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Host–Symbiont Recognition in the Environmentally Transmitted Sepiolid Squid–*Vibrio* Mutualism

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ABSTRACT

Associations between environmentally transmitted symbionts and their hosts provide a unique opportunity to study the evolution of specificity and subsequent radiation of tightly coupled host–symbiont assemblages [3, 8, 24]. The evidence provided here from the environmentally transmitted bacterial symbiont *Vibrio fischeri* and its sepiolid squid host (Sepiolidae: *Euprymna*) demonstrates how host–symbiont specificity can still evolve without vertical transmission of the symbiont [1]. Infection by intraspecific *V. fischeri* symbionts exhibited preferential colonization over interspecific *V. fischeri* symbionts, indicating a high degree of specificity for the native symbiotic strains. Inoculation with symbiotic bacteria from other taxa (monocentrid fish and loliginid squids) produced little or no colonization in two species of *Euprymna*, despite their presence in the same or similar habitats as these squids. These findings of host specificity between native *Vibrios* and sepiolid squids provides evidence that the presence of multiple strains of symbionts does not dictate the composition of bacterial symbionts in the host.

Introduction

The light organ symbiosis between sepiolid squids (Cephalopoda: Sepiolidae) and their luminous bacterial symbionts (genus: *Vibrio*) is an experimentally tractable system that exhibits advantages not found in other animal–bacterial mutualisms [4, 7, 16, 25]. Specifically, it has been used to combine phylogenetic and physiological data to test evolutionary patterns of parallel cladogenesis and bacterial specificity in the Hawaiian sepiolid squid, *Euprymna scolopes* [20]. In this species of *Euprymna*, intraspecific *V. fischeri* are preferred over interspecific *V. fischeri*, whether they are isolated from other *Euprymna* species or from the surrounding seawater [20]. This preference between intraspecific versus interspecific symbionts mirrored the phylogenetic relatedness between host–symbiont assemblages; that is, sepiolid squids that were more closely related to each other were better colonized by *Vibrio* strains isolated from those same squid species [20]. Although bacterial specificity was measured by the presence or dominance of one strain versus another during colonization (otherwise noted as "competitive dominance"), there have been no further studies that demonstrate whether competitive dominance is present in other

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sepiolid squids. By measuring light organ composition of juvenile *E. tasmanica* and *E. hyllebergi* during competition between symbiotic *V. fischeri* strains, this study tested the hypotheses that (a) selection of particular *V. fischeri* strains has occurred within each taxon, and (b) that the host taxon and not the environment determines specificity in this genus of mutualistic partnerships. This study also examined whether symbiotic *V. fischeri* from other host taxa have the capability to infect and compete against intraspecific *V.fischeri*. Because sepiolid squids from the Indo-West Pacific occupy the same habitat as two other species of light-organ-containing taxa, *Photololigo noctiluca* (a loliginid squid) and *Cleidopus gloriomaris* (a monocentrid fish), this also tested the hypothesis of whether any luminous symbionts obtained from the surrounding seawater were specific for either species of squid or fish.

Methods

Collection of Adults and Maintenance of Egg Clutches

Adult *E. tasmanica* from Botany Bay, New South Wales, Australia, and *E. hyllebergi* from the Bay of Thailand, Rayong, Thailand, were obtained for the production of juvenile *E. tasmanica* and *E. hyllebergi* used in this study (Table 1). Adult *E. tasmanica* were collected at dusk in southern Botany Bay with a 50-m seine net. Animals were primarily caught in sand near or within *Zostera* beds of approximately 1-m depth at low tide. After collection, all animals were sexed and placed in a 100-L holding tank prior to transfer. Adults were then shipped to New Mexico State University where they were maintained in artificial seawater (Instant Ocean, 32 ppt) and were mated. Multiple egg clutches were laid by individual females and were moved to separate aerated tanks containing artificial seawater for 18–21 days until juveniles were ready to hatch. Fully developed clutches were then moved to individual bowls with artificial seawater to ensure that all juveniles used in the beginning of the experiments were aposymbiotic (no symbiotic *V. fischeri* are present in artificial seawater). Animals were used immediately after hatching, also ensuring that no animal had been previously exposed to any infective bacteria.

Adult *E. hyllebergi* were collected in the Bay of Rayong, Thailand, by shallow water trawl at approximately 20-m depth and brought back to the Rayong Coastal Aquaculture Station where adults were placed in 500-L holding tanks. Animals were mated and multiple egg clutches were laid by females. These clutches were then transported to New Mexico State University where they were kept in aerated, artificial seawater (Instant Ocean©, 34 ppt) aquaria that were free of any infective *Vibrio*. Fully developed clutches were then moved to individual bowls containing artificial seawater to ensure their aposymbiotic state until experiments were performed. Once juvenile squids hatched, individuals from several clutches were used for both single-strain infections and competition experiments to compensate for any interclutch variation. Previous experiments measuring infection, colonization, and persistence of symbiotic bacteria in *E. scolopes* juveniles do not show high variation within or between clutches of the same species [11].

Isolation and Inoculation of Vibrio fischeri and Photobacterium leiognathi Symbionts

V. fischeri or *P. leiognathi* isolates were obtained by plating squid light organ contents on Luria Broth high salt (LBS-20% NaCl w/v) agar medium or by using a sterile 22-gauge needle to extract light organ symbionts from live *Cleidopus gloriomaris* [26, 28]. Several strains from each species of host squids and fish were stored in a 40% glycerol stock solution and frozen for future use in the colonization assays. The following experiments also included strains of *V. fischeri* previously isolated from squid taxa collected in other habitats (*Sepiola robusta*, Table 1). Juveniles of *E. tasmanica* and *E. hyllebergi* were inoculated with

Table 1. Host species and symbiotic strains of *Vibrio fischeri* and *Photobacterium leiognathi*

Species of host	Location collected	Bacterial symbiont	Strain designation
Mollusca: Cephalopoda: Sepiolidae			
Euprymna tasmanica[a]	Botany Bay, New South Wales, Australia	*Vibrio fischeri*	ET301
Euprymna tasmanica	Crib Point, Victoria, Australia	*Vibrio fischeri*	ET101
Euprymna tasmanica	Magnetic Island, Queensland, Australia	*Vibrio fischeri*	ET401
Euprymna scolopes	Oahu, Hawaii, USA	*Vibrio fischeri*	ES114
Euprymna hyllebergi	Bay of Thailand, Rayong, Thailand	*Vibrio fischeri*	EH201
Euprymna morsei	Tokyo Bay, Japan	*Vibrio fischeri*	EM17
Sepiola robusta	Banyuls-sur-mer, France	*Vibrio fischeri*	SR5
Chordata: Osteichthyes: Monocentridae			
Cleidopus gloriamaris	Morton Bay, New South Wales, Australia	*Vibrio fischeri*	CG101
Mollusca: Cephalopoda: Loliginidae			
Photololigo noctiluca (previously *Loliolus noctiluca*)	Sydney, Australia	*Photobacterium leiognathi*	LN101

[a] Juveniles used in this study were raised from this population of *E. tasmanica*.

symbionts by exposure in artificial seawater. Approximately 1000 cells/mL of each *V. fischeri* isolate (Table 1) were used during each single infection. Competition experiments used 1000 cells/mL of each strain in colonization assays. Incubation of symbionts with host squid lasted for 12 h to initiate colonization of the light organ [27]. Individual juvenile squids were kept separate in 10-mL glass scintillation vials with 5 mL of artificial seawater. Each morning of the assay period (12 and 36 h) the seawater was changed to remove waste products and bacteria that had been vented with the daily diurnal cycling of bacteria [2]. Juveniles were then rinsed with symbiont-free seawater, and luminescence was monitored via a luminometer (Turner Designs) to ensure that infection had occurred. Aposymbiotic (noninfected) juveniles were kept as negative controls throughout each assay.

Colonization of Symbiotic Strains from Similar Host Taxa

All *V. fischeri* and *P. leiognathi* strains in Table 1 were used in both single inoculations and competition experiments. Approximately 30 animals were used for each individual symbiont infection and 30 for each competition assay between any two symbiont combinations. Juveniles were kept on a 12/12 hour light/dark cycle. Both *E. tasmanica* and *E. hyllebergi* inoculations (single and double) were initiated at room temperature (approximately 24°C) and were maintained at that temperature for the subsequent incubation period (48 h). All single infection and competition experiments were completed in both *E. tasmanica* and *E. hyllebergi* juveniles to determine if the *Vibrio* strains tested were equally capable of infecting both squid hosts, and if intraspecific strain specificity persisted during competition with other interspecific *Vibrios.* Single strain infections in individual squids were measured by homogenizing animals at 12, 24, 36, and 48 h to calculate the number of colony-forming units (CFUs) at each time point to estimate growth rates inside the juvenile light organs during the incubation period. One CFU represents one individual bacterium that was isolated from an individual light organ, and can therefore be used as a quantitative measurement of actual bacterial numbers. Juveniles were rinsed with sterile seawater twice and homogenized for dilution onto LBS agar medium [12]. LBS plates were incubated for 12 h at 28°C, and were subsequently counted for the total number of *V. fischeri* present as CFUs. For competition studies, squid light organs were homogenized at 48 h and the light organ dilutions were plated onto LBS plates. CFUs for each competition were counted, and competing strains were differentiated according to their luminescent phenotypes *in vitro* [21]. Briefly, only strains that were identified by the amount of luminescence produced *in vitro* on LBS medium (nonvisibly luminous, dim, or bright strains) were used in each of the competition experiments so they were easily distinguished and identified from each other. Ratios of competing strains of symbiotic bacteria were calculated by averaging the concentration (in CFUs) of each strain tested in all individual competition experiments and arcsine-transforming the ratios to test for significance [30].

Colonization of Symbiotic Strains from Different Host Taxa

To determine whether the presence of different host taxa affects the colonization and composition of sepiolids in the same habitat, light organ contents in sepiolids using *Vibrio* and *Photobacterium* isolates from those taxa were measured. Juvenile *E. tasmanica* were inoculated with *V. fischeri* strains isolated from *Cleidopus gloriomaris* (a monocentrid fish found in the same habitat as *E. tasmanica*), as well as *Photobacterium leiognathi* strains isolated from *Photololigo noctiluca* (a loliginid squid also inhabiting the same waters *as E. tasmanica*). All strains of *V. fischeri* and *P. leiognathi* were found in Botany Bay as free-living symbionts as well as inside the light organs of their respective hosts. Approximately 30 animals were used for each individual symbiont inoculation and 30 for each competition assay between one intraspecific and one intertaxon isolate. Juveniles were kept on a 12/12 hour light/dark cycle. Both *E. tasmanica* and *E. hyllebergi* inoculations (single and double) were initiated at room temperature (approximately 24°C), and were maintained at that temperature for the subsequent incubation period (48 h). All single inoculations and competition experiments were completed in both *E. tasmanica* and *E. hyllebergi* juveniles to determine whether the symbiotic strains tested were equally capable of infecting both squid hosts, and whether taxon specificity persisted during competition with other intertaxonomic symbionts. Single strain infections in individual squids were measured by homogenizing animals at 12, 24, 36, and 48 h to measure CFUs at each time point to estimate colonization efficiency inside the juvenile light organs during the incubation period. Juveniles were rinsed with sterile seawater twice, and homogenized for dilution onto LBS agar medium [12]. LBS plates were incubated for 12 h at 28°C and were subsequently counted for the total number of bacteria present (CFUs). For competition studies, squid light organs were homogenized at 48 h and the light organ dilutions were plated onto LBS plates. CFUs for each competition were counted, and competing strains were differentiated according to their luminescent phenotypes *in vitro* [21]. Ratios of competing strains of symbiotic bacteria were calculated by averaging the concentration (in CFUs) of each strain tested in all individual competition experiments and arcsine-transforming the ratios to test for significance [30].

Results

Single Inoculations in E. tasmanica and E. hyllebergi Juveniles

For single-inoculation assays, all *V. fischeri* strains isolated from squids of the genus *Euprymna* infected juvenile *E. tasmanica* light organs equally well (Fig. 1A). These included intraspecific *V. fischeri* strains isolated from three populations of *E. tasmanica* (ET101, ET301, ET401) and interspecific *V. fischeri* strains isolated from different species of *Euprymna* (EH201, ES114, EM17; see Table 1 for

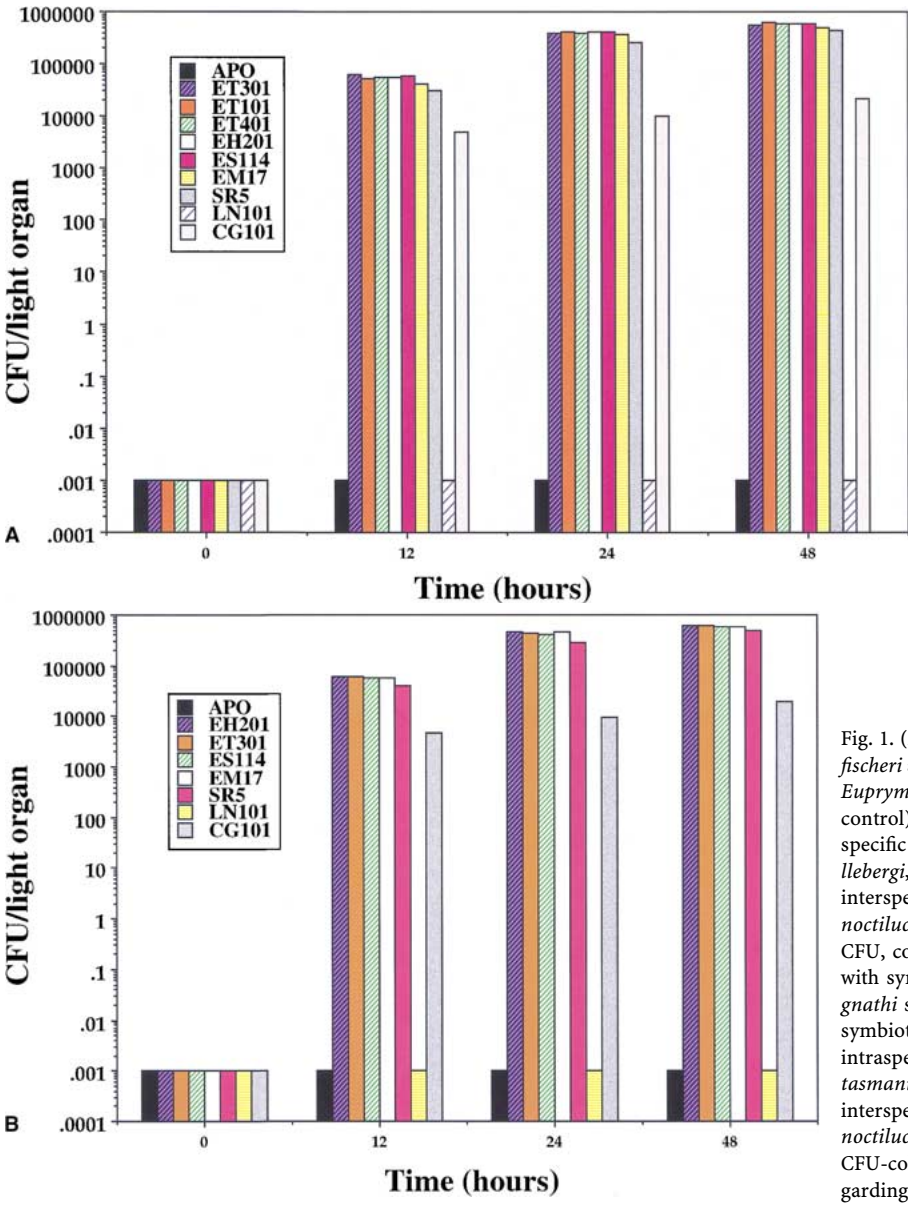

Fig. 1. (A) Single inoculation assay with symbiotic *Vibrio fischeri* and *Photobacterium leiognathii* strains in juvenile *Euprymna tasmanica*. Aposymbiotic (APO-negative control); ET301, ET101, and ET401 (*E. tasmanica* intraspecific isolates); EH201, ES114, EM17, and SR5 (*E. hyllebergi*, *E. scolopes*, *E. morsei*, *Sepiola robusta* interspecific isolates); LN101 and CG101 (*Photololigo noctiluca* and *Cleidopus gloriomaris* intertaxon isolates), CFU, colony-forming units. (B) Single inoculation assay with symbiotic *Vibrio fischeri* and *Photobacterium leiognathi* strains in juvenile *Euprymna hyllebergi*. Aposymbiotic (APO-negative control); EH201 (*E. hyllebergi* intraspecific isolate), ET301, ES114, EM17, and SR5 (*E. tasmanica*, *E. scolopes*, *E. morsei* and *Sepiola robusta* interspecific isolates); LN101 and CG101 (*Photololigo noctiluca* and *Cleidopus gloriomaris* intertaxon isolates). CFU-colony forming units. For more information regarding strains, see Table 1.

descriptions of strain isolates). *V. fischeri* isolate SR5 from *Sepiola robusta* (a Mediterranean sepiolid) had similar concentrations of symbionts/individual within the first 12 h of colonization, but had lower concentrations of symbionts/individual at later periods throughout the assay (Fig. 1A). For intertaxon inoculations, *V. fischeri* symbiont CG101 was able to infect *E. tasmanica* juveniles, but not to the same degree as all other sepiolid symbionts (Fig. 1A). In contrast, *Photobacterium leiognathi* (strain LN101), the light-organ symbiont from the loliginid squid *Photololigo noctiluca*, was not capable of infecting *E. tasmanica* juveniles. For negative controls, all juvenile squids that were not experimentally exposed to bacteria (aposymbiotic) at the beginning of the experimental period exhibited no presence of bacteria in their light organs throughout the 48-h assay period.

Single inoculation assays in *E. hyllebergi* produced similar results to those observed in *E. tasmanica* juveniles. All inoculations performed with strains isolated from any *Euprymna* host species infected juveniles of *E. hyllebergi* to the same degree (Fig. 1B). This included intraspecific strain EH201 and interspecific strains ES114, ET301, and EM17. Again, strain SR5 (from *S. robusta*) did not infect *E. hyllebergi* to the same level as other interspecific strains, as well as the intertaxon strain CG101 (Fig. 1B). Similar results were observed with *P. leiognathi* strain LN101, where no infection of *E. hyllebergi* juveniles was detected (Fig. 1B).

Table 2. Competition of various symbionts in *E. tasmanica* juveniles after 48 h

Symbiont strains	Colony-forming units (CFU)/light organ[a]	Ratio[b]
Competitions with native (ET301) symbiont		
Intraspecific		
ET301 × ET101	3.65×10^5: 2.69×10^5	58 : 42
ET301 × ET401	3.19×10^5: 1.79×10^5	64 : 36
ET101 × ET401	3.27×10^5: 3.3×10^5	50 : 50
Interspecific		
ET301 × ES114	6.89×10^5: 0.63×10^5	92 : 8
ET301 × EM17	5.15×10^5: 0.22×10^5	96 : 4
ET301 × SR5	9.19×10^5: 0.74×10^5	93 : 7
Intertaxon		
ET301 × CG101	4.79×10^5: 0.04×10^5	99 : 1
ET301 × LN101	5.45×10^5: 0	100 : 0
Competitions between nonnative symbionts		
Interspecific		
ES114 × EM17	2.13×10^5: 1.59×10^5	57 : 43
ES114 × SR5	6.19×10^5: 1.75×10^5	77 : 23
EM17 × SR5	3.82×10^5: 1.84×10^5	67 : 33
Intertaxon		
CG101 × LN101	3.10×10^4: 0	100 : 0

[a] Approximately 30 individual squids were used for each combination of symbionts to obtain an average CFU/light organ measurement.
[b] Ratios of CFU/light organ between competing strains of *V. fischeri* or *P. leiognathi* have been converted to percentages and arcsine-transformed to test for significance [30]. All values listed are significant to $P < 0.5$.

Competition Experiments with Intra- and Interspecific Symbionts

For competition experiments in *E. tasmanica* juveniles, intraspecific strain preferences were not observed when isolates of *E. tasmanica* (ET301, ET101, ET401) were placed in competition with each other (Table 2). In contrast, light-organ competition assays with interspecific *V. fischeri* strains (ES114, EH201, EM17, SR5) showed a high degree of specificity for the intraspecific symbiont (strain ET301) compared to the other *Vibrios* (Table 2). In contrast, competition experiments using two interspecific strains of *V. fischeri* displayed a unique hierarchical pattern of specificity. *Euprymna* strains ES114 and EM17 were equally competitive at colonizing and persisting in the light organs of juvenile *E. tasmanica* when inoculated together. Both strains were also capable of exhibiting competitive dominance over all other interspecific and intertaxon symbionts tested (Table 2).

Competition experiments using juvenile *E. hyllebergi* displayed similar results, where native strain EH201 was significantly dominant compared to all other *Vibrios* challenged (Table 3). For competition experiments that included only interspecific strains, EM17 was dominant to both ET301 and ES114 by 93 and 89% whereas strain SR5 was outcompeted by a ratio of 99:1 by EM17 (Table 3). Strains ES114, ET101, and SR5 were similar in their persistence in *E. hyllebergi* light organs when competed against each other, with no strain being completely dominant at the end of the 48-h assay period (Table 3).

Competition between Intertaxon Symbionts from Similar Habitats

When testing the colonization abilities of light-organ symbionts isolated from taxa living in the same habitat, it was observed that host specificity was a stronger determinant of light-organ composition rather than environmental availability (Table 2). For all intertaxon strains isolated from Australia (ET301, CG101, LN101, Table 1), ET301 was the dominant strain in competition assays with either CG101 (*Cleidopus* symbiont) or LN101 (*Photololigo* symbiont, Table 2) in *E. tasmanica* juveniles. Both ET301 and CG101 were capable of completely establishing light organ infections against *P. noctiluca* strain LN101 (Table 2), which was never able to colonize any *Euprymna* light organ (Fig. 1A, 1B). Although CG101 was able to outcompete LN101 during these assays, the number of symbionts/light organ observed were 10-fold lower than those measured between interspecific or intraspecific competitions with sepiolid symbionts (Table 2).

Discussion

The study presented here demonstrates that recognition among native *V. fischeri* strains isolated from either

Table 3. Competition of various symbionts in *E. hyllebergi* juveniles after 48 h

Symbiont strains	Colony-forming units (CFU)/light organ[a]	Ratio[b]
Competitions with native (EH201) symbiont		
Interspecific		
EH201 × ES114	1.12×10^5: 0.03×10^5	97 : 3
EH201 × EM17	0.98×10^5: 0.007×10^5	99 : 1
EH201 × ET301	1.18×10^5: 0.11×10^5	91 : 9
EH201 × SR5	4.57×10^5: 0.04×10^5	99 : 1
Intertaxon		
EH201 × CG101	2.34×10^5: 0.008×10^5	99 : 1
EH201 × LN101	3.85×10^5: 0	100 : 0
Competitions between nonnative symbionts		
Interspecific		
EM17 × ES114	6.08×10^5: 0.43×10^5	93 : 7
EM17 × ET301	4.51×10^5: 0.54×10^5	89 : 11
ES114 × ET301	2.22×10^5: 2.0×10^5	53 : 47
EM17 × SR5	6.09×10^5: 0.21×10^5	99 : 1
ES114 × SR5	3.79×10^5: 3.25×10^5	54 : 46
ET301 × SR5	3.45×10^5: 2.68×10^5	57 : 43
Intertaxon		
CG101 × LN101	2.1×10^4: 0	100 : 0

[a] Approximately 30 individual squids were used for each combination of symbionts to obtain an average CFU/light organ measurement.
[b] Ratios of CFU/light organ between competing strains of *V. fischeri* or *P. leiognathii* have been converted to percentages and arcsine transformed to test for significance [30]. All values listed are significant to $P < 0.5$.

E. tasmanica or *E. hyllebergi* squid hosts are preferentially selected over *V. fischeri* strains isolated from other sepiolid taxa during light-organ colonizations. It also suggests that this preferential exclusion extends to interspecies symbionts as well as intertaxon symbionts found in the same habitat. These findings imply that (i) all intraspecific and interspecific *V. fischeri* strains can colonize sepiolid light organs equally as well when inoculated by themselves with any newly hatched juvenile squid (*E. hyllebergi* or *E. tasmanica*); (ii) not all *V. fischeri* colonize sepiolids equally as well when presented simultaneously; and (iii) *V. fischeri* strains isolated from other symbiotic taxa infect to a lesser degree or are incapable of inoculating sepiolids from the same habitat. This hierarchy of specificity demonstrates how environmentally transferred *Vibrio* bacteria have evolved to become highly specific to their host, possibly due to their mode of transfer. Because sepiolid squids are born aposymbiotic, there must be some mechanism that separates all the other environmentally available bacteria from the selected *Vibrio* strains that have evolved this specificity. If environmental transmission increases the competitive abilities of a particular bacterium to infect only one type of host, then that particular host must have some mechanism(s) that allows the symbiont to increase its fitness as a benefit of the mutualism [4]. This benefit must also outweigh the costs of continually accepting new (and possibly different) symbionts with every new generation of squids. Therefore, the interactions between the host and symbiont must have stronger effects upon the evolution of bacteria specificity than selective pressures from the environment. Since non-symbiotic *V. fischeri* strains are also found in the same habitat [9], selection of a particular symbiont strain before and during colonization must involve several key factors. Such factors might include the ability of the symbiont to locate a particular host species [6], changes in the biochemical milieu of the host light organ to exclude other types of bacteria [5, 22], higher growth rates of symbionts that colonize and persist in the light organ [27], or receptors in the host light organ that are specific for a particular symbiont strain [15]. The result of these changes may develop into further modifications of the host light organ [14] or promote genetic signals that may induce or enhance genes specific for the symbiosis [31]. This includes other symbiotic *Vibrio* and *Photobacteria* from different host taxa (monocentrid fish and loliginid squids) that have probably evolved with their hosts in the same manner as the sepiolids. Lower colonization levels from these bacteria (or none at all; Tables 2, 3) demonstrate that not all symbiotic *Vibrios* found in the same habitat are capable of exhibiting a light organ response in *E. tasmanica*, despite their presence and availability to aposymbiotic juveniles [29]. How quickly different *Vibrio* bacteria are able to adapt to a particular host light organ has not been previously addressed because

of the difficulty of continually culturing hatchlings to the adult stage and subsequently transferring those bacteria to a new generation of hosts. Future studies using new squid culturing techniques will hope to address whether non-native *Vibrios* can eventually adapt to different host light organs to determine which mechanisms are directly responsible for accommodating symbionts to a different light-organ environment. Once these mechanisms are selected for a particular squid–*Vibrio* pair, then the relationship between host and symbiont becomes much more specific and may exclude the ancestral (original) *Vibrio* that was originally obtained from the environment.

The pronounced competitive ability of intraspecific *V. fischeri* strains (ET301, ET101, and ET401 for *E. tasmanica*, and EH201 for *E. hyllebergi*) demonstrates that *Vibrio* specificity does exist between *Euprymna* and its luminescent symbionts. Nearly 100% dominance was observed in these strains when competed against other interspecific and intertaxon strains, but no differences in colonization ability were observed between the intraspecific *V. fischeri* strains (ET101, ET301, ET401), which were isolated from *E. tasmanica* populations at various localities (Table 1). These strains were differentiated by their luminescence phenotype [21], as well as their *gapA* (glyceraldehyde dehydrogenase A) genotype, a hypervariable locus among *V. fischeri* strains [10, 20]. Because these strains did not express a dominant colonization phenotype between populations, other factors may be responsible for the distribution of phenotypically similar but genetically distinct populations of *Vibrio*. Past studies have demonstrated that many species of marine luminous bacteria exhibit phenotypic plasticity between their symbiotic host and the surrounding habitat [23, 28]. Since sepiolid squids vent their symbionts once each day [2], the necessity of adapting to fluctuating conditions may be a dominant force on the survival between free-living and symbiotic stages [19]. *V. fischeri* is globally widespread among ecologically diverse habitats [17], but in certain cases, their symbiotic relationship with sepiolid squids is affected by their abundance and distribution relative to the ecology of the environment [11, 19, 28]. Certain abiotic factors (such as temperature) have been shown to affect the distribution of bacteria surrounding populations of sepiolid squids [19, 28], which may then eventually lead to the evolution of specific host-symbiont relationships. For example, in Mediterranean species of *Sepiola*, squids contain two different species of *Vibrio (V. fischeri* and *V. logei*) in five sympatric species (*S. robusta*, *S. rondoleti*, *S. ligulata*, *S. intermedia*, and *S. affinis*) [13]. Specificity in this case has already evolved with the *Vibrio* genus, yet the presence of multiple species of bacteria with numerous sympatric hosts may be a precursor to the development of specificity for single symbiont–host pairings. Previous phylogenetic analysis within the *Sepiola* clade have suggested that parallel cladogenesis is evident between *V. fischeri* strains isolated from these mixed populations of squids [18, 20]. Future studies will need to address the relationships between *Sepiola* and their *V. logei* symbionts in order to better understand how coevolutionary patterns are formed from dual associations found in closely related taxa, and may help decipher the processes which lead to specificity among mixed species of *Vibrio*.

The competitive hierarchy within *V. fischeri* isolates in *E. tasmanica* and *E. hyllebergi* juveniles also supports previous findings of host–symbiont specificity in the *E. scolopes*–*V. fischeri* mutualism [20]. Not only do these new data support intraspecific recognition of environmentally transferred symbionts, but they also mirror the phylogenetic relatedness between sepiolid squids and their *Vibrio* symbionts [20]. Prior results had shown that phylogenetic relationships among *Euprymna* and *Sepiola* species correlated to the degree of specificity shown in competition experiments with interspecific and intertaxon isolates of *V. fischeri*. This study [20] was the first to examine cospeciation patterns by both phylogenetic analysis of hosts and symbionts and the competitive dominance of *Vibrio* isolates in *E. scolopes* juveniles. The data presented here give additional evidence for specificity evolving among two related species of *Euprymna*, as well as supporting the evolutionary relatedness among the Sepiolidae by creating a pattern of host specificity that is congruent with their phylogeny [18, 20]. Although *E. hyllebergi* was not included in the previous phylogenetic analysis (no tissue samples were available until this study), we have recently extended the sepiolid–*Vibrio* phylogeny to include these taxa as well as additional squids and their symbionts to confer their relationships to the data presented here (Nishiguchi, unpub. results). This study has demonstrated that specificity has evolved between *V. fischeri* and their sepiolid hosts, resulting in the selection of a *Vibrio*-specific, mutualistic association. Although no other study has utilized several related taxa in order to test the degree of specificity that exists in any environmentally transmitted symbiosis, this study hopes to gain insight for understanding the evolutionary process that leads to speciation among host–symbiont pairs. Future studies will need to

examine the microevolutionary processes that select for specificity in these associations, which will help us better understand the molecular mechanisms that drive host–symbiont specificity and eventually cospeciation.

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