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Genetic analysis of sterlet (*Acipenser ruthenus* L.) populations in the Middle and Lower Danube sections

Gorčin CVIJANOVIĆ[1]*, Tanja ADNAĐEVIĆ[2], Ivan JARIĆ[1,3], Mirjana LENHARDT[2] and Saša MARIĆ[4]

1. Institute for Multidisciplinary Research, University of Belgrade, Kneza Višeslava 1a, 11000 Belgrade, Serbia.
2. Institute for Biological Research ''Siniša Stanković'', University of Belgrade, 142 Blvd. Despota Stefana, 11060 Belgrade, Serbia.
3. Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, 12587 Berlin, Germany.
4. Institute for Zoology, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia.
* Corresponding author, G. Cvijanović, E-mail: mitrandir@imsi.rs, tel. +381112078477, fax. +381113055289

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Abstract. Sterlet populations have experienced a decline during the 20th century throughout its range, mainly due to poorly regulated fisheries, pollution, habitat fragmentation and habitat loss. They still represent a species of significant economic importance in the Middle and Lower Danube, so the present study was designed to investigate genetic diversity of wild sterlet populations from the Middle and Lower Danube and Lower Tisza rivers, as a prerequisite for their effective conservation and management. By applying ten microsatellite loci, we determined that pair-wise F_{ST} values were particularly low (0.018-0.038), indicating the presence of a gene flow and a low level of sub-structuring among the assessed locations. This trend can be also observed in allele sharing distance based NJ tree. Genetic variance could be attributed almost entirely to individuals, with no detectable strong population structure. Our analysis revealed considerable variation in the detection of a genetic bottleneck. Point estimate methods revealed different effective population sizes, with the lowest value being 8.1. Our study indicated the need for a careful planning of sterlet stocking programmes.

Key words: microsatellites, gene flow, genetic diversity, population differentiation, bottleneck, effective population size.

Introduction

Sterlet (*Acipenser ruthenus* L.) populations have experienced a decline during the 20th century throughout its range (rivers draining in the Black, Azov and Caspian Sea), mainly due to poorly regulated fisheries, pollution, habitat fragmentation and habitat loss (Jarić et al. 2011b). Secor et al. (2000) reported 80% loss of sturgeon spawning grounds following dam construction on the Volga River, while the Ural River experienced 50% loss with no dam construction, mainly due to sedimentation and pollution. In the Danube River basin, dam construction impacted sterlet spawning grounds through the accumulation of silt (Strelnikova 2012), and Djikanović et al. (2015) reported remarkable reduction of variability in Danube sterlet diet composition after the dam construction. However, while there are indices of the influence of dams on the Danube sterlet environment, there is a lack of data regarding their potential effect on sterlet genetic structure.

In the Middle and Lower Danube, sterlet still represents a species of significant economic importance (Lenhardt et al. 2009), and to compensate for its decline, stocking with larvae, fingerlings and juveniles was carried out by a number of countries along the river (Raikova et al. 2004, Guti 2006, Holčik et al. 2006, Smederevac-Lalić et al. 2011, Lenhardt et al. 2012). Although sterlet is distributed throughout the entire Danube River, their presence in German and Austrian river sections (Upper Danube) is dependent on continuous stocking efforts (Reinartz 2002). According to Frankham et al. (2002), supportive breeding typically has deleterious impact in the long term, and it is likely to lead to a reduction of the effective population size, reduction of reproductive fitness and inbreeding depression. Stocking with non-native specimens can also lead to a dilution and/or an irreparable loss of locally adapted alleles or allelic combinations (Ludwig et al. 2009), through increasing the number of inbred or outbred specimens. Reinartz et al. (2011) suggested that Danube sterlet recovery programs should be based on specimens from the respective river sections, since there is a tendency for a sub-structuring between sample locations. In addition, a lack of dispersal capabilities can be interpreted as a tendency for sub-structuring, because restricted gene flow represents a prerequisite for local adaptation (Kawecki & Ebert 2004). Moreover,

given that most populations (if not all) show some level of genetic structuring or genetic differentiation among localities (Harris & Taylor 2010), and since dam building influences sterlet habitat (Strelnikova 2012, Djikanović et al. 2015) through its loss and reduction, some habitat fragmentation and genetic population differentiation of sterlet in the Danube River should be expected. Consequently, investigation of genetic patterns within and between populations is considered as a prerequisite for a successful conservation. In addition, in an attempt to maximize sustainable harvest levels, the implementation of management strategies based on molecular data can have indirect benefits for population biodiversity, as the main objective of such management plans is to avoid population crashes, which in turn benefits the maintenance of population genetic diversity (Lakra et al. 2007). Furthermore, maintaining sufficient level of genetic diversity is required for populations to evolve in response to environmental changes (Reed & Frankham 2003). Genetic information is also essential for the development of supportive stocking programs, as inadequate genetic structure and diversity of a broodstock used for artificial propagation can result in inbreeding or outbreeding of the supplemented population (Ludwig et al. 2009).

One of the most important decisions for the adequate design of conservation programs is whether to manage for one or several populations. If we define a fish population as a group of individuals of the same species or subspecies that are spatially, genetically, or demographically separated from other groups (Wells & Richmond 1995), samples need to be tested for genetic differentiation. Information about the genetic structure, as the patterning of genetic diversity across multiple populations or demes within metapopulations, is usually measured by Fst, Gst, or related parameters (Wright 1961, Jost 2008). However, Waples (1998) suggested caution when applying statistical tests to determine whether differences among the samples are statistically significant, since statistical tests are not always able to indicate biologically relative differences. This is especially important when the samples are homogenous, with regard to the specimens' age (e.g. when it is consisted only of juvenile fish), due to Allendorf-Phelps effect (Waples 1998). Namely, random assortment of a panmictic fish population into subgroups, such as the one that occurs during spawning, may inflate the chances of observing apparent statistically significant intra-population differentiation (i.e. the Allendorf-Phelps effect) if only progeny of those specimens are sampled, since it will violate the assumption that the sampled individuals have been drawn randomly from the global population (Waples 1998).

The aim of the present study was to investigate genetic diversity of wild sterlet populations from the Middle and Lower Danube and Lower Tisza rivers using microsatellite data, as a prerequisite for their effective conservation and management. Moreover, given the degree of its population decline in this section of the Danube River (Lenhardt et al. 2004), we hypothesized that wild populations may have experienced a genetic bottleneck and a low effective population size.

Material and Methods

Sampling and laboratory procedures

In total, 95 samples were collected during July-November over the course of three years, 2007-2009, from two sites on the Danube River (Bačka Palanka, Serbia, N 45°13'58.89" E 19°22'20.95" and Grindu, Romania, N 45°23'42.59" E 28°16'50.35") and one site on the Tisza River (Novi Kneževac, Serbia, N 46°01'41.37" E 20°04'35.92") (Fig. 1). Individuals from Novi Kneževac and Bačka Palanka locality (n = 41 and 25, respectively) were collected with the help of professional fishermen (by drift nets), while the individuals from Grindu locality (n=29) were collected by electrofishing in cooperation with the researchers from the Danube Delta National Institute (Tulcea, Romania). All assessed individuals were immature, and based on the estimated length at first maturity in the Danube sterlet populations, they were probably less than 3 years old (Kolarević 2004, Kottelat & Freyhof 2007). Anal fin clips were taken non-lethally, and the fish were released back to the river immediately fol-

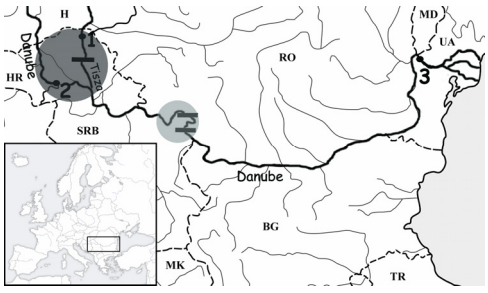

Figure 1 Sampling locations with geographic coordinates. 1 Novi Kneževac (46°01'41.37"N; 20°04'35.92"E), 2 Bačka Palanka (45°13'58.89"N; 19°22'20.95"E), 3 Grindu (45°23'42.59"N; 28°16'50.35"E). Slash marks across waterways indicate dams. Dark and light gray shading represents upper and lower fishing sections, respectively.

lowing the sampling. In order to avoid re-capturing of the same specimens, only those with intact anal fins were sampled. Tissue samples were preserved in 99% ethanol and stored at 4°C. Treatment of animals was conducted in accordance with both national and international animal welfare standards.

DNA was extracted using the standard procedure of the DNeasy Blood & Tissue Kit (QIAGEN, The Netherlands). Microsatellite analysis was conducted on all specimens (n=95). We used ten microsatellites (Afu19, 34, 39, 54, and 68 – May et al. 1997; Spl101, 105 and 173 – McQuowen et al. 2000; Aox23 and 45 – King et al. 2001) of which nine (Aox23 and 45; Spl101, 105 and 173; Afu19, 34, 39 and 68) are polymorphic loci (Reinartz et al. 2011). PCR reaction was performed in a total volume of 10µl, containing 50 mg DNA, 1xPCR reaction buffer [750 mM Tris-HCl (pH 8.8 at 25 °C), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], dNTP mix of 10 mM each, 5 pmol of amplimer and 1 U of *Taq* DNA Polymerase recombinant (Fermentas International Inc. Canada) on a 2720 Thermal Cycler (Applied Biosystems, Boston, CA, USA). Amplification was performed under the following reaction profile: one cycle at 94°C for 3 min, 40 cycles at 94°C for 30 s, 53-59°C annealing temperature for 30 s, 72°C for 30 s; and a final extension at 72°C for 10 min. PCR products were run on 6% non-denaturing polyacrylamide gel electrophoresis for 4h and finally detected using the silver staining technique. Standard molecular weight markers pBR322 DNA/BsuRI Marker, 5 (Fermentas International Inc. Canada) were used in each electrophoretic run. To ensure repeatability of profiles, some of the samples were genotyped twice for each primer combination, and replicate samples were run on separate gels. Only distinct and reproducible, well-marked amplified electrophoretic bands were included in the genetic analysis. Bands were detected and scored using Total Lab v.1.10 software (Phoretix, Newcastle, UK).

Genetic and statistical analysis

Potential presence of genotyping errors, null alleles and allelic dropouts was tested in MicroChecker (Van Oosterhout *et al.*, 2003). Allelic richness (A_r), expected (H_e) and observed (H_o) heterozygosities were calculated with GENETIX 4.04 (Belkhir et al. 1996-2004). FSTAT 2.9.3.2 (Goudet 2002) was used to calculate deviations from the Hardy-Weinberg equilibrium (HWE) and pair-wise F_{ST}, all based on 1,000 permutations. Genetic relationships between individuals were estimated as a proportion of shared alleles at each locus, i.e. allele sharing distances (D_{AS}) (Bowcock et al. 1994). A matrix of D_{AS} was used to construct NJ trees with POPULATIONS software (Langella, 1999). Molecular variance (AMOVA) was used (Arlequin v.3.5 software; Excoffier & Lischer 2010) to examine the amount of genetic variability partitioned within and among studied populations and groups in the whole dataset. Groups were defined with sampling sites Novi Kneževac and Bačka Palanka being one group (Middle Danube group) and Grindu being the other (Lower Danube group). The correlation of pairwise genetic distances and geographic distances between separate populations was evaluated using the Mantel test by the program TFPGA 1.3 (Miller 1997). All Mantel tests implemented 1000 randomizations. Bayesian clustering method implemented in STRUCTURE software v.2.3 (Pritchardt et al. 2000) was used to examine the most likely number of distinct genetic clusters (K) in the dataset. Seven runs were undertaken for each value of K from 1 to 6 and the program was run assuming a model of admixture and correlated allele frequencies. For each value of K, Monte Carlo Markov Chain (MCMC) was run with 100000 replications after a burn-in of 20000 iterations, with and without a priori 'population' information. The LOCPRIOR setting was used to detect cryptic structure by providing priors for the Bayesian assignment process based on the sample location (Hubisz et al. 2009). The LOCPRIOR model allows structure to be detected with lower levels of divergence and is not biased towards detecting structure when it is not present (Hubisz et al. 2009). The most probable number of populations (K) was determined by evaluating the likelihood of the posterior probability [Ln P(D)] and by calculating ΔK by the method described by Evanno et al. (2005), in Structure Harvester v0.6.92 software (Earl & vonHoldt 2012). In order to determine if studied populations experienced effective size reduction (i.e. population bottlenecks), we applied two approaches. We used BOTTLENECK v1.2.02 software (Cornuet & Luikart 1996) which is based on theoretical prediction that a population bottleneck generates faster reduction in allelic diversity compared to heterozygosity, and this in turn generates an excess of heterozygotes in the post-bottleneck population (McEachern et al. 2011). The data were analyzed with recommended settings (Piry et al. 1999) of stepwise mutation model (SMM) and the two-phase model (TPM), with 95% of single-step mutations and the variance mutation size set to 12. One-tailed Wilcoxon signed rank test was used to determine significance of heterozygosity excess over all loci. Additionally, we tested for a bottleneck using M ratio based on the equation of Garza & Williamson (2001), with slight modification according to Excoffier et al. (2005) as implemented in Arlequin v 3.5 (Excoffier & Lischer 2010). The M-test indicates genetic bottleneck according to the proportion of missing alleles in an allele frequency distribution (Schwartz & May 2008). NeEstimator 2.01 software (Do et al. 2014) was used to estimate contemporary effective population size (N_e) for each population and for Serbian samples combined in a single sample, using two single-sample estimation methods, a bias-corrected version of the linkage disequilibrium method (Waples & Do 2008) and an updated version of the heterozygote-excess method (Zhdanova & Pudovskin 2008).

Demographic analysis

Catch data for sterlet fisheries in Serbia were also assessed to obtain potential indices of historic fishing pressures, population abundance and genetic bottlenecks. Data on sterlet catch were acquired from the Statistical Office of the Republic of Serbia, for the period of 1969-1990, as well as from Lenhardt et al. (2004). Data prior to 1969 were excluded from the analysis as only total catch was available, without disaggregated information on an-

nual catch per species. Moreover, as catch data from the last decade of the twentieth century is characterized by a low reliability, due to the period of socio-economic and political transition in the country (Smederevac-Lalić et al. 2012), it was excluded from analysis. Catch data were obtained for the lower part of the Danube River (area of the Iron Gate I and II dams), as well as for the Vojvodina region (upper Danube and Tisza sections in Serbia). Data for sterlet catch in Vojvodina region for 1976 was omitted since the catch data were pooled with data from the other parts of Serbia.

Results

Microsatellite DNA analysis

Mean expected heterozygosity (H_e) at Novi Kneževac, Bačka Palanka and Grindu localities was 0.591, 0.630 and 0.560, respectively (Table 1). These values were below the observed heterozygosity (H_o) at Novi Kneževac (0.648) and Grindu (0.577), while the value for Bačka Palanka (0.614) was above the observed heterozygosity. Allelic richness was 4.8 (Novi Kneževac), 4.9 (Bačka Palanka) and 4.3 (Grindu). F_{IS} values showed significant deviation from HWE (P<0.001) only for Novi Kneževac population (-0.102), while Bačka Palanka and Grindu values were 0.010 and -0.032, respectively and did not deviate from HWE (P>0.05).

Table 1. Summary statistics for the genetic variation of Middle and Lower Danube sterlet (*Acipenser ruthenus*).

Sampling sites	A_r	H_0	H_e	F_{is}
Novi Kneževac	4.8	0.648	0.591	-0.102*
Bačka Palanka	4.9	0.614	0.630	0.010
Grindu	4.3	0.577	0.560	-0.032

A_r = allelic richness; H_0 = observed heterozygosity; H_e = expected heterozygosity; F_{is} = fixation index; * significantly different from 0 (P<0.001).

Population differentiation, bottlenecks and N_e

Pair-wise F_{ST} comparison showed significant differences (P<0.01) among all sampling locations (Table 2), with values being particularly low. Samples from the Danube River (Bačka Palanka and Grindu sampling sites) were most divergent (F_{ST} =0.038), followed by Novi Kneževac and Grindu (F_{ST} = 0.024), with the lowest F_{ST} (0.018) observed between Novi Knjaževac and Bačka Palanka. The D_{AS} values among all sampling sites were also small, indicating that all three populations were closely related. A NJ (neighbor joining) tree constructed from D_{AS} matrix (Fig. 2) showed no differentiation among populations.

Table 2. Paired values of F_{ST} above and D_{AS} below the diagonal for microsatellite marker data. Pairwise significance after Bonferroni-type correction ** P<0.01.

	Novi Kneževac	Bačka Palanka	Grindu
Novi Kneževac		0.018**	0.024**
Bačka Palanka	0.041		0.038**
Grindu	0.032	0.063	

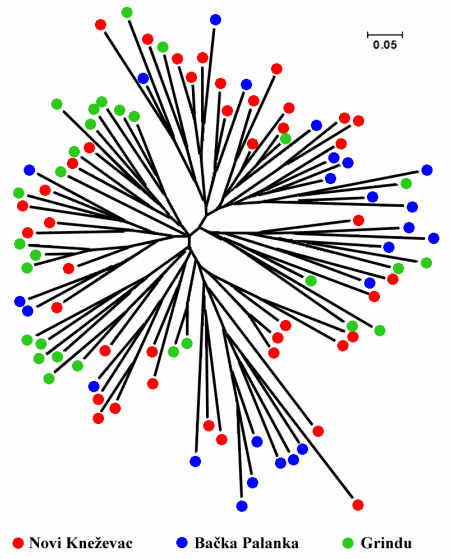

Figure 2 NJ individuals tree based on D_{AS} estimated from 10 microsatellite DNA loci.

The AMOVA indicated that the genetic variance could be attributed almost entirely to individuals, with a low inter-population variability (Table 3). The negative value for genetic variance at intra-population level implied greater differences between two randomly selected individuals from the same population than between two individuals from different populations, and could be the result of great individual variability. The Mantel test for matrix correlation between genetic and geographic distances did not show significant correlation (r = 0.5058, Z = 62.3446, upper tail P = 0.3190 and lower tail P = 0.8440). Therefore, isolation-by-distance model was rejected. The log probability values (Ln P(X/K)) for K, calculated from STRUCTURE analysis of the whole sample, showed the highest value at K=1 (-2235.84 ± 0.15) and the lowest value at K=6 (-2521.09 ± 45.03). ΔK calculation from STRUCTURE HARVESTER showed the highest value at K=4 (9.57), with other values being below 1.00. LOCPRIOR option had similar results, with 4.00 being the highest value of

Table 3. Analysis of molecular variance (AMOVA) of pooled Middle Danube populations (Novi Kneževac and Bačka Palanka sampling sites) and Lower Danube population (Grindu sampling site) of sterlet (*Acipenser ruthenus*) (d.f. - degrees of freedom; * - p<0.001).

Source of variation	d.f.	Sum of squares	Percentage of variation	Fixation indices
Among groups	1	7.068	0.59	F_{CT}=0.02033
Among populations within groups	1	5.818	2.02	F_{SC}=0.00592*
Among individuals within populations	92	225.999	-5.63	F_{IS}=-0.05780
Within individuals	95	262.000	103.02	F_{IT}=-0.03016
Total	189	500.884		

ΔK (for K=4), and other values also being below 1.00. Lack of clear genetic structure was manifest in K=4 population cluster, while K=4 population cluster with LOCIPRIOR option showed evidence of isolation of the Grindu population (Fig. 3). Additionally, every K=4 run for LOCPRIOR option had mean value for *r* (0.2501 - 0.6947).

Recent bottleneck was not detected in any of the assessed populations (SMM and TPM), P<0.05. Mode shift test also did not reveal distortion from L-shape allele frequency distribution in any of the populations. However, values for *M* ratio were 0.305 (Novi Kneževac), 0.282 (Bačka Palanka) and 0.266 (Grindu), which is below the conservative rule of M<0.68 that suggests a bottleneck effect (Garza & Williamson, 2001). Results from NeEstimator v2.01, using the linkage disequilibrium method, showed mean value 132.8 (95% confidence interval, CI 34.1-Inf.) for the Novi Kneževac sample, 8.1 (95% CI 3.2-15.9) for the Bačka Palanka sample and infinity (95% Ci 30.3-Inf.) for Grindu sample. Preforming the heterozygote-excess method, two populations (Bačka Palanka and Grindu) resulted in an infinite value, with only Novi Kneževac population diverging from such pattern, with the mean value 30.1 (95% Ci 3.5-Inf.). Infinity (Inf.) means that a finite result was not possible with the current data, and that the method could not distinguish between large values and infinity (Lecis et al. 2008).

Demographic data

Annual catch of sterlet in the investigated section of the Danube and Tisza Rivers in Serbia is presented in Fig. 4. There was a pronounced decline in catch in the lower Danube section (area of the Iron Gate I and II dams) during the period 1969-1980, while at the same time there was an increase in catch in the upper Danube and Tisza sections in Serbia.

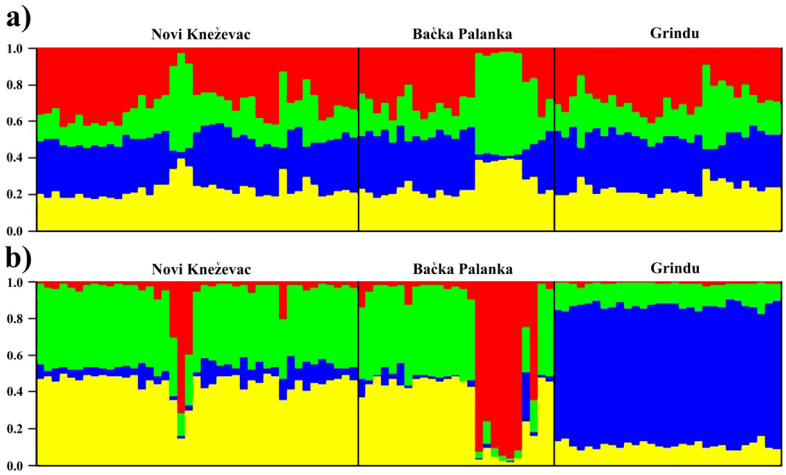

Figure 3. Results of the STRUCTURE analysis, based on 10 microsatellite loci, assuming four population clusters of individuals (K=4): a) an admixture model and b) an admixture model with LOCIPRIOR option.

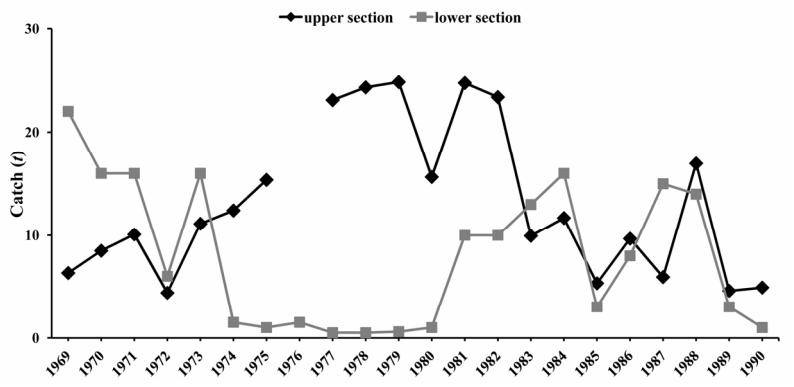

Figure 4. The total annual catch of sterlet: ◊ - upper section (including upper section of the Danube River and lower Tisza River in Serbia) and □ - lower section (lower part of the Danube River in Serbia- area of the Iron Gate I and II dams)

Discussion

Results of the current study did not indicate the presence of a panmictic sterlet population in the Danube River and its tributaries, as suggested by Reinartz et al. (2011). However, results also showed low level of structural differentiation, which may indicate that there is a lack of biologically relevant differences. Findings also may suggest a complex influence of fishing, habitat fragmentation and supportive stocking on the overall population structure.

Population structure in the Middle and Lower Danube sections

As Abbas et al. (2010) suggested, higher level of genetic population differentiation in freshwater fish is produced by a greater number of migration barriers and reduced gene flow. However, results for pair-wise F_{ST} were low (but significantly different; Table 2), which suggests higher levels of inter-population gene flow, and therefore greater dispersal capabilities. This is in concordance with the low F_{ST} values for the Danube sterlet reported by Reinartz et al. (2011). Furthermore, average F_{ST} values in the current study, as well as those reported by Reinartz et al. (2011), were both lower than the average F_{ST} value (0.222) for freshwater fish reported by Ward (2006). However, since pair-wise F_{ST} comparison showed significant differences (P<0.01) among all sampling locations, results of the current study do not support panmixia in the studied population, reported by Reinartz et al. (2011). However, statistically significant differences do not always indicate biologically relevant differences and, given enough data, statistically significant differences can be expected to occur routinely in comparisons of geographic samples, since at least some departures from complete panmixia will generally occur (Waples 1998). Lack of population differentiation can be also observed in D_{AS} distance based NJ tree (Fig. 2). Results of the current study revealed that genetic variance could be attributed almost entirely to individual variability, with a low inter-population variability (Table 3).

Gene flow is important for changing and maintaining the genetic diversity and population structure (Song et al. 2011), but it also hinders local adaptation (Kawecki & Ebert 2004). Amongst large populations that exchange few migrants, and are subjected to strong selective pressures in relatively predictable habitats, local adaptation may be expected, but the scale and extent of local adaptations may be highly variable and not easily inferred from simple measures of the gene flow (Garcia de Leanitz et al. 2007). Since sterlet is able to migrate about 200-300 km from their respective resident river stretches (Ristić 1970), such overlap of sub-populations is likely to ensure constant gene flow, which is in line with the findings of the current study. Current study revealed weak population structure. Although ΔK showed the highest value at K=4 (9.57), it is comparatively smaller than those reported for populations confirmed to have defined structures (Haanes et al. 2011, Adnađević et al. 2012, Marić et al. 2012, Taugbol et al. 2013) where the highest ΔK values are 300, 179, 839 and 130, respectively. Since the method described by Evanno et al. (2005) could not evaluate

ΔK when K=1, and given that the log probability values (Ln P(X/K)) are highest for K=1, there seems to be no real population structure among the sampling sites. Additionally, conservative approach suggested by Pritchard et al. (2000) proposed that in case of 'likelihood plateaus' one should choose the smallest number of *K*. However, when location prior information was applied, it does reveal weak evidence of Grindu population isolation. Moreover, values of *r* near or below 1 imply that the ancestry proportions differed substantially between sampling locations (Hubitsz et al. 2009), but this could be expected since all samples were juveniles, and STRUCTURE tends to overestimate the number of clusters if there is relatedness among some individuals. As discussed earlier, statistical differences regarding the population sub-structuring could also be attributed to Allendorf-Phelps effect, as suggested by Waples (1998). Additionally, as Slatkin (1993) suggested, a matrix of pairwise F_{ST} values and geographic distances between population pairs can reveal information about the importance of historical process, as well as current levels of gene flow. Results of the Mantel test failed to find an evidence of isolation by distance, which in species with relatively low F_{ST} values may indicate that it has recently colonized the study area (Waples, 1998). This can be attributed to reported massive upstream migrations (Janković et al. 1994, Guti & Gaebele 2009) from the Iron Gate region into the investigated section of the Danube and Tisza River. These upstream migrations were indirectly confirmed with available catch data for this region (Fig. 4), where sterlet catch decreased in the lower Iron Gate region, following dam construction, while catch in the upper section simultaneously increased.

Historical fishing impact

Severe decline of sterlet populations in the Danube was reported by various authors (e.g. Reinartz 2002, Lenhardt et al. 2004, Lenhardt et al. 2006, Holčik et al. 2006, Guti & Gaebele 2009, Jarić et al. 2011b, Smederevac-Lalić et al. 2011, Djikanović et al. 2015), but our results did not provide clear evidence of a bottleneck. According to Chapman et al. (2011), depleted populations tend to avoid genetic bottlenecks if the population is able to recover rapidly, gene flow counteracts genetic drift, the species is long-lived, and if the demographic bottleneck is not severe. With the reported maximum age of 26-36 years and the generation time of 7.8 years for sterlet (Froese & Pauly 2013), genetic drift may not have had enough time to erode ancestral genetic variation, since the initial population was probably sufficiently large. Potential recovery of populations in this Danube section may also be attributed to intensive stocking (e.g. Guti 2006, Guti & Gaebele 2009, Smederevac-Lalić et al. 2011, Lenhardt et al. 2012), which represent a process that may mask bottleneck effects (McEachern et al. 2011). However, lack of a bottleneck could also be attributed to the fact that the studied sample (n=25) from the upper Danube section did not mach sample size (n>31-38) proposed by Peery et al. (2012) for bottleneck analysis, but based on other genetic data (e.g. lack of inbreeding, high genetic diversity) this is highly unlikely.

According to Frankham et al. (2014), oftencited N_e = 50 rule for avoiding or minimizing inbreeding depression in short-term time-frame (i.e. 5 generations), and N_e = 500 rule proposed for maintaining evolutionary potential in perpetuity, should be at least doubled. While the studied Tisza population apparently narrowly exceeded short-term N_e, population from the upper section of the Danube River in Serbia experienced severe decline. Lowest short-term N_e (8.1) in this section can be attributed to severe commercial fishing pressure reported by Smederevac-Lalić et al. (2012). However, Waples & Do (2010) suggested that when samples are taken from the age-structured species, the resulting estimate from the LD method can be interpreted as an estimate of the effective number of breeders that produced the cohort(s) from which the sample was taken. Bearing in mind observed shift of sterlet catch towards lower length classes (Lenhardt et al. 2004), N_e from this section could be N_e of a cohort, although it still represent data of concern regarding the conservation of this species. Clearer insight would however require more data and we therefore suggest a more conservative approach and classifying this population as threatened.

Conservation implications

Stocking with larvae, fingerlings and juveniles was carried out by a number of countries along the Danube River (e.g. Raikova et al. 2004, Guti 2006, Holčik et al. 2006, Smederevac-Lalić et al. 2011, Lenhardt et al. 2012). Neff et al. (2011) stated that current breeding programs are too focused on genetic diversity and thereby fail to acknowledge the complexities of the genetic architecture of fit-

ness of wild populations, as hatchery-raised fish show reduced foraging efficiency for live prey, reduced sensitivity to predation risk and lower reproductive success in wild born specimens that had at least one hatchery-bred parent. Moreover, sterlet specimens used for the supportive stocking in the Tisza River in Hungary originated from the Danube River (Rideg, Homokmégy, pers. comm. 2011), which might explain the deviation from HWE in studied samples from the Tisza River. Low level of population differentiation between individuals from the Tisza River and the Middle Danube section, can be also attributed to specimens used for supportive stocking of Tisza River. Additionally, Reed and Frankham (2003) suggested that endangered species typically have lower levels of heterozygosity. High heterozygosity in analyzed wild sterlet from the Lower and Middle Danube section may indicate good population fitness. Moreover, as negative F_{IS} values indicate that mating between close relatives is not common (Chapman et al. 2011), our data suggested that there is no inbreeding within these populations.

According to Ward (2006), natural populations should be examined genetically both before and after release of hatchery-reared juveniles, and we strongly recommend that this should be mandatory for all supportive stocking programs. Carefull evaluation of population genetic structure is also necessary for the identification of the most suitable broodstock specimens. However, as indicated by the current study, effective conservation and management measures will have to incorporate (if available) previous demographic data or alternatively population abundance indices, such as catch time-series. Moreover, if population size fluctuates over time, reductions in population size are not expected to generate a strong reduction in N_e nor a strong bottleneck signature (McEachern et al. 2011). Therefore, regular monitoring of populations is important to differentiate between normal population size fluctuations and those severe enough to warrant conservation measures.

Our findings suggest that decisions based only on genetic data can be unreliable, and that they must include other demographic data, or the data has to span sufficient period of time to allow reliable genetic assessment. Furthermore, although dams on the Middle and Lower Danube and Lower Tisza rivers were constructed only recently (i.e. 30, 37 and 44 years ago, respectively), which was not sufficient time to produce effects in the genetic structure of sterlet populations, they created more lentic conditions that do not suit rheophilous species such as sterlet. As Waples (1998) suggested, patterns of genetic relatedness or differentiation that are consistent across a temporal dimension are unlikely to be caused by sampling artifact. Therefore, future monitoring (e.g. replicate samples over time) will be necessary to support development of appropriate conservation and management efforts.

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