Molecular Phylogenetics and Evolution 89 (2015) 115–129

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Intrinsic and extrinsic factors act at different spatial and temporal scales to shape population structure, distribution and speciation in Italian *Barbus* (Osteichthyes: Cyprinidae) ☆

Luca Buonerba [a,b,*], Serena Zaccara [a], Giovanni B. Delmastro [c], Massimo Lorenzoni [d], Walter Salzburger [b], Hugo F. Gante [b,*]

[a] *Department of Theoretical and Applied Sciences, University of Insubria, via Dunant 3, 21100 Varese, Italy*
[b] *Zoological Institute, University of Basel, Vesalgasse 1, 4056 Basel, Switzerland*
[c] *Museo Civico di Storia Naturale, via s. Francesco di Sales, 188, Carmagnola, TO, Italy*
[d] *Department of Cellular and Environmental Biology, University of Perugia, via Elce di Sotto, 06123 Perugia, Italy*

ARTICLE INFO

Article history:
Received 27 June 2014
Revised 26 March 2015
Accepted 28 March 2015
Available online 14 April 2015

Keywords:
Barbus
Fossil calibration
Population genetics
Hybridization
Introgression
Introduced species

ABSTRACT

Previous studies have given substantial attention to external factors that affect the distribution and diversification of freshwater fish in Europe and North America, in particular Pleistocene and Holocene glacial cycles. In the present paper we examine sequence variation at one mitochondrial and four nuclear loci (over 3 kbp) from populations sampled across several drainages of all species of *Barbus* known to inhabit Italian freshwaters (introduced *B. barbus* and native *B. balcanicus*, *B. caninus*, *B. plebejus* and *B. tyberinus*). By comparing species with distinct ecological preferences (rheophilic and fluvio-lacustrine) and using a fossil-calibrated phylogeny we gained considerable insight about the intrinsic and extrinsic processes shaping barbel distribution, population structure and speciation. We found that timescales of Italian barbel diversification are older than previously thought, starting in the Early Miocene, and involving local and regional tectonism and basin paleo-evolution rather than Pleistocene glacial cycles. Conversely, more recent environmental factors associated with glaciation–deglaciation cycles have influenced species distributions. These events had a more marked impact on fluvio-lacustrine than on rheophilic species by means of river confluence at low sea levels. We show that genetic structure is influenced by species ecology: populations of small rheophilic species inhabiting upper river stretches of large basins are less connected and more differentiated than large fluvio-lacustrine species that inhabit lower river courses. We report the existence of both natural and human-induced interspecific gene flow, which could have great impacts on the evolution and persistence of species involved. In addition, we provide evidence that *B. tyberinus* is genetically distinguishable from all other Italian taxa and that its morphological similarity to *B. plebejus* and intermediacy with *B. caninus* are best explained by recent common ancestry and similar ecology with the former, rather than by hybrid origin involving these two species as previously hypothesized.

1. Introduction

The analysis of molecular variation across space and time provides valuable information regarding the mechanisms responsible for the origin of species as well as their persistence, admixture and extinction. Considerable attention has been given to external factors affecting the geographical distribution and the genetic diversity of temperate taxa. Comparatively less attention has been given to the ecology of species and the impact that ecological preferences may have on genetic structure. The effects of Pleistocene glaciations on distribution and diversification of temperate taxa have been the focus of several studies, with southern peninsulas (e.g. Iberia, Italy) alternating between the roles of refugia or sources of colonizers during glacial and interglacial periods (Taberlet et al., 1998; Hewitt, 1999, 2000). This classical scenario seems to apply less to aquatic than to terrestrial species, due to the confinement of the former to river systems and to insurmountable barriers to dispersal (e.g. the Pyrenees, the Alps). While substantial information has been gathered for northern and central

---

☆ This paper has been recommended for acceptance by G. Orti.

* Corresponding authors at: Department of Theoretical and Applied Sciences, University of Insubria, via Dunant 3, 21100 Varese, Italy (L. Buonerba), Zoological Institute, University of Basel, Vesalgasse 1, 4056 Basel, Switzerland (L. Buonerba and H.F. Gante).

E-mail addresses: luca.buonerba@yahoo.com (L. Buonerba), hugo.gante@unibas.ch (H.F. Gante).

http://dx.doi.org/10.1016/j.ympev.2015.03.024
1055-7903/© 2015 Elsevier Inc. All rights reserved.

European freshwater fishes over the last two decades (e.g. Bernatchez and Wilson, 1998; Kotlík and Berrebi, 2002; Salzburger et al., 2003; Costedoat and Gilles, 2009), considerably less data are available on population structure and speciation of fishes from southern European peninsulas (e.g. Tsigenopoulos et al., 2002; Stefani et al., 2004; Gante et al., 2009; Marchetto et al., 2010).

The Mediterranean primary freshwater fish fauna is characterized by few genera, a high number of species per genus, a high level of endemism and substantial variation in ecology and life history traits, being dominated by cyprinids (Bănărescu and Coad, 1991; Zardoya and Doadrio, 1998; Durand et al., 2002; Kottelat and Freyhof, 2007). Since cyprinids are obligatory freshwater fish restricted to specific drainage systems and show little capacity for trans-watershed dispersal, their biogeographical history closely reflects the geomorphological evolution of hydrographical networks (Reyjol et al., 2007). These characteristics make cyprinids ideal models to study the evolutionary mechanisms driving species diversification and distribution (Briggs, 1995; Zardoya and Doadrio, 1998). Amongst Mediterranean cyprinids, *Barbus* and *Luciobarbus* are the most widespread and diverse freshwater fishes in Europe, comprising more than 30 described species (Kottelat and Freyhof, 2007). Their marked diversity, wide distribution and varied ecology are features that make barbels an ideal evolutionary model of the freshwater fauna in the peri-Mediterranean region (Gante, 2011). Furthermore, *Barbus* and *Luciobarbus* seem to have originated in the Oligocene–Early Miocene (Gante, 2011; Levin et al., 2012), which makes them suitable for studying diversifying mechanisms that act at different timescales.

Barbels are bottom dwellers adapted to a variety of habitats, ranging from small mountain brooks to large and slow-flowing rivers and lakes. Two complementary ecological groups exist, differing in body size and habitat preference: (i) the rheophilic group, characterized by small adult size and preference for colder mountain streams and turbulent stretches of large rivers; and (ii) the fluvio-lacustrine group which comprises larger species (length > 50 cm), inhabits wide calm rivers, with a regular current and warmer temperatures (Tsigenopoulos et al., 1999). Rheophilic and fluvio-lacustrine species often occupy the same rivers, but exploit different stretches therein according to their habitat preferences. This pattern is repeated across species with different pairs co-existing in different geographical regions (Bianco, 1998; Tsigenopoulos et al., 1999). When present, species representing the two ecological groups meet along the same river at a transitional zone between upstream and downstream habitats, which sometimes leads to hybridization (Darius and Berrebi, 1993; Barton and Gale, 1993; Crespin and Berrebi, 1999; Chenuil et al., 2000; Kotlík and Berrebi, 2002; Lajbner et al., 2009).

Within the fourteen peri-Mediterranean ichthyogeographic districts defined on the basis of distinctive assemblages of cyprinids by Bianco (1990), two main regions have been identified in the Italian Peninsula: (i) the Padano-Venetian district (PV), including basins from the Vomano River in Abruzzo Region to the Krka River in Croatia, which all drain into the Adriatic Sea; and (ii) the Tuscano-Latium district (TL), from the Serchio River to the Tiber (Tevere) River, with all basins draining into the middle Tyrrhenian Sea (Fig. 1; Bianco, 1995b). In these districts four species of barbels are described as native (Kottelat and Freyhof, 2007): *Barbus balcanicus* Kotlík, Tsigenopoulos, Ráb and Berrebi, 2002, *Barbus caninus* Bonaparte, 1839, *Barbus plebejus* Bonaparte, 1839 and *Barbus tyberinus* Bonaparte, 1839. *Barbus balcanicus* and *B. caninus*, small-sized rheophilic barbels (total length up to c. 20 cm and 25 cm, respectively), inhabit mountain brooks (Tsigenopoulos and Berrebi, 2000; Kottelat and Freyhof, 2007) of PV and Danubian districts. The latter is endemic to the Po and Brenta river basins while *B. balcanicus*, a species typical of the Danubian district, occurs only in the eastern PV district (Isonzo River basin) (Tsigenopoulos and Berrebi, 2000). In contrast, *B. plebejus* and *B. tyberinus* belong to the large-size fluvio-lacustrine group: *B. plebejus* is widespread in the PV district, inhabiting the Po River basin up to the Adriatic rivers in northern Croatia (Tsigenopoulos et al., 2002; Kottelat and Freyhof, 2007); *B. tyberinus* is endemic to the Italian Peninsula and the only barbel native to the TL district, with a distribution extending to southern Italy (Ketmaier et al., 2009). These two species have very similar morphologies (Bianco, 2003; Livi et al., 2013), which led Gandolfi et al. (1991) and Zerunian (2002) to question the taxonomic validity of *B. tyberinus*. Bianco (1995a) and Lorenzoni et al. (2006) on the other hand, considered *B. tyberinus* a valid species on the basis of morphological characters. Furthermore, Bianco (1995a) suggested that *B. tyberinus* could be of ancient hybrid origin based on intermediate morphology between *B. caninus* and *B. plebejus*.

More recently, human-mediated admixture might have been boosted by restocking of *B. caninus*, *B. plebejus* and *B. tyberinus* out of their natural ranges. In addition to translocations of native species, other barbel species have been introduced into Italian freshwaters. In the last decades massive introduction of the allochthonous European barbel *Barbus barbus* (L.) has occurred and currently this species is well established across the Italian Peninsula (Zerunian, 2001; Bianco and Ketmaier, 2001). It can be found in sympatry with *B. plebejus* and *B. tyberinus* in areas where restocking activities have taken place due to their similar ecological preferences (Livi et al., 2013). Molecular evidence for introgression between *B. plebejus* and *B. barbus* in the Po drainage has recently been reported (Meraner et al., 2013).

Determination of divergence times, genetic diversity and population differentiation among *Barbus* species with contrasting ecologies would provide considerable insight into the factors affecting diversification and distribution of freshwater species in southern peninsulas. In the present work we study populations of rheophilic and fluvio-lacustrine *Barbus* inhabiting different basins and habitats in the Italian Peninsula and adjacent areas. We use mitochondrial and nuclear sequence data to: (i) examine the timescales of Italian barbel diversification, and to address whether speciation occurred during a particular period, which can be related to one major environmental factor, such as Pleistocene glaciations as suggested by Tsigenopoulos and Berrebi (2000) and Livi et al. (2013), or the Messinian 'Lago Mare' phase as suggested by Bianco (1990) and Tsigenopoulos et al. (2003). Otherwise, if Italian barbels have diversified during a wider period of time they were likely influenced by different environmental episodes; (ii) address whether distinct ecological preferences (rheophilic or fluvio-lacustrine habits) impact levels of population structure as proposed by Bănărescu (1998). In particular, if species ecology influences population connectivity, we expect to see greater population genetic structure in *a priori* less connected rheophilic species compared to their fluvio-lacustrine counterparts; (iii) determine differences in the extent and direction of natural and human-mediated interspecific gene flow; and (iv) determine if *B. tyberinus* is distinguishable from *B. plebejus* on the basis of molecular data and evaluate the hypothesis of hybrid origin of *B. tyberinus* between *B. plebejus* and *B. caninus* (Bianco, 1995a). If *B. tyberinus* is of hybrid origin, we would observe a mixture of *B. plebejus* and *B. caninus* alleles.

2. Materials and methods

2.1. Sampling

Samples were collected between 2008 and 2009 by electrofishing across PV and TL ichthyogeographic districts. Species identification was carried out in the field following the criteria proposed by Kottelat and Freyhof (2007). In particular six populations of *B.*

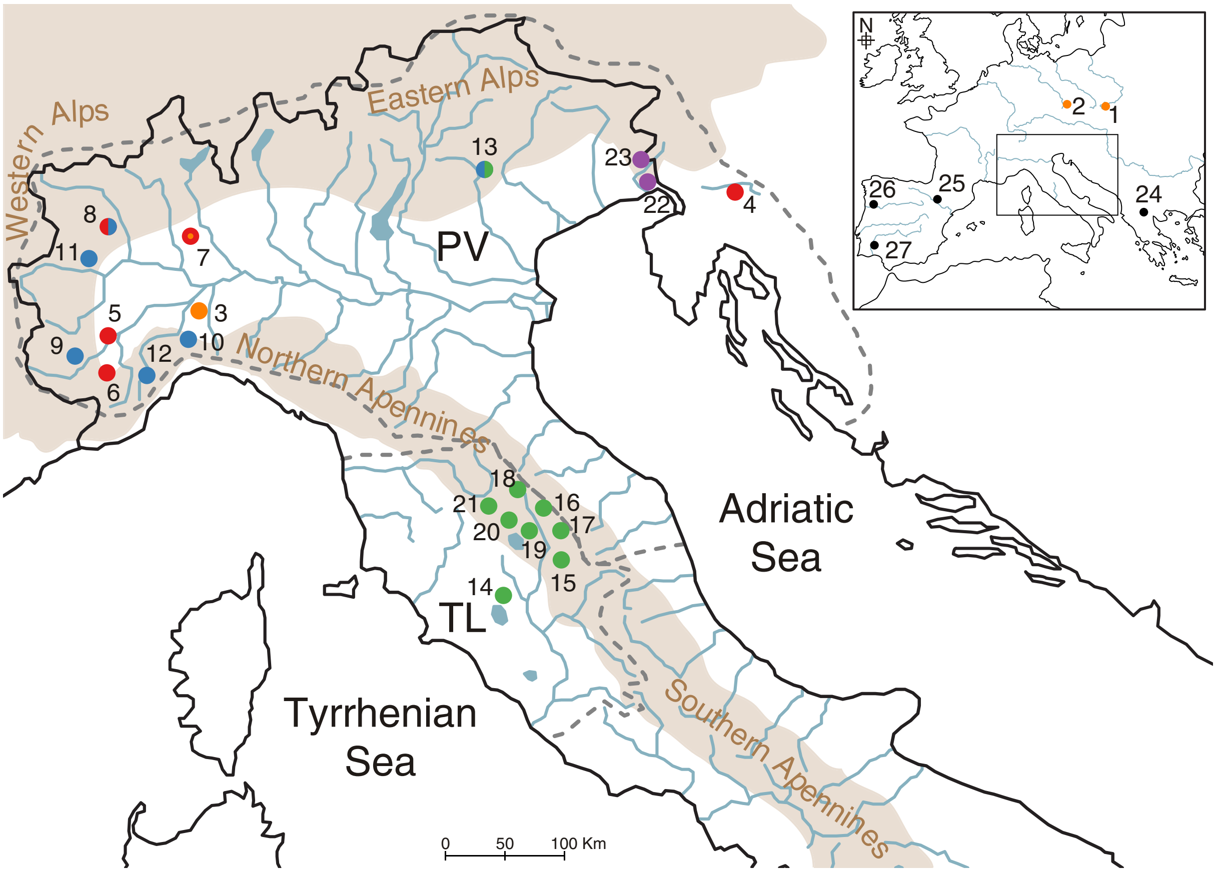

Fig. 1. Map of Europe and the Italian Peninsula showing sampling sites – purple: *Barbus balcanicus*; orange: *Barbus barbus*; blue: *Barbus caninus*; red: *Barbus plebejus*; green: *Barbus tyberinus*; black: other *Barbus* or *Luciobarbus*. Orange center of population 7 denotes hybrid morphology. PV: Padano-Venetian district; TL: Tuscano-Latium district. Further details in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

caninus, five populations of *B. plebejus*, two populations of *B. balcanicus*, nine populations of *B. tyberinus* and one population of the introduced central European *B. barbus* were sampled from Italian and Slovenian freshwaters in a total of 284 specimens (Fig. 1 and Table 1). Five *B. barbus* individuals from the Danubian district were added to the data set: two from Elba River and three from Vistula River (Gante et al., 2011). After identification, a clip of the anal fin was stored in 100% ethanol and kept refrigerated at 4 °C. The majority of specimens were tentatively identified and assigned to species according to morphological criteria. Since fifteen individuals from the Ceronda river and nine from the Cismon river were difficult to assign, due to their small size, final assignment was based on mitochondrial sequences and nDNA Bayesian clustering analysis.

2.2. Molecular data

Total genomic DNA was extracted using a proteinase K digestion followed by sodium chloride extraction and ethanol precipitation (Aljanabi and Martinez, 1997). The entire mitochondrial cyt *b* gene (1141 bp) was amplified by polymerase chain reaction (PCR) using primer pair L15267 and H16461 (Briolay et al., 1998). For each of 289 specimens we analyzed the initial 733 bp-long region of the cyt *b*. Barbels are tetraploid with a chromosome number of 100, therefore we tested the suitability of paralog-specific primers for *S7* ribosomal protein (*S7-1* and *S7-2*) and *growth hormone* (*GH-1* and *GH-2*) genes developed for other European species of *Barbus* and *Luciobarbus* (Gante et al., 2011). Nevertheless, barbels are functional diploids, as chromosomes form bivalents during meiosis (Ohno, 1970) and we have not observed any evidence of recombination between different *S7* or *growth hormone* paralogs. In previous studies it was demonstrated that these loci were sufficiently polymorphic and suitable for phylogenetic and population genetic analyses (Mayden et al., 2009; Moyer et al., 2009; Perea et al., 2010; Gante et al., 2011). Nuclear loci *S7-1*, *S7-2* and *GH-1* were successfully amplified with available primers in Italian barbels (Table 2). An additional internal primer for *S7-2* was designed for sequencing due to the existence of multiple indels that made phasing challenging (Table 2). Regarding *GH-2* reliable amplicons were obtained only for *B. tyberinus* and *B. plebejus*, therefore new primer pairs were designed for *B. caninus* and *B. balcanicus* with an exon-primed intron-crossing strategy (EPIC; Gante et al., 2011; Table 2).

Once the available primer pairs were tested and new ones developed, PCR assays were performed using Multiplex PCR kit (Qiagen) in 10 μL reaction volume containing approximately 10 ng of template DNA and 0.25 μM of each primer pair. Thermal cycling was performed as follows: denaturation of 15 min at 95 °C, followed by 30 cycles (*GH-1*), 35 cycles (*GH-2; S7-2*) and 40 cycles (*S7-1*) of 94 °C for 30 s, 90 s at the appropriate annealing temperature and the extension step at 72 °C for 90 s, the final elongation was at 72 °C for 10 min. Annealing temperature was 55 °C for *GH-2* primers, 56 °C for *S7-1* primers, 60 °C for *GH-1* primers and 62 °C for *S7-2* primers. PCR products (2.0 μl) were purified using Exo-Sap and sequenced in both directions on an ABI 3130xl Genetic Analyzer using Big Dye 3.1 terminators (Applied Biosystem).

The nucleotide sequences of new unique haplotypes of cyt *b* and nuclear alleles were deposited in Genbank database (Tables S1–S10) under accession numbers KC818238–KC818264 and KF963317–

Table 1
Origin of samples analyzed in the present study. ID: Ichthyogeographic Districts, D: Danubian, PV: Padano-Venetian, TL: Tuscano-Latium, G: Greece, IP: Iberian Peninsula. Population numbers refer to Fig. 1.

Population	Species	Localities	River	River Basin	ID	No.	Habitat type
1	*Barbus barbus*	Stará Ľubovňa, Slovakia	Propad	Vistula	D	3	Fluvio-lacustrine
2	*B. barbus*	Sázava	Sázava	Elbe	D	2	Fluvio-lacustrine
3	*B. barbus*	Terzo	Bormida	Po	PV	7	Fluvio-lacustrine
4	*B. plebejus*	Movzazt	Reka	Reka	PV	7	Fluvio-lacustrine
5	*B. plebejus*	Cardè	Po	Po river	PV	9	Fluvio-lacustrine
6	*B. plebejus*	Savigliano	Maira	Po river	PV	15	Fluvio-lacustrine
7	*B. plebejus x B. barbus*	Novara	Terdoppio	Po river	PV	12	Fluvio-lacustrine
8	*B. caninus + B. plebejus*	Varisella	Ceronda	Po river	PV	30	Transitional
9	*B. caninus*	Sanfront	Po	Po river	PV	11	Rheophilic
10	*B. caninus*	Voltaggio	Lemme	Po river	PV	17	Rheophilic
11	*B. caninus*	Trana	Sangone	Po river	PV	15	Rheophilic
12	*B. caninus*	Priola	Tanaro	Po river	PV	15	Rheophilic
13	*B. caninus + B. tyberinus*	Fonzaso	Cismon	Brenta river	PV	14	Transitional
14	*B. tyberinus*	Albergo la Nona	Paglia	Tiber river	TL	19	Fluvio-lacustrine
15	*B. tyberinus*	Scanzano	Topino	Tiber river	TL	8	Fluvio-lacustrine
16	*B. tyberinus*	San Giustino	Tiber	Tiber river	TL	13	Fluvio-lacustrine
17	*B. tyberinus*	Valfabbrica	Chiascio	Tiber river	TL	20	Fluvio-lacustrine
18	*B. tyberinus*	Passano	Lama	Tiber river	TL	6	Fluvio-lacustrine
19	*B. tyberinus*	Baucca	Soara	Tiber river	TL	6	Fluvio-lacustrine
20	*B. tyberinus*	Lupo	Cerfone	Tiber river	TL	19	Fluvio-lacustrine
21	*B. tyberinus*	Arezzo	Teggina	Arno river	TL	6	Fluvio-lacustrine
22	*B. balcanicus*	Costa Bona	Piumizza	Isonzo river	PV	20	Rheophilic
23	*B. balcanicus*	Grojna	Groina	Isonzo river	PV	15	Rheophilic
1	*B. carpathicus*	Hromoš, Slovakia	Propad	Vistula	D	3	Rheophilic
24	*B. prespensis*	Agios Germanos	Agios Germanos	Prespa Lake	G	3	Fluvio-lacustrine
25	*B. haasi*	Zaragoza	Alhama	Ebro	IP	1	Rheophilic
26	*Luciobarbus bocagei*	Amarante	Tâmega	Douro	IP	1	Fluvio-lacustrine
27	*L. comizo*	Brinches	Guadiana	Guadiana	IP	1	Fluvio-lacustrine
27	*L. microcephalus*	Serpa	Guadiana	Guadiana	IP	1	Fluvio-lacustrine

Table 2
Primers used to amplify and sequence nuclear loci.

Primer name	Sequence (5′–3′)	Annealing site	Source
S7RPEX1F	TGGCCTCTTCCTTGGCCGTC	*S7* exon I	Chow and Hazama (1998)
S7RPEX3R	GCCTTCAGGTCAGAGTTCAT	*S7* exon III	Chow and Hazama (1998)
Bs7-1.i1.19F	CCCAGCTAAAGAGTTTTCAAATG	*S7-1* intron I	Gante et al. (2011)
Bs7-1.i1.202R	GCACATGGGGCCCAGTAAT	*S7-1* intron I	Gante et al. (2011)
Bs7-2.i1.19F	CCCAGCTAAAGAGTTATCAAGTT	*S7-2* intron I	Gante et al. (2011)
BS7-2i1F	CGATAAGCTAAGAACATGCTTGGAGTT	*S7-2* intron I	This study
Bs7-2.i1.785Rb	GAAACTGATTTATTAACTCCCAAA	*S7-2* intron I	Gante et al. (2011)
GHe3.min.3F	GACAACCTGTTGCCTGAGGAACGC	*GH* exon III	Moyer et al. (2009)
GHe5.183R	CTACAGGGTGCAGTTGGAATC	*GH* exon V	Moyer et al. (2009)
Bgh-1.i3.79F	GGGGTCTGTGGAAAAGTTTGG	*GH-1* intron III	Gante et al. (2011)
Bgh2.i3.226F	GTACTATAGTAAGCAGAAATGG	*GH-2* intron III	Gante et al. (2011)
Bgh2.i3.226Fb	GTACTAKAGTRRGCAGAAATGG	*GH-2* intron III	This study
Bgh-2.e5.32R	AGTGGSAGGGAGTCATTG	*GH-2* exon V	Gante et al. (2011)
Bgh-2.e5.32Rb	AGTGGSAGGGAGTCGTTY	*GH-2* exon V	This study

KF963512. The new complete cyt *b* sequence of *B. carpathicus* has accession number HG798332. Datasets used in analyses are available at Dryad, http://dx.doi.org/10.5061/dryad.3j8t5.

2.3. DNA polymorphism

Alignment of all sequences was carried out manually in BioEdit (Hall, 1999) to eliminate ambiguities and to check for polymorphic sites. For the nuclear loci, specimens heterozygous for insertions or deletions (indels) were manually phased by analyzing the complementary information carried by the forward and the reverse sequences (Flot et al., 2006). Such procedure is laborious but improves phasing certainty. These manually phased alleles were subsequently used to statistically phase the few remaining SNP heterozygotes in PHASE (Stephens et al., 2001). PHASE input files were generated using seqPHASE (Flot, 2010). Consistency of the inferred haplotypes was assessed in five independent PHASE runs as recommended by the authors.

We then calculated the levels of genetic variation within species and populations. For each locus, we calculated the number of haplotypes (h) using Non Redundant Data Base (NRDB; http://pubmlst.org), and the number of polymorphic sites (S), the average number of nucleotide differences (k), the haplotype diversity (Hd) and the mean number of nucleotide differences (π) using DnaSP 5.0 (Librado and Rozas, 2009).

2.4. Phylogenetic analyses

Phylogenetic analyses were performed independently for each gene on non-redundant haplotypes. In phylogenetic inferences of nuclear loci, previously sampled sequences of *B. barbus*, *B. prespensis* and *B. carpathicus* from central and eastern Europe were added to the dataset and sequences belonging to the genus *Luciobarbus* from Iberian peninsula were used as outgroups (Gante et al., 2011; Table 1). In the mitochondrial phylogeny, one newly generated and 49 complete cyt *b* sequences of circum-Mediterranean *Barbus*, *Luciobarbus* and *Capoeta* retrieved from Genbank (Table S11) were used to root the tree (*Luciobarbus* + *Capoeta*) and provide a larger phylogenetic context to the haplotypes identified in the present study. The Akaike Information Criterion implemented in ModelTest v. 3.7 (Posada and Crandall, 1998) was used to determine the evolutionary model that best fits the data. Maximum likelihood analyses (ML) were performed in PHYML 3.0 (Guindon and Gascuel, 2003; Guindon et al., 2010). A HKY85 model of nucleotide evolution with four substitution rate categories, with estimated transition/transversion ratio, proportion of invariable sites and gamma distribution parameter, and empirical nucleotide frequencies was used. Topologies were estimated using NNI (nearest-neighbor interchange) and SPR (subtree pruning and regrafting) following a BioNJ starting tree. Node support was estimated using approximate Bayes likelihoods and bootstrap.

2.5. Estimation of divergence times

The same 50 complete cyt *b* sequences of *Barbus*, *Luciobarbus* and *Capoeta* used for phylogenetic reconstructions (above; Table S11) were also used to estimate the age of the splits among the species studied, using a Bayesian MCMC approach implemented in BEAST v1.6.1 (Drummond and Rambaut, 2007) and newly available fossil information. *Barbus*, *Luciobarbus* and *Capoeta* fossils from Early Miocene (Burdigalian) to Pleistocene deposits are currently known from several localities in the northern Mediterranean region, from Iberia, across Central Europe, to Anatolia (Mein et al., 1978; Doadrio and Casado, 1989; Doadrio, 1990; Böhme and Ilg, 2003). Fossils consist mostly of disarticulated pharyngeal bones, and more rarely simple dorsal fin rays and vertebrae (Mein et al., 1978; Doadrio and Casado, 1989; Doadrio, 1990; Böhme and Ilg, 2003). Pharyngeal teeth of barbels have a typical three-row arrangement. Furthermore, specific dentary formulas (2, 3, 5–5, 3, 2 versus 2, 3, 4–4, 3, 2) and shape of fourth teeth of outer row (narrow versus wide) are apomorphies of *Barbus* and *Luciobarbus*, respectively. These apomorphies allow assignment of fossils to either genus, but in general preclude assignment to particular species (Doadrio and Casado, 1989; Doadrio, 1990). Alternatively, fossils can be used to calibrate entire monophyletic groups inhabiting a particular region. We used the ages and taxonomic assignment available in Böhme and Ilg (2003), Doadrio (1990) and Přikryl et al. (2014) to calibrate six nodes and the corresponding stems in the barbel phylogeny. We constrained the root of the barbel tree (node A) with the oldest fossil with synapomorphies of *Barbus* (*B. bohemicus* from Czech Republic; fossil age: 18.0–19.0 Ma) and a conservative (very wide) soft upper bound not older than the oldest known cyprinid fossil (48.6 Ma; Patterson, 1993). Within *Luciobarbus* + *Capoeta* we applied four calibration points: one leading to *Capoeta* (node B; oldest *Capoeta* sp. from Turkey; fossil age: 14.45–15.05 Ma), one leading to the entire Iberian *Luciobarbus* (node C; oldest *Luciobarbus* sp. from Spain; fossil age: 5.46 Ma), one leading to the clade composed of Iberian *L. microcephalus* + *L. guiraonisL. graellsii* (node D; *Luciobarbus* sp. from northeastern Mediterranean Spain; fossil age: 3.1 Ma), and the last one leading to Middle Eastern and Asian species that includes *L. esocinus* (node E; *Luciobarbus* cf. *esocinus* from Turkey; fossil age: 12.5–17.0 Ma); within *Barbus* we calibrated the *B. haasi* + *B. meridionalis* clade (node F; oldest *Barbus* sp. from Spain; fossil age: 16.0–17.0 Ma). Because fossils provide the minimum age of clades, we used lognormal priors as suggested by Ho (2007), in which the lower ages fossils are set as hard lower bounds, and the upper ages correspond to a soft bound free to vary. We used lognormal distributions with wide log(means) of 1.0 and log(standard deviations) of 1.1 for the tree root and 1.0 for all others.

BEAST analyses were run three independent times: each run consisted of 30,000,000 generations, sampled every 2500 generations, and the first 1001 trees were discarded as burn-in. A relaxed clock model was used to accommodate for rate heterogeneity among lineages, rates of evolution were calculated using an uncorrelated lognormal distribution (ucld), following a general time reversible (GTR) substitution model and a birth-death speciation prior (Drummond et al., 2006). Third codon positions were treated separately from 1st and 2nd codon positions. Convergence of parameters and age estimates of independent and combined runs was assessed by inspecting the marginal posterior probabilities and effective sample sizes (ESS) using Tracer v1.5 (Rambaut and Drummond, 2003). The combined file of tree and parameter values was produced using LogCombiner v1.6.1 (Rambaut and Drummond, 2007a) with a burn-in of 1001 trees. Posterior probability density of combined tree files was summarized using TreeAnnotator v1.6.1 (Rambaut and Drummond, 2007b). In individual and combined runs, all parameter estimates readily stabilized around the mean and ESS were always well above 200, indicating appropriate mixing and convergence. A summary of tree posterior and root age estimates for each independent run and combined runs is provided in Table S12. A comparison between prior probability distributions with MCMC results obtained with our data and with empty dataset was conducted to confirm the data inform the estimates. Regarding the most important parameter estimates (ages of most recent common ancestors), convergence of the split between Iberian *L. sclateri* and Algerian *L. setivimensis* at 6.55 Ma (95% HPD: 9.522–4.173 Ma), which are thought to have separated during the Messinian Salinity Crisis of the Mediterranean when these two landmasses were connected for the last time (~5.96–5.33 Ma; Krijgsman et al., 1999; Machordom and Doadrio, 2001; Mesquita et al., 2007; Gante et al., 2009), provides an external a posteriori confirmation that the currently used calibration is appropriate to study the timing of barbel speciation.

2.6. Population genetic analyses

For these analyses samples of *B. barbus* from the Danubian District were included (Gante et al., 2011). Covariation among nuclear loci was assessed using the Bayesian clustering program structure v2.3.4 (Pritchard et al., 2000; Falush et al., 2003). Each unique nuclear haplotype per locus was numerically coded, missing data were coded as -9. Structure is useful to detect the presence of distinct genetic clusters, to assign individuals to populations, to identify migrants and admixed individuals. Each structure run consisted of 250,000 MCMC generations as burn-in, followed by 500,000 MCMC replicates to estimate the posterior sample distribution, using the admixture, correlated allele frequency models. To assess reliability of solutions, 20 iterations were run for each *K* clusters (variable depending on the data set; Figs. S5–S9). After an initial analysis of the complete data set, hierarchical analyses of smaller data sets, consisting of samples belonging to the most differentiated clusters, were run in order to highlight potential substructure as recommended by Pritchard and Wen (2002) and implemented successfully in other studies (e.g. Vähä et al., 2007). Unlike the latter, data sets were not at all based on geography, but rather composed of specimens assigned to a cluster with a probability higher than 0.80 irrespective of basin of origin. This procedure prevents that a high proportion of introgressed alleles artificially inflate the estimates of *K* in hierarchical analyses. On the other hand, including as many individuals as possible from each cluster guarantees that sample sizes are adequate for cluster detection (Fogelqvist et al., 2010). Due to low number of individuals we did not examine intraspecific differentiation in *B. barbus*, whose native range lies outside the Italian Peninsula. The number of groups (K) identified by STRUCTURE was estimated by a combination of changes in log-likelihood of consecutive K-values and ΔK (Evanno et al., 2005) using the program STRUCTURE HARVESTER (Earl and von Holdt, 2012). ΔK finds the ad hoc quantity based on the second order rate of change of the likelihood function relative to K. Consensus clustering across iterations was generated using the greedy algorithm in CLUMPP (Jakobsson and Rosenberg, 2007) and plotted using DISTRUCT (Rosenberg, 2004).

Computation of pairwise multilocus F_{ST} values (Weir, 1996) among populations was performed using the software GENETIX v. 4.02 (Belkhir et al., 2001) with 1000 permutations and an allowed level of missing data of 0.05.

We used the coalescence-based program MIGRATE-N 3.6.6. to estimate the mutation-scaled effective population size $\Theta = 4N_e\mu$, where N_e is the effective population size and μ is the mutation rate per generation per locus, of native barbel populations using a Bayesian framework, assuming mutation-drift equilibrium and no introgression (Beerli and Felsenstein, 2001; Beerli, 2006). Three replicate MIGRATE analyses for each population consisted of one "cold" chain and three "heated" chains that were run for 500,000 generations per locus, with a burn-in of 10,000 generations and otherwise default parameters. We excluded any obviously introgressed alleles (those showing deep coalescence) from single individuals, or entire populations where extensive introgression or missing data occur, but we included populations and individuals of *B. tyberinus* with hypothetically alien *B. plebejus* alleles. These analyses allowed us to estimate the effective sizes required to maintain the observed levels of genetic diversity found in each population and to test for introgression. If alleles with shallow divergence found in *B. plebejus* and *B. tyberinus* are shared ancestral polymorphisms, estimates of N_e should not be affected, otherwise if they are shared due to introgression, estimates of N_e should be inflated.

3. Results

3.1. Sequence variation

A total of 29 different haplotypes were identified in the initial 733 bp of the cyt *b* in the 289 individuals analyzed. Sequence analysis of four nuclear loci yielded a 2581 bp-long alignment (*S7-1*: 358 bp; *S7-2*: 582 bp; *GH-1*: 588 bp; *GH-2*: 1053 bp). Several indels (1 bp to 95 bp) were assumed in the alignments to maximize base identity in flanking conserved sequence blocks. When accounting for indels, the nuclear loci exhibited 29, 44, 42 and 81 alleles for *S7-1*, *S7-2*, *GH-1* and *GH-2*, respectively. Numbers of alleles vary with sequence length ($r^2 = 0.97$). Levels of sequence polymorphism for each marker and species are summarized in Table 3 (Table S13 shows polymorphism/population). The allelic diversity varies considerably among species, but in general *B. caninus* shows the highest levels of nuclear polymorphism, whereas *B. plebejus* and *B. tyberinus* show similar levels of polymorphism.

3.2. Phylogenetic analyses

ML phylogenetic analysis of the mitochondrial sequences resulted in a well-resolved tree (−ln likelihood = 9812.397231), with *Barbus* and *Luciobarbus* being reciprocally monophyletic. Four deeper monophyletic clusters are recovered within the former and seven within the latter genus, which show reasonable geographic concordance (Fig. 2; see Gante (2011) for further discussion). All fluvio-lacustrine *Barbus* included in the analysis (*B. barbus*, *B. macedonicus*, *B. plebejus*, *B. thessalus* and *B. tyberinus*) formed a strongly supported monophyletic group, a situation that is not mirrored by rheophilic species, which are recovered as paraphyletic. The Italian *B. plebejus* and *B. tyberinus* are recovered as sister species, and share a common ancestor with a clade containing the other fluvio-lacustrine species: the widely distributed central-eastern European *B. barbus* and species from the Balkans. Rheophilic species instead form different monophyletic groups; relationships retrieved from the analysis reflect the geographic distribution of species. One clade comprises species from southern France and northeastern Iberian Peninsula, *B. meridionalis* and *B. haasi*. A second clade comprises species typical of the Danubian district, *B. carpathicus* and *B. balcanicus* (also present in the Balkan Peninsula and Adriatic basin in Slovenia and Eastern Italy), which are sister to a deep lineage composed of the PV Italian endemic *B. caninus*. The last clade is the most heterogeneous and contains species from the Balkan Peninsula inhabiting rivers that drain the Adriatic and Aegean seas.

Mitochondrial haplotypes found in individuals with *B. barbus*, *B. plebejus* and *B. tyberinus* morphology form three well-supported reciprocally monophyletic groups related by large genetic differences. By comparison, within-group differences are smaller (Fig. 2). Branches leading to rheophilic species are long and well-supported (Fig. 2). Therefore, we interpret any sharing of alleles with deep coalescence as resulting from interspecific hybridization and introgression (Fig. 4).

In contrast, the nuclear gene trees are much less well-resolved than the mtDNA phylogeny and do not recover relationships among the widely distributed fluvio-lacustrine *B. barbus* and Italian species of the same ecological group (Figs. S1–S4). In spite of this lower interspecific resolution, several deeply coalescent monophyletic groups of species-specific alleles can be identified based on its frequency and geography of occurrence.

Following this rationale, several specimens possess mitochondrial and nuclear alleles typical of either parapatric species (e.g. *B. caninus* and *B. plebejus*) or taxa introduced outside their native ranges and now sympatric (e.g. *B. tyberinus*, *B. plebejus* and *B. barbus*; Tables S1–S10). In general, more allele sharing is found among species inhabiting the same habitat type (in particular fluvio-lacustrine), rather than between species adapted to distinct habitats along the river axis. Nevertheless, even species sharing polymorphisms have many private alleles across nuclear loci, for example 26 in *B. tyberinus* and eight in *B. plebejus*, which are likely to be an underestimate due to ongoing introgression (see below).

3.3. Age of Italian barbels

A comparison between prior probability distributions with MCMC results obtained with and without data is shown in Table 4. The intentionally wide confidence intervals of priors could accommodate the joint prior distributions in the analyses ran without data. In turn, the analyses with full dataset confirmed that posterior estimates were effectively informed by the sequence data. Based on our fossil calibration, *Barbus*, *Luciobarbus* and *Capoeta* cyt *b* is estimated to have a mean substitution rate of 0.422% substitutions/site/lineage/MY (SE: 4.288×10^{-6}; 95% HPD: 0.00347–0.00499), with a mean coefficient of variation of 0.269 (SE: 5.521×10^{-4}; 95%HPD: 0.1509–0.3911) across branches. This substitution rate estimate is very similar to another fossil-based estimate (0.52% in Levin et al., 2012) and to the rate determined using the split of *L. setivimensis* from Iberian *Luciobarbus* at the Miocene–Pliocene transition (0.48% in Mesquita et al., 2007). All alternative rates of evolution previously calculated using either various biogeographical events or adopted from other organisms, are much higher (0.76–1.31% in Zardoya and Doadrio, 1999; Machordom and Doadrio, 2001; Durand et al., 2002; Tsigenopoulos et al., 2003, 2010) and lead to erroneous conclusions

Table 3
Sequence polymorphism at mitochondrial and nuclear loci per species. N: number of sequences, *h*: number of haplotypes excluding gaps, *k*: average number of nucleotide differences, *Hd*: haplotype diversity, π: mean number of nucleotide differences, S: number of polymorphic sites, SD: standard deviation.

Locus	Species	Length (bp)	Indel number (size in bp)	*N*	Polymorphism				*S*
					h	*k*	*Hd* ± SD	π ± SD	
Cyt *b*	All	733	–	289	29	38.174	0.866 ± 0.010	0.052 ± 0.001	112
	B. caninus	733	–	78	10	3.487	0.386 ± 0.070	0.004 ± 0.003	68
	B. balcanicus	733	–	35	2	1.976	0.494 ± 0.039	0.002 ± 0.000	4
	B. tyberinus	733	–	106	14	14.638	0.836 ± 0.016	0.019 ± 0.001	42
	B. plebejus	733	–	58	7	5.930	0.339 ± 0.007	0.008 ± 0.002	30
	B. barbus	733	–	12	2	0.303	0.303 ± 0.147	0.000 ± 0.000	1
GH-1	All	533–587	6 (54, 12, 9, 14, 36, 3)	546	34	5.236	0.880 ± 0.008	0.011 ± 0.001	29
	B. caninus	542–578	4 (12, 9, 36, 10)	156	23	2.415	0.883 ± 0.000	0.004 ± 0.000	15
	B. balcanicus	555–569	1 (14)	68	2	2.094	0.349 ± 0.057	0.003 ± 0.001	6
	B. tyberinus	533–587	2 (54, 3)	200	10	2.250	0.681 ± 0.030	0.004 ± 0.000	15
	B. plebejus	533–587	2 (54, 9)	98	12	2.910	0.470 ± 0.062	0.006 ± 0.000	18
	B. barbus	533–587	1 (54)	24	3	2.315	0.554 ± 0.087	0.004 ± 0.001	9
GH-2	All	898–1040	9 (3, 1, 13, 20, 8, 95, 22, 1, 1)	564	62	14.116	0.889 ± 0.009	0.016 ± 0.000	62
	B. caninus	898–1021	7 (5, 6, 3, 95, 22, 1, 1)	150	36	4.195	0.956 ± 0.006	0.004 ± 0.000	32
	B. balcanicus	917–1040	6 (3, 1, 13, 20, 8, 95)	70	8	6.111	0.720 ± 0.047	0.006 ± 0.000	14
	B. tyberinus	898–1029	6 (6, 12, 95, 22, 1, 1)	210	9	6.880	0.521 ± 0.037	0.008 ± 0.000	34
	B. plebejus	898–1029	6 (5, 13, 3, 95, 22, 1)	112	16	5.140	0.608 ± 0.048	0.005 ± 0.001	33
	B. barbus	898–1029	4 (13, 95, 22,1)	22	13	6.013	0.892 ± 0.55	0.006 ± 0.001	28
S7-1	All	329–353	7 (1, 1, 2, 4, 6, 3, 7)	560	25	6.567	0.886 ± 0.006	0.021 ± 0.000	32
	B. caninus	344–353	3 (1, 7, 1)	154	7	1.425	0.678 ± 0.025	0.004 ± 0.000	12
	B. balcanicus	329–343	4 (1, 1, 28, 12)	68	5	1.556	0.561 ± 0.041	0.004 ± 0.001	12
	B. tyberinus	342–344	7 (1, 1, 2, 4, 6, 3, 7)	208	9	1.657	0.691 ± 0.025	0.005 ± 0.000	18
	B. plebejus	342–353	6 (1, 1, 4, 6, 3, 7)	112	15	3.940	0.692 ± 0.041	0.011 ± 0.001	24
	B. barbus	342–344	3 (4, 5, 7)	18	4	2.778	0.595 ± 0.109	0.008 ± 0.003	13
S7-2	All	537–569	7 (5, 2, 2, 2, 3, 27, 6)	570	36	5.358	0.891 ± 0.006	0.010 ± 0.000	35
	B. caninus	537–564	7 (5, 2, 2, 2, 3, 27, 6)	154	10	2.015	0.736 ± 0.031	0.003 ± 0.000	13
	B. balcanicus	569	–	70	3	0.292	0.188 ± 0.061	0.000 ± 0.000	2
	B. tyberinus	558–564	4 (5, 2, 3, 6)	208	16	1.541	0.637 ± 0.035	0.002 ± 0.000	16
	B. plebejus	537–564	7 (5, 2, 2, 2, 3, 27, 6)	114	8	2.903	0.653 ± 0.039	0.005 ± 0.000	15
	B. barbus	558–564	7 (9, 2, 5, 2, 3, 6, 3)	24	12	4.275	0.899 ± 0.041	0.007 ± 0.001	19

regarding the historical biogeography of barbels that are inconsistent with the age of the oldest fossils (see Gante, 2011 for further discussion).

Using this calibration, we estimate a divergence time of circa 19.0 Ma (95%HPD: 16.3–22.4) for the most distantly related *Barbus* found in the Italian Peninsula, i.e. between the fluvio-lacustrine group composed of *B. barbus*, *B. plebejus* and *B. tyberinus* and the rheophilic group composed of *B. carpathicus*, *B. balcanicus* and *B. caninus* (Fig. 3). The latter group shared a common ancestor at 13.4 Ma (95%HPD: 9.7–17.4), while the former shared a common ancestor at 7.8 Ma (95%HPD: 4.9–11.0). The two most closely related species, *B. plebejus* and *B. tyberinus*, started diverging around 4.1 Ma (95%HPD: 2.2–6.2). Overall, this defines the divergence times of Italian barbels between Early Miocene and the Plio-Pleistocene.

3.4. Population structure and effective sizes

The intrabasin values of F_{ST} vary from zero in *B. balcanicus* and 0.156 in *B. tyberinus* (Table S14). Despite values of F_{ST} among population of the same species from different river basins being very low in some cases (i.e. *B. plebejus*), statistical tests for genotypic differentiation show significant differences ($p < 0.05$) in several comparisons. All comparisons among *B. caninus* populations are statistically significant, which is not true for the other rheophilic species *B. balcanicus* or the fluvio-lacustrine *B. plebejus*. As in *B. plebejus*, *B. tyberinus* shows low F_{ST} values, yet most are statistically significant.

The Bayesian cluster analysis on the complete nDNA dataset identifies five genetically differentiated major groups ($K = 5$; Figs. 4 and S5), corresponding to the five taxa at the species level. These clusters (i.e. species) show good geographical concordance with biogeographical districts. Noteworthy exceptions are the presence of ‘pure’ *B. barbus* and *B. tyberinus* in the Padano-Venetian District. When each of these five clusters is analyzed separately, finer differentiation is visible only within rheophilic *B. caninus* ($K = 4$; Figs. S6–S9). Genetic clusters in this species show a fair concordance with geography. No intraspecific genetic sub-division is detected by STRUCTURE in *B. balcanicus*, *B. plebejus* or *B. tyberinus*, which is consistent with low pairwise population F_{ST}s.

Median estimates of Theta (Θ) were scaled using a general eukaryotic mutation rate (μ) in the order of 10^{-8} per locus per generation (Baer et al., 2007) to calculate the median effective population sizes (N_e) using the equation $\Theta = 4N_e\mu$. Effective population sizes estimated from each locus were extremely variable, indicative of different demographic and/or selective forces acting in distinct regions of the genome (Table S15). Effective population sizes estimated from the four loci combined were generally greater in rheophilic species than fluvio-lacustrine species, except for some populations of *B. tyberinus* (Table 5).

3.5. Hybridization and introgression

Besides the identification of distinct genetic clusters, STRUCTURE detected admixture between several of these groups in individuals with a membership coefficient lower than 0.9. Most evidence of admixture is found between fluvio-lacustrine species native to different biogeographical districts, either to the Italian Peninsula (*B. tyberinus* and *B. plebejus*) or to central European freshwaters (*B. barbus*) and introduced outside their native ranges. There is also clear evidence of introgression between rheophilic and fluvio-lacustrine species, in particular in samples originating from transitional habitats (e.g. Ceronda River; Fig. 4). Interestingly, many

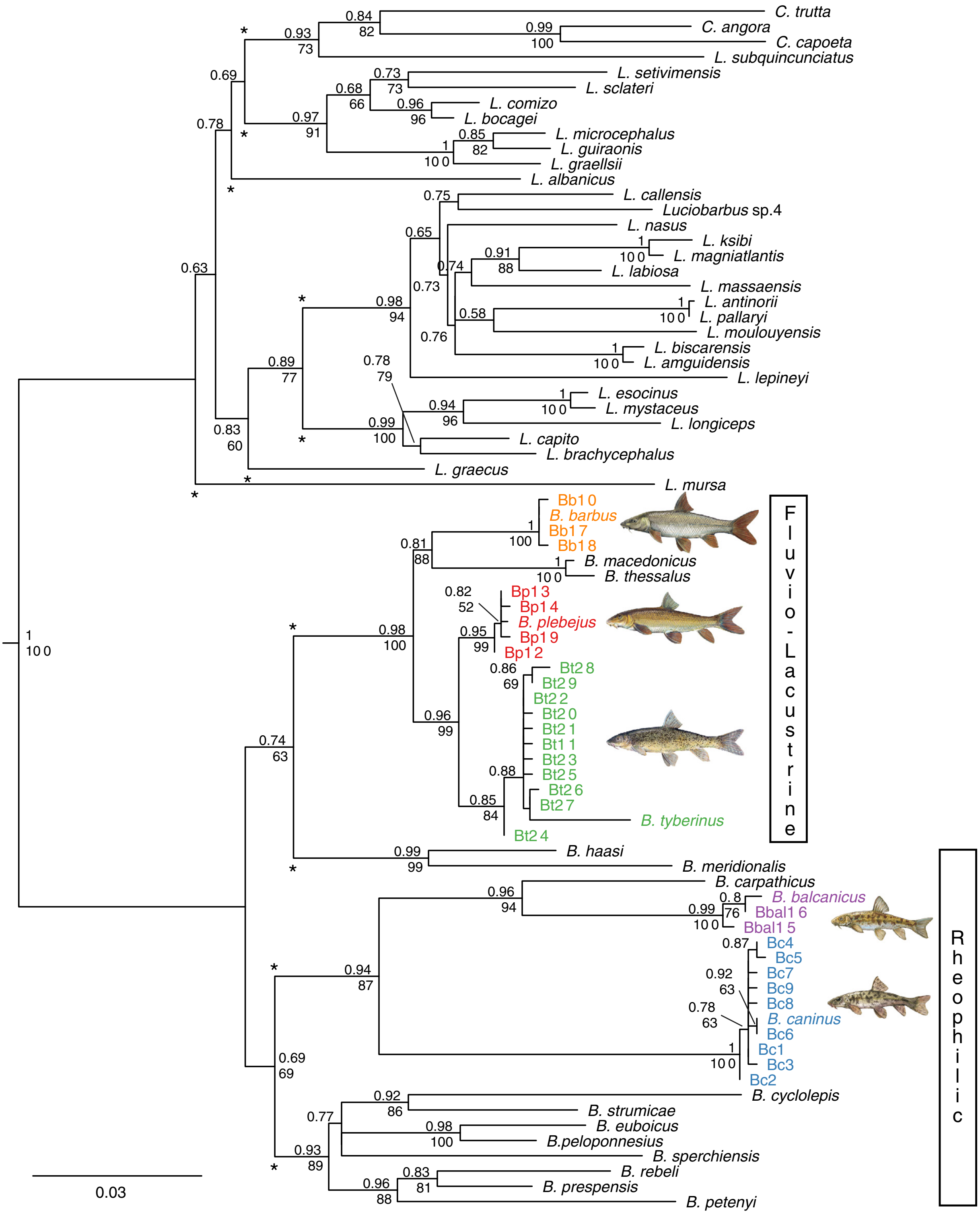

Fig. 2. Maximum Likelihood phylogeny of barbels based on unique cyt *b* haplotypes. Species-specific haplotypes of species found in Italy are color-coded. Asterisks highlight deep lineages with reasonable geographical concordance. Values above and below branches refer to approximate Bayes and likelihood ratio test support, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

admixed specimens show genetic contribution from up to three species in their nuclear genomes. A final evidence of admixture is the observed mismatch between nuclear and mitochondrial genomes in several specimens (e.g. Terdoppio, Cismon and Cerfone; Fig. 4). Interestingly, no introgression of mtDNA of small rheophilic species is detected.

4. Discussion

4.1. Timescales and mechanisms of barbel diversification

Our understanding of the factors promoting divergence within and between barbel species and of the mechanisms shaping

Table 4
Prior probability distributions and posterior probability densities of barbel fossil calibrations. Ages in Ma. CI: 95% confidence interval, HPD: 95% highest posterior density, ESS: effective sampling sizes.

Calibration node	Prior medians [CI]	MCMC medians [HPD], no data	ESS	MCMC medians [HPD], posterior	ESS
Tree root (A)	20.720 [41.480,18.310]	23.423 [37.638,18.108]	2267.117	29.877 [36.726,24.251]	14322.668
Capoeta (B)	17.170 [33.750,14.830]	16.228 [19.836,14.565]	10327.402	15.794 [18.280,14.558]	19328.777
Iberian *Luciobarbus* (C)	8.178 [24.760,5.843]	8.259 [15.236,5.582]	5816.268	17.745 [21.578,14.444]	14850.769
L. microcephalus + *L. guiraonis* + *L. graellsii* (D)	5.818 [22.400,3.483]	4.843 [8.093,3.215]	8713.012	10.523 [13.851,7.281]	13975.499
Middle Eastern and Asian species including *L. esocinus* (E)	15.220 [31.800,12.880]	14.572 [18.942,12.605]	9344.949	14.412 [17.143,12.654]	13212.028
B. haasi + *B. meridionalis* (F)	18.720 [35.300,16.380]	17.524 [21.200,16.098]	10062.996	17.146 [19.377,16.084]	24463.378

historical distribution patterns relies heavily on knowledge about their timing of speciation. The influence of the Mediterranean Basin geomorphological evolution on freshwater fish distribution and speciation has long been hypothesized, although the exact colonization routes and the timing of colonization events are different between biogeographical models (Almaça, 1976, 1988; Bănărescu, 1960, 1992; Bianco, 1990). The wide distribution of barbel fossils of Miocene age was recently interpreted as evidence that diversification within *Barbus* and *Luciobarbus* happened much earlier than previously thought and had already started by the Middle Miocene (Gante, 2011). Thus colonization of new areas around the Mediterranean during the Messinian Salinity Crisis (Bianco, 1990) seemed an unlikely scenario. An exception could be the Italian Peninsula, although only *B. plebejus* was included in a previous analysis (Gante, 2011). In the present work we study all species native to Italian freshwaters, allowing us to further evaluate this hypothesis.

Based on our time-calibrated phylogeny we conclude that colonization of Italian freshwaters and subsequent speciation occurred at distinct times for different species and was not driven by only one factor. It is likely the ancestor of *B. plebejus* and *B. tyberinus* reached the Italian Peninsula during the Messinian 'Lago Mare' phase of the Mediterranean (5.96–5.33 Ma), supporting Bianco's (1990) hypothesis for this particular region. This period is coincident with the emergence of the Northern Apennine Chain and several continental (hinterland) basins formed (Ghelardoni, 1958; Bonini, 1998). One such basin is the Tiber (Tevere) River, the largest basin inhabited by *B. tyberinus*, formed during the Early-Middle Pliocene (Bonini, 1998; Ascione et al., 2008). By comparison, the Po Basin is of post-Pliocene age and resulted from tectonic collision between the Alps' passive margin to the north and the actively thrusting Apennines to the south (Ori, 1993) and its present extent and configuration was achieved only recently (Garzanti et al., 2011).

The estimated age of split between *B. plebejus* and *B. tyberinus* inferred here based on fossil calibrations is older than previously estimated without the use of fossils (Livi et al., 2013), suggesting that speciation was mediated by local tectonism during the formation of those basins rather than Pleistocene glacial cycles as previously proposed. It is coincident with a major uplift phase of the Apennines' External Sector starting in the Upper Pliocene and responsible for the emergence, growth and continentalization of the Adriatic slope (Ghelardoni, 1958; Simoni et al., 2003; Scarselli et al., 2006; Ascione et al., 2008), involving captures of Tyrrhenian and Adriatic rivers on opposite sides of the Apennines (Ghelardoni, 1958; Nesci and Savelli, 2003; Simoni et al., 2003; Bonini and Tanini, 2009).

Regarding the rheophilic species, we suggest that *B. caninus* (endemic to the Po and Brenta basins) split from the common ancestor of *B. carpathicus* and *B. balcanicus* (inhabitants of the Carpathian and Dinaride mountains) during a phase of orogenic uplift of the Alpine mountain range in the Middle Miocene. Conversely, *B. balcanicus*, originated in the Late Miocene but is likely a recent immigrant in the Padano-Venetian region from the Dinarides, a scenario supported by low overall nucleotide diversity and suggested in previous studies (Kotlík and Berrebi, 2002; Tsigenopoulos et al., 2002), as rivers in karstic regions readily undergo changes in direction of flow (Semeraro, 2000). Bănărescu (1990) was among the first to hypothesize a recent faunal dispersion from the Danubian district to the south through a Slovenian corridor. Past connections in this area with an opposite direction have also been documented in Danubian populations of bullhead (*Cottus*; Šlechtová et al., 2004) and *Telestes* (Salzburger et al., 2003; Gilles et al., 2010) where haplotypes belonging to the Adriatic basin were found.

Overall, the timing of diversification of Italian *Barbus* varies greatly, suggesting that several external factors have influenced colonization and diversification in the southern peninsula. We do not find support that speciation events of Italian barbels occurred in one particular period (Pleistocene glaciations as suggested by Tsigenopoulos and Berrebi, 2000 and Livi et al., 2013, or the Messinian 'Lago Mare' phase as suggested by Bianco, 1990 and Tsigenopoulos et al., 2003). Diversification of *Barbus* and *Luciobarbus* was instead likely fuelled by distinct episodes in the continent's paleo-geomorphology, climate and associated river basin evolution that started as early as the Oligocene as previously proposed (Gante, 2011; Levin et al., 2012), which was also suggested for other circum-Mediterranean cyprinids (Perea et al., 2010).

4.2. Mechanisms generating population structure and distribution differences in species with distinct ecologies

By comparing species experiencing similar extrinsic factors in the same geographical region, or even in the same river basin, we can better explore the impact of species' intrinsic ecological preferences on their genetic differentiation and distribution. Bănărescu (1998) proposed that ecological preferences of small rheophilic species for colder, more upstream, segments of rivers, would make them less prone to downstream dispersal compared to large fluvio-lacustrine species. This hypothesis predicts higher levels of population structure in rheophilic compared to fluvio-lacustrine species. We found a more pronounced geographic structure in *B. caninus* samples (high and significant F_{STs} and high levels of hierarchical population structure; $K = 4$), than in either *B. tyberinus* or *B. plebejus* (low F_{STs} and non-detectable population structure; $K = 1$), consistent with previous studies that predicted (e.g. Bănărescu, 1998) or observed (e.g. Konopiński et al., 2013) stronger geographic structuring in rheophilic cyprinids than in fluvio-lacustrine species. Hierarchical population structure in rheophilic species is detectable across sub-basins but is not present in very small rivers scales, as exemplified by our samples of *B. balcanicus* ($K = 1$). We predict that this should be the case when comparing populations over large geographic areas and across drainages, as recently reported for its sister species *B. carpathicus* (Konopiński et al., 2013).

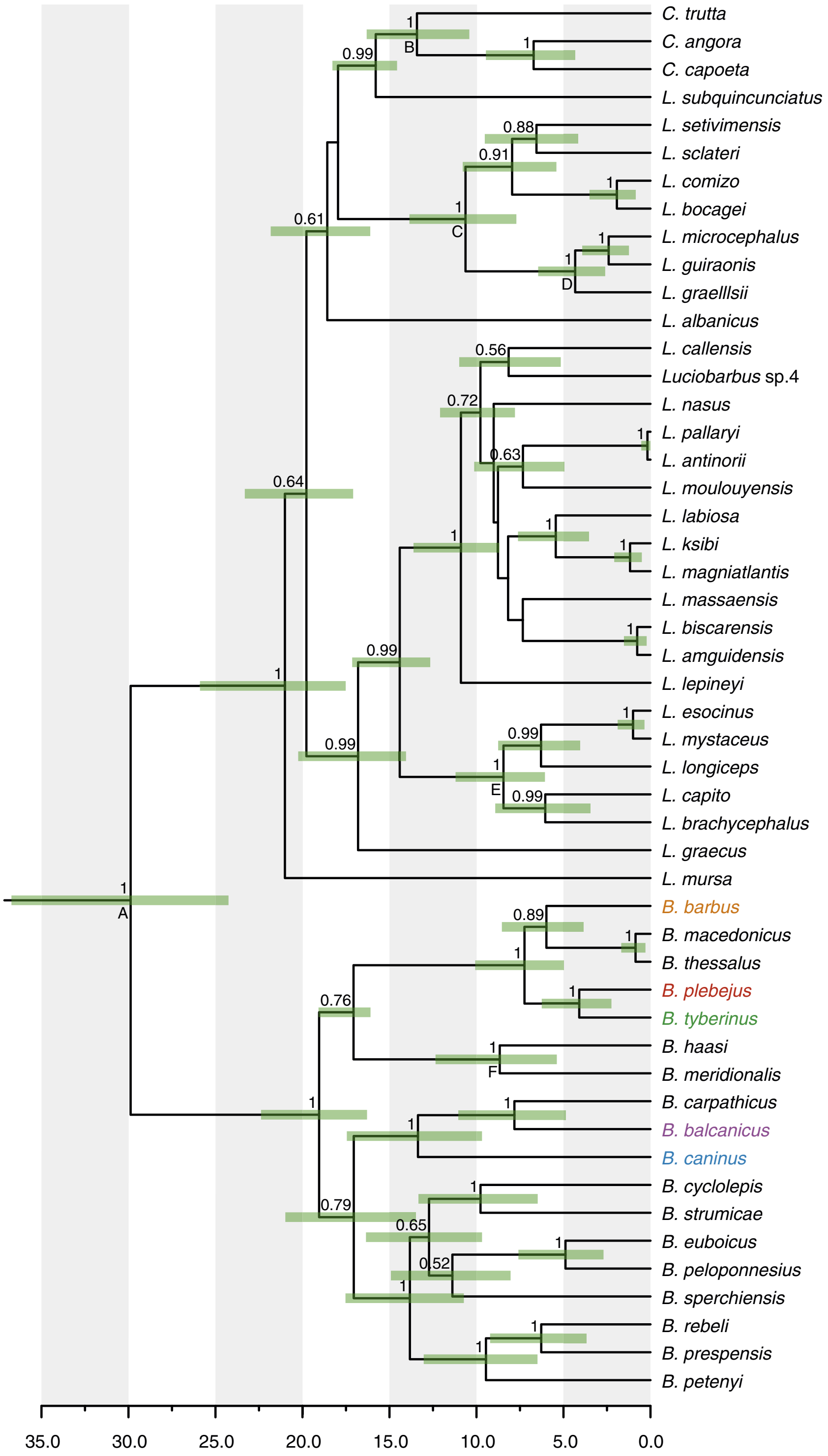

Fig. 3. Bayesian relaxed-clock phylogeny of barbels based on cyt *b*. Values above branches refer to Bayesian support. Green bars show 95% confidence interval. Timescale in Ma.

High F_{ST} levels (i.e. high differentiation) can be due to low gene flow or high genetic drift. Genetic drift is expected to be high among populations with small effective sizes, while low gene flow will eventually lead to differentiation of populations with high genetic variability when given enough time. We observed levels of polymorphism and N_e in *B. caninus* that are higher than those of fluvio-lacustrine species, hence lower levels of gene flow and not smaller effective population size, are responsible for the observed high and significant F_{ST}. These high values of population differentiation in *B. caninus* are comparable to those of other small riverine fishes, such as *Cottus gobio*, *Anaecypris hispanica* and *Telestes muticellus*, that share similar habitat and ecological preferences (Hanfling et al., 2002; Salgueiro et al., 2003; Marchetto et al., 2010). Ecological discontinuities and associated habitat preferences have been found to profoundly affect population connectivity and speciation in several other systems, including plants and insects (Ægisdóttir et al., 2009; Kuss et al., 2008; Baldo et al., 2011).

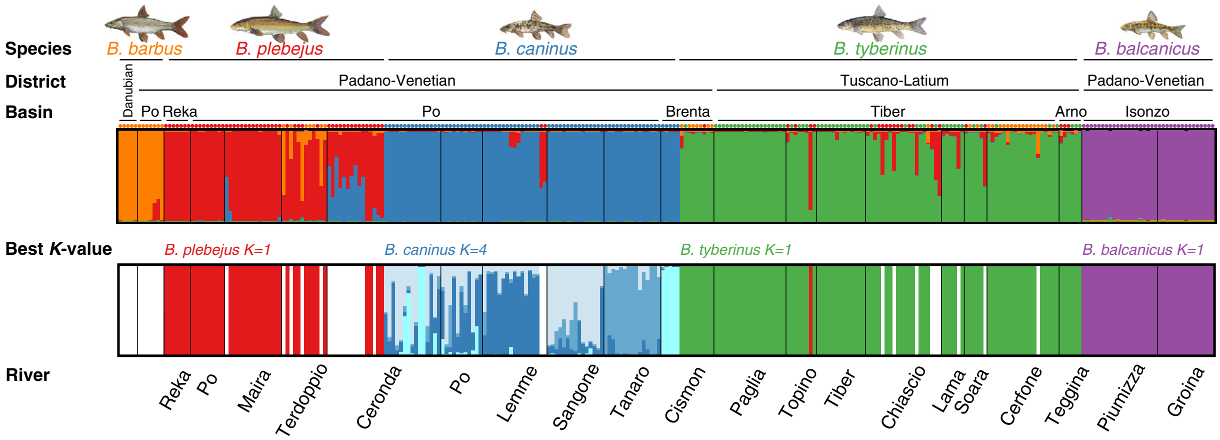

Fig. 4. Structure plots ordered by species, showing ichthyogeographic district and basin/river of origin. Top: analysis of full data set (best solution: $K = 5$); bottom: hierarchical analyses of each K identified in the analysis of the complete data set (excluding *B. barbus* and any individuals with <0.80 assignment probability to one single cluster). Mitochondrial genotype is represented as color-coded circle above the top panel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5
Median estimates of Theta (Θ) and effective population sizes (N_e). HPD: 95% highest posterior density, ESS: effective sampling sizes.

Species	Θ	HPD	ESS	N_e	HPD
B. balcanicus					
Groina	0.00232	0.00067–0.00423	2023562.25	58000	16750–105750
Piumizza	0.00385	0.00123–0.0079	1760012.57	96250	30750–197500
B. caninus					
Po	0.00325	0.0014–0.00523	2403261.36	81250	35000–130750
Sangone	0.00475	0.00243–0.00727	1949661.91	118750	60750–181750
Tanaro	0.00428	0.00197–0.00683	1876358.82	107000	49250–170750
B. plebejus					
Po	0.00195	0.0005–0.00347	2876297.46	48750	12500–86750
Maira	0.00262	0.0011–0.00413	1990336.71	65500	27500–103250
B. tyberinus					
Paglia	0.00252	0.00103–0.004	1915405.73	63000	25750–100000
Topino	0.00488	0.00163–0.0088	3426403.01	122000	40750–220000
Tiber	0.00218	0.00073–0.0037	2179841.08	54500	18250–92500
Chiascio	0.00402	0.00143–0.00707	1695893.67	100500	35750–176750
Lama	0.00335	0.00103–0.0064	3780968.39	83750	25750–160000
Soara	0.00528	0.00187–0.01037	4252127.94	132000	46750–259250
Cerfone	0.00402	0.00153–0.00697	1845582.18	100500	38250–174250

On the other hand, populations of large-sized barbels have lower effective sizes and show very low levels of genetic differentiation across wide geographical ranges that together indicate high dispersal abilities and lower densities. *Barbus plebejus* sampled on opposite sides of the PV district are not differentiated at nuclear loci, despite large geographic distances, in agreement with previous mtDNA results (Tsigenopoulos et al., 2002) and recent microsatellite data (Meraner et al., 2013). We find identical alleles in our wide population sampling from northwest Italy to Slovenia, which is suggestive of recent connectivity among populations from different basins. Indeed it was proposed that fluvio-lacustrine species could more easily disperse via river confluence during lowering of the sea level (Tsigenopoulos et al., 2002). Hence, Pleistocene glacial cycles may have had a homogenizing effect on genetic structure of Padano-Venetian species like *B. plebejus*, while the opposite seems true for southern Iberian *Luciobarbus sclateri* complex (Gante et al., 2009). Although rheophilic species are less apt to disperse through lower river courses, Pleistocene glaciations might have contributed to increase population connectivity by changing favorable habitat along river stretches. Tracking favorable habitat up and down river courses during glaciation cycles is supported also for bullhead (Šlechtová et al., 2004) and Italian vairone (Zaccara et al., 2007), species that share similar ecologies with *B. caninus*.

According to Bianco (1989), the present distribution of freshwater fishes in southern Europe was greatly influenced by geological and hydrological events in the Pleistocene and Holocene. In this particular case, the expansion of the Po Basin during sea level drops down to the Middle Adriatic pitch and the subsequent connection of freshwater networks from both sides of the Adriatic Sea have allowed the dispersal of freshwater organisms via river confluence, such as *Alburnus*, *Barbus*, *Scardinius* and *Telestes* (this study; Tsigenopoulos et al., 2002; Salzburger et al., 2003; Ketmaier et al., 2004, 2009; Marchetto et al., 2010). In this context, the recent finding by Livi et al. (2013) that the southernmost populations of *B. plebejus* from the Marche region are genetically diverse and differentiated deserves further attention and suggests a scenario similar to that of *Telestes muticellus* (Marchetto et al., 2010). Such results could represent reduced connectivity and differentiation of those populations or introgression from *B. tyberinus* after any of the documented Pleistocene river captures (Ghelardoni, 1958; Nesci and Savelli, 2003; Simoni et al., 2003;

Bonini and Tanini, 2009). The use of nuclear sequence data such as those employed here would be greatly beneficial to test these hypotheses.

4.3. Natural hybridization and human-mediated gene flow

In addition to identifying genetically distinct groups at the species level, our analyses detected several individuals of admixed ancestry. Introgression can be suspected from allele sharing and paraphyletic species relationships on gene trees, in particular between otherwise monophyletic groups connected by long branches. Furthermore, multilocus approaches like STRUCTURE are powerful tools in detecting gene flow between differentiated groups. Hybrids between barbel species seem to be common when species meet in sympatry (Machordom et al., 1990; Berrebi and Cattaneo-Berrebi, 1993; Gante, 2009; Lajbner et al., 2009). The present documentation of interspecific gene flow is unique, though, for it reflects both human-mediated and natural secondary contacts. In the latter category we detected late generation backcross hybrids between species of different ecological groups in transitional habitats, i.e. non-F_1 hybrids between *B. balcanicus* and *B. plebejus* at Piumizza and between *B. caninus* and *B. plebejus* at Ceronda, Maira and Lemme. Hybrids between the latter have been previously detected using morphological and low-resolution molecular markers (Betti, 1993; Bianco, 1995c; Tsigenopoulos et al., 2002). Interestingly, we do not observe mtDNA introgression of the small rheophilic species into large fluvio-lacustrine species, which suggests a bias in the preferential direction of hybridization involving female *B. plebejus* and male *B. caninus*. Previous studies have also indicated a preponderance of the larger species acting as the maternal donor (Chenuil et al., 2004; Lajbner et al., 2009; Meraner et al., 2013).

The majority of hybrids detected in the present study belong to the human-mediated category though. They are the result of massive translocation of barbel species between different geographical areas. Meraner et al. (2013) recently documented introgressive hybridization between the Padano-Venetian *B. plebejus* and the allochthonous central European *B. barbus* in the Po River, indicating a displacement of *B. plebejus* from lowlands. Massive spread of *B. barbus* and hybridization occurred in just a few generations, after being first reported in 1994 (Bianco and Ketmaier, 2001). On the other hand, and contrary to recent findings (Meraner et al., 2013) we provide evidence of the presence of *B. barbus* in Brenta River, which is introgressing into *B. tyberinus*. Furthermore, we also found evidence of introgression from *B. barbus* into Tuscano-Latium populations of *B. tyberinus*. Our findings indicate that *B. barbus* is more ubiquitous in Italian freshwaters and potentially inflicting greater ecological and evolutionary impacts on native species than previously acknowledged. Lastly, admixed individuals of both native fluvio-lacustrine species introduced outside of their native ranges in the Italian Peninsula have also been detected. While translocations of *B. plebejus* have been documented (Bianco, 1995c), there were no previous reports of introductions of *B. tyberinus* into the Padano-Venetian district. We found ‘pure’ or ‘mostly pure’ *B. tyberinus* with heterospecific mitochondrial haplotypes in Brenta River. This finding could suggest that *B. tyberinus* is not naturally restricted to the TL district as previously thought. A more likely alternative is the undocumented translocation of *B. tyberinus* outside its native range, as genetic differentiation of the Brenta population would be expected under a natural range expansion. Altogether, introgression between native and translocated species has generated complex hybrids with genetic backgrounds of up to three distinct species in the most varied combinations. While introgressive hybridization has the potential to transfer adaptive variation, leading, in some cases, to the origin of new species from favorable genetic combinations that stabilize over evolutionary times (e.g. Salzburger et al., 2002; Mallet, 2007; Sousa-Santos et al., 2014), it can also negatively impact species persistence by means of fusion of both genetic pools or increased genetic erosion of the rarest species (e.g. Seehausen, 2006; Sousa-Santos et al., 2014). Indeed, Meraner et al. (2013) found significant impacts induced by *B. barbus* on *B. plebejus*’ distribution and genetic integrity.

4.4. Barbus tyberinus is distinguishable and not of hybrid origin

One major point of disagreement among different authors has been the taxonomic validity, and hence distinctiveness, of *B. tyberinus*. In previous studies using molecular data, Tsigenopoulos et al. (2002) and Livi et al. (2013) attempted to clarify the taxonomic status of this taxon using both nuclear and mitochondrial markers (allozymes and cyt *b*, or microsatellites and D-loop sequences, respectively). The authors did not reach a clear conclusion, partly because differences in electrophoretic mobility of the nuclear markers used do not necessarily reflect evolutionary relationships among alleles nor it cannot be determined whether they are identical by state or by descent. In addition, Tsigenopoulos et al. (2002) did not favor the hypothesis of hybrid origin for *B. tyberinus* and instead suggested a close relationship with *B. plebejus* based on population trees, yet *B. caninus*, *B. plebejus* and *B. tyberinus* shared electrophoretic variants at several allozyme loci. In the present study using mitochondrial and nuclear sequence data from all Italian barbel species we could re-address this question. We were able to exclude an origin of *B. tyberinus* through hybridization between *B. caninus* and *B. plebejus*, as this hypothesis predicts that allelic variants typical of both putative parent species should be observed in *B. tyberinus*, which we did not observe. Employing phylogenetic and population genetics approaches we simultaneously confirmed that *B. tyberinus* is genetically distinguishable from either species, it is the closest relative to *B. plebejus* and is readily identifiable based on methods that use multi-locus information (e.g. Bayesian cluster analysis) supporting earlier suggestions by Bianco (1995a), Lorenzoni et al. (2006) and Tsigenopoulos et al. (2002). In spite of sharing genetic variants with *B. plebejus*, *B. tyberinus* shows a large number of private alleles. Human-mediated secondary contact and gene flow between *B. plebejus* and *B. tyberinus* likely added noise to patterns of allele sharing between their genomes in early stages of differentiation. This is supported by effective population sizes larger by a factor of two estimated for *B. tyberinus* in localities where introgression from *B. plebejus* was also detected by other methods (e.g. STRUCTURE). This inflation of effective population size estimates supports allele sharing through introgressive hybridization over ancestral polymorphism. Introgression seems to affect more some loci than others, which is reflected in variable estimates of Theta, as found also in Iberian *Luciobarbus* (Gante et al., submitted for publication). Furthermore, reciprocal monophyly or exclusivity at the majority of nuclear genes is not a reasonable assumption for species discrimination in recent or rapidly radiating lineages (Gamble et al., 2008), or if they have relatively long generation times like barbels (females of different species frequently mature only after four or five years of age). Similar patterns of allele sharing have been observed in *Luciobarbus* of the same age and other recently diversified systems, as nuclear genes take longer to reach monophyly than mtDNA (e.g. Gamble et al., 2008; Pinho et al., 2008; Gante, 2009; Marková et al., 2010).

5. Conclusion

Our study shows that the interplay between extrinsic and intrinsic factors affects the diversification and distribution of

Barbus in the Italian Peninsula. The timescales of Italian barbel diversification are older than previously thought, starting in the Middle Miocene, and implicate local and regional tectonism and basin paleo-evolution rather than Pleistocene glacial cycles. On the other hand more recent environmental factors associated with glaciation–deglaciation cycles have influenced species distributions. Such impacts seem to have been more marked in fluvio-lacustrine than in rheophilic species. We provide molecular evidence that ecology influences population structure, such that less interconnected populations of rheophilic species inhabiting higher river stretches are more genetically differentiated than large fluvio-lacustrine species inhabiting lower river courses. We offer strong evidence for both natural and human-induced interspecific gene flow, which could have great impacts on the evolution and persistence of species involved. Finally, we show that *B. tyberinus* is genetically distinguishable from all Italian species and does not originate from hybridization between *B. caninus* and *B. plebejus* but rather shares a recent common ancestor with *B. plebejus*.

Data accessibility

Sequence alignments are deposited in Dryad: [datasets will be deposited in Dryad as soon as a decision regarding publication is made].

Author contributions

LB designed the study, collected samples, performed laboratory work and data analyses, and wrote the manuscript. SZ and WS designed the study and commented on the manuscript. GD designed the sampling strategy and collected samples. ML collected samples, provided insights on Italian *Barbus* and commented on the manuscript. HFG supervised laboratory work, performed data analyses and wrote the manuscript.

Acknowledgments

This study was supported by grants from the Swiss National Science Foundation to WS. The authors thank Ignacio Doadrio, Petr Kotlík, Giuseppe Moro, Marco Zanetti, Paolo Lo Conte and Alessandro Candiotto for help with acquiring samples. We thank five anonymous reviewers and Brant Faircloth, editor of the Axios Review system (axiosreview.org) for their detailed and helpful comments on the first version of this manuscript. We thank Pedro Fernandes for the scientific illustrations of *Barbus*.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.03.024.

References

Ægisdóttir, H.H., Kuss, P., Stöcklin, J., 2009. Isolated populations of a rare alpine plant show high genetic diversity and considerable population differentiation. Ann. Bot. 104, 1313–1322.

Aljanabi, S.M., Martinez, I., 1997. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucl. Acids Res. 25, 4692–4693.

Almaça, C., 1976. Zoogeografia e especiação dos ciprinídeos da Península Ibérica. Natura Lisboa 4, 3–28.

Almaça, C., 1988. Remarks on the biogeography of Euro-Mediterranean *Barbus* (Cyprinidae, Pisces). Bull. Ecol. 19, 159–162.

Ascione, A., Cinque, A., Miccadei, E., Villani, F., Berti, C., 2008. The Plio-Quaternary uplift of the Apennine chain: new data from the analysis of topography and river valleys in Central Italy. Geomorphology 102, 105–118.

Baer, C.F., Miyamoto, M.M., Denver, D.R., 2007. Mutation rate variation in multicellular eukaryotes: causes and consequences. Nature Rev. Genet. 8, 619–631.

Baldo, L., de Queiroz, A., Hedin, M., Hayashi, C.Y., Gatesy, J., 2011. Nuclear-Mitochondrial sequences as witnesses of past interbreeding and population diversity in the jumping bristletail *Mesomachilis*. Mol. Biol. Evol. 28, 195–210.

Bănărescu, P., 1960. Einige Fragen zur Herkunft und Verbreitung der Süsswasserfischfauna der europäisch-mediterranen Unterregion. Archiv für Hydrobiologie 57, 16–134.

Bănărescu, P., 1990. General Distribution and Dispersal of Freshwater Animals. Aula-Verlag, Wiesbaden.

Bănărescu, P., 1992. Zoogeography of Fresh Waters: Distribution and Dispersal of Freshwater Animals in North America and Eurasia, vol. 2. Aula-Verlag, Wiesbaden.

Bănărescu, P., 1998. On the relations between hydrography and the ranges of freshwater fish species and subspecies. Ital. J. Zool. 65, 87–93.

Bănărescu, P., Coad, B.W., 1991. Cyprinids of Eurasia. In: Winfield, I.J., Nelson, J.S. (Eds.), Cyprinid Fishes. Systematics, biology and exploitation. Chapman & Hall, London, pp. 127–155.

Barton, N.H., Gale, K.S., 1993. Genetic analysis of hybrid zones. In: Harrison, R.G. (Ed.), Hybrid Zones and the Evolutionary Process. Oxford University Press, New York, pp. 13–45.

Beerli, P., 2006. Comparison of Bayesian and maximum likelihood inference of population genetic parameters. Bioinformatics 22, 341–345.

Beerli, P., Felsenstein, J., 2001. Maximum likelihood estimation of a migration matrix and effective population sizes in n subpopulations by using a coalescent approach. Proc. Nat. Acad. Sci. USA 98, 4563–4568.

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N., Bonhomme, F., 2001. Genetix 4.02, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions: CNRS UMR 5000, Université de Montpellier II, Montpellier, France.

Bernatchez, L., Wilson, C.C., 1998. Comparative phylogeography of Nearctic and Palearctic fishes. Mol. Ecol. 7, 431–451.

Berrebi, P., Cattaneo-Berrebi, G., 1993. Natural hybridization of two species of tetraploid barbels: *Barbus meridionalis* and *Barbus barbus* (Osteichtyes, Cyprinidae) in southern France. Biol. J. Linn. Soc. 48, 319–333.

Betti, L., 1993. Prime osservazioni sull'ibridazione tra Barbo comune (*Barbus barbus plebejus* Val.) e Barbo canino (*Barbus meridionalis* Risso). Acta Biol. 70, 23–28.

Bianco, P.G., 1989. Some hypotheses on the origin of primary freshwater fishes in southern Europe. Biol. Gallo-Hellenica 15, 59–64.

Bianco, P.G., 1990. Potential role of the paleohistory of the Mediterranean and Paratethys basins on the early dispersal of Euro-Mediterranean freshwater fishes. Ichthyol. Explor. Freshw. 1, 167–184.

Bianco, P.G., 1995a. A revision of the Italian *Barbus* species (Cypriniformes: Cyprinidae). Ichthyol. Explor. Freshw. 6, 305–324.

Bianco, P.G., 1995b. Factors affecting the distribution of freshwater fishes especially in Italy. Cybium 19, 241–259.

Bianco, P.G., 1995c. Mediterranean endemic freshwater fishes of Italy. Biol. Conserv. 72, 159–170.

Bianco, P.G., 1998. Diversity of Barbinae fishes in southern Europe with description of a new genus and a new species (Cyprinidae). Ital. J. Zool. 65 (Suppl.), 125–136.

Bianco, P.G., 2003. *Barbus tyberinus* Bonaparte, 1839. In: Bănărescu, P.M., Bogutskaya, N.G. (Eds.), The Freshwater Fishes of Europe, vol. 5, Part II. AULA-Verlag, Wiesbaden, pp. 427–449.

Bianco, P.G., Ketmaier, V., 2001. Anthropogenic changes in the freshwater fish fauna of Italy, with reference to the central region and *Barbus graellsii*, a newly established alien species of Iberian origin. J. Fish Biol. 59A, 190–208.

Böhme, M., Ilg, E.A., 2003. fosFARbase, Available from <www.wahrestaerke.com/> (assessed 25.02.15).

Bonini, M., 1998. Chronology of deformation and analogue modelling of the Plio-Pleistocene 'Tiber Basin': implications for the evolution of the Northern Apennines (Italy). Tectonophysics 285, 147–165.

Bonini, M., Tanini, C., 2009. Tectonics and quaternary evolution of the Northern Apennines watershed area (upper course of Arno and Tiber rivers, Italy). Geol. J. 44, 2–29.

Briggs, J.C., 1995. Global Biogeography. Developments in paLeontology and Stratigraphy. Elsevier, Amsterdam.

Briolay, J., Galtier, N., Brito, R.M., Bouvet, Y., 1998. Molecular phylogeny of Cyprinidae inferred from cytochrome b DNA sequences. Mol. Phylogenet. Evol. 9, 100–108.

Chenuil, A., Crespin, L., Pouyaud, L., Berrebi, P., 2000. Movements of adult fish in a hybrid zone revealed by microsatellite genetic analysis and capture–recapture data. Freshw. Biol. 43, 121–131.

Chenuil, A., Crespin, L., Pouyaud, L., Berrebi, P., 2004. Autosomal differences between males and females in hybrid zones: a first report from *Barbus barbus* and *Barbus meridionalis* (Cyprinidae). Heredity 93, 128–134.

Chow, S., Hazama, K., 1998. Universal PCR primers for S7 ribosomal protein gene introns in fish. Mol. Ecol. 7, 1247–1263.

Costedoat, C., Gilles, A., 2009. Quaternary pattern of freshwater fishes in Europe: comparative phylogeography and conservation perspective. Open Conserv. Biol. J. 3, 36–48.

Crespin, L., Berrebi, P., 1999. Asymmetrical introgression in freshwater fish hybrid zone as revealed by a morphological index of hybridization. Biol. J. Linn. Soc. 67, 57–72.

Darius, H., Berrebi, P., 1993. Asymetrie et anomalies morphologiques dans la zone hybride de la Lergue (sud de la France) *Barbus barbus* × *Barbus meridionalis*. Cah. d'Ethol. 13, 243–244.

Doadrio, I., Casado, P., 1989. Nota sobre la ictiofauna continental de los yacimientos de la cuenca de Guadix-Baza (Granada). In: Alberdi, M.T., Bonadonna, F.P. (Eds.), Geología y Paleontologia de la Cuenca de Guadiz-Baza. Trabajos sobre Neógeno y Cuaternario, Madrid, Spain, pp. 139–150.

Doadrio, I., 1990. Phylogenetic relationships and classification of western palaearctic species of the genus *Barbus* (Osteichthyes, Cyprinidae). Aquat. Living Resour. 3, 265–282.

Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7, 214.

Drummond, A.J., Ho, S.Y.W., Phillips, M.J., Rambaut, A., 2006. Relaxed phylogenetics and dating with confidence. PLoS Biol. 4, e88.

Durand, J.D., Tsigenopoulos, C.S., Ünlü, E., Berrebi, P., 2002. Phylogeny and Biogeography of the family Cyprinidae in the Middle East inferred from cytochrome *b* DNA – evolutionary significance of this region. Mol. Phyl. Evol. 22, 91–100.

Earl, D.A., von Holdt, B.M., 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv. Genet. Resour. 4, 359–361.

Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. 14, 2611–2620.

Falush, D., Stephens, M., Pritchard, J.K., 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164, 1567–1587.

Flot, J.F., 2010. SeqPHASE: a web tool for interconverting PHASE input/output files and FASTA sequence alignments. Mol. Ecol. Resour. 10, 162–166.

Flot, J.F., Tillier, A., Samadi, S., Tillier, S., 2006. Phase determination from direct sequencing of length-variable DNA regions. Mol. Ecol. Notes 6, 627–630.

Fogelqvist, J., Niittyvuopio, A., Agren, J., Savolainen, O., Lascoux, M., 2010. Cryptic population genetic structure: the number of inferred clusters depends on sample size. Mol. Ecol. Resour. 10, 314–323.

Gamble, T., Berendzen, P.B., BradleyShaffer, H., Starkey, D.E., Simons, A.M., 2008. Species limits and phylogeography of North American cricket frogs (Acris: Hylidea). Mol. Phylogenet. Evol. 48, 112–125.

Gandolfi, G., Zerunian, S., Torricelli, P., Marconato, A., 1991. I pesci delle acque interne italiane. Ist. Poligrafico e Zecca dello Stato, Roma.

Gante, H.F., 2009. A role for introgressive hybridization in the evolution of freshwater fishes: the case-study of Iberian Barbus (Teleostei, Cyprinidae). PhD Dissertation. Arizona State University, Tempe.

Gante, H.F., 2011. Diversification of circum-mediterranean barbels. In: Grillo, O., Venora, G., (Eds.), Changing Diversity in Changing Environment. InTech, pp. 283–298.

Gante, H.F., Alves, M.J., Dowling, T.E., 2011. Paralog-specific Primers for the Amplification of Nuclear Loci in Tetraploid Barbels (*Barbus*: Cypriniformes). J. Hered. 102, 617–621.

Gante, H.F., Doadrio, I., Alves, M.J., Dowling, T.E., 2015. Semi-permeable species boundaries in Iberian barbels (*Barbus* and *Luciobarbus*, Cyprinidae). BMC Evol. Biol. (submitted for publication).

Gante, H.F., Micael, J., Oliva-Patern, F.J., Doadrio, I., Dowling, T.E., Alves, M.J., 2009. Diversification within glacial refugia: tempo and mode of evolution of the polytypic fish *Barbus sclateri*. Mol. Ecol. 18, 3240–3255.

Garzanti, E., Vezzoli, G., Andò, S., 2011. Paleogeographic and paleodrainage changes during Pleistocene glaciations (Po Plain, Northern Italy). Earth-Sci. Rev. 105, 25–48.

Ghelardoni, R., 1958. Spostamento dello spartiacque nell'Appennino settentrionale in conseguenza di catture idrografiche. Atti. Soc. Toscana Sci. Nat. 65, 25–38.

Gilles, A., Costedoat, C., Barascud, B., Voisin, A., Banarescu, P., Bianco, P.G., Economidis, P.S., Marić, D., Chappaz, R., 2010. Speciation pattern of *Telestes souffia* complex (Teleostei, Cyprinidae) in Europe using morphological and molecular markers. Zool. Scr. 39, 225–242.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59, 307–321.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Hanfling, B., Hellemans, F., Volckaert, A.M., Carvalho, G.R., 2002. Late glacial hystory of the cold adapted freshwater fish *Cottus gobio*, revealed by microsatellites. Mol. Ecol. 11, 1717–1729.

Hewitt, G.M., 1999. Post-glacial re-colonization of European Biota. Biol. J. Linn. Soc. 68, 87–112.

Hewitt, G.M., 2000. The genetic legacy of the Quaternary ice ages. Nature 405, 907–913.

Ho, S.Y.W., 2007. Calibrating molecular estimates of substitution rates and divergence times in birds. J. Avian Biol. 38, 409–414.

Jakobsson, M., Rosenberg, N.A., 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23, 1801–1806.

Ketmaier, V., Bianco, P.G., Cobolli, M., Krivokapic, M., Caniglia, R., De Mattheis, E., 2004. Molecular phylogeny of two lineages of Leuciscinae cyprinids (*Telestes* and *Scardinius*) from the peri-mediterranean area based on cytochrome *b* data. Mol. Phylogenet. Evol. 32, 1061–1071.

Ketmaier, V., Finamore, F., Largiadèr, C., Milone, M., Bianco, P.G., 2009. Phylogeography of bleaks *Alburnus* spp. (Cyprinidae) in Italy, based on cytochrome *b* data. J. Fish Biol. 75, 997–1017.

Konopiński, M.K., Amirowicz, A., Kotlík, P., Kukuła, K., Bylak, A., Pekarik, L., Šediva, A., 2013. Back from the brink: the Holocene history of the Carpathian barbel *Barbus carpathicus*. PLoS ONE 8, e82464.

Kotlík, P., Berrebi, P., 2002. Genetic subdivision and biogeography of the Danubian rheophilic barb *Barbus petenyi* inferred from phylogenetic analysis of mitochondrial DNA variation. Mol. Phylogenet. Evol. 5, 91–100.

Kottelat, M., Freyhof, J., 2007. Handbook of European Freshwater Fishes. Kottelat, Cornol, Switzerland and Freyof, Berlin.

Krijgsman, W., Hilgen, F.J., Raffi, I., Sierro, F.J., Wilson, D.S., 1999. Chronology, causes and progression of the Messinian salinity crisis. Nature 400, 652–655.

Kuss, P., Pluess, A.R., Ægisdóttir, H.H., Stocklin, J., 2008. Spatial isolation and genetic differentiation in naturally fragmented plant populations of the Swiss Alps. J. Plant Ecol. 1, 149–159.

Lajbner, Z., Šlechtová, V., Šlechta, V., Švátora, M., Berrebi, P., Kotlík, P., 2009. Rare and asymmetrical hybridization of the endemic *Barbus carpathicus* with its widespread congener *B. barbus*. J. Fish Biol. 74, 418–436.

Levin, B.A., Freyhof, J., Lajbner, Z., Perea, S., Abdoli, A., Gaffaroğlu, M., Ozuluğ, M., Rubenyan, H.R., Salnikov, V.B., Doadrio, I., 2012. Phylogenetic relationships of the algae scraping cyprinid genus *Capoeta* (Teleostei: Cyprinidae). Mol. Phylogenet. Evol. 62, 542–549.

Librado, P., Rozas, J., 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.

Livi, S., de Innocentiis, S., Longobardi, A., Cataudella, S., Tancioni, L., Rampacci, M., Marino, G., 2013. Genetic structure of *Barbus* spp. populations in the Marches Region of central Italy and its relevance to conservation actions. J. Fish Biol. 82, 806–826.

Lorenzoni, M., Carosi, A., Angeli, V., Bicchi, A., Pedicillo, G., Viali, P., 2006. Individuazione e riconoscimento dei barbi autoctoni nel bacino del fiume Paglia. Provincia di Terni Assessorato alla Programmazione faunistica. Arti Grafiche Iezzi, Terni.

Machordom, A., Berrebi, P., Doadrio, I., 1990. Spanish barbel hybridization detected using enzymatic markers: *Barbus meridionalis* Risso and *Barbus haasi* Mertens (Osteichthyes, Cyprinidae). Aquat. Living Resour. 3, 295–303.

Machordom, A., Doadrio, I., 2001. Evidence of a cenozoic Betic-Kabilian connection based on freshwater fish phylogeography (*Luciobarbus*, Cyprinidae). Mol. Phyl. Evol. 18, 252–263.

Mallet, J., 2007. Hybrid speciation. Nature 446, 279–283.

Marchetto, F., Zaccara, S., Muenzel, F., Salzburger, W., 2010. Phylogeography of the Italian vairone (*Telestes muticellus*, Bonaparte 1837) inferred by microsatellite markers: evolutionary history of a freshwater fish species with a restricted and fragmented distribution. BMC Evol. Biol. 10, 111.

Marková, S., Sanda, R., Crivelli, A., Shumka, S., Wilson, I.F., Vukić, J., Berrebi, P., Kotlík, P., 2010. Nuclear and mitochondrial DNA sequence data reveal the evolutionary history of *Barbus* (Cyprinidae) in the ancient lake systems of the Balkans. Mol. Phylogenet. Evol. 55, 488–500.

Mayden, R.L., Chen, W.J., Bart, H.L., Doosey, M.H., Simons, A.M., Tang, K.L., Wood, R.M., Agnew, M.K., Yang, L., Hirt, M.V., Clements, M.D., Saitoh, K., Sado, T., Miya, M., Nishida, M., 2009. Reconstructing the phylogenetic relationships of the earth's most diverse clade of freshwater fishes order cypriniformes (Actinopterygii: Ostariophysi): a case study using multiple nuclear loci and the mitochondrial genome. Mol. Phylogenet. Evol. 51, 500–514.

Mein, P., Moissenet, E., Truc, G., 1978. Les formations continentales du Néogène Supérieur des Vallées du Júcar et du Cabriel au NE d'Albacete (Espagne). Biostratigraphie et environnement. Doc. Lab. Géol. Fac. Sci. Lyon. 72, 99–147.

Meraner, A., Venturi, A., Ficetola, G.F., Rossi, S., Candiotto, A., Gandolfi, A., 2013. Massive invasion of exotic *Barbus barbus* and introgressive hybridization with endemic *Barbus plebejus* in Northern Italy: where, how and why? Mol. Ecol. 22, 5295–5312.

Mesquita, N., Cunha, C., Carvalho, G.R., Coelho, M.M., 2007. Comparative phylogeography of endemic cyprinids in the south-west Iberian Peninsula: evidence for a new ichthyogeographic area. J. Fish Biol. 71, 45–75.

Moyer, G.R., Remington, R.K., Turner, T.F., 2009. Incongruent gene trees, complex evolutionary processes, and the phylogeny of a group of North American minnows (*Hybognathus* Agassiz 1855). Mol. Phylogenet. Evol. 50, 514–525.

Nesci, O., Savelli, D., 2003. Diverging drainage in the Marche Apennines (central Italy). Quat. Int. 101–102, 203–209.

Ohno, S., 1970. Evolution by gene duplication. Springer-Verlag, Berlin, N.Y..

Ori, G.G., 1993. Continental depositional systems of the Quaternary of the Po Plain (northern Italy). Sediment. Geol. 83, 1–14.

Patterson, C., 1993. Osteichthyes: Teleostei. In: Benton, M.J. (Ed.), The Fossil Record 2. Chapman and Hall, London, pp. 621–656.

Perea, S., Böhme, M., Zupancic, P., et al., 2010. Phylogenetic relationships and biogeographical patterns in Circum-Mediterranean subfamily Leuciscinae (Teleostei, Cyprinidae) inferred from both mitochondrial and nuclear data. BMC Evol. Biol. 31, 265.

Pinho, C., Harris, D.J., Ferrand, N., 2008. Non-equilibrium estimates of gene flow inferred from nuclear genealogies suggest that Iberian and North African wall lizards (*Podarcis* spp.) are an assemblage of incipient species. BMC Evol. Biol. 8, 63.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945–959.

Pritchard, J.K., Wen, W., 2002. Documentation for STRUCTURE software: version 2. Available from <http://pritch.bsd.uchicago.edu>.

Přikryl, T., Gómez de Soler, B., Campeny, G., Oms, O., Roubach, S., Blain, H.-A., Agustí, J., 2014. Fish fauna of the Camp dels Ninots locality (Pliocene; Caldes de Malavella, province of Girona, Spain) – first results with notes on palaeoecology and taphonomy. Hist. Biol., 1–14

Rambaut, A., Drummond, A., 2003. Tracer: MCMC Trace Analysis Tool. University of Edinburgh/University of Auckland, UK/New Zealand, Available at <http://beast.bio.ed.ac.uk>.

Rambaut, A., Drummond, A., 2007a. LogCombiner: MCMC Output Combiner. University of Edinburgh/University of Auckland, UK/New Zealand, Available at <http://beast.bio.ed.ac.uk>.

Rambaut, A., Drummond, A., 2007b. TreeAnnotator: MCMC Output Analysis. University of Edinburgh/University of Auckland, UK/New Zealand, Available at <http://beast.bio.ed.ac.uk>.

Reyjol, Y., Hugueny, B., Pont, D., et al., 2007. Patterns in species richness and endemism of European freshwater fish. Glob. Ecol. Biogeogr. 16, 65–75.

Rosenberg, N.A., 2004. Distruct: a program for the graphical display of population structure. Mol. Ecol. Notes 4, 137–138.

Salgueiro, P., Carvalho, G., Collares-Pereira, M.J., Coelho, M.M., 2003. Microsatellite analysis of genetic population structure of the endangered cyprinid *Anaecypris hispanica* in Portugal: implications for conservation. Biol. Conserv. 109, 47–56.

Salzburger, W., Baric, S., Sturmbauer, C., 2002. Speciation via introgressive hybridization in East African cichlids? Mol. Ecol. 11, 619–625.

Salzburger, W., Branstätter, A., Gilles, A., Parson, W., Hempel, M., Sturmbauer, C., Meyer, A., 2003. Phylogeography of the vairone (*Leuciscus souffia*, Risso 1826) in central Europe. Mol. Ecol. 12, 2371–2386.

Scarselli, S., Simpson, G.D.H., Allen, P.A., Minelli, G., Gaudenzi, L., 2006. Association between Messinian drainage network formation and major tectonic activity in the Marche Apennines (Italy). Terra Nova 19, 74–81.

Seehausen, O., 2006. Conservation: losing biodiversity by reverse speciation. Curr. Biol. 16, 334–337.

Semeraro, R., 2000. A hypothesis of the paleogeography of the western Julian Alps and its role in the karstic development of the Mt. Canin. Ipogea 3, 117–166.

Simoni, A., Elmi, C., Picotti, V., 2003. Late Quaternary uplift and valley evolution in the Northern Apennines: Lamone catchment. Quat. Int. 101–102, 253–267.

Šlechtová, V., Bohlen, J., Freyhof, J., Persat, H., Delmastro, G.B., 2004. The Alps as barrier to dispersal in cold-adapted freshwater fishes? Phylogeographic history and taxonomic status of the bullhead in the Adriatic freshwater drainage. Mol. Phylogenet. Evol. 33, 225–239.

Sousa-Santos, C., Gante, H.F., Robalo, J., et al., 2014. Evolutionary history and population genetics of a cyprinid fish (*Iberochondrostoma olisiponensis*) endangered by introgression from a more abundant relative. Conserv. Genet. 15, 665–677.

Stefani, F., Galli, P., Zaccara, S., Crosa, G., 2004. Genetic variability and phylogeography of the cyprinid *Telestes muticellus* within the Italian peninsula as revealed by mitochondrial DNA. J. Zool. Syst. Evol. Res. 42, 323–331.

Stephens, M., Smith, N.J., Donnelly, P., 2001. A new statistical method for haplotype reconstruction from population data. Am. J. Hum. Genet. 68, 978–989.

Taberlet, P., Fumagalli, L., Wust-Saucy, A., Cosson, J., 1998. Comparative phylogeography and postglacial colonization routes in Europe. Mol. Ecol. 7, 453–464.

Tsigenopoulos, C.S., Berrebi, P., 2000. Molecular phylogeny of North Mediterranean freshwater barbs (genus *Barbus*: Cyprinidae) inferred from cytochrome b sequences: Biogeographic and systematic implications. Mol. Phylogenet. Evol. 14, 165–179.

Tsigenopoulos, C.S., Durand, J.D., Ünlü, E., Berrebi, P., 2003. Rapid radiation of the Mediterranean *Luciobarbus* species (Cyprinidae) after the Messinian salinity crisis of the Mediterranean Sea, inferred from mitochondrial phylogenetic analysis. Biol. J. Linn. Soc. 80, 207–222.

Tsigenopoulos, C.S., Karakousis, Y., Berrebi, P., 1999. The North Mediterranean *Barbus* lineage: phylogenetic hypotheses and taxonomic implications based on allozyme data. J. Fish Biol. 54, 267–286.

Tsigenopoulos, C.S., Kasapidis, P., Berrebi, P., 2010. Phylogenetic relationships of hexaploid large-sized barbs (genus *Labeobarbus*, Cyprinidae) based on mtDNA data. Mol. Phylogenet. Evol. 56, 851–856.

Tsigenopoulos, C.S., Kotlík, P., Berrebi, P., 2002. Biogeography and pattern of gene flow among *Barbus* species (Teleostei: Cyprinidae) inhabiting the Italian Peninsula and neighbouring Adriatic drainages as revealed by allozyme and mitochondrial sequence data. Biol. J. Linn. Soc. 75, 83–99.

Vähä, J.P., Erkinaro, J., Niemelä, E., Primmer, C.R., 2007. Life-history and habitat features influence the within-river genetic structure of Atlantic salmon. Mol. Ecol. 16, 2638–2654.

Weir, B.S., 1996. Genetic Data Analysis II: Methods for Discrete Population Genetic Data. Sinauer, Sunderland.

Zaccara, S., Stefani, F., Delmastro, G.B., 2007. Phylogeographical structure of the vairone *Telestes muticellus* (Teleostei, Cyprinidae) within three European peri-Mediterranean districts. Zool. Scr. 36, 443–453.

Zardoya, R., Doadrio, I., 1998. Phylogenetic relationships of Iberian cyprinids: systematic and biogeographical implications. Proc. Roy. Soc. Lond. 265, 1365–1372.

Zardoya, R., Doadrio, I., 1999. Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. J. Mol. Evol. 49, 227–237.

Zerunian, S., 2001. Condannati all'estinzione? Biodiversità, biologia, minacce e strategie di conservazione dei pesci d'acqua dolce indigeni in Italia. Edagricole, Bologna.

Zerunian, S., 2002. Iconografia dei pesci delle acque interne d'Italia (Iconography of Italian inland water fishes). Min. Ambiente – Ist. Naz. Fauna Selvatica.