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## **Species and hybrid identification of sturgeon caviar: a new molecular approach to detect illegal trade**

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## **ABSTRACT**

Over-exploitation of wild populations due to the high economic value of caviar has driven sturgeons to near extinction. The high prices commanded by caviar on world markets have made it a magnet for illegal and fraudulent caviar trade, often involving low value farmed caviar being sold as top quality caviar. We present a new molecular approach for the identification of pure sturgeon species and hybrids that are among the most commercialized species in Europe and North America. Our test is based on the discovery of species-specific SNPs (single nucleotide polymorphisms) in the ribosomal protein S7, supplemented with the Vimentin gene and the mitochondrial D-loop. Test validations performed in 702 specimens of target and non-target sturgeon species demonstrated a 100% identification success for *Acipenser naccarii*, *A. fulvescens*, *A. stellatus*, *A. sinensis* and *A. transmontanus*. In addition to species identification, our approach

allows the identification of Bester and AL hybrids, two of the most economically important hybrids in the world, with 80% and 100% success, respectively. Moreover, the approach has the potential to identify many other existing sturgeon hybrids. The development of a standardized sturgeon identification tool will directly benefit trade law enforcement, providing the tools to monitor and regulate the legal trade of caviar and protect sturgeon stocks from illicit producers and traders, hence contributing to safeguarding this group of heavily threatened species.

## **1. Introduction**

Sturgeons (Order Acipenseriformes) are large primitive fishes including about 25 species with particular life-history traits. Sturgeons are long-lived organisms, with life-spans up to 100 years, show late maturation (5 to more than 30 years) and do not reproduce annually (2 to more than 10 years between reproductive cycles) [Pikitch et al. 2005]. Multiple levels of ploidy exist in sturgeons, including diploid, tetraploid and hexaploid species with about 120, 240 and 360 chromosomes, respectively [Fontana et al. 2007]. Once widely distributed and abundant in temperate waters of the northern hemisphere, sturgeons exist today in the wild only as fragmented and isolated populations with a limited geographic distribution [Pikitch et al. 2005; Ludwig 2008]. The dramatic decline in sturgeon abundance observed in the last decades has led the International Union for Conservation of Nature (IUCN) to identify sturgeons as the most endangered group of species in

the world, with 85% of sturgeons being at risk of extinction according to the Red List of Threatened Species ([www.iucnredlist.org](http://www.iucnredlist.org)). Together with pollution and habitat degradation, the principal reason for the highly threatened status of sturgeons is over-exploitation of wild populations due to the high economic value of caviar, a luxury delicacy made of refined eggs of sturgeons, one of the most valuable food items of animal origin [Ludwig 2008; Fain et al. 2013; Jahrl 2013].

The severe decline of natural stocks in recent years has prompted a parallel increase of sturgeon aquaculture programs in Europe, Asia and North America with a special interest in those species that either produce high quality caviar or demonstrate enhanced growth rates in aquaculture. Since 2002, import of caviar from aquaculture has increased, while import of caviar from the wild has decreased [Bronzi et al. 2011]. In 2006, global imports of farmed caviar (20 tonnes) were for the first time nearly equivalent to those from wild production (24 tonnes) [Ishihara et al. 2010]. Estimated world production of farmed caviar in 2008 was 110-120 tonnes originating from 16 countries. According to FAO data, current production of meat and caviar in aquaculture largely exceeds production from wild populations [Bronzi et al. 2011].

At present, a substantial portion of aquaculture caviar production comes from sturgeon hybrids. For instance, in China 38% of total biomass production is attributed to farmed hybrids [Wei et al. 2011]. Hybrid production for caviar usually involves species with same level of ploidy, and although hybridization

between species with different degree of ploidy is possible, the resulting hybrids are either sterile or show a reduced production of eggs [Birstein 2002]. Early research into hybrid production focused on Bester, a hybrid between two diploid species: Beluga (*Huso huso*) females and Sterlet (*Acipenser ruthenus*) males. This hybrid produces high quality caviar at a younger maturity age as compared to pure beluga. Many other hybrid combinations are now cultivated. Among these, the following ones obtained from tetraploid species are particularly relevant: AL, a hybrid between Adriatic sturgeon (*A. naccarii*) females and Siberian sturgeon (*A. baerii*) males; Rolik, a hybrid between Russian sturgeon (*A. gueldenstaedtii*) females and Siberian sturgeon males and the bidirectional hybrid between Kaluga sturgeon (*H. dauricus*) and Amur sturgeon (*A. schrenckii*) [Wei et al. 2011]. In recent years, additional hybrids such as *A. schrenckii* x *A. baerii* and *A. gueldenstaedtii* x *A. naccarii* (GUNA) have shown up for the first time in international trade (CITES trade statistics derived from the CITES Trade Database, UNEP World Conservation Monitoring Centre, Cambridge, UK).

The presence of hybrid-origin caviar or meat on the international market poses a challenge to enforcing international trade regulation. Since 1998, sturgeons have been listed under the Convention on International Trade in Endangered Species (CITES; [www.cites.org](http://www.cites.org)). Most species are listed under CITES Appendix II, which allows for sustainable trade under strict guidelines regulated by governments. Trade in sturgeon parts or derivatives must be accompanied by an export permit that indicates the species harvested and is issued on the basis of sustainable

harvest. The European Union has taken measures to fight illegal caviar trade and implemented in 2006 a universal labeling system for caviar traded under CITES that includes species and country of origin (EU regulation No. 865/2006). Hybrid caviar must be labeled and accompanied by an export quota even though CITES does not require the issuance of annual quotas for hybrid caviar in trade.

Illegal harvest and trade of caviar increased dramatically in the 1990s, flooding the international market with illegal, low quality and inexpensive caviar [Knapp et al. 2006]. At present, a large portion of the global caviar trade is thought to be illegal, possibly exceeding legal trade by several times [Knapp et al. 2006].

Concern over fraudulent mislabeling of the species origin of caviar in international trade and domestic markets triggered the development of molecular markers for the identification of caviar products [DeSalle 1996; Birstein et al. 2000; Congiu et al. 2001; Mogue et al. 2008; Doukakis et al. 2012; Congiu et al. 2002]. Most sturgeons can nowadays readily be identified to the species level using mitochondrial genes. However, those fail to identify some closely related and commercially important species such as the ones included in the so-called “*gueldenstaedtii* complex”: Russian (*A. gueldenstaedtii*), Adriatic (*A. naccarii*), Siberian (*A. baerii*) and Persian (*A. persicus*) [Knapp et al. 2006, Doukakis et al. 2012]. Failure of species identification by mitochondrial markers is due to the high genetic variability of all four species of this complex as well as to ancient introgression events [Ludwig, 2008]. Only recently, the identification of a diagnostic polymorphism in the mitochondrial control region allowed distinction of Russian from Siberian sturgeon [Mogue et al. 2008]. Proposed methods for

genetic identification of sturgeon hybrids include Random Amplified Polymorphic (RAPD) and Amplified Fragment Length Polymorphic DNA (AFLP), but their use is discouraged because reproducibility is highly sensitive to experimental conditions and overall too expensive for routine application [Congiu et al. 2001, Congiu et al. 2002; Rozhkovan et al. 2008; Yarmohammadi et al. 2008].

The objective of our study is to establish a new molecular approach for the identification of pure sturgeon species and hybrids of the most commercialized species in Europe and North America. Those included a total of 11 pure sturgeon species, including the four species of the “*gueldenstaedtii complex*” (*A. gueldenstaedtii*, *A. naccarii*, *A. baerii* and *A. persicus*) and two of the most commercially important hybrids in Europe and North America, Bester (*H. huso* female X *A. ruthenus* male) and AL (*A. naccarii* female X *A. baerii* male). The method is based on the discovery of species-specific diagnostic single nucleotide polymorphisms (SNPs) in the first intron of the nuclear encoded S7 ribosomal protein RPS7 and supplementary data from the mitochondrial D-loop and the Vimentin gene. The method is easy and fast, inexpensive, and reliable and reproducible across laboratories. Ultimately, the development of a standardized identification protocol for pure sturgeon species and hybrids provides the tools to control and regulate the global legal trade in sturgeon products and combat illegal and fraudulent caviar trade (e.g., low value farmed caviar sold as top quality hybrid-origin farmed caviar). Besides supporting trade law enforcement, the method is important for the management and conservation of this extremely threatened group of fish.

## 2. Materials and methods

### 2.1 Sampling and DNA extraction

A total of 98 individuals from 11 different sturgeon species (*Acipenser baerii*, *A. gueldenstaedtii*, *A. persicus*, *A. ruthenus*, *A. transmontanus*, *A. naccarii*, *A. fulvescens*, *A. sinensis*, *A. stellatus*, *A. sturio* and *Huso huso*) were selected for sequencing of the first intron RP1 of the RPS7 gene and species-specific primer design (table A1). When possible, samples from different geographical locations were considered in order to have a better representation of the genetic variability of each species. Primer validation was performed for each primer pair on samples of target and non-target species (excluding *A. sturio*) for a total of 686 animals. Results were checked by comparing the band sizes obtained with the expected specific-band sizes as shown in figure 1. Additionally, a preliminary investigation of *A. schrenckii* and *H. dauricus* was conducted on 10 individuals (of aquaculture origin) per species and 90 individuals from 10 non-target species.

Six individuals belonging to three different sturgeon species (1 *A. baerii*, 3 *A. gueldenstaedtii*, 2 *A. persicus*) were selected for Vimentin sequencing and subsequent primer design. Vimentin validation was performed on a total of 309 individuals: 56 *A. baerii*, 96 *A. gueldenstaedtii* (48 from Caspian and 48 from Azov Sea), 48 *A. persicus*, 45 *A. naccarii*, 8 *A. ruthenus*, 8 *A. transmontanus*, 8 *A. fulvescens*, 8 *A. sinensis*, 8 *A. stellatus*, 8 *A. schrenckii*, 8 *Huso dauricus* and 8 *H. huso*.



Finally, a total of 91 individuals from two commercially important hybrids were used to develop a hybrid identification tool: 71 Bester (*H. huso* female X *A. ruthenus* male) and 20 AL (*A. naccarii* female X *A. baerii* male).

Genomic DNA was extracted from fin clips, muscle or caviar eggs (50 ng) using the standard protocol of the EuroGOLD Tissue DNA Mini Kit (Euroclone).

## 2.2 Development of RP1 marker

Initially, the first intron (RP1) of the nuclear encoded S7 ribosomal protein (RPS7) was amplified from three individuals of three different species (*A. gueldenstaedtii*, *A. transmontanus* and *H. huso*) using the universal primers for fish isolated by Chow and Hazama [1998], S7RPEX1F and S7RPEX1R. Following the identification of multiple loci (figures S1 and S2), a new primer was designed to specifically amplify locus A (RP1\_LocusA\_R 5'ATCCAAGTACAAGCTTGAACA3'), which was used in combination with the universal primer S7RPEX1F.

All PCR reactions were performed in a total volume of 20  $\mu$ l, consisting of 0.25  $\mu$ M of each primer, 0.1 mM dNTPs, 1X buffer (500 mM KCl, 15 mM MgCl<sub>2</sub>, 100 mM Tris-HCl pH 8.3, 1mg/ml BSA, 100 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.025 U/ $\mu$ l of Taq DNA polymerase (Sileks or Resnova), and 10 ng of extracted DNA template. All amplifications were performed on an Applied Biosystem GeneAmp<sup>®</sup> PCR System 9700 set as follows: 2 min at 94°C, 33-35 cycles at 94°C for 45 s, 59°C for 45 s and 72°C for 45 s, followed by a 7 min extension at 72°C. Products were checked by 1.8% agarose gel electrophoresis, purified using ExoSAP-IT<sup>®</sup> and directly

sequenced on an ABI Prism 3730XL or an ABI 3100 automatic sequencer at BMR Genomics.

Sequences presenting no polymorphism were considered as single alleles (homozygote individuals). For sequences with double peaks, the consensus sequence was edited according to the IUPAC code. Heterozygote individuals, presenting insertions/deletions that resulted in chromatogram shifting, were cloned in order to identify all alleles for each individual. Cloning procedure was performed in JM109 competent cells using the P-GEM-T Easy Vector (Promega) following the manufacturer's recommendations. Considering that sequencing of cloned PCR products is known to generate errors through single-base mutations [Kobayashi et al. 1999], sequences were considered to be true alleles only if the comparison between cloned sequences and observed peaks in the genomic sequence profiles confirmed the presence of each polymorphism [Barbisan et al. 2009]. Moreover, unclear sequences were cloned again from a second independent PCR. Locus A was sequenced for a total of 98 individuals from 11 species to detect species-specific SNPs. Sequences were edited using FinchTV (<http://www.geospiza.com/finchtv/>) and aligned using ClustalW (<http://www.ebi.ac.uk/Tools/clustalw/index.html>) [Thompson et al. 1994].

A multi-alignment was generated using all sequences obtained and species-specific single nucleotide polymorphisms (SNPs) were identified. A panel of nine species identification primers were developed using Oligo-Explorer (Gene Link) and FastPCR (Primer Digital) [Kalendar et al, 2009]. Diagnostic mutations were used for primer design following three general criteria: first, the last nucleotide at

the 3' primer-end must be complementary to the diagnostic mutation; second, the second-last nucleotide should be not complementary to the target sequence in order to establish a mismatch of two nucleotides if paired with non-target sequences, which causes a detachment from the target DNA [Mugue et al. 2008]; third, primers were chosen so that different species would differ in PCR product size and could be easily distinguishable on 2% agarose gel. All primer pairs were optimized and checked against target and non-target species in uniplex reactions for a total of 702 animals. Validation experiments were performed independently in two laboratories located in Italy and Russia, respectively. The same PCR conditions reported above were used for all primer pairs in order to facilitate multiplexing.

### **2.3 Development of Vimentin marker**

With the original aim of developing a marker to investigate a supposed ancient introgression of *A. baerii* into *A. gueldenstaedtii* in the Caspian Sea [Mugue et al. 2008] (thus expected to be absent in *A. gueldenstaedtii* from Azov Sea) a second nuclear marker (Vimentin) was analyzed. The Vimentin sequence of *A. baerii* (AJ493266) was aligned to the *Danio rerio* sequence (NP\_57194) and exon-intron boundaries were predicted. The primer pair AbVim\_F (5'GTCTACAATGAATCGGCAGTCGTC3') and AbVim\_R (5'TCCCCAAGGTTGTCTCGGTCTAC3') was designed to amplify the first coding exon.

PCR products were amplified from one *A. baerii*, three *A. gueldenstaedtii* (Azov and Caspian Seas) and two *A. persicus* individuals, ligated in P-GEM-T Easy Vector (Promega) and cloned in *E. coli*. A multi-alignment was generated using all sequences obtained and polymorphisms were identified. One primer pair consisting of Bae154B7F (5'TCCAGGGTTTCCTACACCAGCCAAT3') and Bae154B7R (5'CCACCCTCGCCTTTTCGTTGGTTTG3') was designed to amplify alleles present in *A. baerii* but rarely found in *A. gueldenstaedtii* individuals from Azov. The criteria for selective primer construction were the same as for RP1 with the reverse primer designed on the selected SNP. The expected amplification product is 373 bp long.

Vimentin mix reactions were the same as reported for RP1 with MgCl<sub>2</sub> increased to 2.5 mM. Amplifications were performed with the following cycle: 2 min of denaturation at 94°C, 30 cycles at 94°C for 15 s, 61°C for 30 s and 72°C for 30 s, followed by a 10 min extension at 72°C. As in RP1, Vimentin was validated independently in two laboratories.

#### **2.4 Hybrid detection tools**

In the case of Bester, the paternal contribution was identified using primer RutBae\_RP1F designed for Siberian (*A. baerii*) and Sterlet (*A. ruthenus*) sturgeons in combination with the reverse primer specific for RP1-Locus A (RP1\_LocusA\_R). This amplification was multiplexed with the positive amplification control following the same thermal cycle as for the uniplex-PCR. In the multiplex reaction, 0.25 µM of each primer forward and 0.5 µM of the reverse

primer were used. The maternal contribution was identified by mitochondrial (mtDNA) DNA analysis following Mogue *et al.* [Mogue *et al.* 2008].

In the case of AL, the paternal (*A. baerii*) contribution was identified using the primer pair Bae154B7F / Bae154B7F for Vimentin. The maternal (*A. naccarii*) contribution was identified using the primer pair Nac\_RP1F / NacFul\_RP1R. The maternal contribution can be confirmed by analysis of the mitochondrial control region and comparison with the full list of existing haplotypes identified for *A. naccarii* (GenBank accession numbers KF771109 - KF771115) [Congiu *et al.* 2011], with an additional haplotype referred to a putative case of sequence heteroplasmy.

## **2.5 Estimation of hybrid detection power**

While for pure species, the expected identification power of the markers is directly inferred from the percentages observed in the validation experiments, the expected power in the case of hybrids depends on the probability that the diagnostic marker for the corresponding paternal species is inherited, which depends on its frequency and on the ploidy level. Dealing with dominant bi-allelic markers (presence/absence of a given band), the allelic frequencies of the "absence" alleles can be estimated, in pure species, from the frequencies of the complete homozygous genotypes (e.g. individuals not presenting the amplification product) by applying the square root for diploids (for tetraploids the fourth root should be applied). By doing this, we accepted the error given by the assumption of Hardy Weinberg equilibrium in samples composed by individuals

from different geographical areas. Based on allelic frequencies and taking into account that hybrids may inherit one or two paternal chromosomes (in diploids and tetraploids, respectively), we estimated the identification power as the proportion of hybrids expected to have inherited at least one copy of the “presence” allele from the paternal species.

### **3. Results**

#### **3.1 RP1 Characterization**

Amplification of the RP1 by universal primers resulted in two bands for each individual distinguishable on agarose gel, of around 950 and 700 bp, respectively. After cloning of the PCR products, sequencing and cluster analysis showed 3 groups of sequences, A, B and C (figures S1 and S2). The highest band on the agarose gel corresponded to the group B, while groups A and C presented the same size, but were characterized by different insertions/deletions as shown in figure S1.

The presence of at least one sequence per group in each individual suggests the existence of 3 different loci (A, B and C in figure S2). All subsequent analyses were focused on the characterization of locus A. In order to selectively amplify this group of sequences, a new locus A-specific reverse primer was designed (RP1\_LocusA\_R) to be used in combination with the universal primer (S7RPEX1F).

A maximum of four different alleles for tetraploid species and two alleles for diploid species were observed, which points to the selective amplification of a

single locus. A multi-species alignment of 583 bp was obtained including over 150 different sequences obtained from 11 species. A simplified alignment of one sequence per species is reported in figure S3.

### 3.2 Species-specific primers set and test validation

Diagnostic species-specific SNPs were detected for 5 species (*A. naccarii*, *A. fulvescens*, *A. stellatus*, *A. sinensis*, *A. transmontanus*), in addition to a 10 bp deletion shared by *A. baerii* and *A. ruthenus* (table 1). On the basis of the diagnostic polymorphisms found, a panel of primers for species identification was designed. Primer combinations, species identified and expected band size are detailed in table 2. Additionally, a common primer for all sturgeon species studied to be used as positive control (pc\_RP1F) was designed on the basis of a conserved region shared by all individuals sequenced. No specific SNPs were identified for *A. gueldenstaedtii*, *A. persicus* and *H. huso* although 9 specific polymorphisms were detected for *A. sturio* (table 1 and figure S3; *A. sturio* not studied further here).

A 100% identification success was obtained for the primer pairs designed to amplify *A. naccarii* (66 out of 66 individuals), *A. fulvescens* (26/26), *A. sinensis* (13/13), *A. stellatus* (46/46) and *A. transmontanus* (16/16) (table 3; figure 1A). For each of these five specific primer pairs, no amplification was observed for any of the non-target species after a rigorous examination of (on average) 215 individuals: *A. naccarii* (0 amplifications out of 306 individuals of non-target species tested), *A. fulvescens* (0/178), *A. sinensis* (0/191), *A. stellatus* (0/204)

and *A. transmontanus* (0/198). For the primer pair designed for *A. ruthenus/A. baerii* on the basis of the shared 10 bp deletion, the identification success was 67.6% (171 out of 253 individuals). The efficiency was 96% for *A. ruthenus* (48/50) and 60.6% for *A. baerii* (123/203). This incomplete efficiency was unpredicted from the reference sequence data but was confirmed a posteriori after sequencing the animals that gave unexpected results. Considering non-target species, positive amplifications were observed in only 2.3% *A. gueldenstaedtii* (6/258) and 27.1% *A. persicus* (13/48).

An extra primer was designed to specifically amplify Kaluga sturgeon (*H. dauricus*) and Amur sturgeon (*A. schrenckii*) on the basis of a common shared 20 bp deletion (tables 1 and 2 and figure S3). Although results are preliminary due to the low number of individuals tested and need further validation, a 100% positive amplification was obtained for both species (10/10 *A. schrenckii* and 10/10 *H. dauricus*), while no amplification was observed in 90 individuals of 9 non-target species (table 3). Note that primer pc\_RP1F designed as positive control for sturgeon species does not work for *H. dauricus* and *A. schrenckii* due to a deletion in the region in which the primer was designed that prevents annealing (figure S3).

Finally, the Vimentin primer tested on *A. baerii* and *A. gueldenstaedtii* individuals collected from Azov Sea showed that 44 out of 56 *A. baerii* (78%) and 1 out of 48 *A. gueldenstaedtii* (2%) resulted positive to the amplification of the expected band of 373 bp. On the contrary, *A. gueldenstaedtii* from Caspian Sea yielded



positive amplification from 18 out of 48 individuals, limiting the possibility to use this marker for the identification between Russian and Siberian sturgeon only to individuals from Azov Sea. With regards to the other species, no amplification was observed in *A. naccarii* (0/45), *A. fulvescens* (0/8), *A. sinensis* (0/8), *A. transmontanus* (0/8), *A. ruthenus* (0/8), *A. schrenckii* (0/8) and *H. dauricus* (0/8). However, positive amplifications were observed in *A. stellatus* (7/8), *H. huso* (8/8) and *A. persicus* (16/48).

### **3.3 Assays for hybrid identification.**

Bester (*H. huso* female x *A. ruthenus* male) and AL (*A. naccarii* female x *A. baerii* male) hybrids were identified using a combination of RP1 and mtDNA, supplemented in the case of AL with a second nuclear gene (Vimentin).

In the Bester identification (figure 2), the RP1 assay is able to distinguish Bester (two bands of 169 bp and 306 bp) from *H. huso* (one single band of 306 bp) but not from *A. ruthenus* (same two bands as Bester). However, Bester and *A. ruthenus* can be distinguished by mtDNA analysis, since Bester presents a 374 bp band and *A. ruthenus* presents an exclusive 190 bp band. The expected detection power for Bester based on the estimated allele frequencies is 80%. Since *A. ruthenus* is a diploid species [Fontana et al. 2007], only one chromosome of this species is inherited by the hybrid thus the frequency of the band expected in Bester corresponds to the “presence” allele frequency in *A. ruthenus*. In the validation test, observed results were similar to expectations and 53 out of 71 (74.6%) Bester samples were correctly identified using the combined mtDNA and

RP1 approach. The undetected hybrids (25.4%) presented the typical pattern of *H. huso* individuals, missing the *A. ruthenus* specific band of 169 bp for RP1.

With regard to AL, a hybrid between Adriatic sturgeon (*A. naccarii*) females and Siberian sturgeon (*A. baerii*) males, the identification power is given by the application of RP1, totally effective in detecting the *A. naccarii* contribution, which is supplemented by the Vimentin marker for the identification of the *A. baerii* contribution (figure 2). Since all AL commercialized world-wide have been produced in a single Italian hatchery by crossing Adriatic sturgeon females with the males of a limited stock of Siberian sturgeons, the expected power of AL detection directly depends on the percentage of correct identification on this paternal stock. The Vimentin marker amplification performed on all the 51 pure Siberian sturgeons composing the above stock yielded a clear band of the expected size from all individuals. Consequently, also the expected detection power on AL hybrids is 100%. In fact, in the AL identification (figure 2), the RP1 assay can distinguish *A. naccarii* (2 bands of 227 bp and 306 bp) from *A. baerii* (single 306 bp) but AL hybrids present the same two bands as *A. naccarii*. AL and *A. naccarii* can be distinguished using Vimentin, with AL presenting a single 373 bp band, while no band is amplified in *A. naccarii*. Validation was performed on 20 AL individuals. All individuals presented the two RP1 bands at 227 bp and 306 bp, respectively, plus the 373 bp Vimentin band, confirming their hybrid status. As a final step, the maternal *A. naccarii* contribution can be confirmed by mitochondrial control region analysis and comparison with the 7 existing *A. naccarii* haplotypes.

Finally, we estimated the expected probability of correct hybrid detection for other hybrids not experimentally validated in our study, based on the positive and negative identification of target and non-target species in the validation tests using RP1. Hybrids between species for which no specific polymorphism was detected are not distinguishable, such as the ones between *A. baerii* and *A. gueldenstaedtii* observed in the Volga River [Jenneckens et al. 2000] and of increasing interest for aquaculture [Ludwig 2008].

For all hybrids with *A. naccarii*, *A. fulvescens*, *A. stellatus*, *A. sinensis* or *A. transmontanus* as paternal species, a 100% identification success is expected. A 80% success is expected for those hybrids with *A. ruthenus* as paternal species.

Although results are preliminary and need to be validated in more individuals, a 100% identification success is also expected for those hybrids obtained by crossing either *A. schrenckii* or *H. dauricus*.

## **4. Discussion**

### **4.1 New DNA markers for sturgeon identification**

We present a new molecular marker for fast, inexpensive and reliable identification of sturgeon species and hybrids, which represents a major step in the development of a standardized protocol for identifying illegal trade of sturgeon caviar and derivatives. The method is based on simple PCR amplification of a single locus nuclear marker (RP1), the first intron of the RPS7 gene, which

has traditionally been used for detecting polymorphisms at the intraspecific level due to its high mutation rate (e.g. atherinids) [Francisco et al. 2008]. In sturgeons, the retention of species-specific single nucleotide polymorphisms can be attributable to their slowed evolutionary rate [Krieger & Fuerst, 2002], predicted on the basis of particular life history traits (e.g., long generation time, large body size, ectotherm, late sexual maturation, low metabolic rate) and molecular data (i.e., limited karyotype and genetic differentiation). "The approach here proposed is based on SNP-specific primers and is rapid, cheap and suitable for all laboratories; however, the same polymorphisms can also be genotyped following alternative approaches (e.g. pyrosequencing) that are less sensitive to DNA quality and become especially convenient in the case of high numbers of samples."

In regards to pure species identification, besides full identification power for *A. fulvescens*, *A. sinensis*, *A. transmontanus* and *A. stellatus*, we present for the first time a molecular tool to distinguish the Adriatic sturgeon with 100% accuracy. Until now, *A. naccarii* was indistinguishable from the rest of species of the "gueldenstaedtii complex" (*A. gueldenstaedtii*, *A. baerii* and *A. persicus*) using the mitochondrial cytochrome b and D-loop [Birstein et al. 2008]. The complete reliability of the *A. naccarii* identification by both mtDNA and RP1 is guaranteed by the fact that all *A. naccarii* available worldwide descend from the stock analyzed in our study [Congiu et al. 2011], allowing the complete description of all existing mitochondrial haplotypes. The Adriatic sturgeon is considered to be

possibly extinct in nature, its persistence being strictly linked to the *ex situ* conservation of a single captive broodstock of 43 animals currently decimated to 13 individuals that represent the last remaining population of Adriatic sturgeon of certain wild origin [Congiu et al. 2011]. Following the successful reproduction in captivity of the original F0 stock, all the Adriatic sturgeons reared worldwide for aquaculture purposes are F1 groups that directly descend from this limited stock. Since all *A. naccarii* specimens used in the present study are either F0 individuals or F1 broodstock from the original stock, we can expect 100% reliability in correctly identifying *A. naccarii*.

The variability at the RP1 locus was only partially explored in our study. For example, several specific SNPs were available for *A. sturio* but primers were not developed because this species is not traded or reared for commercial purposes. It is reasonable to expect diagnostic SNPs for its sister species *A. oxyrinchus*, which is the subject of reintroduction in Europe [Ludwig et al. 2008], but for which samples were not available. Limited sample availability was also an issue in the case of *H. dauricus* and *A. schrenckii* and we only conducted a preliminary analysis. Further samples are needed for validation, but if the 100% of identification power of the RP1 primer is confirmed, it would represent the first reliable nuclear marker to distinguish these two commercially important sturgeon species from all others.

## 4.2 First molecular marker for hybrid identification

Besides species identification, one fundamental application of the RP1 and Vimentin markers is hybrid identification, significantly improving the identification power for Bester (from 0 to 80%) and AL (from 0 to 100%) and providing the tools to identify many other hybrids.

Bester is the most important hybrid in the world from a commercial point of view, produced by crossing Beluga (*H. huso*) females and Sterlet (*A. ruthenus*) males.

The maternal species produces one of the most valuable caviar in the world known as Beluga, which is not distinguishable from Bester hybrid caviar using only the maternally-inherited mtDNA, as both are produced by *H. huso* females.

However, by combining mtDNA and RP1 analysis we increased the identification success of Bester hybrids from 0 to 80%. Moreover, the possibility to analyze bester caviar obtained from unrelated females can significantly increase the probability of detecting the Sterlet paternal contribution in at least one of them.

With only two or three available females, the detection power increases to 96% and 99.2%, respectively. The RP1 primer used for *A. ruthenus* detection also yields a band of the same size in 60.6% of Siberian sturgeons (*A. baerii*) as expected and in a very low fraction of *A. gueldenstaedtii/persicus* (6.15%).

However, with regards to Bester caviar identification, this is not relevant since both *A. baerii* and *A. gueldenstaedtii/persicus* are not crossed with *H. huso* for hybrid caviar production due to different ploidy levels [Fain et al., 2013]. The co-occurrence of the above band in *A. ruthenus* and *A. baerii* also makes their hybrids not identifiable. Again, the different ploidy level makes these hybrids,

recently observed in the Danube River [Ludwig et al. 2009], not interesting for caviar production.

AL is another hybrid intensively produced for both caviar and meat production due to its high nutritional value [Vaccaro et al. 2005], resulting from crossing Adriatic sturgeon (*A. naccarii*) females and Siberian sturgeon (*A. baerii*) males. Our approach combining RP1, Vimentin and mtDNA is the first tool that allows the recognition of a hybrid between two of the most difficult species to identify, both included in the "*Gueldenstaedtii complex*" [Ludwig, 2008, Knapp et al. 2006]. In this case, the identification success increased from 0 to 100%. One caveat of using the Vimentin gene is its positive amplification in other sturgeon species including *H. huso*, *A. stellatus* and some populations of *A. gueldenstaedtii*. However, these species are not successfully hybridized with *A. naccarii* females for caviar production, and do not interfere with AL identification. In any case, we suggest the use of Vimentin only when both mtDNA and RP1 have previously shown to be positive for *A. naccarii*.

Other newly-produced hybrids involving *A. naccarii* as parental species have shown up recently in international trade and can be potentially identified using the RP1 marker and mtDNA with a 100% accuracy. This is the case of GUNA hybrids, obtained by crossing Russian sturgeon (*A. gueldenstaedtii*) females and Adriatic sturgeon (*A. naccarii*) males. Similarly, in the case of ADAM hybrids, obtained in Italy by crossing Adriatic sturgeon (*A. naccarii*) females with aquaculture-produced white sturgeon (*A. transmontanus*), a 100% identification

accuracy is expected using RP1. Furthermore, any additional hybrids produced using *A. naccarii*, *A. fulvescens*, *A. stellatus*, *A. sinensis* or *A. transmontanus* as paternal species could also be potentially identified with a 100% identification power with the species-specific RP1 markers developed in our study, supplemented by mtDNA. For example, in a previous conservation study, some animals with *A. transmontanus* mitochondrial haplotypes were detected in a *A. naccarii ex situ* brood-stock reared for conservation purposes. These animals, hypothesized to be hybrids with the Adriatic sturgeon based on microsatellite information [Congiu et al. 2001], could have been unambiguously identified with RP1.

#### **4.3 A molecular approach to regulate legal trade and combat fraudulent caviar trade**

The development of a new molecular approach for the identification of pure sturgeon species and hybrids of the most commercially important species in Europe and North America based on diagnostic polymorphisms in the RPS7 gene ultimately offers new opportunities to regulate and control the international legal and illegal trade in caviar. Since the inclusion of all sturgeon species in the appendices of CITES, a number of conservation measures have been put in place to improve law enforcement of caviar trade. Despite these efforts, mislabelling of caviar products is common and it is frequent to find low value farmed caviar being sold as top-quality farmed caviar of hybrid origin [Knapp et al. 2006]. It is thus essential for custom officials and trade authorities to have access to an



accurate and reliable method of authenticating the caviar labelling system. The RP1 method allows for the identification of the source of caviar in order to assess the validity of the product label. Importantly, the method enables not only the identification of caviar at the species level but for the discovery of hybrid species, which have proven to be unidentifiable using other DNA-based methods. Our new molecular approach, based on the concordance between mtDNA and nuclear markers, is already suitable for law enforcement.

The expanding criminal caviar trade is a serious threat to the survival of wild sturgeon populations, diminishes the effectiveness of management and conservation programs, undermines legal trade and threatens the sustainable production of caviar. Hence, the introduction of RP1 as a suitable marker for the identification of pure species and hybrids is not only useful to certify and corroborate the correct labelling of legal caviar by authorized producers, processors and traders, but will also have a strong dissuasive effect against fraudulent activity and could be a valuable support to conservation programs in which the purity of the species is an essential prerequisite. Improving the control and monitoring of caviar trade will ensure its sustainability and will be the first step toward the rescue and safeguard of this group of threatened species.

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## Author contribution

Boscari E. and Congiu L. had a major role in designing the experiments based on S7 markers, performing experiments and writing the paper. Barmintseva A. and Mugue N. designed the experiments based on Vimentin and performed experiments. Pujolar JM had a relevant role in planning part of the experiments and in writing the manuscript. Doukakis P. contributed to writing the manuscript and to frame it in the context of CITES enforcement.

## Data Accessibility

DNA sequences:

Adriatic sturgeon control region haplotypes, GenBank accessions: KF771109 - KF771115.

RP1 sequences, GenBank accessions KF771068 - KF771095.

Locus A sequences GenBank accessions KF771096 - KF771108.

Sampling information for each species: Table S1 (Supporting information).

Alignments for figures S2 and S3 are available on Dryad entry DOI: 10.5061/dryad.t3jd5

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## FIGURE CAPTIONS

**Figure 1.** Expected profiles and corresponding band sizes on agarose gel for pure species identification using RP1 including (1) *A. fulvescens*, (2) *A. stellatus*, (3) *A. sinensis*, (4) *A. transmontanus*, (5) *A. naccarii*, (6) *A. baerii* and (7) *A. ruthenus*. The 306 pb band represents the positive control with slight variable sizes due to different deletions in the different species. The band of 223 bp identified as a potential marker for *A schrenckii* and *H. dauricus* was not included.

**Figure 2.** Expected band patterns for Bester and AL hybrid identification using RP1 and mtDNA (Bester) and RP1 and Vimentin (AL).

**Table 1.** Diagnostic single nucleotide polymorphisms (SNPs) for species identification (in bold). SNPs shaded in grey were further selected for primer design. Numbers associated with each SNP refer to the nucleotide position in the multi-species alignment (figure S3). The symbol (-)<sub>n</sub> indicates a deletion of n bases.

Nucleotide position (bp)	62	75	83	112	126	172	204	223	255	306	334	400	406	442	443	473	533	534	
Species	2	5	3	2	6	2	4	3	3	4	4	0	6	7	0	9	0	2	2
<i>A. fulvescens</i>	T	<b>G</b>	T	C	C	T	T	T	G	C	G	<b>G</b>	C	G	G	<b>A</b>	G	<b>T</b>	
<i>A. naccarii</i>	*	T	*	*	*	*	*	*	*	<b>T</b>	*	*	*	*	*	C	*	<b>*</b>	
<i>A. transmontanus</i>	*	*	*	*	*	*	<b>C</b>	*	*	*	*	<b>*</b>	*	*	*	C	*	G	
<i>A. sinensis</i>	<b>C</b>	*	*	*	*	*	*	*	*	*	<b>*</b>	*	*	*	*	C	*	G	
<i>A. stellatus</i>	*	*	*	*	<b>A</b>	*	*	*	*	*	*	A	*	*	*	C	*	G	
<i>A. ruthenus</i>	*	*	*	*	*	*	*	*	*	*	*	A	(-) <sub>10</sub>	-	*	C	*	G	

<i>H. huso</i>	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	C	*	G
<i>A. baerii</i>	*	*	*	*	*	*	*	*	*	*	*	A	(-) <sub>10</sub>	-	*	C	*	G
<i>A. gueldenstaedtii</i>	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	C	*	G
<i>A. persicus</i>	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	C	*	G
<i>A. sturio</i>	*	*	<b>C</b>	<b>T</b>	*	<b>C</b>	*	(-) <sub>5</sub>	*	*	<b>T</b>	A	*	<b>A</b>	<b>A</b>	<b>T</b>	<b>C</b>	G
<i>A. schrenckii</i>	*	*	*	*	*	*	*	*	(-) <sub>20</sub>	-	*	A	*	*	*	C	*	G
<i>H. dauricus</i>	*	*	*	*	*	*	*	*	(-) <sub>20</sub>	-	*	A	*	*	*	C	*	G

**Table 2.** Details of primers designed for sturgeon species identification including sequence, primer pairing, expected PCR product size and target species.

Primer	Sequence	Used with	bp	Specificity
<b>Reverse</b>				
<b>NacFul_RP1R</b>	ACACGAGTTCCGATTTTATTATA			<i>A. naccarii</i> ; <i>A. fulvescens</i>
<b>SinTran_RP1R</b>	GGTCTGTAACACAGGTAATTATC			<i>A. sinensis</i> ; <i>A. transmontanus</i>
<b>Forward</b>				
<b>Nac_RP1F</b>	GCCAATTTCAAAGTGATTTTGATT	NacFul_RP1R	227	<i>A. naccarii</i>
<b>Ful_RP1F</b>	CAGTGATCTGATTTTAAAGTAAAAAAG	NacFul_RP1R	521	<i>A. fulvescens</i>
<b>Ste_RP1F</b>	TGTCACCTTTCAAATTTGGTA	RP1_LocusA_R	479	<i>A. stellatus</i>
<b>RutBae_RP1F</b>	TTACATTAATTACCTGTGTTACAGATAG	RP1_LocusA_R	169	<i>A. ruthenus</i> ; <i>A. baerii</i>
<b>Sin_RP1F</b>	GGAATTTTACAGTGATCTGATTAC	SinTran_RP1R	386	<i>A. sinensis</i>
<b>Tran_RP1F</b>	TGTGAAATTTAGATGATTTTAGTGC	SinTran_RP1R	246	<i>A. transmontanus</i>
<b>SchDau_RP1F</b>	GCCCATTTCAAATGTATTGGGG	RP1_LocusA_R	223	<i>A. schreckii</i> ; <i>H. dauricus</i>
<b>pc_RP1F</b>	TGCAGGTAGTTATAAATAATTACTCTGC	RP1_LocusA_R	309	Positive control

**Table 3.** Validation tests of all primers pairs performed in 10 different sturgeon species presented as fraction of positive amplifications on the total number of individuals tested (also including animals tested in the RP1 primer development phase as first term of the denominator). Cells corresponding to target species for each primer are shaded in grey. Validation tests for the preliminary study of *A. schrenckii* and *H. dauricus* are also included. Accuracy refers to the validation success of the primer to identify the target species.

Species tested Primer pairs	<i>A. naccarii</i>	<i>A. baerii</i>	<i>A. gueldenstaedtii</i>	<i>A. persicus</i>	<i>A. ruthenus</i>	<i>A. transmontanus</i>	<i>A. fulvescens</i>	<i>A. sinensis</i>	<i>A. stellatus</i>	<i>H. huso</i>	<i>A. schrenckii</i>	<i>H. dauricus</i>	IDENTIFIED SPECIES	ACCURACY (%)
	Nac_RP1F NacFul_RP1R	$\frac{66}{6+60}$	$\frac{0}{14+16}$	$\frac{0}{20+83}$	$\frac{0}{3+48}$	$\frac{0}{10+16}$	$\frac{0}{10+6}$	$\frac{0}{10+16}$	$\frac{0}{3+10}$	$\frac{0}{12+19}$	$\frac{0}{8+12}$	$\frac{0}{2+8}$	$\frac{0}{2+8}$	<i>A. naccarii</i>
Ful_RP1F NacFul_RP1R	$\frac{0}{6+10}$	$\frac{0}{14+23}$	$\frac{0}{20+30}$	$\frac{0}{3}$	$\frac{0}{10+8}$	$\frac{0}{10+6}$	$\frac{26}{10+16}$	$\frac{0}{3+10}$	$\frac{0}{12+11}$	$\frac{0}{8+4}$	$\frac{0}{2+8}$	$\frac{0}{2+8}$	<i>A. fulvescens</i>	100



<b>Ste_RP1F</b> <b>Stor1aIS7</b>	$\frac{0}{6+15}$	$\frac{0}{14+8}$	$\frac{0}{20+21}$	$\frac{0}{3+24}$	$\frac{0}{10+8}$	$\frac{0}{10+6}$	$\frac{0}{10+16}$	$\frac{0}{3+10}$	$\frac{46}{12+34}$	$\frac{0}{8+12}$	$\frac{0}{2+8}$	$\frac{0}{2+8}$	<b><i>A. stellatus</i></b>	<b>100</b>
<b>RutBae_RP1F</b> <b>RP1_LocusA_R</b>	$\frac{0}{6+15}$	$\frac{123}{14+189}$	$\frac{6}{20+238}$	$\frac{13}{3+48}$	$\frac{48}{10+40}$	$\frac{0}{10+6}$	$\frac{0}{10+16}$	$\frac{0}{3+10}$	$\frac{0}{12+19}$	$\frac{0}{8+16}$	$\frac{0}{2+8}$	$\frac{0}{2+8}$	<b><i>A. ruthenus</i></b>	<b>96</b>
<b>Sin_RP1F</b> <b>SinTran_RP1R</b>	$\frac{0}{6+10}$	$\frac{0}{14+23}$	$\frac{0}{20+30}$	$\frac{0}{3}$	$\frac{0}{10+8}$	$\frac{0}{10+6}$	$\frac{0}{10+16}$	$\frac{13}{3+10}$	$\frac{0}{12+11}$	$\frac{0}{8+4}$	$\frac{0}{2+8}$	$\frac{0}{2+8}$	<b><i>A. sinensis</i></b>	<b>100</b>
<b>Tran_RP1F</b> <b>SinTran_RP1R</b>	$\frac{0}{6+10}$	$\frac{0}{14+23}$	$\frac{0}{20+30}$	$\frac{0}{3}$	$\frac{0}{10+8}$	$\frac{16}{10+6}$	$\frac{0}{10+16}$	$\frac{0}{3+10}$	$\frac{0}{12+11}$	$\frac{0}{8+4}$	$\frac{0}{2+8}$	$\frac{0}{2+8}$	<b><i>A. transmontanus</i></b>	<b>100</b>
<b>SchDau_RP1F</b> <b>RP1_LocusA_R</b>	$\frac{0}{6+10}$	$\frac{0}{14+10}$	$\frac{0}{20+10}$	$\frac{0}{3}$	$\frac{0}{10+10}$	$\frac{0}{10+10}$	$\frac{0}{10+10}$	$\frac{0}{3+10}$	$\frac{0}{12+10}$	$\frac{0}{8+10}$	$\frac{10}{2+8}$	$\frac{10}{2+8}$	<b><i>A. schrenckii</i></b>	<b>100</b>
<b>pc_RP1F</b> <b>RP1_LocusA_R</b>	$\frac{66}{6+60}$	$\frac{203}{14+189}$	$\frac{258}{20+238}$	$\frac{51}{3+48}$	$\frac{50}{10+40}$	$\frac{16}{10+6}$	$\frac{26}{10+16}$	$\frac{13}{3+10}$	$\frac{46}{12+34}$	$\frac{38}{8+30}$	$\frac{0}{2+8}$	$\frac{0}{2+8}$	<b>All species tested</b>	<b>100</b>



