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Phylogenetic evidence for a new species of *Barbus* in the Danube River basin ☆

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ABSTRACT

Three species of small-sized rheophilic *Barbus* fishes are endemic to and widely distributed throughout the mountain regions in the Danube River basin. In Hungary, barbels referred to as *B. petenyi* occur in streams in the foothills of the Carpathians near the borders with Slovakia, Ukraine and Romania. However, up to now, no genetic investigations were carried out on rheophilic barbels in this region. This study aims to clarify the taxonomic identity and distribution of the rheophilic barbels in the Hungarian plain based on molecular and morphological analyses. Two mitochondrial genes (cytochrome *b*, ATPase 6/8) and one nuclear gene (beta-actin intron 2) were sequenced and several morphometric and meristic characters were recorded. Phylogenetic and morphological analyses revealed that there are four genetically distinct lineages among the rheophilic barbels in the Carpathian Basin. The results demonstrated that North-Hungarian *Barbus* populations belong to *B. carpathicus* and that *B. petenyi* presumably does not occur in Hungary. As expected, *B. balcanicus* was only recorded in samples from the Balkans analyzed for reference. A distinct species, new to science, was discovered to be present in Sebes-Körös River (Crişul Repede) in eastern Hungary and western Romania and is formally described here as *B. biharicus* Antal, László, Kotlík – sp. nov.

1. Introduction

Petenyi's barbel (*Barbus petenyi* Heckel, 1852) is a small-sized rheophilic cyprinid species endemic to mountain regions of the Carpathian Basin (Kotlík et al., 2002; Bănărescu and Bogutskaya, 2003). The species was originally described from Transylvania in the present day Romania and was named after János Salamon Peté-nyi, an eminent Hungarian zoologist (Bănărescu and Bogutskaya, 2003). Since the original description the taxonomic status of the species was controversial, with some authors considering it a sub-species of *B. meridionalis* Risso, 1827, or of *B. peloponnesius* Valenciennes, 1842 (Bănărescu, 1964; Karaman, 1971; Doadrio, 1990). Despite superficial morphological similarity of barbels from the different parts of the Danube River basin, separate evolutionary lineages were described within *B. petenyi* based on the high genetic divergence of mitochondrial DNA (mtDNA) sequences (Tsigenopoulos and Berrebi, 2000; Machordom and Doadrio, 2001a; Kotlík and Berrebi, 2002). Later on, Kotlík et al. (2002) formally described two new species from the Danube River basin, distinct from *B. petenyi*, which they named *B. carpathicus* Kotlík, Tsigenopoulos, Ráb and Berrebi, 2002, and *B. balcanicus* Kotlík, Tsigenopoulos, Ráb and Berrebi, 2002. Further phylogenetic analyses of nuclear and mitochondrial genes confirmed the presence of the three different species, which most likely diverged from each other in the Miocene (Marková et al., 2010; Gante, 2011; Berrebi et al., 2013; Konopiński et al., 2013; Buonerba et al., 2015). The Petenyi's barbel inhabits the Eastern and Southern Carpathians and the Stara Planina Mountains, the Carpathian barbel *B. carpathicus* occurs in the Western and Eastern Carpathians, and the large spot barbel *B. balcanicus* lives in the Dinaric and Western Stara Planina Mountains (Kotlík et al., 2002; Kottelat and Freyhof, 2007) (Fig. 1).

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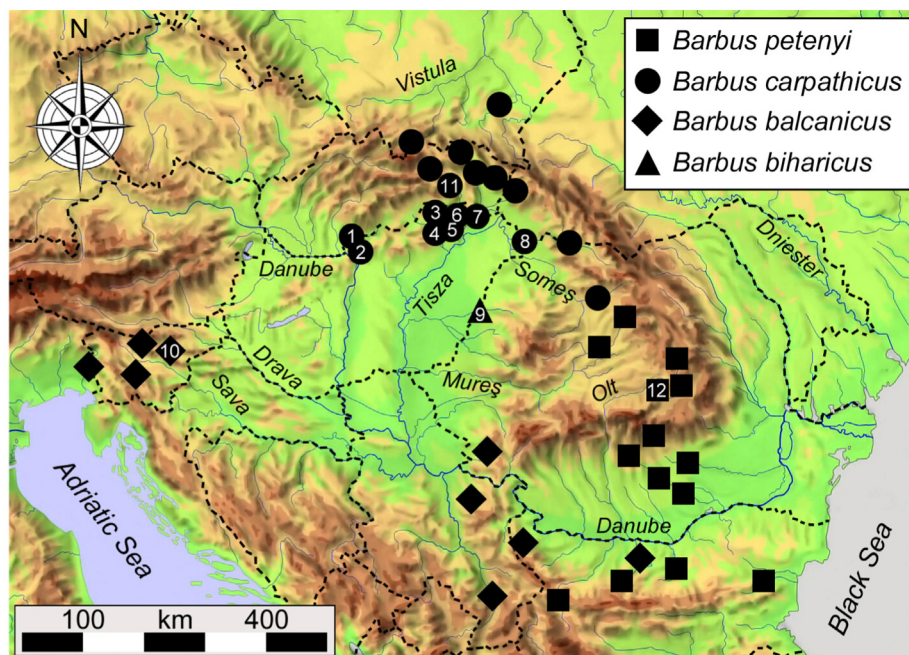

Fig. 1. Map of the Carpathian (Pannonian) Basin showing the geographic location of sampling sites of rheophilic barbels. Symbols without numbers indicate the original records of three *Barbus* species based on Kotlík et al., 2002. Symbols with numbers represent the sampling sites of the present study: 1. Kemence stream (samples HU006-008), GPS: 48.0461°N 18.9185°E; 2. Török stream (HU018-020), 47.8371°N 19.0062°E; 3. Bódva River (HU001-002), 48.3330°N 20.7306°E; 4. Sajó River (HU012-014), 48.1509°N 20.8071°E; 5. Vadász stream (HU021-023), 48.3227°N 20.8863°E; 6. Hernád River (HU003-005), 48.4198°N 21.2130°E; 7. Kemence stream (HU009-011), 48.4652°N 21.4963°E; 8. Tisza River (HU015-016), 48.1072°N 22.8315°E; 9. Sebes-Körös River (HU024-028), 47.0146°N 21.6255°E; 10. Hudinja River (SLO001-004), 46.2323°N 15.2778°E; 11. Torysa River (SK001-004), 48.9775°N 21.2539°E; 12. Aita stream (RO001-004), 45.9838°N 25.6509°E.

Due to their preference for mountain habitats, the rheophilic barbels are largely absent from the Hungarian (Pannonian) plain (Fig. 1). In Hungary, barbels referred to as *B. petenyi* occur in streams in the foothills of the Carpathians near the borders with Slovakia, Ukraine and Romania (Halasi-Kovács and Harka, 2012; Fig. 1). Due to the limited distribution in the country, *B. petenyi* is listed as strictly protected species in Hungary. However, up until now, no genetic investigations were carried out on rheophilic barbels in Hungary and their taxonomic status is therefore an open question. This study aims to clarify the taxonomic identity and distribution of the rheophilic barbels from the Hungarian plain based on molecular and morphological analyses.

2. Materials and methods

2.1. Sampling

Altogether, 28 individuals provisionally identified as *B. petenyi* were collected from nine geographical locations in Hungary, from all regions where the species was assumed to occur. Altogether 12 individuals of *B. petenyi*, *B. carpathicus* and *B. balcanicus* were collected as references from their habitats in Romania, Slovakia and Slovenia, respectively (Fig. 1). Samplings were carried out using electric sampling equipment (HansGrassl, Germany) in Hungary and hand nets in the other countries, between 2010 and 2012. Some individuals and the tissues samples were stored in 96% ethanol at −20 °C until DNA extraction. The collection and storage of the samples were approved by the National Inspectorate for Environment, Nature and Water, Hungary (permission number: 14/4620-3/2010.).

In 2015, additional 27 individuals of the suspected new species were collected from Sebes-Körös River (Crişul Repede) near Bratca, Romania (46.9279°N 22.5934°E), for the morphometric and meristic examination.

2.2. Molecular methods

DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen, Germany) from tissue samples. Two mitochondrial genes and one nuclear gene were analyzed by polymerase chain reaction (PCR) with high fidelity AccuTaq™ DNA polymerase (Sigma–Aldrich, USA). Partial sequence of mitochondrial cytochrome *b* (Cyt *b*) gene (598 bp) was amplified with primers L15267 (5′AAT GACTTGAAGAACCACCGT3′) and H15891 (5′GTTTGATCCCGTTTCGT GTA3′) designed by Briolay et al. (1998). The same Cyt *b* fragment was used in previous studies (Tsigenopoulos and Berrebi, 2000; Kotlík and Berrebi, 2002; Tsigenopoulos et al., 2002), which allowed direct comparison with published data. The complete mitochondrial ATPase 6 and 8 genes (842 bp) were amplified with primers ATP8.2 (5′AAAAGCRTYRGCCTTTTAAGC3′) and Co3.2 (5′GTT AGTGGTCAKGGGCTTGGRTC3′) designed by Machordom and Doadrio (2001b). The second intron of the nuclear-encoded beta-actin (Act-2) gene (496 bp) was amplified with primers Act18U21 (5′GCTCCAGAAAAACCTATAAGT3′) and Act554L21 (5′CTCACTGAA GCTCCTCTTAAC3′) described by Marková et al. (2010). Species from the genus *Barbus* are evolutionarily tetraploid, so that the primers were designed to selectively amplify a single paralogous copy of Act-2 (Marková et al., 2010). Briefly, the two paralogs (corresponding to the two ancestral diploid chromosome sets) were separated by electrophoresis based on the length difference between them, gel-purified and sequenced, and specific primers were designed to match regions differing in sequence, allowing selective amplification and sequencing of only the longer paralog in different species (Marková et al., 2010). The use of locus-specific primers is essential because it allows distinguishing allelic variation segregating at a single locus from variation between paralogous gene duplicates (Marková et al., 2010; Gante et al., 2011). The average uncorrected distance between the Act-2 paralogs (12.0%) largely exceeds average levels within the paralogs (1.6% and 1.1%;

147 Marková et al., 2010), allowing easy verification of the paralog
148 specificity of the obtained sequences (e.g. Yang et al., 2015).

149   Amplified PCR products were purified using the QIAquick Gel
150 Extraction Kit (Qiagen) after agarose gel electrophoresis. Direct
151 cycle sequencing was carried out with the BigDye® Terminator
152 v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA). The
153 amplicons were bidirectionally sequenced on an ABI PRISM® 310
154 Genetic Analyzer (Thermo Fisher Scientific) automated sequencer.

155 *2.3. Genetic data analysis*

156   Sequences were checked and aligned using the BioEdit version
157 7.2.5. software (Hall, 1999) and multiple alignments were manu-

158 ally adjusted using the GeneDoc version 2.7 software (Nicholas
159 et al., 1997). The new sequences were aligned with published
160 homologous sequences (phased haplotypes in case of Act-2;
161 Marková et al., 2010) for *B. petenyi*, *B. carpathicus* and *B. balcanicus*
162 as well as for a few outgroup species (Zardoya and Doadrio, 1999;
163 Tsigenopoulos and Berrebi, 2000; Machordom and Doadrio, 2001a;
164 Kotlík and Berrebi, 2002; Tsigenopoulos et al., 2002; Marková et al.,
165 2010; Levin et al., 2012; Konopiński et al., 2013; Buonerba et al.,
166 2015; for GenBank accession numbers see Fig. 2). Sequences
167 obtained in this study were deposited in GenBank under accession
168 numbers KT733654 to KT733670.

169   Phylogenetic relationships among the sequences were recon-
170 structed using the maximum likelihood optimality criterion. The
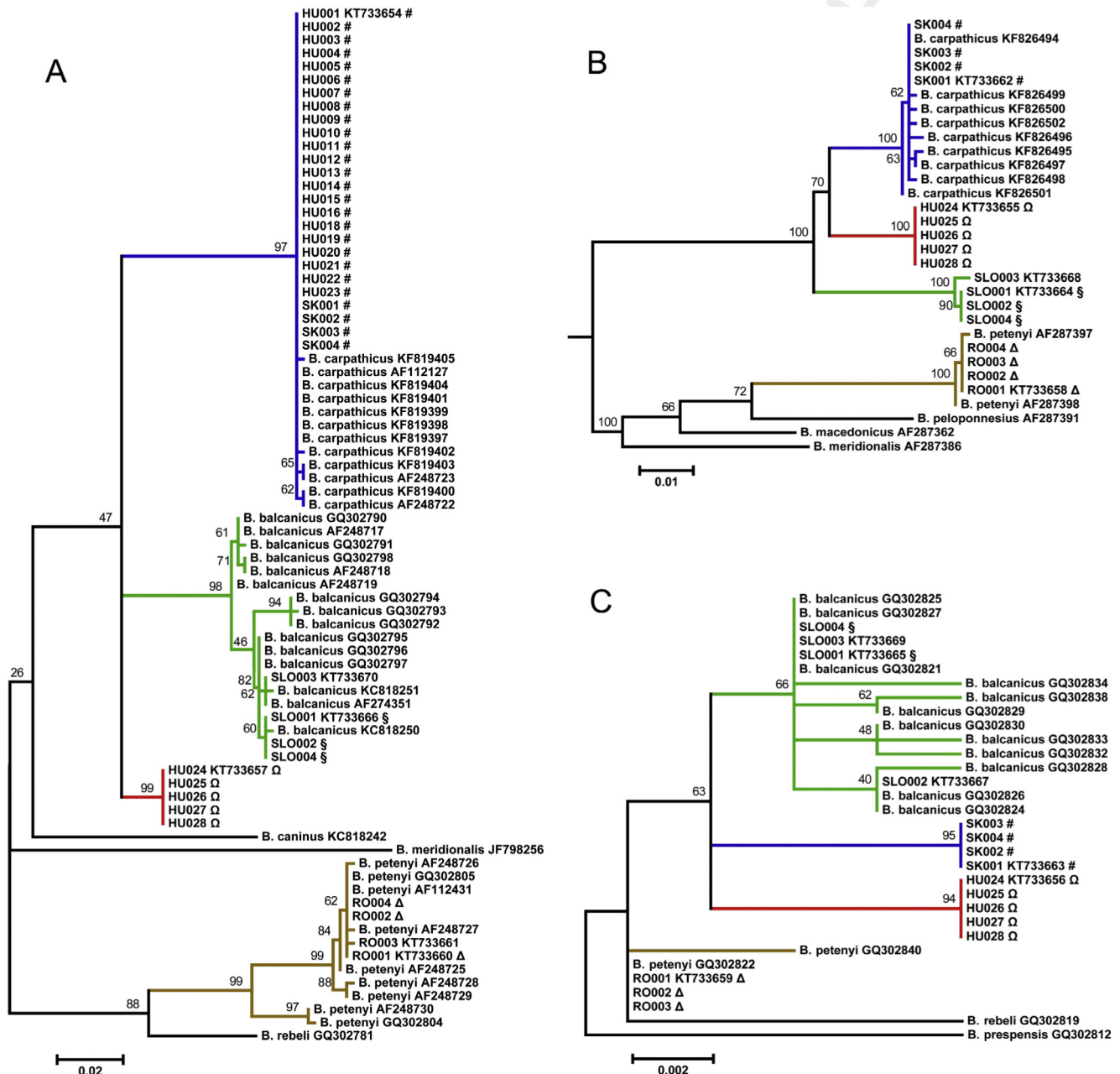

Fig. 2. Phylogenetic relationships of the rheophilic barbels from the Carpathian Basin. All trees are maximum-likelihood reconstructions with branch support estimated based on 1000 bootstrap replicates. (A) Cytochrome *b* gene (TN93+G model). (B) ATPase 6/8 gene (TN93+I). (C) Beta-actin gene intron 2 (T92). Previously published sequences are labelled with the species name and GenBank accession number. New sequences are labelled with the number of the individual sequenced. GenBank accession number is given for those individuals whose sequence was deposited in GenBank, with individuals carrying identical sequences indicated by symbols. Note that in (C) SLO003 differs from SLO001 by a single heterozygous site. Color coding: brown – *B. petenyi*, blue – *B. carpathicus*, green – *B. balcanicus*, red – *B. biharicus*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

TN93+G model of sequence evolution (Tamura and Nei, 1993; where G stands for the gamma-distributed rates across sites) was determined to be the appropriate model for the Cyt *b* dataset according to the Bayesian information criterion used as a measure of goodness of fit of different models to the data (Posada and Buckley, 2004; Tamura et al., 2011). A TN93+I model (where I is the proportion of invariable sites) was identified as the best-fit model for the ATPase 6/8 data set and a T92 model (Tamura 92) for the Act-2 dataset. Maximum-likelihood phylogenetic analyses were performed using the NNI (nearest neighbor interchanges) and SPR (subtree pruning and regrafting) tree search algorithms. Branch support for the phylogenetic partitioning in the trees was quantified by bootstrap analysis with 1000 random replicates (Felsenstein, 1985). The calculations were performed with both the MEGA6 (Tamura et al., 2013) and PhyML software (Guindon et al., 2010). Model-corrected pairwise genetic distances between species were calculated with MEGA6.

2.4. Morphological data analysis

Morphological analysis was performed to reveal any morphological differences between the *Barbus* species. The morphological characters (altogether 27) were scored based on Kotlík et al. (2002) and Žutinić et al. (2014). In the case of *B. petenyi*, *B. carpathicus* and *B. balcanicus* the dataset of Kotlík et al. (2002) was used.

In order to eliminate the effect of size, the morphometric data were standardized according to Elliott et al. (1995). Canonical Variance Analysis (CVA) was performed on the entire standardized dataset ($N = 85$) to test the interspecific differences with STATISTICA 12.0 software (StatSoft, 2013). Subsequently, Spearman rank correlation analysis were used to quantify the importance of each morphological feature (i.e., which are primarily responsible for the separation). In the cases of morphometric characters which proved to be significant additional ANOVA post-hoc (Tukey HSD) test were used to determine differences between species.

3. Results and discussion

3.1. DNA sequence analysis

There were six different haplotypes among the 39 aligned 546-bp sequences of Cyt *b* and five haplotypes among the 17 aligned 842-bp sequences of ATPase 6/8. Six unique sequences were identified among the 16 aligned 496-bp sequences of Act-2. All new Act-2 sequences were homozygous over the entire length, except for two *B. balcanicus* (SLO002 and SLO003), which were both heterozygous at the same site, with an additional site being heterozygous in SLO002. The sequences of SLO002 and SLO003 were analyzed as heterozygous with the heterozygous sites encoded with IUPAC symbols.

Phylogenetic relationships were reconstructed independently for each gene, with the analyses using MEGA6 (Fig. 2) yielding virtually identical results to those obtained with PhyML (not shown). The analysis combining new Cyt *b* data with *B. petenyi*, *B. carpathicus* and *B. balcanicus* sequences from GenBank unambiguously recovered the three clades known from the previous studies to correspond to these species (Fig. 2A). The majority of samples collected in different Hungarian populations were genetically closely related to *B. carpathicus* (HU001 to HU023) except for five fish (HU024 to HU028) collected in Sebes-Körös River (Crişul Repede), which formed a distinct clade (Fig. 2A). All of the new *B. carpathicus* sequences carried the same haplotype while two haplotypes were identified in the new samples of each *B. balcanicus* and *B. petenyi*, and another single haplotype was shared by all individuals in the new clade (Fig. 2A).

To test whether the Cyt *b* differentiation of barbels from Sebes-Körös River is reflected also by divergence at other genes, a subset of samples were selected for analyses of additional mtDNA and nuclear loci. The mitochondrial ATPase 6/8 and the nuclear Act-2 genes were amplified and sequenced in samples of the three species and in the samples from Sebes-Körös River. In all analyses, the samples of the three species and the Crişul Repede samples segregated into four distinct clades (Fig. 2B and C). In the case of *B. balcanicus*, two haplotypes of ATPase 6/8 and three haplotypes of Act-2 (two of them heterozygous; see above) were identified, while in the other species only one haplotype was found for each gene. The present study is the first to report the sequences of ATPase 6/8 genes for *B. balcanicus* and of Act-2 for *B. carpathicus*.

From these results it can be concluded that the rheophilic barbels from North-Hungary belong to *B. carpathicus* and are closely related to *B. carpathicus* from Slovakia. However, the results suggest that *B. petenyi* does not occur in Hungary. Instead, the phylogenetic analyses suggest that the rheophilic barbels from Sebes-Körös River in eastern Hungary belong to a distinct genetic lineage with a differentiation as great as that among other species (Table 1), thus representing a new, fourth species in the Carpathian Basin.

3.2. Morphological analysis

According to the result of CVA, the three known species and the new species show remarkable morphological differences based on the 27 recorded characters (Table 2). The first canonical axis of CVA explained 71.6% of the total variance (Fig. 3; Eigenvalue = 5.58; Canonical $R = 0.92$; Wilks' $\lambda = 0.03$; Chi^2 test $P < 0.0001$). The second axis accounted for 17.9% of the total variance (Eigenvalue = 1.39;

Table 1
Genetic distances between the rheophilic species of *Barbus* of the Danube River basin. Average distance between species is given above the diagonal and net distance below. Mean distance within each species is given in bold along the diagonal. All estimates are expressed as percentages.

Gene	Species	B. petenyi	B. carpathicus	B. balcanicus	B. biharicus
Cyt *b*	B. petenyi	**1.4**	10.7	10.9	9.2
	B. carpathicus	10.0	**0.1**	6.0	5.2
	B. balcanicus	9.7	5.5	**0.9**	4.3
	B. biharicus	8.5	5.2	3.8	**0.0**
ATPase 6/8	B. petenyi	**0.1**	11.6	11.1	11.3
	B. carpathicus	10.2	**0.2**	4.7	3.3
	B. balcanicus	9.9	4.3	**0.2**	4.5
	B. biharicus	10.1	3.1	4.2	**0.0**
Act-2	B. petenyi	**0.2**	0.9	0.7	0.9
	B. carpathicus	0.8	**0.0**	1.0	1.2
	B. balcanicus	0.4	0.9	**0.3**	1.0
	B. biharicus	0.8	1.2	0.9	**0.0**

Table 2

Meristic and morphometric characteristics of *Barbus petenyi*, *B. carpathicus*, *B. balcanicus* and *B. biharicus*. Data for *B. petenyi*, *B. carpathicus* and *B. balcanicus* are from Kotlík et al., 2002.

	Barbus petenyi		Barbus carpathicus		Barbus balcanicus		Barbus biharicus	
Meristic characters								
Rays in dorsal fin	III/7–8		II–III/7–8		II–III/7–8		II–III/7–8	
Rays in anal fin	I–II/4–5		I–II/4–5		I–III/4–5		I–II/4–5	
Scale counts	52 8–11/8–10 59		51 8–11/8–9 64		51 8–11/8–10 61		51 9–11/8–10 56	
Morphometric characters								
	Neotype NMW–94602	$N = 13$ Mean ± SD Range	Holotype NMW–94604	$N = 20$ Mean ± SD Range	Holotype NMW–94609	$N = 18$ Mean ± SD Range	Holotype HNHM–ICH 2015.51.1.	$N = 28$ Mean ± SD Range
SL (mm)	135.0	122.2 ± 11.4 110.0–143.0	110.0	151.4 ± 23.2 110.0–186.0	130.0	136.8 ± 16.3 114.0–174.0	79.3	90.9 ± 15.7 55.2–115.3
In percent of SL								
Total length	119.3	117.3 ± 3.5 107.1–120.2	117.3	115.9 ± 2.2 111.1–120.3	113.1	115.5 ± 1.8 111.5–118.5	119.4	117.4 ± 1.2 115.1–120.7
Head length	24.8	23.4 ± 1.1 20.8–24.8	24.8	24.6 ± 0.6 23.1–25.6	24.6	24.2 ± 1.1 22.0–26.1	23.7	23.5 ± 1.2 21.3–25.9
Maximum body depth	21.6	21.2 ± 1.4 19.1–23.1	21.7	20.8 ± 1.0 18.6–22.6	26.1	21.5 ± 1.6 18.8–26.1	22.0	20.9 ± 1.2 18.7–23.4
Predorsal distance	51.9	48.7 ± 2.5 43.7–51.9	48.8	49.7 ± 1.2 46.3–52.2	50.2	49.6 ± 2.2 45.5–54.2	51.8	51.5 ± 1.5 48.4–54.3
Preanal distance	69.9	69.7 ± 2.5 64.4–74.1	71.5	73.8 ± 1.6 71.5–77.4	72.0	73.0 ± 2.6 67.6–77.7	76.2	77.5 ± 1.3 74.9–80.8
Preventral distance	53.6	50.7 ± 2.1 45.9–53.6	53.1	53.2 ± 1.2 50.7–55.3	51.2	52.4 ± 1.8 50.2–56.6	56.6	56.7 ± 1.4 54.1–59.2
Distance between pectoral and ventral fin bases	30.4	29.5 ± 1.5 26.6–32.8	29.5	29.0 ± 1.3 26.2–31.7	29.3	29.6 ± 1.0 28.0–32.5	34.9	33.8 ± 2.1 26.8–36.7
Distance between pectoral and anal fin bases	48.9	49.2 ± 2.6 44.5–53.3	49.5	49.9 ± 1.7 46.6–54.5	50.2	51.3 ± 1.4 49.5–54.4	54.0	54.6 ± 2.2 48.7–58.4
Length of caudal paduncle	18.1	17.9 ± 1.1 15.8–19.2	19.5	18.3 ± 1.1 17.0–21.4	18.9	18.3 ± 1.4 15.9–21.2	17.6	16.7 ± 1.1 13.2–18.9
Minimum body depth	9.3	9.6 ± 0.4 9.0–10.4	9.5	9.0 ± 0.6 7.5–9.8	10.5	9.5 ± 0.8 7.9–10.9	9.5	9.2 ± 0.3 8.5–9.8
Depth of caudal paduncle	12.9	11.5 ± 0.7 10.5–12.9	11.7	10.8 ± 1.0 9.0–13.6	12.0	10.5 ± 0.7 9.3–12.0	11.3	11.0 ± 0.5 10.3–12.3
Length of dorsal fin base	12.2	12.1 ± 0.9 10.4–13.8	12.1	13.0 ± 0.9 10.8–14.6	12.1	11.8 ± 0.7 10.2–12.8	12.5	12.5 ± 0.5 11.5–14.2
Depth of anal fin	18.3	18.8 ± 2.4 15.3–23.3	17.4	20.3 ± 2.6 15.9–24.1	20.2	22.0 ± 1.7 19.7–24.9	15.4	15.0 ± 1.7 12.7–19.3
Length of anal fin base	6.4	6.7 ± 0.8 4.9–7.9	7.5	7.7 ± 0.5 6.9–8.8	7.2	7.6 ± 0.9 6.1–8.9	6.8	6.7 ± 0.5 5.3–8.3
Length of ventral fin	15.4	14.4 ± 0.8 13.1–15.4	14.9	13.9 ± 0.8 12.6–15.9	13.7	13.9 ± 0.8 12.0–15.5	15.5	14.8 ± 0.7 13.2–15.9
Length of pectoral fin	18.5	18.2 ± 0.8 16.3–19.3	17.6	17.3 ± 0.9 16.0–19.1	16.6	16.9 ± 1.4 13.0–19.2	21.2	20.3 ± 1.1 16.1–21.6
Length of upper caudal lobe	23.7	22.1 ± 2.0 18.4–24.7	23.2	21.0 ± 1.0 19.4–23.2		20.0 ± 1.7 16.9–23.1	22.3	21.3 ± 0.9 19.3–23.1
Length of middle caudal part	10.2	9.0 ± 0.9 7.1–10.3	10.3	8.4 ± 0.8 6.9–10.3	8.4	8.2 ± 0.9 6.4–10.2	9.3	9.0 ± 0.7 7.9–11.1
Length of lower caudal lobe	23.5	22.0 ± 2.0 17.9–24.4	23.2	19.4 ± 1.6 16.5–23.2	18.7	18.8 ± 1.5 16.8–23.1	24.0	21.1 ± 1.9 12.9–24.0
Body width	13.8	12.7 ± 1.4 10.9–15.4	14.3	12.5 ± 1.2 9.9–15.0	14.4	14.2 ± 2.1 11.1–17.5	15.8	15.0 ± 0.9 11.8–16.6
Head width	14.8	14.5 ± 1.3 12.6–17.7	14.0	13.9 ± 0.7 12.5–14.9	15.2	14.4 ± 1.7 11.9–16.9	14.3	14.2 ± 0.5 13.0–15.3
Preorbital distance	11.0	9.9 ± 0.7 8.8–11.0	11.1	11.4 ± 0.5 10.3–12.4	9.4	10.5 ± 0.9 8.5–11.8	8.6	8.8 ± 0.3 8.3–9.3
Postorbital distance	11.0	10.6 ± 1.2 9.6–11.4	9.9	10.3 ± 0.4 9.8–11.3	10.7	10.5 ± 0.6 9.4–11.7	10.2	9.9 ± 0.7 8.7–11.4
Horizontal eye diameter	4.9	4.1 ± 0.5 3.2–4.9	4.7	3.8 ± 0.4 3.1–4.7	4.3	3.9 ± 0.4 3.1–4.6	4.9	4.9 ± 0.5 3.9–5.9
Interorbital distance	6.4	6.7 ± 0.5 6.0–7.5	7.0	7.2 ± 0.5 6.4–8.3	7.2	7.2 ± 0.5 6.1–8.0	9.2	8.4 ± 0.5 7.2–9.5
Head depth	15.2	14.1 ± 0.7 13.3–15.4	14.7	14.2 ± 0.5 13.5–14.7	16.6	15.4 ± 0.7 14.2–16.8	17.4	16.4 ± 0.9 15.0–17.7

Canonical $R = 0.76$; Wilks' $\lambda = 0.23$; Chi^2 test $P < 0.0001$). The correlation analyses confirmed that the head depth ($r = -0.79$), the interorbital distance ($r = -0.78$), and the preanal distance ($r = -0.78$) played the most important roles in the morphological separation of the species. These morphological characters showed strong negative correlation with the first axis and were the highest ($P < 0.0001$) in the new *Barbus* species, with the exception of the head depth which did not differ significantly ($P = 0.2465$) between the new species and *B. balcanicus*. The preorbital distance ($r = 0.58$), and the length of upper ($r = 0.56$) and lower ($r = 0.48$) caudal lobe positively correlated with the first axis. The preorbital distance was the lowest ($P < 0.0001$) in the new species.
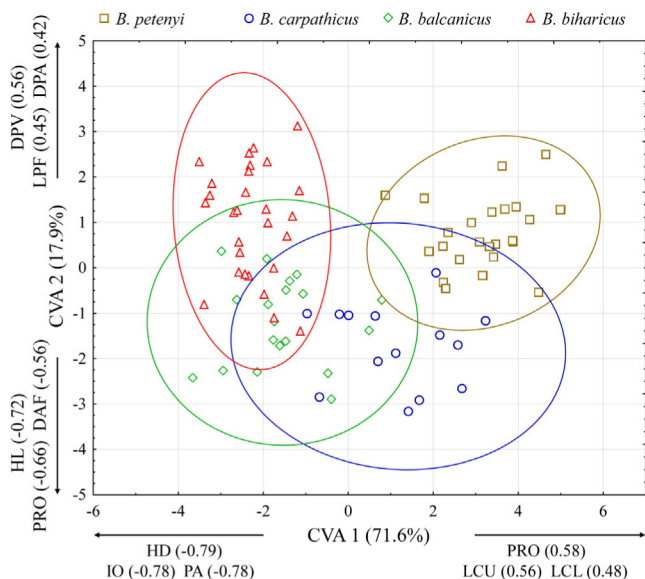
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Fig. 3. Discriminant analysis for comparison of the morphology of *Barbus* species. The isodensity circles contain the 95% of the specimens classified into a certain group. *Abbreviations:* HL: head length; PRO: preorbital distance; DAF: depth of anal fin; DPV: distance between pectoral and ventral fin bases; LPF: length of pectoral fin; DPA: distance between pectoral and anal fin bases; HD: head depth; IO: interorbital distance; PA: preanal distance; LCU: length of upper caudal lobe; LCL: length of lower caudal lobe.

In contrast to the morphometric characters, meristic characters did not show significant differences between the four species. Numbers of rays in the dorsal and anal fins as well as scale counts (numbers of pored scales along lateral lines and numbers of scale rows above and below lateral lines) were nearly the same for all species (Table 2).

3.3. Historical biogeography

The isostatic uplift of the Apuseni Mountains (Munţii Apuseni) in the Miocene (Late Badenian–Early Sarmatian) might play an important role in the isolation of the *Barbus* species (Thamó-Bozsó et al., 2002). The Apuseni Mountains form an important barrier to gene flow between the species because there is an insurmountable geographical barrier between the main streams of the area (Szamos/Someş River, Maros/Mureş River, Körös/Criş Rivers). The separation of the main streams therefore likely contributed

to the genetic and morphological differentiation of the new species from *B. carpathicus* and *B. petenyi* in that it prevented effective gene flow via hybridization, which is known to occur between other *Barbus* species (Berrebi et al., 2013; Gante et al., 2015).

The congruence between the molecular phylogenetic and the morphometric analyses provides evidence that barbels from Sebes-Körös River in eastern Hungary and western Romania are a species new to science. A formal description of the new species follows.

3.4. Barbus biharicus Antal, László, Kotlík – sp. nov.

3.4.1. Holotype

The holotype of *B. biharicus* (Fig. 4) was deposited in the Hungarian Natural History Museum, Ichthyological Collection, under lot HNHM-ICH-2015.51.1; adult male; 79.3 mm SL; Romania: Danube River basin: Sebes-Körös River (Crişul Repede), tributary of Tisza River, at Bratca, 46.9279°N 22.5934°E; collected by I. C. Telcean and L. Antal, 24 June 2015.

3.4.2. Paratype

The paratype of *B. biharicus* was deposited in the Hungarian Natural History Museum, Ichthyological Collection under lot HNHM-ICH-2015.48.1. (formerly HU026); adult male; 82.5 mm SL; Hungary: Danube River basin: Sebes-Körös River (Crişul Repede), tributary of Tisza River, at Körösszakál, 47.0146°N 21.6255°E; collected by L. Antal, A. Mozsár & I. Czeglédi, 30 September 2010.

3.4.3. Diagnosis

Barbus biharicus is distinguished from the other species by three diagnostic nucleotide substitutions in the studied 546-bp region of Cyt *b* [position 12 A (adenine) → G (guanine); position 186 C (cytosine) → T (thymine); position 435 A → G; GenBank accession number KT733657], by 8 diagnostic nucleotide substitutions in the studied 842-bp region of ATPase 6/8 [position 14 A → G; position 60 C → T; position 423 C → T; position 483 C → T; position 551 A → G; position 692 C → G; position 707 A → G; position 728 A → G; GenBank accession number KT733655] and by three diagnostic nucleotide substitution in the studied 496-bp region of Act-2 [position 157 T → A; position 300 G → C; position 373 T → C; GenBank accession number KT733656]. Although there is overlap in most meristic and morphometric characters (Table 2), *B. biharicus* has significantly larger interorbital and preanal distance than *B. balcanicus*, *B. carpathicus* and *B. petenyi*, and significantly deeper head than *B. carpathicus* and *B. petenyi*. It also has a ten-

Fig. 4. *Barbus biharicus*, HNHM-ICH-2015.51.1., holotype, male, 79.3 mm SL; Romania: Sebes-Körös River (Crişul Repede) at Bratca. (A) Living individual. (B) Individual fixed in formalin and preserved in 70% ethanol (photo: L. Antal).

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dency to have shorter, rounded snout, slightly longer pectoral fins, but slightly shorter anal fin than the other three species. *Barbus biharicus* has small dark spots on dorsal region of body and flanks and, to a lesser extent, head, and dark pigments on the fins occasionally form dark spots (the other species have heavy dark spots, which typically are arranged into rows on dorsal and caudal fins).

3.4.4. Description

General body shape and appearance are shown in Fig. 4 and the meristic and morphometric characters are given in Table 2. There are 2 (in 11 individuals) or 3 (in 16 individuals) unbranched rays and 7 (1) or 8 (26) branched rays in the dorsal fin, which has a slightly concave outer margin. There are 1 (2) or 2 (25) unbranched rays and 4 (14) or 5 (13) branched rays in the anal fin, which when depressed can reach to (male) or beyond (female) the middle of caudal peduncle. There are 51–56 (mean 53.7, SD 1.3) pored scales along the lateral line; 9 (9), 10 (16) or 11 (2) scale rows above the lateral line; 8 (3), 9 (22) or 10 (2) scale rows below the lateral line. Coloration of living specimens is dark greyish-brown on dorsal region of body and head, yellowish brown on flanks, yellowish white on ventral region, and yellowish[1] on fins (Fig. 4).

3.4.5. Sexual dimorphism

Anal fin of female is markedly longer than that of male.

3.4.6. Distribution

Barbus biharicus is presently known only from Hungarian and Romanian sections of Sebes-Körös River (Crişul Repede), a tributary of Tisza River, which is a tributary of the Danube River. The species presumably lives also in the Fekete-Körös River (Crişul Negru) and Fehér-Körös River (Crişul Alb).

3.4.7. Etymology

Named after the Bihar Counties (there are counties with this name in both Romania and Hungary), where the species is almost exclusively distributed. Suggested common name: Biharian barbel.

Acknowledgments

References

Bănărescu, P.M., 1964. Fauna Republicii Populare Romine [Fauna of the People's Republic of Romania, vol. 13]. Pisces – Osteichthyes. Acad. Rep. Pop. Romine, Bucuresti (in Romanian).

Bănărescu, P.M., Bogutskaya, N.G. (Eds.), 2003. The Freshwater Fishes of Europe. Cyprinidae 2. Pt. II: Barbus, vol. 5/II. AULA-Verlag, Wiebelsheim.

Berrebi, P., Chenuil, A., Kotlík, P., Machordom, A., Tsigenopoulos, C.S., 2013. Disentangling the evolutionary history of the genus *Barbus sensu lato*, a twenty years adventure. In: Alves, J. (Ed.), Professor Carlos Almaça (1934–2010) – Estado da Arte em Áreas Científicas que Desenvolveu. Museu Nacional de Historia Natural e da Ciencia, Lisboa, pp. 29–55.

Briolay, J., Galtier, N., Brito, R.M., Bouvet, Y., 1998. Molecular phylogeny of Cyprinidae inferred from cytochrome *b* DNA sequences. Mol. Phylogenet. Evol. 9, 100–108.

Buonerba, L., Zaccara, S., Delmastro, G.B., Lorenzoni, M., Salzburger, W., Gante, H.F., 2015. Intrinsic and extrinsic factors act at different spatial and temporal scales to shape population structure, distribution and speciation in Italian *Barbus* (Osteichthyes: Cyprinidae). Mol. Phylogenet. Evol. 89, 115–129.

Doadrio, I., 1990. Phylogenetic relationships and classification of western palaearctic species of the genus *Barbus* (Osteichthyes, Cyprinidae). Aquat. Living Resour. 3, 265–282.

Elliott, N.G., Haskard, K., Koslow, J.A., 1995. Morphometric analysis of orange roughly (*Hoplostethus atlanticus*) off the continental slope of southern Australia. J. Fish Biol. 46, 202–220.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Gante, H.F., 2011. Diversification of circum-mediterranean barbels. In: Grillo, O., Venora, G. (Eds.), Changing Diversity in Changing Environment. InTech, Rijeka, Croacia, pp. 283–298.

Gante, H.F., Alves, M.J., Dowling, T.E., 2011. Paralog-specific primers for the amplification of nuclear loci in tetraploid barbels (*Barbus*: cypriniformes). J. Hered. 102, 617–621.

Gante, H.F., Doadrio, I., Alves, M.J., Dowling, T.E., 2015. Semi-permeable species boundaries in Iberian barbels (*Barbus* and *Luciobarbus*, Cyprinidae). BMC Evol. Biol. 15, 111.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59, 307–321.

Halasi-Kovács, B., Harka, Á., 2012. How many fish species are existing in Hungary? Zoogeographic and taxonomic review and evaluation of the Hungarian fishfauna. Pisc. Hung. 6, 5–24 (in Hungarian).

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Karaman, S.M., 1971. Süsswasserfische der Türkei. 8. Teil: Revision der Barben Europas, Vorderasien und Nordafrikas. Mitt. Hamburg. Zool. Mus. Inst. 67, 175–254.

Konopiński, M.K., Amirowicz, A., Kotlík, P., Kukuła, K., Bylak, A., Pekarik, L., Šediva, A., 2013. Back from the Brink: the Holocene History of the Carpathian Barbel *Barbus carpathicus*. PLoS One 8, e82464.

Kotlík, P., Berrebi, P., 2002. Genetic subdivision and biogeography of the Danubian rheophilic barb *Barbus petenyi* inferred from phylogenetic analysis of mitochondrial DNA variation. Mol. Phylogenet. Evol. 24, 10–18.

Kotlík, P., Tsigenopoulos, C.S., Ráb, P., Berrebi, P., 2002. Two new *Barbus* species from the Danube River basin, with redescription of *B. petenyi* (Teleostei: Cyprinidae). Folia Zool. 51, 227–240.

Kottelat, M., Freyhof, J., 2007. Handbook of European Freshwater Fishes. Publications Kottelat, Cornol, Switzerland.

Levin, B.A., Freyhof, J., Lajbner, Z., Perea, S., Abdoli, A., Gaffaroglu, M., Ozulug, M., Rubenyan, H.R., Salnikov, V.B., Doadrio, I., 2012. Phylogenetic relationships of the algae scraping cyprinid genus *Capoeta* (Teleostei: Cyprinidae). Mol. Phylogenet. Evol. 62, 542–549.

Machordom, A., Doadrio, I., 2001a. Evolutionary history and speciation modes in the cyprinid genus *Barbus*. Proc. R. Soc. Lond. Biol. 268, 1297–1306.

Machordom, A., Doadrio, I., 2001b. Evidence of a cenozoic betic-kabilian connection based on freshwater fish phylogeography (*Luciobarbus*, Cyprinidae). Mol. Phylogenet. Evol. 18, 252–263.

Marková, S., Šanda, R., Crivelli, A., Shumka, S., Wilson, I.F., Vukić, J., Berrebi, P., Kotlik, P., 2010. Nuclear and mitochondrial DNA sequence data reveal the evolutionary history of *Barbus* (Cyprinidae) in the ancient lake systems of the Balkans. Mol. Phylogenet. Evol. 55, 488–500.

Nicholas, K.B., Nicholas Jr., H.B., Deerfield, D.W., 1997. GeneDoc: analysis and visualization of genetic variation. EMBnet News 4, 14.

Posada, D., Buckley, T.R., 2004. Model selection and model averaging in phylogenetics: advantages of akaike information criterion and bayesian approaches over likelihood ratio tests. Syst. Biol. 53, 793–808.

StatSoft, Inc., 2013. STATISTICA (Data Analysis Software System), Version 12.0. <www.statsoft.com>.

Tamura, K., 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. Mol. Biol. Evol. 9, 678–687.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Thamó-Bozsó, E., Kercsmár, Zs., Nádor, A., 2002. Tectonic control on changes in sediment supply: quaternary alluvial systems, Körös sub-basin, SE

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[1] For interpretation of color in Fig. 4, the reader is referred to the web version of this article.

Hungary. In: Jones, S.J., Frostick, L.E. (Eds.), Sediment Flux to Basins: Causes, Controls and Consequences, vol. 191. Geol. Soc. London Spec. Publ., pp. 37–53.

Tsigenopoulos, C.S., Berrebi, P., 2000. Molecular phylogeny of North Mediterranean freshwater barbs (genus *Barbus*: Cyprinidae) inferred from cytochrome *b* sequences: biogeographic and systematic implications. Mol. Phylogenet. Evol. 14, 165–179.

Tsigenopoulos, C.S., Kotlik, P., Berrebi, P., 2002. Biogeography and pattern of gene flow among *Barbus* species (Teleostei: Cyprinidae) inhabiting the Italian Peninsula and neighbouring Adriatic drainages as revealed by allozyme and mitochondrial sequence data. Biol. J. Linn. Soc. Lond. 75, 83–99.

Yang, L., Sado, T., Hirt, M.V., Pasco-Viel, E., Arunachalam, M., Li, J., Wang, X., Freyhof, J., Saitoh, K., Simons, A.M., Miya, M., He, S., Mayden, R.L., 2015. Phylogeny and polyploidy: resolving the classification of cyprinine fishes (Teleostei: Cypriniformes). Mol. Phylogenet. Evol. 85, 97–116.

Zardoya, R., Doadrio, I., 1999. Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. J. Mol. Evol. 49, 227–237.

Žutinić, P., Jelić, D., Jelić, M., Buj, I., 2014. A contribution to understanding the ecology of the large spot barbel – sexual dimorphism, growth and population structure of *Barbus balcanicus* (Actinopterygii; Cyprinidae) in Central Croatia. North-West. J. Zool. 10, 158–166.