Phylogenetic Relationships of Amur Sturgeon

Acipenser schrenckii Brandt, 1869 Based on 18S rDNA Sequencing

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Received July 25, 2008

Abstract—The phylogenetic relationships of Amur sturgeon A. schrenckii Brandt, 1869 with related species have been analyzed based on sequencing of the 18S rDNA small subunit. The complete sequence (1746 bp) of 18S rDNA has been estimated in seven individual A. schrenckii clones. The results show that the rDNA mutation profile of A. schrenckii 18S is very similar to that of A. fulvescens (Genbank data). Structural–functional and phylogenetic analyses allowed us to identify a presumably expressed variant, as well as taxon-specific mutation (adenine insertion after position 658 bp), for A. schrenckii 18S rDNA. Phylogenetic reconstructions performed with various approaches (NJ, MP, ML and Bayesian) support the monophyly of the genus Acipenser and point (1), based on which, in accordance with the classification based on ecological and morphological data (Artyukhin, 2006), the Amur sturgeon is closer to the sterlet than the Baltic sturgeon and (2) to substantial differentiation between North American (A. fulvescens) and Eurasian (A. schrenckii, A. ruthenus, and A. sturio) species of Acipenseridae.

Key words: 18S rDNA, Amur sturgeon, phylogeny, Acipenseridae.

DOI: 10.1134/S1990519X09020072

Abbreviations: bp, base pair; PCR, polymerase chain reaction; ML, maximum likelihood; MP, maximum parsimony; MST, minimal spanning tree; NJ, neighbor-joining; 18S rRNA, small subunit ribosomal RNA.

Acipenseriformes are notable among other vertebrates due to their unique properties; they are ancient and have a relatively low rates of evolution (Bemis et al., 1997; Krieger and Fuerst, 2002). Another feature of this group is that all living representatives of Acipenseriformes are listed in CITES (Ludwig, 2008). The main causes of their status are environmental pollution, dam constructions in spawning areas, and overfishing. In addition, Acipenseriform species are characterized by late sexual maturation (at 10–15 years), reproduction in up to five-year intervals (Krykhtin and Gorbach, 1994; Grunwald et al., 2002), and interspecific and intergeneric hybridization (Krykhtin and Gorbach, 1994; Birstein et al., 1997). Knowledge of Acipenseriformes phylogeny is necessary to develop measures for their preservation. However, the classification of Acipenseriformes is very contradictory; phylogenetic studies based on morphological data are not always confirmed by molecular-genetic evidence (Artyukhin, 2006).

Most molecular taxonomic and phylogenetic studies of Acipenseriformes have been performed with mitochondrial DNA (mtDNA) markers (Zhang et al., 2000; Ludwig et al., 2001; Birstein et al., 2002; Peng et al., 2007; Krieger et al., 2008) and the results are mainly similar. Thus, further studies based on mitochondrial genes can hardly systematically clarify Acipenseriformes; therefore, it is necessary to use nuclear genes. However, their analysis is complicated by a high level of ploidy in sturgeons; Acipenseridae genomes have 120–500 chromosomes that may be arranged in series of $4n–8n–16n$ (Ludwig et al., 2001).

A molecular marker generally applicable for phylogenetic investigations of many animals and plants is nuclear 18S rDNA (Luan et al., 2004; Yoon and Kim, 2006; Hines et al., 2007; etc.). However, the sturgeon species has diverse 18s rRNA alleles (Krieger and Fuerst, 2002, 2004; Krieger et al., 2006), which hampers the estimation of interspecies relationships (Buckler et al., 1997; Krieger and Fuerst, 2002); therefore, the application of 18S rDNA for Acipenseridae phylogeny is only possible with preliminary sequence analysis (Abouheif et al., 1998; Xia et al., 2003).

The aim of our work was to find reliable molecular markers for Acipenseridae phylogenetic studies. The main purpose was to define phylogenetic relationships
of sturgeon *Acipenser schrenckii* Brandt, 1869 with other sturgeon species based on the data of 18S rDNA complete sequence. A comparison of the results obtained with phylogenetic evidence based on other traits showed that the maker we applied was adequate to specify the interrelationships between sturgeon species.

**MATERIALS AND METHODS**

Reagents: thermophilic DNA-dependent Pfu (Fermentas, Lithuania) and Taq (Medigen, Russia) DNA polymerases; Long PCR Enzyme Mix (Fermentas, Lithuania); InstI/Aclone PCR Product Cloning Kit (Fermentas, Lithuania); sequencing kit with BigDye Terminator (Applied Biosystems, United States).

The full-sized gene of a small ribosome subunit (about 1800 bp) was amplified by a polymerase chain reaction (PCR) with primers (Table 1) developed for the North-American lake sturgeon *Acipenser fulvescens* (Krieger and Fuerst, 2002) and Long PCR Enzyme Mix (Fermentas, Lithuania). The PCR reaction mixture was 90 ng genome DNA, 5 µl 10× Long PCR buffer, 17 mM MgCl₂, 2.5 mM dNTP, 1.5 units of enzyme mix for long PCR and 0.5 mM of each primer. The temperature regime was as follows: 94°C–3 min (94°C–1 min, 55°C–2 min, 68°C–4 min), 35 cycles, 72°C–15 min. Amplified products were purified by precipitation with 70% ethanol.

Complete 18S rDNA gene was cloned with InstI/Aclone PCR Product Cloning Kit (Fermentas, Lithuania) according to the manufacturer’s instructions. *E. coli* Dh5α strain was used for preparation of competent cells.

To amplify cloned 18S rDNA sequences, colonies about 2 mm² were picked with sterile tips and placed into the mixture composed of 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 0.25 mM of each dNTP, 0.25 mM of each primer, 1.5 mM MgCl₂ and one unit of Taq/Pfu (to minimize PCR errors). The temperature regime was as follows: 95°C–3 min (95°C–1 min, 72°C–4 min), 35 cycles.

Sequencing was performed with the automatic laser sequencer machine ABI PRISM 310 in IBSS FEB RAS. Sequencing reaction was conducted in 15 µl of reaction mixture composed of 10 ng plasmid DNA with an insertion, 1 pM primer, and 2.5 µl BigDye Terminator v. 3. The temperature regime was as follows: 96°C–1 min (96°C–30 s, 50°C–15 s, 60°C–4 min), 35 cycles. Amplification products were purified by precipitation with 70% ethanol.

Assembly and alignment of 18S rDNA sequences, as well as the estimation of genetic distances and phylogenetic reconstructions (by methods of neighbor joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian), were carried out with Mega v. 4 (Tamura et al., 2007), Arlequin v. 3.11 (Excoffier et al., 2006) and MrBayes v. 3.1 (Ronquist and Huelsenbeck, 2003) software. The choice of an optimal mathematical model to calculate genetic distances and phylogenetical reconstruction was performed with AIC information criterion (Akaike, 1974) by Modeltest v. 3.06 software (Posada and Crandall, 1998). In addition to our own data, 18S rDNA sequences for the following species: *Acipenser fulvescens*, (lake sturgeon), *A. sturio* (Baltic sturgeon), *A. ruthenus* (sterlet), *Polyodon spathula* (paddlefish), and *Oncorhynchus mykiss* (rainbow trout) were retrieved from the GenBank and used for phylogenetic analysis as the outgroup.

**RESULTS AND DISCUSSION**

To estimate the phylogenetic relationships of Amur sturgeon with other Acipenseridae species, we sequenced seven clones with complete 18S rDNA sequences (1746 bp) with low 486 nucleotide variability in 960–1144 bp position (Chelomina et al., 2008). The analysis of insertions and deletions confirmed the presence of six hot spots in *A. schrenckii*, i.e., sites with insertions or deletions in all analyzed sequences identified previously in Acipenseriformes within two 18S rDNA regions with total lengths of 788 bp (Krieger et al., 2006). In addition, in *A. schrenckii*, we revealed a specific adenine insertion after 658 bp position that was not found in the region examined previously (Table 2; Fig. 1).

Allele variants of Amur sturgeon 18S rDNA are distinct for 14 transitions, three transversions, nine insertions, five deletions, and two complex mutations (involving more than three nucleotides). As a whole, the mutation profile of *A. schrenckii* is similar to that of *A. fulvescens* (GenBank data, Fig. 2). Amur and lake

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**Table 1.** List of primers used for amplification of sturgeon complete 18S rDNA gene

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>CRN5A</td>
<td>5’-GGTTGATCTCCTGCCAGTAG-3’</td>
</tr>
<tr>
<td>373C</td>
<td>5’-GATTCCGGAGAGGGAGCCT-3’</td>
</tr>
<tr>
<td>892C</td>
<td>5’-GTCAGAGGTTGAAATCCTTG-3’</td>
</tr>
<tr>
<td>570</td>
<td>5’-GCTATTTGGAGCTTGAATTAC-3’</td>
</tr>
<tr>
<td>1262</td>
<td>5’-GAACGGCCATGCAACCAC-3’</td>
</tr>
<tr>
<td>SSU2</td>
<td>5’-CCGCGGCCGGATCCTGATCCCCTCGCAGTTAC-3’</td>
</tr>
</tbody>
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