



# Defining criteria for the reintroduction of locally extinct populations based on contemporary and ancient genetic diversity: The case of the Adriatic Beluga sturgeon (*Huso huso*)

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## Abstract

**Aim:** The restoration of the extinct Adriatic population of Beluga sturgeon, an iconic species with economic and traditional relevance, is a priority in upcoming conservation strategies but it must not occur without deep prior knowledge on the current diversity distribution. We defined informed criteria for the reintroduction of Beluga in Italian rivers by analysing its contemporary and ancient diversity based on a multi-markers approach.

**Location:** Ponto–Caspian and Adriatic basins.

**Methods:** We examined the distribution pattern of genetic diversity of the extant beluga populations by conducting genetic analysis on the mitochondrial dLoop and 27 nuclear microsatellites in 119 individuals from 3 geographical basins (Azov, Black and Caspian seas) and genomic analysis on 893 SNPs isolated through the 2bRAD approach in a subgroup of 92 samples. Mitochondrial information was also used to evaluate the variability of the extinct Adriatic population by analysing a few available museum samples.

**Results:** The historical Adriatic sample cannot be traced back to any of the contemporary ones laying to hypothesize the presence of a past isolated population. Instead, mitochondrial data did not reveal any geographically based clustering possibly reflecting the deep paleogeographical changes experienced by those areas. The genomic approach allowed us to depict for the first time a clear and supported genetic differentiation between two areas (the Black–Azov and the Caspian basins) but microsatellites also revealed a signal of differentiation of the Azov sample, possibly related to historical management activities in that area.

**Main conclusions:** The outcomes of the study revealed an unprecedented amount of information that can provide great benefits to the establishment of ex situ Beluga broodstocks and support any future translocations. The provided guidelines should be taken as a reference for the upcoming restoring of the Italian extinct Beluga

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population and also for any future management activity of this species in its entire distribution range.

#### KEYWORDS

conservation, genetic diversity, genomics, guidelines, Ponto–Caspian and Adriatic regions, population genetics, reintroduction, risk of extinction, sturgeons

## 1 | INTRODUCTION

The Beluga sturgeon (*Huso huso*) is an iconic species with high economical and traditional relevance actually managed through trade control and hatchery supplementation programs (Doukakis et al., 2010). Historically, this anadromous species was common in both Ponto–Caspian and Adriatic regions (Doukakis et al., 2010; Dudu et al., 2014). As a consequence of its migratory behaviour, dam construction represents a key factor for the decline of this species, drastically altering its habitat by precluding access to the majority of historical spawning grounds, therefore reducing the reproductive success (Birstein et al., 1997). The species is considered Critically Endangered (CR) by the International Union for Conservation of Nature (IUCN), after a decline of 90% of the populations in only 3 generations (60 years) (Gessner et al., 2010) across its entire distribution range.

Its distribution is currently confined to the Black, Azov and Caspian seas and their main tributaries, (Danube, Don and Kuban, Volga and Ural rivers); its presence in the above rivers mostly relies on continuous releases of juveniles born in captivity (Gessner et al., 2010).

In Italy, it was known to be present only in the Adriatic Sea with a spawning migration in River Po up to its middle reaches (D'Ancona, 1924). No accurate historical population assessments are available (Vecsei et al., 2002) but a massive decline of the Italian population mostly due to overfishing became evident in the catches since the early 1920s, with fewer and fewer sightings until the 1980s. Approximately 10 years after the impoundment of the Isola Serafini Dam, the population was extirpated (Bianco, 2014). However, other spawning sites, downstream the dam, have always been reachable, indicating that the extinction was caused not only by habitat loss but also by overharvesting (Rossi et al., 1991). Nowadays, sturgeon fishing is totally banned and poaching is better control thanks to an agreement recently signed by the four regions of the Po basin to act against illegal fishing. Moreover, a fish passage was completed within the EU-LIFE project ConfluPo in (2012–2017). Even if the dimensions are not fully suitable for the migration of sexually mature Beluga longer than 2 m, a retrofitted design could possibly overcome this problem. As such, river connectivity to the upper stream of River Po and therefore the extension of suitable habitat for an effective Beluga reintroduction remain to be verified. Once these obstacles are solved, the major prerequisite for a reintroduction of the species in River Po and Adriatic Sea would be the establishment of adequate broodstocks, in terms of purity, relatedness and geographical origin of the animals.

Currently, the Beluga sturgeon is reared and reproduced for caviar production by aquaculture facilities in Northern Italy. However, these animals do not descend from the extinct Italian population, but originate from other zoogeographical sources. Consequently, in the context of action plans aimed at the reintroduction of the species in the Adriatic area, the careless use of animals reared in Italy, without appropriate genetic analyses should be avoided as differentiated management units might exist and should be kept separated in establishing captive broodstocks.

Accordingly, the IUCN directive for reintroductions (IUCN, 2013), the Pan European action plan for sturgeon approved by the Bern Convention (Fredrich et al., 2019), the WSCS “Vienna”—and “Ramsar declarations on global sturgeon conservation” (Rosenthal et al., 2018; Rosenthal & Pourkazemi, 2005) and the Italian Guidelines for the introduction of fauna species (AA.VV., 2007), indicate as a priority that reintroduced individuals are as genetically similar as possible to the extinct population. In fact, individuals from different river systems often differ in morphological, ecological and life history traits (e.g. age of sexual maturation) (Birstein, 1993), while subpopulations within catchments have shown expressed specificities with regard to timing and duration of migration and selection of spawning sites (Berg, 1948). However, no individuals of the historical Adriatic population are available anymore. Only a few museum specimens originally caught in the Adriatic Sea in the second half of the 19th century can serve as a reference sample for this area.

Anyhow, little is known about the genetic diversity of this species. Some genetic differences between the Black and Caspian Sea populations were claimed to be detected in the cytochrome b gene (Dudu et al., 2009) by analysing a very low number of reference individuals. At nuclear level, Pourkazemi (2008) and Ghadirnejad et al. (2008) identified a moderate genetic diversity between different populations in the Caspian Sea. However, this does not allow to infer any conclusion on the remnant genetic diversity of this species across the entire distribution area.

The present study aims at providing the first complete picture of the geographical patterns of genetic variation for this species across its residual distribution range through the analyses of both mitochondrial and nuclear markers. Moreover, by including also the available museum samples from the Adriatic region, we aim at gathering useful information for the suitability of extant populations for the reintroduction in the Adriatic region.

Here we used mitochondrial (dLoop sequencing, on museum and contemporary samples) and nuclear (microsatellites and SNPs, only on contemporary samples) information to specifically evaluate: (a) the mitochondrial genetic variability of the extinct Italian population;

(b) the genetic diversity and phylogeography of Beluga populations across the present distribution range; (c) the identification of the best suitable source population for future reintroductions; and (d) the recovery strategies for the remaining populations based upon the detected level of structure and diversity.

The study produced here is of unprecedented accuracy and will be of high relevance for informed management of the Beluga sturgeon in Italy and elsewhere. The outcomes will also be of great help to draw up guidelines for the planning of conservation actions, maintenance of biodiversity through the selection of optimal breeders in release programs and thus strengthening the sturgeon recovery measures.

## 2 | METHODS

### 2.1 | Study area, samples collection and DNA extraction

In museum collections throughout the Italian country, a total of 10 specimens were identified as *Huso huso* from the Adriatic Sea: four Formalin-Fixed (FF) samples from the Museum of Natural History of Florence (n. 5720, 5920, 5921, 6474) and six (two FF and four dried samples) from the Museum of Natural History of Venice in Italy (MSNVE-1307, MSNVE-1316, MSNVE-21460, MSNVE-21443, MSNVE-20312, MSNVE-21337). Tissues of gill and abdominal cavity were collected from the FF samples with sterile tools and preserved in absolute ethanol. Bony tissues of dorsal and cranial scutes were sampled from dried specimens by a miniature Dremel drill instead (the external layer was removed prior to the collection of bone powder in order to avoid contamination). Total ancient DNA (aDNA) was extracted as described in Appendix S1 (Supplementary information).

A total of 119 fresh samples originating from the Azov Sea (15), Black Sea basin (20 from Black Sea and 44 from Danube River) and Caspian Sea basin (20 from Caspian Sea and 20 from Ural River) were collected. Total genomic DNA was extracted using the standard protocol of the EUROGOLD Tissue-DNA Mini Kit (EuroClone). Before any genetic analyses, all samples were tested for pureness with dedicated nuclear diagnostic markers (Boscari et al., 2014, 2017) thus confirming that no specimens of hybrid origin are present.

### 2.2 | DLoop amplification and mitochondrial analyses

Foreseeing a high level of DNA degradation in aDNAs, nine overlapped primer pairs were designed to amplify and sequence the dLoop region from museum samples. All details are extensively reported in Appendix S1 (Supplementary Information). While for the 119 fresh samples, a standard amplification was performed as also described in Appendix S1.

In addition to the 119 samples processed here, further 138 Beluga sequences by Mugue et al. (*under publication*) were included

for a total of 257 dLoop sequences subdivided in three groups according to their basin of origin: Azov Sea (87), Black Sea basin (53, Black Sea/Danube River), Caspian Sea basin (117, Caspian Sea/Ural River) and corresponding to a total of 96 haplotypes accessible at GenBank (Accession Nos: *\*to be provided upon acceptance\**)

The Blast analysis and alignments were performed with MEGAX (Kumar et al., 2018), and each polymorphism was checked on the corresponding chromatogram when available.

The overall and within population genetic variability was estimated with ARLEQUIN ver.3.5 (Excoffier & Lischer, 2010) through: haplotype diversity ( $h$ ), nucleotide diversity ( $\pi$ ), relative haplotype frequencies,  $\phi_{st}$  indices and non-hierarchical analysis of molecular variance (AMOVA, Excoffier et al., 1992).

Haplotypes and their relationships (i.e. representation of gene genealogies based on a maximum parsimony approach) were organized in a network with the PopART software (Leigh & Bryant, 2015; <http://popart.otago.ac.nz>) on the bases of the TCS network inference methods (Clement et al., 2000) with and without sequences from museum samples.

### 2.3 | Microsatellite genotyping and genetic analysis

After a first screening on 38 microsatellites, genotypes were obtained successfully for 111 individuals (16 from the Azov Sea, 56 from the Black Sea basin and 39 from the Caspian Sea basin) at 27 loci (see Appendix S2 for details).

Prior to population structure analyses, the data set was tested for the presence of null alleles with MICROCHECKER (version 2.2.3, Van Oosterhout et al., 2004) and for the neutrality of markers with ARLEQUIN ver. 3.5.2.1 (Excoffier & Lischer, 2010) and BAYESCAN 2.01 (Foll & Gaggiotti, 2008). Only loci identified by both methods as under positive selection were considered outliers (see details of the methods in Appendix S2, Supplementary Information). The statistical power to detect differentiation signal was also assessed with POWSIM 4.1 (Ryman & Palm, 2006). Extensively details are reported in Appendix S2 (Supplementary information).

Basic descriptive genetic statistics were estimated. Specifically, allelic richness ( $A_R$ ) and private  $A_R$  ( $pA_R$ ) for each locus and population were calculated considering a standardized number of 8 individuals with HP-RARE software version 1.1 (Kalinowski, 2005). For each locus, the number of alleles ( $n$ ), the average unbiased expected ( $H_e$ ) and observed ( $H_o$ ) heterozygosity was calculated using ARLEQUIN. Linkage disequilibrium between loci was evaluated, and departure from Hardy–Weinberg equilibrium (HWE) within populations for each locus and overall loci was estimated using the Markov chain method (dememorizations = 10,000; batches = 500; iterations = 10,000) in GENEPOP ver 4.7.2 (Raymond & Rousset, 1995; Rousset, 2008). The significant threshold was set to 0.05 and adjusted by the Benjamini and Hochberg (1995) correction for multiple tests.

Differentiation within and between populations was estimated computing pairwise  $F_{ST}$  indices and performing non-hierarchical analysis of molecular variance (AMOVA; Excoffier et al., 1992).

Significance was assessed by 10,000 permutations for all comparisons, and *p*-values were corrected for multiple tests as above described for the HWE.

The software STRUCTURE VER. 2.3.4 (Falush et al., 2003, 2007; Pritchard et al., 2000) was used to infer the number of genetically differentiated clusters (*K*) with the highest posterior probability and to estimate individual admixture proportions. Each run was performed with 100,000 length of burnin period and 1,000,000 of Markov chain Monte Carlo (MCMC) repeats. An admixture model and correlated allele frequencies were used with no prior information. Ten replicates per *K* were performed testing for *K* = 1–5. STRUCTURE HARVESTER (Earl & vonHoldt, 2012) was used to infer the most likely number of clusters through the calculation of  $\Delta K$  values following the method proposed by Evanno et al. (2005). CLUMPAK (Kopelman et al., 2015) was used for detailed inspections of convergence between independent runs for each *K* and graphical interpretations of the results.

We tested for a bottleneck signature in our populations using BOTTLENECK version 1.2.02 (Cornuet & Luikart, 1996; Piry et al., 1999) (Supplementary Information, Appendix S3).

Relatedness was verified within each population with ML-RELATE (Kalinowski et al., 2006).

## 2.4 | Genotyping by sequencing of SNPs and genomic analysis

A subgroup of high-quality RNA-free DNA was selected to be processed for the genotyping by sequencing of single nucleotide polymorphisms (SNPs) with the 2b- Restriction site Associated DNA (2bRAD) approach (Wang et al., 2012), discarding samples with low-quality DNA. A total of 92 samples (14 from the Azov Sea, 40 from the Black Sea basin and 38 from the Caspian Sea basin) was processed following the 2bRAD protocol steps with a few optimisations as described in Boscari et al. (2019).

The pool of libraries was sequenced twice allowing for the recalibration of the relative amount of target bands coming from different individuals as described by Paterno et al. (2017). The pools were sequenced in the first run on an Illumina NextSeq 500 platform and in the second run on an Illumina HiSeq 2500 with a single-end 50 bp read module (SR50 High Output mode) by Genomix4Life S.r.l. (Baronissi, Salerno, Italy), which also performed demultiplexing by individual barcode and quality filtering. Demultiplexed reads were checked for quality by FastQC ([www.bioinformatics.babraham.ac.uk/projects/fastqc/](http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)). Then, the raw reads obtained for each individual library were processed using a custom-made Python script that allowed filtering for the presence of the specific CspCI restriction site and adaptors' trimming (see Boscari et al., 2019 for details).

Limited to NextSeq output, a fraction of reads showed a base substitution within the expected recognition site of the enzyme due to the higher error rate of this type of sequencing (Ambardar et al., 2016; Reuter et al., 2015). These substitutions were corrected

using a second home-made script, allowing for an increase of usable information by retaining also reads with a maximum of one mismatch in the recognition site for further trimming.

The trimmed, high-quality reads were processed with the STACKS software (Catchen et al., 2013) allowing for the assembly of loci and genotyping for which “denovo\_map.pl” pipeline was employed. Polymorphic loci shared by all the animals and containing only 1 SNP were filtered out by minor allele frequency ( $MAF \geq 0.01$ ) and HWE following the method described in Boscari et al. (2019) and finally converted into genepop format for statistical analysis.

All statistical analysis (presence of null alleles, neutrality tests, statistical power to detect differentiation, disequilibrium and basic descriptive statistics) and tests for population differentiation estimations were implemented with the final data set including 893 high-quality polymorphic SNPs for 73 individuals (11 from the Azov Sea, 24 from the Black Sea basin and 38 from the Caspian Sea basin) following the same protocols and parameter settings reported for the microsatellite markers. Tests for demographic changes were implemented using DIYABC version v2.1.0 (Cornuet et al., 2014) (Supplementary Information, Appendix S3).

Moreover, a further STRUCTURE run (parameter settings as above) and assignment tests with GENECLASS2 (Piry et al., 2004) were also conducted with a subset of 24 putatively diagnostic SNPs showing  $F_{ST}$  values higher than 0.1 to verify their reliability in the allocation procedures. The assignment tests were carried through the Bayesian exclusion method of Rannala and Mountain (1997) following the criteria for the final assignment as reported in Boscari et al. (2019).

## 3 | RESULTS

### 3.1 | Mitochondrial analysis to identify putative source population

The mitochondrial data set of adult Beluga individuals consists of 257 sequences (119 here obtained and 138 from GenBank) 591 bp long. Fifty haplotypes were identified (13 for the Azov Sea, 22 for the Black basin and 36 for the Caspian basin) 13 of which shared among basins. Basic descriptive statistics per geographical basin and pairwise  $\phi_{ST}$  are shown in Table 1.

For the samples from the ancient Adriatic population, only the QIAamp DNA FFPE Tissue Kit provided reliable purified DNA from 6 out of 10 museum samples (2 Formalin-Fixed FF and 4 dried samples/bony scutes). PCR products suitable for sequencing and reliable chromatograms were obtained from 3 out of 4 dried tissues/bony scutes only (samples MSNVE-21337, MSNVE-21443 and MSNVE-21460), while FF samples did not provide any successful results. For two museum samples, MSNVE-21443 and MSNVE-21460, the positive amplification obtained with 8 primer pairs out of 9 and the successful sequence of all the PCR replicates allowed us to generate two consensus sequences about 7% shorter than the sequences obtained from contemporary samples (553 bp out of 591 bp, the primer pair number 6 in Table S1 of supplementary

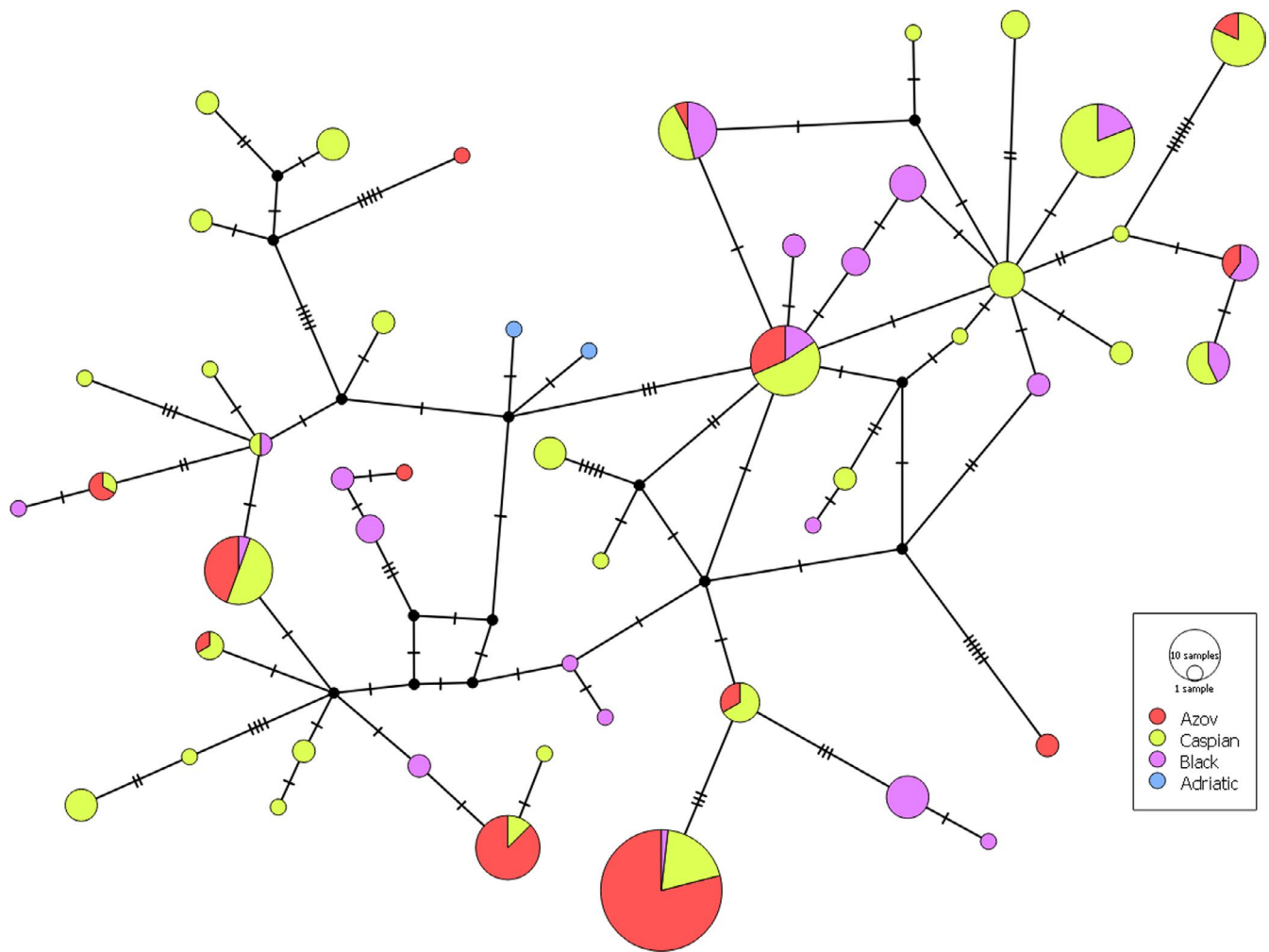
**TABLE 1** Summary basic descriptive statistics based on the 71 identified haplotypes of *Huso huso* grouped according to their geographical origin from the Azov, Black and Caspian seas and respective matrix of pairwise  $\phi_{st}$  comparisons (below the diagonal) with their associated  $p$ -value (above the diagonal)

Basic descriptive statistics					Pairwise $\phi_{st}$		
	N	$N_h$	$h$	$\pi$		Azov	Black
Azov	87	13	$0.698 \pm 0.047$	$0.015 \pm 0.008$	Azov	–	<b>&lt;0.00001</b>
Black	53	22	$0.948 \pm 0.012$	$0.012 \pm 0.006$	Black	0.18693	<b>0.00040</b>
Caspian	117	36	$0.945 \pm 0.009$	$0.017 \pm 0.009$	Caspian	0.11065	0.03515

Note: Overall  $\phi_{st} = 0.107$  ( $p$ -value <0.00001).

Abbreviations:  $h$ , haplotype diversity;  $N$ , number of individuals;  $N_h$ , number of haplotypes;  $\pi$ , nucleotide diversity.

Bold values of  $\phi_{st}$  are significant after correction for multiple tests.



**FIGURE 1** Haplotype network, obtained with PopART software, showing relationships among haplotypes detected in the extant Beluga populations and in the museum samples from the Adriatic basin. Only the shared portion of 553 bp of the alignment was considered. The size of pie charts is proportional to the corresponding haplotype frequency, while the colour indicates the geographical origin

information did not amplify). These two sequences differ from each other only for two mutations. The third sample, MSNVE-21337, for which only a shorter fragment of 347 bp was obtained, was identical in this portion to the MSNVE-21443 thus suggesting it may be the same haplotype. Interestingly, the comparison

between extant populations and the two Adriatic haplotypes from aDNA revealed that the latter ones were not detected in the contemporary populations, as the closest haplotype differs for three mutations, and can be therefore assumed to be Adriatic-private haplotypes (Figure 1).



Another noteworthy observation is that the haplotype network in Figure 1 does not reveal any geographically based clustering confirming the observation by Dudu et al. (2014). The distribution pattern observed here does not differ significantly when considering the entire dLoop region (591 bp), available only for contemporary samples.

### 3.2 | Basic descriptive statistic and population structure based on nuclear markers

The microsatellite data set includes 111 individuals genotyped at 27 loci. The software MICROCHECKER suggested the presence of null alleles, across sites, for a few loci (i.e. locus 31601, 12159, AoxD241, Aox45, LS-54, LS-19, Ag09, Aox32). The mean null allele frequency across the 27 loci, estimated with FreeNA, ranged between 0 and 0.132. The *t* test ( $p = .16$ ) showed that putative null alleles have a negligible effect on the genetic structuring analysis; therefore, all loci were used in subsequent analysis. No outlier loci putatively under positive selection have been detected with both the ARLEQUIN and BAYESCAN softwares.

POWSIM results showed that the population sample size, the number of loci, the number of alleles per locus and their frequency distributions were sufficient to detect  $F_{ST}$  values as small as 0.005 with a statistical power ranging from 95.5% to 100% under all the tested conditions of effective population sizes and number of generations of drift (see Supplementary Information to see paramet settings).

No signs of linkage disequilibrium were detected in the data and genetic diversity estimates showed that all loci were polymorphic, with the number of alleles per locus ranged from 3 (Anac\_c22096) to 32 (LS-54). Summary basic statistics for each locus are resumed in Table S4 (Appendix S4 Supplementary information).

The SNPs data set includes 73 individuals genotyped at 893 loci. Neutrality tests revealed 64 loci putatively under positive selection with ARLEQUIN, while no markers that deviate from the hypothesis of neutrality were detected with BAYESCAN. Given that no marker, resulted to be putatively under selection, were shared by the two software, we decided to retain all of them for the genetic analyses.

The 200 simulations performed by POWSIM showed that the population sample size, number of loci and their frequencies have sufficient statistical power to detect  $F_{ST}$  values as small as 0.005 with a power ranging from 57% but rapidly approaching to 100% under all the tested conditions of effective population sizes and number of generations of drift. No signs of linkage disequilibrium were detected.

Summary basic statistics over all loci for each population are resumed in Table 2 for both the microsatellites and SNPs data sets.

The non-hierarchical AMOVA performed on the three populations sampled showed that 2.59% for microsatellites and 1.15% for SNPs of the variation occurred among geographical basins, with a statistically significant global  $F_{ST}$  ( $p < .0001$ ) indicating the presence of a high genetic structure across the study area. Pairwise  $F_{ST}$

**TABLE 2** Summary basic descriptive statistics over all loci of nuclear markers in *Huso huso* from Black, Azov and Caspian origin (see Table S4 for detailed statistics per locus and per sampling site)

Geographical basin	N	$A_R$	$pA_R$	$H_0$	$uH_e$
Azov	16	4.50	0.54	0.6040	0.6549
Black	56	4.93	0.75	0.6215	0.6627
Caspian	39	5.02	0.89	0.6289	0.6732
Azov	11	1.65	0.08	0.1835	0.1586
Black	24	1.65	0.08	0.1766	0.1532
Caspian	38	1.67	0.12	0.1809	0.1552

Note: The statistics above the 'fourth raw' are estimated based on 27 microsatellite loci, while data below the 'third raw' are referred to the panel of 893 SNPs.

Diversity:  $N$ , number of individuals;  $A_R$ , Allelic richness;  $pA_R$ , private Allelic richness,  $H_0$ , observed Heterozygosity;  $uH_e$ , unbiased expected Heterozygosity. Allelic richness was calculated on the bases of the minimum number of gene copies.

confirmed that all comparisons are highly significant using both data sets (Table 3).

The optimal number of clusters for the whole microsatellite and SNPs data sets obtained with STRUCTURE was two ( $K = 2$ ). Results for  $K = 2$  using microsatellites (Figure 2a) revealed the division into two differentiated clusters without a clear pattern of subdivision: the first cluster corresponds to most individuals from the Azov Sea as well as some others from the Black Sea basin, and the second cluster includes most individuals of the Black Sea basin and almost all individuals from the Caspian population. Highest  $K$  (Figure 2a) revealed a confused sub-structuring even maintaining the Azov population separated from the other basins. The differentiation of the Azov population sample is discordant with the results obtained with the SNPs panel, as shown later (Figure 2b). In order to verify whether this discrepancy could be due to a certain degree of relatedness within the Azov basin, the analysis of relatedness among individuals has been implemented in ML-relate. The percentage of comparisons between two related individuals (full sibs or half sibs) calculated on the total of pairwise comparisons within each basin resulted equal to: 30.8% in the Azov basin, 9.2% in the Black basin and 10.9% in the Caspian basin, respectively. Compared to what obtained with the microsatellites, STRUCTURE results for  $K = 2$  using SNPs (Figure 2b) revealed a much clearer subdivision into two well differentiated clusters according to their geographical location: one cluster includes the populations of the Azov and the Black Sea basins, and another homogenous cluster corresponds to the Caspian Sea basin. Only two individuals from the Azov Sea were assigned with the Caspian ones and one individual from the Caspian Sea showed an admixed membership probability.

Bottleneck signatures were revealed in all populations with both the microsatellite and SNPs data set. Results are reported in details in Supplementary Information (Appendix S3).

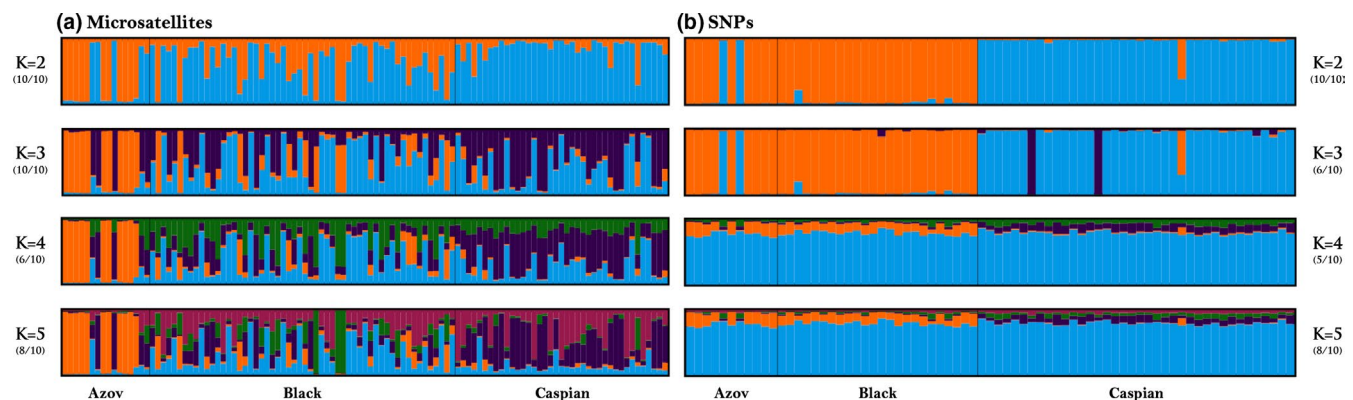
In order to develop an allocation procedure based on a few informative loci possibly analysed without the need of a next generation sequencing approach, we selected the 24 loci showing a  $F_{ST}$  higher than 0.1 and evaluated their discrimination ability through the

**TABLE 3** Pairwise genetic differentiation ( $F_{ST}$ ) among three geographical population samples of *Huso huso* from Black, Azov and Caspian seas obtained with 27 microsatellite loci and 893 SNPs, respectively

Population (sampling sites)	Microsatellites			SNPs		
	Azov (AZ)	Black (BS and DR)	Caspian (CS and UR)	Azov (AZ)	Black (BS and DR)	Caspian (CS and UR)
Azov (AZ)	–	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	–	<b>0.0003</b>	<b>&lt;0.0001</b>
Black (BS and DR)	0.0291	–	<b>&lt;0.0001</b>	0.0045	–	<b>&lt;0.0001</b>
Caspian (CS and UR)	0.0362	0.0215	–	0.0179	0.0107	–

Note: Estimates of pairwise  $F_{ST}$  are reported below the diagonal; associated  $p$ -values are reported above the diagonal. Significant values after correction for multiple tests (Benjamini & Hochberg, 1995) are reported in bold. Global  $F_{ST}$  0.0259 ( $p < .0001$ , Microsatellites) and 0.0115 ( $p < .0001$ , SNPs).

Abbreviations: AZ, Azov Sea; BS, Black Sea; CS, Caspian Sea; DR, Danube River; UR, Ural River.



**FIGURE 2** Major modes of the CLUMPAK-averaged Structure output for 10 independent runs of  $K$  ranging from 2 to 5. Numbers under each  $K$ -value indicated the proportion of runs that converged to the solution presented. Minor unsupported modes are not reported as they did not provide further information. (a) Analyses performed using 111 individuals of three population samples genotyped at 27 microsatellite loci. (b) Analyses performed using 73 individuals of three population samples genotyped at 893 SNPs

software STRUCTURE and assignment tests. Results confirmed that the basin of origin could also be detected with only 24 SNP markers (Appendix S5, supplementary information). These loci could be, therefore, considered putatively diagnostic to allocate animals to their basin of origin.

## 4 | DISCUSSION

This is the first application of different classes of genetic markers to explore population differentiation patterns of the most iconic among extant sturgeon species, the Beluga.

Limited to mitochondrial information, it was possible to compare the few available museum samples representing the extinct Italian population, with the extant populations of the Black, Azov and Caspian seas.

The analysis of nuclear diversity allows to define a general picture of diversification among contemporary population samples. More locally, as extensively described later, the results allow to propose criteria for the reintroduction of *H. huso* into the Adriatic-Po basin. Overall, the distribution pattern of genetic differentiation

detected among contemporary population samples are consistent with a genetic structuring explained by their geographical subdivision in two main biogeographical regions, the Black Sea–Azov basin and the Caspian Sea basin. However, some discrepancies between the results obtained by different markers and different patterns of admixture were observed and are discussed based upon historical data on supplementation programs.

### 4.1 | Mitochondrial diversity

Despite a significant differentiation among populations and the presence of a few shared mtDNA haplotypes among basins, their relationships revealed that there is not a clear differentiation between basins. This scenario, concordant with previous inferences on Beluga (Doukakis et al., 2010) and also on other sympatric species such as the Russian (*Acipenser gueldenstaedtii*) and the Sterlet (*A. ruthenus*) sturgeons (Dudu et al., 2014), seems to bear the signature of past reiterated admixtures. The Ponto–Caspian area, indeed, experienced deep historical paleogeographical changes that may have played a relevant role on distribution and demography

of aquatic species (Kotlik et al., 2008). The current scenario is presumably the result of multiple alternating periods of isolation and interconnection that have occurred in the paratethys in the last 5 million years (Tomilova et al., 2020). The latest natural contact between the two basins dates back to 15.000–11.000 years ago following the last glaciation of the Quaternary (Zubakov, 1988) through the Manych–Kerch Spillway. At the same time, spreading of animals and gene flow among basins may have favoured multiple introgressions that are likely the main reason why geographical differentiation is lacking. This was first detected by Dudu et al. (2014) using non-recombinant mtDNA and is confirmed here with a larger, population samples.

Besides the above paleogeographical changes, also human-driven translocations took place as a consequence of the demographic collapse of wild populations caused by overharvest and habitat loss through the construction of dams on all major sturgeon rivers in the Black and Azov seas. A series of restocking activities, mainly driven by the economic relevance of the species for caviar production, resulted in translocations between basins. Most translocations occurred from the Caspian basin (Ural and Volga rivers) to the Azov Sea (e.g. 18.2 million Caspian juveniles were released into the Azov Sea from 1976 to 1986, Balandina, 1983; Chebanov et al., 2002), thus exposing this natural population to a series of possible introgression events. After the ban on introduction of the fertilized eggs originated from the Caspian basin in 1985, ex situ conservation activities in the Azov Sea are relying on autochthonous breeders only, to respect the genetic composition of the populations and to limit the risk of introgressions and outbreeding.

The analysis of museum samples from the extinct Adriatic population revealed the presence of only two very similar haplotypes not shared by any other specimens from contemporary population samples which otherwise showed high haplotype diversity.

For what concerns the two Adriatic haplotypes, no certain conclusion can be brought up about the homogeneity of the extinct population given the low number of museum animals successfully analysed. We could, however, speculate that the uniqueness of the Adriatic haplotypes points to the presence of a population historically isolated and possibly founded by a limited number of animals without regular contacts with the closest Black Sea population. Therefore, the possibility of it being strongly adapted to local conditions is high. Unfortunately, at present we cannot infer the best suitable populations for the recruitment of animals to be employed as breeders and the low quality of aDNAs prevents us from analysing any nuclear markers.

## 4.2 | Nuclear diversity in contemporary populations

The analyses of microsatellite and SNPs yielded apparently contradictory results with general evidence of more information provided by the genomic approach. Microsatellites, indeed, failed in detecting a clear differentiation among basins (Black and Caspian basins),

except for the Azov population sample which resulted clearly differentiated from the Black Sea. This cluster composition, however, was not confirmed by analysing over 850 SNPs which revealed two clearly differentiated groups (the Black–Azov group and the Caspian one).

This cluster subdivision is very clear and is supported by the almost full concordance with the geographical distribution of samples, also confirmed by the assignment tests.

The ability to assign animals according to their population of origin, here shown by SNP markers, is extremely important in the establishment of geographically homogeneous ex situ broodstocks. However, the genome-wide approach fits well to the analysis of many samples simultaneously, but it wastes time and costs for routine analyses which are usually required only for a few samples. For this reason, in future studies, we strongly encourage the use of the 24 here detected diagnostic loci as a single-locus genotyping PCR after validation by mapping their short sequences as soon as the Beluga genome will be available. The availability of such genome would also allow to deepen the role played by the different loci of our data set and in particular by the 64 ones that resulted to be putatively under selection with ARLEQUIN but not with BAYESCAN.

Looking at the historical events that shaped the landscape of the Ponto–Caspian basin so far, the clear differentiation of the Caspian group from the Black–Azov one allows us to argue that the past translocations of million animals did not significantly impact the genetic composition of the different populations probably due to a negative selection on the translocated animals for many possible reasons. Firstly, the animals were usually released at an age of a few weeks when the expected mortality rate is still high. Secondly, the environmental conditions and in particular the temperatures during the reproductive season in the basin of the source population (the Caspian Sea, and the Volga and Ural rivers) are significantly lower compared to the receiving basin (the Azov Sea); lower temperatures lead to a postponed reproduction and a late transfer of fertilized eggs to the Kuban River where larvae and juvenile stages are not exposed to optimal rearing temperatures and as a result become maladapted (M. Chebanov, 2020, personal communication). Thirdly, a maladaptation of the species following the onset of feeding in the new environment to the succession of local food sources (Cushing, 1990; Thackeray et al., 2010). A transfer of these organisms into a system with different successions results in a mismatch of feed requirements and feed availability, restricting performance and survival. This was described both in herring (*Harengus harengus*) and cod (*Gadus morhua*) in the North Sea under the impacts of climate change recently (Cushing, 1995).

On the other hand, the differentiation of the Azov Sea population revealed by microsatellites can be explained by a high ability of multiallelic markers compared to biallelic ones to resolve fine-scale population structuring (Lemopoulos et al., 2019) and consequently signals of the probable recent human impact on this population. Accordingly, due to the interruption of migratory routes by damming the main tributaries of the Azov Sea (the



Don and Kuban rivers), the last natural spawning of Beluga in the Azov Sea basin was reported in the Don River in 1963 (Makarov & Balandina, 2000). Between 1956 and 1970, a few wild breeders of Azov origin were caught every year and used for the production of about 400 thousand juveniles/year that were released in the wild. More recently, a captive broodstock of native origin is maintained in captivity for controlled propagation. However, even if of native origin, the broodstock includes about 50 females also born in captivity from a wild generation of no more than 12 females (M. Chebanov, 2020, personal communication). This human activity, even if conducted using autochthonous breeders, has certainly deeply modified allelic frequencies compared to the previous natural reproduction of thousands of animals, possibly explaining the high level of relatedness detected in the Azov Sea, with a note of caution due to the low sample size. This human impact might be revealed by the presence of a bottleneck signature. However, given the very long generation time of the species which exceeds the 50 years (IUCN sturgeon specialist group pers. comm.), animals reproduced in that period might be just one generation older than the samples here analysed making this human-driven bottleneck undetectable. Accordingly, the only signature of bottleneck detected by D<sub>Y</sub>ABC dates back to post-glacial population decline.

#### 4.3 | Implications for management

This study reveals, for the first time, a clear picture of the differentiation pattern among beluga populations, consistent with the contemporary hydrography of the region, by identifying two fundamental conservation units (the Black–Azov and Caspian basins) which must be respected in planning management, restocking or reintroduction activities. Further and more cryptic differentiation in local subpopulations cannot be excluded as the population sample sizes are not adequate for fine phylogeographical analyses. For this reason, we suggest adopting a precautionary approach in transferring individuals between different locations, even within the same main basin, possibly considering ecological and climatic compatibility of source and target ecosystems.

Also, the establishment of ex situ broodstocks should include a prior genetic allocation of the founders to one of the two main conservation units in order to retain the gene pools separated. This can be challenging as captive mature beluga are often transferred many times between even distant rearing facilities and mixed with animals of different origins. Moreover, the late sexual maturity and the low diversity in captive stocks make the collection of an adequate number of breeders a long process of many years during which the candidates must be checked for purity, origin and reciprocal relatedness.

Another aspect emerging from our results is the risk of genetic erosion in the Azov population. We strongly suggest a complete characterization of the captive broodstock using the microsatellite loci presented here, and a comparison with our data to verify how much the described impoverishment is due to the small number of

breeders. If confirmed for the entire stock, conservation managers should try to decrease the level of relatedness by recruiting, if possible, additional unrelated native breeders.

#### 4.4 | Guidelines for the reintroduction in the Adriatic basin

Given that the genetic investigation does not allow to identify a best suitable source population for the collection of individuals to be used as founders, alternative approaches should be applied, based on ecological evaluations. However, the main drawback is the scarcity of specific information about the ecology of the historical population. The only option in this case would be the comparison of the hydrological, hydrobiological and ecological features of the target system with potential donor systems searching for the more similar one in terms of migration distance, temperature, hydrology and prey organisms. As such, this approach also is of limited reliability.

Additional considerations can be advanced based on the observed pattern of differentiation between the Black–Azov and the Caspian seas. Indeed, the negligible effect of past translocations on the gene pools testifies that ecological aspects are crucial for the success of the restocking programs and that the animals from the two basins are probably pre-adapted to different conditions. Therefore, management decisions should avoid mating animals coming from different basins to prevent the population from incurring in outbreeding depression. As all extant populations could be potentially suitable from a genetic point of view, different captive broodstocks should be established using animals homogeneous for geographical origin. The rapid global warming of the last decades contributes to increase the uncertainties in identifying a priori a best suitable source population (Lassalle et al., 2010). Changes in hydrological and thermal regimes of rivers might have a direct impact on freshwater ecosystems (van Vliet et al., 2013) and relying on source populations pre-adapted to different conditions might represent an additional value, increasing the adaptive potential of the released stocks.

The simultaneous release in River Po of Beluga stocks coming from different conservation units and pre-adapted to different conditions might represent an additional value possibly increasing the chances that at least one of them can adapt to the new environment (River Po/Adriatic Sea). Given the very long generation time and the anadromous life cycle of the species, the possibility that animals descending from different populations will hybridize upon maturation will become realistic not earlier than in 15 years, during which the natural selection acts. If the reintroduction will be successful, three different scenarios are possible: (a) only one of the released origins will adapt to the characteristics of the River Po /Adriatic system while the others will go extinct in the long run; (b) different lines will adapt to the local environment with a reproductive continuity merging in a self-sustaining Adriatic population; and (c) the different stocks will result not to be interfertile for physiological, ecological or

behavioural incompatibilities, leading to two coexisting and possibly competing forms of Beluga. All these scenarios would represent an important success.

The process of foundation of a sufficiently diverse captive broodstock, however, does not have to exclusively consider the animal origin but must also carefully evaluate the purity of the animals for which markers are available (Boscari et al., 2014, 2017; Havelka et al., 2017), their possible relatedness and the amount of genetic variability (Rosenthal et al., 2018). The purity is especially relevant for sturgeons because animals used as breeders are often collected in aquaculture plants where many other sturgeon species as well as interspecific hybrids are often reared and the detection of hybrids in “pure” conservation stock is not infrequent (Boscari & Congiu, 2014).

Also, the relatedness among breeders represents a critical aspect dealing with sturgeons. The difficulty in finding animals to be included in conservation programs and the ban on collecting wild sturgeons, often forces the use of animals reared in aquaculture plants for commercial purposes (meat/caviar production). However, these stocks are made up of hundreds or thousands of full sibs and the random recruitment of animals leads with high probability to select highly related breeders with possible inbreeding problems in subsequent generations. Beside avoiding inbreeding, the use of an adequate number of unrelated breeders allows to increase the adaptive potential of the released individuals in the long run.

The reintroduction of the Beluga in the Adriatic Sea has already been started within a recent LIFE Project granted by the European Community to the Ticino River Park. The results of this paper were not available at that time, and the choice of the animals to be released or to be retained as future breeders was not carried out by informed decision. However, the process of reintroduction of a species with a generation time of more than 25 years requires decades and, being just at the beginning, there is room to easily incorporate the outcomes of this study into management plans which are underway already. Besides the above project, officially approved by public institutions, other much less well-considered actions were carried out in Italian waters by sportfishing associations that released a few hundred juvenile individuals into the waters of River Po, without any preliminary evaluation being conducted on the released animals.

The first recommendation that we would like to make is that this kind of activities, even if driven by a sincere conservation spirit, must be coordinated in the context of an informed management of recovery actions.

## 5 | CONCLUSIONS

The present study, thanks to an intense collaboration among different research institutes engaged in sturgeon research and recovery, represents an unprecedented advance of the knowledge of one of the species at great risk of extinction. Several countries are putting in place a considerable joint effort to recover this species as evidenced by a recent meeting held in Galati, Romania, where

representatives of 18 countries reached an agreement and signed the “Galati Declaration” underlining the importance of ex situ conservation for the future of the species. Furthermore, urgent emphasis has been placed upon the need to establish a network of ex situ facilities working in collaboration. In this context, our study represents a milestone for the knowledge of this species showing a clear genetic distinction among basins that must be treated as such.

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## DATA AVAILABILITY STATEMENT

Sequencing raw data available from SRA-NCBI (BioProject ID: PRJNA686848).

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## BIOSKETCH

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Author contributions: EB and LC conceived the ideas and designed the experiments, analysed the data and wrote the manuscript. EB performed the experiments. CC and ML analysed the museum samples. IAMM and NM contributed to data analysis. LC contributed reagents, materials and analysis tool. NM, RS and DO contributed to the sampling. All authors contributed in reviewing and approving the final version of the manuscript.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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