

Article

Genetic Assessment of Remnant Sub-Populations of Sterlet (*Acipenser ruthenus* Linnaeus, 1758) in the Upper Danube

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Abstract: While the potamodromous sterlet was common in the past throughout the Upper Danube in Germany and Austria, it nearly vanished in the second half of the 20th century. Until recently, only one small and isolated reproductive sub-population is known from the German–Austrian border. However, isolated remnants in another section downstream of Vienna, near the Austrian–Slovakian border, were discovered in 2014. An assessment of the population size is one of the most important prerequisites for conservation management. This study aims to assess the population sizes at both sites, using genetic pedigrees and comparison to mark–recapture data. A total of 193 samples collected from these populations between 2011 and 2021 have been investigated. In addition, 59 samples from captive stocks, 38 wild fish from downstream, and 247 genetic profiles from previous studies were used for comparison. Results show close relationships and intermittent reproduction on one site. Estimated populations based upon genetic pedigree are very small, and are consistent with mark–recapture results. Small population sizes of remnant populations have only limited, sporadic reproduction, as well as continual losses to outmigration support conservation actions for sturgeons in the Upper Danube, including the restoration of functional migration corridors.



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1. Introduction

Sturgeons are the world's most threatened group of species, as most populations have collapsed in the last few centuries due to anthropogenic impacts, such as overfishing, the construction of migration barriers, and habitat degradation [1]. Six sturgeon species once thrived in the Danube, which can be separated into three parts along its course: the Lower Danube (LD), from its mouth into the Black Sea to the Iron Gate Gorge between Serbia and Romania, the subsequent Middle Danube (MD), until the Austrian–Slovakian border, and the Upper Danube (UD), between the border and its source in Germany [2]. In the past, the sturgeons' range extended until Ulm, in the UD. Presently, more than 30 barriers in the historic range deny the unrestricted use of the Danube by sturgeons [3]. Hydropower plants (HPP) constructed at the Iron Gate in 1969 and 1984 made the Upper and Middle Danube inaccessible for anadromous species [4].

At present, the potamodromous sterlet (*Acipenser ruthenus* Linnaeus, 1758) is the only species still occurring in small numbers in the UD [5]. The species has patterns of a pan-mictic population in the Danube [6], but the fractured range with the barriers being mostly impassable for the species may lead to genetic separation of sub-populations, as gene flow is restricted to downstream drift and outmigration of juveniles and adults. The remnant sub-population in the UD within Austria is estimated to be less than 1000 reproductive adults [7]. While the sterlet is classified as “endangered,” with a decreasing trend on the IUCN Red List [8], it is classified as “critically endangered” by the Austrian Red List [7]. Currently, only one small reproductive sub-population below the HPP Jochenstein at the

German–Austrian border is known [9], with sporadic captures of 1+ and 2+ proving reproduction on a small scale. However, the size of the reproductive cohort remains unknown.

The last records of juveniles and, hence, the last evidence of natural reproduction further east, in the area of Vienna, date back to 1986, before the construction of the HPP Freudenuau [3,9]. A remnant sub-population consisting of large adults (Figure 1) was encountered during monitoring with trammel nets downstream of the HPP Freudenuau in 2014. Subsequent monitoring through 2021 provided mark–recapture data to assess the size of the local sub-population [10].



Figure 1. Large female *A. ruthenus* of the Freudenuau remnants (TL 860 mm, weight 4900 gr).

The main objective of this study was to increase our understanding of the size and structure of the panmictic sterlet population in the Danube. In addition, we attempted to characterize the status of inter- and intra-specific hybridization within populations discovered earlier [11,12], as nine other sturgeon species are farmed in the catchment, with *A. baerii* and *A. gueldenstaedtii* being the most common. The study provides the first population estimate based on genetic data of two remnant sub-populations in the Upper Danube, and provides a direct comparison of genetic population estimates with a mark–recapture assessment of the Freudenuau sub-population [10].

2. Materials and Methods

The study sites comprise the Upper Danube sections between the hydropower plants Jochenstein and Aschach (hence called Jochenstein) on the German–Austrian border, and the free-flowing section downstream of Vienna to the Slovakian border (hence called Freudenuau). Sampling of the fish took place directly below the power plants at the upstream end of the sections (Figure 2). The study site Jochenstein is situated below the HPP Jochenstein, in the head of the 41-km-long impoundment Aschach. The annual mean discharge at the nearest gauging station is 1440 m³/s. The sampling site Freudenuau is located below the spill gates of the HPP Freudenuau, at the upstream end of the 48-km long-free-flowing section along the NP, with the 50-km-long impoundment Gabčíkovo following downstream of Bratislava in Slovakia. The annual mean discharge at the nearest gauging station, 20 km upstream, is 1910 m³/s.

Between 2011 and 2021, sampling of the fish was conducted using set benthic trammel nets, as described previously [10]. Genetic samples were either obtained by fin clipping with sterilized scissors or by the Tissue Sampling Unit (TSU) by Biomark, with the fish released immediately afterwards. Samples were stored in 90% ethanol within the refrigerator. The standard protocol of the DNeasy Blood and Tissue Kit (Qiagen) DNA was used for DNA extraction [11].

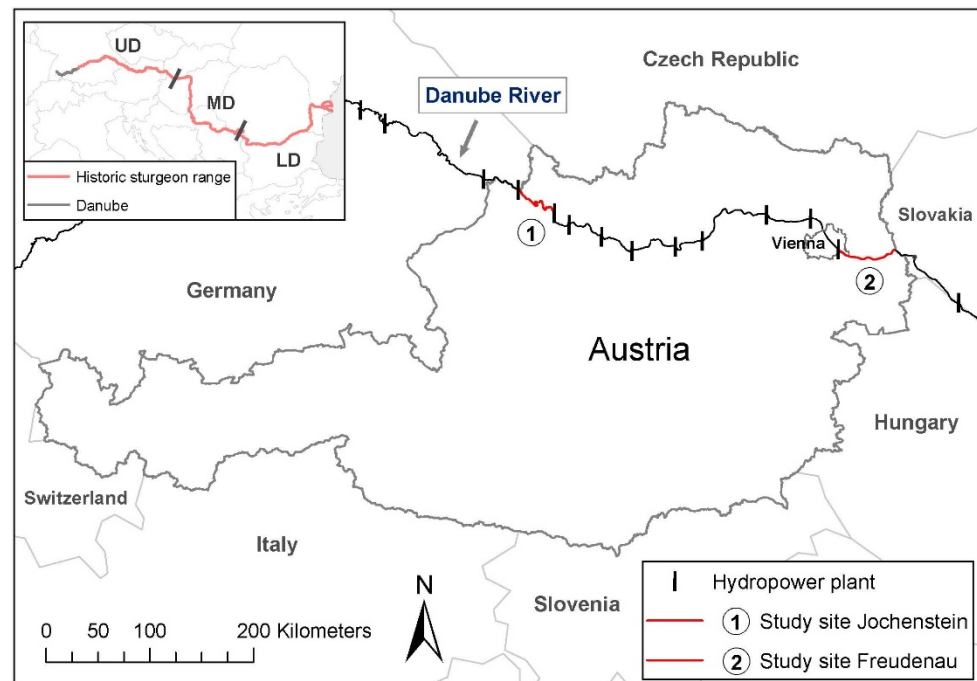


Figure 2. Location of the study sites.

In total, 152 samples of the Jochenstein sub-population and 41 samples of the Freudenau sub-population were obtained (Table 1). For comparison, 59 samples from captive stocks of several countries and 38 samples from wild fish in Slovakia, Bulgaria, and Romania were included. In addition, 247 sterlet genotypes were used from previous studies [6,11]. With step down analysis, we mostly followed the original procedures described in the corresponding papers [13,14]. However, some modifications were made for hybrid destination. PCR reactions of a hypervariable mitochondrial control region fragment were conducted with primers described in [11], under the following setup: reaction volume was 25 μ L, and the reaction mixture included 0.5 U FastStart Taq Polymerase (Roche), 50 mM Tris-HCl pH 8.3, 10 mM KCl, 5 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgCl_2 , 1.6 mg/mL BSA, 0.4 μ M of each primer, and 0.2 mM of each dNTP. PCR fragments for sequencing were cleaned with Exonuclease/FastAP (Thermo Scientific) and sequenced using the BigDye v1.1 Terminator method (Life Technologies) on an ABI 3130xl Genetic Analyzer. Microsatellite genotyping was performed with nine formerly established markers: Afu19, Afu34, Afu39, Afu68 [14], Aox23, Aox45 [13] and Spl101, Spl105, and Spl173 [15]. The Type-it Microsatellite Kit (QIAGEN) was used according to the manufacturer instructions, except the cycling protocol. We conducted the same touchdown program for all markers but Aox23 (the latter one with a temperature reduced protocol: first annealing at 58 $^{\circ}$ C and loops with 50 $^{\circ}$ C): denaturation and activation of the enzyme for 10 min at 95 $^{\circ}$ C; first annealing at 63 $^{\circ}$ C (this temperature was reduced by 2 $^{\circ}$ C for the first 5 cycles) for 1 min 30 s; elongation at 71 $^{\circ}$ C for 30 s; denaturation at 94 $^{\circ}$ C for 30 s; loop 30 cycles at 55 $^{\circ}$ C, all other conditions as above; final elongation at 60 $^{\circ}$ C for 30 min. Genotyping was carried out with fluorescence-labeled primers and the Red 500 size standard (NimaGen) on the ABI 3130xl Genetic Analyzer, using the GeneMapper v. 3.7 software (Applied Biosystems, Foster City, CA 94404, USA).

Hybrids with other species and fish with >25% Volga genotypes originating from stocking and escapes, as well as F1 hybrids between Danube and Volga specimens and F2 backcrosses (~25%), were excluded from downstream analysis. The 25% threshold was introduced in [6], considering both the intraspecific hybridization and the ancestral polymorphism of the species. The 25% threshold decreases the number of non-native genotypes and ensures the conservation of Danube fish. Assignment tests based on Q-values were conducted with STRUCTURE v. 2.3.4. [16].

Table 1. Total number of samples analyzed for both populations, with year of sampling and samples used as comparative material (light), as well as the number of individuals included in the population assessments and detected numbers of intraspecific, interspecific, and Volga genotypes. Intraspecific hybrids below the 25% threshold are also included in the population assessment.

Year	Country	Location	Number	Incl. in Population Assessment	Intraspec. Hybrids	Volga Genotype	Interspec. Hybrids
2011	AT	Jochenstein	1	1			
2012	AT	Jochenstein	1	1			
2013	AT	Jochenstein	16	11	3	1	1
2014	AT	Jochenstein	30	19	6	6	
	AT	Freudenau	3		1		
2015	AT	Jochenstein	25	16	4	6	1
	AT	Freudenau	1				
2016	AT	Jochenstein	8	5	1	2	
	HU, GE, CZ, IT	CAPTIVE	18	/	5	5	
	SK, RO	WILD	8	/			
2017	AT	Jochenstein	4	3	3		
2018	AT	Jochenstein	24	23	2		
	AT	Freudenau	9	5	3	1	
	BG	WILD	30	/	1	1	
2019	AT	Freudenau	8	6	2		
2020	AT	Jochenstein	9	8	1		
	AT	Freudenau	8	7	1		
	BG	CAPTIVE	40	/	3	2	
2021	AT	Jochenstein	14	14			
	AT	Freudenau	8	7	2		
Total samples analyzed			265	/	37	23	2
Total samples Jochenstein			132	101	20	15	2
Total samples Freudenau			37	29	9	1	
TOTAL samples Aquaculture			58	/	8	6	
TOTAL samples wild comparison			38	/	1	1	

Abbreviations: AT (Austria); BG (Bulgaria); CZ (Czech Republic); GE (Germany); HU (Hungary); IT (Italy); SK (Slovakia)

In order to assess the size of the reproductive populations in both Austrian sections, estimates for assuming both random and non-random mating were calculated using the full likelihood method in COLONY v. 2.0.6.8 [17], inferring parentage and sibship from codominant/dominant marker data [18].

3. Results

3.1. Hybrids & Non-Native Species

Sixteen samples from Jochenstein belonged to the non-native *A. baerii*, and another to *A. gueldenstaedtii*. These were excluded from further analysis. Three of the Jochenstein samples and four of the Freudenau samples did not amplify and were disregarded.

Furthermore, fifteen and sixteen samples of the Jochenstein sub-population and one and eight samples of the Freudenau sub-population were excluded from the analysis, due to their assignment to non-native Volga sterlet genotypes or F1/F2 > 25% threshold intraspecific hybrids, respectively. Two interspecific hybrids, between *A. ruthenus* and *A. baerii* from Jochenstein, were also detected and eliminated from consideration (Table 1 and Table S1). The additional analysis of aquaculture stocks from multiple farms in 2016

showed a high level of non-native genotypes (~50%), while fish from a single farm in Bulgaria are of ~90% Danube heritage (Table 1).

3.2. Population Genetic Analysis

While it cannot be ruled out that sub-populations once existed, contemporary data supports a panmictic population of sterlets through the course of the Danube, based upon the wild samples sequenced in this study and available samples from previous studies from the Danube [6,19]. Two dominant native groups of genotypes were identified in the new samples, one of them being present mostly in samples in Jochenstein before 2017, and the other dominating in Jochenstein since 2017, as well as in all samples further downstream (Figure 3; Q values are listed in Supplementary Materials).

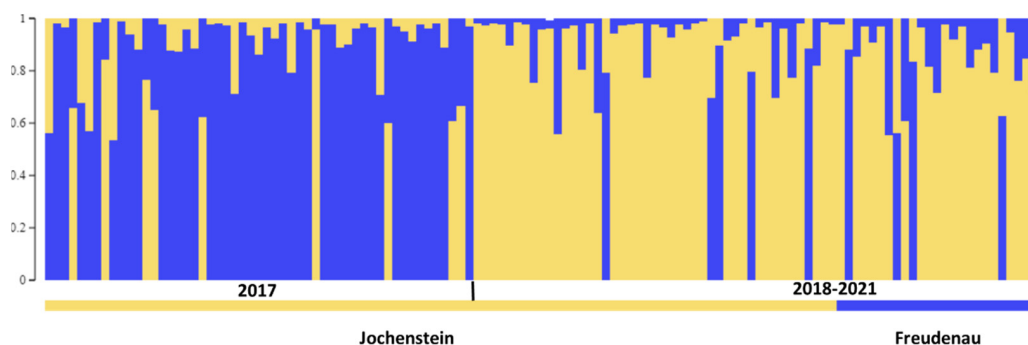


Figure 3. Assignment of 123 sterlets from Jochenstein (J) and Freudenu (F), considering only samples above the 75% threshold and calculated in Structure 2.3.4. ($K = 2$). Q values, sample numbers, and details for the samples are given in Supplementary Table S2.

3.3. Reproductive Population Sizes Jochenstein & Freudenu

Based on the genetic pedigree of the analyzed fish population, estimates are given considering inbreeding in both populations. The reproductive population size of the Jochenstein section was estimated to be 99 individuals (95% CI = 74–136) for assumed random mating and 60 individuals (95% CI = 41–85) for nonrandom mating. The assessment of the fish from the Freudenu lead to an estimate of 75 (95% CI = 46–146) for random mating and 57 (95% CI = 34–110) for non-random mating.

3.4. Relationship

Pedigree assignments with COLONY 2 showed 16 full siblings and an additional 71 closely related individuals within the 126 investigated fish of the Jochenstein sub-population. In comparison, only 3 of 35 analyzed fish of the Freudenu sub-population are closely related. Of interest is the close relationship between five Jochenstein and Freudenu specimens, with nine HPPs between the sub-populations.

4. Discussion

The results of our study underline the results by [6] that the sterlet, at present, exhibits a panmictic population in the Danube. While sub-populations may have been present in the past, genetic structures could have been blurred through stocking over the last decades. While the previous study [11] and the data from the Jochenstein sub-population show a 20–30% share of non-native sterlet genotypes and intraspecific hybrids in the Danube, this number dropped in the samples from 2017 onwards to below 10%. This development may be linked with a decrease in stocking activities with fish of unknown origin in both Bavaria (Germany) and Austria, compared to the 1990s and early 2000s. In the Freudenu sub-population, this share is approximately 20%. This may be based on stocking activities in early 2001 to 2005, with the length of the fish (TL 790–930 mm) also easily corresponding to an age of more than 10 years. The higher number of a second genotype being present before 2017 and subsequently decreasing in Jochenstein supports this hypothesis, as does

the high number of very closely related animals, especially before 2017. However, a close relationship of 1+ (TL 295–365 mm) and 2+ (TL 375–450 mm), caught in 2018 and assigned to the genotype dominant from 2018 onwards, points towards limited natural reproduction in 2016 and 2017. While non-native sturgeon species are present in the Jochenstein samples, with more than 10% in the samples, so far none have been encountered in Freudenu. Of interest are the two encountered interspecific hybrids in Jochenstein, in line with the findings in [11].

Based on mark–recapture analysis between 2018 and 2021, comprising 38 individuals, 35 of which were also included in the genetic analysis of this study, [10] estimated the population size of the Freudenu population to be 48 individuals (SE = 4.98, 95% CI = 42–63) for a closed population model [20], and the superpopulation to be 53 individuals (SE = 8.36, 95% CI = 43–80) for a POPAN open population model [21,22]. As net-sampling in the Upper Danube is mainly restricted to the tailwater of HPPs, the traditional mark–recapture approach only covers a small part of the available habitat, and may, therefore, slightly underestimate the population size. The estimates of the population size based on genetic criteria are slightly higher than in the mark–recapture models; therefore, both methods complement each other and give a very similar picture of the rather small effective size of this remnant population.

Overall, both the reproductive sub-population in Jochenstein and the remnant sub-population in Freudenu can only exhibit gene flow in a downstream direction. Available fish passage facilities along the Danube are not suitable for sturgeons, based on their autecology and backed by monitoring data, and, therefore, hamper immigration from downstream. Telemetry data for both sub-populations clearly shows outmigration of adults over downstream dams [23,24] and successive loss of individuals for the local sub-population. Similarly, drift of larval stadiums may lead to both quantitative mortalities within reservoirs [25] and passive outmigration. Combined with the low numbers of mature sterlets in both sub-populations, and sporadic reproduction in only one of them, an urgent need for the re-opening of the migration corridors for the species in the Danube, as well as habitat protection and restoration, are of the utmost importance as a prerequisite to restoring self-sustaining populations. The additional risk of escaped or deliberately introduced non-native sturgeon species and genotypes must be addressed on multiple levels. Releases of artificially reproduced progeny to support declining populations must not further decrease genetic diversity [26]. We recommend that the use of native genotypes be ensured, and that the remaining genotypes of the Upper Danube be included in captive breeding programs with conservative genetic mating schemes. These recommendations are currently being implemented for the Freudenu sub-population within the LIFE-Sterlet and LIFE-Boat 4 Sturgeons projects, which aim to support depleted populations of the remaining four Danube sturgeon species by living Genebanks, supportive releases, and the use of a standardized population monitoring scheme throughout the catchment.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d14100893/s1>, Table S1: Details of samples, Q values and mt haplotype data (+HTs from this study; DTHT01-05 are from Cvijanovic et. al. 2015 [19]). Previous samples from Reinartz et al. 2011 [6] are marked with reddish colour.

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Data Availability Statement: The data that support these findings are available from the corresponding author upon reasonable request.

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