

Molecular systematics of Scaphirhynchinae: an assessment of North American and Central Asian Freshwater Sturgeon Species

By C. B. Dillman¹, R. M. Wood¹, B. R. Kuhajda², J. M. Ray¹, V. B. Salnikov³ and R. L. Mayden¹

¹Department of Biology, Saint Louis University, St Louis, MO, USA; ²Department of Biological Sciences, University of Alabama, Tuscaloosa, AL, USA; ³National Institute of Deserts, Flora and Fauna, Ministry of Nature Protection of Turkmenistan, Ashgabat, Turkmenistan

Summary

The sturgeon subfamily Scaphirhynchinae contains two genera of obligate freshwater sturgeon: *Scaphirhynchus* and *Pseudoscaphirhynchus*, from North America and Central Asia, respectively. Both genera contain morphologically variable species. A novel data set containing multiple individuals representing four diagnosable morphological variants for two species of *Pseudoscaphirhynchus*, *P. hermanni* and *P. kaufmanni*, was generated. These data were used to test taxonomic hypotheses of monophyly for the subfamily Scaphirhynchinae, monophyly of both *Scaphirhynchus* and *Pseudoscaphirhynchus*, monophyly of *P. hermanni* and *P. kaufmanni*, and monophyly of the recognized morphological variants. Monophyly of the subfamily Scaphirhynchinae is consistently rejected by all phylogenetic reconstruction methodologies with the molecular character set while monophyly of both river sturgeon genera is robustly supported. The molecular data set also rejects hypotheses of monophyly for sampled species of *Pseudoscaphirhynchus* as well as monophyly for the recognized intraspecific morphological variants. Interestingly both *Scaphirhynchus* and *Pseudoscaphirhynchus* demonstrate the same general pattern in reconstructed topologies; a lack of phylogenetic structure in the clade with respect to recognized diversity. Despite rejection of monophyly for the subfamily Scaphirhynchinae with molecular data, reconstructed hypotheses from morphological character sets consistently support monophyly for this subfamily. Disparities among the data sets, as well as reasons for rejection of monophyly for Scaphirhynchinae and species of *Scaphirhynchus* and *Pseudoscaphirhynchus* with molecular characters are examined and a decreased rate of molecular evolution is found to be most consistent with the data.

Introduction

The Acipenseriformes are an ancient lineage containing both fossil and extant taxa. Extant Acipenseriform diversity is represented by 27 species in two families: Polyodontidae and Acipenseridae. The two extant families have fossil records dating back to the Mesozoic, with Polyodontidae known from the Early Cretaceous and Acipenseridae known from the Late Cretaceous (Hilton and Grande, 2006). Polyodontidae consists of two genera, each monotypic, and Acipenseridae has four genera that contain the remaining 25 species. Acipenseridae is subdivided into two subfamilies: Acipenserinae and Scaphirhynchinae and each have two recognized genera. Acipenserinae contains 19 species, two in genus *Huso* and 17 in genus

Acipenser. The subfamily Scaphirhynchinae is comprised of six species, three in *Scaphirhynchus* from North America, and three in *Pseudoscaphirhynchus* from Central Asia (Nelson, 1994; Mayden and Kuhajda, 1996). Life history strategies differ between these subfamilies (Bemis and Kynard, 1997) with members of Acipenserinae exhibiting variable life history strategies (i.e. anadromous, amphidromous, and potamodromous; Bemis and Kynard, 1997), while members of Scaphirhynchinae complete all aspects of their life history as obligate freshwater river inhabitants (potamodromous; Bemis and Kynard, 1997).

The river sturgeon subfamily Scaphirhynchinae exhibits high levels of intraspecific phenotypic variability. In the Asian *Pseudoscaphirhynchus* the two species endemic to the Amu Darya River (*P. kaufmanni* and *P. hermanni*) each have two morphologically diagnosable forms while the species endemic to the Syr Darya River (*P. fedtschenkoi*) has three forms (Kuhajda, 2002). Variation within *P. kaufmanni* was first noted by Sagitov (1969) who described a 'common' and a 'dwarf' form of this species. The common form is characterized by a larger overall size, a lighter dorsum, and greater longevity than the smaller 'dwarf' form (Sagitov, 1969; Tleuov and Sagitov, 1973; Kuhajda, 2002). Intraspecific variation within *P. hermanni* has also been noted by previous investigators and includes both long and short snout forms (Kuhajda, 2002). In general the long snout form attains a larger adult size and is lighter in coloration than the short snout form. For a complete treatment of the taxonomic history of these species see Kuhajda (2002).

Phenotypic variability is also present in the two North American *Scaphirhynchus* (*S. albus* and *S. platyrhynchus*, Mayden and Kuhajda, 1996; Murphy et al., this volume) inhabiting the Missouri and Mississippi River basins. Recent studies have confirmed that in addition to morphological variation there is also a significant level of genetic variation and geographic substructure within both species (Ray et al., this volume; Schrey and Heist, this volume). Critical habitat alteration and loss in both the Nearctic (e.g. damming and channel management of the Missouri and Mississippi Rivers and Mobile Basin tributaries) and Palearctic (e.g. draining of the Aral Sea basin via its tributaries, the Amu Darya and Syr Darya rivers, for irrigation) have prompted biologists to list two species in North America as endangered (*S. suttikusi* and *S. albus*) and all three species in Asia as either endangered (*P. kaufmanni*) or critically endangered (*P. hermanni* and *P. fedtschenkoi*, with the latter species possibly extinct; IUCN, 1996; Mayden and Kuhajda, 1997a,b; Salnikov, 1999a,b). In

addition to the negative impacts of habitat modification on Scaphirhynchinae, their roe is prized as a delicacy; and as such they have been historically over-fished and are currently still taken illegally in spite of regulations in place to protect them (Auer, 2004). Given the morphological variation present within the genera, and the conservation implications, it is important to understand the underlying phylogenetic relationships among the genera, species and morphs.

Several alternative phylogenetic hypotheses have been presented for Scaphirhynchinae. Osteological characters of these species unite *Scaphirhynchus* and *Pseudoscaphirhynchus* as monophyletic and sister taxa (Bemis et al., 1997; Findeis, 1997). The recent discovery of the well-preserved sturgeon fossil †*Psammorhynchus* and the re-evaluation of other Acipenseridae fossil materials place this new genus in its own subfamily †*Psammorhynchinae*, which is basal to the subfamily Acipenserinae, which now contains *Acipenser*, *Huso* and the tribe Scaphirhynchini (*Scaphirhynchus* and *Pseudoscaphirhynchus*; Grande et al., 2002; Grande and Hilton, 2006). External morphology also indicates that the river sturgeons form a natural group, but *Pseudoscaphirhynchus* is paraphyletic with respect to the monophyletic *Scaphirhynchus* based on phylogenetic analysis of morphological data (Maiden and Kuhajda, 1996; Kuhajda, 2002). Birstein et al. (1997, 2002) using partial DNA sequences from four to five genes recovered the Scaphirhynchinae as paraphyletic with respect to sampled members of the Acipenseriformes. Simons et al. (2001) using mtDNA sequence data from the cytochrome *b* (cyt *b*) gene and control region (CR) recovered a well-supported clade of *Scaphirhynchus*. Billard and Lecointre (2001) presented a combined molecular and morphological data phylogeny in which Scaphirhynchinae was recovered as monophyletic and the two genera were reciprocally monophyletic. Despite the high level of attention given to sturgeon at the generic and family level of phylogeny, there has been no attempt to provide a robust test of monophyly for genus *Pseudoscaphirhynchus* or quantify variation at the intraspecific level between the two extant species of the genus using a molecular character set. Given that species of genus *Pseudoscaphirhynchus* are arguably the most imperiled members of the order Acipenseriformes we feel it is of tremendous importance to discover as much as we can about the diversity of these fishes while they are still extant.

Given these circumstances the purpose of the current study is to: (i) investigate the monophyly of the genera *Scaphirhynchus* and *Pseudoscaphirhynchus*; (ii) determine whether the observed morphological variants within *Pseudoscaphirhynchus* are genetically distinct and monophyletic and (iii) determine whether the increased taxonomic sampling contained within this study has any affect on phylogenetic relationships within Scaphirhynchinae.

Materials and methods

Fin clips were used to extract total genomic DNA from both the 'common' and 'dwarf' forms of *P. kaufmanni* and the long and short snout forms of *P. hermanni*. Samples of both species were taken from the Amu-Darya River near Kerki, Turkmenistan from 1997–2005. Voucher specimens are deposited at the National Institute of Deserts, Flora and Fauna, Ashgabat, Turkmenistan. Genomic DNA was extracted using QIAGEN's DNEasy tissue kit. PCR amplification of cyt *b* and CR was completed using the primers given in Simons et al. (2001). Amplifications consisted of 45 cycles with an initial 4 min

denaturation at 94°C, followed by 45 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min with a final extension of 72°C for 4 min. Successfully amplified products were gel extracted with the QIAGEN Qiaquick gel extraction kit and eluted to a final volume of 30 µl.

Amplified mtDNA regions were sequenced in both directions with the dye-labelled di-deoxynucleotide chemistry of Beckman-Coulter and run on a CEQ8000 DNA sequencer. Sequences in both directions were aligned using BIOEDIT v7.0 (Hall, 1998) and adjusted by eye. Complete nucleotide sequence alignment was done for each region separately using CLUSTALX (Thompson et al., 1997), and gaps in control regions were manually adjusted for optimal sequence alignment. Sequence data for the three species of *Scaphirhynchus* is from Simons et al. (2001). Sequences for outgroup taxa used to test monophyly of Scaphirhynchinae were downloaded from GenBank and include representatives of *Polyodon*, *Huso* and *Acipenser* (see Table 1). Sequences for outgroup taxa used to root trees were also downloaded from GenBank and include *Amia* and *Lepisosteus* (Table 1).

Phylogenetic analyses and reconstructions were completed using Maximum Parsimony in PAUP* v4.0b10 (Swofford, 2002) and Bayesian reconstruction with MRBAYES v3.0 (Huelsenbeck and Ronquist, 2001) on both separate and combined data alignments. Only results from the combined runs are

Table 1
Taxa and associated GenBank numbers used in this study

Taxa examined	GenBank number	
	Cytochrome <i>b</i>	Control region
<i>Amia calva</i>	NC_004742	Not used
<i>Lepisosteus osseus</i>	NC_008104	NC_008104
<i>Atractosteus spatula</i>	NC_008131	NC_008131
<i>Polyodon spathula</i>	NC_004419	NC_004419
<i>Acipenser baerii baerii</i>	AF238644	AF168469
<i>A. baerii baicalensis</i>	AF238625	AF168496
<i>A. baerii stenorrhynchus</i>	AF238655	AF168495
<i>A. brevirostrum</i>	AJ245828	AF402848
<i>A. dabryanus</i>	AY510085	NC_005451
<i>A. fulvescens</i>	AJ245829	AF402847
<i>A. gueldenstaedtii gueldenstaedtii</i>	AF238677	AF238738
<i>A. gueldenstaedtii colchicus</i>	AF238681	AY847788
<i>A. medirostris</i>	AF184105	AF184106
<i>A. naccarii</i>	AF217208	AJ275214
<i>A. persicus persicus</i>	AF238614	AF238716
<i>A. stellatus</i>	AY847791	Not used
<i>A. sturio 1</i>	AF217209	AF402849
<i>A. sturio 2</i>	AJ245839	Not used
<i>A. transmontanus</i>	AF184107	AF184108
<i>Huso huso</i>	AJ245840	AY846679
<i>H. huso maeoticus</i>	AY846708	AY846667
<i>Pseudoscaphirhynchus hermanni</i> (Short rostrum S-1)	EF484338	EF484345
<i>P. hermanni</i> (Short rostrum S-2)	EF484342	EF484349
<i>P. hermanni</i> (Long rostrum L-2)	EF484341	EF484348
<i>P. kaufmanni</i> (Common Form C-1)	EF484340	EF484347
<i>P. kaufmanni</i> (Common Form C-2)	EF484343	EF484350
<i>P. kaufmanni</i> (Dwarf Form D-1)	EF484339	EF484346
<i>P. kaufmanni</i> (Dwarf Form D-2)	EF484344	EF484351
<i>Scaphirhynchus albus 1</i>	U56987	U43741
<i>Scaphirhynchus albus 2</i>	U56983	U43740
<i>S. platorynchus 1</i>	U56988	U43897
<i>S. platorynchus 2</i>	U56986	U43744
<i>S. platorynchus 3</i>	U56985	U43743
<i>S. suttkusi</i>	U55994	U43679

reported here. The parsimony search was conducted with random sequence addition for 10 replicates holding 10 trees at each step. Branch swapping was completed with the TBR algorithm and the steepest descent option in effect. The final tree was constructed using the strict consensus of all equally most parsimonious trees. In addition, bootstrap support (Felsenstein, 1985) and decay values (Bremer, 1994) were calculated in *TREEROT* v2 (Sorenson, 1999) to determine support for recovered nodes. Bayesian reconstruction was completed by first partitioning the data set and selecting the model of evolution for each codon position for *cyt b* and as a unit for CR with *MrModelTest* (Nylander, 2004) and then completed with two independent runs of 2×10^6 generations each. Likelihood scores were plotted against generation number to determine when stationarity was reached. All trees retained after stationarity were used to construct a 50% majority rule consensus tree with Posterior Probability Scores (PPS) used to describe support for recovered nodes.

Genetic distance measures of divergence were used to document the level of sequence variation in the recovered clades of Acipenseriformes using *MEGA* (Kumar et al., 2004).

The recovered clades of Acipenseriformes, excluding *Polyodon*, were coded based on the recovered topology under the Maximum Parsimony criterion and subsequently used to estimate intra- and inter-clade divergences under the Tamura 3-parameter model of nucleotide evolution with an estimated gamma shape parameter of 0.5.

Results

A total of 827 aligned nucleotides of the CR and 1140 nucleotides of *cyt b* were generated for three *P. hermanni* ($N = 2$ short snout, $N = 1$ long snout phenotype) and four *P. kaufmanni* ($N = 2$ 'common', $N = 2$ 'dwarf'). Of 1967 total nucleotide characters, 1057 (53.7%) were constant, 247 (12.5%) were variable but uninformative for parsimony, and 663 (33.7%) were parsimony informative. Phylogeny reconstruction under parsimony recovered 641 equally parsimonious trees at 1949 steps (Fig. 1; CI = 0.646; RI = 0.801), with sampled sturgeon falling out into five well-supported clades (A, B, C, D, E). Scaphirhynchinae is not recovered as monophyletic, though monophyly for both river sturgeon

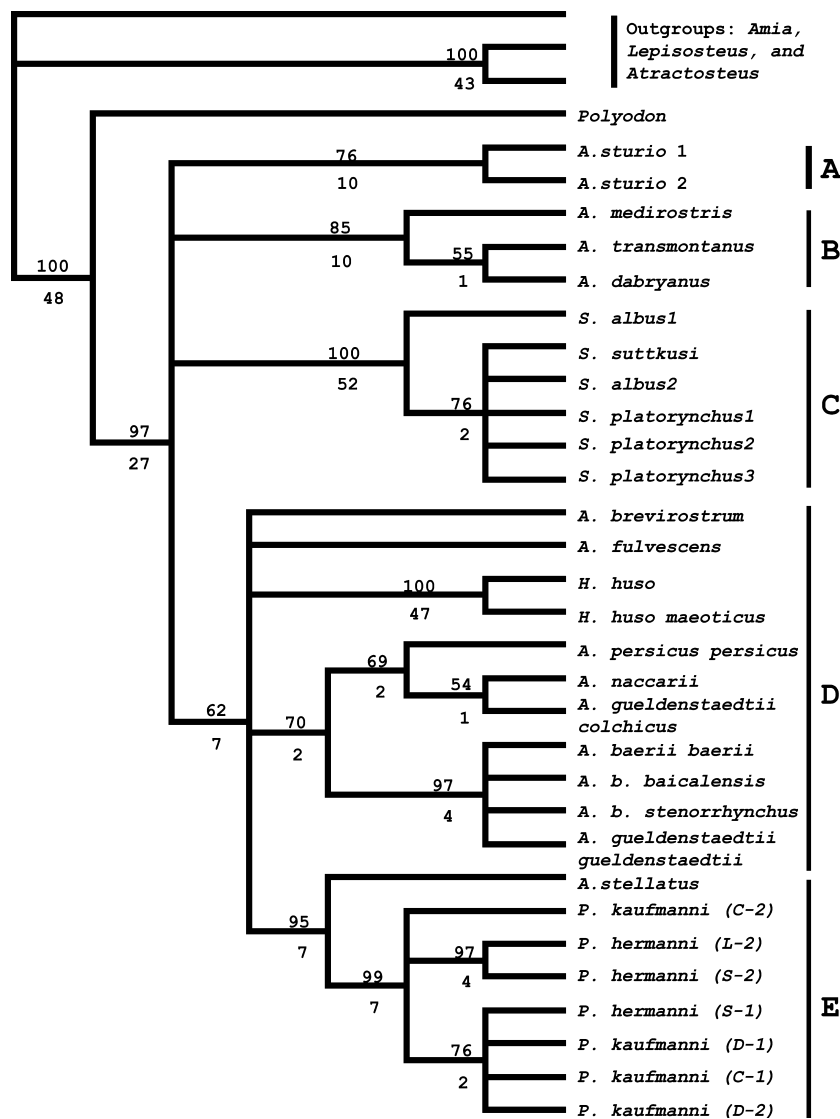


Fig. 1. Strict Consensus hypothesis from 641 recovered Maximum Parsimony trees at 1967 steps for combined *cyt b* and CR mitochondrial DNA sequence data. Numbers above branches are bootstrap support values for each node and numbers below branches are Bremer support indices. Scaphirhynchinae is not recovered as monophyletic. Sampled species and morphotypes of *Pseudoscaphirhynchus* are also not monophyletic

Table 2

Pairwise comparisons of interclade divergences (below diagonal) among the 4 recovered clades [letters correspond to figures 1 and 2] of sturgeon using the Tamura-3-parameter model of nucleotide substitution with a gamma shape parameter of 0.5. Intraclade divergence estimates are also given (with standard error estimates). All calculations were completed using MEGA v3.0

	<i>A. sturio</i> [A]	<i>Acipenser</i> [B]	<i>Scaphirhynchus</i> [C]	<i>Acipenser</i> [D]	<i>Pseudoscaphirhynchus</i> and <i>A. stellatus</i> [E]
Intraclade divergence	0.000	0.049 (0.013)	0.003 (0.002)	0.036 (0.008)	0.009 (0.004)
Interclade divergence					
<i>A. sturio</i> [A]	–				
<i>Acipenser</i> [B]	0.094	–			
<i>Scaphirhynchus</i> [C]	0.128	0.115	–		
<i>Acipenser</i> [D]	0.097	0.078	0.112	–	
<i>Pseudoscaphirhynchus</i> and <i>A. stellatus</i> [E]	0.104	0.072	0.091	0.047	–

3.6%, respectively. Pairwise interclade divergence values (Table 2) ranged from a low of 4.7% between Clades E and D to a high of 12.8% between clades A and C.

Discussion

We tested genus and species level taxonomic hypotheses for *Scaphirhynchus* and *Pseudoscaphirhynchus*, monophyly of known morphological variants within *Pseudoscaphirhynchus*, and relationships among members of subfamily Scaphirhynchinae using newly generated sequence data from two mitochondrial loci. Our results support monophyly for both *Scaphirhynchus* and *Pseudoscaphirhynchus* but do not support: (i) monophyly of the subfamily Scaphirhynchinae; (ii) monophyly of any species within these genera or (iii) monophyly of any morphological species variants. While Birstein et al. (2002) have suggested the non-monophyletic status of subfamily Scaphirhynchinae, our data set is the first to include multiple individuals of species assigned to genus *Pseudoscaphirhynchus*. Interestingly, Simons et al. (2001) failed to recover either *S. albus* or *S. platyrhynchus* as monophyletic with these same mtDNA loci, though like species of *Pseudoscaphirhynchus*, species within genus *Scaphirhynchus* are morphologically diagnosable. The subfamily Scaphirhynchinae does not appear to be a natural group based on the mtDNA sequence characters used for reconstruction here, or based on mitochondrial genes in general (Birstein et al., 2002).

In contrast to the mitochondrial DNA hypotheses, researchers investigating Scaphirhynchinae, and Acipenseriformes in general, using morphological characters have recovered monophyly for the subfamily or tribe (Mayden and Kuhajda, 1996; Bemis et al., 1997; Findeis, 1997; Kuhajda, 2002; Grande and Hilton, 2006). Bemis et al. (1997) and Findeis (1997) recovered a monophyletic tribe Scaphirhynchini in which both *Scaphirhynchus* and *Pseudoscaphirhynchus* are monophyletic; however, their studies included only one representative of each genus. While Mayden and Kuhajda (1996) and Kuhajda (2002) also recovered a monophyletic subfamily Scaphirhynchinae and genus *Scaphirhynchus*, neither study recovered a monophyletic *Pseudoscaphirhynchus*. Bemis and Kynard (1997) in a study that included full taxon sampling for the genera, as well as fossil taxa, also found a monophyletic tribe Scaphirhynchini based on morphological data. Of all studies published to date, only those studies that use mtDNA data alone refute monophyly of tribe Scaphirhynchini or subfamily Scaphirhynchinae. The morphological and mitochondrial DNA data sets do not provide a consistently resolved hypothesis for members of the

Scaphirhynchinae. Conflicting hypotheses derived from different data sets are analogous to the problem addressed by Wiley (1981, p. 140) for conflict among characters. If we consider the morphological data set as one character and the mtDNA sequence data set as a second character, we can reliably infer that at least one of these two data sets does not reflect the true history of these species, in point of fact both character sets may be erroneous but at best only one can be correct.

A recent survey of animal mtDNA studies (Funk and Omland, 2003) indicated several potential mechanisms that can lead to either paraphyly or polyphyly of taxa at the species level. Logically however, these same arguments should apply to any monophyletic assemblage, even those occurring at higher taxonomic levels. Among mechanisms noted are hybridization, incomplete lineage sorting, taxonomic inaccuracy, and a lack of phylogenetically informative characters in the mitochondrion (Funk and Omland, 2003). While each of these represents a plausible explanation for paraphyly or polyphyly in some taxa we feel that one explanation provides the most plausible explanation for the results we have obtained. This is the lack of phylogenetically informative characters in the mitochondrion. The reason we opt for this explanation at this time centres on the pattern obtained in genus *Scaphirhynchus*.

Simons et al. (2001) recovered the allopatrically distributed *S. suttikusi* as monophyletic with respect to *S. albus* and *S. platyrhynchus*. However, given the amount of time since the hypothesized isolation of *S. suttikusi* from remaining species of *Scaphirhynchus* we find what we consider to be a very reduced rate of molecular evolution. The separation of the Mobile Basin (to which *S. suttikusi* is endemic) from the upper Tennessee River and thus the remainder of the eastern highlands in North America (which contains both *S. albus* and *S. platyrhynchus*) is hypothesized to have occurred in the Miocene approximately 5 million years before present (Mayden, 1987). Peng et al. (2007) date the speciation event separating *S. suttikusi* from *S. albus* at 4.1 million years (with 95% confidence intervals ranging from 0.1 to 17.1 million years) based on molecular dating. This is a strikingly good agreement with the dates postulated for the vicariant event hypothesized to have separated the areas containing these species. What is noteworthy, however, is that *S. suttikusi* is far <1% divergent from all other *S. albus* or *S. platyrhynchus* sampled for these mtDNA loci (Simons et al., 2001). Comparisons with other freshwater fishes that span the same divide between the Mobile Basin and other eastern highlands basins, and which are most likely to have experienced the same vicariant event as *S. suttikusi* and remaining members of

genus *Scaphirhynchus* vary between 7% and 10% sequence divergence for the *cyt b* gene. This is based on a comparison of closely related species of darters that span the same geographic divide generated in the lab of one of the authors (R.M. Wood) and available on GenBank. Assuredly sturgeon and darters are two very different groups of fishes. Yet they are also two groups containing members that likely experienced the same vicariant event and yet show an order of magnitude difference in the sequence divergence of the same gene across the same divide. We feel that the shallow divergence observed at this locus in members of genus *Scaphirhynchus* is supportive of a reduced rate of molecular evolution in North American river sturgeon relative to other species of fishes, and is a finding consistent with Krieger and Fuerst (2002) and Robles et al. (2004) for Acipenseriformes in general. In order to fully test the generality of the reduced rate of molecular evolution hypothesis for species of genus *Pseudoscaphirhynchus* genetic material from *P. fedtschenkoi* is required as this is the only member of the genus allopatrically distributed with respect to *P. hermanni* and *P. kaufmanni*. Unfortunately, acquisition of *P. fedtschenkoi* for this purpose is not possible due to its presumed extinct status. Nevertheless, the similar levels of DNA sequence divergence determined for sampled members of *Pseudoscaphirhynchus* and *Scaphirhynchus* are strongly suggestive of the generality of this hypothesis for members of the Acipenseriformes as hypothesized by the authors above.

In spite of the lack of molecular evolution within Acipenseriformes in general and genus *Pseudoscaphirhynchus* in particular, it is still possible given the pronounced morphological variation within described species of *Pseudoscaphirhynchus* that we are underestimating diversity in this genus. If there are more species than the three currently recognized, and if the alternative morphs of *P. kaufmanni* and *P. hermanni* actually harbour additional cryptic taxa, we would expect the observed non-monophyletic classification of these species and morphs based on the molecular data.

While the mitochondrial DNA characters do not recover a monophyletic Scaphirhynchinae morphology consistently does. If the morphological hypothesis is correct then decreased rates of evolutionary change in the *cyt b* and CR loci (mtDNA loci in general) for clades of *Scaphirhynchus* and *Pseudoscaphirhynchus* would provide an additional character supporting the monophyly of Scaphirhynchinae. Morphologically, as with the molecular data, it is interesting to note that either, no synapomorphies (Findeis, 1997), or very few synapomorphies (Mayden and Kuhajda, 1996) unite members of *Acipenser*, and that there are few, if any, differences between recognized extant and extinct sturgeon taxa (Hilton and Grande, 2006; and references therein). The lack of phylogenetically informative characters has made the inference of phylogeny using morphology problematic as well. Given these circumstances we feel strongly that the alpha-level taxonomy of sturgeon species should not be altered on the basis of any single data set including the one presented herein. While there is obviously more biodiversity within sturgeon than currently recognized by formally named taxa we do not feel that the data sets assembled to date provide sufficient resolving power to alter any currently recognized genera. It is imperative that the number of studies conducted on sturgeon species be increased and completed on both morphological and molecular data sets. Due to the lack of phylogenetically informative characters it is obvious that more data are needed to fully understand the history of this taxonomic group. These data are not only

necessary to establish a set of diagnostic markers specific to the known morphological variants, but are also required for effective management by conservation groups in the recovery of these imperilled fishes.

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- Author's address:** R. M. Wood, Department of Biology, Saint Louis University, 3507 Laclede Avenue, St Louis, MO 63103, USA.
E-mail: wood2@slu.edu