Current status of the brown trout (*Salmo trutta*) populations within eastern Pyrenees genetic refuges

- **SALMONID SYMPOSIUM GIRONA**-

Araguas, R.M. 1*a*
Vera, M. 1*a*
Aparicio, E. 1
Sanz, N 1
Fernández-Cebrián, R. 2
Marchante, C. 3
García-Marín, J.L. 1

1 Laboratori d’Ictiologia Genètica, Department of Biology, Faculty of Sciences, Universitat de Girona. Campus of Montilivi, E-17071 Girona, Spain
2 Geneaqua S. L. C/ Primavera, Nº 40, 1ºB. E-27002 Lugo, Spain
3 Direcció General del Medi Natural i Biodiversitat, DAAM, Generalitat de Catalunya, C/Dr. Roux 80, E-08017 Barcelona, Spain

* to whom correspondence should be addressed

TLF +34 982 82 24 28
Fax +34 982 82 24 28
e-mail: rosa.araguas@udg.edu

*a These two authors contributed equally to this work and are first co-authors

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Abstract

Since the end of the 20th century some headwaters of rivers in the eastern Pyrenees have been designated as genetic refuges to protect remaining native brown trout (Salmo trutta) diversity. The declaration was based on limited or no evidence of genetic impact from released non-native Atlantic hatchery fish. Hatchery releases were completely banned into the genetic refuges but pre-existing fishing activities were maintained. Specific locations in each refuge have been monitored every 2-3 trout generations to update genetic information to accurately assess the contribution of these reservoirs to the preservation of native brown trout gene pools. This work updates genetic information to year 2014 in 3 of these locations (in Ter, Freser and Flamisell rivers). Previous studies identified hatchery introgressed populations within refuges, and suggested discrepancies between the underlying intention of the genetic refuges and the gene pools detected. Therefore, we also examined genetic divergences among locations inside refuge river segments. Combined information at five microsatellite and the lactate dehydrogenase C (LDH-C*) loci showed reduced but significant temporal native allele frequency fluctuations in some of the above specific locations that did not modify overall levels of local diversity and river divergences. Bayesian clustering analyses confirmed the presence of differentiated native units within each genetic refuge. Some locations of the Freser River within the genetic refuge area showed high hatchery impact of non-native fish (over 20%). We discuss additional local actions (releases of native fish, selective removals and fishery reinforcement with sterile individuals) to improve the conservation objective of genetic refuges.
Preservation of genetic diversity within species maintains their evolutionary potential and thereby the long-term conservation of the species (Ryman et al. 1995; Hurt & Hedrick 2004; Utter 2004). However, rates of anthropogenic hybridization and introgression are increasing dramatically worldwide because of intentional translocations of organisms and habitat modifications by humans (Allendorf et al. 2001; Champagnon et al. 2012; Chunco 2014). This situation is especially significant in game species, where captive-bred animals derived from native, alien, or hybrid stocks are often released in large numbers into the wild with the intention of reinforcing exploited populations (Mamuris et al. 2001; Negro et al. 2001; Vernesi et al. 2003; Barilani et al. 2005). The loss of native gene pools through hybridization is particularly widespread in aquatic species (Moyle & Leidy 1992; Hanfling 2007). The problem is well known in salmonids, where for several decades hatchery-reared fish have either escaped or have been released deliberately into wild populations (Allendorf et al. 2001; Valiquette et al. 2014) with the aim to enhance recreational opportunities (Brown & Day 2002; Arlinghaus & Mehner 2005; Cowx et al. 2010). Salmonids have experienced declines on native freshwater biodiversity (Lewin et al. 2006; Naish et al. 2007) due to exotic species introductions, overfishing and/or release of non-native stocks (Cowx & Gerdeaux 2004). Also, on-going climate change has increased the vulnerability and endangered status of salmonid species worldwide (e.g. Hari et al. 2006; Almodovar et al. 2012; Vera et al. 2013).

Mediterranean brown trout populations have evolved through complex evolutionary processes involving secondary contacts between ancient lineages, and local adaptations (Sanz et al. 2002; Aparicio et al. 2005; Snoj et al. 2008; Vera et al. 2010). However, these populations have been extensively compromised with genetically divergent North-eastern Atlantic stocks to support recreational fisheries in the entire territory, and the displacements of native gene pools are well documented (Poteaux &
Berrebi 1997; García-Marin et al. 1998; Marzano et al. 2003; Jug et al. 2005; Sanz et al. 2006; Apostolidis et al. 2008). In the eastern Pyrenees, successful hatchery releases in wild populations have been estimated to increase the average individual introgression rate between 1% and 5% in a single year (García-Marin et al. 1999, Araguas et al. 2004). Thus, continuation of these hatchery releases with foreign stocks is presumably going to dramatically erode native diversity and population structure in this century (Fernández-Cebrián et al. 2014).

In order to preserve the native gene pools remaining in wild populations from eastern Pyrenees river basins, the Autonomous Government of Catalonia changed fishery policies in 1997 to exploit new approaches that balanced harvest and conservation of wild genetic resources. With the aim to address both conservation of remnant native gene pools and transition toward self-sustained recreational fisheries, several river segments were designated as ‘genetic refuges’ based on the genetically demonstrated native status of their brown trout populations. Within each refuge, the previous fishery status was maintained, but releases from hatchery stocks were completely banned. In addition, the stocking ban was accompanied with measures to promote a self-sustaining fishery, with restriction on the number of captures and on the length of removed fish (revised in Araguas et al. 2008). This management action is different from genetic sanctuaries defined by Poteaux and Berrebi (1997), which are areas where neither stocking nor fishing are permitted. Genetic refuges have also been implemented in French Mediterranean brown trout populations since 2005 (Caudron et al. 2011, 2012).

Genetic monitoring of trout populations within genetic refuges is essential to assess the contribution of the management strategy to the ultimate objective of preserving native brown trout gene pools. Such an approach was undertaken in the eastern Pyrenees from a set of 10 selected locations (hereafter reference locations). Results from 1993 to 2006 monitoring are summarized in two papers by Araguas et al. (2008, 2009). Their
findings indicated that the establishment of a genetic refuge did not reduce the average regional abundance of the foreign stock alleles, though the policy of genetic refuges controlled the increase of introgression from 1993 to 1999, and maintained major trends in the pattern of population structure. Similar results were reported from wild French trout populations in the Mediterranean Alpine rivers where genetic refuges policies were also implemented (Caudron et al. 2011, 2012).

At local scale, some monitored reference locations increased abundance of hatchery alleles as a consequence of upstream migration of admixed or released hatchery fish from non-refuge areas (Araguas et al. 2008). Therefore, the monitoring of genetic refuges using the information from only a few locations could result in an incomplete picture where some highly introgressed populations within refuges are protected, representing a serious threat for native populations in surrounding river sections (Araguas et al. 2008, 2009). Thus, it is important to maintain the monitoring and the collection of genetic information from trout populations both within genetic refuges and in adjacent areas. Unfortunately, after 2006 no more monitoring studies have been done in these eastern Pyrenees populations. This present work updates to year 2014 genetic information within three refuges (including reference locations used to define genetic refuges) in Ter, Freser and Flamisell rivers with the aim: i) to assess changes on levels of introgression at these populations after 17 years of the designation of the first genetic refuges and ii) to examine genetic divergences among locations from different river segments within refuges.
**Material and Methods**

**Sampling sites**

A total of 603 individuals from 15 locations distributed across three genetic refuges (Ter, Freser and Flamisell drainages, Table 1, Fig. 1) were collected in 2014. Temporal data from previous studies were available for four sampled locations (Vallter in Ter, Nuria and Queralbs in Freser, and Manyanet in Flamisell, 576 individuals) mainly from reference locations used to establish the genetic refuges in the studied area (see Table 1). Finally, 90 individuals from the Baga hatchery stock were included. This stock is commonly used for releases on the studied rivers (Araguas et al. 2004; Fernández-Cebrián et al. 2014).

**Analyses of molecular markers**

For the 2014 collections whole genomic DNA was obtained from a piece of adipose fin using the Chelex® Resin procedure described by Walsh et al. (1991). These samples were genotyped at microsatellite loci and LDH-C*. Based on published genotype information from 2006 collections at nine loci (Fernández-Cebrián et al. 2014), we designed a single PCR multiplex of five loci (SsHaeIII14.20, Str591INRA, Str73, Ssa85 and SSoSL438) which produced similar estimates on diversity and introgression indices. According to previous studies on trout populations of the region (Sanz et al. 2009), these five loci detected significant divergence among wild populations ($F_{ST}$ range: 0.109 locus Str73 - 0.393 locus Str591INRA) and among wild and hatchery fish ($F_{ST}$ range: 0.082 locus Ssa85 - 0.382 locus Str73). Amplifications were performed in 10 µL volumes which consisted of 1 µL template DNA (~30 ng) in 1X PROMEGA GoTaq®G2 Hot Start Colorless Master Mix (containing GoTaq®G2 Hot Start DNA Polymerase, Multiplex PCR Buffer kit, MgCl₂, dNTP mix, 0.1 µM of both forward and reverse PCR primer for SsHaeIII14.20, Ssa85, Str73 and SSoSL438, and 0.4 µM of both forward and reverse PCR primer for Str591INRA). Thermal cycling was conducted on a Verity ™ 96-Well Thermal Cycler (Applied Biosystems) as follows:
initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 95 °C for 1 min, 54 °C (annealing multiplex temperature) for 1 min, and extension at 72 °C for 1 min. There was a final extension step at 72 °C for 15 min. PCR products were resolved by using an ABI PRISM® 3730 automatic sequencer (Applied Biosystems). The LDH-C* genotypes for all these samples were obtained following the primers and protocol described by Chat et al. (2008), which allows the analysis of this marker together with microsatellite loci in an automatic sequencer. Allele scoring was performed with GeneMapper 4.0 software (Applied Biosystems).

*Genetic diversity, introgression impact and population structure studies*

Allele frequencies and genetic diversity within-populations (average number of alleles per locus \(N_a\), average allelic richness \(A_r\), observed \(H_o\) and expected heterozygosity \(H_e\)) were estimated using FSTAT 2.9.3 (Goudet, 2001) for all loci (microsatellites and LDH-C*). Allelic richness was standardized to the smallest population sample in our data set using the rarefaction method (Elmousadik & Petit, 1996) implemented in FSTAT. Deviation from Hardy–Weinberg (HW) expectations for each locus in each population was estimated using GENEPOP 4.0 (Rousset, 2008). MICROCHECKER 2.2.3 (Van Oosterhout et al. 2004) was used to check for genotyping accuracy and to detect homozygote excess evenly distributed among homozygote size classes at specific locus, which may be interpreted as evidence of null alleles.

Introgression levels into each wild location were estimated by the LDH-C*90 allele frequency and the proportion of introgressed genome \(q\) in each sampled wild fish, calculated using microsatellite genotypes. These \(q\) values were calculated by the Bayesian Markov Chain Monte Carlo (MCMC) approach method using the STRUCTURE Program ver. 2.1 (Pritchard et al. 2000), following the incomplete baseline method assuming an admixture model with two populations (hatchery and native) as described by Sanz et al. (2009). Individuals from Baga hatchery formed the
reference collection for hatchery gene pools. A burn-in period of 50,000 steps followed by 200,000 Monte Carlo replicates was used in these runs. For each sample, ten replicates of the STRUCTURE run were obtained to assess the reproducibility of the estimated q values.

In addition, hybridization between native and hatchery fish was examined by the Bayesian statistical method implemented in the NEWHYBRIDS (Anderson & Thompson 2002). This method estimated the posterior probability (P) that each individual in a wild sample falls into different parental (native (Pnat) or hatchery (Phat)) or hybrid classes (F1, F2, Bnat, Bhat). For each data set, which included the wild populations and the Bagà hatchery genotypes, posterior probabilities were evaluated after 100,000 iterations of the Monte Carlo Markov Chains. The program ran without any prior information about the hybrid status of collected individuals and populations, and with the ‘Uniform’ prior option for both mixing proportions and allele frequencies.

Population differentiation was investigated from pairwise $F_{ST}$ using FSTAT and a significance test of 10,000 permutations. Analysis of the molecular variance (AMOVA; Excoffier et al. 1992) was used to study the distribution of genetic variation within and among genetic refuges according to geographical and temporal hierarchical models of population grouping. We also determined the number of genetically homogenous population groups (K) by minimizing Hardy–Weinberg and linkage disequilibrium using the Bayesian MCMC approach implemented in STRUCTURE v2.3.1 (Pritchard et al. 2000). Analysis for each genetic refuge was carried out under the admixture ancestral model with correlated allele frequencies, without prior population information with a burn-in period of 50,000 steps and 200,000 MCMC replicates. Ten independent runs were conducted for each tested K value (from “1” to “number of sampling sites within genetic refuge + 1”). The most likely K value was estimated following Evanno et al. (2005) recommendations using the program STRUCTURE HARVESTER v 0.6.92 (Earl & vonHoldt 2012). A factorial component analysis (FCA) using individual
genotypes was performed using GENETIX 4.05 (Belkhir et al. 2004). FCA uses individuals as operational units without the necessity of making assumptions of HWE and linkage equilibrium, so it can be useful to reveal cryptic structure among groups of populations in a scenario of highly differentiated populations as expected in brown trout (Araguas et al. 2004; Sanz et al. 2006; Vera et al. 2013).
Results

Genetic diversity and introgression impacts in Eastern Pyrenees locations

All microsatellite loci were polymorphic in all locations analysed (Table 2). Over all analysed loci (microsatellites and LDH-C*), the average number of alleles (Na) ranged from 2.5 in NU93 (Freser drainage) to 6.2 in AIG14 (Flamisell drainage). In 2014 collections the lowest average Na was 3.0 in MY14 (Flamisell drainage). Allelic richness (Ar) and gene diversity (HE) were minimum at TE06 (Ar = 2.170; HE = 0.358) and maximum at QB14 (Ar = 3.935; HE = 0.663). Concordant with the results found for Na, MY14 showed the lowest Ar (2.508) and HE (0.448) values for the 2014 collections.

After Bonferroni correction, 18 out of 156 HW tests were significant. HW deviations were mainly detected at the SsoSL438 (in TE14, NU14, PLA14, CAP14, LPM14, SEN14, ERI14 locations) and at the Str591INRA loci (in NU04, NU06, NU14, CAP14, ERI14, MY04 locations). MICRO-CHECKER analyses suggested homozygote excess as the most plausible explanation for HW deviations at the SsoSL438 locus, where null alleles, stuttering and drop-out, were not detected. Homozygote excess and presence of null alleles were suggested at Str591INRA locus, but neither stuttering nor drop-out was detected. At the rest of microsatellite loci no genotyping errors were indicated by MICRO-CHECKER results. No more than two loci presented HW deviations per location. Inbreeding coefficients (FIS) at each collection were low, except for NU14 (0.190), PLA14 (0.174), MY04 (0.095) which indicated heterozygote deficit (i.e. homozygote excess). All samples conformed to Hardy-Weinberg expectations for LDH-C* locus except NU14 due to heterozygote deficit (FIS = 0.870).

Estimates of hatchery introgression (q) calculated by STRUCTURE software from genotypes at the 5 microsatellite loci had higher background noise than the ones computed from 9 loci (Table 2). For instance, the pure native brown trout population in Vallter location showed values from 0.015 (TE14) to 0.023 (TE06). This range
overlapped with current estimates obtained in populations where previously analyses
recognized low impact of hatchery fish (e.g. collections from Nuria stream in years
2004 and 2006). However in these less impacted collections, bar plots of hatchery
ancestry showed some individuals with an estimated introgression rate that largely
exceeded the average q-value (Fig. 2), clearly confirming the existence of fish with
some degree of hatchery ancestry. In addition, the LDH-C genotypes confirmed
hatchery impact in all these locations. Thus, the combined information from the 5
microsatellite and the LDH-C* locus permitted to identify pure and hybrid trout
populations in the studied Pyrenean drainages. A significant positive correlation was
found between both introgression indices (frequency of LDH-C*90 allele and q values)
for natural locations (Spearman’s correlation coefficient (ρ) = 0.740, P < 0.001).
Estimates of hatchery introgression in 2014 collections from reference locations
suggested the preservation of pure Mediterranean trout populations in Vallter (TE14)
and the putative restoration of a pure native population in Manyanet (MY14) after the
hatchery introgression detected in 2004 and 2006 collections. In Nuria, it was a slightly
increasing of the hatchery introgression levels observed in 2004 and 2006. Exact
probability tests at the LDH-C* locus detected significant temporal changes in the allele
frequencies in Nuria and Manyanet locations (P < 0.05). The frequency of the LDH-
C*90 allele in these locations in 2004 suggested hatchery impacts between 1993 and
2004 rather than low detection in 1993 because reduced sampled sizes. In 2014, this
allele was not detected in Manyanet collection, but its frequency increased in Nuria
(Table 2).

Low hatchery introgression (<10%) was observed in 2014 in Ter and Flamisell basins,
where native fish predominated in all locations (Table 2). In the Freser River, results
indicated moderate (10-30%) impact of hatchery releases throughout the basin, but a
population of introduced hatchery fish was present in the tributary Segadell stream at
Pardines (PAR14). Accordingly, NEWHYBRIDS assigned a higher number of
individuals to hatchery and hybrid classes (i.e. $F_1$, $F_2$ and backcrosses) in Freser refuge than in Ter and Flamisell where hatchery fish or hybrids were practically undetected (Table S1). At a local level, collections with higher introgression impacts also had higher diversity levels, due to the significant genetic divergence among hatchery and native Iberian gene pools. In spite of that, there were not significant temporal changes on overall diversity indexes and gene diversity despite the $LDH-C^*90$ allele being not detected in 1993 collections (Table 3).

Genetic differentiation and population structure

All 2014 collections except PAR14 showed high and significant genetic differentiation with the BA hatchery stock ($F_{ST\ PAR-BA} = 0.040$). Large genetic divergence among the studied genetic refuges was also observed by pairwise $F_{ST}$ values (Table S2) and AMOVA analysis (Table 4). When BA location was excluded in the AMOVA analysis, the percentage of genetic differentiation was reduced from 24.41% to 14.34 % (the same value was obtained when PAR14 was excluded from the analysis). This observation reflected the high genetic differences among native Mediterranean and hatchery gene pools.

Comparisons between temporal collections in Ter, Nuria, Queralbs and Manyanet locations resulted in the lowest pairwise $F_{ST}$ values, which indicated small temporal fluctuations of gene pools at these locations (Table S2). Hierarchical AMOVA analysis of reference locations (Ter, Nuria and Manyanet sampling sites), assigned the lowest percentage of variation among temporal collections (1.41%) and the highest percentage of variation among genetic refuges (30.63 %) (Table 4). Over all sampled collections of the year 2014, AMOVA analyses indicated that the lowest but highly significant percentage of variation was among locations within genetic refuges (Table 4).

FCA analysis basically grouped individuals by basin (Fig. 3). However, individuals from PAR location and any other with estimated high proportion of hatchery genome were
placed close to the BA fish. STRUCTURE plots confirmed a higher introgression impact in the Freser refuge than in Ter and Flamisell refuges (Fig. 2). Several fish collected in the Freser River basin showed large proportion of ancestry of the BA cluster. Freser and Ter refuges, which are hydrographically closer (Fig. 1), were also genetically more similar in comparison with Flamisell refuge.

When basins with multiple sampling locations were analysed separately, STRUCTURE suggested the presence of three clusters in Freser drainage among 2014 collections: one corresponded to a hatchery genome and the other two to native ones. The proportion of hatchery genome was high in PAR14 and moderate in QA14 and QB14 (Fig. 2b). In Flamisell, STRUCTURE identified two genetic groups (Fig. 2c), none of them associated to a hatchery genome according to levels of hatchery introgression at these locations by microsatellites and the LDH-C* locus. The first cluster mostly distributed in the main stream in the Flamisell River and the other one in its tributary (Bóssia). It should be mentioned that when loci in HW disequilibrium (SsoSL438 and Str531) were removed from the STRUCTURE analyses, these native substructures faded away, probably due to the lower discrimination power of analyses based in just three microsatellite loci.

Discussion

Current status of brown trout populations in genetic refuges

Genetic analysis in 2014 on the reference locations of the studied genetic refuges showed disparate results. The most optimistic situation was perceived in the Ter River basin, where the genetic integrity of native brown trout population was preserved during 24 years of monitoring. However, according to the discussion below, this result should be taken with caution, because only one location has been analysed within Ter genetic refuge.

A positive situation was also suggested in Flamisell genetic refuge from results in temporal sampling of Manyanet location, where the moderate levels of introgression
noticed at 2004 collection disappeared in 2014. In addition, all the other 2014 Flamisell studied locations, within and outside of the genetic refuge, presented low levels of introgression and only three individuals were identified as possible hybrids using the method implemented in NEWHYBRIDS program (see Table S1). However, available genetic data from Filia River (a Flamisell tributary within the genetic refuge and not included in this study, see Fig. 1), indicated high introgression levels along temporal monitoring initiated 1993 and stopped in 2006 ($LDH-C^*90 > 0.300$; Araguas et al. 2008).

A different situation was found in the Freser genetic refuge, where a significant increasing of introgression levels and the detection of hatchery and hybrids fish (Table S1) occurred in several 2014 collections (including NU14 reference sample). Especially concerning was the situation in the Segadell stream at Pardines (PAR14 collection), where our results detected a naturalized hatchery population. This location, placed in one of the first genetic refuges established in 1997 (Araguas et al. 2004; 2008), has never been genetically studied before.

All these observations exemplify the limitations of sampling few or a single reference location to define and monitor genetic refuges. Genetic monitoring of few reference locations can give a rough impression of the average status of trout populations at regional scales, but is not informative of genetic changes at the local level (Fernández-Cebrián et al. 2014). The above situation described in the Freser basin shows how local events as described in PAR14 might be responsible for changes at a river scale. Thus, larger genetic surveys from different and distant locations along river basins are necessary to accurately define the situation and the future perspectives of genetic refuge areas. Such surveys would help to define strategies to avoid situations as described in the Freser, where a highly introgressed population is presently protected and threatens the surrounding native populations (García-Marín et al. 1998; Hitt et al. 2003; Araguas et al. 2009). Introgressed populations could also disturb inferences on
the native population structure (Sanz et al. 2011). Geographically extended surveys may be useful to detect genetically distinct native gene pools within a single genetic refuge, as observed in Flamisell and Freser refuges (Fig. 2, Fig. 3, Table S2), and hence to introduce local actuations even at intra-drainage level within a refuge. Significant genetic divergence among brown trout collections at short hydrological distances (few kilometres) within a drainage have been described in other rivers from the studied region (Sanz et al. 2011; Fernández-Cebrián et al. 2014), as well as in other European basins (e.g. Carlsson & Nilsson 2000). Restricted gene flow between trout populations of the main stream and nearby tributaries in the Norwegian Nordre Finnvikelv River were reported by Carlsson et al. (1999).

**Management recommendations**

In the eastern Pyrenees, the current Spanish legislation on biodiversity conservation (Article 52.2, Law 42/2007 of Natural Heritage and Biodiversity) forbids the releases of foreign stocks into freshwater ecosystems. In particular, the legislation prohibits the introduction of alien species, subspecies or geographic races and illegal transplantation of individuals when they are capable of competing with native wildlife, altering its genetic purity or the ecological balance. This restriction prevents future introductions and reinforces the role of genetic refuges for the conservation of brown trout native resources but, what happens with hybridized or hatchery populations already established? In spite of some isolation degree was revealed by contrasting levels of introgression at NU and QA locations in the absence of physical barriers, several studies support the spreading of hatchery genes from highly introgressed salmonids populations to neighbouring locations (García-Marín et al. 1998; Hitt et al 2003; Araguas et al 2008). Then, the dispersal of individuals from hybridized populations cannot be ignored as a possible threat that might contribute to the increase of hatchery introgression in adjacent populations. For instance, the highly introgressed collection of PAR14 in Segadell river might contribute to the introgression of neighbouring
populations, such as PLA and RF, due to the lack of hydrological barriers among them. Similarly, these trout could reach populations located upstream of small weirs in the main stream, such as QA and QB, because it is observed that some larger trout easily overpass these physical obstacles (Ordeix et al. 2011). Nevertheless, the highest introgression levels detected at QA and QB suggested that additional processes (e.g. direct releases or transplantations) are also involved in the observed introgression. Illegal transplantations were suspected in other locations of the region (Araguas et al. 2009).

The establishment of genetic refuges in Mediterranean rivers has not resulted in a rapid and significant decrease of hatchery alleles at regional scale (Araguas et al. 2008, 2009; Caudron et al. 2011, 2012). The maintenance of fishery pressure in some river stretches has prevented the increase of introgression from hatchery releases due to selective angling on released non-native fish (Mezzera & Largiader 2001; García-Marín et al. 1998; Harkonen et al. 2014). However, maintaining fishery within genetic refuges has not been effective enough for recovering native gene pools in introgressed populations (Araguas et al. 2008, 2009; Caudron et al. 2012). Thus, while genetic refuges have the potential to prevent further introgression, additional measures should be implemented to recover native gene pools, particularly within protected areas (Araguas et al. 2008, 2009; Caudron et al. 2012). In these situations, different approaches can be used to the restoration of native gene pools; the most popular are the supplementation with native individuals and/or the selective removal of hybrids and hatchery naturalized fish.

Native stock reinforcement following habitat recovery is an accepted measure within the European Inland Fisheries Advisory Commission (EIFAC) code of practice for recreational fisheries (FAO 2008). The supplementation of native individuals could be achieved by stocking with local native breeding stocks and/or by translocations of wild native individuals. Both actions have been reported to be successful in northern French...
Alps brown trout populations. For instance, stocking with fry from a native stock has led to the restoration of a functional Mediterranean trout population in the Ugine River (Caudron et al. 2006). The translocations of native Mediterranean trout also resulted in a significant decrease in the percentages of non-native alleles in the Borne River (Caudron et al. 2012).

A simulated supplementation program using native brown trout stocks predicted the recovery of native diversity in populations highly impacted by releases of a foreign hatchery stock (Fernández-Cebrián et al. 2014). Nevertheless, the supplementation with native individuals appears to be ineffective on self-sustaining non-native or hybrid populations, where the release of native fish did not restore the native gene pool and led to further introgression (Caudron et al. 2012). Theoretical studies demonstrated reduced effective population size \( (N_e) \) and fitness of wild populations following supportive breeding (Wang & Ryman 2001; Ford 2002), and a decline in local populations following long-term supplementation by native domestic stocks (Satake & Araki, 2012). In spite of these limitations, supplementation from native stocks might balance social benefits of angling and biological damage to native diversity in the regional river basins (Fernandez-Cebrián et al. 2014).

Selective removal of non-native individuals and hybrids seems necessary to recover native gene pools in populations such as the Pardines (PAR14), within the Freser genetic refuge. In the case of hatchery naturalized populations, this action can be carried out promptly. However, in other cases the implementation of this strategy is predicted to be more difficult, as it requires a quick and easy tool to distinguish between Mediterranean native and hatchery and hybrid fish in the wild. Several phenotypic characters such as the spotting pattern, the size of the spots on body flanks, the number and shape of parr marks, and the colour of margins of the anal and dorsal fins have been proven to be useful for a visual distinction between Atlantic and Mediterranean trout (Lascaux 1996; Mezzera et al. 1997; Aparicio et al. 2005).
classification tree model proposed, using three variables of coloration and spotting pattern very easily measurable in the field, could be used as a tool to distinguish among Mediterranean, Atlantic and hybrid fish in low and moderate introgressed populations (Aparicio et al. 2005). Despite total removal of exotic alleles seeming impractical over a short time, a significant reduction of the introgression rate is expected if individuals classified as non-native and hybrid are removed from population. To demonstrate the efficiency of these selective removals, several years are needed and shallow stretches have to be selected as candidates due to the limited fish catchability by electrofishing in deep water (Carmona-Catot et al. 2010; Caudron & Champigneulle, 2011).

The Spanish legislation regulating recreational fishing activities considered sustainable management essential to ensure that the exploitation of the resource harmonizes with its optimal conservation. To achieve this goal and avoid introduction of alien specimens, some authors suggested that fishery reinforcements should be done with sterile individuals such as triploid (Piferrer et al. 2009). Certainly, the use of controlled sterile individuals allows reaching equilibrium between recreational fishing (i.e. economical resources) and conservation of wild resources. The production of triploids is simple and cheap, involving either physical treatment (temperature or pressure shocks) or the application of chemicals (Thresher et al. 2014). Moreover, because triploidy does not involve manipulations of individual chromosomes or genes, they are not widely considered to be "genetically modified". A protocol of optimization and production of triploid individuals in S. trutta have been recently described (Preston et al. 2013). Nonetheless, triploids are not always 100 % sterile (Normand et al. 2008). While triploid fish females are always 100% sterile because their ovaries never develop, triploid males can produce functional spermatozoa and attempt to spawn with females, competing with wild males (Piferrer et al. 2009, Fjelladal et al. 2014). Legislation introduced by the British Environment Agency to protect wild trout and preserve the
recreational fisheries of the species allows stocking practises in enclosed waters using
either sterile triploid all-female stocks or fish from breeding programs using local
broodstocks (EA, 2009). Similar practises could be also applied over the Pyrenean
brown trout populations. No differences in performance and behaviour have been
suggested between stocked diploid and triploid individuals (Budy et al. 2012). However,
density-dependent mortality has been described in brown trout (Lobón-Cervia 2012,
Richard et al. 2015), indicating that releases of individuals, both sterile or not, would be
expected to disturb wild populations. Therefore, more research on the ecological
impact of stocking triploids into the wild is still necessary (Preston et al. 2013, Fjelldal
et al. 2014).

Several guidelines to improve management based on genetic refuges were previously
presented in Araguas et al. (2009). However, according to the genetic results
presented in this work some new recommendations are necessary. First, large
geolectrically extended surveys covering the entire basin are essential to identify
genetic entities within refuge areas and to monitor genetic changes occurred to
populations within and surrounding the refuge areas. Second, long term monitoring and
action in highly introgressed populations is necessary to recover native gene pools (i.e.
selective removal, releases by native stocks, translocations) to prevent genetic
changes resulting in loss of local adaptations. Third, despite sterile triploid trout being
within the current legislative restrictions on the use of foreign stocks to reinforce
recreations fisheries, such releases should be monitored to avoid detrimental effects
(e.g. ecological competition, introduction of diseases, etc.) on recipient wild brown trout
populations. Finally, to limit expenses, we suggest an optimization of the molecular
tools to the minimal number of informative loci needed to assess management actions.

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Figure Captions

**Figure 1.** Geographical situation of sampled locations, shaded areas delineate refuges. Codes are defined in Table 1. 1) Ter and Freser. 2) Flamisell genetic refuges. Triangle indicates the geographical situation of Filia location (not analysed in this study, but see text). Physical barriers (mostly small dams below 3 m) are also represented.

**Figure 2.** Bayesian STRUCTURE results. a) All 2014 collections and Baga hatchery stock, $K=3$ ($K=2$, mean $\ln P=-10277.32$; $K=3$, mean $\ln P=-9316.29$; $K=4$, mean $\ln P=-9077.26$) b) 2014 collections from the Freser genetic refuge, $K=3$, ($K=2$, mean $\ln P=-2893.34$; $K=3$, mean $\ln P=-2752.84$; $K=4$, mean $\ln P=-2734$) c) 2014 collections from the Flamisell genetic refuge, $K=2$, ($K=1$, mean $\ln P=-4512.7$; $K=2$, mean $\ln P=-4412.74$; $K=3$, mean $\ln P=-4337.02$). Each individual is represented by a vertical bar partitioned into segments according to the proportion of the genome assigned to each of the identified clusters. Codes are defined in Table 1.

**Figure 3.** Factorial Component Analysis (FCA) among studied individuals. Blue, orange, green and red colours represent respectively fish sampled at Freser, Ter, Flamisell refuges and Baga hatchery. Location codes are shown on Table 1.
Table 1. Locations and number of individuals (N) analysed in the present study.

| Locations          | Drainage          | Genetic Refuge  | Year sampled | Code | N   |
|--------------------|-------------------|-----------------|--------------|------|-----|
| Vallter*           | Ter               | Since 1997      | 1990         | TE90 | 15  |
|                    |                   |                 | 2004         | TE04 | 55  |
|                    |                   |                 | 2006         | TE06 | 65  |
|                    |                   |                 | 2014         | TE14 | 53  |
| Nuria*             | Nuria-Freser      | Since 1997      | 1993         | NU93 | 28  |
|                    |                   |                 | 2004         | NU04 | 71  |
|                    |                   |                 | 2006         | NU06 | 155 |
|                    |                   |                 | 2014         | NU14 | 41  |
| Queralbs           | Freser            | Since 1997      | 2004         | QA04 | 14  |
|                    |                   |                 | 2014         | QA14 | 39  |
| QueralbsB          | Freser            | Since 1997      | 2014         | QB14 | 41  |
| Planoles           | Rigard-Freser     | Since 1997      | 2014         | PLA14| 38  |
| Pardines           | Segadell-Freser   | Since 1997      | 2014         | PAR14| 10  |
| Ribes de Freser    | Freser            | Since 1997      | 2014         | RF14 | 34  |
| Capdella           | Riqüerna-Flamisell| Since 2002      | 2014         | CAP14| 47  |
| Aiguabella         | Flamisell         | Since 2002      | 2014         | AIG14| 49  |
| La Plana de Montrós| Flamisell        | No              | 2014         | LPM14| 35  |
| Senterada          | Flamisell         | No              | 2014         | SEN14| 32  |
| Erinya             | Flamisell         | No              | 2014         | ERI14| 41  |
| Avellanos          | Valiri-Flamisell  | Since 2002      | 2014         | AVE14| 23  |
| Sarroca de Bellera | Bossia            | No              | 2014         | SAR14| 48  |
| Manyanet*          | Manyanet-Flamisell| Since 2002      | 1993         | MY93 | 23  |
|                    |                   |                 | 2004         | MY04 | 46  |
|                    |                   |                 | 2006         | MY06 | 104 |
|                    |                   |                 | 2014         | MY14 | 50  |
| Baga Hatchery      |                   |                 | 2003         | BA   | 90  |

* Locations initially used to define Genetic Refuges
Table 2. Gene diversity and estimates of introgression levels for locations analysed in the present study.  

| Collection | N  | Na | AR | HE | FIS | PIW | q Value | LDH-C’90 |
|------------|----|----|----|----|-----|-----|---------|----------|
| TE90       | 15 | 2.7| 2.330| 0.427| -0.014| 0.686| 0.020 (0.003) | 0.000^A |
| TE04       | 55 | 3.3| 2.285| 0.386| 0.039| 0.630| 0.017 (0.005) | 0.000^A |
| TE06       | 65 | 2.8| 2.170| 0.358| 0.005| 0.648| 0.023 (0.004) | 0.000^A |
| TE14       | 53 | 3.7| 2.308| 0.379| -0.012| 0.002| 0.015 | 0.000 |
| NU93       | 28 | 2.5| 2.254| 0.382| -0.028| 0.789| 0.021 (0.002) | 0.000^A |
| NU04       | 71 | 4.8| 2.703| 0.430| 0.055| 0.000| 0.026 (0.032) | 0.035^A |
| NU06       | 155| 5.8| 2.660| 0.426| 0.030| 0.000| 0.016 (0.024) | 0.021^A |
| NU14       | 41 | 4.7| 3.252| 0.504| 0.190| 0.000| 0.034 | 0.110 |
| QA04       | 14 | 4.0| 2.982| 0.474| -0.055| 0.272| 0.054 | 0.038 |
| QA14       | 39 | 5.5| 3.818| 0.659| 0.028| 0.260| 0.190 | 0.319 |
| QB14       | 41 | 5.8| 3.935| 0.663| 0.048| 0.018| 0.223 | 0.264 |
| PLA14      | 38 | 5.2| 3.471| 0.592| 0.174| 0.000| 0.033 | 0.129 |
| PAR14      | 10 | 3.5| 3.210| 0.523| 0.078| 0.272| 0.802 | 1.000 |
| RF14       | 34 | 5.2| 3.393| 0.543| 0.014| 0.021| 0.030 | 0.136 |
| CAP14      | 47 | 5.8| 3.743| 0.618| 0.065| 0.000| 0.034 | 0.053 |
| AIG14      | 49 | 6.2| 3.681| 0.589| 0.008| 0.063| 0.031 | 0.014 |
| LPM14      | 35 | 5.5| 3.497| 0.533| 0.027| 0.000| 0.036 | 0.014 |
| SEN14      | 32 | 5.2| 3.545| 0.565| -0.017| 0.006| 0.028 | 0.017 |
| ERI14      | 41 | 6.0| 3.729| 0.565| 0.082| 0.001| 0.049 | 0.013 |
| AVE14      | 23 | 3.7| 3.017| 0.491| 0.050| 0.010| 0.031 | 0.023 |
| SAR14      | 48 | 5.0| 3.043| 0.502| 0.042| 0.254| 0.027 | 0.000 |
| MY93       | 23 | 2.8| 2.488| 0.423| -0.044| 0.681| 0.022 (0.005)| 0.000^A |
| MY04       | 46 | 4.2| 2.873| 0.497| 0.095| 0.000| 0.053 (0.049)| 0.141^A |
| MY06       | 104| 3.7| 2.599| 0.436| 0.002| 1.000| 0.019 (0.008)| 0.024^A |
| MY14       | 50 | 3.0| 2.508| 0.448| 0.004| 0.216| 0.016 | 0.000 |
| BA         | 90 | 5.0| 3.152| 0.493| 0.039| 0.093| 1.000 | 1.000 |

^A from Araguas et al. 2008
Table 3. Genetic diversity pattern, in space and time, from reference locations (Ter, Nuria and Manyanet). \(N\): total sampled individuals (minimum local sampled size), \(A_R\): allelic richness, \(H_T\): total gene diversity, \(H_S\): average local gene diversity, \(F_{ST}\): population divergence, \(LDH-C^*90\): average frequency of the hatchery allele.

| Year | \(N\)  | \(A_R\) | \(H_T\) | \(H_S\) | \(F_{ST}\) | \(LDH-C^*90\) |
|------|--------|---------|---------|---------|------------|--------------|
| 1993 | 66 (15)| 2.625   | 0.600   | 0.407   | 0.313      | 0.000        |
| 2004 | 172 (46)| 3.154   | 0.637   | 0.434   | 0.309      | 0.052        |
| 2006 | 324 (65)| 2.912   | 0.622   | 0.414   | 0.331      | 0.015        |
| 2014 | 143 (41)| 3.194   | 0.636   | 0.438   | 0.308      | 0.037        |
Table 4. AMOVAs analyses grouping samples by Genetic Refuges.

|                         | F-statistic | Variance component | % Variation |
|-------------------------|-------------|--------------------|-------------|
| **2014 collections**    |             |                    |             |
| Among Genetic Refuges \((F_{CT})\) | 0.24406*** | 0.54639            | 24.41       |
| Among locations within Genetic Refuge \((F_{SC})\) | 0.07189*** | 0.12166            | 5.43        |
| Within locations        |             | 1.57070            | 70.16       |
| **2014 collections without BA** |             |                    |             |
| Among Genetic Refuges \((F_{CT})\) | 0.14337*** | 0.28572            | 14.34       |
| Among locations within Genetic Refuge \((F_{SC})\) | 0.07114*** | 0.12146            | 6.09        |
| Within locations        |             | 1.58577            | 79.57       |
| **2014 collections without BA and PAR14** |             |                    |             |
| Among Genetic Refuges \((F_{CT})\) | 0.14920*** | 0.29458            | 14.92       |
| Among locations within Genetic Refuge \((F_{SC})\) | 0.05376*** | 0.09030            | 4.57        |
| Within locations        |             | 1.58952            | 80.51       |
| **Reference locations for Genetic Refuge** |             |                    |             |
| Among Reference Locations \((F_{CT})\) | 0.30630*** | 0.56911            | 30.63       |
| Among temporal replicates within Reference Location \((F_{SC})\) | 0.02028*** | 0.02614            | 1.41        |
| Within locations        |             | 1.26276            | 67.96       |

*** \(P < 0.001\)
a)

b)

c)
