

What is the smallest distance of genetic structuring in the brooding ophiuroid *Amphipholis squamata* from the Western Mediterranean?

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ABSTRACT: Our goal was to assess the smallest scale of genetic differentiation in a minute ophiuroid considered as a cosmopolitan species, which was already shown to be differentiated at a relatively small scale (about 1 km) in the Mediterranean Sea – Medes Islands. *Amphipholis squamata* is hermaphroditic and broods its young. Both auto- and allo-fertilization occur. Morphs of several colors exist, and the species is bioluminescent. RAPD markers were used to assess the genetic structuring at a very small scale (a 90×90 cm square consisting of 3×3 adjacent quadrates of 30 cm side length each). Very strong genetic differentiation was found between quadrates, it was not correlated to the distance between quadrates. An equally strong differentiation was observed between color morphs (pooling individuals of the 9 quadrates). To check if the observed differentiation between quadrates was due to genetic differentiation between color morphs, associated with heterogeneous distribution of “colors” among quadrates, we tested for differentiation between quadrates, using individuals of two morphs taken one by one, namely the “green” morph, and the “gray” morph. There was still a strong differentiation between quadrates. No relationships can be deduced between genetic entities and color morphs.

1 INTRODUCTION

In spite of the absence of a larval phase, the simultaneous hermaphrodite (Nisolle 1990), brood protecting and bioluminescent ophiuroid *Amphipholis squamata* (Delle Chiaje 1828) is regarded as a cosmopolitan species. It constitutes dense local populations often occupying (cryptic) microhabitats. There is a paradox given an extremely wide distribution of a species that lacks a larval stage and often shows an aggregative spatial distribution. *A. squamata* presents a great anatomical homogeneity throughout the world (Hyman 1955). As early as 1965, Tortonese raised the question of the authenticity of such a pandemic species, which shows a great variability of colours and bioluminescent capabilities (Binaux & Boquet 1971, Mallefét et al. 1992, Deheyn et al. 1997, Deheyn & Jangoux 1999, Dupont et al. 2000). Using molecular markers (PCR-RAPD), Poulin et al. (1999) demonstrated the co-existence of selfing and outcrossing in this species. These authors also revealed a high genetic differentiation of *A. squamata* from the Western Mediterranean over a very short distance (1 km in Medes Islands, Catalanian Sea). Deheyn et al. (1997, in local populations of Normandy) and Féral et al. (2001, in very close local populations of Sicily isolated in neighbouring lagoons) showed a link between polychromatism and

bioluminescence. Each recognized color variety possesses its own luminous capabilities. For the latter, a link between polychromatism and genetics within local populations was established, but no clear genetic differences were demonstrated between color variants in the different lagoons. The question that arose was whether color morphs corresponded to different species. Chauffer (1999) and Dupont et al. (2000) have shown that even within a given morphotype, very distant local populations (Channel, Mediterranean, Pacific) may be highly differentiated and may correspond to different species.

At a different scale, Sponer et al. (1999) showed that very distant (1000s km) populations are genetically highly differentiated and also hypothesized that different 16S mtDNA clades likely represent different species. In a more recent paper, Sponer et al. (2001) observed that at a smaller geographic scale, color variability was not congruent with genetic variability. These authors considered that the different morphotypes were not corresponding to different species.

A. squamata has been assigned to approximately 25 different species since its discovery. Deheyn et al. (2000) and Féral et al. (2001) hypothesized, respectively, on bioluminescence data and on genetic data, that *A. squamata* might not represent a single taxon. Despite its status as “common species”, new

investigations are necessary to determine the status of this taxon and its genetic structure. The aim of this pilot-study is to determine the minimum distance of genetic differentiation.

2 MATERIAL AND METHODS

Mediterranean specimens of *Amphipholis squamata* were collected by means of SCUBA diving at the Île Grosse (Bay of Banyuls, France). *A. squamata* was found associated with the calcareous alga *Corallina officinalis*, between 2 and 4 meters of depth. Figure 1 describes the fine-grained sampling method used: a 90 cm square consisting of 3 × 3 adjacent quadrates of 30 cm side length each.

Total genomic DNA extractions were done with the CTAB protocol (Winnepenninckx et al. 1993).

Random Amplified Polymorphic DNAs (RAPD) provide a rapid detection of genomic polymorphism (Williams et al. 1990) using a single short oligonucleotide primer of arbitrary sequence in a Polymerase Chain Reaction (PCR). PCR was carried out to generate a reproducible array of strain-specific products that were subsequently analyzed by gel electrophoresis [*Ready To Go* kit (Pharmacia Biotech) using 2 different decamer primers: primer “2” (5′-d[GTTCGCTCC]-3′) and primer “6” (5′-d[CCCGTCAGCA]-3′)].

DNA profiles were analysed using Scion Image software v. beta 4.0.2 (2000). Cluster analysis of RAPD-PCR patterns in individuals were performed with RAPDPLOT v. 3.0. program (Black 1995), using Nei and Li’s (1985) genetic similarity index [$S = 2NAB/(NA + NB)$ where NAB is the number of bands that individuals A and B share and NA is the number of bands in individual A and NB the number of bands in individual B], developed for RAPD markers.

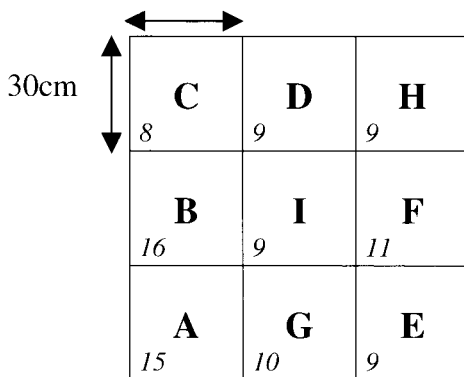


Figure 1. Diagram of the sample area. Ophiuroids were sampled from a 90 cm square consisting of 3 × 3 adjacent quadrates of 30 × 30 cm. The digit in italics at the bottom left corner of each square is the number of analyzed specimens.

Cluster analysis was done using the Neighbour-Joining method [NEIGHBOR program in PHYLIP 3.5c package (Felsenstein 1993)]. A single unrooted tree was generated and plotted using DRAWGRAM in the same package.

Genetic differentiation was estimated by the analysis of RAPD-PCR assuming that fragments that co-migrate arise from identical alleles [Apostol et al. 1996, using the RAPDFST software (Black 1995)]. F_{ST} index and its significance were assessed by comparison to a distribution generated by 1000 permutations. A Mantel test was performed (GENETIX v. 4.02, Belkhir et al. 2001) to compare genetic and spatial distances using a “geographical” distance matrix made after the calculation of distance between the barycentre of every small square (A to I).

3 RESULTS

3.1 Genetic differentiation and spatial distribution

Primer “2” and “6” gave 81 DNA polymorphic (present or absent) fragments usable to analyze the genetic structure of the 96 individuals of *Amphipholis squamata*. Individuals are distributed in each elementary square sampled according to Figure 1. None individual had the same genotype.

Figure 2 displays genetic relationships among individuals using RAPD-PCR markers after the neighbour-joining tree construction. We noticed a patchy distribution of the individuals among the clusters. Indeed, they are not randomly distributed. Some clusters reflect the observed spatial arrangement and neighbourhood of individuals in the sampled squares. For instance, 7 out of 10 individuals from quadrate G are in the same sub-cluster, the sister group of which is made of 7 out of 16 individuals of square B. These 2 sub-clusters are themselves clustering with 7 out of 9 individuals of quadrate I and 2 other individuals of quadrate B. Other strong connections are observed for individuals of both quadrates A and C that were not directly in contact when sampled. On the other hand, individuals of different quadrates may also be found in any cluster.

In order to test the observed genetic structure, the frequency distribution of F_{ST} values of RAPD loci was calculated. Results are shown in Figure 3. The differentiation is very high ($F_{ST} > 0.250$, $P = 0.000$) or high ($0.250 \geq F_{ST} \geq 0.150$, $0.003 \geq P \geq 0.000$) for 34 loci (41% of loci), moderate ($0.150 \geq F_{ST} \geq 0.005$, $0.792 \geq P \geq 0.000$) for 47 loci (59%).

All RAPD multiloci F_{ST} by pair, considering quadrates while pooling colours (or the reverse) are significant ($P = 0.000$).

The Mantel test was not significant, indicating that the very high genetic differentiation observed does not

seem correlated with distance between individuals, at the scale of the present study (about 1 m²).

3.2 Color morphs and genetic differentiation

Analyzed individuals were classified in 5 main color categories: greenish, yellowish, gray or flecked gray,

orange and bordeaux. Genetic differentiation between color morphs was investigated, computing the significance levels of the F_{ST} between morphs, pooling individuals of the 9 quadrates. At this spatial scale, genetic structuring, as revealed by means of RAPD loci, does not seem to be strongly related to color morphs. F_{ST} values are highly significant only at 13 loci ($0.006 \geq P \geq 0.000$) (16% of loci). No structuring is demonstrated for 68 loci (87% of loci). We also checked if genetic differentiation and color morphs between quadrates was still observed within color morphs for the 2 most abundant ones: the greenish ($N = 37$) and the gray ($N = 34$) morphs. In both cases, genetic structuring appears to be strongly related to spatial distribution of the individuals (Figure 4), although sample sizes were reduced for these analyses within color types (relative to the analyses pooling all morphs).

Among the greenish individuals, the differentiation is very high ($F_{ST} > 0.250$, $0.010 \geq P \geq 0.000$) for 30.5% of loci (24/79), or high ($0.250 \geq F_{ST} \geq 0.150$, $0.233 \geq P \geq 0.029$ for 28 loci (35.5% of loci). For 34% of loci (27/79), it is moderate ($0.150 \geq F_{ST} \geq 0.005$, $0.960 \geq P \geq 0.001$). A similar observation is made for the gray + flecked gray groups.

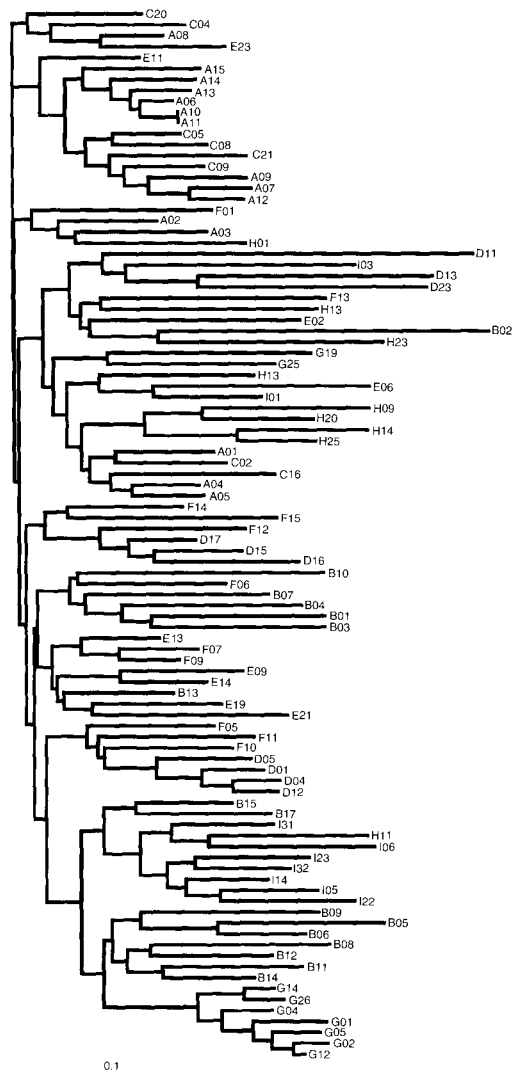
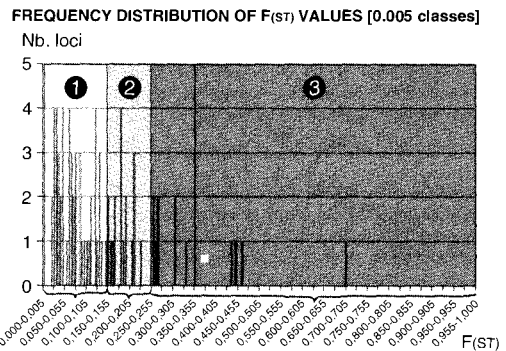


Figure 2. Neighbor-joining tree inferred from a distance matrix calculated after Nei and Li (1985). Letters are the names of the sampled squares (cf. Figure 1). Individuals are not always randomly distributed. Some clusters reflect the observed spatial arrangement and neighborhood of individuals in the quadrates.



DIVERSIFICATION [F_{ST}] OF RAPDLOC

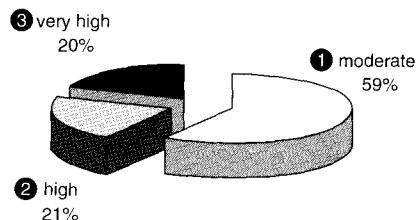


Figure 3. Frequency histogram of F_{ST} values, between quadrates, for each RAPD locus. The sector graph below shows that a highly significant genetic differentiation exists at 41% of the studied RAPD loci (2 & 3), and a moderate one at 59% of these loci (1).

4 DISCUSSION AND CONCLUSION

F_{ST} analysis from dominant marker are made assuming Hardy-Weinberg equilibrium within population, and also assuming equilibrium between mutation and drift. Although this hypothesis is probably violated in this partially selfing species, there is no reason to expect a systematic overestimation of F_{ST} values and significance levels. RAPD revealed an important geographical structuring of *Amphipholis squamata* in a very small area, with a strong differentiation on an extremely tiny scale (less than one meter), and between color morphs. The fact that F_{ST} values between colors, pooling quadrates, are globally less significant than F_{ST} between quadrates, pooling colors, and that strong F_{ST} are still observed for a given color between quadrates strongly suggests that a spatially heterogeneous distribution of color morphs is not the cause of the strong genetic differentiation at such a small spatial scale. It is difficult to separate the respective roles of the absence of dispersal [no larval phase, but proven possibility of “rafting” of juveniles and adults (Highsmith 1985, Alvà & Vadon 1989, Murray 1989)] and of selfing, in generating this differentiation, which is revealed at all spatial scales. But it is very likely that at a very small scale, selfing and discrete events have a higher influence as suggested by the lack of correlation between genetic distance and spatial distance. At this scale the movement of individuals as they seek appropriate habitat away from the parent is important. The data also show that a quadrate is not necessarily occupied by a (unique) “family”.

It is therefore important to use co-dominant markers to estimate the selfing rate in the natural populations, for instance starting from the calculation of heterozygote deficiency. Such an approach must be accompanied by an estimate of the correlation between geographical and genetic distances, to evaluate the scale at which this possible correlation no longer exists. Only within this scale, one will be able to apply the simple relation, which makes it possible to estimate the selfing rate, starting from the F_{IS} estimate. Sample sizes were too small to draw a definitive conclusion about the link between coloration and genetic structure. Very likely, different taxa coexist on a small surface (as also revealed by means of mtDNA, Le Gac 2002), however, it does not seem that morphotypes defined by color are directly related to them. Crossing experiments should be performed.

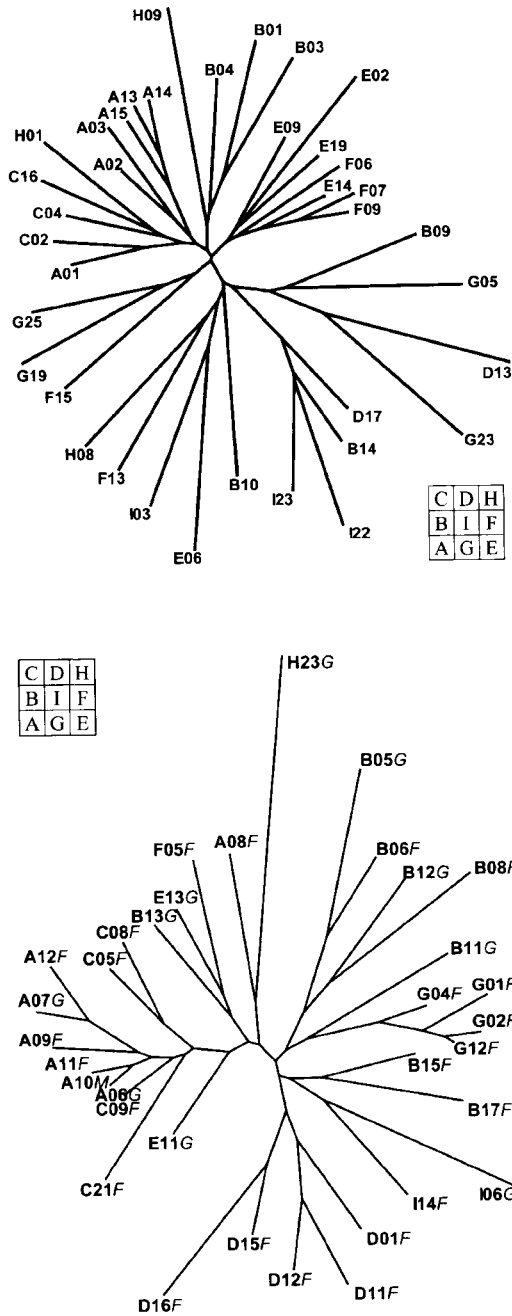


Figure 4. Unrooted trees inferred from distance matrix of Nei and Li (1985) calculated from the RAPD patterns (79 DNA fragments) of 37 greenish individuals [top] and of 34 gray (G) and flecked gray (F) individuals [bottom]. Genetic structuring seems to be strongly related to spatial distribution of the individuals. The squares represent the sampled area.

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