

ABSENCE OF COSPECIATION BETWEEN DEEP-SEA MYTILIDS AND THEIR THIOTROPHIC ENDOSYMBIONTS

YONG-JIN WON,^{1*} WILLIAM J. JONES^{2,3} AND ROBERT C. VRIJENHOEK²

¹Division of EcoScience, Ewha Womens University, Seoul, Korea, 120-750; ²Monterey Bay Aquarium Research Institute, Moss Landing, California 95039; ³Present Address: Environmental Genomics Core Facility, University of South Carolina, Columbia, South Carolina 29208

ABSTRACT Molecular phylogenetic analyses revealed no evidence for cospeciation between deep-sea bathymodiolin mussels (Bivalvia: Mytilidae) and their associated thiotrophic (sulfur-oxidizing) bacterial endosymbionts. Host and symbiont tree topologies were not congruent and inferred time-depths of the gene trees were inconsistent, as expected if the mussel hosts are infected by local strains of the symbiont. Evolutionary divergence among the thiotrophs is correlated with geographical distances among sample locations. Apparently these bacteria established a global distribution long before contemporary oceanic barriers achieved their current positions, and before evolutionarily younger mussel hosts diversified into presently recognized species.

KEY WORDS: Bathymodiolinae, symbiosis, sulfur-oxidizing bacteria, phylogeny

INTRODUCTION

Bathymodiolin mussels (Mytilidae: Bathymodiolinae) are dominant members of deep-sea hydrothermal vent, cold seep, and wood- and whale-fall communities worldwide (Jones et al. 2006). These mussels are mixotrophic, retaining functional guts and hosting thiotrophic (sulfur-oxidizing) or methanotrophic endosymbionts in their gill tissues (Page et al. 1990). Nearly half of the bathymodiolin species are only known to host thiotrophs, three species are only known to host methanotrophs, and six species have dual symbiosis involving thiotrophs and methanotrophs (Table 1). *Bathymodiolus azoricus* Cosel & Comtet, 1999, a Mid-Atlantic vent mussel with dual symbiosis, acquires thiotrophic bacteria from the environment in which it grows (Won et al. 2003). Infection probably occurs through gill epithelial cells, which appear to engulf bacteria by endocytosis (Le Pennec et al. 1988, Won et al. 2003). However, these cells may also shed bacteria by exocytosis (Salerno et al. 2005), providing a mechanism for horizontal transfer among adult mussels (Kádár et al. 2005). Nevertheless, vertical transmission may also occur. According to a preliminary study that mentioned having detected symbionts RNA in gonadal tissues of a Pacific species, *B. thermophilus* Kenk and Wilson 1985, transovarial transmission mechanism in the symbiont may operate in these vent mussels (Cary & Giovannoni 1993). Furthermore, the gill tissues of freshly settled, early postlarvae of *B. azoricus* already contain bacterial cells that are identifiable as thiotrophs and methanotrophs (Salerno et al. 2005), which is consistent with population genetic evidence that dispersing larvae may transport bacteria from their natal sites (Won et al. 2003).

Modes of symbiont transmission—ranging from strictly vertical (heritable) transmission to completely horizontal (infectious) transmission (sensu Werren & O'Neill 1997)—can profoundly affect the genetic structure and evolution of microbes (Moran 1996, Lambert & Moran 1998, Peek et al. 1998a, Wernegreen & Moran 1999, Newton et al. 2007). Strictly vertical transmission *via* the host's eggs will generate associations (disequilibria) between cytoplasmic genes belonging to the host and cotransmitted symbiont genomes (Sanchez et al. 2000, Hurtado et al. 2003, Hurst & Jiggins 2005). Expanded to

evolutionary time-scales, cotransmission leads to cospeciation (parallel host and symbiont phylogenies) as seen in deep-sea vesicomid clams and their thiotrophic endosymbionts (Peek et al. 1998a). In contrast, infectious symbionts such as the thiotrophs hosted by vestimentiferan tubeworms (Nussbaumer et al. 2006) vary independently of the hosts (Di Meo et al. 2000, Vrijenhoek et al. 2007), and the symbiotic partners do not exhibit cospeciation (Feldman et al. 1997, Nelson & Fisher 2000, McMullin et al. 2003). However, cases are known where geographical covariates of diversification in the host and symbiont might create an illusion of cospeciation. For example, Atlantic *versus* Pacific sepiolid squids host geographically corresponding strains of luminescent *Vibrio* symbionts that are acquired environmentally (Nishiguchi et al. 1998). Such appearances of congruence are expected if comparisons of host and symbiont phylogenies examine only a small number of taxa from very distant locations. Geographical covariates of diversification must always be considered as a factor in studies of host-symbiont cospeciation (Di Meo et al. 2000).

Our purpose in this study is to conduct a global-scale phylogeographic analysis of chemosynthetic endosymbionts associated with bathymodiolin mussels and to assess evidence for cospeciation with the host species. First, we updated an earlier phylogeny of bathymodiolin mussels (Jones et al. 2006) with newly published sequences from two mitochondrial genes (ND4 and COI) and one nuclear gene (28S rRNA). Second, we generate a phylogeny for the thiotrophic endosymbionts based on their 16S (small subunit) ribosomal RNA (rRNA) gene sequences. Third, we test for cospeciation between the thiotrophs and mussel hosts. Finally, we examine potential geographical sources of covariation between host and symbiont genetic distances matrices. Methanotrophic symbionts were not considered in these analyses, because of their restricted phylogenetic distribution among bathymodiolin species (Table 1).

MATERIALS AND METHODS

Bathymodiolin Taxonomy

Bathymodiolin taxonomy is in a state of flux because of recent discoveries of new species. The name *Bathymodiolus*

*Corresponding author. E-mail: won@ewha.ac.kr

TABLE 1.
Endosymbiont types associated with bathymodiolin mussel operational taxonomic units (OTUs).

Bathymodiolin OTU	Region (location) ¹	Hab. ²	Type ³	Method ⁴	References ⁵
Pacific Ocean					
<i>B. brevior</i>	N. Fiji Basin	V	T	IMT	(Dubilier et al. 1998) *
<i>B. septemdiarium</i>	IOA (Myojin Knoll)	V	T	MT	(Fujiwara et al. 2000) *
<i>B. thermophilus</i>	IBA (Mariana Trough)				
<i>B. aff. thermophilus</i>	EPR (Galapagos Rift)	V	T	IM	(Kenk & Wilson 1985) *
<i>B. aff. thermophilus</i>	EPR (32°S)	V	T	M	*
' <i>B.</i> ' <i>aduloides</i>	IHR (off Kikajima Island)		T	I	(Yamanaka et al. 2000)
' <i>B.</i> ' sp. Manus	MB (Manus Basin)	V	T	M	*
' <i>B.</i> ' <i>platifrons</i>	IHR (North Knoll)		M	MT	(Fujiwara et al. 2000, Yamanaka et al. 2000)
' <i>B.</i> ' sp. Lihir	PNG (Lihir Seamount)	V	?		
' <i>B.</i> ' <i>hirtus</i>	Kuroshima Knoll (IBA)	S	?		
' <i>B.</i> ' <i>securiformis</i>	Kuroshima Knoll (IBA)	S	?		
' <i>B.</i> ' <i>tangaroa</i>	KERM (Cape Turnagain)	S	T	M	*
<i>G. gladius</i>	KERM (Rumble III Seamount)	V	T	M	*
<i>I. washingtonia</i>	California margin	W	T	IT	(Deming et al. 1997)
<i>Be. lignicola</i>	KERM (Chatham Rise)	W	?		
<i>b.</i> sp. NZ-3	KERM (Macaulay Cone)	V	T	M	*
Atlantic Ocean					
<i>B. azoricus</i>	IHR (North Knoll)				
<i>B. azoricus</i>	MAR (Menez Gwen)	V	TM	IEMT	(Pond et al. 1998, Fiala-Medioni et al. 2002, Won et al. 2003, Salerno et al. 2005, Duperron et al. 2006)
<i>B. brooksi</i>	GOM (Alamiños Canyon)	S	TM	M	(Duperron 2005)
<i>B. boomerang</i>	Barbados accretionary prism	S	TM	T	(Cosel & Olu 1998)
<i>B. heckerae</i>	GOM (W FL Escarp.), Blake Ridge	S	TM	MT	(Duperron et al. 2005, Salerno et al. 2005)
<i>B. puteoserpentis</i>	MAR (Snakepit)	V	TM	MT	(Cavanaugh et al. 1992, Distel et al. 1995, Won et al. 2003, Duperron et al. 2006)
' <i>B.</i> ' <i>childressi</i>	GOM (Louisiana Slope)	S	M	MT	(Distel & Cavanaugh 1994)
' <i>B.</i> ' <i>mauritanicus</i>	Gabon continental margin	S	TM	MT	(Duperron et al. 2005)
<i>I. macdonaldi</i>	GOM (Louisiana Slope)	S	T	M	*
<i>T. fisheri</i>	GOM (Louisiana Slope)	S	?		
Indian Ocean					
<i>B. marisindicus</i>	CIR (Kairei Field)	V	T	IMT	(Van Dover et al. 2001) *

¹ Sample location, Regions: CIR = Central Indian Ridge; EPR = East Pacific Rise; GOM = Gulf of Mexico; IBA = Izu-Bonin Arc; IHR = Iheya Ridge; IOA = Izu-Ogasawara Arc; KERM = Kermadec Arc; MAR = MidAtlantic Ridge.

² Habitat type: S = cold seep; V = vent; W = wood and bone.

³ Endosymbiont type: M = methanotrophic; T = thiotrophic; TM = dual symbiosis; ? = undetermined.

⁴ Methods: E = enzyme assays; I = stable isotopes; M = molecular; T = transmission electron microscopy.

⁵ 16S sequences obtained for this study may be from locations that vary from previously cited studies (see Table 2).

* This study.

sensu lato is often applied arbitrarily to unrelated members of this subfamily, and a genus-level revision is warranted (Gustafson et al. 1998, Cosel 2002). Here we apply *Bathymodiolus* sensu stricto to the *B. thermophilus* species group, a monophyletic clade previously defined by Jones et al. (2006). We enclose "*Bathymodiolus*" in double quotes to denote members of the "*B.*" *childressi* Gustafson et al. 1998 species group, which is morphologically distinct (Cosel 2002) and paraphyletic to *Bathymodiolus* (Jones & Vrijenhoek 2006). Based on the present findings, we enclose '*Bathymodiolus*' in single quotes to denote a questionable assignment of '*B.*' *aduloides* Hashimoto & Okutani 1994. For unnamed species that do not fall into known clades, we assign placeholder names (e.g., '*bathymodiolus* sp. NZ-3') based on geographic location (Table 1).

Molecular analyses have also identified synonymous names for what may be single widespread species (Jones & Vrijenhoek 2006, Jones et al. 2006). For example, *Bathymodiolus septem-*

dierum Hashimoto & Okutani 1994 and *B. marisindicus* Hashimoto 2001 may be junior synonyms for *B. brevior* Cosel et al. 1994 (cf. Van Dover et al. 2001). Named members of other geographical species-pairs e.g., "*B.*" *mauritanicus* Cosel, 2002 versus "*B.*" *childressi*, and *B. heckerae* Gustafson et al. 1998 versus *B. boomerang* Cosel & Olu 1998 are very closely related and may be conspecific (Olu-Le Roy et al. 2007, Génio et al. 2008). Informative morphological characters and additional genetic markers are needed to resolve these problems. For the purposes of the following analyses, we treat named species as distinct operational taxonomic units (OTUs).

Molecular Methods

The present host phylogeny was based on NADH dehydrogenase 4 (ND4), cytochrome-*c*-oxidase subunit I (COI) and large subunit ribosomal RNA (28S) sequences (Table 2) that were previously published (Miyazaki et al. 2004, Iwasaki et al.

TABLE 2.

Operational taxonomic units (OTUs) examined for thiotrophic endosymbionts. Associated GenBank accession numbers original to this study in boldface type.

Bathymodiolin OTU	Location (abbreviation) ¹	Latitude; Longitude	Depth Meters	Host Sequences			Thiotroph 16S
				ND4	COI	28S	
Pacific Ocean							
<i>B. brevior</i> (LBA)	Lau Basin (LBA)	23°13'S; 176°38'W	1750	AY046277	AY275544	AY781143	DQ321714
<i>B. septemdiarium</i> (MT)	Mariana Trough (MT)	18°13'N; 144°42'E	3589	AY649806	AY649799	AY781150	DQ321713
<i>B. thermophilus</i> (GAR)	Galapagos Rift (GAR)	0°48'N; 86°13'W	2460	AY649807	AF456285	AY781141	DQ321716
<i>B. aff. thermophilus</i> (32S)	EPR, 32°S (32S)	31°52'N; 112°3'W	2331	AY649809	AF456317	AY781140	DQ321717
<i>'B.' aduloides</i>	Off Kikaijima Island (IBA)	28°26'N; 130°19'E	1451	AB175324	AB170055	—	—
<i>'B.' sp. Manus</i> (MB)	Manus Basin (MB)	3°44'S; 151°40'E	1629	AB175320	AB101431	—	EU326223
<i>"B." hirtus</i>	Kuroshima Knoll (IBA)	24°08'N; 124°12'E	637	AB175299	AB170047	—	—
<i>"B." japonicus</i>	Sagami Bay (IBA)	35°00'N; 139°14'E	1170	AB175284	AB101422	—	—
<i>"B." platifrons</i>	Sagami Bay (IBA)	36°00'N; 139°14'E	1180	AB175287	AB101419	—	—
<i>"B." securiformis</i>	Kuroshima Knoll (IBA)	24°88'N; 124°12'E	641	AB175294	AB170048	—	—
<i>"B." sp. Lihir</i>	Lihir Seamount (MB)	3°19'S; 152°35'E	1598	—	DQ317307	—	—
<i>"B." tangaroa</i> (CT)	Cape Turnagain (CT)	40° 26' S; 178°58' E	920	AY649811	AY608439	AY781134	EU326222
<i>G. gladius</i> (RIII)	Rumble III (RIII)	35°44'S; 178°30'E	300	AY649813	AY649802	AY781149	EU326224
<i>I. washingtonia</i>	Monterey Bay, CA (MON)	36°37'N; 122°26'W	2980	AY649815	AY275546	AY781146	—
<i>Be. lignicola</i>	Chatham Rise (CR)	34°41'S; 177°14'W	826	AY649817	AY275545	AY781131	—
<i>b. sp. NZ-3</i> (MC)	Macauley Cone (MC)	30°13'S; 178°27'W	200	AY649812	AY608440	AY781133	DQ321718
Atlantic Ocean							
<i>B. azoricus</i>	Menez Gwen (MG)	37°17'N; 32°15'W	866	AF128534	AY649795	AY781148	DQ321711
<i>B. brooksi</i>	W. Florida Escarpment (WFE)	26°02'N; 84°55'W	3314	AY649805	AY649798	AY781135	—
<i>B. heckerae</i>	Alamiños Canyon, TX (AC) W. Florida Escarpment (WFE)	26°21'N; 94°29'W 26°02'N; 84°55'W	2222 3314	AY130247 AY130246	AY649797 AY649794	AY781136 AY781138	AM236328
<i>B. puteoserpentis</i>	Snakepit (SP) Blake Ridge (BR)	23°22'N; 44°56'W 32°30'N; 76°12'W	3023 2155	AF128533 AY130245	AY649796 AY649793	AY781151 AY781139	DQ321712 —
<i>"B." childressi</i>	Alamiños Canyon, TX (AC)	26°21'N; 94°29'W	2222	AY130248	AY649800	AY781137	—
<i>"B." mauritanicus</i>	Gabon Cont. Margin (GCM)	0°53'N; 5°28'W	1000	AY649810	AY649801	AY781144	AJ745718
<i>I. macdonaldi</i>	LAS, Garden Bank (LAS)	27°50'N; 92°10'W	650	AY649816	AY649804	AY781145	EU326225
<i>T. fisheri</i>	LAS, Garden Bank (LAS)	26°21'N; 94°29'W	2222	AY649814	AY649803	AY781132	—
Indian Ocean							
<i>B. marisindicus</i>	Central Indian Ridge (CIR)	23°53'S; 69°36'E	3289	AY046279	AY275543	AY781147	DQ321715

¹ Abbreviations, as used in Figures 1–3.

2006, Jones & Vrijenhoek 2006, Jones et al. 2006). The present symbiont phylogeny was based on 7 published and 12 new small subunit ribosomal RNA (16S) sequences (Table 2). The thiotrophic endosymbiont from four vesicomid clams, *Calyptogena pacifica* Dall 1891, *Calyptogena elongata* (Dall 1916), *Vesicomya gigas* Dall 1896, and *Ectenagena extenta* Krylova & Moskalev 1996, (GenBank acc. no. AF035723, AF035719, AF035726, and AF035725 respectively), were used as outgroups (see Peek 1998).

Purification of bacterial DNA from host gill tissue followed an established protocol (Goffredi et al. 2005). PCR used eubacteria-specific 16S primers (Lane et al. 1991) under the following conditions: 30–100 ng of template DNA, 2.5 units of *AmpliTaq* polymerase in 5 µL 10 × buffer (Applied Biosystems Inc., Foster City, CA), 5 µL MgCl₂ (2.5 µM), 2 µL of each primer (10 µM final conc.), 5 µL of a 2 mM stock solution of dNTPs, and sterile H₂O to a final-volume of 25 µL. PCR involved an initial denaturation step at 94°C for 10 min. followed by 25 cycles

at 94°C/40 sec., 55°C/1 min., and 72°C/1 min., followed by a final extension at 72°C/7 min. PCR products were purified with the Qiagen PCR purification Kit (Qiagen Inc. Valencia CA), and template DNA was cloned with a TOPO-TA protocol (Invitrogen, Carlsbad, CA) and sequenced using Big Dye Terminator cycle sequencing (Applied Biosystems Inc., Foster City, CA) on an ABI Prism 3100 DNA sequencer (Applied Biosystems Inc., Foster City, CA). M13 forward and reverse primers were used for sequencing (Invitrogen, Carlsbad, CA). We sequenced 16 clones bidirectionally for each mussel. The sequences were coarsely aligned with Sequencher v. 4.2 (Gene Codes Corporation Inc. Ann Arbor, MI) and finely aligned by eye.

Phylogenetic Analyses

We used MrBayes v. 3.12 (Ronquist & Huelsenbeck 2003) to conduct phylogenetic analyses of mussel hosts and thiotrophic

endosymbionts. Monte Carlo Markov chains (MCMC)—length of 1.1×10^6 generations with nine chains—were sampled every 100 generations. MCMC convergence was assessed visually by inspecting sample paths of model parameters (to establish a 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 analyzed graphically with Tracer, v. 1.3 (Rambaut & Drummond 2003). We used the last 5,000 sampled trees to estimate Bayesian posterior probabilities (BPP). 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). Therefore, nodes with less than 0.95 BPP were collapsed to polytomies in the figures. Divergence time among OTUs was estimated with BEAST v. 1.0.3 (Drummond & Rambaut 2003).

We analyzed 16S rRNA sequences from the thiotrophic endosymbionts. Stem and loop structures were predicted from the *E. coli* model (Brodsky et al. 1992). The MCMC log-likelihood values and associated parameters for sampled trees stabilized after $\sim 5 \times 10^5$ generations. An estimate of divergence time was based on the assumption that 16S substitutions occur at a rate of about 1% per 50 million years (Ochman & Wilson 1987).

The combined analysis of ND4, COI and 28S sequences for mussels coded absent sequences (see Table 2) as missing data (see Ronquist & Huelsenbeck 2003). *Benthomodiolus lignicola* was used to root the tree. To remain consistent with previous phylogenetic analyses (Jones & Vrijenhoek 2006, Jones et al. 2006), we applied the codon model to ND4 and COI and partitioned 28S by stem and loop structures, as predicted by GeneBee (Brodsky et al. 1992). MCMC log-likelihoods and associated parameters for ND4 and COI stabilized after $\sim 2-3 \times 10^5$ generations, and for 28S after $\sim 3-4 \times 10^5$ generations. An estimate of divergence time was based on the assumption that COI sequence substitutions in deep sea mussels occur at a rate of about 0.5% per million years (Jones et al. 2006).

We tested for cospeciation between hosts and symbionts by examining a subset of mussels that used thiotrophs (alone or in dual symbiosis). The program PARAFIT (Legendre et al. 2002) assessed the correlation between principle components derived from genetic distance matrices for the thiotrophic symbionts and mussel hosts. Uncorrected “*p*” distance was used for the symbionts and a general time-reversible (GTR) distance model was used for the hosts, both generated by the program PAUP* (Swofford 1998). Finally, we assessed whether host and symbiont genetic distances were correlated with geographical distance among sample locations. Two geographical scenarios were investigated: (1) Great Arc distances between pairs of locations were estimated from the geographical coordinates listed in Table 1; and (2) Minimum oceanic distance around continents. Great Arc distances and Mantel tests of correlation were estimated with the program R-PACKAGE (Casgrain & Legendre 2000). Minimum oceanic distance around continents and islands was estimated using GOOGLE EARTH v.4 (<<http://earth.google.com/>>). The shortest paths between all pairs of locations were traced around present-day continental shelf margins that intervened between locations.

RESULTS

Bathymodiolin Evolution

We conducted a combined Bayesian phylogenetic analysis involving published ND4, COI, and 28S sequences for bathymodiolin mussels (Fig. 1). We did not include published 18S rRNA sequences in this study, because this gene provides no phylogenetically informative variation for the ingroup taxa (Distel et al. 2000, Jones et al. 2006). Slight differences exist between the present combined tree and an earlier analysis by Jones et al. (2006). Differences resulted mostly from inclusion of new taxa with incomplete sequence data—'*Bathymodiolus aduloideus* (ND4 + COI) and “*B.*” sp. Lihir (COI). Lower Bayesian posterior probabilities (BPPs) were obtained for clades that involved taxa with missing data, but this is a known problem for combined phylogenetic analyses (Smith et al. 2004, Yoshizawa et al. 2006). Notwithstanding, the present mussel tree is composed of a number of highly supported monophyletic groups (BPPs = 1.0). The first grouping (I) contains all the taxa, except for bathymodiolin sp. NZ-3 and the outgroup *Benthomodiolus lignicola* Dell 1987. Nested within group I are groups II (*Idas*) and III (a polytomy involving four groups IV–VII). Hierarchical relationships within groups IV (*Bathymodiolus* s.s.) and VII (the “*Bathymodiolus*” *childressi* group) are well resolved. Divergence time between *Benthomodiolus lignicola* and the ingroup taxa was estimated to be about 60 MY (range 48–74 million years) using the conservative 0.5% per million years clock.

Symbiont Condition

The symbiont condition associated with each mussel species (Table 1) was inferred from a literature survey and original data. Endosymbionts have not been reported for *Benthomodiolus lignicola*, and our attempts to amplify bacterial 16S from the gill tissues of specimens in our possession resulted in no detectable product (two attempts with each of three individuals available to us). Similarly, we were unable to amplify bacterial 16S from our *Idas washingtonia* Bernard 1978 specimens (two attempts with each of two individuals). The symbiont conditions of *Tamu fisheri* Gustafson et al. 1998, “*Bathymodiolus securiformis* Okutani et al. 2004, “*B.*” *hirtus* Okutani et al. 2004, and “*B.*” sp. Lihir, have not been reported and we did not have gill tissues from these specimens. It should be noted that the presence of methanotrophs in dual symbiosis may be missed with PCR-based approaches; consequently the distribution of methanotrophs among mussel hosts may be underestimated (Duperron 2005). Nevertheless, the present information suggests that the thiotrophic condition is more widespread among bathymodiolins.

Phylogeny of Thiotrophic Endosymbionts

Our Bayesian phylogenetic analysis of 16S rRNA sequences from thiotrophic endosymbionts revealed a well-resolved clade that is related to the thiotrophs found in vesicomid clams and used here as outgroup taxa (Fig. 2). Divergence time between the outgroup and ingroup taxa was estimated to be about 138 MY (range 112–166 million years), based on a conservative molecular clock calibration of 1% per 50 MY for 16S rRNA sequences (Ochman & Wilson 1987).

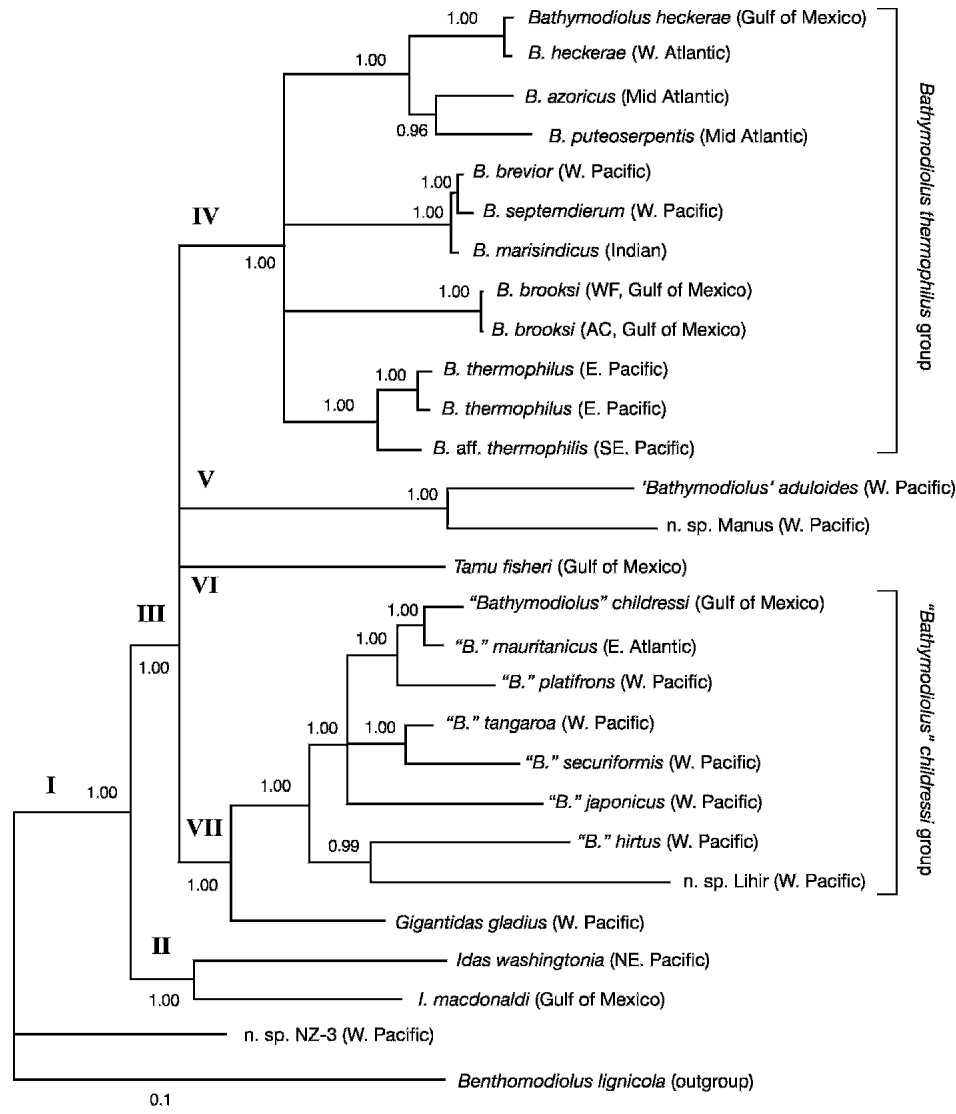


Figure 1. Bayesian tree of bathymodiolin mussels based on combined dataset for ND4, COI and 28S. Scale bar indicates percent sequence divergence. Nodes with Bayesian posterior probabilities (BPPs) less than 0.95 were collapsed into polytomies. Roman numerals define well-supported clades discussed in the text.

The thiotroph phylogeny suggests that some of these bacteria are clustered geographically and not according to similarity of host species. For example, closely related (and possibly conspecific) mussels in the *Bathymodiolus brevior* complex (*B. brevior*, *B. septemdierum*, and *B. marisindicus*) host some of the most divergent thiotroph lineages in the tree (Fig. 2). Except for the Gulf of Mexico thiotrophs, which did not group together, the remaining symbionts clustered mostly according to their residence in the West Pacific, East Pacific, Atlantic, and Indian oceans (Fig. 2).

Tests of Host, Symbiont, and Geographical Congruence

Host and symbiont tree topologies were not congruent (Fig. 3). A comparison of host and symbiont patristic distances for the subset of thiotrophic mussels (Table 3) revealed no relationship based on tests applied with PARAFIT ($P = 0.37$). Similarly, a Mantel test of correlation between host and sym-

biont genetic distance matrices revealed no significant association (Mantel's $r = 0.064$, $P = 0.374$).

Symbiont genetic distances were correlated, however, with great arc distances among the sample locations (Mantel's $r = 0.459$, $P < 0.001$). Minimum oceanic distances, traced along continental margins, were not correlated with symbiont distances (Mantel's $r = 0.032$, $P = 0.360$). Among the mussel hosts, genetic distances were not correlated with great arc (Mantel's $r = 0.020$, $P = 0.425$) or minimum oceanic distances (Mantel's $r = 0.044$, $P = 0.304$). The geographic distances used in our analysis would change slightly if complete ranges of the mussel species were known, but most of the ocean benthos remains unsampled and unexplored (Sibuet & Olu 1998, Van Dover et al. 2002). Regardless, the Mantel test procedures used in our analyses convert geographical distances to rank-order distances, so range inaccuracies should not significantly affect correlations between the symbionts and geography, or mask a potential correlation between mussels and geography.

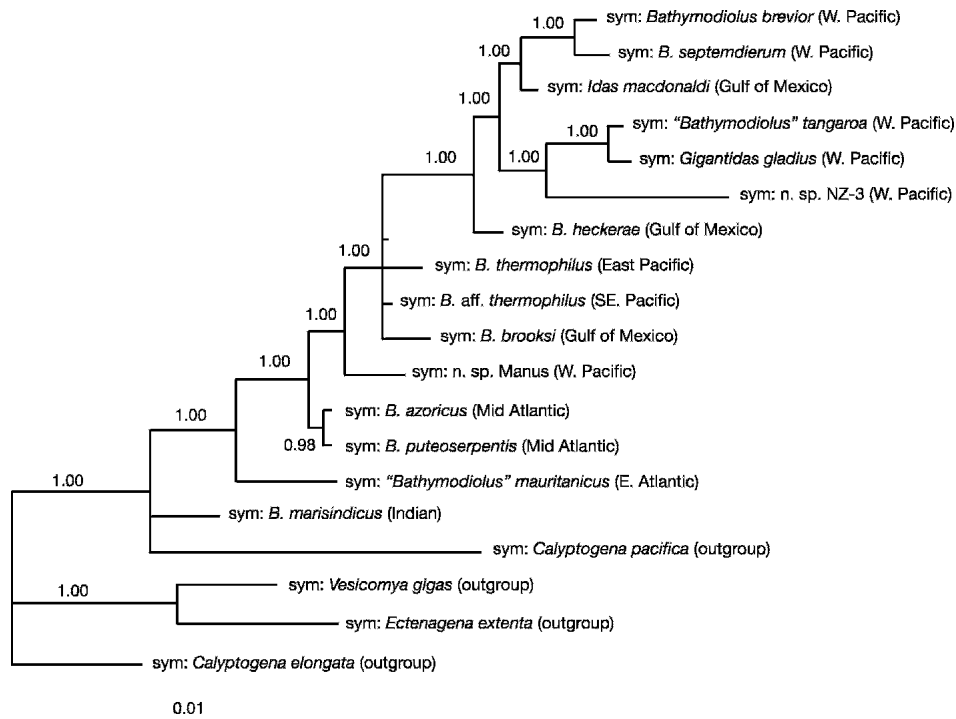


Figure 2. Bayesian tree of bathymodiolin thiotrophic endosymbionts based on 16S rRNA sequences. Scale bar indicates percent sequence divergence. Nodes with Bayesian posterior probabilities (BPPs) less than 0.95 were collapsed into polytomies.

DISCUSSION

An updated bathymodiolin phylogeny generated from published DNA sequences (Table 2; Figure 1) provided a framework for characterizing evolutionary associations with symbiotic bacteria (Table 1). Occurrence in a particular ocean basin appears to play a primary role in determining the symbiotic condition of these mussels. All the Atlantic species, except for *Idas macdonaldi* Gustafson et al. 1998, are known to host methanotrophic symbionts in the dual or single conditions. Conversely, all Pacific species, except for two ("*B.*" *platifrons* Hashimoto & Okutani, 1994 and "*B.*" *japonicus* Hashimoto & Okutani, 1994, are known to host thiotrophs. It is not apparent, therefore, that phylogenetic constraints limit the ability of bathymodiolins to host methanotrophs. The remarkable success of bathymodiolins in a variety of chemosynthetic environments (hydrothermal vents, cold-water hydrocarbon seeps, and wood- and whale-falls) probably results from their mixotrophic capabilities, which includes suspension feeding and the ability to adopt chemosynthetic bacteria that can use alternative energy sources (Page et al. 1990, Won et al. 2003). Additional studies with *Benthomodiolus lignicola*, *Tamu fisheri*, and other species that remain to be characterized for symbionts are needed to portray a more complete picture of the evolutionary associations with thiotrophic and methanotrophic endosymbionts.

Like all other sulfur-oxidizing endosymbionts associated with vent and seep invertebrates, the 15 thiotrophic mussel endosymbionts examined in this study (Fig. 2) fall into a well-supported monophyletic clade of γ -subdivision of the Proteobacteria (Distel et al. 1988, Peek 1998). Unlike the vertically transmitted thiotrophs associated with vesicomid clams (Hurtado et al. 2003), mussel thiotrophs did not exhibit

cospeciation with their hosts (Fig. 3). In addition, the time depths for diversification of the mussel and thiotroph phylogenies appeared to be dissimilar. Evolutionary rates are accelerated in some maternally transmitted endosymbiotic bacteria, including the thiotrophs associated with deep-sea vesicomid clams (Peek et al. 1998b, Woolfit & Bromham 2003). Severe population bottlenecks that occur during vertical transmission reduce the variance effective size (N_e) of the bacterial population, but this acceleration apparently has not occurred in the thiotrophic endosymbionts of horizontally transmitted symbionts. Cloned 16S rRNA sequences originating from environmental bacteria in Suiyo Seamount hydrothermal plumes appear to represent the closest free-living bacterial relatives of the thiotrophs hosted by vent mussels (see Figure 1 Sunamura et al. 2004). Branch lengths among these clones were similar to those among mussel endosymbionts, which suggests that accelerated substitution rates in the symbionts are unlikely. The molecular clock calibration that we used to date the diversification of symbiotic thiotrophs should be refined in future studies. Nevertheless our estimated age of this thiotroph radiation (~138 MY, range 112–166 million years) appears to significantly predate the estimated age for the bathymodiolin radiation (~60 MY, range 48–74 MY). A similar lack of congruence in tree topologies and evolutionary time-scales is found in vestimentiferan tubeworms and their environmentally acquired thiotrophic endosymbionts (Feldman et al. 1997, Nelson & Fisher 2000, McMullin et al. 2003).

Despite the absence of evidence for cospeciation, our findings cannot be interpreted as evidence against some component of vertical transmission (cf. Cary & Giovannoni 1993, Won et al. 2003, Salerno et al. 2005). A 'leaky' vertical system of transmission would produce a similar lack of congruence.

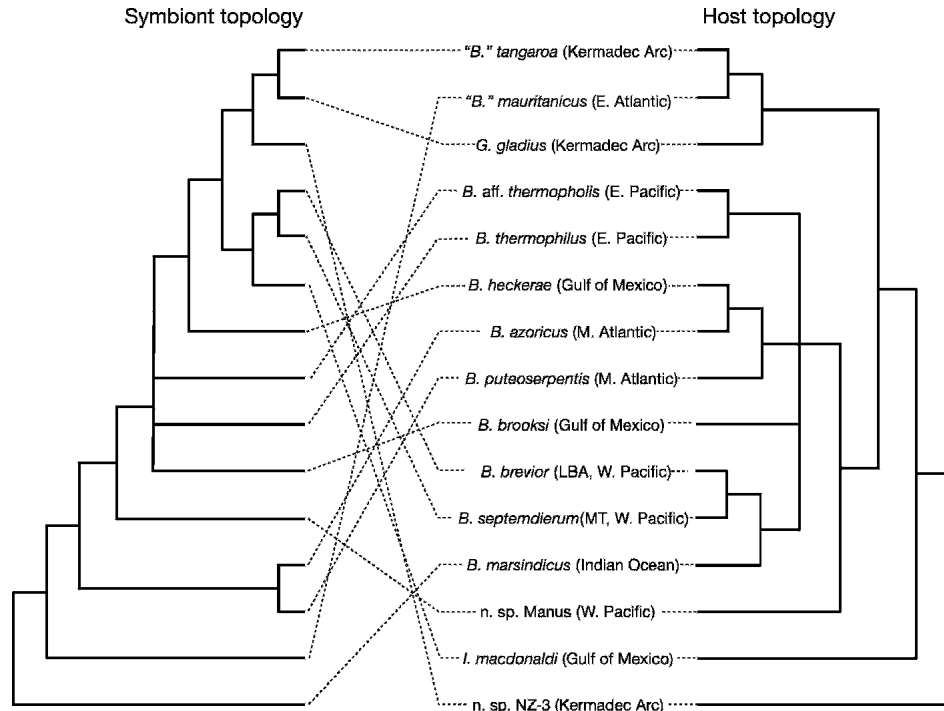


Figure 3. Coevolutionary relationships of thiotroph symbiont 16S rRNA (left) and mussel ND4/COI/28S (right) phylogenetic trees. Mussel and thiotroph symbiont associations are indicated by connecting lines. Crossed lines indicate incongruent evolutionary host-symbiont relationships.

Occasional losses of infection, reinfections, and host shifts occur with vertically transmitted *Wolbachia* symbionts of insects and result in a similar lack of host-symbiont congruence (West et al. 1998, Shoemaker et al. 2002, Kikuchi & Fukatsu

2003, Reuter et al. 2004). It seems that the thiotrophic symbionts of bathymodiolins can be lost if the host is starved of H_2S and reacquired by horizontal transmission if the host is exposed to H_2S and infected individuals (Kádár et al. 2005).

TABLE 3.

Genetic distance matrices. Pairwise genetic distances among bathymodiolin mussels (below diagonal) and associated thiotrophic symbionts (above diagonal).

Species (locality)	Pacific								Atlantic						Indian	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16*
1. <i>B. brevior</i> (LBA)		0.005	0.020	0.023	0.019	0.034	0.019	0.017	0.023	0.023	0.019	0.013	0.012	0.041	0.047	0.059
2. <i>B. septemdiarium</i> (MT)	0.005		0.023	0.026	0.022	0.029	0.022	0.020	0.027	0.027	0.022	0.015	0.015	0.044	0.049	0.061
3. ' <i>B.</i> ' sp. Manus (MB)	0.232	0.229		0.026	0.029	0.043	0.014	0.012	0.012	0.012	0.012	0.022	0.018	0.029	0.034	0.060
4. ' <i>B.</i> ' <i>tangaroa</i> (CT)	0.135	0.134	0.245		0.000	0.022	0.018	0.017	0.028	0.028	0.023	0.020	0.015	0.058	0.061	0.083
5. <i>G. gladius</i> (RIII)	0.130	0.134	0.241	0.104		0.030	0.028	0.026	0.032	0.032	0.030	0.019	0.015	0.046	0.047	0.061
6. b. sp. NZ-3 (MC)	0.176	0.182	0.296	0.164	0.157		0.039	0.037	0.043	0.043	0.041	0.026	0.030	0.057	0.030	0.063
7. <i>B. thermophilus</i> (GAR)	0.092	0.091	0.218	0.125	0.115	0.158		0.003	0.012	0.012	0.007	0.016	0.010	0.030	0.035	0.070
8. <i>B. aff. thermophilus</i> (32S)	0.094	0.091	0.219	0.131	0.122	0.170	0.029		0.010	0.010	0.004	0.013	0.008	0.027	0.032	0.067
9. <i>B. azoricus</i> (MG)	0.086	0.092	0.230	0.135	0.137	0.177	0.092	0.102		0.000	0.012	0.020	0.015	0.021	0.027	0.061
10. <i>B. puteoserpentis</i> (SP)	0.088	0.094	0.239	0.138	0.136	0.177	0.092	0.105	0.058		0.012	0.020	0.015	0.021	0.027	0.061
11. <i>B. brooksi</i> (AC)	0.091	0.094	0.253	0.132	0.135	0.167	0.108	0.102	0.095	0.100		0.017	0.015	0.030	0.035	0.065
12. <i>B. heckerae</i> (WFE)	0.088	0.094	0.256	0.137	0.143	0.175	0.099	0.104	0.064	0.070	0.104		0.012	0.034	0.037	0.056
13. <i>I. macdonaldi</i> (LAS)	0.156	0.157	0.237	0.154	0.149	0.175	0.149	0.156	0.160	0.164	0.162	0.163		0.044	0.056	0.077
14. ' <i>B.</i> ' <i>mauritanicus</i> (GCM)	0.129	0.139	0.259	0.047	0.112	0.161	0.123	0.132	0.130	0.126	0.128	0.130	0.154		0.028	0.065
15. <i>B. marisindicus</i> (CIR)	0.005	0.009	0.227	0.130	0.129	0.174	0.093	0.089	0.091	0.092	0.089	0.091	0.156	0.131		0.051
16. outgroup†	0.211	0.225	0.352	0.234	0.206	0.211	0.207	0.215	0.228	0.235	0.198	0.223	0.236	0.218	0.220	

* Symbiont outgroup, *Calyptogena pacifica* symbiont.

† Host outgroup, *Benthomodiolus lignicola*.

Consequently, horizontal transfers of symbionts might have occurred between mussel species that co-occur geographically. For example, *Bathymodiolus azoricus* and *B. puteoserpentis* Cosel et al. 1994 co-occur and hybridize at a contact zone on the MidAtlantic Ridge, and some individuals are coinfecting by symbiont strains that otherwise differ between the host species (Won et al. 2003). Host transfers might also explain associations of closely related thiotrophs with highly divergent mussel hosts (Fig. 2), such as *Gigantidas gladius* Cosel & Marshall 2003 from the Kermadec Arc, "*Bathymodiolus*" *tangaroa* Cosel & Marshall 2003, and the undescribed bathymodiolin sp. NZ-3, all from the western Pacific.

Diversification among the mussel thiotrophs increased with geographical distance among the sample locations, a pattern that also occurs in the horizontally acquired symbionts of vestimentiferan tubeworms (Di Meo et al. 2000). However, divergence among the mussel hosts was not correlated with geographical distance. Continental barriers presently inhibit dispersal of these organisms among ocean basins, but minimal distances around continental barriers were not correlated with symbiont or mussel genetic distances. This result is not surprising for the symbionts if we conditionally accept their evolutionary age (112–160 MY), because their diversification began before the continents acquired their modern positions (~50 MY). Therefore, related thiotrophs hosted by western Pacific mussels (*B. brevior* and *B. septemdiarum*) and Gulf of Mexico

mussels (*Idas macdonaldi*) might reflect bacterial dispersal that occurred long before the final closure of the Tethys Sea, 15–20 MY ago (Steininger & Rögl 1984). Similarly, related symbionts hosted by eastern Pacific *B. thermophilus* and Gulf of Mexico *B. brooksi* (Gustafson et al. 1998) might reflect a recent connection through the Isthmus of Panama, which finally closed about ~3.5 MY ago (Coates et al. 1992). These barriers might have closed earlier with respect to the bathymetric limits for dispersal of these deep-sea organisms (Hessler et al. 1988), but we presently do not know these limits. If 'leaky' vertical transmission exists in these mussels, the hosts might also have contributed to dispersal of symbionts. Though the biogeographic history of these thiotrophic bacteria is unknown, the present phylogenetic analyses provide evolutionary and geographical frameworks for characterizing new mussel species and their thiotrophic endosymbionts as they are discovered.

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