Genetic characterization of *Vimba vimba persa* (Pallas, 1814) in Southern parts of the Caspian Sea using microsatellite markers

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Received: April 2011 Accepted: September 2011

Abstract

Population genetic structure of *Vimba vimba persa* was investigated using microsatellite markers from 40 regions along the Iranian coastline of the Southern Caspian Sea (Anzali lagoon and Havigh River in Gilan province, BabolRoud River in Mazandaran province and GorganRoud River in Golestan province). Genomic DNA from 121 specimens was extracted from fin tissue by the Phenol-Chlorophorm method and PCR reaction was accomplished with 17 microsatellite primers, out of 17 microsatellite primers 13 loci were amplified, in which 10 of them were amplified with reasonable polymorphism and 3 were monomorphism. A total of 302 alleles were identified on average 7.55. Observed and expected heterozygosity averages were 0.80 and 0.77 respectively. Most cases significantly deviated from Hardy-Weinberg equilibrium ($p \leq 0.01$). The estimation of *F*st ($p \leq 0.01$) revealed significant population structuring and an estimation of the four population of *Vimba vimba persa* was identified in the Caspian Sea in which restocking of these species should be considered.

Keywords: *Vimba vimba persa,* Population genetic, Microsatellite, Caspian Sea, Iran

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[DOR: 20.1001.1.15622916.2012.11.2.10.8]

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Introduction

The Caspian Vimba, *Vimba vimba persa* (Pallas, 1814), is one of the valuable stocks of the Caspian Sea and migrant species, and it has been listed as an endangered species by the IUCN classification (Kiabi et al. 1999), This species has a semi-migratory form that enters fresh water for reproduction in spring and after spawning, it migrates to estuaries and brackish water for feeding until the next reproductive season (Berg, 1948). Fishing, along with other factors such as rivers regulation, pollution, destruction of habitat and blockage of migration routes have resulted in the extinction of this fish species in the Caspian Sea (CEP, 1998). Also these activities have reduced the genetic variation of many fish populations (Ferguson et al., 1994). Reduction of genetic variation in fish is part of a larger global concern for the genetic resources of the biosphere. For this reason, molecular genetics research should be supported (Park and Moran, 1994). Since the development of molecular biology techniques in the early 1990s, different DNA markers, such as microsatellite DNA (Liu et al., 2004) have been used to investigate the genetic variation of species (Meng et al., 2009). The development of highly polymorphic genetic markers such as microsatellites has provided an essential tool for identifying pedigree and obtaining genetic characterization and information in aquaculture selection programs (O'Reilly and Wright, 1995; Divu et al., 2009). The great advantage of these co dominant DNA markers, which are ubiquitously distributed within genomes, are their high level of polymorphism, relatively small size and rapid detection protocols (Crooijmans et al., 1997; Aliah et al., 1999; Wei et al., 2001; Li et al., 2007; Yue et al., 2009). Barinova et al. (2004) showed that the microsatellite markers described here are conserved and polymorphic in a wide range of taxa, indicating that they will be valuable tools for population genetic analysis in *Leuciscus idus* and *Rutilus rutilus* and other fish from the Cyprinidae family. Rezvani Gilkolaei et al (2009) revealed the population genetic structure of Cobia (*Rachycentron canadum*) using microsatellite markers, Yue et al. (2009) estimated genetic variation and population structure of Asian seabass (*Lates calcarifer*) in the Asia-Pacific region and Aung et al. (2010) mentioned that microsatellite DNA markers revealed genetic population structure among captive stocks and wild populations of *Cirrhinus cirrhosus* in Myanmar. Meng et al. (2009) suggested that for revealing any underlying genetic structure on a small geographic scale, microsatellite markers are too conservative and they are polymorphic in a wide range. Thus the general aim of these studies was to analyze the population structure of *Vimba vimba persa* from four regions in the Iranian coastal of the Caspian Sea based on microsatellite markers to assist conservation in restoration goals and sustainable harvest of these populations. Due to having little information about *Vimba vimba persa*'s genetic population in the Caspian basin and importance of conserving its genetic variation, the study

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of this species has become one of the main goals of sustainable stock management programs.

Materials and methods

Fish samples and DNA extraction

Fish specimens were caught by beach seine from four different areas of the Iranian coastline of the Caspian Sea where this species migrates for spawning during spring (mid April to mid June), including 43 samples from Anzali Lagoon, 18 samples from Havigh River, 30 samples from BabolRoud River and 30 samples from GorganRoud River. The positions of these rivers are illustrated in Figure 1.

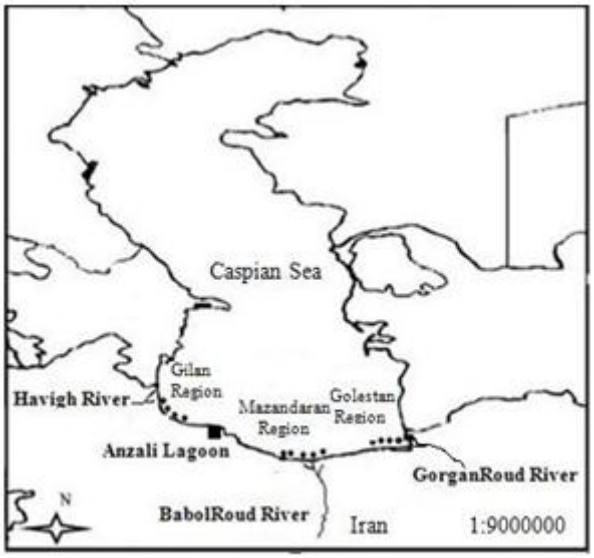


Figure 1: Map shows the sampling sites of *Vimba Vimba persa* in the Iranian coastline

A total of 2-3gr of dorsal fin tissue of 121 samples of *Vimba vimba persa* were collected and stored in 1.5ml Eppendorf tubes with 96% ethanol. In the laboratory genomic DNA was extracted from fin tissue following the method of Phenol-Chloroform described by Hillis and Moritz (1990), the quality and quantity of DNA were assessed by 1% agarose gel electro-phoresis and spectrophotometer (model CECIL DE2040), and then stored in -20°c until use.

PCR amplification and electrophoresis

Nuclear DNA was amplified using 17 microsatellite primers (Table1). Polymerase Chain Reaction (PCR) conditions, especially the annealing temperatures, for each primer set was optimized for *Vimba vimba persa*. The PCR reactions were performed in a 25µl reaction volume containing 100ng of the template DNA, 0.5-1pm of each primer, 200mM of dNTPs, 1U *Taq* DNA polymerase, 1.5 µl reaction buffer(10X), and 1-2.5mM MgCl2 and double distilled water to a final volume of 25µl, concentrations were indicated for all loci, samples were subjected to an initial denaturation step at 95°C for 3 min, followed by 30 cycles of 95°C for 30 seconds, at annealing temperature for 45

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seconds, 72°C for 1 min and a final extension at 72°C for 10 min. The PCR products were electro-phoresed on 8% polyacrylamide gel at 150 W for 3 hours with a ladder marker (pBR322 DNA/AluI Marker, 20, MBI Fermentas) and the DNA fragments were stained by silver nitrate.

Table 1: Primer sequence, Accession number and Reference of 17 used primers

locus	Primer sequence	Accession number	Refrence
CA3	GGACAGTGAGGGACGCAGAC TCTAGCCCCCAAATTTTACGG	AF277575	(Dimsoski et al., 2000)
CA5	TTGAGTGGATGGTGCTTGTA GCATTGCCAAAAGTTACCTAA	AF277577	(Dimsoski et al., 2000)
CA7	ACACGGGCTCAGAGCTAGTC CAAATGTCAGGAGTTCTCCGA	AF277579	(Dimsoski et al., 2000)
CA9	ATCAAGCCTGCCATGCAC ATCACTGTAGACTGCGACCAG	AF277581	(Dimsoski et al., 2000)
Lco1	CACGGGACAATTTGGATGTTTAT AGGGGGCAGCATACAAGAGACACTA	AY318777	(Turner et al., 2004)
Lco3	GCAGGAGCGAAATCATAAAT AAACAGGCAGGACACAAA	AY318779	(Turner et al., 2004)
Lco4	ATCAGGTCAGGG GTGTCACG TGTTTATTTGGGGTCTGTGT	AY318780	(Turner et al., 2004)
Lco5	TTACACAGCCAAGACTATGT CAAGTGATTTTGCTTACTGC	AY318781	(Turner et al., 2004)
Lid-11	CTCCTGATTCTTTGTCTGACT TTATTATTTCCTGTGGTGATTG	AB112736	(Barinova et al., 2004)
Lid-1	TAAAACACATCCAGGCAGATT GGAGAGGTTACGAGAGGTGAG	AB112732	(Barinova et al., 2004)
Rru-2	TTCCAGCTCAACTCTAAAGA GCACCATGCAGTAACAAT	AB112738	(Barinova et al., 2004)
Z21908	CTCCTGATTCTTTGTCTGACT TTATTATTTCCTGTGGTGATTG	G40277	(Shimoda et al., 1999)
Z3,4	TTTGACAAGTGAGTGTCCGC TAGGACCAGCTTCTGCCTGT	-	(Shimoda et al., 1999)
Z8145	TTCCAGCTCAACTCTAAAGA GCACCATGCAGTAACAAT	G40625	(Shimoda et al., 1999)
Z7,8	CTCCTGATTCTTTGTCTGACT TTATTATTTCCTGTGGTGATTG	-	(Shimoda et al., 1999)
Z9,10	TTCCAGCTCAACTCTAAAGA GCACCATGCAGTAACAAT	-	(Shimoda et al., 1999)
MFW1	GTCCAGACTGTCATCAGGAG CAGGTGTACACTGAGTCACGC	DQ377202	(Crooijmans et al., 1997)

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Microsatellite analysis

Microsatellite alleles were sized using UVI DOC Version V.99.04 software (Table 2). In order to calculate allelic and genotype frequencies, observed (Ho) and (He) expected heterozygosity, deviations from Hardy-Weinberg expectations (HWE), Fst value and AMOVA (Analysis of Molecular Variance) were conducted using the GenAlex 6.2 software (Peakall and Smouse, 2005), genetic distance and genetic identity between two populations was estimated from Nei standard genetic distance and genetic similarity index (Nei,1972), UPGMA was computed in TFPGA version 1.3 and the presence of null alleles was checked using Microcheker (Version 2.2.3).

Table 3: Variability of 10 microsatellite loci in four population of the Caspian Sea

Pop		CA3	CA7	Lco1	Lco3	Lid11	Z21908	Z8145	Z7.8	Z9.10	Rru-2	Average
Anzali	Na	16	8	9	4	11	9	3	8	10	11	8.9
	Ho	0.767	0.465	0.698	0.791	0.930	0.977	0.140	1.000	1.000	1.000	0.77
	He	0.911	0.766	0.844	0.666	0.853	0.825	0.511	0.805	0.836	0.882	0.80
	P	**	***	***	ns	*	***	***	***	**	***	-
Havigh	Na	12	7	6	4	6	7	3	6	7	6	6.4
	Ho	0.778	0.611	0.833	0.722	1.000	0.833	0.333	1.000	1.000	1.000	0.81
	He	0.890	0.836	0.764	0.554	0.741	0.716	0.549	0.798	0.799	0.721	0.73
	P	***	*	**	ns	***	*	***	***	ns	*	-
BabolRoud	Na	8	9	8	6	9	7	3	6	6	9	7.1
	Ho	0.800	0.767	0.500	0.833	1.000	0.967	0.233	1.000	1.000	1.000	0.81
	He	0.819	0.861	0.847	0.762	0.852	0.797	0.565	0.786	0.778	0.881	0.79
	P	*	***	***	ns	***	**	***	***	***	*	-
GorganRoud	Na	11	8	5	5	8	8	4	9	9	11	7.8
	Ho	0.867	0.900	0.467	0.900	1.000	0.900	0.167	1.000	1.000	1.000	0.82
	He	0.888	0.847	0.746	0.687	0.818	0.760	0.542	0.782	0.822	0.888	0.77
	P	ns	ns	***	ns	***	***	***	***	***	***	-

Na: Number of alleles; Ho: Observed heterozygosity; He: Expected heterozygosity; p: *p*-values of x^2 tests for Hardy-Weinberg equilibrium, statically significant are marked with asterisks, *<0.05, **<0.01, ***<0.001 and none significant ns.

Results

Our results showed that the microsatellite loci characterized from another genus of Cyprinidae was suitable for microsatellite amplification of *Vimba vimba persa*. Out of 17 loci, polymorphism of amplified fragment length was observed in 10 loci, 4 sets (CA5, CA9, Z3,4 & Lco4) didn't show any flanking sites and 3 sets (lco5, MFW1, lid1) were monomorphism. The results showed the number of alleles per

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locus for ten microsatellite loci which was between 3 at Z8145 to 16 at CA3. Out of 302 observed alleles 243 occurred at frequencies of p≤0.05 in all samples, the highest and the lowest mean number of alleles per locus (8.9 and 6.4) was observed in Anzali Lagoon and in Havigh River respectively (Table 3). The expected and observed heterozygositiy average was 0.77 and 0.80 respectively and the highest observed heterozygosity was estimated in GorganRoud River with a 0.82 mean and the lowest with a mean of 0.77 was estimated in Anzali Lagoon. Significant deviations from Hardy–Weinberg equilibrium were observed in all loci (p≤0.01) except Lco3 in Anzali Lagoon, Lco3 and Z9.10 in Havigh River, Lco3 in BabolRoud River and CA3, CA7 and Lco3 in GorganRoud River (Table 3). The genetic differentiation (Fst) value was observed among all populations (p≤0.01). The Highest Fst (0.092) was observed between Havigh and BabolRoud Rivers also Anzali Lagoon and GorganRoud River showed the lowest and most significant Fst (0.023) (Table 4). Analysis of the genetic structure of the populations with AMOVA method revealed that 5% of the genetic diversity was distributed among the populations and 95% occurred among individuals within the populations. The genetic (D) distance between each pair of collection ranged from 0.14 to 0.48 (Table 4). By using Microcheker, null alles in ca3, ca7 and Lco1 were found. There was no evidence for scoring error due to stuttering and no evidence for large allele drop-out.

Genetic dendrogram of Caspian Vimba in four regions of the South Caspian Sea showed three clarifies cluster (Fig. 2). The sample region of the BabolRoud River showed separate cluster.

Table4: Pairwise estimates *F*st Values (figures above the diagonal) detected at 10 microsatellite loci with (p≤0.01). The figures below the diagonal show genetic distance (D) between pairs of *Vimba vimba persa*

		*F*st			
	Sample	Havigh.R	Anzali.L	Babol.R	GorganRoud.R
Genetic distance	Anzali. L		0.052	0.038	0.023
	Havigh. R	0.24		0.092	0.065
	BabolRoud. R	0.23	0.48		0.069
	GorganRoud.R	0.14	0.30	0.40	

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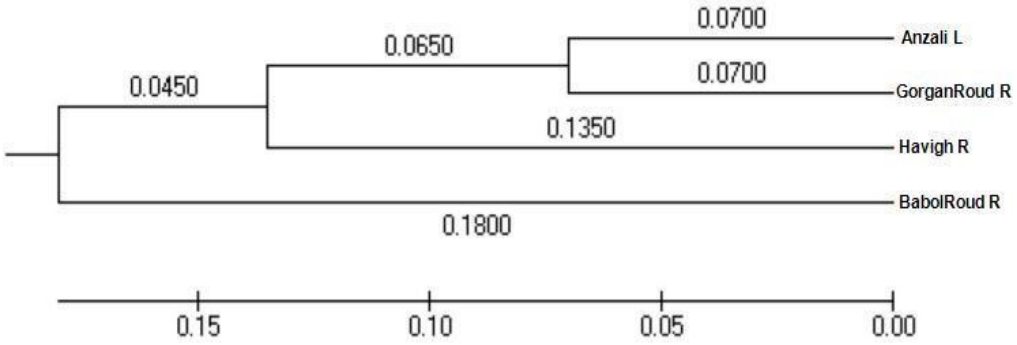


Figure 2: UPGMA dendrogram based on the genetic distance computed by Nei's (1972) between *Vimba vimba pesra* populations, according to microsatellite DNA analysis

Discussion

Microsatellites as genetic markers have been extensively used in studying genetic characterization in fish species (Chistiakov et al., 2006). There is no information on the genomic sequence of *Vimba vimba persa* species, with use of 17 microsatellite primers developed from another species of the Cyprinidae family the population genetic structure of Caspian Vimba was estimated. Shao et al. (2002) used 14-21 microsatellite primer pairs from *Scaphirhynchus platorynchus* in *Acipenser sinenis* and 10 primer pairs were highly polymorphic and liu et al. (2004) with 45 primer pairs from the common carp found 6 highly polymorphic microsatellite loci in Grass carp. In the present study, out of 17 primers 14 of them were amplified in *Vimba vimba persa.* All these results have shown that microsatellites are used in the related species because of its flanking regions for these loci among a related wide range (Norouzi et al., 2009). The statistical data of 524 microsatellite loci of 78 species reported by Dewoody and Avis (2000), revealed that Ho of anadromus fishes were 0.68, whereas the mean values of Caspian vimba were 0.8. The results obtained in this study were similar to the report of Ying-Chun et al. (2006) which analyzed genetic polymorphism of microsatellite DNA in Two Populations of Northern Sheatfish (*Silurus soldatovi*) and Triantafyllidis et al. (2002), which study the genetic population structure of native and translocated Aristotle's catfish (*Silurus aristoltelis*) using the microsatellite markers as fresh water fishes, they found that He was 0.64 and 0.77, respectively. Since the Iranian Fisheries Research Organization (I.F.R.O) does not have any enhancement stocks by artificial reproduction in this "species", there is no report on the releasing fingerlings in the Caspian Sea by I.F.R.O, so inbreeding in population reduced at the most and the high heterozygosity showed high variation. Significant deviation from HWE was found at some loci in all populations, explanations of deviation from Hardy-Weinberg equilibrium might be explained by the presence of null alleles that do not amplify, low levels of polymorphism, population structuring or

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because this fish species population decreased dramatically in the Caspian Sea, therefore the population size might have been too small, causing a deviation in the number of samples. Fst value is a useful measure of genetic differentiation among populations that helps us understand the degree of population differentiation within species (Peakall and Smouse, 2005), both Fst analysis and AMOVA showed significant genetic differentiation between populations. According to Wright (1978) Fst obtained below 0.05 shows little genetic differentiation, between 0.5-0.15 shows moderate and more than 0.15 shows high genetic differentiation. Mainly the different range of Fst between populations is because of geographic isolation, the gene flow and the effect of genetic drift (Li et al., 2007). In the present study, the Fst value in all four sampling regions was moderate (0.054) and statistically significant ($p<0.05$), suggesting that the four populations are genetically different and do not represent a single panmictic population. Because the Caspian vimba decreased dramatically recently, it is a candidate for enhancing and restocking, therefore consideration of differences is useful for gathering brood stock from four different regions to improve its genetic variation in the future. The maximum Fst values were obtained from two regions such as BabolRoud and Havigh River and the minimum values were obtained from Anzali lagoon and GorganRoud River, although the geographic distance between BabolRoud and Havigh is less than Anzali Lagoon and GorganRoud river but the genetic differentiation between these two regions was the most, also Rezvani Gilkolaei et al. (2010) obtained low Fst values between SefidRoud and Tajan rivers with high geographic distance in the Rutilus frisii kutum species. It seems that *Vimba vimba persa* has lost its role in GorganRoud River and only migrates from other places especially Anzali Lagoon for feeding, to this place or perhaps these populations are related to other rivers or neighboring coasts. The average of Nm between populations was 6.45, the differentiations were caused by genetic drift and gene flow, when $Nm>1$, the gene flow was the main factor and when $Nm<1$, the genetic drift was the main factor of genetic variation (Li et al., 2007), so the present results showed that in this study, differentiation is caused by gene flow. The distance values between four regions in this study were in the range of 0.14 – 0.48, Shakla et al. (1982) and Thorpe et al. (1994) suggested that Genetic distance values (Nei, 1972) for conspecific population ranged from 0.002-0.07 and for congeneric species ranged from 0.03-0.61. In the present study the distance values fall within the average value of congenerics. Genetic dendrogram of the Caspian Vimba in four regions of the South Caspian Sea showed three clarified cluster (Fig. 2). The sample region of BabolRoud River showed separate cluster while other regions were genetically more closely stocks. It could be explained that BabolRoud River showed greater background than the other stations and could be counted as their ancestor. Hänfling et al. (2010) which showed Shallow phylogeographic structuring of *Vimba vimba* across Europe with mtDNA sequencing method suggests two distinct refugia during the last glaciations

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mentioned that there were two main clusters between samples of *Vimba vimba* that the first cluster comprises all haplotypes from northern, eastern and central Europe and also the Azov Sea and the second cluster includes haplotypes from the Caspian Sea.

In conclusion, our study provides useful information on the level of genetic variability and differentiation of *Vimba vimba persa* in the Iranian coastline of the Caspian Sea for enhancing this species in the Caspain Sea by IFRO's plan. For this case, catching brood stock of species is important to conserve genetic stocks of this species. This study illustrated four different populations which are living in the South Caspian Sea (Iranian coastline), IFRO should catch brood stock from four different regions and often artificial reproduction should release the fingerlings to reverse differentiate to which brood stock belong.

Acknowledgements

This study was supported by the Iranian Fisheries Research Organization (I.F.R.O) and performed in the Biotechnology laboratory of the Ecology Research Institute of the Caspian Sea. We are grateful to Dr. Pourgholam head of institute and Mr. Laloei for their great co-operation and support and we would like to thank Mr. kor and Mr. Taleshian for providing a part of the samples.

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[DOR: 20.1001.1.15622916.2012.11.2.10.8]

شناسایی ساختار ژنتیکی ماهی استخوانی سیاه کولیدر حوزه ی جنوبی دریای خزر با استفاده از نشانگرهای ریزماهواره

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چکیده

ساختار ژنتیکی جمعیت ماهی سیاه کولی دریای خزر (*Vimba vimba persa*)در چهارمنطقه از سواحل جنوبی دریای خزر (تالاب انزلی و رودخانه حویق واقع در استان گیلان، رودخانه بابلرود واقع در استان مازندران و رودخانه گرگانرود واقع در استان گلستان) با استفاده از نشانگرهای ریزماهواره مورد مطالعه قرار گرفت. در این بررسی استخراج ژنوم DNA از بافت باله ۱۲۱ ماهی با استفاده از روش فنول- کلروفرم صورت گرفت و واکنش PCR با استفاده از آغازگرهای ریزماهواره انجام شد. از ۱۷ جفت آغازگر استفاده شده ۱۳ جایگاه شناسایی شد که ۱۰ جایگاه پلی مورف و 3 جایگاه مونومورف بود. در مجموع 302 الل مورد شناسایی قرار گرفت که میانگین اللی در هر جایگاه ۷/۵۵ بدست آمد. میانگین هتروزایگوسیتی مشاهده شده و مورد انتظار بترتیب ۰/۸۰ و ۰/7۷ بود. بیشتر مناطق انحراف از تعادل هاردی- واینبرگ را نشان دادند، مقادیر محاسبه شده Fst چهار جمعیت مجزا که از نظر آنالیز آماری معنی دار هستند از ماهی *Vimba vimba persa* در سواحل جنوبی دریای خزر را نشان داد.از اینرو مدیریت برای حفاظت ذخایر این ماهی باید مورد توجه قرار گیرد.

واژگان کلیدی: سیاه کولی، ژنتیک جمعیت، ریزماهواره، دریای خزر، ایران

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