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Direct selection on allozymes is not required to explain heterogeneity among marker loci across a *Mytilus* hybrid zone

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Abstract

Unequal differentiation between two types of loci (allozyme and DNA markers) across a *Mytilus* hybrid zone has recently been claimed as evidence for direct selection on some llozyme loci. We provide here a counter-example: a noncoding DNA locus that exhibits as much differentiation as the incriminated allozymes do. The levels of genetic differentiation varied widely among both allozymes and noncoding DNA markers and no clear difference emerged between the two types of markers. This suggests that the strong interlocus variance in genetic differentiation has been confounded with a discrepancy between marker types as a result of an insufficient and unbalanced locus sampling. Heterogeneity in differentiation among neutral loci can be created by stochastic variance during the allopatric divergence preceding a secondary contact. In hybrid zones, a further source of variance is differential introgression among chromosomal regions after the secondary contact owing to the local influence of selected genes on more or less distant markers. However, the degree of differentiation alone gives no way to distinguish indirect pseudo-selection (a regular and ubiquitous feature of hybrid zones) from direct selection. More generally, we suggest that comparative neutrality tests based on discrepancies among marker types have to be applied with caution when the presence of semi–permeable genetic barriers to gene exchange is suspected.

Keywords: allozymes, hybrid zones, introgression, Mytilus, neutral hypothesis, selection

Introduction

The status of allozyme markers with regard to natural selection has long been debated (Eanes 1999). To infer selection on allozymes, an approach is to use a neutral control on which direct selection is very unlikely. Non-coding DNA markers have provided such neutral controls for at least the last decade and the comparison of geographical variation at allozymes and DNA markers has become a test of the neutral

status of allozymes (McDonald 1994). However, such tests are often crippled by an insufficient number of loci or by unbalanced sampling regarding the two types of loci. Neutral loci may differ greatly in their amount of differentiation across a barrier to gene flow. It is common knowledge that drift itself generates a large stochastic variance in the degree of differentiation between two allopatric populations. When secondary contacts occur, gene flow can be restrained by partial genetic isolation, whose effect is twofold. Firstly, it delays the homogenization process, exactly as a physical barrier would do (Barton 1986), which increases the chance of observing remnants of the initial variance in differentiation acquired in allopatry. Secondly, it can accentuate heterogeneity among neutral loci because of differential introgression rates (Barton & Hewitt 1985; Harrison 1990). Indeed, according to the theory of hybrid zones, the genes directly involved in reproductive isolation will form stable clines, while the homogenization of neutral markers is slowed down in proportion to their linkage disequilibrium with the former (Barton 1986). Introgression rates at neutral markers therefore often vary according to their physical linkage with selected genes, as can be observed in sunflower species for instance (Rieseberg *et al.* 1999). Riginos *et al.* (2002) compiled data on allele frequency differences at five allozyme loci, a mtDNA locus and four nuclear DNA loci, between a *Mytilus edulis* population of the Skagerrak (North Sea) and a *M. trossulus* population of the Baltic Sea. These authors contrasted the five non-allozyme markers, which showed relatively little differentiation, to the five, much more differentiated, allozyme loci. This was interpreted as evidence of selection on the five allozyme loci. Here, we expanded the genetic dataset on mussels from Baltic-Sea and Skagerrak sites by adding two noncoding nuclear markers, *mac-1* (Daguin *et al.* 2001) and *EFbis* (Bierne *et al.* 2003). Also, we took into account all 18 allozyme loci examined in mussel samples from the Øresund hybrid zone area (Bulnheim & Gosling 1988; Väinölä & Hvilsom 1991). We conclude that: (i) a continuum in the degree of differentiation exists among loci within both the allozyme and nonallozyme markers; (ii) there was no statistical evidence that the ranges of differentiation differed between marker types; (iii) the five allozymes used by Riginos *et al.* (2002) are a biased sample of allozymes which only represents the upper range of the distribution. Therefore the conclusion of direct selection on allozymes is unwarranted. We instead propose, as did Väinölä & Hvilsom (1991), that the neutral hypothesis, which involves the stochasticity of drift and the heterogeneity in the rate of introgression is consistent with the data.

Materials and methods

Mytilus spp. adult individuals were collected in Flødevigen (Norway, Skagerrak) and Gdansk (Poland, Baltic Sea). We also used a sample from Helgoland (Germany, North Sea) as reference for *M. edulis*, and samples from Gaspésie (Canada, Atlantic Ocean) and Hog Island (Tomales Bay, California, eastern Pacific Ocean) as references for *M. trossulus*. North Sea and eastern Pacific mussels have traditionally been used as references for, respectively, *M. edulis* (Coustau *et al.* 1990; Daguin *et al.* 2001) and *M.trossulus* (Rawson & Hilbish 1995; Borsa*et al.* 1999). Two intron-length polymorphic markers were used; *mac-1* (Daguin *et al.* 2001) and *EFbis* (Bierne *et al.* 2003). These markers have first been developed for *M. edulis* and *M. galloprovincialis* but new primers have been designed from *mac-1* sequences (M. Ohresser, unpublished data) to obtain more efficient PCR-amplifications in *M. trossulus*. We used the following primer pairs: a newly designed *forward* primer (5 '-GCTGTATTTCCATCAATTGTTGG−3') in association with the *reverse* primer of Daguin *et al.* (2001) for locus *mac-1*; *Fbis-F* (5'-ACAAGATGGACAATACCGAACCACC-3') and *EFbis-R2* (5'-CCTTCTGGATTTCCATGAATCGG-3') for locus *EFbis*. Comparison among loci can be hampered by variation in diversity (McDonald 1994; Hedrick 1999), especially when the differentiation is strong as is often the case in hybrid zones. To circumvent this problem, pooling alleles has been recommended (McDonald 1994). Alleles at a single locus were therefore pooled into species-specific compound alleles according to their frequencies in samples from each species. Synthetic alleles at a locus, that were characteristic of *M. edulis* populations, were called *E*. To measure differentiation, we used the difference in *E* allele frequencies, Δp (Barton 2000), between the two populations considered.

Homogeneity of allele frequencies between pairs of populations was tested by an exact test using the GENEPOP software (Raymond & Rousset 1995).

Results

Allele frequencies at loci *EFbis* and *mac-1* are given in Table 1. The electrophoresis method we used does not allow us to measure bands precisely and only a relative scale can be used. The nomenclature for *EFbis* size-alleles follows Bierne *et al.* (2003). We here describe a new major class of size-alleles we have named *Ti*, that is around 100 base pairs (bp) smaller than the *Ei* class. *T0* was the most frequent allele in our Baltic Sea, western Atlantic and eastern Pacific samples of *Mytilus trossulus* (Table 1). Other size-alleles were numbered consecutively by size, each increment presumably corresponding to one base pair. The nomenclature for *mac-1* size-alleles was an extension of the one presented in Daguin *et al.* (2001). Locus *EFbis* was almost differentially fixed ($\Delta p > 0.98$; $P < 0.001$) between eastern Pacific *M. trossulus* and North Sea *M. edulis* used as references for each species (Table 1). It was also almost diagnostic ($\Delta p = 0.97$; $P < 0.001$) between Baltic Sea *M. trossulus* and North Sea (Germany) *M. edulis*, but a remarkably high frequency of *trossulus* alleles (60%) was found in *M. edulis* populations from Skagerrak. A similar pattern was observed for an allozyme locus, *Aap*, suspected to be under selection by Riginos *et al.* (2002). *Aap* was almost dagnostic ($\Delta p > 0.95$) between Baltic Sea *M. trossulus* and northern Atlantic (Iceland) *M. edulis*, but *M. trossulus Aap* alleles were found in the Skagerrak (17%). The genetic structure observed at locus *mac-1* was more complex. At this locus the *M. edulis* reference sample from the North Sea sharply differed from its *M. trossulus* equivalent, from the eastern Pacific ($\Delta p = 0.87$, $P < 0.001$). However, inconsistencies were observed when using synthetic alleles assigned with these reference samples in the Skagerrak-Baltic Sea comparison. For instance, *mac-1* allele *a3*was more frequent in the North Sea than in the eastern Pacific, but it was more frequent in the Baltic Sea than anywhere else (Table 1). This allele would be assigned as *edulis* using the sample from the eastern Pacific as reference for *M. trossulus*, and the conclusion would be that Baltic Sea *M. trossulus* populations carry an *edulis* allele at a higher frequency than Skagerrak *M. edulis* populations. Such a pattern is not only visible at locus *mac-1* but also at some enzyme loci [e.g. allele *100* at locus *Ap* for the Tvärminne population in the Baltic Sea (McDonald & Koehn 1988) compared with Skagerrak mussels (Varvio *et al.* 1988)]. It seems improbable that such alleles have introgressed into *M. trossulus* and then decreased by genetic drift in North Sea *M. edulis* populations to the point that allele frequencies are now reversed compared to the initial situation. More parsimoniously, we propose that eastern Pacific *M. trossulus* should not be considered as representative of the initial state of the conspecific European populations. This is illustrated by the strong genetic differentiation observed at loci *EFbis* and *mac-1* between our *M. trossulus* samples from the northwestern Atlantic and reference eastern Pacific *M. trossulus* (*EFbis*: $\Delta p = 0.37$, $P < 0.001$; *mac-1*: $\Delta p = 0.41$, $P < 0.001$). This differentiation applies to alleles not found in *M. edulis* (e.g. allele *EFbis-T-10*). In conclusion, in the absence of valid reference samples, it seems hopeless to disentangle the effects of the initial state vs. the introgression that could have occurred after secondary contact in determining the current allelic composition of Baltic *M. trossulus* populations. Table 2 presents Δp estimates between Skagerrak (*M. edulis*) and Baltic Sea (*M. trossulus*) populations, Δp estimates between North Sea (*M. edulis*) and eastern Pacific (*M. trossulus*) populations and the frequencies of *E* compound alleles in Baltic Sea *M. trossulus* samples, across six nuclear DNA and 18 allozyme loci. The lack of data for less discriminative allozymes illustrates how four or five discriminating allozyme loci, which may well belong to the same linkage group (Beaumont 1994), have been consistently used in the *Mytilus* literature, neglecting the information given by other loci and therefore adding to the sampling bias we are attempting to point out in the present paper. No discrepancy between allozyme and nonallozyme markers was apparent from the results of the Skagerrak-Baltic Sea comparison presented in Table 2. First, the suggestion that all the nonallozyme markers exhibit large frequencies of *M. edulis* alleles into *M. trossulus* Baltic ea populations, allegedly because they are not under selection (Riginos *et al.* 2002), can now be refuted by one counterexample: that of locus *EFbis* for which the frequency of compound allele *E* was

as low as for the most extreme allozyme locus. Second, although the most discriminating markers were allozyme loci, one should notice that the allozymes screened (18) outnumbered the DNA markers (6). Actually, the least differentiated markers were also allozymes. Using the reasoning of Riginos *et al.* (2002), balancing selection should be suspected for the latter. In short, the final picture was a continuous gradient of differentiation, independent of marker type (Wilcoxon two-sample test, $P > 0.10$).

Discussion

Variable rates of admixture and introgression characterize *Mytilus* spp. assemblages in western Europe (Skibinski *et al.* 1983; Väinölä & Hvilsom 1991). The two mussel populations considered here are separated by a hybrid zone that features concordant narrow clines of allele frequencies (Väinölä & Hvilsom 1991). Whatever the status of the allozymes screened, the strong linkage disequilibria observed within the zone (Väinölä & Hvilsom 1991) suggest a strong barrier to neutral gene flow. Neutral DNA markers are also affected by the barrier, as shown by significant nonzero Δp-values across the Øresund (Table 2). The barrier to gene exchange therefore seems to be active at the scale of the whole genome. However, introgression does occur, at least for some markers (Väinölä & Hvilsom 1991; Borsa *et al.* 1999). In such conditions, the cumulative effect of precontact differentiation variance and postcontact differential introgression rates can create strong heterogeneity among neutral loci (Harrison 1990). Of course one cannot exclude the possibility that some of the genes observed may be under selection: the most discriminative allozyme loci are possible candidates for disruptive selection, as are the less discriminative ones with regard to balancing selection. However, we have shown that allele-frequency differentials said to be typical of directly selected allozymes were also found in one noncoding DNA marker (*EFbis*). This is sufficient proof that variation at the five allozyme loci did not depart from the expectations for neutral loci, which are under indirect pseudo-selection through their linkage to loci involved in reproductive isolation. Moreover, it should be noted that none of the five allozyme loci suspected to be under direct selection were fully diagnostic between the two mussel species. Direct selection would have to take a very peculiar form to explain why favoured alleles did not reach fixation on either side of the hybrid zone. We are aware that selection at sites tightly linked to some markers (for example an exon of the *EF1-α* gene of which we scored an intron) is possible. However, linkage to a selected site and direct selection are different issues: the existence of indirect selection of variable intensity in various markers within a hybrid zone has been well established for decades (Barton & Hewitt 1985), while it remains ground-breaking to demonstrate the direct action of selection on a polymorphism. Heterogeneity in the rate of differentiation across a hybrid zone has therefore been hastily interpreted as evidence of direct selection. Could the same misinterpretation have occurred in other studies? The case of the American oyster *Crassostrea virginica* in Florida is an instructive example. Direct selection on allozymes was claimed (Karl & Avise 1992) but was subsequently re-evaluated (McDonald *et al.* 1996). Karl & Avise (1992) found drastic differences in allele frequencies between the Gulf of Mexico and the Atlantic Ocean at four anonymous nuclear-DNA markers, whereas allozyme polymorphisms were said to be fairly uniform all along the same area (Buroker 1983). This led to the conclusion that allozymes were under balancing selection (Karl & Avise 1992). However, the discrepancy among marker types disappeared when six more nuclear-DNA markers were added to the analysis of oysters from the same area (McDonald *et al.* 1996). The picture finally obtained instead was a gradient of differentiation without clear discrepancies between types of markers (see Table 2 of McDonald *et al.* 1996). Further analyses revealed that *C. virginica* population structure in Florida has the characteristics of a hybrid zone (Hare & Avise 1996, 1998). Interestingly, geographical structures consistent with the presence of a hybrid zone have recently been described with microsatellite loci in the Atlantic cod, *Gadus morhua* (Nielsen *et al.* 2003) and the acorn barnacle, *Semibalanus balanoides* (Dufresne *et al.* 2002), two taxa where selection on some protein loci has previously been suspected (*Gadus*: Mork *et al.* 1985; Pogson *et al.* 1995; *Semibalanus*: Holm & Bourget 1994; Schmidt & Rand 1999). However, some evidences of selection were inferred from the analysis of geographical variation outside the hybrid zones, making the conclusion of direct selection more robust in

these cases. Nonetheless, genetically differentiated entities have been recognized with neutral markers. Geographic and/or reproductive isolation must therefore have occurred in the past and is probably still operating. Under these circumstances the variance in neutral evolution can be much larger than predicted under the singlepopulation coalescence theory (Wakeley 2000; Irwin 2002; Hudson & Turelli 2003), as it has recently been illustrated by strong heterogeneity in genealogical patterns across loci in the *Drosophila pseudoobscura* complex of species (Machado *et al.* 2002). Even though some of the genetic markers used here and in other studies may eventually prove to be under direct selection, rejecting the neutral hypothesis based on discrepancies in geographical variation among loci is hazardous, especially in the case of hybrid zones. Because genetic barriers to gene exchange (i.e. hybrid zones) may remain undetected, heterogeneous levels of differentiation should always be interpreted with caution.

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All co-authors have interest in the population genetics and evolution of marine organisms. This study was a by-product of a project aimed at examining the biogeography and the structure of genetic barriers to gene flow in the *Mytilus edulis* complex of species.

Table 1 Allelic frequencies at nuclear-DNA loci *EFbis* and *mac-1* in *Mytilus edulis* and *M. trossulus*; frequency of *M. edulis* compound allele (*E*) given for each population in bold; *N*, sample size

Locus	Allele	North Sea Helgoland (*edulis*)	Skagerrak Flødevigen (*edulis*)	Baltic Gdansk (*trossulus*)	Atlantic Gaspésie (*trossulus*)	Pacific Hog Island (*trossulus*)
EFbis	(N)	(38)	(48)	(55)	(48)	(40)
	E0	0.97	0.38	0.01	—	—
	E-1	—	0.01	—	—	—
	E-2	0.01	—	—	—	—
	G2	—	0.01	—	—	—
	T5	—	—	0.01	—	—
	T4	—	—	0.01	—	—
	T2	—	—	—	0.05	—
	T0	0.02	0.60	0.95	0.83	0.55
	T-1	—	—	0.01	0.01	—
	T-7	—	—	—	—	0.02
	T-10	—	—	0.01	0.08	0.43
	T-12	—	—	—	0.01	—
	T-13	—	—	—	0.01	—
	E	**0.98**	**0.40**	**0.01**	**0.00**	**0.00**
mac-1	(N)	(67)	(47)	(47)	(44)	(31)
	t9 (T)	—	—	—	0.01	—
	t8 (T)	—	—	—	0.02	—
	t7 (T)	—	—	—	0.03	0.04
	t6 (T)	—	—	—	—	0.02
	t1 (T)	—	—	0.21	0.11	0.20
	t2 (T)	—	—	0.10	0.25	0.41
	t3 (T)	—	—	0.03	0.08	0.15
	t4 (T)	—	—	—	—	0.02
	t5 (T)	—	—	—	0.01	0.02
	c4 (E)	0.04	0.05	—	—	—
	a0.4 (T)	—	—	—	0.01	—
	a0 (E)	—	0.02	—	—	—
	a1 (E)	0.01	0.06	—	—	—
	a2 (E)	0.20	0.10	—	—	—
	a3(T)	0.25	0.28	0.47	0.28	0.13
	a4 (T)	0.01	0.07	0.07	0.08	—
	a5 (E)	0.48	0.39	0.12	0.10	—
	a8 (T)	—	—	—	—	0.03
	a9 (E)	0.01	0.01	—	—	—
	d (E)	—	0.01	—	—	—
	E	**0.74**	**0.64**	**0.12**	**0.10**	**0.00**

Table 2 Frequency of compound *Mytilus edulis* allele (*E*) in Baltic mussels and relative difference in allele frequency, Δp, calculated between Skagerrak *M. edulis* and Baltic Sea *M. trossulus* and between North Sea *M. edulis* and eastern Pacific *M. trossulus* (in brackets: Δp-value for the northern Atlantic -Baltic Sea comparison). Loci were classed by type (nuclear-DNA vs. allozyme) and ranked by increasing Δp (Skagerrak-Baltic comparison); ***bold type***, allozyme loci previously suspected to be under direct selection; *ND*, no data

Locus	*E* Frequency in Baltic Sea	Δp Skagerrak-Baltic	Δp North Sea-Pacific	Reference
DNA markers				
PLIIa	0.72	ND	ND	(1)
ITS	0.70	ND	ND	(1)
MAL-1	0.37	ND	ND	(1)
Glu-5'	0.52	0.48	1.00	(2)
mac-1	0.12	0.53	0.87	(3)
EFbis	0.01	0.39 (0.97)	0.98	(3)
Allozymes				
Ark	0.13	0.03	ND	(4)
Pgk	0.02	0.06	ND	(4)
Me-1	0.06	0.07	ND	(4)
Ap	0.17	0.08	0.18	(4)+(5)
Idh-2	0.83	0.08	ND	(4)
Aat-1	0.77	0.22	ND	(4)
Fdp	0.57	0.42	ND	(4)
Ald	0.05	0.45	ND	(4)
Aco-2	0.21	0.49	ND	(4)
Lap	0.33	0.56	0.33	(4)+(5)
Idh-1	0.16	0.60	ND	(4)
Odh	0.33	0.60	0.66	(4)+(5)
Gpi	0.03	0.78	0.56	(4)+(5)
Mpi	0.11	0.82	1.00	(4)+(5)
Aap	0.00	0.83 (0.95)	0.77	(5)+(6)
Est-D	0.03	0.87	0.96	(4)+(5)
Pgm	0.09	0.87	0.57	(4)+(5)
Aco-1	0.03	0.91	ND	(4)

(1) Riginos *et al.* (2002); (2) Borsa *et al.* (1999); (3) present study; (4) Väinölä & Hvilsom (1991); (5) McDonald & Koehn (1988); (6) Bulnheim & Gosling (1988).