



Characterizing Photosymbiosis Between Fraginae Bivalves and *Symbiodinium* Using Phylogenetics and Stable Isotopes

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Photosymbiotic associations between heterotrophic hosts and photosynthetic algae play crucial roles in maintaining the trophic and structural integrity of coral reef ecosystems. The marine bivalve subfamily Fraginae contains both non-symbiotic and photosymbiotic lineages, making it an ideal comparative system to study the origin and evolutionary adaptations of photosymbiosis. The symbiotic species exhibit unique morphological adaptations to photosymbiosis. However, the basic biology of these photosymbiotic relationships, such as symbiont diversity and nutritional benefits, has not been thoroughly characterized. In this study, we examined the general morphology of four Fraginae species occupying different depths (0–10 m): *Corculum cardissa*, *Fragum fragum*, *Fragum scruposum*, and *Fragum sueziense*. Abundant symbionts were found in the mantle, gill, and part of the foot, contained in tubular networks within host tissues. We used molecular phylogenetics to investigate the algal symbiont community of these Fraginae species. Results showed that symbionts from all four species are dinoflagellates belonging to the *Symbiodinium* clade C and we did not detect any host-specific or geographic-specific genetic structures within the symbionts. We also used stable carbon isotope analyses to examine whether the cockles are directly utilizing photosynthetically derived carbon sources. All species show less depleted ¹³C compared to filter-feeding bivalves, suggesting at least part of their organic carbon is derived directly from the symbionts. However, ¹³C depletion of *Fragum sueziense* collected from deeper habitats are less distinguishable from filter-feeding bivalves. This indicates that species in deeper habitats may rely less on photosymbiosis due to the reduced light availability. Given that the symbiotic fragines exhibit varying morphologies, habitats, and utilization of symbiont photosynthesis, the subfamily represents an ideal model system to study differential adaptations to photosymbiosis.

Keywords: symbiosis, *symbiodinium*, dinoflagellate, *cardiidae*, *fragum*, *corculum*, *tridacna*, coral reef

INTRODUCTION

Photosymbiotic associations between heterotrophic hosts and photosynthetic algae play crucial roles in maintaining the trophic and structural integrity of coral reef ecosystems (Cowen, 1988; Trench, 1993; Stanley and Lipps, 2011). The algae provide photosynthetic products to the host and gain shelter and inorganic nutrients in return, allowing the symbiosis to flourish in nutrient deficient habitats. Such associations have evolved in diverse marine lineages, including Foraminifera, Porifera, Cnidaria, and Mollusca (Gast and Caron, 2001; Stanley Jr, 2006; Hill et al., 2011; Vermeij, 2013). Among marine bivalves, photosymbiosis has been documented in at least four groups (Vermeij, 2013; Kirkendale and Paulay, 2017). However, obligate associations are found in only two cockle (Cardiidae) subfamilies, Tridacninae (i.e., giant clams), and Fraginae (i.e., heart cockles and relatives), where the former is relatively well studied. The tridacnids possess dinoflagellates (i.e., zooxanthellae) belonging to the genus *Symbiodinium*. A combination of photosymbiosis and filter feeding allows them to reach great sizes up to 1.2 m in width (Yonge, 1936; Fitt and Trench, 1981; Hawkins and Klumpp, 1995). The second group, Fraginae cockles, is much less understood despite their remarkable morphological adaptations presumably facilitating photosymbiosis (Kawaguti, 1941; Schneider and Carter, 2001; Kirkendale, 2009).

The subfamily Fraginae is composed of more than fifty morphologically diverse cockle species inhabiting tropical seas worldwide. Unlike the exclusively photosymbiotic Tridacninae giant clams (Fitt and Trench, 1981), Fraginae contains both non-symbiotic and symbiotic species. Seven of the ten modern genera are entirely non-photosymbiotic, while the remaining three are photosymbiotic (*Fragum*, *Corculum*, and *Lunulicardia*; Kirkendale, 2009). Recent molecular phylogenies (Kirkendale, 2009; Herrera et al., 2015) revealed a paraphyletic *Fragum* giving rise to *Corculum* and *Lunulicardia*, suggesting a single origin of Fraginae photosymbiosis, although the possibility of evolutionary loss in the non-symbiotic lineages cannot be rejected (Kirkendale and Paulay, 2017).

Some photosymbiotic fragines exhibit unique morphologies that are hypothesized to be adaptations to photosymbiosis, including large shell surface to volume ratio, highly flattened posterior shells, and transparent shell microstructures to increase light penetration (Watson and Signor, 1986; Ohno et al., 1995; Carter and Schneider, 1997; Schneider and Carter, 2001; Kirkendale, 2005; ter Poorten, 2009). Certain species also show behavioral strategies presumably to maximize sunlight exposure, such as posterior valve gaping (Persselin, 1998; Kirkendale, 2005). To date, the anatomy of symbiont-containing tissues has been studied in a single species, *Corculum cardissa* (Linnaeus, 1758) (Farmer et al., 2001). The bivalve harbors extracellular symbionts within the lumen of a tertiary tubular network, originating from the digestive system and extending into the mantle and gill tissues. The tubular structure is superficially analogous to that of the giant clams, although giant clams only harbor symbionts in the mantle (for more detailed descriptions of Fraginae morphology, see ter Poorten, 2009).

The diversity of Fraginae-associated symbiont communities are under explored (Kirkendale and Paulay, 2017). The majority of symbiotic algae in marine organisms are dinoflagellates belonging to the genus *Symbiodinium*, which has been divided into nine distinct clades (A-I) base on molecular phylogenies (Pochon and Gates, 2010). Different lineages in *Symbiodinium* vary greatly in their geographical distributions (Finney et al., 2010; LaJeunesse et al., 2010), physiological tolerance (Lien et al., 2007), and host specificity (Baker, 2003; Pochon et al., 2006; Yorifuji et al., 2015). Some lineages (e.g., clades A and C) are generalists that occupy a diverse array of hosts from different phyla; others (e.g., clade H) are relatively host-specific (Pochon et al., 2006). Existing literature on *Symbiodinium* is most comprehensive for coral-associated photosymbioses. Within Mollusca, tridacnid-associated symbionts are relatively well studied, covering 8 out of 12 currently recognized Tridacninae species. Their symbionts are mostly placed into *Symbiodinium* clade A, C, and occasionally D (Kirkendale and Paulay, 2017; see comprehensive literature list in **Supplementary Table 2**) and a single host individual can sometimes possess multiple symbiont lineages (DeBoer et al., 2012). Within Fraginae, the symbiont community from four species have been examined to date using molecular markers: *Fragum fragum* (Linnaeus, 1758), *Fragum unedo* (Linnaeus, 1758), *Corculum cardissa*, and *Corculum monstrosum* (Gmelin, 1791) (Carlos et al., 1999, 2000; Baillie et al., 2000; LaJeunesse, 2001; Dreier et al., 2014). Symbionts freshly isolated from the hosts mostly belong to *Symbiodinium* clade C, while symbionts cultured from host tissues are from clade A (Carlos et al., 1999). This discrepancy indicates possible selection bias, certain *Symbiodinium* strains may grow well in culture medium even though they are not the dominant strains within the host (Rowan, 1998). Therefore, to fully capture the symbiont diversity in Fraginae, freshly isolated symbionts from a more comprehensive suite of host species need to be examined.

The nature of Fraginae photosymbiosis has not been thoroughly examined across the subfamily, especially given that different photosymbiotic species show great variations in habitat choice and morphological traits (Kirkendale, 2009). For example, species in the genus *Corculum* are epifaunal and found in shallow reef flats with their extremely flattened posterior shells fully exposed (although often fouled). In contrast, many other species are shallowly buried in the sand like typical cardiids. Some populations of certain species can even live in deeper, more turbid environments like subtidal lagoons, e.g., *Fragum sueziense* (Issel, 1869). While some photosymbiotic species have morphologies that are uncommon in extant bivalves, others exhibit shell morphologies that do not differ greatly from non-photosymbiotic fragines. The different forms observed within the photosymbiotic Fraginae are suggestive of a variable reliance on photosymbiotically derived carbon sources. Therefore, it is important to verify whether the bivalve-algal relationship is nutritionally based and truly mutualistic, as research on gastropod-algal associations have suggested possible parasitic interactions between the adult animal and the algal symbionts (Banaszak et al., 2013).

Stable carbon isotope ratios have been useful in assessing trophic interactions in the marine environment

(Robinson and Cavanaugh, 1995). The ratios are represented by $\delta^{13}\text{C}$ values, calculated as $(R_{\text{sample}}/R_{\text{std}}-1) \times 1000$, where $R_{\text{sample}} = {}^{13}\text{C}_{\text{sample}}/{}^{12}\text{C}_{\text{sample}}$ and R_{std} refers to the Vienna Pee Dee Belemnite Standard. Marine phytoplankton typically exhibit depleted ${}^{13}\text{C}$ (i.e., negative $\delta^{13}\text{C}$) in their tissues compared to zooplankton, as they use ribulose 1,5-bis-phosphate carboxylase/oxygenase (RubisCO) to catalyze carbon fixation and all forms of RubisCOs fix ${}^{12}\text{CO}_2$ more rapidly than ${}^{13}\text{CO}_2$ (Hayes, 2001). This signature is reflected in heterotrophic organisms that consume phytoplankton. For example, common filter-feeding cardiid exhibit $\delta^{13}\text{C}$ values between -24‰ and -22‰ (Rossi et al., 2004). Peridinin-containing dinoflagellates, including *Symbiodinium*, use form II RubisCO that discriminates less against ${}^{13}\text{C}$ than most other forms of RubisCO (Cavanaugh and Robinson, 1996; Rowan et al., 1996). Thus, organisms relying largely on *Symbiodinium*-derived carbon sources will be more ${}^{13}\text{C}$ enriched (i.e., show less negative $\delta^{13}\text{C}$ values) than those who consume diverse phytoplankton. For instance, the photosymbiotic giant clam *Tridacna gigas* (Linnaeus, 1758) exhibits $\delta^{13}\text{C}$ value of -16‰ (Black and Bender 1976). This differential isotope signature may be used to assess the carbon sources of *Fragum*. If *Fragum* uses symbiotically derived carbon, their tissues should show less depleted ${}^{13}\text{C}$ than other filter-feeding cardiid.

The diverse spectrum of habitats, morphologies, and the inclusion of non-symbiotic species in Fraginae suggest that species in this subfamily could represent early evolutionary establishments of photosymbiotic partnerships. Therefore, understanding the origin of photosymbiosis in Fraginae will provide a better picture of how marine metazoans and photosynthetic algae develop symbiotic associations. It is unclear whether the two photosymbiotic cardiid groups, Tridacninae and Fraginae, share a common photosymbiotic ancestor or independently evolved the associations, as the evolutionary relationships between the two groups are currently unresolved (Herrera et al., 2015; Kirkendale and Paulay, 2017). Thus, it is important to examine the morphology and ecology aspects of the two groups to gain more comparative data, especially on the less studied subfamily Fraginae. Here, we characterized photosymbiosis in four representative fragines to deepen our understanding of this subfamily.

Specifically, we identified the phylogenetic relationships of the dinoflagellate symbiont communities from the Fraginae species and synthesized current knowledge on symbiont diversity in Fraginae and Tridacninae to contextualize these findings. We also investigated the stable carbon isotopes of the fragines to evaluate their potential food sources. These results add to our knowledge of the enigmatic bivalve group Fraginae and shed more light on photosymbiotic evolution in the Cardiidae.

MATERIALS AND METHODS

Specimen Collections

Four photosymbiotic Fraginae species from different habitats were chosen for this study (Table 1). Three of them were collected from Guam: *Fragum fragum*, *Fragum scruposum* (Deshayes, 1855), *Fragum sueziense*; and one from Australia: *Corculum*

TABLE 1 | Information of bivalve species collected for this study.

Species	Locality	Depth (m)	Association with algae
<i>Fragum fragum</i>	Guam	0.5–1	Yes
<i>Fragum scruposum</i>	Guam	0.5–1	Yes
<i>Fragum sueziense</i>	Guam	6–10	Yes
<i>Corculum cardissa</i>	Australia	<1	Yes
<i>Fulvia</i> sp.	Guam	6–10	No

cardissa, *F. fragum*, and *F. scruposum* were collected from shallow sandy habitats at depth 0.5–1 m at East Agana Bay, Guam, by sieving through sand while snorkeling. *F. sueziense* were collected from sandy bottoms at depth 6–10 m via scuba diving at Outhouse Beach, Guam. One co-occurring heterotrophic cardiid species (*Fulvia* sp.) was also collected from Outhouse Beach as a control group. Specimens were processed at the University of Guam Marine Laboratory. Gill, mantle, and foot tissues of the specimens were fixed in 4% paraformaldehyde for histological study or dried for stable isotope analysis (see General morphology and histology and Phylogenetic analysis). Remaining specimens were preserved in 95% ethanol and deposited in the Museum of Natural History, University of Colorado Boulder (Supplementary Table 1). The collecting was done under the banner of the University of Guam Marine Laboratory permits for scientific collection as provided by the Government of Guam Department of Agriculture.

One specimen of *Corculum cardissa* was collected from intertidal reef habitat during the Western Australia Museum expeditions to Rowley Shoals in October 2014. Mantle and gill tissues were preserved in RNAlater. The rest of the specimen was preserved in 95% ethanol and deposited in the Western Australian Museum.

General Morphology and Histology

The general morphology of *Fragum fragum* ($n = 5$), *F. scruposom* ($n = 5$), and *F. sueziense* ($n = 2$) was examined to identify and characterize symbiont-containing tissues. Slices of gill and mantle tissues were removed and the symbionts were examined *in hospite* (i.e., within host) using light microscopy.

To examine the organization of the symbionts within the bivalve, gill tissues were embedded in paraffin and sectioned using standard histology protocols. Sections (10 μm) were placed on glass slides and stained with 0.05% toluidine blue solution for 20 min to highlight cell nuclei; rinsed with distilled water and air dried. Slides were then cleared in two changes of xylene and Canada balsam was used to fix the cover slips. Slides were examined using an AxioZoomV16 Microscope (Harvard Center for Biological Imaging).

Phylogenetic Analysis

Total DNA (host and symbiont) was extracted from approximately 3 mm^2 of the symbiont-containing bivalve gill tissues using the Omega E.N.Z.A. Mollusc DNA kit. Gill tissues were homogenized in 1.5 ml microcentrifuge tubes using plastic pestles in 200 μL of ML1 buffer provided in the kit. An

additional 150 μ L of buffer was added to the homogenates and DNA was extracted according to manufacturer's instructions.

Four different *Symbiodinium* genetic markers were analyzed in this study: nuclear 18S rRNA, nuclear 28S rRNA, chloroplast 23S rRNA, and mitochondrial COI. Genes were amplified using *Symbiodinium*-specific primers following published protocols (Table 2). PCR amplifications were performed using Promega GoTaq Green Master Mix. Amplicons were visualized on a 1% agarose gel, purified using the Omega E.N.Z.A. Cycle Pure Kit, and directly sequenced at the Dana-Farber/Harvard Cancer Center DNA resource core. Sequence data were deposited in Genbank (Supplementary Table 1).

Sequences from this study were aligned with *Symbiodinium* sequences retrieved from Genbank (Supplementary Table 1) in Geneious 8.1.3 (Biomatters) using the built-in Geneious algorithms with default parameter settings. A maximum likelihood phylogeny was constructed for each gene with 100 bootstrap replicates using the RAxML 7.7.1 (Stamatakis et al., 2008) online server hosted at RAxML BlackBox (<http://embnet.vital-it.ch/raxml-bb/index.php>). The best scoring trees were selected to represent the phylogenies.

Bayesian phylogenies were established for each individual gene and the concatenated dataset using MrBayes v3.2.6 (Ronquist et al., 2012). The concatenated dataset only included *Symbiodinium* strains that were represented by more than two gene markers. Gene substitution models were selected using PartitionFinder (Lanfear et al., 2012) based on Bayesian information criterion as follows. 23S and 28S: GTR+I+G; 18S: K80+I+G; COI codon 1: HKY+G; COI codon 2, and 3: F81+I. For each dataset, two independent MCMC runs were conducted simultaneously for 10 million iterations and sampled every

1,000 iterations. Convergence was visually examined for each analysis in Tracer V1.6.0 (Drummond et al., 2012). A 50% majority rule consensus phylogeny was generated with 25% burnin. All Bayesian analyses were conducted on the CIPRES science Gateway (<http://www.phylo.org>). All phylogeny data were deposited in TreeBase (Study ID: 21845).

Stable Carbon Isotopes Analyses

Symbiont-containing mantle and symbiont-free foot tissues of *Fragum fragum* ($N = 5$), *F. scruposom* ($N = 9$), and *F. sueziense* ($N = 1$) were dissected and rinsed in filtered seawater. The mantle and foot tissues of the non-photosymbiotic cardiid (*Fulvia* sp., $N = 6$) collected from the same locality as *F. sueziense* were also harvested. Due to the small sizes of *Fulvia* specimens, we had to combine foot and mantle tissues together for the analysis. Tissues were placed on pieces of aluminum foil and dried for 48 h at 40°C. Dried tissues were acidified and stable carbon isotopes were measured using IsoPrime isotope ratio mass spectrometer at the Boston University Stable Isotope Laboratory. $\delta^{13}\text{C}$ values (‰) were reported as $(R_{\text{sample}}/R_{\text{std}}-1) \times 1000$, where $R_{\text{sample}} = {}^{13}\text{C}_{\text{sample}}/{}^{12}\text{C}_{\text{sample}}$ and R_{std} refers to the Vienna Pee Dee Belemnite Standard. For technical details on isotope analysis, please refer to the Boston University Stable Isotope Laboratory quality assurance webpage: <https://www.bu.edu/sil/quality.htm>. Stable carbon isotopes were not determined for *Corculum cardissa* as the specimen available was not preserved for isotope analysis.

A Welch's *t*-test was used to statistically compare $\delta^{13}\text{C}$ values between foot and mantle tissues of the Fraginae species, as well as between Fraginae and *Fulvia* tissues. Values for *F. sueziense* were not used in the test due to small sample size.

TABLE 2 | *Symbiodinium* specific primers and PCR protocols used in this study.

Gene	Length (bp)	Primers	Sequence 5'-3'	Protocol	References
28S	755	ls1.5 (F) ls1.3 (R)	CGCTGAAATTAAGCATATAAGTAAG AACGATTTGCACGTCAGTATC	94°C/2 min; 30 cycles: 94°C/15 s, 52°C/20 s, 72°C/45 s; Extension: 72°C/7 min	Wilcox, 1998
18S	881	ss5zA/B (F) ss3z (R)	GCAGTTATARTTTATTTGATGGTCRCTGCTAC AGCACTGCGTCAGTCCGAATAATCACCGG	30 cycles: 94°C/1 min, 55°C/2 min, 72°C/3 min	Rowan and Powers, 1991
23S	890	23S1M13 (F) 23SS2M13 (R)	CACGACGTTGTAAAACGACGGCTGTAACATAACGGTCC GGATAACAATTTACACAGGCCATCGTATTGAACCCAGC	95°C/1 min; 35 cycles: 95°C/45 s, 55°C/45 s, 72°C/60 s; Extension: 72°C/7 min	Santos et al., 2002
COI	475	DINOCOX1F DINOCOX1R COX1.DINO.F COX1DINO.2.R	AAAAATTGTAATCATAAACGCTTAGG TGTTGAGCCACCTATAGTAAACATTA GAATTTGGAGGTGGCACNGGNTGGACNYT CCCATCGTATACATRTGRTGNCCCCANAC	3 min at 94°C; 35 cycles: 94°C/30 s, 48°C/30 s, 72°C/45 s; Extension: 72°C/7 min	Zhang et al., 2007 Stern et al., 2010

Forward and reverse primers are indicated by F and R. Note that the COI gene is amplified via nested PCR using the two sets of primers respectively.

RESULTS

Overall, we found abundant symbionts in the mantle, gill and part of the foot tissues in the photosymbiotic fraginae, and the symbionts were extracellular and contained in tubules within host tissues. Symbiont sequences all fell in *Symbiodinium* clade C based on our molecular phylogenies. All three *Fragum* species exhibited less depleted C^{13} compared to filter-feeding cockles, although *F. sueziense* showed $\delta^{13}C$ values closer to those of the filter-feeding bivalves (see details below).

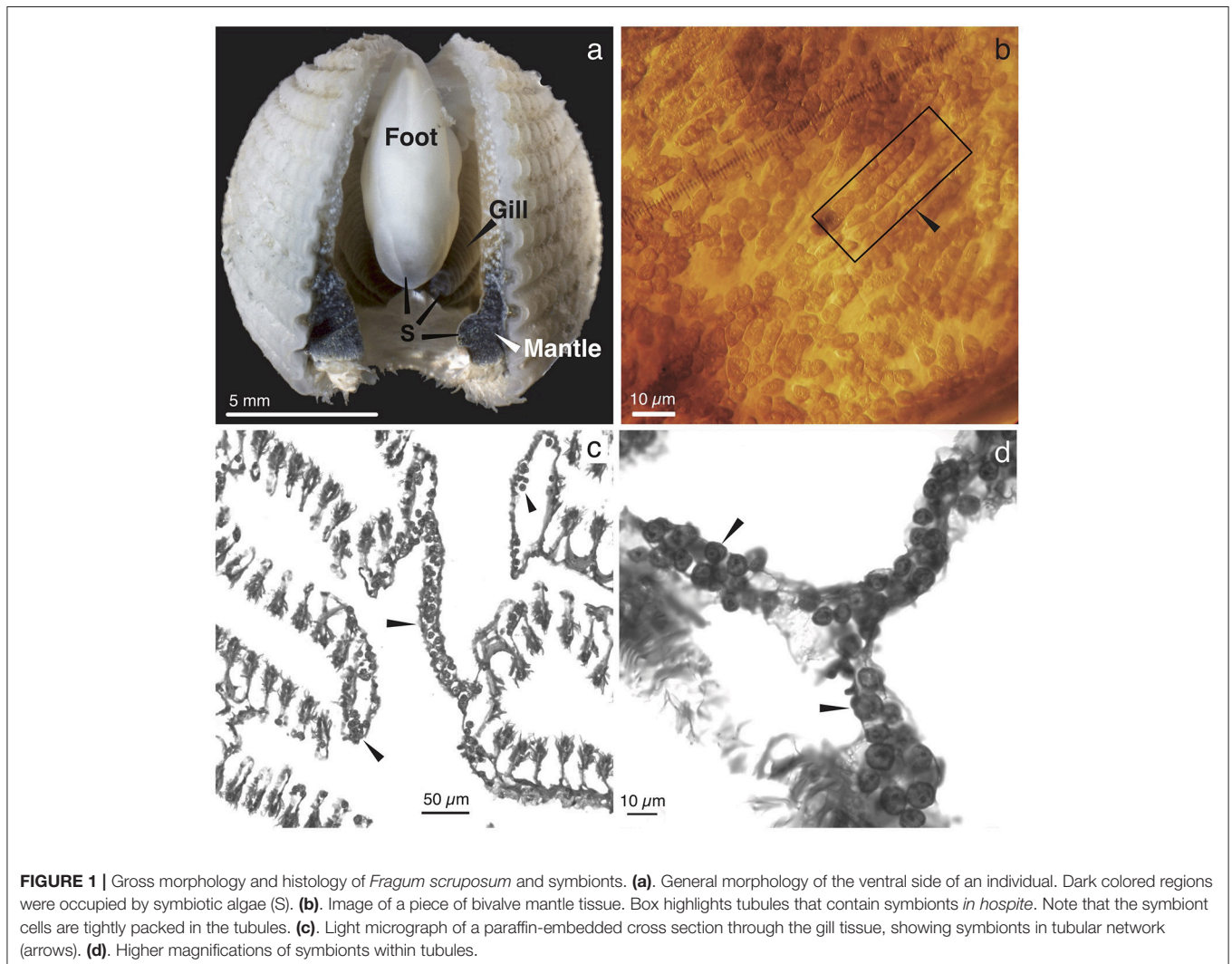
General Observations and Morphology

Abundant single-cellular golden brown algae were found in most of the mantle, the entire gill, and the heel portion of the foot of the three *Fragum* species (*F. fragum*, *F. scruposum*, and *F. sueziense*) that were observed alive (**Figure 1a**). The symbiont-containing structures within the tissues were similar among all three species, and images of *F. scruposum* are shown as representatives. In all symbiont-containing tissues, the algae were extracellular, and tightly packed within the lumen of tubular

structures (**Figures 1b–d**). The tubules appeared to be narrow enough to accommodate only one or two rows of algal cells. When observed *in hospite*, the algal cells exhibited great flexibility and mostly conformed to the shape of the tubules (**Figure 1b**). Only when the cells were squeezed out of the tissues after fixation, did they resume the typical spherical shape of the coccoid stage found in other invertebrate hosts.

Phylogenetic Position of Fraginae Symbionts

All *Symbiodinium* genetic markers were successfully amplified from tissues of all four Fraginae species: *Fragum fragum* ($N = 7$), *F. scruposum* ($N = 5$), *F. sueziense* ($N = 1$), and *Corculum cardissa* ($N = 1$). Both maximum likelihood (ML) and Bayesian analyses recovered well-supported clades (A–H) that are largely congruent with current *Symbiodinium* phylogenies (Pochon et al., 2012, 2014). For simplicity, only Bayesian topologies are shown here (**Figure 2**). ML phylogenies are available in TreeBase (ID: 21845).



Sequences amplified from symbiont communities from all four Fraginiae species fell in *Symbiodinium* clade C. The placement was consistent for the concatenated phylogeny (Figure 2) and all four individual gene trees. Clade A symbionts were not detected in our samples, although their existence is reported from other studies (Carlos et al., 1999, 2000; Baillie et al., 2000; LaJeunesse, 2001). Symbiont sequences showed low variations among different host species for the more conservative

genes (18S, 28S, and 23S). The mitochondrial COI gene revealed some substructures among different Fraginiae symbionts, but the symbionts did not cluster based on host species or locality. The overall genetic variation in the entire clade C was relatively low compared to other clades such as A, D, and F.

Currently known Fraginiae-associated symbiont phylogenetic diversity based on our results and five other molecular studies (Carlos et al., 1999, 2000; Baillie et al., 2000; LaJeunesse, 2001; Dreier et al., 2014) are summarized (Table 3, Supplementary Table 2). All freshly isolated symbiont communities (i.e., non-cultured) were from clade C, except for one individual of *Fragum unedo*, which harbored clade A symbionts (Baillie et al., 2000). In contrast, all symbiont communities cultured from host tissues belonged exclusively to clade A (refer to Supplementary Table 2 for details on the host species, geographical locations, genetic markers used, and references). To compare symbiont diversity between Fraginiae and Tridacninae, a summary of symbiont communities for Tridacninae was also included in Table 3 and Supplementary Table 2.

Stable Isotope Analyses

Stable carbon isotope ratios of the bivalve species studied here are shown in Table 4, and compared with $\delta^{13}C$ values for related filter-feeding bivalves in Cardiidae (Figure 3). The stable carbon isotope ratios of the symbiont-containing mantle and symbiont-free tissues of the two shallow water photosymbiotic species, *Fragum fragum* and *F. scruposum*, were relatively similar with $\delta^{13}C = -13.1$ to -16.5% . Both mantle and foot values were statistically different ($P < 0.001$) from that of the filter-feeding cardiid, *Fulvia* sp. ($\delta^{13}C = -20\%$). The deeper species,

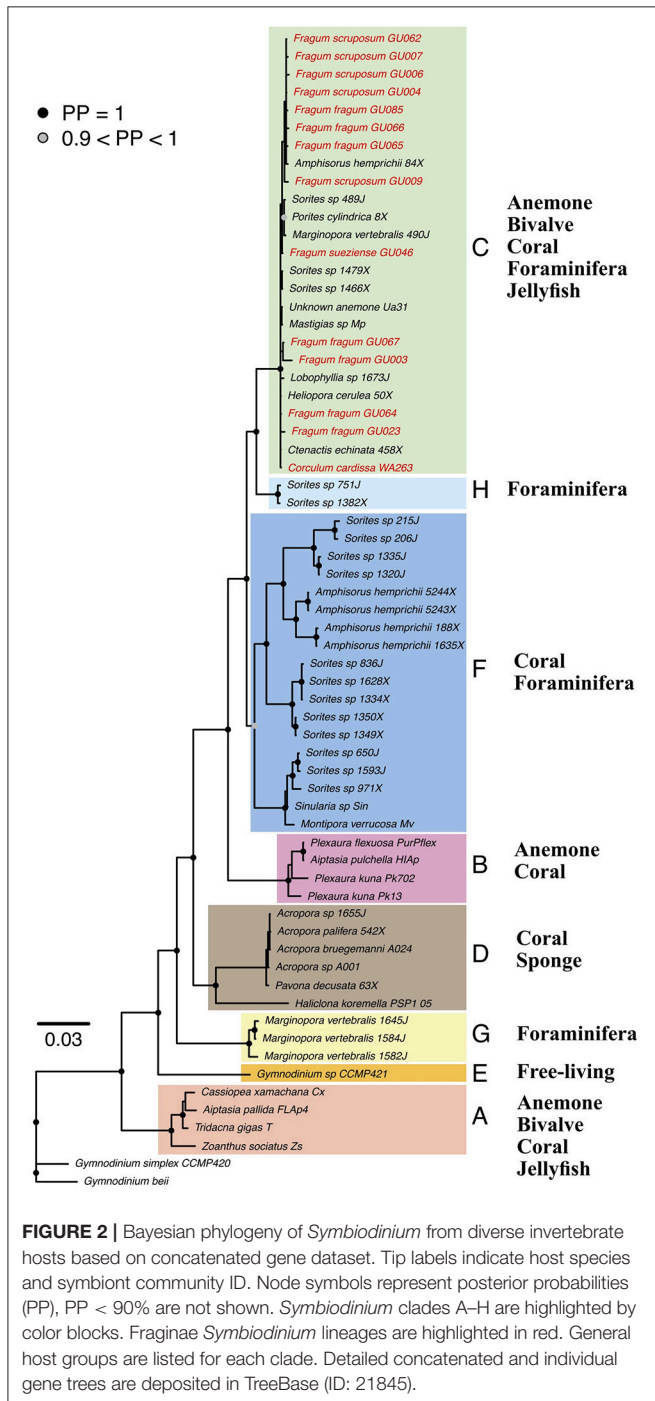


TABLE 3 | Phylogenetic identity of Fraginiae and Tridacninae associated *Symbiodinium* based on this study and literature review.

Subfamily	Host species	Sample size	<i>Symbiodinium</i> clade	
			Freshly isolated	Cultured
Fraginiae	<i>Corculum cardissa</i>	5	C	A
	<i>Corculum monstrosum</i>	1	–	A
	<i>Fragum fragum</i>	9	C	–
	<i>Fragum scruposum</i>	5	C	–
	<i>Fragum sueziense</i>	1	C	–
	<i>Fragum unedo</i>	4	A,C	A
	Tridacninae	<i>Hippopus hippopus</i>	9	C
<i>Hippopus porcellanus</i>		3	C	A
<i>Tridacna crocea</i>		264	A,C,D	A,C
<i>Tridacna derasa</i>		5	C	A
<i>Tridacna gigas</i>		9	C	A
<i>Tridacna maxima</i>		58	A,C,D	A
<i>Tridacna squamosa</i>		166	A,C,D	A
<i>Tridacna squamosina</i>		13	A	–

Letters indicate the *Symbiodinium* clade type. See Supplementary Table 2 for detailed information on each study, including geographical distribution, study methods, genetic markers used, and reference.

TABLE 4 | Tissue $\delta^{13}\text{C}$ values of *Fragum fragum*, *F. scruposum*, *F. sueziense*, and the filter-feeding *Fulvia* sp.

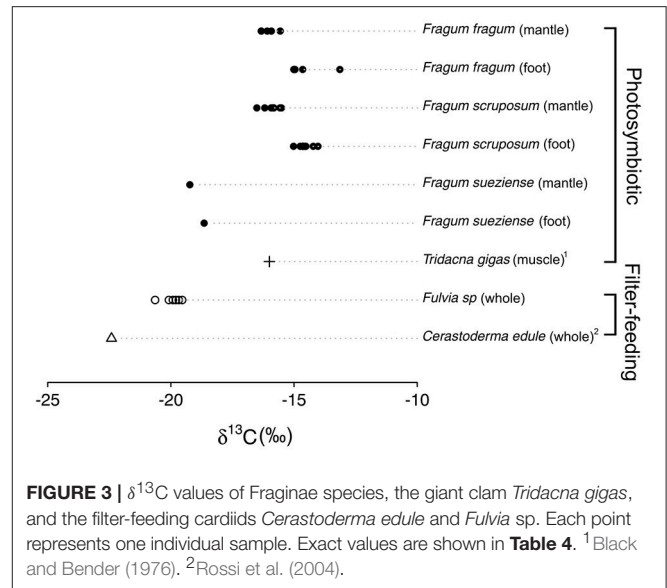
Species	Sample size	Tissue	$\delta^{13}\text{C}$ (‰)	
			Average	Range
<i>Fragum fragum</i>	5	Foot	-14.6 ± 0.8	-13.1 to -15.0
	5	Mantle	-16.1 ± 0.3	-15.6 to -16.3
<i>Fragum scruposum</i>	9	Foot	-14.6 ± 0.3	-14.0 to -15.1
	8	Mantle	-15.9 ± 0.3	-15.51 to -16.5
<i>Fragum sueziense</i>	1	Foot	-18.7	NA
	1	Mantle	-19.2	NA
<i>Fulvia</i> sp.	6	Mantle and foot	-20.0 ± 0.4	-19.6 to -20.6

F. sueziense, was more depleted in ^{13}C than the shallower *Fragum* species, with a mean $\delta^{13}\text{C} = -19\text{‰}$, closer to that of *Fulvia* sp. For the three symbiotic *Fragum* spp., the foot tissue was enriched over the mantle tissues in ^{13}C , averaging 1.4 and 0.5 ‰ for the two shallow and one deep species, respectively. The $\delta^{13}\text{C}$ differences between foot and mantle tissues were statistically significant ($P < 0.01$) for *F. fragum* and *F. scruposum* (*F. sueziense* could not be tested due to small sample size). Overall, the $\delta^{13}\text{C}$ values of all three *Fragum* species were largely comparable with the photosymbiotic cardiid, the giant clam, *Tridacna gigas*, but more enriched in ^{13}C than those of the filter-feeding cardiids, *Fulvia* sp. and *Cerastoderma edule* (Figure 3).

DISCUSSION

Our study on representative Fraginiae species indicates that they share similarities with the giant clams in terms of symbiont diversity and general symbiont-containing structures. Stable isotope analyses indicate that these bivalves utilize photosymbiosis to obtain at least part of their food sources.

The symbiont-containing morphologies of the three *Fragum* species are consistent with that of a previously examined Fraginiae species *Corculum cardissa* (Farmer et al., 2001). This is consistent with the preliminary conclusion that fragines may all share similar tubular systems. Farmer et al. (2001) reported that *Corculum cardissa* contains symbionts in both mantle and gill tissues. We found additional symbionts in foot tissues of all *Fragum* species tested. The symbionts are mostly concentrated in the heel portion of the foot, which is directly under the siphonal opening of the animal in its natural condition (i.e., exposed to more light); while the rest of the foot is symbiont-free. This is the first published observation of photosymbionts housed in the foot of a bivalve. This new information can be interpreted as evidence that symbiont locations are relatively flexible in Fraginiae and the development of symbiont-containing tubules could potentially be influenced by levels of light intensity. The ephemeral nature of zooxanthellal tubules has been observed in *Tridacna*,



with tubules only developing in juveniles in the presence of zooxanthellae (Fitt and Trench, 1981). Tridacnids also exhibit tertiary tubule atrophy following bleaching experiments, suggesting that these structures may be regenerative following bleaching incidents (Norton et al., 1992). Coupling with our observations on Fraginiae tubular structures, it is obvious that a research gap exists concerning how this inducible tubular system functions and is maintained at the molecular and cellular levels.

Fraginiae symbiont cells that were examined *in hospite* show surprising shape flexibility. The algae were in full contact with each other and within tubules, exhibiting cylinder shapes due to the tight packing. *Symbiodinium* in other invertebrate hosts are typically reported as having a spherical (coccoid) shape maintained by a multiple-layered cell wall (Trench et al., 1981; Wakefield et al., 2000). Although we did not examine the Fraginiae symbiont cell wall structure in detail, it is likely that the pleomorphic shapes observed here may be a result of reduced cell wall thickness, as it is known that *Symbiodinium* cell wall development can be somewhat flexible and symbionts cultured outside of their hosts develop significantly thicker cell walls (Palincsar et al., 1988). In addition, artificially removing cell walls can cause *Symbiodinium* to have pleomorphic shapes (Levin et al., 2017). Therefore, symbiont cell wall construction within host may be suppressed or regulated to facilitate nutrient exchange and/or host communication. It is also possible that reports on symbionts in other hosts (Trench et al., 1981) are affected by tissue fixation artifacts, as even within-tubule symbionts in our samples showed spherical shapes after being fixed in 4% paraformaldehyde (Figures 1c,d).

Phylogenetic analyses recovered exclusively clade C *Symbiodinium* sequences from all four Fraginiae species and the symbiont communities exhibited low levels of genetic variation among different hosts (Figure 2). Although the COI phylogeny

showed some structure, support values for the subclades were relatively low. Based on genetic markers used in this study, the symbiont communities do not seem to be Fraginae-specific and do not show any signature of co-diversification with the hosts. Clade C *Symbiodinium* is the most dominant clade in the Indo-Pacific region and can be found in diverse groups of invertebrate hosts (LaJeunesse, 2005; Pochon et al., 2006). Given that the photosymbiotic Fraginae is a relatively young clade (late Miocene, Vermeij, 2013), existing symbionts in clade C likely established associations with fragines via host expansion. It is worth noting that even though we did not identify any Fraginae-specific *Symbiodinium* subclades within clade C, a more variable genetic marker (e.g., ITS) may reveal a different picture. Species identification for *Symbiodinium* requires dense population-level sampling; both morphological examination (e.g., Lee et al., 2015) and more genetic markers are needed to clarify host specificity of the diverse *Symbiodinium* lineages.

In coral symbioses (LaJeunesse et al., 2004), it is quite common to have multiple *Symbiodinium* lineages in one host species, as “stocking” multiple ecologically diverse symbiont lineages may facilitate the hosts’ ability to cope and adapt to fluctuating environments (Jones et al., 2008; LaJeunesse et al., 2009). Therefore, although we only detected clade C *Symbiodinium* in our study, symbionts from other clades may coexist within the fragines, but are not the dominant strains. Clade A *Symbiodinium* have been frequently found in symbiont communities cultured from Fraginae and Tridacninae hosts, but not from freshly isolated symbionts. If this is not a result of the presence of non-intercellular algae (e.g., environmental free-living algae on host body surface), it suggests that a small proportion of Clade A symbionts exist in the host but the numbers are not high enough to be identified by standard PCR methods. To capture these potential rare stains in Fraginae, more sensitive methods that can detect multiple lineages per host (e.g., clade-specific primers, qPCR, cloning, high-throughput sequencing, etc.) will be needed in the future. In addition, our sequences were obtained through standard PCR amplifications from a community of symbionts within each host. Therefore, it is unavoidable that the amplicons are composed of mixed sequences from different symbiont individuals, and these mixed signals could generate artifacts (e.g., chimeric sequences) in the Sanger sequencing process. Therefore, it is important to note that these sequences may represent symbiont community amplicons instead of gene identities of individual symbionts.

Stable carbon isotope ratios suggest that *Symbiodinium* supplies photosynthates for the *Fragum* species (Table 4, Figure 3). The tissue carbon isotope ratios of the two shallow water species *Fragum fragum* and *F. scruposum* ($\delta^{13}\text{C} = -13.1$ to -16.5‰) are consistent with those of other zooxanthellae symbioses, including the giant clam *Tridacna gigas*, corals, and sea anemones with transfer of symbiont-fixed carbon to host documented in a number of these species (Black and Bender, 1976; Baker et al., 2015). Symbioses with zooxanthellae are typically enriched in ^{13}C relative to filter-feeding invertebrates since *Symbiodinium* sp. fix CO_2 using a form II RubisCO which

discriminates against ^{13}C less than form I RubisCOs of most free-living algae (Rowan et al., 1996). Further, the symbiont-free foot tissues of *F. fragum* and *F. scruposum* are ^{13}C enriched on average 1.3 to 1.5‰ compared to their symbiont-containing mantle. Such enrichment is typical of the small metabolic fractionations that occur during producer—consumer interactions (DeNiro and Epstein, 1978). $\delta^{13}\text{C}$ of the symbiont-containing mantle reflects a value combination of both the algae and the host (producer and consumer). $\delta^{13}\text{C}$ of the foot tissue, on the other hand, only reflects value of the host (consumer). So the difference is partially a result of the isotope composition difference between the symbionts and the bivalve. It is known that animal consumers take on isotopic composition of their food with a small ^{13}C enrichment about 1–2‰ (DeNiro and Epstein, 1978). This enrichment pattern has been confirmed in chemosymbiotic bivalves (Conway et al., 1989). Therefore, the observed ^{13}C enrichment in the bivalve foot over mantle tissues further suggests that the bivalves are utilizing symbiont produced organic carbon.

Tissues of the deeper photosymbiotic *Fragum sueziense* exhibit an isotopic signature comparable to some photosymbiotic organisms (Baker et al., 2015), with a 0.5‰ ^{13}C enrichment of foot relative to mantle tissue. However, its $\delta^{13}\text{C}$ values are only slightly less negative than that of the co-occurring filter-feeding cockle *Fulvia* sp. A probable explanation is that due to the lower light intensity at depth, *F. sueziense* obtains a smaller proportion of organic carbon from photosymbiosis than *F. fragum* and *F. scruposum*, and that this host species likely supplements its nutrition obtained via filter feeding. A similar trend is seen in corals with $\delta^{13}\text{C}$ values increasing with depth (Muscatine et al., 1989). However, our sample size for *F. sueziense* is small due to the rarity of this species, hindering our ability to conduct statistical analysis. To further explore this phenomenon, additional sampling of Fraginae species across a broader depth gradient (ter Poorten, 2009), coupled with measurements of photosynthetic activity and conventional feeding, are needed to clarify the host nutritional and the stability/plasticity of the fragine-zooxanthellae trophic interactions.

Our results demonstrated several similarities between the Tridacninae and photosymbiotic Fraginae bivalves. Like Tridacninae, the Fraginae species examined appear to rely on photosynthetically derived carbon sources from the symbionts as a food source. Both can be associated with clades C or A *Symbiodinium* and harbor them in similar tubule systems. The question remains as to whether these similarities evolved independently or were inherited from a common ancestor. The two groups obviously differ in photosymbiotic ecology and general morphology. The fossil record of tridacnine bivalves can be traced back to Oligocene while symbiotic fragines have only be found extending to late Miocene (Vermeij, 2013). If photosymbiosis was acquired separately by the two subfamilies, mechanisms that generate a convergent evolution of the symbiont harboring tubule systems need to be illustrated. If photosymbiosis is an ancestral trait, losses of symbiotic relationship within the non-symbiotic fragines and possibly other cockle lineages

need to be explained (Kirkendale and Paulay, 2017). In addition, given the late appearance of photosymbiotic Fraginae morphology in the fossil record, even if the symbiotic associations are ancestral, the adaptive shell phenotypes must have evolved later and might be driven by habitat shifts and/or expansion to light-abundant environments. Further studies will use comprehensive phylogenetic and functional genomic approaches to illuminate the evolution and origin of symbiotic associations between bivalve hosts and their algal symbionts.

ETHICS STATEMENT

Protocols for studying preserved invertebrate animals were exempt from Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

JL and CC designed the study. JL, MV, and LK conducted the fieldwork. JL and MV performed the experiments. JL, LK, and CC wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2018.00045/full#supplementary-material>

Supplementary Table 1 | Information of all symbiont sequences used in this study, including host species, genbank ID and voucher ID.

Supplementary Table 2 | Current knowledge on Fraginae and Tridacninae-associated symbiont diversity, including host species, geographical distribution, symbiont type, and genetic markers used.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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