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Evolution in the Dinarids: Phylogeography, Diversity and Evolutionary History of the Endemic Genus Delminichthys (Actinopteri; Leuciscidae)

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ABSTRACT: The origin of exceptionally rich fish communities harboured within the freshwater systems of southern Europe is usually explained by allopatric speciation due to a long isolation of water basins. On the other hand, hybridization events have been recorded in several fish species, but they role in the speciation of freshwater fishes in the Southern Europe has not received significant attention. Contrary to most species within the Leuciscidae family, the genus *Delminichthys* inhabits a geographically restricted area (middle and southern Dinarides) and consists of only four endemic species. This study analysed the population genetic structure and demographic history of each *Delminichthys* species as a contribution to the understanding of the evolutionary peculiarities in Dinaric water systems. The obtained results revealed pronounced mito-nuclear and nuclear-nuclear discordance, likely the result of incomplete lineage sorting, as well as nuclear introgression observed in the Ombla River population in southernmost Croatia. In addition to allopatric speciation, ancient hybridization might have played an important role in the evolutionary history of this genus. The origin of the genus *Delminichthys* can be dated back to the Oligocene/Miocene boundary, to a period of significant tectonic activity in the Mediterranean region, and its ancestor likely inhabited the region of the central Dinarides. Intrageneric divergences occurred in the lower Miocene and Pliocene. Similarly, as previously proposed for *Delminichthys adspersus*, traces of underground migrations were found among *Delminichthys ghetaldii* populations, implying adaptations to underground life to be characteristic for the genus. All *Delminichthys* species express high levels of genetic diversity, likely as a consequence of their old origin. Size of *D. adspersus* is currently decreasing, while the remaining three species appear stable.

Keywords: Dinaric freshwater systems; Evolutionary history; Genetic composition; Genus Delminichthys; Nuclear introgression; Underground migration

1. Introduction

The freshwater systems of the Dinaric karst region maintain exceptionally rich fish communities with a high portion of endemic species [1]. Several events and phenomena have been proposed as possible explanation for such diversity [2–5]: geographic isolation of rivers and their underground connections, geological history, ice-age refugia and specific mutation rates. Allopatric speciation, as a consequence of long-term isolation of water basins, has been considered the most important divergence mode in southern Europe (e.g., [6]), including the Dinaric karst region [5]. Evidence of parapatric speciation is very rare (e.g., [6]) and disputed, while hybridization events, although locally recorded (e.g., [7]) were not considered as important for the speciation of freshwater fishes in this area. Nevertheless, for two cyprinid species from the Iberian Peninsula, *Iberocypris alburnoides* (Steindachner, 1866) and *I. palaciosi* Doadrio, 1980, hybridization profoundly affected their evolutionary histories [8]. Hybridizations and mixed genetic material in a single population, originating from two species, was also found in the *Squalius svallize* Heckel & Kner, 1858 population from southern Croatia [9]. Furthermore, several cases of mitochondrial introgressions have been

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reported among cyprinid fishes in southern Europe (e.g., [9–11]). However, data on the evolutionary history and recent genetic composition of species distributed in the Dinaric freshwaters remain scarce.

The genus *Delminichthys* Freyhof, Lieckfeldt, Bogutskaya, Pitra & Ludwig, 2006 is endemic to the freshwater systems of the Dinaric karst in Croatia and Bosnia and Herzegovina. Its closest relative, the genus *Pelasgus* Kottelat & Freyhof, 2007 [3], is distributed in the southern Balkans, primarily in Greece with one species in Lake Ohrid [8]. Unlike most genera within the Leuciscidae family, *Delminichthys* inhabits a very restricted geographic area [12]. It consists of only four endemic species: *D. krbavensis* (Zupančič & Bogutskaya, 2002), restricted to the Krbavsko karstic field in Croatia; *D. jadovensis* (Zupančič & Bogutskaya, 2002), inhabiting the small Jadova River in Croatia; *D. adspersus* (Heckel, 1843), found in the Imotski Field, Matica River (in the Jezero Field), Norin River, Kuti and Baćinska Lakes in Croatia, as well as in the Trebižat River and Krenica Lake in Bosnia and Herzegovina; and *D. ghetaldii* (Steindachner, 1882), found in the Popovo, Ljubomirsko, Dabarsko and Fatničko karstic fields in Bosnia and Herzegovina, and in the Ombla River in Croatia. All four *Delminichthys* species are endemic species with very restricted distribution ranges and all four species are considered endangered. *Delminichthys jadovensis* and *D. krbavensis* are considered critically endangered (CR), based on the IUCN Red List, whereas *D. adspersus* and *D. ghetaldii* are listed as vulnerable species (VU).

Though *Delminichthys* represents an ideal model system for investigating the evolution in the karstic river systems of the Dinarides, it has not yet been comprehensively studied. Ref. [3] suggested that the *Delminichthys*/*Pelasgus* lineage is one of the basal lineages inside Leuciscidae, and its origin was estimated to around 14 MYA (million years ago). Ref. [12] also recognized *Delminichthys* as the remnant of the first great Leuciscidae radiation. Ref. [4] investigated the population genetic structure of *D. adspersus* and found evidence of its underground migrations. The lack of the stygobiontic characters was explained by the limited time this species annually spends in the underground environment. On the other hand, ref. [3] reported the presence of reductive characters in both *Delminichthys* and *Pelasgus*.

With an aim of contributing to the understanding of evolutionary peculiarities in Dinaric water systems, this study examines the population genetic structure and demographic history of each *Delminichthys* species. Considering that recent genetic structure and polymorphism bear witness to the past geology and evolutionary history of a certain species, we wanted to look for evolutionary responses and adaptations to specific conditions of karstic freshwaters. Another goal was to further investigate underground migrations in the genus *Delminichthys*. Namely, if adaptations to underground life are characteristic to genus, this should be visible in the intraspecific structure of *D. ghetaldii* (another species occurring at more than one locality), and perhaps also in the genetic polymorphism of *D. jadovensis* and *D. krbavensis.* The two latter species are located in an area that was affected by glaciations led to bottleneck effects in previously investigated freshwater fishes [13].

2. Materials and Methods

Samples of all four *Delminichthys* species were collected (Table 1, Figure 1) and used for phylogenetic and population genetic analyses. A total of five molecular markers were analysed: mitochondrial cytochrome *b* (cyt *b*) and control region (CR), and nuclear recombination activating gene 1 (RAG1), gene for interphotoreceptor retinoid binding protein (IRBP) and gene for beta-actin (BA). All but CR are protein coding genes and CR is a non-coding DNA with function in RNA and DNA synthesis.

Total genomic DNA was extracted from fresh and deep-frozen fin tissue using a standard extraction product (DNeasy tissue kit, Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) amplifications were performed in a 50 µL reaction volume containing 25 µL AmpliTaq Gold Master Mix (Applied Biosystems, Waltham, MA, USA), 2 µL of each primer and 4 µL of template DNA. Amplification protocols and primers, as well as sequencing primers are presented in the Table 2. Sequencing was carried out by Macrogen Europe Service Centre (Amsterdam, The Netherlands).

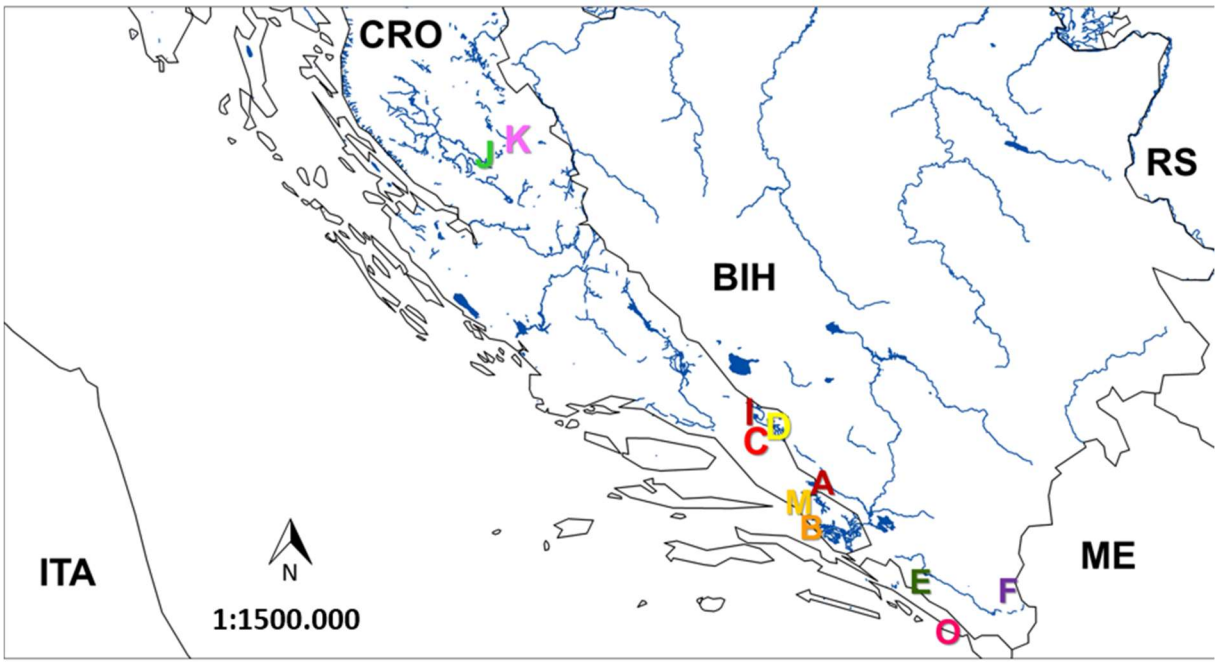

Figure 1. Map of the study area indicating the distribution ranges of *Delminichthys* species and sampling localities. J—Jadova River, K—Krbavsko Field, D—Vrljika River, I—Prološko blato Lake (in the Imotsko Field), C—Sija, A—Rastočko Field, M—Matica River (in the Jezero Field), B—Baćinska Lakes, E—Popovo Field, F—Bilećko Lake, O—Ombla R. Abbreviations: CRO—Croatia, BIH—Bosnia and Herzeovina, ME—Montenegro; RS—Republic of Serbia, ITA—Italy.

Table 1. Number of samples from each locality used for analyses, as well as haplotype codes and GenBank accession numbers. Country codes: CRO—Croatia, BIH—Bosnia and Herzegovina.

Species	Locality (Country Code)	No. of Sequences	Haplotypes	Accession Numbers
		cyt *b*		
D. krbavensis	Krbavsko Field (CRO)	14	KRB1–9	PQ329256-298
D. jadovensis	Jadova River (CRO)	14	JAD1–5	
	Baćinska Lakes (CRO)	4	ADS1, ADS10	
	Matica River (CRO)	18	ADS1–7	
	Vrljika River (CRO)	11	ADS1, ADS12–15	
D. adspersus	Prološko blato Lake (CRO)	1	ADS8	
	Rastočko Field (CRO)	1	ADS1	
	Sija (CRO)	1	ADS9	
	Popovo Field (BIH)	1	ADS11	
	Bilećko Lake (BIH)	10	GHE1–9	
D. ghetaldii	Ravno Field (BIH)	1	GHE13	
	Ombla River (CRO)	9	GHE10–14	
		CR		
D. krbavensis	Krbavsko Field (CRO)	11	CRKRB1–4	PQ329299-329316
D. jadovensis	Jadova River (CRO)	15	CRJAD1, 2	
D. adspersus	Baćinska Lakes (CRO)	4	CRADS1–3	
	Matica River (CRO)	14	CRADS2, 4–6	
	Vrljika River (CRO)	11	CRADS4	
D. ghetaldii	Bilećko Lake (BIH)	7	CRGHE1–5	
	Popovo Field (BIH)	1	CRGHE1	
	Ombla River (CRO)	8	CRGHE1, 6	
		RAG1		
D. krbavensis	Krbavsko Field (CRO)	24	RAGKRB1–3	PQ341976-342000
D. jadovensis	Jadova River (CRO)	20	RAGJAD1–4	
	Baćinska Lakes (CRO)	6	RAGADS6, RAGJAD1	
D. adspersus	Matica River (CRO)	34	RAGADS1, 7–10, RAGJAD1	
	Vrljika River (CRO)	8	RAGADS2–5, RAGJAD1	
	Prološko blato Lake (CRO)	2	RAGJAD1	
D. ghetaldii	Bilećko Lake (BIH)	10	RAGGHE8, RAGJAD1	
	Ombla River (CRO)	16	RAGGHE1–7	
		IRBP		

D. krbavensis	Krbavsko Field (CRO)	24	IrbpKRB1, 2	PQ356685-708
D. jadovensis	Jadova River (CRO)	2	IrbpJAD1	
	Baćinska Lakes (CRO)	6	IrbpADS1, 2	
	Matica River (CRO)	24	IrbpADS1, 5–12	
D. adspersus	Vrljika River (CRO)	6	IrbpADS1, 3, 4,	
	Prološko blato Lake (CRO)	2	IrbpADS1	
	Rastočko Field (CRO)	4	IrbpADS1	
	Bilećko Lake (BIH)	8	IrbpGHE2–7, IrbpJAD1	
D. ghetaldii	Popovo Field (BIH)	2	IrbpGHE1, 2	
	Ombla River (CRO)	4	IrbpGHE8, IrbpJAD1	
		BA		
D. krbavensis	Krbavsko Field (CRO)	20	BAKRB1–4	PQ356709-729
D. jadovensis	Jadova River (CRO)	24	BAJAD1, 2	
	Baćinska Lakes (CRO)	10	BAADS4, 9, 10, BAJAD1	
D. adspersus	Matica River (CRO)	34	BAADS4, 6–8, BAJAD1	
	Vrljika River (CRO)	12	BAADS1–4, BAJAD1	
	Prološko blato Lake (CRO)	2	BAADS5, BAJAD1	
D. ghetaldii	Bilećko Lake (BIH)	10	BAGHE1–3, BAADS5, BAJAD1	
	Ombla River (CRO)	6	BAGHE4, 5, BAJAD1	

Table 2. PCR protocols, amplification and sequencing primers used to amplify and sequence for investigated genetic markers.

Genetic Marker	PCR Protocol	PCR Primers	Sequencing Primers
cyt *b*	10 min at 95 °C; 30 cycles of 1 min at 94 °C, 30 s at 55 °C and 1 min at 72 °C; 7 min at 72 °C	L15267 & H16526 [14]	L15267 & H16526 [14]
CR	10 min at 95 °C; 40 cycles of 1 min at 92 °C, 1 min at 50 °C and 90 s at 72 °C; 10 min at 72 °C	LN20: ACCACT AGCACCCAAAGCTA HN20: GTGTTA TGCTTTAGTTAAGC	LN20: ACCACT AGCACCCAAAGCTA HN20: GTGTTA TGCTTTAGTTAAGC
RAG1	10 min at 95 °C; 5 cycles of 40 s at 94 °C, 1 min at 60 °C and 2 min at 72 °C; 35 cycles of 30 s at 95 °C, 1 min at 56 °C and 2 min at 72 °C	RAG1F & RAG9R [3]	RAG3F, RAG1F [3] & internal primer RAG6R (5′ATGGCTTTCCGCTC TGCTAC 3′)
IRBP	10 min at 95 °C; 35 cycles of 30 s at 94 °C, 40 s at 55 °C and 90 s at 72 °C; 7 min at 72 °C	IrbpF: AACTACTGC TCRCCACAAAARC IrbpR: GGAAATGCA TAGTTGTCTGGAA [15]	IrbpF: AACTACTGC TCRCCACAAAARC IrbpR: GGAAATGCA TAGTTGTCTGGAA [15]
BA	10 min at 95 °C; 35 cycles of 40 s at 95 °C, 40 s at 55 °C and 65 s at 72 °C; 7 min at 72 °C	Bactfor: ATGGATGAT GAAATTGCCGC Bactrev: AGGATCTTC ATGAGGTAGTC [15]	Bactfor: ATGGATGAT GAAATTGCCGC Bactrev: AGGATCTTC ATGAGGTAGTC [15]

Homologous regions of the investigated genes were aligned manually. Chromatograms and alignments were checked visually and were found to contain no gaps or stop codons. Haplotype variants of nuclear genes in heterozygous individuals were reconstructed with the Bayesian statistical method implemented in PHASE 2.1 software [16,17]. To test whether all mutations were selectively neutral, statistical tests D* and F* (proposed by [18]) and that of Tajima [19] were conducted using DnaSP v5 [20].

Phylogenetic relationships of the genus *Delminichthys* were investigated using two methods of phylogenetic reconstruction: maximum parsimony (MP) and maximum likelihood (ML) implemented in PAUP (version 4.0b10; [21]). For MP analyses, a heuristic search mode with 100 replicates was used, with randomized input orders of taxa, and tree bisection-reconnection (TBR) branch-swapping with all codon sites and nucleotide substitutions types weighted equally. ML analyses were performed under the heuristic search option using the TBR branch-swapping algorithm. Branch support (BS) was assessed by nonparametric bootstrapping (1000 pseudoreplicates, ten additional sequence replicates). Each gene was analysed separately, due to differences in their phylogenetic performance (Table 3). Sequences of species belonging to other Leuciscidae lineages (as defined by [3]) were also included in the phylogenetic reconstruction with the aim of describing the position of the genus *Delminichthys* within the subfamily. List of included sequences with their GenBank accession numbers and references is presented in Supporting information (Table S1). Sequences of *Cyprinus carpio* were used as outgroups. Namely, since in the analyses we have also included

representatives of other Leuciscidae species, the rooting could be accomplished by a species that is outside Leuciscidae family, but inside Cypriniformes and for which sequences of all investigated genetic markers are available, so *Cyprinus carpio* was chosen as an outgroup.

Table 3. Length and phylogenetic performance of genetic markers.

Genetic Marker	Total Characters	Parsimony Informative Characters (in %)	Length of the Parsimony Tree	Consistency Index	Homoplasy Index	Retention Index
cyt *b*	1140	333 (29)	1156	0.5164	0.4836	0.7599
CR	320	174 (54)	586	0.7986	0.2014	0.6731
RAG1	1300	53 (4.1)	240	0.7958	0.2042	0.8717
IRBP	849	38 (4.48)	205	0.7317	0.2683	0.6821
BA	460	248 (54)	910	0.7681	0.2319	0.7736

Estimation of divergence times was conducted with the Bayesian MCMC coalescent method, using BEAST 1.7.0 software [22]. The rate homogeneity across phylogenetic lineages was assessed by the log-likelihood ratio test. The likelihood of phylogenetic trees (reconstructed by maximum likelihood approach using PAUP) with and without molecular clock enforcement were compared. Since they were the same in both cases, a strict molecular clock was applied. Branch rates were drawn from an uncorrelated log-normal distribution and a Yule speciation prior with random starting tree. The applied substitution model was HKY with Gamma site heterogeneity model. We used default prior distributions for kappa, frequencies and alpha, whereas substitution rate parameters were unlinked across codon positions. The number of MCMC steps (chain length) was three million. Molecular clock calibration was conducted based on the divergence rate of cyt *b* gene in Leuciscidae, of 0.4% per lineage per million years [3]. Since nuclear genes did not enable resolution of *Delminichthys* species, they were not used for divergence time estimation or for ancestral ranges reconstruction. Likewise, due to the lack of adequate data set for investigation of evolutionary history based on CR, this genetic marker was not included in the divergence times estimations.

In order to reconstruct ancestral geographic distributions, we performed Statistical dispersal-vicariance analysis implemented in the S-DIVA program [23]. This method reconstructs the ancestral distribution in a phylogeny by optimizing a three-dimensional cost matrix, in which extinctions and dispersals "cost" more than vicariance and determines statistical support for ancestral range reconstruction [23]. A total of 11 geographic localities inhabited by *Delminichthys* were denoted (concordant with the sampling sites shown in Figure 1). A set of trees used for ancestral distributions reconstruction was obtained by BI analysis of cyt *b* haplotypes. This, thereafter, resembles the maternal ancestral ranges, while further study is needed on more nuclear markers to describe ancestral ranges of paternal populations. Similarly as with divergence times estimations, this analysis was based on cyt *b* data set.

Intrapopulational and intraspecific genetic diversity was described using several measures of genetic polymorphism calculated using the DNAsp software [21]: number of haplotypes (h), number of polymorphic sites (S), haplotype diversity (Hd), average number of nucleotide differences (k), and nucleotide diversity (π).

In order to detect possible gene flow among populations of the same species (*D. adspersus* and *D. ghetaldii*, because those two species comprise more than one population) and to test the hypothesis of underground migrations, we examined the interactions among populations using maximum likelihood approach [24,25] implemented in MIGRATE 3.2.1 [26]. The computer programme MIGRATE calculates the profile likelihoods of the population parameters using genetic data. We determined the immigration rates between populations, as well as the number of migrants in one generation. Immigration rates (more specifically—mutation scaled immigration rates) are defined as a measure of how much more important immigrations are over mutations in bringing new variants into populations. In MIGRATE, this parameter is considered as a long-time average over the genealogy of the individuals in the sample [26]. Immigration rates were calculated both as mutation-scaled effective immigration rates and as the number of immigrants per generation. Due to low resolution of nuclear genes and their inability to clearly distinguish *Delminichthys* species, migration rates were calculated based only on the mitochondrial genetic markers (cyt *b* and CR).

Changes in past effective population sizes were analysed using Bayesian skyline plots (BSP), implemented in Beast 1.7.0. The BSP model was employed on the cyt *b* data set and it estimates the history of changes in effective population size from the variability among sampled haplotypes, assuming a mutation rate of 0.4%/MY. The BSP settings were the same as in the divergence time estimations with the tree prior set to Coalescent: Bayesian Skyline.

3. Results

A total of 84 cyt *b* sequences of a length of 1140 base pairs (bp), 71 CR sequences of a length of 320 bp, 120 RAG1 sequences (from 60 individuals) of a length of 1300 bp, 82 IRBP sequences (from 41 individuals) of a length of 849 bp and 118 BA sequences (from 59 individuals) of a length of a 460 bp were obtained. No stop codons, insertions or deletions were recorded; sequences of the same gene were of the same length in all *Delminichthys* species. Number of sequences was determined by the availability of samples in the field sampling campaigns.

Of the 60 samples sequenced for RAG1, 20 (33%) were heterozygous. The number of heterozygous individuals for IRBP gene was 23 (56%), whereas in the BA data set there were 30 heterozygous individuals (51%). The samples provided 43 unique cyt *b* haplotypes, 18 CR haplotypes, 25 RAG1 haplotypes, 23 IRBP haplotypes and 21 BA haplotypes. Neutrality tests conducted on all data sets suggested that generally all species are in mutation-drift equilibrium. Only exception was *D. adspersus* for which neutrality tests implied some deviation from mutation-drift equilibrium since calculated statistics (Fu and Li's D* and F* statistics, as well as Tajima's D) for its cyt *b* and BA data sets turned out to be statistically significant ($p < 0.05$), whereas statistics for CR and IRBP genetic markers implied mutation-drift equilibrium. The number and proportion of variable and parsimony informative characters was much higher in mitochondrial genetic markers than in nuclear genes, with the exception of BA (Table 3). Even though investigated BA data set provides high amount of variable and parsimony informative characters (comparable with mitochondrial markers), those characters are mostly present in other species included in the data set and not in *Delminichthys*, yielding phylogenies in which positions of *Delminichthys* haplotypes are not well resolved.

The phylogenetic reconstruction of cyt *b* sequences revealed clear resolution of the four investigated species (Figure 2) and corroborated the monophyletic origin of all *Delminichthys* species, which are most closely related to *Pelasgus prespensis* (Karaman, 1924). Based on MP phylogenetic reconstruction, two sublineages can be observed within the *Delminichthys* lineage, each comprised of two geographically more closely located species (where *D. adspersus* is a sister species to *D. ghetaldii*, while *D. jadovensis* and *D. krbavensis* form a second species pair). Strong support for the separation of two species pairs is suggested by the 100% bootstrap value. ML analysis, however, implied an earlier separation of *D. ghetaldii* and its distinct position. There is, however, no obvious intraspecific structuring that could be related to different geographic localities or pertinence to separate populations. Similar situation was observed in CR phylogenies (Figure 3), but with the exception of *D. ghetaldii* position. Three *Delminichthys* species, *D. adspersus*, *D. jadovensis* and *D. krbavensis* form separate lineages and are monophyletic, with *D. jadovensis* and *D. krbavensis* forming sister pair. Position of *D. ghetaldii* was not resolved well by CR. *Delminichthys ghetaldii* sequences form soft polytomy in the CR phylogenies, event though all of them clearly belong to *Delminichthys* genus (Figure 3).

The RAG1 gene tree, on the other hand, implied a polyphyletic origin of *Delminichthys* (Figure 4). Samples from all localities clustered together, with the exception of the Ombla River, as a sister lineage to *P. prespensis*. Haplotypes from the Ombla River (*D. ghetaldii*) were included into different lineage and appear to be more closely related to other Leuciscidae, in particular to *Telestes pleurobipunctatus* (Stephanidis, 1939), than to the remaining *Delminichthys*, including other *D. ghetaldii* populations. It is important to mention that 75% of the Ombla samples are heterozygous for RAG1, but both alleles of all samples clustered with *T. pleurobipunctatus* and not with the remaining *Delminichthys* samples. Sequences of the second investigated *D. ghetaldii* population (from Bilećko Lake) clustered with the remaining *Delminichthys* species and no structuring or species differentiation can be noted within that lineage. Moreover, the same haplotypes were found in samples belonging to different species.

The remaining nuclear genes (phylogenetic trees presented in the Supporting information, Figure S1) did not enable clear resolution of the position and relationships of four *Delminichthys* species inside the Leuciscidae phylogenetic tree. Yet they resulted in soft polytomies of *Delminichthys* haplotypes. Moreover, some of the haplotypes are present in more than a single species (Table 1). Nevertheless, these markers clearly pointed out monophyletic position of all *Delminichthys* species and no traces of hybridogenetic origin or introduction of DNA belonging to other species could be noticed from phylogenies based on IRBP and BA. Interestingly, in IRBP and BA data sets sharing of haplotypes among various species was noticed. In both data sets, a haplotype with the highest frequency (IrbpJAD1 and BAJAD1) was found in the Jadova River and in both *D. ghetaldii* populations (Bilećko Lake and Ombla River). BA most widespread haplotype (BAJAD1) was found besides in *D. jadovensis* and *D. ghetaldii*, also in *D. adspersus*.

Due to the observed situation, intraspecific structures, divergence time and ancestral ranges estimations were calculated based only on the mitochondrial data sets.

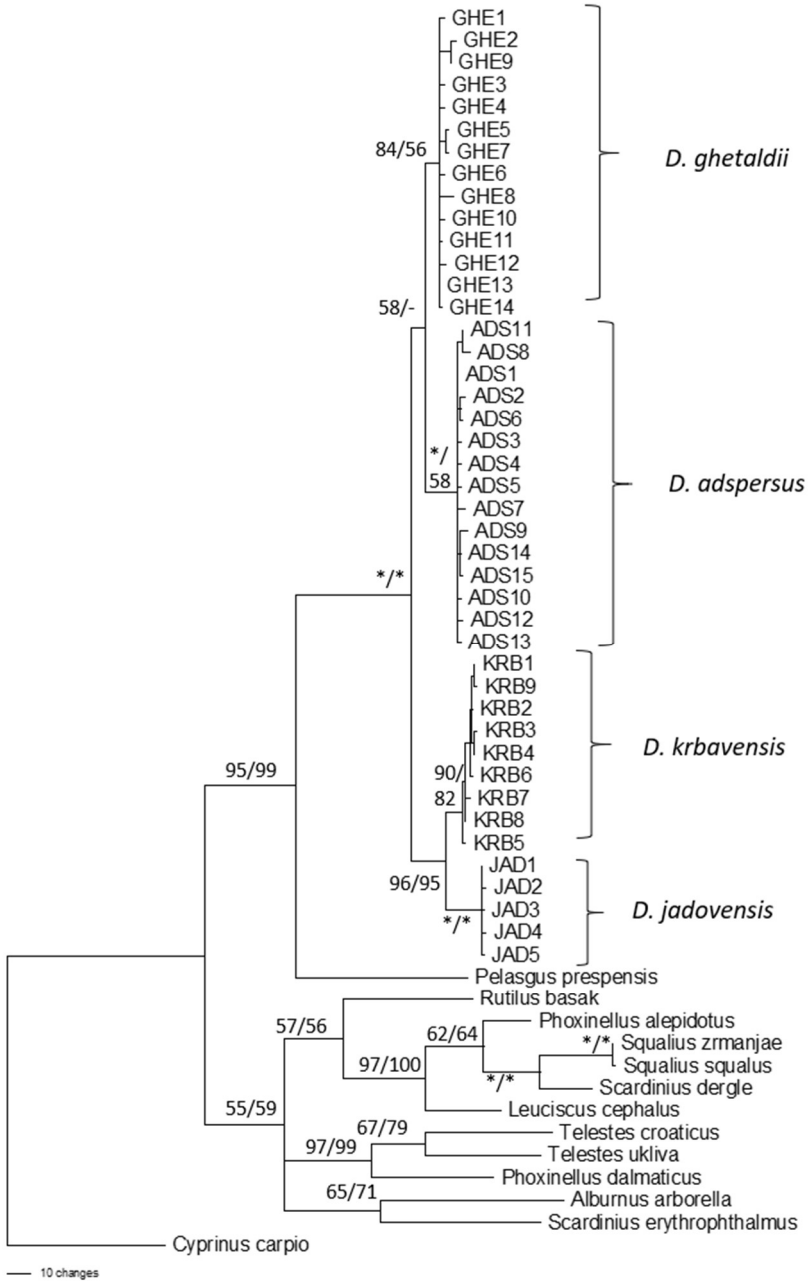

Figure 2. MP phylogenetic tree of *Delminichthys* cyt *b* sequences. Numbers at nodes represent MP and ML branch support. Asterisk denotes full branch support (100).

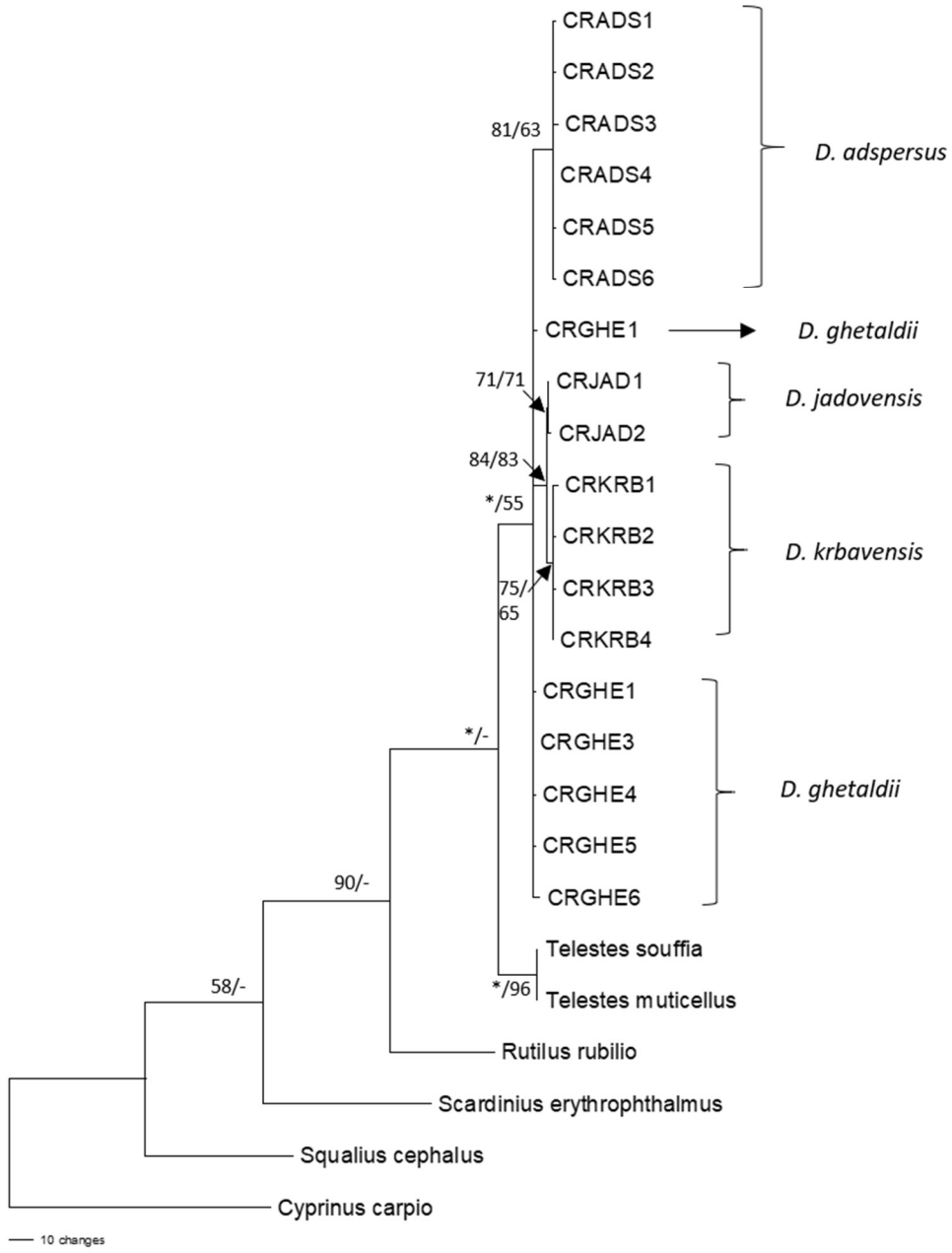

Figure 3. MP phylogenetic tree of *Delminichthys* CR sequences. Numbers at nodes represent MP and ML branch support. Astersik denotes full branch support (100).

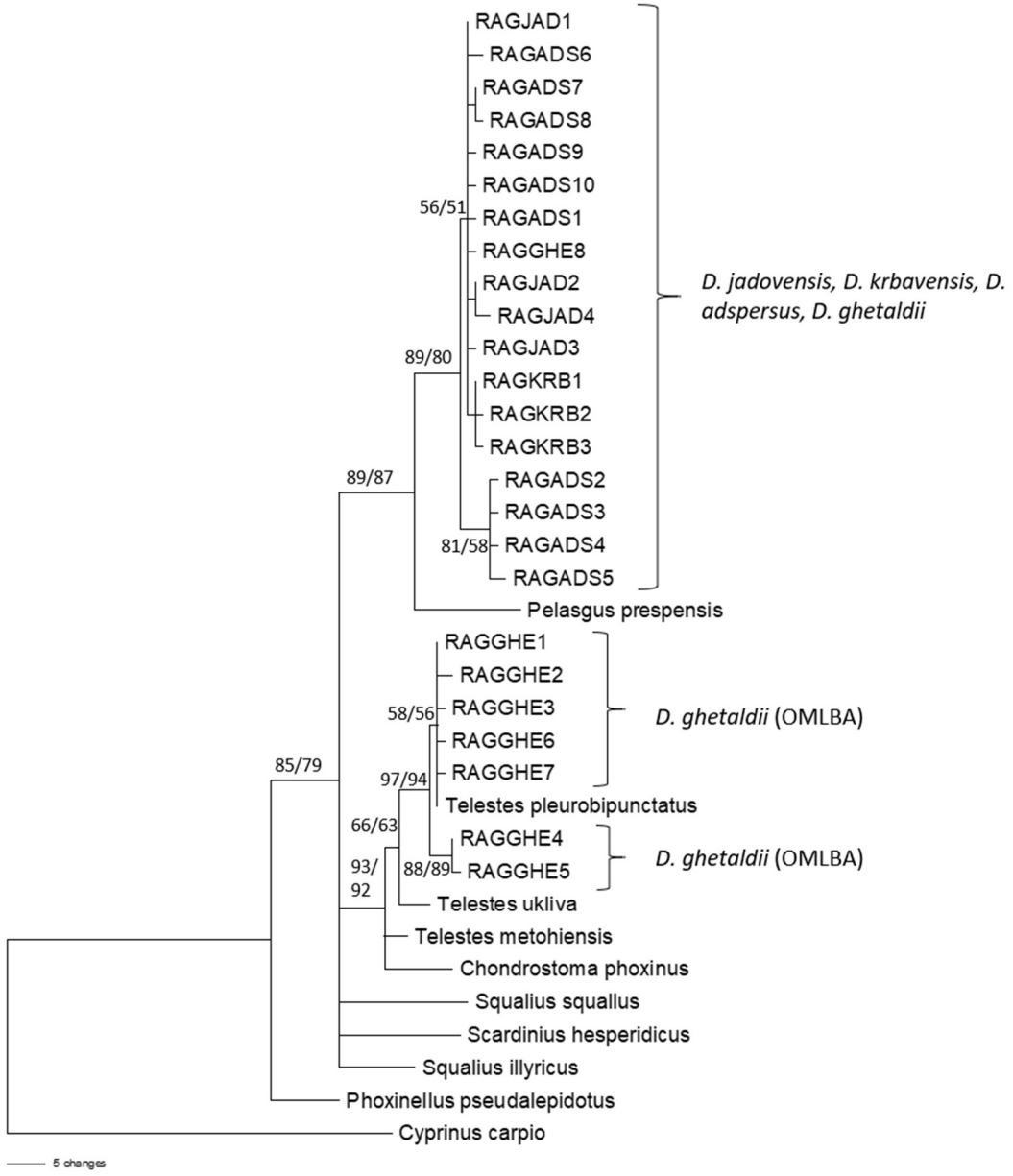

Figure 4. MP phylogenetic tree of *Delminichthys* RAG1 sequences. Numbers at nodes represent MP and ML branch support.

The origin of the genus *Delminichthys*, i.e., its separation from *Pelasgus*, probably already occurred in the Upper Miocene (Figure 5). The intrageneric divergences, however, are much younger, with two sublineages (*D. jadovensis/D. krbavensis* and *D. adspersus*/*D. ghetaldii*) separating in the Lower Miocene, and the species are of Pliocene origin. Even though the ancestral distribution analysis (Figure 5) did not yield clear results for earlier nodes, it seems likely that the ancient *Delminichthys* population, as the ancestor of all the present day species, was distributed in the western and middle Dinarides region, concordant with today's Gorski Kotar, Dalmatian hinterland and southern Bosnia and Herzegovina. The Matica River in the Jezero Field most likely harboured the ancestral population of *D. adspersus*, while the origin of *D. ghetaldii* might be connected with the karstic fields of eastern Herzegovina.

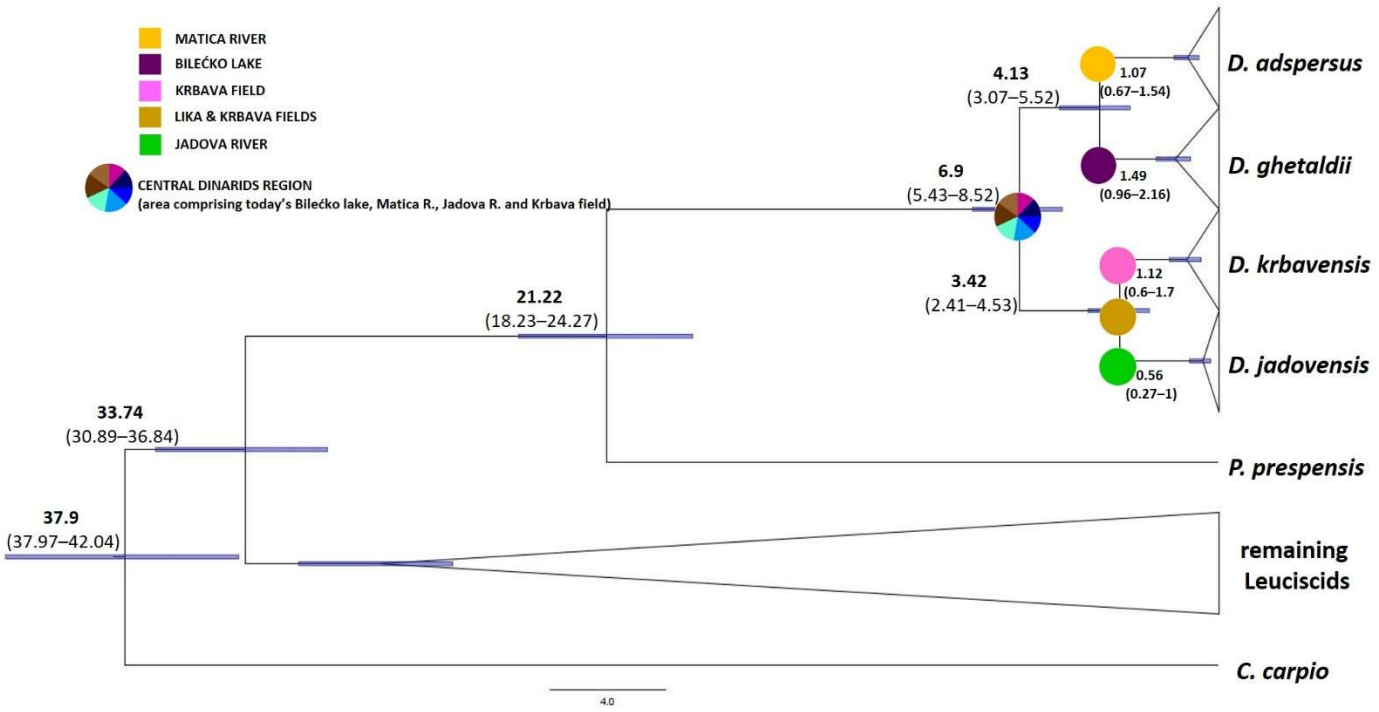

Figure 5. Divergence time estimations within the genus *Delminichthys* based on cyt *b* sequences. The timing of splitting events is presented by mean values and the 95% credibility range (in brackets), in million years ago. Reconstruction of ancestral distribution ranges is marked by the nodes.

BSPs were estimated to analyse the changes in past population sizes of *Delminichthys* species (Figure 6). *Delminichthys adspersus* seems to presently be in a reduction period, which was preceded by significant growth that lasted for about 220,000 years, while the BSPs of *D. ghetaldii* and *D. krbavensis* imply stability and minimal growth in the last period of their evolution. *Delminichthys jadovensis* also appears to be stable.

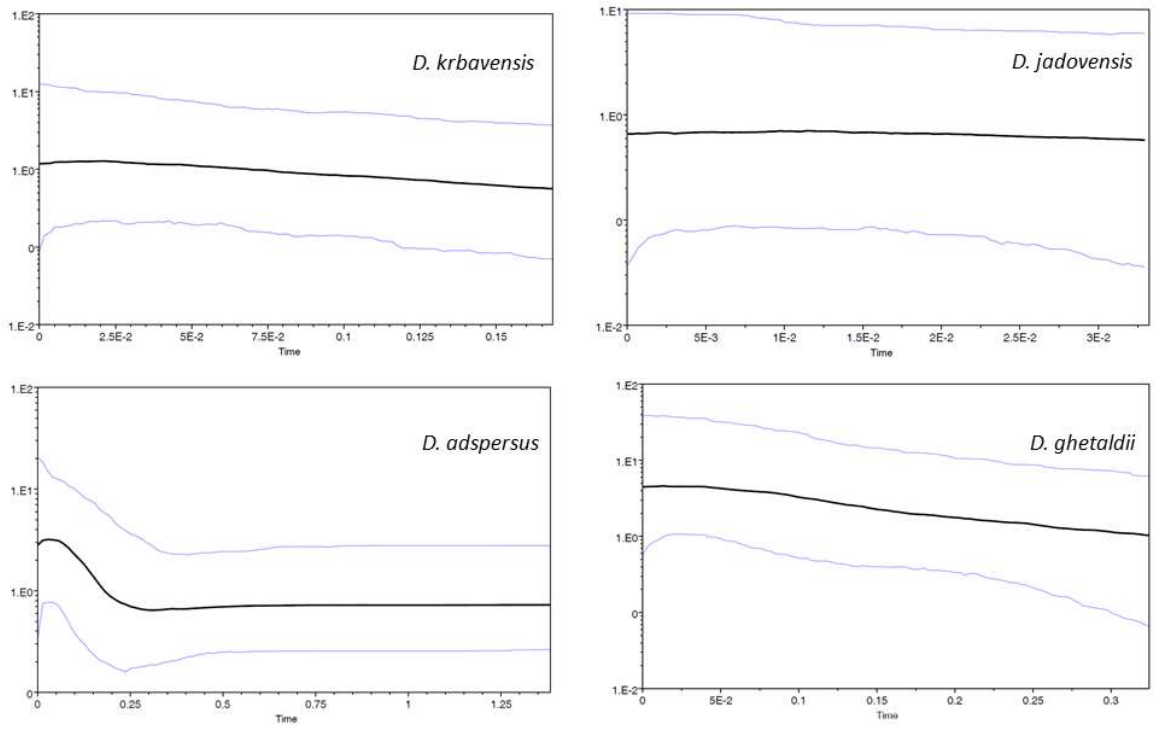

Figure 6. Bayesian skyline plots based on cyt *b* sequences. Changes in the effective population size (millions of individuals on a log scale; Y-axes) are depicted over time (in million years; x-axes). The black central lines represent the median values of Ne, while the purple lines represent the 95% highest posterior density of the Ne estimates.

Analysis of the intraspecific structure based on the cyt *b* gene revealed small migrations among *D. adspersus* populations (from Baćinska Lakes to Matica River, M = 5809.32, Nm = 4.5), and migrations among *D. ghetaldii* populations

(from Ombla River to Bilećko Lake, M = 341.44, Nm = 4.5; from Ombla River to Popovo Field intensive migration with M = 24019). Estimates for migrations among the remaining population pairs were not significantly different from 0. Even though control region data set was employed for gene flow estimations, no reliable results were obtained.

The measures of genetic polymorphism based on cyt *b*, as the most relevant genetic marker, revealed that moderate to high levels of genetic diversity are characteristic for all species and populations (Table 4). *Delminichthys ghetaldii* and *D. krbavensis* contain higher genetic diversities than the other two species, whereas genetic polymorphism of *D. jadovensis* is the lowest within this endemic genus. CR pointed to similar conclusions, but overall levels of genetic diversity are lower and differences among populations and species even more pronounced. Based on CR, *C. jadovensis* express low level of genetic diversity and the remaining species contain moderate levels of genetic polymorphisms. As expected, the RAG1 gene yielded smaller values of genetic polymorphism, with populations and species containing small to moderate levels of genetic diversity. Contrary to the situation with mtDNA, *D. krbavensis* contains a very small polymorphism of RAG1 (only 3 haplotypes found in the sample of 24 sequences, Hd = 0.163). Despite the low genetic diversity of *D. adspersus* based on RAG1, it is moderate in the Vrljika population. Higher values of nuclear polymorphism of *D. ghetaldii* are visible in the number of polymorphic sites, haplotype diversity and average number of nucleotide differences. The remaining nuclear markers, IRBP and BA expressed highly variable results for different populations and species. The haplotype diversity in the IRBP data set ranged between 0 (recorded in *C. jadovensis* despite significant sample number) and 1 (recorded in the Bilećko Lake population of *D. ghetaldii*). Generally, species with distribution ranges located more to the south (*D. adspersus* and *D. ghetaldii*) have higher genetic polymorphisms based on nuclear markers, whereas *D. jadovensis* express lower or no genetic diversity in nuclear markers. RAG1 is an exception because, besides enabling better phylogenetic resolution, implied different results regarding genetic polymorphism measures (high in *D. ghetaldii*, low to moderate in the remaining species).

Table 4. Measures of genetic polymorphism of the investigated populations and species. As it is not possible to calculate genetic diversity measures for populations where a single individual was sampled, those localities are not included in this table. However, when calculating the genetic diversity of a species, all individuals belonging to that species were included.

Population/Species	N	h	S	Hd	k	π	Haplotype Frequencies
					cyt *b*		
D. jadovensis	14	5	4	0.758	1.176	0.001	JAD1 = 43%, JAD2 = 7%, JAD3 = 29%, JAD4 = 14%, JAD5 = 7%
D. krbavensis	14	9	12	0.923	3.407	0.003	KRB1 = 7%, KRB2 = 21%, KRB3–5 = 7%, KRB6 = 21%, KRB7 = 14%, KRB8, 9 = 7%
D. adspersus	37	16	42	0.827	3.138	0.0028	
Matica River & Baćinska Lakes	22	8	11	0.654	1.468	0.0013	ADS1:59%, ADS2 = 9%, ADS3–5 = 4.5%, ADS6 = 9%, ADS7 = 4.5%, ADS10 = 4.5%
Vrljika R.	10	5	4	0.822	1.556	0.0014	ADS1 = 10%, ADS12 = 20%, ADS13, 14 = 30%, ADS15 = 10%
D. ghetaldii	20	14	24	0.937	4.089	0.0036	
Bilećko Lake	10	10	19	0.982	5.018	0.0044	GHE1,2 = 10%, GHE3 = 20%, GHE4–9 = 10%
Ombla River	9	5	5	0.722	1.556	0.0014	GHE10 = 11%, GHE11 = 56%, GHE12–14 = 11%
Total sample	84	43	107	0.955	25.906	0.0227	
					CR		
D. jadovensis	14	2	1	0.143	0.143	0.0005	CRJAD1 = 93%, CRJAD2 = 0.07%
D. krbavensis	11	4	2	0.764	1.018	0.0032	CRKRB1 = 18%, CRKRB2 = 18%, CRKRB2 = 18%, CRKRB4 = 0.36%
D. adspersus	29	6	4	0.650	0.803	0.0025	
Matica River & Baćinska Lakes	18	6	4	0.719	1.052	0.0033	CRADS1 = 6%, CRADS2 = 50%, CRADS3 = 6%, CRADS4 = 22%, CRADS5 = 6%, CRADS6 = 11%
Vrljika R.	11	1	0	0	0	0	CRADS4 = 100%
D. ghetaldii	16	6	4	0.733	1.333	0.0042	
Bilećko Lake	7	5	3	0.905	1.476	0.0046	CRGHE1 = 29%, CRGHE2 = 14%, CRGHE3 = 29%, CRGHE4 = 14%, CRGHE5 = 14%
Ombla River	8	2	1	0.536	0.536	0.0017	CRGHE1 = 63%, CRGHE6 = 38%
Total sample	70	18	19	0.890	6.904	0.0216	
					RAG1		
D. jadovensis	20	4	4	0.489	1.168	0.0009	RAGJAD1 = 70%, RAGJAD = 5%. RAGJAD3 = 5%, RAGJAD4 = 20%
D. krbavensis	24	3	2	0.163	0.167	0.0001	RAGKRB1 = 92%, RAGKRB2 = 4%, RAGKRB3 = 4%

D. adspersus	50	11	16	0.394	1.304	0.0010	
Matica River & Baćinska Lakes	40	7	7	0.322	0.442	0.0003	RAGJAD1 = 83%, RAGADS6 = 3%, RAGADS7 = 5%, RAGADS8–11 = 3%
Vrljika R. & Prološko blato	8	5	9	0.786	4.036	0.0031	RAGJAD1 = 60%, RAGADS2–5 = 10%
D. ghetaldii	26	9	31	0.794	13.923	0.0107	
Bilećko Lake	10	2	1	0.200	0.200	0.0002	RAGJAD1 = 10%, RAGGHE8 = 90%
Ombla River	16	7	9	0.742	1.833	0.0014	RAGGHE1 = 50%, RAGGHE2 = 6%, RAGGHE3 = 19%, RAGGHE4–7 = 6%
Total sample	120	25	47	0.757	7.641	0.0059	
					IRBP		
D. jadovensis	28	1	0	0	0	0	IrbpJAD1 = 100%
D. krbavensis	26	3	12	0.440	1.948	0.0023	IrbpKRB1 = 79%, IrbpKRB2 = 21%
D. adspersus	42	14	10	0.670	1.682	0.0020	
Matica River & Baćinska Lakes	30	12	8	0.715	1.768	0.0021	IrbpADS1 = 53%, IrbpADS2 = 3%, IrbpADS5 = 10%, IrbADS6 = 10%, IrbpADS7 = 7%, IrbpADS8–12 = 3%
Vrljika R. & Prološko Lake	8	5	5	0.786	2.071	0.0024	IrbpADS1 = 67%, IrbpADS2 = 17%, IrbpADS3 = 17%
D. ghetaldii	14	11	10	0.967	3.110	0.0036	
Bilećko Lake	8	8	10	1	3.893	0.0045	IrbpJAD1 = 13%, IrbpGHE2 = 25%, IrbpGHE3–7 = 13%
Ombla River	4	2	3	0.667	2.000	0.0023	IrbpJAD1 = 50%, IrbpGHE8 = 50%
Total sample	110	27	29	0.846	2.455	0.0028	
					BA		
D. jadovensis	24	2	1	0.391	0.391	0.0009	BAJAD1 = 25%, BAJAD2 = 75%
D. krbavensis	20	4	3	0.537	0.642	0.0014	BAKRB1 = 65%, BAKRB2 = 25%, BAKRB3 = 5%. BAKRB4 = 5%
D. adspersus	58	11	13	0.445	0.828	0.0018	
Matica River & Baćinska Lakes	44	7	9	0.365	0.613	0.0013	BAJAD1 = 80%, BAADS4 = 9%, BAADS6-10 = 2%
Vrljika R. & Prološko Lake	14	6	8	0.681	1.505	0.0033	BAJAD1 = 43%, BAADS1–3 = 7%, BAADS4 = 14%, BAADS5 = 7%
D. ghetaldii	16	7	25	0.625	5.242	0.0114	
Bilećko Lake	10	5	11	0.667	2.356	0.0051	BAJAD1 = 60%, BAADS5 = 10%, BAGHE1–3 = 10%
Ombla River	6	3	17	0.600	8.867	0.0193	BAJAD1 = 67%, BAGHE4 = 17%, BAGHE5 = 17%
Total sample	118	21	39	0.715	2.386	0.0052	

N—number of sequences, h—number of haplotypes, S—number of polymorphic sites, Hd—haplotype diversity, k—average number of nucleotide differences, π—nucleotide diversity.

4. Discussion

4.1. Evolutionary History of Delminichthys

Although mito-nuclear discordance was observed, mitochondrial DNA corroborated a clear separation and monophyly of the four *Delminichthys* species. As already proposed by [3], the closest relative of the genus *Delminichthys* is *Pelasgus*. Assuming a lineage divergence rate of 0.4%, the origination of the genus *Delminichthys* can be dated back to the Oligocene/Miocene boundary, which is earlier than proposed by [3], but similar to the results obtained by [12]. This was a period of increased continentalization in Europe and significant tectonic activity in the Mediterranean region [27], which, together with a warm climate, might have induced ancient divergences within freshwater systems. However, intrageneric divergences are of a much younger origin. More than 15 "million years of silence" presents a period with events that did not leave a trace in the recent genetic structure of this genus. A period from the lower Miocene or early Pliocene until the middle Pleistocene has been proposed as important for divergences of freshwater fishes in the Adriatic basin, and this period also brought divergences within the *Delminichthys* genus. Connection of the ancestor of recent *Delminichthys* species with the central Dinarides region was corroborated by the ancestral ranges' reconstruction. Separation of two sister species pairs is concordant with the separation of *Cobitis bilineata* Canestrini, 1865 (from the Zrmanja River) and the ancestor of *C. dalmatina* Karaman, 1928 and *C. narentana* Karaman, 1928 (Cetina and Neretva River basins) [13], which was likely induced by changes in the Dinaric Lake system (DLS). This system of tectonic freshwater lakes originated in the early Miocene within the western Dinaric belt [28–30]. In accordance with reports that evolution of DLS during favourable Miocene climatic conditions enabled

diversification of several snail genera [29], as well as the freshwater fishes of the genus *Cobitis* [13] and *Telestes* [5], it might have shaped early divergences within the genus *Delminichthys*. Furthermore, ancestral geographic ranges fall within the geographic range of the DLS. It seems that the spectacular Miocene radiation [29] comprised even more animal taxa than previously considered and might be one of the most important evolutionary events for freshwater fishes in the Adriatic basin. Divergence of *D. adspersus* and *D. ghetaldii* occurred more than a million years earlier than the separation of *T. miloradi* Bogutskaya, Zupančič, Bogut & Naseka, 2012 and *T. metohiensis* (Steindachner, 1901) [5], species that are distributed in similar areas. The youngest divergence event, the separation of *D. jadovensis* and *D. krbavensis*, occurred in the Middle Pliocene. Interestingly, their separation is somewhat younger than the separation of *Telestes croaticus* (Steindachner, 1866) and *T. fontinalis* (Karaman, 1972), distributed in the same area [5]. Thereafter, it can be concluded that although several geological events generally influenced the evolutionary history of cypriniform fishes recently distributed in the Dinaric region, it is also evident that the evolutionary history of the endemic *Delminichthys* genus is not completely concordant with other fishes.

4.2. Peculiar Case of Nuclear Introgression

Hybridization phenomenon, leading to the pertinence of genetic material of two species in a single individual or population, has been repeatedly observed in fishes (e.g., [31–34]). There is even evidence that in some cases, significant proportions of genomes are derived by hybridization [34] or that hybridization led to speciation [35]. Even though hybrid biotypes (considered as any taxonomic or ecologic units originated by past or current hybridization events) blur the species borders and obscure the definition of species as taxonomic units, they certainly present an amazing part of biodiversity that is still vastly unknown and without adequate protection. With only a few reported exceptions, hybrid biotypes that succeeded in reproducing and persisting for a longer period originated as a result of the interbreeding of closely related species (e.g., [32,36]). Furthermore, although widely observed in the central and northern European freshwater basins, hybrid biotypes are the exception in the Adriatic watershed, which has been explained by the long-term isolation of these river basins (see [13]).

Based on the genetic structure of *Delminichthys* species revealed here, the existence of hybrid lineages in the Adriatic watershed can be hypothesized and the possibility that, in addition to allopatric speciation, hybridization might also played yet unnoticed, but nevertheless important role in the evolution of biodiversity in this ara. The mito-nuclear discordance observed within *Delminichthys*, with mitochondrial markers implying monophyletic origin of the four *Delminichthys* species and RAG1 discovering polyphyletic origin of *D. ghetaldii* in the Ombla River, whereas nuclear markers in general did not enable species distinction, is the probable result of two phenomena. Incomplete lineage sorting is the likeliest explanation that the nuclear markers, having lower mutation rates, did not enable resolution of intrageneric structuring of *Delminichthys*, yet all species (with the exception of *D. ghetaldii* from the Ombla River, based on the RAG1) share the same or similar nuclear haplotypes. However, extensive nuclear introgression of RAG1 observed in the Ombla population invokes the possibility of its hybridogenetic origin. Mitochondrial haplotypes of the Ombla population are closely related to those from Bilećko Lake and, together with morphological determination, corroborate the pertinence of the Ombla population to *D. ghetaldii*. On the other hand, both RAG1 alleles of all specimens from the Ombla River are very distinct from the remaining *Delminichthys* sequences. They are the most similar, though not entirely, to *T. pleurobipunctatus* RAG1 haplotypes. Even in heterozygous individuals, both alleles clustered with *T. pleurobipunctatus*, which is very unusual since *T. pleurobipunctatus* is currently distributed in Greece. The time, locality and pathway of their connection are not known. The possiblet explanation for this peculiar case of nuclear DNA capture is ancient hybridization that caused mosaicism in the nuclear genome, although further, preferably genomic investigations are required to distinguish between possible ancient hybridization and incidental cross-generic gene flow. Noteworthy, pattern of mitochondrial isolation with nuclear introgression is highly unusual. The reversed situation of mitochondrial introgression has been reported among different taxa of freshwater fishes and occurs much more frequently than the observed nuclear introgression (e.g., [10,11]). Namely, cytoplasmically inherited genes are more likely to cross species boundaries than nuclear ones [37,38]. Accordingly, there are only several reports on nuclear introgression causing cytonuclear and/or nuclear/nuclear discordance [39,40]. It is thought that nuclear genes may be prone to introgression in cases with different incompatibilities between species, depending on which species is the female parent [39], or that some loci are selectively advantageous in the “wrong” species and not closely linked to genes causing intraspecific incompatibility so they might more easily cross species boundaries [40]. Since there are no evidences for nuclear introgressions of IRBP and BA genes, we can suspect that hypothesis on selective advantage of certain nuclear genes is more likely. The hypothesys that the RAG1 pattern is a retention of an ancestral polymorphism

is not supported by our phylogenetic analysis that revealed distant relations among the two parental taxa. On the other hand, presence of the same haplotype in various species, as observed in nuclear markers, is a likely example of ancestral polymorphism retention. This is also corroborated by higher frequencies of those haplotypes, which is usually characteristic for ancestral haplotypes.

4.3. Subterranean Migrations

One of the most astonishing adaptations of freshwater fishes for life in karstic rivers might be their ability to survive in and migrate through underground waters. Namely, due to the carbonate terrain that makes up the karstic surfaces in the Dinarides Mountains area, surface water flows are scarce. The hydrographic network of the Adriatic watershed is fragmented, comprised of short, isolated rivers. The underground water network is, on the other hand, well developed, complex and widespread [41], providing possible shelter grounds, migration and/or colonization routes for fishes. Nevertheless, evidence of underground migrations have only been provided for *D. adspersus* [4]. No significant underground migrations among *D. adspersus* populations investigated here were detected (with migration observed only between the Matica River and Baćinska Lakes that are connected by an artificial tunnel), while migrations were found among *D. ghetaldii* populations that have no surface connections. Adaptation to underground life, thereafter, seems to be characteristic to this genus, developed during the specific evolutionary history in the Dinaric karst freshwater systems. The revelation of morphological characters responsible for this adaptation should assist in explaining the evolutionary history of this species. Results of the migration between the Ombla River and Popovo Field populations are not likely precise, due to low sample size from the Popovo Field. Nevertheless, the indication of the Ombla population as a source of immigrants is very important for two reasons. First, since individuals migrate upstream, it provides evidence that underground migrations within this genus are active. Secondly, this knowledge is important as it indicated the need for immediate, effective conservation of the Ombla population.

4.4. Genetic Diversity

No significant reduction of genetic polymorphism was observed in species distributed in areas affected by glaciations (*D. jadovensis* and *D. krbavensis*), which might also be a consequence of its ability to survive in underground shelters. High genetic diversity contained within all *Delminichthys* species is most likely a consequence of their old origin. Recent genetic diversity presents a reservoir for coping with future environmental changes and a guarantee of the viability of a species. It does seem possible that hybridization led to an increased mutation rate [42,43] and higher genetic variability. Namely, genetic polymorphism of the RAG1 gene within the Ombla River population is unexpectedly high (more than threefold higher than in the conspecific Bilećko Lake population, higher than the cyt *b* polymorphism of this population), which is contrary to what was expressed by the cyt *b* gene. Lower genetic diversities observed in IRBP and BA are probable consequences of their lower mutation rates and ancestral polymorphism retention phenomenon. High genetic diversity of the majority populations and species, and the stability observed by BSPs are favourable when discussing the extinction risk of the *Delminichthys* species. Their restricted distribution ranges and high level of endemicity, nevertheless, require maximum care and effective protection.

Supplementary Materials

The following supporting information can be found at: https://www.sciepublish.com/article/pii/285, Table S1: Accession numbers and references of the sequences retrieved from the GeneBank; Figure S1: MP phylogenetic trees of *Delminichthys* IRBP and BA sequences. Numbers at nodes represent MP and ML branch support.

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Author Contributions

Conceptualization: I.B.; Data curation: I.B., Z.M., M.Ć. and R.Š.; Formal analysis: I.B., S.R.; Investigation: I.B., Z.M., M.Ć., R.Š. and S.R.; Methodology: I.B.; Resources: I.B. and R.Š.; Software: I.B. and S.R.; Writing—original draft: I.B., Z.M. and M.Ć.; Writing—review & editing: I.B., Z.M., M.Ć., R.Š. and S.R.

Ethics Statement

This study was conducted entirely in accordance with the ethical standards and Croatian legislation, and was approved by the Ethics Committee of the Faculty of Science, University of Zagreb. Sampling permits were issued by the competent authorities: for localities within Croatia, the permit was issued by the Ministry of Nature Protection; and for localities within Bosnia and Herzegovina the permit was issued by the Republic Department for the Protection of Cultural, Historical and Natural Heritage in Banja Luka.

Informed Consent Statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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