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Limited contribution of hatchery-produced individuals to the sustainment of wild marble trout (*Salmo marmoratus* Cuvier, 1829) in an Alpine basin

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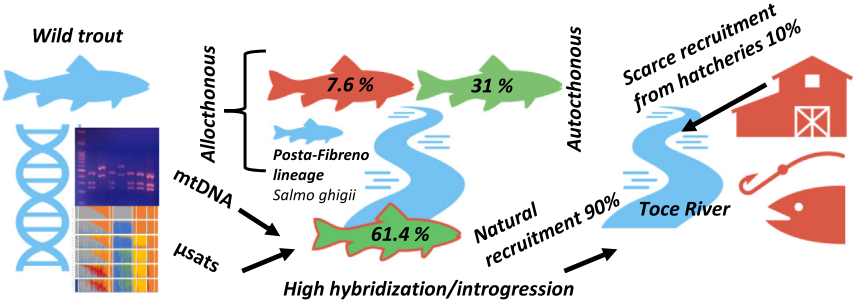
HIGHLIGHTS

- *Salmo marmoratus* is a fish species negatively affected by restocking.
- Three *Salmo* species have been stocked in the Toce River since 5 decades.
- *S. marmoratus* showed uncomplete hybridization with non-native brown trouts.
- Hatched *S. marmoratus* stocks showed high levels of allochthonous introgression.
- The contribution of hatched trout to *S. marmoratus* recruitment is neglibile.

GRAPHICAL ABSTRACT



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ABSTRACT

Fish stocking constitutes a common management practice in freshwaters all over the world, to enhance fisheries or to support threatened fish populations. Pervasive detrimental effects may affect the real effectiveness of stocking programs. However, studies assessing the real impacts and relative contribution of stocked trout in wild populations are surprisingly few.

The marble trout, *Salmo marmoratus* (Cuvier 1829), is a critically endangered sub-endemic salmonid in Northern Italy, and an iconic species for recreational fishing and conservation, also representing an emblematic case of species negatively affected by restocking. For instance, marble trout inhabiting the Toce River, the second largest tributary of Lake Maggiore, has been stocked with different hatchery congener trout belonging to the *Salmo trutta* complex (putative marble trout, Atlantic trout *Salmo trutta* Linnaeus 1758 and putative Mediterranean trout *Salmo ghigii* Pomini 1941) over the last decades. Using mitochondrial (D-loop) and nuclear (12 microsatellites and LDH-C1*) markers, we characterised the genetic variability and gene flow among the wild and hatchery individuals of marble trout of this basin, to investigate the effectiveness of stocking activities on the native residual population.

Despite extensive hybridization of marble trout with non-native brown trout stocks was shown, the presence of individuals belonging to pure native stock has been detected as well. However, concerns could be advanced regarding its future persistence, due to climatic and hydraulic instabilities or loss of environmental heterogeneity. Moreover, despite ongoing yearly massive stocking activities, a negligible contribution of reared putative marble trout in the wild sample has been documented, suggesting that natural recruitment represents the greatest source of this wild population sustainment. Important adaptive differences between wild and domestic trout are present, likely due to the deleterious long-term effects of the close breeding system of hatcheries. Finally, possible implications for stocking management improvement have been discussed.

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1. Introduction

Fish stocking, the introduction into wild populations of hatchery-produced fish, constitutes a common management practice in many rivers and lakes worldwide, to enhance freshwater fisheries or support threatened fish species or populations. Strong and pervasive detrimental effects, however, may affect the real effectiveness of stocking programs. Those effects include genetic risks, such as introgression with non-native stock and loss of genetic variability (Araki et al., 2007; Brown et al., 2005; Pante et al., 2001; Ruzzante et al., 2001), and loss of fitness and behavioural changes induced by domestication (i.e. keeping a strain in hatchery through generations for breeding purposes) (Araki et al., 2008; Kopack et al., 2015; Lorenzen et al., 2012; McClure et al., 2008; Price, 2002; Tatara et al., 2021). In some cases, alterations in interactions among resident species were found (Uusi-Heikkilä et al., 2018).

The marble trout, *Salmo marmoratus* Cuvier 1829, is an iconic salmonid of angling and conservation interest in southern Europe (Freyhof and Kottelat, 2007). It is a sub-endemism within the Adriatic drainages (located in the northern Mediterranean basin), distributed in the orographic left tributaries of the paleo-Po in Northern Italy, Slovenia, Bosnia-Herzegovina, and Montenegro. It is a large salmonid, inhabiting preferentially rivers and subalpine lakes and showing a characteristic marbled colour pattern in adults, which may reach a maximum size of about 120 cm up to 20 kg (Meraner and Gandolfi, 2017).

Marble trout belongs to the highly polymorphic *Salmo trutta* L., 1758 complex, but it is distinguishable from other brown trouts by some key morphological and biological peculiarities as well as by the genetic make-up (Gratton et al., 2014). Marble trout is phylogenetically distinct from other *Salmo* species (e.g., Pustovrh et al., 2011; Pustovrh et al., 2014; Segherloo et al., 2021). This specie represents a monophyletic group that diverged from other brown trout taxa about 1.5 Myr ago (Pustovrh et al., 2014), showing significant population differentiation at the macrogeographic scale in the northern Adriatic basin (Gratton et al., 2014; Pujolar et al., 2011). Moreover, marble trout populations from the northern Adriatic basin (Italy and Slovenia) are fixed for the mitochondrial clade *marmoratus* (MA) (Bernatchez, 2001; Giuffra et al., 1994; Meraner et al., 2007), one of the 5 main *S. trutta* complex lineages with Atlantic (AT), Mediterranean (ME), Danubian (DA) and Adriatic (AD) (Bernatchez et al., 1992). However, the mtDNA MA clade is neither fixed nor private in marble trout throughout its entire distribution range (Meraner and Gandolfi, 2017 (and refs therein); Splendiani et al., 2020). Marble trout populations are threatened by anthropogenic pressures, such as habitat loss, river fragmentation, overfishing, water withdrawal for agriculture and energy production (Rondinini et al., 2022). Ongoing climate changes, with rising spring and summer temperatures, may represent an additional pressure for poorly thermotolerant species (Simčič et al., 2015). Furthermore, the genetic integrity of the marble trout populations is substantially threatened by the hybridization and gene flow from non-native brown trout resulting from fish stocking (Meraner and Gandolfi, 2017; Povz, 1995). Specifically, introgressive hybridization occurs with the non-native Atlantic trout *Salmo trutta* (Giuffra et al., 1996; Meraner et al., 2007, 2010), widely introduced in the Adriatic region at least since the second half of the 19th century (Meraner and Gandolfi, 2017). At present, no pristine marble trout populations have been detected in Italy (Baraldi et al., 2010; Meraner et al., 2007, 2010; Molina, 2019). Due to the remarkable numerical and spatial contraction of marble trout populations this species is listed in Annex II of EU Habitat Directive (92/43/EEC) and it is classified as "critically endangered" in the Italian Red List of vertebrates (Rondinini et al., 2022), and it has been subject to many conservation projects (e.g. LIFE NAT/IT/007268 Salmo Ticino "Conservazione di *Salmo marmoratus* e *Rutilus pigus* nel Fiume Ticino", LIFE15 NAT/IT/000823 IdroLIFE "Conservation and management of Freshwater Fauna of EU interest within the ecological corridors of Verbano Cusio Ossola", "MarmoGen - Genotipizzazione delle trote (genere *Salmo*) nelle acque principali dell'Alto Adige") in the whole northern Italy. Conservation of marble trout is mainly carried on by river defragmentation and/or supportive breeding. Additionally, due to its importance as game fish, marble trout stocking is mostly performed by fishing associations (Polgar et al., 2022) that are committed to do this by institutions and, in some cases, directly by public authorities. The selection of spawners used for artificial reproduction, traditionally based on by phenotypic selection (marbled pattern), does not prevent the presence of hybrids, as shown by molecular analyses (Baraldi et al., 2010; Meraner et al., 2010; Penserini et al., 2010). However, despite the great interest in marble trout conservation, assessment of the real contribution of stocking to wild populations have been rarely investigated so far.

To fill this gap of knowledge, in this work we used the case study of the Toce River, a large subalpine glacial river in Piedmont (North Italy), to investigate the genetic make-up of the marble trout population and assess the contribution of stocked fish (putative marble trout, non-native Atlantic trout and putative Mediterranean trout *Salmo ghigii* Pomini 1940) on the genetic structure of wild stock. Nuclear (*LDH-C1** and 12 microsatellites) and mitochondrial (ca. 1000 bp of the mtDNA control region) genetic markers were here used to: i) investigate the genetic diversity and structure of wild fish caught in the river Toce; ii) assess the rates of introgressive hybridization with non-native salmonid taxa; iii) assess the genetic diversity of reared broodstocks; iv) estimate the contribution of stocked fish to the wild population in terms of genetic diversity.

2. Material and methods

2.1. Study site

The Toce River (Ticino basin) is located in the Italian north-western Alps, province Verbano-Cusio-Ossola, Piedmont region. It is the second larger tributary of Lake Maggiore (length 83.6 km; catchment area ~ 1780 km^2; Regione Piemonte, 2018) (Fig. 1), and is strongly impacted by the presence of several small hydropower plants, altering the waterflow of the river.

2.2. Samples selection and DNA extraction

A total of 213 trout were collected by electrofishing in the Toce catchment (Toce) (8 sampling sites, Fig. 1) and 14 in the Lake Maggiore (Lake). One hundred-eighty-two fish samples were also obtained from four hatcheries belonging to the local fishing associations and used as source for fish stocking in the basin. Specifically, hatchery samples belonged to two reared putative Mediterranean trout stocks (HATghig1, $n = 17$; HATghig2, $n = 50$), a reared Atlantic trout stock (HATtrut, $n = 15$) and a reared putative marble trout (HATmarb, $n = 100$). The latter strain had been maintained in captivity for at least 10 years, without any input from wild fish.

Additionally, 40 trout samples caught in the river Ticino basin, upstream of Lake Maggiore (Swiss) (Molina, 2019) and 14 samples of pure Slovenian marble trout (SLO) (Fumagalli et al., 2002), used as reference for the species, have been analysed (Tables 1 and 2).

DNA was extracted from fish samples using a DNeasy Blood and Tissue Kit (Qiagen, Germany), following manufacturer's instructions, from a piece of caudal fin stored in 96 % alcohol. A total of 463 individuals were analysed.

2.3. Mitochondrial DNA markers

A PCR-RFLP (*Restriction Fragments Length Polymorphisms*) and SSCP (*Single Strand Conformation Polymorphisms*) analysis were performed to screen mitochondrial genetic variability. The mitochondrial control region (D-loop) was amplified using the primers LN20 (5'-ACCACTAGCACCCAAAGCTA-3') (Suárez et al., 2001) and HN20 (5'-GTGTTATGCTTTAGTTAAGC-3') (Bernatchez and Danzmann, 1993). The D-loop amplification was performed in 25 μl reaction volume containing: 1× buffer (with MgCl2 15 mM) (Biotech Rabbit, Berlin, Germany), 0.3 μM of each primer, 0.1 μM dNTPS, 1 U of Taq polymerase (Biotech Rabbit, Berlin, Germany) and 2 μl of genomic DNA. The thermal protocol consisted of an initial

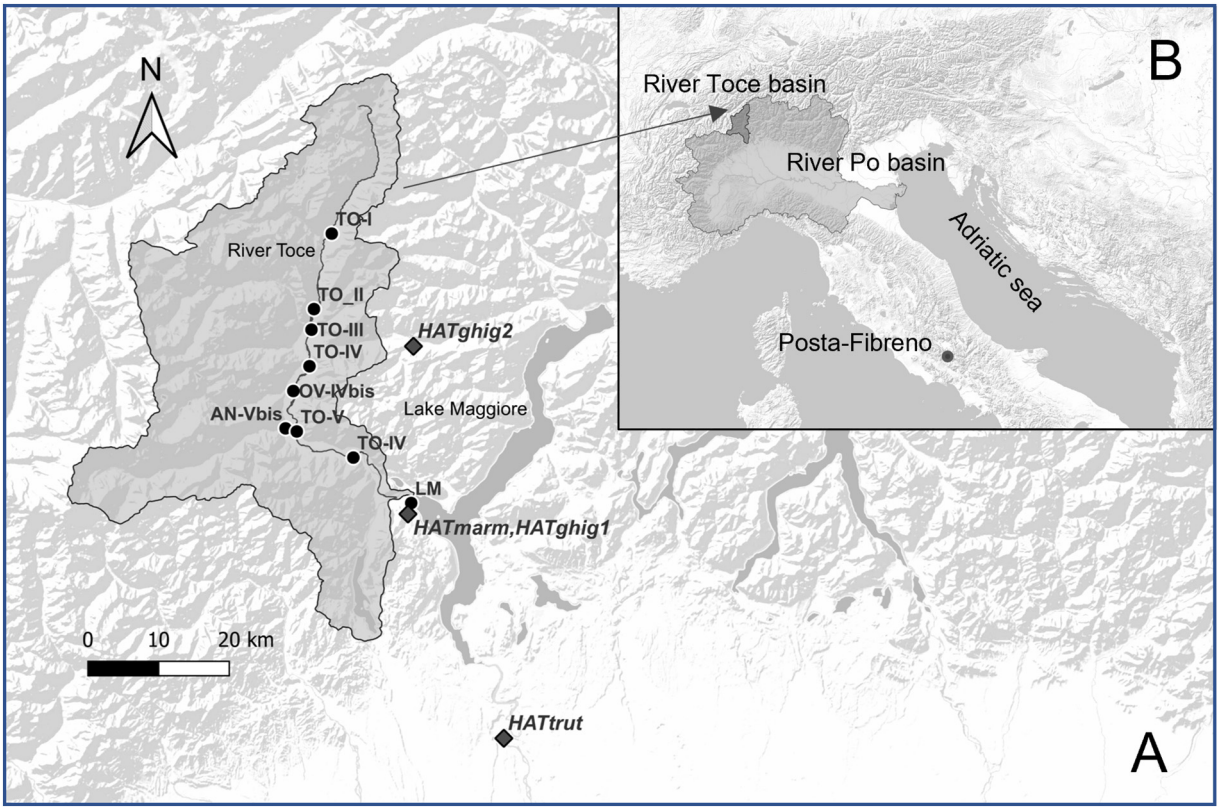

Fig. 1. Map of the sampling sites (black dots) for wild trout populations alongside Toce river and main tributaries in the basin (A). Grey area represents the Toce river basin, LM indicates collection sites of samples caught by professional fishermen in the Lake Maggiore. Grey diamond points indicate the location of the three hatcheries which provided trout samples. In the inset (B) the geographical location of the Toce River basin respect to the main River Po and the Adriatic basins is represented. The location of the Posta-Fibreno basin, which is the native location of one haplotype found in one hatchery, is also indicated.

denaturation step at 95 °C for 1 min; followed by 35 cycles with denaturation at 92 °C for 60 s, annealing at 50 °C for 60 s, and extension at 72 °C for 90s, with a final elongation step at 72 °C for 10 min. Amplicons were digested with *Alu*I (Thermo Fisher, Germany) following manufacturer's protocol. Digestion products were diluted 1:5 with pure water and 5 μl was added to 4 μl loading buffer (98 % formamide, 10 mM, EDTA (0,5 M, pH 8), 0.05 % bromphenol blue, 0.05 % xylene cyanol), heated to 95 °C for 5 min and immediately chilled on ice. A vertical electrophoresis run was performed in a nondenaturing polyacrylamide gel (8 % acrylamide/polyacrylamide [37.5:1], 10 % glycerol), at 250 V for 12 h at room temperature, with 1 × TBE as the running buffer. Sanger sequencing of the entire D-loop (~1 Kbs) was performed on both directions on a subsample for each detected SSCP pattern, using the same primers of amplification. Sequences were manually checked and aligned in BioEdit (Hall, 1999) and the diagnostic sites of the major mitochondrial lineages of the *S. trutta* complex were identified, aiming at assessing the frequency of allochthonous (AT, AD, ME, DA lineages) and native (MA lineage) haplotypes. Haplotypes denomination was defined by applying the same codes already described for other *S. trutta* D-loop sequences available in GenBank. A statistical parsimony network, providing a 95 % plausible set for all haplotype linkages, was constructed using the software TCS v. 1.21 (Clement et al., 2000), using default settings. Levels of genetic introgression for each sample were estimated by calculating the percentage of allochthonous haplotypes.

2.4. Microsatellites

Twelve non-coding microsatellite loci (di- and tetra-nucleotide repeats) were labelled with fluorescent dyes and genotyped in multiplex reactions, as reported in Table SM1. Three PCR reactions were performed, each was carried out in 15 μl containing 1× Qiagen Multiplex PCR Master Mix

Table 1
MtDNA lineages and haplotypes frequencies for whole dataset. Toce sample include all sub-populations reported in Fig. 1, which did not resulted significantly divergent each other.

Sample	MtDNA lineage			MtDNA haplotype													
	AD	AT	MA	AD-porh1	AD-Tyrrh1	AD-Posta Fibreno	Hap.1	Hap.2	Hap.3	Hap.4	A10	Strutta 1DupB	ATcs54	Ma1a	Masl1	Ma2b	Ma2c
SLO[a]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
HATmarb		33.3	66.7							33.3				3.4	1.2	62.1	
HATghig1	15.4	61.5	23.1	15.4						38.4	23.1					23.1	
HATghig2	24.4	66.7	8.9	8.9	8.9	6.7		8.9		4.4	53.3					8.9	
HATtrut		81.8	18.2				9.1			72.7						18.2	
Swiss[b]		66.7	33.3					47.6		4.8		14.3				33.3	
Toce	0.6	23.3	76.1	0.6				8.7	3.3	10.1	0.6		0.6	26.1	12.7	36.7	0.6
Lake	8.3	50.0	41.7	8.3				8.3	33.4		8.3			16.7	8.3	16.7	

[a] Sample from Fumagalli et al., 2002
[b] Samples from Molina, 2019

Table 2
Descriptive statistics of genetic diversity based on 12 nuclear microsatellite loci for whole dataset.

Sample	River/hatchery	N	H_{exp}	H_{nb}	H_{obs}	F_{IS}	Sign.	NA mean	AR mean
SLO	Soca[a]	14	0.448 (0.36.2)	0.467 (0.378)	0.289 (0.253)	0.392	*	4.083	3.610
HATmarb	*S.marmoratus* hatchery	96	0.671 (0.239)	0.674 (0.240)	0.630 (0.250)	0.067	*	9.000	5.356
HATghig1	*S.ghigii* hatchery1	17	0.674 (0.208)	0.694 (0.214)	0.662 (0.292)	0.048	NS	6.417	5.170
HATghig2	*S.ghigii* hatchery2	50	0.690 (0.255)	0.697 (0.257)	0.637 (0.259)	0.086	*	9.667	5.693
HAT trut	*S.trutta* hatchery	15	0.699 (0.185)	0.724 (0.192)	0.709 (0.243)	0.02	NS	7.833	5.997
Swiss	Ticino basin[b]	38	0.780 (0.166)	0.791 (0.168)	0.715 (0.202)	0.097	*	11.917	6.888
Toce	Toce	158	0.763 (0.188)	0.766 (0.189)	0.678 (0.216)	0.115	*	16.000	7.121
Lake	Lake Maggiore	14	0.763 (0.162)	0.791 (0.168)	0.618 (0.250)	0.226	*	8.917	7.099

n = sample size, H_{exp} = expected heterozygosity, H_{nb} = unbiased expected heterozygosity, H_{obs} = observed heterozygosity, F_{IS} = inbreeding coefficient, NA = mean number of alleles, AR = allelic richness, Sign * = significant F_{is} (indicative adjusted nominal level (5 %) is: 0.00052).
[a] Sample from Fumagalli et al., 2002
[b] Samples from Molina, 2019

(Qiagen, Germany), 0.2 μM of each primer and 2 μl of genomic DNA. The PCR thermal protocol consisted of an initial activation step at 95 °C for 15 min; then 30 cycles with denaturation at 94 °C for 30s, annealing specific for each multiplex (see Table SM1) for 90s, and extension at 72 °C for 60s, with a final elongation step at 60 °C for 30 min. The PCR products were sent to Macrogen Europe for genotyping.

We used PeakScanner v.2 (Thermo Fisher Scientific) to score and FlexiBin (Amos et al., 2007) to bin microsatellite allele sizes. The presence of null alleles and potential scoring errors was investigated both using Microchecker 2.2.3 (Van Oosterhout et al., 2003) using the default setting, and ML-Null freq (Kalinowski and Taper, 2006) software, to reduce the potential detection of false negatives (Dąbrowski et al., 2015). Moreover, the FreeNA software (Chapuis and Estoup, 2006) was used to investigate if null alleles affected the genetic differentiation estimates. The bootstrap 95 % confidence intervals (CI) for the global F_{ST} values were calculated using 1000 replicates over all loci.

Genetic differentiation among samples was investigated firstly by estimating pairwise F_{ST} using FSTAT (Goudet, 2001). Because no significant differentiation resulted between samples within the Toce River, we considered it as a single group. Then, the genetic structure was investigated with an exploratory Principal Component Analysis (PCA) carried out using the *adegenet* R package (Jombart, 2008) and with the Bayesian model-based clustering implemented in the software STRUCTURE 2.3.4 (Pritchard et al., 2000). The analyses were based on 20 serial runs for each number of clusters (K) between 1 and 9. The Admixture model and correlated allele frequencies model were set. All analyses were run for 6×10^5 generations after a burn-in of 1×10^5. The correct number *k* was individuated using both the Evanno method (delta K) (Evanno et al., 2005) and the four statistics: MedMeaK, MaxMeaK, MedMedK, and MaxMedK. These last statistics were estimated by the web software StuctureSelector (https://lmme.ac.cn/StructureSelector/) (Li and Liu, 2018). We assessed the outputs changing thresholds (0.5 and 0.7) to decrease the chances of detecting spurious clusters. Furthermore, two more cluster analyses were performed using the same settings on restricted datasets. In the first case, to simplify the analysis and obtain a clearer output, we excluded Swiss and HATghig1 samples and all hybrid individuals showing an intermediate assignment value ($0.1 > q < 0.9$) for K = 2 from the previous Structure analysis. The second was conducted on marble trout only ($q \geq 0.9$) from SLO, Toce, Lake and HATmarb samples.

Genetic diversity was assessed for each population considering both the entire dataset and a restricted dataset (obtained excluding hybrid individuals ($0.1 > q < 0.9$) detected by STRUCTURE for K = 2). Expected heterozygosity (H_e), unbiased expected heterozygosity (H_{nb}, Nei, 1978), and observed heterozygosity (H_{obs}) and the mean number of alleles by locus (NA) were calculated using GENETIX version 4.05 (Belkhir et al., 1996). We also calculated allelic richness (AR) and the deviation from HW equilibrium (F_{IS}) with FSTAT.

Current migration rates (*m*) and direction of gene flow were estimated for samples using the Bayesian approach, based on individual multi-locus genotypes and Markov chain Monte Carlo techniques, as implemented in BayesASS 3.0.4 (Wilson and Rannala, 2003). We performed 10 independent runs with different random seed values setting 10,000,000 Markov chain Monte Carlo iterations after a burn-in of 2,000,000, sampling the chain every 100 iterations. The parameters delta M (mixing parameter for migration rate), delta A (mixing parameter for allele frequencies) and delta F (mixing parameter for inbreeding coefficients) were adjusted respectively to 0.20, 0.50 and 0.60 to change the acceptance rate fitting, following the software manual instructions. We excluded the less informative samples HATghig1 and Swiss from the analysis, given the analysis might be affected by the number of populations and individuals (Meirmans, 2014). The MCMC convergence and the consistency of the estimates between independent runs were examined in TRACER v 1.7.1 (Rambaut et al., 2018). The Bayesian Deviance Criterion (DIC) (Spiegelhalter et al., 1998) estimated in R following Meirmans (2014) was used to find the run that provided the lower Bayesian deviance value, indicating the best fit of the data to the model (Faubet et al., 2007). Rough 95 % confidence intervals (CIs) were estimated as migration rates mean ± 1.96 × SD, and values not including zero were considered significant. Sib-ship relationships, within and between reared (HATmarb) and Toce samples, were investigated using the maximum likelihood method. Using the software COLONY 2.0.6.1 (Jones and Wang, 2010) we calculated the probability that any two sampled individuals are either full or half-siblings. We performed three runs to confirm the reliability of the results. Program settings were: allelic dropout rate = 0.0000, other error rate = 0.0001, polygamous mating system, with inbreeding, no priors for sibship assignments, long length runs and high likelihood precision.

2.5. LDH genotyping

Closed reproductive cycles and multiple generations of backcrossing, as may happen in hatcheries, can affect the result of genetic structure analysis by masking potential hybrids (Meraner et al., 2008; Meraner and Gandolfi, 2018). Therefore, restricted to the HATmarb samples, a PCR-RFLP of a 440 bp segment of the protein-coding locus LDH-C1*, was performed using restriction enzyme *BseLI* (Thermo Scientific™) following McMeel et al. (2001). The LDH-C1* locus is biallelic and is a useful marker to detect introgression of the Atlantic trout lineage in trout populations of different origins. In northern Europe and most of the farm stocks the allele *90 predominates, whereas the allele *100 is fixed in many wild populations from the Mediterranean area. In the present study, levels of genetic introgressive hybridization were estimated by calculating the percentage of heterozygotes and homozygotes for the allochthonous allele (LDH-C1*90).

3. Results

3.1. Mitochondrial marker (Dloop)

Overall, the SSCP analysis and Sanger sequencing identified 14 haplotypes (Table 1). Reference samples from Slovenia (SLO) had already been assigned to MA lineage by a previous study (Snoj et al., 2000). The observed

haplotypes belonged to three main mtDNA lineages: MA, AD and AT (sensu Bernatchez, 2001; Bernatchez et al., 1992). The MA lineage was represented by four haplotypes already described in literature: Ma2b (DQ841190), Ma1a (DQ841191) and Ma2c (JQ582461) (Meraner et al., 2007, 2013), and MAsl1 (MK948036; Splendiani et al., 2020). Considering the AD lineage, three already described haplotypes were observed: ADporh1 (MK948034; Splendiani et al., 2020) native from the southwestern Alps, AD-PostaFibreno (JQ314219; Gratton et al., 2013) endemic from the Posta-Fibreno basin in central Italy and AD-tyrrh1 (KX450257; Berrebi et al., 2019), widely distributed along the Italian Tyrrhenian side rivers, Corsica and Sardinian islands. Finally, the AT lineage was represented by seven haplotypes, four of them were already detected in Mediterranean rivers and classified as haplotypes of hatchery origin: haplotype 1 (AF273086), haplotype 2 (AF273087), haplotype 3 (AF274574) and haplotype 4 (AF274575) (Cortey and García-Marín, 2002); A10 (HQ848361; Kohout et al., 2012) recorded in central Europe in Medvědí and Celní streams (Danubian basin) (Kohout et al., 2012) and in the Warm Bode river (Elbe Bain) (Schmidt et al., 2015). Two haplotypes were described for the first time in this study, that we named Strutta1DupB (GenBank Accession No OQ544590) and ATcs54 (GenBank Accession No OQ544591). Strutta1DupB showing an 82 bp perfect tandem repeat located in the 3′ end of the CR and is highly similar, except for one base mutation, with the Strutta1Dup (EF530536) detected in Spain (Wetjen et al., 2017). Both these haplotypes revealed the haplotype 1 (AF273086; Cortey and García-Marín, 2002) excluding the tandem repeat structures. Haplotype ATcs54, instead, differs from haplotype ATcs53 (MK330940; Berrebi et al., 2020) by a single base mutation and from ATcs49 (EF530509, Cortey et al., 2009) by two base mutations. The ATcs53 was observed only in Chuzenji hatchery, Jigoku stream and in the upper part of the Azusa river in Japan (Berrebi et al., 2020), while the ATcs49 was detected in the Coquet river, British Isles (Cortey et al., 2009) and does not belong to the contemporary hatchery haplogroup. Presence of ATcs53 haplotype in Japanese strain has been related to the importation of trout eggs from Scotland and England at the end of the 19th century (Berrebi et al., 2020).

The MA and AT lineage has been detected in all samples (Fig. SM 1). The MA lineage dominates in the Toce River (76.1 %) and marble hatchery sample (HATmarb, 66.7 %). The most common MA haplotype was Ma2b (Toce 36.7 %; HATmarb 62.7 %; Lake 16.7 %). Ma1a and MAsl1 were detected at quite lower frequencies within the Toce (26.1 % and 12.78 %, respectively) and Lake samples (16.7 % and 8.3 %), while they were detected at minimal frequency within HATmarb (3.5 % and 1.2 %) sample. Finally, Ma2c was detected just in one specimen from Toce.

On the contrary, the AT non-native haplotypes showed an evident high abundance within both Mediterranean trout hatchery (HATghig1 61.5 %, HATghig2 66.7 %), HATtrut (81.8 %), Swiss (66.7 %) and Lake (50 %) samples (Table 1). The haplotype 4 was present in all samples except for Lake and dominant within the HATtrut sample (72.7 %). The haplotype3 dominated in the Lake (33.3 %), while haplotype 2 in Swiss and A10 haplotype in HATghig2 (53.3 %), respectively. This last haplotype was also detected within HATghig1 (23.1 %) and lake samples (8.3 %). The new Strutta1DupB haplotype was detected only in the Swiss sample.

Finally, the AD lineage, contrary to expectations, was detected in just few specimens, all within the two *S. ghigii* hatchery samples (HATghig1 15.4 % and HATghig2 24.4 %). All three haplotypes were detected within the HATghig2 at similar frequencies, while only the ADporh1 was detected in HATghig1. This latter haplotype was recorded for the first time limited to two individuals within the Toce River and one in the Lake Maggiore, attributable to stocking activities.

3.2. Nuclear markers (microsatellites and LDH genotyping)

After removing individuals showing >20 % missing data, the dataset contained a total of 402 individuals, which were genotyped using 12 polymorphic microsatellite markers (Table 2).

The number of alleles per locus ranged from 5 (*Str60*) to 40 (*OMM1064*). Comparison between MICRO-CHECKER and ML-Null results detected null allele in 33 tests over 192. The number of loci showing null alleles per site ranged from 0 (sampling sites AN-Vbis, and OV-IVbis in the Toce sample) to 6 (SLO). Loci *Ssa410UOS* and *SsoSL438* were affected by null alleles in seven samples, loci *SSaD190* and *Ssa408UOS* did not show null alleles, while the rest of the loci showed null alleles in at least two samples. However, the occasional presence of null alleles in this study should not impact the population genetic differentiation estimates, as global F_{ST} values, including and excluding the ENA correction method, gave comparable results, 0.096 (CI 0.067–0.134) and 0.094 (CI 0.064–0.131) respectively.

Finally, the PCR-RFLP analysis of locus LDH*C1 revealed a high hybridization rate within the marble hatchery sample. Out of 96 reared marble trout individuals, 51.6 % showed homozygosis for the native allele (*100/*100), 4.3 % were homozygotes (*90/*90) for the non-native allele, while 44.1 % of individuals were heterozygotes (*90/*100).

3.3. Genetic structure

Generally, the microsatellite data recovered a significant genetic structure between marble and non-native trouts. Pairwise F_{ST} differentiation indexes detected no significant genetic differentiation among Toce sites (Table SM 2); thus, all the Toce River has been considered as a single group. All pairwise F_{ST} values were highly significant, except for one comparison (HATtrut vs Lake) (Table 3). The Slovenian marble trout sample (SLO) was the most divergent population, compared to both the rest of the wild and the hatchery samples, showing a mean F_{ST} value of 0.244. These pure marble trout showed the lowest divergence when compared to the HATmarb (F_{ST} = 0.138) and Toce (F_{ST} = 0.130). HATmarb and Toce were on average moderately divergent from the other hatchery populations, (0.153–0.205 and 0.086–0.124, respectively). Toce population showed the lowest differentiation with HATmarb (0.034). Finally, differentiation was generally low for comparisons among HATtrut, both HATghig samples, Swiss, and Lake samples (0.015–0.087).

The PCA (Fig. 2) first and second principal components represented 15.4 % and 3.6 % of the total variation, respectively, distinguishing two principal genetic clusters, which correspond to marble (SLO) and non-

Table 3
Pairwise F_{ST} based on 12 microsatellite loci between 4 wild trout samples (Toce samples were grouped) and 4 domestic trout samples (below diagonal); significance (*) was obtained after 560 permutations (above diagonal), Indicative adjusted nominal level (5 %) for multiple comparisons is 0.001786.

	Hatcheries				Wild			
	HATmarb	HATghig1	HATghig2	HATtrut	Swiss	Toce	Lake	SLO
HATmarb		*	*	*	*	*	*	*
HATghig1	0.153		*	*	*	*	*	*
HATghig2	0.185	0.042		*	*	*	*	*
HATtrut	0.205	0.087	0.066		*	*	NS	*
Swiss	0.137	0.056	0.049	0.038		*	*	*
Toce	0.034	0.086	0.118	0.124	0.073		*	*
LAKE	0.076	0.056	0.053	0.047	0.015	0.027		*
SLO	0.138	0.299	0.319	0.348	0.260	0.130	0.217	

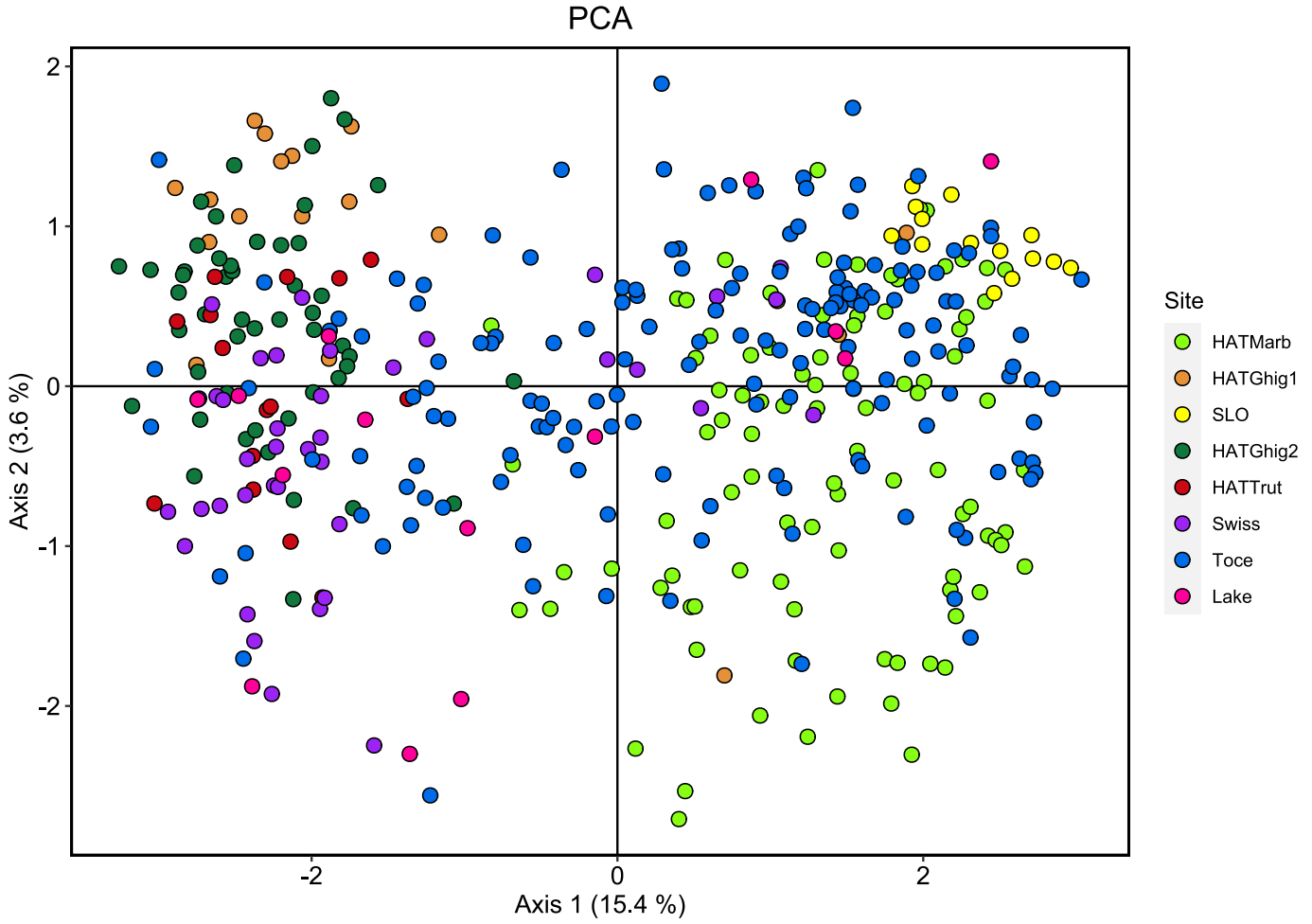

Fig. 2. Principal component analysis (PCA) of nuclear microsatellite diversity among the 402 trout individuals. Dots represent single individuals, coloured according to the sampling site.

native (HATtrut, HATghig1 and HATghig2) samples. Among these groups, there is a continuous range of variation and no clear limits consisting of hybrid individuals, principally represented by Toce, Swiss, Lake and HATmarb samples. The STRUCTURE clusters corroborated the PCA result (Fig. 3). When the uppermost structure (K = 2, 20 runs out of 20), detected by DeltaK (Fig. 3A), was investigated, a clear division between marble and non-native lineage was found (Fig. 3C), with introgressive hybridization (0.1>q< 0.90), affecting particularly wild samples. SLO and HATmarb showed the dominance of individuals assigned to the first group showing a q value ≥0.9, 100 and 91.7 %, respectively. Just 8 individuals from the HATmarb sample showed q values lower than 0.90 (range 0.615–0.897). Contrarily, the larger part of trout from HATtrut (100 %), HATghig1 (82.4 %), HATghig2 (94 %), Swiss (76.3 %) and Lake (57.1 %) samples were assigned to the second group (q ≤ 0.1). A percentage of 14.3 % (Lake), 17.6 % (HATghig1) and 7.9 % (Swiss) were assigned to the first group and the rest were identified as hybrids among the two groups, showing intermediate assignment values. Most individuals (57 %) were identified as hybrids in Toce sample, 34.2 % were assigned to the first group and 8.9 % were assigned to the second one. The threshold applied (q ≥ 0.9) to define 'purebred' individuals was based on Slovenian purebred reference. However, it can overestimate purebred detection under several generations' scenarios, including backcrossing (Meraner et al., 2008; Meraner and Gandolfi, 2018). Misclassification of hybrids as purebreds, under this threshold, is evidenced by the occurrence of three specimens with a brown trout phenotype and four showing AT MtDNA lineage, both assigned to the marble trout group.

When the fine structure was investigated, using MedMeaK, MaxMeaK, MedMedK, and MaxMedK, the best number of groups were identified as K = 6 (threshold = 0.5) (Fig. 3B) and K = 5 (threshold = 0.7). In both structures, the non-native group detected for K = 2 was split in two: reared Mediterranean samples (HATghig1, HATghig2) split from the Atlantic one (HATtrut). Within the marble cluster, three or four clusters were detected. Two groups appeared, in both structures, within the HATmarb sample. For K = 5 wild marble trout (Toce, Swiss and Lake) were gathered with the SLO sample, while for K = 6 a new cluster was observed grouping hybrid wild individuals (Toce, Swiss and Lake). The main mode for K = 5 was supported by only 8 runs out of 20 while the main mode for K = 6 was observed in 14 runs out of 20. Then we selected the latter as the best structure. Generally, wild marble trout were grouped in the same group, with just two individuals caught in Toce assigned to the HATmarb hatchery. The Slovenian sample was identified as an isolated group for K = 7 (Fig. 3C).

Cluster analysis on restricted datasets, excluding hybrid individuals, corroborated the previous results and made it possible to better identify the genetic structure within the marble group (Fig. SM1). The first analysis detected the uppermost structure for K = 2 (Delta K, 20 runs out of 20), corroborating the previous result, and fine structure for K = 5 (other statistics, threshold = 0.5) and K = 4 (other statistics, threshold = 0.7). As before, we selected K = 5 because all 20 runs supported the same structure. For K = 5 non-native samples (HATtrut and HATghig2) clustered in a single group, while within the marble trout 4 clusters were detected. Specifically, SLO represented the first group, the second gathered the wild marble trout from Toce and Lake and two groups (third and fourth) were detected within

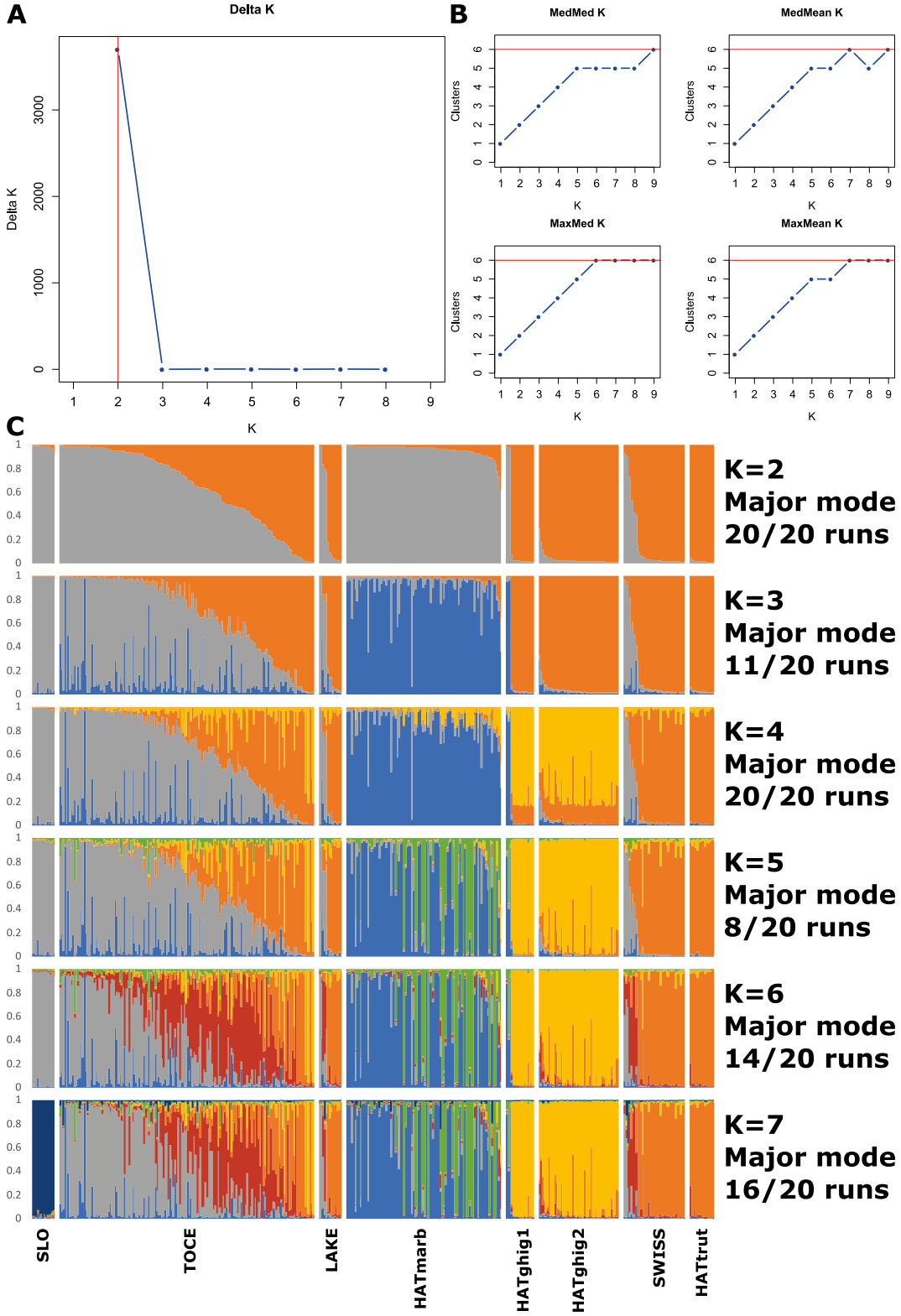

Fig. 3. STRUCTURE assignment plot based on whole dataset and 12 microsatellite loci. (A) delta K plot. (B) MedMeaK, MaxMeaK, MedMedK, and MaxMedK plot. (C) STRUCTURE barplot for K 2–7 indicating the genetic composition of all samples in the current study. Colours indicate the relative contribution of each genetic cluster recovered from the data for each individual (column) in each sampled population.

the HATmarb. Only three specimens from Toce were grouped within the third group with HATmarb. This structure was corroborated by the cluster analysis using only marble trout, where both deltaK and other statistics identified K = 4 (20 runs out of 20) as the best number of groups, reproducing the same structure just described.

The observed pattern of genetic introgression, based on both mitochondrial and nuclear markers, revealed that complete panmixia is not reached, although hybrids represent the largest portion (61.4 %) compared to marble (31 %, MA MtDNA lineage and $q \geq 0.9$) and Atlantic (7.6 %; AT MtDNA lineage and $q \leq 0.1$) ones.

Population genetic variability parameters are summarized in Table 2. Comparison between the entire dataset and restricted dataset estimation suggested that genetic variability was correlated with the introgression levels. The highest genetic diversities, based on the entire dataset, were observed in the wild locations with the highest nuclear introgression (Toce, Lake and Swiss). Lower diversity, in terms of unbiased expected heterozygosity (H_{nb}) and allelic richness (AR) was observed in the SLO sample (H_{nb} = 0.47 and AR = 3.61). Hatchery populations showed intermediate values ($0.67<H_{nb}<0.72$; $5.17<AR<6.0$). In the Toce River, excluding hybrid and non-native individuals, heterozygosity (H_{nb}) decreased from 0.77 to 0.63 and AR from 7.12 to 5.74 (Table SM3).

High levels of self-recruitment (97.7 % – 85.9 %) were detected in five populations but Lake (68.3 %). No contemporary migration (95 % CI overlapping with zero) was detected in most, except in five, comparisons (Fig. 4). In these cases, migration rates were asymmetric and directed to Toce and Lake populations. Specifically, *m* values were relatively high from HATtrut to Toce (0.091 ± 0.026) and Lake (0.157 ± 0.073) and from Toce to Lake (0.099 ± 0.066) while were very low from HATmarb to Toce (0.024 ± 0.018) and from HATghig2 to Toce (0.022 ± 0.015).

Among sib-ship pairwise estimation within HATmarb 1.23 % were fullsibs against 0.09% in wild sample (Toce and Lake). No full-sibs and 0.36 % halfsib were shared between hatchery and wild sample. Fourty-nine full-sibship relationships were estimated within HATmarb sample (1.07%) ($p > 0.5$) and all individuals identified as full-sibs were assigned to one of the same genetic clusters found within the hatchery sample. Only 7 full-sibship relationships ($p \geq 0.5$) were detected among wild individuals from Toce and Lake together (0.05 %). At the same time, more half-sibs (4.32 %) were detected within HATmarb (2.37 %, $p > 0.5$) against 1.77 % in wild sample (1.14 %, $p > 0.5$).

4. Discussion

4.1. Extensive hybridization in marble trout of the Toce River

This study represents the first detailed genetic investigation on the trout population inhabiting Toce River. Recently, a study detected a high degree of non-native introgression in a very limited number of phenotypically selected wild individuals (Gibertoni et al., 2014). Marble trout was historically present along the whole Ticino basin, but it is currently ascertained limited to the Toce River and its main tributaries and the Ticino river downstream of Lake Maggiore (Turin et al., 2006). The absence of marble trout populations in the Ticino basin upstream of the lake, except for little traces in the Melezza Occidentale River, was corroborated by molecular analysis (Molina, 2019). Information on Toce's marble trout population genetic make-up remained very scarce until now. For instance, some marble trout samples from Toce River have been used just as a reference on a brown trout survey in north Italy (Giuffra et al., 1994, 1996; Molina, 2019). The observed pattern of genetic introgression, based on both mitochondrial and nuclear markers, revealed that complete panmixia is not reached, although hybrids represent the largest portion. This hybridization pattern has been usually described in northern Italian basins, where the conservation of the marble trout population is critically threatened by a high rate of non-native introgressive hybridisation. Genetic structure analysis excludes the presence of marble trout parental individuals as the result of ongoing yearly stocking activities, clustering wild and hatchery samples in two different genetic groups.

In-stream experiments did not provide evidence of a reproductive barrier between marble and other trout (e.g Atlantic trout such as *S. trutta*) (Meldgaard et al., 2007), while the absence of complete replacement of native with hybrid population has been already observed in other north Italy basins (Adige River, (Meraner et al., 2010). This pattern has been justified

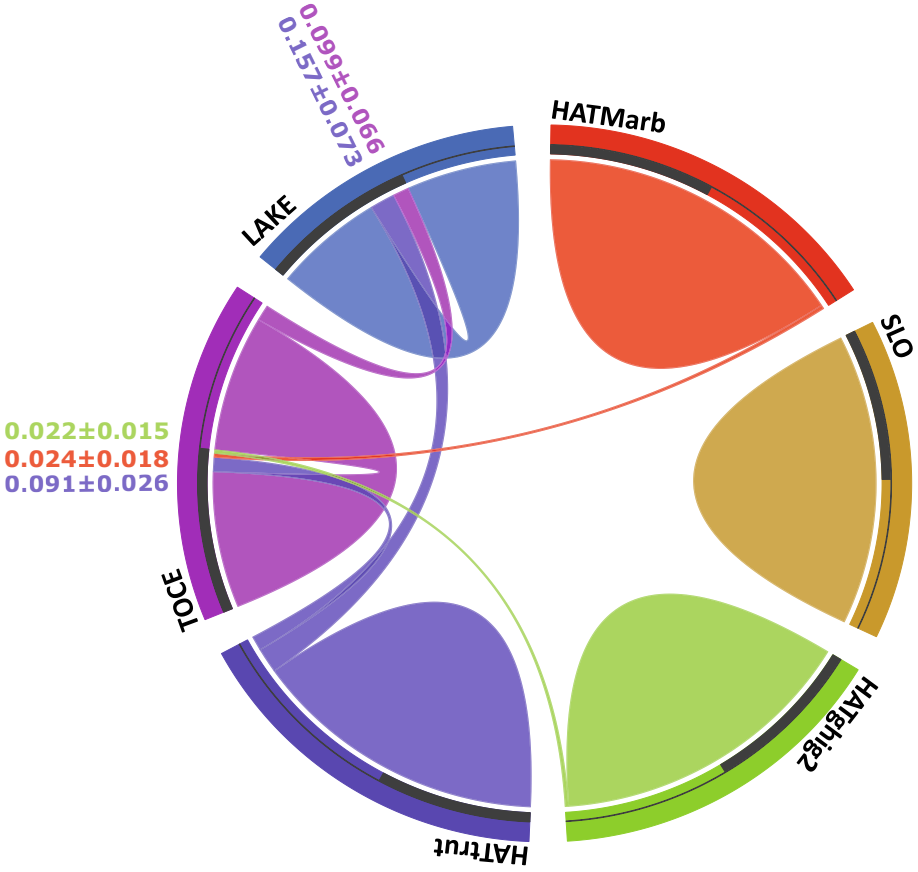

Fig. 4. Migration rates between the Toce River (TOCE), Lake Maggiore (LAKE), hatcheries (HATtrut, HATMarb, HATghig2) and reference Slovenian trout populations (SLO), as estimated by BayesASS 3.0.4. Values whose 95 % CI did not overlap the zero are reported.

by the marginally overlapping spawning period (end of November) between marble and brown trout. The same pattern has been advocated even within admixed Apennine trout populations, whose reproductive isolation, caused by timing mismatch coupled with spatial isolation, partially prevents introgressive hybridization among native and Atlantic lineages (Splendiani et al., 2019). However, the observed individual assignment probabilities (*q*-values) distribution reveals a complex pattern of ongoing hybridization and backcrossing. The finest structure that gathers hybrid individuals, in a separate cluster, revealed a hybrid cohort (post-F1 hybrids population), suggesting interbreeding between hybrid and backcrossing between its parent populations. However, we cannot exclude that the identified cluster could be spurious (Li and Liu, 2018). It could also be an allochthonous trace of origin different from the reference used, due to the presence of non-native haplotypes unshared with hatchery strains. Observed backcrossing occurs bidirectionally, both towards non-native and marble trout. Conversely to previous detection (Meraner et al., 2010), the presence of specimens highly assigned to the wild marble cluster, carrying AT lineage, revealed that interbreeding between hybrids and native marble trout occurred in Toce River. A complex interplay of hybridization and backcrosses are acting on the Toce River marble trout, which may be susceptible to future shifts towards a complete loss of native genotypes. A series of factors, increasing the chance of complete loss of native genotypes can be listed, which may be of concern in the studied river basin in the near future. For instance, loss of environmental heterogeneity and habitat anthropization has been found to cause the relaxation of reproductive isolating mechanisms (Hasselman et al., 2014; Seehausen et al., 2008). These kinds of perturbations are widely spread along the Toce River and may determine fitness advantages for hybrid cohorts, as demonstrated in highly changing and altered ecosystems (Hasselman et al., 2014). Climatic and water-flow instability had been also related to the introgressive hybridization spreading in native trout populations (Almodóvar et al., 2006; Splendiani et al., 2016; Vera et al., 2018, 2023) and may become a key factor, increasing hybridization rates.

Native marble trout individuals still inhabit the Toce River at present. The most diffuse MA haplotype was the indigenous Ma2b, observed elsewhere within the Po River drainages (Meraner et al., 2007, 2013). Moreover, the presence of the Masl1 haplotype, also found recently in a specimen from a Southwestern alpine trout population (Stura di Lanzo River; (Splendiani et al., 2020)), may represent a relict haplotype restricted in the western portion of the marble trout distribution range. Ma1a was already reported in the Toce basin by Giuffra et al. (1994) (MA1) and in the upper Adige River system (Meraner et al., 2007). Being this haplotype fixed in the Slovenian population (Meraner et al., 2007) is not still clear if its presence in Northern Italy is either a relict of a previously wider distribution or the result of stocking with Slovenian marble trout (Meraner et al., 2007). The wild marble trout from Toce River forms a clearly differentiated genetic cluster, related to the pure marble trout reference from Slovenia, confirming the presence of substructure between marble trout populations in the whole distribution area (Gratton et al., 2014; Pujolar et al., 2011).

The AD lineage within Toce River is described here for the first time. However, it was previously found in a museum lacustrine specimen from Lake Maggiore, dated 1879 (Splendiani et al., 2017) and ascribed to ADcs1 haplotype. Our specimens, however, showed AD-PostaFibreno haplotype, endemic from the Posta-Fibreno Lake and its tributary in central Italy, whose presence is attributable to recent stocking using reared Mediterranean trout. In support of our findings, the presence of single trout carrying the A10 and AD-PostaFibreno haplotypes was detected in a Mediterranean trout hatchery strain investigated. Mediterranean trout is non-native in the Toce basin, being marble trout the only native trout of the Italian Alpine region (Meraner et al., 2013; Polgar et al., 2022); except for some south-western tributaries of the Po River, where native brown trout populations have been recently described (Splendiani et al., 2020). Sporadic observations of mitochondrial Adriatic haplotypes have been traced back to stocking actions in the Lake Maggiore basin (Gandolfi, personal communication), as well as the presence of a structured population of Mediterranean trout in the Frua stream (Toce tributary) at 2000 m a.s.l. Such trouts were translocated from the Turin province (Gibertoni, 2018). Our detection of AD lineage only in hybrid individuals highlights the potential role of the hybrid swarm in favouring introgressive hybridization.

4.2. Introgressive hybridization within marble and brown trout hatchery strains

Several genetic investigations have demonstrated that hatchery trout stocks, in the Mediterranean area, usually represent taxa that are different from the native ones, mostly being originated from central Europe stocks. Frequently, when the stocked strain corresponds with the native one, they represent hybrid stocks with non-native traits (Penserini et al., 2010; Splendiani et al., 2019). Mitochondrial lineage distribution revealed that all hatchery broodstocks investigated here were affected by introgression, and that the AT lineage was detected in all of them. The introduction of allochthonous traits within the marble trout hatchery is due to the phenotypic selection of starting spawners, a condition which do not warrant selection of pure marble trout individuals (Meraner et al., 2010; Meraner and Gandolfi, 2017; Penserini et al., 2010). The reared marble trout stock analysed in this work makes no exception. The observed haplotype frequencies within the marble hatchery strain, coupled with its little differentiation (F_{ST}) with the wild marble sample suggest its local origin. However, the presence of non-native haplotypes and the LDH-C1*90 allele identified it as a hybrid population; and just 32.3 % of specimens show only native traits ($q \geq 0.9$, mtDNA MA and LDH-C1 100/100). On the contrary, high microsatellites assignment values ($q \geq 0.90$) to the marble cluster were found for most individuals when $K = 2$. The apparent failure of assignment analysis to detect introgression, based solely on microsatellite markers, is related to the hatcheries' closed breeding system, backcrossing and sibship among reared individuals. In presence of backcrossed specimens, this approach carries the risk of misclassifying post-F1-hybrids as purebreds (Meraner et al., 2008; Vähä and Primmer, 2006). A simulation performed by Meraner and Gandolfi (2018) showed that backcrossed breeding populations have comparable q-values with the initial population, after just three generations, although all individuals should be classified as hybrids, due to their pedigree.

Marble trout broodstock, moreover, resulted poorly but significantly differentiated from wild stock, representing a separate genetic group, and showed fixation for the Ma2b mtDNA haplotype. Many half and full-sib relationships were detected by parentage analysis, compared to the wild samples. The family effect also explains the presence of two clusters within this stock, identifying members assigned to the same genetic group as half- and full-sibs. The loss of genetic variability in the hatchery stock and its differentiation is attributable to small effective genetic size, linked to the founding event and subsequent genetic drift, adding unintended selection during hatchery closed breeding procedures (Aho et al., 2006; Hansen and Jensen, 2005; Vuorinen, 1984). Therefore, the current hatcheries' marble trout stock is not suitable for supportive breeding programs in Toce River, due to the risk of threatening the genetic pool integrity in the wild population.

The two Mediterranean trout strains, that are used for stocking by fishing associations and are, misleadingly, considered native, are dominated by the non-indigenous AT haplotypic variant (A10) native to central Europe (Kohout et al., 2012; Schmidt et al., 2015) and that was never described in Mediterranean populations. Moreover, the mixed origin of these stocks has been revealed by the presence of two AD haplotypes. The AD-PostaFibreno haplotype is endemic from the Posta-Fibreno lake and their tributary in Central Italy and AD-tyrrh1 is widely distributed along the Italian Tyrrhenian side rivers, Corsica and Sardinian islands (Berrebi et al., 2019) but both were never detected before and are non-native in the Adriatic side and Po basin. Our work is the first to detect its presence in northern Italy.

4.3. Limited contribution of the hatchery trout to wild trout population

The fate of stocked fish is a critical aspect for stocking practices. Our genetic analysis on wild and domestic trout clearly identified the origin of fish

collected in the wild and show a negligible contribution of domestic trout in the wild sample, despite ongoing yearly massive stocking activities. In 2020, ~ 799,500 domesticated marble trout and ~ 1,202,155 non-native brown trout were stocked in the VCO province. The finest genetic structure revealed that just over 10% of individuals from Toce samples had a hatchery origin (1.3 % HATmarb, 7.6 % HATtrut and 1.9 % HATghig). The remaining 89.2 % was represented by hybrids and wild marble specimens, suggesting that natural recruitment represents the greatest source of this wild population sustainment, as suggested by the relatively low recent migration rates detected from hatcheries towards the Toce River. Very limited presence of hatchery origin fish has been observed in a recent study investigating regular stocking contribution to the Shetland sea trout (*S. trutta)* population (King et al., 2021).

The limited presence of domestic trout in the wild may be an effect of a selective fishing harvest or reduced survival of domestic trout in the wild. Current regional angling regulation allows to capture up to 8 non-native (brown) trout over 22 cm long and 2 marble trout or its hybrids over 35 cm, daily, per person. For marble trout, there is a limit of 10 fish per year per person (Regione Piemonte, 2012) This regulation aims to protect marble trout and its hybrids. Consequently, while angling could have a role in reducing non-native trout abundance in the Toce, it may not hold for domestic marble trout, given the more restrictive fishery regulation. Stocked trout have been shown to have a greater susceptibility to being caught by anglers, compared to wild trout (Almodóvar and Nicola, 2004; García-Marín et al., 1998). However, only a limited contribution of 22–23 % of marked stocked trout has been estimated in total anglers' catches, against natural recruitment (78 %) (Champigneulle and Cachera, 2003). Less than 25 % of rainbow trout (*Oncorhynchus mykiss*) stocked each month was caught by fisherman (Baker and Sammons, 2021). Baer (2004) found that only 12–19 % of stocked yearling brown trout were recaptured after six months; compared to 40–70 % of 1+ and up to 100 % of older wild trout. Thus, factors other than angling may have a more relevant role in reducing the population size of stocked trout. It should be assumed that important adaptive differences exist between wild and reared trout, which are probably unfit to most wild natural conditions. Lower survival or persistence of captively reared fish in natural environments have been largely documented (Aarestrup et al., 2014; Baker and Sammons, 2021; Brignone et al., 2022; Flick and Webster, 1964; Fraser, 2008; McGinnity et al., 2003; Skaala et al., 2012; Weiss and Schmutz, 1999). The hatchery environment and close breeding system, causing loss of genetic diversity, inbreeding depression, accumulation of new mildly deleterious mutations, and genetic adaptation to captivity, can elicit phenotypic and genetic changes which can result in maladapted individuals for survival in the wild (Bryden et al., 2004; Christie et al., 2012, 2014, 2016; Heath et al., 2003; Le Luyer et al., 2017; Sundström et al., 2004). Fitness decline can be quick, sometimes after few generations of captive rearing (Araki et al., 2007; Christie et al., 2016; Jackson and Brown, 2011). Offspring of farmed Atlantic salmon (*Salmo salar* L., 1758) and steelhead trout (*Oncorhynchus mykiss* (Walbaum, 1792)) showed lower survival under wild conditions, compared with offspring of wild stocks (McGinnity et al., 2003; Blouin et al., 2021). Rearing under farm conditions can result in many behavioural changes. Reared brown trout display reduced territory holding (Sundström et al., 2004) and lower ability to obtain food (Bachman, 1984; Kahilainen and Lehtonen, 2001; O'Grady, 1983; Sundström and Johnsson, 2001); those individuals also preferentially feed on items captured near the surface (Teixeira and Cortes, 2006). Domestication impairs anti-predatory behaviours and favours bold behavioural responses too (Alvarez and Nicieza, 2003; Biro et al., 2004; Huntingford, 2004; Jackson and Brown, 2011; Petersson and Järvi, 2006).

4.4. Management strategy to improve marble trout conservation

Stocking programs constitute the larger part of fisheries' management tools (Epifanio, 2000), due to the considerable economic value of recreational angling (Arlinghaus et al., 2002; Hutt et al., 2013; Rolfe and Prayaga, 2007) and the entrenched idea that in the absence of constant fish stocking the recreational fishery would collapse. Stocking practices with captively reared individuals are implemented in Toce River by angler associations, to support intermittent and put-grow-take fisheries (harvest supplementation) and the marble trout population (population supplementation). However, the coexistence of supplementation using non-native and marble strains is a stark contradiction, due to the unambiguous detrimental effect that admixture may have on native marble populations. Our investigation reveals that the current management actions are ineffective, both for stock enhancement and conservation, due to the negligible proportion of domestic fish detected in the wild and evidence of extensive introgressive hybridization. Stocking could provide a short-term increase in trout populations (Brignone et al., 2022) but may lead to a longer-term decline (Satake and Araki, 2012). Survival and breed of some domestic individuals are erroneously taken as an indication that stocking is beneficial to increase the total number of fish in the wild. The stocked fish may survive and breed at the expense of an equal, or even greater, number of wild fish. Hybrids may have lower survival and fitness (outbreeding depression) compared to pure wild individuals (McGinnity et al., 2003), this results in a lowering of fitness in the wild population as a whole and a reduction in the number of individuals available for exploitation and breeding (Ferguson et al., 2007). Managing actions, firstly, must pose no threat to the wild natural self-sustained population, not only for conservation purposes but also because natural recruitment greatly supports recreational fishery (Champigneulle and Cachera, 2003).

Stocking of both non-native Atlantic and Mediterranean trout must be stopped also in the upper Toce tributaries. Several studies have shown a decrease in the level of domestic gene introgression, over subsequent years, after the stop of stocking. For example, in Soča River, where brown trout stocking has been banned since 1996, the proportion of domestic lineage in the highly introgressed marble population dropped regularly, by about 1–2 % each year (Berrebi et al., 2022). Genetic drift and selection can both act together, after stocking ban, with the possibility of a return to a nearby wild genetic state after stocking cessation (Leitwein et al., 2020; Létourneau et al., 2018). The use of Mediterranean trout, currently advertised as a conservation-friendly alternative to the Atlantic brown trout (Polgar et al., 2022; Splendiani et al., 2019), must be avoided since the absence of scientific evidence of the natural presence of Mediterranean trout populations in this area (Polgar et al., 2022), because of the hybridisation with wild individuals and the unknown or mixed origins of these stocks. Sold “Mediterranean trout” stocks are admixture from several peninsular and insular Italian locations, including domesticated progeny, often hybridized with non-native Atlantic stocks (Splendiani et al., 2019; and this study). Moreover, reducing angling pressure on marble trout, by implementing a strict ‘no-kill’ strategy, and increasing brown trout catches, by eliminating bag limits, could help to improve marble trout populations. Angling techniques are highly size-selective against adults (Tiberti et al., 2017) thus, may contribute to reduce the number of non-native spawners in the wild.

The availability of suitable habitats is another key factor in supporting a structured and resilient population. The Toce River has a good ecological and chemical status (Regione Piemonte, 2018), showing, however, a moderate level of morphological quality (IQM) (http://www.arpa.piemonte.it/approfondimenti/temi-ambientali/geologia-e-dissesto/profluviali/monitoraggio-morfologico-dei-corsi-dacqua/valutazione-dellindice-di-qualita-morfologica-iqm-in-piemonte). This river is affected by anthropogenic pressures such as water abstractions and flow alterations, morphological changes of the riverbed and the coastal area, alterations of the riparian area (Regione Piemonte, 2018); which may lead to habitat changes and consequently affect the marble trout population. Anthropogenic modifications of flow regimes (e.g. induced by dams or diversions) and higher fine sediment loads in rivers (e.g. induced by changes in land use) can decrease availability of salmonid spawning site, due to clogging gravels (Acornley and Sear, 1999; Schälchli, 1992). Both the chemical and physical effects of high concentrations of fine sediment were shown to reduce survival of early life stages in salmonids (Chapman, 1988; Ingendahl, 2001; Jonsson and Jonsson, 2011; Louhi et al., 2011; Olsson and Persson, 1986; Rubin and Glimsäter, 1996; Sternecker and Geist, 2010). For instance,

alterations in the hydro-morphological structure of the riverbed caused the reduction in the suitable habitats to support structured populations of marble trout in Brenta river, nullifying management efforts including restocking (Tenci et al., 2019). Therefore, habitat rehabilitation should take priority over stocking. Environmental improvements give much greater, and longer-term, returns than stocking. When the stocking programme was stopped, and habitat restoration was carried out trout densities increased (Fjellheim et al., 2003).

Finally, when an identifiable problem prevents the natural population from reaching its full capacity, a supportive breeding strategy can be adopted to protect and enhance declining wild trout populations. However, based on our analysis, the investigated marble hatchery strain is not suitable for supporting breeding programs. Despite the low level of divergence with the wild population, high hybridization and individuals' relatedness do exist, and potential adaptive differences are also likely. Genetic (bottleneck, family effect, drift) and domestication issues must be minimised in supporting breeding programs. Fish farming finalised to higher offspring production, should be avoided. Gametes must be obtained regularly from wild native marble trout under a strict phenotypic and genetic screening (Meraner et al., 2010), and permanence in captivity should be reduced to decrease domestication. The absence of pure pristine Italian marble populations, however, makes the selection of breeders very challenging. Cryopreservation can represent an efficient system for long-term sperm preservation and storage of sperm until it is genetically tested (Horváth et al., 2014). Moreover, creation of natural nurseries, as implemented by the Forest and Fauna Service on the Rio Ischielle (Trentino), in the three years 2013–2016 (Pontalti, 2020) can represent a strategy to prevent the domestication of offspring, increasing their survival in the wild.

5. Conclusion

Fish stocking with hatchery reared individuals is frequently practised in many rivers and lakes to artificially increase the fishing harvest and recreational opportunities as well as to mitigate the decline of threatened populations. In this paper we showed that this practice has not necessarily positive effects on natural populations, and its implementation should be contrasted against alternative strategies, such as those mitigating the limiting factors to the natural recruitment potential. Indeed, by evaluating the genetic differentiation between the wild and hatchery marble trout, we detected a high level of introgressive hybridization of non-native traits in both wild and hatchery samples and a very low contribution of the stocked fish to the wild marble trout population. The limited presence of both marble and non-native stocked trout in the wild strongly suggests a very limited survival of these fish and the ineffectiveness of restocking activities to increase the stock and angler opportunities. Moreover, because of the high non-native introgressive hybridization within marble trout domestic strain, these hatchery stocks should not be selected for supporting breeding programs. In this perspective, the genetic characterization of strains used for stocking appears as an essential prerequisite to check for breeders suitability, taking into account the local genetic makeup of populations, and imposing a periodic turnover of breeders set to limit genetic bottleneck and domestication.

CRediT authorship contribution statement

Tommaso Righi: Investigation, Methodology, Analysis, Writing- Original draft preparation, Reviewing and Editing. **Emanuele Fasola**: Investigation, Methodology, Analysis, Writing- Original draft preparation, Reviewing and Editing**; Mattia Iaia**: Conceptualization, Investigation, Methodology, Data curation; **Fabrizio Stefani**: Conceptualization, Methodology, Analysis, Investigation, Writing- Reviewing and Editing; **Pietro Volta**: Conceptualization, Methodology, Investigation, Funding acquisition; Project administration; Writing- Reviewing and Editing.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.164555.

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