Molecular Ecology (2000) **9**, 1325–1338

Mitochondrial haplotype variation and phylogeography of Iberian brown trout populations

A. MACHORDOM,*† J. SUÁREZ,* A. ALMODÓVAR,* and J. M. BAUTISTA‡
*Instituto Madrileño de Investigación Agraria y Alimentaria, El Encín. Apdo. 127, 28800 Madrid, Spain, †Museo Nacional de Ciencias Naturales, José Gutiérrez Abascal, 2, 28006 Madrid, Spain, ‡ Departamento de Bioquímica y Biología Molecular IV, Facultad de Veterinaria, Universidad Complutense de Madrid, Avda. Puerta de Hierro s/n, Madrid, Spain

Abstract

The biogeographical distribution of brown trout mitochondrial DNA haplotypes throughout the Iberian Peninsula was established by polymerase chain reaction-restriction fragment polymorphism analysis. The study of 507 specimens from 58 localities representing eight widely separated Atlantic-slope (north and west Iberian coasts) and six Mediterranean drainage systems served to identify five main groups of mitochondrial haplotypes: (i) haplotypes corresponding to non-native, hatchery-reared brown trout that were widely distributed but also found in wild populations of northern Spain (Cantabrian slope); (ii) a widespread Atlantic haplotype group; (iii) a haplotype restricted to the Duero Basin; (iv) a haplotype shown by southern Iberian populations; and (v) a Mediterranean haplotype. The Iberian distribution of these haplotypes reflects both the current fishery management policy of introducing non-native brown trout, and Messinian palaeobiogeography. Our findings complement and extend previous allozyme studies on Iberian brown trout and improve present knowledge of glacial refugia and postglacial movement of brown trout lineages.

Keywords: conservation units, introgression, mtDNA, PCR-RFLP, phylogeography, Salmonidae

Received 11 February 2000; revision received 11 April 2000; accepted 11 April 2000

Introduction

Genetic studies performed on brown trout (*Salmo trutta* L.) strongly suggest that a large proportion of the evolutionary diversity of this widespread species corresponds to southern European countries bordering the Mediterranean (Giuffra *et al*. 1994; Apostolidis *et al*. 1996; García-Marín *et al*. 1999). However, the genetic diversity and phylogeographic structure of these southern forms has not yet been well-established, and this diversity may be threatened by the introduction of hatchery reared trout from western and northern European populations to restock Spanish populations. Herein, we report the findings of a general mitochondrial DNA (mtDNA)-based survey of brown trout in drainage systems throughout the Iberian Peninsula, aimed at establishing the distribution of native and non-native mtDNA lineages. The resultant information was used to assess the impact of short-term restocking, and the long-term phylogeographic history of the distribution of brown trout populations in the Iberian Peninsula.

Although one of the most genetically polymorphic fish species (Ferguson 1989), showing multiple genetic boundaries within a broad natural distribution (Behnke 1986), the brown trout is considered a single species. Moreover, the phenetic variation of the brown trout constitutes a complex mosaic, involving geographical morphs and considerable life history variation (Behnke 1972, 1986), and has even led some authors to consider *S. trutta* to be a complex species (revised in Kottelat 1997).

Two regional groups of brown trout have been described according to the frequency of alleles at an allozyme locus (*LDH-5**): *LDH-5*100* predominates in southern European specimens, and *LDH-5*90* is found in fish from central and north-west European regions, where trout populations have probably only existed since the end of the last glaciation (Ferguson 1989; Hamilton *et al*. 1989; Guyomard 1991; Bernatchez & Osinov 1995; García-Marín & Pla 1996). Bernatchez *et al*. (1992) sequenced two regions of

Correspondence: Annie Machordom. Museo Nacional de Ciencias Naturales, José Gutiérrez Abascal, 2, 28006 Madrid, Spain. Fax: + 34 91 564 50 78; E-mail: annie@mncn.csic.es

the mitochondrial D-loop representing a large part of the brown trout found in Europe and described five mtDNA haplotype groups. Regional associations were indicated by use of the following names: Atlantic, Mediterranean, Adriatic-1, Adriatic-2 and Danube. However, these authors observed no variation between specimens from Atlantic river basins, whereas extensive variation is suggested by restriction fragment length polymorphism (RFLP) analysis of total mtDNA and the ND1, 5 and 6 (NADH dehydrogenase subunits) and 16S rRNA genes (Hall & Nawrocki 1995; Hansen & Loeschcke 1996; Hynes *et al*. 1996).

The Iberian Peninsula has both Atlantic and Mediterranean draining rivers and can be characterized by: (i) the possible presence of anadromous trout in the Cantabrian Sea and Galician region [these are absent southwards from about latitude 42°N (Hamilton *et al*. 1989)]; (ii) allozymic differentiation between Atlantic and Mediterranean forms (García-Marín & Pla 1996); and (iii) minor allozymic differences between specimens of different basins (García-Marín & Pla 1996; Machordom *et al*. 1999). However, allozyme data do not suggest strong biogeographical relationships (García-Marín & Pla 1996; Morán *et al*. 1996). The allele *LDH-5*100* appears to characterize native Spanish populations, while the presence of *LDH-5*90* always corresponds to introduced fish or their descendants (García-Marín *et al*. 1991; Martínez *et al*. 1993).

In Spain, the reduction or total loss of natural populations has prompted a restocking programme involving the introduction of foreign trout mainly from central (Germany) (Hatcheries' staff personal communication) or northern Europe (Denmark). Molecular markers have shown that hatchery-reared salmonids can interbreed with wild fish (Taggart & Ferguson 1986; García-Marín *et al*. 1991; Hindar *et al*. 1991; Martínez *et al*. 1993; Arias 1996; Machordom *et al*. 1999), but the success of wild reproduction of brown trout strains introduced into the Iberian Peninsula has not been rigorously assessed.

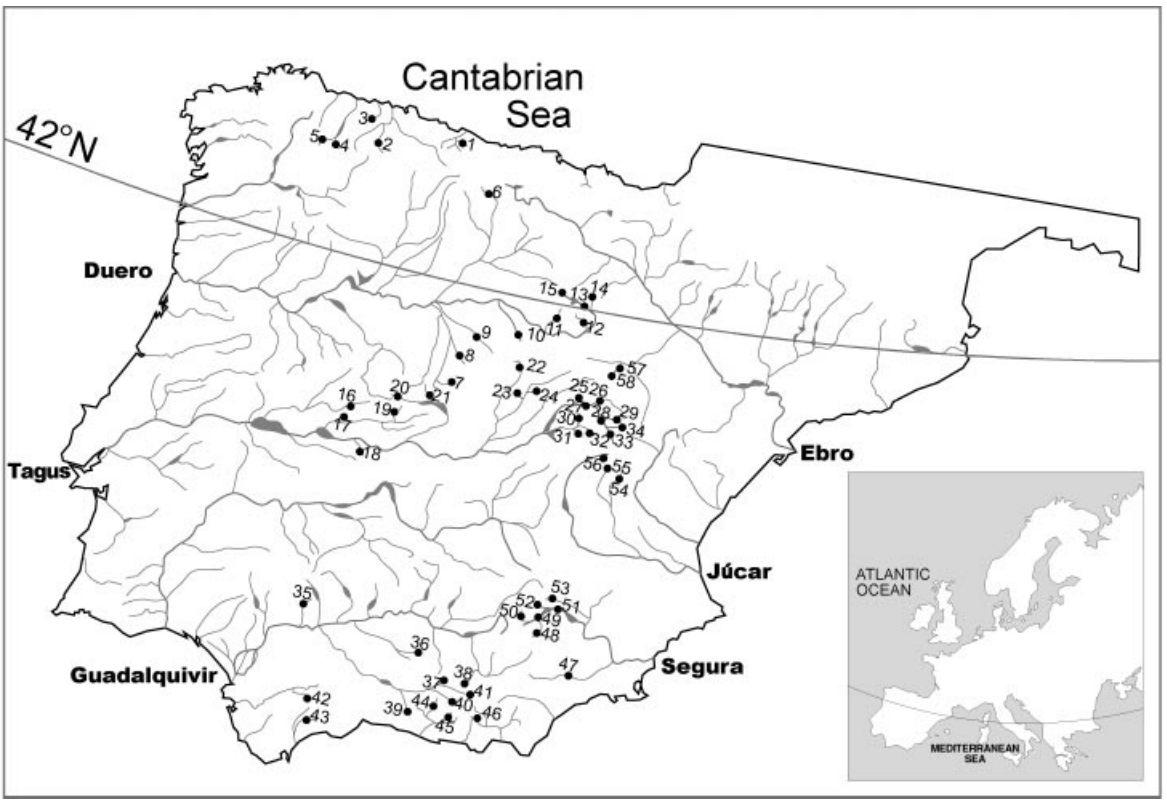

Fig. 1 Sampling localities (**slope**, basin, number and name of river): **Cantabrian**: Sella (1-Dobra), Nalón (2-Pigüeña), Esba (3-Moras), Navia (4-Moia, 5-Lamas). **Atlantic**: Duero (6-Pisuerga, 7-Moros, 8-Eresma, 9-Cega, 10-Aguisejo, 11-Abide, 12-Mazo, 13-Duero-Hinojosa, 14-Tera, 15-Duero-Duruelo de la Sierra); Tagus (16-Jerte, 17-Tiétar, 18-Ibor, 19-Del Arenal, 20-Alberche, 21-Garganta de la Iruela, 22-Jarama, 23-Bornova, 24-Dulce, 25-Ablanquejo, 26-Gallo, 27-Tagus-Buenafuente, 28-Tagus-Peralejos, 29-Cabrillas, 30-Arandilla, 31-Escabas, 32-Guadiela, 33-Tagus-Tres Mojones, 34-Hoz Seca); Guadalquivir (35-Ribera de Hueznar, 36-San Juan, 37-Maitena, 38-Fardes, 39-Cebollón, 40-Dilar, 41-Genil), Guadalete (42-Bosque); Morocco: 59-Ifni Lake; **Mediterranean**: Genal (43-Genal), Guadalfeo (44-Lanjarón, 45-Trévelez), Andarax (46-Andarax), Segura (47-Guadalentín, 48-Zumeta, 49-Arroyo Endrinales, 50-Madera, 51-Segura, 52-Tus, 53-Paterna), Júcar (54-Cabriel, 55-Laguna del Marquesado, 56-Arroyo Almagrero), Ebro (57-Mesa-Mochales, 58-Mesa-Algar de Mesa). Hatcheries (60–63).

This paper describes extensive sampling of Iberian brown trout populations from the main Spanish drainage systems, and from central Europe (from hatcheries) and Morocco. Evolutionary lineages were explored by restriction fragment analysis of mtDNA. This technique has been empirically demonstrated to distinguish between brown trout of different geographical origins (Hall & Nawrocki 1995; Hansen & Loeschcke 1996; Hynes *et al*. 1996).

The main objectives of the present study were: (i) to determine whether mtDNA markers may discriminate foreign from native Iberian brown trout; (ii) to compare mtDNA lineage distribution to the Iberian biogeographic structure previously suggested by chorological and allozyme data, and possibly find markers able to distinguish haplotypes at the basin, river or even population level; (iii) to test hypotheses regarding glacial refugia and later recolonizations from southern Europe; and (iv) to apply these results to management policy in terms of Operational Conservation Units (OCUs, Doadrio *et al*. 1996).

Materials and methods

Most of the trout specimens were obtained by electrofishing methods. Liver or skeletal muscle samples were dissected and preserved in the field in liquid nitrogen. Alternative tissue samples were fin clips preserved in 70% ethanol.

We analysed 507 specimens corresponding to 58 populations of Spanish brown trout. Corresponding areas, basins and rivers analysed were allocated a sample site number (see Fig. 1), hereafter shown in square brackets. Five of the populations sampled represented rivers of the Cantabrian slope [1–5], 37 to Atlantic rivers [6–42], and 16 to Mediterranean rivers [43–58] (Fig. 1). We also examined one population ($n = 10$) from Morocco [59] (Atlantic slope), and four different hatchery stocks [60–63] ($n = 38$) as references for non-native trout. A *Salmo salar* and a *Oncorhynchus mykiss* specimen were used for outgroup analysis.

Basin sample sizes were based on the size of the basin and differences in available habitat, and ranged from a maximum of 19 populations ($n = 242$) taken from the Tagus basin [16–34] and a minimum of one ($n = 4$) from the Andarax basin [46] (see Table 1).

Total DNA extraction was performed according to standard phenol–chloroform procedures (Sambrook *et al*. 1989). Primers designed for the mtDNA sequencing of rainbow trout (Zardoya *et al*. 1995) were used to amplify the region between the NADH-5 ('1920Fw4': 5'GCAGCTATGCACCCGACTACACT3') and the cytochrome *b* ('*PvuII*16Rev': 5'GGCAAACAGAGGAGAAAGCTGTT3') genes. The second primer was used for preliminary amplifications but was subsequently replaced by a primer specific for brown trout ('*PvuII*16St': 5'GGCAAACAGAGGAAAAGGCTGTT3').

The PCR mixture contained approximately 50–150 ng of DNA, 150 ng of each primer, 200 μM of each dNTP, 1.5 U *Tth* polymerase (XL, Perkin Elmer) and corresponding buffer, 1.5 mM $MgCl_2$ and H_20 in a final volume of 50 μL. Amplification was performed in a thermal cycler (Perkin Elmer 2400) programmed as follows: 94 °C for 5 min, 40 cycles at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min 30 s, followed by a final extension at 72 °C for 10 min. PCR amplification yielded a fragment of approximately 2700 bp, which was visualized by ethidium bromide in 0.8% agarose gel.

The amplified fragments were subsequently screened for polymorphism using the restriction endonucleases: *Alu*I, *Hin*cII, *Msp*I, *Rsa*I and *Sau*3AI. All these enzymes recognize tetranucleotide palindromic sequences except *Hin*cII which recognizes a hexanucleotide sequence. Seven or 8 μL of each PCR product were digested overnight with 5 U of the restriction enzyme in a final volume of 18–20 μL. Restriction fragments were visualized under UV light on 1.5% agarose gels stained with ethidium bromide. The program 'Kodak Digital Science 1D 2.0' was used to calculate the molecular size of the restriction fragments, based on comparisons with a comigrating 100 bp ladder molecular weight marker.

The RFLP pattern produced by each endonuclease was assigned a letter so that each composite mtDNA genotype was defined by a five letter code.

Non-native haplotypes were established according to distance and parsimony analysis using hatchery samples of foreign trout as reference. Where possible, allozyme (Machordom *et al*. 1999 and unpublished data) and mtDNA data permitted us to corroborate the foreign origin of specimens. Once the non-native brown trout haplotypes had been defined, we were able to analyse the biogeographical distribution of natural populations and their degree of differentiation based on comparisons between native mtDNA haplotypes.

The ARLEQUIN program (Excoffier *et al*. 1992; Schneider *et al*. 1997) was used for F-statistics and to estimate haplotype frequencies and molecular variance (AMOVA) in each population. Distances between haplotypes or nucleotide sequence divergence (Nei & Li 1979; Nei 1987) were determined using the REAP program (McElroy *et al*. 1992). As *O. mykiss* showed no common product following *Hin*cII digestion of the PCR-amplified segment, a hypothetical common fragment profile was established (Table 2, bold characters) to permit the calculation of nucleotide divergence (d). This index is computed separately for each restriction enzyme and an overall weighted estimate of evolutionary divergence is then generated (McElroy *et al*. 1992). If two operational taxonomic units (OTUs) or composite haplotypes do not share a common

Table 1 Percentage of haplotypes detected in each population. The number of the populations are described in Fig. 1. Two basins were abbreviated: Glte = Guadalete and Anax = Andarax. The population frequency of the foreign allele *LDH-5*90* is indicated when available

	Slope	Cantabrian Sea					Atlantic Ocean …																												
	Basin	Sella		Nalón		Esba	Navia		Duero									Tagus																		
	Pop.	1		2		3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Haplotype	*n* =	8		8		11	11	11	4	6	10	21	10	6	5	15	5	6	10	9	10	6	8	5	12	19	20	12	10	12	10	20	9	13	20	12	25
AAAAA		25		25		91	45	45	—	—	70	—	—	17	—	—	60	—	—	—	20	—	—	20	—	—	35	—	20	8	—	5	—	31	—	—	—
AAAEA		—		—		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	—	—	—	—	—	—	—	—	—	—
ABACA		75		75		9	55	55	—	17	20	29	—	—	—	—	—	50	40	56	80	67	—	—	33	37	25	100	80	92	100	95	100	—	95	100	100
ABACC		—		—		—	—	—	—	—	—	—	—	—	—	—	—	—	60	—	—	—	100	20	67	63	—	—	—	—	—	—	—	—	—	—	—
BAAAA		—		—		—	—	—	—	—	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
BBABB		—		—		—	—	—	100	83	—	71	100	83	100	100	40	50	—	44	—	33	—	60	—	—	—	—	—	—	—	—	—	—	—	—	—
BBACA		—		—		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	46	—	—	—
BBBDA		—		—		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	15	—	—	—	—	—	—	—	—	—	—
BBCDA		—		—		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	20	—	—	—	—	—	—	—	—	—	—
CBBDA		—		—		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	23	5	—	—
*LDH-5*90*		0		0		0	0	0	0	4	2.2	2.7	21.1	17	0	0	18	2	0	0	6.1	0	0	15	0	2.9	10	0	7.5	2.5	2.5	8.3	0	27.8	26.4	20	6.2

	Slope	… Atlantic Ocean								Mediterranean Sea																Foreign trouts				
	Basin	Guadalquivir							Glte	Genal	Guadalfeo		Anax	Segura							Júcar			Ebro		Ifni	Hatcheries			
	Pop.	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63
Haplotype	*n* =	2	4	2	2	3	4	1	2	1	4	3	4	4	4	9	2	4	14	3	12	8	12	11	13	10	17	11	9	1
AAAAA		100	—	—	—	—	—	—	100	100	75	33	—	—	—	—	50	—	—	100	8	50	—	—	—	—	100	100	67	100
AAAEA		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	12	—	—	—	—	—	—	33	—
ABACA		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100	—	—	—	—
ABACC		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
BAAAA		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
BBABB		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
BBACA		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
BBBDA		—	—	—	—	—	—	—	—	—	—	—	—	—	—	100	—	—	100	—	92	37	100	100	100	—	—	—	—	—
BBCDA		—	100	100	100	100	100	100	—	—	25	67	100	100	100	—	50	100	—	—	—	—	—	—	—	—	—	—	—	—
CBBDA		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
*LDH-5*90*		—	—	—	—	—	—	—	—	—	—	—	—	—	—	0	—	—	2.5	—	25	81.6	2.6	7.5	7.5	—	100	100	42.1	—

Table 2 Matrix of restriction fragments presence/absence for the different patterns found for each endonuclease. For the explanation of the bold column, see the text

*Alu*I		*Hinc*II		*Msp*I		*Rsa*I		*Sau*3A I	
A	000100011110010	**A**	0100001101	**A**	00110010100	**A**	010000011001	**A**	100000000100101
B	100000010110010	**B**	0101000001	**B**	00110001101	**B**	010000011010	**B**	010000000100111
C	010000010110010	**C**	1000100011	**C**	00011110100	**C**	100000011001	**C**	000110000100101
D	001100001110110	**D**	0011010001	**D**	10010010000	**D**	000110011001	**D**	001000011001000
E	000011100011001			**E**	01010000110	**E**	010001001001	**E**	000001101110000
						F	010000010100		
						G	001010100000		

fragment, the resulting evolutionary distance may be artificially increased. The error introduced by including the hypothetical character is most likely far smaller than the error resulting from a lack of shared characters (McElroy *et al*. 1992). The presence/absence matrices of each restriction fragment were processed by both distance and parsimony (Dollo method) analysis using PHYLIP version 3.5 (Felsenstein 1993) and PAUP* version 4.0b2 programs (Swofford 1999). An heuristic search with tree bisection reconnection (TBR) branchswapping and 10 replicates of random addition of taxa was performed. The relative robustness of each dichotomy was established by bootstrap analysis (1000 replicates). Neighbour-joining algorithms served to construct trees based on interhaplotype and interpopulation distances. In the latter case, the distance matrix included haplotype frequencies corresponding to each population and the distances between each haplotype (DA program, REAP package, McElroy *et al*. 1992).

Results

Twelve mtDNA haplotypes were identified in the populations. The outgroups, *Oncorhynchus mykiss* and *Salmo salar* showed the haplotypes DCDFD and EDEGE, respectively. The different endonucleases recognized one to six restriction sites. Corresponding RFLP patterns are shown in Fig. 2. The size of fragments under 100 bp could not be reliably estimated. RFLP patterns corresponding to the Spanish samples reflected the gain/loss of a single restriction site. Only two fragments were shared by all haplotypes. Thirty-seven fragments were autapomorphic, and 23 represented phylogenetically informative characters.

Autochthonous vs. allochthonous haplotypes

The four hatchery strains of foreign brown trout showed the haplotypes AAAAA and AAAEA. The BAAAA pattern was observed in a single specimen from the river Eresma [8], a population containing several trout with the non-native AAAAA haplotype. This suggests that the BAAAA haplotype represents a mutation in the hatchery reference strain. These three haplotypes clustered together in the parsimony and distance analyses (see below).

Twenty-four of the Spanish populations showed foreign haplotypes with frequencies ranging from 5% to 100% (Table 1). Mitochondrial analysis of populations from the Tagus-Tres Mojones [33], Mesa-Mochales [57] and Mesa-Algar de Mesa [58] was only performed on specimens considered to be autochthonous based on previous allozyme data (unpublished results, Table 1). The Cantabrian [1–5] populations showed 25% to 90% AAAAA, suggesting a significant contribution of non-native trout. Allozyme analysis however, indicates that all Cantabrian trout are homozygous for the autochthonous diagnostic allele (*LDH-5*100*), and there is no indication

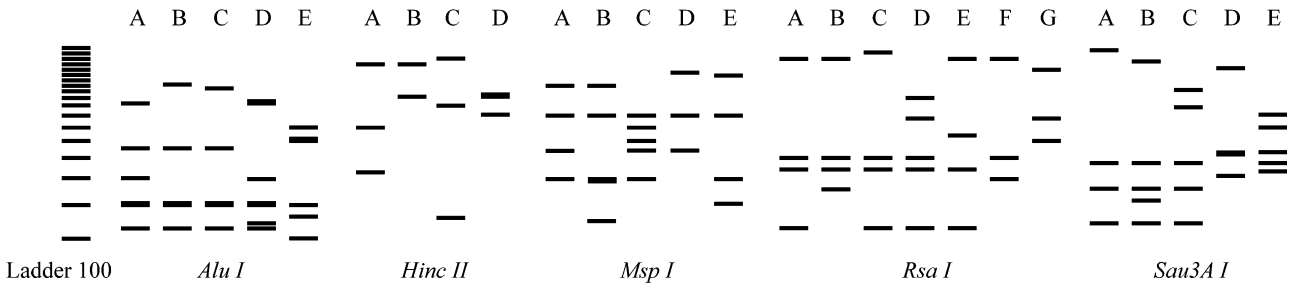

Fig. 2 Random fragment length polymorphism patterns yielded by the five restriction enzymes. 'Ladder 100' is the molecular weight reference.

Table 3 Analysis of variance of pairwise differences and haplotype frequencies among native trouts, estimated by AMOVA (Schneider *et al*. 1997). *P* is the probability that the random value was greater than the observed value from 1000 iterations

	Pairwise differences				Haplotype frequencies			
	Variance	% total	Φ	*P*	Variance	% total	*F*	*P*
Among basins (Φ_{CT}, F_{ST})	2.863	70.58	0.706	< 0.001	0.248	55.90	0.559	< 0.001
Among populations within basins (Φ_{SC}, F_{IS})	0.592	14.61	0.497	< 0.001	0.105	23.59	0.535	< 0.001
Within populations (Φ_{ST}, F_{IT})	0.601	14.81	0.852	< 0.001	0.091	20.51	0.795	< 0.001

of non-native alleles at other loci (P. Martínez and P. Morán, personal communications, Table 1). Thus, allozyme data strongly suggest that the Cantabrian [1–5] populations sampled here should be considered autochthonous or, at least, they provide no evidence of recent hybridization with restocked non-native trout. In subsequent analyses, the haplotype AAAAA was considered autochthonous for Cantabrian [1–5] populations. In the remaining populations, AAAAA, AAAEA and BAAAA were considered non-native and were excluded from the analysis of genetic diversity and biogeographic structure.

In general, the number of non-native mtDNA haplotypes observed in the different Iberian populations is low, although locally abundant in some areas (Table 1). For example, of the 10 populations representing the Duero [6–15] (Atlantic slope), seven ($n = 67$) only showed the native mtDNA haplotypes (mainly BBABB followed by ABACA), but two of the populations ($n = 15$) were characterized by 60% or more non-native haplotypes. Obviously, it is possible that some of these so-called 'non-native' haplotypes represent the Cantabrian AAAAA autochthonous form. However, the absence of the AAAAA haplotype in as many as seven populations leads us to speculate that its occurrence in trout from the Duero [6–15] reflects local stocking with non-native fish. In the other main Atlantic-draining basin, the Tagus [16–32, 34], 11 out of 18 populations ($n = 140$) showed only native mtDNA haplotypes (mainly ABACA followed by ABACC), and the proportion of non-native haplotypes in the remaining seven populations ($n = 90$) ranged from 5% to 40%. Although samples sizes were considerably smaller for the Mediterranean slope channels, the best-sampled river, the Segura [47–53], showed a similar trend. Five out of seven populations ($n = 35$) only showed the native mtDNA haplotypes, BBCDA or BBBDA, and the remaining two populations had 100% ($n = 3$) and 50% ($n = 2$) AAAAA haplotypes.

It is of interest that samples showing relatively high proportions of non-native mtDNA haplotypes corresponded to rivers or lakes known to have been stocked with non-native hatchery fish i.e. the rivers Eresma [8] (70% non-native haplotypes), Tera [14] (60%), and the Marquesado lagoon [55] (50%). Allozyme markers confirmed the mtDNA diagnosis of non-native trout at these locations (Machordom *et al*. 1999 and unpublished data, Table 1). Although two Atlantic (Ribera de Hueznar [35] and Bosque [42]) and two Mediterranean (Genal [43] and Paterna [53]) rivers could only be associated with the non-native haplotype AAAAA, the corresponding sample sizes were too small ($n = 1–3$) to be informative.

Haplotype and population diversity

Nucleon diversity shown by the 51 native populations, of which we analysed more than one autochthonous trout, ranged from 0 (40 populations) to 0.7121 (river Dulce [24]) with an average of 0.1549 ± 0.2365 (s.d.), while nucleotide diversity values averaged 0.0177 ± 0.0303 (s.d.) with a maximum of 0.0978 (river Duruelo [15]).

When the Iberian populations were grouped according to basin of origin (i.e. the 12 basins shown in Table 1 after exclusion of the Guadalete [42] and Genal [43] basins, where only three specimens were analysed and found to bear the AAAAA haplotype), intergroup (interbasin) variation was 55.90% when F_{ST} were calculated from haplotype frequencies ($P < 0.001$), and 70.58% when pairwise differences (ϕ_{CT}) were considered ($P < 0.001$) (Table 3). Populations were also stratified according to geographical region and shared haplotypes to give the following regional assemblages: North (Sella [1], Nalón [2], Esba [3] and Navia [4–5] basins), South (Guadalquivir [35–41], Guadalfeo [44–45] and Andarax [46] basins), and Mediterranean (Ebro [57–58] and Júcar [54–56] basins). In this case, intergroup variation showed a slight increase yielding values of $F_{ST} = 57.98$ ($P < 0.001$), and $\phi_{CT} = 72.10\%$ ($P < 0.001$).

Table 4 shows the indices of nucleotide divergence between haplotypes. The distances from the outgroup (*O. mykiss* and *S. salar*) and remaining haplotypes were clearly the greatest ($\bar{d} = 0.1012$). Minimum divergence was shown between the haplotypes AAAAA and AAAEA ($d = 0.0037$), BBBDA and CBBDA ($d = 0.0037$), and ABACA and ABACC/BBACA ($d = 0.0058$ and 0.0061, respectively). Remaining values for Spanish native trout ranged from $d = 0.0113–0.0315$.

Table 4 Nucleotide divergence (Nei & Li 1979; Nei 1987) between the composite haplotypes found

	AAAAA											
AAAAA	0.0000	**AAAEA**										
AAAEA	0.0037	0.0000	**ABACA**									
ABACA	0.0113	0.0154	0.0000	**ABACC**								
ABACC	0.0171	0.0215	0.0058	0.0000	**BAAAA**							
BAAAA	0.0058	0.0100	0.0179	0.0245	0.0000	**BBABB**						
BBABB	0.0245	0.0296	0.0228	0.0249	0.0179	0.0000	**BBACA**					
BBACA	0.0179	0.0226	0.0061	0.0126	0.0118	0.0154	0.0000	**BBBDA**				
BBBDA	0.0263	0.0315	0.0195	0.0269	0.0198	0.0249	0.0126	0.0000	**BBCDA**			
BBCDA	0.0263	0.0315	0.0195	0.0269	0.0198	0.0249	0.0126	0.0120	0.0000	**CBBDA**		
CBBDA	0.0263	0.0315	0.0195	0.0269	0.0245	0.0307	0.0175	0.0037	0.0166	0.0000	**DCDFD**	
DCDFD	0.0669	0.0752	0.0726	0.0746	0.0828	0.0824	0.0917	0.1102	0.0962	0.1102	0.0000	**EDEGE**
EDEGE	0.1287	0.1287	0.1176	0.1202	0.1263	0.1176	0.1149	0.1026	0.1026	0.1026	0.1485	0.0000

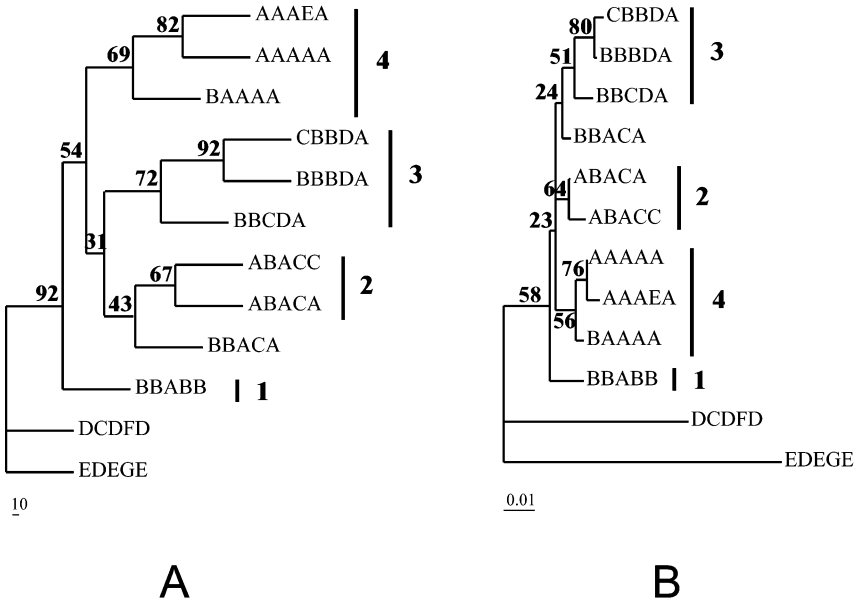

Fig. 3 Parsimony and distance trees of the 12 haplotypes detected. (A) Dollo parsimony analysis with presence/absence of each fragment. (B) Neighbour-joining tree based on nucleotidic divergence. In both trees, numbers on the branches indicate percentage support in 1000 bootstrap replications and the numbers next to the vertical lines represent the clusters cited in the text.

Distance and parsimony analyses of haplotypes yielded equivalent clusters, with the exception of BBACA which clustered with group 2 in the parsimony analysis and with group 3 in the distance analysis, but with no supporting Bootstrap values (see Fig. 3). Four groups were established according to the main haplotypes corresponding to each basin (Fig. 3, and clusters indicated in Table 5). Thus, group 1 defined the BBABB haplotype, predominant in trout from the Duero basin [6–15]. Group 2 represented fish collected from the Cantabrian [1–5] and Atlantic [6–34] rivers, and group 3 characterized the southern [35–46] and Mediterranean [47–58] rivers. Group 4 showed the foreign trout and Cantabrian [1–5] haplotypes.

The distribution and frequency of the main Atlantic haplotype (ABACA) was: 51% in the Cantabrian slope [1–5], 15.8% in the Duero [6–15], 72% in the Tagus [16–34], and 100% in Morocco [59] (Tables 1 and 5). This haplotype was absent in fish from one of the Atlantic basins, the Guadalquivir [35–41], which in the present study was genetically classed as a southern basin. The main haplotype corresponding to the Duero basin [6–15] (BBABB, 84.2%) was only present in 4% of specimens from the Tagus basin [16–34]. Southern populations (from the Guadalquivir [35–41] on the Atlantic side, to the Andarax [46] flowing into the Mediterranean) showed the unique BBCDA haplotype, while the Mediterranean populations (Ebro [57–58] and Júcar [54–56] basins) only showed the BBBDA haplotype. The Segura [47–53] was inhabited by trout of both southern (36.1%) and Mediterranean (63.9%) haplotypes. This river would appear to represent the penetration limit of both haplotypes. However, these 'non-Atlantic' haplotypes were also found in specimens of the Tagus basin (river Dulce [24]) and represented 3.1% of the native haplotypes observed.

Table 5 Distribution of the presumed native haplotypes in the different basins analysed. n_1 = total number of individuals analysed, n_2 = number of individuals not presenting foreign haplotypes. For numbers in square brackets see Fig. 1. Cluster numbers correspond to Fig. 3

Slope	Basin (n_1, n_2)	Haplotypes	Percentage	Cluster
Northern slope	Sella [1]	ABACA	75.0	2
	(8)	AAAAA	25.0	4
	Nalón [2]	ABACA	75.0	2
	(8)	AAAAA	25.0	4
	Esba [3]	AAAAA	90.9	4
	(11)	ABACA	9.1	2
	Navia [4, 5]	ABACA	54.5	2
	(22)	AAAAA	45.4	4
Atlantic slope	Duero [5–15]	BBABB	84.2	1
	(88, 76)	ABACA	15.8	2
	Tagus [16–34]	ABACA	72.6	2
	(242,223)	ABACC	15.7	2
		BBABB	4.0	1
		BBACA	2.7	2–3
		BBCDA	1.8	3
		CBBDA	1.8	3
		BBBDA	1.3	3
	Guadalquivir [35–41]	BBCDA	100	3
	(18, 16)			
	«Morocco» [59]	ABACA	100	2
	(10, 10)			
Mediterranean slope	Guadalfeo [44, 45]	BBCDA	100	3
	(7, 3)			
	Andarax [46]	BBCDA	100	3
	(4, 4)			
	Segura [47–53]	BBBDA	63.9	3
	(40, 36)	BBCDA	36.1	3
	Júcar [54–56]	BBBDA	100	3
	(32, 26)			
	Ebro [57, 58]	BBBDA	100	3
	(24, 24)			

Inter-population divergence (D_A) indices were used to construct a population neighbour-joining tree (Fig. 4). The first dichotomy separates the populations of the Duero [6–15] basin from remaining populations. The main groups define the geographical or basin origin of each population, with the exception of the three populations from the Tagus basin (rivers Tiétar [17], Del Arenal [19] and Iruela [21]), that also showed the Duero basin haplotype, BBABB. Two populations of the Tagus basin (rivers Escabas [31] and Dulce [24]), which showed haplotypes common to or derived from those of the Mediterranean populations (CBBDA), were also separated from the Tagus cluster. In addition, one Duero basin population (river Eresma [8]) was grouped with those of the Tagus basin. Finally, the Moras river [3] population (Cantabrian slope) showed slight variation compared to other populations of the same area, as its main haplotype was AAAAA.

Discussion

Autochthonous vs. allochthonous haplotypes

The genetic identification of non-native and autochthonous haplotypes of brown trout throughout the Iberian Peninsula should help to improve the management of this species. The AAAAA haplotype was found in 92.1% of foreign hatchery trout (the AAAEA haplotype completed the total) and typically identifies brown trout or their descendants, that have been introduced into the Iberian Peninsula. In most cases, allozyme data confirmed the AAAAA haplotype of introduced fish as it was associated with the allozyme allele *LDH-5*90* (Table 1), known to be allochthonous in the Iberian Peninsula (Machordom *et al*. 1999 and unpublished results). This general rule, however, does not hold true for the Cantabrian [1–5] populations. In rivers of the north coast of Spain, the AAAAA haplotype

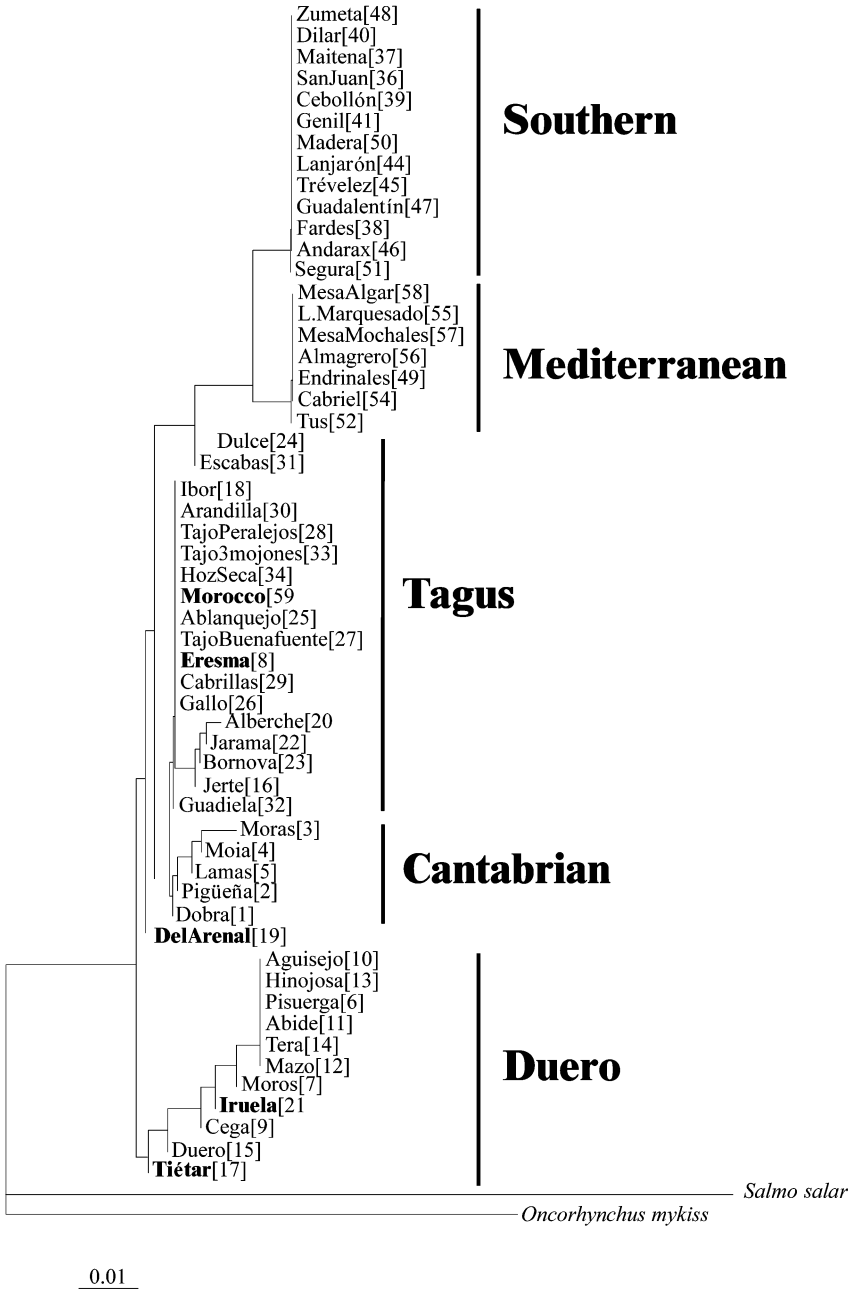

Fig. 4 Neighbour-joining tree of nucleotide divergence between the populations analysed according to haplotype frequency distributions and haplotype nucleotidic divergence (estimated using REAP). The basins or slope of each group are indicated. The bold characters mark the populations that cluster outside their geographical area.

is very frequent, although the specimens analysed were classified as native through their association with the *LDH-5*100* allele (the autochthonous form of the *LDH-5** locus corresponding to the Iberian Peninsula) and the absence of other foreign allozyme markers. Thus, AAAAA, AAAEA and BAAAA are considered non-native haplotypes except for the Cantabrian [1–5] populations (where AAAAA is related to autochthonous forms).

The natural Iberian distribution limit of the AAAAA haplotype is almost certainly associated with latitude 42°N, the southern migration limit of anadromous brown trout (Hamilton *et al*. 1989) (Fig. 1). Our results suggest that the north-European mtDNA haplotype (AAAAA) has penetrated Iberian brown trout populations to a greater extent than nuclear markers representing these northern forms of the species (*LDH-5*90*). It is proposed that the differentiation of *LDH-5** took place around the last glaciation (Ferguson 1989; Hamilton *et al*. 1989). Thus, nuclear penetration of *LDH-5*90* might have occurred much more recently than differentiation of the mitochondrial haplotypes associated with the Iberian Peninsula [see below, where we date mitochondrial differentiation as 0.4–6.3 million years ago (Ma)], and is probably associated with trout dispersal following the last glaciation. The fact that

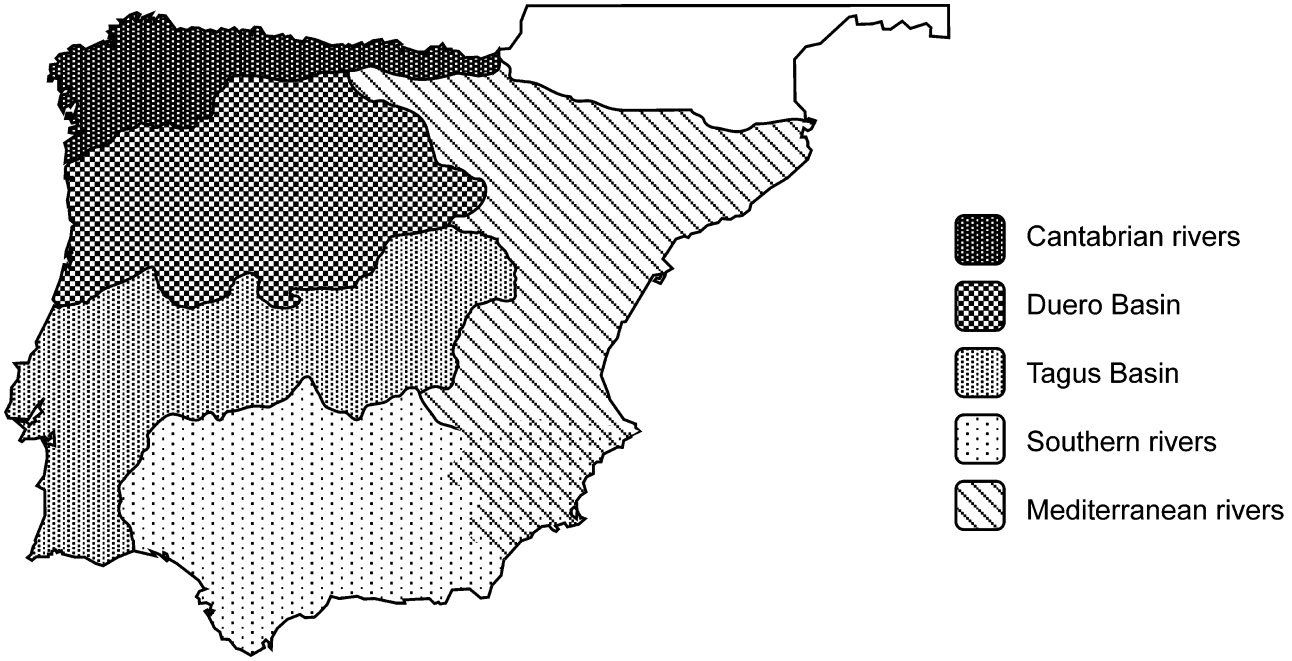

Fig. 5 Biogeographical areas and proposed Operational Conservation Units (OCUs) based on the presence and frequencies of haplotypes in the populations analysed. The Segura basin shows overlapping because it shares Mediterranean and Southern haplotypes.

female migration might be greater than that of males (Campbell 1977; Jonsson 1985) should also be taken into account as a possible explanation for the increased southward penetration of mitochondrial vs. allozyme genotypes.

Non-native mtDNA haplotypes are widespread across the Iberian Peninsula but vary greatly in frequency. Many of the present populations only showed autochthonous mtDNA haplotypes, while a much smaller number of populations bore relatively high proportions of non-native mtDNA haplotypes. The patchy distribution of non-native fish haplotypes across the Iberian Peninsula is not surprising given the long but localized tradition of restocking with brown trout of north and central European origin. Excluding the Guadalfeo basin [44–45] (57.14%), where only seven specimens were collected, non-native haplotypes were observed in under 20% of fish in each basin: Júcar [54–56] (18.75%), Duero [6–15] (13.64%), Tagus [16–32, 34] (8.26%), Guadalquivir [35–41] (11.11%) and Segura [47–53] (10%).

Haplotype and population diversity

Divergence values for the autochthonous haplotypes ranged from 0.37% to 3.07%. These are slightly higher than values quoted for conspecific salmonids (e.g. Bernatchez *et al*. 1992, 0.16–1.92%; Cronin *et al*. 1993, < 1%; Bembo *et al*. 1994, 0.03–0.13%; Bernatchez & Osinov 1995, < 1.43%), but similar to those reported for Greek brown trout (Apostolidis *et al*. 1996, 0.21–3.42%). The mean value between allochthonous and autochthonous haplotypes was in the same range (2.25%). Mean divergence between *Salmo trutta* and its congener *S. salar* was 11.62%, higher than values provided by other authors: 6.18–6.52% (Gyllensten & Wilson 1987) or 5.44–6.44% (Bernatchez *et al*. 1992). These differences are probably attributable to the fact that here, RFLP was detected using restriction enzymes showing polymorphism, with its inherent bias. F-statistics analysis yielded higher values for the mitochondrial marker than those previously reported in allozymic studies, permitting the more accurate detection of interbasin population structure differences.

Phylogeography

Nucleotide sequence divergence is often useful for describing the phylogenetic relationships of haplotypes, but not necessarily those of populations or species (Bermingham & Avise 1986; Pamilo & Nei 1988). Nonetheless, and in contrast with the findings of similar studies (Hansen & Loeschcke 1996; Morán *et al*. 1996; Apostolidis *et al*. 1997), our results suggest a high level of congruence between haplotype distribution and Iberian biogeography. The general pattern did not hold true for a few populations, although human management strategies generally served to explain these discrepancies. A strong geographical component was shown by the phylogenetic relationships of the autochthonous haplotypes. This was represented by the five clusters corresponding to the populations: (1) Cantabrian [1–5], (2) Duero [6–15], (3) Tagus [16–34], (4) Southern [35–53] (both Atlantic and Mediterranean), and (5) Mediterranean [47–58] (Fig. 5).

Fish from the Cantabrian [1–5], Duero [6–15] and Tagus [16–34] basins shared the haplotype ABACA, indicating a historical relationship between Atlantic rivers (as far south as the Atlantic Moroccan coast [59]). However, the main haplotype found in the Duero [6–15] basin (BBABB, 84.2%) is a local haplotype. This differentiation might be related to the geological features of the Duero Basin, found at the greatest altitude in the Iberian Peninsula (around 800 m). Marked changes in slope (Arenillas Parra & Sáenz Ridruejo 1987) may have led to isolation of the river headwaters from its lower reaches and thus to population isolation. mtDNA haplotypes were also restricted to populations of the Tagus basin [16–34] (e.g. ABACC, 15.7%).

Different forces appear to have shaped the genetic structure of Atlantic and Mediterranean drainage systems after the differentiation of the populations of these regions. All the Mediterranean populations analysed shared one haplotype, except those of the Segura basin [47–53], which showed two haplotypes (Mediterranean and Southern). The Southern populations also formed a distinct group because a unique diagnostic haplotype was present in both Atlantic and Mediterranean Andalusian slopes. This might be attributable to the existence of a Betic-Riffian common ancestral genotype as shown by different fish species (Perdices *et al*. 1995; Doadrio *et al*. 1996; Machordom *et al*. 1998). This region, which includes the south Iberian area (from the Guadalquivir basin) and part of North Africa, was isolated from the Iberian Peninsula during the Palaeogene and has been connected since the Miocene/Pliocene (5.5 Ma) (López-Martínez 1989; Barbadillo *et al*. 1997). Unfortunately, due to the scarce presence of trout off the Maghreb Mediterranean coast (we were unable to obtain a single sample) we could not test this hypothesis. The low diversity of the Mediterranean populations was nevertheless evident and might be explained by the recent, rapid recolonization and/or a drastic reduction in population size. Even though a certain degree of polymorphism in the Mediterranean populations analysed was suggested by the allozyme markers, it must be remembered that the effective population size for mtDNA is a quarter of that corresponding to nuclear DNA (assuming equal sex ratios). The different behaviour of nuclear and mitochondrial markers is particularly relevant in the study of salmonids because, as a result of natal homing in specific tributaries, the breeding population size may be in the hundreds. Thus, the difference in effective population size corresponding to nuclear DNA and mtDNA would be significant (Ferguson *et al*. 1995).

The present findings based on mitochondrial haplotyping are in general agreement with those of allozymic studies conducted in the Iberian Peninsula (García-Marín & Pla 1996; Machordom *et al*. 1999), although here the separation between the different areas was clearly apparent. However, genetic data corresponding to the southern Iberian Peninsula were insufficient and, to date, no diagnostic or unique alleles have been found in Andalusian populations (García-Marín & Pla 1996). Hence, the present mitochondrial analysis is the first indication of the singularity of the southern Iberian trout populations.

Correlation between nucleotide diversity and time of separation of taxa may be high as, given the close nature of the taxa or populations under study, a similar evolutionary change rate would be expected. According to general models (Brown *et al*. 1982; Moritz *et al*. 1987; Kocher *et al*. 1989; Bembo *et al*. 1994; Bermingham *et al*. 1997), this correlation may be estimated at 1%–2% divergence per million years (Myr) although a more accurate estimation for salmonids (also based on RFLP analyses) would be 0.5–0.9% substitutions per Myr (Martin & Palumbi 1993). Using this last criterion, the time of divergence between the different haplotypes (d = 0.37–3.15% excluding outgroups, Table 4) would be 0.41–3.5 Myr (0.9% per Myr), or 0.74–6.3 Myr (0.5% per Myr). These values are in agreement with those reported for Greek brown trout (Apostolidis *et al*. 1997), whose haplotypes were also estimated to differentiate during Messinian or Early Pliocene times (2.5–6.0 Ma). Moreover, both Bernatchez *et al*. (1992) and Apostolidis *et al*. (1997) suggested that the different haplotypes found in their respective study areas appeared at the same time, due to the short length of the branches that join them. This is also evident in our analysis. For the Greek and Iberian populations, the Messinian Crisis, when contact between the Atlantic Ocean and the Mediterranean Sea was interrupted, might have brought about the isolation and subsequent differentiation of the different haplotypes currently shown by brown trout.

The biogeographical history of brown trout has been explained by several hypotheses. The present findings are inconsistent with the idea of a lack of differentiation of populations from different Spanish drainage systems (Morán *et al*. 1996), and with the suggestion by the same authors that all Spanish specimens are of the Mediterranean type (*sensu* Bernatchez *et al*. 1992). Nevertheless, the present relationships between haplotypes indicate the contemporary appearance of four major groups (plus derivative or sister haplotypes): the AAAAA haplotype, the general Iberian Atlantic haplotype, ABACA, the Duero haplotype BBABB, and the Mediterranean and southern haplotypes, BBBDA and BBCDA, respectively.

Although our results suggest the probable appearance of these haplotypes during the Miocene, several authors relate the current distribution of the different genetic units of brown trout to a dispersal phenomenon after the last glaciation, indicating that the ancestral form is that corresponding to southern and Mediterranean areas (trout bearing the *LDH-5*100* allele shared by species

such as *S. salar*). Four major lineages associated with four possible refugia during the last glaciation in Europe were proposed by García-Marín *et al*. (1999) and correspond to: north Atlantic and Iberian Atlantic drainage systems, the Mediterranean Sea and the Black-Caspian-Aral seas. This hypothesis is highly congruent with the data shown here for the areas under study because the differentiation of both Atlantic and Mediterranean lineages is clear and the existence of a different group of haplotypes from the north or central European area was also observed. Moreover, García-Marín *et al*. (1999) also indicate the possible routes of postglacial migration, including north, east and west routes for an initial lineage of southern British and adjacent continental brown trout. Consistent with our mitochondrial results, we could propose two alternative hypotheses: First, the AAAAA haplotype would be distributed between the North Atlantic and the Cantabrian coasts, before the last glaciations; and second, this haplotype, from the northern lineage, reached the Cantabrian drainage system through southward migration from the putative British refuge. A second lineage associated with northward migration from Iberian Atlantic and Cantabrian areas was proposed by García-Marín *et al*. (1999), but our results indicate the coexistence of two haplotypes in the Cantabrian region, probably representing the limit of mitochondrial marker penetration of the two lineages proposed for the Atlantic coast. According to the distribution of the ABACA haplotype (from the Cantabrian Sea to the Moroccan coasts), we propose that the second ('south-Atlantic') refuge is centred to the south of that indicated by García-Marín *et al*. (1999).

Management implications

Given that in some rivers hundreds of thousands of specimens (several times the native trout population size) are restocked each year, the number of non-native fish recorded herein is surprisingly low. We, therefore, propose that Spain's restocking policy requires reconsideration, not only based on the lack of increase in population sizes, but also in an attempt to avoid genetic contamination and potential loss of locally adapted genomes.

If Spain is to discontinue restocking with non-native fish, both biogeographical areas and mtDNA endemism should be taken into account, and the regional differences demonstrated for brown trout preserved. The present authors propose a limit of five OCUs [continuous area limited by geographical boundaries, and inhabited by one or more populations sharing the same genetic pattern, Doadrio *et al*. 1996) or evolutionarily significant units (Moritz 1994)] corresponding to: Cantabrian rivers, Duero basin, Tagus basin, southern rivers, and Mediterranean rivers (Fig. 5). These biogeographic divisions generally coincide with those established by chorological studies on freshwater ichthyofauna of the Iberian Peninsula (Doadrio 1988). Slight discrepancies are probably due to the historical anadromous nature of brown trout.

Acknowledgements

The authors wish to thank I. Doadrio, P. Martínez, P. Morán, and J. Carmona for the providing brown trout specimens from Morocco, Galicia, Cantabria and Andalucía, respectively. We are also grateful to A. F. Alonso for his provision of further samples from rivers in central Spain, to R. Zardoya for his help and interest at the outset of the project, to S. Pérez-Benavente for skilful technical assistance and to A. Perdices for her helpful suggestions. We are especially indebted to E. Bermingham for his constructive, critical review of the manuscript. L. Ashcroft checked the English of a first version and A. Burton reviewed the final draft. J.S. was sponsored by a predoctoral grant from the INIA. The study was funded by the INIA Project SC95/005.

References

Apostolidis AP, Karakousis Y, Triantaphyllidis C (1996) Genetic differentiation and phylogenetic relationships among Greek *Salmo trutta* L. (brown trout) populations as revealed by RFLP analysis of PCR amplified mitochondrial DNA segments. *Heredity*, **77**, 608–618.

Apostolidis AP, Triantaphyllidis C, Kouvatsi A, Economidis PS (1997) Mitochondrial DNA sequence variation and phylogeography among *Salmo trutta* L. (Greek brown trout) populations. *Molecular Ecology*, **6**, 531–542.

Arenillas Parra M, Sáenz Ridruejo C (1987) *Guía Física de España. 3. Los Ríos*. Alianza Editorial, Madrid.

Arias J (1996) *Análisis alozímico de la estructura genética de la trucha común* (Salmo trutta *L.*): *implicaciones para la gestión de los recursos de esta especie en Galicia*. PhD Thesis. University of Santiago de Compostela, Lugo, Spain.

Barbadillo LJ, García-París M, Sanchiz B (1997) Orígenes y relaciones evolutivas de la herpetofauna ibérica. In: *Distribución y biogeografía de los anfibios y reptiles en España y Portugal* (ed. Pleguezuelos JM), pp. 47–100. Universidad de Granada, Granada.

Behnke RJ (1972) The systematics of salmonid fishes of recently glaciated lakes. *Journal of Fisheries Research Board of Canada*, **29**, 639–671.

Behnke RJ (1986) Brown trout. *Trout*, **27**, 42–47.

Bembo DG, Weightman AJ, Beverton RJH, Cresswell RC (1994) Mitochondrial DNA variation in River Usk brown trout, *Salmo trutta*. *Journal of Fish Biology*, **44**, 717–723.

Bermingham E, Avise JC (1986) Molecular zoogeography of freshwater fishes in the southeastern United States. *Genetics*, **113**, 939–965.

Bermingham E, McCafferty SS, Martin AP (1997) Fish biogeography and molecular clocks: Perspectives from the Panamanian Isthmus. In: *Molecular Systematics of Fishes* (eds Kocher TD, Stepien CA), pp. 113–126. Academic Press, San Diego.

Bernatchez L, Guyomard R, Bonhomme F (1992) DNA sequence variation of the mitochondrial control region among geographically and morphologically remote European brown trout *Salmo trutta* populations. *Molecular Ecology*, **1**, 161–173.

Bernatchez L, Osinov A (1995) Genetic diversity of trout (genus *Salmo*) from its most eastern native range based on mitochondrial DNA and nuclear gene variation. *Molecular Ecology*, **4**, 285–297.

Brown WM, Prager EM, Wang A, Wilson AC (1982) Mitochondrial DNA sequences of primates: Tempo and mode of evolution. *Journal of Molecular Evolution*, **18**, 225–239.

Campbell JS (1977) Spawning characteristics of brown trout and sea trout, *Salmo trutta* L., in Kirk Burn, River Tweed, Scotland. *Journal of Fish Biology*, **11**, 217–229.

Cronin MA, Spearman WJ, Wilmot RL (1993) Mitochondrial DNA variation in chinook (*Oncorhynchus tshawytscha*) and chum salmon (*O. keta*) detected by restriction enzyme analysis of polymerase chain reaction (PCR) products. *Canadian Journal of Fisheries and Aquatic Sciences*, **50**, 708–715.

Doadrio I (1988) Delimitation of areas in the Iberian Peninsula on the basis of freshwater fishes. *Bonner Zoologische Beiträge*, **39**, 113–128.

Doadrio I, Perdices A, Machordom A (1996) Allozymic variation of the endangered killifish *Aphanius iberus* and its application to conservation. *Environmental Biology of Fishes*, **45**, 259–271.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.

Felsenstein J (1993) PHYLIP *(Phylogeny Inference Package), Version 3.5*. Department of Genetics, University of Washington, Seattle, WA.

Ferguson A (1989) Genetic differences among brown trout, *Salmo trutta*, stocks and their importance for the conservation and management of the species. *Freshwater Biology*, **21**, 35–46.

Ferguson A, Taggart JB, Prodöhl PA *et al.* (1995) The application of molecular markers to the study and conservation of fish populations, with special reference to *Salmo*. *Journal of Fish Biology*, **47** (Suppl. A), 103–126.

García-Marín JL, Jorde PE, Ryman N, Utter F, Pla C (1991) Management implications of genetic differentiation between native and hatchery populations of brown trout (*Salmo trutta*) in Spain. *Aquaculture*, **95**, 235–249.

García-Marín JL, Pla C (1996) Origins and relationships of native populations of *Salmo trutta* (brown trout) in Spain. *Heredity*, **77**, 313–323.

García-Marín JL, Utter FM, Pla C (1999) Postglacial colonization of brown trout in Europe based on distribution of allozyme variants. *Heredity*, **82**, 46–56.

Giuffra E, Bernatchez L, Guyomard R (1994) Mitochondrial control region and protein coding genes sequence variation among phenotypic forms of brown trout *Salmo trutta* from northern Italy. *Molecular Ecology*, **3**, 161–171.

Guyomard R (1991) Diversité génétique et gestion des populations naturelles de truite commune. In: *La Truite, Biologie et Écologie* (eds Baglinière JL, Maisse G), pp. 215–235. Institut National de la Recherche Agronomique, Paris.

Gyllensten U, Wilson AC (1987) Mitochondrial DNA of salmonids. Inter- and intraspecific variability detected with restriction enzymes. In: *Population Genetics and Fishery Management* (eds Ryman N, Utter F), pp. 301–317. University of Washington Press, Seattle.

Hall HJ, Nawrocki LW (1995) A rapid method for detecting mitochondrial DNA variation in the brown trout, *Salmo trutta*. *Journal of Fish Biology*, **46**, 360–364.

Hamilton KE, Ferguson A, Taggart JB, Tomasson T, Walker A, Fahy E (1989) Post-glacial colonization of brown trout, *Salmo trutta* L. *Ldh-5* as a phylogeographic marker locus. *Journal of Fish Biology*, **35**, 651–664.

Hansen MM, Loeschcke V (1996) Genetic differentiation among Danish brown trout populations, as detected by RFLP analysis of PCR amplified mitochondrial DNA segments. *Journal of Fish Biology*, **48**, 422–436.

Hindar K, Ryman N, Utter F (1991) Genetic effects of cultured fish on natural fish populations. *Canadian Journal of Fisheries and Aquatic Sciences*, **48**, 945–957.

Hynes RA, Ferguson A, McCann MA (1996) Variation in mitochondrial DNA and post-glacial colonization of north western Europe by brown trout. *Journal of Fish Biology*, **48**, 54–67.

Jonsson B (1985) Life history patterns of freshwater resident and sea run migrant brown trout in Norway. *Transactions of the American Fish Society*, **114**, 182–194.

Kocher TD, Thomas WK, Meyer A *et al.* (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the USA*, **86**, 6196–6200.

Kottelat M (1997) European freshwater fishes. An heuristic checklist of the freshwater fishes of Europe (exclusive of former USSR), with an introduction for non-systematists and comments on nomenclature and conservation. *Biologia, Bratislava*, **52** (Suppl. 5), 1–271.

López-Martínez N (1989) Tendencias en Paleobiogeografía. El futuro de la biogeografía del pasado. In: *Paleontología, Nuevas Tendencias* (ed. Aguirre E), pp. 271–296. Consejo Superior de Investigaciones Científicas, Madrid.

Machordom A, Bouhadad R, Doadrio I (1998) Allozyme variation and evolutionary history of North African populations of the genus *Barbus* (Osteichthyes, Cyprinidae). *Diversity and Distributions*, **4**, 217–234.

Machordom A, García-Marín JL, Sanz N, Almodóvar A, Pla C (1999) Allozyme diversity in brown trout (*Salmo trutta*) from Central Spain. Genetic consequences of restocking. *Freshwater Biology*, **41**, 707–718.

Martin AP, Palumbi SR (1993) Body size, metabolic rate, generation time, and molecular clock. *Proceedings of the National Academy of Sciences of the USA*, **90**, 4087–4091.

Martínez P, Arias J, Castro J, Sánchez L (1993) Differential stocking incidence in brown trout (*Salmo trutta*) populations from Northwestern Spain. *Aquaculture*, **114**, 203–216.

McElroy D, Moran PE, Bermingham E, Kornfield I (1992) REAP: an integrated environment for the manipulation and phylogenetic analysis of restriction data. *Journal of Heredity*, **83**, 157–158.

Morán P, Pendás AM, García-Vázquez E (1996) Mitochondrial DNA variation in wild and hatchery brown trout (*Salmo trutta* L.) populations from Spain. *Aquaculture*, **141**, 59–65.

Moritz C (1994) Applications of mitochondrial DNA analysis in conservation: a critical review. *Molecular Ecology*, **3**, 401–411.

Moritz C, Dowling TE, Brown WM (1987) Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annual Review of Ecology and Systematics*, **18**, 269–292.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences USA*, **76**, 5269–5273.

Pamilo P, Nei M (1988) Relationships between gene trees and species trees. *Molecular Biology and Evolution*, **5**, 568–583.

Perdices A, Machordom A, Doadrio I (1995) Allozyme variation of African and Iberian populations of the genus *Cobitis*. *Journal of Fish Biology*, **47**, 707–718.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: a Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Schneider S, Kueffer JM, Roessli D, Excofier L (1997) *Arlequin: A software for population genetic data analysis, Version 1.1*. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Switzerland.

Swofford DL (1999) *PAUP*: Phylogeny Analysis Using Parsimony (*and Other Methods), Version 4.0b2*. Sinauer Associates Inc., Sunderland, Massachusetts.

Taggart JB, Ferguson A (1986) Electrophoretic evaluation of supplemental stocking programme for brown trout, *Salmo trutta* L. *Aquaculture and Fisheries Management*, **17**, 155–162.

Zardoya R, Garrido-Pertierra A, Bautista JM (1995) The complete nucleotide sequence of the mitochondrial DNA genome of the rainbow trout, *Oncorhynchus mykiss*. *Journal of Molecular Evolution*, **41**, 942–951.

This study represents the joint efforts of two different research teams: one from the Instituto Madrileño de Investigación Agraria and the other from the Molecular Biology Department of the School of Veterinary Sciences, Complutense University, Madrid. Annie Machordom is a postdoctoral research scientist mainly interested in the use of molecular genetics to address questions on phylogeny, evolution and conservation of freshwater fish. Juan Suárez is preparing his PhD on the molecular differentiation and evolution of Iberian populations of *Salmo trutta*. Ana Almodóvar, who leads the IMIA group, presently focuses her research efforts on the ecology, life history and management of salmonids and other fish families. José M. Bautista heads a research team devoted to the study of molecular evolution and systematics through mitochondrial genes, and also investigates the structure/function relationship of human G6PD variants.