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Genetic structure in anzali wetland's pike (*Esox lucius*) using microsatellite molecular method

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Abstract

In this research, Pike (*Esox lusius*) one of the most valuable commercial marine species has been evaluated for genetic structure in Anzali wetland using microsatellite markers. 60 specimens of adult pikes were sampled from two spawning seasons, winter and spring in Anzali wetland. Five pairs of microsatellites, tested on the genomic deoxyribonucleic acid (DNA). All loci of microsatellite produced polymorphic bands as polymorphic loci were used to analyze the genetic variation of the pike. Analyses revealed that average of alleles per locus were 10.8 (range 9 to 13 alleles). All sampled seasons contained private alleles. The average observed and expected heterozygosity was 0.914 and 0.885, respectively. The inbreeding coefficient values of five microsatellite loci were negative. With the exception of a locus in spring, all loci significantly deviated from H-W equilibrium ($P<0.01$). Based on AMOVA, R_{ST} and F_{ST} values were significant between seasons ($P<0.01$). The genetic distance between populations was 0.442, which indicates that the genetic difference among the studied populations is significant. These results support the existence of different genetic populations in spawning seasons in this area.

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Introduction

Pike (*Esox lucius* L), is one of the freshwater fishes. In the south Caspian Sea, Anzali wetland and estuary of the rivers ending to the wetland are the main habitats of this fish (Khodadoust *et al*., 2013). Pike almost lives lonely and during spawning period prefers to live in shallow or full of nutrient regions. Late February to May is the spawning period of this fish in Anzali Wetland (Abdoli and Naderi, 2009). Pike growth is considerably significant to be one of commercial species. In addition, it has significant fishing fans. However, its population is getting decreased recently due to destruction of natural habitats (water pollution and destruction of canebrakes). In this situation, it is classified as endangered species requiring appropriate protection (Abdoli and Naderi, 2009). The Anzali Wetland, which is one of the registered wetlands in Ramsar Convention (Mazandaran Province, Iran), has considerable importance including valuable aquatic animals, is getting destroyed because of pollutants, sediments and nutrient contaminations. If this situation continues, this wetland and its inhabitants would be dull much earlier than their determined fate (Zebardast and Jafari, 2011). Therefore, studying genetic structure of Anzali Wetland inhabitants like pike will reflect the impact of current situations on survival of these organisms. In addition ecological and population structure investigation of commercial fishes are essential for protection of their reservoir and sustained fishing (Wang *et al*., 2007). Genetic change can make one species or population competent to survive by providing fitness against environmental changes. Thus, genetic variation is necessary for long term survival of species (Bataillon *et al*., 1996), and crucial for management and protection of aquatic resources, and also serves as first pioneer for maintenance of population fitness in variable conditions of the environment (Diz and Presa, 2009). In addition, by increasing the knowledge about populations and interspecies variation, the protection of putative species becomes successful. Microsatellite markers that can be applied in such purposes are able to reflect high levels of polymorphism (Chistiakov *et al*., 2005). Microsatellites are copiously distributed across the genome, demonstrate high levels of allele polymorphism and can easily be amplified with polymerase chain reaction (PCR). Microsatellites recently have used as popular marker indicator in a wide variety of genetic investigations (Sekar *et al*., 2009). These features provide the underlying basis for the successful application of microsatellites in a wide range of fundamental and applied fields of fisheries and aquaculture (Sekar *et al*., 2009). Microsatellite-applied studies on pike were used in some studies (Reading *et al*., 2003, Nicod *et al*., 2004, Jacobsen *et al*., 2005, Launey *et al*., 2005, Larsen *et al*., 2005, Lucentini *et al*., 2009, and Wang *et al*., 2011).

In this study we used pike reservoir of Anzali Wetland to determine genetic population structure using microsatellite markers. Pike has different populations in winter and spring spawning seasons (Abdoli and Naderi, 2009). Since genotype and allele frequencies of each population are different from each other, sampling has been done in two different spawning seasons to identify the populations and genetic distinction between them by using microsatellite markers and evaluation of different management measures necessities on reservoirs of this fish in the Caspian Sea.

Material and methods

Sample Collection

Pike samples (fin tissue) were obtained during two spawning seasons including winter and spring with 30 samples for each season. The fin tissue were cut and fixed in 96% ethanol and transferred to genetic lab and maintained at -70 °C till DNA extraction.

DNA Extraction

genomic DNA was extracted using a high pure PCR Template preparation kit (Roach, Germany) according to manufacturer guidelines. The quantification and qualification of extracted DNA

were analyzed by spectrophotometer (CECIL model CE2040) and 1% agarose gel electrophoresis.

PCR (Polymerase Chain Reaction)

PCR was applied using 5 sets of microsatellite primers (Table 1) and BIO-RAD thermocycler.

The PCR was carried out in a final volume of 25 µl containing 2.5 µl of reaction buffer, 0.2 µl of dNTPs, 1.5 µl of $MgCl_2$ (25 mM), 1 unit of Taq DNA Polymerase, 1 µl of mixed primers, and 100 ng of template DNA. Sterile distilled water was added to the mixture to 25 µl in total volume.

Amplification was performed as follow: 35 cycles of denaturation at 95°C for 45s to 2 min, annealing at 57 to 61 °C for 40s to 2 min, primer extension at 70 to 72°C for 45s to 2 min (table 3). The PCR products were analyzed on an 8% polyacrylamide gel electrophoresis and silver nitrate staining, and the results were analyzed by Uvitec software (http://www.labtech-equipment.com/UV/UV.html).

Table 1. Characteristics of 5 polymorphic microsatellite loci for northern pike (wang *et al.*, 2011).

locus	Primer sequences(5′-3′)	Repeat motif
Eluc002	F:TGATGACACTGTCCGTGTGT R:AGCCATCTGTTCCTGCAA	(GT)7N2(TG)17N2(TG)8
Eluc004	F:TGATTGTAACTGCACAGCGG R:TAGCGCAGACACACTACTGGA	(TG)12N2(GT)9
Eluc027	F:TCTCTGTCTAACACGAGCGA R:GTGTGTGTGCAGGTTCACAT	(CA)10
Eluc041	F:GTGTGTAGACTTTGGCTCGAT R:ACCCAGACAGAAACAAAGACC	(GT)20
Eluc045	F:AGCATCAGGGAGTAGTTGCA R:CAGGTAAGCGTCCAGGTAAA	(CA)19

Data Analysis

Allelic frequencies, observed (Ho) and expected (He) heterozygosities, genetic distance, genetic identity, F_{IS}, F_{ST}, and F_{ST} value, Nm, Hardy-Weinberg (HW) tests of equilibrium, AMOVA (analysis of Molecular Variance) were computed in GeanAlex 6.7 software.

Results

Amplification and Banding Patterns

In order to investigate the population genetic structure of Anzali Wetland's pike, five sets of microsatellite primers were applied that after amplification and polyacrylamide electrophoresis all the primers made polymorphic bands. All of loci have at least one band and in some cases two bands were observed (Fig. 1-3). In this study overall 64 alleles were identified. In the winter samples 50 alleles were identified that 26 of them have 0.05 or less frequency. In the spring samples 58 alleles were identified that 22 of them have 0.05 or less frequency. The most allele frequency (0.217) was observed on Eluc004 and Eluc045 loci with five alleles from winter and spring seasons, respectively (Table 2).

Genetic Variation Analysis

Overall 13 specific alleles were found. The spring and winter samples have 4 and 9 specific alleles respectively with 0.05 frequency that it has not been observed during other sampling seasons. The Eluc004 locus has five specific alleles including 2 alleles from winter samples with 0.150 and 0.160 frequencies, respectively; and 3 alleles from spring samples with 0.100, 0.133 and 0.133 frequencies, respectively. The Eluc045 locus has 5 specific alleles including 1 allele from winter samples with 0.200 frequency and 4 alleles from spring samples with 0.100, 0.100, 0.100 and 0.217 frequencies, respectively. The Eluc027 locus has 2 specific alleles from spring samples with 0.083 and 0.067 frequencies. The Eluc002 locus has 1 specific allele from winter samples with 0.117 frequency. The Eluc041 locus has not any specific allele (table 2). The means of total number (Na) and efficient alleles (Ne) were 10.8 and 8.7, respectively. The allele

range was achieved among 9 to 13 alleles (Table 3). Many alleles were found in 0.05 or less frequencies.

Table 2. PCR product size (bp) and allele frequency in winter and spring seasons.

Loci	Allele size	samples		Loci	Allele size	Samples	
Eluce002	**(bp)**	**winter**	**spring**	**Eluce004**	**(bp)**	**Winter**	**spring**
	244	0.00	0.050		238	0.00	0.100
	248	0.133	0.083		240	0.00	0.133
	250	0.150	0.183		242	0.00	0.133
	252	0.133	0.167		244	0.133	0.050
	254	0.083	0.083		243	0.033	0.050
	274	0.050	0.00		248	0.083	0.050
	276	0.100	0.083		254	0.150	0.00
	278	0.083	0.100		258	0.167	0.00
	280	0.100	0.200		262	0.067	0.067
	282	0.050	0.050		264	0.017	0.117
	284	0.117	0.00		268	0.017	0.117
					270	0.100	0.117
					272	0.270	0.067
					274	0.017	0.00
Eluce027	142	0.00	0.050	Eluce041	210	0.033	0.083
	144	0.00	0.083		212	0.083	0.067
	146	0.00	0.067		214	0.083	0.100
	148	0.00	0.050		246	0.067	0.083
	150	0.083	0.117		218	0.117	0.083
	152	0.183	0.050		220	0.067	0.033
	154	0.150	0.100		222	0.050	0.050
	156	0.117	0.067		228	0.083	0.167
	158	0.050	0.033		230	0.183	0.117
	162	0.067	0.083		232	0.133	0.100
	164	0.050	0.117		234	0.00	0.033
	166	0.150	0.100		236	0.100	0.083
	168	0.150	0.083				

Table-2-continuation

Loci	Allele size	Samples	
Eluce045	**(bp)**	**Winter**	**spring**
	170	0.00	0.100
	172	0.00	0.100
	174	0.00	0.100
	176	0.00	0.217
	178	0.00	0.033
	182	0.017	0.033
	186	0.133	0.017
	188	0.183	0.067
	190	0.083	0.067
	192	0.100	0.067
	196	0.017	0.067
	200	0.200	0.00
	202	0.117	0.117
	204	0.150	0.017

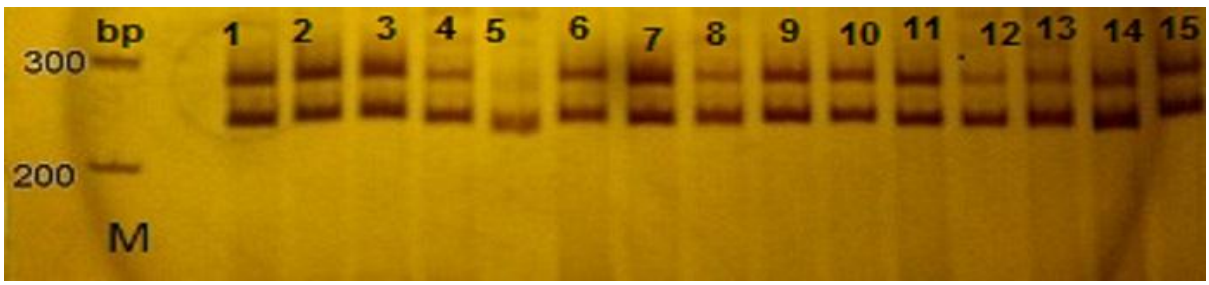

Fig. 1. Microsatellite banding profile of *Esox lucius* from Anzali wetland using primer pair Eluc002.

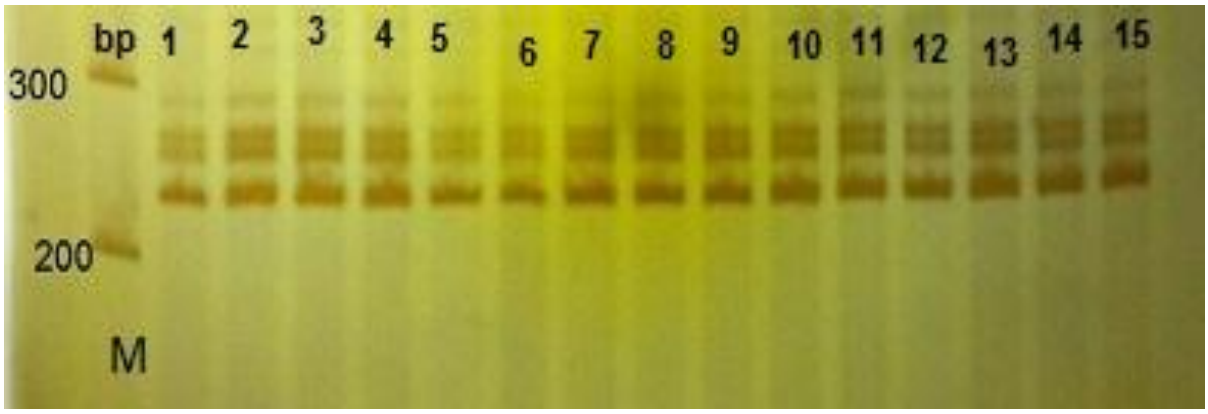

Fig. 2. Microsatellite banding profile of *Esox lucius* from Anzali wetland using primer pair Eluc004.

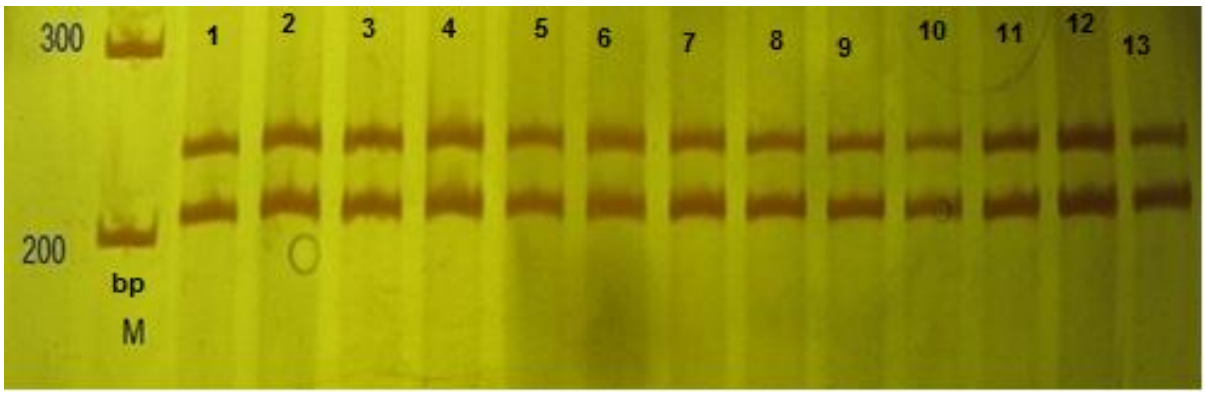

Fig. 3. Microsatellite banding profile of *Esox lucius* from Anzali wetland using primer pair Eluc041.

Table 3. Numbers of alleles observed within 2 sampling sites using 5 sets of microsatellite primers. Number of alleles (Na), effective allele (Ne), Observed (Ho) and expected (He) heterozygosities, were calculated at 5 loci in two sampling sites. Loci in accordance with H-W equilibrium, *P < 0.05; **P < 0.01; ***P < 0.001; ns= not significant.

	Season Samples		**Ta (°C)**
Locus	**Winter**	**spring**	**Allele range (bp)**
Eluc002			58
Na(Ne)	10(9)	9(7.2)	244-248
Ho(He)	1(0.889)**	(0.863)** 0867	
Eluc004			57
Na(Ne)	11(7.2)	11(9.7)	238-272
Ho(He)	0733(0.862)***	0.967(0.897)**	
Eluc027			59
Na(Ne)	9(7.6)	13(11.6)	142-168
Ho(He)	0.833(0.869)***	0.933(0.914) ns	
Eluc041			59
Na(Ne)	11(9.2)	12(10.1)	210-236
Ho(He)	1(0.892)***	1(0.902)	
Eluc045			61
Na(Ne)	9(6.8)	13(9)	170-204
Ho(He)	0.933(0.855)***	**0.8 (0.889)	Total
Average: Na(Ne)	10(8)	11(9.5)	10.8(8.7)
Average: Ho(He)	0.9(0.873)	0.913(0.893)	0.907(0.883)

In this study, the Ho range between two sampling seasons in all loci was 0.733 to 1 and its means was 0.913. The Ho mean in winter and spring seasons was 0.900 and 0.913, respectively. The He range was between 0.855 to 0.914 and its mean was 0.883. The He means in winter and spring seasons were 0.873 and 0.893, respectively (Table 3). All of the loci were deviant from Hardy-Weinberg Equilibrium (P≤0.001) with the exception of Eluc027 locus among spring samples (Table 3).

The F_{ST} value obtained 0.023 based on allele frequency indicating low genetic distance (Balloux *et al.*, 2002). The F_{ST} and R_{ST} values according to AMOVA test were significant (P<0.01) and the calculated rate of gene follow based on RST was 1.19. The inbreeding coefficient or F_{IS} values in all microsatellite loci were 0.026 and its range was between 0.033 in the Eluc004 locus to 0.115 in the Eluc041 locus (Table 3). The positive values of F_{IS} reflect the decrease of heterozygocity. The Eluc004 locus with the lowest value of F_{IS} showed the highest heterozygocity among all loci (Table 4).The genetic distance according to Nei (Nei, 1972) was 0.442 and genetic similarity was 0.643.

Table 4. F-Statistics and Estimates of F_{IS} over All Populations for each Locus using 5 sets of microsatellite primers.

	Locus					
	Eluc002	**Eluc004**	**Eluc027**	**Eluc041**	**Eluc045**	**)SE (Average**
F_{ST}	0.010	0.040	0.014	0.005	0.046	0.023(0.008)
F_{IS}	-0.065	0.033	0.009	-0.115	0.006	-0.026(0.028)

Discussion

The Anzali Wetland is one of the valuable ecosystems that due to increase in pollutants originating from industry expansion, unruly increase in crowd along the wetland, applying insecticide and fertilizer as consequences of agriculture growth, entrance of industrial and urban waste to the wetland made the aquatic organisms especially pike, one the most valuable fishes of this ecosystem, endangered. Therefore, protection of its important reservoirs is essential. Awareness of genetic reservoirs and genetic variations among the individuals of one species is important goal of reservoir management and eugenic affairs. Study of population genetic structure of economical fishes for protection of their populations and keeping sustained fishing is important (Wang *et al.*, 2007). Currently, reduction of the reservoir of aquatic organisms all over the world attracted the researcher's attention toward precise molecular methods for management of their reservoir (Lin *et al.*, 2000).

The allele number and heterozygocity are important indexes of genetic variation of populations during environmental changes (Frankham, 2008) and determine the competition and survival abilities of organisms in natural biomass (Hakansson and Jensen, 2005). The results of this study show that the obtained mean of allele number (10.8±0.49) is in announced range (9.1±6.1) for freshwater fishes (DeWoody and Avis, 2000). Wang *et al.*, (2011) got 7.67 for obtained allele number and 2 to 13 for allele range. Reading *et al.*, (2003) obtained 5 for average of allele number and 2 to 11 for allele range. Jacobsen *et al.*, (2005) reached 7 to 11 for allele range. In this study we obtained 10.8 for allele number and 9 to 13 for allele range. Although the obtained allele number is in the range for freshwater fishes, many alleles with low frequency have been found. The presence of many alleles with low frequency indicates the genetic bottleneck or impact of inbreeding (Alarcon *et al.*, 2004).The decrease of genetic changeability in this species comes from immethodical fishing especially in protected areas, pollution of environment, destruction of biome, and lowering water level of Caspian Sea in recent years that these factors lead to significant decline of pike reservoir in Anzali Wetland. If this process continues, its result would

decrease the genetic variation and consequently susceptibility to various diseases and other selective factors (Shen and Gong, 2004) and eventually considerable decrease in populations of the species will appear.

The heterozygocity is an index to evaluate genetic variation and is important for study of population structure of species since it creates different genotypes as a response to fitness during changeable environments. Also most of economical features like growth rate, fertilization and resistance to diseases are affected by heterozygocity (Beardmore *et al.*, 1997). Our results show that mean observed heterozygocity is 0.9 (±0.052) that is higher than announced value (0.54 ±0.25) for freshwater fishes (DeWoody and Avis, 2000). In two sampling seasons, the average of observed heterozygocity was higher than expected heterozygocity. Wang *et al.*, (2011) reported 0.154 to 0.846 for observed heterozygocity and 0.145 to 0.817 for expected heterozygocity. Reading *et al.*, (2003) calculated the observed heterozygocity between 0.190 to 0.917 and expected heterozygocity 0.194 to 0.850. Jacobsen *et al.*, (2005) obtained the heterozygocity range between 0 to 0.852 and expected heterozygocity 0 to 0.777. We reached observed heterozygocity range between 0.733 to 1 and expected heterozygocity range from 0.855 to 0.914. Perhaps, the increase of heterozygocity in some loci is due to presence of null alleles that are ranked falsely.

Since all samplings have been conducted in Anzali Wetland, the increase of heterozygocity rate is probably due to presence of unrelated individuals. According to statistics, the significant decrease of pike reservoir in recent years is due to genetic bottleneck including high rate of fishing and destruction of natural biomes. The rivers carry industrial waste and hospital materials to the wetland. These materials contain various pollutants like heavy metals, nutrients and so on. The water drainage of rice fields has nutrients and agricultural pesticides that increase the eutrophication phenomenon or gradual destruction of wetland. On the other hand, entrance of solid sediments to the wetland decreases its depth and cause the annihilation of wetland (Abdoli and Naderi, 2009). Some studies show that Anzali Wetland faced with decrease of area and increase of nutrition-orientation during recent decade (Zebardast and Jafari, 2011).

Our results show that during two sampling seasons all loci are deviant from Hardy-Weinberg equilibrium ($P \leq 0.001$). This could be due to existence of null alleles that their appearance in microsatellite inheritance in pike has been approved (Wang *et al.*, 2011; Aguilar *et al.*, 2005; Lucentini *et al.*, 2009; Reading *et al.*, 2003; Larsen *et al.*, 2005). It appears that mixing of populations is the most important effective factor for deviation from Hardy-Weinberg equilibrium.

In general, the F_{ST} and R_{ST} are applied in description of population distinction in different levels of genetic structure (Balloux and Lugan, 2002). In this study the F_{ST} value based on allele frequency was 0.023 indicating low genetic distinction (Balloux *et al.*, 2002). According to AMOVA test, the F_{ST} and R_{ST} values between two sampling seasons were significant ($P<0.01$). It appears that at least two different genetic populations exist in Anzali Wetland. The low value of F_{ST} is due to high polymorphism (because of mutation) in microsatellites and migration in various regions of wetland that probably outcomes from lack of ecological or physical barriers and leads the populations to be in the close contact and can efficiently decrease the F_{ST} value (Balloux and Lugan, 2002). Shaklee *et al.,* (1982), Thorpe and Sol-Cave (1994) reported 0.3 (with range between 0.03 to 0.61) for average value of genetic distance for population distinction that is consensus with observed genetic distance of this study (0.442) and implies the genetic distinction among populations (Nei, 1972).

Conclusion

Collectively, the genetic variation of pike reflects the existence of genetic bottlenecks for this species; however, it is possible to protect genetic variations in

each population. The decrease of pike population during recent years shows the importance of protective measures for revival of pike reservoir. Maintenance of complete genetic variation of pike populations is important, therefore, identification of population structure will aid the design of appropriate revival programs. This study shows the reasons and primitive results of existence of different populations of pike in different spawning seasons and also their genetic bottlenecks. Protection of genetic reservoir of pike is essential and requires serious actions. In fact, the pike population decreases each year due to unbalanced fishing and pollution of wetland which demands urgent actions for controlled fishing and revival of its reservoir.

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