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

Elisa Boscari [a], Anna Barmintseva [b, c], Shuhuan Zhang [d], Huamei Yue [d], Chuangju Li [d], Sergei V. Shedko [e], Dietmar Lieckfeldt [f], Arne Ludwig [f, g], Qiwei W. Wei [d], Nikolai S. Mugue [b, c], Leonardo Congiu [a, g, *]

[a] Department of Biology, University of Padova, Padova, I-35131, Italy
[b] Russian Federal Research Institute of Fisheries and Oceanography, Moscow, 107140, Russia
[c] Institute of Developmental Biology RAS, Moscow, 117808, Russia
[d] Key Laboratory of Freshwater Biodiversity Conservation, Ministry of Agriculture of China. Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, 430223, China
[e] Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, 690022 Russia
[f] Leibniz-Institute for Zoo and Wildlife Research, Department of Evolutionary Genetics, Berlin, 10252, Germany
[g] IUCN - Sturgeon Specialist Group

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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 L8*. 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, &

* Corresponding author. Department of Biology, University of Padova, Padova, I-35131, Italy.
E-mail address: leonardo.congiu@unipd.it (L. Congiu).

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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 extra-specific 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.

transcriptomes of three sturgeon species (*A. fulvescens, A. naccarii* and *A. stellatus*) (Hale, McCormick, Jackson, & DeWoody, 2009; Vidotto et al., 2013, 2015) against three available genomes of teleosts (*Takifugu rubipres, Latimeria chalumnae* and *Danio rerio*) (Amemiya et al., 2013; Brenner et al., 1993; Howe et al., 2013), as reported in detail in Boscari et al. (2017). Out of the 1867 predicted introns, ten were selected for sequencing and primer pairs matching the corresponding exon-flanking regions designed. After a preliminary characterization conducted on up to 10 individuals (5 *A. schrenckii* and 5 *H. dauricus*), the fourth (RP4) and fifth (RP5) introns of the nuclear coding gene *Ribosomal Protein L8* (RPL8) showed putatively diagnostic polymorphisms and were further investigated. The primer pairs used to amplify the two introns were: RP4L8_F – RP4L8_R (5′GAAAGTAATCTCCTCTGCCAAC3′ - 5′AATACGACCACCACCAGCAA3′) (Fig. 1) and RP5L8_F – RP5L8_R (5′GTCCGTGGTGTGGCTATGAA3' - 5′AATGTGCTGATGGTTACCACC3′) (Fig. 2).

The amplification of the intron RP4L8 was performed using the following settings: 2 min at 94 °C, 35 cycles at 94 °C for 30″, 60 °C for 45″ and 72 °C for 60″, followed by a 7′ extension at 72 °C.

To amplify the intron RP5L8, the thermal profile was optimized as follows: 2 min at 94 °C, 35 cycles at 94 °C for 30″, 58 °C for 30″ and 72 °C for 30″, followed by a 7′ extension at 72 °C.

In order to characterize all different alleles in each specimen and identify possible duplicated loci, PCR products obtained by each sample were cloned in JM109 competent cells using the P-GEM-T Easy Vector (Promega) following the manufacturer's recommendations.

A total of 20 clones *per* locus were sequenced after purification of the PCR products. All sequences obtained from each intron were aligned using MEGA6 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) and checked for the presence of species-specific mutations.

2.2.2. Characterization of the RP4L8 and RP5L8 introns

Exploring the interspecific variability at the RP4L8 intron, two SNPs putatively diagnostic for the species *H. dauricus* were detected. To this regard, two primers were designed with their 3'-end matching the two nucleotides apparently fixed in all the Kaluga sequences: RP4L8_H.dau_F (5′CAAGTTCAGAACACAAACAAAGGA3′) and RP4L8_H.dau_R (5′TGGACTATTTTCTCAAGACAAATGC3′) (Fig. 1). The primers were built with the second to last nucleotide at the 3'-end as not complementary to the target sequence ensuring a double-nucleotide mismatch if paired to non-Kaluga DNAs. The size of the amplification products in Kaluga samples is expected to be 213 bp, while no bands is expected in Amur specimens.

At the RP5L8 intron, the forward primer RP5L8_groupA_F (5′TCCGTGGTGTGGCTATGATT3′) has been projected to pair with the reverse primer RP5L8_A.sch_R (5′AGTGCAATAAACTACTTCTGTG3′) (Fig. 2). The last one is complementary to an insertion putatively specific for the Amur sturgeon. A positive amplification showing a band 146 bp long is expected in Amur individuals while no amplification should be observed in Kaluga samples.

The following thermal profile was finally set up for both markers: a first denaturation step at 95 °C for 3′, 35 cycles at 95 °C for 30″, 61 °C for 20″, 72 °C for 30″ and a final elongation step at 72 °C for 7'.

Thus, the detection power of both introns was evaluated in different laboratories (Table 1) on 171 Kaluga and 327 Amur specimens, and on 31 animals of sure hybrid origin. Both primer pairs were also tested on other 10 sturgeon species. All PCR results were checked by 1.8% agarose gel electrophoresis stained with GelRed®.

2.3. Microsatellite analysis

In order to evaluate if a multi-locus approach may increase the frequencies of correct identification between the Amur and Kaluga species, we checked results of our RPL8 markers with two microsatellite loci (An20 and AfuG41), already proposed by Barmintseva and Mugue (2013), with a good detection power for the two species.

The locus An20 is known for having alleles 137 and 169 with strongly unbalanced frequencies in the Amur and in the Kaluga species. The allele 169 has been detected with an allele frequency of 0.5 in Kaluga sturgeon populations. This corresponds to a low incidence of about 7% (6.25%) of individuals in which the allele cannot be detected (Barmintseva & Mugue, 2013). On contrary, the allele 137 has been found with a frequency of 0.8 in Amur populations, that makes expectable the presence of at least one copy of this allele in almost all *A. schrenckii* genotypes Barmintseva and Mugue (2013).

The locus AfuG41 behaves as presence/absence marker and the absence of amplification is expected only from pure *H. dauricus* while the presence is always observed in other species.

The above microsatellites were used to screen 249 Amur, 110 Kaluga individuals and 31 hybrid samples.

All loci were amplified following the conditions reported in their original reference and genotyped at the external service BRM genomics (http://www.bmr-genomics.it/). The scoring was performed using the GeneMarker software version 1.95 (Soft Genetics LLS®). The final concentrations of the PCR reagents are the same above reported.

3. Results

3.1. Preliminary investigations on sampling

The obtained sequences of mitochondrial Control Region and/or the mitochondrial specie-specific bands resulted after the amplification with the mtDNA tool, proposed by Mugue and colleagues in 2008, confirmed the presence of the declared species origin in all samples used in the present study, providing also an indication of the maternal lineage (data not shown).

Moreover, the RP1S7 Amur/Kaluga specific-band at 223 bp was successfully amplified in all the declared Amur and Kaluga individuals as expected. The above result guarantees the presence of at least one of the two target species in those samples, even if the presence of hybrids or introgression signals cannot be excluded. No amplification was obtained from all the other sturgeon species with this marker, as expected.

3.2. Results of intron characterization and validation

3.2.1. The RP4L8 intron

The amplification of the intron RP4L8 produced a fragment of about 570 bp in sturgeons (Fig. 1) against the 118 bp in *T. rubipres*, 3121 bp in *L. chalumnae* and 1585 bp in *D. rerio*.

The RP4L8 sequences showed two polymorphisms in positions 113 and 278 of the alignment in Fig. 1. These mutations were selected to design *Kaluga*-specific primers expected to amplify a band 213 bp long specifically from Kaluga samples, as described in the paragraph 2.2.2.

As expected, all the 176 Kaluga and 31 hybrid samples checked for the presence of the Kaluga-band yielded positive amplification. Unexpectedly, however, also the 8.7% (29 out of 332) of Amur samples showed the same band, that corresponds to an allele frequency of about 0.02 in the tetraploid Amur sturgeon. These animals represent the 7.8% of the wild animals (20 out of 254) and the

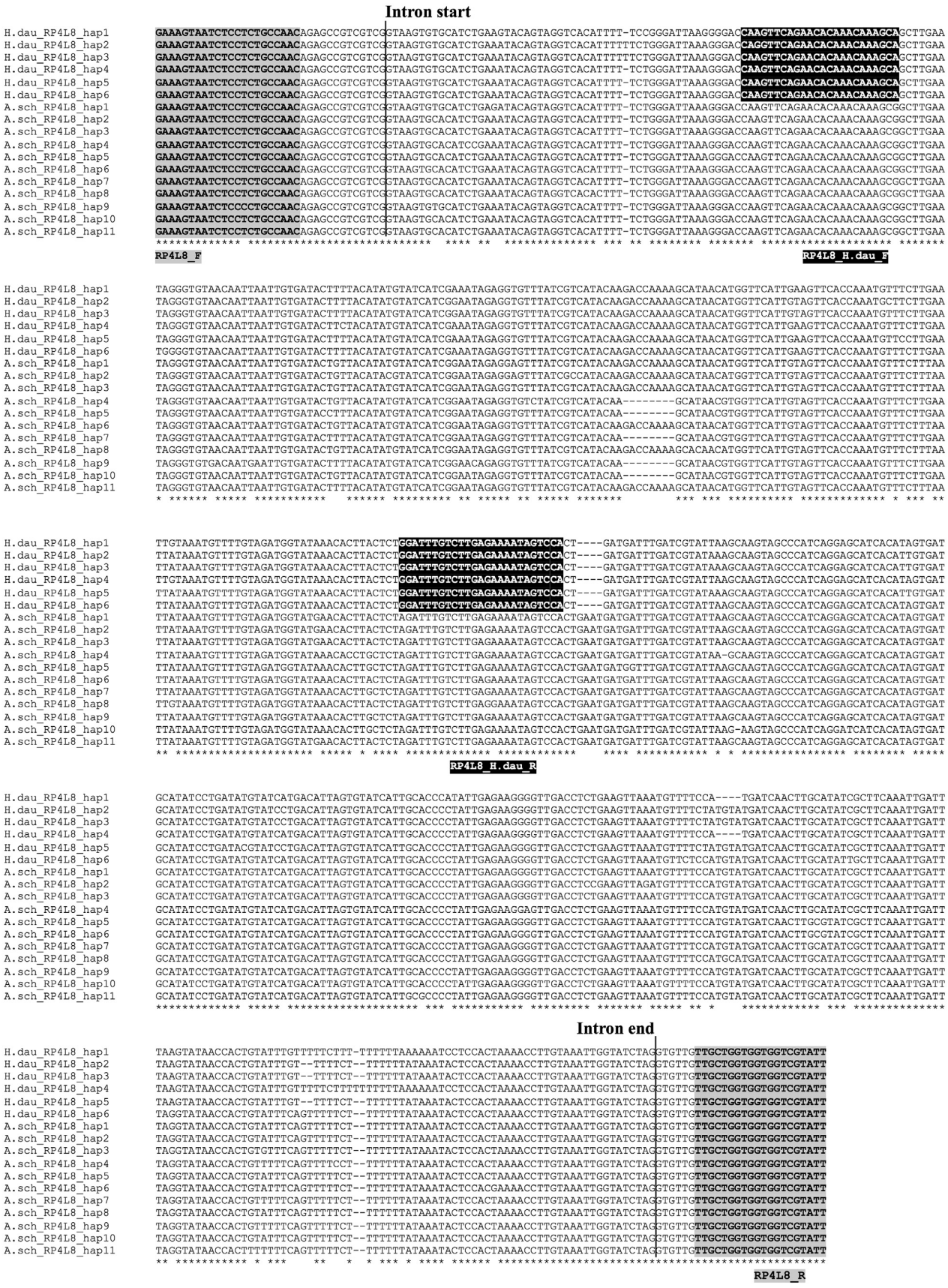

Fig. 1. Alignment among all the different haplotypes detected at the fourth intron of the *Ribosomal Protein L8* gene (RP4L8) in the Amur (*Acipenser schrenckii*) and Kaluga (*Huso dauricus*) species (Accession numbers: MF429895-MF429911). Shaded regions correspond to primers projected to amplify this locus (the name of each primer is reported below the corresponding region while the original sequence of each primer is reported in the paragraph 2.2.1 and 2.2.2).

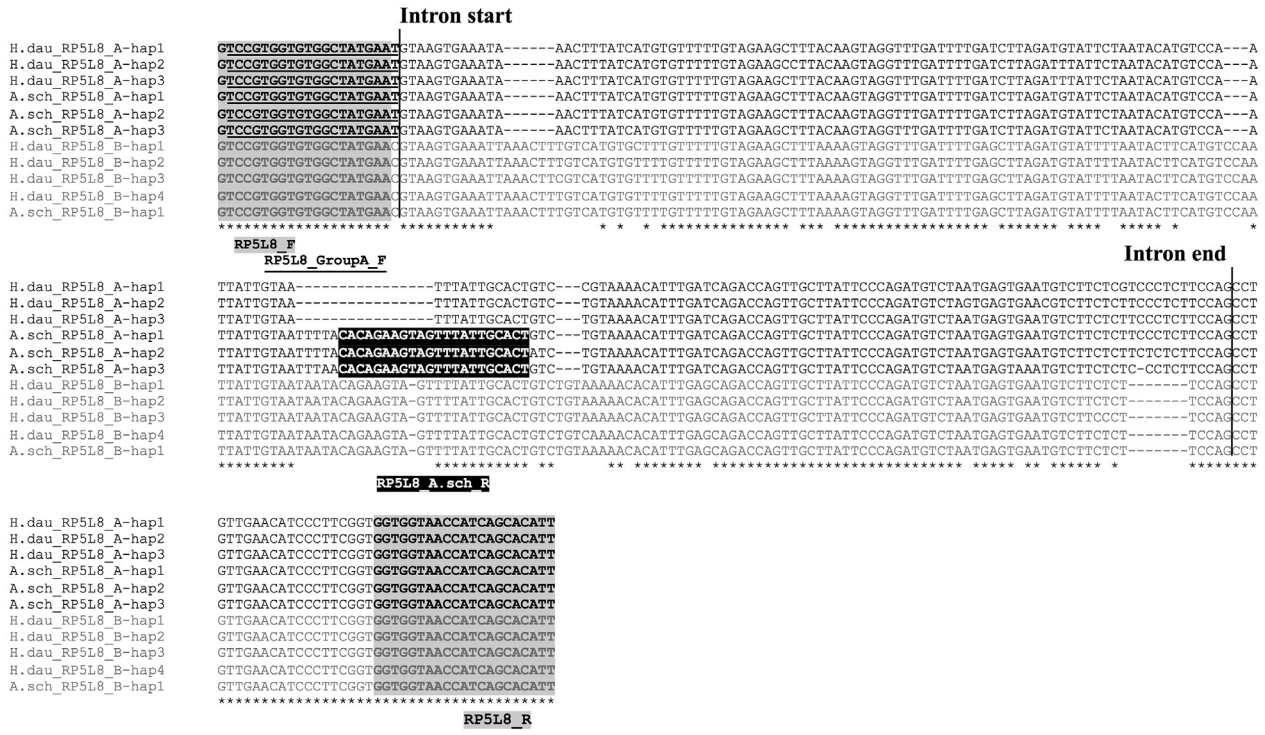

Fig. 2. Alignment among all the different haplotypes detected at the fifth intron of the *Ribosomal Protein L8* gene (RP5L8) (group A and group B) in the Amur (*Acipenser schrenckii*) and Kaluga (*Huso dauricus*) species (Accession numbers: MF429912-MF429922). Shaded/underlined regions correspond to primers projected to amplify this locus (the name of each primer is reported below the corresponding region while the original sequence of each primer is reported in the paragraph 2.2.1 and 2.2.2).

13.8% (9 out of 65) of the aquaculture samples.

The good specificity of the RP4L8 Kaluga-marker however, is confirmed by its absence from all the 317 individuals of the other 10 non-target species.

3.2.2. The RP5L8 intron

The amplification of the region RP5L8 showed a short PCR fragment of 280 bp in sturgeons (Fig. 2) against the 941 bp in *T. rubipres*, 2371 bp in *L. chalumnae* and 89 bp in *D. rerio*.

After cloning, the RP5L8 showed two clearly distinct groups of sequences (group A and group B, Fig. 2), probably indicating a duplication of this region. This result is similar to what observed in other introns of the S7 and S6 sturgeon genes encoding for ribosomal proteins (Boscari et al., 2014, 2017). The hypothesis of duplication is supported by the co-occurrence of both types of sequence in the same individual (data not shown). In the group A of the RP5L8 region, a putatively specific insertion of 16 bp was detected for the Amur species, as shown in Fig. 2. In that position, a primer was designed to selectively amplify Amur samples when paired with a forward one which was indeed selective for the groupA-sequences.

The expected band at 146 bp was observed in all the 332 Amur and 31 hybrid samples; however, also the 13.1% (23 out of 176) of Kaluga samples yielded positive amplification at this locus corresponding to an allele frequency of 0.03 in the tetraploid Kaluga sturgeon. These animals represent the 19.3% of the wild animals (17 out of 88), the 3.8% (2 out of 52) of the aquaculture samples and the 11.1% (4 out of 36) of samples with unknown origin that however include all caviar samples that are likely of aquaculture origin.

Among the other 10 sturgeon species tested with the RP5L8 Amur-marker, the following individuals yielded positive amplification: 20/65 *H. huso*, 21/38 *A. gueldenstaedtii*, 0/5 *A. persicus*, 26/52 *A. baerii*, 19/26 *A. stellatus*, 36/36 *A. naccarii*, 40/40 *A. fulvescens*, 11/11 *A. transmontanus*, 16/29 *A. ruthenus* and 0/15 *A. sinensis*.

3.3. Microsatellites results

Since in the case of frequency-based diagnostic markers with suboptimal identification power the chance to detect the contribution of the different species involved increases by using several unlinked loci, most samples used for the validation of the RPL8 tool were also analysed at two microsatellite loci: An20, known to have strongly unbalanced frequencies in the two species and AfuG41 expected to not amplify at all in Kaluga samples.

The genotyping of An20 showed the Amur allele (137 bp) in all the Amur samples, and in 17 out of the 110 Kaluga individuals; while the Kaluga allele (169 bp) has been detected in all the Kaluga samples, and in 9 out of 249 Amur sturgeon individuals. The above results confirm that, also at locus An20, the segregation of alternative alleles in the two species is not complete. For what concerns the 31 known hybrids checked, only 14 animals showed the expected co-presence of the two alleles, while for 17 individuals the hybrid condition was not correctly detected by microsatellites. In fact, 11 animals presented only the dominant Kaluga-allele, while 6 presented only the Amur one. However, it is worth noting that for frequency-based markers a lower percentage of individuals presenting a diagnostic band is expected in hybrids. This is because only half of the allele copies are transmitted to the hybrid by each parental species, thus halving the chances of inheriting the expected band.

In agreement to what observed in Barmintseva and Mugue (2013), no amplification was obtained at locus AfuG41 from 88 out of the 110 declared Kaluga samples, confirming their pure origin. For the 22 individuals that yielded an amplification product, some degree of hybridization with other sturgeon species can be hypothesized as shown later by the multi-locus comparison. All the

249 declared Amur animals showed, as expected, the positive amplification at the locus AfuG41 that however, as explained above, is not Amur-specific.

3.4. Multi-locus comparison

Most unexpected positive amplifications obtained with different diagnostic markers and interpreted as signals of a sub-optimal detection power for the Amur and Kaluga species were concordant across markers.

Comparing results among mtDNA and the three nuclear markers (RPL8 tool, An20 and AfuG41), 307 out of 390 tested samples hypothesized to be pure, showed concordant results among the four markers. In detail, while for 300 animals (Line 1, 8 and 13 in Table 2) all markers confirmed the declared species (or hybrid), for 7 animals declared to be pure Kaluga or pure Amur sturgeons, the three nuclear markers detected hybrid condition (Line 2 and 9 in Table 2).

For the remaining 83 animals an incomplete concordance across markers was observed, probably due to an incomplete detection power and/or to the presence of samples with different degrees of hybridization. We decided to follow the criterion that when at least two markers were discordant with the declared species, an alternative classification was proposed. Here below, a detailed description of cases reported at lines 4, 5, 6, 7, 10 of Table 2 is provided.

Out of the 22 Kaluga that presented an amplification product at locus AfuG41, the three animals reported at line 4 are the only ones for which this discordance from the expectance is not confirmed either by RPL8 or by An20. According with the above criteria of at least two discordant markers, the classification of these three animals was not changed, however, this would be the first time to our knowledge that this locus gives an amplification product in pure Kaluga. On the other hand, the presence of an amplification product at locus AfuG41 is observed in most other species. For this reason, the possibility that these samples are hybrids between a Kaluga female and a male of an unidentified species cannot be excluded. In any case, the purity of these three animals should be considered with caution.

At lines 5, 6 and 7, the presence of the RP5L8 band, the allele 137 at locus An20 and the "presence" allele at locus AfuG41 indicate that these 13 animals are almost surely not pure Kaluga specimens. In particular, the "presence" allele at locus AfuG41 clearly indicate a hybrid condition while the RP5L8 at line 5 and the allele 137 at lines 6 and 7, which are both highly frequent in the Amur sturgeon suggest that these animals are actually *H. dauricus X A. schrenckii*. However, since these two markers are also observed in other species with different frequencies, those animals might also be hybrids between a Kaluga female and a male of a different species than the Amur sturgeon. For this reason, the presence of a prudent question mark in the proposed classification in Table 2 is necessary.

At line 10, the presence of the RP4L8 band in individuals declared to be Amur sturgeons should be carefully interpreted as possible trace of hybridization. As better explained in the discussion section, the occurrence of this band not only in the Kaluga species but also in some individuals of the Amur sturgeon is, in our opinion, a trace of the multiple contacts occurred between the two species. That is why we decided to purpose an alternative classification, even though only Kaluga marker showed discordance with the declared species.

All markers used in the present study are arranged in a table as supplementary information, reporting the estimated allele frequencies and the observed percentage of individuals showing each marker in Kaluga and Amur sturgeons (Suppl. Table 1). Moreover, in order to assist the application of the multi-locus approach proposed in the present paper, a workflow diagram is also available as supplementary material (Suppl. Fig. 1).

Table 2
Results of multi-locus genetic characterization for the 249 Amur, 110 Kaluga and 31 hybrid samples analysed at all loci. The 15 observed multi-locus combinations (genotypes) are reported in different rows. Columns report the declared species or hybrid; the species assessed by mtDNA; the observed alleles at locus RPL8; the observed allele at An20 microsatellite; the presence (p) or absence (a) of amplification product at AfuG41 microsatellite locus. The proposed classification based on multi-locus analyses is also provided, as well as the number of samples (N) showing each multi-locus combination (T = Tissue; C = Caviar). The origins of samples are reported as wild (W), aquaculture (A) or unknown (U). Shaded cells highlight discordances with the declared species.

	Declared species	mtDNA	Nuclear markers (specificity)					Proposed classification	N.	W/A/U
			RPL8		An20		AfuG41			
			RP4 (Kaluga marker)	RP5 (Amur marker)	169 (Kaluga allele)	137 (Amur allele)	p/a (a → Kaluga)			
1	Kaluga	Kaluga	X	–	X	–	a	Kaluga	66T, 8C	35W/18A/21U
2	Kaluga	Kaluga	X	X	X	X	p	Kaluga X Amur	5T, 1C	3W/2A/1U
3	Kaluga	Kaluga	X	X	X	–	a	Kaluga	13T, 1C	11W/3U
4	Kaluga	Kaluga	X	–	X	–	p	Kaluga	3T	2W/1U
5	Kaluga	Kaluga	X	X	X	–	p	Kaluga X ?	3T	3W
6	Kaluga	Kaluga	X	–	X	X	p	Kaluga X ?	5T	5U
7	Kaluga	Kaluga	X	–	–	X	p	Kaluga X ?	5T	5U
8	Amur	Amur	–	X	–	X	p	Amur	202T, 10C	185W/14A/13U
9	Amur	Amur	X	X	X	X	p	Amur x Kaluga	1T	1W
10	Amur	Amur	X	X	–	X	p	Amur x Kaluga	28T	19W/9A
11	Amur	Amur	–	X	X	X	p	Amur	6T	6W
12	Amur	Amur	–	X	X	–	p	Amur	2T	2W
13	Kaluga x Amur	Kaluga	X	X	X	X	p	Kaluga X Amur	9T, 5C	14A
14	Kaluga x Amur	Kaluga	X	X	X	–	P	Kaluga X Amur	8T, 3C	11A
15	Kaluga x Amur	Kaluga	X	X	–	X	P	Kaluga X Amur	4T, 2C	6A

Declared species - name of the nominal species of the samples (Kaluga = *Huso dauricus*; Amur = *Acipenser schrenckii;* Kaluga x Amur = *H. dauricus* female *x A. schrenckii* male).
mtDNA - mitochondrial DNA.
RPL8 - *Ribosomal Protein L8 gene;* RP4 - fourth intron of the *Ribosomal Protein L8 gene* corresponding in the text to the Kaluga-marker; RP5 - fifth intron of the *Ribosomal Protein L8 gene* corresponding in the text to the Amur-marker.
An20 - microsatellite locus An20; 170 – Kaluga-specific allele; 137 – Amur-specific allele.
AfuG41 - microsatellite locus AfuG41; p – presence; a – absence that is interpreted as Kaluga specific.
W/A/U – Wild Origin/Aquaculture origin/Unknown.

4. Discussion

The original goal of this study was the characterization of two introns, RP4L8 and RP5L8, with the final aim of isolating new markers for the identification of the Amur and Kaluga sturgeons, two partially sympatric species with emerging role in the international caviar market. Even though no fixed diagnostic markers have been detected, two single nucleotide polymorphisms were identified within intron RP4L8 that showed promising specificity for the Kaluga sturgeon. Diagnostic primers designed on these SNPs allowed the amplification of a 213 bp fragment from all the Kaluga individuals. No amplification product was obtained from any of the other species with the only exception of few Amur sturgeons. The marker isolated on RP5L8, with the aim of specifically identify the Amur sturgeon, successfully amplified the expected band from all the Amur sturgeons but showed a lower specificity, as its amplification was observed in most species analysed with variable frequencies.

The two RPL8 markers usefully complemented the information yielded by the microsatellite loci, formerly proposed as identification tools by Barmintseva and Mugue (2013). This allowed disclosing probable hybrids among the animals considered to be of pure origin and used as reference samples. Specifically, some discordances between the nominal species and the results obtained with the RPL8 marker were confirmed by the two microsatellites, raising perplexities about the purity of the sample used. We think that the incomplete identification power shown by our markers as well as by the ones previously proposed for Amur and Kaluga sturgeons are, at least in part, due to some degree of hybridization among the two species. This hybridization could be either human driven or due to past events occurred in nature and documented for the Amur and Kaluga species (Chelomina et al., 2008; Krykhtin & Svirskii, 1997; Shedko & Shedko, 2016; Wei et al., 1997).

The massive production of hybrids between the two species for aquaculture purposes and their translocation in different Chinese provinces started in the late 1990s (Wei et al., 2011). During these 25 years, the progeny of these fertile hybrids probably reached the third generation of uncontrolled admixture. Hybrids are fertile and can be crossed among each other or backcrossed with parental species. Presently, a heterogeneous variety of animals with different degrees of hybridization probably exists in captivity. These hybrids represent a relevant risk of genetic contamination of natural population, especially if reared in floating cages like the ones that in September 2016 caused a massive escape of non-autochthonous species and of interspecific hybrids in the Yangtze River (Wei Qiwei personal communication).

For what concerns the situation in the wild, hybrids between the Amur and the Kaluga sturgeons were intentionally released in the Amur River (Chelomina et al., 2008) with a negative effect on the genetic integrity of the two parental species.

Predating the impact of human activities in the Amur River basin, the occurrence of a certain rate of ancestral natural hybridization between the two largely sympatric species cannot be excluded as observed for other sturgeon species (Ludwig, Lippold, Debus, & Reinartz, 2009).

For the above reasons, the genetic boundaries between the two species are not well defined. Consequently, the identification of private genetic traits to be used as forensic markers on commercial products is difficult if not impossible.

The only way forward to increase the probability of detecting signals of remote hybridizations is the simultaneous genotyping of different nuclear loci. In this perspective, the identification of additional loci with a certain identification power remains a priority.

The presence of hidden genetic admixture and the consequent uncertainty of purity represent a critical problem also for the choice of individuals used as reference samples. In fact, animals supposed to be pure might hide some hybridization event in their genealogy. This could explain the trouble encountered in this work to identify the two species with a complete detection power. Without certainty of purity of the reference samples, like in the case of the Amur and Kaluga specimens, it becomes very hard to understand if the unexpected presence of a putative diagnostic marker in a non-target species reflects a real incomplete specificity or, alternatively, if it is due to horizontal transfer through hybridization. To solve this ambiguity, the analyses of the marker in several other species can be of help. In fact, the occurrence of a given marker in several species with different geographical distributions and without ecological or aquaculture contacts, represents a clear evidence of aspecificity. This is the case of the RP5L8 marker, which was originally isolated with the aim of selectively amplify the Amur sturgeon and not the Kaluga. Extending the analyses to other species, RP5L8 occurrence was observed in most tested species with different frequencies, clearly showing an aspecific pattern of distribution among sturgeons.

On contrary, the RP4L8 here designed for the Kaluga identification showed a very interesting specificity, being present in all Kaluga individuals and absent from all other species with the only exception of few Amur sturgeons. A possible explanation for this result is that the marker is actually Kaluga-specific; its occurrence, being limited to a small minority of animals of a sympatric species with several ecological and aquaculture contacts, could represent a trace of hybridization. The combined application of RP4L8 marker here developed with the RP1S7 markers (Boscari et al., 2014) can be used to identify the Amur sturgeon by nuclear markers as follows. First, the positive amplification of 223bp band at RP1S7 unambiguously identifies the presence of Kaluga and/or Amur genomes; second, the concomitant absence of RP4L8 band, excludes the presence of Kaluga sturgeon, indirectly detecting the presence of Amur sturgeon.

Following the same criterion, Kaluga and Amur can be also identified as paternal species in hybrids with other species. In this case, the 223 bp band at RP1S7 indicates the Kaluga or Amur as paternal species while the mtDNA detects a different maternal contribution. The paternal species can be further specified either through the multi-locus approach in the case of Kaluga, or by the absence of RP4L8 band in the case of Amur, as above described. The latter case is especially interesting since other important hybrids between Siberian (*A. baerii*) or Russian (*A. gueldenstaedtii*) females and Amur males are also utilized in aquaculture (Wei et al., 2011; Shen et al., 2014).

Moreover, the 100% of positive amplification of RP4L8 in the Kaluga and of RP5L8 in the Amur sturgeon ensure their suitability as validation markers on the F1 hybrids between the two species (Amur x Kaluga or Kaluga x Amur) as well as on their caviar. As confirmed by the analyses performed on 31 known hybrids, the presence of both bands specific for the two parental species is always expectable. This does not mean that the presence of both bands is diagnostic for one of the above hybrids: also a small percentage of Kaluga presents the two bands and they are both expected also in hybrids between Kaluga and the many other species, in which the RP5L8 band have a certain frequency. However, if caviar is labelled as Amur x Kaluga or Kaluga x Amur, the two bands must both be present and the absence even of one of them should be considered as mislabeling evidence.

5. Conclusion

Notwithstanding the panel of markers here used represents the more effective tool for the genetic identification of *Acipenser*

schrenckii and *Huso dauricus* and in spite of their classification into different genera and their pronounced morphological differences, distinguishing the two species resulted to be an unexpected challenge. Our hypothesis is that the genetic boundaries between the two species have been violated either by natural hybridization encouraged by the extreme depletion of the two species, or by artificial production of hybrids with aquaculture purposes, followed by careless or accidental releasing of hybrids into the wild. The new markers here presented, in combination with the ones already available, provide a powerful tool, even though not 100%, for discriminating pure Kaluga, Amur and their hybrids with other species, strongly improving the current ability to distinguish their commercially relevant products.

Moreover, the new markers are fully efficient for the validation of tissues and caviars obtained from hybrids between Amur and Kaluga and *vice versa*. The successful application to DNA purified from single eggs confirms the good sensitivity of these approach also on samples purified from a low number of cells, usually target of forensic tests performed on commercial caviar.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2017.07.001.

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