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The use of physiological data to corroborate cospeciation events in symbiosis

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Summary. The symbiotic association between sepiolid squids (Family Sepiolidae) and luminous bacteria (Genus *Vibrio*) provides an unusually tractable model to study the evolution and speciation of mutualistic partnerships. Both host and symbiont can be cultured separately, providing a new avenue to test phylogenetic congruence through molecular and physiological techniques. Combining both molecular and morphological data as well as measuring the degree of infectivity between closely related pairs can help decipher not only patterns of co-speciation between these tightly linked associations, but can also shed new light on the evolution of specificity and recognition among animal-bacterial associations.

Introduction

The presence of symbiotic associations between eukaryotic hosts and their microbial partners has long intrigued biologists who are interested in evolution and cospeciation between the individuals in the relationship. The diversity and biogeographical range of each host-symbiont pair demonstrates how each partner has co-evolved to exploit a new ecological niche, providing a new capability or function for the newly adapted symbiosis and their respective populations. A number of contemporary examples indicate that the formation of a symbiotic relationship allows the host or its symbiont to radiate into newly formed niches; these include the chemoautotrophic bacteria residing within tissues of hydrothermal vent or sewage outfall invertebrates (Cavanaugh, 1994), nitrogen-fixing bacteria in the nodules of leguminous plants (Wilkinson et al., 1996), endosymbiotic bacteria within the body cavities of aphids, termites and whiteflies (Smith and Douglass, 1987; Nardon and Grenier, 1989; Moran and Telang, 1998), and luminous bacteria within the light organs of monocentrid and anomolopid fish and sepiolid and loliginid squid (McFall-Ngai and Ruby, 1991; Haygood and Distel, 1993). Although these symbiotic partnerships may have evolved as part of an adaptation to a particular ecological niche, the question of whether the ecology or the specificity of the partnership drives the evolutionary radiation of the hosts and their symbionts has yet to be answered. Understanding how a particular symbiosis has diverged from one association into many has led to the development of several techniques that allow the elucidation between strict co-speciation or symbioses that are more promiscuous among the partners.

Studying eukaryotic-prokaryotic symbiosis has been difficult for many biologists; in many cases the symbiont cannot be cultured outside the symbiosis and therefore many of the physiological properties that are used to characterize the prokaryotic partner are not measurable or functional without the eukaryotic host. In these instances the symbiont may not retain certain morphological characters or biochemical processes that delineate it from similar free-living species. Without proper identification of specific symbionts, systematists studying co-evolving associations have had to rely on parts of the fossil record, which has provided some evidence about species interactions (Moran and Telang, 1998). Molecular techniques, which allow the amplification of specific loci from culturable and non-culturable symbionts, also allow scientists to further probe the history of symbiotic events. Using present-day specimens, one can measure characters (morphology and molecules) from both host and symbiont, and then use this information to predict how long interacting lineages have been associated. Phylogenetic analyses provide a means to assess whether symbiotic associations have evolved in parallel, or have progressively adapted so that different hosts have evolved with their symbionts, irrespective of their relationship to each other. Parallel cladogenesis would imply patterns of co-speciation, and would become more evident from the phylogenetic analyses of both partners.

Reconstructing host-symbiont phylogenies

Comparing data sets from both host and symbiont phylogenies is one method to determine if parallel cladogenesis exists between the partners. In an ideal case, unique specificity may exist among the two systems, with every single host species having one single parasite and *vice-versa*. But it is clear that other cases might exist where some hosts have no symbionts, some symbionts occur in more than one host, or hosts have more than one symbiont (Schuh, 2000). In order to test whether cospeciation is occurring among two systems, independent phylogenetic analyses can be compared using two separate phylogenies, such that cospeciation events can be inferred. In the example of many strict phylogenies, such as the aphid/*Buchnera* symbiosis, symbionts are transmitted vertically, that is, from maternal lineage to each of the offspring (Munson et al., 1991; Baumann et al., 1997). Combining molecular and morphological data of hosts and their respective symbionts, each phylogenetic tree is constructed and compared to test for phylogenetic congruence amongst the host-symbiont pairs. In species where symbiotically linked genes have been identified, those loci are ideal for further phylogenetic analyses (Lee and Ruby, 1994) and may provide greater sensitivity for developing phylogenies in parallel.

In vertically transmitted symbioses, little or no evidence exists for host-switching of partners, and the relationships tend to be strictly congruent in their phylogenetic patterns. Environmentally (or horizontally) transmitted symbioses (where hosts obtain their symbionts anew with every generation),

would predict non-specific patterns of co-speciation, where symbionts from the environment will be found in hosts from the same area. Although the assumption of this pattern seems evident, it has been shown that certain environmentally transmitted symbioses still suggest strict patterns of parallel cladogenesis. In the example of the sepiolid squid-*Vibrio* mutualism, host phylogenies parallel the respective symbiont trees in a strict consensus pattern, despite the fact that the symbiosis is transmitted environmentally (Nishiguchi et al., 1998; Fig. 1). Using combined molecular data from both host and symbionts, this parallel phylogeny demonstrates that specificity can still evolve between hosts and symbionts, despite the fact that juvenile squids obtain their partners from the environment at every generation.

The program TREEMAP (Page, 1995) offers the possibility of analyzing both host and symbiont characters simultaneously and robustness of the evolutionary patterns can be tested, particularly for host-symbiont specificity and host-switching. The number of shared characters between different competing symbionts are tested, and these data are matched *a priori* for each association that is presently found in modern taxa. The number of host-switching events can then be calculated, to accommodate host-symbiont pairs that may not be strictly evolving in parallel. Thus, each node tested provides a measurement of whether host symbiont pairs are derived and co-speciated, or whether certain partners have been promiscuous in their symbiotic relationships. The robustness of the data set is then determined statistically by determining if each host-symbiont pairing is significant, compared to the other possible pairings among all the associations tested.

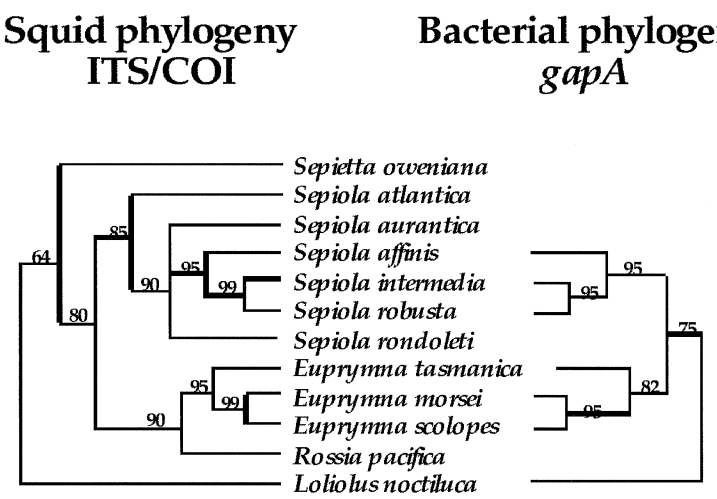

Figure 1. Phylogenetic tree of squid host species and their symbiotic bacteria isolates. Trees were inferred from unambigously aligned positions of ITS/COI or *gapA* data sets. Numbers above nodes represent bootstrap values. For details see Nishiguchi et al. (1998).

Once parallel cladogenesis is established among the host species and their respective symbionts (using molecules and/or morphology), competition experiments between symbionts can be used to test the robustness of the prior phylogenetic analyses, which is the focus of this chapter.

Competitive hierarchy and strain specificity

In sepiolid-*Vibrio* symbiosis, newly born aposymbiotic (without symbionts) juvenile squids are infected with individual symbiont strains isolated from closely related squid taxa, and are used for measuring the individual degree of colonization and subsequent host specificity. Bacterial strains are mixed at a concentration of 1000 cells/mL seawater using one or, for competition experiments, two strains of symbiotically competent bacteria. The juvenile squids are then placed in this inoculated seawater for approximately 12 h to initiate the light organ infection (Ruby and Asato, 1993). Once infected, the juvenile squids contain enough bacteria that their luminescence can be detected with a luminometer (Turner Designs 20/20), ensuring colonization has occurred (Ruby and Asato, 1993). Single bacterial strains that are presented to the juvenile squids alone, as well as squids that are kept aposymbiotic (no symbionts), provide stable controls for the competition experiments between two different symbiont strains or species. Since bacterial cells from light-organ homogenates have approximately 100% plating efficiency, the extent of colonization can be calculated from the number of colony-forming units (CFUs) arising from diluted aliquots of whole juvenile squid homogenates that are plated on seawater tryptone agar (SWT) medium (Boettcher and Ruby, 1990; Ruby, 1996). Using two different *Vibrio* strains or species that express different luminescent phenotypes in culture, quantification is achieved by comparing the ratio of one luminescent phenotype to the other (Nishiguchi et al., 1997). When quantifying symbionts with the same luminescent phenotype, differentiation can be accomplished by using a 16S rDNA probe for a particular variable region (VA1; Fidopiastis et al., 1998) which differentiates between *V. fischeri* and *V. logei*. Using colony blot techniques, the number of colonies from each squid homogenate can be quantified for both competing symbionts (Nishiguchi, 2000).

Colonies grown from the homogenized individuals will produce various concentrations of each symbiont. The proportion of each symbiont in the juvenile squid light organ is dependent upon whether specificity exists between the symbionts and the particular squid species used in the competition (Nishiguchi et al., 1998). When two species of symbiont are presented to a hatchling juvenile, several outcomes can be expected. First, if neither symbiont is capable of infecting the host squid itself, then the competition assay between these bacterial strains will not be different from the control animals and no colonization will occur from competition (initiation, Fig. 2). If both symbiont strains are capable of infecting and colonizing the squid light organ by themselves, then

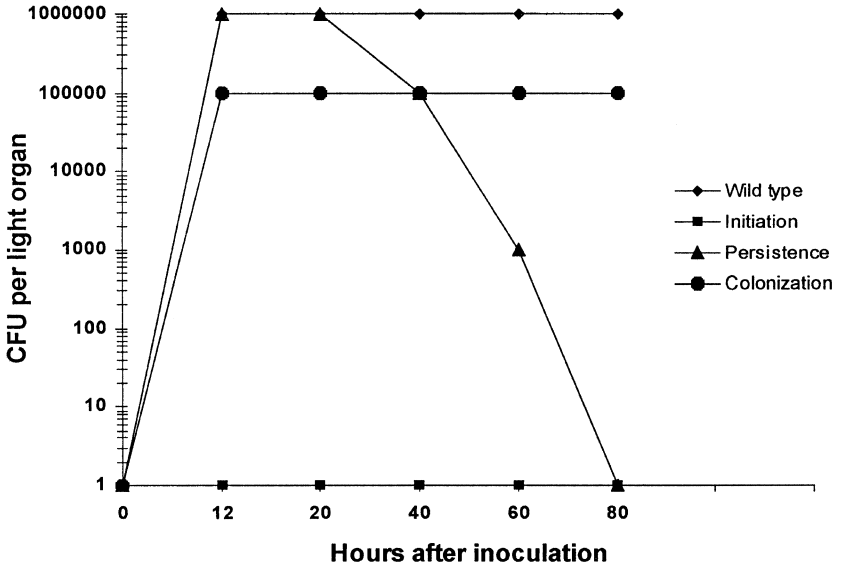

Figure 2. Classes of symbiosis-defective strains of *V. fischeri*. Schematic illustration of the colonization patterns of strains that either fail to infect ("initiation strains"), infect to a diminished extent ("colonization strains"), or are incapable of maintaining the infection ("persistence strains"), compared to the wild-type pattern. CFU: colony-forming units.

both strains will be present in equal concentrations in the light organ during the assay period. This would indicate that each symbiont is equally capable of persisting inside the host light organ equally well, and neither one can be differentiated by host recognition or specificity. In contrast, if two equally competent strains of symbiont are used to infect a newly hatched juvenile, and only one is present or dominant at the end of the assay period, then this would indicate that some preference or recognition exists for the more predominant symbiont (Nishiguchi, 2000; Nishiguchi et al., 1998).

Using specificity to delineate symbiotic relationships

Initially, if one were to compare the evolutionary history and lineages of host-symbiont pairings, the assumption would be that each partner has shared some common ecological or environmental past. Nealson and Hastings (1991) initially hypothesized that those associations between marine animals and luminous bacteria were initiated in a shared environment, where the ecology of the association would predict the evolutionary history of the partners. This is the presumed case for environmentally transmitted symbioses, where hosts must obtain their symbionts from the surrounding seawater and bacteria are available for uptake and proliferation within the host light organ. Over evolution-

ary time, a symbiosis has the capacity to evolve species specificity, in which symbionts are more likely to be associated with a particular host species. Examples of both ecologically determined and host-specific symbioses can be observed within the sepiolid squid-luminous bacterium symbiosis (Nishiguchi et al., 1998; Nishiguchi, 2000). During the initiation of the symbiosis, juvenile squids obtain their symbionts environmentally within the first few hours after hatching (McFall-Ngai and Ruby, 1991; Nishiguchi, 2000). From this inoculum (which can be as little as 10 bacteria), the symbionts are able to successfully colonize the juvenile squids and maintain populations inside the light organ throughout the duration of the squid's life history. The occurrence of two species of *Vibrio* symbiont in one host exemplifies the notion that this environmentally transmitted symbiosis is selective for specific types of bacteria (*Vibrio*), but the population and concentration of each *Vibrio* species are affected by abiotic factors such as temperature (Nishiguchi, 2000). These subtle changes in symbiont composition may lead to the eventual differentiation and radiation of a particular host-specific association, where one host is affiliated with one type/strain of symbiont. In Mediterranean squid species of the genus *Sepiola*, the symbiosis is comprised of two species of luminous bacteria, *Vibrio fischeri* and *V. logei* (Fidopiastis et al., 1998). Both species of *Vibrio* are abundant in coastal environments where Mediterranean sepiolids are also abundant. *V. fischeri* and *V. logei* are both found in free-living populations throughout the Mediterranean, but the more psychrophilic of the two species, *V. logei*, is more common among species of sepiolids that inhabit colder temperatures (Nishiguchi, 2000). For example, in the Bay of Banyuls, France, where both symbionts reside in many of the *Sepiola* species examined, *V. logei* is the more predominant of the two bacterial species in light organs of *S. robusta*, *S. intermedia* and *S. ligulata* species. These sepiolid species are commonly found at depths below 20 m and at temperatures ranging between 10 °C to 16 °C (Nishiguchi, 2000). These squids are not affected by seasonal fluctuations in temperature due to the fact that the summer thermocline prevents changes in water temperature below 20 m. In contrast, the shallow-water *S. affinis* resides between 0–20 m in depth and is exposed to temperatures ranging between 14 °C to 24 °C throughout the year. The population of vibrios that reside in *S. affinis* changes with respect to temperature changes; in the summer, *V. fischeri* is the more predominant species in *S. affinis* light organs, where surrounding environmental temperatures are between 18–22 °C at the shallower depths (0–20 m; Tab. 1). During the winter months, when the summer thermocline disappears and temperatures drop to between 12–16 °C, *S. affinis* individuals mostly host *V. logei* in their light organs (Tab. 1). Although both species of *Vibrio* are always present in all *Sepiola* species tested thus far, there is a definitive ecological boundary that establishes whether *V. logei* or *V. fischeri* is present in higher concentrations in the light organs of sepiolid squids. *V. logei* also exhibits higher growth constants *in vitro* and in certain *Sepiola* species compared to *V. fischeri*, where depressed temperatures of the symbiotic habitat have an effect on species composition of the association (Nishiguchi, 2000).

Table 1. Total light organ concentrations of *V. fischeri* and *V. logei* from field-caught adult *Sepiola*

Squid species*	Depth (m)/Temp (°C)	*V. fischeri***	*V. logei***	Ratio (Vf:Vl)	Month caught
S. affinis (1)	15/18	6.1×10^9	2.4×10^9	72: 28	May, 1999
S. affinis (1)	20/22	2.4×10^9	6.3×10^8	79: 21	July, 1995
S. affinis (7)	10/22	$7.5 \times 10^9 \pm 6.6 \times 10^8$	$9.1 \times 10^8 \pm 2.1 \times 10^7$	89: 11	July, 1999
S. affinis (2)	20/18	$5.1 \times 10^8 \pm 4.3 \times 10^8$	$3.2 \times 10^8 \pm 1.2 \times 10^7$	61: 39	Sept, 1995
S. intermedia (2)	75/16	$4.8 \times 10^8 \pm 5.4 \times 10^7$	$8.3 \times 10^9 \pm 3.4 \times 10^9$	6: 94	July, 1999
S. intermedia (2)	60/12	$8.4 \times 10^7 \pm 4.6 \times 10^2$	$2.1 \times 10^9 \pm 1.2 \times 10^8$	4: 96	Sept, 1995
S. intermedia (1)	55/12	1.0×10^9	3.8×10^9	21: 79	Sept, 1998
S. ligulata (1)	40/16	2.1×10^{10}	7.9×10^{10}	21: 79	July, 1999
S. ligulata (3)	55/10	$7.1 \times 10^{10} \pm 3.2 \times 10^7$	$1.8 \times 10^{11} \pm 2.9 \times 10^6$	28: 72	Sept, 1998
S. robusta (1)	60/12	5.1×10^9	4.2×10^{10}	11: 89	July, 1995
S. robusta (2)	60/10	$5.4 \times 10^9 \pm 1.5 \times 10^9$	$7.1 \times 10^{10} \pm 4.5 \times 10^9$	7: 93	Sept, 1998

* Numbers in parentheses indicate number of adult animals used to calculate bacterial concentrations. All concentrations are in CFU (colony-forming units)/adult light organ.

** Values represent CFU/light organ ± standard deviation for animals with more than one representative specimen.

Vf = *V. fischeri*, Vl = *V. logei*

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

Strains used in competition	No. of animals tested[2]	Mean proportion found in light organs at 48 h[3]
ES114 and EM17	37	98:2
ES114 and ET101	35	95:5
EM17 and ET101	17	75:25
ET101 and SA1	30	90:10
ES114 and LN101	21	100:0

[1] Bacterial isolates from squid species are as follows: ES114-*E. scolopes*, EM17 = *E. morsei*, ET101-*E. tasmanica*, SA1-*S. affinis*, *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 $P < 0.05$.

Thus, ecological factors of the association can be important in establishing the symbiotic composition of the partnership, demonstrating a need for understanding abiotic factors that influence the evolution of the association.

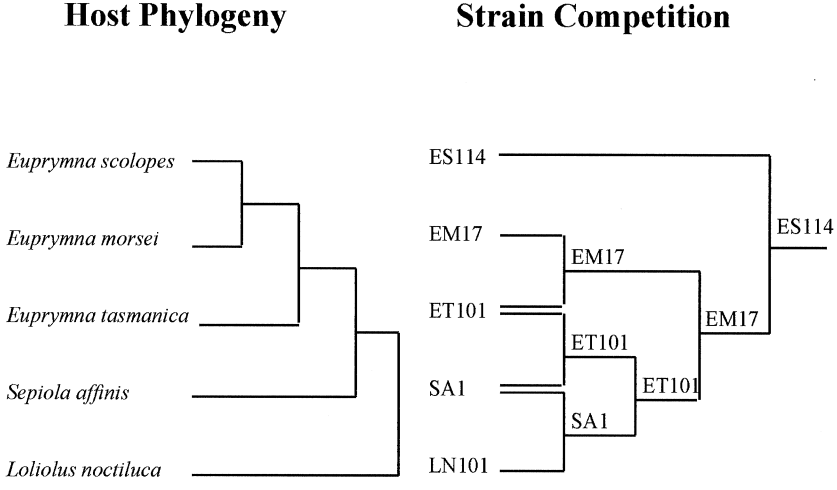

Figure 3. Comparison of species from the sepiolid host phylogeny (based on ITS and COI sequence data), to the symbiont competition experiments. According to symbiont competition experiments completed in *E. scolopes* juveniles, native ES114 (*E. scolopes* symbiont) always dominates the light organ colonization, compared to any other symbiont in a competition. EM17, the symbiont isolated from *E. morsei*, is the next best strain to dominate in a competition experiment, and complements the molecular phylogenetic data which suggest that *E. scolopes* and *E. morsei* are sister taxa. Following the phylogenetic relatedness of host squids, respective symbiont strains colonize the light organ in the same hierarchical manner as the molecular phylogenies. ES114-*Euprymna scolopes* symbiont; EM17-*Euprymna morsei* symbiont; ET101-*Euprymna tasmanica* symbiont; SA1-*Sepiola affinis* symbiont; LN101 *Photololigo noctiluca* (*Loliolus noctiluca*) symbiont.