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Genetic Variations and Structure of Common Carp, (*Cyprinus carpio*) Populations by Use of Biochemical, Mitochondrial and Microsatellite Markers

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Abstract: Wild common carp populations from southern Caspian Sea were examined for transferrin polymorphism, PCR-RFLP and microsatellite DNA variations. Transferrin variability (1-2) allele per locus and observed heterozygote was 0.1 at 3 loci that was lower than RFLP in which the average haplotype was 0.48 for ND-3/4 and ND-5/6 gene and much lower than microsatellite variability (5.5-13 alleles per locus, Expected heterozygocity 0.694-0.856 at five loci). There was very low variation in transferrin variations, but more pronounced at microsatellite loci. Genetic diversity showed that for microsatellite loci most of variation was due to the within population component. 13 and 9 different haplotype in ND-3/4 and ND-5/6 gene of common carp were detected Therefore by applying transferrin and mitochondrial marker could be well characterized generally, genetically and geographically and it could be used for breeding program as well as comparing the differences of races of quite different population, while the microsatellite are better suited to detect the detail structural genetic of population and loss of variation due to inbreeding and departure from Hardy-Weinberg Equilibrium.

Key words: Transferrin polymorphism · PCR-RFLP · Microsatellite DNA · Common carp

INTRODUCTION

Common carp (*Cyprinus carpio* L) is among the most important freshwater which are distributed in all the word. They are presented in brackish water of Caspian Sea as a commercially important fish which are caught by the fisherman of north of Iran.

Common carp of Caspian Sea is an anadromic form of Common carp that spawn in the rivers of Iran and some other countries surrounding the Caspian Sea. Fingerlings of Common carp migrate to rivers. Degradation of the natural environment, through industry activity and agricultural pollution as well as construction of dams and power plant, that reduce the water current, all of these problems cause inadequate condition for carp migration and consequence cased reduction of spawning grounds and habitats suitable for reproduction of Common carp. To Compensate of natural reproduction, enhancement of Common carp through the release to Caspian Sea of millions artificially fingerlings have been performed. Common carp of Caspian Sea is able to grow in brackish water, to due very few genetically studies have been published, while it is intended to start special management programs of this species in brackish water ecosystems, using wild breeders and juvenile dissemination. The success of these programs depends on the knowledge of the genetic structure of the fish population to be managed and on breeders selection.

Genetic variations of common carp population or strains have been investigated by scientists using different molecular markers. Recently allozyme and microsatellite were used for Dutch carp [1], French and Czech carp [2] transferrin marker, RAPD and microsatellites for Hungarian carp[3, 4], Chines carp [5,6]. Serum transferrin (TF) is a single monomeric glycoprotein that are used in common carp genetic study and a number of different alleles have been identified by polyacrylamide gel electrophoresis (PAGE) of sera collected from different carp breeding stocks, with some deviations from expected mendelian frequency distributions noted Innazarov and Blatowas[7].

Mitochondrial DNA molecules of fishes were widely used in the studies of population genetics for the advantages of compaction in size, rapid evolution, maternal inheritance and lack of recombination[8]. In the present report, PCR-RFLP analysis of mtDNA ND5/6 and

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ND3/4 were performed among common carp belonging to three geographically part of Caspian Sea for elucidating genetic relationships among this species and finding molecular genetic markers that might discriminate the three geographically different.

Among different techniques in analysis genetic variations, microsatellite, has been successful in detecting allele frequency difference in different morphotypes. The higher level of allele variation at microsatellitte markers make them useful for addressing questions related to genetic structure [9].

Microsatellites have been found suitable for a variety applications in fisheries and aquaculture research, particularly where genetic differentiation within and between populations may be limited.

The common carp of Caspian Sea is a native and geographically isolated from other population. It is very valuable fish of north of Iran, but despite the importance of this species, genetic data of common carp of Caspian Sea stocks are relatively scare. Analysis of protein polymorphisms was performed on this population [10,11] and recently its genotype with mitochondrial DNA method (PCR-RFLP), [12] and microsatellite markers [13]. On the basis of this consideration and in order to extend our knowledge to southern Caspian Sea, this paper addresses to compare different genetically variation and structure of Caspian common carp by Biochemical, Mitochondrial and Microsatellite differentiation of several population inhabiting central, eastern and western Caspian Sea.

MATERIAL AND METHODS

Sampling of Specimen: The study was performed for TF at 2004, mtDNA 2008 and 2009 for microsatellite. The common carp were selected from south Caspian Sea. Marketable size fish was caught n=30 by gillnet and immediately were subjected to biochemical and molecular analysis.

For transferrin analysis, serum samples for typing of TF allelic polymorphism were collected from n=90 individuals. Total serum proteins were separated by non-reducing polyacrilamide gel electrophoresis, under circumstances particularly suited to visualize TF protein [10]. Sample of 5µl were applied on running by gel. Electrophoresis was carried out in running buffer (72 mM tris, 26 mM boricacid, pH=8) at 90 V for 30 min followed by 220 V for 2 h. The gel were stained for 45 min with 0.04% of amidoblack dissolved in ethanol. The gel was visually analyzed and the position and number of allele were scored.

For DNA isolation and RFLP assay, fin clips were cut from the fish and were placed in 1 ml ethanol. The samples then were digested with 0.5µg/µl proteinase K at 55ºC overnight. After centrifuge the solution, the supernatant precipitated in ethanol and dissolved in 1ml 1×TE buffer. The genomic DNA was isolated by phenol - chloroform [14]. The quality and concentration of DNA from both sources were assessed by 1% agarose gel electrophoreses is then sample were stored at 4ºC until use.

The PCR- reaction for amplifying ND-3/4 and ND-5/6 mt DNA molecule were performed as described in Gross *et al.* [15]. The following primer were tested for ND-3/4:

Forward Primer: 5`- AAA GTT AGT ACA AGT GAC TTV CAA - 3`
Reverse Primer: 5` - TTT TGG TTC CTA AGA CCA ACG GAT - 3`And for ND-5/6
Forword Primer: 5`- AAC AGT TCA TCC GTT GGT CTT AGG - 3 ó
Reverse Primer: 5` TAA CAA CGG TGG TTC TTC AAG TCA - 3

PCR was performed by the use of 2 mM dNTP mix 1 U Taq polymerase, 50 mM $MgCl_2$, 10 ng primers, 50-100 ng DNA plus PCR buffer and BD water up to final volume of 50 µl. RFLP reaction were run in a PCR thermo cycler (Auto-Q, Quanta biotech company). RFLP products (20 µl load) were separated on 2% agarose gels containing 0.5 µg/ml ethidium bromide. The pattern of bands was photographed under UV light. The enzymatic digestion of PCR products were used by 15 RE enzyme include: *Hinf 1, Mae I, BsuR I, Hha I, Alu I, Hpa II, Mbo I, Hin 6I, Taq I, Xba I, Eco 47I, Ava II, Dpn I, Rsa I, Tha I*. For this purpose 3-5 µl PCR product, 1µl RE, 2µl enzyme buffer in final volume of 20µl placed in 37 ºC incubator for 2-3 h.

Total genomic DNA was extracted from ethanol-preserved fin tissues using traditional proteinase-K digestion and a protocol of phenol: chloroform extraction with slight modifications [16]. For amplification of microsatellite loci, five pairs of microsatellite primers designated for common carp(Syp4, Ca3/4, Lidll, Z9/10, Loc5), were used in this study. PCR amplifications were performed in a 12.5 µl volume containing 10-50 ng DNA, 1×PCR buffer, 120 µmol/l dNTPs, 0.15 µmol/l primers and 0.5 U taq DNA polymerase. The reaction were performed on a thermal cycle and the cycle were as follows: a pre-denaturation at 94°C for 300s; followed by 38 cycles of denaturation at 94°C for 35s, annealing at proper

temperature for 40s and elongation at 72°C for 40s and a final elongation at 72°C for 600s. PCR products were separated on 7.5% non-denaturing polyacrylamide gels using 1×TBE buffer in the gel and reservoirs at 200V for 2-3 h according to alleles size, stained with ethidium bromide in water and visualized with ultraviolet. The size of alleles was scored by comparison with pBR322 DNA/Msp I markers (Sino-American, Luoyang, China) combined with image analysis as described by Liao *et al.* [6].

Data Analysis: The TF patterns were visually analyzed and scored from photographs. For the analysis and comparison of the patterns a set of distinct, well separated band were selected After identification of transferrin alleles the following parameters were determined: the frequency of alleles; the frequency of genotypes; the relationship of observed genotype frequencies to the Hardy-Weinberg distribution with the chi-squared test; and the proportion of homozygous and heterozygous genotypes.

Composite haplotype file was obtained by numbers of restriction enzyme and data of haplotype. Restriction enzyme file was obtained by digestion fragments and discrimination sites of restriction enzymes. Analysis of mitochondrial DNA PCR(RFLP) were performed using Reap soft and x2-test based on were [17]. Indices of genetic diversity for populations, e.g. The observed number of alleles (*A*), effective number of alleles frequency (*Ne*), allele frequency (*P*), observed heterozygosity (*Ho*) expected heterozygosity (*He*) [18], gene diversity, were calculated and deviations from Hardy-Weinberg Equilibrium (HWE) was estimated ÷2 test, Fst and Nm between populations were given by the software of GeneAlex [19].

RESULTS

Two transferrin alleles were found in the 3 carp stocks, collected at East, Middle and West of Southern part of Caspian Sea. For all the three stocks 2 transferrin alleles were present, namely A, B (Table 1). The three transferrin genotypes have been observed in the 3 stocks of carp determined by these two alleles. Allele B was found in all stocks. The frequency of allele. B was markedly high. Allele A was present at high frequency in the West stocks while at quite low frequency (0.25) in east and zero in Middle of southern Caspian Sea.

Table 1: Observed and expected frequency of different genotypes, frequency of transferrin alleles in Caspian carp

	Total number of individual	100%	Observed	Expected
East of basin	AA		0	0.01
	BB	27	0.9	0.81
	AB	3	0.1	0.08
Middle of basin	AA		0	0.01
	BB	30	1	0.81
	AB		0	0.08
West of basin	AA	1	0.03	0.01
	BB	24	0.8	0.81
	AB	5	0.17	0.08

Homozygous/heterozygons (%) 82 : 8

Frequency of transferrin alleles (A= 0.06; B= 0.94)

Table 2: Restriction hyplotypes of 3 stocks of common carp digested by restriction enzymes

Gene	Haplotype	South-East	South-Midlle	South-West
	AAAAAA	18	25	29
	CCBBAA	-	1	2
	CABBAB	1	1	3
	BBBBBB	2	2	5
	CCABAA	-	-	-
	BAAAAA	2	1	-
ND3/4	BBBBBA	-	-	1
	AACCAB	1	1	-
	CCACAC	3	3	-
	CCCAAA	3	3	-
	CCAACC	-	2	-
	AACACC	1	-	-
	AACCCA	2	2	-
	AAAAAAA	20	30	29
	AABAAAB	-	1	2
	BABAAAA	1	1	3
	BBBAABB	2	2	5
ND5/6	AABABAA	-	-	1
	AAABAAB	1	1	-
	ABAAAAA	-	2	-
	AABBAAA	4	1	-
	AABAABA	2	2	-

The homozygous BB genotype had the highest frequency in all Caspian Sea carp populations. The frequency of the homozygous AA genotype was quite low. The Homozygous AA genotypes were represented only in the West of the basin. As a result, homozygous genotypes predominated in all populations.

The approximately 2.4 kb mtDNA ND3/4 and 2.6 kb mtDNA ND5/6 segments were amplified from all samples of three group without size difference among all individuals . Digestion patterns of , *BsuRI, Eco 47I, Rsa I, Hinf, Hpa II* for ND-3/4 and *Hpa II, Mbo I, Bsu I, Hinf Rsa I,Alu I,Eco47I* for ND-5/6 were polymorphism among all individuals of 3 stocks. Diagnostic restriction yielded totally 13 and 9 hyplotype for ND-3/4 and ND-5/6 respectively (Table 2).

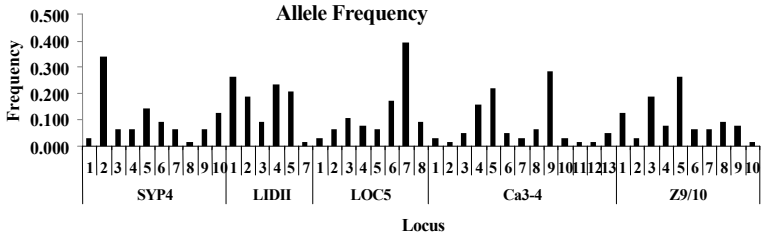

Fig. 1: Allele frequency at 5 polymorphic microsatellite loci

Table 3: Nucleotide and haplotype variation between 3 sampling station in South Caspian Sea

	Haplotype Variation		Nucleotide Variation	
Sampling station	ND3/4	ND5/6	ND3/4	ND5/6
South-West	0.461	0.461	0.066	0.025
South-Middle	0.623	0.438	0.055	0.023
South-East	0.693	0.545	0.062	0.035

Table 4: Thenucleotide differences (%) between common carp of between 3 sampling station at south Caspian Sea

	South-West		South-Middle		South-East	
Sampling station	ND3/4	ND5/6	ND3/4	ND5/6	ND3/4	ND5/6
South-West	0.00	0.00				
South-Middle	0.20	0.001	0.00	0.00		
South-East	0.15	0.02	0.14	0.004	0.00	0.00

Evaluation of two ND3/4 and ND5/6 gene illustrate the significant between East and West (P<0.05).

The maximum haplotype variation for ND3/4 and ND5/6 gene was 0.69 and 0.43 belong to the South-East sample (Table 3) as well as maximum nucleotide variation in case of ND5/6 gene, while higher nuclear variation of ND3/4 was belong to South-West.

As it is illustrated in table 3, nucleotide and haplotype variation was higher in ND3/4 than ND5/6 in all sampling station. Differences between nucleotide of sampling station are presented in table 4.

All five pair primer Syp4, Ca3/4, LidII, Lco5 and Z9/10 related to microsatellite sites were applicable and polymorphic in Caspian common carp. These loci all were variable in population. The number of allele ranged from 6 at LidII to 13 at Ca3/4. The highest allelic frequency was 0.391 in at Loc5 loci (Fig. 1).

Genetic variations within population in experimental groups for each locus were tested and average observed hetrozygosity ranged from 0.469 to 1. The lowest observed genetic variation was at Loc5 and highest at Z9/10 (Table 3)

Average expected hetrozygosity ranged from 0.782 to 0.849 (Table 3). The exact test for fitness to Hardy-Weinberg equilibrium (HWE) on all loci indicated that, non of the loci were found to be in HWE (P<0.01;Table 5). For determining polymorphism of loci by the real and effective number of allele, the highest effective allele was 9.06 and the lowest were 4.592. The highest real allele was in case of Syp4and Z9/10 (Table 4).

Table 5: Comparison of haplotype frequency of the ND3/4 and ND5/6

Comparing Station	ND5/6	ND3/4
West to Middle	P=0.239	P= 0.057
West to East	P=0.037*	P=0.008*
East to Middle	P=0.724	P=0.969

* Significant difference at the level of 95%

Table 6: The expected (*He*) and observed heterozygosity (*Ho*)at five microsatellite loci of common carp

	N=32	
Loci	*Ho*	*He*
Syp4	1.000	0.821
LidII	0.938	0.789
Loc5	0.469	0.782
Ca3/4	0.906	0.834
Z9/10	0.906	0.849

Table 7: Effective number of alleles (*Ne*) in five loci of common carp

	n=32	
Loci	*Ne*	*Na*
Syp4	5.840	10
LidII	4.741	6
Loc5	4.592	8
Ca3/4	6.041	13
Z9/10	6.606	10

Table 8: The results of test of Hardy-Weinberg at different loci in two groups of common carp

loci	df	Chi-square	Prob.	Sig.
SYP4	45	119.363	0.001	***
LIDII	15	54.268	0.000	***
LOC5	26	108.719	0.001	***
Ca3/4	78	128.211	0.001	***
Z9/10	45	98.690	0.000	***

Table 9: The estimate Fst and Nm between the loci

Loci	SYP4	LIDII	LOC5	Ca3-4	Z9/10	Mean
Nm	7.750	4.35	11.696	10.59	22.817	8.619
Fst	0.031	0.055	0.021	0.023	0.011	0.029

In study of Population genetic differentiation, Fst were larger than the standard with no genetic differentiation between two groups of study. The average of Fst was (0.028) and the average of *Nm* between two group was 8.619 (Table 6). The difference between two group was 98% and within each group was 2%. The genetic relationship among samples analyzed by multidimential scaling analysis of Nei, analysed by GeneAlex and the value was 0.794 shows the two groups were distinct.

DISCUSSION

Despite the important of Common carp as a food fish world wide, our knowledge on the genetic background of natural or farmed populations is generally not very extensive.

Conservation of genetic variation of strain in live gene banks is a high priority task, especially for basic population such as the fishes of Caspian Sea. To study the genetic structure is the essential information in this work by applying molecular study, the population could be characterized.

This investigation represented the first contribution of comparing different methods of molecular genetic, to the status of genetic variability of Caspian Sea population and may be of interest for characterization, conservation and breeding programs for this species. The procedure employed allowed the application of the microsatellite technique on a few fish scales to assess polymorphism pattern through an economic, rapid and non-invasive method and without a quality loss in PCR procedure.

By application transferrin analysis only two alleles were determine in all sampling site allele B had the dominant and significant number among 3 different region of Caspian Sea. The fish of Caspian sea also did not showed allelic variation and allele A, having been the rare in all population. Most of the present studies of common carp by using transferrin analysis were focused on selective breeding. In the study of 15 populations of carp7 allele and twenty transferrin genotypes have been observed by Csizmadia *et al.*[3]. Alleles G, D,E had the highest frequency in the study of Csizmadia. The detection of 7 allele was due to analyzing higher number of different stocks but in a distinct population variation among population is very low. This result is in agreement with Csizmadia *et al.* [3] and Kohlmann and kersten, [20] that isoenzyme analysis of Common carp sample indicated that the genetic diversity between stocks originating from the same geographic region is small.

Genetic divergences among some strains of common carp in Europe and Asia were studied by Kohlman and kersten, [21] and Gross *et al.* [15], that analyzed the same population using allozyme markers and mtDNA ND 3/4 and ND5/6 markers. However the strains of common carp they mainly analyzed by them were partial wild and domestic strain in Europe and two Asian strains, Red River wild common carp in Vietnam and Amur River wild common carp that was introduced by Russian, genetic distance revealed that genetic divergences exist between 3 subspecies analyzed by them while in the present study we observed small genetic distance (except comparing east to west of Caspian Sea). Low polymorphism in carp population of Caspian sea at mitochondrial ND-3/4 and ND-5/6 genes that is consistent with the results of Froufe *et al.* [22] and Kohlman *et al.* [21],who worked on European carp. They assumed a relatively recent bottleneck event in the history of European carp which most probably is the result of postglacial colonization of the Danube drainage from the Caspian basin refugee. Caspian Sea comparing to several study have been done in Europe and Asia.

DNA markers are more likely to detect small differences between populations due to their higher levels of allelic variation, for example, mtDNA and microsatellite

DNA markers have been shown the genetic structure of population [13] to detect significant of alleles between wild and farmed strains [23]. In the present study we observed relatively high variation among the population even one sample site. Genetic variability was less pronounced at allozyme loci than at microsatellite loci. Microsatellite proved to be powerful in reclassifying specimen to continent of origin in the assignment procedure. The result of this study is in agreement with Desvignes *et al.* [2] ,who observed that microsatellite were more efficient in detecting subtle differences between closely related French and Czech stock than isoenzymes. Our publication deals with DNA marker - based genetic analysis of full broodstocks two fish farms.

In fact the clear differences found in this study in alleles frequencies between different part of Caspian Sea sampling, as well as the specific alleles detected, will be of value in assessing the relative contribution the mixing population in this lake, in the other hand DNA marker, specially microsatellite were more suited to show the genetic variation, however the genetic variation in carp of Caspian Sea is relatively low and on the basic of the transferring, RFLP and microsatellite data of several stocks tested shows a large number of similar genotype, which would be typical for a highly uniform population.

CONCLUSION

In conclusion, the genetic markers grouped population into three divergent clusters: East, Middle and west population. Thus, our present genetic data supported the three different stocks formerly distinguished on the basis of morphomeristic and electrophoretic differences. Preliminary results of mtDNA analyzing indicate a divergence between three groups. The high degree of genetic differentiation in South-east and South-west of Caspian sea showed at the present study, suggest that further differences might be exist in two region . In this respect, detailed genetic studies are needed in order to conform or reject the existence of the supposed different subspecies of common carp in Caspian Sea.

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