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A Preliminary Study on Phylogenetic Relationship between Five Sturgeon Species in the Iranian Coastline of the Caspian Sea.

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Abstract: The phylogenetic relationship of five sturgeon species in the South Caspian Sea was investigated using mtDNA molecule. Sequence analysis of mtDNA D-loop region of five sturgeon species [Great sturgeon (*Huso huso*), Russian sturgeon (*Acipenser gueldenstaedtii*), Persian sturgeon (*Acipenser persicus*), Ship sturgeon (*Acipenser nudiventris*), Stellate sturgeon (*Acipenser stellatus*)] and DNA sequencing of two *Escherichia coli* cloned DNA fragments sized 758 bp and 922 bp was carried out. Sequence comparison of the mtDNA D-loop region between the five sturgeon species showed 100 % sequence identity. However comparison between a short (758 bp) and long (922bp) mtDNA D-loop region within Russian sturgeon showed a 5 bp differences. On the contrary a partial sequence of the mtDNA ND5 gene region showed high sequence and organic base variability in the five species. These results indicate that direct sequencing of ND5 gene provide more information for phylogenetic or population level studies in sturgeon species. The present study revealed a distinct classification among the five sturgeon species. On the basis of the phylogeny tree it is clear that a close evolutionary relationship exists 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 Persian and Russian sturgeon indicates that these two species have a closer evolutionary relationship. This study also showed a 2.2 % sequence divergence in sequence data for ND5 gene between Persian sturgeon and Russian sturgeon which indicated that these two species diverged from each other approximately one million years ago. However the verification of this calls for DNA sequencing of different genes.

KEY WORDS: Sturgeon. Caspian Sea, Phylogenetic, Iranian Coastline, PCR.

Introduction

Since the development of the PCR technique, the detection and manipulation of specific DNA fragments has become easier and faster than conventional cloning

techniques. The amplification of a specific DNA segment makes it possible to amplify hundreds of samples in a short period and to sequence directly the PCR product easily. There are many methods to analyse PCR-amplified DNA, including restriction endonuclease mapping, hybridisation, Restriction Fragment Length Polymorphism (RFLP), sequencing and so on. Among these, DNA sequencing provides more and comprehensive information. RFLP analyses are based on the presence or absence of a restriction site, reveal little about the kinds of nucleotide substitutions. In the past, most of the work on population structure and phylogenetic relationship of many fish species was based on RFLP analyses of mtDNA data (reviewed in Meyer, 1993). However, direct sequencing of PCR amplified fragments might substitute RFLP studies. This is primarily because of the greater sensitivity of sequencing in detecting polymorphism and also in providing information about patterns of nucleotide substitution. Many different strategies have been envolved for sequencing of PCR products. Each of them has specific requirements and limitations (see Mitchell *et al.*, 1994, table 1). However, the direct sequence of the PCR product is preferred over sequencing of cloned amplification products (it is cheaper and faster).

Several PCR-based amplifications of genomic or mitochondrial DNA followed either by cloning or direct sequencing have been done in recent years. Palsson and Arnason (1994) amplified the cytochrome b genes in three salmonid species including Arctic charr (*Salvelinus alpinus*), Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) from Iceland. They found four haplotypes within Atlantic salmon, however no variation was reported within Arctic charr or brown trout. Lockwood *et al.*, (1993) investigated the phylogenetic relationship among members of the Coregoninae (salmonidae) fish 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 substitution. In another study Shedlock *et al.*, (1992) analysed the evolutionary relationship of eight species of the Pacific salmon (genus *Oncorhynchus*) and Atlantic salmon, the mountain whitefish and the Atlantic grayling using PCR-direct sequencing of the mitochondrial DNA D-loop region. They found that the D-loop of these eleven taxa show typical vertebrate D-loop organisation. The UPGMA clustering analysis agreed with previous parsimony analysis that had been done for six species of Pacific salmon based on mtDNA coding region sequences. However it contrasted with previous classical analyses by placing the rainbow cut-throat species pair within the core of the

genus *Oncorhyncus* and showed pink salmon as the sister taxon of the remaining species examined within this genus.

Lee *et al.* (1995) amplified and sequenced the mitochondrial DNA D-loop region from 23 species representing six families of teleost fishes. They found that the length of this segment is highly variable due to the presence of tandemly repeated sequences and insertions of large unique sequences. They reported that the longest control region was observed in pleuronectids and shortest in cichilds and gadoids. However, the control region of sturgeon species is even shorter than these fish.

In this study the mitochondrial DNA D-loop region of five sturgeon species from the South Caspian Sea was amplified using the PCR technique. Two different lengths of PCR product of Russian sturgeon were cloned. This was followed by sequencing of entire fragments (758, 922 bp). The PCR product of four other sturgeon species (Ship, Persian, Great and Stellate) was directly sequenced. The mitochondrial DNA ND 5/6 gene region was also amplified and a proportion of the NADH subunit 5 gene was sequenced. The sequence of these two parts of the mtDNA molecule (D-loop and ND5 regions) were compared in five sturgeon species from the South Caspian Sea. The objectives of the present study were to compare the phylogenetic relationship between sturgeon species from the South Caspian Sea using the D-loop region and partial ND5 gene sequences.

Materials and Methods

Total genomic DNA of four samples from each of Stellate, Russian, Persian, Ship and Great sturgeon species was extracted using the phenol-chloroform method as described in Pourkazemi (1996). The mitochondrial DNA D-loop region was amplified using L-tRNA ^{pro} and H-740 primers (Pourkazemi, 1996) and the mitochondrial DNA ND5 gene was amplified using primers A and B. The PCR conditions and reaction mixture were exactly the same as described in Pourkazemi (1996). For PCR-direct sequencing of the D-loop and ND 5/6 gene the primer H-740 and primer A were ligated with biotin at the 5' end to make ssDNA. A primer (primer C) was designed within ND5 gene by alignment of the entire ND5 gene sequence of Cow, Frog and Carp using the Seqnet, Genebank (GCG program) (see also Pourkazemi 1996). The primer sequence was 5'-GATTCCGACGCCTT CTCA-3'. The primer C and primer A amplified a PCR fragment of 475 bp in the mitochondrial ND5 gene region.

PCR products of four samples of Russian sturgeon which carried different types of mtDNA size classes were selected and 50 µl of PCR product was washed with QIAquick PCR purification kit(QIAGEN) to remove the unincoporated nucleotide, primers and polymerase. The PCR fragment was then cloned using the protocol described in Pourkazemi (1996).

A white colony that did not show any sign of blue colour was picked off from the plate with a sterile glass Pasteur pipette. The colony was blown out into 20 µl of H_2O in a 0.5ml tube. About 70µl of mineral oil was then overlaid on this mixture and heated to 95°C for 5 mins. The mixture was spun for 5 mins and 10 µl of the mixture under the mineral oil layer was used as a template in the PCR reaction using T3 and T7 primers which flank the region of insertion side in the Bluescript plasmid or using the insert PCR amplification primers (primer L and primer H-740).

In the present study, sequencing was performed both on the PCR-cloned fragment and the PCR amplified fragment for the mitochondrial DNA D-loop and ND5 gene regions of five sturgeon species from the South Caspian Sea. Two different lengths of PCR-cloned fragments of Russian sturgeon were sequenced using the dideoxynucleotide chain-termination methods (Sanger *et al.*, 1977) and T7 polymerase (United State Biochemical). The double-stranded sequencing method was performed using T7 gene 6 exonuclease with plasmid T3 and T7 primers and single stranded method using the biotinylated T7 primer. The three following internal primers were used for sequencing of the mtDNA D-loop region: L2265'-CATCTACCATTAGATGATATACA-3',L384-5'TTACTGGCAT CTGGTTCCTATTT-3', L551-5'TGCAGGGCCTTCAGAAAA-3'. Primer C (see above) was also used as internal primer for sequencing of the ND5 gene. The methods of PCR-cloned fragment sequencing and PCR-direct sequencing were presented separately in Pourkazemi (1996).

Sequence analyses were performed using Genetic Computer Group's (GCG) programs Seqnet. The mtDNA D-loop and ND5 gene region sequences of sturgeon from the South Caspian Sea were compared with published sequences in Genbank using the FASTA program. The multiple sequence alignment were done using the PILEUP and BESTFIT [gap (3.0) and gap length penalties (0.1)] programs. PHYLIP (Phylogeny Inference package, version 3.2) (Felsenstein, 1994) was used for phylogenetic analysis using different programs including: SEQBOOT, NEIGHBOR-JOINING(NJ),UPGMA, Genetic distance (GENEDIS), DNAPARSs, DNACOMP, DNAPENNY and DNA maximum likelihood (DNAML).

Results

PCR amplification of the mtDNA D-loop region revealed length heteroplasmy in all five sturgeon species from the South Caspian Sea. Two different lengths of PCR fragments (758 and 840 bp) were visualised after silver staining in polyacrylamide gel. Both methods of PCR amplification, white colonies and digestion of extracted plasmid, confirmed the insertion of PCR fragments in the plasmid. Excising the PCR band from heteroplasmic individuals is revealed as an effective method for PCR-direct sequencing of heteroplasmic individuals.

The comparison of the mtDNA D-loop sequence between five sturgeon species from the South Caspian Sea did not reveal any nucleotide differences between species. This result indicated that the mitochondrial DNA D-loop sequence is not informative for phylogenetic study in the southern Caspian Sea sturgeon species. Partial mtDNA ND5 gene sequences of five sturgeon species from the South Caspian Sea were determined using the PCR-direct sequencing method. A total of 252 bp sequences were determined for the three species Ship, Great and Persian sturgeon. However for the Russian and Stellate sturgeon the sequence was determined only for 142 bp. This is because of the presence of an extra faint fragment in the PCR products of Russian and stellate sturgeon PCR amplifications that interfered with interpretation of the sequencing gel. The sequence comparison of the mtDNA ND5 gene with the analogous sequences of other animals from GenBank (using FASTA) revealed the sequence identity of the ND5 gene in the different species. A 77.8 % sequence identity was detected with Rainbow trout (*Oncorhynchus mykiss*), 75.0% with Common carp (*Cyprinus carpio*), 70.9% with frog (*Xenopus leavis*) and 70.0% with Rat (*Rattus norvegicus*). Surprisingly, the level of variation of mtDNA, for the ND5 gene partial sequences of the five sturgeon species was higher than the level of variation in the mtDNA D-loop region. The alignment of the mtDNA ND5 gene sequence of five sturgeon species from the South Caspian Sea and the homologous sequence of the Rainbow trout (as an outgroup) is shown in fig. 1.

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          1                                                        50
I     GCTGTGACTA GGGTAATTAT AGCGATCAGG AATAGGAGTA GATATTTGAA
II    GCTGTGACTA GGGTAATTAT AGCGATCAGG AATAGGAGTA AATATTTGAA
III   GCTGTGACTA GGGTAATTAT GGCAATCAAG AATAGGAGCA GATATTTGAA
IV    .......... .......... .......... .......... ..........
V     .......... .......... .......... .......... ..........
VI    .........C CTTTTTGTCT TCCTAGACCA AGGAACCGAA ACTATCGTGA

          51                                                      100
I     GAATCGGTTT ATGTTGGGGT CTGAGTGTAT GTATCATGAG GC.AAATTCT
II    GAATCGGTTT ATGTTGGGGT CTGAGTGTAT GTATCATGAG GC.AAATTCT
III   GAATCGGTTT ATGTTGGGGT CTGAGTGTAT GTATCACGAG GC.AAATTCT
IV    .......... .......GGT CTGAGTGTAT GTATCACGAG GC.AAATTCT
V     .......... .......GGT CTGAGTGTAT GTACCATGAG GC.AAATTCT
VI    CTAACTGACA ATGAATAAAC ACCACAACCT TTGACATTAA CCTTAGCTTT

          101                                                     150
I     AAGATTGATC A......AGT TACGTAGAGG GCCACGGGTG TAAAGATAA.
II    AAGATTGATC A......AGT TACGTAGAGG GCCACGGGTG TAAAGATAA.
III   AAAATTGATC A......AGT TACGTAGAGG GCTACGGGTG TAAAGATAA.
IV    AAAATTGATC A......AGT TACGTAGAGG GCTACGGGTG TAAAGATAA.
V     AAAATTGACC A......AGT TACGTAGAGG GCTACGGGTG TAAAGATAA.
VI    AAATTTGACC ACTACTCCAT TATTTTTACC CCTATTGCCC TGTACGTAAC

          151                                                     200
I     TTGAGTATTG GTCAAATTTG AAGCTGATGT TGATGTCAAA GGTGGCAATA
II    TTGAGTATTG GTCAAATTTG AAGCTGATGT TGATGTCAAA GGTGGCAATA
III   TTGATTATTG GTCGAATTTG AAGCTGATGT TAATGTCAAA GGTGGCAATA
IV    TTGAGTATTG GTCAAATTTG AAGCTGATGT TAATGTCAAA GGTGGCAATA
V     TTGAGTATTG GTCAAATTTG AAGCTGATGT TAATGTCAAA GGTGGCAATG
VI    CTGATCTATT CTAGAATTCG CATCCTGATA TATACACGCC GACCCCAACA

          201                                                     250
I     TTTATTCAAT GTCAGTTAGT AGTAATGACT TCTATGCCTT GGTCAAAGAA
II    TTTATTCAAT GTCAGTTAGT AGTAATGACT TCTAGGCCTT GGTCAAAGAA
III   TTTATTCAAT GTCAGTTAGT AGTGATGACT TCTATGCCTT GGTCAAAGAA
IV    TTTATTCAAT GTCAGTTAGT AGTAATGACC TCTATGCCTT GGTCAAAGAA
V     TTCATTCAAT GTCAGTTAGT TGTAATGACT TCCATGCCTT GGTCAAAGAA
VI    TAAACCGATT .....CTTTA AGTACCTCCT CCTCTTCCTA ATCGCCATAA

          251
I     TACTCGAAGG
II    TACTCGAAGG
III   TACTCGAAGG
IV    TACTCGAAGG
V     TACTCGAAGG
VI    TTATTTTAGT
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Fig. 1 : Partial sequence alignment of mitochondrial DNA ND5 gene region of five sturgeon species from the South Caspian Sea (where I= Ship, II=Great, III= Persian, IV= Russian V= Stellate sturgeon) and Rainbow trout (VI). Variable nucleotides in sturgeon are marked in bold.

Twenty nucleotide positions in this region vary among five different sturgeon species from the South Caspian Sea. There are eight base pair additions and five base pair deletions in the sequence of Rainbow trout in comparison with the homologous sequence of southern Caspian Sea sturgeon. Heteroplasmy was also found in the sequence of some sturgeon species (ship sturgeon).

In this study two series of phylogenetic analyses were performed using partial sequence of the ND5 gene region. First, construction of different phylogenetic trees using the mtDNA ND5 gene sequence of the Rainbow trout (*Oncorhynchus mykiss*) as an outgroup and second without the outgroup. Genetic distance between sturgeon species in both series of analyses (with and without outgroup) using DNADIST program revealed similar results (table 1). A minimum of 0.53% genetic distance was found between the Ship and Great sturgeon. The maximum genetic distance was observed in pairwise sequence comparison of stellate sturgeon with two other (Great and Persian) sturgeon species.

Table 1 : 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.

Species	Ship	Great	Persian	Russian	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	******

In the present study all phylogenetic trees were constructed using the sequence data that were bootstrapped 100 times. The bootstrapped genetic distance matrices were used for the construction of UPGMA and NEIGHBOR-JOINING (NJ) trees. However the input data for other programmes in the PHYLIP package was the actual sequence data. The majority of phylogenetic trees which were constructed using Rainbow trout sequence data (as an outgroup) showed clustering of the Ship and Great sturgeons as one group. This assemblage was significant almost in all

using Rainbow trout sequence data (as an outgroup) showed clustering of the Ship and Great sturgeons as one group. This assemblage was significant almost in all trees with the minimum bootstrap number of 84 out of 100 (NJ method) to a maximum 99 out of 100 resamplings in the Maximum Likelihood method (ML). However, when the clustering analysis was carried out without including the Rainbow trout sequence data as an outgroup, the majority of phylogenetic trees, besides clustering the Great and Ship sturgeon in one assemblage also clustered the Persian and Russian sturgeon as a group (figures 2). The significance level of clustering of the Russian and Persian sturgeon assemblage was not as strong as the Great and Ship sturgeons clustering. In a minimum of 67 and maximum 77 times out of 100 this clustering was observed in the UPGMA and in the DNAPARS-DNACOMP programme. This result indicated that including the outgroup sequence in the phylogenetic analyses may alter the actual assemblage between different species but not the significant level.

Discussion

The partial sequences of mtDNA ND5 gene in all five sturgeon species exhibit a higher level of variability than the D-loop region sequences (polymorphism at 20 nucleotide positions out of 252 bp). Such a level of variability, in comparison with the D-loop region sequences, may suggest that the mtDNA D-loop region is not an informative region for evolutionary studies of closely related sturgeon species. A similar result in relation to the use of the mitochondrial DNA D-loop region in population and phylogenetic studies was obtained for salmonid species (Park *et al.*, 1993 ; Cronin *et al.*, 1993 and Hall, 1993).

Another aspect of this study is the evolution of the NADH-dehydrogenase sub-unit 5 molecule itself. Unfortunately, only a few phylogenetic studies have been conducted on the Caspian Sea sturgeons by means of molecular or morphological data, (RezvaniGilkolaei, 1997).

Sequence alignment of five closely related sturgeon species shows reasonable clustering between sturgeon from the South Caspian Sea. An analysis of tree topologies (using the sequence data of Rainbow trout as an outgroup), revealed that the Great and Ship sturgeon are more closely related species (average 95 times out of 100 bootstrapping over six different phylogenetic trees). On the other hand, Persian and Russian sturgeons exhibited another clustered assemblage almost in the majority of phylogenetic tree (5 out of 6 phylogenetic trees).

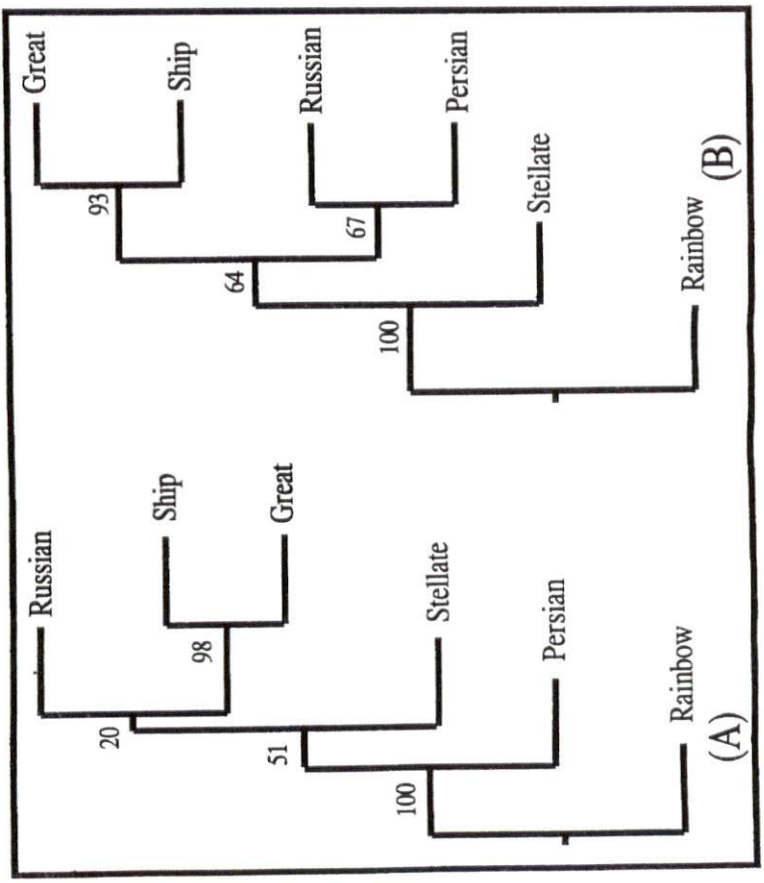

Fig. 2 : Phylogentic trees constructed using DNACOMP (A) and UPGMA (B) methods. The number at the forks represents the number of times that nodes occurred among the 100 replicates (mtDNA ND5 gene).

Such phylogenetic relationships may agree with the morphologically based classification. The phylogenetic analyses of mtDNA ND5 gene also revealed another finding in clustering of Persian and Russian sturgeon as closely related species. This finding can be informative, because up to 1974, sturgeon taxonomists believed that Persian sturgeon (*Acipenser persicus)* is a subspecies of Russian sturgeon (*Acipenser güldenstaedti*) known as (*Acipenser güldenstaedti persicus)* (Holcik, 1989). However Luk'yanenko *et al.* (1974) using an immunological approach found that the Persian sturgeon is a separate species and named it as *Acipenser persicus* Borodin.

In this study the sequence data for ND5 gene revealed a 2.2% sequence divergence between Russian and Persian sturgeons which indicates that these two species diverged from each other approximately one million years ago. However, direct estimates of genetic distance must be used with caution owing to the biased rate of substitution in the ND5 gene and the relatively short length of DNA analysed (Kocher *et al.*, 1989).

In summary, PCR-based cloned fragments and direct sequencing of mtDNA D-loop region revealed no variation in this region among five sturgeon species from the South Caspian Sea. However the mtDNA ND5 gene sequence exhibited some variation which was informative in the phylogentic relationship analyses.

This study suggested that direct sequencing of the entire mtDNA ND5 gene may show significant differences in closely related sturgeon species and may also be useful for population differentiation analysis. The mtDNA D-loop sequence revealed that all five sturgeon species exhibit length heteroplasmy in association with the presence of an 82 bp tandem repeat. This finding in connection with significant level of heteroplasmy suggested that the study of mtDNA inheritance in sturgeon species might be useful.

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