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ORIGINAL PAPER

Unexpected structured intraspecific diversity of thioautotrophic bacterial gill endosymbionts within the Lucinidae (Mollusca: Bivalvia)

Terry Brissac[1,2,3] · Dominique Higuet[1] · Olivier Gros[2] · Hervé Merçot[1]

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Abstract In chemoautotrophic associations, sequence comparison of 16S rRNA has been the method of choice to study bacterial diversity in the context of host/symbiont coevolution. However, the relative low rate of evolution of 16S rRNA has been shown to result in a diminished capacity to discriminate between closely related bacterial strains or species. Within chemoautotrophic associations, as described in several studies, the use of other genetic markers may reveal previously unobserved strain diversity among gill endosymbionts. Herein, we conducted a survey of symbionts harbored by six species within the Lucinidae family using five genetic markers (*dnaE*, *gyrB*, *aprA*, *cbbL* genes and the 16S–23S internal transcribed spacer). Thus, within the already described SoLuc_1 bacterial species shared by six host species we observed an obvious bacterial strain diversity. This diversity is structured in function of the geographic location of the hosts and not in function of ecological parameters or host phylogeny. Interestingly at the local level (same seagrass bed environment), we observed an unexpected specificity in host species/bacterial strain association (i.e., all individuals of the same host species harbor the same symbiotic sequence type). This specificity of association implied that there was a control of symbiont strain acquisition by the host, which was confirmed by a cross-infection experiment of starved adults performed in our study. Based upon our results and other evidence from the literature, we hypothesize that this pattern may be due to a "capture/escape" type antagonist evolution of the two partners.

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✉ Terry Brissac
tbrissac@uab.edu

[1] UMR 7138 – Evolution Paris-Seine, Equipe Evolution du génome eucaryote, C.N.R.S, Institut de Biologie Paris-Seine, Sorbonne Universités Paris VI, Paris, France

[2] UMR-CNRS-UPMC 7138 "Evolution Paris Seine", Equipe Biologie de la mangrove, Département de Biologie, UFR des Sciences Exactes et Naturelles, Université des Antilles, B.P. 592, 97159 Pointe-À-Pitre Cedex, Guadeloupe, France

[3] Present Address: Department of Microbiology, School of Medicine, University of Alabama at Birmingham, 845 19th St S. BBRB 673, Birmingham, AL 35294, USA

Introduction

From ecology to morphology and enzymology, comparative studies have increased our understanding of the associations between chemoautotrophic bacteria and marine invertebrates (Felbeck et al. 1981; Fisher 1990; Stewart et al. 2005; Dubilier et al. 2008). In these associations, the characterization of symbionts has been performed using 16S rRNA gene sequences (Cary et al. 1993; Polz et al. 1994; Dubilier et al. 1995; Polz and Cavanaugh 1995; Gros et al. 1996; Blazejak et al. 2006; Duperron et al. 2008). According to this marker, the majority of symbionts involved in these interactions belong to the gammaproteobacteria subdivision. This is despite that alpha-, delta-, epsilonproteobacteria and spirochetes are also retrieved (Dubilier et al. 2008). Moreover, 16S rRNA provides information on the evolutionary history of host/symbiont couples by comparing diversity of the symbionts versus that of the host (using 18S and 28S rRNA genes) (Distel et al. 1988, 1994; Feldman et al. 1997; Peek et al. 1998; Brissac et al. 2011).

Commonly, species-specific associations are maintained by vertical transmission of the symbiont, from parents to offspring via gametes (Peek et al. 1998). In these cases, hosts and symbionts present congruent evolutionary histories. This

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is due to the coevolution of the two partners and leads to the specific couples observed today, for example, as described within bivalves of the Vesicomyidae family (Peek et al. 1998). However, when symbionts are not transmitted vertically, phylogenies of hosts and symbionts are decoupled. This has been demonstrated to be the case in vestimentiferan tube worms and lucinid bivalves, where different host species can be associated with the same symbiont species (Durand and Gros 1996; Gros et al. 1996; Feldman et al. 1997; Gros et al. 2000, 2003a). In these cases, hosts become associated with free-living symbiosis-competent bacterial species present in the environment. Such bacteria are acquired by juveniles or even adults of the host (Gros et al. 1996, 2012). Thus, host species inhabiting the same (or close) environments harbor the same (or close) symbiont species.

To date, all lucinid genera analyzed harbor thioautotrophic gammaproteobacteria within specialized cells of the gills called bacteriocytes (Fisher 1990; Williams et al. 2003; Brissac et al. 2011). Bacterial colonization begins at the juvenile stage after metamorphosis, as previously described in the tropical shallow-water species *Codakia orbicularis* (Gros et al. 1996) and in the deepwater species *Lucinoma aequizonata* (Gros et al. 1999), and as suggested for other Caribbean lucinids (Gros et al. 1998), symbiosis-competent bacteria being acquired from a free-living stock present in the environment (Gros et al. 2003b). Recent studies have demonstrated that adult lucinids can also acquire symbionts from their environment throughout their life (Elisabeth et al. 2012; Gros et al. 2012).

Concerning the 16S rRNA gene, there is a perfect sequence identity among the symbionts harbored by the six lucinid host species living in *Thalassia testudinum* seagrass beds of the Lesser Antilles (Durand and Gros 1996; Gros et al. 1996, 2000, 2003a; Brissac et al. 2011). However, and as discussed above, the relatively low rate of evolution of this marker may have led to an inability to discriminate between closely related bacterial strains or species (Lukjancenko et al. 2010). In this paper, we sought to analyze the genetic intraspecific diversity of the symbionts shared by these six lucinid host species, belonging to four genera, inhabiting seagrass beds of Guadeloupe and Martinique (Lesser Antilles, French West Indies). We used five different markers to measure strain diversity. Thereafter, we sought to determine whether this diversity was structured according to host diversity, geography and/or environmental factors. This study was performed at two levels: (1) a large-scale analysis of symbionts harbored by host lucinids of the Lesser Antilles and (2) a local analysis using hosts inhabiting the same location, colonizing the same seagrass bed and consequently exploiting the same stock of free-living lucinid gill endosymbionts.

Materials and methods

Lucinid collection

Individuals of *Anodontia alba* (Link, 1807) and *Codakia pectinella* (Adams, 1852) were collected in Guadeloupe ("Ilet Cochon", 16°12′53.76″N 61°32′05.74″W). Individuals of *C. orbicularis* (Linné, 1758) and *Codakia orbiculata* (Montagu, 1808) were collected in Guadeloupe ("Ilet Cochon") and in Martinique ("Anse Macabou", 14°31′01.52″N 60°49′00.86″W). Individuals of *Divaricella quadrisulcata* (Orbigny, 1842) were collected in la Désirade ("plage des galets", 16°17′46.88″N 61°04′53.77″W). Individuals of *Lucina pensylvanica* (Linné, 1758) were collected in Martinique ("Anse Macabou"). Subadult individuals of *C. orbicularis* (~0.5 cm shell length) were also collected in *Thalassia testidunum* seagrass beds from Guadeloupe ("Ilet Cochon"). All individuals (3 per host species) were collected by hand in *T. testudinum* seagrass beds (from November 2005 to January 2006). All collection sites are presented in Fig. 1. Gills (which harbor symbionts) and other symbiont-free tissues (*e.g.,* foot) were dissected and conserved in absolute ethanol at –20 °C before investigation.

Sediment collection

Sediments samples were collected by coring from three different locations at "Ilet Cochon" (16°12′53.76″N 61°32′05.74″W, 16°12′54.17″N 61°32′09.08″W, 16°12′55.85″N 61°31′11.61″W). From each core, three samples were collected from the surface layer of the seagrass bed (0–5 cm depth).

DNA preparation

Template DNA was extracted from symbiont-containing gills and symbiont-free foot tissue of each bivalve host according to the CTAB method (Ishaq et al. 1990). DNA concentration was determined by spectrometry (BioPhotometer, Eppendorf), and quality of DNA was verified on a 0.8 % agarose gel in comparison with a concentration marker (Smart Ladder, Eurogentec).

Polymorphism analysis

Three kinds of markers were used in this study: (1) two housekeeping genes already used for typing purposes in the literature: alpha subunit of DNA polymerase III (*dnaE*) (Byun et al. 1999) and B subunit of DNA Gyrase (*gyrB*) (Holmes et al. 2004), (2) the 16-23S internal transcribed spacer (*ITS1*), a hypervariable region comprised between the 16S and 23S rRNA genes and (3) two metabolic genes

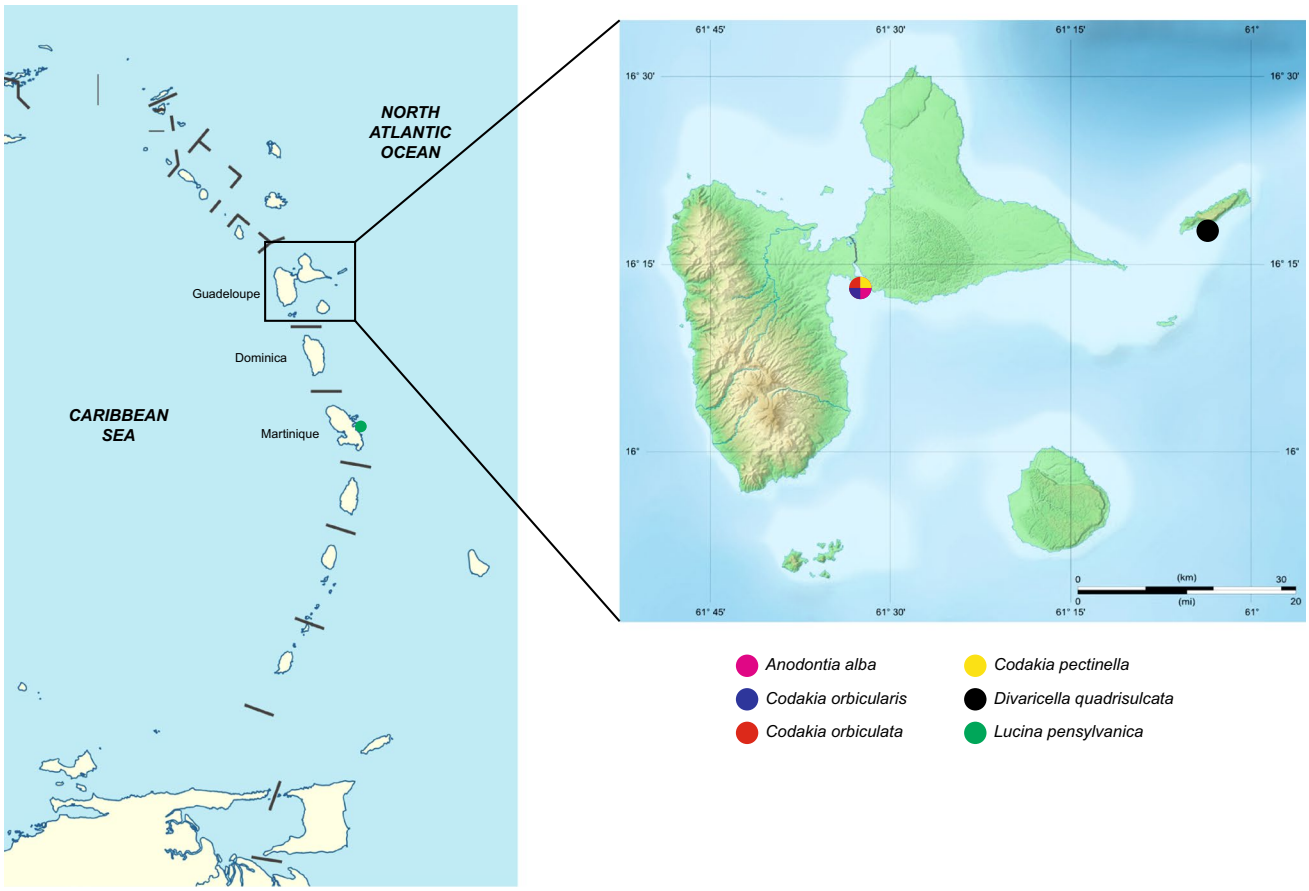


Fig. 1 Specimen collection maps. *Circles* represent sampling locations and *colors* the host species. *Purple* for *A. alba, blue* for *C. orbicularis*, *red* for *C. orbiculata, yellow* for *C. pectinella, black* for *D. quadrisulcata and green* for *L. pensylvanica*

previously used to characterize symbiont metabolic pathways in chemoautotrophic associations: Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO [*cbbL*]) for chemoautotrophy and alpha subunit of adenylyl-1,5-bisphosphate reductase (APS Reductase [*aprA*]) for sulfur-oxidizing metabolism (Duperron et al. 2007). Due to the lack of genomic data for lucinid gill endosymbionts, sequences of the chosen markers were obtained by nested PCR on *C. orbicularis* (see below) except for RubisCO for which published primer sequences were used (Elsaied and Naganuma 2001).

Nested-PCR amplifications

Two-step nested-PCR amplifications were performed with DNA extracted from *C. orbicularis* gills. The first step was performed in 25 µL, using degenerated "nested-primers" (Table 1) and 10–25 ng of symbiont + host template DNA mixture. The second amplification was performed in the same conditions with "degN primers" instead of "nested-primers" and 1 µL of a 100-fold to 1000-fold dilution of the first amplification as a template. Degenerated "nested" and "degN" primers were designed in conserved sites flanking more variable regions. These sites were determined from an alignment of 50 bacterial sequences using SVARAP 2.0 (Colson et al. 2006).

Specific PCR amplifications

Specific primers were designed using Primer3 software (Rozen and Skaletsky 2000; http://primer3.ut.ee/). Bacterial sequences were amplified from host/symbiont DNA extracts using the primers and PCR conditions listed in Table 1. Amplification was performed in a final volume of 50 µL using 10–25 ng of DNA template, 1X Taq buffer (Invitrogen), 3 mM $MgCl_2$ (Invitrogen), 0.4 mM of each dNTP, 1 µM of each primer and 2 units of recombinant Taq DNA polymerase (Invitrogen). Amplicons were then directly sequenced (GATC biotech, www.gatc-biotech.com). Symbiont sequences for each gene were analyzed using DNAsp4 software (Rozas et al. 2003). Haplotype networks were realized using NETW4510© software (Fluxus Technology Ltd.) using the median-joining method (Bandelt et al. 1999).

Table 1 Primers and PCR conditions used for each gene analyzed

Primers				PCR conditions				
Name		Séquence (5′ → 3′)	Size	Expected size (bp)	Targeted gene	Optimal Tm (°C)	Cycles	Ref[b]
Host	18S-5′f	CTGGTTGATYCTGCCAGT	18	1000	18S rRNA	54	30	1
	18S-1100r	CTTCGAACCTCTGACTTTGG	20					2
	28S_LSU-900f	CCGTCTTGAAACACGGACCAAG	22	700	28S rRNA	52	40	3
	28S_LSU-1600r	AGCGCCATCCATTTTCAGG	19					2
Symbiont[a]	16Suniv-27f	AGAGTITGATCMTGGCTCAG	20	1465	*rrs*	54	30	4
	16Suniv-1492r	TACGGYTACCRRGTTACGACIT	21					
	ITS-degN-1242f	GCTACACACGTGCTACAATGG	21	~1000	*ITS1*	54	35	5
	ITS-degN-196r	TCTCGGTTGATTTCTTTTCTT	21					
	ITS-Symb-282f	GGAGTGGGTTGNAAAAGAAG	20	712		58	30	
	ITS-Symb-954r	ATCGCAAGCTACAACGTCCT	20					
	dnaE-nested-1069f	CCDGTDGGICCDGGICGYGGC	21	1171	*dnaE*	46	35	5
	dnaE-nested-2279r	TGNGATTTRTTRAANCCRTA	20					
	dnaE-degN-1180f	CCIGARCGKGTITCIATGCC	20	857		60	35	
	dnaeE-degN-2076r	YTGCATNACYTGYTCYTGRTA	21					
	dnaE-Symb-201f	GAAGATGATCCCCTTCGAGA	20	667		64	30	
	dnaE-Symb-810r	GTAGGAGACCTTGGCACGAC	20					
	gyrB-nested-217f	GHAARCGYCCDGGYATGTA	19	1409	*gyrB*	56	35	5
	gyrB-nested-1809r	CVACRTCVGCATCBGTCAT	19					
	gyrB-degN-277f	GGTDTTYGARRTDGTDGATAA	21	1130		56	35	
	gyrB-degN-1590r	CHGCNGARTCMCCYTCMAC	19					
	gyrB-Symb-185f	AGCCACCCGAAGAGAAGAA	19	932		58	30	
	gyrB-Symb-1150r	TGGCAGACCGGCTATGT	17					
	CBBL-1B	CACCTGGACCACVGTBTGG	19	800	*cbbL*	60	30	6
	CBBL-2C	CGGTGYATGTGCAGCAGCATGCCG	24					
	aprA-Symb-23f[c]	GCAACGACCTGATGGGTATC	20	532	*aprA*	58	33	5
	aprA-Symb-555r[c]	GTCGGTACGAAGCGGTTTT	19					

[a] "nested" and "degN" primers correspond to degenerated primers used in nested PCR. "Symb" primers correspond to specific primers designed using sequences obtained by nested-NCR

[b] References: 1 (Winnepenninckx et al. 1998), 2 (Williams et al. 2003), 3 (Olson et al. 2003), 4 (Lane 1991), 5 this study, 6 (Elsaied and Naganuma 2001)

[c] These primers were designed using sequence specific for symbiont a lucinid symbiont and obtained using method derived from TE walking (Piednoel and Bonnivard 2009) based on a "core sequence" amplified using APS-FW and APS-RV primers (Deplancke et al. 2000)

Sediment analysis

Total DNA from each core was extracted following protocols described by Zhou et al. (1996). A three-step nested PCR was performed using 50 ng of DNA template and "nested" primers (Table 1) as a first step. A 100-fold dilution of this PCR product was then used as a template for a second amplification with "degN" primers (Table 1), and the procedure was repeated for a last step with specific primers. Fragments of interest were then eluted from 1 % agarose gel using Nucleospin® Extract II kit (Macherey–Nagel) and cloned with pGEM-T® TA cloning kit (Promega) according to manufacturer's instructions. This vector was used to transform Dh5α-competent *Escherichia coli* (Promega), and plasmids from positive clones were collected using the Nucleospin® plasmid kit (Macherey–Nagel). Inserts were then sequenced directly using an ABI PRISM automated sequencer (Genome express, www.gexbyweb.com).

Host sequences

DNA extracted from symbiont-free foot tissue was used in order to amplify 18S rRNA and 28S rRNA genes. PCRs were performed in the same conditions as for symbiont-specific PCR amplification. PCR products were then directly sequenced (GATC biotech, gatc-biotech.com).

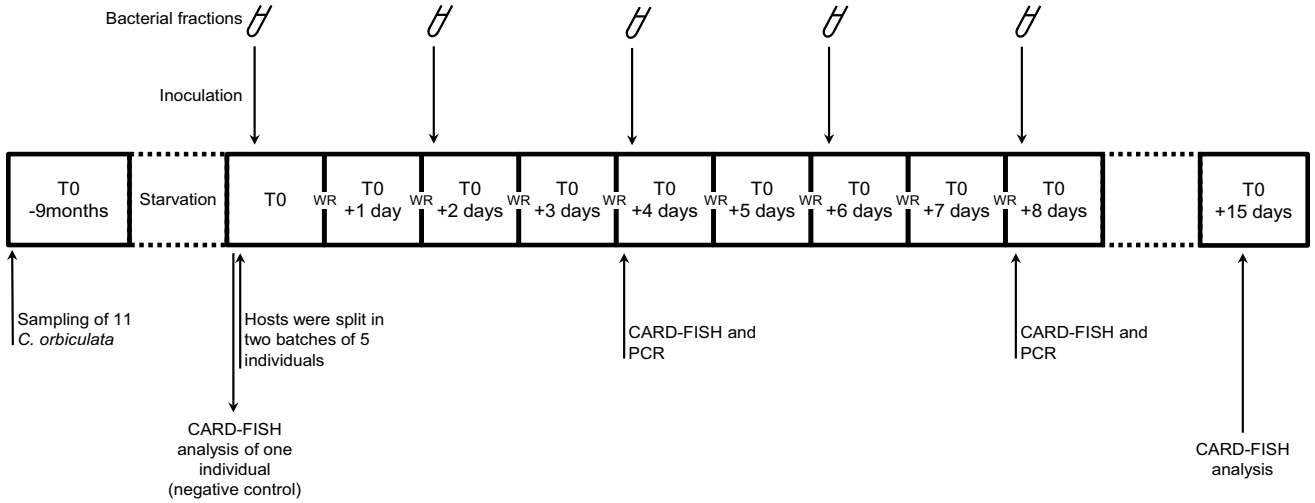


Fig. 2 Cross-infection experiments. Individuals of *Codakia orbiculata* starved for nine months (i.e., without detectable gill endosymbionts) were used in this experiment. These hosts were then separated in two batches and exposed to gill endosymbiont purified fractions from *C. orbicularis* or *C. orbiculata*, performed as Caro et al. (2009). Symbiont exposures were performed on alternate days, after water renewal (WR), using bacterial fractions prepared from freshly collected individuals. Every fourth day, one individual by batch, randomly chosen, was killed for molecular and CARD-FISH analyses as previously described (Brissac et al. 2009)

Phylogenetic analysis

Phylogenetic analyses were performed as described in Brissac et al. (2011).

Cross-infections experiments

We used eleven 9-month starved individuals of *C. orbiculata* (i.e., without gill endosymbionts detected) maintained in oxygenated tanks with no sulfur addition as previously described (Brissac et al. 2009; Caro et al. 2009). One of them was checked by PCR and CARD-FISH to ensure no detectable gill endosymbionts. The ten remaining symbiont-free hosts were separated into two batches and exposed to gill endosymbiont purified fractions from *C. orbicularis* or *C. orbiculata* prepared according to Caro et al. (2009). All purified fractions were tested using the *dnaE* marker in order to control the gill endosymbiont type present in each inoculum. The detailed protocol of the experiment is presented in Fig. 2.

Results

Symbiont 16S rRNA sequence analysis

All PCR amplifications performed from gills of *A. alba, C. orbicularis, C. orbiculata, C. pectinella, D. quadrisulcata* and *L. pensylvanica* yielded a single 16S rRNA bacterial sequence per host, without any ambiguity according to ABI chromatograph analysis. All sequences presented 100 % identity (1466 bp) with the *C. orbicularis* gill symbiont 16S rRNA sequence already described:X84979.1 (Durand and Gros 1996; Gros et al. 1996).

Host phylogeny

For all amplifications of 18S rRNA and 28S rRNA genes, a single band of the expected size (~1000 bp and ~700 bp, respectively) was observed. We analyzed the six host species mentioned above with species of Thyasiridae and Crassatellidae, two sister groups of Lucinidae (Williams et al. 2003), chosen as outgroups. According to this phylogeny (Fig. 3), the six host lucinids fall in the different clades described by Williams et al. (2003): Anodontia clade for *A. alba*, clade A for *C. orbicularis* and *C. orbiculata* and Clade B for *C. pectinella*, *D. quadrisulcata* and *L. pensylvanica*.

Symbiont polymorphism analysis

Polymorphism results are presented in Table 2. As expected, *ITS1*, *aprA* and *cbbL* variable markers present higher sequence variability than *dnaE* and *gyrB* housekeeping genes (2.14, 1.42, 1.96, 0.83 and 0.79 % of analyzed sites, respectively). An intraspecific bacterial diversity is demonstrated by haplotype networks constructed using sequences of 18 individuals (three per host species), which revealed four to six haplotypes per marker (Fig. 4). The different haplotypes were not randomly distributed among the 18 individuals analyzed. A common pattern was observed for all markers (Fig. 4 and Table 3): (1) a single,

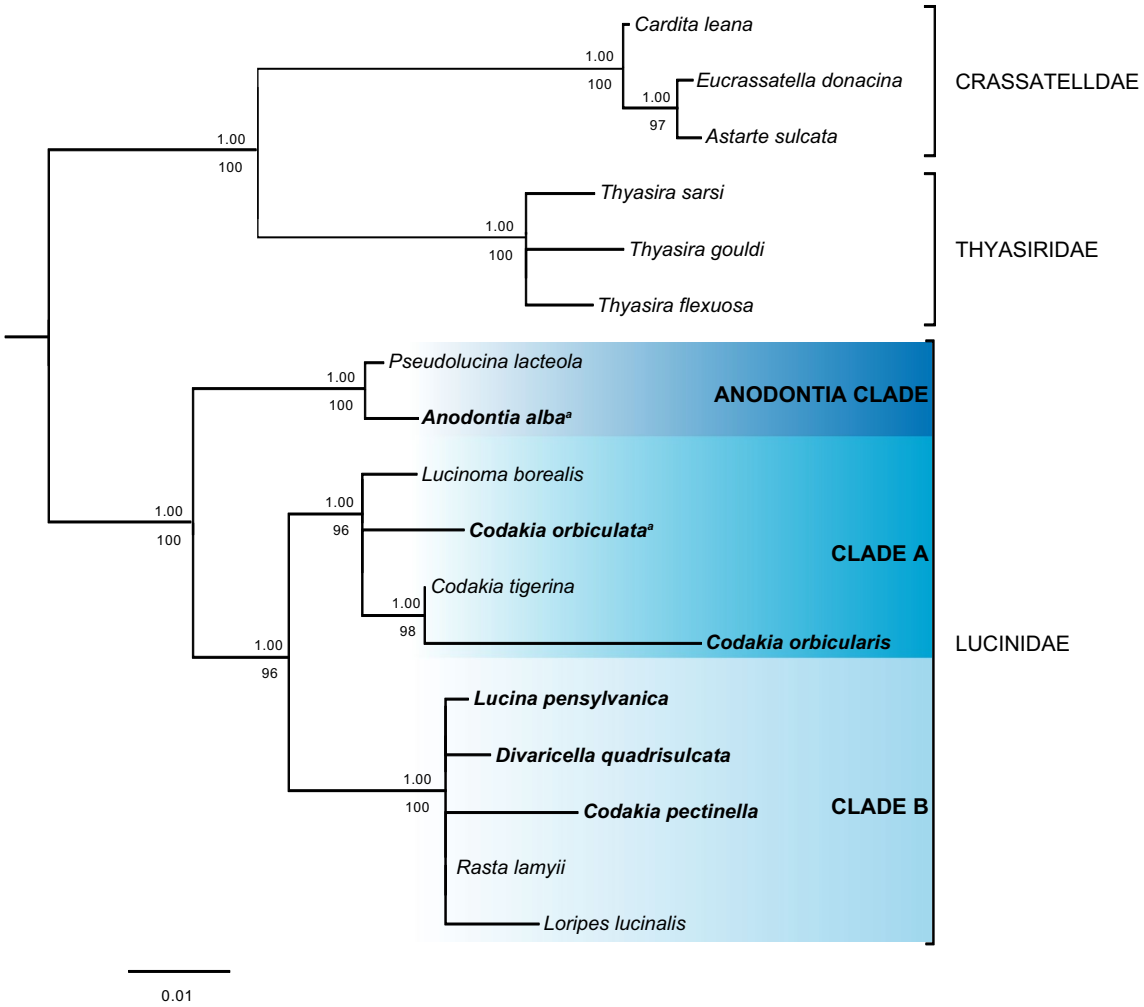


Fig. 3 Molecular phylogeny of Lucinoidea based on concatenated sequences from 18S and 28S rRNA genes (1391 bp). Tree reconstructed using maximum likelihood (ML) and Bayesian inference (BI) methods under TrNef + Γ (ML) and K80 + Γ (BI) evolutionary models with Crassatellidae and Thyasiridae as outgroups. For clade robustness, only bootstrap values (ML, 100 replicates [under branches]) and posterior probabilities (BI [above branches]) greater than 80 % were displayed. Sequences obtained in this study are in *bold*, with other sequences from Williams et al. (2003). [a]Sequences obtained herein that correspond to those previously published

Table 2 Polymorphism analysis data

Gene[a]	Sequence		Polymorphic sites[b]	Haplotypes	Haplotypes accession numbers
	Nb	Length (bp)			
dnaE (~3.5 kb)	18	481	4 (0.83 %)	4	KX250229/KX250230 KX250231/KX250232
gyrB (~2.4 kb)	18	764	6 (0.79 %)	5	KX250233/KX250234/KX250235 KX250236/KX250237
ITS1 (~550 pb)	18	512	11 (2.14 %)	5	KX250238/KX250239/KX250240 KX250241/KX250242
aprA (~1 kb)	18	491	7 (1.42 %)	6	KX250217/KX250218/KX250219 KX250220/KX250221/KX250222
cbbL (~1.5 kb)	18	612	12 (1.96 %)	6	KX250223/KX250224/KX250225 KX250226/KX250227/KX250228
Total	90	2860	40 (1.4 %)		

[a] gene names. *dnaE*: DNA polymerase III α-subunit, *gyrB*: DNA gyrase β-subunit, *ITS1*: 16S/23S internal transcribed spacer, *aprA*: adenylyl-1,5-bisphosphate reductase α-subunit, *cbbL*: Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit

[b] Percentages represent number of polymorphic sites in relation to the length of the considered fragment

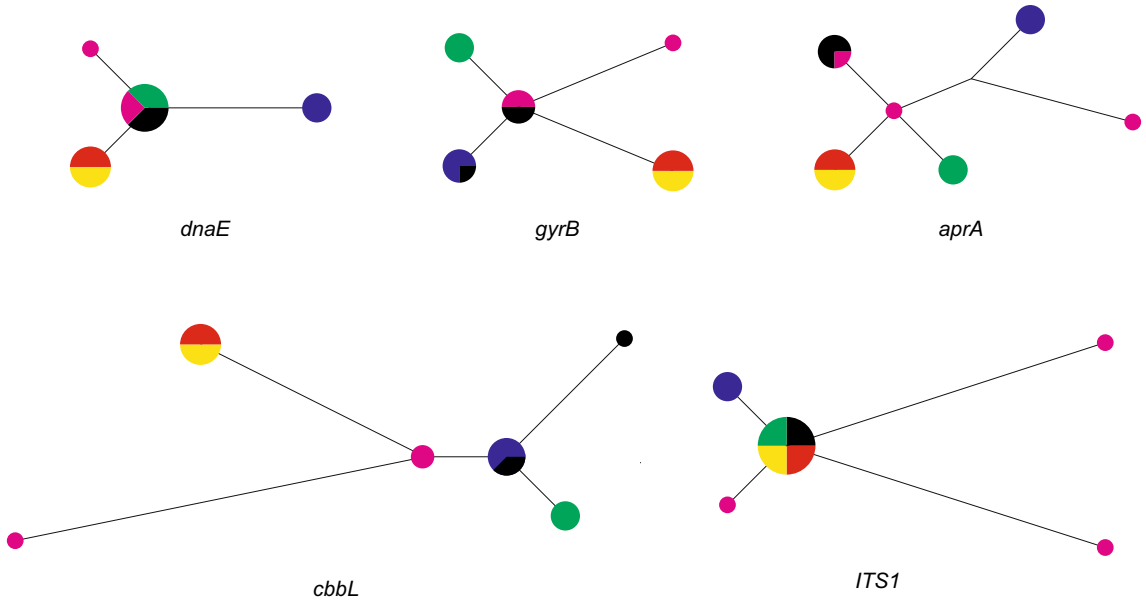


Fig. 4 Bacterial haplotype networks prepared based on sequences from MLST genes. Networks were reconstructed using median-joining method. *Circles* represent haplotypes, with size proportional to the number of sequences for each haplotype. *Colors* represent host species as in Fig. 1. *Branch length* represents the number of substitution between haplotypes

Table 3 Symbiotic type observed

Host species	Symbiont type[a]		
	Nb	Name	Haplotypes
Anodontia alba	3	ST_{ALBA}	a1 c3 d3 g3 i2 a3 c3 d3 g3 i4 a6 c2 d1 g4 i5
Codakia orbicularis	1	ST_{CIS}	a5 c4 d4 g2 i1
Codakia orbiculata	1	ST_{CATPEC}	a2 c1 d2 g6 i3
Codakia pectinella	1	ST_{CATPEC}	a2 c1 d2 g6 i3
Divaricella quadrisulcata	2	ST_{DIV}	a1 c3 d3 g3 i3 a1 c5 d3 g2 i3
Lucina pensylvanica	1	ST_{PEN}	a4 c6 d3 g1 i3

[a] Symbiont type (ST) is determined in function of the haplotype present in each individual for each gene marker: a: *aprA*, c:*cbbL*, d:*dnaE*, g:*gyrB*, i:*ITS1*

host-specific symbiont sequence in *C. orbicularis* and in *L. pensylvanica* symbionts (ST_{CIS} and ST_{PEN}, respectively), (2) a symbiont polymorphism in *A. alba* and in *D. quadrisulcata* (ST_{ALBA1} ST_{ALBA2}, ST_{ALBA3}, ST_{DIV1} and ST_{DIV2}) and (3) a single, shared bacterial haplotype found in *C. orbiculata* and *C. pectinella* (ST_{CATPEC}).

Symbionts of *C. orbicularis* and *C. orbiculata* from Martinique had different *dnaE* sequences than symbionts from the same host species found in Ilet Cochon (two and four mismatches in relation to ST_{CIS} and ST_{CATPEC}, respectively).

Sequencing of *dnaE*, *gyrB* and *aprA* genes for three *C. orbicularis* subadults (0.5 cm shell length) indicated that subadults harbored exactly the same symbiont type (ST_{CIS}) as adults (100 % of sequence identity).

The sediment layer comprised between 0 and 5 cm was also analyzed using the *dnaE* marker. After sequencing, we observed clones with sequences corresponding to *C. orbicularis* symbiont from Ilet Cochon (ST_{CIS}).

Cross-infections

After nine months of starvation, no symbiont could be detected in *C. orbiculata* gills by PCR or CARD-FISH (Fig. 5b) attesting to the loss of symbionts as previously described (Gros et al. 2012). After four days of exposure, gills from starved *C. orbiculata* individuals exposed to *C. orbiculata* symbionts were colonized (Fig. 5d), while gills from *C. orbiculata* exposed to *C. orbicularis* symbionts appeared to be totally symbiont-free (Fig. 5c). The same pattern was observed at $T_0 + 8$ days (Fig. 5e, f) and at $T_0 + 12$ or 15 days (data not shown).

Sequences obtained by PCR/sequencing from purified fractions of *C. orbicularis* symbionts were identical to those observed during polymorphism analysis (i.e., ST_{CIS}). The observation was the same for purified fractions obtained from *C. orbiculata* hosts (ST_{CATPEC}).

Post-experiment control performed by PCR/sequencing of *C. orbiculata* reinfected gills revealed the presence of a bacterial sequence identical to those obtained during

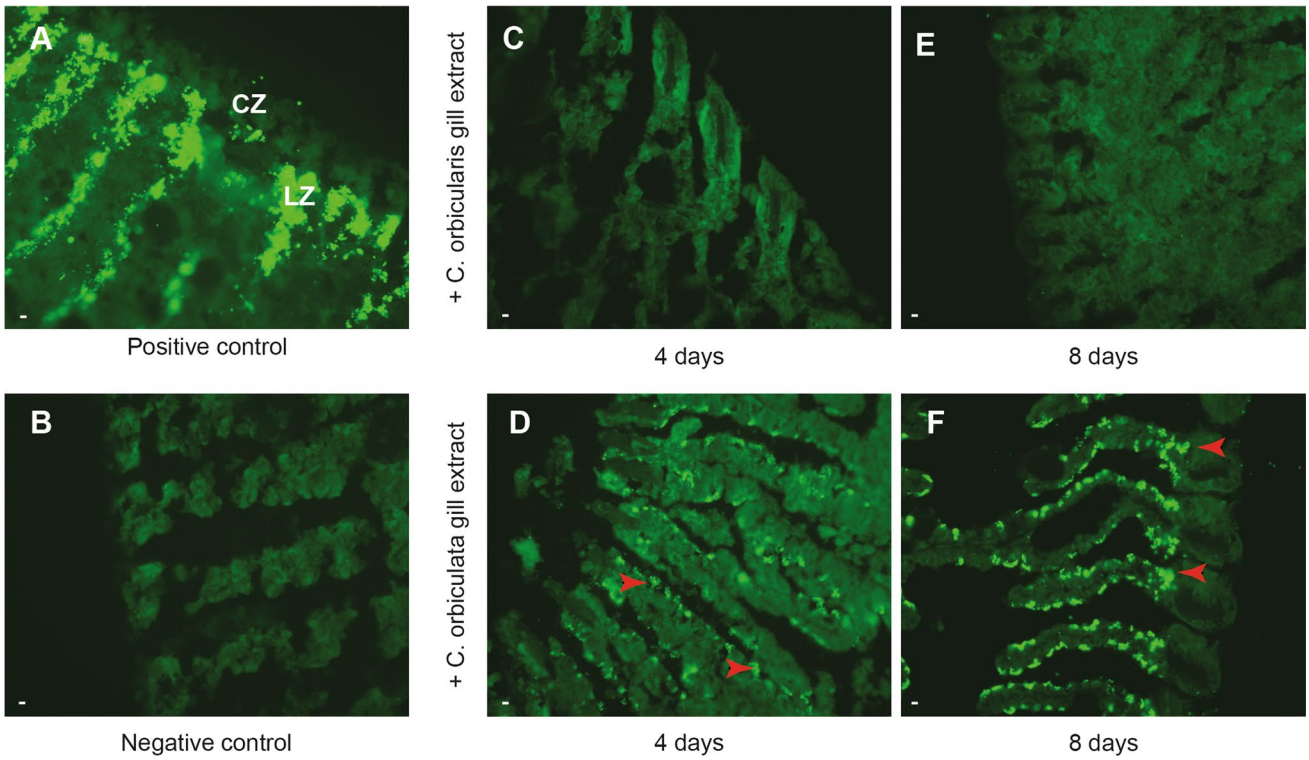


Fig. 5 CARD-FISH using symco2A probe on starved *Codakia orbiculata* individuals after exposure of purified symbionts from *C. orbicularis* or *C. orbiculata*. **a** Gill section of freshly collected *C. orbiculata* (CARD-FISH *positive control*). **b** Gill section of *C. orbiculata* after 9 months of starvation (*negative control*). **c** and **d** Gill sections of starved *C. orbiculata* after addition of *C. orbicularis* gill extract after 4 days (**c**) or 8 days (**d**). **e** and **f** Gill sections of starved *C. orbiculata* after addition of *C. orbiculata* gill extract after 4 days (**e**) or 8 days (**f**). *Scale bars* = 10 µm. Ciliated zone (CZ). Lateral zone (LZ). *Arrows* shows fluorescently labeled symbionts

polymorphism and inoculum analyses (i.e., ST_{CATPEC}). Within *C. orbiculata* gills remaining free of symbionts despite being exposed to purified fractions from *C. orbicularis*, no amplification was obtained.

Discussion

The purpose of this study was to determine the extent genetic diversity of sulfur-oxidizing gill endosymbionts associated with six lucinid species inhabiting *T. testudinum* seagrass beds in Lesser Antilles. We performed analyses at two scales: (1) a geographic level, since sampling was conducted in three islands separated by 50–200 km (i.e., La Désirade, Martinique and Guadeloupe) and (2) a local level since four host species (*A. alba, C. orbicularis, C. orbiculata* and *C. pectinella*) are sympatric within Ilet Cochon seagrass bed. We obtained almost complete 16S rRNA gene symbionts' sequences and confirmed that the same bacterial species is harbored by these six host species (Durand and Gros 1996, Gros et al. 1996; 2000, 2003a; Brissac et al. 2011).

A structured strain genetic diversity is evident among lucinid gill endosymbionts from *T. testudinum* seagrass beds from Lesser Antilles

We used five markers in this study, classified in three categories according to Cooper and Feil (2004): (1) two housekeeping genes, (2) two core metabolism genes and (3) one hypervariable gene. Unlike data obtained by sequencing 16S rRNA gene, the use of these five markers allowed us to observe an expected sequence divergence among the lucinid symbionts investigated. Mean polymorphism was near 1.5 %, which is consistent with literature data (Zhang and Hewitt 2003) even if sequence diversity can be underestimated due to the technique used here (i.e., PCR product direct sequencing): in the event of multi-infections within a host individual, it is possible that only the most abundant symbiont would be detected. However, as described previously (Won et al. 2003), direct sequencing of PCR purified templates can allow the observation of multi-infections. Thus, we assume that the diversity observed here is real and not biased and no multi-infection occurs within Lucinidae as previously suggested (Brissac et al. 2011).

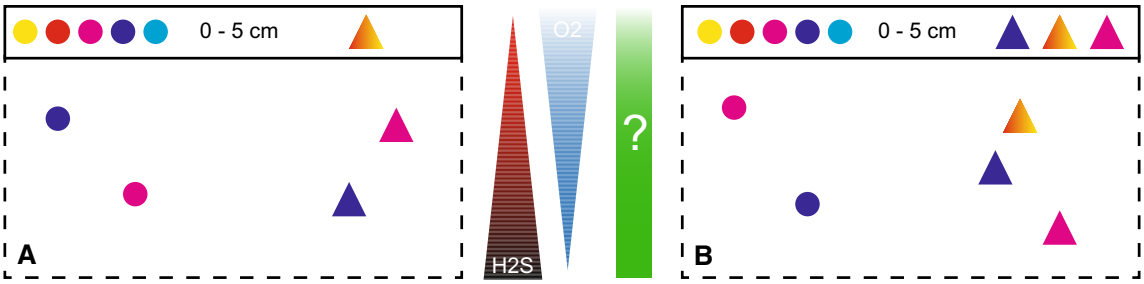


Fig. 6 Hypothetical distributions of symbionts in *Thalassia testudinum* seagrass beds from Ilet Cochon (Guadeloupe). **a** According to the ecotype hypothesis, the bacterial population is structured in function of oxygen, sulfur or other gradient. **b** Alternative distribution of the bacterial population as our experiments revealed no structuration of the bacterial population. *Circles* correspond to hosts and *triangles* to symbionts. *Circles and triangles* colors correspond to host species as in Fig. 1, with *pale blue* for juveniles of *Codakia orbicularis*

To examine whether the diversity of sulfur-oxidizing gill endosymbiont in lucinids from Lesser Antilles is structured, we built haplotype networks marker sequences. We found that bacterial diversity is structured since, regardless of the marker, haplotypes are not randomly distributed among individuals (Fig. 4). Moreover, even if haplotype networks were not entirely congruent, similar patterns were observed for each of them. The hosts *C. orbiculata* and *C. pectinella* shared the same symbiont type (i.e., ST_{CATPEC}). *C. orbicularis* and *L. pensylvanica* were specifically associated with their own symbiont type (i.e., ST_{CIS} and ST_{PEN}, respectively). *Anodontia alba* and *D. quadrisulcata* individuals harbored different symbiont types (i.e., ST_{ALBA1}/ST_{ALBA2}/ST_{ALBA3} and ST_{DIV1}/ST_{DIV2}, respectively).

How can the structuration of the symbiotic diversity associated to Lucinidae from Lesser Antilles be explained?

The bacterial diversity associated to lucinids from Lesser Antilles being structured; we sought to determine whether this structuration could be due to geography, environmental conditions and/or host diversity. If the genetic structuration observed is due to geography, host species inhabiting the same geographic location would be expected to share same symbiont type while hosts from different locations would be colonized by different symbiont types. The comparison between symbiont haplotype networks and host geographic locations indicates that the geographic component seems to play a role in the structuration of the bacterial diversity. This is based on our observation that *D. quadrisulcata* and *L. pensylvanica* from la Désirade and la Martinique, respectively, harbor their own symbiont types. This notion is reinforced by the analysis of *C. orbicularis* and *C. orbiculata* specimens collected from Martinique, which harbor different bacterial types from those associated with the same host species collected in Ilet Cochon (Guadeloupe).

Several studies have been conducted on associations between bacterial symbionts and marine invertebrates such as Siboglinidae and Mytilidae. In these studies, the authors describe the influence of geography (Di Meo et al. 2000), host genus (Di Meo et al. 2000) or ecological habitat (Feldman et al. 1997; Di Meo et al. 2000; Won et al. 2003; Vrijenhoek et al. 2007) on host–symbiont relationships. Within the Lucinidae, the geographic distribution of hosts is not sufficient to explain the structuration of the symbiotic diversity since the four sympatric host species from Ilet Cochon do not share the same symbiont type. Consequently, other factors may explain the particular situation observed in Ilet Cochon.

The fact that *C. orbiculata* and *C. pectinella* share the same symbiont strain led us to propose an ecotype hypothesis to explain the structuration of symbiont diversity observed in Ilet Cochon. This hypothesis is built on the assumption that bacterial strains could be clustered according to ecological parameters like porewater sulfur and oxygen concentrations through the sediment or other chemical gradients (Fig. 6a). Thus, the bacterial structuration observed among hosts would mimic the structuration of bacterial populations in the environment. Consequently, hosts localized at the same depth of the sediment column should harbor the same bacterial strain, as is the case for *C. orbiculata* and *C. pectinella* (Fig. 4) which are found only within the first 5 cm of the sediment column due to their short siphon (Allen 1958). Under this hypothesis, only this bacterial type (ST_{CATPEC}) should be retrieved in the upper layer of the sediment or in any clam found in this upper layer. To test this hypothesis, we analyzed the upper layer of sediment and subadults of *C. orbicularis* (up to 5 mm shell length) sampled in this layer. Our results refute the ecotype hypothesis since we have detected free-living forms of *C. orbicularis* symbionts (ST_{CIS}) in the upper layer of sediment. Moreover, juveniles (~0.3 mm in shell length) and subadults of *C. orbicularis* (1–10 mm in shell length) harbor the same bacterial type (ST_{CIS}) as adults (which are

distributed throughout the sediment layer down to a depth of 20 cm). Thus, it seems that bacterial populations are not structured as a function of sediment depth (Fig. 6b).

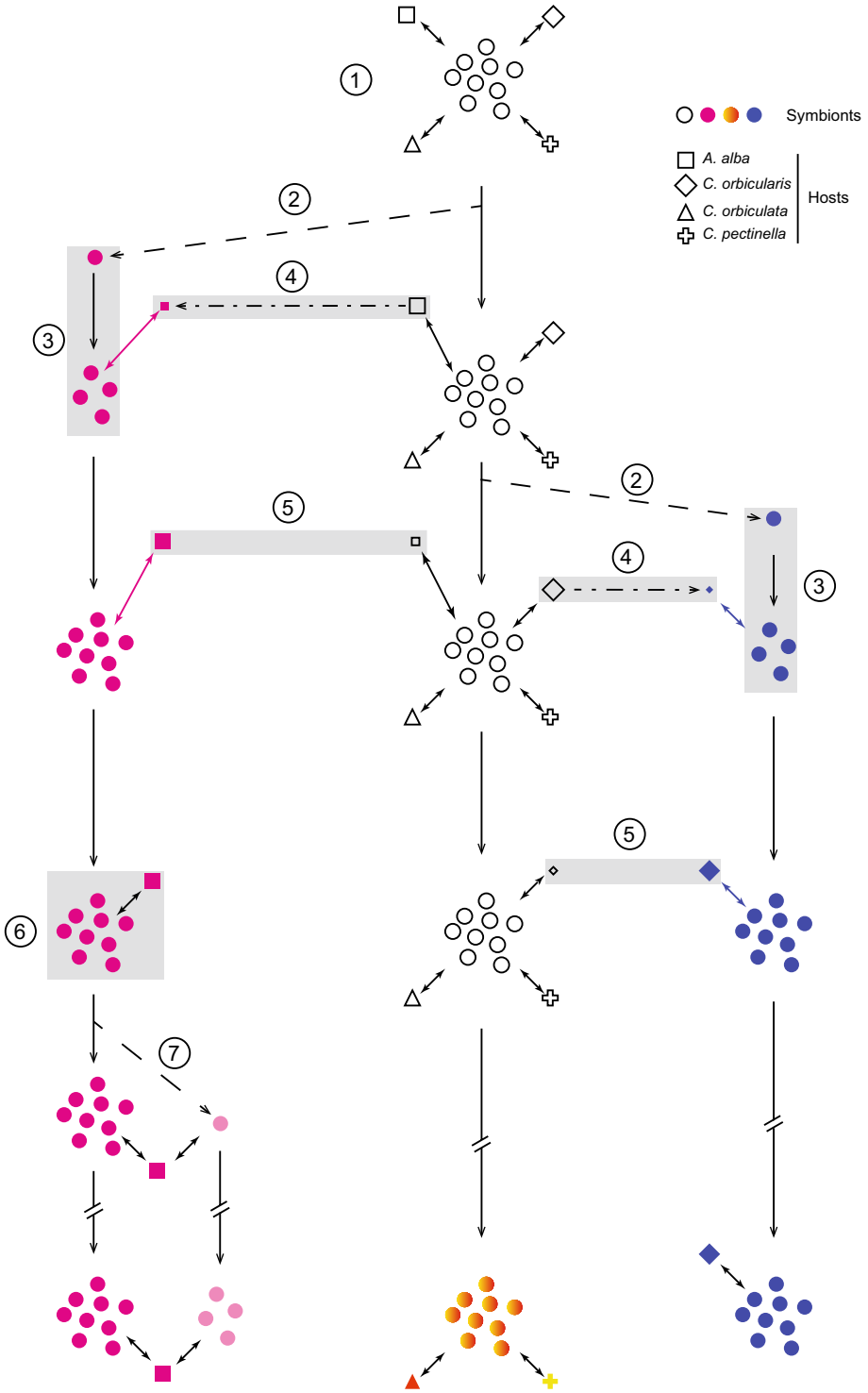
An alternate coevolution hypothesis could explain the structuration of symbiont diversity. According to this hypothesis, we assume that structuration of the bacterial population observed today is the result of ancient associations which have coevolved such that host–symbiont couples were formed. Under this scenario, closely related host species should harbor closely related symbiont strains. This second hypothesis is also rejected here because this pattern is not observed when comparing host phylogeny (Fig. 3) and structured bacterial diversity (Fig. 4). Indeed, the two hosts sharing the same bacterial haplotype (*C. orbiculata* and *C. pectinella* [Fig. 4, yellow/red circle][Table 3, $ST_{CAT-PEC}$]) are not the closest relatives and belong, respectively, to lucinid clades A et B (Fig. 3).

Since "ecotype" or "coevolution" hypotheses fail to explain the observed structuration of bacterial diversity within lucinid hosts from Ilet Cochon seagrass beds, we propose that this specific pattern could be explained by a local selective process between host species and the free-living symbiotic bacterial pool. Figure 7 present a hypothesis explaining the observed structuration. In this case, symbionts evolve to escape from hosts while the latter evolve to keep exploiting symbionts according to an antagonistic evolutionary process.

Our model assumes that there is intraspecific control during the acquisition of symbionts but also the ability to associate with any bacterial strain. In order to test these features, we realized cross-infection experiments using starved *C. orbiculata*. As shown in Fig. 5, we observed a new uptake of symbionts only in starved *C. orbiculata* exposed to *C. orbiculata* gill endosymbiont purified fractions. These results reinforce the intraspecific control hypothesis during symbiont acquisition within lucinid bivalves and suggest the presence, in the lateral zone of the gill filament, of specific bacteriocytes. The latter allow only the recognition and the internalization of specific strains encountered during the first exposure to symbiosis-competent bacterial strains during post-larval development. Evidence in support of this hypothesis comes from a report where the authors studied the cellular organization of the gill filament concurrently with symbiont acquisition/loss kinetics in adult individuals of *C. orbicularis* (Elisabeth et al. 2012). According to this study, there is no obvious apoptosis of bacteriocytes in starved adults but only a decrease in their volume. During symbiont reacquisition phases, these authors observed an increase in the volume occupied by the bacteriocytes (larger than the initial state) linked with massive symbiont acquisition followed by the apoptosis of supernumerary bacteriocytes (compared to the number of bacteriocytes before starvation) in order to recover homeostasis.

Fig. 7 Schematic representation of evolutionary process which could lead to the pattern of structuration observed in *Thalassia testudinum* seagrass bed of Ilet Cochon. The final step shows the structured observed pattern with MLST analysis. Step ①: This first step corresponds to an initial equilibrium state where the four host species exploit the same bacterial pool. Step ②: A mutant who is no longer recognized by the hosts appears. This is could be due to a mutation of the bacterial determinants that are recognized by the host. Step ③: Hosts do not recognize this mutant, and its proportion increases in the environment. In fact, the fitness of this new mutant is superior to the fitness of the original one because this symbiont escapes to the evolutionary dead-end constituted by internalization (Brissac et al. 2009). Step ④: Appearance of mutant hosts which can recognize the new symbionts. Step ⑤: These new host variants exploit the new free-living symbiont bacterial pool, while their conspecifics remain restricted to exploiting the ancient bacterial pool. The frequency of these new host individuals increases due to a selective advantage. This new bacterial strain could be more efficient in carbon fixation, H_2S detoxification or energetic conversion. Therefore, host associated with this new strain have a greater fitness than those associated with the previous strain. Moreover, the new association could decrease "competition for symbionts." Step ⑥: A new specific association is formed between the mutant hosts and symbionts. Step ⑦: Some bacterial mutants for characters that are not involved in the recognition could appear and continue to be captured by the host. This latter step could explain the specificity observed here and the presence of polymorphic symbionts (*e.g.,* within *A. alba*). *Circles* represent bacterial pools. *Squares, crosses, triangles and diamonds* represent host species. *Colors* identify specific associations. *Double arrows* represent established associations. *Dotted double arrows* represent host species where individuals exploit different bacterial pools. *Dashed arrow* represents appearance of a bacterial mutant. *Dashed/dotted arrow* represents appearance of a host capable of exploiting escaped mutants

However, Gros et al. (2003a) tried to infect naive aposymbiotic juveniles of *C. orbicularis* (i.e., obtained from induced spawning in the laboratory) using gill endosymbiont purified fractions obtained from the six lucinid host species used in the present study. In this case, juveniles were successfully colonized whatever the "donor" host species used may be. In such juveniles, large undifferentiated cells distributed throughout the lateral zone could acquire any symbiosis-competent strain and differentiated into mature bacteriocytes (Gros et al. 1997). These undifferentiated cells may be considered as naïve against symbiont strains. Thus, we hypothesize that naive aposymbiotic juveniles may acquire any symbiosis-competent strain due to the presence of undifferentiated cells distributed throughout the lateral zone and considered as naive bacteriocyte precursors (Gros et al. 2003a). Finally, we hypothesize that only bacteriocytes associated with bacterial strains providing the best energetic yield (bacterial metabolism has been shown to support a large fraction of the host's needs (Fisher 1990)) are kept through an apoptosis process similar to those observed during reacquisition (see above). In contrast, in adults, specific bacteriocytes are conserved (with their "memory" for one bacterial strain) during the bivalve's life leading to a tight specificity of association between the host and its specific symbiont strain. Thus, the



putative "plasticity of association" with any bacterial strain by the juveniles could explain the "symbiont switch" ability proposed in our model, the associations being selected in function of the fitness of the "bacteriocyte/bacterial strain" couple and by extension host species/bacterial strain.

In summary, we analyzed strain bacterial diversity associated within lucinids from the same biotope (i.e., seagrass beds) and from different geographic locations in the Lesser Antilles. This analysis revealed that geography influences the structuration of the bacterial diversity, as described within the Mytilidae (Di Meo et al. 2000). Unexpectedly, at the local level (i.e., same seagrass bed), it seems that there is a specificity of association between a host species and a particular bacterial strain. This specificity is neither

due to segregation of symbionts according to ecological parameters nor due to a tight coevolution of hosts and symbionts. However, it seems that an antagonist evolution process could explain this pattern due to selective pressures on hosts and symbionts. In fact, under a "dead-end" evolutionary hypothesis proposed (Brissac et al. 2009) and recently suggested in another symbiotic model involving protists and microalgae (Decelle et al. 2012), symbionts evolve to escape capture by the host. On the opposite side, eukaryotic hosts must evolve to continue exploiting bacterial symbionts. In fact, symbionts confer to the hosts a new autotrophic metabolic ability (i.e.,i chemoautotrophy) usually restricted to photosynthetic eukaryotes.

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Compliance with ethical standards

Conflict of interest Terry Brissac declares that he has no conflict of interest. Dominique Higuet declares that he has no conflict of interest. Olivier Gros declares that he has no conflict of interest. Hervé Merçot declares that he has no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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