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Study of mtDNA Variation of Russian Sturgeon Population from the South Caspian Sea Using RFLP Analysis of PCR Amplified ND5/6 Gene Regions

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Abstract: PCR-based mtDNA analysis (RFLP) was used for the study of population differentiation in the Russian sturgeon (*Acipenser gueldenstaedti*). The mtDNA ND5/6 gene regions were amplified using PCR techniques followed by RFLP analysis. 39 different composite haplotypes were detected among 62 specimens. 29 haplotypes were rare occuring only once in two regions (west and east areas of the Southern Caspian Sea). The average nucleotide and haplotype diversity within populations were estimated to be 0.028727±0.00 and 0.9645±00042 respectively and divergence between populations to be 0.052%. A highly significant differences were observed in the distribution of haplotypes between the west and east areas.

KEY WORDS: mtDNA, Russian sturgeon, South Caspian Sea, RFLP Analysis, PCR, ND5/6.

Introduction

The higher rate of evolution of mtDNA in comparison with the single-copy nuclear DNA makes it a good candidate for evolutionary studies. In mtDNA, approximately 2% sequence divergence occurs per million years (Upholt and Dawid, 1977; Brown *et al.*, 1979; Wilson *et al.*, 1985). This value is five to ten times faster than the rate for nuclear DNA. There is also variation in the evolutionary rate of mtDNA in different taxa in comparison with most higher vertebrates; for example 1/10 as fast in genus *Alosa*, and 1/3 to 1/2 as fast in some salmonids (Bentzen *et al.*, 1988). Brown *et al.* (1993) proposed 2.7% per million years as rate of sequence divergence in the control region and entire mtDNA in the white sturgeon.

The bluegill fish (*Lepomis macrochirus*) was the first fish species which was studied using mtDNA RFLP analysis (Avise *et al.*, 1984). But later, due to the resolving power of mtDNA RFLP analysis, this technique was extended to the population genetic study of other fish species. From the published data it seems that salmonids are the most well

studies group of fish using mtDNA analysis (Stahl, 1987; Palva *et al.*, 1989; Cronin *et al.*, 1993; Park *et al.*, 1993; O`Connell *et al.*, 1995; Neilsen et al, 1998; Apostolidis *et al.*, 1996). MtDNA analysis was also carried out in other commercially important fish and shelfish species, for instance on American eel, *Anguila rostrata* (Avise *et al.*, 1986), Atlantic and Pacific tuna (Graves *et al.*, 1984), Tilapia, *Oreochromis niloticus* (Rognon and Guyomard, 1997), Red Sea bream (Tabata *et al.*, 1997) cod (Smith *et al.*, 1989). To date, the population structure of four species out of twenty five species of the Acipenseridae family have been investigated as follows: white sturgeon, *Acipenser transmontanus* (Brown *et al.*, 1992), lake sturgeon, *Acipenser fulvescens* (Guenette *et al.*, 1993 ; Ferguson *et al.*, 1993), Atlantic sturgeon, *Acipenser oxyrhynchus* (Bowen & Avise, 1990 ; Miracle & Campton, 1995), and stellate sturgeon, *Acipenser stellatus* (Pourkazemi, 1996). The present study on Russian sturgeon population structure from the Caspian Sea which includes more than 70% of sturgeon production in the world (Ivanov, 2000) is the first stock investigation in this species based on DNA technology.

Both ND and ATPase genes are more variable (Johansen *et al.*, 1990) than other mtDNA genes in cod. For ND, this is probably the result of released constraint because it codes some ND subunits by nuclear genes. Therefore because of the greater possibility of discovering variation, it was decided to survey the ND5/6 gene region of mtDNA of Russian sturgeon. Thus, an RFLP analysis based on PCR amplification of genes for NADH dehydrogenase subunit five (ND5) and NADH dehydrogenase subunit six (ND6) of the mitochondrial genome was used for studying population differentiation of Russian sturgeon, *Acipenser gueldenstaedti,* from the South Caspian Sea. To date, the only published data reported on population discrimination of stellate sturgeon based on the above mentioned genes in the Caspian Sea is that of Pourkazemi (1996). No similar study is available for Russian sturgeon. Most of the sturgeon catch in the Caspian Sea, from 40 to 50%, in the past as well as today, is made up of Russian sturgeon (Kosarev & Yablonskaya, 1994). Thus stock assessment studies in this species are of high priority.

Identification and explanation of genetic variation, within and between Russian sturgeon populations in West and East areas of the South Caspian Sea and also investigation of the possibility of using the ND5/6 gene as a marker for discrimination of populations of Russian sturgeon in the Caspian Sea are the objectives of this study.

Materials and Methods

Sample collection:

Russian sturgeon caught for fishery purposes were used for sample collection. Samples were collected directly from the fish. Skeletal muscle from region 1 fish (the west area sample site and geographic location are given in Fig.1) was stored in liquid nitrogen and fin tissue from region 4 fish (the east area) was stored in 95% ethanol then transferred to the UK. All samples were placed in a deep freeze at -70°C in the laboratory.

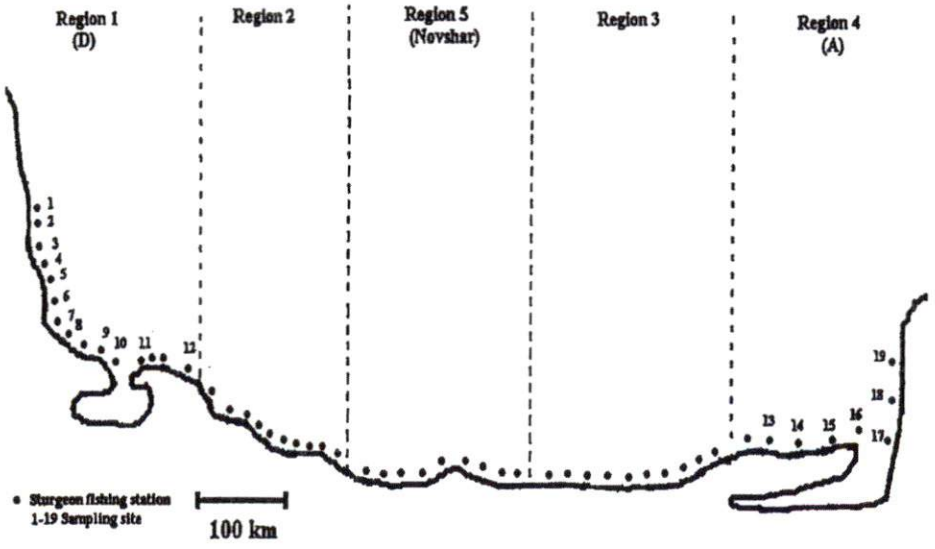

Fig. 1 : The south Caspian Sea sturgeon fishery stations and sampling sites, where A and D are considered as a single population at present study.

DNA extraction and electrophoresis:

DNA was prepared either from fresh muscle tissue (200 mg pieces) (from liquid nitrogen) or preserved fin tissue (50-100 mg) (from alcohol) following the method described by Hillis and Moritz (1990) with some modifications (Rezvani Gilkolaei, 1997).

Approximately 0.5-1 µg of DNA sample and control lambda DNA were run on a 0.8% agarose gel and stained with ethidium bromide (Sigma; 10 mg/ml) to check its quality and approximate quantity. The actual concentration of DNA was measured more accurately in a spectrophotometer (Pharmacia) by taking readings at 260 nm .

Polymerase Chain Reaction (PCR):

The PCR was optimised by changing the reaction, cycle number, annealing temperature and incubation time. The reaction condition found to be optimal is as follows: 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 200 µM each of dATP, dTTP, dGTP, and dCTP (Pharmacia), 20 pmoles of primer, 1.5-2 units of *Taq* DNA polymerase (Promega Biotec), and 50-100 ng of genomic DNA in a final volume of 50 µl. The mixture was overlaid with the same volume of mineral oil. DNA amplification was performed in a thermal cycler (Hybaid, UK). For the first cycle, denaturation, annealing and extension were 94°C for five min, 53°C for 1 min and 72°C for 1.5 min, respectively. Denaturation time was decreased to 30s for the following 28 cycles.

One set of conserved primers from mtDNA including mitochondrial NADH dehydrogenase subunits 5 and 6 (ND5/6) genes were used for PCR amplification. Primers were designed using the comparison of published sequence of cow, *Bos sp.* (Anderson *et al.*, 1982) ; frog, *Xenopus sp.* (Roe *et al.*, 1985) and carp *Cyprinus carpio* (Chang *et al.*, 1994) by Pourkazemi (1996).

Primer (A) from tRNA (Leu):

5'- AATAGTTTATCCAGTTGGTCTTAG-3'

Primer (B) from tRNA (Glu):

5'- TAACAACGATGGTTTTTCATATC A-3'

Digestion of PCR products using restriction enzymes:

The ND5/6 PCR products were digested with 27 restriction endonuclease enzymes, EcoRI, EcorV, HindIII, AvaI, Ava II, PstI, BamHI, XhoI, BstEII, ApaI, ThaI, DdeI,

NcI, MboI, HaeIII, PvuII, CfoI, TagI, MspI, Sau96I, HhaI, BclI, AluI, XbaI, Sau3AI, RsaI and HinfI. The composition of the reaction mix for digestion was as follows:

3	µl	PCR product
14	µl	Distilled water
1	µl	Restriction enzyme
2.	µl	Reaction buffer
20	µl	Total volume

The reaction mix was incubated at the temperature and duration recommended by the manufacturer. The reaction was stopped by adding 3µl of loading buffer (50% v/v glycerol, 0.1 M. EDTA, 1% w/v SDS, 0.1% Bromophenol blue). The digested products (accompanied with standard marker e.g. marker VI (154, 234, 298, 394, 453, 517, 653, 1033, 1230, 1766 and 2176 bp ; Boehringer) and or ΔHindIII fragments (564, 2027, 2322, 4361, 6557, 9416, and 23130 bp ; Promega) in order to measure fragment size were then run on a 6% vertical polyacrylamide gel. The fragments were visualised by silver staining of the polyacrylamide gel.

Data analysis:

Restriction digest profile analysis

The Computer package DFRAG and BIOPROFILE (Vilber-Lourmat Ltd.) were used to analyse the restriction digest profiles. Restriction fragment length sizes (bp) were assigned using the standard marker VI and ΔHindIII fragment ladder and a correlation between distance migrated and band size was calculated for each gel. Different genotypes are described by capital letters. The composite haplotype of an individual is the collection of each of the single enzyme genotypes.

Nucleotide diversity (within and between populations):

Nucleotide diversity (π) is defined as the average number of either nucleotide differences or substitutions per site for a group of DNA sequences (alleles) sampled (Nei and Tajima 1981; Nei 1987). The extent of DNA polymorphism in a population can be measured by nucleotide diversity (π). This can be assigned both within and between populations. When there is polymorphism within populations, the extent of nucleotide divergence between populations for a group of sampled DNA sequences is measured by the average number of net nucleotide substitution per site (dA), where the effect of within-population polymorphism has been subtracted. Both π and dA are

estimated based on the proportion of shared restriction fragments between haplotypes (Nei and Miller, 1990). The nucleotide diversity in a population (π) is calculated by :

$$\pi = 2\sum_{Kj} \frac{d_{ij}}{[n(n-1)]}$$ (Nei and Tajima, 1981)

where: d_{ij} is an estimate of the number of nucleotide substitutions per site between DNA sequences *i* and *j*, and *n* is the number of DNA sequences examined.

Percentage sequence divergence:

The number of nucleotide substitutions between haplotypes (d_{ij}) is based on the total number of restriction sites (m_i and m_{ji}) and the number of shared restriction sites (m_{ij}) between haplotypes *i* and *j*. The d_{ij} was calculated for all enzyme classes, classified according to their nucleotide recognition sequence number (*r*) (Nei, 1987) which in the present study were 4, 5, 5.3 and 6 for different enzymes.

The calculation of shared restriction sites for each enzyme class:

The proportion of shared restriction sites between haplotypes i and j for each enzyme class (S_{ij}) was estimated by:

$$S_{ij} = \frac{2m_{ij}}{(m_i + m_j)}$$

The calculation of $d_{ij(K)}$ for each enzyme class as:

This is computed as :

$$\hat{d}_{ij(k)} = -\left[\log_e S_{ij}\right] / r$$ (Nei and Li, 1979)

where *r* is defined as above and (K) refers to the k^{th} class of restriction enzymes.

The estimation of d_{ij} for two or more enzyme class as:

This is computed as:

$$\hat{d}i_j = \sum_k \overline{m}_k r_k d_{ij(k)} \Big/ \sum_k \overline{m}_k r_k$$

where $\overline{m}_k$ is $\frac{(m_{i(k)} + m_{j(k)})}{2}$ (Nei and Tajima, 1981)

Summation was taken over all different enzyme classes. Standard errors for site data based on the proportion of fragments generated by each enzyme class are computed according to Nei and Tajima (1983); and Nei (1987) eqs. 5.41, 5.44 and 5.51.

Nucleotide divergence:

Nucleotide divergence is the average number of net nucleotide substitution per site between different populations, where the effect of intra-population variation is subtracted. Nucleotide divergence was calculated based on Nei (1987) eq. 10.21 as follows:

$$d_A = d_{xy} - \frac{(d_x + d_y)}{2}$$

where d_X and d_Y are the π values for populations X and *Y*, and d_{xy} is the average number of nucleotide substitution per site between X and *Y*. The d_{xy} value was calculated based on Nei (1987) eq. 10.20.

$$d_{xy} = \sum_{ij} x_i y_j d_{ij}$$

Where d_{ij} is the number of substitutions between the *i*th and *j*th haplotypes and x_i and y_j are the frequencies of the haplotypes in populations X and Y.

Haplotype phylogeny:

In order to determine the phylogenetic relationship between haplotypes, the set of d_{ij} values was bootstrapped 100 times (Seqboot, Phylip 3.2; Felsenstein, 1994). This technique generates new multiple data sets by sampling N characters (restriction sites) randomly with replacement, so that the resulting data set has the same size as the original. These hundred data sets were then used to construct a tree using UPGMA and Neighbour Joining methods (Nei, 1987) to creat a consensus tree. The numbers on the nodes of the tree represent the number of times that the branch to the right of the node was found in the 100 replicates analysed. The Neighbour Joining (NJ) method used here is a phylogenetic estimation procedure proposed by Nei (1987) using a distance matrix of nucleotide divergence values among OUTs (haplotypes). The Unweighted Pair Group Method with Arithmetic averages (UPGMA) (Nei, 1972) used here is another method that estimates the phylogenetic relationship between OTUs based on a distance matrix.

Geographic heterogeneity of haplotypes:

A X^2 test based on Monte-Carlo simulation (Roff and Bentzen, 1989) was used to determine whether there were significant differences in the distribution of mtDNA haplotypes between samples. The technique is recommended for mtDNA analyses where restriction-enzyme surveys often reveal many relatively rare haplotypes. One thousand simulations were performed using the Restriction Enzyme Analysis Package (REAP, version 4.0) (McElory *et al.*, 1992). The estimated probability, p, that sets the significance level is given by $p = \frac{X^2}{N}$, where n is the number of randomisation that generate a X^2 value greater than that observed and where N is the total number of randomised sets.

Results

The Russian sturgeon mtDNA ND5/6 gene region was amplified using the PCR technique. Both digested and undigested PCR products showed that in Russian sturgeon the mtDNA ND5/6 gene region was approximately 2.4 kb in length. Ten of 27 restriction enzymes showed polymorphic patterns. These are BamHI, ThaI, DdeI, NcI, MboI, HaeIII, PvuII, CfoI,, AluI, and HinfI. Using these restriction enzymes, an average of 205 bases were studied. On the assumption that the average mtDNA size in Russian sturgeon is the same as in white sturgeon (16500 bp) the ten enzymes surveyed 1.2% of the genome. Table 1a,b show the restriction band sizes (bp) of Russian sturgeon ND5/6 gene region digested with the 10 restriction enzymes. For example, the NciI restriction enzyme showed three different genotypes, A, B and C. The genotype A has three different bands with a size range from 1200 bp to 100 bp and also it showed a double band with a size of 550 bp. Some of the restriction enzymes showed a monomorphic pattern. For example PstI showed two bands approximately 2100 bp and 300 bp for all individuals. The restriction enzyme MboI revealed three genotypes (A, B and C).

Tabe 1a: Restriction fragment length estimates (bp) of Russian sturgeon ND 5/6 gene region digested with five restriction enzymes. A, B, and C denote different genotypes for each enzyme class. The sign * 2 means the band is probably due to two comigrating fragments.

NciI			DdeI		ThaI		PvuII		BamHI		
A	**B**	**C**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**C**
1200	1250	1150	770	490	1860	1860	1500	1450	1400	1350	1400
550[2]*	600	550[2]*	370	370	540	360	450[2]*	475[2]*	1000	1050	1350
100	550	150	290	290		180					1050
			260	280							1000
			250	260							
			220	250							
			140	220							
			100	140							
				105							

Table 1b: Restriction fragment length estimates (bp) of Russian sturgeon ND 5/6 gene region digested with five restriction enzymes. A, B, and C denote different genotypes for each enzyme class. The sign [3] * means the band is probably due to three comigrating fragments.

HaeIII		MboI			CfoI		AluI		HinfI		
A	**B**	**A**	**B**	**C**	**A**	**B**	**A**	**B**	**A**	**B**	**C**
1150	1050	600[3]*	650[3]*	600	1900	1950	600	600	1050	1000	1050
800	900	400	450	500	500	450	450	465	540	540	540
310	310	200		450			300	300	390	400	390
140	140			400			280	265	340	360	240
				250			250	250	80	100	100
				200			210	210			80
							190	190			
							120	120			

Genotype B occurred together with genotype A in heteroplasmic individuals . Pattern A includes three similar sized band of 600 and 400 and 200 bp bands. Pattern B includes 3 bands of 650 bp and a band of 450 bp. Pattern C includes 6 bands as follows 600, 500, 450, 400, 250 and 200 bp. (Fig. 2).

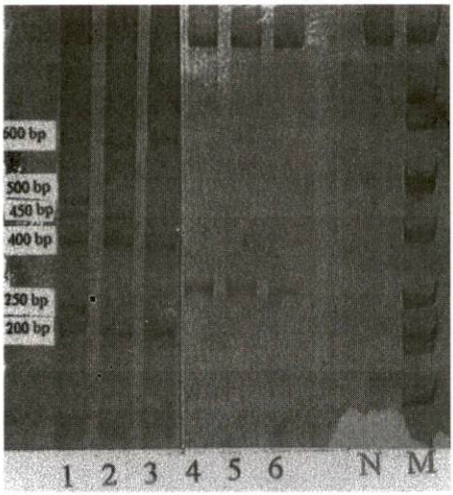

Fig. 2 : Restriction digest patterns observed with MboI (that shows polymophisms and heteroplasmy) and PstI (that shows monomorphism). Lanes 1-3, MboI. Lane 1, genotype C. Lane 2, heteroplasmy for genotypes A and B. Lane 3, genotype A. Lanes 4-6, PstI showing monomorphism. Lane N is PCR product (undigested). Lane M is DNA standard marker (Marker VI).

The frequency of individual enzyme genotypes and their distribution within and between populations are given in Table 2. The composite haplotypes were prepared by combination of the individual enzyme genotypes. The present RFLP analysis showed 39 different haplotypes among 62 specimens of Russian sturgeon from the regions A and D. Of the 39 genotypes, 29 occurred only once (seven from region D and 22 from region A). The frequency of composite haplotypes is given in Table 3. Only three haplotypes BAABBABAAB, BAABAACAAA, and BBAABABAAB are shared between the two populations. Thus the majority of composite haplotypes are specific to either region A or D.

Table 2: The genotype frequencies and sample size (N) within populations

Number of Fishery	Sampling site	N	NciI			DdeI		ThaI		PvuII		BamHI	
Station	**Region D**		A	B	C	A	B	A	B	A	B	A	B
1	Astara	5	1	4	0	3	2	3	2	3	2	3	2
2	Chalevand	4	2	2	0	3	1	3	1	1	3	2	2
3	Lemir	4	3	0	1	0	4	2	2	3	1	3	1
4	Karkan-Rud	6	2	4	0	3	3	4	2	4	2	2	4
5	Shafa-Rud	4	1	3	0	3	1	3	1	4	0	3	1
6	Markazi	7	1	6	0	4	3	6	1	0	7	3	4
7	Golshan	1	0	1	0	1	0	1	0	1	0	0	1
	Sum	31	10	20	1	17	14	22	9	16	15	16	15
	Region A												
8	Goharbaran	10	4	6	0	6	4	6	4	4	6	5	5
9	Amirabad	4	0	3	1	2	2	3	1	1	3	0	4
10	Khajenafas	4	0	4	0	3	1	4	0	0	4	3	1
11	Turkman	13	8	5	0	4	9	4	9	8	5	9	4
	Sum	31	12	18	1	15	16	17	14	13	18	17	16

Number of station	Sampling site	N	HaeIII		MboI			CfoI		AluI		HinfI		
	Region D		A	B	A	B	C	A	B	A	B	A	B	C
1	Astara	5	3	2	1	3	1	4	1	2	3	1	4	0
2	Chalevand	4	3	1	0	3	1	3	1	3	1	1	3	0
3	Lemir	4	2	2	1	3	0	1	3	4	0	1	3	0
4	Karkan-Rud	6	4	2	0	6	0	4	2	5	1	1	5	0
5	Shafa-Rud	4	4	0	1	1	2	1	3	4	0	2	2	0
6	Markazi	7	6	1	1	4	2	6	1	6	1	2	5	0
7	Golshan	1	1	0	0	1	0	0	1	1	0	0	1	0
	Sum	31	10	8	4	21	6	19	12	25	6	8	23	0
	Region A													
8	Goharbaran	10	6	4	4	5	1	6	4	9	1	4	6	0
9	Amirabad	4	3	1	1	3	0	4	0	3	1	1	3	0
10	Khajenafas	4	4	0	1	3	0	4	0	3	1	3	1	0
11	Turkman	13	6	7	8	4	1	11	2	13	0	6	5	2
	Sum	31	17	14	14	15	2	25	6	28	3	14	15	2

Geographic heterogenecity of haplotype frequencies:

A Monte-Carlo X^2 simulation (Roff and Bentzen, 1989) was performed in order to study geographic heterogeneity of composite haplotype frequencies for all samples between populations. To do this, the 20 rare haplotypes from region D and the seven rare haplotypes from region A were pooled. The X^2 test showed highly significant difference in the distribution of haplotypes between regions A and D in the South Caspian Sea (X^2=26.46, P<0.0001). The two regions clearly differ in mtDNA haplotype frequencies.

Haplotype phylogeny:

Using Phylip, two phylogenetic trees were made according to the Neighbour Joining (NJ) (Fig.3) and UPGMA methods (Fig.4). In the Neighbour Joining tree there are three main clusters. Clusters 1 and 2 have approximately 50% haplotypes from each region (region A and D). Cluster 3 includes haplotypes from region D (except haplotypes 22 and 16 that are shared between the two regions). The numbers at the nodes obtained from bootstrapping are less than 50% of bootstrapped trees and thus provide rather poor statistical support for the clusters. However it is significant that cluster 3 has predominantly haplotypes from region D. Three main clusters also appear in the UPGMA tree. Clusters 1 and 2 include haplotypes from both two regions (region A and D). Cluster 3 includes haplotypes from region D (except haplotypes 22 and 16 that are shared between two regions). Thus the structure of the UPGMA tree is similar to Neighbour Joining tree. It can be assumed as a null hypothesis that the populations from which the haplotype derive (A or D) are distributed at random on the terminal nodes of the tree. A Monte-Carlo X^2 test (Roff and Bentzen, 1989) with 10,000 replications can then be carried out to test for significant differences in the ratio of A to D between the three main branches. The p value obtained varies between 0.0327, 0.0018 and 0.0557, 0.0023 suggesting a significant result at the 5% level. An identical result in obtained for the Neighbour Joining tree. This significance is largely due to cluster 3 haplotypes coming from region D. This result thus supports the hypothesis that there is a restriction of on mtDNA gene flow between regions. Divergence between two main branches of the UPGMA tree is 1% suggesting that they diverged about 500,000 years ago. However the error in this divergence time estimate may be considerable.

Nucleotide diversity and divergence within and between populations:

Haplotype and nucleotide diversity is high within each area. Haplotype diversity in region D is 0.9849 ± 0.01336; and in region A is 0.9441 ± 0.02059. Nucleotide diversity in region D is 0.027719 and in region A is 0.029735. The average nucleotide divergence between region A and D is 0.052%.

Table 3: Distribution of haplotype frequencies between region A and B.

No.	Haplotypes	Region D	Region A (east).
1	ABBAABABA	3	0
2	BAABBABAB	1	0
3	BABBAACAA	1	0
4	BAAAABBAB	1	0
5	BBAABABAB	1	0
6	BAAABABAA	1	0
7	BAABBABBA	1	0
8	ABABAACAA	1	0
9	AABBABBAB	1	0
10	ABAABBABA	1	0
11	CBABAABAA	1	0
12	ABBAABBBA	1	0
13	ABABBABAA	1	0
14	AAABBABAA	1	0
15	BABAABBBA	1	0
16	BBAABABAA	1	1
17	BAAABBBBA	1	0
18	BAAABACAA	1	0
19	BAAABABBA	1	0
20	AAAAAACBA	1	0
21	BBBAAAABA	1	0
22	BAABBABAA	3	5
23	BBABBABAB	1	0
24	BAABAACAA	2	3
25	ABBBABABA	1	0
26	BAAABABBA	1	0
27	BAABBABAA	0	3
28	BAABBABAB	0	1
29	BBABBABAB	0	1
30	BAABAACAB	0	1
31	BBABAACAA	0	1
32	BAABBABBA	0	2
33	ABBAABABA	0	4
34	ABBAAAAAA	0	1
35	BABBBABAA	0	1
36	ABBAABAAA	0	2
37	ABBAABAAA	0	2
38	ABBAABAAA	0	2
39	CBBABBAAA	0	1
Total		**31**	**31**

Table 4: A comparison of the annual mean length of Russian sturgeon caught from region 1 and region 4 (Southern Caspian Sea) (Rezvani unpublished)

Age	Region 1 Annual Mean Length(cm)	Region 4 Annual Mean Length(cm)
10	109.00	110.90
11	113.30	115.00
12	117.00	119.10
13	122.30	123.00
14	127.30	130.00
15	134.00	135.70
16	142.30	141.60
17	144.30	147.50
Mean length	125.70	127.40

There were significant differences between annual mean length of Russian sturgeon where n ≥10, caught in region 1 and region 4, pair samples T-test, t-value=3.53, p≤0.01

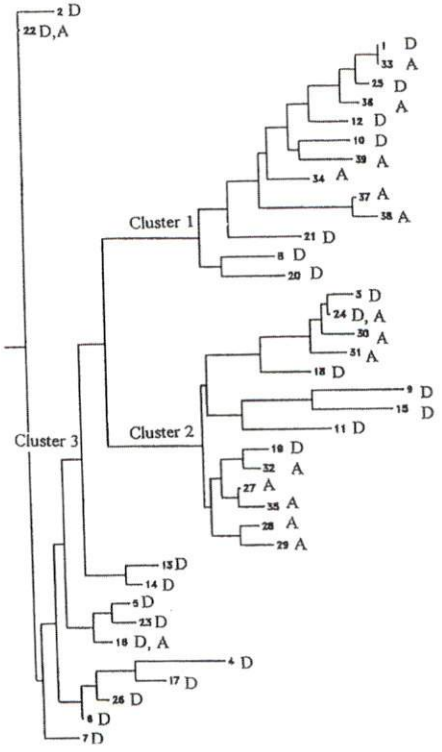

Fig. 3 : Phylogenetic tree constructed using Neighbour Joining method

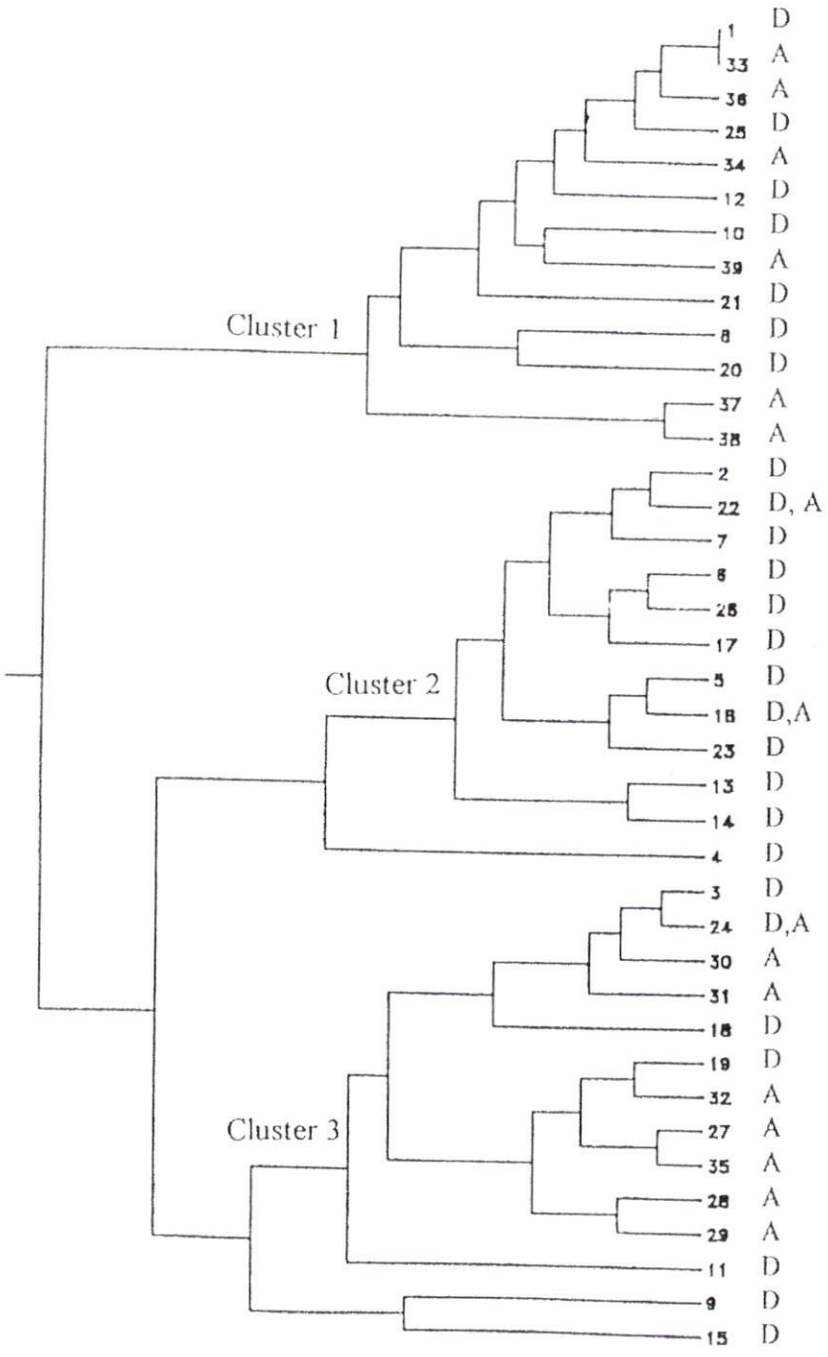

Fig. 4: Phylogenetic tree constructed using UPGMA method

Discussion

The present RFLP analysis on PCR amplification of the mtDNA ND 5/6 gene showed a high level of haplotype and nucleotide diversity within each area. However nucleotide divergence between regions A and D is low. High levels of haplotype diversity have been reported in other sturgeon species. In a recent study, Pourkazemi (1996) reported a similar value for the same gene region for the average haplotype diversity of populations of stellate sturgeon from four geographic regions from the Caspian Sea (D = 0.913). Similarly, Brown (1991) reported a high haplotype diversity in lake sturgeon (0.65) from Lac Saint-Pierre and the Nelson River (Hudson Bay drainage basin). The low sequence divergence between populations reported in this study is also in agreement with earlier studies in sturgeon. For example, Bowen and Avise (1990) reported a low level of sequence divergence between sturgeon populations of the species *A. oxyrhynchus* from the Gulf of Mexico and the Atlantic coasts, with a maximum value of sequence divergence of 0.4%. Much higher values have been reported in other species. The sequence divergence in white sturgeon, *A. transmontanus* based on RFLP analysis of the entire mitochondrial genome was 0.54% (Brown *et al.*, 1992) and 2.27% for the D-loop region (excluding the tandem repeat) (Brown *et al.*, 1993).

Cronin *et al.*, (1993) pointed out that it is important to distinguish between two forms of mtDNA differentiation: nucleotide sequence divergence between haplotypes and the number of different haplotypes and their spatial distribution. The nucleotide sequence divergence can be useful in determining phylogenetic relationship of haplotypes while the number of haplotypes and their spatial distribution may be more useful for assessment of genetic structure and gene flow among populations (Avise *et al.*, 1990). This is because gene flow and colonisation from different source populations may result in the mixing of haplotypes displaying different levels of sequence divergence (Slatkin 1989; Wayne *et al.*, 1990). In the present study sequence divergence is low but haplotype distributions differ between the regions. This suggests a recent gene flow restriction and differentiation by selection or drift.

The rooted trees are consistent with some degree of phylogeographic structure. Thus, all trees show a branch including highly diverged haplotypes from region D and A, which probably represents ancient polymorphism predating the split between area A and D. The maximum divergence of haplotypes on the two main branches is no greater than 1%. Assuming a rate of evolution of 2% per million years (Wilson *et al.*, 1985), puts the divergence of the two main branches at about 0.5 million years ago. However such rate estimates have much error associated with them. The second branch splits

down into two sub-branches. One of them includes haplotypes from area D and the other from areas A and D. This pattern could reflect a preferential migration of fishes from area D to A. Since in region D the sea is deeper and colder than in region A, region A has a more optimal depth for the life style of Russian sturgeon. This is because this species is found in the coastal shallows at depths of 2 to 100-130m, with large number of fish appearing at depths of less than 50m. The warmer temperature in region A would also facilitate a higher density for fish populations in this area. Thus the better conditions in region A (east), would facilitate preferential migration of Russian sturgeon from west to east. Eastern populations in conclusion would represent a mix of native sturgeon and western immigrants. The high value of haplotype diversity and low but significant nucleotide divergence among Russian sturgeon populations may be related to both the life history and contemporary gene flow pattern of this fish. From the point of view of life history, sturgeon from the family Acipenseridae are long lived (>25 years) and represent the largest fish found in freshwater habitats. Russian sturgeon is a large fish, reaching 200 to 210 cm in length with a weight of 60-65 kg. It lives to the age of 40, (Kosarev and Yablonskaya, 1994). From 40% to 50% of the sturgeon catch in the past is made up of the Russian sturgeon, *Acipenser gueldenstaedti*. Thus, one possible explanation for the high sturgeon diversity could be that, due to the large population size and long age of fish, some rare haplotypes that have been observed in this study are derived from an old lineage that is still present within the population .

The difference in haplotype frequencies between region A and D is marked and highly significant. This result contrasts with the study in stellate sturgeon (Pourkazemi, 1996), in which no significant differentiation in haplotype frequencies was observed between populations from the different regions. A possible explanation for this is that since Russian sturgeon is an anadromous species and migrate to spawning sites in different rivers entering the Caspian (Volga, Ural, Terek, Sulak, Kura , Samur, Gorgan and Sefid-Rud), these rivers may represent isolated populations in each geographic location. Since the Russian sturgeon is a migratory fish that travels considerable distance in the sea and there is no geographical barrier between the North, Middle and Southern basin of the Caspian Sea, the different and highly diverged haplotypes observed in this study may came from different reproductively isolated populations from different rivers. If this is so, a further assessment is required by collection of samples from the North and Middle Caspian Sea regions. Russian sturgeon lives in the Caspian Sea (Volga, Kura river), Black Sea (Rioni, Dnieper) and Azov (Don river) all

of them areas in which sturgeons are well differentiated morphologically from each other.

Another possible explanation of the haplotype frequencing differences could be the presence of different ecological forms of the Russian sturgeon in the Caspian Sea. Several sources of evidences support this interpretation. First, immunological analysis of stocks reveal a high degree of antigenic differences between fish from different geographical regions (Umerov and Altuev, 1968; Lukyanenko *et al.*, 1968). Second, several races of summer and winter migratory forms of Russian sturgeon were also reported in the Volga rivers (Barannikova, 1991). Third, a recent biometrics study (Rezvani, unpublished results) on Russian sturgeon in the West and East parts of South Caspian Sea revealed that there were significant differences between annual mean length of Russian sturgeon, caught in region D and region A ($P \leq 0.01$) (Table 4). However these differences might also be the result of the immediate environment. Marti (1940) classified two varieties of Russian sturgeon inhabiting the Black Sea as *Acipenser gueldenstaedti* var. tanaica and *Acipenser gueldenstaedt* var. colchicus. However Berg (1948) considered the latter as subspecies called *Acipenser gueldenstaedti colchicus*. It may be possible that the haplotype differeces between region A and D are caused by the regions differening in the frequences of these two varieties and that the mixing of these two groups of Russian sturgeon in the Southern Caspian Sea is the cause of of the relatively high haplotype diversity in this study.

The results of this study have also relevance for the application of the mtDNA ND 5/6 gene as a genetic marker in stock assessment. For example, a similar approach (PCR amplification of mtDNAND 5/6 gene regions) has been used by Cronin *et al.* (1993) in two salmonid species. They found nine and seven haplotypes in chum and chinook salmon respectively. Sequence divergence between haplotypes was low, ranging from 0.06% to 0.062% in chum salmon, and 0.03% to 0.44% in chinook salmon. They conclude that intraspecific mtDNA sequence divergence was low in chum and Chinook salmon in the areas studies, since common haplotypes were shared between the Yukon river and the pacific Northwest populations. A more recent study on stellate sturgeon from the South Caspian Sea revealed a low level of divergence (0.011%) (Pourkazemi 1996). Thus, although in this study the percentage of divergence in Russian sturgeon is low (0.052%), it is five times higher than the value observed in stellate sturgeon.

In most fish species, distributions with a few common haplotypes and a substantially large number of rare haplotypes that are their mutational derivatives are often found

(Billington and Hebert, 1991). In some earlier studies, sample sizes were not large enough to obtain statistically significant results. However, in the present study an average of thirty one individuals per population was large enough to make statistical analysis and comparison with other mtDNA level studies. Ovenden (1990) stated that haplotypes may also be shared between collection localities because of the use of an insufficient number of informative enzymes to analyse the mtDNA of fish from each locality. However this may not be the case in the present study because no additional polymorphism was found after digestion with seventeen additional enzymes. In conclusion the results of the present study suggest that mtDNA analysis may be very useful in identifying different stocks of Russian sturgeon. Further studies should be made, for example of fish caught regions B and C or caught in rivers lending into the Caspian Sea, before such results are used for the review of or making of new fishing management policies.

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