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Past and Present Environmental Factors Differentially Influence Genetic and Morphological Traits of Italian Barbels (Pisces: *Cyprinidae*)

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Abstract: Local adaptation and phenotypic plasticity can lead to environment-related morphological and genetic variations in freshwater fish. Studying the responses of fish to environmental changes is crucial to understand their vulnerability to human-induced changes. Here, we used a latitudinal gradient as a proxy for past and present environmental factors and tested its influences on both genetic and morphological patterns. We selected as a suitable biogeographic model, the barbels, which inhabit 17 Adriatic basins of the central-southern Italian Peninsula, and explored association among attributes from genetic, morphological, and environmental analyses. The analysis of the mitochondrial DNA control region evidenced a southward significant increase in the number of private haplotypes, supporting the isolation of the southernmost populations related to the Mio-Pleistocene events. In contrast, morphology was mainly affected by changes in the present environmental conditions. Particularly, the number of scales and fish coloration were clearly associated to latitude, and thus thermal and hydrological conditions. Other morphometric and functional traits varied under the selective pressure of other environmental factors like elevation and distance from headwater. These results highlight the sensitivity of barbels to climate changes, which can serve as a basis for future eco-evolutionary and conservation studies.

Keywords: phenotypic plasticity; genetic diversity; geometric morphometrics; functional traits; meristic traits; environmental influence; climate change; latitude; temperature; freshwater fish



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1. Introduction

In lotic ecosystems, environmental conditions (e.g., hydrological regime, channel and substrate characteristics, and water physico-chemical parameters) play an active role in modelling fish morphology relevant to motion and trophic adaptation [1–4]. Different availability of habitats and resources may determine different ecological niches for fish populations inhabiting distinct rivers. According to the niche variation hypothesis [5], niche width variation at the population level, mainly resulting from the increased variation at the between-individual level, is accompanied by greater morphological and genetic variations [6–9]. Phenotypic variations in fish may result in fixed traits (i.e., traits that do not change as fast as the environment [10]), which arise from adaptation following natural selection or other evolutionary forces, or in plastic response to changing environments (phenotypic plasticity) [11] to the point that both populations of fish belonging to the same species or to different species may display both morphological and genetic differences reflecting the environmental conditions of the rivers they inhabit [12–15].

Global changes, particularly the current climate change, are modifying both river habitats and resources [16,17], and could thus lead to changes in the ecological niches

of fish populations. Both air temperature increases and a decrease in low-flow values are causing a relevant increment of water temperature in many rivers [18–22], which is one of the, if not the, most important drivers of phenotypic variations in fish [23–25]. Since temperature and other environmental factors such as hydrology, photoperiod, length of growing season, and productivity vary with latitude [26], the study of morphological and genetic differences of fish populations along latitudinal gradients may provide important indications of the effects of current climate changes.

Variation in meristic traits in fish is mainly expected as a consequence of the current temperature increase and of changes in other environmental parameters such as salinity and dissolved oxygen [27]. In contrast, genetic diversity, particularly in neutral (i.e., not under selection) DNA regions, such as those commonly used for population genetic studies, typically reflect historical demographic evolution due to past climatic and geological changes e.g., [28]. Nevertheless, both genetic and phenotypic variabilities can also be altered by recent anthropogenic activities such as the introduction of exotic species (e.g., following hybridization [29]) and river damming [30–32]. Thus, separating genetic and phenotypic variations induced by local adaptation developed during historical evolution from the variations deriving by relatively recent events, provides valuable insights for both eco-evolutionary processes and for a conservation perspective [33].

Despite its relatively small area (approximately 300,000 km²), the Italian Peninsula is characterized by a high environmental heterogeneity due to the complex structure of its orography (40% of its area is mountainous), and relevant biogeographical and geological events [34]. The wide latitudinal extension of the peninsula (approximately 10 degrees) places it between different climatic zones (continental, temperate, and Mediterranean zones), which affect the hydrography and the hydrological regime of Italian watercourses [35]. In particular, watercourses on the Adriatic side of central-southern Italy are influenced by the presence of the Apennines, are relatively limited in length (<100 km), and flow directly into the sea. Moving southward, these rivers have a hydrological regime increasingly prone to intermittency [36], with climate change also contributing to water scarcity via altered runoff [37]. Changes in conditions of the river environment along the latitudinal gradient may have influenced both morphological and genetic traits of fish, thus making Italy an ideal geographical model to investigate the effects of changes in temperature and hydrological conditions on freshwater fish.

Barbels (genus *Barbus* Daudin, 1805) are among the most widespread primary freshwater fishes inhabiting different geographic districts of Europe including the Italian Peninsula [38]. They consist of a diverse group of medium to large size cyprinids with a high level of endemism and varied ecology [38–40]. Four of the six native barbel species are fluviolacustrine inhabiting middle and lower reaches of the rivers: Padanian barbel *Barbus plebejus* Bonaparte, 1839, Tiber barbel *Barbus tyberinus* Bonaparte, 1839, *Barbus samniticus* Lorenzoni et al., 2021, and *Barbus fucini* Costa, 1853. These species are vicariant in different ichthyogeographic districts: Padano-Venetian, Tuscany-Latium, northern Apulia-Campania, and southern Apulia-Campania, respectively [38,41]. Specifically, three species inhabit the basins flowing into the Adriatic Sea: *B. plebejus* is present from the Krka River in Croatia to the Tronto River in Italy including the Po River basin (i.e., the largest Italian basin, with a 71,000 km² area), *B. samniticus* is present from the Vomano River to the Biferno River, and *B. fucini* was detected in the Fortore and Ofanto basins [38]. Speciation in the mentioned districts and basins started following the Messinian “Lago Mare” phase of the Mediterranean Sea (6.1–5.1 MYA–Miocene), and species distribution was further influenced by Pleistocenic geomorphological events. After the last glacial maximum (Würm glacial phase 15,000–18,000 years ago), when the paleo-Po basin reached the current Vomano basin and biological homogenization occurred, the independent basins flowing into the middle-Adriatic Sea started to isolate from the south to the north due to marine regression [42]. Only a few characteristics allow the discrimination between the four vicariant Italian fluviolacustrine barbels [38,43–45]. Also, the trait mainly examined to discriminate among *B. plebejus* and *B. tyberinus* in the field, i.e., the number (and therefore size) of scales

along the lateral line [46,47], can lead to erroneous species attribution [43,45] since it is likely influenced by environmental conditions like for other meristic traits [38].

Here we used fluviolacustrine barbel populations distributed along the Italian Peninsula to disentangle the influence on morphology and genetic diversity of Mio-Pleistocene isolation of watersheds compared to recent habitat features. This was done by comparing genetic and phenotypic variability along a latitudinal gradient that can be seen as a proxy of changes in river environmental conditions. Thus, we present a study on both the genetic and morphological characterization of barbel populations inhabiting 17 rivers distributed from north to south along the Adriatic side of central-southern Italy (Figure 1) including three out of four Italian ichthyogeographic districts (Padano-Venetian, northern and southern Apulia-Campania). We posited that: i) the genetic variability of the studied populations is related to the geomorphological evolution of the river basins they inhabit with no relation with current environmental conditions, and ii) both intra- and inter-specific morphological variations are related to local environmental conditions, representing a response to the present habitat features potentially varying due to climate change. For this purpose, we employed different approaches including morphological and molecular analyses, and environmental investigation.

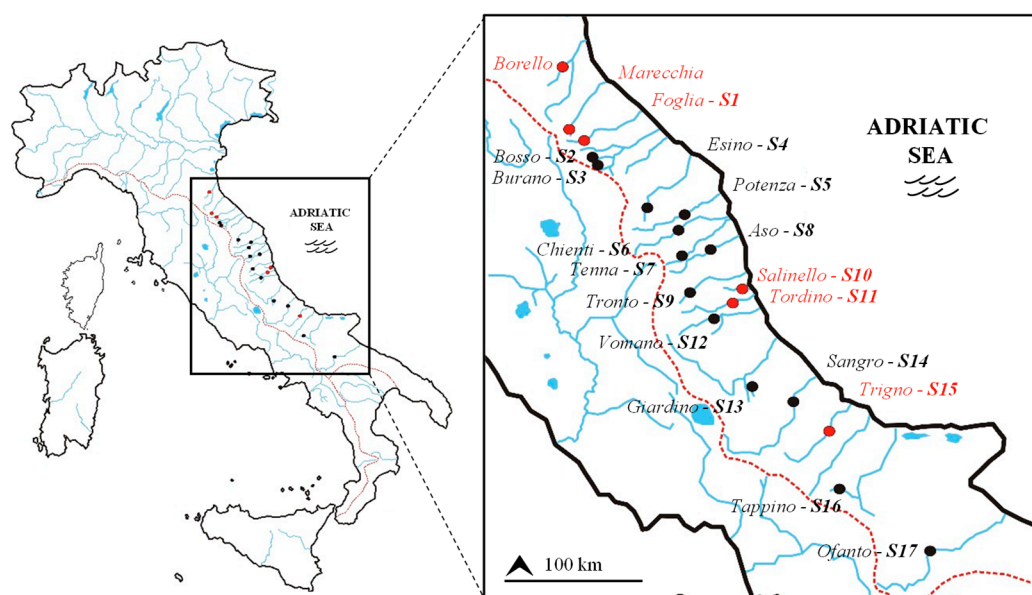


Figure 1. Map of Italy with the new sampling sites (red circles) and the locations of the other purebred barbel populations considered in the present study (black circles). The dashed red line indicates the watershed.

2. Materials and Methods

2.1. Study Area and Barbel Samples

In previous works [41,45], we genetically and morphologically characterized 24 barbel populations from sampling sites within Adriatic basins in central-southern Italy. Since some populations were affected by the invasion of the exotic fluviolacustrine European barbel *Barbus barbus* L. hybridizing with the native Italian species [48–51], thus eroding their genotypes and altering their morphologies [44,51], we tried to find additional purebred populations in vicinity to increase the dataset size. Thus, in August 2020 we sampled barbel populations (approximately 20–30 individuals per population) from six Italian rivers (Borello, Marecchia, Foglia, Salinello, Tordino, and Trigno) located on the Adriatic side of central-southern Italy (Figure 1 and Table 1).

Table 1. Main characteristics of the sampling sites, and genetic attribution of barbel populations. ID = site/population identification code, Ng = number of samples used for genetic (D-loop) analysis, Nm = number of samples used for morphological analysis.

Watercourse	Basin	Latitude (N)	Longitude (E)	Elevation (m)	Distance from Headwater (km)	Species	ID	Ng	Nm	References
Borello	Savio	44°3'8.1"	12°10'47"	77	30	No purebred	-	-	-	This study
Marecchia	Marecchia	43°49'35.2"	12°15'2.4"	335	27	No purebred	-	-	-	This study
Foglia	Foglia	43°45'1.2"	12°27'13.3"	300	25	<i>B. plebejus</i>	S1	13	23	This study
Bosso	Metauro	43°30'49.8"	12°33'42.8"	398	10	<i>B. plebejus</i>	S2	43	10	[45]
Burano	Metauro	43°28'48.3"	12°37'16.4"	341	14	<i>B. plebejus</i>	S3	14	10	[45]
Esino	Esino	43°24'24.4"	12°58'47"	237	19	<i>B. plebejus</i>	S4	26	10	[45]
Potenza	Potenza	43°16'59.8"	13°20'5.1"	150	45	<i>B. plebejus</i>	S5	25	10	[45]
Chienti	Chienti	43°9'35.4"	13°14'37"	268	40	<i>B. plebejus</i>	S6	34	10	[45]
Tenna	Tenna	43°1'10.6"	13°25'34"	363	14	<i>B. plebejus</i>	S7	19	10	[45]
Aso	Aso	43°1'25.5"	13°37'20.1"	185	38	<i>B. plebejus</i>	S8	27	10	[45]
Tronto	Tronto	42°46'12.2"	13°24'29.4"	370	39	<i>B. plebejus</i>	S9	28	10	[45]
Salinello	Salinello	42°47'17"	13°53'28.2"	28	39	<i>B. plebejus</i>	S10	21	29	This study
Tordino	Tordino	42°42'8"	13°49'49.9"	97	44	<i>B. plebejus</i>	S11	19	31	This study
Vomano	Vomano	42°35'21.6"	13°40'51.4"	203	33	<i>B. samniticus</i>	S12	25	10	[45]
Giardino	Aterno-Pescara	42°10'25.8"	13°49'51.4"	247	0.5	<i>B. samniticus</i>	S13	24	10	[41]
Sangro	Sangro	42°3'10.7"	14°20'51.3"	116	93	<i>B. samniticus</i>	S14	23	10	[41]
Trigno	Trigno	41°54'14.2"	14°38'43.7"	121	61	<i>B. samniticus</i>	S15	18	23	This study
Tappino	Fortore	41°33'13.2"	14°52'33.9"	239	30	<i>B. fucini</i>	S16	27	10	[41]
Ofanto	Ofanto	41°4'26.1"	15°32'46.4"	219	74	<i>B. fucini</i>	S17	20	10	[41]

Following their collection by electric fishing, fish were anaesthetized (MS-222) and photographed for morphological analysis from their left side using a Nikon D300 camera (24–85 mm lens) positioned by means of a tripod on a table. Scales were removed from the left side of each fish and stored in 70% ethanol for age determination. Scalimetry was performed by two operators independently [52] under a stereomicroscope coupled with a camera with images stored within an archive built using the image analysis system IAS 2000 (QEA's IASLab software). Determination done by two independent readers allowed to control for potential errors in age determination by applying a quality control approach for which the independent determinations were cross-checked and in case of mismatches, the scale was redetermined or discarded. Although age determination tends to overestimate fish ages [53], this approach allowed us to select fish of relatively similar age (see Section 2.3). A caudal-fin clip was taken, and samples were stored in 100% ethanol for genetic analysis. Following their recovery, all fish were released at their capture sites.

2.2. Genetic Data

Total genomic DNA was extracted from selected individuals using a proteinase K digestion, followed by sodium chloride extraction and ethanol precipitation (i.e., salting out method [54]). First, for excluding populations invaded by *B. barbuis*, the mitochondrial cytochrome b of a few individuals (4–10) per population was amplified through PCR using primers L15267 and H16461 [55], and a fragment of 1121 bp was analyzed. Then, a fragment (867 bp) of the mitochondrial control region (D-loop) locus from 13–21 individuals belonging to purebred populations was amplified using D-loops × F and D-loopd × R [56,57] primers pair (Table 1; Borello and Marecchia populations were excluded from further analysis due to the presence of *B. barbuis*).

Amplifications were performed for both loci using the Q5 High-Fidelity Master Mix (New England Biolabs Inc., Ipswich, Massachusetts, MA, USA) in a 10 µL reaction volume containing 10 ng of template DNA and 0.25 µM of each primer. The PCR profile was set with an initial denaturation at 98 °C for 3 min followed by 35 cycles of 30 s at 98 °C, 90 s at 55 °C, and 90 s at 72 °C, with a final extension step at 72 °C for 10 min. Amplicons were then purified using EuroSAP PCR Enzymatic Clean-up kit (EuroClone s.p.a, Pero, Milan)

and sequenced with single pass by MacroGen Europe B. V. on a 3730XL DNA sequencer. Sequences were aligned using CLUSTAL-W [58], as implemented in Bioedit software [59], and further checked manually to eliminate ambiguities. All the haplotypes detected in this study were deposited in GenBank (ON661665-ON661675 for the D-loop mtDNA). Species attribution was based on the genetic results presented in this and previous studies [41,45].

2.3. Morphological Data

Morphological data were collected from photos taken from adult fish of similar age (2+, 3+, and 4+) to reduce potential allometric bias (Table 1). The sex of the fish was not determined due to the non-lethal nature of our sampling. Thus, potential sources of variation due to sexual dimorphisms were not controlled even though these should be limited to the anal fin [38,47]. For geometric morphometrics, 33 landmarks (Figure 2a) were captured from each photo using the R [60] Geomorph function “digitize2d” [61].

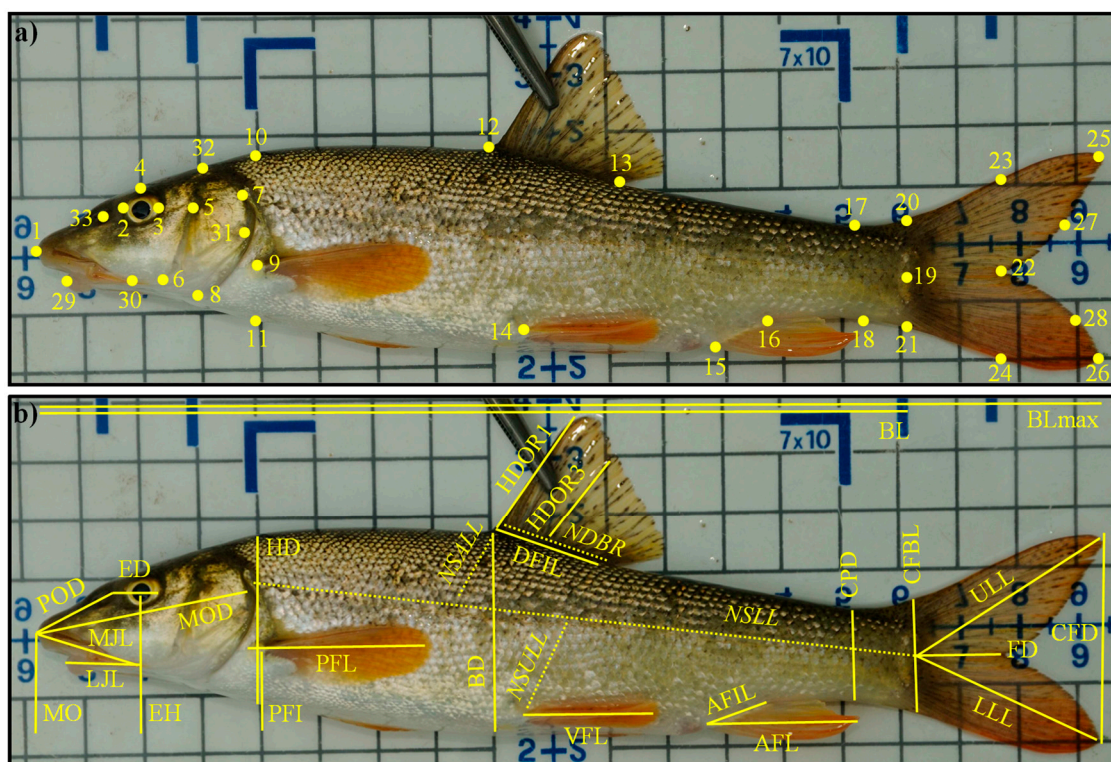


Figure 2. (a) Position of the 33 landmarks used for body shape analysis: (1) anterior tip of snout, (2, 3) anterior and posterior end of the eye, (4) orthogonal projection on the dorsal profile of the eye center, (5) lateral projection of the eye center on the insertion of the operculum, (6) intersection of the operculum at the lateral profile, (7, 8) ventral and dorsal end of gills, (9) anterior insertion of pectoral fin, (10, 11) orthogonal projections on the dorsal and ventral profile of the anterior insertion of pectoral fin, (12,13) anterior and posterior insertion of dorsal fin, (14) insertion of pelvic fin, (15, 16) posterior and anterior insertion of anal fin, (17, 18) anterior attachment of dorsal and ventral membrane of caudal fin, (19) base of middle caudal rays, (20, 21) orthogonal projections on the dorsal and ventral profile of the base of middle caudal rays, (22) fork, (23, 24) orthogonal projections on the dorsal and ventral profile of fork, (25, 26) end of the upper and lower lobe of caudal fin, (27, 28) lateral projections of anterior attachment of dorsal and ventral membrane of caudal fin, (29) opening of mouth, (30) posterior end of jaw, (31) posterior-most edge of opercle, (32) supraoccipital, posteromedial tip (visible as an indentation in dorsal surface), (33) posterior nare, posterior margin. (b) The 25 morphometric traits (BLmax, maximum body length; BL, body (standard) length;

MOD, mouth-operculum distance; POD, pre-orbital distance; HD, head depth; ED, eye diameter; EH, eye position; MO, oral gape position; MJL, maxillary jaw length; LLL, lower jaw length; BD, maximum body depth (i.e., maximum height); HDOR1, height of the dorsal fin ossified ray; HDOR3, height of the third dorsal fin branched ray; DFIL, dorsal fin insertion length; AFL, anal fin length; AFIL, anal fin insertion length; PFL, pectoral fin length; PFI, pectoral fin position; VFL, ventral fin length; CPD, minimum caudal peduncle depth (i.e., minimum height); CFD, maximum caudal fin depth; CFBL, caudal fin base length; FD, fork depth; LLL, length of the lower lobe of caudal fin; ULL, length of the upper lobe of caudal fin) and 4 meristic traits (NDBR, the number of dorsal fin branched rays; NSLL, the number of scales on the lateral line, and on rows above–NSALL–and under–NSULL–the lateral line) considered for morphological analysis.

For common morphological analysis, a total of 40 traits (25 morphometric and 15 meristic traits) were analyzed. In addition to the four meristics showed in Figure 2b, coloration (presence or absence of dots on body–Db, scales–Ds, dorsal fin–Ddf, anal fin–Daf, and caudal fin–Dcf, spots on body–Sb, external pigmentation on scales–EPs, and color of ventral–Cvf, anal–Caf, dorsal–Cdf, and caudal–Ccf–fin) was examined. Fin color was determined independently by two observers following the descriptions reported in previous studies [39,46,47]. Morphometric measurements were standardized to the overall mean standard length [62] to further reduce the effects of size and allometry.

For the analysis of functional traits, 10 ratios of morphological measurements mainly associated with food acquisition and locomotion were taken into consideration [63] (Table 2 and Figure 2b). The knowledge of the relationship between functional morphology and environmental gradients allows for better predictions of the organisms' responses to environmental change [64,65].

Table 2. List of ten functional traits associated with food acquisition and locomotion (slightly modified from Xiang et al. [63], see Figure 2b).

Functional Trait	Code	Formula	Potential Link with Fish Functions	References
Maximum body length	BLmax	BLmax	Size is linked to metabolism, trophic impacts, locomotion ability, nutrient cycling	[66]
Body elongation	BE	BL/BD	Hydrodynamism	[67]
Eye vertical position	EVP	EH/BD	Position of fish and/or of its prey in the water column	[68]
Relative eye size	RES	ED/HD	Visual acuity	[69]
Oral gape position	OGP	MO/BD	Feeding position in the water column	[70,71]
Relative maxillary length	RML	MJL/HD	Size of mouth and strength of jaw	[66]
Body lateral shape	BLS	HD/BD	Hydrodynamism and head size	[66]
Pectoral fin vertical position	PFVP	PFI/BD	Pectoral fin use for swimming	[70]
Pectoral fin size	PFS	PFL/BL	Pectoral fin use for swimming	[72]
Caudal peduncle throttling	CPT	CFD/CPD	Caudal propulsion efficiency through reduction of drag	[73]

2.4. Environmental Data

At each site in Table 1, the percentage coverages of different riverbed substrates, different mesohabitats, shading, plants, and refuges were detected as well as the average depth and width (Table S1). Moreover, geological elements and land use were calculated at basin scale (Table S1) using QGIS [74], and geolithological and land use maps [75,76]. The climate characterizing the different watersheds was determined by downloading data on temperature and precipitation (Table S1) recorded at the meteorological station closest to the barbel sampling sites from SCIA [77]; the average values for the period 1998–2018 were considered for the analysis. In order to consider the water chemistry and benthos status of barbel habitats *sensu* Water Framework Directive (2000/60/EC), the mean values of LIMeco and STAR_ICMi indices [78,79] relative to the river reaches near the sampling sites (Table S1) were collected from reports by regional environmental protection agencies for the period 2015–2017 [80–83]. The total river length and the quotient of average flow at

the river outlet and total basin area (Q/A) were also retrieved [84–87] and the latter was used as indicator of the river hydrological regime (Table S1).

2.5. Data Analysis

2.5.1. Genetic Data

Number of haplotypes (n) and polymorphic sites (S) were calculated for each barbel population, and both nucleotide (π) and haplotype (H) diversity indices were estimated using DnaSP v6.12.01 [88]. Moreover, the relative frequency of population-specific haplotypes (PSh) was used as a proxy of genetic isolation. To visualize the geographical distribution of haplotypes and relative frequencies, a median-joining network was created through the software PopArt [89].

Linear regression of all genetic parameters with latitude was tested using XLSTAT2014 software, and only significant regressions ($p \leq 0.05$) were reported in this study.

2.5.2. Morphological Data

Generalized Procrustes analysis was performed to remove non-shape variations, occurring through variation in position, orientation, and size, as implemented in MorphoJ software [90]. Through the same software, shape variations within the dataset were analyzed by canonical variate analysis (CVA) using populations as a priori groups. Mahalanobis distances (MDs) between populations in the CVA were calculated using permutation tests (10,000 replicates) and then MDs were represented graphically by non-metric multidimensional scaling (NMDS).

Linear regression of NMDS axes and all morphological and functional traits with latitude was tested using XLSTAT2014 software, and only significant regressions ($p \leq 0.05$) were reported. Regressions were examined using both average values and all measured values of the mentioned traits. For meristic traits, the relative frequency of each population was considered.

2.5.3. Environmental Data

Correlations among latitude and the other environmental factors considered in this study were determined using Pearson product moment correlation coefficient. Then, to detect the influence of all environmental factors on morphological and genetic traits of barbel populations, a redundancy analysis (RDA) was carried out. Environmental data were adopted as explanatory variables, and morphometric, functional, and genetic traits as response variables. To reduce the number of variables, principal component analysis (PCA) was performed with data on basin geology and land use, substrate and mesohabitat of sampling sites, climate, and morphometric traits, and the first component (also the second component for morphometric traits) was used in the RDA. All variables were standardized prior to the RDA. The analyses were performed using XLSTAT2014 software.

3. Results

3.1. Genetics of Barbel Populations along a Latitudinal Gradient

Among the six new populations, only four (i.e., Foglia, Salinello, Tordino, and Trigno) were purebred and thus were retained in this study along with other 13 purebred populations from previous works (Table 1). The median-joining network built on the 17 populations identified three main haplotype groups (Figure 3) separated by 16 to 22 mutation steps and corresponding to the native species *B. plebejus*, *B. samniticus*, and *B. fucini*. Among the newly analyzed populations, barbels found in Foglia, Salinello, and Tordino rivers belonged to *B. plebejus* species, while Trigno population consisted of *B. samniticus* specimens (Table 1). *Barbus plebejus* is thus present from Foglia (S1) to Tordino (S11), *B. samniticus* from Vomano (S12) to Trigno (S15), and *B. fucini* in Tappino (S16) and Ofanto (S17) rivers. From *B. plebejus* populations, 34 different haplotypes were detected, 13 from *B. samniticus* populations and six from *B. fucini* populations, for a total of 53 haplotypes. The number of haplotypes per population ranged from two to 11 (Table 3). The *Barbus plebejus* hap-

logroup presented a dumbbell pattern with the two most frequent haplotypes separated by two mutation steps. Several *B. plebejus* haplotypes were shared across different populations with only two populations, namely Tenna (S7) and Aso (S8), characterized, respectively, by two exclusive haplotypes (Figure 3). The *Barbus samniticus* network was characterized by a heterogeneous pattern where the two most frequent haplotypes were connected by up to 12 mutation steps, with Vomano (S12) including four population-specific haplotypes. Haplotypes detected from *B. fucini* were all population-specific haplogroups where four mutation steps were postulated between the major types (Figure 3).

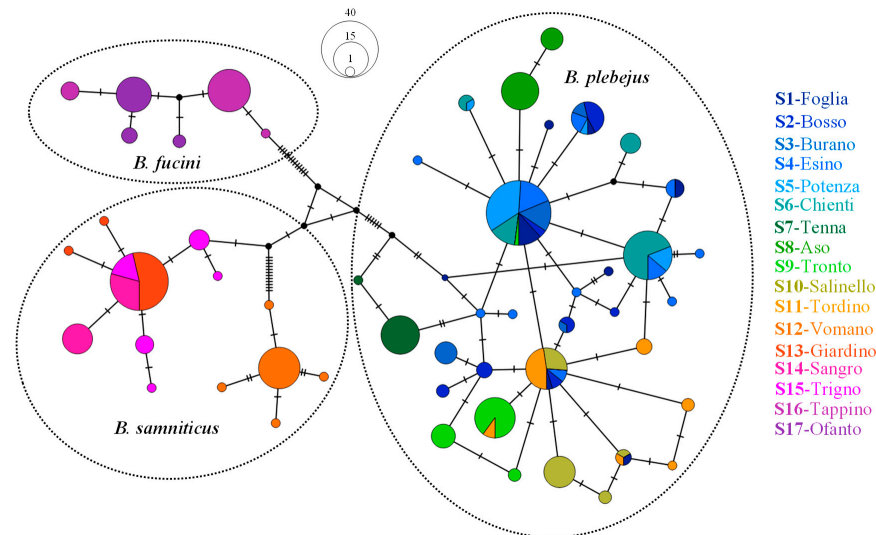


Figure 3. D-loop median-joining network. Circle size is proportional to the observed haplotype frequencies and black points and dashes represent unobserved haplotypes and potential intermediates. Dotted circles show species boundaries (*B. plebejus*, *B. samniticus*, and *B. fucini*).

Table 3. Total number of haplotypes (n), relative frequency of population-specific haplotypes (PSh), number of polymorphic sites (S), and haplotype (H) and nucleotide (π) diversity of the 17 barbel populations investigated along the Adriatic side.

Species	Watercourse	ID	n	PSh	S	π	H
<i>B. plebejus</i>	Foglia	S1	7	0.3	8	0.002 ± 0.001	0.79 ± 0.11
	Bosso	S2	7	0.4	6	0.002 ± 0.001	0.87 ± 0.06
	Burano	S3	4	0.3	5	0.002 ± 0.001	0.68 ± 0.07
	Esino	S4	11	0.5	11	0.002 ± 0.001	0.85 ± 0.05
	Potenza	S5	4	0.0	3	0.0006 ± 0.0006	0.46 ± 0.10
	Chienti	S6	4	0.3	4	0.001 ± 0.001	0.6 ± 0.07
	Tenna	S7	2	1.0	1	0.0001 ± 0.0002	0.10 ± 0.09
	Aso	S8	3	1.0	4	0.001 ± 0.001	0.55 ± 0.08
	Tronto	S9	4	0.5	4	0.001 ± 0.001	0.54 ± 0.09
	Salinello	S10	4	0.5	6	0.001 ± 0.001	0.61 ± 0.09
	Tordino	S11	6	0.5	5	0.001 ± 0.001	0.70 ± 0.10
<i>B. samniticus</i>	Vomano	S12	5	1.0	6	0.0005 ± 0.0005	0.30 ± 0.12
	Giardino	S13	3	0.7	3	0.0003 ± 0.0004	0.16 ± 0.10
	Sangro	S14	2	0.5	3	0.002 ± 0.001	0.52 ± 0.03
	Trigno	S15	5	0.8	4	0.001 ± 0.001	0.76 ± 0.06
<i>B. fucini</i>	Tappino	S16	3	1.0	4	0.001 ± 0.0008	0.32 ± 0.10
	Ofanto	S17	3	1.0	3	0.0007 ± 0.0006	0.43 ± 0.12

The total number of haplotypes (n) detected in each barbel population significantly increased with latitude while the relative frequency of population-specific haplotypes (PSh) significantly decreased (Figure 4). No significant associations with the latitudinal gradient

were observed for the number of polymorphic sites (S), and haplotype (H) and nucleotide (π) diversity indices. However, accounting for the two diversity indices (Table 3), some considerations could be done: (i) populations from S1 to S4 displayed the highest values of both H (0.68–0.87 on average) and π (0.002 on average), indicating populations with a good genetic structure compared to the other populations, and (ii) populations S7, S12, and S13, displayed the lowest values of both H (0.10–0.30 on average) and π (0.0001–0.0005 on average), probably indicating a recent marked demographic contraction.

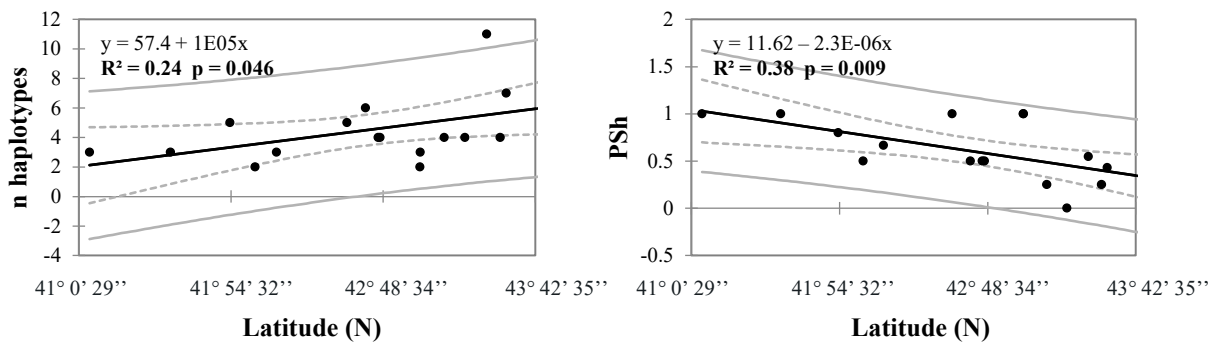


Figure 4. Linear regressions among latitude and the total number of haplotypes (n haplotypes) or the relative frequency of population-specific haplotypes (PSh) detected in each barbel population.

3.2. Morphology of Barbel Populations along a Latitudinal Gradient

Only a few morphological differences were detected along the CVA axes (Figure 5a). These differences were mainly associated to mouth orientation, head length, and the caudal fin shape. Specimens belonging to S15 (Trigno) population were the most morphologically different from the others (Figure 5b), i.e., the average Mahalanobis distance (MD) from the other populations was the highest (9.9). The maximum MD value (12.6) was detected among S15 and S13 (Giardino) populations despite belonging to the same species (*B. samniticus*) and located at close latitudes. No significant relationships were observed among the axes of NMDS displaying MDs and latitude.

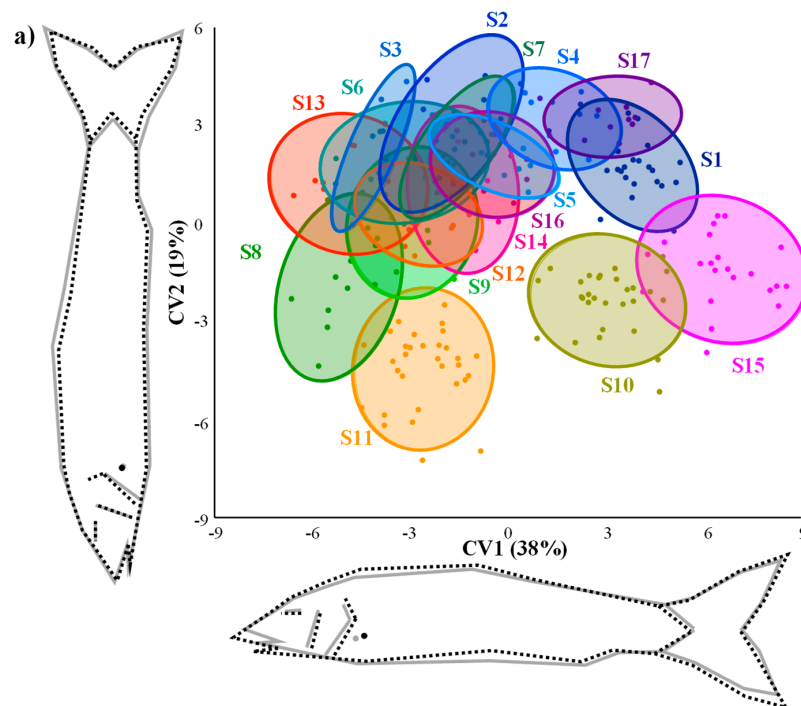


Figure 5. Cont.

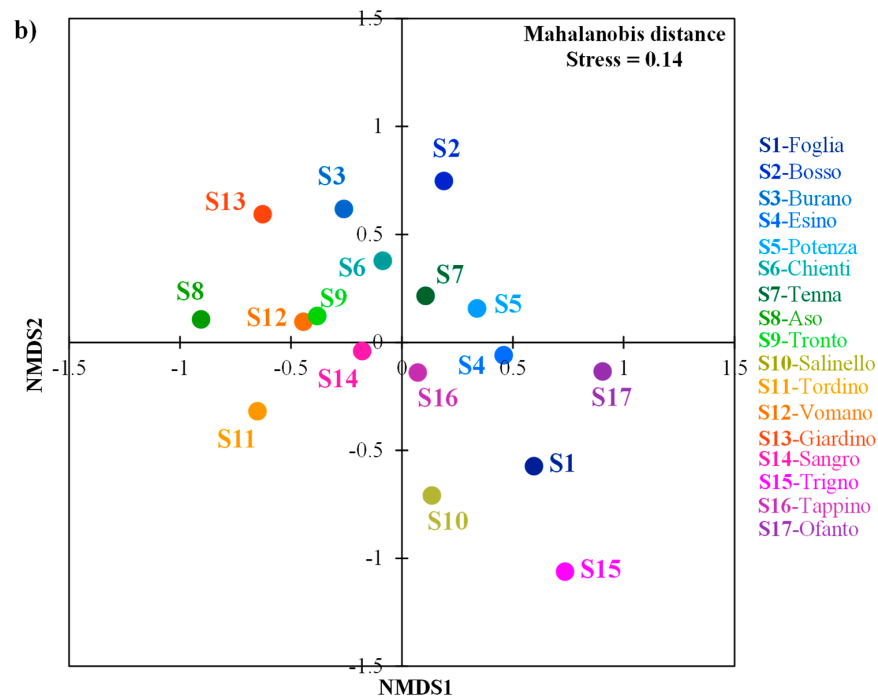


Figure 5. (a) Output of the canonical variate analysis (CVA) on fish body shape carried out between the 17 barbel populations (see Table 1); 90% confidence ellipses were drawn for each population. Wireframe graphs indicate the shape changes along each axis (from grey to dashed black line). (b) Non-parametric multidimensional scaling (NMDS) showing the Mahalanobis distances (MDs) among the populations detected through the CVA.

However, considering morphological traits singularly, some significant linear regressions with latitude were found (Figure 6). On average, the maximum caudal fin depth (CFD) was negatively related to latitude, while the meristic traits associated to the number of scales (i.e., NSLL, NSALL, and NSULL) were positively related to latitude. Also, the relative frequency of individuals having an orange caudal fin (Ccf, the others had an orange/grey caudal fin) increased with latitude while the relative frequency of individuals having dots on scales (Ds) decreased with latitude. If all measured values instead of the average value of each population were considered, other significant linear regressions were detected but R^2 values were low (0.02–0.08, Figure S1). In this case, head depth (HD), minimum caudal peduncle depth (CPD), caudal fin base length (CFBL), and anal fin length (AFL) decreased with increasing latitude while height of the third dorsal fin branched ray (HDOR3), dorsal fin insertion length (DFIL), and anal fin insertion length (AFIL) showed an opposite relationship.

No significant linear regressions were detected among average values of functional traits and latitude. Also, in this case, accounting for all measured values, significant positive relations were found with relative eye size (RES) and maximum body length (BLmax), while significant negative relations were detected with body lateral shape (BLS) and caudal peduncle throttling (CPT) (Figure S1).

Considering only the 11 populations belonging to *B. plebejus* species (intra-species variation), the relationships among latitude and NSLL ($R^2 = 0.72$, $p < 0.001$, average values), NSALL ($R^2 = 0.56$, $p = 0.008$, average values), NSULL ($R^2 = 0.07$, $p < 0.001$, all values), HD ($R^2 = 0.13$, $p < 0.001$, all values), CPD ($R^2 = 0.55$, $p = 0.009$, average values), CFBL ($R^2 = 0.37$, $p = 0.047$, average values), AFL ($R^2 = 0.13$, $p < 0.001$, all values), AFIL ($R^2 = 0.06$, $p = 0.002$, all values), RES ($R^2 = 0.55$, $p = 0.009$, average values), and BLS ($R^2 = 0.12$, $p < 0.001$, all values) were confirmed. Only AFIL and BLS showed opposite relationships between the two datasets.

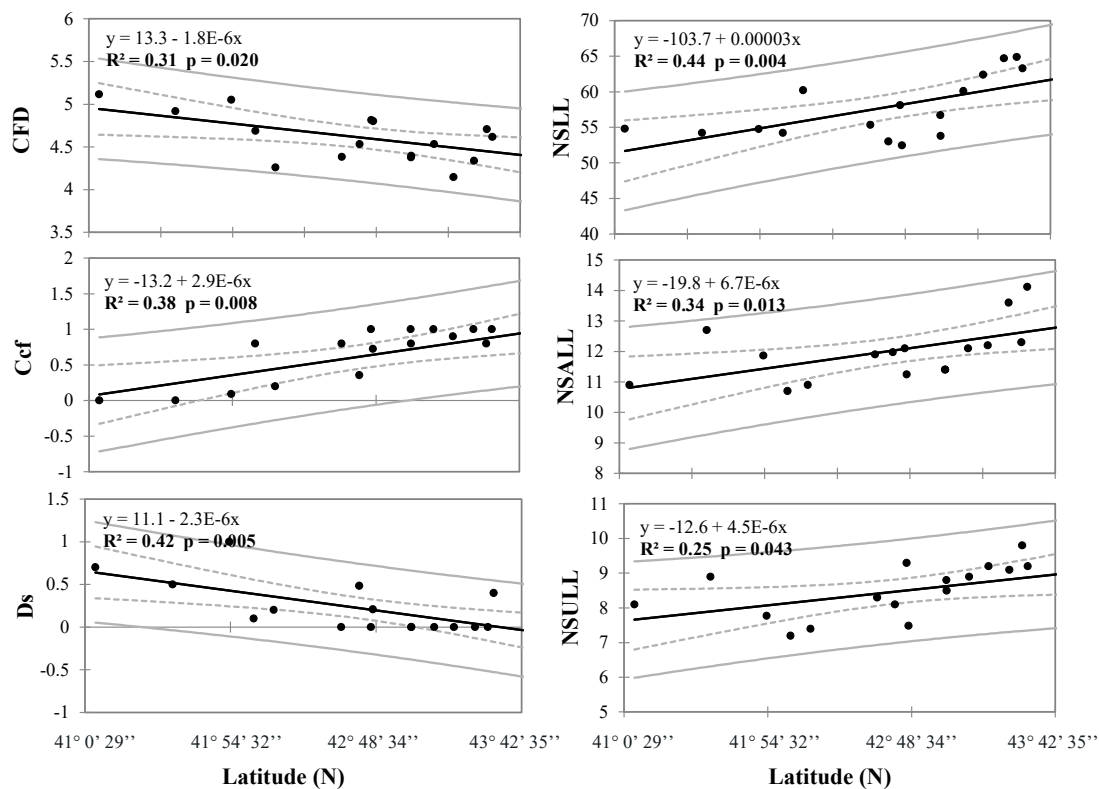


Figure 6. Significant linear regressions among morphological traits (average values) and latitude. CFD = maximum caudal fin depth, Ccf = color of caudal fin (calculated as relative frequency of orange color), Ds = presence of dots on scales (calculated as relative frequency of individuals having dots), NSLL = the number of scales on the lateral line, NSALL = the number of scales on rows above the lateral line, NSULL = the number of scales on rows under the lateral line.

3.3. Environmental Factors Influencing Barbel Populations

Among the environmental factors, latitude was positively correlated with precipitation ($r = 0.71$, $p = 0.001$) and the average flow/basin area (Q/A) ratio ($r = 0.47$, $p = 0.054$).

As shown in the RDA (Figure 7), other environmental factors besides latitude explained the morphological variation of barbel populations. The overall variation explained by both RDA axes was 64%.

Among the explanatory variables, RDA axis 1 was mainly negatively associated to Q/A (score -0.56) and elevation (score -0.42), while positively associated to distance from headwater (score 0.41). Among the response variables, this first axis was positively associated to the first component of the PCA on morphometric traits (i.e., longer pectoral, anal, dorsal, and caudal fins; larger head depth and length; longer jaws; and lower position of mouth, eye, and pectoral fin; Figure S2, score 0.94), body lateral shape (BLS, score 0.74), pectoral fin size (PFS, score 0.74), and relative maxillary length (RML, score 0.71), and was negatively associated to NMDS2 (Figure 5b, score -0.88), oral gape position (OGP, score -0.83), pectoral fin vertical position (PFVP, score -0.83), and eye vertical position (EVP, score -0.75).

RDA axis 2 was mainly negatively associated to elevation (score -0.53), to the first axis of the PCA on substrate (i.e., dominance of larger substrate, Figure S2, score -0.52), and to average depth (score -0.48); these are the explanatory variables. It was also negatively associated to the relative eye size (RES, score -0.80), which is the response variable. Moreover, this second axis was positively associated to the second component of the PCA on morphometric traits (i.e., higher length of fin insertions and ventral fin, and higher body depth, Figure S2, score 0.81).

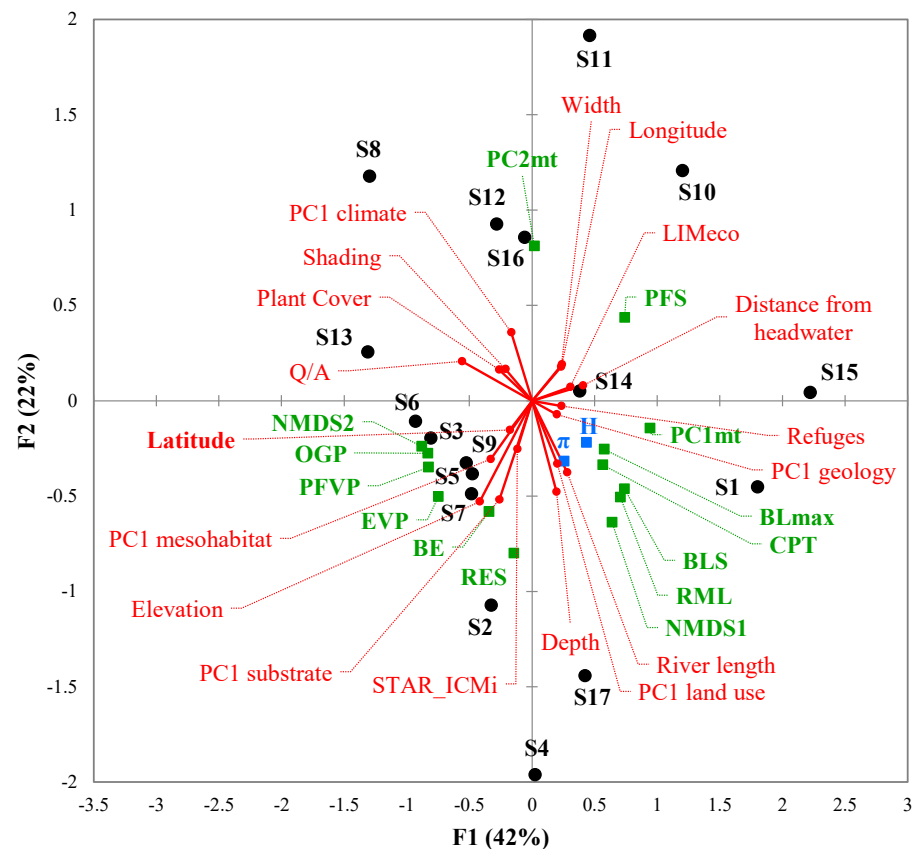


Figure 7. Redundancy analysis (RDA) performed using environmental characteristics as explanatory variables (red circles, for some variables, the first axis of the PCA shown in Figure S2 was used) and morphometric (NMDS axes in Figure 4 and PCA axes in Figure S2), functional (see abbreviations in Table 2) and genetic (H = haplotype diversity, π = nucleotide diversity) traits as response variables (green—morphological or blue—genetic—squares). Different sites/populations are indicated by black circles.

Both the genetic diversity indices were weakly associated to both RDA axes (H scores: 0.44 with F1 and -0.22 with F2; π scores: 0.26 with F1 and -0.32 with F2).

4. Discussion

The primary goal of this study was to investigate the relationships between genetic and morphological variations, detected across 17 Italian barbel populations, and changes in past and present environmental conditions, mainly represented by the latitudinal gradient of approximately 2.5 degrees of the study area. Our previous studies [38,41] have highlighted that Mio-Pleistocenic geomorphological events determined fragmented geographical isolation of the 17 investigated rivers located along the Adriatic side of the Italian Peninsula, leading to barbel speciation. Moreover, a possible cline in meristic traits causing taxonomic identification errors was already pointed out [43,45]. However, the existence of a latitudinal gradient at both morphological and genetic level in Italian barbels was only hypothesized and never specifically tested.

Our results showed that the relative frequency of population-specific haplotypes increased southward, whilst the total number of haplotypes decreased, supporting the isolation of southern barbel populations. These results highlight that the modern-day distribution of freshwater fish throughout multiple rivers reflects the ancient connectivity of their drainages and the subsequent geomorphological history [91], partly supporting our initial hypothesis where a lack of a relationship between genetic variability and local environmental conditions was posited. In contrast, the overall genetic variability expressed by nucleotide and haplotype diversity indices did not offer any clear pattern from the

latitudinal point of view. Within each species, we found populations with relative higher haplotype and nucleotide diversity, and populations with intermediate to low levels of one or both genetic diversity indices, independently from the latitudinal positioning of the inhabited river. The RDA analysis revealed some weak correlations between both nucleotide and haplotype diversity indices and some environmental characteristics, some of which are consistent with previous studies, which found correlations between genetic diversity and ecosystem size (i.e., river length in the RDA analysis [32]), habitat features (i.e., refuges and geology in this study [28]), and hydrological regime (represented in this study by the Q/A [92]). Although these results may suggest an influence of local environmental conditions on genetic variability, the RDA scores of both the genetic indices were too low to support a strong relationship between genetic variability and the environmental parameters. Several factors can determine the lack or non-detection of a relationship with local environmental features [28], especially in case of mitochondrial DNA markers. Mitochondrial DNA markers are less sensitive to recent environmental changes than DNA markers of, or those linked to, adaptive loci. This has prevented us to further speculate on the role of environment in shaping the genetic diversity of Italian barbels, which is thus more likely to reflect the past geomorphological evolution of the river catchments, at least at the DNA markers examined.

The main morphological differences previously detected among the four Italian fluvi-olacustrine barbel species (*B. tyberinus*, *B. plebejus*, *B. samniticus*, and *B. fucini*) were mainly associated to the number of scales on the lateral line and the shape of the caudal fin; the former trait mainly differentiates *B. plebejus* from the other three species while the latter mainly differentiates *B. tyberinus* [38]. *Barbus tyberinus* was, however, excluded from the present study because of its absence in the Adriatic rivers and because of the difficulties in finding purebred populations. Our study confirmed the previous findings and detected a significant increase of some meristics with latitude. These results agree with the Jordan's rule [27,65,93–95], an ecomorphological rule that postulates an inverse relationship between meristic traits and latitude (particularly temperature) in fish. Additionally, we found that individuals at lower latitudes displayed different coloration, supporting our second hypothesis for which morphological traits are related to current environmental conditions rather than past evolution and species attribution. Skin color variations in teleosts play an important role in life history and behavior, such as camouflage, photoprotection, or antipredation, and are influenced by environmental changes [96–99]. These variations can be classified into morphological and physiological: the former include variations in skin pigment cell numbers, as well as changes in density, morphology, and distribution [100], and take days or weeks to complete, with a long-term stimulus following comprehensive adaptation to certain conditions such as background pattern, diet, and UV light [101–104]; the latter are triggered by short-term stimuli that facilitate the dispersion and translocation of skin chromatophores from the periphery to the center [105]. The darker livery of barbels at decreasing latitudes may thus be related to a darker background or to differences in diet or UV light. In southern rivers, this could be also associated to lower flows and water depths, favoring camouflage with the riverbed substrate [106].

The only morphometric trait having a relevant association to latitude was larger caudal fin at lower latitudes. This result was not confirmed by the analysis of the partial dataset which included only *B. plebejus* populations (intra-species variation) but was supported by negative relationships with latitude of other caudal fin measurements. The other consistent result on morphological traits analysis was the increase of visual acuity (i.e., relative eye size) and the decrease of head size at increasing latitudes. However, considering the whole dataset, an increase of the maximum body length at increasing latitudes was also detected, and this agrees with the temperature-size rule, which postulates that ectotherms living in warmer conditions (i.e., lower latitudes) grow faster as juveniles but attain smaller adult body sizes [107], likely due to growth and reproduction trade-offs across temperatures [108]. The relationship between body size and latitude can be controversial in fish, being different

from species to species [109]. Nevertheless, our results were consistent with a previous report dealing with 24 *B. barbuis* populations [110].

Our results thus demonstrate that meristic traits, fish body shape, and some morphometric traits of barbels may be sensitive to climate changes since they have been responsive to an overall limited latitudinal cline. This is an important result given that these traits are not widely studied in this context. Despite several studies assessing the relationship between climate change and phenotypic traits exists (reviewed by Crozier and Hutchings [111]), they focus only on certain “climate-sensitive” traits (e.g., growth, survival, migration, and spawn).

Besides the relationships with latitude, we also found significant relationships between fish morphology and other environmental parameters, which strengthen the potential sensitivity of morphological traits to current climate change. The result of the RDA highlighted an evident role of the ratio between average flow and basin area, elevation, and distance from headwater in shaping fish morphology. Barbel populations were sampled at different elevations (from 28 to 398 m asl) and distance from headwater (from 0.5 to 93 km). Particularly, fin and head size increased at decreasing elevation (in the lower river reaches), while the position of the eyes, mouth, and pectoral fin were lower, indicating a change of the fish position in the water column moving from the headwater to the river outlet. Evidence of the influence of the elevation gradient on morphological traits is widely available in literature for other fish species [112,113].

However, questions remain as to whether the observed morphological variations are a result of phenotypic plasticity (not yet fixed genotypes) or local adaptations (fixed genotypes) and how fast these variations appear across generations. Future experimental studies may shed light on this since understanding these aspects as well as their mechanistic basis is crucial to fully comprehend the vulnerability of fish populations to climate change [114] and other anthropogenic stressors [115].

5. Conclusions

This study proves that the environmental factors, represented by a latitudinal gradient, have an influence on both genetic and morphological traits of three species of freshwater fish belonging to the *Barbus* genus. While genetic variations are mainly related to latitude due to the historical evolution of the landmass and climatic Mio-Pleistocenic events, morphological variations are mainly affected by changes in current environmental conditions. Particularly, meristic traits such as the number of scales and fish coloration are clearly associated to latitude, and thus should not be used for taxonomical identification in the field. Besides latitude, other environmental factors displayed an important influence on fish morphology, which then appear to be potentially sensitive to climate and human-mediated changes. This should be better understood to unravel the vulnerability or adaptability of barbels to these stressors.

Studies like this one provide important information on the biology of freshwater species, information that is also necessary for their conservation and for the correct management of the rivers they inhabit. The management should take into consideration the current and forecasted alterations of both hydrological and thermal regimes due to the synergistic effects of anthropogenic pressure and climate change.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w15020325/s1>, Table S1: List and brief description of the environmental variables considered in this study. Figure S1: Significant linear regressions of morphological and functional traits with latitude. HD = head depth, CPD = minimum caudal peduncle depth, CFBL = caudal fin base length, HDOR3 = height of the third dorsal fin branched ray, DFIL = dorsal fin insertion length, AFL = anal fin length, AFIL = anal fin insertion length, RES = relative eye size, BLS = body lateral shape, CPT = caudal peduncle throttling, BLmax = maximum body length. Figure S2: Principal component analysis of some environmental characteristics of the investigated areas (i.e., geology and land use of basins, substrate and mesohabitat of sampling sites, and climate), and of morphometric traits (see abbreviations in Figure 2).

Author Contributions: Conceptualization, S.Q., V.D.S., M.L., A.C., S.Z. and I.V.; formal analysis, S.Q. and V.D.S.; investigation, M.L., A.C., S.Q. and V.D.S.; data curation, A.C., S.Q., V.D.S. and I.V.; writing—original draft preparation, S.Q. and V.D.S.; writing—review and editing, S.Q., V.D.S., M.L., A.C., S.Z. and I.V.; visualization, S.Q. and V.D.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The genetic dataset generated during the current study is available in GenBank (ON661665-ON661675 for the D-loop mtDNA) while the morphological and environmental datasets are available from the corresponding authors upon reasonable request.

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