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Genetic diversity and taxonomy of *Sabanejewia balcanica* (Osteichthyes: Cobitidae) in the waters of the Czech Republic and Slovakia

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A b s t r a c t . Seven populations (Olšava/Hornád R., Olšava/Topľa R., Ublianka R., Ondava R., Ipeľ R., Malý Dunaj R. and Vlára R.) of Balcan spined loach were subjected to phylogenetic analysis based on variability of the cytochrome *b* gene and mitochondrial specific marker. They were separated in to five groups but these groups were represented by specimens from different populations. Genetic distances among populations and among groups were quite low. Subsequently, analysis of relations among our groups and sublineages of Danubian-Balkanian complex of *Sabanejewia* was made. The first four groups were genetically close to sublineage III, while the fifth one to the sublineage IV. These findings agree with the conclusion about conspecificity of analysed Danubian-Dniester populations belonged to monotypic *S. balcanica*. Phylogenetic analysis showed that the most suitable populations for the restoration of this species in the Bečva River are populations dwelling the Ipeľ and Vlára rivers.

Key words: Balcan spined loach, conservation, cytochrome *b*, mitochondrial marker

Introduction

Sabanejewia balcanica (Karaman, 1922) is a small economically insignificant species and therefore it has been reflected by ichthyologists` a long time. But in the last 10–15 years it has attracted greater attention. The reason was wide expansion of molecular-genetic methods which has been helped by division and description of new species within the larger *Sabanejewia* taxon. Another significant fact is recent emphasis on biodiversity conservation, which enforces identification of actual populations of endangered species.

The genus *Sabanejewia* was separated from the genus *Cobitis* by V l a d y k o v (1929) but the genus name *Sabanejewia* started to be used generally after revalidation by N a l b a n t (1963). However, in 1928 V l a d y k o v already indicated description of new genus based on study of sexual dimorphism of European spined loaches (V l a d y k o v 1928). By this time, two species were included within scope of new genus, those being *S. aurata* (F i l i p p i , 1865) and *S. balcanica* (K a r a m a n , 1922). B e r g (1949) considers only one species (*S. aurata*) to be a valid but he still includes it in the genus *Cobitis*. The validity of *Sabanejewia* as a separate genus was later confirmed by G r o s s u et al. (1971) on the grounds of electrophoretic analysis of muscular proteins and serum proteins. According to recent data this genus includes several species and subspecies described by

different authors based on morphological characters (Nalbant 1957, Karaman 1963, Vasiľeva & Vasiľev 1988, Witkowski 1994, Economidis & Nalbant 1996, etc.). After critical evaluation of European ichthyofauna, Kottelat (1997) considered *S. balcanica* (Karaman, 1922), *S. bulgarica* (Drensky, 1928), *S. larvata* (Filippi, 1859) and *S. romanica* (Bacescu, 1943) as valid species of the genus *Sabanejewia* in Europe (exclusive of the former USSR).

Further advances in species structure of genus the *Sabanejewia* was brought by application of karyological (Vasiľeva & Ráb 1992) and genetic methods (Ludwig et al. 2000, Perdices et al. 2003). Based on analyses of mtDNA, Perdices et al. (2003) defined inside the genus *Sabanejewia* six main evolution lineages, namely: *S. larvata*, *S. romanica*, *S. aurata*/*S. caucasica*, *S. kubanica*, S. *baltica* and the Danubian-Balkanian complex, that was further divided into six sublineages: I. S. *vallachica*/*S. balcanica* from a syntopic population from Romania, II. *S.balcanica*/*S.doiranica* from western Greece, III. S. *bulgarica*/*S. balcanica*/*S. montana* from the middle part of the Danube basin, IV. *S. radnensis*/*S. balcanica* from the Mures basin, V. *S. thrakica*/*S. balcanica* from Greece and VI. *S. balcanica* from the Mur basin.

The taxonomic status of *Sabanejewia* populations in the Danube River basin and in territory of Balkan Peninsula is still ambiguous. Iftime (2002) confirmed this from morphometric analyses. He expressed the opinion that it is not necessary to keep current division to subspecies in the Danube and Dniester river basins.

It is evident that relations among *Sabanejewia* populations in drainages of western Carpathian Bow (left tributaries of the Slovakian Danube, Tisza and Bodrog river basins) as well as in other parts of the Danube River basin are insufficient or totally missing. It is necessary to clear this situation with regards to European conservation legislature, namely Council Directive 92/43/EEC. In supplement II two species *S. aurata* and *S. larvata* are presented. For member countries of EU it is obligatory to define special areas of conservation (SAC) for *Sabanejewia* species. Thus, for national legislature it is needful to clarify species taxonomy of the genus *Sabanejewia*, because in national legislature of the Czech Republic and Slovakia only *Sabanejewia aurata* is mentioned.

Actually, *S. balcanica* occurs in the Czech Republic only in a short lap of the Vlára River near state boundary with Slovakia (Lusk et al. 2002). In the 1950s, this species also dwelt the Bečva River basin, but now it is absent there (Lusk et al. 2000). In territory of Slovakia, *S. balcanica* is a common species in the Tisza River basin, while in western part of Slovakia, which belongs to the Danube River basin, its distribution is restricted to several rivers (Košč o et al. 2006).

Main goals of our study were 1) to define genetic diversity and relations among *Sabanejewia* populations in territories of the Czech Republic and Slovakia, 2) to evaluate relations among these populations and sublineages revealed in the Danubian-Balkanian complex and subsequently to define the taxonomic status of *Sabanejewia* loaches in the Czech Republic and Slovakia, and 3) to identify vanished population from Bečva River and, if possible, to find the most related population(s) based on relationship or similarity for the restoration of this species in the Bečva River.

Materials and Methods

Individuals of *S. balcanica* were caught by electrofishing in 2002–2006. A total number of 77 specimens (Table 1) from seven populations from the Danube and Tisza river basins were

Table 1. Analysed specimens from seven populations from the Danube and Tisza river basins.

Specimen No.	No. of specimens	Drainage system	Collection locality	Accession No.
2727-2730	4	Tisza	R. Oľšava/Hornád (Slovakia)	DQ996468-471
2732	1	Tisza	R. Oľšava/Hornád (Slovakia)	DQ996472
2734-2736	3	Tisza	R. Oľšava/Hornád (Slovakia)	DQ996473-475 EU400433-434
2949	1	Danube	R. Vlára (Czech Republic)	DQ996517
2951-2952	2	Danube	R. Vlára (Czech Republic)	DQ996518-519
3570-3574	5	Tisza	R. Ublianka (Slovakia)	DQ996476-480 EU400439-442
4526-4527	2	Tisza	R. Ondava (Slovakia)	DQ996481-482 EU400443-444
4889-4893	5	Tisza	R. Oľšava/Topľa (Slovakia)	DQ996483-487 EU400435-437
4910-4914	5	Tisza	R. Oľšava/Topľa (Slovakia)	DQ996488-492 EU400438
5022-5023	2	Danube	R. Vlára (Czech Republic)	EU400418-419
5039-5040	2	Danube	R. Vlára (Czech Republic)	EU400420-421
5604-5616	13	Danube	R. Ipeľ (Slovakia)	DQ996493-505 EU400426-428
5621-5624	4	Danube	R. Ipeľ (Slovakia)	DQ996506-509 EU400429
5655-5661	7	Danube	R. Ipeľ (Slovakia)	DQ996510-516 EU400430-432
5662	1	Danube	R. Ipeľ (Slovakia)	EF447286
5667	1	Danube	R. Vlára (Czech Republic)	DQ996520
5668	1	Danube	R. Vlára (Czech Republic)	EU400422
5669-5679	11	Danube	R. Vlára (Czech Republic)	DQ996521-531 EU400423-425
6162-6163	2	Danube	R. Bečva (Czech Republic)	DQ996534-535
6165	1	Danube	R. Bečva (Czech Republic)	EU400445
6166	1	Danube	R. Bečva (Czech Republic)	DQ996536 EU400446
6167-6168	2	Danube	R. Bečva (Czech Republic)	EU400447-448
6600-6601	2	Danube	R. Vlára (Czech Republic)	EF447287-88
6602-6603	2	Danube	R. Vlára (Czech Republic)	DQ996532-533
6964-6965	2	Danube	R. Malý Dunaj (Czech Republic)	EF447289-90
6967-6968	2	Danube	R. Malý Dunaj (Czech Republic)	EF447291-92
6972	1	Danube	R. Malý Dunaj (Czech Republic)	EF447293

analysed on the cytochrome *b* (cyt *b*) gene (Fig. 1). Three individuals from the Bečva River were analysed with partial success. These individuals were caught in 1952 and preserved probably in ethanol. Furthermore, 23 selected individuals and newly eight individuals (Table 1) were analysed on the mitochondrial specific (mt specific) marker (Fig. 1).

Complete genomic DNA was isolated from fin clippings (preserved in 95% ethanol) according to standard phenol-chloroform-isoamylalcohol method (Sambrook et al. 1989) with minor modifications. Entire cyt *b* gene was amplified (1140 bp) with primers and under conditions previously described by Perdices & Doadrio (2001), mt specific

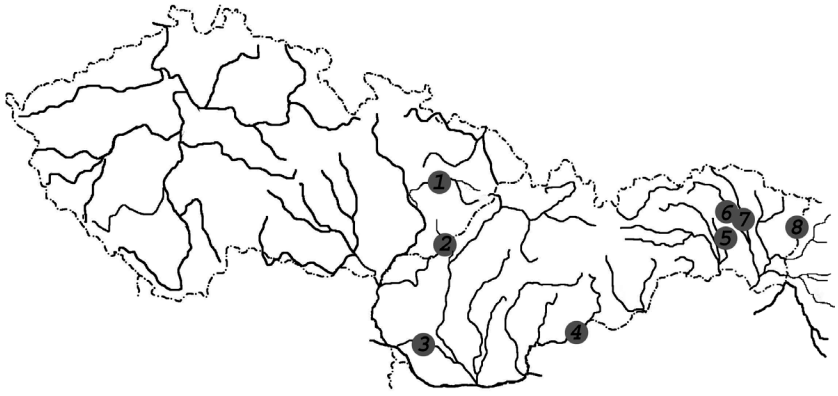

Fig. 1. The map of sample localities in the Czech Republic and Slovakia. Localities are follows: 1-Bečva R., 2-Vlára R., 3-Malý Dunaj R., 4-Ipeľ R., 5-Olšava/Hornád R., 6-Olšava/Topľa R., 7-Ondava R., 8-Ublianka R.

marker (448 bp) was amplified with primers and under conditions according to L u d w i g et al. 2000.

In the case of samples of *S. balcanica* from the Bečva R. we had to divide cyt *b* gene in two parts. The first part was PCR amplified with primers L15267 (5‘-AAT GAC TTG AAG AAC CAC CGT-3‘) and H15891 (5‘-GTT TGA TCC CGT TTC GTG TA-3‘) (B r i o l a y et al. 1998), the second part with primers L15840 (5‘-GAT TCT TCG CAT TCC ACT T-3‘) (B r i o l a y et al. 1998) and H15918R (5‘-CTC CAT CTC CGG TTT ACA AGA C -3‘) (Song et al. 1998). Amplification of these samples proceeded according to the following protocol: denaturation 95 °C 3 min, 35 cycles 90 °C 1 min/46 resp. 38 °C 1 min/72 °C 2 min, final extension 72 °C 7 min. The PCR products obtained were purified and sequenced in an automated ABI PRISM 310 Genetic analyzer.

All sequences were aligned in programme MEGA 4 (T a m u r a et al. 2007) and published in GenBank under accession numbers DQ996468-DQ996536, EF447286-93, EU400418-48. Sequences were translated for verification of correct alignment. No stop codons were found. Further, nucleotide composition and variable sites of sequences were examined. Chi-square homogeneity test of base frequencies was performed in PAUP ver 4.0b10 (S w o f f o r d 2003). Phylogenetic trees were constructed in MEGA 4 with application of Neighbour-joining (NJ) (S a i t o u & N e i 1987) and Maximum parsimony (MP) algorithms. Previously published sequence of *Misgurnus fossilis* (GenBank accession number AF263097, P e r d i c e s & D o a d r i o 2001) was used as outgroup for phylogenetic analyses. FindModel web site (http://hcv.lanl.gov/content/hcv-db/findmodel/findmodel.html) was employed to determine best model of evolution that fits to our data. Consequently, genetic distances were computed and NJ phylogenetic trees constructed using TN93 model with homogenous pattern among lineages and gamma equal 1.5 (T a m u r a & N e i 1993). In MP analysis, only minimal trees were retained. Bootstrap analysis (1000 replicates for NJ and 200 replicates for MP) was used to evaluate robustness of topology, with cut-off level of 60 per cent.

For evaluation of relations among sublineages of the Danubian-Balkanian complex, 30 previously published cyt *b* sequences of various *Sabanejewia* species (P e r d i c e s et al. 2003) were used in some analyses (GenBank accession numbers AY059332, 336, 338-342, 356, 358, 361-363, 365, 368; AF263094; AF499173, 176-177, 179, 182, 184, 188, 189, 191, 193, 195-198, 200).

Results

Molecular characteristics of cyt *b* gene and molecular systematics of *Sabanejewia* populations in the Czech Republic and Slovakia

The molecular characteristics of 77 analysed sequences from two populations from the Czech Republic and five populations from Slovakia are summarized in Table 2. Base frequencies were homogenous across variable sites (chi square = 9.26, df = 204, P = 1.0). Sequence variability was mainly due to transitions and substitutions on the third position. Total of 57 variable sites (from that 35 parsimony informative), which define 47 unique haplotypes, were found. Genetic distances between populations varied from 0.6 to 1.5 %, average was 1.0 % (Table 3).

Phylogenetic analysis identified the presence of five basic groups in territories of the Czech Republic and Slovakia (Fig. 2), with high to moderate bootstrap support (range: <60 – 95%). Most heterogenity was found in the group 2: it comprises of 2 individuals from the Ublianka R., 2 individuals from the Oľšava R. (basin Topľa R.) and 2 individuals from the Malý Dunaj R. Group 1 includes most of individuals from the Ipeľ R., all individuals from the Oľšava R. (basin Hornád R.) and a single specimen from the Vlára R. and Malý Dunaj R. Group 3 includes 18 out of 19 individuals from the Vlára R. and remaining individuals from the Ipeľ R, two individuals from the Malý Dunaj R. and a single specimen from the Ondava R. The group 4 comprised three specimens from the Ublianka R. The most diverged group 5 (mean divergence 1.3 %) contains a single specimen from the Ondava R. and most of individuals from the Oľšava/Topľa R. Average genetic distances between identified groups varied from 0.6 to 1.6 %, total average was 1.1 % (Table 4).

Table 2. Molecular characteristics of the mitochondrial cyt *b* gene in *Sabanejewia balcanica*.

Nucleotide composition			Variable sites	Parsimony informative sites
% T	30.2	First position	0.7 (8)	0.3 (3)
% C	27.3	Second position	0.7 (8)	0 (0)
% A	26.1	Third position	3.6 (41)	2.8 (32)
% G	16.4	Total	5.0 (57)	3.1 (35)

Values represent percentages, with absolute numbers in parentheses.

Table 3. Genetic distances between tested populations computed due to Tamura-Nei TN93 model. Below the line are distances for the Bečva R. population with very high values of differences from the others.

	1	2	3	4	5	6	7
[1] Oľšava/Hornád							
[2] Ublianka	0.007						
[3] Ondava	0.010	0.008					
[4] Oľšava/Topľa	0.012	0.009	0.007				
[5] Ipeľ	0.006	0.008	0.012	0.013			
[6] Vlára	0.014	0.011	0.012	0.015	0.011		
[7] Malý Dunaj	0.010	0.009	0.012	0.014	0.009	0.010	
[8] Bečva	0.112	0.115	0.119	0.120	0.113	0.117	0.112

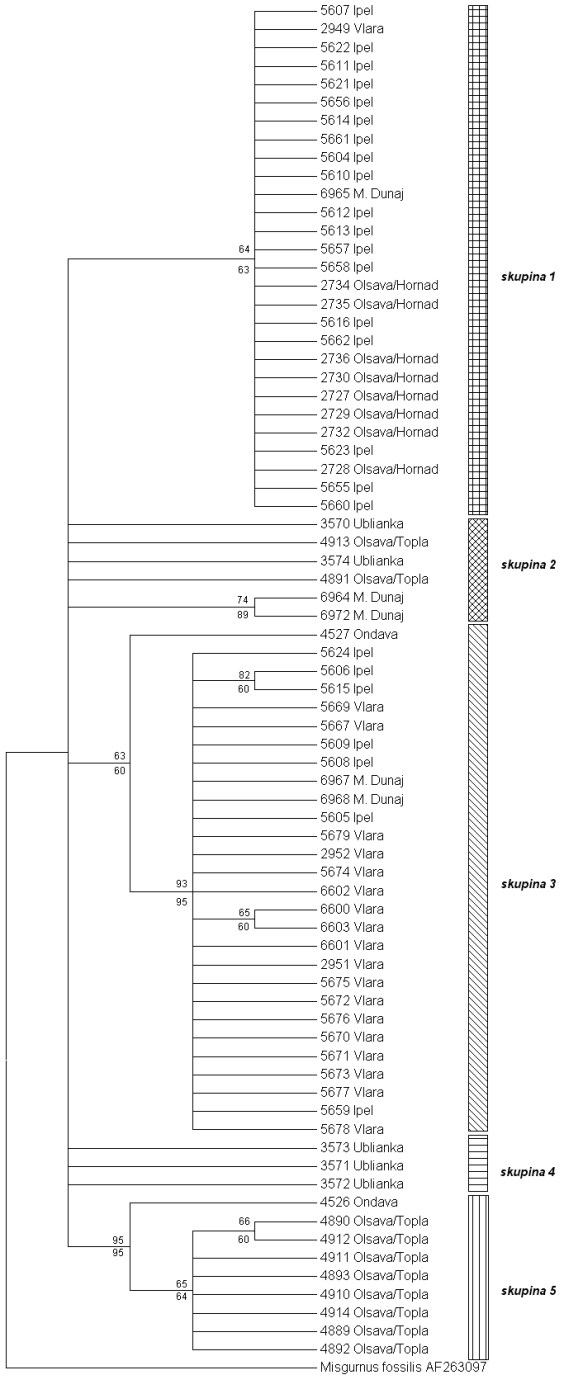

Fig. 2. Phylogenetic relationships of *Sabanejewia balcanica* populations in the Czech Republic and Slovakia based on the entire mitochondrial cytochrome *b* gene (1140 bp). Tree was computed by Neighbour-joining (NJ) analysis based on 1000 replicates and Maximum parsimony (MP) based on 200 replicates. Upper values represent NJ boostrap estimates and lower values represent MP estimates.

Table 4. Genetic distances between detected lineages computed due to Tamura-Nei TN93 model.

	1	2	3	4
Group 1				
Group 2	0.006			
Group 3	0.008	0.008		
Group 4	0.014	0.013	0.009	
Group 5	0.014	0.012	0.011	0.016

Evaluation of relations among examined populations and sublineages of the Danubian–Balkanian complex described by Perdices et al. (2003)

The analysis of relations among examined *Sabanejewia* populations and sublineages defined among Danubian-Balkanian loaches was based on selected individuals from each group mentioned above. These individuals were selected according to the number of specimens and observed heterogenity of given group (6, 5, 7, 3 and 4 individuals, respectively). Sequences of chosen individuals were aligned with sequences used by Perdices et al. (2003) for determination of the Danubian-Balkanian complex of the genus *Sabanejewia*. The phylogenetic analysis suggests that our groups 1–4 can be included in the most heterogenous sublineage III, whereas group 5 belongs to sublineage IV of the Danubian-Balkanian complex (Fig. 3). Further, each of two individuals from the Ondava population belong to the different sublineage (4527 sublineage III, 4526 sublineage IV).

Identification of a vanished spined loach population from the Bečva River

Presently vanished population of *S. balcanica* from the Bečva R. was analysed using museum materials, originally collected probably in 1952. Only three specimens were amplified and sequenced successfully on the cyt *b* gene. However, alignment to other obtained sequences demonstrated their average genetic distances from other populations to exceed 11.5 % (Table 3) and thus correspondent at least to specific level of divergence.

After division of sequence into two parts, first part (1-570 bp) was found to cause this unusually high variability. On average, divergence of the first half to the other populations reached 26.9 %, in contrast to the second half with average sequence divergence of 0.8 %. No sequence similar to the first part of sequence revealed in loaches from the Bečva R. was found either in the genus *Sabanejewia* or in the entire GenBank database. The phylogenetic analysis showed on the grounds of the second part of the gene that the Bečva R. population belongs to group 1. For acknowledging the presence or absence of the pseudogene in the first part mitochondrial specific combination of primers was used. On the mt specific marker only four individuals were analysed successfully. Phylogenetic analysis showed on the grounds of the mt specific marker that the Bečva R. population belongs to group 1 and 3 (Fig. 4) and the analysis also confirmed correct division into the five groups.

Discussion

Our analysis showed that populations of *Sabanejewia balcanica* in areas of the Czech Republic and Slovakia are quite heterogenous and hardly genetically distinguishable.

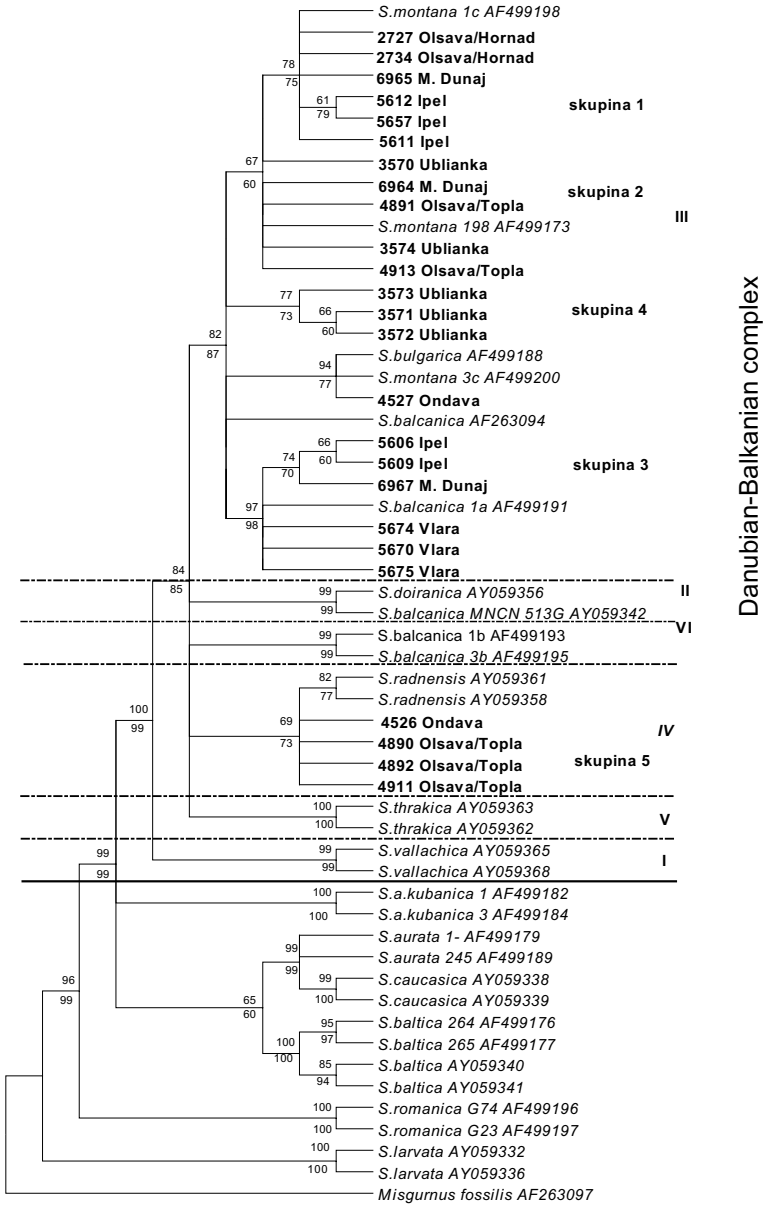

Fig. 3. Relations among our groups 1-5 and sublineages I.-VI. of Danubian-Balkanian complex based on the entire mitohondrial cytochrome *b* gene (1140 bp). Tree was computed by Neighbour-joining (NJ) analysis based on 1000 replicates and Maximum parsimony (MP) based on 200 replicates. Upper values represent NJ boostrap estimates and lower values represent MP estimates. * represents 100 per cent bootstrap support.

Although the phylogenetic analysis separates populations into five groups, this grouping does not correspond to geographic distribution of populations. We found mixed population composition for four of five groups (1+3 Ipeľ and Vlára, 2+4 Ublianka, 2+5 Oľšava/Topľa, 3+5 Ondava, 1+2+3 Malý Dunaj), except for the group 5, which contains Oľšava/Hornád population only. This result agrees with previous findings (P e r d i c e s et al. 2003), having

demonstrated that sublineage III of the Danubian-Balkanian complex, including our groups 1–4, is represented by samples from a large portion of the middle Danube River basin. Furthermore, our group 5 was found to be most related to sublineage IV including loaches which have been described as an independent taxon (*Cobitis aurata radnensis* Bănărescu, Muler et Nalbant, 1960) from the Mures R. basin in Romania (also belongs to the Danube R. basin) but was later considered a younger synonym of *Sabanejewia balcanica* (K o t t e l a t 1997). Further, we found as well as B u j et al. (2008) that individuals from one population (Ondava R., Olšava/Topľa) belong into two different sublineages. Thus we can give support to idea of gene flow among sublineages.

At the same time our data confirm the results of morphological and morphometric studies in *Sabanejewia* populations from Romania and from the Danube and Dniester river

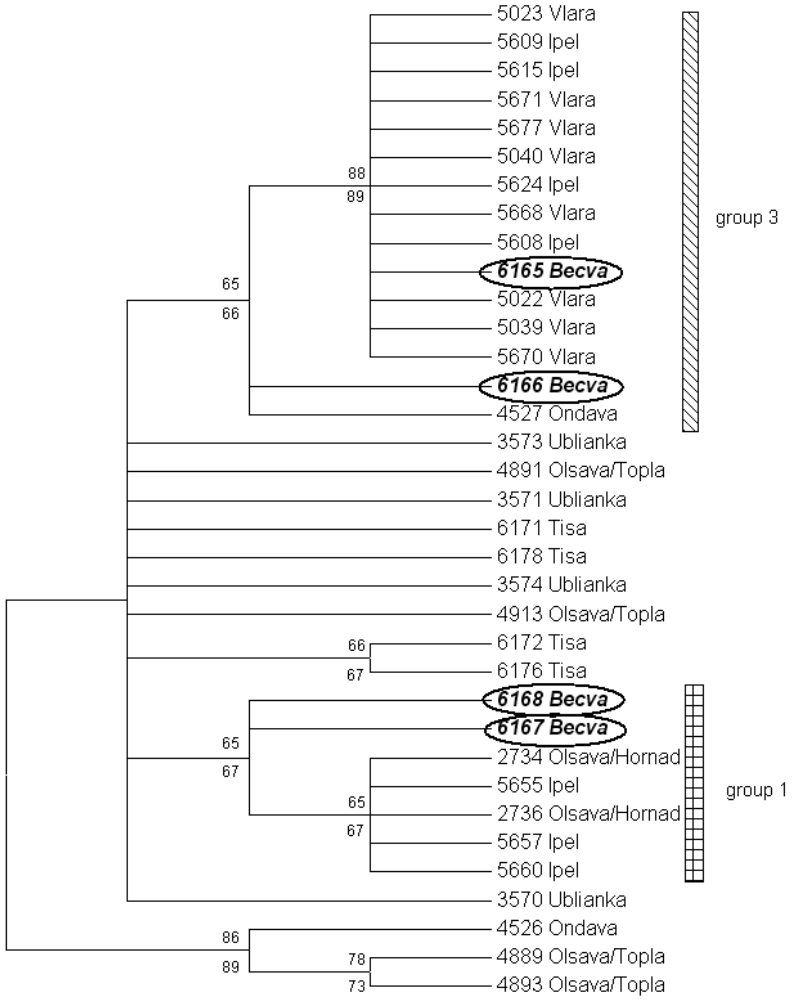

Fig. 4. Relations among the Bečva River population and groups 1-5 based on the mitochondrial specific marker (448 bp). Tree was computed by Neighbour-joining (NJ) analysis based on 1000 replicates and Maximum parsimony (MP) based on 200 replicates. Upper values represent NJ boostrap estimates and lower values represent MP estimates.

basins (I f t i m e 2002). According to recent findings of different authors I f t i m e (2002) concludes that all *Sabanejewia* populations occurring in the Danube and Dniester river basins from Hungary, Romania and Republic of Moldova belong to the same monotypic species *Sabanejewia balcanica*. Thus, nominal Danubian subspecies can be considered as synonyms of *S. balcanica* (K o t t e l a t 1997, I f t i m e 2002). Our findings lead us to similar conclusions: genetic heterogeneity within particular populations, relatively small total variability and geographically overlapping occurrence of close variants do not provide sufficient ground for taxonomic classification into more than one species in areas of the Czech Republic and Slovakia.

By the analysis of the Bečva River population difficulties with amplification occured with one of possible explanations (probably damaged genetic material due to prolonged storage) and subsequently with sequencing (high variability of the first half of the gene only). We examined the possibility of amplification of the nuclear pseudogene of the first part, but the presence of the pseudogene was not confirmed. Our relation among the Bečva River and groups 1–5 was performed based on the second half of the cyt *b* gene and mt specific marker. The Bečva population belongs to group 1 (which is otherwise dominated by Ipeľ River population samples) and 3 (the most samples from Ipeľ and Vlára River popualtions). Therefore, the population of the Bečva River could be restored either from the Ipeľ River, which is most similar genetically, or from the Vlára River, which is also close geographically. There is also genetically similarity between the Vlára and Ipeľ River populations, as they form group 3 together.

A c k n o w l e d g e m e n t

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