Genetic Variation of the Endangered *Ladigesocypris ghigii* Populations Revealed by the RAPD-PCR Technique and the RFLP Analysis of PCR Amplified mtDNA Segments: Implementation to Species Conservation

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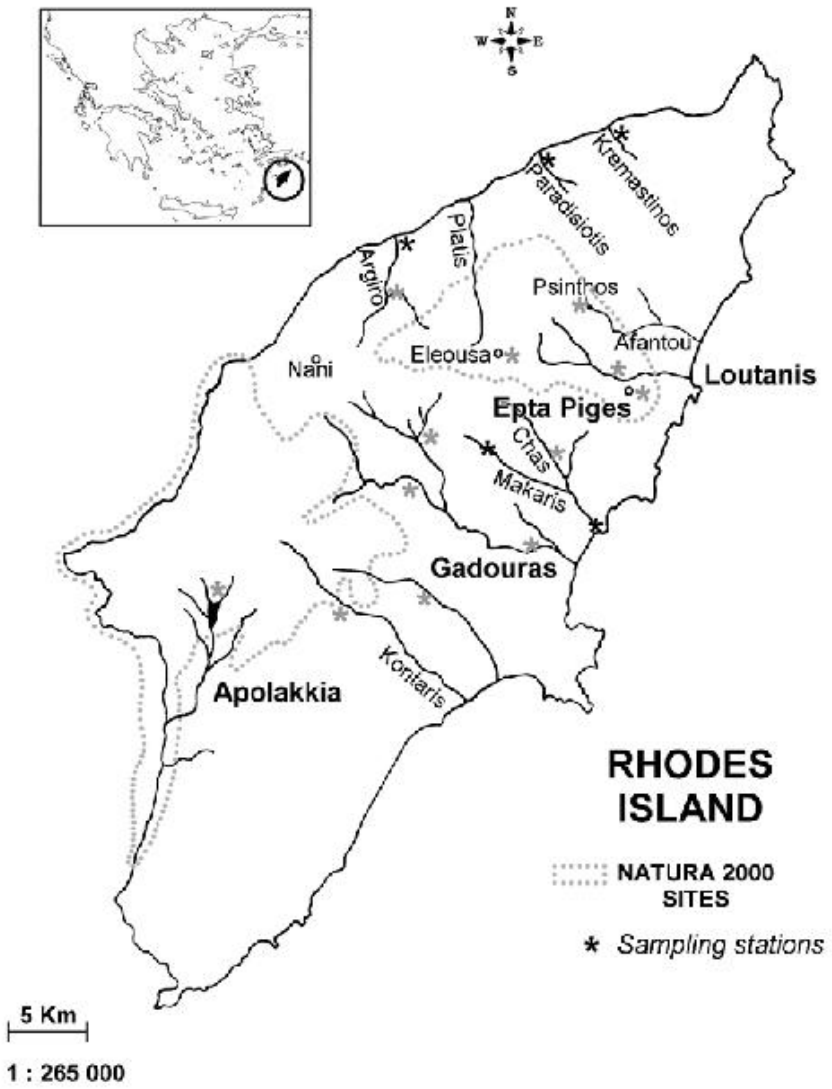
A freshwater fish, commonly known as "gizani"endemic to Rhodes island

A small-bodied cyprinid that inhabits the streams, springs and some small water reservoirs



✓During summers, the majority of the ravines of the island are drying, almost throughout their entire course.

✓Small groups of fish survive in water pockets along the streams and/or near the streams' sources

Loutanis stream, June 2000

Young fishes in Loutanis, June 2000

Gradual decrease of water level in Apolakkia lake

A. April 99

B. June 00

C. September 00

D. May 01

Therefore:

- Gizani is listed as an endangered species of top priority for conservation in ANNEX II of the Habitats Directive (92/43/EEC) of the E.U., concerning the protection of natural habitats and wild fauna and flora

And

- A LIFE-Nature project was undertaken (B4 3200/98/445) aiming at studying the species and implementing measures for its conservation

In the course of this project a biological survey was undertaken to:

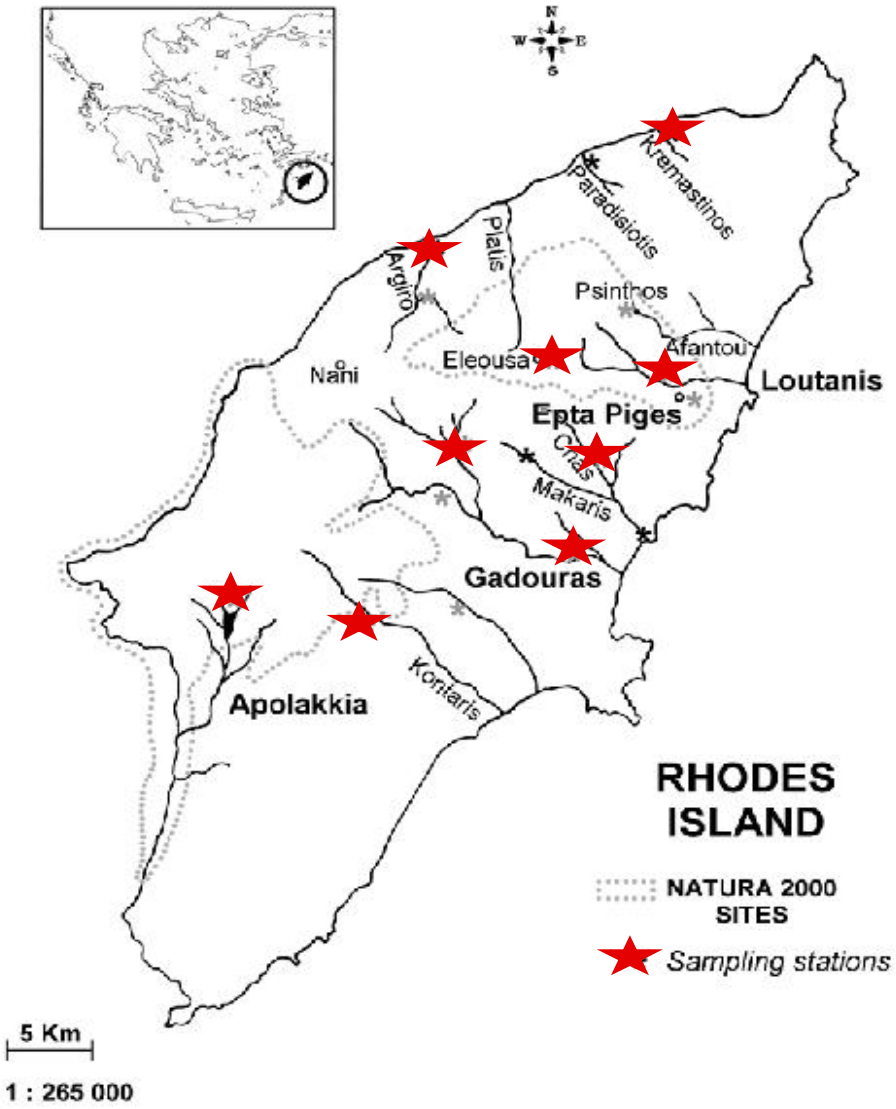
- ✓Create an updated record of existing gizani populations (number and distribution).
- ✓Define the state of the populations and the environmental threats.
- ✓Determine genetic diversity and conservation units.
- ✓Define bottlenecks to recruitment at all life stages.
- ✓Identify options for the conservation of the species.

It was considered essential to obtain **GENETIC INFORMATION** about the gizani populations, to define the conservation units for this species

Nuclear DNA analysis → RAPD method

Mitochondrial DNA analysis → RFLP method

The aquatic systems under study and the sampling stations



RAPD

↓

Sixteen decamer primers

mtDNA

↓

PCR-amplified regions (ND-5/6, COI, 12S-16S rRNA) screened with 20 restriction endonucleases, 4-, 5-, 6-base cutters

RAPD analysis

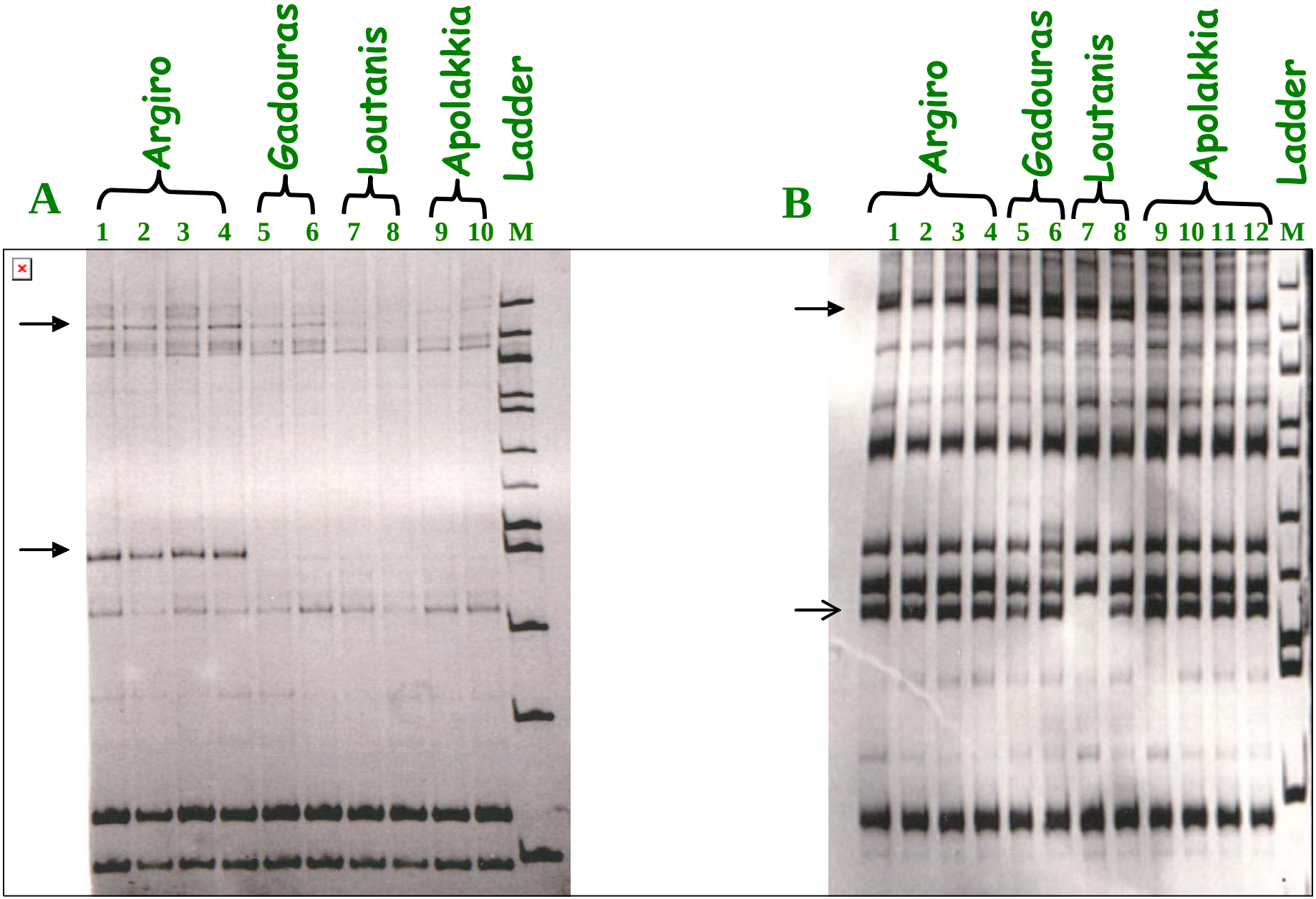
130 bands analysed for all the 16 random primers

6 (4.62%) were polymorphic, observed in different frequencies in the nine populations

112 were monomorphic, constantly present in all individuals

12 were diagnostic (i.e. present in all individuals of one population and absent from all individuals of another population and vice-versa)

RAPD profiles after amplification with two different primers



Numbers of diagnostic bands among the nine populations based on RAPD analysis

Populations	1	2	3	4	5	6	7	8	9
1. Apolakkia	-	7	11	11	6	8	9	5	7
2. Gadouras 1		-	3	7	4	0	2	3	2
3. Loutanis			-	4	5	3	5	6	6
4. Agia Eleoussa				-	5	7	5	8	6
5. Argiros					-	4	6	5	2
6. Gadouras 2						-	2	3	2
7. Kremastnos							-	5	4
8. Kontaris								-	3
9. Chas									-

mtDNA-RFLP analysis

ND-5/6 → 2.50 kb

COI → 1.30 kb

12S-16S rRNA → 2.05 kb

} 40% of the mitochondrial genome

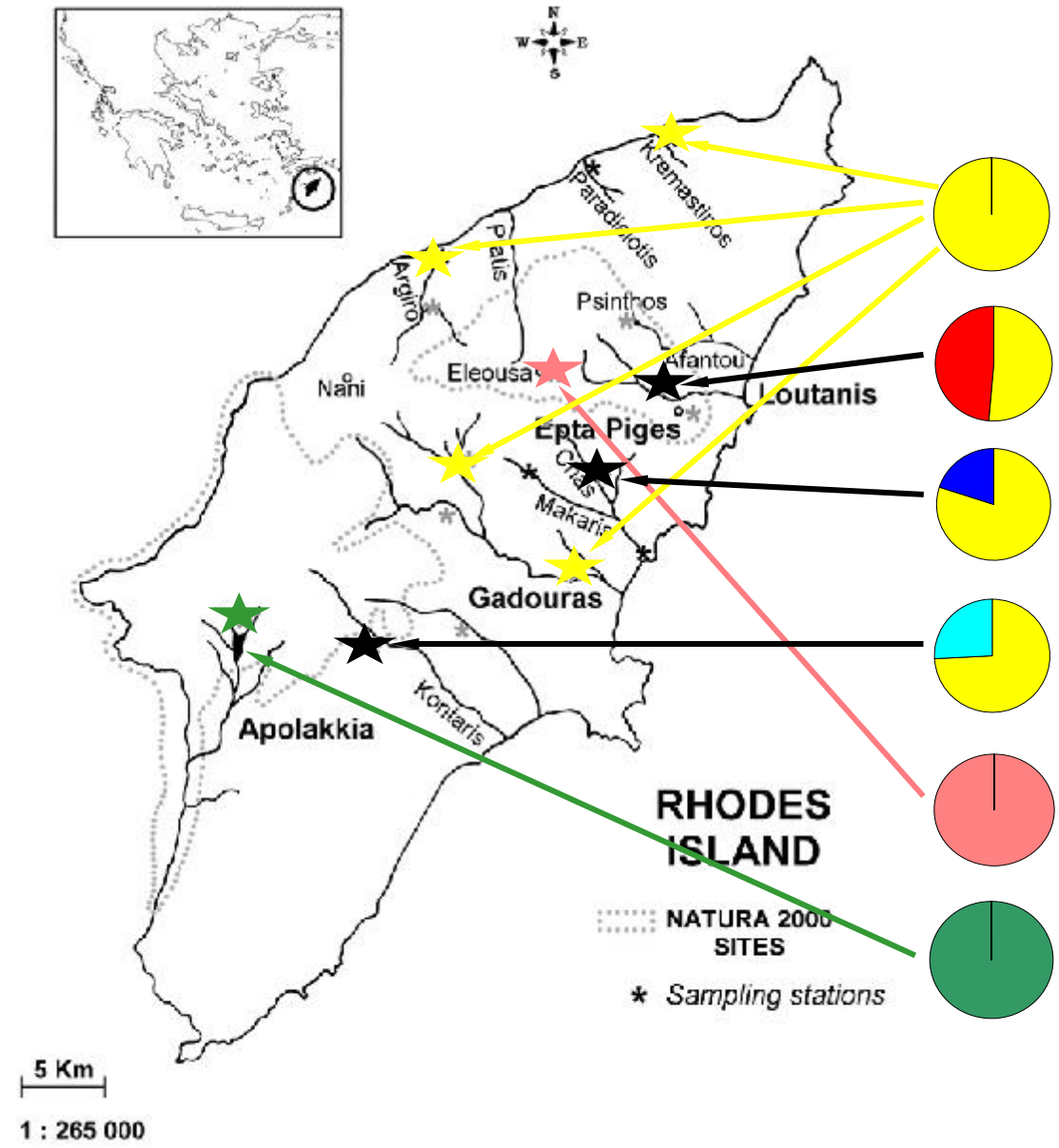
169 restriction sites → 739bp surveyed

mtDNA-RFLP analysis

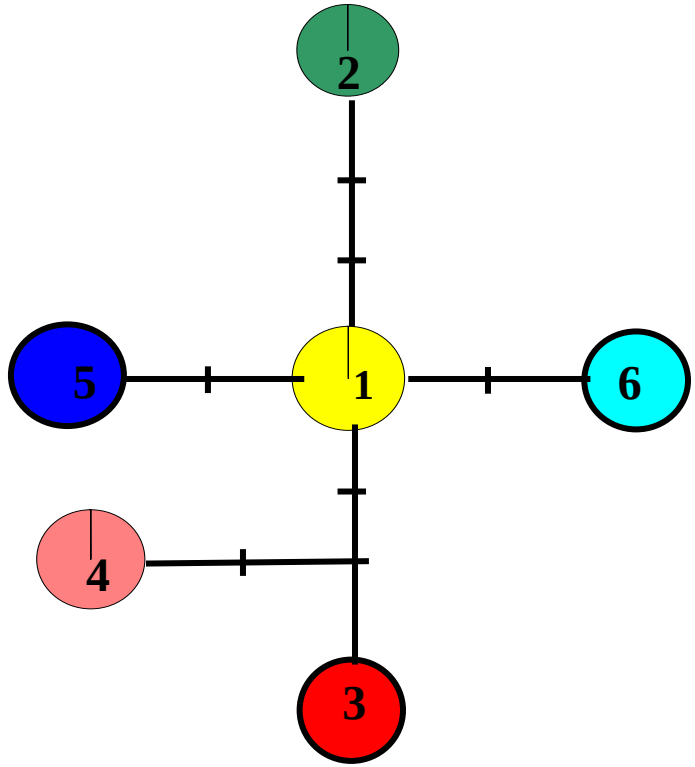
Six haplotypes with very shallow pairwise sequence divergence (x 10^2) (average of 0.500)

Haplotypes	1	2	3	4	5	6
1.Type 1	-					
2.Type 2	0.41	-				
3.Type 3	0.41	0.83	-			
4.Type 4	0.41	0.82	0.69	-		
5.Type 5	0.21	0.63	0.63	0.63	-	
6.Type 6	0.21	0.62	0.62	0.62	0.42	-

Pie diagrams indicating mtDNA haplotype frequencies at each site

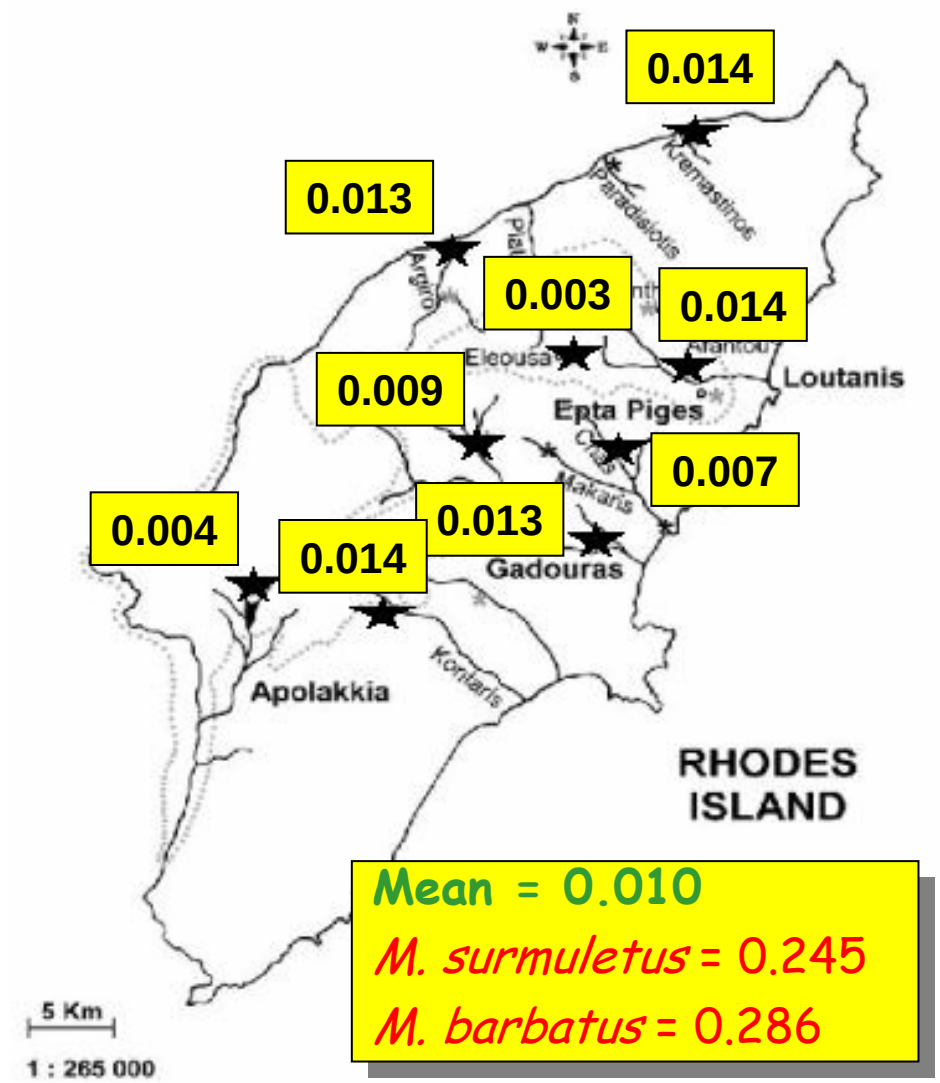


A network indicating the relationships among the 6 haplotypes



A. Low genetic variation within populations

Low values of estimated heterozygosity for the RAPD

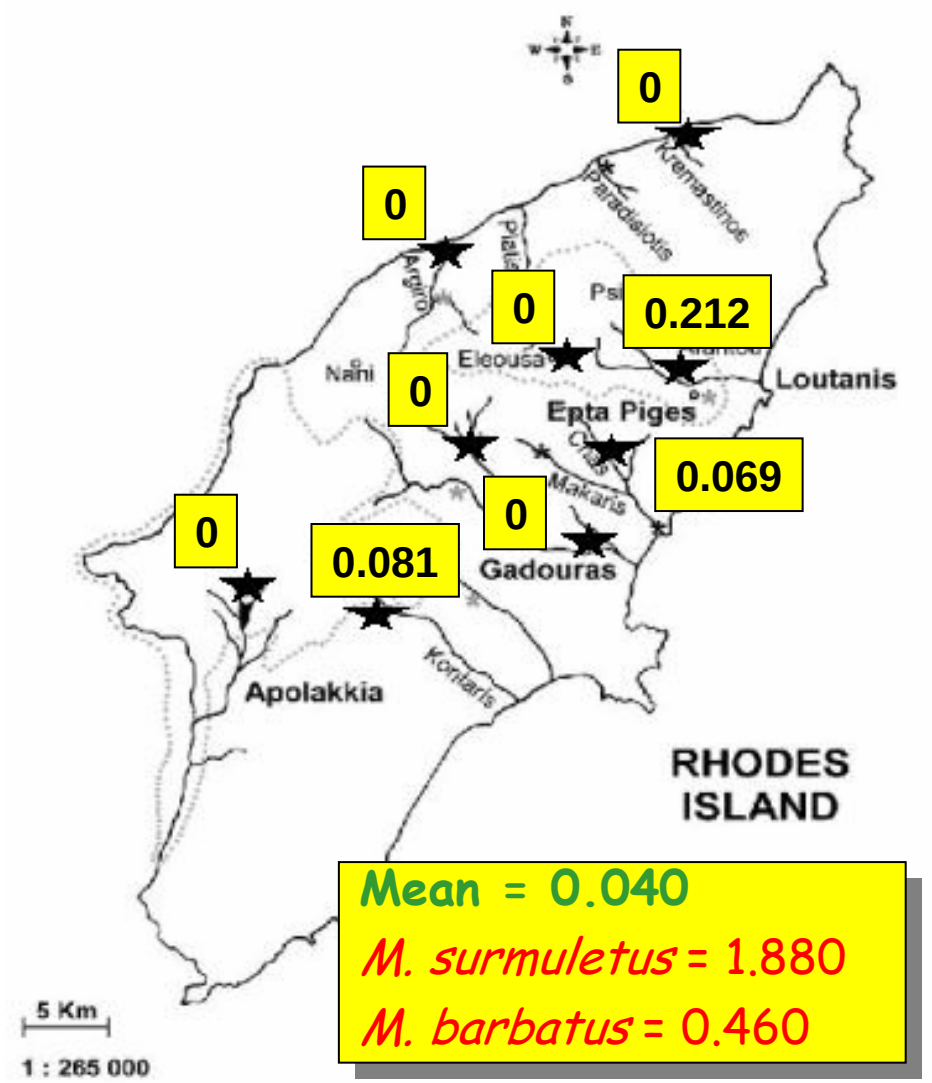


Mean = 0.010

M. surmuletus = 0.245

M. barbatus = 0.286

Low values (%) of nucleotide diversity for the mtDNA



Mean = 0.040

M. surmuletus = 1.880

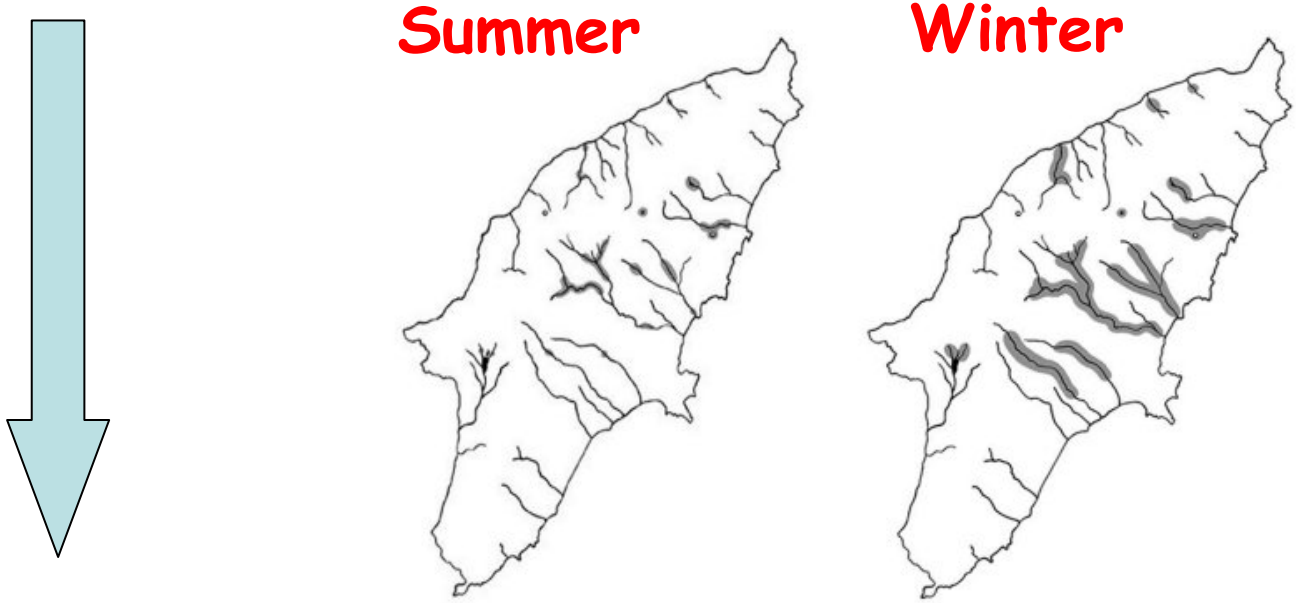
M. barbatus = 0.460

A. Low genetic variation within populations

Species	Mean Heterozygosity (RAPD)	Mean Nucleotide Diversity (mtDNA)
L. ghigii	0.010 *(this study)*	0.040 *(this study)*
Mullus surmuletus	0.245 (Mamuris et al. 1999)	1.880 (Mamuris et al. 2001)
Mullus barbatus	0.286 (Mamuris et al. 1998)	0.460 (Mamuris et al. 2001)
Silurus aristotelis	-	0.046 (Triantafyllidis et al 1999)
Silurus glanis	-	0.034 (Triantafyllidis et al 1999)

Low levels of intra-population variability have been reported for other freshwater fish species in Greece: *Salmo trutta* (Apostolidis *et al.*, 1996), *Leuciscus cephalus* (Imsiridou *et al.*, 1998) and in Europe: *Nannoperca oxleyana* (Hughes *et al.*, 1999), *Chondrostoma lusitanicum* (Mesquita *et al.*, 2001).

Successive bottleneck events evident in shrinkage and expansion of the populations year after year



Complete loss of several genotypes and haplotypes, and an increased degree of inbreeding

Extremely low level of intra-population polymorphism revealed by both nuclear and mitochondrial DNA analysis

B. Strong genetic structuring among populations

Nei's genetic distance based on RAPD (in blue) and nucleotide divergence (x 10^2) for mtDNA analysis (in green),

Lowest value Highest value

Populations	1	2	3	4	5	6	7	8	9
1.Apolakkia	-	0.410	0.509	0.825	0.410	0.410	0.410	0.424	0.420
2.Gadouras1	0.062	-	0.095	0.410	0.000	0.000	0.000	0.013	0.007
3.Loutanis	0.092	0.029	-	0.439	0.095	0.095	0.095	0.107	0.103
4.Eleoussa	0.083	0.051	0.044	-	0.410	0.410	0.410	0.423	0.420
5.Argiros	0.053	0.036	0.046	0.054	-	0.000	0.000	0.013	0.007
6.Gadouras2	0.067	0.002	0.029	0.053	0.033	-	0.000	0.013	0.007
7.Kremastinos	0.081	0.017	0.049	0.037	0.051	0.019	-	0.013	0.007
8.Kontaris	0.046	0.031	0.050	0.064	0.044	0.030	0.050	-	0.020
9.Chas	0.057	0.022	0.051	0.042	0.017	0.017	0.036	0.034	-

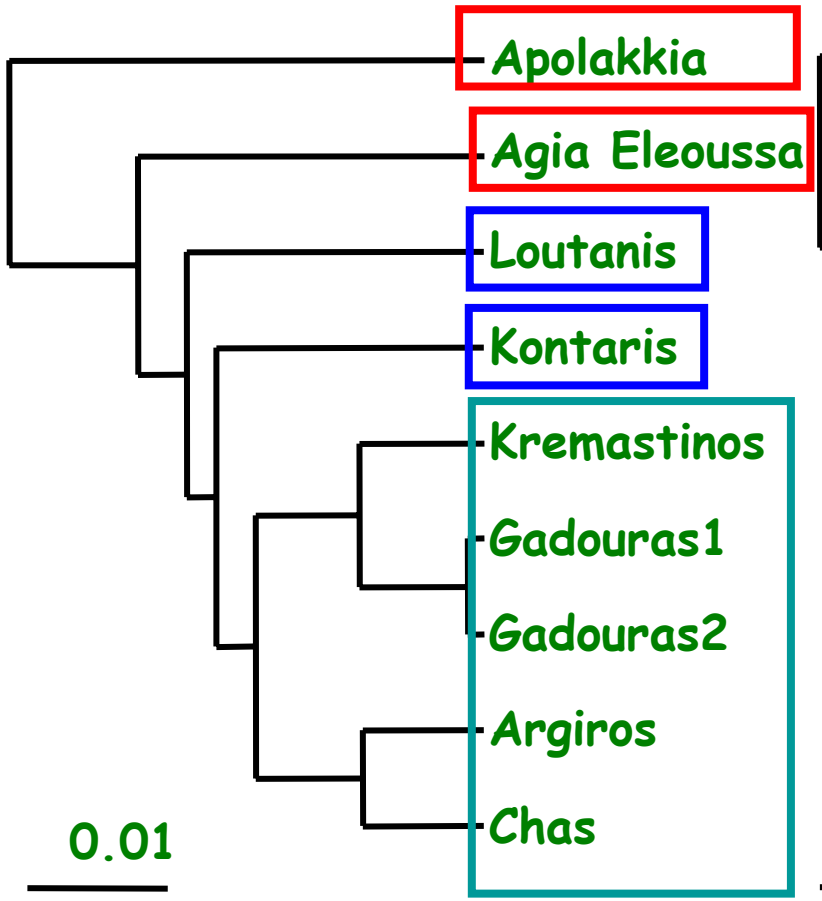
B. Strong genetic structuring among populations

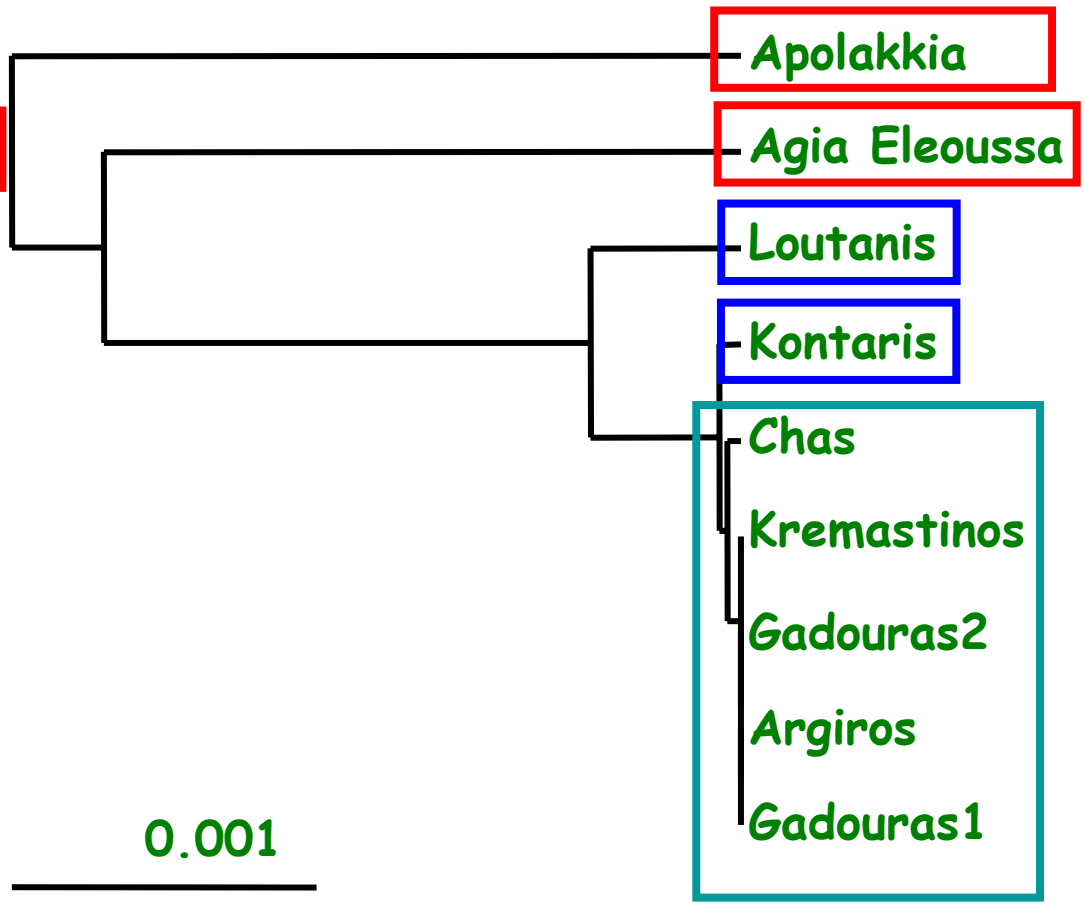
Species	Mean Nei's genetic distance (RAPD)	Mean nucleotide divergence (%) (mtDNA)
L. ghigii	0.045 *(this study)*	0.206 *(this study)*
Mullus surmuletus	0.018 (Mamuris et al. 1999)	0.055 (Mamuris et al. 2001)
Mullus barbatus	0.013 (Mamuris et al. 1998)	0.002 (Mamuris et al. 2001)
Silurus aristotelis	-	0.000-0.110 (Triantafyllidis et al 1999)
Silurus glanis	-	0.000-0.520 (Triantafyllidis et al 1999)

UPGMA phenograms clustering the nine populations

Nei's (1978) genetic distance matrix, RAPD analysis

Net nucleotide divergence matrix mtDNA analysis





It is probable that all haplotypes and/or alleles found here were initially represented in all populations

Long time isolation coupled with successive bottleneck and subsequent genetic drift phenomena

Common mtDNA haplotypes and genotypes among the populations may have become rare or extinct through stochastic lineage loss

Because of the extreme physical isolation between certain sites and the absence of restocking operations

Bottleneck-generated genetic distance could not have been restored by subsequent genetic flow

Suggestions for conservation

The appropriate unit of conservation for an endangered species may be evolutionarily significant units (ESUs)

Units on independent evolutionary trajectories justified by phylogeographic differentiation at the mtDNA level and significant divergence of allele frequencies at nuclear loci

Suggestions for conservation

Fixation of RAPD markers and/or mtDNA haplotypes

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Substantial genetic isolation of populations

↓

The populations may be on independent evolutionary trajectories

↓

Considering that each population appears well adapted so far within each site

IT IS PROPOSED THAT ALL POPULATIONS SHOULD BE MANAGED AND CONSERVED SEPARATELY

Suggestions for conservation

Loss of genetic variability is a major threat to the survival of any species, decreasing the species' potential for adaptation to environmental changes

However

Both RAPD and mtDNA data showed that genetic variability within populations is already extremely low

Therefore

TRANSLOCATIONS OF INDIVIDUALS, ATTEMPTING TO PREVENT A DRAMATIC DECLINE IN POPULATION DENSITIES, ARE NOT RECOMMENDED

They could lead to transmission of pathogens, parasites and locally maladapted individuals or genes, without any certain benefit to increase of genetic variability

Conservation actions concerned mainly:

✓artificial breeding

A female and two males during fertilization in aquaria-indoor installations

✓maintenance of fish stocks

constructions for the refuges in Loutanis stream

✓planning of fish stockings