

Evolution of the mitochondrial control region in Palaeartic brown trout (*Salmo trutta*) populations: the biogeographical role of the Iberian Peninsula

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In order to extend present knowledge of brown trout phylogeography in the Palaeartic, we analysed the complete mitochondrial D-loop sequence (1025–1027 bp) of all mitochondrial haplotypes of *Salmo trutta* found in the Iberian Peninsula and one North African haplotype. Sequence variation in the mitochondrial control region serves to identify four major haplotype groups within the Iberian Peninsula, i.e. Atlantic, Duero, Mediterranean and Andalusian. Including the Iberian haplotypes, the five main European groups previously established were increased to six: (i) an Atlantic group including two different clusters, South European and North Atlantic; (ii) a group representing an endemism restricted to the Duero basin in the Iberian Peninsula; (iii) an Adriatic–Andalusian group found in two vicariant areas including Adriatic–Ionian populations in the Mediterranean and the Andalusian basins of the southern Iberian Peninsula; (iv) a Mediterranean group with a distribution range that extends from the southwestern basins of the Iberian Peninsula to the Ionian basins of the Greek Peninsula; (v) a Danube group of wide distribution in the Black, Aral and Caspian basins; and (vi) a group comprising the *S. t. marmoratus* subspecies confined to the Adriatic Sea. The Iberian Peninsula appears to have acted as a physical boundary between haplotypes corresponding to Atlantic- and Mediterranean-draining rivers. Owing to its geographical position, this area has played a major role in present Palaeartic species distribution, as illustrated by its haplotype diversity.

Keywords: biogeography, D-loop, mtDNA, *Salmo trutta*, Salmonidae.

Introduction

Salmo trutta is one of the most important river fish species in Europe, in social, ecological and economic terms. It is distributed widely across Europe and is present in North Africa and western Asia, constituting a complex mosaic of phenetic variation (Behnke, 1972, 1986). The local genetic structure of brown trout has been investigated throughout Europe (Ferguson, 1989; Hamilton *et al.*, 1989; García-Marín *et al.*, 1999). Electrophoretic studies have defined ‘modern’ (North Atlantic) and ‘ancient’ (South Atlantic and Mediterranean) lineages based on the presence of the *LDH-C*90* and *LDH-C*100* alleles, respectively (Hamilton *et al.*, 1989; García-Marín *et al.*, 1999). Through mitochond-

rial analysis, five phylogenetic assemblages have been identified in Europe i.e. Adriatic, Danube, Mediterranean, *marmoratus* and Atlantic (Bernatchez *et al.*, 1992).

It would appear that the geographical position of the Iberian Peninsula is crucial for brown trout distribution. Its rivers drain both into the Atlantic Ocean and the Mediterranean Sea, and some authors claim that the peninsula acted as a refuge during the last glaciations (García-Marín *et al.*, 1999). Protein electrophoresis performed on Spanish brown trout populations suggests the existence of two distinct lineages associated with Atlantic and Mediterranean drainages (García-Marín & Pla, 1996). This has been confirmed recently using mitochondrial RFLPs by establishing five distinct groups, i.e. the Mediterranean, Andalusian, Atlantic, Duero and Cantabrian haplotypes (Machordom *et al.*,

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2000). However, this distribution pattern has not yet been related to that of other European areas.

The mitochondrial genome has been used widely to investigate phylogenetic relationships. As a consequence of its well-established organization, this genome has been sequenced in many organisms, including *Salmo salar* in the same genus as brown trout. With very few exceptions, the mitochondrial genome has a maternal mode of inheritance and has a mutation rate up to 10 times higher than nuclear single copy coding genes (Meyer, 1993; Avise, 2000). The mitochondrial control region, also called the displacement loop, or D-loop, is located between the genes coding for proline and phenylalanine tRNAs in vertebrates. It is responsible for the transcription of mitochondrial genes and contains the point of origin of heavy strand replication. Several regions of the mitochondrial D-loop show a high degree of variation between populations of the same species, but D-loop variability is not uniform and the region shows several well-conserved sequences among vertebrate species (Brown *et al.*, 1986; Sbisà *et al.*, 1997). The mitochondrial D-loop was selected as the molecular marker for the present study because it has been described as the most variable region of the mitochondrial molecule in terms of substitution rate and length variation (Bentzen *et al.*, 1988; Hoelzel *et al.*, 1991; Meyer, 1993; Cecconi *et al.*, 1995). Furthermore, ample D-loop variability has been reported for brown trout throughout Europe (Bernatchez *et al.*, 1992; Giuffra *et al.*, 1994; Bernatchez & Osinov, 1995; Osinov & Bernatchez, 1996; Apostolidis *et al.*, 1997; Weiss *et al.*, 2000).

The aim of the present study was to analyse the variability of the *S. trutta* mitochondrial D-loop region and to perform a phylogenetic reconstruction of the populations of the Iberian Peninsula. Data from previous genetic studies on brown trout populations are reviewed in the light of present findings to infer phylogenetic relationships among European populations and complete the Palaearctic scenario of *S. trutta* evolution. Based on all of these data, a biogeographical hypothesis for the present brown trout distribution is presented.

Materials and methods

D-loop sequences and specimens

The complete DNA sequence of the D-loop region of 19 brown trout specimens was established (Table 1). Specimens represented all the haplotype groups present in the Iberian Peninsula as identified by RFLPs of a 2.7 kb mitochondrial DNA fragment, that comprised the genes NADH5 to Cyt-*b* (Machordom *et al.*, 2000) plus one haplotype found thereafter (NA6). In most cases, each RFLP haplotype was represented by at

Table 1 Iberian Peninsula specimens analysed. The RFLP pattern and clusters correspond to Machordom *et al.* (2000)

Sample	RFLP pattern	Haplotype distribution	Cluster
NA1	BAAAA	North Atlantic (Foreign)	4
NA2	AAAAA	North Atlantic (Foreign)	4
NA3	AAAAA	North Atlantic (Foreign)	4
NA4	AAAEA	North Atlantic (Foreign)	4
NA5	AAAAA	North Atlantic (Cantabrian)	4
NA6	AEAAA	North Atlantic (Cantabrian)	4
SA1	ABACC	South-European Atlantic	2
SA2	ABACC	South-European Atlantic	2
SA3	BBACA	South-European Atlantic	2
SA4	ABACA	South-European Atlantic	2
SA5	ABACA	Atlantic (Morocco)	2
DU1	BBABB	Duero	1
DU2	BBABB	Duero	1
ME1	BBBDA	Mediterranean	3
ME2	BBBDA	Mediterranean	3
ME3	BBBDA	Mediterranean	3
ME4	CBBDA	Mediterranean	3
AN1	BBCDA	Andalusian	3
AN2	BBCDA	Andalusian	3

least two specimens. Rare or unique RFLP haplotypes were each represented by one D-loop sequence (NA1, NA4, NA6, SA4 and ME4) (See Table 1). In addition to the 19 Iberian sequences analysed here, phylogenetic analysis of the European haplotypes was based on partial D-loop sequences retrieved from the GenBank database. These fragments corresponded to the Phe-tRNA and Pro-tRNA D-loop ends, which resulted in a 621-bp fragment of all the European brown trout haplotypes (GenBank accession numbers M97962-M97985) and one *S. salar* haplotype (GenBank accession numbers M97986, M97987). In all the complete and partial D-loop phylogenetic analyses, *Oncorhynchus mykiss* and *S. salar* (GenBank accession numbers L29771 and U12143, respectively) were used as outgroups.

Structural characterization of the *S. trutta* mitochondrial control region was achieved by aligning 18 complete sequences. These corresponded to the *S. trutta* consensus sequence of the present study, one human (J01415) and 16 teleost sequences (U12067, U12556, NC_002079, AF119321, AF140602, NC_001727, U12059, AF032381, AF06278, NC_001717, NC_002386, U12143, NC_000860). Complete teleost D-loop sequences corresponding to most teleost families were obtained from the GenBank database. Four Salmonidae sequences were similarly retrieved and aligned to identify hypervariable regions.

DNA extraction, amplification and sequencing

Total DNA extraction of frozen and alcohol-preserved muscle and liver tissues was conducted using standard phenol/chloroform protocols (Sambrook *et al.*, 1989). Amplification of the brown trout mitochondrial control region was performed using the primers LN20 (5'-ACCACTAGCACCCAAAGCTA-3') and HN20 (5'-GTGTTATGCTTTAGTTAAGC-3') located in the proline and phenylalanine tRNA genes, respectively (Bernatchez & Danzmann, 1993); 50–150 ng of DNA were used to amplify the entire control region (~1 kb). PCR amplification was performed according to standard procedures (1.25 U BIOTOOLS *Taq*-DNA polymerase, 2 mM MgCl₂, 20 pmol of each primer and 250 µM dNTPs) for 40 cycles (92°C, 60 s; 50°C, 60 s; 72°C 90 s). Cycling was preceded by a 1-min denaturing step at 95°C and followed by a 10-min final extension at 72°C. Direct sequencing of both forward and reverse strands was performed for each amplified fragment in an ABI 377 automated system. Sequences were deposited in the GenBank database under the accession numbers AF253541–AF253559.

Mitochondrial DNA analysis

Sequences were aligned using CLUSTALX v1.5b software (Thompson *et al.*, 1994). Conserved regions were visualized using the GENEDOC v2.3.0 program (Nicholas & Nicholas, 1997). Once the conserved regions had been identified, variable regions were realigned.

Phylogenetic hypotheses were established by minimum evolution (neighbour joining), maximum parsimony

and maximum likelihood approaches. All phylogenetic analyses were performed using PAUP 4.0b3 (Swofford, 2000) including full heuristic searches by tree bisection reconnection (TBR) branch-swapping. *Salmo salar* and *O. mykiss* were run as multiple outgroups. Saturation tests were conducted to assess the reliability of the sequence analysis through all substitution types, and transitions and transversions alone. Likelihood ratio tests performed with MODELTEST 3.0 (Posada & Crandall, 1998), indicated model HKY85 with a gamma distribution and invariable sites as the model that best fitted the data. Nevertheless, the low number of nucleotide substitutions, and the topologies proposed by different variations of the HKY85 model, suggested the use of this model without the variables of rate variation across sites, proportion of invariable sites or gamma parameters, in the phylogeny estimation of brown trout for both distance and maximum likelihood analyses. This model is consistent with the brown trout D-loop sequence and nucleotide substitutions, i.e. sequences with nucleotide frequency bias and two substitution types (transitions vs. transversions). The transition:transversion ratio was estimated by maximum likelihood. Different weighting matrices (unweighted, weighting ts/tv, nucleotide frequencies and combined weighting) were used in the parsimony analyses, yielding similar results. Thus, the trees presented are those corresponding to the simplest, most parsimonious model. Incongruence-length difference tests (Farris *et al.*, 1995) were performed to evaluate whether variable regions and distinct conserved regions resolved the same relationships. Three data partitions were tested (See Fig. 1): (i) The three domains of the mitochondrial

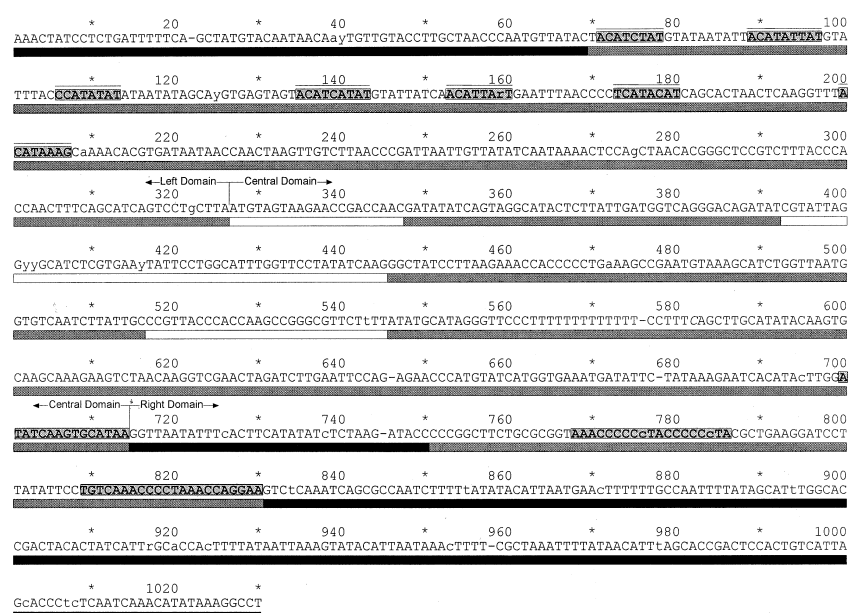


Fig. 1 Mitochondrial D-loop structure of *Salmo trutta*. Black/grey/white boxes below the sequences indicate hypervariable/variable/conserved regions, respectively. The continuous line above the D-loop sequence highlights the TAS elements. The boxes within the sequence indicate CSBs.

control region; (ii) termination associated sequences (TAS), conserved sequence blocks (CSBs) and undefined sequences; and (iii) biologically meaningful elements vs. undefined sequences. Topologies were assessed by bootstrapping with 100 replicates in the distance and parsimony analyses, and by quartet puzzling steps (Strimmer & von Haeseler, 1996) with 1000 replicates in the maximum likelihood approach.

Results

Characterization of the D-loop region

The 19 Iberian D-loop sequences ranged in size from 1025 to 1027 bp. Length variation was produced by single base-pair indels ($n=6$). Among the Iberian haplotypes, the maximum number of substitutions seen corresponded to 16 nucleotide changes between a South European Atlantic and an Andalusian haplotype (Table 2). Base composition in this region (63% A + T) corresponded to that of a typical A + T-rich region (Cecconi *et al.*, 1995). Sequence change resulted predominantly from transitions rather than transversions, with a transition:transversion ratio of 2.39. Nucleotide substitutions occurred at 15 parsimony-informative characters and 16 autapomorphies. No repeated regions were detected in the *S. trutta* D-loop sequence.

Alignment of the *S. trutta* consensus sequence with teleost and human D-loop sequences revealed the general structure found in most vertebrates. The D-loop region was divided into three domains according to the amount of variability: (i) a left domain (Pro tRNA gene end); (ii) a right domain (Phe tRNA gene end); and (iii) a central domain (Fig. 1). TAS elements were found within the right domain and CSBs were found within central and left domains. The results of the incongruence-length difference tests of conserved features vs. variable regions were not significant for the three defined partitions (mitochondrial D-loop domains $P=0.37$, each separate element $P=0.8$, and biologically meaningful elements vs. undefined sequences $P=1$). Although the incongruence-length difference test seems to be conservative with the null hypothesis of homogeneity of partitions (Cunningham, 1997), the P -values obtained for the three defined partitions are far from the significance threshold of 0.05.

Phylogenetic relationships among brown trout

Pairwise comparison of the D-loop sequence of the specimens analysed served to identify two identical South European Atlantic specimens (SA1 and SA2). Three of the Mediterranean sequences (ME2, ME3 and ME4) were also identical, while one presented a unique

Table 2 Number of parsimony-informative characters between D-loop sequence haplotypes and correspondence with the RFLP haplotypes of Machordom *et al.* (2000)

	NA1 = NA2	NA3	NA4	NA5	NA6	SA1 = SA2	SA3	SA4	SA5	DU1	DU2	ME1	ME2 = ME3 = ME4	AN1 = AN2	S. <i>salar</i>
BAAAA/AAAAA															
AAAAA	1														
AAAAE	2	1													
AAAEA	2	1													
AAAAA	2	1	2												
AEAAA	2	1	2	2											
ABACC	4	3	2	2	4										
BBACA	5	4	5	3	5	3									
ABACA	3	4	5	3	5	3	4								
ABACA	6	7	6	6	8	4	7	5							
BBABB	4	3	4	4	4	6	7	7	10						
BBABB	5	4	5	5	5	7	8	8	9	1					
BBBDA	9	8	9	9	9	11	12	10	13	9	8				
BBBDA/CBBDA	10	9	10	8	10	10	11	9	12	10	9	1			
BBCDA	7	6	7	7	7	9	8	10	11	7	6	4			
EDEGE	32	31	32	32	31	34	32	33	34	30	29	30	5	29	
DCDFD	34	33	32	34	33	34	36	35	32	33	32	31	32	32	7

mutation (ME1). All of the remaining sequences, i.e. the North Atlantic (NA1-NA6), Duero (DU1 and DU2), and Andalusian (AN1 and AN2) showed unique substitutions. Estimates of pairwise sequence divergence varied from 0 to 1.58% (mean $\pm$ SD, 0.74% $\pm$ 0.36).

Phylogenetic trees constructed through parsimony, minimum evolution and maximum likelihood analyses were of almost identical topology (Fig. 2). The 19 Iberian *S. trutta* sequences clustered into four monophyletic groups, i.e. Mediterranean, Andalusian, Atlantic and Duero. Character-based analysis resolved a total of 42 equally parsimonious trees (215 steps) in which all differences were shown within the Atlantic group and most within the South European Atlantic cluster.

Distance, parsimony and maximum likelihood analysis of the European D-loop sequences and the sequences presented in this study resolved the same six groups, although the relationships among them could not be fully resolved (Fig. 3). These groups were: (i) Mediterranean; (ii) Adriatic; (iii) Danube; (iv) Atlantic; (v) *marmoratus*; and (vi) Duero. The Mediterranean and Atlantic Iberian haplotypes were included in the Mediterranean and Atlantic European groupings, respectively. Andalusian haplotypes were included in the Adriatic group. Neither *marmoratus* nor Danube haplotypes were found within the Iberian Peninsula.

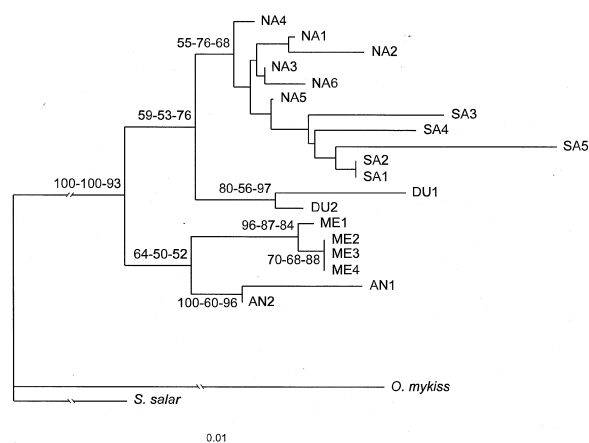


Fig. 2 Neighbour-joining tree based on the complete mitochondrial D-loop sequence of Iberian Peninsula *Salmo trutta* under the HKY85 model of evolution (transition:transversion ratio: 2.39). Numbers on branches represent the bootstrap values obtained for 100 replications corresponding to minimum evolution and maximum parsimony, and puzzling steps for 1000 replications corresponding to the maximum likelihood method. Support values under 50% are not represented. Abbreviations correspond to NA, North Atlantic; SA, South Atlantic; DU, Duero; ME, Mediterranean; AN, Andalusian.

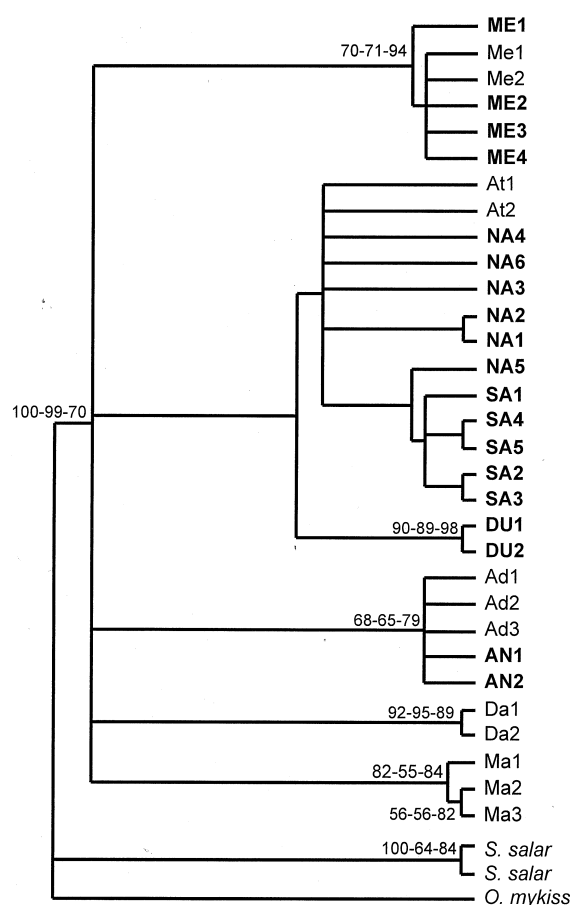


Fig. 3 Maximum parsimony tree based on partial mitochondrial D-loop sequences of European *Salmo trutta*. Numbers on branches represent the bootstrap values obtained for 100 replications corresponding to maximum parsimony and minimum evolution, and puzzling steps for 1000 replications corresponding to the maximum likelihood method. Support values under 50% are not represented. Bold abbreviations as in Fig. 2. Plain text abbreviations represent the European mt D-loop haplotypes described; Bernatchez *et al.* (1992): Me, Mediterranean; At, Atlantic; Ad Adriatic; Da, Danube; Ma, *marmoratus*.

Brown trout biogeography

All of the phylogenetic groups showed a good correlation with geographical distribution (Fig. 4). North Atlantic haplotypes were found from the North Spanish coast to the White Sea. The Duero haplotypes showed a highly restricted distribution area confined to the Duero river, with few populations found in the Tajo. South European Atlantic haplotypes were identified in trout from the Atlantic-draining rivers of the Iberian Peninsula and also in the African specimen. This haplotype showed some overlap with the distribution pattern of the Duero haplotypes in the Duero basin, and with the North Atlantic haplotypes of populations from the

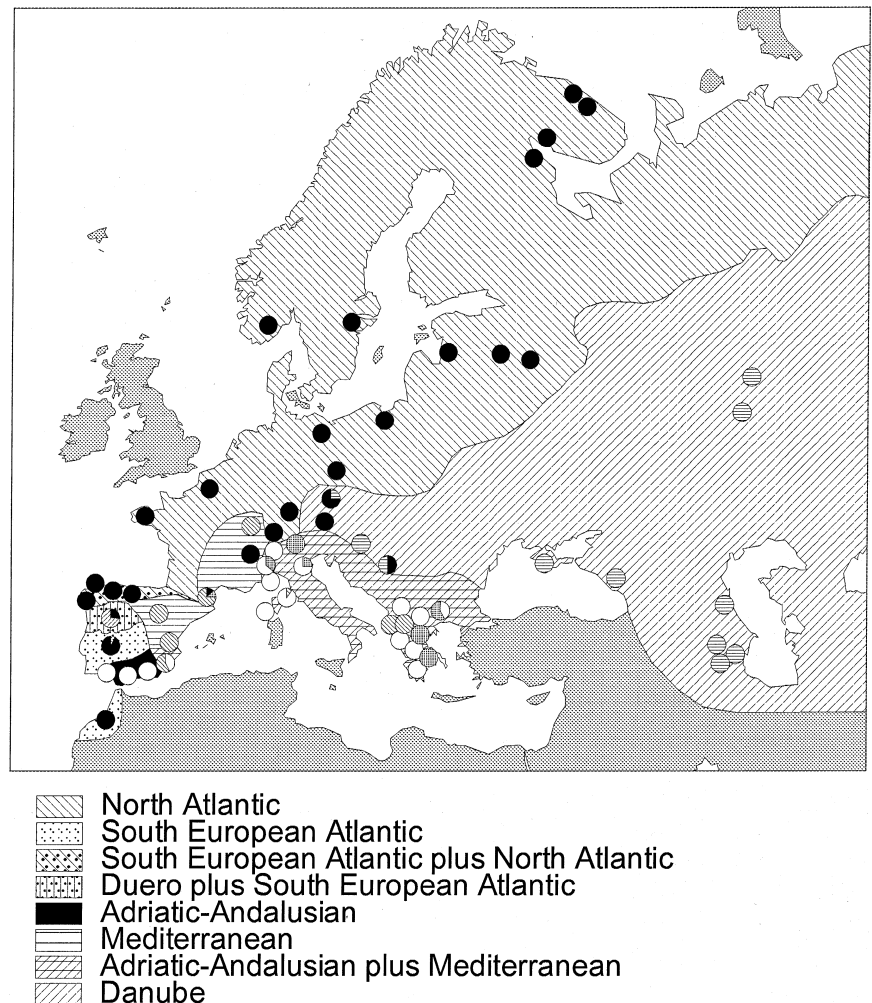


Fig. 4 Model of the biogeographical areas across Europe as indicated by mitochondrial D-loop haplotypes. Dots indicate relative frequencies of each haplotype group: Atlantic (●), Duero (⊙), Mediterranean (⊙), Adriatic – Andalusian (○), *Salmo trutta marmoratus* (⊙) and Danube (⊙).

Cantabrian Sea. Adriatic and Andalusian haplotypes, which constitute a phylogenetic cluster, were identified in specimens from two distinct areas, the Adriatic-Ionian region (Bernatchez *et al.*, 1992; Giuffra *et al.*, 1994; Apostolidis *et al.*, 1997) and the southern Spanish basins of the present study. Mediterranean specimens showed a distribution range from the oriental basins of Spain to the Greek basins of the Adriatic Sea (Bernatchez *et al.*, 1992; Giuffra *et al.*, 1994; Apostolidis *et al.*, 1997). Most of the area boundaries showed haplotype diversity forming contact zones between the different haplotype groups.

Discussion

Phylogenetic relationships among brown trout

The four major haplotype groups found in the Iberian Peninsula (Atlantic, Duero, Mediterranean and Andalusian) were well supported. Although both North and South European Atlantic haplotypes showed low

bootstrap values, the South European Atlantic grouping was resolved as monophyletic in maximum parsimony, minimum evolution and maximum likelihood analyses. This is consistent with previous findings derived from RFLP analysis of the 2.7-kb fragment of mitochondrial DNA (Machordom *et al.*, 2000), in which both groups were also identified as monophyletic. The low sequence divergence within this cluster (mean 0.61%) and the high number of sequences analysed (11) would explain the low bootstrap values of the node separating North and South European Atlantic haplotypes. The topologies presented here show great similarity (Fig. 2) to the phylogeny of Iberian Peninsula populations based on RFLP analysis (Machordom *et al.*, 2000), where identical groups were also defined. Nevertheless, some differences were found with respect to the internal nodes and relationships among groups. Although Mediterranean and Andalusian haplotypes appear as sister groups in both studies, according to RFLP analysis, the Duero cluster is basal to all the remaining haplotypes (Machordom *et al.*, 2000). In the present analysis based

on D-loop sequences, the Duero and Atlantic haplotypes appear as sister groups. The tree branches of these nodes are not well supported in either of these works. The short internodal distance in the RFLP analysis, observed together with the relatively low bootstrap support of the present tree (Fig. 2), indicates that the resolution of this cluster remains unclear. Independently of this cluster's branching position, protein electrophoresis has clearly differentiated brown trout populations of Atlantic- and Mediterranean-draining rivers in the Iberian Peninsula (García-Marín & Pla, 1996), supporting its individuality.

At the European level, the topologies proposed here are consistent with the groups defined in other studies (Bernatchez *et al.*, 1992; Giuffra *et al.*, 1994; Apostolidis *et al.*, 1997) but these studies were unable to resolve the relationships among groups. Andalusian and Adriatic haplotypes constituted an unexpected monophyletic group, which implies a wider distribution of this cluster in southern Europe than previously reported by other authors (i.e. Bernatchez *et al.*, 1992; Giuffra *et al.*, 1994; Apostolidis *et al.*, 1997). Based on the discontinuous geographical distribution of this cluster, we renamed the Adriatic haplotype, Adriatic–Andalusian (see below for further discussion).

Brown trout biogeography

European brown trout populations exhibit biogeographical boundaries that serve to differentiate river basins and drainages as discrete units (Bernatchez *et al.*, 1992; Giuffra *et al.*, 1994; Apostolidis *et al.*, 1997). These boundaries are also present in the Iberian Peninsula, with populations showing a high degree of haplotype diversity in relation to the different drainage systems, including Mediterranean, Atlantic and other endemic haplotypes (Machordom *et al.*, 2000). Through the present D-loop analysis, congruent with the previously cited results, Atlantic haplotypes were segregated into two groups. The North Atlantic haplotypes were distributed from northern Spain to the White Sea, while the distribution of brown trout showing the South European Atlantic haplotype ran from northern Spain to the Atlantic coasts of Morocco. Duero haplotypes, found in populations from the Duero basin and some from the Tajo, represent an endemism of the Iberian Peninsula. Tajo populations are suspected to have acquired these haplotypes through river captures (Machordom *et al.*, 2000). The presence of an Adriatic–Andalusian haplotype in specimens from southern Spain is remarkable, in particular when we consider that this haplotype has not been identified previously in populations of French or eastern Spanish rivers. We propose three hypotheses to explain the distribution of the

Adriatic–Andalusian haplotype: (i) possible contact between Adriatic–Ionian and South Iberian populations through northwestern Africa (Morocco, Algeria and Tunisia); (ii) lineage sorting leading to the present haplotype distribution, with an ancestral polymorphism fixed at separate localities; and (iii) the extinction of Adriatic–Andalusian haplotypes in France and eastern Spain. Brown trout have been found in Algeria and the Mediterranean coasts of Morocco (Behnke, 1984). Hence, the study of these populations may provide evidence for the hypotheses presented here. The Adriatic Sea has been suggested as the origin for the Adriatic haplotypes that subsequently colonized Corsican and other Mediterranean basins via ancestral headwater connections (Bernatchez *et al.*, 1992). However, based on its present distribution, it is impossible to establish an origin for the Adriatic–Andalusian haplotype.

Using the divergence rate of 0.8% per Myr corrected for *Salmo trutta* (Osinov & Bernatchez, 1996), the estimated divergence time between haplotypes corresponding to Atlantic and Mediterranean-draining rivers is in the range 1.2–1.6 Myr. This corresponds to the Pliocene–Pleistocene epoch. Divergence within the *S. trutta* complex was calculated to have occurred approximately 0.7–2 Myr ago (Ma) (Osinov & Bernatchez, 1996), an estimate consistent with the divergence time calculated from complete D-loop sequence alignments. Other approaches have indicated earlier divergence times for this species complex, i.e. 2.6–6.0 Ma (Apostolidis *et al.*, 1997) or 0.4–6.3 Ma (Machordom *et al.*, 2000). Thus, the different analytical methods appear to suggest two sets of divergence times between haplotypes. Estimates based on DNA sequences (Bernatchez *et al.*, 1992; Giuffra *et al.*, 1994; Osinov & Bernatchez, 1996) fall within 2% of the maximum divergence between Atlantic and Mediterranean drainage trout haplotypes, whereas estimates based on RFLPs (Machordom *et al.*, 2000) or pooled DNA sequences and RFLPs (Apostolidis *et al.*, 1997), unexpectedly indicate a value greater than 3% of the maximum divergence between the Atlantic and Mediterranean drainage population haplotypes. Sequence divergence estimates based on complete D-loop region sequences are in agreement with those derived from partial D-loop sequences (Bernatchez *et al.*, 1992) and from pooled partial sequences corresponding to the D-loop, ATP synthase subunit VI and Cytochrome *b* (Giuffra *et al.*, 1994). According to the present data, the Andalusian and Mediterranean haplotypes diverged 0.2–1.1 Ma, while the Duero and Atlantic haplotypes diverged 0.3–1.4 Ma. It is assumed that most significant genetic subdivisions within the brown trout complex are associated with major climatic change and basin isolation. The main Iberian hydrogeographic basins were

established about 1.8–2.5 Ma (Calvo *et al.*, 1993). This could explain present-day brown trout distribution in the Iberian Peninsula.

Glacial history seems to have played an important role in the diversity of North European Atlantic populations of *S. trutta*. Several hypotheses concerning the postglacial colonization of northern Europe have been proposed (Hamilton *et al.*, 1989; García-Marín *et al.*, 1999). Haplotype diversity shown by the southern populations of Europe and the low variability found in the North Atlantic populations suggest that glacial influence on the brown trout distribution was restricted to the north of the continent (Machordom *et al.*, 2000; Weiss *et al.*, 2000). The diversity of haplotypes found in the Iberian Atlantic rivers might represent an approximation to the haplotype diversity present in the northern Europe before the glaciations, when mitochondrial DNA variability would presumably have been higher. As temperatures decreased over the glacial period, the number of populations inhabiting the North decreased to extinction, erasing the haplotype variability of northern Europe; some of these populations survived in several glacial refuges (Hamilton *et al.*, 1989; García-Marín *et al.*, 1999). The Mediterranean populations were not affected by glaciations as much as those in northern Europe, thus maintaining their genetic variability.

In conclusion, the Iberian Peninsula maintains a haplotype variability that is not found throughout Europe. The present findings indicate that the Iberian Peninsula contains three of the five *Salmo trutta* groups previously described, and identifies a further different group, endemic to the Iberian Peninsula. The distribution of North Atlantic Mediterranean and Adriatic–Andalusian haplotypes is extended to the Iberian Peninsula, which could be considered a ‘hot-spot’ for brown trout diversity. Given that different levels of introgression with foreign populations have been recently detected (García-Marín *et al.*, 1991; Morán *et al.*, 1991; Martínez *et al.*, 1993; Bouza *et al.*, 1999; Machordom *et al.*, 1999; Machordom *et al.*, 2000), it is clear that conservation strategies such as avoiding foreign trout stock programmes are urgently needed to protect the endemic Duero and the mainly Iberian South European Atlantic haplotype. If restocking policies continue, trout stocks from all the biogeographical areas will be needed to maintain autochthonous genetic diversity in the Peninsula.

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