

Small differences in Cytochrome-b sequences within the genus *Sabanejewia*

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Abstract. Partial mitochondrial tRNA^{Glu} and Cytochrome-b (Cyt-b) sequences of the following taxa were used for distance calculations: *Sabanejewia romanica*, *S. vallachica*, *S. bulgarica*, *S. radnensis*, *S. montana*, *S. larvata*, *S. sp.* and three populations of *S. balcanica*. Large values of genetic similarity were observed between all analysed golden loaches. Calculations of the Tajima-Nei distances and the algorithms of Neighbor-Joining (NJ) leads to a basal position of *S. larvata*. In a second step, *S. montana* was also separated. The NJ-tree showed two ingroups: 1) *S. romanica*, *S. balcanica* 1+2 and *S. radnensis* and 2) *S. vallachica*, *S. balcanica* 1+3, *S. bulgarica* and *S. sp.* The separation of the ingroups has only limited support by weak bootstrap values. Small sequence differences found in this study were in contrast to morphological characters and pigmentation patterns described in previous studies.

Key words: golden loach, molecular phylogeny, genetic similarity

Introduction

The geographical distribution of golden loaches of the genus *Sabanejewia* includes large parts of southern and eastern Europe. Golden loaches mainly inhabit the middle and upper stretches of medium sized rivers. The populations are often isolated. Some populations are well defined by morphological characters and pigmentation patterns. These populations have been described controversially as species, subspecies or clinal variations. Some of these taxa are involved in a complicated system of sympatric or allopatric occurrence and series of intermediate forms in Rumania (B a n a r e s c u et al. 1972). The phylogenetic relationships of the genus *Sabanejewia* remains unclear, especially outside of Rumania. The major goal of our present study was to bring light into the discussions about phylogenetic relationships using a part of the mitochondrial Cyt-b gene.

Material and Methods

Sampling points are shown in Fig. 1. Specimens used in this study were collected at the following localities: *S. romanica*, River Bratia – a tributary of River Arges; Danube basin (type locality); *S. vallachica*, River Ialomitza, Danube basin (type locality); *S. bulgarica*, channel in the Danube delta; *S. radnensis*, River Mures – a tributary of River Tisza, Danube basin (type locality); *S. balcanica* 1, River Nera – a tributary of River Tisza, Danube basin; *S. balcanica* 2, Baile 1.Mai, system of River Tisza, Danube basin, all points in Rumania; *S. montana*, River Laborec – a tributary of River Tisza, Danube basin, Slovakia; *S. balcanica* 3, River Kolpa – a tributary of River Sava, Danube basin, Slovenia; *S. larvata* caught in a lowland channel of the Adige basin, Italy and *S. sp.*, River Stryi, Dnjestr basin, Ukraine.

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Species was identified by morphology. For the present comparative study all described forms were taken as species, regardless their recent taxonomic assessment (B a n a r e s c u et al. 1972, K o t t e l a t 1997).

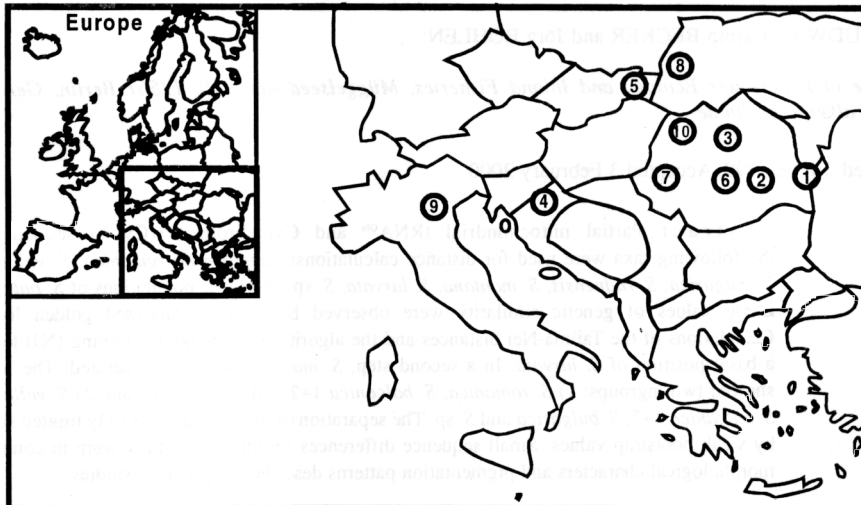


Fig. 1. Map of South-eastern Europe showing the sampling points: (1) *Sabanejewia bulgarica*, (2) *S. vallahica*, (3) *S. radnensis*, (4) *S. balcanica* 2, (5) *S. montana*, (6) *S. romanica*, (7) *S. balcanica* 1, (8) *S. sp.*, (9) *S. larvata* and (10) *S. balcanica* 3. For details of river systems see text.

Tissue samples were used for DNA extraction according to the QIAamp protocols (QIAGEN Inc., Germany). Samples were stored at -80°C . PCR was performed in 50 μl reaction volume containing 2.5 Units AmpliTaq DNA Polymerase, 25mM MgCl_2 , 0.2 mM primers located in the t-RNA^{Glu} gene (L14735 – AAA AAC CAC CGT TGT TAT TCA ACT A) and the Cyt-*b* gene (H15149 – GCC CCT CAG AAT GAT ATT TGT CCT CA) and 25 ng DNA of each sample. PCR-products were amplified in a Perkin-Elmer thermocycler programmed 30 cycles each of 94°C at 10 sec.; 55°C at 10 sec. and 72°C at 2 min, gel extracted and directly sequenced in a 310 Sequencer using BigDye kit. (PEBiosystems, USA). Sequence PCR were performed with the following cycles: 94°C at 5 sec.; 50°C at 10 sec and 60°C at 2 min using the standard protocol for cycle sequencing.

The genetic divergences among samples were calculated with the Tajima-Nei distances (Tajima & Nei 1984) and the Neighbor-Joining (NJ) algorithm (Saitou & Nei 1987). The outcome was not different from that using other distance measures (Tamura-Nei distance – limited to protein coding data – Tamura & Nei 1993; p distance – Saitou & Nei 1987). The Tajima-Nei distance values and the NJ method were calculated with the program MEGA Vers. 1.01 (Kumar et al. 1993).

Results and Discussion

Sequences are available in the EMBL genbank (Accession No.: AJ251440–50). Distance values and number of substitutions as well as partial alignment of sequences used for comparison and genetic distance calculations are shown in Table 1 and Fig. 2. We observed

Table 1. Above diagonal the number of substitutions between the species and below diagonal the Tajima-Nei distance are shown. All codon positions were used.

		2	3	4	5	6	7	8	9	10	11
<i>S. bulgarica</i>		2	3	4	7	3	3	3	13	4	2
2. <i>S. valachica</i>	0.0059			2	5			1	11	4	0
3. <i>S. radnensis</i>	0.0089	0.0030			4	0	0	2	10	3	
4. <i>S. balcanica</i> 2	0.0119	0.0059	0.0030		5			3	11	4	2
5. <i>S. montana</i>	0.0210	0.0149	0.0119	0.0149		4	4	6	12	7	5
6. <i>S. romanica</i>	0.0089	0.0030	0	0.0030	0.0119		0	2	10	3	
7. <i>S. balcanica</i> 1	0.0089	0.0030	0	0.0030	0.0119	0		2	10	3	
8. <i>S. sp.</i>	0.0089	0.0030	0.0059	0.0089	0.0180	0.0059	0.0059		10	3	1
9. <i>S. larvata</i>	0.0396	0.0335	0.0303	0.0335	0.0364	0.0303	0.0303	0.0303		11	11
10. <i>S. balcanica</i> 1	0.0119	0.0119	0.0089	0.0119	0.0210	0.0089	0.0089	0.0089	0.0334		4
11. <i>S. balcanica</i> 3	0.0059	0	0.030	0.0059	0.0149	0.0030	0.0030	0.0030	0.0335	0.0119	

large values of genetic similarity between golden loaches analysed in this study. However, the calculations of the Tamura-Nei distances and the NJ algorithm showed a separation of *S. larvata*. The separation of *S. larvata* is in agreement with previous conclusions (Vasileva & Vasilev 1988) and zoogeographical data. In a second step, *S. montana* was also separated. In the current taxonomy, *S. montana* is considered a synonym of *S. balcanica* (Kottelat 1997). However, the NJ tree showed two ingroups: 1) *S. romanica*, *S. balcanica* 1 + 2 and *S. radnensis* and 2) *S. vallachica*, *S. balcanica* 1 + 3, *S. bulgarica* and *S. spec.* (Fig. 3). This grouping is in opposition to the general acceptance of *S. bulgarica* and

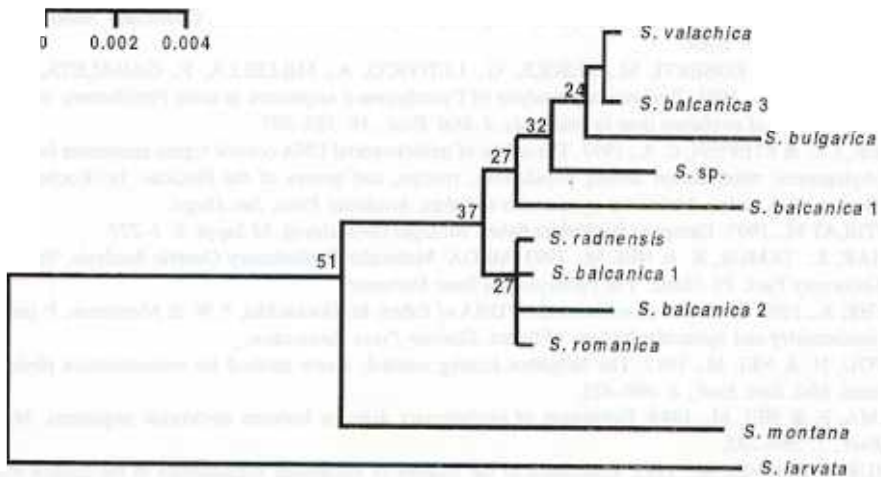


Fig. 3. Neighbor-Joining tree based on the distance matrix of amplified region of the partial tRNA^{val} and the Cyt-b gene. Bootstrap values of 500 replications were calculated in MEGA (Kumar et al. 1993).

S. romanica as independent species (Banarescu et al. 1972, Kottelat 1997). The grouping is rather caused by the great similarity of the Cyt-b sequences among the populations. The separation of the two ingroups had only limited support by bootstrap values below 50 %. These low genetic differences have three possible explanations: 1) the speciation within the genus *Sabanejewia* was rather a “younger” evolutionary event. This explanation contradicts the findings of previous authors on morphological characters and pigmentation or 2) in our state of knowledge, a divergence rate for mtDNA of 2 % per MY has been suggested for bony fishes (Meyer 1993). However, values of 0.5 % were also found (Faber & Stepien 1997, Cantatore et al. 1994). In the present study, sequence divergence ranged from 0 % up to 3.8 %, so it is possible that partial sequences investigated in this study evolve too slowly to result in detectable differences in golden loach, or 3) historical or recent hybridisations among the populations, and possible mitochondrial haplotype sorting following speciation events, may blur the phylogenetic history. On the other hand, genes that are under strong environmental selection (e.g. for pigmentation) would still reflect the special adaptations of the different stocks on their habitats. This leads to a large variability of ecological types and, therefore, it is possible that the genus *Sabanejewia* is oversplit. Unfortunately, single genes or gene complexes involved in expression of pigmentation characteristics are unknown. However, for a final decision between the possible explanations, further studies on the phylogeny of golden loaches are required.

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