Site heteroplasmy in the mitochondrial cytochrome b gene of the sterlet sturgeon *Acipenser ruthenus*

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Abstract

Sturgeons are fish species with a complex biology. They are also characterized by complex aspects including polyploidization and easiness of hybridization. As with most of the Ponto-Caspian sturgeons, the populations of *Acipenser ruthenus* from the Danube have declined drastically during the last decades. This is the first report on mitochondrial point heteroplasmy in the cytochrome b gene of this species. The 1141 bp sequence of the cytb gene in wild sterlet sturgeon individuals from the Lower Danube was determined, and site heteroplasmy evidenced in three of the 30 specimens collected. Two nucleotide sequences were identified in these heteroplasmic individuals. The majority of the heteroplasmatic sites are synonymous and do not modify the sequence of amino acids in cytochrome B protein. To date, several cases of point heteroplasmy have been reported in animals, mostly due to paternal leakage of mtDNA. The presence of specific point heteroplasmic sites might be interesting for a possible correlation with genetically distinct groups in the Danube River.

Key words: sterlet sturgeon, mtDNA, site heteroplasmy.

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(Broughton and Dowling, 1994), *Gadus morhua* (Arnason and Rand, 1992) *Oncorhynchus tschawytscha*, *Oncorhynchus keta* (Cronin et al., 1993, Shigenbou et al., 2005), *Perca fluviatilis*, *Acerina cernua*, *Stizostedion lucioperca* (Nesbø et al., 1998) and *Paralichthys olivaceus* (Shigenbou et al., 2005). Although length heteroplasmy of the control region in sturgeons has been identified (Brown et al., 1996; Zhang et al., 1999; Ludwig et al., 2000), to date, there are no reports on site heteroplasmy.

In this survey, the intention was to describe *A. ruthenus* heteroplasmy and present hypotheses on its origin and evolution.

Small fin fragments from 30 wild individuals of *A. ruthenus* captured in the Lower Danube (Romania). Total DNA was extracted according to the method described by Taggart et al., (1992) with minor modifications.

Amplification was carried out in a 25 µL final volume consisting of 40-50 ng DNA, 10 pmols of each primer, 100 µM of each dNTP, 1.5 mM MgCl₂ and 1 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystems). A combination of two sets of primers designed for cyprinid fish species (Zardoya and Doadrio 1995) was used for amplification of the cytochrome b gene: Glu-F: 5’-gaaagaacccgttgttataca-3’; Cytb-R: 5’-tctttatatgagaartan gggtg-3’; Cytb-F: 5’-cacgaracrggrtcnaayaa-3’; Thr-R: 5’-acctccratctycggattaca-3’. These are versatile primers designed for highly conserved fish mitochondrial DNA sequences around and within the *cytb* gene, and which amplify the two contiguous and overlapping fragments that cover the entire *cytb* gene (1141 bp). The specific annealing temperature for each set of primers, determined by gradient temperature PCR, was 48 °C for Glu-F/Cytb-R and 56 °C for Cytb-F/Thr-R. Amplifications were carried out in the GeneAmp PCR System 9700 (Applied Biosystems), under the following conditions: first denaturation step 10 min at 95 °C, 35 cycles – denaturation (30 s at 95 °C), annealing (30 s at annealing temperature), extension (60 s at 72 °C) and a final polymerization step at 72 °C for 10 min. The PCR products were purified with Wizard SV Gel and PCR Clean-Up System (Promega), sequenced with Big Dye Terminator v3.1 kit (Applied Biosystems) and analyzed on a ABI3130 DNA Genetic Analyzer (Applied Biosystems).

The electropherograms were first visualized and analyzed with Sequence Analysis Software (Applied Biosystems), and the sequences then edited with BioEdit Sequence Alignment Editor (Hall 1999). Multiple alignment of nucleotide sequences were carried out with the Clustal W program (Thompson 1994). We determined the 1141 bp sequence of cytb gene in *A. ruthenus* from the Lower Danube. Through electrophoregram analysis, double-peak sites were identified in several different positions in this gene, in three of the 30 specimens. These double peaks were confirmed by both 5’ → 3’ and 3’ → 5’ strands and are an indication of point heteroplasmy in this gene (Figure 1). The experiment was repeated from DNA extraction until sequences analysis. All the sequences, including double peaks, were identical. Two nucleotide sequences were identified in the heteroplasmic individuals: AruH1 was observed in two of the three heteroplasmic individuals, while AruH2 was observed in one individual (Table 1). The two detected sequences differed by three polymorphic sites. Thus, AruH1 was defined by the heteroplasmic site 867C/T, and AruH2 by 867T. Apart from the polymorphism in position 867, two transitions, 399G/A and 585C/T, were spotted between AruH1 and AruH2 (Figure 2).

Among the 27 non-heteroplasmic individuals, 3 haplotypes were identified based on the polymorphisms corresponding to bases 399 and 585. Thus, one individual presented the haplotype AruH3, three individuals presented the haplotype AruH4 and 23 presented the haplotype AruH5 (Table 1).

A homology search done by using a sequence of *A. ruthenus* from GenBank (AJ249694), facilitated the identification of codons displaying such heteroplasmic sites and the corresponding putative amino acids. Most of the identified heteroplasmic sites are synonymous, which means that the transition to homoplasmy and the fixation of any of the two nucleotide variants will not modify the amino acids sequence in cytochrome B protein (Table 1). The exception is

![Figure 1](image-url) - Partial sequence electropherograms of cytochrome b in *A. ruthenus*. (1) 5’ → 3’ strand; (2) 3’ → 5’ strand. R = A/G; Y = C/T.
Table 1 - Comparison of cytochrome b sequences from the analyzed individuals with similar sequences from other sturgeon species.

|                  | A. ruthenus AnuH1 | A. ruthenus AnuH2 | A. ruthenus AnuH3 | A. ruthenus AnuH4 | A. ruthenus AnuH5 | H. huso GU647228 | A. stellatus GU647226 | A. gueldenstaedtii GU647227 | A. baerii AJ245825* |
|------------------|-------------------|-------------------|-------------------|-------------------|-------------------|------------------|-----------------------|------------------------|-----------------------|
| Site             | 399G              | A                 | A                 | G                 | G                 | G                | A                     | G                      | G                     |
|                  | 585C              | T                 | T                 | C                 | T                 | T                | T                     | T                      | T                     |
|                  | 780C/T            | T                 | T                 | T                 | T                 | C                | T                     | C                      | C                     |
|                  | 786C/T            | C                 | C                 | C                 | C                 | T                | T                     | T                      | T                     |
|                  | 819C/T            | C                 | C                 | T                 | T                 | C                | C                     | C                      | C                     |
|                  | 849C/T            | C                 | T                 | T                 | T                 | C                | C                     | C                      | C                     |
|                  | 858C/T            | C                 | T                 | C                 | C                 | T                | C                     | T                      | T                     |
|                  | 862C/T            | T                 | T                 | T                 | T                 | C                | C                     | C                      | C                     |
|                  | 867C/T            | T                 | T                 | T                 | T                 | C                | C                     | C                      | T                     |
|                  | 870A/G            | G                 | G                 | G                 | G                 | A                | A                     | A                      | A                     |
|                  | 874C/T            | A                 | A                 | A                 | A                 | A                | A                     | G                      | G                     |
|                  | 979A/G            | A                 | A                 | A                 | A                 | A                | A                     | G                      | G                     |
|                  | 984A/G            | A                 | A                 | A                 | A                 | A                | A                     | G                      | G                     |
| Codon            | 133CTG            | CTA               | CTA               | CTG               | CTG               | CTG              | CTG                   | CTG                    | CTG                   |
|                  | 195ATC            | ATT               | ATT               | ATT               | ATT               | ATT              | ATT                   | ATT                    | ATT                   |
|                  | 260AAC/AAT        | AAC/AAT           | AAT               | AAT               | AAT               | AAC              | AAC                   | AAC                    | AAC                   |
|                  | 262CTC/CTT        | CTC               | CTC               | CTC               | CTC               | CTT              | CTT                   | CTT                    | CTT                   |
|                  | 273TAC/TAT        | TAT               | TAT               | TAT               | TAT               | TAC              | TAC                   | TAC                    | TAC                   |
|                  | 283TCC/TCT        | TCT               | TCT               | TCT               | TCT               | TCC              | TCC                   | TCC                    | TCC                   |
|                  | 286AAC/AAT        | AAC/AAC           | AAT               | AAT               | AAC/AAT           | AAC              | AAC                   | AAC                    | AAC                   |
|                  | 288CTA/TTA        | TTA               | TTA               | TTA               | TTA               | TTA              | CTA                   | CTA                    | CTA                   |
|                  | 289GCC/CGGT       | GGT               | GGT               | GGT               | GGT               | GGT              | GCC                   | GCC                    | GCC                   |
|                  | 290GGG/CGGG       | GGG               | GGG               | GGG               | GGG               | GGG              | GGG                   | GGG                    | GGG                   |
|                  | 292CTA/TTA        | TTA               | TTA               | TTA               | TTA               | TTA              | CTA                   | CTA                    | CTA                   |
|                  | 327ACC/GCC        | ACC               | ACC               | ACC               | ACC               | ACC              | ACC                   | ACC                    | ACC                   |
|                  | 328CTA/CTG        | CTA               | CTA               | CTA               | CTA               | CTA              | CTA                   | CTG                    | CTG                   |

| Amino-acids      | 133Leu            | Leu               | Leu               | Leu               | Leu               | Leu              | Leu                   | Leu                    | Leu                   |
|                  | 195Ile            | Ile               | Ile               | Ile               | Ile               | Ile              | Ile                   | Ile                    | Ile                   |
|                  | 260Asn            | Asn               | Asn               | Asn               | Asn               | Asn              | Asn                   | Asn                    | Asn                   |
|                  | 262Leu            | Leu               | Leu               | Leu               | Leu               | Leu              | Leu                   | Leu                    | Leu                   |
|                  | 273Tyr            | Tyr               | Tyr               | Tyr               | Tyr               | Tyr              | Tyr                   | Tyr                    | Tyr                   |
|                  | 283Ser            | Ser               | Ser               | Ser               | Ser               | Ser              | Ser                   | Ser                    | Ser                   |
|                  | 286Asn            | Asn               | Asn               | Asn               | Asn               | Asn              | Asn                   | Asn                    | Asn                   |
|                  | 288Leu            | Leu               | Leu               | Leu               | Leu               | Leu              | Leu                   | Leu                    | Leu                   |
|                  | 289Gly            | Gly               | Gly               | Gly               | Gly               | Gly              | Gly                   | Gly                    | Gly                   |
|                  | 290Gly            | Gly               | Gly               | Gly               | Gly               | Gly              | Gly                   | Gly                    | Gly                   |
|                  | 292Leu            | Leu               | Leu               | Leu               | Leu               | Leu              | Leu                   | Leu                    | Leu                   |
|                  | 327Thr/Ala        | Thr               | Thr               | Thr               | Thr               | Thr              | Thr                   | Ala                    | Ala                   |
|                  | 328Leu            | Leu               | Leu               | Leu               | Leu               | Leu              | Leu                   | Leu                    | Leu                   |

*Ludwig et al. (2000).*
the double peak 979A/G in the first position of codon 327, corresponding to the putative amino acids, Thr or Ala.

Comparative sequence analysis of mitochondrial protein-coding genes in closely related species of salmonids (Doiron et al., 2002) and mammals (Pesole et al., 1999), revealed nucleotide substitution frequencies at non-synonymous sites to be 8 to 30 times lower than at synonymous sites (Shigenbou et al., 2005). Non-synonymous heteroplasmic sites have been observed in fish in the NADH dehydrogenase subunit-5 (nd5) gene of chum salmon and in the NADH dehydrogenase subunit-4 gene (nd4) of the Japanese flounder (Shigenbou et al., 2005). The comparison of amino acid sequences among other sturgeon species revealed that, in the case of the non-synonymous heteroplasmic site, both putative variants of amino acids were identified, thereby showing them to be viable and functional (Table 1).

Although relatively rare, cases of point heteroplasmy have been reported in animals. Amongst others factors, this can be due to interspecific paternal leakage. The biparental inheritance of mtDNA has been revealed in various species, including fish (Guo et al., 2006; Magoulas and Zouros, 1993). Most cases of paternal leakage derive from interspecies hybridization, backcrosses and introgression, probably due to the breakdown of mechanisms recognizing and removing paternal mtDNA (Sutovsky et al., 2003; White et al., 2008). In sturgeons, interspecific hybridization is a relatively frequent phenomenon, mostly due to the temporal and spatial overlapping of breeding areas. Apart from A. ruthenus, three other sturgeon species are currently reproducing in the Lower Danube: A. stellatus, A. gueldenstaedtii and H. huso. Due to both natural and anthropogenic factors, hybridization between these three species and sterlet sturgeons is highly possible (Dudu et al., 2011). Moreover, there have been reported cases of hybridization between A. ruthenus and the exotic species A. baerii (the Siberian sturgeon, bred in local fish farms) in the Danube River (Ludwig et al., 2009). Under these circumstances, heteroplasmic sites are possibly signals of introgressive hybridization. By analyzing the sequence variation shown in Table 1 we consider that the observed heteroplasmy is the result of interspecific hybridization of A. ruthenus individuals in the Danube River. In populations that suffered a bottleneck event, a mitochondrial haplotype from one of the genitor species might become dominant in the cells of the other species, passing through a transitional state of heteroplasmy. This type of mtDNA transfer was proposed by Billard and Lecointre (2001), Doyle (1997) and Maddison (1997) as justifying the close relationship found between A. ruthenus and H. huso, as based on cytb and 12S/16S rRNA sequences (Birstein and DeSalle, 1998).

Due to the risk of detecting heteroplasmic signals in the absence of true heteroplasmy, careful screening should be the rule. The cause of amplification artifacts is correlated with either the existence of nuclear-encoded mitochondrial pseudogenes (or NUMTs), or the presence of duplications within mitochondrial genomes (White et al., 2008). In species harbouthing NUMTs, PCR primers may hybridize to both mitochondrial sequences and nuclear copies. The resulting co-amplifications can be mistakenly recognised as heteroplasmic sites, or lead to inaccurate estimates of heteroplasmy levels (White et al., 2008). This is the first report on point heteroplasmy in a coding mitochondrial region of A. ruthenus. The recombination of mtDNA has a great importance and should be considered for the accuracy of population studies and phylogenetic reconstruction in this group of fish, also taking into account other aspects such as hybridization or introgression. As hybridization among Danubian sturgeons has been the target of several surveys (Ludwig et al., 2009; Dudu et al., 2011), a heteroplasmy description could be a possible aid in its determination. In A. ruthenus the identification of specific point heteroplasmic sites might be interesting for a possible correlation of these haplotypes with genetically distinct groups in the Danube River.

Figure 2 - Partial sequence electropherograms of cytochrome b in A. ruthenus (5’ → 3’ strand) differentiating between AruH1 (1) and AruH2 (2). R = A/G; Y = C/T.
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