Freshwater Biology (2006) **51,** 1175–1187 doi:10.1111/j.1365-2427.2006.01556.x

APPLIED ISSUES

Introgression variability among Iberian brown trout Evolutionary Significant Units: the influence of local management and environmental features

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SUMMARY

1. A comprehensive analysis was carried out on the effects of stocking on the genetic structure of Iberian brown trout evolutionary lineages. Introgression and genetic diversity were estimated from allozyme results of 307 populations based on own data (180) and available literature (127). Stocking records, angling regulations and environmental features related to hatchery trout performance were also analysed to determine the underlying mechanisms of the introgression effects.

2. Fifty per cent of analysed populations showed introgression by genes of hatchery origin. The mean introgression estimated by the single locus approach $\hat{S}$ was 0.134. An increment of both heterozygosity and polymorphism was observed when introgression increased in stream-dwelling populations, which could finally produce a homogenisation of the genetic structure of populations and a decrease of the species' genetic diversity.

3. Introgression rate varied among Iberian evolutionary lineages (Evolutionary Significant Units), and was correlated with the stocking effort, except for the North Atlantic basins. The lack of adaptations for migratory behaviour in hatchery trout could explain the low impact of stocking in North Atlantic rivers where anadromous populations occur.

4. Angling regulation did not seem to influence the survival of hatchery trout. Introgression tends to be higher in heavily stocked localities with fertile waters and stable discharge, which may favour the performance of hatchery trout.

5. Trout management must be based on increasing population size by means of habitat improvement and sustainability of naturally reproducing wild stocks through appropriate angling regulations.

Keywords: conservation, hybridisation, management, *Salmo trutta*, stocking

Introduction

The preservation of genetic diversity is a major task in conservation and evolutionary biology, as genetic variation is the raw material for evolutionary changes within populations (Meffe & Carroll, 1997). The World Conservation Union (IUCN) has recognised genetic diversity as one of the three levels of diversity requiring conservation (McNeely *et al*., 1990). Genetic data, always combined with other ecological and biological factors, may be used as the basis for conservation. The rates of hybridisation and introgression are increasing dramatically worldwide because of translocations of organisms and habitat

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modifications by humans (Allendorf *et al.*, 2001). The harmful effects of hybridisation have led to the extinction of many populations and species (Wolf, Takebayashi & Rieseberg, 2001). Even more, the risk of extinction is probably higher when local populations are introgressed by gene pools from domesticated animals (Lynch & O'Hely, 2001).

A task of major concern in biological conservation is the maintenance of intraspecific genetic diversity in exploited species (Nelson & Soulé, 1987; Ryman, Utter & Laikre, 1995; Reynolds *et al.*, 2001). Several fishing and game species have been restocked using captive-bred individuals, without paying particular attention to the genetic risk to wild populations (Rhymer & Simberloff, 1996). The hybridisation and introgression of exogenous gene pools into the native populations have been observed in game species such as the brown hare *Lepus europaeus* Pallas (Mamuris, Sfougaris & Stamatis, 2001), the wild boar *Sus scrofa* L. (Vernesi *et al.*, 2003) and the red-legged partridge *Alectoris rufa* (L.) (Negro, Torres & Godoy, 2001). The problem is probably most widespread in salmonids, where for several decades hatchery-reared fish have either escaped or been stocked deliberately into wild populations (Allendorf *et al.*, 2001). The release of hatchery fish leads to a reduction of the species' genetic diversity, introgression and eventually, extinction of local populations (Ryman *et al.*, 1995).

In Europe, some of the most detailed genetic monitoring data related to artificial propagation of fish come from brown trout *Salmo trutta* L. populations. The enhancement of populations through stocking has been a common management practice during the past century until now. This management practice has influenced the genetic integrity of native trout, as some genetic studies have revealed (reviewed in Laikre, 1999). For example, Iberian brown trout populations show strong genetic differentiation across drainage systems. Moreover, the Iberian Peninsula played a role as a glacial refuge and therefore is considered a 'hotspot' for brown trout diversity (Machordom *et al.*, 2000; Suárez *et al.*, 2001; Antunes *et al.*, 2002; Sanz, García-Marín & Pla, 2002; Cortey, Pla & García-Marín, 2004). Five Evolutionary Significant Units (ESUs) have been established in this region through polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLPs) of mtDNA (Machordom *et al.*, 2000) and the complete analysis of the mitochondrial D-loop sequence (1025–1027 bp) (Suárez *et al.*, 2001): (1) North Atlantic, distributed northwards the Portuguese border; (2) Duero, an Iberian endemism restricted to the Duero basin; (3) Tajo, which includes the Tajo basin; (4) Southern Iberian rivers, represented by the Guadalquivir basin and minor southern basins and (5) Mediterranean Iberian rivers, distributed northwards the Segura basin (Fig. 1).

The position of the Iberian Peninsula seems crucial for the conservation of brown trout's genetic diversity as a whole, as illustrated by its haplotype and nuclear diversity and its likely role as a physical boundary between Atlantic and Mediterranean lineages. Additionally, populations that occur in marginal habitats may be active sites of natural selection, and hence of adaptive significance to the species (Lesica & Allendorf, 1995). However, the genetic uniqueness of Spanish wild stocks is currently threatened by introgression of foreign genes because of artificial stocking with hatchery-reared fish of central and northern European origin (Elvira, 1995a,b; Machordom *et al.*, 1999; Almodóvar *et al.*, 2001; Elvira & Almodóvar, 2001; Almodóvar, Nicola & Suárez, 2002; Almodóvar & Nicola, 2004). These hatchery stocks genetically diverge from native populations, representing different evolutionary lineages (Grant, García-Marín & Utter, 1999). Despite this, Spanish management planning still includes massive releases of trout to improve fisheries.

The primary aim of the present work was to assess and quantify the impact of hatchery fish of exogenous origin on the evolutionary lineages of brown trout in Spain, using data compiled during 10 years and available from literature. Many estimates of introgression rates of local populations have been obtained throughout Spain in recent years (see Table 1). However, a comprehensive picture for Iberian ESUs has not been achieved until now, despite the fact that introgression seems especially harmful in Mediterranean countries. Furthermore, few studies have evaluated the underlying reasons for differences in introgression rates across the Iberian Peninsula. Another aim of the study was to determine the likely causes of the introgression pattern, by means of analysing the stocking effort, angling regulations and environmental features expected to be important for the survival of hatchery trout in the wild. The ultimate purpose is to provide some guidelines for improving the management of Iberian trout populations.

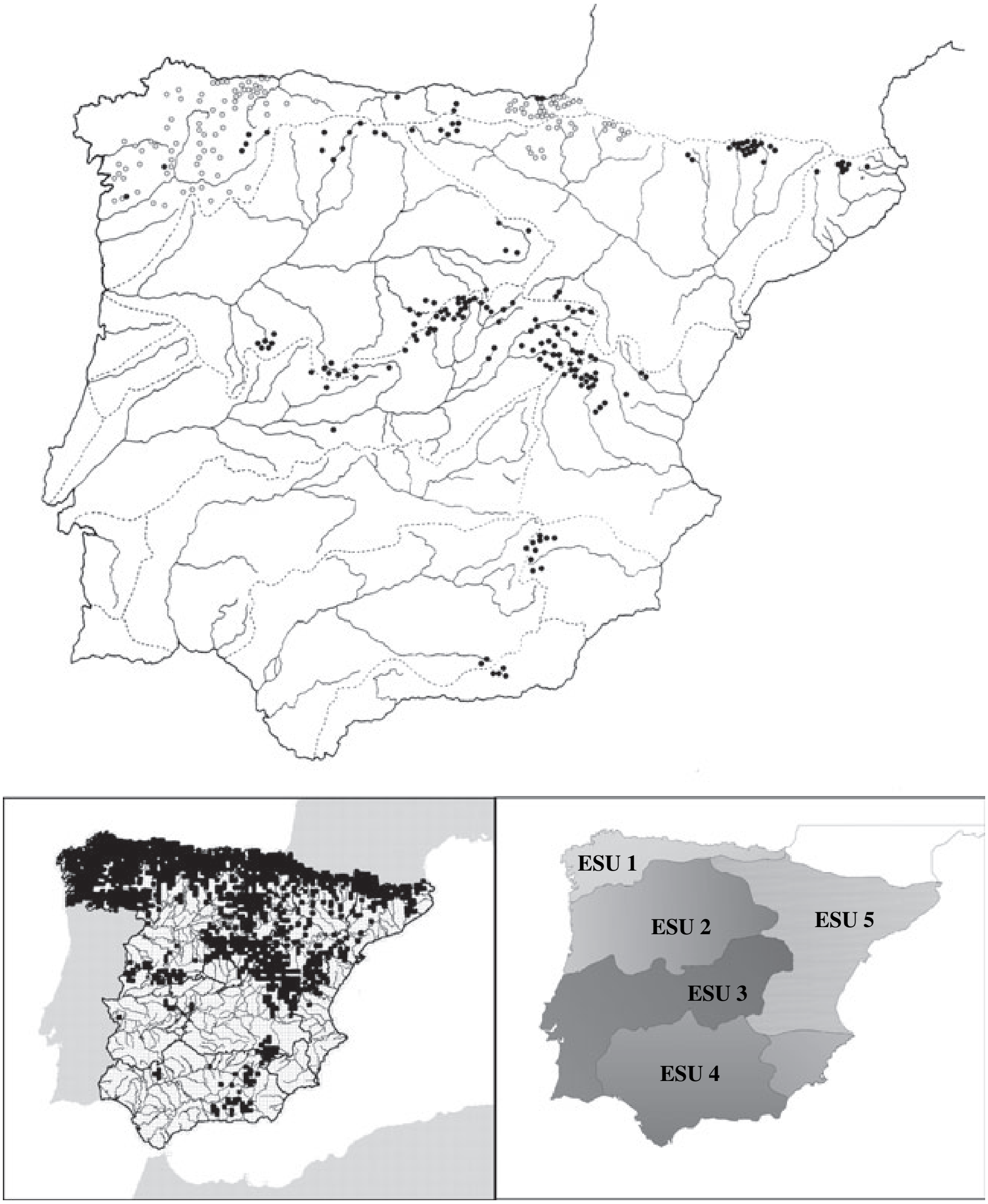

Fig. 1 Map of the sampling localities (black dots: own data, grey dots: literature data). The references for the populations are shown in Appendix 1. Distribution range of brown trout *Salmo trutta* in Spain according to Doadrio (2001) and Evolutionary Significant Units (ESUs) established in the Iberian Peninsula by Machordom *et al.* (2000) through PCR-RFLPs of mtDNA: ESU 1 (North Atlantic), ESU 2 (Duero), ESU 3 (Tajo), ESU 4 (Southern Iberian rivers) and ESU 5 (Mediterranean Iberian rivers). The shaded area in south-eastern Spain corresponds to Segura basin, which shows an overlapping between ESU 4 and ESU 5 because it shares haplotypes from both areas (Machordom *et al.*, 2000).

Methods

Study area and sampling

A total of 3525 brown trout was collected by electrofishing in 180 Spanish streams (Fig. 1, Appendix 1). Trout were anaesthetised with MS-222, immediately frozen on dry ice and stored at −80 °C. Additionally, all published data on genetic interactions between wild and stocked brown trout from 127 Spanish populations were used (3610 trout, Table 1). A mean number of 25 trout were collected from each sampling

Table 1 Mean frequency and range (in brackets) of hatchery markers and a single locus estimation of hatchery genes introgression ($\hat{S}$) found in the wild populations and hatchery stocks analysed in the present study and in the literature within each of the Evolutionary Significant Units of the Iberian Peninsula. The number of studied populations (N_P, own data in brackets), introgressed populations (N_{IP}) and analysed trout (N_T), are also given.

	North Atlantic	Duero	Tajo	Mediterranean	Hatchery
N_P	114 (11)	29 (29)	64 (64)	100 (76)	23 (13)
N_{IP}	28 (25%)	10 (34%)	41 (64%)	75 (75%)	100%
N_T	3476	563	1246	1850	839
*LDH-5*90*	0.050 (0–1.000)	0.039 (0–0.290)	0.129 (0–0.786)	0.197 (0–1.000)	1.000
*G3PDH-2*50*	0.032 (0–0.167)	0.018 (0–0.070)	0.026 (0–0.184)	0.021 (0–0.170)	0.217 (0.118–0.500)
*SMDH-A2*120*	0.008 (0–0.062)	0.026 (0–0.105)	0.017 (0–0.140)	0.037 (0–0.250)	0.205 (0.026–0.400)
*SMDH-B1,2*80*	0.009 (0–0.091)	0.018 (0–0.158)	0.068 (0–0.321)	0.054 (0–0.345)	0.396 (0.207–0.522)
$\hat{S}$	0.016 (0–0.421)	0.033 (0–0.281)	0.068 (0–0.452)	0.137 (0–1.000)	–
References	Present study 1–9	Present study 8–13	Present study 8, 10, 12, 14	Present study 5, 6, 8, 13–16	Present study 1–3, 5–7, 11–16

(1) Arias *et al.* (1995), (2) Martínez *et al.* (1993), (3) Morán *et al.* (1991), (4) Morán *et al.* (1995), (5) Blanco *et al.* (1998), (6) Cagigas *et al.* (2002), (7) García-Marín *et al.* (1991), (8) García-Marín & Pla (1996), (9) Sanz *et al.* (2000), (10) Machordom *et al.* (1999), (11) Almodóvar *et al.* (2001), (12) Almodóvar *et al.* (2002), (13) Sanz *et al.* (2002), (14) García-Marín, Sanz & Pla (1998), (15) García-Marín *et al.* (1999) and (16) Cagigas *et al.* (1999).

locality. The sampling design included representatives from the five ESUs found in the Iberian Peninsula. Thus, ESU 1 (North Atlantic) was represented by 114 samples (3476 trout). ESU 2 (Duero) was sampled in 29 streams (563 trout). Sixty-four collections from the Tajo river basin (1246 trout) typified ESU 3 (Tajo). ESU 4 (Southern Iberian rivers) included four samples from the Guadalquivir River and several southern Spanish rivers (93 trout). The Mediterranean Iberian rivers (ESU 5) comprised 93 streams (1757 trout) (Appendix 1). A recent study (Cortey *et al.*, 2004) suggests that the limits between ESUs 4 and 5 are not as clear as previously thought. Therefore, the two units were pooled and named 'South-eastern Iberian rivers' for analytical purposes. In addition, 23 hatchery samples from central and northern Europe stocks were also screened for comparison.

Genetic analyses

Extracts of eye and muscle tissues were subjected to horizontal starch (11%) gel electrophoresis. Electrophoretic procedures and visualisation of enzyme activity for 59 loci (39 of them being polymorphic) combined the traditional methods of Aebersold *et al.* (1987) and Pasteur *et al.* (1987) modified as indicated in Machordom *et al.* (1999). The following 37 enzymes were studied (Comission number in parentheses): acid phosphatase (3.1.3.2, ACP), aconitate hydratase (4.2.1.3, AH), adenylate kinase (2.7.4.3, AK), alcohol dehydrogenase (1.1.1.1, ADH), aspartate aminotransferase (2.6.1.1, sAAT), creatine kinase (2.7.3.2, CK), diaforase (1.6.4.3, DIA), esterase (3.1.1.1, EST), fructose biphosphatase (4.1.2.13, FBP), fumarate hydratase (4.2.1.2, FH), *β-N*-acetyl-galactosaminidase (3.2.1.53, *β*GALA), *N*-acetyl-*β*-glucosaminidase (3.2.1.30, *β*GLUA), glucose-6-phosphate deshydrogenase (1.1.1.49, G6PDH), glucose-6-phosphate isomerase (5.3.1.9, GPI), *β*-glucuronidase (3.2.1.31, *β*GUS), glutamate dehydrogenase (1.4.1.2, GLUDH), glutathione reductase (1.6.4.2, GR), glyceraldehyde-3-phosphate dehydrogenase (1.2.1.12, GAPDH), glycerol-3-phosphate dehydrogenase (1.1.1.8, G3PDH), guanine deaminase (3.5.4.3, GDA), hydroxybutyrate dehydrogenase (3.1.1.31, HBDB), isocitrate dehydrogenase (1.1.1.42, IDHP), L-lactate dehydrogenase (1.1.1.27, LDH), lactoylglutathione lyase (4.4.1.5, LGL), *α*-mannosidase (3.2.1.24, *α*-MAN), malate dehydrogenase (1.1.1.37, *α*MDH), malic enzyme-NAD (1.1.1.39, ME), malic enzyme-NADP (1.1.1.40, MEP), mannose-6-phosphate isomerase (5.3.1.8, MPI), peptidase leucine-tyrosine (3.4.11.-, PEPLT), proline dipeptidase (phenylalanine-proline substrate, 3.4.13.9, PEPPAP), tripeptidase (leucyl-glicyl-glycine substrate, 3.4.11.4, PEPLGG), phosphoglucomutase (5.4.2.2, PGM), 6-phosphogluconate dehydrogenase (1.1.1.44, PGDH), pyruvate kinase (2.7.1.40, PK), sorbitol dehydrogenase (1.1.1.14, SORD), and superoxide dismutase (1.15.1.1, SOD).

The occurrence of allochthonous genes was estimated by means of the analysis of the diagnostic *LDH-C1** locus (L-lactate dehydrogenase, Enzyme Commission Number 1.1.1.27). The allele *LDH-C1*100* is fixed in Spanish native populations, whereas the hatchery trout employed for stocking are fixed for *LDH-C1*90* allele (García-Marín *et al.*, 1991). We also tested three additional marker loci for hatchery fish, glycerol-3-phosphate dehydrogenase (1.1.1.8, *G3PDH-2**) and malate dehydrogenase (1.1.1.37, *sMDH-A1**, *-A2**, *-B1,2**). Three alleles (*G3PDH-2*50*, *sMDH-A2*120* and *sMDH-B1,2*80*) were apparently absent in Spanish native populations before introductions of the hatchery-reared fish (Sanz *et al.*, 2002). The introgression of hatchery genes in each sample was estimated by the single locus approach ($\hat{S}$), as the average contribution for each of the three allele markers from the expression

$$\hat{S} = \frac{p_{\mathrm{w}} - p_{\mathrm{n}}}{p_{\mathrm{s}} - p_{\mathrm{n}}},$$

where p_{w}, p_{s} and p_{n} are, respectively, the allele frequencies of the wild population, the hatchery stock and the native population before stocking (Taggart & Ferguson, 1986). As these three alleles were not present in native Spanish populations, p_{n} was 0 for all of them (see Sanz, García-Marín & Pla, 2000). For each allele p_{s} was estimated as the average value observed among the available and own data on hatchery stocks.

The 37 studied enzymes were used to analyse within-population genetic diversity by means of expected heterozygosity (H_{e}), percentage of polymorphic loci (P_{95}) and mean number of alleles per locus (N_{a}). Genetic calculations were made using GENEPOP v. 3.2 (Raymond & Rousset, 1995) and GENETIX 4.0 (Belkhir *et al.*, 1998) software.

Management and environmental features

To ascertain management effects on the observed introgression pattern among ESUs, we analysed the stocking records of hatchery-reared brown trout. According to information from the local governments, stocking has been continuous, at least for the last century, in almost all rivers. Stocking records of sampling localities collected by the Spanish Ministry of Agriculture and Fisheries were used to calculate the mean number of stocked trout per year for each locality. To evaluate the genetic effects of harvest we compared introgression levels between exploited and unexploited localities.

Environmental features of sampling localities suspected to be important for trout performance in the wild were considered. Thus, a 10-year series (1993–2003) of daily discharge data for the studied localities was extracted from a database collected by the Spanish Ministry of Environment. The hydrological regime of the studied localities was then characterised by the mean, minimum and maximum annual discharge (as $\mathrm{m}^3\ \mathrm{s}^{-1}$), as well as the discharge irregularity (DI), defined as the coefficient of variation (%) in annual discharge, which gives a measure of the interannual predictability of discharge. Water chemical variables indicative of the stream productivity (mean conductivity in μS, pH and bicarbonates in mg L^{-1}) were calculated for each sampling site from a 10-year series obtained by the Spanish Ministry of Environment and own data.

Statistical analyses

Comparisons of variables between ESUs were performed using parametric (ANOVA) analyses of variance. Multiple *a posteriori* comparisons were then carried out using Scheffé tests (Zar, 1999). Correlations between variables were made using parametric Pearson tests. Stepwise regression analysis was used to explore the relationships of introgression rate with two management and seven environmental variables recorded for each locality in order to build the best predictive model. Assumptions of normality of distributions and homogeneity of variances were verified through Shapiro–Wilk and Levene's tests, respectively. Data were arcsine square root (frequencies) or $\log_{10}$ transformed before the analyses were performed. The significance level for all statistical tests was set at $\alpha = 0.05$.

Results

Differential introgression among ESUs

The frequency of the *LDH-C1*90* allele, the primary indicator of hatchery fish or their descendants, was very variable among Spanish basins and ranged from no hatchery alleles in 154 populations to 100%

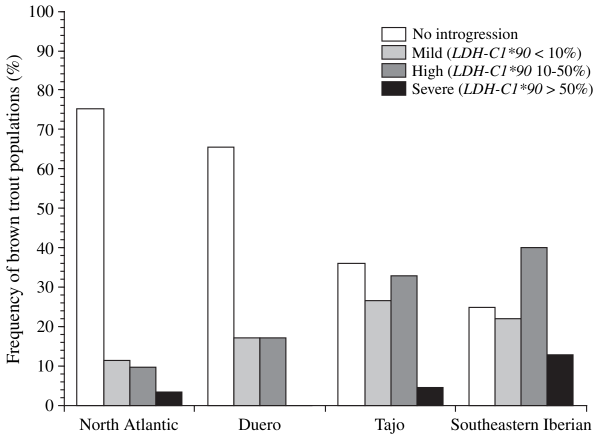

Fig. 2 Distribution of genetic introgression, measured as the percentage of the diagnostic *LDH-C1*90* allele, within each of the Evolutionary Significant Units of the Iberian Peninsula.

hatchery alleles in two populations (Table 1). The hatchery samples were fixed for the *LDH-C1*90* allele. Introgression rates varied significantly among Iberian ESUs (ANOVA, $F_{3,303} = 13.30$, $P < 0.001$) (Table 1), but posterior comparisons revealed no differences between North Atlantic (ESU 1) and Duero (ESU 2) (Scheffé test). Thus, most North Atlantic rivers showed a scarce presence of foreign genes, with little or no introgression in 87% of populations (Fig. 2). The Duero lineage showed a similar pattern, with 83% of populations showing mild (*LDH-C1*90*, <10%) introgression. However, the Tajo lineage showed a higher presence of exogenous genes, with 38% of populations showing high (*LDH-C1*90*, 10–50%) to severe (*LDH-C1*90*, >50%) introgression. The Mediterranean rivers showed average values of introgression around 20%, but 53% of populations were highly or even almost completely introgressed (Fig. 2).

The presence of the alleles *G3PDH-2*50*, *MDH-2*120* and *MDH-1*80* provided additional evidence of the introduction of exogenous genes in some populations. These three alleles were absent from all native populations analysed, but occurred at mean frequencies of 22% (*G3PDH-2*50*), 21% (*MDH-2*120*) and 40% (*MDH-1*80*) in hatchery stocks (Table 1). The *G3PDH-2*50* allele occurred at 35% of the introgressed populations, with mean frequencies between 1.8% and 2.9%. The *sMDH-A2*120* allele was found in 39% of locations, showing an average frequency ranging from 0.7% to 3.7%. The *sMDH-B1,2*80* allele occurred at 35% of populations, with mean frequencies between 0.8% and 6.8% (Table 1). The frequency of these three introgression markers among ESUs showed the same trend as was observed with the *LDH-C1** diagnostic marker. Thus, a significant positive correlation was found between the frequency of these three alleles and the frequency of the *LDH-C1*90* allele (*G3PDH-2**50 versus *LDH-C1*90*, $r = 0.64$, $P < 0.001$; *MDH-2*120* versus *LDH-C1*90*, $r = 0.65$, $P < 0.001$; *MDH-1*80* versus *LDH-C1*90*, $r = 0.67$, $P < 0.001$).

The average contribution of hatchery fish estimated by the single locus approach ($\hat{S}$) presented a similar trend to the *LDH-C1** diagnostic marker (Table 1), showing significant differences among ESUs (ANOVA, $F_{3,217} = 11.44$, $P < 0.001$). Mean introgression ($\hat{S}$) was higher in Tajo and South-eastern Iberian rivers than in North Atlantic and Duero populations, which showed similar low values (Scheffé test).

The level of genetic variation in the analysed populations, as measured by expected heterozygosity (H_e), proportion of polymorphic loci at 95% (P_{95}) and mean number of alleles per locus (N_a), was significantly higher in introgressed populations than in native ones in Duero, Tajo and Mediterranean rivers, whereas in North Atlantic rivers was similar (Table 2). A positive significant correlation was found between the estimated introgression rate and genetic diversity parameters in Duero, Tajo and Mediterranean rivers (Table 3).

Relationships with management and environment

The available data showed that a mean number of 664 000 trout year^{-1} have been released in the studied rivers. The average number of stocked trout showed significant differences among the four ESUs (ANOVA, $F_{3,279} = 19.43$, $P < 0.001$), being particularly lower in the Duero group (Fig. 3). This result is consistent with the low introgression rates observed in this evolutionary unit. The higher stocking numbers observed in the other three ESUs were associated with high observed introgression rates, except for the North Atlantic group. No differences were found in average introgression between exploited and unexploited localities (ANOVA, $F_{1,305} = 0.10$, $P > 0.05$; $N_{\text{exploited}} = 176$, $N_{\text{unexploited}} = 131$).

Some effects of environmental features on the observed introgression pattern were found. A significant negative correlation was found between

Table 2 Mean genetic variability, as measured by expected heterozygosity (H_e), proportion of polymorphic loci at 95% (P_{95}) and mean number of alleles per locus (N_a), in native and introgressed populations within each of the Evolutionary Significant Units considered and in the analysed hatchery stocks

	H_e	P_{95}	N_a
North Atlantic			
Native	0.032	9.942	1.145
Introgressed	0.044	12.500	1.181
	$F_{1,54} = 3.97$, n.s.	$F_{1,54} = 2.28$, n.s.	$F_{1,51} = 2.68$, n.s.
Duero			
Native	0.014	3.751	1.057
Introgressed	0.057	13.887	1.200
	$F_{1,19} = 39.09$***	$F_{1,14} = 53.84$***	$F_{1,12} = 32.04$***
Tajo			
Native	0.012	3.633	1.055
Introgressed	0.048	15.653	1.200
	$F_{1,31} = 37.97$***	$F_{1,21} = 26.31$***	$F_{1,19} = 19.82$***
South-eastern Iberian rivers			
Native	0.033	11.797	1.162
Introgressed	0.065	21.775	1.314
	$F_{1,32} = 25.39$***	$F_{1,32} = 30.10$***	$F_{1,18} = 11.35$**
Hatchery	0.078	21.896	1.270

The results of the analysis of variance (ANOVA) are given (n.s., not significant, **$P < 0.01$, ***$P < 0.001$).

Table 3 Relationship between introgression rate and parameters of genetic variability, i.e. H_e (expected heterozygosity), P_{95} (proportion of polymorphic loci at 95%) and N_a (mean number of alleles per locus) within each of the ESUs considered

	H_e	P_{95}	N_a
North Atlantic	0.15, n.s. (61)	0.12, n.s. (61)	0.15, n.s. (58)
Duero	0.77*** (21)	0.67** (16)	0.71** (14)
Tajo	0.82*** (33)	0.81*** (23)	0.85*** (21)
South-eastern Iberian rivers	0.67*** (39)	0.58*** (39)	0.60** (25)

Correlation coefficients, significance level of correlations (n.s., not significant, **$P < 0.01$, ***$P < 0.001$) and sample size (in brackets) are given.

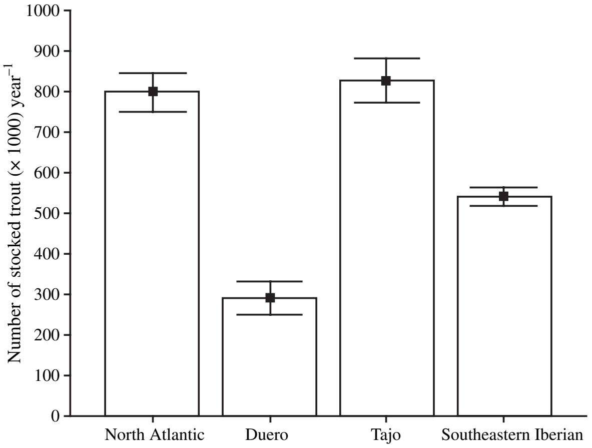

Fig. 3 Mean (±standard deviation) number of brown trout annually stocked in the sampling localities within each of the Evolutionary Significant Units of the Iberian Peninsula. The data refer to the historical stocking records collected by the Spanish Ministry of Agriculture and Fisheries.

introgression rate and both the maximum annual discharge and the discharge irregularity (Table 4). In addition, introgression was found to be positively correlated to conductivity, bicarbonates and pH (Table 4). Thus, introgression tends to be higher in heavily stocked localities with fertile waters and an even discharge. Discharge irregularity (DI) was the variable with the greatest effect on the observed introgression ($\hat{S}$) variability, according to the function $\hat{S} = 0.30 - 0.01\text{DI}$, $F_{1,39} = 6.11$, $r = 0.37$, $P < 0.001$.

Discussion

Introgression rate varied widely among the ESUs studied here. Introgression of exogenous genes reached alarmingly high rates in some Mediterranean rivers, whereas North Atlantic and Duero units showed little or no introgression. A large-scale comparison of European populations of brown trout showed a similar trend. In a recent review, 45% of brown trout genetic studies reported little or no evidence of introgression in rivers where stocking with hatchery-reared trout was intense during many years (Fleming & Petersson, 2001). However, these authors reported massive interbreeding between

Table 4 Mean and range (in brackets) of maximum discharge, discharge irregularity, conductivity, bicarbonates and pH of the study rivers. The correlations between introgression rate and these five variables are also given (correlation coefficient r, significance level of correlation P and sample size n).

	North Atlantic	Duero	Tajo	South-eastern Iberian rivers
Maximum discharge (m^3 s^{-1})	10.8 (0.5–19.3)	24.7 (1.7–87.9)	10.6 (0.4–27.2)	12.0 (0.9–75.7)
$\hat{S}$ × Maximum discharge	$r = -0.23$, $P < 0.01$, $n = 128$			
Discharge irregularity (%)	83.1 (62.1–102.8)	88.0 (52.1–138.1)	59.4 (33.8–121.3)	75.3 (30.6–114.2)
$\hat{S}$ × Discharge irregularity	$r = -0.40$, $P < 0.001$, $n = 128$			
Conductivity (μS cm^{-1})	268.2 (103.0–147.0)	130.5 (18.3–442.4)	431.8 (21.0–1456.0)	332.3 (52.0–1204.3)
$\hat{S}$ × Conductivity	$r = 0.37$, $P < 0.001$, $n = 150$			
Bicarbonates (mg L^{-1})	120.2 (39.2–190.5)	72.9 (11.5–297.7)	135.6 (9–292.5)	129.5 (50.0–367.0)
$\hat{S}$ × Bicarbonates	$r = 0.33$, $P < 0.01$, $n = 92$			
pH	7.8 (6.5–8.4)	7.8 (7.2–8.4)	7.8 (6.7–8.4)	8.1 (7.1–8.5)
$\hat{S}$ × pH	$r = 0.28$, $P < 0.001$, $n = 150$			

hatchery and wild trout and, consequently, high levels of introgression in several populations. Similar studies with other salmonid species have shown a comparable degree of unpredictability on the effect of stocking on introgression (García de Leániz, Verspoor & Hawkins, 1989; Altukhov & Salmenkova, 1990; Crozier, 1993).

The results obtained clearly suggest that the genetic effects of stocking on native trout depend on several site-specific genetic and non-genetic factors. Local stocking practices may also play a role, as high introgression levels were observed in the heavily stocked Tajo and Mediterranean basins. Conversely, the low introgression showed for the Duero basin was related to little stocking. However, in North Atlantic basins the massive release of trout did not result in high introgression rates. Introgressive hybridisation between stocked and wild trout seems to occur more frequently in resident populations than in anadromous ones (Hansen *et al.*, 2000; Utter, 2001; Ruzzante *et al.*, 2004). This fact could partly explain the low impact of stocking in North Atlantic rivers, the only area in the Iberian Peninsula where anadromous trout occur. The lack of complex adaptations for freshwater and marine residence in hatchery trout is suggested as one the main factors supporting this conjecture (Utter, 2001). Natural selection seems to act against hatchery trout and their descendants, including hybrids (Poteaux, Beaudou & Berrebi, 1998; Fritzner *et al.*, 2001; Hansen, 2002). Degree of introgression is thus expected to depend on the strengths of the opposing forces of immigration (stocking) and selection, i.e. the migration-selection balance, as suggested by Hansen *et al.* (1995, 2000). If the immigration rate of hatchery trout on populations is high enough to overcome the rate at which stocked trout are removed by selection, introgression will persist over time and finally replace wild trout.

Angling activity has been proposed to explain introgression differences among rivers, on the basis that introduced trout and their hybrids are more vulnerable to angling than native trout (e.g. Mezzera & Largiadèr, 2001; Champigneulle & Cachera, 2003). However, our findings lend no support to this conjecture; angling regulation did not seem to influence the relative survival of hatchery trout.

Several experimental and field studies have shown that local environmental conditions may be responsible for the lower performance of stocked trout in the natural environment (Einum & Fleming, 2001; Weber & Fausch, 2003). The annual regime of water discharge in rivers plays a role in shaping fish abundance, because of its influence on habitat availability and suitability for fish. Further, water discharge seems to be one of the more relevant environmental features regulating brown trout populations, especially in juvenile stages (Jensen & Johnsen, 1999; Cattanéo *et al.*, 2002). Stepwise multiple regression showed the influence of discharge irregularity on introgression variability, suggesting that more stable flow conditions may improve the survival of hatchery-reared trout. In addition, the positive relationships found between water fertility variables, which are related to geological bedrock, and introgression suggest that stream productivity may favour the performance of hatchery trout. This finding agrees with several comparative reviews (Mann & Penczak, 1986; Kwak & Waters, 1997) and broad-scale studies (Cooper & Scherer, 1967; Scarnecchia & Bergersen, 1987) which have revealed a positive relationship between water fertility, measured as salt concentration, and trout production.

Constant stocking with uniform commercial stocks at the European level results in a reduction of the average genetic distances between regions, and therefore the genetic diversity of brown trout as a whole is reduced. This was confirmed in the present study by the observed positive relationships between both heterozygosity and polymorphism and estimated introgression rate. This entrance of foreign genes into wild populations could eventually homogenise the genetic structure of Iberian populations. A similar pattern has been reported for Mediterranean French populations (Berrebi *et al.*, 2000), where a clear relationship between estimated introgression rate and heterozygosity was found. Further, continuous introgression has also the potential to erode the genetic structure of native brown trout populations. Hatchery-reared trout are certainly replacing native trout in some regions, and a substantial reduction of the gene differences between wild and the hatchery populations after stocking has been observed in some rivers. For example, García-Marín, Utter & Pla (1999) estimated in one eastern Pyrenean river that, despite the reduced reproductive success of hatchery-reared trout in rivers, approximately 5% of the native ancestry is being lost each year. These results were similar to the estimate of 5.5% per year obtained by Arias, Sánchez & Martínez (1995) for a north-western Spanish river and 5.6% for a western Pyrenean river (Blanco *et al.*, 1998).

Brown trout exhibit complex patterns of genetic differentiation both at macro- and microgeographical levels, and several examples demonstrate local adaptations and differences in heritable traits between populations (e.g. Taylor, 1991; Palm & Ryman, 1999; Nicola & Almodóvar, 2002, 2004). At least 43 native allele variants have been reported in the Iberian Peninsula, some of them with a restricted occurrence in only one or a few basins (references in Table 1), where they also tend to occur at fairly high frequencies or sometimes fixed. The distribution of native alleles among evolutionary Iberian lineages reveals a high number of variants in North Atlantic and Duero units, which are the less introgressed ESUs. This finding suggests a possible negative effect of the introduction of exogenous genes on the loss of unique allele variants. Rare alleles contribute little to overall genetic variation, but they may offer unique responses to future evolutionary challenges (Allendorf *et al.*, 2001; Utter, 2001). The uniqueness of formerly isolated Iberian populations may be thus diminished or lost through intraspecific hybridisation with hatchery-reared trout, as may also be their local adaptations and coadapted gene complexes (Jug, Berrebi & Snoj, 2005).

This study shows the unpredictable, diverse and negative genetic effects of stocking practices on native brown trout. As a consequence, the first conservation measure to preserve the genetic integrity of brown trout should be to stop permanently stocking programs with non-native trout. The cessation of stocking in some regions has been a good measure to preserve wild trout (Almodóvar *et al.*, 2001), and similar results have been observed elsewhere in heavily stocked populations (Hansen *et al.*, 1995). However, sometimes population recovery is poorer than expected, as has been observed in France (Poteaux *et al.*, 1998) and Spain (Araguas *et al.*, 2004). Therefore, a long-term monitoring of genetic changes within populations is needed to address their recovery capacity after cessation of stocking. In summary, supplementation of wild populations of fish and game species using domestic individuals is a widespread measure which rarely achieves the objective of increasing production elsewhere. By contrast, the negative genetic effects of these releases are becoming progressively more evident. Large-scale approaches such as the present study could be useful to evaluate the extent of the hybridisation between native and domestic gene pools in other exploited species.

Acknowledgments

M.M. Hansen, G.K. Meffe and two anonymous reviewers considerably improved an early version of this manuscript. This study was partially supported by the Spanish INIA under three research projects and by the Environmental Agencies of Junta de Castilla y León, Junta de Extremadura and Comunidad de Madrid. We are most grateful to the regional Environmental Agencies who have permitted population studies to be conducted over many years.

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(Manuscript accepted 3 March 2006)

Appendix 1 Study rivers and number of sampling sites (own data and literature) within the Evolutionary Significant Units (ESUs)

ESUs River	Number of sampling sites	
	Own data	Literature
North Atlantic (ESU 1) (20 separate river basins)		
La Nive		1
Bidasoa		4
Oiartzun		2
Urumea		4
Oria	2	9
Urola		3
Deva		4
Nervión	2	
Nansa	1	
Nalón		5
Esva		15
Negro		1
Navia		5
Barayo		1
Esqueiro		1
Ferrera		1
Frexulfe		1
Tambre		5
Umia		6
Miño	6	35
Duero (ESU 2) (main river and 11 tributaries)		
Duero	1	
Tera	1	
Mazo	1	
Muriel	1	
Tormes	5	
Riaza	1	
Cega	2	
Adaja	3	
Esla	6	
Pisuerga	2	
Águeda	5	
Huebra	1	
Tajo (ESU 3) (main river and 14 tributaries)		
Tajo	5	
Arandilla	1	
Ablanquejo	1	
Bornova	1	
Gallo	3	
Cabrillas	3	
Hoz Seca	3	
Ompolveda	1	
Guadiela	11	
Jarama	26	
Guadarrama	2	
Alberche	3	
Tiétar	2	
Ibor	1	
Alagón	1	
South-eastern Iberian rivers (ESUs 4 + 5) (10 separate river basins)		
Guadalquivir	3	
Guadalfeo	4	
Segura	9	
Júcar	18	
Turia	2	
Mijares	2	
Ebro	28	25
Ter	6	
Llobregat	2	
Muga	1	