

Molecular phylogeny of Acipenserinae and black caviar species identification.

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Summary

The first phylogenetic tree for the Acipenserinae based on the combined sequence data for fragments of three mitochondrial genes, the cytochrome *b* (650 bp) gene, 12S (150 bp) and 16S (350 bp) rRNA genes is described. Three general conclusions are inferred from the tree: 1) *Scaphirhynchus albus* is the sister-species of all *Acipenser* and *Huso* species; 2) The two species of *Huso* are embedded within the genus *Acipenser*. Therefore, the genus *Huso* is not a separate taxonomic unit; and 3) There are three main clades within *Acipenser*: (a) *A. sturio*-*A. oxyrinchus*, (b) *A. schrenckii*-*A. transmontanus*, and (c) all Ponto-Caspian species plus *A. dabryanus* and *A. brevirostrum*. A method based on the identification of diagnostic nucleotide positions in the cytochrome *b* gene was developed and used to survey the United States and European caviar markets. The survey of 95 lots of caviar obtained at the American market suggests that in December 1995 and April 1996 17% of the designations made by caviar suppliers were mislabeled with respect to species identification. In December of 1996, this figure jumped to 32%. The main commercially harvested species (*Huso huso*, *A. stellatus*, *A. gueldenstaedtii*, *A. schrenckii*, and *H. dauricus*), as well as *A. nudiventris*, *A. persicus*, and *A. baerii* are threatened due to the increased demand of the international caviar market.

Introduction

The order Acipenseriformes is a group of ancient, «living fossil» fishes (Gardiner 1994). According to contemporary views, the extant acipenseriforms form the monophyletic sister-group of all extant Neopterygii (e.g., Lepisosteidae, Amiidae, and Teleostei; Gardiner and Schaeffer 1989; Bemis et al. 1997). Most ichthyologists regard Polypteridae as the sister group of Acipenseriformes plus Neopterygii (Patterson 1982). A comparison of partial sequences of 28S rRNAs supports this relationship (Le et al. 1993). The order consists of two families, Polyodontidae (two extant paddlefish species) and Acipenseridae (25 extant sturgeon species, see Birstein and Bemis 1997). Grande and Bemis (1991, 1996) consider these families to be sister taxa. All workers agree that the Acipenseridae and Polyodontidae diverged prior to the Late Cretaceous (Berg 1948; Yakovlev 1977, 1986; Grande and Bemis 1991; Jin 1995), probably, in the Middle-Late Jurassic.

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.

Unfortunately, all acipenseriform species are endangered, with some on the verge of extinction (Birstein 1993, 1997; IUCN,

1996; Birstein et al. 1997a). One of the main threats to the survival of several species is the demand of the international caviar market, which stimulates poaching mainly in the countries of the former Soviet Union (De Meulenaer and Raymakers 1996; DeSalle and Birstein 1996; Birstein 1997; Taylor 1997).

There is no single agreed upon hypothesis describing the species relationships within the genus *Acipenser*. Artuykhin (1995) published a phylogenetic hypothesis based mainly on the biogeography of the *Acipenser* species and their ploidy (based on chromosome numbers, but not on DNA content). He proposed that a polyploidy event, octaploidization, occurred only once during the evolution of the genus. Results of our work on sequencing fragments of the mitochondrial (mt) cytochrome *b* (*cytb*) gene, 12S and 16S rRNA genes from all living sturgeon species did not support the grouping given in Artuykhin's scheme (Birstein et al. 1997b; Birstein and DeSalle 1998). Because the subfamily Acipenserinae consists of a relatively small number of species, we had a unique opportunity to investigate the relationships between all extant members of a subfamily of «living fossils.» This communication presents an overview of our data on the molecular phylogeny of sturgeons and results of caviar species identification using molecular method based on our phylogeny studies.

Materials and methods

Sequencing and cladistic analysis

The details of DNA preparation, sequencing and cladistic analysis of the DNA sequences are given in Birstein et al. (1998a,b) and Birstein and DeSalle (1998). Three regions of the *cytb* gene with the combined length of 650 bp and fragments of 12S (150 bp) and 16S (350 bp) rRNA genes were investigated. The three variable *cytb* regions correspond to 106-352 bp, 437-562, and 812-962 bp of the *cytb* gene sequence of *A. transmontanus* (Brown et al. 1989). Sequences have been deposited in Genbank under the following accession numbers: 12S, AF004968-AF004983; 16S, AF004954-AF004967; *cytb*, AF006123-AF006188. The mt rDNA sequences were aligned using MALIGN (Wheeler and Gladstein 1993). All phylogenetic analyses were performed with PAUP version 3.1 (Swofford 1993) and using *Polyodon spathula* and *Psephurus gladius* (Polyodontidae) as outgroups. For the combined data set of the three genes we used the method of Agosti et al. (1996) for weighting *cytb* coding regions which involves appending the amino acid sequence to the nucleotide sequence matrix.

For identification of the species of origin for caviar, we used specially designed primer pairs based on the *cytb* sequences from our database (see DeSalle and Birstein 1996; Birstein et al. 1998b). Our method allows us to identify sturgeon species using a single egg¹. We tested 8-10 eggs from each caviar sample for

Drs. Rob DeSalle and Vadim Birstein submitted applications entitled «Methods and Composition for Identification of Species Origin of Caviar» to the United States and European patent offices. The United

reproducibility and to check for possible mixtures. The majority of fresh caviar samples in tin cans or glass jars were purchased from 15 gourmet shops in New York City or ordered by mail from three companies in New Jersey, Massachusetts, and Florida (Table 1). We bought samples three times: in December 1995, in April 1996, and in December 1996. From some of the shops we bought samples twice. Two glass jars were brought from Russia (Lots Nos. 18 and 19). One of the jars (No. 19) was kept in a refrigerator for two years. All American cans or jars were labeled by the retailer as beluga (e.g., *Huso huso*), sevruga (*Acipenser stellatus*) or osetra (*A. gueldenstaedtii*), and four cans were labeled as «American caviar». One lot was labeled «Eastern beluga», and another was labeled as «river beluga». Labels on Russian jars did not identify the species. Additionally, we tested three jars of caviar of unknown origin obtained from Russia. On the whole, we studied 98 samples of caviar bought in the United States and Russia.

Also, in December 1996 we tested nine samples bought in five prominent gourmet shops of London (Harrods, Harvey Nicholls, Fortnam & Masson, Caviar House, Selfridges), and in January 1997, we tested four samples from Hamburg (Caspian Caviar) and five samples from Paris (Persepolis and Black Pearl). All samples were labeled as beluga, sevruga or osetra from Iran. One sample of unknown type of caviar was received from Prof. R. Billard (Muséum National d'Histoire Naturelle, Paris) and was taken from an illegal shipment of Bulgarian caviar seized by French customs.

Results and discussion

Phylogenetic implications

A single parsimony tree was obtained using of the simultaneous analysis of all DNA sequence data with 865 steps; the consistency index is 0.430 and the retention index is 0.525 (see Figure 3 in Birstein and DeSalle 1998). The tree was constructed with the amino acid sequence for *cytb* appended to the DNA sequence data as recommended in Agosti et al. (1996). The following phylogenetic implications can be inferred from the tree:

- 1- *Scaphirhynchus albus*, a representative of the subfamily Scaphirhynchinae, is the sister-species of all species of *Acipenser* and *Huso*;
- 2- The two species of *Huso* are embedded within the genus *Acipenser*. It appears that the genus *Huso* is not a separate taxonomic unit, but belongs to the genus *Acipenser* and is closely related to *A. ruthenus*. *Huso dauricus* is basal to *H. huso* and *A. ruthenus*. The validity of the genus *Huso* is still under discussion (Artuykhin 1995) and our data strongly support the view that *Huso* is not a separate genus. That fact that *H. huso* not only hybridizes easily with *A. ruthenus*, but that these hybrids are also fertile (review in Birstein et al. 1997b), is another genetic evidence of close relatedness of these two species. The extreme morphological difference between these two related species is possibly a result of paedomorphosis, which played an important role in the acipenseriform evolution (Grande and Bemis 1991; Tsessarsky 1992). The existence of larval morphological characters in the adult *H. huso* that disappear in *Acipenser* (Strelkovsky 1957) support this hypothesis.
- 3- There are at least three main clades within *Acipenser*: *A. sturio*-*A. oxyrinchus*, *A. schrenckii*-*A. transmontanus*, and a

clade encompassing all the Ponto-Caspian species plus *A. dabryanus* and *A. brevirostrum*.

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 1977). Since the main geological changes in the North Atlantic Ocean occurred during the Lower and Upper Cretaceous, it is reasonable to assume that the *A. sturio* lineage originated during the Middle Cretaceous, ca. 90 million years ago. Six nucleotide changes in the *cytb* region in the two specimens of *A. sturio* (from the Gironde River and North Sea) indicate an even higher degree of divergence than in the two subspecies of *A. oxyrinchus* which show only one nucleotide change. These data suggest that the specimen from the Gironde River can be considered a representative of the Mediterranean-Black Sea form of *A. sturio*, while that from the North Sea is a representative of the Baltic-North Sea form of *A. sturio* (Birstein et al. 1998a).

Our analysis also indicates that *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), while *A. brevirostrum* is closely related to the Ponto-Caspian species. *Acipenser mikadoi* is the sister-species of the Ponto-Caspian species clade plus *A. brevirostrum*. The position of these latter two species, *A. mikadoi* and *A. fulvescens*, are not robustly supported, and more character information will be needed to confirm placement of these species within the phylogenetic tree.

There are strong trans-Pacific relationships between two Asian species, *A. schrenckii* and *A. sinensis*, and two American species, *A. transmontanus* and *A. medirostris*. Since *A. schrenckii* is the basal species in this cluster, this suggests that the group originated in Asia. Our grouping of *A. transmontanus* with *A. medirostris* within one clade is congruent with their close morphological similarity (Findeis 1993; Artyukhin 1995) and sympatry (Vladykov and Greeley 1963; Scott and Crossman 1973).

Acipenser brevirostrum is basal to the Ponto-Caspian and some Asian sturgeons and has a trans-Atlantic relationship with the Ponto-Caspian species inhabiting the Caspian, Aral, Black and Azov seas (*A. gueldenstaedtii*, *A. stellatus*, *A. nudiventris*, *A. persicus*, and *Huso huso*). All these species are considered the descendants of the fauna of the ancient Tethys Sea and its later derivatives (i.g., Berg 1949). Our data suggest that relationships within this group are complex and these species are closely related to some of the Asian sturgeon species. In our tree *A. baerii* is more closely related to *A. persicus* than to *A. naccarii*. The highly supported grouping of *A. nudiventris* with *A. dabryanus* is an expected result of our analysis. 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, was considered by many authors as the only member of a separate subgenus *Helops* or *Gladostomus* (Berg 1948; Artyukhin 1995; Bemis et al. 1997). Our results suggest that it belongs to the sister group of the *A. dabryanus*-*A. nudiventris* sister pair and, possibly, is closely related to the *A. ruthenus*-*Huso* clade.

There is topological similarity between patterns of ploidy and the DNA sequence phylogeny of the sturgeon species (Figs. 3 and 4 in Birstein and DeSalle 1998). The ancestral ploidy, 4n (reviews in Birstein 1987; 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*. *Acipenser fulvescens* is an octaploid (8n) whose divergence from the ancestral form was accompanied by tetraploidization. All members of the Pacific *A. schrenckii*-*A.*

transmontanus cluster are also octaploids. Tetraploidization in this lineage might have occurred in an Asian ancestor of the group. *Acipenser mikadoi* has an unusually high ploidy (16n). 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. Two ploidy events most likely occurred during the evolution of this lineage as well. *Acipenser gueldenstaedtii*, as well as three species of the *A. baerii*-*A. naccarii* cluster are octaploids. 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 of 4n.

Caviar identification and conservation issues

We determined the species-specific DNA sequence changes in the *cytb* gene region. Using this information, we were able to create species-specific primers for the identification of the main black caviar producing commercial sturgeon species, beluga (*Huso huso*), sevruga (*A. stellatus*), and Russian sturgeon (*A. gueldenstaedtii*) (DeSalle and Birstein 1996; Birstein et al. 1998b). We used this method for a survey of the American caviar market. On the whole, 93 samples were tested (Table 1).

No.	Name of supplier (shop)	Location	Date of purchase	Lot No.
1.	Balducci	New York City	December 1995	1-5
			December 1996	94, 95
2.	Zabars	New York City	December 1995	6-8
			April 1996	34-36
3.	Murray's Sturgeon Shop	New York City	December 1995	9-11
4.	Citarella	New York City	December 1995	12-14
			April 1996	59-61
			December 1996	75-77
5.	Macy's	New York City	December 1995	15-17
6.	Caviarteria	New York City	December 1995	20-22
			December 1996	88-90
7.	Petrossian	New York City	December 1995	23-25
			December 1996	91-93
8.	Vinegar Factory	New York City	April 1996	26-28
9.	Food Emporium	New York City	April 1996	29
10.	Terra Mare	New York City	April 1996	30-33
11.	Marky's	Miami, Florida	April 1996	37-37
			December 1996	71-74
12.	Hansen-Sturm	Bergenfield, New Jersey	April 1996	41-43
			December 1996	78-81
13.	Russ & Daughters	New York City	April 1996	44-46
14.	La Maree	Mamaronek, New York	April 1996	47-48
15.	Grace's Marketplace	New York City	April 1996	49-51
			December 1996	85-87
16.	Caviar Direct	New York City	April 1996	52-54
17.	Dean & DeLuca	New York City	April 1996	56-58
18.	Caviar Aristoff	Los Angeles, California	December 1996	62-64
19.	Connoisseur Brands	New York City	December 1996	65-67
20.	Paramount Caviar	Long Island City, New York	December 1996	68-70
21.	Boyajin	Boston, Massachusetts	December 1996	82-84

Table 1. Purchases of caviar from American suppliers (shops)

We found that in many cases samples were mislabeled, e.g. caviar in a jar or tin was not of the species mentioned on the label (Tables 2, 3). On the average, more than 20% of samples tested were mislabeled. In 1995 and April 1996, the number of mislabelings was less than 20%, but in December 1996 it jumped to 32%. We interpret these data as indicators of overfishing of commercial sturgeons in the Caspian Sea, which is the main area of the exploitation of caviar producing species.

For the identification of species used for the replacement, we sequenced fragments of the *cytb* and compared sequences with our database. Since the sequences of the *cytb* gene regions we examined are similar in the Siberian sturgeon (*A. baerii*), Italian sturgeon (*A. naccarii*) and Persian sturgeon (*A. persicus*), we could not discriminate between the caviar of these species.

The results of our survey show that in several instances the caviar of all commercial species is replaced by the other species on the American market (Table 3). It is not surprising that in some cases (lots 6 and 72) the most expensive caviar (beluga) was replaced by *A. stellatus* and *A. gueldenstaedtii* caviar, which is much cheaper. The instances where we observed replacement

of sevruga or American sturgeon caviar by beluga caviar (lots 43, 62, 81; Table 3) are more puzzling. We suggest that these cases represent the possibility that old caviar or poor quality caviar which could not be sold as beluga was used for such replacements. It is alarming that an extremely endangered ship sturgeon, *A. nudiventris*, is still harvested for caviar production (lots 3 and 54; Table 3). Also, it is evident that another rare species, the Amur sturgeon (*A. schrenckii*), is intensely harvested in Russia or China for caviar (lots 4, 41, 71; Table 3).

Besides labeled cans and tins, we identified five samples of Russian caviar, which lacked labels indicating the species. One was diagnosed as Russian sturgeon, another as sevruga, and three samples as beluga. Since one of the samples was kept in a refrigerator for two years, it is evident that our method can be applied to relatively long-stored caviar. The illegal Bulgarian sample appeared to be sevruga. Currently, the population of *A. stellatus* is the only relatively large sturgeon population in the lower reaches of the Danube River (the main area of poaching in the region) as compared to those of *A. gueldenstaedtii* and *Huso huso* (Birstein 1996b).

Table 2. Total number of stores surveyed, number of shops with mislabelings, total number of samples studied and number of mislabeled samples

Number	December 1995			April 1996			December 1996			Total		
	Total	Mislab eled	%	Total	Mislab eled	%	Total	Mislab eled	%	Samples	Mislabel ed	%
Of stores, USA	7	2	29	12	4	33	11	8	73	-	-	-
Of samples, USA	23	4	17	36	6	17	34	11	32	93	21	23
Of stores, Europe	-	-	-	-	-	-	8	0	0	-	-	-
Of samples, Europe	-	-	-	-	-	-	18	0	0	-	-	-

Table 3. Types of species caviar misrepresentations in American shops. We could not discriminate between *Acipenser baerii*, *A. naccarii* or *A. persicus*

Supplier Designation	Name of supplier (shop)	Lot No.	Date of purchase	Assumed species	Species Identified
1. Beluga	Zabars	6	December 1995	<i>Huso huso</i>	<i>Acipenser stellatus</i>
	Citarella	12		<i>Huso huso</i>	<i>A. baerii</i> (or <i>A. naccarii</i> or <i>A. persicus</i>)
2. Sevruga	Marky's	72	December 1996	<i>Huso huso</i>	<i>A. gueldenstaedtii</i>
	Caviar Direct	54	April 1996	<i>A. stellatus</i>	<i>A. nudiventris</i>
	Caviar Aristoff	62	April 1996	<i>A. stellatus</i>	<i>Huso huso</i>
	Citarella	76	December 1996	<i>A. stellatus</i>	<i>Polyodon spathula</i>
	Balducci	95	December 1996	<i>A. stellatus</i>	<i>A. baerii</i> (or <i>A. naccarii</i> or <i>A. persicus</i>)
3. Osetra	Balducci	3	December 1995	<i>A. gueldenstaedtii</i>	<i>A. nudiventris</i>
	Vinegar Factory	26	April 1996	<i>A. gueldenstaedtii</i>	<i>A. stellatus</i>
	Hansen-Sturm	41	April 1996	<i>A. gueldenstaedtii</i>	<i>A. schrenckii</i>
	Caviar Direct	53	April 1996	<i>A. gueldenstaedtii</i>	<i>A. transmontanus</i>
	Hansen-Sturm	79	December 1996	<i>A. gueldenstaedtii</i>	<i>A. transmontanus</i>
	Connoisseur Brands	66	December 1996	<i>A. gueldenstaedtii</i>	<i>A. baerii</i> (or <i>A. naccarii</i> or <i>A. persicus</i>)
	Marky's	72	December 1996	<i>A. gueldenstaedtii</i>	<i>A. baerii</i> (or <i>A. naccarii</i> or <i>A. persicus</i>)
	Hansen-Strum	78	December 1996	<i>A. gueldenstaedtii</i>	<i>A. baerii</i> (or <i>A. naccarii</i> or <i>A. persicus</i>)
	Grace's Marketplace	86	December 1996	<i>A. gueldenstaedtii</i>	<i>A. baerii</i> (or <i>A. naccarii</i> or <i>A. persicus</i>)
4. Eastern beluga	Balducci	4	December 1995	<i>H. dauricus</i>	<i>A. schrenckii</i>
	Marky's	71	December 1996	<i>H. dauricus</i>	<i>A. schrenckii</i>
5. American sturgeon	Balducci	1	December 1995	<i>A. transmontanus</i>	<i>A. gueldenstaedtii</i>
	Hansen-Strum	43	April 1995	<i>A. transmontanus</i>	<i>Huso huso</i>
	Hansen-Strum	81	December 1996	<i>A. transmontanus</i>	<i>Huso huso</i>
	Dean & Deluca	55	April 1995	<i>A. transmontanus</i>	<i>Polyodon spathula</i>

The situation on the European caviar market differed from that in the United States. We could not identify mislabeling in London, Paris or Hamburg (Table 2). All European shops we checked were selling caviar that originated in Iran. In the United States, Iranian caviar distribution is legally prohibited. Our results suggest that problems with species misrepresentation in the United States are connected with the legal and illegal overfishing in the former Soviet Union. Such a situation could also arise within the United States if a dishonest dealer replaces caviar of Russian species by caviar of the American species (Cohen 1997). Surprisingly, we found out that the same dealer continued replacements after he had been accused in frauds and served his term in prison (see Hansen-Sturm in Table 3). It is evident that only international conservation and legal efforts can save the diminishing commercial sturgeon populations in the countries of the former Soviet Union and protect the non-commercial species that will undoubtedly be substituted for the disappearing commercial species.

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Editorial Note: "The authors attempt to cover two purposes. The first is mainly basic research and concerns a molecular approach to sturgeon phylogeny on the basis of some mitochondrial DNA sequences (three regions of Cytb and the genes for mitochondrial ribosomal subunits 12S and 16S). The second deals with identification of the original species through molecular characterisation of caviar samples. Both approaches are well conducted and the results obtained are significant. However, the reviewers of the manuscript noted that some of the conclusions on phylogenetic affinities of certain species may still be questionable in light of other morphometric, ecological and molecular parameters of recent publications. Additionally, the species status of the Asian green sturgeon (as *A. mikadoi*) on the basis of high cell DNA contradicts some other findings. Although the authors considered it unnecessary to critically discuss the pros and cons of the findings, the editorial committee wish to inform the readership of the contrasting opinion expressed by reviewers. We publish this article as a relevant contribution to the understanding of sturgeon phylogenetic relationships, to stimulate research in this area and thereby enhance our understanding of the general biology of this fascinating systematic group."