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Habitat preference and the marine-speciation paradox

Nicolas Bierne[1,2*], François Bonhomme[1] and Patrice David[2]

[1]*Laboratoire Génome, Populations, Interactions, Adaptation, Université Montpellier II—IFREMER—CNRS, UMR5000, SMEL, 34200 Sète, France*
[2]*CEFE–CNRS, 34293 Montpellier Cedex 5, France*

Marine organisms challenge the classical theories of local adaptation and speciation because their planktonic larvae have the potential to maintain high gene flow. The marine-speciation paradox is illustrated by contact zones between incipient species that are so large that allopatric divergence seems unlikely. For this reason any mechanism preventing sympatric larvae of two incipient species from coexisting in the same habitats can be a powerful promoter of speciation. The contact zone between two hybridizing taxa of mussel, *Mytilus edulis* and *M. galloprovincialis*, in Europe provides an excellent example. Although the zone itself extends over thousands of kilometres, the opportunities for interbreeding are considerably reduced by the small-scale mosaic structure of the zone, where local patches of each taxon alternate at scales of kilometres or less, in response to locally variable ecological factors. Habitat choice by settling larvae would be a less costly mechanism than post-settlement selection to maintain such a mosaic structure. Unfortunately the role of selective settlement has remained hypothetical because larvae could not be scored by classical genetic markers. PCR markers allowed us to study larvae and settlement in ecologically contrasting sites within the zone. We show that only a subset of the genotypes present in the plankton settle in some sites, and that the adults on these sites show the same genetic bias. Genetically based variation in pre-settlement processes therefore accounts for the ecological segregation observed, though it is not the only factor involved in limiting successful interbreeding. The present dataset also supports previous reports of partial spawning asynchrony.

Keywords: habitat preference; selective settlement; marine speciation; hybrid zone; *Mytilus edulis*; *Mytilus galloprovincialis*

1. INTRODUCTION

Many marine invertebrates have a benthic sessile adult phase and disperse only via planktonic larvae. Ecological evidence suggests that larvae can travel over long distances (Scheltema 1988; McQuaid & Phillips 2000; Armonies 2001). This is in agreement with most genetic data for marine species, as very little genetic differentiation is usually observed over a scale of thousands of kilometres (see review in Palumbi 1992). In the long term, such a mode of dispersion should promote random mixing of larvae of different origins and create high gene flow, opposing local adaptation and the speciation process. However, genetic differences between incipient species can be maintained in apparently continuous sea regions, as attested by the persistence of large contact zones (Gardner 1997). Two closely related taxa of marine mussel, *Mytilus edulis* and *M. galloprovincialis*, occur in sympatry and hybridize along the western coasts of Europe over a 2000 km contact zone (Skibinski *et al.* 1983; Coustau *et al.* 1991; Bierne *et al.* 2003*b*). The most surprising feature of this zone is its mosaic structure, in which populations of pure genotypes alternate with mixed populations. The spatial scale of the observed patchiness in genetic variation is sometimes locally far smaller than the scale of presumed larval dispersal, and populations separated by less than a few kilometres maintain genetic differences larger than those usually observed over a scale of thousands of kilometres in marine taxa (Gosling & McGrath 1990; Bierne *et al.* 2002*b*). *Mytilus edulis* and *M. galloprovincialis* are thought to have diverged in allopatry and to have come back into contact. Secondary contact could have put an end to the speciation process if isolation mechanisms were not sufficiently strong. Given the size of the contact zone and the very small distances observed between populations of the two taxa, the persistence of two distinct gene pools in mussels is very puzzling: how has genetic homogenization been slowed down in virtual sympatry? Habitat preference is often mentioned as a key process in such contexts (Bush 1975; Rice 1984). In agreement with this view, partial ecological segregation occurs between adult populations of *M. edulis* (occupying sheltered habitats under freshwater influences) and *M. galloprovincialis* (predominantly found in oceanic exposed habitats). This segregation accounts for most of the small-scale genetic patchiness within the contact zone (Gosling & Wilkins 1981; Skibinski 1983; Gosling & McGrath 1990; Gardner & Skibinski 1991; Gardner 1994; Bierne *et al.* 2002*b*). Differential adaptation seems therefore to be maintained in a fine-grained environment (*sensu* Levins 1968). Two hypotheses, here referred to as the pre-settlement and post-settlement hypotheses, may explain this segregation.

(i) The pre-settlement hypothesis assumes that the segregation occurs before or during settlement. It can be caused by various factors involving either larval mortality or larval behaviour, such as habitat-dependent settlement choice or behavioural responses to flow features.

*Author and address for correspondence: Centre for the Study of Evolution, School of Biological Sciences, University of Sussex, Brighton BN1 9QG, UK (n-bierne@univ-montp2.fr).

(ii) The post-settlement hypothesis assumes that habitat-dependent viability during the post-settlement phase causes the observed segregation.

Post-settlement segregation would be very costly for both taxa because larval movements exceed in scale the grain of habitat heterogeneity. This isolation mechanism therefore entails a large number of selective deaths of mussels recruited in the wrong habitat. However, several marine animals (Burton & Feldman 1983; Powers & Schulte 1998; Lemaire *et al.* 2000; Schmidt & Rand 2001), including mussels (Koehn *et al.* 1980; Hilbish & Koehn 1985), have proven able to pay the cost of local adaptation. One of the best-documented cases is *M. edulis* (in a within-species context). An amino-peptidase allele-frequency cline is maintained by post-settlement habitat-dependent selection between estuarine habitats of Long Island Sound and oceanic coastal zones, despite extensive larval migration (Koehn *et al.* 1980).

The pre-settlement hypothesis has the advantage of sparing the cost of local adaptation. However, while it is accepted that a larva may exploit particular flow features by active vertical movements in the water column (Shanks 1995; Kingsford *et al.* 2002) and determine its final settlement location by testing the substrate and delaying metamorphosis accordingly (Butman *et al.* 1988; Pechenik 1990; Kingsford *et al.* 2002), the role of genetic divergence in settlement preference in the context of speciation has rarely been documented. Marine ecologists have long debated the relative importance of pre- and post-settlement processes in explaining the structure of marine communities (Roughgarden *et al.* 1988; Gaines & Bertness 1992; Olafsson *et al.* 1994; Snelgrove & Butman 1994; David *et al.* 1997), but the debate has rarely been at the species level (Toonen & Pawlik 2001; Bierne *et al.* 2003*a*). Indeed, this level cannot be addressed unless closely related taxa can be distinguished at the larval stage. PCR markers now offer a unique opportunity to tackle this problem (Bierne *et al.* 2003*a*).

2. MATERIAL AND METHODS

(a) *Collection of samples*

We focus on a small area of the blue-mussel hybrid zone in southern Brittany, France (figure 1). Two ecologically and genetically contrasting sites previously studied at the adult stage (Bierne *et al.* 2002*b*) were chosen to study settlement: a sheltered site (protected from surf action by a quay in the Trinité estuary) and an open-sea site (ropes hanging from buoys in the open sea). We initially intended to study settlement in a third site (exposed site, located a few hundred metres away from the sheltered site). However, our spat collectors were unfortunately destroyed and only adults from this exposed site could be analysed. The adult populations from the sheltered, exposed and open-sea sites have already been analysed and described in Bierne *et al.* (2002*b*); all the other analyses are new.

During the spawning season, larvae were collected every two weeks in the middle of the study area (figure 1). Plankton samples were taken by pulling a 200 µm net (only late-larval stages are therefore sampled) 2 m under the water surface, behind a boat. The amount of water sieved was measured using a flux meter. Bivalve larvae were counted and a sample was transferred into 80% ethanol. Larvae were measured and individualized and DNA was extracted as previously described (Bierne *et al.* 2002*a*, 2003*a*). No attempt was made to discriminate *Mytilus* larvae from other species; this was subsequently done at the genetic-analysis stage by using marker primers' specificity. Local currents in this area quickly homogenize water masses through strong tidal back-and-forth movements (Lazure & Salomon 1991). Our larval sample therefore represents a single gene pool from which recruits of both sites originate. At the same time, settled post-larvae were collected from artificial collectors set in the two study sites and renewed at each sampling date. Collectors were composed of a 1 m coconut rope hung vertically from 1 to 2 m under the water surface. At each sampling date, the rope was soaked in 80% ethanol, the fibre was untied into ethanol and falling post-larvae were sorted and measured as for larvae. Post-larvae samples are therefore composed of individuals settled during the two week intervals, with the exception of the last sample in the open-sea site (sample 07/06), which is composed of individuals settled during a one month interval.

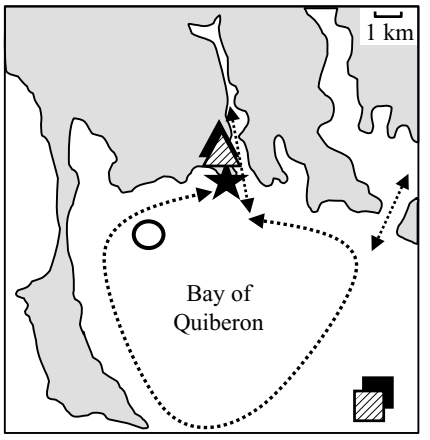

Figure 1. Locations of sampling sites in the Bay of Quiberon, south Brittany, France, and the nomenclature used throughout this article. Dashed arrows indicate local currents that homogenize the zone through strong tidal back-and-forth movements and a gyre created by the geography of the bay. Open circles represent swimming larvae, squares and triangles represent early settled post-larvae (striped) and adults (black) sampled in open-sea and sheltered sites, respectively, and black stars represent adults sampled in the exposed site.

(b) *Molecular markers*

Larvae and post-larvae (total of 448 individuals) were analysed using three previously described PCR-amplified length-polymorphic markers, *Glu-5′* (Rawson *et al.* 1996), *mac-1* (Daguin *et al.* 2001) and *EFbis* (Bierne *et al.* 2002*a*), which differentiate local samples of *M. edulis* from *M. galloprovincialis* (Bierne *et al.* 2002*b*, 2003*b*). Primer specificity eliminates possible biases caused by the co-sampling of other bivalve larvae. The *mac-1* primers used in the present study are specific to *M. edulis* and *M. galloprovincialis* and do not probe even in individuals from the closely related hybridizing species *M. trossulus* (however, a distinct primer set has recently been developed to amplify *mac-1* in *M. trossulus*; C. Daguin, unpublished data). *Glu-5′* primers are specific to the *Mytilus* genus. In the cases where *EFbis* primers probe in other species tested (e.g. *M. californianus*) with the annealing temperature used in our PCR protocol (54 °C), the size of the observed alleles lies outside the range observed in the total sample we have analysed to date.

Nevertheless, identity in the state (length) of alleles and homogeneity in the allele frequencies observed in larvae and adult samples demonstrate that only *Mytilus* larvae have been scored. Moreover, in our analysis we used only individuals for which the three loci had been successfully amplified.

(c) *Data analysis*

The homogeneity of genotypic frequencies between pairs of populations was tested by an exact test using the GENEPOP software (Raymond & Rousset 1995), which allowed us to identify groups of samples having similar genetic compositions. Genetic differentiation was also investigated by correspondence analysis (CA; Benzécri 1982) on the matrix of allele counts per sample using the GENETIX v. 4.03 software (Belkhir *et al.* 2002). CA is particularly well suited to describing the genetic structure in hybrid zones because the genetic differentiation is decomposed in a nested fashion across the axes: the between-species differentiation is apparent on the first axis of the CA (CA_1), whereas differentiation between populations of the same species emerges on secondary axes (Bierne *et al.* 2002*b*, 2003*b*). CA also offers the advantage of simultaneously expressing the genetic differences present in the dataset and the respective contributions of each allele to these differences. Alleles can therefore be pooled, within each locus, into species-specific compound (synthetic) alleles according to their coordinates on CA_1 (Daguin *et al.* 2001; Bierne *et al.* 2002*b*). Synthetic alleles characteristic of *M. galloprovincialis* populations were called *G* and synthetic alleles characteristic of *M. edulis* populations were called *E*. This procedure allowed us to present a summary of the genetic structure in the form of the mean frequencies of synthetic alleles per sample.

Departure from Hardy–Weinberg and linkage equilibrium (HWLE) was analysed according to the maximum-likelihood method of Barton (2000), from which cumulant $\kappa_{1,1}$ corresponds to the average Hardy–Weinberg disequilibrium (HWD) and cumulant $\kappa_{0,2}$ to the average linkage disequilibrium (LD) across loci. The interest of this method is that LD and HWD are estimated jointly. Because Barton's method, as well as the hybrid zone framework in general, considers only disequilibria between pairs of alleles typical of either taxon, synthetic alleles were used for this analysis. Finally, the Metropolis algorithm implemented in Barton's method can be used to obtain the marginal distribution of each parameter through a random walk with parameter *T* (temperature of the simulated annealing) initially set to 1 (Barton 2000). This procedure amounts to Bayesian inference with uniform prior. We therefore obtained 95% confidence intervals (CIs) for the mean frequency of allele *G* and for $\kappa_{1,1}$ (HWD) and $\kappa_{0,2}$ (LD) (for more details see MATHEMATICA v. 3.0 (Wolfram 1996) add-ons provided by N. Barton on his Web site, http://helios.bto.ed.ac.uk/evolgen).

3. RESULTS

(a) *Sampling results*

Larval abundance in plankton collections is illustrated in figure 2. The larvae size was usually between 250 and 350 μm, a size at which metamorphosis is already observed in hatchery-reared larvae (Bierne *et al.* 2002*a*). About 70% of the larvae sampled amplified the three markers. One exception is the sample collected on 25 April, where larvae were smaller than in other samples (less than 250 μm) and could not be amplified. In addition, this sample is between what seems to be two reproductive peaks (figure 2). We therefore suspect this sample to be composed of larvae of another bivalve species. Settlement of post-larvae was continuous throughout the study, although we have no data on recruitment abundance. The post-larvae size ranged between 300 and 1000 μm, corresponding to just metamorphosed larvae (Bierne *et al.* 2002*a*).

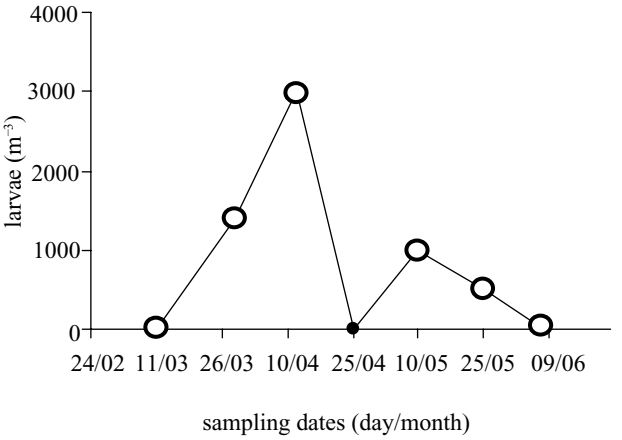

Figure 2. Abundance of bivalve larvae in plankton samples as a function of time. According to genetic analysis, most of these larvae are mussel larvae. Open circles, samples mostly comprising *Mytilus* larvae according to genetic analysis; black circle, a sample of larvae suspected to be composed of bivalve species other than *Mytilus*.

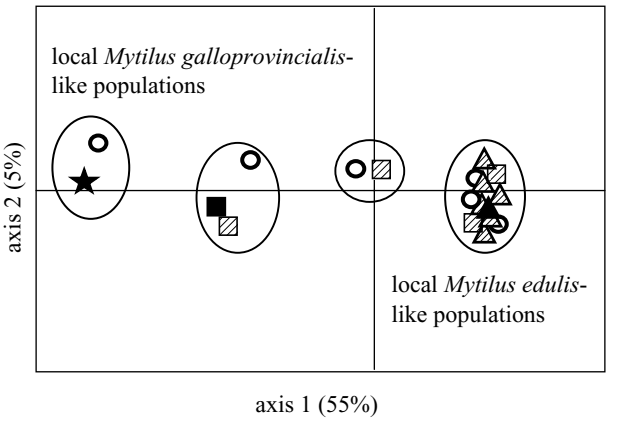

Figure 3. Projection of samples on the first factorial plane of a CA on the matrix of allele counts per sample. Ellipses group samples for which the homogeneity of genotypic frequencies cannot be rejected. See legend to figure 1 for the nomenclature of the symbols.

(b) *Global genetic structure*

Allele frequencies at each locus for each sample are given in electronic Appendix A (available on The Royal Society's Publications Web site). Figure 3 presents the projection of samples on the first factorial plane of the CA. CA_1 reflects the allele-frequency gradient between the two species, from *M. galloprovincialis* on the left to *M. edulis* on the right. Although secondary axes allow the distinction of different populations within the same taxon at a larger spatial scale (Bierne *et al.* 2003*b*), populations were not differentiated on secondary axes at the scale of this study and results can be summarized by the first-axis coordinate (figure 3). The results of tests for the homogeneity of

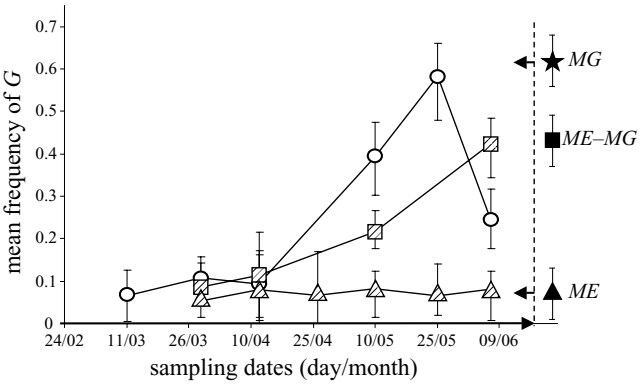

Figure 4. Genetic analysis of recruitment in space and time. Frequency of the *G* compound allele as a function of time (sampling date in day/month). Vertical bars represent 95% CIs. The arrows indicate the frequencies of the *G* compound allele in the local parental populations of adults. Open circles represent swimming larvae, squares and triangles represent early settled post-larvae (striped) and adults (black) sampled in open-sea and sheltered sites, respectively, and black stars represent adults sampled in the exposed site.

genotypic frequencies between pairs of populations (see electronic Appendix B available on The Royal Society's Publications Web site) allowed us to group samples that do not significantly depart from homogeneity into the ellipses shown in figure 3.

Alleles were pooled to form bi-allelic loci at both *mac-1* and *EFbis* according to the outcome of the CA. Ten size-alleles at locus *mac-1* (*a*0, *a*1, *a*2, *a*3, *a*4, *a*5, *a*6, *a*15, *b*6, *c*4) and seven size-alleles at locus *EFbis* (E_{-3}, E_{-2}, E_{-1}, E_0, E_1, E_2, G_3) were pooled to form a compound *E* allele characteristic of *M. edulis*. The remaining eight size-alleles at locus *mac-1* (*b*1, *b*2, *c*1, *c*15, *c*2, *c*3, *a*7, *a*8) and six size-alleles at locus *EFbis* (G_{-10}, G_{-2}, G_{-1}, G_0, G_1, G_2) were similarly pooled to form a compound *G* allele characteristic of *M. galloprovincialis*. The mean frequencies, over the three loci, of the compound *G* allele in the three adult populations are indicated by black symbols on the right of figure 4.

As previously reported, adult populations were different in the three sites (Bierne *et al.* 2002*b*). Importantly for the analysis of settlement patterns, more *M. edulis* genotypes were observed in the sheltered site and more *M. galloprovincialis* genotypes were observed in the open-sea site. We made the following predictions: if the post-settlement hypothesis is valid, recruits from both sites should be genetically similar, and similar to larvae, irrespective of the adult genotypes found in the two sites; if the pre-settlement hypothesis is valid, recruits should differ from larvae and be similar to adult populations.

(c) *Temporal genetic structure in the plankton*

Larval abundance in the plankton collections roughly suggested two reproductive phases (figure 2). The genetic analysis reveals that samples of the first phase (first three sampling dates) were not significantly different from *M. edulis* adult samples (figure 4), while samples of the second phase were either genetically intermediate between *M. edulis* and *M. galloprovincialis* (samples 10/05 and 07/06) or not significantly different from *M. galloprovincialis* (sample 25/05; figure 4).

(d) *Spatial and temporal genetic structure of settled post-larvae*

Settlement in the open-sea site did not depart from that expected based on larvae, taking into account the fact that samples of settled post-larvae represent a two week (or one month for sample 07/06) interval while samples of larvae represent larvae present in the plankton on the day of sampling. Roughly the same temporal structure was observed in settled post-larvae of this site, with *M. edulis* settlement during the first phase and a mixed settlement during the second phase (figure 4).

By contrast, settlement in the sheltered site was exclusively by *M. edulis*, in conformity with local adult populations, even when *M. galloprovincialis* and/or mixed larval populations were observed in the plankton (figure 4).

(e) *Analysis of disequilibria*

The question arises of whether the mixed samples (larvae and settled post-larvae at the open-sea site) were composed of simple admixtures of the two taxa or of hybrid individuals. In larvae, HWDs were small and significant in only one out of the two mixed samples (figure 5*b*). LDs were significant, but their values were half of the possible maximum based on allele frequencies (figure 5*a*). HWD and LD in larvae were therefore not different from those expected following one generation of nearly free hybridization. HWD and LD were slightly stronger in post-larvae of the open-sea site (figure 5) than in larval samples. This is expected because mussels have been recruited over two week intervals during which the larval gene pool changed, producing a temporal Wahlund effect. As shown by the overlapping of CIs, the Wahlund effect alone can account for the disequilibria observed in post-larvae (figure 5). This effect is further illustrated in an example (for clarity of the figure) by the expected disequilibria assuming a simple admixture of two flanking larval samples (dotted curve in figure 5). No further effect of selection is thus required to explain the genetic composition of these samples, although this does not rule out the existence of selection, especially its potential role in the long-term maintenance of the zone.

(f) *Genotypelshell-length associations*

To discriminate further between the competing hypotheses, we have also checked whether correlations exist between individual genotypes as quantified by their hybrid index (the number of *G* alleles per individual) and shell length. None of the correlations was significant at the 5% level in our samples of larvae (11/03: $F_{1,21} = 0.12$; 29/03: $F_{1,21} = 0.39$; 12/04: $F_{1,28} = 1.48$; 10/05: $F_{1,17} = 0.91$; 25/05: $F_{1,18} = 0.11$; 07/06: $F_{1,25} = 0.1$; all $p > 0.05$), in our samples of settled post-larvae in the open-sea site (29/03: $F_{1,20} = 0.07$; 12/04: $F_{1,16} = 0.07$; 10/05: $F_{1,57} = 0.37$; 07/06: $F_{1,27} = 4.12$; all $p > 0.05$) or in our samples of settled post-larvae in the sheltered site (29/03: $F_{1,14} = 2.56$; 12/04: $F_{1,25} = 0.002$; 26/04: $F_{1,18} = 0.3$; 10/05: $F_{1,50} = 1.09$; 25/05: $F_{1,42} = 0.36$; 07/06: $F_{1,32} = 0.00$; all $p > 0.05$). Note, however, that the maximum age of a post-larvae is only 15 days after settlement in our samples. Therefore, if selection has played a role it must have occurred before this age, most probably before settlement itself, and should have removed all the non-*edulis* genotypes during their entrance to the sheltered area for the post-larvae samples to be gen-

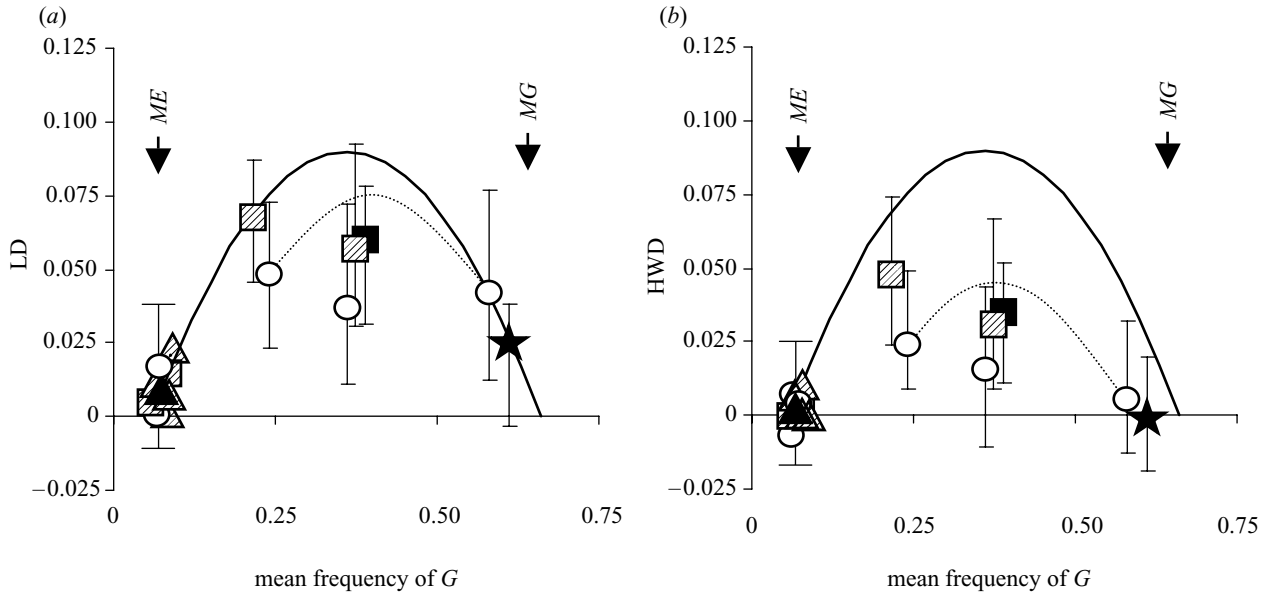

Figure 5. (*a*) LD ($\kappa_{0,2}$) and (*b*) HWD ($\kappa_{1,1}$). Vertical bars represent 95% CIs (for clarity the 95% CIs of the *M. edulis*-like samples are synthesized into a single interval). Disequilibria are given as a function of the frequency of the *G* compound allele because their maximum possible values are a function of the relative frequency of *M. edulis* and *M. galloprovincialis* alleles. The expected maximum value assuming a mixture of two local parental populations without interbreeding is given (solid curve). The proximity of observed values to this curve indicates a lack of genetic introgression within samples. The dotted curve represents the expected maximum value assuming a mixture of the two consecutive samples of larvae that begin and end the curve. Arrows indicate the frequencies of the *G* compound allele in the local parental populations of adults. See legend to figure 1 for the nomenclature of the symbols.

etically so close to the local *M. edulis* adult samples (figures 3 and 4).

4. DISCUSSION

(a) *Habitat preference in a marine hybrid zone*

Our results are consistent with the predictions of the pre-settlement hypothesis, that larvae settle in different places according to their genotype. The existence of non-negligible numbers of hybrid larvae makes things slightly more complicated than a simple partitioning of *M. edulis* in sheltered versus *M. galloprovincialis* in open-sea habitats. However, there is definitely an ecological segregation during settlement, as *M. galloprovincialis* and hybrids seem unable to settle in the sheltered area, leaving *M. edulis* alone in this site.

One might hypothesize that this pattern is caused by a retention of larvae spawned by local adults in the sheltered area through unsuspected micro-currents. However, this hypothesis, in addition to contradicting what is known of local currents (Lazure & Salomon 1991; figure 1), is not consistent with the distribution of adult populations and habitats. The alternation between sheltered and exposed habitats is very rapid in this area, and *M. galloprovincialis* and mixed adult populations are found as soon as the exposed habitat begins, no more than a few hundred metres from the *M. edulis*-recruiting site (Bierne *et al.* 2002*b*; figure 1). Therefore, under the hypothesis of local recruitment, larvae would have to stay within less than 1 km of their site of origin. However, the Trinité estuary, where this site is located, is essentially emptied and filled twice a day by powerful tidal fluxes. In such conditions, it is extremely improbable that the larval pool recruited in our sampled site has been produced *in situ* and has remained uncontaminated by neighbouring *M. galloprovincialis* during five weeks of planktonic life. In addition, in contrast to the local-retention hypothesis, ecologically biased settlement provides the clue to a long-standing paradox in the study of the mussel contact zone (Gosling & McGrath 1990). Two decades of research on the adult stage have consistently revealed an asymmetrical selective advantage, in terms of viability, growth, strength of attachment and reproduction, favouring *M. galloprovincialis* adult mussels (Skibinski 1983; Gardner & Skibinski 1988, 1991; Skibinski & Roderick 1991; Willis & Skibinski 1992; Gardner *et al.* 1993; Wilhelm & Hilbish 1998), even in habitats where *M. edulis* genotypes predominate at the spat stage (Gosling & McGrath 1990). Local retention of larvae cannot reasonably explain the long-term maintenance of *M. edulis* patches in sheltered areas all along the 2000 km hybrid zone. Alternatively, differential settlement might provide a refuge for *M. edulis* in sheltered sites. To what extent it could be sufficient to prevent the elimination of this taxon despite its low adult viability relative to *M. galloprovincialis* remains to be evaluated, but, at least, it diminishes the rate at which *M. edulis* alleles might be eliminated from the region. Our results also show that *M. edulis* settles earlier than *M. galloprovincialis*, which could give an advantage to the former, if recruitment is density dependent (e.g. if late recruitment is reduced owing to a shortage in available space). Note, however, that this effect cannot explain the bias for *M. edulis* in our samples of settled post-larvae from the sheltered site because our collectors were renewed every two weeks, providing free space irrespective of previous recruitment.

Various pre-settlement factors could explain the observed pattern of settlement. Differential settlement could be caused either by the selective elimination of *M.*

galloprovincialis and hybrid larvae when entering the sheltered area or by differential larval responses to settlement cues or flow features. Distinguishing between these possibilities is hardly possible and this problem deserves further study, involving the examination of larvae at each study site, at multiple depths and with increased precision in the temporal sampling scheme. However, several arguments make differential larval behaviour more likely than differential viability. First, *M. galloprovincialis* genotypes exhibit higher viability and growth than *M. edulis* genotypes at the larval stage in hatchery experiments (Bierne *et al.* 2002*a*), even in low salinities (*ca*. 27‰) presumed to favour *M. edulis* (N. Bierne, unpublished data). Second, the correlations between hybrid index and shell length in larvae suggest no advantage to *M. edulis*. The non-significant tendencies were overall in favour of *M. galloprovincialis* genotypes, while the reverse is needed to explain our results. Note that, to explain the *M. edulis* genetic composition of settled post-larvae in the sheltered site, differential larval viability should be extreme, removing all genotypes except pure *M. edulis* before settlement (figures 3 and 4). Unfortunately, little is known about settlement cues in *Mytilus* as recruitment is observed on a large variety of substrates. The principal factor was suggested to be the substratum structure, the preference being for filamentous macro-algae (Bayne 1976). However, settlement also occurs on adult mussel beds (McGrath *et al.* 1988). Recently, the settlement preference of *M. edulis* has been shown to be influenced by the biofilm community of bacteria and diatoms (Dobretsov 1999) and, interestingly, by the presence of conspecifics in the surroundings (Dobretsov & Wahl 2001). Such partial 'gregariousness' would probably favour the maintenance of distinct locally differentiated patches of mussels, although it seems insufficient alone to stabilize them in the long term. Finally, another possible pre-settlement behavioural mechanism would involve the exploitation of particular flow features by active vertical movements in the water column (Shanks 1995). It has been shown that larvae of *M. edulis* are not randomly distributed according to depth (Dobretsov & Miron 2001) and it has recently been shown in the laboratory that *M. edulis* and *M. trossulus* larvae tend to exploit different depths during settlement (Freeman *et al.* 2002).

In apparent contradiction with our results, Gilg & Hilbish (2000) found no evidence of differential settlement among sites in south Cornwall, England, although there was evidence for differential mortality after settlement. However, the frequency of *G* alleles varied only between 0% and 12% in their study and most of the *G* alleles appeared in heterozygous genotypes (Hilbish *et al.* 2002). This genetic context is fairly different from ours: our samples from south Brittany contain from 5% to 60% *G* alleles and exhibit large Hardy–Weinberg and linkage disequilibria (Bierne *et al.* 2002*b*, 2003*b*). This suggests that many genes involved in reproductive isolation are locally segregating (Barton & Hewitt 1985), making it possible to detect their action (in our case, their role in pre-settlement segregation) indirectly through their association with neutral markers. On the other hand, it is likely that only a small subset of the genes involved in reproductive isolation are actually polymorphic in south Cornwall, where the populations are essentially *M. edulis* contaminated by a minority of *M. galloprovincialis* genes. Weaker isolation mechanisms might be at play in south Cornwall than in south Brittany, and therefore pre-settlement processes can be weak or absent in the former while present in the latter. Note that another study, by Gosling & McGrath (1990), on spat collected in Ireland in a similar genetic context to ours, also led to the prediction of the involvement of early-life-stage processes. In summary, we think that post-settlement and pre-settlement processes both exist, and that the diversity of situations allowed by the complexity of the *Mytilus* contact zone provides a unique opportunity to observe various combinations of these mechanisms in different places. In south Brittany, pre-settlement processes are responsible for the fine-scale mosaic structure even if habitat specialization alone would probably be insufficient to explain the long-term maintenance of the two different gene pools (see Bierne *et al.* 2002*b*).

(b) *Implication for speciation in the sea*

Our data provide evidence that genetic variation in pre-settlement processes contributes to the ecological segregation of two hybridizing marine taxa. As explained in § 1, marine ecologists have long debated the importance of pre-settlement processes in the sea. It was also known that marine larvae use sensory cues to find suitable settlement sites (see review in Kingsford *et al.* 2002). However, how well they can efficiently target good habitats is a matter of debate. How fast habitat preference can evolve was even more poorly known because sister species are often indistinguishable at the larvae and post-larvae stages. When genotypes adapted to different environments were observed segregating within one species (the LAP example; Koehn *et al.* 1980), selective settlement was lacking. In addition, the conditions under which selection can favour the evolution of habitat preference are thought to be restrictive when dispersal overwhelms the spatial grain of the environmental variation (Levins 1968) as in marine bivalves with a very dispersive planktonic phase. Selective settlement was therefore previously thought to be unlikely to contribute to the isolation between two incipient or recently formed species. However, the recent access to the larval stage brings this mechanism back into focus. Recently, Appelbaum *et al.* (2002) documented differential settlement between sister species of tropical barnacles. Our study, together with that of Appelbaum *et al.* (2002), points out that habitat preference can evolve during speciation and plays a part in this process, as an isolation mechanism, in sessile invertebrates with planktonic larvae.

One might object that the evolution of a trait such as habitat preference, and its potential involvement in generating new species limits, should ideally be studied at the scale of within-species polymorphisms, before any other isolation mechanism has evolved. However, two arguments contradict this view. First, such polymorphisms are extremely difficult to observe (Toonen & Pawlik 2001) as we usually cannot screen habitat-preference genotypes directly (Bierne *et al.* 2003*a*). Only hybrid zones, thanks to linkage disequilibria, allow one to track habitat-preference genotypes indirectly using neutral markers, as illustrated by the classical *Bombina* hybrid zone (MacCallum *et al.* 1998). Second, speciation most probably relies on the simultaneous emergence of a combination of various isolation mechanisms acting both before and after secondary contact. Hybrid zones are therefore a good place to study

speciation, not only because they point towards the isolation acquired in allopatry, but also because isolation mechanisms may continue to evolve (Butlin 1989) and remain important in sharpening or weakening the incipient species limits in the presence of gene flow (Harrison 1990). In the *M. edulis–M. galloprovincialis* hybrid zone, hybrids are commonly found in some populations (as observed in the present study), and introgression of neutral markers occurs, both at large (Skibinski *et al.* 1983; Daguin *et al.* 2001; Bierne *et al.* 2003*b*) and at small spatial scales (this study; Bierne *et al.* 2002*b*). This suggests that mussels are still somewhere between polymorphism and achieved speciation. According to our data, selective settlement seems to be the main cause of small-scale ecological segregation of the two mussel taxa and hence one of the mechanisms that prevent the two incompletely differentiated gene pools from merging into a single pool. Of course, several other important factors may also promote speciation in the sea. Our data already point to another kind of pre-mating isolation involved in the maintenance of this zone, spawning asynchrony, which is suggested by the pronounced delay observed between the *M. edulis* larval-abundance peak and that of *M. galloprovincialis* and hybrids, in agreement with previous studies (Gardner & Skibinski 1990; Secor *et al.* 2001). Assortative fertilization (Bierne *et al.* 2002*a*), hybrid disadvantage during the larval phase (Beaumont *et al.* 1993; Bierne *et al.* 2002*a*) and post-metamorphic directional selection favouring *M. galloprovincialis* genotypes (see review in Gardner 1994) also enter the list of isolation mechanisms at work in the western European mussel hybrid zone. The multiplicity and intensity of these mechanisms is probably a key to the marine speciation paradox. Molecular analysis now opens a window on the first half of the life cycle of marine animals, which used to be so difficult to observe in the field, but which is so critical to consider in respect to understanding marine biodiversity.

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