

Phylogenetic relationships of hydrothermal vent mussels (Bathymodiolinae) and their symbionts

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ABSTRACT: Deep-sea mussels of the subfamily Bathymodiolinae (Bivalvia, Pteriomorpha, Mytilidae) are dominant members of hydrothermal vent communities across the globe and have been found within every known hydrothermal vent biogeographic province. Thus, the apparent rarity of bathymodiolines at vents of the Juan de Fuca Ridge (JdF) is a notable exception. We examined mussels collected from the JdF over a span of 18 yr to clarify the classifications of mussel hosts and their symbiotic bacteria, and the relationships between sparsely distributed individuals. Several specimens, previously classified as *Bathymodiolus* and *Adipicola*, were reassigned as *Adipicola* sp. JdF based on new phylogenetic evidence and previous morphological studies. *Adipicola* sp. JdF are the most deeply branching members of *Adipicola* identified to date. Ultrastructural, molecular phylogenetic, and stable carbon isotope analyses indicated that *Adipicola* sp. JdF mussels harbor bacterial chemoautotrophic symbionts that appear to be extracellular and that are closely related to symbionts of other bathymodioline hosts. This study demonstrates that a sparsely distributed, yet cohesive, population of *Adipicola* has inhabited JdF vents for at least the past 18 yr. Moreover, the presence of extracellular symbionts in *Adipicola* sp. JdF at hydrothermal vents stands in contrast to the evolutionary patterns proposed for bathymodiolines in general. *Adipicola* sp. JdF represents an exception to the trend of bathymodiolines harboring extracellular symbionts living exclusively in shallow marine wood and bone habitats (<1000 m), indicating that bathymodiolines are more flexible with regard to habitat and symbiont characteristics than previously considered.

KEY WORDS: Symbiosis · *Bathymodiolus* · Mussel · *Adipicola* · Extracellular · Chemoautotrophy · Juan de Fuca

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INTRODUCTION

Bathymodioline mussels (Bivalvia, Pteriomorpha, Mytilidae, Bathymodiolinae) are widespread in deep-sea reducing environments, ranging from hydrothermal vents and cold seeps to wood and whale falls (Cavanaugh et al. 2006, Dubilier et al. 2008). This cosmopolitan distribution may be attributed to their nutritional flexibility, in that they are capable of obtaining nutrition from filter feeding

(Page et al. 1991, Riou et al. 2010a) supplementary to nutrition obtained from single or dual symbioses with sulfide-oxidizing (thiotrophic) and methanotrophic bacteria (Distel et al. 1995, DeChaine & Cavanaugh 2006, Duperron et al. 2006). This mixotrophic lifestyle may allow bathymodiolines to survive fluctuations in energy sources more effectively than other invertebrate symbioses, which in turn may facilitate a broader environmental range (Distel et al. 1995).

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Bathymodiolus is the most widely distributed of the bathymodioline genera, with representatives in every known hydrothermal vent biogeographic province (Van Dover et al. 2002). Newly discovered mussels at hydrothermal vents are typically assigned to this genus based on shell and tissue morphological characters, which serve to distinguish *Bathymodiolus* from other bathymodioline genera (Dell 1987, Gustafson et al. 1998). Recent molecular phylogenetic analyses have refined bathymodioline taxonomy, revealing the paraphyly of genera previously thought to be monophyletic based on morphological characters (*Idas*, *Adipicola*, and *Bathymodiolus*), thus demonstrating the importance of molecular characters in complementing taxonomic assignments based primarily on morphology (Jones et al. 2006, Samadi et al. 2007, Lorion et al. 2010).

Large aggregations of *Bathymodiolus* mussels are conspicuously absent from the spreading centers of the northeastern Pacific Ocean, making accurate phylogenetic characterization of the few sparsely distributed mussels all the more difficult. Several specimens collected in the early 1990s from the Middle Valley segment of the Juan de Fuca Ridge (JdF) were assigned to the genera *Idas* or *Adipicola* based on morphology and tissue ultrastructure (Table S1 in the supplement at www.int-res.com/articles/suppl/m474p147_supp.pdf) (Juniper et al. 1992, Southward 2008). In 1999, a mussel collected from the Endeavor segment (~60 km from Middle Valley) was assigned to *Bathymodiolus* based on a mitochondrial cytochrome *c* oxidase (COI) phylogeny and ultrastructural evidence (McKiness et al. 2005). Subsequent phylogenetic analyses of the mitochondrial data using additional taxa and new phylogenetic techniques found a conflicting phylogenetic placement for the JdF mussel (Duperron et al. 2009, Fujita et al. 2009, Kyuno et al. 2009); however, further molecular analyses were not possible as tissue was no longer available.

The collection of a new JdF specimen in 2008 enabled a revision of the classification of JdF mussels and their symbionts within the broader context of bathymodioline phylogeny using molecular phylogenetic, ultrastructural, and stable carbon isotope evidence. These data revealed that the individual mussels collected in 1990, 1999, and 2008 are all members of a sparsely distributed, yet coherent, species of *Adipicola*. This species, designated *Adipicola* sp. JdF, represents the most deeply branching *Adipicola* described to date and harbors extracellular chemoautotrophic symbionts associated with the surface of the mussel gill.

MATERIAL AND METHODS

A single mussel, here referred to as JdF2008, was discovered in bulk invertebrate collections obtained by the submersible 'Alvin' in July 2008 on the Endeavor segment of the Juan de Fuca Ridge (AT15-34, Dive 4413, 47° 58' N 129° 05' W; ~2189 m depth; Table S1 in the supplement). It had a brown, modioliform shell ~30 mm in length, with large brownish pink, fleshy gills typical of bathymodioline mussels. Portions of the gill were excised aboard ship and frozen at -80°C for molecular and stable isotope analyses. Gill sections for ultrastructural analyses were fixed in 3% glutaraldehyde in 0.1M sodium cacodylate with 0.4M NaCl (pH 7.4), stored at 4°C, post-fixed in 1% osmium tetroxide, stained en bloc in 1% uranyl acetate, and embedded in Spurr's resin. Thin sections were stained with lead citrate (Reynold's stain) and 2% uranyl acetate and examined on a JEOL 2100 electron microscope. The remaining tissue and shell were preserved in 70% ethanol and deposited in the Harvard University Museum of Comparative Zoology mollusk collections (www.mcz.harvard.edu/Departments/Malacology) (MCZ 374309).

We examined bathymodioline host and symbiont phylogeny using sequences from portions of 3 host genes, mitochondrial cytochrome *c* oxidase subunit I (COI; 366 nt), NADH dehydrogenase subunit 4 (ND4; 441 nt), and 18S rRNA (18S; 1604 nt), and 1 symbiont gene, 16S rRNA (16S; 1234 nt). Genomic DNA extracted from foot and gill tissue, using the DNeasy kit (Qiagen), was used for Polymerase Chain Reaction (PCR) amplification of host and symbiont genes, respectively. Primers and PCR conditions are listed in Table S2 in the supplement. Amplicons were purified using the Qiaquick kit (Qiagen), cloned into the StrataClone PCR cloning vector pSC-B (5 clones per gene) (Agilent Technologies), and sequenced at the Dana-Farber/Harvard Cancer Center DNA Resource Core on an ABI3730xl (Applied Biosystems). Sequences were assembled and manually edited in Sequencher v4.7 (Gene Codes Corporation). GenBank/DBJ/EMBL accession numbers for sequences used in this study are listed next to taxon names in phylogenetic trees. The COI sequence (579 nt) for *Adipicola* MV referenced in this study was kindly provided by J. Lorion (Japan Agency for Marine-Earth Science and Technology). Genomic DNA from paratypes of *Benthomodiolus lignicola* fixed in formalin, provided by the Te Papa Tongarewa/Museum of New Zealand (Wellington), was extracted from gill tissue as above, but PCR amplification with universal

bacterial primers for the 16S rRNA gene failed to yield any amplification products.

Bayesian inference was used to determine symbiont and host phylogenetic placement using MrBayes v3.1.2 (see the supplement). A structure-based 16S alignment was conducted using RNAsalsa v0.8.1 (Stocsits et al. 2009) with the *E. coli* 16S RNA structure (J01695) as a reference (Cannone et al. 2002). Nucleotide positions of stems and loops were extracted from the RNAsalsa alignment and used as input for the MrBayes doublet model. Alignments for host genes were performed with ClustalW (Thompson et al. 1994) and edited manually in MacClade 4.08 (Maddison & Maddison 2000). Model selections for each gene used in the MrBayes analyses are listed in Table S3 in the supplement.

JdF2008 mussel tissues were subsampled for stable carbon and nitrogen isotope analyses. Briefly, symbiont-containing gill and symbiont-free foot tissues were flash-combusted to CO₂ and N₂ using standard methods (Sulzman 2007) and analyzed on a GVI IsoPrime at the Boston University Stable Isotope Laboratory. Results are reported relative to Vienna PeeDee Belemite and atmospheric N₂ for carbon and nitrogen standards, respectively.

RESULTS AND DISCUSSION

Phylogenetic, ultrastructural, and stable isotope evidence, in combination with previous morphological studies, demonstrated that mussels collected from the Middle Valley (*Adipicola* MV) and Endeavor (*Bathymodiolus* sp. and JdF2008) segments of the JdF are members of the same species, *Adipicola* sp. JdF, and harbor extracellular chemoautotrophic symbionts. These shared attributes are presented and discussed in detail below.

Phylogenetic evidence indicated that the JdF mussels are a single species, falling within the subfamily Bathymodiolinae. Mytilid 18S and ND4 phylogenies supported placement of JdF2008 within Bathymodiolinae (posterior probability, Pp = 1; Fig. S1 in the supplement) and a sister relationship to all other bathymodiolines (Pp = 0.98; Fig. S2 in the supplement). The close relationship of JdF2008 and *Benthomodiolus lignicola* in the 18S and ND4 phylogenies (Figs. S1 & S2) was likely due to the lack of genetic data for *Bathymodiolus* sp. or *Adipicola* MV in these datasets. The COI phylogeny, with sequences for all 3 JdF mussels, indicated that they cluster separately from *Benthomodiolus* species and from paraphyletic *Adipicola* species (Fig. 1). Further, the COI phylo-

geny supported monophyly of *Adipicola* MV, *Bathymodiolus* sp., and JdF2008 with Pp = 0.99.

The well-supported monophyly and high level of mitochondrial sequence identity among the JdF mussels suggests that they most likely belong to the same species. The JdF mussel COI sequences are 99% identical (362/366 nt), falling within the range found among different populations of the same species (98 to 100% in *Bathymodiolus heckeriae* from the West Florida Escarpment and Blake Ridge), and much higher than the identity between bathymodiolines (69.4 to 88.7%) (Won et al. 2002, Van Dover et al. 2003). Collectively, the phylogenetic analyses and sequence identity support the conclusion that the JdF mussels belong to the same species.

A previous morphological study provides evidence which, considering the ultrastructural and phylogenetic data, suggests that *Adipicola* takes precedence as the genus designation for the JdF mussels. The bathymodioline collected from Middle Valley (*Adipicola* MV) was previously assigned to the genus *Adipicola* based on characters of the pedal and byssal retractor muscles and the relative sizes of the inner and outer demibranchs (Gustafson et al. 1998). Given the current molecular analyses demonstrating that *Adipicola* MV, *Bathymodiolus* sp., and JdF2008 belong to the same species, these mussels are provisionally re-designated as *Adipicola* sp. JdF, and herein each specimen is referred to by its year of collection (e.g. JdF2008).

Ultrastructural observations of the gill of JdF2008 revealed striking similarities to previous analyses of JdF1990 and JdF1999 (McKiness et al. 2005, Southward 2008) gills, underscoring the shared characteristics of *Adipicola* sp. JdF mussels. Electron micrographs of JdF2008 gill filaments revealed gram-negative coccoid bacteria located at the apical end of bacteriocytes, as is typical in bathymodiolines. The symbionts lacked the stacked intracytoplasmic membranes of methanotrophic bacteria which are found in single or dual symbioses in some bathymodiolines (Fig. 2A, B). Cytoplasmic vacuoles enclosing numerous bacteria that appeared to be undergoing degradation were observed in the bacteriocyte cytoplasm (Fig. 2A). Degraded bacteria may serve as a source of carbon for hosts (Streams et al. 1997), or alternatively, hosts may derive carbon from the leaking of symbiont metabolites (Kadar et al. 2008).

The JdF2008 symbionts appeared to be extracellular, associated with microvilli differentiated from the host cell surface (Fig. 2B). The JdF1990 gill exhibited similar symbiont ultrastructure, including the pres-

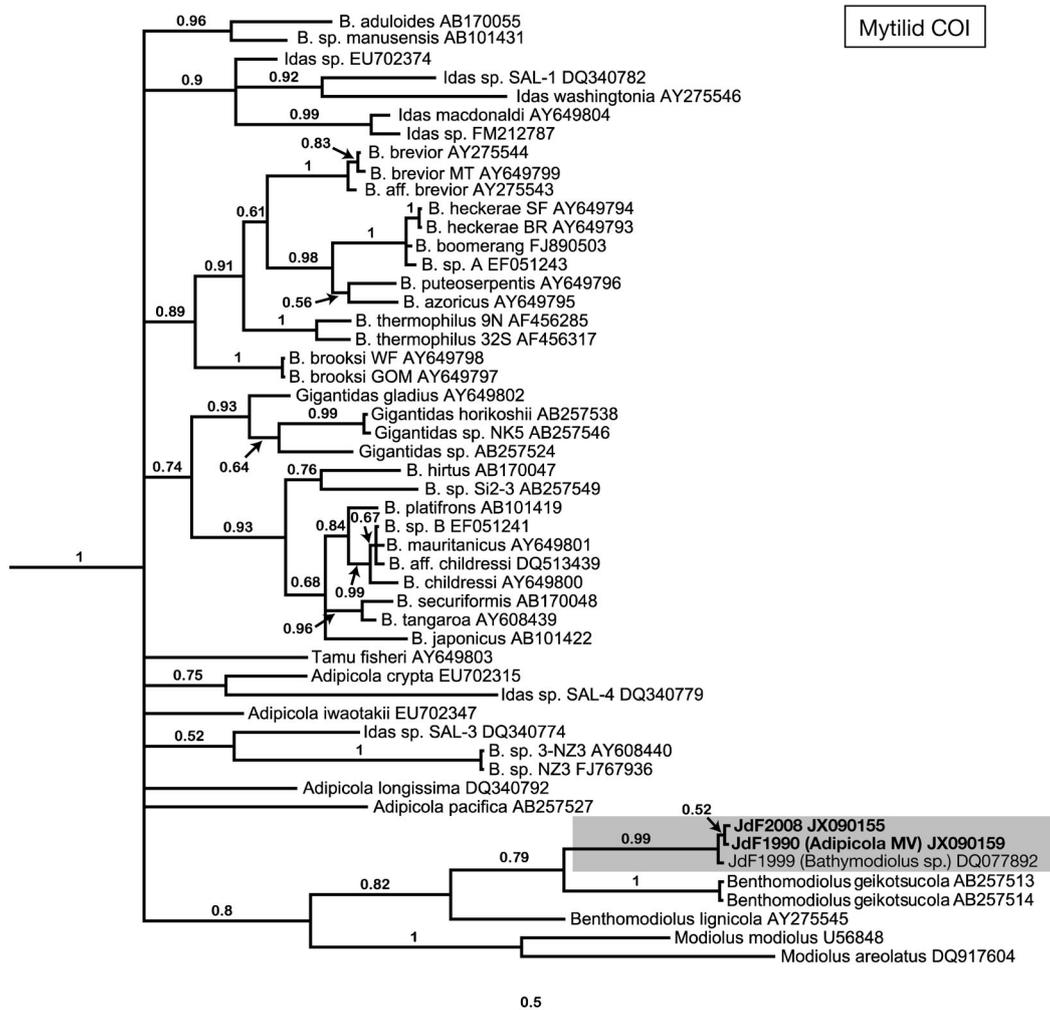


Fig. 1. Bayesian phylogeny of bathymodioline mussels based on host cytochrome *c* oxidase gene (366 bp). JdF2008 clusters with JdF1990 and JdF1999 with strong support (posterior probability $p = 0.99$; grey box). Parentheses: previous taxonomic designations for JdF species. **Bold** taxa: sequences contributed by this study. Outgroup genera *Mytilus*, *Perna*, and *Crassostrea* omitted for illustrative purposes

ence of extracellular bacteria (Southward 2008). The bacteria in JdF1999 are also located at the apical end of bacteriocytes; however, their exact position relative to the host membrane could not be determined due to the poor condition of the gill tissue (McKiness et al. 2005, Southward 2008). These analyses demonstrate that the location of the bacteria and ultrastructure are similar among the *Adipicola* sp. JdF symbionts.

Phylogenetic evidence demonstrates that JdF1999 and JdF2008 harbor symbionts that are closely related to those found in other bathymodioline mussels. Importantly, only a single symbiont phylotype was found in JdF2008 and JdF1999 (McKiness et al. 2005), in contrast to mussels in which multiple minor 'symbiont' phylotypes have been found (Duperron et al. 2007). Symbiont genetic data were not available

from JdF1990, so its relationship to the other JdF symbionts cannot be established. A Bayesian 16S rRNA gene phylogeny of chemoautotrophic bivalve symbionts and close relatives demonstrated that the JdF1999 and JdF2008 form a monophyletic group that falls unambiguously within the clade of thiotrophic bathymodioline symbionts ($P_p = 1$; Fig. 3). In general, as in previous studies (Won et al. 2008), a pattern of geographical clustering of symbionts according to western Pacific, eastern Pacific, Atlantic, and Indian ocean basins was observed in the 16S phylogeny (Fig. 3). This clustering may reflect inherent limits to bacterial dispersal in the deep sea, which is consistent with the observation that diversification among symbionts increases with geographical distance (Won et al. 2008). However, several

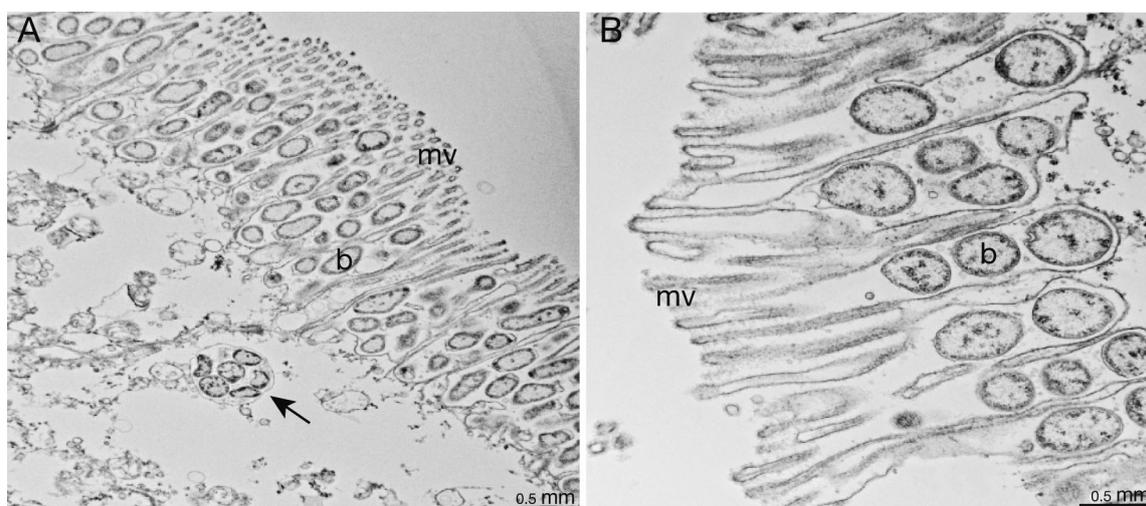


Fig. 2. *Adipicola* sp. JdF2008. Electron micrographs of the gill tissue. (A) Bacteriocytes showing bacteria (b) associated with microvilli (mv). Arrow: multiple bacteria, enclosed by a membrane which may be undergoing degradation. (B) Bacteria that appear to be extracellular are associated with the bacteriocyte microvilli

exceptions to this pattern were identified, suggesting that symbiont diversification cannot be ascribed to dispersal-driven processes alone (Fig. 3).

Stable carbon isotope and enzymatic evidence from previous studies suggest that the symbionts of *Adipicola* sp. JdF mussels are chemoautotrophic, using sulfide as an energy source. The $\delta^{13}\text{C}$ values for JdF2008 (gill = -30.9‰ , foot = -31.3‰) and JdF1999 ($\delta^{13}\text{C} = -26.6\text{‰}$) (McKinness et al. 2005) are within the range of those reported for bathymodioline mussels harboring exclusively thiotrophic symbionts ($\delta^{13}\text{C} = -25$ to -36‰) (Rau & Hedges 1979, Dubilier et al. 1998, Trask & Van Dover 1999) and outside of the range of non-vent filter-feeding bivalves depending on photosynthetically derived carbon ($\delta^{13}\text{C} = -17$ to -22‰) (Van Dover & Fry 1989). Recent studies demonstrated that $\delta^{13}\text{C}$ values of mussel tissue can vary seasonally, among individuals, and among populations and can be enriched by assimilation of photosynthetically derived carbon, which likely underlies the wide range of observed $\delta^{13}\text{C}$ values (Riou et al. 2010a,b). Mussels hosting exclusively methanotrophic symbionts result in more depleted $\delta^{13}\text{C}$ values (e.g. *Bathymodiolus childressi* = -61.8‰ and *B. brooksi* = -61.1‰) (Becker et al. 2010), given the isotopic composition of local methane ($\delta^{13}\text{C} \approx -55$ to -48.4) (Cowen et al. 2002), than are observed in JdF2008. While no stable carbon isotope data are available for JdF1990, sulfide oxidase activity was detected in gill tissues, supporting symbiont thiotrophy in this mussel (Southward 2008). The $\delta^{15}\text{N}$ values for JdF2008 = $+3.8\text{‰}$ and $+4.2\text{‰}$ for the gill and foot, respectively, falling within the range reported for

bathymodioline mussels (-17.2 to $+9.6\text{‰}$) (McKinness et al. 2005). The relatively wide range of $\delta^{15}\text{N}$ values found in bathymodioline mussels may be due to the effect of their reproductive status, environmental conditions, or the use of distinct nitrogen sources (Riou et al. 2010b).

The *Adipicola* sp. JdF mussels are an important lineage that contradicts the evolutionary patterns proposed for bathymodiolines in general. Extracellular chemoautotrophic symbionts have typically been found in species of *Idas* and *Adipicola* inhabiting shallow sunken wood and bone habitats. Extracellular symbionts in *Adipicola* sp. JdF, as well as *Idas washingtonia*, at hydrothermal vents are the only documented exceptions to this pattern (Southward 2008). Thus, the 'progressive evolution' hypothesis of a linear transition from wood- and bone-dwelling species with extracellular symbionts to hydrothermal vent and cold seep species with intracellular symbionts (Fujiwara et al. 2010, Miyazaki et al. 2010) is not supported by these data, as has been demonstrated in previous studies (McKinness et al. 2005, Duperron et al. 2008, 2009). The 'shallow to deep' hypothesis proposes that bathymodiolines evolved progressively into the deep sea (Craddock et al. 1995, Jones et al. 2006, Lorion et al. 2010). However, the basal phylogenetic position of *Adipicola* sp. JdF represents an exception to this pattern. More diverse taxonomic sampling is needed to determine whether this species represents a reversal of the 'shallow to deep' evolution paradigm or whether this scheme of progressive evolution is inappropriate for Bathymodiolinae as a whole.

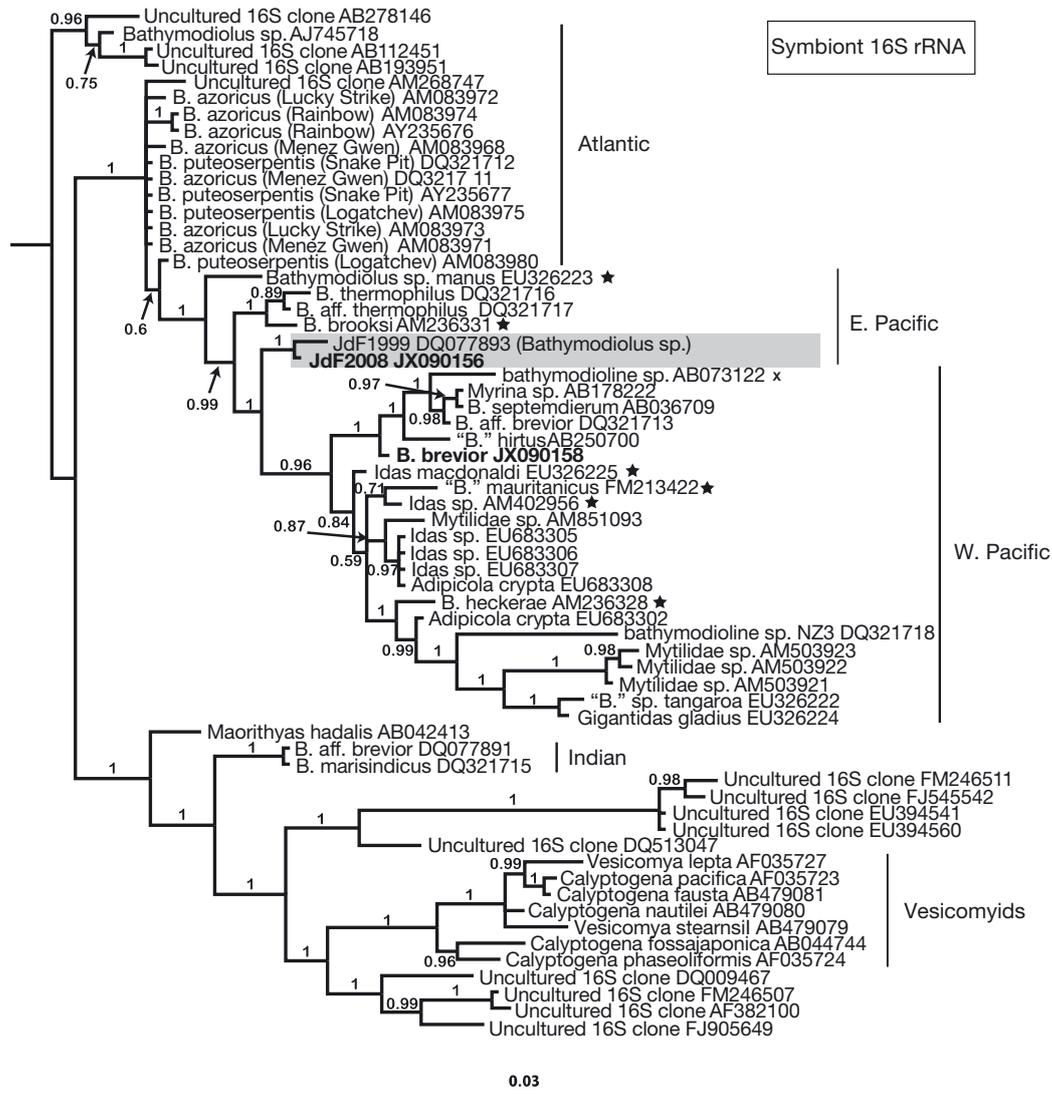


Fig. 3. Bayesian phylogeny of bathymodioline and vesicomiyid symbionts based on 16S rRNA gene (1234 bp). Symbionts are named according to host taxon. Symbiont gene sequence data were not available for JdF1999. The 16S rRNA phylotype isolated from JdF2008 is most closely related to that of JdF1999 with posterior probability = 1. Phylogenetic placement of these 2 sequences within the group of bathymodioline mussel symbionts is strongly supported. Symbionts cluster geographically by ocean basin. *Adipicola* sp. JdF2008. ★: Exceptions to this trend. x: unknown location. Sequence in **bold** contributed by this study. The outgroup (*Thiomicrospira crunogena*) was removed for illustrative purposes

This study reinforces the long-standing mystery of why the northeastern Pacific vents are the only major biogeographic province not known to contain large aggregates of bathymodioline mussels, which are emblematic of hydrothermal vent systems throughout the deep sea. Indeed, the scarcity of mussels at the JdF vents suggests 3 possibilities: unfavorable environmental conditions; barriers to dispersal, impeding mussel colonization; or vents may not be the primary habitat. As the chemical regime of the JdF is similar to chemical regimes found on other hydrothermal vent systems (Seyfried et al. 2004,

Cruse et al. 2008), this hypothesis is unlikely. Fracture zones on the JdF that act as barriers to dispersal for limpets and tubeworms, notably the Blanco Transform Fault, may prevent dispersal of mussel larvae (Johnson et al. 2006, Young et al. 2008). Further, the fracture zones that separate the northern East Pacific Rise from the JdF provide additional barriers, suggesting that barriers to dispersal may play a role in limiting JdF mussel colonization. Finally, the possibility that vents may not be the primary habitat of *Adipicola* sp. JdF is supported by the phylogenetic similarity of *Adipicola* sp. JdF with *Benthomodiolus*

genera inhabiting western Pacific wood and bone habitats (Fig. 3). Possibly, as yet undiscovered vents and seeps may have facilitated dispersal of this species across the Pacific. Alternatively, wood and bone may be the primary habitat of *Adipicola* sp. JdF and may have acted as 'stepping stones' facilitating dispersal of western Pacific mussel species to the JdF, which could explain both the scarcity of JdF mussels at hydrothermal vents and their affinity to western Pacific mussel populations.

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