Straive | Journal Code: AQC | Article ID: 4099 | Dispatch: 00.00.00 | No. of Pages: 26 | CE: Randolf J. Sayon4.4. | ME:

Received: 7 July 2023 | Revised: 18 December 2023 | Accepted: 26 January 2024

DOI: 10.1002/aqc.4099

RESEARCH ARTICLE

WILEY

Population genetics, demography and conservation of Mediterranean brown trout from Sardinia

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Funding information
Progetto di tutela e gestione dei rifugi genetici della trota Mediterranea in Sardegna - registrazione n. 296 del 01.09.2020

Abstract

Brown trout is a species complex (*Salmo trutta* complex, L., 1758) including both widespread invasive (non-native hatchery strains) lineages and endangered local-endemic lineages, among which is the Sardinian trout, the only native salmonid present in Sardinia. Multiple stressors (e.g. the spread of stocked brown trout of Atlantic origin, habitat alteration and climate change) combine to seriously threaten the persistence of wild native populations. In this study, the origin, population genetics and demography of wild Sardinian brown trout populations were extensively investigated. A total of 274 trout individuals collected from 12 hydro-geographical basins were analysed using both mitochondrial (control region) and nuclear (*LDH-C1** locus and 10 microsatellites) markers. Although stocking activities have altered the native genetic makeup of some populations in the study area, several (almost) uncontaminated populations showing strong genetic structure were detected. Eroded intra-population diversity, as well as small effective population size, sometimes associated with a bottleneck signal was also found. The genetic characteristics of Sardinian trout populations described in this study are probably due, at least partly, to the peculiarity of local environmental conditions at the margin of the ecological niche for salmonids. Based on the results of this study, the need for urgent measures of conservation aimed to ensure the near future viability of the last wild Sardinian trout populations was discussed.

KEYWORDS

biogeography, conservation genetics, conservation policy, extinction risk, invasive species, *Salmo trutta*

1 | INTRODUCTION

The delineation of spatial population structure represents a crucial step in understanding the demography and evolution of species (Waples & Gaggiotti, 2006). This implies understanding the spatial scales over which populations are connected through dispersal and gene flow and the role of environmental characteristics underlying the pattern of connectivity between populations. Obtaining this kind of information helps to plan biodiversity management in a rational manner. For example, through the delineation of conservation categories [i.e. conservation units (CUs), evolutionary significant units (ESUs) and management units (MUs)], assessment of population and meta-population viability, and strategic enhancement of landscape connectivity (e.g. Palsbøll et al., 2007; Robertson et al., 2013). Since

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pioneering reflections on protecting species' evolutionary potential (Mayr, 1960), the debate on the delineation of intra-specific entities of conservation and management has become of crucial interest mainly for heavily managed species attracting socio-economic interests, as in the case of the fisheries and/or game–fisheries–species (e.g. Fraser & Bernatchez, 2001). Thanks to a plethora of conservation genetics studies, protection of local populations is nowadays considered pivotal for local managers intending to restore and/or conserve species diversity (e.g. Bruce et al., 2019).

Brown trout (*S. trutta* complex L., 1758) is a fish of great economic (mainly in aquaculture) and recreational value both in its original range and worldwide. Habitat degradation coupled with massive and uncontrolled stocking activities with non-native lineages (mainly from northern Europe) has compromised the conservation status of native populations in several European countries (Araguas et al., 2017; Caputo et al., 2004; Prunier et al., 2021; Splendiani et al., 2019a; Vera et al., 2018; Weiss et al., 2001). Brown trout is an appealing and iconic species for scientists because of taxonomic controversies that are still unresolved, the complex evolutionary history and the intricate patterns of life history traits (Lobón-Cerviá & Sanz, 2018), as well as for its biological conservation needs (Piccolo et al., 2018).

Early phylogenetic studies identified five main mitochondrial (mtDNA) evolutionary lineages: the Atlantic (AT), Mediterranean (ME), marmoratus (MA), Adriatic (AD), and Danubian (DA) lineages (Bernatchez et al., 1992). Subsequently, other lineages were proposed, such as Duero (DU, Cortey et al., 2009; Vera et al., 2010), Tigris (TI, Bardakci et al., 2006), North African (NA, Tougard et al., 2018) and Dades (Snoj et al., 2011). However, mitochondrial lineages often show an overlapping natural distribution, with even more mitochondrial lineages observed in a single population (Hashemzadeh Segherloo et al., 2021). Therefore, if on the one hand, the phylogenetic and phylogeographic approach has failed to resolve taxonomic controversies to date, on the other side, molecular phylogeography has allowed the identification of the paleo-climatic and environmental events that played the most crucial roles in shaping brown trout biogeography (Splendiani et al., 2013; Splendiani et al., 2016a; Splendiani et al., 2020). For this reason and because the identification of brown trout taxonomic status is not the purpose of the present study, only mtDNA lineages and sub-lineages of *S. trutta* will be considered here.

In the Mediterranean area, the Italian Peninsula and its major islands represent a biodiversity hotspot for the genus *Salmo*. Here, at least five valid nominal species have been recognized (*Salmo ghigii* Pomini, 1941; *Salmo cettii* Rafinesque-Schmaltz 1810; *Salmo marmoratus*, Cuvier, 1829; *Salmo carpio*, Linnaeus 1758; and *Salmo fibreni*, Zerunian & Gandolfi, 1990; e.g. Polgar et al., 2022), whose biogeographic history has been moulded by complex colonization routes and ecological adaptation driven by paleo-climatic changes and paleo-hydrological re-arrangements of river networks (Lerceteau-Köhler et al., 2013; Sanz, 2018; Splendiani et al., 2020). A very high genetic differentiation was detected among insular populations (Sardinia and Corsica), especially in Corsican populations (Berrebi et al., 2019). The Corsican trout populations showed a certain degree of similarity with Sardinian brown trout populations when compared with other Italian peninsular trout populations, although Sardinian trout sampling sites were from two river basins only (Flumendosa and Cixerri). More recently, in a genome-wide-based phylogenetic revision, Hashemzadeh Segherloo et al. (2021) highlighted the high distinctiveness of native trout populations from Sardinia with respect to other Mediterranean trout taxa, suggesting to recognize Sardinian trout populations as a distinct species.

Mediterranean brown trout is the only native salmonid in Sardinia. However, since the beginning of the 20th century, notably, from the 1960s onward, stocking activities became a common management practice and introduced into the rivers of this Mediterranean island two exotic species: *S. trutta* from Central Europe (i.e. the Atlantic trout of hatchery origin) and *Oncorhynchus mykiss* from North America (Orrù et al., 2010; Sabatini et al., 2006). The introduction of non-native species was banned in Sardinia since the early 2000s, in compliance with Presidential Decree 357/97.

Habitat/trophic competition and the rapid adaptive plasticity of salmonids coupled with hybridization between native and Atlantic brown trout lineages had progressively reduced local wild populations and altered the original Sardinian gene pool (Sabatini et al., 2006; Sabatini et al., 2011). As a consequence of genetic introgression, habitat alteration, and fishing, the Mediterranean trout is listed as critically endangered in the Italian IUCN Red List (e.g. *S. ghigii*, Rondinini et al., 2022).

Although earlier data from the 20th century (Cottiglia, 1968) reported an almost homogeneous brown trout distribution throughout the island rivers, they were unfortunately not able to distinguish between Mediterranean-native and Atlantic-exotic trout of stocking origin. In subsequent studies (Cau, 1997; Massidda et al., 1996; Zanetti et al., 2007), the presence of native trout populations was proposed for a very small fraction of the investigated sites (11 out of 160). Genetic studies in the last two decades revealed that populations of pure Sardinian trout could be found in the Cixerri, Pula and Flumendosa basins (Berrebi et al., 2019; Hashemzadeh Segherloo et al., 2021; Palmas et al., 2020; Sabatini et al., 2006; Sabatini et al., 2011, 2018; Zaccara et al., 2015). Despite a number of studies focusing on Sardinian trout populations, to date, none has provided a comprehensive characterization of the genetic population structure and diversity, demography and conservation status of wild populations. This is especially relevant as wild Sardinian trout populations are known to inhabit peculiar, sometimes even extreme, environments as, for instance, creeks subject to extreme water flow fluctuations and small ponds characterized by relatively high seasonal temperatures (Mulas et al., 2009; Zaccara et al., 2015). In this Mediterranean island, up to 90% of all streams present a non-perennial hydrological regime (Mulas et al., 2009). In most cases, the hydrology of the streams involved in this study was unstable or even intermittent with frequent severe summer droughts (Table 1). Yearly, during the warmest and driest months, the water discharge is absent and the trout survive in small and isolated pools where the water temperature can exceed 25°C for several days or weeks (Table 1).

TABLE 1 Sites of the 20 wild Sardinian brown trout sampling sites analysed in this study.

Location code		*N*	Region	Stream/River	Basin	Sea drainage	Elevation (m.a.s.l.)	Highest mean summer water temperature (°C)[a]	Barriers
Sardinia	**COG**	7	Sardinia	Riu Bizzolu	Coghinas	Gulf of Asinara	276	23.43 (JL)	W (3)
	PAD	13	Sardinia	Riu de su Piricone	Padrogiano	Tyrrhenian Sea	140	23.86 (SP)	D (1)
	POSa	7	Sardinia	Canale dell'Iserno	Posada	Tyrrhenian Sea	569	23.40 (JL)	WF(1)
	POSb	18	Sardinia	Riu s'Abba e Salinu	Posada	Tyrrhenian Sea	507		
	CED	30	Sardinia	Riu Flumineddu	Cedrino	Tyrrhenian Sea	189	23.54 (JN)	
	CDL	8	Sardinia	Riu Codula de Luna	Riu Codula de Luna	Tyrrhenian Sea	254	19.00 (JN)	
	FLUa	10	Sardinia	Flumendosa	Flumendosa	Tyrrhenian Sea	802	19.80 (JN)	D (1)
	FLUb	9	Sardinia	Riu Bau Mandara	Flumendosa	Tyrrhenian Sea	977	20.32 (JL)	WF (1)
	FLUc	11	Sardinia	Riu Furittu	Flumendosa	Tyrrhenian Sea	390		
	FMCa	8	Sardinia	Riu Cannisoni	Flumini Mannu di Cagliari	Gulf of Cagliari	380	23.90 (JL)	W (4)
	FMCb	12	Sardinia	Riu su Salixi	Flumini Mannu di Cagliari	Gulf of Cagliari	425	20.65 (JL)	D (1)
	PULa	12	Sardinia	Riu Litteras	Pula	Gulf of Cagliari	296	21.90 (JL)	
	PULb1	8	Sardinia	Rio Pula	Pula	Gulf of Cagliari	170		
	PULb2	23	Sardinia	Rio Pula	Pula	Gulf of Cagliari	144		W (1)
	FMPa	30	Sardinia	Riu Piras	Flumini Mannu di Pabillonis	Mediterranean Sea	324	26.27 (JL)	W (19)
	FMPb	17	Sardinia	Riu Sitzedda	Flumini Mannu di Pabillonis	Mediterranean Sea	323		
	TEM	6	Sardinia	Riu Matta Giuanna	Temo	Mediterranean Sea	722	27.00 (JL)	WF (1)
	RMN	10	Sardinia	Riu Mannu	Mare Foghe	Mediterranean Sea	465	22.15 (JL)	WF (1)
	RMF	5	Sardinia	Riu di Mare Foghe	Mare Foghe	Mediterranean Sea	192		
	CIX	30	Sardinia	Riu Is Abius	Cixerri	Gulf of Cagliari	308	21.20 (AG)	F(3), D (1)
Corse	**LTT**	5	Corsica	Lette	Seccu	Mediterranean Sea			
	CTT	5	Corsica	Ciuttare	Liamone	Mediterranean Sea			
	HBT	5	Corsica	Haut Botaro	Liamone	Mediterranean Sea			
	VES	19	Corsica	Ese	Prunelli	Mediterranean Sea			
	VIV	20	Corsica	Speloncello	Vecchio	Tyrrhenian Sea			
Hatc.	**HATa**	26	Central Italy	Hatchery a	Cantiano	Adriatic Sea			
	HATb	20	Central Italy	Hatchery b	Visso	Tyrrhenian Sea			

Note: *N* represents the whole sample size. VES and VIV represent two wild brown trout samples from Corsica analysed in the present study, while LTT, CTT and HBT are Corsican samples from Reynaud et al. (2011) (see Section 2 for more details). HATa and HATb represent two traditional hatchery strains used here as reference samples of the Atlantic genome. Environmental parameters: elevation, mean monthly highest water temperature (JN = June, JL = July, AG = August, SP = September), number (between brackets) of impassable natural and or artificial barriers between the sampling site and the stream/river outflow (W = weir, D = dam, F = ford, WF = waterfall; see also Table S4 for more details), mean summer discharge, duration of drought in days, length in meters of the dry river portion and rivers total length. Demographic parameters: trout density, estimated by applying the two-pass sampling removal method (Zippin, 1956). Protected areas (RP = Regional Park, SCI = Site of Community Importance based on the Habitat Directive.

(Continues)

TABLE 1 (Continued)

Location code	*N*	Region	Stream/River	Basin	Sea drainage	Elevation (m.a.s.l.)	Highest mean summer water temperature (°C)[a]	Barriers

[a]Data provided by Agenzia regionale del distretto idrografico della Sardegna.
[b]Denote protected areas where fishing activities are prohibited (DR n.314/Dec.A9 07.02.2019).
[c]Drought length was evaluated during the summer months (July–September) from 2006 and 2020.

TABLE 1 (Continued)

Location code	Mean summer discharge (m^3 s^{-1}))	Drought duration (days)	Drought length (m)[c]	River length (m)	Trout density (ind m^{-2})	Protected areas
Sardinia	0.0463			16,284		RP
	0.1105			32,190	0.0163	
	0.0213			11,443	0.0047	
				6194	0.0210	
	0.4870	330	10,000	35,097	0.1369	SCI[b]
	0.2025			21,855	0.0257	SCI
	0.0308			147,878	0.0619	
	0.0375			13,689	0.0090	
	0.0290	120	8,848	14,043	0.0504	([b])
	0.0215			9346	0.0179	SCI
	0.0300			4536	0.0750	
	0.0328	120	2641	2848	0.1280	SCI
	0.1950	120	13,282	30,832	0.0083	SCI
	0.1950	120	13,282	30,832	0.0792	RP
		120	6208	12,293	0.2057	SCI ([b])
			4600	7001	0.0653	SCI
	0.0475			12,129	0.0200	
	0.2283			25,160	0.3200	
				33,000	0.0420	
	0.0078	120	2500	3421	0.2816	
Corse						
Hatc.						

Note: *N* represents the whole sample size. VES and VIV represent two wild brown trout samples from Corsica analysed in the present study, while LTT, CTT and HBT are Corsican samples from Reynaud et al. (2011) (see Section 2 for more details). HATa and HATb represent two traditional hatchery strains used here as reference samples of the Atlantic genome. Environmental parameters: elevation, mean monthly highest water temperature (JN = June, JL = July, AG = August, SP = September), number (between brackets) of impassable natural and or artificial barriers between the sampling site and the stream/river outflow (W = weir, D = dam, F = ford, WF = waterfall; see also Table S4 for more details), mean summer discharge, duration of drought in days, length in meters of the dry river portion and rivers total length. Demographic parameters: trout density, estimated by applying the two-pass sampling removal method (Zippin, 1956). Protected areas (RP = Regional Park, SCI = Site of Community Importance based on the Habitat Directive.

[a]Data provided by Agenzia regionale del distretto idrografico della Sardegna.

[b]Denote protected areas where fishing activities are prohibited (DR n.314/Dec.A9 07.02.2019).

[c]Drought length was evaluated during the summer months (July–September) from 2006 and 2020.

Here, samples from various Sardinian rivers generally thought to be representative of the local Mediterranean brown trout variability (plus additional samples from Corsica and from hatcheries of the Italian Peninsula rearing trout of Atlantic origin) were collected and genotyped at multiple molecular markers (mtDNA, *LDH-C1* and microsatellites) with respect to native/exotic lineages and/or fine-scale population distinctiveness. The aims of this study were to (i) infer population genetic structure while controlling for admixture from hatchery-reared Atlantic strains, (ii) provide insight into demography (effective population size, occurrence of bottlenecks) of wild populations and (iii) identify units for management and evaluate their conservation status to provide an appropriate baseline for restoring strategies.

2 | MATERIAL AND METHODS

2.1 | Sampling and DNA extraction

A total of 274 wild brown trout individuals were collected in 20 sampling sites between May and October from 2016 to 2019, representing 12 Sardinian river basins (Table 1 and Figure 1). To introduce comparative (reference) populations, a total of 39 specimens from two pure wild Corsican sites (collected in 2015) and 46 specimens from two hatcheries-rearing Atlantic trout strains (collected in 2006) were also included. Overall, 359 individuals were analysed in this study (Table 1). Unfortunately, the Atlantic strains from local Sardinian hatcheries, used for stocking in recent years were not available, as the only working Sardinian hatchery currently breeds only rainbow trout (*O. mykiss*). However, the Atlantic strains were obtained from two hatcheries in Central Italy, which is an important trout aquaculture region along the Italian Peninsula (ISPRA, 2022). The wild fish were captured by electrofishing and subsequently housed in appropriate tanks during the field job. A small piece from the adipose fin was clipped from every individual and stored in absolute ethanol, before releasing the specimens into nature. Total genomic DNA was extracted using specific cartridge 401 in the *MagCore*® automated Nucleic Acid extractor (*MagCore* ®, *Genomic DNA Tissue Kit*, *n° 401)*.

2.2 | Mitochondrial DNA

The CR sequence was used to detect the diagnostic sites of the major mitochondrial lineages of *S. trutta* complex and therefore to assess the frequency of allochthonous (e.g. Atlantic and Danubian lineages, respectively AT and DA) and native (Adriatic, Mediterranean and marmoratus lineages, respectively AD, ME and MA) Mediterranean haplotypes. A polymerase chain reaction–restriction fragment length polymorphism–single-strand conformational polymorphism (PCR-RFLP-SSCP) analysis was performed to screen mitochondrial DNA (mtDNA) genetic variability. The mitochondrial control region (CR) was PCR amplified using the primers 28RIBa (Sušnik et al., 2001) and HN20

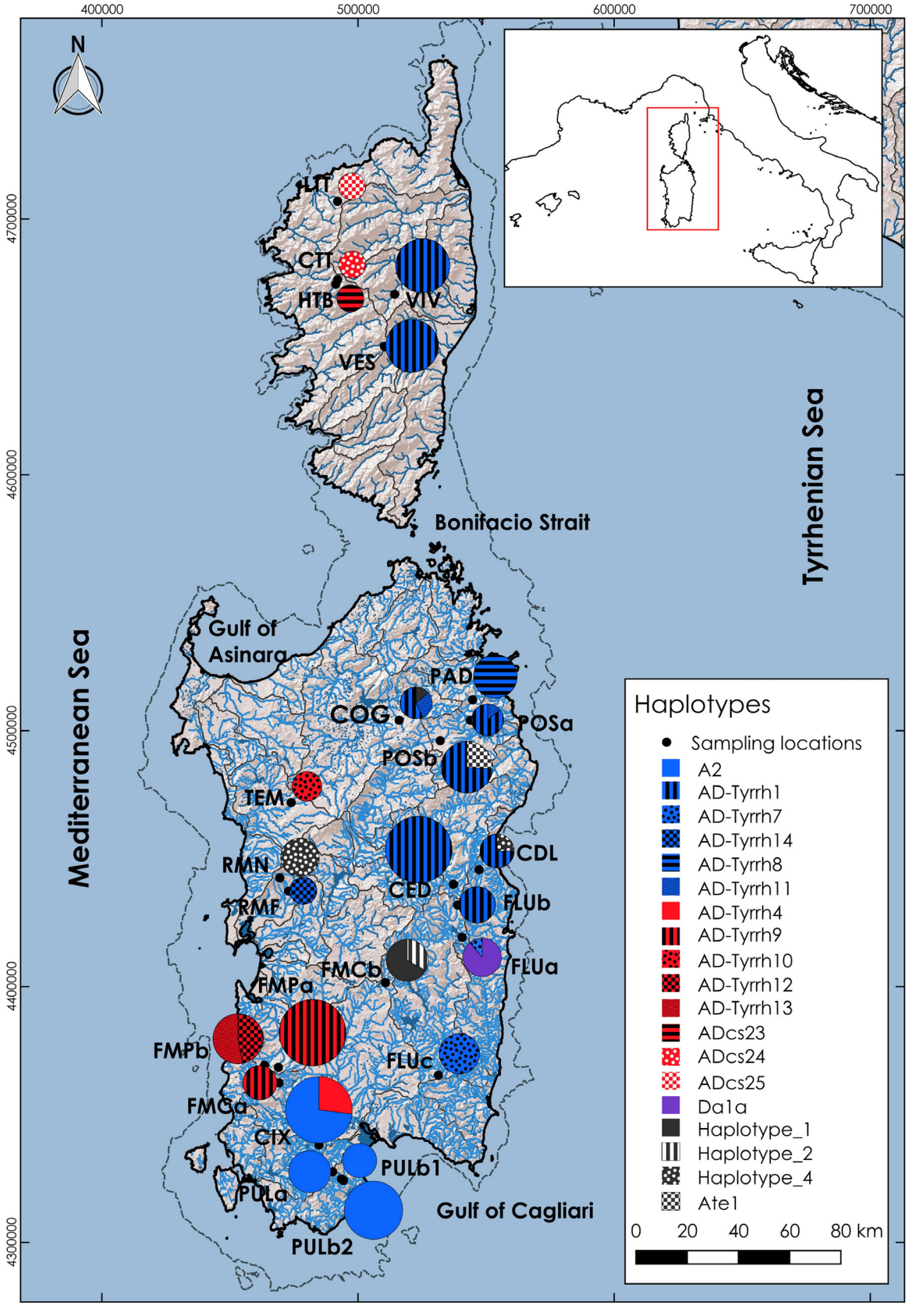


FIGURE 1 Map of the study area showing the brown trout sampling locations from investigated Sardinian and Corsican rivers. Solid lines mark the boundaries of major drainage basins. Dashed line: coastline during the last glacial maximum (LGM); downloaded from Zickel et al. (2016) GIS dataset. Pie charts represent the geographic distribution and frequency of CR mtDNA haplotypes per sampling site. The pie chart size is proportional to the sampling site size.

(Bernatchez & Danzmann, 1993), following procedures described in Bernatchez and Danzmann (1993). Single-strand conformation polymorphisms (SSCP) (Orita et al., 1989) were analysed following the method reported in Righi et al. (2023). Sanger sequencing of the CR (~1 Kbs) was performed, using the same primers of amplification, on a subsample for each different SSCP detected profile on an Applied Biosystems ABI 3730XL DNA by a service facility (BMR-Genomic, Padua). Sequences were aligned using ClustalW (Thompson et al., 1994), checked by eye in BioEdit (Hall, 1999) and assigned to sequences of *S. trutta* available in GenBank using Blast (Altschul et al., 1990). Levels of population genetic introgression were estimated by calculating the cumulative percentage of allochthonous haplotypes in each population. Phylogenetic relationships among 68 CR haplotypes (Table S1) were inferred using two approaches: (i) a 95% parsimony network estimated by the software TCS version 1.18 (Clement et al., 2000) and (ii) a phylogenetic tree using Bayesian inference (BI) as provided in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). For the BI approach the HKY85 substitution model (i.e. the optimal model for our data, as identified by the selection procedure implemented in MEGAX; Kumar et al., 2018), the invgamma rate variation and 5-gamma categories were used. A sequence of *Salmo salar* (GenBank accession number LC012541) was used as an outgroup. Divergence time estimation was carried out in Beast2 v.2.7.3 (Bouckaert et al., 2014). As calibration points, the more recent common ancestor (MRCA) of *Salmo* (*S. immigratus*) and of brown trout (*S. derzhavini*) was used by applying lognormal constraints following Veličković et al. (2023). Moreover, *Salmo orhidanus*, each brown trout lineage (AD, AT, MA, ME and DA) and groups supported by BI posterior probabilities = 1 were treated as *a priori* monophyletic. Divergence time estimations were done with an optimized lognormal relaxed clock (Douglas et al., 2021) and by applying a birth-death (Gernhard, 2008). Computations were performed for three independent runs for 100 million generations sampling every 10,000th generation using the Beagle library (Ayres et al., 2021). Adequate sampling and run convergence were verified in Tracer v.1.7.1 (Rambaut et al., 2018), and then the tree files were combined with LogCombiner. Finally, the maximum clade credibility tree was calculated in TreeAnnotator discharging 1,000,000 states as burn-in. Posterior summaries were only calculated for the nodes having a posterior probability greater than 0.9. The final tree was drawn using FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Finally, isolation and contacts among trout populations, driven by past climate phases enhancing resident or anadromous lifestyle, were investigated using the analysis of molecular variance (AMOVA). Genetic variance was estimated by grouping populations according to (i) 12 river basins and (ii) four sea drainages: Gulf of Asinara, Tyrrhenian Sea, Gulf of Cagliari and the Mediterranean Sea. Tests were carried out with ARLEQUIN version 3.5.1.3 (Excoffier & Lischer, 2010), using conventional ϕ-statistics and testing the statistical significance with 5000 permutations.

A significant and substantial amount of variance explained by differences among river basins would suggest inter-watershed population isolation, which likely occurred during the last glacial maximum, that is when the warmer conditions of the Mediterranean basin resulted in non-optimal environmental characteristics for anadromous Mediterranean trout. Conversely, a large amount of variance explained by differences among sea drainages would imply ancient gene flow among river basins flowing into the same sea drainage. In fact, lower water temperatures during colder climatic phases of the Pleistocene coupled with an anadromous brown trout lifestyle may have favoured migrations along the coast through sea outlets of close river basins (e.g. Splendiani et al., 2016b and references therein). Note that for the above-mentioned mtDNA-based analyses, the dataset was enhanced including CR information of additional 15 trout individuals from three Corsican sites (i.e. LTT, CTT and HBT; see Figure 1, Table 1 and Table 2) from grey literature (Reynaud et al., 2011).

2.3 | Nuclear DNA

A PCR-RFLP analysis of the eye-specific lactate dehydrogenase protein-coding locus (*LDH-C1**) was performed following the procedure described in McMeel et al. (2001). This analysis allows discrimination between diagnostic alleles for the north Atlantic (allele **90*) and Mediterranean populations (allele **100*) of the *S. trutta* complex. Conformity with Hardy–Weinberg equilibrium was tested as described for microsatellite DNA (see below) and levels of genetic introgression were estimated by calculating the percentage of the allochthonous allele **90* in each population.

Ten non-coding microsatellite loci (di- and tetra-nucleotide repeats) were labelled with fluorescent dyes and amplified following Splendiani et al (2019) in two separate multiplex reactions as reported in Table S2. Genotyping was performed using an ABI-PRISM 3130xl Genetic Analyzer (Applied Biosystems), with the LIZ 500 size standard, and allele sizes were manually scored using Peak Scanner™ Software v1.0 (Applied Biosystems).

The microsatellite dataset was screened for false positives, null alleles or other genotyping errors with CERVUS v3.03 (Kalinowski et al., 2007), ML-NUllFreq (Kalinowski & Taper, 2006) and MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004). FreeNA (Chapuis & Estoup, 2007) was used to control the effect of null alleles on F_{ST} estimate. The bootstrap 95% confidence intervals (CIs) for the global F_{ST} value were estimated using 1000 replicates over all loci. The allelic richness (*Ar*) and inbreeding coefficient (F_{IS}) were estimated using FSTAT 2.9.3 (Goudet, 2001). The estimates of *Ar* were adjusted for the smallest sample size, that is COG at locus *Str60* ($n = 3$). The observed (H_o) and expected (H_e) heterozygosities for each sampling site were calculated in ARLEQUIN. The genotypic linkage disequilibrium between loci and population pairs, and the exact test for Hardy–Weinberg equilibrium deviation per population were evaluated using the online software GENEPOP ON THE WEB (Raymond & Rousset, 1995; Rousset, 2008) with 10,000 de-memorizations and 400 batches with 10,000 iterations each. The nominal level of significance (5%) was adjusted following a Bonferroni procedure (Rice, 1989).

TABLE 2 Intra-population genetic diversity obtained by using mtDNA CR sequence analysis, PCR-RFLP analysis of *LDH-C1** gene and 10 microsatellites genotyping on 20 wild brown trout Sardinian sampling sites, two reference samples from wild brown trout Corsican sampling sites and two reference populations for the brown trout Atlantic hatchery stock.

			CR haplotypes (mtDNA)														
Location code		*N*	*A2*	*AD-Tyrrh1*	*AD-Tyrrh4*	*AD-Tyrrh7*	*AD-Tyrrh8*	*AD-Tyrrh9*	*AD-Tyrrh10*	*AD-Tyrrh11*	*AD-Tyrrh12*	*AD-Tyrrh13*	*AD-Tyrrh14*	*ADcs23*	*ADcs24*	*ADcs25*	*Da1a*
Sardinia	**COG**	7	-	0.57	-	-	-	-	-	0.29	-	-	-	-	-	-	-
	PAD	13	-	-	-	-	1.00	-	-	-	-	-	-	-	-	-	-
	POSa	7	-	0.86	-	-	-	-	-	0.14	-	-	-	-	-	-	-
	POSb	18	-	0.74	-	-	-	-	-	-	-	-	-	-	-	-	-
	CED	30	-	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-
	CDL	8	-	0.75	-	-	-	-	-	-	-	-	-	-	-	-	-
	FLUa	10	-	-	-	0.11	-	-	-	-	-	-	-	-	-	-	0.89
	FLUb	9	-	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-
	FLUc	11	-	-	-	1.00	-	-	-	-	-	-	-	-	-	-	-
	FMCa	8	-	-	-	-	-	1.00	-	-	-	-	-	-	-	-	-
	FMCb	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PULa	12	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PULb1	8	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PULb2	23	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	FMPa	30	-	-	-	-	-	1.00	-	-	-	-	-	-	-	-	-
	FMPb	17	-	-	-	-	-	-	-	-	0.47	0.53	-	-	-	-	-
	TEM	6	-	-	-	-	-	-	1.00	-	-	-	-	-	-	-	-
	RMN	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	RMF	5	-	-	-	-	-	-	-	-	-	-	1.00	-	-	-	-
	CIX	30	0.73	-	0.27	-	-	-	-	-	-	-	-	-	-	-	-
Corse	**LTT**	5	-	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-
	CTT	5	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-	-
	HBT	5	-	-	-	-	-	-	-	-	-	-	-	1.00	-	-	-
	VES	19	-	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-
	VIV	20	-	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-
Hatc.	**HATa**	26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	HATb	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: From left: location code; sample size (*N*); frequency of mtDNA control region haplotype(s) observed; *LDH-C1** allele frequencies; Allelic richness (*Ar*); observed heterozygosity (H_O); expected heterozygosity (H_E); fixation index (F_{IS}) with significant adjusted nominal level (5%) ($P < 0.00021$) given in bold; mean admixture coefficient (*q*) and 90% credible intervals (CI); introgression rates (I, pure native trout; II, low introgressed trout; III, moderately introgressed trout; IV, non-native trout) based on admixture (*q*) values and their CIs, frequency of *LDH-C1*90* allele and AT-DA haplotypes; see Section 3.2 for more details. LTT, CTT and HBT are Corsican sampling sites from Reynaud et al. (2011).

TABLE 2 (Continued)

	CR haplotypes (mtDNA)						*LDH-C1**		Microsatellites					
Location code	*Haplotype 1*	*Haplotype 2*	*Haplotype 3*	*Haplotype 4*	*AT-Tyrrh1*	*At1e*	**90*	**100*	*Ar*	H_O	H_E	F_{IS}	*q* (90% CI)	I
Sardinia	0.14	-	-	-	-	-	0.21	0.79	2.71	0.55	0.59	0.078	0.990 (0.933–1.000)	II
	-	-	-	-	-	-	0.12	0.88	2.65	0.61	0.56	−0.097	0.987 (0.917–1.000)	II
	-	-	-	-	-	-	-	1.00	2.83	0.50	0.56	0.118	0.955 (0.885–1.000)	III
	-	-	-	-	-	0.26	0.36	0.64	3.07	0.58	0.61	0.038	0.974 (0.884–1.000)	III
	-	-	-	-	-	-	0.02	0.98	2.06	0.50	0.52	0.048	0.993 (0.964–1.000)	II
	-	-	-	0.25	-	-	0.31	0.69	2.75	0.52	0.54	0.020	0.981 (0.891–1.000)	III
	-	-	-	-	-	-	0.85	0.15	3.43	0.79	0.74	−0.071	0.012 (0.000–0.083)	IV
	-	-	-	-	-	-	-	1.00	2.65	0.54	0.55	0.018	0.919 (0.828–1,000)	III
	-	-	-	-	-	-	-	1.00	1.99	0.49	0.45	−0.089	0.994 (0.967–1.000)	I
	-	-	-	-	-	-	0.13	0.88	2.83	0.52	0.65	0.221	0.992 (0.949–1.000)	II
	0.67	0.33	-	-	-	-	0.83	0.17	3.37	0.72	0.72	−0.013	0.004 (0.000–0.019)	II
	-	-	-	-	-	-	-	1.00	1.77	0.30	0.54	0.475	0.970 (0.925–0.991)	II
	-	-	-	-	-	-	-	1.00	1.36	0.31	0.37	0.176	0.995 (0.978–1.000)	I
	-	-	-	-	-	-	-	1.00	1.28	0.33	0.35	0.027	0.998 (0.993–1.000)	I
	-	-	-	-	-	-	-	1.00	1.52	0.52	0.48	−0.086	0.997 (0.984–1.000)	I
	-	-	-	-	-	-	0.15	0.85	1.92	0.39	0.41	0.042	0.982 (0.912–1.000)	IV
	-	-	-	-	-	-	0.33	0.67	1.87	0.45	0.42	−0.086	0.991 (0.941–1.000)	II
	-	-	-	1.00	-	-	0.77	0.22	3.30	0.65	0.72	0.107	0.875 (0,761-0,922)	III
	-	-	-		-	-	0.30	0.70	2.94	0.64	0.62	−0.036	0.992 (0.955–1.000)	II
	-	-	-	-	-	-	-	1.00	1.48	0.28	0.29	0.056	0.997 (0.987–1.000)	I
Corse	-	-	-	-	-	-	-	-	-	-	-	-	-	
	-	-	-	-	-	-	-	-	-	-	-	-	-	
	-	-	-	-	-	-	-	-	-	-	-	-	-	
	-	-	-	-	-	-	-	1.00	1.82	0.43	0.51	0.081	0.998 (0.987–1.000)	I
	-	-	-	-	-	-	-	1.00	1.80	0.27	0.37	**0.283**	0.981 (0.944–1.000)	I
Hatc.	0.63	-	-	-	0.37	-	0.96	0.04	4.08	0.85	0.82	−0.044		
	0.13	0.74	0.13	-	-	-	1.00	-	4.06	0.75	0.81	0.075		

Note: From left: location code; sample size (*N*); frequency of mtDNA control region haplotype(s) observed; *LDH-C1** allele frequencies; Allelic richness (*Ar*); observed heterozygosity (H_O); expected heterozygosity (H_E); fixation index (F_{IS}) with significant adjusted nominal level (5%) ($P < 0.00021$) given in bold; mean admixture coefficient (*q*) and 90% credible intervals (CI); introgression rates (I, pure native trout; II, low introgressed trout; III, moderately introgressed trout; IV, non-native trout) based on admixture (*q*) values and their CIs, frequency of *LDH-C1*90* allele and AT-DA haplotypes; see Section 3.2 for more details. LTT, CTT and HBT are Corsican sampling sites from Reynaud et al. (2011).

The pairwise genetic differentiation among trout populations (i.e. F_{ST} *sensu* Wright) was computed in FSTAT. As described for mtDNA (see Section 2.2), the analyses of genetic variation (AMOVA) were performed in ARLEQUIN to investigate the partitioning of genetic variance under the two hypothesized hierarchical grouping tested above using CR haplotypes: populations groups were based on (i) the 12 river basins of origin and (ii) four sea drainages (Table 1).

The population genetic structure was investigated using the Bayesian clustering method implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000) using a 'hierarchical STRUCTURE approach' (e.g. Berrebi et al., 2019; García-De León et al., 2020; Marić et al., 2017; Vähä et al., 2007; Warnock et al., 2010) performing subsequent rounds on each subgroup identified by Evanno method. The STRUCTURE parameters were set up as follows: 10 serial runs for each number of clusters (K) between 1 and sampling sites number +1; admixture model with correlated allele frequencies; burn-in period of 50,000 steps followed by 200,000 Monte Carlo replicates. The optimal K was chosen according to the ΔK method (Evanno et al., 2005) as estimated in STRUCTURE SELECTOR (https://lmme.ac.cn/StructureSelector/) (Li & Liu, 2018). Finally, genetic differentiation among individuals and populations was also explored through a discriminant analysis of principal components of genetic variability (DAPC; Jombart et al., 2010), implemented in the package adegenet 2.0 (Jombart, 2008) for the R software (R core team, 2021), by setting sampling locations as pre-defined groups.

Maximum likelihood method implemented in COLONY 2.0.6.1 (Jones & Wang, 2010) was used to evaluate family structure within sites, as it may affect the results of population structure analyses (Anderson & Dunham, 2008). Sib-ship probabilities were estimated by setting: random mating, polygamy for both sexes (e.g. Rossi et al., 2022; Serbezov et al., 2010), no prior for sib-ship assignments, long-length runs and high likelihood precision (other settings were as default). To check for consistency among results, each run was replicated three times.

The effective population size (*Ne*) for each site/drainage was estimated using both the programs NeESTIMATOR 2.01 (Do et al., 2014) and COLONY. The first approach (*Ne*1) is based on linkage disequilibrium and adjusts for missing data (LDNe method implemented in NeESTIMATOR). The *Ne*1 estimation with the lowest allele frequency of 0.02 was reported as recommended for microsatellite markers (Do et al., 2014). The second approach (*Ne*2) uses the sib-ship assignment methods (Wang, 2009) based on the frequencies of sib-ship estimated from a sib-ship assignment analysis, using the multi-locus genotypes of a sample of offspring taken at random from a single cohort in a population.

Recent and substantial demographic reductions were evaluated for each population using BOTTLENECK (Piry et al., 1999) whose method relies on the assumption that the mutation-drift equilibrium is transiently disrupted and the heterozygosity measured at a locus (H_e) will exceed the heterozygosity (H_{eq}) computed from the number of alleles sampled (Cornuet & Luikart, 1996). Both the infinite allele mutation model (IAM, Kimura & Crow, 1964) and the two-phased model (TPM: 90% of single-step mutations with variance set to 30%, Di Rienzo et al., 1994) were applied, as recommended for microsatellite data (Luikart et al., 1998), setting 5,000 replicates. The heterozygosity excess was evaluated according to the one-way Wilcoxon signed-rank test [which is recommended in the event of limited sample sizes and/or loci (Piry et al., 1999) and the allele frequency distribution mode-shift method (Luikart et al., 1998)].

Finally, the association between the amounts of introgression from Atlantic lineages within sampling sites/hatcheries, as revealed by employed diagnostic or semi-diagnostic molecular markers (microsatellites, *LDH-C1** and mitochondrial CR) was investigated using Pearson's linear correlation (*cor.test* function in R). The relationship between measures of genetic diversity (*Ar* and H_e) and introgression of hatchery–Atlantic lineages (as estimated by the frequency of the *LDH-C1*90* allele) across sites/hatcheries was also tested using the *lm* function in R: In this case, a quadratic model was used (second-degree polynomial) as diversity is expected to be higher at intermediate levels of introgression (Rossi et al., 2022).

3 | RESULTS

3.1 | Mitochondrial DNA

A total of 18 CR haplotypes in 359 individuals were detected, belonging to both native and exotic mitochondrial lineages (Table 2). The latter included six AT haplotypes and a single DA haplotype. The AT haplotypes were already observed in European hatcheries—that is *haplotype*-1, 2, 3 and 4 (Cortey & García-Marín, 2002), *AT-Tyrrh1* (Berrebi et al., 2019) and *At1e* (Meraner et al., 2007). The *haplotype*-1 was observed in both reference Atlantic hatcheries (HATa and HATb), and in the wild sites GOG and FMCb, the *haplotype*-2 was observed in HATb and in the wild site FMCb, the *haplotype-3* was observed in HATb, the *haplotype*-4 was observed in the wild sites CDL and RMN, *AT-Tyrrh1* was observed in HATa and *At1e* was observed in the wild site POSb. The single DA haplotype resulted identical to the haplotype *Da1a* (Duftner et al., 2003) and was detected as dominant (90%) in FLUa. As indicated above, this Danubian haplotype was considered to be of stocking origin (see Section 4).

The other 11 haplotypes belonged to the native AD phylogenetic lineage: four were previously described—*A_2* (Zaccara et al., 2015), *AD-Tyrrh1* (Berrebi et al., 2019), *AD-Tyrrh4* [Berrebi et al., 2019, Zaccara et al., 2015 (*C69*)] and *AD-Tyrrh7* (Palmas et al., 2020), while seven haplotypes were detected for the first time in this study (*AD-Tyrrh8–AD-Tyrrh14*, Genbank accession numbers OR972382-OR972391, Table 2). Among AD haplotypes, sequence lengths ranged from 996 to 1324 bp. This polymorphism, observed in 5 (*AD-Tyrrh9–AD-Tyrrh13*) out of 11 haplotypes, was caused by one to five tandem duplications of an 82 bp motif located in the 3′-end of the CR. As the elongation model of this repetition is generally thought to be the result of intra-molecular processes (Buroker et al., 1990; Sell & Spirkovsky, 2004), and the use of the number of repetitions may not be appropriate for phylogenetic reconstruction,

only the first copy was kept in the analysis—but note that after excluding the tandem repeat structures, haplotypes *AD-Tyrrh9* and *AD-Tyrrh13* collapsed into the haplotype *AD-Tyrrh4*. The phylogenetic tree (Figure 2) and the TCS network (Figure 3) roughly provided consistent results. In particular, (1) haplotypes *AD-Tyrrh10*, *AD-Tyrrh4* and *AD-Tyrrh12* formed a strongly supported clade (posterior probability = 1, Figure 2) along with the *ADcs-23/24/25* Corsican haplotypes detected in the west-flowing river basins Seccu and Liamone (e.g. Reynaud et al., 2011, Tables 1 and 2)—given their geographic distribution and remarkable differentiation within the AD lineage, they will hereafter be referred to as belonging to the 'Corso-Sardinian sub-lineage'; (2) other AD haplotypes detected in this study were similar to each other (i.e. showing 1–4 mutations; Figure 3), although mutual relationships were poorly resolved, except for the clade including *AD-Tyrrh8* and *AD-Tyrrh11* haplotypes (BI posterior probability value = 0.77, Figure 2). Time to the most recent common ancestor (T_{MRCA}) of brown trout was dated to 3.82 Ma [95% HPD 1.83–8.54] and T_{MRCA} of AD lineage can be dated to 2.52 Ma [95% HPD 0.85–5.84] (Figure 2, Table S3). The AD lineage appeared ramified into three groups, in which only the Corso-Sardinian sub-lineage was highly statistically supported and its origin was dated around 1.05 Ma [95% HPD 0.24–2.72].

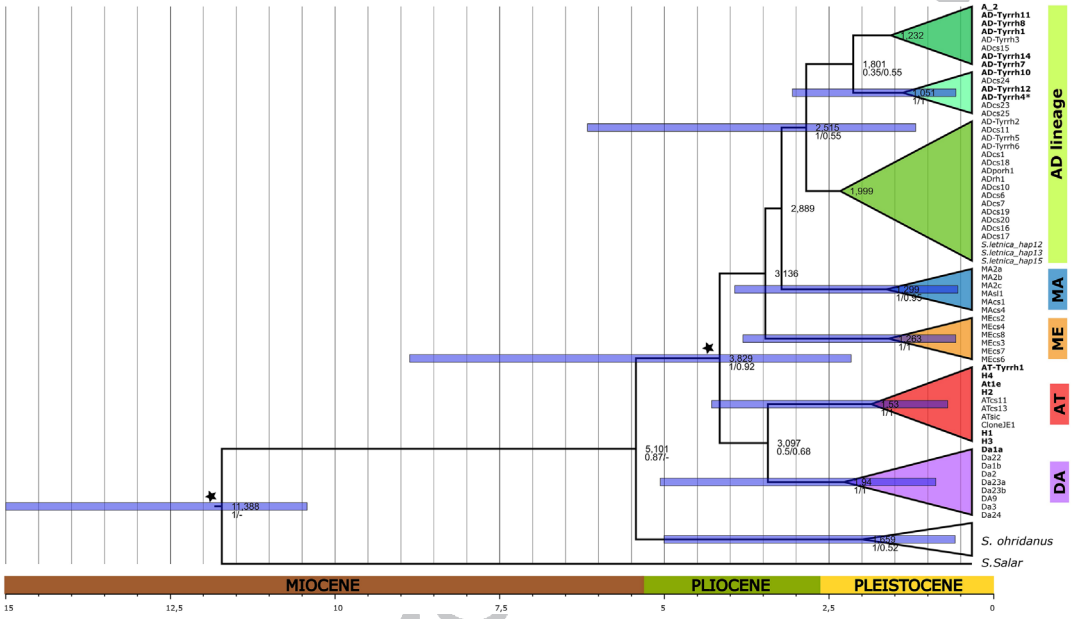


FIGURE 2 Calibrated chronogram of the genus *Salmo* created with an optimized relaxed clock in Beast2. Blue bars at the nodes represent 95% highest posterior density (hpd) intervals; only clades showing posterior probability greater than 0.9 are represented. Median node ages are shown as node labels and Beast/BI posterior probability greater than 0.5 are reported. Time estimates are given in millions of years. Calibration points are indicated by stars. Asterisk: The haplotype *AD-Tyrrh4* includes also the haplotypes *AD-Tyrrh-9* and *13* (see Section 3.1).

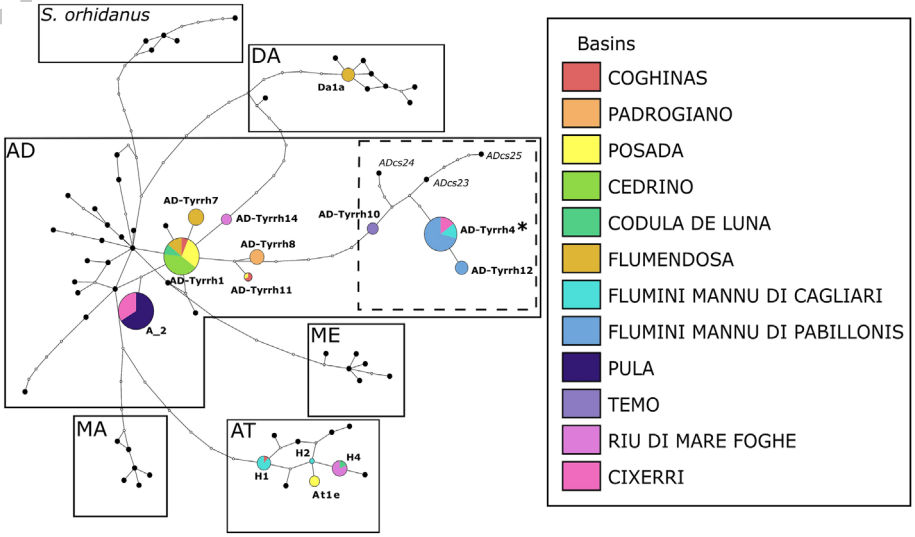


FIGURE 3 Parsimony network (95%) of CR *Salmo trutta* species complex and *S. orhidanus* haplotypes used in this study. In bold, the *S. trutta* CR haplotypes observed in this study. Pie charts indicate the frequency (circle sizes are proportional to observed haplotype frequencies) and distribution of haplotypes across basins (as indicated in Table 1). The white circles along the branches represent the mutational steps. The dashed box includes the CR Corso-Sardinian lineage haplotypes. Asterisk: the haplotype *AD-Tyrrh4* include also the haplotypes *AD-Tyrrh-9* and *13* (see Section 3.1).

TABLE 3 AMOVA hierarchical analysis examining the partitioning of genetic variance of mitochondrial (control region) and nuclear DNA (10 microsatellite loci) according to two hypothesized spatial structures: sites grouped by sea drainages and sites grouped by river basins (as defined in Table 1).

		Control region		Microsatellites	
No. of groups and group composition	**Hierarchical level**	***Variation (%)***	***p***	***Variation (%)***	***p***
12 river basins	among groups	83.37	**0.000**	16.49	**0.000**
COG/PAD/POSa+POSb/CED/CDL/FLUa+FLUb+FLUc/ FMCa+FMCb/PULa+PULb1 + PULb2/FMPa+FMPb/ TEM/RMN/RMF/CIX	among populations within groups	4.64	**0.000**	29.22	**0.000**
	within populations	11.98	**0.000**	54.28	**0.000**
4 sea drainages	among groups	55.82	**0.000**	12.68	**0.000**
COG/PAD+POSa+POSb+CED + CDL + FLUa+FLUb +FLUc/ FMCa+FMCb+PULa+PULb1 + PULb2 + CIX/ FMPa+FMPb+TEM + RMN + RMF	among populations within groups	33.56	**0.000**	34.44	**0.000**
	within populations	10.62	**0.006**	52.88	**0.000**

Note: The amount of variation (%) explained by differences among groups, among populations within groups and within populations, along with the p-value (statistically significant values are in bold) are provided.

A total of 1–3 haplotypes per site were found in Sardinian locations. In a total of 20 sites, 13 and 3 sites were, respectively, entirely, or mainly (>70% frequency) composed of native AD haplotypes, whereas the remaining three sites (i.e. FLUa, FMCb and RMN) showed the prevalence of allochthonous haplotypes. A clear geographic pattern of differentiation was suggested by the distribution of AD haplotypes. The most widespread haplotype was *AD-Tyrrh1*, being detected with high frequencies (from 54 to 100%) in one-third of Sardinian rivers and two Corsican sites (VES and VIV). This haplotype was shared among all of the north-eastern basins investigated apart from the Padrogiano basin (PAD—Table 2). On the other hand, the haplotypes of the Corso-Sardinian sub-lineage (both from this study and from literature) showed a western distribution (Table 2, Table S1 and Figure 1). The other AD haplotypes were found in very restricted areas (1–2 sites each) where they were generally present at high frequencies. In detail, the haplotype *AD-Tyrrh7* was observed only in the Flumendosa basin (FLUa and FLUc). Haplotypes *AD-Tyrrh8* and *AD-Tyrrh11* presented a northern distribution with the haplotype *AD-Tyrrh8* private and fixed in PAD and the haplotype *AD-Tyrrh11* detected in POSa and in COG. Finally, *AD-Tyrrh14* was private in RMF and the haplotype A_2 was fixed in all Pula Basin sampling sites (PULa, PULb1 and PULb2) and the most abundant in CIX (Table 2).

The AMOVAs (Table 3) revealed that grouping samples according to the river basin of origin explained most of the among-group genetic variance (i.e. 83.37%). When sites were grouped according to the location of the catchment outlet, the among-group component decreased to approximately 56%.

3.2 | Nuclear DNA

Besides hatcheries, the exotic Atlantic *LDH-C1*90* allele was found at high frequencies in FLUa (85%), FMCb (83%) and RMN (77%). On the other hand, the *LDH-C1*90* allele was absent in several Sardinian sampling sites Canale dell'Iserno (POSa), Riu Flumineddu (CED—except for one hybrid specimen), Riu Bau Mandara (FLUb), Riu Furittu (FLUc), Pula basin (PULa, PULb1 e PULb2), Riu Piras (FMPa) and Riu Is Abius (CIX). Also, in the Corsican sites (VES and VIV), the *LDH-C1*90* allele was absent. In the remaining Sardinian populations (COG, PAD, POSb, CDL, FMCa, FMPb and TEM), the *LDH-C*90* allele showed moderate frequency (values between 12% and 36%).

Regarding microsatellite data, the presence of null alleles was suggested by all three software used in this study (CERVUS, ML-NUllFreq and MICRO-CHECKER) in 14 tests over 220. The loci Ssa85 and OMM1064 were affected by null alleles in respectively, three (FMCa, PULa and FMPb) and two sampling sites (FMCb and HATb). All other loci showed evidence of null alleles in just one population. However, global F_{ST} values, obtained including or excluding null alleles (i.e. the ENA correction method; Chapuis & Estoup, 2007), returned comparable results by using all loci screened, respectively, 0.422 (CI 0.388–0.465) and 0.428 (CI 0.395–0.470). As null alleles negligibly affected estimates of the population genetic differentiation, all loci for downstream analyses were retained.

Results of genetic variability within populations were reported in Table 2. In total, 198 alleles were detected using 10 microsatellite loci. The number of alleles per locus ranged from 5 (*Str60*) to 38 (*Ssa410UOS*). Measures of genetic diversity substantially differed among Sardinian sites: allelic richness (*Ar*) and expected heterozygosity (H_e) ranged from 1.28 (PULb2) to 3.43 (FLUa) and 0.29 (CIX) to 0.74 (FLUa), respectively. Models revealed that *LDH*-based introgression explained a substantial fraction of both *Ar* ($R^2 = 0.715$, $F_{2,21} = 26.33$, $P < 0.001$) and H_e ($R^2 = 0.675$, $F_{2,21} = 21.82$, $P < 0.001$), although suggesting roughly linear rather than quadratic relationships in our dataset (Figure S1). In other words, intra-population genetic diversity was higher in sites affected by deep introgression from Atlantic strains rather than in purely native sites.

TABLE 4 Effective population size estimates (*Ne*), with 95% confidence intervals based on linkage disequilibrium (NeEstimator, *Ne*1) and sibship approaches (Colony, *Ne*2) and tests of recent events of bottleneck based on Wilcoxon's test and using the allele frequency distribution mode-shift method for 19 wild Sardinian brown trout and two wild Corsican brown trout samples.

	NeESTIMATOR (LD method)			COLONY (random mating method)					
	*Ne*1	Lower 95% CI	Upper 95% CI	*Ne*2	Lower 95% CI	Upper 95% CI	I.A.M Wilcoxon one-way	T.P.M Wilcoxon one-way	L-shaped distribution
COG	∞	8.9	∞	56	16	∞	0.326	0.714	Shifted mode
PAD	∞	71.7	∞	∞	1	∞	0.752	0.997	Normal
POSa	7.4	2.2	162.6	42	12	∞	0.862	0.991	Normal
POSb	25.8	14.9	61.8	29	16	61	0.577	0.958	Normal
CED	42.6	16.5	∞	23	14	44	0.469	0.973	Normal
CDL	∞	9.4	∞	37	14	∞	0.934	0.998	Normal
FLUa	11.6	4.9	44.4	13	6	64	**0.001**	**0.005**	Shifted mode
FLUb	2.8	1.6	11.7	24	10	∞	0.385	0.754	Normal
FLUc	31.5	2.4	∞	28	12	315	0.629	0.987	Normal
FMCa	21.8	3.2	∞	28	11	∞	**0.001**	**0.002**	Normal
FMCb	5.6	2.9	10.2	16	7	50	**0.001**	**0.042**	Normal
PULa	2.6	0.5	∞	12	6	38	**0.008**	**0.040**	Shifted mode
PULb	9.9	1.2	∞	11	6	26	0.563	0.843	Shifted mode
FMPa	5.9	1.6	27.6	12	6	30	**0.016**	0.078	Normal
FMPb	∞	18	∞	20	10	43	0.500	0.898	Normal
TEM	∞	1.8	∞	∞	1	∞	0.980	0.989	Normal
RMN	16.5	6.7	170.8	23	10	299	**0.002**	0.215	Normal
RMF	∞	9.5	∞	20	6	∞	0.179	0.820	Shifted mode
CIX	1.6	0.8	3.7	10	5	28	0.422	0.781	Normal
VIV	10	3.2	30.9	25	14	52	0.629	0.980	Normal
VES	16	2.9	∞	15	7	31	**0.008**	0.055	Normal

Note: In bold, the significant *P*-values ($P < 0.05$) of the Wilcoxon tests.

Significant ($P < 0.05$) deviations from Hardy–Weinberg expectations were observed in three Sardinian (PULa, FMCa and RMF) sampling sites, HATb and one Corsican location (VIV), although only the latter remained significant after Bonferroni correction. Tests for linkage disequilibrium (LD) at the population level revealed three significant associations ($P < 0.001$) out of 1035 comparisons, namely between *Ssa410UOS* and *Ssa408UOS* loci in CIX and HATa, and between *SSsp2213* and *Ssa408UOS* in HATa.

The Wilcoxon one-tailed test revealed the signal of a recent bottleneck in four sampling sites (FLUa, FMCa, FMCb and PULa) when using the TPM model and in seven sites (FLUa, FMCa, FMCb, PULa, FMPa, RMN and VES) in the case of IAM. However, the shifted mode method confirmed the possibility of a bottleneck only in FLUa and PULa, while suggesting a possible bottleneck also for PULb (Table 4).

Both methods of effective population size estimation (Table 4) failed (CIs including infinity) to determine *Ne* in several sampling sites caused by the small sample size. For the rest of the cases, the comparisons of the output from both methods suggest that the Sardinian populations are particularly small ($1.6 \leq Ne1 \leq 25.8$; $10 \leq Ne2 \leq 29$). In general, *Ne* estimations based on the linkage disequilibrium method were lower compared to those based on the sib-ship assignment method. Estimates were partly related among methods (Spearman correlation: $rs = 0.52$, $P = 0.039$), in any event, both tests reported the lowest effective population size for CIX and the highest for POSb.

The global F_{ST} was 0.431 ($P < 0.001$) implying remarkable genetic differentiation among populations. Pair-wise F_{ST} values and their significance are reported in Table 5. The differentiation among sampling sites was substantial ($P < 0.05$ after adjustment for multiple comparisons) in 160 out of 253 comparisons. Lower pair-wise values ($F_{ST} \leq 0.1$) were detected between the two hatcheries, between hatcheries and three wild sites (RMN, FLUa and FMCb), and between Posada Basin sites (POSa and POSb). Notably, three sites (i.e. COG, FLUc and PULa) were not statistically differentiated ($P > 0.05$) from all other sampling sites.

AMOVAs provided similar outcomes, irrespective of the two tested partitioning of sites (Table 3): differentiation among sea drainages and river basins explained approximately 16% and 13% of the overall variance, both significantly ($P < 0.001$); the intra-population differentiation accounted for most of the variation (>52%), as expected when dealing with hypervariable markers.

TABLE 5 Pairwise F_{ST} based on 10 microsatellite loci between 19 wild Sardinian brown trout sampling sites (blue headers), two wild Corsican brown trout populations (orange headers) and two (yellow headers) Atlantic brown trout hatchery strains (below diagonal).

	COG	PAD	POSa	POSb	CED	CDL	FLUa	FLUb	FLUc	FMCa	FMCb	PULa	PULb1	PULb2
COG		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
PAD	0.218		*	*	*	NS	*	*	NS	*	*	NS	NS	*
POSa	0.191	0.176		*	*	NS	*	NS	NS	NS	*	NS	NS	*
POSb	0.174	0.151	0.108		*	*	*	*	NS	*	*	NS	NS	*
CED	0.393	0.269	0.356	0.334		*	*	*	NS	*	*	NS	NS	*
CDL	0.228	0.292	0.280	0.227	0.380		NS	NS	NS	NS	*	NS	NS	*
FLUa	0.266	0.299	0.263	0.258	0.426	0.289		NS	NS	*	*	NS	NS	*
FLUb	0.277	0.287	0.271	0.248	0.407	0.322	0.284		NS	NS	*	NS	NS	*
FLUc	0.419	0.447	0.396	0.385	0.548	0.349	0.381	0.478		NS	NS	NS	NS	NS
FMCa	0.219	0.269	0.221	0.210	0.420	0.270	0.227	0.232	0.397		*	NS	NS	*
FMCb	0.278	0.285	0.266	0.250	0.428	0.294	0.176	0.288	0.419	0.232		NS	NS	*
PULa	0.379	0.440	0.370	0.367	0.558	0.421	0.357	0.404	0.555	0.365	0.429		NS	NS
PULb1	0.473	0.480	0.407	0.394	0.563	0.524	0.454	0.445	0.635	0.431	0.479	0.213		NS
PULb2	0.607	0.572	0.537	0.489	0.625	0.621	0.572	0.565	0.696	0.559	0.591	0.232	0.273	
FMPa	0.551	0.526	0.474	0.447	0.586	0.562	0.533	0.533	0.610	0.434	0.535	0.617	0.621	0.643
FMPb	0.447	0.455	0.393	0.370	0.517	0.463	0.443	0.423	0.545	0.363	0.403	0.553	0.569	0.625
TEM	0.393	0.373	0.310	0.278	0.492	0.471	0.413	0.452	0.614	0.402	0.363	0.648	0.712	0.770
RMN	0.276	0.267	0.233	0.229	0.430	0.294	0.169	0.277	0.382	0.233	0.157	0.405	0.471	0.589
RMF	0.257	0.246	0.218	0.209	0.397	0.284	0.271	0.248	0.423	0.214	0.261	0.431	0.491	0.619
CIX	0.579	0.524	0.534	0.506	0.587	0.616	0.574	0.483	0.691	0.542	0.589	0.561	0.567	0.539
VES	0.454	0.446	0.468	0.395	0.540	0.421	0.471	0.498	0.527	0.463	0.486	0.585	0.654	0.705
VIV	0.514	0.524	0.490	0.437	0.586	0.512	0.478	0.532	0.593	0.479	0.493	0.619	0.673	0.726
HATa	0.232	0.254	0.219	0.216	0.370	0.256	0.093	0.234	0.333	0.162	0.075	0.327	0.381	0.468
HATb	0.261	0.254	0.229	0.220	0.377	0.278	0.101	0.251	0.352	0.178	0.085	0.363	0.407	0.506
C G L	0.026	0.060	0.094	0.128	0.162	0.195	0.229	0.263	0.297	0.331	0.364	0.398	0.432	0.466

Note: *P*-values (above diagonal) were obtained after 5060 permutations; indicative adjusted nominal level-5% for multiple comparisons is 0.000198.
Abbreviation: C G L = F_{ST} colour gradient legend.

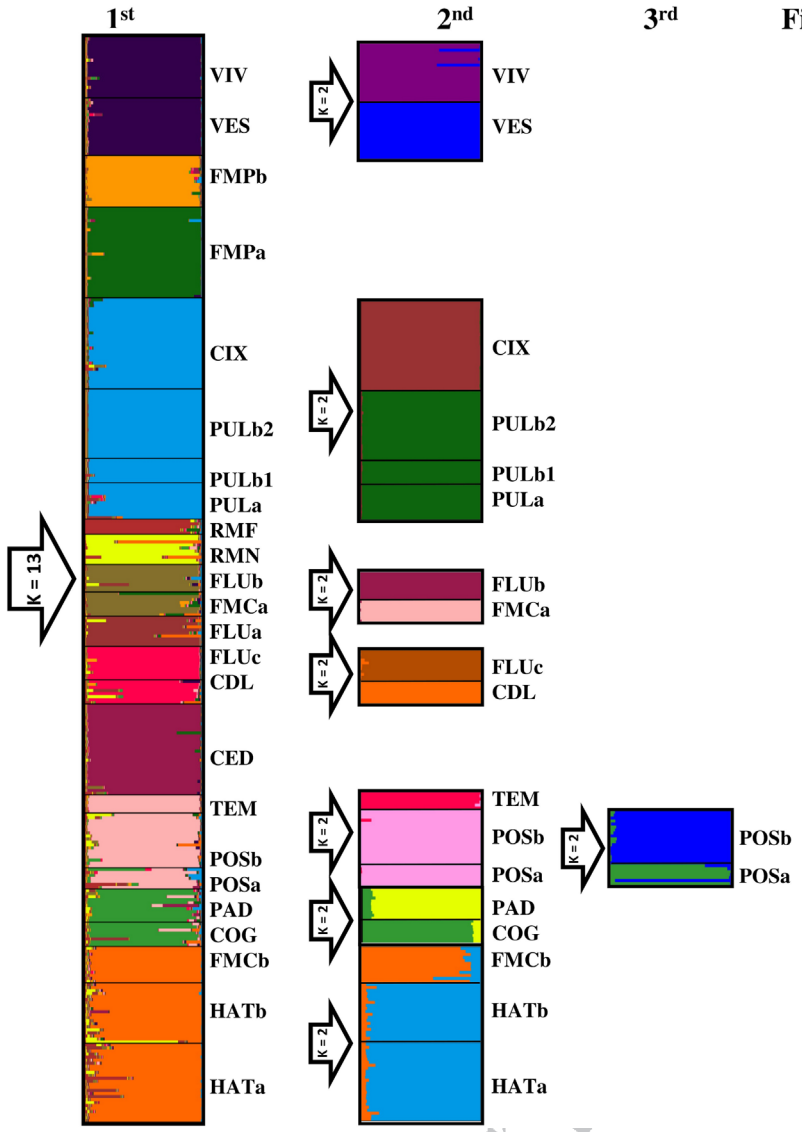
TABLE 5 (Continued)

	FMPa	FMPb	TEM	RMN	RMF	CIX	VES	VIV	HATa	HATb
COG	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
PAD	*	*	*	*	NS	*	*	*	*	*
POSa	*	*	NS	*	NS	*	*	*	*	*

TABLE 5 (Continued)

	FMPa	FMPb	TEM	RMN	RMF	CIX	VES	VIV	HATa	HATb
POSb	*	*	*	*	NS	*	*	*	*	*
CED	*	*	*	*	NS	*	*	*	*	*
CDL	*	*	NS	NS	NS	*	NS	*	*	NS
FLUa	*	*	NS	*	NS	*	*	*	*	*
FLUb	*	*	NS	NS	NS	*	*	*	*	NS
FLUc	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
FMCa	*	*	NS	*	NS	*	*	*	*	*
FMCb	*	*	*	*	NS	*	*	*	*	*
PULa	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
PULb1	*	NS	NS	NS	NS	*	NS	*	*	NS
PULb2	*	*	*	*	*	*	*	*	*	*
FMPa		*	*	*	*	*	*	*	*	*
FMPb	0.550		*	*	NS	*	*	*	*	*
TEM	0.669	0.505		NS	NS	*	*	*	*	*
RMN	0.538	0.403	0.346		NS	*	*	*	*	*
RMF	0.531	0.388	0.394	0.211		NS	NS	*	*	NS
CIX	0.605	0.612	0.744	0.593	0.588		*	*	*	*
VES	0.652	0.583	0.613	0.448	0.473	0.697		*	*	*
VIV	0.645	0.605	0.650	0.458	0.519	0.713	0.584		*	*
HATa	0.425	0.327	0.320	0.109	0.211	0.479	0.408	0.409		*
HATb	0.456	0.355	0.338	0.094	0.205	0.510	0.421	0.420	0.026	
C G L	0.500	0.533	0.567	0.601	0.635	0.669	0.702	0.736	0.770	

Note: *P*-values (above diagonal) were obtained after 5060 permutations; indicative adjusted nominal level-5% for multiple comparisons is 0.000198.
Abbreviation: C G L = F_{ST} colour gradient legend.



Final number of populations

Code; Creek/River; Basin
21) VIV; *Speloncello*; Tyrrhenian Sea
20) VES; *Ese*; Mediterranean Sea
19) FMPb; *Riu Sitzedda*; Mediterranean Sea
18) FMPa; *Riu Piras*; Mediterranean Sea
17) CIX; *Is Abius*; Gulf of Cagliari
16) PULa, PULb1, PULb2; *Litteras, Pula*; Gulf of Cagliari
15) RMF; *Riu di Mare Foghe*; Mediterranean Sea
14) RMN; *Riu Mannu*; Mediterranean Sea
13) FLUb; *Riu Bau Mandara*; Tyrrhenian Sea
12) FMCa; *Riu Cannisoni*; Gulf of Cagliari
11) FLUa; *Flumendosa*; Tyrrhenian Sea
10) FLUc; *Riu Furittu*; Tyrrhenian Sea
9) CDL; *Cadula de Luna*; Tyrrhenian Sea
8) CED; *Riu Flumineddu*; Tyrrhenian Sea
7)TEM; *Riu Matta Giuanna*; Mediterranean Sea
6) POSb; *Riu s'Abba e Salinu*; Tyrrhenian Sea
5) POSa; *Canale dell'Iserno*; Tyrrhenian Sea
4) PAD; *Riu de su* Piricone; Tyrrenian Sea
3) COG; *Riu Bizzolu*; Gulf of Asinara
2) FMCb; *Riu su Salixi*; Gulf of Cagliari
1) HATa, HATb; *Hatchery a, Hatchery b*

FIGURE 4 Hierarchical STRUCTURE analysis based on 10 microsatellites adopted to detect the genetic diversity of 273 wild brown trout from 20 sampling localities from 12 Sardinian river basins, 39 wild brown trout populations from two Corse populations and 46 specimens from two hatchery-reared Atlantic brown trout strains. Black lines separate sampling locations, whose codes (as in Table 2) are reported to the side of each bar plot. Δ*K* outcomes obtained for each hierarchical round of STRUCTURE analysis are reported within the arrows positioned above the corresponding bar plot.

The sequential analysis of genetic structure investigated with STRUCTURE identified a total of 21 genetic cluster (*K*) populations (Figure 4). In the first round of analysis, involving the entire data set, multiple Δ*K* values were supported, therefore, the uppermost structure was chosen corresponding to $K = 13$ (Figure 4). As 7 out of 13 genetic clusters included more than a single sampling location, a second round of STRUCTURE analysis for each 'multi-sample' genetic cluster was conducted: Most of the sampling sites grouped together in the first step were split as single clusters. Finally, a third analysis round allowed distinguishing between POSa and POSb within the 'Posada cluster' identified in the second round of analyses (Figure 4).

To specifically explore the presence of hybrid/Atlantic trout across 20 Sardinian and two Corsican wild sampling sites, while quantifying their admixture degree, a $K = 2$ was forced in the Bayesian STRUCTURE analysis: Because Atlantic/Mediterranean opposition is the first structure in these populations, the individual membership coefficients obtained (i.e. *q* values) were ranked from the highest ($q = 1$, indicating a pure native trout individual in this study) to the lowest ($q = 0$, namely a pure hatchery-Atlantic trout) and their 90% credible intervals (CIs) were plotted against rank (Figure S2). Based on admixture (*q*) values and their CIs, frequency of *LDH-C1*90* allele and AT-DA haplotypes, four groups of individuals were arbitrarily identified. In the first group (*pure native trout*, 25.00% of sites), the mean *q* values were ≈1 with very narrow CIs (the mean lower CI was 0.982); here (FLUc, PULb1, PULb2, FMPa and CIX), neither allochthonous haplotypes nor the *LDH-C1*90* allele were detected. In the second group (*low introgressed trout*, 40.00%), mean *q* values were still high (≈1), while contextually associated with lower

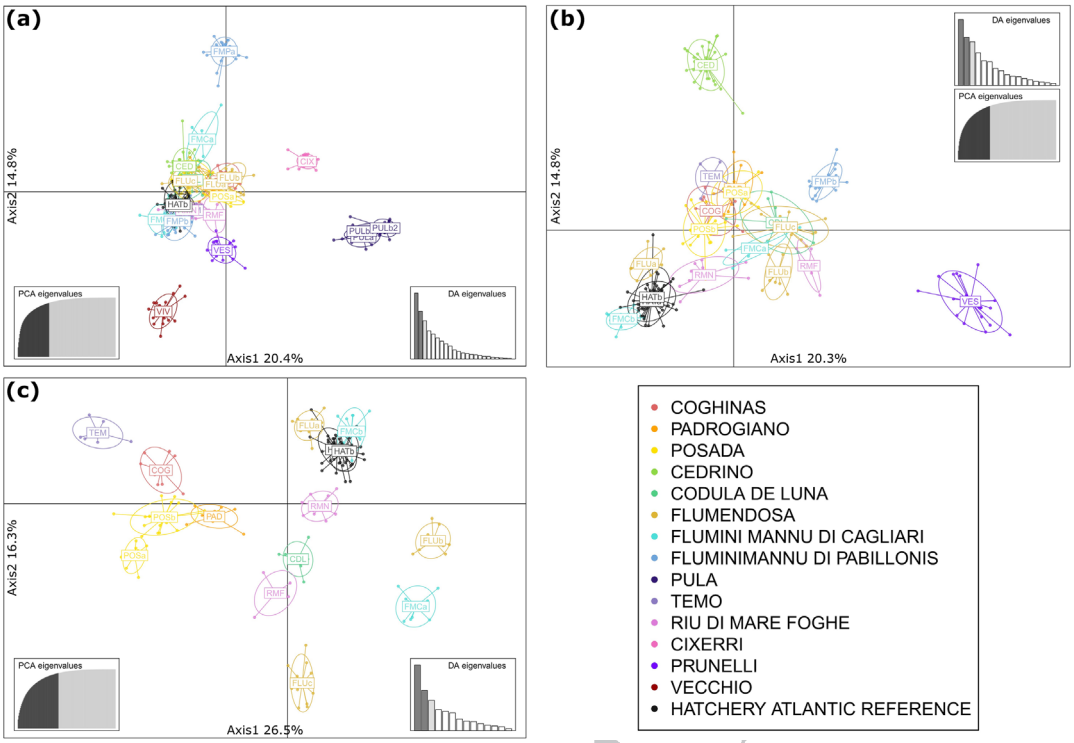


FIGURE 5 Plots showing the two discriminant axes of a hierarchical discriminant analysis of principal components carried out on wild brown trout sampling sites from Sardinia and Corsica and two hatchery strains of Atlantic origin: (a) all sampling sites included; (b) all sampling sites but PULa-b1-2, CIX, VIV and FMPa; and (c) all B step samples but CED, VES and FMPb. Each trout is represented as a dot, and the samples are represented as inertia ellipses.

mean CIs (mean lower CI = 0.912, range 0.912–0.964); here (CED, PAD, FMCa, FMPb, COG, RMF, TEM and PULa), the frequency of allochthonous haplotypes ranged from 0.00 to 0.14 and the frequency of the *LDH-C1*90* allele ranged from 0.00 to 0.33. In the third group (*moderately introgressed trout*, 25.00%), mean *q* values were even lower (mean *q* = 0.94), while the mean lower CI was 0.850 (range = 0.761–0.891); in this group (CDL, POSb, RMN, POSa and FLUb), the frequency of allochthonous haplotypes ranged from 0.00 to 1.00 and the frequency of the *LDH-C1*90* allele ranged from 0.00 to 0.77. The fourth group (*non-native trout*, 10.00%) included pure or almost pure Atlantic trout (FMCb and FLUa), showing mean *q* values ≈ 0; in this latter group, the frequency of allochthonous haplotypes ranged from 0.89 to 1 and the frequency of the *LDH-C1*90* allele ranged from 0.83 to 0.85 (Table 2 and Figure S2).

Estimates of Atlantic brown trout introgression across sites/hatcheries strongly correlated between molecular markers: $r = 0.96$ and $P < 0.001$ for *LDH-C1*90* allele versus Atlantic haplotypes; $r = -0.93$ and $P < 0.001$ for Atlantic haplotypes versus coefficient of hatchery ancestry (*q* of STRUCTURE); $r = -0.88$ and $P < 0.001$ for *LDH-C1*90* allele versus hatchery ancestry.

The DAPC analyses showed a pattern of genetic differentiation quite similar to the scenario depicted by STRUCTURE. The first plot (Figure 5a), which included all sampling sites, pointed to the distinctiveness of Pula River (PULa and PULb1-2), CIX, FMPa and VIV, while the rest of the other sites were grouped together. After removing such distinctive locations (Figure 5b), CED, FMPb and VES diverged from other sites, which were roughly arranged along a gradient: from Atlantic strains in the left (HATa, HATb, FMCb and FLUa) to Mediterranean-native ones at the centre of the plot (e.g. CDL, FLUc, FLUb, FMCa and RMF). The third plot (Figure 5c), which was obtained after removing the most divergent sites of the previous step (i.e. CED, FMPb and VES), highlighted the presence of three groups of populations. Northern populations (TEM, COG, PAD, POSa and POSb), located at the top left part of the scatterplot, form a group well separated from the remaining highly pure populations from the south-eastern side (FLUa, FLUb and FMCb) located at the bottom right portion. At the top centre of the graph, the hatchery-reared Atlantic strains and highly introgressed wild sampling sites FLUa and FMCb are overlapped identifying an omogeneous cluster, quite close to the wild sites RMN, CDL and RMF. Generally, except for FLUa and FMCb, each sampling site was identified as a separated cluster.

The number of families per population identified by the parentage analyses performed with COLONY software identified very few siblings (>0.80 inclusion and exclusion probability in most cases; see Table S3).

4 | DISCUSSION

In this study, the origin, population genetics and demography of wild brown trout populations from Sardinia were investigated, and the role of Sardinia as a hotspot of *Salmo* (genetic) diversity within the

Mediterranean basin was eventually demonstrated. In addition, the presence of a new distinctive Corso-Sardinian mtDNA sub-lineage characterized by haplotypes endemic to the Sardinian and Corsican rivers was described (Figures 2 and 3). Nuclear markers (microsatellites) also pointed out strong differentiation between wild native populations. At the same time, the reduced intra-population genetic variability coupled with small effective population sizes suggested the potentially severe vulnerability of such Sardianian-native populations inhabiting extreme habitats for salmonids. A similar pattern has been observed in Corsica, leading to the same interpretation (Berrebi et al., 2019). The need for the definition of appropriate categories of conservation applicable in the implementation of correct and concrete conservation actions appears crucial for the near future conservation of the last population of Sardinian trout.

4.1 | Population genetic variability and demography

The levels of genetic variability detected within most Sardinian sampling sites appeared generally low. If one takes into account only 'pure' wild locations (i.e. absence of the *LDH-C1*90* allele and AT mtDNA haplotypes, coupled with mean *q*-values ≈ 1; Table 2), a mean value of observed heterozygosity of 0.41 (*SD* = 0.11) and a mean value of allelic richness of 1.86 (*SD* = 0.55) were estimated. Generally, higher values of observed heterozygosity (H_o > 0.60) and allelic richness (A_r > 4.0) are typically observed in the hatchery-reared Atlantic strains (Bohling et al., 2016) or in native Mediterranean brown trout populations highly impacted by the latter (Vera et al., 2023). In fact, similar values of low intra-population genetic diversity have been observed in almost purely native, small and naturally isolated populations from central Italy—such as those inhabiting the Tenna River (Adriatic drainage; Splendiani et al., 2019a) or the Rio Santa Croce (Tyrrhenian drainage, Rossi et al., 2022)—or elsewhere, in the Mediterranean basin: Corsica (Berrebi et al., 2019), the upper part of the Došnica and Konjarska rivers in Macedonia (Aegean drainage; e.g. Marić et al., 2016), two localities from the Mijares and Turia basins (e.g. Vera et al., 2013) and the Ter River (e.g. Araguas et al., 2017) of the Iberian Peninsula. The above cases mostly represent typical freshwater environments where the last native trout populations still survive in the Mediterranean area, such as in small creeks or streams naturally and/or artificially isolated from the other river basins, showing stable hydrological conditions and suitable spawning habitats. Generally, the native trout populations inhabiting these sites benefit from high conservation priority and these habitats are managed, or present themselves to be managed, as genetic refuges. These kinds of river ecosystems are likely to become thermally crucial for the future viability of salmonids in the Mediterranean rivers where, in the next two decades, half of the suitable habitat is expected to be lost (e.g. Almodóvar et al., 2012). However, regarding the present case of study, the water courses where the last pure Sardinian trout populations still survive are very far from the concept of an ideal thermal refuge for brown trout. As described above (Section 1), most water courses investigated presented a non-perennial hydrological regime, with trout populations surviving in small and isolated pools where the water temperature can exceed 25°C for several days or even weeks during the driest months. For brown trout, an upper critical temperature range of 25–30°C with an incipient lethal temperature of approximately 25°C was reported (e.g. Jonsson & Jonsson, 2009). Thermal stress together with low discharge can also affect size, fecundity and population density due to the increased metabolic costs of growth at elevated temperatures in south salmonid habitats (e.g. Jonsson & Jonsson, 2009). Furthermore, intermittent discharge is likely to contribute to the fragmentation of Sardinian trout populations within basins, leading to multiple isolated patches of small effective population sizes.

Estimates of *Ne* (Table 4) resulted dramatically low, irrespective of the adopted method (considering only *Ne* estimates with finite CIs: 1.6 ≤ *Ne*1 ≤ 25.8; 10 ≤ *Ne*2 ≤ 29). Furthermore, *Ne* could be even lower if only native individuals are taken into account, as revealed by previous studies on introgressed populations (Splendiani et al., 2019a). Assuming *Ne* estimates to correspond approximately to ½ of the census population size (according to models based on Novergian river-resident brown trout populations; Serbezov et al., 2012), actual spawners would range between 3.2 and 20 in the smallest population (CIX) and between 51.6 and 58 in the largest population (POSb) according to *Ne*1 and *Ne*2 estimates, respectively. Such a low estimation of the number of spawning adults appears quite realistic and consistent with low densities of trout individuals recorded in the most recent regional freshwater fish census (e.g. AA. VV., 2022, Table 1). Furthermore, also the difficulty encountered during the sampling activities of this study in obtaining a sufficient number of adult specimens in most localities corresponds to the detection in wild Sardinian trout sites of a very low census size.

In addition to generally low levels of genetic diversity and effective population size, some Sardinian trout populations analysed in this study showed signals of a recent bottleneck. In particular, in the Riu Litteras from the Pula River (PULa), a significant excess of heterozygosity and an L-shifted mode of the allele frequency distribution were observed. Here, very low values of effective population size (*Ne*1 = 2.6 and *Ne*2 = 12, Table 4) were observed and the concomitant detection of a recent bottleneck could be related to an extreme flash flooding event that occurred in November 2015 in the area of the Pula River basin (see below, Section 4.3.2). Elsewhere in Sardinia, FLUa also showed both a significant excess of heterozygosity and an L-shifted mode of the allele frequency distribution. This sampling site, however, is largely represented by non-native individuals (DA lineage and individual q values close to 0), so bottleneck signals might be related to a founder effect that occurred by introducing a restricted number of hatchery-origin individuals. Moreover, hybridization can severely influence the outcome of the bottleneck tests (Zhang et al., 2017), so the significant heterozygosity excess of the FLUa is possibly due to hybridization between native and allochthonous stocks as suggested by the co-presence of AD and DA haplotypes.

4.2 | Genetic structure and phylogeographic inferences

Genetic analyses carried out in the present study revealed strong differentiation among the wild Sardinian brown trout populations (global $F_{ST} = 0.43$), which is remarkable even compared to the values observed in similar extreme environments for salmonids as, for example, in trout populations (*Oncorhynchus* sp.) from Northern Sierra Madre Occidental in Mexico ($F_{ST} = 0.33$; Abadía-Cardoso et al., 2021). Considering that several investigated Sardinian sampling sites were collected above artificial barriers and were characterized by an elevated degree of isolation created by an intermittent water flow (Table 1), it could be argued that such a high degree of genetic differentiation can be due to the stochastic effects of strong genetic drift acting on very small populations. Similarly, Pujolar et al. (2011) argued that reduced genetic diversity, low *Ne* sizes and serial bottleneck events revealed in marble trout populations from Slovenia imply a strong impact of genetic drift, limited gene flow and high genetic differentiation, which could have been exacerbated by recurrent mortalities due to flash floods and debris flows. Genetic drift has been proposed also to explain the high level of genetic differentiation observed both between and within the basin level in Mexican trout species of the genus *Oncorhynchus* living at the extreme southern margin of the genus's range (Abadía-Cardoso et al., 2021).

Besides genetic drift, ancient climatic fluctuations (with implications in connectivity among drainage basins) coupled with the anadromous behavior of ancestral Mediterranean brown trout (Splendiani et al., 2016b; Splendiani et al., 2019b) can partly explain the current geographical pattern of genetic structure. Based on the time-calibrated molecular phylogeny of the Sardinian trout, T_{MRCA} suggests that the haplotypes belonging to the Corso-Sardinian sub-lineage (Figure 2, Table S3) originated during the Menapian–Bavelian periods (c. 1.1 Ma; Middle Pleistocene). The alternation of glacial and interglacial phases that characterized the Pleistocene has had an important role in shaping the biogeographic characteristic of Mediterranean trout populations through the alternating promotion of different lifestyle tactics, promoting migratory propensity during the cold phases or a more sedentary lifestyle during the warmest phases. Thus, isolation in thermal refuges during the warmest periods may have promoted the observed haplotype diversification, and colder phases may have played a role in shaping the geographic distribution of the mtDNA diversity. During the colder phases of the Pleistocene, Corsica and Sardinia were connected (Grill et al., 2007), and therefore, the presence of the two routes (west and east) of colonization along the paleo-Corso-Sardinian coasts is conceivable.

The effect of historical colonization patterns and isolation driven by past climatic phases on Sardinian trout genetic diversity is corroborated by AMOVA analysis based on both mtDNA and microsatellites. Significant genetic differentiation among river basins supports the hypothesis of long periods of isolation between trout populations (Table 3). Strong population differentiation was also detected by hierarchical analyses carried out by using both STRUCTURE (Figure 4) and DAPC (Figure 5a–c).

Moreover, AMOVA detected significant genetic variance even when sites were grouped based on the coastal river mouth orientation suggesting also the presence of a geographic genetic structure related to periods of contact between neighbouring rivers that occurred thanks to the anadromous behavior of trouts in defined periods of time. Anadromy, in the Mediterranean basin, appeared periodically during the cold phases of the Pleistocene when the lower part of the river was a more suitable habitat for salmonids (Muñoz & Casedevall, 1997) and seaward migration propensity more likely (e.g. Splendiani et al., 2019b). Contacts were emphasized by the geographic distribution of the mtDNA haplotypes. In particular, Corso-Sardinian sub-lineage showed a western distribution in Sardinia that points to the role played by the last glacial marine regression. During the last glacial maximum, Corsica and Sardinia were connected due to the closure of the Bonifacio strait (Figure 1), and, as a consequence, the populations inhabiting rivers flowing towards the Western Mediterranean Sea were more likely to be interconnected along the western Corso-Sardinian paleo-shoreline. Here, the spread of the Corso-Sardinian sub-lineage probably occurred through migratory trout (i.e. sea trout). In addition, as mentioned above (Section 2.2), sea trout generally feed chiefly in estuaries and along coasts (Jonsson & Jonsson, 2006), and, as a consequence, it is possible to hypothesize that gene flow between Sardinian populations was more likely between populations with a close sea outlet. According to this hypothesis, gene flow between sea trout populations from northern Spain was negatively related to the distance between river mouths (Moran et al., 2005). Furthermore, as regards rivers flowing in a close bay, as in the cases in this study of the Gulf of Asinara and the Gulf of Cagliari, it is reasonable to expect that from an initial population of 'pioneers', a successive source population arises later. This will first colonize the closest rivers in the bay as suggested by shared A_2 haplotype between closer basins Cixerri (CIX) and Pula (PULa, PULb1 and PULb2) and, as was recently observed in brown trout populations from the Kerguelen archipelago in the District of the French Southern and Antarctic Lands, introduced here during the second half of the 20th century (Launey et al., 2010). Moreover, the occurrence of the Corso-Sardinian sub-lineage at mid- to high-elevation Corse sites and above impassable waterfalls (e.g. Berrebi, 2015), suggests a role as refuge played by the Corsican rivers for this sub-lineage during the severe interglacial warming periods of the Pleistocene. Subsequently, during the colder phases of the Pleistocene (the last glacial phase during the late Pleistocene, c. 100,000–15,000 years ago), the Corso-Sardinian sub-lineage could have reached the Sardinian rivers thanks to migratory tactics along the western Corso-Sardinian paleo-shoreline.

Similarly, on the Tyrrhenian side, the distribution of the aplotipe *AD-tyrrh1* (and related ones) appears in accordance with a peri-Tyrrhenian past route of colonization connecting Corsica and Sardinia along the eastern Sardinian–Corsican paleo-shoreline during the last glacial maximum (Figure 1). This haplotype spread mainly along the eastern side of Corsica and Sardinia (e.g. Berrebi et al., 2019 and Figure 1). An exception is the Corsican Ese River (VES), a tributary of the Prunelli River flowing into the western side, where haplotype *AD-tyrrh1* resulted rare both in Sardinian and Corsica (e.g. Berrebi et al., 2019). Here, the presence of this haplotype could either

represent the consequence of the wider past distribution of this Tyrrhenian AD haplotype or, alternatively, the consequence of ancient river captures that occurred between the two sides of the west-Mediterranean and Tyrrhenian catchments, similar to what was suggested elsewhere in the Mediterranean area (e.g. Berrebi et al., 2017; Splendiani et al., 2006).

Finally, the AD sub-cluster formed by the haplotypes *AD-Tyrrh8* and *AD-Tyrrh11* (Figures 2 and 3) showed a north-eastern distribution partially overlapping the distribution of the common haplotype *AD-Tyrrh1*, thus suggesting the occurrence of an eastern biogeographic route adopted by multiple waves of colonization of the AD lineage (Figure 1 and Table 2). Interestingly, the co-occurrence of the above haplotypes in the Coghinas basin (north-western Sardinia; e.g. COG in Figure 1) suggests that waves of colonization involving these AD Tyrrhenian haplotypes are likely to have occurred when, thanks to the sea level rising at the end of the last glacial maximum, the reopening of the Bonifacio strait allowed the formation of a biological corridor for these eastern AD haplotypes. In the southern part of the island, *A_2* represents the sole haplotype observed in the Pula basin and the most common in the Cixerri basin; this haplotype probably reached the Gulf of Cagliari through a further wave of colonization.

4.3 | Major threats acting on native trout populations in Sardinia

4.3.1 | Stocking and fishing activities

This study has revealed the presence of several severe threats to the survival, in the near future, of native trout populations in the Sardinian rivers. A first menace has been highlighted by the detection of clear signals of hybridization between native trout and Atlantic brown trout of hatchery origin. Admixture from Atlantic strains in Sardinian trout has been already observed (Berrebi et al., 2019; Sabatini et al., 2011; Zaccara et al., 2015), although based on a limited number of examined individuals and/or populations, as compared to the present study. Here, two sites comprised almost exclusively allochthonous alleles and/or haplotypes (FLUa and FMCb). Conversely, the rest of the locations revealed genetic introgression from Atlantic gene pools ranging from 0%, in about a third of sampling sites, to low–medium amounts in the rest of the locations (Table 2). In Italy, stocking activities by using non-native species and/or populations have been strictly banned since 2003 (DPR n. 197/2003), although this law has been systematically neglected by local administrations as well as by fishing clubs. (Splendiani et al., 2016a; Splendiani et al., 2019a; Splendiani et al., 2020). More recently (since 2020), as indicated below (Section 4.4), stocking activities using non-native trout are admissible upon an official request to the Italian Ministry of the Environment. However, as far as it is known, only a few regional administrations have obtained this permission, and illegal stocking activities using non-native trout are still popular in some regions (personal communications from local anglers).

Nevertheless, limited evidence of very recent stocking in Sardinia was found, as only a single specimen characterized by a *q* value of 0.03 (corresponding to a pure Atlantic trout) was observed in RMN (Figure S2). However, because of the low effective sizes of wild populations, the deleterious effects of stocking activities should be taken into account more seriously than elsewhere: Even though negative selection is expected to purge exotic maladaptive alleles from wild populations, mildly deleterious alleles may reach fixation in small populations where the action of the purifying selection is weaker as compared to the larger ones (Moran et al., 2021). This implies that particular attention should also be paid in any planning of supportive breeding programs based on native trout populations with very low *Ne* sizes, as in the case of Sardinian trout, because of the concrete risk of promoting (albeit unintentionally) the fixation of deleterious alleles.

Conversely to almost everywhere else in Italy, a relevant proportion of genetically pure native populations in Sardinian rivers were found. It could be argued that the absence of traditional (or intensive) brown trout farming on the island—officially, only a few small family-owned companies exist where the farming of rainbow trout is allowed by law, (Autonomous Region of Sardinia—RAS Det. N.3/22.01.2020) would have facilitated preserving the genetic integrity of wild native populations. In addition, the occurrence of major trout fishing tournaments has been (and still is) rare in Sardinia, when compared with the rest of the Italian Peninsula, probably because the severe environmental characteristics of most Sardinian salmonid waters are inappropriate or unattractive to carry out fishing competitions. As reported in Table 1, most sampling sites of the present study come from streams experiencing long periods of severe droughts during the driest months. If, on the one hand, the risk of stocking activities with allochthonous trout is averted, at least temporarily, other threats related to fishing activities are still present. For example, fishing activities are allowed in most of the sampling sites investigated (Table 1). In Sardinia, a five-fish daily limit is set; however, based on a Regional law ('Decree of the Assessor of the Defense of the Environment' 10.05.1995 n. 412) the fishing of pure native trout individuals is forbidden everywhere.

In addition, in Sardinia, the Autonomous Region designated several river segments as 'genetic sanctuaries' (GS), such as Riu Furittu, Riu Piras, and Riu Flumineddu, and here, fishing activities are totally banned (DR n.314/Dec.A9-07.02.2019). Therefore, based on the outcomes of this study, fishing activities should be totally banned also in those basins hosting exceptionally pure or nearly pure native trout populations that have not yet been ad hoc normative. Therefore, the updating of regional norms regulating fishing activities in freshwaters appears desirable.

4.3.2 | Environmental and climate characteristics

The very low values of effective population size observed in most populations are in accordance with the hydrographic fragmentation of the Sardinian rivers and with the very high summer water

temperatures characterizing these south salmonid waters (e.g. Jonsson & Jonsson, 2009; Shrimpton & Heath, 2003). Moreover, extreme and repeated flood episodes can create demographic and genetic bottlenecks in salmonids (e.g. Pujolar et al., 2011) or even extinction of local populations as in the case of the *Salmo marmoratus* population from Predelica (Soča River) that was extirpated by a landslide triggered by intense rainfall in 2000 (Vincenzi et al., 2016; Vincenzi et al., 2017). In the last two decades (2000–2020), Sardinia has been affected by 13 extreme flooding events, 62% of which involved the Sardinian rivers flowing toward the Gulf of Cagliari (e.g. Faccini et al., 2021), while the others involved the north-eastern part of Sardinia (De Waele et al., 2010): The detection of a bottleneck signal in both Riu Bizzolu (COG) and Flumendosa River (FLUa) appears consistent with such a scenario, although speculative. Similarly, the very low *Ne* values coupled with bottleneck signals in the Pula Basin (see above, Section 4.1) could be related to an extreme flash flooding event that recently occurred in south Sardinia. Forecasts for the near future are even worse, as a 30% increase in extreme precipitation is foreseen. (e.g. Faccini et al., 2021; Marras et al., 2021), Therefore, the need for a comprehensive *Ne* size monitoring of the last Sardinian brown trout populations appears as a crucial and concrete conservation action also in light of the Ne values observed in this study ($1.6 < Ne_1 < 42.6$, mean = 13.2; $10 < Ne_2 < 56$, mean = 23.28) being well below the safe threshold from the 50/500 rule proposed by Frankham et al. (2014). This rule suggests that an effective population size of 50 is desirable to contrast the short-term likelihood of extinction due to the harmful effects of inbreeding depression on population demography, while a *Ne* of 500 is required for mutation to provide genetic diversity back into a population at a similar rate to loss caused by genetic drift, thereby maintaining a population's long-term evolutionary potential.

4.4 | IMPLICATION FOR CONSERVATION

High isolation of Sardinia rivers, due to both natural and anthropogenic factors, is likely to have played a 'Dr. Jekyll and Mr. Hyde' role towards the current status of conservation of wild trout population. The severe degree of isolation of the wild populations likely played a role in hindering the spread of phenomena of introgressive hybridization between native trout and Atlantic trout of hatchery origin; however, at the same time, isolation determined the very low level of genetic variability observed in Sardinian trout populations. Improving river connectivity, through the mapping and removal of those artificial barriers hindering within-basin natural gene flow, is necessary to counteract the low levels of effective population size observed in wild Sardinian trout populations. However, such a process should be carried out carefully since these barriers are also crucial to prevent the spread of alien Atlantic trout (e.g. Splendiani et al., 2019a).

The first step to design appropriate and effective conservation action should be the identification of correct management units. Based on the high genetic differentiation observed in this study, the preservation of Sardinian trout diversity should start from the protection of local populations and the management of wild local populations should be focused on the conservation of genetic diversity at an intraspecific level (e.g. Bruce et al., 2019; Ferguson, 2004; Vera et al., 2023). However, in light of the results obtained, more detailed genetic and/or genomic studies would contribute to the acquisition of sound data in order to support the need for a taxonomic revision of Sardinian trout (e.g. Hashemzadeh Segherloo et al., 2021), the individuation of evolutionarily significant units and the delineation of management units. Within the near future, an advisable long-term conservation strategy of Sardinian brown trout populations should foresee the acquisition of knowledge about the genetic diversity of several wild Sardinian trout populations not yet studied, with as large as possible coverage, as already accomplished for instance in Corsica (>200 sites analysed; e.g. Berrebi, 2015). Moreover, in-depth studies are needed to better understand the pattern of intra-basin genetic diversity, as well as the association between genetic diversity and environmental features of Sardinian salmonid freshwaters.

Together with the delineation of units of conservation and management hopefully, by an authoritative scientific committee, it is of paramount importance that these management units receive a legal value in a similar way to what has been achieved elsewhere, as in Canada where the delineation of conservation units is performed by the Committee on the Status of Endangered Wildlife (e.g. Bernard et al., 2009). On the contrary, in Italy, wildlife species management is still merely based on the definition of Linnean species (e.g. Splendiani et al., 2019c) and furthermore, freshwater fish fauna (as the rest of the ectotherms) is not considered the property of the State, and the management of local fish fauna is mainly delegated to fishing clubs. In this context, the risks of underestimating native trout genetic diversity are significantly high.

Finally, the recent modifications to the Italian national legislation, on the one hand, are open to the introduction of allochthonous fish in nature (decree of 2 April 2020) and, on the other hand, completely ignore the regulation of the management of native species. Therefore, in the present normative context, the legal designation of management units appears of crucial importance.

In conclusion, the need to proceed toward the realization of an international strategy of conservation for Mediterranean salmonids appears therefore clear. A fundamental first step should be the recognition of freshwater fish species as national property of the sovereign states and, consequently, the provision of a legal value to other categories of conservation (i.e. ESUs and MUs). This will significantly help the planning of conservation strategies towards the populations that are most vulnerable to climate change and therefore for which conservation measures should be prioritized.

ACKNOWLEDGEMENTS

Many thanks to the '*Fédération de Pêche de Corse*' and especially to Stéphane Muracciole for providing the Corsican samples. A special thanks to Dr. Stephanie Susan Ames (CSAL Centro di Supporto per l'Apprendimento delle Lingue dell'Università Politecnica delle Marche) for the linguistic revision of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Andrea, S., Tommaso, R., Tatiana, F., Andrea, S., Francesco, P., Christelle, T. et al. (2024). Population genetics, demography and conservation of Mediterranean brown trout from Sardinia. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 1–26. https://doi.org/10.1002/aqc.4099