Genetic analysis of wild common carp, *Cyprinus carpio* L. in the Anzali wetland, the Caspian Sea

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Abstract

The Caspian Sea and its basin (e.g. Anzali wetland) is one of the natural habitats of wild common carp *Cyprinus carpio*. In this study the genetic structure of this species. In the south-west of Caspian Sea (the Anzali wetland) was investigated using PCR-RFLP analysis of D-loop region. Two hundred of mature fish were collected from 5 stations (40 individuals from each station) including Siahkeshim protected area (SK), Selke wild refuge (S), Sorkhankol wild refuge (SO), Abkenar (A) and the Anzali wetland estuary (E) during spawning season. A 420bp fragment of D-loop was amplified and the PCR products were digested with forty endonuclease enzymes. Four out of them: *Tas*I, *Sma*I, *Ssp*I and *Apo*I showed polymorphism. Seven different composite haplotypes were detected among 5 stations and AAAA was the most frequent. F_{ST} ranged from 0.003-0.99. Over all stations, average haplotype and nucleotide diversity were 0.13 and 0.01, respectively. The highest haplotype (0.42) and nucleotide (0.06) diversities were found in (SO) station. AMOVA test showed that the Anzali wetland probably consists of two different populations of wild common carp which are distributed in SK, A-SO-S-E stations. The results of this study will be useful as a guideline for conservation, restocking as well as cultivation purposes of wild common carp in the Caspian Sea.

Keywords: Common carp, *Cyprinus carpio*, Genetic analysis, PCR-RFLP, D-loop region, Anzali wetland, Caspian Sea

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Introduction

Common carp, *Cyprinus carpio* L. is an endemic fish in the Caspian Sea and one of the most commercially important species of freshwater fish in the world (Balon, 1995). Wild populations of common carp inhabit in the Black, Aral and Caspian Sea and their basins, so the Caspian Sea and its basin, especially the Anzali wetland, is one of the most important natural habitats of wild common carp. Recently, wild common carp are extremely endangered or already extinct in many areas of their nature range because of loss of habitats, overfishing, pollution and hybridisation with domesticated carp (Kohlmann et al., 2003; 2005). Wild populations and the preservation of their genetic purity have a key role in conservation of common carp genetic resources. As a first step, the remaining wild populations need to be identified and genetically characterized. Among the different genetic markers available for population identification, mitochondrial DNA (mtDNA) is probably the most widely used, because it follows a maternal inheritance, does not undergo rearrangements or recombination, has a reduced coalescent time and higher mutation rates than nuclear genes (Liu and Cordes, 2004). Among all mtDNA regions, PCR-RFLP of a control region, a highly variable noncoding section of mtDNA has been used to detect genetic variation and population structure of different fish species such as Russian sturgeon, *Acipenser gulendenstaedti* (Pourkazemi et al., 1999), Striped red mullet, *Mullus surmuletus* and Red mullet, *Mullus barbatus* (Mamuris et al., 2001), Japanese rosy bitterling, *Rhodeusocellatus kurumeus* (Kawamura et al., 2001), Skipjack tuna, *Katsuwonus pelamis* (Menezes et al., 2006), common Pandora, *Pagellus erythrinus* (Apostolidis et al., 2009), Atlantic salmon *salmo salar* (Finnegan, 2009) . There are several studies on wild and domesticated strains of common carp based on PCR-RFLP using different mtDNA regions e.g. evolutionary and population investigations in European and West Asian subspecies (Gross et al., 2002), genetic differentiation in *Cyprinus carpio carpio* and *Cyprinus carpio haematopterus* (Zhou et al., 2003) (subspecies of common carp), genetic structure of this valuable species in Turkey (Memis and Kohlmann, 2006) and genetic variations and structure of common carp populations in the southern Caspian Sea (Yousefian and Laloie, 2011). The Anzali wetland as one of the most important wetlands of the Caspian Sea covering approximately 15000 ha and locating in the south-west coast at 37 20' to 37 30' N and 49 15' to 49 40' E (Fig.1). It is one of international wetland under the 1975 Ramsar Convention. There are three main environmental areas including Siah keshim protected area (4500 ha), Sorkhankol (477 ha) and Selke wild refuges (360 ha) which all have a key roles in supporting biological resources of the wetland as well as the Caspian Sea (Mansoori, 1996). Different kinds of wastes are one the major problem for wild common carp in the Anzali wetland (Fallahbagheri, 2010). Knowledge of population genetic structure can provide important data for determining appropriate brood sources for reintroduction and in formulating restoration goals. Currently, there are few

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information about genetic structure of the wild common carp in the southern part of the Caspian Sea (Khalili and Amirkolaie, 2010; Yousefian and Laloie, 2011), but no information is available about genetic structure of this species in different regions of the Anzali wetland as major source of common carp. So, the objective of this study was to determine genetic population structure of wild common carp in the Anzali wetland.

Materials and methods

Sample collection

A total of 200 fish (2-3 g of fin tissue) of sexually mature wild common carp were collected from five stations (40 samples/station) including Siahkeshim protected area (SK), Selke wild refuge (S), Sorkhankol wild refuge (SO), Abkenar (A) and Anzali wetland estuary (E) (Fig.1).

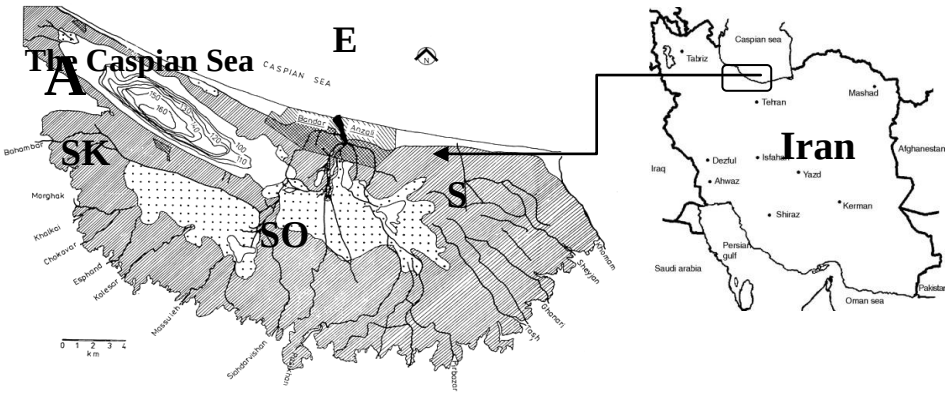

Figure 1: The Anzali wetland and sampling sites for wild common carp. (A): Abkenar, (SK): Siahkeshim protected area, (S): Selke wild refuge, (SO): Sorkhankol wild refuge and (E): Anzali wetland estuary.

All fishes selected from near of main rivers estuary of each separated stations of the Anzali wetland during spawning season in 2010. The samples were preserved in 95% ethanol at 4˚C until used.

DNA extraction

Total genomic DNA was extracted from fin tissue following the salting method described by (Hillis and Mortiz, 1990) with some modifications described in this study. Approximately 50mg of fin tissue was ground in 600µL homogenized buffer (50 mM Tris-HCL; 100mM NaCl; 10 mM EDTA; pH 8.0). Then 30µL of 20% SDS and 5µL of Proteinase K (100 mg mL^{-1}Fermentas,German) were added and the mixture was incubated at 50˚C overnight (24 h) followed by centrifugation at 8000 g for 10 min followed by DNA precipitation with cold absolute ethanol. The DNA was resuspended in 50 µL distilled water. The quality and quantity of DNA was assessed by 1% agarose gel electrophoresis and spectrophotometery method (Nanodrop ND1000,USA).

PCR-RFLP analysis

The mitochondrial D-loop region was amplified using the PCR. The forward primer (LD) was from a conserved region in tRNA-pro gene with the sequence (LD) 5TAC CCC CTG GCT CCC AAA GC3´ and the reverse primer was from the heavy strand (R8) 5AAA TAG GAA CCA GAT GCC AGT AA3´ in the D-loop region of the common carp mitochondrial genome (Haynes et al., 2009).

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Each PCR reaction was carried out in a final volume of 50 µL containing 100 ng genomic DNA , 5µL10X PCR buffer, 2µL $MgCl_2$ 50mM, 10 pM each primers, 0.2 µM dNTPs 10 mM, 5u μL^{-1} *Taq* polymerase (Cinagene, Iran). The PCR reaction profiles included a preliminary denaturation at 95˚C for 5 min, followed by 35 cycles, each consisting of a 30 sec denaturation at 95˚C, 30 sec annealing at 65˚C, 45 sec extension at 72˚C followed by a final 5 min extension at 72˚C.Forty restriction enzymes (*Alu*I, *Nde*I, *Bcl*I, *Vsp*I, *Eco*RI, *Eco*RV, *Pst*I, *Hinf*I, *Hpa*II, *Rsa*I, *Sau3*AI, *Taq*I, *Mbo*II, *Hph*I, *HinC*II, *Mse*I, *Ava*II, *Tas*I, *Hha*I, *Bgl*I, *Bgl*II, *Dra*I, *Sma*I, *AlW26*I, *Nco*I, *Psp*1406I, *Eco*881, *Faq*I, *Xba*I, *Cfr*131, *Pvu*II, *Xho, Mae*III, *Apo*I, *Ssp*I, *Bsu*RI, *Tai*l, *Kpn*I, *Hind*III and *Sal*I) were selected as potential polymorphic enzymes based on initial screening using Gene Runner 3.05 (http://www.generunner.com) and Web Cutter2(http://www.
rna.lundberg.gu.se/cutter2/) software based on D-loop region sequence of wild common carp GU320667 NCBI. The PCR product (4 µL) was incubated with the enzymes (based on recommended amount in enzyme recipe) and separated on a 6% polyacrylamide gel. Four polymorphic enzymes were discovered, where two enzymes recognized a 4-bp restriction site (*Tas*I, *Apo*I) and the others, 6-bp restriction site (*Sma*I, *Ssp*I).

Data analysis

The size of amplified D-loop region and digested fragments were estimated by comparisons of distance travelled by each fragment with distance travelled by known size fragments of molecular weight markers (Lamba DNA digested with *EcoR*I and *Hind*III).

The nucleotide diversity was calculated based on eq.10.21 (Nei, 1978). The nucleotide (Nst) and haplotype (Cst) levels of population differentiation were calculated according to (Crease et al., 1990; Lynch and Crease, 1990) by using the software package REAP, ver. 4.0. Pair-wise exact tests for heterogeneity in haplotype frequencies between the populations were performed using theARLEQUIN,ver.3.1.Genetic differentiation among populations was quantified by analysis of molecular variance AMOVA. (Excoffier and Schneider, 2005). AMOVA input consisted of instance matrix containing genetic distance values for all possible pairs of the seven observed mtDNA haplotypes. Total genetic variation was partitioned into two components, within and among populations. F_{ST} p values ($p < 0.05$) and their significance were calculated for all pair-wise station comparisons of populations by the ARLEQUIN, ver. 3.1 package.

Results

The primers consistently amplified a 420bp DNA fragment of the mitochondrial D-loop that was specific bands.Four restriction endonuclease enzymes (*Tas*I, *Sma*I, *Ssp*I and *Apo*I) exhibited polymorphism in all populations (Table 1). Most of the remaining restriction enzymes (*Alu*I, *Nde*I, *Bcl*I, *Eco*RV, *Pst*I, *Hpa*II, *Sau*3AI, *Taq*I, *Mbo*II, *Hph*I, *HinC*II, *Ava*II, *Tas*I, *Hha*I, *Bgl*I, *Bgl*II, *Dra*I, *Sma*I, *Nco*I, *Psp*1406I, *Eco*881, *Faq*I, *Xba*I, *Cfr*131, *Pvu*II, *Xho, Mae*III, *Apo*I, *Ssp*I, *Bsu*RI, *Tail, Kpn*I, *Hind*III and *Sal*I) did not digest the D-loop region while the other six enzymes (*Alw*26I, *Vsp, Hinf*I, *Rsa*I, *Mse*I and *Eco*RI) digested D-loop region and exhibited monomorphic pattern.

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Table 1: Restriction fragment sizes (bp) in control region of wild common carp, *Cyprinus carpio* produced by the four restriction endonuclease enzymes. A and B are different haplotypes.

*Tas*I		*Sma*I		*Ssp*I		*Apo*I	
A	B	A	B	A	B	A	B
194	197	420	224	420	271	198	400
171	132		196		90	170	20
55	47				59	52	
	44						

The digestion of the mitochondrial D-loop region with the four restriction enzymes resulted in a total of seven different composite haplotypes (Table 2). An average 48.29 bases were studied using four restriction enzymes. The most common haplotype was AAAA in 92.5% of the samples. Haplotypes AABA and ABAB were observed in (A) station and haplotypes BAAA and BAAB in (SK) station, while the BBBA and AAAB haplotypes were only observed in (SO) station and haplotype frequency calculated for each region with divide of each type of haplotype frequency to total haplotype frequency (Fig. 2).

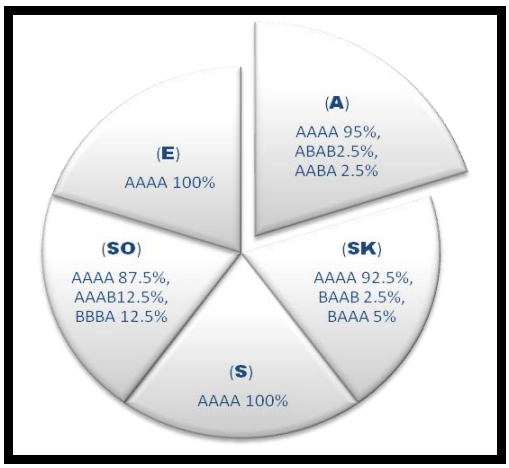

Figure 2: mtDNA haplotype frequencies of the wild common carp, *Cyprinus carpio* for five geographic stations. Each site consists of 40 samples.

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Table 2: The definition of composite mtDNA haplotype of wild common carp, *Cyprinus carpio*, letters refer to restriction fragment pattern that occurs in wild common carp.

Enzyme / Haplotype	TasI	SmaI	SspI	ApoI
AAAA	A	A	A	A
ABAB	A	B	A	B
AABA	A	A	B	A
BAAA	B	A	A	A
BAAB	B	A	A	B
AAAB	A	A	A	B
BBBA	B	B	B	A

The greatest genetic distance (0.35) was observed between the BBBA and AAAB haplotypes while the lowest (0.13) was observed between the BAAA and BAAB haplotypes ($p < 0.05$). Average haplotype and nucleotide diversity were 0.13 and 0.01, respectively over all stations. The greatest haplotype and nucleotide diversity (0.42 and 0.06) was observed in (SO) station. While the lowest (0) was observed in (S) and (E) stations (Table 3).

Table 3:Haplotype and nucleotide diversities of each sampling site. The largest value was observed in Sorkhankol wild refuge(SO) and the lowest observed in Selke wild refuge(S) and Caspian Sea estuary (E).

Sampling site	**Haplotype diversity**	**Nucleotide diversity**
Abkenar (A)	0.0987±0.06381	0.010515
Siahkeshim protected area (SK)	0.1449±0.07369	0.004169
Selke wild refuge (S)	0±0	0
Sorkhankol wild refuge (SO)	0.4167±0.08629	0.056292
Anzali wetland estuary (E)	0±0	0
Average	0.1321±0.00586	0.014196±0.0001145

Nucleotide divergence among populations was measured as 0.03. AMOVA analysis revealed that the majority of mtDNA variation (89.86%) occurred within populations (Table 4). F_{ST} p value ranged from 0.003-0.99 over all stations. This test revealed that there were significant differences between all stations except SO-S, SO-E and SO-A stations ($p<0.05$; Table 5).

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Table 4: Hierarchical nested analysis of molecular variance on genetic distance between populations of *Cyprinus carpio* in five geographic regions in Anzali wetland.

source of variation	d.f.	sum of squares	variance components	% variance
among populations	3	9.15	0.06354	10.40
within population	195	112.60	0.57774	89.86
Total	198	121.75	0.62654	-

Table 5: F_{ST} *p* value (significance level < 0.05) between all stations. High light number shows no significance differences between stations.

A	SK	S	SO	E
A				
SK	0.23±0.0120			
S	0.99±0.0002	0.24±0.0138		
SO	0.044±0.0006	0.06±0.0066	0.0029±0.0016	
E	0.99±0.0002	0.24±0.0101	0.9900±0.0002	0.0029±0.0016

Discussion

The results of this study were revealed that the frequency and variety of haplotypes were significantly different among stations. Generally, the highest diversity was observed in the central station (SO), while the western part (A, SK) is more variable than S and E stations.

Local populations are often considered worth conserving because of their contribution to overall genetic diversity, which may allow individuals to sustain productivity in changing environmental conditions (Hilborn et al., 2003).

Our results showed low level of genetic diversity which is coincided with low level of genetic diversity in the wild common carp in the Caspian Sea as reported by Khalili and Amirkolaie (2010) as well as Yousefian and Laloie (2011). It was in concurrent with low level of genetic diversity of common carp globally (Kirpitchnikov, 1999; Gross et al., 2002). Alternatively, a significant genetic population bottleneck could be another potential reason for low level of genetic diversity of wild common carp in the Anzali wetland, especially in stations such as (S). Different factors can cause bottlenecks in fish population like overfishing, pollution, loss of critical habitats by vacillate in water level in several parts of the wetland especially in (S) station (Mansoori, 1996) which can cause rapid population declines.

Pollution of aquatic ecosystems is recognized as a potential threat to fish population (Belfiore and Anderson, 2001). Presently, several forms of pollution sources including industrial, agricultural and urban sewage have affected the Anzali wetland with varying intensities (Aminiranjbar, 1998). This

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pollution has been resulted in differing water qualities among stations (Sharifi, 1990; Sartaj, 2005). Moreover, interference of the pollutants with nucleotide synthesis cause to abnormalities in DNA and affects genetic diversity over generations (Matter et al., 1992). This hypothesis is supported by Nadig et al. (1998) and Wang et al. (2006) which showed loss of genetic diversity in Redbreast sunfish, *Lepomis auritus* using RAPD markers and Chinese longsnout catfish, *Leiocassis longirostris* by PCR-RFLP following pollution. Water pollutions can affect reproductive success as well as sex ratio of potential brood fish which can decrease the census to effective population size. This can result in increased the risk of loss of genetic diversity increase via genetic drift and inbreeding (Freeland, 2005).

The highest haplotype variation (0.42) and nucleotide diversity (0.06) were observed in the SO station. It could be because of the specific location of SO, as illustrated in Fig. 1, the (SO) is located as a pathway between other stations, by supposition of low level of fish dispersal between different stations, the existence of the highest amount of haplotype and nucleotide diversity is predictable.

There were no significant differences between (S-SO), (SO-E) and (A-SO) stations based on F_{ST} test (p<0.05). It may be due to the close distance between these stations (Fig.1) which increase the potential gene flow by fish dispersal. Another explanation would be the same spawning area of these populations which increase the rate of breeding and decline the genetic differentiation. In many species, the amount of gene flow between populations is inversely proportional to the geographic distances between them because individuals are most likely to disperse to nearby sites furthermore amount of gene flow between populations is direct relative to dispersal ability of species (Freeland, 2005).

AMOVA analysis revealed that most of the total mtDNA variation in the wild common carp at the present study was due to variation in within populations, indicating a very low level of differentiation among populations. The low level of genetic differentiation among populations is also highlighted by low level of nucleotide diversity and divergence among population at present study. It has been reported that a migratory species has 85 and 15% of diversity within and between local populations, respectively. In contrast, a non-migratory species has 67.6 and 32.4% of their diversity within and between local populations, respectively (Vrijenhoek, 1998). The results at the present study are indicating that, these populations are likely to be a migratory population because of low level of genetic differentiation between stations.

It can be concluded that the PCR-RFLP method in mtDNA D-loop region can be used for population studies of the wild common carp in the Anzali wetland and its estuary. In this study, the wild common carp has two populations in (SK), and (A-S-E-SO) stations and there is significant differences between wetland and estuary wild common carp. This study represents a first step towards the use of molecular markers for purposes such as choosing suitable population to employ as resources of reintroduction and in population restoration goal.

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