Ancient Occasional Host Switching of Maternally Transmitted Bacterial Symbionts of Chemosynthetic Vesicomyid Clams

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Abstract

Vesicomyid clams in deep-sea chemosynthetic ecosystems harbor sulfur-oxidizing bacteria in their gill epithelial cells. These symbionts, which are vertically transmitted, are species-specific and thought to have cospeciated with their hosts. However, recent studies indicate incongruent phylogenies between some vesicomyid clams and their symbionts, suggesting that symbionts are horizontally transmitted. To more precisely understand the evolution of vesicomyid clams and their symbionts, we compared the evolution of vesicomyid clams and their symbionts through phylogenetic analyses using multi-gene data sets. Many clades in the phylogenetic trees of 13 host species (*Abyssogena mariana, Ab. phaseoliformis, Akebiconcha kawamurai, Calyptogena fausta, C. laubieri, C. magnifica, C. nautilei, C. pacifica*, *Isorropodon fossajaponicum, Phreagena kilmeri, Ph. okutanii, Ph. soyoae*, and *Pliocardia stearnsii*) and their symbionts were well resolved. Six of the 13 host-symbiont pairs (*C. fausta*, *C. magnifica, C. pacifica, Ph. kilmeri, Ph. okutanii*, and *Ph. soyoae*, and their respective symbionts) showed topological congruence. However, the remaining seven pairs (*Ak. kawamurai, Ab mariana, Ab. phaseoliformis, C. laubieri, C. nautilei*, *I. fossajaponicum*, and *Pl. stearnsii* and their corresponding symbionts) showed incongruent topologies, which were supported by the approximately unbiased and Bayes factor tests. Coevolution analyses indicated that six pairs cospeciated, whereas host switching events occurred in the remaining seven pairs. Markedly, multiple host switching events may have occurred in the lineages from the common ancestral symbiont of *C. pacifica* and *C. fausta.* Our phylogenetic and coevolution analyses provide additional evidence for host switching during the evolution of vesicomyids.

Key words: cospeciation, endosymbiosis, host switching, phylogenetic tree, vertical transmission.

Introduction

Chemosynthetic ecosystems in hydrothermal vents and seeps are dominated by a wide variety of invertebrates, many of which establish symbiosis with chemosynthetic bacteria. The hosts nutritionally depend on the organic compounds synthesized by the chemosynthetic symbionts, as shown by stable isotope analyses (Le Pennec et al. 1995). Therefore, acquisition of symbionts is key for the host's survival (Bright and Bulgheresi 2010). The symbionts are transmitted from one generation to the next through either vertical or horizontal transmissions (Dubilier et al. 2008; Bright and Bulgheresi 2010). In vertical transmission, the symbionts are directly transferred from the parent to the offspring *via* the host's egg or sperm, and most of the symbionts are maternally inherited. In horizontal transmission, the symbiont is acquired from the environment during the host's development. Horizontal transmission of the symbiont is independent of the host's reproduction. The phylogenetic trees of hosts and horizontally transmitted symbionts are incongruent in their topology (Won et al. 2008; Bright and Bulgheresi 2010),

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whereas those of hosts and vertically transmitted symbionts are congruent (Peek et al. 1998; Bright and Bulgheresi 2010). The latter suggests cospeciation of hosts and their symbionts (Peek et al. 1998).

The deep-sea clams belonging to the family Vesicomyidae are endemic to chemosynthetic ecosystems and are dominant members of these ecosystems. Over 100 extant vesicomyid species have been identified worldwide (Krylova and Shaling 2010). Traditionally, Vesicomyidae has been classified into two genera, *Calyptogena* and *Vesicomya*. However, species of *Calyptogena* (*sensu lato*) have recently been classified into as many as 15 distinct genera (Krylova and Sahling 2010; Decker et al., 2012). Vesicomyid clams harbor intracellular sulfur-oxidizing bacterial symbionts in the gill epithelial cells; these symbionts are species-specific and are generally believed to be transmitted to the offspring vertically via eggs (Endow and Ohta 1990; Cary and Giovannoni 1993; Ikuta et al. 2016a). However, Stewart et al. (2008) reported that the positions of several host species and their symbionts in their respective phylogenetic trees were incongruent, suggesting that horizontal transmission may have occurred between host-symbiont pairs in the course of evolution. In addition, two phylogenetically distant vesicomyid species, *Christineconcha regab* and *Laubiericoncha chuni*, which cooccur in the same aggregations, each harbor small populations of the other species' symbionts in the gills, along with much larger populations of their original symbiont species (Decker et al. 2013). These findings suggest that at least a few of the symbionts in vesicomyid clams are occasionally transmitted horizontally, and that not all of the vesicomyid clams and their symbionts have coevolved.

In previously reported phylogenetic trees of both the vesicomyid clams and their symbionts, many nodes were poorly supported, owing to the small gene data sets used for the phylogenetic analyses (Peek et al. 1998). Thus, larger gene data sets are required to reveal the more detailed phylogenetic relationships of the clams and their symbionts. To date, complete genomes of the symbionts from two vesicomyid clams, *Phreagena okutanii* and *Calyptogena magnifica*, have been sequenced (Kuwahara et al. 2007; Newton et al. 2007). In addition, eight gene sequences [16S rRNA, 23S rRNA, UTP-glucose-1-phosphate uridylyltransferase (*galU*), Chaperonin EL subunit (*groEL*), Chaperonin ES subunit (*groES*), transcription-repair coupling factor (*mfd*), excinuclease ABC subunit A (*uvrA*), and DNA helicase II/ATP-dependent (*uvrD* paralog)] have been determined for several other vesicomyid clam symbiont species (Kuwahara et al. 2011; Shimamura et al. 2017). With regard to hosts, the mitochondrial genomes of five vesicomyid clams were sequenced recently (Liu et al. 2016; Ozawa et al. 2017). Mitochondrial genomes are frequently used to resolve phylogenetic relationships within a metazoan group, because they have relatively high mutation rates and generally contain the 13 protein-coding genes, lacking introns, within one compact genome, providing easily accessible gene sequences.

To understand the evolution of vesicomyid clams and their symbionts, the evolutionary events in 13 vesicomyid clams (*Abyssogena mariana, Ab. phaseoliformis, Akebiconcha kawamurai, Calyptogena fausta, C. laubieri, C. magnifica, C. nautilei, C. pacifica*, *Isorropodon fossajaponicum, Phreagena kilmeri, Ph. okutanii, Ph. soyoae*, and *Pliocardia stearnsii*) and their symbionts were analyzed by comparing the phylogenetic trees of concatenated large sequence data sets composed of 11 mitochondrial genes and eight symbiont genes. Our findings strongly suggest that host switching of symbionts has occurred, based on the topological incongruence between the phylogenetic trees of vesicomyid clams and their corresponding symbionts. On the basis of our current knowledge of the intracellular symbiosis between vesicomyid clams and their symbionts, we discuss the possible mechanism of host switching in vesicomyid clams.

Materials and Methods

Sample Collection and DNA Extraction

Ten vesicomyid clam species (*Ab. mariana*, *Ak. kawamurai, C. fausta, C. laubieri, C. nautilei, C. pacifica*, *I. fossajaponicum Ph. kilmeri, Ph. soyoae*, and *Pl. stearnsii*) were collected from seeps, using a Deep Submergence Vehicle (DSV) or Remotely Operated Vehicle (ROV), and immediately stored at −80 °C in a freezer until analysis (table 1). For DNA extraction, after the clam samples were thawed, the mantle and/or gill were dissected and cut into small pieces on ice. Total DNA was isolated from ∼25 mg of each tissue using a DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and isolated DNA was stored at −20 °C. All of these experiments were conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan).

Polymerase Chain Reaction (PCR) Amplification and Sequencing

The mitochondrial genomes in five vesicomyid clams (*Ab. mariana*, *Ab. phaseoliformis, C. magnifica, I. fossajaponicum*, and *Ph. okutanii*) used in the present study were recently sequenced (Liu et al. 2016; Ozawa et al. 2017). We obtained the sequences of 11 mitochondrial genes [ATP synthase F0 subunit 6 (*atp6*), ATP synthase F0 subunit 8 (*atp8*), cytochrome b (*cob*), cytochrome c oxidase subunit I (*cox1*), cytochrome c oxidase subunit II (*cox2*), cytochrome c oxidase subunit III (*cox3*), NADH dehydrogenase subunit 1 (*nad1*), NADH dehydrogenase subunit 3 (*nad3*), NADH dehydrogenase subunit 4 (*nad4*), NADH dehydrogenase subunit 5 (*nad5*), and large subunit ribosomal RNA gene (*rrnL*)] from the remaining eight vesicomyid clams (*Ak. kawamurai, C. fausta, C. laubieri, C. nautilei, C. pacifica*, *Ph. kilmeri,*

Table 1
Vesicomyid Clam Species Used in the Present Study

Species	Date of Collection	Depth (m)	Latitude	Longitude	Location	Collection Method	Dive No	No of Samples
Abyssogena mariana	9/9/2013	5633	11.65 N	143.04 E	Mariana Trench	DSV *Shinkai 6500*	YK13-08 6K #1362	1
Isorropodon fossajaponicum	5/31/2006	6181	40.10 N	144.18 E	Japan trench	DSV *Shinkai 6500*	YK06-05 6K #950	1
Akebiconcha kawamurai	6/13/2005	608	34.08 N	137.79 E	Daini Tenryu Knoll, Nankai Trough	DSV *Shinkai 6500*	YK05-08 6K#881	1
Calyptogena fausta	6/10/1996	1490	34.92 N	138.65 E	Suruga Bay	DSV *Shinkai 2000*	NT96-09 2K#869	1
Calyptogena laubieri	8/23/1997	3761	33.65 N	137.91 E	Nankai Trough	ROV *Kaiko*	KR97-05 Kaiko#45	1
Calyptogena nautilei	6/18/2005	3257	32.58 N	134.69 E	Off Muroto, Nankai Trough	DSV *Shinkai 6500*	YK05-08 6K#884	1
Calyptogena pacifica	3/29/1996	659–683.5	36.77 N	122.05 W	Monterey Bay	ROV *Ventana*	dive #1037	1
Phreagena kilmeri	3/25/1996	900	36.73 N	122.04W	Monterey Bay	ROV *Ventana*	dive #1032	1
Phreagena soyoae	1/14/2010	1171	35.00 N	139.23 E	Off Hatsushima, Sagami Bay	ROV *Hyper-Dolphin*	NT10-01 HPD#1074	1
Pliocardia stearnsii	3/29/1996	659–683.5	36.77 N	122.05 W	Monterey Bay	ROV *Ventana*	dive #1037	1

Ph. soyoae, and *Pl. stearnsii*). To obtain the partial mitochondrial genome sequences, including those of 11 mitochondrial genes, three or four PCR primer sets per vesicomyid species were designed based on the consensus mitochondrial genome sequences (table 2). With regard to the symbionts, we used seven sets of PCR primers for eight symbiont gene sequences [16S rRNA (Lane 1991), 23S rRNA (Kuwahara et al. 2011), and *galU*, *groEL, groES, mfd, uvrA*, and *uvrD* paralog (Shimamura et al. 2017)] from the symbiont of *Ab. mariana* and one primer set for the *galU* gene sequence from the symbiont of *I. fossajaponicum* (table 2). The mitochondrial or symbiont gene fragments were amplified in 25-μL PCR reactions that contained ~100 ng DNA template, 1× LA-Taq buffer (Mg^{2+} plus; TaKaRa Bio, Inc., Shiga, Japan), 0.25 mM each deoxynucleotide (dNTP) solution, 0.2 μM each primer, and 0.625 U LA-Taq polymerase (TaKaRa Bio, Inc.). The PCR reaction mixtures were incubated under the conditions described in table 2 after the initial denaturation step (96 °C for 2 min), using a TaKara TP600 Thermal cycler (TaKaRa Bio, Inc.). To check the size of the PCR-amplified fragments, 2.0-μL aliquots of the PCR reaction mixtures were electrophoresed on 0.6–1.0% agarose gels and stained with 0.06% ethidium bromide. The unincorporated primers and dNTPs in the PCR reaction mixtures were removed as described previously (Werle et al. 1994). The amplified DNA fragments were then directly sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and either an ABI PRIZM 3130xl or 3730xl Genetic Analyzer (Life Technologies), according to the manufacturer's instructions.

Assembly and Analysis of DNA Sequences

The nucleotide sequences of the PCR-amplified fragments were assembled using Sequencher Ver 4.10.1 (Gene Codes Co., MI, USA). The open-reading frames of the protein-coding genes were then determined using Sequencher Ver 4.10.1 and identified using BLASTP against the NCBI nonredundant protein sequence database. The *rrnL* gene was identified using BLASTN against the NCBI nonredundant nucleotide sequence database. The sequences of the host mitochondrial genes and symbiont genes were submitted to the DNA Data Bank of Japan.

Phylogenetic Analyses

Phylogenetic analyses of the 13 vesicomyid clam species (*Ab. mariana, Ab. phaseoliformis, Ak. kawamurai, C. fausta, C. laubieri, C. magnifica, C. nautilei, C. pacifica*, *I. fossajaponicum, Ph. kilmeri, Ph. okutanii, Ph. soyoae*, and *Pl. stearnsii*) were conducted using the concatenated nucleotide sequences of 11 mitochondrial genes (*atp6, atp8, cob, cox1, cox2, cox3, nad1, nad3, nad4, nad5*, and *rrnL*). The accession numbers for mitochondrial gene sequences used in this study are provided in supplementary data S1, Supplementary Material online. The analyses of the corresponding symbiont species were conducted using the concatenated nucleotide sequences of eight bacterial genes (16S rRNA, 23S rRNA, *galU*, *groEL, groES, mfd, uvrA*, and *uvrD* paralog), of which accession numbers are provided in supplementary data S2, Supplementary Material online. A set of five Venerid species (*Meretrix lusoria, M. meretrix, M. petechialis, Paphia euglypta*, and *Venerupis philippinarum*) were included as an outgroup for the phylogenetic analysis of the host species (supplementary data S1, Supplementary Material online). The symbiont of the vent mussel *Bathymodiolus septemdierum*, which is closely related to the vesicomyid symbionts (Ikuta et al. 2016b), was used as an outgroup for the phylogenetic analysis of the symbiont species (supplementary data S2, Supplementary Material online).

Table 2
Primers and PCR Conditions for the Amplification of Vesicomyid Mitochondrial and Symbiont's Gene Regions

Species	Host or Symbiont	Forward Primer	Sequence	Reverse Primer	Sequence	PCR Amplification Condition (30 cycles)	Genomic Region
Abyssogena mariana	Symbiont	27F[a]	AGAGTTTGATCCTGGCTCAG	1492R[b]	GGTTACCTTGTTACGACTT	96 °C 20 s—56 °C 10 s—72 °C 2 min	16S rRNA
	Symbiont	23S_F[c]	GGGAACTGAAACATCTAAGTACC	23S_R[c]	CCCGCTTAGATGCTTTCAG	96 °C 20 s—56 °C 10 s—72 °C 2 min	23S rRNA
	Symbiont	UvrA_F[d]	GCRCGTARTGCTGCRCCAGTGTC	UvrA_R[d]	RCTTGATGGAGGYCCWATTATTGCC	96 °C 20 s—56 °C 10 s—72 °C 5 min	*UvrA*
	Symbiont	UvrDpara_F[d]	AARGYTTCCATGTCAACCCGTAGTG	UvrDpara_R[d]	AAGCATTTGATGCTGGMTGTGATGC	96 °C 20 s—56 °C 10 s—72 °C 5 min	*UvrD* paralog
	Symbiont	mfd_F[d]	ACCAACAACCTCACAACCATCAGC	mfd_R[d]	CGATATTATCACCATCGCTACTAGC	96 °C 20 s—56 °C 15 s—72 °C 5 min	*mfd*
	Symbiont	galU_F[d]	GTTAAGGCTGTATCCCTATATGCAC	galU_R[d]	GCTTGTGGYACTAGTTATAAYGCAG	96 °C 20 s—56 °C 10 s—72 °C 5 min	*galU*
	Symbiont	GroELS_F[c]	YGCACCAGAACAATAYTTAACCAATG	GroELS_R[c]	WAYCCTGCAATTGYYTCATCAACAC	96 °C 20 s—56 °C 10 s—72 °C 4 min	*groES-groEL*
Isorropodon fossajaponicum	Symbiont	Ifos_galU_F	TTAAATAATACCTTGTTGTTTAGC	galU_R[d]	GCTTGTGGYACTAGTTATAAYGCAG	96 °C 20 s—56 °C 10 s—72 °C 5 min	*galU*
Akebiconcha kawamurai	Host	rrnS_F	GGAATTCAGGTACACGTATAGGATC	rrnL_R	TTAATCCAACATCGAGGTCGCAAAC	96 °C 20 s—60 °C 10 s—68 °C 8 min	*rrnS* - *rrnL*
	Host	AkaMit_rrnL_F	TTACAGGGCTGTTAATGGCTGCTC	nad1_R2	AATCAAATGGCGCTCGATTAGTCTC	96 °C 20 s—60 °C 10 s—68 °C 5 min	*rrnL* - *nad1*
	Host	AkaMit_nad1_F	ATTGGCAGAGACTAATCGAGCTC	LNCR_R2	TGATGTATTGCTACATTTGTCGCGC	96 °C 20 s—60 °C 10 s—68 °C 2 min	*nad1* - LNCR
Calyptogena fausta	Host	rrnS_F	GGAATTCAGGTACACGTATAGGATC	rrnL_R	TTAATCCAACATCGAGGTCGCAAAC	96 °C 20 s—60 °C 10 s—68 °C 8 min	*rrnS* - *rrnL*
	Host	rrnL_F	CTACCATAGGGATAACAGCGTTATC	tRNA_Q_R	AGAGAACTTAATCTCATCTCTGACTC	96 °C 20 s—60 °C 10 s—68 °C 6 min	*rrnL* - tRNA ^{Gln}
	Host	CfaMit_nad4_F	AGCCTGTGTTTAACTTGAGAGAACG	nad1_R	ATACTCAACCCTATACCCCGCTAC	96 °C 20 s—60 °C 10 s—68 °C 2 min	*nad4* - *nad1*
	Host	CfaMit_nad1_F	TATCTGGGTGAGTTTGTGATTCTCG	LNCR_R2	TGATGTATTGCTACATTTGTCGCGC	96 °C 20 s—60 °C 10 s—68 °C 2 min	*nad1* - LNCR
Calyptogena laubieri	Host	rrnS_F	GGAATTCAGGTACACGTATAGGATC	rrnL_R	TTAATCCAACATCGAGGTCGCAAAC	96 °C 20 s—60 °C 10 s—68 °C 8 min	*rrnS* - *rrnL*
	Host	ClaMit_rrnL_F	AGATTATGTGGACTATGGTGCCTGC	nad1_R	ATACTCAACCCTATACCCCGCTAC	96 °C 20 s—60 °C 10 s—68 °C 6 min	*rrnL* - *nad1*
	Host	ClaMit_nad1_F	ATGGCTTGTGGCCACATTGGCAG	LNCR_R2	TGATGTATTGCTACATTTGTCGCGC	96 °C 20 s—60 °C 10 s—68 °C 2 min	*nad1* - LNCR
Calyptogena nautilei	Host	rrnS_F	GGAATTCAGGTACACGTATAGGATC	rrnL_R	TTAATCCAACATCGAGGTCGCAAAC	96 °C 20 s—60 °C 10 s—68 °C 8 min	*rrnS* - *rrnL*
	Host	CnaMit_rrnL_F	GTTAGTAGGGTTGATATTGGCCATC	nad1_R2	AATCAAATGGCGCTCGATTAGTCTC	96 °C 20 s—60 °C 10 s—68 °C 6 min	*rrnL* - *nad1*
	Host	CnaMit_atp6_F	TATGCGGGCTGTAGCTCAGAGAG	LNCR_R	AGGCAGRCCTCACGGAGCCACGC	96 °C 20 s—60 °C 10 s—68 °C 8 min	*atp6* - LNCR
Calyptogena pacifica	Host	rrnS_F	GGAATTCAGGTACACGTATAGGATC	rrnL_R	TTAATCCAACATCGAGGTCGCAAAC	96 °C 20 s—60 °C 10 s—68 °C 8 min	*rrnS* - *rrnL*
	Host	CpaMit_rrnL_F	AGAGTGCTTGGTGGGTGTTCTATC	nad1_R2	AATCAAATGGCGCTCGATTAGTCTC	96 °C 20 s—60 °C 10 s—68 °C 6 min	*rrnL* - *nad1*
	Host	CpaMit_atp6_F	GTATGATTCAATTGCGGCAAGGGC	LNCR_R	AGGCAGRCCTCACGGAGCCACGC	96 °C 20 s—60 °C 10 s—68 °C 8 min	*atp6* - LNCR
Phreagena kilmeri	Host	rrnS_F	GGAATTCAGGTACACGTATAGGATC	rrnL_R	TTAATCCAACATCGAGGTCGCAAAC	96 °C 20 s—60 °C 10 s—68 °C 8 min	*rrnS* - *rrnL*
	Host	rrnL_F	CTACCATAGGGATAACAGCGTTATC	nad1_R2	AATCAAATGGCGCTCGATTAGTCTC	96 °C 20 s—60 °C 10 s—68 °C 6 min	*rrnL* - *nad1*
	Host	PkiMit_atp6_F	TGAAGGTTGCTTAGCTTCTTTGCAG	LNCR_R	AGGCAGRCCTCACGGAGCCACGC	96 °C 20 s—60 °C 10 s—68 °C 8 min	*atp6* - LNCR
Phreagena soyoae	Host	rrnS_F	GGAATTCAGGTACACGTATAGGATC	rrnL_R	TTAATCCAACATCGAGGTCGCAAAC	96 °C 20 s—60 °C 10 s—68 °C 8 min	*rrnS* - *rrnL*
	Host	PsoMit_rrnL_F	TTGCCTAAGGCTCATGTGGAGGC	nad1_R2	AATCAAATGGCGCTCGATTAGTCTC	96 °C 20 s—60 °C 10 s—68 °C 6 min	*rrnL* - *nad1*
	Host	AkaMit_nad1_F	ATTGGCAGAGACTAATCGAGCTC	LNCR_R	AGGCAGRCCTCACGGAGCCACGC	96 °C 20 s—60 °C 10 s—68 °C 2 min	*nad1* - LNCR
Pliocardia stearnsii	Host	rrnS_F	GGAATTCAGGTACACGTATAGGATC	rrnL_R	TTAATCCAACATCGAGGTCGCAAAC	96 °C 20 s—60 °C 10 s—68 °C 8 min	*rrnS* - *rrnL*
	Host	PstMit_rrnL_F	AAGAGGTTAGGCCTGCCCGGTG	nad1_R2	AATCAAATGGCGCTCGATTAGTCTC	96 °C 20 s—60 °C 10 s—68 °C 8 min	*rrnL* - *nad1*
	Host	PstMit_atp6_F	GGCATGTGAACTGCTAGTGTAGTG	LNCR_R	AGGCAGRCCTCACGGAGCCACGC	96 °C 20 s—60 °C 10 s—68 °C 5 min	*atp6* - LNCR

[a]Lane (1991).
[b]Turner et al. (1999).
[c]Kuwahara et al. (2011).
[d]Shimamura et al. (2017).

The predicted amino acid sequences of the protein-coding genes from each mitochondrion and symbiont were separately aligned using MAFFT v7.164 b (Katoh and Standley 2013), using the default parameters. On the basis of amino acid alignment, the nucleotide sequences were aligned using the Transalign program in EMBOSS package (Rice et al. 2000). The nucleotide sequences of rRNA genes from both the host mitochondria and symbionts were also individually aligned using MAFFT v7.164 b, with the default parameters. Gaps and insert sequences in the alignments of nucleotide sequences were removed prior to phylogenetic analyses with trimAl 1.2 rev59 (Capella-Gutiérrez et al. 2009). The appropriate evolutionary model for the nucleotide sequence alignment of each gene was selected based on Akaike Information Criteria (AIC) and the Bayesian Information Criterion (BIC) for the maximum likelihood (ML) and Bayesian analyses, respectively, using Kakusan4 software (Tanabe 2011).

ML and Bayesian trees were reconstructed from the alignment of each gene, using an appropriate model for each analysis (supplementary data S3, Supplementary Material online). The ML analyses of the host clams and symbionts were conducted using RAxML version 8.2.10 (Stamatakis 2014), with 40 distinct randomized maximum parsimony starting trees (supplementary data S3, Supplementary Material online), and the ML bootstrap support (BS) was obtained by 100 resamplings. The Bayesian inference was conducted using MrBayes 3.2.6, with the appropriate evolutionary model for each gene (supplementary data S3, Supplementary Material online; Ronquist et al. 2012), and the Bayesian trees for the individual mitochondrial and symbiont genes were generated from random starting trees over one million generations, with one cold and three heated Markov chains and sampling every 100 generations. The first 250,000 generations were excluded as burn-in, and the remaining trees were summarized to obtain Bayesian posterior probability (PP). The phylogenetic trees of respective genes in host and symbiont are shown in supplementary data S4 and S5, Supplementary Material online. The resulting individual gene trees showed no instances in which the taxa were placed in conflicting positions with a zero-branch length or with extremely long branches. Hence, the concatenated alignments of the host mitochondrial genes (10,496 nucleotide sites/18 taxa) and the symbiont genes (15,847 nucleotide sites/14 taxa) were subjected to ML and Bayesian analyses, as described above (supplementary data S3, Supplementary Material online). The alignment data sets are available from Figshare (accession html: https://figshare.com/s/c28afd62efc648d87d5b).

Tree Topology Comparison

The significance of topological difference between the host and symbiont trees was assessed using nonparametric tests. An approximately unbiased (AU) test (Shimodaira 2002) and Bayes factor test were implemented in CONSEL (Shimodaira and Hasegawa 2001) and MrBayes 3.2.6. (Ronquist et al. 2012), respectively. Both the constrained and unconstrained topologies of the host and symbiont trees (table 3) were estimated using RAxML with the GTR + G model. The likelihood of the best constrained tree was compared with the likelihoods of the other trees, thereby providing probability values (*P* values) of AU for each tree. Tree topologies with *P* values of <0.05 were rejected. The marginal likelihoods were estimated by stepping-stone sampling for the hypotheses (Rambaut and Drummond 2009) (table 3), then Bayes factors were calculated by taking the differences between the marginal likelihood values. A log difference of Bayes factors in the range of 3–5 is typically considered to be strong evidence in favor of a model, and a log difference above 5 is considered to be very strong evidence (Kass and Raftery 1995).

Coevolution Analyses

To estimate the most probable coevolution scenario between the host clams and their symbionts, two reconciliation methods, the Jane 4.0 (Conow et al. 2010) and CoRe-PA (Merkle et al. 2010) were used. These topology-based programs assign costs to four coevolution events (cospeciation, duplication, sorting or loss, host switching) that link the two trees. In addition, Jane 4.0 also assigns a cost to failure-to-diverge events (a host speciates while the symbiont does not speciate and remains in both new host species). CoRe-PA calculates the optimal cost values, whereas Jane 4.0 requires the cost values to be inputted. Both methods seek to minimize the total costs of all events that occur. After symbiont phylogeny was mapped onto host phylogeny, several coevolutionary phylogenetic patterns exhibiting minimal total cost were retrieved. Our criteria for selecting a result in coevolutionary phylogenetic patterns took into account only cospeciation and host switching events, as sorting, duplication, and failure-to-diverge events of the symbiont are not expected in a comparison of phylogenetic trees. For the Jane 4.0 analysis, the default cost setting was used, and mapping was performed with 100 generations and a population size of 100. CoRe-PA was performed with automatic estimation of the optimal cost setting and computed reconstructions of 100,000 random data sets. The event costs obtained by CoRe-PA were 0.0999 for cospeciation, 0.3000 for sorting, 0.2999 for duplication, and 0.3000 for host switching.

Results

Phylogeny of Host Vesicomyid Clams

Most clades in the phylogenetic tree reconstructed from the concatenated gene data sets (10,469 nucleotide sites of 11 mitochondrial genes from 18 taxa) could be resolved with high statistical support (fig. 1*A*). Six species (*Ph. soyoae*, *Ph. kilmeri*, *Ph. okutanii*, *Ak. kawamurai*, *C. laubieri*, and

Table 3
Topology Tests of the Phylogenies of 13 Vesicomyid Host Clams and Their Symbionts

Constraint Topology Patterns		AU[a] (*p*)	Log Likelihoods	BF01[b]
(A) Host	Best host topology	1.0000	−79310.243	–
	Constraint topology including *Ak. kawamurai* and *C. laubieri* on symbiont tree	0.0050	−79338.346	12.205
	Constraint topology including *Ak. kawamurai, C. laubieri, Ph. kilmeri, Ph. okutanii* and *Ph. soyoae* on symbiont tree	0.0030	−79337.531	11.851
	Constraint topology including *C. fausta, C. pacifica* and *Pl. stearnsii* on symbiont tree	0.0030	−79365.117	23.832
	Constraint topology including *C. fausta, C. nautilei, C. pacifica* and *Pl. stearnsii* on symbiont tree	0.0020	−79377.612	29.258
	Constraint topology including *Ak. kawamurai, C. fausta, C. laubieri, C. nautilei, C. magnifica, C. pacifica, Ph. kilmeri, Ph. okutanii, Ph. soyoae* and *Pl. stearnsii* on symbiont tree	0.0001	−79404.52	40.944
	Constraint topology including *Ab. mariana* and *I. fossajaponicum* on symbiont tree	0.0000	−79624.583	136.516
	Constraint topology including *Ab. mariana, Ab. phaseoliformis* and *I. fossajaponicum* on symbiont tree	0.0000	−79624.675	136.556
	Best symbiont topology	0.0002	−79715.059	175.809
(B) Symbiont	Best symbiont topology	1.0000	−61438.662	–
	Constraint topology including *Ak. kawamurai, Ph. kilmeri, Ph. okutanii* and *Ph. soyoae* on host tree	0.0160	−61448.226	9.564
	Constraint topology including *Ak. kawamurai, C. laubieri, Ph. kilmeri, Ph. okutanii* and *Ph. soyoae* on host tree	0.0190	−61448.765	10.104
	Constraint topology including *Ab. mariana* and *Ab. phaseoliformis* on host tree	0.0000	−61837.220	398.559
	Constraint topology including *Ab. mariana, Ab. phaseoliformis, C. fausta* and *C. pacifica* on host tree	0.0000	−63718.042	2279.381
	Constraint topology including *Ab. mariana, Ab. phaseoliformis, C. fausta, C. pacifica* and *I. fossajaponicum* on host tree	0.0000	−64030.448	2591.786
	Constraint topology including *Ak. kawamurai, Ab. mariana, Ab. phaseoliformis, C. fausta, C. laubieri, C. magnifica, C. pacifica, I. fossajaponicum, Ph. kilmeri, Ph. okutanii* and *Ph. soyoae* on host tree	0.0000	−64040.829	2602.167
	Constraint topology including *Ak. kawamurai, Ab. mariana, Ab. phaseoliformis, C. fausta, C. laubieri, C. magnifica, C. pacifica, I. fossajaponicum, Ph. kilmeri, Ph. okutanii, Ph. soyoae* and *Pl. stearnsii* on host tree	0.0000	−64041.791	2603.129
	Best host topology	0.0000	−64041.88	2603.218

The AU test was performed in CONSEL. BF test was estimated by MrBayes.
[a]Approximately unbiased test.
[b]Bayes factor test.

C. magnifica) formed a monophyletic clade with ML BS of 58% and Bayesian PP of 0.9959 (fig. 1*A*; clade a). Two monophyletic clades that included either *C. pacifica* and *C. fausta* (fig. 1*A*; clade b), or *Ab. phaseoliformis* and *Ab. mariana* (fig. 1*A*; clade c) were highly supported with ML BS of 98–100% and Bayesian PP of 1.0 (fig. 1*A*), and they formed a monophyletic clade (fig. 1*A*; clade d). *Isorropodon fossajaponicum* was branched with clade d with ML BS of only 45% and Bayesian of PP of 0.9848 (fig. 1*A*; clade e). *Calyptogena nautilei* was the earliest branching lineage within the vesicomyid clam radiation, with ML BS of 100% and PP of 1.0, and the next most basal taxon was *Pl. stearnsii* with ML BS of 80% and PP of 1.0.

Phylogeny of Vesicomyid Symbionts

The phylogenetic tree of the 13 vesicomyid symbionts based on the concatenated gene data set (15,847 nucleotide sites of eight symbiont genes from 14 taxa) is shown in figure 1*B*. The phylogenetic lineages were well resolved at most nodes, showing high statistical support with ML BS of 96–100% and Bayesian PP of 0.9997–1.0 (fig. 1*B*). The symbionts of *Ph. soyoae*, *Ph. kilmeri*, *Ph. okutanii*, *Ak. kawamurai*, *C. laubieri*, and *C. magnifica* (fig. 1*B*; clade a); those of *C. pacifica, C. fausta*, *Pl. stearnsii*, and *C. nautilei* (fig. 1*B*; clade b); and those of *Ab. mariana, I. fossajaponicum*, and *Ab. phaseoliformis* (fig. 1*B*; clade c), respectively, formed monophyletic clades supported with ML BS of 71–100% and Bayesian PP of 0.9681–1.0 (fig. 1*B*). The affiliation of the symbiont of *Pl. stearnsii* with the symbionts of *C. pacifica* and *C. fausta* was low (ML BS of 75% and a Bayesian PP of 0.8440) (fig. 1*B*; clade d).

Comparison of the Host and Symbiont Phylogenetic Trees

A comparison of the host and symbiont phylogenetic trees showed that the phylogenetic lineages of six hosts

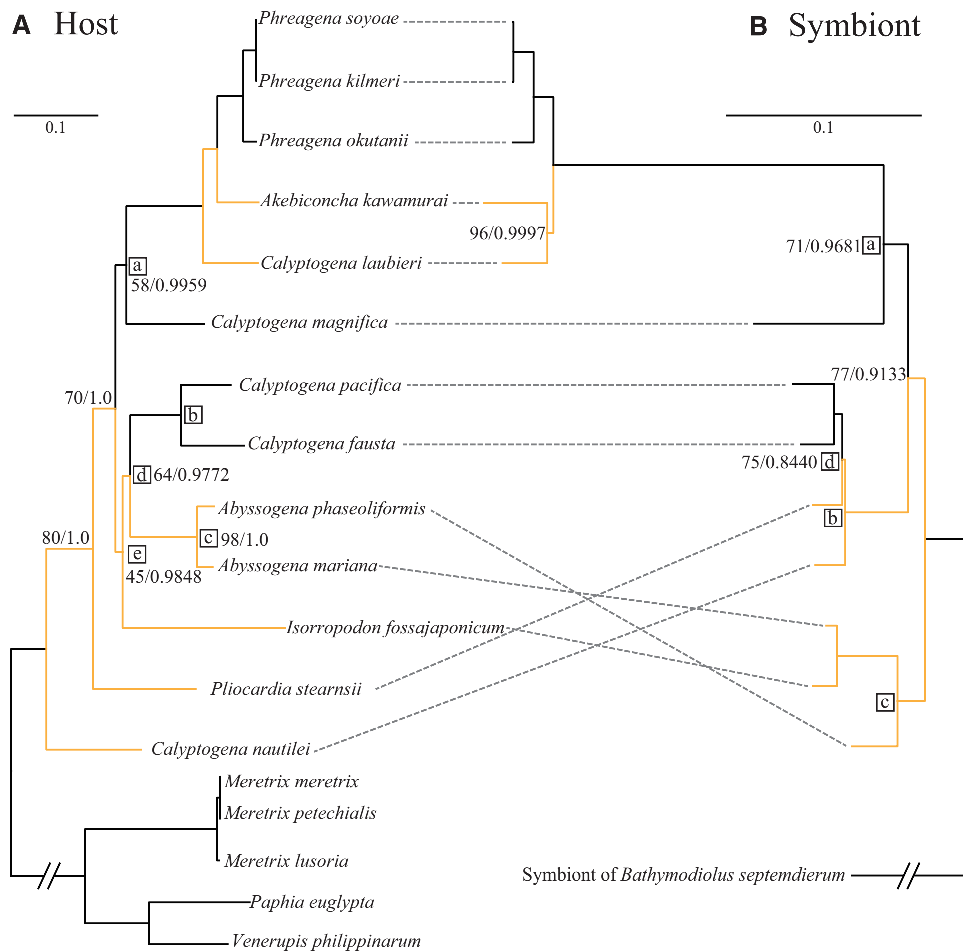

Fig. 1.—Comparison of Maximum Likelihood (ML) phylogenies for 13 species of vesicomyid clams and their symbionts. (A) ML tree of vesicomyid clams based on the concatenated data sets of nucleotide sequences of 11 mitochondrial genes (atp6, atp8, cox1, cox2, cox3, cob, nad1, nad3, nad4, nad5, and rrnL). Venerid species (*Meretrix lusoria, M. meretrix, M. petechialis, Paphia euglypta*, and *Venerupis philippinarum*) were used as outgroups. (B) ML phylogenetic tree of 13 vesicomyid symbionts based on concatenated data sets of the nucleotide sequences of eight genes (16S rRNA, 23S rRNA, galU, groEL, groES, mfd, uvrA, and uvrD paralog). The symbiont of *Bathymodiolus septemdierum* was used as an outgroup. Dotted lines between vesicomyid clam species and their symbionts' trees indicate symbiotic pairs. Numbers at the bipartitions indicate ML bootstrap supports of < 100% (left) and Bayesian posterior probabilities of < 1.0 (right). Unlabeled nodes are fully supported (100% bootstrap support and posterior probability at 1.0). Small letters in the squares of some nodes indicate the monophyletic clades described in the text. The orange branches are those exhibiting incongruence between the topologies of the host clams and symbionts, as indicated by an approximately unbiased (AU) test ($P < 0.05$) and Bayes factor test (>5). Host and symbiont trees were not drawn to the same rate scale, and scale bars indicate the rate of internal nodes except for outgroups.

(*Ph. soyoae*, *Ph. kilmeri*, *Ph. okutanii*. *C. magnifica, C. pacifica*, and *C. fausta*) and of their corresponding symbionts were congruent (fig. 1; black branches). However, those of seven other hosts (*Ak. kawamurai, C. laubieri, Ab. phaseoliformis, Ab. mariana, I. fossajaponicum*, *Pl. stearnsii*, and *C. nautilei*) and their symbionts were not congruent (fig. 1; orange branches). The results of the AU and Bayes factors tests of both constrained and unconstrained topologies in host and symbiont rejected the phylogenetically congruent positions of the seven host-symbiont pairs ($P < 0.05$; BF > 5; table 3 and fig. 1; orange branches).

Coevolution analyses between host clams and their symbionts were performed with Jane 4.0 and CoRe-PA. The Jane 4.0 analysis estimated three coevolution patterns, of which two were selected by our criteria described in the Materials and Methods section (fig. 2*A* and *B*). CoRe-PA yielded six coevolution patterns, only one of which was selected by our criteria; it exhibited the same coevolution pattern as one of the two patterns generated by Jane 4.0 (fig. 2*A*).

The two selected coevolution patterns were essentially similar (fig. 2*A* and *B*) and contained six cospeciation events and six host switching events (fig. 2*A* and *B*). The six cospeciation events (fig. 2*A* and *B*; open circles No. 1–6) and four host switching events (fig. 2*A* and *B*; closed circles a, b, e, and f) in the two selected coevolution patterns were identical. Among the six cospeciation events, three (fig. 2*A* and *B*; open circles

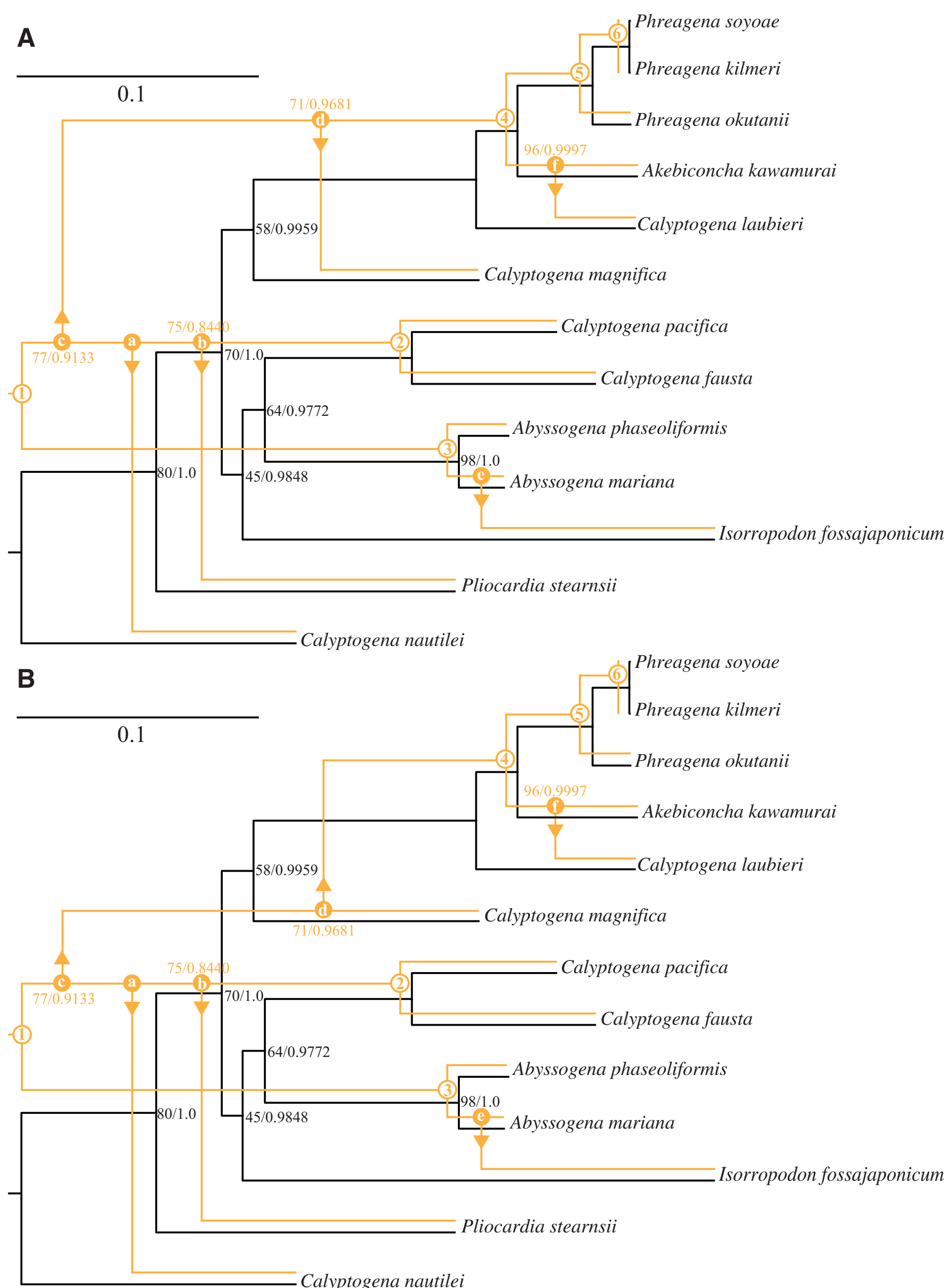

Fig. 2.—Coevolution scenarios between the vesicomyid clams and their symbionts. The coevolution analysis was performed using two reconciliation methods, the Jane 4.0 and CoRe-PA. Two preferred coevolutionary patterns exhibiting minimal total cost were selected, (A) Jane 4.0 and CoRe-PA, and (B) Jane 4.0. The black and orange topologies indicate the topologies of the hosts and symbionts, respectively, and the black and orange numbers at the bipartitions indicate the ML bootstrap supports of $< 100\%$ (left) and Bayesian posterior probabilities of < 1.0 (right) on host and symbiont trees, respectively. Unlabeled nodes are fully supported (100% bootstrap support and posterior probability of 1.0). The numbers in the orange open circles and small letters in the orange closed circles indicate cospeciation and host switching events, respectively.

No. 2, 5, and 6) were also supported by phylogenetic trees (fig. 1); however, the cospeciation of *Ab. phaseoliformis* (fig. 2*A* and *B*; open circle No. 3) was not supported by the phylogenetic trees (fig. 1). Among the four host switching events, two were suggested in the lineage of the common ancestral symbiont of *C. pacifica* and *C. fausta*. Our analyses indicated that the common ancestral symbiont of these two clam species shifted to two other clam species as hosts: *C. nautilei* or its ancestor (fig. 2*A* and *B*; closed circle a), and *P. stearnsii* or its ancestor (fig. 2*A* and *B*; closed circle b). The remaining two

host switching events were suggested to have occurred in the lineage of the ancestral symbionts of *Ak. kawamurai* and *Ab. mariana*. The ancestral symbionts of these two clam species shifted to other clam species as hosts, *C. laubieri* and *I. fossajaponicum*, respectively (fig. 2*A* and *B*; closed circles e and f).

The host switching events associated with the common ancestor of the symbionts of *C. pacifica* and *C. fausta* were suggested to be different between two coevolution patterns. Based on figure 2*A*, the ancestral symbiont of these clam species shifted to the common ancestor of the lineage containing *Ph*. *soyoae*, *Ph*. *kilmeri*, *Ph*. *okutanii*, *Ak. kawamurai*, and *C. laubieri* (fig. 2*A*; closed circle c). After this event, an additional host switching event, to *C. magnifica*, was estimated to have occurred in this lineage (fig. 2*A*; closed circle d). On the other hand, figure 2*B* suggests that the common ancestor of symbionts of *C. pacifica* and *C. fausta* first shifted to the lineage of *C. magnifica* (fig. 2*B*; closed circle c) and then secondarily to the ancestral lineage of *Ph*. *soyoae*, *Ph*. *kilmeri*, *Ph*. *okutanii*, *Ak. kawamurai*, and *C. laubieri* (fig. 2*B*; closed circle d).

Discussion

Occasional Host Switching of the Symbionts of Vesicomyid Clams

The phylogenetic positions of the clams selected in the present study are widely distributed in a previously published phylogenetic tree that was based on sequences of the mitochondrial *cox1* gene (Decker et al. 2012). In the present study, we used large gene data sets (over 10 kbp) to enhance the resolution of the phylogenetic trees of vesicomyid clams and their symbionts, an improvement over previously reported phylogenetic trees (Stewart et al. 2008; fig. 1). The phylogenetic positions of six vesicomyid hosts (*Ph*. *soyoae*, *Ph*. *kilmeri*, *Ph*. *okutanii*. *C. magnifica, C. pacifica*, and *C. fausta*) and their symbionts were congruent (fig. 1). Cospeciation of these host-symbiont pairs was also well supported by coevolution analyses using Jane 4.0 and CoRe-PA, except in *C. magnifica* (fig. 2). However, the tree topologies of seven other host taxa (*Ak. kawamurai, C. laubieri, Ab. phaseoliformis*, *Ab. mariana, I. fossajaponicum*, *Pl. stearnsii*, and *C. nautilei*) and their symbionts were incongruent, and such differences were statistically supported by AU and Bayes factor tests (table 3 and fig. 1). Our coevolution analyses using Jane 4.0 and CoRe-PA estimated that multiple host switching events have occurred. Remarkably, the common ancestral lineage of the symbionts of *C. fausta* and *C. pacifica* was repeatedly associated with such events (fig. 2). Possible host switching may also have occurred between the host lineages of *I. fossajaponicum* and *Ab. mariana* and between those of *Ak. kawamurai* and *C. laubieri*. Indeed, recent studies have reported the possibility of horizontal transfers of symbionts in vesicomyid clams, based on the incongruence between host and symbiont phylogenies and detection of symbionts from other host species along with its own symbiont in the gill using *in situ* hybridization (Stewart et al. 2008; Decker et al. 2013). Therefore, it seems likely that vesicomyid symbionts are predominantly transmitted vertically but also are transmitted horizontally on occasion. The present study also suggests this hypothesis.

Possible Mechanism of Horizontal Transfer Causing Host Switching

Stewart et al. (2008) proposed three mechanisms (i.e., host hybridization, environmental acquisition, and horizontal transmission) that could facilitate the horizontal acquisition (non-maternal transmission) of symbionts that could trigger host switching and result in incongruent tree topologies of vesicomyid clams and their symbionts. The host-hybridization hypothesis states that both the mitochondria and symbionts could be paternally transferred *via* sperm during hybridization between distinct species. In this scenario, hybrid hosts with different symbionts and mitochondrial phylotypes would occur by the displacement or loss of the maternal symbiont or mitochondrion, causing changes in the phylogenetic positions of the resulting symbiont and mitochondrial types. Several bivalve species of Heterodonta, to which vesicomyid clams belong, have a sperm-transmitted mitochondrial genome, along with a standard maternally transmitted one. This mechanism is called doubly uniparental inheritance of mitochondria (Skibinski et al. 1994; Zouros et al. 1994). Bivalve species with doubly uniparental inheritance have two distinct sex-specific mitochondrial DNA types (female-type and male-type) that potentially lead to misunderstanding regarding their evolution (Doucet-Beaupré et al. 2010). The paternal transmission of symbionts has been observed in *Rickettsia*, a facultative symbiont of insects (Watanabe et al. 2014). The symbiont *16S rRNA* sequence was not amplified from the testis of *Ph. okutanii*, suggesting that vesicomyid sperm does not harbor the symbiont (Ikuta et al. 2016a). Paternal symbiont or mitochondrial transfer has not been reported in vesicomyids (Stewart et al. 2008). These data suggest that neither the transmission of mitochondria nor that of symbionts is likely to occur paternally. Thus, the horizontal transfer of mitochondria and symbionts is unlikely to have occurred through hybridization.

The second hypothesis, the environmental acquisition hypothesis, states that symbionts are directly acquired from the environment. However, the genomes of symbionts from *Ph. okutanii* and *C. magnifica* (Kuwahara et al. 2007; Newton et al. 2007) lack genes associated with flagella for motility in the environment and genes for the related type IV secretory system for infection into host cells (Kuwahara et al. 2007). Moreover, vesicomyid symbionts have not been found in bacterial mats or sediments in their hosts' habitats (Moyer et al. 1995; Polz and Cavanaugh 1995). Therefore, this hypothesis is also unlikely to explain the observed patterns.

The horizontal transfer hypothesis states that symbionts are transmitted between distinct hosts without hybridization.

Horizontal transmission could occur through the direct contact between eggs and eggs, eggs and host, or released symbionts and hosts. Symbionts that have no infection system are unlikely to be transferred into other host tissues via direct contact. Moreover, horizontal transmission of symbionts in the gill epithelial cells of host clam is also unlikely, because of their intracellular localization. Recently, Ikuta et al. (2016a) reported that in *Ph. okutanii*, ~400 symbiont cells are located at the external surface of eggs. The symbiont cells are localized at the egg surface in the vitelline coat, which may prevent the symbionts from making direct contact with other eggs or hosts, reducing the frequency of host switching and making such horizontal transmission occasional and historical (Ikuta et al. 2016a). In host switching by direct contact events, horizontally transmitted symbionts must enter the maternal germ cells (such as eggs) of the new host to transfer the symbiont to the next generation. The process by which the symbiont enters both the maternal germ cells and gill epithelial cells may occur during the developmental stage of the host. Thus, among the three hypotheses proposed by Stewart et al. (2008), the most plausible mechanism for the horizontal transfer of symbionts in vesicomyid clams is that the symbionts on the egg surface are transferred by direct contact of eggs of different clam species before the developmental process begins.

The Process of Host Switching

Horizontal transfer of the symbiont from the original host to a new host is considered to be the first step of a host switching event. After the horizontal transfer of a symbiont occurs, the original symbiont may be eliminated by evolutionary change or selection through a possible bottleneck of vertical transmission via egg, and then the horizontally transmitted symbiont speciates in its new host. Recently, Decker et al. (2013) reported that each of two clam species possesses a small population of the symbiont from the other respective species, along with its own symbiont, when the hosts cooccur within the same colony. Cooccurrence of multiple symbionts in a single individual may represent an initial process of host switching via horizontal transfer by direct contact. Moreover, the physical proximity of distinct vesicomyid clam species likely facilitates horizontal symbiont transfer (Decker et al. 2013). Two vesicomyid species, *Ph. okutanii* and *Ph. soyoae*, that coexist in a seep colony in Sagami Bay (Kojima and Ohta, 1997) exhibit synchronized spawning triggered by an increase in water temperature (Fujikura et al. 2007). Although there is currently no evidence of host switching between *Ph. okutanii* and *Ph. soyoae*, it is possible that the extracellular localization of symbionts, such as on the surface of eggs, could facilitate the horizontal transmission of symbionts between cooccurring species. Of the 13 vesicomyids in the present study, *C. pacifica*, *Ph. kilmeri*, and *Pl. stearnsii* are known to cooccur in colonies in Monterey Bay (Barry et al. 2007), and data from the present study suggest that host switching between *C. pacifica* and *Pl. stearnsii* occurred. Other vesicomyids that harbor possible host-switching symbionts are currently isolated geographically; however, it is possible that these clam species lived in close proximity in the past.

Conclusion

Previous studies have proposed that the intracellular symbionts of vesicomyid clams are vertically (maternally) transmitted to offspring and that the hosts and symbionts cospeciated (Endow and Ohta 1990; Cary and Giovannoni 1993; Peek et al. 1998; Ikuta et al. 2016a). However, several recent studies have suggested that the vesicomyid and symbiont phylogenies are decoupled and that at least some symbionts are horizontally transmitted (Stewart et al. 2008; Decker et al. 2013). In the present study, to more closely investigate the coevolution of these vesicomyid clams and their symbionts, the phylogenetic trees were reconstructed using large gene data sets from both the host clam species and their symbionts. Our results suggest that multiple host switching events are highly likely to have occurred during the early evolution of vesicomyids, and such host switching events were most likely mediated by horizontal egg-to-egg transmission. Our data provide strong support for the hypothesis that vesicomyid symbionts are predominantly transmitted vertically but are occasionally transmitted horizontally. These findings contribute to the understanding of the evolutionary process of intracellular symbiosis by which symbionts are generally considered to be vertically transmitted.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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