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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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The role of the 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 the south-western Alps was tested. A total of 412 individuals were analysed 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.

ADDITIONAL KEYWORDS: alpine barrier – biological corridors – conservation genetics – ice cover – *Salmo trutta* complex.

INTRODUCTION

The study of the phylogeographic history of a species represents a fundamental step to understand the factors producing genetic diversity both within and

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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, attempts to identify native distribution of an organism on the basis of phylogenetic studies could be made difficult because of long standing human-mediated natural species range alterations. For instance, in the case of trout, fish stocking erodes genetic diversity or eliminates original local adaptations by hydridization of native populations with domesticated strains (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). Regarding freshwater fish, the first written historical records date back to major human-mediated introductions in Roman times and successively in the medieval period (Sønstebø *et al.*, 2007; Miró & Ventura, 2013). The above ancient freshwater fish translocations were promoted by food purposes, whereas, nowadays, freshwater fish represent 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 among 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 brown trout conservation status as near threatened, and the French red list (IUCN, 2010) considers the trout status as low concern. This is a somewhat optimistic statement 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 seems contradictory. 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 Italian Association of Freshwater Fish Ichthyologists (AIIAD) guidelines for Italian salmonid management proposes to adopt a passive conservation approach for the putative native populations of the south-western Alps (Zanetti *et al.*, 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 for these trout translocations would be related to 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 the 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 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 *Salmo 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 summarized in Supporting Information (Table S1). In this study, for practical reasons, the following terms will be used hereafter: (1) "marble trout" for *Salmo marmoratus* specimens (generally fixed for marmoratus (MA) haplotypes, *sensu* Bernatchez); (2) "native brown trout" for the individuals showing both a fario phenotype (that is, presence of a high number of red and dark spots and presence of parr bands also in adult specimens) and native haplotypes of the Mediterranean area (that are, haplotypes belonging to the lineges Adriatic (AD), Mediterranean (ME) or MA, *sensu* Bernatchez); (3) "Lake Garda Carpione" to indicate *Salmo 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, 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 neighbouring 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 (Fig. 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 (Lab. 1) consisted of a Chelex/Proteinase K-based protocol described by Estoup *et al.* (1996). A small piece of fin was incubated overnight at 56 °C in 195 µL 5% Chelex 100 Resin (Biorad) solution containing 50 mM Tris-HCL (pH 7) and 500 µg/ml 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'-CCCAAAGCT AAAATTCTAAAT-3') and FST (5'-GCTTTAG TTAAGCTACGC-3') primers (Cortey & García-Marín, 2002). Each 50 µL reaction included 0.4 µM each primer (Eurofins MWG Operon), dNTPs (2 mM each), 2 mM $MgCl_2$, 10 µL (5X) PCR buffer, 1 U Taq polymerase (GoTaq® Promega), and about 50 ng 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), 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-3'; HN20, 5'-GTGTTATGCTTTAGTTAAAGC-3'). Screening of mtDNA genetic variability was conducted via Single-Strand Conformation Polymorphism (SSCP) analysis. As 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 the *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 Supporting Information, Table S2, for more details). The genealogical relationship among haplotypes was depicted using a Median-Joining Network (Bandelt *et al.*, 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 v.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 v.3.5 (Excoffier & Lischer, 2010). Briefly, a significantly negative Tajima's D and Fs, and a significantly positive

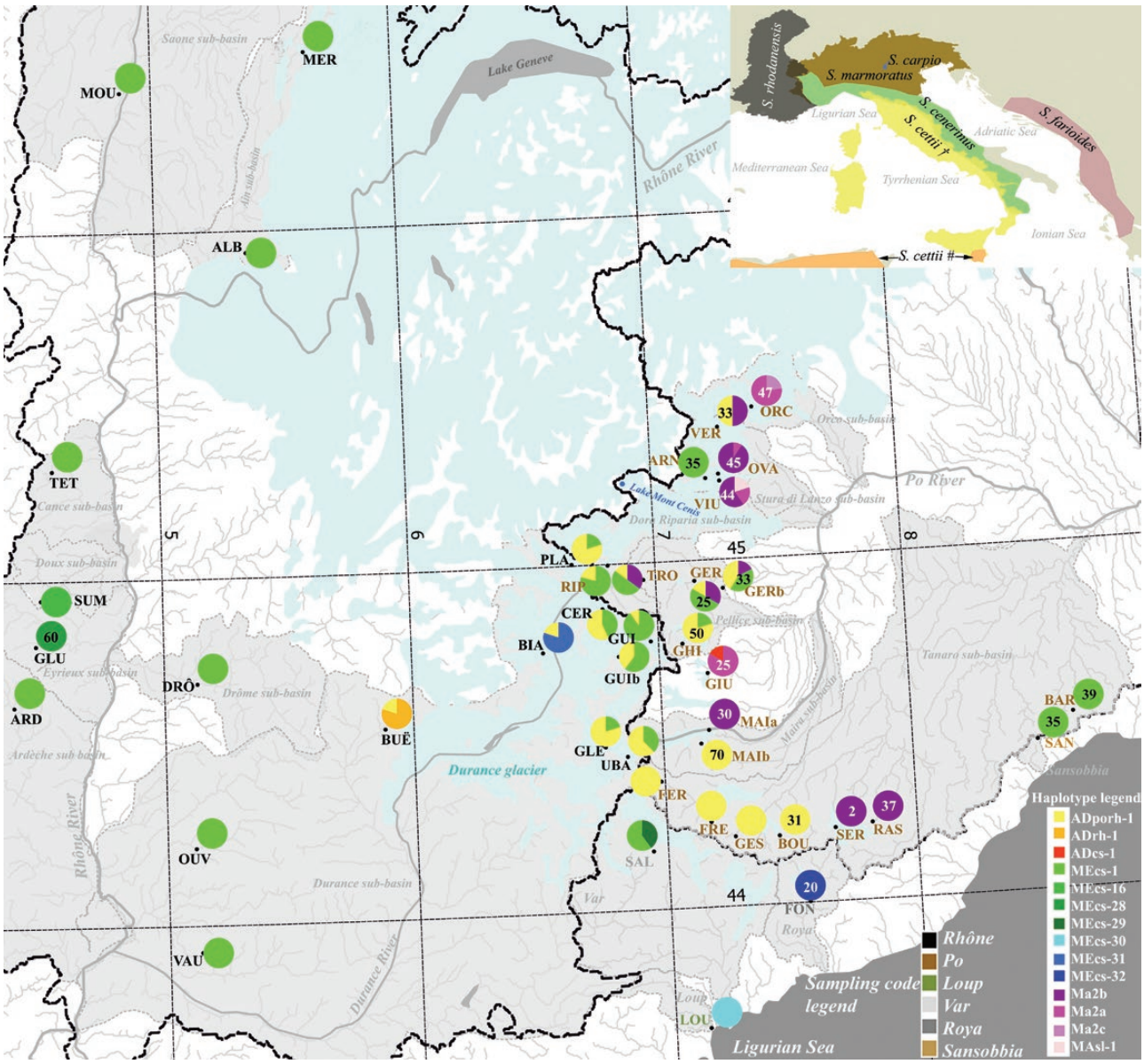

Figure 1. Map showing the sampling locations of brown trout throughout the Rhône and the western part of the Po river basins. Coloured pie charts indicate the mtDNA haplotype frequency distribution of the main brown trout lineages. Numbers in bold within the pie charts represent the Atlantic haplotype frequency (%). The distribution of the Alpine ice cover during the LGM is represented by the light blue area. The box at the top right shows the range of the nominal Mediterranean trout species as reported in Kottelat & Freyof (2007). † The geographic range *of S. cettii* according to Kottellat & Freyhof (2007), # the revised geographic range of *S. cettii* according to Splendiani *et al*. (2019b and references therein).

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 v.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 v.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

Table 1. Collection site information.

Code	Taxon	Sea drainage	Main-basin	Sub-basin	Stream	No.	L	Lat.	Long.	Elevation (m)
MOU	*S.rod*	G	Rhône	Saône	Mouge	5	F	46.40	4.87	176
TET	*S.rod*	G	Rhône	Cance	Riotet	5	F	45.32	4.56	668
SUM	*S.rod*	G	Rhône	Doux	Sumène	5	F	43.98	3.72	203
GLU	*S.rod*	G	Rhône	Eyrieux	Gluyère	5	F	44.81	4.48	568
ARD	*S.rod*	G	Rhône	Ardèche	Thines	5	F	44.64	4.39	217
MER	*S.rod*	G	Rhône	Ain	Merlue	5	F	46.51	5.64	433
ALB	*S.rod*	G	Rhône	Ain	Albarine	5	F	45.94	5.38	258
DRÔ	*S.rod*	G	Rhône	Drôme	Drôme	5	F	44.70	5.13	229
PLA	*S.rod*	G	Rhône	Durance	Clarée	5	F	45.00	6.66	1484
CER	*S.rod*	G	Rhône	Durance	Cerveyrette	6	I	44.87	6.78	2077
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
GUIb	*S.rod*	G	Rhône	Durance	Guil	20	I	44.77	6.97	1779
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
BUË	*S.rod*	G	Rhône	Durance	Petit Buëch	5	F	44.55	5.88	1117
OUV	*S.rod*	G	Rhône	Ouvèze	Ouvèze	5	F	44.22	5.11	222
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
SAL	?	G	Var	Tinée	Tinée	5	F	44.17	6.94	1930
FON	?	G	Roya	Roya	Roya	5	F	44.00	7.55	426
ORC	*S.cen*/*S.far*	A	Po	Orco	Orco	17	I	45.43	7.42	700
VER	*S.cen*/*S.far*	A	Po	Stura di Lanzo	Rio Vercellina	18	I	45.38	7.28	1220
OVA	*S.cen*/*S.far*	A	Po	Stura di Lanzo	Rio d'Ovarda	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
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
GHI	*S.cen*/*S.far*	A	Po	Pellice	Ghiacciard	10	I	44.76	7.09	1440
GIU	*S.cen*/*S.far*	A	Po	Upper Po	Rio Giulian	10	I	44.67	7.19	1120
MAIa	*S.cen*/*S.far*	A	Po	Maira	Bedale di Langra	10	I	44.51	7.18	950
MAIb	*S.cen*/*S.far*	A	Po	Maira	Bedale Intersile	10	I	44.47	7.15	1180
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
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
SER	*S.cen*/*S.far*	A	Po	Tanaro	Rio Serpentera	13	I	44.21	7.67	1280
RAS	*S.cen*/*S.far*	A	Po	Tanaro	Rio Raschera	14	I	44.22	7.82	1100
BAR	*S.cen*/*S.far*	A	Po	Tanaro	Baracca	18	I	44.50	8.65	570
SAN	*S.cet*	L	Sansobbia	Sansobbia	Sansobbia	20	I	44.43	8.50	660

*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.
‡L = Laboratories involved in genetic analyses: F = ISEM, Université de Montpellier (Lab.1), and I = DiSVA, Università Politecnica delle Marche (Lab.2)

(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, and generally more appropriate for trees with shallow roots (phylogenies with a Miocene or more recent root as for *S. trutta*; 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 *et al.*, 2005), a modified Akaike Information Criterion (AICM) as provided in TRACER v.1.6 (Rambaut *et al.*, 2014) was used. The models were run five times for 50 000 000 generations 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 v.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, occurring in the study area, could explain distribution current spatial genetic. 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 with Arlequin v.3.5, using conventional F-statistics and testing the statistical significance of the tests with 5000 permutations.

To depict the biogeographic scenarios underlying the observed haplotype spatial distribution, the mtDNA lineage distribution observed in each population was analysed both by mapping pie charts geographically and using Kernel density (KD) analysis along an elevation, and a 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 matches with plausible scenarios of extinction and recolonization events connected with environmental changes that occurred during the LGM in the south-western Alps. The KD analysis was carried out by using the density function in R software (R Development Core Team, 2013).

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 a 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; 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 *et al.*, 2004). For the ME lineage, seven haplotypes were observed, two of which were already detected in other studies: MEcs-1 and MEcs-15 (e.g. Cortey *et al.*, 2004) and a further five were detected for the first time in this study and named as follows: 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 *et al.*, 2007; Meraner *et al.*, 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, namely: haplotype 1 to haplotype 4 (Cortey & García-Marín, 2002) and haplotype At1e, (Meraner *et al.*, 2007), while one haplotype was described for the first time in this study (haplotype 3b, MK948037). This last haplotype should be also considered of hatchery origin due to high similarity with the domestic variant haplotype 3 (Fig. 2; Table 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) were endemic to the 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 Fig. 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 *et al.*, 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

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

Code	Sub-basin	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
MOU	Saône	Rhône	5																			
TET	Cance	Rhône	5																			
SUM	Doux	Rhône		5																		
GLU	Eyrieux	Rhône			2														3			
ARD	Ardèche	Rhône	5																			
MER	Ain	Rhône	5																			
ALB	Ain	Rhône	5																			
DRÔ	Drôme	Rhône	5																			
PLA	Durance	Rhône	1							4												
CER	Durance	Rhône	9							11												
BIA	Durance	Rhône						4		1												
GUI	Durance	Rhône	3							2												
GUIb	Durance	Rhône	18							2												
GLE	Durance	Rhône	1							4												
UBA	Durance	Rhône	8							13												
BUË	Durance	Rhône								1	4											
OUV	Ouvèze	Rhône	5																			
VAU	Ouvèze	Rhône	5																			
LOU	Loup	Loup					5															
SAL	Tinée	Var	3			2																
FON	Roya	Roya							4										1			
ORC	Orco	Po												7	2			5				3
VER	Stura di Lanzo	Po								6			6				6					
OVA	Stura di Lanzo	Po											10	1				9				
ARN	Stura di Lanzo	Po	3										2					1	4			
VIU	Stura di Lanzo	Po											3	1		1		3	1			
RIP	Dora Riparia	Po	19							5												
TRO	Pellice	Po	10							3			7									
GER	Pellice	Po	3							1			2						3			
GERb	Pellice	Po	5							5			2					2		1	1	
GHI	Pellice	Po	1							4									4	1		
GIU		Po										1		5				2	2			
MAIa	Maira	Po											7						3			
MAIb	Maira	Po								3								1	5	1		
FER	Tanaro	Po								8												
FRE	Tanaro	Po								8												
GES	Tanaro	Po								4												
BOU	Tanaro	Po								9								4				

Table 2. Continued

Code	Sub-basin	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
SER	Tanaro	Po											12						0			
RAS	Tanaro	Po											10					4				
BAR	Tanaro	Po	11																7			
SAN		Sansobbia	13															6	1			

presented a spatial distribution confined to the upper reaches of both the upper Durance River (Rhône basin) and Po River along a transect of the south-western Alps extended from the Cottian to the Maritime Alps (Fig. 1; 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 (Fig. 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 (Fig. 2). These non-native haplotypes for the Mediterranean area showed an evident greater abundance within the Italian samples (Fig. 1; Table 2). 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 Supporting Information, Fig. S1; Table S3) 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 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 Supporting Information, Table S4). The MA lineage appears as the youngest. 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.

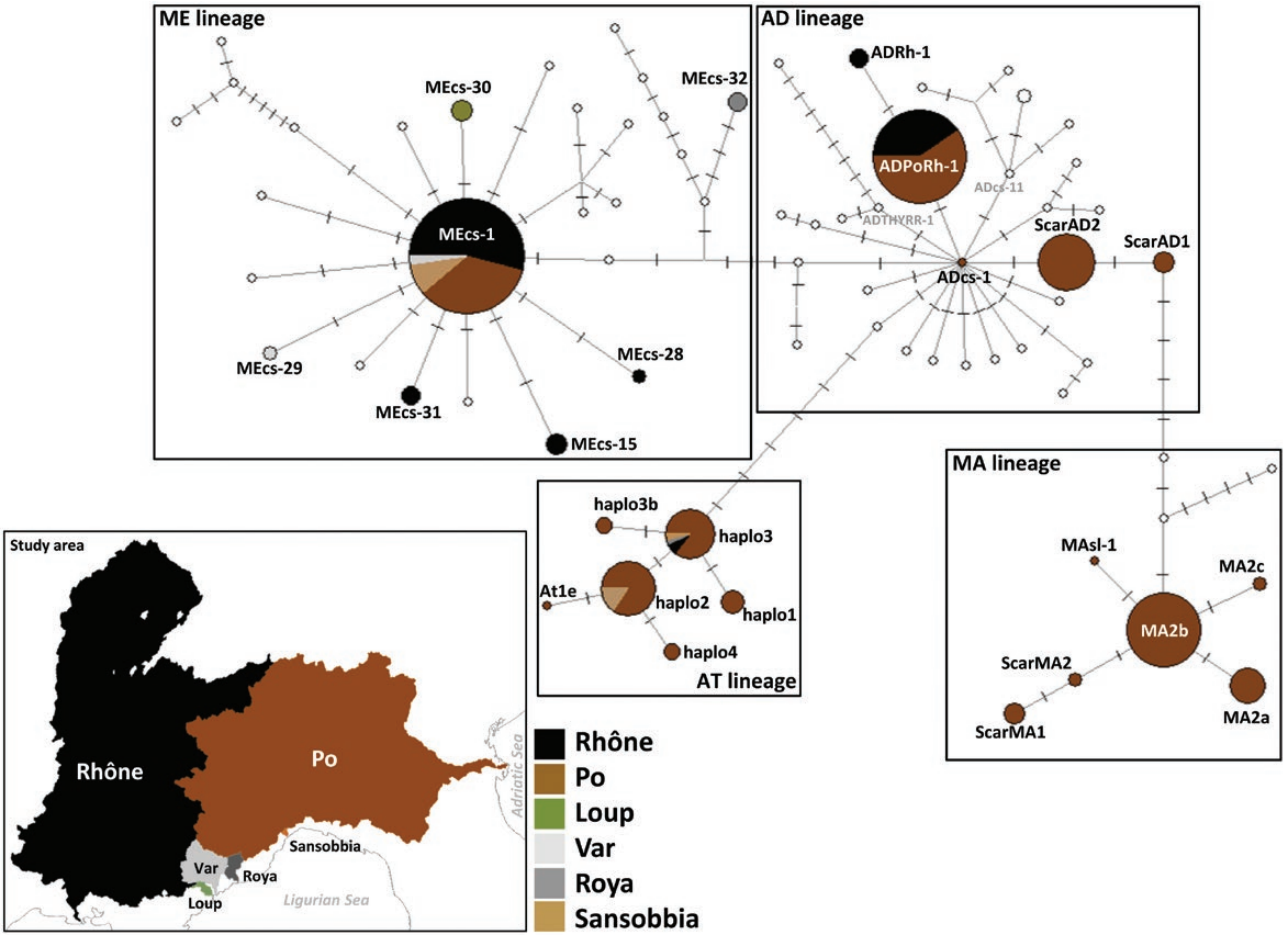

Figure 2. Median-Joining Network showing the phylogenetic relationships subsisting between the 18 native brown trout haplotypes detected in this study (coloured circles) and the brown trout haplotypes observed in previously published studies (grey circles, see also Table 1; the position of the haplotypes ADcs-11 and AD-Tyrrh-1 was reported because they are mentioned in the text). As regards the haplotypes observed in the study area, the size of each circle is proportional to the haplotype absolute frequency.

AMOVA

With both two grouping options (Rhône samples vs. Po samples and Rhône samples vs. Durance and Po samples), 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, AMOVA analyses also showed 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 (Fig. 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 of a mixture of AD and ME haplotypes. In this study, a total of 91 trout originating from the Rhône River basin in France, among them 31 specimens showed the AD haplotype. All these latter trout came from the Durance sub-basin (samples BUË, BIA, CLA, UBA and GUI,

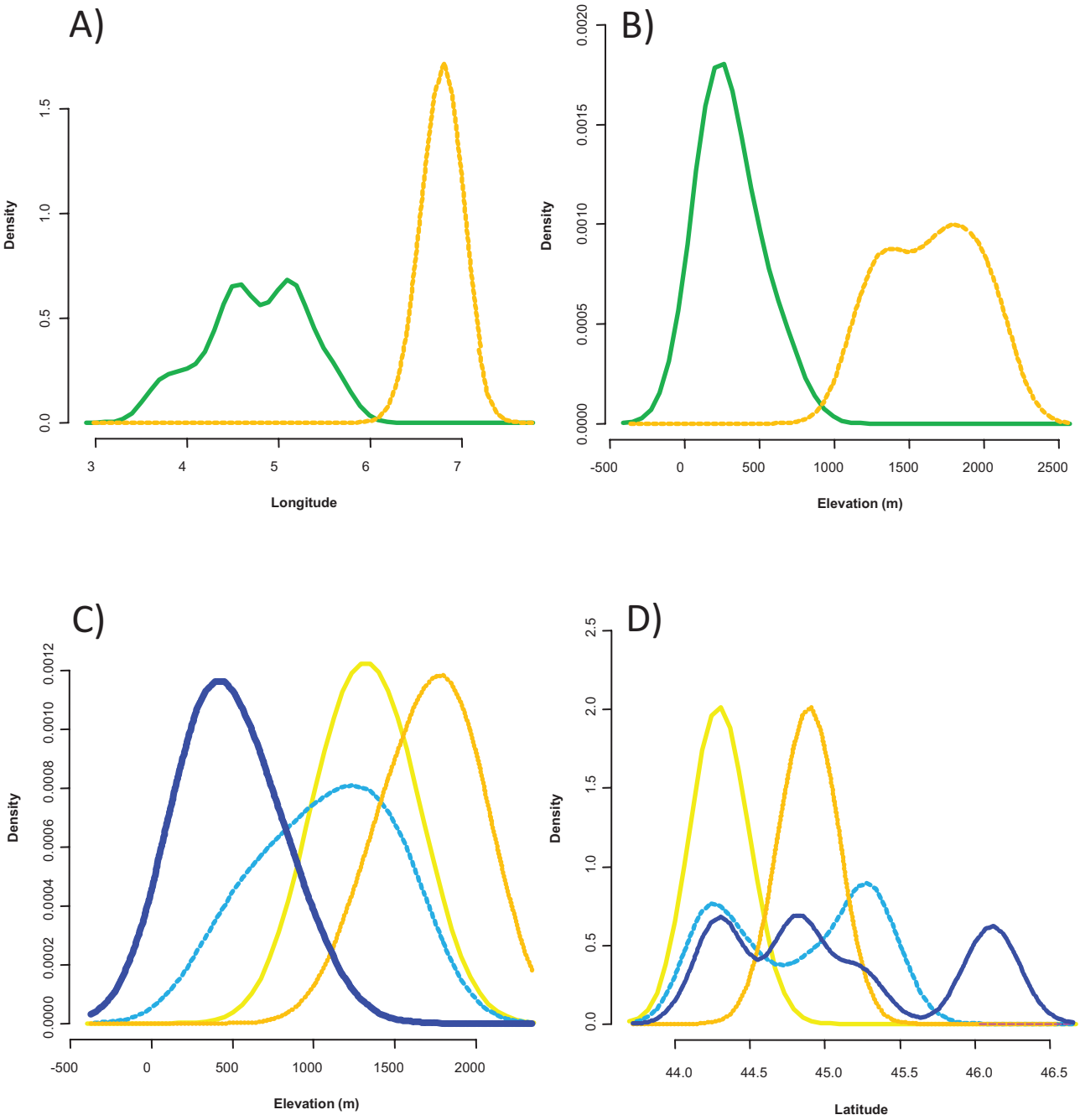

Figure 3. Plot showing the probability density function (density) obtained comparing elevation, longitude and latitude with the mtDNA genetic composition of brown trout populations from the Rhône River basin (A and B) and from the Po River basin (C and D). The mtDNA lineage composition was represented by the following coloured 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.

see Table 2). The last two rivers (UBA and GUI) flow 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 (Fig. 3A). When KD was carried out to relate brown trout lineage 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 (Fig. 3B), whereas admixed populations (AD—ME) showed higher values of probability between 1500 and 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 (Fig. 3C). Around this latter figure, 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 (Fig. 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 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) regarding 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 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 neighbouring 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 should be taken into account with caution for inferring isolation time accurately (Schenekar *et al*., 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 suggests 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 a previous estimation of 190 000 years ago provided by 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 seaward migratory tactics in Mediterranean brown trout populations, as highlighted by several pieces of palaeontological evidence (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 colonization 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) (Fig. 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 Fig. 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 slopes 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 *et al.*, 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 by the presence of biological corridors opened by ancient Mediterranean river captures.

Once the Po River basin was reached, 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, for example, in the 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 KD analysis in *Results* and Fig. 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 have taken place around 267 000–212,000 years ago and therefore nearly simultaneously 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 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) (Fig. 2). Further, the finding of a new AD haplotype (ADporh-1), endemic to 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 substantially (with the exception of the haplotype ADcs-1 observed in a sole trout) the sole AD haplotype observed. This haplotype was found as fixed in samples collected around 1000–1500 m (Fig. 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, such as 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 conditions promoted phases of isolation. The estimated origin for this AD branch of *c.* 151 000–120 000 years ago, corresponding approximately with the Riss-Würm Interglacial. During warmer phases, the 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 a refuge during 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 were covered by massive ice sheets. 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 below).

The Italian side of the Maritime Alps also represented 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 (Fig. 2), we can hypothesize that ADcs-1 first colonized the Po River, and then, during phases of geographic isolation within refugia, new haplotypes, such 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) identified two endemic MA haplotypes (named here ScarMA1 and ScarMA2, see also Fig. 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 refuge last respect to the ME and AD lineages. This hypothesis seems to accord well with the younger origin of the MA lineage with respect to the ME and AD lineages that emerged in this study and also proposed by Oliver (2014). In addition, the altitudinal distribution of the MA lineage appears congruent with the stable or declining demographic scenario found by mismatch analysis. The reduced habitat availability (i.e. most of the upper river reaches were already occupied by the ME and AD lineages) could have contrasted the demographic expansion of this lineage (e.g. Lavery *et al*., 1996; Bernatchez, 2001). It is probable that this mtDNA lineage was fixed in marble trout that inhabited the lower part of the Po River basin, then, when the climate became warmer, this salmonid tried to reach colder habitats at higher elevations. A similar palaeo-historical 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 was 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, 2013; 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 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 LGM (e.g. Fig. 1), which implies the arrival of trout after this period. Interestingly, the ADrh-1 haplotype, a mtDNA variant distant of one mutational step only from the haplotype ADporh-1, was detected only in the Petit Buëch stream, sited at the margin of the Durance glacier. Here, the milder climate conditions of this part of the 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 that 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 palaeo-glacier was one of the most important Alpine glaciers (Cossart *et al.*, 2008) (Fig. 1). On the other hand, along the Italian side of the south-western Alps, the ice cover appeared less extended (Hughes *et al.*, 2006; Szövényi *et al.*, 2009) (Fig. 1). An explanation for the formation of an unidirectional corridor between the two sides of the south-western Alps could be related to 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 Lake Geneva (Rhône River basin) by bullhead (*Cottus gobio*) migrants from the Rhine River basin during the last glacial retreat (Vonlanthen *et al.*, 2007). Furthermore, 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. the 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 from Lake Mont Cenis (1974 m a.s.l.) (Fig. 1), a former small Alpine lake (in 1921 the lake was modified 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 described above, in *Material and Methods*, 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 broach 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: *Salmo rhodanensis* (Rhône River basin) - a contested species (Berrebi & Denys, in prep.), *Salmo cettii* (a non-valid name when used to indicate trout from the Tyrrhenian and Ligurian Sea draining rivers) and *Salmo farioides* or *Salmo cenerinus* (depending on the authors, Adriatic draining rivers) (Fig. 1). At the 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 as in the rest of the Mediterranean rivers. More further 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*), with respect to that shown by rest of the Rhône samples as highlighted by the AMOVA analyses. However, more sound conclusions should be drawn by also analysing nuclear and morphological markers [see Ninua *et al.* (2018) for similar argumentations]. These preliminary results however refute the traditional taxonomic position adopted until now for the Mediterranean trout of the study area.

CONCLUSION

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 the 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 trout from (or found on) the Italian side of 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 exercized 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 5-10 years. In most cases, the outcomes of pivotal genetic screening on local brown trout populations involved in these projects were not published, or may never have been carried out. This probably occurred (and still occurs) in Italy because local administrations have transferred to sport fishing associations the full management of these practices. In these circumstances, the rationale of these putative conservation actions has not been evaluated 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 literature) 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 programmes (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 *et al*., 2009; Schenekar & Weiss, 2017). The situation is far better on the French side of the investigated area. In France, more and more administrative organizations, under the supervision of the Ecology Ministry, adopted "patrimonial" management during the last 20 years. A large proportion of the trout population has been analysed and published in France (https://data.oreme.org/trout/home) with nuclear and mitochondrial markers driving conservation and stocking.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Mismatch distribution of (a) the AD lineage, (b) the ME lineage and (c) the MA lineage.
Table S1. Schematic summary of the Mediterranean taxa in the *Salmo trutta* complex of the study area.
Table S2. Demographic indices calculated for three brown trout mtDNA lineages based on control region sequence analysis.
Table S3. Demographic indices calculated for three brown trout mtDNA lineages based on control region sequence analysis.
Table S4. TMRCA estimates for the Mediterranean brown trout lineages AD, ME and MA and for the sub-clade ADporh-1, ADrh-1 with 95% HPD intervals.