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Evolution of habitat use by deep-sea mussels

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Abstract Previous phylogenetic studies proposed that symbiont-bearing mussels of the subfamily Bathymodiolinae (Bivalvia: Mytilidae) invaded progressively deeper marine environments and evolved from lineages that decomposed wood and bone to specialized lineages that invaded cold-water hydrocarbon seeps and finally deep-sea hydrothermal vents. To assess the validity of the hypotheses, we examined two nuclear (*18S* and *28S rRNA*) and two mitochondrial genes (*COI* and *ND4*) from a broad array of bathymodiolin species that included several recently discovered species from shallow hydrothermal seamounts. Bayesian phylogenetic analysis and maximum-likelihood estimates of ancestral character states revealed that vent species evolved multiple times, and that reversals in vent and seep habitat use occurred within the sampled taxa. Previous hypotheses regarding evolution from wood/bone-to-seeps/vents are supported in that mid-ocean hydrothermal vent species may represent a monophyletic group with one noticeable reversal. Earlier hypotheses about

progressive evolution from shallow-to-deep habitats appear to hold with a few instances of habitat reversals.

Introduction

Bathymodiolin mussels (Mytilidae: Bathymodiolinae) are among the dominant animals found at marine hydrothermal vents and cold-water seeps worldwide. They rely mostly on chemoautotrophic endosymbionts for their nutrition but retain an ability to filter-feed, which together appear to contribute to their broad ecological success (Fisher et al. 1987). It was proposed that the deep-sea vent lineages originated recently from older lineages that occupied cold-seeps and shallow-water environments (Craddock et al. 1995a). Furthermore, it has been proposed that species exploiting sulfide-rich decomposing wood and bone preceded the origin of vent and seep species (Distel et al. 2000). However, the generality of these patterns is not firmly established, because they were derived from phylogenetic studies that examined a limited number of species mostly from the Atlantic Ocean. For example, support for precedence of wood and bone specialists relies entirely on phylogenetic placement of *Benthomodiolus lignicola* as a basal lineage in the bathymodiolin clade. In addition, subsequent phylogenetic studies revealed that some western Pacific mussel species can occur in vents and seeps (Miyazaki et al. 2004) and that vent-to-seep habitat reversals have occurred in some Atlantic mussel lineages (Won et al. 2002).

Nevertheless, broad-scale patterns of habitat evolution (from wood/bone-to-seep/vent and from shallow-to-deep water) may be valid. New opportunities to test these hypotheses are possible, because the number of named species has doubled during the past decade (von Cosel et al. 1994, 1997, 1999; Hashimoto and Okutani 1994; Cosel and Olu 1998; Gustafson et al. 1998; von Cosel 2002; Won et al. 2003b). Particularly relevant to the present analysis is the availability of new DNA se-

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quences from several western Pacific species (Miyazaki et al. 2004) that were not integrated into prior studies. In addition, discoveries of new western Pacific species that live at very shallow cold-seeps and hydrothermal seamounts (Cosel and Marshall 2003; Smith et al. 2004) provide opportunities to test hypotheses about habitat evolution. In this study, we examined DNA sequences from two nuclear ribosomal genes (*18S* rDNA and *28S* rRNA) and two protein-coding mitochondrial genes (*COI* and *ND4*) to estimate a phylogeny of wood, bone, vent, and seep bathymodiolins. We used enhanced statistical methods (i.e., Bayesian analysis) to construct a robust bathymodiolin phylogeny, which in turn was used to test hypotheses about patterns and directions of evolution.

Materials and methods

Specimens used to obtain original DNA sequences for this study were collected from a range of depths and habitats in the Atlantic, Pacific and Indian Oceans (Table 1). GenBank accession numbers for these sequences are listed with each collection locality. To obtain these sequences, we extracted genomic DNA from frozen samples of adductor muscle (50 mg) that was treated with the Qiagen Dneasy™ isolation kit, according to manufacturer's instructions (Qiagen Inc., Valencia, CA, USA). Polymerase chain reaction (PCR) conditions for amplification of the gene regions were as follows: 30–100 ng of template DNA, 5 µl 10× buffer (supplied by manufacturer), 5 µl MgCl₂ (2.5 µM), 2 µl of each primer (10 µM final conc.), 2.5 U of *Taq* polymerase (Promega Inc®, WI, USA), 5 µl of a 2 mM stock solution of dNTPs, and sterile H₂O to a final volume of 50 µl. PCR was performed with a Cetus 9700™ DNA thermocycler (Perkin-Elmer Corp., CT, USA) with the appropriate primers and conditions as listed in Table 2.

Polymerase chain reaction products were purified using Qiagen PCR purification Kit™ (Qiagen Inc. Valencia, CA, USA) or Montage columns (Millipore, Billerica, MA, USA). The purified template DNA was sequenced using Big Dye™ Terminator cycle sequencing reaction kit (PE Biosystems®, Foster City, CA, USA) and ABI Prism 3100™ DNA sequencers (Applied Biosystems Inc., Foster, CA, USA). PCR products were sequenced bidirectionally from each individual sample using the same forward and reverse primers as used in PCR. DNA sequence alignments were initially constructed using Sequencher™ (Gene Codes Corp. Inc., Ann Arbor, MI, USA). Secondary structure of rRNA (i.e., stems and loops) was inferred using the program GeneBee (Brodsky 1992). Amino acid translations of *COI* and *ND4* were performed in MEGA 2.1 (Kumar et al. 1993) using the invertebrate mitochondrial code (GenBank Code 5). Alignments for all four genes analyzed in this study are available in GenBank PopSet (Accession Numbers in Table 1).

In this and in previous studies, we found no evidence for gender-biased mitochondria in bathymodiolin mussels (Won et al. 2003a, b). Gonadal tissue is diffusely distributed throughout the visceral mass and the posterior part of the mantle in bathymodiolins. Our DNA extracts were obtained from a frozen adductor muscle, so we were unable to assess the sex of individuals. Nevertheless, we examined multiple individuals of all species and found no evidence for the degree of intra-specific sequence divergence commonly associated with doubly uniparental inheritance (DUI) of mitochondria in other bivalves (Hoeh et al. 1996, 2002; Passamonti and Scali 2001). In addition, we observed no heteroplasmy, an earmark of DUI (Hoeh et al. 1991), in the present mitochondrial sequences. One case of heteroplasmy was previously observed with *B. thermophilus* (Craddock et al. 1995b). A sample of nine females and ten males from the same location revealed no association between gender and mitochondrial haplotypes (P. Maas, unpublished data).

Phylogeny reconstruction

Nucleotide composition of each gene was estimated using MEGA 2.1 (Kumar et al. 1993). Bayesian relative rates test for each gene was performed with *Benthomodiolus lignicola* as the outgroup using Cadence (ver. 1.08 Wilcox et al. 2004) with the last 1,000 of the post burn-in Bayesian trees (see below).

Saturation plots were constructed for *COI* and *ND4* using uncorrected “p” versus the gene-specific model as determined in Model Test 3.06 (Posada and Crandall 1998). These gene-specific models were used to estimate maximum-likelihood phylogenies in PAUP 4.0b10 (Swofford 1998). Phylogenies were inferred from under the maximum-parsimony and maximum-likelihood criteria as implemented in PAUP 4.0b10 (Swofford 1998). A heuristic approach with ten random additions of the input taxa was used to search for the shortest parsimony trees. The robustness of parsimony trees was assessed by bootstrap analysis (Felsenstein 1985), using the heuristic search procedure in PAUP 4.0b10 with 1,000 replicates and 10 random additions of the input taxa. For all genes surveyed, the maximum-likelihood phylogeny was concordant with the Bayesian phylogeny and are not shown.

The partition homogeneity test (using the same conditions as in the heuristic search) as implemented in PAUP 4.0b10 was used as a proxy to determine the overall concordance between gene trees. There was no significant difference between the overall tree distribution of the two mitochondrial genes ($P=0.57$) or between *28S* rRNA and the mitochondrial genes ($P=0.65$). For estimation of ancestral character states, we used the combined dataset of *28S*, *COI*, and *ND4*.

Bayesian phylogenetic trees were estimated for individual genes using MrBayes version 3.0b4 (Huel-

Table 1 Specimen collection sites, species identifications, and GenBank accession numbers for bathymodioline mussels

OTU	Location/reference	Ocean basin	Latitude, longitude	Depth range (m)	Habitat	GenBank No.			
						18S	28S	ND4	
<i>Bathymodiolus heckeræ</i> BR	Blake Ridge	Atlantic	32°30'N; 76°11'W	2,155	Seep	AY649830	AY781139	AY130245	AY649793
<i>B. heckeræ</i> WF	W. Florida Escarp.	Atlantic	26°02'N; 84°55'W	3,314	Seep	AF221639	AY781138	AY130246	AY649794
<i>B. azoricus</i>	Menez Gwen	Atlantic	37°17'N; 32°15'W	866–2,330	Vent	AY649822	AY781148	AF128534	AY649795
<i>B. puteoserpentis</i>	Snakepit	Atlantic	23°22'N; 44°56'W	3,023–3,510	Vent	AF221640	AY781151	AF128533	AY649796
<i>B. brooksi</i> AC	Alamiños Canyon	Atlantic	26°21'N; 94°29'W	2,222	Seep	AY649826	AY781136	AY130247	AY649797
<i>B. brooksi</i> WF	W. Florida Escarp.	Atlantic	26°02'N; 84°55'W	3,314	Seep	AY649825	AY781135	AY649805	AY649798
<i>B. brevior</i> MT	Mariana Trough	W. Pacific	18°13'N; 144°42'E	3,589	Vent	AY649824	AY781150	AY649806	AY649799
<i>B. brevior</i> LBA	Lau Basin	W. Pacific	23°13'S; 176°38'W	1,750	Vent	AY649827	AY781143	AY046277	AY275544
<i>B. marisindicus</i>	Central Indian Ridge	Indian Ocean	23°53'S; 69°36'E	3,289	Vent	AY649818	AY781147	AY046279	AY275543
<i>B. thermophilus</i> A	9°N EPR	E. Pacific	9°51'N; 104°18'W	2,460–2,747	Vent	AF221638	AY781141	AY649807	AF456285
<i>B. thermophilus</i> B	7°S EPR	E. Pacific	7°26'S; 104°47'W	2,460–2,747	Vent	AY649829	AY781142	AY649808	AF456303
<i>B. aff. thermophilus</i>	32°S EPR	E. Pacific	31°52'S; 112°03'W	2,331	Vent	AY649823	AY781140	AY649809	AF456317
<i>B. childressi</i>	Alamiños Canyon	Atlantic	26°21'N; 94°29'W	540–2,222	Seep	AF221641	AY781137	AY130248	AY649800
<i>B. mauritanicus</i>	West Africa	Atlantic	0°53'N; 5°28'W	1000–1267	Seep	AY649828	AY781144	AY649810	AY649801
<i>Gigantidas gladius</i>	Rumble III	W. Pacific	35°44'S; 178°30'E	300–460	Vent	AY649821	AY781149	AY649813	AY649802
<i>B. tangaroa</i>	Cape Turnagain	W. Pacific	40°26'S; 178°58'E	920–1,205	Seep	AY649820	AY781134	AY649811	AY608439
<i>Tamu fisheri</i>	Garden Banks	Atlantic	27°50'N; 92°10'W	546–650	Seep	AF221642	AY781132	AY649814	AY649803
<i>Idas washingtonia</i>	Monterey Bay	E. Pacific	37°7'N; 122°48'W	960–1,910	Bone, wood	AF221645	AY781146	AY649815	AY275546
<i>I. macdonaldi</i>	Garden Banks	Atlantic	27°50'N; 92°10'W	650	Seep	AF221647	AY781145	AY649816	AY649804
NZ-3	Macauley Cone	W. Pacific	30°13'S; 178°27'W	200	Vent	AY649819	AY781133	AY649812	AY608440
<i>Benthomodiolus lignicola</i>	Chatham Rise	W. Pacific	34°41'S; 177°14'W	826–1,174	Bone, wood	AF221648	AY781131	AY649817	AY275545

Table 2 PCR primers and temperature profiles

Gene	Primer	Sequence	Temperature profile	Reference
<i>mtCOI</i>	HCO-2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	94-4-[94-1-55-2-72-3.5]-72-10 (x35)	(Folmer et al. 1994) (Folmer et al. 1994)
	LCO-1490	5'-GGTCAACAAATCATAAAGATATTGG-3'		
<i>mtCOI</i>	HCO-2148	5'-CCYCTAGGRTCATAAAAAGA-3'	94-4-[94-1-55-2-72-3.5]-72-10 (x35)	This study This study
	LCO-1560	5'-ATRCTDATTTCGWATTGA-3'		
<i>mtNADH4</i>	Arg BL	5'-CAAGACCCTTGATTTTCGGCTCA-3'	94-4-[94-40s-55-1-72-1]-72-10 (x37)	(Bielawski and Gold 1996)
<i>18S rRNA</i>	NAP 2H	5'-TGGAGCTTCTACGTGRGCTTT-3'	94-4-[94-40s-55-1-72-1]-72-7 (x37)	(Arevalo et al. 1994) (Giribet et al. 1996) (Giribet et al. 1996) (Giribet et al. 1996) (Giribet et al. 1996) (Giribet et al. 1996) (Giribet et al. 1996)
	1F	5'-TACCTGGTTGATCCTGCCAGTAG-3'		
	3R	5'-AGGCTCCCTCTCCGGAATCGAAC-3'		
	3F	5'-GTTTCGATTCCGGAGAGGGA-3'		
	5R	5'-CTTGGCAAATGCTTTTCGC-3'		
	5F	5'-GCGAAAGCATTGCGCAAGAA-3'		
	9R	5'-GATCCTTCCGCAGG'TTCACCTAC-3'		
<i>28S rRNA</i>	LSUD1F	5'-ACCCGCTGAATTTAAGCATA-3'	94-5-[94-1-55-1-72-2]-72-7 (x35)	(Scholin and Anderson 1994) (Scholin and Anderson 1994)
	D3AR	5'-ACGAACGATTTGCACGTCAG-3'		

senbeck and Ronquist 2001) using the codon model for *COI* and *ND4* and partitioning by structure (stem/loop) for *18S* and *28S* ribosomal RNAs. An additional series of MrBayes analyses was performed on the combined *28S*, *COI*, and *ND4* dataset using the same parameters as in the individual gene analysis. Parameters are available upon request from the senior author. The Monte Carlo Markov chain (MCMC) length was 1.1×10^6 generations with 6 markov chains, and we sampled the chain every 100 generations to minimize autocorrelation. MCMC convergence was assessed by visually inspecting the sample paths of model parameters (to determine an appropriate burn-in period) and by repeating the analysis at least three times with random initial parameter values (to assess the dependence of posterior distributions on initial conditions). Analyses were run in parallel using two dual processor G5 Macintosh servers.

Parameter estimates were graphically analyzed to assess stability (Tracer ver. 1.0.1, Rambaut and Drummond 2003). Log-likelihood values and associated parameters for sampled trees stabilized after approximately $5-6 \times 10^5$ generations for both *COI* and *ND4*, while it took approximately $4-5 \times 10^5$ generations for the *18S* and *28S* rRNA data sets, respectively. Therefore, we conservatively used the last 5,000 sampled trees to estimate Bayesian posterior probabilities. If $\geq 95\%$ of the sampled trees contained a given clade, we considered it to be significantly supported by our data (sensu Wilcox et al. 2002). Divergence time among taxa was estimated using a Bayesian approach (BEAST version 1.0.3, Drummond and Rambaut 2003).

Maximum-likelihood (Mesquite version 1.04, Maddison and Maddison 2004) and Bayesian (SIMMAP, Bollback 2004) estimates of ancestral character states (Huelsenbeck et al. 2003) for habitat and depth were mapped on the combined *28S*, *COI* and *ND4* dataset. The rate and number of transformations between character states was estimated using SIMMAP

(Bollback 2004) using the last 5,000 post burn-in trees from the combined *28S*, *COI* and *ND4* dataset analysis.

Results

Nuclear *18S* rRNA

The complete aligned *18S* data set consisted of 1,805 base pairs (bp). Altogether, 708 positions were designated as stems and 1,097 positions were designated as loops (Table 3). Thirteen new *18S rRNA* sequences were obtained in the present study. Across both regions, we found 240 variable sites, including 159 that were parsimony informative. In addition to the taxa listed in Table 1, a number of additional taxa were analyzed to determine an appropriate outgroup to root the *28S rRNA* and mitochondrial gene trees (GenBank accession numbers in parentheses): *Adipicola arcuatilis* (AF221644), *Myrina pacifica* (AF221646), *Modiolus auriculatus* (AF117735), *M. americanus* (AF229624), *M. modiolus* (AF124210), *Mytilus edulis* (L33448), *M. californianus* (L33449), *M. galloprovincialis* (L33451), *M. trossulus* (L24490), *Musculus senhousei* (AF124207), *Musculus lateralis* (AF229625), *Hormomya domingensis* (AF117736), *Brachidontes variabilis* (AJ389643), *Geukensia demissa* (L33450), *Lithophaga lithophaga* (AF124208), *L. nigra* (AF124209), *Atrina pectinata* (X90961), *Chlamys islandica* (L11232), *Placopecten magellanicus* (X53899).

The Bayesian analysis generated a topology that was broadly congruent with the maximum likelihood tree obtained by Distel et al. (2000) (Fig. 1a). The wood-degrading specialist, *Benthomodiolus lignicola*, comprises the sister-group to all other sampled deep-sea bathymodiolins. Subsequent analyses only considered members of the Bathymodiolinae with *Be. lignicola* used to root the tree.

Table 3 Base composition and nucleotide substitution patterns for *18S rRNA*, *28S rRNA*, *ND4*, and *COI*

Gene	Nucleotide positions			All
	Loops	Stems		
<i>18S</i>				
%A	25.7	24.5	–	25.2
%C	22.5	22.3	–	22.4
%G	27.0	27.3	–	27.1
%T	24.5	26.0	–	25.3
Variable sites	144	96	–	240
Informative sites	95	64	–	159
TS/TV	1.95	0.96	–	1.11
<i>28S</i>				
%A	25.7	13.7	–	21.0
%C	29.4	37.0	–	32.4
%G	18.9	36.1	–	25.7
%T	26.0	13.1	–	21.0
Variable sites	77	33	–	110
Informative sites	32	11	–	43
TS/TV	2.4	6.8	–	3.45
	First	Second	Third	All
<i>ND4</i>				
%A	23.2	14.7	22.2	20.1
%C	14.1	12.7	12.2	13.0
%G	29.9	22.1	21.6	24.6
%T	32.8	50.5	44.0	42.4
Variable sites	99	50	169	318
Informative sites	74	25	155	254
TS/TV	3.29	1.53	2.19	2.22
Ks	–	–	–	1.17
Ka	–	–	–	0.11
<i>COI</i>				
%A	26.4	14.3	26.6	22.4
%C	15.5	23.6	9.4	16.1
%G	26.5	19.2	19.0	21.5
%T	31.6	43.1	45.1	39.9
Variable sites	44	11	206	261
Informative sites	28	1	167	196
TS/TV	3.54	0.67	3.12	1.8
Ks	–	–	–	1.12
Ka	–	–	–	0.13

Ka is the number of nonsynonymous substitutions per nonsynonymous site; Ks is the number of synonymous substitutions per synonymous site

Nuclear *28S rRNA*

The complete aligned *28S rRNA* data set consisted of 938 bp. Altogether, 358 positions were designated as stems and 580 positions were designated as loops (Table 3). All *28S rRNA* sequences reported in this paper are new. Across both regions, we found 110 variable sites, including 43 that were parsimony informative. The Bayesian analysis generated a topology (Fig. 1b) that was substantially more resolved for bathymodiolins than the *18S rRNA*. Of particular note is the placement of *Bathymodiolus tangaroa* and *Gigantidas gladius* in the same clade as *B. childressi* and *B. mauritanicus*.

Mitochondrial *ND4*

The complete aligned data set of *ND4* consisted of 529 bp, which included 176 amino acids of the coding region. Thirteen new *ND4* sequences were obtained in the present study. The inferred number of amino acid substitutions between taxa averaged 33.0 (range: 0–69). Of the 529 nucleotide positions, 318 (60.1%) were variable and 254 (48.0%) were parsimony informative (Table 3). About half of the substitutions occurred at third positions (53.1%). Compositional biases across all three codon positions were particularly exacerbated for thymine and cytosine at third positions. Thymine was especially biased at second positions.

Parameters were estimated for a Bayesian analysis of *ND4* (available upon request), which revealed a well-resolved bathymodiolin phylogeny (Fig. 1c). The *ND4* Bayesian phylogeny clearly places *Bathymodiolus brooksi* within a clade containing *B. thermophilis*, *B. brevior*, *B. marisindicus*, *B. heckerae*, *B. azoricus* and *B. puteoserpentis*. *ND4* also placed the undescribed New Zealand species NZ-3 as a basal lineage in the Bathymodiolinae. The Bayesian relative rates test identified no statistically significant heterogeneity in *ND4* substitution rates among bathymodiolin taxa.

Mitochondrial *COI*

The complete aligned data set of *COI* consisted of 689 bp, which included 229 amino acids of the coding region. Twelve new *COI* sequences were obtained in the present study. The inferred number of amino acid substitutions between taxa averaged 2.33 (range: 0–8). Altogether, 261 sites (37.9%) were variable mostly at third positions (80%). Of the variable sites, 196 (28.4%) were phylogenetically informative: 28 at first positions; one at second positions; and 167 at third positions (Table 3). A relatively high ratio of transitions to transversions was observed at first positions; however the ratio was lower at second positions. Nucleotide composition resembled that found in mitochondrial *ND4*, with excess thymine and deficiency of cytosine across all positions. Pairwise comparisons involving *Benthomodiolus lignicola* and bathymodiolin taxa revealed possible saturation for third position substitutions (data not shown). However, this problem was not as evident for first and second positions in the codons.

The undescribed New Zealand species, NZ-3, was supported as basal to all other sampled ingroup taxa (Fig. 1d). The two recently described mussel species from New Zealand, *Bathymodiolus tangaroa* and *Gigantidas gladius*, were placed in the same clade as *B. childressi* and *B. mauritanicus*. Inclusion of the partial *COI* sequences (415 bp) from Miyazaki et al. (2004) strongly supported (1.00 BPP) the placement of *B. japonicus* and *B. platifrons* in the same clade as *B. childressi* (data not shown). The remaining well-supported

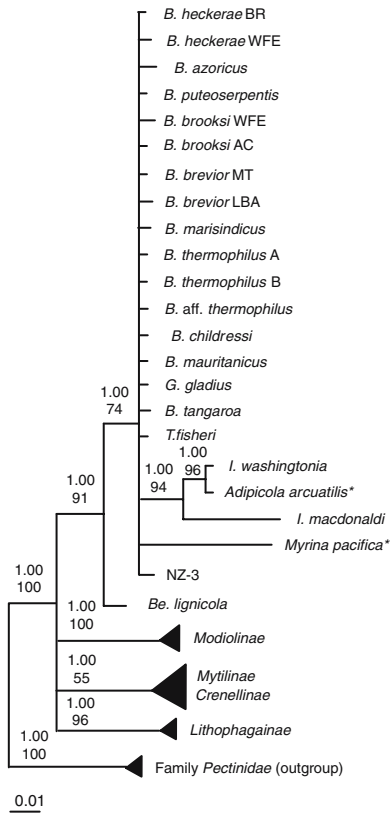
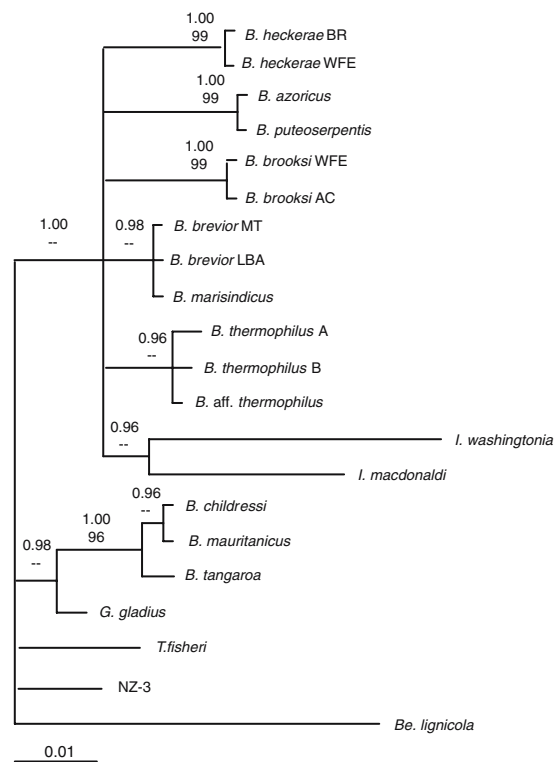
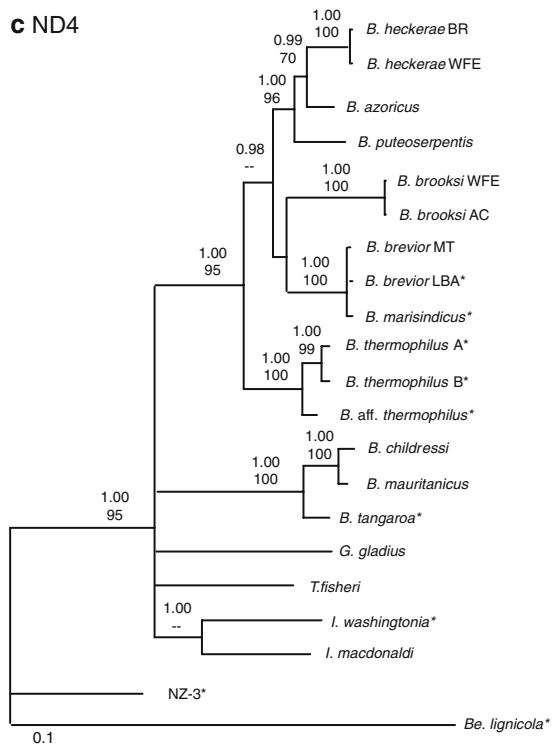
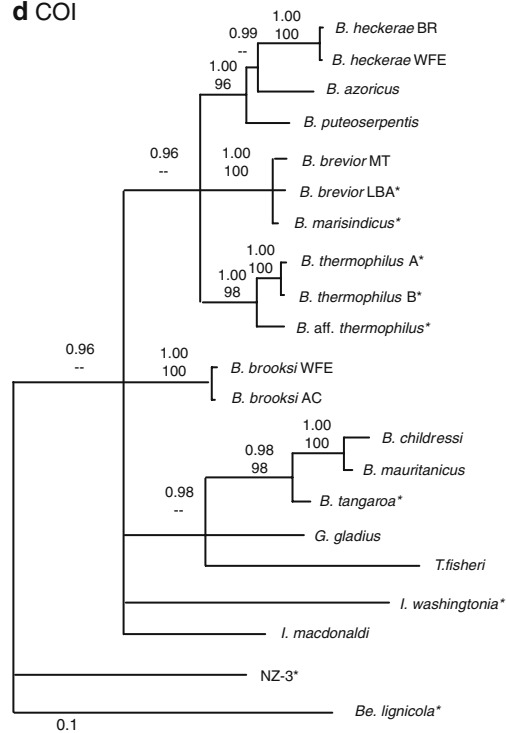
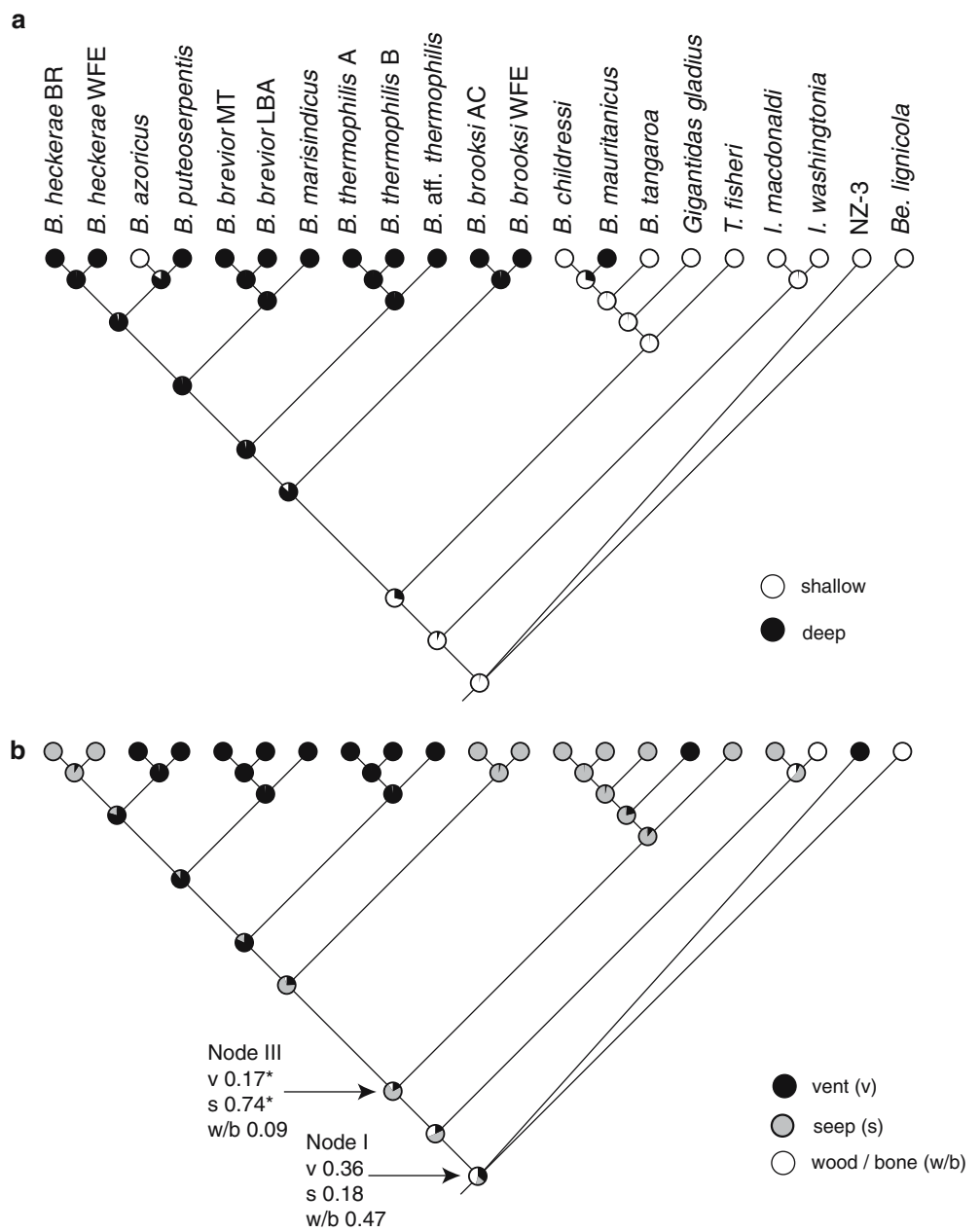
a 18S rRNA**b 28S rRNA****c ND4****d COI**

Fig. 1 Bayesian trees of **a** 18S rRNA, **b** 28S rRNA, **c** ND4, and **d** COI datasets. Previous published data are indicated with asterisks (see Table 1). Scale bar indicates percent sequence divergence. Only Bayesian posterior probabilities (BPP; upper number) greater than 0.95 and maximum-parsimony bootstrap support greater than 70%

are shown (lower number). Nodes with less than 0.95 BPP are collapsed to polytomies. Maximum parsimony bootstrap values less than 70% are indicated by “–”. Additional outgroup taxa 18S rRNA analyzed (see Materials and methods for GenBank numbers) are indicated by the subfamily clade names in Fig. 1a

Fig. 3 Bayesian cladogram of COI, ND4, and 28S (after Fig. 2) with maximum-likelihood estimates of ancestral character states for **a** depth and **b** habitat. Relative support for ancestral character states are indicated by area of pie chart at respective nodes. Values for the three habitat states (*v* vent, *s* seep, *w/b* wood/bone) are given at nodes I and III. *Asterisk* indicates a significant likelihood that the particular character state would have existed at a particular node



Character evolution in deep-sea mussels

The combined phylogeny provides an evolutionary framework against which we can test various hypotheses about character evolution in bathymodiolin mussels. We treated habitat depth and type in each species as discrete character states and examined their traces in the phylogeny. To test Craddock et al. (1995a) hypothesis that these mussels diversified from shallow- to deep-water habitats, we divided the most shallow known depth of each species into those that are greater or lesser than 1,000 m (Fig. 3a). Clearly, the taxa stemming from basal nodes (NZ-3, *Idas* and most of the “childressi” clade) occur in shallow sites. The “thermophilus” clade defined by node IV all tend to live at deep sites, so the general

pattern recognized by Craddock and coworkers appears to be supported. Nevertheless, some notable exceptions exist. One member of this “thermophilus” clade, *B. azoricus*, has also invaded a shallower portion of the Mid-Atlantic Ridge (type locality Menez Gwen, 866 m). Similarly, evolutionary transitions between habitat types appear to be complex. Hydrothermal vent mussels do not comprise a derived monophyletic clade. *Gigantidas gladius* and undescribed species NZ-3 were found on shallow hydrothermal seamounts and they stem from basal lineages. Nevertheless, mussels found at deep-sea hydrothermal vents, the “thermophilus” clade (node IV, Fig. 2) appears to be monophyletic. Thus, hydrothermal habitats have been invaded multiple times, but deep-sea hydrothermal habitats may have been invaded once.

Habitat reversals are evident within the “thermophilus” clade. Two species, *Bathymodiolus brooksi* and *B. heckerae*, have independently invaded deep-water cold seeps.

Distel et al. (2000) argued that mussel species exploiting sulfide-rich decomposing wood and bone preceded the origin of vent and seep species (Distel et al. 2000). *Benthomodiolus lignicola*, a species found on wood and bones, was used to root the present bathymodiolin trees, so it obviously cannot be used to test this hypothesis. The only other species known to occupy wood and bone habitats, *Idas washingtonia*, also stems from a basal branch in the combined tree, but its relative, *I. macdonaldi*, lives in cold seeps. Another basal lineage, undescribed species NZ-3, was found on a shallow hydrothermal seamount. The “childressi” group also is relatively basal in this tree and it mostly comprises cold seep species. Thus, given our current knowledge of deep-sea mussels, it is impossible to assess the habitat state of ancestral nodes in this tree—vent, seep, wood and bone are all probable character states (Fig. 3b)

Evolutionary age of Bathymodilinae

Although prone to saturation across the Mytilidae, *COI* sequences could be used to estimate divergence time for the Bathymodilinae. We used previously published estimates of divergence rates for mitochondrial *COI* in deep-sea mussels (Won et al. 2003b). Using the 1%/MY rate based on divergence between *Bathymodiolus thermophilus* and *B. aff. thermophilus* across the Easter microplate region of the East Pacific Rise (Won et al. 2003b), we estimated the divergence of bathymodiolin mussels from the lineage leading to *Benthomodiolus lignicola* would have occurred about 30.2 MY ago (range: 23.7–37.1 MY). Second, we substituted a more conservative rate of 0.5%/MY for *COI* divergence among invertebrate taxa (Knowlton 2000) that is consistent with the slower rates also seen in some vent–endemic annelids (Chevaldonné et al. 2002). Accordingly, divergence of other bathymodiolin mussels from the lineage leading to *Benthomodiolus lignicola* would have occurred about 60.8 MY (range 48.2–74.3). Despite the wide range of estimates obtained with these Bayesian calibrations (23.7–74.3 MY), molecular evidence clearly places the origin of bathymodilins in the Cenozoic or late Mesozoic.

Discussion

The present analysis of DNA sequences from four gene segments provides the first comprehensive phylogenetic analysis of bathymodiolin mussels from vents, seeps and other reducing environments. To assess the position of the Bathymodilinae within the family Mytilidae, we examined 18S ribosomal DNA sequences from 13 new

OTUs (Fig. 1) with respect to a previous analysis by Distel et al. (2000). The present phylogeny strongly supports the placement of *Benthomodiolus lignicola* as a basal lineage within the monophyletic subfamily Bathymodiolinae. Unfortunately, the phylogeny produced by this highly conserved gene does not resolve relationships among bathymodiolin genera. To obtain better resolution at this level, we generated a combined phylogenetic tree based on sequences from a nuclear gene, 28S rDNA, and two mitochondrial protein-coding genes, *COI* and *ND4*. Restricting our analysis to bathymodilins and rooting with *Be. lignicola* allowed us to circumvent a problem that occurred if the analysis was extended to the family Mytilidae as a whole. The two protein-coding genes exhibited significant saturation of synonymous nucleotide substitutions across the family.

The combined gene tree rooted with *Benthomodiolus lignicola* revealed four well-supported clades. Undescribed species NZ-3 from a shallow hydrothermal seamount off New Zealand branched basally to all other bathymodiolin lineages. Node II links the *Idas* species. Node III links five species placed in three genera: “*Bathymodiolus*” *childressi*, *B. mauritanicus*, *B. tangaroa* and *Gigantidas gladius* and *Tamu fisheri*. Gustafson et al. (1998, p. 89) questioned placement of “*B.*” *childressi*, the first species described from this clade, in the genus *Bathymodiolus*:

“ “*Bathymodiolus*” (their quotes) *childressi* possesses a combination of morphological characters not seen in any previously described deep-sea mytilid genus; however, genetic distance measures (at that time) ...do not clearly separate this species from other members of the genus *Bathymodiolus*. So as to avoid erecting a new mono-specific genus, this species is provisionally placed in *Bathymodiolus*. ... “*Bathymodiolus*” *childressi* differs from all other species referred to *Bathymodiolus* in having multiple separation of the posterior byssal retractors (similar to *Modiolus*), a single posterior byssal retractor scar, and a rectum that enters the ventricle posterior to the level of the auricular ostia.”

Separate analyses of published mitochondrial *COI* sequences (Miyazaki et al. 2004) also allow us to confidently place *B. platifrons* and *B. japonicus* in the “*childressi*” clade. Placement of *Tamu* in this clade is less certain, however. Inclusion of *Tamu* is supported by the *COI* and combined trees, but not by the 28S tree. More studies involving additional gene markers are needed to resolve relationships between the “*childressi*” clade and *Tamu*. Nevertheless, the present analyses clearly reveal that *Bathymodiolus* constitutes a paraphyletic taxon that requires further investigation with detailed morphological and molecular investigations. Until these matters are resolved, we recommend that the provisional use of “*Bathymodiolus*” (in quotes) when applied to members of the *childressi* clade, as follows: “*B.*” *platifrons*,

“*B.*” *japonicus*, “*B.*” *childressi*, “*B.*” *mauritanicus* and “*B.*” *tangaroa*. Depending on the placement of *Tamu* inside or outside this group, either *Tamu* or *Gigantidas* might have priority as the genus name for this group. For now, we suggest that the name *Bathymodiolus* be restricted to the monophyletic group of taxa that stems from the well-supported clade defined by node IV.

A general pattern of progressive evolution from shallow-to-deep water is supported by the combined tree, though reversals occur. Eight of nine basal lineages occur in relatively shallow environments (<1,000 m). In contrast, only one *Bathymodiolus* lineage (node IV) occurs shallower than 1,000 m. *Bathymodiolus azoricus* is found at 866 m at Menez Gwen on the Mid-Atlantic Ridge, though it also is found at deeper sites (1,710–3,350 m). *Idas washingtonia*, a basal lineage, has a remarkable depth range (960–1,919 m) (Baco and Smith 2003). We recently found this species on whale bones at 2,890 m in Monterey Bay, California (R.C. Vrijenhoek, personal observation). It is difficult to trace habitat depth for these mussels because many of the species have been found at only one or a few localities. Continued exploration of shallower vents, seamounts, wood falls and whale bones may significantly alter our limited view of bathymetric limits for many of these taxa.

Furthermore, we suspect that the apparent pattern of evolution from shallow-to-deep habitats may represent a recent re-radiation of mytilids into deep-sea environments. Jacobs and Lindberg (1998) argued that deep-sea vent communities experienced global extinction events associated with oceanic anoxic/dysoxic events at the end of the Mesozoic (Cenomanian–Turonian boundary, 93 MY ago) and early Cenozoic (end Palaeocene, 55 MY ago). Though their argument is broadly consistent with the molecular clock evidence for late Mesozoic and early to mid Cenozoic radiations of the dominant vent and seep invertebrate taxa, fossil evidence suggests that some vent and seep taxa have persisted since the early and mid Mesozoic (Little and Vrijenhoek 2003). The global and complete nature of hypothesized extinction events is debatable; nevertheless, periodic extinctions followed by re-radiation of crown-taxa from stem-ancestors provides a plausible explanation for the discrepancy between fossil and molecular evidence for the ages of vent and seep taxa (Little and Vrijenhoek 2003). Basal bathymodiolins, such as *Benthomodiolus lignicola* and undescribed species NZ-3, might be relics of lineages that survived deep-sea extinction events. However, it is unknown whether they represent lineages that continuously occupied shallow habitats, or if they were derived from deep-sea lineages that secondarily invaded shallow habitats, like *Bathymodiolus azoricus*, part of a deep-sea vent lineage that has invaded a shallow vent near the Azores.

Additional species of bathymodiolin mussels will undoubtedly be discovered as more of the world’s oceans are explored. We anticipate that the present gene trees will provide a robust foundation for adding new branches and testing evolutionary hypotheses. More

significantly, the present results are consistent with the hypothesis that shallow-water hydrothermal seamounts, cold seeps, and hydrocarbon deposits may have served as refugia against large-scale extinction events in deep oceanic basins. We urge comprehensive phylogenetic studies of other animal taxa found on seamounts, vents and seeps to test the hypothesis that such shallow-water habitats may have provided refugia during deep-sea anoxic/dysoxic events and opportunities for re-radiated into deep-sea environments.

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