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Genetic identification of native populations of Mediterranean brown trout *Salmo trutta* L. complex (Osteichthyes: Salmonidae) in central Italy

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Abstract
Italian native populations of Mediterranean brown trout belong to the *Salmo trutta* complex. This species complex includes many mitochondrial lineages and phenotypes that have caused taxonomic controversies over time. The spatial distribution and the genetic diversity of these fishes are threatened by habitat destruction, global warming and, mainly, by the introduction of domestic trout of Atlantic origin. Indeed allochthonous trouts were massively restocked in Italian rivers for a century and they admixed with native populations. In order to identify residual native populations of Mediterranean brown trout, a genetic analysis of specimens collected within Latium region, on the Tyrrhenian slope of central Italy, was undertaken. To this purpose, 210 trout specimens were collected from six different rivers and analyzed for the identification of their nuclear (*LDH-C1** RFLP) and mitochondrial (Control Region sequences) genotypes. Genetic characterization with these molecular markers allowed a quantitative estimate of allochthonous genotypes, which are present in all brown trout populations of the six sites, even if not equally distributed across the sampling area. At least three populations, inhabiting diverse lotic ecosystems (mountain, hilly and coastal streams respectively), are characterized by a high percentage of native nuclear allele **100* at locus *LDH-C1** and typical Mediterranean haplotypes (of AD and ME lineages), which can be considered as different management units (MUs). This finding highlighted the aquatic ecosystems of the Latium Region as an important hotspot of salmonid biodiversity within the Italian peninsula, with important implications from a conservation perspective.

Keywords: *Biodiversity conservation, salmonids, hybridization, restocking, threatened fish*

Introduction

Salmonids include freshwater and anadromous fishes widespread in the Northern hemisphere (Nelson et al. 2016). In the Mediterranean area, the Italian peninsula represents a hotspot of salmonid biodiversity, being inhabited by a huge number of native taxa. Among these, a number of fishes of the genus *Salmo*, commonly known as trout, are included in the national and international red list (Bianco et al. 2013) and partly in annexe II of the European Union "Habitat Directive" (Nonnis Marzano et al. 2016). Their present distribution is the result of the combination of historical natural colonization (Bianco 1990), and recent anthropogenic activities. Indeed the history of trouts of the Mediterranean area was geographically shaped by the different impact of paleoclimatic events (i.e. glaciations) on the isolation of ancestral populations into refuges and subsequent secondary contact (Bernatchez 2001; Cortey et al. 2004, 2009). Since the last century, the original distribution of these fishes has been altered by the massive restocking with domestic brown trout mainly of Atlantic origin, that was translocated for recreational fishery and mixed with natural populations (Splendiani et al. 2016b; Meraner & Gandolfi 2018). The coupling of these domestic brown trout with the native populations has caused introgressive hybridization and reduced the genetic integrity of native trout so that in some geographic areas original populations have been almost completely admixed or replaced by alien trout (Splendiani et al. 2016b, 2019). This context,

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associated with the phenotypic plasticity and ecological adaptation, has contributed to a confusing nomenclature picture and to "taxonomic inflation" (see Tougard et al. 2018) of Italian native trout, with many *morphae* described as species (or subspecies) whose validity is questionable. To date, it is still very difficult to delineate boundaries and unequivocal correspondences between morphological species and evolutionary lineages identified by molecular markers; in order to overcome this difficulty the use of Evolutionary Significative Units (ESUs) has been suggested (Zanetti et al. 2013). However the problem is not completely solved, as Habitat Directive requires species names as labels of protection actions.

The main point of congruence about Italian trouts is that they are part of the *Salmo trutta* complex defined on a molecular basis (Sanz 2018), and include: trout that inhabit the Apennines and the western Alps (AD lineage) that likely corresponds to *S. ghigii* Pomini, 1940; trout present in south eastern Sicily with AT native haplotypes, that should keep the name *S. cettii* Rafinesque, 1810, and whose distribution elsewhere in Italy still needs to be defined; trout from the Tyrrhenian area of the Italian Peninsula, Sardinia and north Sicily, presently without a valid name, and characterized by AD and ME haplotypes; *S. fibreni* (Zerunian & Gandolfi 1990) the endemic trout of the small karstic lake of Posta Fibreno (a split of the AD lineage); *S. marmoratus* Cuvier, 1829 (MA lineage) present in Italian and Slovenian rivers draining into the northern Adriatic; *S. carpio* Linnaeus, 1758 the endemic trout of the Garda Lake (AD and MA haplotypes) (Gratton et al. 2014). In addition to these, allochthonous brown trout corresponding to *Salmo trutta* derived from north European farmed stocks (mainly AT lineage), are present, and frequently hybridized with local native populations (Bernatchez 2001; Nonnis Marzano et al. 2003; Splendiani et al. 2006, 2016b, 2019; Gratton et al. 2014; Fabiani et al. 2018).

In the last decade a 30% decline of native trout populations, and a high rate of hybridization/introgression with domestic trout, have been detected in central Italy (Caputo Barucchi et al. 2015). The only populations partially genetically preserved are those isolated by natural or artificial barriers that prevent or reduce alien fish upstream movements; by contrast the presence of protected areas seems to be irrelevant in genetic conservation (Splendiani et al. 2019).

This work aims to identify and assess the genetic composition of residual native populations of Mediterranean brown trout from different sampling sites within Latium region. Data could provide useful information for conservation strategies, i.e. for the identification of management actions on those populations with higher genetic integrity, and for use in a parallel habitat modelling analysis (Martinoli et al. 2019). To this purpose, two molecular markers that proved to be diagnostic of lineages (Cortey & García-Marín 2002) and geographic origin (McMeel et al. 2001) of brown trout were applied. The mitochondrial marker (Control Region, CR) was used for lineage identification; the nuclear marker (Lactate dehydrogenase C1, *LDH-C1**), to discriminate specimens of European hatchery origin from those of Mediterranean native origin, and to identify their hybrids.

Material and methods

A total of 210 specimens were collected from 6 different rivers/locations (Table I) using electrofishing procedures. In order to minimize the risk of underestimating the local population genetic variability, associated with the sampling of individuals belonging to the same family group (Hansen et al. 1997), each sampling area was extended for more than 20 times the wetted width of the bed of the streams, taking also into account the eventual presence of small tributaries.

Specimens were anaesthetized with a 0.035% MS 222 (Tricaine Methanesulfonate) solution. A small portion of the adipose fin was removed and fixed in 90% ethanol for genetic analysis. The procedures used for fish sampling were carried out in agreement with relevant legislation (CEN EN 14011/2003 - Water quality - Sampling of fish with electricity), avoided animal sacrifice and permitted the live release of sampled specimens after data collection. Fish sampling was authorized by the Direzione Regionale Agricoltura, Promozione della Filiera e della Cultura del Cibo, Caccia e Pesca of the Regione Lazio (Prot. n. 526425).

Total genomic DNA was extracted from fin clips according to Aljanabi and Martinez (1997). For haplotype identification, a 544 base pairs (bp) fragment of the mitochondrial CR was amplified and sequenced in all the 210 specimens (Table I). Amplifications were performed using primers and protocols reported by Cortey and García-Marín (2002). Amplicons were purified and sequenced by using an external service (www.microsynth.ch). Sequences were deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/, accession number MN223698- MN223719) and blasted for similarity searching.

Sequences were aligned using the software Clustal X (Thompson et al. 1997). Seventy-four CR sequences (Supplementary material, Table SI) representative of

Table I. Genetic data of the Mediterranean brown trout populations in the six sampling sites reported from North to South.

River/ stream	Code	Longitude	Latitude	Altitude a.s.l.	Province	*N.*	*CR*				*LDH-C1**		
							H	*AT*	*Hd*	π	90/90	90/100	100/100
Molinaro	MOL	13°17′44.4840”	42°37′58.5480”	892	RI	26	4	2 (24)	0.286±0.112	0.00161±0.00082	13	11	2
Ratto	RAT	13°08′40.7040”	42°30′29.6280”	764	RI	25	5	3 (23)	0.300±0.118	0.00201±0.00097	15	9	1
Simbrivio	SIM	13°13′42.0240”	41°55′32.7000”	798	RM	51	6	1 (2)	0.408±0.083	0.00187±0.00062	6	14	31
Fibreno	FIB	13°38′04.2000”	41°41′35.8080”	290	FR	41	8	1 (2)	0.605±0.080	0.00307±0.00077	0	8	33
Rapido	RAP	13°50′15.5760”	41°28′17.7600”	32	FR	13	6	1 (1)	0.641±0.150	0.00401±0.00159	6	5	2
Santa Croce	SCR	13°42′57.7080”	41°17′13.4160”	20	LT	54	3	1 (1)	0.073±0.049	0.00075±0.00055	0	16	38
					total	**210**	**22**	**6 (53)**	**0.815±0.012**	**0.00713±0.00018**	**40**	**63**	**107**

Province indicate management authority (RI = Rieti, RM = Rome, FR = Frosinone, LT = Latina) and N number of specimens. CR indicates data obtained from mitochondrial control region: total number of haplotypes (*H*), number of Atlantic haplotypes (*AT*) and in parenthesis number of individual showing them, haplotype diversity (*Hd*) and nucleotide diversity (π). *LDH-C1** columns report alleles combination at this nuclear locus

lineages AD, ME, MA and AT available in GenBank were included in the alignment and in subsequent haplotype network reconstruction. DnaSP 5.10 (Librado & Rozas 2009) was used for the identification of the number of haplotypes and haplogroups and to calculate haplotype variability (Hd) and nucleotide variability (π) (Nei & Tajima 1981). To investigate genealogical relationships among mitochondrial CR haplotypes, a parsimony network was constructed using TCS 1.21 (Clement et al. 2000).

For the identification of allochthonous nuclear genotypes, the *LDH-C1** region was amplified and digested using primers and protocols reported by McMeel et al. (2001). Restriction fragments were checked to verify the presence of the allele **90*, fixed or very frequent in north Atlantic populations and European hatchery stocks, or of the allele **100* typical of native Mediterranean populations of the *Salmo trutta* complex.

Results

CR sequences

The analysis of the 210 CR sequences allowed the identification of 22 haplotypes: six belong to the AT, two to the ME and 14 to the AD lineage. Nine of these haplotypes have already been described (Supplementary material, Table SI): among these the most common haplotypes of the ME lineage (Me25) and of the AD lineage (Ad1) (Cortey et al. 2004).

The haplotype network shows that most of the haplotypes observed in this study belong to the AD or ME native lineages, and only a minority fall within the AT lineage (Figure 1). However, the proportion of native/allochthonous haplotypes is not homogeneously distributed among the different sampling locations: the percentage of allochthonous haplotypes ranges from 1.85% in SCR to 92.3% in MOL.

LDH-C1 RFLP*

RFLP analysis of the *LDH-C1** fragments allowed the identification of native (**100/100*), allochthonous (**90/90*) and hybrid (**90/100*) genotypes. The number of native genotypes is very close to the sum of the allochthonous and hybrid ones. Again, there is not a homogeneous distribution of the genotypes among sampling locations.

The comparison of nuclear and mitochondrial data provided a complete pattern, showing all the

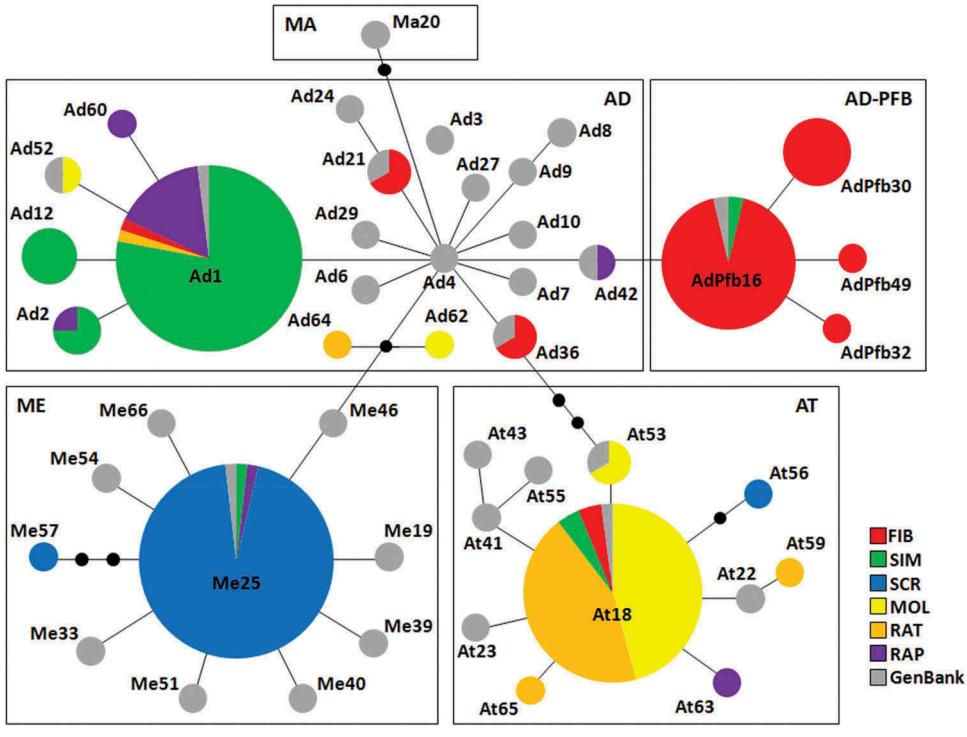

Figure 1. Haplotype network based on CR sequences. Lineages are indicated: AD, Adriatic (AD-PFB haplotypes typical of Fibreno); MA, Marmoratus; ME, Mediterranean; AT, Atlantic. Circle dimension is proportional to the number of individuals showing that haplotype, except for haplotypes recovered from GeneBank.

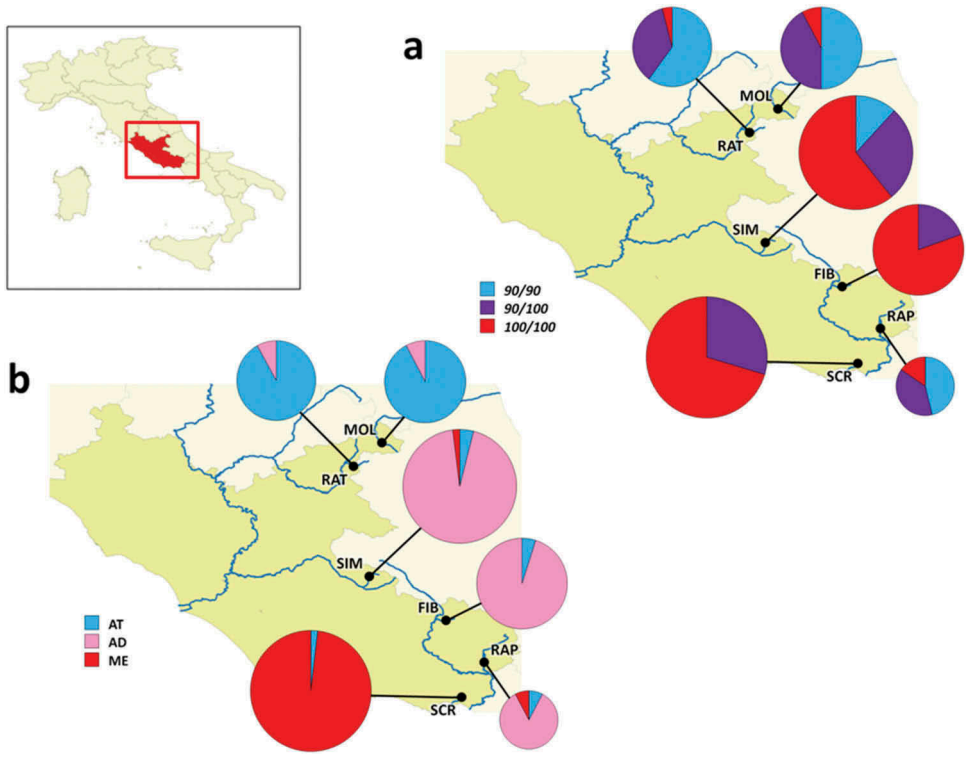

Figure 2. Map of the sampling sites, abbreviated as in Table I. Circles represent (a) genotypes observed at locus *LDH-C1** and (b) CR haplotype lineages. The size of the circles is proportional to the sample size.

possible combination of pure and allochthonous genotypes (Figure 2). In some of the sites, the percentage of pure genotypes (allele **100* and AD or ME haplotypes) reaches 78% (FIB) while in others no pure genotype is present (MOL). Overall, individuals showing allochthonous genotypes (allele **90* and AT haplotypes) represent 13.3% of the total; however, this percentage is much higher in some of the sites, reaching 56% in RAT. In addition to these, traces of hybridization between domestic individuals and native ones are present in all the sites. Allele and haplotype frequencies are reported in Table II.

Table II. Frequencies of nuclear alleles and haplotype lineages of Mediterranean brown trout populations in the six sampling sites. See Table I for site code.

Site	*N.*	*LDH-C1*90*	*LDH-C1*100*	AT	AD	AD-PFB	ME
MOL	26	71.15	28.85	92.31	7,69	-	-
RAT	25	78.0	22.0	92	8	-	-
SIM	51	25.49	74.51	3.92	92.16	1.96	1.96
FIB	41	9.76	90.24	4.88	12.2	82.92	-
RAP	13	65.38	34.62	7.69	84.62	-	7.69
SCR	54	14.81	85.19	1.85	-	-	98.15

Discussion

Results here obtained confirm the deleterious effect of massive introduction of domestic brown trout in Italian rivers already observed in other Italian geographic regions (Nonnis Marzano et al. 2003; Caputo et al. 2004; Splendiani et al. 2016b, 2019) or Latium (Fabiani et al. 2018), with a few exception that characterize some insular areas (Zaccara et al. 2015; Berrebi et al. 2019). The most frequent AT haplotype (At18) corresponds to that originally identified in samples from Norway, Denmark and Spain (Cortey & García-Marín 2002) and already reported in a hatchery stock of Atlantic origin from an Ichthyogenic centre of central Italy (Gratton et al. 2014). These data demonstrate that in spite of law prohibition, stocking practices with domestic trout still continue, spreading among allochthonous genotypes (Splendiani et al. 2019): AT haplotypes and allele **90* are present in all the sites examined in this study, but with a different frequency among them. Atlantic brown trout in some of the sites have replaced almost completely native Mediterranean ones or at least admixed with them. When frequencies of allele **90* and AT haplotype are compared, it is clear that there is no direct correspondence between the two sets of data, as confirmed by Spearman's

correlation analysis (Rs = +0.7714, P = 0.2). The two most admixed sites are MOL and RAT, with 0 and 1 pure individual respectively, and with a high percentage of AT haplotypes. These two sites are part of two different river basins: MOL (892 meters above sea level, a.s.l.) belongs to the Tronto basin, that drains into the Adriatic Sea, while RAT (764 m a.s.l) is a tributary of Velino river that drains into Nera-Tiber River and then into the Tyrrhenian Sea. In spite of this, the two sites are geographically close and are managed by the same province (Rieti), the Local Authority that controls fish introductions. Therefore it is likely that both populations were frequently affected by massive restocking activities.

An intermediate situation is observed in RAP (32 m a.s.l.) that belongs to the Garigliano-Liri basin: this site shows a very admixed trout population with few pure individuals, but the majority of AD haplotypes.

The best-preserved sites, showing a higher percentage of native individuals, are FIB, SIM, and SCR. These sites show both a high percentage of allele **100* and typical Mediterranean haplotypes. Unfortunately, our sequences are shorter than those (1013 bp) that allowed the definition of reference haplotypes by Cortey et al. (2004). For this reason our Me25, mainly characterized by SCR Mediterranean brown trout population, corresponds to 6 different haplotypes (Supplementary material, Table SI), including the one (MEcs1) that is considered basic of this lineages. This haplotype was distributed in Corsica, northern and central Italy in late nineteen century, before common stocking with Atlantic strains (Splendiani et al. 2017) and was already present in prehistoric times in southern Italy (Splendiani et al. 2016a). The same problem of sequence length is present for AD lineages; certainly the most common haplotype of this lineage AD-cs1, originally spread across Italian peninsula and Sardinia (Splendiani et al. 2016a, 2017) is not present in our sampling area. Our results match previous data. Further, Mediterranean brown trout population from Fibreno shows typical genetic features and forms a distinct gene pool from lacustrine *S. fibreni*, although limited hybrid zones are known. The very low level of introgression from Atlantic strain detected in FIB, that is located within a Regional Protected area, was interpreted as the result of conservation measures adopted more than 40 years ago, i.e. cessation of restocking this basin (Gratton et al. 2013). The most common haplotype present in this site (Ad-Pfb16) corresponds to that observed in the 48 specimens examined by Gratton et al. (2013). Other AD haplotypes (Ad36, Ad21 and Ad1) correspond to haplotypes already observed in different sites (ADcs3, ADcs17, and Adcs11 respectively), by Cortey et al. (2004), or by other authors (Ad1, see below). The origin of these haplotypes can be explained by considering two different hypotheses. The first one assumes their presence is the result of natural dispersion. This is quite likely for Ad1, which was identified around the Adriatic area (e.g., Cortey et al. 2004; Sušnik et al. 2007), in Macedonia (Marić et al. 2017) and in central Italy (Fabiani et al. 2018; Berrebi et al. 2019). As regard to ADcs3 and ADcs17, they were originally identified in Spain, so their presence is more difficult to explain unless considering successive waves of colonization. The second hypothesis explains their presence as a consequence of past introduction, congruent with the absence of pure Atlantic individuals, but with their remnants as nuclear hybrid genomes. Concerning SIM and SCR sites, they are geographically close to locations ZLS and CDA, respectively, analyzed by Fabiani et al. (2018), the results obtained being similar in both cases. Indeed SIM is characterized by AD haplotypes, the most common of which is, again Ad1; in addition to this, two further new AD haplotypes (Ad2 and Ad12) are present, plus the most common haplotypes of lineages AD-PFB, ME and AT. Finally, SCR shows almost exclusively ME haplotypes: these are absent in other Latium sites here examined, but were observed in other geographic areas (Cortey et al. 2004). Regarding its features, SCR represents a population with a high percentage of native individuals; these characteristics are shared with CDA (Fabiani et al. 2018), a site geographically very close (about 0.5–1 Km) within the same basin. These two sites are managed by the same province (Latina) and it is likely that both were preserved from the massive introduction of allochthonous trout, although some traces of these activities are still present. Indeed besides a new ME haplotype (Me57), a new AT haplotype (At56) is also present, possibly being a remnant of past introductions (according to the presence of allele **90*). Thus it is also possible that the particular environmental condition of these sites has determined a negative selection of non native brown trouts: CDA and SCR are characterized by cold springs (about 14°C) in lowland and coastal zones (about 20 meters above sea level), very close (< 10 Km) to the stream outlet into the Tyrrhenian Sea.

Overall different Management Units, i.e. conservation units identified on the base of population genetic characters, can be recognized across the sampling area; they include SIM, FIB and SCR, the three sites with the highest percentage of native Mediterranean trout, and with a different abundance of typical AD, AD-PBF and ME haplotypes.

This differential composition and distribution of haplotypes may be associated with the different ecological conditions (e.g. altitude and water temperature) of lotic ecosystems inhabited by these populations. Such a high diversity outlines the aquatic ecosystems of the Latium Region as a unique hotspot of salmonid biodiversity within the Italian peninsula, with important implications from a conservation perspective.

Further analyses are necessary to obtain a clear picture of the genetic distribution of native brown trout populations in the Latium Region and to gain basic data for concrete conservation actions. Such analyses should include "new" populations from other geographic sites and which can be identified on the basis of habitat modeling results (Martinoli et al. 2019).

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Disclosure statement

No potential conflict of interest was reported by the authors.

Supplementary material

Supplemental data for this article can be accessed here.

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