

Genetic differentiation and phylogenetic relationships among Greek *Silurus glanis* and *Silurus aristotelis* (Pisces, Siluridae) populations, assessed by PCR–RFLP analysis of mitochondrial DNA segments

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Mitochondrial DNA diversity of seven *Silurus glanis* populations (six from Greece and one from the Danube Delta) and three populations of the endemic Greek *Silurus aristotelis* was investigated. RFLP analysis of four regions of mitochondrial DNA (cytochrome b, D-loop, ND-5/6) amplified by PCR was used. Ten and nine haplotypes were found in *S. glanis* and *S. aristotelis*, respectively. No haplotype was shared between the two species. Significant geographical substructuring was observed in the distribution of haplotypes, with most populations possessing private haplotypes. These haplotypes can serve as genetic ‘tags’ and therefore warrant protection. Haplotype diversity was very low for all Greek *S. glanis* populations, possibly because the small size and large annual fluctuations of Greek inland waters do not support large fish populations. Nucleotide divergence was in the range of 0.00–0.52% among *S. glanis* populations, and 0.00–0.11% among *S. aristotelis* populations. Historical factors such as glaciations could account for these low values. The value of 6.75% sequence divergence of the two species refutes the classification of the two species in different genera, as proposed by some authors. This study constitutes the first attempt, based on mitochondrial molecular data, to address the complicated evolutionary history of the two species which belong to the widely distributed and economically important Siluridae family.

Keywords: genetic variation, mtDNA, phylogeny, *Silurus aristotelis*, *Silurus glanis*.

Introduction

The Siluridae family consists of more than 100 freshwater fish species with Eurasian distribution (Kobayakawa, 1989; Bornbusch, 1995). In Europe it is represented by only two species: *Silurus glanis*, Europe’s largest freshwater fish, found from the river Rhine eastwards, including Asia Minor, and *S. aristotelis* which is endemic to the basin of the river Acheloos in Greece (Economidis & Banarescu, 1991).

Despite the increasing importance of *S. glanis* in aquaculture, little is known about the genetic characterization of natural populations. Studies on allozyme variation of the two species (Kotsinas *et al.*, 1989; Chombard, 1993; Triantafyllidis *et al.*, 1999) revealed low to moderate variability. Karyotype analysis of the

two species has also shown few differences within the two species (Rab *et al.*, 1994).

Another scientifically interesting problem is the taxonomic classification of the two species. Many authors (Bleeker, 1862; Banarescu, 1989) have reported that the species *aristotelis* belongs to the genus *Parasilurus*. This proposed genus consists of species with an eastern Asian and Japanese distribution. Others have questioned the taxonomic validity of the genus *Parasilurus* and have considered it synonymous with *Silurus* (Kobayakawa, 1989; Bornbusch, 1995).

Mitochondrial DNA has proved to be an excellent tool for examining biogeographical events, above or below the species level (Avise, 1994). It has emerged as a genetic marker able to discriminate stocks (Billington & Hebert, 1991) and to trace past isolation incidents, in a more effective way, in some cases, than the nuclear genome (Taylor *et al.*, 1997).

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This study constitutes the first attempt to analyse mtDNA variation of *Silurus* fish populations using the Restriction Fragment Length Polymorphism technique. The aim of this paper is to describe the geographical distribution of mtDNA variation in the studied populations, evaluate the usefulness of mtDNA analysis in stock identification, compare the results with the previous study on allozyme variation and examine the relationships of the two species in a phylogeographical context.

Materials and methods

Sample collections

Samples from 10 different populations (Fig. 1) of catfishes were collected by traps and/or electrofishing. Three populations are of *S. aristotelis* and the rest belong to *S. glanis*. The two *S. aristotelis* populations from lakes Trichonida (TRI) and Amvrakia (AMV) are native and the third from lake Volvi (VOL) was recently introduced from Trichonida. *Silurus glanis* samples come from five different Greek presumably native unstocked river populations (Evros, EVR; Strymon, STN; Axios, AXI; Aliakmon, ALI; and Pineios, PIN), one hatchery population near the Strymon river (STH,

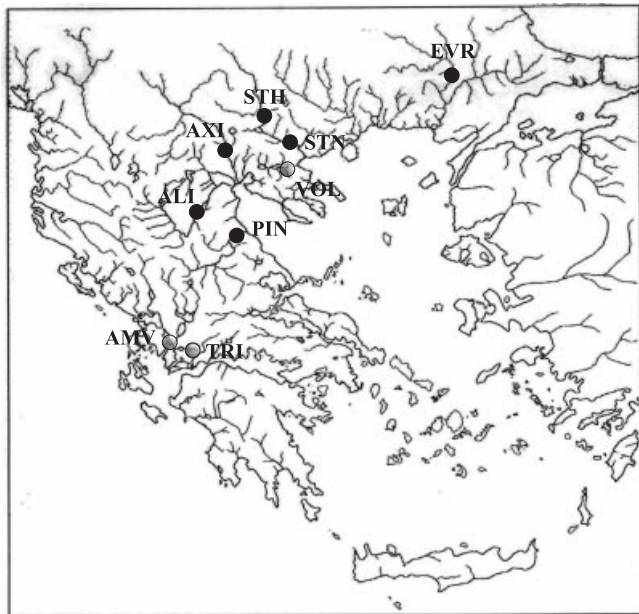


Fig. 1 Sampling sites. *Silurus aristotelis* populations: TRI, Lake Trichonida; AMV, Lake Amvrakia; VOL, Lake Volvi. *S. glanis* populations: EVR, Evros river; STN, Strymon river; STH, Strymon river hatchery; AXI, Axios river; ALI, Aliakmon river; PIN, Pineios river. Sample 10 came from the Danube Delta (DDN) and is not indicated on the map.

probably established from a few individuals from the Strymon river) and one native population from Lake Puiu in the Danube Delta (DDN).

mtDNA RFLP analysis

DNA was extracted from the liver or muscle of 247 individuals using the method of Taggart *et al.* (1992). Two adjacent mtDNA segments were amplified. One (ND-5/6) encompassed the complete NADH-dehydrogenase 5/6 region and the other (HN20) comprised the cytochrome b gene and the control region. PCR conditions were as described in Bernatchez *et al.* (1995). Three to 9 μ L of the PCR product were subsequently digested with 18 restriction endonucleases, electrophoretically separated in 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. The following restriction enzymes were tested: *AluI*, *AseI*, *AvaII*, *BfaI*, *BstNI*, *DdeI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaII*, *MboI*, *RsaI*, *TaqI*, *HincII*, *HpaI*, *NruI* and *PstI*. The last four enzymes had no restriction sites in the ND-5/6 region of *S. glanis* and they were subsequently not used.

Data analysis

Distinct single endonuclease patterns were identified by a specific letter. Each fish was assigned a multi-letter code that described its composite mtDNA genotype-haplotype. The raw data were fragment profiles, but we inferred site differences among haplotypes from changes in fragment profiles as these could be accounted for by the gain or loss of particular restriction sites. The data were analysed using various programs contained in the REAP (McElroy *et al.*, 1991) and the PHYLIP 3.57c (Felsenstein, 1995) computer packages. The significance level in the MONTE program was obtained by 10 000 randomizations. N_{ST} (Lynch & Crease, 1990) was used to estimate the degree of population subdivision at the nucleotide level. A Mantel test (NTSYS computer package, Rohlf, 1990) was used to compare distance matrices calculated from allozyme (Triantafyllidis *et al.*, 1999) and mtDNA data.

Results

Restriction analysis

The sizes of PCR-amplified mtDNA segments for both species were \approx 2.4 kilobases (kb) for ND-5/6 and 2.2 kb for HN20. Routine screening of all populations revealed a total of 130 sites in *S. glanis* and 127 sites in *S. aristotelis* corresponding to 538 nucleotides and 536 nucleotides, respectively, \approx 3% of the mitochondrial

genome. Fragment patterns generated by each enzyme are available upon request. Four (*AluI*, *RsaI*, *HinfI*, *HhaI*) and four (*AluI*, *RsaI*, *HaeIII*, *BstNI*) restriction enzymes out of 14 were polymorphic in the ND-5/6 and HN20 regions of *S. glanis*, whereas the respective values for *S. aristotelis* were two (*HaeIII*, *HhaI*) and four (*AluI*, *HinfI*, *HhaI*, *BfaI*). No haplotype was shared between individuals from the two species. Thus, for better presentation of the results, composite genotypes and corresponding frequencies for the populations of the two species are presented separately in Tables 1 and 2.

RFLP haplotype distribution and diversity

Ten different haplotypes (composite genotypes) were found in *S. glanis* populations (Table 1). The number of observed haplotypes within populations varied from one to six. Eight out of 10 haplotypes were private, i.e. population-specific. Greek populations were dominated by one or two haplotypes, whereas six haplotypes were found in the Danube Delta population. This was reflected in the values of the haplotype diversity index, which varied from 0.00 in most Greek populations to 0.79 in the Danube Delta population (Table 1). Pairwise sequence divergence estimates among these haplotypes varied from 0.10 to 0.73%.

Within the *S. aristotelis* populations nine different haplotypes were found, with seven of them being private (Table 2). Pairwise sequence divergence estimates among these haplotypes varied from 0.09 to 0.39%.

Relationship among haplotypes and populations

The test for geographical heterogeneity in haplotype frequencies among all populations, through the MONTE program of the REAP package, resulted in a significant outcome. All 16 pairwise comparisons between natural populations within the species were also significant ($P < 0.01$). On the other hand, differences between an introduced (Volvi) or a hatchery (Strymon Hatchery) population with its parental (Trichonida and Strymon Natural, respectively) population were not significant.

Nucleotide divergence was in the range of 0.00–0.52% among *S. glanis* populations, and 0.00–0.11% among *S. aristotelis* populations, based on restriction site data. Strong geographical patterning was observed in the distribution of the *S. glanis* mtDNA haplotypes, in both the FITCH (distance) phenogram (not shown) and the majority rule consensus tree of Wagner parsimony trees (Fig. 2). As shown, haplotypes 2g, 3g, 4g and 5g, which are characterized by the patterns *AluI* B in the ND-5/6 region and *AluI* B, C and *RsaI* B in the HN20 region form a cluster. These haplotypes were found in the Greek populations of the Evros, Axios, Aliakmon and Pineios rivers. Bootstrap analysis supports this clustering with 96% confidence. Haplotype 1g was found in the Strymon (Greece) and Danube Delta populations.

Because of the big differences in fragment profiles, site differences could not be inferred in all cases when comparing the two species. Accordingly, fragment rather than site data were used for the analysis of the

Table 1 Composite genotypes (haplotypes), haplotype frequencies, haplotype diversity (*h*) with standard error (SE), nucleotide diversity percentage (π) and sample sizes (*N*) of the studied *Silurus glanis* populations. H_o observed heterozygosity in a previous allozyme study

Haplotypes	Comp. genotypes	Sample locality						
		EVR	STN	STH	AXI	ALI	PIN	DDN
Type 1g	AAAAAAB		1.00	1.00				0.35
Type 2g	BAAABBAB				0.74	0.83	1.00	
Type 3g	BAAABBCB					0.17		
Type 4g	BAAACBAB	1.00						
Type 5g	BABABBAB				0.26			
Type 6g	AAAAAAA							0.06
Type 7g	AAAAADAB							0.06
Type 8g	AAAAACBB							0.18
Type 9g	ABAAAAAB							0.29
Type 10g	AAABAAAB							0.06
<i>h</i>		0.00	0.00	0.00	0.39	0.29	0.00	0.79
SE		0.00	0.00	0.00	0.08	0.09	0.00	0.06
π %		0.00	0.00	0.00	0.04	0.03	0.00	0.17
<i>N</i>		31	30	5	31	30	30	17
H_o		0.030	0.020	0.000	0.035	0.032	0.018	0.050

Composite genotypes are denoted by capital letters in the following order: ND-5/6: *AluI*, *RsaI*, *HinfI*, *HhaI*; HN20: *AluI*, *RsaI*, *HaeIII*, *BstNI*. For the purposes of illustration common haplotypes were omitted.

Table 2 Composite genotypes (haplotypes), haplotype frequencies, haplotype diversity (h) with standard error (SE), nucleotide diversity percentage (π) and sample sizes (N) of the studied *Silurus aristotelis* populations. H_o observed heterozygosity in a previous allozyme study

Haplotypes	Comp. genotypes	Sample locality		
		TRI	AMV	VOL
Type 1a	BCDBBC	0.39		0.24
Type 2a	BCDBBB	0.52		0.67
Type 3a	BCDCBB	0.06		
Type 4a	BCEBBB	0.03		
Type 5a	CCDBBB		0.92	
Type 6a	CCDCBB		0.08	
Type 7a	BDDBBB			0.03
Type 8a	BDDBBC			0.03
Type 9a	BCDBC B			0.03
h		0.598	0.166	0.515
SE		0.053	0.134	0.089
$\pi\%$		0.066	0.016	0.058
N		31	12	30
H_o		0.042	0.035	0.037

Composite genotypes are denoted by capital letters in the following order: ND-5/6: *Hae*III, *Hha*I; HN20: *Alu*I, *Hinf*I, *Hha*I, *Bfa*I. For the purposes of illustration common haplotypes were omitted.

relationship of the two species. The average divergence between the two species based on fragment data was 6.75%. The UPGMA dendrogram showing the relationships between the different populations, based on nucleotide divergence values, is presented in Fig. 3. The differentiation of the two species is evident in this dendrogram. For *S. glanis*, the Evros, Axios, Aliakmon and Pineios populations form one group whereas the rest comprise one second cluster, which is concordant with the previous trees on haplotypes and the UPGMA tree constructed based on sequence divergence values of *S. glanis* populations only (not shown).

Discussion

The present study has revealed low levels of mtDNA divergence and variation within the two European silurid catfishes. Levels of intraspecific mtDNA haplotype sequence divergence for freshwater fish reported in the literature (Billington & Hebert, 1991) were as high as 10%. However, in our study values of intraspecific mtDNA haplotype sequence divergence did not exceed 0.39% and 0.73% in *S. aristotelis* and *S. glanis*, respectively. Many authors (Avice, 1989; Cronin *et al.*, 1993) suggest that this pattern may occur in areas that have recently been colonized following glaciation, because of bottlenecks at the time of population origin.

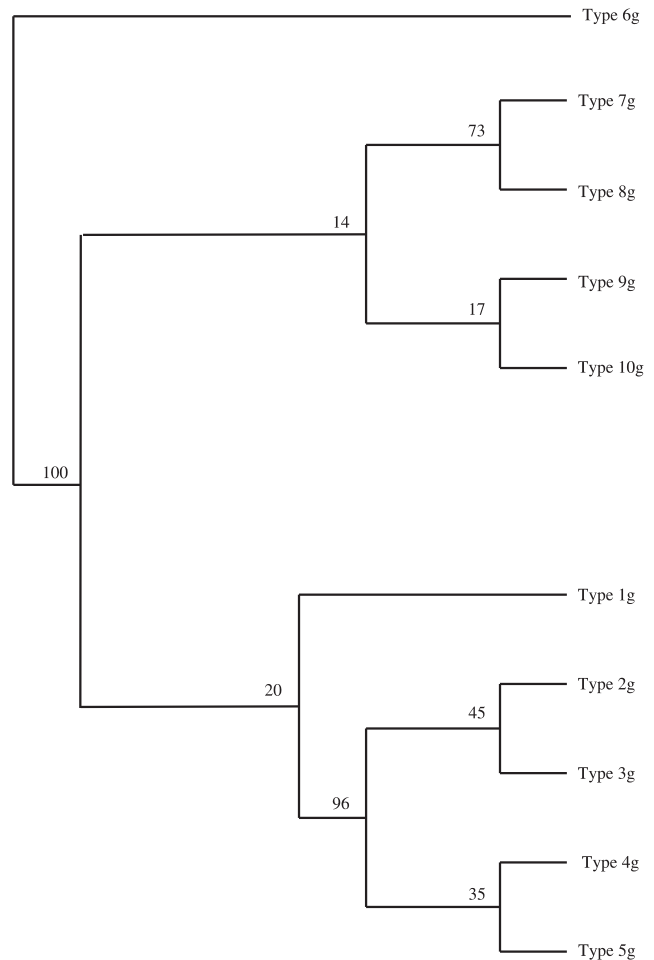


Fig. 2 Majority rule consensus tree clustering the 10 *Silurus glanis* mtDNA haplotypes described in Table 1. Numbers at the forks indicate the percentage of times the group consisting of the haplotypes located to the right of the fork occurred among the trees, out of 1000 trees. The tree was rooted with haplotype 6, which is found in the Danube Delta population.

Ecological characteristics can also play significant roles in determining the distribution and genetic structure of modern-day populations. Wilson & Hebert (1996) also discovered comparably low levels of mtDNA divergence in lake trout (*Salvelinus namaycush*) populations in eastern North America. Apart from the importance of glaciation events, they emphasized that lake trout as a top predator (as is *S. glanis*) may be expected to have smaller population sizes than species at lower trophic levels, which could account for low levels of mtDNA variation. However, additional sampling throughout the distribution of *S. glanis* is required to test if these low diversity values could be considered a species-specific trait.

Despite the low levels of divergence among haplotypes, considerable genetic differentiation was observed

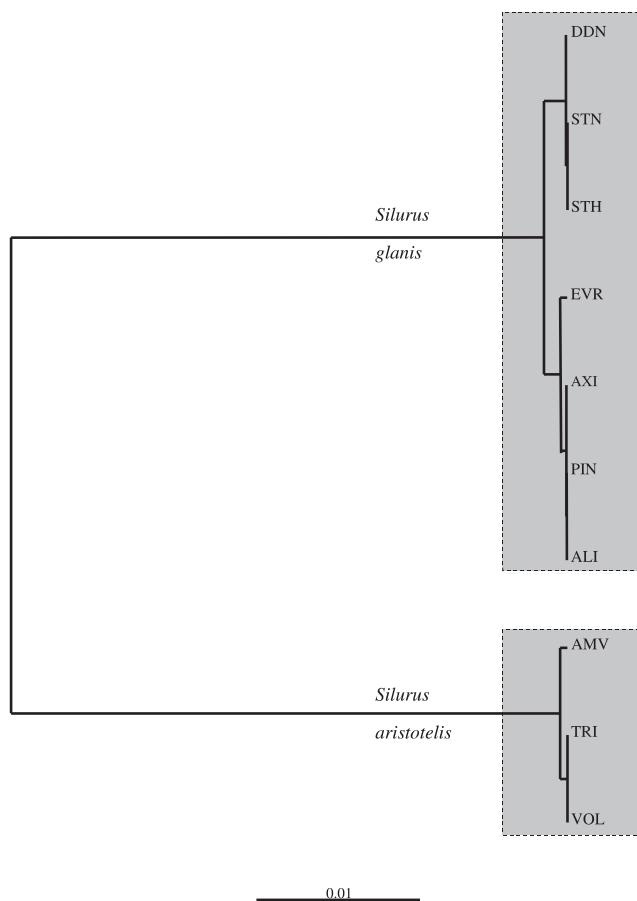


Fig. 3 UPGMA phenogram clustering seven *Silurus glanis* and three *S. aristotelis* populations according to the distance matrix of percentage sequence divergence, based on restriction fragments.

among populations. Eight out of 10 haplotypes for *S. glanis* and seven out of nine haplotypes for *S. aristotelis* were private. As a result, most populations can easily be distinguished because some samples do not share any haplotypes. This is reflected in the significant outcome of all pairwise comparisons between native populations for geographical heterogeneity of mtDNA haplotype distribution.

The differentiation of the wild *S. glanis* population is also evident by the N_{ST} estimate of 0.84, according to which only about 15% of the overall genetic diversity observed was within populations. This value is higher than those reported for various groups of invertebrates and vertebrates (range 0.03–0.075; compiled in Lynch & Crease, 1990). However, such high N_{ST} values have been reported after RFLP analysis of the mtDNA diversity of two more Greek freshwater fish: *Salmo trutta* (Apostolidis *et al.*, 1996) and *Leuciscus cephalus* (Imsiridou *et al.*, 1998). Long-time isolation of the populations coupled

with bottleneck and subsequent genetic drift phenomena, which resulted in stochastic loss of haplotypes, were proposed as a possible explanation.

This strong geographical patterning in the distribution of the mtDNA haplotypes is evident in all trees constructed from relationships either of haplotypes or of populations (Figs 2 and 3). The populations (or haplotypes) of Evros, Axios, Aliakmon and Pineios rivers seem to form one monophyletic group, which diverged from the rest and has remained isolated. The Strymon population does not cluster with the rest of the Greek populations. Loss of haplotypes, characteristic of the other populations, because of stochastic forces and/or introgression of the Danubian origin haplotype 1g, after movement of fish from the Danube through river captures or human influence, could explain this pattern.

Levels of congruence between mtDNA and allozyme variation data

MtDNA haplotype diversity is considered equivalent to heterozygosity of protein polymorphism (Nei, 1987) and as such it is interesting to note that there was agreement with an allozyme variation analysis on the same populations (Triantafyllidis *et al.*, 1999). In both studies, the central European population of the Danube Delta showed the highest value (Table 1). Additionally, the three Greek *S. glanis* populations (of the Pineios, Strymon and Evros rivers) with zero haplotype diversity also exhibited the lowest values of observed (allozyme) heterozygosity. Congruence was also noticed for *S. aristotelis* populations (Table 2).

However, greater differentiation between populations was observed at the mtDNA level than at nuclear loci. Allozyme analysis did not reveal alleles which were characteristic of populations or, if it did, these alleles were of low frequency (<0.05). On the other hand, many mtDNA haplotypes were private for some populations, even fixed in certain cases. This pattern has been reported before (Taylor *et al.*, 1997). It has been associated with the heightened sensitivity of mtDNA as a marker of population structure, because of its fourfold lower effective population size. Consequently, in populations of recent origin the effects of genetic drift are first evident at the mtDNA level.

Another discrepancy between the two methods regards relationships between populations as indicated by genetic distance. The Mantel test, employing 10 000 permutations, showed that there is no correlation between the genetic distance matrices calculated with the two methods ($r = -0.021$, $P = 0.48$). Differences were also observed in the phylogenetic relationships between populations, because the Strymon population does not cluster with the rest of the Greek populations

in the present analysis. Discordance between the two methodologies has been reported before (e.g. Degnan, 1993; Bernatchez & Osinov, 1995). Therefore, this study emphasizes once more the need for multidisciplinary genetic analyses (Triantaphyllidis *et al.*, 1997).

Taxonomic and phylogenetic relationship between the two species

Average sequence divergence between the two species based on fragment data is 6.75%. When fragment data were used to estimate the genetic divergence within species, the values obtained were $\approx 20\%$ lower than those obtained with restriction site data. Some authors (Bernatchez *et al.*, 1991) also suggest that at above 5% divergence, undetectable homoplasy in either restriction site or fragment data analyses leads to an underestimation of the real value. Thus, the estimated value of 6.75% divergence should be considered a minimum. Even so, the value should be within the range of 1.4–16% reported for congeneric freshwater fish (Billington & Hebert, 1991). Therefore, our data support previous morphological studies on catfish (Kobayakawa, 1989; Bornbusch, 1995) according to which the genus *Parasilurus* should be synonymized with *Silurus*.

Estimating the time of divergence between phylogenetic assemblages requires certain assumptions on which there is not always agreement. For instance, Bernatchez *et al.* (1991) assume a rate of 2% nucleotide divergence per million years for *Coregonus* fish, whereas Martin & Palumbi (1993) propose a rate of 0.5–0.9% for salmonid fish. Even if the most conservative value is used, the time of divergence calculated is sometime during the Middle to Late Miocene. Our results are concordant with other authors' views (Bianco, 1990; Economidis & Banareescu, 1991) that the ancestral *Silurus* population was established in central Europe at around that time.

Silurus aristotelis is found only in western Greece. The fish fauna of western Greece comprises species that are believed to have reached the area and remained isolated there since Miocene or at least early Pliocene times (Economidis & Banareescu, 1991). It is proposed therefore that the ancestors of *S. aristotelis* dispersed from the original *Silurus* stock in central Europe, somewhere around that time, and moved south reaching Greece. Based on the low divergence values between *S. glanis* haplotypes, *S. glanis* seems to have spread in Europe later, during the Pleistocene.

The results of this study suggest that protection measures and a conservation programme of the two silurid species be undertaken, for both financial and ecological reasons. First, a number of private haplotypes, which were sometimes fixed in Greek populations, were revealed. These haplotypes can serve as 'genetic

tags' for stock identification in future aquaculture programmes. Secondly, low levels of mtDNA diversity were found, a result in agreement with previous allozyme analysis. This impoverishment of genome variability could be a threat for the future survival of the species.

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