Phylogeographic Congruence Between mtDNA and rDNA ITS Markers in Brown Trout

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Variation in the internal transcribed spacer (ITS) of rDNA was examined throughout the range of the brown trout (*Salmo trutta*) to analyze the usefulness of this molecular marker for phylogeographic analysis. The results were compared with those previously obtained with mtDNA, a region exhaustively analyzed along the brown trout distribution. ITS2 was essentially conserved at all populations sampled, no informative characters being detected across the main lineages described in this species. Conversely, ITS1 showed a greater homogenization than other genetic markers at a microgeographic scale, with variation partitioning into several major phylogenetic groups. Phylogeographic patterns were partially congruent between both ITS1 and mtDNA. The main discrepancies were the detection of intra-individual variation and putative recombinant ITS1 sequences in hybridization areas between genetically different, yet historically overlapping, assemblages. Also, the existence of an ancient ITS1 sequence in the Mediterranean-southeastern area (rMEDA), not revealed by mtDNA analysis, was evidenced after rDNA ITS1 analysis.

Introduction

The phylogeographic approach has been used to assess patterns of genetic variation with reference to geographic distribution of a species with respect to historical changes in gene flow and diversification among populations (Avise 2000). The majority (>80%) of phylogeographic studies have relied upon the analysis of mitochondrial (mtDNA) variation, mainly because of processes of evolution that are conducive to providing a strong geographic signal (Avise 2000). The reliability of such studies, however, has often been criticized because the mitochondrial genome represents only a maternal perspective of the total historical record of sexually reproducing organisms (Degnan 1993; Palumbi and Baker 1994). Circumventing this constraint necessitates a test for phylogeographic congruence across nuclear and mitochondrial genes (Avise 2000). Increasingly attempts are being made to estimate nuclear gene genealogies in a phylogenetic context (Aquadro et al. 1991; Bernardi, Sordino, and Powers 1993; Burton and Lee 1994; Palumbi and Baker 1994). Yet, intra- and interspecific genealogic information from nuclear genes remains limited (Avise 2000). Limitations from nuclear markers in a phylogeographic context are mainly derived from (1) the difficulty in isolating DNA haplotypes, (2) the choice of a genomic region that accumulates mutations rapidly, (3) the greater coalescent time of nuclear sequences as compared with mitochondrial genes, and (4) the potential for reticulate evolution among nuclear alleles at the focussed time scale, due to intragenic recombination (Avise 1998; Bermingham and Moritz 1998).

Key words: *Salmo trutta*, phylogeography, rRNA genes, internal transcribed spacers, mtDNA control region.

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The concerted evolution of tandem repetitive families, such as rDNA make them useful in phylogenetic studies because variation tends to be homogenized within species and populations, whereas divergence is stressed between them (Hillis and Dixon 1991). Additionally, rRNA genes are recombining, biparental markers, which can reveal recent gene flow and hybridization events (Mayer and Soltis 1999). The internal transcribed spacers (ITS) of rRNA genes have been used in phylogenetic studies in closely related species due to their high evolutionary rates. Such studies have mainly focussed on resolving interspecific relationships within genera and occasionally at higher taxonomic levels (Gonzalez et al. 1990; Ritland, Ritland, and Straus 1993; Manos, Doyle, and Nixon 1999), thus being limited to only a few cases of intraspecific investigation (Vogler and DeSalle 1994; Zhuo, Sajdak, and Phillips 1994; King et al. 1999; Mayer and Soltis 1999; Shaw 2000).

The brown trout (*Salmo trutta*) is characterized by a complex genetic structure and a large genetic differentiation, including subspecies, sympatric isolated populations, and ecological forms throughout its distribution (Krieg and Guyomard 1985; Ferguson 1989; Presa et al. 1994; Osinov and Bernatchez 1996; Bouza et al. 2001). This pattern of pronounced population differentiation seems to result from the species habitat fragmentation, homing behavior, and complex evolution during the Pleistocene (Hamilton et al. 1989; Osinov and Bernatchez 1996; García-Marín, Utter, and Pla 1999; Bernatchez 2001). The most salient feature of genetic discontinuity in brown trout was revealed by analysis of mtDNA variation, which distinguished five major evolutionary lineages throughout its distribution (Bernatchez 2001). These lineages exhibited a strong spatial partitioning and seemed to have evolved in allopatry with limited introgression among them, a striking feature taking into account their partially overlapping areas and putative hybridization (Bernatchez 2001).

In this study we conducted an extensive genetic analysis of rDNA ITS variation throughout the natural

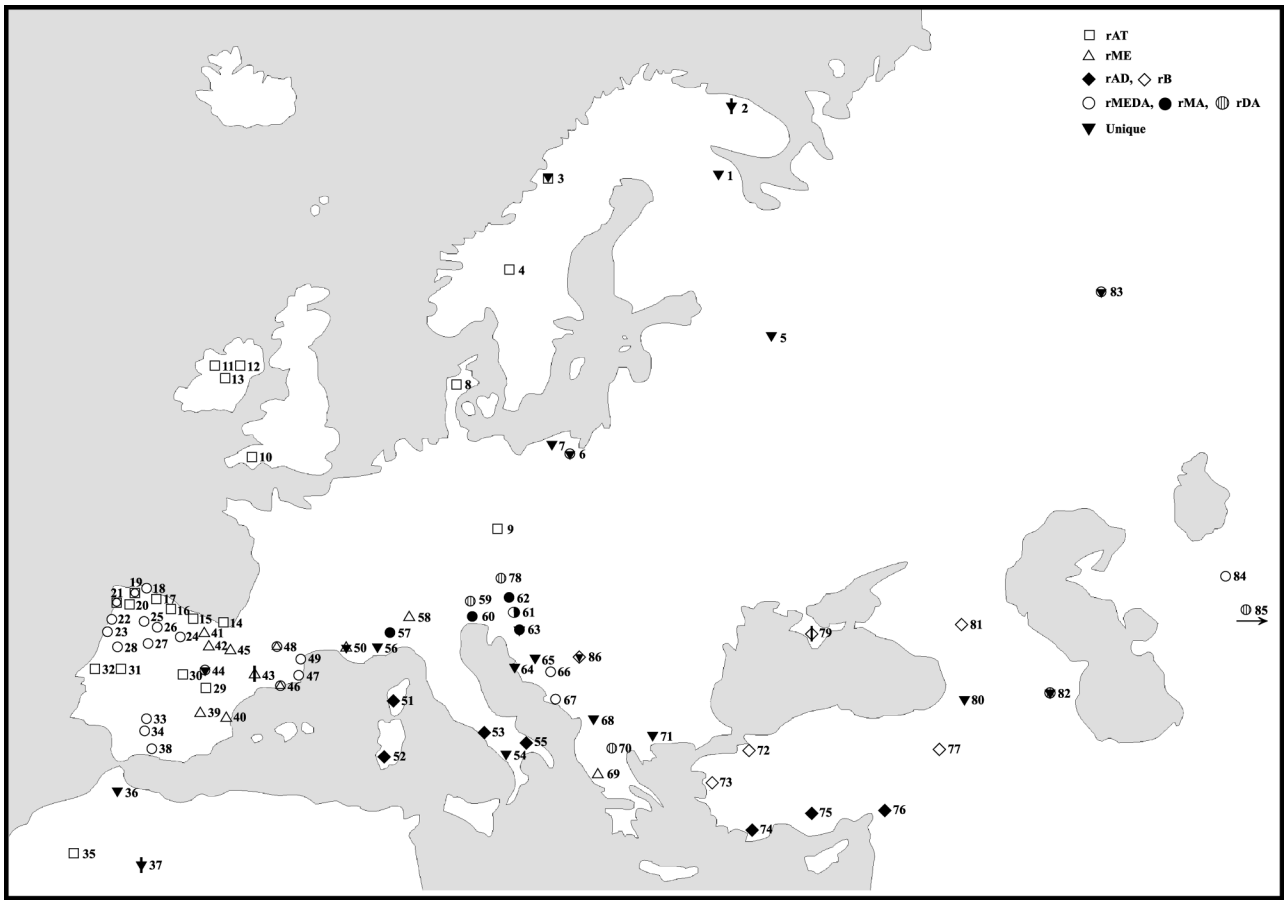

FIG. 1.—Phylogeography of brown trout after rDNA ITS1 analysis. Major ITS1 groups related with mtDNA lineages—rAT, Atlantic; rME, Mediterranean; rAD, Adriatic; rDA, Danubian; rMA, *S. t. marmoratus*; and without mtDNA equivalent—rB (around the Black Sea) and rMEDA (ancestral southern area). The symbols used indicate phylogenetic relationships among these sequences. Unique sequences are represented by solid triangles. Heterozygous individuals with ITS1 sequences defining different groups are indicated by superimposing the two ITS1 symbols or with a middle vertical bar on the symbol when the two ITS1 sequences were unique or pertained to the same group.

distribution of the brown trout. The results were analyzed in comparison with mtDNA data to evaluate the usefulness of rDNA ITS in revealing phylogeographic patterns.

Materials and Methods

Sample Design and ITS Analysis

A macrogeographic sampling scheme was designed to obtain an accurate picture of brown trout rDNA ITS differentiation in relation to previous studies of mtDNA. Eighty-six populations were sampled throughout the natural distribution of the species, i.e., Atlantic, Mediterranean, and southeastern drainages (Black, Caspian, and Aral seas), including some isolated populations (Appendix I and fig. 1). In order to obtain the rDNA ITS variation at a microgeographic scale, part of the samples were collected in the major basins of the Iberian Peninsula, a transition area between Atlantic and Mediterranean populations (Machordom et al. 2000). Samples were collected at sites where no historical record of stocking had been documented. The origin of individuals from the Iberian Peninsula was additionally checked by using starch electrophoresis at several diagnostic isozyme loci (*LDH*-C*, s*MDH*-A2*, *G3PDH*-2*, *IDHP*-1*, *GPI*-A1*; Bouza et al. 1999). To facilitate the identification of the 86 samples analyzed, populations were coded with the numbers presented in Appendix I, which also correspond with their geographical location, from the Scandinavian Peninsula in the Atlantic to the Black Sea, the Caspian Sea, and the Aral Sea in the southeast, along the European coastline (fig. 1).

In order to obtain a preliminary picture of ITS1 and ITS2 divergence in brown trout at both micro- and macrogeographic scales, several individuals from the same river basin as well as from distant geographic areas were examined. Thus, one individual from five populations of each of the two largest river basins in the Iberian Peninsula (Duero and Tajo; fig. 2), as well as from the three isolated forms of Lough Melvin (McVeigh, Hynes, and Ferguson 1995), was analyzed (fig. 1). To check for macrogeographic variation, both ITS1 and ITS2 sequences were studied in one individual from five distant populations (8, 41, 57, 70, and 84; Appendix I and fig. 1) representing the main brown trout lineages described using mtDNA variation (AT, ME, MA, AD, and DA; Bernatchez 2001). These preliminary results showed a high homogeneity of sequences at a local scale, all individuals from the same river basin and from Lough Melvin

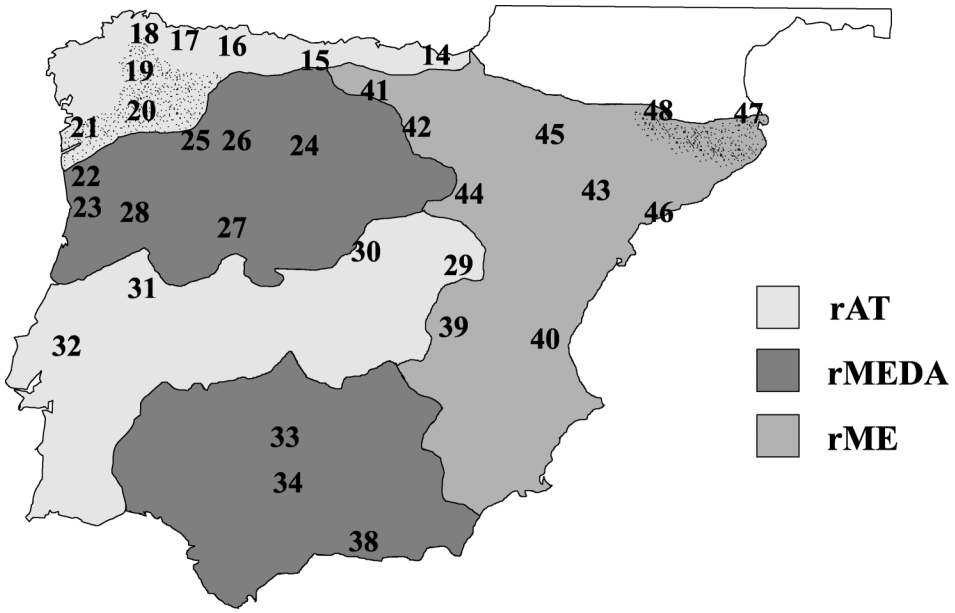

FIG. 2.—Microgeographic distribution of ITS1 variation in brown trout populations of the Iberian Peninsula: Cantabric region and Tajo Basin (rAT1), Duero Basin and southern region (rMEDA), Mediterranean region (mostly rME1). Dotted areas represent putative hybrid regions between rMEDA and rAT1 in northwestern Spain and between rME1 and rMEDA in northeastern Spain.

evidencing the same ITS sequences. Conversely, a moderate yet significant divergence was revealed among the reported brown trout lineages. This picture is in accordance with the high intrapopulation homogeneity observed in brown trout for tandem repetitive segments in the intergenic spacers (IGSs), an rDNA region which usually shows higher intrapopulation variation than ITSs (Morán et al. 1998; Castro et al. 1999). The model of concerted evolution of rDNA (Hillis and Dixon 1991), and also the small effective population size reported for this species (Jorde and Ryman 1995), could explain the high intrapopulation and intrabasin homogeneity observed in brown trout for rDNA. Consequently, one individual from each of the 86 populations sampled was analyzed in an effort to maximize the number of populations examined throughout the range of the species.

DNA Amplification and Sequencing

Samples consisted of fresh, frozen, or ethanol-preserved muscle or liver tissues. DNA was obtained by standard phenol-chloroform extraction (Sambrook, Fritsch, and Maniatis 1989). Sequences of each individual were obtained from total DNA by using double-stranded polymerase chain reaction (PCR) amplifications with the appropriate set of primers (Phillips, Sajdak, and Domanico 1995) located at the highly conserved coding regions of the 18S (kp2, 5′-AAAAAG CTTCCGTAGGTGAACCTGCG-3′) and 5.8S (5.8s, 5′-AGCTTGCTGCGTTCTTCATCGA-3′) genes for ITS1, and at 5.8S (5.8sr, 5′-CTACGCCTGTCTGAGTGTC-3′) and 28S (28s, 5′-ATATGCTTAAATTCAGCGGG-3′) for ITS2.

The PCR amplification mixture (50 μl) contained 150 ng of genomic DNA, 20 pmol of each primer, 0.4 mM dNTPs, 2.5 units of *Taq* DNA polymerase (Amersham Pharmacia Biotech), and 5 μl of 10× reaction buffer (Amersham Pharmacia Biotech). Amplifications were carried out in an MJ Research thermocycler as follows: 95°C for 5 min, and 40 cycles of 95°C for 1 min and 30 s, 55°C for 2 min, and 72°C for 3 min. A final extension step was performed at 72°C for 10 min. Amplified DNA from ITS regions was purified using the Concert™ Rapid PCR Purification System (GIBCO BRL). Both strands were sequenced for accuracy in each individual. Double-stranded DNA sequencing reactions were prepared either using the Thermo Sequenase fluorescent labelled primer cycle Sequencing Kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) for an ALF Express II sequencer or with the BIGDYE terminator method (Applied Biosystems) for an ABIprism 377 sequencer. Calibration on both automatic sequencers was made by sequencing the same five individuals used in the preliminary macrogeographic screening.

Data Analysis

As indicated below, ITS1 sequences were more informative for phylogenetic analysis than ITS2 according to preliminary data, and consequently only sequences from ITS1 were analyzed. Most sequence differences observed for ITS1 among populations analyzed were due to nucleotide substitutions. In addition, changes in 10 positions along the 582 bp length of ITS1 were due to insertion-deletions (indels). In all cases, these indels were detected in regions where short tandem nucleotide repetitions occurred. As previously reported, these areas are prone to mutation by slippage mechanisms both in vitro and in vivo, producing gain or loss of a single nucleotide of the tract, which could weaken the phylogenetic signal (Gonzalez et al. 1990; Schlötterer and

Tautz 1991). Therefore, these indels were treated as missing data.

The 3′ end of the 18S gene, both 5′ and 3′ ends of the 5.8S gene, and the 5′ end of the 28S gene were used to align the sequences using previous information on these regions for salmonid species (Zhuo, Sajdak, and Phillips 1994). The primary sequences from the 86 individuals analyzed were aligned using the Sequence Alignment program of the ALFwin™ Sequence Analyser 2.00 (Amersham Pharmacia Biotech). The alignment yielded a minimum number of evolutionary steps with gaps inserted, producing a final data set of 582 bp per individual suitable for phylogenetic analyses in MEGA 2.0 (Kumar, Tamora, and Nei 1993), PHYLIP 3.5 (Felsenstein 1993), and PAUP 4.0 (Swofford 1998) computer programs.

Nucleotide composition, variable and parsimony informative positions, and transition-transversion rates were estimated using MEGA 2.0. Sequence divergence values were computed with the Kimura two-parameter model (Kimura 1980) using the program DNADIST of PHYLIP. The program PAUP was used to calculate the optimality criteria distance of minimum-evolution (ME) with 10,000 iterations of the Interior-Branch-Length test. The robustness of the phylogenies was assessed by estimating confidence probabilities (*t*-test) and *SBL* (sum of branch length) index. *Salmo salar* (Atlantic salmon) was used as an outgroup to root all trees (AF518876), as this species is considered a basal lineage in the genus *Salmo* (e.g., Gyllensten and Wilson 1987).

Two sequences detected in the putative hybrid areas were excluded from the phylogenetic analysis due to their doubtful genotyping (see first section of Results). Thus, 35 ITS1 sequences out of the 37 obtained in the 86 populations analyzed were used for constructing the phylogeny and for the analysis of recombination. Additionally, the putative influence of recombinant-derived sequences on ITS1 that could weaken the phylogenetic signal suggested analysis of a subset of sequences to evaluate phylogenetic hypotheses. The elimination of recombinant sequences has been applied by different authors when reconstructing rDNA ITS–based phylogenies (Vogler and DeSalle 1994). A global estimation of recombination in the ITS1 sequences was obtained by calculating the value of r (ratio between per-site recombination rate (C) and per-site mutation rate [μ]), taking as a reference the value of r obtained in the mtDNA control region, a presumably nonrecombining genomic region. These estimations were calculated by applying a maximum likelihood approach using Metropolis-Hastings sampling on candidate genealogies (Kuhner, Yamato, and Felsenstein 2000) for both the 35 ITS1 sequences referred to obtained in our study and the 38 mtDNA control region sequences detected by Bernatchez (2001). A more detailed analysis of recombinants was performed by constructing one-step networks both on ITS1 and mtDNA data following the procedure of Templeton, Crandall, and Sing (1992) and using the absolute nucleotide differences obtained from the genetic distance matrix of PAUP 4.0 (Swofford 1998). This network shows the more parsimonious relationships among all sequences analyzed by connecting the ones diverging by a single character state. Following this procedure, the presence of loops in the network would suggest the presence of putative recombinants since a single recombinant event could resolve simultaneously at least two homoplastic events (Aquadro et al. 1986). Also, the existence of multiple homoplasies in the network should be evaluated to look for additional recombinants by combining this information with that obtained in the phylogenetic tree. The ITS1 one-step network performed was also used to contrast and verify the relationships observed in the ME trees.

Results

Composition and Variation of Brown Trout ITSs

The ITS1 length of the most common Atlantic sequence, the putative ancestral one of the species (rAT1), comprised 582 bp with 63.5% G + C richness, whereas the more frequent ITS2 sequence detected had a 376-bp length with 67.5% G + C content (AY057992). These values are very close to those observed within other salmonids (Phillips, Manley, and Daniels 1994; Zhuo, Sajdak, and Phillips 1994; Sajdak and Phillips 1997) and are approximately the mean values reported for ITS sequences in other vertebrates (Gonzalez et al. 1990; Vogler and DeSalle 1994).

The preliminary analysis using both the ITS1 and ITS2 regions and a subset of samples (13 populations; see Materials and Methods) revealed (1) the existence of several phylogenetically informative nucleotide substitutions in ITS1, which suggested its potential usefulness for phylogeographic studies in brown trout; (2) a great homogeneity in ITS2 throughout the species distribution. Most populations analyzed showed the same sequence, with only three noninformative nucleotide substitutions; (3) the homogenization of ITS sequences both among Lough Melvin samples, as well as within each of the Iberian Peninsula basins analyzed (see Duero [24–28] and Tajo [29–32] basins; Appendix I and fig. 2).

Eighteen out of the 86 brown trout individuals analyzed for ITS1 showed intra-individual variation in at least one nucleotide site (Appendix I). These individuals necessarily presented at least two different ITS1 sequences, and their genotypes were inferred according to the following criteria: In 11 samples only one position showed two alternative characters (2, 3, 37, 43, 44, 50, 61, 63, 79, 82, 86), and therefore only a single combination of two different sequences was possible. These individuals were termed as heterozygous only for simplicity, yet these sequences could not be orthologous, considering the multichromosomal location of nucleolar organizer regions (NORs) at specific hybrid areas in brown trout (Castro et al. 2001). In the Guadalfeo sample (38), where previous allozyme analyses suggested stocking (García-Marín, personal communication), the three positions differentiating rAT1 and rMEDA were heterozygous. This individual was assumed to hold the ITS1 characteristic of the Iberian hatchery stock (rAT1) and the native one common in the South Iberian area

(rMEDA; Appendix I and fig. 2). Accordingly, only the native sequence (rMEDA) was considered for population 38 in further analyses (Appendix I). In the remaining six samples, two (6, 83), three (19, 21, 46), or four (48) nucleotide sites showed variation, several genotype arrangements being possible. Their ITS1 composition is only tentative and should be taken with caution. These samples were detected in areas where putative hybridization between two different lineages appeared to occur (see Discussion). These were the cases of Miño Basin (19, 21—between rAT1 and rMEDA), the northwestern Mediterranean area (46, 48—between rME1 and rMEDA), and the neighborhood of Scandinavian Peninsula (6, 83—between rMEDA and rAT1/2). We assumed these individuals to be heterozygous for two ITS1 sequences and followed a conservative criterion to infer their ITS1 constitution, assuming the minimum number of changes with regard to the lineages involved in these hybrid areas. For instance, in population 19 three variable sites were detected, which involved the three differential characters between rMEDA and rAT1 (124, T/A; 368, C/T; and 416, G/A). Eight different combinations of pairs of sequences were possible with this variation. Considering the hybrid condition of Miño Basin between the southerly rMEDA and the northerly rAT1, we assumed this individual to hold both rMEDA and rAT1 sequences. Following these criteria only two new ITS1 sequences were assumed among these six populations (table 1 and Appendix I). Given their doubtful genotyping, we decided to exclude them from phylogenetic analysis because of the distortion they could introduce due to the small number of informative sites for ITS1 in brown trout.

Taking into account the existence of heterozygous individuals in the 86 populations sampled, a total of 103 ITS1 sequences were scored. Thirty-seven different ITS1 sequences were observed among these 103 sequences (table 1). The 10 positions displaying indels (103, 135, 160, 250, 259, 276, 345, 385, 463, 464) were the result of single gains or losses of a single nucleotide in simple repeated tracts, and were obviated as indicated in Material and Methods. Six out of the 22 variable positions detected along the 582 bp of ITS1 corresponded exclusively to the divergence between *S. salar* and *S. trutta*, the remaining 16 variable sites being due to differences among brown trout sequences. In all cases, only two alternative nucleotides were detected at each variable position within brown trout. Ten out of the 16 intraspecific variable positions were phylogenetically informative, the other six showing the alternative nucleotide in only one sequence. Eleven nucleotide changes were due to transitions (68.75%) and five to transversions (31.25%). The transition-transversion ratio for ITS1 (1.75) was slightly higher than that obtained within salmonids for the same region (Phillips, Sajdak, and Domanico 1994; Zhuo, Sajdak, and Phillips 1994) and similar to that observed for the mtDNA control region in brown trout (Bernatchez, Guyomard, and Bonhomme 1992).

Table 1
Alignment and Frequencies of ITS1 Sequences of *Salmo trutta*

Code [a]	Sequence: 1112333 3334444445 55 / 7892498005 6771124561 15 / 4395314058 2581642740 70	Frequency
rAT1	GCTTGCGTGC CCGCCGGTAG AC	16
rAT2	.... G......... ..	4
rME1	.T....A...	6
rME2	.T....A... .T........ ..	3
rME3	.T....A... .T...A.... ..	3
rAD1	..CA..A... .T...A.... ..	2
rAD2	..CA..A... .T........ ..	5
rDA	...AT..... .T...A.... ..	5
rB1	...A..A... .T...A.... ..	3
rB2	...A..A... .T........ ..	2
rB3	......A... .T........ ..	2
rMA1	...A.T.... .T...A.... ..	3
rMA2	...A...... .T...AA... ..	2
rMEDA	...A...... .T...A.... ..	24
2[a]	.... G.C....... ..	1
36	.... G......G.. ..	1
1	.T........ G......... ..	1
3	...A...... G......... ..	1
2[b]	...A...... G.C....... ..	1
37[a]	......AC.. G......... ..	1
37[b]	.T....AC.. G......... ..	1
50	.T........ .T........ ..	1
65	.T.A..A... .T........ ..	1
68	..CA..A... .T.T...... ..	1
64	.TC...A... .T........ ..	1
54	...A.....T .T...A.... ..	1
7	...A...... GT...A.... ..	1
5	...A...... GT........ ..	1
71	.T.A...... .T...A.... ..	1
6	....A.... ..	1
44	.... .T...A.... ..	1
56	...A...... .....A.... ..	1
80	...A...... .T........ ..	1
86	......A... .T...A.... ..	1
63	...A.T.... .T...AA... ..	1
82	A..AT..... .T...A.... ..	1
83	.... .T........ ..	1
Salmo salar	........A. ....T...TA CA	1

NOTE.—Sequences are arranged from top to bottom following both, frequency and similarity. The most frequent sequences were coded in accordance with the terminology applied in mtDNA analysis (Bernatchez et al. 1992).

[a] The superscript letters identify two different unique sequences when detected in the same population.

Genetic Divergence and Phylogeny

Between one and seven nucleotide differences were evidenced among the 37 ITS1 sequences detected in brown trout (mean ± standard deviation [SD] 3.27 ± 0.16). Kimura two-parameter genetic distance ranged between 0.0017 and 0.0123 (mean ± SD 0.0057 ± 0.0003). Fourteen sequences were shared by more than one population, whereas the remaining 23 were unique (table 1 and Appendix I). To facilitate location of the population in tables and figures, unique sequences were identified with the number of the population where they were detected (Appendix I and fig. 1), highlighted in bold characters. Among the most frequent sequences, 11 showed specific character states that defined several subsets of sequences. The good correspondence in the dis-

tribution between mtDNA lineages and these ITS1 sequences in most populations suggested the use of a similar terminology to that applied to brown trout mtDNA lineages (table 1 and Appendix I; Bernatchez, Guyomard, and Bonhomme 1992), namely, Atlantic—rAT (rAT1 and rAT2); Mediterranean—rME (rME1, rME2, rME3); Adriatic—rAD (rAD1 and rAD2); Danubian—rDA; and *marmoratus*—rMA (rMA1 and rMA2). These five ITS1 groups evidenced diagnostic or partial diagnostic characters taking rAT1 as the ancestral reference: rME, T-83; rAD, C-99; rMA, T-191 or A-442 (or both); rDA, T-143. Among the remaining most frequent sequences, the rB group did not show any diagnostic character, but there was a character state common in the Mediterranean area, A/284. Finally, rMEDA was a special ITS1 sequence without an obvious equivalent in mtDNA analysis, remarkable by its high frequency in the populations studied (23%). This sequence and rAT1 (16%) were the most representative ITS1s in brown trout. Furthermore, several sequences showed the same combination of characters of rMEDA (20%) and rAT1 (13%) plus additional ones (i.e., sequences **7, 54,** and **71** showed the three rMEDA characters plus G/362, T/358, and T/83, respectively, taking rAT1 as reference; table 1). It thus appears that rMEDA (43%) and rAT1 (29%) were the core of most brown trout ITS1s.

The minimum evolution (ME) tree obtained for the 35 ITS1 brown trout sequences finally considered plus the outgroup (*S. salar*) is shown in figure 3. Confidence values were in general moderate, the highest ones observed in the clusters defined by the most frequent sequences. Because of the low number of synapomorphic sites in the ITS1 of brown trout, the use of the bootstrapping test provided lower support than did the *t*-test on Interior-Branch-Length tests. A salient and consistent result of this tree was the basal position of rAT1. Four main clusters, in some cases strongly supported, were also evidenced: (1) the rAT2 and closely related sequences, characterized by G/362; (2) the rME group and related sequences characterized by T/83; (3) the rAD group (C/99); and (4) the rMEDA-rDA-rMA group and related sequences (mostly rMEDA plus derived characters). The confidence nodal support separating these groups were generally weak, suggesting the existence of a major Mediterranean-southeastern cluster, where rAD appeared as a consistent sister group to rMEDA-rDA-rMA.

An estimate of the ratio between per-site recombination rate and per-site mutation rate (r) was obtained using the program RECOMBINE (Kuhner, Yamato, and Felsenstein 2000) on both ITS1 and mtDNA data for testing the putative influence of recombination in phylogenetic reconstruction. The value of r for ITS1 (35 sequences; $r = 0.652$) was close to fivefold higher than for the mtDNA control region (38 sequences; Bernatchez 2001; $r = 0.141$), which suggested a higher impact of recombinational events in the origin of ITS1 in brown trout. The estimate of r by this method shows no bias and reasonable accuracy, especially for high values of θ ($4Ne\mu$; moderate-high in the case of brown trout ITS1) and when recombination rate is high (the case of ITS1). Also, when $r = 0$ the estimation is upward biased, which could explain the high value observed for mtDNA (Kuhner, Yamato, and Felsenstein 2000). The recombination rate (C) for ITS1 in our study would be at worst one order lower than μ.

A one-step network method (fig. 4; Templeton, Crandall, and Sing, 1992) was also applied to both the 35 ITS1 sequences considered and the 38 mtDNA haplotypes (Bernatchez 2001) to look for the recombinant origin of specific ITS1 sequences (fig. 4). Several loops of four or more steps were evidenced in the ITS1 network (fig. 4*A*), mainly due to some unique sequences (**5, 86, 80**), which connected the four major phylogenetic groups previously detected (rAT, rME, rAD, and rMEDA-rDA-rMA). In addition, three four-step loops were revealed within groups or connecting closely related groups (**65, rAD1, 63**). In all these cases, a single recombinant event could explain the origin of these sequences, which alternatively would require two homoplastic events (see for instance the four-step loop rMEDA-rMA1-**63**-rMA2). In addition to the loops, in the ITS1 network an important number of homoplastic events were evidenced (e.g., T-83 and A-125), suggesting the presence of additional recombinant sequences, which should also be taken into consideration (Templeton, Crandall, and Sing 1992; for instance the sequence **1** could be the result of recombination between rAT2 and rME, and the sequence **71**, between rMEDA and rME). The situation looked sharply different in the mtDNA network (fig. 4*B*), where only three loops within groups were revealed, and the presence of homoplasies in the net was exceptional.

The most frequent ITS1s constituted the backbones of both the Atlantic and Mediterranean-southeastern groups (fig. 4*A* in bold characters) and were mostly linearly related in the network (only one loop detected: rB1, rB2, rAD1, rAD2). Genetic relationships between the four main clades detected in the ME analysis (fig. 3) were confirmed in this representation. The rAT sequences (rAT1 and rAT2 and related ones) defined a single and well differentiated group, whereas in the Mediterranean-southeastern cluster, the rB sequences connected the three major clusters (rME, rAD, rMEDA-rDA-rMA; fig. 4*A*, shaded groups). The central position of rMEDA within the rMEDA-rDA-rMA group was relevant.

Given the putative recombinant nature of several ITS1 sequences, we performed an additional phylogenetic analysis to check for the influence of recombination by using the 14 most frequent ITS1 sequences, which were determined according to the following criteria: (1) They were the most representative in brown trout (close to 80%). (2) They were mostly linearly related in the one-step network, which suggests no recombination in their origin, and constitute the strongest supported groups in the 35 ITS1 sequences tree (fig. 3). (3) They included the ITS1 sequences homologous to the five mtDNA lineages described. (4) They covered the full geographic range of brown trout. The results of the ME tree obtained (fig. 5) were very similar to those

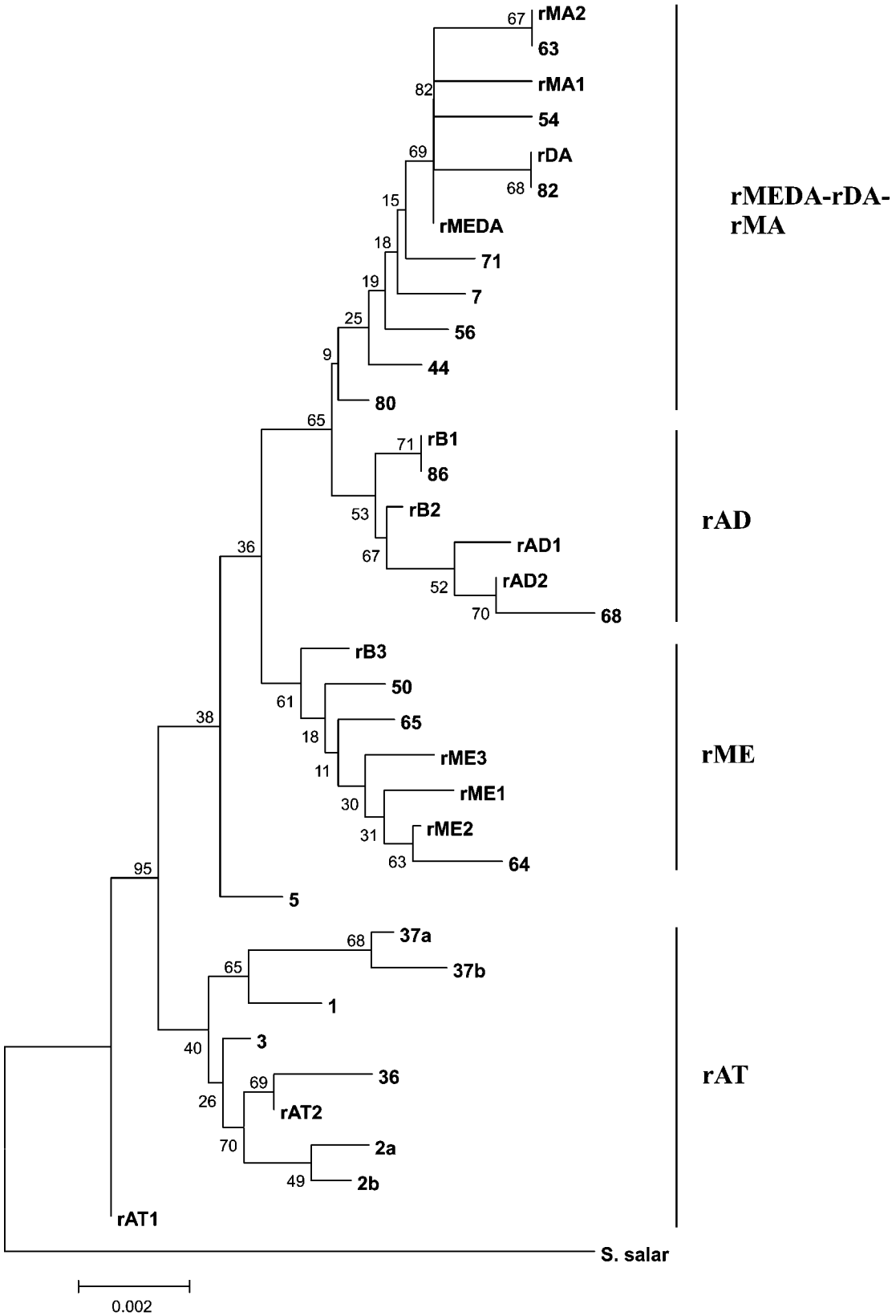

FIG. 3.—Condensed ME tree (SBL = 0.0596) showing nodal confidence probabilities (*t*-test on 10,000 Interior-Branch-Length iterations) using the 35 ITS1 sequences considered in 86 *S. trutta* populations, using *S. salar* as outgroup.

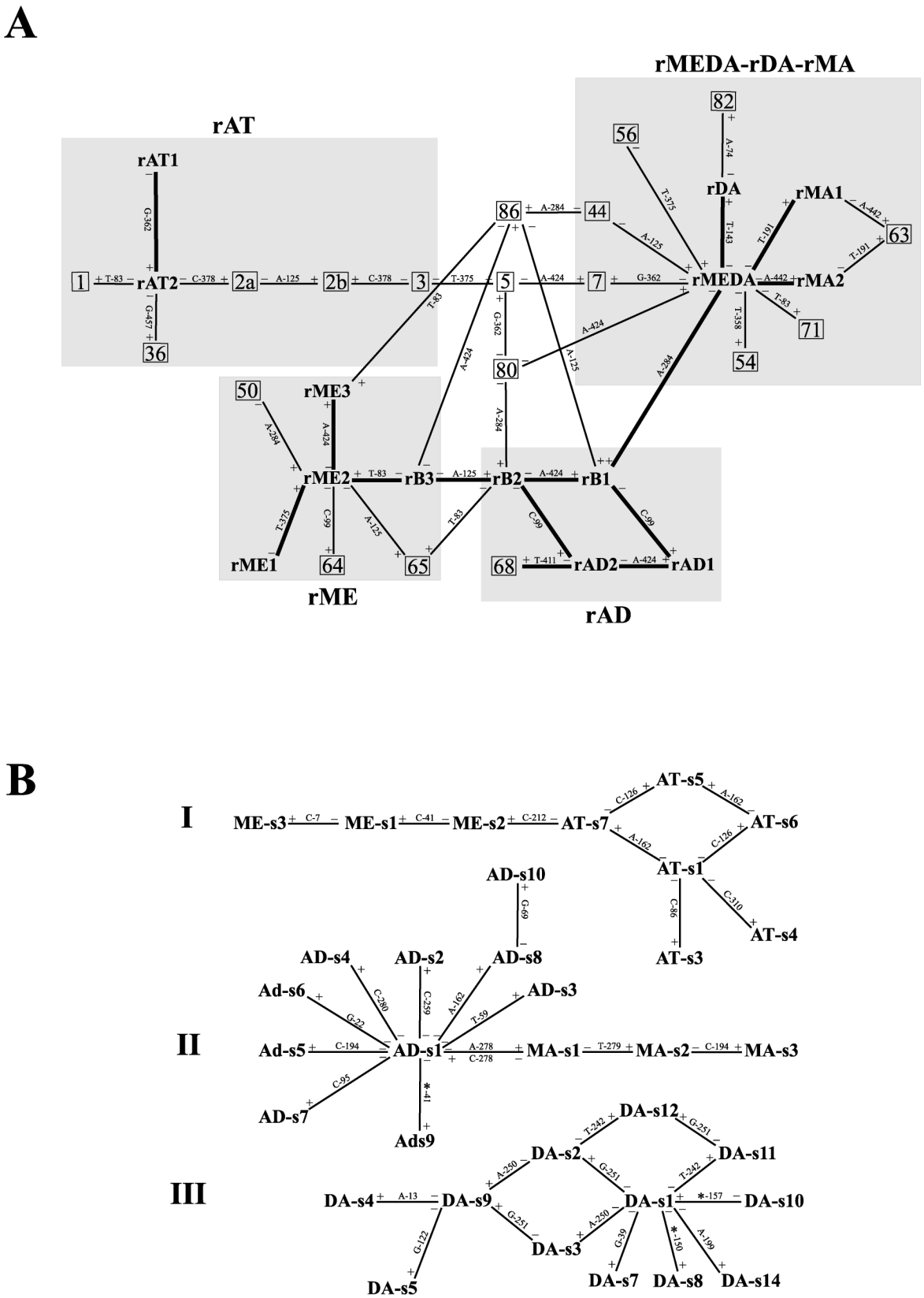

FIG. 4.—One-step networks relating (*A*) the 35 rDNA ITS1 sequences considered for the phylogenetic analysis of *S. trutta* (table 1); and (*B*) the 38 mtDNA control region sequences (Bernatchez 2001) according to the method of Templeton, Crandall, and Sing (1992). In the ITS1 network, the **37a** and **37b** ITS1 sequences constituted a second one-step network not presented here. In the mtDNA network, four sequences (DA-s6, DA-s13, At-s2, AT-s8) showed more than one step with all others, and therefore do not appear in this representation. Each line indicates one mutational event. Nucleotide changes are represented with regard to the ancestral state, symbols + (gain) or − (loss) denote the direction of the change, and asterisks represent indels. The most frequent ITS1 sequences (in bold) are connected by solid lines. The four major ITS1 groups, also recovered by the ME tree, appear shaded (fig. 3; rAT, rME, rAD, and rMEDA-rDA-rMA).

observed with all ITS1 sequences (fig. 3), but in this case the topology contained far larger confidence values. The analyses in figures 4 and 5 both confirmed that rB was not a monophyletic group, containing ITS1 sequences related to the three major Mediterranean-southeastern clusters.

Geography of ITS1 Variants: Hybridization Areas

The most common ITS1 sequences exhibited a differential geographic distribution in good agreement with the reported mtDNA lineages (fig. 1 and Appendix I; Bernatchez 2001). That is, rAT predominated in the Atlantic area, three minor subareas being observed: the Fenno-Scandinavian Peninsula (rAT2), the Iberian Peninsula, with divergent ITS1 sequences (rMEDA and rAT1), and the area between Spain and Scandinavia, where only rAT1 was present. Within the Mediterranean-southeastern area, rME was observed in the western Mediterranean Sea, being distributed in the east of Spain and southeast of France; rAD was observed in the central and eastern Mediterranean regions, from Corsica and Sardinia to Turkey; rMA was only detected in the Po Basin in the northern region of the Adriatic Sea; and rDA ITS1 was mainly observed in the southeastern drainages.

However, these sequences (rAT, rME, rDA, rAD, and rMA) represented approximately half of the 103 ITS1 sequences. Therefore, differences existed between ITS1 and mtDNA data sets. First, within the southern area, the large distribution of rMEDA, ranging from Afghanistan to western Iberia and observed at different locations, was a distinctive ITS1 feature. Secondly, several populations from the southeastern area, which were related with the Danubian mtDNA lineage, appeared here as rB. Thirdly, as evidenced in Appendix I, several discordances were observed between mtDNA versus ITS1 data at specific populations (55 populations with mtDNA and ITS1 information). Fifty-five percent of these populations pertained to the same lineage-group with both data sets (MA, DA, AD, AT, ME). The discrepancies were mostly due to the presence of rMEDA (11%) and rB (11%), sequences without an obvious equivalent in mtDNA analysis, and to the presence of unique ITS1 sequences (18%). Only three samples (5%) evidenced different major lineages-groups between mtDNA and ITS1 analyses.

Finally, the existence of putative hybridization areas between different lineages, evidenced by the presence of heterozygous individuals or putative recombinant sequences, was also revealed by ITS1 analyses (figs. 1 and 2 and Appendix I). These areas were the Miño Basin (northwestern Iberian Peninsula), located between the Cantabric Sea (rAT1) and Duero Basin (rMEDA); the northeastern Iberian area, between Spain and France, where rMEDA (46, 48) and rME1 (Ebro, Júcar, and Segura basins) were prevalent; the Adriatic Sea, in the central part of the Mediterranean Sea, where most brown trout ITS1 sequences were present; and the Fenno-Scandinavian Peninsula, between rAT2 and rMEDA.

The analysis of several samples in the largest river basins of Iberian Peninsula (Miño, Duero, Tajo, Guadalquivir, and Ebro; fig. 2) evidenced a high genetic homogenization at a microgeographic scale in brown trout, excluding the aforementioned hybrid areas. Three major ITS1 groups were detected: the Cantabric drainage and Tajo Basin (rAT1), the Duero and South Iberian basins (rMEDA), and the Mediterranean drainage (Ebro, Jucar, and Segura basins; rME).

Discussion

Despite the assumption of intraspecific rDNA ITS homogenization due to the mechanisms of concerted evolution, the use of this marker for phylogeographic analysis has been successfully applied for several species (e.g., Ritland, Ritland, and Straus 1993; Vogler and DeSalle 1994; King et al. 1999; Mayer and Soltis 1999; Shaw 2000). The study of rDNA ITSs in brown trout has also revealed the existence of genetic variation useful for phylogeographic inference in this species. ITS1 showed more informative variation than did ITS2, as reported in other species (Mayer and Soltis 1999). Sixteen variable sites (2.75%) were detected along the 86 brown trout populations analyzed, 10 of which were phylogenetically informative. The amount of variation detected in *S. trutta* ITS1, although lower than in other species, was congruent with that observed in the mtDNA control region (3.28% variable sites; Bernatchez, Guyomard, and Bonhomme 1992). The evolutionary rate for rDNA ITS1 appears to be roughly half of that of the mtDNA control region, taking into account both the number of informative positions (1.7% vs. 3.3%) and the mean nucleotide divergence among sequences (0.56% vs. 1.2%; Bernatchez, Guyomard, and Bonhomme 1992).

Phylogeographic Congruence Between ITS1 and mtDNA Data Sets

The application of different methods for phylogeographic reconstruction with ITS1 in brown trout revealed patterns of genetic structure that were partially congruent with previous mtDNA analysis (Bernatchez, Guyomard, and Bonhomme 1992; Bernatchez 2001). Several major groups observed with ITS1 analysis most likely correspond to the lineages detected with mtDNA as inferred from their similar geographic distribution. ITS1 groups appeared less differentiated than mtDNA lineages were, being defined by only one or two diagnostic characters. This fact is probably explained by the lower evolutionary rates reported for rDNA ITSs (Gonzalez et al. 1990; Suh et al. 1993; Osinov and Bernatchez 1996). The confidence values supporting ITS1 tree branching were lower than for mtDNA data, as currently observed in similar phylogeographic studies (Manos, Doyle, and Nixon 1999; Mayer and Soltis 1999). However, the phylogenetic relationships obtained with ITS1 in brown trout defined four consistent groups (rAT, rME, rAD, and rMEDA-rDA-rMA), and their relationships were mostly congruent across all reconstruction methods applied. Both mtDNA and ITS1 supported the basal po-

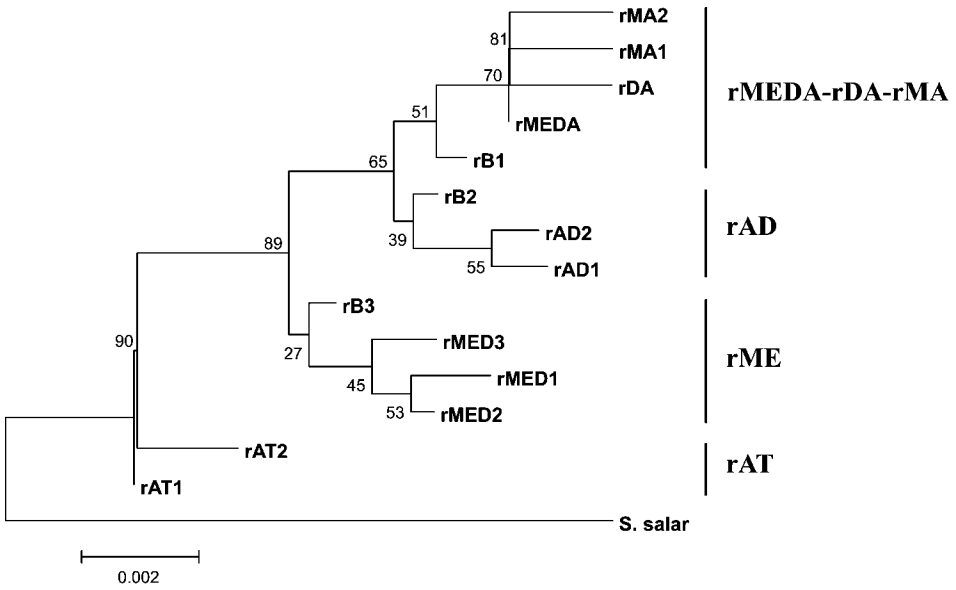

FIG. 5.—Condensed ME tree (SBL = 0.0335) showing nodal confidence probabilities (*t*-test on 10,000 Interior-Branch-Length iterations) of the 14 most common ITS1 sequences of *S. trutta*, using *S. salar* as outgroup.

sition of the Atlantic lineage, the closest one to *S. salar*. However, some discrepancies were evidenced among the Mediterranean-southeastern groups between both data sets, where only rME and rAD appeared as well differentiated clusters in the ITS1 analysis, whereas the consistent mtDNA lineages DA and MA defined a consistent major group with rMEDA (rMEDA-rDA-rMA).

A special ITS1 feature was the ubiquity of rMEDA, a sequence with no apparent equivalent in mtDNA analysis, in the Mediterranean-southeastern area, from the Aral Sea to the Iberian Peninsula. rMEDA and rAT1 represented close to 40% of the ITS1s screened in brown trout, and the specific combination of characters of rAT1 or rMEDA appeared in 72% of the sequences studied. Additionally, rMEDA occupied a central position in the network analysis within the southeastern group and a basal position within the rMEDA-rDA-rMA cluster in the cladograms. All these data suggest the ancestral position of rMEDA in the Southern area. The retention of ancestral haplotypes and the existence of multifurcating rather than bifurcating patterns have been described as characteristics of intraspecific evolution of nuclear sequences. Although these properties can violate the assumptions of phylogenetic analysis (Crandall and Templeton 1993), ITS1 sequence variation provided essential information for phylogeographic reconstruction in brown trout, as reported in other species (Manos, Doyle, and Nixon 1999; Mayer and Soltis 1999). Our data suggest the existence of two ancestral ITS1 sequences in the evolutionary history of brown trout, namely ITS1 at the Atlantic region, and rMEDA at the Mediterranean-southeastern region, most of the remaining lineages detected both with ITS1 and with mtDNA being probably of more recent evolutionary origin.

The use of nuclear sequences within species suggests caution for phylogenetic reconstruction due to possible reticular evolution, where recombination could be an important source of error. Some differences between mtDNA and ITS1 data sets were probably due to the impact of recombination on ITS1. In our study, the estimate of a global value for recombination (*r*; Kuhner, Yamato, and Felsenstein 2000) was fivefold higher for rDNA ITS1 data than for the mtDNA control region, a presumably nonrecombining genome. Also, the identification of specific recombinant ITS1 sequences using the one-step network method (Templeton, Crandall, and Sing 1992) revealed the existence of several sequences connecting the four main clades detected in the phylogenetic tree, and a greater number of homoplastic events. Considering the value obtained for the ratio between per-site recombination rate and per-site mutation rate for ITS1 ($r = 0.652$), it appears more parsimonious a single recombinant event than two independent mutational events (Aquadro et al. 1986) to explain recombinants in the network. However, independent mutational events cannot be fully excluded because the estimation of the ratio between recombination and mutation rates was averaged along the whole sequence, and the existence of mutational hot spots could account for some of these events. In spite of recombination, the phylogenetic analysis performed appeared consistent, and the detection of recombinant sequences could be revealing hybridization and secondary contacts between brown trout lineages.

Hybridization Areas

Evidence of hybridization between divergent ITS1 groups was revealed in our study, with heterozygous individuals and putative recombinants clustered at specific geographic areas throughout the brown trout range (northwestern and northeastern Iberian Peninsula areas, the Adriatic Sea, and the Fenno-Scandinavian Peninsula; see fig. 1). The existence of recombinant haplotypes due to reciprocal recombination and gene conversion events, as well as heterozygous individuals, has been frequently

observed in phylogeographic studies with rDNA ITS1, probably facilitated by hybridization among lineages (Vogler and DeSalle 1994; Sang, Crawford, and Stuessy 1995; Mayer and Soltis 1999). Taking into account the capability of concerted mechanisms to homogenize the brown trout rDNA sequences observed in our study, the existence of heterozygous individuals for ITS1 could indicate their hybrid condition. Notably, rRNA genes in some of these areas studied seem to behave like mobile elements, evidencing unstable multichromosomal location (Castro et al. 1996, 2001; Woznicki et al. 2000). This observation could suggest a degree of genetic incompatibility between some ITS1 groups in brown trout. Ongoing cytogenetic-molecular analyses of these hybrid areas in the Iberian Peninsula suggest different chromosomal NOR locations between these groups, a species-specific characteristic in cytotaxonomic studies (Castro et al., personal communication).

ITS1 Evolution and Phylogeography: Comparison with Other DNA Segments

One main issue related to rDNA evolution is the strength of concerted mechanisms acting on tandem repetitive families. Several studies have cautioned against the generalization of intrapopulation homogeneity of rRNA genes. The critical point focuses on important ITS differences observed within both populations and individuals, probably due to multichromosomal location of NORs (Ritland, Ritland, and Straus 1993). However, little intrapopulation variation could be observed in most ITS studies (Hillis and Dixon 1991; Fritz et al. 1994; Zhuo, Sajdak, and Phillips 1994). Even when a multichromosomal NOR location was evident (Arnheim et al. 1980; Zhuo, Sajdak, and Phillips 1994), the molecular homogenization was achieved very quickly in some cases (Hillis et al. 1991). Our data on brown trout support the homogenization of the rDNA family, at least in stable areas. The analysis of more than 20 samples from the five major river drainages of the Iberian Peninsula and from the three putative isolated forms from Lough Melvin revealed a large genetic homogenization of ITS1 within basins and within regions. This result largely contrasts with the very important genetic differentiation within basins observed for allozymes (Bouza et al. 1999, 2001; Sanz et al. 2000) and the moderate variation observed with mtDNA restriction fragment length polymorphisms (RFLPs) in the same geographic area (Machordom et al. 2000). A pronounced intrapopulation homogenization has also been observed in this species through the use of RFLP analysis of IGSs (Castro et al. 1999), an rDNA region with higher evolutionary rates than those of ITS (Hillis and Dixon 1991). These data suggest that the mechanisms of concerted evolution homogenize rDNA family very efficiently in brown trout, even when small amounts of gene flow exist or has existed in the near past, as has been described in other species (Hillis et al. 1991; Fritz et al. 1994; Zhuo, Sajdak, and Phillips 1994). The situation looks quite different in the putative hybrid areas between divergent lineages, where a multichromosomal NOR pattern linked to the presence of heterozygous ITS1 genotypes has been described (Woznicki et al. 2000; Castro et al. 2001). As outlined before, these results could be an indication of isolation among divergent lineages involved in these areas. A more detailed study would be necessary to understand the dynamics of ITS1 in hybrid areas.

The different modes of evolution of each genome segment caution against phylogenetic reconstruction using a single genomic region (Avise 2000). The concordance between gene tree and species tree is not always evident (Doyle 1992), especially for intraspecific phylogeographic inference (Mayer and Soltis 1999). This fact has been evidenced in the present work, where the degree of concordance between mtDNA and ITS1 data sets, although important, was not complete. Around 45% of populations analyzed showed some disagreement with both genetic markers, mostly due to the detection of ITS1 sequences without obvious equivalent in mtDNA analysis (rMEDA, rB) and to the presence of unique sequences in the ITS1 analysis. This phenomenon has also been reported in other species (Doyle 1992; Manos, Doyle, and Nixon 1999; Mayer and Soltis 1999). The phylogenetic incongruence between markers could be explained by the different modes of evolution of both genome segments. Although point mutation and gene flow are the main forces to explain genetic diversity distribution in the mtDNA genome, the mechanisms of concerted evolution and recombination appear to be the main factors in the case of the rDNA family. The retention of the ancestral rMEDA sequence along the southern area could also indicate a role for selection in the pattern observed in brown trout ITS1.

The combined analysis of mtDNA, a haploid genome maternally inherited, with rDNA ITS1, a nuclear gene family subjected to mechanisms of concerted evolution, has proved to be useful for phylogeographic reconstruction in brown trout. Major phylogeographic events detected with mtDNA analysis have been confirmed after the analysis of ITS1 sequences. However, some other interesting features, such as the existence of specific hybridization areas, revealed by the presence of heterozygous individuals and recombinant sequences, as well as the presence of two putative ancestral brown trout lineages, were revealed after ITS1 analysis.

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APPENDIX I
Location and Genetic Characteristics of *Salmo trutta* Populations

Population	Code Number	COORDINATES		Basin	mtDNA Lineage[a]	ITS1	GenBank Accession Number
		Latitude	Longitude				
Vorob'yeb	1	66°25′N	33°20′E	Barents Sea	AT	1	AF434204
Triasheno	2	69°00′N	36°40′E	Atlantic	AT	$2_a/2_b$	AF434207/AF434208
Tavlaa	3	64°27′N	11°36′E	Atlantic	AT	rAT2/3	AF434209/AF434211
Jegesslerg	4	60°10′N	11°20′E	Baltic Sea	AT	rAT2	AF434205
Medja	5	56°15′N	32°40′E	Baltic Sea	AT	5	AF434245
Wdzydze	6	53°50′N	17°45′E	Wdzydze	na	rMEDA/6	AF434293/AF434226
Swibno	7	54°45′N	18°50′E	Baltic Sea	AT	7	AF434246
Karup	8	56°20′N	09°10′E	North Sea	na	rAT1	AY057991
Vltavaa	9	49°20′N	14°10′E	North Sea	AT	rAT2	AF434210
Riabhaich	10	50°30′N	04°40′W	Atlantic	AT	rAT1	AF434212
Sonaghen	11	54°30′N	08°10′W	Ireland	AT	rAT1	AF434281
Gillaroo	12	54°30′N	08°10′W	Ireland	AT	rAT1	AF434282
Ferox	13	54°30′N	08°10′W	Ireland	AT	rAT1	AF434284
Leizarán	14	43°12′N	02°00′W	Cantabric Sea	na	rAT1	AF434285
Nansa	15	43°15′N	04°25′W	Cantabric Sea	na	rAT1	AF434220
Trubia	16	43°15′N	06°00′W	Cantabric Sea	AT	rAT1	AF434214
Navia	17	43°28′N	06°41′W	Cantabric Sea	na	rAT1	AF434286
AbMiño	18	43°30′N	07°25′W	Atlantic	na	rMEDA	AF434294
SaMiño	19	42°41′N	07°29′W	Atlantic	na	rAT1/rMEDA	AF434287/AF434295
NvMiño	20	42°12′N	07°25′W	Atlantic	na	rAT1	AF434219
LuMiño	21	42°15′N	08°37′W	Atlantic	na	rAT1/rMEDA	AF434288/AF434296
Lima	22	41°50′N	08°20′W	Atlantic	na	rMEDA	AF434297
Ave	23	41°35′N	08°05′W	Atlantic	na	rMEDA	AF434298
PisDuero	24	41°30′N	05°20′W	Atlantic	na	rMEDA	AF434292
SaDuero	25	42°05′N	06°50′W	Atlantic	na	rMEDA	AF434289
SeDuero	26	42°04′N	06°22′W	Atlantic	na	rMEDA	AF434290
TorDuero	27	40°46′N	05°34′W	Atlantic	na	rMEDA	AF434299
TeDuero	28	40°55′N	08°05′W	Atlantic	na	rMEDA	AF434280
HozTajo	29	40°12′N	01°50′W	Atlantic	na	rAT1	AF434221
PelTajo	30	41°14′N	03°03′W	Atlantic	na	rAT1	AF434222
AlTajo	31	40°08′N	04°25′W	Atlantic	na	rAT1	AF434224
ZeTajo	32	40°25′N	07°30′W	Atlantic	na	rAT1	AF434225
SanGuadalquivir	33	36°45′N	06°25′W	Atlantic	na	rMEDA	AF434300
GenGuadalquivir	34	37°12′N	03°50′W	Atlantic	na	rMEDA	AF434291
Oued Oum er Rbia	35	33°05′N	05°12′W	Atlantic	AT	rAT2	AF434206
Oued el Kanar	36	35°14′N	05°05′W	Mediterranean Sea	AT	36	AF434203
Oued Berrem	37	32°40′N	04°47′W	Mediterranean Sea	AT	$37_a/37_b$	AF434201/AF434202
Guadalfeo	38	36°40′N	03°39′W	Mediterranean Sea	na	rMEDA	AF434223
Jucar	39	39°09′N	00°47′W	Mediterranean Sea	na	rME1	AF434215
Turia	40	39°40′N	00°15′W	Mediterranean Sea	na	rME1	AF434217
ClEbro	41	42°15′N	03°02′W	Mediterranean Sea	na	rME1	AF434283
PorEbro	42	42°04′N	02°35′W	Mediterranean Sea	na	rME1	AF434216
AraEbro	43	42°30′N	00°10′W	Mediterranean Sea	ME	rME2/rME3	AF434248/AF434251
MesEbro	44	41°10′N	01°51′W	Mediterranean Sea	na	rMEDA/44	AF434297/AF434227
CarEbro	45	42°15′N	01°00′E	Mediterranean Sea	na	rME1	AF434218
Llobregat	46	42°15′N	01°57′E	Mediterranean Sea	ME	rMEDA/rME3	AF434243/AF434249
Carença	47	42°26′N	02°13′E	Mediterranean Sea	ME	rMEDA	AF434242
Aude	48	42°45′N	02°08′E	Mediterranean Sea	ME	rMEDA/rME2	AF434241/AF434252
Tes	49	43°50′N	03°15′E	Mediterranean Sea	ME	rMEDA	AF434240
Sorgue	50	43°55′N	05°10′E	Mediterranean Sea	ME	rME2/50	AF434253/AF434255
Ascu	51	42°33′N	09°05′E	Corsica	AD	rAD1	AF434256
Pardu	52	39°50′N	09°30′E	Sardinia	AD	rAD2	AF434258
Fibreno	53	41°42′N	13°40′E	Tyrrhenian Sea	AD	rAD2	AF434260
Calore	54	40°46′N	15°00′E	Tyrrhenian Sea	ME	54	AF434234
Sinni	55	40°05′N	15°55′E	Ionian Sea	AD	rAD1	AF434257
Pellice	56	44°50′N	07°38′E	Adriatic Sea	MA	56	AF434279
Villafranca	57	44°50′N	07°10′E	Adriatic Sea	MA	rMA1	AF434228
Garda	58	45°50′N	10°40′E	Adriatic Sea	AD-MA-ME	rME1	AF434254
Socha	59	46°20′N	13°40′E	Adriatic Sea	MA	rDA	AF434274
Zadlascica	60	46°10′N	13°50′E	Adriatic Sea	MA	rMA1	AF434229
Idrija	61	46°00′N	13°55′E	Adriatic Sea	MA	rMA2/rMEDA	AF434231/AF434239
Hudda Grappa	62	46°10′N	14°00′E	Adriatic Sea	MA	rMA1	AF434230
Zala	63	45°55′N	14°05′E	Adriatic Sea	MA	rMA2/63	AF434233/AF434232
Muskovci	64	44°12′N	15°45′E	Adriatic Sea	AD	64	AF434264
Krupa	65	44°10′N	15°52′E	Adriatic Sea	AD	65	AF434265
Cetina	66	43°42′N	16°44′E	Adriatic Sea	DA	rMEDA	AF434238

APPENDIX I
Continued

Population	Code Number	Coordinates		Basin	mtDNA Lineage[a]	ITS1	GenBank Accession Number
		Latitude	Longitude				
Neretva	67	43°40′N	18°00′E	Adriatic Sea	DA	rMEDA	AF434237
Valbona	68	42°20′N	20°05′E	Adriatic Sea	AD	68	AF434259
Voidomatis	69	39°55′N	20°40′E	Adriatic Sea	ME	rME3	AF434250
Tripotamos	70	40°35′N	22°15′E	Aegean Sea	AD	rDA	AF434276
Nestos	71	41°20′N	24°40′E	Aegean Sea	AD	71	AF434244
Gören	72	39°45′N	27°10′E	Marmara Sea	DA	rB3	AF434266
Zeytinili	73	39°40′N	26°59′E	Aegean Sea	DA	rB2	AF434268
Göksu	74	36°40′N	32°38′E	Mediterranean Sea	AD	rAD2	AF434261
Dimcay	75	37°46′N	35°25′E	Mediterranean Sea	AD	rAD2	AF434262
Tohma	76	38°47′N	36°55′E	Persian Gulf	AD	rAD2	AF434263
Fyrat	77	39°35′N	38°40′E	Persian Gulf	DA	rB3	AF434267
Sava	78	46°26′N	13°53′E	Black Sea	DA	rDA	AF434277
Crimea	79	45°30′N	34°20′E	Black Sea	DA	rB1/rB2	AF434271/AF434269
Kodori	80	43°10′N	41°30′E	Black Sea	DA	80	AF434247
Terek	81	43°45′N	44°28′E	Caspian Sea	DA	rB1	AF434272
Sevan	82	40°35′N	45°00′E	Caspian Sea	DA	rDA/82	AF434275/AF434278
Kuterzi	83	53°40′N	58°30′E	Caspian Sea	DA	rMEDA/83	AF434236/AF434213
Sardamiana	84	39°08′N	68°15′E	Aral Sea	DA	rMEDA	AF434235
Oxus	85	36°45′N	72°00′E	Aral Sea	na	rDA	AF434301
Buna	86	43°15′N	17°50′E	Adriatic Sea	na	rB1/86	AF434273/AF434270

NOTE.—Populations are ordered from the northeast in the Atlantic area, following the European coast to the southern area, and then from the west to the east (see fig. 4).

[a] mtDNA lineages from Bernatchez et al. (1992): AT, Atlantic; DA, Danubian; ME, Mediterranean; AD, Adriatic; MA, *marmoratus*. na, not available.