Human-mediated contact zone between endemic and invasive *Barbus* species (Osteichthyes: Cyprinidae) in a regulated lowland river: genetic inferences and conservation implications

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Abstract

Genetic surveys of the endemic Padanian barbel *Barbus plebejus* (Osteichthyes: Cyprinidae) were completed on the lowland Adda River, a heavily regulated Italian Alpine river in which the European *Barbus barbus* is invasive. Both *Barbus* species are of the large fluvio-lacustrine ecophenotype and thus they share habitats in the river. Mitochondrial (N = 35) and nuclear (N = 256) DNA sequences were completed with the aim of estimating the extent and genetic consequences of the *B. barbus* invasion. The phylogenetic analyses, based on *cyt b* mtDNA, *S7* ribosomal protein and growth hormone (*GH*) nuclear genes, revealed 59 haplotypes as *B. plebejus* and 37 as *B. barbus*. Genetic diversity values (H and π) and the mismatch distribution analyses indicated *B. plebejus* was still experiencing demographic radiation from a modest number of founders. The *B. barbus cyt b* haplotypes suggested their original source was the River Danube. Through analysing the nuclear paralog sequences, evidence was provided of consistent genetic admixture between *B. plebejus* and *B. barbus*, being recorded in over 40% of the sampled fishes, indicating that their ecological and biological interactions are disrupting the genetic integrity of *B. plebejus*. Bayesian analysis and factorial correspondence analysis then revealed that the habitat fragmentation of the Adda River is not limiting population connectivity. It was apparent there has been recent and rapid upstream dispersion of *B. barbus*, with individuals overcoming barriers to their migration through using fish passes and/or through translocation between impounded sections by anglers. The potential conservation implications of this for the endemic *Barbus* species are discussed.

Keywords: *Barbus*, genetic structure, *cyt b*, nuclear paralogs, fish passes

Introduction

Freshwater ecosystems represent rich pools of biodiversity at the global scale (Strayer & Dudgeon 2010) but are also hotspots of human use and alteration (Monroe et al. 2009). In particular, the fish diversity of river systems is heavily impacted through disturbances including overexploitation, water pollution, habitat degradation, flow modifications and biological invasions (Allan & Flecker 1993; Naiman & Turner 2000; Malmquist & Rundle 2002; Postel & Richter 2003; Dudgeon et al. 2006). Disturbed habitats can facilitate invasion processes (Hobbs & Huenneke 1992), with invasive populations then often having further negative consequences for native species (Didham et al. 2005; Gozlan et al. 2010; Hermoso & Clavero 2011). These negative consequences, including increased predation pressure and resource competition (Gozlan et al. 2010), are usually increased when there is high niche overlap between the native and invasive species (Strayer 2006). In these situations, and when the species concerned are similar taxonomically, then genetic introgression through hybridisation can also occur (Hanfling et al. 2005).

There are more than 200 endemic freshwater fishes in Mediterranean rivers and, of these, 56% of these have been reported as being under threat (Smith & Darwall 2006). The sustainability of endemic fish
populations in the Northern Adriatic drainage basin are particularly threatened (e.g., Bianco 1995, 1998; Smith & Darwall 2006; Gherardi et al. 2008) with, for example, endemic species of the Cyprinidae family heavily impacted by both hydrologic alterations (e.g., hydroelectric dams impounding sections and regulating flows) and the introduction of over 50 non-native fishes (Bianco 2014). These include species of the Cyprinidae family, such as common bream Abramis brama (L.), silver bream Blöcka bjölkens (L.), roach Rutulus rutulus (L.), bleak Alburnus alburnus (L.) asp Aspius aspius (L.), bitterling Rhodeus amarus (Bloch, 1782), topmouth gudgeon Pseudorasbora parva (Temminck & Schlegel, 1846), gudgeon Gobio gobio (L.), chub Squalius cephalus (L.) and common carp Cyprinus carpio (L.) (for a detailed list of updated morphological identifications assisting plans for interaction with native species have been reported as establishing negative implications of our results for the endemic Barbus species, (iii) examine the population genetic structure, quantifying the hybrid forms between the endemic and invasive Barbus species, and (iv) assess the consequences of interruptions in river connectivity for Barbus population structuring and gene flow. The conservation implications of our results for the endemic B. plebejus are then discussed.

The study area is the Adda River, a main Alpine tributary of the Po River that is heavily regulated. Several barriers, including hydropower dams and weirs, interrupt its longitudinal connectivity. Although strongly hydrologically altered, the lowland stretch of the Adda River is mainly characterised by a fluvio-lacustrine habitat in which the endemic B. plebejus and the invasive B. barbus co-occur, providing a “human-mediated” contact zone (Zerunian 2002; Kottelat & Freyhof 2007; Meraner et al. 2013). In this study, we analysed the Barbus populations present in the lowland Adda River stretch, using mitochondrial DNA (cytochrome b) and nuclear DNA (ribosomal protein (S7) and growth hormone (GH) sequences. The objectives of the study were to: (i) genetically discriminate different haplotypes of each Barbus species, (ii) highlight the historic demographic pattern of the endemic Barbus species, (iii) examine the population genetic structure, quantifying the hybrid forms between the endemic and invasive Barbus species, and (iv) assess the consequences of interruptions in river connectivity for Barbus population structuring and gene flow. The conservation implications of our results for the endemic B. plebejus are then discussed.

Materials and methods

Study species

The Padanian barbel Barbus plebejus, endemic to the Northern Adriatic drainage basin (Bianco 1995), dominates the fish assemblage of the Po river basin and prefers moderately deep, well oxygenated, flowing water on plateaus or foothill regions (fluvio-lacustrine ecophenotype; Bianco 2003). Barbus barbus belong to the fluvio-lacustrine ecophenotype, with total lengths up to 90 cm and a preference for wide and calm rivers but with a regular current (Tsigenopoulos et al. 1999; Kottelat & Freyhof 2007). It is widely distributed in Europe and naturally absent in Mediterranean basins, with the exception of the Lergue River in southern France (Chenuil et al. 2000; Kottelat & Freyhof 2007). Morphological discrimination between B. plebejus and B. barbus is done through the number of scales along the lateral line and the form and microstructure of the dorsal fin; this identification can, however, be unreliable due to the variability of these traits between the species (Bianco 2003; Kottelat & Freyhof 2007). Moreover, interspecific hybridisation is frequently encountered in the Barbus genus, providing further identification problems (Tsigenopoulos et al. 2002; Chenuil et al. 2004; Gante et al. 2008; Lajbner et al. 2009).
**Fish sampling and study area**

Fish were sampled for the genetic analyses using backpack electric fishing conducted between August 2011 and March 2012 from four Northern Adriatic sampling sites (Figure 1). Thirty-four fishes were collected from three localities spread over 50 km of the Adda River that were all in the epipotamal zone (“barbel zone”) and characterised by habitats favouring fish of the fluvio-lacustrine ecophenotype (Figure 1; Table I). The Adda is an Alpine lowland river flowing for 130 km (north to south) with a catchment area of 3400 km², and is part of the Po River drainage, the major basin of the Adriatic Sea catchment area. Mean natural discharge rates are 150–200 m³ s⁻¹ with a channel slope that ranges from 0.01 to below 0.001. Since the 1950s, the Adda has been hydrologically regulated for hydropower generation and irrigation (Figure 1). To test the distribution limit of *B. plebejus* species, samples were also collected from the Brembo River, the main tributary of the Adda (Figure 1). The samples were collected from a site in the rhithral zone (“trout zone”), in which the fast current and gravel riverbeds offer suitable habitat for species of the rheophilic ecophenotype (e.g. *Barbus caninus*) (Zerunian 2002).

Following their capture, the fish were anaesthetized (MS-222) and measured (fork length, nearest mm), and tissue samples (pelvic fin clips) were taken from each individual. These tissue samples were stored in 90% ethanol and then frozen. Reliable species identification from morphology was rarely possible in the field due to the aforementioned issues of similarity in meristic characters in *B. barbus* and *B. plebejus*. Following their processing, the fishes were allowed to recover and then returned to the water.

**Mitochondrial and nuclear DNA sequencing**

Extraction of genomic DNA was completed through a proteinase K digestion followed by sodium chloride extraction and ethanol precipitation (Aljanabi & Martinez 1997). The genetic analyses then included sequencing of (i) mitochondrial DNA Cytochrome b (*cyt b*) gene and (ii) S7 ribosomal protein (*S7*) and growth hormone (*GH*) nuclear genes, as these have

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Figure 1. Map of sampling localities along the Adda River (AD1, AD2, AD3) and Brembo River (BR4) in the Po River basin (North Italy), with the barriers to migration shown. The number of nuclear sequences analysed (N) is indicated for each population, while the number of mitochondrial *cyt b* sequences is detailed in Table I. Pie charts show haplogroup frequency of *Barbus plebejus*, *Barbus barbus* and *Barbus caninus.*
been used previously to discriminate *Barbus* species (Doadrio et al. 2002; Gante et al. 2011; Buonerba et al. 2013). The *cyt b* was amplified and sequenced using primer pair L15267 and H16461 (Briolay et al. 1998), and we followed the methods of Tsigenopoulos and Berrebi (2000).

Since European *Barbus* species are all tetraploid, to avoid the risk of simultaneous amplification of paralogous loci, the amplifications of single-copy *S7* ribosomal protein and *GH* genes were performed using paralog-specific primers (Gante et al. 2011). Following the method of Gante et al. (2011), polymerase chain reaction (PCR) amplification of nuclear loci was performed with Multiplex PCR kit (Qiagen) using primer pairs 1–8 and 10–15 for *S7* paralogs (*S7*-1 and *S7*-2 respectively), and numbers 16–30 (AGT GGS AGG TCG TTY, modified from Gante et al. 2011) and 24–30 for *GH* paralogs (*GH*-1 and *GH*-2 respectively). PCR products were purified using kit Illustra™ Exostar (GE Helthcare) and sequenced in both directions with amplification primers on an ABI 3130xl Genetic Analyzer using Big Dye 3.1 terminator (Applied Biosystem). Only one *GH* paralog (*GH*-1) was sequenced using an internal forward primer (*GH*-1 17b: GTA ATA ACC AAT AGA ATA AAG T).

Mitochondrial and nuclear sequences were manually aligned to eliminate ambiguities and to check polymorphic sites. Specifically, insertions or deletions (indels) for *GH* and *S7* paralog sequences were manually phased using the forward and reverse complementary information, following Flot et al. (2006). The positive feedback of the Flot et al. (2006) method was carried out through cloning and sequencing five samples for each marker. Then, heterozygous alleles for single nucleotide polymorphisms (SNPs) were phased using DnaSP v. 5 (Librado & Rozas 2009). The number of haplotypes was then calculated using Non Redundant Data Base (Gish 2004).

Among mitochondrial and nuclear sequences (*n = 291*), 98 haplotypes were recorded (i.e. 59 haplotypes as *B. plebejus*, 37 as *B. barbus* and two as *B. caninus*) and deposited in GenBank under accession numbers KF923535–KF923632.

### Phylogenetic discrimination of Barbus spp.

Phylogenetic analyses were carried out independently on mitochondrial *cyt b* and on each nuclear marker (*GH* and *S7* paralogs). Computation of phylogenetic reconstructions of haplotypes was performed using two different optimality criteria: maximum likelihood and Bayesian analyses. The former was implemented with phylogenetic analysis using the Maximum likelihood (ML) approach, performed

| Site code | Water course | Altitude (m) | Ecological zone | N | Haplotype distribution | H + SD | π + SD |
|-----------|--------------|--------------|-----------------|---|------------------------|-------|-------|
| BR4       | Brembo       | 333          | Trout           | 1 | 1                      | 0     | 0.5   |
| AD1       | Adda         | 234          | Barbel          | 1 | 1                      | 0     | 0.01  |
| AD2       | Suisio       | 234          | Barbel          | 2 | 2                      | 0.5   | 0.13  |
| AD3       | Trezzo sull’Adda | 187      | Barbel          | 3 | 3                      | 0.16  | 0.11  |
| AD4       | Boffalora d’Adda | 146       | Barbel          | 1 | 1                      | 0     | 0.5   |
| Total     |              |              |                 | 36| 36                     | 0.13  | 0.11  |

SD, standard deviation. *GenBank acc. no. BAR1 = AY331020; BAR2 = AY331017; ** one sample failed.
Demographic pattern of endemic Barbus

Historic demographic expansion was then investigated for native *B. plebejus* population in the Adda River by analysing frequency distributions of pairwise differences (mismatch distribution analysis) between sequences for each marker (*cyt b*, *GH* and *S7* paralogs) (Rogers & Harpending 1992). The sudden stepwise expansion model was tested and the validity of the expansion model was performed using the sum of square deviations (SSD) between the observed and the expected mismatch as a test statistic (Rogers 1995; Excoffier 2004); all calculations were done using Arlequin 3.11 (Excoffier et al. 2005).

The time since *B. plebejus* population growth was also inferred from the *cyt b* mismatch distribution as

$$ t = \left(\frac{\tau}{2\mu}\right) \times \text{generation time (g)} \quad (1) $$

assuming a generation time of approximately 4–5 years (Kottelat & Freyhof 2007). The expansion time ($\tau$) was computed with Arlequin 3.11 while the *cyt b* molecular clock of 0.76% nucleotide substitution per site per million years was retained, as proposed by Zardoya and Doadrio (1999) for European cyprinids.

Genetic admixture and population genetic structure

Following phylogenetic discrimination for each allele retrieved from *S7* and *GH* paralogs, the genetic admixture was quantified as the proportion of native vs. invader haplotypes, discriminating the pure-bred fishes from hybrid forms (*B. barbus vs. B. plebejus*). Then, each unique allele was numerically coded and used to genotype each specimen, and to perform population genetic structure analyses. Genetic variability within populations was then quantified by expected (H_E) and observed (H_O) heterozygosity. Population pairwise $F_{ST}$ values (Weir & Cockerham 1984) and a factorial correspondence analysis (FCA) were performed to visually inspect genetic relations among populations. All these analyses were computed using Genetix v. 4.05 (Belkhir et al. 1996). The most probable number of homogeneous genetic groups (i.e. number of clusters K) was then identified using the Bayesian clustering method implemented in the software STRUCTURE v. 2.3.2 (Pritchard & Wen 2002). Twenty runs for each K value from 1 to 10 were performed to assess the consistency of likelihood estimations. Each run was made of 30,000 MCMC generations as the burn-in, followed by 50,000 MCMC replicates to estimate the posterior sample distribution through GARLI (Genetic Algorithm for Rapid Likelihood Inference) v. 1.0 (Zwickl 2006). Models of sequence evolution, used for each marker (i.e. *cyt b* haplotypes: TIM (transitional mode) (Lanave et al. 1984; Rodríguez et al. 1990); S7 paralog 1 haplotypes: GTR (general time-reversible model) (Lanave et al. 1984; Rodríguez et al. 1990); S7 paralog 2 haplotypes: TrN (TamNei) (Tamura & Nei 1993); GH paralogs haplotypes: K3Puf (Kimura 3-parameter) (Kimura 1981)), were identified by Akaike criteria in the ModelTest 3.7 (Posada & Crandall 1998). Statistical support for the phylogenetic tree nodes was determined through 1000 non-parametric bootstrap replicates. The Bayesian method was implemented in MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003) with a Markov Chain Monte Carlo (MCMC) algorithm. Four simultaneous and independent Markov chains from random trees were started and run for 1,000,000 generations, with the first 25,000 generations discarded as the burn-in when the standard deviation of split frequencies was below 0.01. Twenty runs for each K value from 1 to 10 were performed to assess the consistency of likelihood estimations as the burn-in, followed by 50,000 MCMC replicates to estimate the posterior sample distribution.

Barbus populations of Adda River (Italy)  575
using the admixture and correlated allele frequency models. The most likely K value was evaluated by ΔK, the second-order rate of change of the likelihood function with respect to K (Evanno et al. 2005). STRUCTURE HARVESTER 0.6.8 software was used to visualize the outputs (Dent & Bridgett 2011).

In order to test the interruptions in river connectivity, genetic diversity within and among populations was estimated using an analysis of molecular variance (AMOVA; Excoffier et al. 1992) in Arlequin 3.11, grouping the population in two combinations: (i) Pop. AD1–Pop. AD2 vs. Pop. AD3 and (ii) Pop. AD1 vs. Pop. AD2–Pop. AD3.

## Results

### Mitochondrial and nuclear sequence variation

Thirty-five partial mitochondrial cyt b sequences (1084 bp in length) were obtained from 36 individuals of the sampled Barbus species, as sequencing of one of the fish failed (Table I). Forty variable nucleotide positions were detected, of which three were singletons and 37 were parsimony-informative sites. Sequence alignments did not require deletion or insertion and were grouped in eight unique haplotypes (Table I), two of which were already present in the public database and ascribed to B. barbus (GenBank accession numbers AY331020 and AY331017; Kotlik et al. 2004). Following sequencing and phasing of nuclear genes, a total of 256 sequences were produced. A total of 717 SNPs were found, widely distributed in both S7 ribosomal protein and GH genes. In the alignments, up to 10 indels (from 1 bp up to 104 bp in length) were assumed to maximise base pair identity in conserved sequenced blocks (Table II). Among the 256 nuclear sequences, 90 haplotypes were scored (Table III); only one GH-1 B. barbus haplotype was already in GenBank (FR872190; Gante et al. 2011).

### Discrimination of Barbus spp. through phylogenetic tree reconstruction

The phylogenetic analyses suggested that 23 out of 35 Barbus cyt b sequences fell within the B. plebejus cluster (four PLE haplotypes) and 12 within the B. barbus cluster (four BAR haplotypes) (Figure 2A; Table I). The topology of the cyt b tree obtained with the Bayesian method was concordant with the one obtained by the ML method and was also supported by bootstrap values (Figure 2A).

Among 90 nuclear haplotypes, 55 were congruent with B. plebejus and 33 with B. barbus (Table III). Only analysing S7-2 and GH-2 paralogs, two B. caninus haplotypes were scored, from one fish sampled in the “trout zone” (Brembo river). The nuclear phylogenetic inferences were performed on sequences of reduced bp length (i.e. excluded indels; S7-1: 350 bp; S7-2: 697 bp; GH-1: 583 bp; GH-2: 899 bp). Phylogenetic inferences of nuclear sequences provided very similar trees using both Bayesian and maximum likelihood methods (cf. S7-2 tree; Figure 2B), with three main recognizable lineages: B. plebejus, B. barbus and B. caninus. The B. barbus and B. plebejus clades were related to a large eco-phenotype group of fluvio-lacustrine fishes and the B. caninus clade to a rheophilic eco-phenotype (Zerunian 2002), detected only in the trout zone of the Brembo River. Within the fluvio-lacustrine group, the phylogenetic tree reconstruction supported a significant split between B. barbus and B. plebejus (cf. S7-2 tree Figure 2B).

The gene genealogy inferred from the parsimony probability (95%) and based on the cyt b dataset

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#### Table II. Summary of polymorphisms for S7 and GH paralogs, indicating the number of indels and the number of single nucleotide polymorphisms (SNPs). The number of sequences (N), range of sequence lengths (bp), number of haplotypes (h) and number of segregating sites (S) are indicated.

| Nuclear loci | N    | Length (bp) | h     | S      | Number of indels | Total SNPs |
|--------------|------|-------------|-------|--------|------------------|------------|
| S7_1         | 48   | 673–711     | 25    | 43     | 10               | 178        |
| S7_2         | 70   | 704–718     | 17    | 41     | 10               | 154        |
| GH_1         | 68   | 994–1097    | 19    | 17     | 4                | 125        |
| GH_2         | 70   | 897–1029    | 29    | 55     | 7                | 260        |
| Total        | 256  | 90          | 156   | 31     |                  | 717        |

SD, standard deviation.

#### Table III. Summary of Barbus plebejus and B. barbus polymorphisms for cyt b, S7 ribosomal protein and growth hormone (GH) paralogs. The number of sequences (N), number of haplotypes (h), haplotype (H) and nucleotide (n) diversity are indicated.

| Barbus spp. | N    | h     | H ± SD  | n ± SD |
|-------------|------|-------|---------|--------|
| B. plebejus |      |       |         |        |
| cyt b       | 23   | 4     | 0.38 ± 0.12 | 0.001 ± 0.000 |
| S7-1        | 36   | 16    | 0.87 ± 0.05 | 0.006 ± 0.001 |
| S7-2        | 58   | 8     | 0.65 ± 0.04 | 0.002 ± 0.001 |
| GH-1        | 61   | 13    | 0.57 ± 0.08 | 0.002 ± 0.000 |
| GH-2        | 56   | 18    | 0.64 ± 0.07 | 0.004 ± 0.001 |
| Total       | 234  | 59    |         |        |
| B. barbus   |      |       |         |        |
| cyt b       | 12   | 4     | 0.71 ± 0.10 | 0.001 ± 0.000 |
| S7-1        | 12   | 9     | 0.96 ± 0.05 | 0.011 ± 0.003 |
| S7-2        | 11   | 8     | 0.93 ± 0.07 | 0.006 ± 0.001 |
| GH-1        | 7    | 6     | 0.89 ± 0.11 | 0.002 ± 0.001 |
| GH-2        | 13   | 10    | 1 ± 0.05   | 0.010 ± 0.001 |
| Total       | 55   | 37    |         |        |
yielded two networks that were represented by the *B. plebejus* and *B. barbus* sample sequences. The minimum-spanning networks (MSNs) gave a “radial pattern” around one (*PLE2*) and two (*BAR1* and *BAR2*) widespread haplotypes for *B. plebejus* and *B. barbus* respectively (figure not shown). The genetic diversity values were lower for native *B. plebejus* than for invasive *B. barbus*, as reflected both in mitochondrial and nuclear markers (cf. Table III). For *B. plebejus*, the haplotype and nucleotide diversities values ranged from 0.38 to 0.87 and from 0.0004 to 0.006 respectively, whilst for *B. barbus* they ranged from 0.71 to 1.00 and from 0.001 to 0.011 respectively (Table III).

**Historic pattern of *B. plebejus* in the Adda**

In the Adda River, the historic demographic expansion of the native *B. plebejus* population was highlighted by the mismatch distribution of pairwise nucleotide differences. The mismatch distribution did not deviate significantly from the expected distribution obtained under a sudden expansion model, with the SSD values for *cyt b* and the *S7* paralogs fitting the model (*cyt b* SSD = 0.177; *p* > 0.05; *S7-1* SSD = 0.029, *p* > 0.05; *S7-2* SSD = 0.012, *p* > 0.05). The expansion time inferred from *cyt b* was τ = 0.502 and the time since the demographic expansion was estimated to have occurred between 12,000 and 15,000 years ago.

**Population genetic parameters of Barbus populations**

Following the genotyping of 36 sampled individuals and based on 256 nuclear sequences obtained for four loci from GH and S7 paralogs, 18 pure-bred *B. plebejus* were detected (*n* = 3 in AD1, *n* = 8 and *n* = 7 in AD2 and AD3, respectively), with 18 individuals showing genetic admixture between *Barbus* species: 17 individuals between *B. plebejus* and *B. barbus*, and one individual between *B. plebejus* and *B. caninus*, collected in the trout zone (Figure 3).

No pure-bred *B. barbus* were detected. In order to describe the different hybrid forms, five admixture classes of native vs. invader genotypes were produced, based on the proportion of *B. barbus* alleles to the *B. plebejus* pure-bred: (1) from cl-1 to cl-3 with genetic presence of invader *B. barbus* alleles lower than 50%, (2) a middle class with equilibrated presence of *B. plebejus* vs. *B. barbus* alleles (50%) and (3–5) three classes from cl-5 to cl-7 with *B. barbus* alleles higher than 50% (Figure 3). Thus, the *Barbus* populations were characterised by a complex distribution of hybrid forms in which the higher-admixture forms between *B. barbus* vs. *B. plebejus* (> 50%, cl-5 and cl-6) were collected in downstream AD2 and AD3 populations (Figure 3).

For the three Adda populations, population genetic variability, estimated from nuclear sequences, indicated values of haplotype diversity ranged from 0.56 (GH-1 for AD1) to 0.95 (S7-1 for AD3), and values of \( \pi \) ranged from 0.003 (GH-
The H and \( \pi \) indices, estimated from cyt b sequences, showed congruent values (Table I). Following genotyping, values of \( H_E \) and \( H_O \), averaged over four loci, ranged from 0.81 to 0.88 and from 0.95 to 1.0, respectively. Conversely, values of pairwise \( F_{ST} \) between the three populations were low (~0.004 to ~0.001) and non-significant (\( p < 0.05 \)) (Supplementary material, Table S1). Although Bayesian clustering analysis indicated that the number of subdivisions that captured most of the structure in the data was \( K = 3 \) (Figure 4A), the populations could not be attributed to one of the three groups (Figure 4B), revealing a lack of clear clustering among the populations.

The FCA ordination of samples confirmed the absence of population structure among the Barbus populations (Figure 5). Following the two population combinations (AD1–AD2 vs. AD3 and AD1 vs. AD2–AD3), hierarchical AMOVA indicated that the largest amount of genetic variation was distributed within populations (96% and 99%, respectively), indicating no significant genetic structuring among samples and suggesting high connectivity among them.

### Discussion

**Historic and recent demographic pattern of Barbus species in the lowland Adda River**

In the epipotamal stretch of lowland Adda River (“barbel zone”), the co-occurrence of the endemic and invasive Barbus species has been demonstrated (Tsigenopoulos & Berrebi 2000; Meraner et al. 2013) and the analyses here were able to discriminate between the invasive and native haplotypes. Through the applications of nuclear and mitochondrial markers, large genetic variability was revealed across the fishes, with more than 50 and 30
haplotypes detected for *B. plebejus* and for *B. barbus* respectively.

The gene genealogy of native *B. plebejus*, inferred from *cyt b* sequences, provided a star phylogeny pattern that indicated a starburst radiation from one widespread haplotype (*PLE2*). This might reflect a recent size expansion from a modest number of founders (Avise 2000), with this hypothesis supported by the combination of the relatively high values of haplotype diversity (H) and low values of...
nucleotide diversity ($\pi$) (Grant & Bowen 1998). Population expansion was also supported by the mismatch distributions that suggested significant demographic expansion after recent bottlenecks. Episodes of bottlenecks during Pleistocene glaciations have been largely documented in phylogeographic studies of other European fish species (e.g. Durand et al. 1999; Nesbo et al. 1999; Bernatchez 2001; Kotlik & Berrebi 2001; Kotlik & Berrebi 2002; Hanfling et al. 2002). Thus, the genetic pattern of *B. plebejus* may be interpreted as the result of the post-glacial colonization of the Padano-Venetian district (Bianco 1995) following the last glacial maximum (approximately 20,000 years ago) (Weiss et al. 2002). This biodiversity hotspot area has traditionally been considered one of the most important Southern European glacial refugia (i.e. for the Balkans, Iberia and Italy) from which freshwater fishes colonised Europe in the post-glacial period (Bănărescu 1991; Bianco 1995). The *B. barbus* cyt b mtDNA sequences depicted a radial pattern around two widespread haplotypes, originally found in the River Danube (cf. Kotlik et al. 2004), suggesting that this *B. barbus* invasion originated from stockings of fish from a donor population that originated from the Danube River (Kotlik et al. 2004).

**“Human-mediated” contact zone**

In the 50-km reach of the Adda River that was sampled, it was apparent from the genetic outputs that *B. barbus* and *B. plebejus* were sympatric and thus the river represents a “human-mediated” contact zone between these species (Holsbeek et al. 2008; Aboim et al. 2010). Although this is a recent invasion following introductions that occurred less than 20 years ago (Bianco 1995), the outputs suggested that only 50% of the sampled fishes were native *B. plebejus* (“pure-bred”). Moreover, there were low levels of genetic variability in the native *B. plebejus*, with this also apparent in the invasive *B. barbus*. It was assumed that this represented a loss of genetic diversity in *B. plebejus* that resulted from a decrease in the habitat quality of the river (i.e. the chemical and hydraulic alteration of the lowland river; Meraner et al. 2013). It was also confirmed that there were hybrids present between *B. plebejus* and *B. barbus* that resulted from their interactions in the human-mediated contact zone (Meraner et al. 2013). In addition, it was also shown for the first time that there was hybridisation occurring between *B. plebejus* and *B. caninus* in the ecological “trout zone”, a result of their interactions along a natural transitional zone (Šlechtová et al. 1998; Lajbner et al. 2009).

The current situation of the sympatric presence of the three *Barbus* species along a gradient that moves through fluvo-lacustrine and rheophilic habitats does not exclude the possibility of further anthropogenic disturbances that could further increase the breakdown of behavioural isolation mechanisms in the species by increasing the differential abundance between native and invader species or by increasing habitat overlap (Scribner et al. 2000). Indeed, the data outputs strongly suggested that there was admixture between these species, suggesting interbreeding and hybridisation, and so disrupting the genetic integrity of the endemic *B. plebejus*. Similar patterns of interbreeding between closely related introduced and native fishes have been documented in Northern American fish communities (e.g. Perry et al. 2002; Rubidge & Taylor 2005) that are also compromising the freshwater fish biodiversity through driving species towards their “genomic extinction”.

**Fragmented or river connectivity?**

The Adda River is highly regulated, with six weirs and three dams (named Taccani, Concesa and Rusca) present across the study reach that cause considerable water level variation and habitat fragmentation. Despite this, the hybrid forms between the endemic and the invasive *Barbus* were relatively homogeneous between the sites, ranging from 15% in the upstream population (AD1) to 19% in the downstream population (AD3). Among the nine barriers to migration present in the study reach, longitudinal connectivity for fish is maintained by the presence of six fish passes. The remaining three blockages are due to hydroelectric dams that were considered impassable to *Barbus* without human assistance (Gentili et al. 2011). Whilst a relatively low number of individuals per population were sampled, which may compromise the significance of population structure analyses (Wang 2010), it was found that the major level of genetic variability was within populations, with the output not supporting the presence of structuring between populations that were divided by apparently impermeable dams. This suggests that the *Barbus* species were able to use the fish passes, and/or anglers were moving fish between sections that were separated by the hydroelectric dams.

**Conclusions**

In summary, the combination of anthropogenic disturbances on the Adda River – disconnection in longitudinal connectivity and introduced *B. barbus* – has
had substantial consequences for the endemic *Barbus* species, as shown by Meraner et al. (2013). In the Adda River, the longitudinal disconnection has been partially alleviated by fish pass construction, as the outputs here suggest they are used effectively by the *Barbus* species (Gentili et al. 2011). In doing so, however, this appears to have facilitated the dispersal of the invasive *B. barbus*, leading to their substantial admixture with *B. plebejus*. Consequently, the *B. barbus* introduction into the Adda represents a strong case study of the potential genetic consequences of introducing allochthonous freshwater fish (cf. Bianco & Ketmaier 2001; Zerunian 2002; Gherardi et al. 2008). Accordingly, the status of the endemic *Barbus* species is being eroded and measures to conserve their genetic integrity are required if their populations are to be maintained. Whilst this needs an active focus on increasing the regulation of angling and fishery management practices to prevent the further dispersal of *B. barbus* in this catchment and elsewhere in Italy, the blockages to fish migration within the catchment are also challenging. Nevertheless, fishes were still able to move between some sections of river, for example via fish passes, suggesting that a conservation strategy of preventing further introductions of *B. barbus*, allied with isolating and then culturing and restocking with pure-bred *B. plebejus*, could strongly contribute to the subsequent conservation of these endemic populations.

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