecophysiological traits, numerous subspecies, races and varieties of wild carp

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have been distinguished but their taxonomic status and phylogenetic relationships remain uncertain (Balon 1995) and warrants further studies.

Genetic variation is an essential component for artificial selection programmes that aim to enhance ecological or economically important traits (Wang et al. 2006). Although the relationship between molecular genetic variation at neutral marker loci and quantitative genetic variation is an unresolved issue, measures of quantitative trait and neutral marker divergence have been shown to be positively correlated across different studies, using a variety of marker types (Mabuchi et al. 2008). Genetic characterization methods considered important for other conservation and breeding programmes for the maintenance of common carp strains in the Czech Republic began with allozymes (Hulak et al. 2010). However, allozyme markers were found to be ineffective owing to their low variability, and attention duly shifted to microsatellite markers (Kohlmann et al. 2003). Using microsatellite markers, various genetic population studies were performed to compare carp populations from all over the world (e.g. Kohlmann et al. 2005), or to compare carp strains/populations available within countries (e.g. Liao et al. 2006; Thai et al. 2007; Mondol et al. 2006). Some of the carp strains kept in the Czech Republic were included in other studies (Kohlmann et al. 2005), but complex characterization of all strains using a standardized number of microsatellite markers remains inadequate. In addition, complete sequence of common carp mitochondrial genome was determined, and polymorphism of some of its regions was examined in Asian and European populations (Zhou et al. 2004; Gross et al. 2002).

The present study was undertaken to provide some basic information on the genetic variability and differentiation among populations from the major parts of the Caspian Sea. Genome polymorphic components were identified using polymerase chain reaction with microsatellite markers.

Materials and Methods

Sampling and DNA extraction

A total of 310 common carp individuals were collected from the southern part of the Caspian Sea (Fig. 1; Table 1). Fish were captured using lift and seine nets and tissue samples were obtained as fin clips and preserved in 90% ethanol. The samples were placed into 1 mL STE buffer [10 mM Tris/HCl, 50 mM ethylenediaminetetraacetic acid (EDTA), 200 mM NaCl and 0.5% sodium dodecyl sulfate (SDS); pH 8.0]. Tissue samples were digested with 0.5 $Ag \cdot \mu L^{-1}$ proteinase K at 55 °C overnight; the resulting solution was centrifuged, the supernatant precipitated in ethanol and dissolved in TE (10 mM Tris-HCl and 1 mM EDTA; pH 8.0) buffer. From all of the samples, genomic DNA was isolated by the phenol–chloroform procedure. The quality and concentration of DNA were assessed by agarose gel (1%) electrophoresis and then samples were stored at 4 °C until use. DNA was quantified and diluted to 50 $ng \cdot \mu L^{-1}$ for polymerase chain reaction (PCR) amplifications.

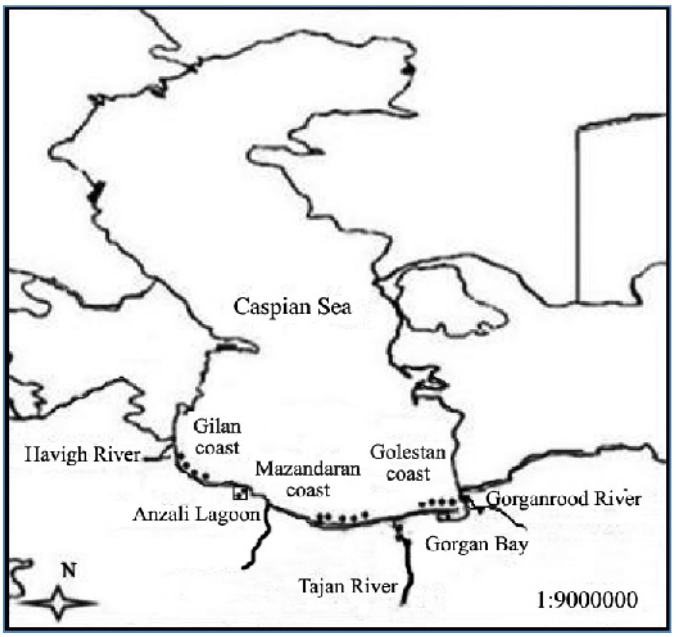

Fig. 1. Map of the Caspian Sea showing the sampling locations of common carp.

Table 1. Locations and sample size for 310 individuals of common carp sampled in this study

Region	Location	Sample size
South eastern of the Caspian Sea	Golestan coast (GC)	40
	Gorganrood River (GR)	40
	Gorgan Bay (GB)	40
South middle of the Caspian Sea	Mazandaran coast (MC)	40
	Tajan River (TR)	40
South western of the Caspian Sea	Gilan coast (GIC)	40
	Sefidrood River (SR)	30
	Anzali Lagoon (AL)	40

Microsatellite marker amplification

Eight primer pairs for microsatellite markers, Ca1-2, Ca3-4, Ca5-6, Ca7-8 and Syp2, Syp4, Syp5 and Syp6 were used in this study (Table 2). PCR was performed in 96-well plates with a total reaction volume of 20 µL. Each reaction consisted of 50 ng genomic DNA, 1.5 mM $MgCl_2$, 0.2 µL of forward and reverse primers, 0.2 mM deoxyribonucleotide triphosphates (dNTP) and 0.5U Taq polymerase. DNA fragments were amplified in a Applied Quanta Biotech (Model Auto Q server) thermal cycler using the following programme: 94 °C for 5 min, then 30 cycles of the following: denaturation at 94 °C for 50 s, annealing temperature for 40 s at different temperatures (Table 1), and extension at 72 °C for 50 s. The programme was finished with a 10-min extension stage at 72 °C. Amplification products were separated by electrophoresis through 8% denaturing polyacrylamide gels. Detection of microsatellite allele sizes obtained by the silver staining method was determined against the size standards (50 bp) marker.

Table 2. Detailed characteristics of amplified microsatellite loci in *C. carpio*.

Locus	Primer sequence	Size (bp)	Annealing temp. (°C)	Accession No.	Reference
Ca1,2	F: AAGACGATGCTGGATGTTTAC R: CTATAGCTTATCCCGGCAGTA	51	112-132	AF277573	Dimsoski et al. 2000
Ca3,4	F: GGACAGTGAGGGACGCAGAC R: TCTAGCCCCCAAATTTTACGG	61	240-285	AF277574	Dimsoski et al. 2000
Ca5,6	F: TTGAGTGGATGGTGCTTGTA R: GCATTGCCAAAAGTTACCTAA	55	140-172	AF277575	Dimsoski et al. 2000
Ca7,8	F: GTGAAGCATGGCATAGCACA R: CAGGAAAGTGCCAGCATACAC	57	132-152	AF277576	Dimsoski et al. 2000
Syp2	F: GCAGGAGCGAAACCATAAAT R: AAACAGGCAGGACACAAAGG	58	204-272	AY318779	Turner et al. 2004
Syp4	F: CACGGGACAATTTGGATGTTTTAT R: AGGGGGCAGCATACAAGAGACAAC	60	204-288	AY318777	Turner et al. 2004
Syp5	F: ATTTTTAGGAGTGATGTTCAGCAT R: CAAGTGTGTCATTGAGGATGTGAG	53	164-200	AY318778	Turner et al. 2004
Syp6	F: TTACACAGCCAAGACTATGT R: CAAGTGATTTTGCTTACTGC	57	132-140	AY318776	Turner et al. 2004

Scoring and statistical analyses

The bands representing alleles at the microsatellite loci were manually scored based on their sizes and designated as A, B, C, D, E, F, G, I, H, J and … from the bottom to the top of the gel. The sizes of individual alleles were determined in relation to a 50 bp DNA size standard (Promega, Madison, WI) using the UVIDOC version 99.04 software (UVItech Limited, UK). The data were checked initially in MICRO-CHECKER (Van Oosterhouse et al. 2004) to identify genotyping errors.

GenAlex software version 6 package (Peakall and Smouse 2006) was used to calculate allele and genotype frequencies observed (H_O) and expected (H_E) heterozygosity and to test for deviations from Hardy-Weinberg equilibrium. Genetic distance between populations was estimated from Nei standard genetic distance and genetic similarity index (Nei 1972). Genetic differentiation between populations was also evaluated by the calculation of pairwise estimates of F_{ST} values. All calculations were conducted using the GenAlex and PowerMarker version 3.0. (Liu 2003). An assignment test was implemented by GeneClass v.1.0.02 software (Cornuet et al. 1999), in order to determine the extent to which individuals could be correctly assigned to their population of origin as a measure of population differentiation and as a means of investigating population mixing. The MEGA4, v. 1.31, software (Tamura et al. 2007) was used to construct neighbor-joining tree.

Results

Genetic variation

The analyses with the MICRO-CHECKER programme rejected the existence of scoring errors, large allele dropout and null alleles in the genotypes. All eight loci were polymorphic and were variable in all populations. Allele frequencies at all loci in all populations are shown in Table 3. In eight common carp populations, the effective alleles ranged from 1.26 to 12.25, and the averages in each population were 4.58 (GC), 4.60 (MC), 4.20 (GIC), 4.26 (SR), 4.39 (TR), 4.47 (GR), 4.37 (AL) and 4.42 (GB). The exact expected heterozygosity ranged from 0.21 to 0.92 with averages of 0.68, 0.68, 0.64, 0.67, 0.67, 0.68, 0.66 and 0.67, by populations. The locus Syp4 had the highest number of alleles (15). The average number of observed alleles in the Gilan coast and Gorgan Bay was the highest observed (7.80), followed by that in the Sefidrood River (7.50). The average number of alleles in the Gorganrood River population was the lowest observed (7.10). The observed heterozygosity of all the populations at loci Ca3-4, Syp5, and Syp6 were lower than the corresponding expected heterozygosity, while the observed heterozygosity at loci Ca1-2 and Syp4 were higher than expected. Average observed heterozygosity ranged from 0.44 in the Golestan coast to 0.52 in the Gilan coast population. Polymorphism information contents (*PIC*) were between 0.08 and 0.86, and the averages were 0.58, 0.60, 0.45, 0.51, 0.56, 0.54, 0.49 and 0.54 in each population. These parameters indicated that the eight populations belonged to the level of middle polymorphism, and genetic variations were moderate.

Deviation from Hardy-Weinberg proportion

In 9 of a total of 64 tests, significant deviations from Hardy-Weinberg expectations (HWE) were detected (Table 4). The test for fit to Hardy-Weinberg proportions revealed that all the strains were found to deviate from Hardy-Weinberg expectations at all the loci, except Syp5 and Syp6 while all of them were found to be in equilibrium at locus Syp6. Only Gorgan Bay samples were found to be in disequilibrium at loci Syp5 and Syp6 and other samples were in disequilibrium at locus Syp6.

Table 3. Polymorphic information of amplified microsatellite loci in eight populations of common carp.

Sites	GC				MC				GIC				SR			
	N_E	H_O	H_E	*PIC*	N_E	H_O	H_E	*PIC*	N_E	H_O	H_E	*PIC*	N_E	H_O	H_E	*PIC*
Ca1,2	3.50	0.92	0.71	0.58	3.63	0.70	0.72	0.59	3.50	1.00	0.71	0.47	2.82	1.00	0.64	0.64
Ca3,4	7.46	0.32	0.87	0.69	5.49	0.40	0.82	0.78	7.67	0.47	0.87	0.51	6.52	0.47	0.85	0.85
Ca5,6	5.20	0.50	0.81	0.71	4.18	0.60	0.76	0.76	5.00	0.37	0.80	0.63	2.13	0.37	0.80	0.80
Ca7,8	2.97	0.45	0.66	0.62	2.77	0.35	0.64	0.61	2.52	0.32	0.60	0.51	2.89	0.22	0.65	0.65
Syp2	4.80	0.70	0.79	0.71	4.48	0.55	0.78	0.69	3.10	0.52	0.68	0.52	4.69	0.52	0.77	0.77
Syp4	9.59	0.52	0.89	0.81	12.75	0.85	0.92	0.86	9.04	0.55	0.89	0.71	8.78	0.55	0.89	0.89
Syp5	1.74	0.15	0.43	0.34	2.03	0.22	0.51	0.31	1.50	0.07	0.33	0.15	1.83	0.07	0.45	0.45
Syp6	1.43	0.35	0.30	0.22	1.44	0.35	0.30	0.25	1.26	0.22	0.21	0.09	1.41	0.22	0.29	0.29
Mean	4.58	0.49	0.68	0.58	4.60	0.50	0.68	0.60	4.20	0.44	0.64	0.45	4.26	0.42	0.67	0.67

Continue Table 3.

Ca1,2	3.37	0.92	0.70	0.62	3.68	0.70	0.73	0.55	3.75	0.77	0.73	0.59	3.79	0.82	0.74	0.54
Ca3,4	6.53	0.45	0.85	0.68	5.65	0.42	0.82	0.63	5.43	0.35	0.82	0.51	7.02	0.37	0.86	0.70
Ca5,6	4.18	0.65	0.76	0.67	4.29	0.62	0.77	0.52	4.86	0.52	0.79	0.59	4.44	0.37	0.77	0.69
Ca7,8	2.61	0.32	0.62	0.59	2.72	0.45	0.63	0.59	2.63	0.35	0.62	0.44	2.81	0.47	0.64	0.54
Syp2	4.95	0.62	0.80	0.71	4.64	0.52	0.78	0.68	3.44	0.55	0.71	0.63	3.72	0.42	0.73	0.64
Syp4	10.19	0.75	0.90	0.82	11.39	0.77	0.91	0.75	11.67	0.80	0.91	0.75	10.39	0.62	0.90	0.82
Syp5	1.97	0.12	0.49	0.33	1.98	0.22	0.49	0.34	1.80	0.12	0.44	0.36	1.86	0.12	0.46	0.36
Syp6	1.36	0.30	0.27	0.12	0.43	0.35	0.30	0.25	1.40	0.32	0.28	0.08	1.33	0.27	0.25	0.09
Mean	4.39	0.52	0.67	0.56	4.47	0.51	0.68	0.54	4.37	0.48	0.66	0.49	4.42	0.44	0.67	0.54

N_E, expected alleles number; H_O, observed heterozygosity; H_E, expected heterozygosity; *PIC*,Polymorphism information contents.

Table 4. Deviation from Hardy-Weinberg genotype frequency expectations in eight different populations of common carp. $\chi 2$ values, followed by degrees of freedom in parentheses.

Population	Locus							
	Ca 1,2	Ca 3,4	Ca 5,6	Ca 7,8	Syp 2	Syp 4	Syp 5	Syp 6
GC	61.35***	185.36***	109.79***	31.47**	138.33***	178.97***	24.51***	1.02**ns**
	(15)	(55)	(15)	(10)	(36)	(91)	(3)	(3)
MC	47.05***	171.64***	90.67***	62.77***	125.35***	197.18***	27.78**	1.51**ns**
	(15)	(45)	(15)	(10)	(45)	(105)	(3)	(3)
GIC	47.49***	160.88***	71.41***	52.84***	139.20***	212.83***	28.67***	1.25**ns**
	(15)	(55)	(28)	(10)	(66)	(105)	(3)	(3)
SR	30.00***	142.19***	41.78***	52.15***	139.29***	147.90***	17.78***	1.20ns
	(10)	(36)	(28)	(15)	(78)	(78)	(3)	(3)
TR	106.55***	181.02***	104.83***	36.61***	162.72***	186.50***	24.08***	0.64**ns**
	(10)	(55)	(21)	(10)	(45)	(78)	(1)	(3)
GR	46.69***	116.54***	87.52***	64.39***	168.09***	202.73***	19.78**	1.80**ns**
	(15)	(36)	(15)	(10)	(45)	(105)	(3)	(3)
AL	50.53***	115.50***	83.62***	68.41***	155.10***	228.06***	19.99***	1.80**ns**
	(15)	(28)	(15)	(10)	(45)	(105)	(3)	(3)
GB	54.79***	197.98***	53.32**	50.47***	182.93***	172.70***	1.37 **ns**	1.80**ns**
	(15)	(55)	(28)	(15)	(78)	(78)	(3)	(3)

Statistically significant values are marked with asterisks. *P <0.05, **P <0.01, ***P <0.001 and ns = not significant.

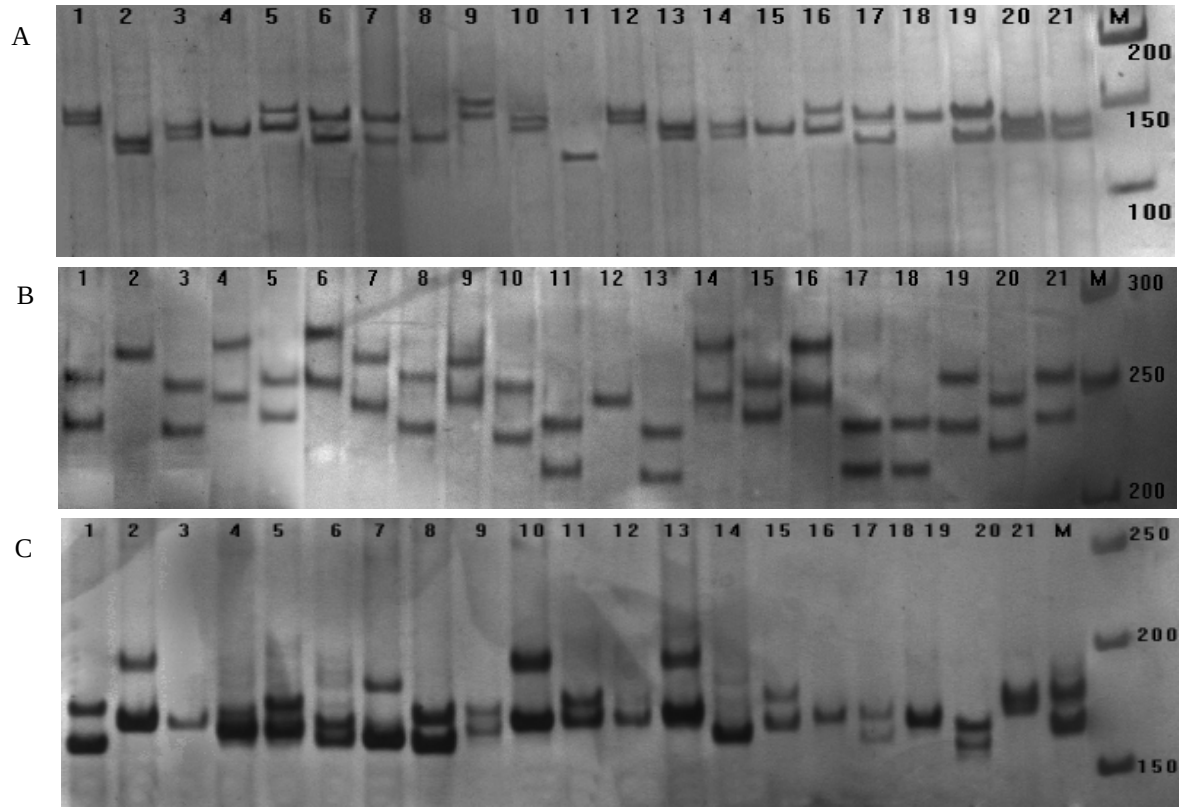

Fig. 2. Microsatellite profiles of *C. carpio* at loci Ca7,8 (A), Syp4 (B) and Syp5 (C). 1-3: GC. 3-5: MC. 6-8: GIC. 9-11: SR. 12-14: IR. 15-17: GR. 18-20: AL. M: molecular weight marker.

Genetic differentiation and relationships among populations

Pair-wise F_{ST} values and genetic distances, calculated on the reduced set of eight microsatellite loci are given in Table 5. The population differentiation (F_{ST}) metric between the Tajan River and the Anzali Lagoon population was the highest (0.128) and significant among the population pair, while the F_{ST} metric between the Golestan and Mazandaran coasts population (0.004) was the lowest and not significantly different (Table 5). The estimated gene flow (Nm) value between the Golestan and Mazandaran coasts population across all the studied loci was the highest (79.55), while the Nm value between the Anzali Lagoon and the Tajan River population was the lowest (7.89) (Table 5). The ability of allelic variation at these loci as a group to distinguish between the eight populations is demonstrated by an assignment test using just these populations. This test resulted in only 29 (9%) of the 281 individuals being misclassified. Overall there is a relatively high proportion of misclassifications, reflecting the generally limited divergence among populations. An analysis of the distribution of genetic variation indicated within population variation is very high (90.0%), while among populations within groups and among groups is low (3.0% and 1.0% respectively) (Table 6).

Table 5. Multilocus Nm (above diagonal) and F_{ST} values (below diagonal) between pairs of *C. carpio* populations across all loci.

	GC	MC	GIC	SR	TR	GR	AL	GB
GC		79.551	18.647	25.518	32.744	58.181	36.166	75.705
MC	0.009		52.086	20.748	17.560	74.702	65.347	58.591
GIC	0.113*	0.005		36.166	19.954	66.316	76.324	58.592
SR	0.010*	0.012	0.009		65.347	15.319	14.974	36.166
TR	0.008	0.014*	0.089*	0.008*		10.538	7.890	69.482
GR	0.008	0.007	0.009	0.124*	0.023*		75.703	33.367
AL	0.009	0.008	0.008	0.016*	0.128*	0.007		29.421
GB	0.007	0.006	0.006	0.009	0.006	0.007	0.008*	

*Statistically significant (P <0.05).

Table 6. Hierarchical nested analysis of molecular variance on genetic distance between populations of *C. carpio* in eight geographic regions.

Source of Variation	d.f.	Sum of Squares	Variance Components	% Variance
Among regions	5	38.312	0.000	1%
Among populations within regions	2	17.142	0.038	3%
Within populations	302	2126.750	7.042	96%
Total	309	2182.203	7.080	100%

Clustering analysis

The neighbor-joining dendrogram constructed on the basis of the D_A distances showed only three major clusters (Fig. 3) (Nei et al. 1983). The highest genetic identity was Mazandaran coast population and Golestan coast population; similarity coefficient was 0.654 (genetic distance *D*s=0.091). The lowest was Gilan population and Golestan coasts population; the similarity coefficient was 0.396 (genetic distance *D*s=0.215). The phylogenetic tree was constructed by the neighbor-joining method using the MEGA4 software.

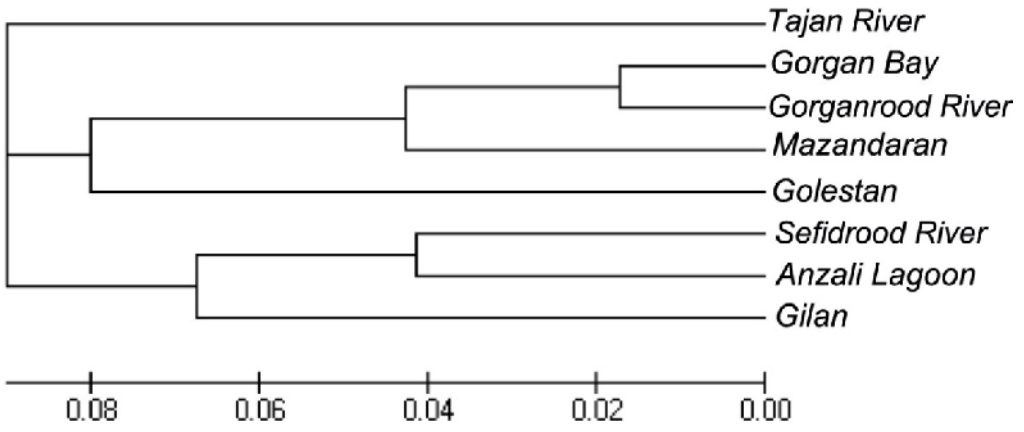

Fig. 3. Clustering of eight common carp populations using the neighbor-joining method.

Discussion

Biological population variation is an important foundation for evaluating species resources. It is a base of the species to adapt to various environments. It is a precondition to make persistent use of the species resource, to keep the highest level of genetic variation. A_E, H_O, H_E and *PIC* are all parameters of population genetic variations. A precise estimation of population structure and genetic distances from microsatellite data is dependent on sample size, number of loci, number of alleles, and range in allele size (Liu and Cordes 2004).

On the basis of the present study, the population variation of the eight common carp populations was high (A_E= 4.41, H_O = 0.56, H_E = 0.67, *PIC* =0.53). Du et al. (2000) reported that the genetic variation parameters H_E=0.65, and A_E=4.91 of the Heilongjiang wild common carp population were lower, when compared to these. However, David et al. (2001) reported that H_E=0.65 and A_E=4.91, when they worked on the differentiations between ornamental common carps and normal ones, using 47 SSR markers. Compared to these, the result here showed that the effective allele was low.

Botstein et al. (1980) indicated that the locus was polymorphic when *PIC*=0.50, low polymorphic when *PIC* = 0.25 and mediate polymorphic when the *PIC* was between 0.25 and 0.50. The average *PIC* here was 0.56, which was at the level of the polymorphic locus. The *PIC* of

Mazandaran population (*PIC* = 0.60) was higher than the polymorphic level, so this population was a high *PIC* population, and the other populations were also very close to this level (*PIC* = 0.45−0.58).

These results showed that although the effective allele was low, the other parameters of polymorphism were still at high levels. The reduction of effective alleles might be on account of the diminishing effective population size, the diminution was mainly caused by environmental degradation, over-fishing, serious water pollution, reduction in spawning areas, and habitat deterioration. Other polymorphism parameters were still at high levels, which could be because of the special genetic background of common carp. With a research on the genome of common carp using microsatellite loci, David et al. (2003) found that common carp had doubled its genome through its long history of evolution. The doubling of genome would be conducive for the enhancement of heterozygosity, but this was the reason why the modern common carp kept higher polymorphism parameters and genetic variation.

Clustering order reflects relationships between populations. According to our research, the genetic identities of populations Mazandaran and Golestan coasts share the highest genetic identity, indicating the nearest relationship. Populations Gilan and Golestan coasts had the lowest genetic identity and the relationship was the farthest. The clustering result is pertinent to their geographic distributing distance. Li et al. (2007) showed that populations that live close to each other have higher genetic identities by the effect of gene flow. The obvious pertinence between geographic isolation and genetic distance shows that the genetic differentiations between populations are mainly because of the geographic proximity. Geographic isolation, environment, population genetic bottleneck problem, gene flow, and selection have large effects on the genetic construction of populations. Heredity and aberrance are necessary to adapt to the changing environment. Population genetic research on a widely distributed species can contribute to the understanding of population genetic distance and potential species differentiation.

According to the expressions of Nm = $(1-F_{ST}) / 4 F_{ST}$ (Li et al. 2007), the average of Nm between populations was 49.54. Theoretically, if the value of Nm was below 1, the genetic drift was the main factor of genetic differentiation. If the value of Nm was greater than 1, the gene flow was the main factor. Different environments induced changes in population genetic structure. Different habitats from south to north caused genetic diversity. Genetic identities of the eight populations I =0.396−0.654 (genetic distance D_s = 0.091−0.215) were lower than the genetic identity of different populations in the species (between populations in one species I = 0.80−0.97, D_s = 0.03−0.20), but larger than the genetic distance (D_s = 0.04−0.17) of the Heilongjiang carp, mirror carp, cold tolerance strain of red carp and Songpu carp (Quan et al. 2005; Sun and Liang 2004), and lower than that (D_s = 0.17−0.23) of three breeds of Jiangxi red carp and Heilongjiang carp (Chang et al. 2004). The genetic identity values were relatively lower for the eight populations; the most probable reasons were lack of gene intercourse, on account of geographic isolation for a long time, significant habitat differentiation, and the relevant genetic differentiations that help them to adapt to their habitats in the course of living and evolution. Li et al. (2007) reported that the different

degrees of differentiations between populations and the variations in populations are mainly because of the effect of the genetic drift and gene flow or the diffusion between populations. The genetic differentiations between populations may be caused by the effect of geographic isolation. The level of genetic variation, high or not, has a relationship with the stability of the habitat (Li et al. 2007). Whether it is the geographic adaptability of the genome or the stochastic process of the statistical events of populations that cause larger genetic differentiations in the wild carp populations, needs further research.

Conclusion

To characterize and distinguish common carp populations microsatellite loci should be preferred because of their generally higher variability and better performance, in particular if populations are within geographical regions. The panel of eight microsatellite loci used in the present study can be used for relatively precise description of genetic diversity and relationships of common carp populations. However, the final number of loci used should depend on the number of populations scored and the expected degree of genetic divergence among populations.

Our study indicated that the eight populations had moderate level of polymorphism and genetic variations. Populations of Golestan and Mazandaran coasts are closely related and there is evidence for a single origin of present day *C. carpio* from a common ancestor in the southern part of the Caspian Sea.

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