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Rare but Not Gone: A Relict Population of the Black Sea Ship Sturgeon *Acipenser nudiventris* Persists in the Rioni River, Georgia

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Abstract: Historically, the ship sturgeon (*Acipenser nudiventris*) occurred in the Aral, Caspian, Azov, and Black Sea basins. However, its numbers decreased dramatically during the 20th century. It is now considered extirpated from the Aral, Azov, and Black Seas, and has almost disappeared in the Caspian Sea. *A. nudiventris* is listed as Critically Endangered on the IUCN Red List and, in Georgia, the species has been undetected for the last three decades. We collected 22 sightings, including nine genetic samples taken from fin clips of ship sturgeon from the Rioni River in Georgia during 2020–2022. For the genetic samples, the mitochondrial DNA control region was used for species identification. Because cases of sturgeon inter-species hybridization have been reported in the Rioni River, we used species-specific diagnostic markers and ship sturgeon-specific microsatellite markers for detecting hybridization with other sturgeon species. In addition, we used a sex-specific marker for sex identification. Based on the maternal identification, all nine individuals are identified as ship sturgeon, representing one haplotype, and the haplotype is different from all other *A. nudiventris* haplotypes available in GenBank. Based on genetic analysis, the specimens did not show signs of hybridization with other locally occurring species. We conclude that ship sturgeon still live in the Rioni River, and are a remnant of an older, preexisting Black Sea ship sturgeon population.

Keywords: *Acipenser nudiventris*; Black Sea; Rioni River; ship sturgeon; Georgia; relict population

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1. Introduction

The ship sturgeon *Acipenser nudiventris* was historically distributed in the Aral, Caspian, Azov, and Black Sea basins. Its numbers decreased dramatically during the 20th century, and the species is categorized as Critically Endangered on the IUCN Red List [1]. The species has been extirpated from the Aral Sea since the 1970s as a result of water pollution, damming of rivers, overfishing, and the introduction of parasites after the stellate sturgeon stocking program in the Aral Sea. Populations in the Azov and Caspian Seas are possibly extinct [2–4].

Regarding the Black Sea basin populations, only a few isolated individuals were reported in the Danube River in 2003 and 2005 [5], and the species is now assumed to be extinct in the basin [6]; ship sturgeon have not been observed in the northern tributaries of the Black Sea rivers for more than 30 years [1]. The Georgian part of the Black Sea and its eastern tributaries were known as suitable habitats for several sturgeon species. However, damming of the rivers, and uncontrolled and continued overfishing led to a dramatic decline in all sturgeon populations in this area [7]. The population of the ship sturgeon, previously described as rare in Georgia [8], was considered extirpated there as well. The species has been unrecorded for the last three decades, at least. A few sightings were reported in the 1980s in the Rioni River, but these reports were not substantiated. Generally, there is a scarcity of knowledge about the ship sturgeon in the Rioni River and the eastern Black Sea.

To support the conservation of ship sturgeon, captive breeding facilities in Iran and the State Centre for Sturgeon Gene Pool Conservation "Kubanbioresursi" (Federal Living Gene Bank in the Krasnodar River) in Krasnodar (Russian Federation) are rearing ship sturgeon from Caspian Sea stocks for a reintroduction program [1]. The reintroduction of the species began in the Kuban River in 2005 [9]. The full mitochondrial genomes of the ship sturgeon stocks used for reintroduction are available in the NCBI (the National Center for Biotechnology Information). These include one haplotype from the Caspian Sea population and one from the Ili River–Balkhash Lake basin population [4]. Ship sturgeon originating from the Aral Sea were introduced to Balkhash Lake during the 1930s, and later successfully colonized the Ili River [10]. Therefore, the Ili River population is probably derived from the Aral Sea ship sturgeon population [1].

Contrary to previous assumptions, however, this study provides evidence that ship sturgeon persists in the Rioni River. Local fishers and Fauna and Flora International team members gathered 22 photographic and video records, including nine genetic samples during 2020–2022. A genetic analysis of the collected samples shows that individuals are ship sturgeon specimens.

2. Materials and Methods

Local fishers and members of the Caucasus Programme of Fauna and Flora International collected 22 records of the ship sturgeon in the Rioni River in Georgia during 2020–2022. All individuals were accidentally caught by fishing rods. Photographs and video footage were taken of all captured individuals, and genetic samples (fin clips) were taken in nine cases. All individuals were released back into the Rioni River. These records were collected in the area of up to 30 km upstream from the river mouth, and around Samtredia municipality, 80–90 km upstream (Figure 1). We used a QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) to extract DNA from fin clips, according to the manufacturer's protocol.

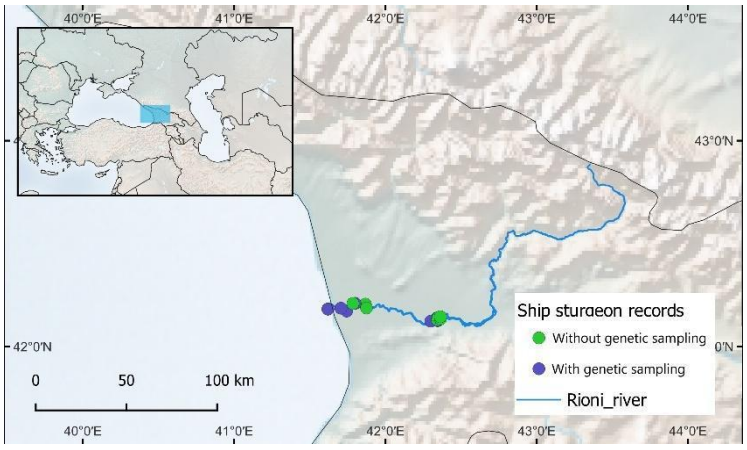

Figure 1. Ship sturgeon sampling sites in the Rioni River in Georgia.

2.1. Study Area

The Rioni River is the largest in western Georgia. It is 327 km long, one of the main eastern tributaries of the Black Sea, and one of the shortest sturgeon spawning rivers. After the construction of the Vartsikhe Dam cascades from 1968–1987, suitable spawning grounds for sturgeon in the middle of Rioni were reduced in length from an estimated 57 km to just 9 km [11].

2.2. Mitochondrial DNA Analysis

We used a mitochondrial DNA (mtDNA) control region sequence to identify species [12]. Samples were sequenced on a 3730xl DNA Analyzer at Macrogen Europe B.V. (Amsterdam, The Netherlands). PCR reactions contained 0.25 uM of each primer, 0.1 mM of dNTPs,

2.5 mM $MgCl_2$ 1x buffer, 1 U Taq DNA polymerase (Promega, Madison, WI, USA), in a 25 µL reaction volume, and 40 ng DNA template for each reaction. Thermal cycling employed the following conditions: 94 °C—5 min; 94 °C—30 s, 56 °C—30 s, and 72 °C—30 s for 34 cycles; and 72 °C—7 min. We used Geneious 8.0 [13] for editing DNA sequences. For sequence alignment and phylogenetic tree reconstruction, we used MEGA 7.0 [14]. We used the NETWORK 5.0 median-joining method to investigate haplotype relationships and genetic distances between ship sturgeons from the Rioni River and ship sturgeon haplotypes obtained from GenBank [15].

2.3. Detecting Hybrids

We used species-specific nuclear primers designed to detect sturgeon hybrids. These primers target diagnostic single nucleotide changes in the sturgeon nuclear genome and identify species-specific genetic contributions in a specimen; the contribution of a species in the sample is detected by the presence/absence of a PCR product [16,17].

Stellate sturgeon-specific test—We used primer pair Ste_RP1F and RP1_LocusA_R [16], which is stellate sturgeon-specific, based on a single nucleotide polymorphism in the ribosomal protein S7, to detect parental contributions from the stellate sturgeon (*Acipenser stellatus*) in our samples. PCR was performed in a volume of 20 µL, with 0.25 uM of each primer, 0.1 mM of dNTPs, 2.5 mM $MgCl_2$, 1x buffer, 1 U Taq DNA polymerase (OxGEn), and 40 ng DNA template for each reaction, with the following PCR cycling conditions: 94 °C—2 min; 94 °C—45 s, 59 °C—45 s, and 72 °C—45 s for 33 cycles; and 72 °C—7 min. PCR products were checked on 1.8% agarose gel.

Russian sturgeon-specific test—To detect potential parental contributions of the Russian sturgeon (*Acipenser gueldenstaedtii*) and Siberian sturgeon (*Acipenser baerii*) in our samples, we used primer pair 395_AB and 395_uni [17]. PCR was performed in a volume of 20 µL, with 0.25 µM of each primer, 0.1 mM of dNTPs, 2.5 mM $MgCl_2$, 1x buffer, 1 U Taq DNA polymerase (OxGEn), and 40 ng DNA template for each reaction, with the following conditions: 94 °C—2 min; 95 °C—45 s, 63 °C—60 s, and 72 °C—60 s for 33 cycles; and 72 °C—12 min. PCR products were checked on 1.8% agarose gel.

Beluga-specific test—We used a marker specific to the beluga sturgeon (*Huso huso*) [18] to detect hybridization with the ship sturgeon. The following primers were used: 153_HHp-153_uni and 153_HHn-153_uni. PCR was performed in volumes of 20 µL, with 0.25 µM of each primer, 0.1 mM of dNTPs, 2.5 mM $MgCl_2$, 1x buffer, 1 U Taq DNA polymerase (OxGEn), and 40 ng DNA template for each reaction, with the following conditions: 5 min for 95 °C; 25 cycles at 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 60 s; and a final extension at 72 °C for 12 min. PCR products were checked on 1.8% agarose gel.

Ship sturgeon-specific microsatellite marker—We used a ship sturgeon species-specific microsatellite marker An20, which amplifies a ship sturgeon-specific allele (153) for the specimens [19]. The marker was used to check for hybridization with any other sturgeon species. Detecting other sturgeon species' specific contribution in the ship sturgeon samples would be an indication of the hybridization of ship sturgeon with other species. For example, if we test the ship sturgeon sample with stellate sturgeon-specific marker and it is positive, we can say that the tested sample is a potential interspecies hybrid between ship and stellate sturgeon. PCR was performed in 10 µL reactions containing 0.25 µM of each primer, a forward primer labeled at the 5′ end with VIC, 2.5 mM $MgCl_2$, 0.1 mM of dNTPs, 1x GoTaq Buffer, 1 U Taq DNA polymerase (Promega, Madison, WI, USA) per reaction, 50 ng of template DNA, and sterile water. Thermal conditions were as follows: 95 °C—5 min; 95 °C—25 s, 53 °C—25 s, and 72 °C—40 s for 34 times; and 72 °C—10 min.

2.4. Sex Identification

We used the AllWSex2 marker for sturgeon sex identification. An mtDNA control region fragment [20] was used in combination with the sex-specific marker as an internal control for each PCR reaction. PCR was performed in a volume of 20 µL with 0.25 µM of each primer (the sex-specific, and the control region), 0.1 mM of dNTPs, 2.5 mM $MgCl_2$,

1x GoTaq buffer, 1 U Taq DNA polymerase (Promega, Madison, WI, USA), and approximately 80 ng DNA template for each reaction. Thermal cycling employed the following conditions: 94 °C—15 min; 94 °C—30 s, 56 °C—30 s, and 72 °C—30 s for 34 times; and 72 °C—5 min. Primers (in combination with internal control) were AllWSex2_F 5′_TGATCAACCTCTTCAGCAATGTC_3′ and AllWSex2_R_5′_TGAGAGCCACTGTACTA ACACA_3′ [21].

3. Results

3.1. Mitochondrial DNA Analysis

Based on analysis of 782 bp of the mitochondrial control region sequence, all nine genetic samples were maternally identified as ship sturgeon (*Acipenser nudiventris*), all having the same haplotype (GenBank accession number: OP903371). We compared the Rioni River haplotype to the Caspian Sea (KU321568) and Ili River (KU321569, Balkhash basin) haplotypes from GenBank, and found two and seven nucleotide differences, respectively (Figure 2).

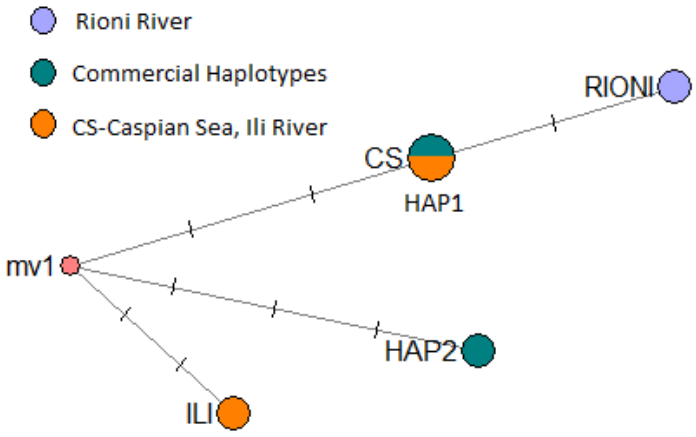

Figure 2. Network analysis of ship sturgeon samples from the Rioni River and ship sturgeon control region DNA sequences downloaded from NCBI (Caspian Sea-KU321568; Ili River-KU321569, (Balkhash basin); HAP0-KU321569; HAP02-KU321568). Analysis was carried out with 782 bp fragments of mitochondrial control region sequences.

Comparing 600 bp of control region sequences between the Rioni River ship sturgeon and the two main commercial haplotypes of ship sturgeon available in GenBank (HAP01 and HAP02, accession numbers KU321569 and KU321568), we found one and six nucleotide differences (Figure 2). Haplotype HAP01 is a common haplotype found in ship sturgeon aquaculture worldwide.

3.2. Detecting Hybrids

We did not detect hybridization of the ship sturgeon with any other locally distributed sturgeon species. Parental contributions from the stellate sturgeon, Russian and Siberian sturgeon, and beluga, were not detected in any of the samples. In all species-specific PCR tests, only positive controls were amplified for each target species. A ship sturgeon-specific microsatellite marker An20 only showed the ship sturgeon-specific allele 153 and we did not detect any other alleles that might be characteristic of other species.

3.3. Sex Identification

Sex-specific marker analysis did not show PCR amplification of the 100 bp female-specific DNA fragment in ship sturgeon samples from the Rioni River. Only the positive controls (the known females *Acipenser gueldenstaedtii*, *Huso huso*, and *Acipenser ruthenus*) were amplified. The control region fragment, used as an internal control for each PCR, was successfully amplified in every test.

4. Discussion

The twenty-two individuals found in the Rioni River were morphologically identified as ship sturgeon. According to our mtDNA sequence analysis of nine ship sturgeon genetic samples, they all shared the same haplotype. A comparison of the Rioni River data with the Caspian Sea (accession number KU321568) and the Ili River (Balkhash Lake basin) haplotypes (accession number KU321569) showed two and seven nucleotide differences, respectively. Therefore, we conclude that the Rioni River ship sturgeon are genetically distinct from the Caspian Sea and the Ili River populations, and represents a remnant of an eastern Black Sea ship sturgeon population. Moreover, the Rioni River specimens have one and six nucleotide differences from the ship sturgeon haplotypes commonly used in aquaculture (accession numbers KU321569 and KU321568). This excludes the possibility that the Rioni River specimens are from the ship sturgeon reintroduction program, which released individuals into the Kuban River in Krasnodar in 2005. We do not know the post-introduction life histories of these fish or how far or where they migrate. However, there is a ~600 km distance between the Krasnodar River and the Rioni River; it is unlikely that the reintroduced fish have migrated from the Azov Sea basin and started reproducing in Georgia in the Rioni River, a conclusion that is reinforced by the observed divergence of haplotypes between the respective populations.

Interspecific hybridization between Russian and stellate sturgeons has recently been reported in the Rioni River [22], which raises concerns about the ship sturgeon in the Rioni River, as it is also capable of hybridizing with these other two species in the wild [8]. For example, ship sturgeon hybrids with Russian sturgeon and stellate sturgeon have been detected in the wild in the Volga River and the mouth of the Safid River in Iran [23]. The ship sturgeon is a diploid species, whereas the Russian sturgeon is tetraploid [24]; their hybrids are supposed to have infertile triploid offspring. However, infertile offspring can participate in spawning and compete with the pure parental species [25]. Moreover, invasive Siberian sturgeon have been recorded by local fishers and FFI team members in the Rioni River close to the localities where ship sturgeon were found. In laboratory conditions, the ship sturgeon has also been shown to hybridize with the Siberian sturgeon [26]. Therefore, the presence of non-native Siberian sturgeon is a potential threat to the ship sturgeon in the Rioni River. In addition, ship sturgeon hybridization with the diploid stellate sturgeon might be a serious threat to both species, as hybridization between diploid sturgeon species can have fertile hybrids [24]. Hybridization of these two species could, in turn, lead to backcrossing with parental pure species. The hybrids may have beneficial traits and compete with their parent species in the natural habitat, or a genetic assimilation of the two separate species may cause rapid extinction of the parental species, both of which are rare [19,27]. Apart from the possible hybridization with locally distributed Russian and stellate sturgeons in the wild, beluga sturgeon is also likely to be present, if rare, in the Rioni River and, as it is also a diploid sturgeon species, hybridization with the ship sturgeon could also lead to fertile hybrid offspring [8].

We used species-specific nuclear markers for the Russian sturgeon, Siberian sturgeon, stellate sturgeon, and beluga, but none of these showed positive amplification in any of the tests [16–18]. Additionally, microsatellite marker An20, which is considered species-diagnostic for the ship sturgeon [19], shows only the ship sturgeon-specific allele (153) and does not exhibit any different alleles.

These markers are designed for the species; the protocols are straightforward and easy to implement in the laboratory. However, there can be uncertainties for Russian sturgeon and Siberian sturgeon-specific markers. These markers differentiate these species from others with 96% and 99% probability, respectively [17], while for stellate sturgeon and beluga sturgeon these assays have shown 100% reliability [16,18]. Based on all these test results, the specimens captured in the Rioni River can be considered pure ship sturgeon, notwithstanding the potential for hybridization with other resident sturgeon species.

A sex-specific marker has recently been designed to detect a female-specific 100 bp DNA sequence in *Acipenser* species. The marker was designed and tested for the Russian

and Siberian sturgeon, sterlet, and beluga, and also successfully identified female specimens of species that had diverged earlier from the common lineage (European sturgeon *Acipenser sturio* and Atlantic sturgeon *Acipenser oxyrinchus*) [21]. Therefore, we assumed that the marker could be used for ship sturgeon sex identification, as the species is within the same clade as Russian and stellate sturgeon. However, none of the nine specimens showed female-specific DNA amplification. Because we did not take any ship sturgeon voucher specimens to physically determine their sex, this finding could mean that either all nine ship sturgeon specimens captured in the Rioni River were indeed males, or that the marker is not working for the species; further research is required to clarify this.

The smallest ship sturgeon, captured in July 2021 in the Rioni River, ca. 25 km upstream from the river mouth, was 10 cm long and thus would have hatched in the summer season. The largest specimen, captured in July 2020 in the same area, was 75 cm long. Most specimens were found from March to August, and one 22 cm specimen was found in October. The sizes of the captured specimens and the timing of the records (Table 1) suggest that several generations of the ship sturgeon occur in the Rioni River.

Table 1. Ship sturgeon captured in the Rioni River from 2020 to 2022.

N	Place	Length (cm)	Capture Date	Species
1	Rioni River, Patara Poti	32	16 March 2020	*A. nudiventris* *
2	Rioni River, Chaladidi village	20	07 April 2020	*A. nudiventris* *
3	Rioni River	39	22 May 2020	*A. nudiventris* *
4	Rioni River, Samtredia	43	19 June 2020	*A. nudiventris* *
5	Rioni River, near Tskhenistskhali River	30	02 July 2020	*A. nudiventris* *
6	Rioni River mouth, north branch	35	07 July 2020	*A. nudiventris* *
7	Rioni River mouth, north branch	40	07 July 2020	*A. nudiventris* *
8	Rioni River, Sagvichio village	75	21 July 2020	*A. nudiventris* *
9	Rioni River, Sagvichio village	10	08 July 2021	*A. nudiventris*
10	Rioni River, Samtredia	60	12 August 2021	*A. nudiventris* *
11	Rioni River, Sagvichio village	22	13 October 2021	*A. nudiventris*
12	Rioni River, Sagvichio village	40	17 May 2022	*A. nudiventris*
13	Rioni River	32	18 May 2022	*A. nudiventris*
14	Rioni River, Patara Poti village	22	24 May 2022	*A. nudiventris*
15	Rioni River, Chaladidi village	26	28 May 2022	*A. nudiventris*
16	Rioni River mouth, north branch	45	07 June 2022	*A. nudiventris*
17	Rioni River mouth, north branch	41	13 June 2022	*A. nudiventris*
18	Rioni River, Sagvichio village	41	17 June 2022	*A. nudiventris*
19	Rioni River mouth, north branch	34	22 June 2022	*A. nudiventris*
20	Rioni River mouth, north branch	47	22 June 2022	*A. nudiventris*
21	Rioni River mouth, north branch	37	22 June 2022	*A. nudiventris*
22	Rioni River mouth, north branch	37	22 June 2022	*A. nudiventris*

* DNA sampling. Other individuals were identified based on morphology.

There is a paucity of demographic data regarding sturgeons in the region, and proper biodiversity assessments have never been conducted in the area. The ship sturgeon, similar to other sturgeon species, is an anadromous species, but some non-migratory populations may remain in a river throughout their lives [8]. The life history of the ship sturgeon in Georgia is not well studied, and it is unknown whether the species migrates to the Black Sea or remains in the river throughout the year. However, the fact that we observed specimens of different sizes (10–75 cm), representing multiple generations of the species indicates that reproduction is still occurring. The samples of *A. nudiventris* identified in this study were roughly clustered, with one set of samples from the coastal region (from 0–25 km inland), and the other samples were found ca. 80 km upstream of the Rioni River mouth. Samples from the coastal region ranged in size from 10 cm to 75 cm, while inland samples ranged in size from 30 cm to 60 cm. However, we have no sure knowledge of the migratory behavior or history of these individuals, or the precise location of spawning grounds for ship sturgeon in the Rioni River, and it would be premature to draw any geographic or biological inference based upon these data. Rather, since ship sturgeon clearly have survived and persisted in the Rioni River; aggressive efforts are needed to monitor ship sturgeon populations in the Rioni and to identify and protect spawning areas. There have

been discussions about updating species conservation strategies and whether species can be considered extinct in the wild after not having records for 50 years, or based on the sighting rates of a species [28–30], or when to consider a species endangered, critically endangered, or extinct in the wild and how to define terms to avoid vagueness [31]. Some species considered extinct have later been rediscovered [3,32]. However, regardless of whether a species is actually extinct in the wild or not, it is of great concern when species that were once widespread are not observed for many years or even decades, especially when their habitat has been altered dramatically [32]. Although we documented evidence of ship sturgeon remaining in the Rioni River, extensive surveys are needed to better understand their status in the region. The rediscovery of the species in the Rioni River, especially with the potential of the population being a remnant of the Black Sea population, highlights the importance of the Rioni River as one of the last remaining spawning rivers for this sturgeon species.

Author Contributions: This study was conceived and executed by T.B., with advice and assistance from F.S. (Fauna and Flora International) and C.A. (Ilia State University). T.E. assisted with sample collection and field observations. T.B. was primarily responsible for drafting the manuscript, with editorial assistance from C.A. and F.S. All authors have read and agreed to the published version of the manuscript.

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