

# Morphological and Molecular Differentiation between Rheophilic Barbels *Barbus* spp. (Cyprinidae) from Headwaters at the Divide between Drainage Areas of the Danube, Vardar and Struma Rivers, Central Balkans

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**Abstract:** External morphology and cytochrome *b* structure of rheophilic barbels from headwaters of the Pčinja, Vrla (*Barbus balcanicus*) and Dragovištica (*B. strumicae*) Rivers in Southern Serbia at the watershed between the drainage areas of the Vardar (Axios), Danube and Struma (Strymon) Rivers, respectively, were examined. Barbel specimens from the Pčinja River were clearly different from both congeners from the other two rivers in the shape of the head and the length of the caudal fin. They were closer in external morphology to *B. strumicae* from the Dragovištica River as compared to *B. balcanicus* from the Vrla River. On the contrary, their relationship inferred from the structure of cytochrome *b* revealed closer relationship between *B. balcanicus* and the rheophilic barbel from the Pčinja River. Despite of the differentiation in morphology found between specimens of the rheophilic barbel, the divergence between them was insufficient for sound taxonomic inferences. Their phylogenetic relationships shed some new light on their evolutionary history in the study area.

**Key words:** *Barbus*, morphometry, cytochrome *b*, peninsular watershed divide, Balkan Peninsula

## Introduction

BANARESCU (1964), KARAMAN (1971), ALMACA (1981, 1984) and ECONOMIDIS (1989) distinguished two groups of *Barbus* spp. based on their morphological traits. One of them is the small-bodied rheophilic group of species with non-serrated, weak (*cyclolepis* type) or very weak (*meridionalis* type) fourth dorsal ray. The members of this group inhabit montane streams and their monophyly has been a subject of controversy (TSIGENOPOULOS et al. 1999). TSIGENOPOULOS & BERREBI (2000) stated, on the basis of examination of the cytochrome *b* (*cyt b*), that “riverine species from the Balkans and eastern Europe constantly produced weakly supported associations”. However, they agreed with the statement of BANARESCU (1990) on the Quaternary dis-

persal of riverine species from the middle Danube area of the former (late Myocene) Panonian basin of the Paratethys to northern Greece via the river catchments of the Južna Morava and Vardar (Axios) Rivers. Contrary to the expectations, that advance of the phylogeography of barbel taxa in the area of the Balkan Peninsula has not been followed by resolving their taxonomic status.

TSIGENOPOULOS & BERREBI (2000) considered rheophilic barbel from the Vardar River as *B. peloponnesius petenyi* Heckel, 1848. In addition, they assigned rheophilic barbel from the Struma River to *B. cyclolepis strumicae* Karaman, 1955, and that from the Danube River drainage area to *B. petenyi* Heckel, 1852. MACHORDOM & DOADRIO (2001) revealed,

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based on results on the structure of three mtDNA genes (cyt *b*, ATPases 8 and 6), that both *B. petenyi* Heckel, 1852 and *B. cyclolepis* Heckel, 1837 consisted of several different groups of populations, similarly to *B. peloponnesius* Valenciennes, 1842, which additionally questioned their taxonomic validity.

For the territory of Serbia, SIMONOVIĆ (2001) anticipated the occurrence of *B. cyclolepis* in the Božica and Lisina Rivers, tributaries of the Dragovištica River from the Struma (Strymon) River drainage area in south-eastern Serbia and assigned rheophilic barbel from the Danube River drainage area to *B. peloponnesius petenyi*. MARIĆ et al. (2004) reported for the first time the occurrence of *B. cyclolepis* in the Dragovištica River and esteemed its national IUCN conservation status in Serbia as vulnerable (VU) due to an ultimately narrow occurrence there (about 12 km of river section) and low abundance. Recently, ECONOMIDIS et al. (2009) denoted the species occurring in the Struma River drainage area as *B. strumicae* Karaman, 1955, following the phylogenetic species concept of CRACRAFT (1987, 1989) and the nomenclature given in KOTTELAT (1997) and KOTTELAT & FREYHOF (2007).

There are no contemporary records about either the occurrence or status of the rheophilic barbel in southern Kosovo or in tributaries of the Lepenac River that belongs to the Vardar River drainage. Nothing is known also about rheophilic barbel in the Nerodimka River that until recently has bifurcated between the drainages of the Danube and Vardar Rivers.

Following DIMOVSKI & GRUPE (1987), SIMONOVIĆ & NIKOLIĆ (1996) and SIMONOVIĆ (2001) also stated that *B. peloponnesius petenyi*, which they have considered occurring in the Danube River drainage area of Serbia, most likely also occurred in the Vardar River drainage of the FYR Macedonia. Relying on the genetic subdivision in the rheophilic barbels of the Danube River drainage area that KOTLIK & BERREBI (2002) have accomplished using morphological traits and mtDNA alleles of the cyt *b* gene, KOTLIK et al. (2002) suggested that the morphology of *Barbus balcanicus*, a new species they described from the Krupaja River (a tributary of the Mlava River in the middle Danube River system, Eastern Serbia), was only slightly distinguishable from *B. petenyi* and *B. carpathicus*, two other rheophilic barbel species from the Danube River drainage area. They also stated that outside the Danube River Basin, populations apparently conspecific with *B. balcanicus* are known from the Gallikos, Vardar and Aliakmon Rivers of the Aegean Sea drainage in northern Greece. VELKOVA-JORDANOSKA et al. (2010) consider the rheophilic

barbel recorded in the FYR Macedonia (Vardar and Crna Rivers and Lake Ohrid) as belonging to *B. peloponnesius*, thus following the nomenclature proposed by TSIGENOPOULOS et al. (2002).

KARAKOUSIS et al. (1993) reported that *B. peloponnesius* and *B. petenyi* significantly differed from *B. meridionalis* from southern France as well as that *B. petenyi* from Central Europe and *B. peloponnesius* from northern Greece were morphologically similar. In addition, morphological differences have been recorded between populations of *B. peloponnesius* from southern and northern parts of Greece (KARAKOUSIS et al. 1995).

The aim of this paper is to examine the morphology and the cyt *b* sequences of rheophilic barbels occurring in highland streams at the peninsular divide between the drainage areas of the Danube, Struma and Vardar Rivers in Serbia in order to advance in resolving their taxonomic status. The sam-



**Fig. 1.** Sampling localities: 1 – Pčinja River; 2 – Dragovištica River housing *B. strumicae*; 3 – Vrla River, where *B. balcanicus* were sampled; 4 – Krupaja River, the type locality of *B. balcanicus* Kotlik, Tsigenopoulos, Rab & Berrebi, 2002; 5 – Gradac River, situated at easternmost slopes of the Dinarid Mts., housing *B. balcanicus*, as well.

ples from the Vrla River in the Danube River drainage area have been considered *B. balcanicus* Kotlik, Tsigenopoulos, Rab & Berrebi, 2002, while those in the same area but from the Struma River drainage have been considered *B. strumicae* Karaman, 1955. The status of the rheophilic barbel from the Vardar River drainage area was difficult to infer. Therefore, three barbel samples from the Vrla, Dragovištica and Pčinja Rivers were assigned as distinct Operational Taxonomic Units (OTUs) (after SOKAL & ROHLF 1981) from Danube, Struma and Vardar Rivers, respectively.

## Materials and Methods

Samples of rheophilic barbels were collected in south-eastern Serbia (Fig. 1) on 26–27 May 2006 through single-pass point-sample electrofishing (PERSAT & COPP 1989) in three streams that flow from the slopes of the watershed situated at the Vlasina highland. Barbel individuals were sampled: (1) at the upper section of the Pčinja River, about 5 km downstream

of the city of Trgovište and about 7 km upstream of the Prohor Pčinjski Monastery ( $n = 7$ ); (2) from the Dragovištica River, about 3 km downstream of the city of Bosilegrad ( $n = 7$ ); and (3) at the Vrla River, 1 km upstream of the city of Surdulica ( $n = 8$ ). Their size in length is given in Table 1.

Character set modified from HOLČIK (1989) comprised 19 continuous morphological characters (Fig. 2): 1 – posterior head height; 2 – predorsal length; 3 – body height; 4 – postanal length; 5 – tail height; 6 – dorsal fin base length; 7 – anterior barb length; 8 – posterior barb length; 9 – anal fin height; 10 – caudal fin length; 11 – pectoral fin length; 12 – ventral fin length; 13 – head length; 14 – posterior head width; 15 – preorbital length; 16 – interorbital distance; 17 – horizontal orbit length; 18 – postorbital length and 19 – upper jaw length. Both posterior head height (1) and posterior head width (14) were measured at the level of the posterior end of the operculum. All characters were measured using digital calliper up to the nearest 0.1 mm. Values were transformed to indices using standard length and the

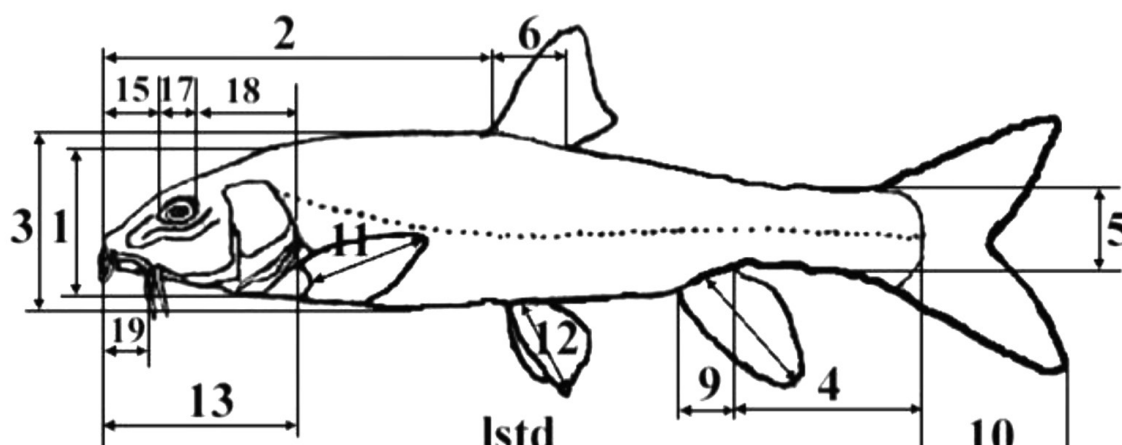


Fig. 2. Character set measured on rheophilic barbel (for explanation of characters, see „Materials and Methods“).

Table 1. Mean ( $\bar{x}$ )  $\pm$  standard error ( $se$ ) in % of standard length ( $SL$ ) for characters that were most variable in the three *Barbus* OTUs (associated numbers in parentheses denote size of samples), their  $SL$  ranges and ANOVA testing ( $df = 2, 18$ ; \* -  $p < 0.05$ ; \*\* -  $p < 0.01$ ).

Character	OTU			ANOVA	
	Struma <i>B. strumicae</i> Dragovištica R. (7)	Vardar <i>Barbus</i> sp., Pčinja R. (7)	Danube <i>B. balcanicus</i> Vrla R. (8)	F	p
$SL$ (mm)	102.4 – 179.8 mm	105.2 – 148.4 mm	73.1 – 152.4 mm		
	$\bar{x} \pm se$	$\bar{x} \pm se$	$\bar{x} \pm se$		
2	49.67 $\pm$ 0.347	51.75 $\pm$ 0.496	51.14 $\pm$ 0.745	3.720	*
3	22.54 $\pm$ 0.348	21.71 $\pm$ 0.581	23.21 $\pm$ 0.438	2.589	
4	18.15 $\pm$ 0.389	19.73 $\pm$ 0.404	19.66 $\pm$ 0.561	3.767	*
10	18.02 $\pm$ 0.446	19.38 $\pm$ 0.599	15.83 $\pm$ 0.815	7.867	**
13	20.08 $\pm$ 0.175	21.89 $\pm$ 0.257	21.22 $\pm$ 0.430	8.986	**
14	12.75 $\pm$ 0.308	13.31 $\pm$ 0.180	15.72 $\pm$ 0.339	30.906	**

arcus-sinus transformation was applied, in order to make the data set closer to the normal distribution. All analyses were made on the transformed character set, except the final descriptive statistics, which was accomplished in percent of standard length.

Principal Component Analysis (PCA) was applied in order to extract the most prominent continuous morphological characters. The dissimilarity between OTUs was studied using Manhattan distances between centroids of PC scores (SOKAL & ROHLF 1995) for each species and their phenetic relationships were analysed using the UPGMA clustering method (SNEATH & SOKAL 1973). The univariate testing for the most prominent characters using PCA by eigenvectors loadings was accomplished on PC scores using ANOVA and *post-hoc* Scheffé test. The analysis was performed using the package STATISTICA v. 5.1. (StatSoft Inc.). Phylogenetic analysis on continuous morphological characters, which were set as unordered, was worked out after the coding of states for characters that either revealed the greatest variability or showed strong discriminating power between OTUs, following the range, divergence and gap coding procedures of COLLES (1980), THORPE (1984) and JOHNSON & MICKEVICH (1977), respectively. The Maximum Parsimony method in construction of the most parsimonious trees was used, with the branch-and-bound search and using tree-bisection-reconnection algorithm for branch swapping. From the most parsimonious trees, the 50% Majority Rule consensus tree was resolved and tested for reliability by bootstrapping with 1000 replicates using the PAUP Version 4.0a158 for Macintosh (SWOFFORD 2017).

Fin clips from eight barbels (two from the Dragovištica River and three from the Pčinja and Vrla Rivers each) were sequenced for the analysis of the *cyt b* structure. Total DNA was extracted using the phenol–chloroform–isoamyl alcohol method (SAMBROOK et al. 1989) and a partial fragment of the *cyt b* (660 bp) was amplified using the primers Glu-F (5'-GAAGAACCACCGTTGTTATTC AA-3') and Cytb-R (5'-TCTTTATATGAGAARTANGGGTG-3') (ZARDOYA & DOADRIO 1998), under the conditions given in DUDU et al. (2012).

For an alignment of *cyt b* sequences of analysed rheophilic barbel OTUs, a sequence of the *cyt b* haplotype from the holotype specimen of *B. balcanicus* (KOTLIK et al. 2002) was used. Preliminary checking of haplotypes in FASTA format was done using the BLAST tool of the GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE=BLASTHome>). Alignment was accomplished using the ClustalW2 (LARKIN et al. 2007). Nucleotide

diversity in haplotypes was examined under the Kimura 2-parameter model and the Tajima's (1989) Neutrality test ( $D$ ) was calculated. Relationships between *cyt b* haplotypes were reconstructed using the Maximum Parsimony method and the tree-bisection-reconnection (TBR) algorithm (NEI & KUMAR 2000). The reliability of the inferred relationships was examined using bootstrap tests with 1000 replicates (FELSENSTEIN 1985) using the MEGA 6.06 software (TAMURA et al. 2013). Relationships between the analysed rheophilic barbel OTUs' haplotypes and those already stored in the GenBank (TSIGENOPOULOS & BERREBI 2000, TSIGENOPOULOS et al. 2002, MARKOVA et al. 2010, GEIGER et al. 2014, BUONERBA et al. 2015; accession numbers given in Fig. 5) were inferred by applying the Maximum Parsimony method, with the branch-and-bound search algorithm, TBR branch swapping with the reconnection limit of eight, unrooted 50% Majority Rule Consensus Tree and Bootstrapping with 1000

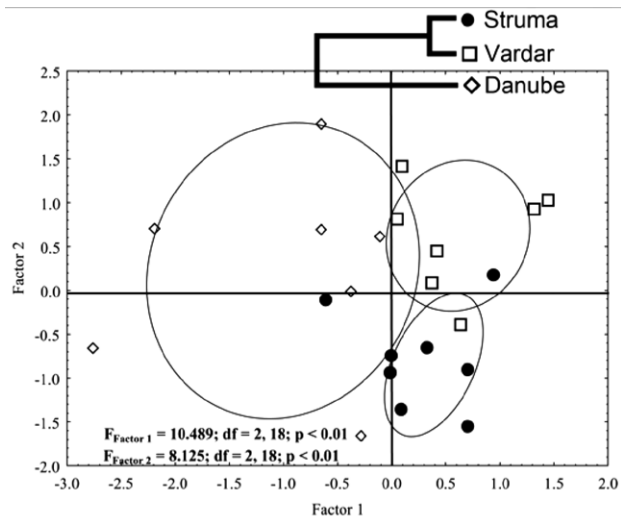


Fig. 3. Relationships between the three species of rheophilic barbels (upper right corner) analysed on their position in the space of 19 eigenvectors and their significantly variable position in the space of first two eigenvectors (Factors 1 and 2).

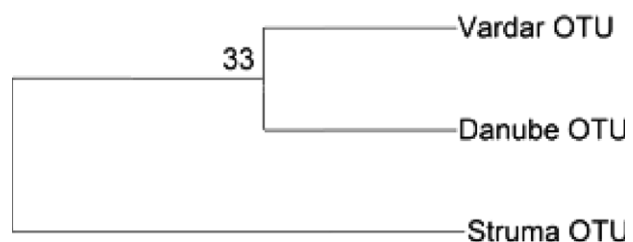
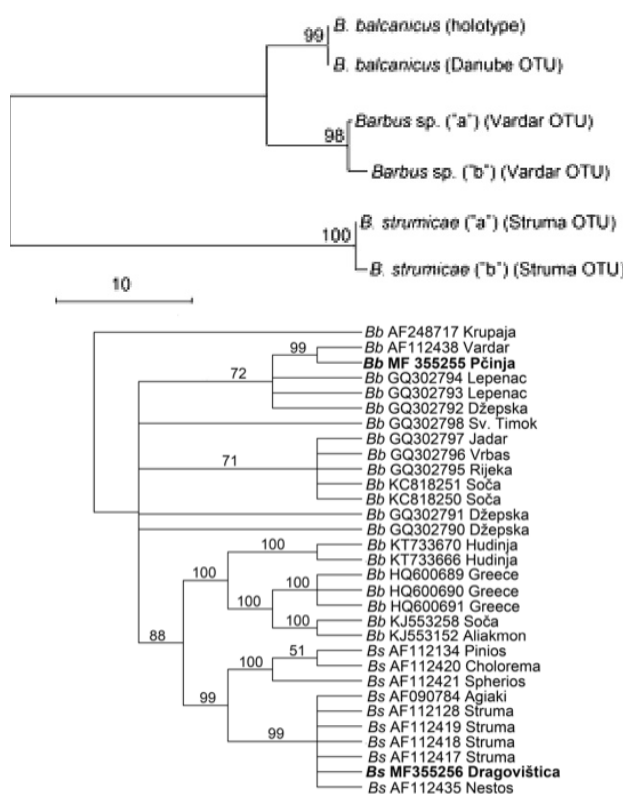


Fig. 4. Phylogenetic relationships inferred from the morphometric characters which either were most variable, or had a strong power of delimiting the three OTUs of rheophilic barbels with the bootstrap value associated to the Struma OTU's clade.



**Fig. 5.** Phylogenetic relationships inferred from the *cyt b* sequences (i.e., haplotypes) between the three rheophilic barbel OTUs with the associated bootstrap values (above) and between the new haplotypes found in the Pčinja (Vardar OTU) and Dragovištica (Struma OTU) Rivers and other *cyt b* haplotypes found in other rheophilic barbel OTUs and stored in the GenBank (below). Labels associated to clades denote nominal barbel species (*Bb* – *Barbus balcanicus*; *Bs* – *Barbus strumicae*), haplotypes' accession numbers and rivers where they were sampled. Numbers associated with clades are bootstrap values.

replicates for testing of the reliability of results, using the PAUP Version 4.0a158 for Macintosh (SWOFFORD 2017).

## Results

The first three eigenvalues explained 75.27% of the variability, but only for scores on Factors 1 ( $F = 10.489$ ;  $df = 2, 18$ ;  $p < 0.01$ ) and 2 ( $F = 8.125$ ;  $df = 2, 18$ ;  $p < 0.01$ ) particular barbel samples appeared as distinct groups. According to the *post-hoc* testing, barbels from the Danube OTU were significantly different for Factor 1 from two other samples that appeared similar to each other, whereas for Factor 2 barbels from the Vardar OTU appeared significantly different from the other samples. Characters 10 (caudal fin length) and 14 (head width) had greatest loadings on the first eigenvector ( $l_1 = 8.11$ , 37.37% of variance), characters 2 (predorsal length) and 13

(head length) on the second eigenvector ( $l_2 = 5.85$ , 29.93% of variance), whereas characters 4 (postanal length) and 3 (maximal height of body) had greatest loadings on the third one ( $l_3 = 2.38$ , 10.97% of variance). Clustering of Manhattan distances between centroids of factor scores on all 19 eigenvectors (Fig. 3) revealed that barbels from the Vardar OTU and those from the Struma OTU are much more similar in external morphology than each of them is to barbels from the Danube OTU.

The univariate analysis of the six most variable characters using ANOVA revealed that for all characters, except for Character 3 (body height), three barbel OTUs were significantly different (Table 1). The *post-hoc* Scheffe test (at the level of  $p < 0.05$ ) revealed that (a) the Struma OTU barbel have significantly shorter predorsal length (Character 2) than those from the Vardar OTU; (b) the Struma OTU barbel have significantly shorter postanal length (Character 4); (c) the Danube OTU barbel have significantly shorter caudal fin (Character 10) than those from both Vardar and Struma OTUs; (d), the Struma OTU barbel had the shortest head (character 13), significantly different from those of the Vardar and Danube OTUs and (e) for posterior head width (Character 14), barbel from the Danube OTU had significantly wider head than those from the other two OTUs (Table 1). Caudal fin length (character 10) and posterior head width (Character 14) had the strong power for delimiting barbel of the Danube OTU and those of the Vardar and Struma OTUs; head length (character 13) delimited barbel of the Struma OTU and those of the Danube and Vardar OTUs and length of upper jaw (Character 19) delimited completely barbel of the Struma OTU and those of the Vardar OTU, whereas delimitation of the Danube OTU was also good (Appendix 1).

Phylogenetic analysis on six continuous morphological characters reconstructed the three most parsimonious trees with none of the characters being parsimony-informative. The bootstrapping of the constructed consensus tree (Fig. 4) revealed very low support (33%) for the inferred relationships between the clades of rheophilic barbels. Two distinct clades were inferred: one for barbels of the Danube and Vardar OTUs who differed by three characters and the other where were those from the Struma OTU, who differed from barbels of the Vardar and Danube OTUs by four and five characters, respectively. The tree topology suggests the ancestral character of the Struma OTU barbel and determines character states in them as plesiomorphic, whereas the most advanced barbels with the autapomorphies in four characters were those of the Vardar OTU,

with the plesiomorphy in characters 4 (postanal length) and 14 (posterior head width) with the barbel of the Struma OTU. In addition to synapomorphies shared with barbels of the Vardar OTU, those from the Danube OTU were autapomorphic in characters 10 (caudal fin length) and 14 (posterior head width).

The partial mtDNA *cyt b* sequencing on two barbels from the Struma OTU and three barbels from the Danube and Vardar OTUs each revealed in total five haplotypes: two from the Dragovištica and Pčinja Rivers, each denoted for both of them “a” and “b”, and one from the Vrla River, which was the same as the haplotype (GenBank accession number AF248717) of the holotype specimen of *B. balcanicus* (Appendix 2). A total of 57 polymorphic sites out of 620 in total were detected. Two haplotypes from the Struma OTU barbels, of which the Struma OTU “a” haplotype was a new one (GenBank accession number MF355256) and that of the Struma OTU “b” was already a known haplotype (GenBank accession number AF112417), differed for only one (285) base place, i.e., 0.17%. Whereas, those from the Vardar OTU barbels, of which the Vardar OTU “b” haplotype was also a new one (GenBank accession number MF355255) and the Vardar OTU’s “a” haplotype was already known (GenBank accession number AF112438), differed for two base places (28 and 51) or 0.34%. The haplotype from the Danube OTU barbel is more similar to haplotypes from the Vardar OTU barbel (1.6 – 2.0% of difference) than to those from the Struma OTU (8.9 – 9.1% of difference). The nucleotide diversity in five *cyt b* haplotypes was  $\pi = 0.0506$  and Tajima’s  $D = 1.3126$ . The evolutionary history between haplotypes inferred using the Maximum Parsimony method revealed that the most parsimonious tree consisted of 58 evolutionary steps and had the Consistency Index CI = 0.982143, the Retention Index RI = 0.982143 and the Homoplasy Index HI = 0.018.

Phylogenetic analysis of 31 rheophilic barbel OTUs for *cyt b* haplotypes accessed and stored in the GenBank (with five haplotypes from the Pčinja, Vrla and Dragovištica Rivers) was reconstructed using 648 parsimony-informative characters (base positions) out of 1141 of them in total, while 465 characters were constant and 28 were parsimony-uninformative. The nucleotide diversity in these 31 *cyt b* haplotypes was  $\pi = 0.3562$  and Tajima’s  $D = 1.7044$ . Bootstrap 50% Majority Rule Consensus Tree (Fig. 5) was of the length of 1032 evolutionary steps, of the Consistency Index CI = 0.923, Retention Index RI = 0.974 and Homoplasy Index HI = 0.077. The main clades that comprised *B. balcanicus* and *B. strumicae* were strongly supported by the bootstrap values. In contrast to them, the bootstrap support for particular clades,

e.g., those of *B. balcanicus* from Greek lakes, the Soča River (western Slovenia) and Aliakmon (central Greece), Hudinja River (northern Slovenia) and *B. strumicae* from the Nestos, Vardar (Axios) and Pčinja Rivers, as well as that of *B. strumicae* from all other streams was much stronger. However, certain rheophilic barbel populations in the Danube River drainage area (e.g. those from Krupaja, Svrlijski Timok and Džepska Rivers) were very different for their *cyt b* haplotype. The polytomy occurring between OTUs within each of clades indicated that the relationships between them remain still unresolved.

The comparison of haplotypes “a” and “b” in barbels from the Vardar OTU to AF112122 and AF112438 haplotypes that TSIGENOPOULOS & BERREBI (2000) found in rheophilic barbels from the tributaries of the Lower Vardar River in Greece revealed that the new Vardar OTU’s haplotype “b” (MF355256) differs in two bases out of 594 or 0.3% from the AF112122 haplotype and only in one base or 0.1% from the AF112438 haplotype. The Vardar OTU haplotype “a” appeared identical to the AF112438 haplotype from the Lower Vardar River. The differences found between sequences of the known and new haplotypes “a” and “b” in rheophilic barbels from the Vardar OTU correspond to those found in the Struma OTU, i.e., the *B. strumicae* clade. The *cyt b* haplotype of the Danube OTU, i.e., the *B. balcanicus* from the Vrla River compared to those that KOTLIK & BERREBI (2002) found in rheophilic barbels from the Upper Sava and Soča Rivers, which they considered conspecifics, differed in 7 out of 594 bases or 1.2%, which was similar to a difference found between haplotypes in rheophilic barbels from Vrla Rivers (the Danube OTU) and Pčinja (the Vardar OTU).

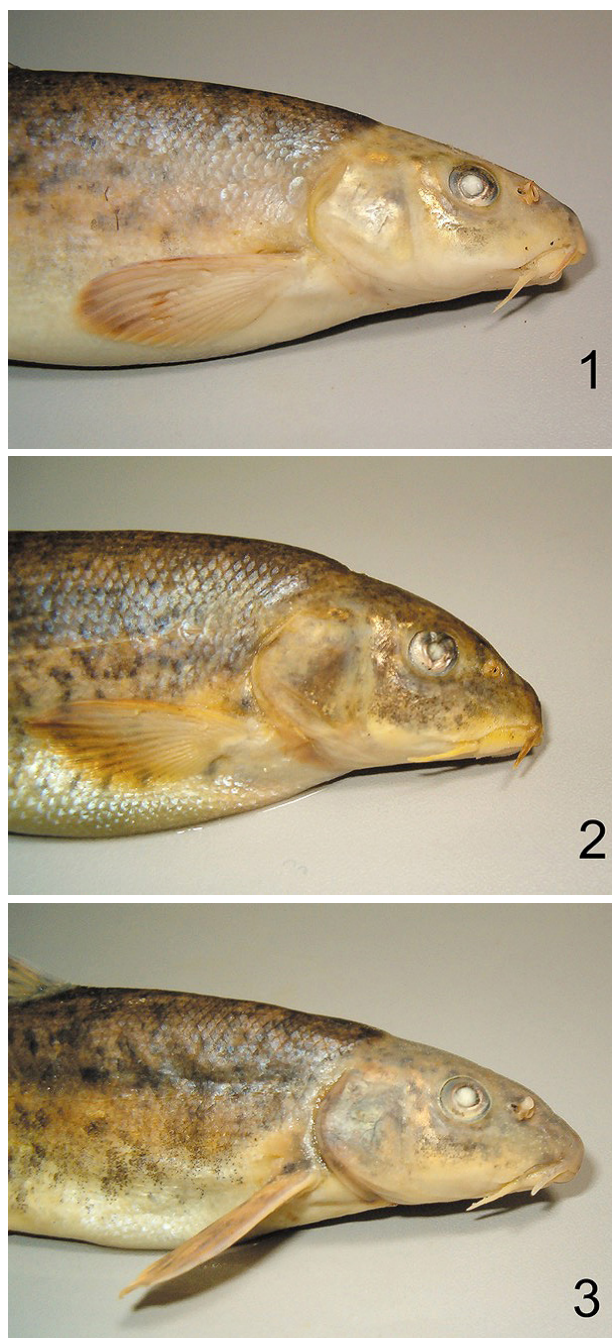
## Discussion

KOTLIK et al. (2002) stated that all rheophilic barbel species considered in this article are very similar by their blunt snout. However, there were obvious differences between rheophilic barbels from the rivers Dragovištica, Pčinja and Vrla relative to their head shape (both length and width) as well as to certain other morphometric characters (Table 1, Appendix 1). The shortest head length in the Struma OTU barbel corresponds to the shortest predorsal length which, combined with their narrowest posterior head width, makes their difference in head shape discernable by bare eye (Fig. 6).

The morphological similarity between barbels from the Struma and Vardar OTUs (Fig. 3) conformed neither to phylogenetic relationships inferred from the same type of characters (Fig. 4) nor to those

inferred from *cyt b* haplotypes. However, that similarity is in line with the report of TSIGENOPOULOS & BERREBI (2000) on the occurrence of the dominant *cyt b* haplotype (denoted as *B. cyclolepis strumicae* – 1) of barbels from the Struma River in those from the Vardar River. KOTLIK & BERREBI (2002) found using *cyt b* that rheophilic barbel from the lower Vardar drainage form sister group of populations to rheophilic barbel in headwaters of Soča River (Isonzo) and upper Sava River from the southern slopes of Alps (Fig. 5); they designated them also as *B. balcanicus*, each of them forming a separate group from *B. balcanicus* of the eastern Dinaric Alps, western Balkan and Rhodopes Mts., which we used here as Danube OTU for comparison. Distances derived from differences in both *cyt b* haplotypes and external morphological characters support the closer phylogenetic relationship between barbel of the Danube and Vardar OTUs than that between barbel of the Struma OTU and two others, which is in contrast to the report of TSIGENOPOULOS & BERREBI (2000) but congruent to the findings of KOTLIK & BERREBI (2002). The close relationship between barbels from the Soča River and Sava River catchments (based on their *cyt b* sequences) is in agreement with the close relationship between west-Danubian (Sava River system) brown trout (identified as *Salmo taleri* Karaman, 1933) and the endemic marble trout *S. marmoratus* Cuv., 1829 from the Soča River drainage demonstrated on the basis of their external morphology (SIMONOVIĆ et al. 2007). Additionally, a similarity in ancient character of their CR mtDNA was ascertained for brown trout of the Da-Vr haplotype from the Vrla River and Ad-Bož haplotype from the headwater of the Dragovištica River originating from the opposite side of the watershed in the Vlasina highland (MARIĆ et al. 2006, SIMONOVIĆ et al. 2017). All these similarities detected on the opposite slopes of watersheds support the hypothesis about the dispersal routes via interconnections between the captures of adjacent headwaters along the watersheds, as long trout fish (FUMAGALLI et al. 2002) and rheophilic barbel (MACHORDOM & DOADRIO 2001) are considered. However, a true character, either convergence or hybridization, of similarity in morphology between Vardar and Struma barbel OTUs (Fig. 3) that might appear as a consequence of dispersal opportunities via these captures cannot be inferred without further studies of their nuclear DNA characters.

ECONOMIDIS (1989) considered that river systems of Balkans home rheophilic barbel which introgressed there after the setting of the recent Danube River, about 700,000 years ago, a long time after the Pannonian remnants of the Parathethys Sea van-



**Fig. 6.** The shape of head of the three rheophilic barbel species: 1 – Struma OTU – *B. strumicae*; 2 – Vardar OTU – *B. balcanicus*; 3 – Danube OUT – *B. balcanicus*.

ished (HSU 1978). The distinctness of the clade of the Struma OTU (i.e., *B. strumicae*) in both external morphology and *cyt b* revealed here (Appendix 1) might suggest the more ancient character of that clade in relation to Vardar and Danube (i.e., *B. balcanicus*) OTUs; this is concordant to the much greater estimation of the time-scale (13.4 million years ago), in which rheophilic barbels shared their common ancestry (BUONERBA et al. 2015). That implicates to the multiple dispersals of rheophilic barbels

**Table 2.** Coding of the continuous morphological characters for character states in the three rheophilic barbel OTUs with the applied coding procedure indicated at each character.

Char-acter	OTU			Coding Procedure
	Struma <i>B. strumicae</i> , Dragovištica R.	Vardar <i>Barbus</i> sp., Pčinja R.	Danube <i>B. balcanicus</i> , Vrla R.	
	Character states			
2	2	1	1	Signifi- cance
4	2	1	1	Gap
10	1	1	2	Range
13	2	1	1	Range
14	1	1	2	Range
19	2	1	2	Range

**Table 3.** Means ( $\bar{x}$ ) and standard deviations (s) of 19 morphometric characters in *Barbus balcanicus* of size range in SL from the Vrla and Krupaja Rivers (Kotlik et al. 2002), as well as means in juvenile (juv.) and adult (ad.) *B. balcanicus* from the Gradac River (Miražić et al. 1996).

	<i>B. balcanicus</i> Vrla R.	<i>B. balcanicus</i> Krupaja R.	<i>B. balcanicus</i> Gradac R.	
	$\bar{x} \pm s$	$\bar{x} \pm s$	$\bar{x}_{\text{juv}}$	$\bar{x}_{\text{ad}}$
SL	8.5 – 12.5 cm	11.4 – 17.4 cm	juv.	adult
1	16.3 ± 1.09	15.4 ± 0.7	15.83	21.37
2	51.1 ± 1.97	49.6 ± 2.2	51.02	50.17
3	23.2 ± 1.16	21.5 ± 1.6	15.47	22.43
4	19.7 ± 1.48	18.3 ± 1.4	18.32	20.37
5	10.8 ± 0.85	10.5 ± 0.7	10.99	10.27
6	13.6 ± 0.69	11.8 ± 0.7	8.14	11.72
7	4.0 ± 0.44	4.0 ± 0.7	3.66	4.28
8	5.5 ± 0.62	5.9 ± 1.1	5.86	5.99
9	7.9 ± 0.57	7.6 ± 0.9	4.98	7.44
10	15.8 ± 2.16	18.8 ± 1.5	19.79	17.38
11	17.8 ± 1.82	16.9 ± 1.4	17.88	16.69
12	15.2 ± 1.12	13.9 ± 0.8	14.80	14.04
13	21.2 ± 1.14	24.2 ± 1.1	21.55	21.23
14	15.7 ± 0.90	14.4 ± 1.7	10.85	13.88
15	10.3 ± 0.82	10.5 ± 0.9	9.09	10.27
16	8.2 ± 0.45	7.2 ± 0.5	6.74	7.53
17	4.6 ± 0.53	3.9 ± 0.4	4.69	4.62
18	8.6 ± 1.11	10.5 ± 0.6	9.82	7.96
19	5.9 ± 0.43	n.a.	6.74	5.65

belonging to these clades. The first dispersal episode might have occurred from the inland freshwater basins being remnants of the ancient Parathetys Sea during the lower Tertiary. It was followed by subsequent isolation that caused by the uprising of mountain chains across and around the Balkans, far before the setting of the recent flow route of the Danube as TSIGENOPOULOS & BERREBI (2000) hypothesized. The recent *B. strumicae* might be the most direct de-

scendant of that lineage. The second dispersal episode might occur after the establishment of the recent Danube River flow route southern of Carpathian Mts. (HSU 1978), with the ancestor of the *B. balcanicus* that dispersed westwards and southwards from the middle Danube to cross over the watershed to the Vardar drainage over the river capture during the Pleistocene interglacials. Based on the available data (KARAKOUSIS et al. 1995, MACHORDOM & DOADRIO 2001, KOTLIK & BERREBI 2002), it is not clear whether there was already an ancient pool of recipient rheophilic barbells, into which more evolved ones have introgressed or they invaded the barbel-free area south-east of the Dinaric Alps and west from the Rhodopes and Balkan Mts. The support for this hypothesis for the secondary introgression and colonization of *B. balcanicus*, which seems to occur throughout the dispersal area of this species, lays in difference of the *cyt b* structure in haplotypes from the same or closely situated populations (e.g., Džepska River; Soča River in Italia and Slovenia; Hudinja River and Rijeka River in the Sava catchment in Slovenia), sufficiently great to separate them to distinct clades (Fig. 5).

The amount of sequence divergence found between two haplotypes in barbels from the Vardar and Struma OTUs is similar to that found by KOTLIK & BERREBI (2002) between rheophilic barbels of the lineage B in the middle Danube that KOTLIK et al. (2002) subsequently considered as the distinct species *B. carpathicus*. The amount of sequence divergence of 8.5–8.7% recorded between two haplotypes of rheophilic barbels of the Struma OTU and Danube OTU and that of 8.9–9.1% recorded between barbels of the Struma and Vardar OTUs are concordant to those found between the haplotypes of barbel lineages A (*B. balcanicus*) and C (*B. petenyi*) as well as of lineages B (*B. carpathicus*) and C (*B. petenyi*) in the middle Danube drainage area (KOTLIK & BERREBI 2002). The smaller amount of sequence divergence between *B. balcanicus*, i.e., barbels of the Danube OTU and that from the Vardar OTU in relation to divergence between *B. balcanicus* and barbels from the Struma OTU *B. strumicae* suggests that the former two OTUs belong to the same lineage assigned to clade A by KOTLIK & BERREBI (2002). However, the low bootstrap probabilities (57–62%) given for basal clade that comprise both *B. strumicae* and *B. peloponnesius petenyi* – *B. caninus* – *B. petenyi* lineages of TSIGENOPOULOS & BERREBI (2000) as well as the geographic distribution and recent taxonomic heterogeneity of the samples of the latter clade question the reliability of the relationship inferred from the *cyt b* sequence structure.

The diversity recorded for the taxa of rheophilic barbels in the area of south-eastern Serbia that comprises the slopes of the Vlasina highland is concordant with that recorded for both nominal taxa and haplotypes of brown trout (*Salmo* spp.) in the same area (MARIĆ et al. 2007, SIMONOVIĆ et al. 2007, 2017). Moreover, an ancient character inferred on unique mtDNA haplotypes about the recent diversity of *Salmo* of Danubian and Adriatic mtDNA lineages (*sensu* BERNATCHEZ 2001) in those headwaters of Vardar, Dragovištica and Južna Morava, respectively, also calls for the special attention to this area as a regional biodiversity hotspot as far as rheophilic fish are considered. It seems that the watershed between the three large river systems (Danube, Struma and Vardar) strongly influences the isolation and local diversification of rheophilic fish species, regardless those events are old or more recent.

*B. balcanicus* from the type locality, the Krupaja River in eastern Serbia (Fig. 1) (KOTLIK et al. 2002), overlaps for great majority of continuous morphological characters with reophilic barbels from the Vrla River (Danube OTU) and for some of them (5, 7, 8, 9 and 15) they are almost identical (Table 3). Moreover, the similarity seems even greater when *B. balcanicus* from Vrla and Krupaja are compared to those from the Gradac River (Table 3), a tributary of the Kolubara River in the lower Sava system (MIRAŽIĆ et al. 1996) (Fig. 1). The morphological similarity, together with the *cyt b* haplotype, confirms the identification of the barbels from Vrla River. This also supports the postulated distribution of *B. balcanicus* throughout the eastern Dinaric Alps and west of the Balkan Mts. (KOTLIK & BERREBI 2002, KOTLIK et al. 2002). However, the differentiation of the external morphology incongruent to relationships inferred using both external morphology and molecular marker as well as the degree of differentiation between *cyt b* haplotypes in rheophilic barbels from the recently isolated rivers Vrla and Pčinja do not justify their recognition as distinct taxa, although differentiation in certain morphological characters might indicate a progress in their divergence.

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**Appendix 1.** Mean ( $\bar{x}$ ), standard deviation ( $s$ ), minimum ( $min$ ) and maximum ( $max$ ) of *Barbus strumicae* from the Dragovištica River (Struma OTU), *B. balcanicus* from the Pčinja River (Vardar OTU) and *B. balcanicus* from the Vrla River (Danube OTU). Size of each sample  $n$  is given below in parentheses, total length  $TL$  and standard length  $SL$  are in mm and numerated characters in % of  $SL$ .

		<i>Barbus strumicae</i> Dragovištica R. ( $n = 7$ )				<i>Barbus</i> sp. Pčinja R. ( $n = 7$ )				<i>Barbus balcanicus</i> Vrla R. ( $n = 8$ )			
		$\bar{x}$	$s$	$min$	$max$	$\bar{x}$	$s$	$min$	$max$	$\bar{x}$	$s$	$min$	$max$
	$TL$	143.17	29.66	102.43	179.80	118.17	14.02	105.16	148.39	116.03	25.83	73.13	152.37
	$SL$	121.59	25.61	87.01	153.24	99.00	12.48	85.09	124.54	100.72	23.23	62.47	135.53
Characters	1	15.30	0.77	14.04	16.23	16.22	0.53	15.67	17.11	16.27	1.09	14.67	17.82
	2	49.67	0.92	48.13	51.05	51.75	1.31	50.02	53.66	51.14	1.97	47.89	53.83
	3	22.54	0.92	21.32	23.76	21.71	1.54	18.98	24.08	23.21	1.16	21.02	24.63
	4	18.15	1.03	16.76	19.22	19.73	1.07	18.06	21.06	19.66	1.48	17.06	21.32
	5	9.86	0.42	9.15	10.53	10.08	0.47	9.54	10.78	10.78	0.85	9.69	11.94
	6	13.02	1.09	11.23	14.63	12.96	0.73	12.15	14.41	13.58	0.69	12.26	14.43
	7	4.29	0.44	3.93	5.09	4.16	0.20	3.78	4.43	4.05	0.44	3.42	4.55
	8	5.59	0.44	5.14	6.43	5.78	0.36	5.36	6.31	5.52	0.62	4.47	6.37
	9	8.85	0.34	8.50	9.45	8.04	0.77	7.03	9.24	7.85	0.57	7.37	8.78
	10	18.02	1.18	16.50	20.31	19.38	1.59	17.98	21.79	15.83	2.16	11.69	17.70
	11	17.94	0.65	17.18	19.26	18.86	0.67	18.13	19.84	17.80	1.82	14.93	19.77
	12	15.25	0.59	14.57	16.31	15.93	0.53	15.23	16.90	15.21	1.12	13.68	16.69
	13	20.08	0.46	19.52	20.82	21.89	0.68	20.67	22.81	21.22	1.14	19.73	23.23
	14	12.75	0.81	11.67	13.98	13.31	0.48	12.69	14.01	15.72	0.90	14.36	16.67
	15	9.38	0.48	8.61	10.19	10.38	0.60	9.36	11.14	10.34	0.82	8.95	11.29
	16	7.42	0.38	6.85	9.88	7.44	0.54	6.96	8.54	8.22	0.45	7.52	8.84
	17	4.16	0.34	3.54	4.59	4.58	0.21	4.35	4.88	4.59	0.53	3.84	5.52
	18	9.05	0.63	8.27	9.90	9.36	0.38	8.65	9.89	8.62	1.11	7.22	10.71
	19	5.25	0.35	4.70	5.66	6.10	0.14	5.88	6.29	5.94	0.43	5.47	6.64

