

Body pigmentation pattern to assess introgression by hatchery stocks in native *Salmo trutta* from Mediterranean streams

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Five qualitative and seven quantitative colouration and spotting pattern features were measured in 23 brown trout *Salmo trutta* populations and two hatchery stocks. Simultaneously, the *LDH-C1**, a diagnostic locus fixed for *90 and *100 alleles in stocking and native populations from southern Europe, respectively, was analysed to classify the brown trout studied according to their origin: native, hatchery stock and hybrids. The three genotypes showed significant differences in the colouration and spotting features and a discriminant function analysis could correctly identify 79% of the individuals. The most discriminating variables were dorsal fin margin colour, number of opercular spots, presence of the preopercular mark and diameter of black spots. Given the low cost, ease and possibility of field identification of native fish, the results indicate great opportunities for the application of morphological-based classification models on the conservation and management of native brown trout stocks.

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Key words: body colouration; conservation genetics; management; phenotype; spotting pattern; tree model classification.

INTRODUCTION

Genetic analyses on brown trout *Salmo trutta* L. have demonstrated the existence of several genetic lineages (Hamilton *et al.*, 1989; Bernatchez, 2001; Presa *et al.*, 2002). Since stocking is a common management option used in brown trout fisheries elsewhere, hybridization between different lineages is an issue of concern for the conservation of species' genetic diversity (Berrebi *et al.*, 2000). Native brown trout distributed across rivers draining the western area of the Mediterranean Sea are heavily affected by stocked hatchery brown trout of Atlantic lineage origin and, recently, introgressive hybridization has been

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identified as a major threat to these populations (Largiadèr & Scholl, 1996; Poteaux *et al.*, 1998; Berrebi *et al.*, 2000; Sanz *et al.*, 2002).

Hybridization and introgression among brown trout strains is mainly studied by genetic techniques, but these are expensive, time-consuming and sometimes involve the sacrifice of fish. On the other hand, the measurement of phenotypic characteristics such as spotting pattern, colour pattern or fin pigmentation are easy to conduct in the field, but few studies have explored their potential for discriminating between native and introduced brown trout strains. They have provided similar information to genetic analysis, however, on several salmonid species (Delling *et al.*, 2000; Kruse *et al.*, 2000; Weigel *et al.*, 2002).

In brown trout, several phenotypic characteristics related to spotting and body colouration have shown a genetic basis (Blanc *et al.*, 1982, 1994; Skaala & Jørstad, 1988; Mezzera *et al.*, 1997) and therefore could be potentially useful to identify individuals belonging to different strains within a population. For example, Skaala *et al.* (1996) used the fine spotting pattern as a morphological marker in addition to an allozyme marker to discriminate between hatchery and wild fish. Some studies have examined phenotypic traits of native brown trout from Mediterranean rivers and their differences from stocked Atlantic brown trout and hybrids. Largiadèr & Scholl (1996) reported that adult Mediterranean brown trout native to the River Doubs (Rhône basin, Switzerland) display four broad black stripes on the body sides. Lascaux (1996) identified a number of spotting and colouration characteristics that differed between native and hatchery brown trout in Mediterranean rivers in south-eastern France. Mezzera *et al.* (1997) observed significant differences in the number and shape of parr marks between native Mediterranean and hatchery brown trout in the River Doubs, and found hybrids between two strains with intermediate morphology.

Natural populations of Iberian brown trout represent a unique and valuable resource to be preserved, but stocking of large numbers of hatchery brown trout of North Atlantic origin has led to high introgression rates on many of these native populations (García-Marín *et al.*, 1999). Awareness on conservation of biological diversity is increasing in Spain and recently some management regulations have arisen for conservation of brown trout genetic integrity. The objective of this research was to develop a cost-effective alternative for assessing introgression on brown trout populations from Mediterranean basins of the Iberian Peninsula. The most informative allozyme locus to discriminate between Mediterranean and North Atlantic brown trout is the *LDH-C1** locus. Brown trout from the Mediterranean group are homozygous for the *LDH-C1*100* allele, while the *LDH-C1*90* allele is found in the North Atlantic brown trout (Hamilton *et al.*, 1989). Domestic strains in Spain belong to this Atlantic type since they are fixed for the *LDH-C1*90* allele (García-Marín *et al.*, 1991; Morán *et al.*, 1991; Martínez *et al.*, 1993; Arias *et al.*, 1995). Hence, the relationship between several external phenotypic measurements and *LDH-C1** genotype was investigated and a predictive model developed to identify native brown trout, hatchery origin brown trout and their hybrids using phenotypic characteristics. This approach could represent a useful tool to assist fishery managers with the restoration and protection of native fish.

MATERIAL AND METHODS

SAMPLING

Sampling was carried out in streams of the northern Mediterranean drainages of the Iberian Peninsula, including the Ter, Tordera and Llobregat Rivers, and several Ebro River tributaries (Segre, Noguera Pallaresa and Aragón). Sites sampled ranged in elevation from 550 to 1620 m. Samples were also obtained from two hatcheries devoted to stocking brown trout waters in Catalonia (Table I). Fish were captured using a pulsed-DC backpack electrofisher at five sites in the Noguera Pallaresa basin in November 1999, and at 18 sites in the remaining basins from May to November 2002. Sampling of stocking individuals was carried out in November 1999 at the Pont de Suert hatchery and in April 2001 at the Bagà hatchery. All brown trout captured were held in buckets of neutral colour until data collection. Fish were anaesthetized with tricaine methanesulphonate (MS-222) and measured (fork length, L_F , to the nearest mm), and afterwards placed in a small portable aquarium to be photographed. The aquarium was made of methacrylate (Plexiglas) and measured 350 mm wide by 200 mm high by 80 mm deep. Direct natural lighting was used and the aquarium was reoriented to minimize glare and reflections. Because of the tradition for taxonomic measurements to be made on the left side of the fish (Behnke, 1992), all fish were photographed facing left. Following photography, the adipose fin of each fish was removed and placed in individual vials with 95% ethanol for genetic analysis. The fish were then placed in a bucket of stream water to recover from the anaesthetic prior to release at the location of capture. Fish from collection sites of Noguera Pallaresa basin were not fin clipped. Instead, after photography they were deeply anaesthetized, individually placed in plastic bags labelled with the site and specimen codes and transferred to carbonic ice for electrophoresis.

GENETIC IDENTIFICATION OF INDIVIDUALS SAMPLED

In Spain, genetic monitoring related to artificial propagation is facilitated by fixed differences between the hatchery stocks and wild populations at the *LDH-C1** locus (García-Marín *et al.*, 1991; Martínez *et al.*, 1993). Genotyping of each individual for this locus was undertaken by standard allozymic methods on retinal tissue for the 1999 collections, while the PCR-RFLP method described by McMeel *et al.* (2001) was used for samples collected in 2002. For statistical analyses of morphological traits of the wild populations, all *100/100 fish were considered as 'native Mediterranean', all *90/90 as 'stocked fish' and the heterozygotes as 'hybrids'. This is a simplification for populations where hybridization between native and hatchery fish is beyond the first generation, but it represents a useful starting point for the analyses.

Genotypic distributions within collections were tested for conformance to Hardy-Weinberg expectations by the exact probability test using the GENEPOP software package (Raymond & Rousset, 1995). For each sample, the observed frequency of the *LDH-C1**90 allele was considered an estimate of the degree of introgression with hatchery fish. All populations were potentially considered as 'native' where this allele was absent, 'low introgressed' where the frequency of the *LDH-C1**90 allele was <0.1, 'moderate introgressed' where the allele frequency ranged between 0.1 and 0.3, and 'highly introgressed' where the *LDH-C1**90 allele was >0.3.

MEASUREMENTS AND COUNTS

Several phenotypic characteristics potentially useful for discriminating between native brown trout and hatchery brown trout were measured or identified from fish photographs. Phenotypic traits that could be influenced by subjective observations, such as pigmentation intensity, background body colouration or shape of spots and marks were avoided.

TABLE I. Geographic and genetic description of samples, and the introgression level based on *LDH-C1*90* allele frequency

River basin	Geographic location			<i>LDH-C1</i> genotypes					Introgression	
	Stream	Altitude (m.a.s.l.)	L_F (mm)	Native	Stocked	Hybrid	<i>LDH-C1*90</i>	Level		
Tordera		41°46' N; 2°23' E	550	0	25	0	1.00	High		
Ter		42° 6' N; 2°22' E	900	18	1	4	0.13	Moderate		
Ter		42°20' N; 2°17' E	1170	20	0	0	0.00	Null		
Ter		42°20' N; 2°24' E	1040	16	0	4	0.10	Moderate		
Ter		42°14' N; 2°10' E	760	9	0	4	0.15	Moderate		
Ter		42°20' N; 2°10' E	1010	30	0	0	0.00	Null		
Ter		42°18' N; 2°05' E	1100	25	0	0	0.00	Null		
Llobregat		42°15' N; 1°55' E	840	12	2	13	0.31	High		
Llobregat		42°15' N; 1°50' E	810	2	7	5	0.68	High		
Llobregat		42°06' N; 1°44' E	960	18	0	3	0.07	Low		
Llobregat		42°08' N; 1°40' E	820	15	0	5	0.13	Moderate		
Ebro		42°22' N; 1°47' E	1030	19	2	3	0.15	Moderate		
Ebro		42°26' N; 1°54' E	1110	11	0	4	0.13	Moderate		
Ebro		42°24' N; 1°48' E	1160	23	0	0	0.00	Null		
Ebro		42°23' N; 1°40' E	1190	10	1	6	0.24	Moderate		
Ebro		42°19' N; 1°47' E	1200	4	3	13	0.48	High		
Ebro		42°39' N; 1°05' E	970	13	0	2	0.07	Low		
Ebro		42°32' N; 1°13' E	840	17	0	1	0.03	Low		
Ebro		42°41' N; 1°14' E	1620	6	0	0	0.00	Null		
Ebro		42°34' N; 1°19' E	1120	19	0	0	0.00	Null		
Ebro		42°20' N; 0°56' E	750	13	0	2	0.07	Low		
Ebro		42°23' N; 0°52' E	930	43	0	0	0.00	Null		
Ebro		42°47' N; 0°31' W	1470	10	0	0	0.00	Null		
Hatchery		42°15' N; 1°51' E	760	0	30	0	1.00	—		
Hatchery		42°24' N; 0°44' E	820	0	22	0	1.00	—		

L_F , fork length; —, not appropriate for captive population.

Five qualitative and seven quantitative variables were used. Qualitative variables were defined as presence or absence of: (1) striped pattern, defined as vertical dark stripes (usually four) on the body sides of fish, visible only on adult individuals; (2) a black mark in the preopercular region, defined as a black spot behind the eye, larger in size than other spots on the gill cover and body; (3) distinct coloured margin on the dorsal fin (absent, white, or black and white); (4) distinct coloured margin on the anal fin (absent, white, or black and white); (5) red spots on the adipose fin. The quantitative variables were: diameters of the largest (1) black and (2) red spot on the body side of fish; (3) number of black spots on the gill cover, defined as the number of spots visible from a lateral view between the rear margin of the eye and the opercular opening, an area that would correspond to the opercular and preopercular bones; number of (4) black and (5) red spots above the lateral line; number of (6) black and (7) red spots below the lateral line. The diameters of spots were measured in the field with callipers on the left side of the specimens and rounded to the nearest 0.1 mm.

Fish of all sizes were captured and processed during field sampling but data measurements were restricted to those fish >140 mm L_F because the characters that discriminate adults did not seem reliable enough for juveniles. To classify young brown trout the suitability of the number of parr marks, a character used by Mezzera *et al.* (1997) to successfully segregate between native and hatchery juvenile brown trout in a Mediterranean basin river, was investigated. Parr marks are a series of distinctive dark bands that run vertically along the sides of the fish. Parr marks may persist into young adults, but disappear in large individuals. All fish with visible parr marks were counted for this variable.

STATISTICAL ANALYSES

Dichotomous variables (presence of striped pattern, a preopercular mark and adipose spots) were analysed with binary logistic regression and polytomous variables (type of margins in the dorsal and anal fins) with multinomial logistic regression, both methods with L_F as the covariate and genotype as the qualitative factor. If the covariate was clearly non-significant ($P > 0.10$) it was excluded from the model and the data were analysed with G -tests of independence to increase statistical power (although the conclusions were generally unaffected). Similarly, differences among the three genotypes in quantitative variables were analysed with ANCOVA or, if L_F was not significant, ANOVA. A nested design was used to examine the effects of tributaries within basins. The importance of the different sources of variation was assessed with measures of effect sizes (odd ratios for qualitative variables and partial eta squared for linear models).

A principal component analysis (PCA) was used to examine the relationships among variables and to see if genotype was the main source of variation. Kaiser–Meyer–Olkin's measure of sampling adequacy (KMO) was used to confirm the usefulness of PCA. KMO ranges from 0 to 1 and should be well above 0.5 if variables are very interdependent and a PCA is useful. Discriminant functions analysis (DFA) was used in the rest of the study because it is recommended when the objective is to identify which, if any, variable discriminates among *a priori* established groups (in this case, genotypes). To describe the percentage of correctly classified individuals, the jackknifed cross-validation was used because, although yielding a lower estimate, it is more realistic. For these multivariate analyses, margins in the dorsal and anal fins were coded as: 0 (absent), 1 (white) or 2 (black and white) (Tabachnick & Fidell, 2001).

As an alternative to DFA, classification tree modelling (CT) (Weigel *et al.*, 2002) was used to develop a simple dichotomous key to identify, in the field, the three genotypes based on simple discriminating characters. Tree models are fitted by a recursive partitioning procedure successively splitting the data to form homogeneous subsets. A standard approach to fitting tree models is to use 'pruning' to simplify the tree and avoid overfitting. The CT is complementary to DFA because it is non-parametric and the results are very simple to apply although it can also be less powerful in some cases. For

the CT variables were used previously identified as discriminating well among genotypes but easy to measure in the field (so the largest spot diameters were avoided).

For all analyses, number of opercular spots, number of spots above or below lateral line and L_F were \log_{10} -transformed [$\log_{10}(x + 1)$, except L_F ($\log x$)] because linearity, homoscedasticity and normality were improved. All analyses were performed with SPSS 11 except the classification tree (S-plus 2000).

RESULTS

GENETIC ANALYSIS

Genotype analysis showed that only eight (35%) out of the 23 populations collected in the wild were found to be pure native Mediterranean based on *LDH-C1** locus. The remaining 15 collections exhibited frequencies of the *90 allele from 0.03% in Cardós to 100% in Tordera (Table I). Tordera seemed to be a naturalized population of hatchery fish, or a population where native fish have been almost completely removed by continuous introductions. Heterozygotes ('hybrid fish') were detected in the other 14 collections where the *LDH-C1*90* allele was observed, but only in the Segre River were genotype proportions significantly deviated from Hardy–Weinberg expectations ($P = 0.0473$) due to heterozygote deficiency. The significance was removed, however, after application of the Bonferroni sequential correction, and therefore hatchery genes were considered to be well established in these wild populations.

RELATIONSHIPS AMONG COLOURATION VARIABLES

Most colouration variables were significantly interdependent (Table II). A PCA was useful in summarizing the correlation matrix ($KMO = 0.75$) and the two first axes overall explained 46.3% of the variance (Fig. 1). The preopercular mark was strongly associated with numbers of red spots, and these three variables were negatively correlated with margins in the dorsal and anal fins. The fish scores showed a curved, continuous configuration suggesting that the two axes should not be interpreted independently; rather, they showed a single gradient from hatchery fish (90/90 genotype) to native fish (100/100 genotype). The stocked (wild 90/90 genotype) and hybrid fish were intermediate and the four groups were distinguishable from each other. In one extreme, hatchery fish were characterized by presence of margins in the dorsal and anal fins and larger red and black spots; in the other, most native fish displayed a preopercular mark, striped pattern, and a higher number of red spots of smaller size above and below the lateral line (Fig. 2).

DISCRIMINATION OF THE THREE GENOTYPES IN THE WILD

All the qualitative variables except margin of dorsal fin significantly depended on L_F (Table III), but there was also a genotype effect that was more important (odd ratios were much larger than for L_F). Native brown trout (genotype 100/100) had a striped pattern and a preopercular mark more frequently than

TABLE II. Correlation matrix of the colouration variables. Below diagonal, Pearson's correlation coefficient; above diagonal, P values. Sample sizes ranged between 464 and 478 (varying due to some missing values). Variables 1–6 were \log_{10} -transformed prior to analysis. Spots above or below, number of spots above or below the lateral line

Variable	1	2	3	4	5	6	7	8	9	10	11	12	13
1 L_F													
2 Opercular spots	0.276	***									0.006	***	0.221
3 Black spots above	0.191	0.397	***								0.003	***	***
4 Black spots below	0.240	0.581	0.644	***							0.004	0.298	0.206
5 Red spots below	-0.098	0.348	-0.165	-0.097	0.034	***	0.036	***	0.807	0.054	***	0.001	***
6 Red spots above	-0.118	0.234	-0.239	-0.240	0.800	0.010	***	***	0.001	***	0.004	0.792	***
7 Striped pattern	0.258	0.345	-0.040	0.096	0.195	0.146	0.001	0.686	***	***	***	0.662	***
8 Black diameter	0.422	-0.153	0.224	0.166	-0.439	-0.473	-0.019	0.637	***	***	0.781	***	***
9 Red diameter	0.445	-0.096	0.088	0.011	-0.152	-0.169	-0.022	0.660	***	***	0.085	***	***
10 Preopercular mark	-0.180	0.297	-0.188	-0.088	0.523	0.534	0.209	-0.507	-0.345	***	***	***	***
11 Adipose spots	0.125	-0.135	-0.133	-0.263	0.132	0.182	-0.013	0.079	0.210	0.089		***	***
12 Anal margin colour	0.166	-0.209	-0.048	-0.159	-0.012	-0.020	-0.163	0.245	0.252	-0.166	0.266		***
13 Dorsal margin colour	0.056	-0.445	-0.058	-0.259	-0.218	-0.226	-0.287	0.420	0.322	-0.390	0.279	0.507	

***, $P < 0.0005$.

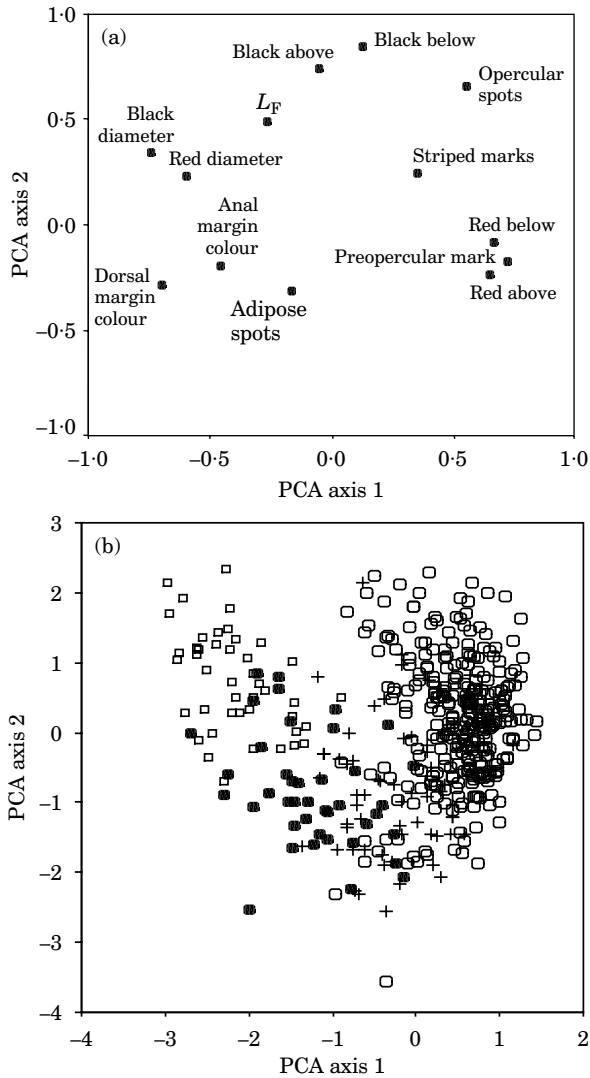


FIG. 1. Principal component analysis of the 12 colouration variables and fork length for the three genotypes plus two hatchery samples. The two axes explained respectively 27.1 and 19.2% of the variance. (a) Factor loadings of the variables. (b) Factor scores of the fish by genotype (\square , 90/90 hatchery; \blacksquare , 90/90 stocked; +, 100/90; \blacksquare , 100/100).

stocked brown trout and less frequently a margin in the dorsal and anal fins (Table IV). Similarly, all the quantitative variables except the number of red spots above the lateral line also varied with L_F (Table V). After adjusting for L_F , there were significant differences among genotypes in all the variables except the number of black spots above the lateral line. Although there was a strong effect of locality, there was no genotype \times locality interaction for six of the seven variables, so the differences among genotypes were similar in different localities.



FIG. 2. Typical morphotypes of (a) native Mediterranean brown trout of studied rivers and (b) stocked brown trout of hatchery origin.

A discriminant function analysis (DFA) of the 12 colouration variables and L_F provided significant functions (Wilks' lambda, $P < 0.001$) which showed a different spotting distribution of three genotypes in the wild (Fig. 3). Native fish displayed a higher number of opercular spots, a preopercular mark and a higher number of red spots of smaller size above and below the lateral line; whereas stocked fish featured the presence of margins in the dorsal and anal fins, more

TABLE III. Effects of fork length and genotype on qualitative variables (excluding fish from hatcheries). The method of analysis was the binary logistic regression (BLR) for binary characters (statistic, Wald), and multinomial logistic regression (MLR) for polytomous characters (statistic, χ^2). r^2 is Nagelkerke's

Variable	Method	L_F			Genotype			r^2
		Statistic	d.f.	P	Statistic	d.f.	P	
Striped pattern	BLR	38.9	1	***	25.9	2	***	0.24
Preopercular mark	BLR	5.84	1	0.016	26.9	2	***	0.17
Adipose spots	BLR	7.64	1	0.006	27.5	2	***	0.16
Dorsal margin	MLR	1.16	2	0.56	141.0	4	***	0.35
Anal margin	MLR	13.5	2	0.001	46.9	4	***	0.20

***, $P < 0.0005$.

TABLE IV. Percentage of fish with presence of certain character states in the four groups: hatchery, stocked, hybrid and native fish

Variable	<i>LDH-CI</i> *genotype			
	90/90 (hatchery)	90/90 (stocked)	100/90 (hybrid)	100/100 (native)
Striped pattern	0	5	12	36
Preopercular mark	4	71	82	96
Adipose spots	2	37	18	7
Black-white dorsal margin	53	68	18	2
White dorsal margin	41	15	40	18
Absent dorsal margin	6	17	43	80
Black-white anal margin	37	51	22	8
White anal margin	61	49	75	87

adipose spots, and less red and black spots but of larger diameter (Fig. 2). The most discriminating variables were dorsal margin colour, number of opercular spots, presence of the preopercular mark and diameter of black spots. The differences among genotypes were larger (partial eta squared) for the number of opercular spots. Native brown trout had a much higher number of opercular spots and a stronger effect of L_F on this variable than hybrid and stocked fish (Fig. 4). A DFA with all the variables identified correctly 79.3% of the individuals (cross-validated), while a DFA with only dorsal margin colour, number of opercular spots and presence of the preopercular mark correctly classified 77.7% (only 46% of the hybrids but 86% and 71% of the native and stocked fish, respectively). The success of DFA prediction was lowest in localities with a mixture of genotypes and very high (usually >90%) in purely native or naturalized populations (Fig. 5).

TABLE V. ANCOVA of the effects of fork length, genotype (G), and locality (L) on quantitative variables (excluding fish from hatcheries). $G \times L$ = interaction. d.f. for the four sources of variation are 1, 2, 22 and 17, respectively; error d.f. = 374. Multivariate tests (Wilks' lambda) were significant for all sources of variation ($P < 0.0005$) except the interaction ($P = 0.10$)

Variable	L_F		G		L		$G \times L$	
	F	P	F	P	F	P	F	P
Opercular spots	97.3	***	29.7	***	4.95	***	1.42	0.13
Black spots above	18.0	***	0.98	0.38	3.38	***	0.42	0.98
Black spots below	28.5	***	3.32	0.037	8.29	***	0.38	0.99
Red spots below	10.5	0.001	7.60	0.001	8.11	***	1.28	0.20
Red spots above	0.02	0.88	3.96	0.020	6.59	***	1.21	0.26
Black diameter	87.2	***	6.90	0.001	11.77	***	1.44	0.12
Red diameter	125.7	***	5.96	0.003	9.34	***	2.30	0.002

***, $P < 0.0005$.

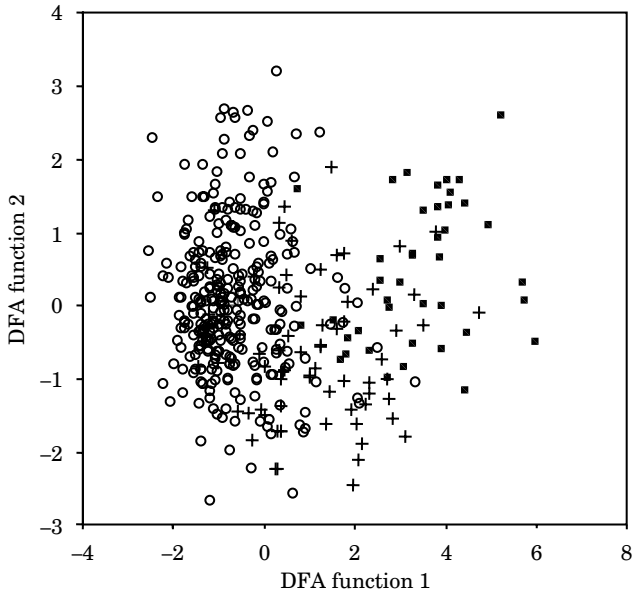


FIG. 3. Discriminant function analysis of the three genotypes (23 localities excluding the two hatchery samples; ■, 90/90; +, 100/90; ○, 100/100) with the 13 variables.

A classification tree model (Fig. 6) with the three most discriminating variables, which are also very easy to measure in the field, agreed with the DFA and allowed identification of the three genotypes with a slightly higher success (83%). Stocked fish usually displayed less than nine opercular spots and a black and white margin in the dorsal fin; native fish had >10 opercular spots or a lower number but without a margin or, more rarely a white margin, in the dorsal fin; hybrids were intermediate. As with DFA, hybrid fish were less successfully identified (50 to 80%) than homozygotes (71 to 95%) with the classification tree (Fig. 6).

Parr marks did not depend on L_F (ANCOVA, $P = 0.82$) and there were clear differences (ANOVA, $P < 0.001$) among the three genotypes (Fig. 7) and no significant genotype \times locality interaction ($P = 0.12$). A DFA of this variable suggested that parr with ≤ 10.2 marks should be considered the stocked genotype, parr with ≥ 11.5 marks the native genotype and intermediate parr should be considered hybrid or uncertain. This criterion correctly classified 89 and 62% of stocked and native parr, respectively.

DIFFERENCES BETWEEN STOCKED AND HATCHERY FISH

Significant differences between stocked (90/90 genotype from natural waters) and hatchery fish (90/90 genotype from hatchery samples) were detected (logistic regression or G -tests, $P \ll 0.05$) on the frequencies of presence of a preopercular mark, margins in the dorsal fin, and adipose spots (Table IV). There were also significant differences between hatchery and stocked fish for all quantitative variables (ANCOVA, $P < 0.001$) except red diameter. A DFA showed that the

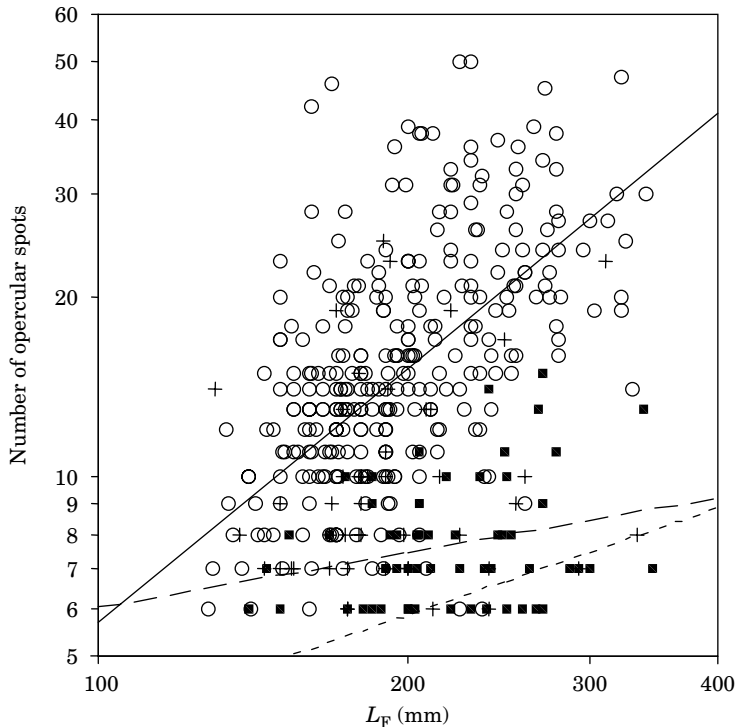


FIG. 4. Effects of the three wild genotypes (■, 90/90; +, 100/90; ○, 100/100) and fork length on the number of opercular spots. ---, 90/90 linear regression line ($r^2 = 0.01$, $P = 0.65$); --, 100/90 linear regression line ($r^2 = 0.01$, $P = 0.36$) and —, 100/100 linear regression line ($y = 0.00806x^{1.4238}$, $r^2 = 0.29$, $P < 0.001$).

most discriminating variables were black spots below the lateral line, red spots above the lateral line and the preopercular mark. A DFA with only these three variables correctly identified 92% of the fish. Stocked fish showed many more black spots below the lateral line (Fig. 8), more red spots and a preopercular mark more frequently than hatchery fish. Similar conclusions were reached from these comparisons when the naturalized fish group was constituted exclusively of fish from the Tordera collection. The fixation of the *LDH-C1*90* allele in the Tordera population suggested that it could be an appropriate control stocked population.

DIFFERENCES AMONG BASINS AND TRIBUTARIES

A nested ANCOVA of the seven quantitative colouration variables (excluding hatchery fish) showed that: (1) the most important sources of variation (largest partial eta squared) were 'tributary within basin' and L_F ; (2) the less relevant sources were 'genotype \times basin interaction' and basin; (3) the genotype \times basin interaction was not significant for the seven variables. Therefore the differences among genotypes were similar in different basins and the geographic variation

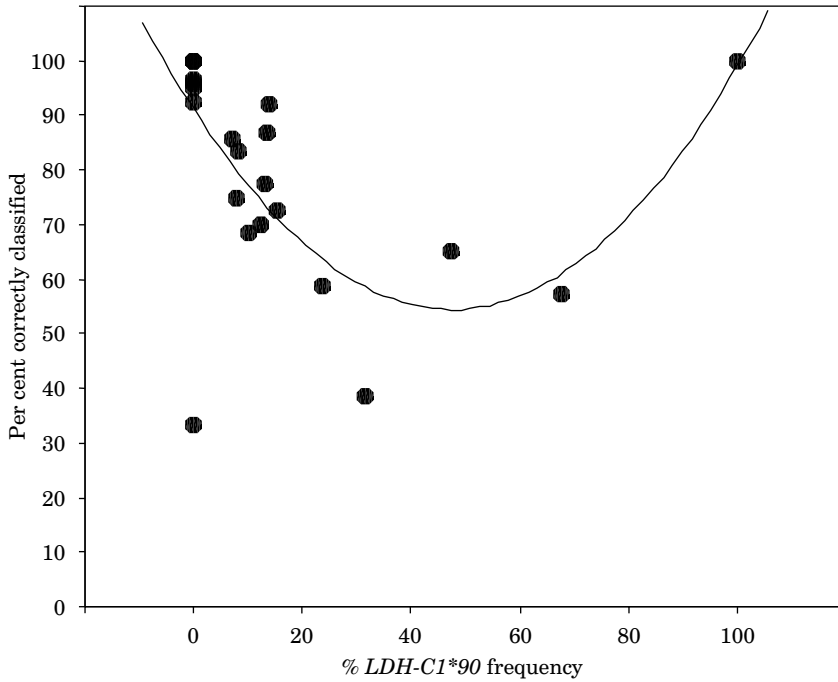


FIG. 5. Per cent of successful classification in the discriminant function analysis of Fig. 4 with genotype frequency in the different localities. The fit of a quadratic polynomial model is shown ($y = 91.1 - 154.2x + 162.0x^2$; $r^2 = 0.41$).

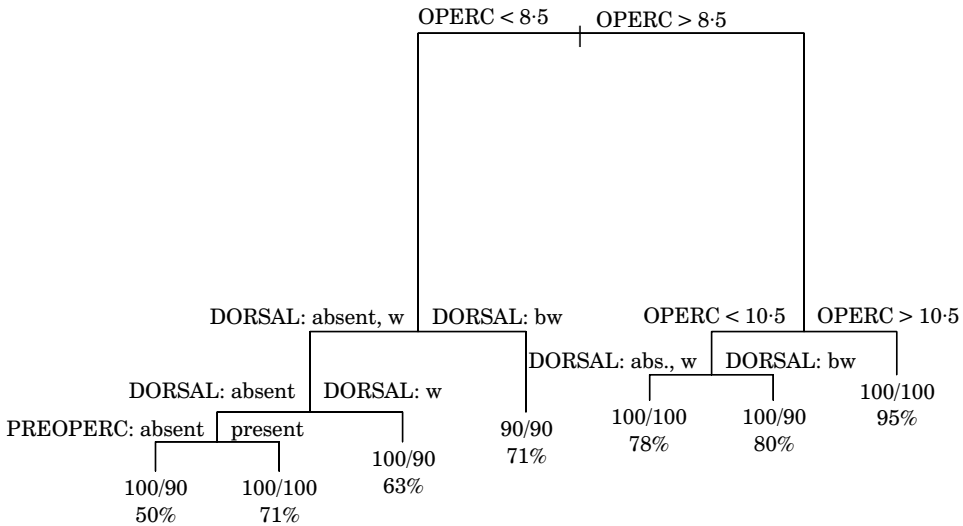


FIG. 6. Classification tree model of the three genotypes (excluding fish from hatcheries) using the number of opercular spots (OPERC), the colouration of the dorsal fin margin (DORSAL: absent, no coloured margin; w, white margin; bw, black and white margin), and the presence of a preopercular mark (PREOPERC). The percentages are the percentages of individuals correctly identified for each node.

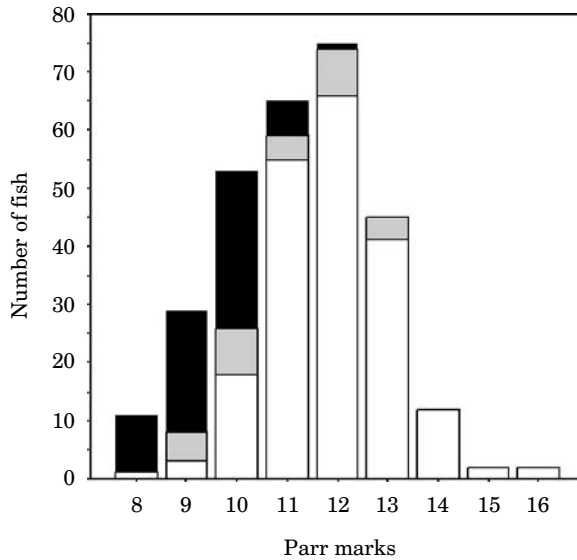


Fig. 7. Frequency of the number of marks in brown trout parr of the three genotypes (■, 90/90; ▒, 100/90; □, 100/100).

(among basins) was less important than the local-scale variation (among tributaries within basins).

DISCUSSION

GENETIC CONSERVATION STATUS OF POPULATIONS STUDIED

Although some fish misclassification suggests that the morphological data cannot be used to make sound inferences on a particular individual, morphological analyses yielded similar proportions of native, stocked and hybrid fish within populations to that reported by the *LDH-C1** locus. Therefore, morphological information could be used to screen the status of many wild populations. Based on the *LDH-C1*90* allele frequency, the average introgression detected in the area under study (12.4%) is similar to that observed in previously analysed Mediterranean populations of Spain (10.9%, Sanz *et al.*, 2002). Only in eight out of the 23 analysed populations (35%) was the hatchery marker *LDH-C1*90* allele absent. These presumptive native populations were found at several headwater sites in the Ter basin and in some high altitude headwater streams of the Ebro basin. In the Ter basin, the sampled area was declared a 'genetic reserve' for native brown trout in 1999 and, since then, the stocking of hatchery brown trout has been banned. Populations with some degree of introgression were the more frequent and reflected the threatened status of the genetic resources of brown trout in the Mediterranean basin (Laikre, 1999). At the Tordera River, the population contained only hatchery forms in a self-sustaining naturalized population. Apparently no natural brown trout population has ever inhabited the sampled stream in this

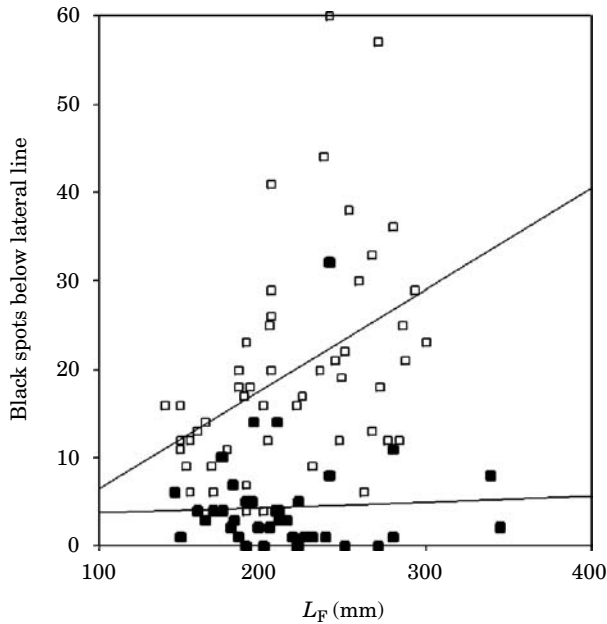


FIG. 8. Differences between hatchery fish (□) and stocked fish (■) (both *LDH-CI*90/90*) in the number of black spots below the lateral line, with length. —, linear regression; hatchery fish ($y = 3.183 + 0.0060x$, $r^2 = 0.0021$, $P < 0.001$) and stocked fish ($r^2 = 0.0021$, $P = 0.78$).

river but natural populations are reported in other tributaries. Introgressed populations usually were located in stretches with annual stocking programmes because of high angling pressure; this is the situation for almost all Llobregat samples. In addition, the location of the Bagà hatchery in the headwaters of this catchment, has contributed, intentionally or accidentally, to the continuous release of hatchery individuals into the river (García-Marín *et al.*, 1999).

GENETIC BASIS OF PHENOTYPE DIFFERENCES BETWEEN NATIVE AND HATCHERY BROWN TROUT

Variation in phenotypic traits among genotypes could be a consequence of genetic differences but also the result of phenotypic plasticity environmentally induced. Fish exhibit higher levels of phenotypic plasticity and lower heritabilities than other vertebrates (Allendorf *et al.*, 1987). Phenotypic plasticity is common in salmonids and artificial environments are known to produce divergence of cultured from wild fishes (Swain *et al.*, 1991; Bernatchez *et al.*, 1992; Fleming *et al.*, 1994; Berejikian *et al.*, 1997). Plasticity may be responsible for the changes detected between hatchery and stocked fish because both groups have a common genetic background. Hatchery brown trout frequently lack the preopercular mark, have a higher number of black spots, mainly below the lateral line, and a lesser number of red spots.

Several types of evidence, however, indicate that the phenotypic variation observed between native and stocked fish (and their hybrids) represents genetic

divergence between both groups. The strong relationship observed between phenotypes and the *LDH-C1** genotypes in all wild populations, suggests a genetic basis for the morphological traits linked to the *LDH-C1** alleles. The lower predictive genotyping in moderate introgressed populations is also consistent with this point of view. In introgressed populations, genetic recombination breaks the above linkage each generation, and produces new gametic associations between the hatchery marker *LDH-C1*90* and native morphological traits (or *vice versa*), that reduces matching scores in the classification of individuals. The intermediate phenotypes observed in hybrid fish for the characters studied, in accordance with similar findings of other studies (Lascaux, 1996; Mezzera *et al.*, 1997), also suggest genetic (additive) variation in morphological traits to explain the divergence observed between native and hatchery brown trout.

GEOGRAPHICAL DIFFERENCES

Significant geographical variation in morphological traits was detected, and nested ANCOVA analyses indicated that variation within basins was more important than among basins. Salmonids develop some morphological and colouration traits in response to environmental factors such as water clarity and bottom structure (Bourke *et al.*, 1997). Without quantitative genetic analyses, however, it is hard to determine what amount of the local divergences represents genetic variation and what reflects local environmental conditions. Using allozymes, Sanz *et al.* (2002) detected large local genetic divergences among Mediterranean brown trout populations that could be related with the above morphological variation, as observed in some species where simultaneous molecular and quantitative studies on population structure have been undertaken (Bonin *et al.*, 1996; Lynch *et al.*, 1999). The spotting pattern and pigmentation traits of native brown trout observed in the present study are common throughout the north-western Mediterranean populations. Thus, native brown trout of Mediterranean rivers from the French Pyrenees were characterized by a fine spotting pattern, high number of black spots on their body sides and gill cover, and usually a white edge on the anal fin (Lascaux, 1996; Poteaux & Berrebi, 1997). Brown trout from the Doubs River (Rhône basin, Switzerland) displayed dark stripes on their body (Largiadèr & Scholl, 1996) and most young brown trout displayed between 11 and 13 parr marks (Mezzera *et al.*, 1997). Mediterranean brown trout from Italy showed a clearly defined, black preopercular spot and a fine spotting pattern on their body sides (Ielli & Alessio, 1996). Differences in the spotting and colouration between native and naturalized trout may therefore have a genetic basis, but local divergences in morphological traits within a basin could be attributed either to genetic or environmental factors. Further research is needed to estimate the genetic components of phenotype variation (and their adaptive value) at microgeographical scale.

POTENTIAL OF DISCRIMINATION OF THE MODEL

Divergence observed in the colouration pattern among native brown trout, stocked fish and their hybrids allows the use of a combination of several

phenotypic characteristics to predict the genetic status of brown trout populations of the area studied with <17% overall error. The three most discriminating characteristics were the number of opercular spots, the dorsal fin margin colour and the presence of a preopercular mark. The first split in the tree model was the number of opercular spots, an easy to measure character useful for separating most fish in the samples, mainly in the mid to large length range. This trait, however, was not a reliable indicator to distinguish hybrid fish and juvenile individuals, where other characters could provide additional information.

The discrimination of individuals according to their genotypes for the *LDH-CI** locus is less powerful in the moderately introgressed populations. As reported for other hybrid populations of brown trout (Delling *et al.*, 2000), the most likely explanation of this result is that after several generations of admixture between native and hatchery fish, recombination occurring between the *LDH-CI** and the loci controlling the morphological traits breaks the association between the diagnostic locus and morphological traits. In spite of the failure to properly discriminate the fish status (native, stocked and hybrid) in these populations, however, the estimates on the population introgression level were similar based either on the *LDH-CI** locus information or in the morphological traits.

The classification model has a major shortcoming because most spotting variables were correlated with L_F and, although this was accounted for in the statistical analyses, the model should not be used beyond the L_F range used to develop it (Table I). Individuals < *c.* 140 mm and > *c.* 350 mm may not be accurately classified by the model. Particularly, native brown trout > *c.* 400 mm L_F seem to deviate from the characteristic spotting pattern shown by medium sized individuals since these old specimens tend to progressively miss spots along their body sides. In addition, although the collections were obtained from a broad geographic area, brown trout populations of Mediterranean basin display high genetic variation (Sanz *et al.*, 2002), therefore some validation is needed before applying the model outside of the studied area.

The effective management of the fish populations needs additional information in multiple areas beyond the genetic approach. Research on life history characteristics, population dynamics, production and behavioural traits requires genetic information for large sample sizes difficult to achieve by genetic analysis, but feasible using phenotypic traits. Therefore the model presented here could help to develop research on these fields. The visible and easily identifiable characteristics of genetically pure brown trout will also help to involve anglers in the conservation of native stocks.

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