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RESEARCH ARTICLE

Introgression despite protection: the case of native brown trout in Natura 2000 network in Italy

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Abstract

Mediterranean brown trout is subject to several serious threats such as pollution, water abstraction, habitat alteration and especially genetic introgression with domestic strains used for stocking activities. Despite this latter issue has largely been debated by scientists, local managers and stakeholders for decades, official stocking practices with domestic trout still persists in several countries (Italy included), even if there are laws explicitly prohibiting introduction of organisms of non-local origin. Probably, the last opportunity to conserve native brown trout populations is represented by protected areas. Therefore, in the present study, we aimed to verify the role of the Nature 2000 network and a national park as valid tools to guarantee the survival of native brown trout in the Apennines. Partial mitochondrial DNA control region sequence analysis and genotyping of the locus *LDH-C1** and 11 microsatellites were used to investigate the genetic diversity of three rivers from central Italy. For all rivers investigated a temporal analysis of introgression was also carried out. The genetic diversity of three domestic stocks was included in the sampling design for comparison. The main results of this study indicated that: (i) the genetic diversity of brown trout in central Italy is very complex and (ii) its conservation is seriously threatened by genetic introgression phenomena still ongoing. The only samples showing no introgression or a decrease in genetic introgression were those isolated by the presence of natural and/or artificial barriers to fish movements rather than protected by inhabiting rivers within the Natura 2000 network. This observation prompts an important reflection on issues concerning fluvial continuity restoration and suggests that barrier removal should be undertaken with caution in order to avoid the concrete risk of domestic trout spreading that could promote additional loss of native brown trout biodiversity.

Keywords Mediterranean native trout · Introgression · Natura 2000 network · Biodiversity conservation

Introduction

The brown trout (*Salmo trutta* Linnaeus, 1758) is a complex of incipient species distributed around the Palearctic region (Kottelat and Freyhof 2007). For Northern populations, glacial peaks that occurred during the Pleistocene represented unfavorable periods, allowing divergence at the margin of the ice sheet, where Ponto-Caspian (DA), Atlantic (AT) (Bernatchez 2001) and Duero (DU) (Suárez et al. 2001; Vera et al. 2010) main mitochondrial lineages differentiated. On the contrary, interglacials represented unfavorable conditions for brown trout populations distributed across the Mediterranean region. In these warm and dry periods, Adriatic (AD), Mediterranean (ME) and *marmoratus* (MA) lineages probably differentiated in an allopatric and/or parapatric context because of isolation in thermal refuges. On the other hand, in the Mediterranean area, coldest phases favored colonization events and an extensive intergradation in the Mediterranean rivers (Cortey et al. 2004). For instance, in Italy AD, ME and MA haplotypes can be found together in the same population (Splendiani et al. 2006).

The *Salmo trutta* complex is characterized by an intricate pattern of phenotypic and geographical forms probably

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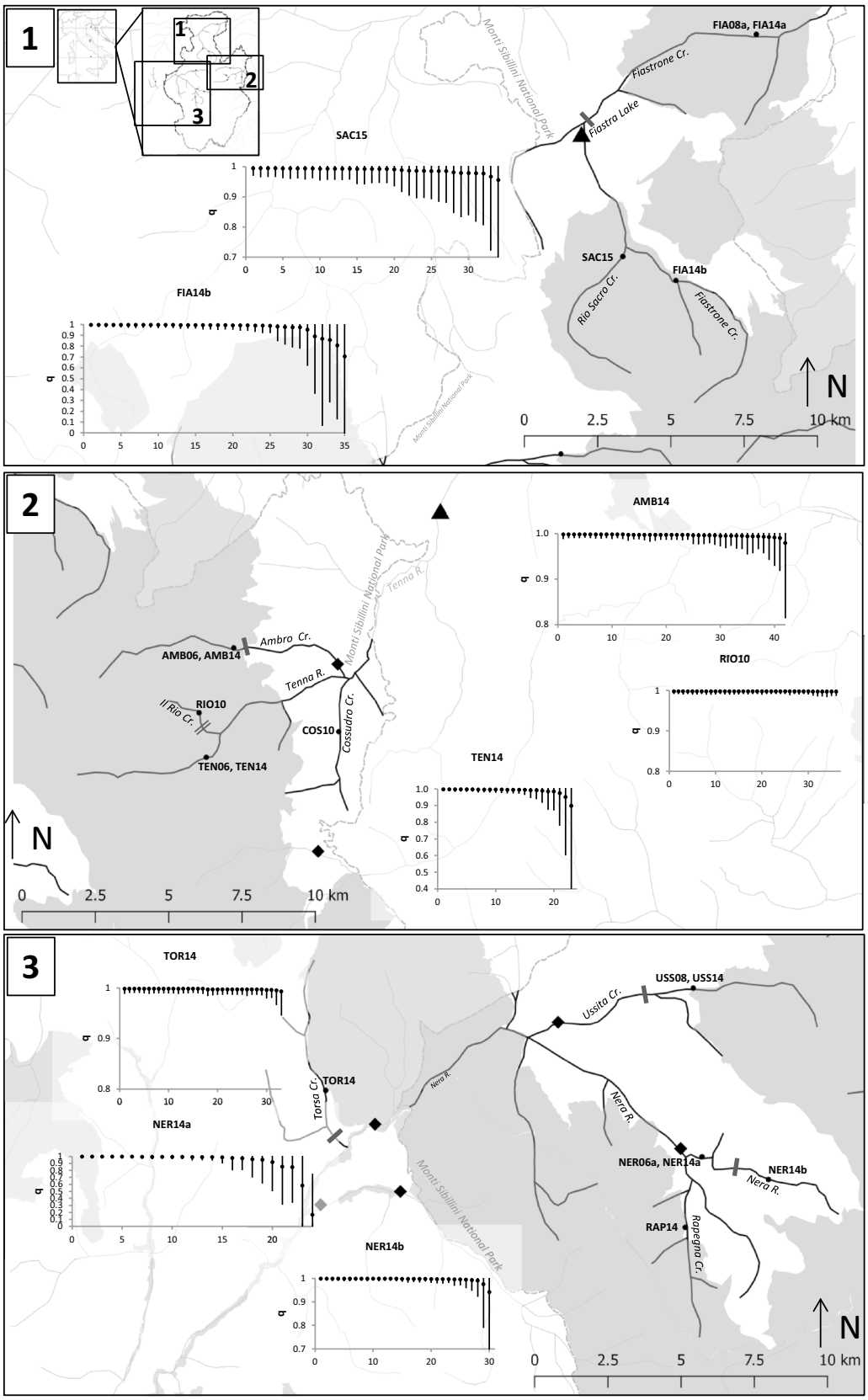
1
2
3
Monti Sibillini National Park
FIA08a, FIA14a
Fiastrone Cr.
Fiastra Lake
SAC15
FIA14b
Rio Sacro Cr.
Fiastrone Cr.
N
0 2.5 5 7.5 10 km
AMB14
RIO10
TEN14
Ambro Cr.
AMB06, AMB14
Tenna R.
Il Rio Cr.
COS10
Cossudro Cr.
TEN06, TEN14
TOR14
USS08, USS14
Ussita Cr.
Torsa Cr.
Nera R.
NER14a
NER06a, NER14a
NER14b
RAP14
Rapegna Cr.
q

◂**Fig. 1** Map showing the location of sampling sites in the Monti Sibillini National Park: (1) Fiastrone stream (upper Chienti River basin), (2) upper Tenna River basin and (3) upper Nera River basin. For each location analysed by 11 microsatellites the plots of individual admixture coefficient (q) values and 90% credible intervals (CI) were also reported. In this study a $q \approx 1$ indicate a pure native trout. Single solid black bar denotes artificial and potentially insurmountable river barriers, double solid black bar denotes waterfalls, triangles denotes fishing playgrounds and squares denotes hatcheries. Grey areas = Natura 2000 network; dashed line = Monti Sibillini National Park borders

underlying the taxonomic inflation reported in literature with the description of nearly 50 *Salmo* species (e.g., Tougard et al. 2018). In Italy, some taxa have been described based on their very restricted geographic range and/or on biological and genetic peculiarities, as in the case of *S. carpio* an endemic species of the Garda Lake (northern Italy) and *S. fibreni* that is endemic to the Posta Fibreno Lake (central Italy) (Gandolfi et al. 1991). The occurrence of other species has been tentatively proposed mainly based on their distribution along the two main peninsular biogeographic districts for fish species. For example, in the case of brown trout populations inhabiting the rivers of the Padano-Venetian district, the following species were proposed: *S. cenerinus* Chiereghini, 1847, *sensu* Kottelat and Freyof (2007), *S. farioides* Karaman, 1938 (*sensu* Bianco 2014)) and *S. ghigii* Pomini, 1941. As for the Tuscano-Latium district and major islands (Sicily and Sardinia), two names are usually adopted for populations belonging to the *Salmo trutta* complex: *S. cettii* Rafinesque, 1810 or *S. macrostigma* Duméril, 1858 originally described for north Africa and then used elsewhere for other Mediterranean populations (Tougard et al. 2018). Finally, in northern Italy, the marble trout, *S. marmoratus* Cuvier, 1829 represents a taxon clearly distinguishable from the above peninsular taxa due to strong morphological and biological peculiarities and also for the genetic make-up (Gratton et al. 2014).

Although the molecular studies carried out on Italian brown trout populations during the last two decades have not provided enough data to clarify the taxonomic disagreement persisting between authors, they demonstrated the persistence of an important level of genetic differentiation between wild populations (Gratton et al. 2013; Fabiani et al. 2018; Berrebi et al. 2019). Despite his extraordinarily rich biodiversity at a very restricted geographic scale, native brown trout populations are now heavily menaced by two main threats: (i) habitat loss and (ii) introgressive hybridization with alien domestic trout of Atlantic origin used for restocking (see Caputo et al. 2004; Lorenzoni et al. 2006). Concerning the second threat, Splendiani et al. (2016) reported that less than 3% of Apennine populations of the Italian native trout were immune by introgression, whereas the remaining populations showed various degrees of hybridization, with some populations totally replaced by alien trout. In addition, non-hybridized populations usually persist in isolated and fragmented headwater habitats, where their long-term persistence is uncertain.

During the activities of the European Project Life + TROTA ("Trout populations recovery in Central Italy", LIFE12 NAT/IT/000940), we had the opportunity to investigate the genetic structure of brown trout populations from the Natura 2000 protection network in central Italy, with particular reference to the territory of the Sibillini Mountains National Park (Fig. 1). The main goal of this work was to highlight possible conservation strategies to improve the management of the remaining native trout populations in a protected area of central Italy. This was accomplished through the description of: (i) intra-population genetic variability, (ii) rates of hatchery introgression and (iii) genetic differentiation between samples, using nuclear (*LDH-C1** and 11 microsatellites) and mitochondrial (310 bp 5′-end of mtDNA control region) markers.

Materials and methods

Study area and possible threats

The Sibillini Mountains National Park (Fig. 1) was established in 1993 and covers an area of approximately 700 km^2 on a predominantly mountainous area, mainly covered with beech woods, whose highest peak is Mount Vettore (2476 m). The geological substrate is mostly represented by limestone and in this area the Apennine chain acts as a watershed between the Adriatic and Tyrrhenian slopes. Four main rivers, belonging to three catchment basins, originate in the territory of the park: Aso, Tenna (including the Ambro branch) and Chienti (including the Acqua Santa, Rio Sacro and Fiastrone branches) flowing leading into the Adriatic Sea; the Nera river (including the Rapegna, Torsa and Ussita branches) is the main tributary of the Tiber River that flows into the Tyrrhenian Sea. The springs of these rivers lie on carbonate geological substrates that are good water tanks (aquifers) that make the flow of these river relatively steady. In the study area, the main threats for native trout populations are represented by: (i) artificial barriers along the river course, (ii) an artificial basin (the Fiastra lake) acting as a tank of allochthonous fish (including trout) and used as a fishing area, (iii) hatcheries and (iv) activities of restocking with domestic trout still performed in the proximity of the Park borders (Fig. 1).

Genetic analyses

A total of 608 wild brown trout were collected by electrofishing from 18 localities (belonging to three catchment basins, namely Chienti, Nera and Tenna) within the Natura

2000 Network (Fig. 1; Table 1) in the years 2006–2015. For a total of six sampling localities a comparison between diachronic samples (with a medium seven years interval) was also possible. In this way a temporal comparison in terms of increased or decreased rates of genetic introgression (by using the *LDH-C1** molecular marker) was possible. A total of 46 brown trout from two hatcheries (HAT-1 and HAT-2) used for stocking in the study area were analyzed for comparison with wild individuals. In addition, 16 specimens were collected form a third hatchery (HAT-3) located in the study area and declaring to breed Mediterranean trout collected from Tyrrhenian water courses. As far as we know, this latter domestic stock of putative Mediterranean origin has never been officially used in the study area for stocking activities.

From each fish, a small fin clip was removed and conserved in 95% ethanol until DNA extraction. Total genomic DNA was extracted following a phenol–chloroform method, as described by Taggart et al. (1992) for the specimens collected before 2014. For the rest of the samples, genomic DNA was extracted using an automated DNA extractor (MagCore® Automated Nucleic acid Extractor in combination with the Genomic DNA Tissue Kit 401). After the extraction of genomic DNA, the control region of the mitochondrial DNA (mtDNA) was PCR-amplified according to Bernatchez and Danzmann (1993) on a subset of samples. To avoid expensive costs for sequencing we selected only one population for each locality. Screening of mtDNA genetic variability was conducted through Single-Strand Conformation Polymorphism (SSCP) analysis. The control region PCR products were digested with *Alu*I restriction enzyme and run on a non-denaturing polyacrylamide gel and subjected to a 12-h electrophoretic run at 5 W in a cool chamber. A segment of 310 base pairs (bp) at the 5′ end of the mtDNA control region (see Aurelle et al. 2001) was sequenced in a sub-sample of individuals with the same SSCP profile (that is, three trout per each SSCP morph). Sequences of 310 bp of the 5′-end mtDNA control region were used to detect the diagnostic sites of the major mitochondrial lineages of *Salmo trutta* complex, and therefore to assess the frequency of allochthonous (AT lineage) and native (AD, ME and MA lineages) haplotypes. The level of introgression was calculated as the percentage of allochthonous haplotypes for each population (Table 1).

A 440 bp segment of the *LDH-C1** nuclear locus was PCR-amplified in all samples. This nuclear locus contains diagnostic alleles for the north Atlantic (allele **90*) and Mediterranean populations (allele **100*) of the *Salmo trutta* complex. The individuals analysed were genotyped as described in McMeel et al. (2001). Conformity with Hardy–Weinberg equilibrium was performed as described for microsatellite DNA (see below). The statistical significance of *LDH-C1** locus allele frequency differences between diachronic samples were tested by both Fisher's exact and Chi square methods using the computer program CHIFISH version 1.3 (Ryman 2006). The statistical significance of *LDH-C1** locus allele frequency differences was also tested between group of samples: (i) above barrier *vs* below barrier (where by barrier we intend those natural and/or artificial barrier that we considered as insurmountable for trout, based on personal field observations) and (ii) samples collected during the years 2006 and 2008 *vs* samples collected recently (years 2014 and 2015).

Microsatellite DNA analysis was conducted on a subset of populations (eight wild and three domestic samples). To avoid expensive costs for microsatellite genotyping we selected one sample for each locality and we excluded also those samples characterized by an almost full alien genetic make-up as revealed by *LDH-C1** and 5′-end mtDNA control region analyses (as for example (USS08-14, RAP14 and COS10)). A total of 11 microsatellite loci (di- and tetra-nucleotide repeats) were labeled with fluorescent dyes and multiplexed in two separated reactions as reported in Table S1. These loci were genotyped using an ABI-PRISM 3130xl Genetic Analyzer (Applied Biosystems).

The incidence of null alleles and other genotyping errors (allele dropout and stutter peaks) were assessed with MICROCHECKER 2.2.1 (Van Oosterhout et al. 2004). In addition, the Dempster algorithm (Dempster et al. 1977), available in FreeNa (Chapuis and Estoup 2007), was used to estimate the effects of null allele frequencies on *F* statistics. Tests for Hardy–Weinberg equilibrium (HWE) were carried out adopting the exact test implemented in ARLEQUIN 3.5 (Excoffier and Lischer 2010). The computer program GENEPOP (Raymond and Rousset 1995) was used to evaluate the presence of genotypic linkage disequilibrium between loci or populations. The nominal level of significance (5%) was adjusted following a Bonferroni procedure (see Rice 1989). The Allelic richness (A_R), Nei's gene diversity (H_e) and the inbreeding coefficient (F_{IS}) were calculated for each population using FSTAT 2.9.3 (Goudet 1994). The statistical significance of F_{IS} values were tested using 10,000 permutations ($\alpha = 0.05$). Finally, we also evaluated effective population size using the sibship assignment method (Wang 2009) available in the computer program COLONY. The analysis of N_e was also carried out after removing from the wild collections specimens with a coefficient membership value for the native cluster ≤ 0.95.

Several methods were employed to investigate genetic differentiation between wild and domestic populations. First, pairwise estimates of F_{ST} (*sensu* Wright) were computed using FSTAT. To test the role of barriers on the protection of brown trout native genetic diversity we carried out also *Fst* comparisons involving group of samples as follow: (i) samples upstream of the barriers (SUpB) *vs* samples downstream of the barriers (SDwB), (ii) SUpB vs hatcheries and (iii)

Table 1 Descriptive population genetic statistics from the analysis of *LDH-C1**locus, partial (≈310 bp) 5′-end mtDNA Control Region and 11 microsatellites for 18 wild and 3 domestic brown trout samples from central Italy

River/Hat.	Location code	Lat.	Long.	n_L	**90*	n_{mt}	ADs-7	ADcs15b	MAs-5	MEs-1
Tenna	AMB06	360108	4756852	41	0.12	24	1.00			
	AMB14	360108	4756852	44	0.11	44	1.00			
	TEN06	359096	4753163	43	0.14	24	0.96			
	TEN14	359096	4753163	24	0.19	24	0.92			
	COS10	363626	4753943	22	1.00	22				
	RIO10	358763	4754582	37	0.00	37	0.78		0.22	
Chienti	SAC08	351772	4763006	22	0.36	NA				
	SAC15	351772	4763006	35	0.46	35	0.26			0.03
	FIA08a	356473	4770442	26	0.62	NA				
	FIA14a	356473	4770442	24	0.54	19	0.26		0.32	0.10
	FIA14b	353564	4762155	37	0.26	37	0.49			
Nera	TOR14	336902	4753175	33	0.02	33	1.00			
	NER06a	349687	4750634	38	0.22	38	0.82			
	NER14a	349687	4750634	23	0.33	24	0.79			
	NER14b	349687	4750604	31	0.18	31	0.81			
	USS08	349515	4756353	45	0.69	NA				
	USS14	349515	4756353	48	0.82	48				
	RAP14	349062	4748252	35	0.33	20	0.40			
Hatcheries	HAT1			26	0.96	26				
	HAT2			20	1.00	20				
	HAT3			16	0.56	16		0.66		

River/Hat.	ATs-1	n_m	A_R	*He*	F_{IS}	*LD*	*Ne* (95% CI)	Ne^* (95% CI)	Mean *q* (CI)
Tenna		NA							
		42	4.83	0.59	−0.03	10 (2)	42 (26–69)	38 (24–65)	0.996 (0.997–1000)
	0.04	23	5.58	0.62	0.07	5 (2)	44 (24–83)	48 (23–140)	0.987 (0.924–1.000)
	0.08	NA							
	1.00	NA							
		36	3.34	0.42	−0.02	1 (0)	27 (16–48)	27 (16–48)	0.998 (0.992–1.000)
Chienti		NA							
	0.71	34	7.50	0.75	0.08	7 (1)	58 (39–98)	47 (26–111)	0.988 (0.920–1.000)
		NA							
	0.32	NA							
	0.51	35	6.98	0.75	**0.10**	6 (1)	50 (32–81)	36 (20–69)	0.966 (0.843–1.000)
Nera		33	4.70	0.66	−0.06	4 (0)	41 (25–68)	41 (25–68)	0.997 (0.988–1.000)
	0.18	NA							
	0.21	24	6.86	0.69	0.09	6 (1)	39 (23–74)	21 (11–47)	0.923 (0.804–0.983)
	0.19	30	4.15	0.52	0.02	4 (1)	33 (18–59)	34 (20–59)	0.994 (0.967–1.000)
		NA							
	1.00	NA							
	0.60	NA							
Hatcheries	1.00	26	7.13	0.80	−0.08	17 (2)	32.9 (19–59)		
	1.00	20	7.63	0.72	0.07	2 (1)	58 (32–180)		
	0.44	16	5.82	0.72	0.08	7 (0)	19 (9–49)		

From left: location information; sample size of *LDH-C1** analysis (n_L); *LDH-C1** allele **90* frequencies; sample size of mtDNA analysis (n_{mt}); haplotype observed; sample size of microsatellite analysis (n_m); Allelic richness (A_R); expected heterozygosity (*He*); Fixation index (F_{IS}) with in bold significant values ($P < 0.001$); number of significant Linkage disequilibrium (LD) tests (in brackets highly significant tests); effective population size (N_e) and 95% credible intervals (CI); effective population size using only pure trout (N_e*) and 95% CI; mean admixture coefficient and credible intervals (mean *q* (CI))

SDwB *vs* hatcheries. The statistical significance of tests was evaluated by 10,000 permutations. Second, genetic differentiation between samples was explored employing a Discriminant Analysis of Principal Components DAPC (Jombart et al. 2010). The DAPC is a multivariate method designed to identify and describe clusters of genetically related individuals. This method does not rely on a specific population genetic model and it is therefore free of assumptions about Hardy–Weinberg equilibrium or linkage disequilibrium. When group priors are lacking, DAPC uses sequential K-means and model selection to infer genetic clusters. K-means relies on the same model as DA to partition genetic variation into a between-group and a within-group component and attempts to find groups that minimize the latter. Third, the level of genetic structure represented in the whole data set was investigated with STRUCTURE 2.3.2.1 (Pritchard et al. 2000; Falush et al. 2003).The STRUCTURE parameters were set choosing the admixture model and considering allele frequencies between samples correlated. Then, we tried to search the best number of genetic clusters (K) testing the probability for K ranging from 1 to 11. For each K, a total of ten replicates were performed adopting a burn-in of 10,000 iterations, followed by 500,000 iterations. Then, to find the best k value we adopted four statistics (MedMeaK, MaxMeaK, MedMedK, and MaxMedK) considered outperforming the popular Evanno method (delta K) specially in cases of uneven sample size (Puechmaille 2016). The STRUCTURE bar plot was visualized by using the web app (http://pophelper.com/) POPHELPER.

STRUCTURE was also used to estimate the admixture coefficient (q) of each individual and their 95% credible intervals (CI) in each wild sample. Each run was performed assuming a K = 2 (i.e. domestic *vs* native). Virtually in each run, the domestic ancestry of each wild sample was calculated incorporating data from the two domestic references for stocking materials in the last years in the study area (HAT-1 and HAT-2).

Results

Overall, five haplotypes were observed: (i) the haplotype ATs1, belonging to the Atlantic phylogenetic lineage (AT, *sensu* Bernatchez 2001) and common in the watercourses of northern Europe and in the domestic stocks, (ii) the new haplotype ADcs-15b (Adriatic phylogenetic lineage, AD), (iii) the haplotype ADs7, very common in the peri-Adriatic area, (iv) the haplotype MAs5 (*marmoratus* phylogenetic lineage, MA), observed so far only in central Italy's watersheds, and (iv) the MEs1 (Mediterranean phylogenetic lineage, ME), widespread in the Mediterranean rivers. The new haplotype observed in this study was classified into the AD lineage based on a further analysis of the complete 5′-end mtDNA control region segment (data not shown) that showed a single nucleotide substitution when compared to haplotype ADcs-15, already observed by Cortey et al. (2004) in Corsican samples. Altogether, two wild samples (COS10 and USS14) out of 18 showed the exclusive presence of alien mitochondrial variants in the study area (Table 1). In the remaining 16 wild populations, the frequency of allochthonous haplotypes showed the highest value in RAP14 (60%), while in AMB06, AMB14, RIO10 and TOR14, only native haplotypes were observed; overall, the presence of mtDNA native genetic variants in the rest of wild populations sites ranged from 29% (SAC15) to 96% (TEN06) (see Table 1). The two hatchery samples (HAT-1 and HAT-2) hosting classical Atlantic domestic trout were characterized, as expected, by the sole presence of the ATs1 haplotype. On the contrary, the hatchery HAT-3 showed a mixture of both Atlantic haplotypes (44%) and Mediterranean haplotypes (66%). These latter can be referred to the haplotype ADcs-15b. From a temporal point of view, AT haplotypes were never recorded in AMB during the sampling period (AMB06 and AMB14, percentage of 0%). On the contrary, an increase of mtDNA introgression was observed between TEN06 and TEN14 (AT haplotypes, respectively from 4 to 8%) and between NER06 and NER14 (from 18 to 21%) (Table 1).

The comparison of the introgression patterns detected with the *LDH-C1** locus and the mtDNA haplotypes showed a high and significant correlation (Spearman's rs = 0.83, $P < 0.0001$). In fact, the *LDH-C1*90* allele was observed with values close to 100% in the same populations where high introgression values were recorded at mitochondrial level. Likewise, the highest frequency of *LDH-C1*100* allele was found in populations nearly fixed for native mtDNA haplotypes (Table 1). Only in one case out of 21 (FIA14b), the observed genotype frequencies showed significant deviation from Hardy–Weinberg expected proportions. However, this significance disappeared when the Bonferroni correction method was applied. Comparisons of temporal collections showed an evident, although not significant ($P_{FISHER} = 0.467$ and $P\chi_2 = 0.331$), decrease of introgression only in Fiastrone stream collections, with the percentage of *LDH-C1*90* that decreased from 61.5% in 2008 (FIA08a) to 54.2% in 2015 (FIA15a). Also for Ambro stream sampling sites a slight decrease (not significant, $P_{FISHER} = 1.000$ and $P\chi_2 = 0.866$), (0.83%) of *LDH-C1*90* allele was observed between 2006 (AMB06) and 2014 (AMB14). On the contrary, for the rest of temporal collections an increase of the *LDH-C1*90* allele was evident. However, these latter temporal comparisons resulted statistically significant only for the diachronic samples of the Nera River: NERA06a *vs* NERA14a ($P_{FISHER} = 0.030$ and $P\chi_2 = 0.018$) and USS08 *vs* USS14 ($P_{FISHER} = 0.005$ and $P\chi_2 = 0.004$). Finally, a significant higher frequency of *LDH-C1*90* allele was observed in case of comparisons carried out by grouping samples as follows:

(i) samples upstream of the barrier *vs* samples downstream of the barrier ($P_{FISHER} = 0.000$ and $P\chi_2 = 0.000$) and (ii) samples collected in years 2006–2008 versus years 2014–2015 ($P_{FISHER} = 0.005$ and $P\chi_2 = 0.005$) (Fig. 2).

Microsatellite allele frequencies distribution, Nei's unbiased gene diversity (*He*), departures from Hardy–Weinberg equilibrium (F_{IS}), and allelic richness (*AR*) are listed in Table 1 and in Table S2. The test performed using MICROCHECKER suggested the presence of null alleles at *Oneμ2* and *Ssa103NVH*, respectively in five samples out of 11 and 8/11. Therefore *Oneμ2* and *Ssa103NVH* were excluded from the F_{IS} estimates that could be biased by null alleles (Van Oosterhout et al. 2004). In total, 244 alleles were detected using 11 microsatellite loci. The number of alleles per locus ranged from four (*Str60*) to 44 (*Ssa410UOS*). In general, samples characterized by a non-native genetic make-up (high frequency of *LDH-C1*90* allele) showed a higher degree of intra-population genetic diversity than the native samples. Globally, the mean allele richness was 5.86, ranging from 3.34 in RIO10 to 7.63 in the hatchery sample HAT2, and similarly, mean *He* ranged from 0.42 in RIO10 to 0.80 in HAT2. A significant excess of homozygotes was detected in SAC15 and NER14a at locus *SsoSL417* and in FIA14b at locus *Ssa410UOS*. All these three samples were characterized by a mixture of Atlantic and native genes (e.g., *LDH-C*90%* in Table 1, respectively, 45.7, 32.6 and 25.7). Over all loci, only in FIA14b a significant F_{IS} value was observed (Table 1 and Table S2). Test for linkage disequilibrium (LD) at population level revealed 69 significant ($P < 0.05$) comparisons out of 363. However, only 11 tests resulted significant after Bonferroni correction. These departures involved all samples but RIO10, TOR14 and HAT3. In no case highly significant LD tests involved the same pair of loci. We interpreted the detection of LD as a signal of a recent genetic admixture between two divergent gene pools instead of a non-random association of alleles at different loci. In fact, apart from the domestic sample HAT3 in which the outcome of LD tests should be taken into account with caution due to the low sample size ($n = 16$), all the rest of samples showing the lowest number of significant LD tests were the wild collections characterized by null or scarce values of genetic admixture with the Atlantic cluster, namely RIO10 (Tenna River) and TOR14 (Nera River) (Fig. 1; Table 1). The estimation of the effective population size (N_e) ranged from 27 (RIO10) to 58 (SAC15) in wild collections and from 19 (HAT3) to 58 (HAT2) in domestic collections. The same test carried out only on "pure" trout (that is, trout with a coefficient of membership for the native cluster ≥ 0.95) showed a clearly

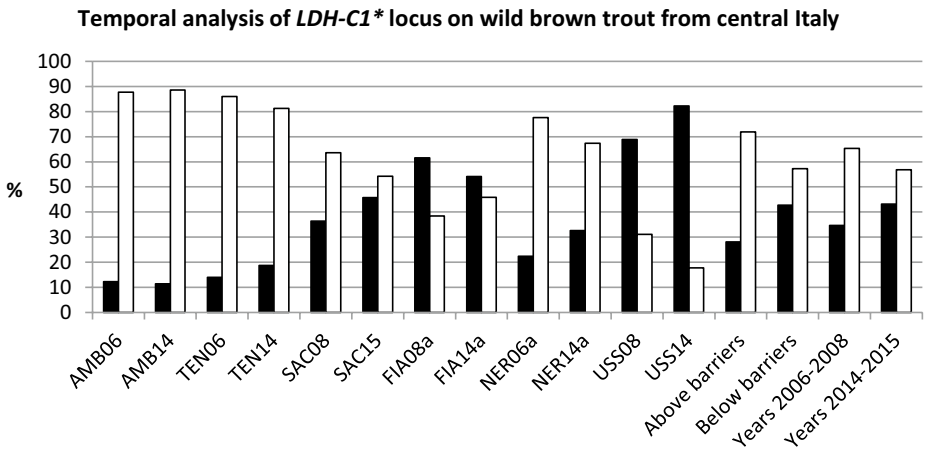

Fig. 2 Temporal analysis of *LDH-C1** locus on six diachronic wild brown trout samples and/or group of samples from central Italy. In white the allele **100*, in black the allele **90*. Allele frequency differences between samples and/or groups of samples were tested by chi square and Fisher's exact test by using CHIFISH (Ryman 2006). Sampling code as in Table 1

Tests	P (Fisher)	SE(P)	χ^2	df	P(χ^2)
AMB06 *vs* AMB14	1.000	0.000	0.028	1	0.866
TEN06 *vs* TEN14	0.780	0.000	0.217	1	0.641
SAC08 *vs* SAC15	0.338	0.002	0.969	1	0.325
FIA08a *vs* FIA15a	0.467	0.001	0.946	1	0.331
NER06a *vs* NER14a	**0.030**	**0.001**	**5.547**	**1**	**0.018**
USS08 *vs* USS14	**0.005**	**0.000**	**8.074**	**1**	**0.004**
Above *vs* Below barriers	**0.000**	**0.000**	**29.498**	**1**	**0.000**
Years 2006-2007 *vs* 2014-2015	**0.005**	**0.000**	**7.997**	**1**	**0.005**

lower effective population size in those samples characterized by higher introgression values (Table 1).

As indicated above, MICROCHECKER indicated the possible presence of null alleles at two loci (*Oneμ2* and *Ssa103NVH*). However, the FreeNA analyses showed similar results in terms of global F_{ST} including or not null alleles (respectively, 0.186 and 0.184). Therefore, for the rest of the analyses these two loci were included.

Almost all the pairwise F_{ST} values resulted highly significant ($P < 0.001$, see Table 2). The naturally isolated wild sample RIO10 (Tenna River), fixed for the *LDH-C*100* and hosting only native haplotypes (see above), resulted the most divergent population respect to both the rest of wild samples and the hatchery collections, showing a mean F_{ST} value of 0.298. The wild samples collected from the Chienti River (SAC15 and FIA14b) showed the lower degree of genetic differentiation (mean ± SD $F_{ST\ \text{Chienti}\ vs\ \text{HATs}} = 0.12 \pm 0.017$) as compared to the domestic samples. On the contrary, the collections from the Tenna River (AMB14 and TEN14) (mean ± SD $F_{ST\ \text{Tenna}\ vs\ \text{HATs}} = 0.20 \pm 0.005$) and Nera River (TOR14, NER14a and NER14b) (mean ± SD $F_{ST\ \text{Nera}\ vs\ \text{HATs}} = 0.20 \pm 0.053$) showed a level of genetic differentiation respect hatchery strains that was about twice that showed above by Chienti River collections (SAC15 and FIA14b). F_{ST} comparisons between group of samples showed the highest average F_{ST} values in the group composed by samples collected upstream of the barriers (SUpB) (mean $F_{ST} = 0.254$). In the comparisons between samples collected downstream of the barriers (SDwB), F_{ST} values were clearly much smaller (mean $F_{ST} = 0.113$). Finally, as expected, the lowest mean F_{ST} value was observed between the hatchery samples ($F_{ST} = 0.037$). The pairwise comparisons between groups was statistical significant only in the case of SUpB *vs* hatcheries ($p = 0.037$).

The scenario depicted above by F_{ST} pairwise estimations is also evident in the DAPC analyses. In the first plot (Fig. 3a) two group of samples clustered together based on their genetic integrity, such that along the first principal component the samples from Tenna River and Nera River were located on the left side of the plot, while hatcheries on the right. The two collections from the Chienti River (SAC15 and FIA14b) partially overlapped with these latter. In this plot, a complete overlap between the two conventional domestic samples (HAT-1 and HAT-2) and the domestic sample of presumed Mediterranean trout (HAT-3) appears also clear. To highlight the presence of a possible genetic substructure in the whole dataset a second DAPC analysis was performed including only wild samples. The second plot (Fig. 3b) showed a clear separation between the collections from the three river catchment investigated (Chienti, Tenna and Nera). In addition, it was also clear an intra-river genetic discontinuity, with RIO10 plotting separately respect to the other collections from the Tenna River. The same was observed for TOR14 with respect to the other samples from the Nera River.

The STRUCTURE analysis provided a pattern of genetic differentiation congruent with the scenario depicted by the above DAPC analysis. The analysis of eight brown trout wild samples and three domestic collections indicated the presence of seven and eight genetic clusters based on, trends in LnP(K) and all four MedMeaK MaxMeaK indices (Fig. S1 and Table S3), respectively. However, in Fig. 4 we reported only the consensus bar plot for K = 8 because after ten runs the clustering options resulted more stables for K = 8 than for K = 7, In details, at K = 8 the genetic differentiation existing between wild and domestic samples appeared clear, with these latter forming two clusters: (i) one including HAT1 and HAT2 (dark blue bars) and (ii) one including HAT3 (blue bars). However, it should be taken into account that for K = 7 almost 50% of clustering solutions grouped the above three domestic samples in a sole genetic cluster (data not shown). On the other hand, as regards wild collections a clear genetic discontinuity was recognizable at both inter and intra-river level. In the Tenna River basin, TEN14 and AMB14 formed

Table 2 Pairwise F_{ST} based on 11 microsatellite loci between 8 wild brown trout samples and 3 domestic brown trout samples (below diagonal)

	Tenna River			Chienti River		Nera River			Hatcheries		
	AMB14	TEN14	RIO10	SAC15	FIA14	TOR14	NER14a	NER14b	HAT1	HAT2	HAT3
AMB14		***	***	***	***	***	***	***	***	***	***
TEN14	0.048		***	***	***	***	**	***	***	**	***
RIO10	0.276	0.273		***	***	***	***	***	***	***	***
SAC15	0.197	0.150	0.207		***	***	**	***	***	***	***
FIA14	0.207	0.180	0.251	0.052		***	***	***	***	***	***
TOR14	0.238	0.195	0.297	0.117	0.100		***	***	***	***	***
NER14a	0.155	0.130	0.290	0.096	0.059	0.122		***	***	***	***
NER14b	0.195	0.181	0.342	0.179	0.151	0.213	0.037		***	***	***
HAT1	0.236	0.207	0.332	0.112	0.097	0.181	0.152	0.268		***	***
HAT2	0.235	0.197	0.347	0.111	0.107	0.187	0.154	0.277	0.029		***
HAT3	0.230	0.199	0.366	0.140	0.136	0.185	0.157	0.274	0.123	0.125	
	0.029	0.069	0.109	0.149	0.189	0.229	0.269	0.309	0.349	0.366	

p values (above diagonal) were obtained after 55,000 permutations, indicative adjusted nominal level-5% for multiple comparisons is 0.000909

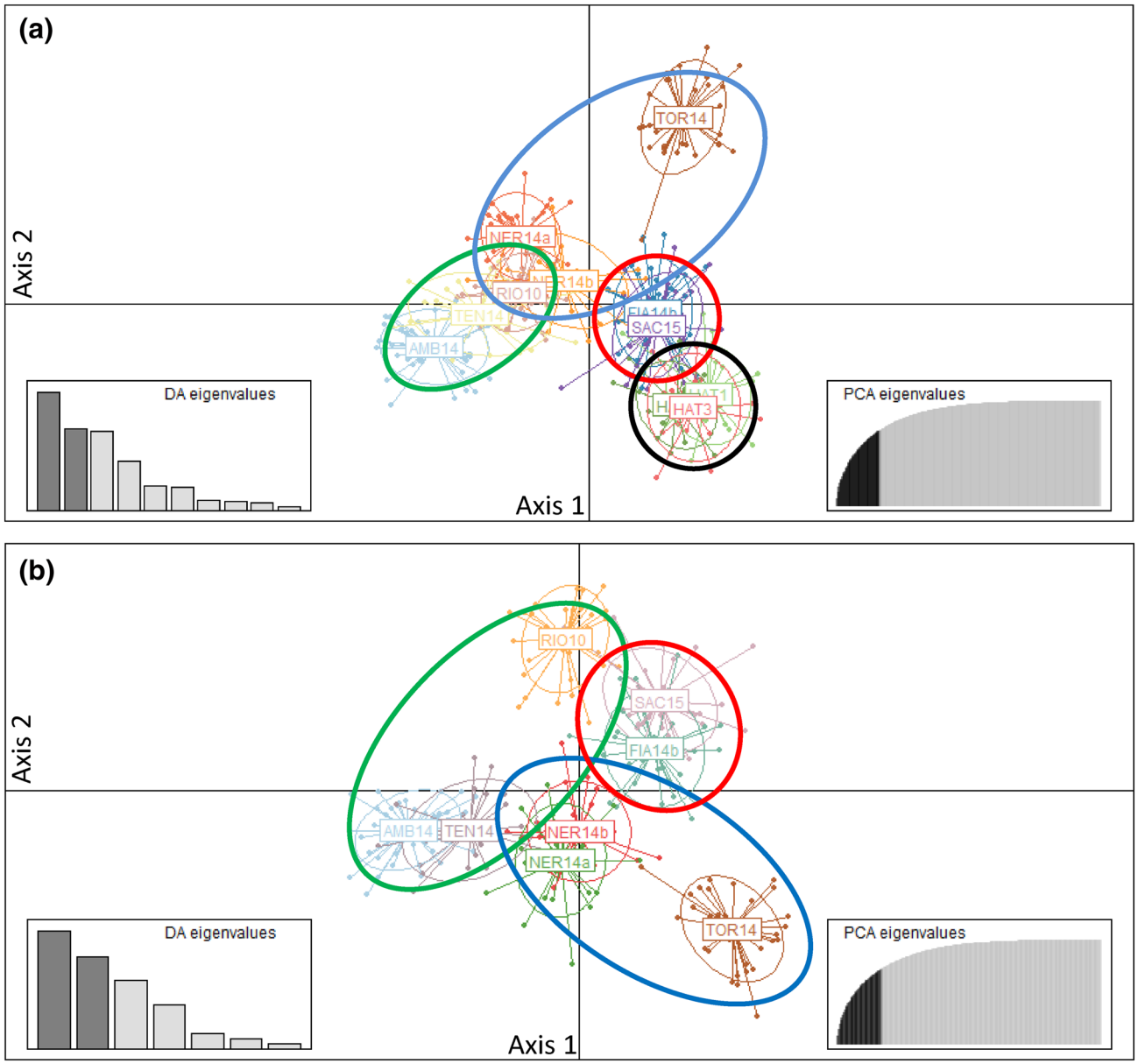

Fig. 3 Discriminant Analysis of Principal Components,, analysis carried out on **a** all samples (wild and domestic), **b** wild samples. Brown trout samples from the same river basin were included in empty colored ovals and/or circles: green: Tenna River basin; blue: Nera River basin; red: Chienti River Basin; black: hatchery. Points represent observed individuals connected to the population centroids and assigned groups are represented as inertia ellipses

a distinct group (light blue bars) respect to the other river basins localities and also respect to the RIO10 (orange bars) sample separated from the location TEN14 by a 40 meters waterfall. A similar scenario could be proposed for the Nera River. Here, the locations NER14a and NER14b, which are very close to each other and were collected from the main stem river, formed a distinct genetic cluster (green bars) respect to the TOR14 sample (light yellow bars) that was collected in a small and artificially isolated stream a few kilometers downstream of the above locations (Fig. 1). Finally, the two collections from the Chienti River (SAC15 and FIA14b) formed two distinct genetic clusters (respectively, red and light green bars).

With the aim to analyze the outcomes of the admixture analysis, the individual values of coefficient admixture (q) were ranked from the highest (1, that in this study indicates a pure wild trout) to the lowest (0, pure domestic trout) and their 95% credible intervals (CI) were plotted against rank. The distribution of q values in a total of 319 wild and three hatchery samples showed four different patterns of

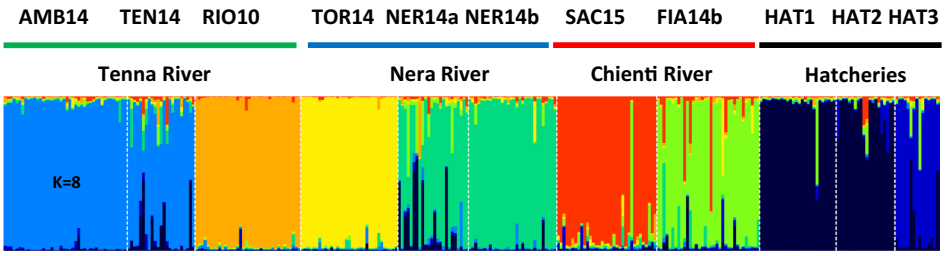

Fig. 4 Bar plots showing the STRUCTURE analysis of 11 microsatellites. White dashed lines separates sampling locations. The wild or domestic origin of each sample is highlighted by colored horizontal lines Green: Tenna River basin; blue: Nera River basin; red: Chienti River Basin; black: hatchery samples

hybridization (Fig. 1). In the two classical hatchery samples (HAT-1-2) almost all individuals presented $0.00 < q < 0.10$ associated with very narrow CI. This result is fully consistent with the exclusive occurrence of hatchery markers (ATs1 haplotype and *LDH-C1* 90* allele $\approx 100\%$) in these samples (plot not showed). The "Mediterranean" domestic stock (HAT-3) when tested for a $K = 2$ together with the Atlantic domestic samples HAT-1 and HAT-2 showed q values ranging from 0.42 to 1 associated with wide CI (plot not showed). In the wild collections, a very variable scenario in terms of patterns of individual coefficient admixture was observed. This scenario was probably influenced by the degree of isolation between the sample locality and any possible source of genetic hybridization (that is, a trout farm and/or a fishing game playground). In Tenna River, for example, the mean q values were ≈ 1 with a very narrow CI (0.992–1.000) in the naturally isolated sample RIO10. As indicated above, the presence of other hatchery molecular markers was null in this location (see Table 1). For the other locations of Tenna River (AMB14 and TEN14) the mean q value was also ≈ 1. However, in these localities the CIs were wider respect to RIO10: from 0.977 to 1.000 in AMB14 and from 0.924 to 1.000 in TEN14, indicating that here the signal of a low level of introgression is still detectable. At the same time, the narrower CIs in AMB14 as compared to TEN14 could be related with the presence of an insurmountable barrier (of anthropic origin) located downstream of AMB14 that probably acted as a barrier to the up-stream migration of stocked trout. In addition, both the *LDH-C1** locus and 5′-end mtDNA control region temporal analyses showed an increase in hatchery diagnostic molecular markers in TEN14, (see Table 1; Fig. 2). This seems to suggest the influence of past rather than contemporary stocking activities on AMB14 as compared to TEN14. High mean q values were also observed in the wild samples from Nera River, as in TOR14 (mean $q \approx 1$ and CI ranging from 0.988 to 1.000) and in NER14b (mean $q = 0.994$ and CI from 0.967 to 1.000). Also in this case the above samples were collected upstream of an insurmountable barrier of anthropic origin. On the contrary, in NER14a, a sampling location situated only a few kilometers downstream of NER14b and very close to a big trout farm, the mean q value was considerably lower (0.92 and CI from 0.804 to 0.983). In addition, the detection of two trout with individual q values of 0.17 (that is an almost pure domestic Atlantic trout) and 0.58 (that could correspond to a F1 or F2 hybrid) could be related with the presence of the above farm acting as a continue source of hybridization, or in alternative to not official stocking activities still in course. Also in this case, as observed above for TEN14, the temporal analysis of diagnostic hatcheries molecular markers (*LDH-C1** locus and 5′-end mtDNA control region) showed an increase in hatchery genes in the period 2006–2014 that is congruent with a still ongoing scenario of hybridization. The samples from Chienti River appeared more altered in terms of genetic introgression. In FIA14b the mean q value was 0.97 with CI ranging from 0.843 to 1.000. In this location a minimum individual q value of 0.70 (probably a F2 hybrid) was also detected suggesting that here the phenomena of introgressive hybridization was recent. This idea was also supported by the significant positive F_{IS} observed in this locality over all 9 microsatellite loci (see Table 1).

Discussion

Hybridization between species is common to all salmonids, and it is higher in areas where fish have been introduced by man than in areas where different species are naturally sympatric (Taylor 2003). This is probably due to the lack of opportunity for historical interactions during mating in nature, thus determining the incompleteness of pre-mating isolation between populations diverging in allopatry (Coyne and Orr 2004; Bettles et al. 2005). This is the case of the Mediterranean trout that have evolved separately from the northern Atlantic lineage during the Pleistocene (Bernatchez 2001). The Atlantic lineage, which has been widely bred in captivity since the second half of the XIX century, was introduced for stocking everywhere in southern Europe during the last century, and the apparent lack of pre-zygotic

isolation determined an extensive introgressive hybridization with resident populations (Caputo et al. 2004; Splendiani et al. 2016) and the spreading into the wild of hatchery genetic markers.

Indeed, our results confirmed that northern Atlantic genetic traits are widespread in wild trout populations of the study area, and this is not surprising considering that the first documents of stocking with domestic trout date back to 1905 (see Splendiani et al. 2013, 2016). Overall, the rate of AT haplotypes ranges from 0 to 100% and the *LDH-C1*90* allele showed a similar pattern. Two populations (COS10 and USS14) out of 18 were almost completely characterized by allochthonous genes. However, as documented by the analysis of 11 microsatellite loci, the pattern of introgression is far from complete panmixia. Rather, the analysis with STRUCTURE clearly indicates a clear-cut distinction between native and domestic clusters. This observation is in line with the inspection of individual admixture proportion, evidencing that in the majority of wild populations (six out eight) a high percentage of individuals were classifiable as pure native (i.e., $q \approx 1$). This result is very interesting considering the long history of stocking with the release of millions of hatchery trout (see Bianco, 1990) and nearly a century (ca 30 generations) of hybridization between domestic and wild trout, which would be expected to result in the deletion of any trace of "pure" native individuals. This separation suggests the existence of partial reproductive isolation between wild and hatchery trout that might be realized by spatial isolation. In fact, it is known that domestic Atlantic trout spawn preferentially at the center of the river bed, more susceptible to floods, rather common in Mediterranean streams, whereas native trout prefers the more protected waters close to the river banks (Champigneulle et al. 2003). On the other hand, previous studies seem to indicate a mechanism of temporal isolation based on the reproductive time in the study area. In fact, wild non-native trout populations, spawn between November and January, with a peak in December for both sexes (Caputo et al. 2010), as it was observed in populations from central Europe (Klemetsen et al. 2003). On the contrary, in Apennine trout populations harboring mostly native genes, analysis of otoliths taken from fry sampled in mid-June evidenced a spawning period between the end of February and the beginning of March (Caputo 2003), as observed in other areas in southern Europe (e.g., Gortázar et al. 2007). The possible involvement of reproductive isolation through isolation by time (IBT) may be common in hatchery-wild trout interactions, as a genetic determination of spawning time differences has been well documented in salmonids (see Hendry and Day 2005). In addition, fish farmers often select brood-stocks for early spawning (see Hansen et al. 2006) and shift in spawning time has been observed in sympatric wild and hatchery salmonids (Shields et al. 2005). As previously suggested by Hansen and Mensberg (2009) for Danish brown trout, we propose that IBT may have contributed to prevent full introgression between native and alien trout in central Italy too. On the other hand, a serious risk for the survival of the last Mediterranean trout populations is represented by the continuation of stocking for recreational fishing or accidental escape from hatcheries of non-native trout. Interestingly, the temporal analyses of *LDH-C1** locus showed a mean increment of *LDH-C1*90* allele of 10.6% over 4 localities, however, statistically significant just for the samples from the Nera River, characterized by an elevated presence of hatcheries (see Fig. 1). In addition, the evidence of wild trout characterized by low values of individual admixture coefficient (q) supports the above concerns.

Unfortunately, in the light of the observed pattern of genetic admixture between native and alien trout, the possibility of an increase in the introgression rates in the study area appears very concrete if no valid management measures are taken. In fact, despite a formal protection guaranteed by the presence of Natura 2000 network and even of a national park, the levels of introgression observed and the ongoing spreading of alien genes into wild trout populations are a clear consequence of ineffective conservation strategies (Bianco 1995). It is noteworthy that the only case ostensibly immune from introgression with alien genes is represented by a strongly isolated wild population (RIO10), probably not reached by massive stocking activities. Indeed, despite stocking have been officially banned from the Sibillini Mountains National Park territory since 2005, the detection of putative F1 or F2 trout, or even pure domestic trout in the wild populations reveals the scarce or null efficiency of conservation measures in the cases involving aquatic game species in Italian protected areas. In fact, even if stocking activities were officially banned in the National Park almost 10 years ago, in the proximity of the Park boundaries, and more precisely in the Chienti River basin (Fig. 1), stocking activities with domestic trout have continued up to the present. In fact, despite strict European, national and regional laws forbid the propagation into the wild of alien species/genetic strains, release of domestic fish in fishing areas are still permitted/tolerated in Italy. The lack of isolation between the upstream sampling localities FIA14b and SAC15 and the downstream fishing area explains very well the detection in these sites of the higher degree admixture observed in the study area by microsatellites analysis (Figs. 1, 4). On the other hand, the higher genetic integrity detected in the samples from Nera and Tenna rivers could be related with a passive obstacle effect operated by both natural and artificial barriers than to the effects of an active conservation strategy. Therefore, it appears clear the occurrence of conditions liable to give rise to a conflict between the aim to restore river continuity, considered a priority in the environmental policy of European Union (e.g., Water Framework Directive (WFD-2000/60/CE)), and the protection of the last pure native trout. In

the Natura 2000 Network and in the territory of Sibillini Mountains National Park there is a considerable number of artificial barriers that obstacle fish migration along the river rod (Fig. 1). This could represent a problem because fragmentation into small isolated reproducing units leads to an increased effect of genetic drift and an accelerated loss of genetic diversity (see Montgomery et al. 2000). Taking into account the very low values of effective population size (N_e) detected in this study, the risks related with the deleterious synergic effects between genetic drift and increase of introgression rates appear as a concrete risk for the long term conservation of native Mediterranean brown trout populations. However, in our case study, artificial obstacles, which interfere with salmonid natural migrations, had a "positive" effect because these barriers apparently sheltered indigenous gene pools from the homogenizing effects of introgression with alien stocks. This paradox is clearly illustrated by two very close populations one of which (NER14b) is isolated by an artificial impassable barrier, the second one (NER14a) is in continuity with the main river rod. Despite in both populations restocking activities were formally stopped soon after the establishment of the Sibillini Mountains National Park, the introgression pattern is very different, with higher q values in NER14b and wider credible intervals in NER14a. This latter also showed the presence of almost pure domestic trout ($q \sim 0$) and putative F1 ($q \sim 0.50$) (Fig. 1). This discrepancy may suggest a purifying action of natural selection in the isolated population (see Araki et al. 2008 for similar consideration), and a significant diffusion of domestic genes in non-isolated one. The role played by the sources of domestic trout is clearly explained considering that the downstream parts of Nera River harbor the highest concentration of trout farms (Fig. 1) of Italian Peninsula. In this context, the absence of migration barriers facilitates the introgression of alien genes into native populations from this river. For example, the high frequency of both *LDH-C1*90* allele and ATcs-1 haplotype in RAP14 could be the consequence of the above phenomena. It is interesting to note that RAP14 is far only a few kilometers from several downstream hatcheries and no apparent barriers seem to occur between this sampling locality and the hatcheries (see Fig. 1).

If on the one hand, the existence of a National Park seems to have had null effects in terms of conservation on native brown trout genetic diversity, on the other hand, the fishing restrictions applied in these protected areas could have even a paradoxical effect. As observed in Spain (García-Marín et al. 2008) the fishing restrictions applied in protected areas could act as a "refuge" for populations characterized almost exclusively by alien genes. In this way, potential source of hybridization will be protected rather than eradicated. In the present study, populations characterized almost exclusively by alien genes were observed in two localities (COS10 and USS08-14, see Fig. 1; Table 1). It is noteworthy that in both these extremely negative situations, the limiting factor seems to be represented by habitat constraints. In the first case, the minor branch of the Tenna River (Cossudro stream) entirely flows on impermeable rocks (sandstone and clays). According to Splendiani et al. (2013), the severe summer droughts affecting these streams would favor phenomena of rarefaction or even local extinction which, in turn, would have favored the progressive displacement of native with domestic trout used in the past stocking activities. On the contrary, for the USS locality (100% of rock permeability) the occurrence of a demographic perturbation affecting a putative preexisting native trout population could be related with periodical episodes of excessive water abstraction as evidenced also during the analyses carried out during the Life + TROTA project (http://www.lifetrota.eu/). In this latter case, a stricter application of the existing law concerning the minimum vital flow would guarantee demographically stable native trout population, more resilient to progressive "invasion" of their genome by alien domestic genes (see Mallet 2005).

Finally, a very critical risk for the long-term survival of native trout is represented by the fish farmers trying to produce the so-called "Mediterranean trout" to meet the needs of stocking for sport fishing by circumventing restrictions due to the ban on the use of alien species, such as the Atlantic brown trout in southern Europe. Indeed, our analysis on one hatchery declaring to produce "Mediterranean trout" (HAT-3) highlighted that this presumed native trout actually derived from the hybridization between Atlantic and native trout (Tables 1, 2; Figs. 3b, 4). Far from resolving the problem of diffusion of alien genes in natural populations, this naïve practice, which comes from a mix of venal interest and poor knowledge of the biogeography of this species, represents a serious possibility for the loss of the partial reproductive isolation mechanisms existing in nature between pure native trout and domestic trout (both for IBT and purifying action of natural selection on hybrids). In addition, the use of wild animals of different Italian provenances for breeding in hatchery can favor translocation phenomena, with a consequent impossibility to clearly delineate the phylogeographic structure of Italian native trout. This eventuality is very concrete, as in HAT-3 we found a haplotype typical of the Tyrrhenian area (ADcs15b). In addition, the use of local populations in supplemental programs is also advisable if we take into account the high degree of genetic differentiation observed at both inter and intra-basin level (mean $F_{ST} = 0.17$; see Table 2; Fig. 3a, b). Probably, such a degree of genetic differentiation is related with the micro phylogeographic history of brown trout in the study area. Central Italy is in fact a biogeographic area where the secondary contact between the three main Mediterranean mtDNA lineages of brown trout occurred as witnessed by the detection of haplotypes belonging to the lineages AD, ME and MA. In addition,

although the use of only 11 putative neutral loci as microsatellites does not permit to find sound information about the adaptive value of the above genetic entities, there are also evidences showing that important genetic adaptation to local conditions can be hosted behind such a degree of genetic differentiation (Schenekar and Weiss 2017).

In conclusion, despite the presence of the Nature 2000 network and of a National Park some important threats for the conservation of native brown trout populations seem to persist in the study area, such as: (i) lack of barrier preventing the upstream migration of alien trout from fishing areas close to the protected area borders ; (ii) the presence of several trout farms, and (iii) naïve tentative of stocking with pseudo-native trout. Until now, the most important protection role has probably been played by the mere presence of physical barriers to migrations and by the combined action of IBT and natural selection. Therefore, it is obvious that long-term goals should focus on the removal of barriers to allow natural migrations and restore the ecosystem, according to WFD-2000/60/CE. However, to prevent additional loss of native brown trout populations and homogenization of genetic diversity, the status and relationship between the adjacent populations should be assessed prior to removing migration barriers. In the evaluation of effective conservation strategies in Italian protected areas, also selective fishing could have a role, considering a major susceptibility of trout of domestic origin to be fished (García-Marín et al. 2008).

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