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SHORT COMMUNICATION

Cytonuclear disequilibrium in a hybrid zone involving deep-sea hydrothermal vent mussels of the genus *Bathymodiolus*

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Abstract

A hybrid zone involving the deep-sea mussels, *Bathymodiolus azoricus* and *B. puteoserpentis*, was recently discovered at Broken Spur hydrothermal vent field (29°10′ N, 43°10′ W) along an intermediate segment of the Mid-Atlantic Ridge axis. Examination of nuclear (allozymes) and cytoplasmic (mitochondrial DNA) gene markers in a new sample from Broken Spur revealed significant cytonuclear disequilibrium caused by an excess of the parental types (coupling phase) and a deficiency of recombinants (repulsion phase). An assignment test of individual multilocus genotypes also revealed an excess of parental genotypes in the admixed population. These results support the hypothesis that the Broken Spur mussel population comprises a nonequilibrium mixture of parental immigrants and hybrid individuals.

Keywords: allozymes, assignment test, *Bathymodiolus*, hybridization, Mid-Atlantic Ridge, mtDNA

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Introduction

Marine hybrid zones may be more common than previously thought (Gardner 1997). Although most examples involve coastal species, O'Mullan *et al*. (2001) reported the first mid-ocean hybrid zone, involving *Bathymodiolus* mussels from deep-sea hydrothermal vents along the Mid-Atlantic Ridge (MAR). The two parental species *Bathymodiolus azoricus* and *B. puteoserpentis* can be distinguished morphologically and genetically (von Cosel *et al*. 1994, 1999; Craddock *et al*. 1995a; Maas *et al*. 1999). *Bathymodiolus azoricus* appears to be restricted to shallower vents (< 2500 m) along a northern region of the MAR axis (36° N to 38° N latitude), and *B. puteoserpentis* occurs at deeper vents (> 3000 m) along a southern region of the axis (14° N to 23° N). The two regions are separated by 1800 km of ridge axis that appears to be inhospitable for mussels (Desbruyères *et al*. 2000a). One vent field in this intermediate region, Broken Spur, houses a small population of mussels that appear to be hybrids between the northern and southern species (O'Mullan *et al*. 2001). Both 'northern' and 'southern' mitochondrial types that distinguish the two species occur together at Broken Spur and allozyme frequencies there are intermediate. The small sample of mussels ($N = 11$) collected from this locality in 1997 exhibited marginally significant cytonuclear disequilibrium, marked by an excess of parental types. O'Mullan *et al*. (2001) hypothesized that the long-distance dispersal capabilities of *Bathymodiolus* larvae (Lutz *et al*. 1979; Craddock *et al*. 1995b; O'Mullan *et al*. 2001; Won *et al*. 2003) would place this intermediate locality within the dispersal range of the two parental forms; thus, the hybrid zone may be a product of immigration of parental forms and some local interbreeding.

In this study, we exploit a new and larger sample of mussels from the Broken Spur vent field to assess better the hypothesis that this population is a product of recent immigration and hybridization. A new expedition to the Broken Spur locality during July 2001 succeeded in collecting 38 of these scarce mussels. We tested the combined

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sample of 49 mussels for cytonuclear disequilibrium of allozyme and mitochondrial DNA markers. We also applied a multilocus (allozyme/mitochondrial DNA) genotypic assignment test to assess the likely origins of individual mussels as immigrants vs. hybrids.

Materials and methods

Adult mussels were collected during *Alvin/Atlantis* expeditions to the MAR during July 1997 and July 2001 (see Fig. 1, O'Mullan *et al*. 2001). We visited all MAR hydrothermal vent localities known to us at the time. Vent names, coordinates, depths and *Alvin* dive numbers for the 1997 expedition were previously described (Table 1, O'Mullan *et al*. 2001). Mussels examined in this study were derived from six MAR localities (from north to south): Menez Gwen (abbreviation MG; 37°50′ N, 31°31′ W); Lucky Strike (LS; 37°17′ N, 32°15′ W); Rainbow (RB; 36°14′ N, 33°54′ W); Broken Spur (BS; 29°10′ N, 43°10′ W); Snake Pit (SP; 23°22′ N, 44°56′ W); and Logatchev (LO; 14°45′ N, 44°58′ W). The July 2001 expedition re-visited the LS, RB, BP, SP and LO localities. During 2001, a new hydrothermal vent named Lost City (30°7′ N, 42°07′ W) was discovered near the Broken Spur vent field (Kelley *et al*. 2001). However, the 2001 and subsequent expeditions to Lost City found no typical hydrothermal vent organisms or mussels that could be a source of immigrants for Broken Spur.

Five allozyme loci were examined that had been recorded as polymorphic in previous studies (Maas *et al*. 1999; O'Mullan *et al*. 2001). Cellulose acetate gel electrophoresis (CAGE) techniques, methods of tissue preparation, sample extraction, and gel staining for *Bathymodiolus* were performed as described previously (Maas *et al*. 1999; O'Mullan *et al*. 2001; Won *et al*. 2003).

Methods for analysis of DNA sequences and restriction fragment length polymorphisms (RFLP) of mitochondrial haplotypes in MAR mussels were previously described (Maas *et al*. 1999). Previous studies revealed two major haplotypes marked by the NADH dehydrogenase subunit 4 gene (ND4) that distinguish the two MAR mussel species: MT*pu* from *Bathymodiolus puteoserpentis* (GenBank accession number AF128533) and MT*az* from *B. azoricus* (AF128534). Minor variants of each haplotype exist, but on average MT*az* and MT*pu* exhibit 10.3% sequence divergence across 646 nucleotide bases (O'Mullan *et al*. 2001). The present study examined species-diagnostic RFLP patterns that distinguish the two major ND4 sequences (Maas *et al*. 1999).

F-statistical analyses and exact tests of population differentiation were conducted with GENEPOP (v. 3.3, Raymond & Rousset 1995b). Exact tests of cytonuclear disequilibrium were conducted with the CNDM program (Asmussen & Basten 1994; Basten & Asmussen 1997). Likelihood ratio contingency tests and other basic statistical tests were performed with JMP (SAS Institute 2000). To assign individuals to parental populations and identify putative hybrids, we applied STRUCTURE (ver. 2.0, Pritchard *et al*. 2000) (http://pritch.bsd.uchicago.edu) as recently implemented

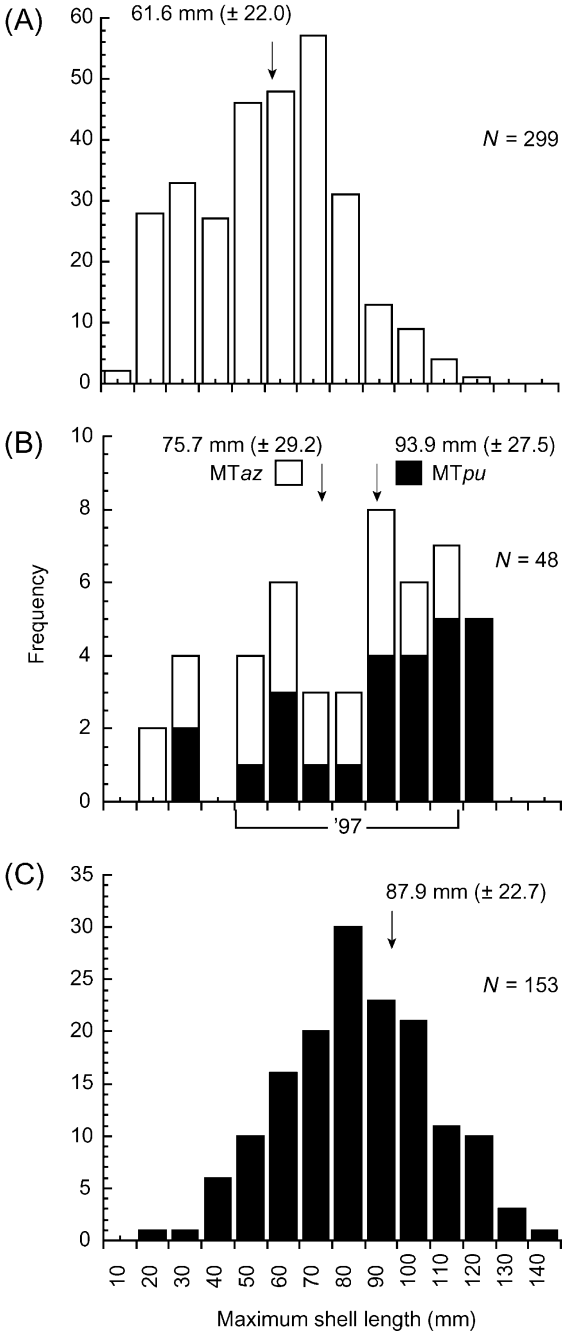

Fig. 1 Size-frequency distributions of deep-sea *Bathymodiolus* mussels from the MAR: (A) the northern species, *B. azoricus*; (B) the Broken Spur hybrid-zone population; and (C) the southern species, *B. puteoserpentis*. For B, the open bars indicate individuals with the MT*az* haplotype, and the black bars represent individuals with MT*pu*. Mean shell lengths (± standard deviations) are indicated with arrows.

Table 1 Observed and expected (in parentheses) combinations of *Lap* and mitochondrial ND4 genotypes in the Broken Spur sample

	Mitochondrial DNA haplotype		
Lap	MT*az*	MT*pu*	Total
Genotypic			
A/A	5 (2.3)*	0 (2.7)*	5
A/B	5 (5.6)	7 (6.4)	12
A/C	5 (2.3)*	0 (2.7)*	5
B/B	5 (6.1)	8 (6.9)	13
B/C	2 (4.2)	7 (4.8)	9
C/C	1 (2.3)	4 (2.7)	5
Total	23	26	49
Allelic			
A	20 (12.7)**	7 (14.3)**	27
B	17 (22.1)*	30 (24.9)*	47
C	9 (11.3)	15 (12.7)	24
Total	46	52	98

For joint allelic counts, numbers of each mitochondrial haplotype were doubled as if they comprised homozygous diploid genotypes (see Basten & Asmussen 1997). Significant single cell deviations are indicated by *$P < 0.05$ uncorrected; **$P < 0.0028$, following table-wide Bonferroni correction (Rice 1989).

in a study of introgression in Scottish wildcats (Beaumont *et al*. 2001). This program uses a Bayesian clustering method to assign each multilocus genotype to one of K probable source populations. Assignment scores (q) correspond to the probability of ancestry in a source population. A Markov chain Monte Carlo (MCMC) method was used to estimate posterior probability distributions for each parameter integrated over all the other parameters. First, it was assumed that prior values of K ranged from 2 to 5 and the means were then estimated for the individual admixture proportions and their 95% probability intervals. Simulations involving 500 000 MCMC iterations followed a burn-in period of 50 000 iterations under a model that allowed the admixture of individuals drawn from more than one source population. Five allozyme plus mitochondrial DNA genotypes were combined to achieve maximum resolving power. The allozyme and mitochondrial data for the parental populations derived from a previous study (O'Mullan *et al*. 2001).

Results and Discussion

Mussels were scarce at BS in 1997, so a concerted effort was made to search this site for more specimens in 2001. Only shell remnants were observed during the first dive (*Alvin* dive no. 3675; 18 July 2001; 29°10.02′ N, 43°10.44′ W; depth 3045 m; G. O'Mullan and S. Hallam, observers). The second dive (no. 3676; 19 July 2001; 29°10.03′ N, 43°10.45′ W; depth 3061 m; C. Van Dover and G. O'Mullan, observers) found and collected 38 individuals from the vicinity of Spire, a 12-m sulphide edifice from which the 1997 sample was taken (O'Mullan *et al*. 2001). Additional scattered mussels were observed just north of Spire on dive 3676, but low battery power in *Alvin* did not allow time for collection. New mussel samples were also obtained in 2001 from parental populations housing *Bathymodiuolus azoricus* (LS and RB) and *B. puteoserpentis* (SP and LO).

The new 2001 sample of mussels was screened for mitochondrial RFLPs from the following numbers of individuals: LS (12); RB (9); BS (38) SP (10); and LO (10). Additional individuals were also examined from the 1997 MG sample (3). Combined with the 1997 samples from O'Mullan *et al*. (2001), total sample sizes for the mitochondrial analysis were: MG (19); LS (28); RB (18); BS (49) SP (22); and LO (26). All mussels from the northern region ($N = 65$) had the *B. azoricus* MT*az* haplotype, and all mussels from the southern region ($N = 48$) had the *B. puteoserpentis* MT*pu* haplotype. The sample of 49 mussels from BS had an even mixture of the two haplotypes (MT*az* = 23 and MT*pu* = 26). Frequencies of the two haplotypes did not change significantly between the 1997 and 2001 samples (likelihood ratio $\chi^2 = 0.919$, d.f. = 1, $P = 0.3379$).

Mussels from the parental regions and hybrid zone differed significantly in size-frequency distributions (Fig. 1). Northern, *B. azoricus*, mussels were smaller on average than those from the hybrid zone and southern, *B. puteoserpentis*, mussels [Tukey–Kramer HSD (Honestly Significant Difference) test, $\alpha = 0.05$]. Size-frequencies were normally distributed in *B. puteoserpentis* (Shapiro–Wilk *W*-test for normality; $P = 0.6490$), but not in *B. azoricus* ($P = 0.0019$) or the hybrid-zone mussels ($P = 0.0027$), which exhibited evidence of size bimodality. Overall, hybrid-zone mussels bearing MT*pu* haplotypes were larger than those bearing the MT*az* haplotypes ($F_{1,46} = 5.003$; $P = 0.0302$; Fig. 1B). Also, the 2001 hybrid-zone sample contained six small individuals (< 40 mm) that might represent new recruits since the 1997 visit, although we do not know how fast these mussels might have grown, or whether similar small individuals might have been overlooked in 1997. Regardless of these facts, the large mussels (> 40 mm) were likely comprised of individuals from generations that overlapped between the 1997 and 2001 samples.

We examined allozyme variation encoded by five polymorphic loci in the new sample ($N = 38$) of mussels collected from Broken Spur in 2001 and compared them to the 1997 sample from the same locality ($N = 11$). Exact tests of genetic differentiation (Raymond & Rousset 1995a) between the 1997 and 2001 subsamples were not significant for single loci or across all five loci ($\chi^2 = 7.061$; $P = 0.720$). No evidence existed for deviations from random mating expectations in the small 1997 sample (total fixation index across loci, $F_{IS} = -0.094$; not significant). However, the larger 2001 sample exhibited significant heterozygote

deficiencies at three loci (f_{Lap} = 0.255, P = 0.014; f_{Pgi} = 0.320, P = 0.029; $f_{Mdh\text{-}1}$ = 0.335, P = 0.030), although the total deficiency across all loci (F_{IS} = 0.205) was not significant (χ^2 = 17.3; d.f. = 10; P = 0.068). Pooling the 1997 and 2001 BS subsamples produced a total sample of 49 that exhibited a marginally significant heterozygote deficiency at one locus ($f_{Mdh\text{-}1}$ = 0.317, P = 0.051), although the total deficiency across all loci (F_{IS} = 0.146) was not significant (χ^2 = 11.6; d.f. = 10; P = 0.315). Because no evidence existed for divergence (i.e. Wahlund effect) between the two subsamples, and because the subsamples overlapped in size (and presumably age classes), we felt confident in combining them for subsequent analyses.

The combined BS sample mussels (N = 49) provided a new opportunity to examine linkage disequilibrium in the hybrid zone. Alternative mitochondrial types are fixed in the two MAR species, and the *Lap* locus exhibits sufficient divergence between two species to warrant a test of disequilibrium in the hybrid zone. All other allozyme loci were similarly tested but significant disequilibria were not observed between pairs of allozyme loci or between allozyme loci and mitochondrial DNA. O'Mullan *et al.* (2001) previously showed that the *Lap*A* and **B* alleles occur in *B. azoricus* (mean frequencies, p = 0.626 and q = 0.358), whereas the *Lap*B* and **C* alleles occur in *B. puteoserpentis* (q = 0. 599 and r = 0.369). Because the *Lap*A* and **C* alleles essentially distinguish the two species, they could be used to assess cytonuclear disequilibrium of the hybrid-zone mussels. Observed and expected combinations of *Lap* and mitochondrial (MT) types are listed in Table 1. Expected values were generated by the contingency method, which assesses the independence of the two markers but not any underlying assumptions about genotypic and allelic equilibrium. We used the CNDM program (Basten & Asmussen 1997) which employs the MCMC method to test the overall departure from random cytonuclear associations. This method also assesses the likelihood of disequilibrium for individual cells in the genotypic and allelic matrices. We relied exclusively on Fisher's exact tests which tend to be more accurate with sample sizes below 100 (Basten & Asmussen 1997). MCMC analysis based on 100 000 replications (100 batches of 1000) of the genotypic matrix (Table 1) rejected the null hypothesis of no overall association between *Lap* genotypes and MT types (P = 0.006). Reconfiguration of the genotypic matrix into a series of 2×2 exact tests revealed that the overall disequilibrium derived mostly from an excess of the *Lap*A*/*A*-MT*az* combination ($D_{AA,az}$ = 0.054, nr^2 = 6.294, P = 0.018) and a corresponding deficiency of *Lap*a*/*a*-MT*az* combinations ($D_{a/a,az}$ = –0.0954, nr^2 = 7.234, P = 0.010), where *a*/*a* represents the alternative genotypes comprised of *Lap*B* and **C* alleles. Likewise, an MCMC analysis of the resulting allelic matrix (Table 1, 100 000 replications) also rejected the null hypothesis of cytonuclear equilibrium (P = 0.003). The rejection derived mostly from an excess of the *Lap*A*-MT*az* combination ($D_{A,az}$ = 0.075, nr^2 = 9.200, P = 0.001) and a corresponding deficiency of *Lap*A*-MT*pu*. These results were not the result of pooling of the temporal subsamples, because the same patterns were evident in the 2001 ($D_{A,az}$ = 0.066, nr^2 = 5.003, P = 0.014, N = 38) and 1997 subsamples ($D_{A,az}$ = 0.087, nr^2 = 3.766, P = 0.167, N = 11), though $D_{A,az}$ was not significant in 1997. Because the *Lap*A*-MT*az* combination defines the coupling phase for *B. azoricus*, whereas *Lap*C* and MT*pu* defines the coupling phase for *B. puteoserpentis*, this excess of coupling types is consistent with the immigration of parental types into the hybrid zone or selection against hybrids.

Direct evidence for the existence of hybrids at the BS locality was found by examining individual genotypes. For example, the *Lap*A* and *Lap*C* alleles are essentially restricted to the northern and southern species, respectively. However, in the BS sample, *Lap*A* and **C* alleles occurred together in five heterozygotes that had northern MT*az* haplotype (Table 1). These heterozygotes are likely to be F_1 hybrids with a northern maternal parent. Evidence for backcrossing was found in individuals with recombinant genotypes. For example, seven individuals carrying the northern allele (*Lap*A*/*B*) had the southern MT*pu* haplotype, and conversely, three individuals carrying the southern allele (*Lap*C*/–) had the northern MT*az* haplotype. Other allozyme loci were not strongly differentiated between the parental species (O'Mullan *et al*. 2001) and, thus, provided limited power for discriminating hybrids.

Analysis of multilocus genotypes with the STRUCTURE program (Pritchard *et al*. 2000) was clearly consistent with the existence of two allopatric species (Fig. 2). This analysis illustrates unique properties of the BS hybrid-zone mussels. With the current multilocus data, we obtained the highest posterior probability for the existence of two clusters (K = 2; $P(K \mid X)$ = 0.982. assuming a uniform prior for K ($K \in [2, 3, 4, 5]$)). Using the admixture model in STRUCTURE, we estimated the probability of ancestry ($\hat{q}$) for each individual in one of the two species. Figure 2 shows the $\hat{q}$ values and 95% probability intervals for each individual (N = 160). Individuals with near zero $\hat{q}$ values and narrow 95% probability intervals are assigned to the southern species and vice versa for northern species. Some of northern mussels had wide probability intervals, which was expected, because the RB and LS localities exhibit evidence for introgression of southern allozymes (O'Mullan *et al*. 2001). On the other hand, southern individuals have narrower 95% intervals, suggesting that introgression from the northern species is unlikely. Except for one northern individual from the RB locality (probability interval 0.243–1), none of the other individuals from the parental populations overlapped in the distribution of their 95% probability intervals for q. In contrast, the Broken Spur hybrid-zone sample of 49 mussels had a large proportion of individuals

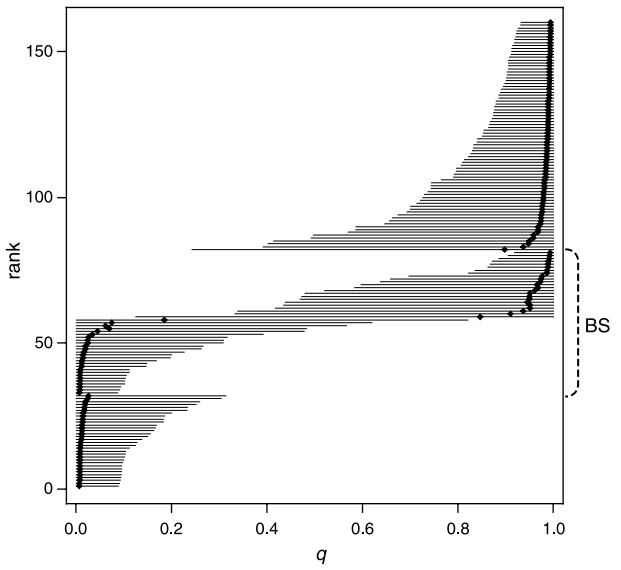

Fig. 2 STRUCTURE analysis of multilocus genotypes from parental and hybrid-zone mussels. Individual values of $\hat{q}$ were separately ranked within northern, hybrid zone, and southern vent fields from top to bottom, respectively. Horizontal lines represent 95% posterior probability intervals for each individual. Diamonds mark the mean values of $\hat{q}$.

that overlapped one or the other parental type and a few individuals that overlapped both and were likely candidates for hybrids.

Conclusions

The present analysis supports the hypothesis that mussels from Broken Spur vent field of the Mid-Atlantic Ridge comprise an admixed population of hybrids and parental forms (O'Mullan *et al*. 2001). The Broken Spur sample contained an excess of parental genotypes from the northern and southern regions and a deficiency of recombinants. Evidence also existed for local reproduction in the genotypes of putative hybrids. Nevertheless, a paucity of hybrids at this locality and an absence of mussels at TAG (Trans-Atlantic Geotravese) hydrothermal mound, which is 375 km to the south, suggest that this intermediate ridge segment is not likely to sustain a stable hybrid zone (*sensu* Barton 1979; Barton & Hewitt 1985). Unlike the parental regions to the north and south, both the BS and TAG hydrothermal vents emit high levels of toxic particulate materials (polymetalic sulphides) that are suspected of fouling the gills of filtering species like mussels (Desbruyères *et al*. 2000a,b). Biological composition varies greatly from year to year at Broken Spur (Murton & Van Dover 1993; Copley *et al*. 1997). Obviously, local geochemical conditions have been conducive to the settlement and growth of mussels at this site and some recruitment may have occurred between our 1997 and 2001 expeditions. In contrast, a mussel population at TAG hydrothermal mound appears to have disappeared. A single living mussel was collected there in 1985 and shell debris was also observed (P. Rona, personal communication), but mussels have not been seen there during subsequent American, French, Japanese, or Russian expeditions through 2001. Hybrid mussel populations are probably formed at these sites during briefly hospitable periods. Given the long-distance dispersal capabilities of *Bathymodiolus* mussel larvae (Lutz *et al*. 1979; Craddock *et al*. 1995b; Won *et al*. 2003), it is likely that most locations along this intermediate ridge segment are within the dispersal range of the parental species. However, it is impossible to tell from the present data whether weak barriers to interspecific matings might limit the number of hybrids that form at these intermediate localities, if hybrids suffer a selective disadvantage, or if colonization of Broken Spur was so recent that mussel hybrids have had little time to form. Finally, we cannot discount the possibility that hybrid mussels immigrated from an undiscovered vent field, because exploration of this intermediate region of the ridge axis has focused mostly on the few known vent fields. Clearly, more detailed exploration of this region of the MAR axis is warranted.

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