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Genetic structure and differentiation among Greek brown trout (*Salmo trutta* L.) populations

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Twenty five loci have been screened in four Greek brown trout (*Salmo trutta* L.) populations using starch gel electrophoresis. A number of alleles which were unique for the Greek populations were found. The degree of genetic differentiation within and among seven Greek brown trout populations was calculated. The values of genetic distance between these seven populations are similar to those reported for other populations of the same species. These results contradict the classification of Greek brown trout populations into five subspecies. Furthermore, the phylogenetic relationships between 19 European populations were investigated. The high values of genetic identity between the Mediterranean and the Black Sea populations indicate that they might be of common origin.

INTRODUCTION

Since the recognition of multiple molecular forms of enzymes (Markert and Moller, 1959), these gene products, called isozymes, have served as probes for the evaluation of the genetic diversity, population structuring and evolutionary relationships of many fish species (Allendorf and Utter, 1979; Ferguson, 1980). This appraisal is particularly useful among members of the family Salmonidae whose striking diversity in morphology, ecology and behaviour is found to reflect their evolutionary relationships and population structuring (Behnke, 1968). Furthermore, genetic variability constitutes a primary biological resource and must be preserved (Smith and Chesser, 1981). For instance, future breeding programmes will require genetically diverse material upon which to act (Vuorinen, 1984). Thus, there is a current awareness of the need to identify and document the genetic diversity, as well as to clarify the complex taxonomic status (Ryman, 1981) as a sound basis for the future management and conservation of the salmonid species.

The genetic diversity and population structure of brown trout (*Salmo trutta*) in lakes and rivers in Scandinavia (Allendorf *et al.*, 1976; Ryman *et al.*, 1978; Ryman, 1983), Ireland (Ferguson and Mason, 1981; Ferguson and Flemming, 1983; Flemming and Ferguson, 1983; Crozier and Ferguson, 1986), France (Krieg and Guyomard, 1983; 1985; Guyomard and Krieg, 1983) and Russia (Osinov, 1984) have been researched. However, only a few studies have been carried out on brown trout populations in Greece, especially on distribution (Economidis, 1973) and biology (Papageorgiou *et al.*, 1984*a*, *b*), even though there are several reasons to study them:

(a) Brown trout is a fish of high preference as regards sport fishing and constitutes a potential species for extensive breeding in Greece.
(b) The populations of brown trout have not been subjected to a massive transplantation programme, so they represent undisturbed natural populations.
(c) Based on morphological characters five subspecies have been recognised: *S. trutta macrostigma*, *S. trutta dentex*, *S. trutta peristericus*, *S. trutta pelagonicus* and *S. trutta macedonicus* (Heckel and Knerr 1858; Dumeril, 1858; Karaman, 1924; 1927; 1937). However, the use of morphological characters in taxonomy has some limitations because they are polygenically inherited with low heritability, so the taxonomic status of these populations is actually unclear. The origin of the populations of brown trout in Greece in relation to other European populations is also unclear.

In a former paper we reported the genetic structure of three populations of brown trout belonging

to the subspecies *S. trutta pelagonicus* (Karakousis and Triantaphyllidis, 1988). The aim of this paper is to expand our previous research including four other populations belonging to different subspecies, in an effort to understand their genetic structure and to clarify their evolutionary relationships. These studies also constitute a necessary step towards the aims of rational management and conservation of this valuable resource.

MATERIALS AND METHODS

Samples of brown trout were collected between 1985 and 1987 from seven streams in N. Greece (fig. 1). Fishes were obtained by electrofishing, specimens were transferred to the laboratory either alive or in ice and stored at −20°C until used.

Samples of skeletal muscle, liver, heart, eye and brain were excised and homogeneised in an equal volume of distilled water, centrifuged and the supernatant used for electrophoresis. Horizontal starch gels were prepared with 10 per cent hydrolysed starch. Buffers, electrophoretic conditions and the staining methods for each system are shown in table 1.

Eleven enzymic systems were studied corresponding to 25 loci. The nomenclature used to designate loci and alleles was that proposed by Allendorf *et al.* 1977) and Taggart *et al.* (1981). The allele frequency estimates result from allele counting. Average polymorphism level, total expected heterozygosity, standard genetic distance and genetic identity (Nei, 1972) were computed. The heterogeneity of the allele frequencies at the polymorphic loci was calculated by the Workman and Niswander (1970) homogeneity test. If there

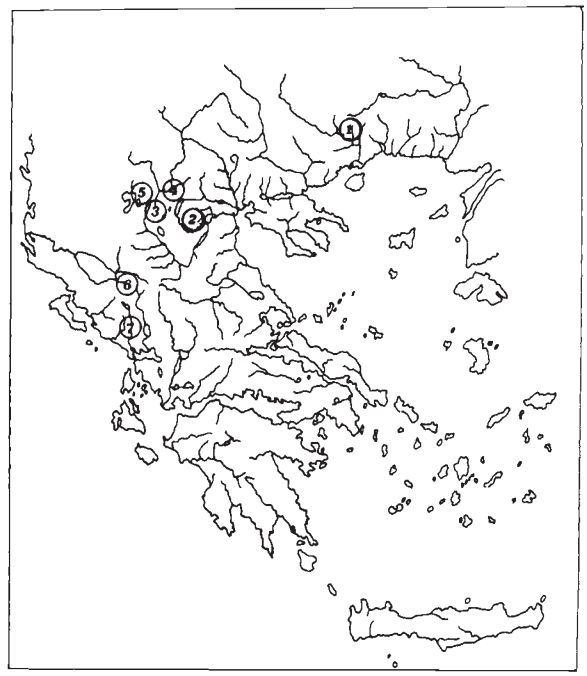

Figure 1 Geographical locations of rivers and streams where brown trout were sampled. 1. Nestos river, 2. Tripotamos stream, 3. Drosopigi stream, 4. Skopos stream, 5. Agios Germanos stream, 6. Voidomatis river, 7. Louros river.

are k alleles at a locus then the χ^2 statistic is given by:

$$2N\left(\sum_{J=1}^{k}\frac{\sigma_{PJ}^2}{\bar{P}_J}\right)$$

where $\bar{P}_J$ and σ_{PJ}^2 are the weighted mean and variance of the frequencies of the Jth allele at the

Table 1 Enzyme systems studied, abbreviations and electrophoretic conditions

System	Abbreviations	EC No	Migration buffer*	Staining methods†	Number of loci
Aspartate aminotransferase	AAT (GOT)	2.6.1.1	AM	3	3
α-glycerophosphte dehydrogenase	α-GPDH	1.1.1.8	TP	1	1
Creatine kinase	CK	2.7.3.2	TCB	2	2
Isocitrate dehydrogenase	IDH	1.1.1.42	AM	1	2
Lactate dehydrogenase	LDH	1.1.1.27	TCB	3	4
Malate dehydrogenase	MDH	1.1.1.37	AM	1	4
Malic enzyme	ME	1.1.1.40	TP	1	3
Phosphoglucomutase	PGM	2.7.5.1	A	3	1
6-phosphogluconate dehydrogenase	6-PGDH	1.1.1.44	AM	1	1
Phosphoglucose isomerase	PGI	5.3.1.9	A	3	3
Superoxide dismutase	SOD	1.15.1.1	A	3	1

* A = Allendorf *et al.* (1977); AM and TCB = Taggart *et at.* (1981); TP = Guyomard and Krieg (1983).
† 1 = Allendorf *et al.* (1977); 2 = Brewer (1970); 3 = Harris and Hopkinson (1976).

Table 2 Allelic frequencies of studied loci, mean heterozygosity (H), proportion of polymorphic loci (P) and interpopulation heterogeneity (χ^2)

Loci	Alleles	Louros $n=75$	Voidomatis $n=35$	Ag. Germanos $n=52$	Paranesti $n=69$	χ^2
Aat-1,2	100	1·00	1·00	0·4706**	0·6463	
	70	0·00	0·00	0·5294	0·3537	85·77++
Aat-4	100	0·5978	0·4848	0·8181	1·00	
	74	0·4022	0·5152	0·1819	0·00	71·61++
α-Gpdh-2	100	1·00	0·7576	1·00	1·00	
	50	0·00	0·2424	0·00	0·00	90·10++
Ck-1	100	1·00	1·00	1·00	1·00	
Ck-2	100	1·00	1·00	0·00	1·00	
	50	0·00	0·00	1·00	0·00	
Idh-1	100	1·00	1·00	1·00	1·00	
Idh-2	100	1·00	1·00	1·00	1·00	
Ldh-1	100	0·94	1·00	0·9904	0·9265**	
	0	0·06	0·00	0·0096	0·0735	10·32+
Ldh-2	100	1·00	0·9143	1·00	0·9044	
	0	0·00	0·0857	0·00	0·0956	24·47++
Ldh-4	100	1·00	1·00	1·00	1·00	
Ldh-5	100	0·00	0·00	0·00	0·0444**	
	105	1·00	1·00	1·00	0·9556	9·67+
Mdh-1,2	100	1·00	1·00	1·00	1·00	
Mdh-3,4	100	1·00	1·00	1·00	1·00	
Me-1	100	1·00	1·00	1·00	1·00	
Me-2	100	0·6933	0·0714	0·00	0·5	
	50	0·3067	0·9286	1·00	0·5**	163·33++
Me-3	100	1·00	1·00	1·00	1·00	
Pgm-1	100	1·00	1·00	1·00	1·00	
6-*Pgdh*-1	100	1·00	1·00	1·00	1·00	
Pgi-1	100	1·00	1·00	1·00	1·00	
Pgi-2	100	1·00	1·00	1·00	1·00	
Pgi-3	100	1·00	1·00	1·00	1·00	
Sod-1	100	1·00	1·00	1·00	1·00	
(H)		0·0407	0·0462	0·0326	0·0541	
(P)		12%	16%	12%	20%	

n = sample size; + significant at the $P=0·05$; ++ significant at the $P=0·01$ level; ** significant at the $P=0·05$ level under the Hardy–Weinberg equilibrium.

locus. For each allele the weighted mean is:

$$\bar{P}=\sum(N_i/N)p_i$$

where p_i is the frequency of the allele in the ith population. The weighted variance is:

$$\sigma_p^2=\sum(N_i/N)p_i^2-p^2.$$

Wright's (1965) standardised genetic variance (F_{ST}) was also calculated.

RESULTS

Most of the enzymic systems (tables 1 and 2) have previously been analysed in other populations (Allendorf *et al.*, 1977; Taggart *et al.*, 1981; Guyomard and Krieg, 1983). Our description and genetic interpretation were in agreement with the previous authors. The enzymic systems examined are discussed below.

AAT. Three polymorphic loci were studied in this enzymic system. The *sAat*-1,2 which are predominantly expressed in skeletal muscle and the *sAat*-4 which is expressed in liver. A variant allele, *sAat*-1,2(70), was observed in this investigation. This allele is probably identical with the *sAat*-1,2(75) allele reported by Osinov (1984), which exists only in Black Sea basin trout.

α-GPDH. Only one population was polymorphic with two alleles *α-Gpdh*-2(50) and *α-Gpdh*-2(100). In all other populations a constant three banded phenotype was observed in the skeletal muscle extracts examined.

CK. Utter *et al.* (1979) reported a common three banded phenotype for brown trout muscle extracts.

These isozymes have been identified as the products of two loci, *Ck*-1 and *Ck*-2. Polymorphism was reported at the *Ck*-1 locus. This polymorphism was not observed in the populations studied. All the populations, except one, showed the same three banded pattern. The population of Agios Germanos showed a two-banded pattern, with the anodal band missing. It is probably due to a different allele at the *Ck*-2 locus, the *Ck*-2(50).

IDH. Two loci appeared in the brain tissues studied. Both loci, *Idh*-1 and *Idh*-2, were monomorphic in all populations studied.

LDH. It is generally agreed upon that in brown trout LDH is coded for by five loci. In this investigation the products of four loci, *Ldh*-1, *Ldh*-2, *Ldh*-4 and *Ldh*-5 were studied (tables 1 and 2). Polymorphism for a null allele was observed at the *Ldh*-1 and at the *Ldh*-2 loci. Null alleles have been reported in other populations (Allendorf *et al.*, 1984; Taggart *et al.*, 1981; Osinov, 1984) at the *Ldh*-1 locus, but not at the *Ldh*-2 locus. Polymorphism has also been observed at the *Ldh*-5 locus, in the population of Nestos, with two alleles, *Ldh*-5(100) and *Ldh*-5(105). It is important that all the other populations studied were fixed for the ancestral allele *Ldh*-(105).

MDH. Very good resolution was obtained for the *Mdh*-1, 2 and *Mdh*-3, 4 loci in the heart tissue samples studied. Polymorphism was not observed in these populations.

ME. Three loci are assumed to code for this tetrameric enzyme. The most anodal locus (*Me*-3) presented a single invariant band in the muscle extracts studied. Polymorphism was detected at the *Me*-2 locus with two alleles, the *Me*-2(100) and *Me*-2(50), as previously described (Karakousis and Triantaphyllidis, 1988).

PGM, 6-PGDH and SOD. A single invariant band has been observed for each of these enzymes. They were the products of the *Pgm*-1, 6-*Pgdh*-1 and *Sod*-1 loci, respectively.

PGI. A constant six banded pattern has been observed in the muscle extracts examined. This phenotype is similar to that reported by Avise and Kitto (1973) and it is the product of three loci, the *Pgi*-1, *Pgi*-2 and *Pgi*-3.

Table 2 shows the alleles observed, their frequencies, the estimates of the average heterozygosity (H) and the proportion of polymorphic loci (P) in the four populations studied. Goodness of fit between the observed and expected genotypes was tested at polymorphic loci using the Hardy–Weinberg equilibrium. From fifteen comparisons only four departures from Hardy–Weinberg expectations were found in the phenotypic distributions (table 2). Thus, the populations seem to be panmictic and no significant deficiency of heterozygotes was observed. The homogeneity test (Workman and Niswander, 1970) shows significant interpopulation differences as regards allele frequencies at the polymorphic loci (table 2).

For an assessment of the level of genetic differentiation between the populations studied in this investigation and the ones studied previously (Karakousis and Triantaphyllidis, 1988), genetic distances and genetic identity were calculated on the basis of the 25 loci (table 3). A UPGMA dendrogram (fig. 2) constructed using this matrix of values (Sneath and Sokal, 1973) reveals an apparent lack of correspondence between geographic distance and genetic identity. This is a feature noted among brown trout populations studied elsewhere (Ryman, 1983). In the same dendrogram the time of divergence of the seven populations, as estimated from the genetic distance (Nei, 1975), is shown.

Table 3 Genetic distance (D) (below diagonal) and genetic identity (I) (above diagonal) of seven populations of brown trout from Greece

	1	2	3	4	5	6	7
Ag. Germanos	—	0·9393	0·9445	0·8978	0·9081	0·9249	0·9062
Voidomatis	0·0626	—	0·9730	0·9431	0·9594	0·9804	0·9514
Paranesti	0·0571	0·0274	—	0·9764	0·9792	0·9859	0·9843
Drosopigi	0·1078	0·0586	0·0239	—	0·9861	0·9813	0·9903
Skopos	0·0964	0·0414	0·0210	0·0139	—	0·9946	0·9918
Louros	0·0781	0·0198	0·0142	0·0189	0·0054	—	0·9896
Tripotamos	0·0985	0·0498	0·0158	0·0097	0·0082	0·0105	—

The gene frequencies used from the populations in italics come from a previous paper (Karakousis and Triantaphyllidis 1988).

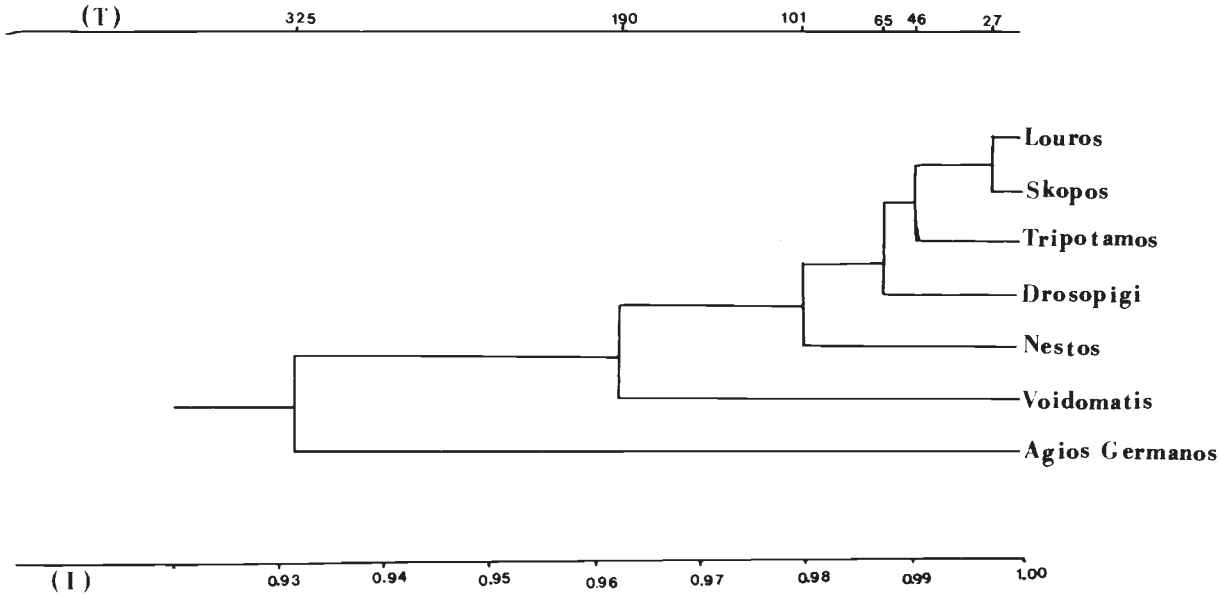

Figure 2 Dendrogram showing genetic relationships among seven brown trout populations from Greece, based on a UPGMA cluster analysis of Nei's (1972) mean genetic identity (I) values. Time of divergence (T), in thousand of years, is also shown.

In an effort to clarify the evolutionary relationships of the European brown trout populations, the genetic identity of 19 populations was calculated using data from the literature (Guyomard and Krieg, 1983; Krieg and Guyomard, 1983; Ferguson and Flemming, 1983; Ryman, 1983; Osinov, 1984; Crozier and Ferguson, 1986). The estimation was based on 17 loci: *Aat*-1,2; *Aat*-4; *α-Gpdh*-2; *Ck*-1; *Ck*-2; *Ldh*-1; *Ldh*-2; *Ldh*-4; *Ldh*-5; *Mdh*-1,2; *Mdh*-3,4; *Pgi*-1; *Pgi*-2; *Pgi*-3. A UPGMA dendrogram has been constructed (fig. 3) based upon the values of the genetic identity.

In order to further analyse the differentiation within and among the seven populations of brown trout from Greece the fixation indices (F_{IS}, F_{IT}, F_{ST}) were calculated (table 4). The mean value of F_{IS} was fairly low and the highest value of F_{IT} was in *Me*-2 locus (table 4). The mean value of F_{ST} was 0·2592, a fairly high value.

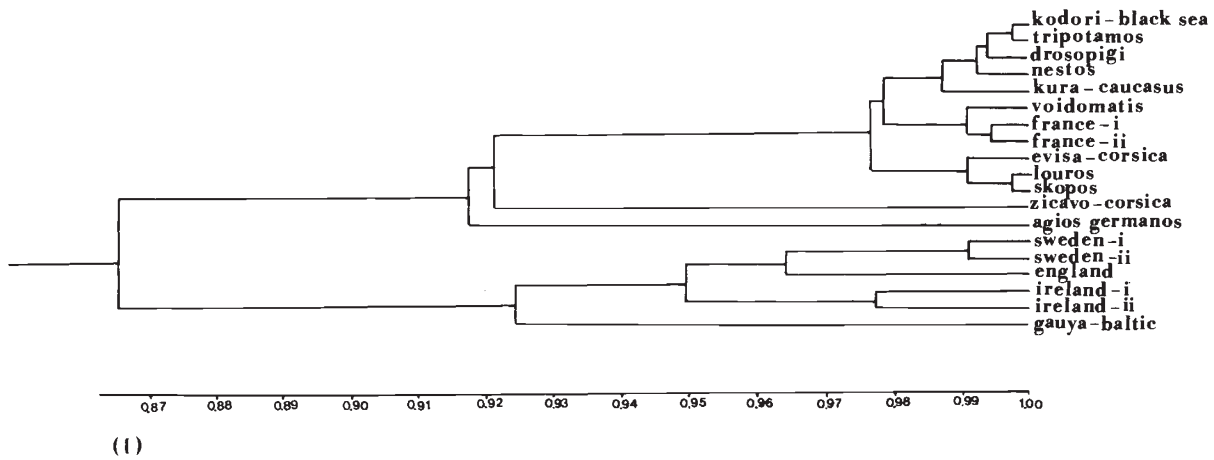

Figure 3 Dendrogram showing genetic relationships among 19 European brown trout populations, based on a UPGMA cluster analysis of mean genetic identity (I) values over 17 gene loci.

Table 4 Results of F-statistic of the polymorphic loci of the seven populations

Loci	F_{IS}	F_{IT}	F_{ST}
Aat-2	−0·1095	0·2862	0·3566
Aat-4	0·0287	0·2919	0·2832
α-gpdh-2	0·0625	0·1030	0·0432
Idh-2	0·2942	0·4148	0·1708
Ldh-1	0·6913	0·7069	0·0506
Ldh-2	0·1676	0·2069	0·0473
Mdh-2	−0·2595	0·1743	0·3444
Mdh-4	−0·2724	0·0914	0·2859
Me-2	0·2970	0·8782	0·8267
Sod-1	0·0243	0·2023	0·1829
Mean values	0·0924	0·3356	0·2592

DISCUSSION

Genetic differentiation

One of the most striking features of brown trout is its genetic diversity. Krieg and Guyomard (1983) reported that 55 per cent of the polymorphism of this species exists between populations. Thus it is not surprising to find alleles which were unique to the populations studied. The alleles *Me*-2(50), *Ldh*-2(0) and *Ck*-2(50) have not previously been reported for other populations and one of these (*Ck*-2(50)) is restricted to one population (Ag. Germanos). Also the allele frequencies found in these populations differ significantly from other European populations, especially from those of Northern Europe. The absence from the Greek populations of the alleles *Aat*-1,2(140) and *Ck*-1(115), which are widespread in other populations, is worth mentioning. Furthermore, the allele *Ldh*-5(105) is fixed in six of the seven populations examined, whereas it is in high frequency in the population of Nestos (table 2). This allele is thought to be the ancestral allele which has been replaced by the *Ldh*-5(100) (Henry and Ferguson, 1985).

For an appraisal of the level of genetic variability of the populations two indicators were used: the proportion of polymorphic loci (P) and the mean heterozygosity per locus (H) (table 2). The values of P and H observed in the present investigation are consistent with those obtained from other brown trout studies (Guyomard and Krieg 1983; Osinov, 1984).

The low mean value of F_{IS} indicates that the populations are not further subdivided. Average levels of differentiation among the brown trout populations of Greece, as measured by mean standardized genetic variance (F_{ST}) (table 4), indicate the low gene flow between these populations. The value $F_{ST} = 0{\cdot}2592$, obtained in the present investigation, is higher than those reported for the Irish (Crozier and Ferguson, 1986) and for the Swedish (Ryman, 1981) brown trout populations and it is also considerably higher than those of *S. salar* (Koljonen, 1989) and other marine species (Ward and Beardmore, 1977).

Origin of the populations and systematic implication

The values of genetic distance (D) and genetic identity (I) (table 3 and fig. 2) between the studied populations are similar to those reported for other populations of the same species (Avise and Smith, 1977; Ayala, 1983). Thus the previous classification, using morphological criteria, of the Greek populations into different subspecies is not supported by the data of this study. These previous classifications were based on few morphological characters *i.e.*, body pigmentation or number of gill rakers. So, these classifications are rather arbitrary. Only the population of Ag. Germanos seems to differ substantially from the other populations. One characteristic present only in this population is the existence of the *Ck*-2(50) allele. All the specimens examined from this population possess this allele (table 2). This population also differs from the other populations in the meristic counts and in the morphometric values (Karakousis *et al.*, in preparation). The population of Ag. Germanos lives in an isolated stream which flows to the lake Megali Prespa (fig. 1) and it has been classified as a different subspecies *S. t. peristericus* (Karaman 1937). The data in this study seems to support this view.

According to Berg (1962) and Balon (1968) the populations of brown trout of the Mediterranean basin are descended from a hypothetical anadromous subspecies, *S. t. mediterranea*, which disappeared from the sea during the last interglacial period (300,000 years BP) leaving neotenic landlocked populations in the streams of the Mediterranean basin. The two dendrograms (fig. 2 and fig. 3) support this hypothesis. Even though there are some limitations to the accuracy of the second dendrogram (fig. 3), namely the small number of gene loci and the different sources of data, it is a good indicator of the probable taxonomic relationships of the natural European brown trout. In this dendrogram (fig. 3) two main groups of populations can be recognised: the Mediterranean and Black Sea group and the Northern European populations group. The value of the genetic iden-

tity between these two groups is $I = 0{\cdot}867$. This value is similar to values reported for different subspecies (Avise and Smith, 1977; Ayala, 1983). The values of genetic similarity between the Mediterranean and the Black Sea populations indicate their common origin. The possible time of divergence between the two groups, namely the Mediterranean–Black Sea group and the Northern European group, is about 700,000 years BP. Balon (1968) based upon the geological changes proposed this time as the time of penetration of brown trout to the Mediterranean basin.

Management implications

Despite their high genetic identity, the populations studied also present genetic differences, as revealed from the fairly high values of F_{ST} (table 4). Every population has its own genetic characteristics which must be preserved. The unique alleles and especially the *Ck*-2(50) allele in the Ag. Germanos population may be used as a genetic tag in future breeding and management programmes.

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