Population Aggregation Analysis of Three Caviar-Producing Species of Sturgeons and Implications for the Species Identification of Black Caviar

VADIM J. BIRSTEIN, PHAEDRA DOUKAKIS,* BORIS SORKIN, AND ROB DeSALLE

Molecular Laboratories, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024, U.S.A.

Abstract: *We describe a reliable method for the identification of the species source for caviar. The assay is based on the identification of diagnostic nucleotide positions in the mitochondrial cytochrome b gene from a sequence database of 20 of 25 living sturgeon species. The collection of attribute data is polymerase chain reaction (PCR)-based and can readily identify the character states of several diagnostic nucleotide positions in the cytochrome b gene for the three main commercial species of caviar-producing sturgeons (Acipenser gueldenstaedtii, A. stellatus, Huso huso). Over 20 individuals from each of the three species from a wide range of geographic regions were used to determine which of the potentially informative sites are diagnostic of phylogenetic lineages through use of the population aggregation analysis method. We also report results from a survey of 95 lots of commercially available caviar in the New York City area. Overall, 23% of the designations made by caviar suppliers were mislabeled with respect to species identification. Recent declines in population size of the three commercial species, combined with an increase in the demand for caviar on the international market, could have caused the observed mislabeling. Replacement of commercial species with endangered and threatened species indicates possible illegal harvest and poaching; action should be initiated to ensure the survival of all threatened Acipenseriformes. The use of our PCR assay might aid in conservation efforts in the United States and internationally.*

Análisis de Agregación de Poblaciones de Tres Especies de Esturiones Productoras de Caviar y sus Implicaciones en la Identificación de Especies de Caviar Negro

Resumen: *Describimos un método confiable para la identificación de las especies fuente de caviar. La prueba se basa en la identificación de posiciones diagnóstico de nucleótidos en el gen citocromo b de una base de datos de secuencias de 20 de las 25 especies de esturiones vivientes. La colección de datos se basa en PCR y puede identificar fácilmente los estados de diversas posiciones diagnóstico de nucleótidos del gen citocromo b de las tres especies comerciales de esturiones mas importantes productoras de caviar (Acipenser gueldenstaedtii, A. stellatus y Huso huso). Cerca de 20 individuos de cada una de las tres especies provenientes de un amplio rango de regiones geográficas fueron usados para determinar cuales de los sitios potenciales de información sirven como diagnóstico de linajes filogenéticos, usando el método de análisis de agregación poblacional. Tambien reportamos resultados de un estudio de 95 lotes de cavier comercialmente disponible en el área de la ciudad de Nueva York. Alrededor de un 23% de las designaciones realizadas por los proveedores de caviar estuvieron mal etiquetadas con respecto a la identificación de especies. Recientes disminuciones en los tamaños poblacionales de las tres especies comerciales combinadas con un incremento en la demanda de caviar en el mercado internacional han causado la mala etiquetación observada. La renovación de especies comerciales por especies en peligro o amenazadas es un indicativo de la posible cosecha y sacrificio ilegal, se*

766

deberán iniciar acciones para asegurar la supervivencia de todos los Ascipensiformes amenazados. El uso de nuestro análisis de PCR podría contribuir con los esfuerzos de conservación en los Estados Unidos e internacionalmente.

Introduction

Accurate species identification is the first step in any conservation program (Avise 1989; 1994). In cases where endangered or threatened species are commercially sought, the problem is even more acute (Cherfas 1989; Baker & Palumbi 1994; 1996; Baker et al. 1996). Currently, two phylogenetically based methods are used in conjunction with DNA sequence analysis to determine species identification of commercial materials. The first involves the sequencing of phylogenetically informative stretches of DNA followed by phylogenetic tree building to identify the sister taxon of unknown individuals. This method has been used by Baker and Palumbi (1994, 1996) and Baker et al. (1996) to identify the species of origin of whale tissues sold in Asian markets. A similar method was used to determine the origin of green sea turtles (Encalada 1994). The second method involves the use of diagnostic nucleotide positions in DNA sequences as indicators of species origin (Cherfas 1989; Bartlett & Davidson 1992; Amato & Gatesy 1994; Unseld et al. 1995; DeSalle & Birstein 1996). Both methods require the development of a data base for implementation. In the tree building approach, the data base is needed to provide a representation of all possible taxa that the unknown may be related or identical to. In the diagnostic nucleotide position method, this database also needs to include a number of individuals from different geographic locations to determine if potential sites are entirely diagnostic (population aggregation analysis [PAA] method of Davis and Nixon [1992]). We elaborate upon the diagnostic method that we have previously reported (DeSalle & Birstein 1996) for commercial caviar-producing sturgeon. In addition, we present a more detailed and larger analysis of commercially available caviar from the New York City area.

Sturgeons (Acipenseriformes) are the primary producers of the black caviar sold on the world market. Overfishing and habitat degradation threaten the survival of many species (Birstein 1993; 1996*a*; Bemis & Findeis 1994; Waldman 1995; Birstein et al. 1997*a*), and almost all of the 27 members of this group are endangered (Birstein 1993; World Conservation Union 1996; Birstein et al. 1997*a*). The three commercial Russian species inhabiting the Volga River–Caspian Sea basin—the beluga sturgeon (*Huso huso*, the source of beluga caviar), Russian sturgeon (*Acipenser gueldenstaedtii*, the source of osetra caviar), and sevruga (*A. stellatus*, the source of

sevruga caviar)—are especially vulnerable because of the great commercial value of their roe. Recent reports suggest that the population sizes of these three species are at historically low levels. Because the commercial demand for caviar has not diminished, commercial use is a factor in the continuing decline of sturgeon species and increases the potential for illegal harvest and poaching. Thus, an accurate method for species identification of caviar is needed to aid in the enforcement of current fisheries regulations and to ensure the conservation of this ancient group of fishes.

Methods

Tissue Samples and Caviar Lots

Species and tissue samples used for the cytochrome *b* (cyt *b*) sequence database and population survey of the three commercial species (Birstein & DeSalle 1997) and for extraction of control DNA are given in Table 1. The majority of caviar samples in tin cans or glass jars were purchased from 15 gourmet shops in New York City or were ordered by mail from three companies in New Jersey, Massachusetts, and Florida. We bought samples in December 1995 (lots 1–17), April 1996 (20–25, 26–61), and December 1996 (62–95). We bought samples twice from the same location in some instances. Two glass jars (lots 18 & 19) were brought from Russia; one had been refrigerated for 2 years. All American cans or jars were labeled by the retailer as beluga (*Huso huso*), sevruga (*Acipenser stellatus*), or osetra (*A. gueldenstaedtii*), and four cans (lots 1, 30, 55, & 81) were labeled as "American caviar." Lot 4 was labeled "Eastern beluga," and lot 71 was labeled "river beluga." Labels on Russian jars did not identify the species. We also received a sample of an unknown type of caviar (lot 96) from R. Billard (Muséum National d'Histoire Naturelle, Paris). This sample was taken from an illegal shipment of Bulgarian caviar seized by French customs.

Primer Design

We used a three-step process to develop primers for species identification (Fig. 1). The first step is to obtain a large and preferably exhaustive data base for a group of species to assess the location of potential diagnostic nucleotide positions. Primers are then designed that will

Table 1. Sample type of acipenseriform species used as controls.

Species	Geographic area of sampled specimens	Name of collector
Family Acipenseridae		
Subfamiy Acipenserinae		
Genus *Acipenser*		
A. baerii[a]	Lena River (Siberia, Russia)	V. Birstein (USA)
A. brevirostrum[b]	Connecticut River (Massachusetts, USA)	B. Kynard (USA)
A. dabryanus[c]	Yangtze River (China)	Q. Wei (China)
A. fulvescens[d]	Great Lakes (Wisconsin, USA)	F. Binkowski (USA)
A. gueldenstaedtii[a,b,c]	N. & S. Caspian Sea, Black Sea, Sea of Azov	Y. Altufiev (Russia), V. Birstein (USA), T. Gulyas (Hungary), M. Pourkazemi (Iran), R. Suciu (Rumania), A. Vlasenko (Russia)
A. medirostris[a]	Columbia River (Oregon, USA)	J. North (USA)
A. mikadoi[c]	Tumnin River (Russian Far East)	E. Artyukhin (Russia)
A. naccarii	Po River (Italy)	P. Bronzi (Italy)
A. nudiventris[a]	Aral Sea (Uzbekistan, Central Asia)	V. Birstein (USA)
A. oxyrinchus desotoi[c]	Pearl River (Louisiana, USA)	J. Waldman (USA)
A. oxyrinchus oxyrinchus[c]	St. Lawrence (Quebec, Canada)	J. Waldman (USA)
A. persicus[c]	Caspian Sea, Southern part (Iran)	M. Pourkazemi (Iran)
A. ruthenus[a]	Volga River (Russia)	V. Birstein (USA)
A. schrenckii[c]	Amur River (Siberia, Russia)	V. Svirskii (Russia)
A. sinensis[c]	Yangtze River (China)	Q. Wei (China)
A. stellatus[a,b,c]	N. & S. Caspian Sea, Black Sea, Sea of Azov	Y. Altufiev (Russia), V. Birstein (USA), T. Gulyas (Hungary), M. Pourkazemi (Iran), R. Suciu (Rumania), A. Vlasenko (Russia)
A. sturio[c]		
(a) A specimen from the Gironde River population	Gironde River (France)	P. Williot (France)
(b) A specimen from the North Sea	North Sea, near the Dutch coast[d]	L. Debus (Germany)
A. transmontanus[a]	Columbia River (Oregon, USA)	J. North (USA)
Genus *Huso*		
Huso dauricus[c]	Amur River (Siberia, Russia)	V. Svirskii (USA)
H. huso[a,b,c]	N. & S. Caspian Sea, Black Sea, Sea of Azov	Y. Altufiev (Russia), V. Birstein (USA), T. Gulyas (Hungary), M. Pourkazemi (Iran), R. Suciu (Rumania), A. Vlasenko (Russia)
Subfamily Scaphirhynchinae		
S. albus[a]	Yellowstone River (Montana, USA)	H. Bollig (USA)
Family Polyodontidae		
Polyodon spathula[a]	Moscow Aquarium	V. Birstein (USA)
Psephurus gladius[c]	Yangtze River (China)	Q. Wei (China)

[a]*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.*
[b]*Egg samples were from freshly obtained eggs fixed at 96% ethanol.*
[c]*Tissue samples were from small pieces of muscle fixed in 96% ethanol.*
[d]*This fish was caught in 1993 (Timmermanns & Melchers 1994).*

recognize the diagnostic nucleotide position so that a positive polymerase chain reaction (PCR) reaction (the presence of a band of correct length after PCR) will indicate the presence of the diagnostic base in the target template. The initial utility of the primer is assessed using a set of template DNA samples made from vouchered tissues of the group of species. If the primer passes this test, it is then examined for polymorphism among large sample sizes from populations of species from a wide geographic range. The diagnostic position must be present in all members of the species it is designed for and cannot amplify target DNA from any other species. If the primer passes this test, it is kept as a diagnostic.

The design of the species-specific primers used in this study involved the four major steps outlined above. First, several regions of three mitochondrial DNA genes were sequenced for all living species of Acipenserinae (20 of 27 extant acipenseriforms; Birstein et al. 1997*b*; Birstein & DeSalle 1998). Short regions of the 16S (350 bp) and 12S (250 bp) rRNA genes, and three regions of the cyt *b* gene (for a total 650 bp) were sequenced for the taxa shown in Table 2. All of these sequences have been deposited in GENBANK (Table 2). The data provided us with several nucleotide positions in the cyt *b* gene unique to specific species and potential candidates for species diagnosis. We then designed several primers to be specific for unique nucleotide changes for each of
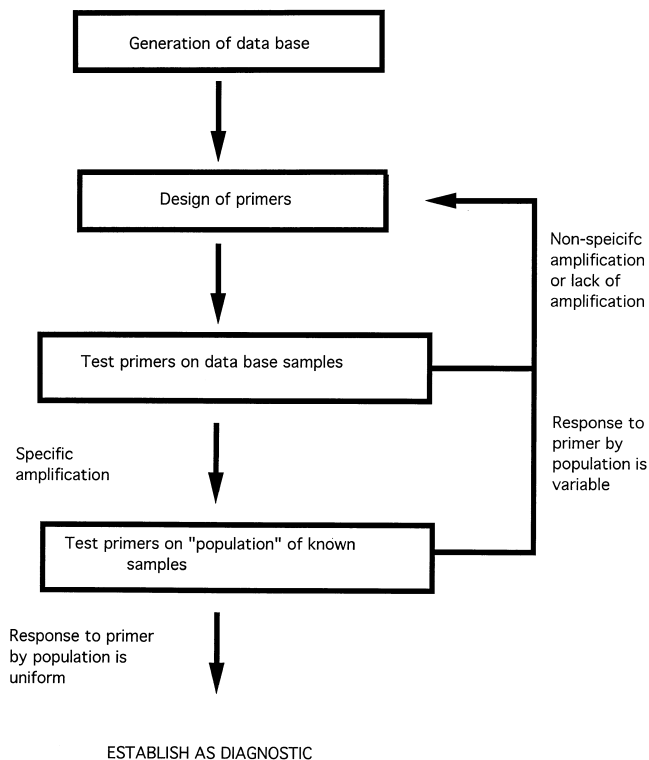
Figure 1. The approach to and criteria for developing primers for species identification (PCRASS method of Amato and Gates [1994]). "Specific amplification" of the proper samples establishes a primer pair as a diagnostic. Nonspecific amplification, lack of amplification, or nonuniform response of the primers to the population of samples results in rejection of that primer pair as diagnostic and requires a redesign of the primer system.

the Caspian Sea commercial species (*Huso huso*, *Acipenser gueldenstaedtii*, and *A. stellatus*). These primers were designed to have similar AT:GC ratios and to be of similar length. Anchor primers (Table 2) universal for all sturgeon species were chosen to be no more than 200 base pairs away from the species-specific primers so that even highly degraded DNA could be used as PCR template (Table 2). The primers were designed so that a high-stringency PCR reaction with primers for a given species gives a positive reaction (i.e., a band of the correct length on an agarose gel) and a primer pair that is not specific for a particular species does not amplify (a lack of the PCR band of correct length). This is essentially the PCRASS (Amato & Gatesy 1994) (Fig. 1) or PASA (Sommer et al. 1992) method. To ensure that the lack of a positive PCR reaction was not due to contamination or inhibition, we also designed several universal primer pairs approximately 150 bases apart that amplify all species of Acipenserinae and served as PCR controls.

The second step verified that species-specific primers were functional in reactions with that species only.

Template DNA from each of the 18 species of Acipenserinae (Table 1) were used in both control and species-specific reactions. When a particular diagnostic primer showed amplification for only the species for which it was designed, we then proceeded to the next step.

The third step involved testing whether diagnostic primers amplified all members of the species but no members of the other species. To accomplish this we obtained samples from a wide geographic range for the three commercial caviar-producing species (Table 1). Template DNA from these specimens was PCR-amplified with all diagnostic primer pairs. Any primer pair that amplified a band of correct size for all samples of that species and did not amplify a single individual from either of the other two commercial species or any of the other species in Table 1 was then kept as a diagnostic.

DNA Isolation and Manipulation

DNA from single caviar eggs was prepared from unwashed eggs, from eggs washed with 10% chlorox or homogenization buffer, or from adult tissue. DNA was isolated from a single egg by crushing the egg in 100 μl of homogenization buffer followed by Proteinase K (1 mg/ml final concentration) digestion for 1 hour at 60° C. Standard phenol-chloroform extraction (DeSalle et al. 1993) followed by two ethanol precipitations was performed to purify the DNA preparation for PCR. PCR template was also prepared by the chelex method (Walsh et al. 1991) and by crushing the egg in 1 × PCR buffer followed by centrifuging at 10,000 rpm in a microcentrifuge to remove debris. DNA preparation blanks were also run in parallel to assay for contamination. Performance of the various washes and template preparations was assessed by the presence of a PCR product. PCR was performed using the various primer pairs at 10 μM in 25-μl reactions. The PCR conditions were 94° C for 1 minute, 55° C (53° C for osetra primers) for 1 minute, and 72° C for 1 minute for 25 cycles for all primer pairs. Control PCR reactions using universal Acipenserinae primers were run first on all samples to ensure the quality of the DNA extraction. PCR products were assayed on 1.5% agarose gels in 1 × TBE (Tris Borate EDTA) run at 140 V for 20 to 30 minutes in a BioRad miniature submersible electrophoresis rig. DNA was visualized under UV light after the gel was stained in Ethidium Bromide. For all figures in this paper the gels were photographed with Polaroid Type 57 film and scanned into Adobe Photoshop. The presence of a PCR product of correct length was taken as evidence that the base position targeted by the primer existed in the target template.

Sequencing PCRASS Ambiguous Samples

When a sample was diagnosed as osetra (with the G3 and G4 primer pairs) or when none of the primer pairs

Table 2.   Primer pairs used in the diagnosis of beluga, sevruga, and osetra caviar.[a]

Primer pair	Position in the cytochrome b gene[b]	Specific for the following species	Primer sequence
B2	26	*Huso huso* (beluga)	gcaaacggggcctcctc
B2a	402	B2 anchor	cagaatgatatttggcctca[c]
B3	177	*Huso huso* (beluga)	cactacacagctgacatc
S1	465	*Acipenser stellatus* (sevruga)	ctttctgccttcccgtat
S1an	536	S1 anchor	gaagaaagtggaaggcg
S2	884	*Acipenser stellatus* (sevruga)	ggagtcctagccctcctg
S2an	998	S2 anchor, control primer	cctccaattcatgtgagtact
G3	871	"*A. gueldenstaedtii*" (Russian sturgeon or osetra)	aataaactaggtggagtt
S2an	998	G3 anchor	cctccaattcatgtgagtact
B7[d]	739	control primer	cctaacctcctaggagaccaga

[a] *Primers were designed using information from 650 bases of the mitochondrial cytochrome* b *gene for 24 species of Acipenseriformes (Birstein & DeSalle 1998; GENBANK accession Nos. AF006123–AF006188).*
[b] *Position refers to the published cytochrome* b *sequence of* A. transmontanus *(Brown et al. 1989).*
[c] *Sequence refers to partial H15149 primers listed by Kocher, et al. (1989).*
[d] *The B7 primer was designed by J. Groth, Department of Ornithology, American Museum of Natural History, New York, New York.*

diagnosed the sample, control fragments were generated from universal Acipenserinae primers, and sequences of these fragments were used to perform the diagnosis or verify osetra identification. PCR products for sequencing were GeneCleaned (BIO 101) and sequenced with an ABI Model 373 automated sequencer. Sequences were transferred to Sequencher software and analyzed with this program. Diagnostic positions in our sturgeon data base were then used to determine the species of origin for these ambiguous samples.

Results

Design and Utility of Primer Systems

We designed our primers by visual comparison of sequences in our sturgeon cyt *b* data base. To illustrate this approach, we show an example of a short region of the cyt *b* gene region for all sturgeon species that contains a nucleotide position that is potentially diagnostic for *A. stellatus* (Fig. 2). In this example a 20-bp PCR primer was designed so that the first 3′ base exactly matched the *A. stellatus* sequence. A second primer was designed approximately 150 bases away (not shown in Fig. 2) from the potential *A. stellatus* specific primer. Table 2 shows the six primer systems that were eventually designated as diagnostic for this study. The primers specific for sevruga (S1 and S2) and beluga caviar (B2 and B3) were designed by inspection of the aligned cyt *b* sequences. Diagnostic primers for *A. gueldenstaedtii* (osetra) were difficult to design because of the close relation of this species to other members of the Eurasian sturgeon clade (*A. baerii, A. persicus, A. naccarii, A. nudiventris*, and *A. dabryanus*; Birstein & DeSalle 1998) (Table 2). Hence, we designed two primer systems for osetra caviar that were diagnostic when used in combi-

nation. The G3 primer system is specific for *A. gueldenstaedtii, A. baerii, A. persicus, A. naccarii,, A. nudiventris, A. dabryanus*, and *A. brevirostrum*. The G4 primer system is specific for *A. gueldenstaedtii, Scaphirhynchus albus, Polyodon spathula*, and *Psephurus gladius*. As noted above, all cases of osetra designation were confirmed by DNA sequencing.

As an example of testing for the species specificity of primers, use of the S2 primer system on several species of Acipenserinae shows that only *A. stellatus* DNA amplifies with this primer system (Fig. 3). Because these same DNA preparations were successfully amplified with cyt *b* control primers, DNA preparation inhibition did not cause the observed results. We further tested the S2 primers on over 60 individuals from various geographical locations for the three commercial species. Again, control primers amplified all samples and only DNA of *A. stellatus* amplified with this primer system (Fig. 4).

Diagnosis of PCRASS Ambiguous Samples

The methods we describe here result in unambiguous species assignment to caviar lots. Ambiguity due to impure DNA and subsequent lack of PCR product was eliminated by using universal Acipenseridae control primers to initially test DNA preparation quality. When all diagnostic primer systems failed to amplify the DNA of a lot of caviar, or when only one of the G primer systems gave a positive PCR reaction, we assumed that the species of caviar origin was not one of the three commercial species and that the positive control PCR fragment was sequenced. The sequences were then compared to the sturgeon sequence data base, and the most likely species of origin was identified by means of diagnostic sites for those species.

Ambiguity resulting from the mixing of eggs from different species in the same lot of caviar was controlled
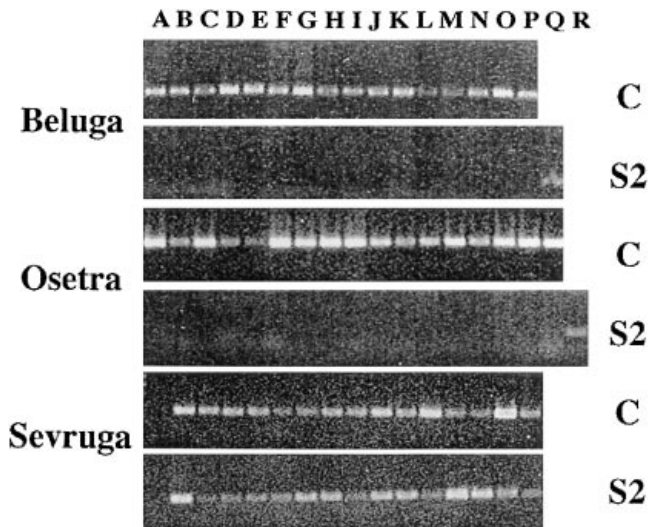
```
A   A. baerii         T C A C T A A C C T C C T C T C C G C C T T T C C G T A T A T C G G C G A C A C A C T A G T A C A A
    A. brevirostrum   . . . . . . . . . . . . . T . . . . . . . . C . . . . . C . . . . . . . . . . . . . T . . . G . . .
    A. dabryanus      . . . . C . . . . . . . . T . . . . . . . . C . . . . . C . . . . . . . . . . . . . . . . . . . . .
    A. fulvescens     . . . . . . . . . . . . . . . . . . . . . . C . . . . . C . . . . . . . . . . . . . T . . .
    A. transmontanus  . . . . . . . . . . . . . T . . . . . . . . C . . C . . C . . . . . . . . . . . . . T . . .
    A. gueldenstaedtii . . . . A . . . . . . . . T . . . . . . . . C . . . . . C . . C . . . . . . . . . . . . . . . . . .
    A. mikadoi        . . . . . . . . . . . . . . . . . . . . . . C . . . . . C . . . . . . . . . . . . . T . . .
    A. naccarii       . . . . C . . . . . . . . . . . . . . . . . C . . . . . C . . . . . . . . . . . . . . . .
    A. nudiventris    . . . . C . . . . . . . T . A . . . . . . . C . . . . . C . . . . . . . . . . . . . . . .
    A. oxyrinchus     . T . . . . . . . . . . . . . . . . . . . . C . . A . . C . . . . . . . . . . G . . . . G T . . G
    A. persicus       . . . . C . . . . . . . . . . . . . . . . . C . . . . . C . . . . . . . . . . . . . . . .
    A. ruthenus       . T . . . . . . . . . . . . . . . . . . . . C . . . . . C . . . . . . . . . . . . . . . .
    A. shrenckii      . . . . . . . . . . . . . . . . . . . . . . C . . . . . C . . . . . . . . . . . . . T . . .
    A. sinensis       . . . . . . . . . . . . . . . . . . . . . . C . . . . . C . . . . . . . . . . . . . T . . .
    A. stellatus      . . . . C . . . . . . . . T . (T) . . . . . . C . . . . . C . . . . . . . . . . . . . . . . .
    A. sturio         . T . . . . . . . . . . . . . . . . . . . . C . . A . . C . . . . . . . . . . . . . G . . .
    A. medirostris    . . . . . . . . . . . . . T . . . . . . . . C . . . . . C . . . . . . . . . . . . . T . . .
    H. dauricus       . T . . . . . . . . . . . . . . . . . . . . C . . . . . C . . . . . . . . . . . . . . . .
    H. huso           . T . . . . . . . . . . . . . . . . . . . . C . . . . . C . . . . . . . . . . . . . . . .
    S. albus          . . . . A . . . . . . . . . . . . . . . . . C . . C . . C . . . . . . . . . . . . . . . .
    P. spathula       . T . . C . . . . . . . . T . . . . . . . . C . . C . . C . . . . . . G . . . . . C . . . . .
    P. gladius        . . . . A . . . . . . . . T . . . . . . . . C . . C . . C . . . . . A . . . . . . . . .
```

B   *A.stellatus* specific primer design
```
    5' T C A C T A A C C T C C T C T C T 3'
```

C   *A. gueldenstaedtii* template
```
        A G T G A T T G G A G G A G A G G C G G C G G A A A G G C A T A T A G C C G C T G T G T G A T C A T G T T
     5'T C A C T A A C C T C C T C T C
                                      T
                                      3'
                              NO EXTENSION
    A. stellatus template
        A G T G A T T G G A G G A G A G A C G A C G G A A A G G C A T A T A G C C G C T G T G T G A T C A T G T T
     5'T C A C T A A C C T C C T C T C T ─────────────────────────────────────────────────────────────────▶
                                      EXTENSION
```

Figure 2. An example of the design and use of a species-specific primer. First, we collected a partial sequence of the cytochrome b *gene for 20 species of Acipenseridae and 2 species of Polyodontidae (a). Second, we then constructed a primer by using the illustrated* A. stellatus–*specific primer (b). When this primer was used in diagnostic PCR amplification, extension and amplification only occurred with the* A. stellus *template (c).*

for by making multiple DNA preparations from single eggs from the same lot. Mixtures of eggs from different species were not found in any of the lots we examined.

A Survey of Commercial Caviar Lots from the New York City Area

The primers designed in this study were used to determine the species of origin for commercially obtained lots of caviar in the New York City area. We examined 71 lots of caviar in addition to the 25 lots assayed by De-Salle and Birstein (1996). Our analysis detected 17% of all lots bought in December 1995 (Nos. 1-25) and April 1996 (lots 26–61) and 32% of lots bought in December 1996 were mislabeled (Table 3). On average 23% of the caviar lots we examined in 1995-1996 were mislabeled. In December 1995 we found mislabeled caviar in 2 of 7 stores, and in April 1996 in 4 additional shops out of the 12 sampled. Two shops that previously sold mislabeled goods in 1995 continued selling such products in December 1996, and one company that we discovered selling mislabeled lots in April again mislabeled two of four types of its caviar in 1996. Mislabeled caviar also ap-

Figure 3. Performance of the S2 primer system on 20 species of the family Acipenseridae and one species of the family Polyodontidae. Cytochrome b *positive control PCR fragment for all species and PCR amplification using the* A. stellatus–*specific S2 primer pairs (Panel C and S, respectively). Abbreviations: A, negative control; B, Acipenser baerii; C,* A. brevirostrum; D, A. fulvescens; E, A. gueldenstaedtii; F, A. medirostris; G, A. mikadoi; H, A. naccarii; I, A. nudiventris; J, A. oxyrinchus; K, A. persicus; L, A. ruthenus; M, A. schrenckii; N, A. sinensis; O, A. stellatus; P, A. sturio; Q, A. transmontanus; R, Huso dauricus; S, H. huso; T, Pseudoscaphirhynchus kaufmanni; U, Scaphirhynchus albus; V, Polyodon spathula.*

*Figure 4. Performance of the S2 primer system for samples of beluga (*Huso huso), *Russian sturgeon (*Acipenser gueldenstaedtii), *and sevruga (*A. stellatus). *For each species cytochrome* b *positive control PCR fragment and S2 primer pair PCR amplification are shown (Panel C and S2, respectively). Abbreviations in the Beluga sturgeon panels: A-B, H, Sea of Azov; C-E, Iranian (southern) part of the Caspian Sea; F, Ural River; G, Black Sea; I-P, Russian (northern) part of the Caspian Sea; Q, positive control. In the osetra sturgeon panels: A-F, Russian (northern) part of the Caspian Sea; G-H, Black Sea; I-J, Sea of Azov; K-Q, Iranian (southern) part of the Caspian Sea; R, positive control. In the sevruga sturgeon panels: A, negative control; sevruga from B, N, Ural River; C-E, O, Black Sea; F-G, P, Russian (northern) part of the Caspian Sea; H-I, Sea of Azov; J-M, Iranian (southern) part of the Caspian Sea.*

peared in December 1996 in four shops that had sold authentic caviar in April 1995. Although direct, statistically significant comparisons cannot be made between sampling periods, mislabeling has apparently become more frequent.

Sequencing of PCR products obtained from the eggs of mislabeled samples allowed us to identify the species used for replacement (Table 3). In most of these cases the caviar of commercial sturgeon species was replaced by the caviar of the Persian (*A. persicus*), Adriatic (*A. naccarii*), or Siberian (*A. baerii*) sturgeon.

Discussion

Utility of Primer Systems and the Rationale of PCRASS Diagnosis

The primer system we describe is identical to the PCRASS method reported by Amato and Gatesy (1994)

and the PASA method by Sommer et al. (1992). The theory behind our system application is based on population aggregation analysis (PAA; Davis & Nixon 1992), which differentiates between characters and traits among attributes (such as DNA sequences) in populations, where the former can be used as a diagnostic for phylogenetic distinctiveness and the latter cannot. Criticism of this method is based on sampling problems that could result in the overdiagnosis and underdiagnosis of phylogenetic lineages (Davis & Nixon 1992). Lineage underdiagnosis tends to occur when too few attributes are sampled. We sampled over 600 bases of the cyt *b* gene and over 500 bases of mitochondrial ribosomal sequences (Birstein & DeSalle 1998), so it is unlikely that we have underdiagnosed lineages in this study. Overdiagnosis occurs if any of the three commercial sturgeon species are polymorphic for these variable and presumably diagnostic sites and if the variation is undersampled. Detection of such polymorphism will negate the reliability of these primers as diagnostic tools. This problem is circumvented in our study by the use of individuals from every living sturgeon species (Table 1, Fig. 3) and large numbers of individuals of the three main caviar-producing species sampled (Table 1, Fig. 4).

Conservation Aspects

Populations of all three commercial species of the Caspian Sea, beluga, Russian sturgeon (osetra), and sevruga, are heavily exploited by both legal and illegal fisheries because of the high demand for caviar on the international market (De Meulenaer & Raymakers 1996). These three species produce 80–90% of this caviar (Barannikova et al. 1995). Estimates from Dieckmann & Hansen caviar trading company (S. Taylor, personal communication) indicate that the needs of the international market for black caviar were almost triple the legal production in 1996. U.S. Commerce Department figures indicate that caviar imports have increased 100% since 1991 (De Meulenaer & Raymakers 1996).

Increasing commercial pressure in conjunction with environmental degradation of the Caspian region (Dumont 1995) has caused a decline in the population size of commercial Caspian Sea sturgeon species during the last several years (Khodorevskaya et al. 1997). Beluga sturgeon have been especially affected: in 1995 only 83 beluga females were caught in the northern part of the Caspian Sea basin (E. Artyukhin, personal communication), whereas in 1990 and 1991 more than 2000 females were caught each year (Barannikova et al. 1995). Moreover, beluga sturgeon have not reproduced in the Volga River since the 1950s, when the Volgograd dam construction impeded the spawning migration of this species. Artificial breeding conducted during the Soviet period (Barannikova et al. 1995) has practically stopped because of the economic crisis in Russia and because of

Table 3.   Species designations for 95 commercially available lots of caviar.[a]

Result of identification	Type of caviar			
	Supplier designation			
	Beluga	Sevruga	Osetra	Other
		Number of lots		
Confirmed designations	2, 9, 15, 21, 23, 27, 31, 35, 37, 40, 44, 47, 49, 52, 58, 60, 64, 67, 68, 70, 75, 80, 83, 87, 90, 92, 94	5, 8, 11, 14, 17, 19, 20, 25, 28, 29, 33, 36, 39, 42, 46, 50, 57, 59, 65, 69, 74, 78, 84, 85, 88, 91	7, 10, 13, 16, 18, 22, 24, 32, 34, 38, 45, 48, 56, 61, 63, 73, 82, 89	
Mislabeled	6,[b] 12,[c] 72[d]	54,[e] 62,[f] 76,[g] 95[c]	3,[e] 26,[b] 41,[b] 53,[i] 66,[c] 77,[c] 78,[i] 78,[c] 86,[c] 93[c]	1,[j] 4,[k] 30,[j] 43,[l] 55,[m] 71,[n] 81[l]

[a] *Most lots were unambiguously identified by means of the primer pairs shown in Table 2. Lots that yielded negative results for all primer pairs were identified by sequencing diagnostic regions of the cytochrome* b *positive control PCR fragment. Lots 1–25 were previously reported in De-Salle and Birstein (1997).*
[b] *Sevruga (*A. stellatus*).*
[c] *Persian (*A. persicus*), Adriatic (*A. naccarii*), or Siberian (*A. baerii*) sturgeon.*
[d] *Russian sturgeon (*A. gueldenstaedtii*).*
[e] *Ship sturgeon (*A. nudiventris*).*
[f] *Beluga (*Huso huso*).*
[g] *American paddlefish (*Polyodon spathula*).*
[h] *Amur River sturgeon (*A. schrenckii*).*
[i] *White sturgeon (*A. transmontanus*).*
[j] *American sturgeon diagnosed as Osetra (i.e.,* A. gueldenstaedtii*).*
[k] *Eastern beluga (i.e., kaluga* Huso dauricus*) diagnosed as Amur River sturgeon (*A. schrenchkii*).*
[l] *American sturgeon diagnosed as Beluga (i.e.,* Huso huso*).*
[m] *American sturgeon diagnosed as Paddlefish (*Polyodon spathula*).*
[n] *River beluga (kaluga?) diagnosed as Amur River sturgeon (*A. schrenckii*).*

insufficient numbers of spawners in breeding programs. Because there is no natural reproduction of beluga in any other area it inhabits (Birstein 1996*b*), the survival of beluga in the wild is threatened by contemporary harvesting pressure. During the last few years, poaching in the lower Volga River has also led to the decline in natural reproduction of two other commercial species, sevruga and Russian sturgeon (Khodorevskaya et al. 1997). Only captive and hatchery-stocked populations of these and other Caspian Sea sturgeon may exist in the future (Dumont 1995).

The mislabeling reported in this study can be examined in terms of motivating factors and the potential impact on the conservation of all Acipenserinae. Caviar designated by several of the suppliers as beluga, was often found to be mislabeled (Table 4, lots 6, 12, & 72) and replaced with caviar from sevruga, Siberian, and Russian sturgeons, respectively. This is not surprising because the availability of beluga females in the wild is nearly zero, whereas the prices for beluga caviar are extremely high ($20–65 per ounce for the beluga we purchased). Caviar from two endemic Amur River sturgeon species, the Amur River sturgeon (*A. schrenckii*), and kaluga (*Huso dauricus*), the only close relative of the beluga (its trade name in the United States is "Eastern beluga"), have also reportedly been sold in the United States as cheap alternatives to Caspian Sea beluga (Fabricant 1993). These two species presently are intensively overfished from both the Russian and Chinese sides of the river. The legal catch of sturgeons in the Amur River dropped drastically during the 1990s in both China and Russia. In 1990, 340 and 65 metric tons of sturgeon were caught by these countries, respectively, whereas in 1993 only 170 and 47.3 metric tons of sturgeon were caught, respectively. At the same time, poaching has increased enormously in both countries (Krykhtin & Svirskii 1997). Our data also indicate the alarming use of the Amur River sturgeon, as a replacement for caviar of other sturgeon species (lots 4, 41, & 79).

Other cases of mislabeling further illustrate the threat posed by the caviar trade to endangered species. In two cases (lots 3 & 54) we discovered replacement of Russian sturgeon caviar with that of the ship sturgeon (*A. nudiventris*). Ship sturgeon populations are declining rapidly; the Aral Sea population of *A. nudiventris* is already extinct (Birstein 1993) due to of the drying of the Aral Sea (Smith 1994). The Black Sea and Sea of Azov populations of *A. nudiventris* have also practically disappeared. Due to the 1964 ban on catching sturgeon, the Caspian Sea–Ural River (Kazakhstan) population of

A. nudiventris was the only relatively large population remaining in the early 1990s (Avetisov 1992). Currently, this is the only place where *A. nudiventris* is still caught commercially. Ship sturgeon caviar labeled as Russian sturgeon caviar could have been shipped to the United States from Kazakhstan, but this remains uncertain.

Samples labeled as "American sturgeon" were identified as Russian sturgeon (lots 1 & 30) and beluga (lots 43 & 81). There is currently an overproduction of poor-quality *A. gueldenstaedtii* caviar in Russia that is rejected by the European caviar market (S. Taylor, personal commununication). This may have caused the observed marketing in some New York gourmet stores of Russian caviar mislabeled as "American" caviar (which is usually made of the paddlefish [*Polyodon spathula*] roe; lot 55). Most American caviar is considered inferior to Russian caviar and sells for much less ($10 an ounce) than Russian caviar. In December 1996 we found that paddlefish caviar was used as a replacement for sevruga caviar (lot 76). The size and color of the eggs of both species are similar, but the paddlefish caviar has a distinctive taste. In two cases (lots 53 & 79) we identified caviar to be of the American white sturgeon (*A. transmontanus*) instead of the Russian sturgeon, as labeled by the distributor, indicating that white sturgeon caviar—as in the past (O'Neil 1993, Cohen 1997)—is still sold in the United States as an illegal replacement for the caviar of Russian sturgeon species.

In December 1996 we detected an unusually high number of misrepresentations of Russian sturgeon caviar (lots 66, 77, 86, & 93). We were able to determine that those samples were not the caviar of *A. gueldenstaedtii*, but we were unable to determine whether these samples were caviar of *A. naccarii*, *A. persicus*, or *A. baerii*, which are, according to our previous data (Birstein & DeSalle 1998), closely related species. We had this same identification problem with one sevruga caviar sample (lot 95). We are currently developing methods to differentiate among these three species.

Our procedure allowed us to diagnose samples undesignated by suppliers: lot 18 was identified as osetra, and lots 19 and 96 were identified as sevruga. Lots 18 and 19 were bought in Russia, where sturgeon species are not identified on the labels of caviar jars and cans sold within the country. Moreover, the samples (lot 19) that had been refrigerated for 2 years showed that our method could be used for old caviar samples. It is not surprising that the illegal Bulgarian sample (lot 96) appeared to be sevruga because populations of *A. stellatus* are the only relatively large populations in the lower reaches of the Danube River (the main area of poaching in the region), compared to those of *A. gueldenstaedtii* and *Huso huso* (Birstein 1996*b*).

All populations of the main European commercial sturgeon species (*A. gueldenstaedtii*, *A. stellatus*, *H. huso*), as well as the Amur River species (*A. schrenckii*, *H. dauricus*) were included in the 1996 IUCN Red Data Book with different threatened status (World Conservation Union 1996). Many Acipenseriformes are also awaiting listing by the Convention on International Trade in Endangered Species. A rapid decline in the population size of these "living fossils" during the 1990s is a result of environmental degradation and unprecedented levels of poaching in the countries of the former Soviet Union and in China. The apparent rise in the number of shops replacing caviar of the three commercial Caspian Sea species with other sturgeon and paddlefish species possibly reflects the decreasing availability of these species in nature. The decrease in caviar production seems to be so significant that even reputable shops are starting to sell mislabeled caviar. It is possible that most shops are unaware of the replacements and are being cheated by Russian suppliers.

Only international conservation and legal efforts can save the declining commercial sturgeon populations and protect the noncommercial species that will be increasingly substituted for the disappearing commercial species. Our PCR method of caviar species identification provides a simple and quick tool for monitoring the production and importation of black caviar throughout the world.

Acknowledgments

Note Added at Proofs

Drs. R. DeSalle and V. Birstein have submitted a patent application in the United States and Europe entitled "Method and Composition for Identification of Species Origin of Caviar." U.S. patent rights for this method have been assigned to the American Museum of Natural History (AMNH), New York, New York. Information regarding use of this method in the United States can be obtained from Dr. Rob DeSalle at the corresponding address for this article or from desalle@amnh.org. European patent rights have been assigned to the Karl-Schmitz-Scholl-Fonds for Environmental Law and Policy (Bonn, Germany). Those interested in use of the method

in the EU, Romania, or Russia should contact Dr. Wolfgang Burhenne, Karl-Schmitz-Scholl-Fonds for Environmental Law and Policy, Adenauerallee 214, D-53113, Bonn, Germany, tel: +49/228/2692212, fax: +49/228/2692251.

Since the submission and acceptance of this publication, all 27 Acipenseriformes species have been listed under Appendix I and II of CITES.

Literature Cited

Amato, G., and J. Gatesy. 1994. PCR assays of variable nucleotide sites for identification of conservation units. Pages 215–226 in B. Schierwater, B. Streit, G. P. Wagner, and R. DeSalle, editors. Molecular ecology and evolution: approaches and applications. Birkhäuser, Basel, Switzerland.

Avetisov, K. B. 1992. Present status of the ship sturgeon (*Acipenser nudiventris*) in its area. Pages 3–15 in V. P. Ivanov, editor. The reproduction of acipenserids, salmonids, and some less valuable fishes. VNIRO Publishing, Moscow. (In Russian.)

Avise, J. C. 1989. A role for molecular genetics in the recognition and conservation of endangered species. Trends in Ecology and Evolution **4:**279–281.

Avise, J. C. 1994. Molecular markers, natural history and evolution. Chapman and Hall, New York.

Baker, C. S., and S. R. Palumbi. 1994. Which whales are hunted? A molecular genetic approach to monitoring whaling. Science **265:**1538–1539.

Baker, C. S., and S. R. Palumbi. 1996. Population structure, molecular systematics, and forensic identification of whales and dolphins. Pages 10–49 in J. C. Avise and J. L. Hamrick, editors. Conservation genetics: case histories from nature. Chapman and Hall, New York.

Baker, C. S., F. Cipriano, and S. R. Palumbi. 1996. Molecular genetic identification of whale and dolphin products from commercial markets in Korea and Japan. Molecular Ecology **5:**671–685.

Barannikova, I. A., I. A. Burtsev, A. D. Vlasenko, A. D. Gershanovich, E. V. Makarov, and M. S. Chebanov. 1995. Sturgeon fisheries in Russia. Pages 124–130 in A. D. Gershanovich and T. I. J. Smith, editors. Proceedings of international symposium on sturgeons, 6–11 September, 1993. VNIRO Publishing, Moscow.

Bartlett, S. E., and W. S. Davidson. 1992. FINS (forensically informative nucleotide sequencing): a procedure for identifying the animal origin of biological specimens. BioTechniques **12:**408–411.

Bemis, W. E., and E. K. Findeis. 1994. The sturgeon's plight. Nature **370:**602.

Birstein, V. J. 1993. Sturgeons and paddlefishes: threatened fishes in need of conservation. Conservation Biology **7:**773–787.

Birstein, V. J. 1996a. Sturgeons may soon disappear from the Caspian Sea. Russian Conservation News **7:**15–16.

Birstein, V. J. 1996b. Sturgeons in the lower Danube: a trip to Romania. The Sturgeon Quarterly **4(1–2):**10–11.

Birstein, V. J., and R. DeSalle. 1998. Molecular phylogeny of Acipenserinae. Molecular Phylogenetics and Evolution **9:**141–155.

Birstein, V. J., W. E. Bemis, and J. R. Waldman. 1997a. The threatened status of acipenseriform fishes: a summary. Pages 423–431 in V. J. Birstein, J. R. Waldman, and W. E. Bemis, editors. Sturgeon biodiversity and conservation. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Birstein, V. J., R. Hanner, and R. DeSalle. 1997b. Phylogeny of the Acipenseridae: cytogenetic and molecular approaches. Pages 127–155 in V. J. Birstein, J. R. Waldman, and W. E. Bemis, editors. Sturgeon biodiversity and conservation. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Cherfas, J. 1989. Science gives ivory a sense of identity. Science **246:** 1120–1121.

Cohen, A. 1997. Sturgeon poaching and black market caviar: a case study. Pages 419–422 in V. J. Birstein, J. R. Waldman, and W. E. Bemis, editors. Sturgeon biodiversity and conservation. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Davis, J. I., and K. C. Nixon. 1992. Populations, genetic variation, and the determination of phylogenetic species. Systematic Biology **41:** 421–435.

De Meulenaer, T., and C. Raymakers. 1996. Sturgeons of the Caspian Sea and investigation of the international trade in caviar. TRAFFIC International, Cambridge, United Kingdom.

DeSalle, R., and V. J. Birstein. 1996. PCR identification of black caviar. Nature **381:**197–198.

DeSalle, R., A. K. Williams and M. George. 1993. Isolation and characterization of animal mitochondrial DNA. Pages 176–204 in E. A. Zimmer, T. J. White, R. L. Cann, and A. C. Wilson, editors. Methods in enzymology. Volume 224. Molecular evolution: producing the biochemical data. Academic Press, San Diego.

Dumont, H. 1995. Ecocide in the Caspian Sea. Nature **377:**673–674.

Encalada, S. E., S. A. Eckert, and B.W. Bowen. 1994. Forensic application of mitochondrial DNA markers: origin of a confiscated green turtle. Marine Turtle Newsletter **66:**1–3.

Fabricant, F. 1993. And knowing what to look for. The New York Times, **December 29:**C1.

Khodorevskaya, R. P., G. F. Dovgopol, O. L. Zhuravleva, and A. D. Vlasenko. 1997. Present status of commercial stocks of sturgeons in the Caspian Sea basin. Pages 209–219 in V. J. Birstein, J. R. Waldman, and W. E. Bemis, editors. Sturgeon biodiversity and conservation. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Krykhtin, M. L., and V. G. Svirskii. 1997. Endemic sturgeons of the Amur River: kaluga, *Huso dauricus*, and Amur sturgeon, *Acipenser schrenckii*. Pages 231–239 in V. J. Birstein, J. R. Waldman, and W. E. Bemis, editors. Sturgeon biodiversity and conservation. Kluwer Academic Publishers, Dordrecht, The Netherlands.

O'Neil, M. 1993. Caviar distributor and 2 fishermen charged with evading limits on scarce U.S. roe. The New York Times **April 4:** 40.

Smith, D. R. 1994. Change and variability in climate and ecosystem decline in Aral Sea basin deltas. Post-Soviet Geography **35:**142–165.

Sommer, S. S., A. R. Groszbach, and C. D. K. Bottema. 1992. PCR amplification of specific alleles (PASA) is a general method for rapidly detecting known single-base changes. BioTechniques **12:**82–87.

Timmermanns, G., and M. Melchers. 1994. Desteur in Nederland. Natura **7:**155–158. (In Dutch.)

Unseld, M., B. Beyermann, P. Brandt, and R. Hiesel. 1995. Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. PCR Methods and Applications **4:** 241–243.

Waldman, J. R. 1995. Sturgeons and paddlefishes: a congruence of biology, politics, and greed. Fisheries **20:**20–21, 49.

Walsh, P. S., D. A. Metzger, and R. Higuchi. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques **10:**506–513.

World Conservation Union. 1996. 1996 IUCN red list of threatened animals. Gland, Switzerland.