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Habitat reversal in vent and seep mussels: seep species, *Bathymodiolus heckerae*, derived from vent ancestors

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Introduction

Bathymodiolus mussels are conspicuous members of deep-sea hydrothermal vent and cold-water seep communities. Phylogenetic studies revealed that the ancestors of this genus first exploited decaying wood, whale bones, and other sites of organic enrichment and diversified at progressively deeper environments in sulphide/hydrocarbon seeps (Craddock et al., 1995; Distel et al., 2000). Occupation of hydrothermal vents appears to have happened relatively recently. Nevertheless, it remains unclear from these studies whether the vent mussels comprise a specialized monophyletic group, or if habitat evolution is reversible (i.e. from seep to vent and back to seep). Different tree construction methods and the choice of molecular and morphological characters provided different answers to this question (Craddock et al., 1995).

Herein, we address this question with DNA sequences from mussels recently acquired from the Blake Ridge off the South Carolina coast. *Bathymodiolus* mussels associated with methane hydrate seeps at this locality were first observed in 1995 during the Ocean Drilling Program (ODP) (Paull et al., 1996). We examined samples of these mussels obtained recently (25-28 September 2001) by C. L. Van Dover during DSRV *Alvin* dives (3709-3712) at Blake Ridge (32°29.6'N; 76°11.5'W; 2155 m). The depth and location of Blake Ridge raised intriguing questions about evolutionary relationships between its mussels and morphologically similar mussels from the Mid-Atlantic Ridge vents and Gulf of Mexico cold seeps. We compared new DNA sequences from the mitochondrial Nicotinamid Adenine Dinuclotide dehydrogenase subunit 4 gene (ND4) from Blake Ridge mussels with published sequences from the Mid-Atlantic species, *B. azoricus* Von Cosel et al., 1999 and *B. puteoserpentis* Von Cosel & Metivier, 1994, the Central Indian Ridge species, *B.* aff. *brevior*, and the western Pacific vent species, *B. brevior* Von Cosel & Metivier, 1994 (Maas et al., 1999; O'Mullan et al., 2001; Van Dover et al., 2001), and new sequences from Gulf of Mexico species, *B. heckerae* Turner et al., 1998, *B. brooksi* Gustafson et al., 1998, and *B. childressi* Gustafson et al., 1998, and East Pacific Rise species, *B. thermophilus* Kenk & Wilson, 1985. Preliminary results revealed that seep mussels from Blake Ridge are nearly identical to *B. heckerae* from the West Florida Escarpment and, thus, are considered conspecific. However, *B. heckerae* is closely allied with the two MAR vent species, to the exclusion of *B. brooksi* and *B. childressi* from the Gulf of Mexico. These results refute the hypothesis that vent mussels comprise a natural (i.e., monophyletic) group, and they are consistent with the hypothesis that evolutionary habitat transitions from seeps to vents are reversible in these mussels.

Materials and methods

Blake Ridge mussels used in this study were collected by C. L. Van Dover during DSRV *Alvin* dive 3709 (25 September 2001; Table 1). Reference materials were collected during

Table 1. Collection localities for deep-sea hydrothermal vent and cold-water methane/sulphide seep mytilids.

Habitat and sampling site		Latitude; Longitude	Dive # A: *Alvin*; BL: *BioLau* J: *Jason*	Depth (m)	OTU
Blake Ridge	BL	32°30'N; 76°11'W	A3709	2155	*B.* aff. *heckerae*
Mid-Atlantic vents					
Lucky Strike	LS	37°17'N; 32°15'W	A3120	1710	*B. azoricus*
Snake Pit	SP	23°22'N; 44°56'W	A3129	3480	*B. puteoserpentis*
East Pacific Rise					
9°N	9N	9°51'N; 104°18'W	A2498	2525	*B. thermophilus*
Western Pacific vent					
Lau Back Arc	LBA	23°13'S; 176°38'W	BL5	1750	*B. brevior*
Central Indian Ridge					
Edmond	ED	23°53'S; 69°36'E	J301	3289	*B.* aff. *brevior*
Gulf of Mexico seeps					
W. Fl. Escarpment	FL	26°02'N; 84°55'W	A2196	3314	*B. heckerae*
Alamiños Canyon	AC	26°21'N; 94°30'W	A2211	2222	*B. brooksi*
Alamiños Canyon	AC	26°21'N; 94°30'W	A2211	2222	*B. childressi*

previous expeditions (see, Craddock et al., 1995; Maas et al., 1999; Van Dover et al., 2001). Samples used for DNA analyses were frozen immediately (-70°C) on the support vessel, transported on dry ice to a land-based laboratory, and stored at -80°C until DNA extraction. Genomic DNA was extracted from a small portion (~50 mg) of adductor mussel that was treated with the DNeasy Tissue Kit (QIAGEN Inc, Valencia, CA) according to manufacturer's instructions. A 780-bp fragment of a mitochondrial ND4 was amplified with DNA primers described in Mass et al. (1999). We used the Big Dye™ (PE Biosystems, Foster, CA) cycle sequencing kit and an ABI Prism 3100 (Applied Biosystems Inc, Foster, CA) to obtain DNA sequences. Sequences were obtained from both strands from two or more individuals representing each OTU (operational taxonomic unit). Sequences were aligned with Sequencher (Gene Codes Corporation Inc. Ann Arbor, MI), and variable sites were confirmed by sequences of the opposite strand of the DNAs. For *B. azoricus*, *B. puteoserpentis, B. brevior* and *B.* aff. *brevior*, we used previously published (Maas et al., 1999; Van Dover et al., 2001) sequences (GenBank accession #s: AF128534, AF128533, AY046277, and AY046279). Estimates of pairwise sequence divergence and phylogenetic analyses were conducted by PAUP employing maximum parsimony (MP), neighbor-joining (NJ), and maximum likelihood (ML) methods (v. 4.0b8, Swofford, 1998). Pairwise differences of amino acid sequences were inferred by an invertebrate mitochondrial genetic code table using MEGA (v. 2.1, Kumar et al., 2001). We determined the best-fit model of molecular evolution to be Hasegawa-Kishino-Yano (HKY85) + gamma (Hasegawa et al., 1985) by using MODELTEST (Posada & Crandall, 1998).

Results

The full length PCR product was about 780 bp including two transfer RNAs (~200 bp) and the first third of the coding region for ND4. Confident sequence alignments from nine OTUs were obtained for 507 bp of the ND4 coding sequence. Percentage of nucleotide divergence, *d*, between sequences, following HKY85 + gamma model with shape parameter $\alpha = 0.4149$, ranged from 1.4 % to 30.6%. The inferred number of amino acid substitutions, according to mitochondrial codon usage in mussels (Hoffmann et al., 1992), ranged from 1 to 39 of the 169 amino acid sites (Table 2). Blake Ridge mussels were very closely related to *B. heckerae* from the West Florida Escarpment ($d = 1.44$). Despite the small preliminary sample sizes ($N = 2$ from each OTU), the close similarity of Blake Ridge mussels and *B. heckerae* from the West Florida Escarpment suggests they are conspecific. Significantly higher distances existed between the named species (range of *d*: 15.3-66.9%). *B. childressi* from Alamiños Canyon clearly was the most divergent of the species examined in this study.

Table 2. Pairwise genetic distances and number of inferred amino acid substitutions (above diagonal) among mussels examined in this study. Genetic distances were calculated according to the Hasegawa-Kishino-Yano substitution model (HKY85) with gamma correction ($\alpha = 0.4149$).

	BL	*B. az.*	*B. pu.*	*B. th.*	*B. bre*	ED	*B. he.*	*B. br.*	*B. ch.*
Blake Ridge		13	14	24	21	20	2	24	39
B. azoricus	0.1457		14	20	22	21	13	23	36
B. puteo-serpentis	0.2120	0.1863		21	22	21	14	23	38
B. thermophilus	0.4569	0.3805	0.3638		22	22	24	28	36
B. brevior	0.3313	0.2794	0.3526	0.4903		1	21	24	37
Edmond	0.3267	0.2973	0.3747	0.5226	0.0081		20	23	36
B. heckerae	0.0144	0.1525	0.2099	0.4659	0.3457	0.3496		24	39
B. brooksi	0.4079	0.3810	0.4560	0.5780	0.3599	0.3464	0.4588		34
B. childressi	0.6124	0.6054	0.6067	0.6634	0.6077	0.6148	0.6691	0.5327	

Of 507 nucleotide positions, 218 (43.0%) were variable and 124 (24.5%) were parsimony informative. An exhaustive search identified a single most parsimonious tree of 364 steps. Two tree construction methods (MP and NJ with HKY85 + gamma) yielded the same single most parsimonious tree. Here, we present the NJ tree (Fig 1). Also, bootstrap analysis of phylogenetic tree constructed by amino acid sequences supported four nodes of the parsimonious tree from DNA sequences. ML method (HKY85 + gamma, with $\alpha = 0.4149$) yielded a slightly different result locating the *B. thermophilus* as an another long branch apart from all taxa. The Shimodaira-Hasegawa

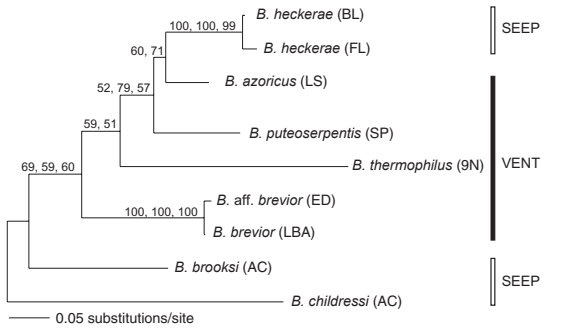

Figure 1. The single most parsimonious tree found based on ND4 sequences. Numbers on tree branches indicate the percentages of bootstrap samplings derived from 1,000 replications supporting by ≥50% in the order of neighbor-joining, maximum parsimony method, and, if any, parsimony analysis of amino acid sequences. Branch lengths are drawn proportional to the inferred amount of change along the branch (scale shown) by neighbor-joining distance method. Root is placed on the branch of *B. childressi* as an outgroup. Each OTU is shown with its species name and location (in parenthesis, see Table 1). Right side vertical bar represents habitat type.

(SH) test, however, could not reject the null hypothesis of no difference between the best-fit ML tree and the MP tree (p value = 0.300). A root for the tree was provided by a reference sequence from *B. childressi*, a Gulf of Mexico seep species that based on morphological and allozyme evidence should serve as an appropriate outgroup (Craddock et al., 1995; Gustafson et al., 1991).

To test an hypothesis of monophyly of vent taxa from other seep taxa, we examined Hasegawa-Kishino (HK) test. The shortest tree obtained by MP method was compared to an hypothetical tree which consists of basal seep taxa and monophyletic vent taxa with similar topology to MP tree as following tree constraint; (*B. childressi*, *B. brooksi*, Blake Ridge, *B. heckerae* (Edmond, *B. brevior* (*B. thermophilus* (*B. puteoserpentis*, *B. azoricus*)))). The HK test rejected the null hypothesis of no difference between the two trees (p value <0.0001). The present results are consistent with the hypothesis (Craddock et al., 1995) that the seep species *B. heckerae* is most closely affiliated with, and probably derived from, a Mid-Atlantic vent species, *B. azoricus* or *B. puteoserpentis*. However, the phylogenetic trees are equivocal regarding the sister-species for *B. heckerae*. Bootstrap values, 60% in NJ and 71% in MP tree, weakly supported the *heckerae-azoricus* pair. On the other hand, ML tree supports *heckerae-puteoserpentis* pair with 62% bootstrap value.

Discussion

Recent discovery of *Bathymodiolus* mussels from methane hydrate seeps on the Blake Ridge raised questions about its evolutionary affinities with known seep and vent mytilids in Atlantic Ocean and Gulf of Mexico. Craddock et al. (1995) used allozymes and morphology to examine evolutionary relationships among deep-sea mytilids from this region. Phylogenetic trees constructed by the distance-Wagner method (based on allozymes) and parsimony (based on allozymes and morphology) methods supported the hypothesis that seep mytilids were broadly ancestral to vent species. However, a discrepancy in tree topologies existed between the two methods. The discrepancy was a consequence of saturation of allozyme differences at higher levels of divergence. Although useful for assessing relationships within and among closely related species, allozymes have limited value for deciphering higher systematic relationships (Hillis et al., 1996). Thus, the earlier study could not determine whether evolutionary habitat transitions proceeded unidirectionally (i.e., from seep to vent) or reversibly (i.e., from seep to vent to seep). Resolving this matter was of considerable interest to deep-sea biologists, because it appears that vestimentiferan tubeworms (Annelida: Siboglinidae) endemic to vents might comprise a monophyletic group derived from seep ancestors (Black et al., 1997). In contrast, clams (Bivalvia: Vesicomyidae) that occupy hydrothermal vents have evolved multiple times from seep ancestors, but it is unclear whether the process is reversible (Peek et al., 1997).

The present molecular data generated a single most parsimonious tree revealing reversible evolutionary transitions between vent and seep habitats. The tree topology based on ND4 sequences broadly supports the earlier parsimony tree (Fig. 1B, Craddock et al., 1995) based on total evidence from allozymes and morphology. However, the new sequence data revealed that the seep species *B. heckerae* and *B. brooksi* are not sister-species. Instead, *B. heckerae* is more closely related to the Mid-Atlantic hydrothermal vent mussels, *B. azoricus* and *B. puteoserpentis*.

Although Blake Ridge is relatively close to the Mid-Atlantic vent localities of *B. azoricus* and *B. puteoserpentis*, the mussels from the Blake Ridge are very similar to, and probably conspecific with, *B. heckerae* from the West Florida Escarpment seeps. This result warrants further investigation with additional genetic markers (e.g., allozymes, nuclear DNAs, etc.) and morphometric analyses (e.g., Maas et al., 1999). In the interim, and in the absence of conflicting evidence, we recommend that Blake Ridge mussels be referred to as *B. heckerae*. Although its ancestors probably lived at hydrothermal vents, we do not understand the factors that limit this species to seep environments. Perhaps tight associations with vent- vs. seep-specific endosymbionts are involved. Detailed studies of associations with sulfide- vs. methane-oxidizing endosymbionts of these mussels may shed light on this matter.

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