

# Origins and relationships of native populations of *Salmo trutta* (brown trout) in Spain

J. L. GARCÍA-MARÍN\* & C. PLA

Laboratori d'Ictiologia, F.C.E. Universitat de Girona, Plaça Hospital 6, E-17071-Girona, Spain

Allele frequency variation at 25 protein-coding loci was compared among collections from 24 naturally spawning brown trout populations in Spain. Evidence of introgression was apparent but alleles of indigenous fish predominated in each of the nine populations sampled in areas where exogenous hatchery fish had been released. Native Spanish gene pools were characterized by strong individuality based on distinct allele frequencies of both adjacent and distant rivers ( $G_{ST} = 0.64$ ), the locally high frequency of rare and previously unreported alleles, and low heterozygosities ( $H_S = 0.031$ ). Two major Spanish lineages are proposed within a previously postulated 'ancestral' grouping of populations existing in southern Europe, based on predominant alleles at the *CK-A1*\* locus. An 'Atlantic' group (comprising populations in Atlantic flowing streams) extends to unglaciated areas of the southern British Isles and Brittany, and is characterized by high frequencies of the \*115 allele. Mediterranean flowing streams are populated by a 'Mediterranean' group extending eastward through the Caspian basin and characterized by high frequencies of the \*100 allele.

**Keywords:** brown trout, evolution, isozymes, *Salmo trutta*.

## Introduction

The evolution and often coexistence of multiple ecological and morphological varieties of brown trout, *Salmo trutta*, have confused the taxonomy of the species throughout its native Eurasian distribution, as reflected in now obsolete designations such as *S. trutta trutta* for anadromous and *S. trutta fario* for freshwater varieties (Behnke, 1986). A better understanding of ancestral groupings of brown trout arose following development of biochemical and molecular methods for identifying allelic variations at multiple defined loci (e.g. Ferguson, 1989; Skaala & Nævdal, 1989; Karakousis & Triantaphyllidis, 1990; Hindar *et al.*, 1991). However, disproportionate sampling of more northern areas has limited possible conclusions about inferred relationships of brown trout at the species level based on the accumulation of biochemical genetic information throughout the species range. Two major groups, distinguished primarily by different common alleles at the *LDH-C*\* locus, have indicated distinct southern ('ancestral') and northern ('modern') populations (Hamilton *et al.*, 1989). Despite the clarity of

this separation, much uncertainty persists about the underlying evolutionary history because of limited sampling of southern populations. For example, previous studies of Spanish wild populations were restricted to small areas or few collection sites (García-Marín *et al.*, 1991; Martínez *et al.*, 1993; Morán *et al.*, 1995).

This study's purpose is to understand better relationships among indigenous Spanish brown trout. Based on variation at 25 polymorphic protein-coding loci, we identify introgression from exogenous ('modern') populations, and tentatively distinguish two subgroups within the 'ancestral' grouping. The implications of these observations to the pre- and postglacial history of brown trout are discussed.

## Materials and methods

### Samples

Brown trout were collected between 1988 and 1992 from 24 Atlantic and Mediterranean drainages throughout the Iberian Peninsula (Fig. 1). The primary criterion for collection locations was the likelihood (based on discussions with management personnel in the sampled areas) that populations

\*Correspondence.

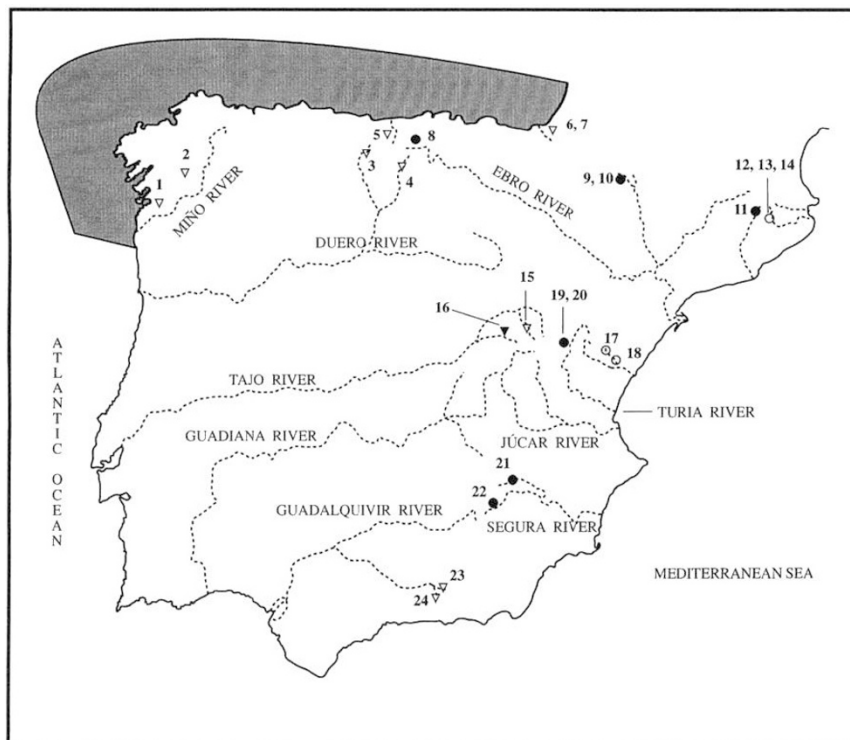
represented descendents of indigenous rather than introduced hatchery fish of exogenous origins. Legal limitations restricted sample sizes to a maximum of 25 individuals per collection, and numbers of some collections were further restricted by the availability of fish. Sampling was by electrofishing within a linear distance not less than 400 m upstream. Following collection, fish were immediately frozen on dry ice, and were transferred to a  $-80^{\circ}\text{C}$  freezer in the laboratory.

### Electrophoresis

Electrophoretic procedures including tissue extraction from skeletal muscle, liver and eye and visualization of enzyme activity were as described in García-Marín *et al.* (1991). Nomenclature for the designation of loci and alleles follows Shaklee *et al.* (1990). The activities of 33 enzymes resolved gene products of 49 loci (Enzyme Commission number and loci in parentheses): acid phosphatase (3.1.3.2, *ACP\**), aconitate hydratase (4.2.1.3, *AH-2\**), alcohol dehydrogenase (1.1.1.1, *ADH\**), aspartate aminotransferase (2.6.1.1, *sAAT-4\**), creatine kinase (2.7.3.2, *CK-A1\**, *-A2\**), esterase (3.1.1.1, *EST-1\**, *-2\**), fumarate hydratase (4.2.1.2, *FH-3\**),  $\beta$ -N-acetylgalactosaminidase (3.2.1.53, *bGALA-2\**), N-acetyl- $\beta$ -glucosaminidase (3.2.1.52, *bGLUA\**), glucose-

6-phosphate isomerase (5.3.1.9, *GPI-A\**, *-B1\**, *-B2\**),  $\beta$ -glucuronidase (3.2.1.31, *bGUS-2\**), glutamate dehydrogenase (1.4.1.2, *GLUDH\**), glyceraldehyde-3-phosphate dehydrogenase (1.2.1.12, *GAPDH-1\**, *-2\**, *-3\**), glycerate dehydrogenase (1.1.1.29, *GLYDH\**), glycerol-3-phosphate dehydrogenase (1.1.1.8, *G3PDH-2\**), guanine deaminase (3.5.4.3, *GDA\**), hydrogen peroxide oxidoreductase (1.11.1.7, *POD\**), isocitrate dehydrogenase (1.1.1.42, *IDHP-1\**, *-2\**, *-3\**), L-lactate dehydrogenase (1.1.1.27, *LDH-A1\**, *-A2\**, *-B2\**, *-C\**), lactoylglutathione lyase (4.4.1.5, *LGL\**),  $\alpha$ -mannosidase (3.2.1.24, *aMAN\**), malate dehydrogenase (1.1.1.37, *sMDH-A1\**, *-A2\**, *-B1,2\**), malic enzyme-NAD (1.1.1.39, *ME\**), malic enzyme-NADP (1.1.1.40, *MEP-1\**, *-2\**, *-3\**), mannose-6-phosphate isomerase (5.3.1.8, *MPI-2\**), peptidase leucine-tyrosine (3.4.11.-, *PEPLT\**), proline dipeptidase (phenylalanine-proline) (3.4.13.9, *PEPPAP\**), dipeptidase (valine-leucine) (3.4.13.\*, *PEPVL\**), tripeptidase (leucyl-glycyl-glycine) (3.4.11.4, *PEPLGG\**), phosphoglucomutase (5.4.2.2, *PGM-1\**), 6-phosphogluconate dehydrogenase (1.1.1.44, *PGDH-1\**), superoxide dismutase (1.15.1.1, *SOD-1\**), xanthine oxidase (1.1.3.22, *XO\**).

Most loci were codominantly expressed, permitting direct counts of allele frequencies from gel phenotypes. Frequencies for the *LDH-A2\*100QL* allele, described in greater detail in the results, were



**Fig. 1** Geographical locations of rivers and streams where brown trout were sampled, and the main river systems in the Iberian Peninsula (Guadiana and Júcar rivers were not sampled). Darkened portion in the Atlantic ocean represents the area where anadromous fish are present. Map codes correspond to streams: (1) Tea, (2) Bubal, (3) Carrión, (4) Pisuerga, (5) Vendúl, (6) Gorrotola, (7) Santolatz, (8) Guares, (9) Ordisa, (10) Otal, (11) Riutort, (12) Ter, (13) Nuria, (14) Massanes, (15) Hocesecca, (16) Valsalobre, (17) Linares, (18) Villahermosa, (lower part of the Linares stream), (19) Guadalaviar, (20) Noguera, (21) Madera, (22) Mundo, (23) Genil and (24) Dilar. Symbols:  $\circ$  Mediterranean flowing river,  $\nabla$  Atlantic flowing river. Filled symbol means potential presence of hatchery fish in the sampled area as indicated by forestry guards or presence of the *LDH-C\*90* allele.

estimated by the square root of the homozygous phenotypes, assuming Hardy–Weinberg equilibrium. Allele frequencies for the isoloci *sMDH-B1,2\** were allocated to individual loci, *sMDH-B1\** and *sMDH-B2\**. All variation of the \*80 allele was assigned to the *sMDH-B1\** locus, and frequencies were estimated from the square root of the homozygous \*100/100 phenotypes, assuming Hardy–Weinberg equilibrium. Frequencies of the \*75 allele could be calculated directly from electrophoretic phenotypes and were assigned to either the *sMDH-B1\** or the *sMDH-B2\** loci as indicated in Fig. 2.

### Data analysis

Phenotypic distributions of all codominantly expressed loci were tested for agreement with Hardy–Weinberg expectations by the exact probabilities test (Hernández & Weir, 1989). For loci with more than two alleles, the variant alleles were lumped; such lumping was required for only four (three triallelic and one tetra-allelic) out of 140 tests. Allele frequency differences among samples were tested by contingency chi-square analysis (Sokal & Rohlf, 1981). The sequential Bonferroni technique (Rice, 1989) was used to adjust significance levels for multiple simultaneous comparisons. To test for random mating in populations introgressed with hatchery fish, a study of pairwise gametic phase equilibria involving alleles of hatchery and indigenous origin was made using Burrow's disequilibrium parameter *D* (Campton, 1987).

Hierarchical gene diversity analysis (Nei, 1973; Chakraborty *et al.*, 1982) was used to partition the total genetic variation ( $H_T$ ) into its components within streams ( $H_S$ ), between streams within the main river ( $G_{SR}$ ), between rivers within the drainage

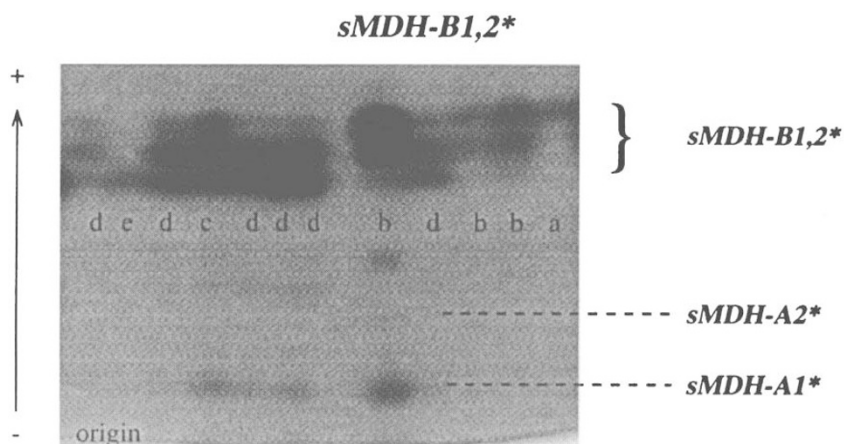
( $G_{RD}$ ), and between Atlantic and Mediterranean drainages ( $G_{DT}$ ). Pairwise multilocus comparisons between samples were calculated by Nei's measure of genetic identity and its associated genetic distance (Nei, 1972). A dendrogram was constructed by the unweighted pair-group method with arithmetic averages (UPGMA, Sneath & Sokal, 1973) from the matrix of distances; confidence of the branching points was estimated by bootstrapping of the polymorphic loci over 1000 replicates. In addition, patterns of variation were assessed from the matrix of identities (rather than the non-Euclidean distance measure) by a principal coordinate analysis (Gower, 1966).

## Results

### Interpreting LDH-A2\* variation

We observed variation of LDH patterns from muscle extracts involving a reduced intensity of the homomeric band for *LDH-A2\** (Fig. 3, phenotypes b). Similar variation has previously been described in populations from Greece by Karakousis & Triantaphyllidis (1990), who interpreted the variant phenotype to reflect individuals heterozygous for a null allele at the *LDH-A2\** locus. The high frequency of this phenotype (>90 per cent) in some Spanish samples considerably exceeds expectations of heterozygotes under Hardy–Weinberg equilibrium, and has forced us to consider an alternate explanation. We therefore interpret the b phenotypes in Fig. 3 to be from homozygous individuals for a variant allele at the *LDH-A2\** locus having altered banding intensities that reflect a reduced level of activity of this allele. Following Shaklee *et al.* (1990), we designate this allele \*100QL.

**Fig. 2** MDH zymogram from brown trout muscle extracts showing phenotypic variation as a result of a previously unreported allele in the *sMDH-B1,2\** isoloci. Genotype interpretations: a, *sMDH-B1,2\** 100/100/100/100 (common phenotype); b, *sMDH-B1,2\**\*75/100/100/100; c, *sMDH-B1,2\**\*75/75/100/100; d, *sMDH-B1,2\**\*75/75/75/100; e, *sMDH-B1,2\**\*75/75/75/75.

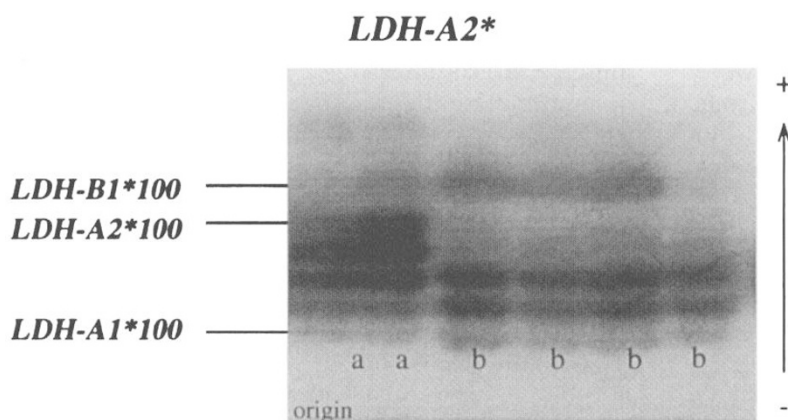


### Genetic variation within populations

Allelic variation was detected at 25 of the 49 protein-coding loci examined (Appendix). Among individual populations, this overall polymorphism ranged from 0 to 36 per cent, and heterozygosity ranged from 0 to 6.1 per cent. Allelic variants *ADH\*0*, *ACP\*115*, *bGALA-2\*90*, *GPI-B2\*50*, *IDHP-1\*85*, *LGL\*90*, *aMAN\*90*, *sMDH-B1,2\*75*, *ME\*140*, *MEP-3\*90*, *PGM-1\*80* and *SOD\*120* do not appear to have been reported previously in other countries. However, although side-by-side gel comparison was not made, our *sMDH-B1,2\*75*, *MEP-3\*90* and *SOD-1\*120* alleles seem to be equivalent to the previous rare alleles *MDH-3\*80* (Martinez *et al.*,

1993), *ME-4\*75* (Krieg & Guyomard, 1985) and *SOD-2\*135* (Riffel *et al.*, 1995).

Genotypes of five individuals from collection (11) Riutort Creek, and six from collection (22) River Mundo, differed particularly from other concurrently collected fish at these locations. Most notable was the fixation of the *LDH-C\*90* allele, typical of exogenous fish from Spanish hatcheries (García-Marín *et al.*, 1991) that was absent in other fish of these collections. Tests for gametic phase disequilibrium (*D*) were made with these collections, both including and excluding these 11 fish, as well as with collections from other areas where hatchery fish had been reported (Fig. 1, Table 1). Significant *D*-values were restricted to (11) Riutort Creek, and (22)



**Fig. 3** LDH zymogram from brown trout muscle extracts showing a phenotypic variation interpreted as a variant allele at the *LDH-A2\** locus (see text).

**Table 1** Gametic phase disequilibrium analysis in populations of brown trout planted with hatchery-reared fish

Population	<i>LDH-C*90</i> <i>G3PDH-2*50</i>	<i>LDH-C*90</i> <i>sMDH-A2*120</i>	<i>G3PDH-2*50</i> <i>sMDH-A2*120</i>	<i>LDH-C*90</i> Indigenous allele
8 Guares	N.c.	N.c.	N.c.	-0.000
9 Ordisa	N.c.	-0.006	N.c.	+0.029
10 Otal	N.c.	-0.010	N.c.	-0.023
11 Riutort (all fish)	+0.042*	+0.097***	+0.025**	-0.262***
11 Riutort (indigenous fish)	-0.003	N.c.	N.c.	N.c.
16 Valsalobre	+0.005	+0.030	+0.039*	+0.018
19 Guadalaviar	+0.002	N.c.	N.c.	-0.071*
20 Noguera	+0.017	-0.002	-0.007	-0.067
21 Madera	N.c.	N.c.	N.c.	-0.002
22 Mundo (all fish)	+0.032	+0.108	+0.031	-0.438***
22 Mundo (indigenous fish)	N.c.	N.c.	N.c.	-0.135***

Single-test significance probabilities are: \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

Indigenous allele is: *ACP\*115* in population (8); *bGALA-2\*95* in population (9); *IDHP-3\*130* in populations (10) and (22); *aMAN\*90* in populations (11) and (16); *ADH\*0* in populations (19) and (20); and *ME\*140* in population (21).

'N.c.' indicates that the disequilibrium parameter (*D*) is not computable because one or both loci are monomorphic in the population.

River Mundo, and all but one involved inclusion of these 11 fish; the other involved *LDH-C\*90* and *IDHP-3\*130* in the (22) River Mundo collection excluding these fish. These disequilibria are consistent with these 11 fish representing a distinct hatchery population. The (11) Riutort Creek and (22) River Mundo samples are therefore each listed twice in the Appendix, with and without these fish of apparent hatchery origin.

Tests for expected binomial (Hardy–Weinberg) genotypic frequencies within loci and other subsequent analyses of this study excluded these 11 fish of presumed hatchery origin. No significant deviations from expected Hardy–Weinberg genotypic frequencies ( $P < 0.05$ ) were observed.

#### Genetic variation among populations

Large differences in allele frequencies were observed among populations (Table 2, Appendix). Frequencies of the \*100 allele ranged from 0.0–1.0 for eight loci (*CK-A1\**, *bGALA-2\**, *LGL\**, *aMAN\**, *ME\**, *MEP-3\**, *PEPLT\**, *PGM-1\**) and the range exceeded 0.5 for 11 others. The magnitude of variation was apparent both in the significance of heterogeneity among samples for all loci but *EST-2\**, and in a level of significance less than 0.001 for all other loci except *G3PDH-2\**. Variant alleles, when present, tended to occur at fairly high frequencies; however, their distributions among streams within main rivers sometimes failed to show a clear pattern.

**Table 2** Distribution of electrophoretically detectable gene diversity among Spanish populations of brown trout (standard errors in parentheses)

Locus	Frequency range of *100 allele	NPOP	Absolute gene diversity		Relative gene diversity (%)			
			Total $H_T$	Within populations $H_S$	Within populations $H_S/H_T$	Differentiation among populations ( $G_{ST}$ )		
						$G_{SR}$	$G_{RD}$	$G_{DT}$
<i>ACP*</i>	0.75–1.00	2	0.029	0.023	81	11	8	0
<i>ADH*</i>	0.33–1.00	2	0.098	0.039	40	0	55	5
<i>sAAT-4*</i>	0.42–1.00	4	0.063	0.036	57	23	19	1
<i>CK-A1*</i>	0.00–1.00	18	0.497	0.127	26	2	8	64
<i>EST-2*</i>	0.97–1.00	2	0.006	0.005	97	1	2	0
<i>bGALA-2*</i>	0.00–1.00	9	0.186	0.084	45	23	25	7
<i>bGLUA*</i>	0.16–1.00	12	0.205	0.129	63	16	17	4
<i>GPI-B2*</i>	0.22–1.00	3	0.111	0.040	36	34	25	5
<i>G3PDH-2*</i>	0.94–1.00	4	0.018	0.017	96	1	2	1
<i>IDHP-1*</i>	0.33–1.00	1	0.054	0.019	34	34	28	4
<i>IDHP-2*</i>	0.04–1.00	7	0.185	0.052	28	15	48	9
<i>IDHP-3*</i>	0.10–1.00	10	0.174	0.100	58	28	12	2
<i>LDH-A2*</i>	0.06–1.00	10	0.357	0.152	43	10	45	2
<i>LDH-C*</i>	0.79–1.00	11	0.135	0.101	75	11	11	3
<i>LGL*</i>	0.00–1.00	2	0.152	0.000	0	0	89	11
<i>aMAN*</i>	0.00–1.00	7	0.386	0.045	12	14	70	4
<i>sMDH-A2*</i>	0.86–1.00	4	0.035	0.032	90	6	4	0
<i>sMDH-B1*</i>	0.02–1.00	9	0.215	0.067	31	1	62	6
<i>sMDH-B2*</i>	0.34–1.00	2	0.093	0.039	42	1	51	6
<i>ME*</i>	0.00–1.00	10	0.324	0.131	40	16	26	18
<i>MEP-3*</i>	0.00–1.00	5	0.224	0.040	18	2	79	11
<i>MPI-2*</i>	0.22–1.00	13	0.250	0.130	52	12	32	4
<i>PEPLT*</i>	0.00–1.00	5	0.169	0.017	10	1	78	11
<i>PGM-1*</i>	0.00–1.00	4	0.111	0.025	23	42	29	6
<i>SOD-1*</i>	0.52–1.00	3	0.081	0.053	66	12	18	4
Average			0.085 (0.017)	0.031 (0.006)	36 (4)	12 (2)	39 (6)	13 (7)

NPOP, number of populations with allelic variants.

$G_{SR}$  differentiation between populations (streams) within main river,  $G_{RD}$  differentiation between main rivers within drainage and  $G_{DT}$  differentiation between drainages (Atlantic vs. Mediterranean).



The projection of the first two principal coordinates of the genetic identity matrix (PCO, Fig. 5) also linked the similar paired populations from common rivers, and indicated such outlying individual populations as (6) Gorrotola, (11) Riutort and (14) Massanes. However, PCO added resolution not apparent in the cluster analysis by assigning positive values of coordinate 1 to populations of Atlantic drainages and negative values to the Mediterranean collections. In addition, the PCO projections plots tended to draw together most of those populations from areas where hatchery plants have been reported.

## Discussion

Interpretation of the data of this study is limited by legally imposed sampling restrictions resulting in reduced accuracy and precision of allele frequency estimates based on five to 31 individuals. For instance, estimates of actual allele frequency values other than 0, 0.10, 0.20, etc. would inevitably be distorted in a sample of 10 genes from five individuals. Such distortions also tend to bias downwards estimates of polymorphic loci and heterozygosity, and to inflate components of gene diversity. In spite of these limitations, the observed variation provides some useful insights into the genetic population

structure of Iberian brown trout populations because of its qualitative nature, reinforced by the low levels of polymorphisms detected in most of the larger samples, i.e. (2) Bubal, (3) Carrión, (4) Pisuerga, (5) Vendúl, (6) Gorrotola, (11) Riutort, (15) Hocesecca, (17) Linares, (18) Villahermosa, (23) Genil and (24) Dilar.

### *Influence of hatchery fish*

The exclusion from analyses of 11 fish of apparent hatchery origin or ancestry does not eliminate the possibility of introgression from hatchery fish among the remaining fish. The fixation of the *LDH-C\*90* allele in hatchery populations used in Spain and its apparent absence from Iberian populations prior to introductions of hatchery fish (García-Marín *et al.*, 1991; Martínez *et al.*, 1993; Morán *et al.*, 1995) has provided a useful genetic marker for seeking such introgression. This characteristic allele of the 'modern race' of northern European brown trout (Hamilton *et al.*, 1989) was observed only in populations from each of the areas of collection where hatchery fish had been reported. Other alleles occurring at appreciable frequencies (>0.10) in Spanish hatchery stocks but rare in indigenous populations were also observed in these areas, including *G3PDH-2\*50*, *sMDH-A2\*120* and *sMDH-B1\*80* (García-Marín *et al.*, 1991; Martínez *et al.*, 1993).

Cumulatively, these data strongly infer the presence of hatchery genes in these areas. Similar introductions of hatchery genes have been reported in Mediterranean French populations (Barbat-Leterrier *et al.*, 1989). The lack of significant departures from Hardy-Weinberg expectations and only a single observation of gametic phase disequilibrium among wild fish collected in areas of hatchery releases suggests that these exotic genomes are assimilated within several random mating wild brown trout populations of Spain. Clearly, indigenous 'ancestral' and exogenous 'modern' lineages (Hamilton *et al.*, 1989) have not evolved full reproductive isolation. Although the low frequencies of the introduced *LDH-C\*90* allele in each location (range 0.02–0.21) reflect a persisting predominance of native genes in these areas, hatchery populations have completely displaced native ones in other areas of Spain (García-Marín *et al.*, 1991).

### *Patterns of genetic differentiation*

The high  $G_{ST}$  value (0.64) indicates a particularly high genetic divergence among Spanish populations. The above-noted exaggeration of divergence because

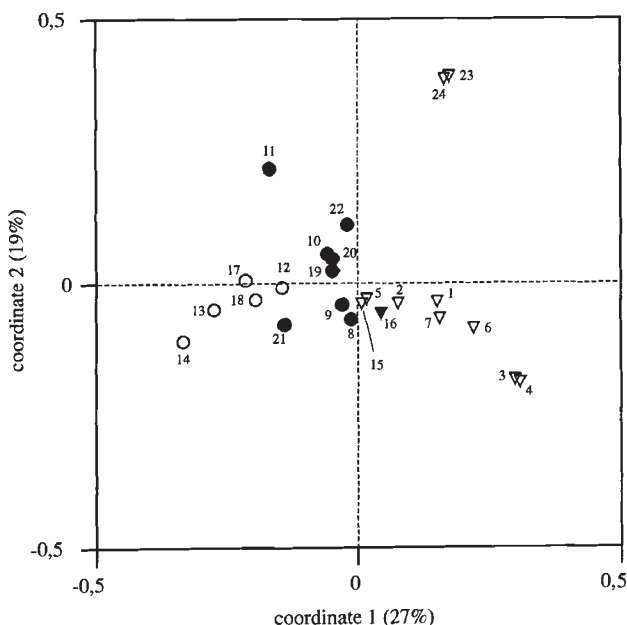


Fig. 5 Principal coordinates analysis of Spanish brown trout populations based on Nei's genetic identity matrix. Populations are projected onto the plane formed by the first two principal coordinates axes (symbols as in Fig. 4).

of small sample sizes is offset by reduced divergence through apparent introgression from a common exogenous population in nine collections. Consequently, this high value is considered to be biologically meaningful.

Some of this distinctness certainly reflects a highly restricted gene flow among populations and extensive genetic drift within them, typical also of brown trout studied in Scandinavia (Ryman, 1983) and the British Isles (Ferguson, 1989). In addition, the considerable number of previously unreported alleles in these populations is consistent with the opportunity for accumulation of genetic variation in this region that is not available to recently repopulated postglacial regions (Ferguson, 1989). Under these conditions, it is not surprising to find populations of rivers with unique alleles, resulting in the high degree of differentiation between main rivers within the Atlantic and Mediterranean drainages ( $G_{RD} = 0.39$ ). Such limited gene flow is also consistent with the general lack of geographical and genetic correspondence in the UPGMA cluster analysis, and with the low average heterozygosities ( $H_S = 0.031$ ) we observed. We propose that the brown trout in Spanish rivers have been isolated since the end of the Würmian glaciation in northern Europe. We envisage the existence of larger and possibly anadromous populations during glacial times whose sizes and migratory potential became reduced as increased temperatures restricted them to upstream regions. In this sense, our  $G_{RD}$  value is nearly twice that observed among Spanish populations when anadromous fish are present ( $G_{RD} = 0.21$ , Morán *et al.*, 1995).

#### *Ancestral origins*

Another isolating component of Spanish populations is superimposed on that resulting from restricted migration and drift. A distinct separation between unstocked populations of Atlantic and Mediterranean drainages is apparent in the first principal coordinate (Fig. 5). This grouping implies the existence of subdivisions within the 'ancestral race' proposed by Hamilton *et al.* (1989). The interfacing of these groups in Iberian rivers further contributes to the overall heterogeneity ( $G_{DT} = 0.13$ ) in this region.

This distinctness between Atlantic and Mediterranean drainages is primarily a reflection of the respective predominance of the *CK-A1\*115* and *CK-A1\*100* alleles in Atlantic and in Mediterranean rivers. The 'Atlantic' subdivision is proposed to extend to unglaciated areas of the southern British

Isles and Brittany based on the predominance of the *CK-A1\*115* allele and the *LDH-C\*100* allele (the marker allele of the 'ancestral race') reported in unstocked populations in these areas (Krieg & Guyomard, 1985; Hauser *et al.*, 1991). The 'Mediterranean' subdivision is proposed to extend eastward through the Black and Caspian Seas based on the predominance of *CK-A1\*100* and *LDH-C\*100* throughout this area (Krieg & Guyomard, 1985; Osinov, 1988; Barbat-Leterrier *et al.*, 1989; Karakoussis & Triantaphyllidis, 1990).

Our hypothesis of distinct 'ancestral' subgroups of brown trout in Spain raises additional issues that cannot presently be resolved. The presence of high frequencies in the two Guadalquivir River samples (23, 24) of the *LDH-A2\*100QL* and *MEP-3\*90* alleles found otherwise only in many (but not all) Mediterranean streams is puzzling. Perhaps these sampling locations represent areas of merging between Atlantic and Mediterranean groups, as suggested by similarities of other ichthyofauna (Doadrio, 1988; Bianco, 1991). The absence of similar data sets from other areas inhabited by native brown trout such as the Atlas Mountains in northern Africa, leaves open the possibility of the existence of additional major lineages. Conclusions derived from the present data provide a valuable framework for further insights into the evolutionary history of brown trout as information from nuclear loci and mitochondrial DNA (e.g. Bernatchez *et al.*, 1992) accumulates to resolve these and other issues.

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**Appendix** Observed frequencies at brown trout polymorphic loci and locus sample size. For populations 11 and 22, estimated frequencies are given both excluding and including (in parentheses) the presumed hatchery fish (variation at *GPI-A\** locus resulted from hatchery fish). Population codes refer to Fig. 1

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
<i>ACP*</i>	6	25	29	31	29	26	22	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23	
*100	1.00	1.00	1.00	1.00	1.00	1.00	0.75	0.90	1.00	1.00	1.00 (1.00)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00	
*115							0.25	0.10																	
<i>ADH*</i>	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	24	27	5 (11)	22	23	
*100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.42	0.33	1.00	1.00 (1.00)	1.00	1.00	
*0																			0.58	0.67					
<i>sAAT-4*</i>	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	26	27	5 (11)	22	23	
*100	1.00	0.42	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00 (1.00)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.96	0.94	1.00 (0.95)	1.00	1.00	
*50		0.58								0.06										0.04	0.06	(0.05)			
<i>CK-A1*</i>	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23	
*100	1.00	0.34	1.00	1.00	0.60	0.02	0.17	0.58	0.73	0.89	1.00 (1.00)	1.00	1.00	1.00	1.00	0.03	1.00	1.00	0.94	0.91	0.98	0.80 (0.77)	1.00	1.00	
*115	1.00	0.66	1.00	1.00	0.40	0.98	0.83	0.42	0.27	0.11					1.00	0.97			0.06	0.09	0.02	0.20 (0.23)	1.00	1.00	
<i>EST-2*</i>	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23	
*100	1.00	1.00	1.00	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00	
*92					0.03			0.03																	
<i>bGALA-2*</i>	6	25	29	31	29	26	23	30	15	18	25 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23	
*100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.87	0.53	0.94	1.00 (0.83)	0.77	0.92	0.68	1.00	0.94	1.00	1.00	0.97	0.91	1.00	1.00 (1.00)	1.00	1.00	
*95					0.47			1.00			(0.17)	0.23			0.06										
*90							0.13					0.08	0.32						0.03	0.09					
<i>bGLUA*</i>	6	25	29	31	29	25	23	30	15	18	25 (30)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23	
*100	1.00	1.00	0.50	0.82	1.00	0.16	0.85	0.87	1.00	0.94	1.00 (0.98)	0.83	1.00	1.00	1.00	0.92	1.00	1.00	0.87	0.94	1.00	0.90 (0.64)	0.89	1.00	
*150			0.50	0.18		0.84	0.15	0.13		0.06	(0.02)	0.17			0.08				0.13	0.06		0.10 (0.36)	0.11		
<i>GPI-A*</i>	6	25	29	31	29	26	23	29	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23	
*100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (0.98)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00	
*105											(0.02)														
<i>GPI-B2*</i>	6	25	29	31	29	26	23	29	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23	
*100	1.00	0.22	1.00	1.00	1.00	1.00	0.46	0.93	1.00	1.00	1.00 (1.00)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00	1.00	
*135		0.78						0.07																	
*50							0.54																		
<i>G3PDH-2*</i>	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23	
*100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.98 (0.95)	1.00	1.00	1.00	1.00	0.94	1.00	1.00	0.96	0.94	1.00	1.00 (0.95)	1.00	1.00	
*50											0.02 (0.05)				0.06				0.04	0.06		(0.05)			
<i>IDHP-1*</i>	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23	
*100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00	1.00	1.00	0.33	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00	
*85															0.67										
<i>IDHP-2*</i>	6	25	29	31	29	26	23	30	15	18	26 (31)	14	6	11	19	18	28	27	26	26	27	5 (11)	22	23	
*100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.90	1.00	1.00 (0.98)	0.04	0.75	0.09	1.00	1.00	1.00	1.00	1.00	0.96	0.85	1.00 (0.95)	1.00	0.98	
*160									0.10		(0.02)	0.96	0.25	0.91					0.04	0.15		(0.05)		0.02	
<i>IDHP-3*</i>	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	26	27	5 (11)	22	23	
*100	0.42	1.00	1.00	1.00	1.00	1.00	1.00	0.95	1.00	0.28	1.00 (1.00)	0.80	1.00	1.00	1.00	1.00	0.84	0.93	0.94	0.98	0.96	0.10 (0.59)	1.00	1.00	
*130	0.58						0.05			0.72	0.20	0.20				0.16	0.07	0.06	0.02	0.02	0.04	0.90 (0.41)			

Appendix Continued

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
LDH-A2*	6	25	29	31	29	26	23	30	15	18	25 (30)	14	6	11	19	18	28	27	25	27	27	5 (11)	22	23
*100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.53	0.06 (0.14)	0.73	1.00	1.00	1.00	1.00	0.58	0.81	0.47	0.61	1.00	0.37 (0.57)	0.09	0.14
*100QL										0.47	0.94 (0.86)	0.27					0.42	0.19	0.53	0.39		0.63 (0.43)	0.91	0.86
LDH-C*	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23
*100	1.00	1.00	1.00	1.00	0.98	0.98	1.00	0.93	0.83	0.83	0.92 (0.77)	1.00	1.00	1.00	1.00	0.81	1.00	1.00	0.79	0.83	0.95	0.90 (0.41)	1.00	1.00
*90										0.02	0.04	0.17				0.19			0.21	0.17	0.05	0.10 (0.59)		
*110					0.02	0.02			0.05	0.13														
LGL*	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23
*100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00
*90																								
aMAN*	6	25	29	31	29	24	23	30	15	18	24 (29)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23
*100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (0.17)	1.00	1.00	1.00	1.00	0.17	0.27	0.30	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00
*90											1.00 (0.83)					0.83	0.73	0.70						
sMDH-A2*	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23
*100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.93	0.97	1.00 (0.93)	1.00	1.00	1.00	1.00	0.86	1.00	1.00	0.94	0.94	1.00	1.00 (0.86)	1.00	1.00
*120									0.07	0.03						0.14				0.06				
sMDH-B1*	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23
*100	1.00	0.94	0.03	0.02	1.00	1.00	1.00	0.77	0.97	1.00	1.00 (0.97)	1.00	1.00	1.00	1.00	0.94	1.00	1.00	0.94	0.90	0.78	1.00 (0.85)	1.00	1.00
*80		0.06						0.23	0.03		0.03					0.06			0.06	0.10	0.22	0.15		
*75			0.97	0.98																				
sMDH-B2*	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23
*100	1.00	1.00	0.48	0.34	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00
*75			0.52	0.66																				
ME*	6	25	29	31	29	25	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23
*100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.53	0.90	1.00	1.00 (0.98)	0.70	0.17	1.00	1.00	1.00	0.39	0.48	0.94	0.98	0.26	1.00 (0.95)	1.00	1.00
*140								0.16			0.30	0.83	1.00				0.57	0.52		0.74				
*120								0.26	0.10		0.02						0.04			0.02				
*80								0.05																
MEP-3*	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23
*100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.27 (0.35)	0.90	0.75	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00
*90											0.73 (0.65)	0.10	0.25											
MP1-2*	6	25	29	31	29	23	23	30	15	18	24 (29)	12	6	11	19	18	28	27	25	20	27	5 (11)	22	23
*100	0.25	1.00	1.00	0.98	0.98	0.22	0.26	0.87	1.00	0.84	0.98 (0.95)	0.96	1.00	1.00	1.00	0.81	1.00	1.00	0.74	0.82	1.00	0.80 (0.77)	1.00	1.00
*105	0.75			0.02	0.02	0.78	0.74	0.13		0.16	0.02 (0.05)	0.04				0.19			0.26	0.18		0.20 (0.23)		
PEPLT*	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23
*100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.83	1.00	0.97	1.00 (1.00)	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00
*70											0.03													
PGM-1*	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23
*100	1.00	1.00	1.00	1.00	0.95	1.00	1.00	0.97	1.00	1.00	1.00 (1.00)	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00	1.00 (1.00)	1.00	1.00
*80											0.33													
SOD-1*	6	25	29	31	29	26	23	30	15	18	26 (31)	15	6	11	19	18	28	27	26	27	27	5 (11)	22	23
*100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00 (0.98)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.77	0.70	0.52	1.00 (1.00)	1.00	1.00
*120																			0.23	0.30	0.48			
*50											0.02													