COSPECIATION BETWEEN HOSTS AND SYMBIONTS:
The Sepiolid Squid- Vibrio Mutualism

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I. Introduction

Mutualistic associations between eukaryotic hosts and their microbial partners have long been an interest of ecologists and evolutionary biologists due to the importance of understanding phylogenetic congruence, coevolution, host specificity, infectivity, and maintaining the precarious balance between pathogenicity and innocuousness. Each partner has an important function in the life history of the symbiosis; the "super organism" functions as a result of combining both lifestyles of host and symbiont, enabling the entire association to take advantage of a broader ecological niche than either one could accomplish independently. These types of functional interactions between host and symbiont can only be established when an avenue for the exchange of information has evolved specifically within the symbiosis. Once this occurs, the discourse between the partners (physiological, genetic) allows the association to flourish and become a competitive and dynamic system in an environment where it may not normally exist. The presence of such interactions between highly divergent taxa provides an excellent opportunity to study the interactive roles of coevolving species in processes such as horizontal gene transfer, interspecies gene regulation, allopatric and sympatric routes of speciation, and the development of population structure and dynamics.

A number of examples exist that indicate that the formation of a symbiotic relationship allows the host or its symbiont to radiate into newly formed niches: chemoautotrophic bacterial residing within tissues of hydrothermal vent or sewage outfall invertebrates (Cavanaugh, 1994); nitrogen fixing bacteria in the nodules of leguminous plants (Wilkinson and Parker, 1996); endosymbiotic bacteria within the body cavities of aphids, termites, and weevils (Moran and Telang, 1998; Nardon and Grenier, 1991; Smith and Douglass, 1987); and luminous bacteria within the light organs of monocentrid and anomolopid fish and sepiolid and loliginid squids (Flaygood and Distel, 1993; McFall-Ngai and Ruby, 1991). Although these mutualisms have evolved as part of a new "evolutionary innovation" (Margulis, 1989), there resides the question of how the association is directly affected by either specific host-symbiont interactions, or whether environmental or ecological factors play an important role in the formation of the partnership. Various hypotheses have alluded to the fact that transmission of the symbiosis, whether vertical or horizontal, would also have a major influence in determining whether host-symbiont pairs display parallel modes of speciation or promiscuous patterns of affiliation. Does specificity drive the cospeciation of host-symbiont pairings, or does the fidelity of vertical transmission have a more dramatic effect on determining the coevolution of the mutualism? Does the evolution of specific recognition factors help determine whether a symbiont will maintain fidelity or be promiscuous? Do environmental or ecological factors influence the onset of the symbiosis as well as continually having an additional effect on evolving specificity and recognition?

2. Association, cospeciation, congruency and fidelity of host-symbionts

2.1. UNDERSTANDING THE BASIS OF COOPERATIVE ASSOCIATIONS

Until recently, there has been little evidence that addresses questions of evolution and cospeciation of both partners in a symbiosis. Many symbiotic relationships are obligate; in other words, the host or symbiont depends entirely upon the other for some type of capability or function which they do not possess (Baumann *et al.*, 1997; Cavanaugh, 1994). This co-dependence has made most physiological and biochemical studies of the separated organisms nearly impossible to complete *in vitro*. Microscopic studies of symbionts residing in host tissues have provided only a few clues as to the nature of these relationships and how they were initially established (Buchner, 1965). The inability to culture many of the microscopic partners outside of the symbiotic niche still frustrates biologists, since it does not allow the separate study of either organism without the presence or influence of the other (Distel, 1998). Studies involving the use of antibiotics or other biochemical methods to clear animals of symbionts were inconclusive, since most hosts suffered drastic physiological changes due to the loss of the symbionts (Baumann *et al.*, 1997; Ohtaka and Ishikawa, 1991). Recently, the advent of molecular techniques has allowed investigators to define and identify the nature of the symbioses, characterize the type of symbiont associated with the partnership, and determine whether the symbionts are transmitted vertically or horizontally (Bourtzis and O'Neill, 1998; Cary, 1994; Cary and Giovannoni, 1993; Distel and Cavanaugh, 1994; Kreuger and Cavanaugh, 1997; Munson *et al.*, 1991). These techniques have led to some progress toward an understanding of how symbiotic associations have evolved and whether the presence of a particular symbiont has an influence on host speciation and symbiont recognition.

2. 1. 1. The nature of cospeciation and congruency

Although a number of symbioses have been widely studied, few have proven to be effective models for answering questions concerning the coevolution and establishment of symbiotic associations. Whether the ancestral lineage between host and symbiont holds any clues to the origin of the symbiotic character (i.e., commensalistic, mutualistic or pathogenic), only detailed analyses of genes that are responsible for specific mechanisms within the symbioses can be examined which may uncover any details of the interactions among the individuals involved. Since many of these "symbiotic" genes are yet unknown or may vastly differ between symbiotic organisms, other markers have been chosen to delineate symbiotic lineages that may be evolving in parallel. Phylogenetic analyses of phenotypic characters as well as molecular sequence data obtained from both symbiont and host are helpful in establishing any congruence that may be occurring along these parallel lineages. Previous investigations with various symbiotic associations using molecules and morphology have revealed congruent phylogenetic patterns between the partners (Baumann *et al.*, 1997; Hinkle *et al.*, 1994; Nishiguchi, 2001). These patterns have resolved questions of whether the symbionts were evolving in parallel (cospeciation/coevolution) or have expressed patterns of promiscuity or host switching over their evolutionary lineages. Understanding the evolution of host-symbiont specificity, as well as the effects of the association on life history patterns and interactions between the individual partners has only just begun to be explored among those well studied systems.

2. 1.2. Promiscuity or fidelity?

The assumption that patterns of coevolution between symbiotic partners were strictly parallel was primarily based upon which type of symbiotic transfer occurred between host and symbiont. Associations where symbionts were transferred directly (vertically) through parental inheritance, assumed that strict patterns of cospeciation would occur

due to the intimate transfer between parent (usually maternal, through the ova) and offspring. It was believed that this direct infection of host offspring with the symbiotic population would eventually form a highly specific association, and that this relationship would produce strict patterns of cospeciation among host species and their particular symbionts. The presence of these vertically transmitted symbioses would also allow the establishment of a hierarchy of geographically distinct populations, where host/symbiont pairs have diverged from a particular ancestral species, but have retained their specificity due to their mode of transfer. An example of this type of tightly congruent association is the evolution of aphids and their bacterial symbionts (Baumann *et al.*, 1997; Moran and Telang, 1998; Munson *et al.*, 1991). Molecular phylogenies based on l6S rDNA sequences of the bacterial symbionts show that the primary endosymbionts have descended from a single common ancestor (Munson *et al.*, 1991). This result corresponds with a number of insect-bacterial studies (Aksoy, 1995; Bandi *et al.*, 1995; Clark *et al.*, 1992; Munson *et al.*, 1993; Schröder *et al.*, 1996), in which the symbionts form a monophyletic group, indicating that each clade was founded by an endosymbiont. Although insect endosymbionts have arisen a number of times from their free-living relatives, there are strict patterns of cospeciation observed among those that represent the symbiont clades (Aksoy, 1995; Bandi *et al.*, 1995; Moran *et al.*, 1993; Munson *et al.*, 1991; Schröder *et al.*, 1996;). The congruency observed is strong evidence for parallel cospeciation that has evolved from the prolonged and highly specific vertical transmission of symbionts among hosts. Along with molecular studies, microscopic studies and observations of maternal transmission (Buchner, 1965) have never shown that horizontal transmission is present among these groups of insects. Thus, parallel cladogenesis reflects not only the origin of the ancestor of insect-bacterial symbioses, but also implies that those ancestors were living at the same time.

In contrast, environmentally transferred symbioses allow new symbionts from the free-living population to infect each new generation of hosts (i.e., promiscuous). Therefore, the hosts are obtaining their partners from a "symbiont" pool that may or may not be directly related to the previous generation of symbiotic associates. With this type of infection behavior, one would predict that environmental transfer would provide a variety of closely related strains or species that are promiscuous between the hosts, with the ability to infect each new generation of hosts equally as well. With this assumption, the host would not be able to differentiate between any of the symbionts, and the chances of obtaining any one of the symbionts is equal between all symbionts in the free-living population (given that they are present in equal concentrations). But in reality, most environmentally transmitted symbioses are specific to a particular type of bacterium and a particular host species (Cavanaugh, 1994; Nishiguchi *et al.*, 1998). In these systems where parallel cladogenesis is evident, the underlying hypotheses did not predict the strict phylogenetic nature of the symbioses, thereby creating a paradigm in the 'traditional' symbiotic theory. Although correlating traits between interacting organisms are often evidence for corroborating strict cospeciation, the presence of species specificity in associations where the symbiont is transmitted environmentally invalidates this theory (Nishiguchi *et al.*, 1998). Whether the biogeography, ecology, or the life history of the host is a determining factor in the evolution of a specific symbiotic relationship, little is understood about how this specificity arises among symbionts with environmental modes of transfer.

2.1.3. Ancestry and cospeciation

The specificity of each symbiotic association and how the individual partners have accommodated and adapted to each other's presence can be an indicator of how cooperative associations have evolved from ancient lineages. Along with an obligate life history pattern, the mechanism of symbiont transfer between generations may also affect the patterns of parallel cladogenesis. Although transfer mechanisms are not

completely understood in many groups of animals, there are examples where both hypotheses of transmission strategies have been proposed. For example, the presence of symbionts in the reproductive tissues of two families of chemosynthetic bivalves (Vesicomyidae and Solemyidae) suggests that the bacteria may be transferred vertically through the gametes (Cary, 1994; Cary and Giovannoni 1993; Krueger *et al.*, 1996). However, experiments with another family of bivalves (Lucinidae) suggest that the symbionts are obtained anew with every generation, that is, transferred environmentally. Comparisons of host and symbiont phylogenies using 16S rRNA analysis for the symbionts and morphological data from the hosts have shown species specific associations within the chemoautotrophic containing bivalves indicating congruence (Distel *et al.*, 1988, 1994; Distel and Cavanaugh 1994; Krueger and Cavanaugh, 1997). This finding supports the mechanism of vertical transfer among host-symbiont pairs. However, when the phylogenies of these symbionts are compared to other symbionts of chemoautotrophic invertebrates, the associations are much more complex, with some of the bivalve symbionts being more closely related to vestimentiferan worm symbionts (Distel 1998; Distel *et al.*, 1994; Feldman *et al.*, 1997; Krueger 1996). Because all these bacterial symbionts have been classified within the same subdivision of the γ Proteobacteria (Distel, 1998), the evolution of chemoautotrophic symbioses in a number of animal phyla suggests that the history of the associations is complex, and the specialization of these bacteria was established prior to the diversification of host organ isms.

2.1.4. A contradiction of theories

Whether host-symbiont specificity has occurred between intimately associated partners cannot be predicted based on transmission strategies alone. If vertical transmission did occur between host and symbiont, and a high degree of fidelity between the partners was observed, then cospeciation patterns would be evident and their respective phylogenies would mirror each other. Additional evidence from the fossil record of host and symbiont may also corroborate the duplicity of the phylogenies (Distel, 1998; Moran and Telang, 1998). Rarely do we find stringent congruencies; usually phylogenetic signals are confounded by rare transfer between hosts species (Goff *et al.*, 1996, 1997) or the establishment of new associations (Wilkinson *et al.*, 1996). Many problems arise in the interpretation of evolutionary relationships amongst pairs of organisms, versus organisms within a single lineage. The symbiotic association has additional aspects to consider when interpreting phylogenetic congruence; not only do the individual lineages of each organism be taken into consideration, but the history of the association must be regarded as well. Even when the evolutionary pasts have been teased apart, there can always be room for promiscuity, that is, the loss or gain of new symbionts, the symbionts switching to an entirely new host, or the re-infection of a new type of symbiont. All these factors can also confound the relationships between a host and its symbiont if the phylogenetic composition of the association is not strictly parallel.

3. A case of congruency and specificity: The Sepiolidae-Vibrio symbiosis

One association that is shedding more light on the evolutionary history of symbiotic associations is the partnership between a group of shallow-water benthic squids (Family Sepiolidae) and their luminous bacterial symbionts (Genus *Vibrio*, Table 1). This system offers several advantages as an experimentally tractable system in which to study the coordinated influence of bacteria on the parallel evolution and specificity of closely related symbiotic species (Nishiguchi *et al.*, 1998). Unique features that render this symbiosis attractive for such studies are: (i) host animals are easily obtained in quantity, for example, in the Mediterranean Sea where several species of sepiolid squids

Table 1. *Euprymna* and *Sepiola* species

Species	**habitat**	**Location**	**Symbiont**
Euprymna morsei (Verrill, 1881)	continental shelf	Seto Sea, Japan	*V.fischeri*
Euprymna scolopes Berry, 1913	continental shelf	Kane'ohe Bay, HI	*V. fischeri*
*Euprymna stenodactyl*a (Grant, 1833)	continental shelf	Solomon Islands	*?*
Euprymna tasmanica (Pfeffer, 1884)	continental shelf	Crib Point, Australia	*V.fischeri*
Sepiola affinis Naef, 1912	continental shelf	Mediterranean	*V.fischeri* *V. logei*
Sepiola atlantica d'Orbigny, 1839	shelf-edge of slope	NE Atlantic	*?*
Sepiola auranhiaca Jatta, 1896	outer shelf-upper bathyal	S. Norway-Mediterranean	*?*
Sepiola intermedia Naef 1912	continental shelf	Mediterranean	*V. fischeri* *V. logei*
Sepiola knudseni Adam, 1984	inner shelf	NW and WAfrica	*?*
Sepiola ligulata Naef, 1912	shelf-edge of slope	Mediterranean and Adriatic	*V. fischeri* *V. logei*
Sepiola pfefferi Grimpe, 1921	continental shelf	W. Africa and Mediterranean	*?*
Sepiola robusta Naef, 1912	Outer shelf	Mediterranean	*V. fischeri* *V. logei*
Sepiola rondeleti Leach, 1834	shelf and upper bathyal	Mediterranean	*?*
Sepiola steenstrupiana Levy, 1912	upper sublittoral	Mediterranean	*?*

co-exist (Mangold and Boletzky, 1988); (ii) both the hosts and the bacterial symbionts are easily cultured and maintained independently under laboratory conditions (McFall-Ngai, 1999; McFall-Ngai and Ruby, 1998; Ruby, 1999a); (iii) the initiation of the association can be maintained under experimental control (Wei and Young, 1989); (iv) molecular genetics can be applied to the bacterial partner (Graf *et. al.*, 1994; Visick and Ruby, 1996); and, (v) the association represents the only known animal symbiosis where cross-colonization experiments can be used to determine the extent of specificity (or symbiotic competence) existing among bacterial symbionts from different host species (McFall-Ngai and Ruby, 1991; Nishiguchi *et al.*, 1998). Along with these characteristics, the symbiosis is environmentally transmitted, which in turn allows the study of whether transmission has a role in the origin of symbiotic lineages and parallel speciation.

Symbioses occur between species of luminous bacteria and squids in two families of cephalopods, the Loliginidae and Sepiolidae, the latter of which consists of four light organ genera: *Euprymna*, *Sepiola*, *Rondeletiola*, and *Semirossia* (Herring *et al.*, 1981; Mangold and Boletzky, 1988; Fig. 1A). In all species within these genera, the bacteria are maintained in a morphologically complex bibbed light organ that lies within the center of the animal's mantle cavity, where it is continually bathed with seawater as a result of normal ventilatory activity (Ruby and McFall-Ngai, 1992; Visick and McFall-Ngai, 2000; Fig. 1B). Observations of the behavior of the host organisms suggest that the bacterially produced light is used in antipredatory behavior (McFall-Ngai, 1990). Emission of the bacterial luminescence is controlled in two ways: (i) by a host modulated, diel restriction on the luminescent output per bacterial cell (Boettcher *et al.*, 1996); and, (ii) by a series of accessory tissues (Montgomery and McFall-Ngai, 1992) which are functionally analogous to the tissues that control light quality in the eye (Fig. IC). Although some minor differences exist in light organ morphology, the functionality remains the same among sepiolid species (Foster and McFall-Ngai, 1998; Mangold and Boletzky, 1988).

Bacterial cell numbers in the crypts is controlled in part by diel venting of approximately 90% of the bacterial culture through the lateral pores of the light organ which lead into the mantle cavity and are flushed out into the environment (Boettcher *et al.*, 1996). This venting behavior provides a sufficiently high density of symbiotic- competent vibrios in the water column to promote colonization of the next generation of juvenile squids (Lee and Ruby, 1994a). The remaining 10% of bacteria are capable of establishing a new complement of symbionts during the daylight hours when the squids are buried in the sand and quiescent (Boettcher *et al.*, 1996). It is during this time that the squids do not use any bacterially produced luminescence and may have a decreased metabolic demand from the few symbionts remaining in the light organ (Nyhoim and McFall-Ngai, 1998). Bacteria that remain in the light organ are spatially organized near or adhered to the microvilli layer of the crypt epithelium (Lamarcq and McFall-Ngai, 1998; Fig. 1D), enabling them to maintain contact within the tissues of the juvenile squid light organ. However, when two different strains of *Vibrio* are placed in direct competition with each other, the host squid will displace a non-native strain by preferentially venting the non-native *Vibrio* (Nishiguchi *et al.*, 1996). Once inside the light organ, the native strains are closer to the microvilli and crypt epithelium of the light organ compared to their non-native competitors (Nishiguchi *et al.*, 1996).

Within the first few hours after hatching, juvenile squids are colonized by competent *Vibrio* cells, but little is known about the recognition and specificity involved in determining light organ composition. During the initial part of the infection process, symbiosis-competent *Vibrio* strains from the ambient seawater must enter the juvenile light organ through the lateral pores and travel down narrow, ciliated ducts that lead into the epithelium-lined crypts (Montgomery and McFall-Ngai, 1993). These *Vibrio* strains are active participants during the infection process, since the bacteria are not simply swept along by epithelial cell ciliary movement of the host (Montgomery and McFall-

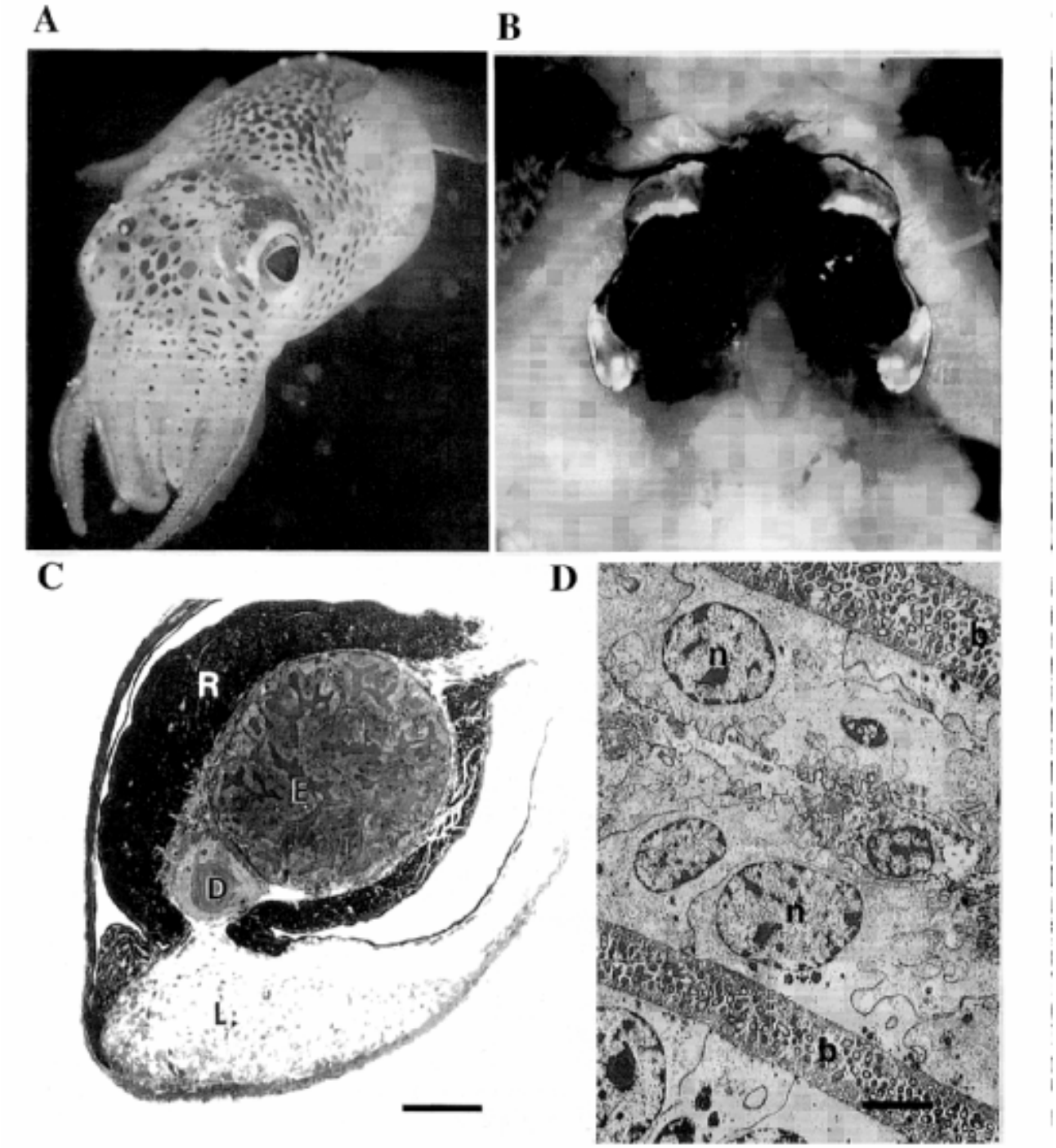

Figure I. Images of the sepiolid squid-luminous bacterium symbiosis. (A) An adult specimen of E. scolopes (5cm in length). (B) A ventral dissection of the bilobed light organ located within the mantel cavity. (C) A histological section of one lobe (bar =1 mm), revealing several tissues:
(e) epithelial central core tissue housing bacteria, (r) reflector, (I) lens, (d) diverticula. (D) A transmission electron micrograph of an area of the epithelium-line crypts containing symbiotic bacteria: (n) = nucleus of squid, (b) = bacteria in crypts (bar = 5 um). Photos: (A) W. Ormerod; (B) S.V. Nyholm; (C) M.J. McFall-Ngai; (D) M.K. Montgomery.

Ngai, 1993), but actually propel themselves into the crypts of the nascent light organ (Graf et al., 1994). Studies identifying the symbiotic bacteria from a variety of Mediterranean sepiolids indicate that each species of sepiolid harbors two species of *Vibrio* (*V. fischeri* and *V. logei*), and that no other species of bacteria are found inside the adult light organ (Fidopiastis *et al.*, 1998; Nishiguchi, 2000). Since the light organ pores are continually open to the surrounding seawater providing access for any type of bacterium, the presence of only *V. fischeri* or *V. logei* in the light organ illustrates very strong species-specific interactions between these bacteria and their host (Nishiguchi *et al.*, 1998).

3.1. EVIDENCE FOR THE PARALLEL EVOLUTION OF ENVIRONMENTALLY TRANSMITTED HOST-SYMBIONT ASSOCIATIONS

Free living, symbiotically competent vibrios have been found in the surrounding environment, as well as other luminescent vibrios that are not able to infect the light organ of the sepiolid squids and monocentrid fishes (Lee and Ruby, 1994a; 1994b). Along with the biogeographical separation among the Indo-west Pacific sepiolid squids, the distinction between related host genera and their specific *Vibrio* symbionts could be hypothesized as evolving from the influences of the surrounding environment of the association (ecology) and not the specific host partner. This hypothesis was first proposed by a number of researchers who believed that the specific *Vibrio* symbionts had originated from the surrounding seawater, and then diverged along with their host partner (Nealson and Hastings, 1991). If this was the case, then we would expect to see similarly related bacterial symbionts from different host organisms found living in the same coastal waters. Previous studies investigating monocentrid fishes found in the same location as sepiolid squids have shown that although the symbionts of both monocentrids and sepiolids are *Vibrio fischeri*, these strains are genetically distinct and cannot cross infect other hosts (Lee and Ruby 1994b; Nishiguchi *et al.*, 1998; Nishiguchi, unpub. data). What is interesting about this preference is the degree of specificity; among the *Vibrio fischeri* strains tested in competition, all *V. fischeri* strains isolated from sepiolid squids can infect juvenile *Euprymna scolopes*, whereas *V. fischeri* isolated from the monocentrid fish *Cleidopus gloriamaris* cannot infect the light organs of *E. scolopes* to the same concentration, even when presented alone. Since the symbioses of both sepiolids and monocentrids are transmitted environmentally, how has the evolutionary radiation among strains of vibrios become so species specific, providing the foundation for patterns of parallel cladogenesis among the phylogenetically analyzed partners?

Phylogenetic congruence has been previously used in other symbiotic systems to test the fidelity of host-symbiont relationships (Baumann *et al.*, 1997; Haygood and Distel, 1993; Hinkle *et al.*, 1994). Because sepiolids in general have been very difficult to identify morphologically (Bello, 1995), molecular methods have recently been used to establish phylogenetic information about the relationships between the existing species complexes (Nishiguchi *et al.*, 2001; 1998). Genetic markers such as the cytochrome *c* oxidase subunit I (COI) and the internal transcribed spacer region of the ribosomal repeat (ITS), have been employed to delineate population level relationships, as well as genus and species level associations (Nishiguchi *et al.*, 1998; Nishiguchi, unpub. data). These data combined with glyceraldehyde phosphate dehydrogenase (*gapA*) sequence data from the symbiotic bacterial partners provide molecular evidence for parallel cladogenesis (Fig. 2). Competition experiments between various symbiotic strains of Vibrio and juvenile sepiolids corroborate the phylogenetic data, where a genetic hierarchy exists among closely related symbiotic strains that matches the phylogenetic evidence (Nishiguchi *et al.*, 1998, Table 2). Using a standard squid colonization assay (Ruby, l999b; Ruby and Asato, 1993), two different strains of *Vibrio* symbionts are mixed in equal concentrations and are presented to the newly hatched aposymbiotic

squids. The infection and growth of the bacteria are monitored over time, and at the end of the assay period (48 hours), the number and strain type of bacteria are quantified by counting the two different competitor strains plated from each juvenile light organ (Nishiguchi 2001; Nishiguchi *et al.*, 1997). Since each strain or species used in the competition experiments can colonize a juvenile light organ equally well when presented by themselves, the existence of possible recognition factors responsible for differentiating native from non-native symbionts supports the hypothesis that specificity has evolved between each host-symbiont pairing.

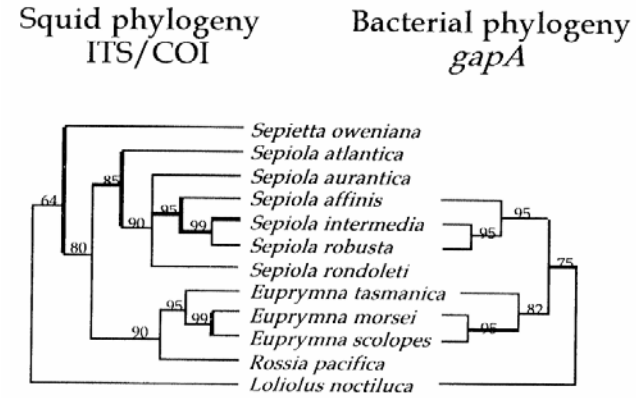

Figure2. Phylogenetic tree of squid host species and their symbiotic bacteria isolates. Each tree was inferred by comparison of the unambiguously aligned positions of either the ITS or the *gapA* sequence. Identical branching patterns were obtained for each locus when analyzed by either the parsimony or maximum- likelihood methods. The values given at each node are the percentages of 100 bootstrap re-samplings and subsequent heuristic searches that support the relationships between the clades.

TABLE 2. Infection of juvenile *E. scolopes* with mixed inocula of squid light organ symbionts.

Strains used in competition[1]	No. of animals tested[2]	Mean proportion found in light organs at 48 h[3]
ESll4andEMl7	37	98:2
ES114andET10135		95:5
EM17 and ET101	17	75:25
ET101 and SAl	30	90:1
ESll4andLNl	21	100:0

[1]Bacterial isolates from squid species are as follows: ES114 - *E. scolopes*, EM17 - *E. morsei*, ET101 - *E. tasmanica*, SA1 - *S. affinis*, LN101 - *Photololigo noctiluca.*

[2] Total number of animals tested in three or four experiments for each pair of strains. All of the animals became colonized by at least one strain in these experiments.

[3] Mean averages of individual ratios of bacterial strains were arc sine transformed (Sokal and Rohlf, 1981) to determine the significance of each treatment. All mean values were significantly different to within $P<=0.05$.

3. 1.1. Ecology vs. specificity

As mentioned previously, the underlying hypothesis regarding luminous bacterial symbioses has suggested that the environment determined the type of symbiont each host species acquired (Nealson and Hastings, 1991). Initially, this assumption must be correct, since the partnership must have evolved from two separate organisms living in close proximity prior to the initiation and radiation of the symbioses. If so, are there present day examples where the ecology is driving the speciation of symbiotic

associations? If such associations do exist, then to what level has the partnership evolved such that the symbiont is specific to one host and is also found in many closely related species?

The Mediterranean Sea hosts a large group of luminous sepiolid squid species that offer unique examples of how ecologically driven patterns of evolution might be a precursor to host specificity and parallel cladogenesis. Previously, all luminous bacterial-animal mutualisms were thought to be monotypic, i.e., that only one species of bacterium was associated with a particular host taxon (Nealson and 1-lastings, 1991; Ruby, 1996). However, Fidopiastis *et al.* (1998) have recently discovered a different species of *Vibrio* (*V. logei*) that resides in the light organs of several *Sepiola* squid species of the Mediterranean, giving rise to a two species consortium (Nishiguchi. 2000). This unique and interesting finding is the first observation of two *Vibrio* species of bacteria residing in the light organs of sepiolid squids (Fidopiastis *et al.*, 1998). What is peculiar about the dual-symbiont relationship is that the two species of *Vibrios* differ not only in their 16S rDNA genotype, but in some of their physiological characteristics as well (Fidopiastis *et al.*, 1998; Lickliter and Nishiguchi, unpub. data). Most notably, growth rates of *V. logei* isolates are higher than *V. fischeri* isolates at lower temperatures (18°C), whereas at higher temperatures (26°C), *V. fischeri* isolates grow at a faster rate (Nishiguchi, 2000; Table 3). Another major difference between the two symbiotic species is their luminescence at different temperatures. Again, *V. logei* isolates are more sensitive to higher temperatures, with decreased luminescence at temperatures above 18°C. The addition of decyl aldehyde to *V. logei* strains grown above 18°C enhanced their luminescence, indicating a temperature sensitivity for the capacity to synthesize natural aldehyde substrate for the luminescence reaction (Fidopiastis *et al.*, 1998). Therefore, *V. logei* symbionts are the more psychrophilic species of the two observed symbionts in *Sepiola* species. Because there are several species of Mediterranean *Sepiola* living in the same coastal habitats as each other, this provides an opportunity to test how environmental factors effect the nature of the symbioses. Does the variety of *Sepiola* species have an effect on the distribution and abundance of *Vibrio* symbionts, or do ecological factors (i.e., temperature) have a greater affect on the distribution and cospeciation of symbiotic associations?
At present, there are ten recorded species of endemic *Sepiola* living in the Mediterranean Sea (Mangold and Boletzky, 1988; Table 1). Of these ten species, five can be found within the same coastal waters off the southernmost part of France (Banyuls-sur-Mer), providing an ideal habitat in which to examine whether specificity or ecology is influencing symbiont distribution. To test whether an ecological (temperature) parameter has a direct effect on the colonization and specificity of symbiotic recognition, several symbiotic strains were analyzed for their competitive ability in infecting light organs of juvenile *Sepiola* squids (Nishiguchi, 2000). Initially, individual strains of *V. fischeri* or *V. logei* were isolated from adult specimens of *S. robusta*, *S. affinis, S. ligulata, and S. intermedia*. These isolates were used to test whether the species of host determined the symbiotic composition, or if temperature was a significant factor in establishing symbiotic competence. Strains of *V. fischeri* or *V. logei* were added in a 1:1 ratio (approximately 10^3/ml total bacteria) to either juvenile *S. affines* or *S. ligulata* at 18°C and at 26°C. After 48 hours, juvenile squids were homogenized, and plated on seawater tryptone plates to visualize the number and types of vibrios present (Lee and Ruby 1 994a). Identification between strains of *V. fischeri* and *V. logei* were accomplished by direct colony lifts (Lee and Ruby, 1992), and probed for the variable region (V1) of the 16S rRNA gene (Fidopiastis *et al.*, 1998). All eight isolates of *V. fischeri* or *V. logei* tested could infect either squid species light organ when the infection was initiated by a single strain (Nishiguchi, 2000). For all competitions held at 26°C, all *S. affinis* and *S. ligulata* juveniles were infected primarily with *V. fischeri* isolates. All light organ competitions held at 18°C resulted in colonization by mostly *V. logei* symbionts in either *S. affinis* or *S. ligulata* juveniles

(Nishiguchi, 2000). Thus, the ecology (in this case, temperature) determines the symbiont composition of the sepiolid light organ, and not the host species. These results are in contrast to observations within the Indo-west Pacific species of *Euprymna*, where symbiont composition was determined by host species, and not ecology (Nishiguchi *et al.*, 1998).

Table 3. Growth Constants of *V. fischeri* and *V. logei* isolated from European *Sepiola* species (from Nishiguchi, 2000).

Bacterial strain and species		**Host squid**	**Growth rate constant at 18°C (per hr)**	**Growth rate constant at 26°C (per hr)**
SA1	(*V. fischeri*)	*S. affinis*	0.6	0.8
SA8	(*V. fischeri*)	*S. affinis*	0.6	1.0
SA6	(*V. logei*)	*S. affinis*	0.9	0.7
SA12	(*V. logei*)	*S. affinis*	0.8	0.7
SL2	(*V. fischeri*)	*S. ligulata*	0.4	1.3
SL8	(*V. fischeri*)	*S. ligulata*	0.5	1.3
SL4	(*V. logei*)	*S. ligulata*	1.2	0.9
SLI2	(*V. logei*)	*S. ligulata*	1.2	0.9
SI2	(*V.fischeri*)	*S. intermedia*	0.6	1.1
SI4	(*V. fischeri*)	*S. intermedia*	0.6	1.0
SI5	(*V. logei*)	*S. intermedia*	0.9	0.7
SI7	(*V. logei*)	*S. intermedia*	0.9	0.7
SR5	(*V.fischeri*)	*S. robusta*	0.4	0.9
SR10	(*V.fischeri*)	*S. robusta*	0.5	0.9
SR1	(*V. logei*)	*S. robusta*	1.4	1.0
SR18	(*V. logei*)	*S. robusta*	1.3	0.9

3.1.2. Assessment of wild-caught sepiolids

Along with laboratory experiments, we have sampled a number of sepiolid squid populations throughout the year, to determine if there is a correlation between temperature and light organ composition in the natural habitat. Adult light organ contents from four species of *Sepiola* revealed that both species of *Vibrio* were present, as mixed assemblages, with one species dominating in concentration over the other (Nishiguchi, 2000). What is intriguing about the *Vibrio* species composition in each squid light organ is the manner in which it varied depending on the season and depth at which the individual was caught. During the summer months (June-September), the Mediterranean near Banyuls-sur-Mer experiences a formation of a thermocline at approximately 20 meters, in which an abrupt change in temperature occurs. Above the thermocline, temperatures average at approximately 23°C, whereas below the thermocline, temperatures drop to approximately 13°C. During winter months, the thermocline disappears, and temperatures on average range between 8-16°C (unpublished data, Laboratoire Arago database). Along with this distinct temperature boundary, one species of sepiolid, *S. affinis*, is mostly found at depths ranging from 5- 15 meters. All other species of *Sepiola* sampled thus far (*S. robusta*, *S. intermedia,* and *S. ligulata*) are found at depths ranging from 30-80 meters. Thus, three of the four species of sepiolid squids collected reside at temperatures below 16°C.

Specimens that were primarily populated by *V. fischeri* or were dominated by *V. fischeri* versus *V. logei* were obtained between the first part of July until early September, and were collected at depths above the thermocline (< 20 meters). These specimens were all *S. affinis* individuals whose habitat is found above this thermocline

boundary. Sepiolid squids that were populated primarily by *V. logei* or were dominated by *V. logei* versus *V. fischeri* were collected in late September until late spring (May), during which the thermocline disappears due to winter conditions which consist of wind-driven mixing of surface waters with those below. All specimens that were collected at all depths had a larger proportion of *V. logei* symbionts than *V. fischeri* symbionts. Although the Bay of Banyuls is mainly coastal shelf (approximately 0-150 meters), the change in temperature due to seasonality may be a strong factor influencing symbiont distribution in the squid species that are affected by changes in temperature (*S. affinis*). Since S. affinis is found above the thermocline boundary year around, this squid was found to be more susceptible to changes in symbiont composition due to environmental changes rather than host specificity. Sepiolids which reside at depths below the thermocline (S. intermedia, S. ligulata, S. robusta) are rarely found in habitats above the thermocline; therefore these species may be more prone to form species alliances with psychrophilic V. logei strains. Although previous in vitro experiments with juvenile squids did not show any differences in the infection capability between *S. affinis* or *S. ligulata* juveniles (Nishiguchi, 2000), there have not been sufficient data collected to test host specificity with the other species of squids or their symbiotic vibrios. Future work is planned to include all species of sepiolids found in the Mediterranean, Adriatic, and Atlantic Seas, and to test how concentrations of environmentally transmitted bacteria are related to the presence and distribution of various host squids.

4. Deciphering the mechanisms which drive specificity and parallel cladogenesis

In the preceding paragraphs, I have given examples of how various symbiotic associations either follow expected patterns of cospeciation between the partners or are influenced by abiotic factors such as temperature. Because a particular mode of transmission cannot be used to predict whether two organisms have evolved in parallel with each other, other components such as environmental fluctuations or host-symbiont specificity must be considered. In the examples where there is promiscuity between symbionts and their hosts, there is much speculation about how specific host-symbiont assemblages evolved, and why there is so much diversity expressed in associations where there is a high degree of dependence for some physiological function or capability. It has been previously hypothesized that the benefit of obtaining a symbiont would allow the adaptation to a new environment to exploit a different ecological niche (Saffo, 1992). Obviously, these types of adaptations would increase the survival and fecundity of both organisms. Evolving with a new symbiotic partner can also affect other biochemical interactions not necessarily linked to nutrient exchange (Huger *et al*., 1985), and may alter sex ratios of hosts with either beneficial or detrimental effects (Nardon and Grenier, 1991). Developmental changes can also be induced with the onset of symbioses (Doino and McFall-Ngai, 1995; Lemus and McFall-Ngai, 2000; Schwemmler, 1989; Visick and McFall-Ngai, 2000), increasing the ability of the symbionts to become an integral part of the host life cycle and therefore having an effect on the overall success of the entire symbiosis. Thus, many of the interactions that occur between host and symbiont have caused one or both partners to undergo complicated and extensive life history changes to accommodate the other's existence.

4.1. ADAPTATION IN PARALLEL?

If several ancestral symbionts adapted to similar host species, thereby allowing independent lineages of symbiotic associations to evolve, then why do we see congruency in host-symbiont pairs where environmental transmission would allow more diversity? Is specificity a less deleterious mode of antagonistic behavior that has been

modified between the host and the symbiont to allow coexistence between the partners? Why have we observed examples where expected congruency is promiscuous (as in vertical transmission) and parallel cladogenesis is evident in environmentally transmitted symbiosis? Do environmental factors have direct effects upon the degree of specificity found in symbiotic associations, or do they magnify the initiation and continuation of a specific host-symbiont relationship?

In the sepiolid-squid mutualism, there are two populations of genera that exhibit both specificity and promiscuity. Law (1985) makes the prediction that mutualistic associations should express less specialization between symbionts that are found in similar host species. Therefore, one would expect that allopatric populations are more prone to divergence than sympatric sister taxa, and that their symbionts would diverge in parallel. Support for this prediction comes from the competitive hierarchy observed between strains of symbionts with closely related host taxa (Nishiguchi *et al.*, 1998). For example, the Indo-west Pacific genus *Euprymna* has several biogeographically isolated species, and their symbiotic partners display a high degree of specificity, even though all symbionts equally infect juveniles of a different host squid when presented alone (Nishiguchi *et al*., 1998). Although the hosts obtain their symbionts anew with every generation, other mechanisms must influence the precise identification and retention of a particular symbiotic strain. These mechanisms could include: specific receptors on the host epithelia which recognize specific bacterial strains (Hensey and McFall-Ngai, 1992; McFall-Ngai *et al.*, 1998); various adhesin proteins on the bacteria that allow it to settle inside the squid light organ (Jacob-Dubuisson *et al.*, 1993); adaptation to the biochemical milieu of the light organ (Small and McFall-Ngai, 1999; Visick and Ruby, 1998; Weis *et al.*, 1996); the ability of the bacteria to colonize the squid tissues effectively and without competition from other types of bacteria (Graf and Ruby, 1998) and the communication between symbionts and host to enable the symbiosis to effectively work in unison (Doino and McFall-Ngai, 2000; Foster and McFall-Ngai, 1998). Deciphering these mechanisms will shed more light on how symbiotic bacteria and their particular hosts are capable of evolving intricate patterns of specificity and coevolution.

Despite the specific associations shown in *Euprymna*, the genus *Sepiola* has different patterns of physiological specificity. Specificity is observed within the symbiont genus *Vibrio*, and it appears that the most influential factor determining symbiont composition between *Vibrio* species is the ecology of the association. Previous studies investigating the influence of temperature acclimation in *E. coli* (Mongold *et al.*, 1996; Bennett and Lenski, 1993) have shown that the selection criterion of a particular strain has no discernable effect on the future adaptation to a new or different environment. But, in contrast, the evolutionary history of a bacterial strain will influence the future fitness of a strain, particularly when it is acclimated to a temperature which that species has been previously adapted to (Bennett and Lenski, 1998). Assuming that this holds true for bacteria in symbiotic associations, one may conclude that symbiotically competent vibrios capable of infecting squids at certain temperatures are not under any less selection pressure than other species of *Vibrio*, but may have the ability to infect different host species at a better rate or increase their persistence more than strains not acclimated to the infection temperature. This may provide some insight as to how environmental factors can influence the distribution of infective bacteria in a particular host taxon and therefore have direct effects on the biogeography and population genetics of host-symbiont pairs.

Finally, the type of transfer among numerous symbiotic taxa has traditionally been thought of as a good predictor of cospeciation. Recently, however, a number of examples have demonstrated that the systematics underlying these symbioses are not as clear as we have traditionally thought. The conflict between patterns of symbiont transmission arises when there is competition between closely related symbionts; migration leads to this competition from other closely related symbionts, and may have

potential side effects on the hosts (Frank, 1996a). Possible explanations for the variation in patterns of cospeciation have been complicated; the fundamental difference between transfer modes may be linked to the selection pressure on the host's fitness and how the symbiont may or may not affect future generations of host-symbiont pairs (Sniegowski *et al.*, 1997; Frank, 1996a). As well as host fitness, population size, and the availability of hosts that can be infected will affect the dynamics of host-symbiont pairs and the specificity that these pairs will eventually express (Bull *et al.*, 1991, Turelli, 1994; Turner *et al.*, 1998;). The presence of two different species of symbiont that can equally infect different host species may be an initial step in establishing host symbiont congruency prior to host-symbiont specificity. But how the natural balance between host fitness and symbiont virulence is established, remains to be determined (Frank, l996b). Because there are several sympatric host species of Sepiola living in the area sampled, the possibility of host switching between squids and the establishment of host-symbiont specificity can be studied. Future studies will need to address what degree ecological and/or genetic factors control patterns of cospeciation and congruency, and how we can better predict which patterns arise from these factors. Whether the symbionts determine new avenues for host evolution and radiation into different ecological habitats in this family of squids is just one of the many questions that need to be addressed in future studies.

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