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

Terry Brissac¹, Hervé Mercôt¹ & Olivier Gros²

¹UMR 7138 UPMC-CNRS-MNHN-IRD ‘Systématique, Adaptation, Évolution’, Equipe: ‘Génétique & Evolution’, Université Pierre et Marie Curie, Paris, France; and ²UMR 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 Médecine, 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.
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**

<table>
<thead>
<tr>
<th>Specimen code</th>
<th>Species</th>
<th>Location</th>
<th>Sampling site</th>
<th>Station</th>
<th>Depth (m)</th>
<th>Accession numbers*</th>
</tr>
</thead>
<tbody>
<tr>
<td>OG 18</td>
<td>Myrtea tanimbarensis (Von Cosel &amp; Bouchet, 2008)</td>
<td>Bohol Sea</td>
<td>Off Balicasag Island</td>
<td>CP 2340</td>
<td>271–318</td>
<td>FN869533FN869534FN869535</td>
</tr>
<tr>
<td>OG 57</td>
<td>Dulcina sp. (Von Cosel &amp; Bouchet, 2008)</td>
<td>Bohol Sea</td>
<td>Off Balicasag Island</td>
<td>CP 2355</td>
<td>1764–1775</td>
<td>FN869536FN869537FN869538</td>
</tr>
<tr>
<td>OG 78</td>
<td>Undetermined genus and species</td>
<td>Bohol/Sulu seas sill</td>
<td>Off Balicasag Island</td>
<td>CP</td>
<td>397–372</td>
<td>FN869550FN869551FN869552</td>
</tr>
<tr>
<td>OG 101</td>
<td>Cardiolaria quadrata (Prashad, 1932)</td>
<td>Bohol Sea</td>
<td>Off Balicasag Island</td>
<td>CP 2388</td>
<td>762–786</td>
<td>FN869554FN869555</td>
</tr>
<tr>
<td>OG 103</td>
<td>Myrtea flabelliformis (Prashad, 1932)</td>
<td>Bohol Sea</td>
<td>Off Balicasag Island</td>
<td>CP 2390</td>
<td>762–786</td>
<td>FN869545FN869546</td>
</tr>
</tbody>
</table>

*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.

---

---

**FEMS Microbiol Ecol 75 (2011) 63–76**
Thus, *Cardiolucina quadrata*, *Gloverina rectangularis*, *Myrtea flabelliformis* and *Myrtea tanimbarenensis* 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 CaCl2 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 (Jean-guillaume 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.
Amplification was performed in 50 µL using 25–50 ng of DNA template, 5 µL of 10 × Taq buffer (Invitrogen), 3 mM MgCl₂ (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://www.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 ML. Posterior 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 chromatogram 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 ‘Lucinidea 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 Cras- satelloidea, which are sister-groups of *Lucinidae*, were used.

---

**Table 2. Primers and PCR conditions**

<table>
<thead>
<tr>
<th>Sequence (5’ → 3’)</th>
<th>Temperature (°C)</th>
<th>Name</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symbiont</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGAGTTTGTATCAGGGCTCAG</td>
<td>54</td>
<td>16S_UNIV-8f</td>
<td>16S rRNA gene</td>
<td>Lane (1991)</td>
</tr>
<tr>
<td>TACGGCTACCTGTTACGACT</td>
<td>58</td>
<td>16S_UNIV-1492r</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGGCCAGATMATGATYMACGGG</td>
<td>18S-5’F</td>
<td>APS-FW</td>
<td>APS reductase</td>
<td>Deplancke et al. (2000)</td>
</tr>
<tr>
<td>GGGCCGTAACGCCCTTGA</td>
<td>54</td>
<td>18S-1100R</td>
<td>18S rRNA gene</td>
<td>Williams et al. (2003)</td>
</tr>
<tr>
<td>Host</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCGTTCTGAGACCGGACCAAG</td>
<td>52</td>
<td>LSU-900f</td>
<td>28S rRNA gene (large subunit)</td>
<td>Olson et al. (2003)</td>
</tr>
<tr>
<td>ACGCCATCCATTTCCAGG</td>
<td>52</td>
<td>LSU-1600r</td>
<td></td>
<td>Williams et al. (2003)</td>
</tr>
<tr>
<td>CTGGTTGATGCTGCCAGT</td>
<td>54</td>
<td>18S-5’F</td>
<td></td>
<td>Winnepenningh et al. (1998)</td>
</tr>
<tr>
<td>CTTCGAACCCTGACTTGG</td>
<td>54</td>
<td>18S-1100R</td>
<td></td>
<td>Williams et al. (2003)</td>
</tr>
</tbody>
</table>
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...
*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. rectangula*) 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).

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

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.000</td>
<td>ID</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.984</td>
<td>ID</td>
<td>0.95</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.957</td>
<td>ID</td>
<td>0.971</td>
<td>0.961</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.966</td>
<td>ID</td>
<td>0.97</td>
<td>0.966</td>
<td>0.966</td>
<td>0.959</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.969</td>
<td>ID</td>
<td>0.97</td>
<td>0.969</td>
<td>0.969</td>
<td>0.969</td>
<td>0.985</td>
<td>ID</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.966</td>
<td>ID</td>
<td>0.97</td>
<td>0.969</td>
<td>0.969</td>
<td>0.969</td>
<td>0.969</td>
<td>0.969</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.970</td>
<td>ID</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.969</td>
<td>ID</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.969</td>
<td>ID</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.971</td>
<td>ID</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.971</td>
<td>ID</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.971</td>
<td>ID</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>0.971</td>
<td>ID</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.971</td>
<td>ID</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.971</td>
<td>ID</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0.971</td>
<td>ID</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>0.971</td>
<td>ID</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Identity matrix for the symbiont 16S rRNA gene (pairwise deletion). ID, identical.
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 / 2.5 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 Gammaproteobacteria (Figs 5c, 6d and 7c, d). The bacteria in Dulcina sp. and C. quadrata (Fig. 5f–h) are large (4 μm long x 1.5 μm wide), in M. tanimbarensis (Fig. 6d) they are longer and thinner (6 μm long x 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).
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 symbionts, 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. tanim-barensis) 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,
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.
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
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 (H₂S)], 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 H₂S 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 Electronique de l’IFR 83-Biologie Integrative CNRS (Paris, France)’ for TEM facilities. We thank Dominique Huguet for his fruitful comments and Denis Poinset 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**


