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

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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.

Introduction

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 &

52 Berrebi 1997; García-Marín et al. 1998; Marzano et al. 2003; Jug et al. 2005; Sanz et
53 al. 2006; Apostolidis et al. 2008). In the eastern Pyrenees, successful hatchery
54 releases in wild populations have been estimated to increase the average individual
55 introgression rate between 1% and 5% in a single year (García-Marín et al. 1999,
56 Araguas et al. 2004). Thus, continuation of these hatchery releases with foreign stocks
57 is presumably going to dramatically erode native diversity and population structure in
58 this century (Fernández-Cebrián et al. 2014).

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

74 Genetic monitoring of trout populations within genetic refuges is essential to assess the
75 contribution of the management strategy to the ultimate objective of preserving native
76 brown trout gene pools. Such an approach was undertaken in the eastern Pyrenees
77 from a set of 10 selected locations (hereafter reference locations). Results from 1993 to
78 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.

100 **Material and Methods**

101 *Sampling sites*

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

110 *Analyses of molecular markers*

111 For the 2014 collections whole genomic DNA was obtained from a piece of adipose fin
112 using the Chelex® Resin procedure described by Walsh et al. (1991). These samples
113 were genotyped at microsatellite loci and *LDH-C*.* Based on published genotype
114 information from 2006 collections at nine loci (Fernández-Cebrián et al. 2014), we
115 designed a single PCR multiplex of five loci (*SsHaeIII14.20*, *Str591INRA*, *Str73*, *Ssa85*
116 and *SSoSL438*) which produced similar estimates on diversity and introgression
117 indices. According to previous studies on trout populations of the region (Sanz et al.
118 2009), these five loci detected significant divergence among wild populations (F_{ST}
119 range: 0.109 locus *Str73* - 0.393 locus S*tr591INRA*) and among wild and hatchery fish
120 (F_{ST} range: 0.082 locus *Ssa85* - 0.382 locus *Str73*). Amplifications were performed in
121 10 µL volumes which consisted of 1 µL template DNA (~30 ng) in 1X PROMEGA
122 GoTaq®G2 Hot Start Colorless Master Mix (containing GoTaq®G2 Hot Start DNA
123 Polymerase, Multiplex PCR Buffer kit, $MgCl_2$, dNTP mix, 0.1 µM of both forward and
124 reverse PCR primer for *SsHaeIII14.20*, *Ssa85*, *Str73* and *SSoSL438*, and 0.4 µM of
125 both forward and reverse PCR primer for *Str591INRA*). Thermal cycling was
126 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 (*Na*), average allelic richness (*Ar*), observed (*Ho*) and expected heterozygosity (*He*)) 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

154 reference collection for hatchery gene pools. A burn-in period of 50,000 steps followed
155 by 200,000 Monte Carlo replicates was used in these runs. For each sample, ten
156 replicates of the STRUCTURE run were obtained to assess the reproducibility of the
157 estimated *q* values.

158 In addition, hybridization between native and hatchery fish was examined by the
159 Bayesian statistical method implemented in the NEWHYBRIDS (Anderson &
160 Thompson 2002). This method estimated the posterior probability (*P*) that each
161 individual in a wild sample falls into different parental (native (*Pnat*) or hatchery (*Phat*))
162 or hybrid classes (*F1*, *F2*, *Bnat*, *Bhat*). For each data set, which included the wild
163 populations and the Bagà hatchery genotypes, posterior probabilities were evaluated
164 after 100000 iterations of the Monte Carlo Markov Chains. The program ran without
165 any prior information about the hybrid status of collected individuals and populations,
166 and with the 'Uniform' prior option for both mixing proportions and allele frequencies.

167 Population differentiation was investigated from pairwise F_{ST} using FSTAT and a
168 significance test of 10,000 permutations. Analysis of the molecular variance (AMOVA;
169 Excoffier et al. 1992) was used to study the distribution of genetic variation within and
170 among genetic refuges according to geographical and temporal hierarchical models of
171 population grouping. We also determined the number of genetically homogenous
172 population groups (*K*) by minimizing Hardy–Weinberg and linkage disequilibrium using
173 the Bayesian MCMC approach implemented in STRUCTURE v2.3.1 (Pritchard et al.
174 2000). Analysis for each genetic refuge was carried out under the admixture ancestral
175 model with correlated allele frequencies, without prior population information with a
176 burn-in period of 50,000 steps and 200,000 MCMC replicates. Ten independent runs
177 were conducted for each tested *K* value (from "1" to "number of sampling sites within
178 genetic refuge + 1"). The most likely *K* value was estimated following Evanno et al.
179 (2005) recommendations using the program STRUCTURE HARVESTER v 0.6.92
180 (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).

187 **Results**

188 *Genetic diversity and introgression impacts in Eastern Pyrenees locations*

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

197 After Bonferroni correction, 18 out of 156 HW tests were significant. HW deviations
198 were mainly detected at the *SsoSL438* (in TE14, NU14, PLA14, CAP14, LPM14,
199 SEN14, ERI14 locations) and at the *Str591INRA* loci (in NU04, NU06, NU14, CAP14,
200 ERI14, MY04 locations). MICRO-CHECKER analyses suggested homozygote excess
201 as the most plausible explanation for HW deviations at the *SsoSL438* locus, where null
202 alleles, stuttering and drop-out, were not detected. Homozygote excess and presence
203 of null alleles were suggested at *Str591INRA* locus, but neither stuttering nor drop-out
204 was detected. At the rest of microsatellite loci no genotyping errors were indicated by
205 MICRO-CHECKER results. No more than two loci presented HW deviations per
206 location. Inbreeding coefficients (F_{IS}) at each collection were low, except for NU14
207 (0.190), PLA14 (0.174), MY04 (0.095) which indicated heterozygote deficit (i.e.
208 homozygote excess). All samples conformed to Hardy-Weinberg expectations for *LDH-*
209 *C** locus except NU14 due to heterozygote deficit (F_{IS} = 0.870).

210 Estimates of hatchery introgression (*q*) calculated by STRUCTURE software from
211 genotypes at the 5 microsatellite loci had higher background noise than the ones
212 computed from 9 loci (Table 2). For instance, the pure native brown trout population in
213 Vallter location showed values from 0.015 (TE14) to 0.023 (TE06). This range

214 overlapped with current estimates obtained in populations where previously analyses
215 recognized low impact of hatchery fish (e.g. collections from Nuria stream in years
216 2004 and 2006). However in these less impacted collections, bar plots of hatchery
217 ancestry showed some individuals with an estimated introgression rate that largely
218 exceeded the average *q*-value (Fig. 2), clearly confirming the existence of fish with
219 some degree of hatchery ancestry. In addition, the *LDH-C* genotypes confirmed
220 hatchery impact in all these locations. Thus, the combined information from the 5
221 microsatellite and the *LDH-C** locus permitted to identify pure and hybrid trout
222 populations in the studied Pyrenean drainages. A significant positive correlation was
223 found between both introgression indices (frequency of *LDH-C*90* allele and *q* values)
224 for natural locations (Spearman's correlation coefficient (ρ) = 0.740, $P < 0.001$).
225 Estimates of hatchery introgression in 2014 collections from reference locations
226 suggested the preservation of pure Mediterranean trout populations in Vallter (TE14)
227 and the putative restoration of a pure native population in Manyanet (MY14) after the
228 hatchery introgression detected in 2004 and 2006 collections. In Nuria, it was a slightly
229 increasing of the hatchery introgression levels observed in 2004 and 2006. Exact
230 probability tests at the *LDH-C** locus detected significant temporal changes in the allele
231 frequencies in Nuria and Manyanet locations ($P < 0.05$). The frequency of the *LDH-*
232 *C*90* allele in these locations in 2004 suggested hatchery impacts between 1993 and
233 2004 rather than low detection in 1993 because reduced sampled sizes. In 2014, this
234 allele was not detected in Manyanet collection, but its frequency increased in Nuria
235 (Table 2).

236 Low hatchery introgression (<10%) was observed in 2014 in Ter and Flamisell basins,
237 where native fish predominated in all locations (Table 2). In the Freser River, results
238 indicated moderate (10-30%) impact of hatchery releases throughout the basin, but a
239 population of introduced hatchery fish was present in the tributary Segadell stream at
240 Pardines (PAR14). Accordingly, NEWHYBRIDS assigned a higher number of

individuals to hatchery and hybrid classes (i. e. *F1*, *F2* 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 ($F_{ST\ PAR\text{-}BA}$= 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

295 noticed at 2004 collection disappeared in 2014. In addition, all the other 2014 Flamisell
296 studied locations, within and outside of the genetic refuge, presented low levels of
297 introgression and only three individuals were identified as possible hybrids using the
298 method implemented in NEWHYBRIDS program (see Table S1). However, available
299 genetic data from Filia River (a Flamisell tributary within the genetic refuge and not
300 included in this study, see Fig. 1), indicated high introgression levels along temporal
301 monitoring initiated 1993 and stopped in 2006 (*LDH-C*90* > 0.300; Araguas et al.
302 2008).

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

310 All these observations exemplify the limitations of sampling few or a single reference
311 location to define and monitor genetic refuges. Genetic monitoring of few reference
312 locations can give a rough impression of the average status of trout populations at
313 regional scales, but is not informative of genetic changes at the local level (Fernández-
314 Cebrián et al. 2014). The above situation described in the Freser basin shows how
315 local events as described in PAR14 might be responsible for changes at a river scale.
316 Thus, larger genetic surveys from different and distant locations along river basins are
317 necessary to accurately define the situation and the future perspectives of genetic
318 refuge areas. Such surveys would help to define strategies to avoid situations as
319 described in the Freser, where a highly introgressed population is presently protected
320 and threatens the surrounding native populations (García-Marín et al. 1998; Hitt et al.
321 2003; Araguas et al. 2009). Introgressed populations could also disturb inferences on

322 the native population structure (Sanz et al. 2011). Geographically extended surveys
323 may be useful to detect genetically distinct native gene pools within a single genetic
324 refuge, as observed in Flamisell and Freser refuges (Fig. 2, Fig. 3, Table S2), and
325 hence to introduce local actuations even at intra-drainage level within a refuge.
326 Significant genetic divergence among brown trout collections at short hydrological
327 distances (few kilometres) within a drainage have been described in other rivers from
328 the studied region (Sanz et al. 2011; Fernández-Cebrián et al. 2014), as well as in
329 other European basins (e.g. Carlsson & Nilsson 2000). Restricted gene flow between
330 trout populations of the main stream and nearby tributaries in the Norwegian Nordre
331 Finnvikelv River were reported by Carlsson et al. (1999).

332 *Management recommendations*

333 In the eastern Pyrenees, the current Spanish legislation on biodiversity conservation
334 (Article 52.2, Law 42/2007 of Natural Heritage and Biodiversity) forbids the releases of
335 foreign stocks into freshwater ecosystems. In particular, the legislation prohibits the
336 introduction of alien species, subspecies or geographic races and illegal transplantation
337 of individuals when they are capable of competing with native wildlife, altering its
338 genetic purity or the ecological balance. This restriction prevents future introductions
339 and reinforces the role of genetic refuges for the conservation of brown trout native
340 resources but, what happens with hybridized or hatchery populations already
341 established? In spite of some isolation degree was revealed by contrasting levels of
342 introgression at NU and QA locations in the absence of physical barriers, several
343 studies support the spreading of hatchery genes from highly introgressed salmonids
344 populations to neighbouring locations (García-Marín et al. 1998; Hitt et al 2003;
345 Araguas et al 2008). Then, the dispersal of individuals from hybridized populations
346 cannot be ignored as a possible threat that might contribute to the increase of hatchery
347 introgression in adjacent populations. For instance, the highly introgressed collection of
348 PAR14 in Segadell river might contribute to the introgression of neighbouring

349 populations, such as PLA and RF, due to the lack of hydrological barriers among them.
350 Similarly, these trout could reach populations located upstream of small weirs in the
351 main stream, such as QA and QB, because it is observed that some larger trout easily
352 overpass these physical obstacles (Ordeix et al. 2011). Nevertheless, the highest
353 introgression levels detected at QA and QB suggested that additional processes (e.g.
354 direct releases or transplantations) are also involved in the observed introgression.
355 Illegal transplantations were suspected in other locations of the region (Araguas et al.
356 2009).

357 The establishment of genetic refuges in Mediterranean rivers has not resulted in a
358 rapid and significant decrease of hatchery alleles at regional scale (Araguas et al.
359 2008, 2009; Caudron et al. 2011, 2012). The maintenance of fishery pressure in some
360 river stretches has prevented the increase of introgression from hatchery releases due
361 to selective angling on released non-native fish (Mezzera & Largiader 2001; García-
362 Marín et al. 1998; Harkonen et al. 2014). However, maintaining fishery within genetic
363 refuges has not been effective enough for recovering native gene pools in introgressed
364 populations (Araguas et al. 2008, 2009; Caudron et al. 2012). Thus, while genetic
365 refuges have the potential to prevent further introgression, additional measures should
366 be implemented to recover native gene pools, particularly within protected areas
367 (Araguas et al. 2008, 2009; Caudron et al. 2012). In these situations, different
368 approaches can be used to the restoration of native gene pools; the most popular are
369 the supplementation with native individuals and/or the selective removal of hybrids and
370 hatchery naturalized fish.

371 Native stock reinforcement following habitat recovery is an accepted measure within
372 the European Inland Fisheries Advisory Commission (EIFAC) code of practice for
373 recreational fisheries (FAO 2008). The supplementation of native individuals could be
374 achieved by stocking with local native breeding stocks and/or by translocations of wild
375 native individuals. Both actions have been reported to be successful in northern French

376 Alps brown trout populations. For instance, stocking with fry from a native stock has led
377 to the restoration of a functional Mediterranean trout population in the Ugine River
378 (Caudron et al. 2006). The translocations of native Mediterranean trout also resulted in
379 a significant decrease in the percentages of non-native alleles in the Borne River
380 (Caudron et al. 2012).

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

393 Selective removal of non-native individuals and hybrids seems necessary to recover
394 native gene pools in populations such as the Pardines (PAR14), within the Freser
395 genetic refuge. In the case of hatchery naturalized populations, this action can be
396 carried out promptly. However, in other cases the implementation of this strategy is
397 predicted to be more difficult, as it requires a quick and easy tool to distinguish
398 between Mediterranean native and hatchery and hybrid fish in the wild. Several
399 phenotypic characters such as the spotting pattern, the size of the spots on body
400 flanks, the number and shape of parr marks, and the colour of margins of the anal and
401 dorsal fins have been proven to be useful for a visual distinction between Atlantic and
402 Mediterranean trout (Lascaux 1996; Mezzera et al. 1997; Aparicio et al. 2005). A

403 classification tree model proposed, using three variables of coloration and spotting
404 pattern very easily measurable in the field, could be used as a tool to distinguish
405 among Mediterranean, Atlantic and hybrid fish in low and moderate introgressed
406 populations (Aparicio et al. 2005). Despite total removal of exotic alleles seeming
407 impractical over a short time, a significant reduction of the introgression rate is
408 expected if individuals classified as non-native and hybrid are removed from
409 population. To demonstrate the efficiency of these selective removals, several years
410 are needed and shallow stretches have to be selected as candidates due to the limited
411 fish catchability by electrofishing in deep water (Carmona-Catot et al. 2010; Caudron &
412 Champigneulle, 2011).

413 The Spanish legislation regulating recreational fishing activities considered sustainable
414 management essential to ensure that the exploitation of the resource harmonizes with
415 its optimal conservation. To achieve this goal and avoid introduction of alien
416 specimens, some authors suggested that fishery reinforcements should be done with
417 sterile individuals such as triploid (Piferrer et al. 2009). Certainly, the use of controlled
418 sterile individuals allows reaching equilibrium between recreational fishing (i.e.
419 economical resources) and conservation of wild resources. The production of triploids
420 is simple and cheap, involving either physical treatment (temperature or pressure
421 shocks) or the application of chemicals (Thresher et al. 2014). Moreover, because
422 triploidy does not involve manipulations of individual chromosomes or genes, they are
423 not widely considered to be ‘‘genetically modified’’. A protocol of optimization and
424 production of triploid individuals in *S. trutta* have been recently described (Preston et al.
425 2013). Nonetheless, triploids are not always 100 % sterile (Normand et al. 2008). While
426 triploid fish females are always 100% sterile because their ovaries never develop,
427 triploid males can produce functional spermatozoa and attempt to spawn with females,
428 competing with wild males (Piferrer et al. 2009, Fjelldal et al. 2014). Legislation
429 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 geographically 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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698 **Figure Captions**

699

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

704

705 **Figure 2**. Bayesian STRUCTURE results. a) All 2014 collections and Baga hatchery
706 stock, *K*=3 (*K*=2, mean ln *P*=-10277.32; *K*=3, mean ln *P*=-9316.29; *K*=4, mean ln *P*=-
707 9077.26) b) 2014 collections from the Freser genetic refuge, *K*=3, (*K*=2, mean ln *P*=-
708 2893.34; *K*=3, mean ln *P*=-2752.84; *K*=4, mean ln *P*=-2734) c) 2014 collections from
709 the Flamisell genetic refuge, *K*=2, (*K*=1, mean ln *P*=-4512.7; *K*=2, mean ln *P*=-4412.74;
710 *K*=3, mean ln *P*=-4337.02). Each individual is represented by a vertical bar partitioned
711 into segments according to the proportion of the genome assigned to each of the
712 identified clusters. Codes are defined in Table 1.

713

714 **Figure 3**. Factorial Component Analysis (FCA) among studied individuals. Blue,
715 orange, green and red colours represent respectively fish sampled at Freser, Ter,
716 Flamisell refuges and Baga hatchery. Location codes are shown on Table 1.

717

718

719 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

720 * Locations initially used to define Genetic Refuges

721

722

723 Table 2. Gene diversity and estimates of introgression levels for locations analysed in
724 the present study. *N*: number of individuals, *Na*: mean number of alleles per locus, A_R:
725 allelic richness, H_E: mean expected heterozygosity, F_{IS}: inbreeding coefficient, P_{HW}:
726 Hardy-Weinberg equilibrium tests, *q* Value: proportion of introgressed genome on the
727 five microsatellites (within parentheses hatchery introgression estimates based on 9
728 loci), *LDH-C*90*: frequency of the hatchery allele.

Collection	*N*	*Na*	A_R	H_E	F_{IS}	P_{HW}	*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

729 [A] from Araguas *et al.* 2008
730

731 Table 3. Genetic diversity pattern, in space and time, from reference locations (Ter,
732 Nuria and Manyanet). *N*: total sampled individuals (minimum local sampled size), A_R:
733 allelic richness, H_T: total gene diversity, H_S: average local gene diversity, F_{ST}:
734 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

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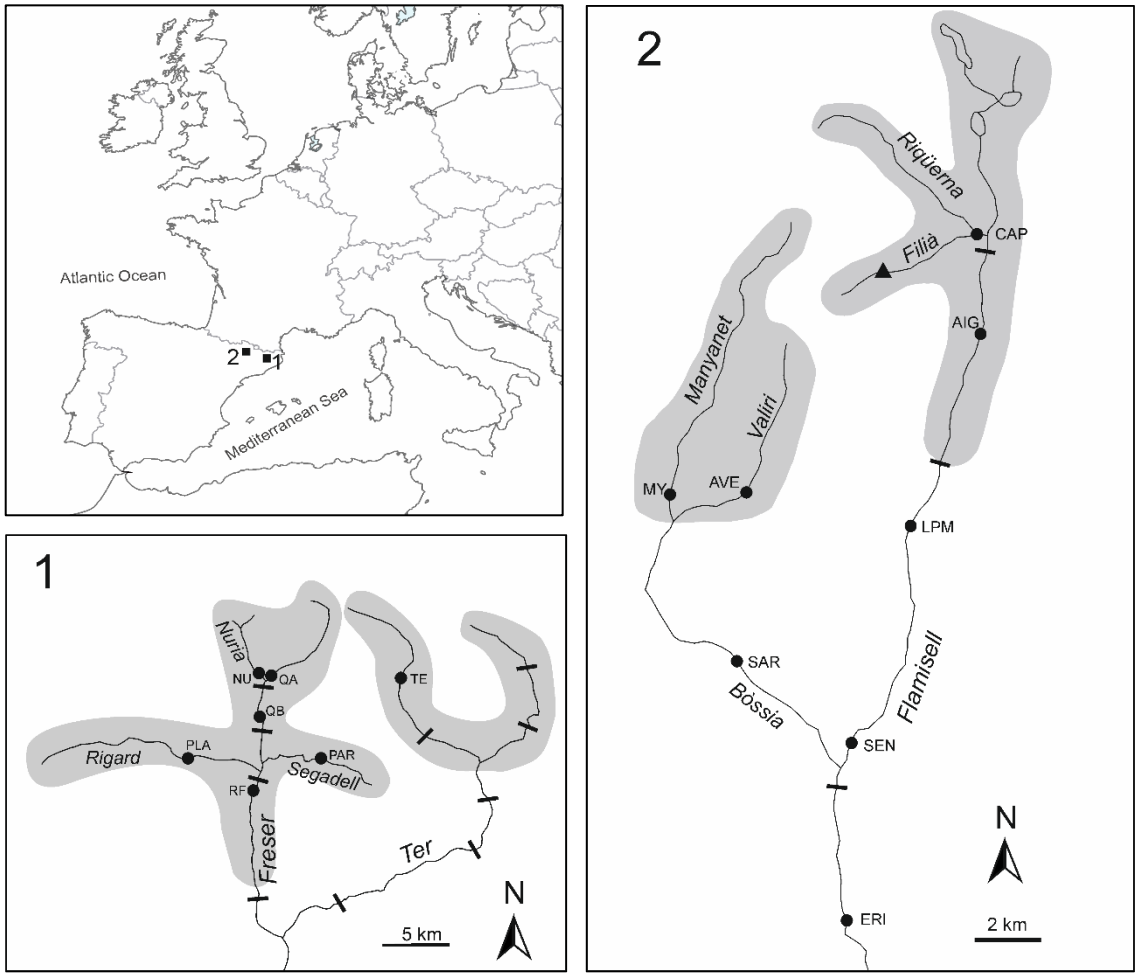
737 Table 4. AMOVAs analyses grouping samples by Genetic Refuges.

738

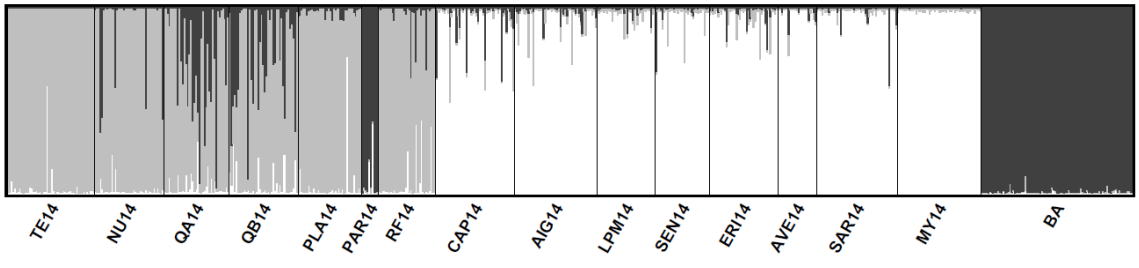
	F-statistic	Variance component	% Variation
2014 collections			
Among Genetic Refuges (F_{CT})	0.24406***	0.54639	24.41
Among locations within Genetic Refuge (F_{SC})	0.07189***	0.12166	5.43
Within locations		1.57070	70.16
2014 collections without BA			
Among Genetic Refuges (F_{CT})	0.14337***	0.28572	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

739 *** P < 0.001


Atlantic Ocean
Mediterranean Sea
2
1
1
Nuria
NU
QA
QB
TE
Rigard
PLA
PAR
Segadell
RF
Freser
Ter
5 km
N
2
Riqüerna
Fillà
CAP
AIG
Manyanet
Valiri
MY
AVE
LPM
SAR
Bòssia
Flamisell
SEN
ERI
2 km
N

747

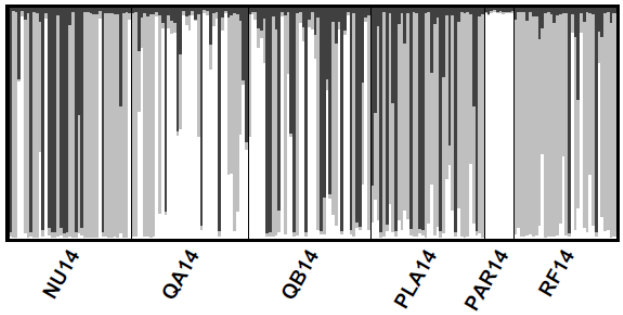
748 **a)**



749

750

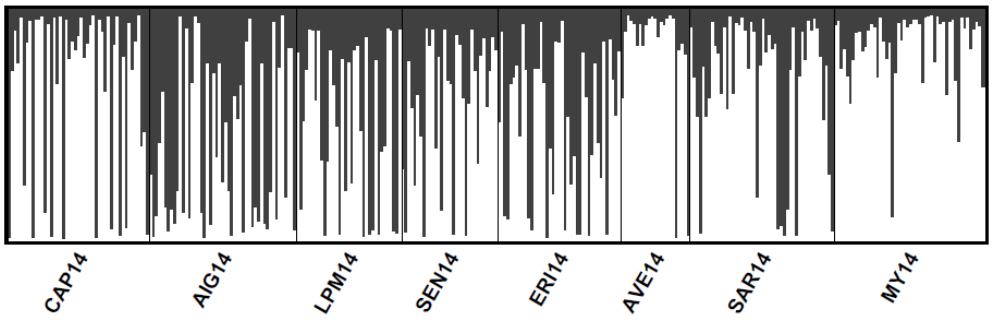
751 **b)**



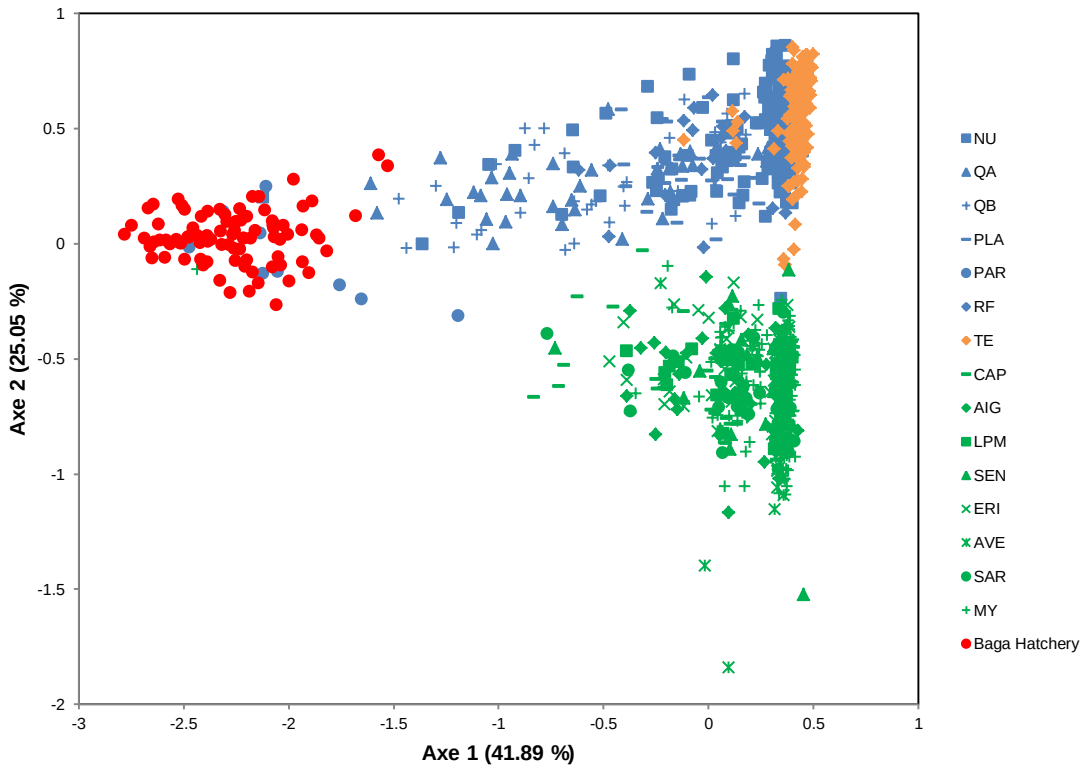
752

753

754 **c)**



755


Axe 2 (25.05 %)
Axe 1 (41.89 %)
1
0.5
0
-0.5
-1
-1.5
-2
-3
-2.5
-2
-1.5
-1
-0.5
0
0.5
1
NU
QA
QB
PLA
PAR
RF
TE
CAP
AIG
LPM
SEN
ERI
AVE
SAR
MY
Baga Hatchery

756