Molecular taxonomy, phylogeny, hybridization and population genetic structure of sturgeon species in Georgia

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Statement

As an author of this doctoral thesis, I confirm that this compilation thesis is my original work and does not include the materials of other authors that have already been published, accepted for publication or submitted for a degree, which have not been cited in an accepted manner.

Tamar Beridze ----------

აბსტრაქტი

XX საუკუნის ბოლოს, მთელ მსოფლიოში, გადაჭარბებული მოპოვებისა და ჰაბიტატების განადგურების გამო, ზუთხისებრების პოპულაციების რაოდენობა მკვეთრად შემცირდა. შეცვლილი საბინადრო გარემოს მიუხედავად, საქართველო კვლავ რჩება ზუთხისებრების ბიომრავალფეროვნების ცხელ წერტილად. ჩვენი კვლევის დაწყებამდე არ არსებობდა განახლებული, გენეტიკური კვლევებით გამყარებული ინფორმაცია საქართველოში გავრცელებული ზუთხის სახეობების შესახებ. ამიტომ, ჩემი სადოქტორო კვლევის განმავლობაში შევისწავლიდი მდინარე რიონსა და შავ ზღვაში გავრცელებულ ზუთხისებრების პოპულაციებს. კვლევების განმავლობაში გამოვიყენეთ მოლეკულური მარკერები სახეობების იდენტიფიცირებისთვის, ჰიბრიდიზაციის დადგენისთვის, ზუთხისებრების სქესის იდენტიფიცირებისთვის და პოპულაციურ-გენეტიკური ანალიზისთვის. ჩვენმა კვლევებმა აჩვენა, რომ ისტორიულად ცნობილი 5-7 ზუთხისებრი სახეობიდან, სულ მცირე, სამი სახეობა (რუსული ზუთხი, ტარაღანა, ჯარღალა) ჯერ კიდევ ქვირითობს მდინარე რიონში, ხოლო მეოთხე - სვია, ჯერ კიდევ შემორჩენილია საქართველოს შავი ზღვის აკვატორიაში. ასევე, მდინარე რიონში და საქართველოს შავი ზღვის სანაპიროზე მოხდა არა-ადგილობრივი (ინვაზიური) სახეობის, ციმბირული ზუთხის აღმოჩენა. სადოქტორო კვლევის განმავლობაში გამოვიყენეთ გარემოდან მოპოვებული დნმ-ის ანალიზი ზუთხისებრების პოპულაციების მონიტორინგისთვის. კვლევის განმავლობაში გამოყენებული მოლეკულური მარკერები გამოსადეგი იქნება საქართველოში გავრცელებული ზუთხისებრების მონიტორინგისთვის. ასევე, ველური და კომერციული ინდივიდების გენეტიკური დახასიათებისთვის და მონიტორინგისთვის. სადოქტორო კვლევის შედეგები აჩვენებს ზუთხისებრების პოპულაციების მდგომარეობას საქართველოში და შეიძლება დაგვეხმაროს კონსერვაციული ერთეულების იდენტიფიცირებაში.

ძირითადი საძიებო სიტყვები: ზუთხები საქართველოში, მდინარე რიონი, სახეობების იდენტიფიცირება, პოპულაციური გენეტიკა, პოპულაციების მონიტორინგი, საფრთხის ქვეშ მყოფი სახეობები.

Abstract

Sturgeon species populations decreased dramatically because of overexploitation and habitat destruction at the end of the last century. Georgia, in terms of sturgeon diversity, is a global sturgeon diversity hotspot despite the altered environment. There was no current information about sturgeon populations, and no genetic analysis has been done for sturgeon in Georgia prior to this study. Therefore, during my doctoral studies, I studied extant sturgeon species and the current state of sturgeon populations in the Rioni River and the Black Sea. Molecular markers were used for sturgeon species identification, hybrid identification, sturgeon sex determination, and population monitoring and genetic analysis. Our results show that from historically known 5-7 sturgeon species, at least three sturgeon species (Stellate surgeon, Russian sturgeon, Ship sturgeon) are still spawning in the Rioni River, and a fourth species (Beluga sturgeon) persists in the eastern Black Sea, in Georgian waters. One non-native Siberian sturgeon was identified in the Rioni River and the Black Sea coast. Environmental DNA analysis was optimized for sturgeon population monitoring. Optimized molecular markers will be useful for ongoing sturgeon monitoring. These methods will help identify illegally traded sturgeon and characterization of wild and commercial sturgeon species in Georgia. The study results provide evidence of current sturgeon species' conservation status, identify conservation units in Georgia, and will help sturgeon species conservation.

Key words: Sturgeon in Georgia, Rioni River, Species identification, Population genetics, Population monitoring, Endangered species.

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Abbreviations

PCR – Polymerase Chain Reaction

MtDNA-Mitochondrial DNA

CITES - Convention on International Trade in Endangered Species of Wild Fauna and Flora

IUCN - International Union for Conservation of Nature

eDNA-Environmental DNA

BS – the Black Sea

RM - Rioni River mouth

RR - Rioni River

List of publications:

1. Interspecific hybridization in natural sturgeon populations of the Eastern Black Sea: the consequence of drastic population decline?
2. Rare but Not Gone: A Relict Population of the Black Sea Ship Sturgeon Acipenser nudiventris Persists in the Rioni River, Georgia
3. Fish diversity assessed by eDNA detection methods in the Rioni River
4. Decades of Global Sturgeon Conservation Efforts Are Threatened by an Expanding Captive Culture Industry
5. Draft: Molecular data demonstrate the provenance of Sturgeon sold in Georgia
6. Ship sturgeon rediscovered in the Rioni River in Georgia
7. Russian sturgeon in the eastern Black Sea basin, Georgia

General Introduction

The Acipenseridae family includes 25 sturgeon species distributed in the northern hemisphere. They are sometimes mentioned as 'living fossils' because of their primitive morphology (Banarescu and Holcik 1989; Berg 1962). According to fossil records, these species appeared around 200 MYA and have not significantly changed their morphological characteristics (Ludwig 2006; Peng et al. 2007). They are cartilaginous species with bony plates on the skull, and body covered with 5 rows of the bony scouts, with a protrusive jaw, and heterocercal tail (Banarescu and Holcik 1989; Berg 1962). Most Acipenser species are anadromous, sexually mature after 6-25 years, and spawn once in several years, depending on species (Banarescu and Holcik 1989; Berg 1962). Sturgeon species are characterized with long life cycle, and different ploidy levels: diploids (~120 chromosomes) and tetraploid (~240 chromosomes) plus macrochromosomes (Lanfredi et al. 2001).

For centuries, sturgeon species have been valued for producing black caviar, which is a valuable delicacy, and one of the most expensive wildlife products in the world (Ludwig 2006). Therefore, wild sturgeon stocks were exploited worldwide. Around 30 000 metric tons of sturgeon were captured in 1977 alone, of which 90% were from the Soviet Union, from the Black and Caspian Seas (Banarescu and Holcik 1989; Bronzi et al. 2011; Bronzi et al. 2019). Besides overexploitation, for anadromous fish species survival, spawning rivers have critical importance. Therefore, habitat disruption, and damming of sturgeon spawning rivers has also contributed to dramatic declines and near extinction worldwide during the second half of the last century (Billard and Lecointre 2000; Ludwig 2006; Congiu, Gessner, and Ludwig 2023). According to the IUCN reassessment of sturgeon species, populations decreased worldwide and 17 of them are listed as Critically Endangered (Congiu, Gessner, and Ludwig 2023). Besides direct anthropogenic threats for the sturgeon species, altered environment and decrease of spawning grounds raised another threat, which is interspecies hybridization (Linhartová et al. 2018; Ludwig et al. 2009; Fopp-Bayat,

Nitkiewicz, and Chandra 2021). Moreover, sturgeons are characterized with high genetic plasticity, with ability to hybridize with other sturgeon species and produce fertile or in some cases sterile offspring in nature (Havelka et al. 2016; Káldy et al. 2020; 2020; Linhartová et al. 2018). Hybridization is perhaps even common for some natural sturgeon populations (Berg 1962), however for rarer species, interspecies hybridization might contribute to extinction, over several generations (Wolf, Takebayashi, and Riesebrg 2001). In addition, sturgeon, interspecific hybridization might cause disruption of adaptive genetic variation (Ludwig et al. 2009).

The Rioni River and eastern part of the Black Sea represent a sturgeon diversity hotspot. Despite their critical conservation status and importance in world sturgeon conservation, these populations still are not well understood, and illegal trade still takes place. Therefore, it is important to understand both native sturgeon population status, and define appropriate conservation units.

Literature review

Historically, Georgia represented high sturgeon diversity; based on the different literature sources, there were 6 or 7 sturgeon species historically foundd in Georgia (Zarkua et al. 1998; Ninua and Guchmanidze 2013).

1. European sturgeon – *Acipenser sturio*
2. Russian sturgeon – *Acipenser gueldenstaedtii*
3. Colchic sturgeon – *Acipenser colchicus, A. Gueldenstaedtii colchicus.*
4. Persian sturgeon - *A. persicus colchicus*
5. Ship sturgeon – *Acipenser nudiventris*
6. Stellate sturgeon – *Acipenser stellatus*
7. Beluga sturgeon – *Huso huso*

Worth to mention that Colchic sturgeon taxonomic status is under the question, according to some resources the species is described as *Acipenser gueldenstaedtii ssp. colchicus* possibly synonymous with Russian sturgeon-*Acipenser gueldenstaedtii* (Birstein, Doukakis, and DeSalle 2000; Berg 1962), others mention Colchic sturgeon as Caucasian Black Sea (Kolkhida) sturgeon - *Acipenser persicus colchicus* (Zarkua et al. 1998) or as *Acipenser colchicus*, as a separate species endemic and the most abundant sturgeon species in Georgia (Ninua and Guchmanidze 2013). Persian sturgeon separate taxonomic status is also questioned recently (Ruban 2015, IUCN 2022).

The studies about sturgeon in Georgia were carried out according to morphological analysis and never verified with genetic data. Also, information about sturgeon species populations have not been updated for the last two decades with new data. There is a lack of information about current distribution and species composition. There was no clear information about taxonomic status of the species currently distributed in Georgia, or their current population status, and how viable those populations are. Therefore, the aim of the research was to study the current statement of the sturgeon populations in Georgian waters. The main questions of the study: a) Which sturgeon species are currently distributed in Georgian waters? b) What is their population state? Specific areas of research include:

1) Molecular identification to see which sturgeon species remain.
2) Phylogenetic connections between these species and population state of remaining populations.
3) Improved molecular tools to distinguish commercial and wild individuals.
4) Use the data generated in items 1-3 to develop population monitoring methods for natural populations.

These investigations were carried out in close collaboration with Fauna & Flora Caucasus programme. With Fauna & Flora, we initiated an evidence-based sturgeon research and developed sturgeon research team, which will be working on sturgeon conservation in the region. Genetic research methods assist in sturgeon species conservation; they create reliable knowledge

of sturgeon species' presence and population structure, which is essential to monitor and manage natural populations, and detect illegal trade of sturgeon in Georgia.

Section 1 – Sturgeon species identification—Stellate and Russian sturgeon interspecific hybridization in Georgia, Non-Native Siberian sturgeon

Introduction

Different literature cited different number of species distributed in the Eastern Black Sea region. European, Stellate, Russian, Ship, and Beluga sturgeon populations were known as well established (Banarescu and Holcik 1989; Berg 1962), when Colchic and Persian sturgeon presence was mainly under the question (Ninua and Guchmanidze 2013; Zarkua et al. 1998).

European sturgeon is extinct from the Black Sea basin, it was last recorded in 1991 in the Rioni River (IUCN 2020; Kolman 2011). **Russian sturgeon** (*Acipenser gueldenstaedtii*) was listed as one of the least abundant species, single individuals described to be found in the Rioni River and the Black Sea by Zarkua and Ninua (Ninua and Guchmanidze 2013; Zarkua et al. 1998). However according to the other sources, the species was common for the Rioni, Enguri Rivers (Banarescu and Holcik 1989; Berg 1962; IUCN 2019a). Other species which Zarkua and Ninua described in Georgia are **Colchic sturgeon** (*Acipenser colchicus*) and **Persian sturgeon** (*Acipenser persicus colchicus*) of which taxonomic status are not clear (Ruban et al. 2011; IUCN 2019b; Berg 1962). **Ship sturgeon** was about to list as extinct in wild because of the species disappearance for the last two-three decades not only from Georgia but worldwide (Ninua and Guchmanidze 2013; Mugue et al. 2016), we will discuss about the species in the next section. **Stellate sturgeon** (*Acipenser stellatus*) was one of the abundant species in Georgia (Ninua and Guchmanidze 2013) entering to the Rioni River and other eastern Black Sea tributaries for spawning (Banarescu and Holcik 1989; Berg 1962). **Beluga sturgeon** also was one of the abundant sturgeon species distributed in the Rioni River, Enguri River and other east Black Sea tributaries (Ninua and Guchmanidze 2013; Berg 1962; Banarescu and Holcik 1989).

At the beginning of our studies we did not know if those species still remain in Georgian waters, and what was their population status. We aimed to identify species and understand their population status in the region.

Methods

In collaboration with Fauna & Flora Caucasus porgramme and local anglers, we collected 189 tissue samples in total from natural sturgeon populations, Black Sea and the Rioni River.

Figure 1. Geographic locations of sampled individuals (green = *A. stellatus*, yellow = *A. gueldenstaedtii*, and blue = *H. huso*). The inset map shows a continental view where the sampling area in the southeastern Black Sea is framed (Beridze, et al. 2021a).

We used multiple molecular methods—PCR amplification, mitochondrial DNA sequencing, nuclear markers, and sex-specific markers--to investigate the taxonomy, phylogeny, sex ratios, hybridization and population status of multiple sturgeon taxa. Sampling details and laboratory methods and protocols can be found in (Beridze, et al. 2021a; Ananiashvili et al. 2023) and in Appendix I (Table A 1-4), and Appendix II. Figure 1. shows the regions sampled for these studies.

Results

From historically known six-seven species, four were detected in our studies Russian, Ship, Stellate, Beluga sturgeon, Table 1.

Table 1. Locations of identified taxa, numbers of each taxon are shown in brackets.

Black Sea	Black Sea and Rioni River	Rioni River
1. **Beluga** (*Huso huso)* [25]	2. **Russian sturgeon** *(A. gueldenstaedtii)* [119] 3. **Stellate sturgeon** (*A. stellatus)* [35]	4. **Ship sturgeon** *(A. nudiventris)* [17]

Detailed analysis of selected samples showed that Russian sturgeon and Stellate sturgeon still use the Rioni Rive for spawning, as juvenile individuals were found in the Rioni River and its mouth. Ship sturgeon were found only in the Rioni River, the detailed analysis will be discussed in the next section (see page 11). Beluga sturgeon have been only found in the Black Sea Georgian aquatory, juvenile beluga individuals have not been found during this research.

Russian sturgeon is the most abundant species in the region. This taxon also exhibits more haplotype diversity. Stellate sturgeon also exhibits relatively high haplotype diversity, however is represented in lower numbers (Beridze et al. 2021a).

Six Russian and Stellate sturgeon hybrids were found in the Rioni River and the Rioni River mouth, see Table 3. Russian sturgeon is the maternal parent for all hybrid individuals. The six maternal lineages among them showed four different haplotypes, indicating at least four independent hybridization events occurred in 2018-2022. Comparing percentages, the number of hybrids is ca.5 % of the Russian sturgeon and ca.16 % of the stellate sturgeon samples found in the region. See details in (Beridze et al. 2021a).

Table 2. hybrid Stellate and Russian sturgeon found in the Rioni River during the doctoral research, information about the first four samples is published in (Beridze et al. 2021a).

#	Place	Date	Length (cm)	Sex
1	Ac112-Rioni River mouth	15.08.2018	11	♀
2	Ac160-Rioni River	15.04.2020	20	♀

3	Ac169-Rioni River mouth	06.06.2020	28	♀
4	Ac175-Black Sea, near Poti port	18.06.2020	22	♀
5	Ac248-Rioni River mouth, Nabada	17.07.2021	30	♂
6	Ac302-Rioni River, Pirveli Maisi village	01.06.2022	19	♂

Selected samples were tested with sex-specific markers (Kuhl et al. 2021), detailed protocol in Appendix I (Table A. 2) and Appendix II. Russian sturgeon and Beluga sturgeon exhibit roughly 1:1 sex ratio, see Table 4. Analysis for *A. stellatus* and *A. nudiventris* needs further verification, because the marker we used has not definitively been tested for these two species.

Table 3. Sturgeon sex identification. BS – the Black Sea, RM-Rioni River mouth, RR-Rioni River. Samples collected during 2018-2021. Results for *A. stellatus* and *A. nudiventris* is given in gray color, these results need further validation, as the marker is not tested for those two species.

Species	Individual	BS	RM	RR	♀	♂
Wild						
A. gueldenstaedtii	48	11 (6♀5♂)	32 (14♀18♂)	5 (2 ♀3♂)	22	26
A. stellatus	26	23	2	-	-	26
A. nudiventris	9	-	-	9	-	9
H. huso	18	18	-	-	11	7
Total:	101					

During our research we found three individuals of non-native Siberian sturgoen (*Acipenser baerii*) in the Rioni River and the Black Sea coastline (Grigoleti). Two juveniles and one adult individuals were found. The adult individual was 90 cm long, found close to the potential sturgeon spawning grounds (close to Samptredia) in August 2020 (White et al., 2023).

We did not detect European (*A. sturio*), Persian (*A. persicus colchicus*) or Colchic sturgeon (*A. colchicus*) using sturgeon species identification markers.

Summary and Recommendations

At the beginning of our studies we did not know if/which sturgeon species remained in Georgian waters, and if they persist, what their population status would be. Based on our research, 4 historically known species (Russian, Ship, Stellate, and Beluga sturgeon) still occur in the region and 3 of them (Russian, Ship, Stellate sturgeon) are still spawning, as demonstrated by the presence of early-stage juveniles was found.

Besides species composition, one of the most important findings of this doctoral research is a clearer understanding of the population status of remaining sturgeon species.:

- Russian sturgeon is the most abundant sturgeon species remained in the region (Beridze et al. 2021a; Ananiashvili et al. 2023).
- Stellate sturgeon status is more critical, it is less abundant in the randomly captured specimens sampled in this study, 16% of the captured individuals were hybrids with Russian sturgeon, presumably because this species cannot always find conspecific mates (Beridze et al. 2021a).
- Ship sturgeon in Georgia will be discussed in detail in the following section.
- Beluga sturgeon population status is still under question, only adult individuals were reported form the eastern Black Sea, no juveniles were detected. Presumably the number of the species is so low that we did not encounter them in our field work, fishing, or with local fishers. It may be that the species is not using the Rioni River for reproduction anymore; this species' population status in Georgia needs further research.
- European, Colchic, or Persian sturgeon were not identified in any of the samples we collected.
- Non-native Siberian sturgeon was detected in the Rioni River and the Georgian Black Sea coast.

Our findings show that there are spawning populations of Russian, Ship, and Stellate sturgeon in the region, which lends urgency for strengthening the conservation actions to protect reproductively active populations.

We recommend supporting conservation actions for the three native sturgeon species (Russian, Ship, and Stellate sturgeon) still spawning in the region, and supporting research to understand Beluga sturgeon population status in Georgia. More research is needed to detect scale of distribution of the non-native Siberian sturgeon in the region. The methods used in our research was carried out in at Ilia State University, we recommend using the capacity and existing sturgeon research team in Georgia to use advanced methods for sturgeon research. Ilia State University is already working closely with Fauna & Flora Caucasus programme and scientists from the USA, who support developing the sturgeon research team in Georgia.

Section 2 – Ship sturgeon in Georgia

Introduction

Having previously identified three sturgeon species (Russian, Stellate, and Beluga sturgeon) still remaining in Georgian waters, with Fauna & Flora's successful awareness-raising programs and the help of local fishers, we encountered a fourth species in the region, Ship sturgeon (*Acipenser nudiventris*). Ship sturgeon was previously described as rare in the Rioni River and the Black Sea basin (Ninua and Guchmanidze 2013). This species had not been detected during the last three decades, was considered regionally extinct in the Black Sea basin, and almost extinct in the Caspian Sea basin (Mugue et al. 2016). Only an Aral Sea population introduced in the Ili River in Kazakhstan remained (Zholdasova 1997). After the rediscovery of ship sturgeon in the Rioni River we, we characterized multiple genetic markers useful to identify the species, and determine whether the specimens from the Rioni River originated there, or if they were immigrants from a Krasnodar ship sturgeon breeding program.

Methods

In total, 22 ship sturgeon sightings were collected, including nine genetic samples (Table 3.) all with help of FFI and local fishers. See Figure 2, which shows the locations of the ship sturgeon samples and sightings. Samples were tested with genetic markers for species identification, hybrid detection, and sex identification. Detailed protocols and analysis methods in (Beridze et al. 2022).

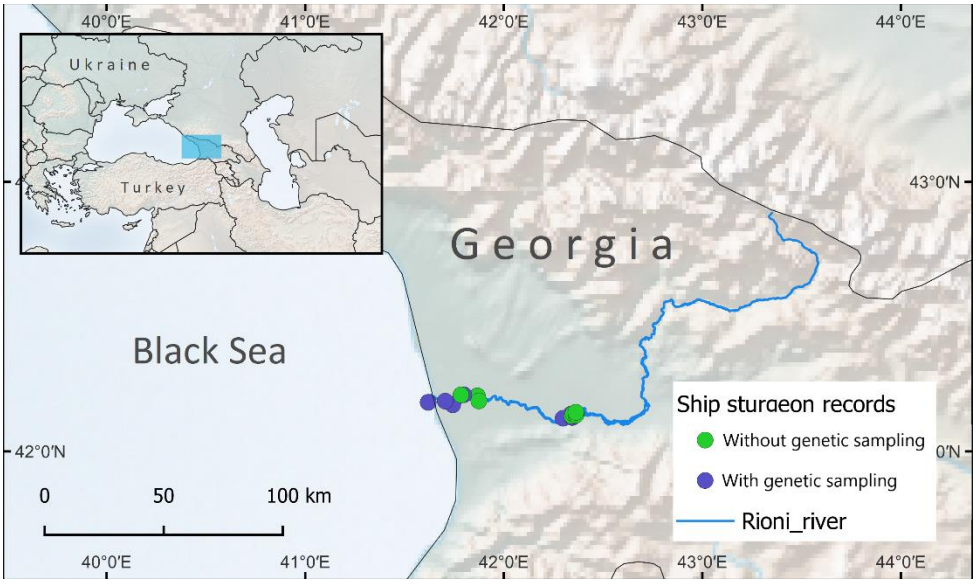

Figure 2. Sampling locations of ship sturgeon in the Rioni River, Georgia

Results

Based on our genetic analysis, the species is still present in the Rioni River. Mitochondrial analysis shows that the all nine specimens represent one haplotype, and are different from the Aral Sea and the Caspian Sea ship sturgeon haplotypes. Sizes of individuals varied from 10 cm to 75 cm, see table 3. Nuclear, sturgeon-specific diagnostic markers do not show signs of hybridization with other sturgeon species, see detailed description of findings in (Beridze et al. 2021b; Beridze et al. 2022).

N	Place	Length (cm)	Capture Date	Species
1	Rioni River, Patara Poti	32	16.03.2020	*A. nudiventris**
2	Rioni River, Chaladidi village	20	07.04.2020	*A. nudiventris**
3	Rioni River	39	22.05.2020	*A. nudiventris**
4	Rioni River, Samtredia	43	19.06.2020	*A. nudiventris**
5	Rioni River, near Tskhenistskhali River	30	02.07.2020	*A. nudiventris**
6	Rioni River mouth, north branch	35	07.07.2020	*A. nudiventris**
7	Rioni River mouth, north branch	40	07.07.2020	*A. nudiventris**
8	Rioni River, Sagvichio village	75	21.07.2020	*A. nudiventris**

9	Rioni River, Sagvichio village	10	08.07.2021	*A. nudiventris*
10	Rioni River, Samtredia	60	12.08.2021	*A. nudiventris**
11	Rioni River, Sagvichio village	22	13.10.2021	*A. nudiventris*
12	Rioni River, Sagvichio village	40	17.05.2022	*A. nudiventris*
13	Rioni River	32	18.05.2022	*A. nudiventris*
14	Rioni River, Patara Poti village	22	24.05.2022	*A. nudiventris*
15	Rioni River, Chaladidi village	26	28.05.2022	*A. nudiventris*
16	Rioni River mouth, north branch	45	07.06.2022	*A. nudiventris*
17	Rioni River mouth, north branch	41	13.06.2022	*A. nudiventris*
18	Rioni River, Sagvichio village	41	17.06.2022	*A. nudiventris*
19	Rioni River mouth, north branch	34	22.06.2022	*A. nudiventris*
20	Rioni River mouth, north branch	47	22.06.2022	*A. nudiventris*
21	Rioni River mouth, north branch	37	22.06.2022	*A. nudiventris*
22	Rioni River mouth, north branch	37	22.06.2022	*A. nudiventris*

Figure 3. Ship sturgeon sightings (photos and videos) recorded from the Rioni River in 2020-2022. Asterisks indicate individuals that provided genetic data (Beridze et al. 2022).

Summary and Recommendations

In our research, we demonstrated that the Ship sturgeon distributed in Georgia is genetically distinct from the Caspian Sea and the Ilia River populations (Accession numbers: Caspian Sea-KU321568; Ili River (Balkhash basin)-KU321569) also different from known commercial Ship sturgeon haplotypes (Accession numbers: HAP01-KF974767; HAP02-KF974768). This indicates that the individuals we sampled did NOT originate from the Centre for Sturgeon Gene Pool Conservation, Krasnodar, or from farmed Ship sturgeon. Based on these results, we conclude that the ship sturgeon from the Rioni River are remnants of the native Black Sea Ship sturgeon population. Further analysis of our research shows no signs of interspecific hybridization between Ship sturgeon and any other locally distributed sturgeon species in the Rioni River. Moreover, the sizes of the captured individuals show that different generations occur in the region. Multiple generations present indicates that reproduction still occurs locally (Beridze et al. 2022).

Our research shows that the ship sturgeon remains in the Rioni River, potentially the only natural population worldwide; this finding highlights the importance of the Rioni River as one of the last remaining spawning rivers, more extensive studies and conservation measures are needed to protect ship sturgeon in Georgia.

Section 3 – Wild *versus* Commercial Sturgeon

Introduction

Because of the dramatically decreased stocks in nature, and high demand for sturgeon products, sturgeon rearing in aquaculture has become more common (Bronzi, Rosenthal, and Gessner 2011; Bronzi et al. 2019). Sturgeon species' ability to hybridize easily (Lanfredi et al. 2001; Fontana, Tagliavini, and Congiu 2001) has been exploited in sturgeon aquaculture, with cultivated hybrid sturgeon species selected for desirable properties of parent species, such as early sexual maturation, and fast growth (Havelka et al. 2017). Sturgeon commercial aquaculture programs started rising in popularity in Russia from 1970. Later, sturgeon farming developed in Europe, USA, and China, from the early 2000s, with programs still expanding today (Bronzi, Rosenthal, and Gessner 2011; Bronzi et al. 2019). Commercial sturgeon aquaculture is legal, controlled, and regulated activity under the Convention on International Trade in Endangered Species of Wild Fauna and Flora-CITES (2021).

A more recently apparent threat for wild sturgeon populations is the release or escape of commercially-bred sturgeon into natural sturgeon habitats (White et al. 2023; Ludwig et al. 2009). Farmed sturgeon interbreeding with native populations might cause genetic admixture between local and farmed populations, which disturbs local genetic adaptations, even if the local and captive fish are the same species. It also raises other issues such as competition between local and captive-bred individuals, interspecific hybridization and introduced parasites (Ludwig et al. 2009; Zholdasova 1997; White et al. 2023).

During our research we detected four sturgeon species (Russian, Ship, Stellate, and Beluga sturgeon) still occurring in the region, three of them still spawning. Concurrently, there are at least four farms rearing sturgeon, of which one is operating along the Tekhuri River, which is the Rioni River tributary. Therefore, it raises concerns about the effects these farms might have on the local sturgeon species if not strictly regulated (White et al., 2023). In parallel, it is important to have tools to identify and track wild and commercial sturgeon. Uncontrolled release or escape

of commercial sturgeon individuals into nature, and hybridization of native and non-native lineages will make it difficult to identify local sturgeon species and monitor markets, if those two groups are admixed (White et al., 2023)

Due to the high complexity of sturgeon genomes, there is currently no single test or assay for identifying interspecies hybrids, or illegally traded individuals. Only a few sturgeon identification markers (mitochondrial and nuclear) are intended specifically for species and hybrid identification, although nearly all sturgeon species can be identified with high certainty through the use of multiple markers (Barmintseva and Mugue 2013; Boscari et al. 2014; Boscari et al. 2017; Havelka et al. 2019; Ludwig et al. 2021; Reinartz et al. 2011). However, several species are problematic, for example Russian Sturgeon (*A. gueldenstaedtii*) and Siberian sturgeon (*A. baerii*) can be differentiated from every other sturgeon species, but not from each other if they are hybridized (Havelka et al. 2018). At the same time, both of the species are involved in commercial propagation, and are frequently hybridizedfor aquaculture (Bronzi et al. 2011). Also, ability to hybridize makes it difficult to identify individual origins, which requires either multiple molecular genetic markers, isotope analysis, and/or morphological identification to reliably identify illegally traded sturgeon (Ludwig et al. 2021). In this research component we investigatedwhich sturgeon species were regularly encountered in commercial markets in Georgia. In addition, we compared the genetic characteristics of wild and commercially propagated sturgeon within Georgia, which provided insight into the presence of wild-caught individuals in commercial settings.

Methods

Mitochondrial DNA sequencing and microsatellite analysis methods were used to detect which species were present in Georgian fish markets and farms and infer their provenance. See details in Beridze et al, 2023 (MS in prep, see attached draft).

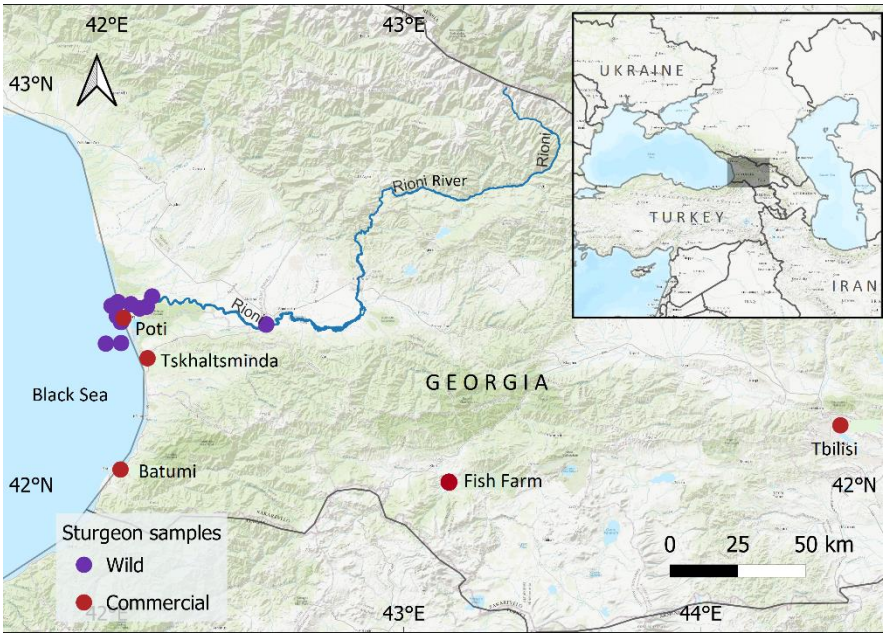

Figure 4. Wild and Commecial sturgoen samples collection location from 2016-2020.

Results

Four sturgeon species (Russian sturgeon, Stellate sturgeon, Beluga sturgeon, Sterlet) were identified from markets and farms in Georgia. Russian sturgeon (*A. gueldenstaedtii*) is the most common species sold (almost 92%). Four microsatellite markers were used: Afug41, An20, Aox45, and AoxD165, see Table A 1 and Appendix II. Analysis of 4 microsatellite markers differentiated wild-caught from commercial Russian sturgeon specimens from fish markets and fish farms. These findings were supported with mitochondrial DNA analysis. Based upon mtDNA haplotypes, there were some specimens collected in coastal markets which clustered with wild samples, possibly indicating wild individuals present in fish markets. See details in Beridze et al, 2023 (MS in prep, see attached draft).

Summary and Recommendations

Four microsatellite markers were able to differentiate wild and commercial Russian sturgeon individuals from each other (Beridze et al., 2023, Ms. in prep). This method, augmented with additional markers, can be used for wider baseline studies to understand current population status of the native sturgeon species, and characterize commercial sturgeon stocks in the country, until

natural production increases in the region. Russian sturgeon appears to be the preferred taxon for human consumption, most frequently appearing in fish markets and fish farms in Georgia. Russian sturgeon is one of the most valuable economic sturgeon species (Bronzi et al. 2017), therefore we assume those individuals are legally provided on Georgian fish markets and farms. However, 11 individuals possessed wild Russian sturgeon haplotype and exhibiting wild genrtypes with microsatellite analysis which indicates potential wild origin of the species found on fiah markets.

Supporting sturgeon conservation in the regions where commercial aquaculture is developing is crucial for native sturgeon populations conservation. We recommend additional research to study local populations and their regional genetic variation (Russian, Ship, Stellate, and Beluga), and to further characterize commercially reared sturgeon in Georgia, in order to more easily accurately distinguish and monitor wild and commercial fish and fish markets.

Specifically, we recommend the following for routinely monitoring wild and commercial sturgeon in Georgia.

(a) Microsatellite analysis and mitochondrial DNA sequencing methods are relatively cheap, and provide species identification and genotype data.
(b) Stable isotope analysis is more expensive and requires specific expertise and equipment, which is not currently in use in the region. However, this technology can provide reliable information about sturgeon provenance and natal sites, irrespective of where the animal's location when it is caught or sampled. Because of their protected status, samples intended for stable isotope analysis will need to be permitted for transport to countries with the requisite equipment and expertise.

Section 4 – Environmental DNA for sturgeon monitoring in Georgia

Introduction

Environmental DNA (eDNA) methods have been used to study species distribution, presence/absence, for monitoring of rare populations, and the investigation of invasive species (Rees et al. 2014). It is a non-invasive method and does not require catching the individual itself (Shu, Ludwig, and Peng 2020). eDNA is genetic material living organisms leave behind during their lifetime; it can be scales, feces, or bodily fluids that are shed by the organism, which remain floating in the aquatic environment until they decay. Water samples are collected to extract DNA, which is then used for genetic testing on targeted organisms (Thomsen et al. 2016; Shu, Ludwig, and Peng 2020). We pioneered environmental DNA metabarcoding methods for sturgeon population monitoring in Georgia, to guide conservation efforts.

Methods

We used eDNA metabarcoding methods to attempt detection of multiple *Acipenser* species in the Rioni River water samples, from habitats where these fish historically were found. 12 water samples were collected, water was filtered according to the manufacturer's protocol by NatureMetrics, UK. Samples were shipped to and processed by NatureMetrics for eDNA metabarcoding using the "eDNA Survey – Fish" pipeline (NatureMetrics, UK). See details in (Beridze et al. 2023).

Results

We did not detect sturgeon DNA in the Rioni River water samples. However, we detected many other fish species, and these data comprise a species composition list for the Rioni River in the areas we sampled. This data represents the most recent fish taxonomic survey and first eDNA survey in Georgia. We compared the eDNA-based taxonomic composition to the known faunal composition within the Rioni River. We found that the method detected 75% of the expected total fish fauna in the Rioni River. Several new species occurrences were detected, including three

invasive species (*Carassius gibelio, Pseudorasbora parva, Rhinogobius lindbergi*) in the Rioni River Basin and a new country record of the ninespine stickleback (genus *Pungitius*) for Georgia. See detailed results in (Beridze et al. 2023).

Summary and Recommendations

Environmental DNA monitoring method is non-invasive which is important when working on highly critically endangered species such as sturgeon. eDNA method was successfully used for rediscovering and monitoring of multiple amphibian species in Brazil (Lopes et al. 2021). Our previous research shows that there are at least three species of sturgeon (Russian, Ship, Stellate sturgeon) spawning in the Rioni River, by detecting juvenile species at different life stages. For our research we identified potential reasons why we could not detect sturgeon in the Rioni River water samples as follows: (1) the species might not inhabit the selected sampling sites; (2) volume of water filtered for DNA extraction was insufficient for detecting sturgeon DNA; and (3) sample number and water volume were too small. We think that the method should be able to effectively detect sturgeon DNA in the Rioni River samples, if sampling methods and sample numbers were improved. Based on our eDNA results we recommend the following:

(a) Metabarcoding analysis is informative and provides information about multiple species, However, using a commercial sequencing service for eDNA is expensive and needs specific expertise. Therefore we recommend adapting the method in the country, using sturgeon-specific PCR-based detection methods and Taqman probes (Anderson et al. 2018; Schenekar, Schletterer, and Weiss 2020) which are cheaper and more sensitive.

(b) The Rioni River is very turbid, which makes filtering larger volumes of water difficult (we could not filter more than 800 ml in any one sample, most were much less). Therefore, we recommend using bigger pore (1.2 μm or more) size of the filter (Thomas et al. 2019; Shu, Ludwig, and Peng 2020) (instead of 0.8 μm pore size we used) and using filtration apparatus and pumps that can filter higher volumes of water (2-5 litres, Thomas et al. 2018).

(c) Optimize sampling methods to collect from different water depths (Thomas et al. 2018), especially the water closest to the bottom, since the sturgeon species are benthic organisms (Banarescu and Holcik 1989).

(d) Using multiple analysis controls, field and laboratory blank samples from the field, DNA extraction control, and PCR reactions negative controls to check the confidence of the analysis (Shu, Ludwig, and Peng 2020).

We recommend further development and deployment of eDNA methods for the sturgeon species in the region. Potentially, the method could be routinely (and cheaply) applied in the Rioni River and the Black Sea tributaries. Fauna & Flora Caucasus programme and Ilia State University are currently working on sturgeon-specific eDNA monitoring optimization, for detecting sturgeon species in the region.

Section 5 – Concluding remarks and conservation implications

During the doctoral research we answered the prior questions we had about sturgeon species composition and population status in Georgia. We identified four (Russian, Ship, Stellate, and Beluga sturgeon) sturgeon species remaining in Georgian waters. Of these, three (Russian, Ship, and Stellate sturgeon) are still spawning in the Rioni River. A fifth species, non-native Siberian sturgeon, several individuals (*n=3*) were also found in the Rioni River and along the Black Sea shore.

For each species the conditions appear different. For example, **Stellate sturgeon** is still spawning in the Rioni River and juveniles are found in the Rioni River and its mouth. However, this species is hybridizing with the more abundant and sympatric Russian sturgeon, which underscores the extinction threat to Stellate sturgeon in the Eastern Black Sea region.

Russian sturgeon is the most abundant species in the Rioni River and the Black Sea, and juveniles occur in the Rioni River and its mouth. Genetic analysis did not detect **Colchic sturgeon** or **Persian sturgeon,** mentioned in the historical literature as abundant species in the region. Samples whose photographs were morphologically identified as Colchic sturgeon (Dr. Nargiza Ninua pers. comm), genetically assayed as Russian sturgeon, Colchic sturgeon taxonomic identification as a separate species needs more complex genomic research, to prove whether it is a separate species, or a form of Russian sturgeon characteristic for the eastern Black Sea. Regarding **Persian sturgeon** (*Acipensr persicus*), its status as a separate species is questioned because of the genetic similarities with Russian sturgeon; it is considered by many as a conspecific (IUCN 2019b). Persian sturgeon, together with Russian sturgeon, Adriatic sturgeon (*A. naccarii*) and the Siberian sturgeon (*A. baerii*) are included in the so-called 'gueldenstaedtii complex' of species, due to the genetic relatedness of these four taxa (Ruban et al. 2011; Birstein, Doukakis, and DeSalle 2000; Doukakis et al. 2012).

Ship sturgeon is still spawning in the region, and based on our research, it is a remnant of the Black Sea ship sturgeon population, and the rediscovered population is likely the last surviving wild ship sturgeon population worldwide.

Beluga sturgeon is found only as adult individuals in the Black Sea, it is still unknown if the species is spawning in the region. More research is needed to understand Beluga migration patterns and distribution, and if this species still spawns in Georgian rivers.

One of the more important findings was the discovery of non-native **Siberian sturgeon** in our sampling area; three individuals were found in the Rioni River and the Black Sea shore (Grigoleti). The individual found in the Rioni River close to the Samptredia municipality was a 90 cm long individual, male, in August 2020. This site is a potential sturgeon spawning ground, and the invasive sturgeon might be a threat to native sturgeon populations. Besides, this species is highly commercial species, it might have escaped or been released from sturgeon aquaculture. It will be important to study how widespread this species is in the Rioni River, and the eastern Black Sea, and evaluate its potential affects on the native sturgeon species.

Using molecular tools, we have been able to differentiate wild and commercial Russian sturgeon species individuals and demonstrate that wild genotypes were sold in Georigan fish markets. Such sales are illegal, and highlights the urgency of monitoring sturgeon fish markets and commercial settings for preventing illegal sturgeon trade.

We also attempted eDNA metabarcoding analysis for sturgeon population monitoring. We have so far not been able to detect sturgeon DNA in the Rioni River water samples via eDNA methods; however, this was an important preliminary attempt for planning the next steps for sturgeon eDNA monitoring, route to reliable and cheap sturgeon detection.

The methods mentioned in our studies are optimized and in use in Georgia, at Ilia State University Genetic laboratory. The next generations of biologists can use the technologies and get training in those methods in country which will help to sustain the sturgeon species research and conservation in the region. Strong ties with American and European scientists, different institutions are established and supporting sturgeon research in Georgia. The goal of future research is to identifying sturgeon spawning grounds in the region.

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Appendix I

Sturgeon species identification markers

Table A 2. Mitochondrial DNA markers used during the doctoral reseach.

Name	Sequence 5'-3'	PCR product size (bp)	Reference
Control Region			
Acipenser_Pro1F	CACCCTTAACTCCCAAAGC	850	Congiu et al., 2011
Acipenser_Phe1R	CCCATCTTAACATCTTCAGT		
F.for	GCA TCT GGT TCC TAT TTC AGG TCC	~300	Ludwig et al., 2009
F.rev	TAT TAG GCT TGT TTC GGC GTA AGG		
Cytochrome b			
cytb-for1	CGTTGTHWTTCAACTAYARRAAC	1141	Ludwig et al., 2000
cytb-rev1	CTTCGGTTTACAAGACCG-3'		
cyt*b*: B1	CCATCCAACATCTCTGCTTGATGAAA	790	Doukakis et al., 1999
S2A	CCTCCAATTCATGTGAGTACT		
L14735	AAAAACCACCGTTGTTATTCAACTA	500	Wolf et al., 1999
H15149	GCCCCTCAGAATGATATTTGTCCTCA		

Table A 3. Sturgeon Sex Identification marker

Sex-identification marker			
Name	Sequence 5'-3'	PCR product size (bp)	Reference
AllWSex2_F	TGATCAACCTCTTCAGCAATGTC	~100-150	Kuhl et al., 2021
AllWSex2_R	TGAGAGCCACTGTACTAACACA		

Table A 4. Nuclear markers for sturgeon identification and interspecies hybrid detection.

Sturgeon specific diagnostic markers				
Name	**Sequence 5'-3'**	**PCR product size (bp)**	**Detected species**	**References**
Ste_RP1F	TGTCACCTTTCAAATTTGGTA	479	*Acipenser stellatus*	Boscari et al., 2014
RP1_LocusA_R	ATCCAAGTACAAGCTTGAACA			
395_AB	CCACAAAACAACAAAACATATGGAG	395	*Acipenser gueldenstaedtii/ A. Baerii*	Havelka et al, 2018
395_uni	CCTTGGGCTAGTCTTCATGCC			
153_HHp	GATCTGAACATCAGCCACTGC	153	*Huso huso*	Havelka et al., 2017
153_uni	TACTGTGCCTGTATGTCTCC			
153_HHn	GATCTGAACATCAGCCACTGG			
247_ARp	TAAGGGTCCATGCATGCAG	247	*Acipenser ruthenus*	
247_uni	TTTTAGCTGCACCGTGGC			

247_ARn	TAAGGGTCCATGCATGCCT			Havelka et al., 2018
RP2S6_huso-F	CATAACATTGCACTGAATGTTATA	194	*Huso huso*	Boscari et al., 2017
RP2S6_groupA_R	CTTTCGTTGATTTAGGGAAATGGT			
RutBae_RP1F	GATCCAAGTACAAGCTTGAACA	169	*Acipenser ruthenus*	Boscari et al., 2017
RP1_LocusA_R	GATCCAAGTACAAGCTTGAACA			

Table A 5. Microsatellite markers optimized for sturgeon population studies.

Name	Primer sequence 5' - 3'	Repeat motif	Dye	Size	Reference
Afug41	F: TGACGCACAGTAGTATTATTTATG	(GATA)9TA(GATA)3	5' - 6-FAM	200-260	Welsh et al., 2003
	R: TGATGTTTGCTGAGGCTTTTC				
AoxD161	F: GTTTGAAATGATTGAGAAAATGC	(CTAT)15	5' - 6-FAM	118-148	Henderson-Arzapalo and King 2002
	R: TGAGACAGACACTCTAGTTAAACAGC				

An20	F: AATAACAATCATTACATGAGGCT R: TGGTCAGTTGTTTTTTTATTGAT	(ATCT)10(TG)5	5' – VIC	108-204	Zane et al., 2002
Afug51	F: ATAATAATGAGCGTGCTTTCTGTT R: ATTCCGCTTGCGACTTATTTA	(AAAC)6(AC)2(AAAC)8	5' - PET	230-260	Welsh et al., 2003
AoxD165	F: TTTGACAGCTCCTAAGTGATACC R: AAAGCCCTACAACAAATGTCAC	(CTAT)13CTAC(CTAT)2	5' - NED	118-148	Henderson-Arzapalo and King 2002
Aox45	F: TTGTCCAATAGTTTCCAACGC R: TGTGCTCCTGCTTTTACTGTC	(AAT)20	5' - VIC	109–154	King et al., 2001
LS39	F: TTCTGAAGTTCACACATTG R: ATGGAGICATTATTGGAAGG	(GTT)10	5' - 6-FAM	700	May et al., 1997
Aox23	F: CAGTGTGCTAGCTTCTCAATA	(ATT)2(ACT)10	5' - 6-FAM	91–133	King et al., 2001

	R: GTTAGCTTAACCATGAATTGTG	(AAT)5			
LS-54	F: CTCTAGTCTTTGTTGATTACAG	(GATA)	5' - 6-FAM	216-284	May et al., 1997
	R: CAAAGGACTTGAAACTAGG	(GACA)			
LS-19	F: CATCTTAGCCGTCTGTGGTAC	(TTG)9	5' - 6-FAM	133 (113 – 136)	May et al., 1997
	R: CAGGTCCCTAATACAATGGC				
LS-68	F: TTATTGCATGGTGTAGCTAAAC	(GATA)13	5' - 6-FAM	120 (116-144)	May et al., 1997
	R: AGCCCAACACAGACAATATC				

Appendix II

Sturgeon identification protocols optimized during the research

MtDNA – Control Region (Congiu et al. 2011):

PCR protocol:

25ul volume, 10x Reaction Buffer (OxGEn) or 5x Reaction Buffer (Promega), 25 mM $MgCl_2$, 10 mM dNTP's mix (100µm ATP, GTP, CTP, TTP end concentrations), each primer 10 pmols/microliter, 0.2 µL of (OxGEn, Promega) *Taq* polymerase (5U/µL, 1 units/reaction), ca. 100 ng of template DNA, and sterileH_2O.

Cycler-TEC: Ac-Dloop (2h) Thermal cycler conditions:
94°C - 5 min
94°C - 45 s
56°C - 30 s
72°C - 45 s - 34 times
72 °C - 5 min

MtDNA – Control region fragment (Ludwig 2008):

PCR protocol:

25ul reaction volume, 10x Reaction Buffer (OxGEn) or 5x Reaction Buffer (Promega), 25 mM $MgCl_2$, 10 mM dNTP's mix (100µm ATP, GTP, CTP, TTP end concentrations), each primer 10 pmols/microliter, 0.2 µL of (OxGEn, Promega) *Taq* polymerase (5U/µL, 1 units/reaction), ca. 100 ng of template DNA, and sterileH_2O.

TEC: Ac-Dloop (2h), Thermal cycler conditions:
94°C - 15 min
94°C - 30 s
56°C - 30 s
72°C - 30 s - 34 times
72 °C - 7 min

Sex-specific marker -(Kuhl et al. 2021):

In combination with mitochondrial control region gene fragment (Ludwig 2008).

PCR protocol:

20ul reaction volume, 10x Reaction Buffer (OxGEn) or 5x Reaction Buffer (Promega), 25 mM $MgCl_2$, 10 mM dNTP's mix (100µm ATP, GTP, CTP, TTP end concentrations), each primer 10 pmols/microliter, 0.2 µL of (OxGEn, Promega) *Taq* polymerase (5U/µL, 1 units/reaction), ca. 100 ng of template DNA, and sterileH_2O.

TEC: Ac-Dloop (2h) Thermal cycler conditions:
94°C - 5 min
94°C - 40 s
56°C - 30 s
72°C - 40 s - 34 times
72 °C - 5 min

Beluga/Sterlet-specific markers-(Havelka et al. 2017)

PCR protocol:

20 ul reaction volume, 10x Reaction Buffer (OxGEn) or 5x Reaction Buffer (Promega), 25 mM $MgCl_2$, 10 mM dNTP's mix (100µm ATP, GTP, CTP, TTP end concentrations), each primer 10 pmols/microliter, 0.2 µL of (OxGEn, Promega) *Taq* polymerase (5U/µL, 1 units/reaction), ca. 100 ng of template DNA, and sterileH_2O. **Stellate sturgeon-specific marker-** (E. Boscari et al. 2014):

PCR protocol:

20 ul reaction volume, 10x Reaction Buffer (OxGEn) or 5x Reaction Buffer (Promega), 25 mM $MgCl_2$, 10 mM dNTP's mix (100µm ATP, GTP, CTP, TTP end concentrations), each primer 10 pmols/microliter, 0.2 µL of (OxGEn, Promega) *Taq* polymerase (5U/µL, 1 units/reaction), ca. 100 ng of template DNA, and sterileH_2O.

Fragment length: 479

TEC: Acipense.Ste (2:4 h:m) Thermal cycler conditions:
94°C - 5 min
94°C - 45 s
58°C - 45 s
72°C - 45 s 33 times
72 °C - 5 min

Russian sturgeon-specific marker-(Havelka et al. 2019):

PCR protocol:

20 ul reaction volume, 10x Reaction Buffer (OxGEn) or 5x Reaction Buffer (Promega), 25 mM $MgCl_2$, 10 mM dNTP's mix (100µm ATP, GTP, CTP, TTP end concentrations), each primer 10 pmols/microliter, 0.2 µL of (OxGEn, Promega) *Taq* polymerase (5U/µL, 1 units/reaction), ca. 100 ng of template DNA, and sterileH_2O.

Fragment length: 395

Cycler: ACP-GUE (2:21 h:m) Thermal cycler conditions:
95°C - 2 min
95°C - 45 s
63°C - 60 s
72°C - 60s 33 times
72 °C - 12 min

Microsatellite markers

Afug 41, Afug 51-(Welsh, Blumberg, and May 2002)

LS39-(Jenneckens et al. 2001)

Aox45-(King, Lubinski, and Spidle 2001)

AoxD161-AoxD165-(Henderson-Arzapalo and King 2002)

An20-(Zane et al. 2002)

PCR protocols

10u10x Reaction Buffer (OxGEn) or 5x Reaction Buffer (Promega), 25 mM $MgCl_2$, 10 mM dNTP's mix (100µm ATP, GTP, CTP, TTP end concentrations), each primer 5 pmols/microliter, 0.2 µL of (OxGEn, Promega) *Taq* polymerase (5U/µL, 1 units/reaction), ca. 100 ng of template DNA, and sterileH_2O.

Thermal cycler conditions:

Mix3: **Afug41, AoxD161, An20**
95°C - 1 min
95°C - 25 s
50°C - 25 s
65°C - 40 s 33 times
65 °C - 10 min

Cycler: ST-MSAT (1:47 h:m)

Mix4: **Aox45, AoxD165**
95°C - 2 min
95°C - 25 s
55°C - 25 s
72°C - 35 s 30 times
72 °C - 5 min
4°C - 5 min

Cycler: ST-MSAT- (1:35 h:m)

LS39:
94°C - 5 min
94°C - 30 s
57°C - 30 s
72°C - 30 s 33 times
72 °C - 5 min
Cycler: ACP_LS (1:39 h:m)

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SHORT COMMUNICATION

Interspecific hybridization in natural sturgeon populations of the Eastern Black Sea: the consequence of drastic population decline?

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Abstract
The eastern part of the Black Sea and its tributaries are suitable habitats for several sturgeon species, among which *Acipenser gueldenstaedtii*, *A. stellatus*, *A. nudiventris*, *A. persicus*, *A. sturio*, and *H. huso* are well documented. However, different threats have led these species to a dramatic decline, all of them are currently listed as *Critically Endangered*, and some *Locally Extinct*, in that area. We tested 94 wild sturgeon samples from the Black Sea and Rioni River by analyzing the mitochondrial Control Region and nuclear markers for hybrid identification. The data analyses (1) assessed mitochondrial diversity among samples, (2) identified their species, as well as (3) indicated instances of hybridization. The data collected, besides confirming a sharp decrease of catches of Beluga and Stellate sturgeon in recent years, also revealed four juvenile hybrids between Russian and Stellate sturgeon, providing the first evidence of natural interspecific hybridization in the Rioni. The present communication raises concerns about the status of sturgeon species in this area and underlines the urgent need for conservation programs to restore self-sustaining populations.

Keywords Sturgeons · Interspecific hybrids · *Acipenser gueldenstaedtii* · *Acipenser stellatus* · Rioni · Black Sea

Introduction

Sturgeons are among the most endangered species groups in the world according to the International Union for Conservation of Nature (IUCN 2010), with some of the most imperiled species distributed in the Palearctic region. The Eastern part of the Black Sea and a major tributary in the Caucasus, the Rioni River, are known to have hosted in historical times at least five sturgeon species. The Russian sturgeon (*Acipenser gueldenstaedtii*), the Stellate sturgeon (*A. stellatus*), the Beluga sturgeon (*Huso huso*), the European sturgeon (*A. sturio*), and the Ship sturgeon (*A. nudiventris*) (Variadilis et al. 1998; Guchmanidze 2009). All of these are listed by IUCN as *Critically Endangered*, with European and Ship sturgeons also believed to be *Locally Extinct*, with wild populations considered to be extirpated from the Black Sea basin (Gessner et al. 2010; Mugue et al. 2016).

The population decline is mainly caused by habitat degradation, including river damming and consequent high sediment flushing, overfishing, and pollution. Accurate historical or present assessments of population sizes are not available, but there are indications that sturgeon populations in the region have been in steep decline since the early 20th century (Beridze et al. 2021). Historically, the Rioni River in Georgia is known as one of the main sites for sturgeon spawning in this area (IUCN, 2010, www.iucnredlist.org) and it currently is the only remaining functional sturgeon

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spawning river of the Eastern Black Sea. This was confirmed by monitoring research on sturgeon recruitment in the Rioni conducted by Fauna & Flora International (FFI) between 2018 and 2020. Intensive field surveys, annually held from March until October, led to the discovery of multiple juvenile sturgeon specimens of various species. In addition, data collection with associated anglers on the Rioni led to the discovery of eight specimens of *A. nudiventris* in 2020. Their possible origin from an ongoing captive breeding program in Krasnodar (River Kuban) has been excluded, supporting the hypothesis of a relict reproductively active population of *A. nudiventris* in the Rioni River (Beridze et al. 2021).

This study reports the results of the genetic characterization of sturgeons collected within the first three years of these monitoring activities. Monitoring is ongoing, with the aim of evaluating the state of sturgeon populations of this area and verifying abundance of the different species, the purity of the animals and whether the sampled individuals come from restocking activities or if they result from natural reproduction.

The collected data has unexpectedly indicated the occurrence of interspecific hybrids between two species of sturgeon historically known to reproduce in the Rioni River. The occurrence of interspecific hybridization events is discussed in light of implications for the conservation of natural sturgeon populations.

Materials and methods

Between 2018 and 2020, a sturgeon sampling campaign was carried out in the Georgian part of the Black Sea (BS) and Rioni River (RR = Rioni River; RM = Rioni mouth) (Fig. 1A) by FFI. A total of 94 tissue samples were collected (Table 1); captured animals, mostly juveniles, were immediately released after sampling.

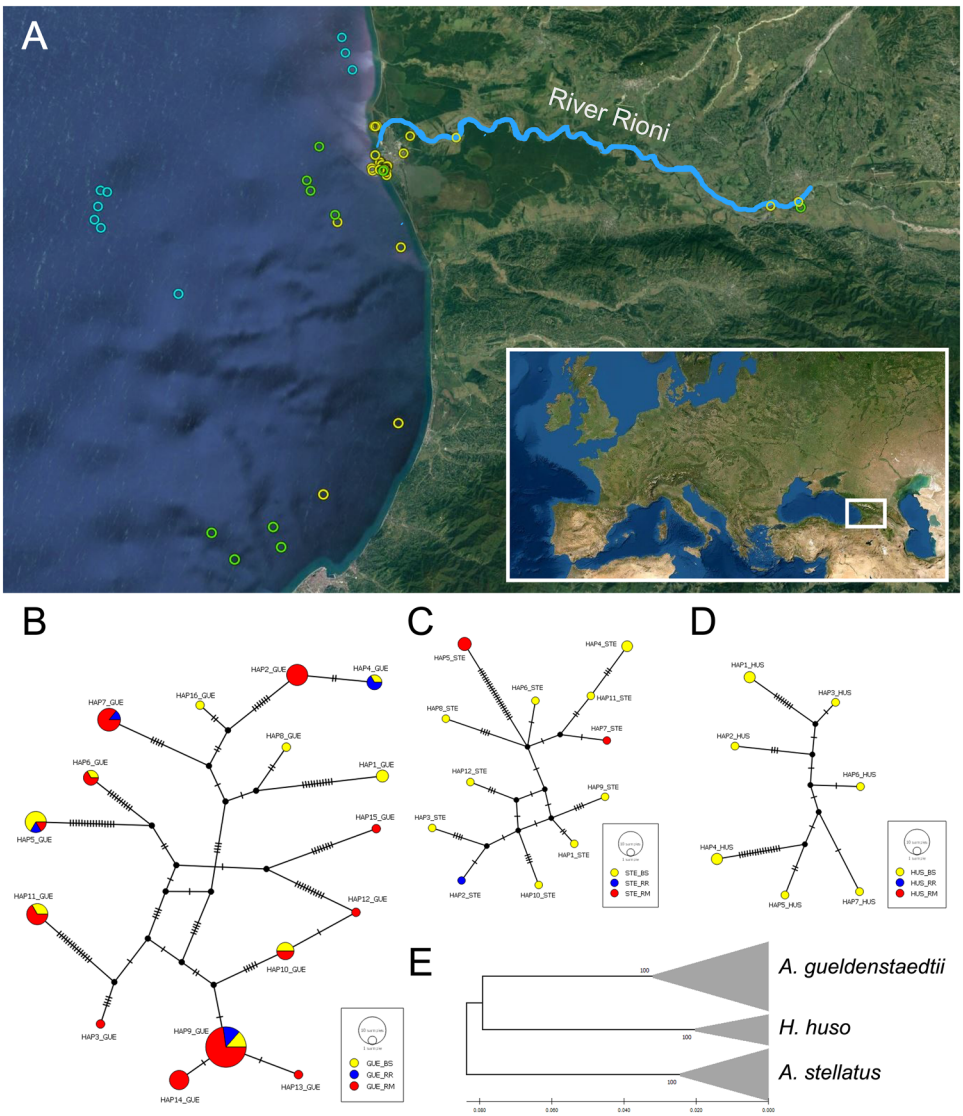

Fig. 1 Study area, sampling locations, and haplotype relationships. **A** Geographic locations of sampled individuals (green = *A. stellatus*, yellow = *A. gueldenstaedtii*, and blue = *H. huso*). The inset map shows a continental view where the sampling area in the south-eastern Black Sea is framed. Satellite image obtained from Google Earth Pro V. 7.3.3.7786 (Google LLC, California, USA). **B**–**D** Haplotype networks, obtained with PopART 1.7 software, showing relationships among haplotypes in the three species detected. The size of pie charts is proportional to the corresponding haplotype frequency while colours indicate the sampling origin (Yellow = BS – Black Sea; Blue = RR – Rioni River, Red = RM – Rioni Mouth). Capital letters B, C, and D show haplotype relationships of *A. gueldenstaedtii* (GUE), *A. stellatus* (STE), and *H. huso* (HUS), respectively. E) Schematic NJ tree representing haplotypic distances between species

Table 1 Summary of the results from mitochondrial control region and diagnostic nuclear markers which specifically identify *A. stellatus* (ste), *A. gueldenstaedtii* (gue) and *H. huso* (hus)

Year	SampleID	mtDNA (present study)	Match with previously known haplotypes		Nuclear markers		
			Hap/Acc.n.	Occurence	ste	gue	hus
2018	**Ac91_BS**	ste_Hap1	*STE-HAP21*	AS			
	Ac86_RR	ste_Hap2	*STE-HAP108*	CS			
	Ac89_BS	ste_Hap3	/	/			
	Ac92_BS, Ac105_BS	ste_Hap4	/	/			
	Ac94_RM, Ac114_RM	ste_Hap5	*STE-HAP83*	AS			
2019	**Ac137_RM**	ste_Hap5	*STE-HAP83*	AS			
	Ac101_BS	ste_Hap6	AF168525*	/			
	Ac115_RM	ste_Hap7	*STE-HAP62*	AS			
	Ac123_BS	ste_Hap8	AF168528*	/			
	Ac139_BS	ste_Hap9	AF168535*	/			
	Ac157_BS	ste_Hap10	*STE-HAP15*	CS/UR			
	Ac156_BS	ste_Hap11	*STE_HAP64*	DR/UR			
2020	**Ac193_BS**	ste_Hap12	*STE_HAP9*	AS			
2018	**Ac70_BS, Ac76#_BS**	gue_Hap1	/	/			
	Ac93_RM, Ac95_RM	gue_Hap2	*GUE_HAp137*	BS			
	Ac96_RM	gue_Hap3	/	/			
	Ac112_RR	gue_Hap4	/	/			
	Ac113_RR	gue_Hap5	HQ7304710° *GUE_HAP195*	BS/CS			
2019	**Ac125_BS**	gue_Hap4	/	/			
	Ac120_BS	gue_Hap5	HQ7304710° *GUE_HAP195*	BS/CS			
	Ac116_RM, Ac136_RM	gue_Hap6	*GUE_HAP101*	AS/BS			
	Ac118_RR, Ac144_RM, Ac145_RM	gue_Hap7	/	/			
	Ac119_BS	gue_Hap8	*GUE_HAP1*	BS/CS			
	Ac121_BS, Ac135_RR, Ac143_RM, Ac146_RM, Ac147_RM, Ac148_RM	gue_Hap9	AF238725° *GUE_HAP11*	AS/BS			
	Ac127_BS	gue_Hap10	*GUE_HAP140*	DR			
	Ac128_BS, Ac140_RM	gue_Hap10	*GUE_HAP140*	DR			
	Ac133_BS	gue_Hap11	*GUE_HAP135*	BS			
	Ac150_RM	gue_Hap11	*GUE_HAP135*	BS			
2020	**Ac198_RM, Ac200_RM, Ac203_RM, Ac205_RM**	gue_Hap2	*GUE_HAp137*	BS			
	Ac196_RM	gue_Hap4	/	/			
	Ac189_BS, Ac194_BS, Ac210_BS, Ac201_RM	gue_Hap5	HQ7304710° *GUE_HAP195*	BS/CS			
	Ac199_BS	gue_Hap6	*GUE_HAP101*	AS/BS			
	Ac171_RM, Ac182_RM, Ac185_RM, Ac188_RM	gue_Hap7	/	/			
	Ac204_BS, Ac209_BS, Ac165_RR, Ac161_RM, Ac163_RM, Ac168_RM, Ac172_RM, Ac174_RM, Ac177_RM, Ac179_RM, Ac181_RM,	gue_Hap9	AF238725° *GUE_HAP11*	AS/BS			
	Ac183_RM, Ac187_RM, Ac202_RM, Ac208_RM						
	Ac160_RR	gue_Hap9	AF238725° *GUE_HAP11*	AS/BS			
	Ac197_RM	gue_Hap10	*GUE_HAP140*	DR			
	Ac175_BS	gue_Hap11	*GUE_HAP135*	BS			
	Ac162_RM, Ac164_RM, Ac173_RM	gue_Hap11	*GUE_HAP135*	BS			
	Ac167_RM	gue_Hap12	*GUE_HAP140*	DR/UR			
	Ac169_RM	gue_Hap13	/	/			
	Ac170_RM, Ac176_RM, Ac180_RM, Ac186_RM, Ac192_RM	gue_Hap14	*GUE_HAP185*	CS			
	Ac207_RM	gue_Hap15	/	/			
	Ac212_BS	gue_Hap16	*GUE_HAP141*	DR/CS			
2018	**Ac72_BS, Ac90_BS**	hus_Hap1	*HUS_HAP60*	DR/CS			
	Ac73_BS	hus_Hap2	*HUS_HAP73*	DR			
	Ac74_BS	hus_Hap3	/	/			
2019	**Ac98_BS, Ac99_BS**	hus_Hap4	/	/			
	Ac100_BS	hus_Hap5	*HUS_HAP12*	AS/BS/CS			
	Ac102_BS	hus_Hap6	/	/			
	Ac104_BS	hus_Hap7	*HUS_HAP75*	DR			

Table 1 (continued) Samples are sorted by collecting year and by haplotype. Any correspondence with known haplotypes and their distribution is also reported. Codes in italics indicate haplotypes detected in the Russian sturgeon broodstocks. For each sample, the amplification of diagnostic nuclear markers is shown in grey-filled cells. Amplification of nuclear markers from four individuals failed, indicated in the table by the empty cells

BS Black Sea, *RR* Rioni River, *RM* Rioni mouth, *AS* Azov Sea, *DR* Danube River, *CS* Caspian Sea

*Doukakis et al 1999; °Birstein et al. 2000; #= A. gueldenstaedtii baerii-like haplotype

Genomic DNA was purified using the Qiagen DNeasy Blood & Tissue Kit. All samples were genetically analyzed for species and hybrid identification by amplifying and sequencing the mitochondrial control region, and by checking the presence/absence of sturgeon diagnostic nuclear markers.

Primer pairs, PCR amplifications, and thermocycler conditions for the control region are as reported in the original reference by Congiu et al. (2011). PCR reactions were performed on Applied Biosystem GeneAmp®PCR System 9700 and MJ Research PTC-225 thermal cyclers. All PCR products were purified with ExoSAP- IT® according to the manufacturer's protocol, and directly sequenced on an ABI Prism 3730XL or an ABI 3100 automatic sequencer at Eurofins Genomics (Germany) or an ABI Prism 3730XL automatic sequencer at Macrogen Europe B.V. (Netherlands).

Mitochondrial sequences were aligned using ClustalW in MegaX (Kumar et al. 2018) and BLAST (Basic Local Alignment Search Tool, Altschul et al. 1990) searches were performed to determine the maternal species; most individuals were juveniles, making morphological identification more difficult. Mitochondrial genetic variation among collected samples was also evaluated. Haplotype diversity (h) and nucleotide diversity (π) were estimated with ARLEQUIN ver.3.5 (Excoffier and Lischer 2010) for each group of species detected by the BLAST searches.

Haplotypes and their relationships (i.e., representation of gene genealogies based on a maximum parsimony approach) were organized in networks with the PopART 1.7 software (Leigh and Bryant 2015; http://popart.otago.ac.nz) based on TCS network inference methods (Clement et al. 2000). A schematic neighbor-joining tree based on p-distance was generated by MegaX. Haplotypes were also compared with available datasets, including information on haplotype diversity of wild and captive sturgeon populations collected over the past years (personal communication by N. Mugue).

Focusing on sturgeon species that more likely could hybridize in the Black Sea and Rioni River, available diagnostic nuclear markers for *A. stellatus* (Ste_RP1F and RP1_LocusA_R, Boscari et al. 2014), *A. gueldenstaedtii* (395_AB_for and 395_uni, Havelka et al. 2019), and *H. huso* (RP2S6_huso-F and RP2S6_groupA_R, Boscari et al. 2017) were also used to test samples for interspecific hybridization. Experimental protocols were as reported in the original references.

The presence/absence of diagnostic products (479 bp for *A. stellatus*, 395 bp for *A. gueldenstaedtii*, and 194 for *H. huso*) was checked on 1.8% agarose gel stained with GelRed (BIOTIUM, GelRed™ Nucleic Acid Stain).

Results and discussion

BLAST performed with mitochondrial data revealed three species: *A. gueldenstaedtii* (Accession numbers: MZ665962-MZ665977), *A. stellatus* (MZ665978-MZ665989), and *H. huso* (MZ665990-MZ665996) (Table 1), with 74% of haplotypes indicating *gueldenstaedtii* species identification. Among samples collected from the Black Sea (BS), 10 animals presented *A. stellatus* haplotypes, 17 *A. gueldenstaedtii*, and nine *H. huso*. In the Rioni River and its mouth (RR and RM) only *A. stellatus* (one RR samples and four RM samples) and *A. gueldenstaedtii* (six RR samples and 47-RM samples) haplotypes were found.

For each sample, Table 1 shows year of collection and the previous detection of each haplotype in wild populations or captive stocks; results of tests for interspecific hybridization performed with diagnostic nuclear markers are also indicated. Table 2 describes mitochondrial diversity for the three species. Figure 1B–E shows the relationships among haplotypes in the three species (12 haplotypes for *A. stellatus*, 16 for *A. gueldenstaedtii*, and seven for *H. huso*). For the three species, two, nine, and three haplotypes respectively were never observed before.

Four individuals with *A. gueldenstaedtii* haplotypes (one young of the year caught in 2018 and three 1 year olds caught in 2020), were positive for the diagnostic nuclear marker for *A. stellatus*, strongly pointing to their hybrid

Table 2 Summary data based on control region sequences

Control Region summary basic statistics					
Maternal species	N	N_h	Ps	h	π
A. stellatus	15	12	52	0.962 ± 0.040	0.019 ± 0.010
A. gueldenstaedtii	70	16	87	0.867 ± 0.029	0.023 ± 0.011
H. huso	9	7	36	0.944 ± 0.070	0.017 ± 0.009

N number of individuals, *Nh* number of haplotypes, *Ps* Polymorphic sites, *h* haplotype diversity, *π* nucleotide diversity

origin. It is worth noting that, for one of these animals, an informal identification as *A. stellatus* was provided before release by the FFI team members who collected it. The clear discordance between this *a priori* morphological classification and the haplotype sequence likewise indicates hybrid origin of that individual. Moreover, even though morphological indications for the other three detected hybrids were not provided, the *A. stellatus* diagnostic marker has never given false positive amplification in 11 other sturgeon species, including amongst 41 specimens of *A. gueldenstaedtii* from outside of the Black Sea (Boscari et al. 2014), strongly corroborating the reliability of this result. Given the underrepresentation of reference Russian sturgeons from the Black Sea, we cannot exclude that the allele frequency differs across locations such that *A. gueldenstaedtii* in the Black Sea might naturally carry the *A. stellatus*-diagnostic allele. However, commercial controls for species purity we routinely performed also included many *A. gueldenstaedtii* caviar samples from the Black Sea which have never shown the *A. stellatus* allele (data not shown).

The four haplotypes of the hybrid animals were not detected in the Russian sturgeon broodstocks used to generate juveniles for restocking in North Eastern Black Sea (Nikolai Mugue, pers. comm), suggesting that the hybrids found in this study were likely the offspring of wild breeders. Additionally, the four putative hybrids exhibited different haplotypes, indicating that each had a different *A. gueldenstaedtii* mother, and that hybridization involved at least four females. Sturgeons are known for their ability to hybridize in captivity and several species combinations have been generated in aquaculture for production purposes (Boscari et al. 2014). To our knowledge however, hybrids between *A. gueldenstaedtii* and *A. stellatus* are not used in aquaculture, and this species combination is not produced. Furthermore, no hatchery producing *A. stellatus*, either as pure species or as hybrids, is present in the area, excluding the possibility that the detected hybrids represent accidental escapees from aquaculture plants. This is not the first evidence of interspecific hybridization in nature between sturgeon species; for example, natural interspecific hybrids between *A. ruthenus* and *A. baerii* were found in the Danube River following careless release of the allochthonous *A. baerii* (Ludwig 2009). In this instance, it appears that hybridization occurs between indigenous species.

Although, a certain rate of hybridization may have always occurred, the present low density of populations (in particular of the Stellate sturgeon) might increase this phenomenon. Population decline may in fact promote interspecific hybridization due to the scarcity of conspecific mates. This phenomenon, known as Hubb's 'desperation' hypothesis (Hubbs 1955), adduces the urgent need for sturgeon conservation measures in the Eastern Black Sea and Rioni drainage. Additional concerns are raised by the possible impact that the presence of interspecific hybrids might have on the already seriously compromised natural populations (Havelka et al. 2011). In our specific case, the two parental species have respectively about 240 (Russian sturgeon) and 120 chromosomes (Stellate sturgeon) and the resulting hybrids, having an intermediate chromosomal set, are expected to be sterile (Birstein 2002; Linhartová et al. 2018). However, the sterile condition does not prevent the animals from taking part to spawning as adults, competing with breeders of pure species with adverse effects on their reproductive success (Arcella et al. 2014; Fjelldal et al. 2014). Furthermore, a relict population of Ship sturgeon (*A. nudiventris*), whose chromosomal set is compatible with the Stellate sturgeon (*A. stellatus*), also inhabits the Rioni River (Beridze et al. 2021). An interspecific admixture between these species would result in fertile hybrid which might backcross with the parental species, potentially compromising their genetic integrity. This would be particularly harmful for the Ship sturgeon, on the verge of being classified as extinct in the wild, and for which a last spawning site of the entire distribution area was recently recorded in the River Rioni (Beridze et al. 2021).

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Authors' contributions TB and EB equally contributed to this study. FS, CA substantially contributed to the design of the work. TB and EB performed all the experiments and data analyses. TB, EB and LC contribute to results interpretation and wrote the manuscript. All the authors critically revised the manuscript and accepted the final version for publication.

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Code availability Not applicable.

Declarations

Conflicts of interest/Competing interests No conflict of interest.

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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.

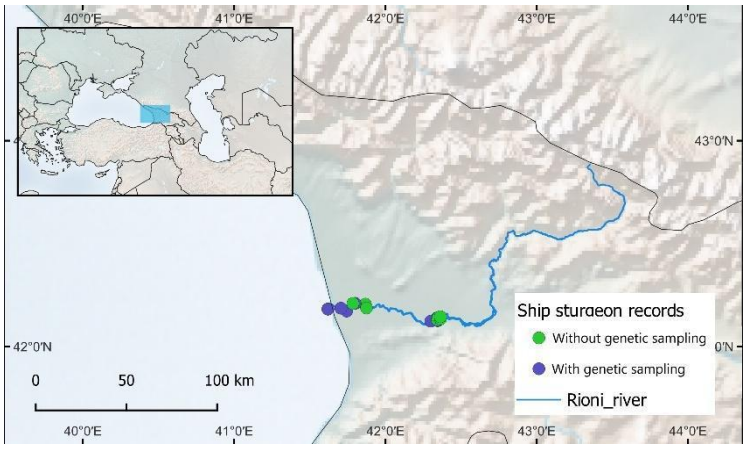

Figure 1. Ship sturgeon sampling sites in the Rioni River in Georgia.

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).

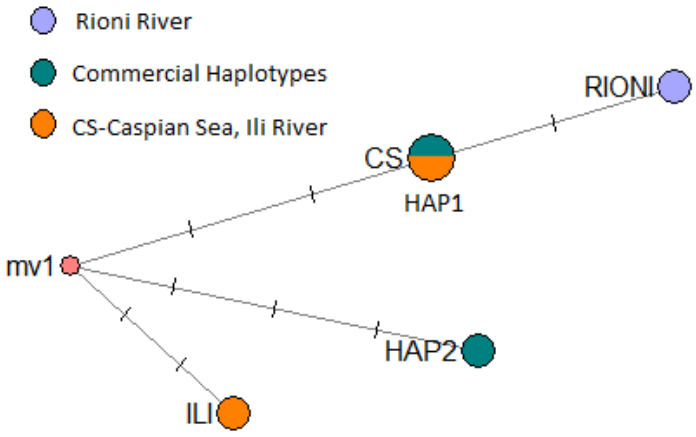

Figure 2. Network analysis of ship sturgeon samples from the Rioni River and ship sturgeon control region DNA sequences downloaded from NCBI (Caspian Sea-KU321568; Ili River-KU321569, (Balkhash basin); HAP0-KU321569; HAP02-KU321568). Analysis was carried out with 782 bp fragments of mitochondrial control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

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Applied Study

Fish diversity assessed by eDNA detection methods in the Rioni River

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Abstract

Due to anthropogenic influences, habitat degradation and a continuous loss of biodiversity in freshwater ecosystems are occurring on a large scale, while these ecosystems constitute invaluable natural resources. Therefore, it is essential to study and monitor freshwater ecosystems to guide conservation efforts. Freshwater ecosystems are one of the less-studied fields in Georgia. Studies about the species distribution of many taxa and/or regions carried out during the last century have not been updated for decades. Here, we report the results of an environmental DNA (eDNA) metabarcoding exercise, based on samples collected from the Rioni River, a tributary to the Black Sea and a crucial aquatic ecosystem regionally and globally. The only comprehensive review of the fish of the Rioni River dates back to 1956. We compared the eDNA-based taxonomic composition to the known faunal composition within the Rioni River and found that the eDNA-based taxonomic coverage approached 75% of the expected total fish fauna. A number of new species occurrences were also found, including the first detection of three invasive alien species (*Carassius gibelio*, *Pseudorasbora parva*, *Rhinogobius lindbergi*) in the Rionis River Basin and a new country record of the ninespine stickleback (genus *Pungitius*) for Georgia. In spite of the usefulness of the eDNA metabarcoding approach, the sparsity of the fish DNA barcode reference library for the region emerged as a limitation to this study. However, our findings still represent a great leap forward in updating fish status on the Rioni River and testing the effectiveness of the eDNA sampling for aquatic species.

Key words: Caucasus, eDNA, fish diversity, Rioni River

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Introduction

Even though the Republic of Georgia is a part of the internationally-recognised Caucasus Biodiversity Hotspot, harbouring tertiary relic flora and fauna (Milne and Abbott 2002; Mittermeier et al. 2004; Habel et al. 2019), its biodiversity is still poorly characterised and conservation measures are needed to protect this diversity (Mumladze et al. 2020). Within Georgia, the Rioni River is one of the largest rivers in the Black Sea Basin, housing the last remaining eastern Black Sea breeding populations of at least three sturgeon species (Beridze et al. 2022a, b) and, thus, is a critical habitat for the conservation of this most

endangered vertebrate group globally (IUCN). This alone makes the Rioni River a focus of global attention. In addition to threatened sturgeon taxa, the Rioni and its tributaries are home to a number of endemic fish species from the Colchic refugium (*Rhodeus colchicus*, *Barbus rionicus*, *Oxynoemacheilus phasicus* etc), for which the Rioni River and its tributaries encompass a major part or entirety of a species' distribution (Bogutskaya and Komlev 2001; Baycelebi et al. 2015; Freyhof et al. 2021). As such, the Rioni is of enormous importance for aquatic biodiversity in the Caucasus. At the same time, the river is subject to ongoing heavy anthropogenic pressure, such as hydro-power development, pollution, mining and poaching (Caruso et al. 2012; Japoshvili et al. 2021; Suciu et al. 2021a). For the conservation of aquatic biodiversity in the face of these challenges, ongoing species monitoring in the Rioni watershed is essential. However, the last comprehensive assessment of the Rioni River fish community is more than half a century old (Elanidze 1956) and, since then, only occasional sampling directed mainly at the biology of an individual fish species has taken place (Levin et al. 2018; Epitashvili et al. 2020; Freyhof et al. 2021).

In the past decade, metabarcoding of environmental DNA (eDNA) has become a promising technique for effective biodiversity monitoring in fresh and marine waters (Bohmann et al. 2014; Pfleger et al. 2016; Cristescu and Hebert 2018). The applications of eDNA techniques include the evaluation of species richness and the monitoring of rare and threatened species (Thomsen et al. 2012; Shaw et al. 2016; Evans and Lamberti 2018). Moreover, eDNA methods are able to detect species that are otherwise difficult to find with traditional sampling (Thomsen et al. 2016; Suarez-Menendez et al. 2020) or can be used for early detection of invasive species (Ficetola et al. 2008; Goldberg et al. 2011). It is already clear that eDNA methods are amongst the most cost-effective for biodiversity monitoring (Rees et al. 2014; Thomsen and Willerslev 2015). Publicly-available and ever-increasing DNA reference libraries, such as GenBank (Benson et al. 2012) or BOLD systems (Ratnasingham and Hebert 2007) are crucial to the usefulness of eDNA technology. However, the lack of reference sequence barcode data in many parts of the world still impedes the effective use of the eDNA metabarcoding approach in those areas. Thus, a concerted effort to improve the eDNA technology and availability of relevant infrastructure and also develop regional DNA barcode inventories is necessary to advance DNA-based biodiversity studies, which will in turn allow for more cost effective, accurate and wider-ranging biodiversity assessments and monitoring (Pawlowski et al. 2018; Weigand et al. 2019).

Despite being a biodiversity hotspot, Georgia and the Caucasus ecoregion as a whole still lack effective biodiversity inventory and monitoring programmes, based on both traditional collection methodologies and new technologies. Thus, our goal was to set a precedent in the Caucasus biodiversity hotspot by using modern techniques in biodiversity inventorying, while also evaluating the effectiveness of the eDNA sampling in assessing the diversity of fish species in the Rioni River.

In the present study, we provide the first eDNA analysis results, based on water samples collected in the Rioni River and compare the obtained data on fish species diversity to those known from literature based on 20th century collections (Elanidze 1956) and to the updated species list of fishes of Georgia (Kuljanishvili et al. 2020, 2021). We demonstrate the utility of eDNA technology to deliver fish biodiversity

information, from a region that lacks records of DNA barcodes for native species with the exception of recent work by (Epitashvili et al. 2020). Due to the success of our pioneering eDNA work in the Rioni River, we encourage further eDNA-based research on aquatic ecosystems in the Caucasus biodiversity hotspot.

Materials and methods

Study area and sampling

The Rioni River is the longest river in Georgia (length – 327 km, annual discharge – 13.37 km^3) and its diverse freshwater community includes a number of endemic fish taxa unique to the region. Along the Rioni River, there are a number of artificial constructions, some of which are insurmountable barriers for freshwater animals. Industrial development of the Rioni River has led to habitat degradation, fragmentation and loss, with artificial barriers formed by dams and weirs posing a particular threat to migratory and diadromous species. One such barrier is the Vartsikhe Hydropower Plant. Along with other anthropomorphic pressures (e.g. poaching, gravel mining, pollution), this dam has significantly reduced the historical spawning area of Black Sea sturgeons from ca. 90 km to 9 km downstream of the River (Suciu et al. 2021b). As a result, sturgeon populations are now on the verge of extinction.

To mitigate the risk of sturgeon extinction in the Rioni and improve their habitat quality, a number of projects have been initiated. One of those projects, led by Fauna & Flora International (FFI), investigated different aspects of surviving sturgeon populations and habitats (Suciu et al. 2021b; Beridze et al. 2022b). As part of this initiative, FFI piloted eDNA sampling between 2018 and 2019 to detect sturgeon species in the river. Water samples were collected during September 2018 and March 2019 from the river mouth to approximately 90 km upstream (Fig. 1). As part of the effort to detect sturgeon, these samples simultaneously generated a catalogue of non-targeted species whose DNA was present in the samples, providing a snapshot of species assemblages at the various sampling locations.

In total, 12 water samples each up to 0.8-litre volume were collected using the NatureMetrics eDNA filter kits. Using a polyethersulphone filter with a 0.8 µm pore size, water was filtered and eDNA preserved according to the manufacturer's protocol (NatureMetrics, UK). The specific volume of water used for each sample was dictated by water turbidity (minimum 150 ml, maximum 800 ml). More precisely, high turbidity precluded higher volumes (Table 1). Samples were collected mostly from the water surface and, in two cases, at a depth of 3 m. Field control samples were not collected for the survey. Samples were shipped to and processed by NatureMetrics for eDNA metabarcoding using the "eDNA Survey – Fish" pipeline (NatureMetrics, UK).

DNA processing

Samples were processed by NatureMetrics company, following the eDNA survey – Fish pipeline, including DNA extraction, amplification, sequencing and DNA analysis. DNA was extracted from 12 filters using a DNeasy Blood and Tissue Kit (Qiagen). PCR inhibitors were removed from extracted DNA using DNeasy

Table 1. The volume of water filtered and the resultant concentration of purified DNA and index PCRs.

Sample ID	Sampling data	Coordinates	Sampling depth	Filtered volume	DNA (ng/µl)	Index (ng/µl)	Species
1	15-Jun-2019	42.14962, 41.68107	3 m	150 ml	> 20	11.1	1
2	22-Mar-2019	42.14962, 41.68107	3 m	250 ml	> 20	17.6	15
3	22-Mar-2019	42.21298, 41.79929	Surface	500 ml	5.26	17.6	24
4	31-Oct-2018	42.20775, 41.80520	Surface	450 ml	2.86	10.2	22
5	25-Sep-2018	42.20504, 41.80986	Surface	500 ml	9.6	4.46	24
6	22-Mar-2019	42.15894, 42.16789	Surface	500 ml	6.26	19.8	22
7	31-Oct-2018	42.14581, 42.18570	Surface	550 ml	0.842	11	24
8	24-Sep-2018	42.14491, 42.18603	Surface	500 ml	4.36	3.26	21
9	23-Apr-2019	42.11546, 42.29542	Surface	650 ml	11.2	12.7	23
10	22-Mar-2019	42.14172, 42.28985	Surface	800 ml	3.04	11.4	19
11	22-Mar-2019	42.11837, 42.33069	Surface	750 ml	6.06	18.6	19
12	21-Mar-2019	42.15686, 42.38307	Surface	750 ml	5.84	10.8	24

PowerClean Pro Cleanup Kit (Qiagen). A hypervariable 12S rRNA gene fragment was amplified in twelve PCR replicates using vertebrate primers with expected 140–200 bp amplicon sizes, excluding primers (Miya et al. 2015). Both negative and positive controls were used alongside all PCRs. The mock community with a known African fish species composition was used, as such species were not expected in the region. No contamination between the mock community and analysed samples was detected. Amplification products were checked on gel electrophoresis. All PCR replicates were pooled and purified and adapters were added before sequencing. The success of this step was checked using gel electrophoresis and quantified using a Qubit high-sensitivity assay. The index PCRs were pooled into the final library and sequenced on Illumina MiSeq V.2 kit at 12 pM with a 10% PhiX spike in. Sequence data were processed using a custom bioinformatics pipeline developed at NatureMetrics (NatureMetrics, UK) passing though quality filtering, dereplication, denoising and taxonomic assignment steps. The bcl2fastq software was used for demultiplexing the sequences and USEARCH (Edgar 2010) was used for merging paired-end FASTQ reads for each sample. Primers were removed from the forward and reverse reads using cutadapt (Martin 2011). Sequences between 140–200 bp length were retained in the analysis after removing primers. Sequences with an expected error rate per base of 0.05 or below were quality filtered using USEARCH (Edgar 2010) and were dereplicated. Unique reads were denoised using UNOISE (Edgar 2016). ZOTUs (zero-radius OTUs) were clustered at 99% similarity with USEARCH. All dereplicated reads were mapped for each sample to the ZOTU representative sequences at 97% identity threshold. Taxonomy assignments were made via BLAST (Altschul et al. 1990; Camacho et al. 2009) searches of the representative sequences against the NCBI nucleotide database and with the custom in-house taxonomic database of 12S fish sequences at the NatureMetrics (NatureMetrics, UK). Identifications of the sequences were based on the highest available percentage sequence identity with a minimum e-score of 1e-20 and a hit length of at least 80% of the query sequence. For the species-level

identification, a sequence identity cut-off of 97% was used. Confident species IDs were made at ≥ 98%, sequences between 97 and 98% similarities were retained for species identification and interpreted, based on local knowledge. In case of multiple hits meeting these criteria, more conservative higher taxonomic classification was selected. Low abundance detections (< 0.05% or < 10 reads) per sample were excluded. All samples were pooled together and summarised, based on their taxonomic assignments. The OTUs identified as originating from human, food or livestock were removed from the database.

Results

DNA sequences

The average total DNA yield from samples was 7.94 ng/µl and ranged from 0.842 ng/µl (Tsilori Oct 18, Sample ID #7-Table 1) to > 20 ng/µl (Market Bridge Mar 19, Sample ID #12 and Poti Market Bridge, Sample ID #5-Table 1). Amplification was successful for all 12 samples. A total of 747,622 high-quality sequences were generated and used for taxonomic assignment.

Sample composition

A total of 34 fish taxa were detected across the 12 samples (excluding non-metazoan and contaminant taxa), of which 22 could be confidently identified to species level (Table 3). The remainder were identified at the above/species taxonomic level. The identified fish species belong to two classes, 11 orders, 15 families and 32 genera (Table 2). The average species richness (per sample) was 20 and ranged from 1 (location 1) to 24 (localities 3, 5, 7 and12) and the diversity is summarised in Table 1. A nase species belonging to the genus *Chondrostoma* (possibly *C. colchicum* or *C. cyri*), accounting for 18% of the total sequence reads was the most abundant in terms of DNA sequences.

In the entire dataset, DNA of four alien species was detected: (1) rainbow trout (*Oncorhynchus mykiss*); (2) mosquitofish (*Gambusia holbrooki*); (3) bighead carp/silver carp (*Hypophthalmichthys nobilis*/*H. molitrix*) and; (4) grass carp (*Ctenopharyngodon idella*). In addition, there were a number of other species (eight in total) that we initially identified as non-native. However, these taxa are most probably native species of the Rioni River, closely related to other congenerics represented in the NatureMetrics reference database (Table 2). For example, we detected barbel (*Barbus barbus*) at four locations, but this species is not listed amongst the Georgian fish according to Kottelat and Freyhof (2008) and Kuljanishvili et al. (2021). Only schneider – *Alburnoides* sp. was detected in every sample. Nine other species were detected in 11 samples: Barbel (*Barbus* sp.), Khramulya (*Capoeta capoeta*), Prussian carp (*Carassius gibelio*), Nase (*Chondrostoma* sp.), Goby (*Ponticola* sp.), Topmouth gudgeon (*Pseudorasbora parva*), Bitterling (*Rhodeus* aff. *colchicus*), Roach (*Rutilus rutilus*) and Chub (*Squalius cephalus*). No sturgeon was detected in any sample, while only Schneider (*Alburnoides* sp.) was found in the Poti Market Bridge sample (location 1) with a significant number of palmate newt (*Triturus helveticus*) and edible dormouse (*Glis glis*) sequences. The lower diversity of fish DNA detected here is probably due to the smaller volume of water filtered.

Table 2. Species composition in the Rioni River according to Elanidze (1956) and after eDNA investigation. Species names according to modern taxonomy are given in the first column. Note that some of the species were not recorded neither by Elanidze (1956) nor by eDNA survey, but were known from other sources (indicated by asterisk).

Taxonomy according to Kuljanishvili et. al. (2020, 2021); Epitashvili et al. (2020)	Records by Elanidze (1956)	Detected by eDNA
Anguillidae		
1. *Anguilla anguilla*[1]	–	–
Acheilognathidae		
2. *Rhodeus colchicus*	as *R. sericeus*	as *R. sericeus*
Acipenseridae		
3. *Huso huso*	as *H. huso*	–
4. *Acipenser nudiventris*	as *H. nudiventris*	–
5. *Acipenser gueldenstaedtii*	as *H. gueldenstaedtii*	–
6. *Acipenser sturio*	as *H. sturio*	–
7. *Acipenser stellatus*	as *H. stellatus*	–
Atherinidae		
8. *Atherina caspia*	as *A. mochon*	–
Carangidae		
9. *Trachurus mediterraneus*	–	as *T. mediterraneus*
Clupeidae		
10. *Alosa tanaica*	as *Caspialosa paleostomi*	–
Cobitidae		
11. *Cobitis satunini*	as *C. taenia*	as *Cobitis* sp.
Cyprinidae		
12. *Barbus rionicus*	as *B. tauricus*	as *B. barbus*
13. *Capoeta sieboldii*	as *Varicorhinus sieboldii*	as *C. capoeta*
14. *Cyprinus carpio*	as *C. carpio*	as *C. carpio*
15. *Carassius gibelio*	–	as *Carassius* sp.
Engraulidae		
16. *Engraulis encrasicolus*	–	as *E. encrasicolus*
Esocidae		
17. *Esox lucius*	as *E. lucius*	as *E. lucius*
Gobiidae		
18. *Babka gymnotrachelus*	as *Mesogobius gymnotrachelus*	–
19. *Ponticola constructor*	as *Neogobius* (*C.*) *constructor*	as *Ponticola* sp.
20. *Neogobius melanostomus*	as *N. melanostomus*	–
21. *Neogobius fluviatilis*	as *N. fluviatilis*	as *N. fluviatilis*
Gobionidae		
22. *Gobio artvinicus*	as *G. gobio*	as *G. gobio*
23. *Pseudorasbora parva*	–	as *P. parva*
Leuciscidae		
24. *Petroleuciscus borysthenicus*	as *Leuciscus borysthenicus*	–
25. *Leuciscus aspius*	as *Aspius aspius*	as *Leuciscus* spp.

Taxonomy according to Kuljanishvili et. al. (2020, 2021); Epitashvili et al. (2020)	Records by Elanidze (1956)	Detected by eDNA
26. *Chondrostoma colchicum*	as *C. colchicum*	as *C. nassus*
27. *Alburnus derjugini*	as *Chalcalburnus chalcoides*	as *A. chalcoides*
28. *Alburnus alburnus*	as *A. alburnus*	as *A. alburnus*
29. *Alburnoides fasciatus*	as *A. bipunctatus fassciatus*	as *A. bipunctatus*
30. *Blicca bjoerkna*	as *B. bjoerkna*	–
31. *Abramis brama*	as *A. brama*	as *A. brama*
32. *Rutilus* spp.	as *R. rutilus*	as *R. rutilus*
33. *Squalius orientalis*	as *Leuciscus cephalus*	as *S. cephalus*
34. *Scardinius erythrophthalmus*	as *S. erythrophthalmus*	–
35. *Vimba vimba*	as *V. vimba*	as *V. vimba*
Mugilidae		
36. *Mugil cephalus*	as *M. cephalus*	as *M. cephalus*
37. *Chelon auratus*	as *Mugil auratus*	–
38. *Chelon saliens*	as *Mugil salines*	–
Nemacheilidae		
39. *Oxynoemacheilus phasicus*[2]	as *Nemachilus* sp.	–
Oxudercidae		
40. *Rhinogobius lindbergi*	–	+
Petromyzontidae		
41. *Lampetra ninae*[3]	–	as *Lampetra* sp.
Percidae		
42. *Sander lucioperca*	as *Lucioperca lucioperca*	–
43. *Perca fluviatilis*	as *P. fluviatilis*	as *P. fluviatilis*
44. *Gymnocephalus cernua*	–	–
Poeciliidae		
45. *Gambusia holbrooki*	as *G. affinis*	as *G. holbrooki*
Salmonidae		
46. *Salmo labrax*	as *S. fario* and *S. labrax*	as *S. labrax*
47. *Oncorhynchus mykiss*	–	as *O. mykiss*
Scombridae		
48. *Scomber scombrus*	–	as *S. scombrus*
Siluridae		
49. *Silurus glanis*	as *S. glanis*	as *S. glanis*
Syngnathidae		
50. *Syngnathus abaster*	as *S. nigrolineatus*	–
Pleuronectidae		
51. *Platichthys flesus*	as *P. flesus*	–
Xenocyprididae		
52. *Ctenopharyngodon idella*	–	as *C. idella*
53. *Hypophthalmichthys nobilis/molitrix*	–	as *H. nobilis/molitrix*
Gasterosteidae		
54. -	–	as *Pungitius pungitius*

[1,3]Elanidze (1983); [2]Freyhof et al. (2021); [4]Epitashvili et al. (2020).

Table 3. Species DNA sequence representation in each of the 12 water eDNA samples collected from September 2018 to March 2019. Species names are given after adjusting the NatureMetrics results to the up-to-date fish list of south Caucasus provided by Kuljanishvili et al. (2020) and subsequent literature.

Species\Samples	1	2	3	4	5	6	7	8	9	10	11	12
Engraulis encrasicolus	x	x									x	
Cobitis satunini	x		x			x	x	x	x		x	
Abramis brama		x		x	x			x	x			
Alburnoides fasciatus	x	x	x	x	x	x	x	x	x	x	x	x
Alburnus alburnus		x		x	x			x	x	x	x	
Alburnus derjugini	x		x	x	x	x	x	x	x	x		
Barbus rionicus	x	x	x	x	x	x	x	x	x	x	x	
Capoeta sieboldii	x	x	x	x	x	x	x	x	x	x	x	
Carassius gibelio	x	x	x	x	x	x	x	x	x	x	x	
Chondrostoma colchicum	x	x	x	x	x	x	x	x	x	x	x	
Ctenopharyngodon idella	x		x			x	x	x			x	
Cyprinus carpio	x		x	x		x	x	x	x		x	
Gobio artvinicus				x	x			x	x		x	
Hipophthalmichthys nobilis/molitrix	x	x		x	x	x	x	x	x		x	
Leuciscus aspius	x		x	x	x	x	x	x	x	x	x	
Pseudorasbora parva	x	x	x	x	x	x	x	x	x	x	x	
Rhodeus colchicus	x	x	x	x	x	x	x	x	x	x	x	
Rutilus sp.	x	x	x	x	x	x	x	x	x	x	x	
Squalius orientalis	x	x	x	x	x	x	x	x	x	x	x	
Vimba vimba	x		x	x	x	x	x	x	x	x	x	
Gambusia holbrooki	x		x			x	x					
Esox lucius			x			x						
Pungitius sp.			x			x						
Neogobius fluviatilis	x					x	x				x	
Ponticola constructor	x	x	x	x	x	x	x	x	x	x	x	
Rhinogobius lindbergi				x				x	x	x	x	
Mugil cesphalus	x					x	x					
Trachurus mediterraneus		x										
Perca fluviatilis			x			x		x				
Scomber scombrus				x						x		
Oncorhynchus mykiss	x		x	x	x	x	x	x	x	x	x	
Salmo labrax	x						x		x	x	x	
Silurus glanis	x			x				x	x	x		
Lampetra ninae					x							

In addition, DNA of the ninespined stickleback – *Pungitius* was also detected at sampling locations 3 and 6 (Fig. 1) with over 98% similarity to *P. pungitius*. These sequences belong to a taxon that apparently has never been detected in the Georgian aquatic environment and, thus, is a new species record for the country.

The detected taxonomic diversity showed a positive relationship with the water sample size (Fig. 2). In particular, less than 400 ml water resulted in an

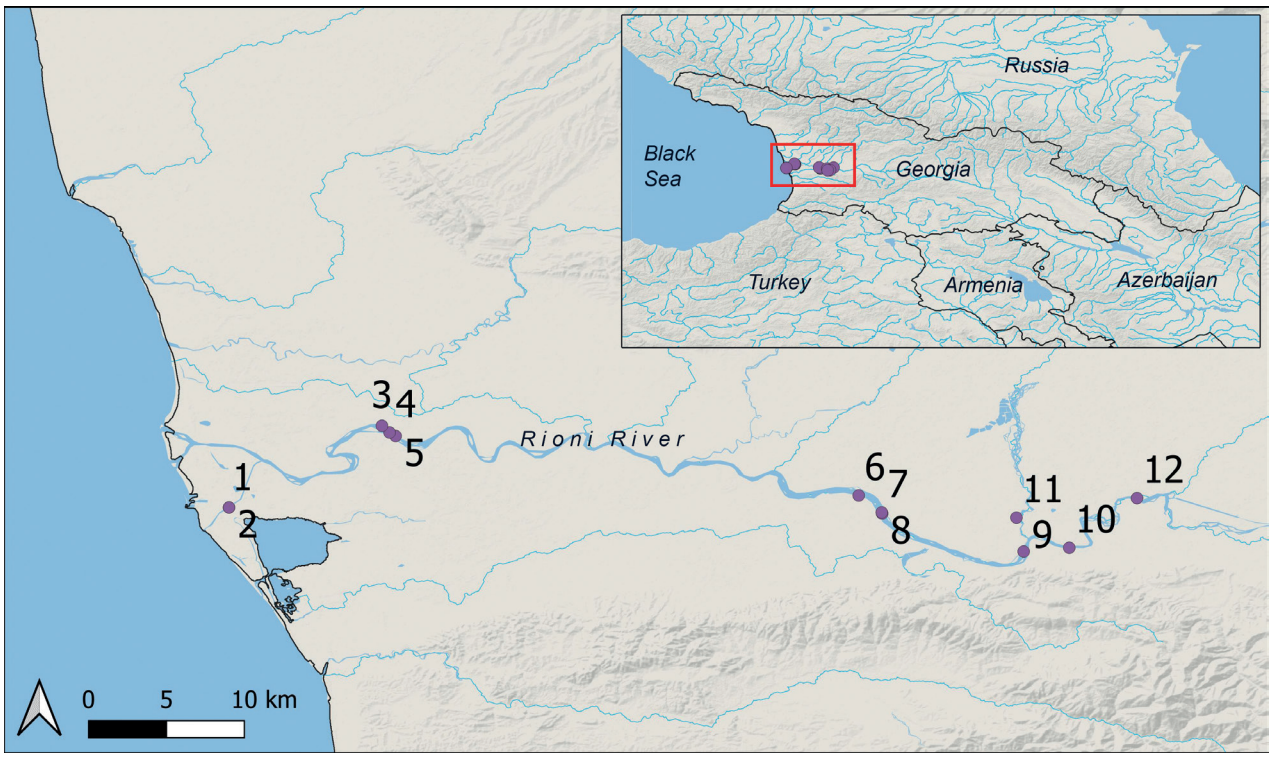

Figure 1. Sampling locations on the Rioni River. Note that samples 1 and 2 are collected from the same site, albeit at different times. Inset map shows the Caucasus region for context.

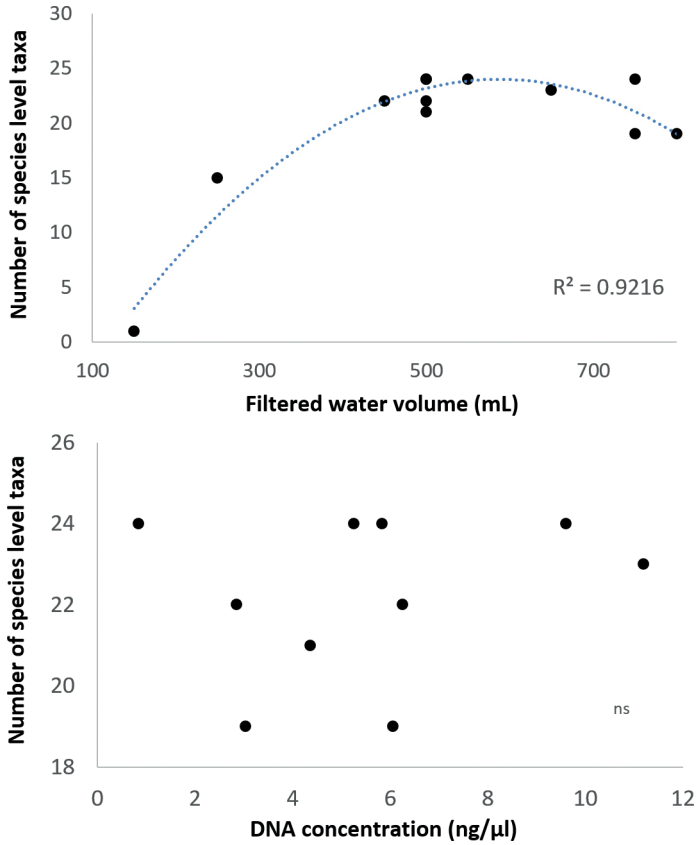

Figure 2. Dependences of detected species number on the water volumes filtered (upper panel) and the DNA concentration in filtrates (lower panel). Note that the concentration of DNA for first and second samples is not included in the graph on the lower panel, because inexact numbers (i.e. > 20) were indicated in the report.

apparent drop in detected taxonomic diversity. On the other hand, no further increase in diversity is evident above the 500 ml volume of filtered water. In contrast, eDNA concentration did not have any visible effect on taxonomic diversity (Fig. 2).

Discussion

Fish fauna of the Rioni River

The first (and only) systematic investigation of the fish fauna of the Rioni River was carried out by (Elanidze 1956), who reported records for 46 species-level taxa. Considering the synonymy due to outdated nomenclature given in that publication, the actual species list given by (Elanidze 1956) is equivalent to 41 currently-recognised species (Table 2). It is noteworthy that a systematic study of the ichthyofauna of the Rioni River has not been conducted since then, with only a few publications reporting new findings, including reports of *Lampetra ninae* and *Anguilla anguilla* from the Rioni River Basin (Elanidze 1983). In addition, very recently, two new species were added to the Rioni River fish list, including the newly-described species – *Oxynoemacheilus phasicus* (Freyhof et al. 2021) mentioned by (Elanidze 1956) as *Nemachilus* sp. and Epitashvili et al. (2020) as *Oxynoemacheilus* sp. – and one alien species – *Gymnocephalus cernua* (Epitashvili et al. 2020). Thus, prior to our current study, 44 species were known for the Rioni River (Table 2).

Careful examination of the eDNA data provides evidence of at least nine additional species in the Rioni River. This includes three invasive alien species: *Carassius gibelio*, *Pseudorasbora parva* and *Rhinogobius lindbergi*, which are widespread and generally abundant in the South Caucasus Region (Shoniya et al. 2011; Japoshvili et al. 2013, 2020; Kuljanishvili et al. 2021). As already reported by Kuljanishvili et al. (2021), *R. lindbergi* is a recent introduction for western Georgia (and for the eastern Black Sea Basin). This small-bodied species is a cryptic invader and its discovery is rather difficult due to morphological similarities with native gobies. This species was also detected with the help of DNA barcoding in eastern Georgia (Epitashvili et al. 2020; Japoshvili et al. 2020). Finding *R. lindbergi* in five sampling locations out of 12, indicates that the species is already widely established in the Rioni River Basin. Most probably, the species is already in other eastern Black Sea rivers, for which additional research is needed.

The other three alien species from the Xenocyprididae family, such as *Ctenopharyngodon idella*, *Hypophthalmichthys molitrix*/*H. nobilis* and the salmonid *Oncorhynchus mykiss*, seem to be robustly represented in the Rioni River. Kuljanishvili et al. (2021) indicated that these species are subject to regular stocking in the region and not yet established. At least no self-sustaining populations are known yet. Finding DNA evidence in 6, 9 and 10 sampling locations out of 12 for *C. idella*, *H. molitrix*/*nobilis* and *O. mykiss*, respectively and, in some cases, a dominant proportion of total eDNA, indicates a significant presence of these species within the study area. However, further research is needed to clarify eDNA sources and evaluate how established these populations are in the River. Nevertheless, the invasive status and the high risks of establishment related to all these non-native species as suggested by Kuljanishvili et al.

(2021) and Mumladze et al. (2022) are fulfilled and, thus, care must be taken to prevent or mitigate the potential threats for the native fauna and ecosystems.

Perhaps the most interesting finding in this study is the detection of the DNA sequence of *Pungitius pungitius*. This species is usually known from the northern regions of Eurasia and America (Kottelat and Freyhof 2008). From the northern Black Sea and Azov Sea regions, another species *P. platygaster* is known that was not previously recorded from the south and eastern Black Sea regions. Based on our results, we cannot confidently say if the sequences in our samples belong to this latter species instead. While the detection of *Pungitius* in Georgia is a new country record, further study is needed to resolve the species identity.

Lastly, the DNA detection of three marine species in the Rioni River – Atlantic mackerel (*Scomber scombrus*), anchovy (*Engraulis encrasicolus*) and Mediterranean horse mackerel (*Trachurus mediterraneus*) is not very surprising. On the one hand, these species can be considered contaminants since they are the main market fish widely available all along the Rioni River settlements. Thus, there is a chance that these commercially targeted species DNA in the river arrived via wastewater effluent. Furthermore, they are often sold and consumed in the Rioni River area and nearby communities. On the other hand, all three species are suggested to frequently migrate at the lower reaches of the Rioni River (Elanidze 1956, 1983) and, thus, the occurrence of their DNA at sampling localities close to the river mouth (one and two localities on the map) could be a sign of their actual presence.

Fresh and brackish water fish DNA library and eDNA-based detection efficiency

From the 34 taxa discovered amongst the sampled eDNA reads, 17 (51%) taxa were correctly identified to species level. Identification ambiguity related to the remaining 17 taxa is mainly due to gaps in the barcode reference library, while in a few cases, unresolved taxonomy also played a role. For instance, species complexes of roaches (*Rutilus*) or Caucasian gobies (Gobiidae) are still waiting for comprehensive investigation. The current CO1 (Cytochrome Oxidase 1) barcode library for Georgian fresh and brackish water fishes includes only 52% of species at the time of writing this article (excluding taxa that are usually considered marine species, for example, *T. mediterraneus*, *E. encrasicolus*, *S. scombrus*) (Epitashvili et al. 2020) and the 12S marker library is likely to be much less complete. Thus, the eDNA-based discovery of 32 fresh/brackish water species, of which 51% were correctly identified at species level, is in line with the current development of the regional fish DNA barcode reference library.

Species that were not detected during our eDNA survey, but are historically known for the Rioni River (e.g. Elanidze (1956)) fall into three categories. First are the rare/threatened species, populations that have either declined in recent decades likely due to anthropogenic influence (e.g. *Acipenser* spp.) or naturally occur in very low population densities in the rivers (*Anguilla anguilla*, *Blicca bjoerkna*). The continued existence of some threatened species in the Rioni River is questionable. For instance, *A. nudiventris* was considered locally extinct in the River until recent targeted field-based sampling revealed the presence of at least three species of sturgeons Stellate Sturgeon, Russian Sturgeon and Ship Sturgeon in the Rioni River (Beridze et al. 2022a, b). The second category

includes species that are brackish-water species (*Alosa tanaica*, *Platichthys flesus*, *Syngnathus abaster*, *Chelon* spp., *Atherina caspia*) occurring in rivers (usually near the river mouth) with low densities (Elanidze 1956). The third category includes species that are predicted for the region and which should occur in the Rioni River, for example, *Neogobius melanostomus*, *Petroleuciscus borysthenicus*, *Oxynoemacheilus phasicus* and *Sander lucioperca* (Elanidze 1956, 1983; Ninua and Japoshvili 2008). Small-bodied *O. phasicus* is widespread in the middle part of the Rioni River and its tributaries, but no dense populations have been reported (Freyhof et al. 2021), nor is the species known to range in the lower reaches of the River. Thus, these species might not inhabit the sampling area. Similar arguments are hard to devise for the other three species, the absence of their DNA might be indicative of the insufficiency of sampling, either because the locations were not adequate or the volume of water was insufficient—in other words, improper sampling strategies related to fish life history. Thus, the potential reasons for lack of detection within these three categories could be due to: (1) the species might not inhabit the sampling area or might simply not have been active in that environment during sampling; (2) volume of water was insufficient when sampling; and (3) sample size was small.

Concluding remarks

In spite of some complications, such as a poorly-developed DNA barcode reference library, limited sampling (only 12 samples, all from the lower parts of the river and limited coverage of the depth gradient) and small volumes of water filtered per sample, the eDNA survey recovered more than 70% of the known fish taxa and also detected new invasive and market species. Although the study lacks true field replicates and field controls which limit our ability to interpret the data, we show that eDNA is very effective in assessing fish species assemblages in the Rioni River and the methodology has great potential as a means to assess fish communities either for species inventory or monitoring purposes.

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Conflict of interest

The authors have declared that no competing interests exist.

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Data availability

All of the data that support the findings of this study are available in the main text.

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PERSPECTIVE

Decades of Global Sturgeon Conservation Efforts Are Threatened by an Expanding Captive Culture Industry

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After centuries of overexploitation and habitat loss, many of the world's sturgeon (Acipenseridae) populations are at the brink of extinction. Although significant resources are invested into the conservation and restoration of imperiled sturgeons, the burgeoning commercial culture industry poses an imminent threat to the persistence of many populations. In the past decade, the number and distribution of captive sturgeon facilities has grown exponentially and now encompasses diverse interest groups ranging from hobby aquarists to industrial-scale commercial facilities. Expansion of sturgeon captive culture has largely fallen outside the purview of existing regulatory frameworks, raising concerns that continued growth of this industry has real potential to jeopardize conservation of global sturgeon populations. Here, we highlight some of the most significant threats commercial culture poses to wild populations, with particular emphasis on how releases can accelerate wild population declines through mechanisms such as hybridization, introgression, competition, and disease transmission. We also note that in some circumstances, commercial captive culture has continued to motivate harvest of wild populations, potentially accelerating species' declines. Given the prevalence and trajectory of sturgeon captive culture programs, we comment on modifications to regulatory frameworks that could improve the ability of captive culture to support wild sturgeon conservation.

INTRODUCTION

Sturgeon (Acipenseridae) are one of the most ancient and unique clades of extant fishes. With little morphological change in their circa 200-million-year history, the 25 extant species of sturgeons are frequently referred to as living fossils for their primitive scutes and cartilaginous skeletons (Gardiner 1984). However, the natural history of these fishes has been anything but static. Subsistence fisheries by Indigenous peoples and early settlers had limited effect on populations and commercial interest for sturgeon products remained low throughout much of the 1800s. By the turn of the 20th century, increased efficiency in capture, storage, and transportation methods inspired the growth of a global fishing industry for sturgeon and demand for caviar and flesh intensified. This enterprise was short-lived, as serial depletion of regional and global stocks subsequently led to collapse of many of the world's sturgeon populations in less than 100 years (Saffron 2002). Today, sturgeon are considered one of the world's most imperiled groups of fishes (IUCN 2022) and the majority of species are afforded regulatory protection within their native waterways (see Table 1). Despite these conservation measures, most populations have been slow to recover from legacy effects of overharvest and continue to be threatened by ongoing habitat loss and anthropogenic activity. Accordingly, most sturgeons have continued to decline despite conservation actions (IUCN 2022).

In the mid-1990s, amidst rising consumer demand for caviar and dwindling abundance of wild populations, the sturgeon culture industry saw a rise in the number and success of production facilities (Saffron 2002; Bronzi et al. 2019). Originally promoted as a means to alleviate harvest pressure on wild populations, commercial aquaculture for sturgeon is now a global enterprise that serves numerous consumer interests, including caviar and meat production, pet trades, leather smithing, and isinglass manufacturing. Despite fluctuations in market value and biomass production in the past decade, today there are over 2,300 commercial sturgeon facilities spread across at least 60 countries (Figure 1), with at least 13 of 25 known sturgeon species and numerous hybrids in captive production (Bronzi et al. 2019).

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Table 1. Description of policies and resolutions that protect worldwide sturgeon populations against habitat loss, overharvest, and illegal trade.

Resolution	Description	Application to Sturgeon	Resources
Convention on the International Trade in Endangered Species of Wild Fauna and Flora (CITES)	Multinational agreement through which countries work together to ensure that international trade is not detrimental to the survival of the species in the wild. Member nations to CITES regulate and monitor international trade (import and export) through permits and certifications to ensure sustainable use. The CITES Secretariat is not a law enforcement authority and does not conduct investigations; instead, each country is to investigate within-county allegations if potential criminal activity lies with the relevant national law enforcement authorities of that country.	International trade led to a CITES listing of all species of Acipenseridae; since 1998, all sturgeon trade is regulated and all parts or derivates (e.g., caviar, meat, skin, etc.) require a CITES permit or certificate. Only Shortnose Sturgeon *Acipenser brevirostrum* is listed under CITES Appendix I (threatened by extinction with trade permitted only in exceptional circumstances). All other sturgeons are listed in Appendix II (trade could threaten species persistence if not controlled).[1]	The Convention on the International Trade in Endangered Species of Wild Fauna and Flora provides country-specific contacts for reporting violations and information regarding illegal trade[2]; in the USA, the U.S. Fish and Wildlife Service (USFWS) is the official CITES authority and provides opportunity to report violations.[3]
U.S. Endangered Species Act (ESA)	Under the ESA, the National Marine Fisheries Service (NMFS) and the USFWS are required to conserve the ecosystems upon which endangered and threatened species depend on, provide a program for listed species conservation, and take appropriate steps toward recovery. Section 9 of the ESA outlines prohibitions, including illegal "take" of a listed species, which includes harm, capture, and harassment, and Section 10 describes penalties for illegal take. Under Section 7, federal actions are required to undergo a consultation to assess and reduce potential interactions with listed species and their designated critical habitat helps to conserve listed species. Limited federal dollars are also available for research. The ESA primarily protects foreign species by restricting trade and may prohibit certain activities, including import, export, take, commercial activity, interstate commerce, and foreign commerce. By regulating these activities, the United States ensures that people under its jurisdiction do not contribute to the further decline of a listed species.	Eight of the nine sturgeon species in the USA are currently listed under the ESA as either threatened or endangered and a review on the status of the Lake Sturgeon *Acipenser fulvescens* is scheduled for 2024. There are also eight sturgeon species that are not native to the United States listed under the ESA[4]. As described, the introduction of a nonnative species could harm listed sturgeon.	The National Oceanic and Atmospheric Administration (NOAA) is mainly responsible for marine wildlife[5] and USFWS for terrestrial and freshwater organisms[6]. Violations of the ESA should be reported to either NOAA by calling 1–800–853-1964 or the USFWS via their online reporting[7]; photos and videos are encouraged.
United Nations Convention on the Conservation of Migratory Species of Wild Animals (CMS)	An environmental treaty of the United Nations designed to conserve migratory species, with special emphasis on protection of habitats and migration routes. The CMS agreements range from legally binding treaties to less formal agreements.	European Sturgeon *Acipenser sturio* is listed under Appendix I (threatened with extinction) and 18 species are listed under Appendix II (species that would benefit from international cooperation).	
Bern Convention on the Conservation of European Wildlife and Natural Habitats	A binding international legal agreement among 50 countries and the European Union designed to conserve wild flora and fauna and their natural habitats, with special attention given to endangered and vulnerable species.	Adriatic, European, and Mediterranean populations of Beluga *Huso huso* Sturgeon are listed as strictly protected, while Sterlet *Acipenser ruthenus*, Stellate Sturgeon *A. stellatus*, and remaining Beluga Sturgeon populations are listed as protected.	
International Union for the Conservation of Nature (IUCN)	Global environmental network of government and civil society organizations that uses expert panels to inventory the status of biological species. Using a set of precise criteria, these experts evaluate the extinction risk of thousands of species and subspecies. Species at risk of extinction are placed on the IUCN Red List.	The Sturgeon Specialist Group has over 50 experts contributing to conservation of sturgeon and paddlefish. As of the 2022 status assessment, all 26 species are imperiled to some degree, with 17 sturgeons identified as critically endangered, a listing that represents organisms at extremely high risk of extinction in the wild.	
Species at Risk Act of Canada	Canadian legislation that provides legal protection for wildlife species and provides measures to assist in the recovery of threatened and endangered species.	Classification is often population-specific, but at least some populations of Atlantic Sturgeon *Acipenser oxyrinchus*, White Sturgeon *A. transmontanus*, Green Sturgeon *A. medirostris*, Lake Sturgeon, and Shortnose Sturgeon are listed under Schedule 1 indicating populations that are endangered, threatened, or of special concern. Endangered populations are afforded federal protections of critical habitats and prohibitions on individual harm.	

(Continues)

Table 1. (Continued)

Resolution	Description	Application to Sturgeon	Resources
U.S. Lacey Act	Administered by NMFS and the USFWS, the Lacey Act makes it unlawful to any person to import, export, transport, sell, receive, acquire, or purchase fish, wildlife, or plants that were taken, possessed, transported, or sold in violation of any law or regulation of any state.	Illegal trade of sturgeon is punishable by felony fines with no innocent owner exceptions.	Violations of the Lacey Act can be reported at fws_tips@fws.gov or at 1–844–397-8477

[1]https://bit.ly/3U6XibC
[2]https://bit.ly/3DIjq6O
[3]https://bit.ly/3fjcQKj
[4]https://bit.ly/3SLsllR
[5]https://bit.ly/3NdZV98
[6]https://bit.ly/3ff1qr3
[7]https://bit.ly/3gQlZe3

Sturgeon are also becoming more prevalent in conservation aquaculture programs. These programs, which use careful genetic and demographic planning to aid in species recovery, have been instrumental in the restoration of several sturgeon populations. However, conservation aquaculture is not the focus of this manuscript, as threats to wild populations are most likely to occur when individuals are released or escape from captive populations that have not been bred and reared for the explicit purposes of population restoration. As such, this manuscript focuses on the increasing prevalence in releases from captive populations that occur in commercial, private, and/or other research facilities.

We contend that growth of sturgeon captive culture has real potential to countermand decades of global conservation efforts and accelerate declines of many critically imperiled sturgeons. Moreover, given the projected expansion in the size, distribution, and scope of commercial aquaculture facilities, existing regulatory frameworks (Table 1) may be insufficient to protect future wild sturgeon populations. Here, we highlight some of the most significant threats that the captive culture industry presents to native sturgeon populations. We then discuss modifications to existing regulatory frameworks that could help support the collective goals of conservation and sustainable consumerism of sturgeons.

CAPTIVE STURGEONS IN THE WILD

As the number of sturgeon culture facilities has increased, so too has the number of reported incidents of sturgeon outside of their native waterways. The release of captive fishes, be it through intentional stocking or accidental release, has left one of the biggest footprints on global fisheries conservation (Lockwood et al. 2019). Yet, there are still few answers to the catastrophic declines in native fish communities that commonly follow the establishment of nonnative ichthyofauna. Physical, chemical, and genetic tools are available to control the spread of aquatic invasive species and populations, but these require significant resource investment and can result in further harm to native species. Even then, efforts largely focus on management of the nonnative population, as complete eradication is often impossible, particularly in large river systems and marine environments (Gozlan et al. 2010). Therefore, the best tool for limiting the spread of nonnative species is to minimize introduction pathways.

Below, we highlight the major introductory pathways for captive sturgeons into wild populations. Importantly, while unsanctioned release of captive sturgeon from research, commercial, and private facilities has been documented, limited monitoring and difficulty sampling sturgeon populations likely allows many incidences to go undetected. Moreover, many pathways that lead to captive sturgeon introductions may receive little attention as they involve release of relatively few individuals. However, the invasion histories of other species provide cautionary tales that colonization and spread of nonnative species can occur from small founding populations (Rachels 2021). In addition, the shared habitat requirements among sturgeons and the low abundance of many native populations suggest that invasion success of released captive sturgeon could be high.

Commercial Culture

The potential for commercial culture operations to negatively affect wild sturgeon populations is already being realized, as we have witnessed repeated incidences of accidental release of captive sturgeons from commercial facilities (Ludwig et al. 2009). In one example, a catastrophic flood in 2016 resulted in the escape of over 9.8 million kg of captive fish, including five nonnative sturgeon species, several sturgeon hybrids, and a nonnative paddlefish, into the Yangtze River, China (Ju et al. 2020). Escapees vastly outnumbered native species, including the critically endangered Chinese Sturgeon *Acipenser sinensis*, making hybridization and competition significant concerns (Gao et al. 2017). A similar event was documented in the United States in 2017, when flooding in the state of Idaho resulted in the release of approximately 3,000 captive adult White Sturgeon *A. transmontanus* into a nearby river, of which most were not recaptured (Idaho Power Company 2018).

In the Rioni River and Black Sea in the country of Georgia, nonnative Siberian Sturgeon *A. baerii* have been recently documented multiple times. Although the origin of these individuals is unknown, it is believed they were released or escaped from commercial aquaculture facilities. How Siberian Sturgeon will influence the conservation of four native sturgeons (Russian Sturgeon *A. gueldenstaedtii*, Stellate Sturgeon *A. stellatus*, Beluga Sturgeon *Huso huso*, and Ship Sturgeon *A. nudiventris*) is still under investigation. However, because native Rioni River sturgeons already exist in critically low abundance, presence of relatively few Siberian Sturgeon could result in significant declines and/or extirpation of native sturgeon (Scheele, written communication; see below for potential mechanisms of interaction between Rioni River sturgeons).

Effects of commercial culture are not always restricted to release of captive fishes. For example, investigations by Fauna and Flora International show that Georgian Black Sea fishers sometimes sell live Beluga Sturgeon and Stellate Sturgeon to local ponds, where owners mix wild and captive individuals with the goal of releasing offspring into the wild

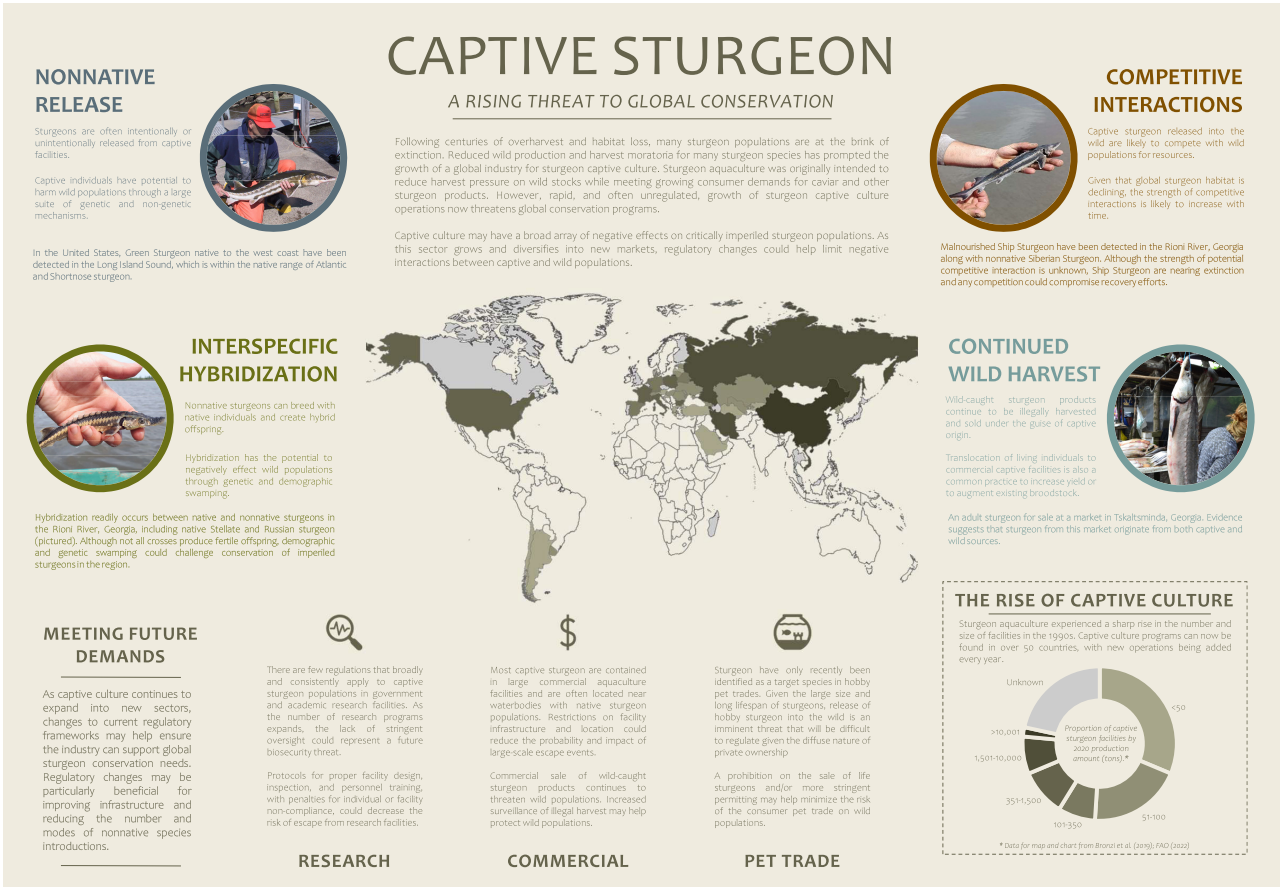

Figure 1. The expanding scale and scope of sturgeon captive culture (center map; data from Bronzi et al. 2019; FAO 2022) presents an emerging threat to wild sturgeon populations around the world. Changes to existing regulatory frameworks would increase opportunities for captive culture to support wild sturgeon conservation.

(Fleur Scheele, Fauna and Flora International, written communication). Effects of these releases are unlikely to be realized for several decades; however, even moderate amounts of interbreeding and competition may jeopardize the survival of several endemic, critically endangered sturgeons in the region.

Research and Government Laboratories

Damage to captive infrastructure typifies the catastrophic threats that high biomass commercial facilities pose to native fish communities. However, these events are generally isolated, well documented, and often followed by increased management and surveillance. Conversely, poor biosecurity and uncontrolled trade likely present a more chronic and cryptic threat to wild populations. By nature, public documentation of unpermitted introductions is sparse. However, credible anecdotes implicate individuals, including career biologists, with the unsanctioned release of captive sturgeon, including the introduction of species outside of their native range. In the United States, a federally threatened Green Sturgeon *A. medirostris,* native to the Pacific coast of North America, was collected on April 23, 2010 in the Long Island Sound, an estuary of the Atlantic Ocean (Figure 1). Due to the conservation status of Green Sturgeon, the species is not permitted in private or commercial aquaculture. As such, it has been hypothesized that the nonnative Green Sturgeon was introduced from a nearby research facility; however, it is unclear when and how many individuals were released and how many may still be alive.

Although introductions are most frequently associated with nonnative species, the life history of sturgeon raises unique concerns about the invasion potential of captive individuals that are released within their native range but are genetically dissimilar to the local population. The philopatric tendency of sturgeon can create strong spatial genetic structuring among nearby populations, potentially leading to local adaptation to the unique physiochemical environments found in each river system (Schreier et al. 2012). As a result, the conservation value of a nonnative population of an otherwise native species is unknown. This question is currently being debated after a nascent population of Atlantic Sturgeon *A. oxyrinchus oxyrinchus* (a taxon listed under the United States Endangered Species Act) was discovered in the Connecticut River—a waterway where native Atlantic Sturgeon were thought to have been extirpated several decades ago (ASSRT 2007). Genetic analyses suggested the contemporary Connecticut River population was likely founded by individuals that originated from a population in the southeastern United States (Savoy et al. 2017). Atlantic Sturgeon are largely philopatric and show strong patterns of genetic isolation by distance, leading to genetically unique populations among most rivers (White et al. 2021). Therefore, while we cannot rule out the possibility that the Atlantic Sturgeon captured in the Connecticut River strayed from the southern Atlantic, it is more plausible that fish were released from a nearby research facility. At present, it is unclear if the population will adapt to the physiological requirements of a more northern climate.

However, if the disjunct population persists, it may complicate future conservation efforts by conflating results of individual assignment and mixed-stock analyses, both of which are extensively used to understand regional and range-wide threats to Atlantic Sturgeon recovery (e.g., Kazyak et al. 2021).

Private Pet Trade

A rapidly emerging, and generally unregulated, threat to sturgeon conservation is the increased circulation of numerous sturgeons, including species of conservation concern, in hobby pet trades. The scale and scope of the pet trade for sturgeons is largely undocumented, and so their effect on native populations is unknown. However, ad hoc monitoring of popular consumer-to-consumer websites and online pet stores by the authors found numerous instances of both native and nonnative sturgeons being openly traded in the United States and abroad. Exotic pet trade has been unequivocally implicated with widespread biodiversity loss (Lockwood et al. 2019; Morton et al. 2021), and surveys of private aquarists have shown that up to 10% of fish owners admit to deliberately releasing aquarium fish into the wild (Chang et al. 2009; Strecker et al. 2011). Release of hobby sturgeon is a likely outcome, as sturgeon rapidly attain sizes that are too large for aquaria and small ponds and pet owners are often averse to euthanizing otherwise healthy animals (Holmberg et al. 2015). As such, the buying, selling, and transportation of hobby sturgeon is an expected pathway for nonnative invasions, as has already been documented by a Dutch public database (https://steuren.ark.eu/). This website monitors observations of native European Sturgeon *A. sturio* in the Netherlands, but has also documented nearly 50 occurrences of nonnative Siberian Sturgeon, Russian Sturgeon, and Sterlet *A. ruthenus* since 2010, with most occurring since 2020. The three nonnative species are likely derived from breeding facilities in eastern Europe and Asia, were sold in Europe as pond fish, and were subsequently released into the wild. This finding is further corroborated by Brevé et al. (2022), who noted the occurrence of 11 nonnative sturgeon species in the Rhine–Meuse River delta, most of which could be attributed to unintentional and aided escape from garden and angling ponds.

THREATS OF STURGEON CAPTIVE CULTURE TO WILD POPULATIONS

The full extent how captive sturgeon may impact wild populations is still unknown, as we have only recently started to document the invasion of captive sturgeons into wild populations (e.g., Ludwig et al. 2009). The outcome of an introduction likely depends on the number of individuals released, density of competitors, and habitat suitability. Therefore, we likely have not yet observed the full suite of potential negative interactions that may occur between captive and wild populations. Moreover, given the late maturation and long lifespan of sturgeon, it may take several decades before the consequences of present-day captive releases are fully realized. This underscores the importance of proactive regulation of captive sturgeon populations, as it may be too late to mitigate negative effects once declines in wild populations are detected.

Hybridization

A significant concern with release of captive individuals is the potential for interbreeding between domestic and wild lineages. The most significant threat is likely that of hybridization—the mating of individuals from different species or, rarely, different genera or families—which can cause rapid loss of native population fitness. Hybrid offspring are often sterile, and so the effect of hybridization may be restricted to loss of reproductive effort. For large populations, temporary reduction in fitness is unlikely to have significant, long-term genetic or demographic effects. However, in populations where few spawning individuals remain, as is the case in many imperiled sturgeon populations, hybridization has real potential to result in demographic swamping leading to collapse of local populations and even whole species extinction (Wolf et al. 2001).

When hybrid offspring are fertile, concerns arise over the potential for increased fitness of hybridized individuals relative to either parental species (i.e., hybrid vigor; Shivaramu et al. 2020). Hybrid vigor can lead to rapid displacement and loss of genomic signatures of the native species, ultimately resulting in declines in native populations through genetic swamping. One of the best documented cautionary tales of hybrid vigor comes from the prolific cutbow trout—a fertile hybrid from the mating of Cutthroat Trout *Oncorhynchus clarkii* and Rainbow Trout *O. mykiss*. Cutbow trout have physically displaced many populations of native Cutthroat Trout, including the Westslope Cutthroat Trout *O. clarkii lewisi*, which has experienced rapid declines in abundance and distribution due to habitat loss and erosion of genetic integrity from hybridization (Muhlfeld et al. 2009). Substantial resources are invested into ongoing efforts to identify and eradicate hybrids in an attempt to restore pure populations of Yellowstone Cutthroat Trout *O. clarkii bouvieri*. This case study underscores the potential long-term biologic and economic costs that can occur following release of nonnative species and the potential for hybridization to result in permanent genetic effects to native populations.

Hybridization has been well documented in wild and captive sturgeon populations, with over 20 different hybrid crosses reported in the literature (Table S1), including interfamilial hybridization between Russian Sturgeon and American Paddlefish *Polyodon spathula* (Káldy et al. 2020). Moreover, hybrids can be produced between species with differing ploidy levels, as exemplified by nonnative Siberian Sturgeon (~240 chromosomes) and native Sterlet (~120 chromosomes) hybrids in the Danube River (Ludwig et al. 2008). Although offspring of this cross were found to be sterile, hybrids from native Russian Sturgeon (~240 chromosomes) and Stellate (~120 chromosomes) in the Rioni River (Ludwig et al. 2009; Beridze et al. 2022; Figure 1) are viable. This success of hybrid sturgeons in wild environments is not well understood; however, hybridization between native Russian Sturgeon and nonnative Siberian Sturgeon in the Caspian Sea (Jenneckens et al. 2000), and subsequent laboratory studies documenting hybrid vigor (Shivaramu et al. 2019) suggest that genetic swamping is a possible outcome of hybridization in sturgeons.

Sturgeon hybridization may occur readily in natural environments because many species have similar life history characteristics and spawning habitat requirements. In addition, sturgeon are broadcast spawners, which is a mode of reproduction that is associated with high hybridization rates in other taxa. Together, the documented ease and high probability of hybridization in sturgeon in captive and wild environments suggests that continued unregulated release of nonnative sturgeons has real potential to lead to population declines through demographic and/or genetic swamping. In addition, it is often difficult to discern hybrid individuals from purebred species

using morphologic traits or genetic techniques, particularly after multiple generations of admixture and backcrossing. As the number of hybrid sturgeon in captive and wild populations increases, it may become increasingly challenging to identify purebred individuals in the field and trace the origin of commercial sturgeon products, both of which will complicate conservation efforts for native populations.

Anthropogenically Mediated Gene Flow

In addition to hybridization, sturgeon may also be prone to introgression, which we define as breeding between individuals of the same species but belonging to different populations. Introgression can improve population viability by increasing genetic diversity and long-term evolutionary potential. When executed successfully, as in many conservation aquaculture programs, introgression between wild and captive populations can be an effective conservation strategy for genetic rescue.

However, unintended introgression between wild and captive populations is likely to reduce fitness and survival of native populations. Wild-caught broodstock and juveniles are infrequently used in commercial production, and so generations of artificial selection can render captive individuals maladapted for wild environments. Under this scenario, introgression can lead to outbreeding depression and subsequent reduction in the fitness and survival of future generations—a phenomenon that has been well documented in salmonids (Araki et al. 2007). Moreover, introgression across large spatial scales can result in genetic homogenization and diminished long-term adaptive capacity. Together, loss of contemporary fitness and evolutionary potential may jeopardize the ability of native populations to persist and could severely undermine current efforts aimed at demographic recovery. Because many commercial facilities rear sturgeon outside of their native range, the threat of introgression is likely lower relative to hybridization. However, aforementioned examples of released captive Atlantic Sturgeon and White Sturgeon suggest the risk of introgression in contemporary populations is not negligible, and could increase with expansion in the size, scope, and location of commercial facilities.

Competition and Depredation

Negative effects of captive release can occur in the absence of reproduction, including the potential for resource competition and juvenile depredation. Given significant knowledge gaps about many aspects of sturgeon life history and ecology, the strength and outcome of competitive interactions may be difficult to predict. However, significant resource overlap among species and the global decline in sturgeon habitat suggests competition for already limited resources is likely to increase in future decades.

Although not causally linked to competition, malnourished Ship Sturgeon have been discovered during recent surveys in the Rioni River (Scheele, written communication); a potential indicator of negative interactions between native and introduced sturgeons (Figure 1). This is a discouraging finding, as Ship Sturgeon was believed to be extirpated from the Black Sea basin (Beridze et al. 2021) and now the long-term viability of the relict breeding population may be jeopardized by low fitness and survival.

Nonnative species, including sturgeon, but also other non-sturgeon species, can pose significant risk to recruitment through depredation. Although sturgeon develop bony scutes to deter predators, younger life stages have few natural defenses and are vulnerable to predation (Flowers et al. 2011). The risk of depredation may be particularly high when nonnative fishes are released near freshwater spawning and nursing habitats.

Pathogens and Parasites

Indirect effects of captive culture can be observed through the introduction of disease and parasites. High densities of fish in aquaculture facilities and mixing of multiple taxa in hobby aquaria increases the prevalence of viral, bacterial, and parasitic infections in captively reared individuals. Although many pathogens commonly found in captive facilities also occur in wild environments, human transport of captive fish across river basins introduces novel sources of disease to which native populations may have little natural immunity. Once released, captive individuals can then spread pathogens through entire ecosystems as they move through different habitats to complete their life cycle. Therefore, the pathogenic consequences of a single captive release may manifest across vast spatial scales, particularly for anadromous species like sturgeon.

Introduction of aquatic diseases during intentional stocking events has been implicated in population declines, loss of entire spawning year-classes, and even complete extirpation of species in some areas (Zholdasova 1997). Presently, the largest disease risks in captive sturgeon populations appear to be bacterial (e.g., *Streptococcus iniae*, which also poses a disease risk to humans [Mugetti et al. 2022]) and viral infections, including herpes viruses, White Sturgeon iridovirus, and potentially species of *Ranavirus* normally associated with amphibian declines (Waltzek et al. 2014). Although the threat of *Ranavirus* remains unclear, herpes viruses and White Sturgeon iridovirus are highly transmittable and have been correlated to necrotic infection and large mortality events. Viral infections of wild and captive sturgeon populations have been detected in multiple species and countries (e.g., Kurobe et al. 2010; Hofsoe-Oppermann et al. 2020), suggesting that more widespread outbreaks could be forthcoming as the captive industry continues to expand.

Continued Harvest of Wild Populations

With most sturgeon species under strict harvest moratoria, captive facilities are now the most viable, and often only legal, source for caviar and other sturgeon products. However, large body size and late age of maturation can make sturgeon difficult to raise in captivity. Commercial fish culturists looking to reduce the space and resource requirements needed to support a self-sustaining captive sturgeon population or needing to compensate for incidental loss may continue to harvest individuals from wild populations. In Eurasia, wild sturgeon, particularly gravid females, are sometimes translocated to commercial facilities and temporarily held before being used as broodstock and/or harvested. This practice, which is illegal in many regions, perpetuates the stress on wild populations, and specifically monetizes removal of individuals during critical life stages. However, limited oversight and lack of critical inspection of many food labels still provide abundant opportunity for individuals to directly harvest or translocate individuals from wild populations (Dolan and Luque 2019; Figure 1).

SUPPORTING STURGEON CONSERVATION IN THE ERA OF CAPTIVE CULTURE

Demand for caviar and other sturgeon products remains high, and continued loss and restriction of wild fisheries are likely to compel further development of the captive culture

industry. Expansion of research laboratories, commercial facilities, and private pet trades presents serious challenges for conservation, as the scope and location of many captive facilities will likely fall outside the purview of current regulatory frameworks (see Table 1 for a list of policies that pertain to global sturgeon conservation). Specifically, the majority of current regulations aim to protect critical habitats, minimize individual harm, and regulate commercial harvest and transport of sturgeon and sturgeon products. While these challenges will remain into the future, emerging threats are likely to develop as the taxa's user group continues to expand. How this new era of sturgeon captive culture will affect wild populations remains to be seen; however, more stringent, effective, and targeted regulation would likely provide more opportunities for captive culture to be apposite to global conservation. Below, we outline three major sectors of sturgeon captive culture and discuss possible regulatory changes that could improve the outlook of sturgeon conservation:

(1) **Commercial culture**. Commercial facilities account for the majority of captive sturgeon populations (Bronzi et al. 2019) and are likely to be the source of most nonnative sturgeon introductions. While many regulations already pertain to captive culturists (Table 1), there are still opportunities for improved oversight of this sector. Increased governance on allowable infrastructure, including restricted use of flow-through systems and banning facilities in flood-prone areas, would likely reduce the probability of high biomass escapes. Additional oversight on the location of large facilities may be particularly important given that catastrophic flood events are likely to increase under future climate change scenarios. Another potential mechanism for reducing introduction pathways could be to severely restrict or prohibit the commercial sale of live sturgeon. However, across all commercial markets, more stringent investigation and castigation for mislabeled products is likely to reduce illegal harvest and minimize impacts on wild populations.

(2) **Research and government laboratories**. There is an expectation that fisheries professionals will uphold the highest standards of conservation. However, multiple observations presented in this article are consistent with unsanctioned releases of captive sturgeon from scientific facilities. In addition, fisheries research facilities are often highly trafficked outdoor environments, which increases the biosecurity risk associated with these captive populations. Despite recent expansion in the number of sturgeon research programs, there are surprisingly few regulations that pertain to the proper handling of captive fish in these environments. For example, while academic research facilities in the United States operate under the oversight of animal care and use committees and may seek accreditation from the Association for the Assessment and Accreditation of Laboratory Animal Care International, accreditation is not mandatory for all federal research facilities. As such, within the United States there are no universal protocols pertaining to proper transport, handling, and disposal of captive individuals in laboratory environments. Likewise, there is no mechanism to assure that captive sturgeons are eliminated following laboratory testing, which is a requirement under federal permitting in the United States. Overall, the absence of consistent regulatory frameworks for academic and federal research facilities highlights a significant biosecurity gap, and an opportunity to enhance protocols pertaining to proper infrastructure, facility inspection, and personnel training. For example, in Canada, captive facilities must satisfy requirements pertaining to disease transfer, husbandry methods, culture equipment, and fish holding densities, with more stringent requirements for listed species. The adoption of similar protocols by other countries may be particularly critical given that many research facilities are located near large waterbodies, and vulnerable infrastructure (i.e., flow-through systems) and insufficient maintenance has significant potential to be a source of future unintentional captive escapes.

(3) **Pet trade**. Although this sector has not yet been documented to have an impact on wild populations, rising popularity of sturgeon in the hobby pet trade foreshadows future negative consequences of private captive culture. This sector is very challenging to directly regulate due to the diffuse and poorly documented nature of private culture. Due to the challenges with regulating individual owners, and the near-certain probability that sturgeon growth will surpass all indoor aquaria, the strongest regulatory mechanism may be to prohibit all sale. Even with restrictions on sales, it may still be beneficial to increase surveillance for illegal trade of sturgeons. Automated approaches for monitoring the internet for trade of invasive species (e.g., Great Lakes Detector of Invasive Aquatics in Trade; https://bit.ly/3DIJ5Mu) have been developed and might provide a useful model for tracking the trade of sturgeons.

The captive culture industry represents an emerging threat to the recovery and conservation of critically imperiled sturgeon populations. Negative effects of captive culture programs, including intentional and unintentional release and ongoing harvest of wild populations, have already manifested in some populations. Under the current trajectory of the captive culture industry, nonnative invasion, introgression, and hybridization have real potential to reverse decades of conservation and drive one of the most ancient and globally revered groups of fishes further toward extinction. Increased attention to these emerging issues may help improve the outlook for sturgeon conservation programs.

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SUPPORTING INFORMATION

Additional supplemental material may be found online in the Supporting Information section at the end of the article.

Table S1 List of known sturgeon hybrids that appear in wild and captive environments along with ploidy levels inferred by Ludwig et al. (2001). Of note, many hybrid crosses that exist in the wild also appear in captive populations. AFS

diversity

MDPI

Type of the Paper (Article)

Genetic evidence for the presence of wild-caught sturgeons in commercial markets in Georgia

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Abstract: Sturgeons (Family: Acipenseridae) are among the most endangered taxa, worldwide. Significant resources have been invested into the conservation of global sturgeon populations, including development of commercial aquaculture programs. These programs are intended to improve conservation outcomes by reducing harvest of wild populations while still meeting commercial demand for sturgeon products. However, there is growing concern that commercial aquaculture programs may contribute to wild population declines through continued, illegal harvest and the escape and/or release of captive individuals into wild environments. These concerns may be particularly acute in the country of Georgia which, despite its small territory and altered landscape, is a globally significant hotspot for sturgeon diversity. In order to understand the potential threat of captive culture on wild sturgeon populations in Georgia, we used mitochondrial DNA sequencing and microsatellite analyses to identify the species and origin of sturgeon encountered in commercial settings. Microsatellite analyses showed significant differentiation between wild and commercial Russian sturgeon populations and highlighted the potential for wild-caught individuals to be present in coastal markets in Georgia. Analyses of mitochondrial haplotypes also suggested that commercial markets may contain sturgeon species that are not native to the region. Overall, our results suggest that wild sturgeon populations may still be exploited to support commercial propagation, and that aquaculture programs may pose an ongoing, cryptic threat to wild sturgeon populations in the region.

Keywords: sturgeon1; commercial aquaculture 2; conservation 3; Georgia 4; illegal trade 5.

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1. Introduction

Sturgeons (Family: Acipenseridae) are among the oldest living animal taxa with evolutionary records dating species as far back as 200 million years ago [1,2]. Although populations were once widely distributed and abundant in the northern hemisphere, sturgeon populations have been declining over the last century, with most extant species now considered to be at risk of extinction by the International Union for the Conservation of Nature (IUCN) [3]. Overharvest is one of the major factors contributing to precipitous population declines, with 14 of 25 extant species being recognized as commercially important for caviar and meat [4]. Over the last century, demand for sturgeon products has risen, with average landings reaching 28 900 mt (metric tons) between 1976 and 1983 [5].

Of this, 90% of sturgeon harvest occurred in the Soviet Union, with most landings concentrated in the Caspian and Black seas [5,6].

In response to global population decline, sturgeon commercial aquaculture programs began rising in popularity in Russia in 1970. By the 2000s, sturgeon aquaculture had also developed in France, Germany, Italy, Hungary, USA, and China. Programs are still under development today [5,7], with expansion into new continents such as South America and Australia [8]. Although commercial sturgeon aquaculture may be intended to support recovery of sturgeon populations through reduced harvest of wild individuals and is a legal, controlled and regulated activity under Convention on International Trade in Endangered Species of Wild Fauna and Flora-CITES (2021), the propagation and trade of captive sturgeon remains a significant threat to wild populations [9]. For example, there have been reported cases where wild sturgeons have been provided to fish markets for direct sale or used to increase captive production [10]. Pilot poaching monitoring has also shown that 23 wild sturgeon from three different species (Russian sturgeon [*Acipenser gueldenstaedtii*] stellate sturgeon [*A. stellatus*], and beluga sturgeon [*Huso huso*]) were harvested from December 2017 to July 2018 in the eastern Black Sea [11]. Overall, these reports suggest that illegally harvested wild sturgeon are likely being sold in commercial markets under the guise of commercial production. However, because it is largely impossible to visually differentiate the origin of wild and captive individuals, these reports remain largely anecdotal evidence and it remains unclear the extent to which wild sturgeons are being sold in commercial settings.

In addition to illegal sale, escape or release of captive individuals may pose a threat to wild populations through genetic admixture, interspecific hybridization, resource competition, and introduction of parasites and pathogens [9,12]. This is of particular concern with regards to the release of interspecific hybrids, which are being commercially grown to optimize growth and early maturation [13–15] and, if released, may readily outcompete native populations[16].

Despite its small size, the country of Georgia is a global diversity hotspot for sturgeon. The Rioni River, a tributary to the eastern Black Sea, historically supported spawning populations of at least five, and potentially six species of sturgeon, including Russian, stellate, beluga, European (*A. sturio*), ship sturgeon (*A. nudiventris*), and Colchic sturgeon (*A. colchicus*; of which the taxonomic status is not clear) [17,18]. Overfishing and habitat degradation greatly reduced sturgeon abundance in the Rioni River, with landings decreasing from 100 mt in the 1930s to just 12 mt tons in the 1960s [17], ultimately leading to a harvest moratorium in 1967. Despite the cessation of legal harvest, contemporary populations remain threatened, with evidence that only three species (Russian, ship, and stellate sturgeons) still spawn in the Rioni River [18]. In addition, only adult beluga sturgeon have been found in the eastern coast of the Black Sea, suggesting limited juvenile recruitment [19]. Of the aforementioned species, four (Russian, ship, stellate, and beluga sturgeons) are listed as critically endangered (CR) by the IUCN (IUCN Red List of Threatened Species), with the European sturgeon considered extirpated in the Black Sea.

It is presently unclear the extent to which captive facilities may threaten wild populations in the Rioni River. However, according to the surveys carried out by Fauna & Flora Caucasus Programme, there are currently four fish farms rearing sturgeon within the Rioni River watershed in western Georgia. This includes one farm that is located close to the Tekhuri River which joins the Rioni River [20]. The surveys suggest that sturgeon in these farms are mostly Russian and Siberian sturgeons (*A. baerii*). Notably, Siberian sturgeon are non-native to the Black Sea and originated from broodstock sourced from Armenia. Studies have shown that Siberian sturgeon readily hybridize with native sturgeons in captive and wild environments [16], highlighting the potential for hybridization to negatively impact wild sturgeon populations. One obstacle for understanding the threat that captive facilities may pose on wild populations is that hybridization and transportation of individuals among regions has made it difficult to readily identify the source, and even

species, of individuals that are sold in markets. However, genetic monitoring may present a viable tool for addressing the questions and monitoring the effect of aquaculture on wild populations. In particular, because acipenserids are philopatric [6,21] populations tend to show a high degree of genetic differentiation among geographical regions, river systems, and even commercial facilities [22–25]. Therefore, it may be possible to use genetic tools to discern the natal origin of individuals and ultimately determine whether wild individuals are present in commercial facilities or markets.

In this study, we used complementary genetic tools to understand the species and source of sturgeons sold in commercial markets in Georgia. Our first objective was to use mitochondrial DNA sequencing analysis to identify the species present in commercial environments. We then used microsatellite analyses to ascertain the likely source (wild vs. captive) of sturgeons sold in Georgian markets. Results of this study provide early insights into the potential threats that commercial captive culture may pose on wild sturgeon populations in the Rioni River.

2. Materials and Methods

2.1 Sample Collection

We collected fin clips from sturgeon that were being commercially sold in fish markets in Tbilisi, Batumi, Poti, and Tskhaltsminda and from the fish farm in Georgia from January 2016 to December 2019 (Figure 1). In total, 72 tissue samples were collected from individuals of reported captive origin, including 66 Russian, 2 stellate, 1 beluga, and 3 sterlet sturgeons (*A. ruthenus*; Table 1).

We also analyzed tissue samples from 84 wild sturgeon captured in the Rioni River and the Black Sea collected by the Fauna & Flora Caucasus Programme Sturgeon Conservation Team from August 2018 to December 2020 (Figure 1, Table 1). All wild-caught individuals were immediately released after tissue collection. DNA extraction was carried out using QIAamp Blood & Tissue Mini Kit, according to the manufacturer's protocol (QIAGEN, Hilden, Germany).

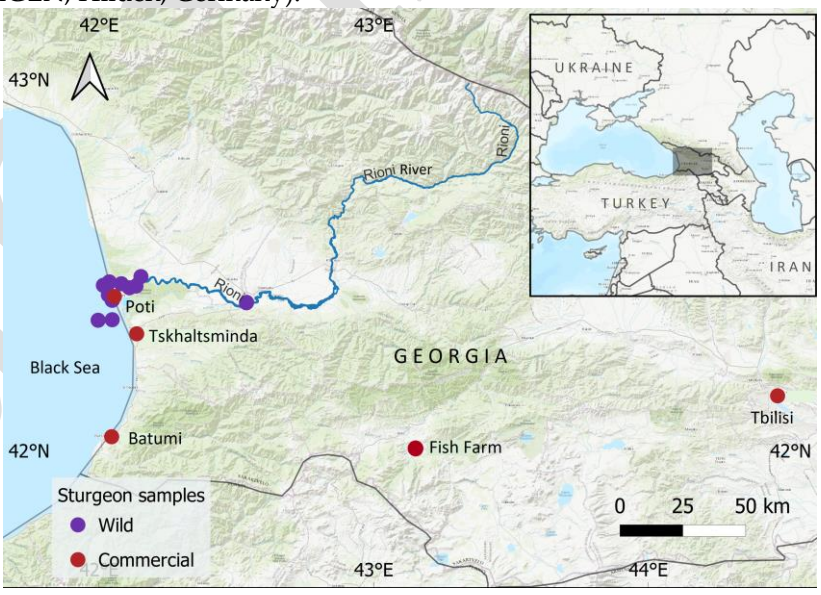

Figure 1. Locations where tissue samples were collected from commercial markets (red) and wild populations (purple).

2.2 Mitochondrial analysis

We used mitochondrial DNA sequencing [26] for species identification of both wild and captive samples, with 716 bp of the mitochondrial control sequenced. PCR was

performed in the volume of 20 ul, with 0.25 uM of each primer, 0.1 mM of dNTP's, 1x buffer, 0.1 U/ul Taq DNA polymerase (OxGEn and Promega), and approximately 80 ng/ul DNA template for each sample. We used the following primer pairs: Acipenser Pro1-F: 5′-CACCCTTAACTCCCAAAGC-3′ and Acipenser Phe1-R: 5′-CCCATCTTAACATCTTCAGT-3′ [25] with the following conditions: 94°C – 2 m; 94 °C – 45 s, 56 °C – 45 s, 72 °C – 45s for 33 times; 72 °C – 5 m. All samples were sequenced on a 3730xl DNA Analyzer (Thermo Fisher Scientific, MA, United States) at Macrogen Europe B.V. (Amsterdam, The Netherlands).

We used MEGA 11[26] for multiple sequence alignment using the program ClustalW and phylogenetic tree reconstruction. Trees were constructed using the maximum likelihood statistical method with the Tamura 3-parameter model with rates of change between sites following a gamma distribution. The best model was selected in MEGA 11 as best fit to available sequence data. Trees were examined to assess phylogenetic relationships between wild caught and commercial sturgeon individuals. We expect that genotypes will reflect the provenance of the individuals tested; wild individuals sold in markets will cluster with other wild specimens, and farmed fish will have haplotypes that reflect their origins, and different from those found in wild fish. We also included DNA sequences from sturgeons available from GeneBank [27]. Phylogenetic haplotype relationships and distances between wild and commercially sold specimens were investigated using the NETWORK 5.0 Median-Joining method [28].

Table 1. Number of individuals from each species that was sampled from wild and commercial environments. Numbers represent sample size used for microsatellite analyses, of which a subset (shown in parentheses) were used for DNA sequencing analyses. When possible, species identification was made using mitochondrial analyses, otherwise it was inferred from morphological characteristics.

Species	Wild-caught			Commercial
	Rioni River	Rioni River Mouth	Black Sea	
Russian sturgeon	6 (6)	40 (34)	9 (6)	66 (64)
Ship sturgeon	4 (4)	2 (2)	-	-
Stellate sturgeon	1 (1)	5 (4)	9 (6)	2 (2)
Sterlet sturgeon	-	-	-	3 (3)
Beluga sturgeon	-	-	8 (4)	1 (1)
Total		84 (67)		72 (70)

2.3 Microsatellite analysis

We genotyped tissues samples at four microsatellite markers including *Afug41*, *An20*, *Aox45*, and *AoxD165* [29–32]. PCRs were performed in 10 µl reactions containing 0.25 µM of each primer, forward primers labeled at 5' end with either VIC or 6-FAM dye, 2.5 mM MgCl2, 0.1 mM of dNTPs, 1x GoTaq Buffer, (Promega, pH 8.5, 50 mMTris-HCl, 50 mM NaCl), and 0.1 µl of Promega Go Taq polymerase (5U/µl, 1 unit/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, 72 °C – 40 s for 34 times; 72 °C – 10 min. The PCR reactions were analyzed using 3130xl Genetic Analyzer (Thermo Fisher Scientific, MA, United States). Genotyping Software GeneMapper 5 (Thermo Fisher Scientific, MA, United States) was used for allele calls. Tetraploids were assessed based on individual genotypes showing more than two alleles per marker.

We performed a hierarchical STRUCTURE analysis to visualize patterns of differentiation within- and among-species and identify possible patterns of hybridization among groups [33,34]. Others have shown that STRUCTURE can reliably recover patterns of differentiation in mixed-ploidy groups [35]. Therefore, the first level of our analysis included

all five species, with two alleles in diploid species coded as missing. Based on results from this analysis we grouped collections with similar patterns of differentiation and then performed another STRUCTURE analysis on each of those groups independently. We continued this iterative process until each species was represented as a unique genetic cluster or there was no evidence of further substructuring within the group. For all STRUCTURE analyses, we ran a recessive alleles model assuming admixture, correlated allele frequencies, and allelic ambiguity [34]. For each level of the analysis we retained 200,000 repetitions after a burn-in of 200,000 steps and ran 10 replicates for each value of K = 1 to G + 1 (where K was the number of genetic clusters and G was the number of groups in each level of the STRUCTURE analysis). Results from STRUCTURE were visualized using STRUCTURESelector [36], with appropriate values for K selected using the ΔK method [37].

3. Results

3.1. Mitochondrial DNA analysis

Based on the mitochondrial DNA sequencing analysis, we identified four sturgeon species in the market and aquaculture samples (Table 1). The majority of samples (almost 92%) were identified as Russian sturgeon. The remaining samples were identified as stellate sturgeon (n=2), and sterlet (n = 3), beluga sturgeon (n = 1).

Among the wild specimens, four species were identified based on mitochondrial DNA sequencing. The majority of these individuals (almost 70%) were identified as Russian sturgeon. The three other taxa were observed less frequently: stellate sturgeon (n = 11), ship sturgeon (n = 6; all from the Rioni River) and beluga sturgeon (n = 4; all from the Black Sea), see table 1.

On the phylogenetic tree and Network analysis (Figure 2, Figure 3), ship, stellate, beluga, and sterlet are grouped in separate species groups. There was a separation between wild and commercial Russian sturgeon haplotypes. However, there were some market samples that were grouped with wild samples, potentially representing wild haplotypes present in fish markets.

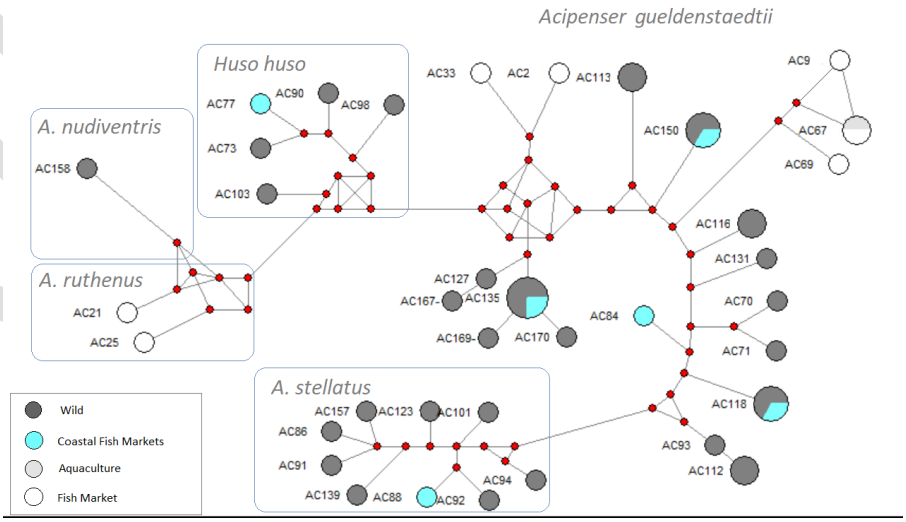

Figure 2. Network analysis of wild sturgeon samples from the Rioni River, the Rioni River mouth, and the eastern Black Sea (in gray) and commercial samples from Georgian fish markets and farm (blue, light gray, white). Each circle represents a single haplotype and the colors represent the origin of the sample(s).

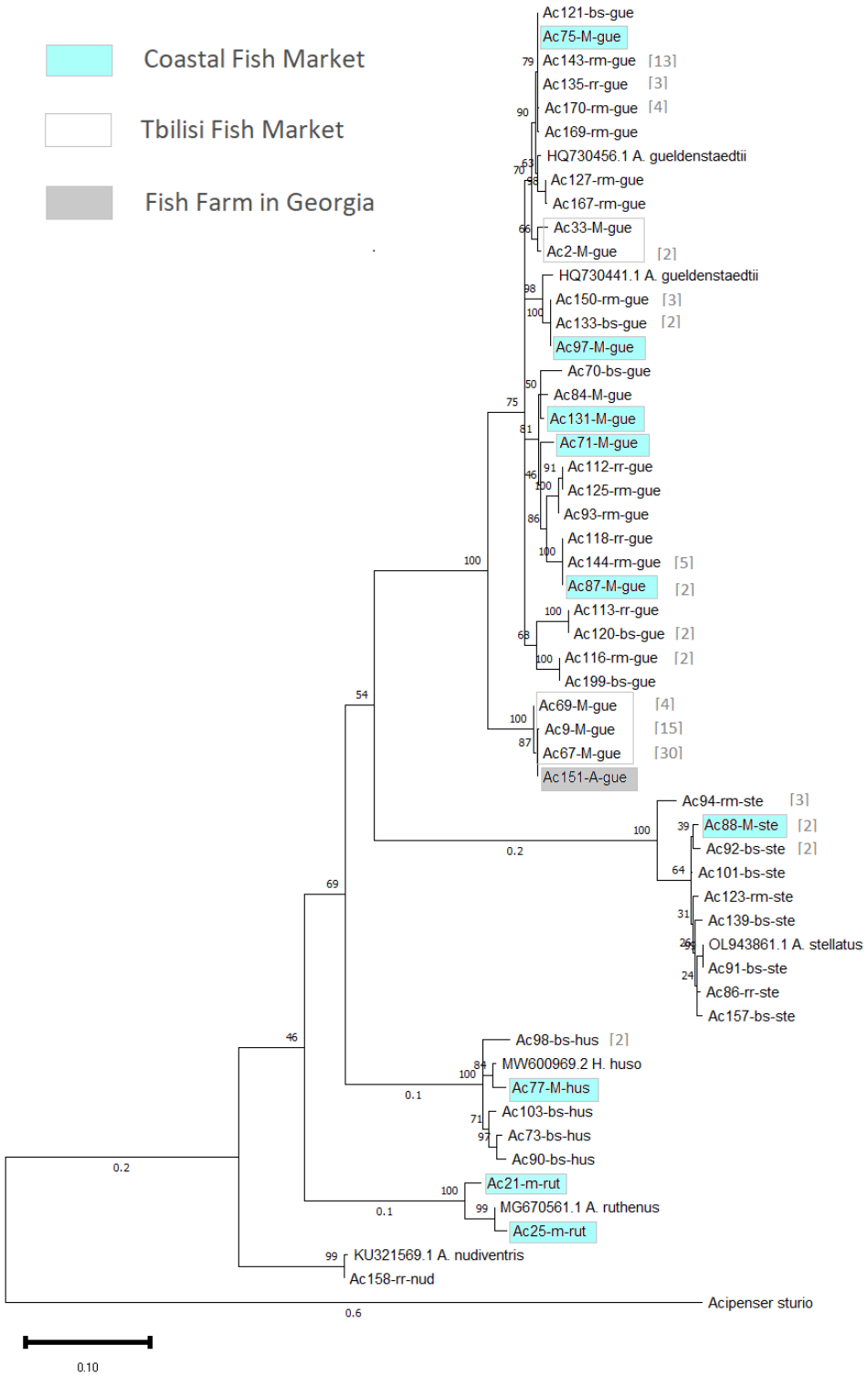

Figure 3. Phylogenetic tree of wild (with codes bs- Black Sea, rm-Rioni River mouth, rr – Rioni River) and commercial samples (with codes M-market, A- aquaculture from Tbilisi, Batumi, and other Coastal markets), and haplotypes from GenBank. Trees were constructed using the maximum likelihood method with the Tamura 3-parameter model with rates of change between sites following a gamma distribution (G) and 100 bootstrapped replicates per tree. Species codes: gue-Russian sturgeon; ste-stellate sturgeon; nud-ship sturgeon; rut-sterlet; hus- beluga sturgeon. Numbers above the

branches show boot strap support, numbers below indicate branch lengths. The haplotypes with no numbers are single haplotypes in analyzed samples.

3.2. Microsatellite analysis

The first level of the hierarchical STRUCTURE analysis supported K=3 groups, which separated wild-caught and commercial Russian sturgeon from the other four species in our analysis. A separate STRUCTURE analysis on only the Russian sturgeon generally differentiated wild from commercial individuals. However, this analysis also suggested that several individuals from the markets may have originated from a wild source (Figure 4). In particular, the coastal markets in western Georgia which included markets in Batumi, Tskhaltsminda, and Poti had 11 of 74 individuals assign to the wild-caught cluster with a q-score of at least 0.25, highlighting the potential for a large proportion of commercial Russian sturgeon in those markets to either be of direct wild descent or be a first- or second-generation hybrid with a captive individual. Additional STRUCTURE analyses on the remaining four species were able to differentiate ship and beluga sturgeons, but could not discriminate between stellate sturgeon and sterlet. In addition, the analysis could not distinguish between wild and market individuals of these species (Figure 4). Notably, individuals from the market that clustered with the wild-caught collection also grouped to wild haplotypes on the phylogenetic tree.

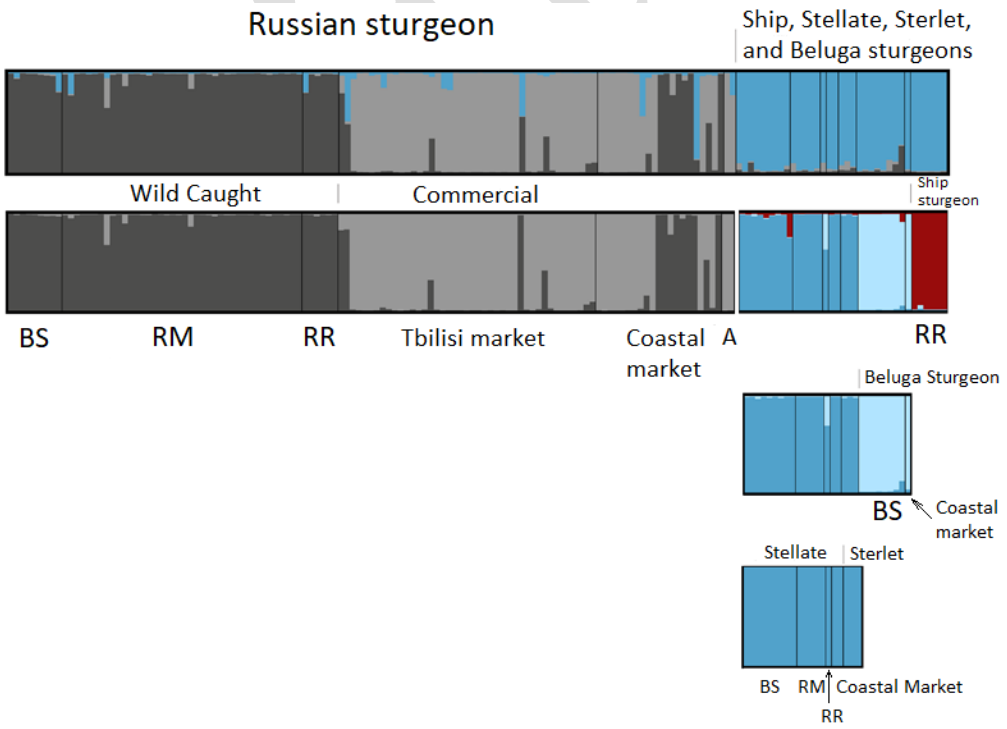

Figure 4. Results of hierarchical STRUCTURE analysis of wild-caught (BS- Black Sea, RM- Rioni River Mouth, RR- Rioni River) and commercial Russian, ship, stellate, sterlet, and beluga sturgeons.

4. Discussion

Identifying the species and provenance of sturgeons in fish farms and markets can be challenging due to species' similar morphologies and the mixed ancestry of many captive populations [38,39]. Through molecular analyses, we identified the species and origin of sturgeons encountered in farmed, market, and wild environments in Georgia. All three environments had similar species composition, including Russian, stellate, and beluga sturgeon, However, we only detected sterlet sturgeon in fish markets and ship sturgeon

were found only in the Rioni River. Both mitochondrial and microsatellite analyses suggested that wild-caught Russian sturgeon may either be directly sold in coastal markets or used as broodstock to support captive culture. Taken together, these results suggest commercial aquaculture may present a cryptic, yet significant threat to the conservation of wild sturgeon populations in Georgia. If allowed to continue, on-going harvest of wild populations has the potential to contribute to demographic declines and reduce conservation efficacy.

Due to the largely unregulated nature of Georgian public markets combined with the sale of largely unidentifiable sturgeon flesh, it can be difficult to accurately identify the species and source of sturgeons available in market environments. Processed sturgeon parts generally lack distinguishing morphological characteristics [10,40], making it nearly impossible to use morphometrics or meristics for species identification. While genetic assays could aid in species recognition, the high complexity of sturgeon genomes has generally limited broad application of genetic tools for monitoring of sturgeon trade. Recent studies have increased the number and types of genetic markers that can be used for species and hybrid identification, and today nearly all sturgeon species can be identified with high certainty [13,15,38,41,42]. However, several species remain problematic, including Russian and Siberian sturgeons. These two species can be differentiated from other sturgeon species, but it can be difficult to distinguish between the two species or detect hybrid individuals [42, 43]. This can present a challenge for monitoring commercial trade, as both species are regularly used in commercial propagation, and Russian x Siberian sturgeon hybrids are commonly reared in aquaculture [5]. Moreover, sturgeons are characterized with high genetic plasticity, with ability to hybridize with other sturgeon species and produce sterile and fertile offspring [15,44,45]. This high rate of hybridization can reduce the reliability of morphology for species identification, and ultimately highlights the utility of more complex methods such genetic tools or isotope analyses for monitoring sturgeon trade [38].

Based on maternal lineage analysis of commercial specimens, market samples in our analyses were predominantly Russian sturgeon. This is not surprising, as Russian sturgeon are of high commercial value relative to many other sturgeon species. [5,7,38]. Moreover, our phylogenetic tree and network analysis show that most of the market samples that maternally identified as Russian sturgeon grouped into three haplotypes (Ac67, Ac69, and Ac9), which are grouped separately from wild-caught Russian sturgeon that were sampled from the Rioni River, Rioni River mouth, and the Black Sea. Overall, this suggests that most Russian sturgeon that we sampled from markets did appear to be of captive origin. However, several coastal market samples (Ac71, Ac75, Ac84, Ac87, Ac97, Ac131) grouped with wild-caught specimen haplotypes, which suggest that wild Russian sturgeon may also occasionally be sold in markets. This finding was supported by microsatellite genetic analysis, which revealed clear differentiation between wild and commercial Russian sturgeon and highlighted the presence of some individuals in market samples that clustered most strongly with the wild-caught individuals.

Although the source of wild-caught individuals in the market is unknown, reports of sturgeon poaching in the Rioni River are not uncommon and sturgeon poaching equipment has been found and confiscated along the river [11,45]. Given that some individuals in STRUCTURE analyses has intermediate q-scores, it is also possible that wild-caught Russian sturgeon are being used as brood stock for fish farms, as has been reported for stellate sturgeon in western Georgia [20]. However, the lack of significant admixture and strong differentiation between wild and captive populations lends limited support for ongoing, large-scale use of wild individuals in commercial propagation.

In addition to Russian sturgeon, we also detected three additional species in Georgian fish markets including stellate (n=2), sterlet (n=3), beluga (n=1) sturgeons. Due to limited power from low sample sizes and limited microsatellite markers, our analyses could not distinguish between wild and market individuals from these species. Nonetheless,

their presence in market samples is still of interest. For example, while sterlet is not native to Georgia, it is frequently reared on commercial farms [5,42] and so it was not surprising to find this species in commercial settings. Converscly, critically endangered populations of stellate and beluga sturgeon are native to Georgia and the eastern Black Sea; however, their origin in market environments is presently unclear. Recent surveys of Georgian sturgeon farms suggested that none of the nearby commercial facilities rear these two species, and only a single farm had a one stellate sturgeon that was originally captured in the Black Sea as bycatch [20]. Thus, further investigation of the source of these species that were being sold in commercial settings appears warranted.

Although captive sturgeon propagation has been promoted as a means to reduce pressure on wild populations, our findings suggest that without careful monitoring and enforcement, captive sturgeon propagation could contribute to further erosion of critically endangered populations. This may be of particular concern for Russian sturgeon, which appears to be numerically dominant in the commercial settings that we surveyed. Our results suggest that wild and commercial populations may not be fully isolated, and release or escape of non-native Russian sturgeon in the Rioni River systems likely present a direct threat for the native Russian sturgeon population in Georgia. Admixture of native and non-native species or lineages could cause erosion of native diversity and result in the loss of adaptive genetic diversity necessary for the survival of native species [16]. In addition, admixture between wild and captive Russian sturgeon populations could make it more difficult to apply genetics tools to identify illegal trade of sturgeon and sturgeon products [38].

Genetic characterization and population genetic studies of extant wild sturgeon populations and commercial stocks is essential for future monitoring of natural populations and commercial markets. For example, studying origin of commercial sturgeon present in the Georgian markets. On the other hand, studies of local, natural population structure may help identify genetically distinct populations and determine the spatial scale in which conservation actions should be applied [24]. On-going monitoring efforts may also be important for early detection of non-native species introductions and the expansion of inter- and intra-specific hybridization in wild and captive populations.

Future analysis and monitoring efforts may benefit from the development and use of more advanced genetic and genomic tools. Inferences in this study were made from a modest number of microsatellite loci, which was necessary as genetic markers remain underdeveloped for many sturgeon species. However, the high ploidy levels can make analyses possible with relatively few loci. For example, Russian sturgeon are a polyploid species [46,47] and, because polyploid species have more alleles per locus and a higher mutation rate than diploid species, differences between populations may accumulate more rapidly and enable analyses with relatively few loci [48]. However, future studies could explore the use of stable isotope analysis, which may be a useful and reliable tool for identifying individual natal origin (e.g., farmed/wild; [38]). For example, using stable isotope analyses, Avigliano et al., 2023 [49] were able to determine the source of introduced Siberian and Russian sturgeon in South American waterways. However, stable isotope analyses may still require the use of genetic methods to avoid species mis-identification. For example, using stable isotopes, a captive American paddlefish (*Polyodon spathula*) in Ukraine that was fed a diet of wild forage and was later assigned as native individual [38]. Combining geochemical and genetic analyses for source stock identification may be particularly important when determining natal origins of Georgian Russian sturgeon, as it is the main species available in markets and the widely caught sturgeon in Georgia.

Our study highlights the utility of molecular tools for assessing the species and provenance of sturgeon in wild, aquaculture, and market environments, and documents the potential, continued threat that commercial propagation may have on conservation of native sturgeons in Georgia. Our work also underscores the challenges of trying to identify the origin of critically imperiled sturgeon species, where very low sample sizes offer

limited statistical power to resolve potential differences. Collectively, these findings highlight the importance of developing genetic baselines for wild and farm-reared sturgeons in order to enable future assessments of the provenance of sturgeons. Future studies may be warranted to better understand wild sturgeon genetic diversity in the Rioni River and the eastern Black Sea. For example, genetic characterization of wild and commercial sturgeon populations could help understand the natural population genetic diversity and differentiation and ultimately improve the ability to monitor fish markets for illegal harvest and trade.

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Conservation news

Ship sturgeon rediscovered in the Rioni River in Georgia

Preliminary findings indicate that the ship sturgeon *Acipenser nudiventris,* long thought to have been extirpated from the Black Sea basin, in fact survives, and is still spawning in Georgia. The ship sturgeon was historically found in the Black, Azov, Caspian and Aral Sea basins. Overfishing, destruction of spawning grounds, and habitat degradation combined to cause a catastrophic decline of all sturgeon populations worldwide (Ludwig, 2006, *European Journal of Wildlife Research*, 52, 3–8). The ship sturgeon was no exception; its population has decreased so dramatically that it has been considered extinct in the Black Sea basin, and Azov and Aral Seas, and dramatically reduced in the Caspian Sea (Mugue et al., 2016, *Mitochondrial DNA Part B*, 1, 195–197). It is categorized as Critically Endangered on the IUCN Red List.

After decades without confirmed evidence of ship sturgeon in the Rioni River, Fauna & Flora International collected photographic evidence and genetic samples from eight ship sturgeons in the Rioni River in 2020. Taking into account the biology of the fish, and the apparent maturity of these eight individuals (20–75 cm in length) the species appears to survive in the Rioni River. Initially, we suspected these individuals were releases from an ongoing captive breeding programme in the Kuban River in Krasnodar. In this breeding programme, ship sturgeons bred from Caspian Sea stocks are hatched and released into the Kuban River (N. Mugue, pers. comm., 2020). We therefore presumed the individuals from the Rioni River were most likely captive-bred individuals that had dispersed to the Rioni River after their release into the Kuban River c. 950 km distant. However, mitochondrial DNA sequence data indicates that the Rioni specimens are genetically different from the Kuban River breeding stocks. This, in turn, suggests that the Rioni River individuals are in fact from a surviving breeding population that spawns in the Rioni River, and that the species, once thought to be extinct in the Black Sea basin, has persisted. It is therefore likely that the Rioni River still hosts native stock of the ship sturgeon.

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Status of *Cassine koordersii*, a tree endemic to East Java and last collected in 1898

Cassine koordersii (Celastraceae) is an endemic tree known only from the Puger area in Jember Regency, East Java, Indonesia. In 1998, the tree was categorized as Critically Endangered on the IUCN Red List because of its small geographical range (WCMC, 1998, dx.doi.org/10.2305/IUCN.UK.1998.RLTS.T37405A10050197.en). The tree is known from herbarium collections made in 1898 by Koorders from Watangan Mountains in Puger (Kostermans, 1986, *Gardens' Bulletin Singapore*, 39, 188–189). Since 1898 there have been no additional records of this tree. It is currently known only from two ex situ living collections in Bogor Botanic Gardens, which were propagated from seeds of a former mature tree that died in 2003.

To gather data for an updated conservation assessment of *C. koordersii* we conducted a survey in August–September 2020 in the Watangan Mountains. A total of seven localities were surveyed: from the western extent of the mountains at Puger Watangan Nature Reserve, through the central areas of Igir Pletes, Watu Susu, Maelang, Klatakan and Papuma, to the eastern mountains at Tanggul Asri, over an elevation range of 0–391 m. We were, however, unable to locate *C. koordersii*. We observed many charcoal production sites in the areas surveyed, and we believe this, together with timber extraction, is the most likely cause of our failure to relocate *C. koordersii*. In addition, the forest lies on periodically dry soil of weathered coral limestone, susceptible to frequent wildfires that could reduce the survival of *C. koordersii*.

Based on our findings, we have reassessed *C. koordersii* as Critically Endangered based on criteria A2c, B1ab(iii)+B2ab(iii) (Possibly Extinct in the Wild) using IUCN Categories and Criteria version 3.1. The species remains assessed under criterion B, as at present, with an area of occupancy and extent of occurrence of 8 km² and continuing decline in the area and quality of the habitat, but for the updated assessment criterion A is also used. Given the threats to the species, which have caused a decline in area of occupancy, extent of occurrence and/or habitat quality, the population size is likely to have decreased by at least 80% in the last three generations. This is inclusive of the original year in which the species was collected. The forest of Watangan Mountains continues to be affected by timber extraction and wildfires, and our updated assessment is an urgent call for the conservation of this endemic species.

Ex situ conservation is in progress for *C. koordersii*. There have been several attempts to propagate the species from the two living collections, including grafting and shoot cutting. Grafting has been successful, with three of four individuals surviving after 6 months. For shoot

Conservation news

Next generation of global conservation leaders awarded funding and support

The Conservation Leadership Programme (CLP)—an initiative of Fauna & Flora, BirdLife International and the Wildlife Conservation Society—has announced its 2023 award winners. In total, 17 groups of young conservationists have been granted vital funding, and will also be provided with invaluable training and skills development, to strengthen their species-saving projects. This year's award winners are based across the globe—from Honduras to Ghana to Indonesia—and focus on a broad range of species, including the tuco-tuco, a burrowing rodent in Argentina, the Javan slow loris and Sharpe's longclaw, a bird native to Kenyan grasslands.

CLP trains and supports the next generation of conservationists. The programme invests in teams of people at the beginning of their career who are working to protect threatened species in low- and middle-income countries.

Through its 2023 award programme, which is funded by Arcadia, a charitable fund of Lisbet Rausing and Peter Baldwin, and the March Conservation Fund, CLP will provide funding, worth up to a total of USD 280,000, alongside training and support to the 17 projects: six in Africa, five in Asia Pacific and six in Latin America and the Caribbean.

See the full list of projects at conservationleadershipprogramme.org/news/2023-team-awards-announced-latest-conservation-projects.

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Russian sturgeon in the eastern Black Sea basin, Georgia

All five species of sturgeon in Georgia, including the Russian sturgeon *Acipenser gueldenstaedtii*, are categorized as Critically Endangered on the IUCN Red List. The latest study carried out by Fauna & Flora and Ilia State University shows that the Rioni River is still the only remaining sturgeon spawning river in the eastern Black Sea. From a total of 117 Russian sturgeon tissue samples (taken from individuals subsequently released into the river from where they were captured) collected from the Black Sea and the Rioni River during August 2018–June 2022, we detected juveniles only in the Rioni River and the mouth of the Black Sea, underlining the importance of the Rioni River as a spawning ground. We captured only 13 adults, all in the Black Sea.

Our findings also provided further evidence of hybridization of the Russian sturgeon and stellate sturgeon *Acipenser stellatus* in the Rioni River (Beridze et al., 2022, *Conservation Genetics*, 23, 211–216). Of the 117 samples, six were identified as hybrids (which produce infertile offspring). In all cases, stellate sturgeon males had mated with Russian sturgeon females, suggesting the stellate sturgeon may be encountering difficulty finding individuals of its own species for mating. Additionally, we found three invasive Siberian sturgeon *Acipenser baerii* (known to be farmed in the region) in the Rioni River. They could further hybridize with and outcompete native sturgeon species. There was an almost 1:1 sex ratio in our 117 Russian sturgeon samples (60 females, 57 males), which is common in juvenile populations but not adult populations (Fortin et al., 1993, *Canadian Journal of Zoology*, 71, 638–650) and suggests individuals may not be surviving to sexual maturity.

Threatened sturgeon species in the eastern Black Sea are facing critical challenges. Hybridization is a clear threat, not only to the Russian and stellate sturgeons but also to the ship sturgeon *Acipenser nudiventris*, which shares the same spawning habitat. Although recruitment is occurring in the Rioni River, individuals may not be surviving to maturity. Given the fact that sturgeons only reach maturity at 7–9 years old, they are extremely vulnerable to extinction. Understanding the structure and status of sturgeon populations in this region will help to target conservation measures to protect the Black Sea ecosystem and some of the evolutionarily oldest living fish species.

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First global summit on human–wildlife conflict and coexistence

The International Conference on Human–Wildlife Conflict and Coexistence took place from 30 March to 1 April 2023 in Oxford, UK. It was organized by the IUCN Species Survival Commission (SSC) Human–Wildlife Conflict & Coexistence Specialist Group (hwctf.org) and co-hosted