

# Polymerase Chain Reaction (PCR) and Direct Sequencing of mtDNA from the ND5/6 Gene Region in Persian Sturgeon *Acipenser persicus* from the Southern Caspian Sea

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**Abstract:** A partial sequence of the mtDNA ND5 gene region was used for population study in Persian sturgeon (west and east areas of southern Caspian Sea). The result showed that although this approach was informative for phylogenetic study in sturgeon, it was less informative for population study in Persian sturgeon.

**KEY WORDS:** Polymerase Chain Reaction, mtDNA, sturgeon, Caspian sea

## Introduction

The advent of new approaches in molecular biology such as PCR, gene cloning and sequencing has mostly increased our understanding about the gene and its function in different organisms. Development of PCR technique as well as detection and manipulation of specific DNA fragments have become easier and faster. On the other hand amplification of a specific DNA segment makes it possible to amplify hundreds of samples in a short period of time and to sequence the PCR product directly and easily.

Giuffra *et al.*, (1994) studied the population structure of brown trout *Salmo trutta* from North Italy, using PCR direct sequencing and restriction fragment length polymorphism (RFLP) analysis of D-loop and protein coding regions such as cytochrome b and ATP subunit 6. They found extensive genetic heterogeneity among morphologically identical brown trout populations. Lockwood *et al.* (1993) investigated the phylogenetic relationship among members of the Coregonidae (salmonidae) fishes using direct sequencing of PCR-amplified mitochondrial DNA cytochrome b gene. They found a minimum of 0.0% and a maximum of 5.8% sequence divergence among the taxa with no amino acid replacement. Due to reduced functional constraints, in the mitochondrial DNA D-loop region, some portions of control region evolve much faster than the average

for mtDNA sequence (Brown, 1983). Accordingly, many studies on population and phylogenetic relationship have focused on this region. The size of the mitochondrial DNA control region is not the same in different species. For example in salmonids it is 1128 bp; (Shedlock *et al.*, 1992) and in white sturgeon 976 bp (Buroker *et al.*, 1990). Lee *et al.* (1995) studied 23 species representing six families of teleost fish. They reported that the longest control region was observed in Pleuronectids and the shortest in cichlids and gaoids. However, the control region of sturgeon species is even shorter. Pourkazemi, (1996) reported the sequences of two different length of mitochondrial DNA D-loop region in Russian sturgeon *Acipenser guldenstaedti* (758 and 922 bp) due to tandem repeats in that study. A proportion of the NADH (Nicotinamide Adenine Dinucleotide Hydrogenase) subunit 5 gene was then sequenced in five species of sturgeon from the South Caspian Sea. The results indicated that the variation level of mtDNA, for ND5 gene partial sequences of the five sturgeon species was higher than the variation level in the mtDNA D-loop region. The objectives of our study were as follows:

- 1) A population study in Persian sturgeon based on the comparison of the partial ND5 gene sequences between two regions west (D) and east (A) in the Southern Caspian Sea.
- 2) A study of the usefulness PCR direct sequencing methods of mtDNA for phylogenetic study (between species) and population study (within species) of the Caspian Sea stocks.

## Materials and Methods

### *PCR amplification of ND 5/6 gene regions*

Total genomic DNA of twenty samples of Persian sturgeon from region A (west) and region D (east) was extracted using the phenol-chloroform methods as described by Rezvani Gilkolaei (1997). The mitochondrial DNA ND5/6 gene was amplified using primers A (5'-AATAGTTTTATCCAGTGGTCTTAG-3') and B (5'-TAACAACGATGGTTTTTCATATCA-3') to give a 2400 bp product (Rezvani Gilkolaei, 1997). The PCR conditions and reaction mixture were same as described by Rezvani Gilkolaei (1997). For PCR direct sequencing the ND5/6 primer A was ligated with biotin at the 5' end to make single strand DNA (ssDNA). Another primer (C) was designed with in ND5 gene by alignment of the entire ND5 gene sequence of cow, frog and carp (see Rezvani Gilkolaei, 1997).

The sequence of C is 5'-GATTCCGACGCCTTCTCA-3'. The primer A and primer C amplified a fragment of 475 bp in the mitochondria ND5 gene region.

#### *Analysis of PCR product*

PCR products, due to amplification of Persian sturgeon using the primer A (biotin labelled and forward), primer B (reverse) and primer C (reverse), were run on an 8% polyacrylamide gel with marker fragments to determine the presence and size of amplified products. Polyacrylamide gels were stained with nitrate silvered as described by Rezvani Gilkolaei (1997).

#### *Staining and purification of PCR products*

Amplification using primer A and C showed an additional band of approximately 80 bp. Therefore, in order to supply enough pure PCR products for sequencing, polyacrylamide gels were stained with ethidium bromide (10 mg/ml) for 25 min. The band of interest were cut out under the long wave (365 nm) UV transilluminator and placed in spin-X centrifuge filter tubes (Costat UK Ltd). The samples were kept at -70°C overnight and then spun in Spin-X filter tubes to purify the DNA at 13000 g for 5 min.

#### *Reamplification of target DNA fragment*

Purified DNA (40-60 ng) was reamplified using the same primers A and C using the initial amplification conditions. Reamplified PCR product was cleaned as follows:

- 1) One to ten volumes of STE and an equal volume of 4M ammonium acetate and 2.5 volumes of absolute ethanol was added, in turn, to the PCR product.
- 2) The mixture was immediately spun down at 13000 g at room temperature for 10 min to precipitate the DNA.
- 3) Ethanol was pipetted off and then evaporated in a centrifugal evaporator (Jouan Ltd.). The pellet was resuspended in 50 µl sterile distilled water.

#### *Cycle sequencing optimization*

Three samples of Persian sturgeon were sequenced by the cycle sequencing method using Thermo Sequence Fluorescent Labelled Kit (Amersham). In order to optimise the experiment for the different length PCR products (474 bp and 2400 bp), different concentration of DNA (50 ng, 100 ng, 150 ng, 200 ng and 300 ng), two different primer concentrations (5 and 10 pmol) and several PCR

programs which differed in annealing temperature (48°C, 51°C and 55°C ) were used. The optimised reaction was used in this study was as follows:

DNA template (PCR product with length of 474 bp)	150 ng (1-2 µl)
Primer C (Reverse)	10 pmol (2 µl)
Distilled Water	21-22 µl
	The total volume reaches to 25 µl

Four 1.5 ml PCR ependorfs were labelled A, C, G, and T. Then 2 µl of each thawed A, C, G and T reagents were added into the tubes.

DNA template was mixed briefly by pipeting. Then 6 µl of this template was added into each A, C, G, and T reagent tubes. The PCR tubes were then spun for about 2 min. Then reactions were then incubated as follows:

Denaturing at 95°C, 5 min, Cycle 1; Denaturing at 95°C, 45 sec; Annealing at 51°C, 45 sec; Extension at 72°C, 1 min; Cycle 40; Extension at 72 °C, 5 min. This PCR product was used for sequencing reaction after adding 4µl of stop solution. 6µl of reaction was loaded into the appropriate wells of a sequencing gel.

In addition to this sequencing protocol used for 3 individuals, sequencing reaction was prepared for two individuals using the Solid Phase Autoload™ sequencing kit. This method was based on comb sequencing approach as described in the kit protocol (Pharmacia, UK). The difference between the two method/approaches was not very clear.

#### *Automated electrophoresis and analysis of sequencing reactions*

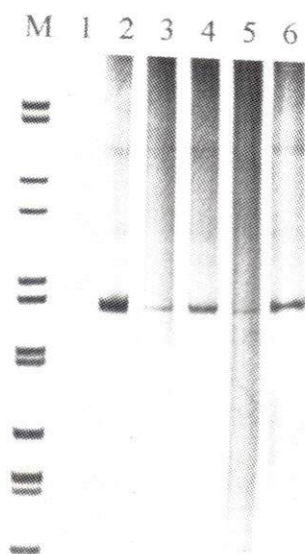
A sequencing experiment was carried out using A.L.F. DNA sequencer (Pharmacia). The visualisation and analysis of fluorescently labelled dideoxy chain-terminated fragments by electrophoresis was carried out in this system using a scanning laser machine. The DNA fragments in each lane migrate down through the gel during electrophoresis. The laser beam excites the fluorescently labelled molecule and the light emitted is detected by photo detectors behind the gel. The four signals for each sample are converted into serial digital data that is sent to the computer and stored in a file. Using the standard dideoxy sequencing methods the fluorescent primer is extended by T7 DNA polymerase in four separate dideoxy reactions (A,C,G and T), creating four separate populations of fluorescent molecules separated in four adjacent lanes on a sequencing gel .

A 6% premixed, ready-to-use polyacrylamide gel (Ready mix gel, A.L.F. grade, Pharmacia) was used and 450  $\mu$ l 10% (w/v) ammonium perulfate was added when the gel mix came to room temperature. The gel was then mixed gently and poured into the gel mould and the comb inserted. The gel was left to polymerise at room temperature for about 60-90 minutes. After polymerisation the samples were applied to the gel attached to A.L.F. sequencer. Upper buffer reservoir was attached to the notched glass plate side of the gel cassette and tightened. The electrophoresis run was continued for 300-400 min. under the control of dedicated software. The sequence data was stored on file for later processing.

## Results

### *PCR amplification analysis*

Using the forward primer A (biotin labelled) and reverse primer B, Persian sturgeon genomic DNA was amplified using the PCR program as described in Rezvani Gilkolaei (1997) and revealed a band of 2400 bp. Also primer A with reverse primer C gave a band of 475 bp and also an extra band of about 80 bp.



**Fig.1:** PCR amplification of mtDNA ND5 region from Persian sturgeon using primers A and C. Lane M, DNA marker (pGEM). Lane 1, negatives control. Lanes 2-6, PCR product (475 bp) from five individuals

The purified 475 bp was reamplified band using primer A and C to give single band (Fig.1). This DNA was used for sequencing.

### *Sequencing analysis*

Partial mtDNA ND5 gene sequences of five individuals of Persian sturgeon three samples from the west (region D) and two samples from the east (region A) were obtained. This sequence from the ND5 corresponds with that analysis in the study of Pourkazemi (1996). A total of between 213 and 280 bp of sequence were determined for the five samples (region D, 254 bp and 254 bp for the auto-load solid phase method; and 213 bp and 233 bp from region A and 280 bp from region D for the cycle method, (Fig.2). The sequences align were satisfactory although there were a number of gaps. These could not be resolved by repeated sequencing experiments. However because this is a coding sequence and gaps would cause a frame-shift the gaps at these positions have been ignored for calculation of distances between the sequences. Only the base substitution is considered.

### *Phylogenetic tree*

A total of 171 bp of the five sequences overlapped completely and were analysed using the DNADIST program which employs the Jukes-Cantor distance correction. This analysis gave a 5×5 matrix of nucleotide distance values (Table 1). Four of the sequences are the same. The fifth shows two substitutions towards the 3' end. Thus the UPGMA tree constructed using these data has a very simple structure (Fig.3).

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D280   TCTGAGTGTATGTATCACGAGGCAAATTCTAAAATTG. ATCAAGTTACGT
A213   ..... TCTAAAATTG. ATCAAGTTACGT
d224   ..... AAATTGGATCAAGTTACGT
D254   .... AGTGTATGTATCACGAGGCAAATTCTAAAATTG. ATCAAGTTACGT
A233   .....

D280   A... GAGGGCTACGGGTGTAAGATAAATTGAGTATTGGTCAAATTTGAAG
A213   A... GAGGGCTACGGGTGTAAGATAAATTGAGTATTGGTCAAATTTGAAG
d254   GTTAGAGGGCTACGGGTG. AAAGATAATTGAGTATTGGTCAAATTTGAAG
D254   A... GAGGGCTACGGGTG. AAAGATAATTGAGTATTGGTCAAATTTGAAG
A233   ..... AATTGAGTATTGGTCAAATTTGAAG

D280   CTGATG. TTAATGTCAAAGG. TGGCAATATTTATTCAATGTCAGTTAGTA
A213   CTGATGGTTAATGTCAAAGGGTGGCAATATTTATTCAATGTCAGTTAGTA
d254   CTGATGGTTAATGTCAAAGGGTGGCAATATTTATTCAATGTCAGTTAGTA
D254   CTGATG. TTAATGTCAAAGG. TGGCAATATTTATTCAATGTCAGTTAGTA
A233   CTGATG. TTAATGTCAAAGG. TGGCAATATTTATTCAATGTCAGTTAGTA

D280   GTGATGACTTCTATGCCTTGGTCAAAAAATACTGCAAGGGGGAGTAGGCT
A213   GTGATGACTTCTATGCCTTGGTCAAAAAATACTGCAAGGGGGAGTAGGCT
d254   GTGATGACTTCTATGCCTTGGTCAAAAAATACTGCAAGGGGGAGTAGGCT
D254   GTGATGACTTCTATGCCTTGGTCAAAAAATACTGCAAGGGGGAGTAGGCT
A233   GTGATGACTTCTATGCCTTGGTCAAAAAATACTGCAAGGGGGAGTAGGCT

D280   AGCATAGAAGGCTGTTTGAAGTCCGGTTTTGACATGGGTGGTTGCCCAT
A213   AGCATAGAAGGCTGTTTGAAGTCCGGTTTTGACATGGGTGGTTCCCCC.
d254   AGCATAGAAGGCTGTTTGAAGTCCGGTTTTGACATGGGTGGTTGCCCAT
D254   AGCATAGAAGGCTGTTTGAAGTCCGGTTTTGACATGGGTGGTTGCCCAT
A233   AGCATAGAAGGCTGTTTGAAGTCCGGTTTTGACATGGGTGGTTGCCCAT

D280   TTTTGTTGAGAGGGCTGGGGTTGAGGG. ATACGATAA
A213   .....
d254   TTTTGTTGAGAGGGCTGGGGTTGAGGGGATACG. T
D254   TTTTGTTGAGAGGGCT
A233   TTTTGTTGAGAGGGCTGGGGTTGAGGG. ATACGATAAGGGGATATGTCAG

D280   .....
A213   .....
d254   .....
D254   .....
A233   AAGAATGAAAATGATTAGG

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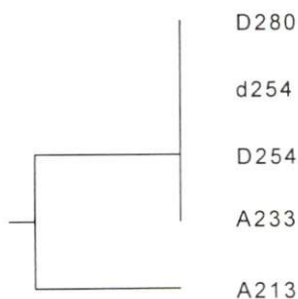
Note: D or d on the left side mean fish is from region D (west area) and A means fish is from region A (east area). The length of sequence is given next to A, d or D

**Fig. 2:** Partial sequence alignment of mitochondrial DNA ND5 gene from five individuals of Persian sturgeon from west and east areas of South Caspian Sea

**Table 1:** Pairwise genetic distance comparison between five individuals Persian sturgeon from the South Caspian Sea calculated from the partial sequence of mitochondrial DNA ND5 gene region.

Individuals	D280	A213	d254	D254	A233
D280	0.0000				
A213	0.0119	0.0000			
d254	0.0000	0.0119	0.0000		
D254	0.0000	0.0119	0.0000	0.0000	
A233	0.0000	0.0118	0.0000	0.0000	0.0000

**Fig. 3.** Phylogenetic tree constructed using UPGMA method



### Database search

The sequence comparison of mtDNA ND5 gene between d254 with homologous sequences from genbank (using FASTA) revealed the sequence identity of the ND5 gene with that of other species. A 72.1% sequence identity was detected with rainbow trout *Oncorhynchus mykiss* 70.2% with *Oncorhynchus gorbusha* and 64.8% with *Ecaballus*. Also it showed a 93.4% identity with published sequence of Persian sturgeon Pourkazemi (1996) (Fig.4). It should be noted for reasons given above that the sequence comparisons were made ignoring gaps.



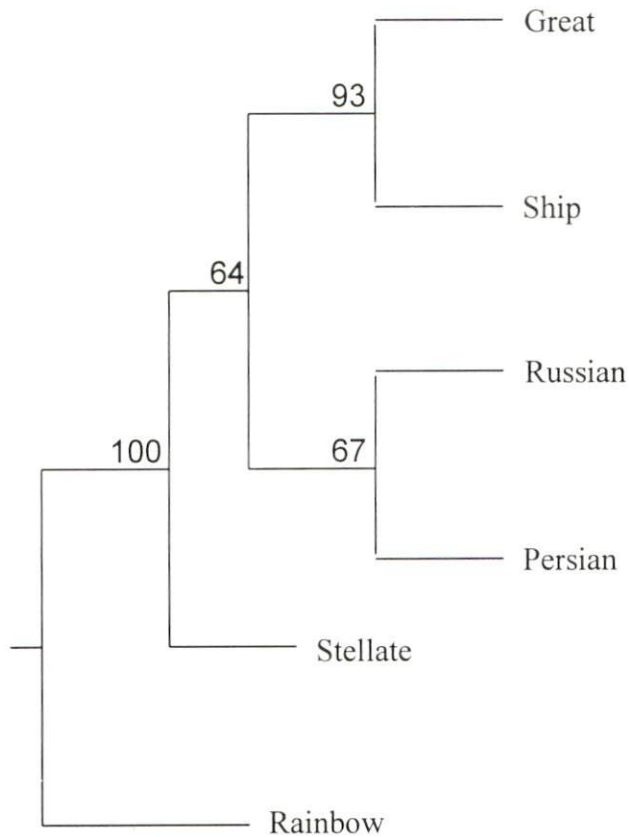
51	gaatcggtttatgttggggtctgagtgatgtatcaagaggcaaatteta	100
1	.....A	1
101	aaattgatcaagttac..gtagaggctacgggtgtaagataattgatt	148
2	AATTGGATCAAGTTACGTGTAGAGGGCTACGGGTG. AAAGATAATTGAGT	50
149	attggtcgaatttgaagctg..atgttaatgtcaaa. ggtggcaatattt	195
51	ATTGGTCAAATTTGAAGCTGGATGGTTAATGTCAAAGGGTGGCAATATT	100
196	attcaatgtcagttagtagtgatgacttctatgccttggccaagaatac	245
101	ATTCAARGTCAGTTAGTAGTGATGACTTCTATGCCTTGGTCAAAAAATAC	150
246	tcgaagg..... 252	
151	TGCAAGGGGAGTAGGCTAGCATAGAAGGCTGTTTGAAGTGGGTTT	197

**Fig. 4:** Comparison between sequence A213 (bottom) and published mtDNA ND5sequenceregion from Persian sturgeon shows 93.4% similarity

## Discussion

Two base substitutions were detected in five Persian sturgeon individuals from the west and east regions of the South Caspian Sea. The sample size is small for a population study and the approach used here might have been more informative in a study with large sample size. Using the partial sequence analysis of the mtDNA ND5 from five sturgeon species of the Caspian Sea for phylogenetic study, by Pourkazemi (1996) showed that these results were more informative (Fig.5 and Table 2). In the present study the genetic distance between the one different sequence and others is only 1 %.

The result of this preliminary study suggests that : i) the partial sequence of ND5 is more informative for phylogenetic studies but less informative for population studies, ii) base substitution of this part of mtDNA between species is higher than within species, iii) although a small sample size from each species might be enough, larger sample size is needed for populations studies. Direct sequencing of PCR products was also had limited success in other population studies.



**Fig. 5:** Phylogenetic tree constructed using UPGMA method. The number at the forks represent the number of the times that nodes occurred among 100 replicates (after Pourkazemi, 1996).

**Table 2:** Pairwise genetic distance comparison between five sturgeon species from the South Caspian Sea and Rainbow trout (as an outgroup) calculated from the partial sequence of mitochondrial DNA ND5 gene region (after Pourkazemi 1996)

Species	Ship	Great	Persian	Russia	Stellate	Rainbow
Ship	*****					
Great	0.0053	*****				
Persian	0.0374	0.0430	*****			
Russian	0.0265	0.0320	0.0212	*****		
Stellate	0.0484	0.0541	0.0541	0.0429	*****	
Rainbow	1.6058	1.6655	1.4970	1.5785	1.6091	*****

For example, sequencing of small regions of the cytochrom b gene and D loop region (which are supposed to be variable) failed to reveal significant levels of variation in salmonids (Hall, 1993). Such results have been obtained for marine fish populations (Carr and Marshall, 1991a,b). However, Bartlett and Davidson (1991) were successful in finding high levels of variation within four species of tuna. Two problems of low variation with sequencing has led other workers to concentrated a large sequences of mtDNA and digestion with restriction enzymes rather than sequencing. Hall (1993) and O'Connell (1993) amplified a large region (72kb) of ND genes using this approach for trout and salmon respectively. Variation was revealed in the trout study, however all of 69 animals analysed in the salmon study from four different rivers had the same RECP patterns. Thus, even large DNA segments can be studied using direct sequencing, with a set of restriction enzymes is not guarantee that variation will be discovered in population studies. Nevertheless the present study and that of Pourkazemi (1996) does show the potential of sequencing mtDNA gene regions in future studies of phylogeny of sturgeons.

In conclusion our result indicated that:

- 1- Conserved partial sequence from ND5/6 (mt DNA) of cow, frog and carp are available to amplify DNA from different species of sturgeon.
- 2- There is a high differentiation between five sturgeon species in ND5 region sequence of mtDNA molecule but there is no significant differentiation between five fish species from two regions.
- 3- ND5 sequence is more informative for phylogenetic study in sturgeon but is less informative in population study.

## Acknowledgments

The present study has been financed by the Iranian Ministry of Jihad-e-Sazandegi through Iranian Fisheries Co., that I am greatly indebted to.

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