RESEARCH ARTICLE

Lucinidae/sulfur-oxidizing bacteria: ancestral heritage or opportunistic association? Further insights from the Bohol Sea (the Philippines)

Terry Brissac[1], Hervé Merçot[1] & Olivier Gros[2]

[1]UMR 7138 UPMC-CNRS-MNHN-IRD 'Systématique, Adaptation, Évolution', Equipe: 'Génétique & Evolution', Université Pierre et Marie Curie, Paris, France; and [2]UMR 7138 UPMC-CNRS-MNHN-IRD 'Systématique, Adaptation, Évolution', Equipe: 'Biologie de la mangrove', Département de Biologie, Université des Antilles et de la Guyane, Pointe-à-Pitre, Guadeloupe, France

Correspondence: Present address: Terry Brissac, INSERM U1002 'Pathogeny of Systemic Infections', Faculté de Medecine, Necker – Enfants malades, 156 rue de Vaugirard, 75015, Paris, France. Tel.: +33 1 40 61 53 74; fax: +33 1 40 61 56 77; e-mail: terry.brissac@inserm.fr

Received 4 January 2010; revised 23 August 2010; accepted 3 October 2010.
Final version published online 22 November 2010.

DOI:10.1111/j.1574-6941.2010.00989.x

Editor: Patricia Sobecky

Keywords
Bivalvia; comparative molecular phylogeny; energy-filtered transmission electron microscopy analysis; electron energy loss spectroscopy; hemoglobin; symbiosis.

Abstract

The first studies of the 16S rRNA gene diversity of the bacterial symbionts found in lucinid clams did not clarify how symbiotic associations had evolved in this group. Indeed, although species-specific associations deriving from a putative ancestral symbiotic association have been described (coevolution scenario), associations between the same bacterial species and various host species (opportunistic scenario) have also been described. Here, we carried out a comparative molecular analysis of hosts, based on 18S and 28S rRNA gene sequences, and of symbionts, based on 16S rRNA gene sequences, to determine as to which evolutionary scenario led to modern lucinid/symbiont associations. For all sequences analyzed, we found only three bacterial symbiont species, two of which are harbored by lucinids colonizing mangrove swamps. The last symbiont is the most common and was found to be independent of biotope or depth. Another interesting feature is the similarity of ctenidial organization of lucinids from the Philippines to those described previously, with the exception that two bacterial morphotypes were observed in two different species (*Gloverina rectangularis* and *Myrtea flabelliformis*). Thus, there is apparently no specific association between *Lucinidae* and their symbionts, the association taking place according to which bacterial species is present in the environment.

Introduction

Associations between chemoautotrophic bacteria and marine invertebrates were discovered in hydrothermal vents by Felbeck *et al.* (1981). Later, they were described in shallow water sediments (Berg & Alatalo, 1984; Schweimanns & Felbeck, 1985), cold seeps (Juniper & Sibuet, 1987), whale bones (Deming *et al.*, 1997) and sunken woods (Gros & Gaill, 2007; Duperron *et al.*, 2008). This type of association is present in several host taxa and involves several bacterial clades with different kinds of metabolisms (Dubilier *et al.*, 2008). One of these associations includes the *Lucinidae* (*Bivalvia*), the most diverse and very common bivalve family that lives in a large variety of marine habitats and is widespread (Taylor & Glover, 2006). To date, all lucinid genera studied harbor sulfur-oxidizing symbionts that are *Gammaproteobacteria* according to 16S rRNA gene sequences. They are typically located in bacteriocytes, specialized cells distributed throughout the lateral zone of gill filaments (Fisher, 1990).

Within the *Lucinidae*, the capacity to establish symbiotic relationships appears to be ancestral: symbiosis may have been acquired before the divergence of the Lucinoidea superfamily, which is composed of *Lucinidae* and *Fimbriidae* (Taylor & Glover, 2006). This ancestral capacity is reinforced by recent and fossil records of lucinid, which attest to symbiosis as an ancient way of life (Taylor & Glover, 2006). Such symbiotic associations led Reid (1990) to consider that the onset of symbiosis was the most important factor in the subsequent evolution of the Lucinacea. Nevertheless, the origins and evolutionary histories of current symbiotic partnerships remain obscure and ambiguous (Distel *et al.*, 1994; Durand & Gros, 1996). In fact, both partners may or may not have coevolved from an ancestral symbiotic couple.

FEMS MICROBIOLOGY ECOLOGY

One way to distinguish between these alternatives is a comparison of the molecular phylogenies of hosts and symbionts. In the case of coevolution, the two partners must share a strong common evolutionary history and the two phylogenies must be congruent (Peek *et al.*, 1998a).

Separate molecular data on *Lucinidae* or their symbionts have already been published, but sequences of hosts and symbionts have not yet been compared directly. Molecular data of the hosts have been used to investigate the relationships among Lucinoidea (Williams *et al.*, 2003). 16S rRNA gene sequences of symbionts were used to analyze the diversity of such symbioses (Distel *et al.*, 1988, 1994; Durand & Gros, 1996; Durand *et al.*, 1996). Distel *et al.* (1988, 1994) showed specifically that each host species was associated with a unique bacterial ribotype. Subsequently, Durand & Gros (1996), Durand *et al.* (1996) and Gros *et al.* (2000, 2003) observed six host species sharing the same symbiont (100% of identity on 16S rRNA gene sequences). Thus, although the first observations suggested unique species-specific associations due to coevolution between lucinid clams and their symbionts, more recent studies do not support this hypothesis. However, in these studies, the molecular diversity of symbionts was compared with host morphological taxonomy and not with host molecular phylogeny. Moreover, sampling strategies were very different: Distel *et al.* (1988, 1994) studied specimens from various ecosystems and geographic localizations and their sampling was more heterogeneous than that of Durand & Gros (1996), Durand *et al.* (1996) or Gros *et al.* (2000, 2003), who studied lucinid specimens from *Thalassia testudinum* sea-grass beds of the Lesser Antilles.

In the present paper, we use both original molecular data on *Lucinidae* from the Philippines and their bacterial symbionts as well as published data of various geographic origins, to study the evolutionary history of present-day symbiotic couples and to determine the true scale of the diversity of bacterial species associated with *Lucinidae*. We compare the molecular phylogenies of diverse *Lucinidae* hosts (using 18S rRNA and 28S rRNA genes) and their associated symbionts [using 16S rRNA and APS (adenosine 5′-phosphate) reductase (*aprA*) gene sequences]. In addition, we conduct ultrastructural (gills and symbionts) and cytochemical (occurrence of hemoglobin) analyses of our samples from the Philippines.

Materials and methods

Sampling

Philippine bivalves (Table 1) were collected during the PANGLAO cruise in May 2005 (chief scientist: P. Bouchet) in the Bohol Sea (8.3–9.6°N, 123–124°E) using a beam trawl at depths of 219–1775 m. Species determination was performed according to their morphological characteristics

Table 1. Sampling of Philippine lucinids during the PANGLAO 2005 cruise

Specimen code	Species*	Sampling site	Location	Station	Depth (m)	Accession numbers†			
						16S rRNA gene	APS reductase	18S rRNA gene	28S rRNA gene
OG 18	*Myrtea tanimbarensis* (Von Cosel & Bouchet, 2008)	Bohol Sea Off Balicasag Island	9°29.4′N 123°43.7′E	CP 2340	271–318	FN869533	FN869534	–	FN869535
OG 57	*Dulcina* sp. (Von Cosel & Bouchet, 2008)	Bohol Sea	9°24.3′N 124°10.7′E	CP 2355	1764–1775	FN869536	FN869537	FN869538	FN869539
OG 78	Undetermined genus and species	Bohol/Sulu seas sill	8°49.9′N 123°37.6′E	CP 2360	357–372	FN869549	FN869550	FN869551	FN869552
OG 101	*Cardiolucina quadrata* (Prashad, 1932)	Bohol Sea	9°26.9′N 123°34.5′E	CP 2388	762–786	FN869540	FN869541	FN869542	FN869543
OG 103	*Myrtea fiabelliformis* (Prashad, 1932)	Bohol Sea	9°26.9′N 123°34.5′E	CP 2388	762–786	FN869544	–	–	–
OG 108‡	*Gloverina rectangularis* (Von Cosel & Bouchet, 2008)	Bohol Sea	9°27.4′N 123°43.1′E	CP 2390	627–645	FN869545	FN869546	FN869547	FN869548

*Species were identified by John D. Taylor (NHM, UK) based on morphological characteristics.
†Because of poor tissue preservation, some sequences could not be obtained for some specimens.
‡Microscopy analysis could not be performed for this specimen.

(Von Cosel & Bouchet, 2008). Thus, *Cardiolucina quadrata*, *Gloverina rectangularis*, *Myrtea flabelliformis* and *Myrtea tanimbarensis* were identified even if two specimens remained uncertain (Fig. 1). Samples were processed on the ship within 1 h of collection. The gills of each lucinid specimen were dissected and treated according to the following protocol: the first gill piece was stored in 100% ethanol for the molecular investigation of bacterial diversity and host phylogeny. The second gill piece was prepared for electron microscopy. In addition, *Codakia orbicularis* and *Phacoides pectinatus* (also called *Lucina pectinata*) were sampled by hand, respectively, in *T. testudinum* sea-grass beds of Ilet Cochon (Guadeloupe, French West Indies) and mangrove swamps of La manche à eau (Guadeloupe, French West Indies) in 2006.

Transmission electron microscopy (TEM)

Gills were prefixed onboard for 1 h at 4 °C in 2.5% glutaraldehyde in 0.1 M pH 7.2 cacodylate buffer and adjusted to 900 mOsM with NaCl and $CaCl_2$ to improve membrane preservation. Gills were briefly rinsed in the same buffer and stored in the same buffer at 4 °C until they were brought to the laboratory after 2–3 weeks. Samples were fixed for 45 min at room temperature in 1% osmium tetroxide in the same buffer, then rinsed in distilled water and postfixed with 2% aqueous uranyl acetate for 1 h before embedding, sectioning (60 nm thick) and observation using a Leo-912 microscope at 80 kV.

Energy-filtered TEM (EFTEM) analysis: parallel electron energy loss spectroscopy (PEELS) and electron spectroscopic imaging (ESI) procedures

The EFTEM observations were performed using a LEO 912 Omega transmission electron microscope (LEO Electron Optics GmbH, Oberkochen, Germany) at 120 kV. Acquisition was accomplished using the ESIVISION program (version 3.0, Soft-Imaging Software, SIS, GmbH, Münster, Germany). For PEELS acquisition, the primary magnification was set to a small area delimited by the entrance aperture of the spectrometer. We used the three-window method for ESI acquisition and to minimize the radiation damage (Jeanguillaume *et al.*, 1978; Reimer *et al.*, 1991), as described in Lechaire *et al.* (2008).

DNA template preparation

Template DNA for PCR amplifications was extracted from symbiont-containing gills of each bivalve host according to the cetyltrimethyl ammonium bromide method (Ishaq *et al.*, 1990). The DNA concentration was determined using spectrometry (BioPhotometer, Eppendorf, Germany) and on a 1% agarose gel in comparison with concentration markers (Smart Ladder, Eurogentec).

PCR amplifications

Bacterial (16S rRNA and APS reductase) or host (18S rRNA and 28S rRNA) genes were amplified from a host–symbiont DNA mixture using the primers listed in Table 2.

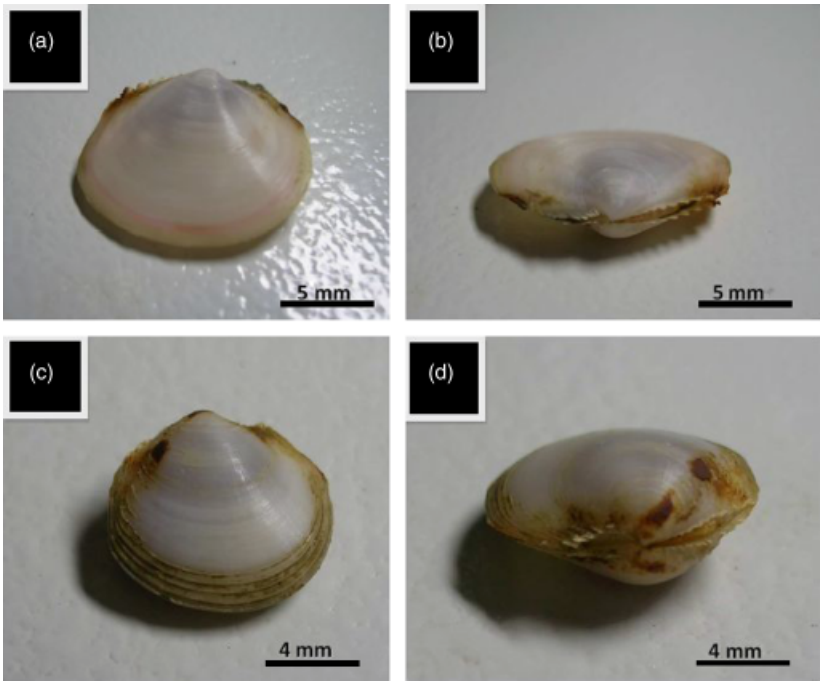


Fig. 1. Shell characters of the two undescribed lucinids of this study collected during PANGLAO cruise. (a, b) Living individual of specimen OG57 belonging to the genus *Dulcina*. The ventral margin of the quite smooth valves appears pink. Cardinal and lateral hinge teeth are well defined. (c, d) Living individual of the specimen OG78. This shell has small, but well-defined hinge teeth (cardinals and laterals) in both the left and the right valves. Concentric growth lamellae parallel to the margin are present on both valves. (a, c) Right valve; (b, d) laterodorsal view.

Table 2. Primers and PCR conditions

Sequence (5′ → 3′)	Temperature (°C)	Name	Target	Reference
Symbiont				
AGAGTTTGATCATGGCTCAG	54	16S_UNIV-8f	16S rRNA gene	Lane (1991)
TACGGCTACCTTGTTACGACT		16S_UNIV-1492r		
TGGCAGATMATGATYMACGGG	58	APS-FW	APS reductase	Deplancke *et al*. (2000)
GGGCCGTAACCGTCCTTGAA		APS-RV		
Host				
CCGTCTTGAAACACGGACCAAG	52	LSU-900f	28S rRNA gene (large subunit)	Olson *et al*. (2003)
AGCGCCATCCATTTTCAGG		LSU-1600r		Williams *et al*. (2003)
CTGGTTGATYCTGCCAGT	54	18S-5′F	18S rRNA gene	Winnepenninckx *et al*. (1998)
CTTCGAACCTCTGACTTTGG		18S-1100R		Williams *et al*. (2003)

Amplification was performed in 50 μL using 25–50 ng of DNA template, 5 μL of 10 × Taq buffer (Invitrogen), 3 mM $MgCl_2$ (Invitrogen), 0.4 mM of each dNTP, 1 mM of each primer and 1 U of recombinant Taq DNA polymerase (Invitrogen). Target DNA was amplified in 30 cycles [94 °C for 1 min, optimal temperature for 1 min (Table 2), 72 °C for 1 min (1 min 30 s for 16S rRNA gene)], with an initial denaturation step at 94 °C for 10 min and a final elongation step at 72 °C for 10 min. PCR products were controlled on a 1% agarose gel and directly sequenced using an ABI Prism automated sequencer (GATC-biotech, http://www.gatc-biotech.fr).

Phylogenetic analyses

Sequences were controlled by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned with CLUSTALW (Thompson *et al*., 1994) with default parameters. The alignment was then checked visually and corrected if necessary. Gap positions and ambiguous sites of the alignment were removed using GBLOCKS 0.91b (Castresana, 2000). Alignment was then imported in TOPALI v2.5 software (Milne *et al*., 2004) to define the substitution model to be used for each dataset, using MODELGENERATOR (Keane *et al*., 2006). The best model for each dataset was chosen using the Bayesian information criterion (Schwarz, 1978). Phylogenetic analyses were then performed using TOPALI v2.5. Trees were chosen by maximum likelihood (ML) using PHYML (Guindon & Gascuel, 2003) and by Bayesian inference (BI) using MRBAYES 3 (Ronquist & Huelsenbeck, 2003). Support for individual clades was evaluated for each node using nonparametric bootstrapping (Felsenstein, 1985) from 100 replicates for MLPosterior probabilities for BI.

Results

Symbiont phylogeny

All PCR amplifications of 16S rRNA gene or *aprA* sequences yielded fragments of the expected size (∼1500 and ∼300 bp, respectively). Direct PCR product sequencing and chromatograph analysis show only one single detectable sequence per amplification. Phylogenetic trees of symbionts (Figs 2 and 3) were obtained using 1263 sites for 16S rRNA gene and 273 sites for *aprA*. For the 16S rRNA gene (Fig. 2), two free-living marine *Gammaproteobacteria* were used as an outgroup. This tree aligns the symbiont sequences of Philippine clams in a monophyletic clade that corresponds to 'Lucinoidea symbionts'. We observed that three different bacterial species (16S rRNA gene identity < 97%) occur within *Lucinidae* (Fig. 2, clades A, B and C). According to this tree and based on a pairwise identity matrix (Table 3), all symbionts analyzed here belong to the same bacterial species (Fig. 2, clade A) with a 16S rRNA gene identity > 97% (97.7%). Moreover, within this defined species, we can distinguish two different bacterial types harbored by the Philippine clams. The first type (Fig. 2, clade A, group I) is formed by symbionts of *M. tanimbarensis*, OG78 and *G. rectangularis*, with a mean 16S rRNA gene sequence identity of 99.9%, and the second type (Fig. 2, clade A, group II) includes symbionts of *Dulcina* sp., *C. quadrata* and *M. flabelliformis* (98.6% mean identity). Thus, the six Philippine lucinid clams appear to share two different variants of the same bacterial species. The sulfur-oxidizing metabolism of these symbionts is confirmed by the detection of the gene for APS reductase (*aprA*) by PCR. According to the phylogenetic tree based on the sequences of this gene (Fig. 3), these symbiont sequences fall in the lineage II of *aprA* in the sulfur-oxidizing bacteria clade. Unfortunately, because of poor tissue preservation, it was not possible to obtain the *aprA* sequence from the specimen of *M. flabelliformis*.

Host phylogeny

In all amplifications, only one fragment of the expected size was obtained for 18S rRNA and 28S rRNA genes (∼1000 and ∼700 bp, respectively). These fragments yielded unique sequences in each sample analyzed. Thyasiroidea and Crassatelloidea, which are sister-groups of *Lucinidae*, were used

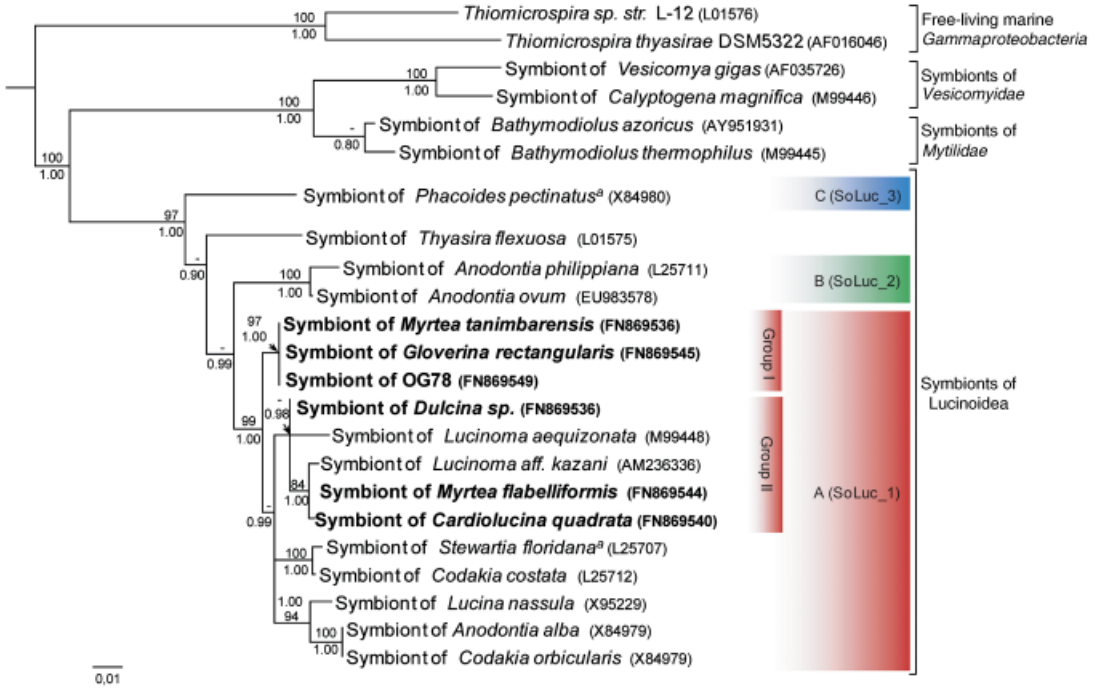


Fig. 2. Symbiont phylogenetic tree based on 16S rRNA gene sequences (1263 bp). The tree was constructed using ML and BI under K80+I+Γ evolutionary models. The robustness of clades was evaluated and only bootstrap values [ML, 100 replicates (above branches)] and posterior probabilities [BI (under branches)] up to 80% were displayed. Data for taxa in bold are from this study. [a]Also called *Lucina pectinata* (*Phacoides pectinatus*) and *Lucina floridana* (*Stewartia floridana*).

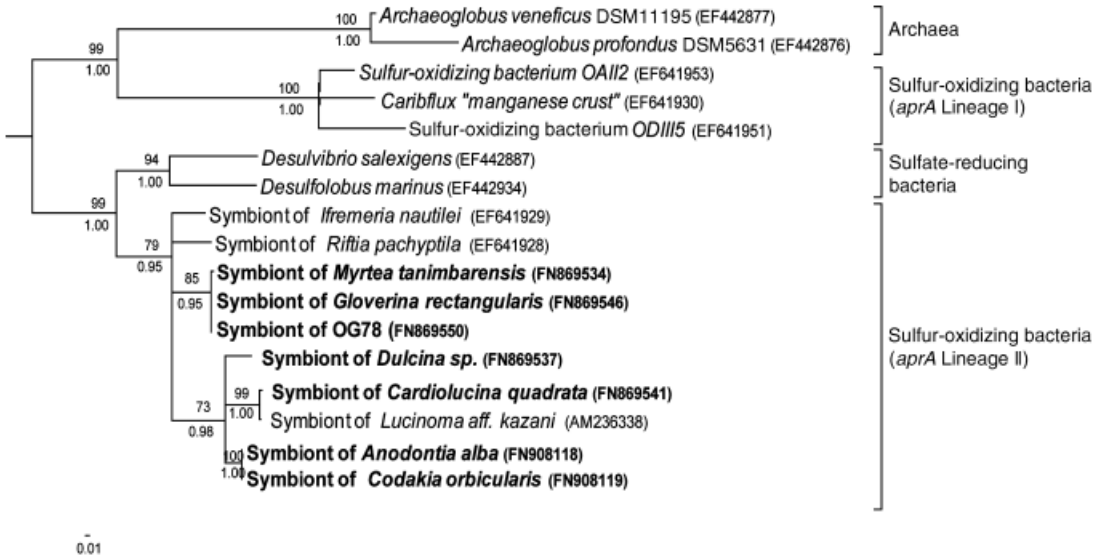


Fig. 3. Symbiont phylogenetic tree based on *aprA* sequences (273 bp). The tree was constructed using ML and BI under TrNef+Γ (ML) and K80+Γ (BI) evolutionary models. The robustness of clades was evaluated and only bootstrap values [ML, 100 replicates (above branches)] and posterior probabilities [BI (under branches)] up to 70% were displayed. Data for taxa in bold are from this study.

as the outgroup. The host phylogeny built using concatenated sequences of these two genes (1252 sites, Fig. 4) aligns these samples in the *Lucinidae* clades described by Williams *et al.* (2003). Except for clade A, the samples analyzed here were distributed in all of the previously described clades. Specifically, *G. rectangularis* belongs to the *Myrtea* clade, *C. quadrata* belongs to clade B, and OG78 and *Dulcina* sp. belong to the *Anodontia* clade. The 18S rRNA gene for

Table 3. Identity matrix for the symbiont 16S rRNA gene (pairwise deletion)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1: *Codakia orbicularis* symbiont	ID																
2: *Anodontia alba* symbiont	1.000	ID															
3: *Lucina nassula* symbiont	0.984	0.984	ID														
4: *Codakia costata* symbiont	0.975	0.975	0.979	ID													
5: *Stewartia floridana** symbiont	0.971	0.971	0.981	0.996	ID												
6: *Dulcina* sp. symbiont	0.966	0.966	0.971	0.976	0.978	ID											
7: *Cardiolucina quadrata* symbiont	0.970	0.970	0.973	0.982	0.980	0.985	ID										
8: *Myrtea flabelliformis* symbiont	0.970	0.970	0.973	0.978	0.978	0.980	0.993	ID									
9: *Lucinoma aff. kazani* symbiont	0.969	0.969	0.973	0.981	0.978	0.982	0.994	0.990	ID								
10: *Lucinoma aequizonata* symbiont	0.960	0.960	0.963	0.968	0.966	0.981	0.976	0.973	0.974	ID							
11: *Myrtea tanimbarensis* symbiont	0.977	0.977	0.978	0.979	0.976	0.988	0.981	0.984	0.977	0.969	ID						
12: OG78 symbiont	0.971	0.971	0.972	0.973	0.972	0.985	0.978	0.978	0.973	0.968	0.999	ID					
13: *Gloverina rectangularis* symbiont	0.969	0.969	0.970	0.971	0.972	0.986	0.976	0.976	0.971	0.969	0.999	0.999	ID				
14: *Anodontia ovum* symbiont	0.951	0.951	0.959	0.957	0.959	0.952	0.953	0.952	0.951	0.944	0.960	0.955	0.953	ID			
15: *Anodontia philippiana* symbiont	0.947	0.947	0.955	0.949	0.951	0.946	0.947	0.946	0.946	0.938	0.954	0.949	0.946	0.990	ID		
16: *Thyasira flexuosa* symbiont	0.942	0.942	0.944	0.945	0.942	0.941	0.941	0.939	0.940	0.933	0.950	0.944	0.942	0.947	0.945	ID	
17: *Phacoides pectinatus** symbiont	0.947	0.947	0.949	0.941	0.939	0.933	0.933	0.932	0.933	0.926	0.941	0.936	0.934	0.945	0.943	0.941	ID

*Also called *Lucina floridana* (5) and *Lucina pectinata* (17). ID, identical.

M. tanimbarensis was not obtained; however, the 28S rRNA gene sequence of this specimen falls in clade B of a 28S rRNA gene tree (data not shown). No sequences were obtained for *M. flabelliformis*. The sequences obtained here for *C. orbicularis* and *P. pectinatus* correspond to those published by Williams *et al.* (2003).

Comparison of host/symbiont phylogenies

Host and symbiont phylogenies appear to be totally incongruent, the topologies (i.e. branching order) and time of splitting events (i.e. branch length) being different. Thus, close relative host species do not harbor any close relative (or the same) bacterial variant (e.g. OG78 and *Dulcina* sp.). However, *C. quadrata* and *Dulcina* sp., which are different host species, appear to harbor the same bacterial type. More interestingly, the three host species that harbor the same bacterial strain (*M. tanimbarensis*, OG78 and *G. rectangularis*) belong to three different host lineages (clade B, *Anodontia* and *Myrtea*, respectively). Thus, no species-specific host/symbiont association was found within the *Lucinidae*.

Special features of ctenidial cells of the Philippine lucinids

All analyzed individuals presented ctenidial characteristics that are common within *Lucinidae*, as described by Frenkiel & Mouëza (1995), especially concerning the ciliated zone (Figs 5a, 6a and 7a). However, two individuals displayed some special features. In *M. tanimbarensis*, bacteriocytes (Fig. 6a and b) contained numerous dark granules of an unknown nature inside the cytoplasm. In *M. flabelliformis*, the deep-red color of the gills associated with the dark aspect of the cytoplasm with finely granular electron-dense areas suggests that intracellular hemoglobin was present inside the bacteriocytes (Fig. 5a and b). This was confirmed by PEELS analysis, in which iron was detected in the cytoplasm of each bacteriocyte tested (Fig. 5d), with a specific iron spectrum (edge close to 730 eV) obtained after background subtraction (Fig. 5e). The ESI method was used to locate iron in the bacteriocyte cytoplasm. When the iron-distribution image was superimposed onto the high-contrast image recorded at 250 eV (Mixmap image, Fig. 5d), iron was observed localized homogeneously inside the cytoplasm of the bacteriocyte, whereas bacteria remained free of iron (Fig. 5d). The presence of sulfur was tested using the PEELS method in the same area and no significant signal was obtained (data not shown).

Finally, the lateral zone of *M. tanimbarensis* collected from Balicasag Island contained numerous granule cells (Fig. 6c) similar to those described in several tropical lucinids belonging to the genera *Codakia* and *Divaricella* (Frenkiel & Mouëza, 1995; Gros *et al.*, 2000).

Fig. 4. Host phylogenetic tree based on concatenation of 18S rRNA and 28S rRNA gene sequences (1252 bp). The tree was reconstructed using ML and BI under TrNef+Γ (ML) and SYM+Γ (BI) evolutionary models. The robustness of clades was evaluated and only bootstrap values [ML, 100 replicates [above branches)] and posterior probabilities [BI (under branches)] up to 80% were displayed. Data for taxa in bold are from this study. All other sequences originate from Williams *et al*. (2003). [a]Sequences obtained for these two species are identical to those published by Williams *et al*. (2003). [b]Also called *Lucina pectinata*.

Ultrastructure of the gill endosymbionts

Bacteria are individually enclosed within vacuoles (Figs 5b, f, h, 6b and 7b). These endosymbionts are rod-shaped, with the characteristic double membrane of gram-negative bacteria belonging to the *Gammaproteobacteria* (Figs 5c, 6d and 7c, d). The bacteria in *Dulcina* sp. and *C. quadrata* (Fig. 5f–h) are large (4 μm long × 1.5 μm wide), in *M. tanimbarensis* (Fig. 6d) they are longer and thinner (6 μm long × 0.5 μm wide) and in OG78 and *M. flabelliformis* (Fig. 7c and d) both size categories are found. Some ovoid-shaped figures can be observed, but these are probably due to the orientation of the section. Symbionts are generally abundant in bacteriocytes, with the exception of *M. flabelliformis*. Bacterial cytoplasm contains numerous non-membrane-bound irregular inclusions (up to 50 nm in diameter) appearing as black dots in TEM sections, which can be considered storage granules (Fig. 7c and d), either glycogen-like granules or polyphosphates according to the high amounts of phosphorus detected in electron-dense granules of the bacterial symbionts of *Lucinoma aequizonata* using Electron Diffraction X-Ray (EDX) analysis (Arndt-Sullivan *et al*., 2008). Electron-lucent granules (sulfur granules) are not obvious in these gill endosymbionts, in contrast to bacterial symbionts encountered in the gills of tropical shallow water lucinids such as *C. orbicularis* (Lechaire *et al*., 2008).

Discussion

To date, host and symbiont diversity within *Lucinidae* has been investigated in several studies, which have drawn opposing conclusions. Distel *et al*. (1988, 1994) concluded that species-specific associations were due to coevolution of the two partners. Later, the association of six different host species with the same bacterial symbiont was described, attesting to an association that is dependent on the bacteria present in the local environment (Durand & Gros, 1996; Durand *et al*., 1996; Gros *et al*., 2000, 2003). However, these studies did not compare the molecular phylogeny of hosts with that of symbionts. In the present paper, we compare host and symbiont molecular phylogenies to gain a better understanding of the specificity (or lack thereof) of *Lucinidae*/symbiont associations.

Evolution of host/symbiont relationships

We analyzed host diversity using 18S rRNA and 28S rRNA gene sequences. Specimens from the Philippines analyzed here belong to the *Lucinidae* monophyletic group (Fig. 4) as described in Williams *et al*. (2003). As in all lucinids described to date, Philippine clams harbor symbiotic bacteria in their gills. According to 16S rRNA gene phylogeny, these symbionts belong to a well-supported monophyletic clade formed by already described lucinid symbionts (Fig. 2). Moreover, PCR detection in our specimens of the APS reductase gene attests to the fact that these symbionts possess a sulfur-oxidizing pathway even if sulfur or sulfur granules are not detected on gill sections by microscopic analyses. The symbionts analyzed in this study all belong to the same bacterial species (Fig. 2, clade A) based

on the sequence identity matrix showing a > 97% mean 16S rRNA gene sequence identity in this clade. Thus, as described in Durand & Gros (1996), different host species can harbor the same bacteria. Our results refute the hypothesis that *Lucinidae* host/symbiont associations are species specific due to coevolution of the two partners. It appears to us that the identity of the bacterial symbiont in lucinid is dependent on the bacterial species present in the local environment. Therefore, the specificity of this association described by Distel *et al.* (1988, 1994) could be an artifact due to widely varying ecosystems and geographic locations in their study.

What could then be the reason that within geographically diverse lucinids, identical bacterial species are harbored? The Philippine specimens investigated here come from different locations in relation to the species with which they are compared (*C. orbicularis*, *L. aequizonata* or *Lucinoma aff. kazanii*, *Stewartia floridana*). These latter lucinids were collected from the Mediterranean Sea (*L. aff. kazanii*, Duperron *et al.*, 2007), Florida (*S. floridana*, Distel *et al.*, 1994), California (*L. aequizonata*, Distel *et al.*, 1994) and the Caribbean (*C. orbicularis*, Durand & Gros, 1996), respectively. Two hypotheses could explain the low level of genetic diversity we found among the symbionts: (1) the geographic divergence of symbiotic populations is too recent to detect genetic divergence and (2) a low evolution rate of the marker (16S rRNA gene). We favor the second hypothesis because Peek *et al.* (1998b) showed that the 16S rRNA gene sequences of environmentally acquired symbionts (such as lucinid symbionts) evolve more slowly than those of vertically transmitted symbionts, as environmentally acquired symbionts do not experience a severe bottleneck when they pass from one host generation to the next.

How many bacterial species occur within the *Lucinidae*?

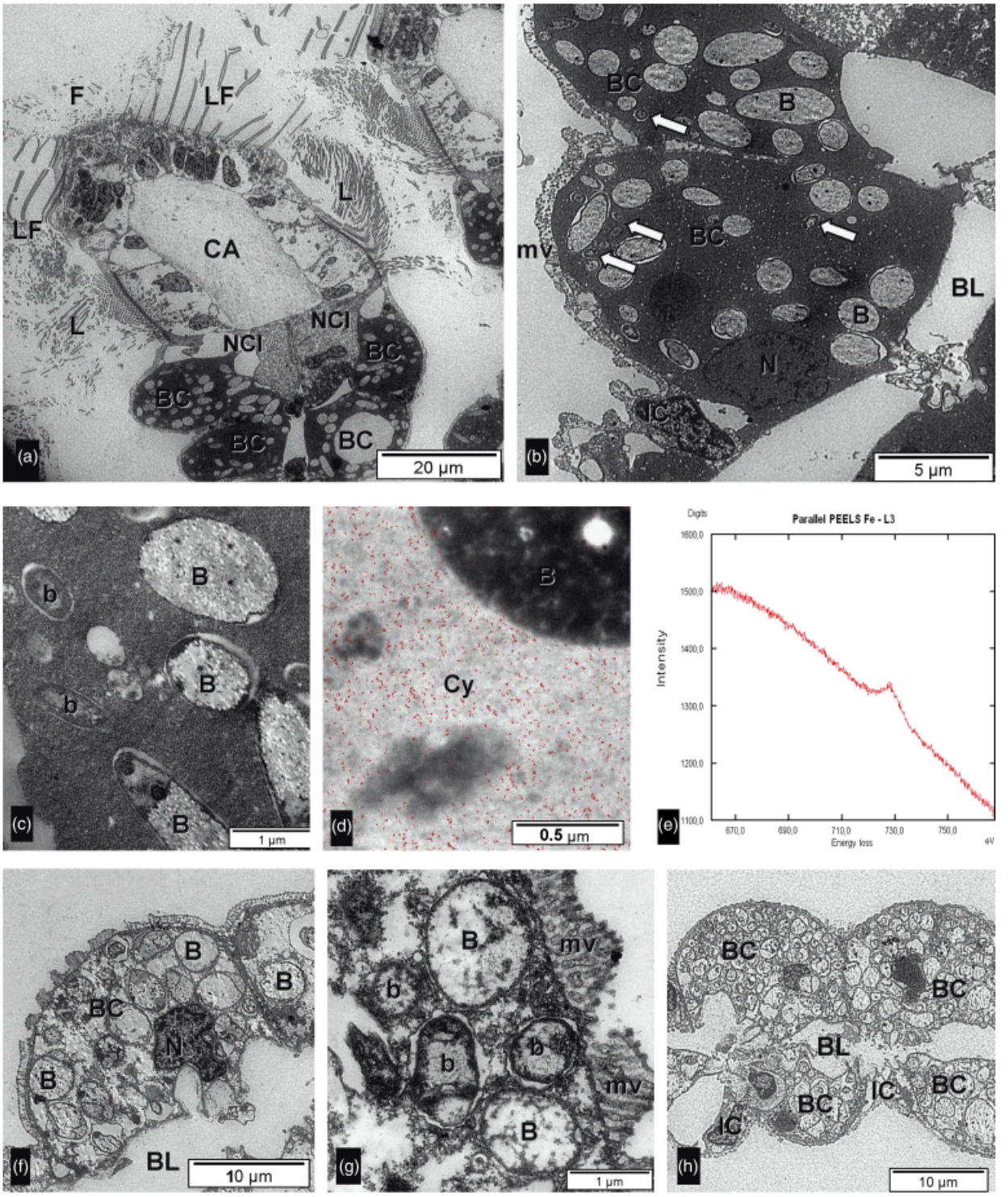
Even if no species-specific association is found within the *Lucinidae*, there is more than one bacterial species associated with the *Lucinidae*. The main bacterial species (named SoLuc-1 for symbiont of *Lucinidae* 1) corresponds to clade A (Fig. 2), but there are also two other species, one harbored by *P. pectinatus* (SoLuc-3) and the other by host species, which belong to the *Anodontia* clade, with the exception of *Anodontia alba* (SoLuc-2, Fig. 2, clade B). The latter two bacterial species appear to be more closely related to symbionts present in hosts inhabiting high sulfide environments (e.g. mangrove swamps) in comparison with shallow water sea-grass beds or low sulfide sediments. This finding supports the idea that lucinid clams do not coevolve with their symbiont, but instead associate with the symbiosis-competent bacteria present in their environment.

Does multi-infection occur within the *Lucinidae*?

According to symbiont phylogeny, we can distinguish two different groups in the unique bacterial species observed in Philippine lucinids (Fig. 2, groups I and II, clade A). They probably correspond to the two bacterial morphotypes viewed in TEM analysis. The large morphotype (observed in *C. quadrata* and *Dulcina* sp.) would correspond to group II, and the longer and thinner one (observed in *M. tanimbarensis*) to group I. Interestingly, only one bacterial sequence was detected in OG78 and *M. flabelliformis*, even though the two morphotypes are present in their gills. Thus,

Fig. 5. TEM observations from freshly collected individuals of *Myrtea flabelliformis* (OG103 [a–e]), *Cardiolucina quadrata* (OG101 [f–g]) and a *Dulcina sp.* (OG57 [h]). (a) Low magnification of gill filaments from adult individuals dissected immediately upon recovery. Each gill filament was characterized by a ciliated zone (CZ) separated from the lateral zone (LZ) by a single non-ciliated intermediary cell (NCI). The ciliated is composed by frontal (F), laterofrontal (LF), and lateral (L) ciliated cells organized along a collagen axis (CA). Bacteriocytes (BC) filled with chemoautotrophic-symbionts represent the most prevalent cell of the lateral zone. (b) Bacteriocytes (BC), which were the most prevalent cells in the gill filament, had an electron-dense cytoplasm, a basal nucleus (N), and a rounded apical pole harboring short microvilli (mv) in contact with the pallial sea-water. The cytoplasm was crowded by envacuolated bacteria which were usually individually enclosed inside the bacteriocyte vacuole and occupied the whole bacteriocyte volume. Two bacterial morphotypes could be distinguished, a large one (B) and a 10 times smaller one (white arrows) depending on section orientation. IC, intercalary cells, BL: blood lacuna. (c) Higher magnification focusing on the two morphotypes of gill-endosymbionts encountered in the bacteriocyte of *Myrtea flabelliformis.* Bigger bacteria (B) were characterized by an electron luscent cytoplasm while smaller intracellular symbionts (b) possesed a dark cytoplasm. The size difference is probably not due to the section orientation. (d) Mixmap image : the net iron map is superimposed on the High Contrast Image [contrast appears inversed compared to (b) & (c)]. The iron distribution (red dots) is homogenous through the cytoplasm of the bacteriocyte (Cy) while the bacterial cytoplasm appears free of iron (B), and consequently probably free of hemoglobin. (e) A specific iron spectrum (Fe-L3 edge around 730 eV) is obtained from the cytoplasm of the bacteriocyte, confirming the existence of intracellular hemoglobin in such lucinid species. (f) Bacteriocytes (BC) from the lateral zone of gill filaments of *C. quadrata*, which are the most prevalent cells in the gill filament, have a basal nucleus (N) and a rounded apical pole developing a broad contact with pallial seawater. The cytoplasm is crowded by intracellular bacterial symbionts (B), which are individually enclosed in vacuoles produced by the host. BL, blood lacuna. (g) Higher magnification focusing on the two morphotypes of gill-endosymbionts encountered in *C. quadrata*. Large bacterial cells (B) were characterized by a clear cytoplasm, whereas smaller intracellular symbionts (c) possessed a dark cytoplasm. mv, microvilli of the apical pole of the bacteriocyte. (h) TEM view of the lateral zone of a gill filament from an undescribed lucinid collected at 1770m depth (Bohol sea) showing several bacteriocytes (BC) filled with intracellular bacteria and few intercalary cells (IC). Only one bacterial morphotype is distinguishable inside the bacteriocytes. The blood lacuna (BL) of the filament is irregularly partitioned.

multi-infection by two different bacterial types might occur in our samples, but escapes detection by PCR direct sequencing because this method yields only the major sequence. A more concerted effort concerning these two lucinids (OG78 and *M. flabelliformis*) including the analysis of a 16S rRNA gene clone library established from gill DNA as well as FISH analysis of the symbionts will be necessary to confirm the dual symbiosis hypothesis.



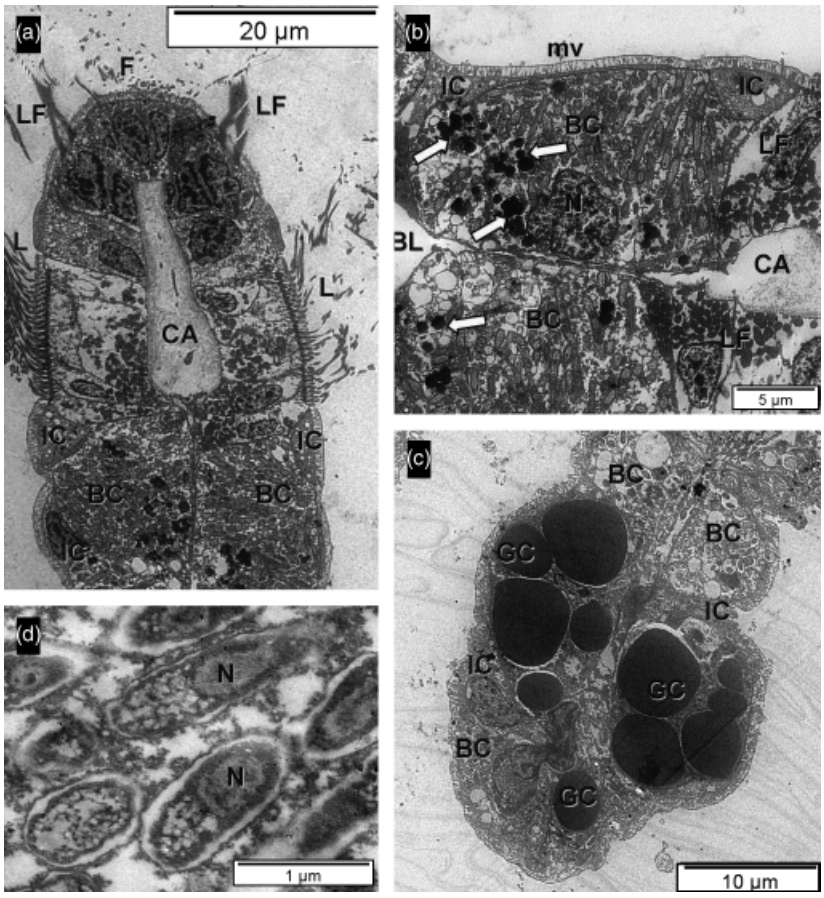


Fig. 6. TEM observations from freshly collected individuals of *Myrtea tanimbarensis* (OG18 [Balicasag island]). (a) The ciliated zone of the gill filament from adult individuals is composed of frontal (F), laterofrontal (LF), and lateral (L) ciliated cells organized along a collagen axis (CA). The intermediary zone is not obvious in this species. The lateral zone mostly contains bacteriocytes (BC) which are filled with chemoautotrophic bacteria. Intercalary cells (IC), characterized by a trumpet shape and an apical nucleus, are regularly interspersed among bacteriocytes. (b) The cytoplasm of the bacteriocytes (BC) was characterized by a basal nucleus (N), a rounded apical pole harboring short microvilli (mv) and numerous pigment granules (white arrows) distributed in most of the bacteriocytes. Bacterial symbionts are very long. BL: blood lacuna; CA, collagen axis; IC, intercalary cells; LF, laterofrontal cells from the ciliated zone in direct contact with the lateral zone. (c) Abfrontal end of the gill filament. Some bacteriocytes (BC) and granule cells (GC) characterized by homogeneous osmiophilic granules. Granule cells represent the third cell type of the lateral zone which devoid of bacterial symbionts. Few intercalary cells (IC) can also be observed interspersed with bacteriocytes and/or granule cells. (d) Higher magnification focusing on the endocellular bacteria tightly enclosed inside bacteriocyte vacuoles. The bacterial cytoplasm contains numerous non membrane-bound granules (probably sulfur ones). The nucleoid (N) is located at one pole of the bacterial cell.

Recently, a multiple symbiosis partnership was suspected in the lucinid *Anodontia ovum* based on TEM observations. In fact, the two morphotypes described in this lucinid differ in size (there are larger and smaller ones) and in the way they are housed in the bacteriocytes (the larger bacteria are housed individually and the smaller ones are clustered together in vacuoles). However, molecular data could not confirm these results as only one identical 16S rRNA gene sequence was obtained from each of the 62 clones analyzed (Ball *et al*., 2009).

In the case of lucinid from the Philippines, all bacteria are always housed individually in vacuoles. Hence, the two bacterial morphotypes inside a bacteriocyte could be related to the DNA content of new phagocytosed symbionts and old ones.

Recently, Caro *et al*. (2007) showed, using single-cell approaches including flow cytometry, that the sulfur-oxidizing gill endosymbiont population of *C. orbicularis* was genomically heterogeneous, ranging from one genome copy for the smaller cells to more than four for the larger ones, and up to 20 copies when the host was kept under starvation conditions (Caro *et al*., 2009). This multiplication of genomic DNA content while cell division is suspended by the host could explain the heterogeneous size of a single bacterial species inside the same bacteriocyte. Thus, recently recruited symbionts (the small bacterial symbionts) may be

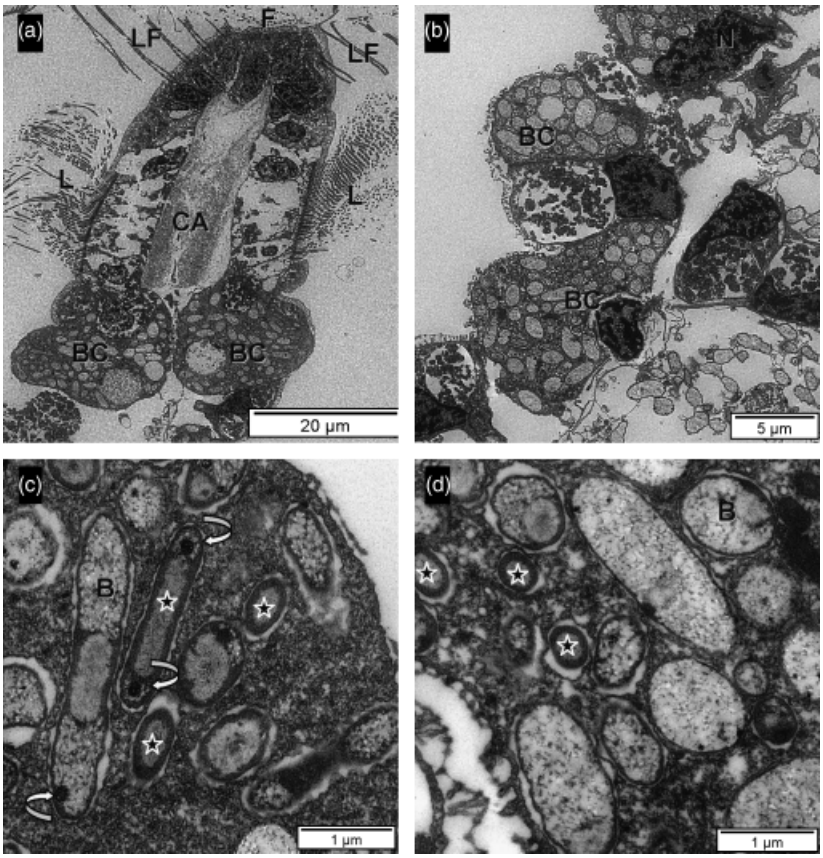


Fig. 7. TEM observations from freshly collected *Gloverina rectangularis* (OG108) collected at 640m depth. (a) General view of the ciliated zone and the adjacent part of the lateral zone with a few bacteriocytes (BC) which are filled with chemoautotrophic bacteria. Frontal (F), latero-frontal (LF), and lateral (L) ciliated cells are the main cell-types of the ciliated zone. The ciliated part of the filament is stiffened by a collagen axis (CA), whereas the axis of the lateral zone is occupied by a blood lacunar space. (b) In the innermost part of the lateral zone, bacteriocytes (BC) are filled with gill-endosymbionts, whereas intercalary cells are characterized by very large secondary lysosome-like structures (stars) occupying the whole cytoplasm and a basal nucleus (N). There appear to be two morphotypes of bacterial symbionts inside a single bacteriocyte. The larger ones are electron-lucent, whereas the smaller ones look darker (arrows). BL: blood lacuna. (c) Two morphotypes of gram-negative symbionts (stars), both individually enclosed in vacuoles, are present in the bacteriocytes. Electron-dense granules (curved arrows) located in the cytoplasm of both morphotypes probably correspond to glycogenic storage. Even if intracellular symbionts appear to be morphologically different, they could represent strains of a single bacterial species colonizing this lucinid. (d) Higher magnification focusing on the endocellular bacteria tightly enclosed inside bacteriocyte vacuoles. The bacterial cytoplasm of the larger symbionts contains numerous non-membrane-bound granules (probably sulfur storage). Small ovoid shapes (stars) correspond to transverse sections of the long and thin morphotype observed in (C) and represented by similar stars.

located near the apical pole and are abundantly supplied with metabolic substrates by the host [probably to maintain an active bacterial metabolism to allow nutrition of the host or detoxification of the environment against hydrogen sulfide (H_2S)], whereas their division is inhibited. As new symbionts are recruited, the older ones migrate toward the basal pole, becoming larger with an increase of their genome (Caro *et al.*, 2007, 2009).

Hemoglobin, sulfur and symbiosis

According to TEM analysis, there is no evidence of sulfur granules in gill endosymbionts of lucinids from the Philippines, in contrast to the situation described, for example, in the symbionts of *C. orbicularis* (Lechaire *et al.*, 2008). Yet, the symbionts we found were indeed sulfur oxidizing, as the detection of APS reductase gene attests, which makes the absence of sulfur granules surprising.

Another unusual feature can be found in the clam *M. flabelliformis* (OG103). This species has a dark bacteriocyte cytoplasm and a low number of gill endosymbionts. Both the deep-red gills and the dark color of bacteriocyte cytoplasm suggest the presence of hemoglobin in the gill tissues of this host, which was confirmed by PEELS and ESI analyses. Hemoglobin, which is an oxygen and also a H_2S transporter (Kraus, 1993), is scarce within *Lucinidae*, even

though its presence has been detected in gill tissues of some species such as *Anodontia edentula* (Lebata, 2001), *P. pectinatus* (Kraus & Wittenberg, 1990; Gavira *et al.*, 2008) or *Myrtea spinifera* (Dando *et al.*, 1985), and suggested in *A. ovum* (Ball *et al.*, 2009). It has been proposed in the literature that hemoglobin contributes to the detoxification of sulfide (which is toxic for the respiratory chain and could harm tissues) in high sulfide environments such as hydrothermal vents (Arndt *et al.*, 2001). This role of hemoglobin in detoxification together with sulfide-oxidizing bodies has also been suggested for the shallow water lucinid *P. pectinatus* (Liberge *et al.*, 2001). However, sulfide concentrations in the environment of *M. flabelliformis* specimens are unknown. In addition, hemoglobin has been described in another species of the genus *Myrtea* (*M. spinifera*) that inhabits *low* sulfide environments (Dando *et al.*, 1985). Finally, there is no evidence of sulfur-oxidizing bodies in gill sections of *M. flabelliformis*.

As a role of hemoglobin in sulfur detoxification cannot be proved for this species, two other hypotheses could explain the high hemoglobin concentration we found in its cytoplasm. According to Dando *et al.* (1985), hemoglobin could function as oxygen storage during burrowing because of its high affinity for oxygen or it could protect the sulfur-oxidizing endosymbionts against an excess of oxygen because sulfur-oxidizing bacteria are frequently microaerophiles (Jorgensen, 1982). With our data, it is not possible to distinguish between these two hypotheses.

Conclusion

Our analyses based on six lucinid species from the Philippines as well as on literature data support the hypothesis that the *Lucinidae* and their symbionts have not coevolved from an ancestral symbiosis. On the contrary, lucinids appear to establish associations as a function of the potential symbionts present in their environment. Moreover, according to the 16S rRNA gene, the family *Lucinidae* appears to be mainly associated with only one bacterial sulfur-oxidizing species (SoLuc-1). However, hosts inhabiting high sulfide environments (*A. ovum*, *Anodontia philippiana* and *P. pectinatus*) could also harbor other bacterial species (SoLuc-2 and SoLuc-3).

Acknowledgements

The material from the Philippines was obtained during the PANGLAO 2005 deep-sea cruise aboard M/V *DA-BFAR*. We thank Dr P. Bouchet and L. Labe, the coprincipal investigators, for their invitation. Our participation was made possible by an MNHN grant (Museum National d'Histoire Naturelle) under the program 'Status and Phylogenetic Structure of Recent and Fossil Biodiversity' led by Dr P. Janvier. Labwork was supported within the framework of the DIWOOD project. The authors wish to thank the 'Service de Microscopie Électronique de l'IFR 83-Biologie Intégrative CNRS (Paris, France)' for TEM facilities. We thank Dominique Higuet for his fruitful comments and Denis Poinsot for English revision. We also thank John D. Taylor and Emily Glover for species determination based on morphological characteristics and two anonymous reviewers for their helpful comments, leading to an improvement of this manuscript. T.B. was funded as a PhD student by an MESR grant (Ministère de l'Enseignement Superieur et de la Recherche).

References

Arndt C, Gaill F & Felbeck H (2001) Anaerobic sulfur metabolism in thiotrophic symbioses. *J Exp Biol* **204**: 741–750.

Arndt-Sullivan C, Lechaire J-P & Felbeck H (2008) Extreme tolerance to anoxia in the *Lucinoma aequizonata* symbiosis. *J Shellfish Res* **27**: 119–127.

Ball AD, Purdy KJ, Glover EA & Taylor JD (2009) Ctenidial structure and three bacterial symbiont morphotypes in *Anodontia* (Euanodontia) *ovum* (Reeve, 1850) from the Great Barrier Reef, Australia (Bivalvia: Lucinidae). *J Mollus Stud* **75**: 175–185.

Berg CJJ & Alatalo P (1984) Potential of chemosynthesis in molluscan mariculture. *Aquaculture* **39**: 165–179.

Caro A, Gros O, Got P, De Wit R & Troussellier M (2007) Characterization of the population of the sulfur-oxidizing symbiont of *Codakia orbicularis* (Bivalvia, Lucinidae) by single-cell analyses. *Appl Environ Microb* **73**: 2101–2109.

Caro A, Got P, Bouvy M, Troussellier M & Gros O (2009) Long term starvation of a host bivalve (*Codakia orbicularis*, Lucinidae) and effects on its symbiont population. *Appl Environ Microb* **75**: 3304–3313.

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* **17**: 540–552.

Dando PR, Southward AJ, Southward EC, Terwilliger NB & Terwilliger RC (1985) Sulfur-oxidizing bacteria and hemoglobin in gills of the bivalve mollusk *Myrtea spinifera*. *Mar Ecol-Prog Ser* **23**: 85–98.

Deming JW, Reysenbach AL, Macko SA & Smith CR (1997) Evidence for the microbial basis of a chemoautotrophic invertebrate community at a whale fall on the deep seafloor: bone-colonizing bacteria and invertebrate endosymbionts. *Microsc Res Techniq* **37**: 162–170.

Deplancke B, Hristova KR, Oakley HA, McCracken VJ, Aminov R, Mackie RI & Gaskins HR (2000) Molecular ecological analysis of the succession and diversity of sulfate-reducing bacteria in the mouse gastrointestinal tract. *Appl Environ Microb* **66**: 2166–2174.

Distel DL, Lane DJ, Olsen GJ *et al.* (1988) Sulfur-oxidizing bacterial endosymbionts: analysis of phylogeny and specificity by 16S rRNA sequences. *J Bacteriol* **170**: 2506–2510.

Distel DL, Felbeck H & Cavanaugh CM (1994) Evidence for phylogenetic congruence among sulfur-oxidizing chemoautotrophic bacterial endosymbionts and their Bivalve hosts. *J Mol Evol* **38**: 533–542.

Dubilier N, Bergin C & Lott C (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat Rev Microbiol* **6**: 725–740.

Duperron S, Fiala-Medioni A, Caprais JC, Olu K & Sibuet M (2007) Evidence for chemoautotrophic symbiosis in a Mediterranean cold seep clam (Bivalvia: Lucinidae): comparative sequence analysis of bacterial 16S rRNA, APS reductase and RubisCO genes. *FEMS Microbiol Ecol* **59**: 64–70.

Duperron S, Laurent MC, Gaill F & Gros O (2008) Sulphur-oxidizing extracellular bacteria in the gills of Mytilidae associated with wood falls. *FEMS Microbiol Ecol* **63**: 338–349.

Durand P & Gros O (1996) Bacterial host specificity of Lucinacea endosymbionts: interspecific variation in 16S rRNA sequences. *FEMS Microbiol Lett* **140**: 193–198.

Durand P, Gros O, Frenkiel L & Prieur D (1996) Phylogenetic characterization of sulfur-oxidizing bacterial endosymbionts in three tropical Lucinidae by 16S rDNA sequence analysis. *Mol Mar Biol Biotech* **5**: 37–42.

Felbeck H, Childress JJ & Somero GN (1981) Calvin–Benson cycle and sulphide oxidation enzymes in animals from sulphide-rich habitats. *Nature* **293**: 291–293.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Fisher CR (1990) Chemoautotrophic and methanotrophic symbioses in marine invertebrates. *Rev Aquat Sci* **2**: 399–613.

Frenkiel L & Mouëza M (1995) Gill ultrastructure and symbiotic bacteria in *Codakia orbicularis* (Bivalvia: Lucinidae). *Zoomorphology* **115**: 51–61.

Gavira JA, Camara-Artigas A, De Jesus-Bonilla W *et al.* (2008) Structure and ligand selection of hemoglobin II from *Lucina pectinata*. *J Biol Chem* **283**: 9414–9423.

Gros O & Gaill F (2007) Extracellular bacterial association in gills of 'wood mussels'. *Cah Biol Mar* **48**: 103–109.

Gros O, Frenkiel L & Felbeck H (2000) Sulfur-oxidizing endosymbiosis in *Divaricella quadrisulcata* (Bivalvia: Lucinidae): morphological, ultrastructural, and phylogenetic analysis. *Symbiosis* **29**: 293–317.

Gros O, Liberge M & Felbeck H (2003) Interspecific infection of aposymbiotic juveniles of *Codakia orbicularis* by various tropical lucinid gill endosymbionts. *Mar Biol* **142**: 57–66.

Guindon S & Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**: 696–704.

Ishaq M, Wolf B & Ritter C (1990) Large-scale isolation of plasmid DNA using cetyltrimethylammonium bromide. *Biotechniques* **9**: 19–20.

Jeanguillaume C, Trebbia P & Colliex C (1978) About the use of electron energy-loss spectroscopy for chemical mapping of thin foils with high spatial resolution. *Ultramicroscopy* **3**: 237–242.

Jorgensen BB (1982) Ecology of the bacteria of the sulphur cycle with special reference to anoxic–oxic interface environments. *Philos T Roy Soc B* **298**: 543–561.

Juniper SK & Sibuet M (1987) Cold seep benthic communities in Japan subduction zones: spatial organization, trophic strategies and evidence for temporal evolution. *Mar Ecol-Prog Ser* **40**: 115–126.

Keane TM, Creevey CJ, Pentony MM, Naughton TJ & McLnerney JO (2006) Assessment of methods for amino acid matrix selection and their use on empirical data shows that *ad hoc* assumptions for choice of matrix are not justified. *BMC Evol Biol* **6**: 29.

Kraus DW (1993) *Heme-Proteins in Sulfide-Oxidizing Bacteria Mollusk Symbioses*, pp. 112–120. American Society of Zoologists, Los Angeles.

Kraus DW & Wittenberg JB (1990) Hemoglobins of the *Lucina pectinata*/bacteria symbiosis. I. Molecular properties, kinetics and equilibria of reactions with ligands. *J Biol Chem* **265**: 16043–16053.

Lane DJ (1991) 16S/23S sequencing. *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds), pp. 115–175. John Wiley and Sons, New York.

Lebata MJ (2001) Oxygen, sulphide and nutrient uptake of the mangrove mud clam *Anodontia edentula* (Family: Lucinidae). *Mar Pollut Bull* **42**: 1133–1138.

Lechaire J-P, Freibourg G, Gaill F & Gros O (2008) *In situ* characterization of sulfur in gill endosymbionts of the shallow water lucinid *Codakia orbicularis* (Linné, 1758) by cryo-EFTEM microanalysis. *Mar Biol* **154**: 693–700.

Liberge M, Gros O & Frenkiel L (2001) Lysosomes and sulfide-oxidizing bodies in the bacteriocytes of *Lucina pectinata*, a cytochemical and microanalysis approach. *Mar Biol* **139**: 401–409.

Milne I, Wright F, Rowe G, Marshall DF, Husmeier D & McGuire G (2004) TOPALi: software for automatic identification of recombinant sequences within DNA multiple alignments. *Bioinformatics* **20**: 1806–1807.

Olson PD, Cribb TH, Tkach VV, Bray RA & Littlewood DT (2003) Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *Int J Parasitol* **33**: 733–755.

Peek AS, Feldman RA, Lutz RA & Vrijenhoek RC (1998a) Cospeciation of chemoautotrophic bacteria and deep sea clams. *P Natl Acad Sci USA* **95**: 9962–9966.

Peek AS, Vrijenhoek RC & Gaut BS (1998b) Accelerated evolutionary rate in sulfur-oxidizing endosymbiotic bacteria associated with the mode of symbiont transmission. *Mol Biol Evol* **15**: 1514–1523.

Prashad B (1932) The Lamellibranchia of the Siboga Expedition. Systematic Part. II Pelecypoda (exclusive of the Pectinidae). *Siboga-Expeditie, Uitkomsten op Zoologisch, Botanisch, Oceanographisch en Geologisch Gebeid Vezameld in Nederlandsch Oost-Indie* 1899–1900, **53c**: 1.

Reid RGB (1990) Evolutionary implications of sulphide-oxidising symbioses in bivalves. *The Bivalvia – Proceedings of a Memorial Symposium in Honour of Sir Charles Maruice Yonge,*

Edinburgh, 1986 (Morton B, ed), pp. 127–140. Hong Kong University Press, Hong Kong.

Reimer L, Rennekamp R, Fromm I & Langenfeld M (1991) Contrast in the electron spectroscopic imaging mode of a TEM. IV. Thick specimens imaged by the most-probable energy loss. *J Microsc* **162**: 3–14.

Ronquist F & Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Schwarz G (1978) Estimating the dimension of a model. *Ann Stat* **6**: 461–464.

Schweimanns M & Felbeck H (1985) Significance of the occurrence of chemoautotrophic bacterial endosymbionts in lucinid clams from Bermuda. *Mar Ecol-Prog Ser* **24**: 113–120.

Taylor JD & Glover EA (2006) Lucinidae (Bivalvia) – the most diverse group of chemosymbiotic molluscs. *Zool J Linn Soc* **148**: 421–438.

Thompson JD, Higgins DG & Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680.

Von Cosel R & Bouchet P (2008) Tropical deep-water lucinids (Mollusca: Bivalvia) from the Indo-Pacific: essentially unknown, but diverse and occasionally gigantic. *Mém Mus Natl Hist Nat* **196**: 115–213.

Williams ST, Taylor JD & Glover EA (2003) Molecular phylogeny of the Lucinoidea (Bivalvia): non-monophyly and separate acquisition of bacterial chemosymbiosis. *J Mollus Stud* **70**: 187–202.

Winnepenninckx BM, Reid DG & Backeljau T (1998) Performance of 18S rRNA in littorinid phylogeny (Gastropoda: Caenogastropoda). *J Mol Evol* **47**: 586–596.