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Morphologic and genetic variability in the *Barbus* fishes (Teleostei, Cyprinidae) of Central Italy

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Abstract

Italian freshwaters are highly biodiverse, with species present including the native fishes *Barbus plebejus* and *Barbus tyberinus* that are threatened by habitat alteration, fish stocking and invasive fishes, especially European barbel *Barbus barbus*. In central Italy, native fluvio-lacustrine barbels are mainly allopatric and so provide an excellent natural system to evaluate the permeability of the Apennine Mountains. Here, the morphologic and genetic distinctiveness was determined for 611 *Barbus* fishes collected along the Padany-Venetian (Adriatic basins; PV) and Tuscany-Latium (Tyrrhenian basins; TL) districts. Analyses of morphological traits and mitochondrial DNA sequence data explored the natural and anthropogenic factors that have shaped their distribution ranges. Over 100 alien *B. barbus* were recorded in the Tiber basin (TL district) and Metauro basin (PV district). Comparisons of genetic and morphometric data revealed that morphometric data could identify alien *B. barbus* from native *Barbus*, but could not differentiate between *B. tyberinus* and *B. plebejus*. Genetic analyses revealed approximately 50 D-loop mtDNA haplotypes and identified a distinct *Barbus* lineage present only in the Vomano River at the southern boundary of PV district. Demographic expansion and molecular variance analyses revealed a lack of geographic structuring across the sampling regions. Whilst the contemporary *B. plebejus* distribution has been driven primarily by anthropogenic fish translocations, the dispersal of *B. tyberinus* has been via natural dispersion, including their crossing of the Apennine Mountains via temporary river connectivity. The results also revealed that the *Barbus* fishes of the mid-Adriatic region of Europe have a complex pattern of local endemism. To conserve these patterns of genetic uniqueness, especially in the mid-Adriatic basins, *Barbus* fishes should be managed by treating them as unique evolutionary units and ceasing translocations of all *Barbus* fishes between river basins.

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1 Introduction

Italian freshwaters are within the Mediterranean Biodiversity Hotspot, one of the world's most diverse biogeographic regions (Geiger et al., 2014). Together with Iberia and the Balkans, the Italian peninsula was one of three main European Pleistocenic refugia that enabled species' persistence during the glacial periods. Moreover, within species, these regions have promoted divergence between lineages (Hewitt, 1999). The Italian peninsula is isolated from continental Europe by the Alps, but the dispersion of its freshwater fauna is also limited by the Apennine Mountains that separate the Tyrrhenian and Adriatic river basins. This north-south barrier strongly influences biogeographic distinction between two ichthyogeographic districts; the Padany-Venetian district (PV) that includes basins draining into the Adriatic Sea and covers the Vomano River to the Krka River in Croatia, and the Tuscany-Latium district (TL), with basins that drain into the middle Tyrrhenian Sea, from the Serchio River to the Tiber River (Bianco, 1995a).

The cyprinid fish genus *Barbus* includes species adapted to a variety of freshwater habitats, ranging from small mountain streams to large and slow-flowing rivers and lakes (Kottelat & Freyhof, 2007). As for all primary freshwater fishes (Berrebi, 1995; Zardoya & Doadrio, 1999; Reyjol et al., 2007; Sousa-Santos et al., 2016), their distributions are often restricted to specific drainage systems, indicating a low capacity for dispersal between watersheds (Bianco, 1998; Banarescu, 1998; Tsigenopoulos & Berrebi, 2000; Machodrom & Doadrio, 2001; Gante et al., 2009). In central Italy, native *Barbus* species are fluvio-lacustrine, sharing habitat preferences of larger, slower flowing rivers characterised by laminar flows and relatively warm temperatures (Kottelat & Freyhof, 2007), and show an allopatric distribution. The common barbel (*Barbus plebejus* Bonaparte, 1839) is naturally widespread in the Adriatic basins (PV district), with an approximate southern limit of its range localised between the Tronto and Vomano rivers (Bianco, 1994; Bianco, 2003a; Kottelat & Freyof, 2007). Conversely, Tiber barbel (*Barbus tyberinus* Bonaparte, 1839) is naturally distributed in the main Tyrrhenian basins and in those of the Adriatic slope on the south of the Vomano River (Bianco, 2003b). Recently, it was demonstrated through fossil calibration that the two species split in the Upper Pliocene (around 4M years ago; Buonerba et al., 2015) following the geomorphological evolution of the hydrographic networks. The phylogenetic split occurred in conjunction with a major uplift phase of the Apennines' External Sector, when the Tyrrhenian and Adriatic rivers started to flow on opposite sides of this mountain range (Ascione, Cinque, Miccadei, Villani, & Berti, 2008). Despite this east-west orographic barrier, local tectonism and geomorphological processes have since affected drainage patterns (Ghelardoni, 1958; Nesci & Savelli, 2003; Simoni, Elmi, & Picotti, 2003) and might have facilitated natural dispersion of both native species. This would have been likely to promote local population admixture and, potentially, introgression between the fishes (Livi et al., 2013, Buonerba et al., 2015; Zaccara, Antognazza, Buonerba, Britton, & Crosa, 2014).

From a conservation perspective, these *Barbus* fishes are both classed as 'vulnerable' on the Red List of Italian Vertebrates (Rondinini, Battistoni, Peronace, & Teofili, 2013), and are included on the IUCN Red List as 'near threatened' (*B. tyberinus*) and 'least concern' (*B. plebejus*) (Freyhof, 2011a, b). Threats to their populations are two-fold, habitat fragmentation and alien fish species. Habitat fragmentation has occurred via the presence of anthropogenic structures, such as hydropower dams and weirs, which disrupt longitudinal connectivity within basins. Fisheries management activities have then exacerbated these issues through releases of alien fishes (Bianco, 2014). Releases of non-indigenous fishes have primarily been European barbel (*Barbus barbus* Linnaeus, 1758) and Spanish barbel (*Luciobarbus graellsii* Steindachner, 1866). In particular, *B. barbus* has established populations, and has negatively interactions with native *Barbus* spp. as they share similar fluvio-lacustrine habitats (Bianco & Ketmaier, 2001; Lorenzoni et al., 2006; Livi et al., 2013; Buonerba, Pompei & Lorenzoni, 2013; Zaccara et al., 2014; Bianco, 2014; Carosi, Ghetti, La Porta & Lorenzoni, 2017). This is resulting in local introgressive hybridization, especially with endemic *Barbus plebejus* (Zerunian, 2002; Kottelat & Freyhof 2007; Meraner et al. 2013; Zaccara et al., 2014).

The protection of native and endemic species requires conservation strategies that reflect the complexity of their distribution patterns and highlight the anthropogenic disturbances that affect their population status. The development of conservation management units thus enable strategies to be implemented at more local levels that are relevant to the species in question (e.g. Salducci et al., 2004; Freyhof, Kottelat, & Nolte, 2005; Gilles et al., 2010; Geiger, Schreiner, Delmastro, & Herder, 2016). Given that the native *Barbus* of central Italy have a primarily allopatric natural distribution, the aim of this study was thus to utilise morphological and molecular analyses to ~~accurately~~ decipher the complex pattern of Italian barbel distribution ranges, ~~. Correspondingly, objectives were to~~ with assessment of the natural versus anthropogenic processes that have shaped their contemporary distribution patterns. Correspondingly, the objectives were to determine the morphological distinctiveness between the *Barbus* species, and assess the spatial distribution of alien *B. barbus* in the river basins of central Italy. The use of population genetics and morphologic analyses on samples collected from a wide spatial area then provides opportunities for developing conservation strategies and defining the priority basins for conservation actions. These objectives are achieved through the application of morphological characteristics and mitochondrial DNA sequence data on samples collected from the Adriatic and Tyrrhenian basins of central Italy.

2 Materials and methods

2.1 Field sampling

During sampling periods completed in 2015 and 2016, 611 specimens of *Barbus* spp. were collected from 34 sampling sites across river basins in central Italy. There were 15 sites in TL district (Tyrrhenian side) and 19 sites in PV district (Adriatic basins) (Table 1, Figure 1). All the 15 TL sampling sites were in the Tiber River basin, where three sub-basins were recognised: Upper Tiber (sites 1-7), Paglia River (sites 8, 10-15), and Topino River (site 9). On the Adriatic side, 10 basins were sampled: from the northern Metauro (sites 16-24), Cesano (site 25), Esino (sites 26-27), Potenza (site 28), Chienti (site 29), Tenna (sites 30-31), Aso (site 32), Tronto (site 33), up to the southern Vomano (site 34) basin, that represent the southern boundary of the PV district (Bianco, 1995a). Each fish was identified to species level through their phenotypic characters (e.g. colouration pattern, spot form and size, fin colour), and scale conformation for native species, and the upper margin shape of the dorsal fin and the robustness of its ossified ray for *B. barbus* (Bianco, 1995b; Kottelat & Freyhof, 2007; Bianco 2003a,b; Lorenzoni et al., 2006).

Following their collection by electric fishing, fish were anaesthetised (MS-222) and, for morphological analyses, photographed from their left side using a Nikon D300 camera (24-85 mm lens) positioned by means of a tripod on a table with a millimetric scale. A sample of fin tissue was also collected from each fish and stored in 90% ethanol for subsequent molecular analysis. Following their recovery, all fish were released at their site of capture.

2.2 Morphological analysis

For testing species and phenotypic discrimination using morphological traits, a series of external additional characters (morphometric and meristic traits) were selected for measurement. Following Lorenzoni et al. (2006), seven morphometric traits were chosen: i) eye diameter (ED), ii) pre-orbital distance (POD), iii) mouth-operculum distance (MOD), iv) length of pectoral fin (LPF), v) length of ventral fin (LVF), vi) length of anal fin (LAF), and vii) height of the third dorsal fin (ossified) ray (HDOR). To reduce the effects of size and allometry, data were standardized to the overall mean standard length according to the following formula (Beacham, 1985):

$$D_s = D_o \left(L_{mean} / L_o\right)^b$$

where D_s = standardized distance; D_o = observed distance; L_{mean} = overall mean standard length; L_o = observed standard length; and b = regression coefficient of $\ln D_o$ on $\ln L_o$.

Four meristic traits were also considered: three related to lateral line scale counts (the number of scales on the lateral line (NSLL), and on rows above (NSALL) and under (NSULL) the lateral line) and the number of dorsal fin branched rays (NDBR).

Co-variation among morphometric and meristic traits was assessed using principal components analysis (PCA), reducing multidimensionality of the dataset into new principal components (orthogonal variables). The variance-covariance matrix of standardized morphometric and meristic traits was used. Then, analysis of variance (ANOVA) was performed, followed by the Tuckey post-hoc test, to identify the traits with highly significant differences ($P < 0.05$). These analyses were performed using PAST software (Hammer, Harper & Ryan, 2001).

Geometric morphometric analysis of body shape was then performed placing on the digital images 19 landmarks (LMs): (1) anterior tip of snout, (2 and 3) anterior and posterior end of the eye, (4) orthogonal projection on the dorsal profile of the eye centre, (5) posterior end of neurocranium, (6 and 7) anterior and posterior insertion of dorsal fin, (8 and 9) anterior attachment of dorsal and ventral membrane of caudal fin, (10 and 11) end of the upper and lower lobe of caudal fin, (12) “furca” of caudal fin, (13) base of middle caudal rays, (14 and 15) posterior and anterior insertion of anal fin, (16) insertion of pelvic fin, (17) orthogonal projection on the ventral profile of the (anterior) insertion of pectoral fin, (18 and 19) ventral and dorsal end of branchiae. (supporting information Figure S1). Their *x,y* coordinates were collected using the Geomorph function ‘‘digitize2d’’ (Geomorph package; Adams, Collyer & Kaliontzopoulou, 2018) and processed with MorphoJ (Klingenberg, 2011). Non-shape variation, introduced through variation in position, orientation and size, was mathematically removed using generalized procrustes analysis (Rohlf & Slice, 1990). This minimised the sum of squared distances between corresponding LMs by scaling, translating and rotating specimens onto a mean consensus configuration calculated from all specimens. Shape variations were analysed by canonical variate analysis (CVA). Mahalanobis distances (Mahalanobis, 1936) were estimated through permutation tests (10,000 replicates).

2.3 Molecular analysis

Total genomic DNA was extracted from all individuals using a proteinase K digestion, followed by sodium chloride extraction and ethanol precipitation (i.e. salting out method; Aljanabi & Martinez, 1997). A fragment (867 bp) of the mitochondrial control region (D-loop) locus was amplified using D-loopsxF and D-loopdxR (Rossi et al., 2013; Antognazza, Andreu, Zaccara & Britton, 2016) primers pair. PCR assay was performed using Multiplex PCR kit (Qiagen) in 10 μl reaction volume containing approximately 10 ng of template DNA and 0.25 μM of each primers pair. Thermal cycling was performed as follows: denaturation of 15 min at 95 °C, followed by 35 cycles at 94 °C for 30 s, 55 °C for 90 s and the extension step at 72 °C for 90 s; the final elongation was at 72 °C for 10 min. PCR products were purified using ExoSAP-IT^{TM} (USB, Cleveland, USA) and directly sequenced by MACROGEN Inc (Amsterdam, The Netherlands; http://www.macrogen.org) using a 3730XL DNA Sequencer. All new haplotypes generated in this study were deposited in the GenBank database (Acc. N° MG717942-718018).

2.4 Phylogenetic analysis and minimum spanning network

All sequences were aligned using Clustal W (Thompson, Higgins, & Gibson, 1994), as implemented in Bioedit (Hall, 1999) software. Then, alignments were visually checked and adjusted. For phylogenetic analysis, identical sequences were collapsed into haplotypes in order to facilitate computational processes, as implemented in DnaSP v 5.0 (Librado & Rozas, 2009) software. Computation of phylogenetic tree reconstructions of haplotypes was performed using maximum likelihood (ML) and Bayesian (BI) analyses. The former was conducted in GARLI v 2.0 (Zwikl, 2006; Bazinet, Zwickl & Cummings, 2014) software. The best evolutionary model, identified under the Akaike's information criterion (AIC), as implemented in JModelTest v.2.1.10 (Darriba, Taboada, Doallo & Posada, 2012), was HKY+I+G (Hasegawa, Iida, Yano, Takaiwa & Iwabuchi, 1985). Statistical support for the phylogenetic tree nodes was estimated as bootstrap probability (btp) value over 1,000 replicates.

Bayesian analyses were performed using four independent runs of four Markov Monte Carlo coupled chains of 4 x 10^6 generations each to estimate the posterior probability (pp) distribution, as implemented MrBayes v 3.1.2 (Ronquist et al., 2012) software. Topologies were sampled every 100 generations, and the majority-rule consensus tree was estimated after discarding the first 25% of generations. *Barbus meridionalis* (AJ388417) was used as outgroup. As indicated by Doadrio, Carmona, & Machordom (2002), pairwise uncorrected mean divergences between taxa (uncorrected p-distance) derived from mtDNA D-loop were used as a surrogate for levels of species divergence.

Then, Minimum Spanning Networks (MSNs) were created from the multiple D-loop alignment using a statistical parsimony criterion as implemented in TCS v 1.18 (Clement, Posada & Crandall, 2000) software.

2.5 *Genetic diversity, demography and population structure*

Nucleotide and haplotype diversity parameters were estimated for each species using DnaSP v 5.0. To detect signature of demographic expansion and visualize historical demographic trends of native species, mismatch analyses were performed by examining frequency distributions of pairwise differences between sequences, as implemented in Arlequin v.3.5 (Excoffier & Lischer, 2010) software. The sudden demographic expansion model was tested by the probability of obtaining sum-of-squared deviation values (SSD) and Harpending's Raggedness index lower than observed (Rogers & Harpending, 1992) in a coalescent algorithm simulation over 1,000 pseudo-replications with statistical significance ($P < 0.05$).

In order to compare the connectivity between populations (within and between hydrographic districts), genetic differentiation was tested among populations using the fixation index Φ_{ST} (Weir & Cockerham, 1984) and assessing their significance ($P < 0.05$) by permuting haplotypes between populations 3024 times, as implemented in

Arlequin v 3.5. Overall differences between PV and TL district were also examined. Evidence for genetic hierarchical population structure was then assessed by an analysis of molecular variance (AMOVA), as implemented in Arlequin v 3.5, with the aim of determining the genetic variability within and among populations of Adriatic and Tyrrhenian districts.

3 Results

3.1 Phenotypic attribution

Phenotypic classification of 611 specimens enabled their attribution to *B. plebejus* (n = 113); *B. barbus* (n = 102) and *B. tyberinus* (n = 157) (Table 1, Figure 3a). The remaining 239 sampled *Barbus* had been collected in the southern PV Adriatic basins (SPV, from Burano (site 24) to Vomano (site 34)). These fish were characterised by their distinctive pigmentation and spot pattern that was similar to *B. tyberinus* and so were referred to as named *Barbus tyberinus*-like (*B. tyb*-like) (see supporting information Figure S2A). Phylogenetic analysis of D-loop sequences for these fish identified 214 individuals as *B. plebejus* (named *B. plebejus* in SPV), but with 25 specimens identified as an independent *Barbus* lineage, named *Barbus* in Vomano, as it was recorded exclusively in the most southern PV basin (site 34) (*cf.* Table 1).

3.2 Analysis of morphological data

The PCA of morphometric and meristic traits explained 49% of the observed variation within the first two PCs and did not reveal any specific groups, thus not providing a clear visual separation even between the Italian endemic species (*B. tyberinus* and *B. plebejus*) and the allochthonous *B. barbus* (supporting information Figure S2B and Table S1). However, most *B. barbus* specimens had positive PC1 values, whereas *B. plebejus* and *Barbus* in Vomano specimens were instead distributed on the opposite side on respect to the PC2.

Excluding mismatches between phenotypic and genetic attribution (2% of samples for *B. barbus*, 11% for *B. plebejus* and 30% for *B. tyberinus*) (supporting information Table S2), ANOVA revealed significant morphological differences between the attributed species ($P < 0.05$), especially between *B. barbus* and native *Barbus*. In particular, *B.*

barbus has higher LVF, POD and HDOR traits on average, and different NSLL (57 on average, except for *B. tyberinus*). The most distinctive traits between *B. plebejus* and *B. tyberinus* were the numbers of scales on lateral line, higher in *B. plebejus* (NSLL=63 and NSALL=13 on average) than in *B. tyberinus* (NSLL=57 and NSALL=12 on average). In addition, *B. tyb*-like groups differed from both *B. plebejus* (i.e. NSLL, NSALL, and MOD) and *B. tyberinus* (i.e. POD, MOD, and LAF), and between each other (i.e. ED, LAF, NSLL, and NSULL).

Geometric morphometric analyses provided partial visual separation in body shape morphology among the five *Barbus* groups (Figure 2), supported by permutation tests of Mahalanobis distances (all $P < 0.05$), ranging between 2.33 and 4.75. The position of the five groups within the CVA plot revealed that the *B. plebejus* and *B. tyberinus* groups overlapped, while *B. barbus* and the *Barbus* in Vomano lineage were partially separated along the CV1 and CV2 axis, respectively. The *B. tyb*-like *(B. plebejus* SPV) group mainly overlapped with *Barbus* in Vomano specimens. The shape variations along the CV1 (40%) were mainly associated with the overall fish body shape, while those along the CV2 (34%) mainly resulted from the eye diameter, the shape of the caudal peduncle and the length of the caudal fin lobes.

3.3 Mitochondrial phylogeny and Minimum Spanning Networks (MSNs)

A total of 611 D-loop sequences of 867 base pair (bp) length were aligned. There were 86 variable nucleotide positions detected, of which 61 were parsimony informative sites and with 75 haplotypes scored. ML and BI phylogenetic analysis resulted in a congruent phylogenetic tree structure in which *B. plebejus*, *B. tyberinus* and *B. barbus* haplotypes (i.e. 32, 18 and 20, respectively) clustered independently (Figure 3A). Then, five haplotypes, all recorded exclusively in the Vomano River (site 34, Figure 1) and characterised by *B. tyb*-like phenotype, clustered within *B. tyberinus* lineage (named *Barbus* in Vomano) (Figure 3A). The uncorrected p-distance between lineages ranged from 2.2% to 4.6% (supporting information Table S3).

The network analysis of the mitochondrial data connected *B. plebejus* to *B. tyberinus* haplotypes with 14 mutational steps, while *B. tyberinus* was linked to Vomano haplotypes by 15 mutational steps (Figure 3B). *Barbus plebejus* and *B. tyberinus* showed high haplotype richness and both included two main haplotypes. In *B. plebejus*, MSN had a dumbbell pattern, where the two most frequent and widespread haplotypes

(*Bpleb01* and *Bpleb02*) differentiated by one mutational step. In *B. tyberinus*, six mutational steps were apparent between the two widespread haplotypes: *Btyb17*and *Btyb11*. *Barbus barbus*, grouped in an independent network (>14 mutational steps), had high haplotype richness that radiated around the most frequent haplotype (*Bbar01*) (Figure 3B).

3.4 Genetic diversity and demography

Overall, genetic diversity had values of nucleotide diversity (π) of 0.003 in both native species, and haplotype diversity (H) of 0.86 and 0.90 in *B. tyberinus* and *B. plebejus* respectively. The values of haplotype and nucleotide diversity in *B. barbus* were 0.72 and 0.002 respectively; in the 25 fish belonging to Vomano River, the haplotype and nucleotide diversity were 0.30 and 0.001 respectively.

The mismatch distribution analysis does not reject the sudden expansion model for both *B. tyberinus* and *B. plebejus* lineages. In *B. plebejus*, the unimodal peak and non-significant sum of square deviations (SSD = 0.003, $P < 0.05$) and Harpending's raggedness index (RAG = 0.036, $P < 0.05$) values are indicative of recent demographic expansion (Rogers & Harpending, 1992). Values of sum of square deviations (SSD=0.021, $P < 0.05$) and Harpending's raggedness index (RAG = 0.066, $P < 0.05$) confirm similar expansion patterns in the *B. tyberinus* fishes.

3.5 Haplotype distribution and population structure

In the MSNs, the geographic distribution of all barbus taxa revealed a largely complex pattern along both Adriatic and Tyrrhenian basins (see Figure 3B). In detail, the haplotype distribution of the native fishes (Supporting Information Table S4) revealed *B. plebejus* was widespread in all sampling sites in the Adriatic basins (PV district). With Tyrrhenian basins, they shared nine haplotypes localised in three populations of upper-Tiber (Pop1, Pop2 and Pop5), four populations in Paglia basin (Pop8, Pop10, Pop13 and Pop14) and in Topino basin (Pop8) (Table 1). In the distribution of *B. tyberinus* haplotype, six haplotypes were shared between both districts and localised in three Adriatic basins: Metauro (Pop16, Pop19 and Pop29), Cesano (Pop25) and Esino (Pop26 and Pop27) rivers. Only two haplotypes (Btyb03 and Btyb10) were exclusively present in the Cesano (Pop25) and in Metauro (Pop19 and Pop21) river. *Barbus barbus* was prevalently in Tyrrhenian basins (90 % of sites), equally distributed in upper-Tiber (Pop2, Pop3, Pop5, Pop6 and Pop7) and in Paglia basin (Pop10, Pop11, Pop13 and Pop14) with *Bbar01* haplotype as dominant (57%). Conversely, along the Adriatic basins, *B. barbus* was present exclusively in Metauro basin (Pop17, Pop18, Pop19 and Pop20) (cf. Supporting Information Table S4).

Within *B. plebejus* and *B. tyberinus*, overall genetic differentiation between PV and TL districts was $\phi_{ST} = 0.09$ ($P < 0.05$) and $\phi_{ST} = 0.10$ ($P < 0.05$) respectively. Detailing the genetic differentiation among populations of native species within their original district, three *B. tyberinus* populations (Pop4, Pop12 and Pop15) were differentiated (ϕ_{ST} ranging from 0.20 to 0.83; $P < 0.05$) within TL district, while 15 *B. plebejus* populations were differentiated in the Adriatic basins (ϕ_{ST} ranging from 0.06 to 0.84; $P < 0.05$) (supporting information Table S5 and Table S6, respectively).

Within *B. plebejus* and *B. tyberinus*, the genetic differentiation among populations inhabiting basins of the opposite side (Adriatic vs. Tyrrhenian) revealed a complex pattern: in *B. plebejus* ϕ_{ST} values ranged from 0.20 up to 1.00 ($P < 0.05$) while in *B. tyberinus* ϕ_{ST} values range from 0.13 up to 0.91 ($P < 0.05$) (supporting information Table S5 and Table S6, respectively). The AMOVA analyses of *B. tyberinus* and *B. plebejus* revealed that, between the groups (TL vs PV), ϕ_{CT} values were not significantly different, detecting the higher percentage values of molecular variance within populations (60-65%) (supporting information Table S7), and rejecting that there was genetic structure among the districts for both native *Barbus*.

4 Discussion

The sampling of over 600 *Barbus* fishes from 34 sites from across the Adriatic and Tyrrhenian basins of central Italy revealed a highly variable structure regarding their genetics, morphology and biogeography. It is generally assumed that *Barbus* fishes in Italian rivers occur in allopatry, with their ranges being discrete and separated according to biogeographic barriers (Kottelat & Freyhof, 2007). However, the results here do not support this, with *B. plebejus* and *B. tyberinus* apparently both being present in the Tyrrhenian and Adriatic regions (Bianco, 1995b; Buonerba et al., 2015). The genetic data suggest that this current spatial distribution was achieved relatively recently, given that both species had weak genetic differentiation between the two districts, supporting the sudden demographic expansion model (mismatch distribution).

There are a series of hypotheses that can help potentially explain these results. The first is associated with the natural dispersal of the fishes, with both species apparently crossing the Apennine Mountains in an east-west direction during periods of temporal permeability. Support for this relates to the high seismic activity and intensive tectonic movements along the Apennines (Sorgi, Deffontaines, Hippolyte & Cadet, 1998; Pizzi & Galadini, 2009; Garzanti, Vezzoli & Andò, 2011) that have historically shaped the Adriatic and Tyrrhenian catchment areas. This has resulted in the formation of short periods during which novel hydrological links were formed, enabling mixing of populations across this orographic barrier (Bianco, 1994). Indeed, trans-Apennine river connections have been suggested to have been used for historical dispersal by primary fish, such as vairone *Telestes muticellus* Bonaparte 1837 (Zaccara, Stefani & Delmastro, 2007; Marchetto, Zaccara, Muenzel, & Salzburger, 2010) and bullhead (*Cottus gobio* Linnaeus, 1758) (Lorenzoni et al., 2018). The second hypothesis is that the current *Barbus* distribution has resulted primarily from anthropogenic influences, with the fish being translocated between basins in recent decades, mainly for recreational angling purposes. Although this has been mainly reported for the alien *B. barbus*, there is also some evidence of some alteration of the original distribution pattern of the endemic *B. plebejus* (Meraner et al., 2013) and *B. tyberinus* (in Brenta River – Adriatic basin; Buonerba et al., 2015). It is, therefore, likely that the current *Barbus* distribution in the two districts was achieved through both of these mechanisms, with support available for both hypotheses.

A final hypothesis relates to dispersal of *B. tyberinus* between the two regions entirely through natural means and on a more permanent basis than suggested by Hypothesis 1. (i.e. the natural dispersion hypothesis). This is proposed for the Cesano and Esino basins (Pop 25 and Pop27), and requires *B. tyberinus* from a TL basin (e.g. the Topino basin) dispersing into both Adriatic rivers at the limit of the connection between the two districts (Bartolini & Pranzini, 1988; Bianco, 1991). The occurrence of this local trans-Apennine river connection is at least partially supported by both the absence of alien species in these rivers and by genetic differentiation with other TL populations. This hypothesis does not, however, get support from the *B. plebejus* populations. In TL, these fish revealed a genetic signature suggesting anthropogenic dispersal, including their co-occurrence with *B. barbus*, with native *Barbus* translocations occurring in the last century and *B. barbus* since 1950 (Bianco & Ketmaier, 2001; Zerunian, 2002). Moreover, the translocation of *Barbus* populations between Italian geographical areas has favoured the invasion of *B. barbus*, especially in the Po River basin where its range now completely overlaps with *B. plebejus*, where the introgression process between both species has been widespread in the lowland part of the Po basin (Meraner et al., 2013; Zaccara et al., 2014) and in some upper Adriatic basins (Livi et al., 2012). There is now also a high risk of this occurring in the Tiber basin, where high *B. barbus* presence suggests it is becoming widespread throughout central Tyrrhenian rivers, where introgression processes may also occur (Bianco & Ketmaier, 2001; Lorenzoni et al., 2006; Geiger et al., 2016; Carosi et al., 2017). In contrast, their distribution remains more constrained in the mid-Adriatic basins due to minimal river connectivity, resulting in their dispersal being restricted to some tributaries of the Metauro basin where the genetic admixture between *B. barbus* and *B. plebejus* might be just on-going.

Although the presence of putative hybrid forms between native and *B. barbus* cannot be excluded, the results did reveal that *B. barbus* could be discriminated from native *Barbus* fishes by the identification of specific morphological characteristics, including the number of scales along the lateral line, eye diameter and position, and body depth. This is consistent with previous studies on these fishes that suggest *B. barbus* retains its peculiar traits and morphological features in its invasive range (e.g. Lorenzoni et al. 2006; Livi et al., 2013). Conversely, these morphological characteristics had high overlap between the native *Barbus*, thus did not provide clear discrimination between them, probably due to both their evolutionary relationships and their sharing of geographical origin (Italian peninsula) where the ecological uniformity of the rivers results in the selection of similar traits (Livi et al., 2013; Buonerba et al., 2015).

Along the mid-Adriatic basins from Cesano River to the Vomano River, the *Barbus* fishes revealed some novel phenotypic and morphological characters. Meristic and morphological characters, plus geometric morphometry, identified some local distinctiveness, as previously anticipated by Livi et al. (2013). The *B. plebejus* populations inhabiting the most southern part of the PV district (from Cesano River to Vomano River) had characteristics relatively similar to *B. tyberinus* (hence, '*B. tyb*-like'), but also with some distinct morphological characters (such as smaller eye, shorter POD and MOD, number of minor scales). In the Vomano River, these fish were highly genetically differentiated from *B. plebejus* populations (p-distance > 2.3%) and were termed here as *Barbus* in Vomano. This morphological and phenotypic variability, and population genetic differentiation, was highly localized, being restricted to the Southern Padany-District (SPV). This supports the hypothesis that this distribution was achieved through temporary and historical periods of river connectivity between basins (Barraclough & Nee, 2001; Brito, 2005; Burridge, Craw, Jack, King & Waters, 2008). As also suggested by other studies, this primary freshwater fish dispersal pattern would have been achieved by periodic extension of the Po basin (Paleo-Po) up to the last natural event of glacial regression during the Pleistocene (e.g. Bianco 1990; Hewitt, 1999; Bianco & Ketmaier, 2001; Stefani, Galli, Zaccara & Crosa, 2004; Zaccara et al., 2007; Ketmaier, Finamore, Largiadèr, Milone & Bianco, 2009; Garzanti et al., 2011). The subsequent geographical isolation of these mid-Adriatic rivers has since enabled the *Barbus* to evolve to the local conditions and in a separate manner to other basins (Markova et al., 2010; Livi et al., 2013; Buonerba et al., 2015; Antal et al., 2016). This scenario is similar to *T. muticellus*, a cyprinid fish with similar fluvio-lacustrine ecological traits (Marchetto et al., 2010). However, this result contrasts to patterns recorded in the *Barbus* spp. of the northern PV district. There, the *B. plebejus* populations have been homogenised by the Pleistocene glacial cycles that increased their population connectivity across the northern Adriatic basins (Meraner et al., 2013).

The study has thus revealed that the distribution, morphology and genetics of the *Barbus* fishes of central Italian rivers have been shaped by a combination of biogeographic factors and, more recently, by anthropogenic translocations, particularly in the Tiber and Matauro River basins. The translocations of both native and alien *Barbus* spp. have now resulted in a complex barbel community where the possibility of introgression is elevated, undermining the integrity of the natural conditions of local populations. Despite this complex pattern, morphological and genetic analyses were sufficient here to discriminate between the native and alien *Barbus* in the samples, so providing a more detailed map of *B. barbus* invasion. Moreover, these analyses identified high genetic and morphological variability that was focused in the mid-Adriatic region, indicating an area of high local endemism where the alien *B. barbus* has yet to reach. Consequently, conservation strategies and actions are required in mid-Adriatic region to conserve this local endemism. This could be done via managing the populations as one genetic unit. Management actions to then promote the conservation of the genetic uniqueness would be to cease all fish translocations between Italian river basins. Steps should also be taken to reduce the risk of further natural dispersal of alien *B. barbus*, although it is acknowledged that this remains challenging. However, in doing so, it will help protect the endemic *Barbus* of the mid-Adriatic rivers, conserving their genetic and morphological uniqueness.

In conclusion, this study revealed high complexity in these *Barbus* fishes at both morphological and genetic scales that offer future opportunities for investigations using nuclear markers. These future analyses should be capable of detecting introgression and hybridization events so that individuals with specific morphological traits can be matched with their parent species (invasive or native), and the mechanisms involved in these processes can also be better understood. Finally, these analyses should also assist in the identification of different evolutionary units that can be applied to conservation management.

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TABLE 1 Description of 611 *Barbus* populations (N = sampling size) collected along 34 sampling sites, including ID, district (PV = Padany-Venetian, TL = Tuscany-Latium), river basin, watercourse, altitude and distance from source. Field (Lorenzoni et al., 2006) and genetic (D-loop mtDNA lineages) attribution are detailed.

								Filed attribution			**mtDNA lineages**		
ID	**District**	**Sampling site**	**Watercourse**	**Basin**	**Altitude (a.m.s.l.)**	**Distance from source (km)**	***N***	***B. plebejus***	***B. tyberinus***	***B. barbus***	***B. plebejus***	***B. tyberinus***	***B. barbus***
1	TL	Pieve S.Stefano	Tiber	UpperTiber	434	16.81	13	4	9		3	10	
2	TL	Baucca	Soara	UpperTiber	349	11.05	20	3	17		8	10	2
3	TL	S.Lucia	Tiber	UpperTiber	280	54.24	15		5	10			15
4	TL	S.Secondo	Aggia	UpperTiber	299	14.87	9		9			9	
5	TL	Umbertide	Assino	UpperTiber	238	23.16	13	1	5	7	2	3	8
6	TL	Montecorona	Tiber	UpperTiber	228	82.47	20		2	18			20
7	TL	Ponte pattoli	Tiber	UpperTiber	208	98.30	17		3	14			17
8	TL	La Casella	Chiani	Paglia	209	26.47	35		35		2	33	
9	TL	Borgo Trevi	Clitunno	Topino	211	5.20	7		7		3	4	
10	TL	Torre alfina	Paglia	Paglia	319	35.61	15		3	12	2	3	10
11	TL	Allerona	Paglia	Paglia	150	48.07	13			13			13
12	TL	La Cavella	Carcaione	Paglia	263	3.98	16		16			16	
13	TL	Cava Bagioli	Romealla	Paglia	140	12.47	16		15	1	3	12	1
14	TL	Ciconia	Paglia	Paglia	116	57.23	19			19	1		18
15	TL	Gabelletta	Montacchione	Paglia	207	9.58	11		11			11	

16	PV	Sompiano	Meta	Metauro	517	6.30	21	20	1		20	1	
17	PV	Borgo Pace	Auro	Metauro	573	7.80	17	16		1	15		2
18	PV	S.Vincezo-Furlo	Candigliano	Metauro	201	41.03	7	1		7	1		6
19	PV	A monte di Piobbico	Candigliano	Metauro	362	19.38	20	18	2		14	4	2
20	PV	Cagli	Burano	Metauro	225	26.98	23	5	17	1	17	4	2
21	PV	Piobbico	Biscubio	Metauro	394	3.96	14	14			13	1	
22	PV	Piannello	Bosso	Metauro	398	10.31	18	18			18		
23	PV	Certano	Certano	Metauro	544	7.15	8	8			8		
24	PV	Cantiano	Burano	Metauro	341	13.57	14		14[†]		14		
25	PV	Ganga	Cesano	Cesano	253	22.53	19	3	16[†]		10	9	
26	PV	Genga	Esino	Esino	237	18.73	27		27[†]		26	1	
27	PV	Sassoferrato	Sentino	Esino	297	24.93	16		16[†]		7	9	
28	PV	Passo di Treia	Potenza	Potenza	150	45.32	25		25[†]		25		
29	PV	Belforte	Chienti	Chienti	268	39.65	34	2	32[†]		34		
30	PV	Molino Smerillo	Tenna	Tenna	363	13.62	19		19[†]		19		
31	PV	S.Ruffino	Tenna	Tenna	309	18.63	10		10[†]		10		
32	PV	Aso	Aso	Aso	185	37.73	27		27[†]		27		
33	PV	Acquasanta Terme	Tronto	Tronto	370	38.65	28		28[†]		28		
34	PV	Leognano	Vomano	Vomano	203	32.95	25		25[†]			25[‡]	
		Total					**611**	**113**	**396**	**102**	**330**	**165**	**116**

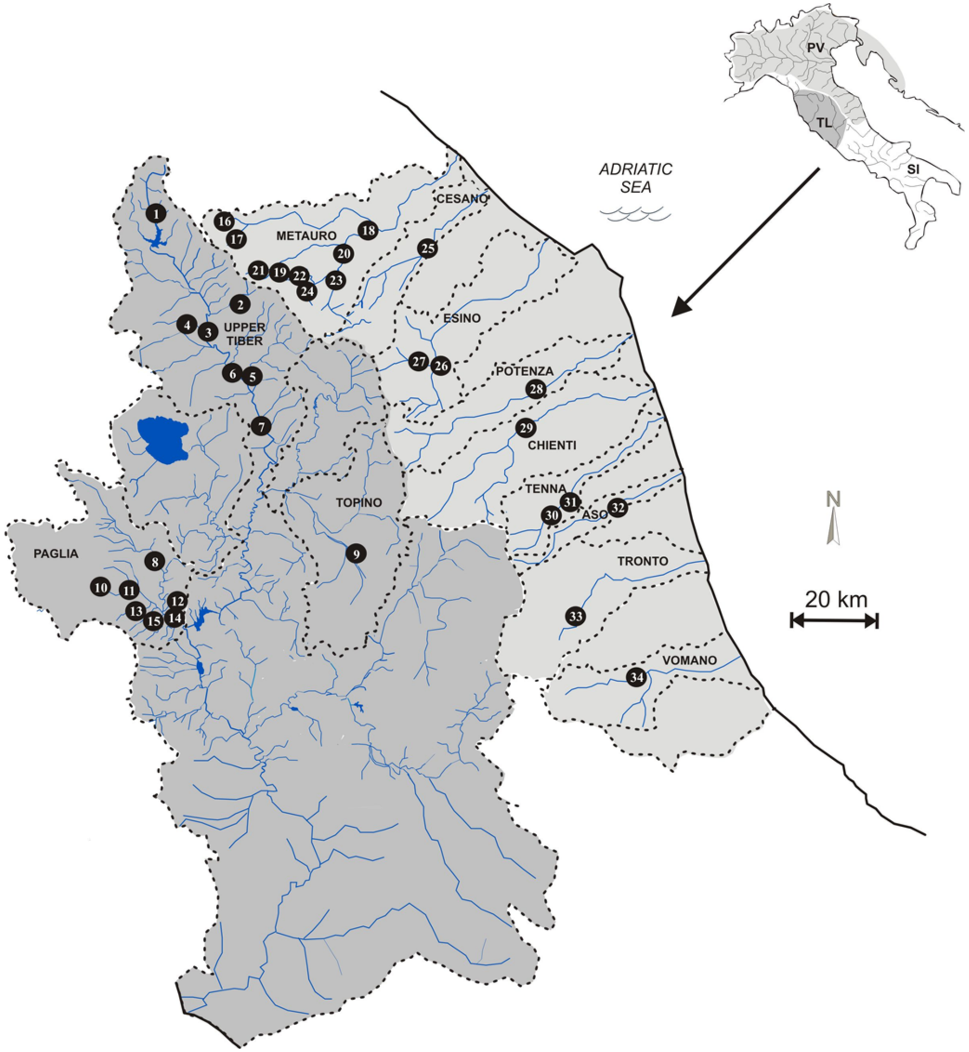
[†] *B. tyberinus*-like; [‡] *Barbus* recorded only in Vomano basin (Figure 5)

Figure legends

FIGURE 1 Map of the 34 sampling sites (ID code is detailed in Table 1). Dashed lines represent river basins. The ichthyo-geographic districts are indicated according to Bianco, (1995a): light grey for Padany-Venetian district = PV; dark grey for Tuscany-Latium district = TL.

FIGURE 2 CVA output of the overall morphological comparisons of the *Barbus* groups: *B. barbus* (orange), *B. plebejus* (green), *B. tyberinus* (light blue) and *B. tyb*-like livery for *B. plebejus* in SPV (red) and for *Barbus* in Vomano (purple) (see supporting information Figure S2A). The pattern described by the first two discriminant axes is shown. Wireframe graphs indicate the shape changes along each axis (from dark to light grey along the CV1 and from light to dark grey along the CV2).

FIGURE 3 A. Maximum likelihood phylogeny of D-loop mitochondrial haplotypes rooted on *Barbus meridionalis*. Bootstrap and Bayes posterior probability are given next to relevant nodes; B. D-loop haplotype (TCS) networks. Circle size is proportional to the observed haplotype frequencies and black points represent unobserved haplotypes and potential intermediates. Mutations between native *Barbus* haplotypes groups are indicated with numbers on branches linking haplotypes. Dot-boxes show phylogenetic lineages (*B. tyberinus*; *B. barbus*, *B. plebejus*, *Barbus* in Vomano). Colours represent Adriatic and Tyrrhenian basins (*c.f. T*able 1).


PV
TL
SI
ADRIATIC SEA
CESANO
METAURO
ESINO
POTENZA
CHIENTI
TENNA
ASO
TRONTO
VOMANO
UPPER TIBER
TOPINO
PAGLIA
N
20 km
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
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19
20
21
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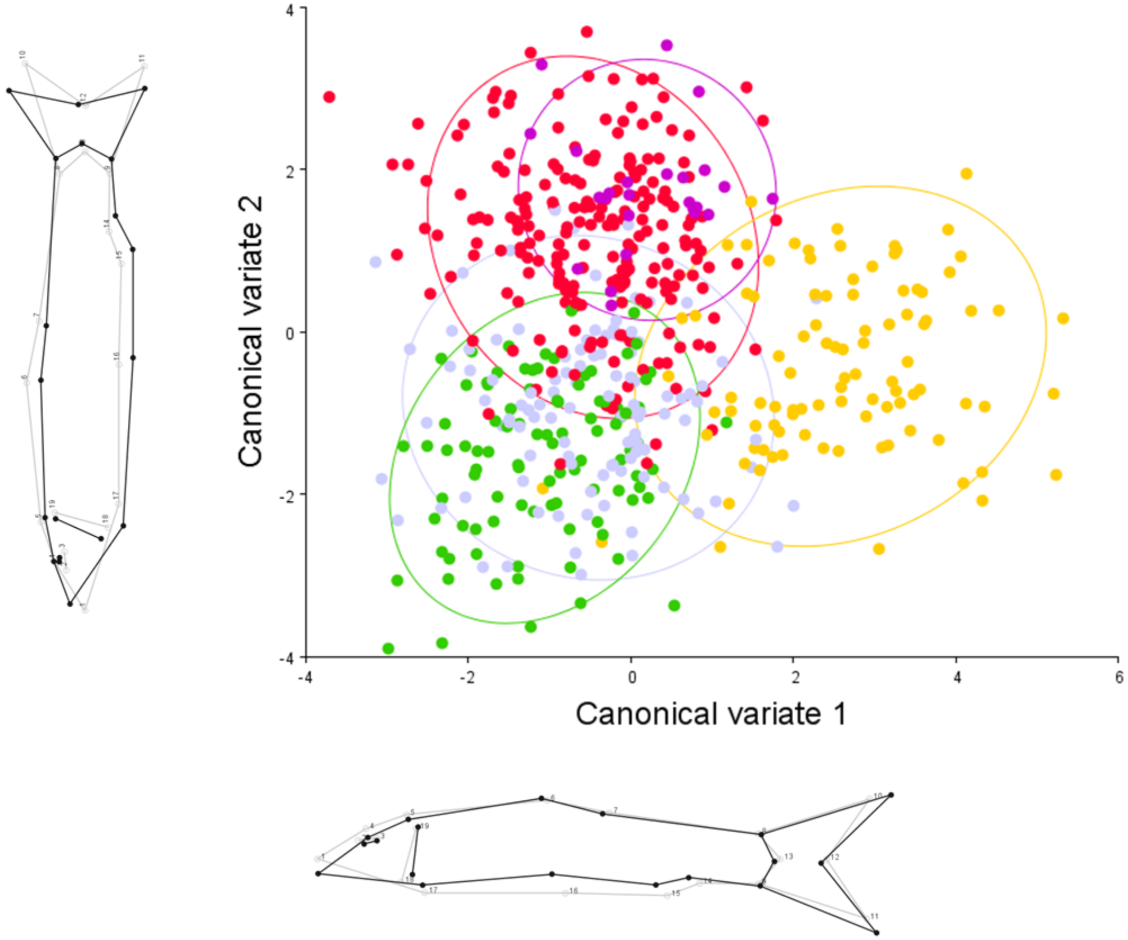
31
32
33
34

Figure 1.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Canonical variate 1
4
2
0
-2
-4
-4
-2
0
2
4
6

Figure 2

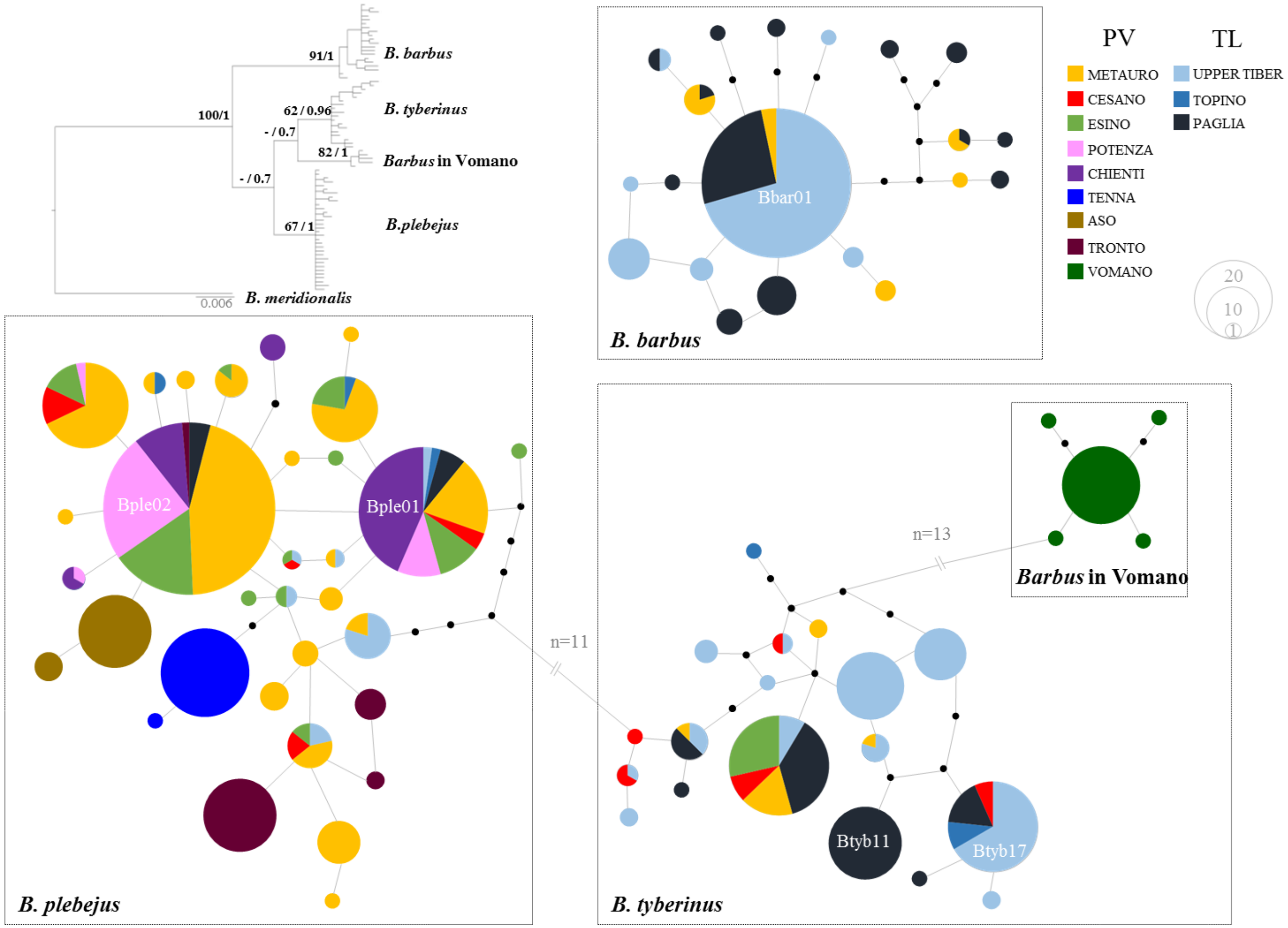
91/1
B. barbus
100/1
62 / 0.96
B. tyberinus
- / 0.7
82 / 1
Barbus in Vomano
- / 0.7
67 / 1
B.plebejus
0.006
B. meridionalis
Bbar01
B. barbus
PV
TL
METAURO
CESANO
ESINO
POTENZA
CHIENTI
TENNA
ASO
TRONTO
VOMANO
UPPER TIBER
TOPINO
PAGLIA
20
10
1
Bple02
Bple01
n=11
B. plebejus
n=13
Barbus in Vomano
Btyb11
Btyb17
B. tyberinus

Figure 3.