Mitochondrial DNA variation in Pleistocene and modern Atlantic salmon from the Iberian glacial refugium

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Abstract

Current understanding of the postglacial colonization of Nearctic and Palearctic species relies heavily on inferences drawn from the phylogeographic analysis of contemporary generic variants. Modern postglacial populations are supposed to be representative of their Pleistocene ancestors, and their current distribution is assumed to reflect the different colonization success and dispersal patterns of refugial lineages. Yet, testing of phylogeographic models against ancestral genomes from glacial refugia has rarely been possible. Here we compare ND1 mitochondrial DNA variation in late Pleistocene (16 000–40 000 years before present), historical and contemporary Atlantic salmon (*Salmo salar*) populations from northern Spain and other regions of western Europe. Our study demonstrates the presence of Atlantic salmon in the Iberian glacial refugium during the last 40 000 years and points to the Iberian Peninsula as the likely source of the most common haplotype within the Atlantic lineage in Europe. However, our findings also suggest that there may have been significant changes in the genetic structure of the Iberian refugial stock since the last ice age, and question whether modern populations in refugial areas are representative of ice age populations. A common haplotype that persisted in the Iberian Peninsula during the Pleistocene last glacial maximum is now extremely rare or absent from European rivers, highlighting the need for caution when making phylogeographic inferences about the origin and distribution of modern genetic types.

Keywords: ancient mtDNA, Atlantic salmon, glacial refugium, phylogeography, potsglacial evolution

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Introduction

The genetic legacy of Quaternary glaciations on modern intraspecific biodiversity is the subject of much debate (Hewitt 2000). A central tenet in phylogeography is that contemporary lineages living in or near formerly glaciated areas can be traced to ancestral lineages from glacial refugia (Avise *et al.* 1987). A second, albeit implicit, assumption is that the genetic character of refugial stocks can be inferred from modern distributions of variant types (Bernatchez & Wilson 1998). Yet, both assumptions have rarely been tested against known

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ancestral genomes, and are difficult to resolve with studies of contemporary genetic variation (Leonard *et al.* 2000; Pääbo 2000).

Insight into these issues can potentially be gained by comparing modern and ancestral genomes of Nearctic and Palearctic species such as Atlantic salmon (*Salmo salar*), whose historical distribution lies largely within areas covered by ice at the time of the last glacial maximum ≈ 22 000–18 000 years before present (BP) (MacCrimmon & Gots 1979). In Europe, the species' main glacial refugia are thought to have been in the Iberian Peninsula, the North Sea, and the ice lakes east of the Baltic Sea, with perhaps minor refugia in unglaciated areas along the ice edge (Koljonen *et al.* 1999; Verspoor *et al.* 1999; Nilsson *et al.* 2001). Following deglaciation, refugial salmon populations may

have been able to recolonize formerly glaciated areas, but the number and location of such refugia, as well as the pattern of recolonization, remain uncertain.

Nowadays, some of the most endangered Atlantic salmon populations are those inhabiting former southerly glacial refugia, located farthest away from the centre of the species' current geographical range (Parrish *et al.* 1998; Kellogg 1999). At the southern end of that range, post-glacial warming from about 11 500 years BP to the present (Courty & Vallverdu 2001; Ellwood *et al.* 2001) may have constituted a strong selective force for refugial salmon populations, that is likely to intensify in the near future (McCarthy & Houlihan 1997). Yet, the likely genetic response to past and current climatic change is poorly understood (Minns *et al.* 1992; McCarthy & Houlihan 1997).

Much could therefore be learned through the study of Atlantic salmon from Pleistocene glacial refugia, the main source of which is likely to come from archaeological sites occupied by humans since Upper Palaeolithic times (Jochim 1983). In this context, the narrow coastal strip between the Cantabrian coast and the Cordillera of northern Spain is particularly rich in such late Pleistocene archaeological sites, of which Altamira is perhaps the best known (González Sainz & González Morales 1986; Straus 1992). Along with red deer, wild goat and horse remains, salmonid-like bones have commonly been found (Casteel 1976; Straus *et al.* 1980), although positive species identification of prehistoric faunal remains has often proved problematic on morphological grounds (Gobalet 2001). Fortunately, this problem can now potentially be resolved with the advent of molecular archaeology (Brown & Brown 1992). Application of techniques for ancient DNA (Greenwood *et al.* 1999; Leonard *et al.* 2000) has opened the possibility of amplifying genomes up to 100 000 years old (Hofreiter *et al.* 2001), although we know of no attempt to examine the DNA of

salmonids more than a few decades old (Nielsen *et al.* 1999).

Here we compare mitochondrial DNA (mtDNA) variation in Palaeolithic (16 000–40 000 BP), historical and modern Atlantic salmon populations from northern Spain, a presumed glacial refugium for the species during the last ice age. Ancestral salmon DNA was extracted from vertebrae collected at El Mirón Cave (Cantabria), an archaeological site in the valley of the River Asón with an extensive series of accelerator mass spectroscopy (AMS) and conventional radiocarbon dates, as well as palaeoclimate estimates (Ellwood *et al.* 2001, Straus & González Morales 2001, Straus *et al.* 2001). Historical scales were obtained from adult salmon returning to the river Asón since 1948, and modern samples were collected from the Cantabrian rivers Asón, Pas, Nansa and Deva from 1996 to 2000. Comparisons with other Atlantic salmon populations (Verspoor *et al.* 1999; Nilsson *et al.* 2001) allowed us to reassess the phylogenetic relationships of lineages at glaciated and refugial areas, and to contrast existing hypotheses about the evolution of salmon mitochondrial haplotypes across Europe.

Materials and methods

Pleistocene samples

Thirty salmonid vertebrae (100–2088 mg) ranging in age from 3250 to 41 000 years BP (Fig. 1) were collected directly by the archaeologists from El Mirón Cave (Straus & González Morales 2001), using rubber gloves and were stored at 4 °C in sealed polythene bags until analysis. Radiocarbon dating was performed on bone and charcoal samples at Geochron Laboratories in conjunction with Lawrence Livermore Laboratories for AMS determinations (Straus *et al.* 2001). Diagnostic Fourier Transform Infra-red (FTIR) spectrometer peaks were detected around 1647,
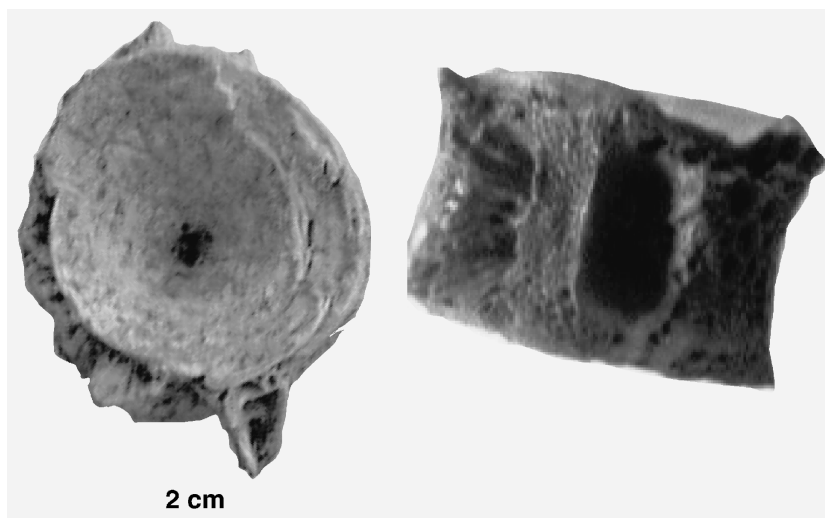

Fig. 1 Vertebral centra of adult Atlantic salmon (*Salmo salar*) from the Upper Palaeolithic collected at El Mirón Cave, near the River Asón (Cantabria, northern Spain). Such vertebrae provided the oldest salmonid mtDNA sequences reported to date, and demonstrate the presence of Atlantic salmon in the Iberian glacial refugium during the last 40 000 years.

1539 and 1425 wave numbers (per cm), indicating that bone collagen was relatively well preserved, although some vertebrae also showed evidence of light charring and the presence of humic acids (Shahack-Gross & Weiner, unpublished report). The sizes of the fish from which vertebrae were derived were estimated from measurements of vertebral centra (Wise 1980).

To reduce the risk of exogenous contamination, we adopted stringent protocols for ancient DNA (Kwok & Higuchi 1989; Greenwood *et al.* 1999), and carried all manipulations prior to polymerase chain reaction (PCR) in a forensic laboratory free of salmon DNA and physically separated from post-PCR procedures (Leonard *et al.* 2000). Vertebrae were pretreated with UV light for 10 min before being pulverized in liquid nitrogen, and divided into two subsamples. To extract DNA, we employed three different methods: DNAce (Bioline), Geneclean (Bio-101) with overnight preincubation (5 μL ethylenediaminetetraacetic acid 0.5 M pH 8.0, 200 μL sodium dodecyl sulphate 10%, 200 μL 20 mg/mL proteinase K), and the Mix-and-Clean method (Scholz & Pusch 1997). Concentration with Microcon 50 was performed to remove PCR inhibitors when necessary (Hagelberg 1994). Extractions and amplifications were replicated at least twice for each subsample. Risk of contamination was further minimized by UV irradiation of tubes and solutions (Kwok & Higuchi 1989) and by employing specific sets of Atlantic salmon primers (Knox *et al.* 2002) amplifying 100–110 bp fragments at known variable restriction sites (*Hae*III, *Hin*fI, *Rsa*I) in the mtDNA ND1 region. Two additional sets of primers (Cronin *et al.* 1993; Palumbi 1996; Knox *et al.* 2002) amplifying larger fragments in the same ND1 region were used to test the authenticity of the ancient DNA (Pääbo 1989). North American salmon DNA (Saint John River, Canada), which has a different *Hae*III/*Rsa*I haplotype from most European fish (Verspoor *et al.* 1999), was used as a positive control to reveal cross-contamination across samples.

Replicated, independent PCR-hotstart amplifications (5 μL; 35 cycles) of each subsample were carried out in 50 μL volumes (Verspoor *et al.* 1999), with the addition of 1.3 mg/mL bovine serum albumin to overcome inhibition (Höss & Pääbo 1993). One microlitre was re-amplified (15 cycles) in a volume of 50 μL without bovine serum albumin, and was visualized in 2% agarose stained with ethidium bromide. Extraction and PCR-negative controls were performed in all cases. Samples that yielded a single faint band of the expected fragment size were subjected to a third 30-cycle amplification to increase DNA yield for sequencing. PCR products were excised from a 2% agarose gel, purified with Prep-A-Gene (BioRad), and both strands were sequenced on an Applied Biosystems 373A DNA sequencer. Replicated sequences of the right size were obtained in each case and deposited in the GenBank database (accession nos AF385752–AF385759).

Historical scales and modern samples

Total DNA from historical scales (1948–89) of 121 adult salmon from the River Asón was extracted in 5% Chelex (Beacham & Dempson 1998), 10 μL of the extracted DNA was then amplified (hot-start, 40 cycles) with the short *Hae*III, *Hin*fI and *Rsa*I primers (Knox *et al.* 2002) and digested with restriction enzymes. For modern samples, total DNA was extracted from adipose fins (Taggart *et al.* 1992) of 828 adult salmon collected in the rivers Asón, Pas, Nansa and Deva during 1996–2000. One microlitre of the extracted DNA was used to amplify a 1400-bp fragment in the same ND1 region with the 16sRNA (Palumbi 1996) and ND1 (Cronin *et al.* 1993) primers, followed by digestion with restriction enzymes (Verspoor *et al.* 1999). Ten individuals from the R. Asón were sequenced as above to allow comparisons with ancient samples.

Analysis of mtDNA variation

To examine the relationship between the Pleistocene salmon from the R. Asón and contemporary anadromous Atlantic salmon populations, we used previously reported mtDNA ND1 haplotypic frequencies for *Hae*III, *Hin*fI and *Rsa*I across Europe (Verspoor *et al.* 1999; Nilsson *et al.* 2001). A total of 36 populations were included in the analysis, including the 31 wild anadromous samples from the original Nilsson *et al.* (2001) dataset ($n = 1448$), plus new data on four extant ($n = 828$) and one ancient ($n = 6$) Iberian populations from Cantabria. We excluded from the comparative analysis those samples that consisted of known hatchery-reared fish or were land-locked (nonmigratory) salmon, as these are known to differ from wild migratory populations (Verspoor 1988, 1994).

Levels of haplotype (h) and nucleotide (π) diversity within populations (Nei 1987) were estimated using DNASP (Rozas & Rozas 1999), based on the published sequences of 34 fish from northern Europe (Nilsson *et al.* 2001) plus new sequences of 10 modern fish from the Iberian Peninsula (Fig. 2).

Phylogenetic analysis and spatial distribution of haplotypes

The relationship between ancient and modern populations was estimated by two pairwise genetic distances generated from haplotype frequencies: D_A distance (Nei *et al.* 1983) using DISPAN (Ota 1993), and chord distance (Cavalli-Sforza & Edwards 1967) using PHYLIP (Felsenstein 1993). UPGMA dendrograms were then constructed from distance matrices.

We attempted to reconstruct the phylogeny of mitochondrial haplotypes by constructing a majority rule radial consensus tree bootstrapped 1000 times for maximum parsimony, based on sequence data (Nilsson *et al.* 2001;

and Fig. 2) using PHYLIP (Felsenstein 1993) and assuming that the newly reported DAA haplotype (Fig. 2) differed from the AAA only at the *Hae*III restriction site. Brown trout (GenBank AF11716) and North American Atlantic salmon (Nilsson *et al.* 2001) were used as outgroups. The relationship between haplotypes was also examined by statistical parsimony (TCS program, Clement *et al.* 2000) and minimum spanning network (ARLEQUIN package, Schneider *et al.* 2000) to account for relatively low levels of intraspecific genetic variation (Posada & Crandall 2001).

The current geographical distribution of haplotypes was examined with an unbiased linear kriging smoother (Bucci & Vendramin 2000) using SYSTAT 9.0 (SPSS 1998) and by calculating 95% confidence geographical centroids for each haplotype, weighed by sample frequencies. 'Kriging' is an interpolation technique commonly used in geostatistics that uses local information around sampling points to extrapolate complex and irregular spatial patterns. It produces the best linear unbiased estimation of a stochastic process by generalized least-squares, fitting a surface to irregularly spaced, spatially autocorrelated data (Isaaks & Srivastava 1989). Associations between haplotypes were tested by Bonferroni-adjusted Pearson correlation coefficients on arcsin transformed sample frequencies. Along with the phylogenetic and network analysis, these allowed us to infer the oldest haplotypes, which under coalescent theory should be those that are the most frequent and most broadly distributed (Posada & Crandall 2001).

Temporal changes in Iberian populations

To address the small sample size of Pleistocene vertebrae, we conducted a resampling analysis of ancient and contemporary Cantabrian samples. We generated 1000 random samples of six individuals from the contemporary Cantabrian data set ($n = 828$), and employed an exact test to compare the distribution of composite haplotypes between ancient and modern Cantabrian samples under the null hypothesis of no difference.

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(a)  Hae-III      4447                                                                                                          4546
Modern R.Asón     GTGCACCCTT TGACCTTACA GAAGGAGAAT CAGAATTAGT CTCCGGATTT AATGTAGAAT ACGCCGGAGG GCCCTTCGCC CTCTTCTTTC TAGCCGAATA
V59 (17050±60 BP) .......... .......... .......... .......... .......... .......... .......... .A........ .......... ..........
V62 (16960±80 BP) .......... .......... .......... .......... .......... .......... .......... .A........ .......... ..........
V67 (~17000 BP)   ....NN.... .......N.N .NN....N.. .N.N..NN.. N.N.N.....N. ....N....N .......... .N........ .......... ..........
V69 (17050±60 BP) .......... .......... ........N ..N..N.N.. .N.NN...NN ....NNNNN. .NNN..NN. .......... .A........ .......... ..........
V70 (16130±250 BP).......... .......... .......... .......... .......... .....NN... N.NN...... .A........ .......... ..........
V71 (41280±1120 BP).N.....NN .....NN.N. N.......... .......N. NNN.N..N.. N.N..N..N .......... ..NN...... .......... ...N.....
Baltic salmon     .......... .......... .......... .......... .......... .......... .......... .A........ .......... ..........
N.America salmon  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Salmo trutta      .C........ .......... ...C...... .......... .......... .......... .......... .......... .......... ..........
Salvelinus alpinus .......T.. ......C... .......... .......... .......... T..A.....C .C....... .......T.. ......A... .......... ..C. .G..T.....
Human             .AA.C..... C......G.C .......... ...G..G. .C...C.... ...A..C..C .CA.C.... ......C.. C........ ..A.....CA ..........
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(b)  Hinf-I       4850                                                                                                          4959
Modern R. Asón    GGACCACCTT GATAGCGTGG CTGATAGGGG TTCAAGTCCC CTCAATTCTA GAGAGAAGGG ACTCGAACCC ATCCTCAAGA GATCAAAACT CTTGGTGCTT CCACT-ACACG
V59 (17050±60 BP) ---------- ------..... .......... .......... .......... ..N.......N .....NN... .......... .......... .......... ...-..N.
V62 (16960±80 BP) --...N...N N.NN...N.. N.......... .......... NN.N...... ....N.N.N. ......N... ......... .......... .......... ...-...N.
V67 (~17000 BP)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
V69 (17050±60 BP) NN..N--NN .NN--NNNN- N--N.NNNNN N.NNNN....N N.N....NNN NNNN....N ...N...N..N-NN .NNNN--N-- NNN.NNNN--N .NNNNN.N.N ...NN-N..N
V70 (16130±250 BP)---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
V71 (41280±1120 BP)-.-....... .......... .......... .......... ..NN...... ...N..N..N ......N... NN..NNNN.N ....NNN.N .NN..N.... N..N.-N..
Baltic salmon     .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ...-.....
N.America salmon  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ...-.....
Salmo trutta      .......... .......... .......... .......... .......... .......... .G........ .......... .......... .......... ...-.....
Salvelinus alpinus .......... .......... .......... .......... .......... .......... .T........ .......... .......... .......... ...-.....
Human             .AGTT..T.. .....A..AA A.A....A. C.T..AC... ..T.T..... .GACT.T.A. .A....... ......CTG... ATC.....T. ..CC....CA ..TA.C....
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(c)  Rsa-I        3914                                                                                                          4023
Modern R. Asón    CGCTTTCCTC AC-CTTACTCG AACGAAAAGT CCTTGGATAT ATGCAACTTC GGAAAGGGGC AAACATCGTC GGTCCATACG GATTGCTTCA ACCTATCGCG GACGGCCTAA
V59 (17050±60 BP) .N..N..... ..-......N .N.N.N.. .......... .......N.... .N........ .......... .......N.. .......... .......... ..........
V62 (16960±80 BP) -NN....... ..-...N... .......... .......... ...N...N... N.......... .......... ...N..... .......... .......... ..........
V67 (~17000 BP)   .......... N.-..NN.... ...N....... ...N...... .N.....NN. ..N.N..... .......... ...N..... N....N.N.. .......... ..........
V69 (17050±60 BP) ---------- ---------- ---- .NNNNN ...N.NN... NNN.NN.... .....N.N.. N......... ...NN .N..... .......... .......... ..........
V70 (16130±250 BP)---------- ---------- ------..N. N...NNN.NN N.NN..N... .......... .......N.. ...N..... .......N.. .......... ..........
V71 (41280±1120 BP)......... .......N... .......N... ....N..... ...NN.... .NN.NN.N ..N...N. NNNN..N... ..NN..N.N. N.NN..NNNN .N.NNN...N
Baltic salmon     .......... ..-....... .......... .......... .......... .......... .......... ...G...... .......... .......... ..........
N.America salmon  .......... ..-....... .......... .......... .......... .......... .A........ ...G...... .......... .......... ..........
Salmo trutta      T......... ..-....... .......... .......G. ..A...... .A....A. .......... ...G.T.. ...A...... .......... ..........
Salvelinus alpinus T..C.....A ..-..C..... .......... ......G... .......... .A....A. .C........T ..C.G.T.. ...A...... .......... ..........
Human             G..A.....A ..TG....... ........A. T..A..C.. ..A....A. .C.....C. C...G.T..A ..C.C.... .GC.A..A. ...CT....T ...C.A...
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Fig. 2 Mitochondrial ND1 sequences for six large Atlantic salmon (*Salmo salar*) vertebrae from the Upper Palaeolithic (V59–V71) collected at El Mirón Cave, near the River Asón (Cantabria, northern Spain): (a) *Hae*III fragment, positions 4447–4546; (b) *Hin*fI fragment, positions 4850–4959; (c) *Rsa*I fragment, positions 3914–4023 (Hurst *et al.* 1999). Identity with modern River Asón salmon sequences ($n = 10$) is indicated by dots, absence of amplification product is indicated by a dash, and ambiguous bases are indicated by an N. ND1 haplotypes for the six vertebrae (DAA, DAA, A-A, DAA, D-A and A-A) were derived from known restriction sites (Verspoor *et al.* 1999), shown underlined. Palaeolithic sequences totalling 165–294 bp (GenBank accession numbers AF385752–AF385759) were compared with those of extant Atlantic salmon populations from the River Asón (AAA), the Baltic (DAB), and North America (NAB), as well as with those of brown trout (*Salmo trutta*; GenBank AF405331, AF405334, AF405337), arctic char (*Salvelinus alpinus*; GenBank AF154851) and humans (GenBank AF347008). The North American control salmon is from the Saint John River in Canada (GenBank AF405329, AF405333, AF405336), and the Baltic salmon is from the River Torne, Finland (GenBank AF405330, AF405332, AF405335). The Palaeolithic sequences obtained correspond in all cases to Atlantic salmon.

Table 1 Characteristics of the Palaeolithic fish vertebrae (Vert.) collected in El Mirón Cave (Cantabria, northern Spain), and size, weight and mtDNA haplotypes of the six adult Atlantic salmon (*Salmo salar*) from which they originated

Vert. ID	Vert. type*	Centrum length (mm)	Centrum weight (mg)	Location of vertebrae			AMS/^{14}C age (yr BP)	Estimated fish length (cm)	Estimated fish weight (kg)	mtDNA ND1 Haplotype†			Total fragments sequenced (bp)
				Strata	Sector	Transect				*Hae*III	*Hin*fI	*Rsa*I	
V59	pc	10.59	730	117	V8d	24	17 050 ± 60	107	10.7	D	A	A	289
V62	pc	8.98	496	119	V8a	28	16 960 ± 80	91	6.8	D	A	A	294
V67	ac	12.37	839	118	V8c	25	~17 000	112	12.1	A	—	A	178
V69	ab	9.34	476	117	V8d	22	17 050 ± 60	94	7.6	D	A	A	182
V70	pa	7.33	409	110	V7b	12	16 130 ± 250	74	4.0	D	—	A	165
V71	pa	8.40	2088	130	W10c	26	41 280 ± 1120	85	5.7	A	A	A	244

Mitochondrial haplotypes were read directly from the DNA sequences shown in Fig. 2., fish sizes were estimated from measurements of vertebral centra (Wise 1980), and fish weight was calculated from the fork length–weight regression observed in the extant River Asón salmon population (WT = $10-5 \times 4.253 \times FL^{2.6604}$; $r^2 = 0.827$, $n = 6809$). Vertebrae most likely correspond to six different individuals based on their different radiocarbon dates, different estimated fish size, and/or different three-dimensional location within the excavation site.
*pc, postcranial; ac, anterocaudal; ab, abdominal; pa, postabdominal.
†Haplotype designation follows that of Verspoor *et al.* (1999).

Temporal stability in haplotype frequencies from the River Asón over the last 50 years was examined using the MONTE program in the REAP package (McElroy *et al*. 1991). Historical trends in water temperature, population size and mitochondrial frequencies were examined by the Cochran's test of linear tend, Kruskall–Wallis nonparametric analysis of variance and Spearman rank correlation (Sokal & Rohlf 1995), using angling catches as a proxy for population size and pooling the haplotypes according to the *Hae*III variant site (A or D).

Results

Species identification of Pleistocene vertebrae

The amplification success rate for Pleistocene vertebrae was low (20%), as one would expect from degraded DNA. Only six of the largest vertebrae extracted with the Geneclean kit gave positive results after the second amplification (35 + 15 cycles). The remaining 24 vertebrae gave negative results because of insufficient DNA or the presence of PCR inhibitors, which the DNAce Forensic kit and the Mix-and-Clean method were unable to remove. Vertebrae that did amplify had a greater weight (mean = 840 mg) than those that did not amplify (mean = 429 mg; Mann–Whitney, $P = 0.015$), but they did not differ in age, colour, or degree of evident charring from the other samples (Mann–Whitney, $P > 0.3$).

We succeeded in sequencing three mtDNA fragments totalling 289–294 bp in two vertebrae, and 165–244 bp in each of the four remaining vertebrae (Fig. 2). All the vertebral sequences correspond to Atlantic salmon DNA, but to neither human contamination, nor brown trout or arctic char, the two other western European salmonids with which Atlantic salmon bones might potentially be confused.

Vertebral sequences also differ at the *Rsa*I restriction site from the North American salmon used as a positive control. Attempts to amplify longer fragments (471 bp and 1100 bp) in the same ND1 region proved unsuccessful, except for sample V67, which amplified the 471 bp region with a faint band. Replicated sequences from duplicate amplifications gave identical results, though many bases could not be read, and combined haplotypes (*Hae*III, *Hin*fI, *Rsa*I) could only be obtained for four individuals (Table 1). The vertebrae most likely correspond to six different adults, based on their different radiocarbon dates, different fish size, estimated from measurements of vertebral centra (Wise 1980), and/or different location within the excavation site.

mtDNA analysis

The DAA haplotype detected in three of the Pleistocene Iberian samples (Table 1) is extremely rare, and provides a further argument in support of our contention for authenticity. It was not reported previously in a screening of over 120 European salmon populations (Verspoor *et al* 1999; Nilsson *et al*. 2001), and has only been detected recently in a nearby Spanish river at a marginally low frequency (0.004, Table 2). Another vertebra also shows the 'D' type for *Hae*III (Table 1), consisting of a single base substitution (A → G) with respect to the most common, southern 'A' variant. The remaining two Palaeolithic fish show an 'A' variant for both the *Rsa*I and *Hin*fI regions and include an individual with the AAA composite haplotype, this being the most common haplotype in Iberian salmon populations today. Thus, both the rare DAA and the common AAA types seem to have coexisted in the Iberian peninsula during the last ice age.

Table 2 Frequencies of mtDNA ND1 haplotypes and haplotypic (*h*) and nucleotide (π) diversity (Nei 1987) in modern and ancient Atlantic salmon populations from the Iberian glacial refugium and in other anadromous populations from Europe grouped by region. Known hatchery-reared fish have been excluded from the analysis

River/ region	*n*	mtDNA ND1 haplotype (*Hae*III, *Hinf*I, *Rsa*I)						Pooled D-type	(SD in parentheses)	
		AAA	AAB	DBB	DAB	DAA	SAB		*h*	π
Cantabrian populations (Iberian Peninsula)										
Asón	177	0.764	0.230	0.000	0.006	0.000	0.000	0.006	0.365 (0.035)	0.00091 (0.00009)
Pas	245	0.914	0.033	0.000	0.049	0.004	0.000	0.053	0.162 (0.032)	0.00056 (0.00012)
Nansa	240	0.863	0.117	0.000	0.021	0.000	0.000	0.021	0.250 (0.034)	0.00070 (0.00010)
Deva	166	0.928	0.036	0.012	0.024	0.000	0.000	0.036	0.138 (0.036)	0.00056 (0.00026)
Average Cantabria	*828*	*0.867*	*0.104*	*0.003*	*0.025*	*0.001*	*0.000*	*0.029*	*0.229 (0.034)*	*0.00068 (0.00012)*
Asón-Pleistocene	6	0.250*	0.000	0.000	0.000	0.750*	0.000	0.667†	—	—
European anadromous populations										
Baltic Sea‡	591	0.034	0.000	0.070	0.896	0.000	0.000	0.966	0.107	0.00013
Iceland§	50	0.230	0.000	0.140	0.630	0.000	0.000	0.770	0.528	0.00077
Scandinavia‡	367	0.548	0.120	0.084	0.229	0.000	0.019	0.313	0.480	0.00064
White and Barents Seas‡§	214	0.714	0.134	0.046	0.106	0.000	0.000	0.152	0.282	0.00045
British Isles‡§	150	0.410	0.357	0.147	0.087	0.000	0.000	0.233	0.652	0.00089
South Europe§¶	905	0.805	0.076	0.034	0.085	0.001	0.000	0.120	0.324	0.00072

*$n = 4$.
†$n = 6$.
‡Nilsson *et al.* (2001).
§Verspoor *et al.* (1999).
¶Including four extant Cantabrian populations listed above.

Relationships between populations

The analysis of modern and ancient mtDNA ND1 frequencies confirms the existence of two distinctive phylogenetic groups amongst anadromous salmon populations in Europe (Verspoor *et al.* 1999; Nilsson *et al.* 2001): one encompassing the Baltic and Icelandic populations, and the other encompassing most of the Atlantic populations (Fig. 3). We obtained identical topologies regardless of the genetic distance method used (D_A or chord distance; not shown). The Baltic group has significantly lower haplotypic (*h*) and nucleotide (π) diversities than the Atlantic group (Mann–Whitney, $P = 0.012$ and $P = 0.002$, respectively), as well as a higher frequency of the *Hae*III D-type (Mann–Whitney, $P < 0.001$). Modern Iberian populations (rivers Asón, Pas, Nansa, Deva and Narcea) fall within the Atlantic cluster and generally group together, along with other 'open-sea' populations from southern Europe, the British Isles, Scandinavia and the arctic regions of Russia. On the other hand, although the high frequency of the *Hae*III D-type observed in the ancient vertebrae (0.667) appears more characteristic of Baltic populations (0.909 ± 0.037) than of Atlantic ones (0.174 ± 0.037), the Pleistocene

Iberian sample is located at the root of the Atlantic lineage (Fig. 3).

Comparisons between Palaeolithic and extant Cantabrian salmon populations suggest that Iberian salmon may have undergone significant genetic changes since the last ice age. The frequency of the D-variant observed in the ancient River Asón stock (0.667) is significantly greater (Fisher's exact test $P < 0.001$) than in modern populations (River Asón = 0.006; Cantabria = 0.029), but more similar to frequencies still observed in the Baltic region (Table 2). Resampling analysis designed to reduce the effect of stochastic sampling errors produced identical results, and indicates that — bearing in mind the small Pleistocene sample size — ancient and modern Cantabrian populations probably differ significantly (exact test on repeated samples of six individuals $P = 0.000$).

Relationships between haplotypes

Maximum parsimony phylogenetic analysis (Fig. 4a) suggests that the Atlantic haplotypes (AAA, AAB, DAA) diverged before the Baltic ones (DAB, DBB). Thus, the most parsimonious tree places the ancient AAA haplotype
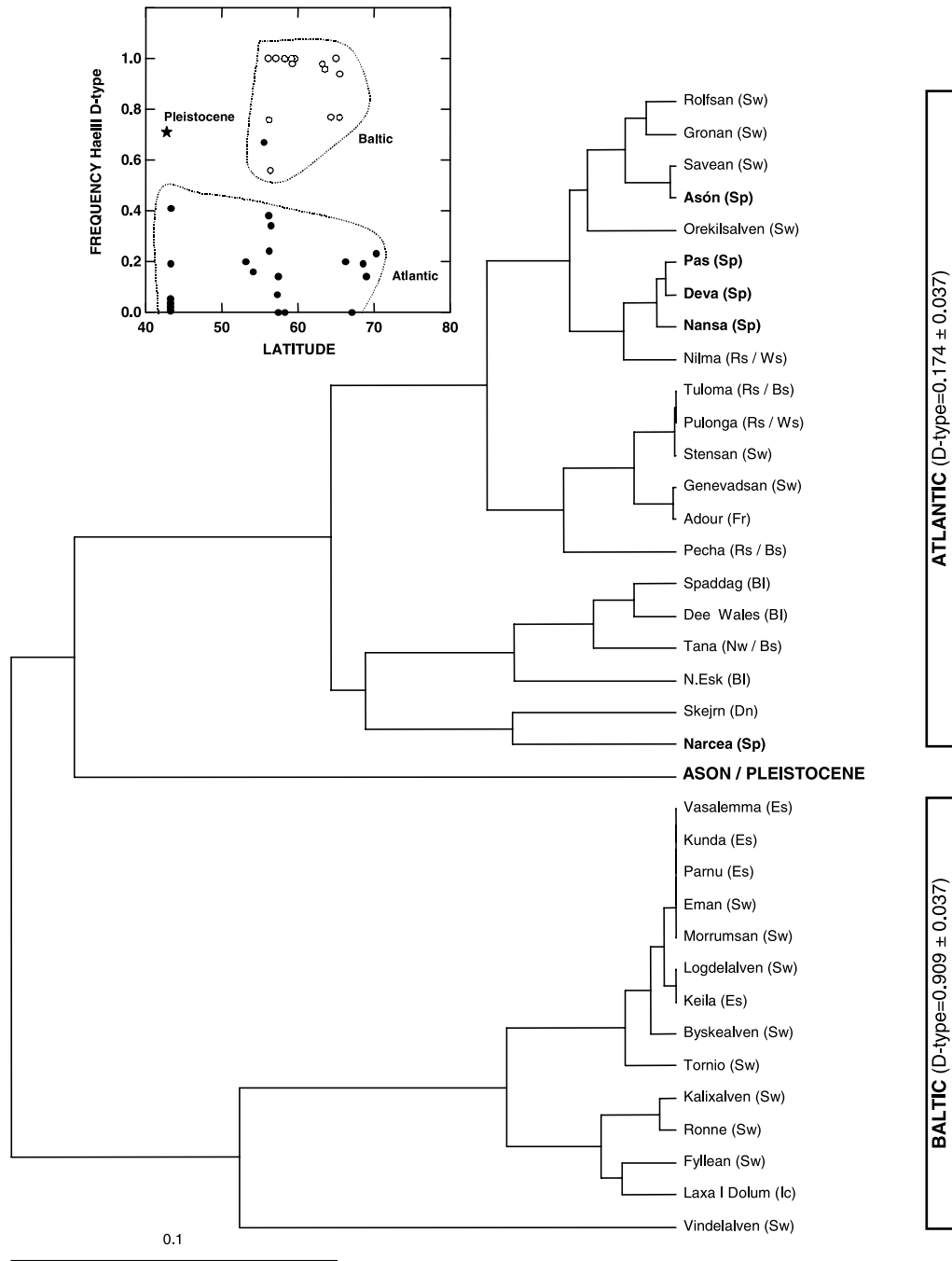
Fig. 3 UPGMA dendrogram based on Nei's D_A distances showing clustering of ancient (Asón-Pleistocene) and modern Cantabrian populations from the Iberian glacial refugium (Asón, Pas, Nansa, Deva), and other anadromous Atlantic salmon populations from Europe (Verspoor *et al.* 1999; Nilsson *et al.* 2001). Insert shows relationship between latitude and frequency of the *Hae*III D-type. Country abbreviations are as follows: Sweden (Sw), Spain (Sp), Russia-White Sea (Rs/Ws), Russia-Barents Sea (Rs/Bs), France (Fr), Denmark (Dn), British Isles (BI), Norway-Barents Sea (Nw/Bs), Iceland (Ic), Estonia (Es).

common in the Iberian Peninsula at the first branching node, followed by the AAB type and the other haplotype with a known Pleistocene ancestry (DAA). On the other hand, the Scandinavian SAB type and the two Baltic types (DBB and DAB) appear to have diverged more recently, although the increasingly low bootstrap values make the relative divergence of the Baltic types less clear. Network analysis (Fig. 4b) seems to confirm the early divergence of Atlantic haplotypes (AAA and AAB), but is unable to resolve their ancestry. Thus, while brown trout is most likely connected to the AAA type, North American salmon appears most closely related to the AAB type. Both
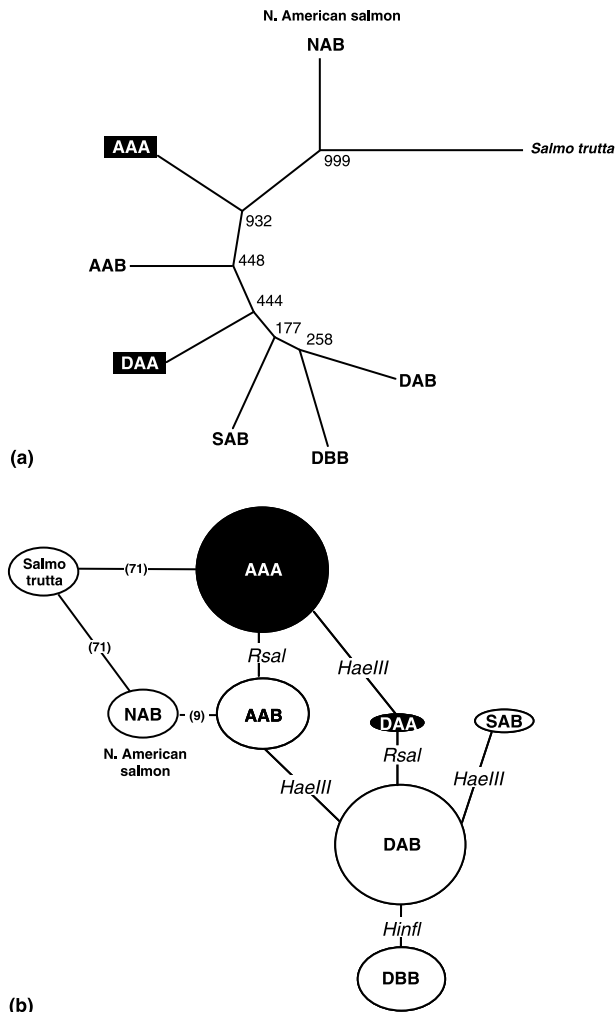
Fig. 4 Reconstructed phylogeny of Atlantic salmon mtDNA ND1 haplotypes in Europe estimated by (a) majority rule radial consensus tree for maximum parsimony and (b) minimum spanning network. The figures at each node denote the number of bootstraps (*n* = 1000) that originated each group. Shapes in the network are proportional to the number of European populations that share that haplotype. The two haplotypes detected in Pleistocene salmon vertebrae from the Iberian Peninsula are shown in black. Restriction site mutations between European haplotypes, as well as distances from outgroups (number of base substitutions), are also indicated.

haplotypes are in turn connected by one-step substitutions with the DAA and DAB types, forming a network loop. The same results are obtained regardless of the network method employed (minimum spanning or statistical parsimony; not shown). Spatial analysis (Fig. 5a,b) indicates that the most widely distributed of the common haplotypes (and also the most southerly one) is the AAA type that dominates within the Atlantic lineage, and which we know predates the last glacial maximum. Furthermore, significant negative correlations exist between frequencies of the DAB haplotype, which predominates in the Baltic Sea, and the
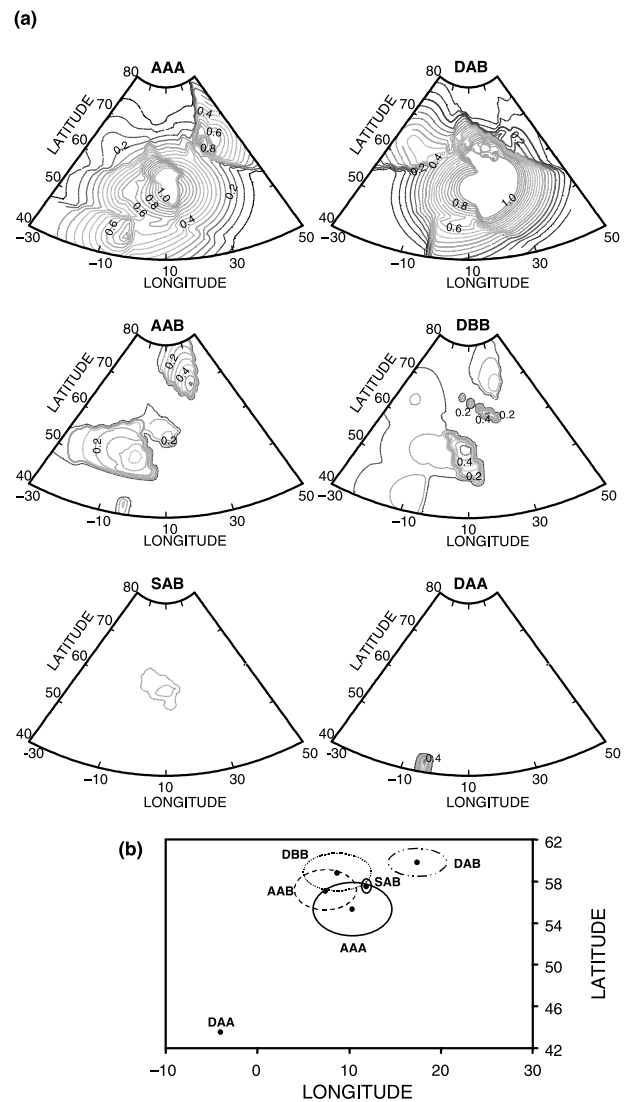
Fig. 5 Spatial distribution of Atlantic salmon mtDNA ND1 haplotypes examined by (a) kriging smoothing of haplotypic frequencies and (b) 95% confidence geographical centroids (latitude/longitude) weighed by sample frequencies. Numbers at each contour represent interpolated haplotype frequencies.

frequencies of both the AAA (*r* = −0.813, *P* = 0.000) and AAB (*r* = −0.607, *P* = 0.002) haplotypes, that predominate in the Atlantic. This suggests a different evolutionary origin from separate refugia for Atlantic and Baltic lineages. A significant south to north decrease in both the number of haplotypes and in nucleotide diversity can be observed (Fig. 6a,b), although the highest diversity corresponds to the centre of the distribution located around the British Isles.

Temporal changes in Iberian populations

Analysis of historical scales from adults returning to the River Asón indicates that there has been a significant
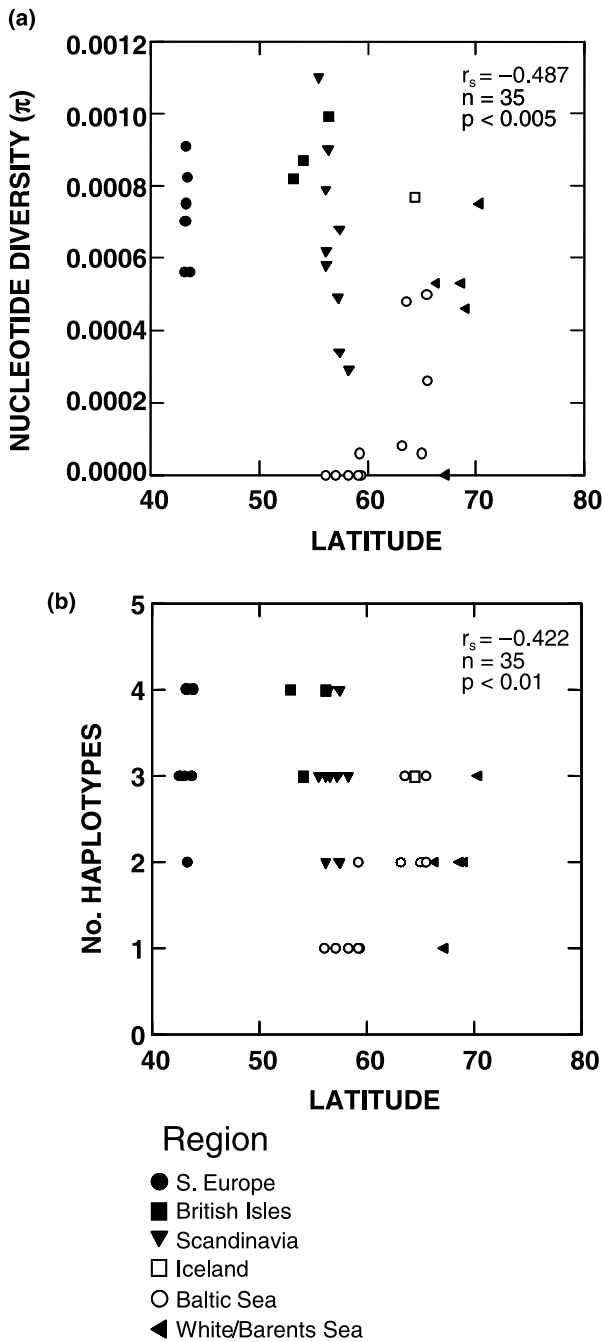
(a)

(b)

Region

- ● S. Europe
- ■ British Isles
- ▼ Scandinavia
- □ Iceland
- ○ Baltic Sea
- ◀ White/Barents Sea

Fig. 6 Relationship between river latitude and (a) nucleotide diversity (π) and (b) number of mtDNA ND1 haplotypes grouped by region.

change in haplotype composition during the last 50 years (Table 3; $\chi^2 = 35.89$, $P < 0.001$). The frequency of the D-type was already low 50 years ago, and may have continued to decline (Cochran's test of linear trend, $P = 0.066$), although the reasons for this are not clear. Average water temperature in the River Asón increased by 2.0 °C during the period AD 1950–2000, close to the estimated global warming rate of 0.5 °C/decade (McCarthy & Houlihan 1997),

whereas population size, as inferred from angling catches, decreased by 92%. Both trends are highly significant (water temperature: Kruskall–Wallis = 16.213, $n = 523$, d.f. = 4, $P = 0.003$; angling catches: Kruskall–Wallis = 36.542, $n = 50$, d.f. = 4, $P < 0.001$) and indicate that there have been changes both in the river environment and in population structure. A significant negative association may also exist between water temperature and the frequency of the D-type since the last glaciation ($r_s = -1.00$, $n = 5$, $P < 0.05$), if we assume that water temperature for northern Spain during the last glacial maximum was at least 5 °C lower than today (Ellwood *et al*. 2001).

Discussion

The poor amplification success rate, low DNA yield, and failure to amplify fragments of increasing size, characteristics typical of degraded DNA (Hagelberg 1994; Handt *et al*. 1994), strongly suggest that the replicated ancient salmon sequences are genuine. Contamination of vertebrae with modern salmon DNA seems unlikely; most vertebrae gave no results, there was a lack of amplification in negative controls carried out in a salmon-free laboratory, and one of the haplotypes revealed is uncharacteristic of modern salmon populations. Instead, it seems that large adult salmon, lightly cooked by humans from the Magdalenian period possibly on hot stone slabs (Cleyet-Merle 1990), dried rapidly, and some vertebrae preserved enough bone collagen at El Mirón Cave for several thousand years.

The vertebrae analysed, although severely limited in number, provide the oldest fish sequences reported to date, and demonstrate the feasibility of genetically identifying prehistoric fish to species at archaeological sites. They also demonstrate the presence of Atlantic salmon in the River Asón (Cantabria, northern Spain) during the last 40 000 years, and confirm the Iberian Peninsula as an ice age refuge for the species during the Pleistocene last glacial maximum.

The DAA haplotype detected in the Pleistocene salmon from the Iberian Peninsula constitutes a previously unreported ancient type, seemingly on the decline in modern Iberian populations and absent from the rest of Europe. Thus, while serving to confirm the ancient provenance of the vertebral DNA, our results justify a reassessment of current models on the origin and distribution of Atlantic salmon mtDNA variation across Europe (Verspoor *et al*. 1999; Nilsson *et al*. 2001). First, our extension of the data strengthens support for the division of European populations into two main lineages — Baltic and Atlantic (Verspoor *et al*. 1999; Nilsson *et al*. 2001) — but indicates that such division is attributed largely to a single base substitution at the ND1 *Hae*III restriction site. Second, when ancient and contemporary Iberian populations are included in the analysis,

Table 3 Ancestral, historical and contemporary mitochondrial ND1 haplotype frequencies in the River Asón (Cantabria, northern Spain)

		mtDNA ND1 haplotype (*Hae*III, *Hin*fI, *Rsa*I)						Pooled
Period	*n*	AAA	AAB	DBB	DAB	DAA	SAB	D-type
Pleistocene	6	0.250*	0.000	0.000	0.000	0.750*	0.000	0.667
1950s	25	0.960	0.000	0.040	0.000	0.000	0.000	0.040
1960s	36	0.972	0.000	0.000	0.028	0.000	0.000	0.028
1980s	60	0.767	0.217	0.000	0.017	0.000	0.000	0.017
1990s	177	0.764	0.230	0.000	0.006	0.000	0.000	0.006

*$n = 4$.

a greater degree of discrimination between lineages becomes apparent. The Pleistocene Iberian sample appears at the root of the Atlantic lineage, as one would expect if Atlantic rivers had been recolonized from the Iberian glacial refugium. Although this contention is based on a limited Pleistocene sample size, the inverse relationships detected between latitude and both the number of haplotypes and nucleotide diversity strengthen support for colonization from southern glacial refugia (Bernatchez & Wilson 1998; Smith *et al*. 2001). However, despite this latitudinal trend, the highest nucleotide diversity is currently found in the centrally located British Isles, which may have acted as a contact zone for colonizers from multiple refugia. Alternatively, the higher diversity found in the British Islands may simply reflect the declining status of many southern European populations (Parrish *et al*. 1998).

The ancient provenance of the Iberian Pleistocene haplotypes (DAA, AAA) seems at variance with the view that the four common European haplotypes were derived from the AAB type, as suggested in previous studies (Verspoor *et al*. 1999; Nilsson *et al*. 2001). Results from phylogenetic, network and spatial analysis show that there is considerable uncertainty in the phylogeny of Atlantic salmon mitochondrial haplotypes in Europe, but consistently point to the Iberian Peninsula as the most likely origin for the AAA haplotype that dominates within the Atlantic lineage. From the Iberian Peninsula, the AAA type (but apparently not the DAA) would have radiated northwards with the retreat of the ice-sheet and the re-establishment of the North Atlantic Drift, some 11 500 years ago (Adams *et al*. 1999; Lynch-Stieglitz *et al*. 1999). Such 'southern' variants would have first colonized the British Isles, and then spread into Iceland, Scandinavia and the White and Barents Seas, eventually entering the Baltic. Similar Atlantic colonization patterns originating from an Iberian glacial refugium have been proposed for several other species (Hewitt 1999), including the brown trout (García-Marín *et al*. 1999).

In our view, the appearance of the AAB type within the Atlantic lineage could have resulted from a second, more recent mutation of the AAA type at the *Rsa*I restriction site. Like the AAA variant before, the AAB mitochondrial type would have colonized Iceland, Scandinavia, and the arctic coast of Russia, perhaps during a second, more recent, expansion from the British Isles (where the AAB haplotype is currently centred). Historical data from the River Asón (Table 3) suggest that the marginal incidence of the AAB type in southern Europe (Table 2) may constitute a recent event, possibly resulting from the stocking of foreign hatchery fish of British and northern European origin over the last 20 years (García de Leániz *et al*. 1989; Verspoor & García de Leániz 1997).

While our data demonstrate the existence of an Iberian glacial refugium for Atlantic salmon, and provide some support for a southern origin of the most common Atlantic mitochondrial type (AAA), the origin and postglacial distribution of the Baltic types (DAB and DBB) remain obscure. Nilsson *et al*. (2001) have argued strongly against an Atlantic origin of the Baltic salmon as suggested by Verspoor *et al*. (1999), invoking instead the existence of an 'eastern' glacial refugium. Our results suggest that the Baltic haplotypes (DAB, DBB) constitute the most recent ND1 mutations, and that Baltic salmon probably originated from separate refugia, either in the North Sea as suggested by Verspoor *et al*. (1999) or in the eastern glacial lakes as suggested by Nilsson *et al*. (2001). Whatever its precise origin, our study clearly indicates that the frequency and distribution of contemporary haplotypes may not necessarily reflect those of refugial populations. For example, the Iberian DAA type — which predates the last glacial maximum — may have also existed in more northern refugia, giving rise to the DAB and DBB Baltic types through two-step substitutions.

The apparent genetic change detected amongst the Iberian salmon population from the late Pleistocene to the present day could have been the result of genetic drift (as suggested by the observed decrease in population size), or by selection (as suggested by the observed inverse correlation with water temperature). The small number of ancient Pleistocene samples precludes more definite conclusions at this stage. Whatever the reasons for such a change, our results suggest that a common haplotype that persisted in the Iberian Peninsula during the Pleistocene last glacial maximum is now extremely rare or absent from rivers in

the region. This highlights the need for caution when using the phylogeographic approach (Avise *et al.* 1987), and questions whether modern populations in refugial areas are representative of ice age populations. The former is an assumption implicit in studies using modern haplotype distributions to infer patterns of postglacial recolonization (Taberlet *et al.* 1998; Nilsson *et al.* 2001) and the latter is an issue relevant to the potential impact of past and future climatic change (Minns *et al.* 1992; McCarthy & Houlihan 1997) and the development of conservation plans for Atlantic salmon (Dodson *et al.* 1998). More extensive genetic characterization of the Pleistocene fish remains found at archaeological sites should help to address these issues.

Acknowledgements

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