

# Mitochondrial control region and protein coding genes sequence variation among phenotypic forms of brown trout *Salmo trutta* from northern Italy

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## Abstract

The Pô River basin of northern Italy is the home of distinctive and endemic morphological forms of brown trout *Salmo trutta*. We used PCR-direct sequencing and RFLP techniques to study variation in the mitochondrial control region of 225 trout in order to assess genetic relatedness among 18 populations from that region. The distribution analysis of these genotypes among north Italian populations confirmed the phylogenetic differentiation of marbled trout *Salmo trutta marmoratus* populations and the postglacial origin of *S. t. carpio*. Extensive genetic heterogeneity was observed among morphologically identical *S. t. fario* populations. Introgression with domestic strains of Atlantic basin origin was detected in all forms. In order to assess the phylogenetic congruence detected in coding and noncoding regions of the mitochondrial genome, we also analysed sequence variation in segments of the cytochrome *b* and ATPase subunit VI genes among representatives of all variants detected in the analysis of the control region. Variation in protein coding genes was only slightly less than that observed in the control region of the same individuals, both in terms of number of variants detected and of pairwise sequence divergence estimates among variants. Phylogenetic analysis based on protein coding genes sequences identified the same phylogenetic groupings defined by the control region analysis and also allowed a partial resolution of their phyletic relationships that was previously unresolved. However, coding and noncoding segments differed substantially in the transition-transversion ratio (17:0 in coding segments vs. 17:6 in control region segments).

*Keywords:* control region, mitochondrial DNA, phylogeny, polymerase chain reaction, protein coding genes, *S. trutta*

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## Introduction

Throughout its natural distribution range, the brown trout *Salmo trutta* L. is composed of numerous geographic forms which exhibit complex patterns of phenotypic diversity. Based on the extent of morphological differentiation, numerous attempts have been made to interpret the evolutionary history and resolve phylogenetic relationships among brown trout populations (Behnke 1968, 1972).

Nevertheless, there are still many uncertainties and inadequacies in the classification of the different forms, and consequently, considerable taxonomic confusion in this population complex still persists in contemporary literature (Lelek 1987). Thus, various morphological variants are still recognized either as full species, subspecies or morphs depending on authorship. The lack of understanding on the relationships and significance of phenotypic differentiation among brown trout populations has practical drawbacks for their conservation and management in the face of increasing exploitation and habitat alterations. For example, several genetic studies have demonstrated the polyphyletic origin of several ecological variants, such as anadromous and sedentary forms

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(Behnke 1986; Skaala & Naevdal 1989; Hindar *et al.* 1991; Skaala 1992). Conversely, it has been demonstrated genetically that morphologically similar populations may belong to very distinct evolutionary lineages (Guyomard 1989; Bernatchez *et al.* 1992). In both situations, management decisions solely based on levels of morphological distinction would fail to recognize the fundamental genetic basis of differentiation among these populations.

The Pò River basin of northern Italy is the home of several distinct forms of trout, some of which are highly distinctive and endemic to the Adriatic basin. These forms are considered as threatened in Europe (Lelek 1987). One of them is the marbled trout *Salmo trutta marmoratus*. It inhabits larger tributaries of this region where it is found in parapathy with the more typical fluvial landlocked form of trout, *S. trutta fario*, from which it can readily be distinguished by a complete lack of black and red spots on the body, the presence of a unique light-coloured marbled patterning and anatomical characteristics such as body shape (Sommani 1948, 1961). Based on its high morphological distinctiveness, it has been hypothesized that the marbled trout represents the earliest branching in the *S. trutta* complex and deserves specific status (Behnke 1968). Another morphologically unique population is endemic to Lake Garda and known as *carpio*, or *S. trutta carpio*. It is a small deep-water, bottom-spawning trout and, unlike any other trout population, it exhibits peculiar adaptations for a benthic mode of life (D'Ancona & Merlo 1958).

In a recent PCR-direct sequencing analysis of mitochondrial DNA sequence variation among European brown trout populations, levels of genetic differentiation did not correlate with the extent of morphological distinction of these forms (Bernatchez *et al.* 1992). Genetic data did not support the hypothesis that *marmoratus* represents the oldest branching in the *S. trutta* complex, and rather suggested that its branching does not appear earlier than that of any of the other major population groupings identified. The form *carpio* could not be associated with any distinct genotype but represented a mixture of different mtDNA groupings. However, because only a limited sampling of northern Italy was included in the study and these results were based only on variation of the noncoding, control region, it is possible that the apparent lack of congruence between phylogenetic hypotheses based on levels of morphological and genetic differentiation was due to an insufficient genetic characterization of these populations.

In this paper, we used PCR and direct sequencing techniques to compare mitochondrial DNA sequence variation observed among morphologically distinct *marmoratus* and *carpio* populations, and Pò River basin *fario* populations by analysing segments of two coding genes, the cytochrome *b* and the ATPase subunit-VI genes, in addition to segments of the mitochondrial control region studied by Bernatchez *et al.* (1992). This allowed us to: (a) test the congruence in

phylogenetic relationships among brown trout populations inferred from the analysis of coding and noncoding regions of the mitochondrial genome, (b) improve the resolution of the intraspecific phylogenetic tree previously obtained for *S. trutta*, (c) present a first detailed analysis of the genetic variation among north Italian brown trout populations and, (d) document the levels of genetic contamination among native populations due to the stocking programs in northern Italy.

## Materials and methods

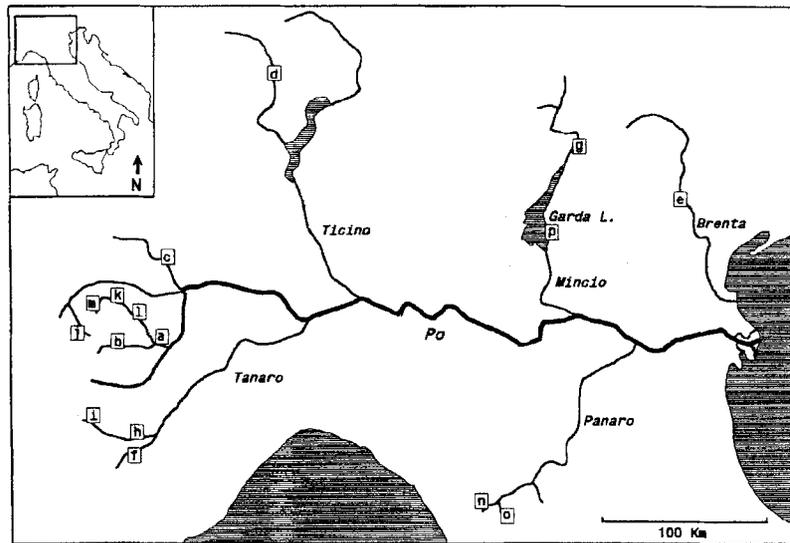
### Sample collections

A total of 225 trout were collected between 1990 and 1992. These samples represented eight populations of the form *marmoratus* distributed throughout its distribution range, seven populations of the form *fario* from the same or adjacent tributaries and the unique *carpio* population (Table 1, Fig. 1). The identification of specimens to a given morphological form was based on diagnostic characteristics, namely skin colour pattern and body shape. Most samples were obtained from apparently unstocked locations with domestic strains, unless otherwise indicated in Table 1. In order to detect the potential genetic contamination of native populations resulting from stocking, we also characterized mtDNA variation of individuals from the two major domesticated populations used in stocking programs in the Piemonte region and of eight additional individuals from a fish farm from Gournay, France.

**Table 1** Origins, phenotypic identification, and sample sizes (*N*) of trout populations sampled in the Pò River basin from northern Italy. Asterisks indicate populations currently stocked with domestic strains of Atlantic basin origin

Population	<i>N</i>	Phenotype
a Pellice Villafranca	10	<i>marmoratus</i>
b Pellice Torrepellice	10	<i>marmoratus</i>
c Stura di Lanzo	20	<i>marmoratus</i>
d Toce	10	<i>marmoratus</i>
e Brenta (*)	12	<i>marmoratus</i>
f Gesso	17	<i>marmoratus</i>
g Sarca (*)	8	<i>marmoratus</i>
h Stura di Demonte Vinadio	15	<i>marmoratus</i>
i Stura di Demonte Sambuco	15	<i>fario</i>
j Ripa	6	<i>fario</i>
k Chisone P. Argentina (*)	12	<i>fario</i>
l Chisone S. Germano (*)	10	<i>fario</i>
m Chisone Fenestrelle	15	<i>fario</i>
n Fontanaccia (*)	15	<i>fario</i>
o Tagliole (*)	12	<i>fario</i>
P Garda Lake	16	<i>carpio</i>
q Dom. Perosa Argentina	12	<i>domestic</i>
r Dom. Cuneo	10	<i>domestic</i>

**Fig. 1** Geographic locations of trout populations sampled in the Pò River basin for mtDNA sequence analysis. Shaded area indicates the distribution of the morphologically distinct form *Salmo trutta marmoratus* (after Sommani 1961).



#### DNA extraction

DNA was extracted from frozen muscle or liver following the protocol of mtDNA extraction of Bernatchez *et al.* (1988) or the protocol for total DNA extraction of Kocher *et al.* (1989). For two individuals, DNA was obtained from muscle of museum specimens fixed in formalin and conserved in ethanol (70%) after Pääbo (1990), adding a dialysis of the minced tissue in a microconcentrator (Centricon 30, Amicon).

#### DNA amplification and sequencing

Double-stranded PCR amplifications of the control region were performed in a programmable thermocycler (Prem III<sup>tm</sup>, LEP Scientific) using the same primers and protocols reported in Bernatchez *et al.* (1992). For museum specimens an additional primer (H2: 5' CGTTGGTCGGTTCCTAC-3') reported in Bernatchez & Danzmann (1993) was used in conjunction with primer L19 of Bernatchez *et al.* (1992) to amplify a region of approximately 350 bp at the 5' end of the control region. Moreover, BSA (bovine serum albumin, 0.1 µg/mL from Sigma, St Louis, MO) was added to the PCR mix used for museum specimens in order to overcome the inhibitory activity, of unknown origin, occasionally present in old material extracts (Pääbo 1990).

The primers L14841 and H15149 described in Kocher *et al.* (1989) and modified as McVeigh *et al.* (1991) were used to amplify a 308-bp segment at the 5' end of the cytochrome *b* gene. PCR reaction mix composition was identical to that described in Bernatchez *et al.* (1992). The thermal profile used for the cytochrome *b* gene segment was as follows: one denaturation (95 °C, 1 min) followed by 40 cycles of strand denaturation (92 °C, 1 min), primer annealing (55 °C, 1 min), and DNA extension (72 °C, 45 s).

For the ATPase subunit-VI gene, we used primers H9208 (5'-TATGCGTGTGCTTGGTGTGCCA-3') and L8558 (5'-AGCTTCTTCGACCAATTTATGAG-3') designed by E. Giuffra and P. Presa (Laboratoire de Génétique des Poissons, INRA, 78352 Jouy-en-Josas, France) from sequence homology between Pacific salmon (genus *Oncorhynchus*) and distant taxa (*Xenopus*, *Caenorhabditis*, *Strongylocentrotus* and human). This set of primers amplifies a 697-bp segment comprising the full gene. PCR conditions were as followed: a first denaturation step (95 °C, 1 min), followed by 35 cycles with denaturation at 92 °C (1 min), annealing at 62 °C (1 min), and extension at 72 °C (1 min) with a final elongation step (72 °C, 10 min).

Amplified DNA was purified either on Na45 nitrocellulose membranes (Scliecher & Schuell) according to the supplier's protocol or by selective alcohol precipitation (Benmansour, personal communication; Laboratoire de Pathologies des Poissons, INRA, 78352, Jouy-en-Josas, France) as follows:

- 1 extraction in 100 µL with 1 vol. chloroform-isoamyl alcohol (24:1);
- 2 10 minutes' precipitation in 1.4 M ammonium acetate (pH 5.0), 70% isopropanol and 15 min centrifugation;
- 3 DNA pellets rinsed in 200 µL of ethanol (70%) and centrifuged for 10 min; and
- 4 vacuum-dried pellets resuspended in 10 µL of sterile, distilled water.

Double-stranded DNA sequencing procedures were as described Bernatchez *et al.* (1992) with the following modifications. Sequencing of the 5' end of the control region was done using the internal primer H2. For coding genes segments, both light and heavy strand primers were used to obtain complementary sequences. Furthermore, the termination reaction tubes contained 1.5 µL each of ddNTPs mixture and 1.0 µL of the extension mix in order to elongate the readable portion of the sequence.

*RFLP analysis of the control region*

Based on the sequencing results of brown trout mitochondrial control region, we identified a diagnostic restriction site combination that allowed us to assign 88 additional individuals to one of the major mtDNA groupings described in Bernatchez *et al.* (1992), without the need of performing complete sequencing (Table 5). To do so, PCR-amplified DNA was purified by phenol/chloroform extraction, precipitated in 70% ethanol and resuspended in sterile, distilled water. *AluI* and *AsnI* restriction enzymes were used as recommended by the supplier to detect the presence or absence of the four restriction polymorphic sites that characterized the major mtDNA groupings.

*Sequence analysis*

Sequence data obtained for coding and noncoding gene segments were separately subjected to both distance and character-based analyses. The distance measure used was the estimate of nucleotide substitution calculated under the Kimura two-parameter model (Kimura 1980). The resulting matrices of pairwise distance comparisons were used to build trees using the Neighbour-Joining algorithm (Saitou & Nei 1987) on which confidence estimates were obtained from 1000 bootstrap replicates. This procedure was carried out by the program *SNJBOOT* provided by J. M. Cornuet (Laboratoire de Biologie et Génétique Évolutive, Université d'Orsay, France). Character-based analysis was realized using the *PHYLP* 3.5c computer package (Felsenstein 1993). Sequence data were used to generate phylo-

genetic trees according to a maximum parsimony criterion (analogous to Wagner trees) using the *DNAPARS* program. Majority-rule consensus trees were constructed using the *CONSENSE* program and confidence statements on branches were estimated by running *DNAPARS* on 1000 bootstrap replicates obtained by *SEQBOOT* program. These same analytical procedures were then performed on coding and noncoding segment, from data sets pooled together to generate overall phylogenetic trees. In all cases, trees were rooted using Atlantic salmon (*Salmo salar*) (obtained from Matredal hatchery, Norway) sequence as an outgroup.

Intra- and interpopulation genetic diversity was measured by the maximum likelihood estimation of the average number of nucleotide substitutions per site within and between populations (Nei 1987). We constructed a matrix of net interpopulation nucleotide divergence which was used to build a phenogram (*NEIGHBOUR*, option *UPGMA*; *PHYLP* 3.5c) relating all populations analysed.

**Results***Sequence variation and diversity of mtDNA genotypes*

The sequence of a 310-bp segment at the 5' end of control region (homologous to the one studied by Bernatchez *et al.* (1992)) was determined for 137 trout. A total of seven genotypic variants were identified for this segment among Italian populations (Table 2). All of them were previously detected among European populations and they represented four of the five phylogenetic groupings described in Bernatchez *et al.* (1992); Atlantic (AT), Adriatic (AD), Mediterranean (ME) and Marmoratus (MA).

Pop'n	(a) Sequence							(b) Sequence + RFLP			
	AT1	ME1	ME2	AD1	MA1	MA2	MA3	AT	ME	AD	MA
a						8					10
b						1		1			9
c						8		1			19
d					5 (2)	5					10
e	3					8 (1)		4			8
f	1					8 (1)		1			16
g						8					8
h						12 (1)					15
i				8 (2)						15	
j			5 (2)	1 (1)					5	1	
k			1	7		4			1	7	4
l								6			4
m			3	7 (2)		5			3	7	5
n		1 (1)						7	8		
o		1 (1)						4	8		
p			2	7 (2)		1 (1)	1 (1)	1	2	9	4
q	8 (1)							12			
r	8 (1)							10			

**Table 2** (a) Absolute frequency distribution of seven mtDNA variants detected among 137 *Salmo trutta* control region sequences; (b) absolute frequency distribution of the four major mtDNA groupings among 225 trout from the Pô River basin detected either by sequence or RFLP analyses. Genotype and mtDNA groupings designation refers to nomenclature used in Bernatchez *et al.* (1992); Atlantic (AT), Adriatic (AD), Mediterranean (ME) and *marmoratus* (MA). Numbers in parentheses refer to individuals used for cytochrome *b* and ATPase subunit-VI genes sequence analyses

We then subsampled 20 individuals representing each of these major groupings to determine sequence variation for a segment of both the cytochrome *b* and ATPase subunit VI genes (Table 2). We also extended the coding regions sequence analysis to four additional trout (for a total of 24 individuals) representing other control region genotypic variants (DA1, DA2, AD3 and AT2) reported in Bernatchez *et al.* (1992) (AD2 unavailable) in order to perform a more complete phylogenetic analysis of coding and

noncoding mtDNA sequences. The cytochrome *b* gene segment was homologous to the one analysed for *S. salar* by McVeigh *et al.* (1991). The ATPase subunit VI segment was homologous to that of rainbow trout *Oncorhynchus mykiss* analysed by Thomas & Beckenbach (1989). However, the sequence alignment of *S. trutta* and *S. salar* with that species revealed the insertion of four additional codons (Fig. 2). A minimum of 295 bp and 315 bp could be determined for each segment respectively (Fig. 2 a,b).

#### ATPase subunit VI

	1				50
<i>S. salar</i>	.....G..	.....A.....	.....G..	.....T..	
<i>S. trutta</i>	CGAAACCAGC	CCACCGCCGC	CCTCGGCCAT	CTCCTGCCTG	AAGGAACCCC
		<u>1</u>			
	51				100
<i>S. salar</i>	.C....A...	.....T..	.T.....	.....	.....
<i>S. trutta</i>	CGTTCCCTA	ATCCCCGTCC	TCATTATTAT	CGAGACAATT	AGCCTTTTTA
		<u>2</u>	<u>3</u>	<u>4</u>	
	101				150
<i>S. salar</i>	.....	.....	.....C..	....C..T..	.....
<i>S. trutta</i>	TCCGCCCCCT	CGCCCTTGGT	GTACGACTTA	CAGCTAACCT	CACAGCAGGC
	151				200
<i>S. salar</i>	.....A..	.....T..	.....	..A.....	.....
<i>S. trutta</i>	CACCTCCTCA	TTCAACTAAT	CGCCACAGCA	GCCTTTGTCC	TCCTACCTAT
				<u>5</u>	<u>6</u>
				<u>7</u>	
	201				250
<i>S. salar</i>	C..A.....	.....	.....	.....C...	..A..T....
<i>S. trutta</i>	AATGCCTACA	GTAGCAATCC	TAAGTTCTAT	CGTCCTATTC	CTGCTCACCC
		<u>8</u>			<u>9</u>
	251				300
<i>S. salar</i>	.....	.....	..A.....	.....	..T.....
<i>S. trutta</i>	TCTTGAAAT	TGCCGTTGCC	ATGATCCAAG	CCTATGTATT	TGTCCTACTC
	<u>10</u>		<u>11</u>		
	301	315			
<i>S. salar</i>	.....	.....			
<i>S. trutta</i>	CTAAGCCTCT	ACCTA	.....	H9208	

#### Cytochrome *b*

	1				50
<i>S. salar</i>	.C.....	.....C..	.....	.....C..	.....G..
<i>S. trutta</i>	CTTTGGCTCA	CTCTTAGGCT	TATGTCTAGC	CACCCAAATT	CTTACCGGAC
			<u>12</u>	<u>13</u>	
	51				100
<i>S. salar</i>	.....	.....	.....	.....	T.....
<i>S. trutta</i>	TCTTCCTAGC	CATACACTAC	ACCTCCGATA	TCTCAACAGC	CTTTTCCTCT
		<u>14</u>	<u>15</u>		
	101				150
<i>S. salar</i>	.....	.....	.....T..	.....	.....T.....
<i>S. trutta</i>	GTTTGCCACA	TTTGCCGAGA	TGTTAGCTAC	GGCTGACTCA	TCCGAAACAT
			<u>16</u>		<u>17</u>
	151				200
<i>S. salar</i>	.....	.....	.....	.....	.....C....
<i>S. trutta</i>	TCACGCCTAAC	GGAGCATCTT	TCTTCTTTAT	CTGTATTTAT	ATACATATCG
	201				250
<i>S. salar</i>	.....	T..T.....	.....T....	.....	.....
<i>S. trutta</i>	CCCGAGGACT	CTACTATGGT	TCCTACCTAT	ATAAAGAAAC	CTGAAATATC
	251				295
<i>S. salar</i>	.....T....	..T.....	.....	..T.....	..A....
<i>S. trutta</i>	GGAGTCGTAC	TGCTACTTCT	CACTATAATA	ACCGCCTTCG	TGGGC

Fig. 2 DNA sequences (L strand) of segments of the cytochrome *b* (Fig. 2a), and ATPase subunit-VI (Fig. 2b) mtDNA genes, from *Salmo trutta* type AT1 (domestic strain) and *S. salar*. Nucleotides are given for *S. salar* when different from *S. trutta*, while identity is indicated by dots. Variable positions among brown trout mtDNA variants are underlined and numbered. The four additional codons found by aligning the sequence of the ATPase subunit VI gene with that of rainbow trout are shadowed. The sequences presented in this paper (cf. Table 3) have been entered into GenBank under the accession numbers X74240 to X74245.

**Table 3** Variable nucleotide positions of cytochrome *b* and ATPase subunit VI genes observed among individuals representing 11 *Salmo trutta* mitochondrial control region variants described in Bernatchez *et al.* (1992). Numbers above sequences refer to variable nucleotide positions in Fig. 2. Nucleotide at each position is given for genotypes AT(1,2). For other genotypes, nucleotides are given when different from AT, and identity is indicated by dots. Abbreviations above sequences refer to amino acids. (\*) The non-silent mutation resulted in an Isoleucine – Valine replacement.

	ATPase subunit VI											Cytochrome <i>b</i>				
	1	2	3	4	5	6	7	8	9	10	11	12–13	14	15	16	17
	Gln	Leu	Ile*	Glu	Leu	Leu	Pro	Ala	Thr	Leu	Met	Leu	Ala	Tyr	Asp	Arg
'AT1,2'	CAG	CTA	ATT	GAG	CTC	CTA	CCT	GCA	ACC	CTT	ATG	TTA	GCC	TAC	GAT	CGA
ME1	..A	...	...	..A	...	..G	..C	..G	...	..C	...	C..G	..T	...	..C	...
ME2	..A	...	...	..A	...	..G	..C	...	...	..C	...	C..G	...	...	..C	...
AD1	..A	T..	...	..A	...	..G	..C	...	...	..C	...	...	...	...	..C	...
AD3	..A	T..	...	..A	..T	...	..C	...	...	..C	...	..G	...	...	..C	...
AD4	..A	T..	...	..A	...	..G	..C	...	...	..C	...	..G	...	...	..C	...
'MA1,2,3'	..A	...	G..	..A	...	..C	...	..T	..C	...	...	..G	...	...	..C	...
'DA1,2'	..A	...	...	..A	...	..G	..C	...	...	..A	...	..G	...	..T	..C	..G

Levels of variation detected among coding regions were slightly less than that reported for the control region, both in terms of number of mtDNA variants detected and sequence divergence estimates among variants. The cytochrome *b* gene segment represented the least variable region, with six substitutions detected, compared to eleven in the ATPase subunit-VI gene (Table 3) and 17 in the 5' end segment of the control region analysed among the same 24 individuals. Sequence variation in both coding gene segments was strictly due to transitional events (TS\TV ratio; 17:0). Fourteen transitions occurred at the third base of a codon, while the three others involved first positions. Only one substitution in the ATPase subunit VI gene involved a conservative amino acid replacement (Table 3). In the 5' and 3' end segments of the control region, the transition:transversion ratio was nearly 3:1 (17:6) and three deletions were found (Bernatchez *et al.* 1992). By combining the variable positions for coding segments, a total of eight genotypic variants were detected as opposed to 11 in the control region (Table 3). Several control region genotypic variants collapsed into a same coding segment variant, namely MA1, MA2 and MA3, DA1 and DA2, AT1 and AT2. In contrast, there was only one instance (AD4) where individuals with identical control region sequence (one AD1 genotype individual) differed in a coding segment sequence (Table 3).

Pairwise sequence divergence estimates among mtDNA variants detected in coding gene segments varied from 0.17 to 1.70% (mean  $\pm$  SD:  $0.93 \pm 0.40\%$ ), implying one to nine mutational steps ( $5 \pm 2.10$ ) among 610 bp between genotypes (Table 4). These estimates were slightly less than those reported by Bernatchez *et al.* (1992) for the control region: divergence estimates ranged from 0.16 to 1.92% ( $1.20 \pm 0.44\%$ ); mutational steps among 640 bp from 1 to 12 ( $7.5 \pm 2.8$ ).

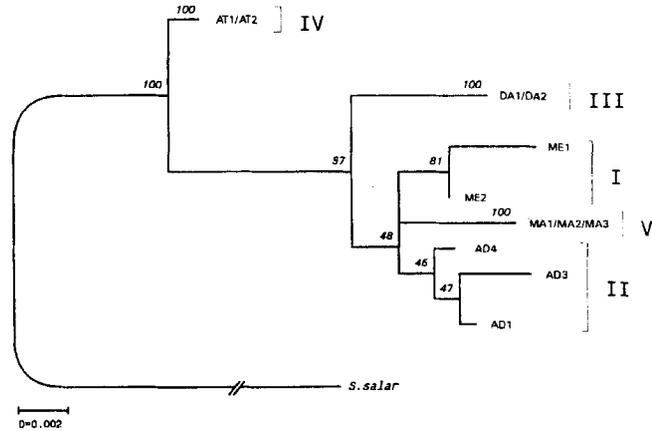
**Table 4** Matrix of pairwise percentage sequence divergence estimates (below main diagonal) and observed number of mutational events differences (above main diagonal) in the 295-bp cytochrome *b* and 315-bp ATPase subunit VI gene segments among 24 trout representing 11 mitochondrial control region genotypic variants described in Bernatchez *et al.* (1992)

	AT1,2	ME1	ME2	AD1	AD3	AD4	'MA 1,2,3'	'DA 1,2'
'AT1,2'		10	8	7	8	8	8	9
ME1	1.70		3	6	7	5	7	7
ME2	1.35	0.33		4	5	3	5	5
AD1	1.18	0.84	0.50		4	2	6	6
AD3	1.18	1.18	0.84	0.33		3	5	7
AD4	1.35	0.67	0.33	0.17	0.50		5	5
'MA1,2,3'	1.35	1.01	0.67	0.84	0.84	0.67		7
'DA1,2'	1.52	1.18	0.84	1.01	1.35	0.84	1.18	

#### Molecular trees

Both neighbour-joining tree and the majority-rule consensus tree resulting from the parsimony analysis of cytochrome *b* and ATPase subunit VI data had the same topology. The parsimony analysis resolved a minimum of 11 equally parsimonious trees and the consensus tree required 23 mutational steps with a consistency index of 0.74. In both analyses, all individuals that clustered into five separate groupings (AT, AD, MA, ME, DA) in the control region analysis also clustered distinctively in the coding regions analysis (Fig. 3). Similarly to the results of Bernatchez *et al.* (1992), the AD grouping was the least supported with a bootstrapping value of 45. Most branching pattern among these groupings also remained weakly supported. A major difference was in the resolution of the branching pattern of the AT grouping for which the early divergence from other groupings was supported by a high

**Fig. 3** Neighbour Joining tree clustering individuals representing 11 brown trout mtDNA control region variants reported in Bernatchez *et al.* (1992) according to sequence variation in their cytochrome *b* and ATPase subunit VI gene segments. Clusters are numbered as in Bernatchez *et al.* (1992). Numbers above branches indicate the number of bootstrap replicates (as a percentage) for which the groupings were supported. The topology of the majority rule consensus network resulting from parsimony analysis had identical topology to the Neighbour Joining tree.



bootstrap value (97) and by the rooting position between that grouping and the others in the coding region analysis. The distinction of the AT grouping was mainly due to homology at four nucleotide positions with *S. salar* that differed in all other *S. trutta* genotypes.

We then pooled sequence data obtained for both the control region and the coding genes to generate overall phylogenetic trees based on 1250 bp (Fig. 4). Both the neighbour-joining and the majority-rule consensus trees had identical topology. Five major groupings were identified and supported by bootstrap values varying from 84 to 100, thus confirming that they represent phylogenetically distinct assemblages. These groupings were well differentiated as indicated by the net sequence divergence estimates among them that varied from 0.79 to 1.24% (mean = 1.05%). They were also completely congruent to those identified solely on the basis of the control region analysis. The early branching of the AT grouping was still supported by the rooting position. Branching patterns among other groupings were not significantly supported although moderate bootstrap values (69 and 72) were in-

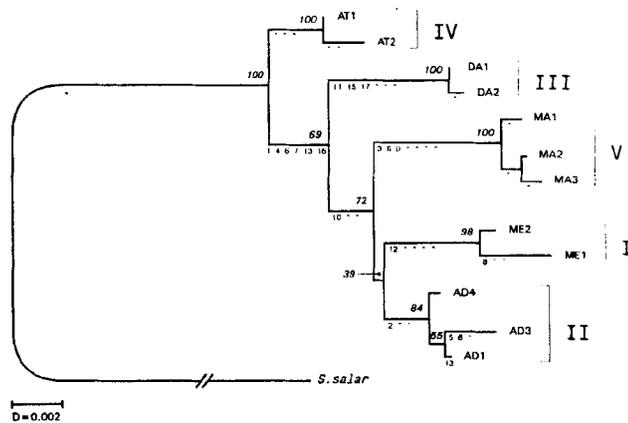
dicative that the DA grouping has a sister group relationship with the remaining three groupings (MA, ME, AD). The branching pattern among these three groupings still represented an unresolved trichotomy.

*mtDNA variation among north Italian brown trout populations*

Because sequence information obtained from both coding and noncoding segments was congruent in defining major mtDNA phylogenetic groupings, we used data from the control region only to document their distribution among trout populations from the P6 River basin. Beside the 137 individuals that were sequenced, we analysed an additional 88 trout by diagnostic enzymatic restriction assays (Table 5).

All *marmoratus* populations were clearly distinguished from others (Table 2). Thus, 93% ( $n = 86$ ) of the 92 *marmoratus* analysed belonged to mtDNA grouping MA. The other six *marmoratus* possessed the AT genotype, which was characteristic of all fish from the two Italian

**Fig. 4** Neighbour Joining tree among individuals representing 12 brown trout mtDNA variants resolved by the combination of cytochrome *b*, ATPase subunit VI, and control region segments sequence data ( $n = 1250$  bp). The five groupings resolved are identical to those obtained from the control region analysis alone. Confidence statements (in percent) estimated from 1000 bootstrap replicates are given above branches. The majority-rule consensus tree resolved from parsimony analysis had identical topology and comparable confidence statements; numbers of the variable coding region sites are drawn on each branch (cf. Table 3), while arrowheads refer to the positions of the variable control region sites as reported by Bernatchez *et al.* (1992).



and the French domestic strains surveyed. The AT grouping was naturally absent from southern drainages (Bernatchez *et al.* 1992), and has been introduced in northern Italy from domestic strains originating from the Atlantic basin.

In contrast to *marmoratus* populations, the *carpio* population was not specifically associated with any given genotype, and was characterized by all four major mtDNA groupings, including that of domestic origin.

High levels of heterogeneity in mtDNA variation were observed among *fario* populations in which all four major mtDNA groupings were observed with variable frequencies (Table 2). The domestic (AT grouping) genotype was found at relatively high frequencies in most populations known to be heavily stocked, except in the Sarca and Chisone P. Argentina populations. Furthermore, the domestic genotype was observed in the Gesso population, not reported to have been stocked. For other mtDNA groupings, some populations, such as Stura di Demonte R. vs. Fontanaccia and Tagliole R. were respectively fixed for AD and ME mtDNA groupings. Conversely, samples from Ripa R. and Chisone R. possessed both AD and ME groupings. In addition, the MA grouping, that dominated *marmoratus* samples was also present among Chisone R. samples.

The distance matrix of net interpopulation nucleotide divergence was used to construct a UPGMA tree relating all 18 trout populations studied (Fig. 5). The populations

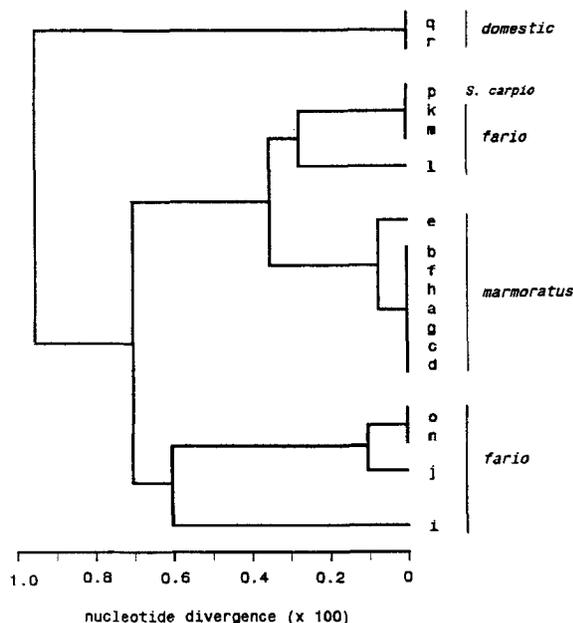


Fig. 5 Population tree (UPGMA) clustering 18 *S. trutta* populations from the Pô River basin according to the distance matrix resulting from the maximum likelihood estimation of the net average number of nucleotide substitutions per site among populations based on the 5' end of the control region (310 bp). Letters refer to populations listed in table 1.

Table 5 Polymorphic restriction sites from the control region for restriction enzymes *AsnI* and *AluI* that discriminate the four major mtDNA groupings found among trout populations from the Pô River basin. Numbers refer to variable sites reported in Bernatchez *et al.* (1992). The presence of a given cut site for a particular mtDNA grouping is indicated by the complete restriction site recognition sequence. Nucleotides are given when different from the recognition sequences and identity is indicated by dots

	Enzyme		
	<i>AsnI</i> (5)	<i>AluI</i> (12-13)	<i>AluI</i> (20)
'AT1,2'	. . . . G .	AGCT	. . . A
'MA2,3'	. . . . G .	. AT .	AGCT
MA1	. . . . G .	. GT .	AGCT
'AD1,3,4'	. . . . G .	. GG .	. . . A
'ME1,2'	ATTAAT	AGCT	. . . A

clustered into several distinct groupings. All *marmoratus* populations formed a distinct cluster, while morphologically identical *fario* populations did not compose a monophyletic grouping but clustered into three different branches, reflecting the heterogeneous distribution of all major mtDNA groupings among these populations. The *carpio* population did not differentiate from *fario* populations, and clustered with the three *fario* samples from Chisone R. (k, l, m), also characterized by the mixture of AD and MA genotype groupings.

## Discussion

### Congruent levels of variation in protein coding genes and control region

Globally, the sequence analysis of coding ( $n = 610$  bp) and noncoding ( $n = 640$  bp) regions were comparable both in terms of mtDNA variants detected (8 vs. 11) and in sequence divergence estimates among the variants (mean: 0.93% vs. 1.20%). Congruence in the level of variability was also found at the interspecific level. The average coding gene sequence divergence estimates between *S. trutta* and *S. salar* (6.17–7.50%) were comparable to those observed for the control region (5.44–6.44%) and almost identical to those reported by Gyllensten & Wilson (1987) in an RFLP analysis (6.18–6.52%). However, they contrasted with previous observations for intraspecific variation in mammals reporting that the rate of sequence divergence in the control region (for the homologous segments analysed in the present study) is about five to 15 times greater than that of the coding gene region (Brown *et al.* 1982). Our results also contrasted with a recent study of Brown *et al.* (1993) which reported that the rate of sequence divergence in the control region of the white stur-

geon (*Acipenser transmontanus*) was comparable to that reported for mammals, being four to five times that observed over the entire mtDNA molecule by RFLP study. As a result, Brown *et al.* (1993) resolved twice as many variants in the control region analysis as in the RFLP study performed on the same individuals. Conversely, Bernatchez & Danzmann (1993) found that the mutation rate in the control region of brook charr (*Salvelinus fontinalis*, Salmonidae) was less than twice that observed over the entire mitochondrial genome by RFLP analysis, and therefore comparable to that observed in brown trout.

MtDNA variation in the control region of these salmonids also differed from that observed in mammals and sturgeon in the type of mutations observed. It has been generally documented that at the intraspecific level, there is a strong mutational bias favouring transitional events that outnumber transversions by a 10:1 to 30:1 ratio both in the control region (e.g. Aquadro & Greenberg 1983; Brown *et al.* 1993), and in the protein coding gene regions (e.g. Beckenbach *et al.* 1990; Carr & Marshall 1991; Smith & Patton 1991; Birt-Friesen *et al.* 1992). Similarly, we observed a strong transitional bias (ratio of 17:0) in mtDNA protein coding genes (cytochrome *b* and ATPase subunit VI genes) of brown trout. In contrast, ratios of 17:6 and 8:3 were observed in the control region of brown trout and brook charr (Bernatchez & Danzmann 1993), respectively. Such low ratios of transition/transversion events at the intraspecific level appears to be very unusual among vertebrates, and to our knowledge have been reported only for one other vertebrate species (Fajen & Breden 1992). The ratio of transitional mutations to transversions in the mtDNA molecule is generally believed to decrease towards higher taxonomic levels because undetectable multiple transitional events at more variable sites accumulate as a function of time since divergence. For instance, it has been reported in primates that the ratio may be as high as 32:1 at the intraspecific level, decreasing to a ratio of 3:1 between species that diverged 8 millions years ago, and reaching 1:1 when time of divergence exceeds 25 million years (Brown *et al.* 1982). In a sequence analysis of mitochondrial protein coding genes, Thomas & Beckenbach (1989) documented for *Oncorhynchus* species (Salmonidae) a transition: transversion ratio of 12:1 at the intraspecific level, 9:1 among the most closely related species, and 5:1 among the most distantly related Pacific trout and salmon. The same tendency was observed recently in an interspecific comparison of sequence variation of the control region in *Oncorhynchus* (Shedlock *et al.* 1992). In the present study, we observed comparable transition-transversion ratio for the coding gene (25:11) and control region (25:7) segments in interspecific comparison between *S. salar* and *S. trutta*. Therefore, while the strong transitional bias (17:0) in the mtDNA protein coding genes of brown trout was comparable to intraspecific values reported in

other vertebrates, the transition:transversion ratio (17:6) observed in the control region of the same individuals was closer to those reported for interspecific comparisons. As was reported in Bernatchez & Danzmann (1993) for *S. fontinalis*, our results suggest fundamental differences in patterns of mutation in the control region of *S. trutta*.

#### *Phylogenetic resolution of coding sequences*

A major objective of the present study was to verify the congruence of mtDNA sequence variation among coding (cytochrome *b* and ATPase subunit VI genes) and non-coding (control region) segments in order to re-examine the phylogenetic relationships among morphologically distinct trout populations from northern Italy. The analysis of coding gene sequences gave additional support to the definition of the five phylogenetically distinct mtDNA trout assemblages previously found among European trout populations (Bernatchez *et al.* 1992). It also allowed a better resolution of branching patterns among mtDNA groupings, namely in establishing the phylogenetic position of the AT grouping. It is noteworthy that the branching pattern of the AT mtDNA grouping was determined by identity at four nucleotide positions with *S. salar* that differed in all other *S. trutta* genotypes. The choice of a distant outgroup may cause apparent reversions of substitutions due to homoplasy which could explain the apparent homology between *S. salar* and AT grouping. In such a case, however, one would expect homoplastic events to be scattered randomly among different groupings, as was observed in the control region (Bernatchez *et al.* 1992). Thus, it appears more likely that synplesiomorphy can better explain the homology between *S. salar* and the AT grouping at these nucleotide positions. We can only speculate at present on the possible explanations for the retention of ancestral sequence in the AT grouping. Namely, stochastic lineage extinction could have determined the fixation of this genotypic grouping in early times of differentiation between *S. salar* and *S. trutta* from a common ancestor (Avice *et al.* 1983, 1987; Avice, Neigel & Arnold 1984). It could also be evidence of a deviation from the constant rate of molecular evolution (Gillespie 1984, 1986) or of selective processes (MacRae & Anderson 1988).

#### *MtDNA variation among trout populations in northern Italy*

Our results clearly demonstrated that all *marmoratus* populations are monophyletic in origin, and represented a distinct evolutionary lineage among the brown trout population complex. Based on sequence divergence estimates, and tentatively applying the '2% sequence divergence per million year molecular clock' (Brown *et al.* 1979), *marmoratus* would appear to have evolved independently

in the order of 500 000 years. Because there is growing evidence that the mtDNA mutation rate is highly variable among vertebrates but probably lower in fish than reported for higher vertebrates, we do not consider this value as absolute but only as a minimal estimate of the time of divergence for *marmoratus*. Protein electrophoretic data also revealed a strong differentiation of this form in the nuclear genome, and provided more evidence that *marmoratus* represents a distinct, monophyletic assemblage (Giuffra *et al.* 1991; Giuffra 1993).

In contrast, the origin of the phenotypically distinct *carpio* population appear much more recent than that of *marmoratus* as it was not associated with any phylogenetically distinct branching but composed of mtDNA groupings observed in other phenotypic forms of trout. These groupings most likely evolved in allopatry during the Pleistocene glaciations (Bernatchez *et al.* 1992). Their intermixing observed in *carpio* probably occurred postglacially, which would support the hypothesis of a recent morphological differentiation of that population (D'Ancona & Merlo 1958). Thus, *carpio* possibly represents another example of rapid morphological radiation among salmonid fishes during postglacial times (Behnke 1972). The other extreme of uncoupling between genetic and phenotypic differentiation was observed in *fario*, for which morphologically indistinguishable populations were as genetically differentiated as they were from the highly distinct *marmoratus*, revealing the presence of the ME (Mediterranean) grouping also in the Pô River basin and a marked introgression with the MA (*marmoratus*) grouping in the Chisone River samples.

The presence of the AT (Atlantic) grouping among many populations demonstrated that domestic strains introgressed with all native forms (*fario*, *marmoratus*, *carpio*). Consequently, these results show that the actual stocking program in northern Italy may significantly alter the genetic integrity of unique gene pools of trout found in that region. We are currently examining whether the pattern of genetic variation found in mtDNA of these populations is reflected in nuclear genes.

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