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Genetic diversity of trout (genus *Salmo*) from its most eastern native range based on mitochondrial DNA and nuclear gene variation

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Abstract

Russia and western Asia harbour trout populations that have been classified as distinct species and subspecies, most often on the basis of morphological and ecological variation. In order to assess their origins and to verify whether traditional taxonomy reflects their evolutionary distinctiveness, we documented their genetic relationships on the basis of mitochondrial DNA (mtDNA) RFLP, mtDNA sequence analysis, and allozyme variation. Both mtDNA and nuclear gene variation defined two ancient phylogenetic assemblages of populations distributed among northern (Baltic, White, Barents), and southern (Black, Caspian, Aral) sea basins, between which gene flow has been possible but limited in postglacial times. These results supported the traditional taxonomic differentiation between populations of these two regions. They provided weak support for the taxonomic distinction of southern brown trout (*Salmo trutta*) populations based on their basin of origin. They also refuted the hypothesis that L. Sevan trout (*Salmo ischchan*) diverged from a primitive brown trout ancestor. Nevertheless, all trout populations from southern sea basins possessed private alleles or mtDNA genotypes and were genetically distinct. Therefore, they represent unique gene pools that warrant individual recognition for conservation and management.

Keywords: allozymes, conservation, mitochondrial DNA, phylogeny, *Salmo*, taxonomy

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Introduction

The application of molecular systematics revealed that the sole analysis of phenotypic characters may lead to erroneous interpretations of evolutionary history (e.g. Avise & Nelson 1989; Bowen *et al.* 1991; Bernatchez *et al.* 1991; Bernatchez & Dodson 1994). The problem is particularly acute at specific and infraspecific levels where taxonomic recognition has often been based on limited numbers of morphological and ecological traits (Bernatchez 1995). In fish, the problem is enhanced by the propensity of many phenotypic characters to homoplasy, either because of plasticity or selective pressures generating convergence of expression (Svärdson 1979; Lindsey 1981). The growing concern that the conservation units of biodiversity should be evolutionarily significant (e.g. Waples 1991), stresses the need for a better understanding of the evolutionary history of populations.

Brown trout, *Salmo trutta* L., is a wide-ranging species complex native to Eurasia and north Africa. It exhibits a complex mosaic of phenotypic forms, involving distinct geographic morphs and considerable life history trait variation (Benhke 1972, 1986). Genetic studies, largely based on electrophoretic analysis of allozyme variation, have been most useful in assessing the origins of diversity among trout populations inhabiting western Europe (reviewed in Guyomard 1989; Ferguson 1989). They have also demonstrated in some instances the inadequacy of prevailing taxonomy in reflecting the evolutionary history of brown trout (e.g. Guyomard *et al.* 1984; Hindar *et al.* 1991).

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More recently, the analysis of mtDNA sequence variation among geographically and phenotypically remote populations from western and central Europe revealed the existence of five major phylogenetic groupings of populations that were geographically disjunct, indicating their possible allopatric origins (Bernatchez *et al.* 1992; Giuffra *et al.* 1994). In contrast, no mtDNA studies assessed the relationships and origins of populations inhabiting the most eastern part of the species range, that is, Russia and western Asia. This vast area harbours many forms of trout (Berg 1948). Based on morphological and life-history trait variation, populations from different sea basins have been classified into distinct taxa. Thus, populations from the Barents, Baltic, and White sea basins are recognized as *S. t. trutta*, those from the Black sea basin as *S. t. labrax*, those from the Caspian sea basin as *S. t. caspius*, and those from the Aral sea basin as *S. t. oxianus*. In addition, distinct ecophenotypic forms from Lake Sevan are recognized as a distinct species, *S. ischchan*, presumably derived from the primitive ancestor of all brown trout populations (Benhke 1986).

The results of previous analyses of isozyme variation among these populations were partially concordant with traditional taxonomy (Osinov 1984, 1989, 1990a,b). They suggested a postglacial radiation of Sevan trout (*S. ischchan*) populations, and long-term isolation among populations of the northern and southern sea basins. However, those analyses provided limited information regarding the origins of these populations, and their relationships to more western populations. This was partly related to the fact that genetic data from different regions were not analysed together. It was also due to the lack of variation in some instances, and to the absence of information about allelic relationships.

Because of its faster mutation rate and its capacity to provide phylogenetic information among alleles, the analysis of mitochondrial DNA variation may enhance the understanding of evolutionary history of these populations. The previous identification of five major phylogenetic population groupings further increases its usefulness to assess relationships in the species.

In this paper, we combined analyses of allozyme and mtDNA variation in order to (1) compare levels of differentiation revealed by both approaches among populations from the White, Barents, Baltic, Black, Caspian, and Aral seas basins, (2) test competing hypotheses regarding the origins of these populations, and their relationships to western populations, and (3) assess the congruence between genetically and phenotypically derived population assemblages.

Materials and methods

Sample collections

A total of 649 trout were collected between 1979 and 1993 from sampling sites representing drainages of six seas from Russia and western Asia (Table 1, Fig. 1). For allozyme analysis, samples of eye, liver, and white muscle tissue were kept frozen (–20 °C) for 0–12 months before analysis. Samples used for mtDNA consisted of either liver or white muscle tissues kept frozen (–20 °C) for one to 10 years, and then ethanol-preserved for one to two years before analysis. All samples were obtained from unstocked populations or from hatcheries maintaining local breeders.

Allozyme analysis

Electrophoresis was carried out in polyacrylamide gel (PAGE) following standard protocols (Davis 1964; Peacock *et al.* 1965), with the modifications of Osinov (1984). The enzymes examined in this study, their abbreviations, International Union of Biochemistry enzyme classification numbers (EC), the loci that code for them, and the tissues used are listed in Table 2. Designation of isoenzyme loci,

Sample		Population	Basin	Taxon	Year	Sample size	
						mtDNA	allozyme
1	Ar.1	Sardamiana R. (s)	Aral	*oxianus*	1987	6	14 (14)
2	Ar.2	Sofidaron R. (s)	Aral	*oxianus*	1987	7	53 (39)
3	Ca.1	Terek R. (a)	Caspian	*caspius*	1982, 1991	10	102 (19)
4	Ca.2	Arpa R. (s)	Caspian	*caspius*	1982	8	44 (10)
5	Se.1	Sevan L. (s)	Caspian	*ischchan*	1979, 1982	6	92 (11)
6	Bl.1	Kodori R. (a)	Black	*labrax*	1982	9	20 (18)
7	Bl.2	Crimea (s)	Black	*labrax*	1992	5	6 (6)
8	Br.1	L. Triasheno (a)	Barent	*trutta*	1985	6	57 (24)
9	Wh.1	Nil'ma R. (a) Vorob'yev Br. (a)	White	*trutta*	1982, 1984 1986, 1990	7	170 (30)
10	Wh.2	Svyatoe L. (a)	White	*trutta*	1982	–	56 (15)
11	Ba.1	Medja R. (s)	Baltic	*trutta*	1993	8	28 (23)

Table 1 Origins, subspecific designation (specific for *S. ischchan*), years of collection and sample sizes of trout populations analysed. Letters in brackets refer to anadromous (a) or sedentary (s) forms. Taxonomic designation followed that of Berg (1948). Samples for Ca.1 were hatchery fry produced from wild breeders. For Wh.1, all samples for allozymes were from Vorob'yev Br. while samples for mtDNA were an unidentified mixture of Nil'ma R. and Vorob'yev Br. Number in brackets indicates sample size analysed for MEP-1* and GPI-1*

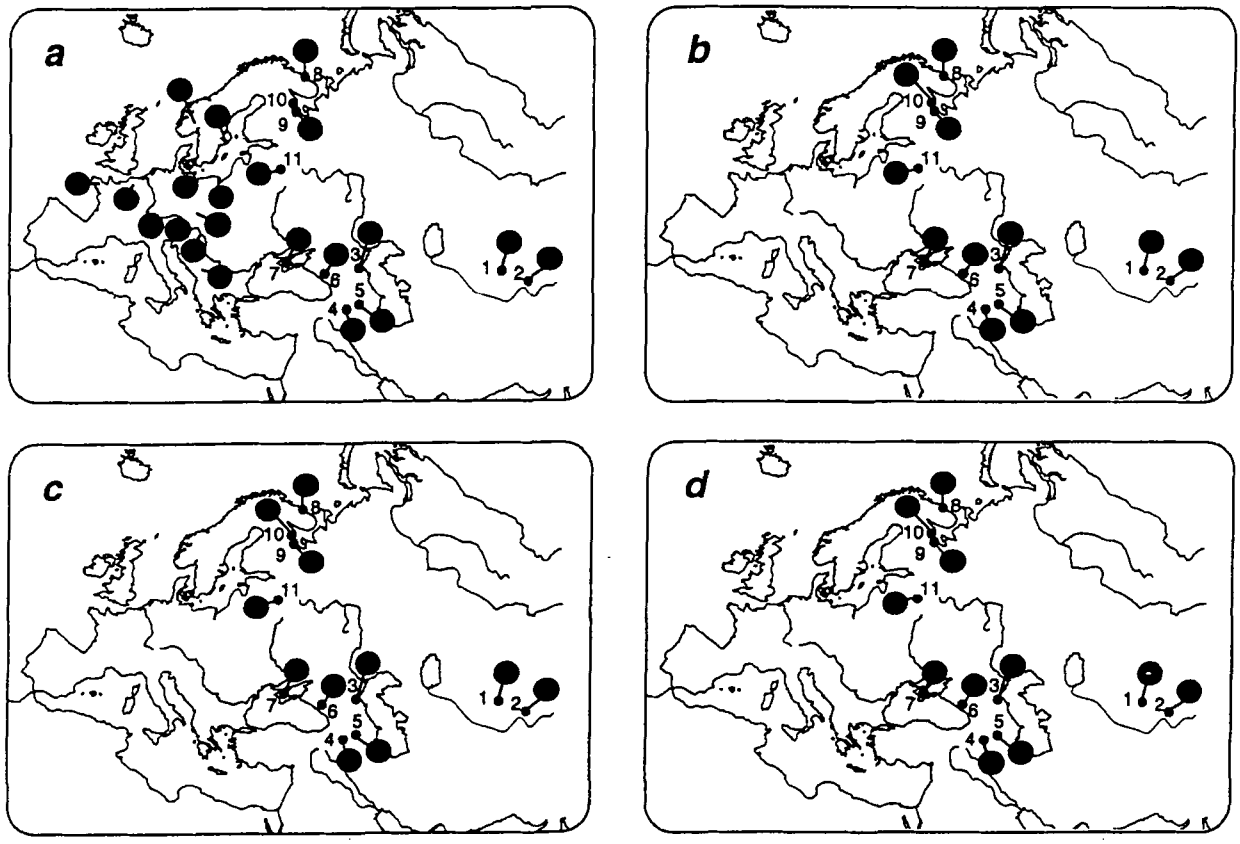

Fig. 1 Maps illustrating the geographic distribution of allelic variants detected in mtDNA and allozyme analyses: (a) mtDNA phylogenetic grouping IV (in black), and III (grey); (b) MEP-1*, allele 100 (black), and 80 (grey); (c) MDH-2*, allele 100 (grey) and 118 (black); (d) LDH-5*, allele 90 (black), and 100 (grey). Numbers refer to population designation in Table 1. Symbols without number for mtDNA data refer to native populations characterized by mtDNA groups III and IV, and reported in Bernatchez *et al.* (1992).

and allelic nomenclature followed those of Allendorf & Utter (1979) with modifications according to Shaklee *et al.* (1989).

Allele frequencies were estimated by direct allele counts. For isoloci (AAT-1,2*, MDH-3,4*; EST-5,6*; FH-2,3*), we used a genetic model that assumed equal frequencies of the common allele at both loci, as it conformed to Hardy–Weinberg (HW) expectations for all samples, except for loci EST-5,6* in Arpa R. In this case, a one polymorphic locus model best conformed to HW expectations. Good resolution of MEP-1* and GPI-1* phenotypes could not be obtained for all samples. Consequently, sample sizes at these loci were often smaller than for others (Table 1).

Levels of genetic variation and differentiation within and among populations were characterized from estimates of mean heterozygosity per locus (H), proportion of polymorphic loci (P; 99% criterion), mean number of alleles per locus (n_a), and standard genetic distance (Nei 1972). The extent of genetic differentiation among populations within and between regions was also evaluated from F_{st} estimates with corrections for sampling error (Wright 1978). The matrix of pairwise standard genetic distance values was used to construct a phenogram relating all populations analysed using the unweighed pair-group method of arithmetic averages (UPGMA; Sneath & Sokal 1973). Significance of differences in allele frequencies among samples was estimated by using the Snedecor-Irvin test of homogeneity (Workman & Niswander 1970).

Mitochondrial DNA analysis

Mitochondrial DNA variation was analysed both by sequencing and restriction fragment length polymorphism (RFLP) performed on PCR amplified products. Sequencing was done on the 5′-end 310 bp segment of the control region studied previously in brown trout (Bernatchez *et al.* 1992; Giuffra *et al.* 1994). The primers used in PCR and sequencing reactions were LN20, HN20, and H2 reported in Bernatchez & Danzmann (1993). Technical procedures of mtDNA purification, amplification, and sequencing are detailed in Bernatchez *et al.* (1992).

RFLP analysis was performed on two adjacent PCR amplified segments. One encompassed the complete ND-

Table 2 Enzymes, International Union of Biochemistry enzyme classification numbers (EC), loci, and tissues of expression for trout. Dashes indicate incomplete enzymatic nomenclature

Enzyme	EC No.	Locus	Tissue (a)
Aspartate aminotransferase	2.6.1.1	sAAT-1,2* (b)	m
Malic enzyme (NADP+)	1.1.1.40	mMEP-1,2* (c)	m
Malate dehydrogenase	1.1.1.37	sMDH-1,2* (c)	e,l
		sMDH-3,4* (b)	m,e
L-Iditol dehydrogenase	1.1.1.14	IDDH-1,2* (c)	l
Lactate dehydrogenase	1.1.1.27	LDH-1,2* (c)	m
		LDH-3,4* (c)	e,l
		LDH-5*	e
Esterase	3.1.1.–	EST-1*	l
		EST-2*	m
		EST-5,6* (b)	m
Esterase-D	3.1.–.–	EST-D*	m
Glycerol-3-phosphate dehydrogenase	1.1.1.8	G3PDH-2,3* (c)	m
Creatine kinase	2.7.3.2	CK-1,2* (c)	m
Superoxide dismutase	1.15.1.1	mSOD-1*	m,l,e
		sSOD-2,3* (c)	m,l,e
Octanol dehydrogenase	1.1.1.73	ODH*	l,m
Alcohol dehydrogenase	1.1.1.1	ADH*	l
Phosphogluconate dehydrogenase	1.1.1.44	PGDH*	m,l
Phosphoglucomutase	5.4.2.2	PGM-2*	m,l
Glucose-6-phosphate isomerase	5.3.1.9	GPI-1,2* (c)	m
		GPI-3*	m,l
Fumarate hydratase	4.2.1.2	FH-2,3* (b)	m
Diaphorase	–.–.–.–	DIA-1*	l
		DIA-2*	l

(a) tissues: m, white muscle; l, liver; e, eye.
(b) presumably duplicate and isoloci; most common alleles (100) are indistinguishable.
(c) presumably duplicate; the common alleles (100) are different.

5/6 region (approximately 2.4 kb) and was amplified with the primers of Cronin *et al.* (1993). The other segment (approximately 2.1 kb), comprised the cytochrome *b* gene, and the control region (D-loop), and was amplified using the complementary primer of the 3′-end primer of the ND-5/6 segment, and HN20. PCR conditions were as described by Bernatchez *et al.* (1995). As both regions were adjacent, restriction digests were done on pooled aliquots of 2–7 μL of each segment, and as recommended by suppliers (Gibco BRL, Pharmacia, Promega). Resulting fragments were electrophoretically separated on 1.2% agarose gels, ethidium bromide stained, and photographed under UV light.

The choice of restriction enzymes to be used was made by first screening a total of 19 enzymes on 12 genotypes found among five phylogenetic groupings previously identified from sequence analyses of coding (cytochrome *b*, and ATPase subunit VI genes) and noncoding (D-loop) mtDNA segments (Bernatchez *et al.* 1992; Giuffra *et al.* 1994). These enzymes were either multihexameric (*Ban*I, *Ban*II, *Hae*II, *Hinc*II), multipentameric (*Ava*II, *Nci*I), or tetrameric (*Alu*I, *Bsp*1286I, *Dde*I, *Hae*III, *Hha*I, *Hin*fI, *Hpa*II, *Mbo*I, *Mbo*II, *Msp*I, *Rsa*I, *Sau*3AI and *Taq*I).

We then pooled RFLP and sequence mutational sites detected among mtDNA genotypes in order to generate phylogenetic trees according to a maximal parsimony criterion, using the program MIX of the PHYLIP 3.5c computer package (Felsenstein 1993). A majority-rule consensus tree and confidence statements on branches were obtained using the CONSENSE program performed on 10 000 different trees generated by MIX from bootstrap replicates produced by the SEQBOOT program. This procedure allowed to select a set of six enzymes (*Hin*fI, *Hpa*II, *Mbo*I, *Nci*I, *Rsa*I, and *Taq*I) that were the most informative in assigning new mtDNA genotypes to either one of the major phylogenetic groupings.

Distinct single endonuclease patterns generated by these enzymes were identified by a specific letter in order of appearance, and used in combination with sequence variation to describe RFLP/sequence composite genotypes. Matrices of restriction sites presence-absence, resolved from mtDNA fragment patterns (Bernatchez & Dodson 1991), and sequence variation were then used to estimate phylogenetic relationships among mtDNA genotypes according to the maximal parsimony criteria described above. Intra- and interpopulation genetic diversity was quantified from the frequency distribution of mtDNA genotypes, and their pairwise divergence estimates by the

maximum likelihood estimation of the average number of nucleotide substitutions per site within and between populations (Nei 1987). Genetic differentiation among populations was also evaluated from F_{st} estimates (Wright 1978) by considering the mitochondrial genome as a unique locus with mtDNA genotypes corresponding to distinct alleles (Chapman 1989). The matrix of nucleotide diversity among populations was used to build a phenogram with the program NEIGHBOUR (option UPGMA; PHYLIP 3.5c). A randomized chi-square contingency analysis was used to test for heterogeneity of mtDNA genotypes among populations (Roff & Bentzen 1989).

Results

Allozymes

A total of 38 loci, corresponding to 16 enzymatic systems were examined (Table 2). Polymorphism was detected at 23 of these, for an overall P estimate of 61% (Table 3). Intrapopulation levels of genetic variation were highly variable, as exemplified by observed heterozygosity (H), proportion of polymorphic loci ($P_{0.99}$), and mean number of alleles per locus (n_a) (Table 3). Populations from the Aral Sea basin (Ar.1, Ar.2), and Sevan L. (Se.1) showed extremely reduced levels of polymorphism whereas the highest variability was observed in White (Wh.2) and Caspian (Ca.1) Sea basin populations. Tests of fit between observed and expected genotype distribution revealed that all samples were in Hardy–Weinberg equilibrium at all polymorphic loci, with the exception of population Ca.1 (1991 sample) that deviated from equilibrium at loci EST-5,6*. Furthermore, statistically significant changes in allele frequencies at some loci (G3PDH-2*, EST-2*, LDH-1*, LDH-5*, MDH-2*) were recorded among temporal samples in populations Wh.1 and Ca.1.

Many loci revealed a strong pattern of geographic differentiation in allele frequencies. Near fixation for alternate alleles was observed at MEP-1* between northern (White, Barents, Baltic), and southern (Caspian, Black, Aral) sea basin populations (Fig. 1). This geographic dichotomy was enhanced by the presence of alleles unique to each region at nine other loci (EST-5*, EST-6*, MDH-2*, IDDH-1*, IDDH-2*, G3PDH-2*, CK-1*, and FH-2,3*) and by differences in allele frequency at locus LDH-5*. Consequently, both regions formed two distinct populations clusters diverging by a Nei's standard genetic distance of 0.077 (Fig. 2a). Important genetic differentiation among all samples was also reflected by an overall F_{st} estimate of 0.478.

High levels of genetic variation were also found among populations within each region (Table 3). All pairwise comparison of allelic frequency distribution among populations were statistically significant ($P < 0.05$). Within the

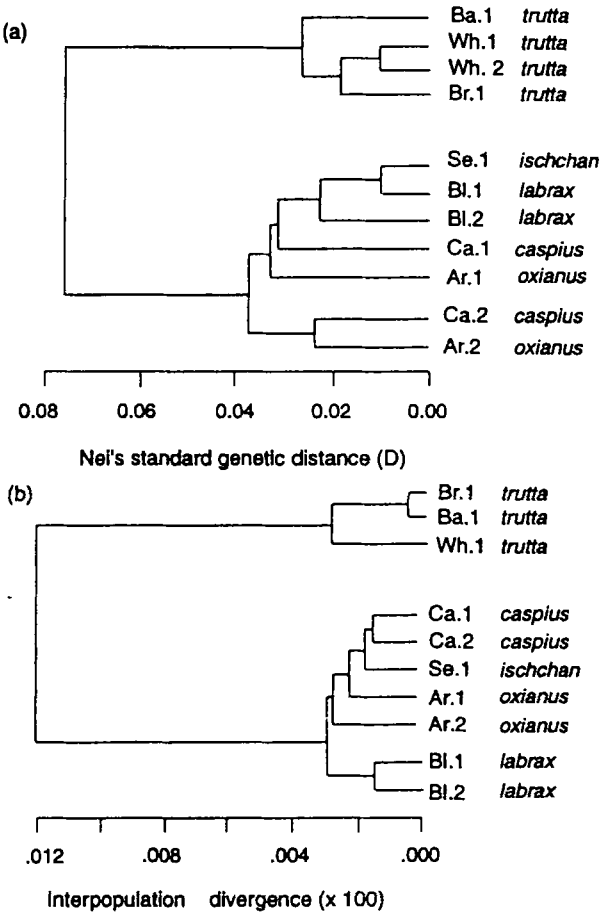

Fig. 2 (a) Allozyme phenogram showing genetic relationships among 11 trout populations, based on a UPGMA cluster analysis of Nei's pairwise standard genetic distance matrix estimated from allele frequency at 38 loci; (b) MtDNA population tree (UPGMA) clustering 10 populations trout populations according to the distance matrix resulting from the estimation of interpopulation nucleotide diversity. Complete names of populations are given in Table 1.

northern region, the Baltic Sea population (Ba.1) was the most differentiated, lacking alleles common to other populations at four loci (AAT-1,2*, LDH-1*, LDH-5*). Restricted gene flow among samples was also indicated by an overall F_{st} estimate of 0.150.

Interpopulation genetic diversity was even more important among southern populations as indicated by the overall F_{st} estimate of 0.392. Private alleles were found at five loci among three populations (Ca.1, Ca.2, Ar.1). Populations from the same sea basin were highly differentiated. Thus, fixation for alternate alleles at two loci (EST-2*, EST-5*), and highly significant allele frequency differences ($P < 0.001$) at two others (IDDH-2* and ODH*) were found between the two samples from Amu-Darya river system (Ar.1 and Ar.2). Significant allelic differentiation ($0.05 < P < 0.001$) was also observed between Bl.1 and Bl.2

Table 3 Allele frequency distribution, average heterozygosity (H), proportion of polymorphic loci ($P_{0.99}$), and mean number of alleles per locus (n_a) calculated for 38 enzyme loci in brown and Sevan trout populations (Table 1)

		Population										
Locus	Allele	Ba.1	Br.1	Wh.1	Wh.2	Bl.1	Bl.2	Ca.1	Ca.2	Ar.1	Ar.2	Se.1
AAT-1,2*	100	1.00	0.989	0.849	0.661	0.940	0.917	1.00	1.00	1.00	0.943	0.986
	120	–	0.011	0.151	0.339	–	–	–	–	–	–	0.002
	70	–	–	–	–	0.060	0.083	–	–	–	0.057	0.012
MEP-1*	100	1.00	1.00	1.00	1.00	†	0.083	–	–	–	–	†
	80	–	–	–	–	1.00	0.917	1.00	1.00	1.00	1.00	1.00
MEP-2*	100	1.00	1.00	1.00	1.00	1.00	1.00	0.970	0.818	1.00	1.00	1.00
	90	–	–	–	–	–	–	–	0.182	–	–	–
	70	–	–	–	–	–	–	0.030	–	–	–	–
MDH-2*	100	0.870	0.419	0.745	0.554	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	118	0.130	0.581	0.255	0.446	–	–	–	–	–	–	–
MDH-3,4*	100	1.00	0.993	1.00	1.00	1.00	0.958	1.00	0.989	1.00	1.00	1.00
	50	–	–	–	–	–	–	–	0.011	–	–	–
	120	–	0.007	–	–	–	0.042	–	–	–	–	–
LDH-1*	100	1.00	0.961	0.967	0.804	1.00	0.917	1.00	0.943	1.00	1.00	0.982
	OQ	–	0.039	0.033	0.196	–	0.083	–	0.057	–	–	0.018
LDH-4*	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.987	1.00	1.00	1.00
	120	–	–	–	–	–	–	–	0.013	–	–	–
LDH-5*	100	–	0.206	0.284	0.598	1.00	0.917	0.962	0.670	1.00	1.00	1.00
	90	1.00	0.794	0.716	0.402	–	0.083	0.038	0.330	–	–	–
EST-2*	100	1.00	1.00	1.00	1.00	1.00	1.00	0.426	0.295	1.00	–	1.00
	90	–	–	–	–	–	–	0.402	–	–	–	–
	110	–	–	–	–	–	–	0.172	0.705	–	1.00	–
EST-5*	100	1.00	1.00	1.00	1.00	0.684	1.00	0.534	0.307	–	1.00	1.00
	90	–	–	–	–	0.316	–	0.466	0.693	1.00	–	–
EST-6*	100	1.00	1.00	1.00	1.00	0.684	1.00	0.534	1.00	1.00	1.00	1.00
	90	–	–	–	–	0.316	–	0.466	–	–	–	–
IDDH-1*	100	0.643	0.754	0.944	0.836	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	-80	0.357	0.246	0.056	0.164	–	–	–	–	–	–	–
IDDH-2*	100	1.00	1.00	1.00	1.00	0.947	0.250	0.446	1.00	0.810	1.00	1.00
	70	–	–	–	–	0.053	0.750	0.554	–	0.190	–	–
G3PDH-2*	100	0.839	0.548	0.943	0.902	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	75	0.161	0.452	0.057	0.098	–	–	–	–	–	–	–
CK-1*	100	0.357	0.908	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	115	0.643	0.092	–	–	–	–	–	–	–	–	–
SOD-1	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.864	1.00	1.00	1.00
	117	–	–	–	–	–	–	–	0.136	–	–	–
ODH*	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.820	1.00	1.00
	85	–	–	–	–	–	–	–	–	0.180	–	–
GPI-1*	100	1.00	1.00	1.00	1.00	0.944	1.00	0.526	1.00	1.00	1.00	1.00
	70	–	–	–	–	0.056	–	0.474	–	–	–	–
GPI-3*	100	0.929	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	120	0.071	–	–	–	–	–	–	–	–	–	–
FH-2,3*	100	0.839	0.898	0.725	0.720	0.882	1.00	0.899	0.881	1.00	1.00	1.00
	104	0.161	0.102	0.273	0.280	0.015	–	–	–	–	–	–
	92	–	–	0.002	–	0.103	–	0.101	0.119	–	–	–
	H	0.055	0.062	0.063	0.091	0.045	0.034	0.082	0.063	0.016	0.006	0.002
	$P_{0.99}$	0.184	0.315	0.237	0.237	0.211	0.211	0.237	0.289	0.053	0.053	0.079
	n_a	1.18	1.32	1.29	1.24	1.26	1.21	1.26	1.29	1.05	1.05	1.13

†The existence of allele MEP-1* 100 is not ruled out.

(Black Sea) at three loci (EST-5*, EST-6*, IDDH-2*), and between Ca.1 and Ca.2 (Caspian Sea) at eight loci (MEP-2*, LDH-5*, EST-2*, EST-5*, EST-6*, IDDH-2*, SOD-1*, GPI-1*).

While all populations were genetically differentiated, they did not cluster geographically or according to their taxonomy (Fig. 2a). Of particular interest is the lack of genetic distinctiveness of Lake Sevan trout (*Salmo ischchan*) from the clustering of *S. trutta* populations.

mtDNA RFLP variation among major phylogenetic groupings

The 19 restriction enzymes used to screen representatives of the five major phylogenetic groupings generated 133 restriction sites, corresponding to an estimated additional 576 bp analysed. Five of these (*Ban*I, *Ban*II, *Bsp*1286I, *Hae*II, *Hha*I) were monomorphic. Twenty-seven synapomorphic sites were detected among the other 14 enzymes, and these were used in combination with the 32 synapomorphies found in sequence analysis (Giuffra, Bernatchez & Guyomard 1994) to generate an overall majority-rule consensus tree (Fig. 3a). As in previous sequence analysis, genotypes clustered into five highly differentiated (net sequence divergence estimates among groupings varying from 0.0070–0.0149) and significant (confidence statements on group branches > 99%) phylogenetic groupings (I to V). The subset of six most informative enzymes generated 13 nonhomoplasic, synapomorphic sites, that were used with D-loop mutational sites to assign new genotypes to either one of the five major phylogenetic groupings with confidence > 99% (Fig. 3a).

Classification of new mtDNA genotypes to major phylogenetic groupings

A total of 310 bp was determined at the 5′-end of the control region of which 12 nucleotides were variable (Fig. 4). RFLP analyses generated a total 61 restriction sites, and 26 were variable. Twenty-two mtDNA genotypes were defined among 72 trout analysed (Table 4). All clearly belonged to two of the five phylogenetic groupings defined previously. Genotypes At1, At2, and At3 belonged to group IV (Atlantic) as they shared identical character states at the five diagnostic positions for this grouping and none with those of others (Fig. 3b). Genotypes At1 and At2 were the same as defined in Bernatchez, Guyomard & Bonhomme (1992). Genotypes Da3 to Da21 were not observed before, and belonged to group III (Danube) sharing identical character states at all diagnostic positions for this grouping, and none with others. No genotypes belonging to other groupings [I (Mediterranean); II (Adriatic); V (*marmoratus*)] were observed.

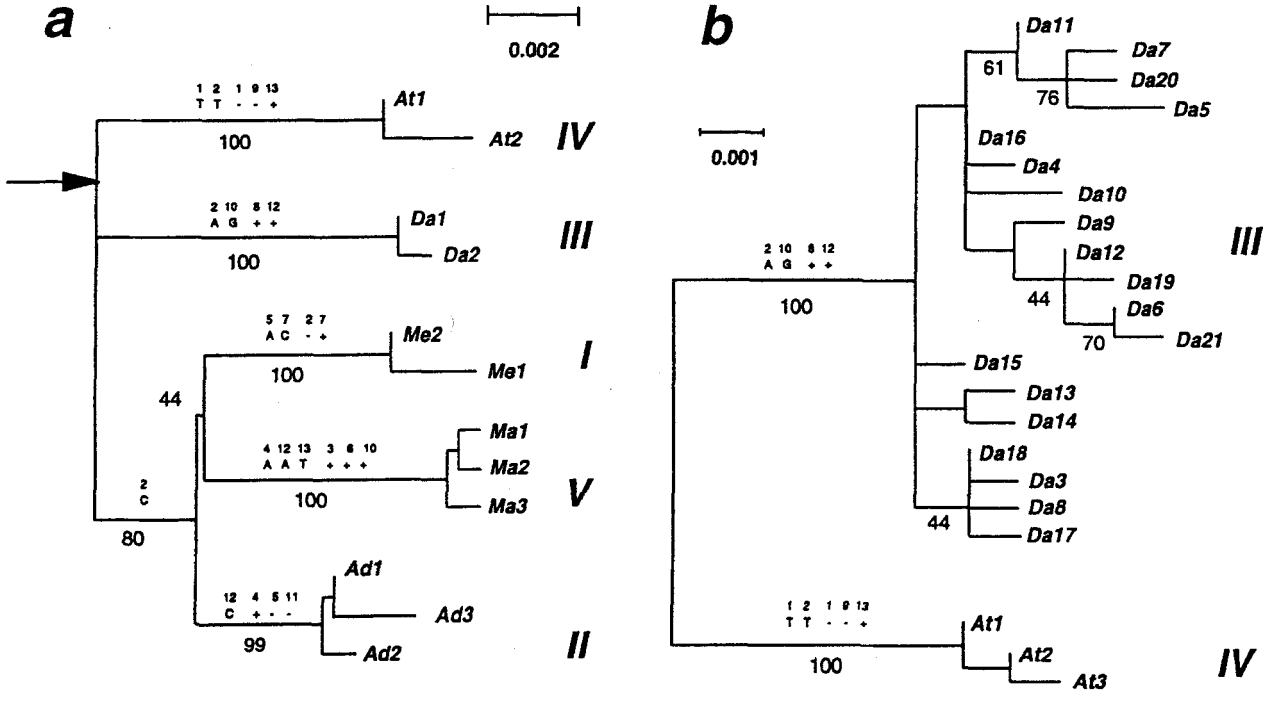

Fig. 3 (a) Majority-rule consensus tree relating five trout mtDNA phylogenetic groups (12 mtDNA genotypes), from the combination of 32 synapomorphic sites detected previously in sequence analysis of 1250 bp (Giuffra *et al.* 1994) and 27 synapomorphic sites detected in PCR-RFLP analysis with 19 restriction enzymes. Roman numbers refer to group designation from those studies. Character states of 13 diagnostic positions analysed in this study are indicated above branches, both for the D-loop sequence (capital letters indicating nucleotides), and restriction site data (+ = site gain, – = site loss). For the D-loop, numbers refer to variable positions reported in Bernatchez, Guyomard & Bonhomme (1992). For restriction site data, numbers refer to distinct enzymes: *Hin*fI (1, 2, 3), *Hpa*II (4), *Mbo*I (6), *Nci*I (7), *Rsa*I (8, 9, 10), and *Taq*I (11, 12, 13). Bootstrap estimates (as a percentage) are indicated below branches. Branch lengths are expressed in nucleotide substitution per site according to estimates from Neighbour-joining analysis. The arrow indicates the root position (*Salmo salar*) as determined by Giuffra, Bernatchez & Guyomard 1994; (b) Majority-rule consensus tree relating the 22 brown trout mtDNA genotypes identified among populations from six Russian and Asian sea basins. Genotype At1 was used as an outgroup taxon in assessing phylogenetic relationships within group III.

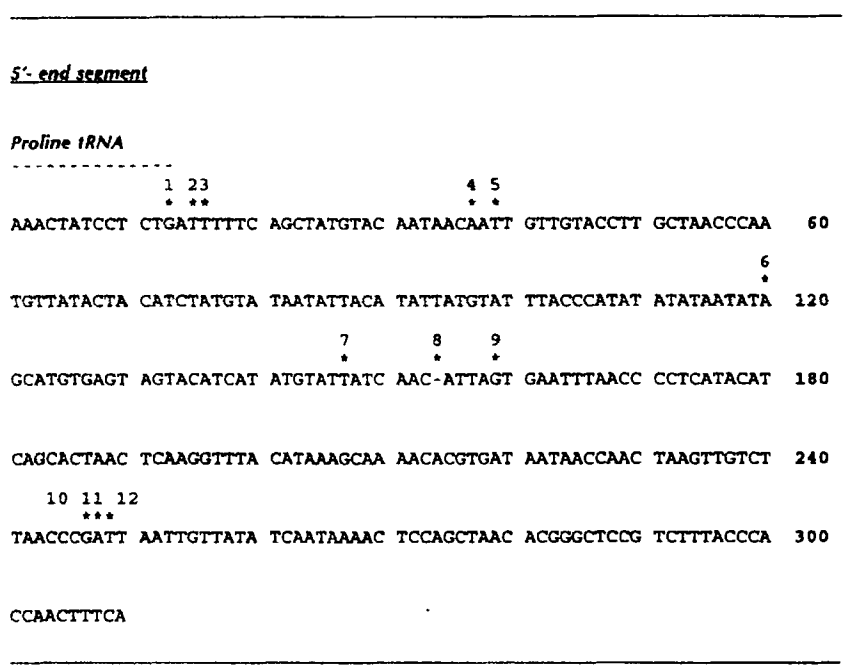

Fig. 4 Sequence for a 5′-end, 310-bp segment of the mtDNA control region, type At1, from *S. trutta*. The sequence shown is for the light strand, and includes part of the proline tRNA gene. Asterisks and numbers above mark the 12 variable positions among the 22 genotypes observed in this study. The sequences presented in this paper (cf. Table 4) have been entered into GeneBank under the accession numbers U18198 to U18205.

Phylogenetic relationships and sequence divergence among new mtDNA genotypes

In a first evaluation of phylogenetic relationships, no distinct clustering was observed beside the two major groups III and IV. This was apparently due to high levels of homoplasy at two adjacent positions (10 and 11) observed in the control region (Table 4, Fig. 4). The omission of these non informative sites in subsequent analyses and the use of genotype At1 as an outgroup revealed subclades within group III that were supported by moderate bootstrapping estimates (Fig. 3b). Thus, the position of the root separated genotypes Da3, Da8, Da13, Da14, Da15, Da17, and Da18 from other genotypes. Within these, Da3, Da8, Da13, and Da14 shared a unique apomorphy.

Genotype	RFLP fragment pattern	Control region variable sites											
		1	2	3	4	5	6	7	8	9	10	11	12
At1	AAAAAA	G	T	T	A	T	A	T	*	G	G	A	T
At2	BAAAAB	–	–	–	–	–	–	–	–	–	A	G	–
At3	BCABAB	–	–	–	–	–	–	–	–	–	A	G	–
Da3	DAAADC	–	C	–	–	A	–	–	–	–	A	–	G
Da4	CBAADC	A	C	–	–	A	–	–	–	–	A	–	G
Da5	CBACBC	–	C	–	–	A	G	–	–	–	A	G	G
Da6	EBADDC	–	C	–	–	G	–	–	–	A	–	–	G
Da7	CBACBC	–	C	–	G	A	–	–	–	–	–	–	G
Da8	DAAADC	–	C	–	–	A	–	del	–	–	–	–	G
Da9	GBAADC	–	C	–	–	A	–	–	–	–	A	G	G
Da10	CBAACC	–	C	–	–	A	–	–	A	–	–	–	G
Da11	CBAABC	–	C	–	G	A	–	–	–	–	–	–	G
Da12	GBADDC	–	C	–	–	A	–	–	–	–	–	–	G
Da13	FAAADC	–	C	–	–	A	–	–	–	–	–	–	G
Da14	GAAAEC	–	C	–	–	A	–	–	–	–	–	–	G
Da15	CAAADC	–	C	–	–	A	–	–	–	–	A	G	G
Da16	CBAADC	–	C	–	–	A	–	–	–	–	–	–	G
Da17	IAAADC	–	C	–	–	A	–	–	–	–	–	–	G
Da18	DAAADC	–	C	–	–	A	–	–	–	–	–	–	G
Da19	CBADDC	–	C	–	–	A	–	–	–	–	–	–	G
Da20	HBACDC	–	C	–	–	A	–	–	–	–	–	–	G
Da21	EBADDC	–	C	–	–	A	–	–	–	–	–	–	G

Table 4 Genotype designation, composite definition from PCR-RFLP analysis, and variable positions in the control region sequence analysis among 22 *S. trutta* mtDNA genotypes. The order of fragment patterns corresponds to restriction enzymes *Hinf*I, *Hpa*II, *Mbo*I, *Nci*I, *Rsa*I, and *Taq*I. For control region sequence analysis, numbers refer to position in Fig. 4. Nucleotide at each position is given for genotype At1. For other genotypes, nucleotides are given when different from At1, while identity is indicated by slashes. 'del' indicates a deletion and the asterisk indicates a site of insertion.

Among the remaining genotypes Da5, Da7, Da11, and Da20 also shared a common apomorphy, and clustered separately from others. Finally, genotypes, Da6, Da12, Da19, and Da21 clustered distinctively.

Pairwise estimates of base pair substitutions between groups III and IV were high, varying from 0.0089–0.0166 (Table 6). These values are comparable to those observed in sequence analysis alone (Giuffra *et al.* 1994). Within-group variation was much lower, varying between 0.0014 and 0.0044 in group IV, and between 0.0007 and 0.0057 in group III.

Geographic distribution of mtDNA genotypes and population differentiation

MtDNA genotypes representing phylogenetic groups III and IV exhibited a strong geographic pattern of distribution. All fish collected from northern sea basins belonged to phylogenetic group IV whereas phylogenetic group III was fixed in Black, Caspian, and Aral sea basins (Fig.1, Table 5). Consequently, both regions composed two very distinct population clusters diverging by a mean inter-population diversity of 0.0125 (Fig. 2b).

Interpopulation diversity within each region was much lower, never exceeding values of 0.003. Nevertheless, and despite low sample sizes available for mtDNA analysis ($n < 10$ per population), significant substructuring ($P < 0.05$) was observed among populations. In northern sea basins, an overall F_{st} estimate of 0.590 reflected the important differentiation among samples. The White Sea sample (Wh.1) was dominated by genotype At1 not observed among other samples. The Baltic and Barents Sea samples (Ba.1 and Br.1) showed different genotype frequency distribution, although not statistically significant ($P = 0.06$).

Even more important was the genetic differentiation observed among southern sea drainages as indicated by an overall F_{st} estimate of 0.640, and the heterogeneous frequency distribution of genotypes. Among 19 detected genotypes, 18 were private, that is observed in a single location (Table 5). Consequently, significant differences in the frequency distribution of genotypes ($P < 0.05$) were observed among all populations. The geographic distribution of genotypes also partly correlated with their phylogenetic relationships (Fig. 3b). All trout from the Black Sea basin (*S. t. labrax*) belonged to the subclade composed by genotypes Da5, Da7, Da11, and Da20 not observed elsewhere. All trout but two from the Caspian Sea basin (*S. t. caspius* and *S. ischchan*) belonged to the subclade composed by Da6, Da12, Da19, and Da21 that was confined to this region. Trout from Sofidaron R. (*S. t. oxianus*) were characterized by the subclade comprising Da3, Da8, Da17, Da18 restricted to this population. As for allozymes, L. Sevan trout (*S. ischchan*) did not cluster distinctively from *S. trutta* populations, although characterized by genotypes Da6 and Da21 observed nowhere else.

Table 5 Frequency distribution of 22 mtDNA genotypes among 10 populations of trout from Russian and Asian sea basins.

	Population									
Genotype	Ar.1	Ar.2	Ca.1	Ca.2	Se.1	Bl.1	Bl.2	Br.1	Wh.1	Ba.1
At1	—	—	—	—	—	—	—	—	0.857	—
At2	—	—	—	—	—	—	—	1.00	0.143	0.625
At3	—	—	—	—	—	—	—	—	—	0.375
Da3	0.143	—	—	—	—	—	—	—	—	—
Da4	—	0.166	—	—	—	—	—	—	—	—
Da5	—	—	—	—	—	0.333	—	—	—	—
Da6	—	—	—	—	0.167	—	—	—	—	—
Da7	—	—	—	—	—	0.333	—	—	—	—
Da8	0.143	—	—	—	—	—	—	—	—	—
Da9	—	0.166	—	—	—	—	—	—	—	—
Da10	—	0.166	—	—	—	—	—	—	—	—
Da11	—	—	—	—	—	—	0.800	—	—	—
Da12	—	—	0.800	—	—	—	—	—	—	—
Da13	—	—	0.100	—	—	—	—	—	—	—
DA14	—	—	0.100	—	—	—	—	—	—	—
Da15	—	0.166	—	—	—	—	—	—	—	—
Da16	—	0.333	—	—	—	—	—	—	—	—
Da17	0.428	—	—	—	—	—	—	—	—	—
Da18	0.286	—	—	—	—	—	—	—	—	—
Da19	—	—	—	1.00	—	—	—	—	—	—
Da20	—	—	—	—	—	0.333	0.200	—	—	—
Da21	—	—	—	—	0.833	—	—	—	—	—

Table 6 Pairwise number of nucleotide substitutions per site averaged for mtDNA PCR-RFLP and control region sequence analysis (below main diagonal), and pairwise estimates of Nei's standard genetic distance for allozymes (above main diagonal) among 10 populations of trout from Russian and west Asian sea basins. Samples for Wh.2 were not available for mtDNA

Population	Ar.1	Ar.2	Ca.1	Ca.2	Se.1	Bl.1	Bl.2	Br.1	Wh.1	Ba.1
Ar.1		0.057	0.032	0.023	0.029	0.018	0.038	0.095	0.081	0.106
Ar.2	0.0013		0.042	0.021	0.027	0.034	0.043	0.093	0.078	0.104
Ca.1	0.0009	0.0017		0.034	0.034	0.021	0.028	0.102	0.086	0.113
Ca.2	0.0007	0.0023	0.0007		0.032	0.026	0.048	0.084	0.070	0.091
Se.1	0.0019	0.0029	0.0008	0.0014		0.006	0.016	0.063	0.049	0.074
Bl.1	0.0017	0.0033	0.0028	0.0023	0.0038		0.020	0.071	0.055	0.082
Bl.2	0.0008	0.0023	0.0018	0.0014	0.0028	0.0006		0.073	0.059	0.083
Br.1	0.0113	0.0122	0.0127	0.0124	0.0141	0.0134	0.0124		0.012	0.019
Wh.1	0.0088	0.0093	0.0099	0.0096	0.0112	0.0106	0.0096	0.0021		0.019
Ba.1	0.0118	0.0124	0.0130	0.0127	0.0143	0.0137	0.0127	0.0001	0.0022	

Discussion

Levels of congruence between nuclear gene and mtDNA variation

Results obtained from the analysis of mtDNA and allozyme variation showed elements of congruence and discrepancies. Both data sets demonstrated a strong genetic partitioning between trout populations from northern (White, Barents, Baltic), and southern (Black, Caspian, Aral) sea basins. The importance of this dichotomy was also stressed by the magnitude of genetic distance values. Both data sets also discriminated populations within each region, and revealed lower levels of genetic diversity among populations within than between each region.

MtDNA diversity was generally much higher than that of allozyme. Furthermore, there was no correlation between levels of intrapopulation diversity observed in the mitochondrial and nuclear genome. Thus, no mtDNA diversity was found in two populations (Ca.1 and Br.1) which were among the highest values of nuclear heterozygosity ($H = 0.063$ and 0.062). Reciprocally, mtDNA diversity was maximal ($h = 0.788$ and 0.545) in Aral Sea basin populations (Ar.1 and Ar.2) where nuclear heterozygosity was minimal ($H = 0.016$ and 0.006).

Population relationships within region was also different for both data sets. In the northern region, the Barents and Baltic sea samples clustered closer in mtDNA analysis while the Baltic sea population was the most distant in the allozyme survey. In the southern region, population samples had the tendency to group by sea basins based on mtDNA differentiation whereas there was no evidence of such grouping with allozymes.

Several reasons can explain differences observed between the analyses of mtDNA and allozyme variation. We cannot ignore the potential stochastic effects of low sample sizes which could be important for mtDNA. This may be partly responsible for the extremely high numbers of private genotypes observed among southern populations. Nevertheless, this cannot explain all differences observed. For instance, increasing samples sizes would not reduce levels of intrapopulation diversity for those with already high diversity values detected at low sample sizes.

Another explanation for the lack of congruence between both methods may be attributed to differences in analytical approaches. While the power to discriminate populations was comparable for both analyses, allozymes may be less informative than mtDNA to infer population relationships. Phylogenetic treatment of allozyme is complicated by the impossibility to relate differences in electrophoretic mobility to discrete character state changes. In contrast, variation observed in DNA sequence analysis (in this case mtDNA) is generally attributed to neutral single base pair substitutions which can be interpretable readily as gene genealogies.

Strong genetic drift related to population bottlenecks could also have resulted in random branching patterns of these populations. Such population bottlenecks is evidenced by the severely reduced allozyme diversity observed in some populations (e.g. Ar.1, Ar.2, Se.1; see also Osinov 1990a,b). In this case, difference in mutation rates may partly explain the high number of genotypes detected in mtDNA, although postglacial temporal frame (10 000 years) appears limited to restore such variation.

Origins of trout populations from Russian and Asian sea basins

Hypotheses proposed to explain the origins of the different forms of trout inhabiting Russia and western Asia can be summarized into two alternative scenarios. First, many investigators considered brown trout as a member of northern ichthyofauna which originated from the Atlantic basin and recently penetrated the Black, Caspian, and

Aral sea basins, following the last glacial retreats, some 15 000 years ago (e.g. Berg 1928; Balon 1968; Kuderskiy 1974). According to this scenario, recent colonization was possible by headwaters connections of the Palaeo-Rhine and Danube, as well as other rivers of southern and northern sea basins that connected via postglacial lakes which may also have served as glacial refugia for trout and other fishes. Besides, it has been proposed that brown trout may also have penetrated the Black Sea from the Mediterranean basin (e.g. Berg 1928). Alternatively, some authors hypothesized that Caucasian trout populations constituted the primitive form from which all populations of southern sea basins radiated and expanded their range (Derzhavin 1934; Vladimirov 1944, 1948).

The complexity of glacial history in these regions has hampered the usefulness of biogeographic, palaeontological, and geological data in identifying centres of origin for trout populations and testing alternative scenarios of recent and ancient colonization events. Thus, northern seas were intermittently covered with ice while in the south, rapid regressions caused important decreases in sea area. Furthermore, interdrainage connections between northern and southern sea basins may have developed several times. Consequently, the dynamics of change in distribution of trout in that area must have been very complex, and substantially different from its contemporary range.

Previous analyses of allozyme variation favoured the hypothesis of ancient divergence between trout from southern and northern sea basins (Osinov 1989, 1990a). High levels of divergence observed in the present study between populations from northern and southern sea basins clearly reinforced this view. This is particularly obvious from the pattern of mtDNA differentiation which showed a fixed distribution of two highly divergent mtDNA phylogenetic groupings. MtDNA analysis also provided additional knowledge regarding the origins of trout populations from the eastern part of the species range and their relationships to the more western ones. Based on their geographic distribution among trout populations from Europe (Fig. 1), the two mtDNA groupings observed in this study were described as the 'Atlantic' (Group IV) and 'Danube' (Group III) groupings (Bernatchez, Guyomard & Bonhomme 1992). Among the five phylogenetically distinct mtDNA groupings that were observed in that study, the Atlantic grouping has been the only one observed in the Atlantic basin, from France to Sweden, which suggested that the whole Atlantic basin was recolonized by this single group which may have diverged from all others more than 500 000 years ago (Bernatchez *et al.* 1992). Trout from the Baltic, Barents, and White Sea basins also clearly belonged to the Atlantic grouping, giving further support to the hypothesis of an ancient allopatric origin of all trout from the Atlantic basin. Phylogenetic group III (Danube) was originally observed in several individuals from remote headwaters of the Danube drainage in Austria, Germany, and Slovenia, where it mixed with fish from the Atlantic grouping (Bernatchez, Guymard & Bonhomme 1992). Thus, it was not possible to confirm the allopatric origins of these groups. The fixed distribution of group IV within the Black, Caspian, and Aral Sea basins indicated that it most likely evolved in isolation of other groupings in that vast hydrographic system for a very long period of time.

MtDNA data also refuted the hypothesis of recent, postglacial colonization of the Black Sea by trout from the Mediterranean basin. Previous analyses showed that mtDNA genotypes found in native, unstocked populations through the whole Mediterranean basin, from Spain to Turkey belonged to either one of the three major phylogenetic groupings, beside groups III and IV (Bernatchez *et al.* 1992; Giuffra *et al.* 1994; Bernatchez 1995). The important sequence divergence and disjunct geographic distribution of these groupings indicated their long isolation from trout populations associated with either the Atlantic or Danube grouping. Because they have not been found to overlap in distribution anywhere with mtDNA genotypes characteristic of trout from Black, Caspian, and Aral Sea basins, postglacial origins of trout in these basins by recolonization from the Mediterranean connections appears very unlikely.

While mtDNA data provided convincing evidence for the ancient divergence of northern and southern sea basins trout populations, sample sizes were too small to assess the possibility of secondary contact among these in postglacial times. While no mixing of phylogenetic groupings III and IV were observed in this study, this had been the case in native, unstocked populations of the Danube headwaters, indicating that interdrainage gene flow was possible in that area (Bernatchez *et al.* 1992).

In contrast, allozyme data provided strong support for secondary intergradation between both regions. Thus, alleles which were characteristic of one region were also detected at low frequencies in the other part of the range for many loci (MEP-1*, AAT-1,2*, MDH-3,4*, LDH-1*, LDH-5*, FH-2,3*). Of particular interest is the allelic variation observed at the LDH-5* locus. This locus has previously been the most informative marker to assess origins of trout among western populations (Hamilton *et al.* 1989; Guyomard 1989). Two alleles dominate at this locus. The LDH-5*100 allele is symplesiomorphic, being found in 12 salmonid species, while the LDH-5*90 allele is unique to brown trout. The LDH-5*100 allele was fixed in the Aral Sea basin, approached fixation or dominated among populations of Black and Caspian Seas, was less frequent than the LDH-5*90 allele in the Barents and White Sea basins, and was at low frequency or absent in the Baltic. (see additional data in Osinov 1984, 1989; Hamilton *et al.* 1989). Such pattern is fully consistent with the hypothesis postu-

lating a recent origin of the LDH-5*90 allele in one glacial refugium from north-western Europe followed by its postglacial dispersal through the range. There is direct and indirect evidence that the spread of the LDH5–90* allele may have been enhanced by selective advantage (Osinov 1984; Henry & Ferguson 1985; Hamilton *et al.* 1989).

In summary, the present study supported the hypothesis of an ancient monophyletic origin for all populations from northern and southern sea basins. It also indicated that gene flow among northern populations and those from Black and Caspian seas has occurred in postglacial times. The fact that the smallest genetic distances observed between northern and southern populations were among those from the White and Black seas suggested that the main dispersal route between northern and southern sea basins connected these two seas. Additional sampling and analyses will be necessary for verifying this hypothesis as well as for documenting with more scrutiny the rate and direction of gene flow, and the level of introgressive hybridization between both phylogenetic assemblages.

Levels of congruence between genetic variation and taxonomic designation

Based on slight differentiation in meristic counts and body proportion, Berg (1948) recognized trout populations from northern sea basins (Baltic, White, Barents) as a distinct subspecies (*S. t. trutta*) from more southern populations. Both mtDNA and allozymes supported this view by demonstrating an ancient monophyletic origin of these two groups. Therefore, distinct taxonomic recognition of the northern populations clearly reflects their evolutionary distinctiveness.

Populations from different southern sea basins (Black, Caspian, Aral) have been recognized as distinct subspecies solely based on criteria of slight morphological variation, namely differences in modal gill-raker counts (Berg 1948). The analysis of allozyme variation did not support the hypothesis that populations from each basin represent distinct evolutionary assemblages, as no diagnostic alleles were found, and genetic variation within basins was comparable to that among basins. In contrast, mtDNA data provided partial support for this hypothesis. However, given the limited confidence estimates obtained on the mtDNA gene tree and the low divergence estimates observed among sea basin populations, a firmer assessment of their relationships may require the use of finer analytical approaches. This study also refuted the hypothesis that L. Sevan trout (*S. ischchan*) is a species derived from the primitive ancestor of all brown trout populations (Benhke 1986). Clearly, L. Sevan trout represents morphologically and ecologically unique populations that evolved recently and belong to the same evolutionary lineage as all other trout from the Caspian, Black and Aral Sea basins.

While our results did not clearly support taxonomic partitioning among southern trout populations, they demonstrated that each of them were genetically very distinct, possessing private alleles or mtDNA genotypes in all instances. Therefore, they represent unique gene pools that warrant individual recognition for conservation and management.

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This study was accomplished in an attempt to further increase our understanding of the evolutionary history of the polytypic species that represents brown trout. This was done by pooling complementary effort and expertise on the analysis of mtDNA (LB) and nuclear gene (AO) polymorphisms. The major interests of both authors are in the understanding of patterns and processes of molecular and organismal evolution, as well as their significance to conservation.
