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Integrative study of population structure of *Telestes dabar*, the strictly endemic cyprinid species from the Dinaric karst on the Balkan Peninsula

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Abstract

Diversification of the genus *Telestes* (Cyprinidae), endemic to the Euro-Mediterranean biogeographic subregion, mirrored an evolutionary footprint of complex biogeographical history. Hence, hidden diversity and noticeably high endemism of freshwater ichthyofauna were discovered in the Dinaric karst on the Balkan Peninsula. One such example includes two steno-endemic Dinaric karst (Bosnia and Herzegovina) cyprinid species, *Telestes dabar* and *T. metohiensis*. Although the conservation status of *T. metohiensis* has been evaluated, *T. dabar* from Dabarsko Polje has not yet been assessed for the IUCN Red List due to its taxonomically ambiguous position and data deficiencies. Our first aim was to evaluate the resolution of taxonomic ambiguities of the two putative species, *T. dabar* and *T. metohiensis*, by the complementary use of two mitochondrial DNA markers (*cytb* and *COI* mtDNA). The results clearly demonstrated the evolutionary independence of *T. dabar* (Dabarsko Polje) and *T. metohiensis* (Nevesinjsko Polje) and confirmed the usefulness of both markers for assessing the species borders. In addition, population structure of putative *T. dabar* was analyzed using *cytb* and *COI* mtDNA and morphological parameters (linear measurements and landmark-based geometric morphometric data). A high genetic admixture and homogeneity in *T. dabar* from Dabarsko Polje were found and confirmed using both morphometric approaches as no statistically significant differences were consistently registered between different samples.

Keywords *cytb* mtDNA · *COI* mtDNA · Geometric and linear morphometrics · Population structure · Cyprinidae

Introduction

The freshwater biodiversity of the Mediterranean Basin Hotspot is globally recognized for its species richness and extraordinary high level of endemism (Myers et al. 2000; Smith

and Darwall 2006). Among the seven main riverine fish fauna biogeographical regions in Europe, the central Peri-Mediterranean (the Balkans and the Italian Peninsula) is particularly highlighted, with over 60% of its fish species being endemic (Reyjol et al. 2007). Belonging to south-west Balkan corridor, the Dinaric karst also represents a home of a noticeably high endemism of freshwater ichthyofauna (Jelić et al. 2016) and a region of distinct hydrological and hydrogeological phenomena with complex biogeographical history (Bianco 1990). Namely, many karstic streams, springs, and the associated fields (=polje) that contain marshes or small lakes characterize the heterogeneous karst landscape of complex interconnectedness of groundwater and surface water (Bonacci 1987).

The geographical distribution of many Dinaric karst fish (especially in the underground hydrological network) is still unknown and, coupled with the complex species ecology and the limited source of data used for their description and delimitation, contributes to long debates in fish systematics, as evidenced by the case of cyprinids (see Bianco 1990). One such example includes the cyprinid genus *Telestes*, which

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comprises 14 species (Buj et al. 2017) that are distributed in eight ichthyological districts of the Mediterranean and Black Sea freshwater drainage systems (Bianco 1990). Its evolution started in the Early/Middle Miocen (16.3–14.3 million years ago) (Buj et al. 2017), but the main diversification is considered to have occurred during the freshwater Messinian “Lago Mare” phase of the Mediterranean Sea (7.9–3.25 MYA, Ketmaier et al. 2004). Diversification of this genus, mainly distributed in the Euro-Mediterranean biogeographic subregion, mirrored an evolutionary footprint of intense tectonic activity, followed by multiple vicariant events, dispersion, colonization, and secondary contact of freshwater fishes (Ketmaier et al. 2004; Gilles et al. 2010; Perea et al. 2010; Dubut et al. 2012; Buj et al. 2017). Consequently, the biogeographical pattern of species composition of the genus *Telestes* is characterized by hidden diversity, leading to the controversial taxonomic position of certain taxa.

A recent taxonomic revision of the samples originating from the karst fields in Eastern Herzegovina (Bosnia and Herzegovina) discovered three cryptic species within morphologically nominated striped pijaon, *Telestes metohiensis* (Bogutskaya et al. 2012). *Telestes miloradi* Bogutskaya, Zupančič, Bogut and Naseka, 2012 was described based on the differences in the number of branched anal fin rays of museum samples from Ljuta River in Konavle Polje (Croatia) collected in 1901 by J. Kolombatovic (Bogutskaya et al. 2012). However, this species was considered extinct until it was recorded in 2013 (Jelić and Jelić 2015). Meanwhile, a severe destruction of Dragić Stream in Krbavsko Polje (Croatia), the locality of the rediscovered population of *T. miloradi* (Jelić and Jelić 2015), as well as the introduction of non-indigenous fish species in Konavosko (=Konavle) Polje, Croatia (Mrakovčić et al. 2006), resulted in the main population and habitat of the species being lost (Jelić and Jelić 2015). In addition, another taxonomic dilemma arose around the status of *T. dabar* Bogutskaya, Zupančič, Bogut and Naseka, 2012 and *T. metohiensis* (Steinachner, 1901) (Bogutskaya et al. 2012). Namely, according to Bogutskaya et al. (2012), morphological characteristics which differ in *T. dabar* and *T. metohiensis* are, respectively, the count of rays in the dorsal fin ($8\frac{1}{2}$ compared to $7\frac{1}{2}$), the number of gill rakers (9 or 10 gills in comparison to 7–10, usually 8), and the dark line on the ventral side of the body (which does not reach the caudal peduncle versus reaching caudal peduncle). Although the populations from Dabarsko Polje have just tentatively been recognized as *T. metohiensis* (Zupančič and Bogutskaya 2002; Bogutskaya and Zupančič 2003), Bogutskaya et al. (2012) subsequently confirmed the presence of the cryptic species, *T. dabar*, within this locality. Furthermore, it was suggested that *T. dabar* is restricted to Dabarsko Polje, while the allopatric species *T. metohiensis* is distributed in Gatačko Polje (Mušnica River), Cerničko Polje (Ključka River in Vilina Pečina), and Nevesinjsko Polje

(Zalomka River) in Bosnia and Herzegovina (Bogutskaya et al. 2012). Until now, *T. dabar* was considered as abundant species in rivers Vrijeka and Opačica (Dabarsko Polje), being an exclusive native fish in Opačica River, while occurring in sympatry with *Delminichthys ghetaldii* in Vrijeka River (Bogutskaya et al. 2012).

Morphological traits were used for taxonomic revisions and generic species assignments (Zupančič and Bogutskaya 2002; Bogutskaya and Zupančič 2003), species description of *T. miloradi* and *T. dabar* and their distinction from *T. metohiensis* (Bogutskaya et al. 2012), as well as for the morphometric characterization of the red blood cells of *T. metohiensis* in Dabarsko Polje (Dekić et al. 2012). However, Geiger et al. (2014) reported *T. dabar* and *T. metohiensis* as species which shared a common *COI* mtDNA haplotype. Hence, an integrative approach based on the complementary use of multilocus genetic data and morphological traits was suggested for the recognition of the closely related species, cryptic species, and recently diverged species such as *T. dabar* and *T. metohiensis* (Gilles et al. 2010; Palandačić et al. 2017).

Conservation implications of cryptic diversity and high level of endemism have been recognized by the International Union for Conservation of Nature (IUCN; <http://www.iucnredlist.org>). Indeed, among 12 (Vuković and Sofradžija 1986) or 35 (taking into account endemism in a broader sense; Glamuzina et al. 2011) endemic fish species in the Adriatic basin of Bosnia and Herzegovina, several have been listed as endangered/vulnerable. For example, *T. metohiensis* is categorized as vulnerable B1ab(ii,iii) + 2ab(ii,iii) ver3.1. It is also noted in Red List of Republic of Srpska, Bosnia and Herzegovina (<http://www.nasljedje.org>), and as regionally extinct (RE) in Croatia (Jelić et al. 2008). However, strictly restricted endemic species *T. dabar* from Dabarsko Polje has not yet been assessed for the IUCN Red List due to its taxonomically ambiguous position and data deficiencies regarding its molecular and phenotypic diversity, ecology, and biology.

Therefore, our first aim was to test genetic borders between putative species *T. dabar* and *T. metohiensis*. We studied four populations from Dabarsko Polje (a locality where *T. dabar* and *T. metohiensis* were both suggested to be present according to Bogutskaya and Zupančič 2003) and one population from Nevesinjsko Polje (Zalomka River, a typical *T. metohiensis* locality). Given the fact that accurate species identification is fundamental for the biodiversity assessment of vulnerable ichthyofauna from unique and species-rich Dinaric karst ecosystem, the complementary approach of two mitochondrial DNA markers, cytochrome *b* (*cytb*) and cytochrome *c* oxidase subunit I (*COI*), was implemented in this study. By developing informative DNA barcode database, we aimed to provide a firm basis for designing a long-term sustainable conservation strategy of these intriguing cyprinoids on the Balkans.

Our second aim was to perform an integrative study of population structure of steno-endemic cyprinid *T. dabar* from Dabarsko Polje (Opačica, Vrijeka, Pribitul, and Suški Potok) by using *cytb* mtDNA and *COI* mtDNA, and traditional (linear measurements) and geometric morphometrics (size and shape). In addition, an adaptive potential based on molecular and phenotypic diversity of *T. dabar* was evaluated, closing the crucial gap in the assessment of the species' status.

Material and methods

Sample collection

Electrofishing was conducted with transportable electroshockers (IG 600, 1.2 kW, and ELT 62 II GI, 3 kW) and collecting nets with different diameters. Electrofishing based on the induction of short-term stress caused galvanotaxis and galvanonarcosis, followed by a rapid recovery of fish. A short time of shock was used for taking samples for DNA analysis and photographing. After taking the data and tissue samples (part of dorsal fin), individuals were returned to their original habitat. This method did not interfere neither with the population count nor with the health status of fish. The permission for sampling was issued by the Ministry of Agriculture, Forestry and Water Management of the Republic of Srpska. Samples were collected in 2015 (18.-19.03.2015.), 2016 (21.04.2016., 22.12.2016.), and 2017 (18.08.2017.) by R. Dekić and M. Lukač. The specimens were determined to the species/genus level using morphological keys (Vuković and Ivanović 1971; Kottelat and Freyhof 2007), a morphological study by Bogutskaya et al. (2012), and the results of molecular analysis of this study.

A total of 70 adults of putative *T. dabar* collected from four localities of Dabarsko Polje (Opačica-16 individuals, Pribitul-14, Suški Potok-21, Vrijeka-19) were used in morphometric analyses, while 47 and 40 specimens were used for the analyses of *cytb* and *COI* mtDNA variability, respectively. Putative *T. metohiensis* sample collected from Zalomka river (Nevesinjsko Polje) was used for molecular analyses (*cytb* mtDNA-15 specimens, *COI* mtDNA-9) (Fig. 1; Table 1, Supp. Table S1). Since preliminary analysis did not reveal the presence of sexual dimorphism, specimens of both sexes of putative *T. dabar* were pooled together for molecular and morphometric analyses.

DNA analyses

Total genomic DNA was extracted from 64 fish tissue samples using NucleoSpin® Tissue DNA extraction kit (MACHEREY-NAGEL, Düren, Germany) and following the manufacturer's protocol. Fish tissue and extracted DNA were stored at -20°C . The *cytb* mtDNA was amplified using Glu-F (5'-GAAG

AACCACCGTTGTTATTCAA-3')/Thr-R (5'-ACCT CCRATCTYCGGATTACA-3') primer pair (Zardoya and Doadrio 1998), while the amplification of the 5' end of *COI* mtDNA was done by LCO-1490 (5'-G GTCAACAAATCATA AAGATATTGG-3')/HCO-2198 (5'-TTAAACTTCAGGGT GACCAAAAATCA-3') primer pair (Folmer et al. 1994). PCR reactions were performed using an Illustra PuReTaq Ready-To-Go PCR Beads kit (GE Healthcare Life Sciences, Buckinghamshire, UK). PCR conditions for *cytb* and *COI* mtDNA amplification were as described in Palandačić et al. (2012) and Milankov et al. (2009), respectively. To check the success of reactions, amplification products were separated on a 2% agarose gel. PCR products were then purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Vilnius, Lithuania) and bidirectionally sequenced on ABI3730XL by Macrogen (Netherlands).

Chromatograms obtained by mtDNA sequencing were edited in Chromas 2.6 (Tehnelysium Pty Ltd) for erroneously called bases, while sequence alignment was performed in BioEdit 7.2.5 (Hall 1999). Identification of variable positions and number of haplotypes and calculation of uncorrected *p* distance among within- and between-species haplotypes was done using MEGA7 (Kumar et al. 2016). Haplotype networks were constructed in Network 5.0.0.0. (Fluxus Technology Ltd.) using the median joining approach.

To assess the species boundary between putative *T. dabar* and *T. metohiensis* and to study the degree of genetic differentiation among the *T. dabar* samples, we implemented individual- and population-based approaches. For both molecular markers, analysis of molecular variance (AMOVA; Excoffier et al. 1992) was estimated using ARLEQUIN, version 3.5 (Excoffier and Lischer 2010). Total genetic variance was partitioned into covariance components at different hierarchical levels: (1) we computed the proportion of genetic variation between and within the species defined according to Bogutskaya et al. (2012) species assessment criteria, and (2) we quantified the proportion of genetic variation among and within conspecific populations of *T. dabar* to investigate its geographical structure. Ten thousand permutations were used to determine the significance of variance components.

In addition, we applied the Bayesian model-based clustering algorithm implemented in BAPS 6.0 (Corander and Tang 2007) to infer interspecific differences. Clustering of individuals was performed using clustering with linked loci and codon linkage model. We ran BAPS for values of *K* ranging from 1 to 10, performing five replicates for each *K* (*K*). The number of iterations was set to 100, the number of reference individuals per population to 200, and the number of iterations per reference individual to 20. The degree of genetic differentiation between species was determined separately on *cytb* and *COI* mtDNA dataset using the identical settings.

To infer the *T. dabar* intraspecific differentiation, the Bayesian clustering method employed in GENELAND 4.0.3

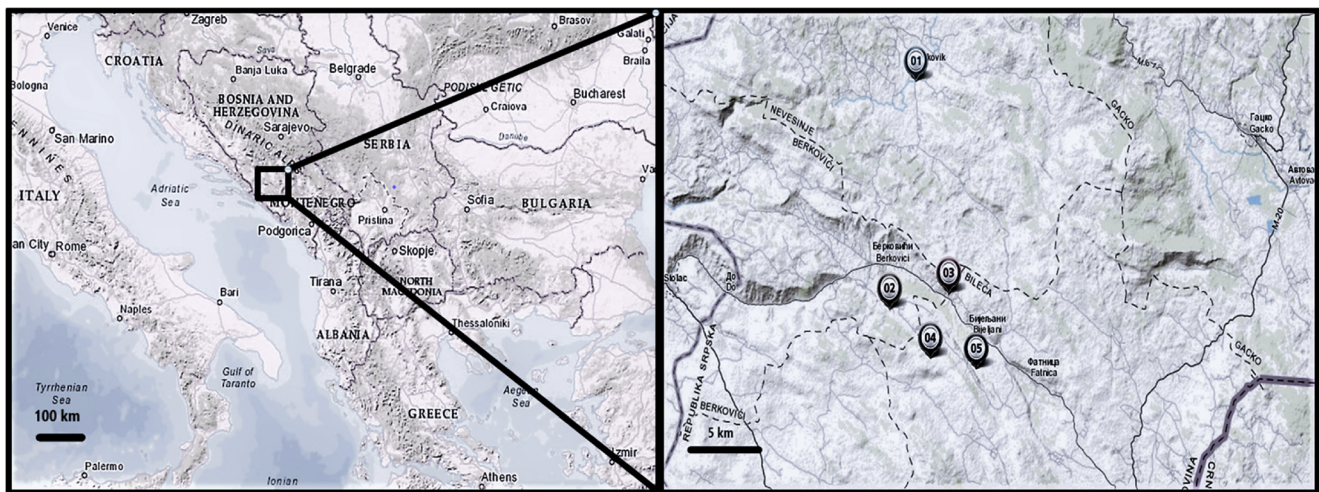


Fig. 1 Map of the Balkan Peninsula (left) and Bosnia and Herzegovina (right) with the studied localities: Nevesinjsko Polje (1. Zalomka River); Dabarsko Polje (2. Opačica River; 3. Pribitul River; 4. Suški Potok; 5. Vrijeka River)

(Guillot et al. 2005), implemented in R (v3.4.3; R Core Team 2017), was used. We ran the algorithm to infer the number of genetic clusters within the samples and the assignment of individuals to the clusters with 10 independent runs with 1,000,000 MCMC iterations, a thinning of 100, a maximum rate of Poisson process fixed to 100, K allowed to vary between 1 and 5, and the maximum number of nuclei in the Poisson-Voronoi tessellation fixed to 300. This was done using the Dirichlet model where allele frequencies are assumed to be independent (Guillot 2008).

Morphometric analyses

Two morphometric approaches were chosen for several reasons. Firstly, although traditional morphometrics using linear distances and ratios of distances has some disadvantages compared to geometric morphometrics (e.g., distances, ratios, and angles capture relatively little information about shape; Maderbacher et al. 2008; Zelditch et al. 2012), previously published morphological characterization of *T. dabar* was based on linear measurements and meristic counts (Bogutskaya et al. 2012). Thus, to be comparable with

T. dabar type material, herein we applied the traditional morphometric approach. Secondly, it has already been proven that geometric morphometrics is a statistically powerful method for analyzing morphological variability, allowing the visualization of shape changes (Zelditch et al. 2012). Finally, the simultaneous utilization of both linear and geometric morphometrics gave us an opportunity to compare their potential and usefulness in quantifying phenotypic variability of focal populations.

For all individuals, the lateral image of the right side was taken with a Nikon D7100 digital camera with 50-mm f/1.4D objective mounted on a tripod stand and positioned perpendicular to the surface. The fish were photographed with a ruler graduated in millimeters and, prior to the digitization procedure, the scale factor was set for each individual.

Linear measurements

Nine linear phenotypic characteristics were measured (in mm) on digital images using the mode for taking linear measurements in tpsDig 2.26 (Rohlf 2016). The measures used are standard for describing cypriniform fish (Armbruster 2012):

Table 1 Sampling localities and sample size of *Telestes dabar* and *T. metohiensis* used for mtDNA and morphometric analyses

Species	Locality	MtDNA analyses		Morphometric analyses	
		<i>COI</i> mtDNA (N_t/N_p)	<i>cytb</i> mtDNA (N_t/N_p)		
<i>Telestes dabar</i>	Dabarsko Polje	Opačica River	10 (3/0)	14 (3/0)	16
		Pribitul River	10 (3/0)	15 (4/1)	14
		Suški Potok	10 (3/0)	9 (4/1)	21
		Vrijeka River	10 (3/0)	9 (3/0)	19
<i>Telestes metohiensis</i>	Nevesinjsko Polje	Zalomka River	9 (2/2)	15 (3/3)	–
Total			49	62	70

N_t , total number of haplotypes, N_p , number of private haplotypes

total length (TL), standard length (SL), orbit length (OL), snout length (SNL), postorbital head length (PHL), head length (HL), body depth (BD), caudal-peduncle depth (CPD), and caudal-peduncle length (CPL) (Fig. 2). In order to remove any length-related influence, linear measurements were transformed in two ways. Firstly, to be comparable with the published data for type material (Bogutskaya et al. 2012), the percentage ratio of measures and SL (for OL, SNL, HL, BD, CPD, and CPL), HL (for OL, SNL and PHL), and CPL (for CPD) was calculated. Secondly, the size component was removed from a multivariate data set of measured distances following Elliott et al. (1995) procedure that transformed absolute measurements to size-independent (adjusted) using formula:

$$M_{adj} = M (L_s/L_o)^b$$

where M is the original morphometric measurement, M_{adj} the size-independent measurement, L_s the overall mean of SL for all the fish in each population, L_o the SL of the fish, b the slope (allometric coefficient) of regression of $\log M$ on $\log L_o$ calculated for each population. To confirm that the effect of size was removed, correlation coefficients between adjusted size-independent variables and SL were calculated. Non-significant correlation was found for each measure.

Prior to statistical analyses, deviation from normality of percentage ratios and size-adjusted measurements were performed using both Shapiro-Wilk test for univariate normality (performed for each measure separately) and Doornik and Hansen omnibus test for multivariate normality (performed on both matrices of size-independent measurements and percentage ratios of measurements).

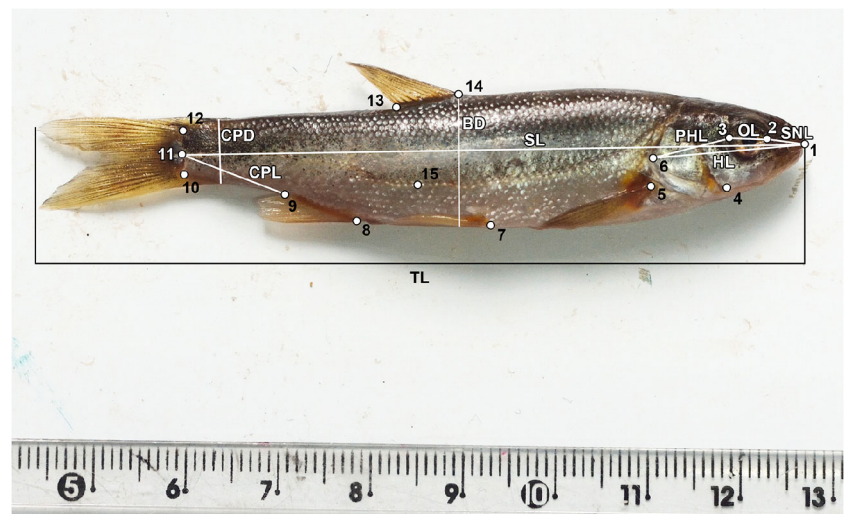
Differences among populations were tested using analyses of variance (ANOVA), followed by the post hoc Tukey honest significant difference test. Alternatively, for measurements that deviate from normal distribution, the non-parametric

ANOVA (Kruskal-Wallis test followed by Mann-Whitney pairwise post hoc test) was used. Also, matrix of seven size-adjusted measurements was subjected to principal component analysis (PCA). Statistical data analysis was performed in PAST (Paleontological Statistics) software, version 3.20 (Hammer et al. 2001).

Geometric morphometrics

A total of 14 homologous landmarks (Fig. 2) were digitized on the images using tpsDig 2.26 (Rohlf 2016): (1) snout tip, (2) most anterior point of the eye outline, (3) most posterior point of the eye outline, (4) lower position on preopercular, (5) superior insertion of pectoral fin, (6) most posterior edge of operculum, (7) anterior insertion of pelvic fin, (8) anterior insertion of anal fin, (9) posterior insertion of anal fin, (10) ventral insertion of caudal fin, (11) posterior extent of lateral line, (12) dorsal insertion of caudal fin, (13) posterior insertion of dorsal fin, and (14) anterior insertion of dorsal fin. In order to remove the effect of unnatural bending of specimens, an additional landmark (15) was positioned on the lateral line half-way between landmarks 6 and 11 (Fig. 2). Using option ‘unbend specimens’ implemented in tpsUtil 1.74 (Rohlf 2017), four landmarks were aligned (1, 6, 11, 15). Landmark 15 was subsequently removed from further analyses. Landmarks’ coordinates were then superimposed using a full Procrustes fit procedure (Dryden and Mardia 1998; Klingenberg and McIntyre 1998) and shape information (Procrustes coordinates) was extracted. Prior to the unbending procedure, centroid size (CS; the square root of the sum of squared distances between each landmark and centroid, Zelditch et al. 2012) as size variable was also calculated. Also, to test the presence of allometry, the relationship between size and shape, a multivariate regression of Procrustes coordinates against centroid size on pooled within-group

Fig. 2 Nine linear measurements taken for linear morphometric analysis: TL-total length, SL-standard length, OL-orbit length, SNL-snout length, PHL-postorbital head length, HL-head length, BD-body depth, CPD-caudal-peduncle depth, CPL-caudal-peduncle length. White dots indicated position of 14 homologous landmarks (landmarks 1–14) and one semilandmark (landmark 15) digitized for geometric morphometric analysis. Landmark 15 was used only for ‘unbend specimens’ procedure



(pooled by population) variation was conducted. The significance of the allometry was calculated with a permutation test with 10,000 iterations.

To examine interpopulation body size differences, analysis of variance (ANOVA) on centroid size was performed, followed by the post hoc Tukey honest significant difference test. Pairwise significance test was adjusted by a sequential Bonferroni correction. To determine differences in body shape, the set of shape variables (a matrix of Procrustes coordinates) was subjected to multivariate analysis of variance (MANOVA)/canonical variates analysis (CVA). Pairwise differences were quantified using Procrustes distances and compared with a permutation test with 10,000 iterations. The reliability of the discrimination was assessed using leave-one-out cross-validation implemented in discriminant function analysis (DFA). Statistical analysis was performed in MorphoJ 1.06d (Klingenberg 2011) and PAST (Paleontological Statistics) version 3.20 (Hammer et al. 2001) software.

Results

Molecular identification and interspecific variation of *Telestes dabar* and *Telestes metohiensis*

A total of eight *cytb* mtDNA haplotypes (1094 bp long) were found among the 62 analyzed individuals (Supp. Table S1). There were 12 variable positions defining *cytb* mtDNA haplotypes, which diverged from each other up to eight bases. Five haplotypes (HI–HV) were found only in *T. dabar* populations, whereas the remaining three haplotypes (HVI–HVIII) were unique to *T. metohiensis* (Fig. 3; Table 1). Interspecific

sequence divergences (uncorrected p divergence as a percentage) ranged from 0.38 to 0.73%, while intraspecific p distance ranged from 0.09 to 0.46% and 0.09 to 0.18% in *T. dabar* and *T. metohiensis*, respectively (Supp. Table S2).

Moreover, the molecular identity of the analyzed taxa was determined using *COI* mtDNA 670 bp-long 5' end fragment of 49 individuals (Supp. Table S1). The *COI* mtDNA sequences yielded five haplotypes (A–E) with six variable positions in total. As was the case with *cytb* mtDNA, the *COI* mtDNA haplotypes were also species-specific: haplotypes A, B, and C were retrieved from *T. dabar* samples, while D and E were unique to *T. metohiensis* (Fig. 3; Table 1). Interspecific sequences differed on average by 0.60% (range 0.30–0.90%) (Supp. Table S3). *COI* mtDNA sequence differences of the *T. dabar* haplotypes ranged from 0.15 to 0.45%, while two haplotypes unique to *T. metohiensis* differed in a single substitution (p distance = 0.15%) (Supp. Table S3).

In both *cytb* mtDNA and *COI* mtDNA, an overlapping of inter- and intraspecific p distance was observed which implied a lack of a barcode gap for *T. dabar* and *T. metohiensis*. For instance, intraspecific p distances among *T. dabar* haplotypes at *cytb* mtDNA (HII vs HIV = 0.46%) and *COI* mtDNA (A vs B = 0.45%) were equal or even higher than some interspecific p values (Supp. Tables S2, S3). Contrary to the distance-based approach, distinguishing between the two closely related species was possible by using the character-based method. Indeed, four and two diagnostic substitutions at *cytb* mtDNA (T → C; G → A; A → G; G → A) (Supp. Table S4) and *COI* mtDNA (C → G; T → C) (Supp. Table S5) confirmed the species borders between *T. dabar* and *T. metohiensis*.

Population-based approach was used to gain insight into the patterns of *T. dabar*/*T. metohiensis* genetic differentiation.

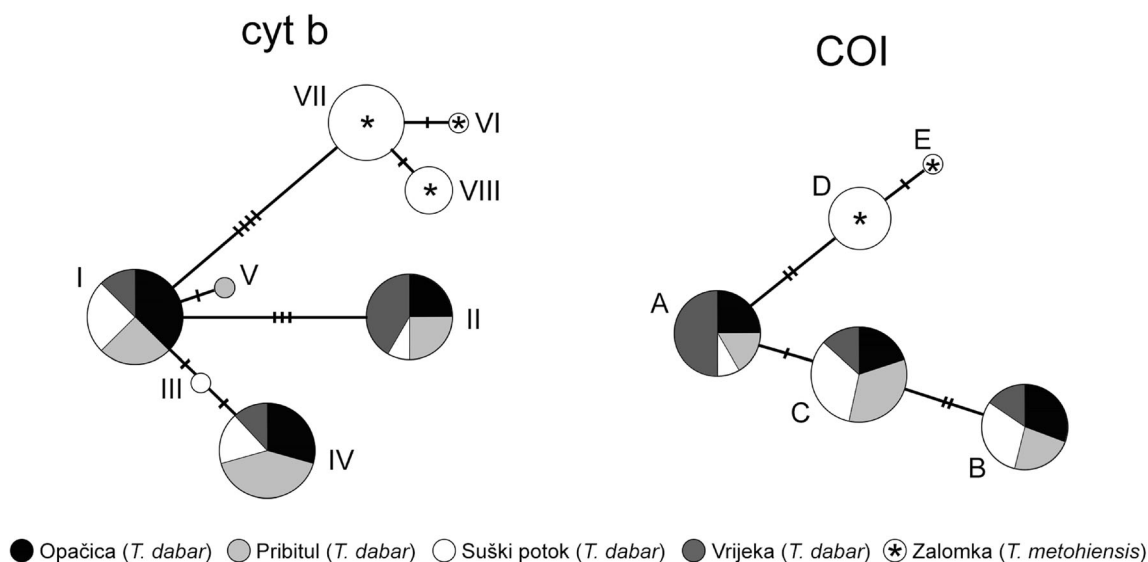


Fig. 3 Spanning network of mitochondrial DNA sequence haplotypes of *Telestes dabar* and *T. metohiensis*, constructed using the median-joining method. Each circle represents one haplotype, the size of the circle is

proportional to the overall number of individuals with that haplotype. Thick marks on lines connecting haplotypes represent single-nucleotide substitutions

AMOVA tests were statistically significant between species defined according to Bogutskaya et al. (2012) species assessment criteria. Approximately 75% of variation was found between the taxa based on *cytb* mtDNA marker. Similarly, AMOVA tests of *COI* mtDNA variation showed that 70% of total genetic variation was allocated between the species' populations (Table 2).

Finally, individual-based analyses performed in BAPS retrieved two genetic clusters ($K = 2$) using both *cytb* and *COI* mtDNA sequence datasets (Supp. Fig. S1). All *T. dabar* specimens were organized in a single cluster, while *T. metohiensis* individuals were grouped into the other cluster, confirming the existence of interspecific differences based on both molecular markers.

Intraspecific molecular and phenotypic variation of *Telestes dabar*

Testing the number of genetic clusters within the *T. dabar* data set in GENELAND analyses conducted separately on *cytb* mtDNA and *COI* mtDNA haplotypes resulted in an inferred $K = 1$ in the *T. dabar* data set (data not shown). Ten independent MCMC runs resulted in the consistent assignment of all individuals to a single population.

AMOVA analyses showed no significant differences among *T. dabar* populations. AMOVA analysis of *cytb* mtDNA failed to uncover any significant intraspecific differentiation in *T. dabar* (Table 2). Likewise, for *COI* mtDNA, only 0.86% of the variation was attributed to differences among populations, while a large proportion of the variation (99.14%) was partitioned within populations (Table 2).

Hence, variation at both markers indicated that populations represent a largely homogeneous group.

Morphometric analysis based on linear measurements The standard length of *T. dabar* specimens collected from the four locations ranged from 50.55 to 105.89 mm. Ratios of linear morphometric measurements are summarized in Table 3, concurrently with the previously published morphometric data for type material (Bogutskaya et al. 2012). Between the four populations we analyzed, the ranges (min-max values) of all the measurements overlapped, while mean values slightly varied. However, data comparison showed that the ranges of snout and head length measures [SNL(%SL), SNL(%HL), HL(%SL)] for type material were higher than values recorded in populations we studied. Moreover, although mean values for standard length of type specimens were lower than for samples we studied, mean values of all measures except OL(%HL) and PHL(%HL) were higher (Table 3).

Likewise, the ranges of the adjusted, size-independent measurements highly overlapped among samples, while mean values slightly varied (Supp. Table S6). Among the analyzed samples, Pribitul was distinguished by the smallest values for all measures except SNL_{adj}. On the other hand, none of the populations was separated from the others based on consistently higher values. For instance, Opačica was characterized with the highest mean values of adjusted measures related to the head region (OL_{adj}, SNL_{adj}, HL_{adj}), while Vrijeka had the highest mean values for body depth (BD_{adj}) and caudal-peduncle depth (CPD_{adj}) (Supp. Table S6).

Univariate analysis of normality suggested that all seven size-adjusted measures were normally distributed (Shapiro-Wilk test, $p > 0.05$ for all measurements). The multivariate

Table 2 AMOVA results based on mtDNA markers for *Telestes dabar* and *T. metohiensis* species

Sample groups	Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	Fixation index F_{ST}
<i>Cytb</i>						
<i>T. dabar</i> (Dabarsko Polje)/ <i>T. metohiensis</i> (Nevesinjsko Polje)	Between species	1	50.98	2.21	74.79	0.747*
	Within species	60	14.67	0.74	25.21	
	Total	61	95.65	2.95		
Intraspecific <i>T. dabar</i>	Among populations	3	2.91	0.007	0.86	0.008
	Within populations	43	37.89	0.881	99.14	
	Total	46	40.80	0.888		
<i>COI</i>						
<i>T. dabar</i> (Dabarsko Polje)/ <i>T. metohiensis</i> (Nevesinjsko Polje)	Between species	1	19.93	1.32	69.77	0.698*
	Within species	47	26.84	0.57	30.23	
	Total	48	46.77	1.89		
Intraspecific <i>T. dabar</i>	Among populations	3	1.95	-0.002	-0.25	-0.002
	Within populations	36	24.00	0.667	100.25	
	Total	39	25.95	0.665		

* $p < 0.001$

Table 3 Population mean, standard deviation (SD), and minimal and maximal values of morphometric traits of *Telestes dabar*

Linear measures	Data obtained in this study						Data published in Bogutskaya et al. (2012)					
	Opačica River		Pribitil River		Suški Potok		Vrijeka River		Females		Males	
	Ranges	Mean ± SD	Ranges	Mean ± SD	Ranges	Mean ± SD	Ranges	Mean ± SD	Ranges	Mean ± SD	Ranges	Mean ± SD
TL (mm)	73.09–125.22	98.30 ± 13.99	60.24–122.30	88.50 ± 14.07	85.04–127.38	97.66 ± 11.29	80.08–121.74	92.93 ± 11.67	–	–	–	–
SL (mm)	61.73–105.89	82.37 ± 11.87	50.55–104.33	74.36 ± 12.26	70.34–103.88	80.99 ± 9.02	66.94–102.08	77.69 ± 9.85	60.3–81.8	67.8	55.5–69.0	61.1
OL(%SL)	5.26–6.55	6.01 ± 0.45	5.37–7.00	6.17 ± 0.46	5.25–6.51	5.86 ± 0.35	5.46–6.70	5.97 ± 0.35	5.9–7.2	6.6 ± 0.4	6.3–7.8	6.8 ± 0.4
OL(%HL)	22.88–27.38	24.85 ± 1.52	22.84–29.06	25.47 ± 1.79	21.57–27.75	24.50 ± 1.77	22.16–27.41	25.69 ± 1.37	22.1–26.8	24.5 ± 1.5	22.1–26.8	24.3 ± 1.4
SNL(%SL)	5.49–6.88	6.12 ± 0.36	5.36–6.98	6.09 ± 0.51	4.51–6.57	5.74 ± 0.64	4.63–6.52	5.47 ± 0.48	7.4–9.2	8.1 ± 0.5	7.8–9.0	8.5 ± 0.4
SNL(%HL)	22.64–27.72	25.31 ± 1.29	23.15–27.73	25.11 ± 1.44	20.69–26.32	23.91 ± 1.66	21.04–26.33	23.49 ± 1.34	27.4–32.8	29.7 ± 1.3	28.5–31.7	30.3 ± 1.1
PHL(%HL)	50.30–56.59	52.20 ± 1.69	49.88–54.27	52.14 ± 1.44	51.12–57.08	53.91 ± 1.74	50.28–54.68	52.69 ± 1.44	48.8–54.0	52.2 ± 1.4	47.1–53.0	50.2 ± 1.9
HL(%SL)	23.01–25.10	24.16 ± 0.63	22.76–26.06	24.23 ± 1.11	21.46–26.05	23.98 ± 1.24	21.87–25.02	23.27 ± 1.07	25.6–28.7	27.1 ± 0.9	26.6–29.6	28.1 ± 1.0
BD(%SL)	18.64–22.06	20.37 ± 0.90	18.80–26.76	21.06 ± 2.12	18.61–22.43	20.46 ± 1.02	19.61–23.33	21.14 ± 1.00	21.6–26.0	23.6 ± 1.3	21.7–25.0	22.6 ± 0.9
CPLD(%SL)	8.61–9.96	9.17 ± 0.41	8.88–10.27	9.54 ± 0.49	8.08–10.19	9.36 ± 0.50	9.06–10.73	9.81 ± 0.41	9.8–12.4	11.0 ± 0.7	10.0–12.4	11.2 ± 0.7
CPLD(%CPL)	40.56–52.23	44.77 ± 2.99	38.26–53.30	46.47 ± 3.97	37.89–52.80	45.58 ± 4.08	40.15–51.42	46.30 ± 3.41	47.7–59.6	53.6 ± 4.3	44.6–62.2	52.9 ± 5.2
CPL(%SL)	19.01–23.14	20.54 ± 1.11	18.50–23.94	20.63 ± 1.40	18.50–23.47	20.62 ± 1.23	19.82–23.68	21.26 ± 1.33	18.5–21.9	20.5 ± 0.8	19.2–23.5	21.2 ± 1.1

TL total length, ST standard length, OL orbit length, SNL snout length, PHL postorbital head length, HL head length, BD body depth, CPL caudal-peduncle depth, CPD caudal-peduncle length

normality test also revealed non-significant deviation (Doornik and Hansen omnibus test, $Ep = 20.76$, $p = 0.11$). Also, all but $BD(\%SL)$ and $CPL(\%SL)$ morphometric ratios were normally distributed (Shapiro-Wilk test, $p > 0.05$ for all measurements), while multivariate normality test revealed significant deviation (Doornik and Hansen omnibus test, $Ep = 178.6$, $p < 0.001$).

ANOVA/Kruskal-Wallis test performed on measurement percentage ratios showed that four analyzed populations significantly differed in $SNL(\%SL)$, $SNL(\%HL)$, $PHL(\%HL)$, $HL(\%SL)$, and $CPD(\%SL)$ (Supp. Table S7). In addition, ANOVA performed on each adjusted measurement showed statistically significant differences among the four populations. Tukey's post hoc pairwise test revealed 1–4 significant comparisons, indicating that there is a lack of consistency in interpopulation differences among the measurements. Namely, significant differences for Opačica/Pribitul pair were calculated for all the adjusted measurements except BD_{adj} , while significant differences for the other sample pairs were found in four or less adjusted measurements (Supp. Table S7). PCA revealed that more than two thirds of the variation in this set of size-independent measures was explained by the first two principal components (PC1 42.2%, PC2 28.4%). The scatterplot for the first two PC axes showed that although with a slight overlap, Pribitul was separated from the other populations along PC1 and Opačica was separated from Vrijeka along PC2 (Fig. 4). The measures with the highest loading on PC1 and PC2 were HL_{adj} (0.63) and BD_{adj} (0.72), respectively.

Geometric morphometrics ANOVA of centroid size among the four analyzed *T. dabar* populations revealed no statistically significant differences ($F_{(3,66)} = 1.83$, $p = 0.15$). Multivariate regression of shape variables on centroid size was non-significant ($p = 0.07$) and accounted for 3% of the

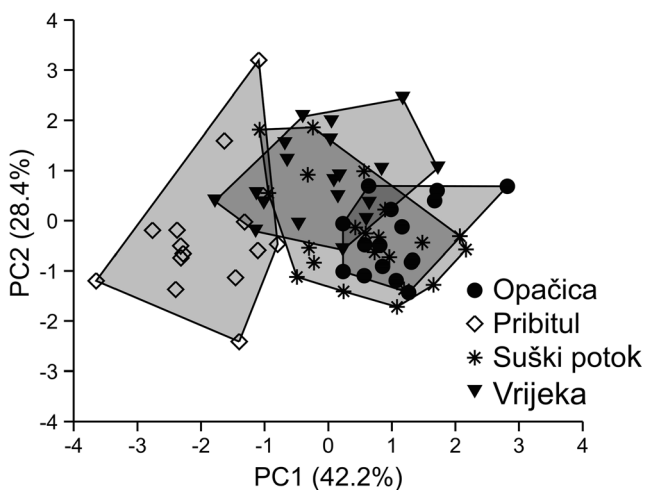


Fig. 4 Principal component analysis of size-independent morphometric data, depicting body shape variation in the samples of *Telestes dabar*

overall shape variation. However, MANOVA/CVA conducted on Procrustes coordinates revealed significant shape differences (Wilks' $\Lambda = 0.097$, $F_{(72,129.4)} = 2.12$, $p < 0.001$). Pairwise comparisons assessed by the permutational test showed that the mean shape configuration of the samples collected from Vrijeka River was significantly different from Opačica (Procrustes distance = 0.0156, $p = 0.003$), Pribitul (Procrustes distance = 0.0178, $p < 0.001$) and Suški Potok (Procrustes distance = 0.0120, $p = 0.005$) populations. The first canonical axis (CV1), accounting for 60.8% of total shape variation, separated Vrijeka River population from the other ones. Percentage of correct classification was 38.6%. Shape changes along the CV1 were mostly associated with the displacement of the landmarks which influenced the width of individuals: 8, 9, 13, and 14 (Fig. 5).

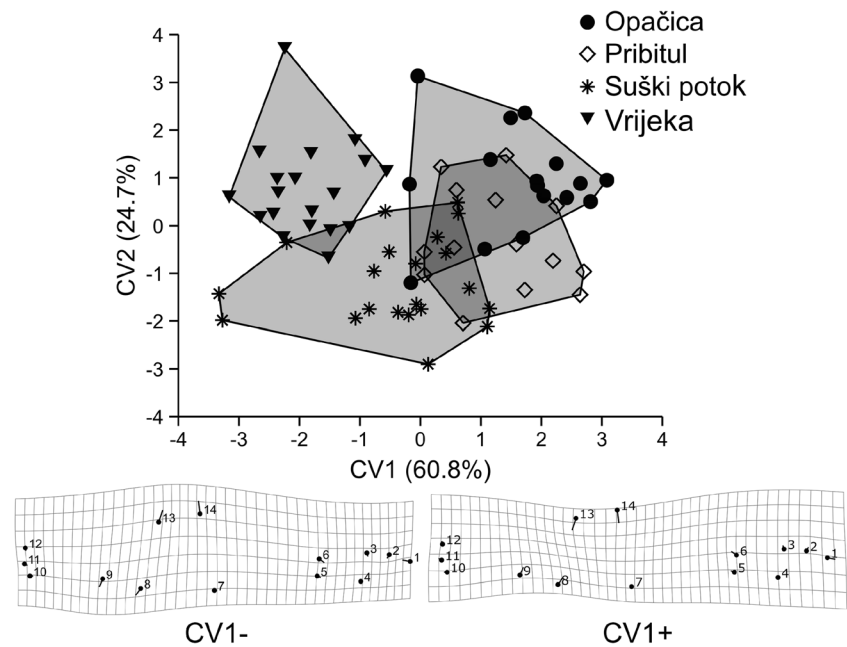
Discussion

Molecular distinction of *Telestes metohiensis* and *Telestes dabar*

In the present study, the hypothesis of the *T. dabar*/*T. metohiensis* species border was tested by combining two molecular markers of mtDNA (*cytb* and *COI*). According to Bogutskaya et al. (2012), only *T. dabar* specimens inhabit Dabarsko Polje, while Bogutskaya and Zupančič (2003) suggested that the two species occur in sympatry within this habitat. When genetic structuring among *Telestes* samples was studied using population-based method (AMOVA test), we revealed differentiated genetic clusters corresponding to the fish sampled in Nevesinjsko Polje and Dabarsko Polje. Furthermore, the observed absence of common haplotypes between the samples from Dabarsko and Nevesinjsko fields suggests a lack of gene flow.

In addition, the estimated p distances between the focal species pair is congruent with the previous research according to Buj et al. (2017) (*T. dabar* and *T. metohiensis*: 0.4–1.0%). However, divergence between *T. dabar* and *T. metohiensis* was significantly lower than the distances observed between *T. metohiensis* and *T. miloradi* species pair (2.4–2.6%; Buj et al. 2017), *T. karsticus* and *T. polylepis* (3.2–4.1%; Marčić et al. 2011), *T. souffia* and *T. muticellus* (8.0%), the “Italian” and “French” clades of *T. muticellus* (12.9%; Zaccara et al. 2007), and the other *Telestes* species pairs (2.4–10.7%; Buj et al. 2017) and *Phoxinus* genera (2.0–11.0%; Palandačić et al. 2015). These differences in values of interspecific p distances and the consequent barcoding gap reflect different divergence times, speciation modes, and processes responsible for shaping the evolutionary history of the European cyprinoids. This is in line with the notion that there is no standard threshold value in the species delineation (Lipscomb et al. 2003; Will and Rubinoff 2004), which further implies the use of

Fig. 5 Scatterplot of individual scores from canonical variate analysis (CVA) and deformation grids of body shape differences between the samples of *Telestes dabar*. The amount of variation explained by each axis is given in parentheses. Numbers in the deformation grids refer to the landmarks shown in Fig. 2



“flexible” thresholds for species identification when using distance-based methods (Krishnamurthy and Francis 2012). On the other hand, if there is a lack of the standardized species-delimitation threshold for the specific group species, such as the cyprinids of the Balkan karst region, character-based approach seems to be an appropriate method for biodiversity assessments (Damm et al. 2010). Indeed, to detect recently diverged species, such as the species pair *T. dabar* and *T. metohiensis*, the use of diagnostic substitutions within the barcoding fragment is a more reliable method than the genetic-distance approach (Fišer et al. 2018). This was evidenced by our results, where in the case of both molecular markers, no clear p distance-based barcoding gap was retrieved for the species delimitation. Contrarily, by using the character-based approach, the diagnostic set of characters or specific nucleotide positions (e.g., DeSalle et al. 2005; Rach et al. 2008; Damm et al. 2010) was identified. Indeed, consistent nucleotides in four and two diagnostic positions at *cytb* mtDNA (Supp. Table S4) and *COI* mtDNA (Supp. Table S5), respectively, differentiated *T. dabar* and *T. metohiensis*. We conclude that in the case of recently diverged taxa, *T. dabar* and *T. metohiensis*, fixed nucleotide state differences were taxonomically more informative than the quantitative approach. Thus, our results reject the sympatric distribution of the two species in Dabarsko Polje (the species border sensu Bogutskaya and Zupančič 2003), instead confirming the species border suggested by Bogutskaya et al. (2012). The evaluation of the species status of *T. dabar* and *T. metohiensis* will be further evaluated by an ongoing study of comprehensive analyses of multilocus and phenotypic data.

Diversification of freshwater fishes in the Mediterranean Biodiversity Hotspot region is tightly linked to dynamic

tectonic activity and hydrogeographic history (Karamata 2006; Buj et al. 2017). Owing to the alteration of glacial and interglacial phases and local plate-tectonics during Pliocene and Pleistocene, isolation and interconnection of rivers’ system in the peri-Mediterranean region resulted in the split of the main lineages within the genus *Telestes* around 5 million years ago (MYR) (Zaccara et al. 2007; Buj et al. 2017) during the Messinian salinity crisis (“Lago Mare” phase according to Bianco 1990). Buj et al. (2017) suggested that the ancestor of the three closely related species, *T. dabar*, *T. metohiensis*, and *T. miloradi*, became isolated from the other *Telestes* species around 14 MYR (Langhian phase in Miocene), followed by the separation of *T. croaticus* from the congeneric species and ultimately *T. metohiensis* 5.6–7.9 MYR and 3.25 MYR, respectively (Ketmaier et al. 2004). Furthermore, the split between *T. metohiensis* and *T. miloradi* happened during the Pliocene (3.6–2.5 MYR). Finally, the divergence time between *T. dabar* and *T. metohiensis* inferred from the substitution rate of 1.0% (e.g., Durand et al. 2002; Ketmaier et al. 2004) and 0.8% per million years (Perea et al. 2010) (around 600 thousand years, KYR and 750 KYR, respectively), corresponds to the period of major glaciations in Pleistocene (Dubut et al. 2012; Perea et al. 2010). Thus, the fragmentation of ancestral populations probably occurred during glaciation cycles in Middle Pleistocene, which is considered as an important period for diversification of the genus *Telestes* (Gilles et al. 2010; Dubut et al. 2012).

However, understanding the processes responsible for historical and contemporary biodiversity pattern is a demanding task in the case of the Dinaric karst due to its complex of subterranean hydrological networks (Bonacci 1987). Since underground interconnection of rivers belonging to the Neretva

tributary (Zalomka River-Nevesinjsko Polje; Mušnica River-Gatačko Polje, Cerničko Polje, and Trebišnjica River-Popovo Polje) (Palandačić et al. 2015) and its isolation from Dabarsko Polje, it seems reasonable that contemporary allopatric distribution of *T. dabar* (Dabarsko Polje) and *T. metohiensis* (Nevesinjsko, Gatačko, and Cerničko Polje) reflects an allopatric speciation model. Since this whole region was unglaciated during the Pleistocene, the Neretva watershed was refugia for freshwater species, promoting further diversification and, thus, high regional endemism (Oikonomou et al. 2014). Owing to steno-endemic species, such as *T. dabar* and *T. metohiensis*, the Neretva watershed has been proposed as a distinct biogeographical region separated from the northern Adriatic Sea region (Oikonomou et al. 2014).

Molecular and phenotypic heterogeneity of *Telestes dabar*

This study provides the first integrative study of molecular and phenotypic variation of the comprehensively sampled *T. dabar*, the species limited to Dabarsko Polje and its adjacent streams. Using both individual- (spatial analyses in GENELAND) and population-based (AMOVA) approaches to evaluate the population structure, we revealed that the samples of *T. dabar* from four rivers (Opačica, Vrijeka, Pribitul, and Suški Potok) represent a single genetic cluster. The observed low values of p distance among the five *cytb* mtDNA haplotypes ($p = 0.09\text{--}0.46\%$) and three *COI* mtDNA haplotypes ($p = 0.15\text{--}0.45\%$) further indicate an existence of a single panmictic unit of *T. dabar*, which is in concordance with the published results of haplotype differentiation at *cytb* mtDNA within *T. dabar* (0.1–0.6%), *T. metohiensis* (0.2–0.5%), and *T. miloradi* (0.3%) (Buj et al. 2017), *T. karsticus* (0.1–0.8%), and *T. polylepis* (0.1–0.4%) (Marčić et al. 2011). Still, some incongruence was found in our study between *cytb* mtDNA and *COI* mtDNA regarding genetic diversity. For instance, private *cytb* mtDNA haplotypes were found in two rivers (Pribitul and Suški Potok), while all three haplotypes of *COI* mtDNA were present continuously across Dabarsko Polje. Similar to our results, some incongruence was previously found for *cytb* mtDNA and *COI* mtDNA in cyprinids (Perea et al. 2010; Palandačić et al. 2017). As a rapidly evolving gene, *cytb* mtDNA has already been proposed as an appropriate marker for studying population structure (Palandačić et al. 2012).

Population structure based on phenotypic variation was tested by linear and geometric morphometric approaches. The linear measurements of the morphological traits of the *Telestes* species have already been studied (e.g., Zupančič and Bogutskaya 2002; Bogutskaya and Zupančič 2003; Bogutskaya et al. 2012), but geometric morphometrics, a precise and sophisticated method for quantification of phenotypic diversity, has been performed only in a few cases (Bravi et al. 2013; Tancioni et al. 2013). We found herewith an

inconsistence in population differentiation obtained by linear morphometrics (Pribitul vs. the other samples and Vrijeka vs. Opačica) and geometric morphometrics (Vrijeka vs. the others). It likely resulted from different power of methods for the detection of phenotypic variation and complex of factors underlining the studied phenotypic traits. Still, Vrijeka sample was slightly phenotypically different from the other samples, which is not in the line with the molecular data. Hence, morphological heterogeneity obtained in this study is likely influenced by habitat variation presented in different streams of Dabarsko Polje. This seems reasonable because the estimated genetic homogeneity of *T. dabar* was reached by gene flow operating among fishes from different, but connected streams. It is important to highlight that the pattern of phenotypic variation, especially of body shape, is tightly linked to environmental variables (soil pH, water velocity, temperature, etc.), differences in diet, and predation pressures (Collin and Fumagalli 2011). Likewise, it has been suggested that body shape variation in *Phoxinus* fishes is highly affected by habitat and, consequently, different modes of swimming and foraging (Ramler et al. 2017). Still, the influence of complex evolutionary factors on the population heterogeneity in *P. phoxinus* suggested a need for the comprehensive study of both molecular markers and morphological traits in association with ecological and geographical data (Collin and Fumagalli 2015). Although our study lacks ecological data concerning different rivers where the fish were sampled, it is the first study of intraspecies phenotypic variation of the recently described species *T. dabar*, as well as the first instance where geometric morphometrics was used for the research of the *Telestes* fish from the Dinaric karst. Hence, our analyses should be taken as the preliminary data of the shape and size variation of these intriguing, vulnerable endemic freshwater fish. Therefore, deeper insights into the pattern of the distribution of phenotypic variability and the processes that shape population structure will be obtained in future studies.

Conservation implications

Findings on *T. dabar* population heterogeneity in phenotypic traits and mtDNA genes presented in this study suggest the existence of phenotypic plasticity and adaptive potential of this recently described species. In addition, a lack of molecular differentiation among the samples from different rivers of Dabarsko Polje confirms their connection through gene flow, which is essential for maintaining contemporary genetic diversity of this minnow with extremely restricted range. Another finding of conservation concern includes new localities of the species. Up to date, *T. dabar* has been found only in Opačica and Vrijeka Rivers (Bogutskaya et al. 2012) and, thus, the data on samples from Pribitul and Suški Potok expanded our knowledge on its distribution. Integrative data from this comparative study of molecular and phenotypic

variation is also valuable because of the data deficient status of *T. dabar* according to the IUCN (<http://www.iucnredlist.org>). Indeed, understanding the spatial distribution of intraspecific variation is a prerequisite task for assessing conservation status of the focal taxon. Hence, to improve conservation strategy for the freshwater fish in the vulnerable karst habitat, we provoke to change the IUCN assessment of both *T. dabar* and *T. metohiensis* species.

Furthermore, the evidence of species boundary between minnows allows a new insight into the species diversity of ichthyofauna of the Dinaric karst. Among 249 endemic freshwater fish species in the Mediterranean Biodiversity Hotspot, 56% are considered as threatened and 16% as Data Deficient (Smith and Darwall 2006). Diversity of the genus *Telestes* is especially high in the Eastern Adriatic coast (Buj et al. 2017) and might be significantly higher due to the hidden diversity. Indeed, the level of biodiversity is considered to be highly underestimated as a result of cryptic diversity (Fišer et al. 2018), and, thus, 35 endemic fish species recorded so far in Adriatic Basin of Bosnia and Herzegovina (Glamuzina et al. 2011) likely are an underrepresentation of the total species diversity. Hence, our data significantly contribute to the future DNA barcode studies of endemic cyprinid fish diversity as valuable baseline data in the reference libraries of both *cytb* mtDNA and *COI* mtDNA proposed by FishBOL (<http://www.fishbol.org>).

Finally, to evaluate conservation priorities in the region of the conservation importance, such as the unique Dinaric karst, knowledge regarding accounts and distribution of endemic and endangered taxa is essential. Since *T. dabar* exclusively inhabits small karstic streams belonging to Dabarsko Polje, it should have a high priority in further conservation actions. This is of great importance in the light of climate changes (increasing temperature and decreasing precipitation), pollution and water extraction, drought, and invasive alien species (Smith and Darwall 2006), which strongly affect the ground-water ecosystems in the Dinaric karst environment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This work did not involve human subjects or experiments on animals.

Informed consent Informed consent statement does not apply to this work since it did not involve human subjects.

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