Knowl. Manag. Aquat. Ecosyst. 2019, 420, 46
© S. Marić *et al.*, Published by EDP Sciences 2019
https://doi.org/10.1051/kmae/2019035

www.kmae-journal.org

Knowledge & Management of Aquatic Ecosystems

Journal fully supported by Agence française pour la biodiversité

RESEARCH PAPER OPEN ACCESS

Genetic characterisation of European mudminnow (*Umbra krameri*) populations from the Sava River system

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Received: 21 February 2019 / Accepted: 25 September 2019

Abstract – Two new populations of the European mudminnow (*Umbra krameri* Walbaum, 1792) were discovered in the Sava River system, one in its middle part (Bosnia and Herzegovina) and the other in a tributary to the Kupa River (Croatia). The Croatian population is the most upstream mudminnow discovery in the Sava River system known to date. The genetic structure of the newly recorded mudminnow populations was examined using mitochondrial DNA and microsatellite loci. By adding these new populations to the analysis of the population genetic structure of mudminnow from the Sava River system, previously unresolved relationships between the populations from the upper and the lower Sava were clarified: the middle Sava populations were shown to be well outside the hybridisation zone with the Danubian clade, meaning the upstream boundary of this zone is confined to the lower Sava. The results also suggest that mudminnow populations in the Sava River system are less isolated than previously believed. Namely, intermediate gene-flow was detected when comparing the uppermost Sava population with the lower Sava populations. Taking these results into account, appropriate guidelines are proposed to preserve mudminnow populations from the Sava River system.

Keywords: Conservation / European mudminnow / microsatellites / mitochondrial DNA / Sava River system

Résumé – Caractérisation génétique des populations de poisson chien européen (*Umbra krameri*) du système de la rivière Sava. Deux nouvelles populations de poisson chien européen (*Umbra krameri* Walbaum, 1792) ont été découvertes dans le système de la rivière Sava, l'une dans sa partie centrale (Bosnie-Herzégovine) et l'autre dans un affluent du fleuve Kupa (Croatie). La population croate est la découverte de poisson chien européen la plus en amont connue à ce jour dans le système de la rivière Sava. La structure génétique des populations de poisson chien européen nouvellement observées a été examinée à l'aide d'ADN mitochondrial et de loci microsatellites. En ajoutant ces nouvelles populations à l'analyse de la structure génétique des populations de poisson chien européen du système de la rivière Sava, on a clarifié les relations non résolues entre les populations de la Sava supérieure et de la Sava inférieure : les populations de la Sava moyenne se trouvaient bien au-delà de la zone d'hybridation, le clade danubien ayant démontré que la limite amont de cette zone est limitée à la Sava inférieure. Les résultats suggèrent également que les populations de poisson chien européen dans le système de la rivière Sava sont moins isolées qu'on ne le croyait auparavant. A savoir, un flux intermédiaire de gènes a été détecté en comparant la population de Sava la plus élevée avec les populations de Sava les plus basses. Compte tenu de ces résultats, des directives appropriées sont proposées pour préserver les populations de poisson chien européen de la rivière Sava.

Mots-clés : Conservation / poisson chien européen / microsatellites / ADN mitochondrial / système de la rivière Sava

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1 Introduction

The European mudminnow (*Umbra krameri* Walbaum, 1792, hereinafter mudminnow) is endemic to the Danube and Dniester river drainages (Wanzenböck, 2004; Freyhof and Brooks, 2011; Sekulić *et al.*, 2013). The species inhabits wetlands rich in aquatic vegetation, and densely overgrown backwaters (Povž *et al.*, 2015). This species is highly endangered due to the specificity and rarity of these habitats that are disappearing both naturally and due to anthropogenic impacts, such as pollution, water regulation, draining or even dredging (Delić *et al.*, 1997; Mrakovčić *et al.*, 2006; Kuehne and Olden, 2014).

Owing to habitat loss, the distribution of mudminnow has become patchy with constant declines in population size (Vuković and Ivanović, 1971; Maitland, 2000; Simonović, 2001; Wilhelm, 2003; Freyhof, 2011; Freyhof and Brooks, 2011; Kuehne and Olden, 2014; Takács *et al.*, 2015). Consequently, mudminnow has been given high protection at both the international and national levels in all countries throughout its range (Freyhof, 2011; Freyhof and Brooks, 2011).

In general, this species occurs in the lowlands of the Danube, Sava, Drava, Tisza, Prut and Dniester systems (Movchan, 1995; Wanzenbock, 2004; Kottelat and Freyhof, 2007; Kuehne and Olden, 2014 and references therein). However, the magnitude of the species areal reduction is critical, and is especially evident in Serbia, where all 10 historically (1860–) known populations disappeared by 1995 (Sekulić *et al.*, 2013). On the other hand, some previously unrecorded populations have been detected over the last 30 yr: two in the Danube drainage in Serbia (Sekulić *et al.*, 2013; Miljanović *et al.*, 2016), several in the Drava and Mura River systems in Croatia and Slovenia (Mrakovčić and Kerovec, 1990; Povž, 1990; Delić *et al.*, 1997; Govedič, 2010), three in the Sava River system in Croatia, Bosnia-Herzegovina and Serbia (Zanella, 1997; Sekulić *et al.*, 1998; Petronić *et al.*, 2010) and two in the Timiş and Jiu River systems in Romania (Covaciu-Marcov *et al.*, 2018). As a small bodied fish, mudminnow is not easy to find (Movchan, 1995), and therefore, it is more likely that these populations were simply overlooked rather than newly established. Furthermore, these "new" populations were mostly recorded in well preserved localities (also historically). These findings suggest that there may be more unrecorded populations.

To study the phylogeography and inter-population variation of mudminnow, a recent genetic survey of the species was performed across its entire range (Marić *et al.*, 2017), revealing three large mitochondrial clades geographically corresponding to the: (1) Danube–Drava–Dniester River systems, (2) Sava River system, and (3) Tisza River system. According to microsatellite analysis (Marić *et al.*, 2017), a clear differentiation was recorded between the populations from the upper and the lower Sava River. However, the middle Sava was not included in the analyses, as there was no data on the species present in this part of the river.

Habitat loss and fragmentation leads to formation of small populations with little to no gene flow (Marić *et al.*, 2017). In such populations, random genetic drift leading to loss of genetic diversity and inbreeding may impair the evolutionary potential of the species, pushing it toward local extinction risk. Therefore, the discovery of each new population and the protection of the corresponding habitat is an extremely beneficial contribution to the protection of the species.

In this study, we present two newly recorded mudminnow populations found in 2016. Both were discovered in the Sava River system, one in the middle and the other in the upper part of the river. As these two populations may be a possible source for re-establishing populations in areas where they have become extinct (Leiner, 1995; Povž, 1990), we examined their genetic constitution using mtDNA and microsatellite makers. By combining these new data with the data presented in Marić *et al.* (2017), we also re-evaluate the population structure of the mudminnow Sava clade.

2 Material and methods

2.1 Description of localities

In the middle Sava, the mudminnow was first discovered in the Kraljica stream (tributary to the Matura River, near the town of Srbac in Bosnia and Herzegovina) (Čolić, 2018). Later surveys also detected the species in the lower Matura River system, including the tributaries Karavida, Glibača and Adžaba, and in numerous springs in that area (Fig. 1).

The second newly recorded population inhabits the upper Odra River, just one kilometre below the confluence of the two streams that form this 45 km long river, which flows *via* the Kupa River into the Sava River near the town of Sisak (Fig. 1). The mudminnow was found at just one of six comprehensively studied sampling sites. This is the most upstream mudminnow discovery in the Sava system to date.

2.2 Samples

Using electrofishing and landing nets, 19 specimens were collected from the Kraljica stream (Kraljica-3; 45°04′35″N, 17°23′25″E) in spring 2016, and 22 from the Odra River (Odra-1; 45°42′04″N, 16°09′07″E) in winter 2018.

A partial sample-set from Marić *et al.* (2017), representing the sampling locations from the Sava drainage (Šuma Žutica (Šuma Žutica-2; upper Sava), Gromiželj (Gromiželj-4) and Bakreni Batar (Bakreni Batar-5; lower Sava)) and two from the Danube drainage, Kraljevac (Kraljevac-6) neighbouring the Sava mouth, and Lugomir (Lugomir-7) upstream in the Danube River, were also incorporated in the study (Fig. 1, Tab. 1).

2.3 Molecular analyses

Fin clips were sampled and stored in 96% ethanol. Total DNA was isolated using the phenol–chloroform–isoamyl alcohol method (Sambrook *et al.*, 1989).

2.3.1 Mitochondrial DNA

The cytochrome b (cyt b) gene was PCR-amplified from 15 specimens from the Kraljica stream and 22 from the Odra River (Tab. 1), using GluF and ThrR primers and the PCR conditions as described in Machordom and Doadrio (2001). Bidirectional sequencing was carried out on an ABI Prism 3130xl DNA sequencer using the same primers.

Cytochrome b sequences were edited and aligned using the programs Chromas Lite 2.01 (http://www.technelysium.com.au/chromas.html; Technelysium Pty Ltd, Australia) and

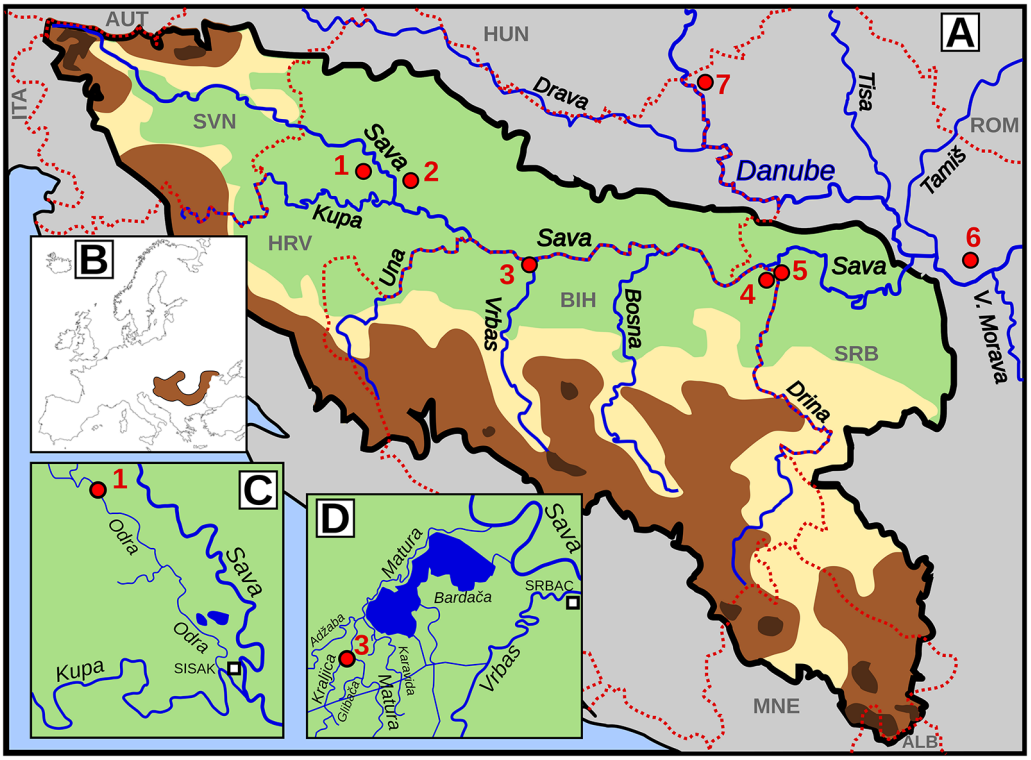

Fig. 1. (A) Map of sampling locations (Odra-1, Šuma Žutica-2, Kraljica-3, Gromiželj-4, Bakreni Batar-5, Kraljevac-6, Lugomir-7). The Sava River system is delineated with a thick, black solid line, while the borders between countries are shown with thin, red dotted lines. (B) Distribution of *Umbra krameri* in Europe according to Wanzenböck (2004). (C) Detailed map of the lower Kupa River system. (D) Detailed map of the lower Matura River system.

Table 1. Sampling sites (asterisks denote populations described in Marić *et al.*, 2017), mtDNA haplotypes, number of individuals sampled (N), expected heterozygosity (H_E), allelic richness (A_R) and average values of effective population sizes calculated from IM model estimation (θ).

Population – river system	mtDNA - Cyt b haplotypes					Microsatellite DNA			
	N	Da1	Da2	Da3	Sa1	N	H_E	A_R	θ
Odra-1 – Sava	22				22	22	0.516	4.61	1.27
Šuma Žutica-2 – Sava*	11				11	19	0.378	3.43	0.88
Kraljica-3 – Sava	15				15	19	0.579	6.29	5.06
Gromiželj-4 – Sava*	10				10	20	0.678	8.02	20.79
Bakreni Batar-5 – Sava*	10				10	20	0.766	8.03	10.02
Kraljevac-6 – Danube*	10	5		3	2	24	0.762	8.63	11.64
Lugomir-7 – Danube*	10	9	1			19	0.634	5.86	0.92
	∑ 88					143			

Clustal X (Thompson *et al.*, 1997). All sequences were compared to the reference database from Marić *et al.* (2017), comprising of ten recorded haplotypes (seven Danube, two Tisza and one Sava haplotype) in 182 individuals from 17 populations (eight Danube populations, three Drava, two Tisza, three Sava and one Dniester).

2.3.2 Microsatellites

Seven microsatellite loci (*UkrTet1, UkrTet3–UkrTet8*) were amplified from all specimens from the newly recorded locations, according to the protocols of Winkler and Weiss (2009). Fragment analysis was performed on a 3130xl Genetic Analyzer and genotyped using Gene-Mapperv4.0 (Applied Biosystems).

Microsatellite loci were analysed in order to test for the presence of null alleles (Microchecker v2.2.3; Van Oosterhout *et al.*, 2004), to determine the parameter of genetic diversity (H_E) (GENETIX 4.04; Belkhir *et al.*, 1996–2004), to test for Hardy-Weinberg equilibrium (HWE), to calculate F statistics and allelic richness (FSTAT 2.9.3.2; Goudet, 2002) and the most probable number of genetic groups (K) based on a two-step hierarchical STRUCTURE analysis (STRUCTURE v2.3; Pritchard *et al.*, 2000) – in the first step, all localities were

analysed together, while in the second step, pure Danubian population were removed to allow for a focus on only those populations carrying at least some amount of Sava genes. The ΔK method (Evanno *et al.*, 2005) was applied to estimate the most probable K (Appendix A).

To investigate whether genetic differentiation may be influenced by stepwise mutations (R_{ST}), we used an allele size randomization procedure (10 000 permutations) in SPAGeDi v.1.3 (Hardy and Vekemans, 2002) across all loci and genetic clusters. A R_{ST} significantly larger than the permuted R_{ST} (pR_{ST}) suggests that stepwise mutations have had an important influence on the current differentiation. IMa2 software (Hey and Nielsen, 2007) was used to determine the gene flow and effective population size.

As IMa2 only assumes a stepwise mutation model (SMM) for microsatellite loci, two separate data sets were used and compared – one including all microsatellite loci and the other including only those five loci in which the individual permutation test and comparison of the R_{ST} and F_{ST} values were clearly in favour of SMM (*UkrTet1*, *UkrTet3*, *UkrTet4*, *UkrTet5* and *UkrTet7*, see Sect. "Results"); in both datasets the HKY model of sequence evolution was applied to the mitochondrial sequences. Gene flow was estimated among all neighbouring populations and from several additional combinations selected according to biological rationale. Six parameters were estimated from each combination (using all seven and only five microsatellite loci): descendent (for both populations; θ_1, θ_2) and ancestral population sizes (θ_{ANC}), relative time since divergence (t) and two population migration rates ($2N_1m_{2\rightarrow1}$ and $2N_2m_{1\rightarrow2}$). For methodological details regarding microsatellite data analysis, see also Marić *et al.* (2011, 2017). Besides inferring historical gene-flow with IMa2, we have also used BayesAss Edition 3.0.4 (Wilson and Rannala, 2003) to evaluate contemporary migrations that occurred only in recent (1–3) generations. The analysis incorporated different delta values to ensure that proposed changes between chains at the end of the run were between 20 and 40% of the total chain length; for BayesAss analysis all microsatellite loci were considered and the analysis was set in line with suggestions from Meirmans (2014). The populations were analysed in two separate analyses to reduce the number of possible combinations for gene flow estimation; in one we estimated gene flow between the new populations (Odra-1 and Kraljica-3), Šuma Žutica-2 and Gromiželj-4 and in the other gene flow between Bakreni Batar-5 and the two Danubian populations (Kraljevac-6 and Lugomir-7). Three different sets of delta values (ΔA 0.40–0.60, Δm 0.20–0.30, ΔF 0.65–0.75) were selected in the accepted proportion of proposed changes (A 24–38%, m 28–37%, F 33–37%; 100 million iterations, 20 million burn-in and sampling frequency of 4000). All analyses were run in two repeats using different random seeds. Convergence of all parameter estimates was checked with Tracer (Rambaut *et al.*, 2018).

3 Results

3.1 Mitochondrial DNA

A total of 1085 bp of the cyt b gene were resolved with sequence analysis in 37 individuals (15 from Kraljica-3 and 22 from Odra-1). After sequence alignment, all 37 sequences collapsed into a single haplotype. Following the nucleotide database search using BLAST, this haplotype was found to correspond with 100% nucleotide identity to the Sa1 haplotype (GenBank acc. no. KP898876), previously detected in the Sava drainage (Marić *et al.*, 2017) and characterizing the Sava clade. This haplotype differed from the Da1 haplotype, which is the dominant Danubian haplotype recorded at the localities Lugomir-7 and Kraljevac-6, in seven nucleotide positions (0.65%). On the other hand, the Da1 haplotype differed from Da2 in a single nucleotide position (0.09%) and from Da3 in two nucleotides (0.18%).

3.2 Microsatellites

The Kraljica-3 and Odra-1 populations were found to be in HWE. No null alleles were detected. Expected heterozygosity and allelic richness were 0.516 and 4.61 in the Odra-1 population, and 0.579 and 6.29 in the Kraljica-3 population (Tab. 1).

Pair-wise F_{ST} was 0.274 between Odra-1 and the geographically close Šuma Žutica-2 population, 0.065 between Kraljica-3 and the first downstream Gromiželj-4 population, and 0.142 between the Odra-1 and Kraljica-3 populations. Both newly recorded populations show the lowest F_{ST} values in relation to Gromiželj-4, and the highest in relation to the Lugomir-7 population (Tab. 2).

Genetic differentiation of the whole sample set was assessed using hierarchical STRUCTURE analysis (Pritchard *et al.*, 2000; Vähä *et al.*, 2007). The most probable K values were $K=2$ for the 1st and $K=4$ for the 2nd step. In the 1st step, two genetic groups were detected, where one characterized the populations from the middle and upper Sava (Odra-1, Šuma Žutica-2 and Kraljica-3) and the second the Danubian Lugomir-7 population, while the populations from the lower Sava (Bakreni Batar-5 and Gromiželj-4) and Danubian Kraljevac-6 population, located near the mouth of the Sava River, were admixed with different proportions of parental genes. In the next step, the genetically uniform Lugomir-7 was excluded, as this is a Danubian population showing no introgression with the Sava lineage (unlike the Danubian Kraljevac-6 population where clear admixture with Sava lineage is evident both from mitochondrial and microsatellite data). After excluding the Lugomir-7 population, four genetically homogenous units emerged in the 2nd step (Kraljevac-6, Kraljica-3, Šuma Žutica-2 and Odra-1), whilst the populations of the lower Sava (Bakreni Batar-5 and Gromiželj-4) showed different levels of genetic mixing of those four genetic units with the dominant participation of the neighbouring populations (Fig. 2 and Appendix A).

The observed R_{ST} value of the whole sample set was 0.374, with a pR_{ST} value of 0.159 ($P=0.0000$) and F_{ST} value of 0.2007. The significantly higher R_{ST} than pR_{ST}, and considerably higher value than F_{ST}, suggested that SMM contributed to genetic differentiation. However, when analysing the loci individually, this mutational influence was not apparent at two loci: for *UkrTet6* and *UkrTet8,* the R_{ST} (0.072 and 0.291, respectively) was considerably lower than both pR_{ST} (0.323 and 0.338, respectively) and F_{ST} (0.346 and 0.430, respectively).

Table 2. Paired values of F_{ST} for microsatellite marker data and their significance ($^{*}P < 0.05$; $^{***}P < 0.001$).

	Odra-1	Šuma Žutica-2	Kraljica-3	Gromiželj-4	Bakreni Batar-5	Kraljevac-6
Odra-1						
Šuma Žutica-2	0.274***					
Kraljica-3	0.142***	0.238***				
Gromiželj-4	0.114***	0.213***	0.065***			
Bakreni Batar-5	0.126***	0.208***	0.106***	0.028*		
Kraljevac-6	0.194***	0.281***	0.196***	0.127***	0.038***	
Lugomir-7	0.372***	0.437***	0.341***	0.276***	0.194***	0.176 ***

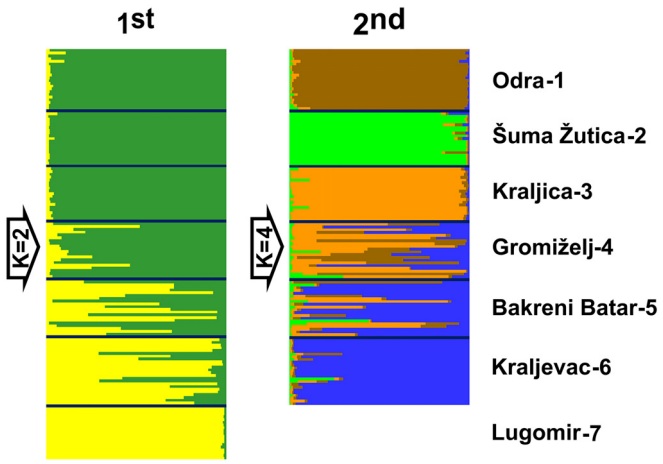

Fig. 2. Estimated population structure as inferred by hierarchical STRUCTURE analysis of microsatellite marker DNA data. Black lines separate sampling sites. The most probable K for the analysed samples shown in the arrows is based on the ΔK method; no further structures were detected in subsequent rounds (after the second step) and within the excluded clusters ($K = 1$).

No large discrepancies were observed between IM estimations with only five or all seven microsatellite loci; however, in some cases it was not possible to estimate the ancestral θ when considering all loci (Tab. 3). When comparing estimations done with five or seven loci, those with a narrower confidence interval were considered more reliable. In general, a narrower confidence interval (CI) was observed when using seven loci, and only population migration rates that differed significantly from zero were considered.

IM analysis of neighbouring populations revealed low to intermediate levels of historical migrations between the lower (Gromiželj-4 and Bakreni Batar-5) and the upper/middle Sava (Odra-1; Šuma Žutica-2 and Kraljica-3) mostly in the downstream direction towards the lower Sava: with the highest migrations from the Odra-1 and Šuma Žutica-2 towards the lower Sava (Gromiželj-4) (6.87 and 1.40 individuals per generation, respectively), followed by migrations primarily suggesting gene flow from peripheral Sava populations (Odra-1 and Gromiželj-4) to the central Sava population, Kraljica-3 (1.07 and 1.04 individuals per generation, respectively). A low historic population migration rate was also observed from the lower Sava (Bakreni Batar-5) to the middle Danube (Kraljevac-6) (1.60 individuals per generation).

BayesAss estimation revealed contemporary gene flow going only from Bakreni Batar-5 to Kraljevac-6 and from Gromiželj-4 to Kraljica-3 (24.01% [1.99–33.21] and 11.28% [1.58–20.64], respectively), while all other migrations did not differ significantly from zero.

The IM model showed that Gromiželj-4 and Bakreni Batar-5 were the largest populations in the Sava River watershed, with θ values of 20.79 (averaged over three estimations) and 10.02, respectively. Of the new Sava populations, the highest θ values were recorded at Kraljica-3 (5.06, averaged over three estimations), while Odra-1 (1.27, averaged over three estimations) and Šuma Žutica-2 (0.88, averaged over two estimations) were considerably smaller and of comparable size. In addition, the relative times since divergence calculated by the IM model were generally young between neighbouring populations and old when comparing the split between the Danubian and Sava watersheds (t_0 between Bakreni Batar-5 and Kraljevac-6 was 5.87); the only exception was the t_0 between Odra-1 and Gromiželj-4; however, estimations of this parameter did not converge nicely even after discarding the first 2.5 M generations.

4 Discussion

The finding of the Sa1 cyt b haplotype in the middle Sava, represented by the Kraljica-3 population, confirmed the previous assumption (Marić *et al.*, 2015, 2017) that this haplotype characterizes the Sava phylogeographic lineage while also leaving a trace in the Danube River, at least 50 km downstream of the Sava River mouth (Kraljevac-6 sampling site), where it was still detected to a minor extent (Tab. 1). The relatively high divergence between the Sa1 haplotype and the Danubian haplogroup, represented by the Da1, 2 and 3 haplotypes characterizing two Danubian populations, suggests long lasting and ancient separation of the Sava and Danubian mtDNA lineages, which according to Marić *et al.* (2017) dates back to the Middle Pleistocene. As inferred from microsatellite data, this is in line with only low historic population migration rate, observed in the direction from the lower Sava (Bakreni Batar-5) towards the middle Danube (Kraljevac-6). The presence of the Sava haplotype in the Danubian location Kraljevac-6 and the absence of the Danubian haplotypes in the Sava locality correspond with the observation that contempo-

Table 3. Migration estimates performed with IMa2 and BayesAss for each tested population pair. IMa2: time since divergence (t_0), effective population sizes of ancestral and the two descendent populations (qANC, q1, q2), historical population migration rates ($2N_1m_{2\to1}$, $2N_2m_{1\to2}$) between the first (1) and second (2) populations with the lower and upper boundaries of the 95% highest posterior density (HPD) interval is given in brackets. All parameters except population migration rates are scaled by mutation rate. BayesAss: contemporary migration evaluations ($M_{2\to1}$, $M_{1\to2}$) between the first (1) and second (2) populations occurring only in recent (1–3) generations given in the percentage of migrants.

	t_0	q_{ANC}	q_1	q_2	$2N_1m_{2\to1}$	$2N_2m_{1\to2}$	$M_{2\to1}$ [%] (1–3 gen)	$M_{1\to2}$ [%] (1–3 gen)
Kraljica-3 – Š. Žutica-2: 5 loci	1.30 (0.54–)[†‡]	46.36 (19.00–)[†]	9.96 (4.92–18.20)	1.32 (0.52–3.00)	0.00 (0.00–1.78)[§]	0.00 (0.00–0.44)[§]	–	–
Kraljica-3 – Š. Žutica-2: 7 loci	0.68 (0.38–)[†‡]	38.12 (16.22–)[†]	5.50 (2.82–10.28)	0.84 (0.34–1.74)	0.00 (0.00–0.36)[§]	0.00 (0.00–0.05)[§]	–[§]	–[§]
Kraljica-3 – Odra-1: 5 loci	0.58 (0.21–)[†]	35.08 (19.64–)[†]	8.60 (3.16–15.96)	1.88 (0.68–4.76)	0.54 (0.00–1.39)[§]	0.01 (0.00–2.95)[§]	–	–
Kraljica-3 – Odra-1: 7 loci	0.81 (0.21–9.17)	27.96 (13.96–)[†]	4.76 (2.12–9.32)	1.56 (0.52–3.24)	1.07 (0.15–3.86)	0.10 (0.02–1.49)	–[§]	–[§]
Odra-1 – Š. Žutica-2: 5 loci	0.32 (0.16–)[†]	11.88 (0.28–72.68)	2.20 (0.84–4.76)	1.16 (0.36–2.60)	0.26 (0.00–0.93)[§]	0.02 (0.00–0.36)[§]	–[§]	–[§]
Odra-1 – Š. Žutica-2: 7 loci	0.30 (0.13–)[†]	5.72 (0.00–74.44)	1.48 (0.60–3.16)	0.92 (0.28–2.04)	0.34 (0.00–0.96)[§]	0.03 (0.00–0.36)[§]	–[§]	–[§]
Kraljica-3 – Gromiželj-4: 5 loci	?	29.48 (11.32–78.04)	7.00 (2.12–14.52)	24.20 (10.76–57.24)	1.04 (0.12–3.36)	0.05 (0.00–7.23)[§]	–	–
Kraljica-3 – Gromiželj-4: 7 loci	1.07 (0.77–)[†]	18.73 (8.17–45.02)	4.92 (2.12–8.52)	19.00 (9.72–33.48)	0.77 (0.00–2.11)[§]	0.04 (0.00–3.86)[§]	11.28 (1.58 –20.64)	1.85 (0.00–5.34)[§]
Odra-1 – Gromiželj-4: 5 loci	1.20 (0.28–8.53)	4.92 (0.20–28.40)	1.00 (0.44–3.32)	49.72 (28.60–)[†]	1.11 (0.20–2.30)	6.87 (0.07–19.26)	–	–
Odra-1 – Gromiželj-4[¶]: 7 loci	4.83 (3.09–)	?	0.76 (0.36–2.12)	26.29 (11.32–75.96)	0.41 (0.03–1.24)	10.05 (2.38–88.40)	–[§]	–[§]
Š. Žutica-2 – Gromiželj-4: 5 loci	2.58 (1.41–)[†]	4.36 (0.12–50.36)	1.40 (0.52–2.92)	31.88 (18.20–76.76)	0.03 (0.00–0.41)[§]	1.40 (0.24–9.37)	–	–
Š. Žutica-2 – Gromiželj-4: 7 loci	5.38 (2.81–)[†]	?	0.52 (0.20–1.48)	17.08 (8.44–70.60)	0.10 (0.00–0.32)[§]	4.78 (0.88–31.86)	–[§]	–[§]
B. Batar-5 – Kraljevac-6: 5 loci	?	16.12 (0.12–60.84)	17.24 (6.04–37.24)	14.92 (6.92–29.72)	0.00 (0.00–5.07)[§]	1.79 (0.98–6.31)	–	–
B. Batar-5 – Kraljevac-6: 7 loci	5.87 (0.95–)[†¶]	?	10.20 (5.32–19.24)	9.80 (5.40–18.52)	0.40 (0.00–3.14)[§]	1.60 (0.29–4.62)	5.49 (0.00–27.15)[§]	24.01 (1.99–33.21)
Kraljevac-6 – Lugomir-7: 5 loci	0.73 (0.52–)[†‡]	19.24 (0.20–64.44	21.96 (12.20–39.88)	1.40 (0.52–3.32)	0.61 (0.00–5.08)[§]	0.65 (0.19–1.50)	–	–
Kraljevac-6 – Lugomir-7: 7 loci	0.58 (0.41–)[†‡]	19.32 (1.32–64.36)	13.48 (6.52–23.00)	0.92 (0.36–2.36)	0.00 (0.00–2.24)[§]	0.37 (0.03–1.63)	–[§]	–[§]

[†]Multiple peaks were present in some of the runs, only runs with a clear younger peak were used for final estimations with *L* mode; t_0 was estimated from the younger peak.
[‡]Upper boundary for population size could not be calculated as posterior density does not reach low levels.
[§]Gene-flow estimations were not significantly different from zero.
[¶]Multiple peaks were present in some of the runs, only runs carrying a clear older peak were used for final estimations with *L* mode; t_0 was estimated from the younger peak.

rary gene flow is only in the direction from Bakreni Batar-5 to Kraljevac-6. For more phylogenetic considerations and the historical processes that shaped the geographic haplotype distribution of *U. krameri*, see Marić *et al.* (2017).

On the basis of microsatellites, locations from the middle and upper Sava, including the new ones (Kraljica-3 and Odra-1), host well defined populations displaying high to moderate F_{ST} values, while the reanalysis of the two neighbouring locations from the lower Sava (Gromiželj-4 and Bakreni Batar-5) confirmed the conclusion from Marić *et al.* (2017) that they host a single population. By adding the Kraljica-3 and Odra-1 populations to the analysis of the population genetic structure of mudminnow from the Sava River system, previously unresolved relationships between the populations from the upper (Šuma Žutica-2) and the lower Sava (Bakreni Batar-5 and Gromiželj-4) were clarified: the existence of a hybrid zone between the Sava and the Kraljevac-6 sampling sites in the Danube river system was confirmed, as previously suggested by Marić *et al.* (2017), and it was found that the upstream boundary of this zone extends well below the middle Sava (Kraljica-3 sampling site) (Tab. 1, Fig. 2).

Furthermore, STRUCTURE analysis revealed additional genetic sub-structuring in the Sava, which separated the three populations from the middle and upper Sava into three genetic sub-clusters that are also present in the admixed lower Sava population in varying proportions. However, such a genetic structure could also be a result of the founder effect coupled

with the small sizes of the upper populations, as small populations undergo a reduction in genetic diversity more quickly than larger ones, due to the effects of genetic drift (also see Marić *et al.*, 2017). Therefore, an alternative explanation is that the presence of genes from the two upper and middle Sava sub-clusters in the lower Sava is not due to gene-flow, but is instead a consequence of the lower Sava acting as a donor for the founders of the upriver populations. Over time, these small populations may have succumbed to an even higher loss of genetic diversity until they became almost monomorphic, while the large lower Sava populations retained their diversity and the signal of the founder genes are still present.

The comparison of the H_E and A_R values of the newly discovered Kraljica-3 and Odra-1 populations to the values of the entire species range (Marić *et al.*, 2017) reveals that the Kraljica-3 population is characterized by an average level of genetic diversity while this is somewhat lower in the Odra-1 population. The genetic structure and diversity of mudminnow populations across the Sava River system varies between sampling sites, indicating the genetic spatial structure of the species. At first glance, genetic diversity appears to increase gradually from the upper towards the lower river system by geographic distance, with the Kraljica-3 population taking an intermediate position. However, the reliability of this assumption, based only on five populations, is questionable, also because the Šuma Žutica-2 population, located downstream from the Odra-1 population, deviates from the assumed trend, showing the lowest genetic diversity within the Sava River system (Tab. 1). On the other hand, the level of genetic diversity of all the Sava populations is positively associated with their effective population sizes (Tab. 1) – the higher the Ne, the higher the genetic diversity – which may suggest that population size is the main factor shaping the observed distribution of genetic diversity among populations. Nevertheless, in the case of the Kraljica-3 population ($\theta = 5.06$), relatively high values of its diversity parameters may also indicate its communication with other adjacent populations in the Matura River system (see description of localities).

Inter-population differentiation measured by F_{ST} values (Tab. 2) also implies that geographic distance is the primary driver of the genetic structure of populations. F_{ST} values are generally low to moderate, showing temperate inter-population gradual differentiation, indicating a certain amount of communication and gene exchange between populations. This observation was also supported by the population migration (IM) analysis (detailed explanation below). Here, the Šuma Žutica-2 population is again an exception, as it was found to be considerably and more or less equally differentiated from other Sava populations, even in comparison to the Odra-1 population, less than 30 km away. Also, no statistically significant gene-flow was detected between the Šuma Žutica-2 and the other populations. This observation along with the low genetic diversity and relatively high pairwise F_{ST} values of the Šuma Žutica-2 population indicates its localized distribution and restricted gene flow, which likely explains its pronounced position among the Sava populations.

Finally, we showed that mudminnow populations in the Sava are less isolated from each other than previously believed (Marić *et al.*, 2017). Namely, intermediate gene-flow and population differentiation were also detected when comparing the uppermost Sava population (Odra-1) with the lower Sava (Gromiželj-4, Bakreni Batar-5), though it appears that the differentiation of the Šuma Žutica-2 population from the upper Sava is an exception to this pattern.

Converted per-generation population migration rates between these populations generally correspond to the migration of about one individual per generation, in the downstream direction from the upper (Odra-1) or middle Sava (Kraljica-3) towards the lower Sava (Gromiželj-4, Bakreni Batar-5). On the other hand, contemporary migrations between the lower and middle Sava population were detected flowing in the opposite direction – upstream from the lower to the middle Sava, indicating a potential change in the migration pattern. However, caution is advised when disentangling demographic, especially migration parameters using molecular markers. For IM models, Quinzin *et al.* (2015) demonstrated that while it is possible to distinguish between scenarios with or without gene flow, estimating its extent when different from zero is associated with relatively high error rates, while increasing the number of loci or sample size reduces the variance and credible interval of the estimates.

Furthermore, in this case, it should also be recognised that the 95% highest posterior density (HPD) intervals of contemporary (BAYESASS) immigrations and emigrations are relatively wide and overlap to at least some degree in all population pairs where such gene flow was detected (Tab. 3). To finally resolve whether a change in the direction of migration pattern truly happened, more microsatellite loci or other nuclear markers are required.

In the Sava River system, mudminnow populations from the upper Sava system are characterised by low values of genetic diversity and very small effective population sizes. These two populations likely became fragmented due to human impacts, thus their genetic structure may not reflect the natural evolutionary process but rather random drift. Marić *et al.* (2017) stated that it is questionable whether such small populations represent genetically viable entities with a good prospect of long-term survival without appropriate management. As inferred from the results of this study, the relatively large population size and genetic diversity of the Kraljica-3 population suggest its solid fitness and evolutionary potential. Furthermore, its genetic similarity with the upper Sava mudminnows and their geographic proximity that also frequently assures adaptive similarities of the adjacent populations make the Kraljica-3 population a suitable source for potential re-establishment or even genetic rescue (Whiteley *et al.*, 2015), an under-used management option that shows great promise also in management of isolated freshwater fish populations (Robinson *et al.*, 2017). However, despite this possibility, conservation efforts should be directed towards ensuring a favourable habitat status for mudminnow populations throughout its distribution range.

The genetic characterisation of European mudminnow populations from the Sava River system provides important findings applicable to the genetic management of the focal species. However, beyond that, we demonstrated that molecular genetic methods can serve as an effective tool for the basic guidelines for protection, conservation and sustainable management of other small-bodied and commercially unimportant freshwater species of high conservation value, which are often characterized by specialized requirements

(habitat and/or diet), low fecundity, population size, dispersal capability, and are often geographically isolated or live in fragmented habitats (Reynolds *et al.*, 2005; Sekulić *et al.*, 2013; Kuehne and Olden 2014; Arthington *et al.*, 2016).

Acknowledgements. This study received financial support from the Slovenian Research Agency and the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 173045). RŠ was fully supported by the Ministry of Culture of the Czech Republic: project NAKI II (DG16P02B038) and DS by Slovenian Ministry of Education, Science and Sport and the European Regional Development Fund: Operational Programme for the Implementation of the EU Cohesion Policy in the period 2014–2020 (5442-15/2016/18).

Appendix A. Hierarchical steps in estimating K (the number of genetic clusters) from STRUCTURE runs using the ΔK method. $L(K)$ – posterior probability of K; stdev – standard deviation of $L(K)$ from seven independent runs; ΔK – an ad hoc quantity, predictor of the real number of clusters (Evanno *et al.*, 2005), best ΔK are in bold.

	K	L(K)	stdev	ΔK
I step – all populations	1	–3955.10	0.29	
	2	**–3612.51**	**0.74**	**203.73**
	3	–3420.99	8.46	2.10
	4	–3247.25	0.57	139.12
	5	–3152.56	0.98	84.074
	6	–3140.49	3.19	22.55
	7	–3200.28	21.93	0.33
	8	–3252.80	14.42	
II step – Kraljevac-6,	1	–3222.51	0.28	
Bakreni Batar-5,	2	–3022.24	1.06	30.45
Gromiželj-4, Kraljica-3,	3	–2854.29	4.31	15.62
Šuma Žutica-2 i Odra-1	**4**	**–2753.60**	**1.91**	**71.89**
	5	–2790.00	47.54	0.54
	6	–2851.99	20.71	2.34
	7	–2865.50	51.98	

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Cite this article as: Marić S, Stanković D, Šanda R, Ćaleta M, Čolić S, Šukalo G, Snoj A. 2019. Genetic characterisation of European mudminnow (*Umbra krameri*) populations from the Sava River system. *Knowl. Manag. Aquat. Ecosyst.*, 420, 46.