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Eggs of echinoids separated by the Isthmus of Panama harbor divergent microbiota

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ABSTRACT: Relationships between animals and their associated microbiota are dependent on both the evolutionary history of the host and on the environment. The majority of studies tend to focus on either one of these factors but rarely consider how both determine the community composition of the associated microbiota. One 'natural experiment' to test how evolutionary history, shared environments, and the interaction between these factors drive community composition is to compare geminate species pairs. Echinoids separated by the Isthmus of Panama are suitable for this comparison due to their known evolutionary history and differences in the oceanographic characteristics of the Caribbean Sea and the Pacific Ocean. By comparing the bacterial communities of the eggs of *Echinometra* and *Diadema* geminate species pairs, we show that each pair of geminate species associates with a distinct bacterial community in a pattern consistent with phylosymbiosis, and that the interaction between the evolutionary history of the host and the environment best explains differences in these communities. Moreover, we found that the relative abundance of particular bacterial taxa differed considerably between the 2 bodies of water and that the 2 Caribbean *Echinometra* species were dominated by unclassified bacterial taxa within the phototrophic *Oxyphotobacteria*. Taken together, data presented here support the hypothesis that the bacterial communities associated with geminate species are another characteristic of these species that have diverged in ~2.8 million years of isolation.

KEY WORDS: Sea urchin · Symbiosis · Microbiome · Host–microbe · Phylosymbiosis · Geminate species · *Echinometra* · *Diadema*

1. INTRODUCTION

Relationships between animals and their associated microbiota are generally hypothesized to be driven by 2 primary factors: the evolutionary history of the host and the environment (Bordenstein & Theis 2015). In the former, animals associate with species-specific microbial communities that often co-vary with host phylogeny (Schmitt et al. 2012, Brooks et al. 2016, Carrier & Reitzel 2018, Pollock et al. 2018, O'Brien et al. 2019, Lim & Bordenstein 2020). In the latter, the composition of host-associated microbiota depends on the abiotic and biotic environments, such that bacterial communities may differ, for example, due to temperature, salinity, and diet, to form a community with specific functional properties (Soto et al. 2009, Webster et al. 2011, Kohl & Carey 2016, Carrier & Reitzel 2017).

Studies assessing the relative importance of the host's evolutionary history and the environment have shown that both influence community composition, but one of the 2 factors is commonly more influential. In scleractinian corals (Pollock et al. 2018) and sponges (Easson & Thacker 2014, Thomas et al. 2016), for example, host phylogeny can best explain compositional differences in these microbial communities. Shifts in symbiont composition due to the abiotic environment have been observed in barnacles (Aldred

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& Nelson 2019), echinoderms (Carrier & Reitzel 2020), and sponges (Webster et al. 2011). In select cases, however, the influence of the host's evolutionary history and the environment are comparably similar (Mortzfeld et al. 2016).

Discerning the impacts of evolutionary history and adaptation to different environmental conditions is a challenge in many marine species because phylogenetic relationships are often unknown, and environmental conditions for closely related species tend to overlap. One type of species pair where both factors are known and the contributions of phylogeny and environment can be teased apart are geminate species, or sister species pairs that formed as a result of geological events (Jordan 1908). These geological events are often dated from independent evidence and allow for the rates of molecular divergence between geminate species to be calibrated (Lessios 1979). Moreover, isolation by such geological events also provides a basis for understanding the factors responsible for the evolution of each species in these isolated environments (Lessios 2008, O'Dea et al. 2016). Geminate species are thus an important and potentially powerful system to compare the divergence of organismal traits and determine how these factors relate to communities of host-associated microorganisms (Wilkins et al. 2019).

One geographic change that resulted in multiple geminate species was the formation of the Isthmus of Panama. Until the Miocene, the Caribbean Sea and Tropical Eastern Pacific were continuous, with fauna that spanned the region (Lessios 2008, O'Dea et al. 2016). The emergence of the Isthmus of Panama isolated these 2 bodies of water and also affected the physical conditions of these 2 oceans, causing multiple groups of marine fauna to undergo independent evolutionary trajectories. Those that did not become extinct have since diverged and formed geminate species pairs (Lessios 2008, O'Dea et al. 2016, Wilkins et al. 2019).

One group of well-studied geminate species that resulted from the Isthmus of Panama are echinoids (phylum Echinodermata). The intertidal and subtidal waters off the Panamanian coast feature many geminate pairs, including sea urchins in the genera *Echinometra* and *Diadema*. Following the formation of the Isthmus of Panama, *Diadema* split into the Pacific *D. mexicanum* and the Caribbean *D. antillarum* (Lessios et al. 2001, Hickerson et al. 2006, Lessios 2008). *Echinometra* diverged into *E. vanbrunti* in the Pacific and *E. lucunter* and *E. viridis* in the Caribbean, with the speciation event of the 2 Caribbean species occurring after the emergence of the Isthmus of Panama (McCartney et al. 2000, Lessios 2008). Since the rise of the Isthmus, these geminate pairs have diverged in several life-history characters, including egg size and biochemical composition (Lessios 1990, McAlister & Moran 2012), larval morphology and feeding ecology (McAlister 2008), and the timing of reproductive events (Lessios 1981, 1984). In other echinoids, these life-history characters are correlated with specific bacterial communities (Carrier & Reitzel 2020). For example, eggs of 3 confamilial echinoids are associated with a phylogenetically diverse and species-specific bacterial community that shifts gradually over the course of development and in response to food availability (Carrier & Reitzel 2018, 2019a). Due to the parallels with other echinoids, we hypothesize that members of these geminate pairs would also associate with distinct bacterial communities.

The microbiota associated with geminate species pairs were recently hypothesized to have diverged during their independent evolutionary trajectories, either as a product of the host genetics or environmental differences (Wilkins et al. 2019). For the echinoid geminate species pairs on the Panamanian coast, relatedness of microbial communities would either mirror the evolutionary history of the host or form distinct clades unique to species found in each ocean (Fig. 1). To test this hypothesis, we sampled eggs of all 3 *Echinometra* and 2 *Diadema* species. Bacterial communities of the eggs of these 5 species were then profiled using amplicon sequencing. We found that the bacterial communities are distinct be-

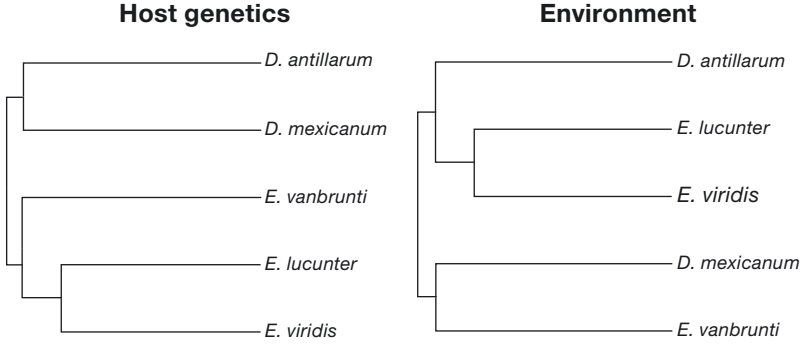

Fig. 1. Hypothetical microbial dendrograms for *Echinometra* and *Diadema* geminate species pairs if host genetics (left) or the environment (right) were the primary factor contributing to community assembly. *D. antillarum*, *E. lucunter*, and *E. viridis* are found in the Caribbean Sea while *D. mexicanum* and *E. vanbrunti* are found in the Pacific Ocean

tween geminate species and reflect an interactive effect between the evolutionary history of the host and the environment as well as a pattern consistent with phylosymbiosis.

2. MATERIALS AND METHODS

2.1. Echinoid collections and spawning

Adult *Diadema mexicanum* and *Echinometra vanbrunti* were collected by SCUBA diving off Isla Taboguilla near Panama City, Panama, in July and August of 2019. Spawning of *D. mexicanum* follows a lunar cycle (Lessios 1981), so collections were made during full moon phases. Adults were transferred to and spawned at the Smithsonian Tropical Research Institute (STRI) Naos Island Laboratories in Balboa. *D. antillarum*, *E. lucunter*, and *E. viridis* were collected at Galeta Marine Laboratory near Colón, Panama, in the Caribbean Sea at new moon phases in July and August of 2019. Of the Caribbean species, *E. lucunter* and *E. viridis* were brought to and spawned at Naos, while *D. antillarum* was spawned at Galeta because the adults often spawn or perish during transport.

Adult sea urchins (n = 15 per species; Table S1 in Supplement 1 at www.int-res.com/articles/suppl/m648p169_supp/) were spawned by intracoelomic injection of 0.50 M KCl. Eggs of all 3 *Echinometra* species were shed into 5.0 µm filtered seawater from their site of origin, while *Diadema* eggs of both species were shed onto and collected from the aboral surface of the mother. Approximately 250 eggs per individual were collected with a sterile pipette and transferred to a 1.5 ml Eppendorf tube, where they were concentrated using a microcentrifuge, and the supernatant was removed. The eggs were preserved in RNAlater in the Eppendorf tube and frozen at −20°C for long-term storage.

The environmental microbiota from the seawater where the sea urchins were collected was also sampled. For both Pacific and Caribbean populations, ~0.5 l of seawater was filtered onto a 0.22 µm Millipore filter to retain the environmental microbiota (n = 3). Full filter disks were preserved in RNAlater and frozen at −20°C.

2.2. Profiling bacterial communities

Total DNA was extracted from sea urchin eggs, seawater samples, and DNA kit/reagent blanks (n = 3) at the University of North Carolina at Charlotte, USA, using the GeneJet Genomic DNA Purification Kit (Thermo Scientific). DNA was quantified using a Qubit Fluorometer (Life Technologies) and diluted to 5 ng μl^{-1} using RNase/DNase-free water. Bacterial sequences were then amplified using primers for the V3/V4 regions of the 16S rRNA gene (Table S2; Klindworth et al. 2013). Products were purified using the Axygen AxyPrep Mag PCR Clean-up Kit (Axygen Scientific), indexed using the Nextera XT Index Kit V2 (Illumina), and then purified again. At each clean-up step, fluorometric quantitation was performed using a Qubit, and libraries were validated using a Bioanalyzer High Sensitivity DNA chip (Agilent Technologies). Illumina MiSeq sequencing (v3, 2 × 300 bp paired-end reads) was performed in the Department of Bioinformatics and Genomics at the University of North Carolina at Charlotte.

2.3. Computational analysis

Raw reads along with quality information were imported into QIIME 2 (v. 2019.1; Bolyen et al. 2019), where adapters were removed, forward and reverse reads were paired using VSEARCH (Rognes et al. 2016), filtered by quality score, and denoised using Deblur (Amir et al. 2017). QIIME 2-generated 'features' were analyzed as amplicon sequence variants (ASVs; Callahan et al. 2017) and were assigned taxonomy using SILVA (v. 132; Quast et al. 2013). Sequences matching to Archaea or found in DNA kit/reagent blanks were filtered from the data, and samples with <1000 reads were discarded (Table S1). The filtered table was then rarified to 1027 sequences per sample (i.e. the read count for the sample with the fewest remaining reads), a depth at which the taxonomic and phylogenetic diversity for our samples had essentially plateaued (Fig. S1 in Supplement 2 at www.int-res.com/articles/suppl/m648p169_supp/).

To test whether community membership and composition were species-specific, we calculated unweighted and weighted unique fraction (UniFrac) values (Lozupone & Knight 2005) and compared them using principal coordinate analyses. Results from these analyses were then recreated in QIIME 1 (v. 1.9.1; Caporaso et al. 2010) and stylized using Adobe Illustrator. We then used a permutational multivariate ANOVA to test for differences in membership and composition between species and, subsequently, performed pairwise comparisons. We also calculated 4 measures of alpha diversity: total ASVs, Faith's phylogenetic diversity, McIntosh dominance,

and McIntosh evenness. We compared these values using a 1-way ANOVA for *Echinometra* and a Student's *t*-test for *Diadema*. Lastly, we summarized the cumulative bacterial profiles associated with all sea urchin species as well as the mean number of shared and species-specific ASVs.

Using the weighted UniFrac values, we constructed microbial dendrograms in QIIME 2, where samples were collapsed by host species by pooling sequence data from all samples for each host. These were then used to test for phylosymbiosis by comparing topological congruence with the cytochrome c oxidase subunit 1 (COI) gene tree for these geminate species pairs (McCartney et al. 2000, Lessios et al. 2001). The host COI tree was constructed using BEAST (v. 1.8.4; Drummond et al. 2012) by starting from a random coalescent tree and running for 10^7 steps, with recordings every 10^3 steps. We then used Tracer (v. 1.7.1; Rambaut et al. 2018) to verify that effective sample size values for all parameters exceeded 4260. A maximum credibility tree, with support of 1 for all nodes, was then estimated using TreeAnnotator (v. 10.10.4). Patterns of phylosymbiosis were tested using the Robinson-Foulds metric in TreeCmp (Bogdanowicz et al. 2012) and matching cluster metrics with 10 000 random trees. This analysis was performed using the Python script of Brooks et al. (2016).

Our QIIME-based pipeline used to convert raw reads to ASVs for visualization is presented in detail in Supplement 3 at www.int-res.com/articles/suppl/m648p169_supp/. The 16S rRNA gene reads are accessible in the Dryad Digital Repository (doi.org/10.5061/dryad.2z34tmphq).

3. RESULTS

3.1. Community relatedness and diversity

The bacterial communities associated with the eggs of these 2 geminate species pairs were species-specific in both community membership and composition, except in 1 case (unweighted UniFrac: $p < 0.001$; weighted UniFrac: $p < 0.001$; Fig. 2; Table S3). The 2 Caribbean *Echinometra* species, *E. lucunter* and *E. viridis*, associated with comparatively similar bacterial communities in both membership and composition (unweighted UniFrac $p = 0.055$; weighted UniFrac: $p = 0.069$; Fig. 2; Table S3). Relatedness of these bacterial communities suggests that the Pacific *E. vanbrunti* groups with the Pacific *Diadema mexicanum* and Caribbean *D. antillarum*, while Caribbean *E. lucunter* and *E. viridis* group separately (Fig. 2). Moreover, comparison of host phylogeny and the bacterial dendrogram suggest that the topological congruence for these trees is non-random, even though they do not fully mirror each other. Phylosymbiosis was thus supported for these geminate species pairs (both Robinson-Foulds and Matching Split: $p = 0.062$, normalized score: 0.0; Fig. 3; Table S4).

Echinometra-associated bacterial communities were, on average, more diverse in individual taxa and phylogenetic breadth than those of *Diadema*

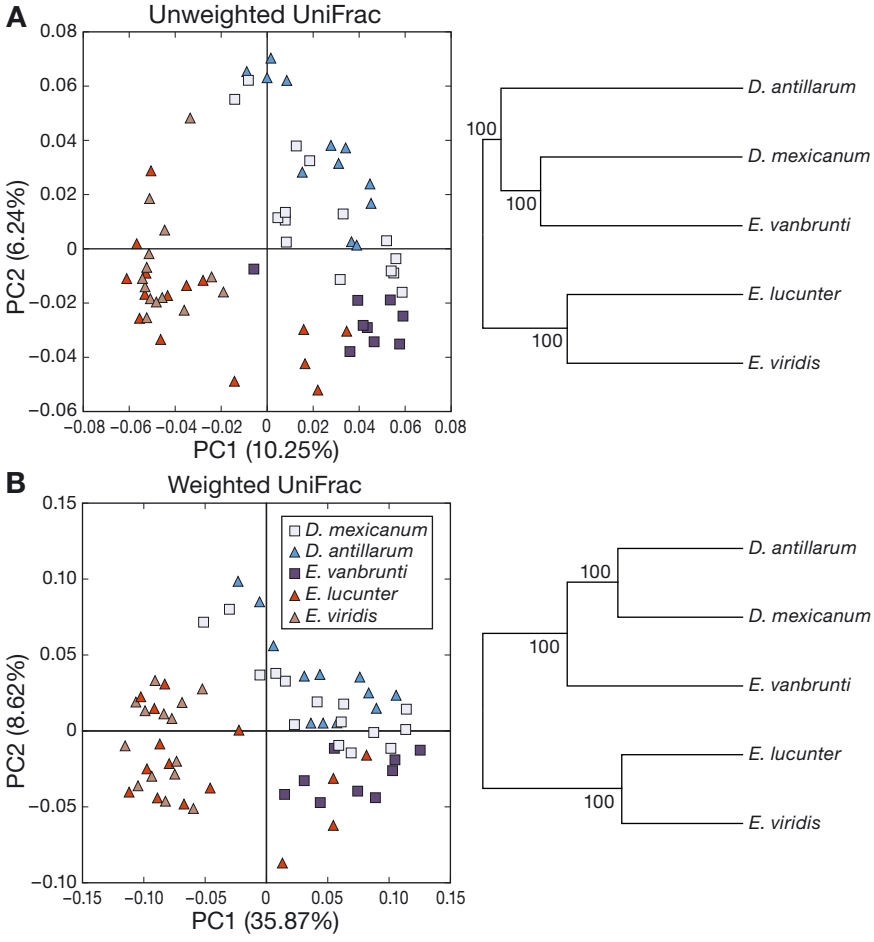

Fig. 2. Similarity between the bacterial communities of *Echinometra* and *Diadema* geminate species pairs based on (A) membership (unweighted UniFrac) and (B) composition (weighted UniFrac) with corresponding microbial dendrograms

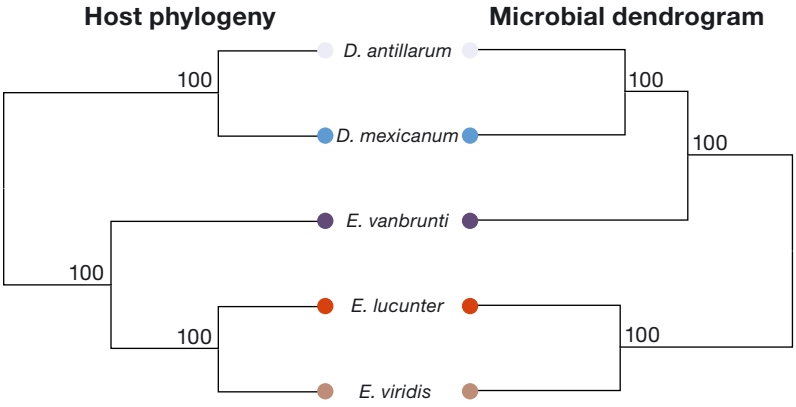

Fig. 3. Phylosymbiosis for geminate sea urchin species pairs. Gene tree using COI for *Echinometra* and *Diadema* geminate species pairs and microbial dendrograms based on community composition (i.e. weighted UniFrac). COI sequences for *Echinometra* are from McCartney et al. (2000) and those for *Diadema* are from Lessios et al. (2001)

(Fig. 4A,B; Table S5). Patterns of community diversity within geminate species pairs differed between genera: the 3 *Echinometra* species were similar to each other, whereas the Pacific *D. mexicanum* was more diverse than the Caribbean *D. antillarum* (Fig. 4; Table S5). Moreover, the bacterial communities of *Echinometra* were more taxonomically dominant and, thus, less even than those of *Diadema* (Fig. 4C,D; Table S5). The eggs of all 3 *Echinometra* species were associated with similarly dominant bacterial communities, while the eggs of *D. mexicanum* had a more taxonomically dominant community than that of *D. antillarum* (Fig. 4; Table S5).

3.2. Taxonomic representation and divergence

Microbiota of both *Echinometra* and *Diadema* eggs were primarily composed of 2 bacterial classes, the *Bacteroidia* (*Bacteroidetes*) and *Gammaproteobacteria* (*Proteobacteria*) (Fig. 5A; Table S6). *Bacteroidia*, on average, represented ~24.4 ± 14.4% (SD) and ~29.6 ± 1.3% of the bacterial community of *Echinometra* and *Diadema*, respectively, while the *Gammaproteobacteria* represented ~35.2 ± 16.8 and ~34.7 ± 1.4% (Fig. 5A; Table S6). The eggs of the Caribbean *Echinometra*, in particular, were also dominated by uncharacterized ASVs within the *Oxyphotobacteria* (phylum *Cyanobacteria*), which represented ~15.2 ± 9.5% of the community (Fig. 5A). These same bacterial taxa were only 2.6 ± 4.4% of the community for 2 *Diadema* species. The *Oxyphotobacteria* were significantly more abundant in the Caribbean *Echinometra* than the Pacific *E. vanbrunti* and represented ~4–5 times more of the community (Fig. 5B; Table S7). In addition to these groups, the eggs of *Echinometra* had 5 other bacterial classes that ranged from ~1.2 to ~6.2% of the community, while *Diadema* had 6 other bacterial classes that represented between ~1.3 and ~12.5% of the community (Fig. 5A; Table S6).

There was a total of 404 ASVs in the 3 *Echinometra* geminate species. Of these, 137 (33.9%), 86 (21.3%),

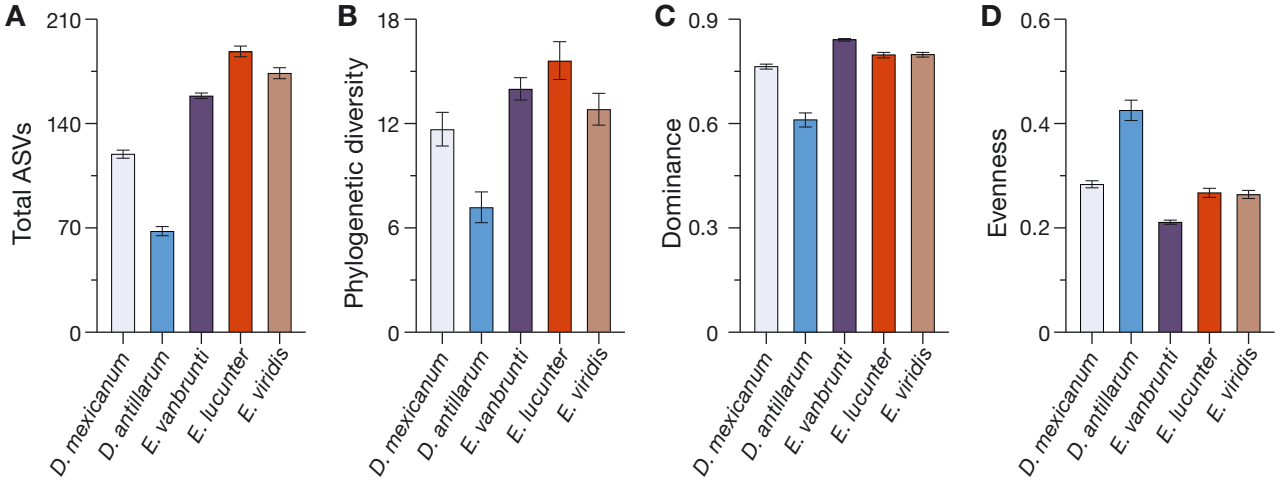

Fig. 4. Diversity of the bacterial communities associated with eggs of *Echinometra* and *Diadema*, as estimated by (A) enumerating total amplicon sequence variants (ASVs), (B) Faith's phylogenetic diversity, (C) McIntosh dominance, and (D) McIntosh evenness. Error bars for each represent SE

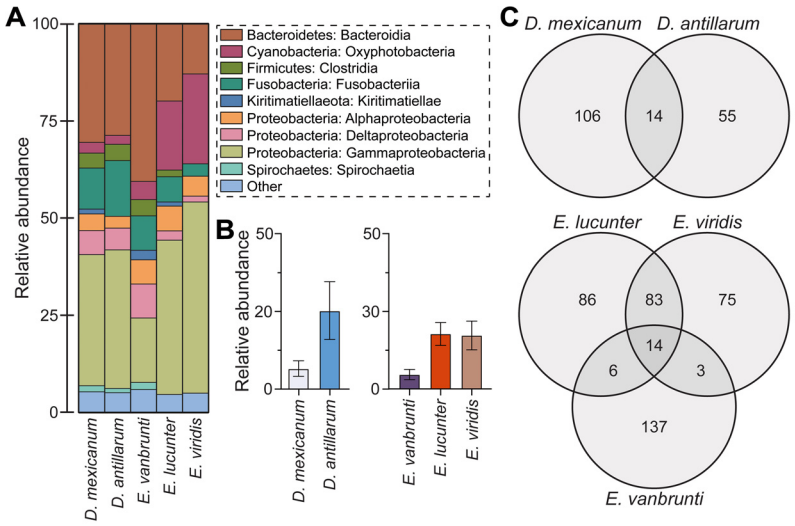

Fig. 5. Bacterial taxa associated with each sea urchin species. (A) Class-level profiles of the bacterial communities associated with the *Echinometra* and *Diadema* species. (B) Differential abundance (mean ± SE) in a *Kistimonas* amplicon sequence variant (ASV) associated with *Diadema* species (left) and *Oxyphotobacteria* associated with *Echinometra* species (right). (C) ASVs of the *Diadema* (top) and *Echinometra* (bottom) geminate species pairs

and 75 (18.6%) were specific to *E. vanbrunti*, *E. lucunter*, and *E. viridis*, respectively, while 14 (3.5%) were shared between these species (Fig. 5C; Fig. S2; Table S7). Notably, a total of 97 (24.0%) ASVs were shared between the Caribbean *E. lucunter* and *E. viridis,* while 17 (4.2%) or 20 (5.0%) ASVs were shared between either species of Caribbean *Echinometra* and *E. vanbrunti* (Fig. 5C; Fig. S2; Table S7). The *Diadema* geminate species, on the other hand, associated with 175 ASVs; 106 (60.6%) and 55 (31.4%) were specific to *D. mexicanum* and *D. antillarum*, respectively, and 14 (8.0%) were shared. The most abundant ASV for both *Diadema* species belonged to *Kistimonas*. This ASV represented ~25.3 ± 32.3% of the community associated with *D. antillarum* eggs, was significantly more abundant in *D. antillarum* than in *D. mexicanum*, and was barely present (<0.01 ± 0.03%) in the *Echinometra* bacterial communities (Fig. 5B; Table S8).

4. DISCUSSION

The microbiota with which animals associate is often attributed to their evolutionary history or to the environment (Carrier & Reitzel 2017, Lim & Bordenstein 2020). Studies assessing the relative importance of a host's evolutionary history or the environment suggest that both influence microbial community composition, but one factor is commonly more pronounced. One 'natural experiment' to test whether either factor or the interaction between these factors primarily drives community composition is to compare geminate species pairs (Jordan 1908). One example are echinoids separated by the Isthmus of Panama (Lessios 2008, O'Dea et al. 2016, Wilkins et al. 2019).

By comparing the egg-associated microbiota for the Echinometra and Diadema geminate species pairs, we reach 3 main findings. First, both pairs of geminate species associated with distinct bacterial communities that reflect a relationship consistent with phylosymbiosis. Second, the relatedness of these microbiota — based on both membership and composition — supports the hypothesis that the interaction between the evolutionary history of the animal host and the environment best explains differences in these communities. Third, particular microbial taxa (e.g. *Oxyphotobacteria* and *Kistimonas*) differed considerably between oceans.

Like the developmental stages of many marine invertebrates, echinoid embryos and larvae associate with species-specific bacterial communities that are composed of hundreds of taxa (Carrier & Reitzel 2018, 2019a,b, 2020). Presently, no study has compared the microbiome of echinoids across their evolutionary history, but the microbiota associated with 3 confamilial echinoids showed a phylogenetic signal (Carrier & Reitzel 2018, 2019a). Multiple studies do, however, suggest that feeding environment and geographic locations with distinct oceanographic conditions influence the composition of echinoid-associated bacterial communities (Carrier & Reitzel 2018, 2019b, 2020, Carrier et al. 2019).

Since their separation ~2.8 million yr ago, members of echinoid geminate species pairs have diverged in several aspects of their biology and ecology, including egg size and biochemical composition (Lessios 1990, McAlister & Moran 2012), larval feeding ecology (McAlister 2008), and reproductive ecology (Lessios 1981, 1984). In this study, we have shown that the bacterial communities with which they associate are another biological difference between these species pairs. Specifically, members of Pacific and Caribbean geminate pairs shared between ~4 and 8% of their microbiota. This fraction of shared bacterial taxa is similar to differences between the micro-

biota of *Strongylocentrotus droebachiensis* larvae from multiple geographical locations (Carrier et al. 2019). Differences in these communities may reflect common taxonomic divergences amongst echinoid species and/or between populations.

One exception to interspecific differences in egg microbiota is our finding regarding the 2 Caribbean *Echinometra* species, *E. lucunter* and *E. viridis*. These species diverged from each other ~1.6 million yr ago (McCartney et al. 2000) and, in this time, have maintained a ~36.3% overlap of their bacterial taxa. This lower level of taxonomic divergence has not resulted in species-specific bacterial communities, which is hypothesized to be a fundamental property of animal-associated microbiota (Gilbert et al. 2012, McFall-Ngai et al. 2013, Bordenstein & Theis 2015). Recently, however, notions for universal rules governing animal–microbe symbioses have been called into question (Hammer et al. 2019). The comparison between these 2 Caribbean *Echinometra* species may provide initial evidence against a general expectation of species-specific microbiota.

In marine and terrestrial taxa, the relatedness of the microbial community tends to mirror the evolutionary history of the host (i.e. phylosymbiosis; Brooks et al. 2016, Lim & Bordenstein 2020). When comparing host phylogeny and microbial dendrograms for the *Echinometra* and *Diadema* geminate species pairs, we found evidence for phylosymbiosis (Brooks et al. 2016, Lim & Bordenstein 2020). Although these trees were not fully congruent, observing phylosymbiosis suggests that the influence of the host's evolutionary history was strong for these echinoids, despite the species having evolved in contrasting and isolated environments (Figs. 1 & 2). Relatedness of these bacterial communities did not fully reflect either factor; instead, what was observed was an intermediate between a host- and environment-driven pattern (Figs. 1 & 2).

Symbioses between animals and microbes are a product of the interaction between the host genotype (G_H), the microbial metagenome (G_M), and the environment (E) (Zilber-Rosenberg & Rosenberg 2008, Bordenstein & Theis 2015, Carrier & Reitzel 2017). This tripartite interaction ($G_H \times G_M \times E$) was evident in the *Echinometra* and *Diadema* geminate species pairs, where the Pacific *E. vanbrunti* grouped with the *Diadema* geminate species pair and the 2 Caribbean *Echinometra* species grouped separately. Provided that both evolutionary history and environment contribute to the composition of echinoid bacterial communities, the known history of the geminate species can shed light on whether symbionts have a deep common history with the host (e.g. co-speciation; Peek et al. 1998, Funkhouser & Bordenstein 2013, Moeller et al. 2016). Moreover, this may also determine whether novel symbiotic partnerships formed following the emergence of the Isthmus of Panama (Lessios 2008, O'Dea et al. 2016).

Two potential candidates that most closely mirrored host evolution or environmental differences are the *Oxyphotobacteria* and *Kistimonas*. *Oxyphotobacteria* are a group of cyanobacteria that perform oxygenic photosynthesis (Soo et al. 2017). In our data, this bacterial class was, on average, ~4–5 times as abundant in the Caribbean *Echinometra* than in the Pacific *E. vanbrunti.* The Caribbean is oligotrophic relative to the eastern Pacific. This environmental difference has been hypothesized to drive the evolution of a number of life history traits that are presumed to be adaptations for life in a lower-productivity environment (Lessios 2008). Multiple echinoderms living in oligotrophic seas have been observed to associate with bacterial lineages known to perform photosynthesis (Bosch 1992, Galac et al. 2016, Carrier et al. 2018, Carrier & Reitzel 2020); however, the function of these bacteria remains unknown. *Kistimonas* is a recently identified bacterial lineage that is most closely related to *Marinobacter* and *Endozoicomonas* (Choi et al. 2010, Lee et al. 2012, Ellis et al. 2019), the latter of which is known to contribute to the health of marine sponges and corals (Neave et al. 2016). The apparent abundance of this bacterium on the eggs of *D. antillarum*, but not *D. mexicanum*, may suggest that *Kistimonas* are of functional importance.

Taken together, the data presented herein support the hypothesis that the bacterial communities of echinoid geminate species pairs have diverged since the formation of the Isthmus of Panama. Moreover, the relatedness of these bacterial communities suggests that this divergence is a product of both the evolutionary history of the host and of subsequent evolution in their respective environments. The functional importance of these bacterial communities and, consequently, whether they are adapted for each oceanographic regime, remain open questions (Wilkins et al. 2019). Determining whether performance is enhanced under different environments may be addressed by profiling what genes these bacteria have and the conditions under which they are expressed (Moitinho-Silva et al. 2014, Slaby et al. 2017, Domin et al. 2018, Carrier & Reitzel 2020). The function and physiology of these bacterial symbionts may then be determined by isolating and culturing individual taxa. Add-back experiments may then be

used to determine the importance of each bacterium to the echinoid host (Moitinho-Silva et al. 2014, Slaby et al. 2017, Domin et al. 2018, Carrier & Reitzel 2020).

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