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

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

Abstract

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

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

INTRODUCTION

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

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

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

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

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

MATERIAL AND METHODS

SAMPLING DESIGN

The *S. trutta* complex is characterized by a puzzling pattern of geographical forms probably underlying the taxonomic inflation reported in literature with the description of nearly 50 *Salmo* species (Tougard *et al.*, 2018). The description of the native trout taxa previously identified (*e.g.*, Kottelat & Freyof, 2007) in the study area have been summarised in Table S1. In this study, for practical reasons, the following terms will be used hereafter: (1) ‘‘marble trout’’ for *S. marmoratus* specimens (generally fixed for MA haplotypes), (2) ‘‘native brown trout’’ for the individuals showing both a fario phenotype and native haplotypes of the Mediterranean area (that are, haplotypes belonging to the lineages AD, ME or MA), (3) “Lake Garda Carpione” to indicate *S. carpio* specimens and (4) ‘‘Atlantic brown trout’’ to indicate *S. trutta* specimens hosting domestic and non-native haplotypes (AT lineage). The sampling efforts were mainly focused along the south-western Alps, as for example, i) in the upper Durance, that belongs to the Rhône basin, but is located adjacent to the Po River basin along the French side of the south-western Alps, and ii) along the main sub-basins of the Po River basin, as regards the Italian side of the south-western Alps. For comparisons, further samples from other sites of the Rhône River basin and from neighbour minor Mediterranean independent rivers were also included, for example, the rivers Var, Loup, Roya and Sansobbia. The sampling size ranged from 4 to 24 with a mean value of 10 trout per sample. The total number of analysed *S. trutta* was 412 specimens that were collected from 42 sites (Figure 1, Table 1).

DNA EXTRACTION

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

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

POPULATION STRUCTURE, DEMOGRAPHIC HISTORY, AND MOLECULAR DATING

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

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

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

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

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

RESULTS

HAPLOTYPE CLASSIFICATION

Before starting with sequence analyses, the PolyT region of the CR was considered of a constant length of 14bp in all sequences. In fact, such region is likely to be unstable and thus characterized by a high mutation rate, showing frequent 14-T variants. In this circumstance, sequence stretch length identity, could be the mere consequence of homoplasy and would not represent a real phylogenetic signal. On the whole, an alignment of 981 bp was obtained, from which 21 haplotypes emerged (Table 2). The observed haplotypes belonged to four main mtDNA lineages: AD, ME, MA and AT (*sensu* Bernatchez *et al.*, 1992 or Bernatchez, 2001). The AD lineage was represented by three haplotypes: ADporh-1 and ADrh-1, observed for the first time in this study (GenBank accession numbers, respectively, MK948034 and MK948035) and ADcs-1 already described in literature (Cortey, Pla & García-Marín, 2004). As far for the ME lineage, seven haplotypes were observed, two of which already detected in other studies, that are: MEcs-1 and MEcs-15 (*e.g.*, Cortey et al., 2004) and the other five were detected for the first time in this study and named as follow: MEcs-28 to MEcs-32 (from MK948029 to MK948033). The MA lineage displayed a new haplotype named MAsl-1 (MK948036), and three mtDNA variants already detected in previous studies, namely Ma2a, Ma2b and Ma2c (Meraner, Baric, Pelster, & Dalla Via, 2007; Meraner, Gratton, Baraldi & Gandolfi, 2013). Finally, the Atlantic lineage was represented by six haplotypes, five of them were already detected in Mediterranean rivers and classified as haplotypes of hatchery origin, that are: haplotype 1 to haplotype 4 (Cortey & García-Marín, 2002) and the haplotype At1e, (Meraner *et al.*, 2007), while one haplotype was described for the first time in this study (haplotype 3b, MK948037). This latter haplotype should be also considered of hatchery origin due to high similarity with the domestic variant haplotype 3 (Table 2, Figure 2).

HAPLOTYPE SPATIAL DISTRIBUTION

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

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

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

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

MISMATCH ANALYSIS AND DIVERGENCE TIME ESTIMATES

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

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

AMOVA

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

KERNEL DENSITY (KD) BROWN TROUT LINEAGE DISTRIBUTION

Within the Rhône River drainage, the highest density of samples characterized by the sole presence of ME haplotypes was found between 4.5 – 5.5 E longitudes (Figure 3A). Unfortunately, only one sample was characterized by the sole presence of AD haplotypes (BUË), therefore, KD analysis was not applicable in this case. On the contrary, the rest of the Rhône samples (*i.e.*, samples from the Durance sub-basin) were composed by a mixture of AD and ME haplotypes. In this study, a total of 91 trouts originated from the Rhône River basin in France, among them, 31 specimens showed the AD haplotypes. All these latter trouts came from the Durance sub-basin (samples BUË, BIA, CLA, UBA and GUI, see Table 2). The last two rivers (UBA and GUI) are flowing in France directly from the France-Italy boundary. This kind of populations peaked around 7.0 E longitude, corresponding with the upper part of the Durance River (Figure 3A). When KD was carried out to relate brown trout lineages distribution with elevation, a similar net separation between different categories of samples was observed. For example, in the Rhône River, populations fixed for ME haplotypes were most abundant around 0 – 500 m (Figure 3B), whereas admixed populations (AD – ME) showed higher values of probability between 1500 – 2000 m. Within the Po River drainage, the different categories of populations defined based on mtDNA lineage composition, appeared clearly stratified along an altitudinal cline. Pure ME populations were detected only in one case (BAR, Tanaro River, Ligurian Apennine). Admixed AD – ME populations were, however, the most common and reached a density peak around 1600 – 2000 m. Pure AD populations peaked slightly lower, around 1400 m (Figure 3C). Around this latter quote, peaked both pure brown trout samples fixed for MA haplotypes and admixed MA – AD brown trout samples. Further downstream (*c*. 450 m), pure marble trout samples (MA) were abundant (data from Giuffra *et al*., 1994). Finally, a pattern of brown trout mtDNA lineage density distribution along the south-western Alps was also evident along a latitudinal gradient (Figure 3D). For example, pure AD samples appeared most abundant around 44.0 – 44.5 N, roughly corresponding with the Maritime Alps (Italian side), while admixed populations (AD – ME) peaked around 45.0 N (*i.e.*, Cottian Alps).

DISCUSSION

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

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

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

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

The TMRCA analysis suggest a main expansion of the ME lineage around 191,000-267,000 years ago, roughly corresponding with the last (III) Mindel-Riss Interglacial, and resulting slightly more ancient respect previous estimations (Cortey *et al*., 2004). Pleistocene Interglacial warming periods have been regarded as phases of isolation in small headwaters for Mediterranean brown trout populations, thus promoting genetic signatures within lineages (*e.g.*, Sanz, 2018 and references therein). On the other hand, during glaciations, colder climate conditions may have triggered sea-ward migratory tactics in Mediterranean brown trout populations, as highlighted by several paleontological evidences (Muñoz & Casadevall, 1997; Splendiani *et al.*, 2016b; Splendiani *et al.*, 2020). Therefore, thanks to a seaward migratory route, the expansion of brown trout in the Mediterranean area was possible. The colonisation of northern Corsica Island by the ME lineage during the last glaciation is an example of this expansion (Gauthier & Berrebi, 2007). The spatial distribution of the MEcs-1 haplotype suggests a potential eastward dispersion, from the Rhône River outlet to the Var River (SAL, Gulf of Lion) where this haplotype was found and more eastern to the Sansobbia River (SAN, Ligurian Sea) (Figure 1, Table 2). Then, when the ME lineage reached the upper part of the Ligurian rivers, the colonization of the Po River basin was likely possible thanks to river capture events occurred along the Ligurian Apennine chain. This scenario could explain the finding of the MEcs-1 haplotype in the Rio Baracca (BAR, Po River basin, see also Figure 1, Table 2). Interestingly, the role played by the hydrographical captures of the upstream portions of Mediterranean rivers was also proposed in the literature to explain, for example, the exchange of Duero haplotypes between rivers flowing along opposite slope of the Cantabrian mountains (Iberian Peninsula) (Vera *et al.*, 2015), as well as to explain the native occurrence of the Danubian haplotypes within marble trout populations of the Sôca River (Berrebi, Jesenšek, & Crivelli, 2017). Concerning the study area, the phylogeographic history of the Italian vairone (*Telestes muticellus*) in populations of west Liguria (Marchetto *et al.*, 2010) could also be explained invoking the presence of biological corridors opened by ancient Mediterranean river captures.

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

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

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

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

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

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

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

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

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

THE ROLE OF THE SOUTH-WESTERN ALPS AS AN ASYMMETRICAL BIOLOGICAL CORRIDOR FOR BROWN TROUT LINEAGES

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

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

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

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

TAXONOMIC IMPLICATIONS

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

CONCLUSIONS

The main findings of this study highlight that brown trout should be considered native in the south-western tributaries of the Po River basin. In this area, native brown trout survived the extreme climate phases of Pleistocene. In this respect, the biological value of the south-western Alps for the conservation of the last wild native Mediterranean trout population should be considered of primary importance. As a consequence, the non-native statement and the non-intervention approach proposed by the Italian Association of Freshwater Fish Ichthyologists (Zanetti *et al*., 2013), based on a human conjectural man-mediated origin of Italian slope trout from the Rhône River basin should be rejected. In addition, the weakness of the allochthonous hypothesis is also sustained by the lack of historical records describing the occurrence of such practices in the study area (*e.g*., Splendiani *et al*., 2019a).

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

REFERENCES

Arnold ML. 1997. *Natural Hybridization and Evolution*. Oxford University Press, Oxford, UK

Bandelt HJ, Forster P & Rohl A. **1994**. Median-Joining Networks for Inferring Intraspecic Phylogenies. *Molecular Biology* **16**: 37–48.

Bernatchez L. 2001. The evolutionary history of brown trout (*Salmo trutta* L.) inferred from phylogeographic, nested clade, and mismatch analyses of mitochondrial DNA variation. *Evolution; international journal of organic evolution* **55**: 351–79.

Bernatchez L, Guyomard R & Bonhomme F. **1992**. DNA sequence variation of the mitochondrial control region among geographically and morphologically remote European brown trout *Salmo trutta* populations. *Molecular Ecology* **1**: 161–173.

Bernatchez L & Danzmann G. 1993. Congruence in control-region sequence and restriction-site variation in mitochondrial DNA of brook charr (*Salvelinus fontinalis* Mitchill). *Molecular Biology and Evolution* **10**, 1002-1014.

Berrebi P, Povz M, Jesensek D, Cattaneo-Berrebi G & Crivelli AJ. **2000**. The genetic diversity of native, stocked and hybrid populations of marble trout in the Soca river, Slovenia. *Heredity* **85**: 277–287.

Berrebi P, Caputo Barucchi V, Splendiani A, Muracciole S, Sabatini A, Palmas F, Tougard C, Arculeo M & Marić S. **2019**. Brown trout (*Salmo trutta* L.) high genetic diversity around the Tyrrhenian Sea as revealed by nuclear and mitochondrial markers. *Hydrobiologia* **826:**209–231**.**

Berrebi P, Marić S, Snoj A & Hasegawa K. 2020. Brown trout in Japan -introduction history, distribution and genetic structure. *Konoledge and Management of Aquatic Ecosystems* **421**, 18.

Berrebi P, Jesenšek D & Crivelli AJ. **2017**. Natural and domestic introgressions in the marble trout population of Soča River (Slovenia). *Hydrobiologia* **785**: 277–291.

Brandley MC, Schmitz A & Reeder TW. **2005**. Partitioned Bayesian analyses, partition choice, and the phylogenetic relationships of scincid lizards. *Systematic Biology* **54**: 373–390.

Brown RP & Yang Z. **2011**. Rate variation and estimation of divergence times using strict and relaxed clocks. *BMC Evolutionary Biology* **11**.

Budy P, Thiede GP, Lobón-Cerviá J, Fernandez GG, Mchugh P, Mcintosh A, Voøllestad LA, Becares E & Jellyman P. **2013**. Limitation and facilitation of one of the world's most invasive fish: An intercontinental comparison. *Ecology* **94**: 356–367.

Caputo V, Giovannotti M, Nisi Cerioni P, Splendiani A & Olmo E. **2009**. Chromosomal study of native and hatchery trouts from Italy (*Salmo trutta* complex, Salmonidae): Conventional and FISH analysis. *Cytogenetic and Genome Research* **124**.

Casalis G. 1833. Dizionario geografico storico-statisticocommerciale degli Stati di S.M. il Re di Sardegna. Vol. I. Torino: Maspero.

Casalis G. 1852. Dizionario geografico storico-statisticocommerciale degli Stati di S.M. il Re di Sardegna. Vol. XXII. Torino:Maspero.

Cortey M & García-Marín JL. **2002**. Evidence for phylogeographically informative sequence variation in the mitochondrial control region of Atlantic brown trout. *Journal of Fish Biology* **60**: 1058–1063.

Cortey M, Pla C & García-Marín JL. **2004**. Historical biogeography of Mediterranean trout. *Molecular Phylogenetics and Evolution* **33**: 831–844.

Cossart E, Braucher R, Fort M, Bourlès DL & Carcaillet J. **2008**. Slope instability in relation to glacial debuttressing in alpine areas (Upper Durance catchment, southeastern France): Evidence from field data and 10Be cosmic ray exposure ages. *Geomorphology* **95**: 3–26.

Estoup A, Largiader CR, Perrot E & Chourrot D. 1996. Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes. *Molecular Marine Biology and Biotechnology* **5**: 295-298.

Excoffier L. **2004**. Patterns of DNA sequence diversity and genetic structure after a range expansion: Lessons from the infinite-island model. *Molecular Ecology* **13**: 853–864.

Excoffier L & Lischer HEL. **2010**. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**: 564–567.

Fernández-Cebrián R, Araguas RM, Sanz N, García-Marín JL & Fraser D. **2014**. Genetic risks of supplementing trout populations with native stocks: a simulation case study from current Pyrenean populations. *Canadian Journal of Fisheries and Aquatic Sciences* **71**: 1243–1255.

Fernández-García JL, Carranza J, Martínez JG & Randi E. **2014**. Mitochondrial D-loop phylogeny signals two native Iberian red deer (*Cervus elaphus*) Lineages genetically different to Western and Eastern European red deer and infers human-mediated translocations. *Biodiversity and Conservation* **23**: 537–554.

Fu YX. **1997**. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147**: 915–925.

Gauthier A & Berrebi P. 2007. La colonisation de l'ile par différentes souches de truite. In: *Guide de gestion de la truite macrostigma*, 4-10.

Giuffra E, Bernatchez L & Guyomard R. **1994**. Mitochondrial Control Region and Protein-Coding Genes Sequence Variation Among Phenotypic Forms of Brown Trout Salmo-Trutta From Northern Italy. *Molecular Ecology* **3**: 161–171.

Gratton P, Allegrucci G, Sbordoni V & Gandolfi A. **2014**. The evolutionary jigsaw puzzle of the surviving trout (*Salmo trutta* L. complex) diversity in the italian region. a multilocus bayesian approach. *Molecular Phylogenetics and Evolution* **79**: 292–304.

Habit EM, Walde SJ, Zemlak TS, Ruzzante DE & Carrea C. **2010**. Surviving historical Patagonian landscapes and climate: molecular insights from Galaxias maculatus. *BMC Evolutionary Biology* **10**: 67.

Hayashi K. 1991. PCR-SSCP: A simple and sensitive method for detection of mutations in the genomic DNA. *Genome Research* **1**: 34–38.

Hughes PD, Woodward JC & Gibbard PL. 2006. Quaternary glacial history of the Mediterranean mountains. *Progress in Physical Geography* **30**: 334–364.

Kottelat M & Freyhof J (2007) Handbook of european fresh-water fishes. Publications Kottelat, Cornol

Larkin MA, Blackshields G, Brown NP, Chenna R, Mcgettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ & Higgins DG. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947–2948.

Larson G & Fuller DQ. 2014. The Evolution of Animal Domestication. *Annual Review of Ecology, Evolution, and Systematics* **45**: 115–136.

Lavery S, Moritz C, & Fielder DR. 1996. Genetic patterns suggest exponential population growth in a declining species. *Molecular Biology and Evolution* **13**:1106–1113.

Leprieur F, Beauchard O, Blanchet S, Oberdorff T & Brosse S. 2008. Fish invasions in the world's river systems: When natural processes are blurred by human activities. *PLoS Biology* **6**: 0404–0410.

Losos JB, Arnold SJ, Bejerano G, Brodie ED, Hibbett D, Hoekstra HE, Mindell DP, Monteiro A, Moritz C, Orr HA, Petrov DA, Renner SS, Ricklefs RE, Soltis PS & Turner TL. 2013. Evolutionary Biology for the 21st Century. *PLoS Biology* **11(1)**: 1-8.

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

Meraner A, Baric S, Pelster B & Dalla Via J. 2007. Trout (*Salmo trutta*) mitochondrial DNA polymorphism in the centre of the marble trout distribution area. *Hydrobiologia* **579**: 337–349.

Meraner A, Gratton P, Baraldi F & Gandolfi A. 2013. Nothing but a trace left? Autochthony and conservation status of Northern Adriatic Salmo trutta inferred from PCR multiplexing, mtDNA control region sequencing and microsatellite analysis. *Hydrobiologia* **702**: 201–213.

Miró A & Ventura M. 2013. Historical use, fishing management and lake characteristics explain the presence of non-native trout in Pyrenean lakes: Implications for conservation. *Biological Conservation* **167**: 17–24.

Muñoz M & Casadevall M. 1997. Fish remains from Arbreda Cave (Serinya Girona), northeast Spain, and their palaeoecological significance. *Journal of Quaternary Science* **12**: 111–115.

Ninua L, Tarkhnishvili D & Gvazava E. 2018. Phylogeography and taxonomic status of trout and salmon from the Ponto-Caspian drainages, with inferences on European Brown Trout evolution and taxonomy. *Ecology and Evolution* **8**: 2645–2658.

Oliver J. 2014. Evolutionary history of the brown trout, *Salmo trutta* L., in France. First-year MEME research project Université Montpellier II April-June 2014.

Posada D. 2008. jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution* **25**: 1253–1256.

Purvis A & Hector A. 2000. Getting the measure of biodiversity. *Nature* **405**: 212–219.

Rambaut A, Drummond AJ, Xie D, Baele G & Suchard MA. 2018. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology* **67**: 901–904.

Ramos-Onsins SE & Rozas J. 2002. Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution* **19**: 2092–2100.

Redpath SM, Young J, Evely A, Adams WM, Sutherland WJ, Whitehouse A, Amar A, Lambert RA, Linnell JDC, Watt A & Gutiérrez RJ. 2013. Understanding and managing conservation conflicts. *Trends in Ecology and Evolution* **28**: 100–109.

Rondinini C, Battistoni A, Peronace V, Teofili (compilatori) C. 2013. Lista Rossa IUCN dei Vertebrati Italiani. Roma: Comitato Italiano IUCN e Ministero dell'Ambiente e della Tutela del Territorio e del Mare.

Rozas J, Ferrer-Mata A, Sanchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE & Sanchez-Gracia A. 2017. DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution* **34**: 3299–3302.

Sanz N. 2018. Phylogeographic history of brown trout: A review. In: Lobón Cerviá J, Sanz N, editors. Brown trout: Biology, ecology and management. Hoboken, NJ: John Wiley & Sons Ltd. pp. 65–102.

Schenekar T, Lerceteau-Köhler E & Weiss S. 2014. Fine-scale phylogeographic contact zone in Austrian brown trout Salmo trutta reveals multiple waves of post-glacial colonization and a pre-dominance of natural versus anthropogenic admixture. *Conservation Genetics* **15**: 561–572.

Schenekar T & Weiss S. 2017. Selection and genetic drift in captive versus wild populations: an assessment of neutral and adaptive (MHC-linked) genetic variation in wild and hatchery brown trout (*Salmo trutta*) populations. *Conservation Genetics* **18**: 1011–1022.

Shackleton RT, Larson BMH, Novoa A, Richardson DM & Kull CA. 2019. The human and social dimensions of invasion science and management. *Journal of Environmental Management* **229**: 1–9.

Siccardi S. 1996. Elena la Regina mai dimenticata. Torino: Paoline.

Šlechtová V, Bohlen J, Freyhof J, Persat H & Delmastro GB. 2004. The Alps as barrier to dispersal in cold-adapted freshwater fishes? Phylogeographic history and taxonomic status of the bullhead in the Adriatic freshwater drainage. *Molecular Phylogenetics and Evolution* **33**: 225–239.

Snoj A, Marić S, Berrebi P, Crivelli AJ, Shumka S & Sušnik S. 2009. Genetic architecture of trout from Albania as revealed by mtDNA control region variation. *Genetics Selection Evolution* **41**: 22.

Snoj A, Glamuzina B, Razpet A, Zablocki J, Bogut I, Lerceteau-Köhler E, Pojskić N & Sušnik S. 2010. Resolving taxonomic uncertainties using molecular systematics: *Salmo dentex* and the Balkan trout community. *Hydrobiologia* **651**: 199–212.

Sønstebø JH, Borgstrøm R & Heun M. 2007. Genetic structure of brown trout (*Salmo trutta* L.) from the Hardangervidda mountain plateau (Norway) analyzed by microsatellite DNA: A basis for conservation guidelines. *Conservation Genetics* **8**: 33–44.

Splendiani A, Ruggeri P, Giovannotti M, Pesaresi S, Occhipinti G, Fioravanti T, Lorenzoni M, Nisi Cerioni P & Caputo Barucchi V. 2016a. Alien brown trout invasion of the Italian peninsula: the role of geological, climate and anthropogenic factors. *Biological Invasions* **18**: 2029–2044.

Splendiani A, Fioravanti T, Giovannotti M, Negri A, Ruggeri P, Olivieri L, Cerioni PN, Lorenzoni M & Barucchi VC. 2016b. The effects of paleoclimatic events on mediterranean trout: Preliminary evidences from ancient DNA. *PLoS ONE* **11**.

Splendiani A, Fioravanti T, Giovannotti M, Olivieri L, Ruggeri P, Nisi Cerioni P, Vanni S, Enrichetti F & Caputo Barucchi V. 2017. Museum samples could help to reconstruct the original distribution of Salmo trutta complex in Italy. *Journal of Fish Biology* **90**: 2443–2451.

Splendiani A, Palmas F, Sabatini A & Caputo V 2019a. The name of the trout: considerations on the taxonomic status of the *Salmo trutta* L., 1758 complex (Osteichthyes: Salmonidae) in Italy. *The European Zoological Journal* **86**: 432–442.

Splendiani A, Giovannotti M, Righi T, Fioravanti T, Cerioni PN, Lorenzoni M, Carosi A, La Porta G & Barucchi VC. 2019b. Introgression despite protection: the case of native brown trout in Natura 2000 network in Italy. *Conservation Genetics*.

Splendiani A, Fioravanti T, Ruggeri P, Giovannotti M, Carosi A, Marconi M, Lorenzoni M, Righi T, Nisi Cerioni P & Caputo Barucchi V. 2020. Life history and genetic characterisation of sea trout *Salmo trutta* in the Adriatic Sea. *Freshwater Biology* **65**: 460–473.

Stefani F, Anzani A & Marieni A. 2019. Echoes from the past: a genetic trace of native brown trout in the Italian Alps. *Environmental Biology of Fishes* **102**: 1327–1335.

Suchard MA, Lemey P, Baele G, Ayres DL, Drummond AJ & Rambaut A. 2018. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evolution* **4**: 1–5.

Sušnik S, Weiss S, Odak T, Delling B, Treer T & Snoj A. 2007. Reticulate evolution: Ancient introgression of the Adriatic brown trout mtDNA in softmouth trout *Salmo obtusirostris* (Teleostei: Salmonidae). *Biological Journal of the Linnean Society* **90**: 139–152.

Szövényi P, Arroyo K, Guggisberg A & Conti E. 2009. Effects of Pleistocene glaciations on the genetic structure of *Saxifraga florulenta* (Saxifragaceae), a rare endemic of the Maritime Alps. *Taxon* **58**: 532–543.

Tajima F. 1989. Statistical model for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**: 585-595.

Tiberti R & Splendiani A. 2019. Management of a highly unlikely native fish: The case of arctic charr *Salvelinus alpinus* from the Southern Alps. *Aquatic Conservation: Marine and Freshwater Ecosystems* **29**: 312-320.

Tougard C, Justy F, Guinand B, Douzery EJP & Berrebi P. 2018. *Salmo macrostigma* (Teleostei, Salmonidae): Nothing more than a brown trout (*S. trutta*) lineage? *Journal of Fish Biology* **93**: 302–310.

Vera M, García-Marín JL, Martinez P & Bouza C. 2015. Phylogenetic diversity within the endemic brown trout Duero lineage: Implications for conservation and management. *Marine and Freshwater Research* **66**: 1066–1071.

Vera M, Bouza C, Casanova A, Heras S, Martinez P & García-Marín JL. 2019. Identification of an endemic Mediterranean brown trout mtDNA group within a highly perturbed aquatic system, the Llobregat

River (NE Spain). **9**: 277–291.

Vonlanthen P, Excoffier L, Bittner D, Persat H, Neuenschwander S & Largiadèr CR. **2007**. Genetic analysis of potential postglacial watershed crossings in Central Europe by the bullhead (*Cottus gobio* L.). *Molecular Ecology* **16**: 4572–4584.

Waters JM, Craw D, Youngson JH & Wallis GP. **2001**. Genes meet geology: Fish phylogeographic pattern reflects ancient, rather than modern, drainage connections. *Evolution* **55**: 1844–1851.

Zanetti M, Nonnis Marzano F, Lorenzoni M. 2013. I salmonidi italiani: Linee guida per la conservazione della biodiversità. Technical report. Available: http://www.aiiad.it/sito/images/docs/sistematica/GRUPPO%20DI%20LAVORO%20SALMONIDI_RELAZIONE%20FINALE.pdf.

Zemlak TS, Habit EM, Walde SJ, Battini MA, Adams EDM & Ruzzante DE. **2008**. Across the southern Andes on fin: Glacial refugia, drainage reversals and a secondary contact zone revealed by the phylogeographical signal of *Galaxias platei* in Patagonia. *Molecular Ecology* **17**: 5049–5061.

Figure captions

Figure 1

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

Figure 2

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

Figure 3

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

Fig. 1

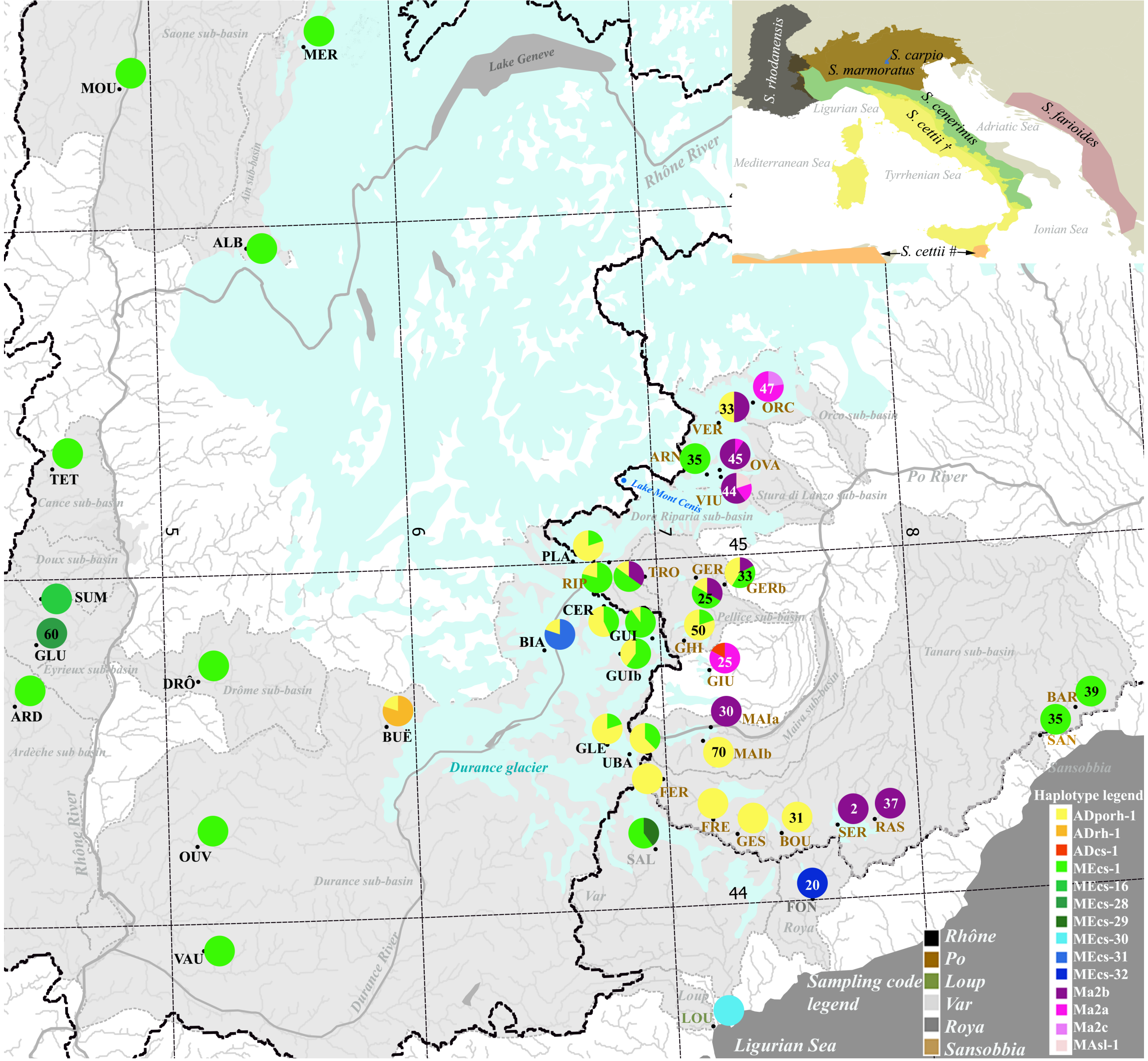

Saone sub-basin
MER
MOU
Lake Geneve
Rhône River
Ain sub-basin
ALB
TET
Cance sub-basin
Doux sub-basin
SUM
60
GLU
Eyrieux sub-basin
ARD
Ardèche sub basin
DRÔ
Drôme sub-basin
BUË
OUV
Durance sub-basin
VAU
Durance River
Rhône River
Durance glacier
Var
Loup
LOU
5
6
7
8
45
44
47
ORC
33
VER
ARN
35
45
OVA
44
VIU
Lake Mont Cenis
Dora Riparia sub-basin
Orco sub-basin
Stura di Lanzo sub-basin
Po River
PLA
RIP
TRO
GER
33
GERb
25
CER
BIA
GUI
50
Pellice sub-basin
GHI
25
GIU
GUIb
30
MAIa
Maira sub-basin
70
MAIb
GLE
UBA
FER
FRE
GES
31
BOU
2
SER
37
RAS
SAL
20
FON
Roya
Tanaro sub-basin
BAR
39
35
SAN
Sansobbia
Ligurian Sea
S. rhodanensis
S. carpio
S. marmoratus
Ligurian Sea
S. cenerinus
S. cettii †
Adriatic Sea
S. farioides
Mediterranean Sea
Tyrrhenian Sea
Ionian Sea
S. cettii #
Sampling code legend
Rhône
Po
Loup
Var
Roya
Sansobbia
Haplotype legend
ADporh-1
ADrh-1
ADcs-1
MEcs-1
MEcs-16
MEcs-28
MEcs-29
MEcs-30
MEcs-31
MEcs-32
Ma2b
Ma2a
Ma2c
MAsl-1

Fig. 2

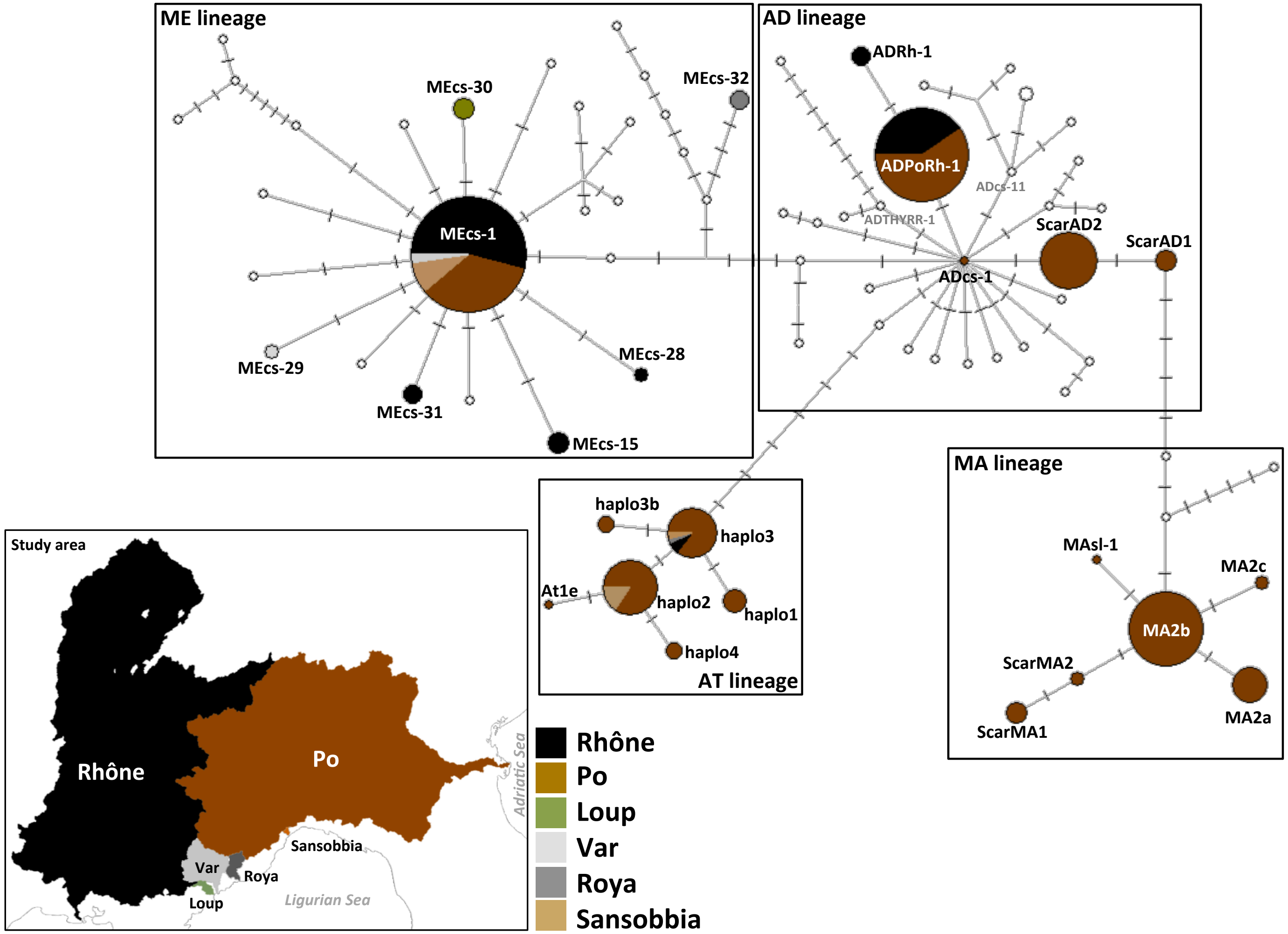
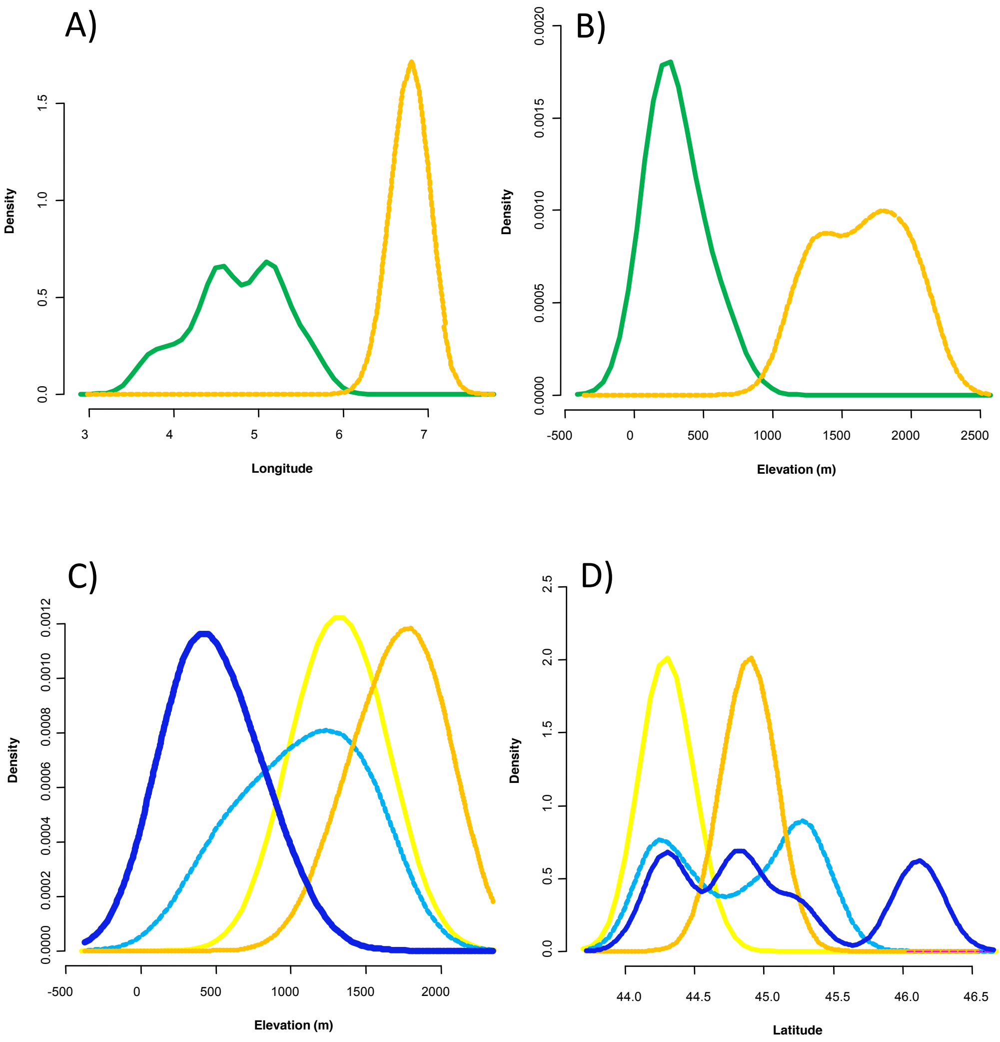

ME lineage
MEcs-30
MEcs-32
MEcs-1
MEcs-29
MEcs-31
MEcs-28
MEcs-15
AD lineage
ADRh-1
ADPoRh-1
ADcs-11
ADTHYRR-1
ScarAD2
ScarAD1
ADcs-1
MA lineage
MAsl-1
MA2c
MA2b
ScarMA2
ScarMA1
MA2a
haplo3b
haplo3
At1e
haplo2
haplo1
haplo4
AT lineage
Study area
Rhône
Po
Adriatic Sea
Sansobbia
Var
Roya
Loup
Ligurian Sea
■ Rhône
■ Po
■ Loup
■ Var
■ Roya
■ Sansobbia

Fig. 3



A)
Density
0.0
0.5
1.0
1.5
3
4
5
6
7
Longitude
B)
Density
0.0000
0.0005
0.0010
0.0015
0.0020
-500
0
500
1000
1500
2000
2500
Elevation (m)
C)
Density
0.0000
0.0002
0.0004
0.0006
0.0008
0.0010
0.0012
-500
0
500
1000
1500
2000
Elevation (m)
D)
Density
0.0
0.5
1.0
1.5
2.0
2.5
44.0
44.5
45.0
45.5
46.0
46.5
Latitude

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	*S. rod*	G	Rhône	Ain	Merlue	5	F	46.51	5.64	433
MOU	*S. rod*	G	Rhône	Saône	Mouge	5	F	46.40	4.87	176
ALB	*S. rod*	G	Rhône	Ain	Albarine	5	F	45.94	5.38	258
ORC	*S.cen/S.far*	A	Po	Orco		17	I	45.43	7.42	700
VER	*S.cen/S.far*	A	Po	Stura di Lanzo	Rio	18	I	45.38	7.28	1220
TET	*S. rod*	G	Rhône	Cance	Riotet	5	F	45.32	4.56	668
OVA	*S.cen/S.far*	A	Po	Stura di Lanzo	Rio	20	I	45.24	7.27	1480
ARN	*S.cen/S.far*	A	Po	Stura di Lanzo	Rio Arnas	10	I	45.24	7.20	1370
VIU	*S.cen/S.far*	A	Po	Stura di Lanzo	Stura di Viù	9	I	45.23	7.28	1540
RIP	*S.cen/S.far*	A	Po	Dora Riparia	Ripa	24	I	45.00	6.81	1900
PLA	*S. rod*	G	Rhône	Durance	Clarée	5	F	45.00	6.66	1484
TRO	*S.cen/S.far*	A	Po	Pellice	Chisone	20	I	44.95	6.95	1835
GER	*S.cen/S.far*	A	Po	Pellice	Germanasca	9	I	44.94	7.15	680
GERb	*S.cen/S.far*	A	Po	Pellice	Germanasca	16	I	44.92	7.27	750
CER	*S. rod*	G	Rhône	Durance	Cerveyrette	6	I	44.87	6.78	2077
GLU	*S. rod*	G	Rhône	Eyrieux	Gluyère	5	F	44.81	4.48	568
GUIb	*S. rod*	G	Rhône	Durance	Guil	20	I	44.77	6.97	1779
GHI	*S.cen/S.far*	A	Po	Pellice	Ghiacciard	10	I	44.76	7.09	1440
BIA	*S. rod*	G	Rhône	Durance	Biaysse	5	F	44.75	6.53	1200
GUI	*S. rod*	G	Rhône	Durance	Guil	5	F	44.73	6.84	1693
DRÔ	*S. rod*	G	Rhône	Drôme	Drôme	5	F	44.70	5.13	229
GIU	*S.cen/S.far*	A	Po		Rio Giulian	10	I	44.67	7.19	1120
ARD	*S. rod*	G	Rhône	Ardèche	Thines	5	F	44.64	4.39	217
BUË	*S. rod*	G	Rhône	Durance	Petit Buëch	5	F	44.55	5.88	1117
MAIa	*S.cen/S.far*	A	Po	Maira	Bedale di	10	I	44.51	7.18	950
BAR	*S.cen/S.far*	A	Po	Tanaro	Baracca	18	I	44.50	8.65	570
MAIb	*S.cen/S.far*	A	Po	Maira	Bedale	10	I	44.47	7.15	1180
GLE	*S. rod*	G	Rhône	Durance	Gleizolles	5	F	44.47	6.77	1319
UBA	*S. rod*	G	Rhône	Durance	Ubayette	15	I	44.44	6.85	1953
SAN	*S. cet*	L	Sansobbi			20	I	44.43	8.50	660
FER	*S.cen/S.far*	A	Po	Tanaro	Rio Ferriere	8	I	44.37	6.98	1480
FRE	*S.cen/S.far*	A	Po	Tanaro	Rio Freddo	8	I	44.24	7.17	1550
RAS	*S.cen/S.far*	A	Po	Tanaro	Rio	14	I	44.22	7.82	1100
OUV	*S. rod*	G	Rhône	Ouvèze	Ouvèze	5	F	44.22	5.11	222
SER	*S.cen/S.far*	A	Po	Tanaro	Rio	13	I	44.21	7.67	1280
GES	*S.cen/S.far*	A	Po	Tanaro	Gesso	4	I	44.20	7.27	1450
BOU	*S.cen/S.far*	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	*S. rod*	G	Rhône	Doux	Sumène	5	F	43.98	3.72	203
VAU	*S. rod*	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	ADporh-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											
MAIa	Po											7						3			
BAR	Po	11																7			
GlE	Rhône	1							4												
MAIb	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, Lerceteau-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**
Salmo rhodanensis 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.).
S. cettii 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 *S. macrostigma* by Splendiani *et al*. (2019).
S. macrostigma (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 *S.cettii* by Splendiani *et al*. (2019).
S. cenerinus 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 *Salmo marmoratus* Cuvier, 1829.
S. farioides 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.
S. carpio L., 1758	Lake Garda	AD, ME, MA	1, 5-7, 9, 13	Considered a subspecies of *S. trutta* by Tortonese (1970)
S. marmoratus Cuvier, 1829	Po River Basin and Balkan Peninsula	MA	1, 5-7, 9, 13-15	Considered a subspecies of *S. trutta* by Tortonese (1970)

Table S1 Bibliography

Bernatchez L. 2001. The evolutionary history of brown trout (*Salmo trutta* L.) inferred from phylogeographic, nested clade, and mismatch analyses of mitochondrial DNA variation. Evolution 55:351–379.

Berrebi P, Caputo Barucchi V, Splendiani A, Muracciole S, Sabatini A, Palmas F, Tougard C, Arculeo M, Marić S. 2019. Brown trout (*Salmo trutta* L.) high genetic diversity around the Tyrrhenian Sea as revealed by nuclear and mitochondrial markers. Hydrobiologia 826:209–231. DOI: 10.1007/s10750-018-3734-5.

Bianco PG, Delmastro GB. 2011. Recenti novità tassonomiche riguardanti i pesci d'acqua dolce autoctoni in Italia e descrizione di una nuova specie di luccio. In: De Filippo G, editor. Researches on wildlife conservation, Vol. 2. USA: IGF publication. pp. 14.(suppl.).

Fabiani A, Gratton P, Zappes IA, Seminara M, D'Orsi A, Sbordoni V, Allegrucci G. 2017. Investigating the genetic structure of trout from the Garden of Ninfa (central Italy): Suggestions for conservation and management. Fisheries Management and Ecology 2017:1–11.

Fruciano C, Pappalardo AM, Tigano C, Ferrito V. 2014. Phylogeographical relationships of Sicilian brown trout and the effects of genetic introgression on morphospace occupation. Biological Journal of the Linnean Society 112:387–398.

Giuffra E, Bernatchez L, Guyomard R. 1994. Mitochondrial control region and protein-coding genes sequence variation among phenotypic forms of brown trout *Salmo trutta* from northern Italy. Molecular Ecology 3:161–171.

Giuffra E,GuyomardR,FornerisG. 1996. Phylogenetic relationships and introgression patterns between incipient parapatric species of Italian brown trout (*Salmo trutta* L. complex).Molecular Ecology 5:207–220.

Gratton P, Allegrucci G, Sbordoni V, Gandolfi A. 2014. The evolutionary jigsaw puzzle of the surviving trout (*Salmo trutta* L. complex) diversity in the Italian region. A multilocus Bayesian approach. Molecular Phylogenetics and Evolution79:292–304. DOI: 10.1016/j.ympev.2014.06.022.

Kottelat M, Freyhof J. 2007. Handbook of European fresh-water fishes. Publications Kottelat, Cornol

Lerceteau-Köhler E, Schliewen U, Kopun T, Weiss S. 2013. Genetic variation in brown trout *Salmo trutta* across the Danube, Rhine, and Elbe headwaters: A failure of the phylogeographic paradigm? BMC Evolutionary Biology 13:176.

Marić S, Sušnik S, Schöffmann J, Kostov V, Snoj A. 2017. Phylogeography of stream-dwelling trout in the Republic of Macedonia and a molecular genetic basis for revision of the taxonomy proposed by S. Karaman. Hydrobiologia 785:249–260.

Meraner A, Gandolfi A. 2018. Genetics of the genus *Salmo* in Italy: Evolutionary history, population structure, molecular ecology and conservation. In: Lobón-Cerviá J, Sanz N, editors. Brown trout: Biology, ecology and management.Hoboken, NJ: John Wiley & Sons Ltd. pp. 65–102.

Sabatini A, Cannas R, Follesa MC, Palmas F, Manunza A, Matta GA, Pendugiu A, Serra P, Cau A. 2011. Genetic characterization and artificial reproduction attempt of endemic Sardinian trout *Salmo trutta* L., 1758 (Osteichthyes, Salmonidae): Experiences in captivity. Italian Journal of Zoology 78:20–26. DOI: 10.1080/11250003.2010.497171.

Schöffmann J, Sušnik S, Snoj A. 2007. Phylogenetic origin of *Salmo trutta* L 1758 from Sicily, based on mitochondrial and nuclear DNA analyses. Hydrobiologia 575:51–55.

Snoj A, Marić S, Sušnik Bajec S, Berrebi P, Janjani S, Schöffmann J. 2011. Phylogeographic structure and demographic patterns of brown trout in North-West Africa. Molecular Phylogenetics and Evolution 61:203–211.

Splendiani A, Giovannotti M, Nisi Cerioni P, Caniglia ML, Caputo V. 2006. Phylogeographic inferences on the native brown trout mtDNA variation in central Italy. Italian Journal of Zoology 72:179–189.

Splendiani A, Giovannotti M, Caniglia ML, Nisi Cerioni P, Battistella S, Caputo V. 2007. Presenza di aplotipi “marmoratus” in popolazioni di trota fario (*Salmo trutta* L., 1758) dell’Italia centrale: Transfaunazione o paleointrogressione? Atti Del Congresso Congiunto AIOL-SItE 2007:89–93.

Splendiani A, Fioravanti T, Giovannotti M, Olivieri L, Ruggeri P, Nisi Cerioni P, Vanni S, Enrichetti F, Caputo Barucchi V. 2017. Museum samples could help to reconstruct the original distribution of *Salmo trutta* complex in Italy. Journal of Fish Biology 90:2443–2451.

Tougard C, Justy F, Guinand B, et al. 2018. *Salmo macrostigma* (Teleostei, Salmonidae): Nothing more than a brown trout (*S. trutta*) lineage? Journal of Fish Biology 93:302–310.

Zaccara S, Trasforini S, Antognazza CM, Puzzi C, Robert Britton J, Crosa G. 2015. Morphological and genetic characterization of Sardinian trout *Salmo cettii* Rafinesque, 1810 and their conservation implications. Hydrobiologia 760:205–223.

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, Túria, 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]Montengro (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, Míjares, 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)

[1] Berrebi, P., Tougard, C., Dubois, S., Shao, Zh., Koutseri, I., Petkovski, S. & Crivelli, A. J. 2013. Genetic diversity and conservation of the Prespa trout in the Balkans. International Journal of Molecular Sciences 14: 23454–23470.

[2] Cortey, M. & García-Marín, J. L. 2002. Evidence for phylogeographically informative sequence variation in the mitochondrial control region of Atlantic brown trout. Journal of Fish Biology 60: 1058–1063.

[3] Cortey, M., Pla, C. & García-Marín, J. L. 2004. Historical biogeography of Mediterranean trout. Molecular Phylogenetics and Evolution 33: 831–844.

[4] Cortey, M., Vera, M., Pla, C. & García-Marín, J. L., 2009. Northern and Southern expansions of Atlantic brown trout (*Salmo trutta*) populations during the Pleistocene. Biological Journal of the Linnean Society 97: 904–917.

[5] Jadan, M., Strunjak-Perović, I., Topić Popović, N. & Čož-Rakovac, R. 2015. Three major phylogenetic lineages of brown trout (*Salmo trutta* Linnaeus, 1758) in the Krka River system (Croatia) revealed by complete mitochondrial DNA control region sequencing. Journal of Applied Ichthyology 31: 192–196.

[6] Kohout, J., Šedivá, A., Apostolou, A., Stefanov, T., Marić, S., Gaffaroğlu, M. & Šlechta, V. 2013. Genetic diversity and phylogenetic origin of brown trout *Salmo trutta* populations in eastern Balkans. Biologia 68: 1229–1237.

[7] Marić, S., Sušnik Bajec, S., Schöffmann, J., Kostov, V. & Snoj, A. (2017). Phylogeography of stream-dwelling trout in the Republic of Macedonia and a molecular genetic basis for revision of the taxonomy proposed by S. Karaman. Hydrobiologia 785: 249–260.

[8] Meraner, A., Baric, S., Pelster, B. & Dalla-Via, J. 2007. Trout (*Salmo trutta*) mitochondrial DNA polymorphism in the centre of the marble trout distribution area. Hydrobiologia 579: 337–349.

[9] Meraner, A., Gratton, P., Baraldi, F. & Gandolfi, A. 2013. Nothing but a trace left? Autochthony and conservation status of Northern Adriatic *Salmo trutta* inferred from PCR multiplexing, mtDNA control region sequencing and microsatellite analysis. Hydrobiologia 702: 201–213.

[10] Sušnik, S., Snoj, A., Wilson, I. F., Mrdak, D. & Weiss, S. 2007. Historical demography of brown trout (*Salmo trutta*) in the Adriatic drainage including the putative *S. letnica* endemic to Lake Ohrid. Molecular Phylogenetics and Evolution 44: 63–76.

[11] Berrebi P, Caputo Barucchi V, Splendiani A, Muracciole S, Sabatini A, Palmas F, Tougard C, Arculeo M, Maric S (2019) Brown trout (*Salmo trutta* L.) high genetic diversity around the Tyrrhenian Sea as revealed by nuclear and mitochondrial markers. Hydrobiologia 826:209–231.

[12] Snoj, A., S. Marić, P. Berrebi, A. Crivelli, S. Shumka & S. Sušnik, 2009. Genetic architecture of trout from Albania as revealed by mtDNA control region variation. Genetics Selection Evolution, 41: 22

[13] Snoj, A., Glamuzina, B., Razpet, A., Zablocki, J., Bogut, I., Lerceteau-Köhler, E., Pojskić , N., Sušnik, S., 2010. Resolving taxonomic uncertainties using molecular systematics: *Salmo dentex* and the Balkan trout community. Hydrobiologia 651, 651, 199–212.

[14] Kohout J., Šedivá A., Apostolou A., Stefanov T., Marić S., Gaffaroğlu M., et al. (2013). Genetic diversity and phylogenetic origin of brown trout *Salmo trutta* populations in eastern Balkans. Biologia, 68, 1229–1237.

[15] Fabiani A, Gratton P, Zappes IA, Seminara M, D'Orsi A, Sbordoni V, Allegrucci G (2018) Investigating the genetic structure of trout from the Garden of Ninfa (central Italy): suggestions for conservation and management. Fishery Management and Ecology 25:1–11.

[16] Gratton, P., Allegrucci, G., Sbordoni, V. & Gandolfi, A. (2014). The evolutionary jigsaw puzzle of the surviving trout (*Salmo trutta* L. complex) diversity in the Italian region. A multilocus Bayesian approach. Molecular Phylogenetics and Evolution 79, 292–304.

[17] Vera, M. (2006). Estudio de las variaciones espaciales y temporales de la diversidad genética de la trucha común, *Salmo trutta*, en ríos de la Península Ibérica. PhD thesis, Girona, 22 de Marzo de 2006.

[18] Vera, M., Bouza, A., Casanova, S., Heras, P., Martínez, J., García-Marín, L. (2019). Identification of an endemic Mediterranean brown trout mtDNA group within a highly perturbed aquatic system, the Llobregat River (NE Spain). Hydrobiologia 827, 277–291.

[19] Jadan, M., Strunjak-Perović, I., Topić Popović, N. & Čož-Rakovac, R. (2015). Three major phylogenetic lineages of brown trout (*Salmo trutta* Linnaeus, 1758) in the Krka River system (Croatia) revealed by complete mitochondrial DNA control region sequencing. Journal of Applied Ichthyology, 31, 192–196.

[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
	Fs	**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

Fs = 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.