

Morphological and genetic diversity of *Sabanejewia balcanica* in Croatia

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Abstract. Taxonomic analyses of four *Sabanejewia balcanica* populations from Croatia included morphological (morphometric, meristic, phenotypical) and mitochondrial DNA analyses. Comparative analyses of 24 morphometric characters revealed some differences between populations. The specimens from the Drava River are significantly larger than specimens from other populations, whereas the individuals from the Voćinska River represent the smallest European *S. balcanica* specimens. Due to similarities in morphometric ratios, in the Tree Diagram of Euclidean distances, the Drava and Petrinjčica Rivers populations formed a separate cluster, while the populations from the Rijeka and Voćinska Rivers formed a second cluster. These morphometrical differences are also corroborated by t-test results. Differences among fishes from different populations also exist in external morphology characters, such as spots on the caudal peduncle and the position of the suborbital spine. Mitochondrial DNA analyses enabled us to infer the phylogenetic placement of four Croatian populations within the genus *Sabanejewia*. All Croatian samples clustered within the two sublineages of the Danubian-Balkan complex. Samples from the Voćinska River, as well as one sample from the Drava River were included in the “*S. balcanica*” (VI) sublineage. The remaining samples, including the two remaining haplotypes from the Drava River, were comprised within “*S. montana-S. bulgarica-S. balcanica*” (III) sublineage.

Key words: Balcan spiny loach, morphology, phylogeny, Danubian-Balkan complex

Introduction

The distribution of the genus *Sabanejewia* stretches from the Aral Sea to Italy and from Asia Minor to the Baltic Sea. This genus is highly diversified in Europe (P e r d i c e s et al. 2003) though the taxonomic position and phylogenetic relationships of taxa have been disputed and are still uncertain. Eight species of this genus have been described for European waters (K o t t e l a t 1997). However, due to morphological variations, some populations have been described controversially as species, subspecies or clinal variations (L u d w i g et al. 2000). In fact, the phylogeny of the genus *Sabanejewia* remains one of the most interesting problems of the family Cobitidae (E c o n o m i d i s & N a l b a n t 1996).

The golden loaches (genus *Sabanejewia*) mainly inhabit middle and upper parts of medium sized rivers and their populations are often isolated (L u d w i g et al. 2000). In Croatia, the goldside loach (*Sabanejewia balcanica*) is widespread in the streams and rivers of the Danube drainage, where it inhabits the Ilova, Toplica, Rijeka, Pakra, Bijela, Voćinska, Petrinjčica and Buzeta Rivers (D e l i ć et al. 2003a).

The objective of this study was to investigate both morphological and genetic diversity among Croatian populations of *S. balcanica* and to infer the phylogenetic position of Croatian populations within the genus *Sabanejewia*.

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Materials and Methods

Sampling

Morphological analyses were conducted on 61 specimens, while mitochondrial DNA analyses included 15 specimens. The specimens were collected by electrofishing from four rivers in continental Croatia (Table 1). All these rivers belong to the Danube watershed; the Drava and Voćinska Rivers form part of the Drava River basin, while the Rijeka and Petrinjčica Rivers belong to the Sava River basin (Fig. 1).

Morphological analyses

In order to investigate differences and similarities in morphological characters among the four *S. balcanica* populations, three types of morphological features (meristic, morphometrical and phenotypical) were examined on specimens preserved in ethanol. The number of specimens included in the morphological analyses varied between 16 and 22 for all population, with the exception of the Drava River. During the three-year sampling period, conducted at regular intervals at several locations on the Drava River in Croatia, only five specimens of *S. balcanica* were caught. Thus, it can be concluded that the population of this species in the Drava River is much less abundant than in other investigated rivers. However, since these specimens represent a population that is geographically separate from other populations, the specimens from the Drava River were also included in morphological analyses.

Analysis of meristic features included the number of unbranched and branched fin rays in dorsal, anal, ventral, pectoral and caudal fin. A total of 23 morphometric characters was measured using an electronic calliper to the nearest 0.1 mm. Morphometric characters assessed include total length (TL), standard length (SL), head length (Lc), distance between head tip and anal aperture (pan), preanal distance (aA), preventral distance (aV), prepectoral distance (aP), caudal peduncle length (lpc), length of dorsal (lD), anal (lA), caudal (lC), pectoral (lP) and ventral fin (lV), distance between ventral fins and anal aperture (Van), head height (hc), maximum (H) and minimum body height (h), head width (laco), maximum body width (lac), distance between eyes (io), eye diameter (o), distance between the beginning of

Table 1. Geographical origin, taxonomic identification, cytochrome *b* haplotype and the sample voucher of the *Sabanejewia* samples included in mtDNA analyses in the study. *Cytb* gene was sequenced for all samples included in the phylogenetic analyses. Samples for which also ATPase 8/6 genes were sequenced are underlined. * refers to the haplotype/sample for which only 680 bp of *cytb* gene were obtained. It differs for the single base substitution from the haplotype DRA3. N= number of individuals included in morphological analyses; Nmt= number of individuals included in phylogenetic analyses.

Locality	River basin	N	Nmt	CYT <i>b</i> Haplotype	Sample voucher
Voćinska River	Drava	18	4	VO1	<u>SBVO1</u> , 2, 5
				VO2	SBVO3
Rijeka River	Sava	22	5	RI1	<u>SBRI1</u> , 3
				RI2	SBRI2
				RI3	<u>SBRI4</u> , 5
Drava River	Drava	5	3	DRA1	<u>SBDR1</u>
				DRA2*	SBDR2*
				DRA3	<u>SBDR3</u>
Petrinjčica River	Sava	16	3	KUP	<u>SBKU1</u> , 3, 4



Fig. 1. Map of continental Croatia with sampling localities.

the head and the beginning of the eye (prO), distance between the end of the eye and the end of the operculum (poO). The percentage ratios of morphometric characters in relations to SL, c and H were analysed. The Students t-test was used to find statistically significant differences in morphometric features among populations at a significance level of $p = 0.05$ and all populations were compared pair-wise. In order to illustrate differences and similarities between populations, a Tree Diagram of Euclidean distances (similarity dendrogram) was constructed based on the mean values of morphometric ratios. The degree of correlation between SL and other morphometric measurements was determined with Pearson's correlation matrices for each population. Linear regression was used to determine the relationship between SL and TL of samples from different populations. The STATISTICA 6.0 and EXCEL 2000 software packages were used for data analyses. In addition to meristic and morphometric characters, overall external morphology was examined for differences between populations.

Mitochondrial (mt) DNA analyses

Mitochondrial DNA was extracted from the fresh or deep-frozen muscle tissues following the procedure of Beckman et al. (1993). Polymerase chain reaction (PCR) amplifications were performed using following primers combinations: L14725 (Hrebek et al. 2004) + H16460 (<http://nmg.si.edu/bermlab.html>), and L8331 (<http://nmg.si.edu/bermlab.html>) + H9236 (<http://nmg.si.edu/bermlab.html>) for the *cytb* gene and the ATPase 8/6 genes, respectively. Sequencing was carried out with the primers H-COB_cyt638 (5' TGA TAC TTT ATC TGC GTC NG 3') and L-Cyp_425 (5' GGA CAA ATA TCC TTT TGA GG 3') for the *cytb* gene, and L8331 for the ATPase 8/6 genes. The *cytb* gene was sequenced from all 15 specimens, while the sequences of ATPase 8/6 genes were obtained only from selected individuals from each population (Table 1). All sequences have been deposited in the GenBank under following accession numbers: EF605289 and EF605330-605347.

Pairwise comparison of uncorrected sequence divergence (p-distances) in the *cytb* gene were analysed with MEGA version 3.1 (Kumar et al. 2004).

Phylogenetic analyses were conducted on concatenated *cytb* (1059 bp) and ATPase 8/6 sequences (828 bp) from this study, and on homologous sequences of the representatives of all main *Sabanejewia* lineages and sublineages of Danubian-Balkanian complex retrieved from GenBank (published by *Perdices et al.* 2003). Sequences of *Cobitis bilineata*, *C. taenia* and *Misgurnus fossilis* were used as outgroups. We employed three methods of phylogenetic inference: maximum parsimony (MP) and maximum likelihood (ML) as well as Bayesian approach, as implemented in PAUP (v 4.0b10, *Swofford* 2002) and MrBayes (version 3.1.2, *Ronquist & Huelsenbeck* 2003), respectively. For ML and Bayesian analyses, the HKY+I+ Γ model was selected as the optimal model of sequence evolution by using the MODELTEST software (version 3.06, *Posada & Crandall* 1998). For MP analysis, we used the heuristic search mode with 100 replicates, randomized input orders of taxa, and tree bisection-reconnection (TBR) branch swapping with all codon sites and nucleotide substitutions types weighted equally. Nonparametric bootstrapping (1000 pseudoreplicates, 10 addition-sequence replicates) was used to assess the branch support. ML analysis was performed under heuristic search option using the TBR branch swapping algorithm. Bayesian analysis consisted of two simultaneous runs and was conducted by running four Markov chains for three million generations sampling trees every 100 generations. The first 20% of 30,000 sampled trees were discarded and Bayesian posterior probabilities (BPP) were estimated from 50% majority-rule consensus tree of the retained trees.

Results

Morphological comparison

The number of fin rays was variable among specimens. The number of fin rays was I-III/6-9 in the dorsal fin, I-II/5-7 in the anal fin, I-II/6-8 in the pectoral fins, I-II/5-7 in the ventral fins and 13–16 in the caudal fin. However, there were no statistically significant differences in fin ray numbers among populations ($p > 0.05$, Student's *t* test).

Table 2. Correlation coefficients of morphometric characters in relation to SL.

population	Lc	pan	pA	Van	pV	pP	lpC
Voćinska R.	0.925	0.976	0.98	0.29	0.925	0.689	0.756
Rijeka R.	0.276	0.981	0.984	0.473	0.984	0.933	0.889
Drava R.	0.99	0.978	0.983	0.839	0.986	0.92	0.899
Petrinjšćica R.	0.873	0.869	0.899	0.764	0.891	0.51	0.46
	ID	IA	IC	IP	IV	hco	H
Voćinska R.	0.576	0.667	0.716	0.873	0.893	0.94	0.93
Rijeka R.	0.787	0.69	0.931	0.92	0.927	0.965	0.948
Drava R.	0.866	0.974	0.972	0.951	0.888	0.89	0.94
Petrinjšćica R.	0.299	0.763	0.592	0.579	0.359	0.76	0.63
	h	laco	lac	io	Oh	prO	poO
Voćinska R.	0.89	0.583	0.8	0.595	0.645	0.925	0.873
Rijeka R.	0.922	0.827	0.682	-0.04	0.249	0.938	0.852
Drava R.	0.985	0.914	0.825	0.8	0.824	0.946	0.95
Petrinjšćica R.	0.62	0.487	0.699	0.123	0.068	0.832	0.754

The highest values of all morphometric characters, except Van, were observed in the specimens from the Drava River. Differences between specimens from other populations were less pronounced, although fishes from the Voćinska River were the smallest. The values of Van were larger in individuals from the Voćinska and Rijeka River. The correlation coefficients between SL and other morphometric characters are presented in Table 2.

Linear regression equations for the relationships between SL and TL for specimens from the investigated populations were as follows: $SL=5.639+1.082TL$ for *S. balcanica* from the Voćinska River; $SL=0.024+1.176TL$ for the Rijeka River population; $SL=1.963+1.154TL$ for specimens from the Kupa River; and $SL=2.592+1.141TL$ for those from the Drava River.

When morphometric characters in relations to SL, c and H were compared, the differences between four populations were smaller (Table 3). Nevertheless, ratios showed greater similarity in the specimens from the Voćinska and Rijeka Rivers on the one hand, and the Petrinjčica and Drava Rivers on the other. In the Tree Diagram of Euclidean Distances, two clusters can be observed (Fig. 2.). The first one comprises the Drava and Petrinjčica River populations, while the second contains specimens from the Rijeka and Voćinska Rivers.

Table 3. Mean values and range of morphometric characters of specimens from four Croatian *S. balcanica* populations.

River	Voćinska		Rijeka		Drava		Petrinjčica	
	mean	range	mean	range	mean	range	mean	range
TL (mm)	60.516	40.75-77.85	66.835	42.8-87.7	84.44	56.7-97.2	70.135	60.95-76.59
SL (mm)	50.737	30.3-65.25	56.804	36.7-74.2	71.758	47.81-82.45	59.094	51.5-64.66
in %SL								
C	0.212	0.187-0.241	0.225	0.19-0.472	0.201	0.192-0.21	0.2	0.187-0.211
pan	0.723	0.678-0.832	0.74	0.681-0.777	0.717	0.69-0.757	0.72	0.662-0.76
aA	0.743	0.677-0.837	0.749	0.703-0.791	0.743	0.719-0.781	0.746	0.695-0.783
Van	0.49	0.22-0.736	0.48	0.27-0.573	0.231	0.196-0.293	0.224	0.154-0.256
aV	0.492	0.399-0.526	0.499	0.461-0.536	0.491	0.473-0.509	0.49	0.444-0.537
aP	0.237	0.148-0.332	0.228	0.201-0.27	0.209	0.194-0.237	0.198	0.13-0.226
lpC	0.204	0.177-0.281	0.179	0.138-0.211	0.185	0.17-0.209	0.171	0.125-0.196
ID	0.1	0.068-0.162	0.097	0.072-0.124	0.098	0.074-0.132	0.101	0.079-0.202
IA	0.079	0.038-0.107	0.079	0.055-0.1	0.074	0.064-0.08	0.076	0.067-0.089
IC	0.185	0.096-0.206	0.188	0.165-0.229	0.179	0.167-0.189	0.189	0.165-0.222
IP	0.158	0.132-0.187	0.154	0.134-0.181	0.143	0.132-0.149	0.153	0.124-0.172
IV	0.139	0.113-0.17	0.134	0.119-0.154	0.129	0.111-0.149	0.137	0.12-0.159
H	0.131	0.113-0.146	0.127	0.113-0.156	0.164	0.148-0.174	0.153	0.137-0.188
h	0.075	0.066-0.089	0.078	0.07-0.091	0.078	0.074-0.082	0.082	0.071-0.092
laco	0.101	0.079-0.19	0.083	0.069-0.102	0.098	0.089-0.109	0.099	0.088-0.116
lac	0.077	0.064-0.112	0.07	0.026-0.083	0.099	0.083-0.116	0.095	0.081-0.111
in Lc%								
io	0.149	0.083-0.206	0.157	0.095-0.207	0.134	0.116-0.171	0.168	0.114-0.286
o	0.195	0.123-0.264	0.191	0.087-0.269	0.163	0.132-0.187	0.162	0.111-0.216
prO	0.435	0.338-0.537	0.429	0.226-0.503	0.433	0.405-0.45	0.45	0.389-0.534
poO	0.461	0.4-0.535	0.444	0.27-0.527	0.491	0.459-0.522	0.474	0.443-0.516
hco	0.591	0.5-0.694	0.559	0.24-0.675	0.528	0.457-0.589	0.661	0.601-0.714
in %H								
h	0.574	0.475-0.736	0.613	0.499-0.812	0.476	0.432-0.508	0.541	0.386-0.648
hc	0.952	0.882-1.009	0.936	0.796-1.11	0.656	0.512-0.787	0.868	0.7-0.963

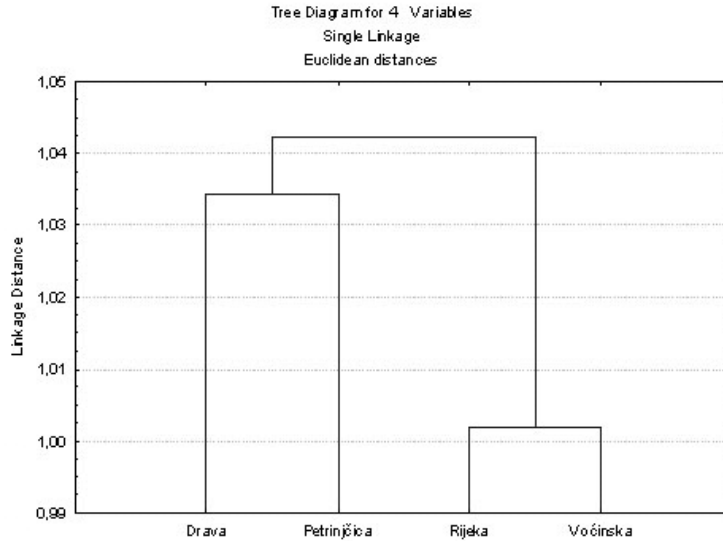


Fig. 2. Tree Diagram of Euclidean distances based on mean values of morphometric ratios for specimens from four Croatian *Sabanejewia balcanica* populations.

Of the 23 calculated morphometric ratios, only two were statistically significantly different ($p < 0.05$; Student's t-test) between the specimens from the Voćinska and Rijeka River: *lpc* and *laco* in relation to *SL* (with $p = 0.006$ and 0.015 , respectively). The differences between the specimens from the Voćinska River and those from the Petrinjčica and Drava Rivers were more pronounced (10 and 8 body ratios, respectively, were significantly different). The significant difference between populations from the Voćinska and Petrinjčica Rivers was found in *c*, *aP*, *Van*, *lpc*, *H*, *h* and *lac* in relation to *SL* ($p = 0.004$, 0.009 , 0.000 , 0.000 , 0.000 , 0.004 and 0.000); *o* and *hc* in relation to *c* ($p=0.000$ in both cases); and also *hc* in relation to *H* ($p=0.002$). The significantly different characters between goldside loaches from the Voćinska and Drava Rivers were: *c*, *Van*, *lp*, *H* and *lac* in relation to *SL* (with the following p values: 0.036 , 0.000 , 0.013 , 0.001 and 0.015); *o* in relation to *c* ($p=0.032$); *h* and *hc* in relation to *H* (with $p = 0.000$ and 0.004 , respectively). When goldside loaches from the Rijeka River were compared with those from the Petrinjčica River, 8 body ratios were significantly different: *Van*, *aP*, *H*, *laco* and *lac* in relation to *SL* ($p = 0.000$, 0.002 , 0.000 , 0.000 , 0.000); *hc* in relation to *Lc* ($p=0.006$); *h* and *hc* in relation to *H* ($p=0.013$ and 0.036 , respectively), while between the Rijeka and Drava River populations, 6 characters were significantly different: *Van*, *H*, *laco* and *lac* in relation to *SL* (p values as follows: 0.000 , 0.000 , 0.011 and 0.004); and also *h* and *hc* in relation to *H* (with $p = 0.000$ and 0.003). Between goldside loaches from the Petrinjčica and Drava Rivers, only four body ratios were statistically significantly different: *io* and *hc* in relation to *c* ($p=0.037$ and 0.007); *h* and *hc* in relation to *H* ($p = 0.009$ and 0.011).

With respect to phenotypic features, the appearance of spots at the base of the caudal fin and the position of the suborbital spine varied among specimens from the different populations. Goldside loaches from the Drava and Voćinska Rivers have two distinctive spots at the base of the caudal fin, whereas in the specimens from the Rijeka and Petrinjčica Rivers, the two spots on the caudal peduncle are connected and form a line (Fig. 3). In the specimens from the Drava, Voćinska and Petrinjčica Rivers, the suborbital spine is hidden

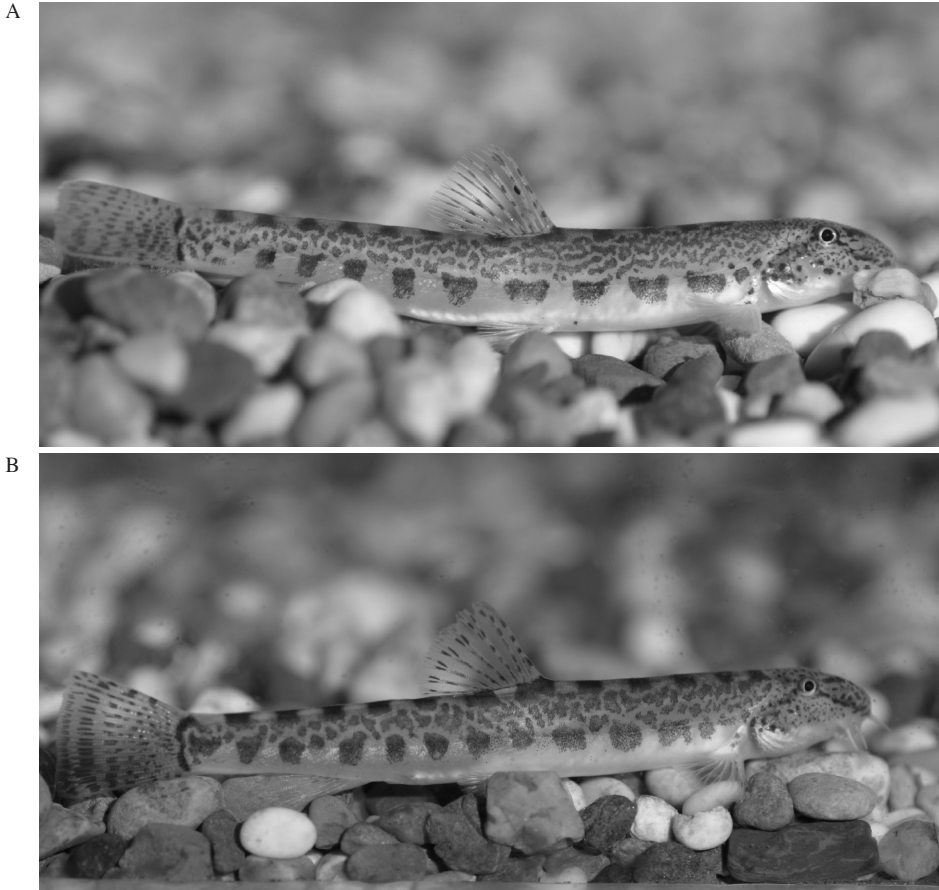


Fig. 3. A) Specimen of *Sabanejewia balcanica* from the Voćinska River. Two distinct spots can be seen at the base of the caudal fin. B) Specimen from the Rijeka River. At the base of the caudal fin, the two spots are connected into a line.

in a skin fold and cannot be observed on the skin surface. In the goldside loaches from the Rijeka River, the suborbital spine was always above the skin surface.

Sequence data

1059 bp of the *cytb* gene was obtained from 14 samples included in the study, as well as 680 bp of an additional sample (Table 1). Including the latter, a total of 9 different haplotypes was found. Additionally, 828 bp of the ATPase 8/6 genes (the complete ATPase 8 and partial ATPase 6 gene) were obtained from 10 individuals (Table 1).

Genetic diversity and phylogenetic relationships

Of the 670 variable sites, 433 were parsimoniously informative. All three methods of phylogenetic inference resulted in congruent topology of the phylogenetic trees (Fig. 4). MP analysis revealed nine equally parsimonious trees (length = 1291, consistency index (CI) = 0.654, retention index (RI) = 0.673, rescaled consistency index (RC) = 0.440). ML

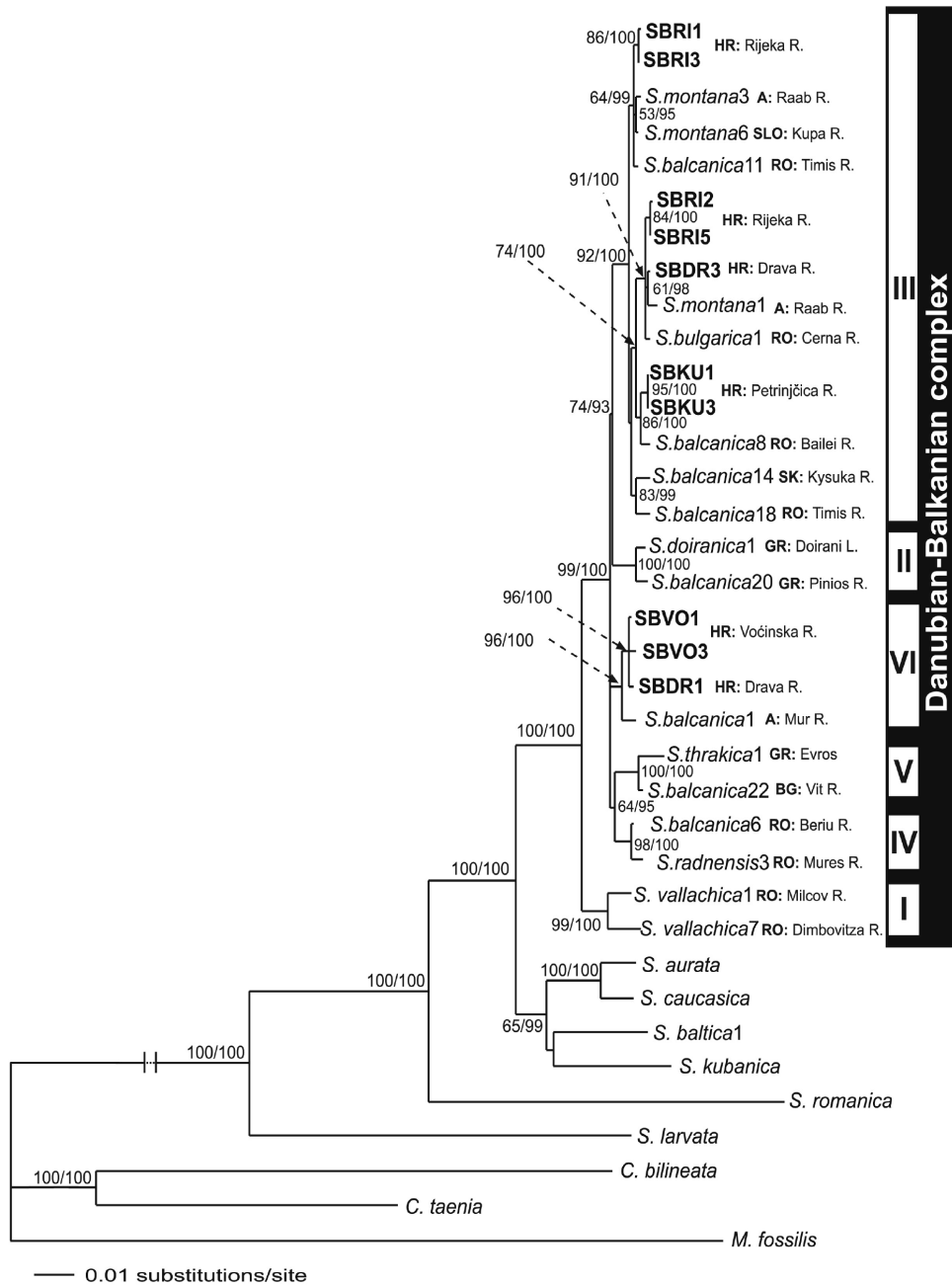


Fig. 4. Phylogenetic position of Croatian populations within the genus *Sabanejewia*. The phylogram is recovered from the ML analysis (combined *cytb* and ATPase 8/6 genes). Numbers by the nodes represent MP bootstrap values and Bayesian posterior probabilities, respectively. Samples from this study are marked in bold. Clade and sublineages designation is the same as in *P e r d i c e s* et al. 2003. Abbreviations: HR = Croatia, RO = Romania, A = Austria, SL = Slovenia, SK = Slovakia, GR = Greece, BG = Bulgaria.

analysis resulted in a single tree ($-\ln L=8539.99009$). All Croatian samples clustered with high bootstrap and Bayesian posterior probabilities support within the two sublineages of the Danubian-Balkan complex of *P e r d i c e s* et al. (2003).

Samples collected from the Voćinska River, as well as one sample from the Drava River (SBDR1) were comprised within the “*S. balcanica*” (VI) sublineage. Uncorrected pairwise sequence divergence (p-distances) in the *cytb* gene within this sublineage (including previously published sequences from the Mur River, Austria) ranged between 0.1–0.6%, while the highest p-distance found between Croatian haplotypes belonging to this sublineage was 0.3%.

The remaining haplotypes found in Croatian populations were comprised within the “*S.montana-S.bulgarica-S.balcanica*” (III) sublineage (Fig. 4). P-distances found between them were in the range of 0.2–0.9%, and overall p-distances within this sublineage were up to 1.6%. Interestingly, unlike the first one, the second sample from the Drava River (SBDR3) also clustered within this lineage. Due to an incomplete sequence, the third sample from the Drava River (SBDR2) was not included in analyses; however, based on the available 680 bp of the *cytb* gene, it has almost identical *cytb* haplotype as SBDR3 sample (Table 1) and will surely also cluster within the “*S.montana-S.bulgarica-S.balcanica*” (III) sublineage. While a single *cytb* haplotype was found in samples from the Petrinjčica River (KUP), two quite different haplotype groups with a maximal p-distance of 0.9% were found in the population of the Rijeka River.

Discussion

Meristic characters of the studied populations showed both intra- and interpopulation variability in the number of fin rays, similar to that shown for other *S. balcanica* populations (D e l i ć et al. 2003b). However, the differences between populations are in the same range as intrapopulation differences and are not statistically significant.

The total length of the investigated individuals of *Sabanejewia balcanica* from Croatia ranged from 40.75–97.2 mm, whereas the standard length was between 30.3–82.45 mm (Table 2). When compared with the measurements found for other investigated populations of this species (G r u p ĉ e & D i m o v s k i 1976, V a s i l e v a & R á b 1992 in D e l i ć et al. 2003b, W i t k o v s k i 1994 in D e l i ć et al. 2003b, E c o n o m i d i s & N a l b a n t 1996, Š u m e r & P o v ž 2000, D e l i ć et al. 2003b), the specimens from the Drava River are among the largest specimens of *S. balcanica* in Europe. Only goldside loaches from the Bijela River, also in Croatia (D e l i ć et al. 2003b), have larger morphometric characters. On the other hand, the specimens from the Voćinska River are the smallest goldside loaches reported in Europe to date.

The highest correlation factors for all investigated populations were between SL/aA and SL/pan. Š u m e r & P o v ž (2000) for *S. balcanica* from Slovenia also found the highest correlation between SL/aA. However, we found very high correlation between SL/aV in the populations from the Rijeka, Petrinjčica and Drava Rivers, indicating that the prefrontal space in the specimens from those populations is longer than in the Voćinska River population. In specimens from the Drava River, the correlation factor was also very high between SL/c and SL/h, thereby indicating it has a longer head and higher body in the area of caudal peduncle in the Drava River specimens than in other populations. It is interesting that the lowest correlation coefficient for the Voćinska River specimens was obtained between

SL/Van, while in the remaining populations, this coefficient is much higher, indicating a larger space between the ventral fins and the anal aperture. In the remaining populations, the lowest contribution to SL variation was found for io, and in the Rijeka and Petrinjčica River populations also for o.

Due to the similarities in morphometric ratios, in the Tree Diagram of Euclidean distances, the Drava and Petrinjčica River populations formed a separate cluster, while the populations from the Rijeka and Voćinska River formed a second cluster. Such clustering indicates that the populations that are geographically located closer together (*S. balcanica* populations from the Rijeka and Voćinska River on one side, and those from the Drava and Petrinjčica River on the other) are more similar to one another, and more pronounced morphometrical differences are seen between these two clusters. These morphometrical similarities are also corroborated by the t-test results. Only two characters (lpc/SL and laco/SL) were statistically significantly different between the populations from the Voćinska and Rijeka Rivers. These two rivers, despite forming parts of two different river basins (Drava and Sava River basins), are in close geographical proximity. Although ecological and habitat studies were not conducted, ecological factors in the Voćinska and Rijeka River are also likely similar, as both are small streams located on Mt. Papuk, which could be the underlying reason for the morphometrical similarities between the species. However, some phenotypical characters (spots at the base of the caudal fin) differ between goldside loaches from the different basins, namely from the Drava River basin (Voćinska and Drava Rivers) and the Sava River basin (Rijeka and Petrinjčica Rivers). This character enables identification of the individuals' pertinence to a basin and it is not likely to be connected to any ecological feature. The position of the suborbital spine could be modified by ecological factors. It is also possible that goldside loaches from the Rijeka River can erect the spine faster or on smaller stimuli than specimens from other populations. However, the morphological differences among populations of *S. balcanica* from Croatia cannot be used to make any conclusions on phylogenetic relationships or taxonomic implications.

In the phylogenetic investigation of the genus *Sabanejewia*, Perdices et al. (2003) found six main phylogenetic lineages. One of these, the Danubian-Balkan complex, has been shown to be subdivided into six further sublineages. All the Croatian samples analysed in this study belong either to the "*S. balcanica*" (VI) or to the "*S.montana-S.bulgarica-S. balcanica*" (III) sublineage of the Danubian-Balkan complex. Perdices et al. (2003) invoked ongoing vicariant separation as the explanation for the geographical pattern observed within the sublineages of the Danubian-Balkan complex. However, among the samples obtained from the same locality, we found mtDNA haplotypes (samples SBDR1 and SBDR3) belonging to two different sublineages (VI and III, Fig. 4). Such overlapping geographical distribution would suggest present (either continuous or occasional) or very recent gene flow between the "*S. balcanica*" (VI) and "*S.montana-S.bulgarica-S.balcanica*" (III) sublineages in this part of Europe. The gene flow between different sublineages of the Danubian-Balkan complex was also corroborated by the findings of Bartoňová et al. (2008). The "*S.montana-S.bulgarica-S.balcanica*" (III) sublineage of the Danubian-Balkan complex is characterised by a great diversity of haplotypes that, however, mostly appear to be poorly geographically correlated. The observed phylogeographic pattern characterised by a lack of geographical sorting of the genetically well differentiated haplotypes is an indication for the lack of long-term barriers to gene flow between populations, and to the absence of the recent bottlenecks.

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