

Microsatellite analysis of the genetic population structure of native and translocated Aristotle's catfish (*Silurus aristotelis*)

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Abstract

The genetic structure of two native *Silurus aristotelis* (one of Europe's oldest surviving freshwater fish species) populations was investigated using eight microsatellite loci. Average values of population heterozygosity were very high, even approaching values reported for marine fishes, possibly due to stable population sizes and the prolonged undisturbed conditions prevailing in the lakes that represent the native distribution of *S. aristotelis*. The success of an attempt to introduce the species into a new environment was also evaluated. No loss of genetic variability in the introduced population was detected. Additionally, assignment tests and trees based on genetic distances among individuals indicated the low differentiation of the translocated population from its donor population, and, differentiated the two autochthonous populations successfully. Comparisons with previous allozyme and mitochondrial RFLP studies were also made and showed that results on relative levels of genetic variability among populations were in good agreement among all methods. However, microsatellite analysis exhibited higher power of statistical tests for differentiation among population samples compared to allozymes. © 2002 Ifremer/CNRS/Inra/IRD/Cemagref/Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Résumé

Analyse de la structure génétique de deux populations natives et une population transplantée de silures d'Aristote (*Silurus aristotelis*) à l'aide de marqueurs microsatellites. La structure génétique de deux populations autochtones de silures d'Aristote (l'une des plus anciennes espèces survivantes de poissons européens) a été décrite à l'aide de huit marqueurs microsatellites. Les valeurs moyennes d'hétérozygotie par population sont élevées, proches de valeurs obtenues chez les espèces marines. Ces fortes valeurs reflètent probablement la stabilité, sur une longue période, des tailles de populations et des conditions rencontrées dans les lacs qui constituent l'habitat naturel de cette espèce. Le succès d'un essai de transplantation de l'espèce dans un nouvel environnement a aussi été évalué. Aucune perte de variabilité consécutive au transfert n'a pu être détectée. Les tests d'assignation et les arbres basés sur les distances génétiques ne font apparaître aucune différence entre la population transplantée et la population donneuse alors qu'ils permettent de différencier très clairement les deux populations autochtones. Ces résultats sont en accord avec ceux des études antérieures de variabilité des allozymes ou de l'ADN mitochondrial par génotypage (PCR-RFLP). Toutefois, les tests basés sur les données microsatellites s'avèrent bien plus puissants que ceux utilisant les données enzymatiques. © 2002 Ifremer/CNRS/Inra/IRD/Cemagref/Éditions scientifiques et médicales Elsevier SAS. Tous droits réservés.

Keywords: Microsatellites; Genetic structure; Translocated populations; *Silurus aristotelis*; Greece

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1. Introduction

The list of European freshwater fish fauna includes only two native silurid (catfish) species (Kobayakawa, 1989), the widely distributed wels (*Silurus glanis* Linnaeus, 1758) and Aristotle's catfish (*Silurus aristotelis* Garman, 1890), which is endemic to Lyssimachia, Trichonida and Amvrakia Lakes, all part of the Acheloos River drainage system in south-western Greece (Economidis, 1991). Both species and especially *S. glanis* have increasing economic importance (Proteau et al., 1996; Linhart et al., 2002), although *S. aristotelis* has not encountered high fishing pressure. During the 1960s and 1970s destruction of spawning grounds and overfishing resulted in very low population sizes of Lake Volvi *S. glanis*, in northern Greece. In the mid 1980s, at least 750 *S. aristotelis* individuals originating from Lake Trichonida were introduced by local fishermen into this lake (Economidis, 1988). This introduction has probably resulted in the extinction of *S. glanis* from Lake Volvi (Economidis et al., 2000).

In general, introduced species often possess low levels of genetic diversity relative to source populations as a consequence of the small effective population sizes associated with founder events (e.g., Amsellem et al., 2000). However, little information exists so far on the genetic variability of the native *S. aristotelis* populations and the success of the attempt to introduce this species into Lake Volvi has not been evaluated, despite its importance for the local economy. Low levels of genetic variation were revealed by previous allozyme (Triantafyllidis et al., 1999b) and mitochondrial DNA restriction fragment length polymorphism (Triantafyllidis et al., 1999a) analyses of two native (Trichonida and Amvrakia) and one translocated (Volvi) *S. aristotelis* populations. Therefore, the limited resolution power of these traditionally used markers does not allow for the genetic characterization of these fish populations, the monitoring of the status of the introduced population and the effective management and conservation of native and transplanted populations.

The advent of new molecular markers, in particular microsatellite DNA, is opening new perspectives for population genetics (Estoup and Angers, 1998). Due to the high levels of variability revealed with microsatellites, this approach has become highly applicable in evolutionary biology, population genetics and ecology (e.g., Bruford and Wayne, 1993). Eight microsatellite loci were used in the present study to investigate the genetic population structure of *S. aristotelis* endemic populations and to document any change in genetic variability due to founder events in the translocated one. Assignment tests were also utilized to demonstrate the resolution power of microsatellites to differentiate individuals according to their donor population. Results were also discussed in comparison to previous work on allozyme and mitochondrial DNA polymorphism.

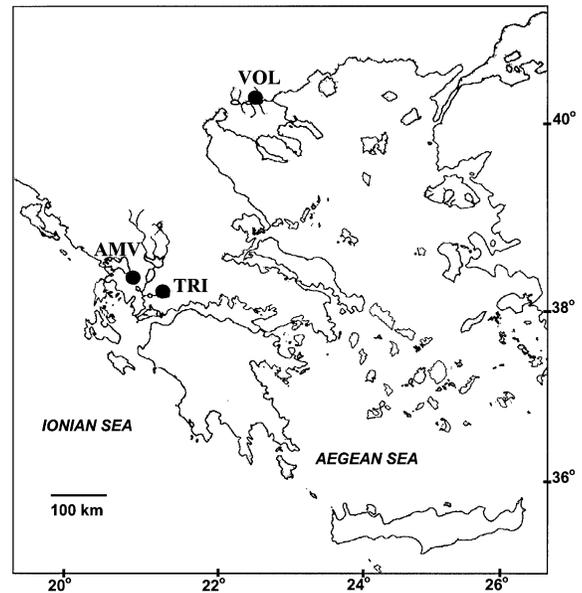


Fig. 1. Sampling sites for two wild (Trichonida-TRI and Amvrakia-AMV) and one translocated (Volvi-VOL) *Silurus aristotelis* populations.

2. Materials and methods

2.1. Sample collection and DNA isolation

Fish samples were collected from three different sites (Fig. 1). The two populations from Lakes Trichonida and Amvrakia are the two main sites of *S. aristotelis* native distribution and the third from Lake Volvi was recently introduced from Trichonida. Fin clips or muscle tissue were collected from 80 *S. aristotelis* individual fish (Table 1), freshly caught by traps and/or electro-fishing, and immediately preserved in 95% ethanol. Whole cell DNA was extracted from individuals according to the method of Taggart et al. (1992).

2.2. Microsatellite analysis

All *S. aristotelis* samples were analyzed at each of eight microsatellite loci, previously isolated and characterized for the European catfish (*S. glanis*) by Krieg et al. (1999). PCR amplifications were performed according to the conditions given in Krieg et al. (1999). Six per cent polyacrylamide sequencing gels were used to resolve amplified products. Microsatellite alleles were identified by their size in base pairs. Allele lengths were determined by reference to control samples.

2.3. Data analysis

Tables of allele frequencies in population samples and genetic variation parameters, i.e. (i) mean numbers of alleles per locus, (ii) unbiased expected heterozygosity (H_e) (Nei, 1987), and (iii) observed heterozygosity (H_o) values, were calculated with the program GENETIX 4.0 (Belkhir, 1999).

Table 1
Levels of genetic variation observed at eight microsatellite loci at three *Silurus aristolis* population samples. Results from previous allozyme (Triantafyllidis et al., 1999b) and mtDNA Restriction Fragment Length Polymorphism, RFLP (Triantafyllidis et al., 1999a) analysis are also presented

Locus		Trichonida	Volvi	Amvrakia	Total
		<i>n</i> = 30	<i>n</i> = 30	<i>n</i> = 20	
S6-95	No. of alleles	19	16	16	24
	He	0.913	0.915	0.923	0.932
	Ho	0.929	1.000	0.889	0.946
S7-E	No. of alleles	3	3	1	3
	He	0.291	0.466	0	0.531
	Ho	0.133*	0.233*	0	0.141
S3-3	No. of alleles	15	14	7	18
	He	0.873	0.868	0.695	0.887
	Ho	0.767	0.900	0.750	0.812
S3-10	No. of alleles	17	17	11	21
	He	0.904	0.905	0.807	0.927
	Ho	0.897	0.793	0.737	0.818
S3-25	No. of alleles	21	12	10	23
	He	0.894	0.841	0.837	0.923
	Ho	0.897	0.867	0.750	0.848
S1-40	No. of alleles	2	1	1	2
	He	0.095	0	0	0.037
	Ho	0.100	0	0	0.038
S1-154	No. of alleles	15	18	11	21
	He	0.902	0.891	0.856	0.917
	Ho	0.929	0.963	0.833	0.918
S7-159	No. of alleles	17	17	3	22
	He	0.900	0.864	0.261	0.864
	Ho	0.933	0.867	0.300	0.750
All microsatellite loci	No. of private alleles	25	16	3	
	Mean No. of alleles	13.6	12.3	7.5	
	Mean He	0.734	0.731	0.562	
	Mean Ho	0.698	0.703	0.532	
Allozyme study	Mean No. of alleles	1.6	1.3	1.1	
	Mean He	0.044	0.041	0.029	
	Mean Ho	0.042	0.037	0.035	
MtDNA RFLP study	No. of haplotypes	4	5	2	
	Haplotypic diversity	0.598	0.515	0.166	

He, unbiased expected heterozygosity (Nei, 1987); Ho, observed heterozygosity.

* Indicates deviation from Hardy-Weinberg equilibrium.

The program GENEPOP 3.3 (Raymond and Rousset, 1995) was used to perform Fisher's exact tests for: (i) conformance of genotypic distribution to Hardy-Weinberg equilibrium (HWE) expectations, for each locus or globally across populations and loci, (ii) linkage disequilibrium between loci (for each pair of populations or globally), and (iii) estimating the significance of allelic differentiation among the entire set or between pairwise population samples. Corrections for simultaneous multiple comparisons were applied using sequential Bonferroni correction (Rice, 1989).

The extent of population subdivision was examined by calculating fixation indices based on an infinite alleles model (IAM) only. The stepwise mutation model (SMM) was not used, since knowledge of the repeat motif is essential for this approach and sequencing of *S. aristolis* alleles in the two loci S3-25 and S6-95 had shown that there were many different single nucleotide substitutions in the repeat region of these loci in comparison to the cloned sequence in *S. glanis* (Guyomard, R., Estoup, A., Angers, B., unpublished results). The computer program FSTAT

2.9.3 (Goudet, 2000) was used to determine: (i) Weir and Cockerham's (1984) theta (θ) statistics (unbiased estimates of Wright's (1951) F -statistics), and (ii) the statistical departure from zero of values of F_{IS} and F_{ST} using 5000 permutations in each case. The genetic distances between populations were estimated using Cavalli-Sforza and Edwards' (1967) chord distance D_{CE} with the program GENDIST, included in the package PHYLIP 3.5 (Felsenstein, 1995).

Genetic affinities among individuals were calculated based on the allele sharing distance of Bowcock et al. (1994) with the program SHAREDIST, found at <http://www.biology.ualberta.ca/jbrzusto> (Paetkau et al., 1998). An Unweighted Pair Group with Arithmetic Means (UPGMA) tree of individuals was constructed using the program NEIGHBOR in PHYLIP 3.5 (Felsenstein, 1995). Finally, assignment of individuals to populations was tested with the program GENECLASS 1.0 using a likelihood-based method, which assigns an individual to the population, in which the individual's genotype is most likely to occur. As recommended by Cornuet et al. (1999), the

Table 2
Results of F -statistics for each locus, in the *Silurus aristotelis* populations studied

Locus	F_{IS}	F_{ST}
S6-95	-0.012	0.006*
S7-E	0.530***	0.545***
S3-3	0.036	0.082***
S3-10	0.091**	0.054***
S3-25	0.033	0.084***
S1-40	-0.033	0.029
S1-154	-0.014	0.030***
S7-159	-0.013	0.209***
All	0.048**	0.128***

* $P < 0.05$;

** $P < 0.01$;

*** $P < 0.001$.

Bayesian approach of Rannala and Mountain (1997) with the 'leave one out' method option was chosen to avoid bias. The Bayesian approach assumes Hardy–Weinberg equilibrium and linkage equilibrium in all locus–population combinations.

3. Results

3.1. Genetic variation within populations

Allele frequencies were calculated for each locus and population (Appendix 1). Estimates of gene diversity for each locus and population are presented in Table 1. A total of 134 alleles were found in the pooled *S. aristotelis* populations, ranging from two for locus S1-40 to 24 for locus S6-95 (Table 1). Forty-four alleles were private, i.e. found in only one population. Many of these private alleles were in low frequencies (<0.05). No population was fixed for a specific private allele. The highest values of mean number of alleles and of expected heterozygosity were detected in the Trichonida population and the lowest in the Amvrakia population (Table 1). The sample from the introduced population of Volvi exhibited similar variation values to the Trichonida sample.

All eight loci used in this study were tested for departure from Hardy–Weinberg equilibrium. Two out of 21 possible tests for HWE were statistically significant ($P < 0.001$). Both departures were found in the S7-E locus in the Trichonida and Volvi populations (the Amvrakia population was monomorphic at this locus). Additionally, values of F_{IS} for each locus (Table 2), showed heterozygote deficiency in this locus, manifested as a statistically significant positive F_{IS} value ($P < 0.001$). No test for genotypic disequilibrium was statistically significant for any of the 28 different pairs of loci across all populations.

Table 3
Values of chord (Cavalli-Sforza and Edwards, 1967) genetic distance (below diagonal) and Weir and Cockerham's (1984) theta (θ) (above diagonal) among the three *Silurus aristotelis* populations examined (Trichonida-TRI, Volvi-VOL and Amvrakia-AMV), based on allele frequency data from eight microsatellite loci

	TRI	VOL	AMV
TRI	–	0.025	0.204
VOL	0.046	–	0.190
AMV	0.099	0.104	–

3.2. Genetic relationships among populations and individuals

Global analysis of *S. aristotelis* populations showed that there are highly significant differences ($P < 0.001$) in the allele frequency distributions at all loci, with the exception of S1-40 locus ($P > 0.05$) and partially of S6-95 locus ($0.01 < P < 0.05$, and therefore not significant after Bonferroni correction). In agreement with this, single-locus F_{ST} values were highly significant except for loci S1-40 and S6-95 (Table 2).

Out of 24 pairwise genetic heterogeneity tests, 14 were significant after Bonferroni correction, the most non significant (5/8) for the Trichonida and Volvi population pair. Pairwise genetic estimates between these two populations also agree on the genetic relatedness of the Trichonida and Volvi populations. All values of chord (Cavalli-Sforza and Edwards, 1967) genetic distances as well as pairwise estimates of Weir and Cockerham's (1984) theta (θ), between these two populations are always smaller to comparisons with the Amvrakia population (Table 3).

Two main clusters are evident in the UPGMA tree built from the matrix of pairwise allele sharing distance among the 65 out of 80 *S. aristotelis* individuals that were scored in all eight loci. The first comprises all Amvrakia individuals and the second all Trichonida and Volvi individuals (Fig. 2). The assignment tests, performed on all 80 individuals, also revealed the potential of multilocus microsatellite genotypes to discriminate *S. aristotelis* populations. Sixty-three individuals were correctly identified (assignment success 78.75%). Out of the 17 individuals misidentified, seven were from the Trichonida population and assigned to the Volvi population and 10 from the Volvi population were assigned to the Trichonida population. The assignment tests were repeated without locus S7-E that is not in Hardy–Weinberg equilibrium, but assignment success remained the same.

4. Discussion

4.1. Genetic variation in *Silurus aristotelis*

Levels of variation found in this study for *S. aristotelis* are very high. It is interesting to note that the mean value of

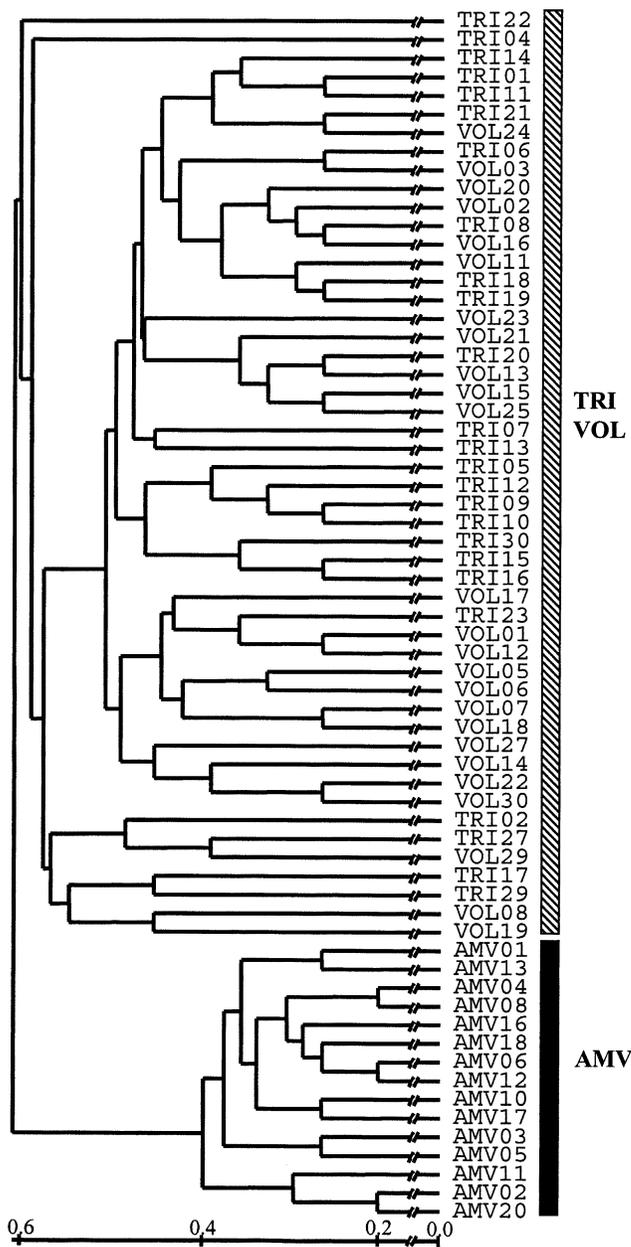


Fig. 2. Unweighted Pair Group Method with Arithmetic means (UPGMA) dendrogram clustering the 65 out of 80 *Silurus aristotelis* individuals that were scored in all eight microsatellite loci, based on the allele sharing distance values (Bowcock et al., 1994).

The per population (0.68) found in this study, is higher than the mean value (0.54) reported by De Woody and Avise (2000) for other freshwater fish species. Conclusions are similar if the Volvi sample is merged with the parental Trichonida sample. Explanations should be sought in the history of the two lakes that represent the native distribution of *S. aristotelis*. The development of both lakes is closely associated to Lake Ohrid, which could be even older than upper Pliocene (Overbeck et al., 1982). *S. aristotelis* could have inhabited this area, which has not been influenced by Pleistocene glaciations, even from those times (Economidis

and Banarescu, 1991). The previous information combined with the low fishing pressure on *S. aristotelis* could have helped it to maintain stable population sizes for thousands of generations. Lake Trichonida is also the largest Greek lake and deeper than Lake Amvrakia, with a surface area of 98.6 km² and a maximum depth of 57 m, whereas corresponding values for Lake Amvrakia are 13 km² and 25 m (Overbeck et al., 1982). Therefore, Lake Trichonida has the potential (due to its size, depth and environmental parameters) to sustain larger populations and the presence of many private alleles (Table 1) in the sample from this lake is in agreement with this hypothesis.

An additional interesting aspect of these results is that variability levels were higher than levels discovered for *S. glanis* from where the loci used in this study were initially cloned. Levels of heterozygosity across 13 wild *S. glanis* populations and using the same eight loci were very close to those reported for freshwater fish species (0.53) (Triantafyllidis et al., 2002). According to Ellegren et al. (1995) there is a potential bias when comparing crosspriming results, since loci cloned in one species tend to be less variable even in closely related taxa, due to a tendency to always clone large and polymorphic loci. However, this does not seem to hold in this study. Possible reasons could be: (i) that the two species are very closely related and therefore deconstruction of these loci has not yet started in *S. aristotelis*, (ii) the selection and cloning was purely arbitrary and therefore the loci chosen were not highly polymorphic from the start, and (iii) the subset of loci studied is small. However, Crawford et al. (1998) have demonstrated that the hypothesis of Ellegren et al. (1995) might not hold. These authors (Crawford et al., 1998) showed, after the analysis of a set of 472 sheep and cattle microsatellites, that sheep microsatellite markers had a longer median allele size regardless of their origin.

However, one should also not forget the major impact that Pleistocene glaciations had on the genetic diversity of *S. glanis* populations (Krieg et al., 2000). Therefore, it is possible that levels of variability are actually not higher in *S. aristotelis* but lower in most *S. glanis* populations. Comparison with the large *S. glanis* population of Volga (Triantafyllidis et al., 2002), which is also the centre of expansion of *S. glanis*, gave similar values of heterozygosity between the two species and corroborates this view.

4.2. Explanations for departure from Hardy–Weinberg equilibrium at the S7-E locus

The microsatellite locus S7-E was the only one that does not conform to Hardy–Weinberg expectations in both polymorphic populations of Trichonida and Volvi (Table 1). Additionally, the overall F_{IS} value of 0.530 ($P < 0.001$) points to a very high heterozygote deficit. Often reported explanations in the literature (e.g., Garcia de Leon et al., 1997) such as inbreeding, existence of subpopulations, or assortative mating phenomena can be ruled out, since they

affect the whole genome and not only one locus. Therefore, apart from problems in gel reading which might always apply, the two most possible explanations are the presence of non-amplifying alleles and selection phenomena. Non-amplifying alleles have regularly been reported in microsatellite studies (Paetkau and Strobeck, 1995; Ishibashi et al., 1996; Jones et al., 2001) in frequencies as high as 25%. They can be traced only in homozygote state. A non-amplifying allele with a frequency of p will lead to a heterozygote deficiency (F_{IS}) approximately equal to $2p$ (when p is small) (Garcia de Leon et al., 1997). In the present study the frequency of the non-amplifying allele would be expected around 26% and approximately four null homozygotes should be found. Assuming that the number of null homozygote follows a binomial distribution, the probability not to find a null/null genotype in the given sample is 0.015. Since no homozygote was found, even though all individuals were scored, selection phenomena acting on this or on a closely linked locus could also be responsible. Slatkin (1995) has shown that a microsatellite locus closely linked to another locus under some selective pressure, will be less polymorphic. Levels of variability of the S7-E locus are actually much lower than the other loci (with the exception of locus S1-40, Table 1).

4.3. The status of the translocated population

Variation is considered by many biologists necessary for populations to evolve (and under changing conditions survive) via natural selection (Ferguson et al., 1995). Therefore, monitoring of the genetic status of introduced populations is essential for the evaluation of the success of such attempts since, slight or moderate decreases in genetic variation together with surprisingly rapid differentiation appear to be common consequences of translocation (Rowe et al., 1998). Levels of variation detected in the translocated Volvi population are very high (Table 1) and very close to the levels found in the parental Trichonida population. This was expected since, based on the equation $H_s = H_o(1-1/2N)$ (Nei, 1987), where $N=750$, the number of *S. aristotelis* individuals translocated into Lake Volvi, H_s and H_o the expected and true heterozygosity in the sample and the original population respectively, we calculate that the new population should have initially contained 99.93% of the variation of the original population. Additional evidence that the (very recently introduced) Volvi population has not undergone any bottleneck events and is not under inbreeding depression due to low population sizes is given by the fact that Hardy–Weinberg and genetic linkage disequilibrium tests were also not significant. Consequently, this data support the notion that *S. aristotelis* has successfully been introduced into Lake Volvi. The high number of private alleles discovered could, in fact, imply that the population is rapidly expanding and new alleles have arisen through new mutations. However, since very high allele number were found in the 30 Trichonida individuals

sampled, more extensive screening of this sample is needed to assure that all existing variation in the parental population has been detected.

4.4. Comparison to previous studies

This study is part of a multidisciplinary analysis of the genetic structure of catfish populations in Europe. The importance of using different genetic markers with different modes of inheritance, mutation rates, etc. to infer safe conclusions on levels of genetic variation and relationships among populations has often been stressed in the literature (e.g., Triantafyllidis et al., 1997; Estoup et al., 1998). Table 1 summarizes levels of genetic variation detected in the three populations of *S. aristotelis* (using the same individuals in many cases) with eight microsatellite loci (present study), 30 allozyme loci (Triantafyllidis et al., 1999b), and from a RFLP analysis of four mitochondrial DNA regions (Triantafyllidis et al., 1999a). As expected, the microsatellite analysis far exceeds the other two techniques in levels of diversity (number of alleles and expected heterozygosity) and consequently potential information content. This is especially true for the comparison to the allozyme technique, where remarkably lower levels of variation were observed (Table 1), a fact which made powerful tests of population structure and stability difficult to apply. However, all three approaches agree on the lower values of variation of the Amvrakia population compared to the other two populations.

The three different approaches also agree on the lower genetic distance among Trichonida and Volvi populations when compared to the Amvrakia sample, either using Cavalli-Sforza and Edwards' (1967) chord distance or Weir and Cockerham's (1984) theta (θ) statistics (Table 3). However, the advantage of the microsatellite DNA approach is its ability to analyze relationships even at the individual level (Roques et al., 1999; Walker et al., 2001). This study has once again revealed the usefulness of microsatellite multilocus genotype analysis to discriminate individuals of different populations. This was illustrated in: (i) the UP-GMA tree constructed based on the allele sharing distance (Bowcock et al., 1994), and (ii) the assignment tests, where individuals from Trichonida and Volvi clearly differentiated from Amvrakia. Mitochondrial DNA, which is more sensitive to genetic drift phenomena due to its effective population size being one fourth of that for nuclear genes (Birky et al., 1983), was also capable of distinguishing the two native populations. In a previous study (Triantafyllidis et al., 1999a), all Amvrakia samples were fixed for a unique mitochondrial haplotype. This clear differentiation is not unexpected since, although Lake Amvrakia belongs to the same drainage system with Lake Trichonida, no subsurface

drainage exists to connect it directly with Trichonida (Overbeck et al., 1982).

5. Conclusion

The results of this study have disclosed a plethora of genetic variation both in native and transplanted populations of the endemic *S. aristotelis*. Despite the “safe” status of these populations based on levels of genetic variation, the warnings (Overbeck et al., 1982) of modern civilization accelerating the aging and the process of eutrophication in Lakes Trichonida and Amvrakia, the two main areas of native distribution of one of the oldest surviving endemic European freshwater fish species, call upon the need for

measures to secure the survival of *S. aristotelis* in the future. The combination of established microsatellite, mitochondrial and allozyme genetic markers can help in this direction.

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Appendix 1

Allele size and frequencies at eight microsatellite loci, in the *Silurus aristotelis* populations examined (population designations are as in Table 3, private alleles are indicated with an asterisk)

		TRI	VOL	AMV			TRI	VOL	AMV
S6-95	220	0.0179	0.0536	0	S3-25	222*	0.0172	0	0
	224*	0.0179	0	0		226*	0.0345	0	0
	228	0	0.0357	0.0278		230	0.0172	0.0167	0
	232	0.0179	0.1071	0.0278		232	0.0172	0	0.1500
	236	0.0536	0.1250	0.0556		234	0.0517	0.1000	0
	240	0.0714	0.0893	0.1111		236	0.0345	0.1500	0
	244	0.1964	0.0893	0.0833		238	0.0517	0.1667	0
	248	0.0179	0.0536	0.0556		240	0.1034	0.1000	0.0250
	252	0.0893	0.1250	0.0833		241*	0.0172	0	0
	256	0.0357	0.0536	0.1111		242	0.0345	0.2833	0.0500
	260	0.0893	0.0357	0.0556		243*	0.0172	0	0
	264	0.0714	0.1071	0		244	0.2414	0.0667	0
	268*	0	0.0536	0		246	0.0517	0	0.1500
	272	0.0893	0	0.1111		247*	0.0172	0	0
	276	0.0536	0	0.0278		248	0.1379	0.0167	0.1000
	280	0.0536	0.0179	0.0556		249*	0.0172	0	0
	284	0.0179	0.0179	0		250	0.0517	0.0500	0.0750
	288	0.0357	0	0.0556		251*	0.0172	0	0
	292*	0.0357	0	0		252*	0.0345	0	0
	296*	0	0	0.0278		254	0.0172	0.0167	0.0750
	300	0.0179	0.0179	0.0833		256	0	0.0167	0.3000
	304*	0	0.0179	0		258	0.0172	0	0.0500
	312*	0	0	0.0278		260	0	0.0167	0.0250
316*	0.0179	0	0	S1-40	98*	0.0500	0	0	
S7-E	203	0.8333	0.6833		0	100	0.9500	1.0000	1.0000
	209	0.1000	0.0667	0	S1-154	287*	0.0357	0	0
	211	0.0667	0.2500	1.0000		291	0.0357	0.0185	0
S3-3	163*	0	0.0167	0		301*	0	0.0185	0
	167	0.0333	0.0833	0		303*	0	0.0185	0
	169*	0.0167	0	0		305*	0	0.0556	0
	173	0.1000	0.0167	0		307*	0	0	0.0833
	175	0.0833	0.1333	0.1250		309*	0.0893	0	0
	177	0.2500	0.1667	0		311	0.1250	0.0370	0.0556
	179	0.1333	0.1500	0.1000		313	0.1607	0.2037	0.0833
	181	0.0500	0.2167	0.0250		315	0.0714	0.1852	0.2222
	183	0.0333	0.0167	0.0250		317	0.0179	0.0741	0.1389
	185	0.1000	0.0500	0.5000		319	0.0714	0.0185	0.2222
	187	0.1167	0.0667	0.0750		321	0.0357	0.0741	0.0278
	189	0	0.0167	0.1500	323	0.0179	0.0185	0.0556	
	191	0.0167	0.0333	0	325	0.1250	0.0185	0.0278	
197	0.0167	0.0167	0	327	0.0357	0.0556	0		
199*	0.0167	0	0	329	0.1250	0.0185	0		
201*	0.0167	0	0	331*	0	0.0926	0		
203*	0.0167	0	0	333	0.0357	0.0556	0		
205*	0	0.0167	0	337	0.0179	0.0185	0.0556		

Appendix 1 (continued)

		TRI	VOL	AMV		TRI	VOL	AMV
S3-10	117	0.0690	0.0690	0	339	0	0.0185	0.0278
	119	0	0.0172	0.0263	S7-159	195*	0.0167	0
	121*	0	0.1034	0	197*		0.0167	0
	123	0.0172	0.0345	0.3421	199	0.1167	0.0500	0.8500
	125	0.0172	0	0.1316		201	0.0167	0.0167
	127	0.0690	0.0862	0.0526		203	0.0333	0.0667
	129	0.0862	0	0.0263		207*	0.0167	0
	131	0.0172	0.0172	0		209	0.0333	0.0500
	133	0.0690	0.0517	0.0263		211	0.0500	0.0167
	134	0.0172	0.0172	0		213*	0.0333	0
	135	0.0517	0.0345	0.0526		215*	0	0.0167
	136*	0.0172	0	0		217	0.0500	0.0667
	137	0.0517	0.1897	0.0263		219	0.0833	0.1000
	138	0.0172	0.0862	0		221	0.2167	0.3000
	139	0.1897	0.0345	0.2105		223	0.0833	0.1167
	140*	0	0.0690	0		225	0.1000	0.0333
	141	0.0862	0.1207	0		227	0.0500	0.0167
	143	0.1379	0.0172	0.0526		229*	0.0500	0
	145	0.0690	0.0345	0.0526		231*	0	0.0667
	146*	0.0172	0	0		233	0.0333	0.0333
	155*	0	0.0172	0		239*	0	0.0167
						243*	0	0.0167
						249*	0	0.0167

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