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Morphometric and meristic differentiation among five populations of *Delminichthys ghetaldii* (Actinopterygii, Cyprinidae) from five karst fields in Eastern Herzegovina

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Abstract: The primary objectives of this study were to advance understanding of the morphological intricacies exhibited by the endemic fish species *Delminichthys ghetaldii*, commonly known as the Trebinje minnow, and to determine intraspecific morphological variability. The study involved the examination of 76 mature specimens collected since 2018 from five distinct karst fields located within the Dinaric region of Eastern Herzegovina, Bosnia and Herzegovina. A comprehensive analysis of 22 morphometric and 8 meristic characteristics was conducted to unravel the underlying patterns of variation. Discriminant function analysis was employed for the thorough investigation of the morphometric traits, whereas canonical correspondence analysis was applied to scrutinize the meristic traits. The discriminant function analysis yielded noteworthy outcomes, revealing substantial differentiations across 17 morphological characteristics within samples from diverse karst fields. Furthermore, it revealed notable dissimilarities in four morphometric characteristics when data including both male and female specimens were examined. Remarkably, these analyses showed that *Delminichthys ghetaldii* specimens inhabiting the Popovo karst field exhibit significant morphological disparities compared to those in the other fields. Conversely, individuals from the Fatničko karst field showed a pronounced morphological affinity with their counterparts from the Mokro karst field. Intriguingly, a resemblance in morphometric characteristics was noted between *Delminichthys ghetaldii* inhabitants of the Dabarsko karst field and those from the Ljubomirsko karst field. The assessment of osteological meristic characteristics across samples from the five distinct karst fields, as well as the differentiation between male and female specimens, did not reveal any statistically significant distinctions.

Key words: Bosnia and Herzegovina, karstified relief, morphological differentiation, Popovo minnow, stenoendemism, subterranean life

1. Introduction

The Eastern Herzegovina region, standing as one of the foremost karstified territories globally, is characterized by deep karst formations, sinking rivers, subterranean river systems, karst fields prone to temporary inundation, and a deficiency of arable land. The karst basins within Bosnia and Herzegovina are host to a multitude of stenoendemic species, which commonly exhibit restricted geographical distributions and often inhabit only select localities. Notably, a substantial proportion of these species belong to the cyprinid subfamily Leuciscinae (Kottelat, 1998). Minnows found inhabiting karstic aquatic ecosystems represent a distinct component of the endemic ichthyofauna. This assemblage encompasses species from three principal genera—Delminichthys, Phoxinellus, and Telestes—collectively known in the region as 'gaovice'. This group shares a distinctive life history strategy linked to the pronounced fluctuations in water level regimes within their respective karstic habitats. It is only during the autumn and spring flood events that these organisms emerge from their subterranean aquatic sanctuaries to surface estavelles. In contrast, they spend the rest of their life cycle within underground karst water streams (Vuković and Ivanović, 1971). Although lacking overt cave adaptations such as regressed eyes and pigmentation, these species exhibit specific traits that potentially correspond to their partially subterranean habitat. These attributes include dermal thickening, reduced cephalic sensory canals, and an increased number of incompletely ossified, deeply embedded scales (Zupančič and Bogutskaya, 2002).

The Popovo minnow, scientifically referred to as *Delminichthys ghetaldii* (Steindachner, 1882), was initially documented as *Phoxinellus ghetaldii* from the caves of the Popovo karst field (Bosnia and Herzegovina).

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Coincidentally, the nearby Trebišnjica River, coursing through the same karstic formation in the vicinity of the city of Trebinje, was documented as home to *Phoxinellus pstrossii* (Steindachner, 1882). An in-depth reassessment of this taxonomic group by Zupančič and Bogutskaya (2002) and Bogutskaya and Zupančič (2003) yielded the division of the ten Phoxinellus species into two distinct groupings. Subsequently, through the scrutiny of nuclear and mitochondrial DNA sequences (Freyhof et al., 2006) the genus Phoxinellus was deemed paraphyletic in nature, encompassing three distinct, unrelated monophyletic units, namely Phoxinellus, Delminichthys, and Telestes. Zupančič and Bogutskaya (2000) merged *Phoxinellus pstrossii* and *Phoxinellus ghetaldii* into a singular taxon, designated as *D. ghetaldii*.

D. ghetaldii is dispersed on distinct karstic fields in Eastern Herzegovina (the Dabarsko, Fatničko, Ljubomirsko, Popovo, and Mokro karst fields) predominantly in the drainage area of the Trebišnjica River, a sinking river of 96.5 km in surface-flow length. With a cumulative surface and subterranean length of 187 km, it is the longest European sinking river and one of the longest in the world. This distinctive watercourse comprises a network of strong karstic wellsprings distributed in two principal aquifer zones that are geographically and hydrologically distinct, despite their relatively close proximity to each other. Groups of underground wellsprings each consist of a number of large, abundant founts. Even during periods of uplift and bottom-level flattening within the karst fields, the process of karstification functioned as an underground drainage mechanism for surface water of the endoreic system of the Trebišnjica River. A succession of sinkholes of varying capacity enabled the absorption of the Trebišnjica River's waters in the upstream succession from the end of the blind valley. During periods of elevated water levels, the ultimate sinkholes were fully active, whereas diminishing water levels rendered them devoid of hydrological impact, their role compensated for by the smaller upstream sinkholes on the riverbed. The river exhibited surface flow characteristics at the time of elevated karst water levels within the submergence zone, referred to as 'stagnant water' by Daneš (1906). Characteristic forms associated with the karst mountains are large flat surfaces, plateaus, and intramontane depressions in which karst fields have developed.

In instances where intense siltation impacted karst fields, certain sinkholes are likely to have become obstructed by transported detritus, resulting in the elevation of the field's base. Subsequently, hanging karst holes, now aligned with the field's bottom or positioned along its slopes, assumed the functional roles previously held by the clogged sinkholes. This transformation marked a shift from their previous intermittent wetness or dryness to the establishment of constantly wet karst zones. Moreover, particular sinkholes located within the karst interior, characterized by their constricted karst channels directed towards the incoming karst network from the karst fields, took on the role of estavelles. These features operated as springs during periods of elevated water levels and as sinkholes during droughts (Spahić, 2015).

Meristic attributes, anchored in osteological structures, serve as the foundation of this study. The growing prominence of osteology in fish systematics stems from the fact that their bone patterns are the most conservative, so less susceptible to environmental influences.

The analysis of morphological traits within endemic species holds notable significance, offering insights into the population status of individuals, and indirectly, into their environmental context. In light of this context, the aim of this study is to discern the impact of distinct isolated karst fields on the morphological variability of *D. ghetaldii* regarding both their morphometric and meristic characteristics, with an extension into an examination of the morphological variability across sexes.

2. Materials and methods

2.1. Sampling

Despite a great sampling effort in the years 2016–2019, which featured a lack of flooding in particular karst fields, only a limited number of fish were sampled between March and May 2018, when all the karst fields were flooded (Figure 1). Geographical and sampling information for each karst field is as follows:

- Fatničko: 43°1′54.12″N, 18°19′0.768″E, 460 m above sea level (asl), 35 individuals (17 males, 18 females)
- Ljubomirsko: 42°46′3.54″N, 18°21′6.804″E, 520 m asl, 24 individuals (10 males, 14 females)
- Dabarsko: 44°57′15.3432″N, 15°18′11.592″E, 470 m asl, 4 individuals of unspecified sex
- Popovo: 42°96′45″N, 18°55′37″E, 240 m asl, 7 individuals (2 males, 5 females)
- Mokro: 42°39′35″N, 18°20′03″E, 270 m asl, 6 individuals (5 males, 1 female)

Fishes were collected from fishermen or caught using standard, battery-powered, backpack electrofishing gear. The sampled fish were fixed in 70% ethyl alcohol at the sampling sites and transported to the laboratory for further morphological analysis. The group of sampled fish from a single karst field was considered an operational taxonomic unit (OTU), as per Sneath and Sokal (1973). For the analysis of vertebral column features, all specimens were radiographed using Pardus, a portable x-ray apparatus in the Natural History Museum of Vienna, Austria.

2.2. Ethical statement

Fish collection and handling followed Animal Care Protocol #30/625-751/18 of the Ministry of Education and Culture of the Republic of Srpska.

Figure 1. Locations of the Popovo, Mokro, Dabarsko, Fatničko, and Ljubomirsko karst fields, showing altitudes in meters above sea level. The blue circles denote the locations where the *Delminichthys ghetaldii* samples were found.

2.3. Data analysis

The phenetic analysis of the morphometric and meristic characteristic data was conducted using R v.4.0.3 (R Core Team, 2020), SPSS 7.0 (Statsoft Inc., 2004), and Excel (97-2003).

2.3.1. Morphometric characteristics

The set of 22 standard morphometric characteristics (Bogutskaya and Zupančič, 2010) were measured using a digital caliper to the nearest 0.1 mm: standard length (SL), head length, horizontal eye diameter, snout length, interocular width, postorbital distance, maximum head width, upper jaw length, lower jaw length, maximum body depth, caudal peduncle length, caudal peduncle depth, dorsal fin base length, dorsal fin depth, pelvic fin length, pectoral fin length, prepelvic length, preanal length, predorsal length, postdorsal length, pectoral–pelvic fin origin distance, and pelvic–anal fin origin distance. Sex was determined by the presence of a genital papilla in females, which looks like a thickened triangular fold with the anus at its base. The genital orifice is located anteriorly near the middle of the papilla, never at the front tip of the fold. The papilla is already developed in juvenile females (39–53 mm SL), though it is narrow and elongated; it is much larger in adult females (53–58 mm SL), with a wide base that extend over the lowermost part of the first anal fin rays (Bogutskaya and Zupančič, 2003).

SL was measured from the most anterior point of the upper lip (not the snout) to the end of the hypural complex. Head length was measured from the most anterior point of the upper lip to the most posterior point of the opercular membrane. Interorbital width was measured including the skin fold. The term 'length of dorsal fin' is used for the span of the dorsal fin's base, and the term 'depth of dorsal fin' is used for the length of the longest ray of the dorsal fin.

Owing to the great variation in fish size among the various karst fields, morphometric characteristics were converted to a fraction of SL and tested for normality of distribution using the Shapiro–Wilk test (Shapiro and Wilk, 1965), both univariately for each characteristic and multivariately for all simultaneously and together, using the mshapiro.test function of the mvnormtest package in R. For both, $p < 0.01$ indicated significance. Multivariate discriminant analysis was used to test the difference between OTUs from the five karst fields and between males and females pooled together from all karst fields. Likewise, it was used to determine which 22 morphometric characteristics had the greatest power to explain the majority of differentiation between OTUs based on the loading of morphometric characteristics on discriminant roots. Mahalanobis D^2 distances were calculated between the discrimination scores of the centroids of the five OTUs, representing the difference in multivariate shape between them and assessed from all discriminant roots. The significances of the revealed differences were assessed using multivariate analysis of variance (ANOVA) F values at the level $p < 0.01$, whereas the relationships between the five *D. ghetaldii* OTUs were inferred using the hierarchical unweighted pair–group of arithmetic averages method of clustering (UPGMA) (Sokal and Rohlf, 1981). The characteristics with the greatest discriminating power were subject to univariate ANOVA to confirm the significance of their discriminating power in five *D. ghetaldii* OTUs. The Tukey HSD post-hoc test (Sokal and Rohlf, 1981) served to determine between which particular OTUs there was significant variation for each of the discriminative characteristics at the level $p < 0.01$, used for identification and differentiation of the OTUs in the field.

2.3.2. Meristic characteristics

Eight meristic characteristics were counted from radiographs of each specimen: number of branched dorsal fin rays, number of branched anal fin rays, number of unbranched fin rays, number of dorsal vertebrae, number of abdominal vertebrae, number of caudal vertebrae, number of precaudal anal fin pterygiophores, and total number of vertebrae (Figure 2). The last two $^1/_2$ branched dorsal and anal fin rays were recorded as 1. The terminology of the meristic traits follows Harrington (1955). The total number of vertebrae includes four Weberian vertebrae and the fused preural–ural centrum as the last one. The abdominal and caudal vertebrae were distinguished by the occurrence of a well-developed haemal spine in the first caudal vertebra; the abdominal vertebrae anterior to the first dorsal fin pterygiophore were considered the predorsal vertebrae, while the rest of the posterior abdominal vertebrae with parapophyses fused to the centra and non-articulated with ribs were considered separately. Canonical correspondence analysis (CCA) is a multivariate exploratory technique for discrete data (Hill and Gauch, 1980; Gauch, 1982), used to reveal an association of particular states of each meristic characteristic with the OTUs and sexes as a source of their variation. The significance of the difference between OTUs in a frequency of occurrence of particular states for each of the meristic characteristics was tested using contingency analysis and was confirmed by pairwise c^2 testing (Petz, 1985) between particular OTUs, with the Yates correction for continuity (Yates, 1934) applied for characteristics where significance was detected.

3. Results

In total, 76 specimens of *D. ghetaldii* were examined for morphometric and meristic traits. The sample was composed of 39 females, 33 males, and four individuals of unspecified sex.

3.1. Morphometric characteristics

Shapiro-Wilk's normality test for 21 morphometric characteristics scaled for each individual to its SL revealed that variation in the majority of characteristics matched the normal distribution. Five characteristics exhibited deviations from the normal distribution: horizontal eye diameter (W = 0.917, p = 1.04e–06), postorbital distance (W = 0.94916, p = 4.16e–03), maximum head width (W = 0.93653, p = 8.93e–04), minimal body height (W = 0.88531, p = 5.023e–06), and dorsal fin depth (W = 95341, p = 7.18e–03). The multivariate testing revealed that whole sample fits into a normal distribution (W = 0.90683, p = 0.05011), rendering the data set suitable for the application of parametric multivariate statistical methods.

Discriminant analysis revealed that the first four discriminant roots explained all (100%) of the distinctions between OTUs. The first discriminant root (l_1 = 13.072,

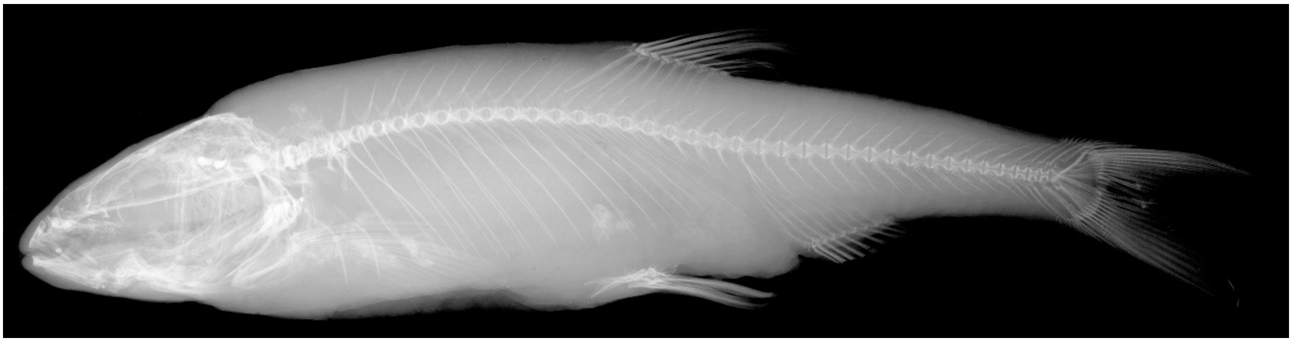

Figure 2. Vertebral column and position of fins in *Delminichthys ghetaldii*.

59.6%) showed interorbital width and maximum body depth to be the most distinctive characteristics; at the second discriminant root (l_2 = 4.845, 22.1%), horizontal eye diameter was particularly prominent. The third discriminant root (l_3 = 2.919, 13.3%) was explained mostly by interorbital width, while the fourth (l_4 = 1.094, 5.0%) was explained mostly by maximum head width (Table 1). The correctness of the a priori set classification was complete (100%), which indicates a strong differentiation, i.e., distinctness among the five *D. ghetaldii* OTUs.

Discriminant scores for the OTUs of *D. ghetaldii* from the five karst fields, presented both pooled together and separated into males and females (Figure 3), revealed that there is a strong differentiation between them in the space of the first three discriminant roots. The most distinct was the OTU from the Popovo karst field, but all OTUs differentiated more along discriminant root 1, with the greatest power of discrimination (59.6%) explained by interorbital width and maximum body depth, than along discriminant root 2 (22.1%), explained by horizontal eye diameter. Discriminant root 3 (13.3%), explained by interorbital width, clearly differentiated the OTUs from the Fatničko and Mokro karst fields, whereas in the four OTUs where both sexes were well represented, discriminant root 2 differentiated them approximately the same as discriminant root 1 did.

UPGMA clustering of Mahalanobis distances D^2 (Table 2) between the centroids of the discriminant scores of males and females from five *D. ghetaldii* OTUs pooled together (Figure 4) revealed three distinct clusters. The Mahalanobis D^2 distances presented in Table 2, along with the clustering shown in the dendrogram, provide a comprehensive model of the differences in morphological characteristics among the *D. ghetaldii* populations, highlighting the statistical significance of these differences. The values above the diagonal in Table 2 represent the Mahalanobis D^2 distances between the centroids of the OTUs, with larger distances indicating more pronounced differences between the OTUs. Conversely, the values below the diagonal display the F values associated with the Mahalanobis D^2 distances, indicating whether the differences between the OTUs are statistically significant at the $p < 0.01$ level. While the *D. ghetaldii* OTU from the Popovo karst field was the most distinct, two other clusters comprised a pair of OTUs each,

Table 1. Factor structure matrix of the first three discriminant roots with the eigenvalues (l) of each and part of the discriminating power (as % of total discrimination) they hold. These explain the discriminative power of roots 1–3, while the morphometric characteristics of each discriminant root explain it the most.

Characteristic	**Root 1**	**Root 2**	**Root 3**	**Root 4**
Snout length	−0.107	−0.171	-0.101	−0.188
Horizontal eye diameter	−0.178	−0.525	0.216	0.142
Postorbital distance	−0.113	0.069	0.008	0.021
Head length	−0.107	−0.127	−0.094	−0.273
Maximum head width	0.169	0.105	0.252	−0.557
Interorbital width	0.482	0.155	0.354	0.167
Upper jaw length	−0.123	−0.039	−0.069	−0.124
Lower jaw length	−0.061	−0.078	−0.063	−0.108
Maximum body depth	0.337	0.070	−0.279	0.098
Caudal peduncle depth	0.219	−0.256	−0.263	0.111
Predorsal length	0.009	0.175	0.081	0.085
Postdorsal length	−0.060	0.084	−0.099	0.035
Prepelvic length	−0.057	0.213	0.117	0.014
Preanal length	−0.059	0.228	0.108	0.059
Caudal peduncle length	0.068	0.021	0.056	−0.259
Dorsal fin length	0.212	−0.136	0.182	0.161
Dorsal fin depth	0.094	−0.211	−0.022	−0.133
Pectoral fin length	0.078	−0.152	−0.238	0.199
Pelvic fin length	0.056	−0.053	−0.069	−0.004
Pectoral–pelvic fin distance	−0.061	0.106	0.002	−0.115
Pelvic–anal fin distance	0.032	−0.152	−0.017	0.360
Eigenvalue (l)	13.072	4.845	2.917	1.094
Proportion of discrimination (%)	59.6	22.1	13.3	5.0
Cumulative proportion	59.6	81.7	95.0	100.0

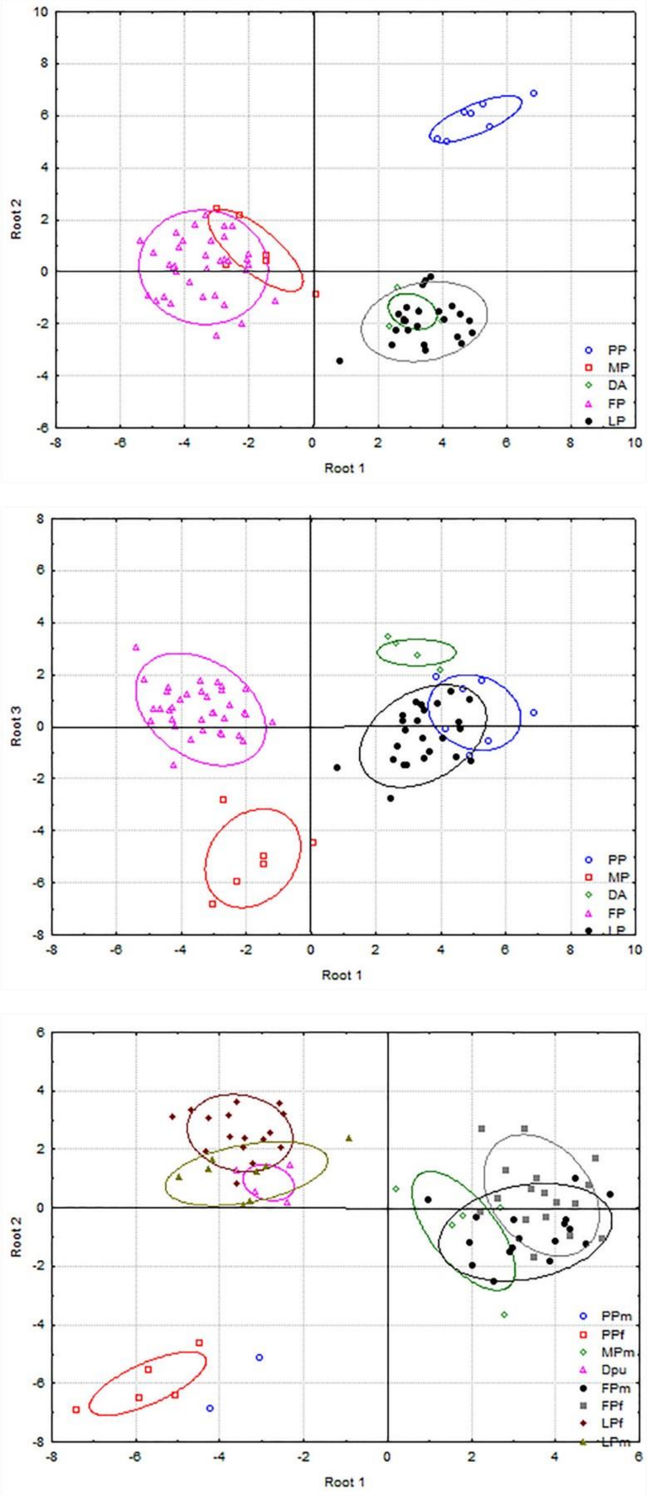

Figure 3. *Pooled* Delminichthys ghetaldii *OTUs in the space of discriminant roots 1–2 (top) and 1–3 (middle), and distinction by sex in the space of discriminant roots 1–2 (bottom; f, females; m, males; u, undefined) from five karst fields (PP, Popovo; MP, Mokro; DA, Dabarsko; FP, Fatničko; LP, Ljubomirsko).*

Table 2. Mahalanobis D^2 distances between the centroids of the *Delminichthys ghetaldii* OTUs from five karst fields (PP, Popovo; MP, Mokro; DA, Dabarsko; FP, Fatničko; LP, Ljubomirsko) (above the diagonal), and their F values (below), which were all significant at p < 0.01.

OTUs	PP	MP	DA	FP	LP
PP		106.124	79.802	103.903	64.112
MP	11.728		97.840	38.328	61.075
DA	6.948	8.032		65.535	29.472
FP	20.732	6.715	8.047		52.635
LP	11.884	10.028	3.456	25.633	

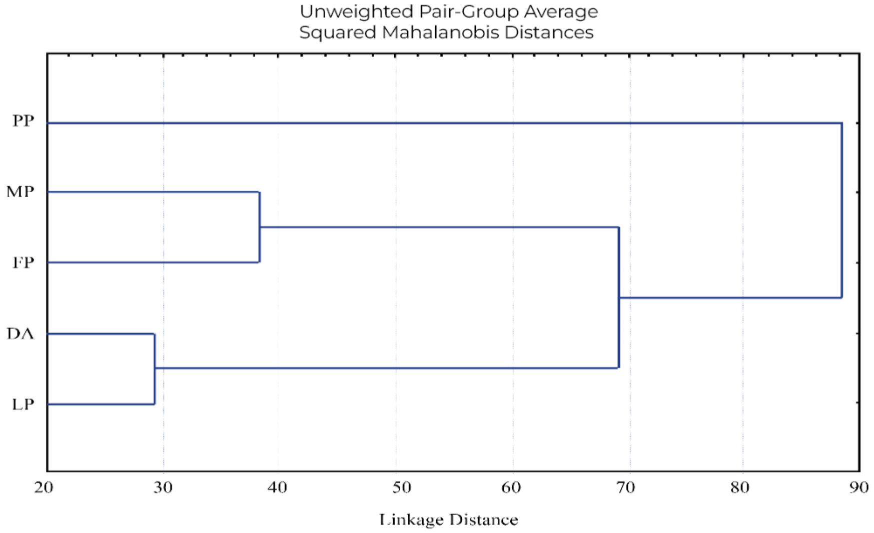

Figure 4. UPGMA clustering of *Delminichthys ghetaldii* males and females from the five karst fields (PP, Popovo; MP, Mokro; DA, Dabarsko; FP, Fatničko; LP, Ljubomirsko) pooled together.

suggesting that the characteristics that mostly explained them, horizontal eye diameter and interorbital width, were in combination important for their distinction (Figure 4). Their relationships and the significance of their D^2 distances suggest a complex pattern of differentiation that might be related to their spatial dispersal in the Trebišnjica River drainage basin.

Multivariate ANOVA revealed significant differentiation in both OTU (F = 12.366, df = 63.147; p < 0.0001) and sex (F = 4.233; df = 21.49; p < 0.0001) of *D. ghetaldii*. The characteristics with the greatest discriminating power were subjected to ANOVA to confirm their discriminating power through significant variation across the five *D. ghetaldii* OTUs. A Tukey HSD post-hoc test was used to determine which specific OTUs had significant variation for each of the discriminating characteristics. This analysis was crucial for identifying and distinguishing the OTUs in the field. Univariate testing using ANOVA revealed that the OTUs were significantly different (df = 3. 70) for 17 of the 21 morphometric characteristics (Appendix 1). These are snout length (F = 3.456; p = 0.021), horizontal eye diameter (F = 47.211; p < 0.0001), postorbital distance (F = 4.014; p = 0.011), head length (F = 6.362; p = 0.0007), maximum head width (F = 8.884; p < 0.0001), interorbital width (F = 71.631; p < 0.0001), upper jaw length (F = 6.314; p = 0.0007), maximum body depth (F = 36.726; p < 0.0001), caudal peduncle depth (F = 16.412; p < 0.0001), predorsal length (F = 3.163; p = 0.029), prepelvic length (F = 5.422; p = 0.002), preanal length (F = 5.739; p = 0.001), dorsal fin base length (F = 16.773; p < 0.0001), dorsal fin height (F = 6.588; p = 0.0005), pectoral fin length (F = 7.635; p = 0.0002), pectoral–pelvic fin distance (F = 3.710; p = 0.015), and pelvic–anal fin distance (F = 3.508; p = 0.020). The Tukey HSD post-hoc test for characteristics that were highly discriminative (both in multivariate discriminant analysis and univariate ANOVA) revealed the following:

- for maximum body depth, fishes of the Dabarsko and Fatničko karst fields were similar and significantly different from all others, while those of the Mokro karst field were significantly different from those of the Popovo and Ljubomirsko karst fields (Appendix 2);

- for head length, fishes of the Popovo karst field were significantly different from those of all other karst fields, although those of the Ljubomirsko karst field were at the very edge of significance, while those of the Mokro and Ljubomirsko karst fields were also significantly different, and fishes of all other karst fields were similar (Appendix 2);
- for maximum head width, fishes of the Dabarsko and Popovo karst fields were similar and significantly different from those of all other karst fields, whereas the fishes of the Mokro, Ljubomirsko, and Fatničko karst fields were similar to each other (Appendix 2);
- for interorbital width, fishes of the Fatničko and Mokro karst fields were significantly different from all others, those of the Ljubomirsko karst field were significantly different from all except the ones of the Dabarsko karst field, and the fishes of the Popovo karst field were significantly different from all the others (Appendix 3);
- for horizontal eye diameter, fishes of the Dabarsko karst field were similar to the ones of the Mokro, Fatničko, and Ljubomirsko karst fields, which were also similar to each other, whereas those of the Popovo karst field were significantly different from all of them (Appendix 3).

Sexual dimorphism (df = 1.70) was found to be connected to four characteristics in the pooled OTU sample (Appendix 4): caudal peduncle length (F = 12.977; $p < 0.001$), pelvic fin length (F = 7.788, p = 0.007), pectoral–pelvic fin distance (F = 11.617; p = 0.001), and pelvic–anal fin distance (F = 26.448; $p < 0.0001$). Namely, males have a longer caudal peduncle than females (23%–25% of SL vs. 21%–23% of SL), a shorter pectoral–pelvic distance (26%–28% vs. 28%–30%), a shorter pelvic–anal distance (16%–17% vs. 17%–19%), and longer pelvic fins (14%–16% vs. 11%–13%). The most specific characteristic and the greatest difference between males and females is the presence of a genital papilla in females. The genital orifice is situated approximately in the middle of its ventral surface, never at the tip of the fold.

3.2. Meristic characteristics

The meristic characteristics analysis revealed that all the *D. ghetaldii* in all the OTUs had three unbranched dorsal fin rays. The majority (96.1%) had seven branched dorsal fin rays, while the remainder (3.9%), all from the Fatničko karst field, had eight. There were seven anal fin branched rays in 93.4% of fishes and eight in 6.6% of fishes, which were again exclusively from the Fatničko karst field. In 72.3% of fishes, the number of predorsal vertebrae was 14, and in 27.7% it was 15. In 30.1% of fishes, the number of abdominal vertebrae was 21, in 61.4% it was 22, and in 8.5% it was 23. In 12.1% of fishes, the number of caudal vertebrae was 17, in 61.4% it was 18, and in 26.5% it was 19. The number of precaudal pterygiophores of the anal fin was two in 15.7%, three in 68.7%, and four in 15.6% of fishes. The numbers of total vertebrae were 38 in 4.8%, 39 in 18.1%, 40 in 53.0%, and 41 in 24.1% of fishes.

The CCA associated discrete states for all meristic characteristics with particular *D. ghetaldii* OTUs in the first two correspondence dimensions, with eigenvalues of $l_1 = 0.371$ and $l_2 = 0.301$. It revealed that the overall differentiation of meristic characteristics is weak, except for total vertebrae number. Testing the differences between OTUs for all meristic characteristics revealed a variation in numbers of occurrence using contingency tables. The discrete states of the five characteristics of branched anal fin rays, predorsal vertebrae, abdominal vertebrae, caudal vertebrae, and precaudal anal fin pterigiophores were tested by comparing the obtained c^2 values of 6.26, 11.36, 12.22, 11.67, and 13.22, respectively, with the critical value of $c^2 = 15.50$ for df = 4 at the probability level of p = 0.05, and this revealed that there were no significant differences in frequency of occurrence. The only meristic characteristic with a significant difference in frequency of occurrence between the five OTUs was total vertebrae number ($c^2 = 19.38$; df = 4; $p < 0.05$), mostly owing to the difference between the Popovo karst field OTU and all other OTUs (Table 3). For the majority of OTUs, total vertebrae numbers of 38 or 39 were the most common states of this characteristic occurring in *D. ghetaldii*. However, the characteristic state of 40 total vertebrae was associated with the *D. ghetaldii* OTU from the Popovo and Dabarsko karst fields, that of 41 with the Ljubomirsko karst field OTU, and that of 42 with the Mokro karst field OTU (Figure 5).

Table 3. Values of the pairwise c^2 test between *Delminichthys ghetaldii* OTUs from the karst fields (PP, Popovo; MP, Mokro; DA, Dabarsko; FP, Fatničko; LP, Ljubomirsko) based on total vertebrae number, the only characteristic that revealed significance in the CCA (above the diagonal) and degrees of freedom for each, with the only significant value ($p < 0.05$) in bold.

	PP	MP	DA	FP	LP
PP		7.252	**0.015**	0.531	1.363
MP	1		1.483	7.804	4.138
DA	1	2		0.812	0.604
FP	3	4	3		3.345
LP	1	2	1	4	

Notably, none of the states for this characteristic were associated with either sex (Figure 6). This suggests that the characteristic in question does not exhibit sexual dimorphism, meaning it is not a distinguishing feature between males and females. This lack of association indicates that this trait is not useful for sex differentiation in this particular context.

4. Discussion

The uneven number of individuals between the samples collected at particular karst fields might be due to the characteristics of the karst terrain and those karst fields specificities, affected by irregular or no flooding. It appears that, along with annual variations in the occurrence of floods, the duration of these flooding periods is greatly decreasing. This restricted opportunities for fishes to exit to the surface, and thus opportunities for sampling, but also affected their feeding and spawning, which is utterly important, considering that *Delminichthys ghetaldii* is already classified as a vulnerable species (VU) on the IUCN Red List.

4.1. General relationships between OTUs

There were obvious differences between *D. ghetaldii* from the five karst fields relative to their head shape (both length and width) and certain other morphometric characteristics (Appendix 1). Comparing to the literature, Dekić et al. (2017) stated that *D. ghetaldii* from the Fatničko and Ljubomirsko karst fields are different from each other in morphometry, while Mustafić et al. (2016) stated the same for those from the Dabarsko and Fatničko karst fields. The characteristics that exert the most substantial influence on the interspecies variation in the Dabarsko and Fatničko karst fields are body height and head length, which is in line with Mustafić et al. (2016). Although the ecological features of those localities differ, subterranean migrations have been postulated but never examined in detail. Our analysis by UPGMA clustering revealed a morphological similarity between the *D. ghetaldii* of the Dabarsko and Ljubomirsko OTUs, as well as between those of the Mokro and Fatničko OTUs (Figure 4).

It is indicative that particular morphometric characteristics that had unique variability, meaning deviation from the normal distribution when tested univariately, were prominent in discrimination. For example, horizontal eye diameter had the greatest discrimination power at discriminant root 2. It distinguished the OTUs of the Mokro and Fatničko karst fields and, in combination with interorbital width, determined the similarity between the two main clusters comprising OTUs of the other karst fields. Likewise, maximum head width distinguished OTUs of the Dabarsko and Popovo karst fields from all others. None

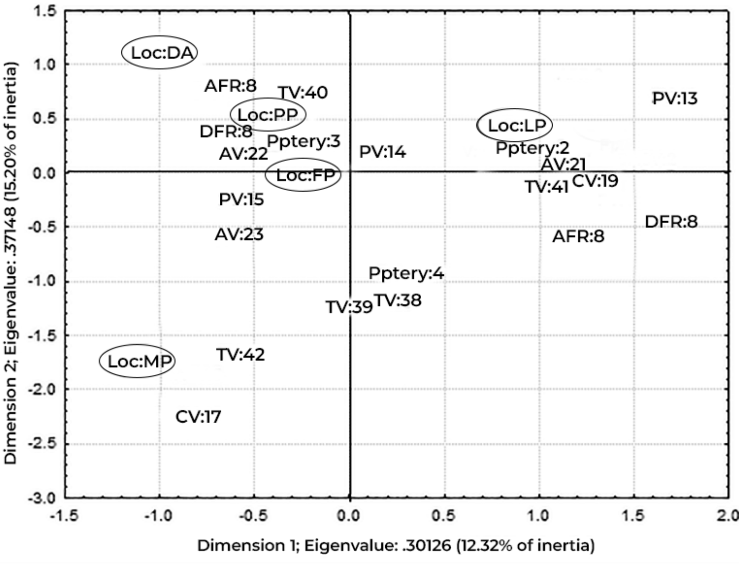

Figure 5. Relationship between *Delminichthys ghetaldii* OTUs (labeled as Loc and assigned as DA, Dabarsko Field; MP, Mokro Field; FP, Fatničko Field; PP, Popovo Field; LP, Ljubomirsko Field) and the discrete values of states (numeric integer values) for their meristic characters' (AFR, number of branched anal– fin rays; DFR, number of branched dorsal-fin rays ; Pptery, number of precaudal anal fin pterigophores; TV, total vertebrae number; AV, number of abdominal vertebrae; PV, number of dorsal vertebrae; CV, number of caudal vertebrae) in the space of the first two corresponding dimensions.

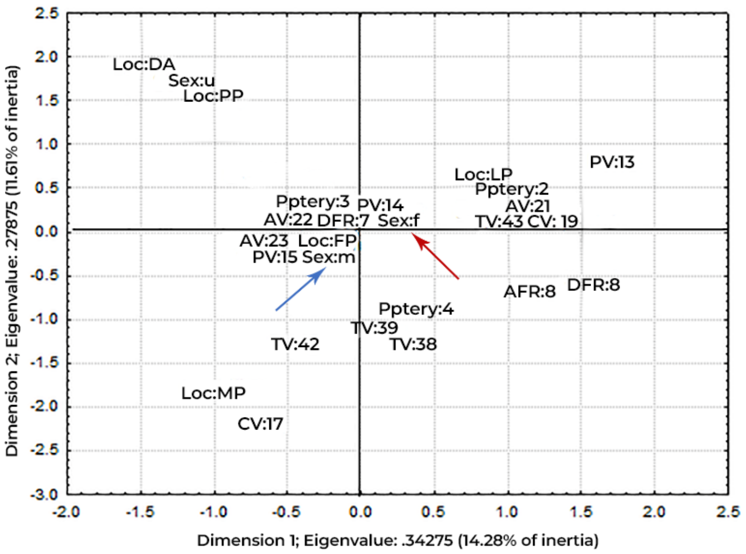

Figure 6. Relationship between sexes (m, males; f, females; u, undetermined; red arrow indicates position of females and blue arrow that of males) in all *Delminichthys ghetaldii* OTUs (labeled as Loc and assigned as DA, Dabarsko Field; MP, Mokro Field; FP, Fatničko Field; PP, Popovo Field; LP, Ljubomirsko Field) and the discrete values of states (numeric integer values) for their meristic characteristics (AFR, number of branched anal– fin rays; DFR, number of branched dorsal-fin rays ; Pptery, number of precaudal anal fin pterigophores; TV, total vertebrae number; AV, number of abdominal vertebrae; PV, number of dorsal vertebrae; CV, number of caudal vertebrae) in the space of the first two corresponding dimensions.

of the characteristics remarkable in sex differentiation deviated significantly from normal distribution, nor did they have great overall discrimination power, indicating weak sexual dimorphism in *D. ghetaldii.*

4.2. Correspondence between geology and morphology

Until the 1960s, the Trebišnjica River stood as Europe's longest sinking river and was possibly among the longest worldwide. It was a complex system of 326 km of interconnected river courses that extended from its source at Dobra Voda to Čemerna and ended in a confluence with the Dubrovnik River, which flowed into the Adriatic Sea. The confluence of the Krupa River with the Neretva River was also part of this intricate system. Along the way, the course alternated between submerged and resurgent stretches. Local inhabitants, unaware of the connections between these segments, bestowed distinct names upon each of these segments (Banjak, 2016). From the Viduša peaks, the confluence descended into the Fatničko karst field, where an underground bifurcation occurred (Milanović, 1979) and those sinking waters split into two branches, one directed toward the Trebišnjica basin and the other toward the Neretva River. This bifurcation appears to explain the similarity observed between *D. ghetaldii* OTUs from the Fatničko and Mokro karst fields. Notably, the Dabarsko karst field is a part of the drainage systems of both the Neretva and Trebišnjica rivers. The most significant sinkhole in this field is the Ponikva. The Vrijeka River, receiving the tributary Pribitul River, constitutes the primary watercourse that disappears in the Ponikva sinkhole (Pecelj, 1989). In the Ljubomirsko karst field, an underground connection to springs exists along the right bank of the Trebišnjica River. The resemblance of OTUs from these locations could be attributed to the sampling sites at the Vrijeka River in the Dabarsko karst field and at the Ljubomirski stream in the Ljubomirsko karst field, both of which have surface-flowing streams. This stands in contrast to the other fields, where *D. ghetaldii* individuals were sampled directly from inundated fields as they emerged due to cave precipitation or estavelles. *D. ghetaldii* of the Dabarsko karst field exhibits some shared continuous morphological characteristics, such as horizontal eye diameter and postorbital distance, with those from the Ljubomirsko karst field (Appendix 2). However, the most distinctive and isolated OTU arises from the Popovo karst field. Morphological differences among *D. ghetaldii* individuals from the Popovo field, especially in head length, maximum head width, interorbital width, and horizontal eye diameter, lend support to the notion of greater similarity between the OTUs of the Mokro and Fatničko karst fields compared to the OTUs of the Dabarsko and Ljubomirsko karst fields (Figure 4). This conclusion aligns with Steindachner (1882), who initially identified and described this species as *Paraphoxinus ghetaldii* while delineating another species in the Popovo karst field as *Paraphoxinus pstrossi*. Hence, he recognized a distinction between species from the Popovo karst field and those from other Eastern Herzegovina karst fields. This distinction persisted until recent molecular analyses, in which Palandačić et al. (2010), utilizing nuclear DNA sequences, established that *D. ghetaldii* of the Ljubomirsko karst field form a sister group to the *D. ghetaldii* populations in the Popovo karst field. The lower course of the Trebisnjica River flows through the Popovo karst field, which is different from other karst fields because it "bends with numerous curves", like a valley of "every other ordinary river" (Absolon, 1916), which correlates with the testimony of Cvijić (1918) that this karst field is a "pure result of river flow" (Spahić, 2015).

4.3. The impact of environmental factors on morphological differences

The primary factors affecting morphological variability in freshwater fish include water flow, the ecosystem they inhabit, predator presence, sexual dimorphism, and diet (Lostrom et al., 2015). Water flow is a key environmental factor influencing fish morphology. In conditions of strong water flow, fishes typically have more elongated and smaller bodies with more pronounced caudal peduncles, as this body shape reduces friction (Lostrom et al., 2015). In this study, individuals from the Ljubomirski stream, where the water flow is the fastest, had the smallest maximum body height and the longest caudal peduncle (Appendix 2).

Ichthyophagous fishes are widely recognized as an important factor shaping the body form of their prey and the composition, structure, and size of fish communities, either directly or indirectly through changes in behavior, habitat selection, or prey choice (Tonn et al., 1990). Body morphology can have an impact on the risk of predation. As examples, some fish species have spines or a greater body height, reducing their likelihood of being preyed upon (Holopainen and Pitkanen, 1985). One common way to avoid predators is through an escape response, with burst swimming being a typical fish reaction when threatened. Domenici et al. (2008) highlighted that fish with greater body height, such as *Carassius carassius*, achieve a higher speed and acceleration during escape responses compared to fish with smaller body height. This increase in body height is accompanied by greater muscle mass, enhancing acceleration performance. Among the studied locations, *D. ghetaldii* only coexist with the predatory *Squalius svalize* (Heckel and Kner, 1858) in the Popovo karst field, where the highest body heights were recorded, and only seven individuals were found, indicating that the density of *D. ghetaldii* is significantly higher in other karst fields without predators. Holopainen and Pitkanen (1985) also demonstrated that in the absence of predators in natural conditions, population density alone can affect fish body shape. Fish with smaller body heights and larger heads are typical in overpopulated communities dominated by a single species (such as small ponds), while fish with greater body heights are generally found in larger lakes with diverse ichthyofauna. Similar results were presented by Tonn et al. (1990), who noted that in high-density locations, individuals with smaller body heights are prevalent. However, transferring these individuals to less populated areas revealed a significant increase in body height even among larger fish that did not grow in length. Fish with smaller body heights were recorded in the Mokro and Ljubomirsko karst fields, where predators were absent and only *D. ghetaldii* occurred. Conversely, fish with greater body heights were recorded in the Popovo karst field, where predators are present and *D. ghetaldii* live in a sympatry with *Phoxinus karsticus* (Bianco and De Bonis, 2015) and *Squalius svalize* (Appendix 2).

Diet might also impact body shape. There is a notable difference between fish that feed on less vagile plankton, which cannot escape, and those preying on amphipods, a highly mobile prey (Parsons and Robinson, 2007). Fish that feed on chironomids typically have a larger head, bigger mouth, taller body, and smaller eyes compared to fish that feed on pelagic prey. *D. ghetaldii* have a broad feeding niche and feed predominantly on macrozoobenthos, but also on zooplankton (Berak Čihorić et al., 2024). The *D. ghetaldii* of the Mokro and Fatničko karst fields, where the diet is dominated by chironomids, have smaller eyes and longer upper and lower lips, whereas individuals of the Dabarsko and Ljubomirsko karst fields, where zooplankton is the main food source, have larger eyes but smaller mouths. Fish from the Popovo karst field feed mostly on nematodes and insect larvae (Berak Čihorić et al, 2024), and they exhibit the widest heads and smallest eyes (Appendix 3).

4.4. Morphometric features

Explaining the underlying causes of morphological distinctions among populations is often a complex task (Poulet et al., 2004). It has been suggested that the morphological characteristics of fish are determined by genetic factors, environmental conditions, and their interactions (Swain et al., 1999). Particularly critical are the environmental influences during early developmental phases, when an individual's phenotype is more susceptible to external stimuli (Pinheiro et al., 2005). Notably, morphological traits exhibit significant plasticity in response to variations in environmental parameters (Wimberger, 1992). As such, the discernible divergence in morphological features across our studied locations could be attributed to the distinct environmental contexts unique to each area. Additionally, differentiation among samples from neighboring stations may be due to geographic isolation. Various authors have extensively discussed the influence of environmental parameters on morphometric attributes in the context of fish population differentiation (Swain et al., 1999).

ANOVA revealed sexual dimorphism in 4 of the 21 morphometric characteristics studied. Specifically, males were discernibly distinct from females by exhibiting shorter pectoral–pelvic and pelvic–anal distances, and longer pelvic fin and caudal peduncle lengths. These findings align with those of Bogutskaya and Zupančič (2003); however, that study also identified sexual dimorphism in longer pectoral fins among males, a trait that fell marginally short of statistical significance ($p = 0.0568$) in this study. Furthermore, a greater depth of the dorsal fin was statistically significant among individuals of unspecified sex, suggesting the presence of distinct sexual dimorphism. In light of these disparities, it is reasonable to assume that three individuals from the Dabarsko karst field, for whom sex was not designated, were males, while one was female (Appendix 4). Overall, this study substantiates the notion that sexual dimorphism is relatively subtle, with males and females appearing nearly identical to the naked eye, save for the expressed genital papilla observed in females. It appears as a thickened triangular fold with the anus at its base on the ventral surface. The genital orifice is positioned roughly in the middle of the ventral surface, never at the tip of the fold. The papilla is already present in juvenile females (39–58 mm SL), though it is narrow and elongated. In adult females (approximately 53–58 mm SL), it is significantly larger, with a wide base and extending over the lower part of the first anal-fin rays.

4.5. Meristic features

There were no significant differences in meristic characteristics between males and females in *D. ghetaldii*. The abdominal region is long, commonly exhibiting a vertebral formula of 22 + 18 or 21 + 18, accompanied by 14–15, and less frequently 13, predorsal vertebrae. Similarly, 21–23 abdominal vertebrae and 17–19 caudal vertebrae typify the species. The range of anal fin pterygiophores in front of the first caudal haemal spine spans from 2 to 4, while the number of total vertebrae is between 38 and 42. These findings align completely with the studies by Bogutskaya and Zupančič (2003), particularly in relation to the prevailing vertebral formulae of 21 + 18 and the frequently observed 14 predorsal vertebrae. Likewise, their documented number of anal fin pterygiophores in front of the first caudal haemal spine is usually 3, sometimes 4, and rarely 2, which is in line with our research outcomes. Principally, uniformity persisted within the dorsal fin's unbranched ray count across all five fields, as did the consistent quantity of branched rays within the anal fin among the compared OTUs. Furthermore, almost the same number of dorsal fin branched rays persisted across the fields. A minor range of variation was evident in the number of precaudal anal fin pterygiophores within all OTUs, as well as the total vertebrae count. Finally, these meristic characteristics collectively suggested a lack of discernible divergence between sexes.

In conclusion, the results of this study underscore the substantive influence of the distinct karst fields on the morphometric variability manifested in *D. ghetaldii*. These findings reveal a heterogenous morphology among the *D. ghetaldii* OTUs across the five Eastern Herzegovina karst fields. In addition, disparities were identified between sexes for four morphometric characteristics. It is of note that meristic osteological characteristics within the five *D. ghetaldii* OTUs, as well as between sexes, did not show significant differentiation. These insightful findings contribute significantly to the enhanced understanding of the biology of this vulnerable species, laying a foundational framework for an effective conservation strategy across the broader area.

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Conflict of interest

The authors declare no conflicts of interest.

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Appendix 1. Arithmetic means ($\overline{x}$) with their standard errors (*SE*) and ANOVA F values (*F*) with the degrees of freedom (df = 3. 70) for 21 morphometric characteristics, with the level of significance (p) denoting significance of difference between OTUs (*n* denotes sample size of each OTU; index at each of sample sizes and means denotes OTUs, i.e., karst fields: PP, Popovo; MP, Mokro; DA, Dabarsko; FP, Fatničko; LP, Ljubomirsko).

Characteristic	$\overline{x}_{PP} \pm SE_{PP}$ ($n_{PP = 7)}$	$\overline{x}_{MP} \pm SE_{MP}$ ($n_{MP=6)}$	$\overline{x}_{DP} \pm SE_{DP}$ ($n_{DA = 4)}$	$\overline{x}_{FP} \pm SE_{FP}$ ($n_{FP=35)}$	$\overline{x}_{LP} \pm SE_{LP}$ ($n_{LP =24)}$	$F_{(3,.70)}$	p
Snout length	0.078±0.0021	0.097±0.0039	0.0950.0019	0.094±0.0018	0.091±0.0013	3.456	0.0209
Horizontal eye diameter	0.044±0.0011	0.065±0.0047	0.079±0.0011	0.081±0.0015	0.079±0.0016	47.211	0.0000
Postorbital distance	0.152±0.0022	0.158±0.0022	0.146±0.0011	0.162±0.0041	0.146±0.0024	4.014	0.0107
Head length	0.286±0.0112	0.355±0.0119	0.340±0.0056	0.330±0.0053	0.317±0.0039	6.362	0.0007
Maximum head width	0.155±0.0048	0.139±0.0027	0.170±0.0014	0.139±0.0014	0.143±0.0017	8.884	0.0000
Interorbital width	0.110±0.0032	0.068±0.0033	0.097±0.0009	0.076±0.0012	0.095±0.0013	71.631	0.0000
Upper jaw length	0.060±0.0014	0.070±0.0029	0.065±0.0016	0.067±0.0012	0.063±0.0010	6.314	0.0007
Lower jaw length	0.071±0.0020	0.083±0.0037	0.078±0.0028	0.078±0.0015	0.077±0.0010	2.074	0.1115
Maximum body depth	0.215±0.0052	0.191±0.0067	0.174±0.0067	0.154±0.0040	0.201±0.0024	36.726	0.0000
Caudal peduncle depth	0.085±0.0007	0.099±0.0031	0.090±0.0034	0.081±0.0017	0.105±0.0026	16.412	0.0000
Predorsal length	0.602±0.0139	0.548±0.0085	0.549±0.0175	0.563±0.0066	0.550±0.0072	3.163	0.0290
Postdorsal length	0.361±0.0107	0.370±0.0137	0.334±0.0074	0.361±0.0058	0.347±0.0054	1.573	0.2035
Prepelviclength	0.575±0.0098	0.525±0.0123	0.523±0.0093	0.5450.0081	0.5050.0083	5.422	0.0021
Preanallength	0.735±0.0118	0.674±0.0119	0.666±0.0117	0.700±0.0087	0.657±0.0085	5.739	0.0014
Caudal peduncle length	0.233±0.0141	0.218±0.0125	0.254±0.0091	0.216±0.0039	0.222±0.0049	1.958	0.1282
Dorsal fin length	0.092±0.0047	0.071±0.0029	0.096±0.0011	0.081±0.0017	0.099±0.0029	16.773	0.0000
Dorsal fin depth	0.148±0.0049	0.166±0.0049	0.188±0.0025	0.157±0.0037	0.180±0.0053	6.588	0.0005
Pectoral fin length	0.146±0.0062	0.174±0.0058	0.138±0.0013	0.149±0.0033	0.174±0.0053	7.635	0.0002
Pelvic fin length	0.113±0.0055	0.119±0.0099	0.116±0.0051	0.109±0.0037	0.122±0.0052	2.702	0.0521
Pectoral-pelvic fin dist.	0.263±0.0103	0.263±0.0065	0.258±0.0051	0.261±0.0047	0.244±0.0043	3.710	0.0154
Pelvic-anal fin distance	0.156±0.0049	0.158±0.0074	0.150±0.0056	0.165±0.0030	0.176±0.0028	3.509	0.0197

Appendix 2. Results of the *post-hoc* Tukey HSD testing between five OTUs of *Delminichthys ghetaldii* from karst fields (PP, Popovo; MP, Mokro; DA, Dabarsko; FP, Fatničko; LP, Ljubomirsko) for characteristics that were with the strongest discriminating power between them (df = 69).

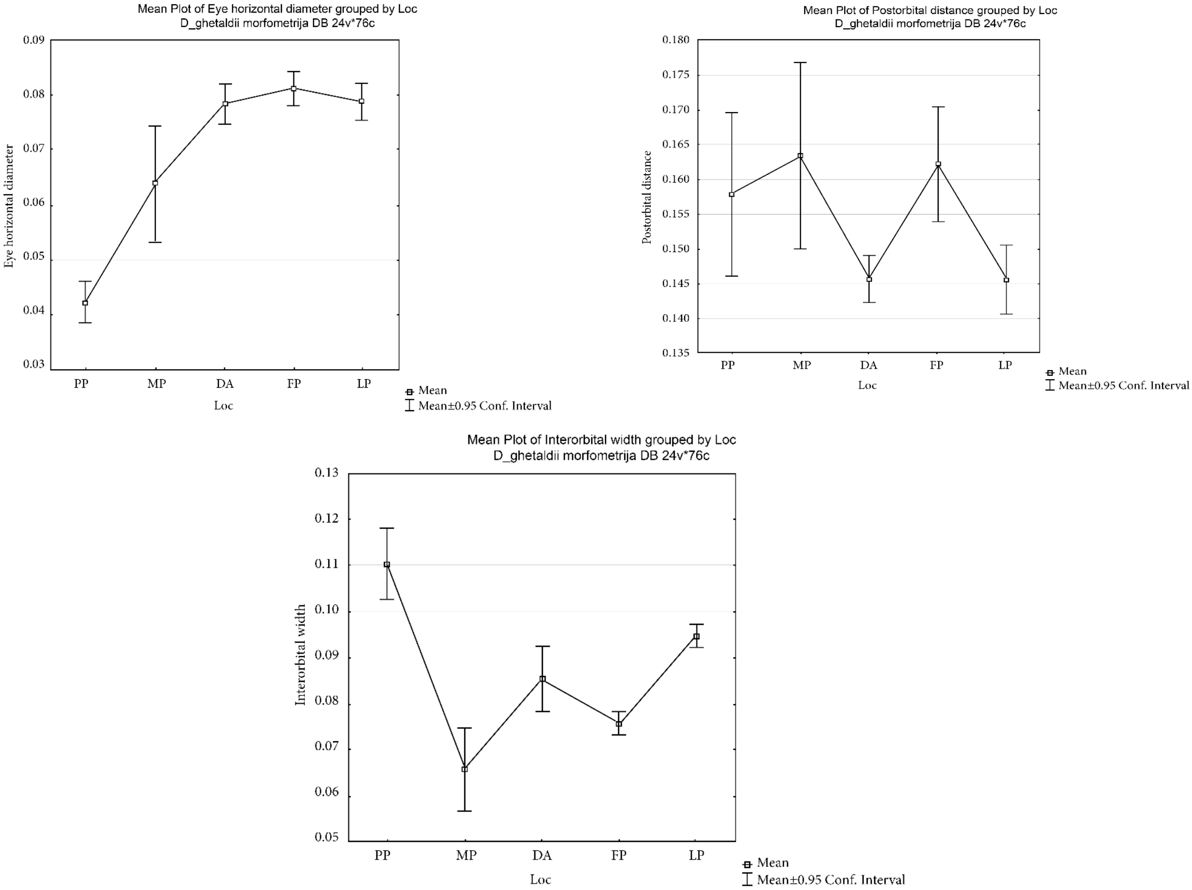
Horizontal eye diameter	PP	MP	DA	FP	LP
PP		0.000	0.000	0.000	0.000
MP			0.141	0.001	0.012
DA				0.966	1.000
FP					0.809
LP					

Head length	PP	MP	DA	FP	LP
PP		0.001	0.017	0.002	0.070
MP			0.923	0.294	0.041
DA				0.948	0.504
FP					0.386
LP					

Maxium head width	PP	MP	DA	FP	LP
PP		0.009	0.061	0.000	0.007
MP			0.000	1.000	0.861
DA				0.000	0.000
FP					0.382
LP					

Interorbital width	PP	MP	DA	FP	LP
PP		0.000	0.023	0.000	0.000
MP			0.000	0.155	0.000
DA				0.000	0.978
FP					0.000
LP					

Maximum body depth	PP	MP	DA	FP	LP
PP		0.190	0.007	0.000	0.368
MP			0.661	0.001	0.839
DA				0.280	0.078
FP					0.000
LP					



Appendix 3. Results of the Box plots graphs five OTUs of *Delminichthys ghetaldii* from karst fields (PP, Popovo; MP, Mokro; DA, Dabarsko; FP, Fatničko; LP, Ljubomirsko) for characteristics that show with the strongest similarity as well as discriminating power between them

Appendix 4. Sexual dimorphism ($\overline{x}$), arithmetic mean; *SE,* standard error of mean; *F*, ANOVA F value, with the degrees of freedom (df = 1. 70); p, level of signigicance; indices "m","f" in subscript denote males and females, respectively) in the pooled OTUs of *D. ghetaldii*, as revealed by univariate ANOVA testing between sexes.

Characteristic	$\overline{x}_{m} \pm SE_{m}$ (Nm=33)	$\overline{x}_{f} \pm SE_{f}$ (Nf=39)	$F_{(1.70)}$	p
Snout length	0.091±0.0016	0.091±0.0017	0.442	0.5084
Horizontal eye diameter	0.075±0.0023	0.076±0.0024	0.873	0.3535
Postorbital distance	0.155±0.0036	0.155±0.0031	0.663	0.4183
Head length	0.329±0.0050	0.317±0.0052	0.256	0.6143
Maximum head width	0.142±0.0019	0.142±0.0013	1.473	0.2289
Interorbital width	0.081±0.0023	0.089±0.0024	0.325	0.5701
Upper jaw length	0.065±0.0011	0.065±0.0011	3.059	0.0847
Lower jaw length	0.078±0.0015	0.077±0.0011	0.016	0.8998
Maximum body depth	0.173±0.0051	0.183±0.0052	0.623	0.4327
Caudal peduncle depth	0.088±0.0026	0.093±0.0025	1.512	0.2231
Predorsal length	0.560±0.0077	0.562±0.0055	0.005	0.9440
Postdorsal length	0.360±0.0056	0.354±0.0055	0.046	0.8299
Prepelvic length	0.531±0.0091	0.534±0.0070	0.455	0.5024
Preanal length	0.683±0.0091	0.690±0.0083	0.519	0.4738
Caudal peduncle length	0.228±0.0045	0.212±0.0035	12.977	0.0006
Dorsal fin length	0.085±0.0028	0.089±0.0022	0.486	0.4882
Dorsal fin depth	0.168±0.0049	0.161±0.0034	3.847	0.0538
*P*ectoral fin length	0.163±0.0045	0.155±0.0037	3.477	0.0665
Pelvic fin length	0.121±0.0041	0.109±0.0034	7.788	0.0070
Pectoral-pelvic fin dist.	0.248±0.0051	0.262±0.0033	11.617	0.0011
Pelvic-anal fin distance	0.158±0.0026	0.176±0.0023	26.448	0.0001