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Genetic introgression on freshwater fish populations caused by restocking programmes

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Abstract

The brown trout (*Salmo trutta* L.) is one of the best studied native salmonids of Europe. Genetic studies on this species suggest that a large proportion of the evolutionary diversity corresponds to southern European countries, including the Iberian Peninsula, where this study is focused. Stocking activities employing non-indigenous hatchery specimens together with the destruction and fragmentation of natural habitats are major factors causing a decrease of native brown trout populations, mostly in the Mediterranean basins of the Iberian Peninsula. The main aim of the present work is to examine the genetic structure of the brown trout populations of the East Cantabrian region, studying the consequences of the restocking activities with foreign hatchery brown trout specimens into the wild trout populations. We have based our study on the Polymerase Chain Reaction and Restriction Fragment Length Polymorphism technique conducted on a mitochondrial fragment of 2700 base pairs and on the lactate dehydrogenase locus of the nuclear DNA. Our results show higher introgression rates in the Ebro (Mediterranean) basin than in the Cantabrian rivers.

Introduction

Most European countries have ratified the Convention on Biological Diversity (Rio de Janeiro 1992), which stresses that 'States are responsible for conserving their biological diversity and for using their biological resources in a sustainable manner.' Since then, biodiversity conservation and survival of species has became a priority for the people involved in the management of natural resources. This principle is more difficult to be applied to those species under exploitation mainly due to economic interests. In this context, a great number of freshwater fishes are seriously threatened with extinction as a direct consequence of human activities: overexploitation by fishing, habitat alteration, introduction of exotic fishes, or stocking activities (Ferguson 1990; Rhymer and Simberloff 1996; Elvira 1998; Cross 2000).

Stocking activities (introductions or reintroductions) with fishes grown in captivity has become a common practice in many countries with the primary aim of getting an increment of angling as well as for the rehabilitation of natural populations. It has been shown that restocking programmes can result in deleterious effects on the natural fish populations that in many cases are the same as those caused by introduction of exotic species: diseases and competition for food and habitat (Lynch and O'Hely 2001). All this might cause a displacement of the local populations, or in extreme cases, their extinction. Furthermore, introduction of fishes of the same species (stocking) but from a different

geographical origin may introduce genetic changes in native populations due to the introgression of allochthonous genes on wild populations (Huxel 1999). It has been shown that natural hybridization has played an important role in the evolution of many animal taxa. However, stocking activities are increasing an anthropogenic hybridization which may reduce the genetic variability of the native populations and in consequence constrains the possibility for future adaptations (Allendorf et al. 2001). In addition, genetic introgression may cause a homogenization of wild populations from different geographical areas because, in most cases, the hatchery stocks form only a part of the total genetic variability of the species.

Brown trout (*Salmo trutta* L.) populations provide an interesting case to study the influence of stocking activities on native populations. Several studies using different genetic markers have demonstrated the existence of a considerable degree of genetic differentiation between brown trout populations throughout its natural distribution range, both at the macro and microgeographical levels (Ferguson 1989; Bernatchez et al. 1992; Hansen and Loeschcke 1996; Hynes et al. 1996). Important differences in the genetic trout diversity between Mediterranean and Atlantic drainages have also been reported (García-Marín et al. 1996). The locus LDH-C* is one of the most important diagnostic genetic marker systems to deal with the study of the population genetics of this species (Ferguson and Fleming 1983; Hamilton et al. 1989). Two major groups of brown trout have been described on the basis of the presence of LDH-C*90 and LDH-C*100 alleles: 'modern' (North Atlantic) and 'ancient' (South Atlantic and Mediterranean) lineages, respectively (Hamilton et al. 1989; García-Marín et al. 1999). Upon mtDNA analysis, Bernatchez et al. (1992) have described five major phylogenetic groups in Europe: Adriatic, Danube, Mediterranean, marmoratus and Atlantic. Furthermore, it has been shown that a large proportion of the evolutionary diversity of brown trout is sited in the south of Europe, including the Iberian Peninsula, where this study is focused (Giuffra et al. 1994; García-Marín and Pla 1996; García-Marín et al. 1999). Some authors have claimed that the Iberian Peninsula acted as a refuge during the last glaciations and proposed the existence of two distinct regional groups associated with Atlantic and Mediterranean drainages. Furthermore, Machordom et al. (2000) have demonstrated the presence of at least five distinct groups in the Iberian Peninsula trout populations: Mediterranean, Andalusian, Atlantic, Duero, and Cantabrian lineages.

Because of economic and sport-fishery interest, the Iberian rivers have been intensively restocked during the last decades employing hatchery strains of brown trout of exogenous origin, mainly from central and northern Europe. Although these stocking activities have been carried out for several decades, most studies indicate that introgression of native populations is limited (McNeil 1991). However, partial displacement of some native populations by hybridization and introgression is very common (Hindar et al. 1991; Martínez et al. 1993).

The aim of the present work was to evaluate the consequences of the restocking activities with allochthonous brown trout specimens into the wild populations. The study area was located in the north of the Iberian Peninsula and included two different drainages: the Mediterranean and Cantabrian–Atlantic basins.

This study was based on Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analyses of the NADH dehydrogenase subunits 5/6 and the cytochrome b segment (ND 5/6-cyt b) of the mitochondrial genome, and the LDH-C* locus of the nuclear DNA. Most hatchery stocks are fixed or show a very high frequency of the allele LDH-C*90 which does not occur naturally in Iberian brown trout populations. In contrast, Iberian native populations are fixed to the LDH-C alleles *100 or *105. Thus, LDH-C* can be considered a good diagnostic marker to estimate the effects of stocking activities, such as genetic introgression, on wild population. On the other hand, the study of mtDNA haplotypes allows us to distinguish between brown trout populations of different geographic origins.

Materials and methods

Sampling

A total of 400 brown trouts were collected by electrofishing between 2001 and 2002 in 20 locali-

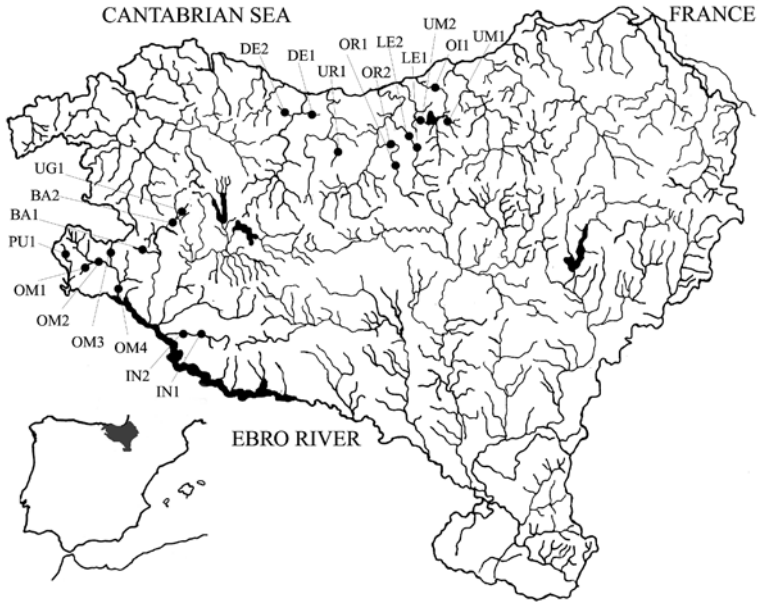

Figure 1. Codes and location of brown trout populations from Cantabrian and Mediterranean drainages in the Iberian Peninsula analysed in this study. See Table 1 for sampling characteristics. OI1: Oiartzun-Altzibar River; UM1: Urumea-Mendaraz River; UM2: Urumea-Urruzuno River; LE1: Leitzaran-Coto River; LE2: Leitzaran-Ameraun River; OR1: Oria-Araxes River (fishing preserve); OR2: Oria-Araxes River (fenced fishing area); UR1: Urola-Matxinbenta River; DE1: Deba-Sallabente River; DE2: Deba-Arranbide River.

ties from 11 different river basins from the north of the Iberian Peninsula (Figure 1). The sampling scheme included brown trout populations from river systems draining to the Cantabrian and Mediterranean (Ebro river system) basins. All of them have been restocked during decades with exogenous hatchery trout of central Europe origin. Two of the Mediterranean rivers included in this study have been regularly restocked up to the sampling years (OM2 and BA1). The rest of the populations have not been restocked since 1996. Samples from the two local fish farms (another 40 specimens), which have been used by local Governments for restocking activities have also been included.

Each captured fish was anaesthetized with etilenglieol (0.05%). A few scales were obtained and preserved in ethanol until their analysis. All fishes were released after handling, resulting in a non-lethal sampling procedure. Total genomic DNA was isolated from scales, using the standard phenol/chloroform–isoamylalcohol protocol after the proteolytic disruption of tissue with proteinase K. DNA was then quantified by fluorimetry and its purity estimated after migration on a 0.7% agarose gel.

Molecular analysis

The locus LDH-C* was PCR amplified according to the protocol reported by McMeel et al. (2001). The 440 bp amplified fragments were digested with the restriction endonuclease *Bsl* I and then electrophoresed on a 2% agarose gel using a 100 bp ladder as a molecular weight standard. According to McMeel et al. (2001), two alleles, LDH-C*90 and LDH-C*100, can be differentiated by this method.

mtDNA haplotypes were characterized by PCR amplification of a segment containing the subunits 5 and 6 of the NADH dehydrogenase gene (ND 5/6) and the cytochrome b gene. PCR conditions followed those described by Machordom et al. (2000). The 2700 bp amplicons were digested with five restriction enzymes (Machordom et al. 2000): *Alu* I, *Hinc* II, *Msp* I, *Rsa* I and *Sau* 3AI. Restriction fragments were visualized in a 2% agarose gel using a 100 bp ladder as a molecular weight standard, to calculate the molecular size of the restriction fragments. Each haplotype was defined by a five letter code following the RFLPs patterns described in Machordom et al. (2000).

According to previous studies carried out in the Iberian Peninsula, wild populations have the diagnostic allele LDH-C*100 fixed in their genetic composition. In this manner, the percentage of introgression was calculated from the mean percentage of the central European alleles at this locus (LDH-C*90).

Results

LDH-C analysis*

Three different genotypes have been observed from the analysis of the LDH-C* locus. LDH-C*90/90 is specific for hatchery stocked brown trout whereas LDH-C*100/100 characterizes Iberian native populations. The heterozygous LDH-C*100/90 genotype identifies hybrid specimens. The whole hatchery specimens of the two stocks studied showed LDH-C*90/90 genotype as was expected for the Central Europe origin of domestic foreign specimens. Heterozygous specimens were not detected in the hatchery stocks (Table 1).

The estimated introgression percentage was very variable among populations (0–65%). There were great differences between the mean introgressions of Cantabrian and Mediterranean populations (9.7 and 30.6%, respectively). The introgression percentage of those populations which were unstocked during the last 6 years was significantly higher in the Mediterranean than in the Cantabrian rivers (Table 1).

Only three of the analysed populations were free of introgression (OI1, UN1, and DE1). Another four showed residual levels of introgression by foreign alleles of hatchery origin (UM2, LE1, OR1 and DE2; LDH-C*90 frequency < 5%). All these populations were located in the Cantabrian region where the domestic foreign alleles occurred at low frequencies (LDH-C*90 frequency < 10%). However, the River Urumea exhibited the highest level of introgression for this region (22%). Hatchery diagnostic allele (LDH-C*90) was not observed in homozygosis in any Cantabrian population; therefore, the introgression detected in this region was only due to hybrid specimens (LDH-C*100/90).

On the other hand, the foreign LDH-C*90 allele was observed in all the analysed Mediterranean localities, including those where the stocking practices stopped before 1996. Unlike the Cantabrian region, high values of introgression were found in the Mediterranean populations unstocked since 1996 (PU = 45%), showing both non-native genotypes LDH-C*90/90 and LDH-C*100/90 in several localities (OM1, OM3, BA2, and UG1). In those Mediterranean populations which were restocked until 2002 (ZA, OM4, and BA1), the estimated introgression rate ranged between 20 and 65%.

mtDNA analysis

A total of four haplotypes were detected among the 20 natural populations analysed. Using capital letters referring to RFLP pattern produced by each endonuclease in the order *Alu* I, *Hinc* II, *Msp* I, *Rsa* I and *Sau* 3AI, the four haplotypes found were AAAAA, ABACA, BBCDA, and BBAAA. According to Machordom et al. (2000), the AAAAA is the haplotype of hatchery reared trouts with Central Europe origin. This haplotype is also shared by the Cantabrian native populations of brown trout. ABACA is the general Iberian Atlantic haplotype and can be considered a diagnostic haplotype for the populations of the Cantabrian slope. BBCDA in the study region of this work is considered a native haplotype for the Iberian–Mediterranean populations. BBAAA is an undescribed haplotype which has been found in four populations of the Mediterranean slope (IN1, IN2, OM3, and OM4).

Every specimen of the two hatchery stocks analysed showed the AAAAA haplotype, which characterizes Atlantic and central European foreign brown trout. ABACA and AAAAA were nearly the only haplotypes present in the Cantabrian populations, with the former showing higher frequencies. Both haplotypes coexisted in several populations (UM1, UM2, LE1, LE2, OR1, OR2, DE1, and DE2). On the other hand, the ABACA haplotype was fixed in one population (OI1), and the AAAAA haplotype was fixed in another one (UR1). Unexpectedly, in the Deba river (DE2) from the Cantabrian slope, the Mediterranean haplotype BBCDA was found in a proportion of 20%. The LDH-C* marker confirmed the Iberian native genotype for these specimens (LDH-C*100/100).

The populations sampled at the Mediterranean drainage were composed of a mixture of the four

Table 1. mtDNA haplotypes inferred from RFLP and allele frequency data detected in the 20 samples of brown trout for Cantabrian and Mediterranean drainages analysed in this study and the two hatchery farms. The sampling areas are indicated as in Figure 1.

Drainage	River	mtDNA haplotype	Locus LDH-C* genotype	Frequencies	Stocking*	*I*
Cantabrian	OI1	ABACA	*100/100	100% (n = 20)	No	0%
	UM1	AAAAA	*100/100	40% (n = 8)	No	0%
		ABACA	*100/100	60% (n = 12)		
	UM2	AAAAA	*100/100	44% (n = 8)	No	2.7% (<5%)
		AAAAA	*100/90	6% (n = 1)		
		ABACA	*100/100	50% (n = 9)		
	LE1	AAAAA	*100/100	25% (n = 5)	No	2.5% (<5%)
		ABACA	*100/100	70% (n = 14)		
		ABACA	*100/90	5% (n = 1)		
	LE2	AAAAA	*100/100	39% (n = 7)	No	5.5%
		ABACA	*100/100	50% (n = 9)		
		ABACA	*100/90	11% (n = 2)		
	OR1	AAAAA	*100/100	40% (n = 8)	No	5%
		ABACA	*100/100	50% (n = 10)		
		ABACA	*100/90	10% (n = 2)		
	OR2	AAAAA	*100/100	60% (n = 12)	No	7.5%
		AAAAA	*100/90	10% (n = 2)		
		ABACA	*100/100	25% (n = 5)		
		ABACA	*100/90	5% (n = 1)		
	UR1	AAAAA	*100/100	55% (n = 11)	No	22.5%
		AAAAA	*100/90	45% (n = 9)		
	DE1	AAAAA	*100/100	25% (n = 5)	No	0%
		ABACA	*100/100	75% (n = 15)		
	DE2	ABACA	*100/100	75% (n = 15)	No	2.5%
		ABACA	*100/90	5% (n = 1)		
		BBCDA	*100/100	20% (n = 4)		
Mediterranean	OM1	BBCDA	*100/100	25% (n = 5)	No	45%
		BBCDA	*100/90	55% (n = 11)		
		BBCDA	*90/90	10% (n = 2)		
		AAAAA	*100/90	5% (n = 1)		
		AAAAA	*90/90	5% (n = 1)		
	OM2	BBCDA	*100/100	53% (n = 9)	No	23.5%
		BBCDA	*100/90	47% (n = 8)		
	OM3	BBCDA	*100/100	10% (n = 2)	No	45%
		BBCDA	*100/90	50% (n = 10)		
		BBCDA	*90/90	5% (n = 1)		
		AAAAA	*100/100	5% (n = 1)		
		AAAAA	*100/90	15% (n = 3)		
		BBAAA	*100/90	15% (n = 3)		
	OM4	BBCDA	*100/100	10% (n = 1)	Yes	65%
		BBCDA	*100/90	30% (n = 3)		
		BBCDA	*90/90	20% (n = 2)		
		BBAAA	*100/100	10% (n = 1)		
		BBAAA	*90/90	30% (n = 3)		
	BA1	BBCDA	*100/100	66% (n = 10)	Yes	20%
		BBCDA	*100/90	20% (n = 3)		

Table 1. Continued.

Drainage	River	mtDNA haplotype	Locus LDH-C* genotype	Frequencies	Stocking*	*I*
		AAAAA	*100/90	7% (n = 1)		
		AAAAA	*90/90	7% (n = 1)		
	BA2	BBCDA	*100/100	65% (n = 13)	No	17.5%
		BBCDA	*100/90	20% (n = 4)		
		BBCDA	*90/90	5% (n = 1)		
		AAAAA	*100/100	5% (n = 1)		
		AAAAA	*100/90	5% (n = 1)		
	UG1	BBCDA	*100/100	39% (n = 6)	No	36.6%
		BBCDA	*100/90	7% (n = 1)		
		BBCDA	*90/90	20% (n = 3)		
		AAAAA	*100/100	7% (n = 1)		
		AAAAA	*100/90	27% (n = 4)		
	INI	BBAAA	*100/100	49% (n = 4)	No	12.5%
		BBAAA	*100/90	13% (n = 1)		
		ABACA	*100/100	25% (n = 2)		
		ABACA	*100/90	13% (n = 1)		
	IN2	BBAAA	*100/100	42% (n = 8)	No	2.5%
		BBAAA	*100/90	5 (n = 1)		
		AAAAA	*100/100	5 (n = 1)		
		ABACA	*100/100	48% (n = 9)		
	PU1	BBCDA	*100/90	35% (n = 7)	No	45%
		AAAAA	*100/100	15% (n = 3)		
		AAAAA	*100/90	45% (n = 9)		
		AAAAA	*90/90	5% (n = 1)		
Hatchery strain	HAT1	AAAAA	*90/90	100% (n = 20)		
	HAT2	AAAAA	*90/90	100% (n = 20)		

I = introgression index related to the LDH-C*90 allele frequency.
*No: unstocked during the last 6 years; Yes: stocked until 2002.

identified mtDNA haplotypes: AAAAA, ABACA, BBCDA, and BBAAA. The stocked populations as well as many of those which were not stocked after 1996 (OM1, OM3, BA1, BA2, UG1, IL1, and PU1) showed different degrees of genetic contamination with the hatchery haplotype AAAAA. Moreover, the ABACA haplotype was found in the Inglares river (IN1, IN2). The LDH-C* analysis indicated the presence of LDH-C*100/100 genotype in most of these 'ABACA specimens'.

Discussion

The three genotypes obtained from the analysis of the LDH-C* locus in the populations studied allow us to differentiate native and stocked brown trouts. The genotype LDH-C*90/90 indicates stocked brown trouts because it was present in all the hatchery samples analysed. According to other studies (García-Marín et al. 1999), the Iberian native populations are homozygous to the allele LDH-C*100. In view of the absence of heterozygotes in the hatchery stocks analysed, we can considerer the heterozygous specimens as hybrids between native and stocked trouts.

Considerably higher introgression rates were detected in the Ebro river (Mediterranean) populations when compared with those of the Cantabrian region. Our results are in agreement with those of other published studies on genetic introgression between wild and stocked brown trouts in Spanish rivers. Thus, the low introgression observed in the Cantabrian rivers was similar and congruent with those found in other rivers located in the north (Morán et al. 1991, 1995;

García-Marín and Pla 1996). Similarly, studies carried out in other Ebro tributaries (García-Marín and Pla 1996; Machordom et al. 1999) have showed introgression and admixture rates equivalent to those found in this study.

These results suggested that environmental conditions of rivers might explain the observed differences in introgression and admixture between the study regions as previously suggested by Almodóvar et al. (2001). In this context, Cantabrian rivers present some common characteristics that would suppose a disadvantage for hatchery stocked trouts. They are short rivers belonging to small basins of narrow valleys with marked torrential character. The average gradient is 11%; they are divided into rapids, deeper sections, and pools. In contrast, Mediterranean rivers present an average slope of 2%; they are seasonal rivers with a low water level and warm temperatures in summer. These environmental differences among rivers of both regions seem to have played an important role and could explain the introgression rate variation detected. It has been shown that the type of environment in which hatchery trout is stocked is important for their survival and reproduction (Poteaux et al. 1998). The variable success of survival and reproduction of stocked fishes is probably the main factor explaining the different introgression rates observed between both regions and could also explain the indirect effect of restocking on the structure of the populations (Poteaux et al. 1998).

On the other hand, several studies reported poor survival and reproduction of stocked trouts in those rivers where high levels of natural reproduction of native trout population occurred (Morán et al. 1991). In this way, trout fry number as well as whole trout populations were much higher in the Cantabrian basin than in the Mediterranean streams analysed. This indicates a higher level of natural reproduction in Cantabrian rivers which could have a negative effect on the survival percentage of stocked trouts.

Although the Cantabrian rivers were strongly restocked over the past decades, this region presented a low introgression rate. Therefore, cessation of stocking for six or more years seemed to have caused a progressive reduction of domestic foreign allele frequencies in the wild populations. However, in the Mediterranean rivers where restocking activities were stopped for the last 6 years, the introgression index was still high. The highest introgression percentages (65%) corresponded to those populations restocked until 2002, probably due to the earliest introductions. These populations might constitute a reservoir of allochthonous genes of hatchery origin that could migrate to other river sections or streams, and cause a negative effect on the genetic integrity of the wild populations.

The observed pattern of haplotype distribution demonstrated great differentiation between the populations of the Cantabrian and Mediterranean regions. The complementary use of mtDNA provided useful additional information about genetic introgression to that obtained with the LDH-C* locus.

The introgression index observed in some Mediterranean populations might have been underestimated by assuming that all sampled trouts with LDH-C*100/100 genotype were native. Some of these 'native trout' presented the AAAAA haplotype and were homozygous for the autochthonous diagnostic allele LDH-C*100. If it is assumed that this haplotype (AAAAA) is non-native for Mediterranean populations, these specimens have to be considered hybrids between hatchery and native breeders. This admixture between a native nuclear genotype and a non-native mitochondrial haplotype might be explained by breeding among heterozygous (LDH-C*100/90) or homozygous (LDH-C*100/100) female hybrids (F1 or F2 proceeding from a hatchery female trout, respectively), and males (both with homo- or heterozygous genotype). Those specimens showing native mitochondrial haplotype (BBCDA or BBAAA) together with allochthonous nuclear allele (LDH-C*90) could have the same hybrid origin. All these results confirmed a high interbreeding index between wild and stocked trouts and their descendants in this region.

The presence of the Mediterranean BBCDA haplotype in one river of the Cantabrian region is difficult to explain. It seems to be a genetic contamination, but its hatchery origin must be dismissed because this haplotype was not detected in the hatchery stocks used for local restocking. It might have come from introductions carried out by uncontrolled anglers from hatcheries working with native Mediterranean trouts, or translocated directly from a Mediterranean river. Another explanation is that the

presence of this Mediterranean haplotype could have been produced by a natural river capture. These natural captures have been demonstrated in different natural systems, and also in the Cantabrian region (Alonso-Otero 1986). In the area studied, Cantabrian and Mediterranean headwaters are close together, and this could facilitate this natural process.

On the opposite side, the ABACA haplotype has been considered until now a Cantabrian and Atlantic hapotype (Machordom et al. 2000). Its presence in the Ebro tributaries cannot be seen as a result of stocking activities because it was not detected in the hatchery stocks analysed. The high proportion estimated in the Inglares river (Mediterranean region) suggested that this haplotype could occur naturally in the native brown trout populations of some upper tributaries of the Ebro basin. Furthermore, a new mtDNA haplotype (BBAAA) was also found in this river and in other close Ebro tributary rivers. These two haplotypes were not detected in other lower tributaries of the Ebro basin. Therefore, their presence appears to be restricted to the upper tributaries of this basin and could indicate the presence of an Iberian-Mediterranean refuge in the Ebro basin. On the other hand, the presence of particular haplotypes in this region may also be due to other reasons as a genetic local adaptation to the particular environment conditions (Taylor 1991) of these rivers. As previously stated by Suarez et al. (2001), the Iberian brown trout maintains a genetic diversity different from that shown by other European trout populations. Restocking policies should take into account several guidelines (Almodóvar et al. 2001) for conservation and management of the Evolutionary Significant Units (ESUs) of native brown trout populations in the Iberian Peninsula rivers.

In conclusion, from the management and conservation points of view, it is obvious that any kind of restocking with foreign specimens should always be avoided to preserve the autochthonous genetic pools. Despite stocking activities being stopped during the last 6 years, the presence of domestic foreign allele (LDH-C*90) demonstrates that hatchery brown trouts reproduced under natural conditions. On the other hand, the Cantabrian rivers' natural conditions seem to prevent or eliminate the negative consequences of such introductions more accurately. Thus, the recovery of the native genetic characteristics of these Cantabrian populations could be possible if the management was adequate.

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