Conservation Genetics of Mediterranean Brown Trout in Central Italy (Latium): A Multi-Marker Approach

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Abstract: Brown trout is considered a complex of incipient species, including several phylogenetic lineages, whose natural distribution in the Mediterranean area has been altered, since the beginning of the 1900s, by massive introductions of domestic strains of Atlantic origin to support fisheries. Introduced trout naturalize in new suitable environments and extensively hybridize with native populations. Here, we characterized putatively neutral and adaptive genetic variability and population structure of Mediterranean brown trout from six river catchments in central peninsular Italy, as revealed by both mitochondrial (Control Region) and nuclear (microsatellites, *LDH-C1*, major histocompatibility complex) markers. We quantified the admixture of wild populations with hatchery strains and evaluated the effects of domestic trout introductions on shaping population genetics. Our analyses indicated: (1) a composite picture of genetic variability in the area, with the presence of all native Mediterranean trout mitochondrial lineages ("Adriatic", "Mediterranean", "*marmoratus*"), various frequencies of allochthonous genotypes and different rates of introgression among sampling sites; (2) asymmetric mito-nuclear introgression; (3) increasing nuclear marker diversity with increasing levels of admixture across populations; (4) strong population structure coupled with relatively low effective population size. Data allowed the identification of five management units and we propose specific actions to support ongoing and future conservation strategies within the examined area.

Keywords: *Salmo trutta* complex; *Salmo ghigii*; population structure; demography; anthropogenic introgression; microsatellites; control region; *LDH-C1*; major histocompatibility complex

1. Introduction

Brown trout (*Salmo trutta* Linnaeus, 1758) is considered an iconic freshwater fish of remarkable economic value, and one of the most broadly distributed (and stocked) salmonids worldwide. The taxon is characterized by an extraordinary genetic and ecological polymorphism, which has often given rise to local phenotypic *morphae* [1,2]. Consequently, the nomenclature and systematic status of the taxon have been long debated and are still unresolved [2–4]. Brown trout is thus considered a complex of incipient species, including several phylogenetically distinct mitochondrial lineages whose origin and spatial distribution are linked to Pliocene and early Pleistocene climatic fluctuations that affected the distribution range and the dispersal of brown trout [2,5].

Since the middle of the 19th century, stock enhancement plans have carried on the introduction of hatchery-reared specimens of Atlantic origin in various geographic areas, including Italy, to support fisheries [1,6]. In the Mediterranean area, introduced domestic trout extensively hybridize with native trout resulting in the massive introgression of Atlantic genes in wild populations [3,7,8].

Brown trout is nowadays considered one of the world's 100 top invasive species [9] while, paradoxically, it is also included in the IUCN Red List of threatened species and the European Habitat Directive [10] that prioritize the conservation of native wild populations. Besides hybridization with human-introduced allochthonous trout, native populations are also exposed to various (interplaying) threats, among which are global warming, habitat fragmentation and degradation. Indeed, upward range shift caused by temperature increases [11] and the occurrence of physical/hydrological barriers contribute to the genetic isolation and fragmentation of wild populations, which are often small and unstructured—i.e., mostly represented by juveniles and young individuals [12]. These factors eventually can promote the erosion of diversity by genetic drift [13].

Cornerstones of modern conservation genetics are evolutionary significant unit (ESU) and management unit (MU) concepts [14,15], that rely on the genetic distinctiveness of populations along with demographic features [16]. Importantly, adaptive genetic distinctiveness should be also taken into account to exhaustively define ESUs and MUs, since the maintenance of adaptive variation is crucial for optimal and effective conservation strategies [17,18]. The ESU approach has the desirable advantage to circumvent "obstacles" and challenges that controversial taxonomies can cause for conservation and management of taxa [19], as in the case of brown trout [20]. Practically, such a goal can be reached by characterizing the genetic diversity of wild populations through multiple classes of genetic markers, each providing a specific evolutionary perspective—such as recent/historical demography, or selection and (local) adaptation [17]—based on their mechanism of inheritance and their polymorphic information content [21], as applied in some threatened native-Italian freshwater fishes (e.g., [22–24]).

In brown trout, fragments of the mitochondrial non-coding Control Region (CR) and various microsatellite arrays have been commonly used to assess neutral genetic diversity across populations. Specifically, the former is primarily employed for the identification of relatively ancient lineages (as defined by [5]), while the latter are extensively used for assessing genetic population structure even at the fine spatial scale [25–28]. When combined, they provide a useful tool to determine the distinctiveness of populations [29]. On the other hand, the nuclear Lactate Dehydrogenase *C1* gene (*LDH-C1*), whose alleles are diagnostic of Mediterranean-native and Atlantic lineages [30], conveniently provides estimates of the amount of introgression from domestic lineages in wild Mediterranean populations (see Section 2 for details). Conversely, adaptive variation can be inferred through loci that are somehow linked to fitness-related traits such as the highly polymorphic genes of the major histocompatibility complex (MHC), which are directly involved in the immune response against parasites [31]. Specifically, signatures of historical and/or current selection have been demonstrated for the MHC-*DAB* gene in brown trout (e.g., [32–34]), hence making it a candidate locus to reveal natural adaptive variation. However, to the best of our knowledge, brown trout ESUs/MUs have never been defined using both neutral and adaptive marker classes to date.

Genetic studies targeting wild Italian brown trout populations frequently revealed remarkable levels of admixture between native and Atlantic (domestic) strains [6,29,35,36]. Most of the Sardinian populations provide an exception [29], as well as some mainland populations isolated by natural and/or artificial barriers preventing fish dispersal [6]. The current distribution of allochthonous brown trout and the admixture degree in wild populations correlate with geological and climate factors, which differently acted in Eastern and Western Alps, Apennines, Sardinia, and Sicily areas [37].

In the peninsular area, a high and complex genetic diversity of brown trout populations has been reported around the Tyrrhenian Sea [29] with central Italy characterized by the coexistence of populations retaining unique genetic features [26,38,39]. In this area, brown trout inhabits mostly mountain freshwaters and, sporadically, lowland cold waters. Previous studies pointed out the presence of all the native Mediterranean mitochondrial lineages (Adriatic "AD", Mediterranean "ME" and *marmoratus* "MA"), together with

Atlantic haplotypes ("AT" lineage) of domestic origin, the abundance of which varies from site to site [6,25,26,39].

In this research, we focused on the central Italy "hotspot" of Mediterranean brown trout genetic diversity. We aimed at characterizing neutral and adaptive genetic variability and population structure as revealed by multiple markers classes (microsatellites, MHC-*DAB*, CR) while quantifying admixture with domestic trout (as assessed by the *LDH-C1* gene). Additionally, we evaluated the effects of natural (drainage basin, altitude) and anthropogenic (*LDH-C1*-based admixture) variables to shape population genetic diversity and structuring. Finally, based on our results, we define MUs and propose specific actions to support ongoing and future conservation within the examined area.

2. Materials and Methods

2.1. Study Area, Sample Collection, and DNA Extraction

During August–October 2019, overall 175 brown trout were caught by electrofishing and released after taking a small fin clip. We gathered specimens from six chosen river catchments (seven sampling locations) covering the major drainage basins in central Italy (Figure 1) and/or showing remarkable hydrographic and ecological variability (Table 1). Fin clips were preserved in 99.8% ethanol at −20 °C, and then genomic DNA was extracted following a salt-extraction protocol [40].
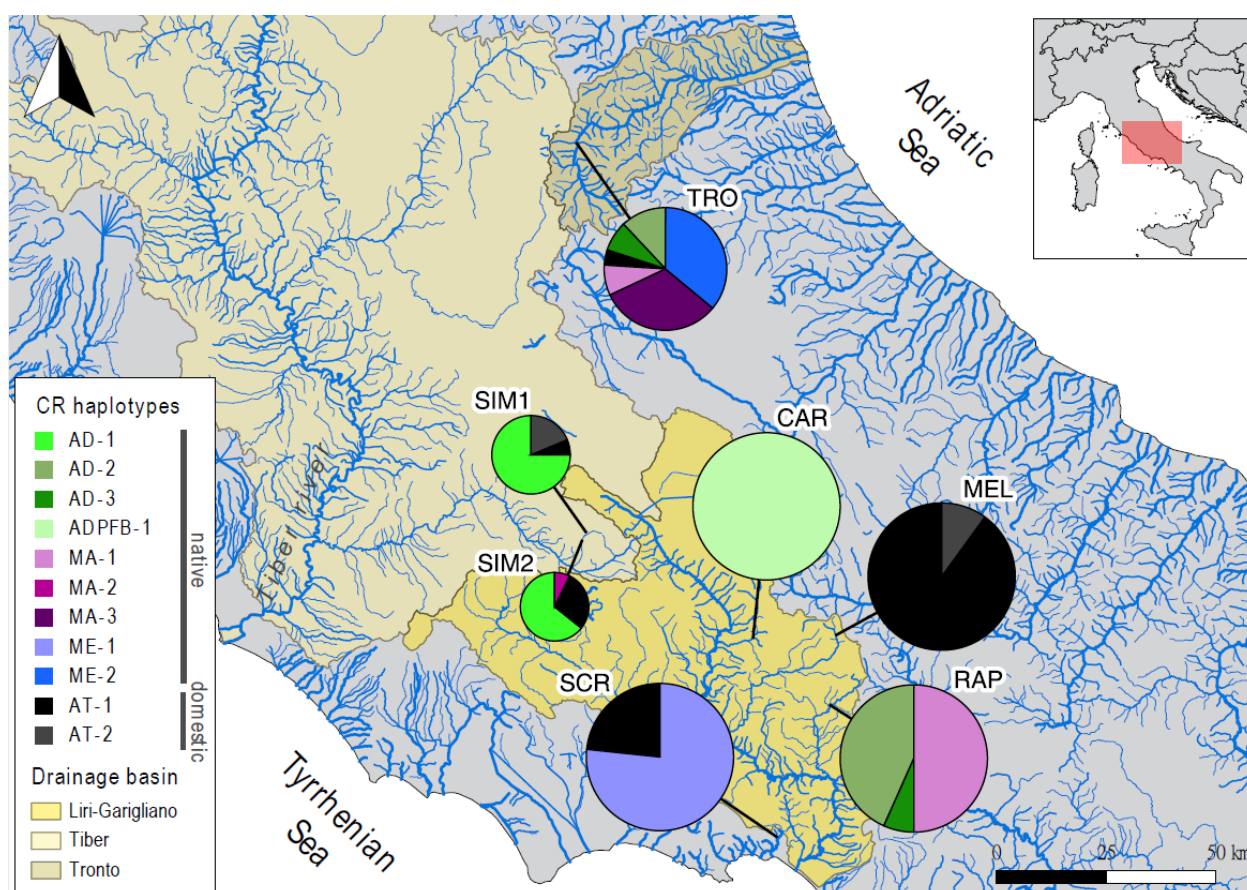
Figure 1. Frequency and geographic distribution of 11 Control Region (CR) haplotypes across seven wild sampling sites from three drainage basins in central Italy. Population abbreviations refer to Table 1; pie charts are proportional to sample sizes.

Table 1. Geographic and ecological features of seven sampling sites (Pop) of Mediterranean brown trout from central Italy. The Datum for geographic coordinates is WGS84; SAC stands for Special Area of Conservation; temperatures of water (Water T°) was measured three times per site in August.

Pop	River Catchment	Drainage Basin (Outlet)	Lat° (N)	Lon° (E)	Elev. (m)	Water T° (± SE)	Fish Assemblage	River Substrate	Protected Area
CAR	Carpello	Liri-Garigliano (Tyrrhenian Sea)	41.70	13.68	293	11.4 (±0.2)	*Gasterosteus aculeatus*	Gravel and aquatic macrophytes	SAC, Regional Natural Reserve
MEL	Melfa	Liri-Garigliano (Tyrrhenian Sea)	41.70	13.91	1159	8.8 (±0.1)	-	Boulder, pebbles, gravel	SAC, National Park
RAP	Rapido	Liri-Garigliano (Tyrrhenian Sea)	41.56	13.88	154	13.6 (±0.0)	-	Pebbles, gravel	-
SCR	Santa Croce	Santa Croce (Tyrrhenian Sea)	41.29	13.71	37	13.7 (±0.2)	*Anguilla anguilla*, *Lampetra planeri*, *Sarmarutilus rubilio*, *Squalius squalus*	Gravel and aquatic macrophytes	SAC, close to Regional Park
SIM1	Simbrivio	Tiber (Tyrrhenian Sea)	41.94	13.24	794	11.3 (±0.1)	-	Boulder, pebbles, gravel	SAC, Regional Park
SIM2	Simbrivio	Tiber (Tyrrhenian Sea)	41.92	13.22	701	12.0 (±0.2)	-	Pebbles, gravel	SAC, Regional Park
TRO	Tronto	Tronto (Adriatic Sea)	42.73	13.26	693	10.7 (±0.1)	-	Pebbles, gravel	-

2.2. Amplification and Genotyping of Mitochondrial and Nuclear Loci

We used a mitochondrial marker and various categories of nuclear loci to comprehensively assess both neutral and adaptive genetic diversity of wild populations and estimate admixture with domestic lineages.

Firstly, the mitochondrial Control Region (CR) was amplified with primer pair PST and FST, following protocols reported in [41], to identify maternal lineages. Amplicons were purified and forward-sequenced by an external service to ultimately obtain a fragment of 544–545 bp. Sequences were visualized and manually corrected using the online software CHROMAS 2.6.6 available at http://www.technelysium.com.au, accessed on 20 September 2021) and aligned in MEGA 7 [42] with the Clustal W algorithm. We retrieved CR haplotypes using DNASP 6 [43] and assigned them to major brown trout lineages (*sensu* [5]) by phylogeny—a Neighbor-Joining tree was built in MEGA 7 including representative sequences of lineages AD, MA, ME, and AT lineages.

Secondly, to deeply characterize population diversity, we genotyped individuals at 11 polymorphic microsatellite loci (STR), namely STR60 and STR73 [44], SSA85 [45], SSOSL417 [46], SSA410UOS and SSA408UOS [47], SSSP2213 [48], OMM1064 [49], SSAD190 [50], Oneµ2 [51], SSA103NVH [52]—see supporting information provided in Talarico et al. (2021) for PCR-amplification and genotyping procedures of STR loci. Note that STR and *LDH-C1* profiles of a subset of individuals have been already published in [34]; data archived in Dryad doi.org/10.5061/dryad.xsj3tx9fg), although used for purposes other than the present study.

Thirdly, individuals were typed for the *LDH-C1* gene through the RFLP method detailed in [30] that is routinely used to distinguish between the *LDH-C1*90* allele (that is widely distributed in wild brown trout populations from northern Europe and, more importantly, it is fixed within European hatchery stocks of brown trout) and the *LDH-C1*100* allele (occurring in wild native populations from the Mediterranean area), thus allowing to estimate admixture between native and domestic lineages.

Lastly, in the final dataset, we included genotypes of the major histocompatibility complex class II *DAB* locus (hereafter MHC-*DAB*) for a subset of 156 trout to account for non-neutral genetic variation, as there was evidence for historical and contemporary positive/balancing selection on this gene (data from [34]).

2.3. Population Genetic Diversity and Relationship with Introgression from Domestic Trout

In GENEPOP on the Web [53] we tested for departures from Hardy–Weinberg expectations (probability test and Markov chain method with default parameters) for each nuclear marker (STR loci, *LDH-C1*, and MHC-*DAB*), and for linkage disequilibrium between pairs of loci across populations. In both cases, *p*-values were Bonferroni-adjusted for multiple testing. We estimated the frequency of null alleles with the EM algorithm implemented in FREENA [54].

Typical indices of population polymorphism were obtained in ARLEQUIN 3.5 [55]: the number of alleles (A) and private alleles (Ap), the expected (He) and observed (Ho) heterozygosity for STR loci and the MHC-*DAB*; the number of haplotypes (H) and private haplotypes (Hp), the haplotype diversity (Hd), and the Kimura-2-parameters based nucleotide diversity (π) for the CR. To compare diversity among populations with unequal sample sizes, we also computed allelic and haplotype richness (Ar and Hr) by rarefaction using the *allel.rich* function of the POPGENEREPORT package in R [56], setting sample size of 12 (24 alleles) for STR and MHC-*DAB*, and 14 (haplotypes) for the CR.

The frequency of the *LDH-C1*90* allele was calculated for each population as a proxy of the introgression of hatchery genes into native populations. We explored the effects of stocking with domestic brown trout on the genetic diversity of wild native populations by testing the relationship between measures of population diversity (allelic/haplotype richness, expected heterozygosity) and the frequency of the domestic *LDH-C1*90* allele. For each marker separately, we fit a non-linear model (second-order polynomial regression) in R, since we expected high diversity associated with intermediate (around 50%) frequency of the *LDH-C1*90* allele, i.e., when admixture between native and domestic lineage is maximized.

2.4. Population Structure and Demography

We estimated marker-specific genetic differentiation between population pairs in ARLEQUIN testing significance with 1000 permutations. Differentiation was computed as Nei's Fst for STR loci and the MHC-*DAB*, and as Φst (Kimura-2 parameters method) for the CR. Matrices of pairwise genetic distances were then visualized by two-dimensions non-metric multidimensional scaling (NMDS) in PAST 4.04 [57]).

To investigate relationships (admixture) among examined populations based on STR loci, we performed a multi-locus Bayesian analysis of population structure in STRUCTURE v2.3.4 [58]. We ran the admixture model with correlated allele frequencies and no population origin priors (500,000 iterations after a burn-in period of 200,000 iterations) five times for each K value in the range 1–8. Results of replicated runs were combined with CLUMPAK [59]. To define the optimal number of clusters (K), we evaluated both the highest log probability of K (LnP method; [58]) and the highest rate of change in LnP between successive K (ΔK method; [60] as implemented in STRUCTURESELECTOR [61].

Additionally, we ran the discriminant analysis of principal components (DAPC) in the R-package ADEGENET v1.3 [62] to identify genetic clusters, as this multivariate approach does not rely on genetic assumptions [63]. We first visually examined the pattern of Bayesian Information Criterion (BIC) values in the range 1–8 clusters (K) using the *find.cluster* function (K-means clustering on PCA-transformed data). After choosing the optimal one(s), we ran the actual discriminant analysis (*dapc* function: Principal Coordinate retained = 90; discriminant functions retained = 3) and obtained membership probabilities of each individual for the K groups previously identified.

The GENECLASS v2.0 software [64] was used to compute the probability that each individual belonged to each reference population. The analysis was performed using the

Bayesian assignment criterion of [65] and a Monte Carlo resampling algorithm with 10,000 simulated individuals and setting the assigned threshold at 0.05.

The analysis of molecular variance (AMOVA) was used to test whether the ecology and geography of sampling localities contribute to explaining the partitioning of genetic variation within and between groups of coherent populations. Specifically, we tested three a priori hypotheses: (1) panmixia (all populations grouped); (2) differentiation between mountain (elevation > 650 m above the sea level, namely MEL, SIM1, SIM2, and TRO) and lowland (<300 m above the sea level, namely CAR, RAP, and SCR) populations, since the elevation provides a proxy of multiple environmental and ecologic variables (e.g., fish assemblage, sediment granulometry, water temperature, slope); (3) differentiation between unrelated major drainage basins as shown in Figure 1 and Table 1 (TRO vs. SIM1/SIM2 vs. SCR vs. MEL/CAR/RAP). AMOVAs were performed in ARLEQUIN (10,000 permutations) for STR loci, the MHC-*DAB* and the CR (setting Kimura-2 parameters distance) separately.

For each population, we inferred the STR-based effective population size (Ne) according to the sibship assignment method implemented in COLONY V. 2.0.0.6 [66] without information on parental genotypes. We assumed non-random mating and polygamy for both males and females [67] and set no priors and a genotyping error rate of 2% to account for erroneous sizing of alleles and allelic dropouts. Finally, we tested for severe recent population size reductions using BOTTLENECK 1.2.02 [68], applying the two-phase mutation model (95% stepwise and 5% non-stepwise microsatellite mutations) with 10,000 iterations and assessing significant heterozygote excess with the one-tailed Wilcoxon test.

3. Results

3.1. Mitochondrial and Nuclear Polymorphism and Population Diversity

We identified 11 CR haplotypes (GenBank accessions: OL830460-70): nine of them belonged to mitochondrial native lineages (4 AD, 2 ME, and 3 MA), while two belonged to the domestic AT lineage (Figure 1). Nine haplotypes matched with previously identified brown trout CR variants (four of them, AD-3, MA-1, MA-2, and MA-3, were never reported in the Latium region before), while one (ME-2) was new. Frequencies and distribution of native and Atlantic (hatchery) haplotypes across populations are depicted in Figure 1 and reported in Table S1 in the Electronic Supplementary Materials (ESI).

Nuclear markers provided genotype frequencies (see Table S2 in ESI for STR genotypes of an additional 19 individuals not examined in Talarico et al., 2021) that did not deviate significantly from Hardy–Weinberg expectations except for the STR locus SSOSL417 in the Rapido (RAP) site (Table S3 in ESI). The FREENA analysis revealed no substantial (frequency >20%) or systematic occurrence of null alleles across loci/populations (Table S4 in ESI). There was statistically significant ($p < 0.05$) linkage disequilibrium in only 5 out of 546 pairwise loci comparisons (three in RAP and two in TRO; Table S5 in ESI), thus suggesting no physical linkage between any loci. Consequently, we safely retained all loci for downstream analyses.

Indices of population genetic diversity are reported in Table 2 for each marker. Specifically, Tronto (TRO)—and to a lesser degree Simbrivio (SIM1 and SIM2)—showed high polymorphism across all marker types. Conversely, low mitochondrial diversity was measured in Carpello (CAR) and Melfa (MEL) where we found a single fixed native haplotype and two domestic haplotypes, respectively (Figure 1). Depleted nuclear DNA variation was revealed in Santa Croce (SCR).

Table 2. Genetic variability parameters for each marker across seven Mediterranean brown trout sampling sites (Pop). N, number of individuals collected and analyzed for CR, *LDH-C1*, and STRs; N_{MHC}, number of individuals analyzed at the MHC-*DAB* locus; H, number of haplotypes and of private haplotypes (Hp); Hr, haplotype richness; Hd, haplotype diversity; π, nucleotide diversity; * *90*, frequency of the Atlantic *LDH-C1*90* allele; A, number of alleles and of private alleles (Ap); Ar, allelic richness; Ne, effective population size.

| Pop	N (N_MHC)	CR				LDH-C1			11 STR loci					MHC-DAB			
		H (Hp)	Hr	Hd (±SE)	π% (±SE)	*90	Ho	He	A (Ap)	Ar	Ho (±SE)	He (±SE)	Ne (95% CI)	A (Ap)	Ar	Ho	He
CAR	30 (28)	1 (1)	1.00	- -	- -	0.13	0.27	0.24	7.09 (1.45)	4.92	0.47 (0.09)	0.52 (0.10)	40 (24–71)	13 (6)	8.35	0.86	0.81
MEL	30 (27)	2 (0)	1.86	0.19 (0.09)	0.034 (0.05)	0.90	0.20	0.18	6.18 (0.45)	5.21	0.69 (0.06)	0.73 (0.05)	34 (21–62)	12 (2)	8.31	0.81	0.84
RAP	30 (25)	3 (0)	2.72	0.58 (0.04)	0.471 (0.289)	0.52	0.43	0.51	7.55 (0.73)	5.95	0.78 (0.08)	0.73 (0.07)	17 (9–35)	12 (5)	8.47	0.88	0.83
SCR	30 (26)	2 (1)	1.99	0.37 (0.08)	0.408 (0.258)	0.05	0.10	0.10	5.09 (1.00)	3.83	0.41 (0.08)	0.41 (0.07)	34 (22–60)	7 (3)	5.28	0.65	0.75
SIM1	16 (15)	3 (0)	2.88	0.43 (0.13)	0.501 (0.314)	0.28	0.44	0.42	8.36 (0.73)	6.93	0.74 (0.06)	0.74 (0.05)	45 (24–95)	15 (3)	10.80	0.60	0.84
SIM2	14 (12)	3 (1)	3.00	0.54 (0.11)	0.591 (0.364)	0.61	0.36	0.49	8.09 (0.73)	6.82	0.68 (0.05)	0.75 (0.06)	88 (35–∞)	14 (5)	11.67	0.75	0.94
TRO	25 (23)	6 (2)	5.12	0.77 (0.05)	0.677 (0.394)	0.44	0.40	0.50	10.09 (1.73)	7.44	0.75 (0.05)	0.77 (0.05)	54 (32–104)	20 (11)	12.89	0.91	0.92

3.2. Extent of Introgression and Its Relationship with Population Diversity

The frequency of the *LDH-C1*90* allele varied considerably across populations (range = 5–90%), from roughly native populations (SCR and CAR) to heavily stocked sites (MEL, SIM2) (Table 2; Table S1 in ESI). Note that the Atlantic component detected through the CR was 3–52% lower than that revealed by the *LDH-C1* in 5 out of 7 sampling sites (see Table S1 in ESI).

Models indicated that the frequency of *LDH-C1*90* allele remarkably contributed to explaining STR and MHC-*DAB* genetic diversity across populations (R^2 ranging from 67% to 91%; Figure 2A–D), with higher population diversity observed at intermediate *LDH-C1*90* frequency (corresponding to the maximum admixture between native and domestic lineages). However, only the model predicting STR-based expected heterozygosity was statistically supported ($p = 0.001$; Figure 2B) while others only indicated a weak tendency ($0.07 < p < 0.11$), likely because of limited observations. Conversely, *LDH-C1* based admixture did not explain CR haplotype richness across populations ($p = 0.25$; Figure 2E).

3.3. Genetic Population Structure and Demography

Genetic differentiation was statistically significant ($p < 0.05$ after Bonferroni correction) for all population pairs except SIM1-SIM2 for all markers, and RAP-TRO for the mitochondrial CR (Table S6 in ESI). Among STRs, values of locus-by-locus Fst indicated that the SSA85 locus mainly contributed to sample differentiation (Table S7 in ESI), as private alleles 101 and 103 were almost fixed in CAR and SCR, respectively (Figure S1 in ESI).
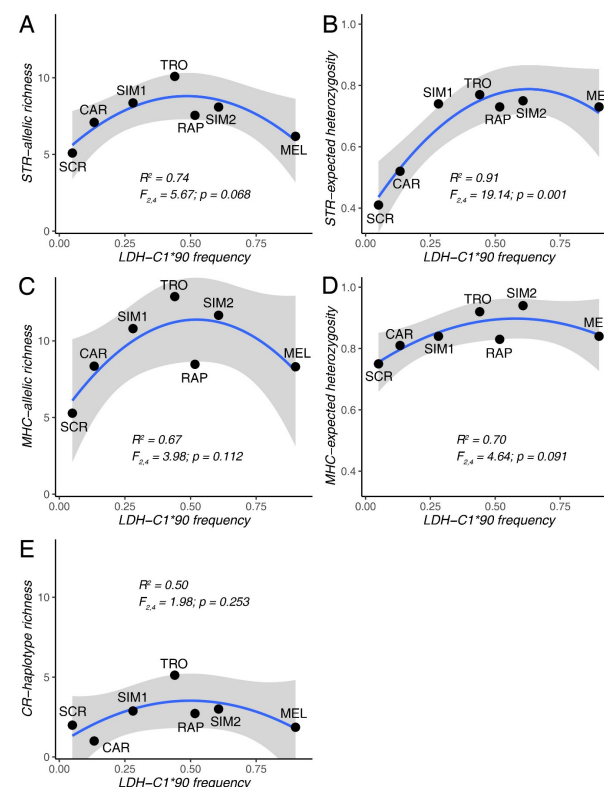
Figure 2. Second-order polynomial regression between the frequency of the domestic *LDH-C1*90* allele and measures of genetic diversity (allele/haplotype richness, expected heterozygosity), based on 11 STR loci (**A**,**B**), the MHC-*DAB* gene (**C**,**D**), and the Control Region (**E**). For each model, the R-squared, the F-statistic, the *p*-value, and the 95% confidence intervals (in grey) are shown. Population abbreviations refer to Table 1.

Irrespective of the marker class, NMDSs revealed differentiation among river catchments with special distinctiveness of Santa Croce (SCR) and Carpello (CAR) populations (Figure 3). Stocked sites (MEL, RAP, SIM1-2, and TRO) showed relative genetic similarity according to only STR-based Fst (Figure 3A), while a more pronounced differentiation was evident in MHC-*DAB* and CR-based NMDSs (Figure 3B,C).
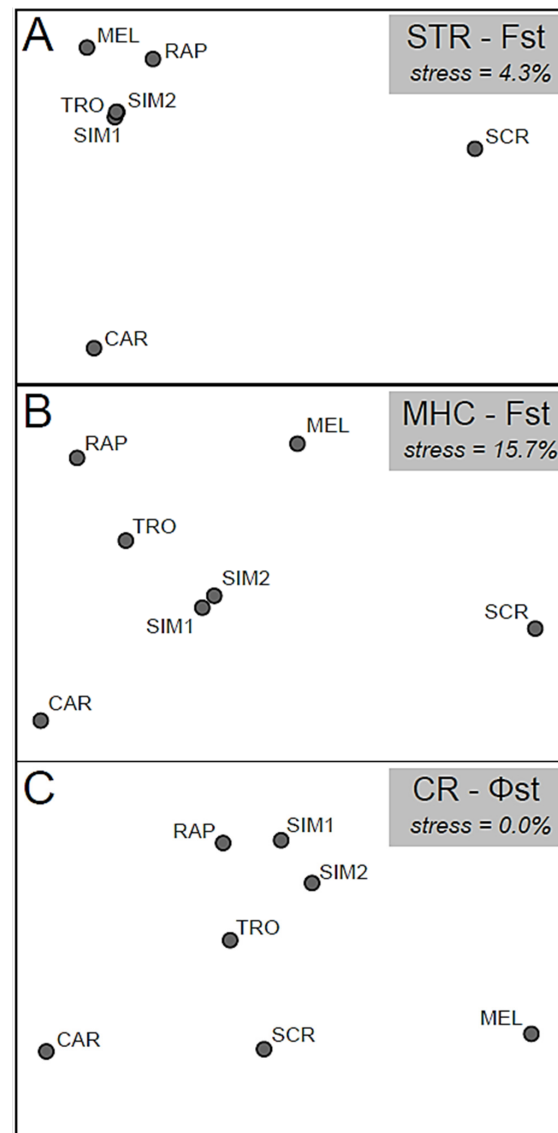
Figure 3. Non-metric two-dimensional scaling of the pairwise genetic distance (Fst or Φst) matrix computed for: (**A**) 11 STR loci; (**B**) the MHC-*DAB* gene; and (**C**) the mitochondrial Control Region. Population abbreviations as in Table 1.

Both LnP and ΔK methods suggested six optimal STRUCTURE clusters (Figure S2 in ESI), clearly corresponding to river catchments (SIM1 and SIM2 formed a single group), with low levels of inter-cluster admixture (Figure 4)—but see Figure S3 in ESI for clustering results when K ranged between 2 and 8. A similar population structure was obtained with DAPC when considering K = 6—but note that the BIC pattern suggested from 5 to 7 optimal clusters with similar support (Figure S4 in ESI)—although there was some degree of admixture, particularly between TRO and RAP. In both analyses CAR, MEL, and SCR formed a "pure" cluster each. Consistently, the GENECLASS analysis correctly assigned 86.3% of the individuals to their actual sampling site (Table S8 in ESI). Only 14 out of 175 individuals were not assigned to any site, likely because of their admixed genome (this

was particularly evident from SMALLCAPS STRUCTURE barplots of individual admixture; see Figure 4). Most of the misassignments occurred between SIM1 and SIM2 sites: when these sites were pooled together, the fraction of correct assignments for SIM increased to 83.3%, while the global one increased to 91.4%.
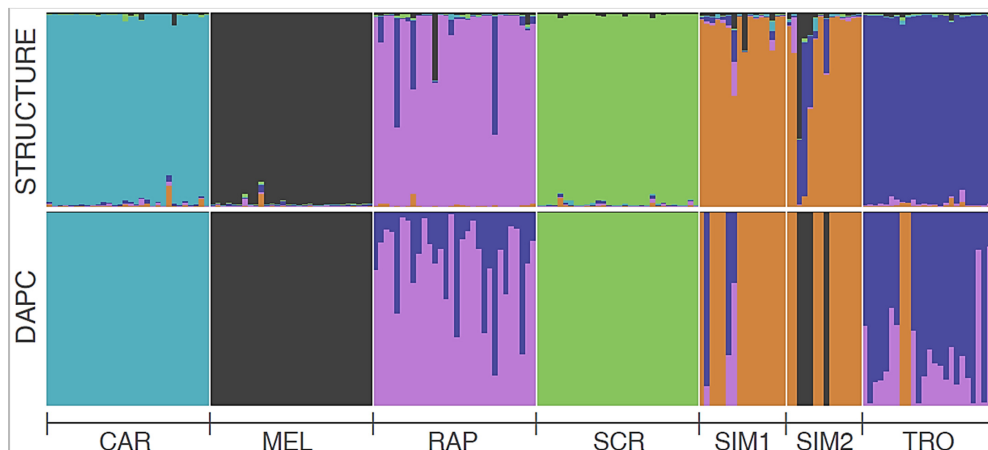
Figure 4. Barplots of individual, STR-based admixture as obtained through the Bayesian clustering analysis in SMALLCAPS STRUCTURE (above) and the DAPC assignment procedure (below) for the optimal number of inferred clusters (K = 6). Population abbreviations refer to Table 1.

AMOVA breakdowns indicated that differentiation within and among populations accounted for the vast majority of genetic variance for each maker class, while variance explained by differences among drainage basins or mountain/lowland river catchments was negligible and statistically non-significant (Table 3).

Table 3. AMOVA hierarchical analysis examining the partitioning of genetic variance of nuclear loci (STR and MHC-*DAB*) and mitochondrial DNA Control Region (CR) according to different hypothesized structures: no structure (one group), drainage basins (four groups), mountain and lowland (two groups). The amount of variation (%) explained by differences among groups, among populations within groups, and within populations, along with the *p*-values (statistically significant values are in bold) are given.

Grouping	Hierarchical Level	nDNA				mtDNA	
		11 STR		**MHC-*DAB***		**CR (K2P)**	
		Variation	*p*	Variation	*p*	Variation	*p*
One group	Among populations	21.96	**0.000**	15.08	**0.000**	62.77	**0.000**
	Within populations	78.04	**0.000**	84.92	**0.000**	37.23	**0.000**
Four groups: drainage basins	Among groups	4.61	0.273	1.05	0.393	−28.78	0.899
CAR/MEL/RAP vs. SCR	Among populations within groups	18.16	**0.000**	14.23	**0.000**	89.13	**0.000**
vs. SIM1/SIM2 vs. TRO	Within populations	77.23	**0.000**	84.72	**0.000**	39.64	**0.000**
Two groups: mountain vs. lowland	Among groups	2.06	0.231	−0.13	0.574	3.53	0.463
CAR/RAP/SCR vs.	Among populations within groups	20.56	**0.000**	15.16	**0.000**	59.78	**0.000**
MEL/SIM1/SIM2/TRO	Within populations	77.38	**0.000**	84.96	**0.000**	36.69	**0.000**

Effective population sizes estimated through the sibship method were generally low (Table 2), ranging between 17 (RAP) and 88 (SIM2). Finally, the SMALLCAPS BOTTLENECK analysis supported a recent population size reduction in MEL (*p* = 0.004).

4. Discussion

We characterized putatively/nearly neutral (STR, *LDH-C1*, and CR) and adaptive (MHC-*DAB*) variation in seven brown trout sampling locations from central Italy, revealing various frequencies of the Atlantic genetic component and different rates of introgression across sites and marker types. We also revealed a well-defined population genetic structure, consistent among markers, eventually allowing identifying distinct MUs and conservation priorities.

4.1. Genetic Diversity

Our analysis of mtDNA showed a dynamic mosaic pattern of native lineages in central Italy revealing the presence of both native and allochthonous haplotypes, consistently with previous studies [26,39]. Below we focus this discussion section on novel insight. First, the occurrence of *marmoratus* (MA) haplotypes provides new information about the spatial distribution of such mitochondrial lineage, so far poorly known in the Italian peninsula. The MA lineage identifies, although not exclusively, *Salmo marmoratus* (Cuvier, 1829) that is endemic of the Alpine watersheds draining to the Northern Adriatic Sea [1,2]. Nevertheless, haplotypes from the MA lineage are also found in wild native populations of Mediterranean brown trout from rivers flowing into the central Adriatic (e.g., Fiastrone, Esino, Aterno), including a tributary of the Tronto river (i.e., the Fosso Tufo) [6,25,28,69] and, more recently, from Corsican rivers [70]. Therefore, to the best of our knowledge, the occurrence of MA haplotypes in Rapido (RAP) and Simbrivio (SIM2) rivers provides the first remarkable records of such lineage in drainage basins of the Italian peninsula flowing into the Tyrrhenian Sea, as well as in the Latium region. Its presence could be either natural, as MA native haplotypes trout have been reported in other areas from central Italy, or may result from the introduction. Particularly, their abundance in the Rapido river (RAP) is somehow surprising, as a previous study did not detect any MA haplotypes in a sampling site located 13 km downstream from RAP [39]. However, (anthropogenic) physical barriers separate both sites, likely reducing their connectivity: natural waterfalls and small weirs are believed effective in preventing brown trout (and other freshwater salmonids) dispersal, thus shaping fine-scale patterns of population structure [71,72]. Further investigation is needed to clarify the native/allochthonous nature of MA haplotypes in RAP and SIM2. Conversely, the occurrence of the MA lineage in the Tronto river (TRO) was expected, because of its previously demonstrated native origin in such area [69]. Second, the nature of the Melfa population is surprising: MEL appears indeed almost entirely represented by domestic trout (100% AT haplotypes and 90% of *LDH-C1*90* allele), despite being located in a poorly accessible creek stretch within the core area of the "Abruzzo, Lazio e Molise National Park". Here introductions are not supposed to occur and there is no official documentation of previous stocking activities (see below for further discussion).

In a general perspective, we found two major patterns concerning the effect of stocking with domestic trout on genetic diversity in wild recipient populations. Firstly, both STR and MHC variation increased with higher *LDH-C1* based admixture, so that maximum diversity was found in RAP, TRO, and SIM sites (Figure 2A–D). Although non-native invasive populations/species often undergo a genetic bottleneck and consequently a stochastic loss of genetic diversity, an increase in genetic variability in recipient wild populations can be observed [73–75], even for non-neutral loci [34]. This may be due to successive introductions from the same or genetically divergent source populations/stocks—in the case of brown trout, high levels of genetic polymorphism and admixture emerged from the analyses of hatchery strains from Europe and North America [76]. Introgressive hybridization with native individuals may further contribute to increasing population genetic diversity, as in the case of natural secondary contact between deeply diverged allopatric lineages, or in the case of stocking with allochthonous individuals [77]. Anyhow, the increase in genetic variability caused by admixture with domestic trout has likely an impact on the original diversity of natural populations as it may disrupt their adaptive makeup, affecting the fitness of the recipient population [78–80]. This well applies to salmonids, as they frequently

show local adaptation [81], thus being particularly sensitive to population admixture [74]. Introgressed populations of Mediterranean brown trout are dramatically common in Italy, being found in the same [26,39] or different geographic areas [6,29,35,69], as well as in other countries [7,73,82]. However, this is not a general rule, and captive introgression sometimes occurs at low rates even in actively stocked ecosystems [83].

The second remarkable finding concerns the usually lower frequency of Atlantic haplotypes as compared to that of *LDH-C1*90* alleles across sampling sites, although not statistically supported (one-way Wilcoxon rank test: W = 6; *p* = 0.109), likely because of the small sample size. This may indicate mito-nuclear discrepancy and asymmetric introgression between markers, as also suggested by the lack of correlation between haplotype richness and the observed degree of admixture (*LDH-C1*90* frequency) across populations (Figure 2E). Such a mismatch may result from various non-mutually exclusive mechanisms: native females preferably mating with domestic males (non-random mating), different fitness and/or migratory behavior associated with lineages and sexes [28,84]. Anyhow, if empirically verified, this would imply an underestimation of the Atlantic component based on the mitochondrial DNA in wild admixed populations.

4.2. Population Structure and Demography

Our analyses agreed to identify a strong genetic structure of wild populations in central Italy, irrespective of the molecular marker from which the analyses relied on. Interestingly, structuring was apparently not linked to geography (i.e., drainage basins) or altitude of sampling sites (see AMOVA results, Table 3). A substantial inter-population differentiation emerged from almost all pairwise comparisons among sites and assignment tests (Tables S6 and S8 in ESI)—this does not apply for Simbrivio sites (SIM1 and SIM2; Tiber River basin) that are quite similar, being only 3 km away from each other. Consistently, both STRUCTURE and DAPC suggested each examined river catchment forming a distinct cluster showing a roughly low admixture degree and high self-assignment percentages (GENECLASS results). The observed structuring is likely influenced by the distribution of informative STR alleles (e.g., those of the SSA85 locus) and mirrors the distribution of the typical Mediterranean genotypes (*LDH-C1*100* allele) and native mitochondrial haplotypes. Indeed, populations characterized by higher frequencies of the Mediterranean component (i.e., SCR and CAR) appeared the most divergent in NMDS plots, especially with respect to STR loci. Additionally, the distinctiveness of Carpello (CAR) and Santa Croce (SCR) was evident at basal subdivisions of STRUCTURE (Figure S3 in ESI): with K = 2, CAR and SCR formed a unique cluster separated from other sites; with K = 3, CAR and SCR formed two discrete clusters, while other sites were grouped together (except for Simbrivio sites). Importantly, the differentiation between CAR and SCR was even more pronounced than that with the almost-pure Atlantic site (MEL)—such distinction emerged only since K ≥ 4 of STRUCTURE partitioning (Figure S3 in ESI). Geographic isolation and limited stocking with domestic strains likely led to and maintained the remarkable distinctiveness of CAR and SCR, as supported by the observed relatively low levels of genetic variability at all examined markers. However, we did not find the signature of bottlenecks, indicating demographically stable populations in the recent past, although coupled with small effective population sizes (Ne = 40 and 34, respectively). Overall, Ne estimates of Latium sites (excluding MEL) are comparable to those reported for eight Apennine populations of Mediterranean brown trout from the Adriatic slope (Ne range: 27–58; Mann–Whitney test: U = 20, *p* = 0.846; [6]) and nine wild brown trout populations in North-western Spain (Ne range: 21–137; Mann–Whitney test: U = 19.5, *p* = 0.410; [27]) using the COLONY software and almost identical analysis parameters. Generally, small brown trout Ne sizes may result from multiple concurring factors, among which the isolation of wild populations and demographic fluctuations associated with water flow fluctuations [27].

The genetic makeup of MEL, almost entirely Atlantic, unambiguously indicates its anthropogenic origin, resulting from the release of hatchery-reared individuals in a natural

site where native individuals were likely originally rare or even absent. We might argue that such introduction is relatively recent, as supported by the evidence of a recent bottleneck. Interestingly, the gene pool of the MEL population is clearly distinguishable from those of the other examined populations (results from the STRUCTURE analysis and the assignment test), including the admixed ones (according to the occurrence of the *LDH-C1*90* allele). This evidence eventually indicates that the source stock of MEL was likely different from most of those used for stocking purposes in the other Latium sites.

Finally, the employment of MHC in the conservation genetics of Mediterranean brown trout deserves a special mention. Distinguishing between domestic (Atlantic) and Mediterranean-native MHC-*DAB* alleles was challenging or even not possible because of non-diagnostic sequence genealogies and the probable widespread allele sharing among Atlantic and native lineages [34]. In other words, the highly polymorphic MHC-*DAB* locus apparently shows limited usefulness for admixture estimates in the examined population, meanwhile being suitable for investigating functional variation and population structure, and thus defining "adaptive" units.

4.3. Management Implications

All in all, our analyses pointed out five MUs on native Mediterranean brown trout in the study area, corresponding with local populations at each river catchment, and characterized by a roughly site-specific genetic makeup: Santa Croce (SCR), Carpello-Fibreno (CAR), Tronto (TRO), Simbrivio (SIM1 and SIM2), and Rapido (RAP). Below we discuss site-specific threats and conservation actions to preserve the local diversity of each MU.

The SCR population should be considered a conservation priority for multiple reasons: (1) typical genetic makeup (e.g., excluding the domestic AT-1, SCR is fixed for the private ME-1 haplotype) and limited introgression from domestic lineages; (2) remarkably reduced genetic diversity even at adaptive genes (Table 1), presumably a signature of ancient bottlenecks [26]—Santa Croce directly flows into the sea, thus the population is likely isolated since the loss of anadromy of Mediterranean brown trout; (3) limited habitat, also potentially vulnerable to consequences of climate change—for instance, the population could not migrate upstream in case of increase in water temperatures [11], risking local extinction; (4) plausible adaptation to peculiar ecological features, such as the relatively hot water temperature of a lowland watercourse. Here, in situ conservation action should limit angling activities, and monitor poaching and illegal stocking with domestic trout. In addition, water abstraction during the dry season should be supervised (e.g., through the maintaining of the minimum river flow) since overexploitation may affect trout survival. Supportive in situ reproduction can be evaluated to sustain the natural population in the case of strong demographic fluctuations due to human activities.

Similarly to SCR, the CAR population is another rare "sanctuary" of highly pure Mediterranean brown trout. Here, multiple studies pointed out the distinctiveness of the population, with local haplotype(s) almost fixed (this study) [26,38,39,85]. The CAR site is located in a Regional protected area where stocking activities stopped decades ago, ensuring the genetic integrity of the population [38]. Nevertheless, local rangers reported (personal communication) increasing trout predation by fish-eating birds (e.g., herons, cormorants) during the breeding period, when trout gather at the confluence between the creek Carpello (the main spawning habitat) and River Fibreno (the large watercourse hosting most of the trout population). Future actions should aim at minimizing over-predation in such a circumscribed area.

Simbrivio (SIM1, SIM2) and Tronto (TRO) populations deserve conservation attention especially because they host high genetic variability (e.g., multiple native lineages and some locally distributed CR haplotypes), possibly resulting from their supposed demographic stability—they are the largest populations so we may expect a limited loss of diversity by genetic drift as compared to smaller populations. Nevertheless, both sites are/were subject to massive stocking with domestic trout, therefore, exotic genes also contribute to

the observed high diversity. For these reasons, conservation actions should aim to increase the frequency of native genes, meanwhile reducing domestic ones. It should be considered to: (1) effectively monitor the (illegal) introduction of Atlantic (hatchery) trout; (2) start programs, one for each MU, of selective breeding and restocking with genetically suitable adult individuals, fry, or fertilized eggs. Furthermore, (3) recreational angling could also contribute to the purpose if controlled and carried on before restocking with native trout, as angling selectively removes recently introduced domestic trout from wild populations: catchability of hatchery trout is believed more effective than that of native individuals [86], although the efficiency of "selective angling" remains controversial [73].

In principle, the RAP population may constitute a rare refugee of the *marmoratus* (MA) lineage in the Tyrrhenian slope. Nevertheless, further investigations (e.g., examining additional sampling sites and molecular markers) are needed to confirm its native origin. If so, Rapido should be treated as a separate MU and a protected area should be set up in consequence. The above-mentioned conservation actions should be applied to preserve native genes and remove domestic genes (*LDH-C1*90* allele frequency is about 52%).

Finally, we invoke future surveys aiming at exploring the occurrence of native populations in the Melfa basin, to be employed for the restocking of the MEL site upon the eradication of the extant naturalized Atlantic population. As the MEL population is located above a 10 m waterfall within a National Park, protection and isolation of the re-founded population would be facilitated, but see [6] for the ineffectiveness of protected areas in Italy to preserve native Mediterranean brown trout biodiversity.

Our data reiterate that conservation genetics provides multiple tools for monitoring threatened salmonid populations/species and identifying donor populations, thus preventing the spread of non-native genes [87]. In addition, whole-genome sequencing enhances the possibility to disclose further hidden biodiversity in the *Salmo trutta* complex [88]. However, as for other species, the opportunity offered by molecular markers still seems dismissed in planning management actions [18].

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