

Original Research

UNCOVERING HIDDEN DIVERSITY OF *Sabanejewia balcanica* IN SERBIA:  
THE DISCOVERY OF NOVEL HAPLOTYPES

DOI: 10.5937/EcoTER26181M

Tamara Mitic<sup>1,a</sup>, Ana Marić<sup>2,b</sup>, Katarina Jovičić<sup>1,c</sup>, Vesna Dikanović<sup>1,d\*</sup>,  
Nikola Marinković<sup>1,e</sup>, Stefan Andus<sup>1,f</sup>, Jelena Čanak Atlagić<sup>1,g</sup>

<sup>1</sup>Department of Hydroecology and Water Protection, Institute for Biological Research  
“Siniša Stanković”, National Institute of the Republic of Serbia, University of Belgrade, Bulevar  
despota Stefana, 142, 11108 Belgrade, SERBIA

<sup>2</sup>University of Belgrade, Faculty of Biology, Studentski trg 16, 11000 Belgrade, SERBIA

<sup>a</sup> <https://orcid.org/0009-0007-4485-9430> <sup>b</sup> <https://orcid.org/0000-0003-1757-3000>

<sup>c</sup> <https://orcid.org/0000-0002-5105-1532> <sup>d</sup> <https://orcid.org/0000-0002-3876-3420>

<sup>e</sup> <https://orcid.org/0000-0002-0360-5393> <sup>f</sup> <https://orcid.org/0000-0002-1102-7984>

<sup>g</sup> <https://orcid.org/0000-0002-5330-1558>

## Abstract

The genus *Sabanejewia* comprises a diverse group of bottom-dwelling loaches with complex phylogenetic relationships and high morphological and genetic variability across European river systems. This study aimed to determine the phylogenetic position of a western Serbian population of *S. balcanica* within the broader Danubian–Balkan framework and assess its genetic distinctiveness. Nine individuals of *S. balcanica* were sampled from the Ribnica River in Serbia (Kolubara River basin), and mitochondrial cytochrome *b* (1,140 bp) sequences were used for phylogenetic reconstruction. A total of 240 variable sites and 128 parsimony-informative sites were recovered by analyzing *cyt b* sequences. Despite the small sample size, two novel mitochondrial haplotypes were identified, which grouped with Serbian sequences of *S. balcanica* retrieved from GenBank. The phylogenetic tree confirmed high diversity and complex relationships in the Danubian-Balkan complex. Newly detected haplotypes suggest high genetic complexity of *S. balcanica* in Serbia, highlighting the importance of continued genetic research to support conservation planning for this protected species in Serbia, as small, localized populations may retain distinct genetic signatures.

**Keywords:** Balkan spined loach, protected species, cytochrome *b*, phylogeny, freshwater.

## INTRODUCTION

The golden loaches of the genus *Sabanejewia* Vladykov, 1929 represent a highly diverse group of rheophilic fish species inhabiting clear, fast-flowing waters. The first comprehensive study on the phylogeny of this genus in Europe by Perdices *et al.* [1] identified six monophyletic mitochondrial lineages, namely, *S. larvata*, *S. aurata*, *S. kubanica*, *S. romanica*, *S. caucasica*, and *S. baltica*. An additional six sublineages comprised a distinct

\* [djiki@ibiss.bg.ac.rs](mailto:djiki@ibiss.bg.ac.rs)

Danubian-Balkan complex consisting of *S. balcanica*, *S. bulgarica*, *S. montana*, *S. vallachica*, *S. doiranica*, and *S. thrakica*, with gene flow between sublineages confirmed [2]. Notably, *S. balcanica* was detected across all six sublineages, highlighting its occurrence throughout the various European drainages, high genetic heterogeneity, and lack of the congruence between geographic distribution and phylogeny [2].

The Balkan golden loach, *S. balcanica*, has been recorded in Serbian rivers of the Black Sea and Aegean Sea basins [3], alongside *S. bulgarica* [4]. Both species display a high level of phenotypic plasticity [5]. This results in overlapping diagnostic morphological traits, making morphology-based identification difficult and somewhat unreliable. More recently, a new species of the genus *Sabanejewia* was identified in Serbia, *S. romanica*, a species native and endemic to Romania [6].

According to the IUCN Red List of Threatened Species [7], *S. balcanica* is currently classified as a taxon of Least Concern (LC), but habitat alterations have made local populations endangered [3]. The primary threats to this species include habitat degradation through river regulation, gravel extraction, pollution, and habitat fragmentation caused by damming, all of which reduce the availability of the clean, well-oxygenated, fast-flowing waters this species depends on. Declared as a strictly protected species according to the national legislation, the collection of *S. balcanica* is prohibited without a special permit [8]. As this species is also included in Annex II of the EU Habitats Directive, the establishment of designated conservation areas is required [9–10]. Given the need for this species' protection, its role as a bioindicator of freshwater ecosystem health should be emphasized, making research on its biology, genetic diversity and distribution in Serbian waters necessary. Therefore, the aim of this study is to identify haplotypes present in the sample from the Ribnica River and to infer the phylogenetic position of these individuals within the genus *Sabanejewia*.

## MATERIALS AND METHODS

### Sampling and DNA extraction

In 2023, nine individuals of the *S. balcanica* were sampled at the Ribnica River (44° 12' 20.916" N, 20° 5' 33.0576" E; Kolubara river basin, Danube watershed) using reversible electronarcosis, and their fin clips were preserved in 96% ethanol until further genetic analyses. Genomic DNA was extracted using the Quick-DNA Miniprep kit (Zymo Research Corporation, USA) following manufacturer's instructions. The mitochondrial gene cytochrome *b* (cyt *b*) has been amplified, as this gene is a commonly used marker in phylogenetic studies [11]. Primers used for amplification of the cyt *b* were P1-F: 5'-GACTTGAAGAACCACCGTTG-3' and P2-R: 5'-CTCCGATCTTCGGATTACAAGAC-3'. The PCR amplification was performed in 30 µl volume under the following conditions: an initial denaturation step at 94°C for 1 min, followed by 30 amplification cycles consisting of denaturation at 94°C for 15 s, primer annealing at 52°C for 15 s, and extension at 72°C for 1 min. A final extension step was carried out at 72°C for 10 min. Amplified fragments were checked on 1.5% agarose gels stained with SybrGreen (Lonza, CH). PCR products were sent to MacroGen Europe for purification and bidirectional sequencing.

## Molecular data analysis

The obtained sequences were manually assembled and compared with existing sequences in the National Center for Biotechnology Information (NCBI) database using the BLAST tool. The *cyt b* sequence of *Sabanejewia larvata* was used as outgroup. To determine the phylogenetic position of sampled *S. balcanica* individuals, 44 sequences of the Danubian-Balkan complex from the Balkans were retrieved from NCBI and included in the analysis.

The following software was used: Chromas 2.6.6 for visualization of raw chromatograms; Clustal X2 for sequence alignment; IQ-TREE 3.0.1 for selecting the most appropriate model of nucleotide substitution and phylogenetic tree reconstruction; FigTree 1.4.4 for visualization and editing of the phylogenetic tree [12–14]. The best-fitting model of nucleotide substitution was the Tamura-Nei model with empirical base frequencies and gamma-distributed rate variation among sites (TN+F+G4). The phylogenetic tree was constructed using the maximum-likelihood (ML) method with 100,000 bootstrap replicates to assess branch support.

## RESULTS AND DISCUSSION

The length of the *cyt b* sequence across all nine samples was 1,140 bp, with 240 variable sites (21.1%) and 128 parsimony-informative sites (11.2%). Utilizing the BLAST tool revealed both previously reported haplotypes (A2105 and 66), as well as two novel haplotypes not present in GenBank. The phylogenetic tree was constructed using sequences of novel haplotypes, as well as 44 sequences obtained from GenBank (Table 1).

**Table 1** Accession numbers of haplotypes used for the reconstruction of the phylogenetic tree. Novel haplotypes from this study are bolded

| Taxon name          | Haplotype name | Accession number | Taxon name                 | Haplotype name | Accession number           |
|---------------------|----------------|------------------|----------------------------|----------------|----------------------------|
| <i>S. balcanica</i> | 66             | AF499192         | <i>S. balcanica</i>        | C90            | HQ291824                   |
| <i>S. balcanica</i> | RJ57           | HQ291787         | <i>S. balcanica</i>        | C92            | HQ291825                   |
| <i>S. balcanica</i> | RJ58           | HQ291788         | <i>S. balcanica</i>        | C358           | HQ291831                   |
| <i>S. balcanica</i> | IL45           | HQ291793         | <i>S. balcanica</i>        | C359           | HQ291832                   |
| <i>S. balcanica</i> | IL46           | HQ291794         | <i>S. balcanica</i>        | C367           | HQ291833                   |
| <i>S. balcanica</i> | A1219          | HQ291795         | <i>S. balcanica</i>        | C824           | HQ291834                   |
| <i>S. balcanica</i> | A1221          | HQ291796         | <i>S. balcanica</i>        | C825           | HQ291835                   |
| <i>S. balcanica</i> | A2097          | HQ291800         | <i>S. balcanica</i>        | C826           | HQ291836                   |
| <i>S. balcanica</i> | A2105          | HQ291803         | <i>S. balcanica</i>        | MNCN<br>388CRR | AY059361                   |
| <i>S. balcanica</i> | A2219          | HQ291805         | <i>S. balcanica</i>        | MNCN<br>354CRA | AY059346                   |
| <i>S. balcanica</i> | A2341          | HQ291809         | <i>S. balcanica</i>        | MNCN<br>340CRA | AY059345                   |
| <i>S. balcanica</i> | A2342          | HQ291810         | <i>S. balcanica</i>        | 105            | AF499185                   |
| <i>S. balcanica</i> | A2793          | HQ291813         | <b><i>S. balcanica</i></b> | <b>RIB1</b>    | <b>Novel<br/>haplotype</b> |
| <i>S. balcanica</i> | A2800          | HQ291814         | <b><i>S. balcanica</i></b> | <b>RIB2</b>    | <b>Novel<br/>haplotype</b> |
| <i>S. balcanica</i> | A2802          | HQ291815         | <i>S. bulgarica</i>        | H41            | MN149891                   |

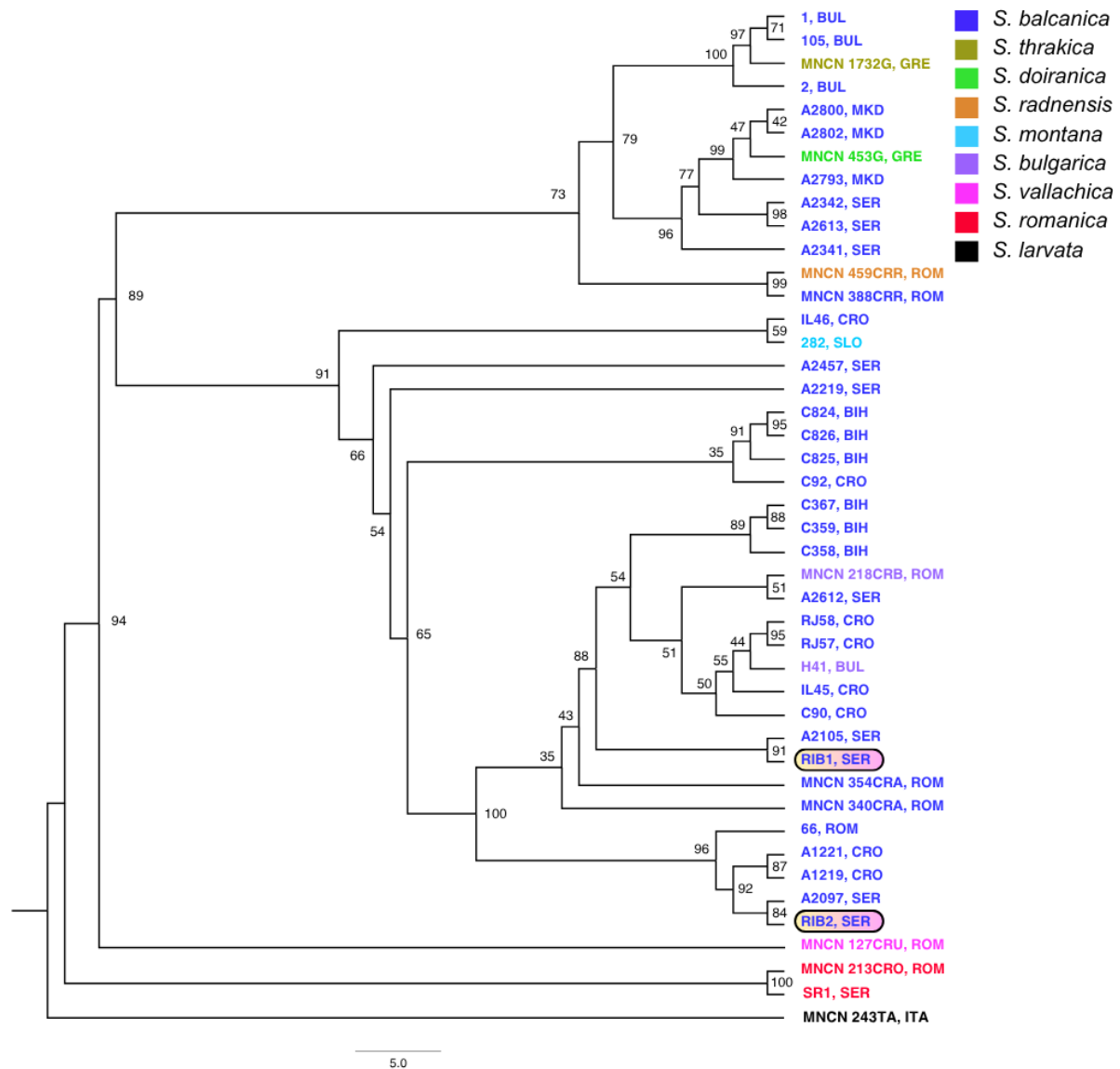
Table 1 continued

|                     |                |          |                      |                |          |
|---------------------|----------------|----------|----------------------|----------------|----------|
| <i>S. balcanica</i> | 1              | AF499171 | <i>S. bulgarica</i>  | MNCN<br>218CRB | AY059353 |
| <i>S. balcanica</i> | 2              | AF499172 | <i>S. vallachica</i> | MNCN<br>127CRU | AY059365 |
| <i>S. balcanica</i> | SR1            | ON866527 | <i>S. doiranica</i>  | MNCN 453G      | AY059354 |
| <i>S. balcanica</i> | MNCN<br>213CRO | AY059337 | <i>S. thrakica</i>   | MNCN 1732G     | AY059362 |
| <i>S. balcanica</i> | A2457          | HQ291818 | <i>S. radnensis</i>  | MNCN<br>459CRR | AY059357 |
| <i>S. balcanica</i> | A2612          | HQ291821 | <i>S. montana</i>    | 282            | AF499175 |
| <i>S. balcanica</i> | A2613          | HQ291822 | <i>S. larvata</i>    | MNCN 243TA     | AY059331 |

According to Perdices *et al.* [1], the most basal branch of the genus is *S. larvata*, which was used as outgroup, followed by the *S. romanica* branch included in this study due to its distribution in the Balkans (Figure 1). The two most diverged lineages originated in the late to middle Miocene, followed by the emergence of the Baltic-Caucasian-Caspian lineages in the Pliocene [1].

The existence of the Danubian-Balkan complex is strongly supported by high bootstrap values, with *S. vallachica* being the most divergent, also reported by Perdices *et al.* [1]. Some of the internal branches show weaker support, indicating shallower divergence between the sublineages of the complex. This may be linked to the more recent emergence of the complex in the Pleistocene, when cycles of glaciation and interglaciation reshaped the river systems [1].

Sequences of *S. balcanica* within the Danubian-Balkan complex have been previously reported across multiple sublineages without clear geographic congruence [2], a pattern also reflected in the present study. According to Fedorčák *et al.* [15], *S. balcanica* and *S. bulgarica* comprise two distinct lineages. However, in the current study, their divergence was less pronounced, with sequences of both species nested within the same clade, consistent with the high degree of genetic heterogeneity and shallow divergence characteristic of the Danubian-Balkan complex. This result should, however, be interpreted with caution, as branch support based on bootstrap values was low to moderate in the central part of the tree. A more comprehensive assessment of the relationship between these two species, incorporating an expanded dataset and morphometric analysis, will be addressed in a subsequent study. The novel haplotypes identified in this study clustered with reference sequences of *S. balcanica* from Serbia, supporting their assignment to this species. This is further supported by morphological identification conducted during sampling. Some of the haplotypes detected in this study had been previously reported for Serbia (A2105) and Romania (66), further highlighting the complex relationships among members of the Danubian-Balkan complex and the lack of strict geographic structuring.



**Figure 1** Phylogenetic tree depicting the position of investigated samples within the genus *Sabanejewia*. Values at the tree nodes represent bootstrap values. Colored taxa names correspond to the species given in the legend. Novel haplotypes from this study are highlighted. Abbreviations of the countries: SER – Serbia, CRO – Croatia, BIH – Bosnia & Hercegovina, ROM – Romania, BUL – Bulgaria, SLO – Slovenia, GR – Greece, ITA – Italy

These findings support a relatively recent Pleistocene diversification of the Danubian-Balkan complex, where shallow genetic differences and unresolved internal branching patterns are consistent with a rapid radiation driven by glacial-interglacial cycles that reshaped river connectivity across the Balkans.

## CONCLUSION

The novel haplotypes detected in this study emphasize the hidden diversity of *S. balcanica* in Serbia and across broader geographic range. Further genetic research on this species will help uncover its diversity, thereby allowing for better inference of phylogenetic relationships within the genus *Sabanejewia* in Europe, underscoring the need for its protection.

## ACKNOWLEDGEMENT

This study was funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, under grant numbers 451-03-33/2026-03/200007 and 451-03-34/2026-03/200178. The study was conducted in accordance with the United Nations Sustainable Development Goals (Agenda 2030), specifically Goal 15 (Life on Land).

## REFERENCES

- [1] Perdices, A., Doadrio, I., Economidis, P.S., *et al.* Mol. Phylogenet. Evol., 2003, 26(2), 289–299.
- [2] Bartonová, E., Papousek, I., Lusková, V., *et al.* Folia Zoologica, 2008, 57(1/2), 60.
- [3] Kottelat, M., Freyhof, J. Handbook of European freshwater fishes. Cornol, Switzerland: Publications Kottelat. Vol. 13, 2007.
- [4] Pehlivanov A.L., Schabuss, M., Szaloky, Z. *et al.* Chapter 9 Fish. In: Liška I., Wagner, F., Sengl M., Deutsch K. & Slobodnik J. (Ed.): Joint Danube Survey 3 – A Comprehensive Analysis of Danube Water Quality. Vienna: International Commission for the Protection of the Danube River, 2015, pp. 126–139.
- [5] Križek, P., Mendel, J., Fedorčák, J., *et al.* In the foothill zone—*Sabanejewia balcanica* (Karaman 1922), in the lowland zone—*Sabanejewia bulgarica* (Drensky, 1928): Myth or reality?. Ecology and Evolution, 2020. 10(14), 7929–7947.
- [6] Marić, S.P., Bănăduc, D., Gajić, Đ.D., *et al.* Acta Zool. Bulg. 2022, 74(3), 369–377.
- [7] International Union for Conservation of Nature (IUCN). (2024). *Sabanejewia balcanica*. The IUCN Red List of Threatened Species.
- [8] Official Gazette of the Republic of Serbia (2016). The Regulation on Proclamation of Strictly Protected and Protected Wild Species of Plants, Animals and Fungi. “Official Gazette RS” No. 98/2016.
- [9] Crvena knjiga faune Srbije V – Ribe (2024) Belgrade: Institute for Nature Conservation of Serbia.
- [10] EU Habitats Directive 92/43/EEC, Annex II and IV.
- [11] Avise, J.C. J. Biol. Sci., 1986, 312(1154), 325–342.
- [12] Larkin, M.A., Blackshields, G., Brown, N.P., *et al.* Clustal W and Clustal X version 2.0. bioinformatics, 2007, 23(21), 2947–2948.
- [13] Wong, T.K., Ly-Trong, N., Ren, H., *et al.* IQ-TREE 3: phylogenomic inference software using complex evolutionary models.
- [14] Rambaut, A. (2018). FigTree v1.4.4. Available from: <https://github.com/rambaut/figtree>.
- [15] Fedorčák, J., Šanda, R., Stefanov, T., *et al.* Fishes, 2023, 8(2), 119.