Fast genetic identification of the Beluga sturgeon and its sought-after caviar to stem illegal trade

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ABSTRACT

Sturgeons are well known for the delicacy of their eggs, the caviar, one of the most valuable products on the food market. The high price of caviar led in the past to a severe overharvest of wild sturgeon species and to an increase in trade of counterfeit products sold with impunity in spite of the strict trade limitations. A priority in the effort to reduce illegal trading is the development of genetic tools in order to identify the species of traded products using a standardized, cheap and rapid approach. We developed the first genetic nuclear marker for the identification of the Beluga sturgeon (*Huso huso*), the most sought-after caviar producer. We explored the interspecific variability at the second intron of the nuclear S6 Ribosomal Protein (RP2S6), selected among 1867 introns, predicted by aligning the transcriptome of 3 sturgeon species with 3 complete fish genomes. The Beluga-specific SNP was identified by cloning and sequencing RP2S6 in 65 individuals of 11 species, validated on 341 additional individuals and tested on 18 caviar samples. Diagnostic primers designed on the SNP successfully amplified the expected band in all Beluga specimens while no PCR product was obtained from other pure species. The marker can also contribute to the identification of interspecific hybrids in which the Beluga is one of the parent species, such as in the case of the Bester, which produces one of the most mislabeled caviars in trade. The complete identification power on this highly relevant species and the proved efficacy on caviar samples represent an essential progress towards a standardized panel of nuclear markers for the control of illegal poaching, smuggling and mislabeling of sturgeons and their products.

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1. Introduction

Sturgeons are an archaic group of fish known, for centuries, as producers of caviar, one of the most valuable food delicacies on the world's market (Fain, Straughan, Hamlin, Hoesch, & LeMay, 2013). Overfishing of wild stocks, encouraged by high profits from illegal markets, is the major cause that has led all sturgeon species to the brink of extinction, inducing the International Union for Conservation of Nature (IUCN) to list them as one of the most imperiled group of animals worldwide (IUCN press release of 18, Mar, 2010, https://www.iucn.org/press/news-releases). Despite protection measures, a significant illegal trade still exists, in which caviar from unsustainable natural sources is sold under false labels (Ogden et al., 2013; Van Uhm & Siegel, 2016). In the last decades, the demographic collapse of sturgeon natural populations and the high demand for caviar on the international market have stimulated a rapid increase of commercial aquaculture programs, mainly aimed at caviar production. The most recent published census of sturgeon aquaculture plants, conducted in 2013 (Bronzi & Rosenthal, 2014), listed 640 sturgeon farms worldwide. This number has more than tripled in four years and over than 2.100 farms are now counted (Paolo Bronzi, personal communication), mostly due to an

exponential increase in Chinese production and in part to the more exhaustive census recently conducted by this country. In parallel, the high performances of interspecific hybrids in terms of growth rate (Jahnichen, Kohlmann, & Renner, 1999) and early gonad maturation (Omoto et al., 2001) prompted a massive increase in their aquaculture production. More than 10 pure sturgeon species and several interspecific hybrids are reared for their caviar, with different yields in terms of production efficiency and product quality. Consequently, mislabeling of caviar is common and often, caviar obtained from less valuable species or hybrids is sold as top quality (Ludwig, Lieckfeldt, & Jahrl, 2015). The most popular brands used to counterfeit commercial caviar are, in order of importance, Beluga, Osietra and Sevruga, respectively produced by *Huso huso*, *Acipenser gueldenstaedtii/persicus* and *A. stellatus*.

In this context, the availability of reliable methods for the validation of the declared species is urgently needed to effectively control illegal trade and commercial fraud in one of the most expensive foods of animal origin (Ludwig, 2008; Ogden et al., 2013). Since 1997, when all sturgeon species were included in the *Appendices* of the Convention on International Trade in Endangered Species (CITES) (Raymakers, 2006), a broad variety of genetic methods have been explored for their applicability to sturgeon species identification (Ludwig, 2008). Currently, mitochondrial regions such as the Cytochrome *b* and the Control Region are used in routine analyses (Johnson & Iyengar, 2015; Krieger, Hett, Fuerst, Artyukhin, & Ludwig, 2008; Ludwig, 2008; Mugue, Barmintseva, Rastorguev, Mugue, & Barminsev, 2008). Mitochondrial genes, however, are usually analyzed by sequencing, making them suboptimal for routine controls in which a cheap and immediate response is required (Ogden et al., 2013); moreover, these markers have incomplete discrimination power for some closely related species (Ludwig, 2008; Ogden et al., 2013) and are not useful for the identification of interspecific hybrids. In fact, mitochondrial markers are maternally inherited and cannot detect paternal contribution, leading to the misclassification of hybrids as belonging to their maternal species. In this way, hybrids can be easily commercialized under the false label of a pure species (Bronzi, Rosenthal, & Gessner, 2011; Mugue et al., 2008). Only in the last few decades have genetic investigations begun to assess the suitability of different nuclear markers for sturgeon species and hybrid identification (Ludwig, 2008). The suitability of these approaches for routine analyses, however, is limited by low reproducibility, high costs, complexity of the protocol or low cross-applicability of isolated markers (Barmintseva & Mugue, 2013; Boscari et al., 2015; Congiu, Fontana, Patarnello, Rossi, & Zane, 2001, 2002; Rozhkovan, Chelomina, & Rachelek, 2008; Yarmohammadi, Shabani, Pourkazemi, & Baradaran Noveiri, 2012).

Recently, Boscari et al. (2014a) developed a Single Nucleotide Polymorphism (SNP)-based method of caviar identification, which looks promising due to its simplicity of application and its applicability across several sturgeon species. The method is based on the application of a panel of primers specifically designed to target species-specific SNPs, identified by the authors within the first intron (RP1) of the nuclear encoded S7 Ribosomal Protein (RP1S7). At present, the method allows for the identification of 7 species (*Acipenser naccarii, A. fulvescens, A. stellatus, A. sinensis, A. transmontanus, A. ruthens* and *A. baerii*) and their hybrids, most of which with 100% accuracy. Unfortunately, using the RPS7 gene, no diagnostic marker was identified for the most valuable sturgeon species, the Beluga sturgeon (*H. huso*), currently detectable only by the mitochondrial DNA analysis.

H. huso is also used for the production of the Bester, one of the most known and commercialized hybrids, widely farmed in Russia, Germany, Hungary, Japan and Italy and sold worldwide (Bronzi et al., 2011; Omoto et al., 2001; Yarmohammadi et al., 2012). The Bester hybrid is obtained by crossing a Beluga female (*H. huso*) with a Sterlet (*A. ruthenus*) male and it is easily misidentified as pure Beluga by mitochondrial DNA analysis.

In the present study, taking advantage of the available transcriptomes of three sturgeon species, *A. fulvescens, A. naccarii* and *A. stellatus* (Hale, McCormick, Jackson, & DeWoody, 2009; Vidotto et al., 2013, 2015), we explored the potential of intronic regions, as suitable markers for species identification in tissue and caviar samples. In particular, we present the results obtained by the analyses at a Single Nucleotide Polymorphism (SNP) located in the second intron (RP2) of the nuclear encoded S6 Ribosomal Protein (RP2S6). This marker complements the RP1S7 tool and represents an important step towards the creation of a standardized and cheap protocol suitable in routine analysis for trade controls for fast identification of sturgeon species and hybrids.

2. Materials and methods

2.1. Sampling and DNA extraction

A total of 406 tissue samples belonging to 11 pure species and 2 interspecific hybrids were analyzed in the present study (Table 1). Individuals of the different species were opportunely selected from different geographical origins or, when possible, based on pedigree information in order to avoid the selection of related specimens. Sixty-five individuals were used for the characterization of the intronic region RP2S6, while a subsequent validation phase was implemented on 341 additional animals. For all tissue samples, genomic DNA was purified from fin clips using the EuroGOLD Tissue DNA Mini Kit (Euroclone) and stored at −20 °C. In addition, 18 caviar samples (Table 1) were used in a sensitivity test. For each caviar, up to three eggs were independently processed and DNA purified using the DNeasy Blood & Tissue extraction kit (Qiagen).

2.2. Intron characterization, cloning and sequencing

The intron RP2S6, characterized in this work, was predicted by aligning assembled transcriptomes of three sturgeon species (*A. fulvescens, A. naccarii* and *A. stellatus*) (Hale et al., 2009; Vidotto et al., 2013, 2015) against three available genomes of teleost fishes (*Takifugu rubripes, Latimeria chalumnae* and *Danio rerio*) (Amemiya, Lander, & Myers, 2006; Brenner et al., 1993; Howe et al., 2013). Fugu genome sequence (fr3 assembly, October 2011) was downloaded from "UCSC Genome Browser web site" (http://hgdownload.soe.ucsc.edu/goldenPath/fr3/bigZips/); while *Latimeria* (latCha1 assembly) and Zebrafish (Zv9 assembly) genome sequences were downloaded from ENSEMBL ftp site (ftp://ftp.ensembl.org/pub/release-75/fasta/).

The transcriptome assembly sequences were aligned on the reference genomes using the program Exonerate (Slater and Birney, 2005) setting the model option to "est2genome". To retrieve intron conservation across multiple genomes we used several homemade perl scripts. The pipeline is composed by different steps. Briefly, Exonerate outputs were parsed, cDNA sequence mapping coordinates as well as teleost genomes introns coordinates were retrieved. In a second step, a comparison within each reference genome was performed. During this step, introns identified by multiple transcriptome libraries were retrieved and checked for consistency. Finally, introns shared by all the genomes and confirmed by a high number of sequences were selected for further analysis.

Two primers were designed on the exon flanking regions (RP2S6_F 5′-TTCATGGGGAAACCCTGCTT3′ and RP2S6_R 5′-ATCCTCTGGGTGAGGAGTG-3′) to amplify and sequence the predicted RP2S6. Touch Down (TD)-PCR conditions were as follows: a