Marine Biology (2006) 149: 1539–1550
DOI 10.1007/s00227-006-0300-x

RESEARCH ARTICLE

**Concetta Tigano · Adriana Canapa · Venera Ferrito
Marco Barucca · Isabella Arcidiacono · Alan Deidun
Patrick J. Schembri · Ettore Olmo**

A study of osteological and molecular differences in populations of *Aphanius fasciatus* Nardo 1827, from the central Mediterranean (Teleostei, Cyprinodontidae)

Received: 20 January 2005 / Accepted: 2 February 2006 / Published online:14 April 2006

Abstract Nine populations of *Aphanius fasciatus* Nardo, 1827 from the central Mediterranean were analysed by examining the mitochondrial control region and the morphology of the bony elements of the skull and vertebral column, to study the degree of intraspecific differentiation of *A. fasciatus* considering the level of isolation of the different populations and the palaeogeographic history of the central Mediterranean area. Both the molecular and morphological analyses differentiate between the populations, even if the topologies of the two trees are different. Molecular and osteological investigations have consistently demonstrated a well-supported differentiation of the south-eastern Sicilian populations both within the same group (Tigano et al. in Ital J Zool 71:1124–1133, 2004a; Tigano et al. in Abstract volume XI European Congress of Ichthyology, Tallin, Estonia, 2004b), and from the populations from western Sicily, Tunisia and the island of Malta. The molecular results show that the nine populations are characterised by haplotypes that are well defined in relation to a probably limited gene flow; while, as regards the morphological data the differentiation found could be explained in terms of the geographic isolation of the various populations, although the influence of environmental factors, which differ greatly between the various sites where the populations live, cannot be ruled out.

Communicated by R. Cattaneo-Vietti, Genova

C. Tigano (✉) · V. Ferrito · I. Arcidiacono
Department of Animal Biology, University of Catania,
Via Androne, 81, 95124 Catania, Italy
E-mail: tigaconc@unict.it
Tel.: +39-95-7306030
Fax: +39-95-327990

A. Canapa · M. Barucca · E. Olmo
Institute of Biology and Genetics,
Marche Polytecnical University, Via Brecce Bianche,
60131 Ancona, Italy

A. Deidun · P. J. Schembri
Department of Biology, University of Malta,
MSD06 Msida, Malta

Introduction

Models of genetic variation relative to the geographic distribution of a species, correlated to changes in gene flow over time and to diversification of the populations, have recently been studied using a phylogeographic approach that has allowed a better interpretation of the micro-evolutionary processes in nature (Avise 2000); studies based on the analysis of variations in mitochondrial DNA, have shown a considerable phylogeographic structure in populations of marine Teleostei (Bernardi 2000; Stepien et al. 2001).

Amongst the vertebrates, cyprinodontids are a group of teleosts that is particularly suitable for the study of micro-evolutionary processes which shape patterns of genetic structuring and geographic variation in natural populations, as well as of those adaptive processes in response to ecological conditions (Villwock 1976). There is a vast amount of literature on the population differentiation of killifish from the morphological, genetic and molecular points of view (e.g. Echelle et al. 1987; Garciamarin et al. 1990; Fernandez-Pedrosa et al. 1995; Strecker et al. 1996; Wilde and Echelle 1997; Duhan and Minckley 1998; Duvernell and Turner 1999; Echelle et al. 2000; Perdices et al. 2001; Torralva et al. 2001; Reichenbacher and Sienknecht 2002; Doadrio et al. 2002; Lussen et al. 2003).

The cyprinodontid *Aphanius fasciatus* Nardo (Fig. 1), is currently distributed in the saline coastal waters of the central and eastern Mediterranean, in salt flats and also occasionally in inland fresh water (Wildekamp 1993). The natural fragmentation of such saline habitats contributes to the non-contiguous coastal distribution of this species, and studies of gene flow between populations suggest that the one-dimensional stepping-stone model proposed by Kimura and Weiss (1964) and Slatkin (1994) might best describe the

Fig. 1 *Aphanius fasciatus* Nardol

mechanisms shaping the genetic structure of this species (Maltagliati 1998b). Studies on the population differentiation of *A. fasciatus* have utilised the osteology of the skull (Tigano and Ferrito 1985; Tigano and Parenti 1988; Tigano 1991; Parenti and Tigano 1993; Tigano et al. 1999, 2001), allozymic variation (Comparini et al. 1984; Maltagliati 1998a, b, 1999, 2002; Maltagliati et al. 2003; Cimmaruta et al. 2003), and cytogenetic variation (Vitturi et al. 1995; Ferrito et al. 2000; Tigano et al. 2003). The allozymic and morphological investigations have all demonstrated a notable differentiation of the populations; analogously, the analysis of the highly variable D-loop tract of the mitochondrial DNA (Tigano et al. 2004a, b) has indicated a strong genetic divergence between three Sicilian populations of this species. On the other hand, the molecular analysis carried out by Hrbek and Meyer (2003) showed that there is limited structuring of *A. fasciatus* populations; however, authors who analysed various species of the genus *Aphanius*, considered mitochondrial genes more useful for studies above the species level. While the allozyme studies indicate that, in some cases, there is indeed genetic divergence of the populations in relation to their geographic distribution (Maltagliati 1998a, 1999), other studies both based on allozymic (Cimmaruta et al. 2003) and morphological data (Tigano et al. 2001; Ferrito et al. 2003), suggest that this divergence does not relate to the geographic distance between the different populations. The aim of the present study is to evaluate the degree of differentiation of nine populations of the killifish *A. fasciatus* from the central Mediterranean, on the basis of molecular and morphological analyses, with reference to the palaeogeographic history of the Mediterranean area.

Materials and methods

Specimens from six different populations of *A. fasciatus* were divided into three groups (two populations in each group), based on their place of origin: western Sicily, Tunisia and the island of Malta; data from a fourth group of three populations from south-eastern Sicily previously studied by Tigano et al. (2004a) (Table 1, Fig. 2) were also used in the statistical analyses of genetic structure and osteological variables.

Meristic and morphometric analysis

A total of 150 specimens of *A. fasciatus* (standard length = 2.1–4.6 cm) were fixed in 5% formalin and then preserved in 70% ethanol. For the study of the skeletal elements, the specimens were treated according to the procedure described by Dingerkus and Uhler (1977) which employs alcian blue for the staining of cartilage and alizarin red S for bone. Nineteen meristic and 25 morphometric characters were examined (Table 2). A diagrammatic representation of the osteological characters considered in this study has been given by Ferrito et al. (2003). The Kruskall–Wallis test was used for analysis of variance while comparisons between the different populations were made using the Mann–Whitney U test. Differences in body shape were analysed using principal component analysis (PCA). To summarise inter-population differences, cluster analysis based on the unweighted pair-group method using arithmetic average (UPGMA) was applied to the matrix of the generalised Mahalanobis distance. Statistical analyses were made using the software package STATISTICA (http://www.statsoft.com).

Table 1 List of the populations examined: sampling areas with geographic co-ordinates, population identification code and number of specimens examined

Groups	Sampling areas	Co-ordinates	Code	Specimens
Sicily south-eastern	Pantano Longarini	36°40′N 15°02′E	LO	n=25: 8 ♂♂ 17 ♀♀
From Tigano et al. (2004a)	Foce Marcellino	37°15′N 15°12′E	FM	n=25: 4 ♂♂ 21 ♀♀
	Pantano Viruca	36°39′N 15°03′E	PV	n=24: 8 ♂♂ 16 ♀♀
Sicily western	Salina Chiusa Trapani	38°01′N 12°28′E	TP	n=25: 5 ♂♂ 20 ♀♀
	Salina Curto Marsala	37°47′N 12°27′E	SMA	n=25: 6 ♂♂ 19 ♀♀
Malta	Ghadira Bird Sanctuary	35°27′N 14°21′E	GHA	n=25: 14 ♂♂ 11 ♀♀
	Salina	35°56′N 14°24′E	SAL	n=25: 11 ♂♂ 14 ♀♀
Tunisia	Lake Tunisi South	36°49′N 10°14′E	LTS	n=25: 6 ♂♂ 19 ♀♀
	Ghar El Milh	37°09′N 10°09′E	GEM	n=25: 11 ♂♂ 14 ♀♀

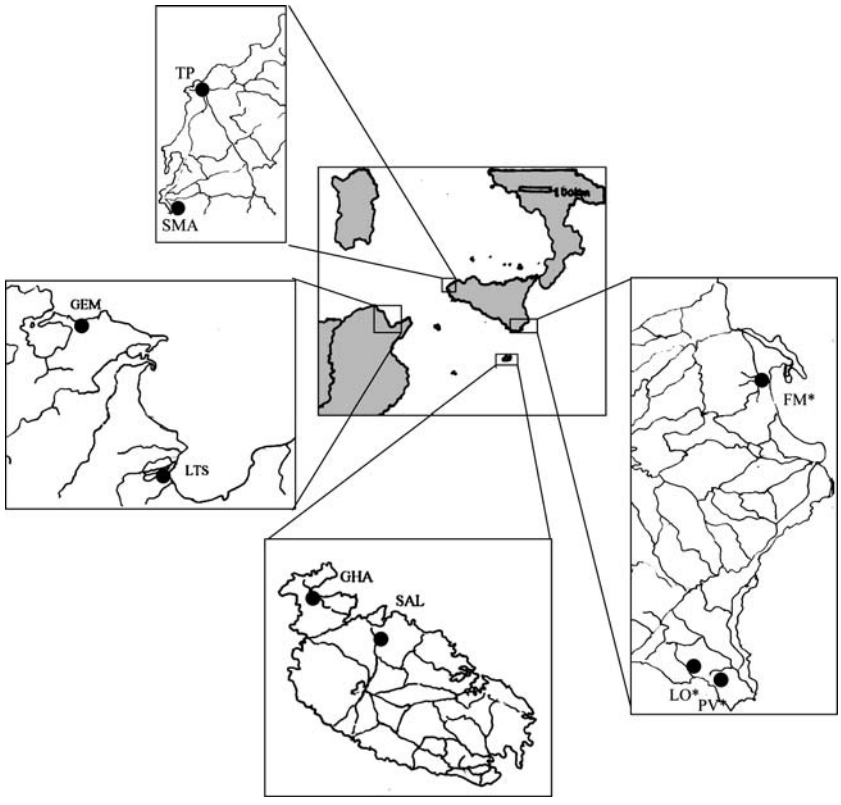

Fig. 2 Sampling areas in Sicily, Malta and Tunisia. The *asterisk* indicates the three south-eastern Sicilian populations (Tigano et al. 2004a). South-eastern Sicily: *LO* Pantano Longarini, *FM* Foce Marcellino, *PV* Pantano Viruca; Western Sicily: *TP* Salina Chiusa Trapani, *SMA* Salina Curto Marsala; Malta: *GHA* Ghadira Bird Sanctuary, *SAL* Salina; Tunisia: *LTS* Lake Tunisi South, *GEM* Ghar El Milh

To remove the size component from the shape measurements (Thorpe 1976), all the measurements of the individual morphometric variables were standardised according to the formula: $M_S = M_O\ (L_S/L_O)^b$, where M_S is the standardised measurement, M_O the measured character length, L_S the overall (arithmetic) mean standard length for all fish from all samples in each analysis, L_O the standard length of specimen; b was estimated for each character from the observed data by the allometric growth equation $M = aL^b$. Parameter b was estimated as the slope of the regression of log M_O on log L_O, using all the specimens from all groups, but allowing the intercept to differ between groups (Elliott et al. 1995).

Mitochondrial control region sequence analysis

Total DNA was extracted according to the method of Jeffreys and Flavell (1977) from 53 specimens of *A. fasciatus*. About ten specimens from each population were analysed. For PCR amplification of the mtDNA control region, the forward primer (5′-ACTATTCTTTGCCGGATTC TG-3′) (Tigano et al. 2004a) and the reverse primer designed by Meyer et al. (1990) were used. The amplification conditions were as follows (30 cycles): 94°C, 1 min; 55°C, 1 min; 72°C, 2 min. The amplified DNA was directly sequenced using an automated DNA sequencer (ABI PRISM 310, from PE Biosystems). Alignments were performed with the CLUSTAL W program (Thompson et al. 1994) set at default parameters. CLUSTAL W is available from the Internet web site http://www.ftp.ebi.ac.uk. The trees were produced using neighbour-joining (NJ) and maximum parsimony (MP) with the PAUP 4.0 beta version (Swofford 1998). The NJ tree was constructed using pairwise distances calculated following the application of Kimura's (1980) two-parameter correction for multiple substitutions. The MP tree was produced using branch and bound search with equal character weighting and random stepwise addition with ten replications, with only minimal trees being retained. Bootstrap values, indicating robustness of nodes, refer to 1,000 replications. The alignment of haplotype sequences (accession numbers from AM184186 to AM184201) with another eight sequences of three populations of *A. fasciatus* from south-eastern Sicily obtained from GenBank (AJ605322-AJ605329) and the sequence of *A. danfordii* used as outgroup (U06062) was 381 nucleotides long. To complement these results, Nested Clade Analysis was performed. The algorithm developed by Templeton et al. (1992) was implemented using the computer program TCS 1.13 (Clement et al. 2000). The permutation tests were carried out using the GEODIS 2.0 program (Posada et al. 2000) and the results were analysed with the inference key provided in Templeton and Sing (1993).

In order to investigate the correlation between molecular and morphological distance matrices, Mantel's test was carried out on matrices of Mahalanobis and D-loop K80 molecular pairwise distances. Probabilities were read directly from the distribution of 5,000 randomised matrices computed by permutation.

Table 2 List of the osteological variables and the codes used in the text

	Code	Osteological variable
1	BB1 b1*	Length of basibranchial 1
2	BB2 b2*	Length of basibranchial 2
3	BS bc*	Length of cartilagonous basihyal
4	BS bo*	Length of ossified basihyal
5	BS n*	Width of the area between cartilage and bone of basihyal
6	CB5 a*	Width of ceratobranchials 5
7	CB5 dt**	Total number of teeth of ceratobranchials 5
8	CB5 x*	Tooth antero-posterior diameter of ceratobranchials 5
9	CB5 y*	Tooth medio-lateral diameter of ceratobranchials 5
10	DE dt**	Total number of teeth of dental
11	MX b*	Length of maxillary
2	MX h*	Width of maxillary
13	MX R*	Ratio length/width of maxillary
14	PA br**	Number of branched rays of anal fin
15	PA rt**	Total number of rays of anal fin
16	PA ur**	Number of unbranched rays of anal fin
17	PB2 df**	Number of tooth rows of pharyngobranchials 2
18	PB2 dt**	Total number of teeth of pharyngobranchials 2
19	PB2 bs**	Number of bicuspid teeth of pharyngobranchials 2
20	PB2 ts**	Number of tricuspid teeth of pharyngobranchials 2
21	PB3 dt**	Total number of teeth of pharyngobranchials 3
22	PB4 tp**	Total number of teeth of pharyngobranchials 4 toothplate
23	PB4bs tp**	Number of bicuspid teeth of pharyngobranchials 4 toothplate
24	PB4ts tp**	Number of tricuspid teeth of pharyngobranchials 4 toothplate
25	PD br**	Number of branched rays of dorsal fin
26	PD rt**	Total number of rays of dorsal fin
27	PD ur**	Number of unbranched rays of dorsal fin
28	PMX b1*	Length of premaxillary
29	PMX df**	Number of tooth rows of premaxillary
30	PMX dt**	Total number of teeth of premaxillary
31	PMX h1*	Width of premaxillary
32	PMX R*	Ratio length/width of premaxillary
33	PRS a*	Width of parasphenoid
34	PRS b*	Length of parasphenoid
35	SPC a*	Width of the anterior bony end of supraoccipital
36	SPC b*	Length of the antero-median cartilagineous strip of supraoccipital
37	SPC e*	Width of the lateral processes of supraoccipital
38	SPC v*	Length of the lateral processes of supraoccipital
39	UR b*	Length of uroyal
40	UR c*	Minimum width of urohyal
41	UR h*	Heigth of hook of urohyal
42	VR n**	Total number of vertebrae
43	VR x*	Length of vertebrae
44	VR y*	Width of vertebrae

*Morphometric characters
**Meristic characters

Results

Meristic and morphometric analyses

The Kruskall–Wallis analysis of variance showed significant differences between all the variables taken into consideration. Results of the Mann–Whitney U test, carrying out a pairwise comparison within each of the four groups of populations, indicated that the south-eastern Sicilian populations are well differentiated within the group; on the other hand, the two western Sicilian populations are the least differentiated.

The pairwise comparison between the four groups of the killifish populations analysed in this study, show that the south-eastern Sicilian populations are very much differentiated from all the other groups, while the western Sicilian populations are well differentiated from those of Malta and, to a lesser extent, from those of Tunisia; finally, the Maltese and the Tunisian populations are the least differentiated (Fig. 3).

Multivariate analysis

A PCA on morphometric measurements normally results in a first principal component characterised by consistently positive loadings, where size is the primary source of variance in the data. However, because we analysed standardised measurements to eliminate the size effect, no such effect is expected in PC1. Principal components 1–5 account for 34.7, 9.3, 7.4, 6.7 and 5.0% of the total variance, respectively; Fig. 4 shows PC1 and PC2. PC1

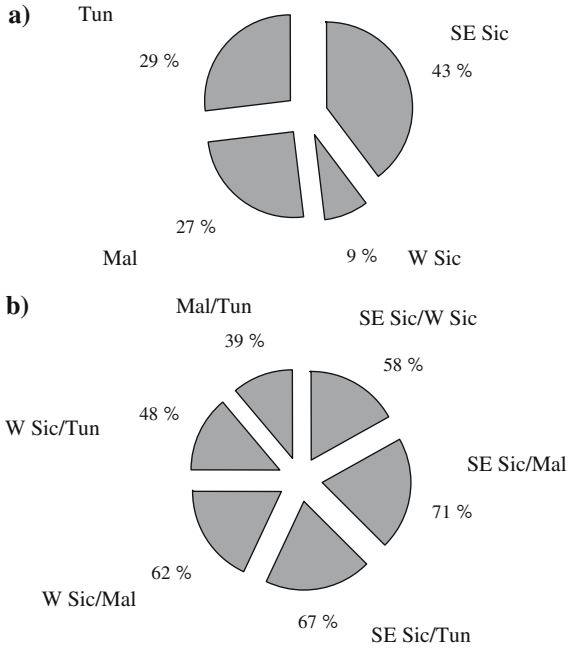

Fig. 3 Diagrammatic representation of the mean percentages of significantly different variables from paired comparisons, by Mann–Whitney U test, in each group of populations (**a**) and between groups of populations (**b**). Malta (*Mal*), South-eastern Sicily (*SE Sic*), Tunisia (*Tun*), Western Sicily (*W Sic*)

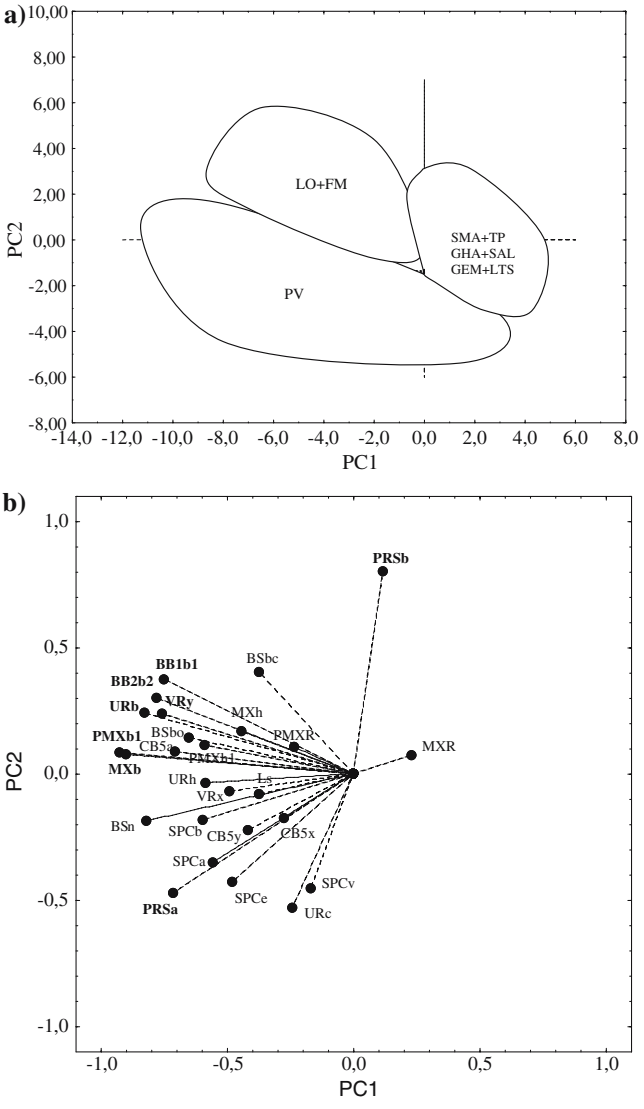

Fig. 4 Results of PCA: **a** PC-scores of the nine populations; **b** correlations of the different morphometric characters with PC1 and PC2; the variables highly correlated with PC1 and PC2 are in bold. South-eastern Sicily: Foce Marcellino (*FM*), Pantano Longarini (*LO*), Pantano Viruca (*PV*); Western Sicily: Salina Chiusa Trapani (*TP*), Salina Curto Marsala (*SMA*); Malta: Salina (*SAL*), Ghadira (*GHA*); Tunisia: Ghar El Milh (*GEM*), Lake Tunisi South (*LTS*). For the code of the variables see Table 2

divides the south-eastern Sicilian populations from the others (Fig. 4a). PC1 scores from the two groups of Sicilian populations (eastern and western) differ significantly from those of Malta and Tunisia. Moreover, PC1 scores for individuals from eastern Sicily differ significantly from those of western Sicily, while PC1 scores for individuals from Malta and Tunisia do not differ significantly (Table 3a). Six measures correlate highly (>0.7) with PC1 (Fig. 4b), of which five are measures of the oral and visceral bony elements: premaxillary and maxillary length, basibranchial 1 and 2 length, and uroyal length; the other measure is the width of the vertebrae.

PC2 distinguishes the bulk of the specimens of the Pantano Viruca population. The parasphenoid length and width are the only measures that are highly correlated with PC2. PRSb is the only measure negatively correlated with PC2 (Table 3b). PC2 scores for individuals from the Pantano Viruca population differ significantly from those of all other populations. Box plots of the variables highly correlated with PC1 and PC2 are shown in Fig. 5.

The first node of the UPGMA cluster analysis, based on the generalised Mahalanobis distance matrix, clearly separates Pantano Viruca from all the other populations, while the second node separates the remaining two south-eastern Sicilian populations (Longarini and Foce Marcellino) from the rest. The western Sicilian group clusters together with the Maltese and the Tunisian groups and shows greatest affinity with the Tunisian populations (Fig. 6).

Molecular analysis

For the molecular study, the sequences of a portion of the D-loop between the tRNA proline and the CSBD zone (length between 376 and 378 nucleotides) were analysed and showed a total of 32 variable sites which defined 24 haplotypes from the 82 specimens examined. The number of haplotypes identified for each population were: two for Pantano Viruca, Salina, Ghar El Milh and Salina Chiusa Trapani; three for Pantano Longarini, Foce Marcellino, and the Ghadira Bird Sanctuary; five for Salina Curto Marsala; and six for Lake Tunis South. The number of segregating sites for different haplotypes

Table 3 Statistical tests on PC scores: (a) test for differences in PC1 scores of individuals from nine populations of *A. fasciatus*; (b) test for differences in PC2 scores (statistical significance Mann–Whitney U test)

	TP	SMA	LO	FM	PV	GHA	SAL	GEM	LTS
(a)									
TP	–								
SMA	n.s.								
LO	***	***	–						
FM	***	***	*						
PV	***	***	n.s.	n.s.	–				
GHA	***	**	***	***	***	–			
SAL	***	***	***	***	**	*	–		
GEM	***	***	***	***	**	n.s.	n.s.	–	
LTS	***	***	***	***	**	*	n.s.	n.s.	–
(b)									
TP	–								
SMA	n.s.	–							
LO	n.s.	n.s.	–						
FM	n.s.	n.s.	n.s.	–					
PV	***	***	***	***	–				
GHA	*	**	**	n.s.	***	–			
SAL	n.s.	*	**	n.s.	***	n.s.	–		
GEM	n.s.	n.s.	n.s.	n.s.	***	*	**	–	
LTS	n.s.	n.s.	*	n.s.	***	*	n.s.	n.s.	–

For the abbreviations of the populations, see Table 1
n.s. not significant
*$P<0.05$
**$P<0.01$
***$P<0.001$

ranges from 3 (Pantano Viruca vs. Pantano Longarini a) to 20 (Salina Curto Marsala vs. Pantano Longarini a) (Table 4). As regards the percentage of divergence, the analysis within the four groups indicates that the south-eastern Sicily populations are the most differentiated (percentage of divergence: minimum 0.5% Pantano Viruca and Pantano Longarini, maximum 5% Foce Marcellino and Pantano Viruca, and Foce Marcellino and Pantano Longarini); within the other three groups, the values of divergence range from 0 to 0.7% among the western Sicilian and the Tunisian populations, and from 0 to 0.5% for Malta. Among the groups, the smallest differentiation was for western Sicily and Malta (percentage of divergence between 0 and 0.5%), while the greatest divergence was for the south-eastern Sicilian group and Tunisia (from 1.8 to 5.3%) (Table 5). The NJ and MP methods gave similar phylogenetic trees (Fig. 7). There is a clear separation of the two Sicilian populations from Pantano Longarini and Pantano Viruca from all the other populations. The second node isolates the third population from south-eastern Sicily, that of Foce Marcellino, which is completely detached from the cluster formed by the western Sicily/Malta/Tunisia populations; within this last group there is a separation of the Tunisian populations, while the populations of western Sicily and Malta are not differentiated.

The Nested Clade Analysis (Templeton et al. 1995; Templeton 1998, 2004) (data not shown) confirms the results of the phylogenetic analysis. In fact, the Pantano Longarini and the Pantano Viruca haplotypes were not inserted in the network due to their high degree of divergence from the other haplotypes. From the nested cladogram constructed using data from the remaining killifish populations, only two clades were significantly separated from the rest (permutational chi-square probability value less than 0.05), revealing continuous range expansion 'for the haplotypes from the Maltese, Tunisian and western Sicilian populations and allopatric fragmentation' of these with respect to the Foce Marcellino population. These findings should be further substantiated by a more detailed study of killifish populations along the whole Sicilian coast, although the high genetic divergence of the Pantano Longarini and Pantano Viruca populations, located geographically close together, is further proof of the effective genetic isolation of the Foce Marcellino population. Mantel's test indicates a significant correlation between the molecular variability and the morphology of the populations examined ($G=2.423$, $P=0.01$).

Discussion

Molecular and osteological studies of populations of *A. fasciatus* from the central Mediterranean have demonstrated a high degree of differentiation in the south-eastern Sicilian populations both within this group of populations (Tigano et al. 2004a), and from three other groups of populations from western Sicily, Tunisia and Malta (this paper). Comparing the dendrograms resulting from the molecular and morphological analyses, it can be observed that both analyses clearly differentiate the south-eastern Sicilian populations from the others, even if the topologies of the two dendrograms are different. This discrepancy, however, does not appear to be significant, and Mantel's test indicates a strong correlation between the two distance matrices based on molecular and osteological characters.

The observed differentiation of the killifish populations studied can be partly explained in terms of the palaeogeographic history of the Mediterranean basin, especially in relation to the evolution of the coastal environments where the species examined here lives. The genus *Aphanius* seems to be of Miocenic origin and studies by Hrbek and Meyer (2003) support a predominantly vicariance-based speciation model for this genus, associated with the closing of the Tethys Sea. During the Messinian *A. crassicaudus* lived in the Mediterranean basin (Gaudant 2002); however, the exact period when *A. fasciatus* first appeared is unknown, nor is it known if an ancestral *A. pliocenii* existed (Gaudant in litteris). Moreover, Hrbek and Meyer (2003) attest that *A. fasciatus* is the only species of the genus that does not fit the hypothesis of Messinian vicariant differentiation. During the early Pleistocene, the Mediterranean did not form a single marine unit but was fragmented into smaller eastern and western basins due to desiccation (Hsu 1972, 1983; Krijgsman et al. 1999); the ongoing westward differentiation of *Aphanius* was most likely driven by this geographic isolation. It is probable that *A. fasciatus*

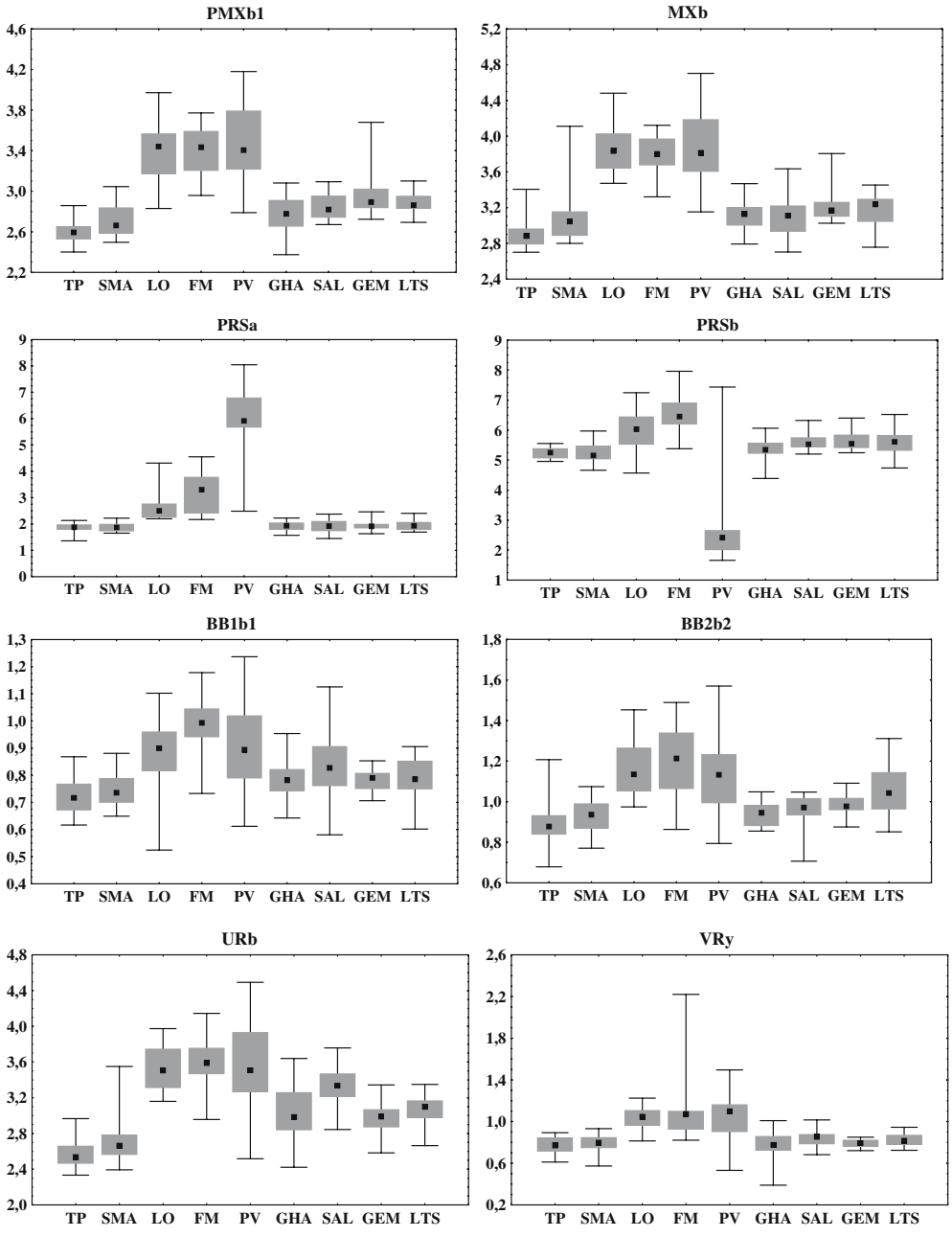

Fig. 5 *Box plots* showing the median (*filled square*), the 25th and 75th percentiles ( ) and the data range ( ) of the variables highly correlated wiith PC1 and PC2. For the abbreviation of the populations see Table 1 and for the code of the variables see Table 2

already became separated from the main ancestral population before the division of the Mediterranean Sea into two basins was complete. During the Pliocene (from 5 to 1.7 million years ago) Sicily consisted of two islands separated from the nearby land masses: one, to the north, that included northern Sicily (equivalent to today's Sicilian provinces of Trapani, Palermo and Messina) and a smaller one that comprised the present Hyblean region (equivalent to today's Sicilian provinces of Ragusa and Syracuse) (Blanc 1942; Pasa 1953; La Greca 1957); the isolation of the Hyblean region was only interrupted for brief periods through a connection with the island of Malta during marine regressions in the Pleistocene, following the last of which, all connections to the land masses around Sicily were definitively interrupted. Therefore, the pronounced differentiation of the three killifish populations from south-eastern Sicily from the other populations studied here, can be explained in terms of the relatively long period of isolation of the Hyblean region. Moreover, the data presented here and that derived from the molecular substitution rate generally accepted for the mitochondrial control region of fish (Stepien et al. 2001) suggest that the Pantano Viruca and Pantano Longarini populations from south-eastern Sicily could be of Pliocenic origin. This marked differentiation of the south-eastern Sicilian group of killifish populations is indicated by the high percentage of sequence divergence (ranging from 1.5 to 5.3%) with respect to the other three groups of killifish populations (Table 5). The range of molecular clock rates generally

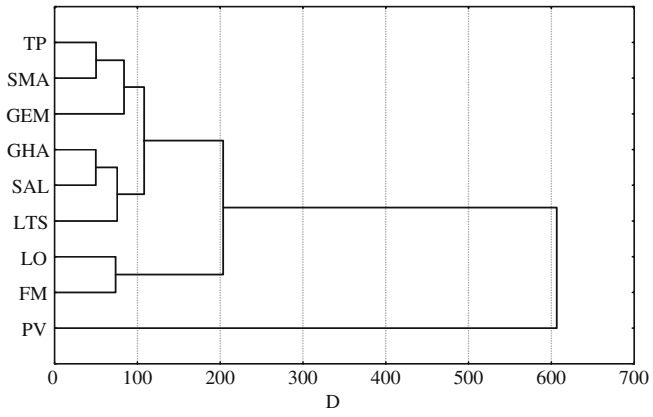

Fig. 6 UPGMA dendogram of the generalised distance (*X* axis). For the abbreviation of the populations, see Table 1

accepted for the control region of fish (from 2 to 10% sequence divergence per million years; Stepien et al. 2001) suggests that the period of isolation of the Hyblean populations took place between 150,000 (fast clock calibration) and 2,650,000 years (slow clock calibration) ago. The Pantano Viruca and Pantano Longarini populations have the highest sequence divergence (between 3.4 and 5.3%) while the third south-eastern Sicilian population (Foce Marcellino) has a sequence divergence ranging between 1.5 and 3.1% with respect to all the other populations examined; furthermore, within the south-eastern Sicilian group, a high divergence between the Pantano Viruca and Pantano Longarini populations and that from Foce Marcellino (5% sequence divergence with a population separation time between 500,000 and 2,500,000 years) is observed; this can be interpreted in the light of palaeogeographic events that took place in the Hyblean region in the Lower Pleistocene (about 1.7 million years ago) (Tigano et al. 2004a). Analogously, the only differentiated population of *A. fasciatus* included in the phylogenetic study by Hrbek and Meyer (2003) was that from Lake Bafa in Turkey, with an average 6.86% sequence divergence from other *A. fasciatus* populations, corresponding to a 3.99 million year separation.

The use of haplotype networks allows a fine discrimination of biological patterns as this technique examines spatial/temporal patterns of genetic variation (Templeton 1998). The values of permutational chi-square probability obtained in our study allow us to reject the null hypothesis of no association between haplotype variation and geography for the two clades that show significant geographical structure caused by "Contiguous Range Expansion" for the haplotypes belonging to the populations from Malta, Tunisia and western Sicily and by "Allopatric Fragmentation" with respect to the south-eastern Sicilian population from Foce Marcellino (the Nested Clade Analysis did not allow us to include the haplotypes from Pantano Viruca and Pantano Longarini within the network because of their high divergence). The bulk of the specimens from Malta and Western Sicily exhbit the h1 and h2 haplotypes that would be considered ancestral for their internal position in the nested cladogram and several Tunisian specimens exhibit the k1 and k2 haplotype (tip position) that seem to be derived from the former. This haplotype pattern could be related to past Pleistocenic climatic events that caused notable changes in coastlines as a consequence of marine regression and transgression. One interesting hypothesis put forward by some authors (e.g. Aprile 1998) is the possible existence during the last Ice Age of an archipelago between Sicily and the North African coasts which could have promoted the range expansion of an ancestral population of *A. fasciatus*. Bathymetric data indicate a continuous submarine platform up to 200 m deep running from northern Tunisia to western Sicily across the Adventure Bank, parallel to the southern Sicilian coast, which borders the westernmost margin of the Hyblean–Maltese platform and which extends south to the Medina Bank; this whole area experienced periodic, alternate emersion–immersion events during the latest Quaternary tectonic movements (Finetti and Morelli 1973; Ogniben et al. 1975; Ben-Avraham and Grasso 1990). The low sequence divergence values (0–0.7%) between the populations of western Sicily and Malta are also reported by Hrbek and Meyer (2003), who employed other mitochondrial genes.

With regards to morphological differences in the killifish populations studied, it is known that environmental factors may play a decisive role in intraspecific differentiation. Studies by Murta (2000) showed that morphometric characters are more susceptible to environmental variations than meristic characters. Intraspecific variation of morphometric and meristic characters was assessed in relation to the salinity of the environment in *A. anatoliae* (Leidenfrost) by Grimm (1979) and in *A. fasciatus* by Boumaiza et al. (1981); in addition, analysis of morphometric data discriminated between the genetically divergent Atlantic and Mediterranean populations of *A. iberus* (Valenciennes) now generally regarded as two separate species: *A. iberus* in the Mediterranean and *A. baeticus* in the Atlantic (Doadrio et al. 2002). Moreover, intraspecific morphological variation of several species of teleosts was found to be related to the exploitation of food resources and habitat, and to predation pressure (Huysseune et al. 1994; Milano et al. 2002; Maltagliati et al. 2003). Although ecological studies of the different habitats sampled in this study were not made, it is evident that at least two environmental factors—salinity (hyperhaline waters, marine water with various contributions of freshwater) and the size of the water body (small coastal ponds, saltsworks and a large coastal lake)—differed greatly between all the sites sampled.

The observed osteological differences in the populations of *A. fasciatus* studied here may be explained in terms of the geographic isolation of the various populations, although the influence of environmental factors, which differ greatly between the various sites, cannot be ruled out; therefore, further studies are necessary to test how much of the observed osteological differentiation could be due to phenotypic plasticity. The slight discrep-

Table 4 Sequences relative to the regions of mitochondrial control of the individuals of the populations examined

Population	Haplotype (Number of individuals)	Nucleotide position in the mitochondrial control region[a]																																
		12	13	17	29	30	96	107	109	113	127	137	138	142	143	166	177	184	188	190	191	218	223	224	227	255	256	257	259	260	286	342	369
LO	a (5)	T	T	T	A	A	G	C	A	G	T	A	T	A	T	C	G	A	T	A	T	C	A	G	T	A	T	T	T	C	A	G	T
	b (4)														C		A	G	C	C													
	b1 (1)					–									C		A	G	C	C													
PV	c (9)														C			G		C													
	d (1)																	G		C													
FM	e (7)	A	C	C			A	T			C	C	C			A	A			T	A		G	C							A	C
	f (1)	A	C	C			A	T		A	C	C	C			A	A			T	A		G	C							A	C
	g (1)	A	C	C				T			C	C	C			A	A			T	A		G	C							A	C
SAL	h1 (6)	A			–	–	A	T				C	C			A	A			T	A		G	A			C		C		A	
	i (2)	A			–	–	A	T				C	C			A	A			T	A		G	A	C		C		C		A	
GHA	h1 (3)	A			–	–	A	T				C	C			A	A			T	A		G	A			C		C		A	
	h2 (2)	A					A	T				C	C			A	A			T	A		G	A			C		C		A	
	j (2)	A					A	T				C	C	G		A	A			T	A		G	A			C		C		A	
LTS	k1 (4)	A					A	T	T			C	C			A	A			T	A		G	A			C		C		A	
	k2 (2)	A				–	A	T	T			C	C			A	A			T	A		G	A			C		C		A	
	l (1)	A					A	T	T			C	C			A	A			T	A		G	A		C	C	C	C	T		A	
	m (1)	A					A	T	T			C	C			G	A			T	A		G	A			C		C			A	
	n (1)	A					A	T	T			C	C			A	A			T	A		G	A		C		–	C			A	
	o (1)	A					A	T	T			C	C			A	A			T	A		G	A					C			A	
GEM	k1 (7)	A					A	T	T			C	C			A	A			T	A		G	A			C		C			A	
	p (2)	A					A	T	T			C	C			A	A			T	A		G	A		C	C	C	C			A	
TP	h2 (7)	A					A	T				C	C			A	A			T	A		G	A			C		C			A	
	q1 (3)	A					A	T				C	C	T		A	A			T	A		G	A			C		C			A	
SMA	h2 (5)	A					A	T				C	C			A	A			T	A		G	A			C		C			A	
	r (1)	A					A	T				C	C			A	A			T	A	T	G	A			C		C			A	
	s (1)	A					A	T				C	C			A	A			T	A		G				C		C		C	A	
	t (1)	A					A	T				C	C			A	A			T	A		G	A			C					A	
	q2 (1)	A				–	A	T				C	C	T		A	A			T	A		G	A			C	C				A	

For the abbreviations of the populations, see Table 1

[a] The position refers to the sequence of the haplotype a

Table 5 Riassuntive table of matrices of the pairwise distances for the mitochondrial control regions (the values of difference are in percentages)

		LO	PV	FM	TP	SMA	LTS	GEM	GHA	SAL
Sicily south-east	LO									
	PV	0.5–0.7								
	FM	3.9–5.0	4.2–5.0							
Sicily west	TP	3.7–4.5	3.9–4.4	1.8–2.3						
	SMA	3.4–4.5	3.7–4.5	1.5–2.3	0.0–0.7					
Tunisia	LTS	3.7–5.3	3.9–5.0	1.8–3.1	0.2–1.3	0.2–1.5				
	GEM	3.9–5.0	4.2–5.0	2.1–2.9	0.2–1.0	0.2–1.3	0.0–0.7			
Malta	GHA	3.7–4.5	3.9–4.4	1.8–2.3	0.0–0.2	0.0–0.5	0.2–1.3	0.2–1.0		
	SAL	3.7–4.5	3.9–4.5	1.8–2.3	0.0–0.5	0.0–0.5	0.2–1.3	0.2–1.0	0.0–0.5	

The maximum and minimum values are shown. For the abbreviations of the populations see Table 1

ancy between the two dendrograms resulting from the molecular and osteological analyses may be attributed to the fact that morphological variation is occasionally a local response to different environments, reflecting only in part genetic differences (Jerry and Cairns 1998).

Finally, the results relative to the Pantano Viruca population from south-eastern Sicily are very interesting in that both morphological and molecular data are in concordance that this population is unique; this population is differentiated by the shape of the parasphenoid as well as being characterised by a haplotype present in nine out of ten specimens examined, which could be the consequence of the founder effect.

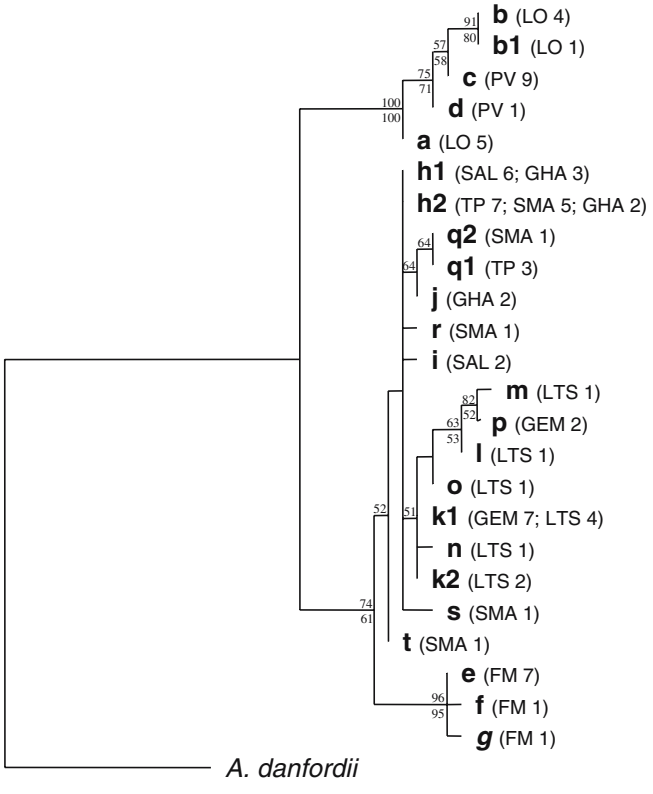

Fig. 7 Tree obtained both with the neighbour-joining and maximum parsimony methods. Values above branches represent NJ bootstrap estimates, those below branches represent MP estimates, based on 1,000 replications in both cases (> 50%). For the abbreviation of the populations, see Table 1

In conclusion, the wide range of the sequence divergence (ranging between 0 and 5.3%), indicates that, in some cases, there is a high degree of differentiation between the killifish populations of south-eastern Sicily and the other populations studied; moreover, differentiation is also evident on a more local scale (e.g. between the populations from Pantano Longarini and Pantano Viruca and that from Foce Marcellino). In other cases (Malta and western Sicily), the molecular analysis indicates an absence of differentiation. The allozyme data reported by Maltagliati (1998a, 1999, 2002), Maltagliati et al. (2003), and Cimmaruta et al. (2003) show a high degree of genetic divergence between thirty Italian populations of *A. fasciatus*, while, on a more local scale, the authors demonstrate the existence of a genetic structure of these killifish populations resulting from occasional gene flow, and from evolutionary forces that are stochastic (drift) or deterministic (natural selection), which act together to shape the gene pool of the species. Analogously, allozymic and morphological studies by Ferrito et al. (2003) indicate well-supported differentiation between two Adriatic and two Sicilian populations of *A. fasciatus*. On the other hand, the molecular data of Hrbek and Meyer (2003) revealed only a limited genetic differentiation in seven populations of *A. fasciatus* and, consequently, a limited genetic structuring of this species. Therefore, the genetic structuring of *A. fasciatus* still appears to be controversial. Data from the present study and from the analysis of the mitochondrial DNA by Costagliola et al. (2003) and by Tigano et al. (2004b) indicate that at least 41% of the studied populations are well differentiated; taking into consideration the ample distribution of this species, it is probable that this percentage could be higher. Therefore, the results reported here, while interesting, indicate the need for more extensive sampling and comparison of populations. Nevertheless, these results, together with those already reported in the literature, provide a valid knowledge base for the understanding of the micro-evolutionary processes acting in *A. fasciatus* populations and for the implementation of management plans aimed at conserving the adaptive

variability in this species, some populations of which are in danger of extinction.

Acknowledgements We wish to thank Prof. G. Lunetta and Dr. A. Mazza from the Dipartimento di Economia e Territorio, University of Catania, Italy, for their valuable suggestions about statistical analysis. Prof. M. Grasso from the Dipartimento di Geologia, University of Catania, for his valuable suggestions about the interpretation of the palaeogeographic events in the Central Mediterranean, and Prof. S. Nouira and Dr. A. Santulli for their help in the collection of samples in Tunis and in Trapani, respectively. We thank the Environment Protection Directorate of the Malta Environment and Planning Authority for permission to work on this protected species in Malta and for access to the Ghadira Bird Sanctuary; we are grateful to Mr Charles Gauci, the warden in charge of the sanctuary, for facilitating our work.

References

Aprile LAA (1998) La diffusione del genere *Homo* in Eurasia e il più antico popolamento extra africano: vie di accesso al continente Europeo, con particolare riferimento a un ipotetico "arcipelago" tunisino-siculo. Degree thesis, University of Catania (unpublished)

Avise JC (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge, MA

Ben-Avraham Z, Grasso M (1990) Collisional zone segmentation in Sicily and surrounding areas in the Central Mediterranean. Ann Tectonicae 4(2):131–139

Bernardi G (2000) Barriers to gene flow in *Embiotoca jacksoni*, a marine fish lacking a pelagic larval stage. Evolution 54:226–237

Blanc AC (1942) Variazioni climatiche ed oscillazioni della linea di riva nel Mediterraneo centrale durante l'Era Glaciale. Geol Der Mere Binnengewasset 5:137–219

Boumaiza M, Ktari MH, Quignard JP (1981) Etude de la variabilité du nombre de vertébres et d'écailles sur la ligne latérale chez *Aphanius fasciatus* Nardo, 1827 (Poisson Cyprinodontidae). Rapp Comm Int Explor Sc Mer Médit 27(5):115–117

Cimmaruta R, Scialanca F, Luccioli F, Nascetti G (2003) Genetic diversity and environmental stress in Italian populations of the Cyprinodont fish *Aphanius fasciatus*. Oceanol Acta 26:101–110

Clement MD, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9:1656–1660

Comparini A, Scattolin N, Rodinò E (1984) Genetic differentiation among some populations of the Cyprinodont *Aphanius fasciatus* Nardo. Nova Thalassia 6:261–268

Costagliola D, Ferrito V, Rocco L, Marsilio A, Pappalardo AM, Tigano C (2003) Analisi dell'mtDNA e RAPD in popolazioni italiane di *Lebias fasciata* (Teleostei, Cyprinodontiformes). Atti del 64° Congresso U.Z.I., Varese 21–25 Settembre 2003, p 105

Dingerkus G, Uhler LD (1977) Enzyme clearing of alcian blue stained whole small vertebrates for demonstration of cartilage. Stain Technol 52:229–232

Doadrio I, Carmona JA, Fernandez-Delgado C (2002) Morphometric study of the Iberian *Aphanius* (Actinopterygii, Cyprinodontiformes), with description of a new species. Folia Zool 51:67–79

Duhan JB, Minckley WL (1998) Allozymic variation in desert pupfish from natural and artificial habitats: genetic conservation in fluctuating populations. Biol Conserv 84:7–15

Duvernell DD, Turner BJ (1999) Variation and divergence of Death Valley pupfish populations at retrotransposon-defined loci. Mol Biol Evol 16:363–371

Echelle AA, Echelle AF, Edds DR (1987) Population structure of four pupfish species (Cyprinodontidae: *Cyprinodon*) from the Chihuahuan Desert region of New Mexico and Texas: allozymic variation. Copeia 3:668–681

Echelle AA, Van Den Bussche RA, Malloy TP Jr, Haynie ML, Minckley CO (2000) Mitochondrial DNA variation in pupfishes assigned to the species *Cyprinodon macularius* (Atherinomorpha: Cyprinodontidae): taxonomic implications and conservation genetics. Copeia 2:353–364

Elliott NG, Haskard K, Koslow JA (1995) Morphometric analysis of orange roughy (*Hoplostethus atlanticus*) off the continental slope of southern Australia. J Fish Biol 46:202–220

Fernandez-Pedrosa V, Gonzalez A, Planelles M, Moya A, Latorre A (1995) Mitochondrial DNA variability in three Mediterranean populations of *Aphanius iberus*. Biol Conserv 72:251–256

Ferrito V, La Paglia L, Sgarlata MT, Mauceri A, Tigano C (2000) Preliminary remarks on NOR location in a Sicilian population of *Lebias fasciata* (Teleostei, Cyprinodontidae). Ital J Zool 67:269–272

Ferrito V, Maltagliati F, Mauceri A, Adorno A, Tigano C (2003) Morphological and genetic variation in four Italian populations of *Lebias fasciata* (Teleostei, Cyprinodontidae). Ital J Zool 70:115–121

Finetti I, Morelli C (1973) Geophisical exploration of the Mediterranean Sea. Boll Geofis Teor Appl 15:263–341

Garciamarin JL, Vila A, Pla C (1990) Genetic-variation in the Iberian toothcarp, *Aphanius iberus* (Cuvier and Valenciennes). J Fish Biol 37:233–234

Gaudant J (2002) La crise messinienne et ses effets sur l'ichthyofaune néogène de la Meéditerranée: le témoignage des squelettes en connexion de poissons téléostéens. Geodiversitas 24:691–710

Grimm VH (1979) Veränderungen in der variabilität von populationen des zahnkarpfens *Aphanius anatoliae* (Leidenfrost, 1912) während 30 jahren: 1943–1974. Sonderdruck aus Z.f.zool. Systematik u. Evolutionsforschung 17(4):272–280

Hsu KJ (1972) When the Mediterranean dried up. Sci Am 227(6):27–36

Hsu KJ (1983) The Mediterranean was a desert. Princeton University Press, Princeton, NJ

Hrbek T, Meyer A (2003) Closing of the Tethys sea and the phylogeny of Eurasian killifishes (Cyprinodontiformes: Cyprinodontidae). J Evol Biol 16:17–36

Huysseune A, Sire JY, Meunier FJ (1994) Comparative study of lower pharyngeal jaw structure in two phenotypes of *Astatoreochromis alluaudi* (Teleostei: Cichlidae). J Morph 221:25–43

Jeffreys AJ, Flavell RA (1977) A physical map of the DNA flanking the rabbit ß-globin gene. Cell 12:429–439

Jerry DR, Cairns SC (1998) Morphological variation in the catadromous Australian bass, from seven geographically distinct riverine drainages. J Fish Biol 52:829–843

Kimura M (1980) A simple method for estimating rate of base substitution through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kimura M, Weiss GH (1964) The stepping stone model of population structure and the decrease of genetic correlation with distance. Genetics 49:561–576

Krijgsman W, Hilgen FJ, Raffi I, Sierro FJ, Wilson DS (1999) Chronology, causes and progression of the Messinian salinity crisis. Nature 400:652–655

La Greca M (1957) Considerazioni sull'origine della fauna siciliana. Boll Zool 24:593–631

Lussen A, Falk TM, Villwock W (2003) Phylogenetic patterns in populations of Chilean species of the genus *Orestias* (Teleostei: Cyprinodontidae): results of mitochondrial DNA analysis. Mol Phylogenet Evol 29:151–160

Maltagliati F (1998a) A preliminary investigation of allozyme genetic variation and population geographical structure in *Aphanius fasciatus* from Italian brackish-water habitats. J Fish Biol 52:1130–1140

Maltagliati F (1998b) Does the Mediterranean killifish *Aphanius fasciatus* (Teleostei, Cyprinodontidae) fit the one-dimensional stepping stone model of gene flow? Environ Biol Fishes 53:385–392

Maltagliati F (1999) Genetic divergence in natural populations of the Mediterranean brackish-water killifish *Aphanius fasciatus*. Mar Ecol Prog Ser 179:155–162

Maltagliati F (2002) Genetic monitoring of brackish-water populations. The mediterranean toothcarp *Aphanius fasciatus* (Cyprinodontidae) as a model. Mar Ecol Prog Ser 235:257–262

Maltagliati F, Domenici P, Fosch CF, Cossu P, Casu M, Castelli A (2003) Small-scale morphological and genetic differentiation in the Mediterranean killifish *Aphanius fasciatus* (Cyprinodontidae) from a coastal brackish-water pond and an adjacent pool in northern Sardinia. Oceanol Acta 26:111–119

Milano D, Cussac VE, Macchi PJ, Ruzzante DE, Alonso MF, Vigliano PH, Denegri MA (2002). Predator associated morphology in *Galaxias platei* in Pantagonian lakes. J Fish Biol 61:138–156

Meyer A, Kocher TD, Basasibwaki P, Wilson AC (1990) Monophyletic origin of Lake Victoria fishes suggested by mitochondrial DNA sequences. Nature 347:550–553

Murta AG (2000) Morphological variation of horse mackerel (*Trachurus trachurus*) in the Iberian and North African Atlantic: implications for stock identification. ICES J Mar Sci 57:1240–1248

Ogniben L, Parotto M, Praturlon A (eds) (1975) Structural model of Italy. Quaderni La Ricerca Scientifica 90:502

Parenti LR, Tigano C (1993) Polymorphic skeletal characters in *Aphanius fasciatus* (Teleostei, Cyprinodontiformes). Copeia 4:1132–1137

Pasa A (1953) Appunti geologici per la paleogeografia delle Puglie. Mem Biogeogr Adr 2:175–286

Perdices A, Carmona JA, Fernandez-Delgado C, Doadrio I (2001) Nuclear and mitochondrial data reveal high genetic divergence among Atlantic and Mediterranean populations of the Iberian killifish *Aphanius iberus* (Teleostei: Cyprinodontidae). Heredity 87:314–324

Posada D, Crandall KA, Templeton AR (2000) GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. Mol Ecol 9:487–488

Reichenbacher B, Sienknecht U (2002) Allopatric divergence and genetic diversity of recent *Aphanius iberus* and fossil *Prolebias meyeri* (Teleostei, Cyprinodontidae) from southwest and western Europe, as indicated by otoliths. Geobios 34:69–83

Slatkin M (1994) Gene flow and population structure. In: Real LA (ed) Ecological Genetics. Princeton University Press, Princeton, NJ, pp 3–17

Stepien CA, Rosenblatt RH, Bargmeyer BA (2001) Phylogeography of the spotted sand bass, *Paralabrax maculatofasciatus*: divergence of gulf of California and Pacific coasts populations. Evolution 55:1852–1862

Strecker U, Meyer CG, Sturmabauer C, Wilkens H (1996) Genetic divergence and speciation in an extremely young species flock in Mexico formed by the genus *Cyprinodon* (Cyprinodontidae, Teleostei). Mol Phylogenet Evol 6:143–149

Swofford DL (1998) PAUP*. Phylogenetic Analysis Using Parsimony (* and other methods.) version 4. Sinauer Associates, Sunderland, MA

Templeton AR (1998) Nested clade analyses of phylogeographic data: Testing hypotheses about gene flow and population history. Mol Ecol 7:381–397

Templeton AR (2004) Using haplotype trees for phylogeographic and species inference in fish populations. Envir Biol Fishes 69:7–20

Templeton AR, Sing CF (1993) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. Genetics 134:659–669

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III cladogram estimation. Genetics 132:619–633

Templeton AR, Routman EJ, Phillips CA (1995) Separating population structure from population history: A cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. Genetics 140:767–782

Thompson JD, Higgins DG, Gibson TG (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Thorpe RS (1976) Biometric analysis of geographic variation and racial affinities. Biol Rev 51:407–452

Tigano C (1991) Il polimorfismo della mascella superiore in *Aphanius fasciatus* Nardo (Pisces Cyprinodontidae). Animalia 18:61–70

Tigano C, Ferrito V (1985) Studio osteologico comparato del cranio di popolazioni di *Aphanius fasciatus* (Nardo 1827) (Pisces: Cyprinodontidae) dell'Adriatico e di fiumi di Sicilia. Animalia 12:13–57

Tigano C, Parenti LR (1988) Homology of the median ethmoid ossifications of *Aphanius fasciatus* and other Atherinomorph fishes. Copeia 4:866–870

Tigano C, Ferrito V, Nicosia R (1999) Morphological analysis of the pharyngeal jaws in two populations of *Lebias fasciata* Valenciennes, 1821 (Teleostei, Cyprinodontidae). J Morphol 241:107–114

Tigano C, Ferrito V, Adorno A, Mannino MC, Mauceri A (2001) Pharyngeal and oral jaw differentiation in five populations of *Lebias fasciata* (Teleostei: Cyprinodontidae). Ital J Zool 68:201–206

Tigano C, Rocco L, Ferrito V, Costagliola D, Pappalardo AM, Stingo V (2003) Chromosomal mapping and nucleotide sequences of ribosomal genes in *Lebias fasciata* (Teleostei, Cyprinodontidae). Genetica 121:95–100

Tigano C, Canapa A, Ferrito V, Barucca M, Arcidiacono I, Olmo E (2004a) Morphological and molecular analysis of three Sicilian populations of *Lebias fasciata* (Teleostei, Cyprinodontidae). Ital J Zool 71:1125–1133

Tigano C, Pappalardo AM, Ferrito V, Messina A, De Pinto V (2004b) Intraspecific variation in killifish *Aphanius fasciatus* Nardo (Teleostei, Cyprinodontidae): evidence from molecules and morphology. In: Saat T (ed) Abstract Volume XI European Congress of Ichthyology, Tallin, Estonia. Estonia Marine Institute Report series 12:128

Torralva M, Oliva Paterna FJ, Andreu A, Garcia Mellado A, Minano PA, Cardozo V, Garcia Alonso J, Fernandez Delgado C (2001) Distribution and conservation status of *Aphanius iberus* (Valenciennes, 1846) in Murcia region (S.E. of Iberian Peninsula. An Biol 23:63–83

Villwock W (1976) A contribution to the understanding of the evolution of the meristic characters, with special reference to Old World Cyprinodontids. Abh NatwissVer Hambg 18/19:11–27

Vitturi R, Catalano E, Colomba MS, Montanino L, Pellerito L (1995) Karyotype analysis of *Aphanius fasciatus* (Pisces, Cyprinodontiformes): Ag-NORs and C-band polymorphism in four populations from Siciliy. Biol Zentralbl 114:329–402

Wildekamp RH (1993) The genus *Aphanius* Nardo. In: Watters BR (ed) A World of killies, atlas of the oviparous cyprinodontiform fishes of the world, vol I. American Killifish Association, Hishawaka Indiana, pp 19–67

Wilde GR, Echelle AA (1997) Morphological variation in intergrade pupfish populations from the Pecos River, Texas, USA. J Fish Biol 50:523–539