ACTA ZOOLOGICA BULGARICA

ACTA ZOOLOGICA BULGARICA

Acta zool. bulg., 74 (3), 2022: 369-377
Published online 23 August 2022
http://www.acta-zoologica-bulgarica.eu/2022/002607

Zoogeography and Faunistics
Research Article

Sabanejewia romanica (Băcescu, 1943) (Actinopterygii: Cobitidae), a new species for the ichthyofauna of Serbia

Saša P. Marić[1], Doru Bănăduc[2], Đorđe D. Gajić[1], Radek Šanda[3] & Tijana Z. Veličković*[4]

[1] Institute of Zoology, Faculty of Biology, University of Belgrade, Studentski trg 16, 11001 Belgrade, Serbia
[2] Applied Ecology Research Centre, Lucian Blaga University of Sibiu, Dr. Ion Raţiu 5-7, 550012 Sibiu, Romania
[3] Department of Zoology, National Museum, Václavské náměstí 68, 115 79 Prague, Czech Republic
[4] Institute of Biology and Ecology, Faculty of Science, University of Kragujevac, Radoja Domanovića 12, 34000 Kragujevac, Serbia

Abstract: *Sabanejewia romanica* was recorded for the first time on the territory of Serbia in three direct tributaries of the Danube River (Rečka River, Slatinska River and Zamna River). This is the first record of the species outside Romania, the westernmost point of its occurrence and the first verified record of the species from the right tributaries of the Danube River. Genetic characterisation of the Serbian specimens showed that they are the most related to the Romanian *S. romanica*, with a high recorded net evolutionary divergence between them. Further studies with included populations from the whole geographical range of *S. romanica* should provide an accurate picture of genetic diversity and phylogenetic relations within the species.

Key words: Romanian golden loach, first record, genetic characterization, distribution, Danube drainage

Introduction

The genus *Sabanejewia* Vladykov, 1929 (golden loaches) includes small freshwater fishes distributed in the Ponto-Caspian basins and some river systems in the Baltic, Aegean and North Adriatic Sea basins. From eight species recognized in Europe (Kottelat & Freyhof 2007), four inhabit the Danube drainage: Balkan golden loach *Sabanejewia balcanica* (Karaman, 1922), Bulgarian golden loach *Sabanejewia bulgarica* (Drensky, 1928), Romanian golden loach *Sabanejewia romanica* (Băcescu, 1943) and Eastern Carpathian golden loach *Sabanejewia vallachica* (Nalbant, 1957). Among listed species, *S. balcanica* has the widest distribution in the Danube drainage, mainly in the middle and upper parts of medium-sized rivers. On the other hand, *S. bulgarica* inhabits the main riverbed of the middle and lower Danube and the lowermost parts of its tributaries. *Sabanejewia vallachica* and *S. romanica* are Romanian endemics. *Sabanejewia vallachica* occurs in Ialomiţa and the Siret river systems. In contrast, *S. romanica*, with its type locality in the Bratia River (Argeş river system), also occurs in Vedea, Olt, Jiu, Topolniţa and Mureş river systems (Bănărescu 1964, Kottelat & Freyhof 2007, Marešová et al. 2011). Moreover, in its Romanian distribution area, *S. romanica* lives in the mountain and hill rivers in the grayling fish zone. It occurs with the Mediterranean barbel *Barbus petenyi* Heckel, 1852, common nase *Chondrostoma nasus* (Linnaeus, 1758) as well as chub *Squalius cephalus* (Linnaeus, 1758). Additionally, *S. romanica* rarely occurs downstream to the barbel fish zone. Instead, it occurs in the parts

*Corresponding author: tijana.velickovic@pmf.kg.ac.rs

of the rivers with the sandy and gravel substrate (Bănărescu 1964).

So far, the most extensive phylogenetic study of *Sabanejewia* based on the mtDNA data (Perdices et al. 2003) identified six main monophyletic lineages inside the genus: *Sabanejewia larvata*, *S. romanica*, *Sabanejewia aurata*, *Sabanejewia caucasica*, *Sabanejewia kubanica*, *Sabanejewia baltica* and the Danubian-Balkanian complex consisting of six sublineages with a dominant position of *S. balcanica* within them (Křížek et al. 2020). However, one common deficit of the performed studies was that they did not include samples from type localities for all the examined species. Moreover, in the region of Central Europe and the Balkans, the taxonomic status of *Sabanejewia* populations in the Danube basin is still uncertain (Erös et al. 2003, Ahnelt & Mikschi 2004, Kováč 2015, Sály 2019, Křížek et al. 2020).

Of the listed *Sabanejewia* species, two have been recorded in Serbia to date: *S. balcanica* in the Black Sea and the Aegean Sea basin (Simonović 2001) and *S. bulgarica* in the main riverbed of the Danube River and lower part of the Nera River (Bammer et al. 2015, Miljanović et al. 2016).

This work presents the first record of the Romanian golden loach *S. romanica* in Serbia.

Materials and Methods

Sample collection and identification

The sampling sites were located at Rečka River (44°29'23.7"N 22°26'01.0"E), Slatinska River (44°24'29.2"N 22°26'33.5"E) and Zamna River (44°19'60.0"N 22°24'12.7"E). All three rivers flow into the Danube from 871 to 883.5 rkm (Fig. 1).

Since sampling sites were stationed between the Djerdap I and Djerdap II Hydropower Plants, in order to determine the potential distribution of *S. romanica* in the broader area of Danube drainage in Serbia, additional field trips were performed. Upstream of the Djerdap I Hydropower Plant, investigation sites were in the Pek River, Brnjička River and two sites in the Porečka river system. Further four sites were in the Timok river system, downstream of the Djerdap II Hydropower Plant (Fig. 1).

Fish sampling was performed with electrofishing appliance type SEN. The length of individuals was taken using a measuring tape to the nearest

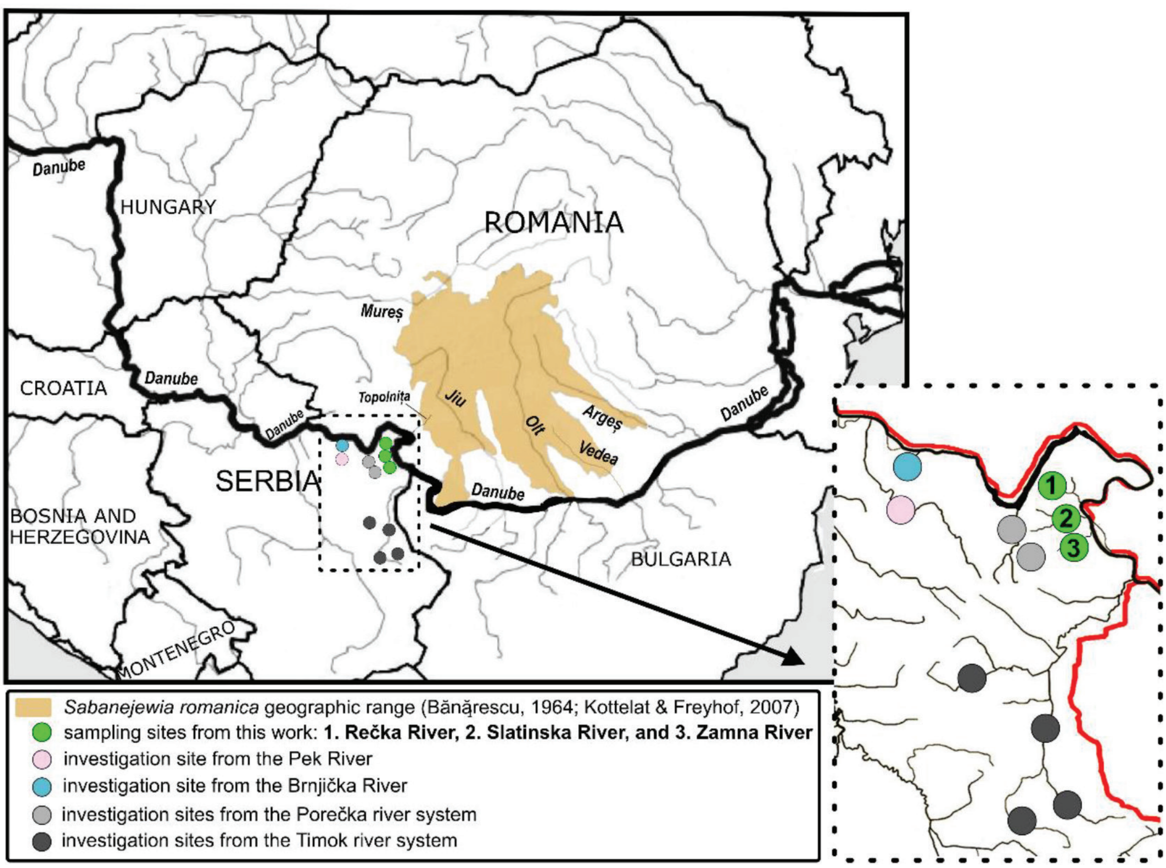

Fig. 1. Distribution of *Sabanejewia romanica*. Green circles represent the *S. romanica* populations recorded in Serbia. With violet, blue and light and dark grey circles are marked investigation sites in Serbia, where the species was not found. The light orange area represents the distribution of this species in Romania based on literature data.

millimetre, while the weight was measured using a digital balance scale to the nearest gram. Sex was determined by dissection. Sampled specimens were photographed using Nikon digital camera. Voucher specimens were deposited in the ichthyological collection of Saša Marić at the Faculty of Biology, Belgrade, Serbia (Catalogue ID: S/2021/1-5).

Two female and one juvenile specimen of *S. romanica* were caught in the Slatinska River (Fig. 2, 3, 4), while in each Zamna and Rečka rivers, one female specimen was caught on August 31st, 2021. For comparison purposes, five and four specimens of *S. balcanica* were sampled from the Slatinska and Porečka rivers, respectively. Fin clips of *S. romanica* and *S. balcanica* specimens were preserved in 96% ethanol for subsequent molecular analysis.

The identification was performed using keys to the species of the genus *Sabanejewia* (Bănărescu 1964, Ivanova & Dobrovolov 1999, Kottelat & Freyhof 2007). In order to avoid misunderstanding of the count of the dorsal and anal fin branched rays, the two last branched rays, which are articulated on a single pterygiophore, were counted as separate.

Genetic characterisation of specimens

Genomic DNA was extracted with the Geneaid™ DNA Isolation Kit (Tissue) (Geneaid Biotech).

Individuals were genotyped by sequencing part of the mitochondrial marker cytochrome b (*cyt b*). A fast mutation rate and the number of informative sites make it a reliable and informative marker, widely used in the population, phylogenetic and taxonomic studies.

Amplification was performed in a total volume of 25 µl by a commercial kit (PPP Master Mix; Top-Bio). *Cyt b* was amplified with the primers BarbusCTb-intF (5'-GGCTC**Y**TA**Y**CT**B**TA**Y**AA**R**GAAAC-3') and ThrR (Machordom & Doadrio 2001) under the conditions described in Šanda et al. (2008). Sequencing reactions were performed by Macrogen Inc with the use of primer BarbusCTb-intF. Sequences were edited and aligned by hand using programs Chromas 2.6.5© Technelysium Pty. Ltd and AliView (Larsson 2014).

Additional *cyt b* sequences representing six main evolutionary *Sabanejewia* mtDNA lineages and the Danubian–Balkanian complex were taken from GenBank (Appendix 1) and used as reference data.

In the MEGA11 software (Tamura et al. 2021), the net evolutionary divergence over sequence pairs between *Sabanejewia* taxa sequences (Appendix 1) was estimated using the Kimura-2-parameter model (Kimura 1980). The rate variation among sites was modelled with a gamma distribution (shape parameter = 1).

Results

The identification of the species was determined by distinctive external morphological characters. The morphological character not observed in any other species of the genus *Sabanejewia* is a thin black dotted line along the flank, positioned between the head and the caudal base. Elliptical or rounded blotches situated on the dotted line in the mediolateral row (10-13) and rectangular blotches on the back row (12-13) were observed (Appendix 2). On the back of the head, there were two dark bands that are ended with V-form. Adipose crests on the caudal peduncle were weakly developed, and two dark semi-circular spots at the caudal base are hardly visible. The number of rays in the fins: D I–II 6–7; A I–II 5–6; P I 7; V I 5–7; C I 11–12 I. All mentioned characters are diagnostic for *S. romanica* except the number of rays in D, A, and V fins, which slightly differed in the number of unbranched rays compared to literature data (Appendix 2; Bănărescu 1964, Ivanova & Dobrovolov 1999). However, the count of fin rays did not contain useful information for discrimination between *S. romanica* and *S. balcanica* specimens. There were recorded minor differences between individuals but also an overlap between the two species on the population levels (Appendix 2).

A total of 776 bp of the *cyt b* gene were resolved with sequence analysis in 14 individuals. Identification of *cyt b* haplotypes showed that five specimens from the Rečka, Slatinska and Zamna rivers carried haplotypes mostly related to the *S. romanica* from Romania (Argeş river system), with an estimated 0.039 net evolutionary divergence between them (Table 1). The remaining nine individuals carried *S. balcanica* haplotypes. Furthermore, considering *S. balcanica* and Serbian *S. romanica* specimens, discrimination was evident with the detected high net evolutionary divergence value (0.148). The overall net evolutionary divergence between *Sabanejewia* species ranged from 0.001 (*S. balcanica* and *S. bulgarica*) to 0.196 (*S. larvata* and *S. romanica* specimens from Serbia) (Table 1), with the notable high values between Serbian and Romanian *S. romanica* in comparison to the other *Sabanejewia* taxa (Table 1).

In the investigated remaining part of the border area between Serbia and Romania, which mostly included direct tributaries of the Danube River in Serbia, *S. romanica* was not recorded (Fig. 1).

In the Serbian part of its distribution, *S. romanica* occurred with chub, spirlin *Alburnoides* cf. *bipunctatus* (Bloch, 1782), Danube barbel *Barbus balcanicus* Kotlík, Tsigenopoulos, Ráb & Berrebi, 2002, stone loach *Barbatula barbatula* (Linnaeus,

Table 1. Estimates of net evolutionary divergence between groups of sequences. The number of base substitutions per site from estimation of net average between groups of sequences are shown below the diagonal. Standard error estimate(s) are shown above the diagonal.

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]
[1] *Sabanejewia romanica* Serbia		[0.008]	[0.018]	[0.019]	[0.018]	[0.018]	[0.018]	[0.019]	[0.019]	[0.017]	[0.017]	[0.018]	[0.018]	[0.021]
[2] *Sabanejewia romanica*	0.039		[0.017]	[0.017]	[0.017]	[0.017]	[0.017]	[0.017]	[0.018]	[0.015]	[0.016]	[0.016]	[0.017]	[0.020]
[3] *Sabanejewia balcanica*	0.148	0.136		[0.001]	[0.005]	[0.002]	[0.004]	[0.002]	[0.001]	[0.009]	[0.010]	[0.010]	[0.009]	[0.017]
[4] *Sabanejewia bulgarica*	0.155	0.142	0.001		[0.006]	[0.003]	[0.005]	[0.003]	[0.001]	[0.010]	[0.010]	[0.011]	[0.010]	[0.017]
[5] *Sabanejewia vallachica*	0.143	0.13	0.021	0.026		[0.006]	[0.007]	[0.005]	[0.006]	[0.010]	[0.010]	[0.011]	[0.011]	[0.018]
[6] *Sabanejewia doiranica*	0.144	0.134	0.006	0.01	0.027		[0.005]	[0.003]	[0.004]	[0.010]	[0.011]	[0.011]	[0.010]	[0.017]
[7] *Sabanejewia thrakica*	0.15	0.141	0.013	0.019	0.032	0.018		[0.004]	[0.005]	[0.010]	[0.010]	[0.011]	[0.010]	[0.018]
[8] *Sabanejewia radensis*	0.154	0.137	0.004	0.009	0.02	0.009	0.015		[0.003]	[0.009]	[0.010]	[0.010]	[0.010]	[0.017]
[9] *Sabanejewia montana*	0.159	0.144	0.002	0.002	0.023	0.012	0.018	0.005		[0.010]	[0.010]	[0.011]	[0.010]	[0.018]
[10] *Sabanejewia aurata*	0.129	0.121	0.053	0.057	0.057	0.059	0.06	0.052	0.056		[0.008]	[0.004]	[0.009]	[0.015]
[11] *Sabanejewia baltica*	0.136	0.131	0.055	0.059	0.059	0.063	0.062	0.053	0.056	0.039		[0.009]	[0.009]	[0.017]
[12] *Sabanejewia caucasica*	0.14	0.132	0.063	0.068	0.065	0.068	0.068	0.061	0.066	0.011	0.048		[0.010]	[0.016]
[13] *Sabanejewia kubanica*	0.143	0.126	0.061	0.067	0.07	0.063	0.07	0.06	0.065	0.047	0.052	0.056		[0.017]
[14] *Sabanejewia larvata*	0.196	0.179	0.145	0.15	0.15	0.151	0.154	0.146	0.15	0.12	0.146	0.129	0.153	

1758) and gudgeon *Gobio* cf. *gobio* (Linnaeus, 1758) in all three rivers, while in Zamna River it was also accompanied with Eurasian minnow *Phoxinus* cf. *phoxinus* (Linnaeus, 1758). Species habitat was characterised by shallow water with slow current and sandy or gravel bottom.

Regarding the other species from the genus *Sabanejewia* in the investigated area, *S. balcanica* was recorded in the Timok river system and in the Porečka River near the confluence with the Danube (Fig. 3a). In contrast, in the Slatinska River, sympatric populations of *S. balcanica* and *S. romanica* were recorded, while in Zamna and Rečka rivers, only *S. romanica* was recorded (Fig. 1).

Discussion

Sabanejewia romanica is a distinct evolutionary lineage formed after one of the more basal split within the genus *Sabanejewia* (see Perdices et al. 2003).

The presence of *S. romanica* on the territory of Serbia represents the first record of the species outside Romania; it is the westernmost point of the occurrence of this species. Moreover, it is the first verified record of the species from the right tributaries of the Danube River (Fig. 1). The newly discovered locations in Serbia belong to the same South-Western Carpathian curvature, which crosses important natural and anthropogenic barriers, the Danube River and the Djerdap I and II artificial big lakes.

In comparison with the Romanian populations, *S. romanica* in Serbia occurs in similar types of habitats with similar fish communities. Identified *S. romanica* specimens from Serbia were clearly discriminated from the other *Sabanejewia* species by distinctive external morphological characters, except for the comparison of meristic characters with *S. balcanica,* which proved not useful. The overlap of meristic characters between these two species on the population levels is not surprising, given that the diagnosis for *S. balcanica* is set in the wide range. Similar observations were made by Křížek et al. (2020) regarding the identification of *S. balcanica* and *S. bulgarica* specimens. Their work reported that difficulties in their identification were due to the overlap of distinguishing features and a wide range of morphological variability in both species.

Additionally, regarding the *S. romanica* specimens from Serbia, minor differences were observed in the number of rays in D, A and V fins compared to the literature; however, they were at the level of already detected meristic variabilities within the Romanian populations (Bănărescu 1964, Ivanova & Dobrovolov 1999). That may be explained by

Fig. 2. Juvenile specimen of *Sabanejewia romanica* from the Slatinska River.

Fig. 3. Representative specimens of *Sabanejewia balcanica* from the Svrljiški Timok (a), *Sabanejewia romanica* from the Slatinska River (b) and *Sabanejewia romanica* from the Cibin River, Olt river system, Romania (c).

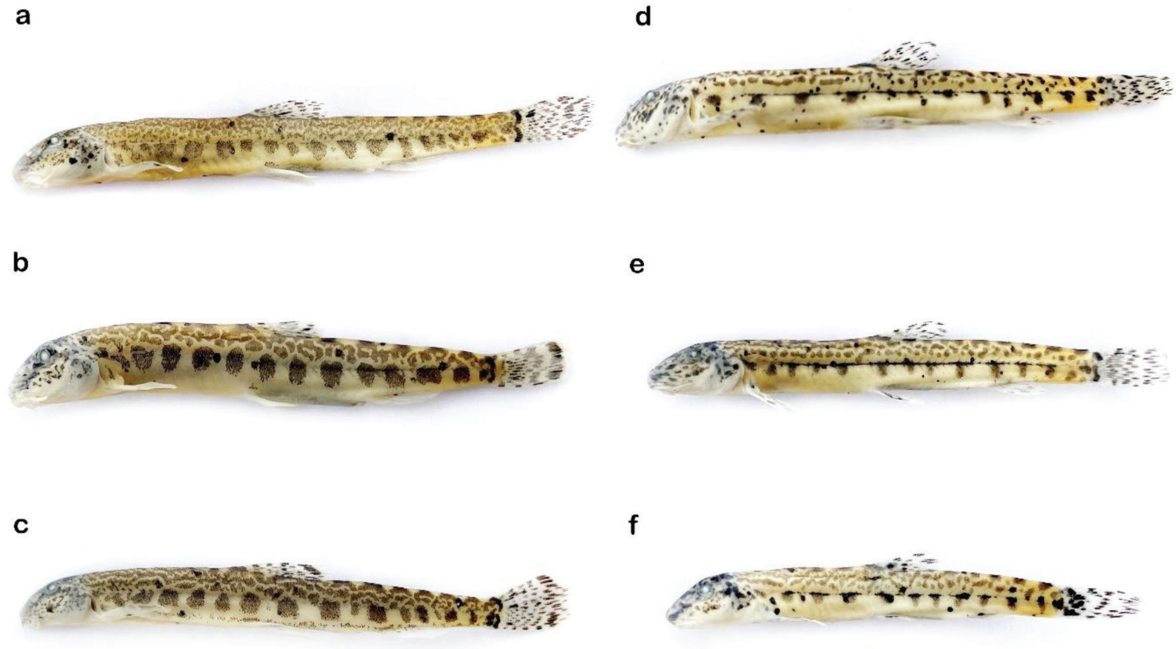

Fig. 4. Representative specimens of *Sabanejewia balcanica* (a, b, c) and *Sabanejewia romanica* (d, e, f) from the Slatinska River after preservation in 96% ethanol.

the species' phenotypic plasticity as well as the geographical isolation of the newly-recorded populations (Norton et al. 1995, Veličković et al. 2020).

Mitochondrial DNA *cyt b* gene analyses showed that the Serbian specimens are the most related to Romanian *S. romanica,* with the high values of evolutionary divergence implying the high diversity within the group. However, the information regarding the position of the newly recorded Serbian specimens should be considered as preliminary. The main difficulty for conclusions is the lack of coverage of the entire area of species occurrence. Previously analysed locations in Romania are in the Argeș river system, the most eastern part of its distribution (Bănărescu 1964, Freyhof & Kottelat 2008; Fig. 1), while the newly identified population in Serbia is in its most western part. Additional sampling and appropriate coverage of the species geographical range from the central and western part of its occurrence in Romania (Vedea, Olt, Jiu, Topolnița and Mureş river systems), would provide an accurate picture of genetic diversity and phylogenetic relationships within the species.

Moreover, since the heterogeneous European *Sabanejewia* taxa have an origin that cannot be traced back to a single event, especially within the Danube drainage (Perdices et al. 2003), the detailed phylogenetic inference would further provide information whether *S. romanica* is a species complex rather than a single homogeneous entity within *Sabanejewia*.

New information provided in this work regarding areas of *S. romanica* occurrence is important for knowing both its actual distribution in Serbia and its global distribution, especially considering that the systematic status of some populations of the genus *Sabanejewia* in Central Europe and the Balkans is still ambiguous (Křížek et al. 2020).

Moreover, *S. romanica* has been listed as a Near Threatened (NT) in the IUCN Red List (Freyhof & Kottelat 2008). However, the species may have an even more threatened status in Serbia because it inhabits only three localities, of which a very poor hydrological status characterises the Zamna and Rečka rivers. Furthermore, due to the great droughts, these two rivers did not have a continuous flow at the time of sampling and the species abundance was very low in them.

To determine and implement future conservation measures, it is necessary to conduct more detailed investigations to assess the population size and threats. In Serbia, the most abundant population of *S. romanica* is recorded in the Slatinska River, where it is about ten times rarer than *S. balcanica*, with which it may potentially hybridise. Furthermore, hybridisation can occur even among different genera of the family Cobitidae. Vasil'eva & Vasil'ev (2019) demonstrated that hybridisation occurred between *Cobitis* and *Sabanejewia*. Therefore, this could be an additional threat for *S. romanica* and, therefore, a subject of future investigation. Additionally, the identified populations are isolated from the main group of populations in Romania and need special management and conservation measures.

Future research should include phylogenetic and population genetic studies to access the genetic diversity of the populations in Serbia. A particular emphasis should be on the inclusion of reference Romanian populations to determine potential colonisation routes and population ancestry as well as to provide information regarding whether *S. romanica* is a species complex or a single entity.

Acknowledgments: Specimens for this study were obtained during field investigations in the frames of the project "Obtaining data and other services related to individual groups of organisms of flora and fauna in order to establish the ecological network of the European Union Natura 2000 in the Republic of Serbia". SM was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 451-03-68/2022-14/200178). TV was supported by the Serbian Ministry of Education, Science and Technological Development (Agreement No. 451-03-68/2022-14/200122). RŠ received further support from the Ministry of Culture of the Czech Republic (DKRVO 2019-2023/6.V.d National Museum, 00023272).

References

Ahnelt H. & Mikschi E. 2004. Zwei Goldsteinbeißerarten (Teleostei, Cobitidae, *Sabanejewia*) in Österreich? Österreichs Fischerei 57 (4): 94–96.

Bammer V., György A., Pehlivanov L., Schabuss M., Szaloky Z. & Zornig H. 2015. 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, pp. 126–139.

Bănărescu P. M. 1964. Fauna Republicii Populare Române, Pisces-Osteichthyes, vol. XIII. Bucureşti: Editura Academiei Republicii Populare Române. pp. 528−545.

Erös T., Sallai Z. & Kotusz J. 2003. Distribution and conservation status of loaches in Hungary. Folia Biologica (Kraków) 51 (Suppl. 3): 17–19.

Freyhof J. & Kottelat M. 2008. *Sabanejewia romanica*. The IUCN Red List of ThreatenedSpecies2008:e. T5036A11109131.https://dx.doi.org/10.2305/IUCN.UK.2008.RLTS.T5036A11109131.en.

Ivanova P. & Dobrovolov I. 1999. Morphological and biochemical comparison of *Sabanejewia aurata balcanica* (Karaman, 1922) and *Sabanejewia romanica* (Băcescu, 1943) (Pisces, Cobitidae). Proceedings of the Institute of Fisheries – Varna 25: 71−82.

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16 (2): 111−120.

Kottelat M. & Freyhof J. 2007. Handbook of European freshwater fishes. Kottelat, Cornol, Switzerland and Freyhof, Berlin, Germany. pp. 326–327.

Kováč V. 2015. Current Status of Fish Communities in the Danube. In: Liska I. (Ed.): The Danube River Basin. The Handbook of Environmental Chemistry Vol. 39. Berlin: Heidelberg: Springer, pp. 359–388.

Križek P., Mendel J., Fedorčák J. & Koščo J. 2020. In the foothill zone – *Sabanejewia balcanica* (Karaman 1922), in the lowland zone – *Sabanejewia bulgarica* (Drensky, 1928): Myth or reality? Ecology and Evolution 10 (14): 7929−7947.

Larsson A. 2014. AliView: a fast and lightweight alignment viewer and editor for large data sets. Bioinformatics 30 (22): 3276−3278.

Machordom A. & Doadrio I. 2001. Evidence of a Cenozoic Betic-Kabilian connection based on freshwater fish phylogeography (*Luciobarbus*, Cyprinidae). Molecular Phylogenetics and Evolution 18 (2): 252−263.

Marešová E., Delić, A., Kostov V., Marić S., Mendel J. & Šanda R. 2011. Genetic diversity of *Sabanejewia balcanica* (Actinopterygii: Cobitidae) in the western Balkans and comparison with other regions. Folia Zoologica, Praha 60 (4): 335−342.

Miljanović B., Jurca T., Vukov D., Simeunović J., Mijić I., Pogrmić S., Živković M., Novković M., Bajić A., Šipoš Š. & Pankov N. 2016. Fisheries Management Program for the fisheries area "Karaš-Nera" for the period 2016-2025. Novi Sad: Faculty of Sciences, University of Novi Sad.

Norton S. F., Luczkovich J. J. & Motta P. J. 1995. The role of ecomorphological studies in the comparative biology of fishes. Environmental Biology of Fishes 44 (1-3): 287−304.

Perdices A., Doadrio I., Economidis P. S., Bohlen J. & Banarescu P. 2003. Pleistocene effect on the European freshwater fish fauna: double origin of the cobitid genus *Sabanejewia* in the Danube basin (Osteichthyes: Cobitidae). Molecular Phylogenetics and Evolution 26 (2): 289−299.

Sály P. 2019. Fishes of the Drava river. In: Lóczy D. (Ed.), The Drava River: Environmental problems and solutions. Cham: Springer International Publishing, pp. 281–297.

Šanda R., Vukić J., Choleva L., Křížek J., Šedivá A., Shumka S. & Wilson I. F. 2008. Distribution of loach fishes (Cobitidae, Nemacheilidae) in Albania, with genetic analysis of populations of *Cobitis ohridana*. Folia Zoologica Praha 57 (1): 42–50.

Sayers E. W., Cavanaugh M., Clark K., Pruitt K. D., Schoch C. L., Sherry S. T. & Karsch-Mizrachi I. 2022. GenBank. Nucleic acids research 50 (D1): D161–D164.

Simonović P. 2001. Freshwater fish of Serbia. Beograd: NNK Internacional, Zavod za zaštitu prirode Srbije, Biološki fakultet (In Serbian). 186 p.

Tamura K., Stecher G. & Kumar S. 2021. MEGA11: molecular evolutionary genetics analysis version 11. Molecular biology and evolution 38 (7): 3022−3027.

Vasil'eva E. D. & Vasil'ev V. P. 2019. Natural hybridization in spined loaches of the genera *Cobitis* and *Sabanejewia* (Cobitidae). Journal of Ichthyology 59 (5): 776−785.

Veličković T., Simić V., Šanda R., Radenković M., Milošković A., Radojković N. & Marić S. 2020. New Record of a Population of *Telestes souffia* (Risso, 1827) (Actinopterygii: Cyprinidae) in Serbia. Acta Zoologica Bulgarica 72 (1): 13−20.

Received: 19.01.2022
Accepted: 26.07.2022

Appendix 1. Sequences of *Sabanejewia cyt b* mitochondrial haplotypes retrieved from the NCBI base (National Center for Biotechnology Information) (Sayers et al. 2022), including the new haplotypes described in this work (marked with an asterisk).

Haplotype	Taxa	Accession number	Haplotype	Taxa	Accession number
SR1*	*Sabanejewia romanica*	ON866527	Timis1	*Sabanejewia balcanica*	AY059346
SR2*	*Sabanejewia romanica*	ON866528	Nera1	*Sabanejewia balcanica*	AY059345
55SAB	*Sabanejewia romanica*	AF263095	Petrinjcica1	*Sabanejewia balcanica*	HQ291797
61SAB	*Sabanejewia romanica*	AF263096	Petricnjica2	*Sabanejewia balcanica*	HQ291798
G74	*Sabanejewia romanica*	AF499196	Petricnjica3	*Sabanejewia balcanica*	HQ291799
G23	*Sabanejewia romanica*	AF499197	*S. bulgarica*	*Sabanejewia bulgarica*	AF499188
213CRO	*Sabanejewia romanica*	AY059337	H43	*Sabanejewia bulgarica*	MN149894
A1857	*Sabanejewia romanica*	EF508595	H44	*Sabanejewia bulgarica*	MN149895
SB1*	*Sabanejewia balcanica*	ON866529	H45	*Sabanejewia bulgarica*	MN149896
SB2*	*Sabanejewia balcanica*	ON866530	H46	*Sabanejewia bulgarica*	MN149897
SB3*	*Sabanejewia balcanica*	ON866531	218CRB	*Sabanejewia bulgarica*	AY059353
Isolate 1	*Sabanejewia balcanica*	AF499171	2c	*Sabanejewia montana*	AF499199
Isolate 2	*Sabanejewia balcanica*	AF499172	282	*Sabanejewia montana*	AF499175
2b	*Sabanejewia balcanica*	AF499194	453G	*Sabanejewia doiranica*	AY059354
3b	*Sabanejewia balcanica*	AF499195	467G	*Sabanejewia doiranica*	AY059355
H13	*Sabanejewia balcanica*	MW239636	436CRR	*Sabanejewia radensis*	AY059360
Hap16	*Sabanejewia balcanica*	HQ291803	459CRR	*Sabanejewia radensis*	AY059357
Hap18	*Sabanejewia balcanica*	HQ291804	1775G	*Sabanejewia thrakica*	AY059363
Hap19	*Sabanejewia balcanica*	HQ291805	1776G	*Sabanejewia thrakica*	AY059364
Hap20	*Sabanejewia balcanica*	HQ291806	127CRU	*Sabanejewia vallachica*	AY059365
Hap21	*Sabanejewia balcanica*	HQ291807	173CRU	*Sabanejewia vallachica*	AY059371
Hap22	*Sabanejewia balcanica*	HQ291808	Isolate 1	*Sabanejewia kubanica*	AF499182
Hap23	*Sabanejewia balcanica*	HQ291809	Isolate 3	*Sabanejewia kubanica*	AF499184
Hap24	*Sabanejewia balcanica*	HQ291810	Isolate 1	*Sabanejewia aurata*	AF499186
Hap25	*Sabanejewia balcanica*	HQ291811	245	*Sabanejewia aurata*	AF499189
Hap26	*Sabanejewia balcanica*	HQ291812	257MO	*Sabanejewia caucasica*	AY059338
Hap36	*Sabanejewia balcanica*	HQ291822	260MO	*Sabanejewia caucasica*	AY059339
Hap37	*Sabanejewia balcanica*	HQ291823	265	*Sabanejewia baltica*	AF499177
KOS51	*Sabanejewia balcanica*	MT543261	266	*Sabanejewia baltica*	AF499178
DRA1	*Sabanejewia balcanica*	EF605341	41TA	*Sabanejewia larvata*	AY059335
CrniTimok1*	*Sabanejewia balcanica*	HQ291818	49TA	*Sabanejewia larvata*	AY059336
Usora1	*Sabanejewia balcanica*	HQ291836			

Appendix 2. Comparative review of the key characters of the *Sabanejewia balcanica* and *Sabanejewia romanica* specimens from the present study and those described by previous authors (Bănărescu 1964, Ivanova & Dobrovolov 1999, Kottelat & Freyhof 2007).

	Sabanejewia romanica						***Sabanejewia balcanica***									
	Slatinska River			**Zamna**	**Rečka**	**Literature**	**Slatinska River**					**Porečka River**				**Literature**
Standard length (SL; mm)	82	68	50	68	59	≤ 105	58	59	54	53	52	76	73	63	63	≤ 90
Weight (g)	7	5.4	3.9	5.2	4.4	-	4.3	4.2	3.9	3.7	3.7	6.2	6.1	5.1	5.2	-
Number of rays in dorsal fin (D)	I 6	I 7	II 6	I 7	I 7	II–III 7–9	I 6	I 6	I 7	I 7	I 7	I 6	I 6	I 7	I 7	II–III 6–9
Number of rays in anal fin (A)	II 5	I 5	I 5	I 5	I 6	II 5–8	I 4	I 5	I 5	I 5	I 4	I 5	I 5	I 5	I 5	II–III 4–6
Number of rays in pectoral fin (P)	I 7	I 7	I 7	I 8	I 7	I 5–9	I 5	I 6	I 7	I 7	I 7	I 11	I 8	I 8	I 8	I 6–9
Number of rays in ventral fin (V)	I 6	I 5	I 5	I 5	I 5	II 6–7	I 5	I 5	I 5	I 6	I 6	I 5 I	I 5 I	I 5 I	I 6	II 5–6
Number of rays in caudal fin (C)	I 11 I	I 12 I	I 12 I	I 12 I	I 12 I	I 10–12 I	I 12 I	I 12 I	I 12 I	I 14 I	I 13 I	I 12 I	I 12 I	I 12 I	/	I (12) I
Number of blotches on the dotted line in mediolateral row	13	12	12	12	11	9–15	12	13	10	13	12	9	15	12	12	10–20
Number of blotches on the back row	13	13	12	12	12	9–14	11	11	10	15	15	9	12	11	11	10–14