Several deep-sea mussels and their associated symbionts are able to live both on wood and on whale falls
Julien Lorion1,*, Sébastien Duperron2, Olivier Gros3, Corinne Cruaud4 and Sarah Samadi1

1Département Systématique et Évolution, Muséum National d’Histoire Naturelle, UMR 7138 UPMC-IRD-MNHN-CNRS ‘Systématique, Adaptation et Évolution’, Equipe Espèces et Spéciation, CP 26, 57 Rue Cuvier, 75331 Paris Cedex 05, France
2UMR 7138 UPMC-IRD-MNHN-CNRS ‘Systématique, Adaptation et Évolution’, Equipe Adaptation aux Milieux Extrêmes, Université Pierre et Marie Curie, 7 quai St Bernard, 75005 Paris, France
3UFR des Sciences Exactes et Naturelles, Département de Biologie, UMR 7138 UPMC-IRD-MNHN-CNRS ‘Systématique, Adaptation et Évolution’, Equipe Symbiose, Université des Antilles et de la Guyane, BP 592, 97159 Pointe-à-Pitre Cedex, Guadeloupe, France
4GENOSCOPE, Centre National de Séquençage, 2 rue Gaston Crémieux, CP 5706, 91057 Evry Cedex, France

Bathymodiolin mussels occur at hydrothermal vents and cold seeps, where they thrive thanks to symbiotic associations with chemotrophic bacteria. Closely related genera Idas and Adipicola are associated with organic falls, ecosystems that have been suggested as potential evolutionary ‘stepping stones’ in the colonization of deeper and more sulphide-rich environments. Such a scenario should result from specializations to given environments from species with larger ecological niches. This study provides molecular-based evidence for the existence of two mussel species found both on sunken wood and bones. Each species specifically harbours one bacterial phylotype related to thioautotrophic bacteria associated with organic falls. Phylogenetic patterns between hosts and symbionts are partially congruent. However, active endocytosis and occurrences of minor symbiont lineages within species which are not their usual host suggest an environmental or horizontal rather than strictly vertical transmission of symbionts. Although the bacteria are close relatives, their localization is intracellular in one mussel species and extracellular in the other, suggesting that habitat choice is independent of the symbiont localization. The variation of bacterial densities in host tissues is related to the substrate on which specimens were sampled and could explain the abilities of host species to adapt to various substrates.

Keywords: Idas; Adipicola; molecular taxonomy; organic falls; symbiosis; thioautotrophy

1. INTRODUCTION

Chemosynthetic bacteria that carry out primary production in the absence of light live in a variety of deep-sea ecosystems, including hydrothermal vents, cold seeps and organic falls (Cavanaugh et al. 2006). Numerous metazoan taxa are endemic to these ecosystems (Sibuet & Olu 1998; Van Dover 2000; Smith & Baco 2003; Rouse et al. 2004), and recent evidence from fossil records suggests overlapping fauna compositions between these ecosystems at least since the Cretaceous period (Kiel & Goedert 2006).

Among the most studied organisms from these ecosystems are the Bathymodiolinae (Bivalvia: Mytilidae), characterized by the occurrence of chemotrophic bacteria associated with specialized cells of the gill tissue (Distel et al. 2000; Duperron 2005). Most species harbour chemoautotrophic bacteria, but other symbionts such as methanotrophs, methylotrophs and Bacteroides were also identified in some species (Duperron et al. 2008a). These mussels rely on symbiont chemotrophy for most of their carbon nutrition, as evidenced by isotopic analyses of their tissues, and by the observation that many hydrothermal vent and cold seep species display reduced digestive tracts (Fisher 1990; von Cosel 2002). Contrary to previous hypotheses, the mussels currently found at deep sea vents have not experienced ‘a long and continuing evolutionary history’ (Newman 1985) and may instead derive from a recent colonization event from shallower environments (Craddock et al. 1995; Jones et al. 2006). Distel et al. (2000) suggested moreover that organic falls may have served as ‘stepping stones’ for the introduction of Bathymodiolinae to deep-sea vents and seeps. In accordance with this ‘wooden step to deep-sea vents’ scenario, several species associated with organic falls display a basal position in mussel phylogenies (Baco 2002; Samadi et al. 2007).

Beyond this phylogenetic pattern that is not yet fully resolved, one can wonder about the processes allowing such a scenario. To get insights into these processes, we examine two points here. First, in this scenario, species able to live in two environments play a hinge role in the colonization process and in the subsequent emergence of specialized species. Whereas most vent and seep species are specialized to one or the other ecosystem, at least two species inhabiting

* Author for correspondence (lorion@mnhn.fr).

2. MATERIAL AND METHODS

(a) Sampling

Two colonization modules containing various organic substrates were deployed near Santo Island in Vanuatu (15°42.24′ S, 167°02.61′ E, 441 m depth). These modules Y and Z were sent to the bottom in October 2005 and in September 2004, respectively, and both were recovered in October 2006. Each module consisted of six baskets containing various woods, seeds, sugar cane and tree fern, but also feathers, sepi shells, turtle and whale bones. Baskets were attached to a common line and separated from each other by 200 m. The module Y yielded 50 mussel specimens from woods and 6 from coconut, while the module Z yielded 2 specimens from turtle bone, 3 from whale bone, 5 from sugar cane and 6 from coconut (table 1; electronic supplementary material 1). Upon recovery, all but seven specimens were directly stored in 90 per cent EtOH. For seven specimens one gill was saved prior to ethanol fixation. Half of each gill was fixed in glutaraldehyde for fixation. Half of each gill was fixed in glutaraldehyde for transmission electron microscopy (TEM) and half was fixed in formaldehyde for fluorescence in situ hybridization (FISH) according to protocols described in Duperron (2005).

From collections of wood-associated mytilids at the Museum National d’Histoire Naturelle were added 25 specimens morphologically close to those collected from experimental devices. These included two noteworthy specimens whose symbiotic relationships were studied in Duperron et al. (2008b).

(b) Molecular analyses

DNA was extracted from gills using the QIAamp DNA Micro Kit (Qiagen). A fragment of the mitochondrial cytochrome

Table 1. List of labels, traps or stations, geographical localities, substrates, depths, genetic clusters and data obtained for bacterial symbionts for all specimens discussed herein. (Bacterial symbionts marked with asterisk in the cluster D were obtained in Duperron et al. (2008b).)

<table>
<thead>
<tr>
<th>label</th>
<th>basket</th>
<th>station</th>
<th>locality</th>
<th>latitude</th>
<th>longitude</th>
<th>substrate</th>
<th>depth (m)</th>
<th>cluster</th>
<th>bacterial 16S</th>
<th>TEM</th>
<th>FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vanu 1–19</td>
<td>Y1</td>
<td>Vanuatu</td>
<td>15,7040 S</td>
<td>167,043 E</td>
<td>wood</td>
<td>441 A</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 20–22</td>
<td>Y5</td>
<td>Vanuatu</td>
<td>15,7040 S</td>
<td>167,043 E</td>
<td>wood</td>
<td>441 A</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 23–24</td>
<td>Z4</td>
<td>Vanuatu</td>
<td>15,7040 S</td>
<td>167,043 E</td>
<td>coconut</td>
<td>441 A</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jap 1–2</td>
<td>CT1</td>
<td>Japan</td>
<td></td>
<td></td>
<td>wood</td>
<td>490 A</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jap 3</td>
<td>CT1</td>
<td>Japan</td>
<td></td>
<td></td>
<td>wood</td>
<td>490 A</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phil 1</td>
<td>CP2355</td>
<td>Philippines</td>
<td>9,4050 N</td>
<td>124,145</td>
<td>wood</td>
<td>1764 A</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 25</td>
<td>Z2</td>
<td>Vanuatu</td>
<td>15,7040 S</td>
<td>167,043 E</td>
<td>turtle bone</td>
<td>441 B</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 26</td>
<td>Z3</td>
<td>Vanuatu</td>
<td>15,7040 S</td>
<td>167,043 E</td>
<td>whale bone</td>
<td>441 B</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 27</td>
<td>Z3</td>
<td>Vanuatu</td>
<td>15,7040 S</td>
<td>167,043 E</td>
<td>sugar cane</td>
<td>441 B</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 28</td>
<td>Z3</td>
<td>Vanuatu</td>
<td>15,7040 S</td>
<td>167,043 E</td>
<td>wood</td>
<td>441 B</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 29</td>
<td>Y5</td>
<td>Vanuatu</td>
<td>15,7040 S</td>
<td>167,043 E</td>
<td>wood</td>
<td>441 B</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 30</td>
<td>Z3</td>
<td>Vanuatu</td>
<td>15,7040 S</td>
<td>167,043 E</td>
<td>whale bone</td>
<td>441 B</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phil 2</td>
<td>CP2673</td>
<td>Philippines</td>
<td>14,9972 N</td>
<td>121,731 E</td>
<td>wood</td>
<td>431 B</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 31</td>
<td>Z2</td>
<td>Vanuatu</td>
<td>15,7040 S</td>
<td>167,043 E</td>
<td>turtle bone</td>
<td>441 C</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 32</td>
<td>Z3</td>
<td>Vanuatu</td>
<td>15,7040 S</td>
<td>167,043 E</td>
<td>whale bone</td>
<td>441 C</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 33–36</td>
<td>Y1</td>
<td>Vanuatu</td>
<td>15,7040 S</td>
<td>167,043 E</td>
<td>wood</td>
<td>441 C</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 37</td>
<td>Y1</td>
<td>Vanuatu</td>
<td>15,7040 S</td>
<td>167,043 E</td>
<td>coconut</td>
<td>441 C</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 38</td>
<td>Z3</td>
<td>Vanuatu</td>
<td>15,7040 S</td>
<td>167,043 E</td>
<td>wood</td>
<td>441 C</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 39–42</td>
<td>AT120</td>
<td>Vanuatu</td>
<td>15,6869 S</td>
<td>167,028 E</td>
<td>wood</td>
<td>431 C</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 43</td>
<td>AT120</td>
<td>Vanuatu</td>
<td>15,6869 S</td>
<td>167,028 E</td>
<td>wood</td>
<td>431 C</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 44</td>
<td>AT121</td>
<td>Vanuatu</td>
<td>15,6630 S</td>
<td>167,027 E</td>
<td>wood</td>
<td>290 C</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 45</td>
<td>AT121</td>
<td>Vanuatu</td>
<td>15,6630 S</td>
<td>167,027 E</td>
<td>wood</td>
<td>290 C</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 46</td>
<td>AT136</td>
<td>Vanuatu</td>
<td>16,0445 S</td>
<td>167,050 E</td>
<td>wood</td>
<td>802 C</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanu 47</td>
<td>AT136</td>
<td>Vanuatu</td>
<td>16,0445 S</td>
<td>167,050 E</td>
<td>wood</td>
<td>802 C</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solo 1</td>
<td>CP2212</td>
<td>Salomon</td>
<td>7,6033 N</td>
<td>157,707 E</td>
<td>wood</td>
<td>400 C</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phil 3</td>
<td>CP2356</td>
<td>Philippines</td>
<td>9,3483 N</td>
<td>124,145 E</td>
<td>wood</td>
<td>1764 D</td>
<td>BC288*</td>
<td>yes*</td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phil 4–7</td>
<td>CP2356</td>
<td>Philippines</td>
<td>9,3483 N</td>
<td>124,145 E</td>
<td>wood</td>
<td>1764 D</td>
<td>BC294.1*</td>
<td>yes*</td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phil 8</td>
<td>CP2356</td>
<td>Philippines</td>
<td>9,3483 N</td>
<td>124,145 E</td>
<td>wood</td>
<td>1764 D</td>
<td>BC294.2*</td>
<td>yes*</td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phil 9–10</td>
<td>CP2356</td>
<td>Philippines</td>
<td>9,3483 N</td>
<td>124,145 E</td>
<td>wood</td>
<td>1764 D</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phil 11</td>
<td>CP2385</td>
<td>Philippines</td>
<td>8,8500 N</td>
<td>123,166 E</td>
<td>wood</td>
<td>982 D</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phil 12</td>
<td>CP2388</td>
<td>Philippines</td>
<td>9,4483 N</td>
<td>123,575 E</td>
<td>wood</td>
<td>762 D</td>
<td></td>
<td></td>
<td>yes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

both were reported (Miyazaki et al. 2004). Data on habitat preference for the small bathymodiolins are scarce, and thus no convincing evidence for ubiquity exists. Moreover, their diversity is largely unexplored and underestimated (Smith & Baco 2003; Samadi et al. 2007; Duperron et al. 2008b). Our first goal was thus to explore the diversity of organic fall-associated bathymodiolin mussels and to test whether some species are able to live on several types of organic substrates. Second, we documented how the relationships between a given ubiquitous host mussel and its symbionts are affected by the different environments. Indeed, symbioses with chemotrophic bacteria extend metabolic capabilities, and therefore ecological niches, of both hosts and bacterial symbionts. In this regard, the transmission strategy is an important clue (Cavanaugh et al. 2006). The second goal of this study was thus to document whether symbionts from such ubiquitous mussel species are substrate- or species-specific by characterizing the symbiotic relationships on the various substrates.
oxidase I-encoding gene (COI mtDNA) was amplified using primers H691 5′-GTRTTAARTGRCATCAAAAAT-3′ and LCO 1490 (Folmer et al. 1994). The domains D1, D2 and D3 of the 28S rRNA nuclear gene were also amplified for hosts, while a fragment of the 16S rRNA gene was amplified for bacterial symbionts. Polymerase chain reaction (PCR) protocols for hosts and symbionts were described in Duperron (2005) and Samadi et al. (2007), respectively, as well as primers for 28S rRNA and 16S rRNA, respectively. PCR products from COI mtDNA and 28S rRNA were sequenced directly using PCR primers, while amplificates of 16S rRNA were purified on columns (Qiagen) and cloned using a TOPO cloning kit (Invitrogen). Inserts from 8 to 14 positive clones were fully sequenced using vector primers M13F and M13R. All specimens sequenced in this study were deposited in BOLD (www.barcodinglife.org, project SUBST), from which sequenced pictures are available, and NCBI (www.ncbi.nlm.nih.gov; electronic supplementary material 1). For host species identified in both plant material and whale bones, symbionts were localized in the gill tissue using (i) electron microscopy and (ii) FISH with Gammaproteobacteria-specific probe GAM42, following protocols described in Duperron et al. (2008b).

(c) Molecular taxonomy and phylogenetics

The specific status of the studied specimens was assessed by a genotypic clustering approach based on the two independent gene fragments. K2P genetic distances were calculated within each dataset with the complete deletion option and neighbour-joining (NJ) trees were built with MEGA v. 4 (Tamura et al. 2007). The robustness of each of the obtained genetic clusters as primary species hypotheses was tested using the phylogenetic criterion. Representative sequences of the different lineages of bathymodiolin mussels were added to the COI mtDNA and 28S rRNA datasets and both single gene and combined datasets were built (electronic supplementary material 2). Host trees were rooted on Benthomodiplus ligmicola (Samadi et al. 2007). Then, in an integrative taxonomy perspective, these primary species hypotheses were evaluated in the light of shell shapes and geographical distributions of specimens.

A dataset including all our 16S rRNA sequences and sequences from thiaotrophic symbionts of mussels from literature was built (electronic supplementary material 3). Trees were rooted on the methanotrophic symbiont of Bathymodiolus brooksi (Duperron et al. 2007).

Best-fitting models of nucleotide evolution were estimated for each dataset using MrAIC (Nylander 2004) and set in maximum likelihood (ML) and Bayesian analyses (BA). ML analyses were performed using PAML and robustness of nodes was assessed with nonparametric bootstrapping with 1000 bootstrap replicates (Felsenstein 1985; Guindon & Gascuel 2003). BA were performed with MrBAYES v. 3.0. Eight Markov chains, 5,000,000 generations and a heating temperature of 0.02 (Ronquist & Huelsenbeck 2003). Convergence between runs was assessed using likelihood curves, standard deviation of split frequencies and potential scale reduction factor (Gelman & Rubin 1992). First, 50 per cent of the samples were discarded for calculations of posterior probabilities (PP). All BA and ML calculations were performed on the cluster developed at the MNHN (17 nodes, 2 GB RAM per node, 30 AMD 64-bit CPUs for the slave nodes and 4 Xeon 32-bit CPUs for the master nodes).

3. RESULTS

(a) Host mussels across substrates

Among the 72 mussel specimens recovered from the deployments, 579 bp COI mtDNA sequences were obtained for 3 specimens from whale bones, 2 from turtle bone and 33 associated with plant material. Three genetic clusters A (24 specimens), B (6 specimens) and C (8 specimens), separated by K2P genetic distances higher than 15 per cent, were detected (electronic supplementary material 4). Species within cluster A were found only on wood samples, whereas specimens from clusters B and C were found either on wood or bone. Examination of the largest specimens allowed us to distinguish three morphs consistent with clusters A, B and C. Among wood-associated specimens from MNHN collections, 4 clustered in A, in B and 10 in C. Ten other specimens from the Philippines, including those already examined by Duperron et al. (2008b), constituted a fourth cluster, D, separated from C by 8.9 per cent, despite both being morphologically very close. The final COI mtDNA dataset included 63 sequences and 579 bp, of which 143 were variable and 138 parsimony informative. The unrooted ML tree also revealed that clusters A and B were both structured into two sub-clusters separated by 1.6 per cent (A1/A2) and 2.7 per cent (B1/B2), respectively. K2P mean genetic distances within sub-clusters A1, A2, B1, B2, C and D ranged from 0 to 1.3 per cent (electronic supplementary material 4 and 5).

After adding 63 representative sequences of the various bathymodiolin lineages (including mussels from sunken organic substrates), the COI mtDNA dataset included 100 specimens and 579 bp, of which 242 were variable and 206 parsimony informative. ML- and BA-based trees displayed similar topologies. Markedly, sequences of Japanese samples included from GENBANK were very close to ours. Indeed sequences of Adipicola iwoatokii from sunken woods clustered within A’ and A”, whereas sequences of Adipicola crypta from whale bones clustered in B’ (figure 1). The four detected genetic clusters A, B, C and D appeared to be phylogenetic lineages robustly supported by bootstrap values. The sub-clusters A’ and B’ were also monophyletic, but the support was weak for B’. Clusters C and D appeared as sister taxa despite low support values, and both clustered into a well-supported lineage entailing some other species attributed to the genus Idas. The relationships among A, B and the whole Idas lineage were not resolved. Previously described phylogenetic patterns between lineages from vents, seeps and sunken wood were recovered (Samadi et al. 2007).

From a subset of specimens from each cluster and sub-cluster identified in the COI analysis, 46 28S rRNA sequences were recovered (electronic supplementary material 1). This dataset included 1011 bp, of which 35 were variable and 23 parsimony informative. Cluster A with its two sub-clusters is recovered (electronic supplementary material 4). Cluster B is also recovered, but B’ and B” shared a unique allele. Alleles from C and D were distinct by a one bp indel. Three specimens of D displayed a one bp frame shift, suggesting that they shared the alleles of C and D. ML and BA trees obtained from the 28S rRNA dataset, including our sequences and representative lineages from vents, seeps and organic falls, were similar to those obtained with COI mtDNA, although less resolved.
The close relationship between C and D was highly supported (95% of bootstraps, PP = 1; result not shown).

Topologies obtained from combined analyses of both genes were similar and also supported the close relationship between C and D (92% of bootstraps, PP = 1; result not shown).

(b) Localization of associated bacteria

Electron microscopy confirmed the occurrence of bacteria of similar morphology in the gills of all examined specimens, with double membranes typical of gram negatives (figure 2b, electronic supplementary materials 6 and 7). All but one specimen displayed bacteria with dark inclusions possibly corresponding to β-polylhydroxybutyrate reserves (figure 2b, electronic supplementary material 7). No sulphur-containing granule was seen.

In specimens from cluster C, bacteria occurred between microvilli located outside of gill epithelial cells, exposed to circulating seawater (figure 3a–d). The thickness of the bacterial layer outside host cells varied considerably between specimens, ranging from 2 to 20 μm (figure 3c,d). Large vacuoles containing many bacteria in one specimen suggest phagocytosis rather than endosymbiosis, since they differed drastically from reported examples of endosymbiosis in mussels displaying usually low numbers of bacteria within a single vacuole (figure 3d).

The presence of a similar association displaying bacteria located in large vacuoles between microvilli of two specimens (table 1), clustering within group D in the current study, was previously reported in Duperron et al. (2008b).

In specimens of sub-cluster B’, the associated bacteria consistently occurred in low numbers in vacuoles which were located within the apical part of host gill epithelial cells (figure 2a,c,d). No layer of extracellular bacteria was seen, and some features reminiscent of phagolysosomes described in vent and seep mussels suggested active endocytosis by host cells (figure 2d). Amounts of bacteria present in host cells varied from few (figure 2a; electronic supplementary material 8) to many (figure 2c,d).

(c) Diversity and phylogeny of associated bacteria

Bacterial 16S rRNA sequences were obtained for ubiquitous clusters B’, B” and C. For all but five, best hits against RDP and GENBANK databases were thioautotrophic symbionts of bathymodiolin mussels. FISH results confirmed the occurrence of Gammaproteobacteria in gill tissue (electronic supplementary material 9). The five minor non-gammaproteobacterial phylotypes, each represented by one or three sequences and unique to a single mussel specimen, were not related to mussel symbionts and may be environmental bacteria (not shown).

Only thioautotrophic symbiont sequences were included in the 16S rRNA phylogenetic dataset, which included 18 sequences and 1418 bp, of which 273 bp were variable and 187 bp parsimony informative. ML and BA trees displayed the same topologies (figure 4).

Specimens from cluster C harboured a single phylotype 1. Compared with the specimens of cluster D investigated by Duperron et al. (2008b), this phylotype 1 was identical to one of the two 16S rRNA partial sequences of one specimen and displayed six differences to that of another. The phylogenetic relationships between symbiont sequences obtained from cluster D and phylotype 1 obtained herein from cluster C were well supported.
Specimens of sub-cluster B" harboured a single phylo-
type 2, while one specimen of sub-cluster Bdisplayed
both phylotypes 1 and 2, phylotype 2 being represented by
seven out of eight full sequences.

Both phylotype 1 and phylotype 2 were included in a
lineage entailing also symbionts from lineage D and from
Adipicola longissima, but the node was poorly supported.
Other known relationships between thiotrophic symbionts
from vents and seeps species were recovered.

4. DISCUSSION

(a) Species and speciation pattern

Previous studies indicate that species delimitation in
bathymodiolin mussels is hindered by high levels of shell
d polymorphism, allometric growth and the occurrence of
cryptic species (Horikoshi & Tsuchida 1984; Won et al.
2003c; Olu-Le Roy et al. 2007). Herein we overcame the
scarcity of taxonomic knowledge by using a comparative
taxonomic approach. Both genetic and morphological
characters were studied and the distribution of their
variability was analysed in an ecological background,
including the type of substrate used and the geographical
location. Using a genotyping clustering approach, including
specimens recovered from experimental devices and from
the MNHN collections, we detected four clusters of

---

**Figure 2.** TEM images of gill filaments and epithelial cells of *A. crypta* colonizing various substrates. (a,b) Gill filaments of the specimen from whale bones. Bacteriocyte (BC) cytoplasm is filled with intracellular bacterial symbionts and secondary lysosome-like particles (Ly). (a) Very few bacteria can be observed at the periphery of the cell, located between microvilli. Higher magnification of intracellular bacteria (b) shows that symbionts are typical Gram-negative bacteria. Their DNA occupies most of the volume of the bacterial cytoplasm. The periplasmic space does not contain sulphur granules (usually appearing as electron-lucent vesicles), but there are numerous electron-dense granules (curved arrows) in the cytoplasm representing β-polyhydroxybutyrate storage granules. Small arrows indicate the microvilli from the host cell contained inside the large phagocytosis vacuole. m: mitochondria (c,d) TEM view of gill filaments from *A. crypta* collected on sunken woods. Bacteria are mostly located at the apical pole of the bacteriocytes; the basal part of the host cell contains mostly secondary lysosome-like structures (Ly). Bacterial symbionts are less numerous per phagocytosis vacuole (stars) with more bacteria located outside the host cell. Such extracellular bacteria probably became enclosed in the vacuoles by phagocytosis (arrows). Bacteria do not possess the dark β-polyhydroxybutyrate granules. BL: blood lacuna; N: nucleus.

---

...
Figure 3. TEM images of gill filaments and epithelial cells of lineage C colonizing various substrates. (a) Gill filaments from an individual collected on whale bone (Vanu 32) at 441 m depth. The thickness of the bacterial layer is greater than the bacteriocyte's thickness. Bacteria, which are located extracellularly between microvilli of the host cells, are mostly ovoid: bacteriocyte cytoplasm possesses few secondary lysosomes (Ly), characterized by their heterogeneous aspect. No bacteria are found inside phagosomes. BL: blood lacuna. N: nucleus. (b) Gill filament of the lateral zone from a wood-inhabiting specimen (Vanu 47) collected at 802 m depth. Bacteriocytes (BC) harbour few layers of extracellular bacteria. In the bacteriocyte cytoplasm, numerous small lysosomes are seen. Extracellular symbionts (arrows) are located on the apical surface of the bacteriocytes in contact with microvilli. (c) Lateral zone of a gill filament from a wood-inhabiting specimen (Vanu 44) collected at 290 m depth. Bacteriocyte cytoplasm contains mostly mitochondria. (d) Lateral zone of a gill filament from a wood-inhabiting specimen (Vanu 46) collected at 802 m depth. The thickness of the extracellular bacteria above the apical pole of the bacteriocytes reaches 15 µm. Similar bacteria can be observed either in the thick layer of extracellular bacteria or inside the phagosomes (stars) without apparent degradation. The bacteriocytes contain various lysosome-like structures in their cytoplasm.

methanotrophic symbiont from Bathymodiolus brooksi

thioautotrophic symbiont from Bathymodiolus aff. brevior

thioautotrophic symbiont from Bathymodiolus septemdierum

thioautotrophic symbiont from Bathymodiolus thermophilus

Figure 4. Bayesian tree obtained from the analysis of the 16S rRNA dataset. Sequences obtained in this study are in bold. Substitution model selected from MrAIC: GTR + [I] + L. Posterior probabilities (PP) and bootstraps values obtained from ML analysis are given above and below nodes, respectively. PP and bootstraps values lower than 0.90 and 50%, respectively, are not shown. Scale bar represents 2% estimated base substitution. The broken branch represents 13% estimated base substitution.
within the *Idas* lineage. We did not depict any morphological difference between lineages C and D, but more detailed morphological studies are forthcoming. Lineages C and D will be thus referred to as *Idas* sp. C and *Idas* sp. D hereafter. The latter was only sampled from woods but, since bones were neither deployed in the Philippines nor collected during cruises which provided material to the MNHN collections from Philippines, this observation may not be relevant.

The 2.7 per cent genetic divergence between subclusters B' and B'' is lower than the 4.4 per cent threshold, but above the range of intraspecific distances documented for Bathymodiolinae, usually lower than 1 per cent (Won et al. 2003c; Smith et al. 2004; Iwasaki et al. 2006; Samadi et al. 2007). Sub-cluster B' is restricted to Vanuatu, whereas B'' includes mostly specimens from Japan and the Philippines, a pattern comparable to that displayed by clusters C and D, respectively. The divergence between B' and B'' could thus be explained by a similar allopatric scenario. However, one specimen from B'' was found on whale bones deployed in experimental devices from Vanuatu. This fact, combined with the low divergence between B' and B'' and their poorly supported monophyly, suggests a shorter time of divergence than between *Idas* sp. C and D. The absence of polymorphism on the nuclear dataset is also congruent with this hypothesis. Specimens of *A. crypta* from GENBANK clustered in B'', together with our own specimens. No consistent morphological character allowed us to distinguish between B' and B'', probably owing to the few specimens examined and their various life stages (see pictures in BOLD). In absence of more morphological and genetic data, cluster B will be referred to as *A. crypta* species complex hereafter.

The 1.6 per cent genetic divergence between subclusters A' and A'', both sampled on woods, is small but nevertheless higher than the documented range of intraspecific distance within bathymodiolin species. It was moreover supported by slight differences between their 28S rRNA alleles and A'' was monophyletic. Both A' and A'' included a specimen identified as *A. twaotauki* from GENBANK. Owing to this and the absence of morphological differences between A' and A'', we suggest that the species name *A. twaotauki* might also correspond to a species complex belonging to the *Adipicola* genus.

**(b) Ubiquitous species do exist**

The deployment of experimental devices permitted us to demonstrate that at least two lineages of bathymodiolini mussels, namely *A. crypta* species complex and *Idas* sp. C, can occur on both sunken wood and whale bones. Adding molecular data from specimens collected from whale bones near Japan (Y. Fujita, H. Matsumoto, Y. Fujiwara, J. Hashimoto, L. O. Martins, S. V. Galkin, R. Ueshima & J. Miyazaki 2007, unpublished data) strengthened this observation for *A. crypta*, while MNHN’s specimens collected from wood did so for *Idas* sp. C. Moreover, no evidence of divergence driven by a specialization for a particular substrate was detected.

While most of the Bathymodiolinae are associated with a single type of ecosystem, ubiquity with regard to substrate has already been pointed out in the cases of *Bathymodiolus japonicus* and *Bathymodiolus platifrons*, two species that occur both at seeps and vents (Miyazaki et al. 2004). In this example, a multi-marker molecular study supported the result. Concerning the mussels associated with organic falls, the best-documented case was *Idas washingtonia* for which the genetic identity of specimens collected on woods and bones was only supported by 18S rRNA data. However, this gene displays almost no polymorphism over all bathymodiolin mussels (Distel et al. 2000; Jones et al. 2006; Samadi et al. 2007). The occurrence of *I. washingtonia* was also recently reported at hydrothermal vents (Southward 2008), while *A. crypta* and *Idas simpsoni* were recorded from both woods and whale bones (Dell 1987; Fujiwara et al. 2007). However no detailed molecular analysis was provided as support.

**(c) Associations with sulphur-oxidizing bacteria and co-speciation patterns**

Bacteria identified in the gills of ubiquitous mussels from the *A. crypta* species complex and *Idas* sp. C are Gammaproteobacteria, most likely sulphur-oxidizers able to fix carbon chemotrophically based on their phylogenetic position. Indeed, the presence of genes encoding form I RubisCO and adenosine 5'-phospho-sulphate reductase was demonstrated in closely related bacteria associated with *Idas*-like mussels from seeps and woods, including two specimens from *Idas* sp. D (Duperron et al. 2008b). Fluorescence in situ hybridization (electronic supplementary material 9) and electron microscopy confirm the abundance of these bacteria in association with the gills.

The localization of the bacteria in host tissue is distinct. Bacteria in specimens from *Idas* sp. C are consistently extracellular. Extracellular symbionts were reported in wood-inhabiting specimens of the sister *Idas* sp. D, in *A. longissima*, and in unidentified specimens from Philippines and Vanuatu (Gros et al. 2007; Duperron et al. 2008b).

Conversely, analysed specimens of the *A. crypta* species complex always display intracellular bacteria located within gill bacterioctyes. Bacterioocyte morphology is similar to that displayed by many hydrothermal vent and cold seep mussels, and by *I. washingtonia*, a species associated with whale bones (Deming et al. 1997; Fiala-Médioni et al. 2002). Thus, both mussel species associated with endo- and ectosymbionts are able to use the two types of environment.

Within each of the two ubiquitous lineages *A. crypta* species complex and *Idas* sp. C, only one major symbiotic lineage was found and a partial congruence between host and symbiont phylogenies was observed. Indeed, (i) *Idas* sp. C and D display a sister-group relationship that is also observed for their associated bacteria and (ii) subclusters of the *A. crypta* species complex display the same dominant bacterial lineages. A similar close relationship between sister species of mussels and their symbionts was reported for *Bathymodiolus azoricus* and *Bathymodiolus puteoserpentis*, *Bathymodiolus heckerae* and *B. aff heckerae*, and *Bathymodiolus brevir and Bathymodiolus septemderium* (Duperron et al. 2006, 2007). However, the phylogenetic congruence is not observed at deeper phylogenetic nodes (Won et al. 2008). Finally, the dominant bacterial 16S rRNA phylotypes associated with the gills of ubiquitous mussel species from the two distantly related *A. crypta* species complex and *Idas* sp. C are closely related since they differ by only 7 out of 1500 nucleotide positions.

Markedly, the unique symbiont phylotype detected in the *Idas* sp. C was detected as a ‘rare sequence’ in clone libraries from a specimen of its putative sister *Idas* sp. D.
Bacterial densities are variable among specimens of the *A. crypta* species complex (Duperron et al. 2008b). Thus, the association of a host lineage to a given bacterial lineage seems less strongly constrained than is the localization of the symbionts in host tissue. The co-occurrence in a single host of two phylogenotypes that are usually found in two unrelated host lineages suggests that symbionts can sometimes coexist. This means that they can possibly compete, and that symbiont replacement is possible over evolutionary time. Moreover TEM pictures displayed evidence of endocytosis. The short-scale co-speciation pattern observed among very close species could consequently be explained either by a predominant vertical transmission mode with rare environmental or horizontal transmission as defined in Cavanaugh et al. (2006), or by an environmental acquisition of symbionts during early developmental stages associated with coevolution of mutual host–symbiont recognition mechanisms (Won et al. 2003a).

**REFERENCES**


Mussels are known for their ability to host a wide variety of symbiotic bacteria. Some of these bacteria are chemoautotrophic, meaning they can produce their own food using chemical reactions, while others are methanotrophic, capable of utilizing methane as an energy source. These symbiotic relationships are crucial for the survival of mussels in deep-sea environments, where food is scarce.

One study, published in *Nature* in 2000, explored the diversity of bacteria associated with deep-sea mussels. The researchers found that these mussels host a complex community of bacteria, including both chemoautotrophs and methanotrophs, which are essential for the mussel's nutrition in the nutrient-poor environment of the deep sea.

Furthermore, another study published in *Proceedings of the National Academy of Sciences* in 2007 discussed the role of endosymbiotic bacteria in the growth and development of deep-sea mussels. The researchers highlighted the importance of these bacteria in the mussel's life cycle, emphasizing the intricate relationship between the host and the bacteria.

In conclusion, the symbiosis between deep-sea mussels and their bacterial endosymbionts is a fascinating example of how marine organisms adapt to their environment. This symbiosis not only provides the mussels with nutrition but also suggests a potential model for understanding the evolution of life in extreme environments.