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Evolutionary history of the Balkan endemic genus *Delminichthys* (Teleostei, Leuciscidae) with an emphasis on the population structure of Southern Dalmatian minnow

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Abstract

The genus *Delminichthys* consists of allopatric species restricted to ephemeral watercourses in the karst fields of the left-bank (*D. ghetaldii*) and the right-bank (*D. adspersus* and *D. jadovensis*) Neretva River basin as well as in the Una River drainage (*D. krbavensis*). The first aim addressed questions of interspecies variation and phylogenetic relationships among *Delminichthys* based on time-calibrated analysis of *cyt* b mtDNA (cytochrome b) and *co1* mtDNA (cytochrome c subunit I) mitochondrial DNA genes. Species-specific haplogroups supported two mtDNA genes as

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suitable barcoding markers, while we additionally proposed character-based and genetic distance threshold-based delimitation of species. We confirmed that the Pliocene and Pleistocene refugium for freshwater species was the Neretva basin, promoting the diversification of the genus *Delminichthys*. Our second aim was to test the population structure of *D. ghetaldii* (Southern Dalmatian minnow) by performing an integrative study using molecular mtDNA data, linear and geometric morphometric data. We consider that the spatial aspects of the population genealogical structure of *D. ghetaldii* mirror the Pleistocene paleodrainages. The differentiated *co1* mtDNA clusters corresponding to the populations from Fatničko Polje+Dabarsko Polje and Ljubomirsko Polje+Konavosko Polje represent geographically discrete genetic entities, which should be addressed through conservation management.

Keywords

biogeographical history – *cyt* b mtDNA – *co1* mtDNA – *Delminichthys* – linear and geometric morphometrics

Introduction

The genus *Delminichthys* belongs to the family Leuciscidae (Actinopterygii: Cypriniformes), the subfamily Leuciscinae (Schönhuth et al., 2018; Tan & Armbruster, 2018). The family consists of more than 300 species distributed in Europe (Reier et al., 2022). Most native cyprinid genera in Europe belong to the tribe Leuciscini (Freyhof et al., 2006), and, thus, the evolutionary history and diversification of leuciscins considerably affected remarkable diversity and extraordinary high endemicity of freshwater ichthyofauna in the Mediterranean Basin Hotspot (Smith & Darwall, 2006). Phylogenetic structure analysis of the Palaearctic leuciscins based on both nuclear (recombination activating gene 1, *RAG1* nDNA) and mitochondrial (cytochrome b, *cyt* b mtDNA) genes confirmed a well-supported monophyletic clade, the genus *Delminichthys* Freyhof, Lieckfeldt, Bogutskaya, Pitra & Ludwig, 2006 consisting of species previously included in the genus *Phoxinellus* (Zupančič & Bogutskaya, 2000; Bogutskaya & Zupančič, 2003; Freyhof et al., 2006; Palandačić et al., 2010; Schönhuth et al., 2018). Moreover, together with one sister clade (*Pelasgus* since Kottlat & Freyhof, 2007) previously belonged to the genus *Pseudophoxinus*, the genus *Delminichthys* formed a separate phylogenetic clade within the Palaearctic leuciscins (Freyhof et al., 2006). Some reductive morphological traits (such as irregular spotted colour pattern, large postcleithrum, increased number of precaudal anal-fin pterygiophores and large genital papilla in females) additionally supported the existence of *Delminichthys* as a distinct evolutionary lineage (Freyhof et al., 2006) that is endemic to the Dinaric Karst of Bosnia and Herzegovina and Croatia (Bogutskaya & Zupančič, 2003; Freyhof et al., 2006; Reier et al., 2022). Furthermore, the

genus *Delminichthys* consists of two lineages, one of which includes the Spotted minnow *Delminichthys adspersus* (Heckel, 1843) and the Southern Dalmatian minnow *Delminichthys ghetaldii* (Steindachner, 1882), and the other the Jadova minnow *Delminichthys jadovensis* (Zupančič & Bogutskaya, 2002) and the Krbava minnow *Delminichthys krbavensis* (Zupančič & Bogutskaya, 2002) (Freyhof et al., 2006).

Several molecular phylogenetic studies (e.g., Ketmaier et al., 2004; Levy et al., 2009; Perea et al., 2010; Dubut et al., 2012; Buj et al., 2017, 2020; Rodriguez et al., 2021) have suggested that the diversification of circum-Mediterranean leuciscins was fostered by a complex of geomorphological and hydrogeological events that led to the dispersal and migration waves of cyprinifom fishes according to the northern river dispersal scenario (Bânârescu, 1992) and the southern sea-dispersal – Lago Mare scenario (Bianco, 1990) which occurred from the late Oligocene (35 MYA) – early Miocene (15 MYA) to the late Pliocene (1.7 MYA), and during the Lago Mare phase (ca. 7.9–3.25 MYA) and the Messinian Salinity Crisis (MSC; ca. 5.7–5.4 MYA), respectively. Multiple dispersals accompanied by a complex of vicariance and fragmentation that riverine fish species experienced during the Quaternary climatic oscillations (from 2.4 MYA to the Last Glacial Maximum 24,000-18,000 YA) additionally triggered the genetic and taxonomic diversity of freshwater ichthyofauna in the heterogeneous Dinaric Karst region (e.g. Buj et al., 2020; Rodriguez et al., 2021; and references therein). For instance, comparative studies of the phylogeographic structure of the endemic genera *Delminichthys* and *Phoxinellus*, and the Dinaric *Telestes* and *Phoxinus* species have yielded congruence between their distributions, *cyt* b mtDNA phylogenetic relationships and paleogeography (Reier et al., 2022), and, thus, support the Miocene refuge hypothesis of Dalmatian karst habitats (Freyhof et al., 2006), the phylogeographic scenario of refugia within refugia (Gomez & Lunt, 2006) and the impacts of Quaternary climatic oscillations (e.g., Dubut et al., 2012; Buj et al., 2020; Rodriguez et al., 2021) on their evolutionary history. Indeed, the diversification of the four *cyt* b mtDNA lineages corresponding to the *Delminichthys* species was started at the end of the Pliocene by the separation of *D. ghetaldii* distributed in the left-bank Neretva River drainage, while the subsequent allopatric speciation, divergence and dispersion of congeneric lineages is correlated with the climatic oscillations during the Pleistocene (Reier et al., 2022).

Being the endemic species of the Dinaric Karst restricted to a few karst fields, the *Delminichthys* species are allopatrically distributed in river drainages belonging to the Adriatic Sea and Black Sea basins. For instance, *D. krbavensis* is exclusively distributed in Krbavsko Polje (Polje = karst field) in the Una River drainage of the Black Sea basin, which further promotes its isolation from the congeneric species that occur in the natural habitats of the Adriatic Sea basin (Ćaleta et al., 2015; Mustafić et al., 2017; Freyhof et al., 2020; Reier et al., 2022). In addition, *D. adspersus* and *D. jadovensis* inhabit the karst fields of the right-bank Neretva River drainage (Tihaljina-Trebižat, Imotsko Polje,

Rastočko Polje, Vrgoračko Polje, Buško Blato) and the karst fields of the Jadova River drainage (Ličko Polje/Lika-Jadova) in Croatia, respectively. On the contrary, *D. ghetaldii* inhabits the karst fields of the left-bank Neretva River drainage (partly Dabarsko Polje), the Trebišnjica River drainage (Dabarsko Polje, Gatačko Polje, Fatničko Polje, Ljubomirsko Polje, Popovo Polje) and Kasindolka Stream (south of Sarajevo) in Bosnia and Herzegovina as well as the Ombla Spring (spring near Dubrovnik) and Ljuta River (Konavosko Polje) in Croatia (Milanović, 2015; Reier et al., 2022; Milanović, 2023).

The strictly endemic *Delminichthys* species faced selection regimes of underground and aboveground environments with geologically unique features in one of the world's most famous karstic regions – Dinarides on the Balkan Peninsula (Reed et al., 2004). Indeed, adaptations of subtroglophiles (adapted to the areas of the underground area with an influx of light, typically found near the cave entrance) or stygophiles (inhabit the completely dark zone of caves, where no light penetrates) – the Dinaric karst endemic cyprinoid genera *Delminichthys*, *Phoxinellus*, and endemic *Telestes* and *Phoxinus* species (Jelić et al., 2016), restricted to ephemeral karstic streams, springs, caves, marshes and/or small lakes in the fields that mainly associated with subterranean hydrological networks (Bonacci, 1987), are regarded as limiting factor for their dispersal potential (Reier et al., 2022). Moreover, the stenoendemic *D. jadovensis* and *D. krbavensis* are considered advanced stygophiles species because they emerge to aboveground habitats only in the spawning season (Ćaleta et al., 2015; Jelić et al., 2016; Reier et al., 2022). Still, *D. ghetaldii* and *Phoxinus karsticus*, *D. jadovensis* and *Telestes croaticus*, and *D. krbavensis* and *Telestes fontinalis* occur sympatrically in the Trebišnjica River (Popovo Polje, Adriatic Sea basin), Jadova River drainage (Ličko Polje, Adriatic Sea basin) and Krbavsko Polje (Una River drainage, Black Sea basin), respectively (Reier et al., 2022).

The unique Dinaric Karst region, as a part of the Central Peri-Mediterranean biogeographical region, is a home of extraordinary freshwater diversity and numerous endemics (e.g., Reyol et al., 2007). Noteworthy, the Neretva tributary, as the Pleistocene refugia for freshwater species and the region of intense speciation and diversification, is recognized as a distinct biogeographical region separated from the Northern Adriatic coast (Oikonomou et al., 2014; Smith & Darwall, 2014). As such, the Dinaric karst is recognized as a region of high conservation priority (Jelić et al., 2016). Moreover, the unique freshwater fish species in this vulnerable karst habitat face various threats affecting the decline and/or extinction of populations and, thus, are recognized by the International Union for Conservation of Nature (IUCN; https://www.iucnredlist.org/). Therefore, studies of endangered ichthyofauna, such as the Balkan endemic *Delminichthys* species, are of fundamental importance for biodiversity assessment. Indeed, species *D. ghetaldii* and *D. adspersus* are classified by the IUCN Red List (https://www.iucnredlist.org/) as Vulnerable (VU) (under criteria B1ab(ii,iii)+2ab(ii,iii) and B2ab(iii), respectively). In the Republic of Srpska, Bosnia and Herzegovina (Službeni Glasnik Republike Srpske br. 65, 2020; https://nasljedje.org/) both species were considered

to be strictly protected wild species, and in Croatia (Red Book freshwater fishes of Croatia; Mrakovčić et al., 2006) as Endangered (EN) and Vulnerable (VU), respectively. Strictly restricted endemic species *D. jadovensis* and *D. krbavensis* are categorized as Critically Endangered (CE) under criteria B1ab(iii,iv) and B1ab (ii,iii,iv)+2ab(ii,iii,iv), respectively (IUCN; https://www.iucnredlist.org/).

To date, traditional morphological traits and nuclear (Recombination-activating gene 1 – *RAG1*; Ribosomal protein gene *S7* – *RPS7*; Rhodopsin – *Rho*; Early growth response 2b–*EGR2B*; Internal transcribed region 1 of ribosomal DNA – *ITS1* rDNA) and mitochondrial (Cytochrome b – *cyt* b; Cytochrome oxidase I – *CO1*) data sets were used to define the phylogenetic position of the genus *Delminichthys* within the Palaearctic Leuciscinae (Bogutskaya & Zupančič, 2003; Freyhof et al., 2006; Palandačić et al., 2010; Perea et al., 2010; Rodriguez et al., 2021). These methods have been successfully used in taxonomy and phylogenetic studies of the related genera such as *Phoxinus* (Palandačić et al., 2015, 2017; Vucić et al., 2018), *Phoxinellus* (Palandačić et al., 2010), *Telestes* (Ketmaier et al., 2004; Gilles et al., 2010; Dubut et al., 2012; Buj et al., 2017; Francuski et al., 2019) and *Squalius* (Buj et al., 2020), for instance. In addition, the time-calibrated *cyt* b mtDNA phylogenetic tree was found to be concordant with the level of molecular and phenotypic interspecific variation and to correspond to the pattern and amount of body shape diversification of Dinaric cyprinoid fishes (Lukač et al., 2023). However, different rates of gene evolution used to resolve phylogenetic relationships, species delimitation and genetic structure of cyprinoids have been observed; *EGR2B*, *Rho* and *RAG1* were suggested as conservative markers (Palandačić et al., 2010, 2015; Buj et al., 2017, 2020, 2021; Rodriguez et al., 2021; but see Palandačić et al., 2017, 2020; De Santis et al., 2021), *cyt* b mtDNA was found to be faster evolving gene than *CO1* mtDNA (Palandačić et al., 2017; Francuski et al., 2019; Ludoški et al., 2021), while microsatellite nDNA loci and *cyt* b mtDNA (Dubut et al., 2012; Palandačić et al., 2012a, b) and AFLP loci (Amplified Fragment Length Polymorphisms) (Collin & Fumagalli, 2011) were considered as fast evolving DNA fragments suitable for population structure analyses. Thus, phylogenetic resolution of different molecular markers was observed in cyprinoid fishes; however, comparative analyses of time-calibrated multilocus phylogenetic trees and phylogenetic relationships among the *Delminichthys* species have not yet been performed.

Considering intraspecific variation within the *Delminichthys* species, comparative analyses of morphometric (linear measurements) and meristic traits suggest that interpopulation and interspecific variation could be influenced by varying habitats (Mustafić et al., 2017). Until now, only a few previous molecular studies on *D. adspersus* have yielded population structure and dispersal patterns by using microsatellite DNA loci (Palandačić et al., 2012a, b), although both molecular markers (*CO1* mtDNA and *cyt* b mtDNA) and phenotypic traits estimated by a geometric morphometric approach (body size and shape) have already been successfully used in studies of phylogeographic structure, cryptic diversity, genetic connectedness and population structure of

European leuciscins (Zaccara et al., 2007; Collin & Fumagalli, 2011; Ramler et al., 2016; Francuski et al., 2019; Buj et al., 2019, 2022; Reier et al., 2022). Therefore, a comprehensive study of the interpopulation and intrapopulation genetic and phenotypic variation of the *Delminichthys* species has been conducted herein.

Given the conservation importance of the Balkan endemic Leuciscinae lineages, in this study we address the question of the phylogenetic relationships of *Delminichthys* species in the context of historical biogeography and evolutionary history. Thus, our first aim was to test the phylogenetic hypothesis of the *Delminichthys* species by implementing the complementary approach of two mitochondrial DNA markers (*cyt* b mtDNA and *co1* mtDNA). The newly obtained data were subjected to time-calibrated phylogenetic analysis and evaluation of the evolutionary history of the *Delminichthys* species across Dinaric Karst by integrating the published data. We reconstruct the Bayesian inference phylogenetic trees (BI) using both *cyt* b mtDNA and *co1* mtDNA sequence datasets in order: (1) to contrast time-calibrated phylogenies, (2) to compare interspecific genetic distances, and (3) to identify genetically distinct lineages. Considering that the distribution of molecular and phenotypic variation within and among species is shaped by historical and contemporary evolutionary processes and underlying mechanisms (Avise, 2000) and, as such, represents fundamental resource data for conservation purposes, we performed a comprehensive population analysis of the focal *Delminichthys* species. Our second aim was to test the population structure of *D. ghetaldii* by performing an integrative study of populations from Dabarsko Polje (Suški Stream, Pribitu Spring and Kanal), Fatničko Polje (Fatničko Lake) and Ljubomirsko Polje (Ljubomirski Stream) by using *cyt* b mtDNA and *co1* mtDNA data, and traditional (linear measurements) and geometric morphometric (size and shape) data. To address the question of phenotypic variation within and among populations, we tested linear (eight measurements) and landmark-based geometric morphometric approaches (body size and shape). The genetic diversity of two mtDNA gene fragments commonly used in phylogenetic and population genetic analyses was assessed in order: (1) to fill the gap of the DNA barcode database of the endemic species in Dinaric Karst, (2) to gather a comprehensive database of population genetics invaluable in designing sustainable conservation management of the threatened minnow species, (3) to test the correlation between interpopulation genetic and phenotypic distances, and (4) to fill the gap in conservation assessment of the endangered ichthyofauna of the Dinaric Karst as a remarkable region of biodiversity within the Mediterranean Basin Hotspot. Finally, a multisource approach to gathering different lines of intraspecies diversity would bridge the gap between the morphological and molecular evolution of this endangered minnow species.

Material and methods

Records of Delminichthys ghetaldii

Delminichthys ghetaldii (Steindachner, 1882)

Syn.: *Paraphoxinus ghetaldii* (Steindachner, 1882), *Phoxinellus ghetaldii* (Steindachner, 1882), *Phoxinellus pstrossi* (Steindachner, 1882)

The genus *Phoxinellus* Heckel (1843) (type species: *Phoxinellus alepidotus* Heckel, 1843 syn. *Paraphoxinus* Bleeker, 1863) included different small Mediterranean leuciscins (Trewavas, 1971; Karaman, 1972; Almaça, 1977; Bânârescu, 1977; Banister, 1980; Economidis & Bânârescu, 1991; Bogutskaya, 1992; Kottelat, 1997). Unlike *P. alepidotus* and *P. ghetaldii* syn. *P. pstrossi*, which were discovered earlier, two new species of *Phoxinellus* (*P. krbavensis* and *P. jadovensis*) were discovered later (Zupančič & Bogutskaya, 2000, 2002; Bogutskaya & Zupančič, 2003). According to Bogutskaya & Zupančič (2003), based on morphological traits *Phoxinellus* species are divided into two groups, each of which includes several species. However, within *Phoxinellus*, based on phylogenetic data, *Delminichthys* is recognized as a separate genus and thus the species *D. adspersus*, *D. ghetaldii, D. jadovensis* and *D. krbavensis* (Freyhof et al., 2006).

Delminichthys (*Phoxinellus*) *ghetaldii* was first found in the caves of Popovo Polje (type locality): Steindachner, 1882, Kosić, 1903; Trgovčević, 1905, 1932; Ćurčić, 1913, 1915a, b; Taler, 1952, 1953; Ristić, 1977; Vuković, 1982. According to Ćurčić (1915a, b) and materials in Natural History Museum Wien (NMW) and Primož Zupančič (PZ) collections, *P. ghetaldii* is distributed north of Popovo Polje, in Ljubomirsko Polje and Fatničko Polje (eastern Herzegovina, all coastal drainage). It was recorded in Dabarsko Polje (Sušica and Ljeljeśnica Caves) for the first time by Ćurčić (1915a) and confirmed by PZ's findings of juveniles of this species in the channel flowing from Ljeljeśnica Cave. Many authors reported *Phoxinellus* (or *Paraphoxinus*) *pstrossii* from the Trebišnjica River near Trebinje, Herzegovina and Dalmatia region, or just the Adriatic basin in Yugoslavia: Kišpatić (1893), Taler (1952, 1953), Sket (1967), Vuković & Ivanović (1971), Ristić (1977), Vuković (1977, 1982), Pocrnjić et al. (1979), Povž et al. (1990), Mikavica (1998), Vuković & Miladinović (1969), Berberović et al. (1969) and Aganović & Kapetanović (1971), while Vuković (1977) reported *P. pstrossii* from Ljubomirsko Polje, Sofradžija & Berberović (1972) from Dabarsko Polje and Ljubomirsko Polje, and Pavlović & Berberović (1978) from Dabarsko Polje. Ćurčić (1915a) found *P. ghetaldii* in Blaća, a small karstic field north-east of Neum (Bosnia and Herzegovina). *Delminichthys* (*Phoxinellus*) *ghetaldii* was found by B. Jalžić and N. Tvrtković for the first time in a cave part of the source of Rijeka Dubrovačka (Ombla) near Dubrovnik and in an isolated spring pool in Stravča near Cavtat (both localities are in Dalmatia) (Croatian Natural History Museum; Zupančič & Bogutskaya, 2002). Based on the available data, individuals of *D. ghetaldii* were found both before and after the construction of dams and the Bileća Reservoir. In Ljubomirsko Polje, Fatničko Polje, Dabarsko Polje and Konavosko Polje they were also recently found (Zupančič & Bogutskaya, 2002; Dekić et al., 2014, 2016; Jelić & Jelić, 2015; Schönhuth et al., 2018; Reier et al., 2022; Lukač et al., 2023). In the Bileća Reservoir and downstream of the Grnčarevo Dam on the Trebišnjica River, individuals have not been detected since 2002 (Zupančič & Bogutskaya, 2002).

Sample collection

A total of 61 *D. ghetaldii* specimens were collected from Dabarsko Polje (26 individuals), Fatničko Polje (13) and Ljubomirsko Polje (22) in the period from 2015 to 2018 (collectors R. Dekić and M. Lukač) using transportable electroshockers (IG 600, 1.2 kW, and ELT 62 II GI, 3 kW) and collecting nets of different diameters (table 1, fig. 1). The permission for sampling individuals was issued by the Ministry of Agriculture, Forestry and Water Management of the Republic of Srpska (19/6-020/961-30/15; 12-03.02-330-1375/18). Species determination was carried out using morphological keys (Vuković & Ivanović 1971; Kottelat & Freyhof, 2007).

Morphometric analyses were performed on all 61 collected *D. ghetaldii* individuals, while genomic DNA was extracted from 41 individuals (table 1, supplementary tables S1 and S2). Specimens of *D. ghetaldii*

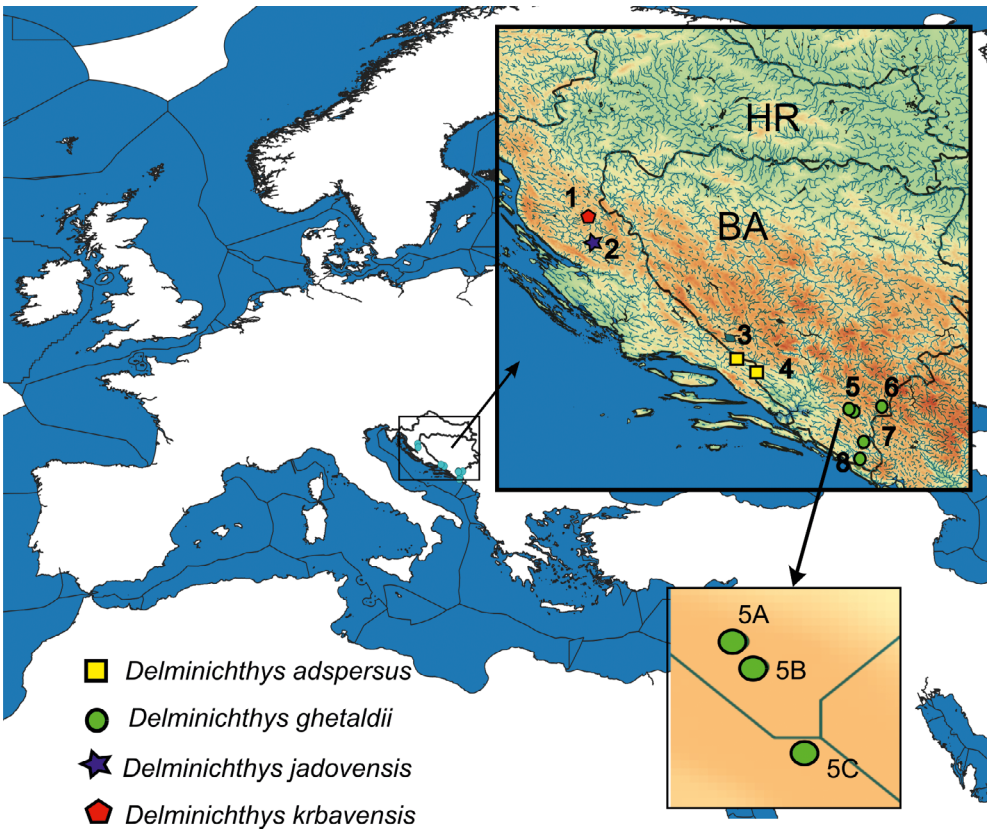


FIGURE 1 Map showing sampled localities for specimens of four *Delminichthys* species (*D. adspersus*, *D. ghetaldii*, *D. krbavensis* and *D. jadovensis*) in the Dinaric Karst region in Croatia and Bosnia and Herzegovina used to generate molecular data (*cyt* b mtDNA and *co1* mtDNA sequences retrieved from GenBank and *de novo* sequenced for all four species) and morphometric data (for *D. ghetaldii*): (1) Krbavsko Polje, (2) Ličko Polje, (3) Imotsko Polje, (4) Tihaljina – Trebižat, (5) Dabarsko Polje (A – Kanal, B – Suški Stream, C – Pribitu Spring), (6) Fatničko Polje, (7) Ljubomirsko Polje, (8) Konavosko Polje

TABLE 1 Sampling locations and sampling size of *Delminichthys ghetaldii* used for molecular and morphometric analysis

	Drainage[a]	Sampling date	Longitude Latitude	Locality	Morphometry	mtDNA	
						cyt b	*co1*
Dabarsko Polje	N/T	18.08.2017.	N 43 °04.346′ E 018 °14.032′	Suški Stream	3	3*	3
		21.04.2016.	N 43 °04.518′ E 018 °13.925′	Pribitu Spring	1	1*	–
		08.08.2018.	N 43 °03.763′ E 018 °14.298′	Kanal	22	9+6*	10
Fatničko Polje	N/T	18.04.2018	N 43 °04.889′ E 018 °31.446′	Fatničko Lake	13	7+6*	10+1*
Ljubomirsko Polje	T	26.03.2015.	N 42 °45.863′ E 018 °20.740′	Ljubomirski Stream	22	13 (12)+5*	10 (9)
Konavosko Polje	L	–	N 42 °36.013′ E 018 °18.666′	Stravča	–	2*	1*
				Overall	61#	29 (28)+23*	33 (32)+2*

a Water from Dabarsko Polje and Fatničko Polje was partly or completely artificially diverted towards the Trebišnjica drainage (Bileća reservoir): N – left-bank Neretva drainage, T – Trebišnjica drainage, N/T – partly Neretva and partly Trebišnjica drainage, L – Ljuta drainage; *cyt* b mtDNA and *co1* mtDNA sequences downloaded from GenBank (Freyhof et al., 2006; Perea et al., 2010; Schönhuth et al., 2018; Reier et al., 2022; Lukač et al., 2023); #Specimens that were included in the previous study of the evolutionary body shape diversification of endemic Cyprinoidei fishes (Lukač et al., 2023). Numbers in parentheses represent the number of *de novo* sequences used for intraspecies analysis (*cyt* b mtDNA sequence OR916349 and *co1* mtDNA sequence OR916373 were excluded from intraspecific analysis due to short length).

were previously included in the study of evolutionary body shape diversification of the endemic Cyprinoidei species (Lukač et al., 2023). For phylogenetic analysis, apart from sequences obtained in this study (*cyt* b mtDNA: 29 sequences, *co1* mtDNA: 33 sequences; table 1), an additional 382 (including one sequence of outgroup taxon) *cyt* b mtDNA sequences and nine (including one sequence of outgroup taxon) *co1* mtDNA sequences were retrieved from the GenBank database (supplementary tables S1 and S2). The database was searched for *cyt* b mtDNA sequences of four *Delminichthys* species: *D. adspersus* (341 sequences), *D. ghetaldii* (23), *D. jadovenis* (7) and *D. krbavensis* (10) (supplementary table S1). The same *Delminichthys* species were searched for *co1* mtDNA sequences: *D. adspersus* (3 sequences), *D. ghetaldii* (2), *D. jadovenis* (1) and *D. krbavensis* (2) (supplementary table S2). The selection was based on the length and purity of the sequences. It should be noted that 11 *co1* mtDNA sequences of four *Delminichthys* species were also found in the GenBank (acc. numbers KJ553317, KJ553338, KJ553492, KJ553408,

KJ553488, KJ553495, KJ553510, KJ553505, KJ553582, KJ553604, KJ553626; Geiger et al., 2014), but the BLAST sequence analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that they all have more than 93% similarity only to *D. adspersus*, and less similarity to the corresponding identified species. Also, some of these sequences are shorter and with unresolved nucleotides, so due to possible misidentification and errors, they are not included in this paper. As an outgroup taxon, we used *Pelasgus minutus* (Karaman, 1924) following the phylogenetic study of Reier et al. (2022) and retrieved sequences from the GenBank database (*co1* mtDNA: MZ230788; *cyt* b mtDNA: MG806851; supplementary tables S1 and S2). Given the lack of both markers for the same individual, whose sequences were retrieved from the GenBank, it was unachievable to concatenate the sequences into one dataset. Therefore, we analyzed individual mitochondrial markers separately. For intraspecific analysis of molecular variation of *D. ghetaldii* datasets included 51 (28 *de novo*) and 34 (32 *de novo*) *cyt* b mtDNA and *co1* mtDNA sequences, respectively (cyt *b* mtDNA sequence OR916341 and *co1* mtDNA sequence OR916373 were excluded from the intraspecific analysis of *D. ghetaldii* due to insufficient length; table 1).

Molecular analysis

DNA extraction and sequence processing

Total genomic DNA was extracted from 41 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 *cyt* b mtDNA was amplified using Glu-F (5′-GAAG AACCACCGTTGTTATTCAA-3′)/Thr-R (5′-ACCT CCRATCTYCGGATTACA-3′) primer pair (Zardoya & Doadrio, 1998). The amplification of the 5′ ends of *co1* mtDNA was done by LCO-1490 (5′-G GTCAACAAA TCATA AAGATATTGG-3′)/HCO-2198 (5′-TT AAACTTCAGGGTGACCAAAAAATCA-3′) primer pair (Folmer et al., 1994) and FISH1F (5′-TCAACCAACCACAAAGACATTG GCAC-3′/FISH1R (5′-TAGACTTCTGGGTGG CCAAAGAATCA-3′) primer pair (Ward et al., 2005). PCR reactions were performed using an Illustra PuReTaq Ready-To-Go PCR Beads kit (GE Healthcare Life Sciences, Buckinghamshire, UK). PCR conditions for *cyt* b mtDNA and *co1* mtDNA amplification were as described in Palandačić et al. (2012a) 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 (Amsterdam, the 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, the number of haplotypes and calculation of uncorrected *p* distance within and between species and populations haplotypes was done using MEGA 11 (Tamura et al., 2021). TCS and Median-Joining haplotype networks were constructed in PopART (Leigh & Bryant, 2015).

To assess the phylogenetic position and evolutionary history of *D. ghetaldii*, Bayesian Inference (BI) phylogenetic trees were established based on the dataset containing a total of 411 *cyt* b mtDNA sequences

(999 bp long) and 42 *co1* mtDNA sequences (619 bp long) belonging to *D. adspersus*, *D. ghetaldii*, *D. krbavensis* and *D. jadovensis* species. The best-fit model of nucleotide substitution was obtained in the program jModelTest v.2.1.10 (Darriba et al., 2012) based on Bayesian Information Criteria (BIC). The calculated model was TPM1+I for both mtDNA datasets and Yule model tree prior was performed.

The BI analysis was conducted using BEAST v1.10.4 (Suchard et al., 2018). The data matrix was initially converted into an XML file, as required by BEAST, using BEAUTI v1.10.4 (Suchard et al., 2018). Four independent runs of 10,000,000 generations for simultaneous Monte Carlo Markov chains (MCMC) with sampling at intervals of 1,000 generations were performed. To diagnose convergence problems by inspecting the individual traces simultaneously of the each runs was assessed visually using Tracer v1.7.2 (Rambaut et al., 2018). Once convergence was confirmed, the first 10% of trees were removed as burn-in, and the remaining trees from all four runs (*cyt* b mtDNA) were combined using LogCombiner v1.10.4 (Rambaut et al., 2018) after resampling every 3,000 generations. For summarizing the information from a sample of trees onto a maximum clade credibility target tree and median node heights with a posterior probability limit of 0.5, TreeAnnotator v.1.10.4 (Rambaut et al., 2018) was used. The target tree was visualized by using FigTree v.1.4.4 (Rambaut et al., 2018). In addition, *cyt* b mtDNA evolutionary rate of the 1.05% divergence per pairwise per million years (0.53% per lineage per million years) estimated for leuciscids (Dowling et al., 2002) and *co1* mtDNA evolutionary rate of the 1% divergence per pairwise per million years (0.5% per lineage per million years) estimated for cypriniforms (Wang et al., 2013; Fang et al., 2022) were applied with a strict clock model in BEAST and visualized time tree with node ages and 95% probability range in FigTree v.1.4.4 (Rambaut et al., 2018; Suchard et al., 2018).

Population genetic structure

To assess the population structure of *D. ghetaldii* for both molecular markers (datasets of 51 *cyt* b mtDNA and 33 *co1* mtDNA sequences) analysis of molecular variance (AMOVA; Excoffier et al., 1992) was estimated using ARLEQUIN v 3.5 (Excoffier & Lischer, 2010). The total genetic variance was partitioned into the proportion of genetic variation among and within conspecific populations of *D. ghetaldii*. One thousand permutations were used to determine the significance of variance components.

Using DnaSP (Rozas et al., 2017) intrapopulation genetic diversity parameters: the number of polymorphic sites (S), number of haplotypes (H), haplotype diversity (h), the average number of nucleotide differences (k) and nucleotide diversity (π) were calculated.

To infer the *D. ghetaldii* intraspecific differentiation, the Bayesian clustering method employed in GENELAND 4.9.2 (Guillot et al., 2005), implemented in R v4.2.2 (R Core Team, 2022), was used. We ran the algorithm to infer the number of genetic clusters (K) within the samples and the assignment of individuals to the clusters with 10 independent runs with 10,000 MCMC iterations, a thinning of 100, a maximum rate of Poisson process fixed to 100, K allowed to vary between 1 and 3, 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). In addition, individual based analysis of both mtDNA datasets were subjected to non-saptial analysis in STRUCTURE 2.3.4 software (Pritchard et al., 2000) (details are given in the supplementary material).

To test the correlation between *D. ghetaldii* interpopulation genetic and phenotypic distance, the matrix representing populations' pairwise values of *p* distances (based on 46 *cyt* b mtDNA and 33 *COI* mtDNA sequences of individuals that were also included in the morphometric analysis) and Procrustes distances (based on shape variables) before and after removing of allometric effect (see below) were assessed using Mantel's test with 10,000 permutations in PAST 4.14 (Hammer et al., 2001).

Morphometric analysis

We applied two morphometric approaches- linear (traditional) and geometric morphometrics. Before analysis, digital photos of the right lateral side were taken with a Nikon D7100 digital camera with a 50-mm f/1.4D objective attached to the tripod stand and focused vertically on the surface of the object. The scale factor is determined for each photo according to the millimetre scale before the digitization process.

Linear morphometrics

Linear morphometric characteristics were measured (in mm) on digital images using measurement mode in tpsDig 2.30 (Rohlf, 2017). According to the standard for cyprinoid fishes (Armbruster, 2012), the total of eight linear measurements used for analysis were: 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*) (fig. 2). To remove the allometry effect, linear measurements were transformed into (1) the percentage ratios of measured distance and *SL* (for *OL*, *SNL*, *HL*, *BD* and *CPD*) and *HL* (for *OL*, *SNL* and *PHL*), and (2) size-independent adjusted measurements according to Elliott et al. (1995):

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

where M is the original morphometric measurement, M_{adj} is the size-independent measurement, L_s *is* the overall mean of *SL* for all fish in the population, L_o is the *SL* of the fish, b is the slope (allometric coefficient) of regression of $logM$ on $logL_o$ calculated for all populations. Correlation coefficients between adjusted size-independent variables and *SL* were calculated to confirm that the size effect was eliminated and a significant correlation was not found.

Before statistical analysis, for both raw and transformed (percentage ratios and size-adjusted) measurement data, a normal distribution was tested using the Shapiro-Wilk test (for univariate normality). Also, for the matrix of size-adjusted measurements, Doornik and Hansen omnibus test for multivariate normality was done.

Permutational ANOVA with 10,000 permutations followed by a post hoc pairwise test (alternatively, Kruskal-Wallis test followed by Mann-Whitney pairwise test when departure from normal distribution was found) was applied to determine differences in variances among three populations. In addition, principal component

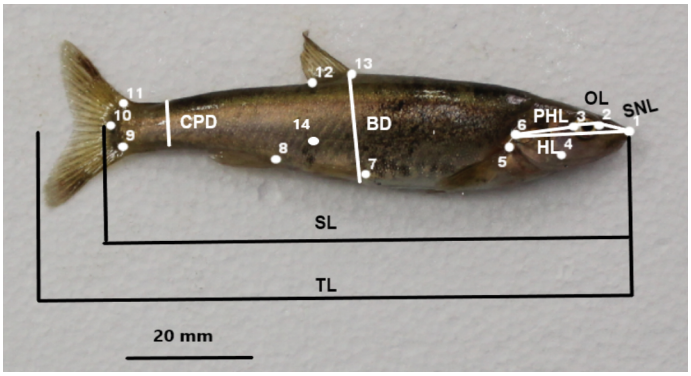


FIGURE 2 The position of eight linear measurements (*TL* – total length; *SL* – standard length; *OL* – orbit length; *SNL* – snout length; *PHL* – postorbital head length; *HL* – head length; *BD* – body depth; *CPD* – caudal-peduncle depth) and 13 landmarks (white dots) and one semilandmark (landmark 14) selected for morphometric analysis of *Delminichthys ghetaldii*. Landmark 14 was used only for the 'unbend specimens' procedure

analysis (PCA) on size-adjusted measurements was done. All statistical analysis on linear measures was performed with PAST 4.14 (Hammer et al., 2001).

Geometric morphometrics

In total, 13 homologous landmarks (fig. 2) were digitized on the images using tpsDig 2.30 (Rohlf, 2017): 1. snout tip; 2. most anterior point of the eye outline; 3. most posterior point of the eye outline; 4. posterior-ventral corner of the preopercula; 5. insertion of pectoral fin; 6. posterior-most edge of operculum; 7. anterior insertion of pelvic fin; 8. anterior point of anal fin; 9. ventral origin of caudal fin; 10. posterior point of lateral line; 11. dorsal origin of caudal fin; 12. posterior insertion of dorsal fin; 13. anterior insertion of the dorsal fin (fig. 2). To remove the effect of unnatural bending of specimens, landmark 14 was added and positioned on the lateral line halfway between landmarks 6 and 10. We applied the unbending procedure implemented in tpsUtil 1.74 (Rohlf, 2017), and four landmarks were aligned (1, 6, 10, 14). Landmark (14) was removed from the following analysis. Procrustes fit procedure was used for superimposing landmarks' coordinates (Dryden & Mardia, 1998; Klingenberg & McIntyre, 1998), and information about shape variables (Procrustes coordinates) was obtained. Before the unbending procedure was applied, centroid size (size variable; CS) was also calculated.

To examine body size differences among defined groups (populations), permutational ANOVA (10,000 permutations) followed by a post hoc pairwise test was performed on CS. Principal Component Analysis (PCA) was performed to explore shape variation within and between populations. Furthermore, to determine differences in mean body shape between a priori-defined groups (populations), Procrustes distances were calculated and compared by permutation test with

1,000 iterations. The matrix of shape variables (Procrustes coordinates) was subjected to Discriminant Function Analysis (DFA) to calculate the percentages of correct classification using a leave-one-out cross-validation procedure (jackknifing). Finally, to estimate the extent of morphological variation of body shape, Procrustes distances between pairs of individuals and Procrustes variance (Zelditch et al., 2012) were calculated for each population and compared between populations with a permutation test with 1,000 iterations.

To test for the presence of allometry (the relationship between size and shape), a multivariate regression of shape variables (Procrustes coordinates) against size (CS) was conducted for each population separately and overall sample size (on pooled by population within-group variation) as well. A permutation test with 10,000 iterations was used to check the significance of the allometry. Since allometry was significant, shape analysis was repeated using residuals from regression (size-independent shape variables).

Geometric morphometric and statistical analyses were performed using MorphoJ 1.08 (Klingenberg, 2011), PAST 4.14 (Hammer et al., 2001), and DisparityBox8i (Sheets, 2007) software.

Results

Phylogenetic analysis and evolutionary history of the genus Delminichthys *based on cyt b mtDNA and* COI *mtDNA*

The analysis included 410 *cyt* b mtDNA sequences (999 bp long) of four *Delminichthys* species and one sequence of *Pelasgus minutus* (outgroup), of which 382 were retrieved from GenBank and 29 were *de novo* sequenced (*D. ghetaldii*) (supplementary table S1). A total of 74 haplotypes from 341 sequences of *D. adspersus* were detected, 18 haplotypes from 52 of *D. ghetaldii*, five from 10 of *D. krbavensis* and two haplotypes from seven of *D. jadovensis* sequences (fig. 3, supplementary table S3). One *D. ghetaldii* haplotype was taken into account but is not visible in the haplotype network (fig. 3) due to the small length of sequences (OR916341). The lowest mean value of pairwise mutational steps was detected between *D. ghetaldii* and *D. adspersus* 17.86 (ranging from 14 to 24) and between *D. jadovensis* and *D. krbavensis* 20.90 (19–23). The highest mean values of mutational steps were observed between *D. jadovensis* and *D. adspersus* (39.56; 37–44) (supplementary table S4). The *p* distances calculated between haplotypes of the *Delminichthys* species pairs are reported in table 2; *p* distance values ranged from 1.50% to 4.66% with the highest values between haplotypes of *D. jadovensis* / *D. ghetaldii* (4.66%), while the smallest values were found among *D. adspersus* / *D. ghetaldii* (1.50%) and *D. jadovensis* / *D. krbavensis* (1.90%) species pairs, which *p* distance ranges also overlapped. As well, overlapping *p* distance range values were observed for *D. jadovensis* / *D. ghetaldii*, *D. jadovensis* / *D. adspersus*, *D. krbavensis* / *D. ghetaldii* and *D. krbavensis* / *D. adspersus* species pairs (table 2).

The analysis of the *COI* mtDNA dataset included 41 sequences (619 bp long) of four *Delminichthys* species and one *Pelasgus minutus* (outgroup taxon), of which nine were retrieved from GenBank and 33 *de novo* sequenced (*D. ghetaldii*)

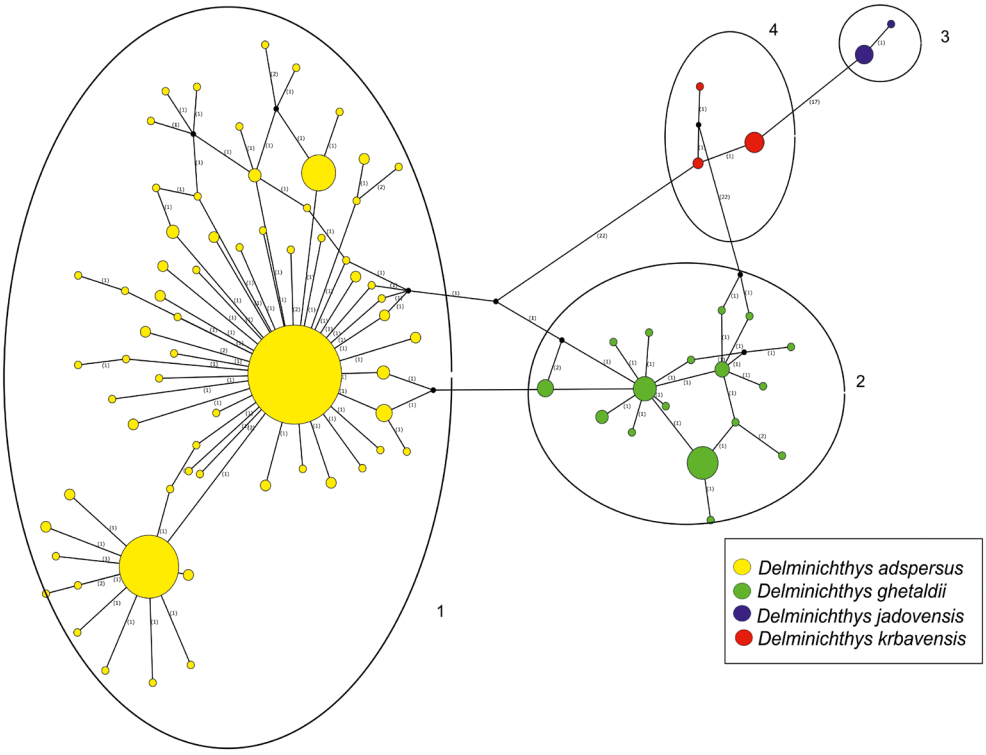


FIGURE 3 TCS network based on *cyt* b mtDNA sequences of four *Delminichthys* species. Haplotypes belonging to a specific species are labeled with numbers: (1) *Delminichthys adspersus*, (2) *D. ghetaldii*, (3) *D. jadovensis* and (4) *D. krbavensis*. Mutational steps are given in parentheses and the size of the circles corresponds to haplotype frequency in the total sample

(supplementary table S2). A total of two haplotypes from three *D. adspersus* specimens were detected, five haplotypes of *D. ghetaldi* (35 specimens), two of *D. krbavensis* (two specimens) and one haplotype of one *D. jadovenisis* specimen (fig. 4, supplementary table S3). The least mean value of mutational steps was detected between *D. jadovensis* and *D. krbavensis* (4.50; ranged from 4 to 5), while the highest was between *D. ghetaldii* and *D. jadovensis* (15.68; 15–18) (supplementary table S4). The *p* distances calculated among the *Delminichthys* species haplotypes ranged from 0.65% for *D. krbavensis* / *D. jadovensis* to 3.07% for *D. ghetaldii* / *D. adspersus* species pair (table 2). With the exception of *D. krbavensis* / *D. jadovensis*, all remaining species pairs had overlapping *p* distance ranges (2.10–3.07%; table 2).

Haplotype networks for both mtDNA markers (figs. 3 and 4) demonstrated a clear differentiation of four haplogroups corresponding to the *Delminichthys* species. More haplotypes were recorded within the *cyt* b mtDNA dataset compared to *CO1* mtDNA, as well as a greater number of mutational steps. The interspecific *p* distances based on *cyt* b mtDNA were also higher compared to the values obtained for *CO1* mtDNA dataset, with the exception of the *D. ghetaldii* / *D. adspersus* pair

TABLE 2 *p* distance values (%) based on mtDNA sequences of four *Delminichthys* species. The range (minimal-maximal) values are given above the diagonal, and mean distance values below the diagonal

Molecular marker	Species	*Delminichthys ghetaldii*	*Delminichthys adspersus*	*Delminichthys jadovensis*	*Delminichthys krbavensis*
cyt b mtDNA	*D. ghetaldii*		1.50–2.44	3.70–4.66	3.30–4.24
	D. adspersus	1.81		3.70–4.40	3.20–4.00
	D. jadovensis	3.94	3.96		1.90–2.30
	D. krbavensis	3.64	3.59	2.09	
COI mtDNA	*D. ghetaldii*		2.26–3.07	2.44–2.93	2.10–2.75
	D. adspersus	2.49		2.11–2.11	2.10–2.26
	D. jadovensis	2.55	2.11		0.65–0.81
	D. krbavensis	2.30	2.18	0.73	

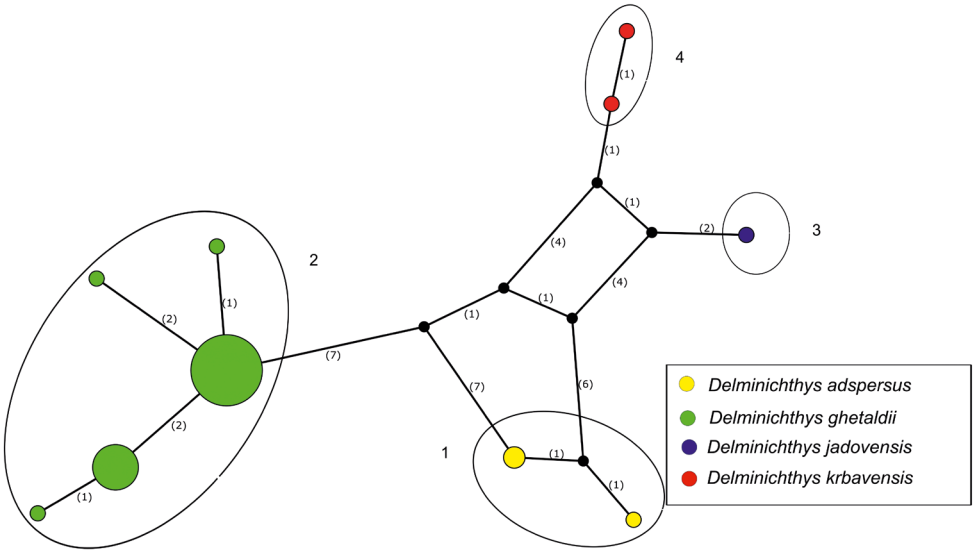


FIGURE 4 TCS network based on *COI* mtDNA sequences of four *Delminichthys* species. Haplotypes belonging to a specific species are labeled with numbers: (1) *Delminichthys adspersus*, (2) *D. ghetaldii*, (3) *D. jadovensis* and (4) *D. krbavensis*. Mutational steps are given in parentheses and the size of the circles corresponds to haplotype frequency in the total sample

(table 2). The lowest *p* distance value was detected for *COI* mtDNA of the *D. jadovensis* / *D. krbavensis* species pairs (table 2).

Concerning the overall *cyt* b mtDNA (411 sequences) and 42 *COI* mtDNA dataset of four *Delminichthys* species (*D. adspersus, D. ghetaldii, D. jadovensis* and *D. krbavensis*) and species *Pelasgus minutus* as an outgroup Bayesian inference (BI) trees were inffered (figs. 5 and 6). Considering

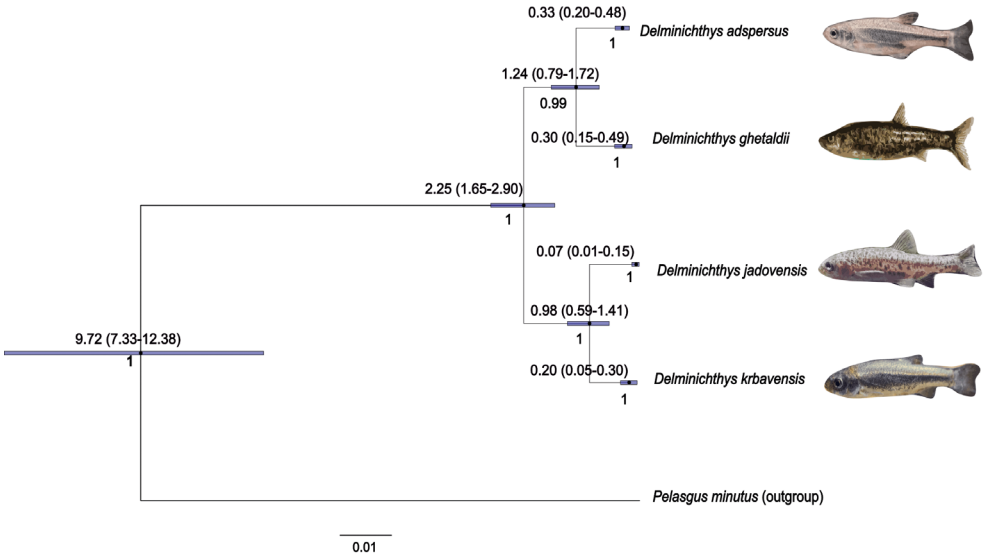


FIGURE 5 Divergence time estimations between and within four *Delminichthys* species based on Bayesian Inference of the phylogenetic tree for *cyt* b mtDNA sequences. Mean values and the 95% credibility range (in parentheses) are presented in a million years above the credibility range line. Values presented below the credibility range line represent posterior probability (PP). Fish images used, except *Delminichthys ghetaldii* (taken by ML), were modified from images attributed to AQUATICA (CC BY-NC 4.0), from FishBase

different mutation rates, the estimated divergence time implies that the split between *Delminichthys* and *Pelasgus* was earlier (ca. 9.72 MYA; Tortonian part of Miocene) according to *cyt* b mtDNA than according to *COI* mtDNA (ca. 4.14 MYA, Pliocene). Branching within *Delminichthys* was estimated to be early Pleistocene (2.26 MYA) by *cyt* b mtDNA and mid-Pleistocene (1.24 MYA) by *COI* mtDNA, which was consistent with different mutation rates. The topology of the phylogenetic trees differs in the position of *D. adspersus*, which represents the first clade separated from other *Delminichthys* species on the *COI* mtDNA tree (not well supported). Clade *D. adspersus* + *D. ghetaldii*, according to the *cyt* b mtDNA phylogenetic tree, diverged from *D. jadovensis* + *D. krbavensis* clade at the beginning of the Pleistocene (2.26 MYA) while the splitting of *D. adspersus* from *D. ghetaldii* was in the middle of the Pleistocene (1.24 MYA). Both phylogenetic trees showed a similar relationship between the sister species *D. jadovensis* and *D. krbavensis* whose divergence time corresponds to different mutation rate. Small support values were registered within *D. ghetaldii* lineage branching in the *cyt* b mtDNA tree, where the divergence time (0.30 MYA) was similar to those on the *COI* mtDNA tree (0.40 MYA) (figs. 5 and 6). However, a supported phylogenetic structure was observed within *D. ghetaldii* clade on the *COI* mtDNA tree, where the haplotypes formed two distinct sublineages (one includes Ljubomirsko Polje and Konavosko Polje with good support and other Dabarsko Polje and Fatničko Polje with low support) (fig. 6).

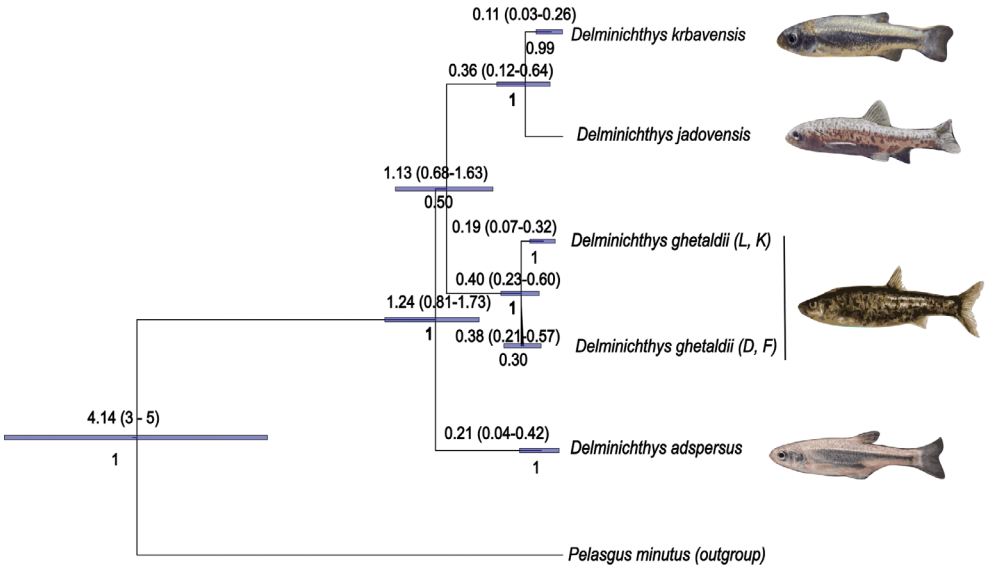


FIGURE 6 Bayesian Inference phylogenetic tree based on *COI* mtDNA sequences of four *Delminichthys* species. Mean values and the 95% credibility range (above the line) are presented in a million years. Values presented below the credibility range line represent posterior probability (PP). Individuals of *Delminichthys ghetaldii* were collected from: Ljubomirsko Polje (L); Dabarsko Polje (D); Fatničko Polje (F); Konavosko Polje (K). Fish images used, except *D. ghetaldii* (taken by ML), were modified from images attributed to AQUATICA (CC BY-NC 4.0), from FishBase

Population genetic structure of **Delminichthys ghetaldii**

A total of 18 *cyt* b mtDNA haplotypes (999 bp long; HI–HXVIII) were found among the 51 analyzed *Delminichthys ghetaldii* individuals (supplementary table S1). There were 21 variable positions defining *cyt* b mtDNA haplotypes, diverging up to seven substitutions from each other (supplementary table S5). Ten haplotypes were found in Dabarsko Polje (HI–HIX and HXIII), eight in Fatničko Polje (HI, HIII, HX–HXIV, HXVII), two in Ljubomirsko Polje (HXV and HXVI) and two in Konavosko Polje (HXV and HXVIII). Unique haplotypes had 15 specimens from the studied populations (Fatničko Polje: HX–HXII, HXIV, HXVII; Dabarsko Polje: HII, HIV–HIX; Ljubomirsko Polje: HXVI; Konavosko Polje: HXVII), while haplotype XV was shared by 17 specimens from Ljubomirsko Polje and Konavosko Polje and common haplotypes HI, HIII and HXIII by 19 individuals from Dabarsko Polje and Fatničko Polje (fig. 7, supplementary table S1). Intraspecific sequence divergences (uncorrected *p* distance) ranged from 0.00% to 0.74%. Minimal and maximal differences were detected between haplotypes that belong to the same as well as different fields. For example, the maximum value (0.74%) was observed for HIII and HX (both from Fatničko Polje), but it was also found for HIV (Dabarsko Polje) and HX (Fatničko Polje) (table 3, supplementary table S5).

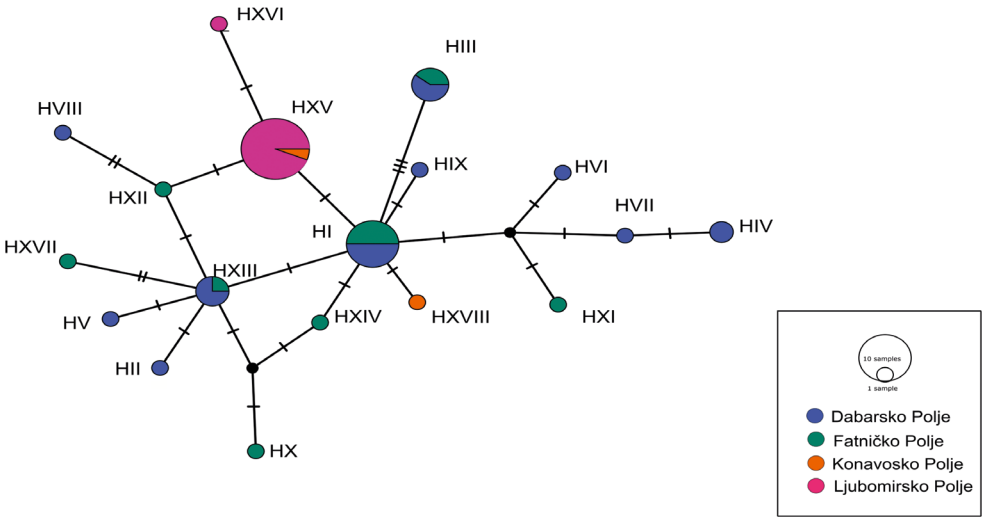


FIGURE 7 Median-joining network based on *cyt* b mtDNA sequences of *Delminichthys ghetaldii*. Mutational steps are given as hatch marks and the size of the circles corresponds to haplotype frequency in the total sample. Haplotypes are marked with Roman numerals

TABLE 3 *p* distance (%) based on mtDNA sequences of three *Delminichthys ghetaldii* populations; minimal and maximal values are given above the diagonal, mean values below the diagonal, and minimal-maximal and mean values (in parentheses) within populations on the diagonal

Molecular marker	Population	Dabarsko Polje	Fatničko Polje	Konavosko Polje	Ljubomirsko Polje
cyt b mtDNA	Dabarsko Polje	0.00–0.70 (0.31)	0.00–0.74	0.10–0.50	0.10–0.50
	Fatničko Polje	0.40	0.00–0.73 (0.39)	0.10–0.53	0.10–0.64
	Konavosko Polje	0.31	0.32	0.20–0.20 (0.20)	0.00–0.30
	Ljubomirsko Polje	0.35	0.34	0.15	0.00–0.10 (0.01)
COI mtDNA	Dabarsko Polje	0.00–0.00 (0.00)	0.00–0.32	0.48–0.48	0.32–0.34
	Fatničko Polje	0.04	0.00–0.49 (0.09)	0.48–0.81	0.32–0.68
	Konavosko Polje	0.48	0.53	0.00–0.00 (0.00)	0.16–0.17
	Ljubomirsko Polje	0.32	0.37	0.16	0.00–0.00 (0.00)

A total of five *co1* mtDNA haplotypes (619 bp long; A–E) among 34 *D. ghetaldii* individuals were detected (supplementary table S2). There were six variable positions detected in *co1* mtDNA haplotypes, diverging up to five nucleotides from each other (supplementary table S6). Three haplotypes (A–C) were found in Fatničko Polje and one in each Dabarsko Polje (A), Ljubomirsko Polje (D) and Konavosko Polje (E). Haplotype A was shared by 22 specimens from Fatničko Polje and Dabarsko Polje, while two private haplotypes were registered in Fatničko Polje (B and C), one in Ljubomirsko Polje (D) and one in Konavosko Polje (E) (supplementary fig. S1, supplementary table S2). The lowest value of *p* distance (0.16%) was detected between haplotypes A (registered in Dabarsko Polje and Fatničko Polje) and C (Fatničko Polje) and D (Ljubomirsko Polje) compared to E (Konavosko Polje), while the highest distance (0.81%) was calculated between haplotypes B (Fatničko Polje) and E (Konavosko Polje) (supplementary table S6).

AMOVA on the *cyt* b mtDNA dataset of *D. ghetaldii* showed higher variation within the population (76.0%) than among populations (24.0%) (table 4). In contrast to *cyt* b mtDNA, AMOVA on the *co1* mtDNA dataset revealed that 11.6% of the variation was attributed to differences within populations, while a higher proportion of the variation (88.4%) was partitioned among populations (table 4).

The Ljubomirsko Polje population had the lowest values of population variability parameters based on *cyt* b mtDNA sequences, in contrast to the high values of Dabarsko Polje, Fatničko Polje and Konavosko Polje, where Dabarsko Polje had the highest number of haplotypes ($H = 10$) and Fatničko Polje of other parameters ($h = 0.919$, $\pi = 0.00313$, $k = 2.95$) (supplementary table S7).

The population genetic variability parameters obtained for the *co1* mtDNA dataset of the overall *D. ghetaldii* sample had lower values for all diversity parameters ($h = 0.524$, $\pi = 0.00185$, $k = 1.09$) compared to the values inferred for *cyt* b mtDNA dataset ($h = 0.845$, $\pi = 0.00259$, $k = 2.27$) (supplementary table S7). Concerning each population separately, based on the *co1* mtDNA data, the most variable is

TABLE 4 AMOVA results for *cyt* b mtDNA and *co1* mtDNA datasets of *Delminichthys ghetaldii* populations (Dabarsko Polje, Fatničko Polje, Ljubomirsko Polje, Konavosko Polje). $^{*}P < 0.05$

mtDNA marker	Source of variation	Degrees of freedom	Sum of squares	Variance components	% of variation	F_{ST}
cyt b	Among population	3	13.656	0.313	24.04	0.240*
	Within population	46	45.444	0.988	75.96	
	Total	49	59.100	1.301		
co1	Among population	3	16.244	0.671	88.40	0.884*
	Within population	31	2.727	0.088	11.60	
	Total	34	18.971	0.759		

Fatničko Polje in terms of the number of retrieved haplotypes (H = 3) and haplotype diversity (h = 0.346), while Dabarsko Polje and Ljubomirsko consist only one haplotype (supplementary table S7).

Determining the number of genetic clusters within the *D. ghetaldii* performed in GENELAND (individual-based analyses) was conducted separately on the *cyt* b mtDNA and *COI* mtDNA datasets. Analysis of the *cyt* b mtDNA data showed no structuring (K = 1; data not shown). However, based on *COI* mtDNA data, two genetic clusters (K = 2) were recognized; populations from Dabarsko Polje and Fatničko Polje belong to one cluster, while Ljubomirsko Polje and Konavosko Polje to the second one (supplementary fig. S2). In addition, non-spatial analysis performed in STRUCTURE supports the results from GENELAND (for more details, see supplementary material).

Population phenotypic structure of **Delminichthys ghetaldii**

Linear morphometric traits

Specimens of *D. ghetaldii* collected from three karst fields ranged from 32.5 to 124.9 mm in standard length; mean standard length values were 46.4 mm for Dabarsko Polje, 109.2 mm for Fatničko Polje, and 83.0 mm for Ljubomirsko Polje (table 5). Among the analyzed samples, Dabarsko Polje was distinguished by the lowest values recorded for all adjusted measures and three ratio measures (for results regarding raw measures, see supplementary material). In contrast, Fatničko Polje was characterized by the highest values and/or the highest mean values of adjusted measures, and $PHL(\%HL)$, $BD(\%SL)$ and $CPD(\%SL)$ as well (table 5). Individuals from the Dabarsko Polje and Fatničko Polje differ in all adjusted measurements, while individuals from Fatničko Polje and Ljubomirsko Polje overlap in some of them (OL_{adj} and SNL_{adj}). By comparing the values of the measurements between the samples from Dabarsko Polje and Ljubomirsko Polje, it was found that the ranges of all adjusted measures do not overlap. The ranges of ratio measures greatly overlap between all three populations (table 5).

To assess differences between populations (Fatničko Polje, Dabarsko Polje and Ljubomirsko Polje), the ANOVA/Kruskal Wallis test was applied to transformed measurements (size-adjusted measurements and percentage ratios of measurements). Statistically significant differences between group means were found ($P < 0.05$; table 5); pairwise comparison revealed significant differences between all population pairs for all adjusted measurements and three ratio measures. Pairwise test showed a lack of significant differences between Ljubomirsko Polje and Fatničko Polje in $SNL(\%SL)$, $HL(\%SL)$, $BD(\%SL)$ and $CPD(\%SL)$, and between Dabarsko Polje and Fatničko Polje in $SNL(\%HL)$ (table 5).

PCA revealed that the majority of the variation in the set of size-independent measures (adjusted measures) was explained with the first principal component (PC1 97.35%, PC2 2.09%). The scatterplot for the first two PC axes showed a clear separation of all the three populations along PC1 (fig. 8). The measures with the highest loading on PC1 were BD_{adj} (0.67), HL_{adj} (0.59) and PHL_{adj} (0.34). Based on the total variance within each population, the population from Fatničko

TABLE 5 Population mean, minimal and maximal values of the linear morphometric traits of *Delminichthys ghetaldii* from three Karst fields.

Linear measures	Dabarsko Polje		Fatničko Polje		Ljubomirsko Polje		ANOVA[a]	pairwise comparison[a]
	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD		
SL (mm)[a]	32.5–80.9	46.4 ± 12.7	94.2–24.9	109.2 ± 9.5	67.8–115.9	83.0 ± 12.4	***	all pairs
OL(%*SL*)[a]	5.6–8.9	7.4 ± 0.8	4.0–6.2	5.2 ± 0.6	5.1–6.6	5.8 ± 0.4	***	all pairs
OL(%*HL*)	22.6–30.3	27.4 ± 2.3	17.1–23.6	20.5± 1.8	21.3–27.1	23.8 ± 1.8	***	all pairs
SNL(%*SL*)	5.7–8.5	7.3 ± 0. 7	5.2–7.6	6.6 ± 0.7	5.8–8.0	6.9 ± 0.5	**	F/D, L/D
SNL(%*HL*)	23.4–33.5	27.0 ± 2.3	19.7–30.6	26.2 ± 2.6	25.3–31.8	28.3 ± 1.5	*	L/F, L/D
PHL(%*HL*)	44.0–58.8	49.0 ± 3.2	50.6–57.8	54.1 ± 2.1	48.7–54.3	51.4 ± 1.8	***	all pairs
HL(%*SL*)	24.1–30.1	26.9 ± 1.7	23.2–27.4	25.2 ± 1.5	20.1–27.0	24.3 ± 1.5	***	F/D, L/D
BD(%*SL*)[a]	14.8–21.5	17.3 ± 1. 6	18.3–26.2	22.7 ± 2.1	20.0–26.3	21.7 ± 1.4	***	F/D, L/D
CPD(%*SL*)	6.63–10.58	8.0 ± 0.8	7.7–10.6	9.1 ± 0.8	8.9–10.5	9.5 ± 0.4	***	F/D, L/D
OL_{adj}[a]	3.0–3.7	3.4 ± 0.2	4.4–6.7	5.6 ± 0.6	4.3–5.4	4. 8 ± 0.3	***	all pairs
SNL_{adj}[a]	2.9–3.9	3.4 ± 0.3	5.7–7.9	7.2 ± 0.7	5.0–6.9	5.7 ± 0.4	***	all pairs
PHL_{adj}[a]	5.5–7.1	6.1 ± 0.4	13.7–16.6	14.9 ± 1.1	8.5–12.2	10.4 ± 0.8	***	all pairs
HL_{adj}[a]	11.3–13.5	12.4 ± 0.6	25.4–29.9	27.6 ± 1.5	16. 9–22.5	20.2 ± 1.2	***	all pairs
BD_{adj}[a]	7.4–9.3	8.1 ± 0.4	21.2–29.5	24.8 ± 2.1	16.5–20.4	18.1 ± 1.0	***	all pairs
CPD_{adj}[a]	3.2–4.3	3.7 ± 0.3	9.0–11.9	9.9 ± 0.8	7.4–8.7	7.9 ± 0.4	***	all pairs

SL – standard length, *OL* – orbit length, *SNL* – snout length, *PHL* – postorbital head length, *HL* – head length, *BD* – body depth, *CPD* – caudal-peduncle depth; *SD* – standard deviation; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; D – Dabarsko Polje; F – Fatničko Polje; L – Ljubomirsko Polje; [a]Measurements that deviate from normal distribution were tested using the non-parametric ANOVA (Kruskal-Wallis test) and Mann-Whitney pairwise post hoc test.

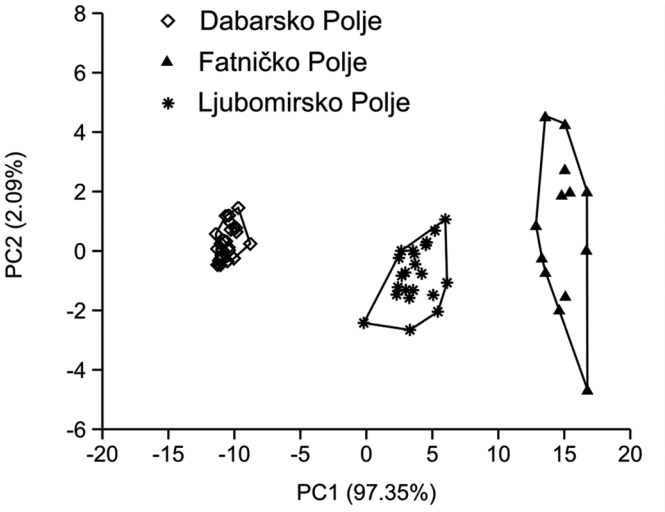


FIGURE 8 Principal component analysis of the size-independent adjusted measurements of *Delminichthys ghetaldii* populations. The percentage of explained variance of each PC is in parentheses

Polje has the greatest morphological variance (9.4672), followed by Ljubomirsko Polje (3.5498) and Dabarsko Polje (0.9187).

Body size and shape variation

Permutational ANOVA on centroid size (CS) showed statistically significant differences between populations (F = 133.5; P < 0.001). Pairwise comparisons revealed a statistically significant difference in mean CS for all pairs of populations (P < 0.001). The highest CS was detected in Fatničko Polje (143.3 ± 11.8 mm) and the smallest in Dabarsko Polje (60.3 ± 17.0 mm), with intermediate values in Ljubomirsko Polje (108.7 ± 16.0 mm). Principal component analysis (PCA) was applied to explore the body shape variation between all studied populations. PCA plot of the studied populations along the first axis showed overlapping of specimens from Fatničko Polje and Ljubomirsko Polje, and the partial separation of a large number of individuals from Dabarsko Polje (fig. 9). PCA reveals that the PC1 axis describes 47.48% and PC2 15.70% of the total variance. More than 2/3 of the total variability was explained by the first three PC axes (72.90%). Along PC1, shape differences occur in body depth (landmarks 7, 8, 12 and 13), head length (1, 3, 5 and 6) and caudal fin area (9, 10 and 11), while along PC2 in the head region (1, 5 and 6) and the dorsal fin area (12 and 13) (fig. 9). Statistically significant Procrustes distances (P < 0.001) for all of compared population pairs were found (supplementary table S8). The percentage of correct classification was 77.1%; the confusion matrix is presented in supplementary table S9. Within-population variance analysis showed that Procrustes distance among the most divergent specimens is found among specimens from Fatničko Polje (0.0012), then in Dabarsko Polje and

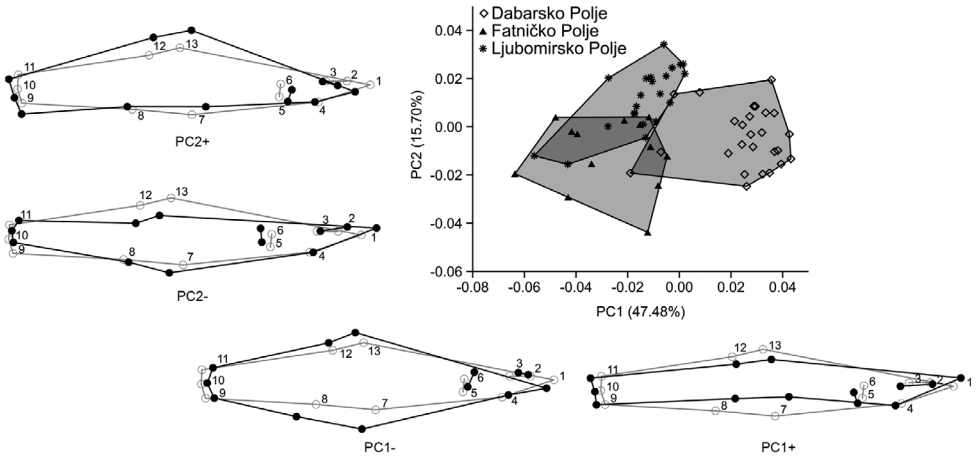


FIGURE 9 Principal component analysis based on body shape variables of *Delminichthys ghetaldii* individuals. The percentage of explained variance is in parenthesis. The wireframe graphs represent body shape changes (black lines) from consensus configuration of landmarks (gray lines) along first two PC axes, and numbers refer to landmarks shown in fig. 2.

Ljubomirsko Polje (0.0009) (supplementary table S10). However, Procrustes variances did not differ significantly among populations (supplementary table S11).

Multivariate regression of shape variables on centroid size showed significant allometry in populations from Dabarsko Polje (21.78%, $P < 0.001$) and Ljubomirsko Polje (8.74%, $P = 0.04$), but not from Fatničko Polje (10.25%, $P = 0.38$). Statisticaly significant allometry that accounted for 6.67% of the overall shape variation ($P = 0.001$) was also found when multivariate regression was conducted on overall sample pooled within-group variation (pooled by population).

As allometry was found to explain a significant fraction of the phenotypic variance, it was further considered in subsequent analyses. PCA after removal of the allometric effect reveals that the PC1 axes showed 25.63% and PC2 20.09% of the total variance. The first five-PC axis encompasses 74.00% of the total shape variance. PCA plot indicates an overlap (to a lesser or greater degree) between all three populations (supplementary fig. S3). Body shape variation is associated with changes in body depth (landmarks 7, 8, 12 and 13) and length (9 and 11), as well as the head length (1 and 6) (supplementary fig. S3). Based on size-corrected shape variables, Procrustes distances ($P < 0.001$) among all population pairs were statistically significant (supplementary table S8). The percentage of correct classification was 72.1%; the confusion matrix is presented in supplementary table S12. Within-population variance analysis based on residuals from multivariate regression showed that the most divergent specimens were in Fatničko Polje (0.0014), then in the remaining two populations (supplementary table S10). Procrustes variances of populations were statistically significant between Dabarsko Polje and Fatničko Polje populations ($P < 0.0001$) and Fatničko Polje and Ljubomirsko Polje ($P < 0.05$) (supplementary table S11).

The correlation analysis between the matrices of *p* distances based on *co1* mtDNA and Procrustes distances among *D. ghetaldii* populations was performed using Mantel's test and showed no statistical significance before ($R = -0.73$, $P = 0.84$) and after the removal of the allometric effect ($R = -0.91$, $P = 0.84$). Likewise, Mantel's test did not display a significant correlation between *p* distances based on *cyt* b mtDNA and morphometric distances between populations before ($R = 0.99$, $P = 0.17$) and after the removal of allometric effect ($R = 0.90$, $P = 0.17$).

Discussion

Comparative phylogenetic relationships and evolutionary history of the genus Delminichthys

In this study, our first aim addressed questions of interspecies variation and phylogenetic relationships among four species of the endemic *Delminichthys* genus based on time-calibrated phylogenetic analysis of *cyt* b mtDNA and *co1* mtDNA genes. A comparative study of molecular variation revealed four haplogroups for both genes corresponding to species *D. adspersus, D. ghetaldii, D. jadovensis* and *D. krbavensis*, while a different number of haplotypes were detected at *cyt* b mtDNA (*D. adspersus*: 74 haplotypes out of 341 specimens; *D. ghetaldii*: 18/52; *D. krbavensis*: 5/10; *D. jadovensis*: 2/7) and *co1* mtDNA (*D. adspersus*: 2 out of 3 specimens; *D. ghetaldii*: 5/34; *D. krbavensis*: 2/2; *D. jadovensis*: 1/1). Noteworthy, the interspecific variation of *cyt* b mtDNA was found to be at least 50% higher than *co1* mtDNA variation between the closely related species *D. krbavensis* / *D. jadovensis* (*p* distance: 2.09% vs 0.73%) as well as the distantly related species *D. adspersus* / *D. krbavensis* (3.59% vs 2.18%), *D. adspersus* / *D. jadovensis* (3.96% vs 2.11%), *D. ghetaldii* / *D. krbavensis* (3.64% vs 2.30%) and *D. ghetaldii* / *D. jadovensis* (3.94% vs 2.55%), which further supports the already suggested different rates of mitochondrial genes evolution in European *Phoxinus* (Palandačić et al., 2017), Dinaric *Telestes* species (Francuski et al., 2019) and *Aulopyge huegelii* (Ludoški et al., 2021). In contrast, *co1* mtDNA variation was found to be greater than *cyt* b mtDNA between *D. adspersus* and *D. ghetaldii* (*p* distance: 1.81% vs 2.49%), which accordingly affects the topology of the phylogenetic tree (see later). Hence, species-specific haplogroups revealed differentiated *Delminichthys* lineages that (again) support both *cyt* mtDNA (e.g., Ketmaier et al., 2004; Freyhof et al., 2006; Marková et al., 2010; Perea et al., 2010; Marčić et al., 2011; Palandačić et al., 2015, 2017; Buj et al., 2017, 2019, 2020; Vucić et al., 2018; Rodriguez et al., 2021; Reier et al., 2022; Lukač et al., 2023) and *co1* mtDNA genes (Geiger et al., 2014; Perea et al., 2010; Palandačić et al., 2017, 2020; Francuski et al., 2019) as suitable barcoding markers for the delimitation of distantly related species, recently diverged species and cryptic evolutionary clades of the Balkan cyprinoidei fishes. However, Geiger et al. (2014) found that species *D. ghetaldii, D. jadovensis* and *D. krbavensis*, as well as *Telestes dabar* and *T. metohiensis*, and several other species pairs share the same *co1* mtDNA haplotypes (Geiger et al., 2014: Suppl. C) suggesting limited taxonomic value of barcoding fragments in the case of closely related freshwater fishes in the

Mediterranean region. Still, an integrative approach of broadly-ranged populations of the *Delminichthys* species (Perea et al., 2010; results of this study) and *T. dabar* and *T. metohiensis* (Francuski et al., 2019) confirmed the *CO1* mtDNA as a suitable marker for the biodiversity assessment of the Balkan freshwater ichthyofauna.

Besides the lack of common haplotypes among species, a clear *p* distance-based barcoding gap (the largest intraspecies *p* distance is lower than the smallest interspecies haplotype *p* distance according to Hebert et al., 2003) was observed within *D. ghetaldii* (max *p* distance for *cyt* b mtDNA: 0.74%; *CO1* mtDNA: 0.81%) and between closely related species pairs *D. adspersus / D. ghetaldii* (min *p* distance for *cyt* b mtDNA: 1.50%; *CO1* mtDNA: 2.26%) and distantly related species pairs *D. ghetaldii* / *D. krbavensis* (min *p* distance for *cyt* b mtDNA: 3.30%; *CO1* mtDNA: 2.10%) and *D. ghetaldii* / *D. jadovensis* (min *p* distance for *cyt* b mtDNA: 3.70%; *CO1* mtDNA: 2.44%), which is congruent with the *cyt* b mtDNA data according to Reier et al. (2022) (min *p* distance *D. adspersus* vs *D. ghetaldii*: 1.8%; *D. krbavensis* vs *D. jadovensis*: 2.1%; *D. adspersus/ D. ghetaldii* vs *D. krbavensis* / *D. jadovensis*: 4%). Since *cyt* b mtDNA and *CO1* mtDNA have been suggested as suitable barcoding markers (references herein), we additionally proposed that both character-based (= based on discrete nucleotide substitutions or character state) and genetic distance threshold-based delimitation of species are appropriate taxonomic approaches for assessing the biodiversity of *Delminichthys* lineages.

In addition to the diagnostic utility of the markers and methods used in accounting for the endangered Balkan ichthyofauna biodiversity, we tested the phylogenetic hypothesis of the *Delminichthys* species by contrasting time-calibrated *cyt* b mtDNA and *CO1* mtDNA phylogenies. In contrast to the *cyt* b mtDNA time-calibrated tree that had good support for all the species nodes (*PP* > 0.99), the *CO1* mtDNA time-calibrated tree showed low support for the split of the *D. ghetaldii* lineage and *D. krbavensis* + *D. jadovensis* clade (*PP* = 0.50). Thus, due to better phylogenetic resolution and good node support, we consider that *cyt* b mtDNA time-calibrated tree is more reliable for inferring interspecific relationships within the *Delminichthys* genus than the *CO1* mtDNA phylogeny. Predictably, we observed that the molecular variation is in concordance with the time-calibrated *cyt* b mtDNA phylogenetic tree, meaning that the species *D. krbavensis* and *D. jadovensis* are clustered together against phylogenetically distant species pair *D. adspersus* and *D. ghetaldii*. Our finding regarding the closest relationship between *D. krbavensis* and *D. jadovensis* is in line with the previously obtained *cyt* b mtDNA phylogenetic tree (Freyhof et al., 2006; Perea et al., 2010; Reier et al., 2022). When *cyt* b mtDNA (Freyhof et al., 2006; Reier et al., 2022; this study) and *CO1* mtDNA (Perea et al., 2010; this study) phylogenetic trees are considered, there is partial incongruence in their topology (especially in the case of *D. adspersus* and *D. ghetaldii*) as a result of their both different diversity and level of evolutionary changes. Moreover,

compared to mtDNA phylogenetic trees, a multilocus nDNA (*Rho, ITS1*, EGR2B) phylogenetic tree showed discrepancies in relationships among the *Delminichthys* species (Palandačić et al., 2010), although some species were missing from phylogenetic studies (Perea et al., 2010: *D. jadovensis* for *CO1* mtDNA and nDNA; Rodriguez et al., 2021: *D. krbavensis* and *D. jadovensis* for nDNA and *cyt* b mtDNA). The observed conflict between nDNA and mtDNA phylogenetic trees is not surprising given differences in their inheritance modes (biparental nDNA vs uniparental mtDNA), mutational rate and/or the effects of incomplete lineage sorting and introgression among nuclear genes, for instance (e.g., for review see Funk & Omland, 2003; Palandačić et al., 2010; Choleva et al., 2014; Rodriguez et al., 2021). Considering that previous phylogenetic analyses were based solely on *cyt* b mtDNA (Freyhof et al., 2006; Reier et al., 2022) or nDNA genes (Palandačić et al., 2010), or included only certain *Delminichthys* clades (Perea et al., 2010 for *CO1* mtDNA and nDNA markers; Rodriguez et al., 2021), our comparative study of *cyt* b mtDNA and *CO1* mtDNA phylogenies of the *Delminichthys* genus provides a basis for a comprehensive multilocus assessment of evolutionary relatedness of the freshwater ichthyofauna from the Dinaric Karst.

The diversification of lineages of the family Leuciscidae provides an evolutionary footprint of multiple vicariance, dispersion and recolonization that the species experienced during the complex paleoclimatological, geomorphological and hydrogeological history of the Mediterranean region (e.g., Levy et al., 2009; Perea et al., 2010; Dubut et al., 2012; Buj et al., 2017; Rodriguez et al., 2021; Reier et al., 2022). According to the Miocene refuge hypothesis of Dalmatian karst habitats (Freyhof et al., 2006; Reier et al., 2022), the extensive Dinaric orogenesis (Reed et al., 2004) promoted diversification within leuciscin cyprinids during the Early (ca. 15 MYA) and Middle (ca. 10 MYA) Miocene, while the genus *Delminichthys* split from the *Squalius* + *Telestes* + *Phoxinellus* clade around 10 MYA (Zardoya & Doadrio, 1998; Freyhof et al., 2006; Perea et al., 2010; Lukač et al., 2023) and from its sister genus *Pelasgus* as well (Perea et al., 2010; this study; but see Rodriguez et al., 2021). In line with previous studies of evolutionary history of the genus *Delminichthys* (Palandačić et al., 2010; Perea et al., 2010; Rodriguez et al., 2021; Reier et al., 2022), our findings also suggested that climatic oscillations at the end of Pliocene through the late Pleistocene (2.58–0.12 MYA) promoted further diversification within the *Delminichthys* genus. Indeed, according to the phylogeographic scenario refugia within refugia (Gomez & Lunt, 2006), the Neretva watershed as an unglaciated region during the Pleistocene promoted the speciation of freshwater species and thus high regional endemism (Oikonomou et al., 2014). According to Reier et al. (2022) allopatric speciation occurred within the Neretva drainages, and it started with the split between the left-bank *D. ghetaldii* and the rest of right-bank *Delminichthys* species, followed by vicariance of *D. adspersus* from the ancestor who dispersed into the Una drainage (through Danube

River belongs to the Black Sea basin) and ended with the youngest allopatric species *D. jadovensis* and *D. krbavensis*.

Population genetic structure corresponds to evolutionary relatedness and biogeographical history of **Delminichthys ghetaldii**

In this study, we used a comparative approach to reveal the *cyt* b mtDNA and *co1* mtDNA diversity among populations from Dabarsko Polje (Suški Stream, Pribitu Spring, Kanal), Fatničko Polje (Fatničko Lake), Ljubomirsko Polje (Ljubomirski Stream) and Konavosko Polje (Stravča Spring) with the aim to understand historical and contemporary evolutionary processes and mechanisms underlying the population genetic structure of *D. ghetaldii*. Negligible interpopulation difference and genetic structure was observed at the *cyt* b mtDNA gene in spite of private haplotypes registered in Dabarsko Polje (in the sample of 19 specimens out of 10 haplotypes 7 were unique), Fatničko Polje (13/ 8 /5), Ljubomirsko Polje (17 / 2 /1) and Konavosko Polje (2 / 2 /1). Indeed, using both individual-based (spatial analyses in GENELAND) and population-based (AMOVA) approaches we detected the genetic connectedness of the analyzed populations representing a single genetic cluster. On the contrary, *co1* mtDNA, as a slowly evolving gene, expressed low variation considering that the populations from Dabarsko Polje and Ljubomirsko Polje were found to be monomorphic, while the population from Fatničko Polje possessed three haplotypes, two of which are private. Moreover, population-based analyses of genetic structure (AMOVA) indicated significant differences among populations of *D. ghetaldii*, while F_{ST} was 0.21 in the case of *cyt* b mtDNA compared to 0.88 inferred for *co1* mtDNA. Likewise, the individual-based Bayesian clustering method (GENELAND) using *co1* mtDNA data clearly supported that Dabarsko Polje + Fatničko Polje and Ljubomirsko Polje + Konavosko Polje formed two non-overlapping genetic clusters. Thus, the spatial distribution of *cyt* b mtDNA could not be registered apart using this faster evolving marker in diagnosing species borders, while the *co1* mtDNA barcoding fragment as a slowly evolving marker could, however, is of informative value for understanding the evolutionary footprint of historical evolutionary processes. Noteworthy, the highly polymorphic (both mtDNA markers) population from Fatničko Polje, which possessed unique haplotypes (five in *cyt* b mtDNA and two in *co1* mtDNA), is connected with another polymorphic population (based on *cyt* b mtDNA marker) that inhabits Dabarsko Polje.

Therefore, the *co1* mtDNA genealogical partitions distinguished the populations belonging to the left-bank Neretva drainage basin (Fatničko Polje and Dabarsko Polje) from the hydrogeologically and genetically discrete entity that includes populations from Ljubomirsko Polje and Konavosko Polje belonging to the Trebišnjica drainage system and Ljuta River tributaries, respectively. Although karst fields might be permanently or temporarily connected by surface water flows (karst springs, streams and rivers) and an underground drainage system, Ljubomirsko Polje is a quiet, isolated field which is mainly connected with the right bank of Trebišnjica River via underground

karst channels, especially in periods of low groundwater levels. The Trebišnjica River crosses the vast karst plain of the Trebinje Forest and Popovo Polje *sensu stricto* (both belong to the Popovo Polje, "the most famous karst field in the world" according to Milanović, 2023: 8) through the underground karst channels further feeds the Ombla Spring, which continues towards the submerged river valley- the Dubrovnik River, and finally discharges into the Adriatic Sea near Dubrovnik city. Together with the population from Ljubomirsko Polje, the sample from Konavosko Polje, which is restricted to underground karst channels and small springs closed to Brotnice, Stravča and Duba Konavoska that form temporary stream Kopačica, a branch of temporary Ljuta Stream (according to PU 6109, 2023: Jelić et al., 2008; Zupančič, 2008, 2012; Bogutskaya et al., 2012), belong to the distinct *CO1* mtDNA cluster in our study. Along the Adriatic seacoast, hydrogeologically isolated from the Neretva drainage system but close to the Trebišnjica drainage basin, is situated Konavosko Polje (Konavli), whose hydrogeographic network consists of the Ljuta River and Konavočica and Kopačica tributaries. Although Konavoska Ljuta is considered a seepage spring, part of the water discharges into the Adriatic Sea through an artificial canal for irrigation at the Pasjača inlet (Milanović, 2023). Still, there is a lack of data on whether these tributaries of Trebišnjica River and Konavoska Ljuta are interconnected when great fluctuations in their water levels occur (N. Lovrić, pers. comm.). In this study, another *CO1* mtDNA cluster consists of two populations originating from Dabarsko Polje and Fatničko Polje. Hydrogeologically, both karst fields (Dabarsko Polje and Fatničko Polje) belong to the Neretva drainage system, which includes complex springs, streams, rivers and watersheds, as well as a subterranean network. Along the reverse fault, both fields were mainly created from Tertiary sediments, while Cretaceous limestone was also present. Besides the permanent springs Vrijeka (considered the most important spring) and Pribitu, Dabarsko Polje also includes the temporary spring Sušica, one of the several smaller springs. Given that summer is a dry period, almost all the waters of the Opačica (the most significant river that causes the flooding of Dabarsko Polje), Vrijeka and Pribitu flow through the underground system to the springs of the Bregava River; thus, hydrologically, Dabarsko Polje belongs to the Bregava watershed and Neretva drainage basin. However, part of the water from the Dabarsko Polje has been artificially re-routed into the Trebišnjica watershed with the construction of the Hydroelectric Power Plant on Trebišnjica (the first phase began in 1959). Consequently, the Dabarsko Polje is naturally connected to the Neretva basin via the Bregava watershed and to the Trebišnjica drainage system via the artificial channel linked with the Bileća accumulation. Likewise, a part of the water of Fatničko Polje (10–15%) naturally flows underground toward Bregava Spring and through the Bregava watershed discharges into the Neretva drainage system. Moreover, Dabarsko Polje and Fatničko Polje are artificially connected by the Dabar – Fatnica tunnel (length of 3.24 km) constructed inside the limestone ridge Ljut between them, while the water from the Fatničko Polje further flows toward the Bileća reservoir via the Fatnica – Bileća

tunnel, which passes through karstified Cretaceous limestone. Noteworthy, Fatničko Polje has been considered the most complex hydrogeological and hydrological system in the karst of the Dinarides and, as such, is of exceptional importance for water management and hydropower regulation in East Herzegovina (Milanović, 2023). Accordingly, the complex belowground and underground water systems of Dabarsko Polje and Fatničko Polje naturally and artificially belong to the Neretva and Trebišnjica basins, respectively.

Therefore, the spatial aspects of population *co1* mtDNA genealogical structure and the higher genetic diversity at both the *cyt* b mtDNA and *co1* mtDNA genes (in the case of Fatničko Polje) in populations from Dabarsko Polje and Fatničko Polje that belong to the Neretva drainage system suggesting shared historical biogeographical influences. This is indeed supported by the distribution of shared *cyt* b mtDNA haplotypes HI, HIII and HXIII between the populations from Fatničko Polje and Dabarsko Polje, as well as the common *cyt* b mtDNA haplotype HXV in the populations from Ljubomirsko Polje and Konavosko Polje. Likewise, the majority of specimens from Fatničko Polje and Dabarsko Polje shared *co1* mtDNA haplotype A, while solely haplotypes E and D were found in Ljubomirsko Polje and Konavosko Polje, respectively. Indeed, findings regarding the intraspecific genetic structure of *D. ghetaldii* are compatible with the conclusion of Reier et al. (2022) and data in the current study related to the time-calibrated phylogenetic tree of the genus *Delminichthys* highlighting the Neretva drainage basin as a regional biodiversity hotspot. We consider that the populations from Fatničko Polje and Dabarsko Polje acted as a source or evolutionary core population (according to Avise, 2000) of south-eastward migrants into Ljubomirsko Polje and Konavosko Polje. Given the tree-calibrated phylogeny, the dispersal of *D. ghetaldii* followed by allopatric isolation due to climatic change and fragmentation occurred during the Pleistocene (ca. 0.30 and 0.40 MYA based on *cyt* b mtDNA and *co1* mtDNA trees, respectively).

Phenotypic heterogeneity does not correspond to evolutionary relatedness and biogeographical history of Delminichthys ghetaldii

In this study, we tested linear morphometric and landmark-based geometric morphometric approaches in estimating the phenotypic population structure of *D. ghetaldii*. With respect to our second goal, a consistent pattern of phenotypic heterogeneity was observed for both linear measurements, and centroid size and body shape variation. It was found that the population from Dabarsko Polje differs significantly from the population from Fatničko Polje, while the population from Ljubomirsko Polje overlapped in all traits and mainly all traits (except adjusted measurements) with the populations from Fatničko Polje and Dabarsko Polje, respectively. Geometric morphometrics detected sophisticated variation as a statistically significant difference in mean centroid size and Procrustes distances (i.e., shape differences) was detected between populations from Fatničko Polje and Ljubomirsko Polje. Noteworthy, the

population from Dabarsko Polje is characterized by the smallest values of all linear measurements (with the exception of a few ratio measures) and centroid size, as well as the smallest intrapopulation variation obtained for both adjusted measures and body shape. Contrary to that, the population from Fatničko Polje was distinguished by the highest mean values of size measures (including all raw and adjusted linear measures, some ratio measures, and centroid size) but also by the highest variance using both adjusted measures and shape data. Interpopulation body shape differences were associated with the variation in landmarks that defined body depth, head length and caudal fin area.

Besides the fact that no morphological variation supported the distinctiveness of the spatially isolated population from Ljubomirsko Polje in relation to hydrogeographically interconnected Fatničko Polje and Dabarsko Polje, we found that phenotypic heterogeneity did not correspond to evolutionary relatedness (obtained by molecular data) and biogeographical history as well. It seems reasonable that the evolutionary footprint of common hydrogeographic history, pattern of dispersion and vicariance, and mechanisms operating among fishes from permanent and temporary (mostly underground) connected streams, watersheds, tributaries and fields is integrated into genetic population structure and evolutionary history of *D. ghetaldii*, while morphological heterogeneity could be attributed to environmental variables and habitat variation. It should be taken into account that fishes in our study originated from different sampling seasons, and, thus, year-on-year variation of environmental variables (supplementary table S13) and available food (data not available) may influence variation in morphologically optimized phenotypes, which has already been suggested (e.g., Treer et al., 2008; Mihinjač, 2018). Our results related to phenotypic plasticity are complementary to the findings of studies of European minnows and several other European freshwater fishes (e.g., *Phoxinus*: Collin & Fumagalli, 2011, 2015; Ramler et al., 2017; *Delminichthys*: Mustafić et al., 2017; *Telestes dabar*: Francuski et al., 2019; *Aulopyge huegelii*: Ludoški et al., 2021; *Salmo trutta*: Zavorka et al., 2020; *Sarmarutilus rubilio*: Petrosino et al., 2022). For instance, the sensitivity of body size to environmental conditions and size-related shape variation (allometry) that significantly affects body shape has been observed in cyprinids (Bravi et al., 2013; Rossi et al., 2016; Ramler et al., 2017; Lukač et al., 2023). Indeed, a significant allometry revealed in this study (6.67%) could also suggest population-specific adaptations and plastic responses of *D. ghetaldii*. To conclude, although a phylogenetic signal of body shape variation of some endemic cyprinoids has been reported (Lukač et al., 2023), our study did not support the hypothesis of correlation between morphological heterogeneity, and evolutionary relatedness and biogeographical history of *D. ghetaldii*.

Summary: Conservation implication

The unique Dinaric Karst region of the Balkan Peninsula harbours extensive freshwater biodiversity and a remarkable

number of endemic species facilitated by cycles of vicariance and dispersal occurring at the end of Pliocene to mid-Pleistocene (references herein). Stenoendemic cyprinids of the genus *Delminichthys* contribute to the notable freshwater fish biodiversity of the regional hotspot Dinaric Karst that belongs to the south-west Balkan corridor of the central Peri-Mediterranean fish fauna biogeographical region in Europe (Reyjol et al., 2007). Considering the Miocene refuge hypothesis, the genus *Delminichthys* was trapped in the Dinaric karst region during the Alpine orogenesis (Freyhof et al., 2006), while the allopatric isolation of four evolutionary lineages (defined based on *cyt* b mtDNA and *co1* mtDNA sequences) corresponding to *D. adspersus, D. ghetaldii, D. jadovensis* and *D. krbavensis* occurred at the end of Pliocene through the late Pleistocene (Freyhof et al., 2006; Reier et al., 2022; results herein). Our findings confirmed that the Pliocene and Pleistocene refugia for freshwater species were the Neretva drainage basin ("the eight-named river" according to Bonacci et al., 2013: 963), promoting diversification of the genus *Delminichthys* as it has been already suggested for other cyprinoids genera (e.g., Gilles et al., 2010; Dubut et al., 2012; Francuski et al., 2019; Buj et al., 2020; Reier et al., 2022). Therefore, we would like to strongly encourage long-term sustainable conservation management planning for the threatened species richness of the Dinaric karst by prioritizing the Neretva tributary as a key biodiversity hotspot and a distinct biogeographical region (Oikonomou et al., 2014).

This study of the population structure of *D. ghetaldii* implemented an integrative approach based on the complementary use of multilocus genetic data (*cyt* b mtDNA and *co1* mtDNA), traditional (linear measurements) and geometric morphometrics (centroid size and body shape). We consider that the spatial aspects of the population genealogical structure of *D. ghetaldii* mirror the Pleistocene paleodrainages, while the differentiated *co1* mtDNA clusters corresponding to the populations from Fatničko Polje + Dabarsko Polje and Ljubomirsko Polje + Konavosko Polje represent geographically discrete genetic entities. The conservation implication of findings in this study concerns genetic uniqueness, indicating the presence of two distinct evolutionary lineages that should be addressed through conservation management as Evolutionarily Significant Units (ESU) or Management Units (MU) (according to Ryder, 1986; Moritz, 1994; Fraser & Bernatchez, 2001).

Moreover, apart from the historical outcomes, contemporary activities might strongly affect the vulnerable heterogeneous karst region of complex interconnectedness of aboveground and underground hydrological networks. For instance, Trebišnjica watershed has already been hugely modified by the creation of the Hydroelectric Power Plant on Trebišnjica (first phase began in 1959), which consists of seven hydroelectric power plants with six reservoirs, six dams, six tunnels with a total length of 59.7 km, several access tunnels, concrete 62.5 km long canal through Popovo Polje, the canal through Dabarsko Polje (6.75 km) and Fatničko Polje (2.77 km), tunnel-canal system towards Dubrava, and the Bileća Reservoir (Milanović, 2023). Consequently, water flows have been significantly altered; for instance, the Fatnica – Bileća

tunnel connects the temporarily flooded Fatničko Polje and the Bileća Reservoir, while the Dabar – Fatnica tunnel transfers water from the Dabarsko Polje to the Fatničko Polje and from the Bregava River catchment area into the Trebišnjica River catchment area (Milanović, 2023). In addition, the construction of facilities for the Hydroelectric Power Plant on Trebišnjica released the flood in large part of Popovo Polje, while the Trebišnjica riverbed was concreted (1978), which therefore closed many estavelles and great numbers of estavelles along the edge of Popovo Polje were disconnected from the river course, interrupting the life cycle of the *D. ghetaldii* fishes (Lučić, 2012; Milanović, 2023). Populations of *D. ghetaldii* have already disappeared from many locations and are threatened by habitat loss due to pollution, drying, or channelization of karstic watercourses, and the introduction of alien invasive species (Crivelli, 2006). For instance, over the past few years, it has been detected an increase in the *Phoxinus* population, while *Delminichthys* is decreasing (Reier et al., 2022) or not detected at all in the Trebišnjica River (pers. obs. by M. Lukač and R. Dekić).

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Author Contributions

ML: conceptualization, sample collection and preliminary species identification, data curation, formal analysis and investigation, writing – original draft, writing – review and editing. JL: conceptualization, data curation, formal analysis and investigation, writing – original draft, writing – review and editing. RD: sample collection and preliminary species identification, writing – review and editing, funding acquisition. VM: conceptualization, data curation, writing – original draft, writing – review and editing, funding acquisition, supervision. All authors read and approved the final manuscript.

Conflict of Interest

The authors have no conflict of interest to declare.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request. GenBank accession numbers for *cyt* b mtDNA and *co1* mtDNA gene sequences obtained in this study are OR916321-OR916382.

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Supplementary material

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