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

9 **Central Italy**

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23 **Running title: Barbels dispersion in central Italy**

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28

29 **Abstract**

30

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

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61 morphometrics; conservation; river connectivity

62 **1 Introduction**

63

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

77 The cyprinid fish genus *Barbus* includes species adapted to a variety of freshwater
78 habitats, ranging from small mountain streams to large and slow-flowing rivers and
79 lakes (Kottelat & Freyhof, 2007). As for all primary freshwater fishes (Berrebi, 1995;
80 Zardoya & Doadrio, 1999; Reyjol et al., 2007; Sousa-Santos et al., 2016), their
81 distributions are often restricted to specific drainage systems, indicating a low capacity
82 for dispersal between watersheds (Bianco, 1998; Banarescu, 1998; Tsigenopoulos &
83 Berrebi, 2000; Machodrom & Doadrio, 2001; Gante et al., 2009). In central Italy, native
84 *Barbus* species are fluvio-lacustrine, sharing habitat preferences of larger, slower
85 flowing rivers characterised by laminar flows and relatively warm temperatures
86 (Kottelat & Freyhof, 2007), and show an allopatric distribution. The common barbel
87 (*Barbus plebejus* Bonaparte, 1839) is naturally widespread in the Adriatic basins (PV
88 district), with an approximate southern limit of its range localised between the Tronto
89 and Vomano rivers (Bianco, 1994; Bianco, 2003a; Kottelat & Freyof, 2007).
90 Conversely, Tiber barbel (*Barbus tyberinus* Bonaparte, 1839) is naturally distributed in
91 the main Tyrrhenian basins and in those of the Adriatic slope on the south of the
92 Vomano River (Bianco, 2003b). Recently, it was demonstrated through fossil
93 calibration that the two species split in the Upper Pliocene (around 4M years ago;
94 Buonerba et al., 2015) following the geomorphological evolution of the hydrographic
95 networks. The phylogenetic split occurred in conjunction with a major uplift phase of

96 the Apennines' External Sector, when the Tyrrhenian and Adriatic rivers started to flow
97 on opposite sides of this mountain range (Ascione, Cinque, Miccadei, Villani, & Berti,
98 2008). Despite this east-west orographic barrier, local tectonism and geomorphological
99 processes have since affected drainage patterns (Ghelardoni, 1958; Nesci & Savelli,
100 2003; Simoni, Elmi, & Picotti, 2003) and might have facilitated natural dispersion of
101 both native species. This would have been likely to promote local population admixture
102 and, potentially, introgression between the fishes (Livi et al., 2013, Buonerba et al.,
103 2015; Zaccara, Antognazza, Buonerba, Britton, & Crosa, 2014).

104 From a conservation perspective, these *Barbus* fishes are both classed as 'vulnerable'
105 on the Red List of Italian Vertebrates (Rondinini, Battistoni, Peronace, & Teofili, 2013),
106 and are included on the IUCN Red List as 'near threatened' (*B. tyberinus*) and 'least
107 concern' (*B. plebejus*) (Freyhof, 2011a, b). Threats to their populations are two-fold,
108 habitat fragmentation and alien fish species. Habitat fragmentation has occurred via the
109 presence of anthropogenic structures, such as hydropower dams and weirs, which
110 disrupt longitudinal connectivity within basins. Fisheries management activities have
111 then exacerbated these issues through releases of alien fishes (Bianco, 2014). Releases
112 of non-indigenous fishes have primarily been European barbel (*Barbus barbus* Linnaeus,
113 1758) and Spanish barbel (*Luciobarbus graellsii* Steindachner, 1866). In particular, *B.*
114 *barbus* has established populations, and has negatively interactions with native *Barbus*
115 spp. as they share similar fluvio-lacustrine habitats (Bianco & Ketmaier, 2001;
116 Lorenzoni et al., 2006; Livi et al., 2013; Buonerba, Pompei & Lorenzoni, 2013; Zaccara
117 et al., 2014; Bianco, 2014; Carosi, Ghetti, La Porta & Lorenzoni, 2017). This is
118 resulting in local introgressive hybridization, especially with endemic *Barbus plebejus*
119 (Zerunian, 2002; Kottelat & Freyhof 2007; Meraner et al. 2013; Zaccara et al., 2014).

120 The protection of native and endemic species requires conservation strategies that
121 reflect the complexity of their distribution patterns and highlight the anthropogenic
122 disturbances that affect their population status. The development of conservation
123 management units thus enable strategies to be implemented at more local levels that are
124 relevant to the species in question (e.g. Salducci et al., 2004; Freyhof, Kottelat, & Nolte,
125 2005; Gilles et al., 2010; Geiger, Schreiner, Delmastro, & Herder, 2016). Given that the
126 native *Barbus* of central Italy have a primarily allopatric natural distribution, the aim of
127 this study was thus to utilise morphological and molecular analyses to accurately
128 decipher the complex pattern of Italian barbel distribution ranges, ~~Correspondingly,~~
129 ~~objectives were to~~ with assessment of the natural versus anthropogenic processes that

130 have shaped their contemporary distribution patterns. Correspondingly, the objectives
131 were to determine the morphological distinctiveness between the *Barbus* species, and
132 assess the spatial distribution of alien *B. barbuis* in the river basins of central Italy. The
133 use of population genetics and morphologic analyses on samples collected from a wide
134 spatial area then provides opportunities for developing conservation strategies and
135 defining the priority basins for conservation actions. These objectives are achieved
136 through the application of morphological characteristics and mitochondrial DNA
137 sequence data on samples collected from the Adriatic and Tyrrhenian basins of central
138 Italy.

139

140 **2 Materials and methods**

141

142 *2.1 Field sampling*

143

144 During sampling periods completed in 2015 and 2016, 611 specimens of *Barbus* spp.
145 were collected from 34 sampling sites across river basins in central Italy. There were 15
146 sites in TL district (Tyrrhenian side) and 19 sites in PV district (Adriatic basins) (Table
147 1, Figure 1). All the 15 TL sampling sites were in the Tiber River basin, where three
148 sub-basins were recognised: Upper Tiber (sites 1-7), Paglia River (sites 8, 10-15), and
149 Topino River (site 9). On the Adriatic side, 10 basins were sampled: from the northern
150 Metauro (sites 16-24), Cesano (site 25), Esino (sites 26-27), Potenza (site 28), Chienti
151 (site 29), Tenna (sites 30-31), Aso (site 32), Tronto (site 33), up to the southern Vomano
152 (site 34) basin, that represent the southern boundary of the PV district (Bianco, 1995a).
153 Each fish was identified to species level through their phenotypic characters (e.g.
154 colouration pattern, spot form and size, fin colour), and scale conformation for native
155 species, and the upper margin shape of the dorsal fin and the robustness of its ossified
156 ray for *B. barbuis* (Bianco, 1995b; Kottelat & Freyhof, 2007; Bianco 2003a,b; Lorenzoni
157 et al., 2006).

158 Following their collection by electric fishing, fish were anaesthetised (MS-222) and, for
159 morphological analyses, photographed from their left side using a Nikon D300 camera
160 (24-85 mm lens) positioned by means of a tripod on a table with a millimetric scale. A
161 sample of fin tissue was also collected from each fish and stored in 90% ethanol for
162 subsequent molecular analysis. Following their recovery, all fish were released at their
163 site of capture.

164

165 *2.2 Morphological analysis*

166

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

$$175 \quad D_s = D_o (L_{\text{mean}} / L_o)^b$$

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

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

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

189 Geometric morphometric analysis of body shape was then performed placing on the
 190 digital images 19 landmarks (LMs): (1) anterior tip of snout, (2 and 3) anterior and
 191 posterior end of the eye, (4) orthogonal projection on the dorsal profile of the eye centre,
 192 (5) posterior end of neurocranium, (6 and 7) anterior and posterior insertion of dorsal fin,
 193 (8 and 9) anterior attachment of dorsal and ventral membrane of caudal fin, (10 and 11)
 194 end of the upper and lower lobe of caudal fin, (12) “furca” of caudal fin, (13) base of
 195 middle caudal rays, (14 and 15) posterior and anterior insertion of anal fin, (16)
 196 insertion of pelvic fin, (17) orthogonal projection on the ventral profile of the (anterior)
 197 insertion of pectoral fin, (18 and 19) ventral and dorsal end of branchiae. (supporting

198 information Figure S1). Their x,y coordinates were collected using the Geomorph
199 function “digitize2d” (Geomorph package; Adams, Collyer & Kaliontzopoulou, 2018)
200 and processed with MorphoJ (Klingenberg, 2011). Non-shape variation, introduced
201 through variation in position, orientation and size, was mathematically removed using
202 generalized procrustes analysis (Rohlf & Slice, 1990). This minimised the sum of
203 squared distances between corresponding LMs by scaling, translating and rotating
204 specimens onto a mean consensus configuration calculated from all specimens. Shape
205 variations were analysed by canonical variate analysis (CVA). Mahalanobis distances
206 (Mahalanobis, 1936) were estimated through permutation tests (10,000 replicates).

207

208 *2.3 Molecular analysis*

209

210 Total genomic DNA was extracted from all individuals using a proteinase K digestion,
211 followed by sodium chloride extraction and ethanol precipitation (i.e. salting out
212 method; Aljanabi & Martinez, 1997). A fragment (867 bp) of the mitochondrial control
213 region (D-loop) locus was amplified using D-loopsxF and D-loopdxR (Rossi et al.,
214 2013; Antognazza, Andreu, Zaccara & Britton, 2016) primers pair. PCR assay was
215 performed using Multiplex PCR kit (Qiagen) in 10 μ l reaction volume containing
216 approximately 10 ng of template DNA and 0.25 μ M of each primers pair. Thermal
217 cycling was performed as follows: denaturation of 15 min at 95 °C, followed by 35
218 cycles at 94 °C for 30 s, 55 °C for 90 s and the extension step at 72 °C for 90 s; the final
219 elongation was at 72 °C for 10 min. PCR products were purified using ExoSAP-IT™
220 (USB, Cleveland, USA) and directly sequenced by MACROGEN Inc (Amsterdam, The
221 Netherlands; <http://www.macrogen.org>) using a 3730XL DNA Sequencer. All new
222 haplotypes generated in this study were deposited in the GenBank database (Acc. N°
223 MG717942-718018).

224

225 *2.4 Phylogenetic analysis and minimum spanning network*

226

227 All sequences were aligned using Clustal W (Thompson, Higgins, & Gibson, 1994), as
228 implemented in Bioedit (Hall, 1999) software. Then, alignments were visually checked
229 and adjusted. For phylogenetic analysis, identical sequences were collapsed into
230 haplotypes in order to facilitate computational processes, as implemented in DnaSP v
231 5.0 (Librado & Rozas, 2009) software. Computation of phylogenetic tree

232 reconstructions of haplotypes was performed using maximum likelihood (ML) and
233 Bayesian (BI) analyses. The former was conducted in GARLI v 2.0 (Zwickl, 2006;
234 Bazinet, Zwickl & Cummings, 2014) software. The best evolutionary model, identified
235 under the Akaike's information criterion (AIC), as implemented in JModelTest v.2.1.10
236 (Darriba, Taboada, Doallo & Posada, 2012), was HKY+I+G (Hasegawa, Iida, Yano,
237 Takaiwa & Iwabuchi, 1985). Statistical support for the phylogenetic tree nodes was
238 estimated as bootstrap probability (btp) value over 1,000 replicates.

239 Bayesian analyses were performed using four independent runs of four Markov Monte
240 Carlo coupled chains of 4×10^6 generations each to estimate the posterior probability
241 (pp) distribution, as implemented MrBayes v 3.1.2 (Ronquist et al., 2012) software.
242 Topologies were sampled every 100 generations, and the majority-rule consensus tree
243 was estimated after discarding the first 25% of generations. *Barbus meridionalis*
244 (AJ388417) was used as outgroup. As indicated by Doadrio, Carmona, & Machordom
245 (2002), pairwise uncorrected mean divergences between taxa (uncorrected p-distance)
246 derived from mtDNA D-loop were used as a surrogate for levels of species divergence.
247 Then, Minimum Spanning Networks (MSNs) were created from the multiple D-loop
248 alignment using a statistical parsimony criterion as implemented in TCS v 1.18
249 (Clement, Posada & Crandall, 2000) software.

250

251 2.5 Genetic diversity, demography and population structure

252

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

262 In order to compare the connectivity between populations (within and between
263 hydrographic districts), genetic differentiation was tested among populations using the
264 fixation index Φ_{ST} (Weir & Cockerham, 1984) and assessing their significance ($P <$
265 0.05) by permuting haplotypes between populations 3024 times, as implemented in

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

271

272 **3 Results**

273

274 *3.1 Phenotypic attribution*

275

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

286

287 *3.2 Analysis of morphological data*

288

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

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

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

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

317

318 3.3 Mitochondrial phylogeny and Minimum Spanning Networks (MSNs)

319

320 A total of 611 D-loop sequences of 867 base pair (bp) length were aligned. There were
321 86 variable nucleotide positions detected, of which 61 were parsimony informative sites
322 and with 75 haplotypes scored. ML and BI phylogenetic analysis resulted in a congruent
323 phylogenetic tree structure in which *B. plebejus*, *B. tyberinus* and *B. barbus* haplotypes
324 (i.e. 32, 18 and 20, respectively) clustered independently (Figure 3A). Then, five
325 haplotypes, all recorded exclusively in the Vomano River (site 34, Figure 1) and
326 characterised by *B. tyb*-like phenotype, clustered within *B. tyberinus* lineage (named
327 *Barbus* in Vomano) (Figure 3A). The uncorrected p-distance between lineages ranged
328 from 2.2% to 4.6% (supporting information Table S3).

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

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

339

340 3.4 Genetic diversity and demography

341

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

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

354

355 3.5 Haplotype distribution and population structure

356

357 In the MSNs, the geographic distribution of all barbus taxa revealed a largely complex
358 pattern along both Adriatic and Tyrrhenian basins (see Figure 3B). In detail, the
359 haplotype distribution of the native fishes (Supporting Information Table S4) revealed *B.*
360 *plebejus* was widespread in all sampling sites in the Adriatic basins (PV district). With
361 Tyrrhenian basins, they shared nine haplotypes localised in three populations of upper-
362 Tiber (Pop1, Pop2 and Pop5), four populations in Paglia basin (Pop8, Pop10, Pop13 and
363 Pop14) and in Topino basin (Pop8) (Table 1). In the distribution of *B. tyberinus*
364 haplotype, six haplotypes were shared between both districts and localised in three
365 Adriatic basins: Metauro (Pop16, Pop19 and Pop29), Cesano (Pop25) and Esino (Pop26
366 and Pop27) rivers. Only two haplotypes (*Btyb03* and *Btyb10*) were exclusively present
367 in the Cesano (Pop25) and in Metauro (Pop19 and Pop21) river. *Barbus barbus* was

368 prevalently in Tyrrhenian basins (90 % of sites), equally distributed in upper-Tiber
 369 (Pop2, Pop3, Pop5, Pop6 and Pop7) and in Paglia basin (Pop10, Pop11, Pop13 and
 370 Pop14) with *Bbar01* haplotype as dominant (57%). Conversely, along the Adriatic
 371 basins, *B. barbatus* was present exclusively in Metauro basin (Pop17, Pop18, Pop19 and
 372 Pop20) (cf. Supporting Information Table S4).

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

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

389

390 **4 Discussion**

391

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

402 There are a series of hypotheses that can help potentially explain these results. The first
403 is associated with the natural dispersal of the fishes, with both species apparently
404 crossing the Apennine Mountains in an east-west direction during periods of temporal
405 permeability. Support for this relates to the high seismic activity and intensive tectonic
406 movements along the Apennines (Sorgi, Deffontaines, Hippolyte & Cadet, 1998; Pizzi
407 & Galadini, 2009; Garzanti, Vezzoli & Andò, 2011) that have historically shaped the
408 Adriatic and Tyrrhenian catchment areas. This has resulted in the formation of short
409 periods during which novel hydrological links were formed, enabling mixing of
410 populations across this orographic barrier (Bianco, 1994). Indeed, trans-Apennine river
411 connections have been suggested to have been used for historical dispersal by primary
412 fish, such as vairone *Telestes muticellus* Bonaparte 1837 (Zaccara, Stefani & Delmastro,
413 2007; Marchetto, Zaccara, Muenzel, & Salzburger, 2010) and bullhead (*Cottus gobio*
414 Linnaeus, 1758) (Lorenzoni et al., 2018). The second hypothesis is that the current
415 *Barbus* distribution has resulted primarily from anthropogenic influences, with the fish
416 being translocated between basins in recent decades, mainly for recreational angling
417 purposes. Although this has been mainly reported for the alien *B. barbus*, there is also
418 some evidence of some alteration of the original distribution pattern of the endemic *B.*
419 *plebejus* (Meraner et al., 2013) and *B. tyberinus* (in Brenta River – Adriatic basin;
420 Buonerba et al., 2015). It is, therefore, likely that the current *Barbus* distribution in the
421 two districts was achieved through both of these mechanisms, with support available for
422 both hypotheses.

423 A final hypothesis relates to dispersal of *B. tyberinus* between the two regions entirely
424 through natural means and on a more permanent basis than suggested by Hypothesis 1.
425 (i.e. the natural dispersion hypothesis). This is proposed for the Cesano and Esino
426 basins (Pop 25 and Pop27), and requires *B. tyberinus* from a TL basin (e.g. the Topino
427 basin) dispersing into both Adriatic rivers at the limit of the connection between the two
428 districts (Bartolini & Pranzini, 1988; Bianco, 1991). The occurrence of this local trans-
429 Apennine river connection is at least partially supported by both the absence of alien
430 species in these rivers and by genetic differentiation with other TL populations. This
431 hypothesis does not, however, get support from the *B. plebejus* populations. In TL,
432 these fish revealed a genetic signature suggesting anthropogenic dispersal, including
433 their co-occurrence with *B. barbus*, with native *Barbus* translocations occurring in the
434 last century and *B. barbus* since 1950 (Bianco & Ketmaier, 2001; Zerunian, 2002).
435 Moreover, the translocation of *Barbus* populations between Italian geographical areas

436 has favoured the invasion of *B. barbuis*, especially in the Po River basin where its range
437 now completely overlaps with *B. plebejus*, where the introgression process between
438 both species has been widespread in the lowland part of the Po basin (Meraner et al.,
439 2013; Zaccara et al., 2014) and in some upper Adriatic basins (Livi et al., 2012). There
440 is now also a high risk of this occurring in the Tiber basin, where high *B. barbuis*
441 presence suggests it is becoming widespread throughout central Tyrrhenian rivers,
442 where introgression processes may also occur (Bianco & Ketmaier, 2001; Lorenzoni et
443 al., 2006; Geiger et al., 2016; Carosi et al., 2017). In contrast, their distribution remains
444 more constrained in the mid-Adriatic basins due to minimal river connectivity, resulting
445 in their dispersal being restricted to some tributaries of the Metauro basin where the
446 genetic admixture between *B. barbuis* and *B. plebejus* might be just on-going.

447 Although the presence of putative hybrid forms between native and *B. barbuis* cannot be
448 excluded, the results did reveal that *B. barbuis* could be discriminated from native
449 *Barbus* fishes by the identification of specific morphological characteristics, including
450 the number of scales along the lateral line, eye diameter and position, and body depth.
451 This is consistent with previous studies on these fishes that suggest *B. barbuis* retains its
452 peculiar traits and morphological features in its invasive range (e.g. Lorenzoni et al.
453 2006; Livi et al., 2013). Conversely, these morphological characteristics had high
454 overlap between the native *Barbus*, thus did not provide clear discrimination between
455 them, probably due to both their evolutionary relationships and their sharing of
456 geographical origin (Italian peninsula) where the ecological uniformity of the rivers
457 results in the selection of similar traits (Livi et al., 2013; Buonerba et al., 2015).

458 Along the mid-Adriatic basins from Cesano River to the Vomano River, the *Barbus*
459 fishes revealed some novel phenotypic and morphological characters. Meristic and
460 morphological characters, plus geometric morphometry, identified some local
461 distinctiveness, as previously anticipated by Livi et al. (2013). The *B. plebejus*
462 populations inhabiting the most southern part of the PV district (from Cesano River to
463 Vomano River) had characteristics relatively similar to *B. tyberinus* (hence, ‘*B. tyb-*
464 *like*’), but also with some distinct morphological characters (such as smaller eye, shorter
465 POD and MOD, number of minor scales). In the Vomano River, these fish were highly
466 genetically differentiated from *B. plebejus* populations (p-distance > 2.3%) and were
467 termed here as *Barbus* in Vomano. This morphological and phenotypic variability, and
468 population genetic differentiation, was highly localized, being restricted to the Southern
469 Padany-District (SPV). This supports the hypothesis that this distribution was achieved

470 through temporary and historical periods of river connectivity between basins
471 (Barraclough & Nee, 2001; Brito, 2005; BurrIDGE, Craw, Jack, King & Waters, 2008).
472 As also suggested by other studies, this primary freshwater fish dispersal pattern would
473 have been achieved by periodic extension of the Po basin (Paleo-Po) up to the last
474 natural event of glacial regression during the Pleistocene (e.g. Bianco 1990; Hewitt,
475 1999; Bianco & Ketmaier, 2001; Stefani, Galli, Zaccara & Crosa, 2004; Zaccara et al.,
476 2007; Ketmaier, Finamore, Largiadèr, Milone & Bianco, 2009; Garzanti et al., 2011).
477 The subsequent geographical isolation of these mid-Adriatic rivers has since enabled the
478 *Barbus* to evolve to the local conditions and in a separate manner to other basins
479 (Markova et al., 2010; Livi et al., 2013; Buonerba et al., 2015; Antal et al., 2016). This
480 scenario is similar to *T. muticellus*, a cyprinid fish with similar fluvio-lacustrine
481 ecological traits (Marchetto et al., 2010). However, this result contrasts to patterns
482 recorded in the *Barbus* spp. of the northern PV district. There, the *B. plebejus*
483 populations have been homogenised by the Pleistocene glacial cycles that increased
484 their population connectivity across the northern Adriatic basins (Meraner et al., 2013).
485 The study has thus revealed that the distribution, morphology and genetics of the
486 *Barbus* fishes of central Italian rivers have been shaped by a combination of
487 biogeographic factors and, more recently, by anthropogenic translocations, particularly
488 in the Tiber and Matauro River basins. The translocations of both native and alien
489 *Barbus* spp. have now resulted in a complex barbel community where the possibility of
490 introgression is elevated, undermining the integrity of the natural conditions of local
491 populations. Despite this complex pattern, morphological and genetic analyses were
492 sufficient here to discriminate between the native and alien *Barbus* in the samples, so
493 providing a more detailed map of *B. barbus* invasion. Moreover, these analyses
494 identified high genetic and morphological variability that was focused in the mid-
495 Adriatic region, indicating an area of high local endemism where the alien *B. barbus* has
496 yet to reach. Consequently, conservation strategies and actions are required in mid-
497 Adriatic region to conserve this local endemism. This could be done via managing the
498 populations as one genetic unit. Management actions to then promote the conservation
499 of the genetic uniqueness would be to cease all fish translocations between Italian river
500 basins. Steps should also be taken to reduce the risk of further natural dispersal of alien
501 *B. barbus*, although it is acknowledged that this remains challenging. However, in doing
502 so, it will help protect the endemic *Barbus* of the mid-Adriatic rivers, conserving their
503 genetic and morphological uniqueness.

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

512

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517

518 **References**

519

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779 **TABLE 1** Description of 611 *Barbus* populations (N = sampling size) collected along 34 sampling sites, including ID, district (PV =
 780 Padany-Venetian, TL = Tuscany-Latium), river basin, watercourse, altitude and distance from source. Field (Lorenzoni et al., 2006) and
 781 genetic (D-loop mtDNA lineages) attribution are detailed.
 782

ID	District	Sampling site	Watercourse	Basin	Altitude (a.m.s.l.)	Distance from source (km)	N	Filed attribution			mtDNA lineages		
								<i>B.</i> <i>plebejus</i>	<i>B.</i> <i>tyberinus</i>	<i>B.</i> <i>barbus</i>	<i>B.</i> <i>plebejus</i>	<i>B.</i> <i>tyberinus</i>	<i>B.</i> <i>barbus</i>
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 alfinia	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 Baglioli	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

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

784 **Figure legends**

785

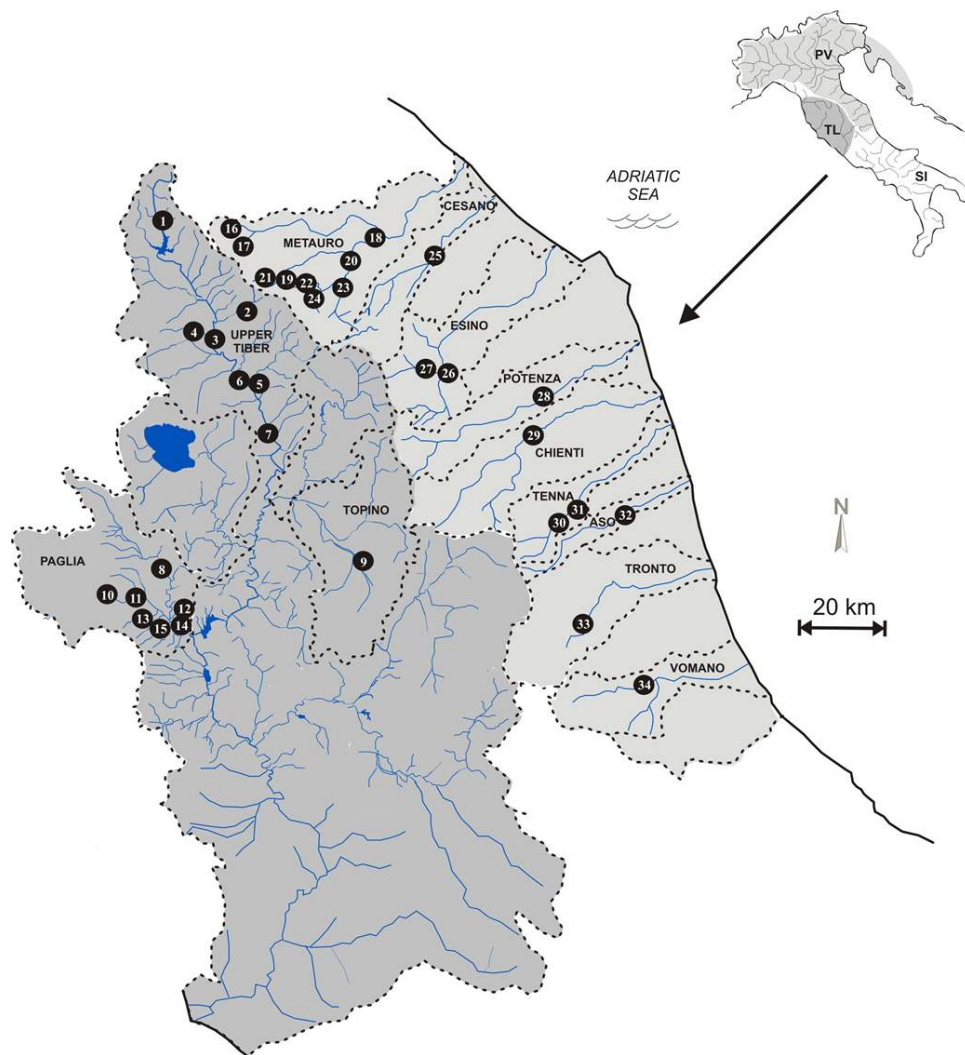
786 **FIGURE 1** Map of the 34 sampling sites (ID code is detailed in Table 1). Dashed lines
787 represent river basins. The ichthyo-geographic districts are indicated according to
788 Bianco, (1995a): light grey for Padany-Venetian district = PV; dark grey for Tuscany-
789 Latium district = TL.

790

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

797

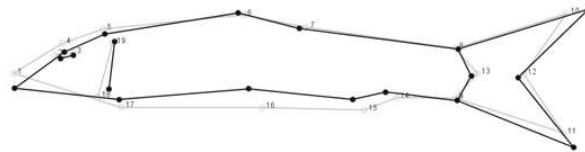
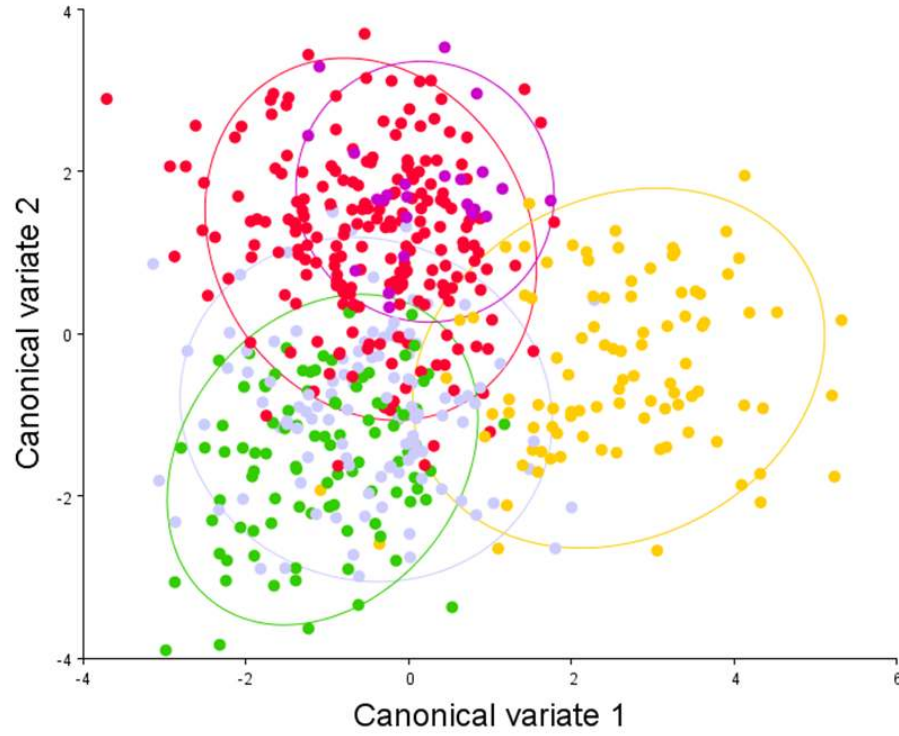
798 **FIGURE 3 A.** Maximum likelihood phylogeny of D-loop mitochondrial haplotypes
799 rooted on *Barbus meridionalis*. Bootstrap and Bayes posterior probability are given next
800 to relevant nodes; B. D-loop haplotype (TCS) networks. Circle size is proportional to
801 the observed haplotype frequencies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



806

807 Figure 1.

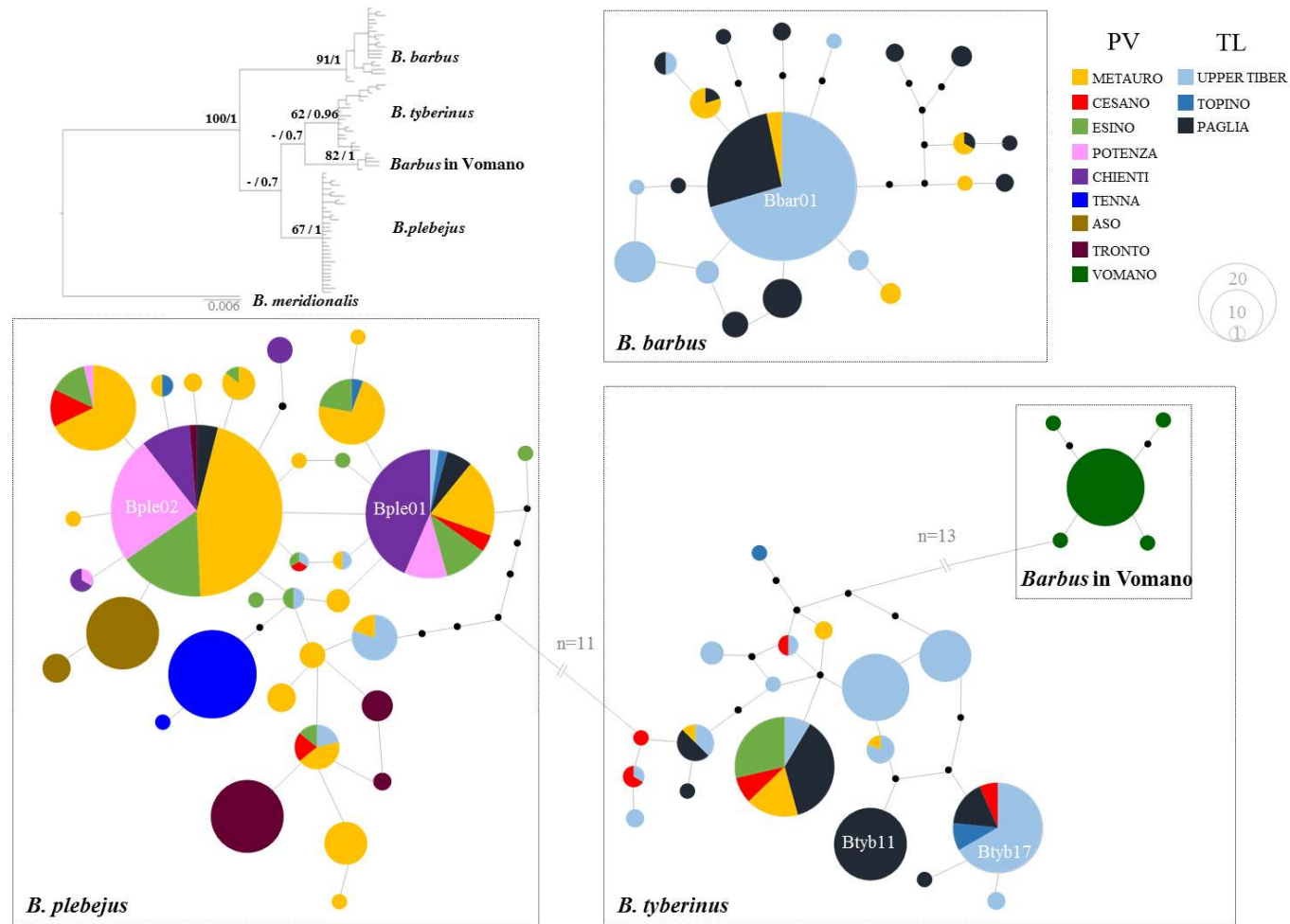
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809

810 Figure 2

811



812
813 Figure 3.