Genetic identification of the caviar-producing Amur and Kaluga sturgeons revealed a high level of concealed hybridization

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ABSTRACT

China has recently become the leader country for sturgeon aquaculture and caviar production, deeply changing the traditional geography of this market in few years. As a consequence, some species originating from the Far East Asia increased their economic relevance, joining the ones traditionally harvested for caviar. In this context, the possibility to reliably and promptly identify these species on the market has increasing importance for the enforcement of control actions against illegal trade or commercial frauds. The present study focuses on two commercially relevant species, massively reared in China not only as pure species but also as reciprocal hybrids: the Amur (*Acipenser schrenckii*) and Kaluga (*Huso dauricus*) sturgeons. We assess the identification power of two putatively diagnostic markers isolated from two predicted introns of the nuclear coding gene *Ribosomal Protein* L*8*. The markers were tested on tissue or caviar of 508 individuals of the two species and 31 hybrids. In order to compare results across loci, most individuals were also checked at two already published microsatellite markers, with a good, even if incomplete, identification efficiency for the two species. No marker showed fixed alternative alleles between Amur and Kaluga sturgeons, confirming the difficulty of distinguishing these two sympatric species in spite of the marked morphological differences and the consequent classification into different genera. So far, the multi-locus panel here used represents the more effective tool for the genetic identification of pure Amur and Kaluga sturgeons and resulted to be fully efficient to validate caviar and tissues obtained from hybrids between the two species.

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1. Introduction

Sturgeons are a group of about 25 fish species widely distributed in the North Hemisphere and mostly appreciated for the delicacy of their eggs, the black caviar, one of the most valuable and refined food of animal origin (Fain, Straughan, Hamlin, Hoesch, & LeMay 2013). Nowadays, natural populations are almost collapsed due to severe overfishing that has led most species to the brink of extinction (International Union for Conservation of Nature - IUCN, http://www.iucnredlist.org). Since 1998, all sturgeon species have been listed in the *Appendices* of the *Convention on International Trade for Endangered Species* (CITES) and strong protection measures have been established to limit harvesting of wild populations. In this context, the interest in sturgeon farming as an alternative source of caviar has grown rapidly. The geography of caviar industry has also changed rapidly and, untied from natural populations and no longer confined to the traditional "caviar" areas, underwent a global diffusion (Bronzi & Rosenthal, 2014; Bronzi, Rosenthal, &

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Gessner, 2011; Fain et al., 2013). The increase of the caviar industry in China and its impact on the international market grew rapidly. According to FAO statistics, this happened in response to the export permissions granted in China since 2006. Estimates showed that in 2014 more than 85% of global sturgeon production comes from China, which currently ranked as first producer country in the world (FAO Fishstat Database). Among the more represented species reared in China, besides the Siberian sturgeon (*Acipenser baerii*), especially relevant are the Amur (*A. schrenckii*) and Kaluga (*Huso dauricus*) sturgeons, two tetraploid species which are cultured as pure species and interbred to produce commercially valuable and fertile hybrids (Wei, Zou, Li, & Li, 2011). In detail, these three species and their hybrids account for more than the 90% of the Chinese sturgeon products (Wei et al., 2011; Shen, Shi, Zou, Zhou, & Wei, 2014) that increased from 14,827 to 75,920 tons in only 8 years (from 2006 to 2014, FAO Fishstat database). Noteworthy, products obtained from Amur and Kaluga sturgeons qualitatively compete on par with the top quality brands. In fact, Kaluga caviar is considered similar to "Beluga" (*Huso huso*), while the caviar produced by Amur sturgeon is comparable to "Osietra" (*Acipenser gueldenstaedtii*).

The two species are endemic of the Amur River with overlapping distributions and compatible reproductive cycles documented by observed events of natural hybridization (Chelomina Rozhkovan, & Ivanov, 2008; Krykhtin & Svirskii, 1997; Shedko & Shedko, 2016; Wei et al., 1997). In spite of the Critically Endangered status of their natural populations, assigned by IUCN in 2010, both the Kaluga and Amur sturgeons are massively reared in aquaculture. They are also used to produce the two reciprocal interspecific hybrids, among which the more common is obtained by crossing Kaluga females and Amur males. The official introduction of valuable products of Amur and Kaluga sturgeons and their hybrids to the world market, never commercialized outside China before 2006, raises the problem of their identification in trade. Presently, the only available approach for the genetic identification of these two species is based on the analyses of mitochondrial DNA. Mitochondrial markers however, for their maternal inheritance, don't allow the identification of the paternal contribution and cannot be applied for the identification of interspecific hybrids, of which they identify only on the maternal species. In order to trace the genetic contribution of both parental species, species-specific polymorphisms located on the nuclear DNA must be identified and used to develop diagnostic tests. Recent efforts in this direction were made by different research groups, allowing to set up cheap and easy-to-use identification tools for several other sturgeon species and hybrids (Barmintseva & Mugue, 2013; Boscari et al., 2017, 2014; Havelka, Fujimoto, Hagihara, Adachi, & Arai, 2017). In the present work, we took advantage from a previous research published by Boscari, Pujolar, Dupanloup, Corradin, and Congiu (2014) in which a diagnostic SNP was identified in the first intron of the nuclear coding gene *Ribosomal Protein S7* (RP1S7). This SNP allows the distinction of the Amur-Kaluga complex from the other commercially relevant species. With the aim of distinguishing the two species one from each other, we examined the intra- and inter-specific variability at two introns of the nuclear coding gene *Ribosomal Protein L8* (RPL8). The existence of these introns were predicted *in silico* by aligning the transcriptome of three sturgeon species against the available genomes of three teleost species.

The genetic heritage of Amur and Kaluga sturgeons is likely shuffled by some degree of admixture both in captivity and in nature. Hybrids, as well as different levels of backcrosses, are massively produced in aquaculture and also animals that based on morphology could be classified as pure species might hide extraspecific genetic contributions. In this context, the detection of

private genetic traits can be challenging and the simultaneous use of different diagnostic markers in a multi-locus approach might be necessary. For this reason, results obtained with the new markers here proposed were compared with two microsatellites already proposed by Barmintseva and Mugue (2013) for their good diagnostic power. This approach revealed the importance of having multiple unlinked markers for species and hybrids identification, especially in context in which genetic boundaries are not clear like between the Amur and the Kaluga sturgeons.

2. Materials and methods

2.1. Sampling and DNA purification

All 508 Amur and Kaluga, and their hybrids, analysed in the present study as tissue (fin clip) or caviar samples are reported in Table 1. In order to provide a more complete information, 317 individuals of 10 commercially important sturgeon species were also analysed (65 *H. huso*, 38 *A. gueldenstaedtii*, 5 *A. persicus*, 52 *A. baerii*, 36 *A. naccarii*, 11 *A. transmontanus*, 40 *A. fulvescens*, 26 *A. stellatus*, 15 *A. sinensis* and 29 *A. ruthenus*) for a total of 825 animals.

Moreover, in order to confirm the presence of at least one of the two target species, all Amur and Kaluga samples were preliminary checked by analysing available markers: the RP1S7 marker specific for the two species (Boscari et al., 2014) and the mitochondrial Control Region (CR) which was either sequenced or analysed by PCR according to the protocol proposed by Mugue, Barmintseva, Rastorguev, Mugue & Barminsev. (2008). For all animals, genomic DNA was extracted using the DNeasy® Blood & Tissue extraction kit (Qiagen), following the manufacture's protocol and stored at −20 °C. For caviar samples, up to three eggs were independently processed and DNA purified using the DNeasy Blood® & Tissue extraction kit (Qiagen).

Prior to the analysis, all DNA samples were checked for quality and quantified by Nanodrop 2000c (NanoDrop Technologies).

2.2. Development of the RPL8 tool

2.2.1. Isolation of loci
Intron prediction was performed by comparing assembled

Table 1
Samples of Amur and Kaluga sturgeons and their hybrids analysed in the present study. For each species, the number of animals are grouped according to the laboratory of origin and laboratory in which the samples were analysed.

Species (Total N)	N	W/A/U	Sample origin	Tested by
H. dauricus (176)	20 T	A	YFI	UNIPD
	32 T	A	YFI	YFI
	19 T	W	VNIRO	UNIPD
	54 T	28W/26U	VNIRO	VNIRO
	10 C	U	VNIRO	VNIRO
	41 T	W	FSCEATB	FSCEATB
A. schrenckii (332)	23 T	A	YFI	UNIPD
	42 T	A	YFI	YFI
	20 T	W	VNIRO	UNIPD
	196 T	193W/3U	VNIRO	VNIRO
	10 C	10U	VNIRO	VNIRO
	41 T	W	FSCEATB	FSCEATB
Hybrids (31)	21 T	A	VNIRO	VNIRO
(H. dauricus X A. schrenckii)	10 C	A	VNIRO	VNIRO

Total N - total number of analysed individuals.
N - number of individuals *per* sample origin; T - Tissue; C − Caviar.
W/A/U − Wild Origin/Aquaculture origin/Unknown.
VNIRO - All-Russia Research Institute of Fisheries and Oceanography.
FSCEATB - Federal Scientific Center of the East Asia Terrestrial Biodiversity.
YFI - Yangtze River Fisheries Research Institute.
UNIPD - University of Padova.