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Vadim J. Birstein[1,*]
Georgii Ruban[2]
Arne Ludwig[3]
Phaedra Doukakis[4] **&**
Rob DeSalle[5]
[1]Sturgeon Conservation International, 331 West 57th Street, #159, New York, NY 10019, USA
[2]A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Leninsky Prospect, 33, Moscow 117071, Russia
[3]Institute for Zoo and Wildlife Research, Department of Evolutionary Genetics, PF 601103, 10252 Berlin, Germany
[4]Pew Institute for Ocean Science, University of Miami, 126 East 56th Street, Level M, New York, NY 10022, USA
[5]Department of Invertebrate Zoology – Molecular Laboratory, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024, USA

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The enigmatic Caspian Sea Russian sturgeon: how many cryptic forms does it contain?

Abstract The Russian sturgeon, *Acipenser gueldenstaedtii*, is closely related to three other sturgeon species (*A. persicus, A. naccarii* and *A. baerii*), with populations in the Caspian Sea containing a cryptic lineage with an *A. baerii*-like mtDNA profile. Using morphological evidence (morphometrics, meristics) and additional genetic analysis (cytochrome *b* gene and control region sequencing), cryptic lineages within the Russian sturgeon and their relation to other closely related species of sturgeons are further examined. These data indicate that three genetic forms exist within what is presently known as *A. gueldenstaedtii.* These forms include the pure *A. gueldenstaedtii* and *A. baerii*-like individuals plus a third rare genetic form whose mtDNA is similar to the mtDNA of the Adriatic sturgeon, *A. naccarii.* Morphological comparison of the three forms and the Yenisei River *A. baerii* indicates that although the three forms of *A. gueldenstaedtii* are not different from one another, all three significantly differ from the Yenisei River *A. baerii*. Competing explanations, including translocation and centre of origin hypotheses are considered. The three genetic forms of *A. gueldenstaedtii* likely colonized different geographic areas during different geological periods, and subsequently evolved in these regions independently into the species currently recognized as *A. gueldenstaedtii*, *A. naccarii* and *A. baerii*.

Key words sturgeon, speciation, mtDNA, caviar, Caspian Sea, Black Sea

Introduction

The Russian sturgeon, *Acipenser gueldenstaedtii* Brandt (1833), is one of the main anadromous Eurasian sturgeon species inhabiting the Caspian, Black and Azov seas and rivers entering into them (Vlasenko *et al*., 1989*a*). *Acipenser gueldenstaedtii* is known to be part of a complex of genetically closely related species that includes the Persian sturgeon, *A. persicus*, the Adriatic sturgeon, *A. naccarii*, and the Siberian sturgeon, *A. baerii* (Birstein *et al*., 2000). Originally, *A. persicus* was described from the Southern Caspian Sea (Borodin, 1897), with its range later extended to also include the northern Caspian Sea (reviewed in Vlasenko *et al*., 1989*b*). It is considered as a valid species by some (Luk'yanenko *et al*., 1974; Putilina, 1983), but as a subspecies of *A. gueldenstaedtii* by others (i.e. *A. g. persicus*; Berg, 1934b; Birstein & Bemis, 1997). Recent molecular analyses did not support a species rank of *A. persicus* (Birstein *et al*., 2000; Birstein & Doukakis, 2001; see also Almodóvar *et al*., 2000). The Adriatic sturgeon is morphologically similar to *A. gueldenstaedtii* and inhabits a small range in the Northern Adriatic Sea (Tortonese, 1989). The Siberian sturgeon differs from these species in that it is a freshwater sturgeon. It inhabits Siberian rivers and Lake Baikal – geographic areas distant and disconnected from the Caspian Sea region by the Ural Mountains (Ruban, 1999).

The Russian sturgeon is thought to have a complex intraspecies structure. Morphological differences observed among sturgeons in different seas and rivers within basins resulted in the rank of subspecies being assigned by some authors to the Caspian and Black/Azov Sea populations (Belyaev, 1932; Marti, 1940; Chugunov & Chugunova, 1964; Movchan, 1967). Seasonal summer and winter spawning races are also recognized within individual river systems (Berg, 1934*a*). An analysis of sturgeon sub-fossils also suggests a particularly complex historic population structure within *A. gueldenstaedtii* as compared with other species in the region (Sokolov & Tsepkin, 1996).

The Caspian Sea has historically harboured the largest and most structurally complex population of the Russian sturgeon, with sturgeon concentrated along the West coast and in the middle of the Southern regions of the sea (Khodorevskaya, 2002). As Russian sturgeon are anadromous, this species utilizes many rivers entering into the sea for spawning, with the Volga River supporting the largest spawning population

* Corresponding author. Email: birstein@pipeline.com

(Berg, 1948; Vlasenko *et al*., 1989a). Four biological groups are recognized within this population, corresponding to the early and late spring races, and the summer and autumn runs within the winter race, although no detailed morphological study of these groups has been conducted (Barannikova, 1957).

The Russian sturgeon population in the Caspian Sea also appears to consist of two genetic forms, the 'typical' or *gueldenstaedtii*-like form, whose mtDNA is similar to that of the Black Sea Russian sturgeon individuals, and the *baerii*-like form, whose mtDNA is similar to the mtDNA of the Siberian sturgeon *A. baerii* (Birstein *et al*., 2000; Jenneckens *et al*., 2000). Our interpretation of these results was that the Russian sturgeon *baerii*-like form, representing about 30% of the whole Caspian Sea population of *A. gueldenstaedtii*, is closely related to the ancestors of the Siberian sturgeon, as the Caspian Sea is likely the centre of origin for many species of sturgeon in the region (Birstein *et al*., 2000). This form might have moved from the Caspian Sea to Siberian rivers and Baikal Lake through peri-glacial lakes that connected these basins during the past geological history of Eurasia. As this hypothesis is based on maternally inherited mtDNA, hypothetically the Russian sturgeon *baerii*-like form could have originated through genetic introgression, i.e. through *A. baerii* (♀♀) × *A. gueldenstaedtii* (♂♂) hybridization (Jenneckens *et al*., 2000). However, although fertile hybrids between the species of *Acipenser* of the same ploidy (most of the species are presumably tetra- and octoploids; discussion in Birstein *et al*., 1997; Ludwig *et al*., 2001; Rodzen & May, 2002) are easily obtained in captivity, they have never been found in the wild (Nikolyukin, 1970; Arefyev, 1998).

In the present study, we perform morphological and genetic comparisons of individuals representing the two genetic forms within the Volga River population of *A. gueldenstaedtii*. We then compare these forms to other closely related species. Our results indicate that individuals representing the two genetic forms of the Russian sturgeon do not differ morphologically, and that the two forms are morphologically distinct from the Siberian sturgeon. Also, a third, rare genetic form of *A. gueldenstaedtii*, whose mtDNA is similar to the mtDNA of the Adriatic sturgeon, *A. naccarii*, is uncovered. Morphologically, the *naccarii-like* form does not differ from the two other Russian sturgeon genetic forms. We further consider all genetic information available for the species complex to examine species diagnostics both in an evolutionary and forensic context and investigate the existence and origins of cryptic lineages within *A. gueldenstaedtii*.

Materials and methods

Sampling

Specimens used for morphological and genetic studies included a total of 34 Russian sturgeon (13 collected between 1 September and 29 September 1999, and 21 collected between 26 April and 18 September 2000). Sturgeon were captured in the Volga River Delta in the fishing area known as Chkalovskaya. The main branch of the Volga River Delta (the Main Bank or *Glavnyi Bank* in Russian) extends in the shallow Northern part of the Caspian Sea as the approximately 60 km-long constructed Volgo–Caspian Channel. Chkalovskaya is the most remote part of the channel, and sturgeons migrating for spawning enter the channel near Chkalovskaya and swim up the Volga River. The Russian sturgeon we used were a part of the catch quota given annually to the Caspian Fisheries Research Institute in Astrakhan (Russia) for scientific purposes. After taking measurements, muscle tissue samples from each specimen were fixed in 96% ethanol.

For comparison of morphometric and meristic characters of the Russian and Siberian (*A. baerii*) sturgeons, measurements for *A. baerii* from the Yenisei River (Siberia) previously collected were used (Ruban, 1999). Only the data for Siberian adult sturgeon of approximately the same size as the Russian sturgeon studied in the present research were included. Specimens from the Yenisei River population were taken as representatives of the species, since subspecies level morphological differences have not been found within *A. baerii* (Ruban, 1999). Siberian sturgeon were collected and measured at the Research Station of the A. N. Severtsov Institute of Ecology and Evolution of the Russian Academy of Sciences located 1420 km upstream from the mouth of the Yenisei River.

DNA extraction, amplification and sequencing

DNA was isolated from all samples using the QIAamp Blood or Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturers instructions. Besides tissue samples from *A. gueldenstaedtii*, we additionally studied a tissue sample of *A. naccarii* from a specimen caught in the Buna River (Albania). Amplification was performed in 100 μl volumes containing: 50 ng genomic DNA, 1 U Taq polymerase (MBI-Fermentas, Vilnius, Lithuania), 5 pmoles of each primer, 0.10 mM Tris-HCl (pH 8.8 at 25 °C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 μg/μl bovine serum albumin (BSA), 0.08% (v/v) Nonidet P40 and 100 μM of each dNTP.

Two regions of mtDNA were amplified and sequenced: the entire cytochrome *b* (cyt*b*) gene (1141 bp) and a fragment (725 bp) of control region excluding the heteroplasmic region. The entire cyt*b* gene was amplified using primers cyt-b.for1 (5′-CGTTGTHWTTCAACTAYARRAAC-3′) and cyt-*b*.rev1 (5′-CTTCGGTTTACAAGACCG-3′) (Jenneckens *et al*., 2000; Ludwig *et al*., 2000). We used four additional primers derived on the basis of partial sequences of acipenseriform species published in Ludwig *et al*. (2000): cyt*b*-L113 (5′-GCCTCTGCCTTRTCAC-3′), cyt*b*-L823 (5′-CTCTTYGCCTACGCCATYC-3′), cyt*b*-H835 (5′-CGTAGGCRAAGAGRAAG-3′), and cyt*b*-H345 (5′-GATRTTTCAGGTYTCTTTTTG-3′). Amplification was accomplished in 30 cycles of the following steps: 60 s at 94 °C, 30 s at 60 °C, 90 s at 72 °C and a 5 min final extension at 72 °C. For the control region fragment, primers dlp 1.5 (Baker *et al*., 1993) and a modified H00651 (5′-ATCTTAACATCTTCAGTC-3′) (Doukakis *et al*., 1999) were used.

PCR products were run on a 1.5% agarose gel at 150 V for 2 h. PCR products were excised from the gel and extracted using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Direct sequencing was performed in both directions

using an ABI 310 (Perkin Elmer, Norwalk, CT) following the manufacturers protocol.

Analysis of the DNA data

For comparison of the total cyt*b* gene (1141 bp) and control region (725 bp) sequences we aligned sequences by eye. For the phylogenetic analysis of the cyt*b* gene sequences, we trimmed the same fragments of this gene (850 bp in total) from the total sequence as in our previous papers (Birstein *et al*., 2000). We further used sequences of these gene regions obtained in our previous study, as indicated in Tables 1 and 2 in Birstein *et al*. (2000). The cyt*b* gene sequence for *A. naccarii* from the Buna River has been published in Ludwig *et al*. (2002). The total data matrix considered included 3 individuals of *A. naccarii*, 79 individuals of *A. gueldenstaedtii*, 11 individuals of *A. persicus* and 35 individuals of *A. baerii*. We used PAUP* 4.0b2 (Swofford, 1999) for this analysis and *A. stellatus* and *Huso huso* for rooting; 10,000 replicates of both bootstrap and jackknife analyses as implemented in PAUP* were used to examine the robustness of clades.

In order to establish diagnostics for the three forms involved in this study, we took a subset of data for *A. gueldenstaedtii*, *A. baerii* and *A. naccarii* and performed population aggregation analysis (Davis & Nixon, 1992) to discover whether species are diagnosably distinct from one another and if species diagnostics exist in mtDNA. We chose only those *A. gueldenstaedtii*, *A. baerii* and *A. naccarii* sequences for which species designation based on morphology and genetics was unambiguous and used representatives from as many different localities for these species as possible (Table 1). We refer to the set of fish used to develop these diagnostics as a 'training set.' These diagnostics were then used to examine any new sample added to the study.

Morphometry

We analysed 22 morphometric (Table 2) and 6 meristic characters (the number of rays: in dorsal fin, D; rays in anal fin, A; dorsal scutes, Dr; lateral scutes, Lr; ventral scutes, Vr; and gill rakers, Sp.br.). These characters are commonly used for morphological comparisons of sturgeons (Ruban, 1999; Elvira & Almodóvar, 2000; Rincón, 2000a, b).

Analysis of morphometric and meristic data

Principal Components Analysis (PCA) was employed to evaluate shape and morphometric variation. While applying this method, one needs to take into consideration the problem of allometric growth – the changes in proportions during ontogenesis (Huxley, 1932; Marcus, 1990). In other words, it is necessary to find the difference in the shape of compared fishes that is not due to differences in size. Variation in shape can be analysed directly using either univariate or multivariate methods or indirectly using shape variables, which are dimensionless ratios or proportions expressed as differences between logarithms, or logarithms of indices – measurements of morphometric characters as a per cent of the total length of the fish (Mosimann, 1970; Mosimann & James, 1979; Mosimann & Malley, 1979; James & McCulloch, 1990). We used a variance-covariance matrix for PCA of our data. Calculations were made using the NTSYSpc ver. 2.1. package (Rohlf, 2000).

Results

mtDNA nucleotide diagnostics with a 'Training Set' of unambiguously identified specimens

Population aggregation analysis (Davis & Nixon, 1992) of the 'training set' of individuals of unambiguous species origin (see Materials and Methods) allowed us to distinguish 10 nucleotides in the cyt*b* gene sequence and 16 in the control region that diagnose *A. baerii* from *A. gueldenstaedtii* and *A. naccarii* (Table 3). Only one diagnostic nucleotide (T in position 345 of the cyt*b* gene) was found for *A. naccarii* relative to the two other species. Surprisingly, no diagnostic nucleotides for *A. naccarii* were found within the control region. If one uses these rules for the analysis of sequence data for samples studied in the present paper, the results coincide with the classification of individuals given in Table 4. These diagnostics can be used in future studies to determine species designations for any wild *A. gueldenstaedtii* caught in the Caspian Sea, as well as to designate the species origin of commercial caviar products.

The diagnostic approach using both cyt*b* and control region sequences indicated that of 13 Russian sturgeon individuals caught in 1999, five were *baerii*-like (Nos 1, 4, 6, 8 and 11), and the others were *gueldenstaedtii*-like (Table 4). Of 21 sturgeon collected and measured in 2000, 8 were *baerii*-like (Nos 17, 21, 23, 25, 27–30), and 11, *gueldenstaedtii*-like (Nos 14–16, 18–20, 22, 26, 31, 33, 34). Only three of the cyt*b* haplotypes (H1, H2 and H4) we found had been described in previous studies (Jenneckens *et al*., 2000). Moreover, in samples 24 and 32 the cyt*b* gene sequence was similar to the *A. naccarii* haplotype. The re-examination of our previous cyt*b* data (Birstein *et al*., 2000) showed that one of the Russian sturgeon from the Black Sea (the individual caught in the mouth of the Dnieper River; AgDn) also belonged to the *naccarii*-like form. The variable nucleotide sites for the haplotypes of the cyt*b* gene are given in Table 5.

Our previous study of cryptic lineages within *A. gueldenstaedtii* resulted in identification of a diagnostic nucleotide separating *A. baerii*-like Russian sturgeon in the Caspian Sea from pure *A. baerii* from Siberian river systems (Birstein *et al*., 2000). This diagnostic, in position 421 of the control region, was not supported in the present study, as individuals 6, 17 and 21 did not contain this diagnostic nucleotide.

Phylogenetic inference

The result of the phylogenetic analysis of the merged databases for the cyt*b* gene sequences and the control region sequences separately are presented in Fig. 1. As in our previous molecular analysis (Birstein *et al*., 2000), for both partitions analysed singly there were two main clades uncovered, corresponding to the Russian sturgeon *gueldenstaedtii*-like and *baerii*-like forms. Individuals of *A. naccarii* and the Russian sturgeon *naccarii*-like form comprised a cluster within the *gueldenstaedtii*-like clade, while the Persian sturgeon *A. persicus* was

Species	Code for figure	Collection location	GenBank accession no. (cyt*b*, D-loop) or paper
A. gueldenstaedtii			
	AgNC2–AgNC9	North Caspian Sea, Russia	AF238662-669, 720-727
	AgC1–AgC2	North Caspian Sea, Russia	Present paper
	AgI2–AgI5, AgI7–AgI8	South Caspian Sea, Iran	AF238673-676, 678-679, 731-734, 736-737
	AgDn	Dnieper River, Ukraine (Black Sea basin)	AF238681, 739
	AgD1–AgD9	Danube River, Romania (Black Sea basin)	AF238682-690, 740-748
	AgBo	Danube River, Romania (Black Sea basin)	AF23869, 749
	AgBl	Black Sea, Romanian	AF238692, 750
	AgK1–AgK4	Sea of Azov, Russia	AF238693-969; 651-754
A. baerii			
	AbB1–AbB8	Lake Baikal, Russia (Siberia), aquaculture	AF238625-631, AF168496-502
	AbL1–AbL12	Lena River, Russia (Siberia), aquaculture	AF238655-658, AF168480-483
	AbO1–AbO11	Ob River, Russia (Siberia)	AF238644-654, AF168469-79
	AbY1–AbY4	Yenisei River, Russia (Siberia)	AF238625-63, AF168496-502
A. naccarii			
	An1–An2	Po River, Italy (Adriatric , Sea basin) aquaculture	AF238659-660, 717-718
	AnBu	Buna River, Albania (Adriatric Sea basin)	Ludwig *et al*., 2002

Table 1 List of specimens used as a 'training set'.

also embedded in the Russian sturgeon *gueldenstaedtii*-like clade. The control region data set was less well resolved and less robust than the cyt*b* data set. The phylogenetic analysis of the combined cyt*b*-control region data sets is shown in Fig. 2. This tree also supports two major clades and is very similar in overall topology to the cyt*b* tree.

Morphometrics

After we had established which Russian sturgeon specimen belonged to the Russian sturgeon *gueldenstaedtii*-like and *baerii*-like genetic forms, we compared the morphometric data. We divided the datasets in two subgroups, the *baerii*-like and *gueldenstaedtii*-like based on the cyt*b* and control region diagnostics discussed above. For 1999, the *baerii*-like subgroup included individuals No. 1, 4, 6 and 11; we excluded No. 8 because the measurements for this individual were not complete. For 2000, in the *baerii*-like subgroup we included all *baerii*-like specimens (Nos 17, 20–21, 23, 25, 27–30). We combined the data for the two study years (1999 and 2000) in the *gueldenstaedtii*-like and *baerii*-like groups.

The projections of the *gueldenstaedtii*-like and *baerii*-like specimens collected in 1999 and 2000 onto the first and second principal components (PC1 and PC2) showed that the distribution of samples of these forms is transgressive and that they cannot be divided into two clusters (Fig. 3). The values of factor loading on the first two components extracted by the PCA of morphometric characters (Table 6) are low. Such morphometric characters as hco, lA and hA are the most important for the division of these two groups.

The projections of the *gueldenstaedtii*-like specimens and Siberian sturgeon onto the PC1 and PC2 showed that these two groups of samples are distributed in the space of the two components as two separate clusters (Fig. 4). According to the values of factor loading, such morphometric characters as R, SO, lc, o and HC are the most important for the division of the *gueldenstaedtii*-like specimens and Siberian sturgeon (Table 6).

The projections of the *baerii*-like specimens and Siberian sturgeon onto the PC1 and PC2 showed that these two groups of samples are distributed as two separate clusters (Fig. 5). According to the values of factor loading, the same morphometric characters as in the previous case (R, SO, lc, o and HC) are the most important for the division of these two groups of samples (Table 6).

The projections of all specimens collected in 1999–2000 onto the PC1 and PC2 showed that the *gueldenstaedtii*-like sturgeon are not separated from the *baerii*-like and both of these forms are well separated from Siberian sturgeon from the Yenisei River. The comparison of means of morphometric ratios (character in per cent of total length) shows that there are no significant differences between the *gueldenstaedtii*-like and *baerii*-like sturgeon (Table 7). The comparison of means of morphometric ratios (character in per cent of total length) show that both the *gueldenstaedtii*-like and *baerii*-like

Number	Character	Abbreviation	1. *A. guelden staedtii*-like, n = 19	2. *A. baerii*-like, n = 12	3. *A. baerii*, n = 42	P 1–2	P 1–3	P 2–3
			Mean ± SD	**Mean ± SD**	**Mean ± SD**			
	Total length, mm	Tl	1220.53 ± 14.31	1272.50 ± 29.65	1201.33 ± 17.54			
1.	Head length	C	17.35 ± 0.20	17.25 ± 0.18	19.20 ± 0.17	> 0.05	< 0.001	< 0.001
2.	Snout length	R	5.18 ± 0.12	5.19 ± 0.16	7.38 ± 0.09	> 0.05	< 0.001	< 0.001
3.	Width of the mouth	SO	5.65 ± 0.08	5.57 ± 0.08	4.69 ± 0.08	> 0.05	0.001	< 0.001
4.	Length of the maximal barbel	lc	3.43 ± 0.11	3.33 ± 0.11	4.57 ± 0.07	> 0.05	< 0.001	< 0.001
5.	Horizontal eye diameter	o	1.27 ± 0.02	1.26 ± 0.04	1.11 ± 0.02	> 0.05	< 0.001	< 0.01
6.	Postorbital distance	op	11.08 ± 0.12	11.09 ± 0.15	10.98 ± 0.11	> 0.05	> 0.05	> 0.05
7.	Interorbital distance	io	5.84 ± 0.09	5.75 ± 0.10	6.56 ± 0.12	> 0.05	< 0.001	< 0.001
8.	Head depth at the nape	HC	11.34 ± 0.20	11.37 ± 0.33	9.05 ± 0.13	> 0.05	< 0.001	< 0.001
9.	Head depth at the centre of eye	hco	5.16 ± 0.09	5.01 ± 0.16	5.05 ± 0.06	> 0.05	> 0.05	> 0.05
10.	Maximum body depth	H	13.27 ± 0.24	13.54 ± 0.18	12.23 ± 0.19	> 0.05	< 0.01	< 0.001
11.	Minimum body depth	h	3.51 ± 0.05	3.48 ± 0.08	3.37 ± 0.13	> 0.05	> 0.05	> 0.05
12.	Antedorsal distance	AD	65.66 ± 0.48	65.61 ± 0.37	63.91 ± 0.35	> 0.05	< 0.01	< 0.01
13.	Anteventral distance	AV	55.96 ± 0.35	55.65 ± 0.67	54.50 ± 0.32	> 0.05	< 0.01	> 0.05
14.	Anteanal distance	AA	70.88 ± 0.43	70.45 ± 0.91	69.88 ± 0.34	> 0.05	> 0.05	> 0.05
15.	Basal length of dorsal fin	lD	10.13 ± 0.22	10.33 ± 0.22	10.86 ± 0.10	> 0.05	< 0.01	< 0.05
16.	Height of dorsal fin	hD	7.88 ± 0.16	7.89 ± 0.13	7.31 ± 0.15	> 0.05	< 0.05	> 0.05
17.	Basal length of anal fin	lA	5.11 ± 0.16	5.59 ± 0.18	5.12 ± 0.11	> 0.05	> 0.05	< 0.05
18.	Height of anal fin	hA	8.16 ± 0.24	7.81 ± 0.32	7.90 ± 0.16	> 0.05	> 0.05	> 0.05
19.	Length of pectoral fin	lP	13.03 ± 0.21	12.31 ± 0.26	13.23 ± 0.22	> 0.05	> 0.05	0.01
20.	Length of pelvic fin	lV	7.79 ± 0.18	7.85 ± 0.21	7.69 ± 0.17	> 0.05	> 0.05	> 0.05
21.	Pectoventral distance	PV	38.15 ± 0.33	37.90 ± 0.54	35.93 ± 0.29	> 0.05	< 0.001	< 0.01

Table 2 Mean values of metric characters for specimens of the *A. gueldenstaedti*-like and *A. baerii*-like forms of *A. gueldenstaedtii* and specimens of *A. baerii* from the Yenisei River. All values (except Total length, Tl, in mm) are expressed in per cent of TL; P is the significance of difference between the mean values estimated by T criteria.

Species	Sequence	Nucleotide and position
A. baerii	cyt*b* gene	A/196, G/306, C/342, C/648, T/685, A/690, A/693, T/735, A/898, G/912
	control region	G/52, A/79, A/98, G/118, G/312, G/316, A/317, A/331, C/388, G/480, T/491, T/513, C/576, T/580, T/607, C/658
A. naccarii	cyt*b* gene	T/345
	control region	None

Table 3 Species-specific diagnostic nucleotides for *A. baerii* and *A. naccarii*.

Sample no.	Genetic form identified using the cyt*b* gene sequence	Haplotype of the cyt*b* gene	Genbank Access No.
I. Catch of 1999			
NAgue 1	*A. baerii-like*	H7	AJ563385
NAgue 2	*A. gueldenstaedtii*-like	H8	AJ563386
NAgue 3	*A. gueldenstaedtii*-like	H9	AJ563387
NAgue 4	*A. baerii-like*	H4	AJ245825
NAgue 5	*A. gueldenstaedtii*-like	H10	AJ563388
NAgue 6	*A. baerii-like*	H4	AJ245825
NAgue 7	*A. gueldenstaedtii*-like	H11	AJ249692
NAgue 8	*A. baerii-like*	H4	AJ245825
NAgue 9	*A. gueldenstaedtii*-like	H10	AJ563388
NAgue 10	*A. gueldenstaedtii*-like	H11	AJ563389
NAgue 11	*A. baerii-like*	H4	AJ245825
NAgue 12	*A. gueldenstaedtii*-like	H11	AJ563389
NAgue 13	*A. gueldenstaedtii*-like	H10	AJ563388
II. Catch of 2000			
NAgue 14	*A. gueldenstaedtii*-like	H1	AJ249692
NAgue 15	*A. gueldenstaedtii*-like	H1	AJ249692
NAgue 16	*A. gueldenstaedtii*-like	H1	AJ249692
NAgue 17	*A. baerii-like*	H16	AJ563390
NAgue 18	*A. gueldenstaedtii*-like	H13	AJ563391
NAgue 19	*A. gueldenstaedtii*-like	H2	AJ245827
NAgue 20	*A. gueldenstaedtii*-like	H17	AJ563392
NAgue 21	*A. baerii-like*	H4	AJ245825
NAgue 22	*A. gueldenstaedtii*-like	H14	AJ563393
NAgue 23	*A. baerii-like*	H4	AJ245825
NAgue 24	*A. naccarii*-like	H19	AJ245826
NAgue 25	*A. baerii-like*	H4	AJ245825
NAgue 26	*A. gueldenstaedtii*-like	H1	AJ249692
NAgue 27	*A. baerii-like*	H15	AJ563394
NAgue 28	*A. baerii-like*	H4	AJ245825
NAgue 29	*A. baerii-like*	H4	AJ245825
NAgue 30	*A. baerii-like*	H18	AJ563395
NAgue 31	*A. gueldenstaedtii*-like	H13	AJ563391
NAgue 32	*A. naccarii*-like	H19	AJ245826
NAgue 33	*A. gueldenstaedtii*-like	H12	AJ563396
NAgue 34	*A. gueldenstaedtii*-like	H1	AJ249692

Table 4 The cyt*b* gene haplotypes identified in 34 individuals of *A. gueldenstaedtii*.

sturgeon significantly differ from the Siberian sturgeon from the Yenisei River by the majority of morphometric characters under the study, especially by C, R, SO, lc, o, io, HC, H, AD, lD, PV (Table 7). Such diagnostic character as fan-shaped gill rakers that are characteristic for *A. baerii*, were found only in the Siberian sturgeon we studied, but not in the Russian sturgeon individuals from the Caspian Sea.

Using PCA, we also compared two individuals 24 and 32 (caught in 2000) that we identified as the Russian sturgeon *naccarii*-like form, with the Russian sturgeon *gueldenstaedtii*-like and *baerii*-like forms, as well as with *A. baerii*. The *naccarii*-like individuals did not differ morphologically from the *gueldenstaedtii*-like and *baerii*-like specimens, but clearly differed from *A. baerii*. A comparison of all

Haplotype	Nos of *A. gueldensraedtii* samples	Nucleotide positions																			
		195	196 (*)	306 (*)	342 (*)	345 (**)	393	429	498	648 (*)	654	685 (*)	690 (*)	693 (*)	735	831	840	898 (*)	912 (*)	916	1053
1. *A. gueldenstaedtii*-like																					
H1	14–16, 26, 34	T	G	A	T	C	T	A	T	T	A	C	G	G	C	C	T	G	A	A	A
H8	2	T	G	A	C	C	T	A	C	C	A	C	G	G	C	C	T	G	A	A	A
H9	3	T	G	A	C	C	T	A	C	T	A	C	G	G	C	C	T	G	A	A	A
H10	5, 9, 13	T	G	A	C	C	T	A	T	T	A	C	G	G	C	C	T	G	A	A	A
H11	7, 10, 12	T	G	A	C	C	T	A	C	T	T	C	G	G	C	C	T	G	A	A	A
A. p.	AJ245837, AF238614	T	G	A	T	C	T	A	C	T	T	C	G	G	C	C	T	G	A	A	A
A. p.	AJ245835, AF238618	T	G	A	T	C	T	A	C	T	A	C	G	G	C	C	T	G	A	A	A
H12	33	T	G	A	T	C	T	A	C	T	A	C	G	G	C	C	T	G	A	A	A
H13	18, 31	T	G	A	T	C	T	A	C	T	A	C	G	G	C	C	C	G	A	A	A
H14	22	T	G	A	T	C	T	A	C	T	T	C	G	G	C	C	T	G	A	A	A
H2	19	C	G	A	T	C	C	G	C	T	A	C	G	G	C	C	C	G	A	A	G
H17	20	C	G	A	T	C	C	G	C	T	A	C	G	G	C	G	C	A	A	A	G
2. *A. baerii*-like																					
H4	4, 6, 8, 11, 21, 23, 25, 28, 29	T	A	G	C	C	T	A	C	C	A	**C**	A	A	T	C	T	A	G	C	A
H7	1	T	A	G	C	C	T	A	C	C	A	T	A	A	T	C	T	A	G	C	A
H15	27	T	A	G	C	C	T	A	C	C	A	T	A	A	T	G	T	A	G	C	A
H16	17	T	A	G	C	C	T	A	C	C	A	T	A	A	T	C	T	A	G	C	A
H18	30	T	A	G	C	C	T	A	C	C	A	C	A	A	T	G	T	A	G	C	A
A. b. (Yenisei)	AbY1, AF238655	T	A	G	C	C	T	A	C	C	A	T	A	A	T	C	T	A	G	A	–
A. b. (Ob)	This study	T	A	G	C	C	T	A	C	C	A	T	A	A	T	C	T	A	G	C	A
A. b. (Baikal)	This study	T	A	G	C	C	T	A	C	C	A	T	A	A	T	C	T	A	G	C	A
3. *A. naccarii*-like																					
H19	24, 32	T	G	A	T	T	T	A	T	T	A	C	G	G	C	C	T	G	A	A	A
Dn	AF238681	T	G	A	T	T	T	A	T	T	A	C	G	G	C	C	T	G	A	A	–
A. n. (Po)	This study	T	G	A	T	T	T	A	T	T	A	C	G	G	C	C	T	G	A	A	A
A. n. (Buna)	Ludwig *et al*., 2002	T	G	A	T	T	T	A	T	T	A	C	G	G	C	C	T	G	A	A	A

Table 5 Variable nucleotide sites in haplotypes of the cyt*b* gene. Nucleotides diagnostic for *A. baerii* are marked with (*) and diagnostic for *A. naccarii* with (**).

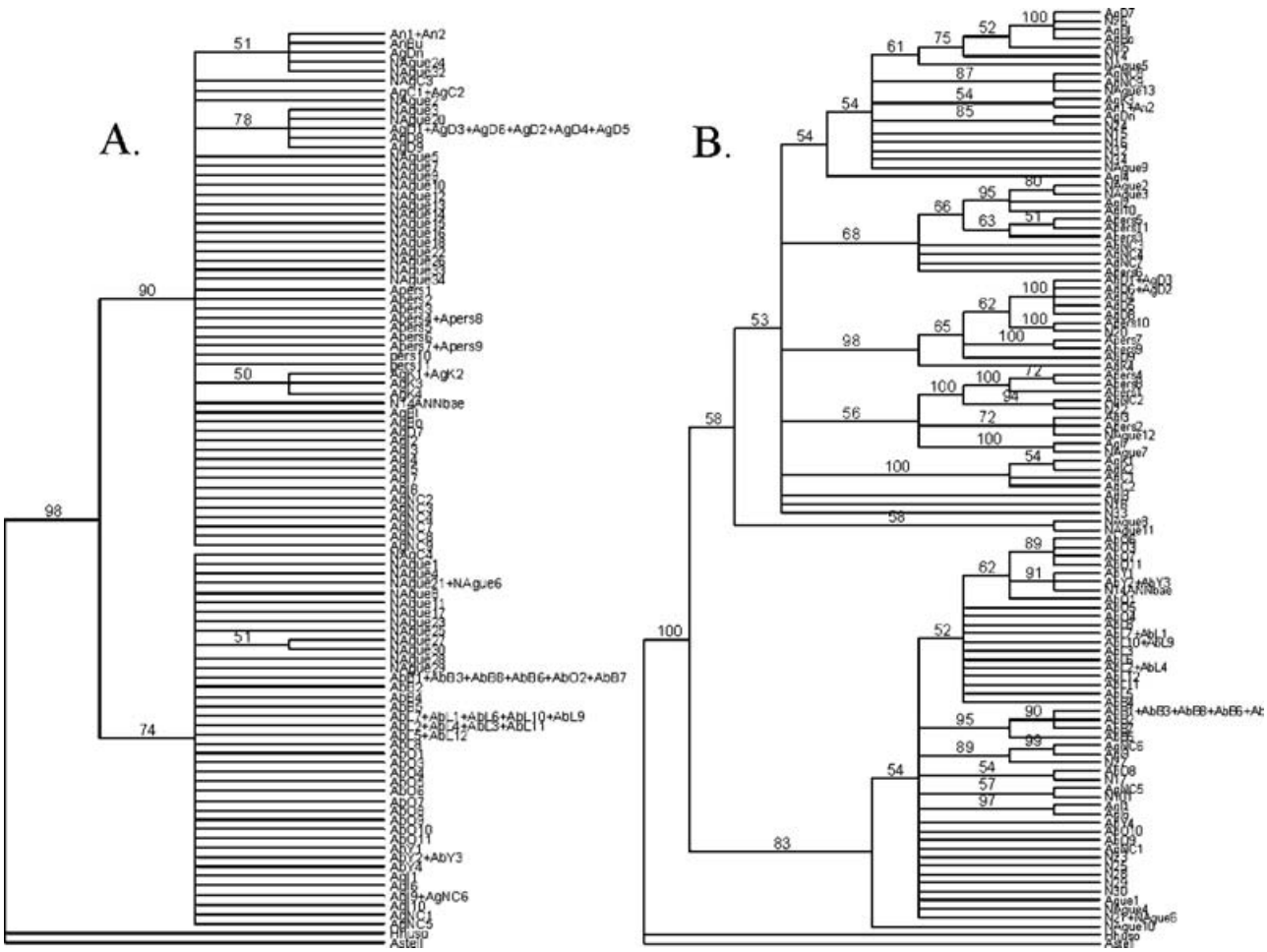

Figure 1 Jackknife analysis of the cyt*b* (panel A) and d-loop (panel B) of all individuals in the study. The jackknife proportions were calculated for each tree using 1000 replicates of jackknifing. The straight parsimony analysis of the cytb data set gave 30 parsimony trees with tree length = 49, Consistency Index (excluding uninformative characters) = 0.7447 and Retention index (RI) = 0.9770. The tree topology of the consensus of these 30 trees is consistent with the jackknife consensus tree shown in this figure. The straight parsimony analysis of the d-loop data set gave greater than 20 000 parsimony trees with tree length = 505, Consistency Index (excluding uninformative characters) = 0.3613 and Retention index (RI) = 0.8653. The tree topology of the consensus of these 20 000 trees is consistent with the jackknife consensus tree shown in this figure.

Russian sturgeon individuals with *A. baerii* is presented in Fig. 6.

Meristic characters

The difference between the average values of all six meristic characters of the *gueldenstaedtii*-like and *baerii*-like specimens was not statistically significant (Table 8).

Discussion

What is the Russian sturgeon, *A. gueldenstaedtii*?

A close genetic relatedness exists for the species known as *A. gueldenstaedtii*, *A. baerii* and *A. naccarii*, as well as for the *gueldenstaedtii*-like and *baerii*-like forms within the Caspian Sea population of *A. gueldenstaedtii* (Birstein & DeSalle 1998; Birstein *et al*., 2000; Birstein & Doukakis, 2001). The present molecular data further support this conclusion and allow us to add a third Russian sturgeon genetic form, the *naccarii*-like form, to the list of biological entities that exists. These forms were not detected in previous RFLP studies of *A. gueldenstaedtii* from the Caspian Sea (Pourkazemi *et al*., 1999; Rezvani Gilkolaei, 2000).

Our analysis of morphometric characters demonstrated that there are no morphological differences between the Russian sturgeon *gueldenstaedtii*-like, *baerii*-like and *naccarii*-like forms. A significant morphological difference does however exist between the three forms of the Russian sturgeon and *A. baerii* from Siberia. These data explain why these genetic forms have remained undetected. Apparently, the Caspian Sea *A. gueldenstaedtii* is one of those rare examples where a previously well-known taxon is later described as consisting of several cryptic forms or species (for instance, Baker *et al*., 1995, 1996; Garcia-Rodriguez *et al*., 1998; Amato *et al*., 1999; Dalebout *et al*., 2002). The recently introduced simplified 'DNA barcoding' system for identifying species (Hebert *et al*., 2003a, b) cannot be applied to such situations.

The structure of the clade *A. gueldenstaedtii–A. baerii–A. naccarii* is unique. We identify a mixture of three morphologically indistinguishable genetic forms of the Russian

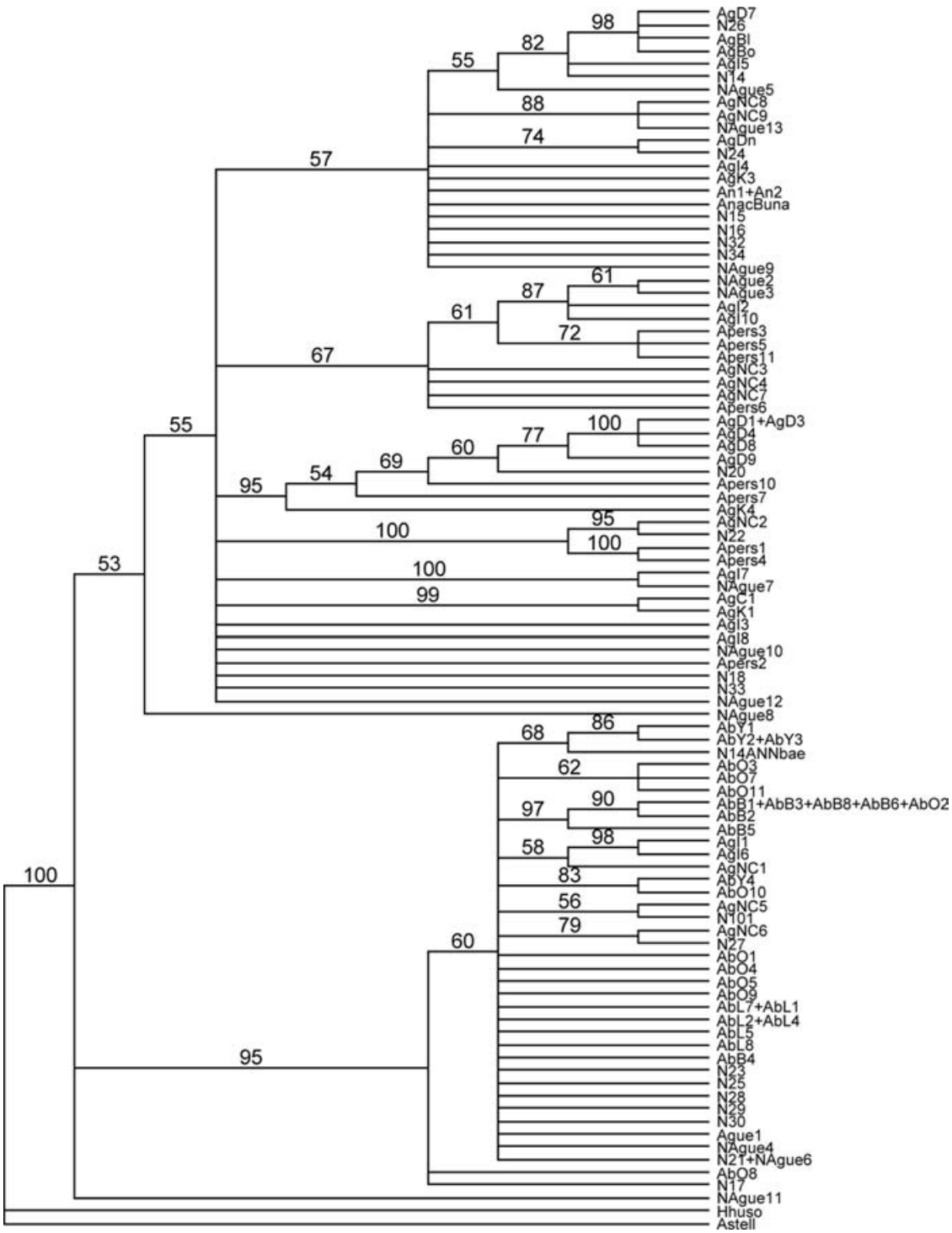

Figure 2 Jackknife parsimony tree for the combined cyt*b* and d-loop analysis. The jackknife proportions are shown on the nodes of the tree. This tree was generated by doing 1000 replicates of jackknifing. The straight parsimony analysis of this combined data set gave greater than 20 000 parsimony trees with tree length = 610, Consistency Index (excluding uninformative characters) = 0.3576 and Retention index (RI) = 0.8661. The tree topology of the consensus of these 20 000 trees is consistent with the jackknife consensus tree shown in this figure.

sturgeon morphotype (the *gueldenstaedtii*-like, *baerii*-like and *naccarii*-like) sympatric in the Caspian Sea, and two forms (the *gueldenstaedtii*-like and *naccarii*-like) in the Black Sea. 'Pure' mtDNA lineages of *A. naccarii* (consisting itself of two main forms) together with the *gueldenstaedtii*-like form exist in the Adriatic Sea (Ludwig *et al*., 2003), and 'pure' *A. baerii* exists in Siberian rivers and Lake Baikal. The geographic distribution of all known forms in the clade is shown in Fig. 7. The morphological similarity of the Russian sturgeon *baerii*-like form with the *gueldenstaedtii*-like form and the morphological difference between the Russian sturgeon *baerii*-like individuals and 'pure' Siberian *A. baerii* were unexpected.

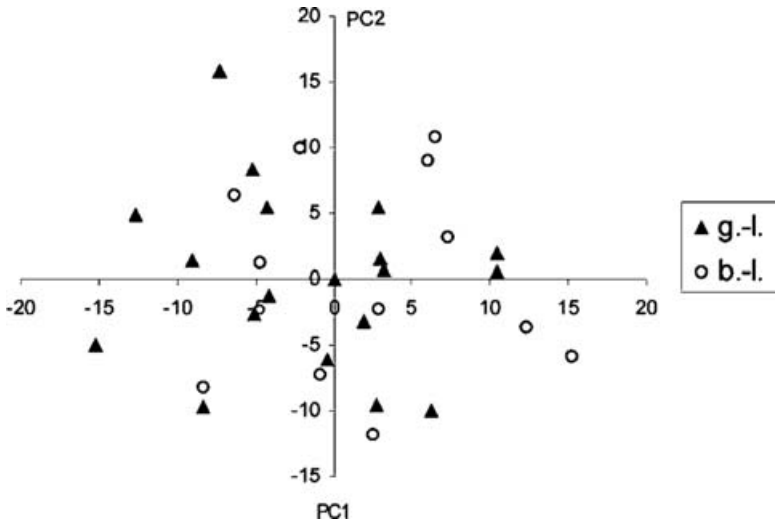

Figure 3 Sturgeon ordination on multivariate morphometric axes from two PCA. PC2 against PC1 extracted from variance-covariance matrix of log transformed measurements. g.-l, the *gueldenstaedtii*-like, and b.-l, the *baerii*-like samples.

Character		To Fig. 3	To Fig. 4	To Fig. 5	To Fig. 6
1.	C	− 0.02023	0.07435	0.07340	0.08053
2.	R	− 0.02678	0.24538	0.22330	0.26368
3.	SO	− 0.01562	− 0.13515	− 0.12009	− 0.13985
4.	lc	− 0.02232	0.21074	0.19647	0.23124
5.	o	− 0.07459	− 0.10006	− 0.07401	− 0.09068
6.	op	− 0.01044	− 0.00998	− 0.00982	− 0.00798
7.	io	− 0.05336	0.07752	0.08358	0.09057
8.	HC	0.01229	− 0.17139	− 0.15774	− 0.17986
9.	hco	− 0.10224	− 0.01582	0.00190	− 0.00327
10.	H	− 0.00120	− 0.05659	− 0.06230	− 0.07021
11.	h	− 0.04649	− 0.05405	− 0.04652	− 0.04486
12.	AD	− 0.01775	− 0.01926	− 0.01710	− 0.02087
13.	AV	− 0.01131	− 0.01774	− 0.01421	− 0.01712
14.	AA	− 0.02072	− 0.01087	− 0.00721	− 0.00947
15.	lD	0.07807	0.04545	0.02463	0.04528
16.	hD	0.01765	− 0.07240	− 0.06852	− 0.06673
17.	lA	0.12282	0.00985	− 0.06210	− 0.02361
18.	hA	− 0.11944	− 0.04701	− 0.01017	− 0.01652
19.	lP	− 0.03172	− 0.00092	0.04277	0.02456
20.	lV	− 0.01705	− 0.01582	− 0.02305	− 0.01851
21.	PV	− 0.00187	− 0.04207	− 0.03369	− 0.04318
22.	VA	− 0.03849	− 0.01159	− 0.00113	− 0.00965

Table 6 Factor loading on the first two components extracted by the PCA of all morphometric characters (for Figs 3–6).

Different explanations could describe this phenomenon. The most likely hypothesis would be that the three genetic forms of the Caspian Sea population are closely related to the ancestral forms of the three 'pure' species. These species might have evolved at first as subdivisions of the original Caspian Sea population and then moved to different geographical areas when the Caspian Sea was connected with the water basins of those areas. These forms still exist in the ancestral regions.

The current distribution of *A. baerii* could be reasonably described by the Pleistocene colonization hypothesis which suggests that migration of species to and from the Caspian Sea occurred through the Volga River and ice-dammed glacial lakes (Berg, 1928; review in Väinölä, 1995). In the last (late Weichselian) glaciation, an ice-dammed lacustrine transgression resulted in the connection of the West Siberian ice-lakes with the Aralo–Caspian basin (merged Caspian and Aral seas) through the Turgai Valley occurring 12–13 500 years ago (Arkhipov, 1998; Grosswald, 1998). The ancestral form of *A. baerii* could have moved from the Aralo–Caspian basin to Siberia through the Turgai Valley and dispersed within basins of different rivers there through the huge lake in West Siberia that overlapped the main river basins. This ancestral form could have evolved morphologically into what we know now as *A. baerii*.

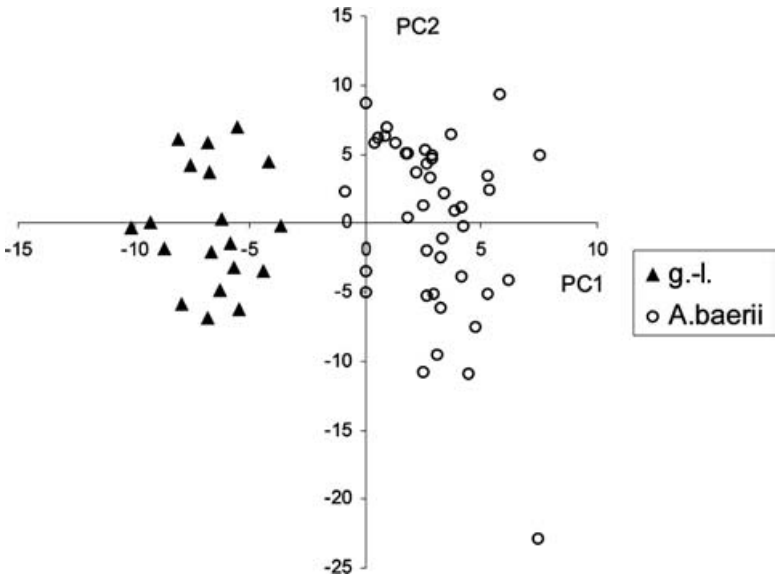

Figure 4 Sturgeon ordination on multivariate morphometric axes from two PCA. PC2 against PC1 extracted from variance-covariance matrix of log transformed measurements. g.-l., the *gueldenstaedtii*-like, and *A. baerii*, the *A. baerii* samples.

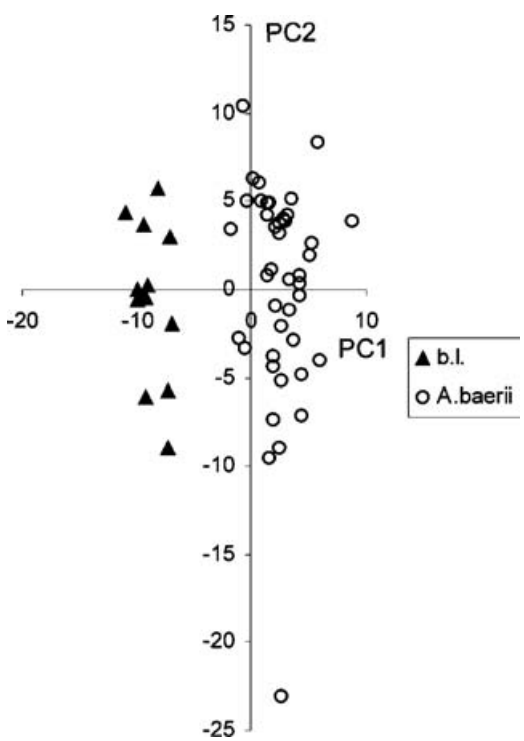

Figure 5 Sturgeon ordination on multivariate morphometric axes from two PCA. PC2 against PC1 extracted from variance-covariance matrix of log transformed measurements. b.l, the *baerii*-like, and *A. baerii*, the *A. baerii* samples.

Another possibility is that *A. baerii* is an old species of unknown origin and affiliation to the other Eurasian species of *Acipenser*. During the last transgression it could have invaded the Caspian Sea and hybridized with the 'typical' ancestral Russian sturgeon. However, this hypothesis is quite problematic. Mature fertile interspecies sturgeon hybrids of any kind have never been caught in the wild. Also, the current high number (30%) of the *baerii*-like individuals within the Russian sturgeon population in the Caspian Sea would be possible if there were numerous females of freshwater *A. baerii* at some time living in the marine conditions of the Caspian Sea. Moreover, it is not known even under laboratory conditions if the *A. baerii* (♀♀) × *A. gueldenstaedtii* (♂♂) hybrids can mature and reproduce. Although we studied only mtDNA – a maternal marker – and did not consider nuclear genes (and, therefore, might not have detected that individuals were in fact hybrids), the morphology of the Russian sturgeon we studied does not support a hybrid origin for the Caspian Sea *baerii*-like and *nacarii*-like sturgeons as we did not find intermediate morphological characters characteristic of sturgeon hybrids (Krylova, 1980a, b).

The Caspian and Black seas were also repeatedly connected during the Plio-Pleistocene geological history of the region (Zubakov, 1988). The last connection occurred 16–8000 years ago, during the Late Khvalynian transgression (Mamedov, 1997). It is presumed that during the late period of this transgression, seven Mediterranean species of invertebrates and three fish species penetrated from the Black into the Caspian Sea (Fedorov, 1988). Possibly, the Russian sturgeon *gueldenstaedtii*-like and *naccari*-like forms also moved to the Black Sea through this connection. Approximately 8000 years ago the Black Sea became connected with the Mediterranean (Gökaşan *et al*., 1997; Ryan *et al*., 1997) and the *naccarii*-like form could have moved to the Adriatic Sea due to the surface water inflow (Zonneveld, 1996).

We did not include new samples of *A. g. persicus* in the present morphological research. However, the addition of new samples of *A. gueldenstaedtii* in the phylogenetic analysis did not change the position of individuals designated as *A. g. persicus* in our trees as this taxa still appears embedded within the Russian sturgeon *gueldenstaedtii*-like form (Fig. 1). Therefore, as we have stated previously (Birstein *et al*., 2000),

		1. *A. guelden-staedtii*-like specimens, n = 19	2. *A. baerii*-like specimens, n = 12	3. *A. baerii*, n = 42			
Character in% of TL		**M ± m**	**M ± m**	**M ± m**	***P* (1–2)**	***P* (1–3)**	***P* (2–3)**
1.	C	17.35 ± 0.20	17.25 ± 0.18	19.20 ± 0.17	> 0.05	< 0.001	< 0.001
2.	R	5.18 ± 0.12	5.19 ± 0.16	7.38 ± 0.09	> 0.05	< 0.001	< 0.001
3.	SO	5.65 ± 0.08	5.57 ± 0.08	4.69 ± 0.08	> 0.05	< 0.001	< 0.001
4.	lc	3.43 ± 0.11	3.33 ± 0.11	4.57 ± 0.07	> 0.05	< 0.001	< 0.001
5.	o	1.27 ± 0.02	1.26 ± 0.04	1.11 ± 0.02	> 0.05	< 0.001	< 0.01
6.	op	11.08 ± 0.12	11.09 ± 0.15	10.98 ± 0.11	> 0.05	> 0.05	> 0.05
7.	io	5.84 ± 0.09	5.75 ± 0.10	6.56 ± 0.12	> 0.05	< 0.001	< 0.001
8.	HC	11.34 ± 0.20	11.37 ± 0.33	9.05 ± 0.13	> 0.05	< 0.001	< 0.001
9.	hco	5.16 ± 0.09	5.01 ± 0.16	5.05 ± 0.06	> 0.05	> 0.05	> 0.05
10.	H	13.27 ± 0.24	13.54 ± 0.18	12.23 ± 0.19	> 0.05	< 0.01	< 0.001
11.	h	3.51 ± 0.05	3.48 ± 0.08	3.37 ± 0.13	> 0.05	> 0.05	> 0.05
12.	AD	65.66 ± 0.48	65.61 ± 0.37	63.91 ± 0.35	> 0.05	< 0.01	< 0.01
13.	AV	55.96 ± 0.35	55.65 ± 0.67	54.50 ± 0.32	> 0.05	< 0.01	> 0.05
14.	AA	70.88 ± 0.43	70.45 ± 0.91	69.88 ± 0.34	> 0.05	> 0.05	> 0.05
15.	lD	10.13 ± 0.22	10.33 ± 0.22	10.86 ± 0.10	> 0.05	< 0.01	< 0.05
16.	hD	7.88 ± 0.16	7.89 ± 0.13	7.31 ± 0.15	> 0.05	< 0.05	> 0.05
17.	lA	5.11 ± 0.16	5.59 ± 0.18	5.12 ± 0.11	> 0.05	> 0.05	< 0.05
18.	hA	8.16 ± 0.24	7.81 ± 0.32	7.90 ± 0.16	> 0.05	> 0.05	> 0.05
19.	lP	13.03 ± 0.21	12.31 ± 0.26	13.23 ± 0.22	> 0.05	> 0.05	0.01
20.	lV	7.79 ± 0.18	7.85 ± 0.21	7.69 ± 0.17	> 0.05	> 0.05	> 0.05
21.	PV	38.15 ± 0.33	37.90 ± 0.54	35.93 ± 0.29	> 0.05	< 0.001	< 0.01
22.	VA	15.44 ± 0.25	15.21 ± 0.34	15.36 ± 0.18	> 0.05	> 0.05	> 0.05

Table 7 Mean values of the morphometric character ratios (in per cent of TL; *P* – significance of the difference between means estimated by the T criteria).

based on the mtDNA data, and in the absence of morphometric data to the contrary, the Persian sturgeon should not be considered a separate species. The existence of morphological differences, but genetic similarity between the Russian sturgeon *baerii*-like and 'pure' *A. baerii*, points to the necessity of a detailed morphological and genetic comparison of the Russian sturgeon *gueldenstaedtii*-like form and *A. g. persicus*. The presence of these forms must also be considered

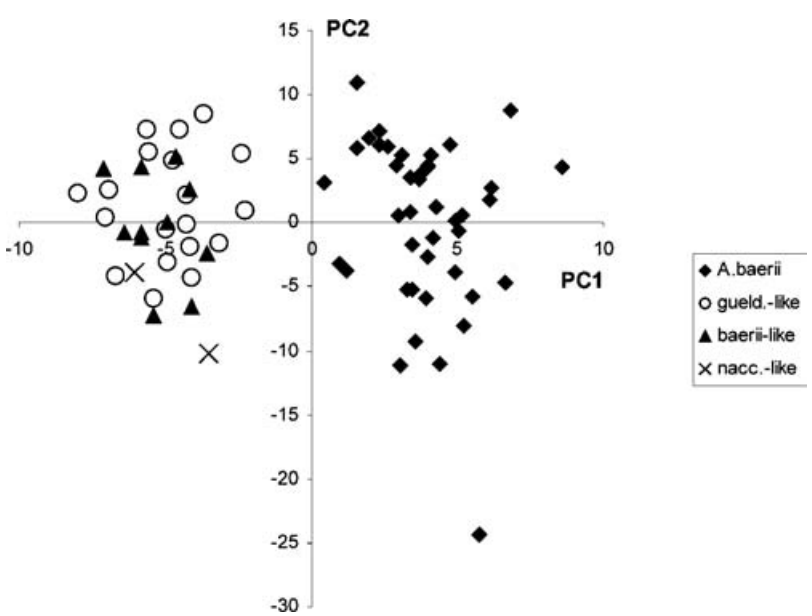

Figure 6 Sturgeon ordination on multivariate morphometric axes from two PCA. PC2 against PC1 extracted from variance-covariance matrix of log transformed measurements. All Russian sturgeon (gueld.-like, the *gueldenstaedtii*-like; baerii-like, the *baerii*-like; and nacc.-like, the *naccarii*-like) and *A. baerii* samples.

Characters	*A. gueldenstaedtii*-like specimens	n	*A. baerii*-like specimens	n	*P*
D	37.42 ± 0.91	19	40.00 ± 1.23	12	> 0.05
A	22.58 ± 0.79	19	23.42 ± 0.67	12	> 0.05
Dr	13.16 ± 0.28	19	12.75 ± 0.37	12	> 0.05
Lr	36.42 ± 1.12	19	36.33 ± 1.31	12	> 0.05
Vr	9.58 ± 0.26	19	9.83 ± 0.25	12	> 0.05
Sp.br.	23.58 ± 0.60	19	23.25 ± 0.76	12	> 0.05

Table 8 Mean values for meristic characters.

in the context of current conservation measures (e.g., stock assessments, CITES conservation programmes, etc.) in the region.

Forensic inference

The present study confirms the conclusion based on previous publications (Birstein *et al*., 2000; Jenneckens *et al*., 2000): up to 30% of black caviar samples coming from the Caspian Sea Russian sturgeon could be mistakenly identified as caviar of Siberian sturgeon. The present study further indicates that this caviar could also be identified as that of the Adriatic sturgeon. In fact, such eggs will come from the *baerii*-like and *naccarii*-like individuals of the Russian sturgeon caught in the Caspian Sea. While we have developed a DNA diagnostic test that will allow one to diagnose three major units – *A. gueldenstaedtii*, *A. baerii* and *A. naccarii* (DeSalle & Birstein, 1996; Birstein *et al*., 1998; Birstein & Doukakis, 2001) – the test and current analytical approaches (Ludwig *et al*., 2002) will not unambiguously discern between 'pure' *A. baerii* and the Russian sturgeon *baerii*-like form or between 'pure' *A. naccarii* and the Russian sturgeon *naccarii*-like form. Only the original morphology combined with DNA sequence data will allow one to discriminate between these forms. Genetic testing of caviar that has been labelled Russian sturgeon ('osetra') caviar will therefore be problematic. However, if caviar labelled 'osetra' comes from the Caspian Sea

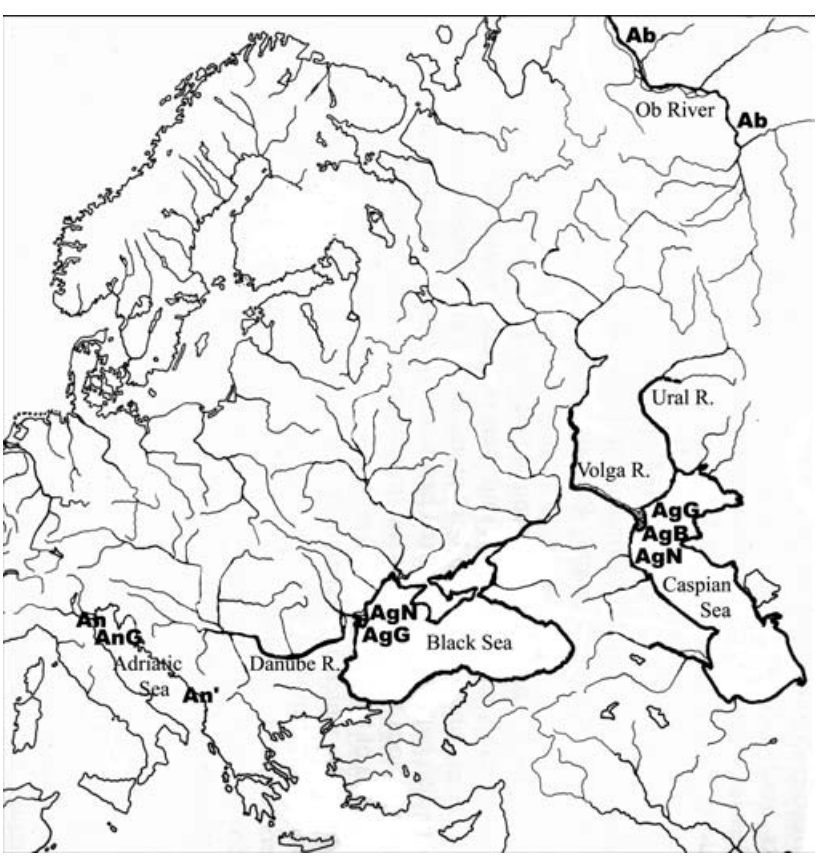

Figure 7 The geographic distribution of three forms of *A. gueldenstaedtii* (**AgG**, *gueldenstaedtii*-like; **AgB**, *baerii*-like; **AgN**, *naccarii*-like), the *A. baerii* population in the Ob River (**Ab**), the *A. naccarii* population in the Po River (**An**, 'pure' *A. naccarii*; **AnG**, *gueldenstaedtii*-like form), and the *A. naccarii* population in the Buna River (**An'**). The current range of the traditionally recognized *A. gueldenstaedtii* in the Caspian and Black seas and main rivers entering into them is shown in heavy black line.

basin but contains eggs with the *baerii*-like or *naccarii*-like forms of mtDNA, it is possible that it is still caviar made of the eggs of morphological Russian sturgeon from the Caspian Sea. There is no caviar production from 'pure' *A. baerii* and especially from 'pure' *A. naccarii* in Russia at present.

Acknowledgements

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