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Intraspecific structure within three caviar-producing sturgeons (*Acipenser gueldenstaedtii*, *A. stellatus*, and *Huso huso*) based on mitochondrial DNA analysis

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Summary

A survey of three mitochondrial DNA regions (control region, NADH5, cytochrome *b*) and comprehensive sequencing of the control region (631–646 bps) was conducted to examine whether subspecies and geographic populations within three species of Eurasian sturgeons, *Acipenser gueldenstaedtii*, *A. stellatus*, and *Huso huso*, are genetically distinct. Neither subspecies nor populations exhibited diagnostic distinction or reciprocal monophyly in any gene region examined. For the control region, molecular variance analyses (AMOVA) indicate that most of the variance is because of differences among haplotypes within subspecies (*H. huso*: 99.6%; *A. stellatus*: 95.0%; *A. gueldenstaedtii*: 81.0%) and populations (*A. gueldenstaedtii*: 76.1%). Significant pairwise *F*-values were found for all pairwise comparisons except for Sea of Azov and Caspian Sea *A. gueldenstaedtii* and Caspian Sea and Black Sea *A. stellatus* and *H. huso*. Only weak genetic differentiation is apparent between select subspecies and populations, reflective of biogeographic and management history. High genetic diversity within *A. gueldenstaedtii* suggests the possibility of additional population structure. Future research and management projects should consider these results.

Introduction

Sturgeons (Order Acipenseriformes) have been the focus of much conservation attention because of their commercial importance and imperilled status (Birstein, 1993). Most of the internationally traded black caviar originates from three sturgeons inhabiting the Caspian and Black seas: Russian (*Acipenser gueldenstaedtii*), stellate (*A. stellatus*), and beluga (*Huso huso*), producers of the highly prized osetra, sevruga, and beluga caviar, respectively. Over the past 40 years, populations of these species have sharply declined, pushing some to the brink of extinction (Khodorevskaya et al., 1997, 2000, 2002; Khodorevskaya and Krasikov, 1999).

A major obstacle in any species conservation program is recognizing and managing the distinct units that exist within a species. Proxies such as allopatric populations and subspecies are useful first hypotheses for identifying these units and genetic data can be used to confirm distinction (Avise, 1994; Moritz, 1994; Vogler and DeSalle, 1994). Within the three main caviar-producing sturgeons, subspecies designations correspond to separate basins within *H. huso* (*H. h. maeoticus*: Sea of Azov; *H. h. ponticus*: Black Sea; *H. h. caspicus*: Caspian Sea) and *A. stellatus* (*A. s. donensis*: Sea of Azov; *A. s. ponticus*: Black Sea; *A. s. stellatus*: Caspian Sea) (Pirogovskii et al., 1989; Shubina et al., 1989). Within *A. gueldenstaedtii*, subspecies correspond to the Black Sea and Sea of Azov combined (*A. g. colchicus*) and the Caspian Sea (*A. g. gueldenstaedtii*) (Vlasenko et al., 1989). Differential protection is currently given to subspecies of *H. huso* (*H. h. maeoticus*: critically endangered; *H. h. ponticus*, *H. h. caspicus*: endangered) and *A. stellatus* (*A. s. stellatus*: vulnerable; *A. s. donensis*, *A. s. ponticus*: endangered) (IUCN, 1996). Molecular studies to date have examined only limited samples of *A. stellatus*, finding no diagnostic differences or phylogeographic structure among subspecies (Doukakis et al., 1999).

Using data from Doukakis et al. (1999) and Birstein et al. (2000), as well as additional samples from *A. gueldenstaedtii* and *H. huso*, we test whether subspecies and allopatric populations represent distinct conservation management units. We use criteria of diagnosis, reciprocal monophyly, and significant intraspecific structure as evidence of distinction.

Methods

Samples of *H. huso* (40), *A. stellatus* (84), and *A. gueldenstaedtii* (33) were examined across the range of each species (Table 1). For *A. gueldenstaedtii*, samples with *A. baerii*-like haplotypes were excluded because the species status of these individuals is questionable (see Birstein et al., 2000).

Standard phenol chloroform protocols were used to extract DNA (see DeSalle et al., 1993). The cytochrome *b* (cyt *b*) and *NADH5* (ND5) gene regions were screened for variability through sequencing using the primers and conditions in Doukakis et al. (1999). Control region amplification used the primers in Doukakis et al. (1999) to amplify the entire region using polymerase chain reaction (PCR). PCR conditions were 94° 1 min, 46° 1 min, 72° 1 min 50 s for 33 cycles in a Perkin-Elmer 480 thermocycler (PE Biosystems, Foster City, CA). PCR products were purified using the BIO 101 Gene Clean system (BIO 101, Inc., La Jolla, CA) before sequencing. Sequencing protocols followed manufacturers specifications for cycle sequencing and ABI 373/377 operation (PE Biosystems). Sequence editing and contig assembly was performed using Sequencer 3.0 (Gene Codes Corporation, Ann Arbor, MI).

A phylogeographic approach (Avise, 1994) compared haplotype 'relationships' to the geographic origin of haplotypes for each gene region. Tree building algorithms implemented in PAUP 4.0b10 (Swofford, 2003) used heuristic parsimony searches with 100 random addition replicates under equal character weighting and Neighbour Joining with a Kimura

Table 1
Mitochondrial DNA control region variation. Percent of polymorphic sites (%P), haplotype diversity (H), and nucleotide diversity (π) are indicated. Information for individual populations and subspecies is listed first followed by information for species overall

Latin name	Location	Genbank no.	n	%P	H (×100) ± SD	π (×100) ± SD
A. g. colchicus	Sea of Azov	AF238751-54	4	7.20	100 ± 17.7	3.91 ± 2.62
A. g. colchicus	Black Sea	AF238739-50, AY847788-89	14	8.37	73.3 ± 12.4	2.65 ± 1.40
A. g. gueldenstaedtii	Caspian Sea	AF238719-20, 23-25, 27-30, 32-33, 35-38	15	12.7	98.9 ± 3.14	3.97 ± 1.79
A. s. donensis	Sea of Azov	AF168546-84, AY847790	40	9.45	98.7 ± 0.99	1.55 ± 0.817
A. s. ponticus	Black Sea	AF168513-45	33	13.1	99.3 ± 0.99	2.03 ± 1.04
A. s. stellatus	Caspian Sea	AF168503-12, AY847791	11	6.47	100 ± 4.47	1.79 ± 0.993
H. h. maeoticus	Sea of Azov	AY846666-67	2	2.66	100 ± 0.50	2.66 ± 2.74
H. h. ponticus	Black Sea	AY846668-79	12	6.55	98.5 ± 4.03	2.03 ± 1.11
H. h. caspicus	Caspian Sea	AY846640-65	26	10.8	99.1 ± 1.33	2.32 ± 1.20
A. gueldenstaedtii	All combined		33	18.1	94.5 ± 3.32	3.69 ± 1.85
A. stellatus	All combined		84	17.6	99.6 ± 0.29	1.83 ± 0.926
H. huso	All combined		40	13.3	99.0 ± 0.082	2.24 ± 1.14

2-paramter model (Kimura, 1980). Population aggregation analysis (PAA) was used to search for fixed diagnostic characters (nucleotide states shared by all individuals) among subspecies within each species (Davis and Nixon, 1992).

For the control region only, haplotype (H) and nucleotide diversity (π) (Nei, 1987) were calculated for each subspecies and species using Arelquin version 2.0 (Schneider et al., 2000). An analysis of molecular variance (AMOVA) (Excoffier et al., 1992) framework was applied to examine genetic variation partitioning using the program Arelquin version 2.0 (Schneider et al., 2000). This program was also used to calculate pairwise *F*-values using a Kimura 2-paramter model (Kimura, 1980) with a gamma distribution of 0.5. Significance tests of *F*-values were calculated using 10 000 permutations. The Sea of Azov *H. h. maeoticus* subspecies was not considered in *F* calculations because of the small number of individuals sampled.

Table 2
F-values for pair-wise population and subspecies comparisons within three Eurasian sturgeons

	Sea of Azov–Caspian Sea	Sea of Azov–Black Sea	Black Sea–Caspian Sea	Sea of Azov and Black Sea–Caspian Sea
A. gueldenstaedtii	0.0179	0.270*	0.283**	0.190**
A. stellatus	0.0750*	0.0547**	0.00809	–
H. huso	–	–	0.0278	–

Significance at the *0.05; **0.01.
Note: Comparison between the Sea of Azov and Black Sea individuals combined and the Caspian Sea individuals was limited to *A. gueldenstaedtii* for purposes of testing subspecies designations [i.e. *A. g. colchicus* (Sea of Azov plus Black Sea) and *A. g. gueldenstaedtii* (Caspian Sea)]. Within *H. huso*, small sample sizes for the Sea of Azov population prohibited comparisons using this population.

Results

A total of 2.1 kb (850 bps cyt *b*, 643 bps NADH5, 631–646 bps control region) was screened for variability using at least 10 individuals per species. The control region was the most variable region (average 10× more nucleotide diversity as compared with cyt *b* and *NADH5*) and was therefore the only region sequenced for all individuals (cyt *b* Genbank nos AY846680-712, AF238661-96; NADH5 Genbank nos AY846713-744, AF238812-47). To avoid the heteroplasmic region, a fragment corresponding to positions 287–922 of the *A. transmontanus* X54348 Genbank sequence (Buroker et al., 1990) was used for the final analysis (Table 1) as length variation and potential heteroplasmy was observed in visualizing control region PCR products (Brown et al., 1996; Ludwig et al., 2000).

Genetic distance between control regions haplotypes varied among species (*H. huso*: range 0.156–4.41%, mean 1.89 ± 0.812; *A. stellatus* range 0.158–4.98%, mean 1.99 ± 0.953; *A. gueldenstaedtii* range 0.156–6.26%, mean 3.66 ± 1.21). Haplotype diversity was generally high within each species and subspecies (Table 1). Nucleotide diversity was highest for *A. gueldenstaedtii* as compared with *H. huso* and *A. stellatus* (Table 1).

No diagnostic differences unique to subspecies or populations were found in any gene region. Tree building and phylogeographic analysis did not recover any monophyletic groups corresponding to subspecies or populations. The AMOVA analysis of control region sequences indicated that most of the variance is because of differences among haplotypes within subspecies (*H. huso*: 99.6%; *A. stellatus*: 95.0%; *A. gueldenstaedtii*: 81.0%) and populations (*A. gueldenstaedtii* only: 76.1%). Pairwise *F*-values were low for *H. huso* and *A. stellatus* (<0.1) and higher for most comparisons within *A. gueldenstaedtii* (Table 2). Statistically significant pairwise *F*-values were found between *A. gueldenstaedtii* subspecies, most pair wise comparisons within *A. gueldenstaedtii*, and *A. s. donensis* and *A. s. ponticus*, and *A. s. donensis* and *A. s. stellatus* (Table 2).

Discussion

Subspecies within the three species under consideration were originally recognized based upon morphometric, meristic, and/or ecological differences, with few authors now considering them valid taxonomic designations (Berg, 1948; Chugunov and Chugunova, 1964; Pirogovskii et al., 1989; Shubina et al., 1989; Vlasenko et al., 1989; Tsvetnenko, 1993; Birstein and Bemis, 1997). As in Doukakis et al. (1999), the present analysis does not support subspecies distinction based upon diagnostic or reciprocal monophyly criteria. Only weak genetic differentiation between these subspecies was detected here for select comparisons. Overall, *A. gueldenstaedtii* appears to harbour the greatest genetic structure (Table 2).

Two mechanisms could be responsible for creating the patterns of differentiation observed. Genetic exchange could be mediated through present water connections or larger scale connections occurring 20 000–13 000 ybp (Osinov, 1984; Zubakov, 1988). Black Sea and Caspian Sea populations of

brown trout (*Salmo trutta*) similarly exhibit only limited genetic structure based upon haplotype frequencies (Bernatchez and Osinov, 1995; Osinov and Bernatchez, 1996). Other sturgeon populations and subspecies isolated for a similar time period also do not display fixed differences (Brown et al., 1993; Guenette et al., 1993).

Gene flow may also have been mediated by hatchery restocking, practiced since the 1960s, with millions of individuals released annually into Caspian, Black and Azov river systems (Secor et al., 2000; Chebanov et al., 2002). In the past, more than 40% of the sturgeons released from Sea of Azov hatcheries were of Caspian Sea origin (Tsvetnenko, 1993; Chebanov et al., 2002). The low genetic distinction between Sea of Azov and Caspian Sea *A. gueldenstaedtii* as compared with other comparisons within this species may be a result of this practice. This practice has also reportedly affected the genetic integrity of *A. stellatus* in the Sea of Azov (Chebanov et al., 2002), although significant *F*-values were found here when comparing these populations.

Acipenser gueldenstaedtii displays high genetic diversity, a characteristic also observed in a study of Southern Caspian Sea individuals of the species (Pourkazemi et al., 1999; Table 1). The comparatively greater variation in this species could be because of differing rates of molecular evolution, management history, degree of historic connectivity, or the presence of additional population structure. An analysis of sturgeon fossils suggests that this species historically contained many distinct population segments (e.g. freshwater, dwarf forms; Sokolov and Tspekin, 1996). Recent works have uncovered morphologically indistinguishable yet genetically distinct lineages within *A. gueldenstaedtii* (Birstein et al., 2000). The high diversity found within this species may be indicative of further as of yet undetected population structure. Similarly, the high diversity found in all three species of sturgeon examined here may indicate further population structure within the sea basins. As these species are anadromous, individuals migrate into rivers for reproduction, but little is known about the degree of homing fidelity to natal river systems. Distinction of these river populations and their mixing within the sea basins could explain the high genetic diversity observed.

Further study on management units of these endangered and economically important species is needed and should utilize nuclear markers and larger samples sizes. Future conservation and management projects (e.g. hatchery supplementation, quota allocation) should consider the genetic differences detected here, especially within *A. gueldenstaedtii*, and the additional population structure that may exist. The effect of hatchery stocking should also be explored, possibly using historic specimens or coalescence analysis.

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