UDC 575
DOI: 10.2298/GENSR1503051C
Original scientific paper

NEW DATA ON STERLET (*Acipenser ruthenus* L.) GENETIC DIVERSITY IN THE MIDDLE AND LOWER DANUBE SECTIONS, BASED ON MITOCHONDRIAL DNA ANALYSES

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Cvijanović G., T. Adnađević, M. Lenhardt, S. Marić (2015): *New data on sterlet (Acipenser ruthenus L.) genetic diversity in the middle and lower Danube sections, based on mitochondrial DNA analyses*.- Genetika, Vol 47, No. 3, 1051 -1062

Poor regulated fishery, pollution, fragmentation and loss of habitat are most important factors influencing decline of sterlet population worldwide. In Middle and Lower Danube region, this species still have significant economic importance since wilde populations are commercially exploited, while Upper Danube populations are dependent on stocking efforts in order to maintain their presence in open waters. Aim of present study is to analyze genetic diversity of sterlet populations from the Middle and Lower Danube and Lower Tisza rivers, as a prerequisite for their effective conservation and management. Analysis of a highly variable D-loop fragment of mitochondrial DNA detected five new haplotypes, while the eight previously identified haplotypes had extended their previous range. Genetic variability could be attributed almost entirely to individuals, with observed lack of population structure. Negative values of neutrality test indicate recent expansion on some sampling locations. Adittionaly, gene flow analysis between Lower and Middle Danube region showed intensive exchange of speciemens. At the same time analysis showed some influence of Tisza dam on gene flow between samples from Tisza and Middle Danube section.Our study indicated the need for a careful planning of sterlet stocking programmes and inclusion of demographic data or catch time-series.

Key words: Acipenser ruthenus; mtDNA; population differentiation; gene flow

INTRODUCTION

Despite the fact that sturgeon species evolved more than 250 million years ago and successfully survived several mass extinction events (JARIĆ *et al.*, 2011a), most of them are nowadays faced with depletion, endangerment, extirpation or extinction (e.g. BEAMESDERFER and

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FARR, 1997; WIRGINE *et al.*, 1997; LENHARDT *et al.*, 2006; JARIĆ and GESSNER, 2012). Migration of sturgeons in the upper part of Danube River was withheld due to river regulation in the Djerdap region during period between 1890 and1896 (PETROVIĆ, 1998). Although sterlet (*Acipenser ruthenus* L.) is a potamodromous resident and the smallest species among Danube sturgeons, it experienced a decline during 20th century being a less important resource regarding caviar production (LUDWIG, 2008). This was mainly due to poorly regulated fishery, pollution, habitat fragmentation and habitat loss (JARIĆ *et al.*, 2011b). Djerdap dams construction were responsible for remarkable reduction of variability in Danube sterlet diet composition (DJIKANOVIĆ *et al.*, 2015) and 50% decrease of sterlet catch (JANKOVIĆ, 1993).

Stocking with larvae, fingerlings and juveniles due to sustaining presence of sterlet in German and Austrian section of Danube River (REINARTZ, 2002) or compensation of sterlet decline in Middle and Lower Danube, is carried out by 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). However, stocking with non-native specimens carries a risk of jeopardizing their adaptation ability (LUDWIG, 2006), and it can also lead to a dilution and/or an irreparable loss of locally adapted alleles or allelic combinations (LUDWIG *et al.*, 2009). Additionally, inbreeding or outbreeding of wild populations can be outcome of inadequate genetic structure and diversity of a broodstock used for artificial propagation (LUDWIG *et al.*, 2009). With this in mind, research of inter- and intra-population genetic patterns should be prerequisite for conservation management plans. Moreover, REINARTZ *et al.* (2011) research of Danube sterlet suggested that recovery programs should be based on specimens from respective river sections.

During the last two decades, extensive molecular studies were conducted on sterlet (e.g. LUDWIG *et al.*, 2000; LUDWIG *et al.*, 2001; DE LA HERRAN *et al.*, 2001; ROBLES *et al.*, 2004; KRIEGER *et al.*, 2008; LUDWIG *et al.*, 2009). However, study by REINARTZ *et al.* (2011), with both mtDNA (D-loop) and nuclear DNA (microsatellite) based techniques, was the only study conducted so far that focused on the sterlet population genetics. Although nuclear DNA (bi-parentally inherited) techniques predominate in studies focused on population identification, mtDNA (maternally inherited) polymorphisms may also be helpful because mtDNA accumulates more substitutions over time than nuclear DNA (LUDWIG, 2008). Additionally, since mtDNA has high substitution rate and smaller effective population size of that of nuclear markers (WARD and GREWE, 1995), along with evidence of genetic stability of sterlet dominant karyotype (BIRSTEIN *et al.*, 1997), recent historical events can be traced without extensive sequencing effort. Moreover, non-coding segments like D-loop exhibit elevated levels of variation relative to coding sequences such as the cytochrome b gene (CHAUHAN and RAJIV, 2010), which makes them suitable for population analysis.

The aim of the present study was to use mtDNA polymorphisms in order to investigate genetic diversity of sterlet populations from the Middle and Lower Danube and Lower Tisza rivers. We also try to assess whether the construction of Danube and Tisza dams had some impact on sterlet migrations and gene flow. Additionally, current research should provide some recommendation for effective conservation and management of wild sterlet.

MATERIAL AND METHODS

During 2007-2009, 32 samples were collected from two sites on the Danube River (Bačka Palanka, N 45^{0}13’58.89” E 19^{0}22’20.95” and Grindu , N 45^{0}23’42.59” E 28^{0}16’50.35”) and one site on the Tisza River (Novi Kneževac, N 46^{0}01’41.37” E 20^{0}04’35.92”) (Figure 1.). Specimens

from Novi Kneževac and Bačka Palanka locality (n = 6 and 10, respectively) were collected with the help of professional fishermen (by drift nets), while the individuals from Grindu locality (n=16) were collected by electrofishing in cooperation with the researchers from the Danube Delta National Institute (Tulcea, Romania). Anal fin clips were taken non-lethally and preserved in 99% ethanol, and fish were released back to the river immediately following the sampling. Treatment of animals was conducted in accordance with both national and international animal welfare standards.

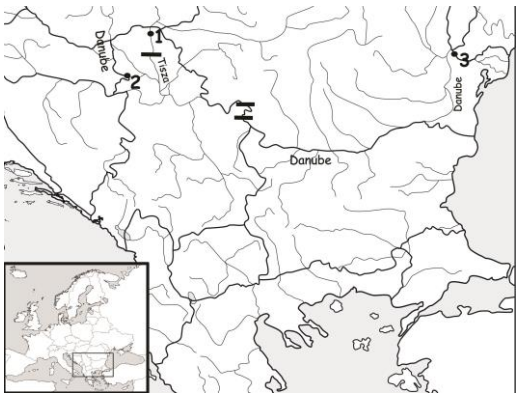

Fig. 1 Sampling locations with geographic coordinates. 1 Novi Kneževac ($46^0$01'41.37"N; $20^0$04'35.92"E), 2 Bačka Palanka ($45^0$13'58.89"N; $19^0$22'20.95"E), 3 Grindu ($45^0$23'42.59"N; $28^0$16'50.35"E). Slash marks across waterways indicate dams.

DNA was extracted using the standard procedure of the DNeasy Blood & Tissue Kit (QIAGEN, The Netherlands). Sequencing of a highly variable D-loop fragment (of 257 bp) was conducted on 32 specimens (Table 1), using primers described by REINARTZ *et al.* (2011). PCR reaction (total volume 15 µl) contained 100 ng DNA, 1xPCR reaction buffer [750 mM Tris-HCl (pH 8.8 at 25 ^{o}C), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], dNTP mix of 10 mM each, 5 pmol amplimer and 0.4 U of *Taq* DNA Polymerase recombinant (Fermentas International Inc. Canada) on a 2720 Thermal Cycler (Applied Biosystems, Boston, CA, USA). The cycle parameters were: one cycle at 94^{o}C for 5 min, 30 cycles at 94^{o}C for 30 s, 56^{o}C for 30 s, 72^{o}C for 30 s; and a final extension at 72^{o}C for 3 min. All the sequencing was performed at Macrogen Inc. (http://www.macrogen.com). Newly described haplotypes were deposited in GenBank (accession number KJ94118-KJ941192). Additional 24 sterlet haplotypes (REINARTZ *et al.*, 2011) from GenBank (accession number KF876135-38, KF876140, KF876142-47, KF876149, KF876151-54, KF876154, KF876157, KF876159-61, and KF876163-66) were included in the analysis.

The sequences were aligned with ClustalX (LARKIN et al., 2007). Overall genetic distance, based on mitochondrial sequences, was calculated in MEGA v.6 (TAMURA *et al.*, 2013), and Kimura's two-parameter gamma model was applied. DnaSp v5 (LIBRADO and ROZAS, 2009) was used to calculate the haplotype diversity (*h*), nucleotide diversity (π) and theta (θ) values based on the number of polymorphic sites. TCS v1.3 software (CLEMENT *et al.*, 2000) was used to build a haplotype network (95% statistical parsimony network) for a better illustration of genetic divergence at the intra-specific level, particularly in cases where multiple haplotypes derive from a

single ancestral sequence (RIVA ROSSI *et al.*, 2012). Hypothesis that all mutations are selective neutral (KIMURA 1983) was evaluated with TAJIMA's (1989) and FU and LI's (1993) in DnaSP v5 (LIBRADO and ROZAS, 2009). Both *D* and *F* and D** is expected to be negative if population has experienced an expansion. Arlequin v.3.5 software (EXCOFFIER and LISCHER 2010) was used to calculate *Φst* (as a measure of population differentiation) and analysis of molecular variance (AMOVA). AMOVA was used 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 from Serbia (Novi Kneževac and Bačka Palanka) being one group and Romania (Grindu) being the other. Estimation of population differentiation (*Gst*; NEI 1973) and number of migrants (*Nm*; NEI 1973) was calculated in DnaSP software (LIBRADO and ROZAS, 2009).

RESULTS

Aligned sequences of 257bp D-loop fragment obtained from 32 individuals grouped into 13 haplotypes, five of which had not been previously described (i.e. DTHT01-DTHT05). Of all the new haplotypes, one (DTHT01) was detected in the Tisza River, one (DTHT02) was detected in the Middle Danube area, while three (DTHT03-DTHT05) were detected in the Lower Danube area. Other eight haplotypes (i.e. HT01, HT03, HT08, HT10, HT12, HT26, HT29, and HT31) had been previously identified in the Danube River drainage of Slovakia, Hungary, Serbia and Romania (REINARTZ *et al.*, 2011). Two haplotypes (HT03, HT12) were present in all sampling locations, while haplotypes HT01, HT08, HT26, HT31 were observed in two locations. Haplotypes HT10 and HT29 and newly detected haplotypes (DTHT01) were detected in a single location (Table 1).

Table 1. Haplotype diversity (HD) and frequency of sampled sterlets.

Haplotype	Novi Kneževac (6)	Bačka Palanka(10)	Grindu(16)
DTH01	0.3333	0	0
DTH02	0	0.3000	0
DTH03	0	0	0.0625
DTH04	0	0	0.0625
DTH05	0	0	0.0625
HT01*	0.1667	0.2000	0
HT03*	0.1667	0.1000	0.1250
HT08*	0	0.1000	0.1250
HT10*	0.1667	0	0
HT12*	0.1667	0.1000	0.1875
HT26*	0	0.1000	0.1250
HT29*	0	0	0.0625
HT31*	0	0.1000	0.1875
HD	0.67	0.91	0.92

The overall genetic distance between described haplotypes was 0.024. Haplotype diversity (HD) was lowest at the Tisza locality (0.67), while the other two locations on the Danube River (Bačka Palanka and Grindu) had similar values of 0.91 and 0.92, respectively (Table 1). The

lowest genetic distance (one mutation, 0.39%) among Serbian samples was between the two Danube haplotypes (DTHT02-HT08), while the highest one (eight mutations, 3.32%) was determined between the Tisza and Danube haplotypes (DTHT01-DTHT04 and DTHT02-HT03).

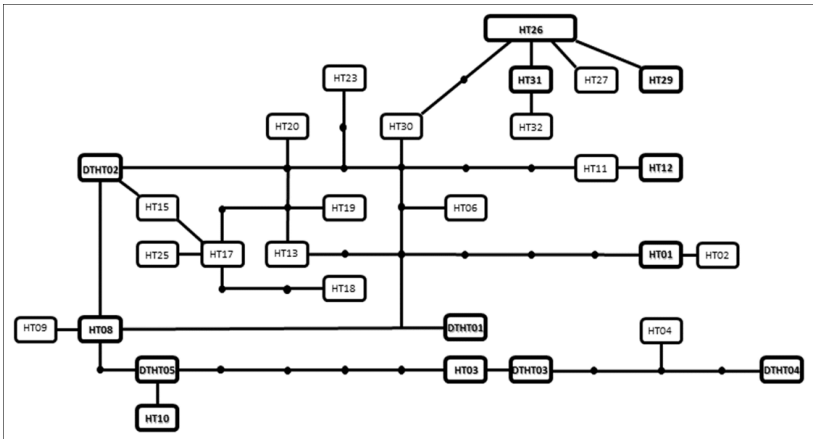

Fig. 2 mtDNA control region based haplotype network relating sterlet with previously published data (Reinartz *et al.* 2011). *Small black circles* represent missing or theorethical haplotypes; *lines*, represent single mutation events linking the haplotypes

Haplotypes from Romania with the lowest genetic distance (one mutation, 0.39%) are both Tisza and Danube haplotypes (DTHT03-HT03 and DTHT05-HT10), while haplotypes DTHT04-HT29 and DTHT04-HT31 differed in 10 mutations (4.22%) which is the highest genetic distance observed among samples. However, the highest genetic distance (11 mutations, 4.68%) was detected between Romanian and Hungarian haplotypes (DTHT04-HT02 and DTHT04-HT32). Summary statistics for sequence polymorphism is given in Table 2. The Novi Kneževac samples had highest π (0.026), while both Novi Kneževac and Grindu samples had same high h (0.933, respectively). The lowest values for π (0.022) and h (0.911) were those from Grindu and Bačka Palanka samples, respectively. Genealogical relationships among the haplotypes (Fig. 2) revealed no differentiation of haplotypes into geographically related groups. There was no significant ($p>0.10$) departure from equilibrium, as determine by TAJIMA's (1989) and FU and LI's (1993) tests. Negative values for both D and D^* and F^* were observed (Table 2.) for samples from middle Danube (Novi Kneževac and Bačka Palanka). Φst comparison showed no significant differences ($P>0.01$) among all sampling locations, with samples from the Danube River (Bačka Palanka and Grindu sampling sites) being most divergent (0.018). Tisza samples (Novi Kneževac) were not differentiated from both Danube samples (Grindu and Bačka Palanka), with Φst values of -0.025 and -0.064, respectively. Calculation of molecular variance showed that 99.94% of genetic variance is among individuals, with almost lack of inter-population variability (Table 3). The negative value for genetic variance at intra-group level implied greater differences between two

populations from the same group than between two populations from different groups, and could be the result of great individual variability. Results of NEI's *Gst* and *Nm* show low pairwise genetic differentiation and high to moderate gene flow. The highest value (*Gst*=0.01533) was between Novi Kneževac and Grindu. Genetic differences between Novi Kneževac and Bačka Palanka were similar (*Gst*=0.00974), while lowest values (*Gst*=0.00396) were between Grindu and Bačka Palanka. The highest gene flow (*Nm*=62.90) was between Grindu and Bačka Palanka. Values of gene flow between Novi Kneževac and Grindu, and Novi Kneževac and Bačka Palanka, were 16.5 and 25.43, respectively.

Table 2. Summary statistic for sequence polymorphism of sterlet at the D-loop region of mtDNA

Location of sterlet	*h*	π	θ	*D**	*F**	*D*
Novi Kneževac	0.933±0.12	0.02594±0.0047	0.02893±0.0149	-0.5777	-0.6448	-0.6487
Bačka Palanka	0.911±0.08	0.02205±0.0036	0.02476±0.0113	-0.2032	-0.3158	-0.5107
Grindu	0.933±0.04	0.02194±0.0022	0.01915±0.0082	0.6549	0.7308	0.5857

h – haplotype diversity ± SD; π – nucleotide diversity ± SD; θ – haplotype polymorphism per site ± SD; *D** and *F** – Fu and Li's (1992) statistic; *D* – Tajima's (1989) statistic

Table 3. AMOVA results for sterlet.

Source of variation	d.f.	Sum of squares	Percentage of variation
Among groups	1	3.531	5.78
Among populations within groups	1	1.646	-5.72
Within individuals	29	83.667	99.94

d.f. - degrees of Freedom; * - p<0.001

DISCUSSION

The present study provides new haplotypes for Danube sterlet. Also, it provides some evidence for anthropogenic influence on population genetics of this species.

With BIRSTEIN *et al.* (2009) reporting genetic distance based on control region of mtDNA between *A. gueldenstaedtii* and *A. baerii* of 6.3-7.9%, and intraspecific distance not exceeding 3%, intraspecific distance of Danube starlet (4.68%) suggest that this species does have long evolutionary history. However, since segment of control region used in this study was 257bp, compared to 643bp reported by BIRSTEIN *et al.* (2009), we suggest additional research in order to clarify this evolutionary event.

Since sterlets are able to migrate about 200-300 km from their respective resident river stretches (RISTIĆ, 1970), overlap of sub-populations is likely to ensure constant gene flow and panmictic population. The findings of the current study, as well as findings of REINARTZ *et al.* (2011), suggest that Danube sterlet should be regarded as single population. In addition, some haplotypes (HT01, HT08, HT10, HT29) that have been previously detected in Slovakia and Hungary (REINARTZ *et al.*, 2011), are now detected in Serbian and Romanian part of the Danube River, as well as in the Tisza River. Also, haplotypes from Serbian part of the Danube River (HT03, HT12, HT31) have been detected in Grindu locality (Romania). Interestingly, specimens from Novi Kneževac (on Tisza River, Serbia) and Radvan (on Danube River, Slovakia; REINARTZ *et al.*, 2011) locations are the only two groups sharing the same haplotype (HT10) despite being almost 700 km apart. Although this could be an ancestral haplotype, or a result of supportive stocking programs in Slovakia and Hungary (HOLČIK *et al.*, 2006; GUTI, 2006), HT10 is still not detected in Hungarian, Serbian and Romanian sections of the Danube River. Additionally, haplotype detected previously only in Slovakia and Hungary (HT29; REINARTZ *et al.*, 2011), which was detected in the current study over 1500 km downstream in Romania, could be an ancestral haplotype, since there are no reports of supportive stocking between these Danube sections. Further evidence for panmictic population is a very low overall genetic distance. Greater genetic diversity detected in Danube populations compared to Tisza population could be the result of the higher ancestral genetic diversity or greater stocking effort on the Danube River. By the same token is the finding of both the lowest and the highest genetic distances detected among Danube haplotypes, as a result of greater genetic diversity when compared to Tisza haplotypes. Evidence for panmictic population could also be found in genetic variance among individuals, with almost lack of inter-population variability (Table 3).

With dams on the Middle and Lower Danube and Lower Tisza rivers being recent (30-, 37- and 44-years old, respectively) and with male and female sterlet reproducing for the first time at 3-5 and 5-8 years respectively (KOTTELAT and FREYHOF, 2007), genetic drift may not have had enough time to erode ancestral genetic variation, or to influence lack of population differentiation. However, different authors (JANKOVIĆ *et al.*, 1994; HENSEL and HOLČIK, 1997; GUTI and GAEBELE, 2009) reported upstream migrations of sterlet in Danube after construction of dams on Danube and Tisza Rivers, which could influence local subpopulations genetics. This could be reason for negative values for both *D* and *D** and *F** at Middle Danube sampling site, since it represent recent expansion of population. Also, with HENSEL and HOLČIK (1997) stating that upstream migration to spawning ground on Tisza River were halted by dams and lack of migration in the Slovak-Hungarian stretch, recent expansion (determine by negative values of *D*, *D** and *F**) of this sampling site were probably due to stocking programs. Low level of population differentiation between individuals from TiszaRiver and Middle Danube section for mtDNA data, can also be attributed to specimens used for supportive stocking of Tisza River, since sterlet speciemens used for the supportive stocking in Tisza River (in Hungary) originate from the Danube River (Hungarian section; ÁERÁD RIDEG, *pers. comm.*).

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 and EBERT, 2004). The findings of current study show intensive gene flow between Lower and Middle Danube sections, despite existence of dams. However, gene flow between Tisza samples and both Lower and Middle Danube samples had lower values. While lower gene flow between Tisza and Lower Danube samples can be attributed to distance and difference in river flow, gene flow between

Tisza and Middle Danube samples is due to dam on Tisza River. However, as GARCIA DE LEANITZ *et al.* (2007) suggest, amongst large populations that exchange few migrants local adaptation may be expected, but its scale and extent may be highly variable and not easily determine by measuring of gene flow. With number of countries along the Danube Rover implement stocking with larvae, fingerlings and juveniles (e.g. RAIKOVA *et al.*, 2004; GUTI, 2006; HOLČIK *et al.*, 2006; SMEDEREVAC-LALIĆ *et al.*, 2011; LENHARDT *et al.*, 2012), identification of most suitable broodstock specimens for future stocking programs should be carefully conducted. Nevertheless, in line with the GARCIA DE LEANITZ *et al.* (2007) suggestion that the implications of ignoring the existence of locally adapted populations when they in fact do exist are much worse than the risk of managing for local adaptations when there are none, we fully support recommendation by REINARTZ *et al.* (2011) that supportive stocking programs should be based on specimens from the respective sections. In addition, as NEFF *et al.* (2011) suggest, current breeding programs are too focused on genetic diversity and thereby fail to acknowledge the complexities of the genetic architecture of fitness of wild populations, so the research prior to stocking programs should be carefully conducted. WARD (2006) suggest that 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.

Overall, our findings suggest that set up for conservation programs should incorporate as much information as can be collected, from brother specter of genetic markers (e.g. microsatellite loci, mtDNA, AFLP), up to available demographic data or abundance indices. Additionally, we imply that dams should be taken in consideration as disrupting influence on population genetic structure despite fact some are recent. Hopefully, future research should include different molecular markers (such as microsatellite loci) and more specimens, in order to fully estimate Danube sterlet population structure, and give more reliable suggestion for future conservation and management projects.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Education, Science and Technological Development of the Republic Serbia, Project No. 173045. The authors would like to thank Radu Suciu and Marian Paraschiv for providing tissue samples. Also, we would like to thank Mr Árpád Rideg for providing data of the supportive stocking in the Tisza River in Hungary.

Received July 03rd, 2015
Accepted October 20th, 2015

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NOVI PODACI O GENETIČKOM DIVERZITETU KEČIGE (*Acipenser ruthenus* L.) U SREDNJEM I DONJEM TOKU DUNAVA, NA OSNOVU ANALIZE MITOHONDRIJALNE DNK

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Izvod

Neki od najznačajnijih faktora koji utiču na ugroženost populacija kečige širom sveta su loše regulisano ribarstvo, zagađenje, gubitak i fragmentacija staništa. U srednjem i donjem toku Dunava ova vrsta predstavlja vrstu koja se privredno eksploatiše, dok je prisustvo prirodnih populacija u gornjem toku Dunava zavisno od programa poribljavanja. Cilj ovog istraživanja je prikaže raznovrsnost gena kečige u srednjem i donjem toku Dunava, kao i donjem toku Tise, kako bi se primenile efikasnije mere njihove zaštite. Analiza izuzetno varijabilnog fragmenta D-petlje mitohondrijalne DNK pokazala je da postoji pet novih haplotipova, uz osam haplotipova koji su ranije opisani a pronađeni na novim lokalitetima. Genska varijabilnost je skoro u potpunosti raspoređena na nivou jedinki, dok populaciona struktura nije detektovana. Negativne vrednosti testa neutralnosti ukazuju na skorašnje širenje populacija na nekim lokalitetima. Analiza protoka gena između srednjeg i donjeg toka Dunava ukazuje na intezivno kretanje jedinki između ovih oblasti, dok je na protok gena između populacija iz Tise i drugih delova Dunava primetan uticaj brane na Tisi. Naša istraživanja ukazuju na neophodnost pažljivog planiranja programa poribljavanja kečiga, kao i korišćenje demografskih podataka i statistika ulova ribe.

Primljeno 03. VII.2015.
Odobreno 20. X. 2015.