Molecular Phylogenetics and Evolution
Vol. 16, No. 1, July, pp. 64–72, 2000
doi:10.1006/mpev.1999.0743, available online at http://www.idealibrary.com on IDEAL®

Phylogenetic Relationships of the North American Sturgeons (Order Acipenseriformes) Based on Mitochondrial DNA Sequences

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Received July 16, 1999; revised October 18, 1999

The evolutionary relationships of the extant species within the order Acipenseriformes are not well understood. Nucleotide sequences of four mitochondrial genes (12S rRNA, COII, $tRNA_{Phe}$, and $tRNA_{Asp}$ genes) in North American sturgeon and paddlefish were examined to reconstruct a phylogeny. Analysis of the combined gene sequences suggests a basal placement of the paddlefish with regard to the sturgeons. Nucleotide sequences of all four genes for the three *Scaphirhynchus* species were identical. The position of *Scaphirhynchus* based on our data was uncertain. Within the genus *Acipenser,* the two *Acipenser oxyrinchus* subspecies were very similar in sequence and found to be basal to the remaining *Acipenser* species examined. Based on our data, *Acipenser transmontanus* and *Acipenser medirostris* were sister taxa, as were *Acipenser fulvescens* and *Acipenser brevirostrum.* Comparison of our results with hypotheses of sturgeon relationships proposed by previous authors is presented. The sequence data presented here are phylogenetically useful and provide a solid foundation of genetic information for the North American Acipenseriformes that can be expanded to include Eurasian species to provide a global picture of sturgeon evolution.

Key Words: **Acipenseriformes; evolution; mitochondrial DNA; molecular phylogenetics; paddlefish; sturgeon.**

INTRODUCTION

The order Acipenseriformes is an ancient group of fish identifiable from the Jurassic (Grande and Bemis, 1991), which possess a highly cartilaginous skeleton, a spiral valve in the gut, a heterocercal tail, and a well developed rostrum with mouth inferior (Sokolov and Berdichevskii, 1989). The group is characterized as monophyletic based on eight osteological characters described in detail by Bemis *et al.* (1997). At present, it consists of approximately 25 extant species of sturgeon divided into two subfamilies and four genera and 2 extant species of paddlefish in different genera but grouped within one family (Birstein, 1993). These fish are anadromous, diadromous, or potamodromous, with a North American and Eurasian distribution (Bemis and Kynard, 1997). In addition, sturgeon and paddlefish have long life spans, the potential to grow very large in size, and slow maturation, as females often require 10 to 20 years before reproduction in nature. Widely exploited for caviar and meat, their popularity as a food source in a number of countries has resulted in frequent overfishing. Recently, the population sizes of many acipenseriform species have decreased to the point at which they are threatened or endangered. The decrease can be traced to three major factors: (1) certain life history characteristics of these fish (slow sexual maturation, females often reproduce only once every few years, and migratory nature); (2) the destruction of spawning habitat by pollution, dams, or other human intervention; and (3) overfishing.

Sturgeons are of interest evolutionarily for a variety of reasons. The "living fossil" status of the group makes them important for understanding vertebrate evolution in general (Gardiner, 1984), and the threatened or endangered status of many of these species indicates that there may be a limited time left to study these organisms. For example, of the 10 species of Acipenseriformes in North America, the IUCN Red List categorizes 6 species as vulnerable, 1 as threatened, and 1 as critically endangered (Birstein *et al.*, 1997a; 1996 IUCN Red List of Threatened Species at the World Conservation Monitoring Centre website: http://www.wcmc.org.uk/species/animals/animal_redlist.html). In addition, information about the relationships of the extant species of sturgeon and paddlefish may be useful in directing conservation efforts toward protecting maximum genetic diversity of sturgeon species (Moritz, 1995). However, the evolutionary relationships are still not clearly understood (Birstein, 1993), and there is limited and sometimes confusing information available from morphological characters for elucidating species-level relationships. The need for additional investigation of this group by morphological, molecular, and other methods has been recently expressed (Bemis *et*

1055-7903/00 $35.00

al., 1997; Findeis, 1997). In particular, regarding the North American species, more information about genetic differences between the two *Acipenser oxyrinchus* subspecies and especially among the three species of *Scaphirhynchus* is needed. Evidence of genetic differentiation is necessary to firmly establish subspecific or specific status for these taxa to ensure protection of maximum acipenseriform diversity within North America. Another idea that has been in question is the existence of a sister species relationship between the North American species *Acipenser brevirostrum* and *Acipenser fulvescens.* Researchers have provided evidence both supporting (Artyukhin, 1995) and refuting (Birstein and Desalle, 1998) a sister species relationship between them, and Choudhury and Dick (1998) have proposed a hypothetical scenario explaining the historical biogeography for these two species that supports a sister species relationship.

To try to help resolve these relevant questions involving North American species and to add to the information already obtained for these important and vulnerable fish (Bemis *et al.,* 1997; Wirgin *et al.,* 1997; Birstein and DeSalle, 1998), we conducted an examination of the phylogenetic relationships among North American acipenseriform species using molecular data, specifically mitochondrial DNA gene sequences. The study has focused on the North American species, not because of an assumption that these species represent a monophyletic group within the Acipenseriformes but rather because our study will shed light on questions which have a predominant or sole emphasis on the North American sturgeon fauna.

The 12S ribosomal RNA (rRNA) gene and the cytochrome *c* oxidase subunit II (COII) gene were chosen for analysis because they have been shown to be useful in phylogenetic studies of other groups (Janecek *et al.,* 1996; Lavergne *et al.,* 1996; Richards and Moore, 1996), and much information is known about the structures and functions of the genes and their products (Brimacombe and Stiege, 1985; Capaldi, 1990; Gutell, 1994). In addition, two transfer RNA gene sequences, the tRNA for phenylalanine ($tRNA_{Phe}$) and the tRNA for aspartic acid ($tRNA_{Asp}$), were examined. They were included in the amplified products which contained the mitochondrial 12S rRNA and COII gene sequences. This study examines the relationships among the 10 extant North American sturgeon and paddlefish species by comparing the nucleotide sequences of the 12S rRNA gene, the COII gene, $tRNA_{Phe}$ gene, and the $tRNA_{Asp}$ gene from each species.

MATERIALS AND METHODS

Specimens. Tissue samples from each of the North American acipenseriform species (Mayden *et al.,* 1992) were provided by various researchers and fishery workers from throughout the United States. Table 1 lists information on the tissue samples. The taxa included were *Polyodon spathula* (North American paddlefish), *Acipenser fulvescens* (lake sturgeon), *A. brevirostrum* (shortnose sturgeon), *A. transmontanus* (white sturgeon), *A. medirostris* (green sturgeon), *A. oxyrinchus oxyrinchus* (Atlantic sturgeon) (Gilbert, 1992), *A. oxyrinchus desotoi* (Gulf sturgeon), *Scaphirhynchus platorynchus* (shovelnose sturgeon), *S. albus* (pallid sturgeon), and *S. suttkusi* (Alabama sturgeon). Blood samples were stored in Queen's lysis buffer

TABLE 1

Species Included in This Study

Species name	Tissue sample	Origin of specimen	Provider of sample
Polyodon spathula	Fin snip	Hatchery fish—parents from Osage River Basin	Blind Pony Lake Conservation Area, Missouri
Scaphirhynchus albus	Fin snip	Hatchery fish—parents from Mississippi and Missouri Rivers[a]	National Biological Services Midwest Science Center, Missouri
Scaphirhynchus platorynchus	Fin snip	Missouri River	National Biological Services Midwest Science Center, Missouri
Scaphirhynchus suttkusi	Muscle	Alabama River (UAIC 1885.01)	University of Alabama at Tuscaloosa
Acipenser brevirostrum	Barbel	Hatchery fish—parents from Savannah River	Bears Bluff National Fish Hatchery, South Carolina
Acipenser fulvescens	Fin snip	Wolf River	Wisconsin Department of Natural Resources
Acipenser medirostris	Muscle	Klamath River	Humboldt State University, California
Acipenser oxyrinchus oxyrinchus	Fin snip	Hudson River	Northeast Fishery Center, U.S.F.W.S., Pennsylvania
Acipenser oxyrinchus desotoi	Blood	Hatchery fish—parents from Suwannee River	University of Florida
Acipenser transmontanus	Fin snip	Snake River	College of Southern Idaho

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

(Seutin *et al.*, 1991), whereas the other types of tissue samples were stored in 95% ethanol.

Assessment of intraspecific variation. To justify the use of one individual specimen to represent each species in this study, an estimate of the intraspecific variation for the mitochondrial COII gene within a sturgeon species was determined. The COII gene was chosen because it was found to be the most variable sequence of the four genes examined among the species studied. For the lake sturgeon (*A. fulvescens*), in addition to one individual from the Wolf River that was sequenced for all four mitochondrial genes and used in the phylogenetic analyses, the COII genes of seven other lake sturgeons were amplified and then sequenced with the internal forward primer, Cox II A. The partial COII gene sequences were compared to identify nucleotide differences among individuals. Two individuals from each of four sampling locations (Wolf River, St. Clair River, Menominee River, and Lake Erie) were included in an effort to incorporate different populations or regions that would be more likely to show differences in mtDNA sequences than fish from the same population.

DNA extraction and gene sequence collection. Tissues other than blood were minced and then digested overnight at 55°C in ABI lysis buffer (Perkin–Elmer Applied Biosystems Inc., Foster City, CA) with proteinase K (Gibco-BRL, Gaithersburg, MD). Cells from the blood samples were already lysed due to storage in Queen's lysis buffer and did not require an additional overnight digestion step. Total DNA was purified from samples by three phenol–chloroform extractions and one chloroform extraction (Sambrook *et al.*, 1982). The DNA was precipitated with 95% ethanol, washed with 70% ethanol, and air dried. DNA was resuspended in 1 × TE buffer, quantified using a spectrophotometer, and stored at 4°C until used.

PCR amplification (Saiki *et al.*, 1988) of mitochondrial 12S rRNA and COII genes from total DNA was carried out in 100-μl reactions, using 2.5 units of *Taq* DNA polymerase (Gibco-BRL) per reaction, for one individual of each species studied. Primers were designed using the computer program Oligo (Rychlik and Rhoads, 1989) based on sequences from the complete mtDNA sequences for three teleost fish species: *Crossostoma lacustre,* GenBank Accession No. M91245 (Tzeng *et al.*, 1992); *Cyprinus carpio,* GenBank Accession No. X61010 (Chang *et al.*, 1994); and *Oncorhynchus mykiss,* GenBank Accession No. L29771 (Zardoya, Bautista, and Garrido-Pertierra, 1994, unpubl.). The 12S rRNA and $tRNA_{Phe}$ genes were amplified using primers T_{thr} and $T_{val\text{-}rev}$ under the following conditions: 3 min at 95°C, then 32 cycles of 1 min at 93°C, 1.5 min at 58°C, and 3 min at 72°C. The COII and $tRNA_{Asp}$ genes were amplified using primers T_{ser} and $T_{lys\text{-}rev}$ 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. Two independent PCR products were pooled for each fragment amplified for each species to compensate for possible replication errors by the *Taq* polymerase that may occur during amplification. Gene sequences were determined as completely as possible by direct cycle sequencing (Gibco-BRL) of PCR products with ^{32}P (ICN Pharmaceuticals, Irvine, CA) end-labeled primers, followed by separation on 6% polyacrylamide gels made with 1× TBE marathon buffer (Gibco-BRL) and exposure of dried gels to X-ray film (Hale Medical Systems, Orient, OH) to visualize the DNA sequence. All of the primers used for PCR amplification and DNA sequencing are listed in Table 2. Six primers were used to sequence the 12 rRNA and $tRNA_{Phe}$ genes, and five primers were used to sequence the COII and $tRNA_{Asp}$ genes. For the COII gene, primer Cox II Ao was used only for the two *A. oxyrinchus* subspecies and primer Cox II Ab was used only for *A. brevirostrum*. This was necessary because primer Cox II A, which was used for the remaining seven species, did not work with *A. oxyrinchus* or *A. brevirostrum*. In addition, primer Cox II Stu-rev was used for all the sturgeon species, but it was necessary to use Cox II E-rev to obtain sequences from paddlefish for this region of the COII gene. Sequences were scored manually, recorded, and depos-

TABLE 2

DNA Amplification and Sequencing Primers Used in This Study

Genes	Primer name and type[a]		Primer sequence (5′ to 3′)
12S rRNA and $tRNA_{Phe}$:	T_{thr}	(A)	AGAGCGCCGGTCTTGTAATCC
	$T_{val\text{-}rev}$	(A)	GCATGGATGTCTTCTCG-GTGTA
	12S.1	(S)	GCCTAGCCACACCCCCACGG
	12S.1-rev	(S)	GGGTGTGGCTTAGCAAGGCGT
	12S.2	(S)	GGTCAATTTCGTGCCAGCCA
	12Sa-rev	(S)	TAGTGGGGTATCTAATCCCAG
	12Sb-rev	(S)	TTGGAGCCTCTCGTATAACCG
	12S.3 Stu	(S)	CCACCTAGAGGAGCCTGTTC
COII and $tRNA_{Asp}$:	T_{ser}	(A)	CCCCATATGCTGGTTTCAAG
	$T_{lys\text{-}rev}$	(A)	CACCAATCTTTGGCTTAAAA
	Cox II A	(S)	TAGGCCACCAATGATATT-GAAG
	Cox II Ao	(S)	GCTATAGGACATCAAT-GATACTGAAG
	Cox II Ab	(S)	AAAGCTATAGGACACCAGT-GATACTGAAG
	Cox II E-rev	(S)	AATTGGGGACNNNATGGG-TACTAC
	Cox II Stu-rev	(S)	TATTCGATGGTCTGCTTC-TAGG
	Cox II J-rev	(S)	GCGTCTTGGAATCCTAATT-GTG

[a] A indicates a primer used for PCR amplification and S indicates a sequencing primer.

ited in GenBank under the following Accession Nos.: aspartic acid tRNA genes (AF125261 through AF125270), phenylalanine tRNA genes (AF125363 through AF125372), 12S rRNA genes (AF125594 through AF125603), and cytochrome *c* oxidase subunit II genes (AF125652 through AF125661).

Sequence alignment and phylogenetic analyses. In addition to the sturgeon and paddlefish sequences determined here, 12S rRNA, COII, $tRNA_{Phe}$, and $tRNA_{Asp}$ gene sequences for *Polypterus ornatipinnis* were included in the analyses as the representative of an outgroup taxon (order Polypteriformes). This order is widely considered to be the closest extant sister group of the other Actinopterygii, within which the Acipenseriformes are believed to be the next basal group (Patterson, 1982; Lauder and Liem, 1983; Bemis *et al.*, 1997). *Polypterus* was previously used as an outgroup by Birstein *et al.* (1997b) in a molecular study of the Acipenseriformes. The *Polypterus ornatipinnis* gene sequences were taken from the complete mtDNA sequence of Noack *et al.* (1996) (Accession No. U62532). Gene sequences were manipulated with the sequence alignment program ESEE (Cabot and Beckenbach, 1989), using additional information. For the 12S rRNA gene, sequences were aligned with the aid of a secondary structure of the 12S rRNA of *Polyodon spathula*, based on the structure of *Bos taurus* (Gutell, 1994). For 12S rRNA gene sequence alignment, regions of the molecule that could not be reliably aligned, due mainly to differences in length, were identified and excluded from the phylogenetic analyses (85 sites in total). Secondary-structure models for the tRNAs (Chang *et al.*, 1994) were used to align the two transfer RNA gene sequences. During alignment of the COII gene sequences, the protein reading frame was used. The sequence alignments for these four genes are available on the laboratory website (http://www.biosci.ohio-state.edu/~pfuerst). Phylogenetic analyses were conducted with a combined four-gene-sequence alignment to maximize the amount of information, using *Polypterus* as the outgroup. A distance tree was constructed using MEGA 1.01 (Kumar *et al.*, 1993), with the neighbor-joining method (Saitou and Nei, 1987) and Kimura two-parameter distances using both transitions and transversions (Kimura, 1980). Maximum-parsimony trees were produced with the exhaustive search option of PAUP 3.0 (Swofford, 1990), using *Polypterus* as the outgroup. Puzzle 4.0 (Strimmer and von Haeseler, 1996) was used for maximum-likelihood analyses.

RESULTS

Survey for intraspecific variation in COII gene sequences within lake sturgeon. A total of 339 bp of COII gene sequence from primer Cox II A was obtained from eight lake sturgeon individuals collected at four different sampling sites. Comparison of these aligned sequences showed no nucleotide variation among any of the fish examined. The COII gene was found to be the most variable gene included in the interspecific study. Therefore, the lack of COII variation among individuals of this one sturgeon species from different populations suggests that intraspecific variation for all four mitochondrial genes examined here is most likely very low, if not nonexistent, within the North American Acipenseriformes.

Phylogenetic analyses with combined data. The phylogenetic analyses were carried out on the set of four mitochondrial gene sequence alignments combined (1721 bp total length) to obtain as much information as possible from the data. This approach is reasonable because all the data involve mitochondrial gene sequences, which are inherited as essentially one linkage group. Figure 1 shows the neighbor-joining tree (1000 bootstraps) and one of two most-parsimonious trees (597 steps, CI = 0.86) constructed from the data. All three *Scaphirhynchus* species have identical nucleotide sequences for all four genes examined, and their sequences were represented as a single sequence in phylogenetic analyses.

The genetic distances (estimated sequence divergence) used to create the neighbor-joining tree (Fig. 1A) are shown in Table 3. To put the support for the relationships suggested by this reconstruction into perspective, an assessment of bootstrap accuracy carried out by Hillis and Bull (1993) showed that bootstrap proportions greater than or equal to 70% usually correspond to a probability greater than or equal to 95% that the corresponding clade is real. In the neighbor-joining tree the paddlefish, *Polyodon spathula*, is basal to all sturgeon species examined here (bootstrap value of 95%). Three sets of sister taxa within the genus *Acipenser* are supported with very high bootstrap support (99–100%): *A. transmontanus* and *A. medirostris*, *A. fulvescens* and *A. brevirostrum*, and *A. o. oxyrinchus* and *A. o. desotoi*. The neighbor-joining tree places the subspecies of *A. oxyrinchus* as basal to the remaining *Acipenser* species with high bootstrap support (82%) and suggests, but with lower bootstrap support (54%), that the species of *Scaphirhynchus* are a sister group to *Acipenser*. A maximum-likelihood tree was identical in topology to the neighbor-joining tree and showed percentage branch support levels similar to the bootstrap values seen in the neighbor-joining tree.

Figure 1B shows one of the two most-parsimonious trees produced from analysis of the data. The trees produced by distance, maximum-likelihood, and parsimony methods all show similar topologies. As in the neighbor-joining tree, the paddlefish is basal to the other sturgeon species examined, and we also see the same three sets of sister taxa within the genus *Acipenser*. However, the results of parsimony analysis

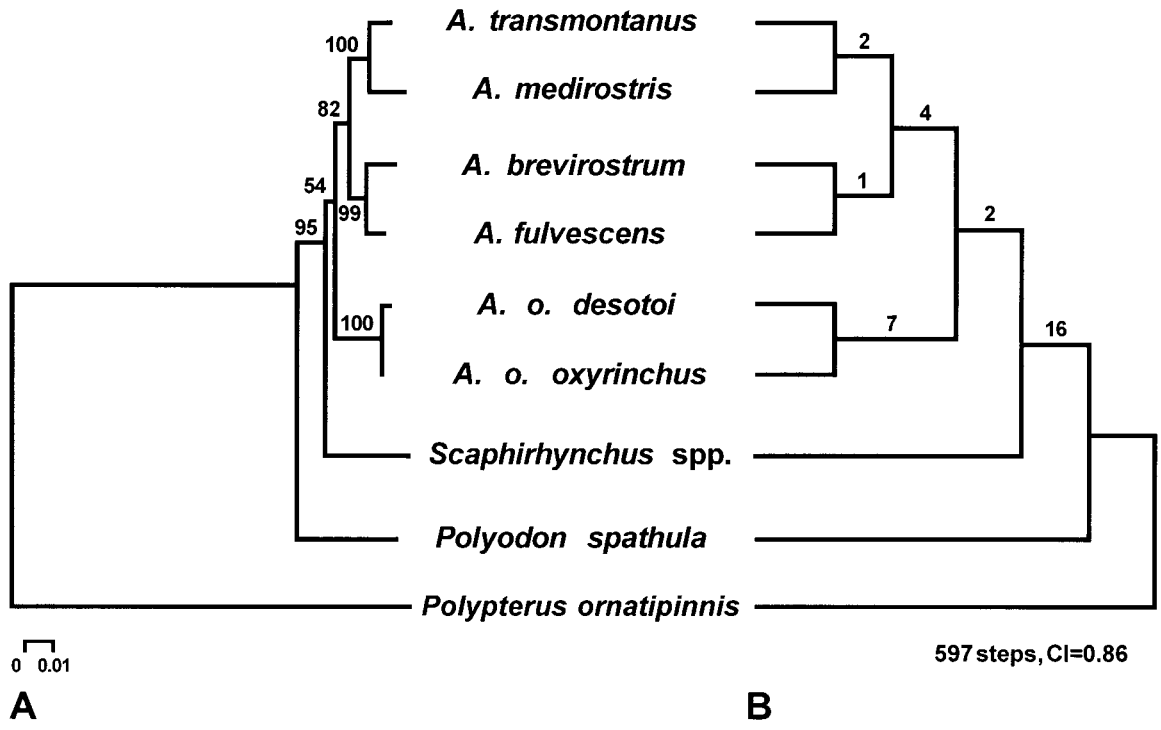

FIG. 1. (A) Phylogenetic tree based on combined mitochondrial 12S rRNA, cytochrome *c* oxidase subunit II, $tRNA_{Phe}$, and $tRNA_{Asp}$ gene sequences created using the neighbor-joining method (Saitou and Nei, 1987) in MEGA (Kumar *et al.,* 1993). Numbers indicate the bootstrap values for 1000 replications. (B) One of four most-parsimonious trees based on mitochondrial 12S rRNA, cytochrome *c* oxidase subunit II, $tRNA_{Phe}$, and $tRNA_{Asp}$ gene sequences produced using the exhaustive search option of PAUP (Swofford, 1990), with *Polypterus ornatipinnis* as the designated outgroup. Numbers represent the number of synapomorphic characters that unite particular clades, determined with MacClade (Maddison and Maddison, 1992). Both trees are based on 1721 bp of sequence from the four genes.

illustrate an uncertainty in the placement of the *Scaphirhynchus* species. The other most-parsimonious tree (not pictured here) shows the clade containing the three *Scaphirhychus* species switched in position with the clade containing the two subspecies of *Acipenser oxyrinchus*. The *A. oxyrinchus* subspecies are basal to the remaining *Acipenser* species in both of the two most-parsimonious trees as well as the distance tree, but there is ambiguous support for the placement of *Scaphirhychus* as a sister group to the *Acipenser* species, as would be expected based on current classification; possibly, the group is actually located within *Acipenser*. Therefore, the relationship of the genus *Scaphirhynchus* to the genus *Acipenser* is not conclusively resolved by our data.

DISCUSSION

Transition–transversion (S/V) ratios can be used to suggest how recently separation of species within the

TABLE 3

Distance Matrix for Four Combined Mitochondrial Gene Sequences[a]

	1	2	3	4	5	6	7	8	9
1. *Polypterus ornatipinnis*		0.2668	0.2604	0.2652	0.2658	0.2644	0.2603	0.2578	0.2726
2. *Polyodon spathula*	0.0149		0.0577	0.0635	0.0653	0.0627	0.0656	0.0630	0.0728
3. *A. fulvescens*	0.0146	0.0061		0.0143	0.0296	0.0253	0.0335	0.0329	0.0416
4. *A. brevirostrum*	0.0148	0.0065	0.0029		0.0314	0.0271	0.0378	0.0366	0.0478
5. *A. medirostris*	0.0148	0.0066	0.0043	0.0044		0.0173	0.0395	0.0377	0.0528
6. *A. transmontanus*	0.0147	0.0064	0.0039	0.0041	0.0032		0.0351	0.0340	0.0484
7. *A. o. desotoi*	0.0146	0.0066	0.0046	0.0049	0.0050	0.0047		0.0018	0.0524
8. *A. o. oxyrinchus*	0.0145	0.0065	0.0045	0.0048	0.0049	0.0046	0.0010		0.0512
9. *Scaphirhynchus* spp.	0.0151	0.0070	0.0051	0.0055	0.0058	0.0055	0.0058	0.0057	

[a] Distances were calculated with the program MEGA (Kumar *et al.,* 1993) using the Kimura two-parameter model (Kimura, 1980) and the pairwise deletion option. Distances are shown in the upper right triangle and standard errors are shown in the lower left triangle.

Acipenseriformes occurred. Values for S/V for the combined mtDNA gene sequences for pairwise species comparisons are approximately 1.4 for comparisons between *Polypterus* and acipenseriform species and range from 3.0 to 10.3 for comparisons between acipenseriform species. This indicates that transition nucleotide substitutions are largely saturated for comparisons between *Polypterus* and sturgeon or paddlefish species but not yet saturated for the comparisons between acipenseriform species. This suggests that the North American Acipenseriformes diverged from one another rather recently, compared to the age of the order as a whole. This is in accord with views expressed by Choudhury and Dick (1998) in their study of the historical biogeography of sturgeons. The authors concluded that "It appears that although the acipenserids are a geologically old group, the historical biogeography of surviving lineages is best explained by more recent geological and climatic changes" (Choudhury and Dick, 1998). It is also possible that the larger transition–transversion ratios seen between acipenseriform species could be the result of the group showing a slower rate of nucleotide substitution in their mtDNA than expected; a slow substitution rate in sturgeons was previously proposed by Brown *et al.* (1996) in his study of mtDNA.

Our results show the paddlefish to be basal to all other sturgeon species. This is expected, as sturgeons and paddlefish are classified as members of two different families. Our studies indicate some uncertainty regarding the relationship of the genus *Scaphirhynchus* to the genus *Acipenser*. Earlier molecular and morphological studies supported the placement of the Scaphirhynchinae as the sister group to the other sturgeons (Mayden and Kuhajda, 1996; Birstein and DeSalle, 1998). Even though the sister group position of *Scaphirhynchus* is only weakly supported by our data, we believe that this is the most likely hypothesis, especially when the other studies are taken into consideration.

Our results indicate that the North American species of *Acipenser* are divided into groups corresponding to their geographical ranges. Based on our data, we find the two eastern North American sturgeons (*A. fulvescens* and *A. brevirostrum*) to be sister species, as well as the two western North American sturgeons (*A. transmontanus* and *A. medirostris*). The two subspecies of *A. oxyrinchus* group together, separated by only three base changes, and their position is basal to the other *Acipenser* species. These relationships agree with the results of Artyukhin (1995), who constructed a phylogenetic tree to describe the relationships among the species of *Acipenser* based on biogeography, morphology, and karyology, and Brown *et al.* (1996), who created trees based on mitochondrial D-loop sequences and whole mtDNA RFLP analysis of four *Acipenser* species (*A. transmontanus, A. medirostris, A. fulvescens,* and *A. oxyrinchus*). Artyhukhin (1995) hypothesized evolutionary relationships that separate the species into what he refers to as "endemic zones." *Acipenser fulvescens* and *A. brevirostrum* are grouped in an eastern American zone, whereas *A. transmontanus* and *A. medirostris* are grouped in a North Pacific zone. In addition, he places the *A. oxyrinchus* species as basal to the other North American *Acipenser* in the North Atlantic zone. Brown *et al.* (1996) also found that the western species clustered together, with *A. fulvescens* branching off followed by *A. oxyrinchus*. Thus, at least for the six North American species of *Acipenser* examined here, biogeography and evolutionary relatedness appear to correlate. This is in contrast to the differences found between Birstein and DeSalle's (1998) partial mitochondrial cytochrome *b*, 12S rRNA, and 16S rRNA gene-based tree and Artyukhin's tree (1995), both of which included Eurasian as well as North American species. Therefore, the correlation between biogeography and evolutionary relatedness may break down when additional (Eurasian) species of sturgeons are examined.

Our trees are in agreement with the tree produced by Birstein and DeSalle (1998) based on the three combined partial mtDNA gene sequences mentioned above, excepting one point: the relationship between *A. fulvescens* and *A. brevirostrum*. Our data strongly support the hypothesis that these are sister species (Fig. 1), whereas Birstein and DeSalle (1998) conclude that the two species are "definitely distantly related" based on their data. Because their study included Eurasian species as well as North American species, we supposed that the difference in tree topology may be due to the difference in species included in the analysis and not due to a difference in signal resulting from the different mitochondrial genes that we examined. The inclusion of Eurasian species may have changed the observed relationship between *A. fulvescens* and *A. brevirostrum*, presumably providing a more accurate indication of relationships. To test this idea, the authors kindly provided us with their sequence alignment for the three partial mitochondrial genes of the North American acipenseriform species that they examined. We used these nucleotide alignments to construct distance, parsimony, and maximum-likelihood trees illustrating the relationships among the North American sturgeon species, using *Polyodon spathula* as the outgroup. Figure 2A shows the neighbor-joining tree (1000 bootstraps) produced with MEGA 1.01 (Kumar *et al.*, 1993) based on their nucleotide sequence data. A maximum-likelihood tree produced with Puzzle 4.0 (Strimmer and von Haeseler, 1996) had the same topology as the neighbor-joining tree. Also, the exhaustive search option of PAUP 3.0 (Swofford, 1990) was used to produce maximum-parsimony trees based on the data. Two of the three most-parsimonious trees showed *A. fulvescens* as basal to *A. brevirostrum*, as

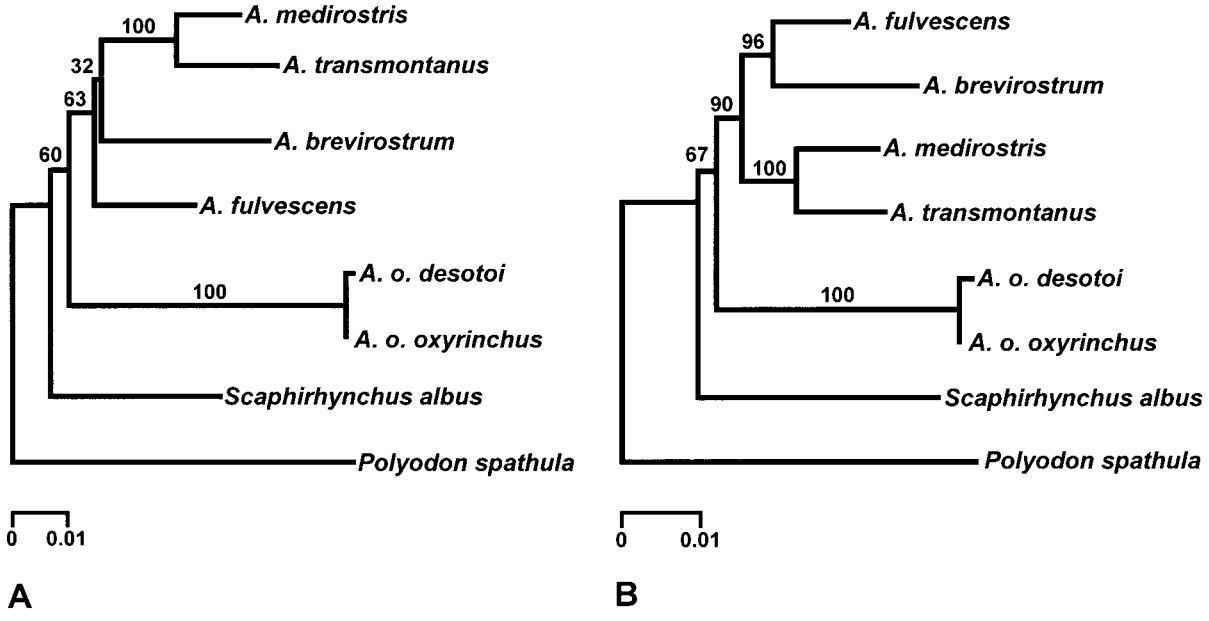

FIG. 2. (A) Phylogenetic tree based on partial combined mitochondrial cytochrome *b*, 16S rRNA, and 12S rRNA gene sequences from Birstein and DeSalle (1998) created using the neighbor-joining method (Saitou and Nei, 1987) in MEGA (Kumar *et al.*, 1993). Numbers indicate the bootstrap values for 1000 replications. (B) "Total evidence" phylogenetic tree based on combined mitochondrial gene sequences (12S rRNA, cytochrome *c* oxidase subunit II, $tRNA_{Phe}$, $tRNA_{Asp}$, cytochrome *b*, and 16S rRNA genes) from this paper and Birstein and DeSalle (1998) created using the neighbor-joining method (Saitou and Nei, 1987) in MEGA (Kumar *et al.*, 1993). Numbers indicate the bootstrap values for 1000 replications.

seen in Fig. 2A, whereas the third most-parsimonious tree showed the two as sister species. This shows that even with the removal of the Eurasian acipenseriform species, the sequence data from these genes supports a distant relationship and not a sister species relationship between the lake sturgeon and the shortnose sturgeon, as indicated by our data. We then combined the mitochondrial nucleotide data of Birstein and DeSalle (1998) with our data to examine trees based on the combined sequences using the same computer algorithms mentioned above. The partial 12S rRNA gene sequences from Birstein and DeSalle (1998) were omitted because our alignment already contained the 12S rRNA gene. The neighbor-joining tree (1000 bootstraps) produced by the combination of all the mitochondrial sequence data is shown in Fig. 2B. The maximum-likelihood tree is identical in topology to the neighbor-joining tree, and both most-parsimonious trees are also similar in topology to the neighbor-joining tree (the difference between the two parsimony trees once again involves the position of *Scaphirhynchus*). All four trees based on the combined data indicate that *A. fulvescens* and *A. brevirostrum* are sister species, as shown by the analysis of our data alone. Based on these observations, the data support the hypothesis that the two eastern North American sturgeon species are indeed closely related, in disagreement with the results of Birstein and DeSalle (1998) based on a primarily different set of mtDNA gene sequences. It would be interesting to determine the COII, 12S rRNA, $tRNA_{Phe}$, and $tRNA_{Asp}$ gene sequences for the Eurasian species that were not included in this study to determine whether the sister species relationship persists in analyses including all extant acipenseriform species.

The detection of genetic differences among threatened or endangered groups of vertebrates is useful for establishing subspecific status to obtain or maintain protection of a group under the Endangered Species Act in the United States (O'Brien and Mayr, 1991). The two subspecies of *Acipenser oxyrinchus,* the Gulf and Atlantic sturgeons, have been designated as subspecies on the basis of differences between habitat range, shape of scutes, length of pectoral fins, and relative head length (Vladykov and Greely, 1963). However, the length of spleen relative to fork length provides the most robust distinction between the two subspecies (Wooley, 1985). We observed three nucleotide differences between the two subspecies *A. o. oxyrinchus* and *A. o. desotoi.* One difference was located in the $tRNA_{Asp}$ gene and two were located in the 12S rRNA gene. Although additional individuals would have to be examined to determine whether these differences are fixed characteristics of the two subspecies, our data give more support to the genetic differentiation between these two subspecies, as described by Ong *et al.* (1996) in their study of the control region of mtDNA of the two species.

In contrast to the differences found in the mitochondrial genes for two subspecies of *A. oxyrinchus,* we

found no nucleotide differences in any of the four genes among any of the three *Scaphirhynchus* species. This is consistent with previous studies that have failed to find much genetic variation among them (Phelps and Allendorf, 1983), despite the morphological and behavioral differences noted among the three species (Bailey and Cross, 1954; Williams and Clemmer, 1991; Mayden and Kuhajda, 1996). Phelps and Allendorf (1983) found that pallid and shovelnose sturgeon were electrophoretically indistinguishable at 37 loci in their allozyme study. A study by Genetic Analyses, Inc. (unpubl.) found some variation among *Scaphirhynchus* species during a comparison of banding patterns produced by PCR amplification of nuclear DNA with three different primer sets (gastrin, prealbumin, and high-mobility-group protein-1 primers). There was greater differentiation between *S. suttkusi* and the other two *Scaphirhynchus* species than between *S. platorynchus* and *S. albus,* and the authors felt that, based on the level of variation that they found, *S. platorynchus* and *S. albus* may not be distinct species. Campton, Bowen, Chapman, and Dryer (unpubl.) examined mitochondrial control region variation in the three *Scaphirhynchus* species. They found a unique haplotype that distinguished the three individuals of *S. suttkusi* that were examined from the other two species, but that haplotype differed from the most common haplotype found in *S. platorynchus* and *S. albus* by only one base substitution. In addition, although *S. platorynchus* and *S. albus* shared haplotypes, significant haplotype frequency differences between the two species were found. Studies like these are difficult to carry out simply because it is hard to locate sufficient numbers of samples for species that are threatened or endangered. Our study of other mitochondrial genes did not find any differences among the three *Scaphirhynchus* species, perhaps because these regions are evolving too slowly to allow the species to be distinguished. Also, the lack of variation must be interpreted while keeping in mind that only a single individual of each species was examined here. But, as evidenced by the small amount of variation found in the mtDNA control region (Campton, Bowen, Chapman, and Dryer, unpubl.) and the nuclear regions analyzed by Genetic Analyses, Inc. (unpubl.), these species may display distinctions in larger studies of control region and/or microsatellite DNA analyses in the future. If no significant interspecific variation is discovered in future studies, it is possible that these species are not reproductively isolated in areas in which they occur together so that hybridization has produced the lack of distinguishable genetic characters or that these species were only recently reproductively isolated so that genetic differences have not yet had time to accumulate (Phelps and Allendorf, 1983).

In conclusion, the accumulation of data on evolutionary relationships among species of the order Acipenseriformes is important since many of these species may soon become casualties of human exploitation. This study represents a solid base of information about the North American species, even though they may not be as vulnerable as some of the Eurasian species and therefore are less likely to be in need of critical immediate attention. The mitochondrial gene regions chosen for this study (12S rRNA, COII, $tRNA_{Phe}$, and $tRNA_{Asp}$ genes) have proven to be useful when used together to infer evolutionary relationships within the Acipenseriformes. We believe that the analysis of these genes from additional Eurasian species of sturgeon and paddlefish will also be useful for clarifying worldwide acipenseriform evolutionary relationships, especially when compared to or combined with other recent results from other genes in this group (Birstein and DeSalle, 1998).

ACKNOWLEDGMENTS

The authors thank those whose efforts helped locate fish tissue samples as well as those that generously provided the samples necessary for this study, including James Brown, Ron Bruch, Frank Chapman, David Davies, Carlos Echevarria, Kim Graham, Jerry Hamilton, Leroy Heman, Bernie Kuhadja, Ron Larson, Edward Little, Richard Mayden, Alan Miller, Jerre Mohler, Tim Mulligan, Terry Patterson, Brady Porter, and Kent Ware. In addition, we thank Rob DeSalle, Vadim Birstein, and Phaedra Doukakis for kindly sharing their mitochondrial DNA sequence data alignment. This work was partially supported by grants from the Theodore Roosevelt Memorial Fund of the American Museum of Natural History and the Ohio Sea Grant College Program/NOAA, by a dissertation improvement award from the National Science Foundation, and by cooperation from the Ohio Department of Natural Resources Division of Wildlife.

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