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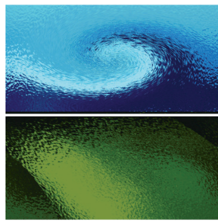

RESEARCH ARTICLE

Low microsatellite variation in *Aphanius fasciatus* from the Tarquinia Salterns

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Abstract

1 - The Tarquinia Salterns (Latium, central Italy) provided the opportunity to analyse the impact of environmental stress on the genetic structure of the resident population of the killifish *Aphanius fasciatus*. Indeed, after the salt production ceased in 1997, the salterns have undergone habitat degradation due to lack of maintenance. The ecological restoration carried out from 2003 to 2006 reverted the environmental conditions to those of ten years before.

2 - The temporal variation of the gene pool of the population of *A. fasciatus* inhabiting the Tarquinia Salterns was investigated using microsatellite markers in samples collected in 1998 and 2003. The results obtained showed a low genetic variability and a genetic homogeneity of the population, which appears not divided in sub-demes.

3 - Microsatellites revealed a surprisingly low level of genetic variability when compared to allozyme data obtained in previous studies. This is likely due to a difference in the time of response of the two markers to environmental degradation. Microsatellites would lose genetic variability earlier and faster because of their usually high polymorphisms. Conversely, allozymes would be more resistant to genetic erosion, being moderately variable markers.

4 - Selection probably contributed in maintaining allozyme polymorphism, while microsatellites, being neutral markers, did not benefit from the action of selection and lost diversity earlier and more rapidly. Accordingly, the population appeared subdivided in distinct demes based on allozyme data but spatially homogeneous following microsatellites results.

Keywords: *Aphanius fasciatus*, microsatellites, genetic variability, genetic erosion, habitat degradation, Tarquinia Salterns

Introduction

The relationships between levels of environmental stress and genetic structure of natural populations have been widely investigated since long time, showing a strong correlation between these two variables (Hoffmann and Willy, 2008; Nevo, 2001; Frankham *et al.*, 2002; Frankham, 2005). This connection is particularly evident in marine coastal habitats, where environmental disturbance frequently occurs (Nevo *et al.*, 1986; Cognetti and Maltagliati, 2000; Larno *et al.*, 2001, Dufresne *et al.*, 2002). Coastal lagoons are a habitat of choice to investigate

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the evolution of genome diversity under environmental stress because they both are subject to wide and rapid variation of their main physical-chemical parameters, and are affected by several human-induced disturbances (overexploitation, habitat alteration, introduction of exotic species and pollution, see for example Cognetti and Maltagliati, 2004).

Tarquinia Salterns, located on the Tyrrhenian coast of central Italy, provide a good opportunity to analyse the impact that environmental stress could have on the genetic structure of resident populations, because of their recent history. After the salt production ceased in 1997, the salterns have undergone remarkable habitat degradation due to lack of maintenance activities. This caused an increased sedimentation and a strong input of organic matter, which in turn generated eutrophication and deterioration of the main ecological parameters such as dissolved oxygen and salinity. The ecological restoration, carried out from 2003 to 2006 within the context of a LIFE-NATURA project, increased water levels and regimented the circulation in the pools. This avoided sedimentation and eutrophication of the salterns, returning to the environmental conditions of ten years before (Cimmaruta *et al.*, 2010).

During the same years (i.e. since 1998), the genetic structure of the killifish *Aphanius fasciatus* Nardo 1827 has been monitored. This is a cyprinodont fish distributed in the central and eastern coastal zones of the Mediterranean Sea; it lives in coastal brackish waters, lagoons and salt marshes and is well adapted to fluctuating environments (Leonardos and Sinis, 1998, 1999). An allozyme survey of *A. fasciatus* from the Tarquinia Salterns carried out throughout time showed that the genetic variability of the killifish strongly reduced, with a remarkable loss of rare alleles and a significant decrease of both heterozygosity and allele richness (Angeletti *et al.*, 2010). The data showed a prominent role of genetic drift in the loss of genetic variation, also suggesting a possible action of selection, based on the allelic distribution pattern observed at some loci (Angeletti *et al.*, 2010).

In this paper the temporal variation of the gene pool of the population of *A. fasciatus* inhabiting the Tarquinia Salterns is investigated using microsatellites (SSRs) and the results obtained compared with previous allozyme data. Both markers (SSRs and allozymes) are biparentally inherited and are codominant, but usually SSRs have higher levels of polymorphism (Chistiakov *et al.*, 2006; Schlötterer, 2000), hence being more sensitive to genetic erosion. Indeed, SSR loci are highly responsive to population size fluctuations and effective population size (Gold *et al.*, 2001; Bérubé *et al.*, 2002; Waples, 2002) and their high polymorphism makes them more susceptible to changes in genetic diversity, since the resolution power of the genetic variability parameters in evidencing a loss of genetic variation increases with the number of alleles detected (Hauser *et al.* 2002; Ryman *et al.* 1995). For the same reason, microsatellites are particularly useful in detecting subtle structuring within populations, evidencing possible heterogeneity in genotype distribution among demes (Falush *et al.*, 2003). Finally, microsatellites are considered neutral markers (even if they can be linked to coding loci possibly under selection, see Chistiakov *et al.*, 2006), so can be usefully compared with allozymes, which can be the target for natural selection.

Stated the different features of allozymes and SSR, their comparison is expected to further evidence the genetic erosion endured by the local population of *A. fasciatus*, to reveal a possible spatial heterogeneity in the distribution of the genotypic variation within the salterns area, and to provide information on the relative relevance of genetic drift

vs. selection in driving the temporal and spatial structure of the gene pool of local *A. fasciatus*.

Materials and methods

Sample collection

The two temporal samples of *A. fasciatus* analysed in this work were collected within the Tarquinia Salterns using fish traps, according to the methodologies described in Angeletti *et al.* (2010). The first sample was taken in 1998 and consisted of 30 specimens trapped in the northern pan of the salterns (Fig. 1). The second sample was collected in 2003 from four pans distributed across the North-South axis of the salterns (Fig. 1). Thirty fishes were collected from each of the four pans, for a total of 120 specimens. All specimens examined in this study underwent allozyme diversity analysis in previous works (Cimmaruta *et al.*, 2003; Angeletti *et al.*, 2010).

DNA extraction and microsatellites analysis

DNA was extracted from muscle tissue following CTAB method (Doyle and Doyle, 1987). We examined microsatellite variation at seven loci: Af7, Af8, Af9, Af18, Af20, Af20b and Af25 (GenBank accession numbers from DQ865156 to DQ865162). PCR conditions followed Babbuci *et al.* (2007), with minor modifications. Amplification products were diluted 1:10 and fragments separated in a 12% polyacrylamide gel. Bands were visualised by silver-staining method.

Statistical analysis of microsatellite data

Allelic and genoptype frequencies were calculated using Biosys-1 (Swofford and Selander, 1981), together with the parameters of genetic variability: the overall number of alleles observed per sample (A_{Tot}), the mean number of alleles per locus (A_{Mean}), the proportion of polymorphic loci according to the criteria of 99% (P_{99}) and 95% (P_{95}), the expected (H_e) and observed (H_o) mean

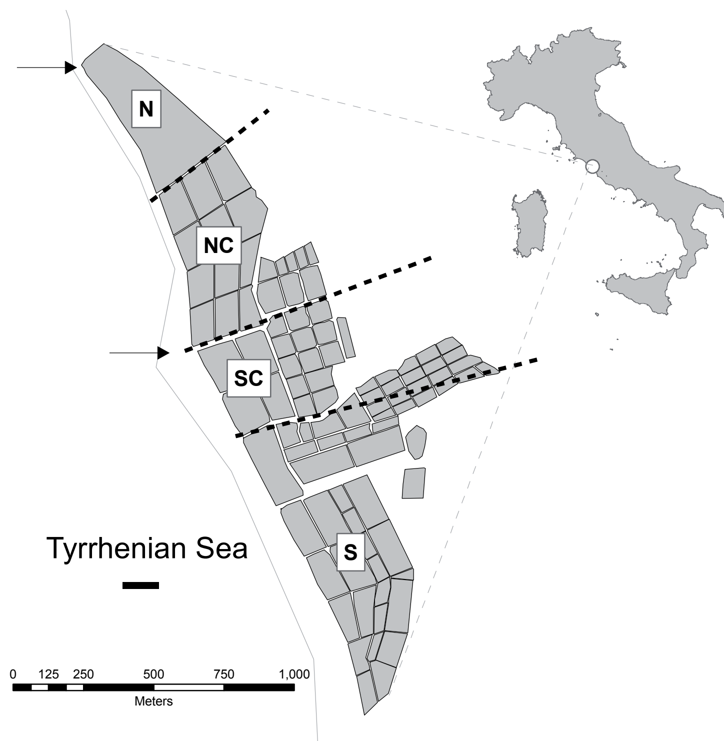

Figure 1. The location and map of the Tarquinia Salterns, showing the four sub-sample areas: North (N); North-Central (NC); South-Central (SC); South (S).

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heterozygosity. The allelic richness (AR) was calculated as implemented in FSTAT (Goudet, 2001).
The software Microcheker was used to check for null alleles (Van Oosterhout *et al.*, 2004). Hardy–Weinberg equilibrium, pairwise linkage disequilibrium among loci and differences in allele and genotype frequencies were tested using Fisher exact test, as implemented in Genepop 3.4 (Raymond and Rousset, 1995; http://www.cefe.cnrs-mop.fr). The occurrence of bottleneck events was tested using the software Bottleneck Version 1.2.02 (Cornuet and Luikart, 1996). Since microsatellites fit better to the IAM (Infinite Allele Mutation) model, this option was picked when running the program (Cornuet and Luikart, 1996; Cristescu *et al.*, 2010).
The possible action of selection on one or more loci was tested using the software LOSITAN, which analyses the relationships between the values of F_{ST} and H_e compared to a set of simulated values (Beaumont and Nichols, 1996; Antao *et al.*, 2008).

Results

The 7 loci analysed were polymorphic, having two to five alleles, and shared the same most frequent allele *100 in both temporal samples (Table 1).
Loci Af25 and Af20 were not in Hardy-Weiberg equilibrium, showing a significant deficit of heterozygote genotypes: Af25 in both temporal samples (P_{1998} = 0.028; P_{2003} = 0.008), and Af20 in the sample of 2003 (P_{2003} = 0.026). The software Microcheker identified the presence of a null allele at locus Af25, explaining the heterozygote deficit recorded in the 2003 sample.
On the contrary, no null alleles were observed at loci Af25 in 1998 and Af20 in 2003. No evidence of linkage disequilibrium was evident after performing Fisher's exact test, so that genotypes at each locus could be considered independent from genotypes at other loci.

Table 1 - Allele frequencies at the 7 microsatellite loci scored for the killifish *A. fasciatus* from Tarquinia Salterns in 1998 and 2003, showed with the number of specimens analysed per locus (*n*).

		Allele frequencies	
Locus	Allele	1998	2003
	(n)	*26*	*120*
Af 7	70	0.288	0.300
	90	0.115	0.067
	100	0.596	0.633
	(n)	*28*	*129*
Af 8*	100	0.518	0.722
	110	0.071	0.000
	120	0.411	0.278
	(n)	*27*	*120*
Af 9	100	0.963	0.978
	120	0.037	0.022
	(n)	*29*	*120*
Af 18	100	0.983	0.989
	120	0.017	0.011
	(n)	*14*	*99*
Af 20	100	0.714	0.777
	120	0.250	0.223
	130	0.036	0.000
	(n)	*27*	*120*
Af 21	100	0.981	0.939
	110	0.019	0.061
	(n)	*25*	*118*
Af 25	70	0.000	0.006
	80	0.260	0.205
	90	0.240	0.199
	100	0.500	0.562
	110	0.000	0.028

* Allelic frequencies significantly different between 1998 and 2003 (P<0.01)

In the sample of 1998 low frequency alleles (< 5%) were present at loci Af9, Af18, Af20 and Af21. Their frequencies decreased in

2003 in most cases (Af9*120, Af18*120), even if Af21*110 increased its frequency. The rare allele Af20*130 disappeared between 1998 and 2003, so as Af8*110, although in this latter the frequency of the lost allele was much higher (7.1%). Accordingly, changes of both allele and genotypic frequencies at this locus were statistically significant according to Fisher's exact test, even when the comparison was made considering only the samples taken from the north pan (the only one sampled at the two times, 1998 and 2003; $P \leq 0.01$). At locus Af25, two rare alleles (Af25*70 and Af25*110) were found in the sample from 2003, while lacking in 1998 (Table 1); it is worth noting that the sample size of 2003 was four times larger than that of 1998.

The comparison between 1998 and 2003 across all loci showed that the population has undergone significant change at both allelic and genotypic frequencies in this time span ($P \leq 0.05$).

A slight decrease in the genetic variability of the population was found from 1998 to 2003, as reported in Table 2, and this drop was more marked when considering only the northern pan (the only one sampled at both times). In both cases, however, the values of heterozygosity and allelic richness did not change significantly between the two temporal samples (t-Student test, $P > 0.05$).

To test for possible genetic heterogeneity among different areas of the salterns, the four sub-samples taken in 2003 have been analysed separately (see Fig.1). The allele frequencies are shown in Table 3: five of the seven polymorphic loci (Af7, Af9, Af18, Af21, Af25) had low frequency alleles, with values between 1.7% and 3.3%. At locus Af25, which showed the higher degree of polymorphism, the allele Af25*60 was present at low frequency only in the deme N (north), while the allele Af25*110 was found only in the demes NC (north-central) and S (south), with a frequency of 3.3% and 6.9%, respectively. The loci Af9 and Af18 showed a rather low degree of polymorphism, being monomorphic in two of the four sub-samples. Significant deviations from Hardy-Weinberg equilibrium have been observed at loci Af25 (in the sample N) and Af20 (sample NC), both showing a significant deficit of heterozygotes ($P = 0.016$ and $P = 0.025$ respectively). The analysis carried out with the software Microchecker did not highlight the presence of null alleles. The possible genetic fragmentation of the four samples was evaluated by means of Fisher's exact test. In all cases the distribution of allelic and genotypic frequencies resulted to be homogeneous at each locus as well as across all loci ($P >> 0.05$), even when the comparison was performed between sample pairs. The values shown by the parameters of genetic variability recorded across the

Table 2 - Parameters of genetic variability estimated in *A. fasciatus* from Tarquinia Salterns: the overall number of alleles observed per sample (A_{Tot}), the mean number of alleles per locus (A_{Mean}), the allelic richness (AR), the proportion of polymorphic loci according to the criteria of 99% (P_{99}) and 95% (P_{95}), the expected (H_e) and observed (H_o) mean heterozygosity.

	Parameters of genetic variability						
Samples	A_{Tot}	A_{Mean} ± SE	AR ± SE	P_{99}	P_{95}	H_o ± SE	H_e ± SE
1998	18	2.6 ± 0.4	2.387 ± 0.283	100	57.1	0.293 ± 0.088	0.336 ± 0.104
2003	18	2.6 ± 0.4	2.152 ± 0.313	100	71.4	0.265 ± 0.078	0.292 ± 0.088

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Table 3 - Allele frequencies at the 7 microsatellite loci scored for the killifish *A. fasciatus* from Tarquinia Salterns as recorded in the four ponds sampled in 2003.

		Pond sampled			
Locus	Allele	N	NC	SC	S
	(n)	*30*	*30*	*30*	*30*
Af 7	70	0.350	0.367	0.300	0.217
	90	0.033	0.067	0.133	0.067
	100	0.617	0.567	0.567	0.717
	(n)	*30*	*30*	*30*	*30*
Af 8	100	0.733	0.667	0.800	0.700
	120	0.267	0.333	0.200	0.300
	(n)	*30*	*30*	*30*	*30*
Af 9	100	0.967	0.967	1.000	0.983
	120	0.033	0.033	0.000	0.017
	(n)	*30*	*30*	*30*	*30*
Af 18	100	0.983	1.000	0.967	1.000
	120	0.017	0.000	0.033	0.000
	(n)	*28*	*26*	*24*	*21*
Af 20	100	0.714	0.885	0.875	0.738
	120	0.286	0.115	0.125	0.262
	(n)	*30*	*30*	*30*	*30*
Af 21	100	0.900	0.967	0.900	0.983
	110	0.100	0.033	0.100	0.017
	(n)	*29*	*30*	*30*	*29*
Af 25	70	0.017	0.000	0.000	0.000
	80	0.259	1.000	0.167	0.224
	90	0.207	0.267	0.200	0.155
	100	0.517	0.600	0.633	0.552
	110	0.000	0.033	0.000	0.069

four demes are quite similar (Table 4). Both heterozygosity and allelic richness values do not show any significant difference between the four 2003 samples, according to a t-student test on sample pairs, indicating a substantial homogeneity of the population in terms of variability. The F_{ST} analysis also confirmed the overall lack of genetic heterogeneity within the salterns during 2003, given the low values recorded (Table 5).

The possible action of selection on one or more loci was tested using the software LOSITAN. The test, run for the 2003 sample on the two data sets (observed and simulated), suggests that all the seven microsatellite loci are selectively neutral.

The Wilcoxon sign-rank test under IAM model was carried out as implemented in Bottleneck, and showed no signatures of recent bottlenecks in the studied samples.

Discussion

The years comprised between 1998 and 2003 have been characterised by notable environmental changes in the habitat of the Tarquinia Salterns (Cimmaruta *et al.*, 2010). The end of salt production in 1997 was preceded by a progressive reduction of extraction activities and was followed by habitat degradation due to the reduced water circulation and to the increased organic sedimentation in the pans. In the end, the basins became affected by eutrophication. These changes had strong impact on the biodiversity at both the community, with a strong reduction of the diversity of the benthic community (5 taxa lost over 21), and at the genetic level, with genetic erosion decreasing the diversity of the killifish *A. fasciatus* (Bellisario *et al.*, 2010; Angeletti *et al.*, 2010). These events provided a unique opportunity to investigate which ecological and evolutionary mechanisms would act on the gene pool of the local killifish population as a response to the environmental changes. Previous studies carried out on the same samples of *A. fasciatus* by means of allozymes showed that its gene pool varied sensitively. The allelic frequencies showed statistically significant differences at 9 allozyme loci, over the 24 analysed, and all parameters of genetic variability (total number of alleles, allele richness and both expected and observed heterozygosity) resulted significantly lower in 2003 with respect to 1998 (Angeletti *et al.*, 2010). The picture offered by the microsatellite analysis reported in this study is in agreement with that shown by allozyme markers, but of lower magnitude. The allelic

Table 4 - Parameters of genetic variability recorded in *A. fasciatus* from Tarquinia Salterns for the four ponds sampled in 2003: the overall number of alleles observed per sample (A_{Tot}), the mean number of alleles per locus (A_{Mean}), the allelic richness (AR), the proportion of polymorphic loci according to the criteria of 99% (P_{99}) and 95% (P_{95}), the expected (H_e) and observed (H_o) mean heterozygosity.

	Parameters of genetic variability						
Samples	A_{Tot}	A_{Mean} ± SE	AR ± SE	P_{99}	P_{95}	H_o ± SE	H_e ± SE
N - north	17	2.4 ± 0.3	2.152 ± 0.255	100	71.4	0.276 ± 0.070	0.319 ± 0.086
NC - n-central	16	2.3 ± 0.4	2.194 ± 0.344	85.7	57.1	0.259 ± 0.101	0.277 ± 0.094
SC - s-central	15	2.1 ± 0.3	2.113 ± 0.265	85.7	71.4	0.302 ± 0.092	0.279 ± 0.086
S - south	16	2.3 ± 0.4	2.081 ± 0.037	85.7	57.1	0.240 ± 0.079	0.280 ± 0.095

frequencies showed significant variation at a single locus (Af8) over the 7 analysed and the parameters of genetic variability did not change substantially. However, the general trend observed using microsatellites was in line with that of the allozymes. There were alleles recorded in 1998 that disappeared in 2003 (Af8*110 and Af20*130) and many parameters of genetic variability decreased their values: allelic richness (AR) lowered from 2.387 to 2.152, observed heterozygosity decreased from 0.293 in 1998 to 0.265 in 2003, and expected heterozygosity declined from 0.336 to 0.292 in the same timespan. The same trend was recorded when considering just the northern pan, the only one sampled in both years, with the values of the genetic variability parameters showing an even more remarkable reduction, although not statistically supported (data not shown). The two genetic markers here considered showed similar temporal trends but did not agree in the strength of their signal. Quite surprisingly, microsatellites did not have statistical support for the pattern of genetic erosion, likely because their level of genetic diversity was lower than that revealed by allozymes. This is a very rare finding, since microsatellites are hypervariable markers, characterized by a mutation rate three to five orders of magnitude greater than that of regular nonrepetitive DNA (Chistiakov *et al.*, 2006). A possible explanation for such a low variation in microsatellites could come from their structure, since it is known that the mutation rate is related to their length (Chistiakov *et al.*, 2006). In our case, however, all the microsatellites analysed have a repeat number well above the threshold of four repeats for (CA/GT) dinucleotides and eight $(A/T)_n$ motifs (see Babbucci *et al.*, 2007), as suggested

Table 5 - The F_{ST} values obtained for the four sub-samples collected in 2003.

Locus	F_{IS}	F_{IT}	F_{ST}
Af 7	-0.028	-0.012	0.016
Af 8	-0.100	-0.087	0.012
Af 9	-0.031	-0.021	0.009
Af 18	-0.029	-0.013	0.015
Af 20	0.223	0.253	0.038
Af 21	-0.094	-0.067	0.025
Af 25	0.143	0.155	0.014
Mean	0.043	0.061	0.018

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by Shinde *et al.* (2003). Another possible explanation could come from the history of the studied population. Microsatellites are highly sensitive to population size, in terms of both numbers of individuals and temporal fluctuations (Waples, 2002). Populations of low effective population size or undergoing fluctuations in their density are rapidly deprived of a relevant part of the genetic variability of highly variable markers, since loss of genetic variation increases with the number of alleles detected, especially if at low frequency (Hauser *et al.*, 2002; Ryman *et al.*, 1995). This refers to microsatellites, which usually exhibit a high number of rare alleles quickly lost if population reaches low effective size (see for example Berubé *et al.*, 2002). Therefore, a possible, although speculative, explanation of the low variability of SSR with respect to allozymes could be that they have lost their genetic variation early, so that in 1998 it was already eroded, while allozymes still retained it. In support to this hypothesis is the fact that the environmental quality of the salterns was already decreasing since 1987, when a flood seriously altered the ponds depth (Cimmaruta *et al.*, 2010). Another support comes from the results of previous studies, showing that the effective size of the Tarquinia Salterns population was very small during the period 1998-2003 (64-136 individuals; Angeletti *et al.*, 2010), likely smaller than under unaltered environmental conditions, i.e. as they were before the environmental degradation due to the stop of extraction activities. Indeed, a significant increase of N_e was recorded during the years immediately following the environmental restoration of the salterns (N_e between 268 and 1064 in 2003-2007), corroborating the idea that in 1998 population size was already decreasing, hence eroding the genetic diversity of highly sensitive microsatellites. In the case of allozymes, the genetic erosion occurred later in time (mainly between 1998-2003) because of a lower incidence of rare alleles. Significantly, both markers agreed in indicating that, in spite of fluctuation of the population size, no bottleneck events occurred (Cimmaruta *et al.*, 2003). Interestingly, the loss of genetic variability decelerated in allozymes after the genetic erosion, between 2003-2007, reaching a rate comparable to that showed by the microsatellites between 1998 and 2003 (hence after the hypothesized genetic erosion of their variability). This observation further supports the idea that the two markers responded to the environmental degradation in a similar way (genetic erosion) but with different timing.

However, a further hypothesis could be added to explain the discrepancy observed. The maintenance of some polymorphism at the allozyme level could be due to the balancing action of selection. Indeed, some allozyme loci such as ADA and GPI-3 showed a correlation between the distribution of alleles and genotypes and either the dissolved oxygen concentration or the salinity (Angeletti *et al.*, 2009). This suggested a role for selection in the maintenance and the distribution of allozyme polymorphisms within the population. Since microsatellites are neutral markers, they did not undergo selection and therefore could loose diversity more quickly and early than allozymes.

This hypothesis is further supported by the finding that the population appeared subdivided in distinct demes according to allozyme data, while spatially homogeneous as to microsatellites data. Allozymes under selection would trace individuals better adapted to (hence more concentrated in) particular microhabitats, differing for the oxygen concentration and the salinity. Neutral microsatellites would not detect such a heterogeneous spatial distribution, being not directly influenced by the environmental variation of the salterns.

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Conclusions

The comparison of the patterns showed by allozyme and microsatellite markers of the population of *A. fasciatus* inhabiting the Tarquinia Salterns revealed a substantial agreement of the data collected across time, indicating a loss of genetic diversity of the population gene pool. On the contrary, some discrepancies emerged between the two markers at the spatial scale and at the genetic diversity level. Surprisingly, genetic variability was relatively low in microsatellites than in allozymes and the latter showed a heterogeneous distribution of alleles and genotypes through the salterns, while microsatellites indicated a substantial homogeneity. This is likely due to a difference in the time of response of these two markers to the environmental degradation and to the different evolutionary forces in action. Microsatellites may have undergone the process of genetic erosion earlier and faster, because of their usually high genetic variability and neutrality. Allozymes were more resistant to genetic erosion, being moderately variable markers and prone to selection. For this reason, in 1998 survey, allozymes still showed genetic variation (subsequently eroded from 1998 to 2003), while microsatellites were already at a low level of genetic variability. In support to this scenario, allozymes slowed down their rate of genetic erosion after 2003, likely because of their already reduced genetic variation, coupled with the ecological rescue of the habitat (Angeletti *et al.*, 2010).

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