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RESEARCH ARTICLE

Phylogeography and genetic structure of the Mediterranean killifish *Aphanius fasciatus* (Cyprinodontidae)

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Abstract The purpose of this study was to determine the phylogeographic structure of the brackish-hypersaline cyprinodont fish *Aphanius fasciatus* (Valenciennes, 1821), using sequencing and RFLP analysis of a 1,330 bp mitochondrial DNA segment containing part of the 16S rRNA gene as well as the genes for tRNA-Leu, NADH subunit 1 and tRNA-Ile. Individuals were collected from 13 different sites in Greece and Turkey, while seven published *A. fasciatus* sequences were also included to cover the area of distribution of the species. Pairwise sequence divergence values ranged from 0 to 4.51%. Congruent phylogenies were recovered with maximum likelihood, maximum parsimony and neighbour-joining methods. All analyses revealed two main groups. The first group consists of populations from almost all localities that drain into the Aegean Sea. The second group comprises the remaining population samples, which in some cases seem to consist of population-specific subgroups. Our results show that vicariant events have predominantly affected the evolution of *A. fasciatus*, with the Messinian salinity crisis having shaped the present genetic structure of its populations. Additionally, the life-history traits of the species, which determine a low potential for dispersal, coupled with the typical fragmentation of brackish-hypersaline water habitats have led to a high degree of isolation of *A. fasciatus* populations, even at restricted spatial scales. Analysis of the partitioning of the total amount of polymorphism with analyses of molecular variance (AMOVA) gave a value of $F_{ST} = 84.6\%$. Potential conservation policies concerning *A. fasciatus* should also consider the low-genetic variability in the majority of its populations and the presence of fixed haplotypes in some of them.

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Introduction

Differences in the levels of genetic substructuring in marine, anadromous and freshwater teleosts have been frequently documented (Gyllensten 1985; Ward et al. 1994). Marine species have the potential for long-distance dispersal of eggs, larvae, juveniles and adults, resulting in low levels of differentiation among populations over large distances. On the other hand, species that inhabit fresh- or brackish-water bodies often exhibit narrow habitat specificity, which in combination with the geographic isolation of the different populations lead to a higher likelihood of evolutionary divergence among populations (Carvalho 1993).

In this respect, the population structure of brackish water species has attracted considerable interest. Brackish-hypersaline waters are spontaneously subject to wide daily and seasonal fluctuations of ecological parameters and are

therefore habitats characterised by a high degree of natural environmental stress. Moreover, when used by man as saltworks, for extensive aquaculture or other activities, an additional source of stressful impacts is imposed. These rapid and wide changes of both physico-chemical and biological features may reduce population sizes, which in combination with genetic drift, inbreeding and founder effects could result in loss of genetic diversity and population differentiation at both the morphological and genetic levels (Cognetti 1994; Hartl and Clark 1997; Cognetti and Maltagliati 2000). Several investigators have reported evidence of moderate to high levels of morphological and genetic divergence among populations of brackish water organisms (e.g. see Muus 1967; Cognetti 1994; Kirchhoff et al. 1999; Ferrito et al. 2003, 2007). Therefore, brackish water species are of great interest not only for the sustainable management of lagoon or estuarine fisheries and the conservation of the respective habitats, but also for the understanding of the evolutionary mechanisms of differentiation.

Aphanius fasciatus (Valenciennes, 1821) is a Mediterranean endemic cyprinodont. It is distributed over the central and eastern coastal zone of the Mediterranean (Whitehead et al. 1986; Fischer et al. 1987). It is able to tolerate a wide range of physico-chemical parameters, such as temperature (5–39°C) and salinity (0–180 ppt). It is usually found in brackish water habitats, such as coastal ponds or lagoons, although it has been recorded in inland waters (Kraiem 1983; Parenti and Tigano 1993). *A. fasciatus* populations have declined dramatically, even driven to extirpation in a few cases, due to problems of habitat degradation, destruction and reduction of saltworks, and introduction of allochthonous fishes. Therefore, this fish species has been listed in the Annex III of the 'Bern Convention', relative to the conservation of wildlife and natural environments in Europe and in the Annex II of the 'Fauna-Flora-Habitat' 92/43/CEE Directive, concerning conservation of natural habitats and wild flora and fauna of the European Union (Boudouresque 1996). However, according to the IUCN red list *A. fasciatus* is widely distributed, abundant and has no major widespread threats (Crivelli 2005).

Life history traits of *A. fasciatus* include external fertilisation, large adhesive eggs, absence of larval dispersal stages, short generation time, high-reproductive rate and rapid population turnover (Leonardos et al. 1996; Leonardos and Sinis 1998, 1999). The dispersal of the species is considered to be minimal and, therefore, is an ideal organism for investigating the effects of genetic drift, selection and gene flow on population divergence. Additionally, this species is of no commercial value and the distribution of genotypes among its populations should not be directly affected by human activities (Leonardos 1996).

Most studies on *A. fasciatus* have documented a high degree of isolation among its populations, marked genetic structuring and morphological differentiation (Kiener and Schachter 1974; Parenti and Tigano 1993; Maltagliati 1998a, 1999; Cimmaruta et al. 2003; Tigano et al. 2004, 2006; Ferrito et al. 2007). Thus, the overall genetic diversity of the species is almost completely determined by the among-population rather than within-population genetic variability (Maltagliati 1998a, b, 1999). The observed low levels of within-population genetic variability have been considered a general characteristic of the species (Maltagliati 1999). However, most studies so far have been restricted to Italian populations of *A. fasciatus*, using allozymes as molecular markers.

Interestingly, in the only geographically extended survey of *A. fasciatus* populations based on sequencing of a mitochondrial DNA segment, Hrbek and Meyer (2003) concluded that there was little genetic differentiation among *A. fasciatus* populations, despite its wide distribution and the potential to have been subjected to Mediterranean vicariance events. However, these authors examined only seven samples.

Since a vicariant-based speciation hypothesis is supported for all other species of *Aphanius*, it is important that Hrbek and Meyer's (2003) suggestive results be further investigated. Given the lack of information on the phylogeographic structure of *A. fasciatus* in the northeastern Mediterranean basin, the purpose of this study was to determine its genetic structure using sequencing and RFLP analysis of a mitochondrial DNA segment. Two segments were initially screened, but only one was eventually analysed (see Results). Data from additional populations were collected to complement/extend the previous study of Hrbek and Meyer (2003), to assess the levels of divergence and to compare the degree of genetic variability among several populations of *A. fasciatus*. The phylogenetic relationships derived from this study should permit inferences regarding the evolutionary history of the *A. fasciatus* populations and provide information on the importance of systematics for effective conservation policies in an area where data on killifish biology are restricted.

Materials and methods

Sample collection

Aphanius fasciatus individuals were collected from 13 different sites in Greece and Turkey (Table 1, Fig. 1). *Aphanius iberus* (Valenciennes, 1846) individuals from Villena saltworks Alicante, Spain were used as outgroup.

Table 1 Localities and draining areas of samples included in this study as well as sample sizes for RFLP (N_R) and sequence analyses (N_S)

	Population[a]	Abbreviation	Locality	Marine basin	N_R	N_S
1	Homa lag	IZM	Izmir, W Turkey	Aegean Sea	–	7
2	M. Embolon sal	MEM	Thessaloniki, N Greece	Aegean Sea	22	7
3	Agiasma lag	AGI	Kavala, N Greece	Aegean Sea	22	7
4	Kalloni sal	KAL	Lesbos Island	Aegean Sea	20	7
5	Polychnitos sal	POL	Lesbos Island	Aegean Sea	–	1
6	Tigaki lag	KOS	Kos Island	Aegean Sea	5	5
7	Almyri spr	ALM	E Peloponnese	Aegean Sea	–	1
8	Meligou lag	MEL	E Peloponnese	Aegean Sea	–	4
9	Mesologgi sal	MES	SW Greece	Ionian Sea	24	7
10	Rebakia riv	REB	Acheloos R (W Greece)	Ionian Sea	20	7
11	Amvrakikos gul	AMV	Louros R (W Greece)	Ionian Sea	21	7
12	Korision lag	COR	Corfu Island	Ionian Sea	5	5
13	Villena sal	IBE	Alicante, Spain	W. Medit. Sea	19	5

[a] *Lag* lagoon, *Sal* saltworks, *Spr* spring, *Riv* river, *Gul* gulf

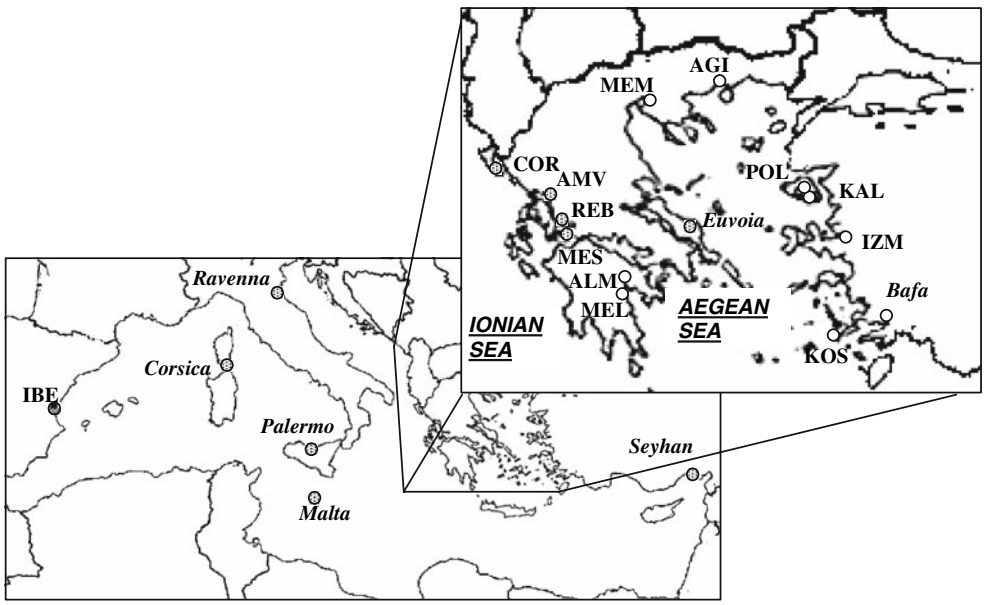

Fig. 1 Map of the Mediterranean Sea showing the geographic location of the *Aphanius fasciatus* populations sampled. Symbols (*open circle* and *filled circle*) refer to the two lineages identified by the phylogenetic analyses. Abbreviated names in bold correspond to populations sampled in this work. Full names in italics correspond to seven samples from Hrbek and Meyer (2003). For abbreviations of population names see Table 1

DNA extraction and amplification

DNA was extracted from individuals using the CTAB method described in Hillis et al. (1996). Initially, two different overlapping DNA segments were amplified (Hrbek and Meyer 2003). The first one (1,330 bp) encompassed part of the 16S rRNA gene as well as the genes for tRNA-Leu, NADH subunit 1 and tRNA-Ile. The second segment (1,280 bp) encompassed the genes for tRNA-Ile, tRNA-Gln, tRNA-Met, NADH subunit 2 and tRNA-Trp. PCR conditions followed Hrbek and Meyer (2003). We used primers L3002 and H4280 for the first segment and L4299 and H5540 for the second segment (Hrbek and Meyer 2003).

Restriction fragment length polymorphism analysis

RFLP analysis was used to study intrapopulation variability in an extended sample size compared to sequencing. Four to seven µl of the PCR product for each segment were digested with nine restriction endonucleases, electrophoretically separated in 2% agarose gels, stained with ethidium bromide, visualised and photographed under UV light. The following restriction enzymes were initially used: *Alu*I,

*Ava*II, *Ban*II, *Hae*III, *Hin*fI, *Msp*I, *Rsa*I, *Ssp*I and *Taq*^{a}I. For molecular weight size standard a 100 bp ladder (New England BioLabs, Beverly, MA, USA) was used. Fragment patterns generated by each enzyme are available upon request.

Sequence analysis

We characterised interpopulational variation within *A. fasciatus* by sequencing the first mtDNA segment, 1,330 bp in length. PCR products were electrophoresed in 1% agarose gels, stained with ethidium bromide and visualised under UV light. DNA fragments were excised from the gels and purified using the NucleoSpin Extract kit (Macherey-Nagel®). Cycle sequencing was performed on a Li-COR 4200 DNA Analyser with the Excell II DNA sequencing kit (EPICENTRE, Madison, WI, USA), using both primers as well as an internal primer (5′-CTATTTTAGGCTCCGG ATGAG-3′). All sequences were deposited in GenBank (accession numbers: DQ923020-DQ923042 and EF640811-EF640857).

Statistical analyses

A specific letter was used to identify distinct, single endonuclease restriction patterns. Each individual was assigned a multi-letter code that described its composite mtDNA genotype. 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. Site inference was aided by seven published *A. fasciatus* sequences (AF449307-AF449313, Hrbek and Meyer 2003).

The genetic software Arlequin 3.01 (Excoffier et al. 2005) was used to calculate haplotype diversity values within populations, to test for population differentiation based on haplotype frequencies (using 10,000 randomisations) and to evaluate the degree of population genetic structure (F_{ST}) through analyses of molecular variance (AMOVA, Excoffier et al. 1992). Throughout the analyses, corrections for multiple comparisons were applied using sequential Bonferroni correction (Rice 1989).

Sequences were aligned with the CLUSTAL X programme (Thompson et al. 1997) and adjusted manually. No indels were found in the final alignment, which included 1,194 bp of the amplified PCR product. The programme PHYML 2.4.4 (Guindon and Gascuel 2003) was used to perform phylogenetic analysis under the maximum likelihood (ML) method. For ML (heuristic search; TBR branch swapping and 100 bootstrap replicates), the best-fit substitution model employed was the TrN + G (Tamura and Nei 1993), as determined by Modeltest 3.7 (Posada and Crandall 1998). The parameters of this model were: unequal base frequencies ($A = 0.26$, $C = 0.28$, $G = 0.16$ and $T = 0.30$), number of substitution types Nst = 3, gamma-shape parameter $\alpha = 0.35$. The MEGA software Version 3.1 (Kumar et al. 2004) was used for the estimation of genetic distances (Tamura and Nei 1993). The obtained pairwise genetic distances were used to construct a neighbour-joining (NJ) tree (Saitou and Nei 1987). The MEGA software was also used for performing maximum parsimony analyses (MP). For MP, trees were generated using heuristic (close neighbour interchange) searches with 100 random taxon additions. Reliability of the inferred NJ and MP trees was assessed by bootstrap analysis using 1,000 and 100 pseudoreplicates, respectively (Felsenstein 1985). In all cases, *A. iberus* was used as an outgroup taxon.

Results

Restriction analysis

The sizes of the two PCR-amplified mtDNA segments were ~1.33 and 1.28 kilobases (kb). Initial screening of the MEM and MES populations (Table 1) revealed a total of 32 cleavage sites in the first segment and 23 sites in the second segment corresponding to 140 and 104 nucleotides, respectively. Seven (*Alu*I, *Ava*II, *Ban*II, *Hae*III, *Hin*fI, *Rsa*I and *Ssp*I) and six (*Alu*I, *Hin*fI, *Msp*I, *Rsa*I, *Ssp*I and *Taq*^{a}I) restriction enzymes detected polymorphism in the first and second segment, respectively. However, no polymorphism was found within the two populations for the second segment. Therefore, for subsequent screening of the polymorphism within populations, only the first segment was analysed. For this analysis, the five enzymes detecting the highest levels of variability were used (*Alu*I, *Ava*II, *Hin*fI, *Rsa*I and *Ssp*I).

Seven haplotypes (composite genotypes) were found in the analysis of eight *A. fasciatus* populations (Table 2). Five of these seven haplotypes were unique (population-specific), but only Af7 haplotype was fixed in the AMV population, whereas all others were in lower frequencies. The *A. iberus* population displayed a different composite genotype (Ai1). Haplotype diversity values were zero in five of the nine populations (including *A. iberus*) analysed by RFLP.

The global test of population differentiation based on haplotype frequencies showed that the results were statistically significant ($P < 0.001$). Nineteen of twenty-eight pairwise comparisons between populations were significant ($P < 0.005$, after Bonferroni correction) and these corresponded to the pairwise comparisons between sites that drain in the Aegean and the Ionian Seas as well as to the comparison of the AMV population with all other populations.

Table 2 Composite haplotypes, haplotype frequencies, sample sizes (*N*) and haplotype diversity (*h*) of the studied *Aphanius* populations, based on restriction sites data for the first mitochondrial segment

The enzymes used were in the following order: *Alu*I, *Ava*II, *Hin*fI, *Rsa*I and *Ssp*I. For abbreviations of population names see Table 1

Composite haplotypes		MEM	AGI	KAL	KOS	MES	REB	AMV	COR	IBE
First segment										
AAAAA	Af1	0.68	0.96	1.00	0.80					
AAABA	Af2	0.32								
AAAEA	Af3		0.04							
AACAA	Af4				0.20					
BBACB	Af5					1.00	0.94		1.00	
BBBCB	Af6						0.06			
CBACB	Af7							1.00		
DBDDA	Ai1									1.00
N		22	22	20	5	24	20	21	5	19
h		0.64	0.09	0.00	0.40	0.00	0.12	0.00	0.00	0.00

Analysis of the partitioning of the total amount of polymorphism with AMOVA gave a value of $F_{ST} = 84.6\%$ (Table 3). More than half (56.16%) of the genetic polymorphism was due to differences between the Aegean and the Ionian marine basins, whereas only 11.78% was due to polymorphism within populations. The remaining 32.05% accounted for differences among populations within marine basins.

Sequence analysis

The sequence of a 1,194 bp part of the first segment of the *Aphanius* mtDNA was determined for 70 individuals (Table 1) representing 13 populations. Seven additional published *A. fasciatus* sequences (Hrbek and Meyer 2003) were also included in the analysis. A total of 262 variable nucleotide sites, 234 of which were parsimony informative were found when including the outgroup and 97 variable and 56 informative characters for *A. fasciatus* alone. The average transition–transversion ratio for the ingroup was 5.4:1. Uncorrected pairwise sequence divergence values for the *A. fasciatus* sequences ranged from 0 to 4.51%, whereas mean sequence divergence between *A. fasciatus* and *A. iberus* sequences amounted to 27.72%.

Congruent phylogenies were recovered with ML (Fig. 2), NJ and MP methods (not shown). All analyses revealed two main groups, strongly supported in most cases. The first group consists of populations from water bodies that flow into the Aegean Sea with the exception of the Euvoia population sample. The second group comprises the remaining populations. Within this group, only one subgroup, including the Palermo, Malta and Corsica populations, is supported by strong bootstrap proportions (>80%); however, other subgroups consisting of population-specific sequences are evident for the AMV and the COR populations. Additionally, all MES and REB sequences cluster together, with the exception of one sequence from each population (Fig. 2). Low-sequence divergence was found within each group (mean 0.14 and 0.55% for the first and the second group, respectively). The highest degree of divergence was detected between the samples from the two groups (mean 3.45%).

Discussion

Research into the evolution of the genus *Aphanius* had until now provided support for vicariant-based speciation hypotheses for each species of the genus with the exception of *A. fasciatus* (Hrbek and Meyer 2003). This was due to little genetic differentiation and population structuring found within *A. fasciatus* analysed in that work. However,

Table 3 Results of the hierarchical analysis of molecular variance for *A. fasciatus* using the Aegean and Ionian marine basins as the levels of structure, or without structure

Structure	Source of variation	% Total variance	Fixation indices	*P*
No structure	Among populations	84.64	$F_{ST} = 0.846$	<0.00001
	Within populations	15.36		
Two major marine basins	Among basins	56.16	$F_{CT} = 0.561$	<0.00001
	Among samples/within basins	32.05	$F_{SC} = 0.731$	<0.00001
	Within samples	11.78	$F_{ST} = 0.882$	<0.00001

The percentage of variation, the *F* statistics as well as the probability (*P*) estimated from permutation tests are given at each hierarchical level (Excoffier et al. 1992)

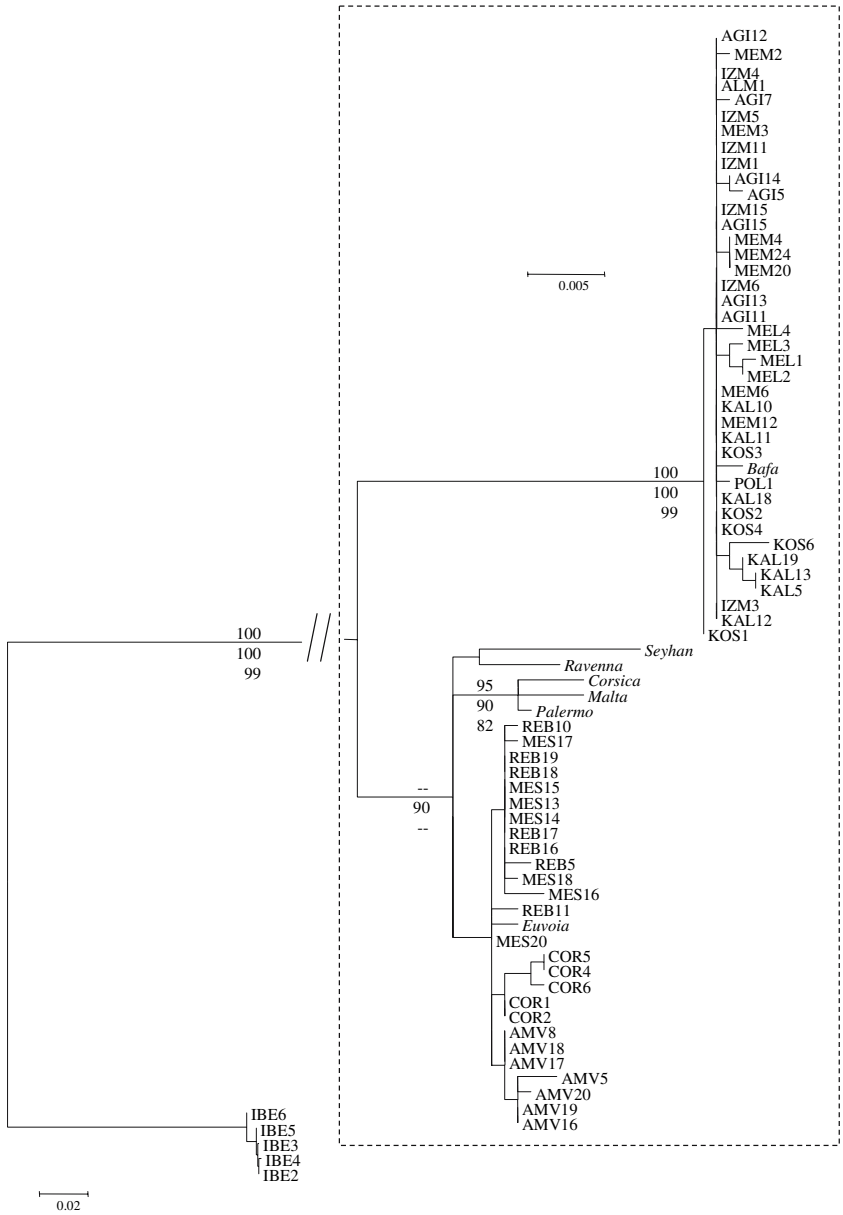

Fig. 2 Maximum likelihood tree showing phylogenetic relationships among *Aphanius* sequences based on a 1,194 bp mitochondrial DNA segment (see text for details). Values at *nodes* indicate bootstrap support for ML (*top*), MP (*middle*) and NJ (*bottom*) methods. Only values higher than 80% are shown. The topology is rooted with *A. iberus*. The branch connecting the outgroup to the ingroup was re-scaled in order to focus on the differences within *A. fasciatus*. For abbreviations of population names see Table 1

the current results provide clear evidence for the presence of two substantially differentiated groups within *A. fasciatus*. These two groups are genetically structured according to a geographical pattern (Fig. 1). The first group contains almost all populations inhabiting localities draining into the Aegean Sea, whereas the second group has a broader distribution.

The presence of diagnostic haplotypes and the degree of genetic divergence found between the two lineages indicate an interruption of gene flow and suggest that the two groups have subsequently evolved independently. This evidence favours the hypothesis that the presence of historical barriers has prevented gene flow, since the two lineages inhabit isolated areas, allowing little or no genetic exchange (see next paragraph). The only deviant sample is the one from Euvoia Island (Lake Prokopis), which does not cluster with the rest of the Aegean group. The sequence of this sample was derived from GenBank. Efforts to acquire additional samples from this area have proven unfruitful.

Mean sequence divergence based on the first mitochondrial segment between the two lineages is 3.45%. Hrbek and Meyer (2003) estimated a sequence divergence value of 6.86% between the single sample (Bafa, Turkey) they analysed from the Aegean group and the rest of the samples belonging to the other group. Based on their estimates for the time of divergence, the timing of separation of the two groups is around 4 million years ago (MYA), sometime after the Messinian salinity crisis (Hsü et al. 1977). This event has played a significant role in the evolution of the present European ichthyofauna (Tsigenopoulos et al. 2003

and references therein) either through the initial formation of distinct refugia or through the subsequent formation of dispersal routes for fishes at the hypothesised Lago Mare phase of the Mediterranean Sea (Bianco 1990). Hrbek and Meyer (2003) have stated that if the Messinian salinity crisis played a role in the structuring of *A. fasciatus* populations, subsequent gene flow erased all traces of this vicariant event. The extended sampling of the present work, however, clearly corroborates the previous work of Hrbek and Meyer (2003) and supports their main conclusion of predominantly vicariant events affecting the evolution of the whole *Aphanius* genus. Our results are also in agreement with the work of Perdices et al. (2001), who estimated a 5-million-year divergence between the Guadalquivir and Balearic populations of *A. iberus* and who also discussed the importance of the Messinian salinity crisis on the evolution of that species. This is also true for other species inhabiting hypersaline environments (Triantaphyllidis et al. 1997, Baxevanis et al. 2006). However, it is also true that the second group includes populations extending from the western to the eastern Mediterranean Sea. Additional sampling might provide more thorough insights into the exact genetic structure and timeframe of diversification of *A. fasciatus*.

Genetic differentiation among populations

Even though low-sequence diversity was found within each group (see results), a high degree of isolation among populations is indicated (even within each lineage) by the number of unique or even fixed haplotypes and sequences that were detected. For example, five unique RFLP haplotypes were found in the populations analysed, with the AMV population fixed for a unique haplotype (Af7, Table 2). As a result, most pairwise comparisons between populations based on RFLP haplotype frequencies were statistically significant and F_{ST} values even within the Aegean or Ionian marine basins were high (Table 3). Additionally, all samples sequenced from AMV and COR populations showed further population-specific mutations that were not always detected by RFLP analysis (Table 2, Fig. 2). Therefore, it seems that cessation of gene flow among populations might be recent, but it appears to be complete in some cases. However, it should be noted that due to the restricted number of samples analysed in some cases, these levels of genetic differentiation might be lower.

The population structuring found in *A. fasciatus* was similar to that in other cyprinodontid fishes. F_{ST} values higher than 0.45 have been reported in populations of various species of brackish- and fresh-water cyprinodontids (Ashbaugh et al. 1994; Dunham and Minckley 1998). Spatial heterogeneity has also been found within the Mediterranean and the Atlantic populations of *A. iberus* (Garcia-Marin et al. 1990; Doadrio et al. 1996; Garcia-Marin and Pla-Zanuy 1999; Perdices et al. 2001). Previous allozyme studies of *A. fasciatus* populations have shown high-genetic differentiation among populations with θ values ranging from 0.302 to 0.507 (Maltagliati 1998b, 1999). AMOVA analysis in this work gave a much higher value of $F_{ST} = 84.6\%$ (Table 3). Under neutral evolution and an island model, the migration rate of mtDNA genomes is four times smaller than nuclear genes (Hillis et al. 1996). So, we could expect a level of genetic differentiation among populations four times higher for mtDNA than nuclear genes (Birky et al. 1983; Triantafyllidis et al. 1999; Krieg et al. 2000). Our results are also in agreement with the recent work of Tigano et al. (2006) who also found numerous unique haplotypes and significant population structuring after sequencing a segment of the D-loop region in central Mediterranean *A. fasciatus* populations.

The genetic substructuring of *A. fasciatus* is not surprising. Apart from the importance of historical biogeography, which has already been discussed, its life-history traits determine a low potential for dispersal. Its biological features account for a limited vagility (benthic eggs and absence of planktonic larval stages) and are coupled with environmental partitioning typical of brackish water habitats. The long-term interruption of gene flow that was detected reflects the strong genetic discontinuity found among the lineages. It should be noted however that, although *A. fasciatus* is considered a non-migratory species, migration of individuals from one habitat to another is possible. In fact, individuals have been recorded in the open sea schooling with juveniles of *Sardina pilchardus* (Walbaum, 1792), 20–70 m from the coast (Torchio 1967 and personal observations). The presence of this species in the marine environment is considered occasional and probably is related to unpredictable extreme events, such as exceptional rainfalls or floods that might flush individuals of *A. fasciatus* away from brackish water habitats into the open sea (Maltagliati 1998a).

Genetic variability within populations

RFLP analyses of the intrapopulation variability in a subset of eight *A. fasciatus* populations have revealed low levels of polymorphism (Table 2). Haplotype diversity values were low or zero in most cases. The results are in agreement with the characteristically low levels of polymorphism in many typical brackish water invertebrate species (Battaglia et al. 1978; Abbiati and Maltagliati 1996). Such low-genetic variation has also been found in previous studies in *A. iberus* (Doadrio et al. 1996) and *A. fasciatus* populations (Maltagliati 1998a, b, 1999). A possible explanation for these low levels of genetic variability in *A. fasciatus* could be the random loss of alleles in isolated local populations

through the effects of genetic drift and additional inbreeding or bottleneck processes. However, an extended allozyme study of 23 *A. fasciatus* populations has found evidence of bottleneck phenomena in only two of them (Maltagliati 2002). Alternatively, the fact that individuals inhabiting high-salinity waters exhibit lower genetic variation, could also be associated with habitat conditions, e.g. be related to the environmental variability of their brackish habitat; among the most extended theories reported for explaining low polymorphism in brackish water species is one related to adaptation to brackish water habitats, which requires tolerance of considerable fluctuation of physicochemical parameters, such as salinity, temperature and oxygen concentration (Cognetti 1994; Naihong et al. 2000).

Conclusions

Results of this study have clearly shown the existence of two differentiated *A. fasciatus* phylogenetic groups. This is particularly important for a species that needs to be protected, such as *A. fasciatus*. In the present case, correct conservation planning should recognise the two lineages as evolutionarily independent entities that should be managed separately. In addition to the above, future management of *A. fasciatus* populations should take into account the low-genetic variability of most populations and the presence of fixed haplotypes in some of them (e.g. the Amvrakikos and the Corfu populations). From a conservation perspective, it has been suggested that heterozygosity estimates should be used in making decisions about the management of populations and species (Vrijenhoek et al. 1985; Leberg 1992). However, the consideration of locally adapted populations as unique entities is also crucial to the preservation of genetic variability.

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