

# Evidence for intragenic recombination within a novel genetic marker that distinguishes mussels in the *Mytilus edulis* species complex

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We have used the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) techniques to design two genetic markers for blue mussels in the *Mytilus edulis* species complex. Both of these markers target the gene encoding the mussel polyphenolic adhesive protein. The first marker, *Glu-5'*, is highly differentiated among and can be used to identify the three blue mussel species, *M. edulis*, *M. galloprovincialis* and *M. trossulus*. The second marker, *Glu-3'*, can identify *M. edulis* and *M. galloprovincialis*. Using these markers we have demonstrated that hybrid mussels from Whitsand Bay, UK carry alleles for this gene that are the products of intragenic recombination. The high frequency (10 per cent) of these recombinant alleles within the hybrid population suggests that recombination is fairly frequent within this gene or that hybridization between *M. edulis* and *M. galloprovincialis* is substantial and has been occurring over considerable evolutionary time. The two novel genetic markers, *Glu-5'* and *Glu-3'* will be invaluable in additional studies regarding the importance of hybridization among blue mussels.

**Keywords:** intragenic recombination, *Mytilus*, population genetics, polyphenolic adhesive protein.

## Introduction

Studies of natural hybridization can reveal patterns of linkage disequilibrium, selection, recombination and introgression between populations which are genetically differentiated, providing valuable insight into the genetic basis of adaptive evolution and speciation (Barton & Hewitt, 1985; Harrison & Rand, 1989). Although hybrid zones appear to be a common phenomenon, the vast majority of well-documented occurrences of natural hybridization are for terrestrial taxa (see Barton & Hewitt, 1985; Hewitt, 1990). However, natural hybridization may also figure prominently in the biology and evolution of many marine taxa, including blue mussels in the *Mytilus edulis* species complex. This complex is composed of three morphologically similar species or subspecies in the Northern Hemisphere, *M. edulis*, *M. galloprovincialis*, and *M. trossulus*, (McDonald *et al.*, 1991). The status of these taxa is actively debated (Koehn, 1991; Gosling, 1992a);

however, allopatric populations of these taxa are genetically distinct despite natural hybridization that occurs wherever any two of the taxa are sympatric (Gosling, 1992b).

Case studies of natural hybridization rely on an extensive suite of well-differentiated molecular and morphological genetic markers for determining the genotypic class to which an individual belongs (Avice, 1994). For *Mytilus* spp., detailed hybrid zone analysis has been complicated by an insufficient number of genetic markers. Allozymes have been used extensively for taxonomic characterization and assessment of selection in natural populations of *Mytilus* spp. (reviewed by Gosling, 1992b). Of the allozymes employed to date, four are well differentiated but not diagnostic between any pair of blue mussel species. Morphological characters can occasionally be used to identify mussel species on a local basis (Freeman *et al.*, 1994) but are less reliable over larger spatial scales (Gosling, 1992b). Thus, to study the importance of hybridization to the evolution of blue mussels it is necessary to develop additional genetic markers that diagnostically identify the three

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species within the *M. edulis* species complex and allow accurate estimation of the frequency of various genotypic classes within mussel hybrid zones.

The advent of modern molecular biological techniques, including the polymerase chain reaction (PCR), provides the opportunity to develop these new genetic markers and to apply these techniques to life stages of mussels that cannot be routinely analysed using allozymes. For example, Corte-Real *et al.* (1994a,b) report the development of a *M. edulis* CaM-1 intron 3 marker using intron-targeted PCR. They further show that this marker is polymorphic within hybrid mussel populations in western Europe, has relatively low levels of local differentiation and is likely to be neutral. However, Corte-Real *et al.* (1994a,b) present no information on whether the CaM-1 marker is differentiated among allopatric populations of the three taxa in the *M. edulis* species complex.

We have used PCR to detect polymorphism at the nuclear locus encoding the mussel polyphenolic adhesive protein. This protein is produced by the endocrine gland in the foot of a mussel and is a key component in the attachment of mussels to the substratum (Waite, 1992). The primary sequence of this protein is highly differentiated between *M. edulis* and *M. galloprovincialis* (Filpula *et al.*, 1990; Inoue & Odo, 1994). We have designed two separate sets of oligonucleotide primers for specific regions in the 5' and 3' ends of the approximately 2.5 kilobase 'giant exon' of this gene. Using these primer sets and PCR, we have designed one assay which identifies all three species within the *M. edulis* complex and a second assay which identifies *M. edulis* and *M. galloprovincialis*. Herein, we report the development of these assays, their application to the study of hybridization in *Mytilus* spp. from south-western England and the identification of individual mussels carrying recombinant genotypes at the polyphenolic adhesive protein locus.

## Materials and methods

We constructed genetic markers for the 5' and 3' ends of a gene that encodes a polyphenolic adhesive protein used by mussels to attach byssal threads to substrate (Waite, 1992). For brevity we have named this the *Glu* locus. The *Glu* gene has been cloned and sequenced for both *M. edulis* (Filpula *et al.*, 1990) and *M. galloprovincialis* (Inoue & Odo, 1994); the last approximately 875 amino acid residues of the protein are encoded by a single large exon (approximately 2.5 kb) and are mainly arranged in tandem decapeptide repeats (Filpula *et al.*, 1990).

### Glu-5' marker

Oligonucleotide primers were designed from the DNA sequences reported by Filpula *et al.* (1990) for *M. edulis*. The first primer (JH-5; GTAGGAA-CAAAGCATGAACCA) corresponds to the extreme 5' end of the 2.5 kb giant exon. For the reverse primer (JH-54; GGGGGGATAA-GTTTTCTTAGG) we aligned the primary sequences for the decapeptide repeats within the giant exon. This primer was designed to anneal to only a subset of these repeats by anchoring most of the primer in the repeat but with the 3' end of the primer annealing to the more variable regions upstream of the repeats. Amplifications were performed by the polymerase chain reaction using 50 ng of total DNA, 2.5 nmol of dNTPs, 50 pmol of each primer and 0.5 U Taq polymerase. Each reaction was conducted in a total volume of 12.5  $\mu$ L using an amplification buffer supplied by the manufacturer (US Biochemical) and 1.5 mM of MgCl<sub>2</sub>. The samples were denatured initially for 3 min at 94°C and then incubated for 30 cycles of 94°C for 20 s, 53°C for 20 s, and 72°C for 45 s. PCR products were then resolved on 2.5 per cent agarose gels.

### Glu-3' marker

A second set of primers was designed to amplify the extreme 3' end of the giant exon. Primer JH-4 (CTTTCAAATGTTTCATCTGTTCCCTC) anneals to a site in the 3' untranslated region (UTR) that Filpula *et al.* (1990), Inoue & Odo (1994) and our own sequence information (data not shown) have indicated is conserved between *M. galloprovincialis* and *M. edulis*. Primer PR-8 (AAGCCAAGT-TATCCTCCAAC) anneals to the region designated as repeat 77 in the *M. edulis* sequence of Filpula *et al.* (1990) and the corresponding repeat in *M. galloprovincialis*. PCR was performed with these primers as described above except that the annealing temperature was lowered to 45°C for 30 cycles.

This assay produced an approximately 220 base pair product in both *M. edulis* and *M. galloprovincialis* but did not amplify any product from *M. trossulus*. Following amplification, 6  $\mu$ L of PCR product was digested with 5 U of the restriction enzyme *Dde*I in 18  $\mu$ L of buffer supplied by the manufacturer (New England Biolabs). Restriction fragments were subsequently resolved on 2.5 per cent agarose gels.

### Reference populations

Adult blue mussels were collected from six intertidal

sites containing allopatric populations of *M. edulis* (Lewes, DE and Cape Anne, MA, USA), *M. galloprovincialis* (San Diego, CA, USA, and Sete, France) and *M. trossulus* (Newport and Port Orford, OR, USA). Total cellular DNA was extracted from individual mussels from each site by digesting a 50 mg subsample of tissue for 3 h at 55°C in 300 µL lysis buffer (50 mM Tris-HCl, pH 8.0; 1 per cent SDS; 25 mM EDTA) with 100 µg of proteinase K. The nucleic acids were isolated by extracting the completely digested tissue once with phenol/chloroform (1:1) followed by precipitation of the DNA with 95 per cent ethanol. The precipitate was washed in 70 per cent ethanol, air-dried and resuspended in 100 µL of TE (10 mM Tris-HCl, 1 mM EDTA). These DNA extracts were then analysed using the *Glu-5'* and *Glu-3'* markers as described above.

#### Comparisons with allozyme data

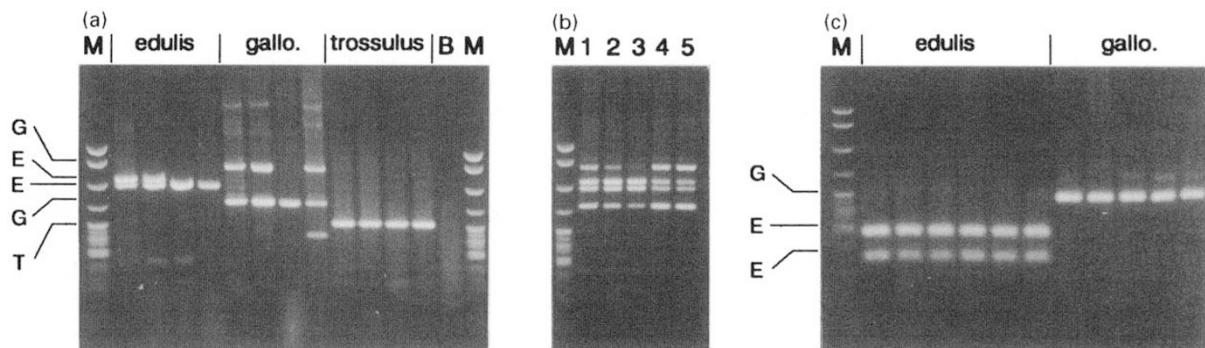
It is well documented that allozyme alleles characteristic of *M. edulis* exhibit a significant decline in frequency with increasing size or age in hybrid mussel populations in south-west England (Skibinski, 1983; Gardner & Skibinski, 1988; Wilhelm, 1993). To determine whether the *Glu-5'* and *Glu-3'* behave in a similar manner, we collected mussels from Whitsand Bay in south-west England where these patterns have been particularly well established. Mussels were sorted into 5 mm shell length size classes and 20 individuals were selected from each size class for genetic analysis. Each mussel was

dissected, tissue samples taken from the gill, mantle and digestive gland and frozen at -80°C. Frozen tissue samples were electrophoresed and stained according to the procedures of Skibinski *et al.* (1980) for *Esterase-D* (*Est*, EC 3.1.1.1) and *Leucine aminopeptidase* (*Lap*, EC 3.4.11) and McDonald *et al.* (1991) for *Mannose-6-phosphate isomerase* (*Mpi*, EC 5.3.1.8). Because each of these loci is multi-allelic, we combined alleles to create compound alleles characteristic of *M. edulis* and *M. galloprovincialis* as described Skibinski *et al.* (1980). Total nucleic acids were extracted from the remaining tissue and assayed for the *Glu-5'* and *Glu-3'* markers as described above.

## Results

#### *Glu-5'* marker

The *Glu-5'* PCR assay produced species-specific banding patterns as shown in Fig. 1a. In *M. edulis*, this assay produced three different banding patterns; approximately 85 per cent of *M. edulis* mussels produced a single 350 bp band, 3 per cent produced a 380 bp band and 12 per cent produced both bands (Table 1). When this assay was applied to *M. galloprovincialis* mussels, two primary bands of 300 and 500 bp were produced. Occasionally *Glu-5'* PCR with some *M. galloprovincialis* individuals produced only the 300 bp band. In contrast, *Glu-5'* PCR with *M. trossulus* consistently produced a single primary band of 240 bp. In many of the PCR reactions with primers JH-5 and JH-54 other bands, such as the



**Fig. 1** (a) PCR products generated using the *Glu-5'* assay for four individuals from *Mytilus edulis* (*edulis*), *M. galloprovincialis* (*gallo.*) and *M. trossulus* (*trossulus*). The size marker (lane M) is pBR322 digested with *Msp*I and the negative control is in lane B. The species-specific banding patterns are indicated on the left (E, *M. edulis* bands; G, *M. galloprovincialis* bands; T, *M. trossulus* bands). (b) *Glu-5'* sensitivity analysis. DNA from *M. edulis* and *M. galloprovincialis* was mixed at ratios of 1:1, 1:3, 1:5, 3:1 and 5:1 and the *Glu-5'* PCR results are in lanes 1–5, respectively. (c) Restriction fragment profiles from the *Glu-3'* assay for six *M. edulis* and five *M. galloprovincialis* individuals. Species designations and marker lanes are the same as in Fig. 1a.

**Table 1** Frequency of individuals with *Glu-5'* or *Glu-3'* banding patterns in allopatric populations of *Mytilus trossulus* (Newport and Pt. Orford), *M. edulis* (Lewes and Cape Anne) and *M. galloprovincialis* (San Diego and Sete)

Location	n	Band size: <i>Glu-5'</i>							Band size: <i>Glu-3'</i>		
		240	350	380	350/380	300	500	300/500	n	214	130/90
Newport, OR	42	100	0	0	0	0	0	0	—	—	—
Pt. Orford, OR	24	100	0	0	0	0	0	0	—	—	—
Lewes, DE	25	0	76	8	16	0	0	0	33	0	100
Cape Anne, MA	36	0	92	0	8	0	0	0	36	0	100
San Diego, CA	64	0	0	0	0	3	0	97	64	100	0
Sete, France	17	0	0	0	0	6	0	94	17	100	0

For the *Glu-5'* marker, only the primary bands were scored (see text). The dashes under the *Glu-3'* columns indicate that this marker could not be amplified for *M. trossulus*

200 bp product for the *M. galloprovincialis* individual shown in Fig. 1, were generated. These were probably produced by the reverse primer JH-54 annealing at other repeat sites within the polyphenolic adhesive gene. However, none of these minor bands was of the same length as the primary bands. Thus, there was some level of band polymorphism within each species but these polymorphisms did not overlap and the primary banding patterns were specific to the allopatric population samples of each species (Table 1).

We have cloned and sequenced the primary *Glu-5'* products generated for each of the three *Mytilus* ssp. in this study. The sequences (Fig. 2) show that the different products resulting from PCR with *M. edulis* are more closely related to each other than they are to the two products from *M. galloprovincialis* and vice versa. From these sequences it is also evident that the annealing site for the *M. edulis* 350 bp band has been significantly disrupted in the 380 bp band. If these banding patterns are treated as separate alleles, the *Glu-5'* genotype frequencies within both *M. edulis* reference populations do not deviate from Hardy–Weinberg equilibrium ( $G = 0.811$ ; NS). We, therefore, feel that these PCR products are produced by two distinct and well differentiated alleles. In contrast, the *M. galloprovincialis* 500 bp product contains an intact annealing site coincident with the annealing site for the *M. galloprovincialis* 300 bp product. We feel that the observed variation in banding pattern among *M. galloprovincialis* individuals may be produced by small differences in nucleotide sequence at the annealing site for primer JH-54. Alternatively, slight variation in PCR conditions may result in the 300 bp band of *M. galloprovincialis* being preferentially amplified. These two possibilities are not mutually exclusive. We have tested this assay repeatedly on a

small set of samples and found that occasionally some individuals produce two primary bands in one reaction and only a single primary band in subsequent reactions and vice versa.

Because of this phenomenon, we used a sensitivity analysis to test whether particular species-specific alleles could out-compete others for priming during PCR with hybrid mussels. We mixed DNA from each of two mussel species at ratios of 1:1, 1:3, 1:5, 3:1 and 5:1 and then conducted the *Glu 5'* PCR analysis. When we mixed *M. edulis* and *M. galloprovincialis* DNA in this manner we obtained both species-specific primary bands at all dilutions, as shown in Fig. 1b. Similar results were obtained for mixtures containing *M. trossulus* and *M. galloprovincialis* or *M. edulis* DNA (data not shown).

#### *Glu-3'* marker

PCR with the *Glu-3'* primers produced a 220 bp product in *M. edulis* and a 214 bp product in *M. galloprovincialis* but failed to amplify any product from *M. trossulus*. These products can be resolved on polyacrylamide gels; however, when the PCR products were digested with the restriction enzyme *DdeI* species-specific restriction fragment patterns that can be separated on agarose gels were generated (Fig. 1c). These patterns were fixed within allopatric populations of *M. edulis* and *M. galloprovincialis* (Table 1). The difference in restriction patterns was the result of a single base change that lies within the coding region of *Glu* for *M. edulis* and within the 3' UTR for *M. galloprovincialis* (Fig. 3). We also tested two other restriction enzymes (*NdeI* and *MseI*) for the capacity to digest differentially the *Glu-3'* PCR product and both produced species-specific restriction patterns between *M. edulis* and *M. galloprovincialis* (data not shown).

M.e. (pub)	TGT	AGGAACAAAG	CATGAACCAG	TATACAAACC	TGTGAAGACA	AGTTATTCCG
M.e. (380)	---	-----JH-5	-----	-----	-----	-----
M.e. (350)	---	-----JH-5	-----	-----	-----	-----
M.g. (pub)	???	?.G.	-----	-----	-----	-----
M.g. (500)	---	-----JH-5	-----	-----	-----	-----
M.g. (300)	---	-----JH-5	-----	-----	-----	-----
M.t. (240)	---	-----JH-5	-----	-----	-----	-----
M.e. (pub)	CACCATATAA	ACCACCAACA	TACCAACCAC	TCAAAAGAA	A---GTGGAC	TATCGTCCTA
M.e. (380)	.....	.....	.....	.....	.....	.....
M.e. (350)	.....	.....	.....	.....	.....	.....
M.g. (pub)	.....	.....T.....	.....	.....	.....	.....A.....
M.g. (500)	.....	.....	.....	.....	.....	.....A.....
M.g. (300)	.....	.....	.....	.....	.....	.....A.....
M.t. (240)	.....	.....	.....	.....	.....CCA.....	.....
M.e. (pub)	CGAAAAGTTA	TCCGCCAACA	TATGGATCAA	AGACAAACTA	TCTACCACCT	GCAAAGAAGC
M.e. (380)	.....	.....	.....	.....	.....G.....	.....
M.e. (350)	.....	.....	.....	.....	.....	.....
M.g. (pub)	.....T.....	.....	.....	.....	.....G.....	.....
M.g. (500)	.....T.....	.....	.....	.....	.....G.....	.....
M.g. (300)	.....T.....	.....	.....	.....	.....G.....	.....
M.t. (240)	---.T....C	.....	.....	.....	.....	.....
M.e. (pub)	TGTCATCTTA	CAAACCTATT	AAGACAACAT	ATAATGCAAA	GACAAATTAT	CCACCAGTTT
M.e. (380)	.....	.....	.....	.....	.....	.....
M.e. (350)	.....	.....	.....	.....	.....	.....
M.g. (pub)	.....	.....	.....	.....	.....	.....
M.g. (500)	.....	.....	.....	.....	.....	.....
M.g. (300)	.....	.....C.....	.....	.....	.....	.....
M.t. (240)	.....	.....	.....	.....	.....G.....	.....
M.e. (pub)	ATAAACCTAA	GATGACTTAT	CCTCCTACAT	ACAAACCAA	GCCCAGTTAT	CCTCCAACAT
M.e. (380)	.....	.....	.....	.....	.....	.....
M.e. (350)	.....	.....	.....	.....	.....	.....
M.g. (pub)	.....	.....	.....A..T..	..T.....T..	.....	.....
M.g. (500)	.....	.....	.....A..T..	..T.....T..	.....	.....
M.g. (300)	.....	.....	.....A..T..	..T.....T..	.....	.....
M.t. (240)	.....	-----JH-54-	-----	-----	-----	-----
M.e. (pub)	ATAAATCAAA	GCCCACATAC	AAACCTAAGA	TAACATACCC	TCCAACATAT	AAAGCAAAGC
M.e. (380)	.....	.....	.....	.....	.....	.....C.....
M.e. (350)	.....	.....	.....	.....	.....	.....
M.g. (pub)	.....C.....	.....	.....	.....GT..T..	AG...T...	.....T..C...T
M.g. (500)	.....C.....	.....	.....	.....GT..T..	AG...T...	.....T..C...T
M.g. (300)	.....C.G.....	.....	.....	.....GT..T..	AG...T...	.....T..C...T
M.e. (pub)	CAAGTTATCC	CTCTTCATAC	AAACCTAAGA	AAACTTATCC	CCCCACATAT	AAACCTAAAC
M.e. (380)	.....T.....	.....T.....	.....G.C.....	T.T.C.C....	T..T.....	.....T.....
M.e. (350)	.....	.....	.....	.....	-----JH-54-----	-----
M.g. (pub)	.....	.....	.....	.....	.....T..A.....	.....
M.g. (500)	.....	.....	.....	.....	.....T..A.....	.....
M.g. (300)	.....	.....	.....	.....	-----JH-54-----	-----
M.e. (pub)	TAAACCTATCC	TCCTACATAT	AAACCAAAGC	CCAGTTATCC	TCCAACATAT	AAACCAAAC
M.e. (380)	-----JH-54-----	-----	.....	.....	.....	.....
M.g. (pub)	.....	.....A.....	.....A.....	.....A.....	.....	.....G.....
M.g. (500)	.....	.....A.....	.....A.....	.....A.....	.....	.....G.....
M.e. (pub)	CAAGTTAT--	-----	-----	-----CC	CCCTTCATAT	AAAACCTAAGA
M.g. (pub)	.....CC	AGCAACTTAT	AAATCCAAGT	CAAGTTAT..	.....	.....
M.g. (500)	.....CC	AGCAACTTAT	AAATCCAAGT	CAAGTTAT..	.....	.....
M.e. (pub)	AAACTTATCC	CTCTTCATAT	AAACCAAAGC	CAAGTTATCC	TCCAACTTAT	.....
M.g. (pub)	.....	.....C.T..A	.....A..C.....	.....T...G...	.....	.....
M.g. (500)	.....	.....	.....	-----JH-54-----	-----	-----

**Fig. 2** Alignment of *Glu-5'* sequences. The three *Mytilus edulis* are designated by the letters M.e. and include the published (pub) genomic sequence from Filpula *et al.* (1990) and one sequence each for the 380 and 350 bp PCR products. The *M. galloprovincialis* sequences are indicated by the letters M.g. and include the published (pub) cDNA sequence from Inoue & Odo (1994) and one sequence each for the 500 and 350 bp PCR products. The single *M. trossulus* sequence is designated as M.t. (240). Annealing sites for the primers JH-5 and JH-54 are indicated by arrows. Alignment gaps are indicated by dashes and dots denote sequence similarity.

### Comparisons with allozyme data

The frequency of *M. edulis* alleles at both the *Glu-5'* and *Glu-3'* markers decreased with size in mussels from the hybrid population at Whitsand Bay as did the frequency of *M. edulis* alleles for each of the three allozyme loci (Fig. 4). The frequency of *M. edulis* alleles at the *Est* locus was significantly correlated with the frequency of *M. edulis* alleles at the *Glu-5'* marker ( $r = 0.780$ ,  $P < 0.05$ ) but not at the *Glu-3'* marker ( $r = 0.472$ ,  $P > 0.05$ ). Similar results were obtained in comparisons between the *Glu*

markers and *Mpi* or *Lap*. This observation was unexpected; because the two *Glu* markers are physically closely linked we anticipated a perfect correlation between species-specific *Glu-5'* and *Glu-3'* allele frequencies across size class. This was not the case as the two markers had unequal allele frequencies and different associations with the allozyme loci. We therefore examined each individual in the sample from Whitsand Bay to determine whether their *Glu-5'* and *Glu-3'* genotypes agreed with one another. Several individuals were detected in which the *Glu-5'* and *Glu-3'* genotypes disagreed, indicat-

M.e. (pub)	ATTGAAGCCA	AGTTATCCTC	CAACATACAA	ATCTAAAACA	AGTTACCCTC	
M.e.	-----	--PR-8--	-----	-----	-----	-----A
rec3(e)	-----	--PR-8--	-----	-----	-----	-----
rec56(e)	-----	--PR-8--	-----	-----	-----	-----
M.g. (pub)	.CCA	-----	-----	-----	-----	-----
M.g.	-----	--PR-8--	-----	-----	-----	-----
rec3(g)	-----	--PR-8--	-----	-----	-----	-----
rec56(g)	-----	--PR-8--	-----	-----	-----	-----

				MseI	DdeI	
M.e. (pub)	CTACATATAA	CAAAAAGATC	AGCTATCCAT	CATCATATAA	AGCTAAGACA	AGTTATCCCTC
M.e.	-----	-----	-----	-----	-----	-----
rec3(e)	-----	-----	-----	-----	-----	-----T
rec56(e)	-----	-----	-----	-----	-----	-----C-----T
M.g. (pub)	..G.....A	.....G	.....G	..CA..T	..TG	.....
M.g.	..G.....A	.....G	.....G	..CA..T	..TG	.....
rec3(g)	..G.....A	.....G	.....G	..CA..T	..TG	.....
rec56(g)	..G.....A	.....G	.....G	..CA..T	..TG	.....

	NdeI		stop			
M.e. (pub)	CAGCATATAA	ACCAACAAAC	AGATATTAAT	CTCAATATTA	AAAGTATTAA	CTAAAATATT
M.e.	-----	-----	-----	-----	-----	-----
rec3(e)	-----	-----	-----	-----	-----	-----
rec56(e)	..A	-----	-----	-----	-----	-----
M.g. (pub)	A.....G	.....C	-----	-----	-----	-----T
M.g.	A.....G	.....C	-----	-----	-----	-----T
rec3(g)	A.....G	.....C	-----	-----	-----	-----T
rec56(g)	A.....G	.....C	-----	-----	.....G	-----

M.e. (pub)	CACATTACTG	TACTACACAT	TTTAACGTTT	GATTATGATG	GGAACAGATG	AACATTTGAA
M.e.	-----	-----	-----	-----	-----	-----
rec3(e)	-----	-----	-----	..G	-----	-----
rec56(e)	-----	-----	-----	..G	-----	-----
M.g. (pub)	..T	-----	-----	..G	-----	-----
M.g.	..T	-----	-----	..G	-----	-----
rec3(g)	..T	..C	-----	..G	-----	-----
rec56(g)	..G	-----	-----	..G	-----	-----

**Fig. 3** Alignment of *Glu-3'* sequences. *Mytilus edulis* and *M. galloprovincialis* sequences are designated as M.e. and M.g., respectively. Also given are the sequences for *edulis* (e) and *galloprovincialis* (g) alleles from two mussels (rec3 and rec56) which carried recombinant genotypes. Annealing sites, for the primers JH-4 and PR-8, alignment gaps and sequence similarity are indicated as in Fig. 2. Restriction sites used in this study are underlined and the stop codon for each taxon is indicated by a box.

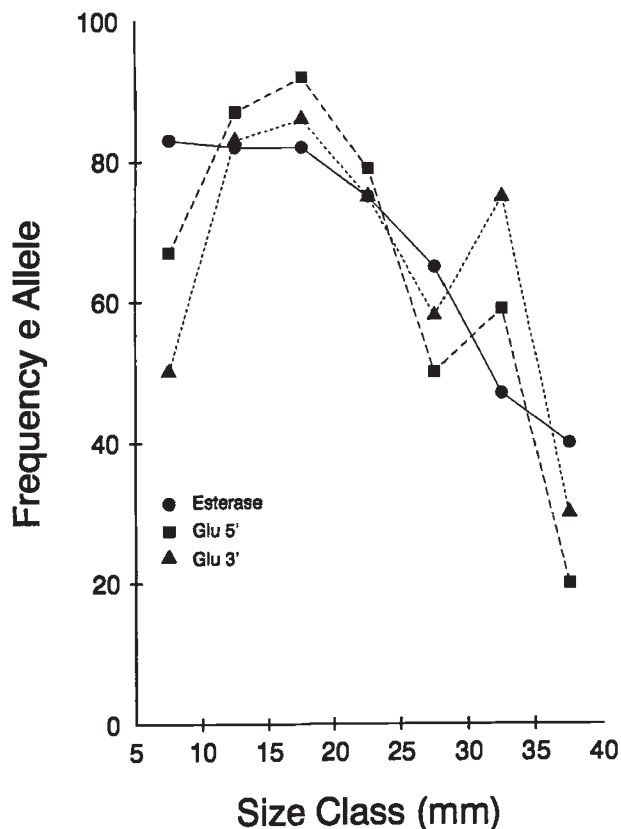
ing that these mussels carried alleles in which recombination has occurred between the 5' and 3' ends of the *Glu* 'giant' exon. This phenomenon was most pronounced in the <10 mm size class (Fig. 5). In cases where the two genotypes disagreed, the majority (57 per cent) were homozygous for an *M. edulis* allele at the *Glu-5'* marker and were heterozygous at the *Glu-3'* marker. Of the remainder, 33 per cent were homozygous for the *M. galloprovincialis* allele at the *Glu 5'* marker and heterozygous at the *Glu 3'* marker whereas 10 per cent were heterozygous at the *Glu-5'* marker and homozygous for *M. edulis* or *M. galloprovincialis* alleles at the *Glu-3'* marker.

## Discussion

We have developed a pair of gene-specific PCR-based genetic markers that reliably identify individual taxa within the *M. edulis* species complex. Both of these markers target the 'giant exon' of the gene encoding the mussel polyphenolic adhesive protein described previously for *M. edulis* (Filpula *et al.*, 1990) and *M. galloprovincialis* (Inoue & Odo, 1994); we have designated this the *Glu* locus. The *Glu* gene has been characterized as a single locus by Filpula *et al.* (1990). The first marker, *Glu-5'*, lies at the 5' end of the giant exon and the variation in banding pattern among the allopatric populations of the three species of *Mytilus* appears to be diagnostic

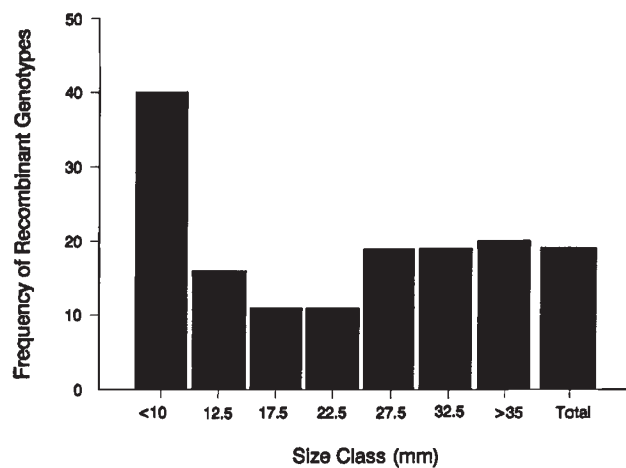
(Table 1). We have analysed the segregation of the *Glu-5'* marker in pair-matings of *M. edulis* and *M. trossulus* provided by Dr E. Zouros and find that the patterns of segregation are consistent with *Glu* being a single autosomal locus. The second marker, *Glu-3'*, lies at the 3' end of the same exon and is highly differentiated among allopatric populations of *M. edulis* and *M. galloprovincialis*. Unfortunately, we have been unable to identify assay conditions that will allow amplification of this marker from *M. trossulus*.

We have genotyped mussels from allopatric populations of *M. edulis* and *M. galloprovincialis* using both the *Glu-5'* and *Glu-3'* markers. In all cases ( $n = 142$ ) an individual's *Glu-5'* genotype was identical to its *Glu-3'* genotype. However, nearly 20 per cent of the mussels collected from the hybrid populations in Whitsand Bay, UK, had genotypes at the two markers that did not correspond, indicating the possibility that recombination or gene conversion has occurred between the 5' and 3' ends of the *Glu* gene. It is possible, however, that rather than being the result of intragenic recombination these genotypes are produced by a single base pair change that eliminates the *DdeI* restriction site from *M. edulis* *Glu-3'* alleles or creates a restriction site in *M. galloprovincialis* *Glu-3'* alleles. We performed two experiments to assess this possibility. First, we cloned and sequenced the *Glu-3'* PCR products for two mussels which were homozygous for alternate alleles at



**Fig. 4** Frequency of *Mytilus edulis* (e) alleles at the *Esterase-D* (*Est*), *Glu-5'* and *Glu-3'* markers as a function of size for the Whitsand Bay, UK, hybrid zone population. For estimating allele frequencies at the *Glu-5'* marker, *M. edulis*-specific 350 bp and 380 bp products were treated as separate alleles whereas the *M. galloprovincialis* 300 bp and 500 bp products were assumed to be produced by a single allele (see text). Thus, mussels with 350 bp, 380 bp, or 350/380 bp banding patterns were scored as *M. edulis* homozygotes, those with 300 bp patterns were scored as *M. galloprovincialis* homozygotes and those with a 300 bp band in combination with either the 350 bp or 380 bp band were scored as heterozygotes.

*Glu-5'* but were putative heterozygotes at *Glu-3'*. These two mussels carried distinct *M. galloprovincialis* and *M. edulis* alleles at the *Glu-3'* marker (Fig. 3) indicating that they each carried recombinant alleles. Secondly, we used the restriction enzymes *MseI* and *NdeI* to analyse PCR products of the *Glu-3'* marker from individuals that exhibited putative recombinant phenotypes. The *Glu-3'* allele from *M. galloprovincialis* contains restriction sites for both *MseI* and *NdeI* which are absent in the *M. edulis* allele. If these animals contain *M. edulis* alleles that have lost the *DdeI* site or *M. galloprovincialis* alleles that have gained the site, they should remain homozygous at



**Fig. 5** Frequency of recombinant *Glu* genotypes as a function of size in the Whitsand Bay, UK, population of *Mytilus*.

the other two restriction sites. If an individual is a heterozygote for the *DdeI* site because of recombination between the *Glu-5'* and *Glu-3'* markers then the *MseI* and *NdeI* sites should also be heterozygous. As these two restriction sites occur on either side of the *DdeI* site it is possible for recombination within the *Glu-3'* marker to generate a heterozygote at one restriction site but not the other. In all cases ( $n = 21$ ), putatively recombinant mussels were heterozygous for both the *MseI* and *NdeI* restriction sites indicating that they represent alleles in which recombination has occurred between the *Glu-5'* and *Glu-3'* markers.

The observed frequency of genotypes that are heterozygous for recombinant *Glu* alleles (approximately 20 per cent) suggests that the frequency of recombinant alleles is approximately 10 per cent. The presence of rare or novel allozyme alleles has been noted in many hybrid zone studies (see Barton & Hewitt, 1985) and rare electromorphs may occasionally attain high frequencies within hybrid populations (Woodruff, 1989). Woodruff (1989) has discussed possible mechanisms for the production of novel alleles in hybrid individuals including post-translational modification of proteins, mutation and intragenic recombination. Recently, Bradley *et al.* (1993) demonstrated that a novel alcohol dehydrogenase allele in *Geomys* was produced through a single point mutation. In our study of *Mytilus*, we have shown that the appearance of a novel *Glu* allele in a hybrid population is not caused by a single base substitution but is probably an intragenic recombination. The mechanisms that maintain the high frequency of novel alleles in hybrid populations, whether arising from mutation or recombina-

tion, are currently being debated. However, the high frequency of recombinant *Glu* alleles in Whitsand Bay suggests that hybridization is relatively common and has been occurring for a substantial period of time. In contrast, intragenic recombination is expected to be rare in hybrid zones that have recently been established or in regions of sympatry where hybridization is infrequent and would lead to a low frequency of novel alleles (Woodruff, 1989).

Each pair of the three sibling species of *Mytilus* is highly differentiated at three to four enzyme-coding genes but none of these loci is completely diagnostic. The *Glu* gene provides an additional marker which is highly differentiated among these sibling species that can be used in the taxonomy of *Mytilus* and in studies of hybridization among species. Hybridization occurs wherever two blue mussel species co-occur and additional genetic markers will be invaluable in assessing the dynamics of these hybrid zones. For example, in mussel populations containing hybrids of *M. edulis* and *M. galloprovincialis* in south-west England, viability selection generates a strong relationship between allozyme allele frequencies and size (Skibinski, 1983; Gardner & Skibinski, 1988, 1991; Wilhelm, 1993). In a hybrid population at Whitsand Bay the *Glu* gene exhibits a virtually identical relationship between allele frequency and size, as do several allozyme loci, indicating that the *Glu* gene is at least as sensitive as enzyme-coding genes in revealing patterns of natural selection in this hybrid population.

The similarity in behaviour between the *Glu* gene and allozyme loci may indicate that these genes are all linked and mark a single section of the genome that is under selection in the hybrid population at Whitsand Bay. Beaumont (1994), for example, demonstrated linkage among some allozyme loci in *M. edulis*, although he did not specifically address linkage among the three allozymes used in this study. It is unlikely that the *Glu* gene is strongly linked to any of the three allozyme loci used in this study. First, the allozyme loci examined here (*Est*, *Lap* and *Mpi*) do not appear to be closely linked; recombinant genotypes are found frequently within hybrid populations (Wilhelm, 1993) and different allozyme alleles do not cosegregate with physiological differences between *M. edulis* and *M. galloprovincialis* to the same degree (Hilbish *et al.*, 1994). Secondly, recombinants between the *Glu* markers and the allozyme genes were frequently observed. Finally, alleles formed by recombination within the *Glu* gene appear to be relatively common, indicating that linkage between this gene and other loci is unlikely. These data suggest that *Glu* is likely to

segregate independently of other gene loci that are highly differentiated between *M. edulis* and *M. galloprovincialis*.

PCR-based assays of genetic markers may also be valuable because they are more sensitive than allozyme methods and can be used in the routine analysis of minute quantities of material, such as mussel larvae or spat. Corte-Real *et al.* (1994a,b) have recently reported a PCR assay of the calmodulin gene and demonstrated that it can be used to analyse individual mussel larvae. Using the procedures described above we have successfully used both *Glu* markers to assay recently settled mussel spat that were preserved in ethanol using the procedures outlined in this paper (data not shown). The PCR-based assay of the *Glu* gene, described here, provides a tool by which the exchange of larvae among genetically differentiated mussel populations and the strength of natural selection within hybrid larval populations can be assessed.

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### References

- AVISE, J. C. 1994. *Molecular Markers, Natural History and Evolution*. Chapman and Hall, New York.
- BARTON, N. H. AND HEWITT, G. M. 1985. Analysis of hybrid zones. *Ann. Rev. Ecol. Syst.*, **16**, 113–148.
- BEAUMONT, A. R. 1994. Linkage studies in *Mytilus edulis*, the mussel. *Heredity*, **72**, 557–562.
- BRADLEY, R. D., BULL, J. J., JOHNSON, A. D. AND HILLIS, D. M. 1993. Origin of a novel allele in a mammalian hybrid zone. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 8939–8941.
- CORTE-REAL, H. B. S. M., DIXON, D. R. AND HOLLAND, P. W. H. 1994a. Intron-targeted PCR: a new approach to survey neutral DNA polymorphism in bivalve populations. *Mar. Biol.*, **120**, 407–413.
- CORTE-REAL, H. B. S. M., HOLLAND, P. W. H. AND DIXON, D. R. 1994b. Inheritance of a nuclear DNA polymorphism assayed in single bivalve larvae. *Mar. Biol.*, **120**, 415–420.
- FILPULA, D. R., LEE, S. M., LINK, R. P., STRAUSBERG, S. L. AND STRAUSBERG, R. L. 1990. Structural and functional repetition in a marine mussel adhesive protein. *Biotechnol. Prog.*, **6**, 171–177.
- FREEMAN, K. R., PERRY, K. L., DIBACCO, T. G. AND SCAR-RATT, D. J. 1994. Observations on two Mytilid species from a Nova Scotian mussel farm. *Can. Tech. Report Fish. Aquat. Sci.*, **1969**, 1–47.



- GARDNER, J. P. A. AND SKIBINSKI, D. O. F. 1988. Historical and size-dependent genetic variation in hybrid mussel populations. *Heredity*, **61**, 93–105.
- GARDNER, J. P. A. AND SKIBINSKI, D. O. F. 1991. Biological and physical factors influencing genotype-dependent mortality in hybrid mussel populations. *Mar. Ecol. Prog. Ser.*, **71**, 235–244.
- GOSLING, E. M. 1992a. Systematics and geographic distribution of *Mytilus*. In: Gosling, E. M. (ed.) *The Mussel Mytilus: Ecology, Physiology, Genetics and Culture*, pp. 1–20. Elsevier, Amsterdam.
- GOSLING, E. M. 1992b. Genetics of *Mytilus*. In: Gosling, E. M. (ed.) *The Mussel Mytilus: Ecology, Physiology, Genetics and Culture*, pp. 309–382. Elsevier, Amsterdam.
- HARRISON, R. G. AND RAND, D. M. 1989. Mosaic hybrid zones and the nature of species boundaries. In: Otte, D. and Endler, J. A. (eds) *Speciation and its Consequences*, pp. 111–133. Sinauer Associates, Sunderland, MA.
- HEWITT, G. M. 1990. Divergence and speciation as viewed from an insect hybrid zone. *Can. J. Zool.*, **68**, 1701–1715.
- HILBISH, T. J., BAYNE, B. L. AND DAY, A. 1994. Genetics of physiological differentiation within the marine mussel genus *Mytilus*. *Evolution*, **48**, 267–286.
- INOUE, K. AND ODO, S. 1994. The adhesive protein cDNA of *Mytilus galloprovincialis* encodes decapeptide repeats but no hexapeptide motif. *Biol. Bull.*, **186**, 349–355.
- KOEHN, R. K. 1991. The genetics and taxonomy of species in the genus *Mytilus*. *Aquaculture*, **94**, 125–145.
- MCDONALD, J. H., SEED, R. AND KOEHN, R. K. 1991. Allozymes and morphometric characters of three species of *Mytilus* in the Northern and Southern Hemispheres. *Mar. Biol.*, **111**, 323–333.
- SKIBINSKI, D. O. F. 1983. Natural selection in hybrid mussel populations. In: Oxford, G. S. and Rollinson, D. (eds) *Protein Polymorphism: Adaptive and Taxonomic Significance*, pp. 283–298. Academic Press, London.
- SKIBINSKI, D. O. F., CROSS, T. F. AND AHMAD, M. 1980. Electrophoretic investigation of systematic relationships in the marine mussels *Modiolus modiolus* L., *Mytilus edulis* L. and *Mytilus galloprovincialis* Lmk. (Mytilidae: Mollusca). *Biol. J. Linn. Soc.*, **13**, 65–73.
- WAITE, J. H. 1992. The formation of mussel byssus: anatomy of a natural manufacturing process. In: Case, S. T. (ed.) *Structure, Cellular Synthesis and Assembly of Biopolymers. Results and Problems in Cell Differentiation*, vol. 19, pp. 55–74. Springer, Berlin.
- WILHELM, R. 1993. *Genotype-Specific Selection within a Hybrid Population of the Mussel Genus Mytilus*. Masters thesis, University of South Carolina, Columbia, SC.
- WOODRUFF, D. S. 1989. Genetic anomalies associated with *Cerion* hybrid zones: the origin and maintenance of new electromorphic variants called hybridzymes. *Biol. J. Linn. Soc.*, **36**, 281–294.