

EXPERIMENTAL
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Polymorphism of the Mitochondrial DNA Control Region in Eight Sturgeon Species and Development of a System for DNA-Based Species Identification

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Abstract—Intraspecific and interspecific nucleotide sequence variations of the mtDNA control region (D-loop) were studied with mtDNAs isolated from tissue specimens of more than 1400 sturgeons of nine species: Russian sturgeon *Acipenser gueldenstaedtii*, Persian sturgeon *A. persicus*, Siberian sturgeon *A. baerii*, Amur sturgeon *A. schrenkii*, Fringebarbel sturgeon *A. nudiventris*, sterlet *A. ruthenus*, stellate sturgeon *A. stellatus*, beluga *Huso huso*, and kaluga *H. dauricus*. The results were used to analyze the interspecific variation of the mtDNA control region in the given set of species and to develop a test system of ten species-specific primers, which allowed species identification from noninvasive tissue samples, spawn, and food products of eight species. The system proved suitable for multiplex PCR. A method was developed for the first time to reliably differentiate the *A. baerii* mitotype and the *baerii*-like mitotype of *A. gueldenstaedtii*. It was found that, although genetically separate, *A. gueldenstaedtii* and *A. persicus* are relatively young species and have common mitochondrial haplotypes, precluding their identification via mtDNA analysis alone. To develop a system for species identification of *A. gueldenstaedtii* and *A. persicus*, it is necessary to study the polymorphism of nuclear markers.

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INTRODUCTION

Sturgeons are a unique ancient group of ganoid cartilaginous fishes and are commercially important. The family Acipenseridae comprises 27 species, including two paddlefish species and 25 sturgeon species of the northern hemisphere. Black caviar and sturgeon products are in great demand and are high-priced, which leads to excessive fishing and poaching. These factors, along with destruction of natural breeding grounds as a result of damming and anthropogenic pollution, have dramatically reduced the population size of most species in both Eurasia and North America. In 1998, all sturgeon species were included in Appendix 1 or 2 of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and, with the exception of two species, in various categories of the International Union for Conservation of Nature (IUCN) Red List [1–3].

Official declaration of the species composition is mandatory for trans-border transportation of all species listed in the CITES appendices and products obtained with such species (including caviar). Since the natural populations have dramatically decreased in size, sturgeon domestication and artificial rearing become widespread in the majority of black caviar-producing countries, including Russia. Species reared in aquaculture are often nonnative for the given locality, which complicates species identification. Examples of broad introduction of sturgeons in aquaculture beyond their spe-

cies region are provided by Siberian sturgeon *A. baerii* (European Russia, Ukraine, Finland, France, the United States, China, etc.), American paddlefish (European countries), and some other species. In addition, rapid and reliable methods of species identification of sturgeon products are essential in view of poaching, illegal production of black caviar, and falsification of trade labels and documentation [4].

In spite of the great ecological plasticity and morphological variation, species identification of adult sturgeons is usually simple and is based on the standard criteria accepted in ichthyology [5], with the exception of green (United States) and Sakhalin sturgeons. In addition, differentiation of Russian, Adriatic, and Persian sturgeons is thought to be problematic. Species identification of juvenile sturgeons is difficult and requires special knowledge.

Traditionally, species identification of black caviar is organoleptic and rather subjective. The caviar taste, color, and roe grain size substantially vary; the grain size ranges of different species overlap and are thus unreliable for species identification. Structural analysis of the egg envelope has shown that roe grains of different species differ in the shape and structure of micropilus fields, but these traits vary and are hardly distinguishable after roe is treated to produce caviar [6].

Attempts to identify the sturgeon species by biochemical methods have not met with success in general. Isoelectric focusing of roe proteins yields some-

what different but overlapping patterns for different species [7, 8]. The difference in albumin spectrum [9] has not found application.

Molecular methods of species identification of commercial products have been developed for more than 15 years [10]. Genetic identification of sturgeons and sturgeon products, including caviar, is now one of the most important proofs acceptable by CITES in importing or exporting the CITES appendix species and relevant products.

The methods of DNA identification can be conventionally divided into two groups addressing nuclear genetic markers and mitochondrial DNA (mtDNA). An advantage of the former is that not only pure species, but also hybrids are identifiable. However, these methods have a drawback of being highly sensitive to the nuclear DNA integrity and, consequently, are hardly suitable for analyzing commercial caviar specimens.

The use of mtDNA for analyzing the species composition has several advantages, since mtDNA has a far greater copy number as compared with nuclear DNA (especially in eggs) and, owing to its circular structure, is more resistant to degradation and is better preserved.

Birstein and colleagues [11–13] were the first to develop a DNA test for species identification of sturgeons. The method took advantage of species-specific substitutions in a region of the *CytB* gene and was patented in the United States and Europe as a set of primers allowing species-specific amplification of the *CytB* gene fragment. A drawback was that many PCRs were to be performed and their results analyzed to examine one specimen. Moreover, almost all primers differed by a single nucleotide substitution, which allowed false test results and required that a reference reaction (positive control) be always carried out for each species.

Ludwig et al. [14] further developed the method based on the *CytB* polymorphism. The method proposed for species identification involved consecutive restriction enzyme analyses of a PCR-amplified fragment of the *CytB* gene with seven enzymes and visualization of the restriction fragments in agarose or polyacrylamide gel.

The above two methods have not been widely employed, and species identification of caviar and other products CITES-regulated species are usually solved by direct sequencing *CytB* fragments and comparing the results with the GenBank reference sequences [15].

We were aimed at developing a method to identify the Russian species of surgeons without sequencing and restriction enzyme analysis. The main requirements for the method were that it be reliable, simple, and inexpensive and yield reproducible results in various laboratories with minimum equipment. We studied the polymorphism of the mtDNA control region (D-loop) in eight sturgeon species in order to identify the species-specific regions for each species and to design the primers suitable for PCR-based species identification.

The mtDNA control region is hypervariable as compared with mitochondrial protein- and RNA-coding genes and, consequently, has not been considered promising for species identification so far. To isolate its species-specific sequences, it is necessary to study its total natural polymorphism and to examine the sequences of many individuals from various localities of the species region. Otherwise, primers based on the individual sequences of one population may yield an artificial result for individuals of another populations. On the other hand, the control region often contains insertions, deletions, and substitutions of several consecutive nucleotides, which is advantageous for genotyping. Such sites are ideal for designing species-specific primers and developing test systems that are less sensitive to the PCR conditions and equipment quality.

MATERIALS AND METHODS

To analyze the natural variation of the mtDNA control region, we used sturgeon roe and tissue specimens (mostly fin fragments) available from the Russian Federal Reference Collection of Genetic Materials. The collection harbors more than 9000 sturgeon tissue specimens, which were collected in various regions of Russia by researchers of the Department of Molecular Genetics of Aquatic Organisms, All-Russian Institute of Fishery and Oceanography or researchers contracted by the institute. Each specimen is labeled with its species name (as identified by ichthyologists on site), collection site, collection time, and the collector's name. The specimens were initially fixed with 96% ethanol in the field and transferred into fresh ethanol in the laboratory. Every specimen of the collection is accompanied by a detail description of the species, collection site, population, sex, age, tissue type, and morphometric parameters. The data were included in the collection protocol, which was signed by the collector. Collection material is stored in ethanol at -70°C .

DNA was isolated by standard phenol [16] or salt [17] extraction after digestion with proteinase K or maxatase.

The full-length mtDNA control region (D-loop) was amplified with primers LproF (AACTCTCAC-CCCTAGCTCCCAAAG) and DL651 (ATCTTAA-CATCTTCAGTG) directed, respectively, to the *tPro* and *tPhe* tRNA genes flanking the control region. Amplification was carried out under standard conditions; the annealing temperature was 52°C . The product size varied from 950 to 1280 bp, depending on the 82-bp repeat number in the haplotype and the species.

Sequencing was carried out with the same primers, using a BigDye 1.1 sequencing kit and an ABI 3100 genetic analyzer (United States). Some individuals were polymorphic for the number of 82-bp repeats at the control region 3' end, flanked by the proline tRNA gene. To avoid a superimposition of the sequencing results obtained with templates differing in repeat num-

Table 1. Control region sequences examined in this work

Species	Number of sequences established	Number of GenBank sequences	Total
Russian sturgeon <i>Acipenser gueldenstaedtii</i> (typical mitotype)	95	56	151
Russian sturgeon <i>A. gueldenstaedtii</i> ("baerii"-like mitotype)	68	9	77
Siberian sturgeon <i>A. baerii</i>	48	35	83
Fringebarbel sturgeon <i>A. nudiiventris</i>	14	2	16
Stellate sturgeon <i>A. stellatus</i>	18	38	56
Persian sturgeon <i>A. persicus</i>	26	28	54
Amur sturgeon <i>A. schrenkii</i>	17	1	18
Sterlet <i>A. ruthenus</i>	10	1	11
Beluga <i>Huso huso</i>	45	25	70
Kaluga <i>H. dauricus</i>	18	2	20

ber, we used additional primer AHR3 (CATAACAT-AATGTTTCATCTACC), which was complementary to the region immediately upstream of the repeat.

In addition to the sequencing data, we used the sequences available from GenBank (NCBI). The total numbers of sequences examined and the GenBank sequences are summarized in Table 1.

We used to SeqMan program to analyze the patterns for sequence alignment and contig assembly, the MegAlign program to construct multiple sequence alignments, and the PrimerSelect program to design the

primers; all programs were of the DNASTar software package (Lasergene).

RESULTS AND DISCUSSION

Variation of the mtDNA Control Region in Sturgeons

We obtained 218 and analyzed 322 nucleotide sequences (including those available from GenBank) of the D-loop. Like in most vertebrates, the sturgeon control region had two hypervariable segments (HVS1 and HVS2) and a relatively conserved spacer between them. HVS2 harbored 2.5–5.5 repeats of 81–82 bp, including the TAS motif, which is presumably involved in initiating mitochondrial heavy-strand synthesis [18, 19]. Many cases displayed heteroplasmy; i.e., one individual had several mtDNAs with different repeat numbers (up to five variants). We studied the mtDNA inheritance in individuals with several mitotypes differing in repeat number and showed that the unit number of the 82-bp repeat may change during lifetime and cannot be used as an informative genetic marker of an individual.

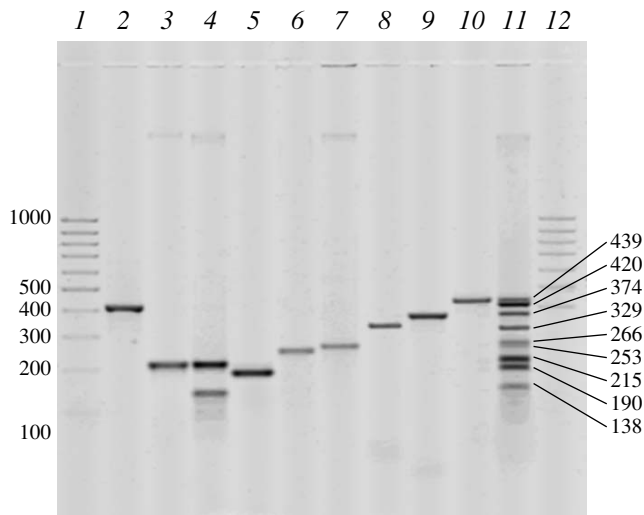
HVS1 varied in size only in *A. schrenkii*. A 12-bp insert was detected in some individuals from the Amur population. The variation of HVS1 was due to point mutations and single nucleotide insertions/deletions in the other species.

Isolation of a conserved region made it possible to design a primer common for all sturgeon species (primer AHR, Table 2).

Phylogenetic analysis of the control region sequences yielded a tree that was similar to earlier dendrograms based on the cytochrome oxidase I gene [20]. The species distinctly clustered into two clades. The Atlantic clade (of a Pontic–Caspian origin) included the *A. gueldenstaedtii*–*A. persicus*–Adriatic sturgeon *A. naccarii* complex, *A. baerii*, *A. nudiiventris*, *A. ruthenus*, *A. stellatus*, and *H. huso*. The group of Pacific species was also monophyletic and included *A. schrenkii*

Table 2. Primers employed in species identification of sturgeons

Primer	Sequence	Used with	Product size, bp	Species specificity
AHR	TATACACCATTATCTCTATGT			All species
AGF	GCACAGACTATGTGGTATCCAGAA	AHR	420	<i>A. gueldenstaedtii</i>
ABF	CAGATGCCAGTAACAGGCTGA	AHR	215	<i>A. gueldenstaedtii</i> ("baerii"-like) and <i>A. baerii</i>
ABRM	TGTCTGTCTAGAACATA _t G	ABF	182	<i>A. baerii</i>
HusF	TATCTATTACCTGCGAGCAGGCTG	AHR	374	<i>H. huso</i>
DauF	CCTCTTATGTACGCGGTGT	AHR	439	<i>H. dauricus</i>
NudF	TGTCTTTTCTGAAGGAGCTTTGC	AHR	329	<i>A. nudiiventris</i>
RutF	GGGAATAACCGTTAATTTGG	AHR	190	<i>A. ruthenus</i>
SteF	GGGGTTCTTGGCATGTTGTGAGCG	AHR	266	<i>A. stellatus</i>
SchF	TGTGGGGTCCACGGA _c TTTACAG	AHR	254	<i>A. schrenkii</i>



Agarose gel electrophoresis of the PCR fragments obtained for various sturgeon species. Lanes: 1, marker (100–1000 bp); 2, *A. gueldenstaedtii* (AGF–AHR, 420 bp); 3, *A. gueldenstaedtii* with the *baerii*-like mitotype (ABF–AHR, 215 bp); 4, *A. baerii* (ABF–AHR, 215 bp and ABF–ABRM, 138 bp); 5, *A. ruthenus* (RutF–AHR, 190 bp); 6, *A. schrenkii* (SchF–AHR, 253 bp); 7, *A. stellatus* (SteF–AHR, 266 bp); 8, *A. nudiventris* (NudF–AHR, 329 bp); 9, *H. huso* (HusF–AHR, 374 bp); 10, *H. dauricus* (DauF–AHR, 439 bp); 11, molecular weight marker of the sturgeon PCR products; 12, marker (100–1000 bp).

and *H. dauricus* of our sample. Thus, analysis of the mtDNA control region supports the polyphyletic origin assumed for the genus *Huso* (*H. huso* and *H. dauricus*) on the basis of a convergent similarity of morphological traits in its species, the largest in Acipenseridae.

Construction of a Panel of Species-Specific Primers

Based on the sequences established in this work and available from GenBank, we identified a strongly consensus sequence with invariable nucleotides for each species. Multiple sequence alignment of the consensus sequences allowed us to determine the species-specific regions of the mtDNA D-loop and to design a set of species-specific primers. The primers were chosen to harbor a region that was characteristic of all individuals of the given species and to lack a region that allowed dimerization of the primer with itself and primer AHR. For the convenience of genotyping, the species-specific primers were at different distances from anchor (common) primer AHR so that the products amplified for different species would differ in size and would be easily distinguishable in 2% agarose gel (figure). The panel of diagnostic primers is shown in Table 2.

Discrimination between the *A. baerii* Haplotype and the *baerii*-Like Haplotype of *A. gueldenstaedtii*

Approximately 30% of *A. gueldenstaedtii* individuals from the Caspian basin are similar in mtDNA to

A. baerii [13, 21]. The *CytB* gene sequence does not allow a discrimination between these two mtDNA types; i.e., the available molecular methods of sturgeon species identification fail to reliably distinguish *A. baerii* and *A. gueldenstaedtii*. Since the control region is four- to fivefold more variable than the *CytB* gene, we studied its polymorphism with 56 mtDNA sequences of *A. baerii* and 45 *baerii*-like sequences of *A. gueldenstaedtii*. One substitution was found to occur in the *baerii*-like haplotype but not in *A. baerii*.

Taking advantage of this substitution, we constructed primer ABRM (Table 2), which annealed exclusively to *A. baerii* mtDNA and, in pair with primer ABF, allowed amplification of a 182-bp fragment.

Verification of the Test System

The panel of primers was tested with several PCR kits most commonly used in Moscow (Dialat, Sileks, Fermentas, etc.), plasticware from several companies, and several thermal cyclers, including Tertsik MS-2 (DNK-Tekhnologiya) and MJ Research PTC-225 (United States) instruments. The PCR mixture for routine analysis contained 1× *Taq* buffer (Sileks, Moscow), 2.5 mM MgCl₂, 2.5 mM each dNTP (Dialat, Moscow), 2.5–5.0 pM primers (according to Table 2), 2.8 μl of Cresol–glycerol (3.5 mM Cresol Red, 50% glycerol in water), 2.5 units of *Taq* polymerase (Sileks), 2 μl of a DNA extract, and water (milliQ) to the final volume of 25 μl.

Amplification included initial denaturation at 95°C for 2 min; 35 cycles of 92°C for 20 s, 57°C for 30 s, and 72°C for 30 s; and last synthesis at 72°C for 10 min.

The sizes of the species-specific PCR products are given in Table 2. An example of electrophoresis of PCR products in 2% agarose gel is shown in the figure.

The primers were designed to allow multiplex PCR; i.e., the reaction mixture can contain more than two primers. The PCR conditions and the concentrations of all primers should be optimized in every laboratory, since they may depend on the instruments, reagents, and plasticware employed in testing. To identify *A. gueldenstaedtii* and *A. baerii*, we routinely perform multiplex PCR with primers AGF, ABF, ABRM, and AHR (1 : 2 : 1.2 : 1) under the same amplification conditions as in single reactions.

The PCR product is applied onto 2% agarose gel (0.5× TBE) and resolved at 15 V/cm for 30 min. The species is inferred from synthesis of the amplification product in the reaction with particular species-specific primers. PCR with other primers is expected to yield no product. The products of all reactions carried out with one specimen can be applied in one well. In this case, the species is inferred from the size of the PCR product. For the convenience of species identification, we obtained a mixture of all possible PCR products (182, 190, 215, 253, 266, 329, 374, 420, and 429 bp). The mixture is applied on gel together with test samples and

Table 3. Examination of the test system with specimens from the Russian Federal Reference Collection of Genetic Materials

Species	Number of specimens examined	Number of correct identifications	%
Russian sturgeon <i>A. gueldenstaedtii</i> (typical mitotype)	480	480	100
Russian sturgeon <i>A. gueldenstaedtii</i> ("baerii"-like mitotype)	152	149	98*
Siberian sturgeon <i>A. baerii</i>	356	356	100
Fringebarbel sturgeon <i>A. nudiventris</i>	20	20	100
Stellate sturgeon <i>A. stellatus</i>	68	68	100
Persian sturgeon <i>A. persicus</i>	180	180	100**
Amur sturgeon <i>A. schrenkii</i>	17	17	100
Sterlet <i>A. ruthenus</i>	15	15	100
Beluga <i>H. huso</i>	120	120	100
Kaluga <i>H. dauricus</i>	25	25	100

Notes: * Three specimens were identified as *A. baerii* (see text for comments).

** *Acipenser persicus* was identified as having the typical *A. gueldenstaedtii* mitotype, while the sympatric Caspian *A. gueldenstaedtii* population displayed the "baerii"-like mitotype in up to 30% of cases and the typical mitotype in 70% of cases.

serves as a molecular weight marker, allowing visual identification of the test product (figure).

The samples and test results are characterized in Table 3. The method did not yield false results in the majority of cases, with the exception of discrimination between the *A. baerii* mitotype and the *baerii*-like mitotype of *A. gueldenstaedtii* (error lower than 1%). The strong specificity of the diagnostic nucleotide substitution detectable in *A. baerii* with primer ABRM was verified with large samples of the two species. The test result was at variance with the declared species in three cases. The mtDNA control region of the three questionable individuals was sequenced. Two individuals had the typical *A. baerii* haplotype and were probably hybrids (the individuals were collected in a fishery farm rearing *A. gueldenstaedtii*, *A. baerii*, and their hybrids for a long time). The third case was a reversion: the mitotype belonged to the cluster of closely related *baerii*-like sequences but had the substitution characteristic of *A. baerii*. Reversions are quite expectable given the high variation of the mtDNA control region, and polymorphism for the substitution does not ensure

species identification with a 100% reliability. With all false-positive results obtained when testing our system, the error of identifying *A. gueldenstaedtii* (both of the mitotypes) and *A. baerii* is approximately 1%, which is allowable is certification of the species composition.

Thus, our test system designed for molecular identification of the eight sturgeon species allows reliable species identification of sturgeons, caviar, and other sturgeon products for all but one species (*A. persicus*). To identify *A. persicus*, it is necessary to develop a test system taking advantage of the polymorphism of nuclear markers.

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