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1 **The Role of the south-western Alps as a unidirectional corridor for**
2 **Mediterranean brown trout (*Salmo trutta* complex) lineages**

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22 **Running title:** The south-western Alps: a corridor for *Salmo trutta*

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31 **Abstract**

32 The role of south-western Alps as a corridor for Mediterranean trout (*Salmo trutta* complex Linnaeus,
33 1758) was evaluated in order to understand the influence of the last glacial events in shaping the spatial
34 distribution of the genetic diversity of this salmonid. For this, the allochthonous hypothesis of a man-
35 mediated French origin (19th century) of the Mediterranean trout inhabiting the Po tributaries in the Italian
36 side of south-western Alps was tested. A total of 412 individuals were analyzed at the mitochondrial control
37 region. The phylogenetic classification was carried out by using a Median-Joining Network analysis.
38 Mismatch pair-wise analysis, molecular dating and Kernel density distribution analysis of the main
39 mitochondrial lineages were evaluated to compare past demographic dynamics with the current spatial
40 distribution of genetic diversity. The main outcomes resulted strongly in agreement with a biogeographic
41 scenario where the south-western Alps acted as a unidirectional corridor that permitted the colonization of
42 the upper Durance (Rhône River basin) by trout from the Po River basin. Therefore, the Mediterranean trout
43 should be considered as native also along the Italian side of the south-western Alps and the allochthonous
44 hypothesis should be rejected.

45 **Key words:** *Salmo trutta* complex, Alpine barrier, ice cover, conservation genetics, biological corridors

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61 INTRODUCTION

62 The study of the phylogeographic history of a species represents a fundamental step to understand the
63 factors producing genetic diversity both within and among species, with particular reference to the role
64 played by past environmental and climatic changes in shaping high levels of genetic complexity as well as
65 their current spatial distribution (Purvis & Hector, 2000). The reconstruction of the recent evolutionary
66 history of an organism is therefore essential to set-up concrete management and conservation actions aiming
67 at protecting its evolutionary potential (Losos *et al.*, 2013). This is particularly true for those organisms
68 impacted by socio-economic interests, where conservation induces conflict with commercial interests
69 (Redpath *et al.*, 2013). In addition, the tentative to state the natural occurrence of an organism on the basis of
70 phylogenetic studies could be made difficult by the long history of the human-mediated natural species range
71 alterations, that may act as a confounding factor that adds further complexity in the study of the already
72 complex evolutionary pathway of an organism (Fernández-García *et al.*, 2014; Sanz, 2018).

73 Although, the human ability in shaping plant and animal distribution is rooted in ancient culture and
74 traditions (Larson & Fuller, 2014), nowadays, human-mediated transport beyond biogeographic barriers has
75 led to the introduction and establishment of alien species (sometimes invasive) in new regions worldwide
76 (Shackleton *et al.*, 2019; Berrebi *et al.*, 2020). About freshwater fish, the first written historical records date
77 back the major human-mediated introductions at Roman time and successively at medieval period (Sønstedt,
78 Borgstrøm, & Heun, 2007; Miró & Ventura, 2013). Basically, the above ancient freshwater fish
79 translocations were promoted by food purposes, whereas, nowadays, freshwater fish represents one of the
80 most important group of animals introduced for sport purposes worldwide (Leprieur *et al.*, 2008). In
81 particular, brown trout (*Salmo trutta* complex) is listed as the 100 of the world's worst invasive alien species
82 (Global Invasive Species Database [2019], downloaded from http://193.206.192.138/gisd/100_worst.php on
83 [13-03-2019](http://193.206.192.138/gisd/100_worst.php)). At the same time, its natural diversity is imperilled in much of its native range (Budy *et al.*,
84 2013).

85 The Italian IUCN red list of vertebrates (Rondinini *et al.*, 2013) classified its conservation status as near
86 threatened, and the French red list (IUCN, 2010) considers the trout status as low concern. It is probably
87 wildly optimistic statements considering that along the Apennine chain less than 3% of native populations
88 were free of genetic introgressive hybridization with Atlantic genes of domestic origin (Splendiani *et al.*,
89 2016a). Further, in the Alpine area, its conservation value appears at least chaotic. The brown trout
90 (regardless of its phylogenetic origin) is “on paper” considered as allochthonous in the Piemonte Region and
91 native in the neighbouring Lombardia Region (based on Regional lists of freshwater fishes). In addition, the
92 AIAD (Italian Association of Freshwater Fish Ichthyologists) guideline for Italian salmonid management
93 proposes to adopt a passive conservation approach for the putative native populations of south-western Alps
94 (Zanetti, Nonnis Marzano & Lorenzoni 2013). Based on the opinions expressed in this document, the

95 Mediterranean trout populations recognizable in this region should be the consequence of historical human-
96 mediated translocations from the Rhône River basin to the Po River basin. The reasons of these trout
97 translocations would be related with the well-known passion for trout fishing of the Queen Elena of Italy
98 (1873 – 1952) (e.g., Siccardi, 1996). Unfortunately, in the above AIIAD document, there is no citation of
99 historical records to sustain this hypothesis. On the contrary, bibliographic records collected in the
100 Geographic dictionary of the Sardinian State (Casalis, 1833, 1852), evidenced a widespread presence of
101 brown trout in the occidental Alps long before Elena’s reign (1900-1946). Interesting, as far as it is known,
102 there are no pieces of evidence of trout translocations from France to Italy, taking into account the above
103 historical literature. On the other hand, historical translocation of freshwater fish across the Alps have been
104 well documented, as the case of the historical (16th century) translocation of *Salvelinus alpinus* from Austria
105 to Trentino Alto Adige Region (Italy) (Tiberti & Splendiani, 2019).

106 Here, a comprehensive molecular data set is used to describe wild Mediterranean brown trout population
107 genetic diversity encompassing from the Rhône River basin to the Po River basin to try to reach several
108 objectives. The first aim of the present study is to provide, for the first time, a focus on brown trout
109 mitochondrial DNA (mtDNA) genetic diversity distribution along a putative contact zone between several
110 lineages of this species complex. The second aim is to find solid hypotheses to correlate spatial distribution
111 of genetic diversity with the main evolutionary forces that could have played a role in shaping the
112 Mediterranean brown trout lineage distribution observed in the study area, that are: i) effects of the last
113 glacial maximum (LGM, that are, ice cover extension, effects of ice flow pattern during deglaciation
114 episodes, localization of glacial refuges, etc), and ii) the role of geomorphologic and hydrogeological
115 characteristics of the mountain relief in the south-western Alps in shaping genetic diversity. Finally, the third
116 aim is to test the hypothesis of non autochthony proposed by AIIAD and as a consequence provide
117 recommendations to preserve, in a rational manner, what remains of the native genetic diversity of brown
118 trout in south-western Alps watercourses.

119 MATERIAL AND METHODS

120 SAMPLING DESIGN

121 The *S. trutta* complex is characterized by a puzzling pattern of geographical forms probably underlying
122 the taxonomic inflation reported in literature with the description of nearly 50 *Salmo* species (Tougaard *et al.*,
123 2018). The description of the native trout taxa previously identified (e.g., Kottelat & Freyof, 2007) in the
124 study area have been summarised in Table S1. In this study, for practical reasons, the following terms will be
125 used hereafter: (1) “marble trout” for *S. marmoratus* specimens (generally fixed for MA haplotypes), (2)
126 “native brown trout” for the individuals showing both a fario phenotype and native haplotypes of the
127 Mediterranean area (that are, haplotypes belonging to the lineages AD, ME or MA), (3) “Lake Garda
128 Carpione” to indicate *S. carpio* specimens and (4) “Atlantic brown trout” to indicate *S. trutta* specimens
129 hosting domestic and non-native haplotypes (AT lineage). The sampling efforts were mainly focused along

130 the south-western Alps, as for example, i) in the upper Durance, that belongs to the Rhône basin, but is
131 located adjacent to the Po River basin along the French side of the south-western Alps, and ii) along the main
132 sub-basins of the Po River basin, as regards the Italian side of the south-western Alps. For comparisons,
133 further samples from other sites of the Rhône River basin and from neighbour minor Mediterranean
134 independent rivers were also included, for example, the rivers Var, Loup, Roya and Sansobbia. The sampling
135 size ranged from 4 to 24 with a mean value of 10 trout per sample. The total number of analysed *S. trutta*
136 was 412 specimens that were collected from 42 sites (Figure 1, Table 1).

137 DNA EXTRACTION

138 From each fish, a small fin clip was removed and conserved in 95% ethanol until DNA extraction. Due to
139 the union of genetic data obtained from two different laboratories (*i.e.*, from the ISEM, Université de
140 Montpellier and from the DiSVA, Università Politecnica delle Marche, hereafter, respectively, Lab.1 and
141 Lab. 2), total genomic DNA was extracted using two methods. The first one (Lab. 1) consisted in a
142 Chelex/proteinase K-based protocol described by Estoup, Largiader, Perrot & Chourrot (1996). A small
143 piece of fin was incubated overnight at 56°C in 195 µl of 5% Chelex 100 Resin (Biorad) solution containing
144 50 mM of Tris-HCL (pH 7) and 500 µg/ml of proteinase K. Samples were then incubated at 95°C for 10 min
145 before centrifugation at 3500 g for 5 min. Supernatants were recovered and frozen at – 20°C until required
146 for use. In the second method (Lab. 2), total genomic DNA was extracted using an automated DNA extractor
147 (MagCore® Automated Nucleic acid Extractor in combination with the Genomic DNA Tissue Kit 401).

148 In Lab. 1, the mtDNA control region (CR) was amplified by PCR using the PST (5'-
149 CCCAAAGCTAAAATTCTAAAT-3') and FST (5'-GCTTTAGTTAAGCTACGC-3') primers (Cortey &
150 García-Marín, 2002). Each 50 µL reaction included 0.4 µM of each primer (Eurofins MWG Operon), dNTP
151 (2 mM each), 2 mM of MgCl₂, 10 µL of 5 9 PCR buffer, 1 U of Taq polymerase (GoTaq® Promega), and
152 about 50 ng of genomic DNA. The PCR conditions included initial denaturation (95°C, 5 min), followed by
153 30 cycles of strand denaturation (94° C, 1 min), primer annealing (52° C, 1 min), and DNA extension (72° C,
154 1 min) cycles, and then by a final extension (72° C, 5 min). All PCR amplifications were performed in
155 Eppendorf Mastercycler thermocyclers. The amplified DNA fragments were run on a 0.8% agarose gel to
156 verify the amplification efficiency. The amplified products were purified and sequenced in both directions to
157 confirm the polymorphic sites in an ABIPRISM 3130/xl/sequencer (Applied Biosystems). In Lab. 2, the
158 mtDNA CR was PCR-amplified according to Bernatchez & Danzmann (1993) (primer sequences: LN20, 5'
159 ACCACTAGCACCCAAAGCTA; HN20, 5' GTGTTATGCTTTAGTTAAAGC). Screening of mtDNA
160 genetic variability was conducted through Single-Strand Conformation Polymorphism (SSCP) analysis.
161 Because shorter fragments are better suited for detection of mutations in SSCP gels (Hayashi, 1991), the CR
162 PCR products of *c.* 1000 bp were first digested with *AluI* restriction enzyme and then run on a non-
163 denaturing polyacrylamide gel for 12-h at 5 W in a cool chamber. Finally, the non-digested segment of *c.*
164 1000 bp was sequenced in a sub-sample of individuals with the same SSCP profile (that is, three-four trout
165 per each SSCP morph detected).

167 The mtDNA CR sequences were aligned using Clustal W (Larkin *et al.*, 2007). In order to assign the
168 sequence haplotypes observed in this study to each of the main brown trout mtDNA lineages, several
169 reference *S. trutta* CR sequences were downloaded from GenBank (belonging to the mtDNA lineages ME,
170 AD, MA and AT) (see Table S2 for more details). The genealogical relationship among haplotypes was
171 depicted using a Median-Joining Network (Bandelt, Forster, & Rohl, 1994) constructed using Network 5
172 (Fluxus Technology Ltd., www.fluxus-engineering.com), considering also gaps and missing nucleotides. The
173 ϵ parameter was set to zero. Historical demography inferences were drawn from three neutrality tests
174 implemented in DnaSP 6 (Rozas *et al.*, 2017): i) Fu's F_S (Fu, 1997), ii) Tajima's D (Tajima, 1989) and iii) R_2
175 (Ramos-Onsins & Rozas, 2002), and from mismatch distribution analysis by using Arlequin 3.5 (Excoffier &
176 Lischer, 2010). Briefly, a significantly negative Tajima's D and F_S , and a significantly positive R_2 indicate a
177 scenario of demographic expansion. In the mismatch analysis, a curve displaying the observed distribution of
178 pair-wise differences within each lineage is compared to an expected curve under a model of population
179 growth-decline. Generally, a curve with a single peak associated with a low number of pair-wise differences
180 indicates expansion, while a curve with two or multiple peaks indicates stability. Differences between
181 observed and expected pair-wise mismatch distribution were evaluated using the sum of squared deviations
182 (SSD) and the raggedness index (r) as implemented in Arlequin 3.5.

183 The estimation of time to the most recent ancestor (TMRCA) of the AD, ME and MA lineages was
184 carried out with a Bayesian coalescent analysis using BEAST 1.10.4 (Suchard *et al.*, 2018) under a HKI + I
185 model as inferred by using jModeltest (Posada, 2008). We adopted two fixed values with a normal prior
186 distribution (0.75 - 1%) of divergence rates (*e.g.*, Sanz, 2018 and references therein), and taking into account
187 that we were interested in the estimation of the separation time between the Mediterranean lineages AD, ME
188 and MA, we adopted the basic strict clock model, as implemented in BEAST, because, potentially
189 outperforming for trees with shallow roots (Brown & Yang, 2011). The strict molecular clock was used in
190 combination with three coalescent models (constant size, exponential growth, expansion growth). To
191 determine the best fitting model of the data (Brandley, Schmitz, & Reeder, 2005), a modified Akaike
192 Information Criterion (AICM) as provided in TRACER 1.6 (Rambaut *et al.*, 2018) was used. The models
193 were run five times for fifty million generation with a 10% burn-in stage. Markov chain convergence was
194 checked visually by the inspection of the traces, while the run stability was measured using the effective
195 sample size ($ESS > 200$ for all parameters) using Tracer. Results of the independent convergent runs were
196 combined with LogCombiner v 1.10.4 (auxiliary program implemented in the BEAST package) to estimate
197 TMRCA and 95% highest probability density intervals (HPD). A consensus tree was then generated using
198 TreeAnnotator 1.10.4 (auxiliary program implemented in the BEAST package) with the following options:
199 maximum clade credibility and mean node heights.

200 Hierarchical analysis of molecular variance (AMOVA) was used to test how the effects of the last glacial
201 events, occurred in the study area, could explain the current spatial genetic distribution. Groupings included:

202 i) Rhône samples vs Po samples and ii) Rhône samples vs Po and Durance samples. This latter grouping was
203 set-up to test the hypothesis of a recent post-glacial origin of brown trout samples from upper Durance
204 related to slope failure phenomena due to last deglaciation events. The above tests were carried out by using
205 Arlequin 3.5, using conventional F-statistics and testing the statistical significance of the tests with 5,000
206 permutations.

207 To depict the biogeographic scenarios underlying the observed haplotype spatial distribution, the mtDNA
208 lineage distribution observed in each population was both analysed by mapping pie charts geographically
209 and using Kernel density (KD) analysis along an elevation, longitudinal and latitudinal gradient. The KD
210 analysis was conducted partitioning the samples as i) samples from the Rhône River basin and ii) samples
211 from the Po River basin. For these tests, brown trout samples were grouped in the following categories: i)
212 samples fixed for AD haplotypes, ii) samples fixed for ME haplotypes, iii) samples fixed for MA haplotypes
213 and mixed samples, iv) samples sharing AD and ME haplotypes, v) AD-MA, vi) ME-MA and vi) AD-ME-
214 MA. The rationale of the above partitioning was to verify if haplotype distribution can match with plausible
215 scenarios of extinction and recolonization events connected with the environmental changes occurred during
216 the last glacial maximum in the south-western Alps. The KD analysis was carried out by using the density
217 function in R software (R Development Core Team, 2017).

218 RESULTS

219 HAPLOTYPE CLASSIFICATION

220 Before starting with sequence analyses, the PolyT region of the CR was considered of a constant length of
221 14bp in all sequences. In fact, such region is likely to be unstable and thus characterized by a high mutation
222 rate, showing frequent 14-T variants. In this circumstance, sequence stretch length identity, could be the
223 mere consequence of homoplasy and would not represent a real phylogenetic signal. On the whole, an
224 alignment of 981 bp was obtained, from which 21 haplotypes emerged (Table 2). The observed haplotypes
225 belonged to four main mtDNA lineages: AD, ME, MA and AT (*sensu* Bernatchez *et al.*, 1992 or Bernatchez,
226 2001). The AD lineage was represented by three haplotypes: ADporh-1 and ADrh-1, observed for the first
227 time in this study (GenBank accession numbers, respectively, MK948034 and MK948035) and ADcs-1
228 already described in literature (Cortey, Pla & García-Marín, 2004). As far for the ME lineage, seven
229 haplotypes were observed, two of which already detected in other studies, that are: MEcs-1 and MEcs-15
230 (*e.g.*, Cortey *et al.*, 2004) and the other five were detected for the first time in this study and named as
231 follow: MEcs-28 to MEcs-32 (from MK948029 to MK948033). The MA lineage displayed a new haplotype
232 named MAsl-1 (MK948036), and three mtDNA variants already detected in previous studies, namely Ma2a,
233 Ma2b and Ma2c (Meraner, Baric, Pelster, & Dalla Via, 2007; Meraner, Gratton, Baraldi & Gandolfi, 2013).
234 Finally, the Atlantic lineage was represented by six haplotypes, five of them were already detected in
235 Mediterranean rivers and classified as haplotypes of hatchery origin, that are: haplotype 1 to haplotype 4
236 (Cortey & García-Marín, 2002) and the haplotype At1e, (Meraner *et al.*, 2007), while one haplotype was

237 described for the first time in this study (haplotype 3b, MK948037). This latter haplotype should be also
238 considered of hatchery origin due to high similarity with the domestic variant haplotype 3 (Table 2, Figure
239 2).

240 HAPLOTYPE SPATIAL DISTRIBUTION

241 The ME lineage dominated in the Rhône River drainage and in the other minor French rivers included in
242 this study. The most common ME haplotype was MEcs-1, the rest of the ME haplotypes (MEcs-28 – 32) was
243 endemic of French Mediterranean rivers. On the other hand, within the Po River drainage and in the
244 Sansobbia River, the ME lineage was represented by the haplotype MEcs-1.

245 The AD lineage was quite common in the Po River drainage, however, the ancestral haplotype of this
246 lineage (ADcs-1, see Figure 2) was observed only in a sole trout (in sample GIU, Po River basin). However,
247 in other studies within the Po River drainage, the haplotype ADcs-1 was detected in both 19th century trout
248 (Splendiani *et al* 2017), as well as in recent samples (Gratton *et al.*, 2014; Stefani, Anzani, & Marieni, 2019).
249 Toward the west part of the Po River drainage, the haplotype ADporh-1 was newly detected (one Mutational
250 Step from ADcs-1). This latter haplotype presented a spatial distribution confined to the upper reaches of
251 both upper Durance River (Rhône basin) and Po River along a transect of the south-western Alps extended
252 from the Cottian to the Maritime Alps (Figure 1 and Table 2, see also next paragraph). Finally, the haplotype
253 ADrh-1 consistently with its position in the Median-Joining Network (one MS from the haplotype ADporh-
254 1) was found only in the western part of the Rhône River drainage, in the Petit Buëch stream.

255 The MA haplotypes were found only within the Po River drainages. The most common haplotype
256 detected for this lineage was Ma2b. This mtDNA variant occupied a central position within the MA lineage
257 (Figure 2). In line with its haplotype network position, this haplotype was observed elsewhere within the Po
258 River drainages in both modern (Meraner *et al.*, 2007, 2013) and museum specimens (from 19th century, *e.g.*,
259 Splendiani *et al.*, 2017).

260 Finally, as expected, the AT haplotypes formed a separate cluster in the Median-Joining Network (Figure
261 2). These non-native haplotypes for the Mediterranean area showed an evident greater abundance within the
262 Italian samples (Table 2, Figure 1). Here, a mean value of AT haplotypes of 38% and a maximum value of
263 70% (in the locality MAIb) were observed. In four localities (TRO, RIP, FER and GES), the AT haplotypes
264 were not observed. In the French samples, the AT haplotypes were observed only in two localities out of 21,
265 GLU (60%) and ROY (20%).

266 MISMATCH ANALYSIS AND DIVERGENCE TIME ESTIMATES

267 Mismatch distribution analysis indicated (see Table S3 and Figure S1) a scenario consistent with a model
268 of demographic expansion (Excoffier, 2004) for the brown trout mtDNA lineages ME and AD and a stable
269 demographic trend for the lineage MA.

270 The AICM suggested that a strict clock under a constant size coalescent model best-fits our data. The
271 TMRCA estimations placed the origin of the AD lineage from 278,000 (95% HPD 170,000 – 391,000) to
272 212,000 (95% HPD 129,000 – 298,000) years ago by adopting, respectively, a substitution rate of 0.75 and
273 1%, the origin of the ME lineage from 267,000 (95% HPD 166,000-372,000) to 191,000 (95% HPD
274 122,000-265,000) years ago, and the origin of the MA lineage from 122,000 (95% HPD 172,000-205,000) to
275 117,000 (95% HPD 51,000-193,000) years ago (see Table S4). MA lineage appears so as the youngest one.
276 Finally, the origin of the AD branch composed by the haplotypes ADporh-1 and ADrh-1 was placed around
277 151,000 (95% HPD 11,000-99,000) and 120,000 (95% HPD 16,000-86,000) years.

278 AMOVA

279 With both two grouping options (Rhône samples vs Po Samples and Rhône samples vs Durance and Po
280 samples), the AMOVA analyses showed that most of the genetic variation was explained at the within
281 population level (53.26 and 51.62%, respectively) and among populations within group level (33.84 and
282 26.84%, respectively). However, the AMOVA analyses showed also that grouping the Durance samples
283 within the Po River group explained much more genetic variation (21.54 %) than grouping the Durance
284 within its main river basin (*i.e.*, the Rhône River basin) (14. 92 %). In both cases, the statistical significance
285 of the source of variation represented among groups was highly significant ($P = 0.0000$).

286 KERNEL DENSITY (KD) BROWN TROUT LINEAGE DISTRIBUTION

287 Within the Rhône River drainage, the highest density of samples characterized by the sole presence of
288 ME haplotypes was found between 4.5 – 5.5 E longitudes (Figure 3A). Unfortunately, only one sample was
289 characterized by the sole presence of AD haplotypes (BUË), therefore, KD analysis was not applicable in
290 this case. On the contrary, the rest of the Rhône samples (*i.e.*, samples from the Durance sub-basin) were
291 composed by a mixture of AD and ME haplotypes. In this study, a total of 91 trouts originated from the
292 Rhône River basin in France, among them, 31 specimens showed the AD haplotypes. All these latter trouts
293 came from the Durance sub-basin (samples BUË, BIA, CLA, UBA and GUI, see Table 2). The last two
294 rivers (UBA and GUI) are flowing in France directly from the France-Italy boundary. This kind of
295 populations peaked around 7.0 E longitude, corresponding with the upper part of the Durance River (Figure
296 3A). When KD was carried out to relate brown trout lineages distribution with elevation, a similar net
297 separation between different categories of samples was observed. For example, in the Rhône River,
298 populations fixed for ME haplotypes were most abundant around 0 – 500 m (Figure 3B), whereas admixed
299 populations (AD – ME) showed higher values of probability between 1500 – 2000 m. Within the Po River
300 drainage, the different categories of populations defined based on mtDNA lineage composition, appeared
301 clearly stratified along an altitudinal cline. Pure ME populations were detected only in one case (BAR,
302 Tanaro River, Ligurian Apennine). Admixed AD – ME populations were, however, the most common and
303 reached a density peak around 1600 – 2000 m. Pure AD populations peaked slightly lower, around 1400 m
304 (Figure 3C). Around this latter quote, peaked both pure brown trout samples fixed for MA haplotypes and

305 admixed MA – AD brown trout samples. Further downstream (c. 450 m), pure marble trout samples (MA)
306 were abundant (data from Giuffra *et al.*, 1994). Finally, a pattern of brown trout mtDNA lineage density
307 distribution along the south-western Alps was also evident along a latitudinal gradient (Figure 3D). For
308 example, pure AD samples appeared most abundant around 44.0 – 44.5 N, roughly corresponding with the
309 Maritime Alps (Italian side), while admixed populations (AD – ME) peaked around 45.0 N (*i.e.*, Cottian
310 Alps).

311 DISCUSSION

312 In the following chapters of this study, a comprehensive phylogeographic analysis of brown trout
313 populations inhabiting adjacent tributaries of the Rhône and Po River basins is proposed for the first time.
314 Based on the biogeographic scenario reconstructed, the allochthonous hypothesis proposed by the Italian
315 Association of Freshwater Fish Ichthyologists (Zanetti *et al.*, 2013) about Mediterranean brown trout
316 populations inhabiting the south-western Po tributaries was evaluated. Substantially, the shared haplotype
317 diversity (AD and ME haplotypes) observed along the two sides of the south-western Alps and, at the same
318 time, the presence of an important ice cover along the western part of the Rhône basin (*i.e.*, the Durance
319 glacier) during the glacial phases, incompatible with the presence of freshwater fish, suggest that native
320 brown trout survived the adverse phases of the upper Pleistocene just in the tributaries of the south-western
321 Po basin. Probably, the erosional events related with the deglaciation phenomena permitted the opening of a
322 biological corridor for brown trout from the Po basin toward the Rhône basin.

323 THE ORIGIN OF THE *S. TRUTTA* ME LINEAGE IN THE STUDY AREA

324 According to previous studies (Cortey *et al.*, 2004; Vera *et al.*, 2019), the Iberian Peninsula would
325 represent the ideal candidate as the centre of the origin of the ME lineage (*e.g.*, Sanz, 2018). However, the
326 ME haplotype diversity detected in the present study partially contrast with the above hypothesis. Within the
327 Rhône River basin and neighbour rivers, five new ME haplotypes were detected. This fact appears in
328 accordance with a western Mediterranean origin of the ME lineage as previously proposed (*e.g.*, Bernatchez,
329 2001; Cortey *et al.*, 2004; Oliver, 2014) but also suggests that the Rhône basin area acted as an important
330 evolutionary centre for ME genetic diversity.

331 Although, the use of the sole mitochondrial control region could be taken into account with caution for
332 inferring isolation time accurately (Schenekar, Lerceteau-Köhler, & Weiss, 2014), an attempt was, however,
333 tried in this study. In this sense, it is interesting to indicate that the pre-defined divergence rates of 0.75 - 1%
334 adopted in this study designed a time since expansion of the major Mediterranean lineages (ME, AD and
335 MA) in accordance with their altitudinal distribution observed in the study area (see below).

336 The TMRCA analysis suggest a main expansion of the ME lineage around 191,000-267,000 years ago,
337 roughly corresponding with the last (III) Mindel-Riss Interglacial, and resulting slightly more ancient respect
338 previous estimations (Cortey *et al.*, 2004). Pleistocene Interglacial warming periods have been regarded as

339 phases of isolation in small headwaters for Mediterranean brown trout populations, thus promoting genetic
340 signatures within lineages (*e.g.*, Sanz, 2018 and references therein). On the other hand, during glaciations,
341 colder climate conditions may have triggered sea-ward migratory tactics in Mediterranean brown trout
342 populations, as highlighted by several paleontological evidences (Muñoz & Casadevall, 1997; Splendiani *et*
343 *al.*, 2016b; Splendiani *et al.*, 2020). Therefore, thanks to a seaward migratory route, the expansion of brown
344 trout in the Mediterranean area was possible. The colonisation of northern Corsica Island by the ME lineage
345 during the last glaciation is an example of this expansion (Gauthier & Berrebi, 2007). The spatial distribution
346 of the MEcs-1 haplotype suggests a potential eastward dispersion, from the Rhône River outlet to the Var
347 River (SAL, Gulf of Lion) where this haplotype was found and more eastern to the Sansobbia River (SAN,
348 Ligurian Sea) (Figure 1, Table 2). Then, when the ME lineage reached the upper part of the Ligurian rivers,
349 the colonization of the Po River basin was likely possible thanks to river capture events occurred along the
350 Ligurian Apennine chain. This scenario could explain the finding of the MEcs-1 haplotype in the Rio
351 Baracca (BAR, Po River basin, see also Figure 1, Table 2). Interestingly, the role played by the
352 hydrographical captures of the upstream portions of Mediterranean rivers was also proposed in the literature
353 to explain, for example, the exchange of Duero haplotypes between rivers flowing along opposite slope of
354 the Cantabrian mountains (Iberian Peninsula) (Vera *et al.*, 2015), as well as to explain the native occurrence
355 of the Danubian haplotypes within marble trout populations of the Sôca River (Berrebi, Jesenšek, & Crivelli,
356 2017). Concerning the study area, the phylogeographic history of the Italian vairone (*Telestes muticellus*) in
357 populations of west Liguria (Marchetto *et al.*, 2010) could also be explained invoking the presence of
358 biological corridors opened by ancient Mediterranean river captures.

359 Once reached the Po River basin, it can be reasonably expected that the ME lineage tried to colonize
360 available salmonid habitats. However, when the climate conditions went colder and the Alpine ice cover
361 expanded, this lineage survived only in refuge areas as, for example, in south-western Alps. Milder
362 conditions in this part of the Alpine chain and the absence of other brown trout lineages may have permitted
363 the colonization of the upper reaches of the western Alps by the MEcs-1 haplotype (see the Kernel density
364 analysis in the Results section and Figure 3).

365 THE ORIGIN OF THE *S. TRUTTA* AD LINEAGE IN THE STUDY AREA

366 The Adriatic-Balkan part of the Mediterranean basin is considered the centre of the origin of the AD
367 lineage (Sanz, 2018). The main expansion of the AD lineage seems to take place around 267,000 – 212,000
368 year ago and therefore nearly simultaneous with the last expansion proposed above for the ME lineage.
369 Although, as already stressed, divergence estimations should be interpreted with caution, mainly with regard
370 to the absolute date of expansion values, more reliable, on the contrary, appears the simultaneous time of
371 expansion of the AD and ME lineages observed in this study. The simultaneous expansion of these two
372 lineages fits well with both their similar peri-Mediterranean spatial distribution and with their phylogenetic
373 complexity (*e.g.*, Sanz, 2018).

374 In north Italy, the central haplotype of the AD lineage (ADcs-1) was observed in two museum specimens
375 (dating back to the end of 19th century) of Lakes Garda and Maggiore and in a modern sample of the Adige
376 Adriatic River (Meraner *et al.*, 2013). According to Splendiani *et al.* (2016a, 2017), the spatial distribution of
377 the Mediterranean trout genetic diversity in north Italy represents a sort of “map” of the potential Alpine
378 peripheral refuges where brown trout survived during the extreme glacial phases. In addition, the role played
379 by the area of the Lake Garda as an important glacial refuge for the genus *Salmo* is also evidenced by the
380 detection of two endemic AD and MA haplotypes in Lake Garda Carpione, an endemic trout of this major
381 Italian lake (Gratton *et al.*, 2014) (Figure 2). Further, the finding of a new AD haplotypes (ADporh-1),
382 endemic of the south-western Alps, suggests that also this area could have acted as both an important glacial
383 and interglacial refuge for brown trout. In the south-western Alps' part of the Po River basin, the haplotype
384 ADporh-1 was the sole AD haplotype observed. This haplotype was found as fixed in samples collected
385 around 1000 – 1500 m (Figure 3), that is slightly lower than the quote where AD-ME mixed populations
386 peaked. Thus, the observed spatial distribution suggests that the ME lineage colonized first the headwaters of
387 the south-western Po River basin, whereas the AD lineage tried to do the same later. Based on molecular
388 dating analyses, both ME and AD lineages showed a similar divergence time and thus it is hard to explain
389 their different altitudinal distribution. However, the proximity of the south-western Alps to an important
390 centre of origin of the lineage ME, as can be considered the Rhône River basin, could explain why the ME
391 lineage reached the highest sites of the south-western part of the Po River basin first.

392 The peculiar AD haplotypes detected across the south-western Alps (*i.e.*, ADporh-1 and ADrh-1
393 haplotypes) probably split from the ADcs-1 ancestor when warmer climate condition promoted phases of
394 isolation. The estimated origin for this AD branch of *c.* 151,000 – 120,000 years ago, corresponding
395 approximatively with the Riss-Würm Interglacial. During the warmer phases, brown trout population may
396 have survived in high altitude habitats of the south-western part of the Po River basin. This region would
397 also have been used as refuge during the colder phases (*i.e.*, the Younger Dryas stadial, *c.* 12,800 and 11,600
398 years BP) when the rest of the high-altitude Alpine streams was covered by a massive ice cover. Later, at the
399 beginning of the Holocene, the extreme erosional events produced by massive episodes of deglaciation
400 promoted the colonization of the upper Durance basin from the adjacent high altitude brown trout
401 populations survived in the south-western Po streams by river captures (see the next paragraph for more
402 information: The role of the south-western Alps as an asymmetrical biological corridor for brown trout
403 lineages).

404 The Italian side of the Maritime Alps represented also an isolated refuge. Interestingly, in this region,
405 both the AD-Tyrrh-1, very common elsewhere within the Tyrrhenian watercourses (*e.g.*, Berrebi *et al.*, 2019)
406 and ADcs-11, very common around the Adriatic Sea rivers (Sušnik *et al.*, 2007; Snoj *et al.*, 2009, 2010),
407 haplotypes were not found. Based on the network haplotype topology (Figure 2), we can hypothesized that
408 ADcs-1 colonized first the Po River, and then, during phases of geographic isolation within refugia, new
409 haplotypes, as ADporh-1 within the Maritime Alps refuge and the *S. carpio* AD haplotypes (ScarAD-1 and

410 ScarAD-2), within the Lake Garda refuge, could split. According to Sanz (2018), the AD lineage was
411 characterized by multiple waves of expansions. Successive expansion opportunities were probably used by
412 individuals carrying haplotypes ADcs-11 (around the Adriatic Sea) and AD-Tyrrh-1 around the Tyrrhenian
413 Sea, these latter ones, however, were unable to colonize the Po River as it was already occupied by both
414 brown trout (showing the ADcs-1, ADporh-1 and MEcs-1 haplotypes) and marble trout (showing the Ma2a,
415 Ma2b, Ma2c and MAsl-1 haplotypes).

416

417 THE ORIGIN OF THE *S. TRUTTA* MA LINEAGE OBSERVED IN THE STUDY AREA

418 Within the Po River basin, the MA lineage showed an evolutionary pathway like that observed in the case
419 of the AD lineage. For example, in the Lake Garda Carpione, Gratton *et al.* (2014) exhibited two endemic
420 MA haplotypes (named here ScarMA1 and ScarMA2, see also Figure 2). Further west, in brown trout
421 samples, a new MA haplotype (MAsl-1) was found, although in a sole specimen (Table 2). In this study, the
422 most diffuse MA haplotype detected in the south-western part of the Po River was Ma2b, also common
423 elsewhere in the Po River basin both in native brown trout and Lake Garda Carpione specimens (*e.g.*,
424 Meraner *et al.*, 2007; 2013). However, the most relevant result was represented by the spatial distribution of
425 the MA lineage as detailed below. In this study, the native brown trout samples from the south-western
426 tributaries of the Po River basin, characterized by the sole presence of MA haplotypes, showed an altitudinal
427 range of distribution intermediate between marble trout (*c.* 450 m) and native brown trout populations
428 characterized by a mix of AD and ME haplotypes (*c.* 1600 – 2000 m). Therefore, based on the altitudinal
429 distribution of the MA lineage it could be hypothesized that this lineage tried to reach the upstream thermal
430 refuges for last. This hypothesis seems to accord well with the younger origin of MA lineage respect to ME
431 and AD lineages emerged in this study and proposed also by Oliver (2014). In addition, the altitudinal
432 distribution of the MA lineage appear congruent with the stable or declining demographic scenario emerged
433 by mismatch analysis. The reduced habitat availability could have contrasted the demographic expansion of
434 this lineage (*e.g.*, Lavery *et al.*, 1996; Bernatchez, 2001). Probably, this mtDNA lineage was fixed in marble
435 trout that inhabited the lower part of the Po River basin, then, when climate become warmer, this salmonid
436 tried to reach colder habitats at higher elevations. A similar palaeohistorical scenario was previously
437 proposed (Berrebi *et al.*, 2000) to explain the spatial distribution of both brown trout and marble trout within
438 the Sôca River basin (Slovenia). The detection, in the present study, of only native brown trout phenotypes in
439 samples characterized by the sole presence of MA haplotypes suggests that the contact between brown trout
440 and marble trout occurred within an ecological contact zone (*i.e.*, ecotonal zone) where the parental
441 Mediterranean phenotype outcompeted (*e.g.*, Arnold, 1997). Alternatively, the evolution of habitat
442 preference of the MA lineage for lower river sections could explain its altitudinal distribution observed in the
443 south-western Alps. However, elsewhere, as for example in the Adige River basin, the MA lineage was able
444 to reach, accessible and formerly glaciated, high altitude sites (around 1000 – 1600 m) (Meraner *et al.*, 2007,
445 2010; Splendiani *et al.*, 2016a).

448 The comparison between the brown trout mtDNA genetic diversity observed along the two sides (east and
449 west) of the south-western Alps highlights the lack of a substantial genetic differentiation between the
450 samples collected in the upper Durance River (Rhône River basin) and the upper reaches of the Po River.
451 Most samples from upper Durance and Po basins were composed by a mixture of the ADporh-1 and MEcs-1
452 haplotypes (Table 2). The similarity in haplotype composition between the native brown trout populations of
453 the two-opposite sides of the south-western Alps was also supported by the hierarchical analysis of
454 molecular variance (AMOVA). In fact, when the samples of the upper Durance were grouped together with
455 the Po River group, the level of genetic variation explained between groups of populations increased from 14
456 to 21%, suggesting that the vicariant events between upper Durance and Po brown trout populations occurred
457 recently. In this regard, it is important to note that all the Durance collection sites extended in elevation from
458 1117 to 2077 m, that is, an altitudinal range occupied by the ice cover during the last glacial maximum (*e.g.*,
459 Figure 1), which implies the arrival of trouts after this period. Interestingly, the private ADRh-1 haplotype, a
460 mtDNA variant distant of one MS only from the haplotype ADporh-1, was detected in the Petit Buëch
461 stream, sited at the margin of the Durance glacier. Here, the milder climate condition of this part of Durance
462 basin could have allowed the maintenance of trout populations and represented a refuge for the haplotype
463 ADporh-1 and the centre of origin for the haplotype ADRh-1.

464 A possible explanation for the high genetic affinity observed between brown trout samples from the upper
465 Durance and Po River basins could be deduced when taking into account the effects, in terms of fish
466 exchange along the Alpine barrier, provided by the last deglaciation events occurred in the south-western
467 Alps. Likely, the first important factor that has drawn the present geographic structure of trout populations is
468 the spatial distribution of the ice cover during the LGM. During this period, the Durance paleo-glacier was
469 one of the most important Alpine glacier (Cossart, Braucher, Fort, Bourlès, & Carcaillet, 2008) (Figure 1).
470 On the other hand, along the Italian side of the south-western Alps, the ice cover appeared less extended
471 (Hughes, Woodward, & Gibbard, 2006; Szövényi *et al.*, 2009) (Figure 1). An explanation for the formation
472 of an unidirectional corridor between the two side of the south-western Alps could be related with the
473 formation of small ephemeral lakes and/or the swelling of connecting streams at the retreating edge of a
474 glacier that may allow watershed crossing and drainage switching by freshwater fish (Waters *et al.*, 2001).
475 This scenario was proposed to explain the colonization of the Lake Geneva (Rhône River basin) by bullhead
476 *Cottus gobio* migrants from the Rhine River basin during the last glacial retreat (Vonlanthen *et al.*, 2007).
477 Further, also the spatial distribution of the genetic diversity of *Galaxias platei* in Patagonia along the Andes
478 also represents a similar example showing the role of glacial retreat events in promoting fish migrations
479 across watersheds (Zemlak *et al.*, 2008; Habit *et al.*, 2010). Therefore, a scenario can be suggested where,
480 first, during the colder phases, brown trout survived in the ice-free tributaries of the south-western Alps (*i.e.*,
481 Maritime and Cottian Alps), and second, during the erosional events related to the ice melting (early

482 Holocene), an unidirectional corridor opened and permitted the colonization of the empty habitats of the
483 adjacent upper Durance river catchment from the Po watershed. Interestingly, along the south-western Alps,
484 the haplotype MEcs-1 was recently detected (Splendiani *et al.*, 2017) in a museum specimen collected in
485 1876 in the Lake Mont Cenis (1974 m a.s.l.) (Figure 1), a former small Alpine Lake (since 1921 the lake was
486 artificialized by the construction of a weir) of post-glacial origin belonging to the Dora Riparia River and
487 located near the divide between the Po and Rhone catchments.

488 Finally, the above scenario could be also proposed to explain the spatial pattern of genetic diversity that
489 has been observed in other freshwater organisms inhabiting the two sides of the south-western Alps. For
490 example, as in the case of the high genetic similarity observed between adjacent populations of *Cottus gobio*
491 (Šlechtová *et al.*, 2004), or similarly, the lack of genetic differentiation observed between adjacent
492 populations of *Austrapotamobius pallipes* (Stefani *et al.*, 2011).

493 TAXONOMIC IMPLICATIONS

494 As suggested above, in material and methods section, the main aim of the present study was not related
495 with the attempt to solve the well-known problem of the *S. trutta* complex systematic (Splendiani *et al.*,
496 2019b). However, the phylogeographic scenario of Mediterranean brown trout that emerged here represents
497 an opportunity to partially face with the above taxonomic issues. The trout from the two sides of the south-
498 western Alps are traditionally classified into three-four nominal species that we have used here for practical
499 reasons: *S. rhodanensis* (Rhône River basin) a contested species (Berrebi *et al.*, in prep.), *S. cettii* (a non-
500 valid name when used to indicate trout from the Tyrrhenian and Ligurian Sea draining rivers) and *S.*
501 *farioides* or *S. cenerinus* (depending on the authors, Adriatic draining rivers) (Figure 1). At mtDNA level,
502 none of the above nominal species showed a genetic distinctiveness able to justify the recognition of
503 different species. For example, the two Ligurian samples (SAN, putative *S. cettii* and BAR, putative *S.*
504 *farioides* - *S. cenerinus*) collected from the two sides of the Apennine chain, were both fixed for the
505 haplotype MEcs-1, that is a haplotype quite widespread in the study area, as well in the rest of the
506 Mediterranean rivers. More north, along the contact zone of the Rhône – Po River basins, the samples of the
507 Durance River (putative *S. rhodanensis*) showed a haplotype composition more similar to that observed
508 along the Italian side (putative *S. farioides* - *S. cenerinus*), respect to that shown by rest of the Rhône
509 samples as highlighted by the AMOVA analyses. Obviously, more sound conclusions should be drawn by
510 analysing also nuclear and morphological markers (see Ninua, Tarkhnishvili, & Gvazava, 2018 for similar
511 arumentations). These preliminary results however refute the traditional taxonomic position adopted until
512 now for the Mediterranean trout of the study area.

513 CONCLUSIONS

514 The main findings of this study highlight that brown trout should be considered native in the south-
515 western tributaries of the Po River basin. In this area, native brown trout survived the extreme climate phases
516 of Pleistocene. In this respect, the biological value of the south-western Alps for the conservation of the last
15

517 wild native Mediterranean trout population should be considered of primary importance. As a consequence,
518 the non-native statement and the non-intervention approach proposed by the Italian Association of
519 Freshwater Fish Ichthyologists (Zanetti *et al.*, 2013), based on a human conjectural man-mediated origin of
520 Italian slope trout from the Rhône River basin should be rejected. In addition, the weakness of the
521 allochthonous hypothesis is also sustained by the lack of historical records describing the occurrence of such
522 practices in the study area (*e.g.*, Splendiani *et al.*, 2019a).

523 In conclusion, caution must be exercised when planning conservation actions. For example, elsewhere, in
524 the Italian Alpine region, the massive introduction of domestic Mediterranean brown trout of Apennine
525 origin (AD, ME and MA haplotypes) started in the last five-ten years. In most cases, the outcomes of pivotal
526 genetic screening on local brown trout populations involved in these projects were not published, or even
527 never done. This probably occurred (and still occurs) in Italy because local administrations have transferred
528 to sport fishing associations the fully management of these practices. In these circumstances, the rationale of
529 these putative conservation actions has not been evaluable by the scientific community. Paradoxically,
530 conservation plans can even represent a further threat for the protection of wild native trout. As far as we
531 know, Mediterranean trout hatchery managers in Italy have not published (even considering grey
532 bibliography) the genetic description of their stocks. Recently, the genetic analysis of one of these putative
533 domestic Mediterranean stocks actually turned out as a mix of Mediterranean and Atlantic brown trout
534 (Splendiani *et al.*, 2019b). The irrational planning of massive stocking activities, even if carried out with
535 native brown trout, can introduce further risks related to the potential deleterious genetic effects of
536 supplementation programs (Fernández-Cebrián *et al.*, 2014). These latter ones can result in the breakdown of
537 the delicate equilibrium persisting in the incipient parapatric speciation process subsisting between native
538 brown trout and marble trout, as for example in south-western Alps (Giuffra *et al.*, 1994), and can affect the
539 adaptive genetic architecture of native genomes (Caputo, Giovannotti, Nisi Cerioni, Splendiani, & Olmo,
540 2009; Schenekar & Weiss, 2017). The situation is far better in the French side of the investigated area. In
541 France, more and more administrative organizations, under the supervision of Ecology Ministry, adopted
542 "patrimonial" management during the last twenty years. For this, a large part of the trout populations have
543 been analysed and published in France (<https://data.oreme.org/trout/home>) with nuclear and mitochondrial
544 markers driving conservation and stocking.

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737
738

739 **Figure captions**

740

741 **Figure 1**

742 Map showing the sampling locations of brown trout throughout the Rhône and the western part of the Po
 743 River basins. Color pie charts indicate the mtDNA haplotype frequency distribution of the main brown trout
 744 lineages. Numbers in bold within pie charts represent the Atlantic haplotype frequency (%). The distribution
 745 of the Alpine ice cover during the last glacial maximum is represented by the light blue area. Box at the top
 746 right shows the range of the nominal Mediterranean trout species as reported in Kottelat & Freyof (2007).
 747 † The geographic range of *Salmo cettii* according to Kottelat & Freyhof (2007), # the revised geographic
 748 range of *S. cettii* according to Splendiani *et al.* (2019b and references therein). Locations code colored
 749 differentially based on the river basin of origin.

750

751 **Figure 2**

752 Median-Joining Network showing the phylogenetic relationships subsisting between the 18 native brown
 753 trout haplotypes detected in this study (colored circles) and the brown trout haplotypes observed in
 754 previously published studies (grey circles, see also Table 1; the position of the haplotypes ADcs-11 and AD-
 755 Tyrrh-1 was reported because commented in the text). As regards the haplotypes observed in the study area,
 756 the size of each circle is proportional with the haplotype absolute frequency. Locations code colored
 757 differentially based on the river basin of origin.

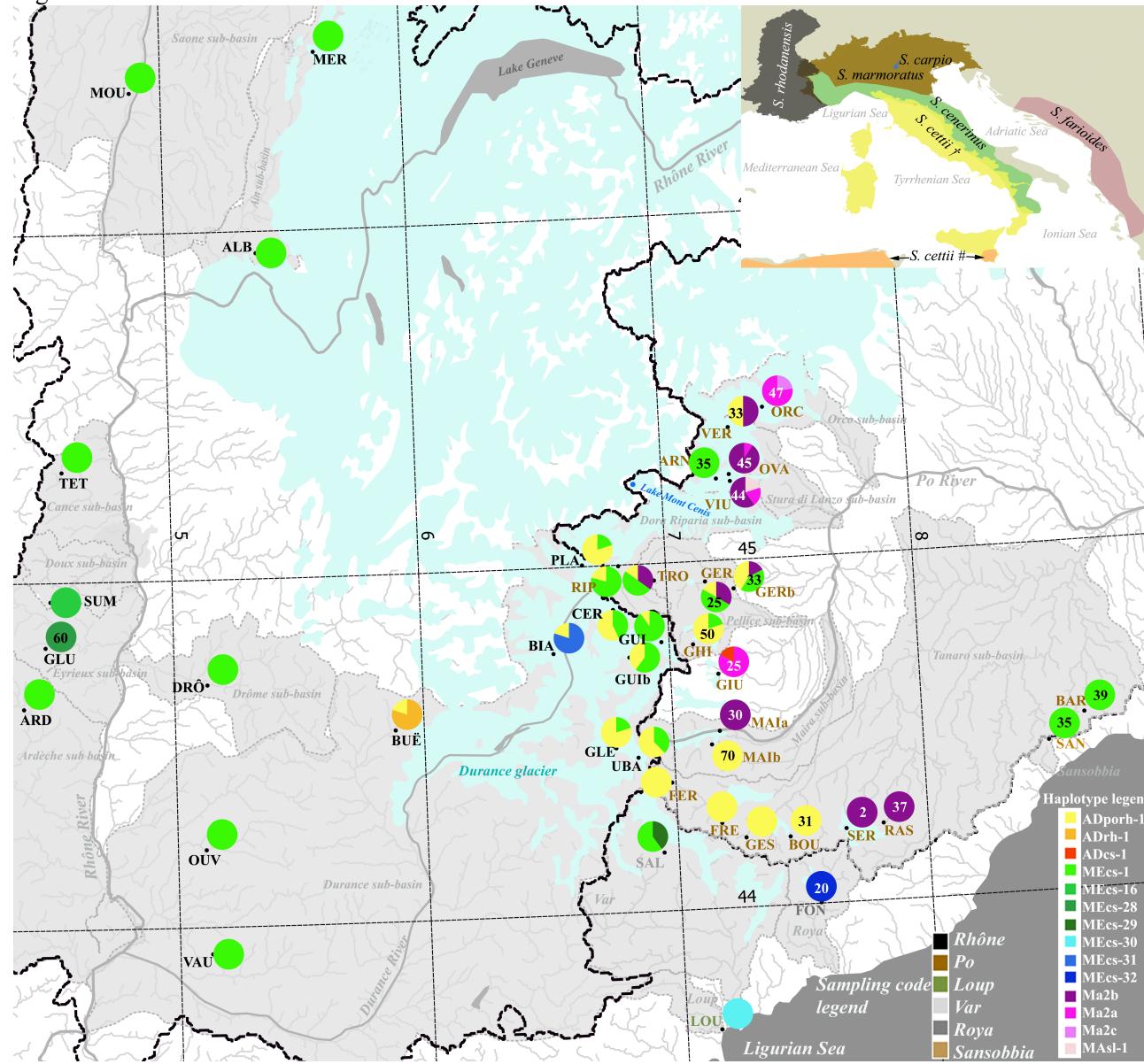
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759 **Figure 3**

760 Plot showing the probability density function (density) obtained comparing elevation, longitude and latitude
 761 with the mtDNA genetic composition of brown trout populations from the Rhône River basin (A and B) and
 762 from the Po River basin (C and D). The mtDNA lineage composition was represented by the following
 763 colored scheme: population characterized by the sole presence of ME haplotypes (Pure ME), in green; pure
 764 AD populations, in yellow; admixed AD-ME populations in orange; admixed AD-MA populations, in light
 765 blue and marble trout samples (from Giuffra *et al.*, 1994) in blue. To avoid confusion, other minor admixed
 766 population were not showed.

767

Fig. 1



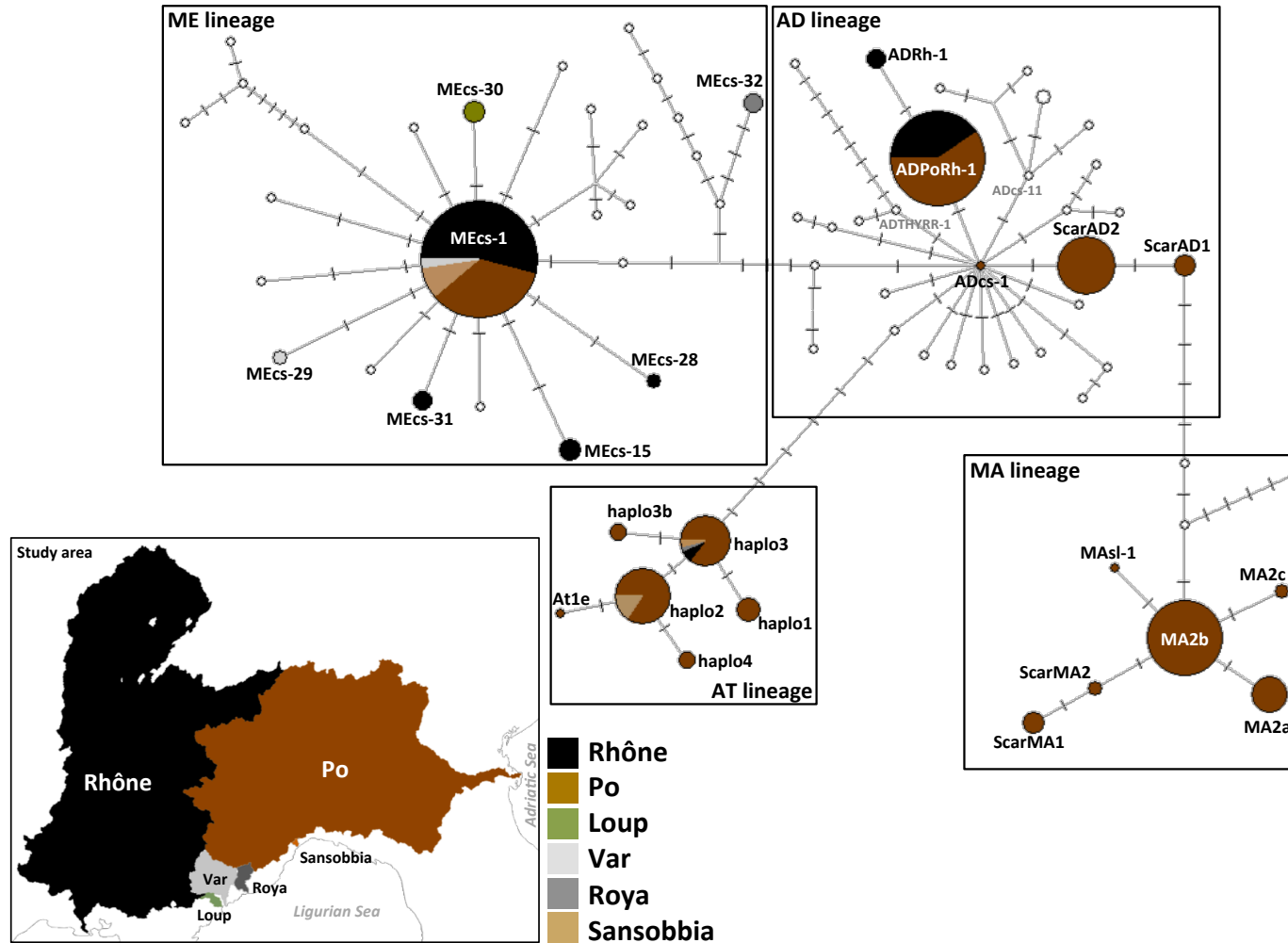


Fig. 3

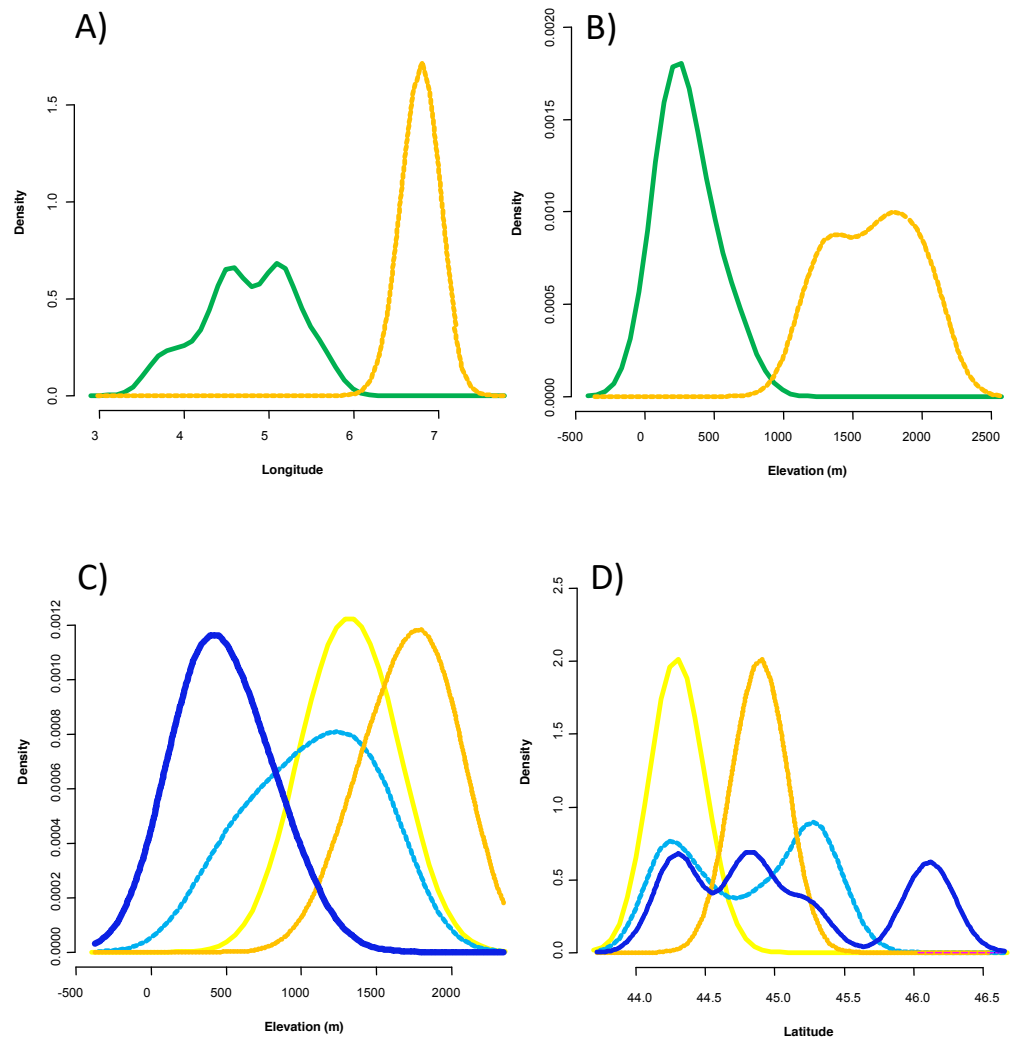


Table 1. Collection site information. Sampling code; Nominal taxon: *Salmo rhodanensis*, *S.rod*; *Salmo cenerinus*, *S.cen*; *Salmo fariodes*, *S. far.*; *Salmo cettii*, *S.cet.*; ?, no nominal proposed in literature; Sea drainage, Gulf of Lion, G; Ligurian, L, Adriatic, A; hierarchical description of the river network investigated (main-basin, sub-basin, stream); Sample size, No; Laboratories (Lab.) involved in genetic analyses, F = ISEM, Université de Montpellier (Lab.1) (Reynaud, Tougard & Berrebi 2011), and I = DiSVA,

Code	Taxon	Sea drainage	Main-basin	Sub-basin	Stream	No	Lab.	Lat.	Lon.	Elevation (m)
MER	<i>S. rod</i>	G	Rhône	Ain	Merlue	5	F	46.51	5.64	433
MOU	<i>S. rod</i>	G	Rhône	Saône	Mouge	5	F	46.40	4.87	176
ALB	<i>S. rod</i>	G	Rhône	Ain	Albarine	5	F	45.94	5.38	258
ORC	<i>S.cen/S.far</i>	A	Po	Orco		17	I	45.43	7.42	700
VER	<i>S.cen/S.far</i>	A	Po	Stura di Lanzo	Rio	18	I	45.38	7.28	1220
TET	<i>S. rod</i>	G	Rhône	Cance	Riotet	5	F	45.32	4.56	668
OVA	<i>S.cen/S.far</i>	A	Po	Stura di Lanzo	Rio	20	I	45.24	7.27	1480
ARN	<i>S.cen/S.far</i>	A	Po	Stura di Lanzo	Rio Arnas	10	I	45.24	7.20	1370
VIU	<i>S.cen/S.far</i>	A	Po	Stura di Lanzo	Stura di Viù	9	I	45.23	7.28	1540
RIP	<i>S.cen/S.far</i>	A	Po	Dora Riparia	Ripa	24	I	45.00	6.81	1900
PLA	<i>S. rod</i>	G	Rhône	Durance	Clarée	5	F	45.00	6.66	1484
TRO	<i>S.cen/S.far</i>	A	Po	Pellice	Chisone	20	I	44.95	6.95	1835
GER	<i>S.cen/S.far</i>	A	Po	Pellice	Germanasca	9	I	44.94	7.15	680
GERb	<i>S.cen/S.far</i>	A	Po	Pellice	Germanasca	16	I	44.92	7.27	750
CER	<i>S. rod</i>	G	Rhône	Durance	Cerveyrette	6	I	44.87	6.78	2077
GLU	<i>S. rod</i>	G	Rhône	Eyrieux	Gluyère	5	F	44.81	4.48	568
GUIb	<i>S. rod</i>	G	Rhône	Durance	Guil	20	I	44.77	6.97	1779
GHI	<i>S.cen/S.far</i>	A	Po	Pellice	Ghiacciard	10	I	44.76	7.09	1440
BIA	<i>S. rod</i>	G	Rhône	Durance	Biaisse	5	F	44.75	6.53	1200
GUI	<i>S. rod</i>	G	Rhône	Durance	Guil	5	F	44.73	6.84	1693
DRÔ	<i>S. rod</i>	G	Rhône	Drôme	Drôme	5	F	44.70	5.13	229
GIU	<i>S.cen/S.far</i>	A	Po		Rio Giulian	10	I	44.67	7.19	1120
ARD	<i>S. rod</i>	G	Rhône	Ardèche	Thines	5	F	44.64	4.39	217
BUË	<i>S. rod</i>	G	Rhône	Durance	Petit Buëch	5	F	44.55	5.88	1117
MAIa	<i>S.cen/S.far</i>	A	Po	Maira	Bedale di	10	I	44.51	7.18	950
BAR	<i>S.cen/S.far</i>	A	Po	Tanaro	Baracca	18	I	44.50	8.65	570
MAIb	<i>S.cen/S.far</i>	A	Po	Maira	Bedale	10	I	44.47	7.15	1180
GLE	<i>S. rod</i>	G	Rhône	Durance	Gleizolles	5	F	44.47	6.77	1319
UBA	<i>S. rod</i>	G	Rhône	Durance	Ubayette	15	I	44.44	6.85	1953
SAN	<i>S. cet</i>	L	Sansobbi			20	I	44.43	8.50	660
FER	<i>S.cen/S.far</i>	A	Po	Tanaro	Rio Ferriere	8	I	44.37	6.98	1480
FRE	<i>S.cen/S.far</i>	A	Po	Tanaro	Rio Freddo	8	I	44.24	7.17	1550
RAS	<i>S.cen/S.far</i>	A	Po	Tanaro	Rio	14	I	44.22	7.82	1100
OUV	<i>S. rod</i>	G	Rhône	Ouvèze	Ouvèze	5	F	44.22	5.11	222
SER	<i>S.cen/S.far</i>	A	Po	Tanaro	Rio	13	I	44.21	7.67	1280
GES	<i>S.cen/S.far</i>	A	Po	Tanaro	Gesso	4	I	44.20	7.27	1450
BOU	<i>S.cen/S.far</i>	A	Po	Tanaro	Bousset	13	I	44.20	7.45	1170
SAL	?	G	Var	Tinée	Tinée	5	F	44.17	6.94	1930
FON	?	G	Roya	Roya	Roya	5	F	44.00	7.55	426
SUM	<i>S. rod</i>	G	Rhône	Doux	Sumène	5	F	43.98	3.72	203
VAU	<i>S. rod</i>	G	Rhône	Ouvèze	Sorgue	5	F	43.92	5.13	89
LOU	?	G	Loup	Loup	Loup	5	F	43.65	7.13	7

Table 2. Haplotype frequency distribution at mtDNA control region in wild brown trout populations from the Rhône, Po and other neighbour Mediterranean rivers. Sampling code as in Table 1.

Code	Basin	MEcs1	MEcs15	MEcs28	MEcs29	MEcs30	MEcs31	MEcs32	ADp orh-1	ADrh-1	ADcs-1	MA2b	Ma2a	MA2c	MAsl-1	hap1	hap2	hap3	hap4	At1e	hap3b
MER	Rhône	5																			
MOU	Rhône	5																			
ALB	Rhône	5																			
ORC	Po												7	2			5				3
VER	Po								6			6				6					
TET	Rhône	5																			
OVA	Po											10	1				9				
ARN	Po	3										2					1	4			
VIU	Po											3	1		1		3	1			
PLA	Rhône	1							4												
RIP	Po	19							5												
TRO	Po	10							3			7									
GER	Po	3							1			2						3			
GERb	Po	5							5			2					2		1	1	
CER	Rhône	9							11												
GLU	Rhône			2														3			
GUIb	Rhône	18							2												
GHI	Po	1							4									4	1		
BIA	Rhône						4		1												
GUI	Rhône	3							2												
DRÔ	Rhône	5																			
GIU	Po										1		5				2	2			
ARD	Rhône	5																			
BUË	Rhône								1	4											
MA1a	Po											7						3			
BAR	Po	11																7			
GIE	Rhône	1							4												
MA1b	Po								3								1	5	1		
UBA	Rhône	8							13												
SAN	Sansob.	13															6	1			
FER	Po								8												
FRE	Po								8												
OUV	Rhône	5																			
RAS	Po											10					4				
SER	Po											12						0			
GES	Po								4												
BOU	Po								9								4				
SAL	Var	3			2																
FON	Roya							4										1			
SUM	Rhône		5																		
VAU	Rhône	5																			
LOU	Loup					5															

Table S1. Schematic summary of the Mediterranean taxa in the *Salmo trutta* complex of the study area. Reference to mtDNA lineages as follows: 1, Bernatchez 2001; 2, Berrebi et al. 2019; 3, Fabiani et al. 2017; 4, Fruciano et al. 2014; 5, Giuffra et al. 1994; 6, 1996; 7, Gratton et al. 2014; 8, Lerceteanu-Köhler et al. 2013; Maric et al. (2017) 9, Meraner & Gandolfi 2018; 10, Sabatini et al. 2011; 11, Schöffmann et al. 2007; 12, Snoj et al. 2011; 13, Splendiani et al. 2006; 14, 2007; 15, 2017; 16, Zaccara et al. 2015; 17, Marić et al. 2017; 18, Tougard et al. 2018.; 19, this study.

Taxon	Geographical range	mtDNA lineages	mtDNA references	Nomenclature change
<i>Salmo rhodanensis</i> Fowler, 1974	Rhône River basin	AD, ME	1, 19	Questioned the validity of specie rank in this study and in Berrebi et al. (in prep.).
<i>S. cettii</i> Rafinesque Schmaltz 1810	Described for the S.E. Sicily but extended to the Apennines (Tyrrhenian side) by Kottelat & Freyhof (2007)	AT (southern clade)	2, 4, 11, 18	Considered a senior synonym of <i>S. macrostigma</i> by Splendiani et al. (2019).
<i>S. macrostigma</i> (Dumeril, 1858)	Described for Algeria, but extended also to Apennines (Tyrrhenian side), Sicily, Sardinia (Italy) and Corsica (France) by Sommani (1951)	AD, MA, ME (According to Sommani, 1951) AT (southern clade) (according to Splendiani et al., 2019)	1-4,7-13, 16	Considered a junior synonym of <i>S.cettii</i> by Splendiani et al. (2019).
<i>S. cenerinus</i> Chiereghini, 1847	Described for the Gulf of Trieste, but extended to the Apennines (Adriatic side) by Kottelat & Freyhof (2007)	AD, MA, ME (according to Kottelat & Freyhof, 2007) MA (according to Bianco & Delmastro (2011)	1, 2, 7, 9, 13-15	Considered by Bianco & Delmastro (2011) as a junior synonym for <i>Salmo marmoratus</i> Cuvier, 1829.
<i>S. farioides</i> Karaman, 1938	Described for south-western Balkans but extended to the Padano-Venetian district by Bianco & Delmastro (2011)	AD	17	Considered a valid name for the native trout of the Drin River basin (S.E. Balkans) by Marić et al. (2017) and extended by Bianco & Delmastro (2011) to the Adriatic slope of the Apennine.
<i>S. carpio</i> L., 1758	Lake Garda	AD, ME, MA	1, 5-7, 9, 13	Considered a subspecies of <i>S. trutta</i> by Tortonese (1970)
<i>S. marmoratus</i> Cuvier, 1829	Po River Basin and Balkan Peninsula	MA	1, 5-7, 9, 13-15	Considered a subspecies of <i>S. trutta</i> by Tortonese (1970)

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Table S2: List of haplotypes retrieved from GenBank, with accession numbers and their distributions.

Haplotype	Accession number	Distribution – country/drainages
haplotype 1 (ATcs1)	AF273086	² Denmark (Skals), Norway (Bjornes Lake, Sima), Spain (hatchery stocks), ⁴ Spain (Garona), France (Gulf of Biscay), Iceland (Skorradalsvatn), British Isles (Coquet, Wear, Lune, Melvin), ¹¹ continental Italy (IT), Sardinia, Corsica
haplotype 2 (ATcs2)	AF273087	² Denmark (Skals, Karup), Norway (Guddal, Sima), Spain (hatchery stocks), ⁵ France (Gulf of Biscay), British Isles (Coquet, Stour, Rother, Fowey, Teifi, Conwy, Loch Romoch), Russia (Nilima, Vorobiev), ¹¹ Sardinia, Corsica
haplotype 3 (ATcs3)	AF274574	² Denmark (Skals), Norway (Bjornes Lake, Guddal, Sima), Spain (hatchery stocks), ⁴ Spain (Garona), France (Gulf of Biscay), British Isles (Coquet, Wear, Rother, Teifi, Conwy, Melvin), ¹¹ continental Italy, Sardinia, Calabria
haplotype 4 (ATcs4)	AF274575	² Denmark (Skals, Karup), Norway (Bjornes Lake, Guddal, Sima), Spain (hatchery stocks), ⁴ France (Gulf of Biscay), British Isles (Lune), ¹¹ Sardinia, Calabria
Hap3b	MK948037	²⁰ Italy (Po)
At1e	DQ841192	⁸ Italy (Adige), ²⁰ Italy (Po)
ADcs1	AY836330	³ Spain (Ter, Ebre, Túría, Segura), ⁶ Bulgaria (Struma, Mesta, Maritza), ^{1, 7} Macedonia (Prespa Lake, Vardar), ²⁰ Italy (Po)
ADcs2	AY836331	³ Spain (Guadalfeo)
ADcs3	AY836332	³ Spain (Ebre)
ADcs4	AY836333	³ Spain (Ter)
ADcs5	AY836334	³ Spain (Guadalfeo)
ADcs6	AY836335	³ Spain (Ebre, Guadalfeo)
ADcs7	AY836336	³ Spain (Ebre)
ADcs8	AY836337	³ Spain (Ebre)
ADcs9	AY836338	³ Spain (Turia)
ADcs10	AY836339	³ Spain (Guadalfeo)
ADcs11	AY836340	³ Greece (Alfios), ¹⁰ Montenegro (Skadar Lake), ¹² Albania (Drin, Skumbini, Cermit), ¹³ Montenegro (Zeta, Morača, Cijevna)
ADcs15	AY836344	⁴ France - Corsica (Corsica stream)
ADcs16	AY836345	³ Spain (Ebre)
ADcs17	AY836346	³ Spain (Ebre)
ADcs18	AY836347	³ Spain (Guadalquivir)
ADcs19	AY836348	³ Spain (Guadalquivir)
ADcs20	AY836349	³ Greece (Tripotamos), ¹³ Bulgaria (Maritza)
ADporh-1	MK948034	²⁰ France (Rhône), Italy (Po)
ADrh-1	MK948035	²⁰ France (Rhône)
AD-Tyrrh1	KX450257	¹¹ Corsica, Sardinia, Calabria, continental Italy (Aniene River)
AD-Tyrrh2	KX450258	¹¹ Corsica, continental Italy (Aniene River)
AD-Tyrrh3	KX450259	¹¹ Calabria (Diga Giulia River)

AD-Tyrrh4	KX450260	¹¹ Sardinia, continental Italy (Aniene River)
AD-Tyrrh5	KX450261	¹¹ continental Italy (Nera River)
AD-Tyrrh6	KX450262	¹¹ continental Italy (Nera River)
AD-zls-01	MG194729	¹⁵ Italy (Liri)
AD-zls-01	MG194729	¹⁵ Italy (Liri)
ScarAD-1 (C208)	KJ834848	¹⁶ Italy (Garda Lake)
ScarAD-2 (C021)	KJ834822	¹⁶ Italy (Garda Lake)
MEcs1	AY836350	³ Spain (Ter, Llobregat, Ebre, Mijares, Palancia, Túria, Segura), ⁵ Croatia (Krka), ²⁰ France (Rhône, Var), Italy (Po, Sansobbia)
MEcs4	AY836353	³ Spain (Ter)
MEcs6	AY836355	³ Spain (Ebre)
MEcs7	AY836356	³ Spain (Ter)
MEcs8	AY836357	³ Spain (Túria)
MEcs9	AY836358	³ Spain (Túria)
MEcs10	AY836359	³ Spain (Ebre)
MEcs11	AY836360	³ Spain (Túria)
MEcs12	AY836361	³ Spain (Ter)
MEcs13	AY836362	³ Spain (Ebre)
MEcs14	AY836363	³ Spain (Ebre, Guadalfeo)
MEcs15	AY836364	³ Spain (Ebre), ²⁰ France (Rhône)
MEcs16	unpublished	¹⁷ Spain
MEcs17	unpublished	¹⁷ Spain
MEcs18	unpublished	¹⁷ Spain
MEcs20	unpublished	¹⁷ Spain
MEcs21	unpublished	¹⁷ Spain
MEcs22	unpublished	¹⁷ Spain
MEcs23	MG970273	¹⁸ Spain (Cardener)
MEcs25	MG970274	¹⁸ Spain (Cardener)
MEcs26	MG970275	¹⁸ Spain (Cardener)
MEcs27	MG970276	¹⁸ Spain (Cardener)
MEcs28	MK948029	²⁰ France (Rhône)
MEcs29	MK948030	²⁰ France (Var)
MEcs30	MK948031	²⁰ France (Loup)
MEcs31	MK948032	²⁰ France (Rhône)
MEcs32	MK948033	²⁰ France (Roya)
ME-nin63	MG194732	¹⁵ Italy (Sisto)
MAcs1	AY836365	³ Slovenia (Soča), ⁶ Greece (Aliakmon), ⁸ Italy (Adige), Italy (Po)
Ma2a	DQ841189	⁸ Italy (Adige), ²⁰ North Italy (Po)
Ma2b	DQ841190	⁸ Italy (Adige), ²⁰ North Italy (Po)
Ma2c	JQ582461	⁹ Italy (Adige), ²⁰ North Italy (Po)
ScarMA1 (C201)	KJ834841	¹⁶ Italy (Garda Lake)

ScarMA2 (C024)	KJ834825	¹⁶ Italy (Garda Lake)
marm1	KJ834770	¹⁶ North Italy (Po, Adige), Slovenia (Soca River)
Mak1	JX846931	¹⁹ Croatia (Krka)
MAsl-1	MK948036	²⁰ North Italy (Po)

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- ²⁰ Present study (new haplotypes)

Table S3. Demographic indices calculated for three brown trout mtDNA lineages based on control region sequence analysis.

	Neutrality test			Mismatch analysis		Model
	F _s	D	R ₂	SSD	r	
ME	-2.874*	-1.897*	0.124***	0.027**	0.066*	Expansion
AD	-2.126	-2.147*	0.121***	0.023**	0.076**	Expansion
MA	-1.019	-1.070	0.188***	0.039	0.111	Stable

F_s = Fu's F statistic, D = Tajima's D statistic, R₂ = Ramos-Osisns and Rozas statistic, SSD = sum of standard deviations of mismatch distribution, r = raggedness index of mismatch distribution.
 ***p<0.001, **p<0.01, *p<0.05.

Substitution rate (%)	Node	TMRCA (MY)	Lower 95% HPD (MY)	Upper 95% HPD (MY)
0.75	ADporh1-ADrh1	0.151	0.011	0.099
	AD	0.278	0.170	0.391
	ME	0.267	0.166	0.372
	MA	0.122	0.172	0.205
1	ADporh1-ADrh1	0.120	0.016	0.086
	AD	0.212	0.129	0.298
	ME	0.191	0.122	0.265
	MA	0.117	0.051	0.193

Table S4. Time to the most common ancestor (TMRCA) estimates for the Mediterranean brown trout lineages AD, ME and MA and for the sub-clade ADporh-1, ADrh-1 with 95% highest probability density (HPD) intervals.