MOLECULAR PHYLOGENETICS AND EVOLUTION
Vol. 9, No. 1, February, pp. 141–155, 1998
ARTICLE NO. FY970443

Molecular Phylogeny of Acipenserinae

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Received August 6, 1996; revised May 23, 1997

The family Acipenseridae consists of 25 extant sturgeon species (19 species of Acipenserinae and 6 species of Scaphirhynchinae). Together with two extant paddlefish species, *Polyodon spathula* and *Psephurus gladius* (Polyodontidae), it composes the order Acipenseriformes, the most numerous of all living "fossil" fishes. This paper presents results of sequencing of three regions of the cytochrome *b* gene (650 bp), and fragments of 12S (150 bp) and 16S (350 bp) rRNA genes, from all extant species of Acipenserinae (species of *Acipenser* and *Huso*) and *Scaphirhynchus albus* (Scaphirhynchinae). The phylogenetic tree obtained for combined data is the first comprehensive treatment of phylogeny within the Acipenserinae. Three general conclusions are inferred from the tree: (1) The pallid sturgeon, *S. albus*, is the sister-species of all species of *Acipenser* and *Huso*. (2) The two species of *Huso* are embedded within the genus *Acipenser*. It also appears that *Huso* is not a separate taxonomic unit. (3) There are at least three main clades within *Acipenser: A. sturio–A. oxyrinchus, A. schrenckii–A. transmontanus,* and all Ponto–Caspian species plus *A. dabryanus* and *A. brevirostrum.* There is congruence between ploidy and the branching patterns of the sturgeon species. A hypothetical evolutionary history of the Acipenseriformes based on the paleontological, geological, and molecular data is discussed. © 1998 Academic Press

INTRODUCTION

The family Acipenseridae consists of 25 extant sturgeon species (Rochard *et al.*, 1991; Birstein, 1993a; Birstein and Bemis, 1997). Together with the family Polyodontidae (two extant paddlefish species, *Polyodon spathula* and *Psephurus gladius*) it composes the order Acipenseriformes, the most numerous among all living "fossil" fishes (Gardiner, 1984). Acipenseriforms inhabit the Northern Hemisphere only, and the present biogeographic distribution of the extant species of this group reflects ancient relationships among fish faunas of Europe, Asia, and North America (Berg, 1909, 1949; Grande and Bemis, 1991, 1996; Grande, 1985, 1994; Wilson and Williams, 1992).

The position of Acipenseriformes in the phylogeny of fishes was discussed in the classic works of Sewertzoff (1926a,b, 1928) and Berg (1948a). Recently this subject has attracted the attention of modern workers because of new paleontological finds and the use of cladistics (Grande and Bemis, 1991, 1996; Jin, 1995; Bemis *et al.*, 1997). According to contemporary views, the extant acipenseriforms form the monophyletic sister-group of all extant Neopterygii (e.g., Lepisosteidae, Amiidae, and Teleostei; Bemis *et al.*, 1997). Most ichthyologists regard Polypteridae as the sister-group of Acipenseriformes + Neopterygii (Patterson, 1982). A comparison of partial sequences of 28S rRNAs supports this relationship (Le *et al.*, 1993). In contrast to other workers (review in Jin, 1995), Grande and Bemis (1991, 1996) conclude that paddlefishes and sturgeons are sister-taxa, and that extinct Mesozoic genera such as *ÜChondrosteus* lie outside this clade. Within Acipenseriformes, all workers agree that the Acipenseridae and Polyodontidae diverged prior to the Late Cretaceous (Berg, 1948a; Yakovlev, 1977, 1986; Grande and Bemis, 1991; Jin, 1995).

Relationships within Acipenseridae (genera *Acipenser, Huso, Pseudoscaphirhynchus,* and *Scaphirhynchus*) are also debated. The subfamily Scaphirhynchinae (three Asian species of *Pseudoscaphirhynchus* and three American species of *Scaphirhynchus*) is usually considered the sister-group of all other sturgeons (Berg, 1904, 1905; Mayden and Kuhajda, 1996) and the oldest group within Acipenseridae (Zograf, 1887; Birstein *et al.*, 1997b). Findeis (1993, 1997), using anatomical characters, concluded that Scaphirhynchinae is a derived group within this family.

There is no single agreed upon hypothesis describing the species relationships within the genus *Acipenser*. Attempts to place the extant species into groups have been made since the early studies on sturgeons (reviews in Dumèril, 1870; Bemis *et al.*, 1997). Our preliminary work on partial sequences of short fragments of the mitochondrial (mt) cytochrome *b* (cyt*b*) gene and 12S and 16S rRNA genes from five *Acipenser* species did not support previous grouping (Birstein *et al.*, 1997b). According to modern paleontological views, the extant species of *Acipenser* belong to different evolutionary lineages which diverged a long time ago,

1055-7903/98 $25.00

possibly in the Upper Cretaceous (Nesov and Kaznyshkin, 1983).

The present paper presents a cladistic analysis of DNA sequence characters from three regions of the cyt*b* gene (650 bp), and fragments of 12S (150 bp) and 16S (350 bp) rRNA genes, for all extant species of Acipenserinae. Because this subfamily consists of a relatively small number of species, we had a unique opportunity to investigate the relationships among all members of a subfamily of "living fossils." The phylogenetic tree obtained for the combined molecular data is the first tree showing relationships among all extant species, including those between American and Eurasian sturgeons. Although the hypothesis of the current distribution of *Acipenser* species based on Wegener's theory of plate tectonics and other geological events was proposed more than 60 years ago (Birstein and Vinogradov, 1934), usually American species of *Acipenser* are still considered a separate group of related species (e.g., Brown *et al.,* 1996; Krieger *et al.,* 1996). Our data point to trans-Pacific and trans-Atlantic relationships between the American and Eurasian sturgeons, which supports the acipenserid distribution hypothesis by Birstein and Vinogradov (1934). We hope our data will stimulate comparative studies of these "living fossils" as a whole group inhabiting both continents, Eurasia and North America.

MATERIALS AND METHODS

Sample collection. The species studied and geographic areas where they were collected are given in Table 1. Almost all samples were taken from live fishes. Names of the sturgeon experts who collected samples are also given in Table 1. Samples of blood in buffer or ethanol-fixed eggs or muscles were used for DNA extraction and amplification. Samples are deposited in the AMNH frozen- and ethanol-preserved tissue collections.

DNA isolation and manipulation. DNA was isolated essentially according to the methods outlined in Birstein *et al.* (1997b). Specifically, blood cells, tissues, or eggs were treated overnight in 1 mg/ml final concentration of Proteinase K, 1% SDS and homogenization buffer. After overnight digestion, a 0.1 vol of 5 M potassium acetate was added. Phenol extraction was performed with an equal volume of phenol followed by an extraction with an equal volume of chloroform. The aqueous phase was ethanol precipitated twice and resuspended in ultrapure water for PCR.

TABLE 1

List of the Blood, Tissue and Egg Samples from the Acipenseriform Species Studied

Species	Geography area of sampled specimens	Name of collector
Family: Acipenseridae		
Subfamily: Acipenserinae		
Genus Acipenser		
A. baerii[1]	Lena River (Siberia, Russia)	Vadim Birstein
A. brevirostrum[2]	Connecticut River (MA, U.S.A.)	Boyd Kynard
A. dabryanus[3]	Yangtze River (China)	Qiwei Wei
A. fulvescens[2]	Great Lakes (WI, U.S.A.)	Fred Binkowski
A. gueldenstaedtii[3]	Caspian Sea, Northern part (Russia)	Anatolii Vlasenko
A. medirostris[1]	Columbia River (OR, U.S.A.)	John North
A. mikadoi[3]	Tumnin River (Russian Far East)	Evgenii Artyukhin
A. nudiventris[1]	Aral Sea (Uzbekistan, Central Asia)	Vadim Birstein
A. oxyrinchus desotoi[3]	Pearl River (LA, U.S.A.)	John Waldman
A. oxyrinchus oxyrinchus[3]	St. Lawrence (Quebec, Canada)	John Waldman
A. persicus[3]	Caspian Sea, Southern part (Iran)	M. Pourkazemi
A. ruthenus[1]	Volga River (Russia)	Vadim Birstein
A. schrenckii[3]	Amur River (Siberia, Russia)	Victor Svirskii
A. sinensis[3]	Yangtze River	Qiwei Wei
A. stellatus[1]	Volga River	Vadim Birstein
A. sturio[3]		
a. A specimen from the Gironde River population	Gironde River (France)	Patrick Williot
b. A specimen from the North Sea	North Sea, near the Dutch coast	Lutz Debus
A. transmontanus[1]	Columbia River (OR, U.S.A.)	John North
Genus *Huso*		
Huso dauricus[3]	Amur River (Siberia, Russia)	Victor Svirskii
H. huso[3]	Caspian Sea, Northern part (Russia)	Anatolii Vlasenko
Subfamily: Scaphirhynchinae		
Scaphirhynchus albus[1]	Yellowstone River (MT, U.S.A.)	Herb Bollig
Family: Polyodontidae		
Polyodon spathula[1]	Moscow Aquarium	Vadim Birstein
Psephurus gladius[3]	Yangtze River (China)	Qiwei Wei

[1] Blood samples were taken, mixed with buffer (100 mM Tris, 100 mM EDTA, and 2% SDS; 0.5 ml of blood and 5 ml of buffer), and the blood cells lysed in this solution were kept in a freezer at −70°C.

[2] Egg samples: freshly obtained eggs fixed in 96% ethanol.

[3] Tissue samples: small piece of muscle fixed in 96% ethanol.

PCR was accomplished for three mt genes. The cyt*b* reactions generated a 1.1-kb fragment using the universal primers cytb1 and cytb14 (Irwin *et al.,* 1989). The 12S primers and 16S primers used are also universal (12Sa/12Sb; 16Sa/16Sb; see Simon *et al.,* 1994). The sequence information was collected in three different ways. In some difficult cases the PCR fragments were cloned using the TA cloning kit (Invitrogen) and three separate clones from each PCR fragment were sequenced to determine the final sequence for the fragment. In the cases where direct sequencing of the double-stranded PCR products was accomplished the PCR product was first cleaned with Geneclean (BIO

101). We used standard direct manual sequencing and automated sequencing on the ABI 373 sequencer to do the direct sequencing.

For the cyt*b* gene fragment, we determined that the most variable regions were between the cytb1 and cytb2 primer pairs, the cytb4 and cytb5 primer pairs, and the cytb7 and cytb14 primer pairs (see Irwin *et al.* (1989) for positions of primers). These primers were used to do all of the cyt*b* sequencing and other regions of the gene were not sequenced. In a similar way it was determined that the entire 12S fragment would not be useful at this level of analysis (i.e., there is no variability in this fragment for these taxa; see Birstein *et al.*, 1997b) and that only the 12Sb end of the 12S fragment would provide useful characters. Therefore, we used the 12Sb primer to accomplish the collection of these sequences. These sequences were all generated by automated sequencing and only the first 150 bases of the sequencing run were used in the data collection. The entire 16S fragment was deemed useful at this level of analysis (see also Birstein *et al.*, 1997b) and so both the 16Sa and 16Sb primers were used to accomplish the sequencing. All sequences have been deposited in GenBank under Accession Numbers AF004968–AF004983(12S), AF004954–AF004967(16S), and AF006123–AF006188(cyt*b*).

Some of our tissue samples were extremely difficult to obtain and to manipulate due to the rarity of the specimens and the manner of storage of tissue for the specimens. In the case of blood samples we had little or no problems in obtaining sequence information for all three genes. We were less fortunate with degraded tissue sources and subsequent DNA isolations. In these cases we resorted to PCR amplification using primers positioned no more than 150 bases apart. One particular sample, from *Acipenser dabryanus,* posed such a problem that for this specimen we were able to obtain data only for the cyt*b* gene. The treatment of this taxon in the data analysis is described below.

Phylogenetic analysis. The mt rDNA sequences were aligned with MALIGN (Wheeler and Gladstein, 1993) using a gap-to-change cost of 4 (see Birstein *et al.*, 1997b). An examination of several gap-to-change costs for the two stretches of structural RNAs (12S and 16S) indicated that there was little alignment ambiguity (Gatesy *et al.*, 1993; Wheeler *et al.*, 1995) in these regions and so we treated our alignment with a gap-to-change cost of 4 as a stable set of primary homology statements (*sensu* de Pinna, 1991; Nelson, 1994).

All phylogenetic analyses were performed with PAUP version 3.1 (Swofford, 1993). The particulars of the phylogenetic analyses using this computer package are articulated below.

Our outgroup choice was based on previous analyses of acipenseriforms where we show several genera as outside of the genus *Acipenser* (Birstein *et al.*, 1997b). As multiple outgroups are preferable for polarizing characters efficiently (Watrous and Wheeler, 1981), we chose two genera as outgroups for the present analysis: *Polyodon spathula,* and *Psephurus gladius* (Polyodontidae).

In our analyses we first opted to apply equal character weights due to our initial assumption of the independence of these three character sets (Nixon and Carpenter, 1996). Since character weighting is a logical part of phylogenetic analysis, we also applied a weighting system that reflects the replacement changes and silent changes in the codon positions of the cyt*b* data. Agosti *et al.* (1996) suggest that a reasonable method for weighting protein-coding regions of a gene is to simply append the amino acid sequence to the nucleotide sequence matrix. This method of data matrix construction allows for the more stable amino acid replacement changes to be upweighted relative to less stable silent positions in accordance with the genetic code (Agosti *et al.*, 1996). To examine the effects of this weighting system we present the results of "unweighted" (i.e., unappended amino acids) and "weighted" (amino acids appended) analyses.

We also opted to represent the robustness of our phylogenetic hypotheses with the decay or Bremer index (Bremer, 1988, 1994; Donoghue *et al.*, 1992). This index gives the number of steps in trees that need to be added for a particular node to collapse in a strict consensus tree.

Two sets of taxa were used in the phylogenetic analysis to reflect the absence of *A. dabryanus* for 12S and 16S rDNA sequences. In the first, *A. dabryanus* was simply removed from the analysis. In the second, *A. dabryanus* was included and the 12S and 16S sequences were coded as missing for this taxon. In general, the removal of *A. dabryanus* did not change the phylogenetic hypotheses generated from the matrix with *A. dabryanus* included. Parsimony analysis was performed using the heuristic search option with 25 repetitions of random addition sequence.

We also partitioned our characters to reflect the different genes involved and calculated the incongruence length difference (ILD) (Mickevich and Farris, 1981; Farris *et al.*, 1995). We partition the character sets as cyt*b* versus rDNA and forego the gene by gene partition. ILD measures are difficult to compute when any of the taxa for one or more of the data partitions are coded as missing. This presents a problem for our study because one taxon (*A. dabryanus*) is coded as missing for all of the rDNA characters. As a proxy for an examination of data partition congruence, we removed *A. dabryanus* from both partitioned data sets and computed the ILD and associated statistical significance. We performed 1000 replications using the ARN program (Farris *et al.*, 1995) to estimate the statistical significance of the partition.

RESULTS AND DISCUSSION

Characteristics of partial sequences of genes studied. Three noncontiguous fragments of the mt cyt*b* gene were sequenced totaling 650 bases that lie within bases 106 through 962 in the published sequence of the cyt*b* gene of *A. transmontanus* (Brown *et al.,* 1989). Also, a 350-bp-long fragment of the mt 16S rDNA and a 150-bp fragment of the 12S rDNA were sequenced. The utility of these mt rDNA regions is discussed in general for animals in Simon *et al.* (1994), and in particular for sturgeon taxa in Birstein *et al.* (1997b). The combined data for three regions of the cyt*b* gene and combined data for 16S plus 12S rRNA genes were used for the analyses.

Incongruence of data partitions. In our examination of the congruence of data partitions we removed *A. dabryanus* from the analysis and partitioned the data into two character sets—mt rDNA and cyt*b*. Figure 1 shows the two phylogenies obtained from such partitioning. Visual inspection of the two cladograms suggests basic incongruence between the two data partitions. The ILD for this comparison is 12 and is significant at $P < 0.05$. We agree with the arguments advanced in Nixon and Carpenter (1996) that suggest data be combined in all instances. We point out, however, that viewpoints advanced by Bull *et al.* (1993), Miyamoto and Fitch (1995), and Huelsenbeck *et al.* (1996) exist that suggest alternative strategies for combining character sets. The significant ILD figure demonstrates incongruence between the two data partitions, suggesting that different phylogenetic signal emanates from the two partitions. Different authors (see above) have suggested various strategies for dealing with this phenomenon with respect to the combination of data partitions and much controversy exists over this issue.

Global parsimony analysis. When equal weights are applied to all nucleotide positions, all data partitions are included in the analysis, amino acids are not appended to the data matrix, and *A. dabryanus* is included, a single parsimony tree is obtained (Fig. 2).

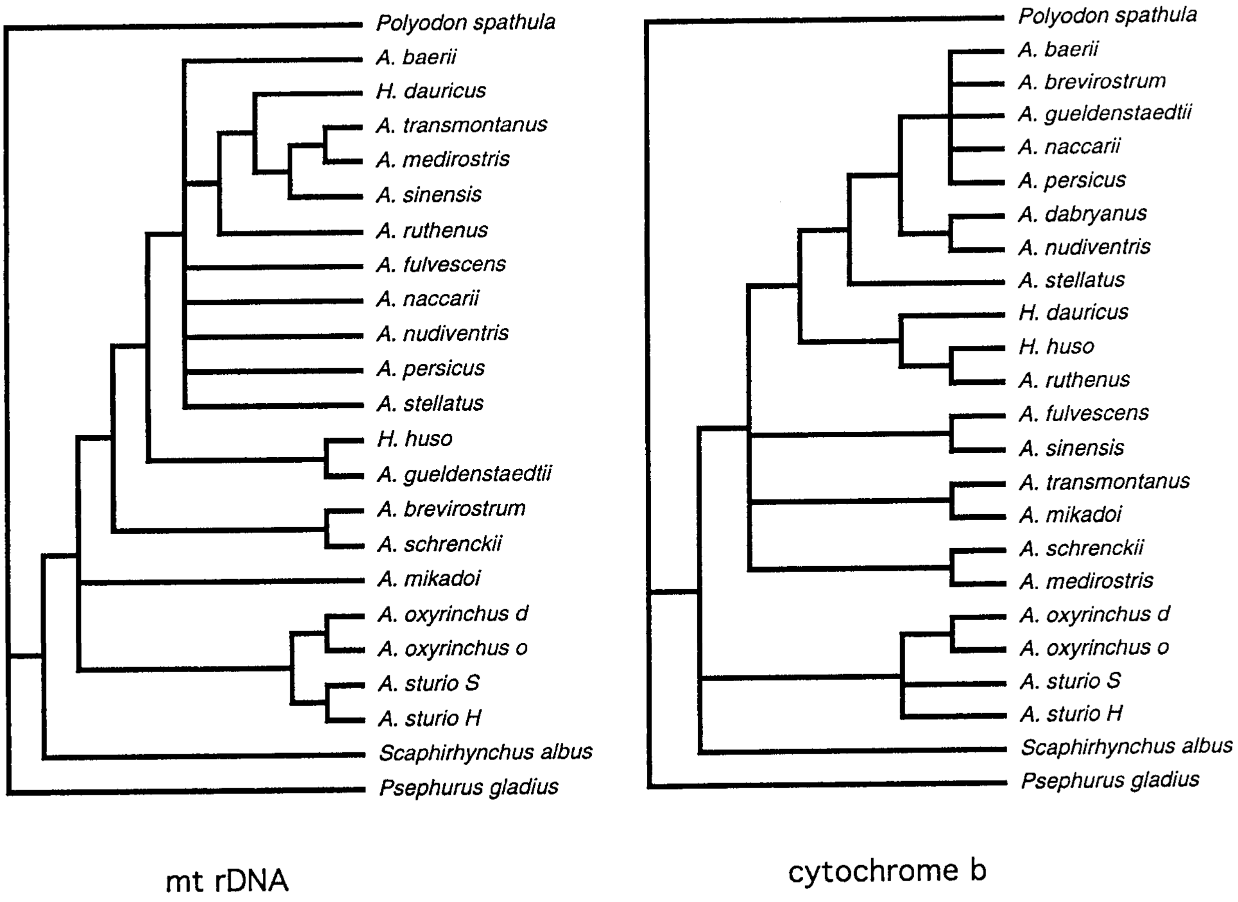

FIG. 1. The phylogeny of the acipenserid mt rDNA (combined data for the 12S and 16S rRNA genes; without *Acipenser dabryanus*) and cytochrome *b* gene constructed by PAUP. *Polyodon spathula* and *Psephurus gladius* were used as outgroups. The total length of the first tree is 312 steps, and the tree is a consensus of 14 trees. The CI value is 0.494, and RI = 0.542. The total length of the second tree is 448 steps, and the tree is a consensus of 24 trees. The CI value is 0.415, and RI = 0.572.

This tree is 787 steps long with a consistency index (CI) = 0.427 and a retention index (RI) = 0.527. When the matrix is weighted by appending amino acids to the data matrix (Agosti *et al.,* 1996), a single alternative parsimony tree is obtained with 865 steps and CI = 0.430 and RI = 0.525 (Fig. 3). The topologies of the two parsimony trees from the different methods of weighting are for the most part congruent.

Comparison of the degree of support for nodes in these two parsimony trees produced with equal weights (Fig. 2) and weighting with amino acids (Fig. 3) shows that the decay indices increase when weighting is applied. In the "unweighted" analysis 7 of the 20 nodes collapse in trees one step longer than parsimony (they have a decay index = 1). In the "weighted" analysis only one of the 20 nodes has a decay index less than 2. The logical necessity of character weighting in phylogenetic analysis, and the objective *a priori* method by which the "appended amino acid" method (Agosti *et al.,* 1996) accomplishes this, renders the phylogenetic hypothesis in Fig. 3 the more reasonable of the hypotheses we present.

Phylogenetic implications. Three robustly supported conclusions can be inferred from the tree presented in Fig. 3:

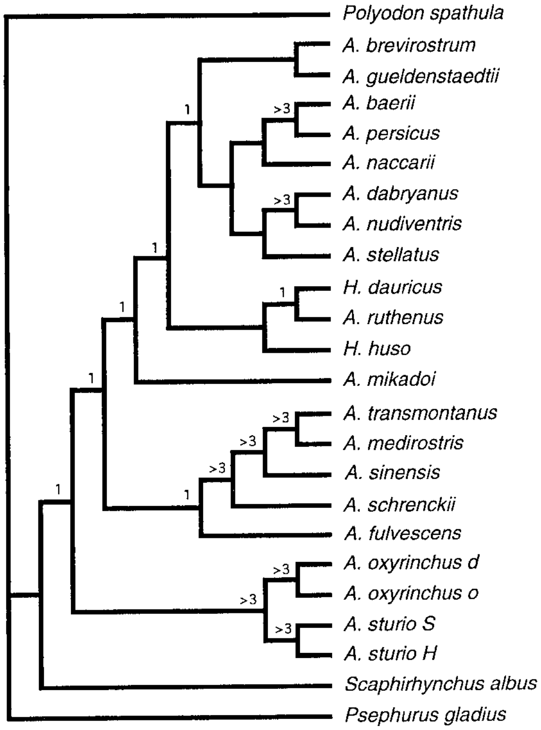

FIG. 2. A tree for the combined "unweighted" data for the cytochrome *b* and 12S and 16S mtrRNA genes of the Acipenseridae generated by PAUP. *Polyodon spathula* and *Psephurus gladius* were used as outgroups. The total length of the tree is 787 steps. The CI value is 0.427, and RI = 0.527. Numbers above branches are decay indices (Bremer, 1988, 1994).

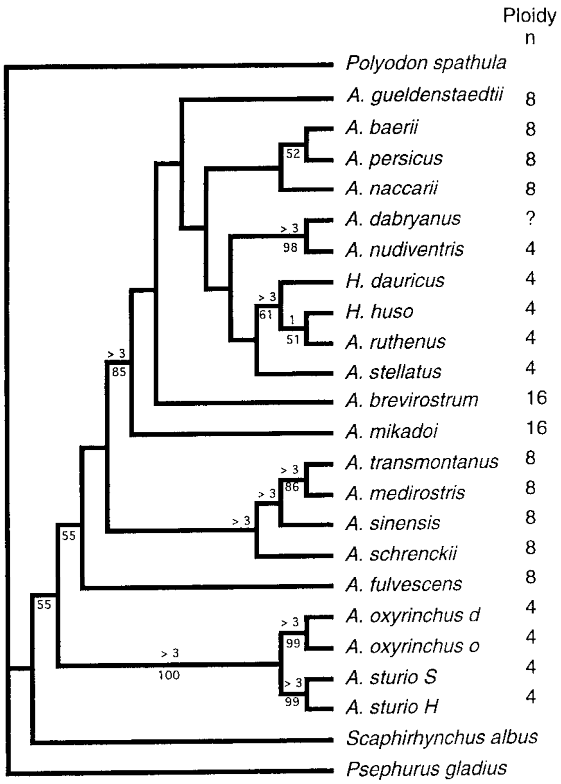

FIG. 3. The phylogeny of the Acipenseridae based on the combined "weighted" data for the cytochrome *b* and 12S and 16S mtrRNA genes and generated by PAUP. *Polyodon spathula* and *Psephurus gladius* were used as outgroups. The total length of this single parsimony tree is 865 steps. The CI value is 0.430, and RI = 0.525. Numbers below the branches indicate the proportion of 100 bootstrap replicates that supported the depicted assemblages; no number below the branch indicates a bootstrap <50%. Decay indices are shown above the branches; no number above the branch on a node indicates a decay index of 2. Ploidy data from Birstein *et al.* (1997b).

(1) *Scaphirhynchus* is the sister-genus of all species of *Acipenser* and *Huso.* The pallid sturgeon, *S. albus,* was used in this study as a representative of the subfamily Scaphirhynchinae. Our tree, however, does not allow discrimination between hypotheses about the ancient origin (Zograf, 1887) or derived status (Findeis, 1997; Bemis *et al.,* 1997) of this subfamily.

(2) The two species of *Huso* are embedded within the genus *Acipenser.* It appears that *Huso* is not a separate taxonomic unit.

From the beginning of the 20th century, the two species of *Huso* were considered belonging to a separate genus (Berg, 1904). Despite the morphological difference between the two species of *Huso* and the species of *Acipenser* (Berg, 1911; 1948b; Antoniu-Murgoci, 1936; Sokolov, 1989a), the validity of the genus *Huso* is still under discussion. Artuykhin (1995) described five morphological and anatomical characters similar in *Huso* and *Acipenser ruthenus.* Our data support the possibility that two species of *Huso* belong to the genus

Acipenser and are closely related to *A. ruthenus*, *H. dauricus* being basal to *H. huso* and *A. ruthenus* (Fig. 3). All species of this clade are tetraploids—they seem to have preserved a karyotype similar to that of *A. sturio*, and, possibly, to the ancestral karyotype (Birstein *et al.*, 1997b).

In contrast, *H. huso* and *A. ruthenus* are considerably different in general morphology and ecology: *H. huso* is the largest (historically up to 5 m) anadromous fish, while *A. ruthenus* is a small, 15–90 cm long, freshwater fish (Pirogovskii *et al.*, 1989; Sokolov and Vasil'ev, 1989b). In classic ichthyology literature *A. ruthenus* was grouped with different species by different authors (Berg, 1911, 1948b; Antoniu-Murgoci, 1942; Sokolov, 1989b; Artyukhin, 1995). The fact that *H. huso* and *A. ruthenus* might be closely related is supported by the ease with which they hybridize. Hybrids between these species are not only viable but also fertile (review in Birstein *et al.*, 1997b). The extreme morphological difference between these two related species is possibly a result of paedomorphosis, which played a very important role in the evolution of acipenseriforms (Yakovlev, 1977; Grande and Bemis, 1991; Tsessarsky, 1992).

(3) There are at least three main clades within *Acipenser.* An *A. sturio–A. oxyrinchus* clade, an *A. schrenckii–A. transmontanus* clade, and a clade encompassing all the Ponto–Caspian species plus *A. dabryanus* and *A. brevirostrum. Acipenser fulvescens* and *A. brevirostrum* are definitely distantly related: *A. fulvescens* is the sister-species of all *Acipenser* species except the *A. sturio–A. oxyrinchus* clade (or belongs to *A. schrenckii–A. transmontanus* clade, see Fig. 2), while *A. brevirostrum* is closely related to the Ponto–Caspian species. *Acipenser mikadoi* is a sister-species of the Ponto–Caspian species clade plus *A. brevirostrum.* The positions of these latter two species, *A. mikadoi* and *A. fulvescens,* are not robustly supported, and more character information will be needed for the robust placement of these species within the phylogenetic tree.

(a) *Acipenser sturio-A. oxyrinchus* cluster. *Acipenser sturio* and *A. oxyrinchus* are closely related species of the same ploidy (Fig. 3). Once considered a subspecies of *A. sturio,* the American Atlantic sturgeons were subsequently split off as a separate species named *A. oxyrinchus* (Vladykov and Greeley, 1963). Then, two subspecies, *A. o. oxyrinchus* and *A. o. desotoi,* were described within *A. oxyrinchus* (Vladykov, 1955; Vladykov and Greeley, 1963). Our data show some genetic difference (one nucleotide change in the cyt*b* gene) between the two subspecies (see also Birstein *et al.*, 1997a). Fixed differences in the nucleotide sequence of the D-loop in these subspecies was found by Ong *et al.* (1996) and Wirgin *et al.* (1997). Six nucleotide changes in the region of cyt*b* analyzed in the two specimens of *A. sturio* are even higher than those in the two subspecies of *A. oxyrinchus*. Possibly, the specimen from the Gironde River can be considered a representative of the Mediterranean–Black Sea form, while that from the North Sea is a representative of the Baltic–North Sea form (Birstein *et al.*, 1997a). The position of *A. sturio–A. oxyrinchus* in our phylogenetic tree supports the hypothesis that *A. sturio* is, probably, a descendant of ancestral forms of *Acipenser* (Nesov and Kaznyshkin, 1983).

(b) *Acipenser fulvescens.* It was believed that the American freshwater *A. fulvescens* which inhabits mostly the Great Lakes basin is closely related to another eastern North American sturgeon, the shortnose sturgeon, *A. brevirostrum* (Lee *et al.*, 1980). Our data show that these two species are distantly related. The basal position of *A. fulvescens* points to a possibility that it is a descendant of a very ancient lineage of sturgeons which gave rise to all other recent acipenserins (except *A. sturio–A. oxyrinchus*).

(c) *Acipenser schrenckii–A. transmontanus* cluster. There are strong trans-Pacific relationships between the two Asian species, *A. schrenckii* and *A. sinensis,* from one side, and the two American species, *A. transmontanus* and *A. medirostris,* from another. Since *A. schrenckii* is the basal species in this cluster, it seems that the group originated in Asia. Our grouping of *A. transmontanus* with *A. medirostris* within one clade is congruent with the morphological similarity (Findeis, 1993; Artyukhin, 1995) and sympatry (Vladykov and Greeley, 1963; Scott and Crossman, 1973) of both species. Our results do not support the opinion of Artyukhin (1995) based on the biogeography data only that *A. sinensis* is closely related to another sturgeon of the Yangtze River, *A. dabryanus.*

(d) *Acipenser mikadoi.* Previously, the Sakhalin sturgeon, *A. mikadoi,* which inhabits the Sea of Okhotsk and small rivers of the Russian Far East, was considered conspecific to the Pacific American green sturgeon, *A. medirostris* (see Birstein, 1994). The only morphological comparative study of these forms showed several meristic differences in dorsal and anal fin rays and dorsal scute rows (Lindberg and Legeza, 1965). Recently, it was shown that the Sakhalin sturgeon has an unusually high ploidy level, 16*n* (Birstein *et al.*, 1993), while the ploidy of the American *A. medirostris* is 8*n* (Blacklidge and Bidwell, 1993). Our molecular data confirm a profound genetic difference between *A. medirostris* and *A. mikadoi.*

(e) *Acipenser brevirostrum* plus Ponto–Caspian taxa. The American shortnose sturgeon of the Atlantic coast, *A. brevirostrum,* is basal to the Ponto–Caspian and some Asian sturgeons (Fig. 3) and definitely has a trans-Atlantic relationship with the Ponto–Caspian species.

Traditionally, anadromous sturgeon species inhabiting the Caspian, Aral, Black, and Azov seas (*A. gueldenstaedtii, A. stellatus, A. nudiventris, A. persicus,* and *Huso huso;* see Table 2) are considered the descendants of the fauna of the ancient Tethys Sea and its later

TABLE 2

Sturgeon Species Belonging to the Ponto–Caspian Group Inhabiting the Caspian, Black, Azov, Aral, and Mediterranean Seas

Species	Historical area	Reference
Acipenser gueldenstaedtii	Black, Azov, and Caspian seas	Berg, 1948b; Vlasenko *et al.*, 1989a
A. nudiventris	Black, Azov, Caspian, and Aral[1] seas	Berg, 1948b; Sokolov and Vasil'ev, 1989a
A. persicus	Caspian and Black seas	Berg, 1948b; Vlasenko *et al.*, 1989b
A. stellatus	Caspian, Azov, Black, and Aegean[2] seas	Berg, 1948b; Shubina *et al.*, 1989
Huso huso	Adriatic,[3] Black, Azov, and Caspian seas	Berg, 1948b; Pirogovskii *et al.*, 1989

[1] *Acipenser nudiventris* is extinct now in the Aral Sea region because of drying of this sea (Salikhov and Kamilov, 1995; Zholdasova, 1997).

[2] *Acipenser stellatus* is practically extinct in the Aegean Sea: there were no reports on it in this sea since the 1970s (Papakonstantinou, 1988).

[3] *Huso huso* is practically extinct now in the Adriatic Sea (Rossi *et al.*, 1991).

derivatives (e.g., Berg, 1949). Our data (Fig. 3) suggest that relationships within this group are complex and these species are closely related to some of the Asian sturgeon species.

Usually *Acipenser gueldenstaedtii* is considered to be similar morphologically to *A. persicus* (Vlasenko *et al.*, 1989b) and *A. naccarii* (Tortonese, 1989) and closely related to *A. baerii* (Sokolov and Vasil'ev, 1989c). In our tree *A. gueldenstaedtii* is basal, whereas *A. baerii* is more closely related to *A. persicus* than to *A. naccarii.*

A highly supported (DI > 3) grouping of *A. nudiventris* with *A. dabryanus* is an expected result of our analysis. *A. nudiventris* is an anadromous species, historically living in the Aral, Caspian, and Black Seas basins (Sokolov and Vasil'ev, 1989a); in the Danube River basin it was represented by a riverine, nonanadromous form (Bănărescu, 1994). Traditionally, *A. nudiventris* was considered as the only representative of the subgenus *Lioniscus* (Berg, 1911, 1948b; Sokolov, 1989b), while Artyukhin (1995) put it into the subgenus *Sterleta* together with *A. ruthenus* and *A. schrenckii*. *A. dabryanus* is a freshwater sturgeon inhabiting the Yangtze River (Wei *et al.*, 1997), and Artyukhin (1995) combined it with *A. sinensis* in the subgenus *Sinosturio*. We assume that an anadromous ancestral form of these species inhabited some ancient sea basin in Central Asia.

Acipenser stellatus, which is plesiomorphic to the other species of the last clade in our tree (Fig. 3), was considered by many authors as the only member of a separate subgenus *Helops* or *Gladostomus* (Berg, 1948b; Sokolov, 1989a,b; Artyukhin, 1995; see also Bemis *et al.*, 1997). Our results suggest that it belongs to the sister-group of the *A. dabryanus–A. nudiventris* cluster and, possibly, is closely related to the *A. ruthenus–Huso* clade (see above the discussion of this clade).

Ploidy. There is topological similarity between patterns of ploidy and the branching patterns of the sturgeon species (Fig. 3). In the following discussion we constrain ploidy events to be restricted to gains only. There are more parsimonious ways to map the ploidy levels if ploidy loss is allowed but since the biological impossibility of such events, losses are not considered (a decrease in chromosome number in tetraploid fishes occurs through a fusion of chromosomes; see Birstein, 1987).

The ancestral ploidy, $4n$ (Birstein *et al.*, 1997b), is characteristic of *A. sturio* and *A. oxyrinchus*. Possibly, these species have preserved karyotypes which are similar to the ancestral ones for the genus *Acipenser. A. fulvescens* is an octoploid ($8n$; Fig. 3) whose divergence from the ancestral form was accompanied by tetraploidization. All members of the *A. schrenckii–A. transmontanus* cluster are also tetraploids ($8n$; Fig. 3). Tetraploidization in this lineage might have occurred in an Asian ancestor of the group. *Acipenser mikadoi* has an unusually high ploidy ($16n$; Fig. 3). It is evident that polyploidization events occurred twice ($4n \rightarrow 8n \rightarrow 16n$) in the course of the evolution of this species (Birstein *et al.*, 1997b). The ploidy of *A. brevirostrum* is also $16n$ (Fig. 3). Two ploidy events most likely occurred during the evolution of this lineage as well. *A. gueldenstaedtii* and three species of the *A. baerii–A. naccarii* cluster are octaploids ($8n$; Fig. 3). Ploidy events perhaps occurred independently in these two lineages because all other species belonging to the clade *A. stellatus–A. nudiventris* (including two species of *Huso*) retained the ancestral ploidy, $4n$.

The most parsimonious mapping of ploidy on the parsimony cladogram is shown in Fig. 4. This figure shows a fully resolved hypothesis and a deresolved hypothesis where nodes not supported by greater than 50% bootstrap values have been collapsed. In either case, ploidy increased a maximum of eight times within *Acipenser* in different lineages of the genus. Addition of ploidy as a character to our matrix with the character coded as an ordered character ($4 \rightarrow 8 \rightarrow 16$) results in the single parsimony tree identical to that shown in Fig. 3. The number of ploidy events may be lower when more robust inferences of phylogeny are obtained from future studies. The implication of Figure 4 is that the radiation of species within a cluster in Fig. 3 occurred without a change of ploidy of an ancestral species which gave rise to the rest of the lineage.

Paleontology, geological history, and a hypothetical evolutionary history of the Acipenserinae. According to our molecular hypothesis (Fig. 3), the European, Asian, and American species have complicated interre-

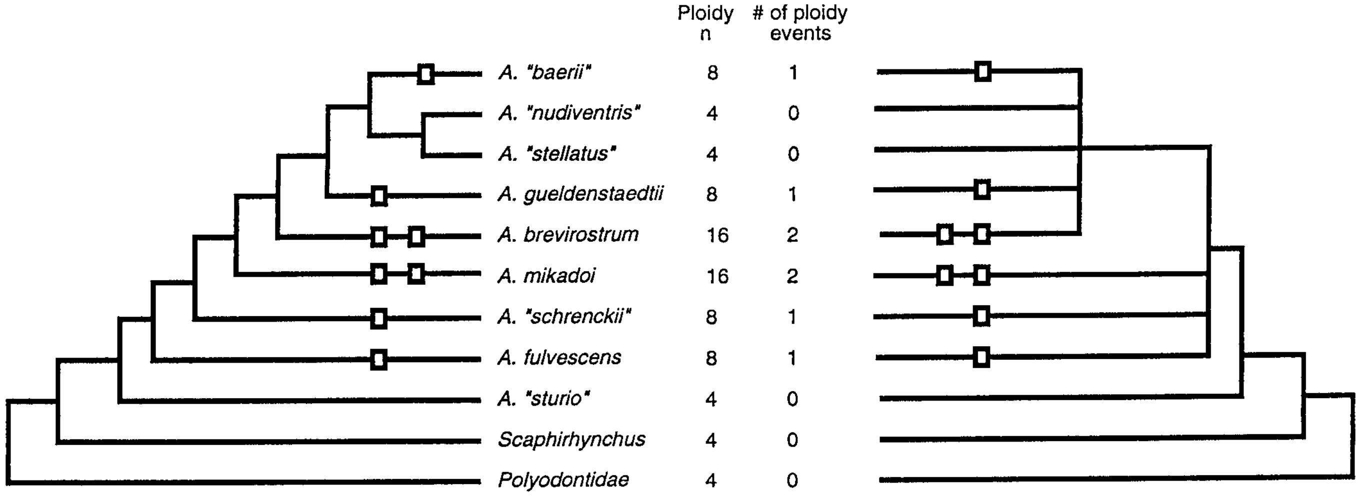

FIG. 4. Ploidy evolution in the Acipenseridae. The two trees in this figure are based on the topology in Fig. 3. Several taxa have been grouped together as in the following; "*A. baerii*" refers to the assemblage of *A. baerii, A. persicus,* and *A. naccarii;* "*A. nudiventris*" refers to the *A. nudiventris–A. dabryanus* pair; "*A. stellatus*" refers to the *H. dauricus, H. huso, A. stellatus, A. ruthenus* assemblage; "*A. schrenckii*" refers to *A. transmontanus, A. medirostris, A. sinensis,* and *A. schrencki* assemblage; "*A. sturio*" refers to the *A. sturio–A. oxyrinchus* pair. The tree on the left is a fully resolved representation of Fig. 3. The tree on the right has several nodes collapsed as explained in the text. Ploidy events are mapped on the tree using open boxes.

lationships which seem to have arisen during the long evolution of the group. Our scheme is at odds with the hypothesis of relationships for these fishes based on the biogeographic data alone (Artuykhin, 1995). The molecular hypothesis allows us to place paleontological and biogeographic information into a phylogenetic perspective (Table 3). In the following section we discuss the utility of the tree topology for interpreting the main divergence and palaeobiogeographic events.

(a) The base of the tree (Jurassic–Upper Cretaceous). Unfortunately, very little is known about the paleontological history of the Acipenseriformes (reviews in Grande and Bemis, 1996; Bemis *et al.*, 1997). The origin and radiation of the order most likely occurred in the Tethys Sea basin which connected the Atlantic with the Indian Ocean (see Smith *et al.*, 1994). The two acipenseriform lineages, Polyodontidae and Acipenseridae, are considered to have diverged in the Jurassic, between 200 and 135 million years (my) ago (Table 3). Their ancestors, the extinct family Peipiaosteidae, are also known from the Late Jurassic (Jin, 1995; Jin *et al.*, 1995; Grande and Bemis, 1996). The two subfamilies within the Acipenseridae, Scaphirhynchinae and Acipenserinae, perhaps also diverged in the Middle–Late Jurassic (Table 3). Therefore, acipenseriforms originated in the region of contemporary Central Asia and radiated during the Jurassic.

The oldest (Upper Cretaceous) fossil representatives of *Acipenser* are known from Central Asia and North America (Appendix 1). In the Oligocene–Miocene (ca. 37–5.0 my ago), the extinct forms of *Acipenser* had a circumboreal distribution. Fossil evidence does not support a connection of the extinct forms with the recent groups of species of *Acipenser.*

Nesov and Kaznyshkin (1983) suggested that *A. sturio* is most likely the descendant of the most primitive forms within the extant lineage of *Acipenser.* Our molecular data support this view. Since the main geological changes in the North Atlantic Ocean area occurred during the Lower (135–95 my ago) and Upper (95–65 my ago) Cretaceous (Smith *et al.*, 1994), it is reasonable to assume that the *A. sturio* lineage originated during the Middle Cretaceous, ca. 90 my ago (Table 3).

(b) The Eurasian–North American intermediate taxa (Upper Cretaceus–Upper Paleocene). *Acipenser fulvescens* colonized its contemporary area (mostly Great Lakes basin) rather recently, after the Wisconsin glaciation (18,000 years ago), from the Mississippian refugium (reviews in Guénette *et al.*, 1993; Ferguson and Duckworth, 1997). Possibly, the Mississippi River basin was the area of origin for the species. This species probably had an European ancestor before the Middle Eocene, when Europe and North America became disconnected (ca. 50–40 my ago, Adams, 1981).

The clade *A. schrenckii–A. transmontanus* most likely originated in Asia: *A. schrenckii,* basal for the clade, inhabits the Amur River (Berg, 1948b), and *A. sinensis* inhabits the Yangtze River (Wei *et al.*, 1997). This group might have appeared in the Upper Cretaceous (80–90 my ago), when transgression of the Tethys Sea was the most advanced in Asia (Smith *et al.*, 1994). The ancestral form of the Pacific American species possibly moved along Beringia, a land bridge which connected

TABLE 3

Hypothetical Chronology of Acipenseriform Evolution

Period	Millions of years ago[1]	Paleontological (P), biogeographic (B), or molecular (M) data	Evolutionary event	Reference to the paleontological, biogeographic or molecular data
Jurassic	200–175	P	Origin of the Acipenseriformes	Grande and Bemis, 1991; Jin, 1995
Jurassic	200–135	P	Divergence of the Polyodontidae and Acipenseridae	Yakovlev, 1977; Grande and Bemis, 1991; Jin, 1995
Middle–Late Jurassic	175–135	P	Divergence of the Scaphirhynchinae and Acipenserinae[2]	Wilimovsky, 1956; Grande and Bemis, 1991, 1996
Middle Jurassic	175–155	P	Origin of *Asiacipenser*[3]	Nesov *et al.*, 1990
Upper Cretaceous	95–65	P	Origin and divergence of *Acipenser*	Berg, 1948a; Wilimovsky, 1956; Nesov and Kaznyshkin, 1983
Upper Cretaceous	95	P, M	Origin of the *A. sturio* lineage	Nesov and Kaznyshkin, 1983; this paper
Upper Cretaceous	90–80	B, M	Origin of the *A. fulvescens* lineage	This paper
Upper Cretaceous	80	B, M	Origin of the *A. schrenckii–A. transmontanus* lineage	This paper
Upper Paleocene	60	B, M	Divergence of the ancestral *A. sinensis* from the ancestral *A. schrenckii*	This paper
Upper Paleocene–Lower Eocene	60–50	M	*A. mikadoi*	This paper
Lower Eocene	50	B, M	Divergence between the *A. brevirostrum–A. gueldenstaedtii* lineages	This paper
Middle Miocene	15	M	Origin of the *A. gueldenstaedtii* lineage	This paper
Upper Miocene–Lower Pliocene	6.0–5.5	M	Origin of the *A. stellatus–A. persicus* lineage	This paper
Lower Pliocene	5.0	M	Origin of the *A. naccarii–A. persicus* and *A. stellatus–A. dabryanus* lineages	This paper
Upper Pliocene	2.2	M	Divergence of *A. transmontanus–A. medirostris*	Brown *et al.*, 1996
Pleistocene	2.0–1.5	B, M	Divergence of *Huso dauricus* from the *H. huso–A. ruthenus* lineage, divergence of the *A. nudiventris* and *A. dabryanus* ancestors	This paper
Pleistocene	1.5 and later	B, M	Dispersion of the Ponto–Caspian species through the Black, Azov, Mediterranean, Aral seas	This paper
Middle–Late Pleistocene	0.9–0.07	B, M	Dispersion of *A. baerii* throughout the Siberian rivers	This paper

[1] Dates are given according to Adams (1981), Howart (1981), and Smith *et al.* (1994).

[2] In this paper we used *Scaphirhynchys albus* as a representative of the Scaphirhynchinae, that is why we do not discuss the question of possible relationships between *Scaphirhynchus* and *Pseudoscaphirhynchus*.

[3] The oldest known sturgeon, *Asiacipenser kotelnikovi*, from the Middle Jurassic (Nesov *et al.*, 1990).

Asia with North America from 66 my to about 2 my ago (Janis, 1993).

(c) The Ponto–Caspian group (Upper Paleocene–Pleistocene). The origin and radiation of the Ponto–Caspian group of sturgeon species (all species in the clade except *A. baerii* and *A. dabryanus*) probably occurred in the Tethys Sea or in its derivate, the Paratethys Sea which covered the contemporary Black, Azov, Caspian, and Aral seas and was formed in the Middle Miocene, about 15 my ago (Hsü, 1978; Jones and Simmons, 1996). *Acipsenser gueldenstaedtii* or its ancestor could have originated at that time, while more speciation events in the group occurred during the Late Miocene–Late Pleistocene as a result of the global geological and environmental changes in this area (Appendix 2). The Caspian Sea has remained the main refugium of sturgeon species common to the Caspian, Aral, Azov, Black, and Mediterranean seas (Table 2) from 15 mya to about 1.5 mya.

The anadromous species *A. gueldenstaedtii, A. stellatus, A. nudiventris,* and *H. huso* probably migrated from the Caspian Sea to the Black–Azov seas (and to the Mediterranean) repeatedly through water connections between the Caspian and Azov–Black seas during the last 1.5 my, when the Black Sea acquired salt water again through its connection with the Mediterranean. About the same time, sturgeons most likely started to use the Danube River for spawning (the Danube be-

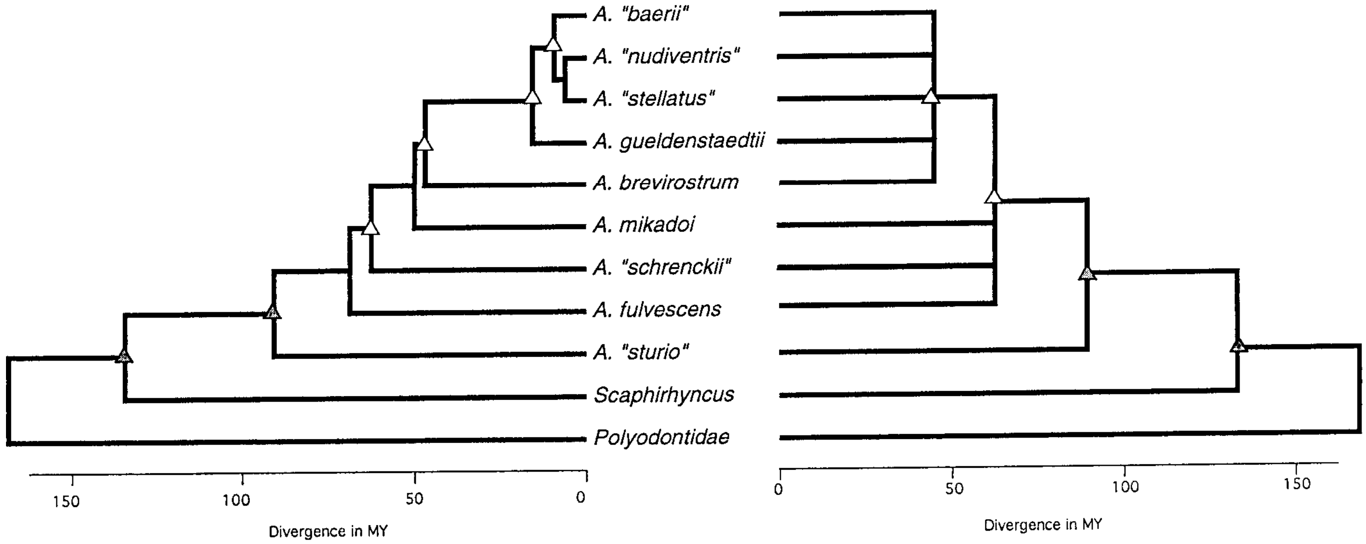

FIG. 5. A hypothetical scheme of the main evolutionary events within Acipenseriformes (based on the data in Fig. 3 and Table 3). Species assemblages (names in quotes) are explained in the legend to Fig. 4. The tree on the left is a fully resolved representation of Fig. 3. The tree on the right has several nodes collapsed as explained in the text. Open triangles refer to divergence dates based on biogeography. Closed triangles refer to divergence dates based on fossil evidence. In the fully resolved tree on the left, the three nodes without triangles do not show divergence times.

came connected with the Black Sea 0.6 my ago). *Acipenser nudiventris* possibly migrated from the Aral to the Caspian and Black seas comparatively recently, when the Aral and Caspian seas were connected through a system of lakes and rivers (ca. 10,000 years ago; Atamuradov, 1994).

Most probably, the ancestor of *A. baerii* moved from the Ponto–Caspian Sea area to Siberian rivers in the Middle Pleistocene through a system of ice-dammed lakes which existed during the glacial maxima (e.g., Berg, 1928). The eastern and east–southern boundary of the contemporary area of this species was probably conditioned by the boundaries of the last glaciation 18,000–20,000 years ago (Velichko *et al.*, 1994). According to the archeological data, during the Late Pliocene–Middle Pleistocene (7,000–1,000 BC) the distribution of *A. baerii* was the same as it is now (review in Tsepkin, 1995).

In the Pacific Ocean, the split between the two American species, *A. transmontanus* and *A. medirostris,* could have occurred about 2.2 my ago (Brown *et al.*, 1996). During the glaciation, a northern population of *A. transmontanus* existed in the Columbia River refugium. Also, the most recent glaciations may have disrupted the ranges of the ancestral European and American Atlantic sturgeons, *A. sturio* and *A. oxyrinchus.* It is assumed that the two subspecies of *A. oxyrinchus* were separated by the emergence of the Florida peninsula and its tremendous expanses during the glacial advances (Rivas, 1954; Avise, 1992; Ong *et al.*, 1996).

(e) General considerations. A hypothetical evolutionary history of the Acipenseridae is presented in Fig. 5. This figure shows a fully resolved hypothesis based on Fig. 3 and a deresolved hypothesis where nodes not supported by greater than 50% bootstrap values have been collapsed. The fully resolved topology allows us to infer six divergence times while the deresolved topology allows us to infer only four. It seems that there were a few periods in formation of the main lineages of the extant sturgeon species. The oldest events, the divergence of the ancestors of *A. sturio,* occurred in the Upper Cretaceous, whereas the other lineages appeared during the Upper Paleocene–Lower Eocene. The ancestor of the Ponto–Caspian group appeared in the Middle Miocene, and this group radiated in the Pliocene. During the Pleistocene, the Ponto–Caspian species moved repeatedly through the connections between the Caspian, Black, Azov, Aral, and Mediterranean seas. At the same time *A. baerii* spread through Siberia and *A. fulvescens,* through its contemporary range, while *A. oxyrinchus* was divided in two subspecies.

Our molecular data support the acipenserid distribution hypothesis developed 60 years ago by Birstein and Vinogradov (1934). These authors found that the current distributions of freshwater decapods, isopods, and acipenserids are similar (Birstein and Vinogradov, 1934; Birstein, 1951). They concluded that this similarity is "a result of the Upper Jurassic transgression, continental movements, and animal migration through the North-Pacific continental bridge" (Birstein and Vinogradov, 1934; p. 63).

APPENDIX 1

The Jurassic–Miocene Fossil Acipenseriforms

Sturgeon name	Epoch	Millions of years ago	Geographic location	Reference
Subfamily Scaphirhynchinae				
Protoscaphirhynchus squamosus	Upper Cretaceous	95–65	Hell Creek beds, Montana (U.S.A.)	Wilimovsky, 1956
Subfamily Acipenserinae				
Asiacipenser kotelnikovi	Middle Jurassic	175–155	Fergana (Central Asia)	Nesov *et al.*, 1990
Acipenser albertensis	Upper Cretaceous	95–65	Red Deer River, Alberta, Canada	Lambe, 1902
A. albertensis	Upper Cretaceous	95–65	Alberta, Canada	Gardiner, 1966
A. albertensis	Upper Cretaceous	95–65	Saskatchewan, Canada	Gardiner, 1984
A. eruciferous	Upper Cretaceous	95–65	Montana (U.S.A.)	Cope, 1876 Lambe, 1902 Estes, 1964 Estes *et al.*, 1969
A. shilini	Upper Cretaceous	95–65	Aral Sea Area, Kazakhstan (Central Asia)	Nesov and Kaznyshkin, 1983
Acipenser sp.	Upper Cretaceous	95–65	Dzhara-Kuduk, Central Uzbekistan (Central Asia)	Nesov and Kaznyshkin, 1983
Acipenser sp.	Upper Cretaceous	95–65	Western Fergana, Tadjikistan (Central Asia)	Nesov and Kaznyshkin, 1983; Nesov and Verzilin, 1983
A. zhylgensis	Upper Paleocene	60	Kazakhstan (Central Asia)	Nesov and Kaznyshkin, 1983
A. lemoinei	Upper Paleocene	60	Epernay, Marne (France)	Priem, 1908; Casier, 1966
A. toliapicus	Lower Eocene	54–50	Isle of Sheppey (England)	Agassiz, 1844; Casier, 1966;
Acipenser sp.	Oligocene	37–25	Aral Sea Area, Kazakhstan (Central Asia)	Formozova, 1949
A. parisiensis	Oligocene	37–25	Paris Basin, France	Lehman, 1966
A. molassicus	Miocene	25–5	Baltringen, Württemberg, Germany	Probst, 1882
A. tuberculosus	Miocene	25–5	Baltringen, Württemberg, Germany	Probst, 1882
A. ornatus	Miocene	25–5	Virginia (U.S.A.)	Leidy, 1856
A. ornatus	Miocene	25–5	Chesapeake Beach, Maryland (U.S.A.)	Wilimowsky, 1956
A. ornatus	Miocene	25–5	Plum Point, Maryland (U.S.A.)	Wilimowsky, 1956

APPENDIX 2

Main Geological and Palaeogeographic Changes in the Ponto–Caspian Area during the Miocene–Holocene[1]

Period	Millions of years ago (mya) or years ago (ya)	Geological or environmental event	Reference
Middle–early Late Miocene	15.5–15.0 mya	Disruption of the Tethys Sea into the southern (Mediterranean Sea area) and northern (Paratethys) parts. The Paratethys gradually becomes a brackish-water inland sea	Hsü, 1978; Adams, 1981; Jones and Simmons, 1996
Late Miocene	7 mya	Formation of the Palaeo–Volga basin; Palaeo–Volga enters the Paratethys	
Late Miocene	6 mya	The Mediterranean Sea area almost completely evaporates	Hsü, 1978; Adams, 1981
Late Miocene–Early Pliocene	5.5 mya	Disruption of the Paratethys into isolated lakes corresponding to the contemporary Black, Caspian, and Aral seas. There are a few salt lakes in the Mediterranean Sea area. Palaeo–Volga and Palaeo–Amu–Darya River enter very small Caspian Sea, Palaeo–Don River enters the Black Sea.	Hsü, 1978; Adams, 1981; Jones and Simmons, 1996
Early Pliocene	5.2 mya	Seawater from the Atlantic enters the Mediterranean area and reaches the Black and Caspian seas. After this the Black Sea gradually loses its salinity and becomes a freshwater lake for the next 4 my	Hsü, 1978; Adams, 1981
Late Pliocene	3.5–1.6 mya	Transgressions (3.4, 2.7, and 2.0 mya) temporarily reestablish connections between the seas and the world's oceans	Jones and Simmons, 1996

APPENDIX 2—*Continued*

Period	Millions of years ago (mya) or years ago (ya)	Geological or environmental event	Reference
Late Pliocene–Late Pleistocene	3.5–0.02 mya	Amu Darya River still enters the Caspian Sea	Atamuradov, 1994
Pleistocene	1.5 mya	Formation of the shelf corresponding to the contemporary shelf of the Black Sea	Fesyunov, 1996
Middle–Late Pleistocene, three major stages of glaciation	1.10–0.60 mya; 0.58–0.30 mya; 0.30–0.05 mya	8 saline water events from the Mediterranean and 7–10 cold water events from the Caspian in the Azov–Black Sea region	Zubakov, 1988
Middle–Late Pleistocene	0.90–0.07 mya	Formation of the West Siberian and other ice lakes which drained toward the Caspian and Aral seas	Väinölä, 1995
Late Pleistocene	0.6 mya	The Danube River takes its course to the Black Sea	Fesyunov, 1996
Three Late Pleistocene periods of transgressions of the Caspian Sea	Baku (0.70–0.35 mya); Khazarian (0.35–0.12 mya); Khvalynian (0.12–0.01 mya)	During transgressions the size of the Caspian Sea reaches 2.5 of its present size, covering almost a half of the contemporary length of the Volga River	Rodionov, 1994; Svitoch and Yanina, 1996
Holocene	0.01 mya	Connection of the Aral and Caspian seas through a system of rivers and lakes (Sarykamysh Lake basin). Connection of the Mediterranean and Black seas through the Bosporus	Hsü, 1978; Atamuradov, 1994
Holocene	6,000–4,000 ya	Amu Darya River enters the Caspian Sea through a big Sarykamysh Lake	Atamuradov, 1994; Salnikov, 1995
Holocene	5,000 ya	Beginning of formation of the contemporary Volga River Delta	Svitoch, 1994
Holocene	4,000 ya	Amu Darya River turns to the Aral Sea	Atamuradov, 1994; Salnikov, 1995

ACKNOWLEDGMENTS

We are extremely grateful to all colleagues who provided us with the samples of tissues or eggs from sturgeon species inhabiting different countries: Drs. Evgenii Artyukhin, Anatolii Vlasenko, Victor Svirskii (Russia); Dr. Patrick Williot (France); Dr. Lutz Debus (Germany); Dr. M. Pourkazemi (Iran); Dr. Qiwei Wei (China); Drs. Fred Binkowski, Herb Bollig, Boyd Kynard, John North, and John Waldman (U.S.A.). Also we thank Xiaobo Yu, Robert Hanner, and Monique Scott for technical assistance with sequencing. Without their kind cooperation this work would not be possible.

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