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ORIGINAL PAPER

Divergence between sea urchins and their microbiota following speciation

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Abstract

Animals have a deep evolutionary relationship with microbial symbionts, such that individual microbes or an entire microbial community can diverge alongside the host. Here, we explore these host-microbe relationships in *Echinometra*, a sea urchin genus that speciated with the Isthmus of Panama and throughout the Indo-West Pacific. We find that the eggs from five *Echinometra* species generally associate with a species-specific bacterial community and that the relatedness of these communities is largely congruent with host phylogeny. Microbiome divergence per million years was higher in more recent speciation events than in older ones. We, however, did not find any bacterial groups that displayed co-phylogeny with *Echinometra*. Together, these findings suggest that the evolutionary relationship between *Echinometra* and their microbiota operates at the community level. We find no evidence suggesting that the associated microbiota is the evolutionary driver of *Echinometra* speciation. Instead, divergence between *Echinometra* and their microbiota is likely the byproduct of ecological, geographic, and reproductive isolations.

Keywords Echinoderm · Echinometra · Evolution · Phylosymbiosis · Symbiosis

Introduction

The process by which one lineage diverges into multiple lineages has been intensively studied for more than a century. Mechanisms that drive speciation events have traditionally been categorized as ecological or reproductive (Turelli et al. 2001; Rundle and Nosil 2005; Sobel et al. 2010). It has become apparent in the last decade that microbes can also serve as the catalyst of, a reinforcement mechanism during, or a life-history trait in speciation events (Brucker and Bordenstein 2012, 2013). If this evolutionary coordination continues over several speciation events, then the host and microbe can display co-phylogeny (Moeller et al. 2016; Pollock et al. 2018; Groussin et al. 2020). If an entire microbial community diverges alongside the host, then the relatedness between species-specific microbiomes can recapitulate host phylogeny (*i.e.*, phylosymbiosis; Brucker and Bordenstein 2013; Brooks et al. 2016). These host-microbe processes have predominately been studied in terrestrial systems (Lim and Bordenstein 2020; Mallott and Amato 2021), but the extent to which these generalizations apply to aquatic systems is little studied and, thus, less clear.

One aquatic system to study how animal-associated bacterial communities change during speciation events are sea

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urchins of the genus *Echinometra*. The Neotropics and Indo-Pacific contain two major extant lineages for *Echinometra*. Following the rise of the Isthmus of Panama (~3 million years ago, MYA) in the Americas, *Echinometra* speciated into *E. vanbrunti* (~3 MYA) in the Pacific Ocean and *E. lucunter* and *E. viridis* (~1.6 MYA) in the Atlantic Ocean (McCartney et al. 2000). These species remain largely isolated from those in the Indo-Pacific due to the Eastern Pacific Barrier. In the last ~1.5 million years, *Echinometra* in the Indo-Pacific speciated into a complex of four closely related species: *E.* sp. A, *E.* sp. B (*E. mathaei senso stricto*), *E.* sp. C, and *E.* sp. D (*E. oblonga*) (Palumbi et al. 1997; Landry et al. 2002). Additional species have more recently been documented in the Pacific Ocean as well as the Indian Ocean and Persian/Arabian Gulf (Bronstein and Loya 2013; Ketchum et al. 2018, 2022).

Due to its relationship with development, the egg has been a cornerstone trait of sea urchin life-history and evolution (Emlet et al. 1987; Wray and Raff 1991; Jaeckle 1995). Eggs are also important for maintaining ecologically and evolutionarily symbioses across generations (*i.e.*, vertical transmission) (McFall-Ngai 2002; Bright and Bulgheresi 2010; Carrier and Bosch 2022). Sea urchins, in particular, transmit bacteria from mother to offspring and these bacterial communities are distinct from the environmental microbiota and between species, can shift in composition and diversity based on the ecological factors that influence the egg, and have functional impacts on host development and ecology (Carrier and Reitzel 2018, 2019, 2020; Schuh et al. 2020; Carrier et al. 2021; Carrier and McAlister 2022). Here, we compare the bacterial communities associated with eggs from multiple molecularly calibrated *Echinometra* speciation events to test whether sea urchins exhibit evolutionary patterns in their symbionts.

Materials and methods

Specimen collection and spawning

Eggs of five species of *Echinometra* were collected from two regions. In Panama, adult *E. lucunter* and *E. viridis* were collected by hand at Punta Galeta near Colón (9.402916 N, 79.860936 W) and *E. vanbrunti* was collected at Isla Taboguilla near Panama City in July and August 2019 (8.801672 N, 79.524555 W; n = 15 per species) (Fig. 1). Individuals were spawned at the Smithsonian Tropical Research Institute's Naos Island Laboratories or Galeta Marine Laboratory (Carrier et al. 2020). In Japan, *E.* sp. A (n = 3) and *E.* sp. B (n = 4) were collected by hand in June 2019 from Okinawa (26.502339 N, 127.843757 E) (Fig. 1) and were spawned at the University of the Ryukyus. Additional attempts were performed to collect eggs from *E.* sp. C and *E.* sp. D, but no reproductive individuals were found.

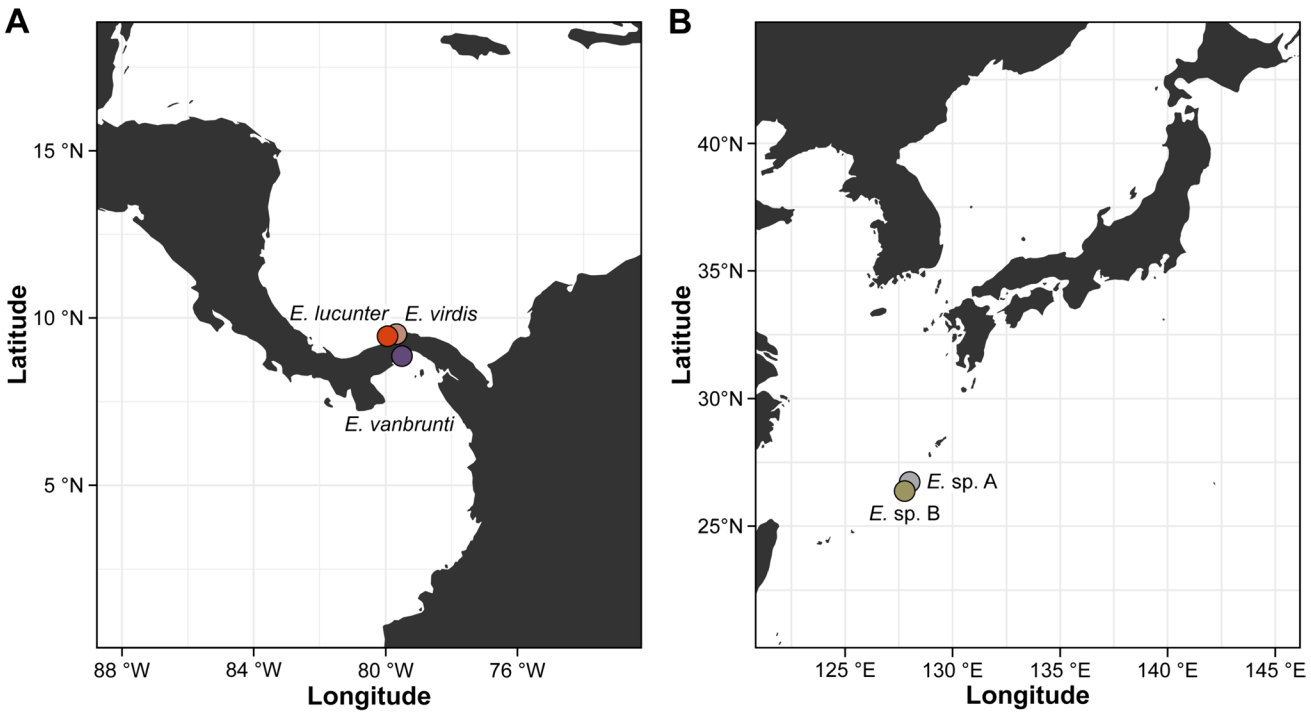

Fig. 1 Map of the sampling locations for (**A**) the three *Echinometra* (*E. lucunter*, *E. vanbrunti*, and *E. viridis*) from Panama and (**B**) the two *Echinometra* (*E.* sp. A and *E.* sp. B) from Japan. These maps were made with ggmap and ggplot in R

Moreover, seawater samples were not taken because it has been shown on multiple occasions that the bacterial communities associated with sea urchin is distinct from those in the environment (*e.g.*, Carrier and Reitzel 2019), including *Echinometra* and their eggs (Carrier et al. 2020).

Adult sea urchins were spawned into filtered seawater using a 2 mL intracoelomic injection of 0.50 M KCl. Approximately 100 eggs per individual were collected using a sterile Pasteur pipette and transferred to a sterile 1.5 mL Eppendorf tube. Eggs were then concentrated into a pellet using a microcentrifuge, and the filtered seawater was removed with a sterile Pasteur pipette. Pelleted eggs were then preserved in RNAlater (Thermo Scientific, Massachusetts, USA) and stored at −20 °C until extraction of nucleic acids.

DNA extraction and sequencing

Genomic DNA was extracted from sea urchin eggs and DNA kit blanks (n = 4) using the GeneJet Genomic DNA Purification Kit (Thermo Scientific). DNA was quantified using a Qubit (Life Technologies) and diluted to 5 ng/µL using RNase/DNase-free water. Bacterial sequences were then amplified using primers for the V3/V4 regions of the 16S rRNA gene (Table S1) (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 Inc.), 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 at the UNC Charlotte.

Bacterial community analysis

Raw reads and quality information were imported into QIIME 2 (v. 2022.11; Bolyen et al. 2019), where forward and reverse sequences 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. 138; Quast et al. 2013). Sequences matching to mitochondria, Archaea, and those that were present in the DNA kit blanks were discarded. These steps filtered our dataset from 434,058 raw reads to 177,537 high-quality reads (mean: 3414 reads; range: 534 to 9118 reads). The filtered table was rarified to 534 sequences (Fig. S1, Table S2).

Four measures of alpha diversity (total ASVs, Faith's phylogenetic distance, McIntosh evenness, and McIntosh dominance) were calculated. These values were compared between host species using a one-way analysis of variance (ANOVA) in Prism (v. 9.0.0) and were followed by a Tukey's post-hoc test for pairwise comparisons. Taxonomy of these communities was then summarized for each sample and then pooled by host species. Unweighted and weighted UniFrac (Lozupone and Knight 2005) distances were calculated in QIIME 2, visualized using principal coordinate analyses in Prism, and stylized in Adobe Illustrator (v. 24.0.1). Permutational analysis of variance (PERMANOVA) and permutational multivariate analysis of dispersion (PERMDISP), and their respective pairwise comparisons, were performed within QIIME 2 to test whether community composition and dispersion varied among host species.

Weighted UniFrac values distances were then compared with the age of each species, as previously calculated using COI divergence (Matsuoka and Hatanaka 1991; McCartney et al. 2000). These were calibrated by the assumption that the differentiation between *E. vanbrunti* and its two Caribbean congeners represents ~3 million years of divergence (McCartney et al. 2000). We compared published species ages for four events: (1) *E.* sp. A and *E.* sp. B (Matsuoka and Hatanaka 1991), (2) *E. lucunter* and *E. viridis*, (3) *E. lucunter* and *E. vanbrunti,* and (4) *E. lucunter* and *E. viridis* (McCartney et al. 2000). Using microbiome distance and species age, we then calculated microbiome distance per million years for each speciation event. Divergence time and microbiome distance was compared using a Mantel test in PASSaGE (v. 2; Rosenberg and Anderson 2011) and microbiome distance per million years compared using a one-way ANOVA in Prism.

Phylosymbiosis

Weighted UniFrac values were also used to construct a dendrogram within QIIME 2, whereby samples were collapsed by host species through pooling reads from all samples for each host species. This dendrogram was then used to compare the topological congruence with a COI gene tree of *Echinometra*. COI sequences for *E.* sp. A (AY262884), *E.* sp. B (AY262915), *E. lucunter* (AF255500), *E. vanbrunti* (AF255539), and *E. viridis* (AF255515) were retrieved from the NCBI database. These sequences were aligned with MUSCLE (Edgar 2004) in MEGA (v. 11.0.9; Kumar et al. 2018). This species level relationship was inferred using maximum likelihood with the optimized DNA substitution model (T92 + G, as determined by BIC criterion) and 1000 bootstraps. Patterns of phylosymbiosis were tested using the Robinson-Foulds and matching cluster metrics in TreeCmp (v. 2.0; Bogdanowicz et al. 2012) with 10,000 random trees (Brooks et al. 2016).

Co-phylogeny

A core microbiome—defined here as ASVs within a bacterial genus being present in all host species—was determined to identify candidate bacterial taxa that may exhibit co-phylogeny with these *Echinometra* species. Sequences for all ASVs within candidate genera were compared to a COI gene tree for *Echinometra*. Specifically, phylogenetic trees of all ASV sequences were reconstructed for each candidate genus using PhyML (Guindon et al. 2010) that was implemented using NGPhylogeny.fr (Lemoine et al. 2019). Co-phylogeny between bacterial genera and host COI gene trees was evaluated through 10 runs with 999 permutations of the ParaFit test (Legendre et al. 2002) from the R package Ape (v. 5.6-2) (Paradis et al. 2004). The null hypothesis is a random distribution of candidate bacterial genera on the *Echinometra* phylogeny. Randomness of each individual association between *Echinometra* and bacterial ASVs was assessed in the ParaFit analysis, and their p-values were adjusted using the Benjamini–Hochberg procedure. The congruence between the *Echinometra* and bacterial trees was visualized through tanglegram and edited with Phytools (v. 1.0-3) (Revell 2012).

Results

Community diversity

Diversity, but not dominance and evenness, of the egg-associated bacterial communities varied significantly between *Echinometra* species (one-way ANOVA for each, total ASVs: $p < 0.001$, phylogenetic diversity: $p < 0.001$, dominance: $p = 0.204$, evenness: $p = 0.305$; Fig. 2; Table S3). The primary difference in total ASVs was that the Indo-Pacific species *E.* sp. A and *E.* sp. B were less diverse than that of *E. lucunter* (Tukey's post-hoc, $p < 0.004$ for each comparison). A nearly significant pattern was observed with the other Caribbean species, *E. viridis* (Tukey's post-hoc, p-value compared to: *E.* sp. A: 0.051, *E.* sp. B: 0.068). Indo-Pacific species were also less phylogenetically diverse than those in Panama (Tukey's post-hoc, $p = 0.045$; Fig. 2; Table S3). These few hundred bacterial ASVs were primarily from four phyla: Proteobacteria ($36.4 \pm 12.3\%$), Bacteroidota ($29.7 \pm 0.7\%$), Fusobacteriota ($11.2 \pm 10.8\%$), and Cyanobacteria ($9.9 \pm 2.6\%$) (Fig. S2).

Community relatedness

The bacterial community associated with the eggs of *Echinometra* species generally had a host species-specific profile in both membership and composition (PERMANOVA, unweighted UniFrac: $p < 0.001$, weighted UniFrac: $p < 0.001$; Fig. 3, Fig. S3; Table S4). The two Caribbean *Echinometra* species, *E. lucunter* and *E. viridis*, were the only case where a species-specific bacterial community was not observed ($p = 0.088$; Fig. 3, Fig. S3; Table S4). Moreover, there were also significant differences in the intraspecific dispersion of the egg-associated microbiota for *Echinometra* species (PERMDISP, unweighted UniFrac: $p = 0.018$, weighted UniFrac: $p < 0.001$; Fig. 3, Fig. S3; Table S4). The primary differences were that *E.* sp. B had less variable bacterial communities than the species from Panama ($p < 0.012$) and that *E. lucunter* exhibited more variable bacterial communities than *E. vanbrunti* and *E. viridis* ($p < 0.028$) (Fig. 3, Fig. S3; Table S4).

Community divergence

There was topological congruence between the *Echinometra* phylogeny and the bacterial dendrogram, suggesting that these trees are non-random, even though they do not fully

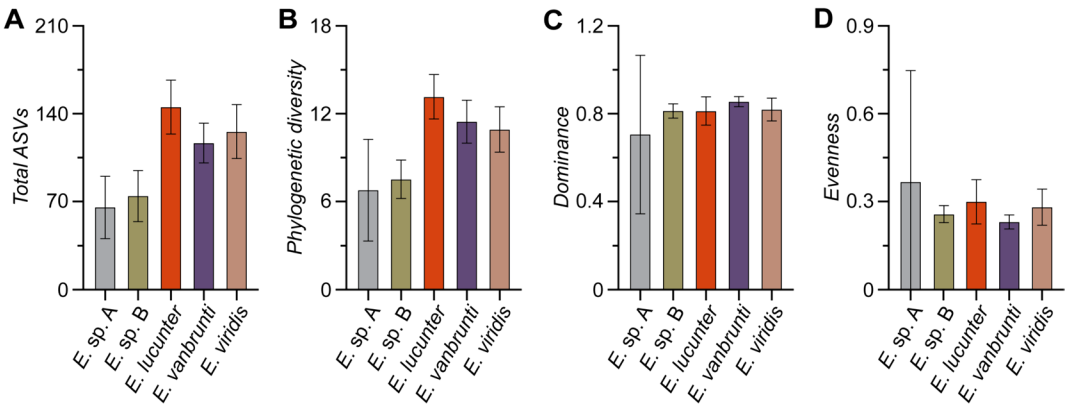

Fig. 2 Diversity of the bacterial communities associated with eggs of species of the sea urchin *Echinometra*, as estimated by (**A**) total amplicon sequence variants (ASVs), (**B**) Faith's phylogenetic diversity, (**C**) McIntosh dominance, and (**D**) McIntosh evenness. Error bars represent 95% intervals

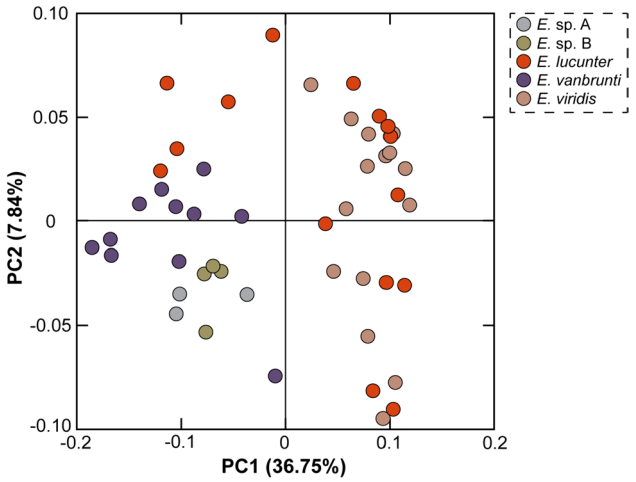

Fig. 3 Species-specificity in the egg microbiota for *Echinometra*. Principal coordinate analysis depicting community relatedness of microbiome compositions (weighted UniFrac) for eggs from the sea urchin *Echinometra*

mirror each other (Unrooted Robinson-Foulds and Unrooted Matching Split, p = 0.069; Fig. 4; Table S6). *E. vanbrunti* was the one species in which the host evolutionary history and microbiome composition were inconsistent. Specifically, host phylogeny for *E. vanbrunti* grouped with the two other *Echinometra* from Panama, while the bacterial dendrograms grouped with the two other *Echinometra* from Japan that are in the Pacific Ocean.

Relatedness in microbiome composition significantly correlated with host divergence time, whereby older speciation events were more divergent than more recent

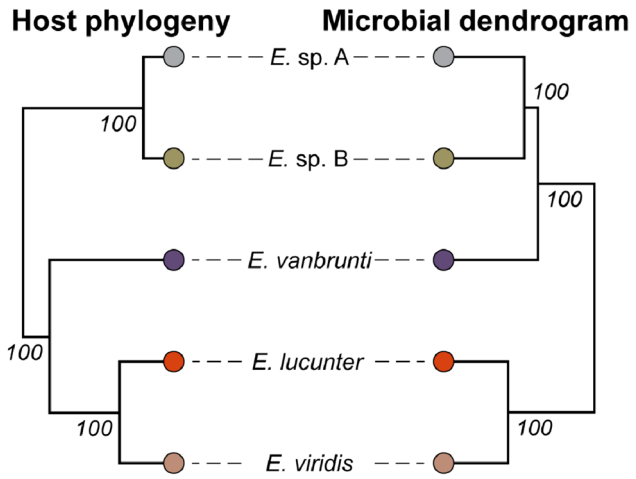

Fig. 4 Phylosymbiosis between *Echinometra* and their egg microbiota. Topology of the host gene tree is congruent with a microbial dendrogram based on community composition, despite that *E. vanbrunti* groups by COI with the neotropical species but by community species with the Indo-West Pacific ones

speciation events (Mantel test, p = 0.004, r = 0.906; Fig. 5; Table S8). When standardized by divergence time, relatedness in microbiome composition per million years was significantly higher for more recent speciation events than older speciation events (ANOVA, F = 244.9, p < 0.0001; Fig. 5; Table S8). Interestingly, a change in the relatedness of microbiome composition per million years was consistent between *E. vanbrunti* and each Caribbean geminate (Tukey's post-hoc, p = 0.155; Fig. 5).

No co-phylogeny

Thirteen ASVs were shared amongst these five *Echinometra* species, with *Photobacterium* and *Vibrio* being the most diverse bacterial genera. These *Echinometra* species collectively associate with a total of 72 ASVs from *Photobacterium* and 171 ASVs from *Vibrio*, of which were studied to explore a co-phylogeny signal. While some ASVs had a significant association with a single *Echinometra* species, we did not observe a co-phylogenic pattern for either *Photobacterium* or *Vibrio* (ParaFit global test, p = 0.181 and p = 0.986, respectively; Figs. S4 and S5). The majority of the *Photobacterium* and *Vibrio* ASVs showed random associations with these *Echinometra* species (Tables S9 and S10).

Discussion

Congruence with host phylogeny based on the relatedness of host-associated bacterial communities varies between taxonomic groups of animals. For example, this relationship appears to be common in corals (Pollock et al. 2018), insects (Brucker and Bordenstein 2013), primates (Moeller et al. 2016), rodents (Weinstein et al. 2021), and sponges (Thomas et al. 2016), while being less common in amphibians (Youngblut et al. 2019), birds (Song et al. 2020), and

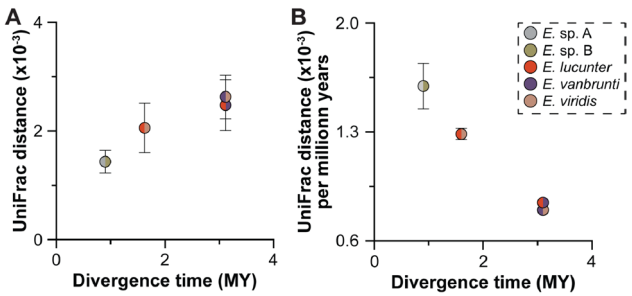

Fig. 5 Co-divergence between *Echinometra* and their egg microbiota. (**A**) Correlation between divergence time (million years, MY) and relatedness of the egg-associated microbiota. (**B**) Microbiome divergence was higher in more recent speciation events exhibit than older speciation events. Each dot (± 95% confidence intervals) represents a speciation event

bivalves (Chiarello et al. 2020). Studies using sea urchins have provided mixed results. Phylosymbiosis is observed, but the strength of this pattern can be influenced by host reproductive strategy (Carrier and McAlister 2022) and ecology (Carrier et al. 2020). Relatedness of the egg-associated bacterial communities for sea urchins in the genus *Echinometra* was congruent with host phylogeny for all but one host species. This species (*E. vanbrunti*) is related with its geminates in the Caribbean but is a distant cousin to the in the Indo-West Pacific species, suggesting that both evolutionary history and ecology are entangled in the specificity and patterns between sea urchins and the bacterial communities associated with their eggs (Carrier and Reitzel 2018; Carrier et al. 2019, 2020, 2021; Ketchum et al. 2021; Carrier and McAlister 2022).

An evolutionary component would seem to operate at the community level, as no shared bacterial groups were identified to have a co-phylogenic pattern. Instead, we find that microbiome divergence rate was higher in more recent speciation events than in older ones. This pattern was also been observed in the microbiome associated with the reproductive organ of cephalopods [*i.e.*, the accessory nidamental gland; (Vijayan et al. 2024)] as well as in some terrestrial taxa [*e.g.*, primates; (Moeller et al. 2014)], suggesting that it may be more widespread in other animals that exhibit phylosymbiosis. In *Echinometra*, this could suggest that microbiota divergence is a byproduct of ecological, geographic, and reproductive isolations. Alternatively, if changes in the microbiota are essential early in speciation events, then these bacterial communities could serve as a reinforcement mechanism. Interestingly, a bacterium that belongs to the Anaplasmataceae (Rickettsiales) and that is transmitted inside the eggs of the sea urchin *Heliocidaris erythrogramma* can encode for a protein that is necessary in the all-or-nothing acrosomal reaction of sperm during fertilization (Carrier et al. 2021; Kustra and Carrier 2022).

The bacterial communities that are provided to sea urchin eggs appears, however, to be influenced more by host ecology than evolutionary history. Unlike strict evolutionary symbioses, the composition of the bacterial communities associated with sea urchin eggs differs between habitats and years, but not between individuals, within a clutch, or with latitude (Carrier and McAlister 2022). Moreover, the diversity of these bacterial communities also differs between individuals, developmental modes, habitats, and years (Carrier and McAlister 2022). A high degree of variation in composition and diversity due to ecological factors may explain why we did not observe a co-phylogenic pattern in any bacterial lineages and why the relatedness of the bacterial communities did not fully recapitulate host phylogeny. Ecology as the driver of which bacteria that mothers provide to their offspring is similarly observed in sponges, which vertically transmit a portion of the microbes that adults acquire from the seawater into the development stages (Björk et al. 2019). Sea urchins that develop using planktotrophic larvae—as is the case for these *Echinometra*—may take a similar strategy for symbiont transmission.

If host ecology is the predominant factor, then this raises several questions about the relationship between sea urchins and which bacterial symbionts that they provide to their eggs. First, what is the source of the bacterial symbionts that sea urchins provide to their eggs and how long do these microbes remain associated with the host? Second, what is the proportional impact of dominant ecological factors (*e.g.*, geography, time, and abiotic environment) on the composition of which microbes that sea urchins provide to their eggs? Lastly, what are the conditions that allow for a co-evolutionary relationship between sea urchins and their microbial symbionts? Regarding the latter, species that develop by lecithotrophy often associate with a dominant endosymbiont (Walker and Lesser 1989; Lesser and Walker 1992; Carrier et al. 2021) and, thus, it has been hypothesized that a co-evolutionary relationship is more likely for this reproductive strategy (Carrier and McAlister 2022). We anticipate that addressing these and related questions will be a fruitful path of future research.

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Author contributions TJC and RNK collected samples. TJC extracted nucleic acids and performed sequencing. TJC, GS, and HAL analyzed the data. AMR provided funding and supervised this study. TJC drafted the manuscript. All authors revised and approved the final manuscript.

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Data availability Amplicon data and the code used here are available on the Dryad Digital Repository: doi.org/https://doi.org/10.5061/dryad.dz08kps3d.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Consent to participate Not applicable.

Consent to publish All authors consent to the publication of this manuscript.

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