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Mosaïcism in the European zone of sympatry and hybridization of *Mytilus edulis* and *M. galloprovincialis*, as revealed by intron length polymorphism at locus *mac-1*

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Intron-size variation at the actin gene locus *mac-1* was used to characterise mussel, *Mytilus* spp., populations in the ~2000-km wide zone of contact and hybridization ('hybrid zone') between *M. edulis* and *M. galloprovincialis* in western Europe. Twenty-five samples were collected in 1995-1999 in locations within the hybrid zone and from reference populations of each species. We used correspondence analysis on the matrix of allelic frequencies to determine which alleles are characteristic of each species, and to characterise samples along the genetic gradient between *M. edulis* and *M. galloprovincialis*. In the hybrid zone, some samples exhibited *mac-1* allele frequencies that were typical of *M. edulis*; other samples were distributed along the *M. edulis* / *M. galloprovincialis* gradient and displayed variable levels of intergradation that were not correlated with geography. Some of the latter samples exhibited significant heterozygote deficiencies. The simple admixture hypothesis (Wahlund effect) could not be rejected for two-fifths of the samples. The hybrid zone thus appeared as a mosaic of populations which are either pure *M. edulis*, or hybrid between *M. galloprovincialis* and *M. edulis*, or a mixture of the foregoing with *M. galloprovincialis* individuals. These results were consistent with published allozyme data, suggesting that they can be extended to the entire nuclear genome. *M. edulis mac-1* alleles were present at moderate frequency in Atlantic *M. galloprovincialis*, and at significantly lower frequency in some Mediterranean samples. This pattern was homogenous over a broad geographic range within each basin. It was not evident that introgression of *M. edulis* into *M. galloprovincialis* presently occurs south of the zone of contact. We propose that the distinctness of the Atlantic *M. galloprovincialis* population results from past introgression by *M. edulis* alleles.

Keywords: *Mytilus edulis*, *Mytilus galloprovincialis*, hybrid zone, intron length polymorphism, heterozygote deficiency, introgression.

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Introduction

Hybrid zones offer opportunities to study the genetics of differentiation and speciation because they allow divergent genomes to meet and interact, informing us about the endogenous and exogenous factors that are involved in maintaining the genetic integrity of a species (Barton & Hewitt, 1989; Harrison, 1993; Arnold, 1997). In marine organisms, these processes are conditioned by peculiar life history traits, such as huge fecundity and dispersing larval stage, which provide potential for high selective mortality, high parent-to-offspring distance, and extensive gene flow.

The best documented cases of hybridization in the marine environment are probably those among pairs of species within the *Mytilus edulis* species complex (*M. galloprovincialis* x *M. edulis*: Skibinski *et al.*, 1983; Coustau *et al.*, 1991; Gardner, 1994; *M. galloprovincialis* x *M. trossulus*: McDonald & Koehn, 1988; Inoue *et al.*, 1997; Rawson *et al.*, 1999; *M. edulis* x *M. trossulus*: Väinölä & Hvilsom, 1991; Comesaña *et al.*, 1999). A number of allozyme surveys have been conducted on the zone of sympatry / hybridization (referred to as 'hybrid zone' throughout this article) between *M. edulis* and *M. galloprovincialis*, which extends from the coasts of Scotland to the Basque Country (Seed, 1992; Gardner, 1994). Abrupt changes in allelic frequencies at allozyme loci that distinguish *M. edulis* from *M. galloprovincialis* have been reported between adjacent localities, as for instance in South West Britain (Skibinski & Beardmore, 1979; Skibinski *et al.*, 1983). These are thought to reflect geographical variation in the opportunities for interbreeding, enhanced by geographically variable levels of selection on recruits (Skibinski & Beardmore, 1979; Skibinski *et al.*, 1983; Gardner, 1994). The distribution of multiple-locus genotypes suggests widespread though incomplete and geographically inconsistent intergradation (Skibinski & Beardmore, 1979). Similar observations have been reported for populations along the French Atlantic coast (Coustau *et al.*, 1991; Comesaña & Sanjuan, 1997), where samples within the hybrid zone displayed allelic frequencies at the two most discriminative loci (*EST-D* and *MPI*) that were intermediate between those of *M. edulis* and *M. galloprovincialis* reference populations, while other samples were genetically similar to *M. edulis*. At other loci (*ODH*, *GPI*), certain alleles appeared in higher frequency in hybrid zone samples as compared to both reference populations. Allozyme data thus depict the European zone of sympatry of *M. edulis* with *M. galloprovincialis* as a geographic mosaic of populations where a site harbours either a pure population of either species, or a hybrid population, or any mixture of the foregoing. *M. edulis* tends to occupy more sheltered habitats than *M. galloprovincialis* (Skibinski *et al.*, 1983).

Outside the hybrid zone, allelic frequencies at loci *AP*, *GPI*, and *ODH* differ between NE Atlantic and Mediterranean *Mytilus galloprovincialis* (Coustau *et al.*, 1991; Sanjuan *et al.*, 1994; Quesada *et al.*, 1995c). An abrupt genetic transition between NE Atlantic and Mediterranean *M. galloprovincialis* is evident at allozyme loci (Quesada *et al.*, 1995c), and for mitochondrial DNAs (Quesada *et al.*, 1995a; Sanjuan *et al.*, 1996). This indicates that NE Atlantic and Mediterranean populations of *M. galloprovincialis* are geographically separated, and that the present hybridization with *M. edulis* exclusively concerns the NE Atlantic form of *M. galloprovincialis*.

Mitochondrial (mt) DNA surveys have shown the introgression of *Mytilus edulis*-like mtDNA into *M. galloprovincialis* in southwest Britain, south of the hybrid zone in the northeastern Atlantic, and even in the Mediterranean (Edwards & Skibinski, 1987; Quesada *et al.*, 1995b; 1998; Rawson & Hilbish, 1995; 1998; Hilbish *et al.*, 2000). MtDNA typical of Mediterranean *M. galloprovincialis* was generally absent in Atlantic *M. galloprovincialis*, being detected in only a putative hybrid population in South-West Britain (Rawson & Hilbish, 1998; Hilbish *et al.*, 2000). Quesada *et al.* (1998) provided similar results: both phylogenetic trees of maternal and paternal mtDNA haplotypes showed two distinct clusters of haplotypes, one dominating Mediterranean *M. galloprovincialis* and occasionally being present in Atlantic *M. galloprovincialis*, and the other containing all haplotypes found exclusively in *M. edulis*, together with haplotypes found in both Atlantic and Mediterranean forms of *M.*

galloprovincialis. This apparently fits the distinction between type *D* and type *A* haplotypes, respectively (Rawson & Hilbish, 1995; Hilbish *et al.*, 2000). That most Atlantic, and a proportion of Mediterranean, *M. galloprovincialis* carry haplotypes of the *A* haplogroup suggests they have been introgressed by *M. edulis*-like mtDNAs. The *A* haplotypes found in *M. galloprovincialis* being however absent from *M. edulis*, which harbours distinct *A* haplotypes (Rawson & Hilbish, 1998), suggests that this introgression is ancient and since then no mtDNA gene flow has been occuring between the two species.

Nuclear-DNA markers are now available to study the genetics of *Mytilus* populations (Côrte-Real *et al.*, 1994; Inoue *et al.*, 1995; 1997; Beynon & Skibinski, 1996; Rawson *et al.*, 1996; Ohresser *et al.*, 1997; Heath & Hilbish, 1998). Studies intending to compare nuclear-DNA with allozyme variation in marine species are interesting because of useful inferences that may arise from contrasting patterns in geographic variation (Karl & Avise, 1992 - but see Hare *et al.*, 1996; Pogson *et al.*, 1995; Lemaire *et al.*, 2000). Introns in particular are highly polymorphic parts of the nuclear genome; these markers are generally presumed to be neutral since they are non-coding. Also, linkage disequilibrium effects are expected to be extremely weak in outbreeding species with very large population sizes such as marine mussels. In contrast, selection has been documented at some allozyme loci in *Mytilus* spp. populations (Mitton & Koehn, 1973; Diehl & Koehn, 1985; Koehn & Hilbish, 1987) and in other bivalves (e.g. Koehn *et al.*, 1988; Gaffney 1994).

The first intron of the *mac-1* actin gene (Ohresser *et al.*, 1997) exhibits considerable variability in length with 23 alleles detected so far in European *Mytilus edulis* and *M. galloprovincialis* (Daguin & Borsa, 1999; 2000). This marker enables the distinction between populations of the two species *M. edulis* and *M. galloprovincialis* (Daguin & Borsa, 1999). *mac-1* therefore appears to be as discriminating as the two allozyme loci (namely, *EST-D* and *MPI*) that best distinguish *M. galloprovincialis* from *M. edulis* (Coustau *et al.*, 1991; McDonald *et al.*, 1991). Moreover, *mac-1* data confirmed that *M. galloprovincialis* populations on either side of the Alboran Sea are genetically distinct (Daguin & Borsa, 1999). The level of variability at locus *mac-1* at once allows it to be useful for the study of genetic variation within each species, while being virtually diagnostic between species. The diagnostic allozyme and other nuclear-DNA loci used so far in *Mytilus* spp. surveys do not have this advantage.

The objective of the present study was to document the geographic and genetic structure of the European hybrid zone using presumably neutral, nuclear-DNA markers. For this, we characterised at locus *mac-1* mussel populations sampled along the European Atlantic coasts, and we compared their allelic frequencies to those of reference populations sampled outside the zone of sympatry, using correspondence analysis. The distribution of heterozygous individuals in samples whose allelic frequencies were intermediate between those of *Mytilus edulis* and Atlantic *M. gallo-provincialis* yielded information about the extent of hybridization *versus* simple admixture. The allele frequency patterns outside the hybrid zone were compared to those expected under scenarios of present or ancient introgression of *M. edulis* into *M. galloprovincialis*.

Materials and methods

Collection of samples

Mytilus spp. samples were collected in 1995-99 in 25 sites along the European and Moroccan Atlantic shores and in the Mediterranean sea (Fig. 1; sample sizes in Table 1). Twelve of these samples were from localities within the hybrid zone of *M. edulis* and *M. galloprovincialis*, as this has been geographically delimited on the basis of allozyme data (Skibinski & Beardmore, 1979; Coustau *et al.*, 1991). Allozyme frequency data have been reported in the literature for samples from some of these localities [POL (Polzeath, Cornwall): sample Polzeath of Beaumont *et al.*, 1989; PVA (Pléneuf-Val-André, Britanny), FOU (La Forêt-Fouesnant, Britanny), NOI (Noir-moutier, Vendée), BTZ (Biarritz, Basque Country): respectively, samples 3, 5, 7 and 9 of Coustau *et al.*, 1991]. In order to assess the degree of genetic intergradation in the populations in the zone of sympatry, it was necessary to analyse reference samples for each species. Thus, samples FLØ

(Flødevigen, Skagerrak), GIL (Gilleleje, Kattegat) and GFP (Grand-Fort-Philippe, North Sea) were chosen as reference *M. edulis* because of their geographic location and according to allozyme data on samples collected nearby (Väinölä & Hvilsom, 1990; Coustau *et al.*, 1991). Samples STB (Setubal, Portugal), FAR (Faro, Portugal) and TEM (Temara, Atlantic coast of Morocco) were similarly chosen as reference *M. galloprovincialis* (see Sanjuan *et al.*, 1994; Quesada *et al.*, 1995c; Comesaña *et al.*, 1998). Although reference *M. gallo-provincialis* are usually sampled in the Mediterranean (e.g. Varvio *et al.*, 1988; Coustau *et al.*, 1991; Rawson *et al.*, 1996), and the type locality for *M. galloprovincialis* is the coast of Roussillon in the Western Mediterranean (Lamarck, 1819), we here used samples from Atlantic (instead of Mediterranean) populations of *M. galloprovincialis* as the reference for this species because of their genetic distinctness (see Introduction). Note that the two types from Linnaeus' collection of *M. edulis* that Bucquoy *et al.* (1898) chose as representative of the species were those collected in Esnandes (Baie de l'Aiguillon, Charente, France) and on the North Sea coast of England.

We here extended the geographical survey of genetic variation in European *Mytilus gallo-provincialis* by analysing samples from the Western Mediterranean [Cartagena, Murcia, Spain (CAR); Banyuls, Roussillon, France (BAN); Sète, Languedoc, France (SET); Ensuès-La Redonne, Provence, France (RED)], the Adriatic Sea [Chioggia, Italy (CHI); Butrintit, Albania (BUT)] and the Black Sea (Agigea, Romania: AGI) (Fig. 1). Allozyme surveys up to now have not detected significant geographic variation among *M. galloprovincialis* populations from the Western Mediterranean, the Adriatic Sea, and the Black Sea (Skibinski *et al.*, 1983; Varvio *et al.*, 1988; Coustau *et al.*, 1991; McDonald *et al.*, 1991; Väinölä & Hvilsom, 1991; Sanjuan *et al.*, 1994; Quesada *et al.*, 1995c). Note that the geographic location of the CAR sample is between Almería and Alicante, a region where no mussel had been found up to now (Quesada *et al.*, 1995c; Sanjuan *et al.*, 1997).

After their collection, the mussels were preserved either alive on ice (samples BOY, BRO, BTZ, SET; Fig. 1) or frozen (POL, AIG, RLB, BUT; Fig. 1) or in alcohol (all the other samples) and sent to our laboratory for analysis.

DNA extraction and amplification by polymerase chain reaction (PCR)

DNAs were first extracted using the Chelex protocol (Walsh *et al.*, 1991), where 0.5-2 mg adductor muscle tissue dissected from each individual was digested at 55°C overnight in a microfuge tube containing 6% Chelex resin (Biorad, Richmond CA, USA) and 12 U proteinase K in 350 μL 0.1 mM Tris-HCl / 0.01 mM EDTA, pH 8.0 buffer. Samples consistently failing to PCR-amplify using Chelex-extracted DNA templates were subsequently subjected to phenol-chloroform DNA extraction, where 3-5 mg of dried adductor muscle tissue was incubated at 55°C overnight in a 50 mM Tris-HCl, 25 mM EDTA, 1% SDS, pH 8.0 buffer containing 18 U proteinase K. This was followed by a two-round phenol-chloroform procedure. The nucleic acid pellet was then resuspended in 200 μL deionised water and frozen at -20 °C until genotyping.

The DNA extracts were used as templates for PCR amplification of a portion of Intron 1 of the actin gene *mac-1* (Ohresser *et al.*, 1997) according to protocols detailed in Daguin & Borsa (1999).

Analysis of data

Correspondence analysis (CA; Benzécri, 1982) was performed using the AFC procedure implemented in BIOMECO (Lebreton *et al.*, 1990) on the matrix of allelic frequencies per sample. This method has been previously used in *Mytilus* by Coustau *et al.* (1991) on multiple-locus allozyme data. The beauty of CA is that it simultaneously expresses the genetic differences present in the data set and sorts out the contributions of each allele to these differences. Guinand (1996) showed that the eigenvalues of each CA's axis are analog to partial *Fst*, Wright's coefficient of genetic differentiation between populations. We thus investigated the genetic relationships among samples within the zone of sympatry, and of the latter to reference *M. edulis* and *M. galloprovincialis*. Mediterranean *M. galloprovincialis* samples were placed as

supplementary variables in the CA to assess their relationships to Atlantic *M. galloprovincialis*. All *mac-1* alleles were taken into account in the analysis. [Coustau *et al.* (1991) pointed out that the occurrence of rare alleles (whose frequency in the total sample was < 0.01) caused distortion on the factorial co-ordinates of samples and alleles. We addressed this potential problem by considering rare *mac-1* alleles as supplementary variables in the CA, and comparing it with the CA where all *mac-1* alleles were active variables. Only negligible differences were noted leading us not to address the problem further.]

The occurrence of samples with allele frequencies intermediate between those of each species' reference samples reflects either intergradation or admixture. Intergradation, resulting from interbreeding, would correspond to Hardy-Weinberg genotype frequencies whereas admixture, defined as the simultaneous occurrence in a sample of individuals of either parental species, would translate into heterozygote deficiency (the Wahlund effect). Admixture may result from the recruitment at a site of swarms of larvae of the two species. Genotype frequency analysis may help to determine which one of these two models is the most appropriate. Correlations of alleles within individuals relative to the population were estimated using Weir & Cockerham's (1984) estimator of *f*, the multiple-allele equivalent of Wright's *Fis*. Random permutations of alleles in the matrix of individuals x genotypes were used to estimate the expected distribution of $\hat{f}$ under the null hypothesis $\hat{f} = 0$. Estimations of *f* and permutation tests were done using the GENETIX 3.3 software (Belkhir *et al.*, 1996).

In order to simulate a Wahlund effect, we constructed two pools of individuals: a *Mytilus edulis* pool, including all individuals from the samples classified as *M. edulis* according to the CA (i.e. FLØ, GIL, GFP, PVA, AIG, BOY, BRO, but not NOI nor RLB) and an *M. galloprovincialis* pool including all individuals from the Atlantic *M. galloprovincialis* samples (BTZ, STB, FAR, and TEM). Then, we generated 10 replicates each of 11 pseudo-samples ($N = 40$) corresponding to 11 proportions of randomly chosen individuals from either pool (from 0% to 100% *M. galloprovincialis* by 10% increment). Weir & Cockerham's *f* was estimated for each replicate and was plotted against the coordinate along axis 1 of the CA for each pseudo-sample (i.e. after averaging allelic frequencies over the ten replicates). A 2nd-order polynomial regression on these points was used as the expected *f* function.

Results

Thirty one size alleles (ranging from *ca.* 220 bp to *ca.* 420 bp) were detected in the total sample. Allelic frequencies per sample are presented in Table 1. While 15 of the alleles were represented in only one or two samples, 11 alleles were present in more than half of the samples. High levels of polymorphism were evidenced in every population sampled as indicated by the range of gene diversity estimates (Table 1).

CA was conducted to explore the variation in allelic frequencies across samples, and to eventually link genetic differences to geography. Figs. 2A and 2B separately present the projections of samples and alleles on the plane defined by the two first factorial axes of the CA. More than 55% of the inertia of the data matrix (this can be considered as an analogue of the total variance in allelic frequency; Benzecri, 1982) was represented by the first axis of CA. All samples except two (NOI and RLB) were positioned along Axis 1, from a *Mytilus edulis* pole (extreme left of Axis 1) to a Mediterranean *M. galloprovincialis* pole (extreme right). Axis 1 thus essentially reflects the allele frequency gradient between the two species. All Atlantic *M. galloprovincialis* samples (STB, FAR, TEM) formed a cluster adjacent to the Mediterranean cluster of samples (BAN, SET, RED, CHI, BUT, AGI). This shows that Atlantic *M. galloprovincialis* are genetically homogeneous but slightly different from Mediterranean *M. galloprovincialis* as formerly reported (Daguin & Borsa, 1999). The estimate of θ (Weir & Cockerham, 1984) between these two groups of samples was $\hat{\theta} = 0.018$ ($P < 0.001$; 2000 random permutations; Belkhir *et al.*, 1996). Sample CAR clustered with the Mediterranean samples as expected from its geographic location, that is, east of the Almería-Oran

oceanographic front (see Quesada *et al.*, 1995c). Among the 12 populations sampled in the hybrid zone, one (BTZ) clustered with the three Atlantic *M. galloprovincialis* reference samples, four (AIG, BRO, BOY, PVA) clustered with the three *M. edulis* reference samples, five (YEU, PAL, POL, FOU, PEN) appeared as genetically intermediate between *M. edulis* and *M. galloprovincialis*, and two (RLB and NOI), recognised as *M. edulis* by their co-ordinates along Axis 1, appeared as distinct along Axis 2. As the sizes of the latter two samples were also the lower, we reiterated the CA on allelic counts instead of allelic frequencies, thereby weighting samples by their size. This did not appreciably affect the positions of samples RLB and NOI relative to the other *M. edulis* samples (data not shown). The originality of these samples is attributable to the higher frequencies of alleles *a*15 and *a*6 as indicated by the contributions of the latter to Axis 2 (Table 2). Allele *a*15 was also present in three (POL, FOU, PEN) of the 5 genetically intermediate samples and in PVA, another *M. edulis* sample from the hybrid zone, but was noticeably absent elsewhere (Table 1).

According to the ranking of samples along Axis 1, the hybrid zone did not appear as a clinal North-to-South transition zone between *Mytilus edulis* and *M. galloprovincialis* but instead as a patchwork of populations.

The positions of alleles on the first CA plane (Fig. 2B) and their relative contributions to Axis 1 (Table 2) indicate which alleles were responsible for the distinction between *Mytilus edulis* and *M. galloprovincialis*. The 31 alleles of the total sample were distributed as two distinct clusters along the first factorial axis, from alleles characteristic of *M. galloprovincialis* (*b*1, *c*1, *c*2 and *a*8 for the most frequent and contributing alleles; see Table 2) to alleles characteristic of *M. edulis* (*a*2, *a*3 and *a*5). This led us to consider that all alleles with positive (respectively, negative) co-ordinates along Axis 1 were characteristic of *M. galloprovincialis* (respectively, *M. edulis*).

Whether the occurrence of five genetically intermediate populations (YEU, PAL, POL, FOU, PEN) reflected intergradation or admixture can be gauged from the distribution of genotype frequencies. Table 1 gives the estimate of *f* for each sample: all five genetically intermediate samples exhibited significant, positive ^*f* values indicating heterozygote deficiencies relative to Hardy-Weinberg expectations; the other significant ^*f* values, all positive, were for samples FLØ, GFP (that is, two of the *Mytilus edulis* reference samples), and BAN. The fact that heterozygote deficiency was already present in reference populations points to the possible existence of null alleles, making the interpretation of the positive ^*f*-values in the intermediate populations uncertain. To test the hypothesis of admixture of NE Atlantic *M. edulis* and *M. galloprovincialis*, we therefore chose to compare the observed ^*f*-values to the pseudo-distribution of ^*f* in a range of pseudo-samples generated by choosing variable proportions of individuals randomly re-sampled within either the pool of *M. edulis* samples (FLØ + GIL + GFP + PVA + AIG + BOY + BRO) or the pool of NE Atlantic *M. galloprovincialis* samples (BTZ + STB + FAR + TEM). Figure 3 shows the expectation of ^*f* for each admixture proportion, plotted against the first factorial co-ordinate of CA. The admixture hypothesis could not be rejected for any sample, except, perhaps, sample POL.

To remove the effect of null alleles --if present-- in *Mytilus edulis* reference populations, alleles characteristic of each species were pooled into compound alleles *E* (for *M. edulis*) and *G* (for *M. galloprovincialis*) and the distribution of genotypes in each sample (Fig. 1) was re-analysed by recalculating ^*f*. Allele-grouping ended up in suppressing heterozygote deficit in *M. edulis* reference samples since these consisted of *EE* and *EG* individuals only (Fig. 1). Here the null hypothesis (H_0) of Hardy-Weinberg equilibrium at a bi-allelic (*E*, *G*) locus was tested, which is equivalent to testing panmixia between *M. edulis* and *M. galloprovincialis*. ^*f*-values remained high for samples FOU (^*f* = 0.263; $p < 0.03$; one-tailed test) and PEN (^*f* = 0.279; $p < 0.05$) and dropped for samples YEU (^*f* = 0.014), POL (^*f* = 0.039) and PAL (^*f* = -0.110). Thus H_0 could be rejected only for samples FOU and PEN. The heterozygote deficiency initially found in sample YEU therefore may have been caused by null alleles. Altogether the above results suggest

that only two samples (FOU, PEN) consist more of some mixture of *M. edulis* and *M. galloprovincialis* genotypes rather than of a hybrid population.

Figure 1 reports the genotypic (*EE*, *EG*, *GG*) composition of these and the other samples. Two reference samples for *Mytilus edulis* (FLØ and GIL) and two Mediterranean *M. galloprovincialis* samples (CHI and AGI) consisted of 100% *EE* and *GG* genotypes, respectively. Atlantic *M. galloprovincialis* samples contained *EG* individuals at a frequency that was geographically homogenous; they possessed *M. edulis* alleles (*a*1, *a*2, *a*3, *a*4, *a*5, *a*6, *b*3, and *c*4) at a total frequency of 0.08 - 0.11. This was at contrast with Mediterranean *M. galloprovincialis*, in which the frequencies of *M. edulis* alleles were marginal if present (Fig. 1; Table 2). Overall, four zones could be distinguished along the European coastline (Fig. 1). (1) A pure *Mytilus edulis* region, which extends from the eastern end of the English Channel to Skagerrak and Kattegat; (2) The zone of sympatry between *M. edulis* and *M. galloprovincialis*, which includes samples from South-West Britain, Britanny, and Charente; this zone consists of a mosaic of populations that include pure *M. edulis* at some localities, putative *M. edulis* x *M. galloprovincialis* hybrid populations at some other localities, the remainder consisting of mixtures of *M. edulis*, *M. galloprovincialis* and their hybrids; (3) A geographically homogeneous Atlantic *M. galloprovincialis* region, which extends from the Basque coastline to northwestern Africa, and where alleles characteristic of *M. edulis* have a frequency comprised between 0.08 and 0.11; (4) The Mediterranean Sea, which harbours a distinct *M. galloprovincialis* form, where *mac-1* alleles characteristic of *M. edulis* have a total frequency ≤ 0.06.

Discussion

Genotypic composition of hybrid zone samples

The present study is the first that uses non-coding nuclear-DNA markers for describing the geographic structure of the hybrid zone of *Mytilus edulis* and *M. galloprovincialis*. Intron-length polymorphism at locus *mac-1* shows a mosaic structure: six out of the 11 samples from the hybrid zone were *M. edulis*-like and the 5 other samples were distributed along the genetic uni-dimensional gradient between *M. edulis* and *M. galloprovincialis*, without congruence with geography.

The genetically intermediate samples found in the zone of sympatry all exhibited significant heterozygote deficiencies. For two of these (FOU and PEN), the heterozygote deficiency reflected some degree of admixture of individuals of each species. Other explanations are needed to account for the heterozygote deficiency in the other genetically intermediate samples of the hybrid zone, as in some samples outside of it (FLØ, GFP, BAN). The null allele hypothesis where apparent excesses of homozygotes would be due to mis-priming was tested in *Mytilus edulis* (FLØ), *M. galloprovincialis* (SET), and in a hybrid zone population (POL) by comparing the individual genotypes with two different primer pairs (Daguin & Borsa, 1999; unpublished). There was no evidence for this type of null alleles, but one cannot exclude the presence of undetected large deletions encompassing both primer sites at either side of the target portion of template DNA. The heterozygote deficiencies may have other causes (e.g. aneuploidy, selection; see Gaffney, 1994 and Raymond *et al.*, 1997, for in-depth discussions of this phenomenon). Since there is evidence of correlation between diagnostic allele frequencies and age elsewhere in the hybrid zone (e. g. Gardner & Skibinski, 1988), the deviations from Hardy-Weinberg proportions may also depend on the age distribution of a sample.

Comparison with allozyme data

The comparison between allozyme (Skibinski *et al.*, 1983; Coustau *et al.*, 1991) and *mac-1* data in the European hybrid zone between *Mytilus edulis* and *M. galloprovincialis* consistently points out a mosaic pattern in the distribution of populations. (1) Both allozyme (Coustau *et al.*, 1991) and *mac-1* data (present study) make it possible to assign the mussels from Noirmoutier, Charente to *M. edulis*. However, unlike locus *mac-1*, allozyme data did not distinguish these from North Sea *M. edulis* [see Fig. 2 of Coustau *et al.* (1991)]. This conclusion held valid out of a CA based

on these authors' *EST-D* and *MPI* data (that is, the two most diagnostic allozyme loci) and using their Atlantic *M. galloprovincialis* sample (Biarritz) as the reference for this species (results not shown). (2) *mac-1* data showed that partial admixture between *M. edulis* and *M. galloprovincialis* occurs at Forêt-Fouesnant, Britanny. This again was consistent with the allelic frequency and heterozygote deficiency data at diagnostic allozyme loci *EST-D* and *MPI* (Coustau *et al.*, 1991; their Fig. 3 and Table 2, respectively). (3) The sample from Pléneuf-Val-André (Saint-Brieuc, Britan-ny), taken from a farmed stock, was *M. edulis* according to *mac-1*. The Saint-Brieuc sample collected in the wild by Coustau *et al.* (1991) was a mixture of *M. edulis*, *M. galloprovincialis*, and their hybrids. *M. edulis* spat are imported into the Bay of Saint-Brieuc every year from Baie-de-l'-Aiguillon (Charente) for cultiv-ation. We here confirmed that farmed stocks in the Bay of Saint-Brieuc are identical to the mussels from Baie-de-l'-Aiguillon (see Fig. 2, samples AIG, BRO, BOY). (4) *mac-1* data showed that the sample POL is more hybrid than mixed, as implicitly suggested by previous allozyme results (Beaumont *et al.*, 1989).

Mosaicism of hybrid zones in Mytilus*: patterns, hypotheses*

Hybridization between *Mytilus* species may show different spatial patterns depending on the taxa involved and on the location. Mosaicism appears to be a common feature of *Mytilus* hybrid zones (Koehn *et al.*, 1984; McDonald & Koehn, 1988; Sarver & Foltz, 1993; Bates & Innes, 1995; Saavedra *et al.*, 1996; Suchanek *et al.*, 1997; Comesaña *et al.*, 1999; Rawson *et al.*, 1999).

Explanations for mosaicism include the following. (1) Endogenous selection against hybrids (tension zone). The coexistence of parental species and hybrids along the hybrid zone would be maintained because hybrid fitness would be reduced; the geographic patchiness of the hybrid zone would be an effect of larval transport, which causes swarms of larvae to land at a place distant from the place of fertilisation. (2) Mosaicism of habitat. It is here assumed that mosaicism arises from adaptation of the two parental forms to different and intermingled environments (Rand & Harrison, 1989; Arnold, 1997). (3) Transplantation. As suggested by Suchanek *et al.* (1997), recurrent transplantation of either species by means of ships may play an important role in maintaining their presence as patches at unfavourable locations, where they do not reproduce; hence their patchy distribution. The data available to date are not sufficient to test the above hypotheses.

Alleles endemic to the hybrid zone

A number of samples from the hybrid zone harboured at substantial frequencies *mac-1* size alleles (*a*6, *a*15) that were absent or rare in both reference *Mytilus edulis* and *M. galloprovincialis*. Allozymes coined hybrizymes by Woodruff (1989) have been found in hybrid zones, where they are endemic.

Selection and genetic drift have been invoked as phenomena responsible for the maintaining of hybrizymes at moderately high frequency (Barton & Hewitt, 1985; Woodruff, 1989), but these do not appear as satisfactory explanations for the increase in frequency of some *mac-1* alleles in mussels. First, this genetic marker is not coding, and there is no reason to suspect that small changes in intron size should be advantageous in the zone of hybridization and only there. Second, genetic drift is unlikely to be perceptible unless a population is isolated and of small effective population size. Alternatively, the presence of these alleles in French Atlantic populations, including some *Mytilus edulis* populations, may simply reflect the genetic distinctness of *M. edulis* in this area.

Genetic variation outside the hybrid zone

South of the hybrid zone, Atlantic *Mytilus galloprovincialis* samples harboured at substantial frequencies *M. edulis mac-1* alleles (*a*2, *a*3, *a*5). These were also present but rare in some Western Mediterranean and Adriatic Sea *M. galloprovincialis* samples. The difference in the frequency of *M. edulis* alleles was mostly responsible for the genetic differentiation between Atlantic and Mediterranean *M. galloprovincialis* populations at locus *mac-1*. A similar trend was evident at allozyme loci *AP* and, perhaps, *GPI* (Daguin & Borsa, 2000) but not at the four other loci (*EST-D*, *LAP*, *MPI*, *ODH*) examined so far in *M. edulis*, Atlantic *M. galloprovincialis*, and Medi-terranean *M.*

galloprovincialis (Coustau *et al.*, 1991; Sanjuan *et al.*; 1994).

If *M. edulis mac-1* alleles were currently introgressing Atlantic *M. galloprovincialis* populations, a regular cline would be expected from Baie de l'Aiguillon where the frequency of *M. edulis* alleles reaches 100%, to the southernmost Atlantic *M. galloprovincialis* sample (Morocco). Instead, *M. edulis* allele frequencies in Atlantic *M. galloprovincialis* populations were homogenous from the Basque Country to Morocco. We therefore propose that the presence of *mac-1* alleles *a*2, *a*3 and *a*5 in, mostly, Atlantic *M. galloprovincialis* results from their past introgression by *M. edulis*. By contrast, the lack of similar pattern for 4/6 allozyme loci suggest that introgression is counter-selected at the latter. We suppose that Mediterranean *M. galloprovincialis* were less affected by this presumed introgression event because of their remoteness (which is consistent with the absence of *M. edulis* alleles in the samples from the more remote regions: northern Adriatic and Black Sea). The differences may also have been enhanced and maintained to the present by the hydrological discontinuity coincident with the Almería-Oran oceanographic front (Tintore *et al.*, 1988; Quesada *et al.*, 1995a, c).

Although the above scenario receives support from mtDNA RFLP and sequence data (Quesada *et al.*, 1998; Hilbish *et al.*, 2000; see Introduction), one cannot exclude inadvertent introduction of alien mussels may have marginally contributed to the current distribution of *mac-1* alleles throughout the area studied.

Conclusion

Allele-frequency data at a non-coding nuclear-DNA marker partly confirmed the main results of former allozyme surveys in showing that *Mytilus edulis* and *M. galloprovincialis* along the Atlantic European coasts meet and interbreed, and that the zone of contact and hybridization overall consists of a geographical mosaic of pure, hybrid, or mixed populations of either species. Despite extensive hybridization at some geographically intermediate locations, presumably neutral *mac-1* alleles do not appear to presently cross the hybrid zone, which therefore likely acts as a barrier to gene flow. The present occurrence of *M. edulis*-like alleles in Atlantic and, to a lesser extent, Mediterranean *M. galloprovincialis* can be explained by past introgression by *M. edulis*. This was also evident at some allozyme loci. At other allozyme loci (Coustau *et al.*, 1991; Sanjuan *et al.*, 1994; Quesada *et al.*, 1995c) the patterns of geographic variation in *M. gallo-provincialis* were consistent with a scenario of vicariance followed by secondary intergradation.

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Table 1 Allelic frequencies at locus *mac-1* for *Mytilus* spp. samples. Alleles were denominated according to their size (Daguin & Borsa, 1999). Abbreviations for samples as in legend to Fig. 1. –: allele absent in sample; ^*H*: gene diversity (Nei, 1987); ^*f*: Weir & Cockerham's (1984) estimator of S. Wright's fixation index; underlined: ^*f*-values found significant by permutation test [Belkhir *et al*. (1996): 1000 permutations]; *N*: sample size. *mac-1* allele frequency data for samples GIL, POL, FAR, TEM and SET have been presented elsewhere (as respectively, samples GILL, POLZ, FARO, TEMA and SETE in Daguin & Borsa, 1999).

	FLØ	GIL	GFP	POL	PVA	PAL	FOU	PEN	YEU	NOI	AIG	RLB	BOY	BRO	BTZ	STB	FAR	TEM	CAR	BAN	SET	RED	CHI	BUT	AGI
*f*1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01	0.02	-	-	-	-	-	-	-	-	-
*f*2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-
*f*3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01
*b*2	-	-	-	0.02	-	0.01	0.01	-	-	-	-	-	-	-	0.02	0.04	-	0.03	0.01	0.06	0.05	0.06	-	0.06	0.03
*b*1	-	-	0.01	0.05	-	0.06	0.05	0.03	0.11	-	-	0.06	-	0.02	0.10	0.15	0.16	0.07	0.26	0.24	0.21	0.16	0.28	0.17	0.22
*b*3	-	-	-	0.02	0.02	-	-	-	-	-	-	-	-	0.02	-	-	-	0.01	0.01	-	-	0.02	-	-	-
*b*4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02	-	-	-	-	-	-	0.01	-	-
*b*5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02	0.01	-	-	-	-	-	-	-	-
*c*0	-	-	-	-	0.01	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*c*1	-	-	0.01	0.06	0.01	0.09	0.07	0.04	0.17	-	-	0.03	-	0.02	0.17	0.10	0.15	0.14	0.04	0.05	0.07	-	0.06	0.08	0.04
*c*15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-
*c*2	-	-	-	0.29	-	0.22	0.22	0.16	0.36	-	0.02	0.06	0.02	0.02	0.42	0.50	0.44	0.5	0.56	0.59	0.54	0.60	0.57	0.57	0.61
*c*3	-	-	-	0.01	-	0.07	0.01	-	-	-	-	0.03	0.01	0.02	0.08	0.02	0.05	0.02	0.01	-	-	-	0.01	0.01	0.01
*c*4	0.05	-	0.02	0.02	0.01	0.05	0.03	0.04	-	-	0.02	0.06	0.04	-	-	-	-	0.01	-	-	-	-	-	-	-
*c*5	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-
*c*6	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01	-	0.01	-	-	-	-
*a*0	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*a*1	0.06	0.02	0.01	0.03	0.02	-	0.04	0.04	0.02	-	0.03	0.06	0.04	-	-	0.02	-	-	-	-	-	-	-	-	-
*a*15	-	-	-	0.01	0.03	-	0.01	0.03	-	0.10	-	0.09	-	-	-	-	-	-	-	-	-	-	-	-	-
*a*2	0.10	0.15	0.20	0.07	0.19	0.06	0.16	0.18	0.15	0.20	0.25	0.13	0.19	0.18	0.01	0.02	0.02	0.02	0.01	0.01	-	-	-	0.02	-
*a*3	0.29	0.31	0.24	0.16	0.29	0.13	0.16	0.19	0.09	0.30	0.25	0.09	0.35	0.33	0.07	0.04	0.05	0.03	0.02	-	0.01	-	-	0.02	-
*a*4	0.07	0.17	0.18	0.03	0.04	0.02	0.05	-	0.03	-	0.03	0.03	0.02	0.08	-	-	0.01	-	-	0.01	-	0.04	-	-	-
*a*5	0.38	0.27	0.29	0.18	0.34	0.17	0.11	0.27	0.05	0.25	0.28	0.25	0.31	0.28	0.01	-	0.01	0.01	0.01	-	-	-	-	-	-
*a*6	-	0.08	0.04	0.02	0.01	0.03	0.03	-	0.02	0.15	0.05	0.09	0.01	0.02	0.02	-	0.01	0.03	0.01	-	0.01	-	-	-	-
*a*7	-	-	-	0.02	-	0.07	0.03	-	0.02	-	0.03	-	-	-	0.03	0.02	0.04	0.06	0.02	-	0.04	0.04	0.03	0.03	0.04
*a*8	-	-	-	0.03	0.01	0.01	-	-	-	-	0.02	-	-	0.02	0.03	0.04	0.05	0.05	0.05	0.03	0.06	0.08	0.03	0.03	0.02
*a*9	0.01	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*a*10	-	-	-	-	-	-	-	-	-	-	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*a*11	-	-	-	-	-	-	-	-	-	-	-	-	0.02	-	-	-	-	-	-	-	-	-	-	-	-
*a*12	-	-	-	-	-	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-
d	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
^*H*	0.76	0.79	0.80	0.85	0.77	0.89	0.88	0.84	0.81	0.816	0.80	0.91	0.75	0.78	0.78	0.72	0.76	0.72	0.62	0.59	0.65	0.61	0.59	0.64	0.58
^*f*	0.24	0.17	0.26	0.12	0.07	0.14	0.27	0.29	0.21	0.40	-0.13	0.25	0.14	0.06	-0.04	-0.01	0.02	0.07	-0.01	0.33	0.12	0.22	0.03	0.06	0.04
N	47	26	42	60	45	43	67	37	33	10	30	16	54	30	46	26	55	43	75	43	68	25	47	62	45

Table 2 Correspondence analysis on *Mytilus* spp. samples characterised at locus *mac-1*: relative contributions of alleles and samples to the two first factorial axes, ranked in decreasing order. Mediterranean samples BUT, CHI, AGI, RED, SET, BAN were introduced as supplementary variables in the analysis. Abbreviations for samples as in legend to Fig. 1.

Allele	Contribution		Sample	Contribution	
	Axis 1	Axis 2		Axis 1	Axis 2
*c*2	0.979	0.001	TEM	0.834	0.000
*a*5	0.922	0.007	FAR	0.815	0.000
*c*1	0.872	0.000	BTZ	0.774	0.001
*b*1	0.842	0.003	STB	0.716	0.003
*a*3	0.828	0.033	PVA	0.653	0.000
*a*2	0.695	0.005	GIL	0.581	0.055
*a*8	0.609	0.010	BRO	0.571	0.107
*b*2	0.545	0.003	GFP	0.569	0.115
*a*7	0.446	0.001	YEU	0.559	0.003
*c*3	0.374	0.016	AIG	0.442	0.002
*f*1	0.306	0.002	BOY	0.434	0.036
*b*5	0.296	0.005	FLØ	0.408	0.138
*a*4	0.289	0.214	NOI	0.350	0.531
*a*1	0.185	0.000	PAL	0.255	0.000
*b*4	0.169	0.004	POL	0.204	0.023
*c*15	0.140	0.000	RLB	0.120	0.573
*f*2	0.135	0.001	PEN	0.106	0.016
*c*4	0.117	0.005	FOU	0.088	0.012
*a*6	0.115	0.575	CAR	-	-
*a*15	0.100	0.835	BAN	-	-
*a*0	0.062	0.117	SET	-	-
d	0.062	0.117	RED	-	-
*a*11	0.050	0.023	CHI	-	-
*a*12	0.050	0.023	BUT	-	-
*c*0	0.043	0.003	AGI	-	-
*a*10	0.038	0.001			
*a*9	0.034	0.095			
*c*5	0.032	0.005			
*c*6	0.007	0.005			
*b*3	0.006	0.017			
*f*3	0.000	0.000			

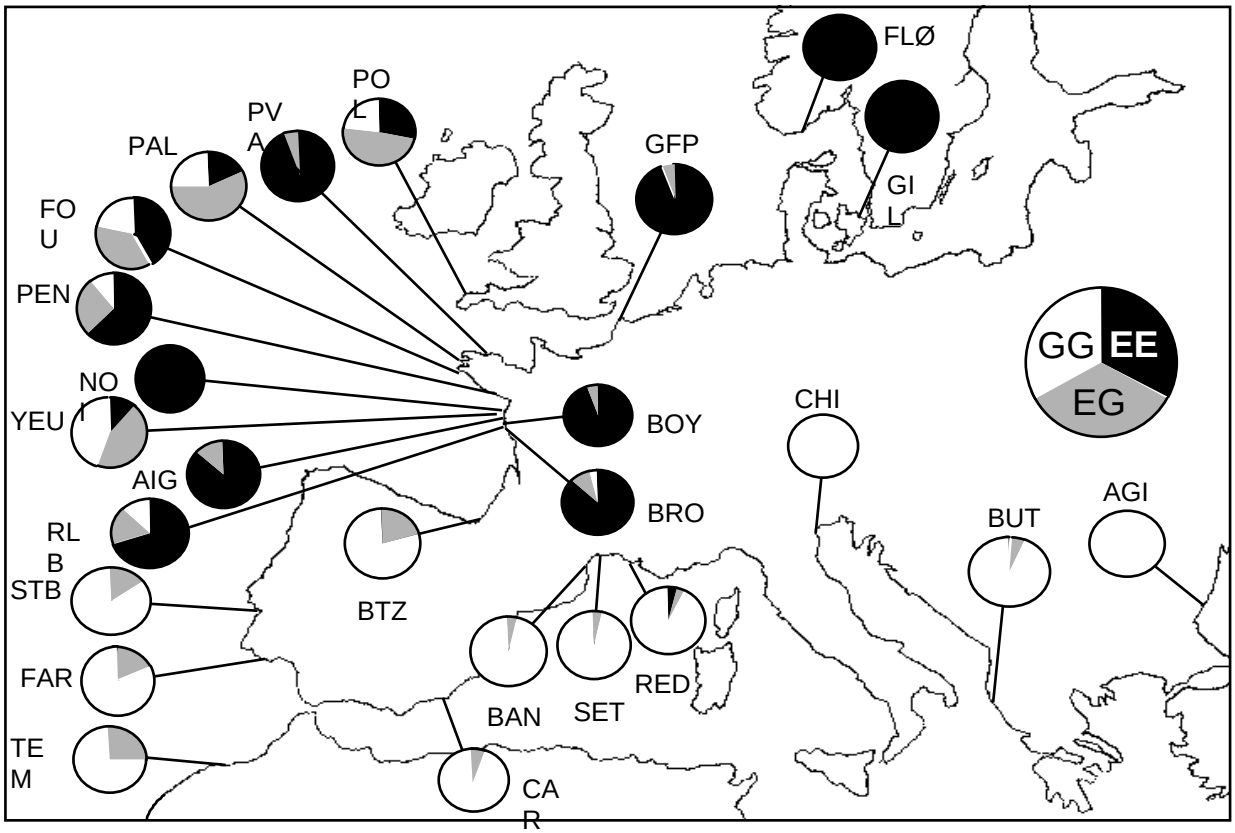

Figure 1 Sampling localities for *Mytilus* spp. in the northeastern Atlantic and the Mediterranean Sea. Genotypic composition of the samples after collapsing alleles characteristic of *M. edulis* and *M. galloprovincialis* (see text and Fig. 2) into classes *E* and *G*, respectively, is also given. Allele *f*3, which was private to sample AGI (Table 1) was included in *G*. *FLØ*: Flødevigen, Skagerrak, Norway; *GIL*: Gilleleje, Kattegat, Denmark; *POL*: Polzeath, Cornwall; *GFP*: Grand-Fort-Philippe, Northern France; *PVA*: Pléneuf-Val-André, Britanny, France; *PAL*: La Palue, Britanny, France; *FOU*: La Forêt-Fouesnant, Britanny, France; *PEN*: Pénestin, Britanny, France; *NOI*: La Guérinière, Ile de Noirmoutier, Vendée, France; *YEU*: Ker Daniau, Ile d'Yeu, Vendée, France; *AIG*: Baie de l'Aiguillon, Charente, France; *BRO*: Brouage, Charente, France; *BOY*: Boyardville, Charente, France; *RLB*: Ronce-les-Bains, Charente, France; *BTZ*: Biarritz, France; *STB*: Setubal, Portugal; *FAR*: Faro, Portugal; *TEM*: Temara (Rabat), Morroco; *CAR*: Cartagena, Spain; *BAN*: Banyuls, Roussillon, France; *SET*: Sète, France; *RED*: Ensuès-La Redonne, France; *CHI*: Chioggia, Italy; *BUT*: Butrintit, Albania; *AGI*: Agigea, Romania.

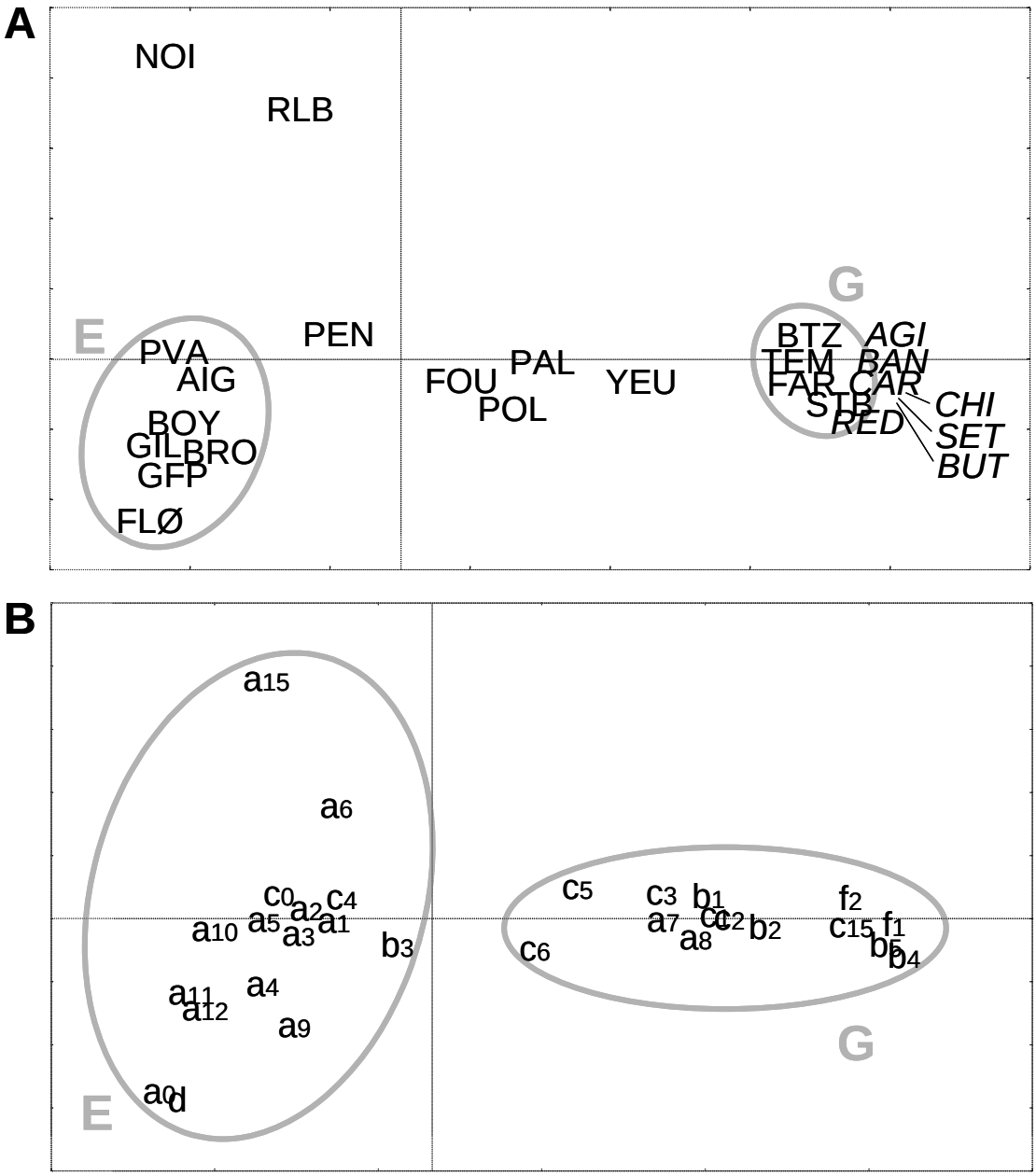

Figure 2 Correspondence analysis (CA, Benzécri, 1982) on the matrix of allelic frequencies of *Mytilus* spp. samples from the northeastern Atlantic and the Mediterranean. Inertias of CA-Axis 1 (horizontal) and CA-Axis 2 (vertical) were 55.3% and 10.0%, respectively. (A) Scatter plot of samples on the first factorial plane. Grey contours delimitate the clusters of *M. edulis* (*E*) and *M. galloprovincialis* (*G*) reference samples. Abbreviations for samples as in legend to Fig. 1. Italics: *M. galloprovincialis* samples introduced as supplementary variables. (B) Scatter plot of alleles on the first factorial plane. Grey contours delimitate the clusters of *M. edulis* (*E*) and *M. galloprovincialis* (*G*) alleles.

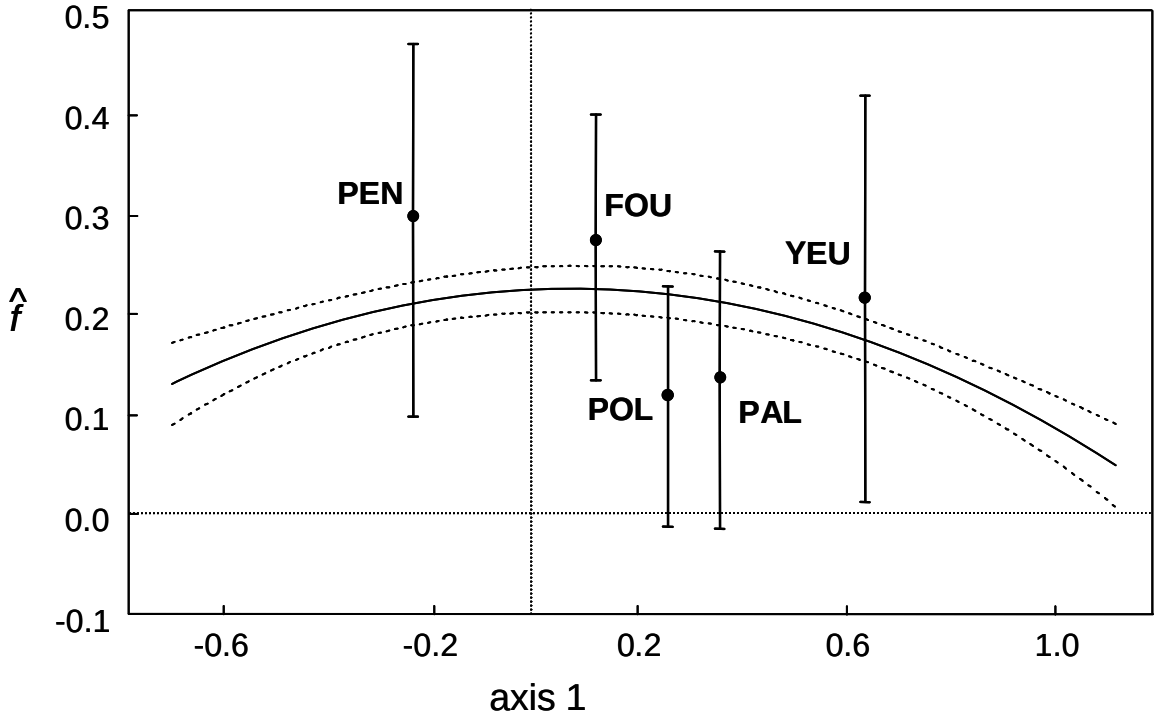

Figure 3 Plot of Weir & Cockerham's (1984) ^*f*-values against coordinate along axis 1 of CA, for five genetically intermediate *Mytilus* spp. samples. Comparison with expected heterozygote deficiency under the admixture hypothesis (solid line, obtained by 2nd-order polynomial regression on a range of pooled random subsamples of reference *M. edulis* and *M. galloprovincialis*). Vertical bars = 95% bootstrap confidence intervals (1000 pseudo-samples). Dotted lines delimitate 95% confidence area around regression line. Abbreviations for samples as in legend to Fig. 1.