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Unusual Intraindividual Variation of the Nuclear 18S rRNA Gene is Widespread Within the Acipenseridae

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Abstract

Significant intraindividual variation in the sequence of the 18S rRNA gene is unusual in animal genomes. In a previous study, multiple 18S rRNA gene sequences were observed within individuals of eight species of sturgeon from North America but not in the North American paddlefish, *Polyodon spathula*, in two species of *Polypterus* (*Polypterus delhezi* and *Polypterus senegalus*), in other primitive fishes (*Erpetoichthys calabaricus*, *Lepisosteus osseus*, *Amia calva*) or in a lungfish (*Protopterus* sp.). These observations led to the hypothesis that this unusual genetic characteristic arose within the Acipenseriformes after the presumed divergence of the sturgeon and paddlefish families. In the present study, a survey of nearly all Eurasian acipenseriform species was conducted to examine 18S rDNA variation. Intraindividual variation was not found in the polyodontid species, the Chinese paddlefish, *Psephurus gladius*, but variation was detected in all Eurasian acipenserid species. The comparison of sequences from two major segments of the 18S rRNA gene and identification of sites where insertion/deletion events have occurred are placed in the context of evolutionary relationships within the Acipenseriformes and the evolution of rDNA variation in this group.

Sturgeons (Acipenseridae) and paddlefishes (Polyodontidae) are members of the order Acipenseriformes, a group of approximately 27 extant species distributed throughout North America and Eurasia (Birstein 1993, 2000). At present, most of the species of this group are threatened or endangered. Their ecological status, the polyploid nature of their genomes, and the "living fossil" status of the group (which makes them important for understanding vertebrate evolution in general—Gardiner 1984) have prompted research on various aspects of their biology, including their phylogenetic relationships and evolutionary genetics (Birstein 2005; Birstein and DeSalle 1998; Birstein et al. 1997, 2002; Fontana et al. 2001; Krieger et al. 2000; Ludwig et al. 2001; Simons et al. 2001).

While conducting a molecular evolutionary study of relationships among the North American acipenseriforms, intraindividual variation for the nuclear 18S rRNA gene was discovered in sturgeons (Krieger and Fuerst 2002). Substantial intraindividual variation in this gene is absent in virtually all other animal species for which 18S rDNA sequences have been obtained. In eukaryotic organisms in which intraindividual variation of the small-subunit rRNA gene has been observed, such as *Plasmodium* (Dame et al. 1984; Gunderson et al. 1987; Qari et al. 1994; Rogers et al. 1995; Waters et al. 1989), *Dugesia mediterranea* (Carranza et al. 1996, 1999), *Acanthamoeba* (Ledee et al. 1998), and *Trypanosoma cruzi* (Stothard et al. 2000), usually only two or three sequence variants are detected. PCR products from the 18S rRNA gene were sequenced for the North American paddlefish, *Polyodon spathula*, eight North American sturgeon species, and representatives of four non-acipenseriform fish groups (orders Polypteriformes, Lepisosteiformes, Amiiformes, and Dipnoi). As anticipated, no intraindividual sequence variation was seen in non-acipenseriform species or in *Polyo. spathula*. However, multiple sequence variants were unexpectedly detected in all nine North American sturgeons—*Acipenser oxyrinchus oxyrinchus*, *Acipenser oxyrinchus desotoi*, *Acipenser fulvescens*, *Acipenser brevirostrum*, *Acipenser transmontanus*, *Acipenser medirostris*, *Scaphirhynchus platorynchus*, *Scaphirhynchus albus*, and *Scaphirhynchus suttkusi*. This is an unusual phenomenon; concerted evolution is believed to ordinarily homogenize the multiple copies of rDNA found within individuals and to promote

Table 1. Species included in this study

Species name	Origin of specimen	Collector/provider
Psephurus gladius	Yangtze River, China	Qiwei Wei
Polyodon spathula	Hatchery fish—parents originally from Osage River Basin, USA	Jerry Hamilton
Scaphirhynchus albus	Hatchery fish—parents from Mississippi and Missouri rivers, USA[a]	Edward Little
Scaphirhynchus platorynchus	Missouri River, USA	Edward Little
Scaphirhynchus suttkusi	Alabama River, USA (UAIC# 1885.01)	Bernard Kuhajda
Pseudoscaphirhynchus kaufmanni	Amu Darya River, Turkmenistan	Vladimir Salnikov
Huso huso	Danube River, Romania	Neculai Patriche
Huso dauricus	Amur River, Russia (Siberia)	Victor Svirsky
Acipenser baerii	Hatchery fish—parents from Lena River, Russia (Siberia)	Patrick Williot
Acipenser brevirostrum	Hatchery fish—parents from Savannah River, USA	Kent Ware
Acipenser fulvescens	Wolf River, USA	Ronald Bruch
Acipenser gueldenstaedtii	Danube River, Romania	Neculai Patriche
Acipenser medirostris	Klamath River, USA	Tim Mulligan
Acipenser mikadoi	Tumnin River, Russia (Siberia)	Eugene Artyukhin
Acipenser naccarii	Po River, Italy	Lorenzo Zane
Acipenser nudiventris	Volga River, Russia	Lutz Debus
Acipenser oxyrinchus oxyrinchus	Hudson River, USA	Jerre Mohler
Acipenser oxyrinchus desotoi	Hatchery fish—Parents from Suwannee River, USA	Frank Chapman
Acipenser persicus	Southern Caspian Sea	Lutz Debus
Acipenser ruthenus	Volga River, Russia	Lutz Debus
Acipenser schrenckii	Amur River, Russia (Siberia)	Victor Svirsky
Acipenser sinensis	Yangtze River, China	Si-Ming Zhang
Acipenser stellatus	Danube River, Romania	Neculai Patriche
Acipenser sturio	Gironde River, France	Patrick Williot
Acipenser transmontanus	Snake River, USA	Terry Patterson

[a] Male parents were from the Missouri River and female parents were from the Mississippi River.

homogeneity of sequences within species (Arnheim 1983). The observations in sturgeons were the first description of intraindividual variation in small-subunit rRNA genes within a vertebrate.

The finding of 18S rDNA intraindividual variation in all North American species of sturgeons examined, but its absence in the North American paddlefish, suggested that the variation may have appeared within the Acipenseriformes after the evolutionary split between the Acipenseridae and Polyodontidae. This split is thought to have occurred in the Jurassic (Grande and Bemis 1991). To further investigate the hypothesis that intraindividual variation originated after the evolutionary split between the Acipenseridae and Polyodontidae, the current study expands the analysis of species by surveying as many Eurasian acipenseriform species as possible (including the Chinese paddlefish, *Psephurus gladius*) for intraindividual variation of the 18S rRNA gene. The results obtained from this survey, including the pattern of presence or absence of insertions or deletions (indels) found within sturgeon species and the variation observed in two segments of the 18S rRNA gene, are discussed in relation to acipenseriform rDNA evolution and evolutionary relationships within the Acipenseriformes.

Materials and Methods

Specimens

Tissue or genomic DNA samples were obtained for as many acipenseriform species as possible from generous researchers in the United States, Europe, and Asia, as summarized in Table 1. Today all sturgeon and paddlefish species are endangered or on the brink of extinction (Birstein 2000). For this reason, we were unable to locate samples of the following three Asian species: two rare *Pseudoscaphirhynchus* species—*Pseudoscaphirhynchus hermanni* from the Amu Darya River (Turkmenistan) and *Pseudoscaphirhynchus fedtschenkoi* from the Syr Darya River (Kazakhstan)—as well as *Acipenser dabryanus* from the Yangtze River (China).

DNA Extraction and PCR

DNA extraction, PCR amplification, and manual radioactive DNA sequencing of the nuclear 18S rRNA gene were carried out previously for nine North American acipenseriform species as described in Krieger and Fuerst (2002). For all tissue samples, genomic DNA extraction was carried out as specified in Krieger et al. (2000). However, some of the methods

Table 2. 18S rRNA gene PCR amplification and sequencing primers used in this study

Primer	Sequence (5′ to 3′)	Orientation
CRN5A	GGTTGATCCTGCCAGTAG	Forward
170	GCATGTATTAGCTCTAGA	Reverse
373	AGGCTCCCTCTCCGGAATC	Reverse
373C	GATTCCGGAGAGGGAGCCT	Forward
570	GCTATTGGAGCTGGAATTAC	Reverse
570C	GTAATTCCAGCTCCAATAGC	Forward
720C	GGGGTCCGAAGCGTTTAC	Forward
892C	GTCAGAGGTGAAATTCTTGG	Forward
1137	GTGCCCTTCCGTCAAT	Reverse
1262	GAACGGCCATGCACCAC	Reverse
1262C	GTGGTGCATGGCCGTTCTTA	Forward
1200	GGGCATCACAGACCTG	Reverse
1200C	CAGGTCTGTGATGCCC	Forward
1/F	CACACCGCCCGTCG	Forward
SSU2A	ATCCTGATCCCTCCGCAGGTTCAC	Reverse

described below for PCR amplification and DNA sequencing differ slightly from those used in the original experiments and refer to protocols used with samples of the Eurasian sturgeon and paddlefish species. The experimental work on these Eurasian species was shared between laboratories at Brown University in Providence, RI, and the Institute for Zoo and Wildlife Research in Berlin, Germany.

PCR amplification (Saiki et al. 1988) of the entire nuclear 18S rRNA genes from total DNA of one individual of each species was carried out either in one piece or in two fragments. If it was not possible to obtain a robust product when amplifying the gene in one unit from a sample, it was then amplified in overlapping fragments; this was the case for *Psep. gladius*, *Acipenser gueldenstaedtii*, *Acipenser schrenckii*, *Acipenser sturio*, *Acipenser ruthenus*, *Acipenser nudiventris*, *Acipenser mikadoi*, *Pseudoscaphirhynchus kaufmanni*, and *Huso dauricus*. Both types of amplifications were carried out in 50-µl reactions using one unit of Platinum *Taq* DNA Polymerase High Fidelity enzyme mix (Invitrogen, Carlsbad, CA). As with the original analysis of the North American species (Krieger and Fuerst 2002), a PCR amplification enzyme mix with proofreading capabilities was used. The lower error rate of Platinum *Taq* DNA Polymerase High Fidelity when compared to *Taq* DNA polymerase used alone reduces the possibility that any sequence variation observed resulted from DNA polymerase errors during amplification. Complete Eurasian acipenseriform 18S rRNA genes were amplified with the primers CRN5A and SSU2A using the following conditions: 2 min at 94°C, then 35 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 68°C. When the gene was amplified as two fragments, the 5′ fragment was amplified with primers CRN5A and 1137 and the 3′ fragment was amplified with primers 892C and SSU2A, both with the following conditions: 2 min at 94°C, then 35 cycles of 1 min at 94°C, 2 min at 52°C, and 3 min at 68°C. The nucleotide sequences of 18S rDNA amplification primers are listed in Table 2. As expected, the size of the gene was approximately 1,800 bp in all species examined here.

Gene Sequence Determination

Prior to sequencing, all PCR products were cleaned with the Qiagen Qiaquick PCR purification kit (Qiagen, Valencia, CA) to remove unincorporated nucleotides and primers, according to instructions. Gene sequences were determined as completely as possible by automated fluorescent DNA cycle sequencing using the Big Dye Terminator kit v1.0 in the United States and the Big Dye Terminator kit v3.1 in Germany (Applied Biosystems, Foster City, CA). Multiple internal sequencing primers designed to obtain overlapping sequence from both strands were used to obtain maximum gene sequence information (see Table 2; in addition, a map of primer locations may be viewed in Krieger and Fuerst 2002). Sequencing products were visualized on either an ABI 377 DNA Sequencer in the United States or an ABI 3100 DNA Sequencer in Germany (Applied Biosystems).

The generated sequences were collected, assembled when possible, and examined. The complete sequence for the Chinese paddlefish 18S rRNA gene, which lacks intraindividual variation, was deposited in GenBank. In addition, sequences from two readable segments of the 18S rRNA gene PCR products, regions of 505 and 283 bp in length, were recorded and submitted for the newly determined Eurasian acipenseriform species as well as for the North American species whose sequences were previously determined (Krieger and Fuerst 2002). These two segments were located between indel events but did contain multiple bases at some sites in their sequences. The accession numbers for all deposited sequences can be found in Table 3.

Results

The variation originally discovered in North American sturgeon species by Krieger and Fuerst (2002) was detectable as multiple bands produced by manual radioactive DNA cycle sequencing of PCR products. The full gene sequence cannot be determined directly because of the multiple products produced by substitution and indel events. Cloning and sequencing of the entire 18S rRNA gene in two *Acipenser* species (*A. fulvescens* and *A. brevirostrum*) confirmed the presence of variation and characterized the location and identity of sequence changes in different versions of the gene present in an individual (Krieger and Fuerst 2002). The current experiments employed automated fluorescent DNA cycle sequencing, producing electropherograms representing a DNA sequence. The electropherogram can be examined for the presence of multiple sequence variants within the individuals studied. On the electropherograms, nucleotide heterogeneity was visible as multiple peaks at a site. In a similar manner, substitutions were identified by observing multiple bands when reading the sequencing ladders of radioactively determined DNA sequence. Indels were identified by the presence of a clear, readable sequence up to the point of the indel, followed by many successive multiple peaks due to the presence of two or more sequences within the genome that differ in the presence of the indel site. The presence of polymorphism within the genome for the indel makes the sequence unreadable

Table 3. GenBank accession numbers for 18S rRNA gene sequences

Species name	GenBank accession numbers
Psephurus gladius	Whole gene—AY544131
Polyodon spathula[a]	Whole gene—AF188371
Scaphirhynchus platorynchus[a]	Partial gene—AF188372
Pseudoscaphirhynchus kaufmanni	Segment 1—AY904474; segment 2—AY904456
Huso huso	Segment 1—AY904473; segment 2—AY904455
Huso dauricus	Segment 1—AY904472; segment 2—AY904454
Acipenser baerii	Segment 1—AY904463; segment 2—AY904445
Acipenser brevirostrum[a]	Segment 1—AY904476; segment 2—AY904458
Acipenser fulvescens[a]	Segment 1—AY904475; segment 2—AY904457
Acipenser gueldenstaedtii	Segment 1—AY904464; segment 2—AY904446
Acipenser medirostris[a]	Segment 1—AY904477; segment 2—AY904459
Acipenser mikadoi	Segment 1—AY904465; segment 2—AY904447
Acipenser naccarii	Segment 1—AY904466; segment 2—AY904448
Acipenser nudiventris	Segment 1—AY904467; segment 2—AY904449
Acipenser oxyrinchus desotoi[a]	Segment 1—AY904480; segment 2—AY904462
Acipenser oxyrinchus oxyrinchus[a]	Segment 1—AY904479; segment 2—AY904461
Acipenser persicus	Segment 1—AY904468; segment 2—AY904450
Acipenser ruthenus	Segment 1—AY928665; segment 2—AY928667
Acipenser schrenckii	Segment 1—AY904469; segment 2—AY904451
Acipenser sinensis	Segment 1—AY904470; segment 2—AY904452
Acipenser stellatus	Segment 1—AY904471; segment 2—AY904453
Acipenser sturio	Segment 1—AY928664; segment 2—AY928666
Acipenser transmontanus[a]	Segment 1—AY904478; segment 2—AY904460

[a] The sequences from these species were determined previously (Krieger and Fuerst 2002).

after the site of the indel event. The use of multiple primers, however, usually allowed sequence determination up to the point of an indel in both directions, barring the presence of another major indel event occurring between the sequencing primer and the original target indel event. This sequencing strategy permitted the determination of the location of major indel events.

Examination of sequences from one individual of each of the 15 different Eurasian acipenseriform species gave the following results. Intraindividual variation consisting of both indels and substitutions was present in all of the sturgeon species. No variation was detected in the Chinese paddlefish, *Psep. gladius*. Lack of intraindividual variation in the North American paddlefish (*Polyo. spathula*) has been confirmed in multiple individuals (Krieger J, unpublished data), but unfortunately, the extreme rarity of *Psep. gladius* did not allow examination of any additional specimens of this species to verify lack of variation.

Examination of the sequences allowed the mapping of the locations of most indel events. Based on the observed locations of indels found in both North American and Eurasian sturgeon species, it is clear that there are six hot spots in the gene where indels occur most often within the Acipenseriformes. The positions of these indel hot spots, with reference to the *Polyo. spathula* 18S rRNA gene sequence, along with information about the presence or absence of an indel at these sites within each species, are shown in Table 4. Most species examined show evidence of indel events at all six sites. Two of the six sites lack indel events in one species each: one is absent in *A. transmontanus* (position 772) and the other in *Acipenser sinensis* (position 1694). Further, two additional indel sites show restricted taxonomic distribution. One indel is specific to *A. transmontanus* (position 1149), and another is specific to the two *A. oxyrinchus* subspecies and *A. sturio* (position 1485).

The three species of *Scaphirhynchus* represent a major exception to the occurrence of the shared indel locations. These three species appear to possess similar variants that have only two indel sites, located near each other in the second half of the gene. These two indel sites in *Scaphirhynchus* (positions 1366 and 1694) are shared with the members of *Acipenser* (see Table 4). This lesser occurrence of indel variation allowed a large portion of the 18S rRNA gene sequence to be read directly (*S. platorynchus*, GenBank accession number AF188372; Krieger and Fuerst 2002). Please note that only *S. platorynchus* is included in the current comparisons and analyses.

The location of indel sites within the 18S rRNA molecule was determined using a *Polyo. spathula* secondary structure model previously constructed utilizing information from accepted small-subunit rRNA secondary structures (Cannone et al. 2002; Gutell 1994). The six common indel regions are spread throughout the 18S rRNA molecule. Five of the six common indel sites are clearly located in hairpin loop regions of the rRNA molecule or in regions with an unidentified secondary structure (as opposed to base-paired stem regions). The *A. transmontanus*–specific indel site is located at the end of a stem region, near the hairpin loop, and the indel site specific to the two *A. oxyrinchus* subspecies and *A. sturio* is located in an internal loop or bulge within a base-paired stem region. The location of indel sites was also compared with an analysis of phylogenetic conservation based on comparison of 1,939

Table 4. Presence or absence of 18S rRNA gene indel events within acipenseriform species possessing intraindividual variation of the 18S rRNA gene

	Nucleotide position[a]							
Species	53	267	772	841	1149	1366	1485	1694
Acipenser baerii	+[b]	+	+	+	−	+	−	+
Acipenser brevirostrum	+	+	+	+	−	+	−	+
Acipenser fulvescens	+	+	+	+	−	+	−	+
Acipenser gueldenstaedtii	+	+	+	N/D	−	+	−	+
Acipenser medirostris	+	+	+	+	−	+	−	+
Acipenser mikadoi	+	+	+	+	−	+	−	+
Acipenser naccarii	+	+	+	+	−	+	−	+
Acipenser nudiventris	+	+	+	N/D	−	+	−	+
Acipenser oxyrinchus desotoi	+	+	+	+	−	+	+	+
Acipenser oxyrinchus oxyrinchus	+	+	+	+	−	+	+	+
Acipenser persicus	+	+	+	+	−	+	−	+
Acipenser ruthenus	+	+	+	+	−	+	−	+
Acipenser schrenckii	+	+	+	+	−	+	−	+
Acipenser sinensis	+	+	+	+	−	+	−	−
Acipenser stellatus	+	+	+	+	−	+	−	+
Acipenser sturio	+	+	+	+	−	+	+	+
Acipenser transmontanus	+	+	−	+	+	+	−	+
Huso dauricus	+	+	+	+	−	+	−	+
Huso huso	+	+	+	+	−	+	−	+
Pseudoscaphirhynchus kaufmanni	+	+	+	+	−	+	−	+
Scaphirhynchus platorynchus	−	−	−	−	−	+	−	+

[a] Indel positions are numbered with reference to the *Polyo. spathula* 18S rRNA gene sequence (GenBank accession number AF188371).

[b] + Indicates presence of an indel event at a site, − indicates no evidence of an indel event at a site, and N/D indicates that the presence or absence of an indel event at that site could not be detected due to technical difficulties in sequencing.

eukaryotic small-subunit rRNAs produced by Cannone et al. (2002). All six common indel hot spots, as well as the two species-specific sites, are located in regions of the molecule that show reduced sequence conservation and higher variability among eukaryotes. Previous results from cloning and sequencing of the 18S rRNA gene variants from *A. fulvescens* and *A. brevirostrum* indicate that indels can and do also occur at other sites within the gene (Krieger and Fuerst 2002). In these two species, however, additional variation appears to be found in rare variant sequences of the 18S rRNA gene and so may not be detected during PCR product sequencing as was carried out here.

Previous studies, especially in *A. fulvescens*, indicated that in addition to the presence of indel events, substitutions that produce intraindividual variation occurred. To assess whether this occurs in other species, we compared the nucleotide sequences of two readable segments from the PCR products of the 18S rDNA gene within the acipenseriform species examined. Segment one, which is 505 bases in length, corresponds to positions 268–771 of the *Polyo. spathula* sequence (GenBank accession number AF188371), and segment two, which is 283 bases in length, corresponds to *Polyo. spathula* positions 890–1172. Electropherograms were examined to determine the possible presence of significant multiple peaks at a site. Thirty-two (4.1%) of 789 homologous sites were identified as showing variability either within species or between species. Twenty-eight of these sites (3.6% of the total alignment) were detected as showing intraindividual polymorphism within one or more species. We note here that this represents a minimal estimate of polymorphism because the detection of polymorphic sites is likely to be dose dependent. The frequency within the repetitive array of rRNA genes in the genome of alleles containing the alternative nucleotides at a particular polymorphic site will affect the ability to identify a site as polymorphic in a mixed PCR product, and so the sites detected and the frequency of variants may differ among individuals. The most likely result of this phenomenon is the underestimation of polymorphic sites. For example, a number of sites that were found to be polymorphic in a study of cloned 18S rRNA gene sequences in *A. fulvescens* (Krieger and Fuerst 2002, 2004) did not appear as polymorphisms in the examination of PCR product sequences carried out here.

Thus, among 789 homologous sites in the two common portions of the 18S rRNA gene sequence that were determined in all species, 28 sites showed intraindividual variability in at least one species. No polymorphic sites were detected in the two paddlefish genera (*Polyodon* and *Psephurus*), and there was no site in the sequence studied here that showed intraindividual nucleotide variation in *S. platorynchus*. Note that we previously determined that the various species of *Scaphirhynchus* display intraindividual variation but that this variation appears to occur at a lower frequency within the 18S rRNA sequence of *Scaphirhynchus* than was seen in comparable portions of the gene from several North American species of *Acipenser* (Krieger and Fuerst 2002). All other species or

Table 5. Number of sites, among 789 total sites in two segments of the 18S rRNA gene sequence, that show intraindividual variation in each of the species included in this study

Species	Number of variable sites
Polypterus senegalus	0
Paddlefish	
Polyodon spathula	0
Psephurus gladius	0
Eurasian sturgeon	
Acipenser baerii	10
Acipenser gueldenstaedtii	9
Acipenser mikadoi	10
Acipenser naccarii	10
Acipenser nudiventris	9
Acipenser persicus	12
Acipenser ruthenus	13
Acipenser schrenckii	11
Acipenser sinensis	11
Acipenser stellatus	11
Acipenser sturio	15
Huso dauricus	9
Huso huso	9
Pseudoscaphirhynchus kaufmanni	18
North American sturgeon	
Acipenser brevirostrum	12
Acipenser fulvescens	10
Acipenser medirostris	9
Acipenser oxyrinchus desotoi	12
Acipenser oxyrinchus oxyrinchus	12
Acipenser transmontanus	9
Scaphirhynchus platorynchus	0

subspecies of sturgeon showed intraindividual nucleotide variation at a number of sites. Table 5 shows the number of sites among the 28 variable sites that were found to show intraindividual variability in each of the taxa, with the number of sites showing intraindividual variation ranging from 9/28 to 18/28 in different species. Six sites showed intraindividual variability for the same two nucleotides in all 20 species or subspecies of *Acipenser*, *Huso*, and *Pseudoscaphirhynchus* examined. Four additional sites show intraindividual variation in the majority of taxa. In contrast, 7 sites were found to be variable in only a single species (three sites each in *Pseu. kaufmanni* and *A. sturio* and a single unique site in *A. brevirostrum*), while 11 additional sites are variable in less than half of the taxa.

The 18S rRNA gene has limited value for phylogenetic studies of acipenseriform taxa due to the intraindividual variation found in most sturgeon species (see Krieger and Fuerst 2002). Paradoxically, the phylogenetic utility of the 18S rRNA genes for this group is further diminished by the low degree of divergence among species that is observed in the readable gene sequence. In phylogenetic analyses of our combined readable segment sequences, the tree topology of acipenseriform taxa was essentially undifferentiated. Thus, aside from the information provided by the presence or absence of specific polymorphic sites among species, sequences from the 18S rRNA gene offer little information useful for elucidating evolutionary relationships among the Acipenseriformes.

Discussion

In this study we have examined the hypothesis that the intraindividual variation in the nuclear 18S rRNA gene arose within the order Acipenseriformes after the presumed divergence of the sturgeon and paddlefish families. Our survey for this unusual genetic characteristic in 24 species of the order Acipenseriformes revealed that the polyodontid species do not possess intraindividual variation but all acipenserid species do possess such variation.

To verify that the *Polyodon* and *Psephurus* form a clade separate from the Acipenseridae, a phylogenetic tree was constructed for the order Acipenseriformes using combined data for five mitochondrial gene sequences (cyt-b, 12S rRNA, COII, $tRNA_{Asp}$, and $tRNA_{Phe}$ genes; details of the phylogeny will be discussed elsewhere, see also Ludwig et al. 2000, 2001). In this analysis, the two paddlefish species do cluster, forming a sister clade to the sturgeon group. Also of note is that *A. sturio* clusters with *A. oxyrinchus*, as expected from previous molecular phylogenetic studies (Birstein and DeSalle 1998; Birstein et al. 2002; Fontana et al. 2001; Ludwig et al. 2000, 2001). This is consistent with the observation that the indel within the 18S rDNA found at position 1485 is unique to *A. sturio*, *A. o. oxyrinchus*, and *A. o. desotoi*, further supporting the close relationship of *A. sturio* and the *A. oxyrinchus* subspecies. Further, within the readable fragments of the 18S rRNA sequence, the three species uniquely share three polymorphic sites, compared to the other taxa, and uniquely lack variation at one other site that is variable in all other *Acipenser* and *Huso* taxa. In other considerations, in our analysis there are two shared, unique polymorphic sites that are detected within *Acipenser stellatus* and *Pseu. kaufmanni*, supporting an unorthodox placement of *Pseu. kaufmanni* within the Acipenserinae and clustered with *A. stellatus* (as first described by Birstein et al. 2002), rather than within the Scaphirhynchinae. In addition, the number and pattern of multiple indel sites seen in the gene and the level of polymorphism found within *Pseu. kaufmanni* for the two gene fragments examined are similar to that found in other species of the Acipenserinae, further supporting its placement with the Acipenserinae as the three *Scaphirhynchus* species show a smaller number of indel events and less polymorphism within their 18S rRNA gene sequences (Krieger and Fuerst 2002).

Our results are consistent with the proposed origin of intraindividual 18S rDNA variation after the split between the Acipenseridae and Polyodontidae. Because no evidence for 18S rDNA variation has yet been found in the Polyodontidae, Polypteriformes, Amiiformes, Lepisosteiformes, Dipnoi, or any other group of vertebrates, the most likely scenario is that the variation arose within the ancestor of the Acipenseridae. It seems unlikely that such a rarely observed genetic characteristic would have evolved independently in 22 different species belonging to four major lineages. The existence of multiple shared indel hot spots/sites, as well as a number of ubiquitous polymorphic sites in sturgeon 18S rRNA genes across species, also supports the concept that the variation in different sturgeon species had a common origin. However, the possibility that the variation arose

before the split between the two acipenseriform families and was later lost in the Polyodontidae cannot be completely ruled out.

It is unclear how exactly rDNA intraindividual variation arose in sturgeons. Krieger and Fuerst (2002) proposed that a polyploid origin of the Acipenseriformes (Birstein et al. 1997; Ludwig et al. 2001) may be linked to the development of this trait. The alternative possibility, hybridization, has been proposed to explain variation in the ITS regions of the rDNA repeat units in a number of polyploid plant species (e.g., Campbell et al. 1997; Sang et al. 1995) and the 5.8S rDNA sequence diversity at both the species and individual levels in the coral *Acropora* (Márquez et al. 2003). But with a very low level of divergence observed in the presumptive functional acipenseriform 18S rRNA genes (0.06%–0.3% difference; Krieger and Fuerst 2004), it is improbable that hybridization could have brought together divergent parental sequence variants in the new species and created the sequence diversity observed in variable sturgeon species.

Apparently, a reduced rate of concerted evolution is characteristic for the acipenserids. This would slow down the loss of any polymorphic variation that arose. A study by De la Herrán et al. (2001) showed that the *Hin*dIII satellite DNA family, which like 18S rRNA genes is a tandemly repeated sequence, also exhibits low rates of homogenization within sturgeon species. In addition to the *Hin*dIII family, a lack of concerted evolution was demonstrated in a number of the *Acipenser* species for the *Pst* satellite DNA family and for the 5S rRNA gene spacer sequences (Robles et al. 2004, 2005).

Future studies should focus on the evolution and diversification of the 18S rRNA genes in individual sturgeon species, on a comparison of the evolutionary trends in different species, and on the selective expression of the gene variants in these fishes (Krieger and Fuerst 2004). As sturgeon and paddlefish species are becoming increasingly imperiled, we hope that the demonstration of the widespread unusual genetic phenomenon described in this paper will attract more attention to these ancient fishes and to the urgent necessity to protect them.

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