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The molecular phylogeny of the order Acipenseriformes revisited

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Summary

As evolutionary relationships within the order Acipenseriformes are not well understood and some classifications are currently controversial, the study of evolutionary relationships, especially based on genetic data, has received much recent attention. In this reanalysis we present a nearly complete proposed phylogeny of the order, including 25 species, based on the maximum likelihood analysis of combined DNA sequence data (4406 base pairs) from five mitochondrial genes sequenced in our laboratories (cytochrome *b*, 12S rRNA, cytochrome *c* oxidase subunit II, $tRNA_{Asp}$ and $tRNA_{Phe}$) and three mitochondrial gene regions sequenced by Birstein et al. (2002) (16S rRNA, NADH5 and control region). Examination of the molecular phylogeny using either maximum likelihood, Bayesian analysis, maximum parsimony or neighbor-joining leads to the following conclusions: (i) the two species of paddlefish do form a clade; (ii) the most basal position within the Acipenseridae remains unresolved, held either by the genus *Scaphirhynchus* or by the clade containing *Acipenser oxyrinchus* and *A. sturio*; (iii) *Huso* is not monophyletic, with the two species of *Huso* found embedded separately within the genus *Acipenser*; (iv) *A. sinensis* and *A. dabryanus* are confirmed as closely related; (v) the previously described Atlantic-Pacific subdivision within the *Acipenser / Huso* complex is supported and (vi) the unexpected placement of *Pseudoscaphirhynchus kaufmanni* within *Acipenser* is supported by this analysis. These results offer further evidence that some revision of acipenseriform classification may be needed to accurately inform conservation efforts and that future phylogenetic studies of this group should focus on the analysis of nuclear genes.

Introduction

The ancient order Acipenseriformes – one of the most primitive lineages of recent vertebrates – is first recognizable in the Jurassic, approximately 200 million years before present (Grande and Bemis, 1991). In current classification it consists of two families: family Polyodontidae, which contains two species of paddlefish, one in North America and the other in China; and family Acipenseridae, the sturgeons, which contains the subfamilies Acipenserinae (including the genera *Acipenser* and *Huso*) and Scaphiyrhynchinae (including the genera *Scaphirhynchus* and *Pseudoscaphirhynchus*) (Berg, 1904, 1940). As most of the approximately 27 extant sturgeon and paddlefish species are considered threatened or endangered (Ludwig, 2008), there has been considerable research focused on them in recent years. Population declines resulting from overfishing for meat and caviar, the damming of rivers used for spawning, and water pollution combined with certain life history characteristics (slow sexual maturation and females that don't reproduce every year) have made the study of artificial propagation, ecology and conservation measures a necessity for many species (Ludwig, 2006). In addition, the phylogeny of the group is also of great interest due to the fragile status of many of these species. A number of features of the classification of the group, historically based on morphological characters, have been called into question due to the results from molecular studies.

However, even the results from different molecular phylogenetic studies have been in conflict. Birstein and DeSalle (1998) published the first molecular phylogeny of Acipenseriformes, based on partial sequences of three mitochondrial genes (650 bases – cytochrome *b*; 350 bases – 16S rDNA; 150 bases – 12S rDNA). Later, Birstein et al. (2002) expanded their work, using a larger data set of partial genes (cytochrome *b*, 12S rRNA, 16S rRNA, NADH5 and control region – 2443 bases in total), while also including four additional sturgeon species. Other studies based on various whole or partial mitochondrial genes (Krieger et al., 2000; – 12 S rRNA, cytochrome *c* oxidase subunit II, $tRNA_{Asp}$ and $tRNA_{Phe}$; Zhang et al., 2000; – ND4L and ND4; Ludwig et al., 2000, 2001; Peng et al., 2007; – cytochrome *b*) did not support several phylogenetic relationships found by Birstein and DeSalle (1998) and Birstein et al. (2002). For example, a distant relationship between the North American *Acipenser fulvescens* and *A. brevirostrum* had been suggested in the maximum parsimony trees of Birstein and DeSalle (1998) and Birstein et al. (2002) (but not by the maximum likelihood tree found in this same paper). In contrast, Krieger et al. (2000) and Ludwig et al. (2000, 2001) found these two species located near one another in their phylogenetic reconstructions. Two Chinese species, *A. sinensis* and *A. dabryanus*, were found to be sister species by Zhang et al. (2000) but not by Birstein and DeSalle (1998). In addition, *A. medirostris* and *A. mikadoi* were identified as sister species by Ludwig et al. (2000, 2001) but not by Birstein and DeSalle (1998) or Birstein et al. (2002). Birstein and DeSalle (1998) also placed the two species *Huso huso* and *H. dauricus* within the genus *Acipenser*, and their trees showed them clustered with *A. ruthenus*. Studies by Zhang et al. (2000) and Ludwig et al. (2000, 2001) agreed that *Huso* belongs within *Acipenser*, but did not find the two species of *Huso* to be closely related. Due to disagreements among the molecular

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phylogenetic studies, we combined the sample sets of our different working groups, plus non-overlapping sequence data of Birstein et al. (2002) to further study the phylogenetic relationships of this fascinating group of fishes. Because many populations of sturgeon are on the brink of extinction, future studies may be limited to the few species / specimens archived in museum collections. A proposed molecular phylogeny of twenty-five species of the order Acipenseriformes was produced by determining and analyzing 4406 bases of sequence from eight combined mitochondrial genes (cytochrome *b* or cyt-*b*, 12S rRNA, cytochrome *c* oxidase subunit II or COII, $tRNA_{Asp}$, $tRNA_{Phe}$, 16S rRNA, NADH5 and control region). The noteworthy features of the resulting phylogeny are compared to those of other recent molecular based hypotheses of acipenseriform evolution and discussed with respect to the ecology of these species.

Materials and methods

Specimens

Samples were obtained for as many acipenseriform species as possible from generous researchers and fisheries in the United States, Europe and Asia. Table 1 shows information on the tissue or genomic DNA samples used for 12S rRNA, cytochrome *c* oxidase subunit II, $tRNA_{Asp}$ and $tRNA_{Phe}$ gene sequencing, as well as the samples of *Psephurus gladius* and *Pseudoscaphirhynchus kaufmanni* used for cytochrome *b* gene sequencing. The information on the remaining samples used for cytochrome *b* gene sequencing may be found in Ludwig et al. (2001). We were unable to locate samples of three Eurasian species: two rare *Pseudoscaphirhynchus* species and *Acipenser dabryanus*, but whole mitochondrial DNA sequence for *A. dabryanus* from D.Q. Wang has recently become available in GenBank (Accession number AY510085).

DNA extraction and PCR amplification

Genomic DNA extraction from tissue samples was carried out by the methods specified in Krieger et al. (2000) or Ludwig et al. (2001). Experimental work on the newly studied sequences was shared between laboratories at Brown University in Providence, Rhode Island and the Leibniz-Institute for Zoo and Wildlife Research in Berlin, Germany.

The entire cytochrome *b* gene (cyt-*b*) and some flanking sequences, comprising 1221 bp (base pairs), were amplified using flanking tRNA primers L-14735 (Wolf et al., 1999) and cytb-rev1 (Ludwig et al., 2000) as well as four additional primers to permit sequencing of overlapping fragments described in Ludwig et al. (2000). Amplifications were carried out with 1 unit *Taq* DNA polymerase (Qiagen, The Netherlands), 10 pmoles of each primer, 50 ng DNA, 0.10 mM Tris–HCl (pH 8.8 at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 $\mu g\ \mu l^{-1}$ BSA, 0.08% (v / v) Nonidet P40 and 100 μM of each dNTP. The following reaction conditions were used: 60 s at 94°C, 30 s at 55°C, 90 s at 72°C for 30 cycles, and a final polymerization at 72°C for 5 min.

PCR amplification of the mitochondrial 12S rRNA and $tRNA_{Phe}$ genes was accomplished using one of two sets of primers, one producing a larger fragment including the D-loop region and the entire $tRNA_{Phe}$ gene (primers Tthr and Tval-rev) and the other amplifying a smaller region containing only the 12S rRNA gene and the 3′ half of the $tRNA_{Phe}$ gene (primers Tphe and Tval-rev). The shorter piece was amplified if a good product could not be produced with the primers targeting the larger region, and this was necessary for *P. gladius*, *Acipenser schrenckii*, *A. nudiventris*, *A. gueldenstaedtii*, *A. sinensis*, *A. naccarii*, *A. mikadoi*, *P. kaufmanni* and *H. dauricus*. Reaction conditions for the larger Tthr and Tval-rev fragment were 3 min at 93°C, then 32 cycles of 1 min at 93°C, 1.5 min at 58°C and 3 min at 72°C, while PCR amplification of the shorter Tphe and Tval-rev region was

Table 1
List of samples of species used for 12S rRNA, COII, $tRNA_{Asp}$ and $tRNA_{Phe}$ gene sequencing

Species name	Origin of specimen	Collector / provider of sample
Psephurus gladius[a]	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[b]	Edward Little
Scaphirhynchus platorynchus	Missouri River, USA	Edward Little
Scaphirhynchus suttkusi	Alabama River, USA (UAIC# 1885.01)	Bernard Kuhajda
Pseudoscaphirhynchus kaufmanni[a]	Amu Darya River, Turkmenistan (Zoological Institute of Academy of Sciences, Russia, Collection number #8420)	Eugene N. Artyukhin
Huso huso	Danube River, Romania	Neculai Patriche
Huso dauricus	Amur River, Russia	Victor Svirsky
Acipenser baerii	Hatchery fish – parents from Lena River, Russia	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	Eugene N. Artyukhin
Acipenser naccarii	Po River, Italy	Lorenzo Zane
Acipenser nudiventris	Volga River, Russia	Lutz Debus
Acipenser oxyrinchus	Hudson River, USA	Jerre Mohler
Acipenser persicus	Southern Caspian Sea	Lutz Debus
Acipenser ruthenus	Danube River, Romania	Neculai Patriche
Acipenser schrenckii	Amur River, Russia	Victor Svirsky
Acipenser sinensis	Yangtze River, China	Si-Ming Zhang
Acipenser stellatus	Donau River, Romania	Neculai Patriche
Acipenser sturio	Gironde River, France	Patrick Williot
Acipenser transmontanus	Snake River, USA	Terry Patterson

[a]These specimens were also used for cytochrome *b* gene sequencing.
[b]Male parents were from the Missouri River and female parents were from the Mississippi River.

carried out using the same conditions but with an annealing temperature of 55°C instead of 58°C. As it was a smaller fragment than any of the other regions studied here, the COII and $tRNA_{Asp}$ genes were able to be amplified from all species using the primers Tser and Tlys-rev under the following conditions: 3 min at 93°C, then 32 cycles of 1 min at 93°C, 1.5 min at 50°C and 2.5 min at 72°C. The Eurasian 12S rRNA, COII, $tRNA_{Asp}$ and $tRNA_{Phe}$ gene amplifications were carried out in 50 μl reactions using 1.25 units of *Taq* DNA Polymerase (Promega, Madison, WI or AmpliTaq, ABI, Foster City, CA).

Gene sequence determination

Prior to sequencing, all PCR products were cleaned according to instructions with the Qiaquick PCR purification kit (Qiagen, Valencia, CA) to remove unincorporated nucleotides and primers. As there were difficulties directly sequencing the 12S rRNA / $tRNA_{Phe}$ gene PCR products for *H. dauricus*, *A. schrenckii*, *A. sinensis*, *A. naccarii and A. nudiventris*, these regions were cloned using TOPO TA Cloning® kit for Sequencing (Invitrogen, Carlsbad, CA). However, when multiple clones from the individual fish representing these five species were examined, no differences in sequence were detected among clones isolated from a species. Gene sequences from PCR products and cloned PCR products 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). 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). Nucleotide sequences of primers used for DNA amplification and sequencing of the five mitochondrial genes in all acipenseriform species studied (North American and Eurasian) are listed in Table 2. Note that not all primers listed in the tables were necessary or used to determine the gene sequences for all species. Some primers were specially designed to obtain sequence from a particular species, and some were needed or not depending on whether manual radioactive or automated sequencing was being used at the time of the sequencing.

Sequences were collected, assembled, aligned and have been deposited in GenBank. The accession numbers for the previously determined gene sequences from acipenseriform species are: cytochrome *b* genes – AJ245825–AJ245841, AJ249692–AJ249694, AJ251451, AJ252186–AJ252187, U55994 (all but *Psephurus* and *Pseudoscaphirhynchus*); 12S rRNA genes – AF125594 through AF125603; cytochrome *c* oxidase subunit II genes – AF125652 through AF125661; phenylalanine tRNA genes – AF125363 through AF125372; and aspartic acid tRNA genes – AF125261 through AF125270. Accession numbers for the acipenseriform gene sequences newly determined here are as follows: cytochrome *b* genes – DQ195872–DQ195873 (*Psephurus* and *Pseudoscaphirhynchus*); 12S rRNA genes – AY544134 through AY544147, AY661695 and DQ202325; cytochrome *c* oxidase subunit II genes – AY547408 through AY547421, AY661696 and DQ202326; phenylalanine tRNA genes – AY550278 through AY550291, AY661698 and DQ202325; and aspartic acid tRNA genes – AY547422 through AY547435, AY661697 and DQ202327. As mentioned above, the complete mtDNA sequence of *A. dabryanus* (accession number AY510085) is available in GenBank and the eight relevant gene sequences used in this study were taken from this entry and included in the phylogenetic analyses. The accession numbers for the partial 16S rRNA, NADH5, and control region gene sequences may be found in Birstein et al. (2002).

Sequence alignment and molecular phylogenetic analyses

Gene sequences from *Polypterus ornatipinnis* were included as a representative of the generally accepted most closely related

Table 2
Mitochondrial DNA primers and their nucleotide sequences used in this study

Genes	Primer name	Primer sequence (5′-to-3′)
12S rRNA and $tRNA_{Phe}$:	Tthr	AGAGCGCCGGTCTTGTAATCC
	Tphe	AGCGTAGCTTAACTAAAGCATAA
	Tval-rev	GCATGGATGTCTTCTCGGTGTA
	12S.1	GCCTAGCCACACCCCCACGG
	12S.4	AGTCCACGGCGTAAAGCGTG
	12S.1-rev	GGGTGTGGCTTAGCAAGGCGT
	12S.2	GGTCAATTTCGTGCCAGCCA
	12Sa-rev	TAGTGGGGTATCTAATCCCAG
	12Sb-rev	TTGGAGCCTCTCGTATAACCG
	12S.3 Stu	CCACCTAGAGGAGCCTGTTC
	Tval-seqR	TCGGTGTAAGGGAGGTGCTTT
COII and $tRNA_{Asp}$:	Tser	CCCCATATGCTGGTTTCAAG
	Tlys-rev	CACCAATCTTTGGCTTAAAA
	Cox II A	TAGGCCACCAATGATATTGAAG
	Cox II Ao	GCTATAGGACATCAATGATACTGAAG
	Cox II Ab	AAAGCTATAGGACACCAGTGATACTGAAG
	Cox II E-rev	AATTGGGGACNNNATGGGTACTAC
	CoxII Pad-rev	GGACTCCATGGGTACTACTA
	Cox II Stu-rev	TATTCGATGGTCTGCTTCTAGG
	CoxII J-rev	GCGTCTTGGAATCCTAATTGTG
	Tser-seqF	TCAAGCCAGCCGCATAAC
	Tlys-seqR	CGCTATTCCCTATTTAGCTTCT
Cytochrome *b*:	cytb-for1	CGTTGTHWTTCAACTAYARRAAC
	cytb-rev1	CTTCGGTTTACAAGACCG
	cytb-L113	GCCTCTGCCTTRTCAC
	cytb-L823	CTCTTYGCCTACGCCATYC
	cytb-H835	CGTAGGCRAAGAGRAAG
	cytb-H345	GATRTTTCAGGTYTCTTTTTG
	L-14735	AAAAACCACCGTTGTTATTCAACTA

outgroup taxon, the order Polypteriformes. The *P. ornatipinnis* gene sequences were taken from the complete mtDNA sequence determined by Noack et al. (1996) (GenBank accession number U62532). The sequence alignment program ESEE (Cabot and Beckenbach, 1989) was used to manually align the sequences with the aid of secondary structure models in the cases of the rRNA (Gutell, 1994) and tRNA genes (Chang et al., 1994) and the protein reading frame in the case of the COII and cyt-b genes. Regions of the 12S rRNA gene that could not be reliably aligned were removed from the alignment (26 sites in total), leaving 944 bases in the final 12S rRNA gene alignment. The sequence from a small region at the 3′ end of the COII gene (24 sites) could not be obtained in all species and so was not included in the analyses, making the final alignment for this gene 667 bases. Due to the necessity in some species of amplifying the 12SrRNA / $tRNA_{Phe}$ gene regions as a smaller fragment by using a primer within the $tRNA_{Phe}$ gene, only sequence from the 3′ end of the $tRNA_{Phe}$ gene could be determined (43 bases). Complete $tRNA_{Asp}$ (72 bases) and cytochrome b (1140 bases) gene sequences were obtained for all species studied, excepting the final base of the cytochrome *b* genes. The16S rRNA and NADH5 gene region sequences (Birstein et al., 2002) were realigned here to include the *P. ornatipinnis* sequences. The acipenseriform mitochondrial control region sequence alignment was composed of 394 bases after removal of the repeat region as determined by Birstein et al. (2002). Sequence from the control region of *Polypterus* was too divergent to create a reliable alignment with the Acipenseriformes, and so could not be used as an outgroup in the eight gene analyses including the control region. The final eight gene alignment excluding *Polypterus* consisted of 4406 sites. However, in order to examine the relationship between the two paddlefish species, seven mitochondrial gene sequences (excluding the control region) were also combined into a seven gene alignment including *Polypterus* (3391 sites). Table 3 gives a summary of the genes analyzed here.

As a heuristic tool to explore the degree of saturation present in the data set, we plotted sequence divergence values (under the Jukes-Cantor 69 model) vs substitution number for all pair-wise comparisons among taxa, for first, second, and third positions in protein genes. If substitutions for a group of sites were saturated, we would expect to see a plateau in such a plot, with little or no additional substitution detectable with increased distance values. The computer package DAMBE (Xia and Xie, 2001) was used for saturation plots.

Four methodologies (Bayesian, maximum parsimony, maximum likelihood and distance) were used for phylogenetic reconstructions in order to compare the consistency of the results produced by different methods. MODELTEST 3.06 (Posada and Crandall, 1998) was used to determine a model of DNA substitution that best fits the data based on hierarchical likelihood ratio tests; in all data sets the HKY85 + I + G model was identified (Hasegawa et al., 1985) and subsequently used in maximum likelihood, Bayesian and distance analyses. TREE-PUZZLE 5.0 (Strimmer and von Haeseler, 1996) was used to carry out maximum likelihood analyses using 10 000 quartet puzzling steps under the HKY85 + I + G model. MRBAYES 3.1 (Ronquist and Huelsenbeck, 2003) was used for Bayesian inferences of phylogeny by conducting Metropolis-coupled Markov chain Monte Carlo sampling for 5 000 000 generations (two simultaneous runs of four MC chains each, chain temperature of 0.2, sample frequency of 100, burn-in of 1 250 000 generations) under the HKY85 + I + G model. The default uniform Dirichlet distribution was used for the base frequencies and the default prior distributions were used for all other parameters. PAUP* v.4.0b10 (Swofford, 2002) was used for maximum parsimony analyses (heuristic searches with TBR branch swapping, MULTREES option effective and 10 random stepwise additions of taxa), as well as for distance based neighbor-joining analysis (Saitou and Nei, 1987) under the HKY85 + I + G model. Bootstrapping (1000 replicates) was conducted in both maximum parsimony and neighbor-joining analyses. In addition, the program MACCLADE (Maddison and Maddison, 1992) was utilized to identify the synapomorphic characters that unite various clades present in the phylogenetic hypothesis produced and examine differences in maximum parsimony tree lengths associated with alternative topologies.

Table 3
Variation and information content of DNA sequence data (excluding *Polypterus*)

Gene region	No. sites	No. variable sites (%)	No. parsimony informative sites (%)
12S rRNA	937	94 (10)	59 (6.3)
16S rRNA	523	81 (15.5)	35 (6.7)
COII	667	175 (26.2)	117 (17.5)
Cyt *b*	1140	339 (29.7)	263 (23.1)
$tRNA_{Asp}$	72	19 (26.4)	12 (16.7)
$tRNA_{Phe}$	43	12 (27.9)	9 (20.9)
Six genes combined	3382	720 (21.3)	495 (14.6)
NADH5	630	207 (32.9)	146 (23.2)
Control region (w / o repeat region)	394	208 (52)	147 (37.3)
Eight genes combined	4406	1135 (25.8)	788 (17.9)

Results

The plots for all characters and the third codon position (protein coding genes) showed a clear bias towards transitions, with a monotonic linear increase against time of divergence (expressed as percentage sequence divergence) up to approximately 11%, illustrating the presence of saturation in the outgroup comparisons with *Polypterus*. The number of transversions, however, showed a linear increase over time without evidence of a plateau in divergence. Saturation was also not evident in protein genes when the numbers of substitutions at both non-synonymous and synonymous sites were plotted over the range of divergences for all pairs of ingroup and outgroup taxa. In the latter instance, synonymous sequence divergence values resulted in a nearly perfect linear increase with $r^2 = 0.99$.

Initial phylogenetic analyses based on the complete eight gene alignment brought our attention to two particular placements within the Acipenseridae, the relationship between *A. ruthenus and H. huso* and the position within the phylogeny of *H. dauricus*. The issue of the placement of *H. dauricus* will be discussed later. In our analyses, *H. huso* is found clustered with *A. ruthenus* (as in the analyses of Birstein and DeSalle, 1998 and Birstein et al., 2002) rather than being basal to the remaining Atlantic species (as found by Ludwig et al., 2001). The *H. huso-A. ruthenus* sister species relationship is seen in trees constructed using all four methods based on the eight gene alignment with varying support levels (Bayesian-100%, NJ-98%, MP-73%, ML-71%). However, when we constructed single gene trees (excluding the $tRNA_{Asp}$ and $tRNA_{Phe}$ genes due to their short lengths), only in the trees constructed using NADH5 and control region do we see the sister species relationship between *H. huso-A. ruthenus*, and with strong

support (100%). This led us to examine the gene sequences of *H. huso* and *A. ruthenus* (Birstein et al., 2002) in more detail. Four nucleotide differences were observed between the control region sequences of the two species, and only one nucleotide difference was found in the NADH5 gene. The number of differences found in the other genes ranged from 12 to 73, except for the very short $tRNA_{Phe}$ gene sequence which showed no differences. We then examined the partial 12S rRNA and cytochrome *b* gene sequences determined by Birstein et al. (2002) (not included in these analyses) for which we have overlapping sequence. We found three differences in our 12S rRNA gene sequences between *H. huso* and *A. ruthenus* in the region covered by Birstein et al. (2002), while the sequences of these two species as determined by Birstein et al. (2002) are identical. Furthermore, the two 12S rRNA sequences determined by Birstein et al. (2002) are identical to the *H. huso* sequence determined here. Three fragments of cytochrome *b* gene sequence were determined by Birstein et al. (2002), and comparison of their *H. huso* and *A. ruthenus* sequences with those determined by Ludwig et al. (2000, 2001) showed no nucleotide differences differentiating between the two species' sequences determined by Birstein et al. (2002) for one region of the gene, but eight differences between the sequences of the two species as determined by Ludwig et al. (2000, 2001) for that region. Once again, the two cytochrome *b* region sequences determined by Birstein et al. (2002) are identical to the *H. huso* sequence determined by Ludwig et al. (2000, 2001). Comparison of the remaining two regions of the cytochrome *b* gene sequence from Birstein et al. (2002) do not show identical *H. huso* and *A. ruthenus* gene sequences. In the second section of cytochrome *b*, four differences are observed between the species (compared to 16 differences between the sequences of Ludwig et al. (2000, 2001)), while 13 differences are observed in the third section (compared to 23 differences in the comparison of sequences by Ludwig et al. (2000, 2001)). Taken as a whole, this evidence suggests that the mitochondrial sequences for the individual (or one of the individuals, as we do not know how many different fish were used for sequencing) determined from *A. ruthenus* by Birstein and DeSalle (1998) and Birstein et al. (2002) may reflect a past introgression of *H. huso* mitochondrial genome into *A. ruthenus*. *H. huso* × *A. ruthenus* hybrids have been reported in natural populations (Birstein et al., 1997), making this scenario plausible. Phylogenetic analyses including these suspect sequences would be misleading, as they artificially cluster *H. huso* and *A. ruthenus*, as seen in the analyses of Birstein and DeSalle (1998) and Birstein et al. (2002) and our eight gene analyses including these *A. ruthenus* sequences. In order to determine the placement of *A. ruthenus* independent of the effects of the sequences reflecting possible hybridization with *H. huso*, we analyzed a six gene alignment including *A. ruthenus,* which excluded the control region and NADH5 gene regions. In all four types of reconstruction, *A. ruthenus* is seen to be the sister species of *A. nudiventris* with good support (Bayesian-100%, NJ-92%, MP-94%, ML-86%).

As a result of the issues with the *A. ruthenus* sequences, phylogenetic analyses were then carried out on the set of eight mitochondrial gene sequences combined, excluding *A. ruthenus*, to maximize the amount of information extracted from the data. Figure 1 shows the tree constructed from maximum likelihood analysis of the eight gene alignment (4406 sites) using the HKY + I + G model (Hasegawa et al., 1985) in TREE-PUZZLE 5.0 (Strimmer and von Haeseler, 1996) (log $L = -17694.14$). This tree is very similar in topology to that produced by Ludwig et al. (2001) based on complete cytochrome *b* gene sequences alone. Bootstrapped trees (1000 replicates) produced by maximum parsimony (50% majority-rule consensus tree: 2252 steps, CI = 0.560, RI = 0.679) and distance methods in PAUP* v.4.0b10 (Swofford, 2002), and by Bayesian methods in MRBAYES 3.1 (Ronquist and Huelsenbeck, 2003) (5 000 000 generations, mean ln $L = -17362.03$) (not shown) are also very similar to the maximum likelihood tree and illustrate two main uncertainties found in these eight gene results: the identity of the most basal clade within the Acipenseridae and the position of *H. dauricus*. One other minor difference between the eight gene maximum likelihood tree and the trees produced by the three other methodologies was that the relationships among the three *Scaphirhynchus* species are not resolved in the Bayesian tree.

In the maximum likelihood, Bayesian and distance trees obtained using HKY85 distances (Hasegawa et al., 1985), the American and European Atlantic sturgeons *A. oxyrinchus* and *A. sturio* are seen as basal to all other *Acipenser* + *Huso* + *Scaphirhynchus* species (76% quartet-puzzling support on the maximum likelihood tree, 99% posterior probability on the Bayesian tree and 72% bootstrap support on the neighbor joining tree). However, in the maximum parsimony tree, the *Scaphirhychus* species are seen as basal to all *Acipenser* + *Huso* species (66% bootstrap support). Sequence analysis using MacClade (Maddison and Maddison, 1992) identified seven sites that support *Scaphirhynchus* branching off first, with the clustering of *A. oxyrinchus* + *A. sturio* and all other *Acipenser* + *Huso* species, while 11 sites were found that support the *A. oxyrinchus* + *A. sturio* branching off first, followed by the clustering of *Scaphirhynchus* with all other *Acipenser* + *Huso* species. Therefore, the evidence we have examined here appears to provide slightly more support for a more basal position of *A. oxyrinchus* + *A. sturio* as compared to *Scaphirhynchus,* in contrast to the currently accepted classification based on morphological data. However, the small difference in levels of support lead us to conclude that the relative positions of the *Scaphirhynchus* species and Atlantic sturgeon clade continues to remain unresolved.

In the maximum likelihood tree in Fig. 1, *Huso dauricus* is seen as clustered with the sister species *A. medirostris* and *A. mikadoi* (62% support). However, the position of *H. dauricus* varies depending on the tree reconstruction method used with this data, the only species to show such instability here. In the Bayesian tree, *H. dauricus* is found basal to the Atlantic species + *Scaphirhynchus* (97%), in the neighbor-joining tree it is positioned basal to the other Pacific species (56%) and in the maximum parsimony tree it is basal to the Atlantic species (66%). To further investigate, we constructed single gene trees (excluding the $tRNA_{Asp}$ and $tRNA_{Phe}$ genes) and found that in analyses of the 12S rRNA, COII and cytochrome *b* genes *H. dauricus* is found clustered with *A. medirostris* and *A. mikadoi*. In NADH5 and control region single gene trees, *H. dauricus* is found clustered with *A. ruthenus* and *H. huso*, and if *A. ruthenus* is omitted from the analysis *H. dauricus* still clusters with *H. huso*, again implicating these two genes. The 16S rRNA gene tree showed no resolution of these relationships. Unfortunately, examination of the nucleotide sequences in this case did not reveal any obvious explanations for the differences in signal from the NADH5 and control region sequences. Analyses of a six gene alignment excluding NADH5 and control region sequences corroborate the clustering of *H. dauricus* with the sister species *A. medirostris* and *A. mikadoi* with strong support (Bayesian-100%, NJ-99%, MP-97%,

ML-99%). Further analysis is required to clarify the positioning of *H. dauricus*.

There are four other characteristics of note in this combined eight mitochondrial gene tree: (i) the two *Huso* species are embedded separately among the *Acipenser* species; (ii) *A. dabryanus* and *A. sinensis* are found to be closely related, with 100% support in all tree types; (iii) *P. kaufmanni* is found clustered with *Acipenser stellatus* (considered an Atlantic species, see below) with generally strong support (84% in the maximum likelihood tree and 100% support in Bayesian, distance and maximum parsimony trees; (iv) the presence of two groups within the *Acipenser / Huso* cluster (excluding the American and European Atlantic sturgeon species) is upheld. The hypothesis that this cluster is split into two monophyletic groups, one containing Atlantic species (*A. ruthenus*, *A nudiventris*, *A.stellatus*, *A. fulvescens*, *A. brevirostrum*, *A. baerii*, *A. persicus*, *A. gueldenstaedtii*, *A. naccarii* and *H.huso*) and the other containing Pacific species (*A. medirostris*, *A. mikadoi, A. transmontanus*, *A. schrenckii*, *A. sinensis* and *H. dauricus*) was put forth by Ludwig et al. (2000, 2001) based on phylogenetic analysis of cytochrome *b* gene sequences alone. Bayesian posterior probabilities, quartet-puzzling, and distance and maximum parsimony bootstrap support for these two clades is as follows: Atlantic clade – 95%, 99%, 100% and 98%; Pacific clade – 100%, 62%, 56% and 79%. The lower support values for the Pacific clade seen in some trees are due to the unstable position of *H. dauricus* within this eight gene alignment (as discussed above). Analysis of the six gene alignment excluding the NADH5 and control region genes showed the following support levels for the Pacific clade – 100%, 94%, 99% and 97%. With the addition of *A. dabryanus* and *P. kaufmanni* to the current analyses, we now find that *A. dabryanus* belongs to the Pacific group and *P. kaufmanni* belongs to the Atlantic group. Examination of the COII, cyt-*b* and NADH5 gene sequences for non-compensatory nucleotide substitutions that distinguish the Atlantic and Pacific clades identified three such sites. The two sites within the cyt-*b* gene are those described by Ludwig et al. (2001): position 690 (C↔A / G) producing a Phe↔Leu substitution at amino acid (AA) position 230, and position 1112 (G↔T) producing a Trp↔Leu substitution at AA position 371. The third site, number 102 (T↔C) in the COII gene alignment, produces a substitution at AA position 34 (Ile↔Met). In addition, one site located at position 874 (G↔A) within the 12S rRNA gene alignment also identifies the Atlantic-Pacific split.

Discussion

Molecular phylogeny

Analysis of the combined mitochondrial gene sequences of cytochrome *b*, 12S rRNA, COII, $tRNA_{Asp,}$ $tRNA_{Phe}$,16S

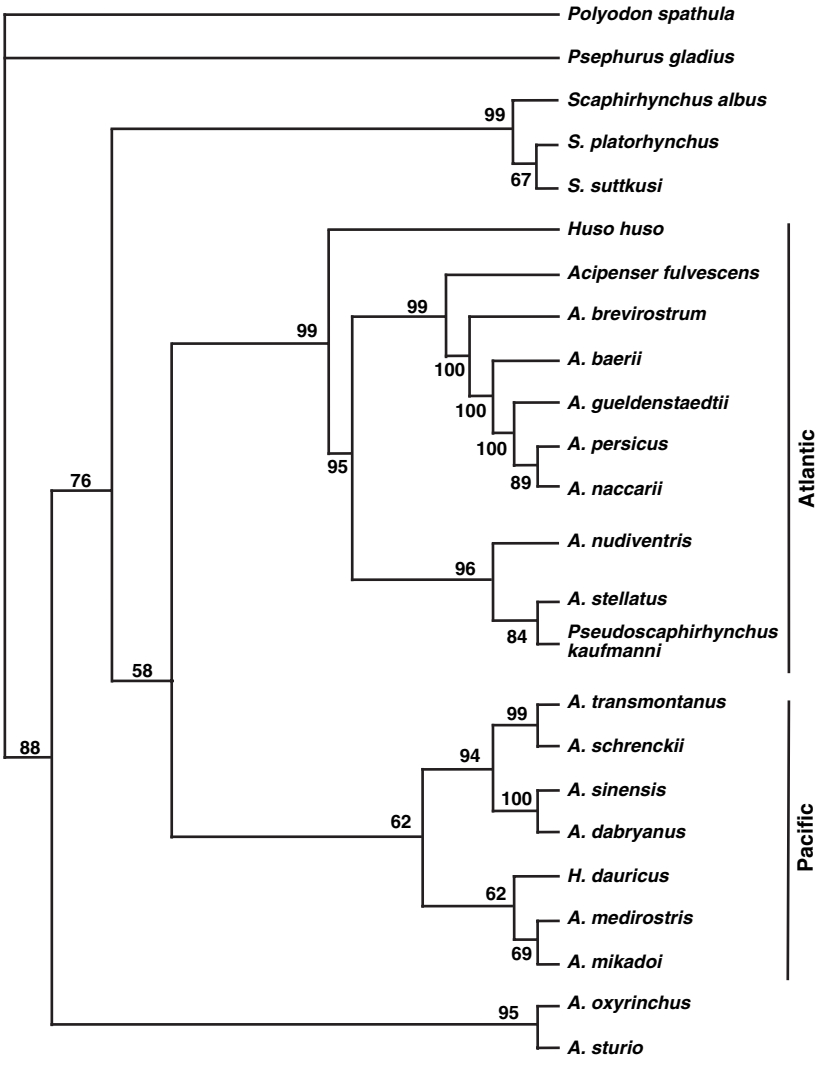

Fig. 1. Phylogenetic tree based on combined mitochondrial cytochrome *b*, 12S rRNA, cytochrome *c* oxidase subunit II, $tRNA_{Phe}$, $tRNA_{Asp}$,16S rRNA, NADH5 and control region gene sequences (eight genes, 4406 sites), excluding *Acipenser ruthenus*, produced using a maximum likelihood approach with 10 000 puzzling steps (log $L = -17694.14$). The numbers located above each branch indicate the quartet puzzling values (as a percent) of the node. Trees produced with Bayesian, neighbor-joining distance and maximum parsimony methods (not shown) were similar in topology (see text)

rRNA, NADH5 and the control region indicates that these mitochondrial sequences are useful for inferring phylogenetic relationships among sturgeon and paddlefish species, with the caveat that sequences that appear to be the products of introgression may exist in the databases. By combining sequences from all eight mitochondrial genes studied in this more comprehensive phylogenetic analysis, the results provide a well-supported and informative picture of evolutionary relationships among the species of the Acipenseriformes. The following phylogenetic conclusions can be drawn from our extended data set: (i) in additional analyses on the seven gene alignment including *P. ornatipinnis* as the outgroup to the Acipenseriformes, the Polyodontidae are seen to be the sister group to the Acipenseridae; (ii) the Atlantic sturgeons (*A. oxyrinchus* and *A. sturio*) form a separate clade having a basal position within the Acipenseridae, however this basal position is shared together with the *Scaphirhynchus* clade; and (iii) all other species of the genera *Acipenser*, *Huso*, and *Pseudoscaphirhynchus* form two clades representing their biogeographic distribution (Atlantic species vs Pacific species). Taken together, all four tree building methods produced strong support (Fig. 1) for the Atlantic species clade (95 / 99 / 100 / 98): *H. huso*, *P. kaufmanni*, *A. stellatus*, *A. nudiventris*, *A. ruthenus*, *A. baerii*, *A. persicus*, *A. naccarii*, *A. gueldenstaedtii*, *A. brevirostrum* and *A. fulvescens* with slightly lower support for the Pacific species clade (eight genes: 100 / 62 / 56 / 79; six genes: 100 / 94 / 99 / 97): *H. dauricus*, *A. medirostris*, *A. mikadoi*, *A. dabryanus*, *A. sinensis*, *A. schrenckii* and *A. transmontanus*.

The results are discussed in more detail below. Most molecular phylogenetic calculations based on the seven gene alignment including *Polypterus* as an outgroup (ML / NJ / MP support values: 91 / 99 / 95) strongly support the separation of the Polyodontidae, and 47 synapomorphic characters were identified that unite this clade. The Bayesian analysis, however, produced only moderate support for this grouping, with a 78% posterior probability. This grouping was also seen in a phylogenetic study by Zhang et al. (2000) of 12 acipenseriform species based on mitochondrial ND4L and ND4 genes.

An issue that continues to be unresolved is the exact position of the *Scaphirhynchus* species as well as of the American and European Atlantic sturgeon species relative to the other Acipenserinae (see also Birstein, 2005). It remains unclear based on this data which group is the most basal within the Acipenseridae, which suggests that the timings of the divergences of the two groups were very similar. Additional, seemingly contradictory, information is provided by the analysis of two nuclear DNA sequences, 18S rRNA genes and *Pst*I satellite DNA. Intraindividual variation of the 18S rRNA gene, including substitutions and indels, was identified in all species of sturgeon examined but not in either polyodontid species (Krieger et al., 2006). Six 'hotspots' were identified where an indel event occurred in most or all sturgeon species, but the three species of *Scaphirhynchus* possess a smaller number of these common indel events (only two indel 'hotspots') than the *Acipenser / Huso / Pseudoscaphirhynchus* species, and show lower amounts of polymorphism within their 18S rRNA gene sequences than all other sturgeon species (Krieger and Fuerst, 2002; Krieger et al., 2006). The sturgeons *A. sturio*, *A. o. oxyrinchus* and A. *o. desotoi* all show a pattern of indel 'hotspots' and polymorphism levels similar to that of the remaining *Acipenser / Huso / Pseudoscaphirhynchus* group. Based on this evidence, it appears that the *Scaphirhynchus* species may be the most basal sturgeon group. However, in their analysis of satellite DNA sequences in acipenseriforms Robles et al. (2004) found that *Pst*I DNA satellite sequences are present in all species of *Acipenser, Huso*, and *Scaphirhynchus* except for *A. sturio* and *A. oxyrinchus*, which suggests that the Atlantic sturgeon clade may be the most basal sturgeon group. Clearly this question continues to remain open.

The small number of differences (four) found in the cytochrome *b* gene sequence of *S. albus* and that shared by *S. platorynchus* and *S. suttkusi*, and the very low levels of differentiation found between *S. platorynchus* and *S. suttkusi* for all eight genes studied leaves the ongoing question of their status as separate species unresolved based on our data (for more detailed discussion of the question see Mayden and Kuhajda, 1996; Wirgin et al., 1997; Campton et al., 2000; Krieger et al., 2000 and Dillman et al., 2007). Most recently, genetic evidence was observed for large-scale hybridization of *S. platorynchus* and *S. albus* (Tranah et al., 2004) suggesting an unresolved species definition of these fishes. Larger sample sizes would of course be necessary to confirm the four differences found among *S. albus* and *S. platorynchus / S. suttkusi* as species diagnostic. In contrast, there are 38 synapomorphic characters that support grouping the American and European Atlantic sturgeon species together, separate from the other acipenserid species. Furthermore, the Atlantic sturgeon species are distinguished from each other by 162 substitutions. American Atlantic sturgeons appear to be further subdivided by three substitutions in just the 12S rRNA, COII, $tRNA_{Asp}$ and $tRNA_{Phe}$ genes separating individuals of the subspecies *A. o. oxyrinchus* and *A. o. desotoi* (Krieger et al., 2000); larger sample sizes are again necessary to confirm these differences. Ong et al. (1996) and King et al. (2001) have provided evidence from mitochondrial control region sequence and microsatellites, respectively, for the separation of these two subspecies.

With regard to the North American species, another unresolved issue was the relationship between the eastern North American species *A. brevirostrum* and *A. fulvescens*, which have long been considered sister species (Lee et al., 1980; Hocutt and Wiley, 1986). Based on their phylogenetic analysis of three mitochondrial DNA genes, Birstein and DeSalle (1998) concluded that these two species were distantly related. Later, Krieger et al. (2000) found *A. brevirostrum* and *A. fulvescens* to be sister species in their phylogenetic study of all ten North American Acipenseriform taxa, based on data from four mitochondrial genes. With the addition of Eurasian species and additional gene sequences in this study, these species, although no longer sister taxa, are seen to be closely related within the same phylogenetic cluster (a group that is supported by three synapomorphic sites), which is found within the Atlantic *Acipenser / Huso* species group (see Fig. 1). This is similar to the findings of Ludwig et al. (2000, 2001) based on cytochrome *b* gene sequences alone. Thus, the current molecular evidence indicates a close relationship between *A. brevirostrum* and *A. fulvescens*, a hypothesis that is also supported by the morphological studies of Artyukhin (2006) and studies of acipenseriform historical biogeography and parasites by Choudhury and Dick (1998, 2001).

A number of findings relate to the Eurasian species. Zhang et al. (2000) identified *A. dabryanus* and *A. sinensis* as sister species, which is also supported by our analyses. We identified 18 synapomorphic sites uniting these species. Zhang et al. (2000) proposed that the two species, both endemic to the Yangtze River, are closely related and that *A. dabryanus* is possibly a landlocked form of *A. sinensis* (Zhang et al., 2000). The validation of this close relationship by our data is

significant because the grouping was not supported in a study carried out by Birstein and DeSalle (1998). The nonmonophyly of the genus *Huso* (Birstein and DeSalle, 1998; Zhang et al., 2000; Ludwig et al., 2001) is again upheld, as the two species are seen placed separately within the genus *Acipenser* and were not found to be closely related in the current analyses. Based on our examination and analyses of the sequences, it appears that *H. huso* is basally located within the Atlantic group and *H. dauricus* is clustered with *A. medirostris* and *A. mikadoi* (a clade supported by three synapomorphic sites) within the Pacific group. This result is similar to that of Zhang et al. (2000), who also found *H. dauricus* clustered with *A. medirostris* and *H. huso* to be basal to a group of Atlantic species. Analysis of our six gene alignment (excluding the problematic NADH5 and control region sequences) in MacClade (Maddison and Maddison, 1992), however, indicates that forcing *H. dauricus* into the basal position in the Atlantic clade increases the maximum parsimony tree length by 23 steps and forcing the two *Huso* species together clustered with *A. ruthenus* (as observed by Birstein and DeSalle, 1998 and Birstein et al., 2002) in the Atlantic clade increases the tree length by 82 steps.

The North American green sturgeon (*A. medirostris*) and Asian Sakhalin sturgeon (*A. mikadoi*) were once considered to be the same species (see Birstein, 1993). Differences in DNA content (ploidy levels) discovered between *A. medirostris* (Blacklidge and Bidwell, 1993) and *A. mikadoi* (Birstein et al., 1993) led Birstein et al. (1993) to propose that the two were actually different species, and then later molecular phylogenetic studies separated these species into two distantly related clades (Birstein and DeSalle, 1998; Birstein et al., 2002). In agreement with Ludwig et al. (2000, 2001), *A. medirostris* and *A. mikadoi* do appear to be sister species based on the current analyses, and this relationship is also supported by 10 synapomorphic sites. The maximum parsimony phylogenies of Birstein and DeSalle (1998) and Birstein et al. (2002) do not indicate a sister species relationship for these two species. When we constructed trees based on single gene sequences (again excluding the $tRNA_{Asp}$ and $tRNA_{Phe}$ genes due to their short lengths), *A. medirostris* and *A. mikadoi* are seen to be sister species in all trees produced, except for that based on the partial NADH5 gene sequences. Examination of the NADH5 gene sequences provides a possible explanation for this difference in topology. Comparison of the *A. medirostris* and *A. mikadoi* NADH5 nucleotide sequences revealed 55 differences between them, compared to just seven differences present between the species in the control region sequence. The large number of differences in the NADH5 gene of *A. medirostris* and *A. mikadoi* may have pulled the two species apart in previous phylogenetic analyses. The control region is non-coding, and 208 out of 394 alignable sites (52%) examined in the control region (without repeat region) are variable. This percentage of variable sites is much higher than that seen in the other gene regions studied here (see Table 3), presumably due to relaxed evolutionary constraints on this region as compared to those genes that code for rRNA or proteins. As a result, we would not expect to see a much higher number of variable sites in the protein coding NADH5 gene than in the control region, which is normally the most rapidly evolving region of mitochondrial DNA. Further scrutiny of the NADH5 sequences revealed that the sequence determined for *A. mikadoi* is identical to that deposited for *A. stellatus*, an unexpected match. Examination of the other seven genes analyzed here show nucleotide differences between *A. mikadoi* and *A. stellatus* ranging from two to 95 differences, so it is unlikely that the NADH5 sequence of *A. mikadoi* is actually identical to that of *A. stellatus*. This anomaly casts doubt on the value of the *A. mikadoi* NADH5 gene sequence for accurate phylogenetic reconstruction and explains the difference in the placement of *A. medirostris* and *A. mikadoi* between studies.

Four Eurasian species found in the Atlantic group, *A. baerii*, *A. persicus*, *A. gueldenstaedtii* and *A. naccarii* (*gueldenstaedtii*-complex), appear to be closely related. Low levels of variation for the eight mitochondrial genes studied here were observed among these species, even for sturgeons, ranging from 17 differences between *A. persicus* and *A. naccarii* to 49 differences between *A. baerii* and *A. naccarii,* with most of the variation found in the control region. The most recent studies of the *gueldenstaedtii*-complex produced a complex pattern of sequences (Ludwig et al., 2003; Birstein et al., 2005), with geographically divergent populations showing levels of variation equivalent to those between some species. However, the levels of variation were not sufficient to change the placement of these four species within the larger phylogenetic analysis.

Finally, the controversial and unexpected placement of *P. kaufmanni* within the Acipenserinae rather than the Scaphirhynchinae, first discovered by Birstein et al. (2002), is corroborated here with analysis of a different *P. kaufmanni* individual. Birstein et al. (2002) and Dillman et al. (2007) also analyzed another species of *Pseudoscaphirhynchus*, *P. hermanni*, and found both species closely related and clustered with *A. stellatus*. In agreement, Hilton (2005) showed that *A. stellatus* and *P. kaufmanni* share several putatively derived similarities in the structure of their skulls, supporting a close relationship between *A. stellatus* and *Pseudoscaphirhynchus*. Birstein et al. (2002) proposed that the morphological similarities that led to the classification of *Scaphirhynchus* and *Pseudoscaphirhynchus* together within the subfamily Scaphirhynchinae separate from *Acipenser* and *Huso* (Acipenserinae) were probably due to convergent evolution resulting from habitation of the two groups in similar environmental conditions on separate continents. This idea was also considered by Bailey and Cross (1954) in their review of the subfamily Scaphirhynchinae, and is now again supported by our molecular data. We also found *P. kaufmanni* to be clustered with *A. stellatus* within the Atlantic *Acipenser / Huso* species group (a relationship supported by 13 synapomorphic sites), further strengthening the argument for the nonmonophyly of the subfamily Scaphirhynchinae.

Convergent evolution and niche realization

With the accumulation of molecular data from the Acipenseriformes, it is becoming increasingly evident that in a number of respects the molecular data is not in agreement with current classification of sturgeon species based on morphological data. Interestingly, in the Atlantic as well as Pacific ingroups the same ecological niches were realized independently by different lineages / species. Firstly, both ingroups contained top-predatory species (*H. huso* – Ponto-Caspian-Adriatic-Mediterranean-regions, *H. dauricus* and *A. sinensis* – Asian Pacific region, *A. transmontanus* – North American-Pacific-region) representing the largest freshwater fish species in their local environment. Secondly, there were always more or less anadromous species within the ingroups (for example: *A. gueldenstaedtii*, *A. stellatus*, and *A. nudiventris*, largest estuarine subpopulations of *A. baerii* from the rivers Ob and Yenissey, *H. huso*, *A. naccarii* – Atlantic species group; *A. medirostris*, *A. sinensis* – Pacific species group). Thirdly, both ingroups contained (predominantly) freshwater species

(*A. baerii*, *A. ruthenus*, *A. fulvescens* – Atlantic species group; *A. dabryanus* – Pacific species group). Neither two top-predatory species nor two or more freshwater species share sympatrically the same river systems. Only *A. baerii* and *A. ruthenus* sympatrically inhabited one single Siberian river system: the Ob River. However, *A. ruthenus* is normally a Ponto-Caspian species; and *A. baerii* is found in the Siberian tributaries of the Atlantic Ocean. Considering the outcome of our extended molecular data set, the ecological adaptation and niche realization leads to the observed morphological convergence found between Atlantic and Pacific species. Nevertheless, the observed phylogeny based on molecular data is strongly supported by the biogeography of the species.

Future prospects

Revision of this classification may be warranted in the future if further investigation of the group continues to provide support for the newly proposed relationships uncovered by molecular analyses. For example, current molecular evidence suggests that the genus *Huso* is not monophyletic, nor is the subfamily Scaphiyrhynchinae, and that both *Huso* and *Pseudoscaphirhynchus* actually belong within the genus *Acipenser*. An accurate picture of the evolution of sturgeon species is necessary in order to effectively plan conservation efforts and provide protection for these unusual and rare fish. A majority of phylogenetic studies on these fish to date have been based on mitochondrial gene sequences, and this study has been the largest study of mtDNA sequences so far. Separate analysis of the cytochrome *b* gene, combined analysis of four other mitochondrial genes (12S rRNA, cytochrome *c* oxidase subunit II, $tRNA_{Asp}$ and $tRNA_{Phe}$) and the combined analysis of all five of these gene sequences produced trees very similar to the results presented here, with good bootstrap values, providing strong support for the resulting phylogenetic hypothesis. The similarity in results using these different data sets indicate that the analysis of additional mitochondrial gene sequences may not be able to provide much more useful information for elucidating phylogenetic relationships in this group. Therefore, the examination of nuclear sequences, although often problematic to due to high ploidy levels and to individual as well as interspecific variability of ploidy levels (Ludwig et al., 2001), should be extended. The few previous studies that used nuclear DNA to investigate phylogeny in the acipenseriforms have examined the 18S rRNA gene (Birstein et al., 1997; Krieger and Fuerst, 2002) or RAPD (randomly amplified polymorphic DNA) markers (Comincini et al., 1998). Birstein et al. (1997) found that maximum parsimony analysis of partial 18S rRNA gene sequences (230 bases) in eight species of acipenseriforms gave little resolution of phylogenetic relationships due to a low degree of sequence divergence among species. A study of intraindividual variation of the 18S rRNA gene discovered in sturgeon by Krieger and Fuerst (2002) included maximum likelihood and distance analyses of partial sequence variants (1212 bases) isolated from four species of sturgeons. They found that the sequence variants from different sturgeon species were intermixed in the trees with no apparent separation by species. It was concluded that the sequences were again not phylogenetically useful, as the intraindividual variation present in these fish affects the ability of this gene to distinguish species or identify relationships (Krieger and Fuerst, 2002). Comincini et al. (1998) carried out a UPGMA analysis of eight RAPD markers on six sturgeon species. The markers were able to correctly distinguish species and did provide some phylogenetic resolution, although the results do not totally agree with more current molecular analyses. To our knowledge, an expansion of the use of the RAPD analysis technique has not been carried out to test whether it would be informative for the inference of relationships within the group as a whole. In currently published research, no valuable nuclear marker analyzing phylogenetic relationships in this group has been found as yet (Robles et al., 2004, 2005; Hett and Ludwig, 2005). *Sox* genes (Hett and Ludwig unpublished data), 5S rDNA and *Hind*III satellite sequences were examined (Robles et al., 2004, 2005; Hett and Ludwig, 2005) and found not to be valuable for phylogenetic classifications. Nevertheless, a phylogenetic tree based on *Pst*I satellite sequences supported the observed Atlantic / Pacific split as well as the basal position of both Atlantic sturgeons (Robles et al., 2004). The extension of nuclear marker investigation should be one of the major goals in the near future.

The evolutionary age of the order Acipenseriformes is one final aspect of evolution that can be addressed by molecular information. As mentioned, the order is first recognizable in the fossil record approximately 200 million years before present in the Jurassic (Grande and Bemis, 1991). An estimate of the divergence times of various sturgeon and paddlefish groups has recently been suggested, based on a subset of the data used here, the cytochrome *b* gene sequences. From the analysis of this single gene, it is estimated that the Atlantic sturgeon lineage originated approximately 171 million years ago (Mya) and that the *Scaphirhynchus* split from the remaining sturgeons about 151 Mya (Peng et al., 2007). Since our analysis of a larger data set is unable to definitively identify which groups form the basal split of sturgeons, it appears that a date of ~170 Mya would be appropriate to apply to the origin of the sturgeon lineage.

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