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Biology and distribution of Danube barbel (*Barbus balcanicus*) (Osteichthyes: Cyprinidae) at the Northwestern limit of its range

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Abstract
Presence of Danube barbel *Barbus balcanicus* was investigated at the westernmost portion of its distribution area (Italian portion of the Isonzo River Basin). Genetic analyses carried out on specimens collected in different watercourses confirmed *B. balcanicus* presence in the same locations where *Barbus caninus* was erroneously classified in the past. More precisely, 4 *Cyt b* haplotypes belonging to the Balkanic species were described, three of which were evidenced for the first time. Analysis of meristic characters also confirmed genetic results and completed the taxonomical assessment. At the same time, a target population was studied to deepen knowledge about the species ecology. In this case, 120 specimens belonging to five age classes (from 0+ to 4+) were collected with monthly frequency in Barbucina Creek, representing the model watercourse inhabited by the species in the study area. Regression between total weight (W) and standard length (S_L) did not differ significantly between males and females, therefore not displaying sexual dimorphism. S_L values showed wide ranges, often overlapping among age classes. Mean condition factor (*Kmean*) decreased significantly with growth and age, as small individuals were in better nutritional condition. From an ecological point of view, biometric parameters seemed to be affected by habitat conditions and, specifically, to the limited space of the creeks, rather than by trophic conditions. Finally, trends of gonadosomatic index (*GSI*) showed that *B. balcanicus* reproductive period stretches between April and late May/early June. Results indicated that sexual maturity is reached at 1+ age, corresponding to a mean standard length equal to 5.50 ± 0.66 for female and 5.71 ± 0.53 for male breeders. In conclusion, experimental results highlighted the presence and adaptability of this "forgotten" species of the Italian fish fauna, which certainly will deserve attention in a future amendment of the Italian IUCN Red List of vertebrates.

Keywords: Barbus balcanicus, *conservation genetics, condition factor, zoogeography, gonadosomatic index*

Introduction

The genus *Barbus* Cuvier and Cloquet, 1816 represents one of richest taxa among teleosts in terms of species number, including more than 300 species widespread over Europe, Asia and Africa, with fossils known since Oligocene period (Tsigenopoulos & Berrebi 1999; Tsigenopoulos et al. 2002). Despite this wide variety of biodiversity, only 27 *Barbus* species are autochthonous in Europe and some of them show restricted distribution patterns within an endemic status from a zoogeographic point of view (Tsigenopoulos & Berrebi 2000; Machordom & Doadrio 2001; Tsigenopoulos et al. 2002; Zaccara et al. 2019). Despite its widespread distribution and ancient origin, knowledge about taxonomy and distribution of the genus *Barbus* is still lacking and it should be improved particularly for Italian populations. Further knowledge is, therefore, necessary for management and conservation plans, in order to protect the most endangered populations. With regard to the Italian territory, there still are critical issues regarding identification of autochthonous

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species of this taxonomic group in freshwater systems (Bianco 1995, 2003a, 2003b, 2003c; Zerunian 2002, 2003; Zaccara et al. 2019). In addition, the complexity of systematic determination is emphasized by hybridization among autochthonous taxa (Betti 1993) or with alien invasive *Barbus barbus* L. 1758 largely widespread over many European countries (Philippart & Berrebi 1990; Gante 2009; Meraner et al. 2013; Piccoli et al. 2017; Carosi et al. 2017).

Four species belonging to the genus *Barbus* are native in Italy: the brook barbel *Barbus caninus* Bonaparte, 1839, which is classified as Endangered by the Italian IUCN Red List (Rondinini et al. 2013), the Italian barbel *Barbus plebejus* Bonaparte, 1839, which is included in the Annexes II and V of the Habitat Directive (Directive 92/43/EEC), the horse barbel *Barbus tyberinus* Bonaparte, 1839 and the Danube barbel *Barbus balcanicus* Kotlík et al., 2002. It must be remarked that *B. tyberinus* and *B. balcanicus* were not listed in the Habitat Directive annexes in 1992, at the time when the Directive was issued. However, similarly to the recent taxonomic splitting of *B. tyberinus* from *B. meridionalis*, also *B. balcanicus* should be treated as a split branch of *B. plebejus* and therefore the same conservation actions should be applied according to the Directive 43/92/ECC.

In particular, *B. balcanicus* was defined a "true" species by Kotlík et al. (2002), as genetic studies revealed that *Barbus petenyi* complex comprises at least three species which have separated during Pliocene (Kotlík & Berrebi 2002); *B. balcanicus* was described as one of those three species based on its unique genetic and morphological characteristics (Buonerba 2010). The original distribution area of *B. balcanicus* includes Slovenia, inland part of Croatia, Bosnia and Herzegovina, Serbia, Southwestern portion of Romania, Bulgaria and Macedonia, besides watercourses of the Aegean basins (Kotlík & Berrebi 2002; Tsigenopoulos et al. 2002; Kottelat & Freyhof 2007; Marić et al. 2012). Within the Italian territory, the species is considered native in the Isonzo River Basin (Buonerba et al. 2015). However, *B. balcanicus* has wrongly been classified as *B. caninus* for a long time, after its first description by Stoch et al. (1992) performed over limited demographic groups within a restricted area of Friuli Venezia Giulia (Northeast Italy), including watercourses of the Isonzo-Torre-Natisone system.

In this context, different aspects dealing with the ecology and taxonomy of the species were analysed in this paper. In particular, it was deemed of interest to clarify whether or not *B. caninus* and *B. balcanicus* share sympatric conditions in the Isonzo basin and if past reports about *B. caninus* resulted from erroneous taxonomical identification of the species. Moreover, due to the lack of knowledge about *B. balcanicus* ecology, which has been scarcely investigated in the past, morphological and morphometric characterization of *B. balcanicus* specimens was carried out together with aspects dealing with growth and reproduction. Due to the supposed erroneous previous species identification, a taxonomic assessment based on a genetic approach was carried out on a subset of samples. Available specimens were therefore correctly identified by means of mitochondrial markers, which are able to discriminate species attribution within the same genus, even if characterized by low genetic differentiation among congeneric taxa. Ecological aspects of the species are herein presented coupled with a molecular classification of samples.

Material and methods

Study area

The Isonzo River (Soča in Slovenian) is a predominantly torrential watercourse, collecting waters from the southern side of the Julian Alps (Kanduč et al. 2008) and flowing through Western Slovenia (100 km) and Northeastern Italy (40 km). The river basin extends for 3300 km^2, 2235 of which are included within the Slovenian borders, while the remaining portion (1065 km^2) is located in Italy (Covelli et al. 2004). Except along the upper sector, where the river flows over carbonate rocks, the riverbed consists of a thick fluvioglacial and alluvial cover (Treu et al. 2017). The average annual flow rate at the river mouth was estimated to be 170 $m^3\ s^{-1}$ (Covelli et al. 2004), however tributaries dissipate a great amount of water during their way across the plain and dry condition can be observed in some streams and rivers, while the Isonzo loses about 26% of its discharge during drought periods (Zini et al. 2013; Treu et al. 2017).

The sampling plan started with a preliminary campaign with the aim of investigating the species distribution within the Italian portion of the Isonzo River basin. Eleven sampling sites were chosen (Figure 1, Table I) on the basis of previous studies reporting the presence of *Barbus caninus*, *Barbus plebejus* and/or *Barbus balcanicus* (Stoch et al. 1992; Pizzul et al. 2006; Buonerba et al. 2015). A single site located in the Barbucina Creek and characterized by an abundant and well-structured population of *B. balcanicus*, (herein confirmed by genetic analyses) was subsequently chosen for further assessments (Figure 1, Table I, site 6). This 8 km length watercourse flows mainly close to the State border

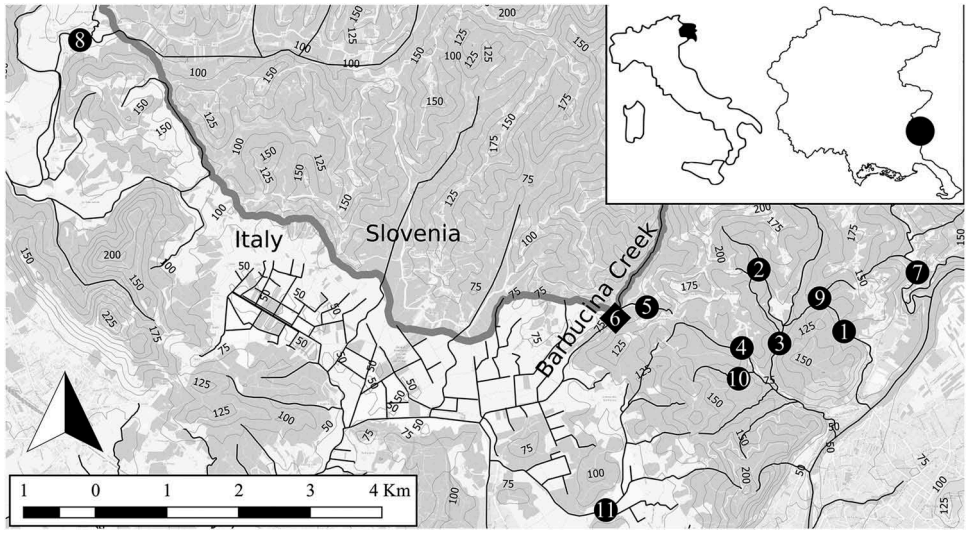

Figure 1. Study area and sampling sites located within the Isonzo River Basin. Barbucina Creek site is indicated by the square symbol (site 6).

Table I. UTM coordinates of the 11 sampling sites monitored during the preliminary sampling campaign. + = site where the presence of genus *Barbus: Barbus plebejus* and/or *Barbus balcanicus* was detected. Six stations did not display any fish population. Grojna Creek is a homonymous tributary of Groina Stream.

		UTM coordinates			
Site		Easting	Northing	Presence of *B. plebejus*	Presence of *B. balcanicus*
1	Pneuma Creek	33T 392257	5091206	+	+
2	Floriano Creek	33T 391082	5092085		
3	Grojna Creek	33T 391354	5091055		
4	Groina Stream	33T 390827	5091002		+
5	Potok Creek	33T 390775	5090575		
6	Barbucina Creek	33T 388471	5090984		+
7	Piumizza Stream	33T 393294	5092007	+	+
8	Reca Stream	33T 381679	5095445	+	+
9	Oslavje Creek	33T 391922	5091674		
10	Kolniac Creek	33T 390775	5090575		
11	Blanchis Creek	33T 388890	5088781		

between Italy and Slovenia and it collects draining waters from the local farming system.

All activities described in the present study were performed according to European (Directive 2010/63/EU), National (D. Lgs. 26/14), and Regional laws (Friuli Venezia Giulia Fishery Authority) dealing with procedures for the protection of animals used for scientific purposes.

Fish sampling

The preliminary sampling campaign was carried out during April 2016 in order to check the distribution of *Barbus balcanicus* within the study area. At each sampling event, captures were performed by wading a watercourse stretch length, proportional to the riverbed width (Forneris et al. 2005), with a battery-powered backpack electrofisher (Model IG200–2: 15–25 A, 150–200 V) manufactured by Hans–Grassl GmbH (Schönau am Königssee, Germany). Electrofishing was used to obtain information about the *B. balcanicus* abundance data and population structure at each site using the removal method (Seber & Le Cren 1967; Seber 1973). Barbels were assessed in 5 sites, while 6 stations did not display any fish at all.

Collected specimens were anaesthetized using tricaine methane-sulfonate MS-222 (Topić Popović et al. 2012), in order to limit damages during manipulation. A little portion of caudal or anal fin was collected for genetic analyses from each specimen and stored in 70% ethanol. After biometric measurements and fin collection, all fish were released at the same site without consequences for their vitality.

In order to deepen analyses regarding meristic/biometric characters, growth and reproduction of *B. balcanicus*, monthly sampling operations were carried out in the Barbucina Creek (site 6, Figure 1, Table I), where the population showed higher density values (0.141 ind m^{-2}). Operations were performed for one entire year from April 2016 to March 2017 and were carried out as indicated for the preliminary collections. At each sampling event, 10 specimens were killed by anaesthetic overdose and placed in frozen bags for the transport to the laboratory to be submitted to abdominal dissection.

Meristic characters, biometric analyses and age determination

Morpho-metric and biological determinations were focused on 120 specimens collected during the monthly sampling operations in the Barbucina Creek. In particular, total weight W ($x \pm 0.001$ g) was obtained and age was determined by scalimetric method performed on five scales (Bagenal 1978; Britton et al. 2004), removed from the central body section above the lateral line. Samples were then analysed using a stereo-microscope (Wild M3) for meristic analyses, considering the following characters: number of dorsal fin rays (*D*), ventral fin rays (*V*), anal fin rays (*A*), lateral line scales (*LLS*), scale rows above lateral line (*SALL*) and scales rows under lateral line (*SBLL*). Unbranched and branched fin rays were considered as reported by Marić et al. (2012). Pictures of each specimen (left side) were also obtained on millimetric table using a digital camera (Canon Powershoot A2200) and used to obtain main morphometric measures ($x \pm 0.01$ mm) (indicated in Figure 2) by means of the ImageJ software (version K 1.45) (Schneider et al. 2012). Ratios between morphometric characters indicated in Figure 2 and standard body length (S_L) were then calculated.

In order to assess the growth rate of *B. balcanicus*, non-linear regressions between total weight W (g) and standard length S_L (cm) were obtained separately for females and males, according to the formula:

$$W = aS_L^b$$

where a is the coefficient and b is the exponent of the arithmetic form of the weight–length relationship, besides being, respectively, the intercept and the slope of the regression line in the logarithmic form.

In addition, the fish condition was examined on the basis of fish body length and weight, using the mean condition factor (K_{mean}), which represents the average condition factor for a given length derived from the respective weight–length relationship (Froese 2006):

$$K_{mean} = 100 \times a \times S_L^{(b-3)}$$

where a and b are the same coefficients described above.

Gonadosomatic index

Fresh gonad weights were obtained immediately after dissection (x ± 0.0001 g) using an analytical balance and gonadosomatic index (*GSI* %) was calculated for all males and females specimens sacrificed during the monthly sampling operations, according to the following formula:

$$GSI = \frac{gonad\ weight}{total\ weight} \times 100$$

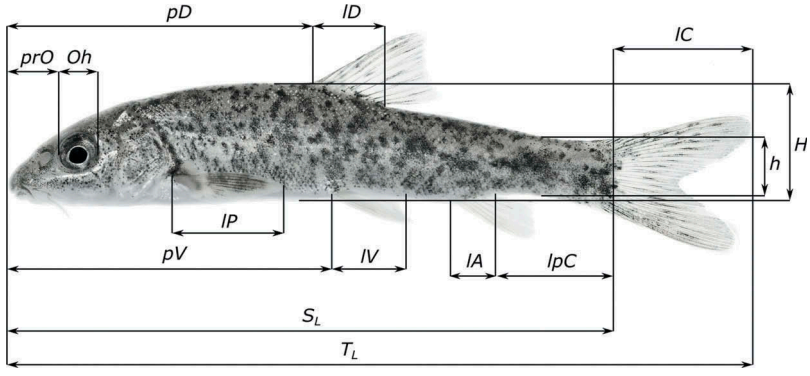

Figure 2. Morphometric parameters measured for *Barbus balcanicus* in the Barbucina Creek (picture by Filippo Bortolon, 2017) (T_L = total length; S_L = standard length; H = maximum body height; h = minimum body height; *lpC* = caudal peduncle length; *pD* = predorsal distance; *pV* = preventral distance; *lP* = length of pectoral fin base; *lV* = length of ventral fin base; *lD* = length of dorsal fin base; *lA* = length of anal fin base; *lC* = length of caudal fin base; *prO* = preorbital distance; *Oh* = horizontal diameter of eye).

At the same time, sex determination was performed during gonad inspection.

Genetic analyses

Forty-five samples were randomly selected for genetic analysis in 3 watercourses among the 11 sampling sites: 8 specimens from Piumizza Stream, 33 from Barbucina Creek and 4 from Reca Stream. High molecular weight genomic (HMWG) DNA was extracted and purified from ethanol-fixed muscle tissue samples stored at −20°C using the Wizard® Genomic DNA Purification Kit (Promega). The cytochrome b (*cytb*) mitochondrial locus was amplified for all 45 samples in a 25 µl total reaction volume containing 1 µl of HMWG DNA, 1 U of GoTaq in 5x Reaction Buffer (Promega Corporation, Madison, WI, USA), 2 mM $MgCl_2$, 0.5 mM of each dNTP, 0.5 µM of Glu-F and Thr-R primer (Zardoya & Doadrio 1998). PCR was set as follows: 35 cycles of 45 s at 94°C, 1 min at 47°C, and 2 min at 72°C, after an initial 3-min denaturation step at 94°C and a final prolonged extension at 72°C for 10 min. Amplicons were then sequenced by means of capillary electrophoresis using a Beckman-Coulter CEQ8000 automatic sequencer and alternatively in outsourcing at Macrogen Europe (Amsterdam) using the same primers cited above (Zardoya & Doadrio 1998). Sequences were manually edited to avoid analytical biases.

For each individual, forward and reverse *cytb* sequences were aligned and compared using the software Mega 7.0 (Kumar et al. 2016) in order to obtain a single consensus sequence for each individual. Sequences from all individuals were thus aligned by means of Clustal-W (Thompson et al. 1994) including *cytb* sequences of *B. plebejus*, *B. caninus* and *B. balcanicus* available from GenBank (see further details for Accession numbers in the Results chapter). Sequences were collapsed into haplotypes that were compared with those previously reported in GenBank. The number of transitions and transversions were also calculated using Mega 7.0 to obtain descriptive data on the genetic differentiation of the species.

A phylogenetic tree was subsequently built on the alignment of unique haplotypes by applying the Maximum Likelihood (ML) analysis implemented in PhyML v. 3.0 (Guindon et al. 2010) and using the substitution model indicated by jModeltest v. 2.1.1 (Darriba et al. 2012). Output data tree was visualized by FigTree 1.3.1 (Rambaut 2009).

Environmental analyses

At each sampling event in Barbucina Creek, values of the main chemical and physical data were recorded in the water column to support biological surveys: in particular, conductivity (µS cm^{-1}), pH (units), temperature (°C) and dissolved oxygen (mg l^{-1}) were registered using field meters (HI 9033 conductivity meter; HI 9125 pH/ORP meter; HI 9147 dissolved oxygen meter; all instruments are manufactured by Hanna Instruments Inc., Woonsocket, Rhode Island, USA). Three values were measured approximately at mid-depth across a representative creek section, to cover different microhabitats. Simultaneously, water samples were collected in sterile containers paying attention to avoid inclusion of sediment particles and immediately frozen and transported to the laboratory. Concentration of NO_3^- (mg l^{-1}) was obtained measuring the absorbance at 525 nm, obtained by adaptation of cadmium reduction method (APHA 1998); concentrations of NH_4^+ (mg l^{-1}) were obtained by an adaptation of the Nessler method, measuring the absorbance at 420 nm (ASTM 2015); in addition, PO_4^{3-} (mg l^{-1}) concentration was obtained with adaptation of the ascorbic acid method (APHA 1998) measuring absorbance at 610 nm. Analyses were carried out using a multi-parameter spectrophotometer (HI83200-02, Hanna Instruments Inc., Woonsocket, Rhode Island, USA) and three replicates were obtained for each parameter.

Statistical analysis

Non-parametric Kruskal–Wallis test was used to detect the presence of significant differences in K_{mean} values among age classes both for males and females, and to check for the presence of significant differences among monthly *GSI* values both for males and females. The Conover-Iman test (Conover & Iman 1979; Conover 1999) was used as post hoc test. Regarding biometric measures, non-parametric Wilcoxon test was used to assess the presence of significant differences between males and females, and to check differences in total length (T_L), standard length (S_L) and K_{mean} for both genders for each age class. Finally, ANCOVA was used to compare *b*-values as slopes of the linear form of the standard length-weight regression equations. All analyses were performed using RStudio software version 1.1.383 (RStudio Team 2017), using a *p*-level of 0.05 to interpret significance for all tests.

Results

Genetic analyses

Preliminary sampling allowed to phenotypically detect the presence of *B. balcanicus* in 5 sites out of the 11 investigated sampling stations (Table I). The species presence was further confirmed in 3 of them by means of genetic analysis. More precisely, a region of 810 bps of mitochondrial *cyt b* locus was successfully sequenced in 45 individuals randomly selected in 3 out of the 11 sampling sites selected during the preliminary campaign (see Table II). Unfortunately, genetic analyses were not carried out in the entire dataset of 11 sites, due to the fact that the molecular approach was not the main topic of this research. In fact, the necessity for a precise taxonomic evaluation emerged during the investigation to verify the correct species identification of barbels. *Cyt b* analyses were therefore carried out to highlight the previous erroneous taxonomic classification (Stoch et al. 1992; Machordom & Doadrio 2001). For the same reason, no assessment of nuclear markers was conducted since the identification of hybrid specimens was not pertinent to the objectives of this study. On the other hand, sympatric populations of *B. plebejus* and *B. balcanicus* were found only in Reca stream (Table II).

All unknown 45 sequences were unambiguously aligned and a total number of 70 polymorphic sites were identified, 61 of which were transitions and 9 transversions. Further descriptive data referred to nucleotide percentages was as follows: 26.95% (A), 28.87% (T), 28.91% (C), and 15.27% (G).

Sequences obtained in our experimental dataset were then compared to 32 reference ones extrapolated from GenBank and referred to *B. balcanicus*, *B. plebejus* and *B. caninus*. The whole dataset (45 experimental and 32 reference) revealed 114 polymorphic sites, 100 of which were transitions and 14 transversions.

Five different haplotypes were identified, and clearly segregating in three major evolutionary clusters (Figure 3). More precisely, according to the positioning of the reference samples obtained from GenBank, three major groups were identified and easily referred to the three species *B. plebejus, B. caninus* and *B. balcanicus*. Among our experimental samples, 42 clustered with *B. balcanicus* reference samples and 3 with *B. plebejus*. None among experimental samples were attributed to *B. caninus* in the investigated streams Barbucina, Piumizza and Reca. The Maximum Likelihood tree confirmed the three major clusters separation supported by nodes robustness with bootstrap values ranging between 0.890 and 0.99 (Figure 3). *Luciobarbus nasus* (Acc. No. KU257539) was inserted as an outgroup.

Among 5 identified haplotypes, 3 were described for the first time in *B. balcanicus* (ISO01, ISO02 and ISO03). In particular, 3 samples from Piumizza and 1 from Reca in hap ISO01; 4 from Piumizza in ISO02; 4 from Barbucina and 1 from Piumizza in ISO03. Twenty-nine samples from Barbucina Creek aligned with haplotype B.caninus1 (first described by Machordom & Doadrio 2001). However, this was an erroneous haplotype original denomination in Barbucina Creek since all 29 samples clearly clustered in the "*balcanicus*" group and were therefore undoubtfully attributed to *B. balcanicus*. At last, 3 samples were attributed to the previously described *B. plebejus* haplotype of Reca stream and correctly clustered in that species group. All haplotypes are listed together with their accession numbers in Table II.

Meristic characters, biometric analyses and age determination

An amount of 120 specimens of *Barbus balcanicus* were collected in the Barbucina Creek. Ten individuals were immature and were then excluded from analyses regarding sex differences. The assessment of meristic and biometric characters was carried out on pictures obtained in a subset of 78 specimens (40 females and 38 males). Results of meristic analyses are reported in Table III and those of biometric measures in Table IV.

Starting from the evidence that regression between total weight W and standard-length S_L (Figure 4) did not differ between males and females (ANCOVA: $F_{1,104} = 0.005$; $p = 0.989$), age determination highlighted the presence of five age classes

Table II. Number of individuals collected in three different streams and their haplotypes (haplotypes in bold were described for the first time in this study). Haplotype *B. caninus*1 was previously erroneously attributed to *B. caninus* by previous authors.

Haplotype	GenBank Accession Number	Barbucina	Piumizza	Reca	Species
ISO01	**MH37916**	–	3	1	*B. balcanicus*
ISO02	**MH37917**	–	4	–	
ISO03	**MH37918**	4	1	–	
***B. caninus*1**	AF287424	29	–	–	
B. plebejus	AY004750	–	–	3	*B. plebejus*

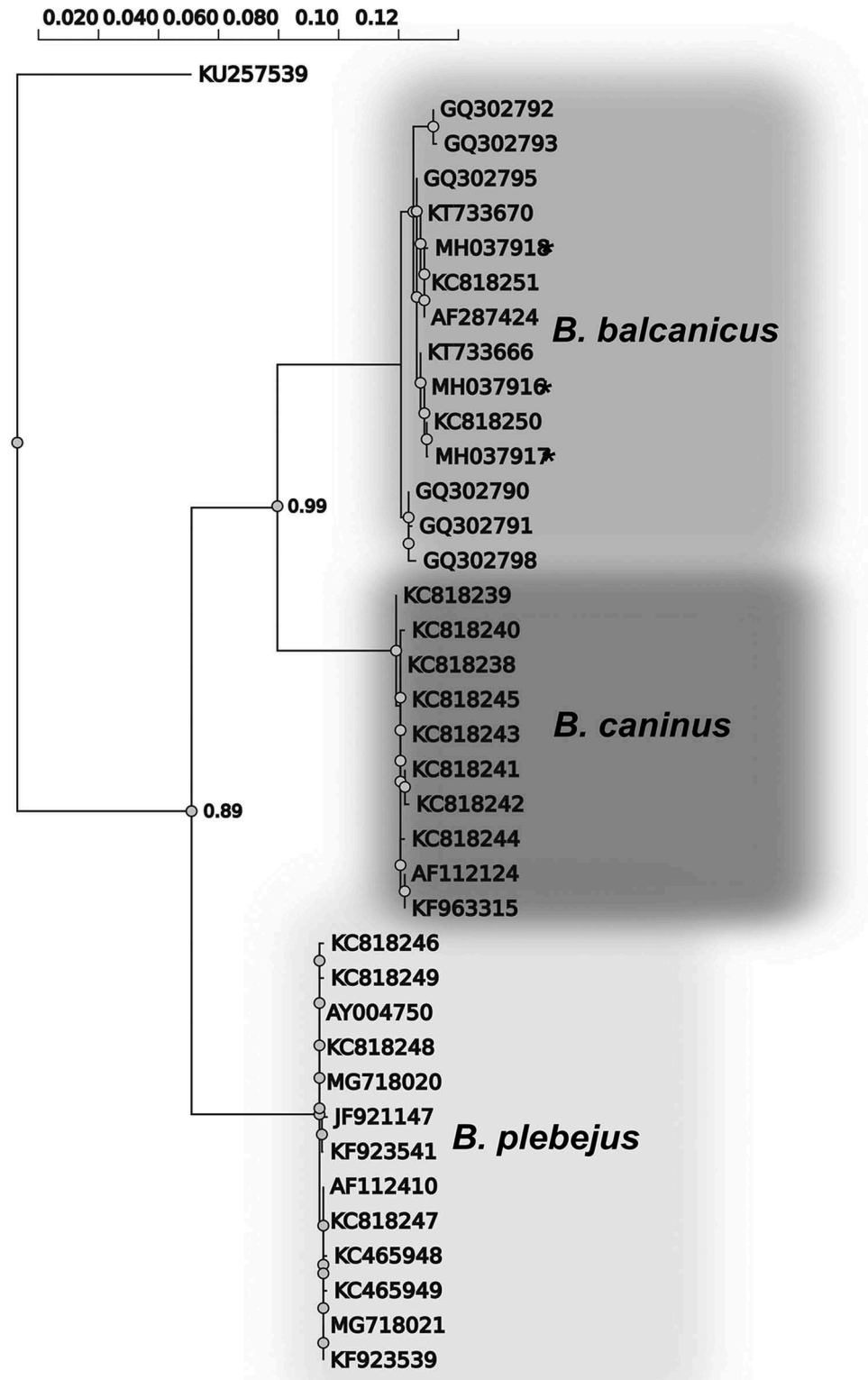

Figure 3. Maximum Likelihood tree of barbels based on 5 *cyt b* haplotypes from the entire dataset (45 experimental samples plus sequences from GenBank). Numbers above and below branches refer to the bootstrap value. Asterisks (*) indicate new haplotypes.

Table III. Comparisons among values of main meristic characters examined on *Barbus balcanicus* specimens collected in the Barbucina Creek and values reported by other authors.

		Present study	Marić et al. (2012)	Kotlík & Berrebi (2002)	Roman (2016)
		n = 78	*n* = 76	*n* = 24	*n* = 4
LLS	Mean	54.55 ± 3.88	55.91 ± 0.46	55.1 ± 2.7	50.7 ± 0.88
	Min	50	50	51	49
	Max	62	70	611	52
SALL	Mean	11.83 ± 1.14	11.9 ± 0.09	10.26 ± 0.92	10.3 ± 0.33
	Min	9	10	8	10
	Max	14	14	11	11
SBLL	Mean	10.17 ± 0.84	9.36 ± 0.09	9.11 ± 0.58	9.3 ± 0.33
	Min	9	8	8	9
	Max	11	11	10	10
D	Mean	8.88 ± 0.58	/	/	11.3 ± 0.33
	Min	7	10	9	11
	Max	10	11	11	12
A	Mean	5.87 ± 0.69	/	/	8
	Min	5	6	5	8
	Max	7	8	8	8
V	Mean	7.71 ± 0.98	/	/	8.3 ± 0.33
	Min	5	/	/	8
	Max	9	/	/	9

LLS = lateral line scales; *SALL* = scale rows above lateral line; *SBLL* = scale rows under lateral line; *D* = dorsal fin rays; *A* = anal fin rays; *V* = ventral fin rays.

(from 0+ to 4+), and among these classes, S_L values showed wide ranges, often overlapping (Table V). As for 0+ class, a proven sex determination was not possible, Table V reports mean values and standard deviations of T_L, S_L and K_{mean} for males and females belonging to classes from 1+ to 4+.

Values reported in Table V did not differ significantly between males and females belonging to the same age class (Wilcoxon test: $p > 0.169$ for all comparisons about T_L; $p > 0.261$ for all comparisons about S_L; $p > 1.169$ for all comparisons about K_{mean}).

K_{mean} decreased with age (Table V); values of K_{mean} always differ significantly among age classes for females (Kruskal–Wallis test: $H = 45.454$, *d.f.* = 3, $p < 0.001$; Conover-Iman test: $p < 0.01$ for all comparisons) while they always differ for males except between 3+ and 4+ classes (Kruskal–Wallis test: $H = 34.664$, *d.f.*= 3 $p < 0.001$; Conover-Iman test: $p < 0.001$ for all comparisons except between 3+ and 4+, $p = 0.427$).

Reproductive cycle

Trends of *GSI* values observed for males ($n = 52$) and females ($n = 58$) in Barbucina Creek during the study period are reported in Figure 5. Monthly values differed significantly both for females and males with wide fluctuations over time (Kruskal–Wallis test: $H = 37.436$; d.f. = 11; $p < 0.001$ e $H = 43.931$; d. f. = 11; $p < 0.001$, respectively).

Female maturation peak was in April as demonstrated by *GSI* in the range 7.5–18%. It decreased significantly in May and kept the same trend until July. The *GSI* started increasing slightly but significantly from values around 2–2.5% in the September–October, November–December and February–March periods (Conover-Iman test: $p < 0.05$ for all comparisons). Male maturation peak was in May with GSI value in the range 9–12%. Values observed for males increased significantly from April to May and decrease in June, July and August; then values increased slightly but progressively from September to January, except for a decrease observed in December (Conover-Iman test: $p < 0.05$ for all comparisons). Sexual maturation is reached at age 1+ in both sexes.

Environmental features

Monthly mean values of both chemical and physical features, with relative standard deviations (SD), were calculated for the whole study period in the Barbucina Creek (Table VI).

Temperature data highlighted a wide variation in relation to seasonal climate conditions. Values were in the range 1–25°C with minimum temperatures during January and maximum during July. Dissolved oxygen ranged between 5.93 and 10.32 mg l^{-1} with maximum concentrations during winter months in accordance with an inverse relationship between the two parameters (temperature

Table IV. Comparisons among values of main biometric parameters (mm) examined on *Barbus balcanicus* specimens collected in the Barbucina creek and values reported by other authors. Unbranched and branched fin rays were considered.

Data source	Present study		Bănărescu and Bogutskaya (2003)						Marić et al. (2012)		Žutinić et al. (2014)	
Basin	Isonzo Basin		Middle Danube				Middle and Lower Danube		Middle Danube Basin		Ilova Basin	
River	Barbucina Creek		Tereblia		Someş		Southern Banath		Drina River		Ilova River	
	78		20		8		13		76		77	
n	Mean ± SD	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
T_L	79.12 ± 15.99	50.00 - 141.00	-	-	-	-	-	-	-	-	-	-
S_L	66.95 ± 13.61	41.00- 121.00	152.30	124.00- 181.00	87.60	81.50- 98.00	104.30	80.00- 166.0	103.81	62.70- 188.0	96.80	46.30- 165.98
% S_L												
H	20.46 ± 4.23	13.06- 28.49	21.43	19.40- 24.70	20.70	18.70- 22.30	21.12	17.4- 22.3	22.58	19.6- 24.6	20.76	15.04- 24.04
h	10.17 ± 1.82	7.13- 13.71	9.31	8.30- 10.00	9.39	8.60- 9.80	10.12	9.10- 11.9	11.1	9.80- 12.7	10.33	8.83- 11.44
lpC	14.49 ± 2.62	10.20- 20.74	19.53	18.30- 26.70	19.91	18.40- 21.20	19.00	16.50- 21.1	19.22	17.00- 21.2	16.50	11.57- 18.84
pD	51.23 ± 7.07	39.05- 64.42	51.39	48.20- 53.60	51.31	50.00- 52.30	50.5	48.90- 54.6	52.4	49.50- 56.0	52.89	49.8- 59.93
pV	56.05 ± 5.76	46.93- 66.68	55.83	53.00- 57.40	53.90	52.50- 57.00	52.58	50.30- 54.0	55.23	51.10- 58.4	55.43	51.66- 61.23
lP	17.35 ± 3.04	12.21- 23.81	18.47	15.30- 21.20	20.50	18.60- 21.00	19.42	17.50- 21.9	19.42	17.10- 21.2	18.48	13.64- 20.64
lV	14.99 ± 2.52	10.26- 19.35	14.43	12.60- 16.80	17.68	16.80- 18.20	15.51	13.60- 16.7	18.53	13.70- 20.7	15.49	13.24- 17.83
lD	13.01 ± 2.68	7.23- 16.98	13.15	12.00- 15.00	13.25	12.10- 14.40	12.13	11.10- 13.4	12.52	10.40- 14.2	11.63	9.61- 14.69
lA	9.25 ± 1.99	4.96- 12.56	8.11	7.00- 8.90	7.72	7.00- 9.00	7.27	5.25- 9.0	6.75	4.60- 8.4	6.82	4.95- 8.59
lC	18.40 ± 2.90	12.74- 26.98	26.99	24.30- 28.80	27.68	26.30- 30.70	26.36	24.80- 28.3		-	21.17	16.56- 26.27
prO	8.12 ± 1.89	5.15- 12.97	-	-	10.99	10.20- 12.40	10.48	8.75- 11.9	12.07	9.80- 14.4	14.23	10.58- 17.97
Oh	5.04 ± 1.11	3.40- 9.05	-	-	4.93	4.60- 5.30	4.67	3.82- 5.6	4.86	4.00- 5.9	6.75	5.03- 8.56

T_L = total length; S_L = standard length; *H* = maximum body height; *h* = minimum body height; *lpC* = caudal peduncle length; *pD* = predorsal distance; *pV* = preventral distance; *lP* = length of pectoral fin base; *lV* = length of ventral fin base; *lD* = length of dorsal fin base; *lA* = length of anal fin base; *lC* = length of caudal fin base; *prO* = preorbital distance; *Oh* = horizontal diameter of eye.

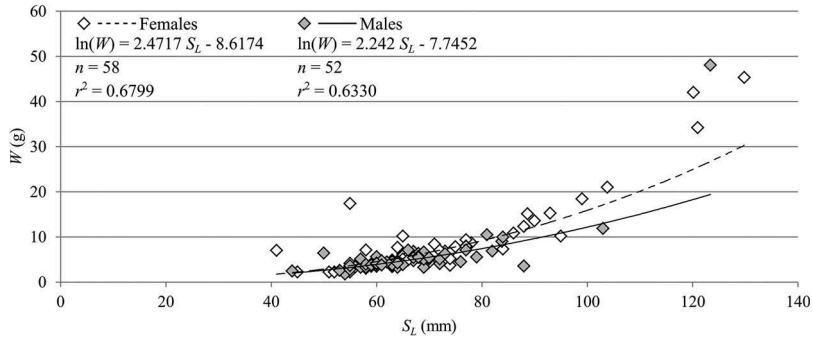

Figure 4. Regressions between standard length (S_L) and weight (W) for males and females of *Barbus balcanicus* in the Barbucina Creek.

Table V. Mean values and standard deviations (*SD*) of total length (T_L), standard length (S_L) and mean condition factor (K_{mean}) for males and females within each age class observed in Barbucina Creek.

Age	Sex	*n*	T_L	S_L	K_{mean}
1+	Females	11	6.39 ± 0.76	5.50 ± 0.66	1.24 ± 0.08
	Males	20	6.70 ± 0.62	5.71 ± 0.53	1.21 ± 0.06
2+	Females	30	8.01 ± 0.60	6.63 ± 0.62	1.09 ± 0.04
	Males	22	7.81 ± 0.46	6.60 ± 0.41	1.11 ± 0.04
3+	Females	10	9.69 ± 0.62	8.14 ± 0.67	0.98 ± 0.03
	Males	7	9.48 ± 0.75	8.17 ± 0.42	0.99 ± 0.05
4+	Females	7	12.70 ± 1.51	10.88 ± 1.46	0.85 ± 0.06
	Males	3	11.34 ± 3.16	10.18 ± 2.22	0.92 ± 0.16

and dissolved O_2). PH variation was in the range of 6.90–9.75. High levels of conductivity, ammonia, nitrates and phosphates were also assessed. In particular, ammonia and nitrates concentrations of 1.20 mg l^{-1} and 34.33 mg l^{-1} were detected in September and August, respectively. In addition, a nitrate peak of 39.33 mg l^{-1} was found in November 2016.

Discussion

Barbus balcanicus was reported by Kotlík et al. (2002) and by Freyhof and Kottelat (2008) to be present in the Italian territory only within the Isonzo River Basin. However, the species has rarely been investigated and often erroneously identified as *Barbus caninus* (Machordom & Doadrio 2001) due to a very similar morphology. As a matter of fact, *Barbus balcanicus* was

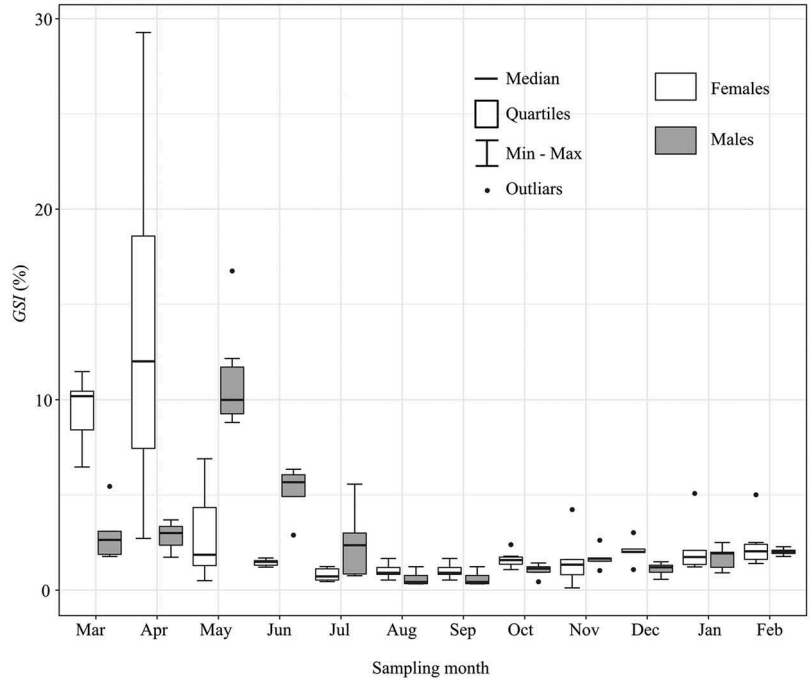

Figure 5. Temporal trends of *GSI* values observed for males and females of *Barbus balcanicus* in the Barbucina Creek.

Table VI. Mean values and standard deviations of chemical and physical parameters monthly monitored in the Barbucina Creek (site 6) between 2016 and 2017.

Month	Temperature (°C)	Dissolved oxygen (mg l^{-1})	pH units	Conductivity (µS cm^{-1})	NH_4^+ (mg l^{-1})	NO_3^- (mg l^{-1})	PO_4^{3-} (mg l^{-1})
April	14.40 ± 0.36	8.66 ± 0.58	6.97 ± 0.15	550.67 ± 3.05	0.20 ± 0.02	15.1 ± 0.85	0.31 ± 0.02
May	22.90 ± 0.17	9.07 ± 0.35	7.11 ± 0.28	497.00 ± 3.00	0.14 ± 0.08	10.4 ± 8.32	0.31 ± 0.09
June	20.60 ± 0.53	8.63 ± 0.25	7.95 ± 0.15	530.67 ± 9.45	0.19 ± 0.03	11.33 ± 1.53	0.56 ± 0.11
July	25.00 ± 0.20	8.27 ± 0.45	6.90 ± 0.09	479.33 ± 4.04	0.31 ± 0.05	1.60 ± 0.98	1.50 ± 0.93
August	17.93 ± 0.30	7.80 ± 0.21	7.20 ± 0.02	489.33 ± 5.03	0.30 ± 0.09	34.33 ± 4.95	0.12 ± 0.02
September	19.70 ± 0.25	5.93 ± 0.15	7.73 ± 0.11	553.33 ± 4.16	1.20 ± 0.03	10.83 ± 0.74	0.17 ± 0.02
October	13.47 ± 0.51	7.14 ± 0.72	7.47 ± 0.15	487.00 ± 6.56	0.63 ± 0.04	20.53 ± 1.96	0.22 ± 0.04
November	11.97 ± 0.15	8.64 ± 0.12	7.92 ± 0.07	638.33 ± 7.23	0.31 ± 0.11	39.33 ± 2.80	0.10 ± 0.04
December	4.96 ± 2.51	9.16 ± 0.30	8.56 ± 0.13	639.80 ± 19.88	0.07 ± 0.03	18.60 ± 1.76	0.71 ± 0.04
January	1.00 ± 0.26	10.32 ± 0.10	9.74 ± 0.05	641.67 ± 3.05	0.29 ± 0.05	29.34 ± 0.74	0.23 ± 0.02
February	6.60 ± 0.10	9.01 ± 0.14	9.75 ± 0.11	592.33 ± 4.72	0.28 ± 0.01	18.63 ± 0.60	0.26 ± 0.04
March	10.33 ± 0.57	9.23 ± 0.55	7.93 ± 0.51	499.67 ± 33.08	0.20 ± 0.02	15.33 ± 1.53	0.22 ± 0.01

completely omitted in the IUCN Italian vertebrates Red List (Rondinini et al. 2013).

Results of genetic analyses reported in the present study showed that *B. balcanicus* colonizes the small tributaries in the right side of the Isonzo River system, near the State border between Italy and Slovenia. Its distribution is probably wider than previously thought considering that in some of these tributaries, *Barbus caninus* was formerly reported (Stoch et al. 1992; Machordom & Doadrio 2001; Pizzul et al. 2006) but this assignment was probably an erroneous taxonomical determination based exclusively on trivial phenotype characteristics. Results of environmental parameters assessed in the present study further highlighted that investigated watercourses (especially the Barbucina Creek) did not fulfil ecological demands of *B. caninus*, which requires oxygenated waters and generally high current speed (Gandolfi et al. 1991; Zerunian 2002, 2004).

In addition, in contrast to Bianco (2014) and Geiger et al. (2016) *cyt b* analyses supported evidence that *B. balcanicus* is autochthonous in the study area. In fact, besides general geographic considerations referred to the Balkanic origin of the investigated portion of the Italian Isonzo River, and its unlikely presence due to introductions (the species has no fishing interest), original mitochondrial haplotypes were discovered for the first time. More specifically, three new *cyt b* haplotypes emerged in Barbucina, Piumizza and Reca streams. Furthermore, the previously described haplotype B. caninus1 in Barbucina Creek resulted in an erroneous assignment (Machordom & Doadrio 2001) since the majority of our samples collected in the same watercourse were assigned to this haplotype, although they correctly clustered in the *B. balcanicus* group at Maximum Likelihood analysis.

A population of *B. plebejus* belonging to haplotype *B. plebejus* was found in Reca Stream in sympatry with *B. balcanicus*. It is noteworthy observing that a previous study by Buonerba et al. (2015) highlighted the presence of late-generation hybrids between *B. balcanicus* and *B. plebejus* in Piumizza stream. In fact, in addition to Reca Stream, sympatry between *B. balcanicus* and *B. plebejus* was also observed in Piumizza and Pneuma waters. The possibility of recent hybridization events between the two species is an important ecological aspect that will have to be better investigated in the future with more specific nuclear markers.

Watercourses where only *B. balcanicus* was observed showed generally common substrates characterized by cobbles and/or flat rocks on the bottom, and reduced cover vegetation. They are affected by low flow values and are subject to strict summer drought conditions. On the opposite, sites where *B. balcanicus* was found in sympatry with *B. plebejus* showed generally constant flow rates with less water shortage (Pneuma Creek, Piumizza and Reca Stream, Table I).

Once correctly identified as *B. balcanicus*, the assessment of meristic characters was in agreement with data reported by Kotlík et al. (2002), Kottelat and Freyhof (2007), Marić et al. (2012) and Roman (2016). Ranges of all meristic characters overlapped those reported by different authors (Table III) with few exceptions, such as *LLS* values reported by Roman (2016), and *D* minimum and *SALL* maximum values. However, *SALL* maximum value is in agreement with Marić et al. (2012).

Values of biometric characters observed in the Barbucina Creek seemed to be smaller than those

reported by other Authors (Table IV). In particular, collected *B. balcanicus* specimens showed smaller size than those observed in the middle and low Danube basin (Bănărescu & Bogutskaya 2003; Marić et al. 2012) and in the Ilova River basin (Žutinić et al. 2014). Despite the smaller size, the Italian specimens were older than those collected in Croatia by Žutinić et al. (2014), as in the Barbucina Creek, five age classes were found (0+–4+). After the 2+ class sex ratio was in favour of females; similarly, Žutinić et al. (2014) reported an exclusive presence of females in the 3+ class.

Growth of *Barbus balcanicus* analysed through regressions between total weight W and standard-length S_L, did not differ between males and females, in agreement with Žutinić et al. (2014). Values of coefficient b obtained from the regressions for males and females were equal to 2.22 and 2.47, respectively, and were lower than those reported by Žutinić et al. (2014) for Croatian specimens and lower than the value indicated by Froese (2006). These results indicate that larger individuals changed their body shape to become more elongated or small individuals were in better nutritional condition at the time of sampling (Froese 2006). The latter hypothesis is the most likely, as Žutinić et al. (2014) reported an increase of K_{mean} values that is in contrast with the results of the present study. However, it is unlikely that changes in body shape due to growth could be different among different zones of the distribution area (Italy and Croatia), as analyses were conducted on the same species. In addition, the Barbucina Creek represents a clearly polluted environment, as highlighted by values of chemical and physical parameters (Table VI), in particular, conductivity, ammonia and phosphates. It must be remarked that Barbucina creek and the other investigated sites are limited water courses collecting drainage waters from the agricultural system. Therefore, they represent narrow creeks and ponds with high productivity but limited space. Reduced space availability certainly limits the morphological dimensions of fish, although habitat trophic conditions are suitable.

Reproductive cycle of *Barbus balcanicus* was formerly little studied. Gonadosomatic index (*GSI*) trends observed in the present work indicated a reproductive period for *B. balcanicus* in the study area between late April and May/early June, while Kottelat and Freyhof (2007) and Žutinić et al. (2014) report the spawning period between May and July. For both sexes, specimens reach sexual maturity at 1+ age with a mean standard length between 5.50 and 5.71 cm.

Overall results of the present study confirm the presence of *Barbus balcanicus* in Italy, in the most western portion of its distribution area, and provide useful information about rarely investigated ecological aspects such as individual growth and reproductive cycle. The investigation highlighted the need to better define the species distribution, often erroneously classified as *Barbus caninus*, and consequently adopt correct management policies. In fact, the Italian portion of the Danube barbel distribution represents a restricted zone, located within a territory subject to significant impacts due to anthropization and especially to the land use. The insertion of *Barbus balcanicus* in the Italian IUCN Red List of vertebrates is, therefore, an urgent need.

Disclosure statement

No potential conflict of interest was reported by the authors.

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