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Molecular Analysis of the Genetic Differentiation among *Aphanius fasciatus* Populations Captured from Tunisian Coastal and Estuary Sites

Ali Annabi*, Abderraouf Ben Faleh, Temim Deli and Khaled Said
Laboratoire de Recherche, Génétique, Biodiversité et Valorisation des Bio-ressources (LR 11/ES 41), Institut Supérieur de Biotechnologie de Monastir, Université de Monastir, Tunisia

Abstract.- *Aphanius fasciatus* is a cyprinodont distributed in the salty coastal water of the central and eastern Mediterranean Sea and occasionally in internal fresh water. In this study, we investigated the genetic structure of three populations of the killifish *A. fasciatus* captured from Tunisian coast. The genetic diversity of mitochondrial NADH-dehydrogenase subunit 1 (965pb) was addressed on 17 specimens of three populations of *Aphanius. fasciatus.*. Phylogenetic analyses (Maximum likelihood (ML) and neighbour-joining (NJ)) were fully congruent and demonstrated the occurrence of two main groups or clades. The first clade comprises coastal populations (Luza and Sfax sites) while the second includes a population from estuary (Oued Hamdoun site). The analysis of molecular variance (AMOVA) showed that the percentage of variation among groups is considerably higher (72.8%) than within populations (34.32%). The overall Fst value (0.65; p=0.01) supports an extensive genetic structure of the two clades. These data represent an attempt towards the geographic diversification of *A. fasciatus* and provide new insights for the knowledge of genetic structural patterns and the evolutionary processes occurring in these species.

Keywords: *Aphanius fasciatus*, genetic differentiation, NADH-dehydrogenase subunit1, Tunisia

INTRODUCTION

Differences in the levels of genetic substructuring in marine, anadromous and freshwater teleosts have been frequently documented (Gyllensten, 1985; Ward *et al.,* 1994). Generally marine species have the potential for long-distance dispersal of eggs, larvae, juveniles and adults, resulting in genetic connection between populations over large distances. Conversely, some characteristics of species living in disjunct habitats, such as fresh or brackish water sites and the nature of the habitats occupied produce a higher likelihood of evolutionary divergence among populations (Carvalho, 1993): benthic eggs, absence of larvae, low mobility of adults, presence of geographical and ecological barriers and habitats fragmentation respectively.

Aphanius fasciatus (Valenciennes, 1821) is a Mediterranean endemic cyprinodont fish, occurring in coastal brackish water habitats (Leonardos and Sinis, 1999), and inhabited a wide range of lowland waters but its distribution is reduced to brackish and hypersaline waters in salt marshes and coastal lagoons. Previous studies showed, a dramatically decline of *A. fasciatus* population, in many cases even to extinction due to problems of brackish-water habitat degradation, introduction of exotic fishes and pollution of continental and coastal waters (Bianco, 1995). In addition, it is a widely distributed coastal species in the Mediterranean area. It has a relatively sedentary life history with large demersal eggs and without larval dispersal stages and the individual populations show strong territoriality (Triantafyllidis *et al.,* 2007).

For the Mediterranean populations of *A. fasciatus*, numerous studies have documented a high degree of isolation among them, marked genetic structuring using allozyme (Maltagliati, 1998a,b, 2002; Maltagliati *et al.,* 2003a) and mitochondrial DNA sequences (Tigano *et al.,* 2004, 2006; Triantafyllidis *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). Additionally, morphological differentiation among *A. fasciatus* populations has been detected particularly from Sicily, Sardinia and the Adriatic Sea (Kiener and Schachter, 1974; Parenti and Tigano, 1993; Maltagliati, 1998a, 1999; Cimmaruta

* Corresponding author: medannabi@yahoo.fr
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et al., 2003; Tigano *et al.*, 2004, 2006; Ferrito *et al.*, 2007).

The NADH-dehydrogenase subunit 1 was a valuable molecular marker for evolutionary relationship reconstructions among populations of fish species (Palumbi, 1996). The mitochondrial DNA presents a good marker to investigate genetic differentiation, among inter or intra-specific investigation, in fish species. In this study, we investigated the patterns of genetic structure between three populations of the killifish *A. fasciatus* captured from Tunisian environment: one population from an estuary (Oued Hamdoun) and two others from the littoral coast (Luza and Sfax population) using mitochondrial NADH-dehydrogenase subunit 1 (ND1, partial Cds) gene. For this purpose, we analyzed for the first time the complete sequence of *Aphanius fasciatus* mitochondrial NADH-dehydrogenase subunit 1 (ND1, partial Cds) gene from specimens collected from Tunisian area.

MATERIALS AND METHODS

Sampling

A total of 17 specimens of *A. fasciatus* were collected from three localities in Tunisian coast (6 samples from Luza, 6 from Oued Hamdoun and 5 from Sfax). 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). The coastal sites Luza (L) and Sfax (S) were selected based on preliminary studies that showed the presence of large population of *A. fasciatus* (Kessabi *et al.*, 2009; Messaoudi *et al.*, 2009). Luza is located approximately 50 km north of Sfax. The third site was Oued Hamdoun (H). It is an estuary and called locally Dkhila coast. It is located in the southern coastal zone of "Sousse" and represents the unique freshwater source feeding this coast. Oued Hamdoun is located approximately more than 150 km north Luza and Sfax sites.

Mitochondrial DNA sequencing

Total DNA was extracted from muscle tissue preserved in ethanol, using a "Wizard Genomic DNA extraction kit" (Promega). NADH1 gene was amplified from total DNA extracts using newly defined primers, respectively designed on the basis of the NADH-dehydrogenase subunit 1(ND1) of *Aphanius fasciatus* (GenBank Accession no. EF640854) (AFND1 Forward: 5'-TGA TTT TAG TAC TTT ATG CAA TTA TTA-3') and (AFND1 Reverse: 5'-GTG GGG GGG CAA GCC AGA-3'). The PCR was performed in a total volume of 50 µl containing: 20 ng DNA, 2.5 mM dNTPs, Buffer Taq (1X), 50 mM $MgCl_2$, 25 pmol from each primer and 0.5 U of Taq DNA polymerase (Promega).The amplification conditions were as follows (35 cycles): 94°C, 3 min; 56°C, 1 min; 72°C, 1 min, and a final extension step 10 min at 72°C. PCR products were visualized following electrophoresis on an agarose gel 1% and staining with ethidium bromide.

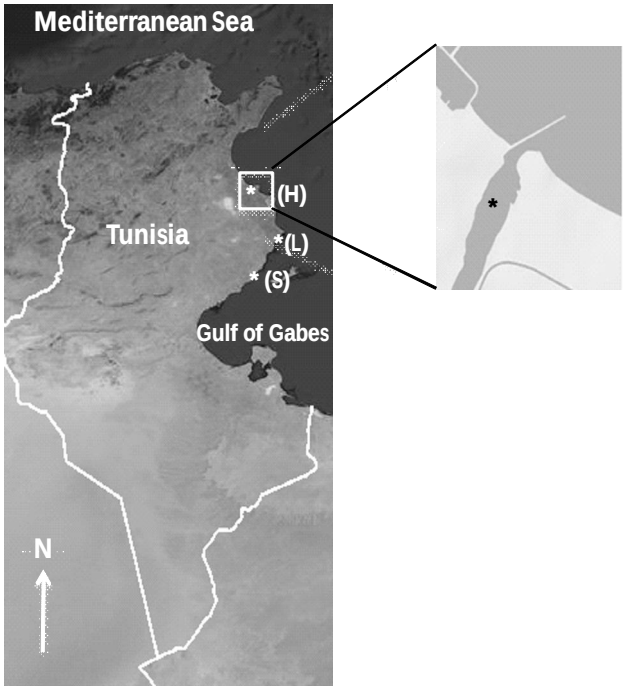

Fig. 1. Geographic overview of the studied sites of of *A. fasciatus* populations. L and S: represents coastal sites while H is an estuary.

Amplified DNA segments encoding NADH1 genes were purified using the "Wizard PCR preps DNA purification kit" according to the manufacturer's instructions (Promega) and then

sequenced. Cycle sequencing was performed by Macrogen (Seoul, Korea) using Automated Applied Biosystems (AB) sequencing and the Taq Dye Deoxy Terminator cycle sequencing kit.

Phylogenetic analysis

Sequence alignments were performed using BioEdit Sequence Alignment Editor (v. 7.0.5.2, Hall, 1999). Neither insertion nor deletion was observed in the dataset. The published sequence of NADH-dehydrogenase subunit 1(ND1) of *A. fasciatus* (GenAcession no. AF449313, Triantafyllidis *et al.*, 2007) was used as an outgroup for phylogenetic analyses. JModelTest (Posada, 2008) was run to determine the most suitable model for DNA evolution through five model selection strategies available in the program. Neighbour Joining (NJ) and Maximum-likelihood (ML) analyses were performed using, respectively, SEAVIEW (Gouy *et al.*, 2010) and PHYML on line at the ATGC Montpellier bioinformatics platform (v3.0, Guindon and Gascuel, 2003).

The genetic variation within groups was then estimated using basic statistics. Haplotype (h), nucleotide (π in percentage) diversities and their standard deviations (± SD) were estimated using DNASP (v4.10.9, Rozas *et al.*, 2003). The MEGA software version 3.1 (Kumar *et al.*, 2004) was used to estimate genetic distances (Tamura and Nei, 1993). Finally, F_{ST} values were calculated and a spatial analysis of molecular variance (AMOVA) was used as implemented in ARLEQUIN (version 3, Excoffier *et al.*, 2006). Variance components of the different hierarchical levels were tested statistically by nonparametric randomization tests using 10.000 permutations. A median-joining network was reconstructed using NETWORK 4.5.1.0 (Bandelt *et al.*, 1999) according to previous published sequences of NADH-dehydrogenase subunit 1 of *A. fasciatus* (Triantafyllidis *et al.*, 2007). Variable sites were differentially weighted reciprocally according to their site-specific mutation rate in the total network. Rooting of the network was done according to previously published NADH-dehydrogenase subunit 1 *A. fasciatus* sequences originating from Turkey, Greece, Spain, Italy and French (Triantafyllidis *et al.*, 2007).

RESULTS

A total of 17 DNA sequences (965 bp) were obtained through the amplification of the NADH1 gene of *A. fasciatus* (Genbank accession numbers: JX273498 to JX273514). Among them, 9 different haplotypes were identified. In total, fourteen sites were variable and six were parsimony informative. The TrN+G model with a gamma distribution shape parameter equal to 0.011 and the proportion of invariable sites equal to 0.242 was the best evolutionary model. The nucleotides frequencies were 24.75%, 26.7%, 15.26% and 33.29 % for A, C, G and T, respectively.

Phylogenetic analyses for the NADH1 gene were fully congruent and evidenced the occurrence of two diverged genetic clades (I and II). Fig. 2 represents the phylogenetic tree corresponding to the ML analysis. *A. fasciatus* sequences were distributed among two main clades. Clade I comprised populations of Luza and Sfax while Clade II included the population of Oued Hamdoun with a very little overlapping (specimen number 17 from Luza; see Table I). Phylogenetic analysis for Clade I showed that haplotype 1 was the most common, including 7 specimens (3 from Luza, 3 from Sfax and one from Oued Hamdoun), and originated from Sfax and Luza locality. For Clade II, the haplotype 5 was represented by 3 specimens from Oued Hamdoun (Table I). These two clades were separated by a moderate value of Tamura and Nei genetic divergence (clade I vs. II: 0.6±0.002%). The mean F_{ST} value between Clades I and II was 0.65 ($p = 0.001$). AMOVA results showed a genetic structuring mainly supported among the two groups (72.8%). Most of the variation was explained by differences among groups (72.8%; $p = 0.0003$) whereas variation within populations was smaller (34.32%). Phylogenetic analysis for the NADH1 gene was fully congruent and showed the occurrence of two diverged genetic clades (I and II). In the Mediterranean context, our data were aligned with 72 haplotypes (507 bp) of mtDNA NADH-dehydrogenase subunit 1 of *A. fasciatus*, published by Triantafyllidis *et al.* (2007). A median-joining network demonstrates the occurrence of two distinct groups (Fig. 3). The first one contains the populations originating from French, Spain Greece,

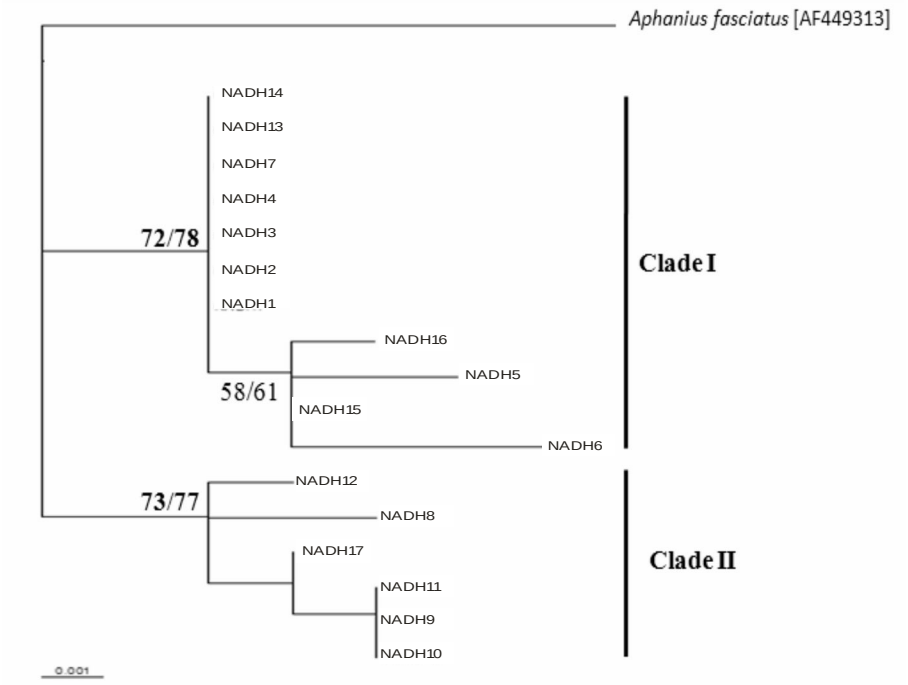

Fig. 2. Maximum likelihood phylogenetic relationships among NADH1 deshydrogenase sequences of 17 specimens of *A. fasciatus.* Numbers aside nodes indicate bootstrap values (% > 50) obtained by the NJ and ML analysis respectively.

Table I.- Geographical locations, sample abbreviations and haplotypes of *A. fasciatus* used in this study.

Sample code	Locality	Latitude	Longitude	Haplotype	Halogroup
1	Luza	35°02'63"0	11°01'35"06	H1	I
2	Luza	35°02'63"0	11°01'35"06	H1	I
3	Sfax	34°38'08"16	10°39'08"64	H1	I
4	Sfax	34°38'08"16	10°39'08"64	H1	I
5	Sfax	34°38'08"16	10°39'08"64	H2	I
6	Sfax	34°38'08"16	10°39'08"64	H3	I
7	Sfax	34°38'08"16	10°39'08"64	H1	I
8	Oued Hamdoun	35°47'20"02	10°41'00"09	H4	II
9	Oued Hamdoun	35°47'20"02	10°41'00"09	H5	II
10	Oued Hamdoun	35°47'20"02	10°41'00"09	H5	II
11	Oued Hamdoun	35°47'20"02	10°41'00"09	H5	II
12	Oued Hamdoun	35°47'20"02	10°41'00"09	H6	II
13	Oued Hamdoun	35°47'20"02	10°41'00"09	H1	I
14	Luza	35°02'63"0	11°01'35"06	H1	I
15	Luza	35°02'63"0	11°01'35"06	H7	I
16	Luza	35°02'63"0	11°01'35"06	H8	I
17	Luza	35°02'63"0	11°01'35"06	H9	II

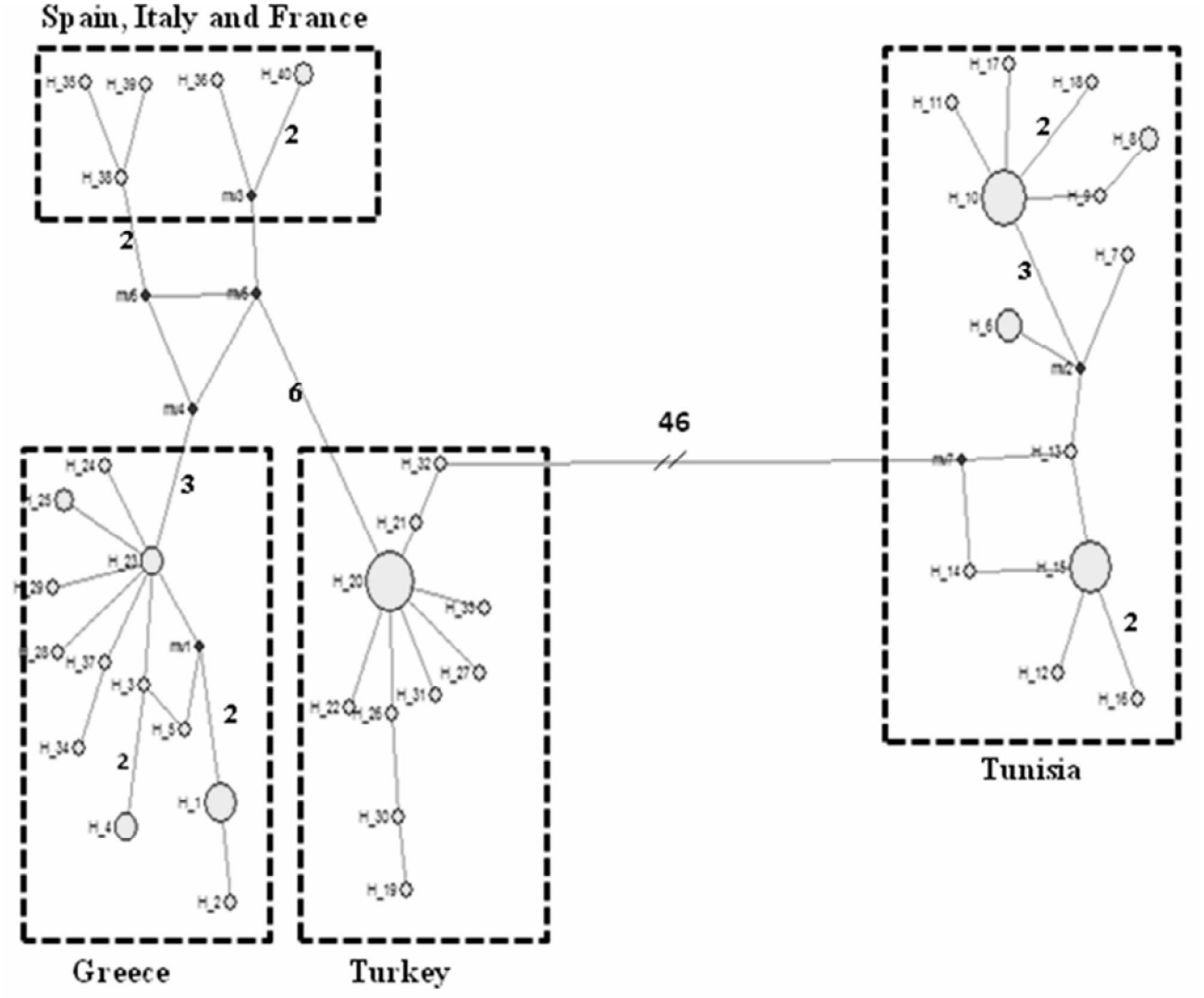

Fig. 3. Median-joining network of mtDNA (NADH-dehydrogenase subunit 1) haplotypes among *A. fasciatus* sequenced in the present study and included sequences published by Triantafyllidis *et al*. (2007). Numbers of mutations (greater than one) between haplotypes are indicated near branches and circle sizes are proportional to the number of similar haplotypes (n) observed in the data set.

Italy and Turkey while the second comprised the Tunisian populations. These two groups were differentiated by 46 mutational steps (Fig. 3).

DISCUSSION

The aim of the present study was to investigate the degree of genetic divergence between natural populations of *A. fasiatus* captured from Tunisian coastal and estuary areas. Our results revealed relatively low levels of genetic polymorphism across Tunisian populations of *A. fasciatus* species and provide clear evidence for the presence of two differentiated clades.

Indeed, the presence of diagnostic haplotypes (H1 and H5) and the high degree of genetic divergence found between the two clades suspect a break of gene flow and suggest that they have subsequently evolved independently. Moreover, these two clades were differentiated by a moderate sequence divergence (0.6%). Our results are in agreement with numerous studies on Italian *A.*

fasciatus populations which indicate morphologic (Tigano and Ferrito, 1985; Tigano and Parenti, 1988; Tigano, 1991; Parenti and Tigano, 1993; Tigano *et al.,* 1999) and genetic differentiation (Maltagliati, 1998a,b, 1999; Cimmaruta *et al.,* 2003; Maltagliati *et al.,* 2003b). Maltagliati (1999) suggest that the observation of low levels within-population genetic variability has been considered as a general characteristic of these species.

In our study the mean F_{ST} value between the two clades was 0.65. This value rather falls within the range of those recovered from intra-specific genetic studies for *Aphanius* species (range 0.00-6.86%, Tigano *et al.,* 2004, 2006; Triantafyllidis *et al.,* 2007). The differentiation, obtained in the present work, was lower with that recorded in *A. fasciatus* populations using molecular tools (0.84) (Triantafyllidis *et al.,* 2007). In this case, higher F_{ST} values (>0.45) have been reported in populations of various species of brackish and fresh-water cyprinodontids (Ashbaugh *et al.,* 1994; Dunham and Minckley, 1998). However, previous studies of *A. fasciatus* populations, based on allozymic analysis, have shown high genetic differentiation among populations with values ranging from 0.302 to 0.507 (Maltagliati, 1998b, 1999). On the other hand, the molecular analysis carried out by Hrbek and Meyer (2003) showed a limited structuring of *A. fasciatus* populations. Numerous unique haplotypes and significant population structuring was found after analysis of D-loop region in central Mediterranean *A. fasciatus* populations by Tigano *et al.* (2006). 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).

The genetic substructuring of Tunisian *A. fasciatus* populations can be explained by the biological characteristics of this species. Indeed, adaptation processes have evolved in response to selection for restricted dispersal populations. Thus the likelihood of reproductive isolation of populations is increased and their genetic differentiation likely (Waples, 1987). Additionally, 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 addition, the high genetic divergence between coastal and estuary populations reported in our study has been described for numerous fish species. A characteristically low level of polymorphism has also reported in many typical brackish water invertebrate species (Battaglia *et al.,* 1978; Abbiati and Maltagliati, 1996).

In this context, the extreme environmental variability of coastal lagoons suggests that physical and ecological factors could contribute to the genetic divergence among populations occurring in coastal and estuary populations. Additionally, coastal lagoons are habitats exposed to wide environmental variations, particularly as regards salinity and temperature, which may cause strong selective pressures on organisms. These characteristics suggest that environmental factors directly modify the genetic patterns of fish species (Gonzalez-Wanguemert *et al.,* 2006, 2009) and could contribute to the genetic divergence among populations, associated with physical and ecological discontinuities between coastal and estuarines populations (Bilton and Bishop, 2002; Iannotta *et al.,* 2008). The natural fragmentation of the brackish-water habitats contributed to the disjunct coastal distribution of *A. fasciatus.* In fact, brackish-water habitats are characterized by rapid and wide changes of both physicochemical and biological features due to natural events, as well as human induced alterations (Cognetti, 1994). The genetic structure of *A. fasciatus*, results from the interaction of life history traits and natural fragmentation of habitats, which determine the observed isolation of local populations. In addition, it is known that environmental factors may play a decisive role in intra-specific differentiation. It is becoming increasingly recognized that estuarine fauna possess patterns of genetic variation that reflect complex population histories (Bilton and Bishop, 2002). Consequently, much remains to be discovered about how the distribution of genetic diversity in these systems is related to the ecology of estuarine organisms. Nevertheless, 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.

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