Genetic relationships of Iranian coastline ship sturgeon (*Acipenser nudiventris*) samples and Ural population based on microsatellite DNA

Safari R.[1*]; Pourkazemi M.[2]; Rezvani Gilkolaei S.[3];

Shabani A.[1]; Bagherian Yazdi A.[1]

Roghi_safari@yahoo.com

1- Gorgan University of Agriculture Science and Natural Resources, P.O.Box: 49138-15739 Gorgan, Iran
2- International Sturgeon Research Institute, P.O.Box: 41635-3464 Rasht, Iran
3- Iranian Fisheries Research Organization, P.O.Box: 14155-6116 Tehran, Iran

Abstract: Genetic relationships among 96 specimens of ship sturgeon, *Acipenser nudiventris*, collected from six locations along the Iranian coastline as well as 8 specimens from northern population (Ural River) were analysed by the restricted maximum likelihood method, as well as two distance analysis method (Nei's and Cavalli-Sforza's distance) of gene frequencies. We have used four SSR makers that produced three polymorphic and two monomorphic loci. Overall results showed that Iranian coastline samples form a monophyletic group (clade) which was different from the northern (Ural) samples. Iranian coastline samples are divided into two groups (clades): Anzali-Kiashahr clade and Sefidrud-Babolsar-Noushahr-Gorgan clade. Bootstrapping test showed monophyleticity of Sefidrud, Babolsar, Noushahr, Gorgan with high (Bootstrap support=93) and Anzali-Kiashahr with middle (Bootstrap support=65) confirmation. Topology of reconstructed trees was in correspondence with geographical distributions of samples.

---

[*] **Corresponding author**

Introduction

Sturgeons stocks throughout the Caspian Sea have declined dramatically mainly due to over fishing, illegal catch, pollution and deterioration of habitats and natural spawning grounds (Pourkazemi *et al.*, 2000). All of the Caspian Sea sturgeons, including ship sturgeon, have been listed in the endangered species of IUCN, CITES (Qasemi *et al.*, 2005). Commercially and biologically regarded as the most valuable fish species, sturgeons have been the focal points of various research interests in Iran in recent years (Pourkazemi *et al.*, 2005; Sattari *et al.*, 2005; Abedian Kenari *et al.*, 2007). These researches have also been extended to genetic, particularly at population level. Nowadays, it is possible to determine the genetic structure of fish through various molecular methods such as RFLP, RAPD and using microsatellite markers. Microsatellites consist of multiple copies of tandemly arranged simple sequence repeats (SSRs) that range in size from 1 to 6 base pairs and are believed to have been caused by polymerase slippage during DNA replication, resulting in difference in number of repeat unit and are inherited in a Mandelian fashion as co-dominant marker. Their abundance and high polymorphism are other characters. Pourkazemi *et al.* (2000) reported phylogenetic relationship of five sturgeon species in the Iranian coastline of the Caspian Sea using mtDNA molecule. They found that direct sequencing of ND5 gene provided more information for phylogenetic or population level studies in sturgeon species. Their study revealed a distinct classification among the five sturgeon species. On the basis of the phylogenetic tree, it is clear that a close evolutionary relationship exist between the great sturgeon and ship sturgeon that was verified in five phylogenetic trees. On the other hand, a close resemblance was also seen between the Persian and Russian sturgeons that indicated these two species have a closer evolutionary relationship. Until now, there has been no documentation of phylogenetic study on *Acipenser* species using microsatellite in Iran. In this study, genealogical relationship of *Acipenser nudiventris* samples from six locations along the southern coastline of the Caspian Sea was analyzed.

Materials and methods

Fin tissue of 104 mature adult ship sturgeon from seven locations (Kiashahr, Anzali, Sefidrud, Noushahr, Babolsar, Gorgan and Ural River) was collected (Fig.

1) Fresh tissues were stored in 95% ethanol solution. Total genomic DNA was extracted using the phenol–chloroform method as described by Pourkazemi (1996). Four sets of microsatellite primers (Table 1), which had been developed for lake sturgeon by May *et al.*, 1997, were used. Amplification was performed in PCR system (Gradient Eppendorf) using a 25µl reaction mixture. Each PCR reaction (final volume 25µl) was composed of 5µl of 10X reaction buffer, dNTPs 10mM, $MgCl_2$ 50mM, primer 20pmol, genomic DNA 100ng and 1.5-2 unit of *Taq* polymerase. The cycling conditions were: initial denaturation in 95°C for five to ten minutes, then 94°C for 30 seconds (denaturation), 60°C for 30 seconds (annealing) and 72°C for 30 seconds to 3 minutes (extension) for 35 cycles, followed by 72°C for 3-10 minutes. PCR products then were run on 6% polyacrylamid gel and bands were scored after visualizing fragment with silver staining (Pourkazemi, 1996). The estimate of genealogical relationships between populations was determined using the restricted maximum likelihood method based on the Brownian motion model. It is based on the model of Edwards and Cavalli-Sforza (Edwards & Cavalli-Sforza, 1964; Cavalli-Sforza & Edwards, 1967). This enables us to use the Brownian motion model that calculates genetic distance between two groups that is just consequence of genetic drift and assumes that each locus evolves independently by pure genetic drift (Felsenstein, 1981). Polarity of characters and transformation series were unknown thus reconstructed trees assumed unrooted.

Unrooted tree was obtained by CONTML (Gene Frequencies and Continuous Characters Maximum Likelihood method) program of the PHYLIP 3.6b computer package (Felsenstein, 2004). Alternatively, genetic relationships between populations were analysed by genetic distance methods (Nei, 1972; Cavalli-Sforza & Edwards, 1967), which were obtained by GENDIST (computes genetic distances from gene frequencies) program of the PHYLIP 3.6b computer package (Felsenstein, 2004). Unrooted tree from distance matrixes reconstructed by Neighbor-Joining and UPGMA methods, NEIGHBOR program of the PHYLIP 3.6b computer package (Felsenstein, 2004). This program implements the Neighbor-Joining method of Saitou and Nei (1987) method of clustering.

Bootstrapping test (Manly, 1991) carried out by SEQBOOT program of the PHYLIP 3.6b computer package (Felsenstein, 2004) with 1000 randomized replicate of data set, consensus tree (Margush & McMorris, 1981) obtained by CONSENSE (consensus tree program) program of the PHYLIP 3.6b computer package (Felsenstein, 2004).

Pattern of genetic distance between samples were displayed using multidimensional scaling techniques, a method that ordinate samples instead of clustering into hierarchical groups (Manly, 1986).
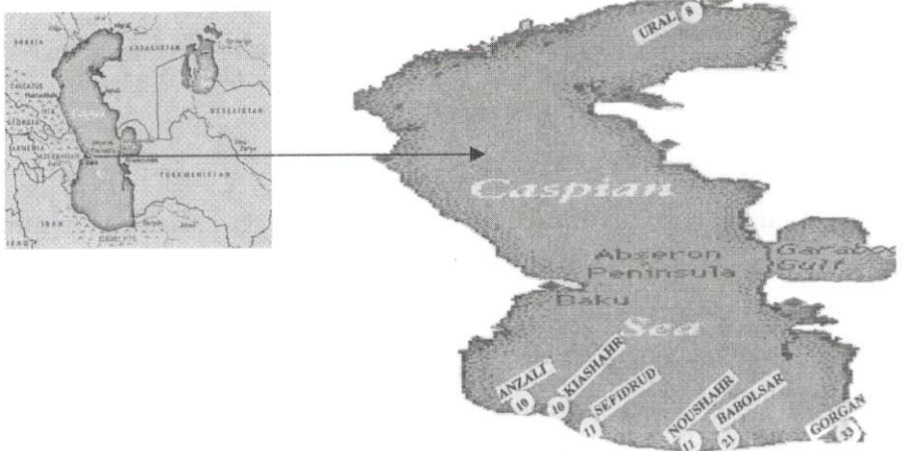

Figure 1: Sampling locations map in the Caspian Sea

Table 1: Characters of used primer (May *et al.*, 1997)

Primer sequences	GenBank accession no.	Repeat motif	Locus	Result
TTATTGGATGGTGTAGCTAAAC AGCCCAACACACGAATATC	U72739	(GATA)13	LS-68	P[*]
GATCAGGAGCGACVAAC CCCTGGATTTGAATTAACAG	U72736	(GAA)29	LS-57	P
GCTTGGTTGCTAGTTTGC GTACAGATGACCAGAGGA	U72730	(TTG)9	LS-19	P& M[**]
CATCTTAG-CCGTCTGTGGTAC CAGGTCCCTAAT/ACA/AT/GGC	U72738	(GACA)7	LS-62	M

[*]P: polymorph   [**]M: monomorph

Results

Four used SSR markers produced three polymorphic and two monomorphic loci (Table 1). SSR marker with GenBank accession no U72739, U72736 and U72730 produced 22, 19 and 17 alleles, respectively. SSR markers with GenBank accession no U72738 and U72736 (in other locus) did not produce polymorph alleles. Allele's frequency at three loci with 22, 19 and 17 polymorphic alleles for seven sampling locations populations and specific alleles for each sampling locations in each locus are indicated in Table 2. Result of distance matrix is shown in Table 3. Reconstruction of phylogenetic tree based on Maximum Likelihood method resulted in one tree (Ln Likelihood = 411.93387) (Fig. 2). In MLtree, Ural samples located in basal node distinct from clade of southern samples that showed monophyleticity of Iranian samples. In Iranian coastline Anzali and Kiashahr samples are located in one clade and another clade consists of Gorgan, Noushahr, Babolsar and Sefidrud samples (bootstrap value = 87).

Consensus neighbour-joining tree using Nei's distance resulted in one clade with high confirmation (BOOTSTRAP SUPPORT=93) consisting of Sefidrud, Babolsar, Noushahr and Gorgan (Fig. 3).

Consensus neighbour-joining tree based on Cavalli-Sforza's distance resulted in three clades in southern groups with moderate confirmation in bootstrapping test (Fig. 4). The first clade consists of Anzali and Kiashahr samples (BOOTSTRAP SUPPORT=65), the second has Babolsar, Sefidrud, Noushahr and Gorgan samples (BOOTSTRAP SUPPORT=71) and the third, one clade with Babolsar and Sefidrud samples (BOOTSTRAP SUPPORT=56).

The multidimensional scaling plot of allele frequency related well to the geography of the rivers. The first two axis (Fig. 5) ordinate specimen in four groups consisting of Ural population, Anzali-Kiashahr, Sefidrud and Noushar-Babolsar and Gorgan samples, respectively.
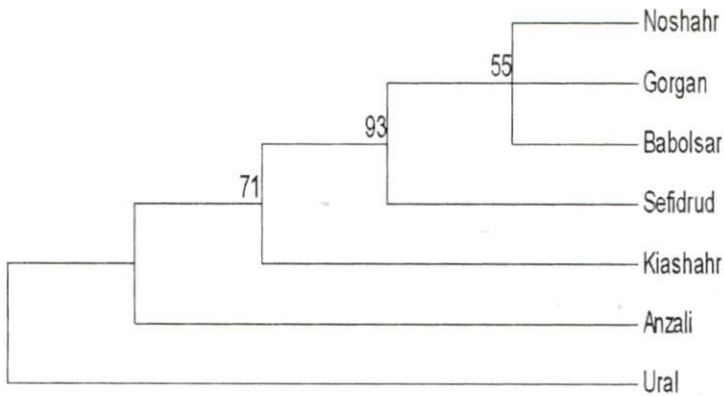

Figure 2: Maximum Likelihood unrooted tree. Numbers are length of each branch

Table 2 : Observed allele frequencies at three loci with 22, 19 and 17 polymorphic alleles for seven populations of ship sturgeon.

Locations	GenBank accession no.	*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Noushahr	UT2739	22	0.05	0	0.1	0.05	0	0	0	0	0.05	0.05	0	0.25	0.05	0.05	0.1	0.05	0.1	0.05	0	0	0.05	0
	UT2736	19	0	0	0.227	0.182	0.045	0	0.137	0	0.091	0	0.054	0.09	0	0.137	0	0	0.045	0	0	0	0	0
	UT2730	17	0	0.067	0	0	0.5	0.1	0.1	0.1	0.15	0.15	0.05	0.1	0.05	0.05	0.05	0.05	0	0	0	0	0.067	0
Anzali	UT2739	22	0	0.067	0	0	0	0	0	0	0.132	0	0.267	0.062	0	0.132	0.267	0.067	0	0	0	0	0.067	0
	UT2736	19	0	0	0.063	0	0.063	0.063	0.063	0.063	0.125	0.125	0.062	0.062	0.062	0.062	0.183	0	0	0	0	0	0	0
	UT2730	17	0	0.1	0.05	0.25	0	0.05	0	0.1	0.15	0.2	0	0.05	0.05	0	0.05	0	0	0	0	0	0	0
Kiashahr	UT2739	22	0	0	0	0	0.059	0	0	0.117	0.117	0	0.295	0	0.059	0.117	0.059	0.059	0.059	0.059	0	0	0	0
	UT2736	19	0	0	0	0	0.185	0.125	0.125	0.125	0.063	0	0.125	0.063	0.063	0.063	0	0	0	0.063	0	0	0	0
	UT2730	17	0	0	0.1	0.025	0.2	0	0	0	0.05	0.15	0.15	0.05	0.05	0.063	0	0	0	0	0	0	0	0
Babolsar	UT2739	22	0	0	0	0.059	0.029	0.059	0.088	0.147	0.059	0.147	0.059	0.119	0.029	0.029	0.029	0.088	0.059	0	0	0	0	0
	UT2736	19	0	0.025	0	0.02	0.15	0.075	0.025	0.125	0.125	0.1	0.025	0.025	0	0.1	0	0.025	0	0	0	0	0	0
	UT2730	17	0.024	0	0.121	0.073	0.097	0.097	0.072	0.25	0.097	0.024	0.024	0.048	0.048	0.024	0	0	0	0	0	0	0	0
Sefidrud	UT2739	22	0	0	0	0	0	0	0	0.056	0.111	0.056	0.384	0.225	0.056	0.056	0.056	0	0	0	0	0	0	0
	UT2736	19	0	0.095	0	0.285	0.095	0	0.048	0.143	0.143	0.095	0	0.048	0	0	0	0.048	0	0	0	0	0	0
	UT2730	17	0	0	0	0	0	0.06	0.2	0.06	0.17	0	0.17	0.22	0.06	0	0.06	0	0	0	0	0	0	0
Gorgan	UT2739	22	0.038	0.038	0.11	0.21	0.038	0.038	0.038	0.034	0.084	0.068	0.102	0.152	0.051	0.152	0.102	0.068	0.068	0.034	0.034	0.034	0	0.017
	UT2736	19	0	0.038	0.097	0.097	0.15	0.12	0.15	0.076	0.11	0.038	0.13	0.056	0.019	0.038	0.019	0	0.019	0	0	0	0	0
	UT2730	17	0	0.038	0.097	0	0	0	0	0.097	0.097	0.038	0.078	0.019	0.019	0	0	0	0	0	0	0	0	0
Ural	UT2739	22	0	0.13	0	0	0	0	0	0.067	0.063	0	0.248	0	0.063	0.063	0.187	0.063	0.187	0.063	0	0	0.063	0
	UT2736	19	0	0.13	0	0	0	0	0	0	0	0	0	0.2	0	0.13	0.266	0	0	0.13	0.067	0	0	0
	UT2730	17	0	0	0	0	0	0.091	0.182	0.091	0	0.272	0.091	0	0	0.182	0	0	0.091	0	0	0	0	0

*: Number of alleles in each locus, Highlighted squares show specific alleles for each location in each locus

Table 3: Distance matrix, upper rectangle is Nei's Distance and lower rectangle is Cavalli-Sforza's.

Noushahr	**0**	**0.822378**	**1.037662**	**0.515078**	**0.571285**	**0.296885**	**0.84088**
Anzali	0.097936	0	0.38047	0.781342	0.722865	0.535389	0.512212
Kiashahr	0.104609	0.066433	0	0.646827	0.669635	0.484657	0.777943
Babolsar	0.068464	0.086647	0.06985	0	0.466469	0.275591	1.141082
Sefidrud	0.07961	0.099959	0.099451	0.055233	0	0.358233	0.88839
Gorgan	0.050032	0.066349	0.061564	0.036909	0.055893	0	0.792141
Ural	0.104117	0.096297	0.106272	0.115878	0.117579	0.095463	0
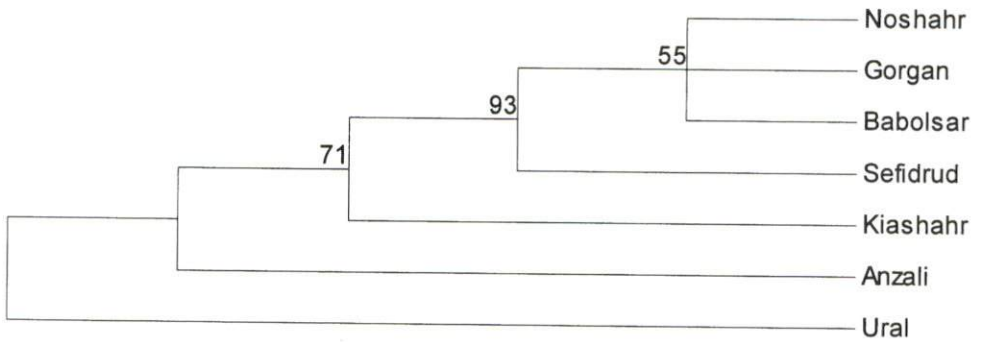
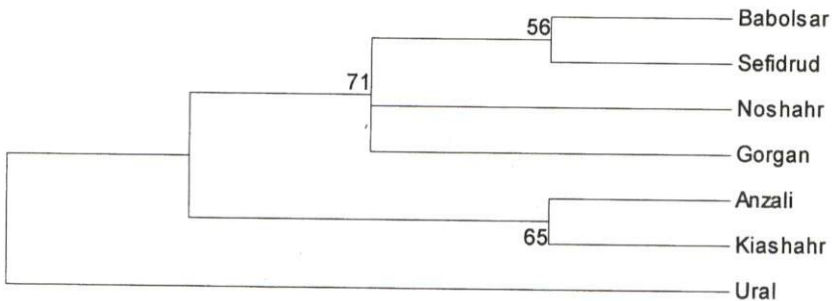Figure 3: Consensus neighbour-joining tree using Nei's distance

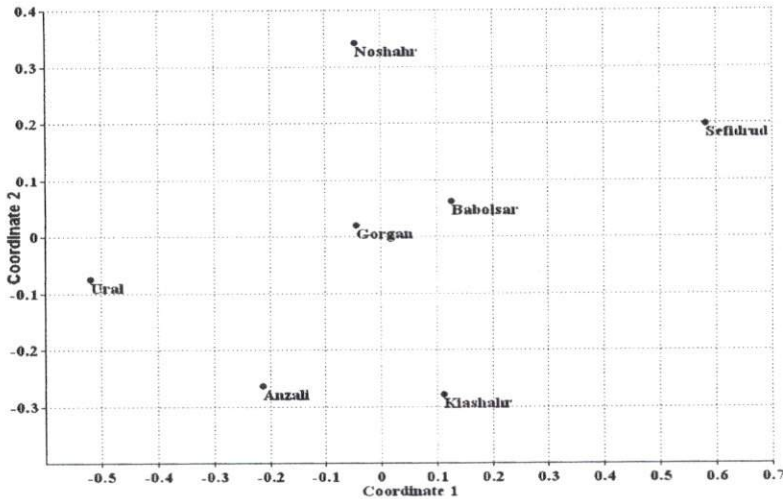Figure 4: Consensus neighbour-joining tree using Cavalli-Sforza and Edward's distance

Figure 5: Multidimensional scaling plots of ship sturgeon based on allele frequencies

Discussion

Maximum Likelihood unrooted tree, consensensus neibor-joining tree using Nei's and Cavalli-Sforza's distance of ship sturgeons from Ural River and South Caspian sea using microsatellite showed monophyleticity of Iranian samples and separation of Ural samples that confirms our previous statistic results by this marker (Safari, 2005). A similar result in relation to the use of microsatellite in population and relationship genetic studies was obtained for *A. stellatus* (Nourozi, 2007) that Nei's distance showed sefidrud samples in one clade; Ural, Kora, Volg's samples in second and Tajan, Gorganrud, Sefidrud and Anzali in third clade. RFLP analysis of ship sturgeons from South Caspian Sea and Ural River also showed significant difference between Iranian coastline and Ural samples. Ural River population, Kora population, Volga population, Sefidrud population were reported in previous studies (Holcik, 1989). This was in contrast to Avetisov (1992) that all populations of ship sturgeons in the Caspian Sea are declining except for Ural population. Pourkazemi *et al.*, (2000) reported phylogenetic relationship of five sturgeon species in the Iranian coastline of the Caspian Sea using mtDNA. They found that direct sequencing of ND5 gene provides more information for phylogenetic or population level studies in sturgeon species. Their study revealed a

distinct classification among five sturgeon species. On the basis of the phylogenetic tree, it is clear that a close evolutionary relationship exist between the great sturgeon and ship sturgeon that was verified in five phylogenetic trees. On the other hand, a close resemblance was also seen between the Persian and Russian sturgeons that indicated these two species have a closer evolutionary relationship.

Study of sturgeon species in Caspian Sea using RAPD by Rezvani Gilkolaei (1997) put Persian sturgeon separate from Great sturgeon, Ship sturgeon and Stellate sturgeon in two different clades. A close relationship was found between Russion sturgeon, Persian sturgeon and Great sturgeon. Another aspect of this study was putting Sefidrud samples in one clade with Babolsar, Gorgan and Noushahr samples (monophyletic) and Kiashahr-Anzali samples clustered in another one. But statistic analyses suggested that Sefidrud samples have significant difference with other locations. No difference was found between Iranian samples using RFLP. Overall results showed that Iranian Coastline samples are clustered in different groups and it shows separations between different locations. This information could be useful in point of future artificial propagations of ship sturgeon in Iran.

Acknowledgements

Reference

Abedian Kenari, A.M., Oveisipor, M.R., Nazari, R.M., 2007. Effects of n3-HUEFA enriched *Daphnia magna* on growth, survival, stress resistance and fatty acid composition of larvae of Persian sturgeon (*Acipenser persicus*). Iranian Journal of Fisheries Sciences, 7(1):1-14.

Avetisov, K.B., 1992. Present status of the ship sturgeon (*Acipenser nudiventris*) in its area. *In*: (ed. V.P. Ivanov), the reproduction of Acipenserids, Salmonids, and some less valuable fishes. VNIRO Publ., Moscow, Russia. pp.3-15.

Cavalli-Sforza, L.L. and Edwards, A.W.F., 1967. Phylogenetic analysis: Models and estimation procedures. American Journal of Human Genetics, 19:233–257.

Edwards, A.W.F. and Cavalli-Sforza, L.L., 1964. Reconstruction of evolutionary trees. In phenetic and phylogenetic classification. (eds. V.H. Heywood and J. McNeill). Systematics Association, London, UK. Vol. 6, pp.67-76.

Felsenstein, J. 1981. Evolutionary trees from gene frequencies and quantitative characters: finding maximum likelihood estimates. Evolution, **35**:1229-1242.

Felsenstein, J., 2004. PHYLIP (Phylogeny Inference Package) version 3.6b. Department of Genome Sciences, University of Washington, Seattle, USA.

Manly, F., 1991. Randomization, Bootstrap and Monte Carlo methods in biology. Chapman and Hall, London, UK. (1st edition 1991, 2nd edition 1997, 3rd edition 2006), 300P.

Margush, T. and McMorris, F.R., 1981. Consensus n-trees. Bulletin of Mathematical Biology, **43**:239-244.

May, B., Krueger, C.C., Kincaid, H.L. 1997. Genetic variation at microsatellite loci in sturgeon: Primer sequence homology in *Acipenser* and *Scaphyrhinchus*. Canadian Journal of Fisheries Aquatic Science, **54**:1542-1547.

Nei, M., 1972. Genetic distance between populations. American Naturalist, **106**:283-292.

Nourozi, M., 2007. Population structure of *A. stellatus* from Caspian Sea using microsatellite. PhD. thesis. Islamic Azad University, Science and Research Branch Tehran. 150P.

Pourang, N., Tanabe, S., Rezvani, S. and Dennis, J.H., 2005. Trace elements accumulation in edible tissue of five sturgeon species from the Caspian Sea. Environmental Monitoring and Assessment, 100(1/3):89-108.

Pourkazemi, M., 1996. Molecular and biochemical genetic analysis of sturgeon stocks from the South Caspian Sea. Ph.D. Thesis. University of Wales, Swansea. 260P.

Pourkazemi, M., Skibinski, D.O.F. and Beardmore, J.A., 2000. A preliminary study on phylogenetic relationship between five sturgeon species in Iranian coastline of the Caspian Sea. Iranian Journal of Fisheries Science, 2(1):1-12.

Qasemi, A., Pourkazemi, M., and Kalbasi, M.R., 2005. Comparision of genetic variation of ship sturgeon (*Acipenser nudiventris*) from south Caspian Sea and Ural River using RFLP. Iranian Scientific Fisheries Journal, 14(4):151-164.

Rezvani Gilkolaei, S., 1997. Molecular population genetic studies of sturgeon species in the South Caspian Sea. Ph.D. Thesis. University of Wales, Swansea. 196P.

Safari, R., 2005. Genetic structure of ship sturgeon from south Caspian Sea and Ural River using microsatellite. M.Sc. thesis. University of Agricultural Sciences and Natural Resources, Gorgan, Iran. 102P.

Saitou, N. and Nei, M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, **4**:406-425.

Sattari, M. and Mokhayer, B., 2005. Occurrence and intensity of and *Huso huso* (Chondrostei: Acipenseridae) from the south-west of the Caspian Sea. Turkish Journal of Veterinary and Animal Sciences, 26(6):1279-1284.