



# Off-axis symbiosis found: characterization and biogeography of bacterial symbionts of Bathymodiolus mussels from Lost City hydrothermal vents

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1 **Off-axis Symbiosis Found: Characterization and Biogeography of Bacterial**  
2 **Symbionts of *Bathymodiolus* Mussels from Lost City Hydrothermal Vents**

3

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16

17 **Running title:** Biogeography of Lost City Symbionts

18

19

## 19 **Summary**

20           Organisms at hydrothermal vents inhabit discontinuous chemical "islands" along  
21 mid-ocean ridges, a scenario that may promote genetic divergence among populations.  
22 The 2003 discovery of mussels at the Lost City Hydrothermal Field provided a means of  
23 evaluating factors that govern the biogeography of symbiotic bacteria in the deep-sea.  
24 The unusual chemical composition of vent fluids, the remote location, and paucity of  
25 characteristic vent macrofauna at the site, raised the question of whether microbial  
26 symbioses existed at the extraordinary Lost City. And if so, how did symbiotic bacteria  
27 therein relate to those hosted by invertebrates at the closest known hydrothermal vents  
28 along the Mid-Atlantic Ridge (MAR)? To answer these questions, we performed  
29 microscopic and molecular analyses on the bacteria found within the gill tissue of  
30 *Bathymodiolus* mussels (Mytilidae, Bathymodiolinae) that were discovered at the Lost  
31 City. Here we show that Lost City mussels harbour chemoautotrophic and  
32 methanotrophic endosymbionts simultaneously. Furthermore, populations of the  
33 chemoautotrophic symbionts from the Lost City and two sites along the MAR are  
34 genetically distinct from each other, which suggests spatial isolation of bacteria in the  
35 deep-sea. These findings provide new insights into the processes that drive  
36 diversification of bacteria and evolution of symbioses at hydrothermal vents.

37

## 37 **Introduction**

38           Recent evidence suggests that microbial populations in spatially and chemically  
39 fragmented habitats exhibit geographic structure (Whitaker et al., 2003; Papke et al.,  
40 2003) rather than being distributed ubiquitously as previously hypothesized (see Finlay  
41 2002; Fenchel 2003). The patchy mosaic of populations in heterogeneous environments  
42 restricts gene flow, while promoting genetic differentiation and local adaptation (Slatkin  
43 1987). Due to the heterogeneous nature of hydrothermal vent environments,  
44 chemosynthetic bacteria inhabiting vents probably have geographically structured  
45 populations as well. If so, this would have direct implications for how topographic  
46 features of the seafloor, deep-ocean currents, and chemically variable environments  
47 impact the evolution and diversity of bacteria, the origin and evolution of bacteria-vent  
48 invertebrate symbioses, and the assemblage of hydrothermal vent communities.

49           The fragmented distribution of deep-sea hydrothermal vents lies in stark contrast  
50 to the uniform conditions of the marine abyssal zone (Tunnicliffe, 1988, 1991;  
51 Tunnicliffe and Fowler, 1996; Van Dover, 2000). Discrete hydrothermal vent fields are  
52 comparable to islands, distributed in a spatially, chemically, and temporally patchy  
53 chain along the deep-sea ridges and remote, off-axis sites (Tunnicliffe, 1988, 1991;  
54 Tunnicliffe and Fowler, 1996; Tunnicliffe et al., 1998; Van Dover et al., 2002).  
55 Differences between ridges in geography, tectonic activity, age of spreading center, and  
56 connectedness of ridge segments likely play a major role in regulating gene flow among  
57 populations (Vrijenhoek, 1997; Van Dover et al., 2002; Hurtado et al., 2003), the  
58 distribution of vent macrofauna (Van Dover, 1995; Tunnicliffe and Fowler, 1996;  
59 Juniper and Tunnicliffe, 1997), and the composition of ecological communities  
60 (Tunnicliffe, 1991). For example, 'fracture zones' (Fig. 1) likely inhibit dispersal of

61 larvae by separating ridge segments that are undergoing independent volcanic evolution  
62 (Van Dover et al. 2002). Though associations between chemosynthetic bacteria and  
63 their invertebrate hosts provide the basis for macrofaunal production at deep-sea  
64 hydrothermal vents, almost nothing is known about the distribution of genetic variation  
65 in the symbionts and how population structure of bacteria affects ecological interactions  
66 and the evolution of symbioses at vents.

67         Most dominant vent macrofauna host endosymbiotic bacteria for the capture of  
68 chemical energy, yielding a constant food source in this stochastic environment (Fisher,  
69 1990; Cavanaugh et al., 2005; Stewart et al., 2005). Typically, the host provides the  
70 symbionts with simultaneous access to oxygen and reduced compounds, and the  
71 bacteria, in turn, supply the host with fixed carbon generated from C<sub>1</sub> compounds.  
72 Along the Mid-Atlantic Ridge (MAR), vents are inhabited by two species of mussels,  
73 *Bathymodiolus azoricus* and *B. puteoserpentis* (Mytilidae; Bathymodiolinae), that host  
74 chemoautotrophic (energy source: reduced compounds such as H<sub>2</sub>S; carbon source:  
75 CO<sub>2</sub>) and methanotrophic (energy and carbon source: CH<sub>4</sub>)  $\gamma$ -Proteobacteria within their  
76 gill tissue (Cavanaugh et al., 1992; Distel et al., 1995; Nelson et al., 1995; Fiala-  
77 Médioni et al., 2002). The unique capacity of some bathymodioline mussels to house  
78 dual endosymbionts permits the host to utilize multiple compounds for energy  
79 acquisition, allowing colonization of diverse environments (Distel et al., 1995; Fiala-  
80 Médioni et al., 2002; DeChaine and Cavanaugh, 2005).

81         The off-axis location, shallow depth (~800 m), distinct chemical environment,  
82 and scarcity of known symbiont-hosting invertebrates at the Lost City Hydrothermal  
83 Field (LC) suggested the possibility that mussels discovered there (*B. aff. azoricus*, T.  
84 Shank unpublished data), host endosymbionts that are different from those on the MAR.

85 For example, the vent fluids of the off-axis Lost City are relatively cool (10-90°C),  
86 alkaline ( $\text{pH} \geq 10$ ), methane-rich ( $0.13\text{-}0.28 \text{ mmol kg}^{-1}$ ), and lie in stark contrast to the  
87 acidic, sulfide-rich effluent of the MAR vents (200-360°C,  $\text{pH}=3\text{-}5$ ) that are ~15 km  
88 east and 2200 m deeper (Kelley, et al., 2001, 2005). The abundance of methane and  
89 hydrogen and low availability of  $\text{H}_2\text{S}$  (due to the high pH of the fluids, given the  $\text{pK}_a$ 1  
90 of  $\text{H}_2\text{S} = 7.04$ ; Budavari 1996) at the LC suggested that mussels found therein might  
91 host primarily methanotrophs, and not thioautotrophs, which would be novel given that  
92 all known vent mussels in the Atlantic Ocean host dual symbionts. Furthermore, the  
93 apparent remoteness of the Lost City provided a unique setting to resolve whether  
94 symbiotic bacterial populations at hydrothermal vents are ubiquitous or structured.  
95 Answers to these questions provide a foundation for understanding the forces that  
96 promote genetic divergence among symbiont populations and thus determine the  
97 biogeography and evolution of bacteria.

98         The objectives of this study were to 1) determine if the bathymodioline mussels  
99 inhabiting the LC hosted symbiotic bacteria and 2) how the symbionts were related,  
100 both phylogenetically and demographically, to those hosted by mussels on the MAR.  
101 First, we employed transmission electron microscopy (TEM) to determine the presence  
102 and morphology of putative symbionts within the Lost City mussel gill tissue and then  
103 resolved the relationship of the Lost City symbionts with known symbiotic and free-  
104 living bacteria using sequence data from the conserved 16S rRNA gene (Woese, 1987).  
105 The bathymodioline chemoautotrophs from the Lost City, and from two MAR fields,  
106 Lucky Strike and Snake Pit, were selected for additional population genetic analyses  
107 because: 1) bathymodiolines apparently acquire their chemoautotrophic symbionts from  
108 the environment each generation (Won et al., 2003), and thus serve as sampling vessels

109 of the free-living bacterial population, 2) the chemoautotrophs only have one ribosomal  
110 RNA operon, precluding concerns over non-orthologous genetic variation (Won et al.,  
111 2003), and 3) chemoautotrophs are more widespread among invertebrate hosts at  
112 hydrothermal vents than methanotrophic symbionts (Cavanaugh et al., 2005), thus  
113 permitting broad genetic comparisons. An intraspecific phylogeny and the demographic  
114 history of each chemoautotroph population (defined by location) were inferred from  
115 sequence data of the rapidly evolving 16S-23S rRNA internal transcribed spacer (ITS;  
116 Antón et al., 1998). By employing both conserved and highly variable markers, the  
117 phylogenetic position of Lost City mussel symbionts was resolved at two scales, within  
118 the  $\gamma$ -Proteobacteria and among populations of chemoautotrophs hosted by vent-  
119 endemic mussels along the northern MAR.

120

## 121 **Results**

### 122 *Characterization of the Lost City symbiosis*

123 This characterization, which constitutes the first description of a symbiosis from  
124 the LC, revealed two morphologically distinct Gram negative bacteria in the mussel  
125 bacteriocytes, gill epithelial cells specialized for housing symbiotic bacteria (Fig. 2a).  
126 As in other vent symbioses, the bacteriocytes were separated by symbiont-free  
127 intercalary cells. Vacuoles within a bacteriocyte harbored either several coccoid bacteria  
128 (~0.3  $\mu\text{m}$  in diameter) or a single, large bacterium (~1.5-2.0  $\mu\text{m}$ ) exhibiting  
129 intracytoplasmic membranes typical of type I methanotrophs (Fig 2b). Based on the  
130 characteristics of endosymbionts in other bathymodioline mussels (Cavanaugh et al.,  
131 1992; Fiala-Médioni et al., 2002; Robinson et al., 1998), the small and large bacteria  
132 were inferred to be chemoautotrophs and methanotrophs, respectively.

133 Phylogenetic analyses of 16S rRNA sequence data corroborated the TEM  
134 observations of dual endosymbionts in the Lost City mussels. First, sequence  
135 alignments revealed that the symbiont phylotypes from the Lost City (Genbank  
136 accession numbers A and B) were identical to two phylogenetically distinct lineages of  
137  $\gamma$ -Proteobacteria, a chemoautotroph and a methanotroph, previously found in both *B.*  
138 *azoricus* and *B. puteoserpentis* on the MAR. The presence of both phylotypes in the gill  
139 tissue of these two MAR mussel species has been verified through *in situ* hybridization  
140 with phylotype-specific probes (Distel et al., 1995; Duperron et al., 2005). In our  
141 analyses, chemoautotrophic and methanotrophic symbionts of the mussels formed  
142 separate, well-supported monophyletic clades that were nested with chemoautotrophs of  
143 vent-endemic vesicomylid clams and free-living methanotrophs, respectively (Fig. 3).  
144 This finding demonstrates the tight ecological and historic specificity of the interaction  
145 between the mussels and two distinct subsets of the  $\gamma$  Proteobacteria. While both  
146 Bayesian and maximum parsimony analyses inferred similar tree topologies, the deep  
147 relationships among the mussel methanotroph, mussel and clam chemoautotroph, and  
148 other symbiont clades remain uncertain (for  $\alpha = 0.05$ ). Finally, the occurrence of both  
149 symbiont phylotypes across the 2-3 mussel species demonstrated a lack of host fidelity  
150 and implied that both methanotrophs and chemoautotrophs were acquired from the local  
151 environment.

152 All mussel individuals harboured several, distinct chemoautotrophic symbiont  
153 ITS-genotypes (Genbank accession numbers X through Y). Twenty-four percent of the  
154 ITS-genotypes from Lucky Strike and 38% from Snake Pit were shared among host  
155 individuals within each of those localities (Fig. 4). In contrast, the two Lost City host  
156 mussels had no chemoautotroph ITS-genotypes in common with each other, possibly



157 owing to the small population size of mussels at that location. The occurrence of  
158 multiple, geographically restricted chemoautotroph ITS-genotypes within an individual  
159 host reinforced the contention that each individual mussel acquired its symbionts from  
160 the local, free-living bacterial community as shown by the distribution of 16S rRNA  
161 phylotypes in this study and previous analyses of ITS variation (Won et al., 2003).

162

### 163 *Biogeography of bathymodioline chemoautotrophic endosymbionts*

164         Analyses of ITS sequence data showed that the chemoautotrophic symbionts of  
165 bathymodioline mussels were not distributed ubiquitously, but rather exhibited  
166 population structure associated with geographic location. This finding, which contrasts  
167 with the observed ubiquity of the 16S rRNA phylotype (above and Duperron et al.,  
168 2005), underscores the need to use highly variable markers in analyses at the population  
169 level. The genetic variation in the ITS region (1.05 % average pair-wise sequence  
170 divergence) permitted resolution of evolutionary relationships among populations of  
171 chemoautotrophic symbionts at hydrothermal fields. Two distinct ITS-clades of  
172 chemoautotrophs were separated by 13 nucleotide substitutions: the *Bathymodiolus*  
173 *puteoserpentis* (Snake Pit) symbionts and the *B. azoricus* - *B. aff. azoricus* clade, which  
174 included symbionts from both Lucky Strike and the Lost City (Fig. 4). Furthermore, the  
175 overall estimates of  $\theta$  (= 6.9) and  $T$  (= 1.6) from MDIV imply that the northern and  
176 southern populations of *Bathymodiolus* chemoautotrophic symbionts in the north  
177 Atlantic (as defined by the ITS-clades) are large and historically have been separated  
178 from one another.

179         Our genetic analyses revealed that populations of chemoautotrophic symbionts  
180 inhabiting different hydrothermal vent fields were isolated and experienced independent

181 demographic histories. First, populations of chemoautotrophs at the Lost City and  
182 Lucky Strike were more genetically diverse, as estimated by  $\theta$  based on the number of  
183 segregating sites ( $W$ ) and the average pair-wise nucleotide diversity ( $\pi$ ) for haploid  
184 genomes, than the population at Snake Pit (Table 1). Moreover, Tajima's  $D$  tests of  
185 neutrality suggest that the populations at the Lost City and at Lucky Strike have been  
186 demographically stable, whereas the symbionts at Snake Pit likely experienced a  
187 population bottleneck (a reduction in population size followed by rapid population  
188 growth; Table 1). We cannot rule out, however, the possibility of a selective sweep for  
189 Snake Pit symbionts, because Tajima's  $D$  does not effectively differentiate between  
190 population processes and selection (Tajima 1989). Finally, based on  $F_{ST}$  estimates of  
191 isolation, our analyses revealed genetic divergence among populations of  
192 chemoautotrophic symbionts at all study locations, irrespective of host species (Table 2)  
193 or distance between sites (no isolation-by-distance,  $p = 0.9$ ).

194

## 195 **Discussion**

196        Though the LC lies distantly off-axis and has a novel chemical environment  
197 (Kelley, et al., 2001, 2005), mussels in the genus *Bathymodiolus* found at the Lost City  
198 host dual symbionts, a methanotroph and a chemoautotroph, with identical 16S rRNA  
199 phylotypes as those along the Mid-Atlantic Ridge (MAR). This result is unexpected  
200 given the paucity of  $H_2S$  (due to the high pH) in the effluent of Lost City vents (D.  
201 Butterfield pers. comm.) and raises the question of whether the chemoautotrophs are  
202 using sources of energy other than sulfur compounds, such as hydrogen that is abundant  
203 at the vent fluids. Indeed, alternate energy sources may be used by many symbionts, as  
204 only *B. thermophilus* found along the Eastern Pacific Rise have been shown to use

205 sulfur (Belkin et al. 1986; Nelson et al. 1995). The discovery and characterization of the  
206 Lost City bathymodioline symbionts, in light of the diversity of chemical environments  
207 inhabited by mussels, underscores the ecological and evolutionary stability of the dual  
208 symbiosis.

209         The occurrence of single phlotypes, for both the chemoautotroph and the  
210 methanotroph, across different host species demonstrated that neither of the symbiont  
211 types was host species-specific. A similar lack of host-species fidelity was shown for  
212 the chemoautotrophic endosymbionts of hydrothermal vent tubeworms that were  
213 inferred to be environmentally transmitted (Feldman et al., 1997; Nelson and Fisher,  
214 2000; reviewed in Cavanaugh et al., 2005). Our analyses revealing the broad  
215 distribution of symbiont phlotypes across multiple host species suggest that *both*  
216 methanotrophs and chemoautotrophs of mussels in the northern Atlantic are acquired  
217 from the environment, rather than being transmitted from mother to offspring each  
218 generation as for the closely related chemoautotrophs of another vent bivalve,  
219 *Calyptogena magnifica* (Cary and Giovannoni, 1993). This finding implies that mussels  
220 acquire symbionts from the local community when they colonize a site and has  
221 implications for local adaptation of symbionts to that environment.

222         Chemoautotrophic symbiont populations hosted by bathymodioline mussels  
223 were inferred to be isolated from each other because no ITS-genotypes were shared  
224 among the three hydrothermal fields. Since *B. azoricus* individuals at the Broken Spur  
225 hybrid zone on the MAR (just south of the Lost City) harboured symbionts from both  
226 northern (*B. azoricus*) and southern (*B. puteoserpentis*) ITS-clades (Won et al., 2003), it  
227 is unlikely that the host organisms affected the distribution of ITS genotypes, though the  
228 host may have selected for certain bacterial phlotypes from the local free-living

229 population. Because the chemical environment of the Lost City is drastically different  
230 from both vent sites on the MAR (Kelley et al. 2001), and symbiont populations from  
231 Lost City and Lucky Strike are closely related phylogenetically while those from the  
232 two MAR sites are not, the chemical environment may not be a large factor in  
233 governing the distribution of bathymodioline symbionts. Rather, geography probably  
234 played a major role in generating isolation among populations.

235         The three sites in this study are separated by fracture zones, depth, distance, and  
236 deep ocean currents, all of which have been implicated as dispersal barriers that could  
237 promote genetic divergence among populations (Van Dover et al., 2002). Though the  
238 distance between the Lost City and Lucky Strike to the north (1253 km) is greater than  
239 the distance between the Lost City and Snake Pit to the south (832 km), the Lost City  
240 chemoautotrophs cluster with those in the north (Fig. 4). Thus, we inferred that distance  
241 did not have as large an effect on isolation as did topographic features that likely  
242 influence deep-ocean currents. For instance, the two ITS-clades (Fig. 4) are  
243 geographically separated by many transform faults that offset the spreading axis,  
244 including the ~6000 m deep Atlantis Fracture Zone, just to the south of the Lost City  
245 and the Kane Fracture Zone just north of Snake Pit (Fig. 1). Smaller fracture zones, such  
246 as the Oceanographer Fracture Zone to the north, are apparently not as strong of barriers  
247 to dispersal, but this remains to be evaluated. Thus, understanding the biogeographic  
248 history of bacteria that inhabit hydrothermal vents provides an empirical basis for and  
249 an independent means of assessing models of deep-ocean currents.

250         Isolation among hydrothermal vent fields has likely led each population of  
251 chemoautotrophic symbionts to experience independent demographic histories, as  
252 inferred through differences in  $\theta$ , Tajima's D tests of neutrality, and the high levels of

253 isolation estimated by  $F_{ST}$ . Because the 16S-ITS-23S spacer is mostly comprised of  
254 seemingly functionless regions (Antón et al., 1998), the possibility that selection caused  
255 the observed patterns of genetic variation is unlikely. Rather, the demographic history of  
256 a symbiont population may depend on the tectonic activity at the site, which, in addition  
257 to supplying the bacteria with reduced compounds for energy production, could  
258 decimate the population in an intense eruption. For example, we inferred that the  
259 population of chemoautotrophic symbionts at Snake Pit was unstable, while the other  
260 two populations were at equilibrium. A long-lived hydrothermal vent field, such as the  
261 Lost City (Früh-Green et al., 2003) may maintain a heterogeneous and stable population  
262 of chemoautotrophs, while shorter-lived or more eruptive sites may generate greater  
263 fluctuations in population size and thus reduce genetic diversity.

264 Our findings fit with the biogeographic model for macrofauna larvae outlined by  
265 Van Dover et al. (2002), which states that the greater degree of faulting along slow-  
266 spreading ridges (e.g., the MAR) should serve to isolate populations. Since symbionts  
267 are acquired from the local environment each generation, the host likely plays little role  
268 in determining the distribution of genetic variation in bacterial populations among  
269 locations. Rather, the strong divergence between northern and southern symbiont  
270 populations and the lack of isolation-by-distance among localities demonstrated that  
271 geographic barriers to dispersal, such as faulting, depth, and other topographic features  
272 of the seafloor, divide bacterial populations. Also, though off-axis sites may be remotely  
273 located, they may be connected (or have a historic connection) via deep-ocean currents  
274 with sites along the ridge, as indicated by the Lost City populations clustering with  
275 those of Lucky Strike. We conclude that topography is a major influence on the  
276 distribution of diversity among populations of symbiotic bacteria at hydrothermal vents,

277 and that additional research is needed to clarify how differences among ridges in  
278 tectonic activity, geography, and physical oceanography have impacted the population  
279 structure of symbiotic bacteria and at what scale.

280         Resolving how populations of bacterial endosymbionts are structured has  
281 important implications for microbial biogeography, bacterial diversity and evolution,  
282 the origin and evolution of prokaryote-eukaryote symbioses, and the ecology and  
283 evolution of life at deep-sea hydrothermal vents. First, studies in microbial  
284 biogeography have revealed that limits to gene flow might yield geographic structure  
285 within microbial taxa (Papke et al. 2003; Whitaker et al. 2003; Kirchman et al., 2005).  
286 Population subdivision implies an increased potential for local adaptation and lineage  
287 diversification. Until now, genetic structure and potential for local adaptation in  
288 chemosynthetic endosymbionts have remained uncertain. Environmentally transmitted  
289 endosymbionts are expected to respond to abiotic selective forces in the environment as  
290 well as experience gene transfer with the hydrothermal vent free-living bacterial  
291 community. This will not only impact the genetic diversity of symbionts, but may  
292 ultimately affect the fitness of the invertebrate host. This and future studies on the  
293 biogeography of symbionts inhabiting deep-sea hydrothermal vents, including  
294 comparisons with the free-living bacterial community, host biogeography, and among-  
295 site variation in environmental factors, will provide a basis for understanding the  
296 processes responsible for the diversification of bacteria and symbioses on this planet.

297

## 298 **Experimental Procedures**

299 *Specimen collection*

300           Mussels were collected using DSV Alvin from the off-axis Lost City  
301 hydrothermal vent field (30°07.40'N, 42°07.24'W; 800 m deep) and from the Lucky  
302 Strike (37°17.26'N, 32°16.50'W; 1693 m deep) and Snake Pit (23°22.10'N,  
303 44°56.91'W; 3492 m deep) vent sites on the MAR (Fig. 1). Specimens were preserved  
304 for ultrastructural analysis or stored at -80°C. Symbiont-bearing gill tissue was fixed,  
305 embedded, and examined by transmission electron microscopy (Distel et al., 1995).  
306 DNA was extracted from the frozen gill tissue of the two Lost City mussels, 20  
307 individuals of *Bathymodiolus azoricus* from Lucky Strike and 20 of *B. puteoserpentis*  
308 from Snake Pit with DNeasy Tissue Extraction Kits (Qiagen, Valencia, CA).

309

310

### 311 *Genetic sampling and analyses*

312           To resolve evolutionary relationships, the symbiont(s) 16S rRNA gene was  
313 amplified using the universal bacteria primers 27f and 1492r (Weisburg et al., 1991),  
314 from multiple specimens of the three vent sites, gel purified (Qiagen Gel Extraction  
315 Kit), and cloned (TOPO TA Cloning Kit; Invitrogen Corp., Frederick, MD). Thirty-two  
316 clones per host population were analyzed (16 for each of the two mussels from the Lost  
317 City and two clones for each of 16 mussels at both the Lucky Strike and Snake Pit  
318 sites). The legitimacy of point mutations in all unique phlotypes was evaluated using  
319 ARB (Ludwig et al., 2004) by assessing complementary base pairing on the 16S rRNA  
320 secondary structure and by following the sequence conservation rule (Acinas et al.,  
321 2004).

322           To estimate within- and among-population genetic variation, sequence data from  
323 the polymorphic 16S-ITS-23S region of the chemoautotrophs was used. The marker was

324 amplified using two symbiont-specific primer combinations: Sym-ITS-830F and Sym-  
325 ITS-23SR; Sym-ITS-1322F and Sym-ITS-23SR (Won et al., 2003). The former primer  
326 set was used to confirm symbiont species identification, because it yielded an 1800  
327 nucleotide sequence including approximately 600 bp of 16S rRNA. The latter pair  
328 provided the ITS sequences for population genetic analyses. Ninety clones from the  
329 Sym-ITS-1322F and Sym-ITS-23SR amplicons were sequenced from each of the two  
330 Lost City mussels. For both the Lucky Strike and Snake Pit populations, 143 clones  
331 were sequenced from 20 host individuals.

332         For each locus, forward and reverse strands were cycle sequenced using the M13  
333 primer pair, the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems,  
334 Atlanta, GA), cleaned with Performa DTR 96-well Std. Plate Kit (Edge BioSystems,  
335 Gaithersburg, MD), and sequenced on an ABI 3730 Gene Analyzer. Sequences were  
336 edited in Sequencher 4.1.2 (Gene Codes Corp.), aligned in ClustalX (Thompson et al.,  
337 1997), and alignments were manually edited in MacClade 4.0 (Maddison and Maddison,  
338 2003).

339         The phylogenetic relationships among the 1303 bp portion of the 16S rRNA  
340 phylotypes from this and previous studies of bathymodioline symbionts (with  
341 *Escherichia coli* as an outgroup; see Table 3 for Genbank accession numbers) were  
342 inferred using maximum parsimony in PAUP 4.10b (Swofford, 2003) and Bayesian  
343 posterior probabilities implemented with MrBayes v3.0b4 (Huelsenbeck and Ronquist,  
344 2001). Maximum parsimony trees were generated on PAUP 4.10b (Swofford, 2003),  
345 with heuristic searches, random sequence addition with 100 replicates, and TBR branch  
346 swapping. Significance was determined from a 1000 replicate bootstrap analysis using  
347 the same search parameters. From the Bayesian analysis, using four-chain Metropolis-



348 coupled Markov chain Monte Carlo (MCMCMC) analysis, a consensus tree of 11,000  
349 post burn-in sampled trees was generated in PAUP 4.10b (Swofford, 2003). Both  
350 Bayesian and parsimony analyses yielded similar inferences of evolutionary history.

351         Intraspecific phylogenies were inferred from the ITS sequences for the  
352 chemoautotrophic symbionts using parsimony implemented in the TCS software  
353 package (Clement et al., 2000) and a combination of Bayesian and maximum likelihood  
354 analyses. Eighty-five nucleotides of tRNA-Ala and 77 bp of tRNA-Ile occurred within  
355 the 16S-ITS-23S sequence. The 1200 bp of ITS included five indels at positions 361-  
356 364, 542-543, 711, 937-939, and 966-982, which were each transformed into a single  
357 polymorphic position (Widmer and Baltisberger, 1999). After converting the indels to  
358 one base substitution each, 48 polymorphic sites were described for the remaining 1178  
359 bp, of which 36 sites were parsimony informative. For the Bayesian analysis, post burn-  
360 in trees were imported into PAUP 4.10b (Swofford, 2003) and sorted to choose the  
361 maximum likelihood tree. The parsimony and maximum likelihood trees were similar  
362 and the few differences did not affect any conclusions.

363         Within-population genetic variation and among-population genetic  
364 differentiation were estimated to test the relationships between the Lost City  
365 chemoautotrophic endosymbionts and the two populations on the MAR. All measures  
366 were averaged across individuals from the population to account for potential PCR bias.  
367 First, an overall measure of genetic diversity for haploid genomes ( $\theta = 2Ne\mu$ ) for all  
368 populations and the amount of genetic divergence (T) between northern and southern  
369 clades (see Results) were estimated using MDIV (Nielsen and Wakeley 2001) assuming  
370 the HKY finite sites model and running the coalescent simulations three times for each  
371 species to evaluate convergence for each parameter. Within-population genetic diversity

372 was estimated based on the average pair-wise nucleotide diversity ( $\theta_s$ ) and the number  
373 of segregating sites ( $\theta_w$ ) for haploid genomes (e.g., Herbeck et al., 2003). In addition,  
374 both estimators of  $\theta$  should be equivalent in a population at equilibrium that is evolving  
375 neutrally. Tajima's D was used to compare the two estimators of  $\theta$  and examine whether  
376 populations were at equilibrium (Tajima, 1989). To test whether or not populations of  
377 bacterial symbionts were isolated, the mean pair-wise differences and degree of  
378 differentiation ( $F_{ST}$ ) among locations were estimated (e.g., Whitaker et al., 2003).  
379 Isolation-by-distance was tested (Rousset, 1997), with distances among sites as follows:  
380 Lost City to Lucky Strike (1253 km), Lost City to Snake Pit (832 km), and Lucky Strike  
381 to Snake Pit (2037 km). All analyses were performed using Arlequin 2.0 (Schneider et  
382 al., 2000).  
383

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391

392

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529

529 Table 1. Summary statistics for ITS sequences from the three populations of  
 530 chemoautotrophic symbionts.

531

| 532 | Location     | Host Species             | $\theta_{\pi}$ (sd) | $\theta_w$ (sd) | Tajima's D |
|-----|--------------|--------------------------|---------------------|-----------------|------------|
| 533 | Lucky Strike | <i>B. azoricus</i>       | 2.4 (1.4)           | 2.8 (1.0)       | -0.63      |
| 534 | Lost City    | <i>B. aff. azoricus</i>  | 2.3 (1.4)           | 2.5 (0.8)       | -0.23      |
| 535 | Snake Pit    | <i>B. puteoserpentis</i> | 0.7 (0.6)           | 1.9 (0.7)       | -1.67*     |

536

537 Estimates of genetic diversity ( $\theta$ ) based on the average pair-wise sequence divergence  
 538 ( $\pi$ ) and number of segregating sites ( $W$ ) are shown, with standard deviations (sd).

539 Estimates of Tajima's D are given for each population (\*denotes  $p < 0.01$ ).

540

540 Table 2. Pair-wise comparisons of populations of chemoautotrophs hosted by  
 541 bathymodioline mussels.

542

543 Mean pair-wise differences

| 544 Location                      | Lucky Strike | Lost City | Snake Pit |
|-----------------------------------|--------------|-----------|-----------|
| 545 Lucky Strike                  |              | 1.2       | 11.7      |
| 546 Lost City                     | 0.34         |           | 12.4      |
| 547 Snake Pit                     | 0.89         | 0.89      |           |
| 548 Population pair-wise $F_{ST}$ |              |           |           |

549

550 Mean pair-wise differences for chemoautotroph ITS sequences are shown above the

551 diagonal, and population pair-wise  $F_{ST}$  values are given below. All  $F_{ST}$  values are

552 significant.

553

553 Table 3. List of bacteria and Genbank accession numbers used to generate the 16S  
 554 rRNA phylogeny for  $\gamma$ -Proteobacteria (Fig. 3).

| 555 | Environment                | Species                           | Genbank accession no. |
|-----|----------------------------|-----------------------------------|-----------------------|
| 556 | Free-living bacteria       |                                   |                       |
| 557 |                            | <i>Achromatium oxaliferum</i>     | L48227                |
| 558 |                            | <i>Beggiatoa alba</i>             | L40994                |
| 559 |                            | <i>Escherichia coli</i>           | J01695                |
| 560 |                            | <i>Halomonas elongata</i>         | X67023                |
| 561 |                            | <i>Hydrogenovibrio marinus</i>    | D86374                |
| 562 |                            | <i>Methylobacter whittenburyi</i> | X72773                |
| 563 |                            | <i>M. capsulatus</i>              | L20843                |
| 564 |                            | <i>M. luteus</i>                  | AF304195              |
| 565 |                            | <i>M. vinelandii</i>              | L20841                |
| 566 |                            | <i>Methylomicrobium agile</i>     | X72767                |
| 567 |                            | <i>M. pelagicum</i>               | L35540                |
| 568 |                            | <i>Methylomonas methanica</i>     | AF150806              |
| 569 |                            | <i>M. rubra</i>                   | AF150807              |
| 570 |                            | <i>Pseudomonas mendocina</i>      | AF232713              |
| 571 |                            | <i>Rhabdochromatium marinum</i>   | X84316                |
| 572 |                            | <i>Thiocystis gelatinosa</i>      | Y11317                |
| 573 |                            | <i>Thiomicrospira thyasirae.</i>  | AF16046               |
| 574 |                            |                                   |                       |
| 575 | Chemoautotrophic symbionts |                                   |                       |
| 576 | Host Taxonomy              | Host species                      |                       |

|     |                 |  |                         |
|-----|-----------------|--|-------------------------|
| 577 | Phylum Annelida |  |                         |
| 578 | Oligochaeta     | <i>Inanidrilus leukodermatus</i>           | U24110                  |
| 579 |                 | <i>Olavius loisae</i>                      | AF104472                |
| 580 | Vestimentifera  | <i>Escarpia spicata</i>                    | U77482                  |
| 581 |                 | <i>Lamellabrachia columna</i>              | U77481                  |
| 582 |                 | <i>Ridgeia piscesae</i>                    | U77480                  |
| 583 |                 | <i>Riftia pachyptila</i>                   | M99451                  |
| 584 | Phylum Mollusca |  |                         |
| 585 | Bivalvia        |  |                         |
| 586 | Lucinidae       | <i>Codakia orbicularis</i>                 | X84979                  |
| 587 |                 | <i>Lucina nassula</i>                      | X84980                  |
| 588 |                 | <i>Lucinoma aequizonata</i>                | M99448                  |
| 589 | Mytilidae       | <i>Bathymodiolus</i> aff. <i>brevior</i>   | DQ077891                |
| 590 |                 | <i>B. puteoserpentis</i>                   | U29163                  |
| 591 |                 | <i>B. azoricus</i> - <i>puteoserpentis</i> | AM083974 and this study |
| 592 |                 | <i>B. septemdierum</i>                     | AB036709                |
| 593 |                 | <i>B. thermophilus</i>                     | M99445                  |
| 594 |                 | <i>B.</i> sp. Gabon Margin                 | AJ745718                |
| 595 |                 | <i>B.</i> sp. Juan de Fuca                 | Z. McKiness unpub. data |
| 596 | Thyasiridae     | <i>Thyasira flexuosa</i>                   | L01575                  |
| 597 | Vesicomysidae   | <i>Calyptogena elongata</i>                | AF035719                |
| 598 |                 | <i>C. fossajaponica</i>                    | AB044744                |
| 599 |                 | <i>C. phaseoliformes</i>                   | AF035724                |
| 600 |                 | <i>C. kilmeri</i>                          | AF035720                |

|     |                          |                                       |                         |
|-----|--------------------------|---------------------------------------|-------------------------|
| 601 |                          | <i>C. magnifica</i>                   | AF035721                |
| 602 |                          | <i>C. pacifica</i>                    | AF035723                |
| 603 |                          | <i>Ectenogena extenta</i>             | AF035725                |
| 604 |                          | <i>Vesicomya gigas</i>                | AF035726                |
| 605 | Phylum Nematoda          |                                       |                         |
| 606 | Desmodoridae             | <i>Laxus</i> sp.                      | U241110                 |
| 607 |                          |                                       |                         |
| 608 | Methanotrophic symbionts |                                       |                         |
| 609 | Host Taxonomy            | Host species                          |                         |
| 610 | Phylum Mollusca          |                                       |                         |
| 611 | Bivalvia                 |                                       |                         |
| 612 | Mytilidae                | <i>B. puteoserpentis</i> M            | U29164                  |
| 613 |                          | <i>B. azoricus - puteoserpentis</i> M | AM083950 and this study |
| 614 |                          | <i>B. childressi</i>                  | U05595                  |
| 615 |                          | <i>B. japonicus</i>                   | AB036711                |
| 616 |                          | <i>B. platifrons</i>                  | AB036710                |
| 617 |                          | <i>B.</i> sp. Gabon Margin M          | AJ745717                |
| 618 |                          |                                       |                         |

618 **Figure Legends**

619 Figure 1. Map of study sites and the Mid-Atlantic Ridge (MAR). The collection sites,  
620 including the Lost City, Lucky Strike, and Snake Pit vent fields, are designated by white  
621 circles and labelled. The MAR and its dominant fracture zones (F. Z.) are highlighted  
622 by black lines. Several fracture zones relevant to the discussion are also labelled to  
623 emphasize the geographically discontinuous nature of the MAR.

624

625 Figure 2. Transmission electron micrographs of endosymbionts within the gill tissue of  
626 a Lost City mussel. A. Chemoautotrophic (C) and type I methanotrophic (M) symbionts  
627 within the apical portion of two bacteriocytes (bc) separated by a symbiont-free  
628 intercalary cell (ic). Scale bar = 2  $\mu\text{m}$ . B. Higher magnification of the two symbiont  
629 morphotypes; note intracytoplasmic membranes of the type I methanotroph. Scale bar =  
630 0.5  $\mu\text{m}$ .

631

632 Figure 3. Phylogeny of chemoautotrophic and methanotrophic endosymbionts hosted by  
633 bathymodioline mussels and free-living  $\gamma$ -Proteobacteria, inferred from 16S rRNA gene  
634 sequences (1303 nucleotides). Posterior probabilities from 11,000 bayesian trees are  
635 shown above branches (significant  $\geq 95$ ) and bootstrap values based on 1000 maximum  
636 parsimony replicates are given below the branches. The two phylotypes in this study (*B.*  
637 *azoricus* - *B. puteoserpentis*) are boxed in gray and lettered (C and M) for  
638 chemoautotrophs and methanotrophs, respectively. Furthermore, the two clades that

639 include mussel symbionts are boxed and labelled (C and M). All symbiotic bacteria are  
640 labelled 'symbiont' while free-living bacteria are designated by taxonomic name alone.

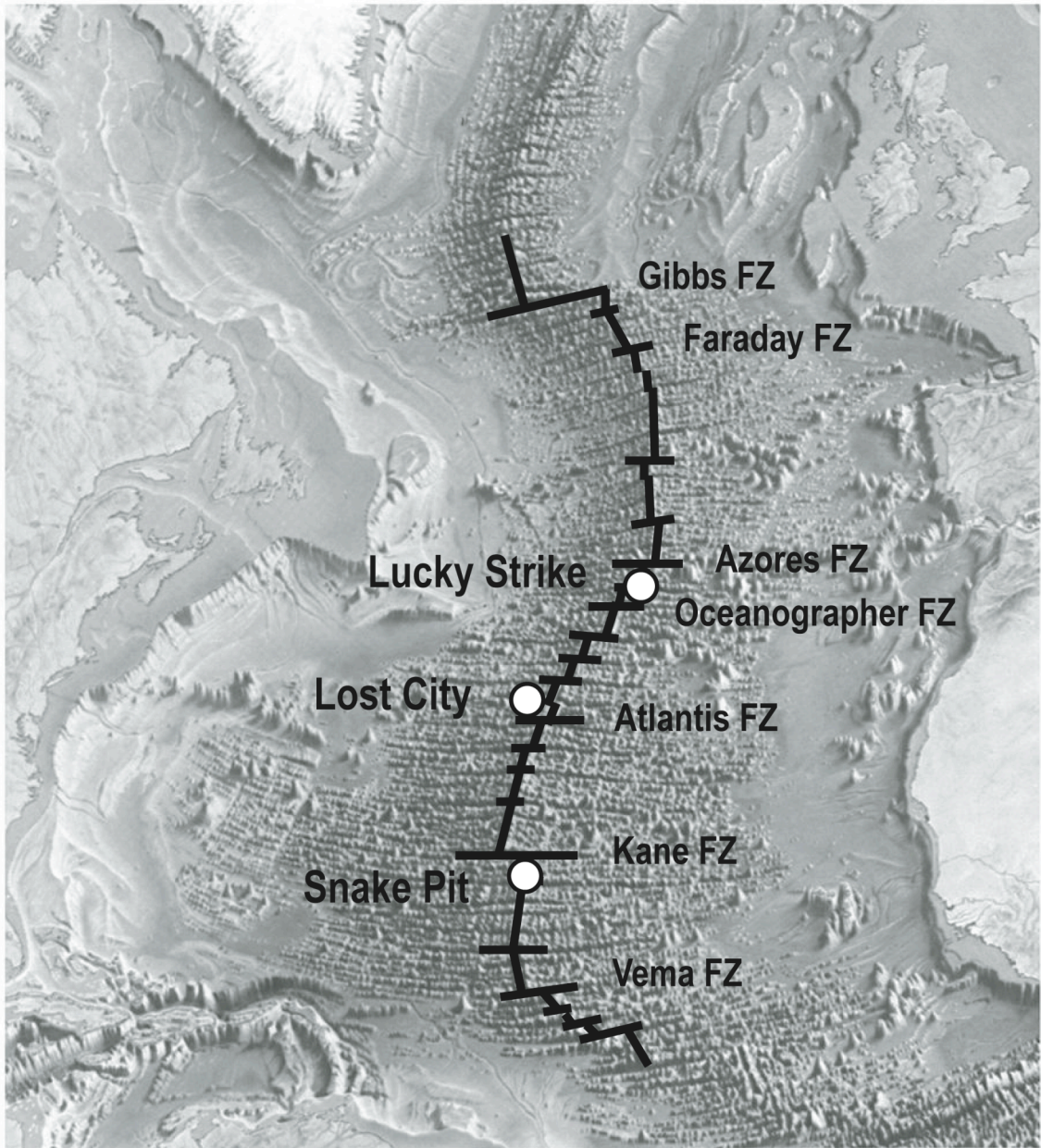
641

642 Figure 4. Parsimony network inferred from 1200 nucleotides of *rrn* internal transcribed  
643 spacer (ITS rRNA) genotypes from chemoautotrophic symbionts of *Bathymodiolus*  
644 mussels collected from the Lost City, Lucky Strike, and Snake Pit hydrothermal fields.  
645 ITS-genotypes are shown as circles, with size indicating relative frequency. Shading  
646 denotes location and the distribution of genotypes within the host mussel population as  
647 follows: Lucky Strike (black = genotypes found in >1 host individual, black  
648 checkerboard = genotypes restricted to only one host individual), Lost City (gray, no  
649 symbionts were shared between the two host individuals), and Snake Pit (white =  
650 genotypes found in >1 host individual, gray checkerboard = genotypes restricted to only  
651 one host individual). Lines connecting genotypes are one nucleotide difference. Small  
652 black dots represent unsampled, hypothetical ancestors. LC1 and LC2 designate  
653 genotype clades from the two individual Lost City mussels. Finally, the northern and  
654 southern 'clades' are boxed and labelled N and S, respectively.

655



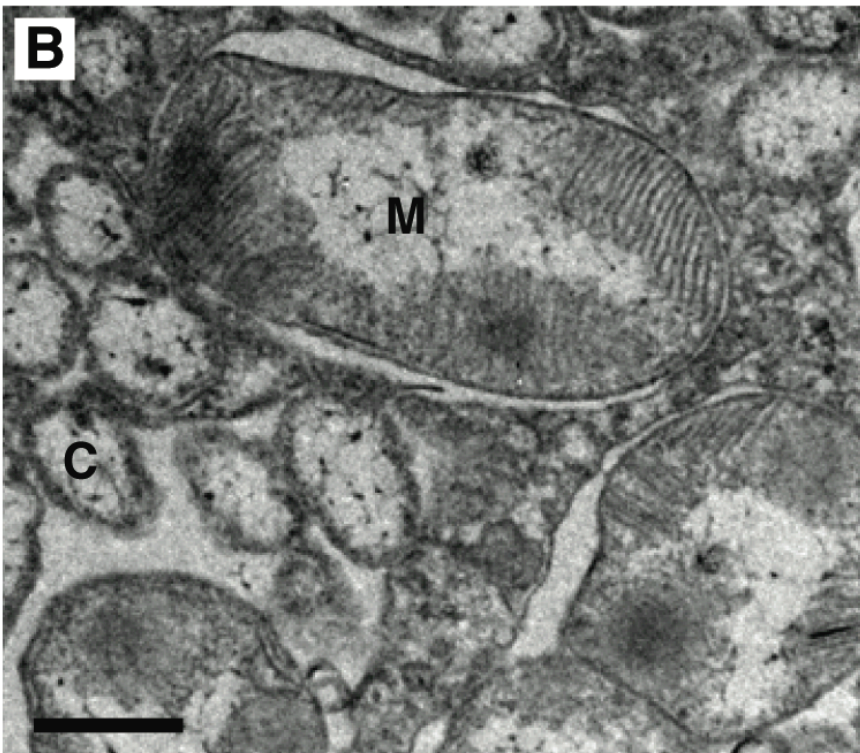
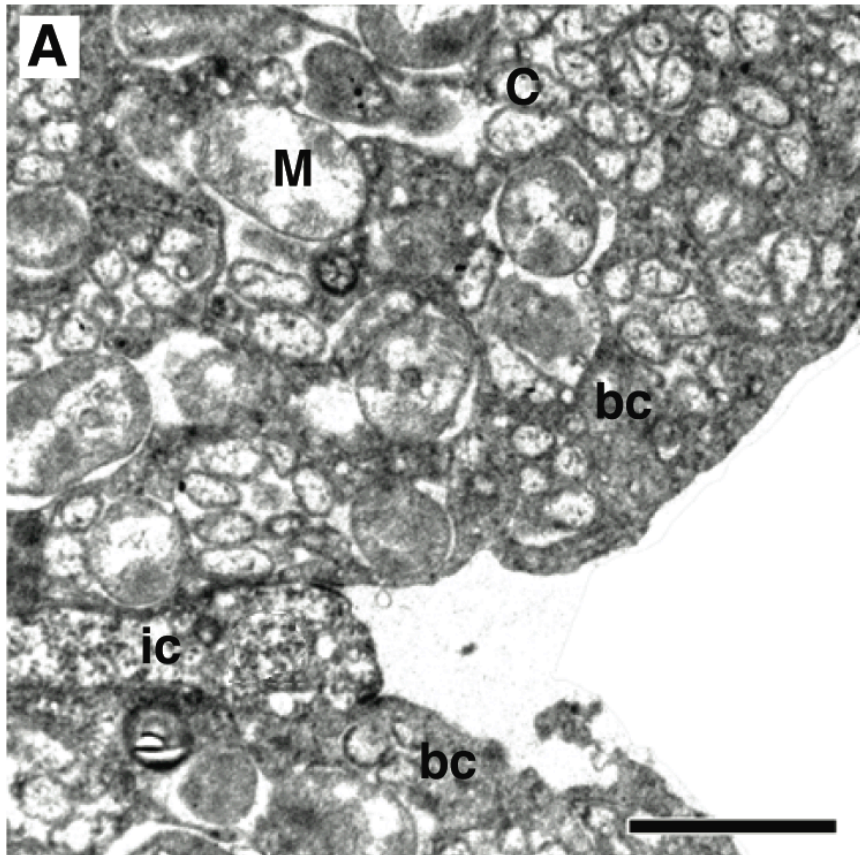
655 Figure 1.



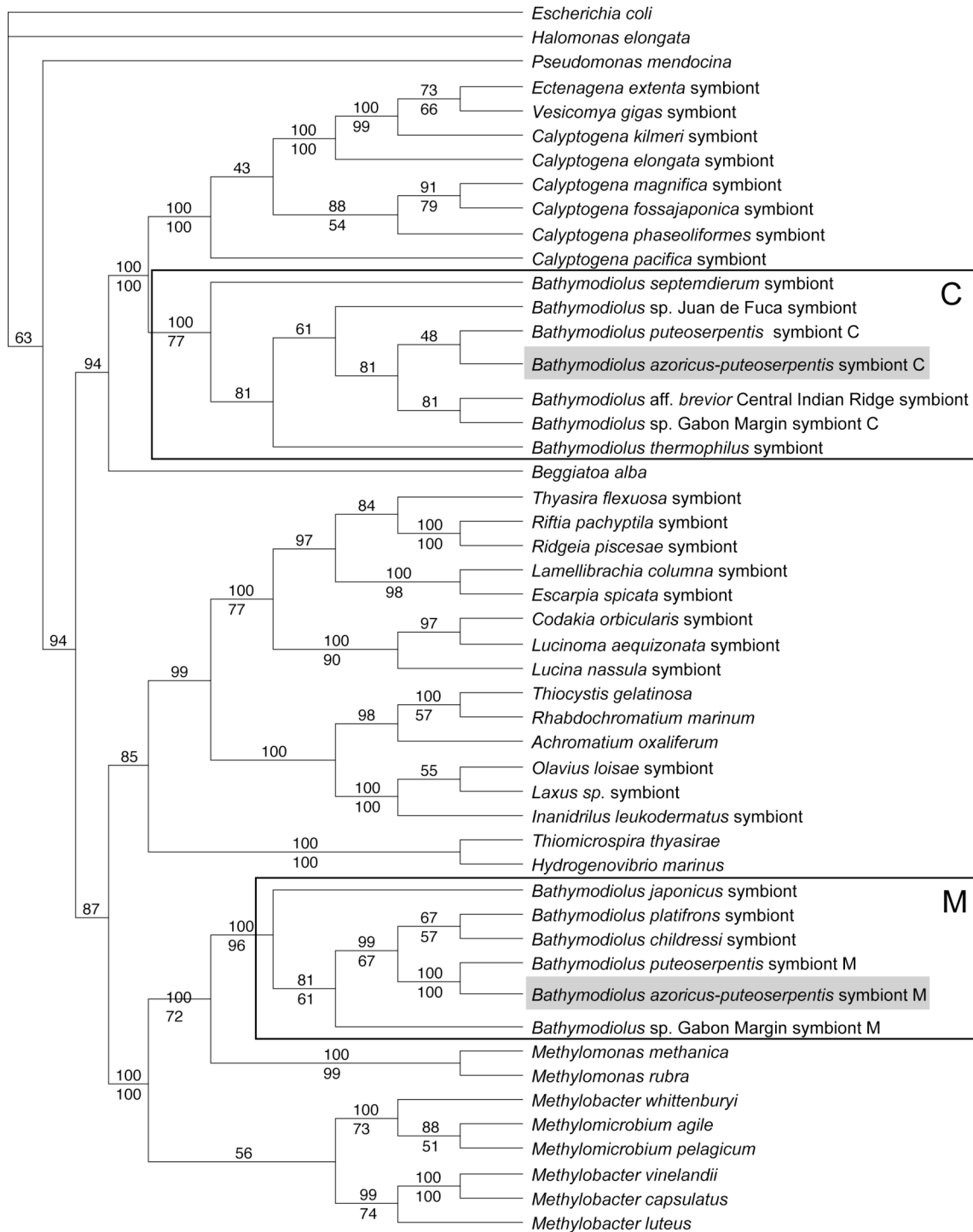
656

657

657 Figure 2.



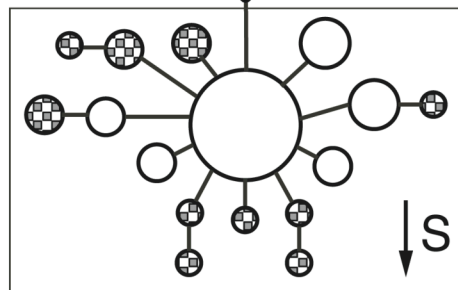
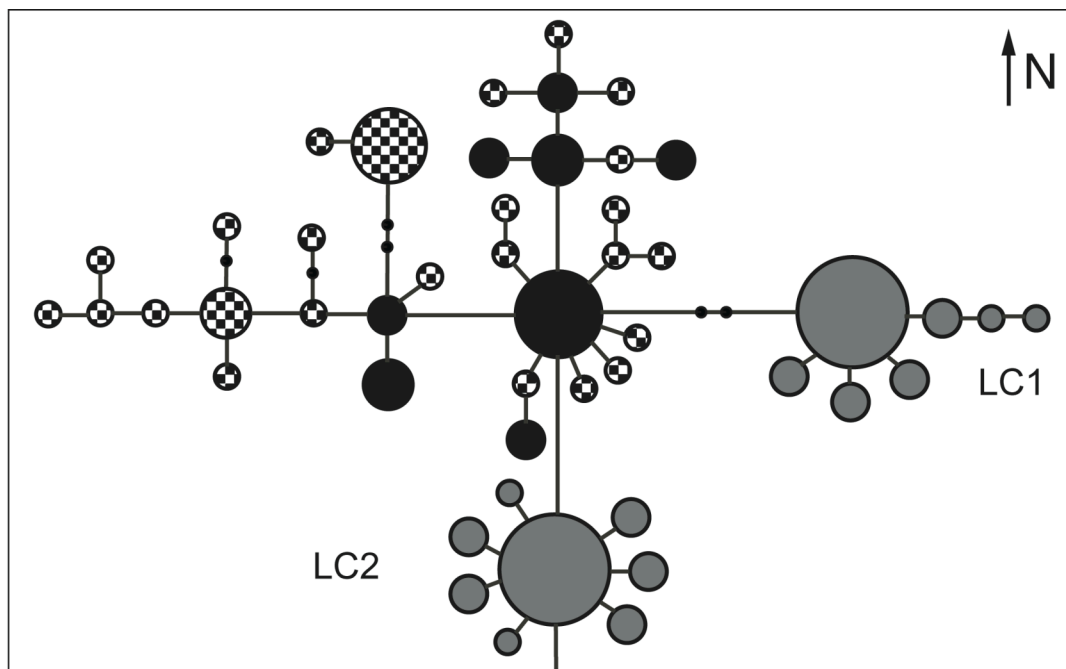
659 Figure 3.



660

661

661 Figure 4.



| Host Mussel                               | Genotype occurrence |           | Genotype Frequency |     |      |       |       |       |     |
|---|---------------------|-----------|--------------------|-----|------|-------|-------|-------|-----|
|   | 1 mussel            | >1 mussel | 1-2                | 3-5 | 6-10 | 11-15 | 15-20 | 21-50 | 51+ |
| Lucky Strike<br><i>B. azoricus</i>        | ⊕                   | ●         | ○                  | ○   | ○    | ○     | ○     | ○     | ○   |
| Lost City (LC)<br><i>B. aff. azoricus</i> | ●                   | n/a       |                    |     |      |       |       |       |     |
| Snake Pit<br><i>B. puteoserpentis</i>     | ○                   | ○         |                    |     |      |       |       |       |     |
| 662 Unsampld                              | •                   |           |                    |     |      |       |       |       |     |

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