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Short Communication

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 heteroplasmic 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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The vertebrate mitochondrial genome presents certain features, such as compact organization, maternal transmission, relatively small size, hundreds to thousands of copies per cell, rapid evolution, and a lack of or much reduced recombination, which recommend it as a useful tool for studies on population genetics, phylogeny and phylogeography. Although mitochondrial DNA (mtDNA) haplotype frequencies were long considered to be primarily controlled by migration and genetic drift, and most intraspecies variation to be selectively neutral (Ballard and Rand 2005), there are more recent indications of the possible recombination of mtDNA in various species (Tsaousis *et al.*, 2005; Guo *et al.*, 2006). Thus, mtDNA variation could be a consequence of simple neutral mutation and genetic drift (Wallace *et al.*, 2003; Ruiz-Pesini *et al.*, 2004). mtDNA heteroplasmy, *i.e.* the presence of multiple mtDNA haplotypes in a single organism, is not an exceptional condition related to mitochondrial disease (Monnat Jr and Loeb, 1985), and many cases of healthy heteroplasmic individuals have been described (Brandstatter *et al.*, 2004; Santos *et al.*, 2005).

Depending on the type of mtDNA mutation, two types of heteroplasmy are known, site (point) and length heteroplasmy. Length heteroplasmy occurs in hypervariable regions of the D-loop as a consequence of poor replication fidelity, and is represented by multiple mtDNA populations of various lengths. Point heteroplasmy is represented by the presence of various mtDNA populations differing from each other at a given nucleotide position. This is a less frequent phenomenon and is considered to be a transient state regarding the fixation of either the wild type or the mutated type (Santos *et al.*, 2005, 2008; Irwin *et al.*, 2009). Apparently, in cattle, mice and humans, heteroplasmy is generally resolved to homoplasmy within a few generations (Chinnery, 2002; Cree *et al.*, 2008; Khrapko, 2008).

The Acipenseriformes constitute one of the oldest groups of fish, having appeared 200 million years ago, during the Jurassic period (Bemis *et al.*, 1997). Since these are among the oldest fish species, having survived several mass extinction events, and are authentic "living fossils" with a slow evolution, sturgeons may constitute a model for studying vertebrate development. *Acipenser ruthenus* Linnaeus, 1758 (Sterlet) is a freshwater species and is the smallest species of sturgeon. Originally it was widely distributed in the tributaries flowing into the Caspian, Black, Azov, Baltic, White, Barents and Kara Seas. The Danube is a stronghold for the species, and in recent years, catches have been reported as far upstream as Austria.

mtDNA heteroplasmy is frequent in fish and has been identified in several species, such as *Cyprinella spiloptera*

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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 $MgCl_2$ 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'-gaagaaccaccgttgttattcaa-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

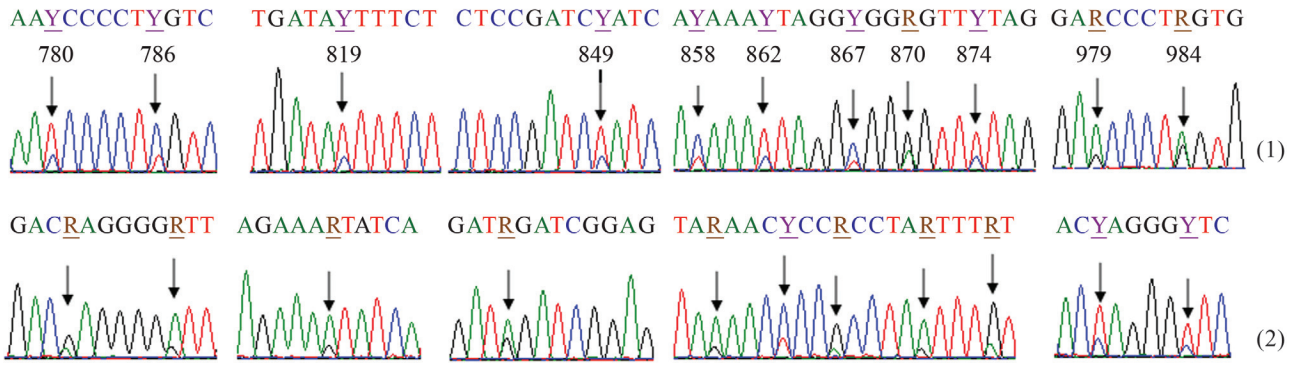

Figure 1 - Partial sequence electropherograms of cytochrome b in *A. ruthenus*. (1) 5' → 3' strand; (2) 3' → 5' strand. R = A/G; Y = C/T.

Table 1 - Comparison of cytochrome b sequences from the analyzed individuals with similar sequences from other sturgeon species.

	A. ruthenus AruH1	*A. ruthenus* AruH2	*A. ruthenus* AruH3	*A. ruthenus* AruH4	*A. ruthenus* AruH5	*A. ruthenus* AJ249694*	*H. huso* GU647228	*A. stellatus* GU647226	*A. gueldenstaedtii* GU647227	*A. baerii* AJ245825*
Site	399G	A	A	G	G	G	G	G	G	G
	585C	T	T	C	T	T	T	T	T	T
	780C/T	C/T	T	T	T	T	C	T	C	C
	786C/T	C/T	C	C	C	C	T	T	T	T
	819C/T	C/T	T	T	T	T	C	C	C	C
	849C/T	C/T	T	T	T	T	C	T	C	C
	858C/T	C/T	C	C	C	C	T	C	T	T
	862C/T	C/T	T	T	T	T	C	C	C	C
	867C/T	T	T	T	T	T	C	C	T	T
	870A/G	A/G	G	G	G	G	G	A	A	A
	874C/T	C/T	T	T	T	T	C	C	C	C
	979A/G	A/G	A	A	A	A	A	G	G	G
	984A/G	A/G	A	A	A	A	A	A	G	G
Codon	133CTG	CTA	CTA	CTG	CTG	CTG	CTG	CTG	CTG	CTG
	195ATC	ATT	ATT	ATC	ATT	ATT	ATT	ATT	ATT	ATT
	260AAC/AAT	AAC/AAT	AAT	AAT	AAT	AAT	AAC	AAT	AAC	AAC
	262CTC/CTT	CTC/CTT	CTC	CTC	CTC	CTC	CTT	CTT	CTT	CTT
	273TAC/TAT	TAC/TAT	TAT	TAT	TAT	TAT	TAC	TAC	TAC	TAC
	283TCC/TCT	TCC/TCT	TCT	TCT	TCT	TCT	TCC	TCT	TCT	TCC
	286AAC/AAT	AAC/AAT	AAC	AAC	AAC	AAC	AAT	AAC	AAT	AAT
	288CTA/TTA	CTA/TTA	TTA	TTA	TTA	TTA	CTA	CTA	CTA	CTA
	289GGC/GGT	GGT	GGT	GGT	GGT	GGT	GGC	GGC	GGT	GGT
	290GGA/GGG	GGA/GGG	GGG	GGG	GGG	GGG	GGG	GGA	GGA	GGA
	292CTA/TTA	CTA/TTA	TTA	TTA	TTA	TTA	CTA	CTA	CTA	CTA
	327ACC/GCC	ACC/GGC	ACC	ACC	ACC	ACC	ACC	GCC	GCC	GCC
	328CTA/CTG	CTA/CTG	CTA	CTA	CTA	CTA	CTA	CTA	CTG	CTG
Amino-acids	133Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
	195Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile
	260Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn
	262Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
	273Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr
	283Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
	286Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn
	288Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
	289Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly
	290Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly
	292Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
	327Thr/Ala	Thr/Ala	Thr	Thr	Thr	Thr	Thr	Ala	Ala	Ala
	328Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu

*Ludwig *et al.* (2000).

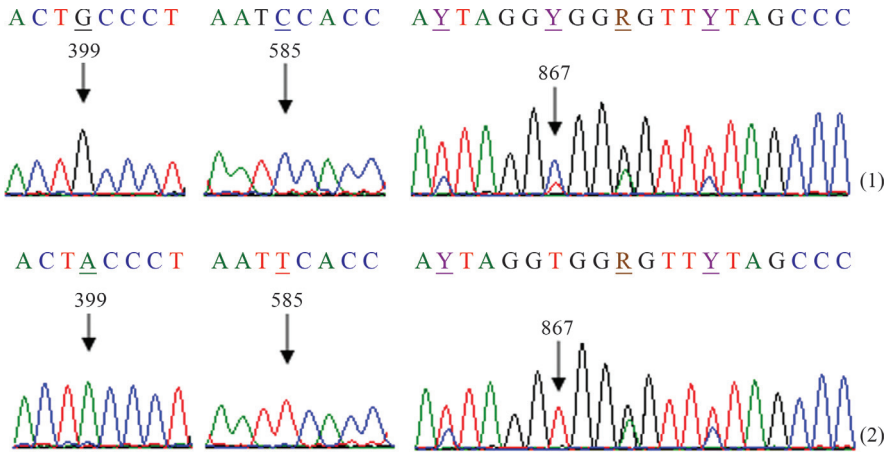

Figure 2 - Partial sequence electropherograms of cytochrome b in *A. ruthenus* (5' → 3' strand) differentiating between AruH1 (1) and AruH2 (2). R = A/G; Y = C/T.

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 heteroplasmy 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 harbouring 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.

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