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Several deep-sea mussels and their associated symbionts are able to live both on wood and on whale falls

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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 corresponding to thioautotrophic bacteria related to other bathymodiolin symbionts. 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 thiotrophic bacteria, but other symbionts such as methanotrophs, methylotrophs and Bacteroides were also identified in some species (Duperron *et al.* 2008*a*). 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

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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.* (2008*b*).)

label	basket station	locality	latitude	longitude	substrate	depth (m)	cluster	bacterial 16S	TEM	FISH
Vanu 1–19	Y1	Vanuatu	15,7040 S	167,0435 E	wood	441	A′			
Vanu 20–22	Y5	Vanuatu	15,7040 S	167,0435 E	wood	441	A′			
Vanu 23–24	Z4	Vanuatu	15,7040 S	167,0435 E	coconut	441	A′			
Jap 1–2	CT1	Japan			wood	490	A′			
Jap 3	CT1	Japan			wood	490	A″			
Phil 1	CP2355	Philippines	9,4050 N	124,1783 E	wood	1764	A″			
Vanu 25	Z2	Vanuatu	15,7040 S	167,0435 E	turtle bone	441	B′			
Vanu 26	Z3	Vanuatu	15,7040 S	167,0435 E	whale bone	441	B′			
Vanu 27	Z3	Vanuatu	15,7040 S	167,0435 E	sugar cane	441	B′	phylotype 1and 2		
Vanu 28	Z3	Vanuatu	15,7040 S	167,0435 E	wood	441	B′			
Vanu 29	Y5	Vanuatu	15,7040 S	167,0435 E	wood	441	B′			
Vanu 30	Z3	Vanuatu	15,7040 S	167,0435 E	whale bone	441	B″	phylotype 2	yes	
Phil 2	CP2673	Philippines	14,9972 N	121,7317 E	wood	431	B″	phylotype 2	yes	yes
Vanu 31	Z2	Vanuatu	15,7040 S	167,0435 E	turtle bone	441	C	phylotype 1		
Vanu 32	Z3	Vanuatu	15,7040 S	167,0435 E	whale bone	441	C	phylotype 1	yes	
Vanu 33–36	Y1	Vanuatu	15,7040 S	167,0435 E	wood	441	C			
Vanu 37	Y1	Vanuatu	15,7040 S	167,0435 E	coconut	441	C			
Vanu 38	Z3	Vanuatu	15,7040 S	167,0435 E	wood	441	C			
Vanu 39–42	AT120	Vanuatu	15,6869 S	167,0284 E	wood	431	C			
Vanu 43	AT120	Vanuatu	15,6869 S	167,0284 E	wood	431	C	phylotype 1		
Vanu 44	AT121	Vanuatu	15,6630 S	167,0273 E	wood	290	C		yes	
Vanu 45	AT121	Vanuatu	15,6630 S	167,0273 E	wood	290	C			
Vanu 46	AT136	Vanuatu	16,0445 S	167,5059 E	wood	802	C		yes	
Vanu 47	AT136	Vanuatu	16,0445 S	167,5059 E	wood	802	C		yes	
Solo 1	CP2212	Salomon	7,6033 N	157,7075 E	wood	400	C			
Phil 3	CP2356	Philippines	9,3483 N	124,1450 E	wood	1764	D	BC288*	yes*	
Phil 4–7	CP2356	Philippines	9,3483 N	124,1450 E	wood	1764	D			
Phil 8	CP2356	Philippines	9,3483 N	124,1450 E	wood	1764	D	BC294.1*, BC294.2*	yes*	
Phil 9–10	CP2356	Philippines	9,3483 N	124,1450 E	wood	1764	D			
Phil 11	CP2385	Philippines	8,8500 N	123,1667 E	wood	982	D			
Phil 12	CP2388	Philippines	9,4483 N	123,5750 E	wood	762	D			

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.* 2008*b*). 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.

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, sepia shell, 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 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 Muséum 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.* (2008*b*).

(b) *Molecular analyses*

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

oxidase I-encoding gene (COI mtDNA) was amplified using primers H691 5′-GTRTTAAARTGRCGATCAAAAAT-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 scaled 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.* (2008*b*).

(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 *Benthomodiolus lignicola* (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 thioautotrophic 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 PhyML 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 with 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 2 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). Specimens 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, 1 in B and 10 in C. Ten other specimens from the Philippines, including those already examined by Duperron *et al.* (2008*b*), 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 NJ tree also revealed that clusters A and B were both structured into two sub-clusters separated by 1.6 per cent (A′/A″) and 2.7 per cent (B′/B″), respectively. K2P mean genetic distances within sub-clusters A′, A″, B′, B″, 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 iwaotakii* 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.

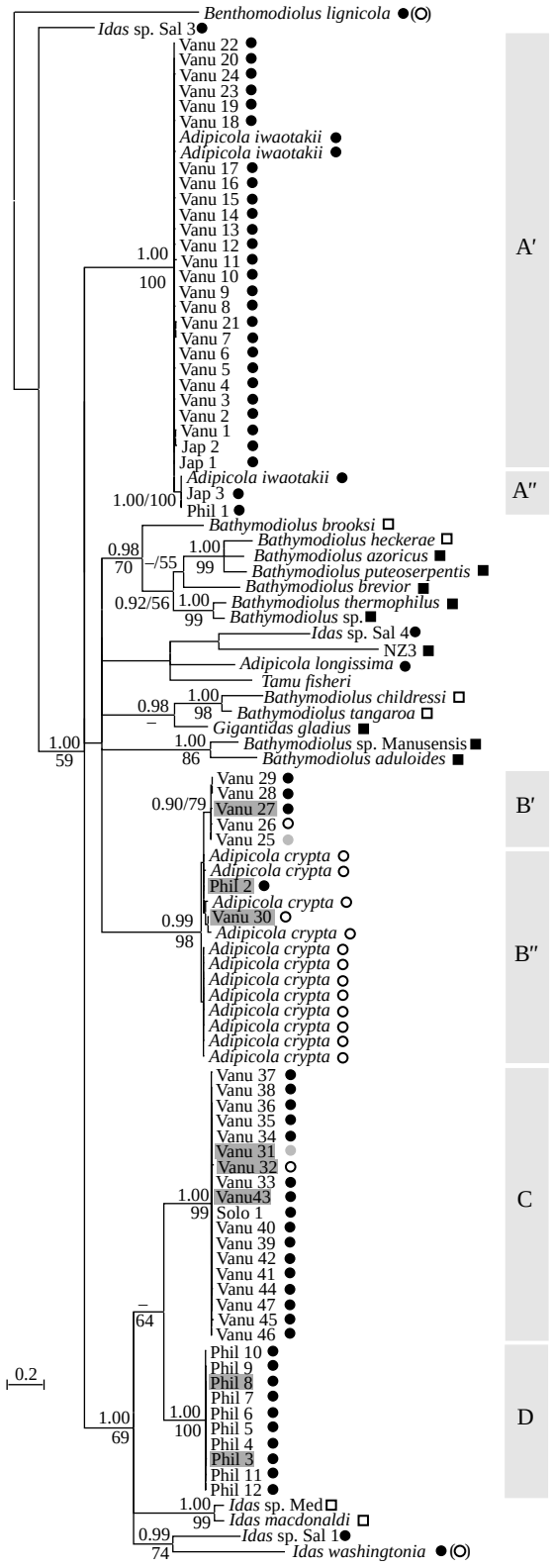

Figure 1. Bayesian tree obtained from the analysis of the COI mtDNA dataset. The substrate from which each specimen was collected is given. Substrate labels within brackets correspond to previous records without molecular support. Labels of newly sequenced specimens are in bold and those used to study symbiotic associations are highlighted in grey. Substitution model selected from MrAIC: GTR+[Γ]+I. 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 10% estimated base substitution. Filled square, vent; open square, seep; grey-filled circle, turtle bone; open circle, whale bone; black-filled circle, wood.

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 2*b*; electronic supplementary materials 6 and 7). All but one specimen displayed bacteria with dark inclusions possibly corresponding to β-polyhydroxybutyrate reserves (figure 2*b*; electronic supplementary material 7). No sulphur-containing granule was seen.

In specimens from cluster C, bacteria occurred between microvillosities located outside of gill epithelial cells, exposed to circulating seawater (figure 3*a–d*). The thickness of the bacterial layer outside host cells varied considerably between specimens, ranging from 2 to 20 µm (figure 3*c*,*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 3*d*). 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.* (2008*b*).

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 2*a*,*c*,*d*; electronic supplementary material 6). 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 2*d*). Amounts of bacteria present in host cells varied from few (figure 2*a*; electronic supplementary material 8) to many (figure 2*c*,*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.* (2008), 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.

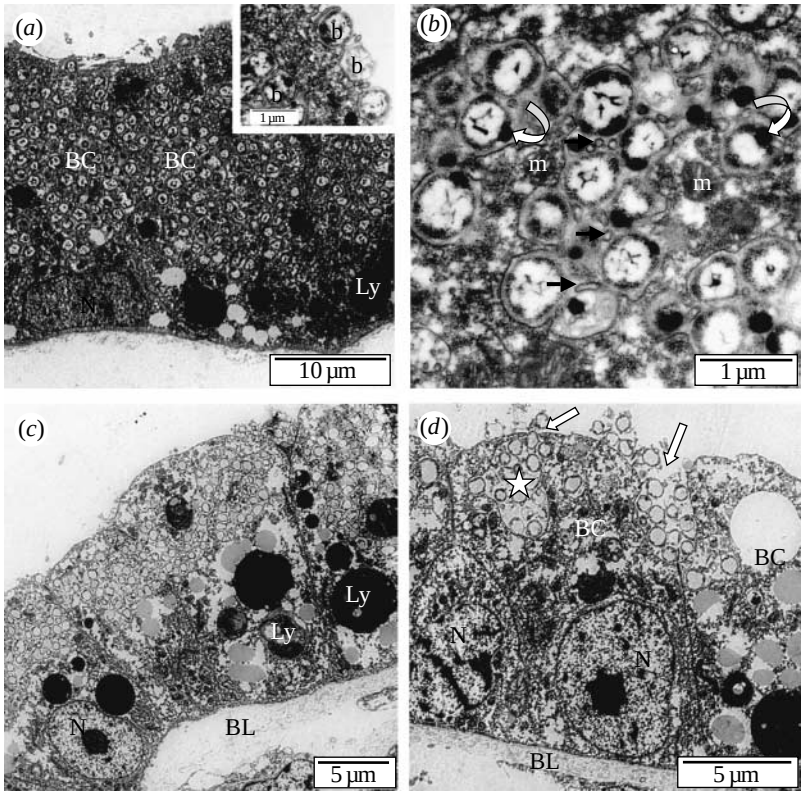

Figure 2. TEM images of gill filaments and epithelial cells of *A. crypta* colonizing various substrates. (*a*,*b*) Gill filaments of the specimen from whale bones. Bacteriocyte (BC) cytoplasm is filled with intracellular bacterial symbionts and secondary lysosome-like particles (Ly). (*a*) Very few 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.

Specimens of sub-cluster B″ harboured a single phylotype 2, while one specimen of sub-cluster B′ displayed 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 polymorphism, allometric growth and the occurrence of cryptic species (Horikoshi & Tsuchida 1984; Won *et al.* 2003*c*; 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 small bathymodiolins inhabiting organic falls. These were referred to as A, B, C and D herein, two of which were subdivided into two sub-clusters.

Cluster C, sampled both on wood and bones, and D, sampled only on woods, differed by a K2P genetic distance of 8.9 per cent. Such a genetic distance is higher than the 4.4 per cent corresponding to the most recent speciation event known between sister species in Bathymodiolinae (Won *et al.* 2003*a*). Moreover C and D form two monophyletic lineages, based on COI mtDNA, which occur in distinct geographical areas, Solomon/Vanuatu and Philippines. A slight polymorphism is observed between specimens of C and D on the 28S rRNA gene fragment, but the two lineages are not completely resolved since some specimens of D share the two 28S alleles. This result suggests either secondary contact and hybridization between the two lineages, as previously shown between two species from the Mid-Atlantic Ridge (O'Mullan *et al.* 2001; Won *et al.* 2003*b*), or an incomplete lineage sorting of ancestral polymorphism. The second hypothesis appears reasonable, given that the 28S rRNA evolves more slowly and has a larger effective size than the COI mtDNA. Thus the pattern is consistent with an allopatric speciation model. Furthermore the combined phylogenetic tree supports that C and D are sister species

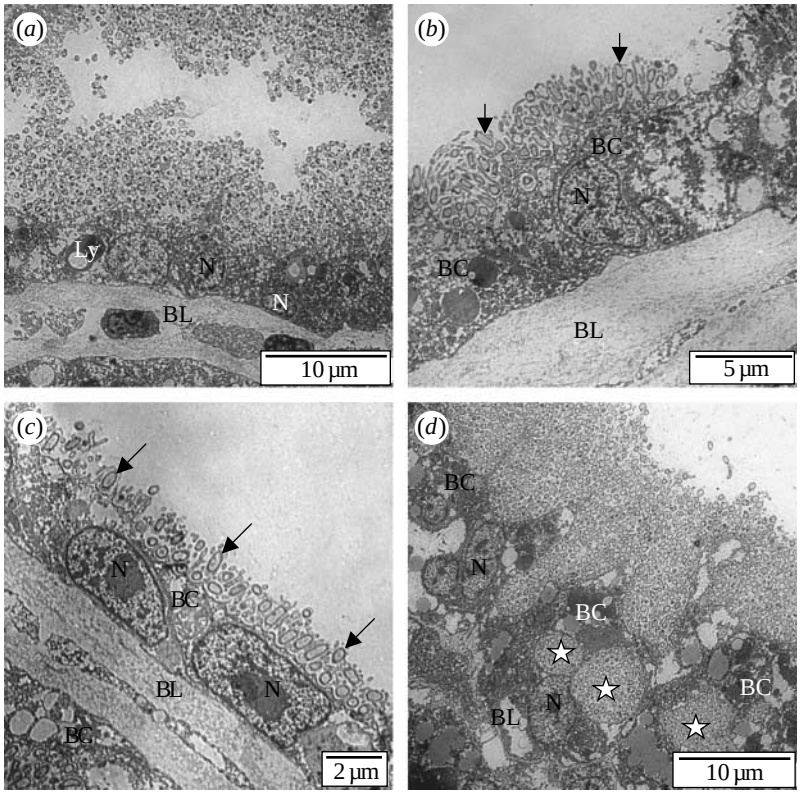

Figure 3. TEM images of gill filaments and epithelial cells of lineage C colonizing various substrates. (*a*) Gill filaments from an individual collected on whale bone (Vanu 32) at 441 m depth. The thickness of the bacterial layer is greater than the bacteriocyte's thickness. Bacteria, which are located 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.

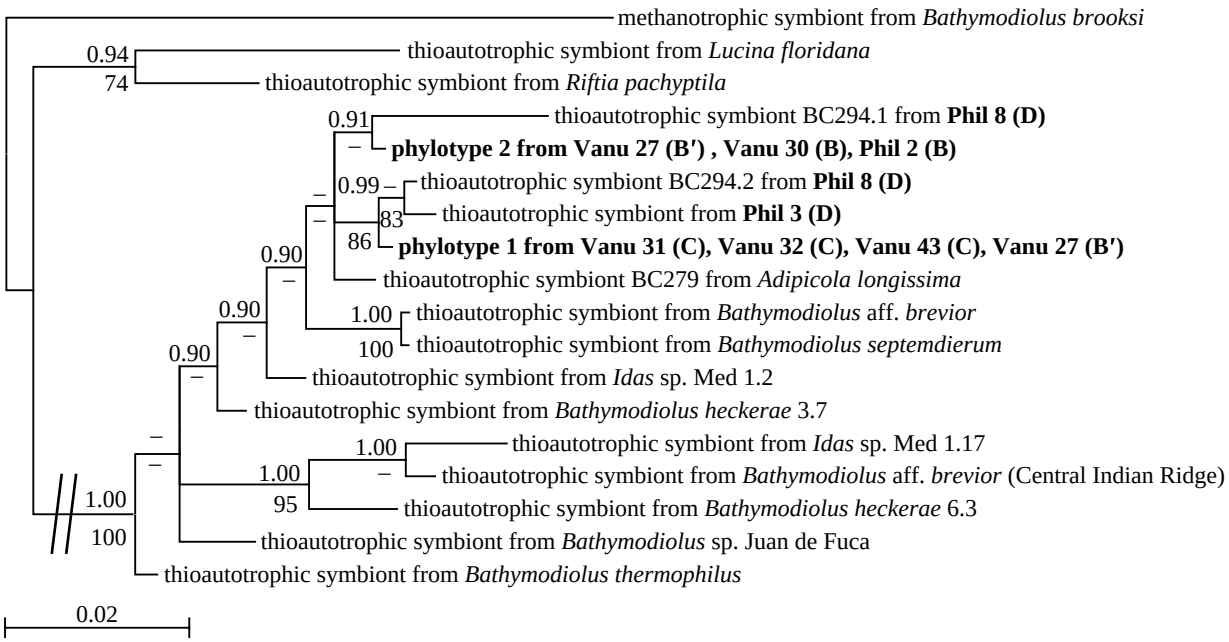

Figure 4. Bayesian tree obtained from the analysis of the 16S rRNA dataset. Sequences obtained in this study are in bold. Substitution model selected from MrAIC: GTR + [Γ] + I. Posterior probabilities (PP) and bootstraps values obtained from ML analysis are given above and below nodes, respectively. 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.* 2003*c*; 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. iwaotakii* from GENBANK. Owing to this and the absence of morphological differences between A′ and A″, we suggest that the species name *A. iwaotakii* 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 bathymodiolin 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′-phosphosulphate 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.* 2008*b*). 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.* 2008*b*). Conversely, analysed specimens of the *A. crypta* species complex always display intracellular bacteria located within gill bacteriocytes. Bacteriocyte 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 brevior* and *Bathymodiolus septemdierum* (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

and also in a specimen from the *A. crypta* species complex (Duperron *et al.* 2008*b*). 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 phylotypes 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.* 2003*a*).

(d) *Some insights into the ability of a host to adapt to different substrates*

Bacterial densities are variable among specimens of the *A. crypta* species complex and *Idas* sp. C, and bacteria are generally more abundant in specimens from whale bones than in specimens from sunken wood. Densities of thioautotrophic symbionts in vent-inhabiting Bathymodiolinae increase quickly in the presence of H_2S (Halary *et al.* 2008). Thus, the differences in bacterial densities observed between specimens on organic falls could correspond to different amounts of H_2S available. H_2S availability varies depending on the amount, nature and degradation state of the organic material. Although direct *in situ* chemical data are not yet available, the degradation of lipid-rich whale bones could provide higher H_2S amounts than the degradation of wood (Smith 2005). The capacity of mussels to inhabit both plant debris, either cellulose- or lignin-rich, and lipid-rich or lipid-poor bones probably lies in the flexibility of bacterial abundances. This would allow mussels to rely more on either chemoautotrophy by their bacteria or filter-feeding, depending on resource availability (Page *et al.* 1991).

5. CONCLUSION

The 'wooden step to deep-sea vents' scenario assumes the existence of mussel species able to live in more than one type of reducing deep-sea environment, which would have played a hinge role in the colonization of the various deep-sea chemosynthesis-based habitats. In this study, we identified at least two species able to live on various organic substrates. Based on these ubiquitous species and on the documented functional diversity of symbionts associated with *Idas*-like mussels from seeps (Duperron *et al.* 2008*a*,*b*), we predict that some mussel species are, or were, able to live associated with both organic substrates and vents or seeps. Allopatric patterns of speciation observed in this study may represent an actual example of a type of process that would initiate the divergence between two species descending from a ubiquitous lineage. Specialization to seep or vent habitats could then result from natural selection.

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