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Microsatellite Polymorphism Reveals Low Genetic Differentiation between Fall and Spring Migratory Forms of Endangered Caspian Trout, *Salmo trutta caspius* (Kessler, 1870)

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ABSTRACT
The main objective of this study was to assess genetic comparison of two migratory forms of Caspian trout *Salmo trutta caspius* namely fall-run and spring-run. Owing to the lack of information on its genetic differences, 5 microsatellite loci were used for 58 sample analyses. Genomic DNA was extracted from caudal fin using Roche DNA extraction kit and each PCR reaction was performed in a 25 µl reaction volume. Results revealed that the most allelic frequencies were observed in fall-runs of Caspian trout. The average observed and expected heterozygosity in fall-runs and spring-runs were 0.7719, 0.6108 and 0.4435, 0.5911, respectively. In both groups except Str543INRA in spring runs, all loci had deviation from Hardy-Weinberg equilibrium. Furthermore except Str543INRA in spring runs, expected heterozygosity in all loci was more than observed heterozygosity. In conclusion microsatellite loci polymorphism in this study reveals low genetic differentiation between fall-runs and spring-runs. In order to increase gene flow between Caspian trout populations of different rivers and to increase the production of these fishes, restoration of rivers habitats, as well as using more breeders originating from various rivers is highly recommended.

Keywords: *Salmo trutta caspius, fall-run, spring-run, genetic differentiation, microsatellite, Caspian Sea.*

INTRODUCTION
The Caspian trout *Salmo trutta caspius* (Kessler, 1870) is an anadromous form and endemic subspecies of the Caspian basin. It is distributed commonly at the western and southern coasts of the Caspian Sea. Caspian trout has two migratory forms namely fall-run and spring-run. These forms migrate for spawning to rivers such as Terek, Kura, Sefid Rud, Nav Rud and mostly Cheshmeh-Kileh (Tonekabon) of the Caspian Sea. Migration of fall forms begins at the end of summer to the middle of fall with rather mature gonads ready for spawning. Spring forms migrate to rivers with immature gonads and remain in rivers until the next fall to reach sexual maturity for spawning. Furthermore, the body of spring forms is more fusiform and silver in color than fall forms. Therefore, there is a hypothesis that these two migratory forms differ from each other morphologically (Kiabi *et al*., 1999).

A loss of intra and inter population genetic diversity through exploitation of brown trout populations, stocking of hatchery bred fish, transfer of fish from other localities, pollution, alteration and degradation of habitats are considered to be the main threats to wild brown trout populations (Laikre *et al*., 1999). *S. trutta caspius* similar to other brown trout populations is at risk of extinction and was listed as threatened in the Red List of International Union for Conservation of Nature (IUCN) (Kiabi *et al*., 1999). As its propagation and stock restoration activities are centered in Iran (Shahid Bahonar center of Kelardasht) focus has been only on the fall runs propagation and no success has been achieved with propagation of spring brood stocks yet. Hence fall run stocks have increased compared to spring runs. Besides, the possibility exists that spring forms are part of fall forms which affected by some interior (physiological- hormonal) and exterior (climates) parameters begin their

migration in spring. Finding genetic diversity across this subspecies is of great importance for the development of aquaculture strains, protection of small-endangered populations and biogeographical inferences (Hassanien *et al.,* 2004). Consequently, there is an urgent need to describe the current genetic diversity of fall run and spring run Caspian trout in order to facilitate proper management based on conservation genetics. As the microsatellites have proven useful in genetic studies of brown trout populations (Cagigas *et al*., 1999; Sonstebo *et al*., 2007; Carlsson & Nilsson, 2000) and no DNA based study of the current genetic structure of these two migratory forms has been conducted, the objective of this study is the analysis of Genetic structure of the mentioned migratory forms of *S. trutta caspius* by using 5 microsatellite loci.

Material and Methods

Study location and fish sampling

Shahid Bahonar Fish Propagation Center is the only salmon propagation center located in Kelardasht of Iran. The brood stock collections of the Caspian trout captured from different rivers in the south Caspian basin are transferred there. Fin clips of *S. trutta caspius* were collected from adult fish during the autumn of 2007 and late winter and spring of 2008. Owing to the decrease of spring runs, 37 fall run and 21 spring run samples were collected in this study.

Microsatellite analysis

Genomic DNA was extracted from caudal fin and stored in 96% ethanol, using Roche DNA extraction kit (Roche, Germany). The quality and quantity of the extracted DNA were determined on 1% agarose gels and spectrophotometer, respectively. (Sambrook, 1989). Five microsatellite loci (table 1) were chosen in this study for their ease of PCR based allele scoring and because of being previously assayed (Sonstebo *et al*., 2007; Hansen *et al*., 2000; Cagigas, *et al*., 1999; Charles, *et al*., 2005) and also their polymorphic status (Rafiee, 2006,; Dorafshan, 2006). Each PCR reaction was performed in a 25 µl reaction volume using 4-40 ng/µL DNA, 0.4 mM primers, 1.5 mM $MgCl_2$, concentration of Taq DNA polymerase 1-1.5U and nucleotides (dNTPs) 0.2 mM and was the same for all loci. PCR conditions were optimized for five microsatellite loci. Thermo-cycling parameters were 5 min at 94 ^oC for an initial denaturation, 35 cycles of denaturation at 95^oC for 1 min, annealing temperature for 30 S and extension at 72 ^oC for 1 min followed by a final extension step at 72 ^oC for 10 min (table 1).

Table 1. Flanking primers, annealing temperature, observed Size range and Genbank Accession number of 5 microsatellite loci

Locus	Primer	Size (bp)	Temp. (°C)	Genbank Accession No.
Strutta58	5'-AACAATGACTTTCTCTGAC-3 5'-AAGGACTTGAAGGACGAC-3'	102-190	57	U60223.1
Strutta 12	5'-AATCTCAAATCGATCAGAAG-3' 5'-AGCTATTTCAGACATCACC-3'	124-216	57	U60220.1
OmyFgt1TUF	5'-AGATTTACCCAGCCAGGTAG-3' 5'-CATAGTCTGAACAGGGACAG-3'	187-263	59	BX9272291.8
Str543INRA	5'-ATTCTTCGGCTTTCTCTTGC-3' 5'-ATCTGGTCAGTTTCTTTATG-3'	119-169	60	AB001062.1
Str591INRA	5'-CTGGTGGCAGGATTTGA-3' 5'-CACTGTCTTTCGTTCTT-3'	146-198	59	AB001066.1

PCR was followed by electrophoresis of products in 6% polyacrylamide gels. DNA fragments were visualized by silver staining method (Sambrook, 1989).

Data analysis

Exact tests of microsatellite deviation from Hardy-Weinberg equilibrium and calculation of allele frequency, the number of allele per locus, observed heterozygosity (H_O) and expected heterozygosity (H_E) were performed with the Gene Alex6 program (Peakal & Smouse, 2006) and GENEPOP 3.2 (Raymond & Rousset 1995, 2004). Genetic differentiation between two immigrant forms was analyzed by calculation of F_{st} values by using Gene Alex6, furthermore

AMOVA test was performed in FSTAT program (Goudet, 2001).

Results

All of five microsatellite loci for both forms showed polymorphism with numbers of alleles ranging from 7 (Str 543INRA, Str 598INRA) to 15 (Strutta12) for the fall form and 2 (Str543INRA) to 7 (Omyfgt1) for the spring form (Table 2).

Table 2. Summary of microsatellite data: number of observed and effective alleles per locus, tests for deviation from Hardy-Weinberg equilibrium, expected (H_E) and observed (H_O) heterozygosity in fall and spring runs of the Caspian trout

Forms	Tests	Strutta58	Strutta 12	OmyFgt1TUF	Str543INRA	Str591INRA
Fall	No. of observed alleles	11	15	١٠	7	7
		5.776	5.358	4.511	2.771	3.991
	No. of effective alleles	** *	** *	** *	** *	** *
		0.8382	0.8245	0.7890	0.6479	0.7597
	H-W. test	0.5405	0.6486	0.6486	0.5135	0.7027
	H_E					
	H_O					
Spring	No. of observed alleles	5	5	7	2	6
		2.911	2.617	3.706	1.446	3.459
	No. of effective alleles	*	**	*	Ns	**
		0.6367	0.5961	0.7150	0.2937	0.7140
	H-W. test	0.4783	0.3478	0.6087	0.3487	0.4348
	H_E					
	H_O					

*Significant at the 0.05 level; ** Significant at the 0.01; ns. not Significant

Number of effective alleles was lower than the number of observed alleles, in all loci. Calculation of allelic richness per locus based on minimum samples for the fall run and spring run were, 6.602-11.998 and 2-7 respectively (Table 3).

Table 3. Calculation of Allelic Richness in fall and spring runs of the Caspian trout (Gene Alex6)

Form / Locus	Fall	Spring	Both
Strutta58	9.113	5	8.463
Strutta12	11.998	5	10.338
Omyfgt1TUF	8.873	7	8.287
Str543INRA	6.602	2	5.856
Str598INRA	6.741	6	6.949

Results revealed that the most allelic frequencies were observed in fall runs. Averages of observed and expected heterozygosity in fall and spring runs were, 0.7719, 0.6108 and 0.4435, 0.5911 respectively. In both groups except Str543INRA in the spring form all loci showed deviation from Hardy-Weinberg equilibrium. Furthermore except Str543INRA in spring runs expected heterozigosity was more than observed heterozygosity.

Application of distance method based on the number of different alleles (Fst) between these migratory forms was 0.025 (Table 4).

Table 4. F Statistics and Gene flow (N_m) of the Caspian trout

Locus / Test	Strutta58	Strutta12	Omyfgt1TUF	Str543INRA	Str598INRA	Mean
F_{IS}	0.282	0.281	0.128	0.056	0.193	0.188
F_{IT}	0.303	0.296	0.141	0.093	0.209	0.209
F_{ST}	0.029	0.021	0.015	0.040	0.020	0.025
N_m	8.299	11.610	16.090	6.059	11.990	9.696

$N_m = 0.25(1 - Fst)/Fst$

Nominal genetic differentiation between fall runs and spring runs were seen. Genetic flow and genetic distance between these groups were, 9.696 and 0.081 respectively. Population assignment for two groups indicated that fall and spring runs weren't distinctive (Figure 1).

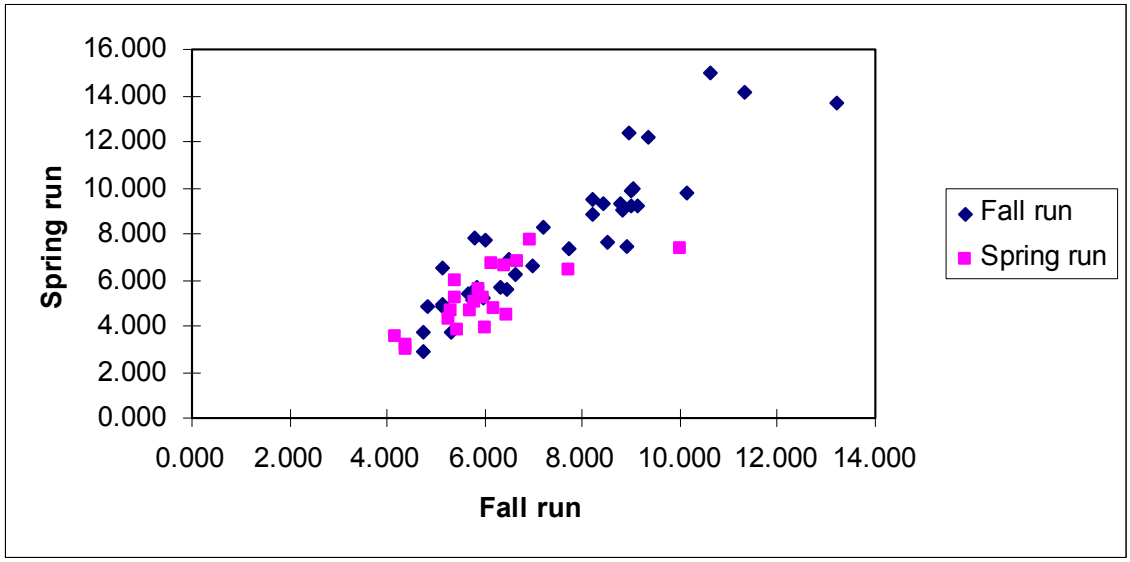

Fig 1. Population assignment for fall and spring runs of the Caspian trout

AMOVA test using FSTAT program showed that there is low differentiation between two migratory forms in which intra population genetic variation (0.97%) was more than their inter population variation (0.03%).

Discussion

In this study no alleles were found that strictly identify the differentiation of fall and spring runs. Highest allelic frequency was related to fall runs. If all alleles have the same frequencies and they are not affected by rare or private alleles (P<0.01), the number of effective alleles in each population will be the same as the actual number of alleles (Nei, 1978). In order to compare lower number of effective alleles with the number of observed alleles in all loci (table 2), we assumed that all alleles of this study have been affected by rare alleles. As average of observed and expected heterozygosity were higher in the fall run than in the spring run (table 2), genetic variation in the fall run was more than that in the spring run. Previous studies revealed that there was genetic variation between all populations in Caspian trout (Navidimoghadam Foumani, 2005, Rafiee, 2006). Furthermore, the mean heterozygosity in this study was significant (0.7143) and all 5 loci were polymorphic. There was genetic diversity between the fall and spring runs. In spite of genetic variation between the fall run and spring run populations, no significant genetic differentiation was found between them (F_{st}= 0.025). In a similar study on *S. trutta*, it was proven that no differentiation between the anadromous and resident forms coexisting in the Oir basin was found but there was a large amount of variation among them (Charles *et al*., 2005). Investigating of genetic variation and population structure of brown trout populations in two Swedish rivers, using microsatellites showed high genetic variation between population while low genetic differentiation within population were observed (Ostergren, 2006). In other study, Banks *et al*. (2000) in order to assess genetic diversity within and among the four runs (winter, spring, fall and late fall) of Chinook salmon (*Oncorhynchus tshawytscha*) in California's Central Valley using 10 microsatellite loci reported that in spite of low genetic differentiation among subpopulations, substantial genetic divergence was found among runs. In this study, AMOVA test using Fstat program

showed that there is genetic variability between the two migratory forms in which intra population genetic variation was more than inter population. On the basis of the assigned test results, only Strutta543INRA in the spring run was in accordance with Hardy-Weinberg equilibrium. Observed deviation from Hardy-Weinberg in other loci could be derived from reduction of heterozygosity (Sonstebo *et al*. 2007; Rafiee, 2006). As expected heterozygosity in all loci except Strutta543INRA in spring run was more than observed heterozygosity, it may be confirmed that inbreeding had occurred among them.

Genetic differences between subpopulations will evolve in the course of time if little or no gene flow exists between them (Chakraborty & Leimar, 1987). Gene flow rates of 10% or less may justify treatments as separate stocks (Carvalho & Hauser, 1995). Consequently, calculation of gene flow indicated that the amount of gene flow between two immigrant forms was high (N_m= 9.696). With high levels of migration and gene flow between populations, the similarity of populations increases (Neigel, 1997) and there isn't considerable genetic differentiation among them.

If amount of F_{st}, as a population distinction index is about 0 - 0.05, genetic differentiation will be considered little (Wright, 1951). In this study, F_{st} indicated weak genetic differentiation between two groups ($F_{st=}$ 0.025). On the other hand, genetic distance between runs was 0.081. It revealed nominal genetic differentiation, too. As F_{IS} parameter was more than zero (0.188), it indicates inbreeding and intermixing (Wright, 1951) of fall and spring runs of Caspian trout fishes. The loss of genetic variation, due to prolonged selection, loss of heterozygosity due to (random) inbreeding or isolation may result in a decrease of the potential adaptability of a population (Ferguson *et al*., 1995).The dangers inherent in subdivision of fish populations are that inbreeding and genetic drift will lead to fixation of genes, loss of fitness (vigor, viability, fecundity, resistance to disease) and ultimately extinction of local populations (Ferguson *et al*., 1995). Allelic richness was positively correlated with effective population size at founding. The results indicate that considerations concerning effective population size in hatcheries must be taken seriously to promote high levels of genetic variation among individuals and minimize loss of genetic diversity (Aho *et al.* 2006). Bottlenecks due to small number of breeders are common in hatchery reared commercial stocks (Verspoor & Jordan, 1989). Consequently, the hatchery produced brood stocks face the risk of reduction of the genetically effective population size (N_e), resulting in excessive inbreeding and loss of genetic variation. Ryman and Laikre (1991), showed how supportive breeding may reduce effective population size and therefore accelerate the loss of genetic diversity within wild populations. This loss may reduce the viability of individuals, for example through reduced heterozygosity, and it may also impact the potential evolution of new adaptations by populations over the long term.

Conclusion and Recommendations

This study indicates high levels of polymorphism in *S. trutta caspius* detectable with microsatellite primers developed from brown trout and rainbow trout. Results of the present study demonstrate that, we may assume the samples are from the same population. It means only one population exists in the southern part of the Caspian Sea. Although 5 sets of microsatellite primers produced replicable amplification in *S. trutta caspius*, they show low genetic differentiation between fall and spring runs. This is a preliminary study and it is necessary to continue it to increase the certainty of the assumption of having one population in the southern part of the Caspian Sea. Therefore, there is a need to assess genetic structure of two migratory forms of *Salmo trutta caspius* using more microsatellite loci and other molecular markers such as AFLP.

Results suggest that there is evolutionary conservation of the flanking regions for these loci among related taxa. The cross amplification between salmon species is consistent with earlier findings that primers developed in one species often work in other related species. Unfortunately, there is a probability that spring runs have decreased because of water pollution, destruction of fish habitats

(spawning grounds), overfishing, illegal catches and use of few brood stocks in artificial fish propagation. It seems that the spring form is probably a part of the fall run population and the deference between them could be attributed to physiological or environmental factors. Artificial breeding practices inadvertently decrease the genetic variation of the Caspian trout population by breeding related individuals or by the use of small numbers of parents as brood stocks and these two migratory forms may have affected bottleneck. Consequently, inbreeding and genetic drift are increased with reduction in effective population size.
It is suggested that maintaining a moderate effective population size per generation and following a strict genetic based mating policy for breeders selected as parents for future generations may control inbreeding. In order to increase gene flow between salmon populations of different rivers and to increase production of these fishes, the restoration of rivers habitats, as well as the use of more breeders originating from various rivers is highly recommended.

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تایید تفاوت محدود ژنتیکی بین ماهیان مهاجر بهاره و پاییزه آزاد دریای خزر از طریق چند شکلی لوکوسهای ریز ماهواره

س. آ. شیرانگی، م. کلباسی، س. درافشان

چکیده:

هدف اصلی از انجام این مطالعه، مقایسه ژنتیکی دو فرم مهاجر بهاره و پاییزه ماهی آزاد دریای خزر بود . با توجه به فقدان اطلاعات در خصوص تفاوتهای ژنتیکی آنها، با انتخاب ۵ لوکوس ریز ماهواره، ۵۸ نمونه جمع آوری شده مورد ارزیابی ژنتیکی قرار گرفتند. در این خصوص دی ان ای ژنومی باله دمی نمونه ها، با استفاده از کیت تخلیص دی ان ای استخراج و واکنش پی سی آر در حجم نهایی ۲۵ میکرولیتر بهینه سازی شد. نتایج بررسی محصولات پی سی آر بیشترین تناوب آللی را در فرم بهاره این ماهی نشان داد. میانگین هتروزیگوسیتی های مشاهده شده و قابل انتظار در فرمهای پاییزه و بهاره به ترتیب معادل ۰/۷۷۱۹ ، ۰/۶۱۰۸ ، ۰/۴۴۳۵ و ۰/۵۹۱۱ بود. در هر دو فرم مورد تحقیق (به غیر از پرایمر str543INRA در فرم بهاره) تمام لوکوسها دارای انحراف از تعادل هاردی واینبرگ بوده و میزان هتروزیگوسیتی مورد انتظار در تمام لوکوسها و برای همه نمونه ها (بجز پرایمر مذکور) بیش از هتروزیگوسیتی مشاهده شده بود.

جمعبندی نهایی نشان می دهد که چند شکلی لوکوسهای ریز ماهواره موید تفاوت محدود ژنتیکی بین فرمهای بهاره و پاییزه ماهی آزاد دریای خزر است و به منظور افزایش جریان ژنی بین جمعیتهای رودخانه های مختلف لازم است تا ضمن اصلاح زیستگاه های طبیعی آنها، از مولدین صید شده از رودخانه های مختلف برای بازسازی ذخائر آنها اقدام نمود.