Investigating the genetic structure of trout from the Garden of Ninfa (central Italy): Suggestions for conservation and management

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Abstract
Mediterranean populations of brown trout (Salmo trutta L. complex) have lost a large part of their genetic distinctiveness, mostly due to massive restocking, and the waters of the Gardens of Ninfa (province of Latina, central Italy, Site of Community Importance since 2013) are regarded as one of a few potential reservoirs of autochthonous trout lineages in the Tyrrhenian drainage of the Italian peninsula. In this study, nuclear and mitochondrial markers were used on brown trout samples from Ninfa to estimate non-Mediterranean influence in the population gene pool, potential changes of genetic structure over time and genetic relationships with other sites known (or suspected) to host native trout gene pools. Striking changes in both microsatellite and mtDNA allele frequencies over a 9-year time span were found and provided evidence of unrecorded stocking from the nearby Lake Fibreno. Results are analysed in the light of potential ecological consequences of such events on a longer time scale and provide a scientific background for fisheries management and conservation programmes in the area.

KEYWORDS
conservation, genetic structure, Salmo cettii, salmonids, stocking

1 | INTRODUCTION

The brown trout, Salmo trutta L. complex, represents one of the most important resources in European freshwater fisheries, for both its economic and scientific values. Popular as food and in recreational fishing, it also exhibits remarkable morphological and life-history variation (Fraser, Weir, Bernatchez, Hansen & Taylor, 2011), which makes this species an interesting model for the study of adaptation (Primmer, 2011; Stearns & Hendry, 2004).

Given its vast morphological plasticity, a large number of taxa have been described within the S. trutta complex, and their delimitation and status are still actively discussed among specialists (e.g. Berrebi et al., 2013; Gratton, Allegrucci, Sbordoni & Gandolfi, 2014; Maric, Bajec, Schoffmann, Kostov & Snoj, 2017 and references therein). In particular, about 20 named species endemic to the Mediterranean basin have been described, most of them regarded as part of the S. trutta complex (Kottelat & Freyhof, 2007). However, a significant fraction of the genetic heritage of the Mediterranean trout populations has already been lost as a consequence of environmental degradation and direct effects of human activities, among which overfishing and stocking with hatchery-reared fish (e.g. Berrebi, Povz, Jesensek, Cattaneo-Berrebi & Crivelli, 2000; Pujolar, Lucarda, Simonato & Patarnello, 2011). This is particularly true in the Italian peninsula, where repeated stocking has been widely and massively practiced for over a century. The first trout hatchery in Italy was set up in 1859 (Borroni & Grimaldi, 1978) and, through the 20th century, a large number of lakes and rivers were stocked with hatchery-reared brown trout of Atlantic origins (Giuffra, Guyomard & Forneris, 1996; Snoj et al., 2002). As a result, the vast majority of indigenous Italian trout populations today exhibit loss of genetic diversity and uniqueness and high levels of introgression
from non-native gene pools (Caputo, Giovannotti, Cerioni, Caniglia & Splendiani, 2004; Meraner, Gratton, Baraldi & Gandolfi, 2013; Nonnis Marzano, Corradi, Papa, Tagliavini & Gandolfi, 2003).

Introductions of non-native species into wild populations represent one of the major threats to biodiversity worldwide (McKinney & Lockwood, 2001), even more so when wild populations are small (Glover et al., 2012). Although introduction of non-native salmonids might provide initial angling benefits, the potential ecological consequences of such events on a longer time scale are difficult to predict and often have unplanned outcomes (Hickley & Chare, 2004; Krueger & May, 1991). Stocking with hatchery fish, even when originating from a native taxon, should also be a topic of major concern. There is evidence that trout are susceptible to fitness loss while in captivity (Araki, Berejikian, Ford & Blouin, 2008) and that captive breeding can have a carry-over effect on the wild population in the generation after restocking, significantly decreasing its effective population size $N_e$ (Araki, Cooper & Blouin, 2009). Finally, populations of freshwater salmonids are often small and fragmented, characteristics that make them particularly prone to the effects of genetic drift. Even natural events such as extreme variations in hydrographic regimes can drastically reduce the size of a population, leaving only few adults able to contribute to the progenies, with a consequent decrease in genetic variation (Pujolar, Vincenzi, Zane & Crivelli, 2016). For these reasons, the documentation of species introductions, the genetic detection of illegal fish stocking and the environmental changes of the habitat should be monitored over a long-term frame. This would help understand natural evolutionary processes over the years and assess how stocking has affected the contemporary genetic structure of populations (Glover et al., 2012; Hansen, Fraser, Meier & Mensberg, 2009).

Phylogeographic studies on European brown trout highlighted five major mitochondrial haplogroups, the commonly used names of which broadly reflect their distribution across geographic regions or morphological taxa: Danubian (DA), mainly found in rivers of the Ponto-Caspian basin; Atlantic (AT), widely spread from Morocco to the White and Barents Sea and whose current distribution in the Italian peninsula is considered a consequence of stocking (e.g. Caputo et al., 2004; but see Schoffmann, Susnik & Snoj, 2007 for Sicily); Marmoratus (MA), loosely corresponding to the marble trout (Salmo marmoratus) of the northern Adriatic basin, but also found in other Mediterranean populations (Apostolidis, Triantaphyllidis, Kouvatsi & Economidis, 1997; Giuffra, Bernatchez & Guyomard, 1994; Gratton et al., 2014; Pustovrh, Snoj & Bajec, 2014); Mediterranean (ME), predominantly found in rivers draining in the western Mediterranean basin (but also in central Italy and Greece: Bernatchez, 2001; Apostolidis et al., 1997); Adriatic (AD), more frequent in the eastern part of the Mediterranean basin, but also present on the west Italian coast, in France and Spain (Bernatchez, 2001; Cortey, Pla & Garcia-Marín, 2004; Giuffra et al., 1994). Although mitochondrial DNA sequences are extremely useful as phylogenetic markers and to track the geographic origins of trout gene pools, they might not provide a comprehensive description of the genetic relationships among individuals and populations, and discordant signals between mitochondrial and nuclear markers would be therefore expected (Toews & Brelsford, 2012). This is often the case in trout (Caputo et al., 2004; Gratton et al., 2014; Meraner et al., 2013; Pustovrh et al., 2014).

A widely cited and relatively recent taxonomic assessment of European trouts (Kottelat & Freyhof, 2007) considers Salmo cettii as the taxon including most of the autochthonous trout of the Tyrrenian basin (with the exception of the restricted endemism Salmo fibreni, from Lake Fibreno, central Italy) and suggests avoiding the name S. (trutta) macrostigma, which was (and still is) often applied to some Italian populations (see Gandolfi, 1991; Iaffaldano, Di Iorio, Manchisi, Esposito & Gibertoni, 2016; Querci, Pecchioli, Leonizio, Frati & Nardi, 2013). There is no current consensus about the validity of S. cettii (sensu Kottelat & Freyhof, 2007) as a species (or even as a distinct taxon within the S. trutta complex) and some authors prefer to simply include it into a more comprehensive “Mediterranean” S. trutta (e.g. Berrebi, 2015; Splendiani et al., 2017). Gratton et al. (2014) analysed a diverse set of mitochondrial and nuclear markers and identified two main native genetic lineages in samples of Italian trouts: a widespread “peninsular” lineage, including samples of Salmo cenerinus, S. cettii and S. fibreni (all sensu Kottelat & Freyhof, 2007) and most of the genetic ancestry of the Garda Lake endem Salmo carpio, and a “marble” lineage, represented by S. marmoratus populations. However, in this study, S. cettii and S. cenerinus (sensu Kottelat & Freyhof, 2007) are used as convenient shorthand to identify the native brown trout from the Tyrrenian and Adriatic drainages of the Italian peninsula, respectively. Salmo fibreni is also used to refer to the morphologically and genetically distinct lineage occurring in Lake Fibreno, as identified by Gratton, Allegrucci, Gandolfi and Sordoni (2013).

The IUCN Red List (The IUCN Red List of Threatened Species. Version 2016-2 www.iucnredlist.org, Downloaded on 17 October 2016) considers S. cettii as Near Threatened and the same populations are listed (as Salmo macrostigma) in Annex II of the Habitats Directive (92/43/EEC). The main threats to these populations include water abstraction and stocking of non-native gene pools (Freyhof & Kottelat, 2008). Indeed, several studies employing genetic markers confirmed that only a few locations might be considered to host “pure” populations of S. cettii (e.g. Gratton et al., 2013; Nonnis Marzano et al., 2003; Querci et al., 2013; Sabatini et al., 2011).

The water bodies of the Garden of Ninfa (central Italy, 41.58 N, 12.96 E, Figure 1, hereafter “Ninfa”) are regarded as a potential reservoir of autochthonous trout lineages in the Tyrrenian drainage of the Italian peninsula (Zanetti, 2016). The lowland streams of Ninfa, fed by a karstic spring with temperatures between 10 and 20°C and abundant submerged macrophytes, represent an ideal habitat for S. cettii (Zanetti, 2016). The ecological requirements of this taxon are thought to differ from those of other Italian salmonids in the S. trutta complex, which usually prefer highland streams with lower temperatures, powerful water flow and scarce or absent submerged macrophytes (Kottelat & Freyhof, 2007). For these reasons, Ninfa has been proposed as a protected area in 1995 and confirmed as a Site of Community Importance (SCI, code IT6040002) in 2013. Direct observations in 2014–2015 showed that the water system is now moderately polluted or, at least, altered (III class Extended Biotic Index, EBI, Seminara, unpublished data). Supportive breeding of trout, with
in-situ hatching and rearing, started in Ninfa, with the first release of about 1,000 fry (1–1.5 cm length) taking place in 2014, and a second in 2015 that reintroduced about 500 small trout (9–12 cm) into the river (Seminara, unpublished data). With the intent of increasing the presence of autochthonous trout, only S. cettii-like phenotypes were selected for hatching, despite previous evaluation of similar phenotypic selection at Posta Fibreno suggested that such practice is highly ineffective as a means for favouring native genotypes (Gratton, Konopinski & Sbordoni, 2008).

Despite the relatively large number of genetic studies on Italian trout populations (Caputo et al., 2004; Giuffra et al., 1994, 1996; Gratton et al., 2013, 2014; Meraner et al., 2013; Nonnis Marzano et al., 2003), the origins of the population of Ninfa are not clear, and its management has been so far lacking an adequate genetic monitoring.

In this study, nuclear and mitochondrial markers were used on trout samples from Ninfa and close-by populations to investigate the local genetic structure and its genetic relationships with other sites known (or suspected) to host native trout gene pools. In particular, trout samples from Ninfa collected in 2005 and 2014 were analysed and compared to newly and previously sampled populations from Lazio and Abruzzo, to (1) assess the level of non-Mediterranean (Atlantic) introgression in the Ninfa population; (2) examine potential changes in population genetic structure and level of introgression over time in Ninfa, as a result of stocking activities, genetic drift and/or selective pressure; (3) explore possible origins of the trout population of Ninfa; (4) provide a scientific background for fisheries management and conservation programmes in the area.

2 | MATERIALS AND METHODS

2.1 | Study area and sample collection

The study area of Ninfa is situated between the Pontine Marshes and the Lepino-Ausonic mountain complex. The high permeability of this fractured rocky complex allows phenomena of groundwater contributions that create almost 40 water springs at the bottom of the Lepino-Ausonic complex. A small group of these water sources, situated at about 30 m above sea level, feed Lake Ninfa and the outflowing Ninfa River, which crosses the Natural Monument Garden of Ninfa (Latina, central Italy). Those karstic resurgences generate a limnocrene spring system (i.e. springs form a pool before flowing into a defined channel) with water of excellent quality, and a dam-wall built in the Middle Ages increases the depth of the lake to maximum level of six metres. The Ninfa River runs for about 0.5 km inside the semi-artificial area of the Ninfa Natural Monument, receiving little tributaries from smaller outlets in the dam-wall, and then gets collected into a peculiar micro-hydrographic system, made of little channels, streams and small pools. The area of Ninfa hosts a population of trout whose census size was estimated as around 130 individuals (Seminara, unpublished data), and the whole water network is accessible to fishes. Once out of the Natural Monument, the Ninfa River crosses a wilder territory (≈2 km) and then rural areas, where it loses its salmonid-stream characteristics and becomes the river Sisto.

Skin samples (<1 cm²) were collected from the dorsal fin of fishes from the Ninfa River and other waters inside the Garden of Ninfa (hereafter NIN). Wild animals were captured in 2005 (NIN05, N = 45) and in 2014 (NIN14, N = 39), using pulsed DC backpack electrofishing equipment (ELT60 IIGI, Scubla S.R.L.). Fish were anaesthetised with a 2-phenoxyethanol solution, measured, weighed, marked with coloured elastomers positioned between the fin rays and then released. Sampling activities followed standard procedures, as suggested within the Habitats Directive, and were approved by the Regione Lazio (Italy). Tissue samples were preserved in 90%–100% ethanol.

Fish samples were also collected in 2014 from two more central Italian sites suspected to harbour native trout populations: Capo d’Acqua (CDA, N = 30), near Formia (LT, Lazio, Figure 1) and Zompo lo Schioppo (ZLS, N = 35), near Avezzano (AQ, Abruzzo, Figure 1).

2.2 | DNA extraction and genotyping

Genomic DNA was successfully extracted from the tissue samples using the DNeasy® Tissue kit (Qiagen, Venlo, the Netherlands) or the GenElute™ Tissue Genomic DNA Purification Kit (Sigma Sigma-Aldrich, St. Louis, MO, USA). After extraction, all samples (Table 1)
were genotyped at eight microsatellite loci: MST79, MST591 (Presa & Guyomard, 1996), BS131, 543AE, T3-13 (Estoup et al., 1998), Str15 (Estoup, Presa, Vaiman & Guyomard, 1993), Strutta12 and Strutta58 (Poteaux, Bonhomme & Berrebi, 1999). PCR conditions followed the original literature, with slight modifications on the annealing temperatures.

All PCR microsatellite products were processed on an automated ABI 3730xl 96-Capillary Genetic Analyzer and analysed for length variation with Peak Scanner™ Software (Applied Biosystems, Carlsbad, CA, USA).

All the microsatellite analyses also included reference data from: a hatchery stock of Atlantic origins from the “Centro Ilttiogenico della Provincia di Roma” in Jenne (JEN: N = 23, Gratton et al., 2014), the “Riserva Naturale Regionale Gole di San Venanzio” in Abruzzo (SVN: N = 24, Gratton, Allegrucci & Sbordoni, 2007) and Posta Fibreno (PFB: N = 105, Gratton et al., 2014), the “Riserva Naturale Regionale Gole di San Venanzio” in Abruzzo (SVN: N = 24, Gratton, Allegrucci & Sbordoni, 2007) and Posta Fibreno (PFB: N = 105, Gratton et al., 2014) near Sora (FR, Lazio) (Figure 1). The latter sample included individuals from two described for Lake Fibreno, S. cetti and the local endemism S. fibreni, whose patterns of genetic differentiation were elucidated by Gratton et al. (2013).

To ensure consistency (i.e. that alleles were identified correctly across different datasets), a sub-set of individuals from the reference samples were re-amplified, a binning procedure was performed with the program FLEXIBIN (Amos et al., 2007), and the alleles were thoroughly re-called.

The mitochondrial DNA (mtDNA) control region was amplified, and both strands were sequenced, using the pair of primers PST and FST (Cortey & García-Marín, 2002), for a total of 58 samples (NIN05: N = 16, NIN14: N = 24, CDA: N = 8, ZLS: N = 10). Raw sequence chromatographs were visualised, edited and aligned using CodonCode Aligner (CodonCode Corporation, Centerville, MA, USA).

To place sequenced mtDNA in the broader context of trout mitochondrial diversity, previously published sequences were included in the analyses: JEN (hatchery, N = 3, Gratton et al., 2014; GenBank: KJ834922–KJ834924), SVN (N = 24, GenBank: KJ834851–KJ834874, Gratton et al., 2007), PFB (N = 55, all identical sequences, GenBank: JQ314219, Gratton et al., 2013) and 39 representative sequences of the AD, ME, AT and MA haplogroups (GenBank: AY836330–AY836349, AY836350–AY836364, AY836327–AY836329, AY836365, Cortey et al., 2004). In addition, sequences were blasted against the GenBank NCBI nucleotide collection to check whether they were identical to already published sequences.

### 2.3 Polymorphism and genetic structure at microsatellite loci

Microsatellite alleles and genotypes were checked for possible typing errors, null alleles, large allele dropout and errors due to stutter peaks with the program MICRO-CHECKER 2.2.3 (van Oosterhout, Hutchinson, Wills & Shipley, 2004). Deviations from Hardy–Weinberg Equilibrium (HWE) were assessed using a Fisher’s exact test and Markov chain method (10,000 dememorisation number, 1,000 batches and 1,000 iteration per batch), as implemented in GENEPOL (Raymond & Rousset, 1995). The same program was also used to test each pair of loci for linkage disequilibrium, and the Holm–Bonferroni correction (Holm, 1979) for multiple comparisons applied, using the P-value calculator from Gaetano (2013).

Number of alleles, number of private alleles, inbreeding coefficient (F<sub>IS</sub>), observed (H<sub>o</sub>) and expected heterozygosity (H<sub>E</sub>) were calculated with the program Genetix 4.0 (Belkhir, Borsa, Chikhi, Rauffaste & Catch, 2004). As the observed number of alleles in a sample is dependent on sample size, allelic richness (A) was also estimated using the rarefaction method implemented in the program Fstat 2.9.3 (Goudet, 1995).

Genetic differentiation between sampled populations was estimated as F<sub>ST</sub> (Weir & Cockerham, 1984), using the program Genetix 4.0 (Belkhir et al., 2004) and applying the correction for multiple comparisons, while the distribution of allele diversity at microsatellite loci was investigated with a principal components analysis (PCA), using the dudi.pca R function (“ade4” package, Dray & Dufour, 2007). The input data consisted of the frequencies of each allele at a given locus (0 = absent, 0.5 = heterozygote, 1 = homozygote) in each sampled individual; therefore, the number of variables for the PCA was equal to the total number of alleles in the dataset (162).

The genetic structure of the sample was further explored using the Bayesian approach implemented in STRUCTURE v2.3.4 (Pritchard, Stephens & Donnelly, 2000). The program uses a Markov Chain Monte Carlo (MCMC) method to fit a model that partitions the genetic variation of the data into K genetically homogeneous clusters and to estimate the proportion of ancestry of each individual into each cluster. Following Falush, Stephens and Pritchard (2003), a model was
assumed with population admixture and correlated allele frequencies and applied. Ten independent runs (1,000,000 iterations each, burn-in = 150,000) for each K between 1 and 10 were carried out, and the estimated log-likelihood (lnPr(D|K)) for each run was examined. K was selected following Evanno, Regnaut and Goudet (2005), whose approach proposed that the value of K corresponding to the maximum rate of increase in the log-likelihood as a function of K (ΔK) is most likely to capture the uppermost level of genetic structure in the data. Although the ΔK approach may underestimate the number of gene pools represented in the data (Kalinowski, 2011; Waples & Gaggiotti, 2006), it can be very useful to detect the most relevant features of the data.

2.4 | Analysis of mtDNA sequences

The phylogenetic relationships of sequenced mtDNA were visualised in the context of the overall trout mitochondrial diversity by building a Maximum Parsimony (MP) tree for the new control region sequences and 121 previously published sequences (see DNA extraction and genotyping section). The MP tree was reconstructed using the R package “ape” with a Random Addition starting tree, SPR optimisation and ACCTRAN criterion for branch lengths.

3 | RESULTS

3.1 | Genetic diversity

The tests run in the program Micro-checker did not find evidence for scoring errors due to stuttering, allele dropout or null alleles in any of the loci analysed. After Bonferroni correction, linkage disequilibrium was detected between 11 pairs of loci (one pair in NIN14, one in JEN, five in PFB and four pairs in NIN05) and significant departure from HWE was found in three loci in NIN14 (ST15, ST58 and ST12) and in one locus in CDA ( locus MST79).

Observed heterozygosity ranged from 0.42 ± 0.2 (CDA) to 0.71 ± 0.40 (JEN); it significantly differed from expected values in NIN14, CDA and ZLS for heterozygosity deficiency, but in JEN for heterozygosity excess (P < .001). Fis values were positive and significant in NIN14, CDA and ZLS, while they were close to zero and not significant in the other populations (Table 1). All sampled populations showed private alleles, from a minimum of four (CDA) to a maximum of 13 (PFB). Mean number of alleles per sampling site varied from 5.37 (CDA) to 10.88 (PFB) and allelic richness from 3.50 (CDA) to 5.59 (NIN14).

3.2 | Population structure

Microsatellite data showed a significant structure among all populations, with an overall Fst index equal to 0.25 (95% CI = 0.19–0.34), and between pairs (Table 2, P < .05, always). A low but significant differentiation (Fst = 0.03) was also found between the two diachronic samples from Ninfa, NIN05 and NIN14 (Table 2).

The PCA on macrosatellite loci revealed a complex genetic structure, with each of the sampled population occupying a well-defined space in the multidimensional space, no obvious geographic structure among the central Italian samples and no single principal component (PC) taking up a very large portion of the variation. Figure 2 shows the position of each individual multigenotype in the space of the six most important PCs. The first two PCs separated the hatchery sample (JEN) from most of the natural samples (Figure 2a); PC1 (17.4% of the total data variation) contrasted JEN with PFB (the large size of the PFB sample, N = 105, might explain why this axis takes up the largest share of the data variation) and PC2 (9.8%) contrasts JEN with SVN, CDA and ZLS. Samples collected in Ninfa in 2005 (NIN05) appear very similar to JEN on both PC1 and PC2, while those collected in 2014 (NIN14) occupy an intermediate position between NIN05 and PFB (Figure 2a). Samples from SVN, CDA and ZLS were well separated from each other along PC3 (6.1%: contrasting CDA from ZLS and SVN, Figure 2b) and along PC 5 (4.2%: contrasting ZLS from SVN).

The ΔK approach (Evanno et al., 2005) indicated K = 3 as the number of clusters most likely to capture the uppermost genetic structure in microsatellite data (Figure 3a). One of the three inferred genetic clusters (cluster 1) prevailed in all genotypes of Posta Fibreno (PFB). A second cluster (cluster 2) made up most of the genotypes in the JEN hatchery samples and in the NIN05 sample. Most individuals from the NIN14 sample appeared as a mixture of clusters 1 and 2. The third cluster (cluster 3) included most of the genetic profiles of the remaining natural sampling sites (CDA, ZLS and SVN) (Figure 3b).

The MP tree of mtDNA D-loop (including the current samples as well as GenBank reference sequences) identified the four well-known lineages AT, AD, ME and MA (Figure 4). As in the microsatellite

<table>
<thead>
<tr>
<th>Sample</th>
<th>NIN14</th>
<th>CDA</th>
<th>ZLS</th>
<th>JEN</th>
<th>PFB</th>
<th>SVN</th>
<th>NIN05</th>
</tr>
</thead>
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<tr>
<td>NIN14</td>
<td>–</td>
<td>0.32</td>
<td>0.18</td>
<td>0.19</td>
<td>0.13</td>
<td>0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>CDA</td>
<td>–</td>
<td>0.26</td>
<td>0.36</td>
<td>0.37</td>
<td>0.33</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>ZLS</td>
<td>–</td>
<td>0.22</td>
<td>0.27</td>
<td>0.18</td>
<td>0.21</td>
<td></td>
<td></td>
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<tr>
<td>JEN</td>
<td>–</td>
<td>0.34</td>
<td>0.32</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PFB</td>
<td>–</td>
<td>0.28</td>
<td></td>
<td>0.24</td>
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<td></td>
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<tr>
<td>SVN</td>
<td>–</td>
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<td></td>
<td>0.26</td>
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</table>

NIN, Ninfa; CDA, Capo d’Acqua; ZLS, Zombo lo Schioppo; JEN, Jenne; PFB, Posta Fibreno; SVN, San Venanzio.
analysis, striking differences were observed between the two samples from Ninfa (NIN05 and NIN14). NIN05 was dominated by the ME mitochondrial lineage (haplotype MEcs01, GenBank: AY836350, n = 14, 87.5%), with a few AT sequences (haplotype 3, GenBank: AF274574, n = 2, 12.5%); conversely, the majority of sequences from NIN14 (n = 18, 78.3%) shared a haplotype belonging to the AD lineage and so far reported only from Posta Fibreno (AD-PostaFibreno, GenBank: JQ314219), while the ME lineage represented only a minor fraction of individuals (haplotype MEcs01, n = 3, 1.3%; new haplotype, GenBank: MG194732, n = 1, 0.4%) and there was one AT sequence (haplotype 3, GenBank: AF274574, 0.4%).

Among the 20 NIN14 individuals for which both microsatellite and mtDNA data were available, a perfect match was observed between the presence of the AD-PostaFibreno haplotype and the estimated membership in the Posta Fibreno-based cluster 1 in STRUCTURE. The 15 individuals carrying AD-PostaFibreno had cluster 1 membership ranging from 0.21 to 0.98, while, for the five individuals carrying other haplotypes, cluster 1 membership ranged from 0.004 to 0.053 (Figure 3b).

Sequences from ZLS represented two new haplotypes in the AD lineage (GenBank: MG194729, n = 4, 40% and MG194730, n = 3, 30%) with three AT sequences (haplotype 3, GenBank: AF274574, 0.4%).
DISCUSSION

The analyses showed that the Ninfa trout population bears deep genetic traces of human intervention and evidenced a dramatic change in the Ninfa gene pool between 2005 and 2014. The NIN05 sample was more similar to the hatchery sample JEN than to any other wild-caught population of the study ($F_{ST} = 0.15$, vs $>0.21$), and NIN05 genotypes were remarkably close to JEN genotypes in the space of principal components (Figure 2). Moreover, the STRUCTURE analysis detected a very high prevalence in NIN05 of the same genetic cluster (96.0%, Figure 3b). It is worth remarking that the JEN sample represents a hatchery (Centro Ittiogenico Provinciale di Roma) that has been regularly used for restocking trout populations in central Italy (although, as far as known not directly those of the area of Ninfa). The JEN sample used in this study has been previously analysed by Gratton et al. (2014) and was found to be similar to samples collected in northern Italy and of known Atlantic/hatchery origins. Moreover, all sequenced mtDNA from JEN showed AT haplotypes. Therefore, the JEN sample is probably a proper benchmark of typical Atlantic S. trutta used for restocking in Italy. The NIN05 sample also showed unambiguous allochthonous mtDNA (12.5% AT lineage, Figure 4). Previous genetic studies on Italian trout have revealed that mtDNA can underestimate the amount of allochthonous genes (e.g. Caputo et al., 2004; Gratton et al., 2007; Marconato et al., 2006; Nonnis Marzano et al., 2003), a pattern that might result from over-representation of males among juvenile trout typically used for restocking (Rasmussen, 1986). Overall, the NIN05 sample appeared to contain a substantial amount of non-autochthonous genes, most likely originating from stocking with hatchery S. trutta of Atlantic origin. By contrast, the proportion of microsatellite gene pool attributable to Atlantic contribution in NIN14 was lower than in NIN05 (Figures 2 and 3b), and NIN14 showed evidence of Posta Fibreno origins at both nuclear and mitochondrial levels. Indeed, the PCA (Figure 2) placed NIN14 genotypes almost midway between NIN05 and PFB, and the most common STRUCTURE cluster in the PFB sample (cluster 1) was essentially absent from NIN05 (3.4%), but accounted for 32.4% of the NIN14 microsatellite gene pool. Moreover, the number of microsatellite private alleles (PrAll) dropped dramatically from NIN05 (PrAll = 11) to NIN14 (PrAll = 1) (Table 1), and the proportion of alleles shared with the PFB sample increased from 0.58 in NIN05 to 0.72 in NIN14. Finally, 75% of NIN14 individuals carried the unique PFB haplotype (GenBank: JQ314219)—a haplotype not present in NIN05 and so far observed only among trouts of Posta Fibreno, where it is apparently fixed (Gratton et al., 2013). Given the fairly large sample sizes for NIN05 and NIN14 (see Methods section), both samples being collected across several different locations within the Gardens of Ninfa, and the relative isolation of Ninfa from other bodies of water suitable for salmonids, it seems extremely unlikely that the differences among the two samples can be explained with undetected spatial genetic structure or sampling error. In theory, a temporal change in microsatellite allele frequencies could be interpreted both as the result of selection against non-native genotypes (e.g. Kovach et al., 2016) and of introduction of individuals from other populations; however, the sudden appearance of PFB microsatellite alleles, of a STRUCTURE
cluster linked to PFB and, most of all, of a mitochondrial haplotype previously found nowhere else than in Posta Fibreno, points to unreported translocation from Posta Fibreno to Ninfa between 2005 and 2014. Moreover, the close correspondence between mtDNA and microsatellite data, where NIN14 individuals with Posta Fibreno mtDNA had a high membership in the STRUCTURE cluster that dominates in the PFB sample (Figure 3b), strongly suggests incomplete recombination and, thus, is consistent with a very recent introduction of trout. Indeed, the STRUCTURE analysis assigned four of the NIN14 individuals almost entirely to the PFB cluster 1 (membership range: 0.91–0.98). The distribution of estimated membership seems to cluster around 0, 0.25, 0.5, 0.75 and 1, suggesting that most NIN14 fish may be F1 offspring or F2 backcrosses, resulting from a recent introduction from PFB. This result is consistent with the 9-year interval separating the collection of NIN05 and NIN14 samples and with a typical trout generation time of 3–4 years.

The Posta Fibreno trout populations are known to show one of the lowest levels of introgression from commercial strains in the central Mediterranean basin (Gratton et al., 2013; Pujolar et al., 2011), probably because the stocking activities into the lake ceased 30–40 years ago (Gratton et al. 2013). These populations consists of two distinct gene pools and two main phenotypic classes (S. fibreni and S. cettii) that are, however, not completely isolated, as hybridisation between the two forms is common, although different ecological specialisation (e.g. temporal and spatial discrepancies in spawning) seems to limit gene flow and maintain genetic differentiation (Gratton et al., 2013). Despite the potential interest of this population as a source for restocking with essentially autochthonous trout, no official records of fish introductions from Posta Fibreno to Ninfa exist, and this study shows the importance of diachronic genetic monitoring of fish populations to reveal unrecorded (and potentially illegal) management.

Despite the strong genetic signatures of introductions from commercial strain of Atlantic origin and from Posta Fibreno, Ninfa gene pool still shows traces of a possible native substrate. In particular, the Ninfa population shared the Mediterranean haplotype MEcs01 with CDA, an area ~70 km distant that shows similar habitat characteristics but is hydrologically disconnected (Figure 1). However, sharing the same haplotype does not necessarily imply having a close genetic relationship, as MEcs01 is a common and widespread variant in the western Mediterranean basin (Bernatchez, 2001). The nuclear gene pools from CDA and Ninfa seem to be quite different, and in both the PCA and the STRUCTURE analyses, CDA does not cluster with Ninfa (Figure 2b and 3b). Indeed, although the current STRUCTURE includes CDA, ZLS and SVN within the same cluster (for K = 3, Figure 3b), pairwise FST values for CDA are all larger than 0.25, and its genetic distinctiveness is also evident in the PCA (Figure 2b). The pairwise FST matrix (Table 2) suggests that the binning of CDA, ZLS and SVN in STRUCTURE is an artefact, likely a result of the hierarchical structure of the data (which contains both Atlantic-originated hatchery and native Italian samples) and of smaller sample sizes of these three sites, compared with those of Posta Fibreno and Ninfa (see Peuchmaire, 2016).

Genetic drift might have had an important effect on the differentiation of CDA from the other populations. Indeed, while the other populations showed expected heterozygosities up to 0.71 and allelic richness up to 5.6, H0 of CDA was only 0.42 and its allelic richness 3.5, all factors that seem to point towards past genetic drift events. Nevertheless, nowadays, the population seems to be in good conditions. A size estimate was not feasible, but the lack of effort in catching the fish for sampling, and the feedback received from local fishermen seem to confirm a large stable (or even increasing) population size. In summary, given the absence of obviously allochthonous alleles, the presence of a widely spread ME haplotype and its apparent genetic uniqueness, CDA might represent a population with autochthonous origins, retaining at least some degrees of naturality and it deserves further attention, especially because of the very small size of the water bodies it depends on.

The ZLS population also appeared unique in the analyses. Unexpectedly, it was not particularly close to PFB, despite the two locations are only ~50 km apart, along rivers within the Liri river hydrographic system (Figure 1). Rather, ZLS shows some similarity with SVN, as they are the last to be separated in the PCA (on PC6, Figure 2c) and are each other’s closest samples in the pairwise FST matrix (Table 2). A possible explanation of this similarity between two populations from two different catchments (ZLS is in the Tyrrhenian side of the peninsular watershed, while SVN is in the Adriatic side, Figure 1) might lie in ZLS and SVN belonging to the same administrative province (L’Aquila). Therefore, the two populations might have received trout stocking from common sources in the past years, or fish originating from SVN might have been introduced in the ZLS area.

San Venanzio itself is regarded as a trout population with a high degree of naturality, and the site has been selected as a Site of Community Importance (SCI, code IT7140096). Historically, the fishing activities in this area have been generally limited, while trout from other sites on the same river Aterno (see Figure 1) have been under stronger fishing and stocking pressure (Seminara, unpublished data). Moreover, trout from SVN do not exhibit Atlantic mitochondrial haplotypes (see Figure 4) and have been previously estimated to possess only a small fraction of allochthonous nuclear alleles (15%, Gratton et al., 2007), suggesting autochthonous origins of the population. The strong differentiation between ZLS and PFB highlights the restricted gene flow along the Liri basin, a pattern that is frequently observed in salmonids (Fumagalli et al., 2002; Pujolar et al., 2011; Vallesstad et al., 2012) and that is consistent with the low quality of the Liri river waters between the two locations (Distretto Idrografico dell’Appennino Meridionale, 2010).

4.1 Management implications

The high prevalence of Mediterranean mitochondrial haplotypes and some variation at microsatellite loci that could not be traced back to known potential sources indicate that the population of Ninfa, despite significant Atlantic admixture and traces of recent translocations from Posta Fibreno, might still retain some unique genetic features. More important, this study revealed unrecorded stocking into Ninfa from Posta Fibreno or from a very closely related source. Although the two areas are very similar, implications of unrecorded management...
could be severe, and potential scenarios should be evaluated. In general, the introduction of individuals with a different gene pool causes genetic homogenisation and can compromise potential adaptations of the local population, with consequent reduction in fitness and loss of genetic diversity (McKinney & Lockwood, 2001; Araki et al., 2008). Therefore, measuring the level of hybridisation and introgression of exogenous gene pools in wild populations is of primary concern in developing conservation and management strategies (Hickley & Chare, 2004; Krueger & May, 1991).

At the same time, carefully planned in- and ex-situ management might be useful when especially valuable populations face a critical threat (e.g. Witzenberger & Hochkirch, 2011). Posta Fibreno freshwater habitats have been undergoing a dramatic change in the last years: the coverage of submerged macrophytes has drastically reduced (from about 80% to 25%, Seminara & D'Orsi, 2011), cultivated areas have been increased around the riparian edges of the river (with consequent reduction of submerged macrophytes has drastically reduced). These factors might be useful when especially valuable populations face a critical threat. Therefore, measuring the level of hybridisation and introgression of exogenous gene pools in wild populations is of primary concern in developing conservation and management strategies (Hickley & Chare, 2004; Krueger & May, 1991).

However, to ensure a successful management programme in Ninfa (and Posta Fibreno), the status of the local population should be continuously monitored over time. Similarly, an analysis on more genetic markers, including the whole Italian peninsula and islands, would help clarifying the origins and connections of the local populations within the network. Finally, a direct and constant involvement of the fishery community has been proven to facilitate successful conservation projects (Granek et al., 2008) and the participation of recreational fishers, anglers associations, together with the correct broadcasting of scientific results (Waples & Cope, 2008) would be of fundamental importance for a long-term conservation programme of Italian trout.

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