

**Preliminary study of the genetic differentiation among natural populations of Mediterranean killifish *Aphanius fasciatus* (Teleostei, Cyprinodontidae)**

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**ABSTRACT**

This study aimed to compare the genetic patterns of three Tunisian populations of *Aphanius fasciatus* by examining for the first time the mitochondrial control region (D-loop) with the available data originating from the Mediterranean Sea. For this purpose, three populations from eastern coastal of Tunisia (L and S coastal site while H represent a coastal site) were collected. Despite, the small size of our samples, the Tunisian populations was differentiated by higher mutational steps compared to those originating from the Mediterranean Sea. These preliminary results support an extensive genetic structure of *Aphanius fasciatus* studied populations and could be explained by the wide environmental variations which may cause strong selective pressures on organisms.

**Key words:** *Aphanius fasciatus*, genetic variation, mtD-loop, 16S ribosomal RNA, Tunisia.

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## 1. Introduction

The analysis of genetic patterns of populations represents a valuable tool to investigate basic questions in evolutionary ecology and to provide reliable models on ecological processes affecting the distribution and dynamics of species (Avice 2000). Studies analyzing the genetic patterns of populations commonly rely on point samples and implicitly assume a relative temporal stability in their genetic structuring (Heath et al. 2002). A growing body of empirical evidence, however, indicates that genetic patterns in vertebrates may sensibly vary over a range of temporal scales (Maltagliati & Camilli 2000). The description of temporal genetic structuring of populations is thus a focal point to verify hypothesis originating from analyses of spatial genetic patterns and to investigate microevolutionary processes affecting the genetic structure of species (Papetti et al. 2005).

*Aphanius fasciatus* (Valenciennes 1821), is distributed in the coastal zone of the central and eastern Mediterranean Sea (Whitehead et al. 1986). In the Mediterranean basin, study based on allozymic (Comparini et al. 1984, Maltagliati 1998a,b, Maltagliati 1999, Maltagliati 2002, Maltagliati et al. 2003, Cimmaruta et al. 2003), morphological (Tigano & Ferrito 1985, Tigano & Parenti 1988, Tigano 1991, Parenti & Tigano 1993, Tigano et al. 1999, Tigano et al. 2001), and cytogenetic data (Vitturi et al. 1995, Ferrito et al. 2000, Tigano et al. 2003) have all demonstrated a notable differentiation between *A. fasciatus* populations. While 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 based both on allozymic (Cimmaruta et al. 2003) and morphological analysis (Tigano et al. 2001; Ferrito et al. 2003), suggest that this differentiation does not relate to the geographic distance between the different populations.

In the same way, the molecular analysis carried out by Hrbek & 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 mitochondrial control region (D-loop) represents useful population markers to estimate the genetic divergence among *A. fasciatus* populations (Tigano et al. 2004, Tigano et al. 2006, Ferrito et al. 2007). In addition, *A.*

*fasciatus* is used as a model because tooth-carps and killifishes are particularly suitable for the study of micro-evolutionary processes (Fuller 2008, Martin and Wainwright 2011). Considering the missing knowledge on the Tunisian populations, we purpose in the present study to analyze the control region of mitochondrial DNA (D-loop) of *A. fasciatus* in order to compare the genetic patterns of Tunisian populations with the previously published sequences in the Mediterranean killifish populations.

## 2. Materials and methods

### 2.1 Sampling Sites

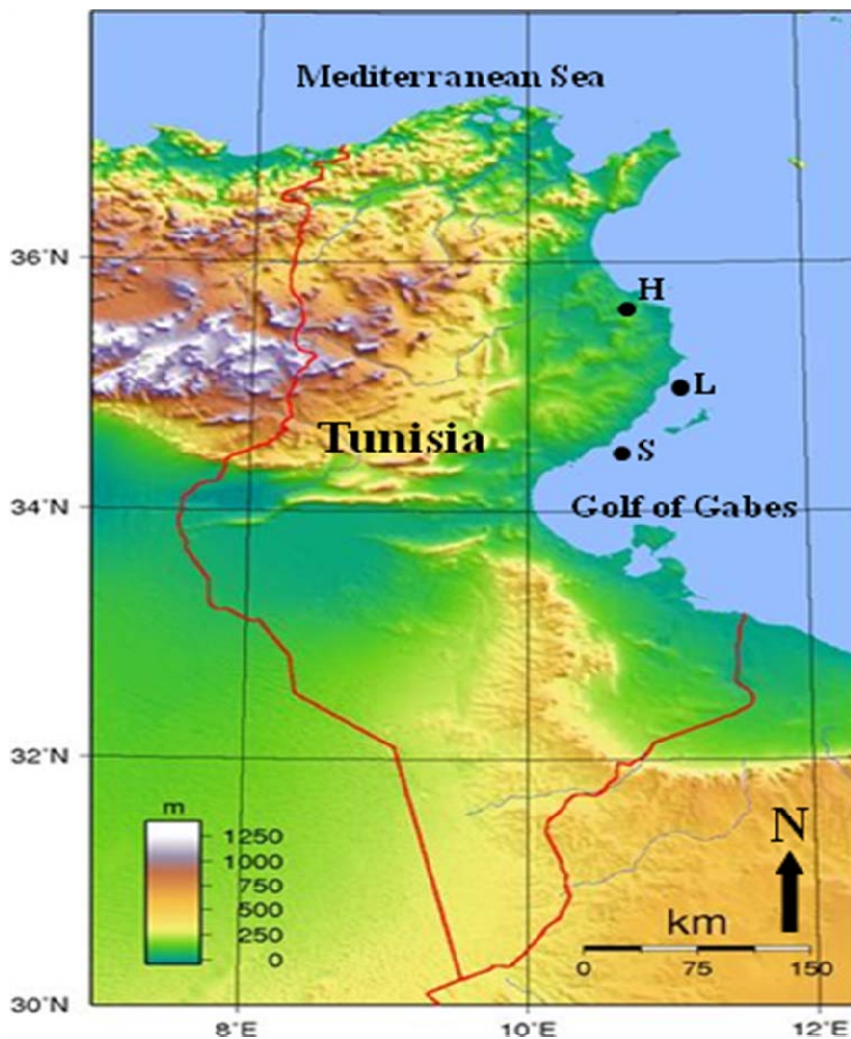
A total of 16 specimens of *A. fasciatus* were collected from three localities in Tunisian coast (5 individu from Luza, 5 from Sfax and 6 from Oued Hamdoun). All specimens were captured in coastal waters (0.5 - 1 m depth) by hand nets during June 2010 along the South Eastern coast of Tunisia (Fig. 1, Table 1). The coastal sites Luza (L) and Sfax (S) were selected based on preliminary studies that showed the presence of large population of *A. fasciatus* (Annabi et al. 2012). The third site Oued Hamdoun (H) and called locally Dkhila coast). The coastal zone of Dkhila is completely open to the sea. The depth of the sampled area does not exceed 10 m. The only estuary feeding the coast of Dkhila with freshwater is the Hamdoun oued (Afli & Ben Mustapha, 2004). Oued Hamdoun is located approximately more than 150 km north L and S sites while L site is located nearly 50 km north of Sfax.

### 2.2 Mitochondrial DNA sequencing and phylogenetic analysis

Total DNA was extracted from muscle tissue preserved in ethanol, using a Wizard Genomic DNA extraction kit (Promega). The D-loop primers were designed according to the mitochondrial partial D-loop of *Aphanius fasciatus* (GenBank Accession no. AM884570). Sequences accession numbers were reported in the results section.

The genetic variation among groups, among populations within groups, and within populations we quantified with an Analysis of Molecular Variance (AMOVA) using ARLEQUIN v.3 (Excoffier et al. 2006). The variance components of the different hierarchical levels were tested statistically by nonparametric randomization tests using 10.000 permutations. Sequence alignments were inspected using the BioEdit Sequence

Alignment Editor (v. 7.0.5.2, Hall 1999). Neither insertion nor deletion was observed in the dataset. The genetic variation was estimated using DNASP (v4.10.9, Rozas et al. 2003). For a genetic analysis among Tunisian and Mediterranean populations, our sequences encoding D-loop region were aligned with the already available sequences in GenBank database (74 sequences respectively for D-loop; Tigano et al. 2006) and a median-joining (MJ) network of-sequences was performed with the software NETWORK 4.5.1.0 (Bandelt et al. 1999).



**Fig. 1. Geographic overview of the geographic studied sites of *A.fasciatus* populations. Luza and Sfax represent coastal sites while Oued Hamdoun is an estuary.**

### 3. Results

A total of 16 sequences of 378 bp were obtained for the D-loop of *A. fasciatus* (Genbank accession numbers: JX406312 to JX406327). Among them, 8 different haplotypes were identified (H1 to H8; Table 1). Fourteen sites were variable and 11 were parsimony informative. The nucleotides frequencies were 37.01, 17.38, 15.79 and 29.82 % for A, C, G and T, respectively. AMOVA results showed that genetic structuring of Tunisian populations was mainly explained by the presence of two main groups. In fact, coastal populations (L and S) represented a separate group I while group II included the population of estuary site (H) with a very little overlapping (Table 1). In addition, most of the variation was explained among the two obtained groups and average 89.7% whereas variation within populations was less important (11.75%) (Table 2).

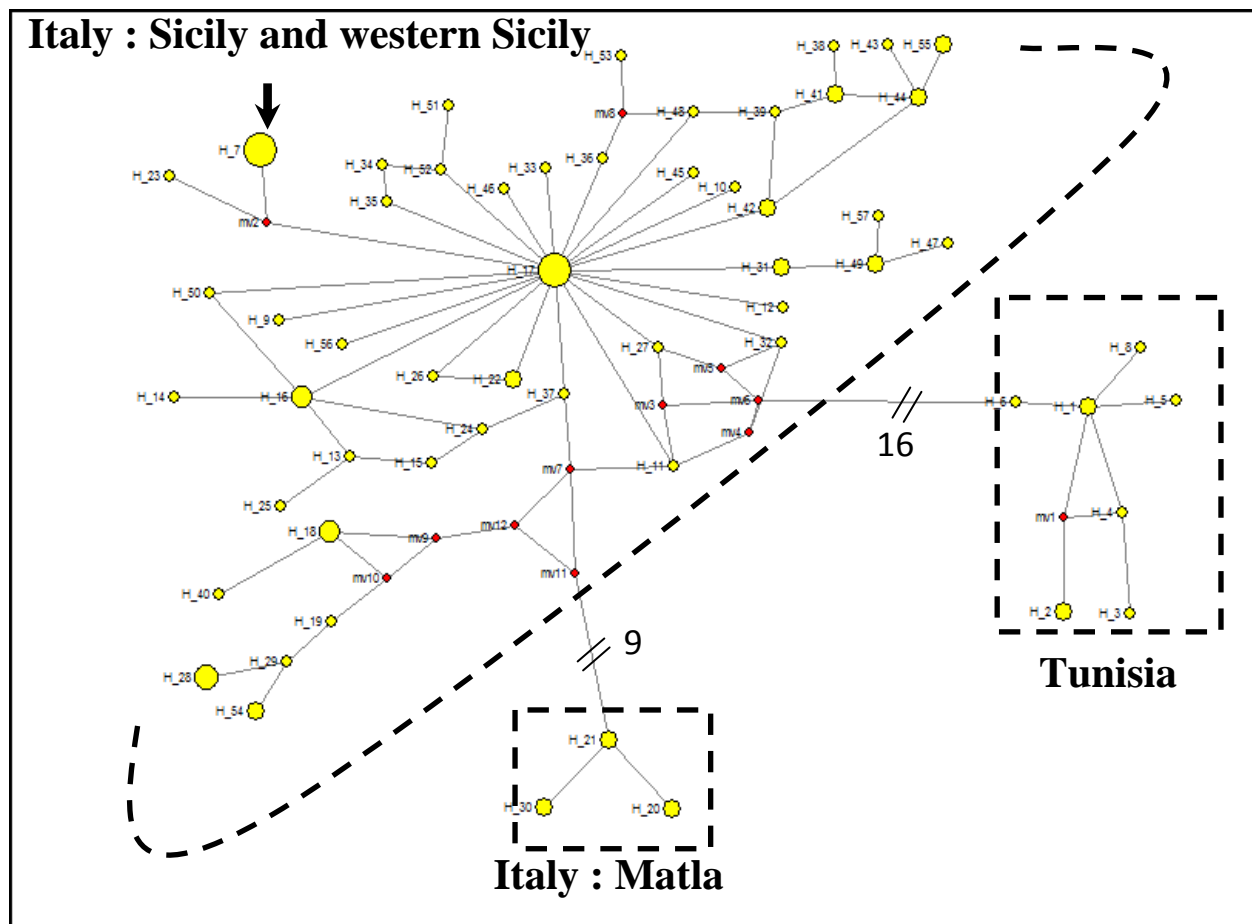
Based on the 74 sequences of mtDNA D-loop of *A. fasciatus* available in GenBank (Tigano et al. 2006), a median joining network was drawn. In the complete network shown in Fig. 2, distinct geographic groups can be readily identified. As mentioned above, D-loop analysis revealed the presence of 8 haplotypes (H1, H2, H3, H4, H5, H6, H7 and H8) in the studied populations (L, S and H sites). Moreover, 7 haplotypes (H1, H2, H3, H4, H5, H6 and H8) represent a distinct group and differed from groups comprising mainly sequences from Italy (Sicily and western Sicily) and Malta samples. Indeed, we showed that this group differs by 16 mutational steps and 9 of the latter two groups (Fig. 2). However, it is important to note that the presence of a diagnostic haplotype 7 (H7), which was only identified in samples from the estuary sites (H), is found belongs the group of samples from Sicily (black arrow in Fig. 2).

**Table 1. Geographical locations of the studied *A. fasciatus* haplotypes**

Locality	Latitude	Longitude	Haplotype
L	35°02'63"0	11°01'35"06	H1
L	35°02'63"0	11°01'35"06	H2
S	34°38'08"16	10°39'08"64	H3
S	34°38'08"16	10°39'08"64	H2
S	34°38'08"16	10°39'08"64	H4
S	34°38'08"16	10°39'08"64	H5
S	34°38'08"16	10°39'08"64	H6
H	35°47'20"02	10°41'00"09	H7
H	35°47'20"02	10°41'00"09	H7
H	35°47'20"02	10°41'00"09	H7
H	35°47'20"02	10°41'00"09	H7
H	35°47'20"02	10°41'00"09	H7
H	35°47'20"02	10°41'00"09	H7
L	35°02'63"0	11°01'35"06	H1
L	35°02'63"0	11°01'35"06	H8
L	35°02'63"0	11°01'35"06	H7

**Table 2. AMOVA results for D-loop sequences of *A. fasciatus* among Tunisian populations. We assessed hierarchical patterns of genetic structure among and within populations (d.f. = degrees of freedom; S.S = sum of square deviations)**

Source of Variance	d.f.	S.S	Variance components	Percentage of
Among groups	1	35.535	4.48354	89.70
Among populations within groups	2	0.728	-0.07262	-1.45
Within populations	12	7.050	0.58750	11.75
Total	15	43.312	4.99842	



**Fig. 2. Median-joining network based on mtDNA of D-loop haplotypes among *A. fasciatus* sequenced in the present study and included sequences published by Tigano et al. (2006). The size of grey circles is proportional to the haplotype frequency.**

#### 4. Discussion

The aim of the present study was to investigate the degree of genetic divergence between natural populations of *A. fasciatus* captured from Tunisian coastal and ones from Mediterranean areas. *A. fasciatus* is widely distributed in coastal and brackish-water habitats of the central and eastern Mediterranean Sea (Wildekamp 1993). In our study, a specific attribute of *A. fasciatus* taking into account that it has no economic use,

and thus the populations are not manipulated but rather reflect the natural genotyp distribution (Maltagliati 1999). *A. fasciatus* therefore serves as model organism in many studies on genetic structures and levels of differentiation based on different methods, i.e. osteological characters (Tigano et al. 1999, 2001, Ferrito et al. 2003, 2007), genetic data (Hrbek and Meyer 2003, Triantafyllidis et al. 2007, Pappalardo et al. 2008) and otoliths (Reichenbacher et al. 2007).

Our results revealed a relatively genetic differentiation among *A. fasciatus* studied populations and provide clear evidence for the presence of numerous unique haplotypes and significant populations structuring using the D-loop region. The results of the present work, however, clearly corroborates with the previous published work and supports their main conclusion of predominantly vicariant events affecting the evolution of the whole *Aphanius* genus (Hrbek & Meyer 2003). Additionally, data from the previous study based on the mitochondrial DNA analysis indicate that at least 41% of the studied populations are well differentiated taking into consideration the ample distribution of this species (Costagliola et al. 2003, Tigano et al. 2004b). These finding was in agreement with numerous researches carried out on Italian killifish populations and indicated a morphologically and genetically differentiation of populations (Tigano 1991, Parenti & Tigano 1993, Tigano et al. 1999, Cimmaruta et al. 2003, Maltagliati et al. 2003). In addition, the analysis of the highly variable D-loop region of the mitochondrial DNA, Tigano et al. 2004 indicated a strong genetic divergence between three Sicilian populations of *A. fasciatus*.

In the evolutionary history, the success of marine teleosts is even more remarkable considering their long freshwater ancestry, since it implies solving the major physiological challenges (e.g osmotic condition of water). The genetic differentiation of *A. fasciatus* populations is not surprising. Indeed, life-history adaptations have evolved in response to selection for restricted dispersal, thus the likelihood of reproductive isolation of populations is increased and their genetic differentiation is likely (Waples 1987). The occurrence of genetic divergence within a species is affected by many factors including population size, time since isolation, and porosity of the isolating barrier or mechanism (Frankham 1995). Genetic drift is faster if populations are small or the isolating barrier is very effective (Leis et al. 2011). In this context, the extreme



environmental variability of *A. fasciatus* habitats (e.g coastal lagoons, estuary) suggests that physical and ecological factors could contribute to the genetic divergence among populations. On the other hand, fish are exposed to wide environmental variations, particularly as regards salinity and temperature, which may cause strong selective pressures on organisms.

The physical connection between coastal sites in the Mediterranean Sea was significantly influenced by many factor and the most important ones was the changing global sea level between glacial and interglacial climate conditions during the Pleistocene (1,8 million years - 11000 years ago) (Lambeck et al. 2002). Several teleost fish species from the Mediterranean Sea, including the silverside *Atherina boyeri* Risso, 1810 (Milana et al. 2012 and references therein) and the goby *Pomatoschistus tortonesei* (Mejri et al. 2009), present a highly genetic differentiation among populations that may be probably linked with Pleistocene sea level falls. According to these studies, distinctive oceanic currents in the Mediterranean Sea represent hydrographic barriers for coastal species and favorise the maintained of differentiation for long periods of time. In our case, the strong sea-level drop (up to -120 m) during the last Pleistocene glaciation (20000 years ago) was suggested to be responsible for the genetic divergence between *A. fasciatus* populations from Italy (Rocco et al. 2007). Moreover, the sea level fall during the early Pleistocene (ca. 1,7 million years ago) represent the most appropriate explanation for the genetic divergence between *A. fasciatus* populations from Sicily, northern Tunisia and Malta (Tigano et al. 2006).

The interpretation of this differentiation represents a stimulating problem that is interesting from the theoretical point of view for the studies of the microevolution of this species, and from that of applied research into the consequent applications for the conservation of *A. fasciatus* species in environmental management. Indeed, life-history traits of *A. fasciatus*, such as benthic eggs, absence of larval stages and habitat preferences, determine a low potential for dispersal, which is consistent with the observed high degree of genetic differentiation among its populations. In conclusion, the present study provides a new insight into the genetic differentiation between Tunisian and Mediterranean populations of *A. fasciatus*. Taking together, these data can provide a valid knowledge base for the understanding of the micro-evolutionary processes, associated to habitat fragmentation acting in *A. fasciatus* populations. For the future,

further investigations based on extensive samples and other molecular markers (e.g nuclear markers) among Mediterranean killifish populations are needed to confirm these results.

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