# Genetic and phenotypic displacement of an endemic *Barbus* complex by invasive European barbel *Barbus barbus* in central Italy

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**Abstract**

Invasions of alien fishes can result in considerable consequences for native biodiversity, including local extinctions of native species through genetic introgression. In Italy, the alien European barbel *Barbus barbus* was first detected in 1994. It has since undergone range expansion, raising conservation concerns on their impacts on endemic *Barbus* species, including *Barbus plebejus* and *Barbus tyberinus*. Here, the genetic and phenotypic consequences of *B. barbus* invasion in the Tyrrhenian and Adriatic basins of central Italy were assessed by comparing 'invaded' with 'uninvaded' river sections that remain free of *B. barbus* due to barriers preventing their upstream dispersal. In both basins, uninvaded sites were confirmed as *B. barbus* free, but the endemic populations had low genetic variability. In the invaded sections, haplotype and nucleotide diversity was relatively high, with introgression skewed towards *B. barbus* genes, with the Barbus populations comprising of only 4 % and 23 % of pure autochthonous *B. tyberinus* and *B. plebejus* respectively. Relatively high morphological differentiation was apparent between pure *B. tyberinus* and hybrid forms, whilst differences were less apparent between pure *B. plebejus* and their hybrid forms. Thus, the endemic *Barbus* only persist in areas that remain free of invasive *B. barbus*, with this only due to river structures that impede their upstream movements. As these structures also limit the effective population size of the endemic *Barbus*, conservation plans must reconcile *B. barbus* dispersal prevention measures with the need to increase the population connectivity of the endemics.

## Introduction

The invasion of freshwater ecosystems by alien fishes can result in considerable consequences for native biodiversity, including local extinctions of endemic and native species (Gozlan et al. 2010; Jackson et al. 2017; Mollot et al. 2017). These consequences can result from the trophic interactions of the invader with native species that lead to increased predation and competition pressure (David et al. 2017; Jackson et al. 2017), the foraging behaviours of the invader that modify the habitat characteristics through ecological engineering (Mollot et al. 2017), and the transmission of novel pathogens (Sheath et al. 2015). In addition, genetic introgression between the invader and native species can result in the loss of genetic integrity of populations of ecologically important native species (Hanfling et al. 2005; Hayden et al. 2010; Meraner et al. 2013; Geiger et al. 2016). Consequently, invasive alien fish represent a considerable global challenge, requiring effective management and regulation (Pimentel et al. 2000; Dlugosch and Parker 2008; Estoup and Guillemaud 2010).

The management and regulation of invasive species can be strongly informed by their invasion genetics (Hänfling 2007). Information on the introduction history of the invader, its biogeographic source, population connectivity, and mixing of the species in both the native and invasive range can inform knowledge on its genetic diversity in the invasive range, how this diversity varies spatially, and help identify the introduction pathways (e.g. Lawson Handley et al. 2011; Bock et al. 2015; Hardouin et al. 2018). A further genetic consideration is where the invasion process is being facilitated by hybridization, where the invader is undergoing introgression with populations of taxonomically similar native species. This can result in the rapid evolution of invasiveness, with a consequent loss of native genetic diversity and locally adapted genotypes (Rhymer and Simberloff 1996; Brennan et al. 2014; Bock et al. 2015; Morais and Reichard 2018). This is particularly common in fish, especially in species of the Cyprinidae family (Scribner et al. 2001), where the widespread

71 incidence of interspecific hybridization among closely related species has been widely observed
72 (Scribner et al. 2001). This potentially leads to new invasive hybrid lineages that may out-compete
73 native parental genotypes through the production of more vigorous hybrids (Hanfling 2007). It can
74 also result in higher adaptive capacity to altered environmental conditions that are driven by
75 anthropogenic exploitation of the freshwater resources (e.g. habitat fragmentation due to dam and
76 weir construction, increased environmental pollution) (e.g. Oziolor et al. 2019).
77
78 These issues of invasion hybridisation and genetic introgression are increasingly apparent in Italian
79 river basins where, during the last century, environmental degradation has increased dramatically at
80 a time when there has also been multiple and recurrent introductions of freshwater fishes, especially
81 of cyprinid fish species (Gherardi et al. 2008; Castaldelli et al. 2013; Bianco, 2014; Carosi et al.
82 2017a; Lanzoni et al. 2018). Introductions of cyprinid fishes have resulted in ecological impacts
83 including trophic niche overlap, habitat shifts, and extirpations of native populations (Vilizzi 2012).
84 There have also been frequent events of genetic introgression between native and exotic species
85 (Kottelat and Freyhof 2007). This is especially the case between co-generic *Barbus* species, with the
86 recent introduction of the exotic European barbel *Barbus barbus* (Linnaeus, 1758) resulting in
87 introgression with endemic *Barbus* species (Meraner et al. 2013; Zaccara et al. 2014). The European
88 barbel, a fluvio-lacustrine cyprinid naturally distributed in central Europe (e.g. Danube basin), has
89 habitat preferences of medium-large flowing rivers that are characterized by laminar flows and
90 relatively warm temperatures (Kottelat and Freyhof 2007). These habitat preferences are shared with
91 endemic Italian barbels (common barbel *Barbus plebejus* Bonaparte, 1839 and Tiber barbel *Barbus*
92 *tyberinus* Bonaparte, 1839). The natural distributions of these Italian endemic barbel vary; *B. plebejus*
93 inhabits the Adriatic basins of Padano-Venetian district (PV), while *B. tyberinus* is present in
94 Tyrrhenian basins within the Tuscany-Latium district (TL) (*sensu* Bianco 1995). *Barbus barbus* was
95 first reported in Italian waters in 1994 in the Po River, with the species surmounting the Alps through
96 'mixed cyprinid stocking' events (Meraner et al. 2013). Its subsequent range expansion and invasion

of several Italian river basins has been assisted by unregulated releases by recreational anglers (Zerunian 2002). In the Po River, impacts of hybridization between *B. barbus* and endemic *Barbus* species has been well documented (Meraner et al. 20013; Zaccara et al. 2014; Piccoli et al. 2017). Since 1998, *B. barbus* has been present in the Tyrrhenian and Adriatic basins of central Italian peninsula (Mearelli et al. 2000), where its hybridization with native *B. plebejus* and *B. tyberinus* is considered likely (Buonerba et al. 2015; Carosi et al. 2017b).

The aim of this study is, therefore, to use the river basins of central Italy that are populated by *B. plebejus* and *B. tyberinus* to assess their genetic and phenotypic responses to the invasion of *B. barbus*. Through molecular and morphological assessment of barbels in these basins, important knowledge on the impact of invasive *B. barbus* will be generated that can then be used by policy-makers and practitioners to limit its further diffusion, including of its hybrid forms.

## Materials and methods

### *Sampling locations and methods*

Pure populations of *B. tyberinus* and *B. plebejus*, and populations in basins where *B. barbus* is present, were sampled in the Tyrrhenian (Tiber River) and Adriatic (Metauro River) basins respectively (Fig. 1, Table 1). In these rivers, both uninvaded and invaded areas have recently been recorded (Zaccara et al. 2019b). In both basins, one invaded and one uninvaded site was selected. In the Tiber basin, the invaded *B. tyberinus* site was in the Paglia River (here after referred as TL*i*), where *B. barbus* has been recorded since 1998 (Carosi et al. 2017b). The non-invaded site in the Tiber river was in the Montacchione Stream (here after referred as TL*p*), a tributary of the Paglia River that is isolated from the main channel by the presence of two weirs with a head of approximately 2 m that prevents the upstream movement of *B. barbus* (Carosi et al. 2017b; Zaccara et al. 2019b). In the Metauro River basin, invaded *B. plebejus* were collected from the Candigliano River, where *B. barbus* has been

123 present since 2005 (Lorenzoni et al. 2006). The non-invaded site was the upper section of the Metauro
124 River basin (i.e. Bosso Stream, here named PV*p*), that was isolated from *B. barbus* invasion by three
125 weirs with heads of between 0.4 and 1 m (Zaccara et al. 2019b). In general, these tributaries are
126 characterised by highly variable flow regimes, especially in summer where flows can be very low
127 due to a combination of drought and abstraction (for irrigation and hydropower production).
128
129 The *Barbus* populations were sampled at each site using electric fishing during July 2019. Following
130 their capture, fish were held in aerated tanks of water. Then, under general anaesthesia (MS-222),
131 fish were photographed (left side; Nikon D300 camera (24–85 mm lens) positioned by a tripod on a
132 table with a millimetric scale), measured (total length, nearest mm), weighed, and a biopsy of the
133 caudal fin taken from a sub-sample of each population (approximately 20 specimens per site). The
134 fin clips were preserved in 90% ethanol and stored at 4°C prior to DNA extraction. Following their
135 recovery to normal behaviour, the fish were released to their approximate location of capture.
136
137 *Morphological analyses*
138 A total of 167 fish were used for morphological analyses. From their images, eight morphometric and
139 four meristic traits were analysed (*sensu* Zaccara et al. 2019a; Supplementary material: Fig. S1A),
140 with their phenotypic characters (spot/dot/pigmentation presence on the body, and all fins and fin
141 colour) also recorded. Twenty-eight landmarks (LMs) were used for geometric morphometric
142 analyses of body shape within the R Geomorph function "digitize2d" (Adams et al. 2018; Fig. S1B).
143 In the images, the positioning of caudal fin was important in ensuring their associated LMs could be
144 used in these analyses (17-28; see supplementary material Fig. S1B). Generalized Procrustes analysis,
145 as implemented in MorphoJ software (Klingenberg 2011), removed any non-shape variation that had
146 resulted from variation in fish position, orientation, and size. In the same software, shape variations
147 between the four populations were analysed by canonical variate analyses (CVA), with Mahalanobis
148 distances calculated using permutation tests (10,000 replicates). Morphometric traits were

149 standardized to the overall mean standard length to reduce the effects of size and allometry (Beacham
150 1985). Pairwise comparison on morphological traits between the four populations was performed
151 using analysis of variance (ANOVA) and Tukey post hoc tests, as implemented in PAST software
152 (Hammer et al. 2001).
153
154 *Molecular analysis and DNA polymorphism*
155 Total genomic DNA was extracted from 102 individuals using a proteinase K digestion, salting-out
156 method (Aljanabi and Martinez 1997). Mitochondrial control region (D-loop) sequences were
157 amplified by polymerase chain reaction (PCR) using D-loopsxF and D-loopdxR (Antognazza et al.
158 2016) primer pairs, with an 869bp length fragment analysed. As *Barbus* species are tetraploid, we
159 sequenced the nuclear DNA (nDNA) growth hormone paralog-2 (GH-2) using specific primers
160 developed for other European species of *Barbus* and *Luciobarbus* (F-
161 GTACTATAGTAAGCAGAAATGG and R- AGTGGSAGGGAGTCGTTC; Gante et al. 2011). The
162 GH-2 *locus* was selected as it is polymorphic and suitable for phylogenetic and population genetic
163 analyses (Moyer et al. 2009; Gante et al. 2011; Buonerba et al. 2015).
164
165 Both PCR reactions were performed using Multiplex PCR kits (Qiagen) in 10 µl reaction volumes
166 that contained approximately 10 ng of template DNA and 0.25 µM of each primer pair. Thermal
167 cycling was performed as follows: denaturation of 15 minutes at 95 °C, followed by 30 cycles (D-
168 loop) and 35 cycles (GH-2) of 30 s at 94 °C, 90 s at 55 °C and the extension step at 72 °C for 90 s,
169 with the final elongation at 72 °C for 10 min. PCR products were purified using ExoSAP-IT™ (USB)
170 and directly sequenced by MACROGEN Inc (http://www.macro gen.org) using a 3730XL DNA
171 Sequencer. The nucleotide sequences of mitochondrial D-loop haplotypes and nuclear GH-2 alleles
172 were deposited in the GenBank database (Accession numbers: MT385872-MT385896 for the D-loop
173 and MT385897-MT385938 for the GH-2).
174

175 Alignment of all sequences was carried out automatically by Clustal W (Thompson et al. 1994), as
176 implemented in Bioedit software (Hall 1999), and further checked manually to eliminate remaining
177 ambiguities. For the nuclear *locus*, the individual fish that were exclusively characterised by single
178 nuclear polymorphisms (SNPs) (i.e. homozygotes for one barbel species) were solved by phasing the
179 sequences using DNAsp (Librado and Rozas 2009), while specimens with alleles of different lengths
180 due to insertions or deletions (indels) (i.e. interspecific heterozygotes) were manually phased by
181 analysing the forward and reverse sequences, as detailed in Flot et al. (2006). Genetic variability was
182 estimated for each species by calculating the number of haplotypes (h), the number of polymorphic
183 sites (S), the haplotype diversity (H), and the mean number of nucleotide differences ($\pi$) for both D-
184 loop mtDNA and the GH-2 nDNA locus, using DNAsp software (Librado and Rozas 2009).
185
186 *Phylogenetic analyses*
187 Maximum likelihood (ML) and Bayesian inference (BI) methods were used for all phylogenetic
188 analyses inferred on both the D-loop and GH-2 datasets. The best-fit nucleotide substitution model
189 was selected by the corrected Akaike Information Criterion (AICc) in jModeltest 2.1.7 (Darriba et al.
190 2012). For the D-loop dataset, the model used was HKY+I+G, while HKY+I was employed for the
191 GH-2 dataset. ML analyses were performed using GARLI software (Zwickl 2006; Bazinet et al. 2014)
192 with 1000 bootstrap replicates (i.e. btp). The BI was applied using MrBayes v.3.2.6 (Ronquist et al.
193 2012), with four independent runs ($10^6$ generations with a sampling frequency of one tree for every
194 100 generations), each with four chains (three hot and one cold). All runs reached convergence
195 (average standard deviation of split frequencies below 0.01). The posterior distribution of trees was
196 summarized in a 50% majority rule consensus tree (burn-in of 25%), with statistical support expressed
197 as posterior probability (i.e. pp).
198
199 To definitively establish the phylogenetic taxonomic attribution of the *Barbus* samples (i.e.
200 differentiating the native and non-native individuals) (Tsigenopoulos et al. 2002), diagnostic

sequences of native *B. plebejus* and *B. tyberinus* (Buonerba et al. 2015; Zaccara et al. 2019b), and of the alien *B. barbus* (detected from pure allopatric populations from English basins (Antognazza et al. 2016) and Italian basins (Zaccara et al. 2019b)) were retrieved from GenBank. These data were included in the analyses of both the mitochondrial and nuclear datasets (see supplementary material Table S1 and Table S2 for D-loop and GH-2 sequences used respectively). This step also enabled possible introgression between the endemic and invasive *Barbus* to be traced. Two rheophilic *Barbus* species were selected as outgroups: *Barbus meridionalis* Risso, 1827 (AJ388417) for D-loop and *Barbus caninus* Bonaparte, 1839 (KF963432) for GH-2. A minimum spanning network was also created from both D-loop and GH-2 multiple alignment using a statistical parsimony criterion, as implemented in PopART v 1.7 software (Leigh and Bryant 2015).

*Population genetic structure*

For each sampling site, allelic polymorphisms, expressed as nucleotide diversity index ($\pi$), were calculated for each species using DNAsp software. To compare the connectivity between populations within the Tyrrhenian and Adriatic basins (*B. tyberinus* and *B. plebejus* respectively), and between invaded Tyrrhenian and Adriatic sampling sites (*B. barbus*), the genetic differentiation was tested using the fixation index $\Phi_{ST}$ (Weir & Cockerham 1984). Its significance ($p<0.05$) was assessed by permuting haplotypes between populations 3,024 times, as implemented in Arlequin v 3.5 (Excoffier and Lischer 2010).

## Results

*Morphological analyses*

The canonical variate analyses (CVA) plot revealed the four populations clearly separated along the CV1 axis, with TL*i* individuals distinct from individuals in the other three groups (Fig. 2). This axis explained shape variations associated with the head, caudal fin and body depth. In TL*i*, the specimens

(identified genetically as hybrids *B. tyberinus* x *B. barbus*) had deeper bodies and longer snouts with a different mouth orientation (i.e. ventral) and longer tail lobes. Specimens from the pure *B. plebejus* and *B. tyberinus* populations (PV*p* and TL*p*, respectively) were separated along the CV2 axis, where shape variations were in head, caudal fin and body depth: TL*p* fish displayed more fusiform and slender bodies, smaller heads and caudal lobes both smaller and more rounded compared to PV*p* fish. Even here, the main source of variation referred to the fish head and caudal fin that was both shorter and more rounded in TL*p* than in PV*p* individuals. The group of fishes from PV*i* partially overlapped with the PV*p* group. The maximum Mahalanobis distance (9.4) was between the TL*i* and the other three populations, while the minimum value (6.6) was recorded between PV*p* and PV*i* populations.

As morphometric traits, pre-orbital distance (POD) was significantly longer in PV*i* and TL*i* specimens than in fish from the other two sites (Tukey, $p<0.05$; Table 2). The length of ventral fin (LVF) and the height of the first dorsal fin ossified ray (HDOR1) differed significantly between all the four populations (Tukey, $p<0.05$), with increasing values from TL*p*, PV*p*, and PV*i*, up to TL*i* fish. The length of the pectoral fin (LPF) was significantly different in the TL*p* fish to the other sites (Tukey, $p<0.05$), except those from TL*i*. The number of scales on the lateral line (NSLL) and above the lateral line was significantly lower in TL*p* and TL*i* specimens (Tukey, $p<0.05$), while NSLL was significantly higher in the PV*p* specimens (Tukey, $p<0.05$) (Table 2).

All of the fish from PV*i* and TL*i* had scales with pigmentation on the edge and most also had dots (Table 3). In contrast, some fish from TL*p* had spots on the body and with the ventral and anal fins being different colours (Table 3); along with almost half of the TL*i* specimens, they also had a grey dorsal fin. Moreover, the caudal fin was mostly grey/orange in these TL*p* individuals, while it was orange in individuals from PV*p* (Table 3).

*Phylogenetic attribution*

253 The complete D-loop alignment, obtained from 102 barbels, consisted of a total length of 869 bp that
254 identified 25 haplotypes. The multiple alignment of 188 GH-2 sequences, obtained from 94 barbels
255 (GH-2 sequencing failed for 8 fish), identified 42 haplotypes. Sequence analyses of the GH-2 nuclear
256 *locus* yielded a 1030 bp-long alignment, where several indels of different length (1 bp up to 95 bp)
257 were assumed to maximize base identity in flanking conserved sequence blocks (see Table 4). The
258 maximum likelihood and Bayesian phylogenetic analyses performed on the D-loop and GH-2 datasets
259 (including 'reference sequences' from GenBank of the native and non-native species; Tables S1, S2),
260 provided congruent tree topology. This revealed three evolutionary lineages that were attributed to *B.*
261 *plebejus*, *B. tyberinus* and *B. barbus* (Fig. 3a, b) and allowed the assignment of our novel sequences
262 to native and non-native barbels. Specifically, the *B. plebejus*, *B. tyberinus* and *B. barbus* clades were
263 largely supported by both the mtDNA and nDNA data (pp > 0.9) (Fig. 3a,b). Among the 25
264 mitochondrial D-loop haplotypes, 7 and 3 haplotypes clustered as *B. plebejus* and *B. tyberinus*
265 respectively, and 15 as *B. barbus*; among the 42 GH-2 haplotypes, 17 were *B. plebejus*, 8 were *B.*
266 *tyberinus* and 17 were *B. barbus* .
267
268 *Genetic variability and Minimum spanning network*
269 The mitochondrial and allelic diversity varied considerably among the species; *B. barbus* had the
270 highest levels of nuclear and mitochondrial polymorphism (H = 0.77 and $\pi$ = 0.50%; H = 0.86 and $\pi$
271 = 0.31 respectively), whereas the lowest levels were recorded in *B. tyberinus* (H = 0.57 and $\pi$ =
272 0.08%; H = 0.12 and $\pi$ = 0.05 respectively) (Table 4). In the network analyses of *B. barbus* D-loop
273 and GH-2 haplotypes (n = 15 and 17 respectively), the most frequent haplotypes (Bbar01 and HBB01,
274 respectively) were shared in both the Adriatic (PV*i*) and Tyrrhenian (TL*i*) invaded sampling sites
275 (Fig. 4). This pattern was also reflected in two more D-loop haplotypes (Bbar09 and Bbar23) (Fig.
276 4). There were 4 and 5 private haplotypes detected at PV*i* in the GH-2 and D-loop dataset respectively
277 (Fig. 4A), whilst 12 and 7 private haplotypes were detected in these at TL*i*, all separated by up to 15
278 mutational steps (Fig. 4B).

279

280 *Status of B. barbus invasion within Tyrrhenian and Adriatic basins*

281 The nuclear and mitochondrial genetic composition of each population are in Figure 1, with the
282 haplotype distribution and frequencies provided in Supplementary material (Table S3 and Table S4
283 for D-loop and GH-2 respectively). Mitochondrial and nuclear sequences obtained from PV*p* and
284 TL*p* populations confirmed the absence of *B. barbus* haplotypes and the exclusive presence of *B.*
285 *plebejus* and *B. tyberinus* haplotypes respectively (Fig. 1, Table S3, Table S4). In contrast, in the PV*i*
286 and TL*i* populations, all of the D-loop sequences (i.e. 26 and 29 respectively) belonged to the *B.*
287 *barbus* clade, while the allelic frequency of GH-2 *B. barbus* sequences ranged between 46 and 79 %
288 respectively (Fig. 1, Table 5). The nuclear sequences thus revealed different admixture between
289 native and alien species, from hybrids (34 % *B. barbus* x *B. tyberinus* in TL*i*; 62 % *B. barbus* x *B.*
290 *plebejus* in PV*i*) to pure strains for *B. barbus* haplotypes (62 % and 15 % in TL*i* and PV*i*,
291 respectively). Only 4 % and 23 % showed both GH-2 alleles for *B. tyberinus* and *B. plebejus*
292 respectively (see Table 5).

293

294 Values of molecular indices (haplotype and nucleotide diversity) were lowest in both native *B.*
295 *plebejus* and *B. tyberinus* pure populations (i.e. PV*p* and TL*p* respectively), and were highest in mixed
296 populations (PV*i* and TL*i*) for both native and exotic alleles (Table 6). Genetic differentiation
297 between pure populations of the native species and introgressed populations were all significant: i) in
298 *B. plebejus* between PV*p* and PV*i* ($\Phi_{ST}$ = 0.22; p<0.001); and ii) in *B. tyberinus* between TL*p* and
299 TL*i* ($\Phi_{ST}$ = 0.24; p < 0.001). Major values of genetic differentiation were also recorded between *B.*
300 *barbus* in PV*i* and TL*i* ($\phi_{ST}$=0.51; p<0.001).

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303

304

## Discussion

The morphological and genetic results confirmed hybridization between the endemic and alien *Barbus* species in the main watercourses of both the Tyrrhenian and Adriatic basins of central Italy. However, in areas of these watercourses that were considered inaccessible to *B. barbus* due to structures in the river preventing their upstream movement, the results revealed the persistence of 'pure' *B. tyberinus* and *B. plebejus* populations, so confirming the uninvaded status of these areas.

A complex of cryptic species, the *Barbus* complex in Italy has high morphological similarity that prevents their straightforward taxonomic differentiation in the field (Geiger et al. 2016; Zaccara et al. 2019a). This similarity is likely to have resulted from an evolutionary lack of divergence that was driven by the ecological uniformity of Italian rivers (Livi et al. 2013; Buonerba et al. 2015; Geiger et al. 2016; Zaccara et al. 2019b). Introductions of the ecologically analogous and alien *B. barbus*, which has high potential for genetic introgression with congeners, generated confusion in taxonomic identification, especially when their hybrid morphological traits are rarely described (see Geiger et al. 2016). While any descriptions of hybrid versus pure species morphologies should be treated cautiously, as they were based on just on a mitochondrial marker and one nuclear genetic locus, there was strong separation between the native fluvio-lacustrine barbel phenotypes that enabled an initial and tentative morphological description of the hybrids to be made. These revealed that the *Barbus* species inhabiting the Tyrrhenian slope (i.e. *B. tyberinus* in TL*p*) were characterized by more fusiform and slender bodies with a smaller head, different mouth orientation (sub-ventral) and shorter and more rounded tail lobes. These morphological variations also distinguished the hybrid phenotypes from the endemic morphotypes (i.e. *B. tyberinus, B. plebejus*), with differences more marked for hybrids in the Tiber River system than those inhabiting the Adriatic slope. Fish in TL*i* showed the greatest morphological differentiation from that of the reference native species (i.e. *B. tyberinus* in TL*p*), while barbels from PV*i* showed little differentiation from the corresponding endemic

morphotype (i.e. *B. plebejus* in PV*p*). For the other morphological traits, the pre-orbital distance and the length of the first ossified dorsal ray and ventral fins were lower in *B. tyberinus* and *B. plebejus*, with the highest values measured in the hybrid morphotypes. Correspondingly, across this morphological gradient, the hybrids tended to have more extreme benthic specialized forms (e.g. having longer snouts and ventral mouths, deeper bodies and longer dorsal, ventral and caudal fins). Similarly, a cline was observed in the number of scales along the lateral line, a commonly used meristic trait for discriminating between *Barbus* species (Bianco 2003a,b; Lorenzoni et al. 2006; Kottelat and Freyhof 2007). The lowest scale number was in the Tiber pure population (i.e. 53-59) and the highest in the *B. plebejus* populations (i.e. 61-67), with hybrids showing intermediate values that match those for invasive *B. barbus* (from literature 53-62; Kottelat and Freyhof 2007). Finally, hybrids were characterized by the pigmentation of the scale edge, a trait typical of the alien *Barbus*, but that was absent in the Italian endemics.

The genetic pattern of both pure populations, characterised by low variability and dominated by just one haplotype, suggest recent periods of low effective population size, promoting local genetic drift (Grant and Bowen 1998). This is supported by general natural population reductions that have resulted from angler exploitation and, especially, from hydrological fluctuations in summer when scarce rainfall and excessive water abstraction cause widespread river droughts. Furthermore, the fish populations in the upstream areas have become increasingly isolated due to the construction of numerous barriers (mainly weirs) that impede their movements. This has limited their spawning migrations and restricted geneflow between downstream and upstream areas, reducing the dispersion of private haplotypes of native species that have remained confined to downstream populations, and generally reducing the genetic variability of upstream populations. Nevertheless, these barriers have also appeared beneficial by preventing the further upstream dispersal of *B. barbus*.

356 Conversely, the genetic signal of invasive *B. barbus* (high H and low π), which was similar in both
357 Adriatic and Tyrrhenian populations, was consistent with a recent invasion history (started in the
358 1990s) that started with several haplotypes. The invasion of both basins probably occurred as a result
359 of the general practice of 'multiple introductions' of fish for angling (i.e. multiple founder events)
360 (Meraner et al. 2013). Although these anthropogenic actions initially favored the fast spread of *B.*
361 *barbus*, its more recent range expansions have been through natural diffusion in the downstream areas
362 of these rivers.
363
364 Although evidence for introgression does not necessarily mean that there has been displacement of
365 one species by another one (or even that it shows the the ability to do so), we did detect that *B. barbus*
366 has invaded and largely displaced native congeners through introgression, and producing only small
367 - but distinct - morphological changes in the invaded populations (as described above). In contrast to
368 the Adriatic basin (i.e. Metauro River, PV*i*), *B. barbus* alleles in the Tyrrhenian basin (i.e. Paglia
369 river, TL*i*) strongly outnumbered the native alleles that were detected exclusively in a low number of
370 fishes. This nearly complete genotype and phenotypic displacement of the endemic Tiber barbel by
371 *B. barbus* may be due to several factors. The first is the hydrographic structure. The Tiber River basin,
372 for which Paglia (TL*i*) is one of the main tributaries, has a dendritic-shaped network extended on a
373 large surface area (17375 $km^2$). This configuration may have favored the natural diffusion of *B.*
374 *barbus* by allowing the fish to spread more easily to large parts of the basin using the hydrographic
375 connections. In contrast, the Metauro River basin (PV*i*) has a relatively limited hydrographic network
376 (1325 $km^2$) and, as with all Adriatic basins of central Italy, it flows independently to the sea, limiting
377 the ability of invasive *B. barbus* to disperse naturally between Adriatic rivers. A second factor may
378 relate to resident time of the alien *B. barbus* in the two basins. The higher number of introgressed fish
379 in PV*i* population is indicative of the more recent hybridization - after 2005 - where first generation
380 (F1) hybrids were dominant (Meraner et al. 2013), which tend to decrease in later hybrid generations
381 (Baack & Rieseberg 2007). Indeed, we detected the highest proportion of pure *B. barbus* in the Paglia

382 River, where the first record of *B. barbus* dated back to 1998. The final factor may relate to degraded
383 water quality and habitat alteration that impacted the sustainability of the natural *B. tyberinus*
384 populations in TL, providing the ecological niche space for the invasive *B. barbus* to utilize. It should
385 be noted that it is likely that it was the interaction of these factors that resulted in these outcomes,
386 rather than one factor acting in isolation.
387
388 In both the Tyrrhenian and Adriatic basins, introgression was skewed toward *B. barbus* mtDNA. This
389 situation has been described as a 'mother species' effect (*sensu* Wirtz 1999), which can be explained
390 by the unequal size between the invader and the native species, where the larger females (i.e. *B.*
391 *barbus*) are favoured in spawning rather than smaller ones (*B. plebejus* and *B. tyberinus*). Indeed, in
392 other hybrids of the *Barbus* genus, the prevalence of mtDNA was observed for the larger females (*B.*
393 *barbus* x *B. meridionalis* (Chenuil et al. 2004); *B. barbus* x *B. carpathicus* (Lajbner et al. 2009). This
394 might be a consequence of a sexual selection mechanism that allows only the larger females to be
395 fecundated or also by a higher relative fecundity of the larger species, given *B. barbus* females may
396 produce more eggs than the native species (Banarescu et al. 2003; Bianco 2003a,b; Meraner et al.
397 2013).
398
399 The pattern of hybridization that resulted from *B. barbus* invasion can lead to adaptation through the
400 establishment of novel genotypes and morphologies, in which the hybrids (especially in Tyrrhenian
401 basin) are showing phenotypic traits outside of the trait range of the endemic parental species, which
402 can be a consequence of an adaptative allele introgression (Whitney et al. 2006), or a transgressive
403 segregation that has resulted in new traits (Rieseberg et al. 1999). The observed morphological
404 changes may be a response to different river characteristics (i.e. level of degradation, flow regime)
405 (e.g. Corse et al. 2009; Samways et al. 2010; Corse et al. 2015) and might be indicative of different
406 trophic resource and habitat uses (Costedoat et al. 2007; Cunha et al. 2009). This potentially results
407 in introgressed *Barbus* populations having a greater adaptive capacity and higher resilience to the

anthropogenically altered rivers than the pure endemic fish, especially as the non-native genes are derived from an ecologically analogous congener. This could help ensure the *Barbus* genus can continue to persist in these modified rivers in future. Indeed, many recent studies allude to the adaptive role of hybridisation (Costedoat et al. 2007; Pfennig et al. 2007; Reyer 2008; Hayden et al. 2010) that can drive biodiversity responses to environmental variation (Scribner et al. 2001). Therefore, it is also possible that the introgression is leading to a species erosion process where the phenotype and genotype of the alien are prevalent when compared to the native ones due to the higher fitness of the invader driving a species substitution process (Ward et al. 2012).

In conclusion, our results emphasize the importance of combining morphological (both with traditional traits and using geometric morphometrics) and genetic (analyzing both mitochondrial and nuclear DNA) approaches in the analysis of cryptic species complexes of cyprinid taxa such as *Barbus* spp., especially when a co-generic invader is present. It was likely that the morphologies recorded in the two populations invaded by alien *B. barbus* (PV*i* and TL*i*) may reflect initial and final displacement stages of the endemic morphotypes and genotypes in the Adriatic and Tyrrhenian basins respectively. This suggests that reliance on using fish body shape to identify the initial invasion stages of *B. barbus* is insufficient, as phenotypic differences might not be evident until the later stages of the invasion. This has important implications for the effective management for this cryptic invasive species, as it suggests it requires the use of molecular tools for its detection in the early invasion stages. Future studies should always analyse the invasion mechanisms, as these shed light on the ecological and trophic factors which facilitate widespread hybridisation. Then, the improvement of detailed morphological and genetic studies should help in identifying the parental hybrid taxa and allow the mapping of the distribution of gene flow between the endemic species and invader. This knowledge could then provide the basis of an adaptive management tool to limit *B. barbus* invasion and contribute to the long-term conservation of endemic barbels.

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631 **Tables**

632 Table 1: Sampling sites of *B. tyberinus* (uninvaded TL*p* and invaded TL*i*) and *B. plebejus* (uninvaded
633 PV*p* and invaded PV*i*) populations, collected in Tyrrhenian (TL) and Adriatic (PV) basins
634 respectively (see Fig. 1). For each site, water course and geographic coordinates are reported. Sample
635 size for morphological and genetic (nDNA and mtDNA) analyses are also indicated.

636

| **Basin** | | **Water course** | **Pop ID** | **Geographic coordinates** | **Morphology** | **mtDNA** | **nDNA** |
|---|---|---|---|---|---|---|---|
| Adriatic | Metauro | Bosso | PV*p* | 43°31'3.14"N 12°33'17.89"E | 41 | 25 | 25 |
| | Metauro | Candigliano | PV*i* | 43°38'8.59"N 12°42'41.32"E | 40 | 26 | 26 |
| Tyrrhenian | Tevere | Paglia | TL*i* | 42°43'38.88"N 12° 7'43.00"E | 42 | 29 | 29 |
| | Tevere | Montacchione | TL*p* | 42°42'44.39"N 12° 5'37.88"E | 44 | 22 | 14 |
| Total | | | | | 167 | 102 | 94 |

637 **Table 2.** List of the measured morphometric and meristic traits, and the mean (±standard deviation)
638 values per site for the pure *B. plebejus* (PV*p*), pure *B. tyberinus* (TL*p*) and their hybrids (*B. barbus*
639 x *B. tyberinus* in TL*i* and *B. barbus* x *B. plebejus* in PVi). Sample size is reported.

| | | **PV*p*<br>N=41** | **PV*i*<br>N=40** | **TL*i*<br>N=42** | **TL*p*<br>N=44** |
|---|---|---|---|---|---|
| **Morphometric traits (cm)** | | | | | |
| Total length | TL | 17.3±4.0 | 14.9±5.9 | 15.9±3.6 | 16.7±5.2 |
| Eye diameter | ED | 0.7±0.1 | 0.6±0.2 | 0.6±0.1 | 0.6±0.1 |
| Pre-orbital distance | POD | 1.3±0.3 | 1.3±0.5 | 1.4±0.3 | 1.3±0.4 |
| Mouth-operculum distance | MOD | 3.5±0.8 | 3.1±1.2 | 3.2±0.8 | 3.3±1.0 |
| Length of pectoral fin | LPF | 2.7±0.7 | 2.2±0.9 | 2.5±0.6 | 2.7±0.8 |
| Length of ventral fin | LVF | 2.1±0.5 | 1.9±0.7 | 2.1±0.5 | 1.9±0.6 |
| Length of anal fin | LAF | 2.3±0.7 | 2.1±0.8 | 2.2±0.6 | 2.5±1.0 |
| Height of the first dorsal fin ossified ray | HDOR1 | 2.4±0.6 | 2.2±0.9 | 2.5±0.6 | 2.2±0.7 |
| Height of the third dorsal fin ossified ray | HDOR3 | 1.9±0.4 | 1.5±0.6 | 1.7±0.4 | 1.7±0.5 |
| **Meristic traits** | | | | | |
| Number of dorsal fin branched rays | NDBR | 8±0 | 8±0 | 8±0 | 8±0 |
| Number of scales on the lateral line | NSLL | 64±3 | 60±4 | 56±2 | 56±3 |
| Number of scales above the lateral line | NSALL | 13±1 | 13±1 | 12±1 | 11±1 |
| Number of scales under the lateral line | NSULL | 9±1 | 9±1 | 8±1 | 8±1 |

640
641

642 **Table 3.** List of phenotypic characters concerning spot/dot/pigmentation presence and fin colour for
643 the barbel populations of the four sites sampled, expressed as percentages (%).
644

| Phenotypic traits | | **PV*p*** | **PV*i*** | **TL*i*** | **TL*p*** |
|---|---|---|---|---|---|
| Dots on body | no | 100 | 100 | 100 | 100 |
| | yes | 0 | 0 | 0 | 0 |
| Spots on body | no | 98 | 92 | 90 | 66 |
| | yes | 2 | 8 | 10 | 34 |
| Scale edge pigmentation | no | 100 | 0 | 0 | 100 |
| | yes | 0 | 100 | 100 | 0 |
| Dots on scales | no | 73 | 0 | 17 | 98 |
| | yes | 27 | 100 | 83 | 2 |
| Dots on dorsal fin | no | 17 | 35 | 45 | 89 |
| | yes | 83 | 65 | 55 | 11 |
| Dots on anal fin | no | 100 | 100 | 95 | 100 |
| | yes | 0 | 0 | 5 | 0 |
| Dots on caudal fin | no | 51 | 40 | 64 | 70 |
| | yes | 49 | 60 | 36 | 30 |
| Ventral fin colour | orange | 100 | 100 | 100 | 27 |
| | grey | 0 | 0 | 0 | 52 |
| | orange/grey | 0 | 0 | 0 | 21 |
| Anal fin colour | orange | 100 | 100 | 100 | 27 |
| | grey | 0 | 0 | 0 | 41 |
| | orange/grey | 0 | 0 | 0 | 32 |
| Dorsal fin colour | orange | 0 | 5 | 5 | 0 |
| | grey | 0 | 5 | 43 | 86 |
| | orange/grey | 100 | 90 | 52 | 14 |
| Caudal fin colour | orange | 80 | 70 | 57 | 11 |
| | grey | 0 | 0 | 0 | 5 |
| | orange/grey | 20 | 30 | 43 | 84 |

645
646

647 **Table 4** Sequence polymorphism at mitochondrial and nuclear loci per species. N: number of
648 sequences, h: number of haplotypes excluding gaps, H: haplotype diversity, π: nucleotide diversity
649 (expressed in %), S: number of polymorphic sites, SD: standard deviation.

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651

652 **Table 5**. Introgression pattern of invaded populations (TLi and Pvi) detailing the mitochondrial (D-loop) and nuclear (GH-2 allelles) combinations

653 of each sample. Haplotypes, taxonomic attribution and GenBank accession number are provided.

| Population | sample ID | hap Dloop | Dloop taxa | GB code | hap GH2_a | GH2_a taxa | GB code | hap GH2_b | GH2_b taxa | GB code | nDNA alleles |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PVi | Mt1 | Bbar09 | *B. barbus* | MT385886 | HBP01 | *B. plebejus* | MT385915 | HBP01 | *B. plebejus* | MT385915 | Bp/Bp |
| PVi | Mt3 | Bbar22 | *B. barbus* | MT385892 | HBP03 | *B. plebejus* | MT385916 | HBB02 | *B. barbus* | MT385914 | Bp/Bb |
| PVi | Mt4 | Bbar01 | *B. barbus* | MT385882 | HBP15 | *B. plebejus* | MT385918 | HBB02 | *B. barbus* | MT385914 | Bp/Bb |
| PVi | Mt5 | Bbar10 | *B. barbus* | MT385887 | HBP03 | *B. plebejus* | MT385916 | HBB02 | *B. barbus* | MT385914 | Bp/Bb |
| PVi | Mt6 | Bbar09 | *B. barbus* | MT385886 | HBB07 | *B. barbus* | MT385915 | HBB07 | *B. barbus* | MT385915 | Bb/Bb |
| PVi | Mt8 | Bbar10 | *B. barbus* | MT385887 | HBP13 | *B. plebejus* | MT385926 | HBB02 | *B. barbus* | MT385914 | Bp/Bb |
| PVi | Mt9 | Bbar10 | *B. barbus* | MT385887 | HBP17 | *B. plebejus* | MT385930 | HBB10 | *B. barbus* | MT385915 | Bp/Bb |
| PVi | Mt10 | Bbar01 | *B. barbus* | MT385882 | HBB01 | *B. barbus* | MT385897 | HBB01 | *B. barbus* | MT385897 | Bb/Bb |
| PVi | Mt11 | Bbar03 | *B. barbus* | MT385883 | HBP05 | *B. plebejus* | MT385918 | HBB10 | *B. barbus* | MT385915 | Bp/Bb |
| PVi | Mt12 | Bbar23 | *B. barbus* | MT385893 | HBP01 | *B. plebejus* | MT385915 | HBP01 | *B. plebejus* | MT385915 | Bp/Bp |
| PVi | Mt16 | Bbar23 | *B. barbus* | MT385893 | HBP06 | *B. plebejus* | MT385919 | HBB01 | *B. barbus* | MT385897 | Bp/Bb |
| PVi | Mt21 | Bbar09 | *B. barbus* | MT385886 | HBB02 | *B. barbus* | MT385914 | HBB14 | *B. barbus* | MT385915 | Bb/Bb |
| PVi | Mt24 | Bbar09 | *B. barbus* | MT385886 | HBP03 | *B. plebejus* | MT385916 | HBB02 | *B. barbus* | MT385914 | Bp/Bb |
| PVi | Mt25 | Bbar15 | *B. barbus* | MT385890 | HBP02 | *B. plebejus* | MT385915 | HBB01 | *B. barbus* | MT385897 | Bp/Bb |
| PVi | Mt26 | Bbar22 | *B. barbus* | MT385892 | HBP01 | *B. plebejus* | MT385915 | HBP01 | *B. plebejus* | MT385915 | Bp/Bp |
| PVi | Mt29 | Bbar10 | *B. barbus* | MT385887 | HBP03 | *B. plebejus* | MT385916 | HBP07 | *B. plebejus* | MT385920 | Bp/Bp |
| PVi | Mt30 | Bbar15 | *B. barbus* | MT385890 | HBP09 | *B. plebejus* | MT385922 | HBB02 | *B. barbus* | MT385914 | Bp/Bb |
| PVi | Mt31 | Bbar22 | *B. barbus* | MT385892 | HBP02 | *B. plebejus* | MT385915 | HBB02 | *B. barbus* | MT385914 | Bp/Bb |
| PVi | Mt32 | Bbar10 | *B. barbus* | MT385887 | HBP10 | *B. plebejus* | MT385923 | HBB02 | *B. barbus* | MT385914 | Bp/Bb |
| PVi | Mt33 | Bbar24 | *B. barbus* | MT385894 | HBP11 | *B. plebejus* | MT385924 | HBB02 | *B. barbus* | MT385914 | Bp/Bb |
| PVi | Mt34 | Bbar01 | *B. barbus* | MT385882 | HBP12 | *B. plebejus* | MT385925 | HBB02 | *B. barbus* | MT385914 | Bp/Bb |
| PVi | Mt36 | Bbar22 | *B. barbus* | MT385892 | HBP01 | *B. plebejus* | MT385915 | HBP01 | *B. plebejus* | MT385915 | Bp/Bp |
| PVi | Mt37 | Bbar15 | *B. barbus* | MT385890 | HBB01 | *B. barbus* | MT385897 | HBB10 | *B. barbus* | MT385915 | Bb/Bb |
| PVi | Mt38 | Bbar22 | *B. barbus* | MT385892 | HBP08 | *B. plebejus* | MT385921 | HBP01 | *B. plebejus* | MT385915 | Bp/Bp |
| PVi | Mt39 | Bbar10 | *B. barbus* | MT385887 | HBP14 | *B. plebejus* | MT385927 | HBB02 | *B. barbus* | MT385914 | Bp/Bb |
| PVi | Mt40 | Bbar10 | *B. barbus* | MT385887 | HBP16 | *B. plebejus* | MT385929 | HBB02 | *B. barbus* | MT385914 | Bp/Bb |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| TLi | PA01 | Bbar11 | B. *barbus* | MT385888 | HBT01 | *B. tyberinus* | MT385931 | HBB11 | *B. barbus* | MT385907 | Bt/Bb |
| TLi | PA04 | Bbar01 | B. *barbus* | MT385882 | HBB04 | *B. barbus* | MT385900 | HBB05 | *B. barbus* | MT385901 | Bb/Bb |
| TLi | PA05 | Bbar16 | B. *barbus* | MT385891 | HBT01 | *B. tyberinus* | MT385931 | HBB06 | *B. barbus* | MT385902 | Bt/Bb |
| TLi | PA07 | Bbar13 | B. *barbus* | MT385889 | HBB01 | *B. barbus* | MT385897 | HBB01 | *B. barbus* | MT385897 | Bb/Bb |
| TLi | PA08 | Bbar05 | B. *barbus* | MT385885 | HBB01 | *B. barbus* | MT385897 | HBB01 | *B. barbus* | MT385897 | Bb/Bb |
| TLi | PA09 | Bbar23 | B. *barbus* | MT385893 | HBB01 | *B. barbus* | MT385897 | HBB01 | *B. barbus* | MT385897 | Bb/Bb |
| TLi | PA10 | Bbar01 | B. *barbus* | MT385882 | HBT01 | *B. tyberinus* | MT385931 | HBB03 | *B. barbus* | MT385899 | Bt/Bb |
| TLi | PA11 | Bbar01 | B. *barbus* | MT385882 | HBT05 | *B. tyberinus* | MT385935 | HBB01 | *B. barbus* | MT385897 | Bt/Bb |
| TLi | PA12 | Bbar01 | B. *barbus* | MT385882 | HBB08 | *B. barbus* | MT385904 | HBB01 | *B. barbus* | MT385897 | Bb/Bb |
| TLi | PA13 | Bbar25 | B. *barbus* | MT385895 | HBB01 | *B. barbus* | MT385897 | HBB09 | *B. barbus* | MT385905 | Bb/Bb |
| TLi | PA15 | Bbar04 | B. *barbus* | MT385884 | HBB01 | *B. barbus* | MT385897 | HBB01 | *B. barbus* | MT385897 | Bb/Bb |
| TLi | PA16 | Bbar01 | B. *barbus* | MT385882 | HBT07 | *B. tyberinus* | MT385937 | HBB08 | *B. barbus* | MT385904 | Bt/Bb |
| TLi | PA17 | Bbar01 | B. *barbus* | MT385882 | HBB03 | *B. barbus* | MT385899 | HBB01 | *B. barbus* | MT385897 | Bb/Bb |
| TLi | PA18 | Bbar01 | B. *barbus* | MT385882 | HBB01 | *B. barbus* | MT385897 | HBB01 | *B. barbus* | MT385897 | Bb/Bb |
| TLi | PA19 | Bbar09 | B. *barbus* | MT385886 | HBT04 | *B. tyberinus* | MT385934 | HBB01 | *B. barbus* | MT385897 | Bt/Bb |
| TLi | PA20 | Bbar04 | B. *barbus* | MT385884 | HBB17 | *B. barbus* | MT385934 | HBB05 | *B. barbus* | MT385901 | Bb/Bb |
| TLi | PA21 | Bbar01 | B. *barbus* | MT385882 | HBT04 | *B. tyberinus* | MT385934 | HBB01 | *B. barbus* | MT385897 | Bt/Bb |
| TLi | PA22 | Bbar01 | B. *barbus* | MT385882 | HBB04 | *B. barbus* | MT385900 | HBB03 | *B. barbus* | MT385899 | Bb/Bb |
| TLi | PA23 | Bbar01 | B. *barbus* | MT385882 | HBB15 | *B. barbus* | MT385911 | HBB03 | *B. barbus* | MT385899 | Bb/Bb |
| TLi | PA24 | Bbar01 | B. *barbus* | MT385882 | HBT08 | *B. tyberinus* | MT385938 | HBB01 | *B. barbus* | MT385897 | Bt/Bb |
| TLi | PA25 | Bbar26 | B. *barbus* | MT385896 | HBT01 | *B. tyberinus* | MT385931 | HBT06 | *B. tyberinus* | MT385936 | Bt/Bt |
| TLi | PA27 | Bbar01 | B. *barbus* | MT385882 | HBB16 | *B. barbus* | MT385912 | HBB06 | *B. barbus* | MT385902 | Bb/Bb |
| TLi | PA28 | Bbar04 | B. *barbus* | MT385884 | HBT01 | *B. tyberinus* | MT385931 | HBB01 | *B. barbus* | MT385897 | Bt/Bb |
| TLi | PA33 | Bbar16 | B. *barbus* | MT385891 | HBB01 | *B. barbus* | MT385897 | HBB01 | *B. barbus* | MT385897 | Bb/Bb |
| TLi | PA34 | Bbar01 | B. *barbus* | MT385882 | HBB12 | *B. barbus* | MT385908 | HBB13 | *B. barbus* | MT385909 | Bb/Bb |
| TLi | PA38 | Bbar09 | B. *barbus* | MT385886 | HBT05 | *B. tyberinus* | MT385935 | HBB01 | *B. barbus* | MT385897 | Bt/Bb |
| TLi | PA39 | Bbar01 | B. *barbus* | MT385882 | HBB01 | *B. barbus* | MT385897 | HBB01 | *B. barbus* | MT385897 | Bb/Bb |
| TLi | PA40 | Bbar01 | B. *barbus* | MT385882 | HBB01 | *B. barbus* | MT385897 | HBB01 | *B. barbus* | MT385897 | Bb/Bb |
| TLi | PA42 | Bbar01 | B. *barbus* | MT385882 | HBB01 | *B. barbus* | MT385897 | HBB01 | *B. barbus* | MT385897 | Bb/Bb |

654

655 **Table 6** Molecular indices calculated for the nuclear GH-2 alleles for pure *B. plebejus* (PV*p*), *B.*
656 *tyberinus* (TL*p*) and their hybrids (*B. barbus* x *B. tyberinus* in TL*i* and *B. barbus* x *B. plebejus* in
657 PVi): haplotype diversity (H), nucleotide diversity (π, expressed in %), with relative standard
658 deviations. N= number of total alleles for sampling sites; in brackets the number of alleles per species.

| **Species** | **Indices** | **PV*p*** ***N=50*** | **PV*i*** ***N=52*** | **TL*i*** ***N=58*** | **TL*p*** ***N=28*** |
|---|---|---|---|---|---|
| *B. plebejus* | π (%) | 0.02 ± 0.01 (50) | 0.30 ± 0.05 (28) | | |
| | H | 0.19 ± 0.01 (50) | 0.88 ± 0.01 (28) | | |
| *B. tyberinus* | π (%) | | | 0.16 ± 0.02 (12) | 0.03 ± 0.01 (28) |
| | H | | | 0.90 ± 0.01 (12) | 0.27 ± 0.01 (28) |
| *B. barbus* | π (%) | | 0.43 ± 0.06 (24) | 0.30 ± 0.06 (46) | |
| | H | | 0.66 ± 0.01 (24) | 0.69 ± 0.01 (46) | |

659

660 **Figure captions**

661

662 **Fig. 1.** Sampling sites of *B. tyberinus* (uninvaded TL*p* and invaded TL*i*) and *B. plebejus* (uninvaded
663 PV*p* and invaded PV*i*) populations, collected in Tyrrhenian (TL) and Adriatic (PV) basins
664 respectively (see Table 1). Pie charts indicate the species frequency according to genetic attribution
665 (mtDNA inner circle and ncDNA outer circle).

666

667 **Fig. 2.** Canonical variate analysis (CVA) output of the body shape comparison between *B. tyberinus*
668 (uninvaded TL*p* and invaded TL*i*) and *B. plebejus* (uninvaded PV*p* and invaded PV*i*) populations.
669 Wireframe graphs indicate the shape changes along each axis (from grey to dashed black).

670

671 **Fig. 3** (a) Bayesian tree for D-loop mtDNA, and (b) Maximum likelihood tree for GH-2 nDNA
672 haplotypes. Statistical support for the major clades is expressed as posterior probability (pp) and
673 bootstrap (btp) values, indicated in bold and italic respectively. Colored bars indicate current species
674 assignation. The haplotypes scored in this study are in bold, whereas the haplotypes retrieved from
675 GenBank are indicated by their accession number (Supplementary material Table S1, S2); * indicates
676 haplotypes previously recorded). Morphology of each lineage is reported (i.e. *B. plebejus* in PV*p*; *B.*
677 *tyberinus* in TL*p*); *B. barbus* is represented by two hybrid forms with *B. tyberinus* and *B. plebejus*
678 (i.e. in TL*i* and in PV*i*, respectively).

679

680 **Fig. 4**. Minimum spanning networks of *B. barbus* mitochondrial (D-loop (A)) and nuclear (GH-2 (B))
681 recorded in Adriatic (PVi) and Tyrrhenian (TLi) invaded population. Circles represent haplotypes
682 and size is proportional to the frequency of each haplotype. Black dots represent missing haplotypes.

683