fishes

Article

The "True Colours" of Golden Loaches (Teleostei: Cobitidae)

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Abstract: Loaches of the genus *Sabanejewia* are model organisms for many ichthyologic studies focusing on morphological and colour variation. The bright lateral spots—a part of the mimicry of this fish—cause a taxonomic puzzle. Common practice is that morphometric and molecular studies lack habitat assessments of species originating from different environments. In this study, we analysed body shape and colour variation within two species of *Sabanejewia* in order to reveal whether they are more affected by phylogeny or environmental conditions. The environmental parameters were obtained using satellite mapping tools, body shape using geometric morphometry, and the molecular identification of specimens using an analysis of mitochondrial DNA (sequence of the *cytochrome b* gene). Generalized linear mixed models were used for the analysis of lateral spot numbers, and principal components analysis to describe the changes in landmark position defined by the permutation tests. Results show a significant impact of the locality elevation and specimen's sex on the change of the body shape. We identified significant differences in the number of lateral spots, which are considered mimicry variation across an elevation gradient. Morphological differences of *Sabanejewia* spp. vary with ecological factors along the river continuum.

1. Introduction

Species delimitation is a key task of systematic biology since species is the basic unit for evolutionary or conservation studies. Most freshwater fish species are restricted to a few river basins, but some, e.g., species of the genus *Sabanejewia*, depart from this general rule. The genus *Sabanejewia* was described based on the sexual dimorphism of males [1] and several studies have analysed their external characters [2–6]. The genus *Sabanejewia* includes ten species, distributed from the Aral Sea to Italy and from Asia Minor to the Baltic Sea [7–11], while the validity of several other nominal *Sabanejewia* species is still unclear [11], the existence of unknown species is possible [12]. From the Danube River basin and Balkan watersheds, the "Danube–Balkan (DB) complex" [13] of sympatric *Sabanejewia* lineages have been described. Within sympatric species, a morphological cline (number of spots, body height, length of caudal peduncle, etc.) reflecting the character of the habitat was observed [3]. Molecular studies on the DB complex have established six mitochondrial lineages [13]. Morphological studies [14,15] of some individuals of the

DB complex showed, however, that their morphological identification does not reflect their genetic identity. Although the morphological cline in the Danube lineages was related to their habitat [3], in most recent taxonomic studies, the influence of habitat has not been considered. Recently, Křížek et al. [15] have confirmed the impact of the microhabitat parameters on morphological characters unrelated to the genetic identity of analysed specimens.

In this study, we evaluate the influence of environmental parameters and sex on the body shape exhibited by two *Sabanejewia* mitochondrial lineages from the DB complex. For the expansion of knowledge within the target genus, we analysed the most widespread lineages of the DB complex: the Balkan golden loach (*S. balcanica* Karaman, 1922) and the Bulgarian golden loach (*S. bulgarica* Drensky, 1928), including samples from type localities of both species and specimens from the adjacent river basins.

2. Materials and Methods

Samples: Individuals of *Sabanejewia balcania* and *S. bulgarica* analysed in this study are preserved in the ichthyological collections of the National Museum in Prague (CZ), the National Museum of Natural History in Sofia (BG) and the Department of Ecology of the University of Prešov (SK) (collection numbers listed in Table S1). All examined individuals were preserved by the same procedure (fixed in 4% formaldehyde solution and later transferred to 70% ethanol for permanent storage). The data set (133 specimens) was sex and length (SL) balanced (the length of both sexes did not differ statistically; $\chi 2 = 0.03$, d.f. = 1, $p > 0.85$) and length balanced between the species ($\chi 2 = 0.13$, d.f. = 1, $p > 0.72$). Out of 133 specimens, we included 111 specimens (24 *S. balcanica* and 87 *S. bulgarica*) in the morphometric analysis and 54 on the *cyt b* analysis (selected specimens were analysed morpologically as well as genetically, Table S1). Analysed specimens originated from the Danube drainage (Sava R., Danube R., Crni Timok R., Great Morava R.—Black Sea drainage) and from the Vardar drainage (Aegean Sea drainage) (Figure 1).
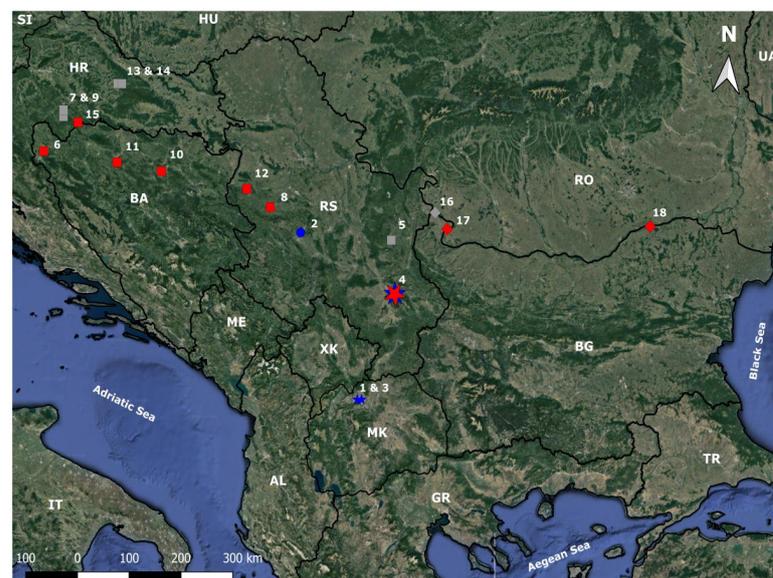

Figure 1. Map showing sampling localities; the numbers (1–18) correspond to the numbers of the localities in Table S1. Colours depict haplotype groups: red—*S. bulgarica*, blue—*S. balcanica*, grey—localities are not included in *cyt b* analysis. Shapes: red square—*S. bulgarica* in Danube River basin; blue circle—*S. balcanica* in Danube River basin; blue/red star—co-occurrence of *S. balcanica* and *S. bulgarica* in Danube River basin (Black Sea drainage); blue stars—*S. balcanica* in Vardar River catchment (Aegean Sea drainage).

The localities represent different habitats at different elevations (Table S1). In addition, we analysed ten specimens of the *S. bulgarica* from the type locality (Danube River in

Vidin) and twelve specimens of *S. balcanica* from the Vardar River catchment (Lepenac R., Treska R.) in Skopje, which are considered part of the type locality [16]. We also analysed specimens of NM Prague (Table S1) representing samples genetically characterised by Marešová et al. [17].

Morphometry: We analysed 11 homologous landmarks defined according to Mousavi-Sabet et al. [18] (Figure 2—excluding the center of the eye) on 111 specimens (Table S1—Geomorph).
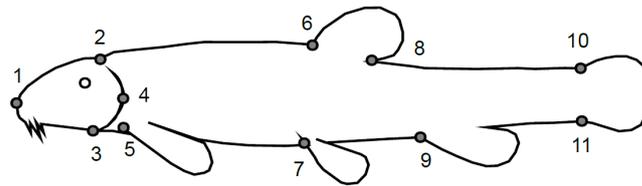

Figure 2. *Sabanejewia* body with 11 landmarks (middle)—1. Tip of snout, 2. Forehead (end of frontal bone), 3. End of operculum (ventral part), 4. End of the operculum (lateral), 5. Dorsal origin of pectoral fin, 6. Origin of dorsal fin, 7. Origin of ventral fin, 8. Termination of dorsal fin, 9. Origin of anal fin, 10. Base of caudal fin (dorsal), 11. Base of caudal fin (ventral).

Photographs of all specimens were taken with a fixed camera on a tripod, setting the height and standard lighting to minimise shooting errors. Standard length (SL; mm) was measured in TPS with graph paper as the calibration scale. The fish samples were pinned on a hard cover to avoid small body deformities. For landmark analysis, we used TPS landmarks based on Rohlf's [19] procedure. Procrustes superimposition of landmarks, which eliminates the effect of different size, position and rotation of compared objects, was performed with the geomorph package [20] in the R statistical environment [21], according to Park et al. [22]. We checked outliers of transformed landmarks using the plot outliers function. Lateral spots were counted on the left side of the body.

Statistical analysis: All analyses were performed in the R statistical environment with a threshold for statistical significance $\alpha = 0.05$. For data evaluation, we used several statistical approaches: (1) We assessed the permutational multivariate analysis of variance (PERMANOVA, Euclid., 9999 perm.) using the function adonis, according to Anderson [20], to define differences in landmark position depending on the explanatory variable (elevation, slope, sex); (2) Principal components analysis (PCA) [20] was performed to plot results of the PERMANOVA and deformation grids; (3) Comparison of the morphological disparity (within/between localities) was done by the function morphol.disparity of the geomorph package [23] under 999 iterations of the permutation test. The probabilities of the posterior population membership of individuals obtained by all grouping methods were converted to between-individual Euclidean distances. We also performed pairwise comparisons of these distance matrices using the Mantel test—ade4 package [24]. (4) For the evaluation of the lateral spot numbers of 111 specimens, we fitted generalised linear mixed models (GLMMs) with the response variable (number of lateral spots) and explanatory variables (elevation, slope, SL, sex, double interactions). Mixed models were fitted with the random factor of locality for correction of the spatial pseudo-replication between specimens from the same locality [25]. The GLMM models were submitted to ANOVA type II (testing the main effect after the other main effects) and III (in the case of significant interaction) [26]. The minimum Akaike information criterion (AIC) was applied for multiple model comparisons and the best model fit (quality/size).

Environmental parameters: We obtained environmental values (river bottom slope and the elevation) of the sampling localities using Google Earth Pro (ver.7.3.2.) according to the GPS locations of the voucher material. For a more accurate estimate of the slope of locality, we calculated the average river slope from 300 m of river transects up to the GPS coordinates.

Molecular analysis: A total of 54 sequences/49 haplotypes (Table S1) of the complete mitochondrial *cytochrome b* (*cyt b*; 1140 bp) of *Sabanejewia* spp. of the lineages *S. bulgarica*

from the Danube River basin ([15,17], this work Table S1) and *S. balcanica* from the Vardar River and Danube River basin [17], together with sequences of the other four lineages of the DB complex ([13], this work Table S1 (OK032158-63)) were used for the construction of a consensus tree. We constructed the consensus tree in PopArt software [27]. The sequence of *S. romanica* (GenBank accession no. AF499196) was used as the outgroup for phylogenetic reconstruction. To avoid potential misidentification based on morphology, the new *Sabanejewia* specimens were not identified to the species level.

For the newly added sequences (Table S1), the total genomic DNA was extracted from a piece of fin tissue using a commercial kit (GT300, Geneaid). The sequences were deposited in the GenBank database (under Accession Nos. OK032158-63). We amplified the *cyt b* gene by PCR with the primer pair GluDG.L [28] and H16460 [29]. We performed 25-μL PCR reactions with the PPP master kit mix (Top-Bio) in a Mastercycler Pro (Eppendorf). The PCR conditions were: one initial denaturation cycle at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 15 s, extension at 72 °C for 1 min and a final extension at 72 °C for 7 min. The amplicons were visualised in 1.7% agarose gel by electrophoresis. The PCR products were purified with the DNA Clean & Concentrator-5 Kit (Zymo Research) and the complete *cyt b* (1140 base pairs) gene was sequenced at Macrogen Europe with the same PCR primers. All PCR amplicons were sequenced in both directions to ensure high quality reads. The DNA sequences were edited and aligned using the SeqMan module within Lasergene 15.0 (DNASTAR Inc., Madison, WI, USA) and also checked manually. Furthermore, as a final quality control, the *cyt b* sequences were translated to verify that they were free of stop codons, frame-shifts and gaps. A detailed list of all studied taxa, their haplotype classification relative to sampling sites, haplotype frequencies and GenBank accession numbers are shown in Table S1.

For the phylogenetic reconstructions, the genetic dataset was analysed by Bayesian inference (BI) using MrBayes 3.1.2 [30], the maximum likelihood (ML) method using PhyML [31] and the neighbour-joining algorithm (NJ) using PAUP* 4.0B.10 [32]. The best-fit model of molecular evolution was determined for the mitochondrial dataset using the Akaike Criterion (AIC) in jModeltest 2.1.4 [33]. MrBayes was run with six substitution types (nst = 6) and considered gamma-distributed rate variation and the proportion of invariable positions (GTR + G + I). For BI, we ran four simultaneous Monte Carlo Markov chains for 2 million generations, with a sampling frequency every 100 generations. The first 5000 trees were excluded as the burn-in. The remaining trees were used to compute a 50% majority-rule consensus tree. For ML analysis, we conducted heuristic searches under the TN93 + G + I model of mutation. The neighbour-joining algorithm (NJ) was used to calculate the genetic distances according to the TN93 model. The robustness of the inferred trees was assessed by bootstrapping (1000 replicates) in ML or NJ analyses and posterior probability values in BI analysis. Branch support values were evaluated in accordance with Yang et al. [34], where good support was defined as bootstrap values of 75–88% and posterior probabilities of 0.85–0.94, strong support as bootstrap values of 89–100% and posterior probabilities of 0.95–1. A number of polymorphic sites (S), nucleotide diversity (π), haplotype diversity (Hd) and neutrality tests were calculated using DnaSP 6 [35].

3. Results

The analysed individuals of *Sabanejewia* spp. originate from 18 localities with an elevational range from 15 to 269 m above sea level and a bottom slope varying from 0.4 to 6.8% (Table S1). The locality parameters represented different environmental conditions from lowland rivers (*metapotamon*) to sub-montane river habitats (*metarithron*).

Body shape at the minima and maxima of the first two PCA axes explained 38.5% of the variability (PC1–23.4%, PC2–15.1%), with visible deformation in the pre-dorsal part represented by landmarks 2 and 6 (Figure 2). According to the permutation test results (Table 1), the significant impact on a specimen's landmark variance was best explained by sex and the locality elevation.

Table 1. PERMANOVA results (Adonis [20]; 9999 permutations) for geometric morphometric data and explanatory variables (sex, elevation, slope) and their double interaction based on Euclidian distances. (Abbreviations: SS–sum of squares, MS–mean sum of squares, d.f–degrees of freedom).

Main Effects	d.f.	SS	MS	Pseudo F	R²
Elevation	13	0.032	0.003	2.468	**<0.001**
Sex	1	0.005	0.005	4.828	**<0.001**
Slope	1	0.0008	0.0008	0.821	>0.076
Residuals	95	0.096	0.001		
Total	110	0.134			
Interaction terms	d.f.	SS	MS	pseudo F	R²
Elevation	13	0.032	0.003	2.543	**<0.05**
Sex	1	0.004	0.005	4.975	**<0.001**
Slope	1	0.0008	0.0008	0.847	**0.033**
Elevation:sex	13	0.016	0.001	1.239	>0.06
Sex:slope	1	0.0008	0.0008	0.783	>0.33
Residuals	81	0.079	0.001		
Total	110	0.134			

Subsequently, we set PCA plot based on sex categories and elevation levels (Table S1). We retained two principal components according to the PCA variance graph, two categories for sex and fourteen categories for locality elevation (Figure 3A,B), responsible for approximately 35.5% of the morphological variation. The deformation grids in PCA plot (Figure 3A,B) showed the importance of the association with the change of landmark position representing the dorsum, the caudal peduncle and the base of the anal fin.
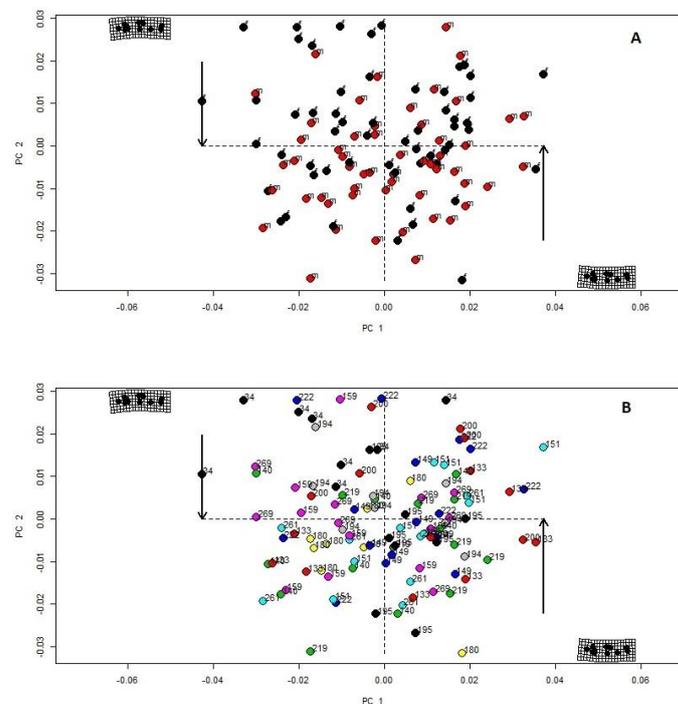

Figure 3. Principal components (PCA) of geometric morphometry based on 11 landmarks (Figure 2) of 111 individuals of *Sabanejewia* spp. based on specimens' sex ((**A**) black dots–females, red dots–males) from 15 localities differing in elevation ((**B**)numbers and colours represent locality elevation). Significant explanatory variables are identified by PERMANOVA (Table 1).

The body shape variability varied with the elevation (Figure 3B, Table 2) and the number of lateral spots decreased with elevation (Figure 4, Table 3).

Table 2. Morphometric variance of 111 individuals of *Sabanejewia* spp. within the groups and pairwise comparisons of group variances (two ways) based on elevation of the localities listed in Table S1. Elevation in m a.s.l.

|		**Observed Variances by Group**												
		0.00053	0.00086	0.00068	0.00056	0.00072	0.00076	0.00077	0.00053	0.00044	0.00087	0.00086	0.00085	0.00066	0.00101
	Elevation	34	133	140	149	151	159	180	194	195	200	219	222	261	269
	34	1													
	133	0.1069	1												
	140	0.4537	0.3722	1											
	149	0.8656	0.1542	0.5719	1										
	151	0.333	0.5124	0.8185	0.4335	1									
	159	0.2417	0.647	0.6647	0.3221	0.8451	1								
Pairwise P-values between variances	180	0.2402	0.6701	0.6458	0.3146	0.8215	0.9811	1							
	194	1	0.1108	0.4723	0.8704	0.3411	0.2494	0.2355	1						
	195	0.6642	**0.0401**	0.2455	0.5447	0.1653	0.1127	0.1062	0.6664	1					
	200	0.1059	0.9546	0.3666	0.1473	0.4917	0.6234	0.6309	0.1118	**0.0436**	1				
	219	0.11	0.9903	0.374	0.1518	0.5194	0.6557	0.6691	0.1147	**0.0456**	0.9473	1			
	222	0.1197	0.9542	0.4058	0.1678	0.5491	0.6929	0.7053	0.1229	**0.0491**	0.914	0.9611	1		
	261	0.4992	0.3404	0.9529	0.6062	0.7781	0.6153	0.6109	0.5072	0.264	0.3318	0.3453	0.3737	1	
	269	**0.0211**	0.4488	0.0964	**0.031**	0.1597	0.2322	0.2305	**0.0215**	**0.0055**	0.4998	0.4412	0.4147	0.0897	1
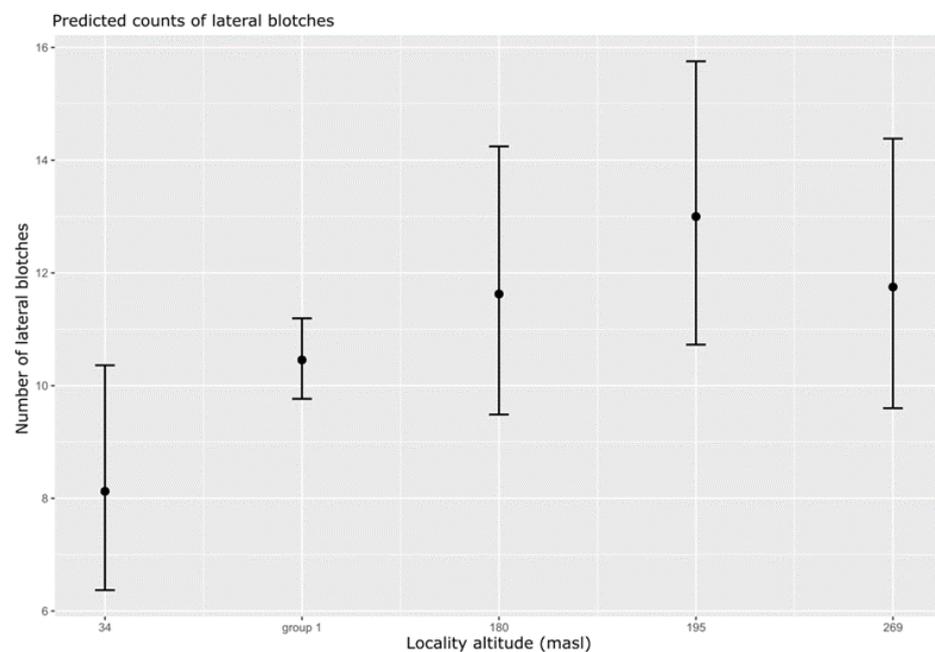
Figure 4. Generalised linear mixed model (GLMM, results of Table 3, Model fit 3) plot showing a significant correlation between the number of lateral spots and elevation (numbers represent locality altitude in meters) (results of Table 3., model 3.) for 111 individuals of *Sabanejewia* spp. Elevation: 34 m–River Danube (type locality Vidin, Vrav, BG); 180 m and 195 m–River Petrinjčica (loc. Miočinovići, Dodoši, HR); 269 m–River Treska (type locality Skopje, NMK), group1–other localities (133–261 m elevation) (Table S1).

Table 3. Summary of GLMM (with mixed model effect of the localities) for lateral blotches (spots) of the 111 *Sabanejewia* spp. and explanatory variables.

GLMM Family	Response	Model Fit	Explanatory	df	Chi^2	*p*	ANOVA	AIC	Df
Poisson	Lateral blotches	3 (ANOVA summary)	Intercept (full model)	1	3.9	**<0.05**	Type 3	500	6
			Elevation2 (groups)	4	10.9	**<0.05**			
			Model summary	**Est**	**SE**	**Pr (>|z|)**			
			Intercept (Elevation 34)	2.09	0.12	**<0.01**			
			(Elevation group1)	0.25	0.13	**<0.05**			
			(Elevation 180)	0.36	0.16	**<0.05**			
			(Elevation 195)	0.47	0.16	**<0.01**			
			(Elevation 269)	0.37	0.16	**<0.05**			
		2	Intercept	1	3.9	**<0.05**	Type 3	517	18
			Elevation	13	13.97	>0.37			
			Sex	1	0.79	>0.77			
			Slope	1	0.58	>0.44			
			SL	1	0.68	>0.40			
		1	Elevation	13	13.97	>0.37	Type 2	569	46
			Sex	1	0.03	>0.86			
			Slope	1	0.16	>0.68			
			SL	1	0.72	>0.39			
			Double int. (all exp.)	no sig. effect of interaction					

Specimens from the type locality of *S. balcanica* in the Vardar River basin (Lepenac R., Treska R.: Table 1 No. 1, 3, elevation 269 m) had a significantly higher number of lateral spots (Table 3—Model fit 3, Figure 4). In contrast, individuals from the *S. bulgarica* type locality in the Danube River (Table S1. No. 17, elevation 34 m) had a lower number of spots (Table S1, Table 3—Model fit 3, Figure 4) and a different body shape (Table 1, Figure 3A). Based on the GLMM results (Table 3), we found a significant correlation between the locality elevation (grouped according to significant levels of the GLMM summary) and the number of lateral spots for the populations from the Danube River (34 m a.s.l.), group 1 of the localities (133–261 m a.s.l.), the Petrinjčica River (180 and 195 m a.s.l.) and the population from the Treska River (269 m a.s.l.). Based on our results (Figure 4) and the field observation, we suggest three ecological groups within the river continuum: (1) ≤132 m a.s.l. (lowland rivers–metapotamon lowland forms/monotypic fine sediments prevalent, lowland ecomorph dominant); (2) 133–260 m a.s.l. (middle river–epipotamon, variability of substrate grain size, high variability of ecomorphs); and (3) >180 m a.s.l. (foothills–hyporithron, fine sediments very rare or absent, increased number of spots to support surface mimicry).

Analysis of the mitochondrial sequences from 54 individuals identified 49 *cyt b* haplotypes based on 85 variable nucleotides and 49 parsimony informative sites. Overall, haplotype diversity was high (0.995 ± 0.003) with relatively low nucleotide diversity (0.0109 ± 0.0007). The values of Tajima's *D* and Fu & Li's *D* neutrality tests were negative (−1.3290 and −1.8483, respectively), but not significant, indicating an excess of low-frequency polymorphisms relative to expectation. The phylogenetic trees clustered analysed *Sabanejewia* haplotypes into six lineages (Figure 5).
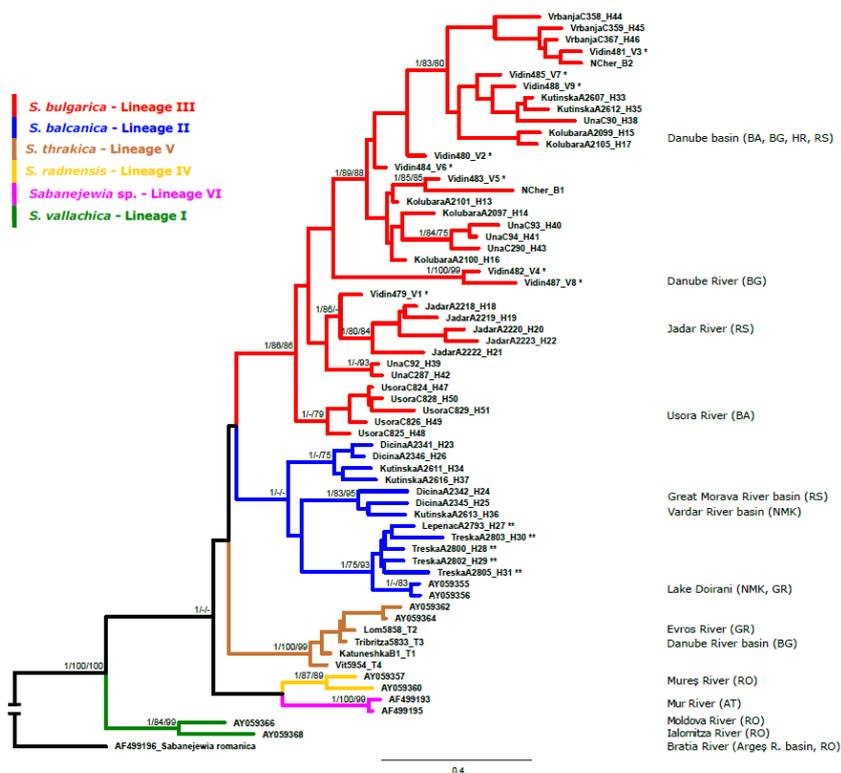
Figure 5. Bayesian consensus tree of *Sabanejewia* DB complex haplotypes resulting from the analysis of *cyt b* sequence data. Bayesian posterior probabilities and ML/NJ bootstrap values are listed above the branches (only value 1 for BI and ≥75% for ML and NJ are shown). The letters in brackets indicate the international country codes. The tip labels are river names and haplotypes or the GenBank accession number (letter H–from [17]; letters AF, AY–from [13], letters V from [15] letters B, T–our samples; see Table S1) * *S. bulgarica* type locality, ** *S. balcanica* type locality.

All analysed samples of *S. balcanica* and *S. bulgarica* belonged to their expected lineages, *S. balcanica* (lineage II) and *S. bulgarica* (lineage III), respectively. The lineages were defined by the presence of sequences of specimens from the type localities of both species. The specimens analysed in this study belonged to subclades II (*S. doiranica–S. balcanica*) and III (*S. montana–S. bulgarica–S. balcanica*) within the DB complex according to Perdices et al. [13]. The mean genetic TN93 distance among lineages included in our study was 0.018 (range 0.009–0.027), while intraspecific/intragroup distances ranged from 0.001 to 0.003 (Table 4). Estimated molecular divergence between the sequences from the two type localities was 0.017.

Table 4. Genetic divergences (by the TN93 model) on *cyt b* sequences between the molecularly studied *Sabanejewia* species. (The number of base differences per site from averaging over all sequence pairs between groups are shown; standard error estimate(s) are shown above the diagonal. Distances within groups are shown diagonally in bold).

	S. bulgarica	*S. balcanica*	"*S. radnensis*"	"*S. thrakica*"	*Lin. VI.*	*S. vallachica*
S. bulgarica	**0.003**	0.003	0.003	0.004	0.003	0.004
S. balcanica	0.018	**0.003**	0.002	0.003	0.003	0.004
"*S. radnensis*"	0.014	0.013	**0.003**	0.003	0.002	0.004
"*S. thrakica*"	0.019	0.018	0.013	**0.002**	0.003	0.005
Lineage VI.	0.015	0.015	0.009	0.014	**0.001**	0.004
S. vallachica	0.027	0.026	0.019	0.026	0.02	**0.004**

4. Discussion

Molecular studies that have used genetic data to infer the phylogenetic relationships of *Sabanejewia* have shown that there is a complex of evolutionary lineages inhabiting the Danube–Balkanian region [11,13,14]; however, morphology does not parallel their taxonomy. Our study evaluates the importance of habitat type (environment) on external morphological characters (body shape and colouration).

The distribution of *Sabanejewia* spp. within a geographic region has been very often characterised by the optimal substrate composition [36,37] and habitats [15,38]. However, the ecological adaptability of the genus representatives is great and suggests genus distribution from river delta [39,40] to streams at an altitude of 750 m a.s.l. in Europe [1], and almost to 2000 m a.s.l. in Asia [41]. Our results showed a geographical gradient of phenotypes similar to those described by Bănărescu et al. [3]. The difference of phenotypes increases at the boundaries (min/max. elevation) within the analysed range of locality elevations (Figures 3B and 4). Furthermore, the local habitat conditions give rise to different phenotypes characterised by body shape (Figure 3B) and the number of lateral spots (Figure 4). The intergradation of the phenotypes described by Bănărescu et al. [3] is evident in streams traversing larger elevation differences (Fedorčák, *pers. obs.*) characterised by diverse microhabitats, reflecting the transition between foothills to lowlands [15]. The cline of the external characters (body shape, lateral spots pattern) reflects the environmental conditions (hydrogeology), which include a range of ecological factors (water velocity, substrate composition, depth, etc.). Based on our data (Figure 4), we identified three ecological groups characterised by a different number of lateral spots and corresponding to the specific habitat conditions: (1) ≤132 m a.s.l. (lowland rivers–*metapotamon*, lowland forms, mean 8 spots); (2) 133–260 m a.s.l. (*epipotamon*, a high variability of forms, mean 10.5 spots); and (3) >180 m a.s.l. (*hyporithron*, sub-montane forms, mean 12 spots). Similar results have been described for populations of the Timiș River [3] (p. 30). Spot groups may overlap (Figure 4) or show a slightly different cline according to the specific environmental gradient in the context of local specifics of the river basin (length of the river basin, hydrology, geology, etc.) [42].

Our results also show that the specimens from the type localities of *S. balcanica* and *S. bulgarica,* analysed by us, represent different phenotypes from different ecological conditions (habitat) characterised by different elevations (Table S1—loc. 1, 3, 17). Moreover, the phylogenetic difference between *S. balcanica* (Figure 5. blue clade, lineage II) and *S. bulgarica* (Figure 5. red clade, lineage III) specimens from their type localities (Figures 5 and 6A,B) is well distinguishable (0.009–0.027).

The haplotypic similarity found in *S. balcanica* among specimens from the type locality (Lepenac and Treska Rivers in the Vardar River basin) and Lake Doirani showed that *S. balcanica* haplotypes are present at both localities (Figure 5). However, in the Danube River basin, the *S. balcanica* haplotypes were observed only in two rivers (Dičina, Kutinska) belonging to the Great Morava River basin in Serbia [17], which are closest in geographic proximity to the Vardar basin. Consequently, we consider the presence *S. balcanica* lineage (II) in the Danube River basin of Bosnia and Herzegovina [44] to be questionable. In contrast, the presence of *S. bulgarica* lineage (III) has never been confirmed south of the Great Morava River basin (Serbia) and the Bulgarian Danube River basin. Furthermore, Križek et al. [15] suppose that *S. bulgarica* (lineage III) is widely distributed in the Danube drainage basin. We also propose that the distribution of lineage III is likely to be broader as it included specimens from the Croatian Danube tributaries recorded by Buj et al. [14]. The specimens from the type locality of *S. bulgarica* (lineage III, Vidin) have a different body shape (Table 1; Figure 3A elevation 34, Figure 6B). The evident morphological differences (bigger body, the shape of the mental lobe, longer barbels, etc.) of *S. bulgarica* specimens from Vidin have been previously documented also by Drensky [45] and Križek et al. [15]. Our samples differ mainly at the pre/post dorsal part (Figure 3A,B and Figure 6B–D). These differences are explained by the elevation of the locality and specimen's sex (Table 2; Figure 3A,B). Križek et al. [15] also showed that the humpback shape (aD) increases with

the presence of fine sediments (the most common sediments of lowland habitats). We hypothesise that *Sabanejewia* spp. from slow-flowing waters (the Danube River in Vidin) generally have laterally compressed and deeper bodies (Figures 2 and 3C), which make them more flexible for maneuvering between river obstacles [46]. Taeniform (ribbon) shape fish are laterally compressed, which is generally advantageous for hiding in bottom shelters (macrophyte roots, river woods, stony particles, etc.). The "hump-backed" appearance pointed out by Iftime [5] is associated with downstream (lowland) forms and hormonal activity during the spawning period. Our results (Table 1; Figure 3B) similarly confirm shape differences affected by sex. We propose that these differences may be caused by an enlargement of the gonads during the spawning season with consequent differences during the season. Changes in the shape of the body of males known as the so-called lateral swelling [1] is linked to spawning behaviour [47].
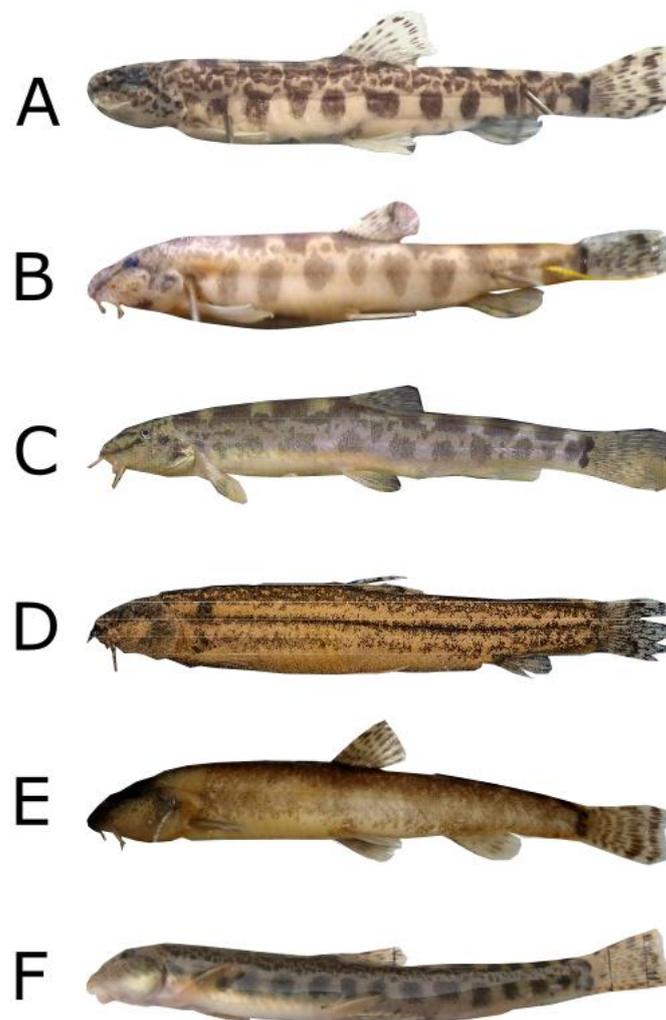
Figure 6. Colouration patterns of the *Sabanejewia* spp. originating from different types of habitat and elevation. (**A**) *S. balcanica*, type locality River Treska in Skopje (269 m a.s.l.); (**B**) *S. bulgarica*, type locality River Danube in Vidin (34 m a.s.l.); (**C**) surface metallic sheen of *S. bulgarica* originating in the Danube delta of Ukraine (Figure A. M. Naseka *in* [6]); (**D**) *S. caspia*–Anzali wetland (28 m a.s.l.) (*in* [40]); (**E**) *S. baltica*–River Ubort near Maidan-Kopishchenskii (154 m a.s.l., river near wetlands) (Figure *in* [43]); and (**F**) *S. baltica*–River Stradomka near Chrostowa (215 m a.s.l.) (Figure Michal Nowak).

Finally, *S. bulgarica* lineage (III) in their type locality (Danube River; Vidin) is externally well distinguishable due to their adaptation for specific habitat parameters given by low locality elevation (Table S1, No. 17). Križek et al. [15] reported the distribution of the *S.*

bulgarica in the large area within the Danube basin, but the type locality (Danube River in Vidin) of this species is located at the lower edge of the elevational distribution (near the Danube River delta). Quite the opposite, *S. balcanica* (lineage II) from the type locality (Vardar River Basin; Figure 3B elevation 269, Figure 6A) does not differ morphologically from other specimens analysed (Figure 3B, elevations 149, 151, 159, 194, 200 m). This means that *S. balcanica* (lineage II) has the same body shape as *S. bulgarica* (lineage III) specimens occurring in a similar environment (environmental conditions at higher elevation). This result is supported by the observation that the body shape of *S. balcanica* (lineage II) from the lower Vardar River (Axios River, Greece) could be much more similar to the body shape of *S. bulgarica* from the type locality (Danube River, Bulgaria). This hypothesis is supported by findings of Economidis and Nalbant [48], who identified lowland morphotypes (seven spot forms) of *S. balcanica* at the streams around the Vardar (Axios) River in the lowlands of Greece. We can confirm a statistically significant difference in the number of lateral spots of *S. balcanica* (lineage II, type locality Vardar River) and *S. bulgarica* (lineage III, type locality Danube River) from different elevations (Table 3, Figure 3). This cline of *Sabanejewia* colour forms has been observed several times [3,4,49]. In addition, Križek et al. [14] confirmed the increase of the number of the lateral spots with the presence of a coarse substrate in foothill river zones. Here, we hypothesise that: (1) the deep habitat and higher turbidity of lowland rivers (*metapotamon*) favour the mechanoreceptor–barbels and affect diurnal activity of spined loaches [50,51], and (2) shallow (sub-mountain) habitats and the coarse-grained bottom of foothill rivers (*metarithron*) favour the photoreceptor (eye) and body mimicry (surface–spots). The high mimicry adaptability of the genus *Sabanejewia* is evident and appears at the boundaries within the elevational distribution and in slow-flowing and stagnant habitats. Among well-known examples is the striped form of *S. caspia* from the wetlands of Iran (Figure 6D) or high colour variation of *S. baltica* (Figure 6E,F) which can be even greater based on actualized distribution [11]. Further, small spotted *S. larvata* [6,52] were recorded in palustrine of the Po River [53] and karst lakes of Croatia [54]. Another visual difference can be caused by the presence of intergeneric crossbreds [55,56] and specific habitat conditions affecting water velocity and the process of particle sedimentation (e.g., distance from a confluence, presence of river obstacles, and flood dynamic change of bottom deposits). Interesting is also the colour peculiarity of lowland *Sabanejewia* characterized by the violet-metallic sheen of the body surface (Figure 6C) observed also by Sivkov [5] in the Arges River (Romania).

5. Conclusions

The results of our work confirm the phylogenetic differentiation between *S. balcanica* (Figure 5. blue clade, lineage II) and *S. bulgarica* (Figure 5. red clade, lineage III). Both species can have various phenotypes depending on the environmental conditions on their habitat. The lowland phenotype is independent of the evolutionary lineage and is found in different molecular clades. Different phenotypes are well distinguished by the lateral colour pattern which reflects the different ecological conditions, mainly elevation and type of the bottom substrate. The colour pattern of cobitid fishes is a character that is more adaptable than body shape and vary across the wide spectrum of the populated environment [57]. The influence of local habitat parameters on the external characteristics of fishes [58] is a key in phenotype adaptability to local habitat conditions [59]. The body shape of individuals of both species also varies depending on specimen's sex. In further morphological studies of *Sabanejewia* DB complex, we recommend to take into consideration the type of habitat and the level of gonadal maturity.

Institutional Review Board Statement: Analysis was carried out on museum material. Sampling of museum material was in accordance with the national law for the collection of fish samples by all participating authors. Details are given in the material and methods section of the manuscript.

Data Availability Statement: Data are be available on request from the corresponding author. Will be applied after acceptance.

Conflicts of Interest: The authors declare no conflict of interest.

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