One of the principal assumptions in symbiosis research is that associated partners have evolved in parallel. We report here experimental evidence for parallel speciation patterns among several partners of the sepiolid squid-vibrio symbioses. Molecular phylogenies for 14 species of host squids were derived from sequences of both the nuclear internal transcribed spacer region and the mitochondrial cytochrome oxidase subunit I; the glyceraldehyde phosphate dehydrogenase locus was sequenced for phylogenetic determinations of 7 strains of bacterial symbionts. Comparisons of trees constructed for each of the three loci revealed a parallel phylogeny between the sepiolids and their respective symbionts. Because both the squids and their bacterial partners can be easily cultured independently in the laboratory, we were able to couple these phylogenetic analyses with experiments to examine the ability of the different symbiont strains to compete with each other during the colonization of one of the host species. Our results not only indicate a pronounced dominance of native symbiont strains over nonnative strains, but also reveal a hierarchy of symbiont competency that reflects the phylogenetic relationships of the partners. For the first time, molecular systematics has been coupled with experimental colonization assays to provide evidence for the existence of parallel speciation among a set of animal-bacterial associations.

Cooperative associations with symbiotic bacteria are a common and ancient theme in the biology of animal and plant life. Thus, it is not surprising that there is often congruency between the evolutionary patterns of closely related host species and their symbiotic partners (1, 6, 11, 13). This congruency, known as parallel cladogenesis, has been revealed by comparing the sequences of genes that have rates of divergence suitable for such studies. However, because most animal-bacterial associations cannot be experimentally initiated, the mechanisms underlying the processes of speciation and host-symbiont specificity have not been explored.

The luminescent organ associations between sepiolid squids and luminous bacteria provide an unusually tractable system for the study of the evolution of symbiosis because (i) both the host and symbiont can be cultured and maintained in the laboratory (10, 19); (ii) newly hatched squids are colonized by symbiotic bacteria from the environment, allowing the association to be initiated and monitored experimentally (for review, see reference 26); and (iii) the numerous sepiolid squid species have a wide biogeographic distribution (22). The ease of studying both the squid host and its bacterial symbiont separately or combined under experimental conditions allows one to examine whether a particular squid host can distinguish its own natural symbiont from those of other sepiolid squid species (17) and whether this specificity reflects the evolution of the partnership. While coevolution has been experimentally studied in certain plant-bacterial systems (31, 33), the squid-vibrio symbiosis offers a unique opportunity to do so among animal-bacterial associations.

In the present study, the sequences of one nuclear locus and one mitochondrial locus have been used to generate phylogenetic trees for several species of sepiolids from Indo-West Pacific, Eastern Pacific, Mediterranean, and Atlantic populations. For a subset of the species, we determined the phylogenetic relationships of their symbiotic bacteria. An analysis of these data showed congruency between the derived phylogenetic trees of the hosts and their symbionts. In experiments in which two symbiotic bacterial strains were both present during the colonization of a representative host species, a hierarchy of competitive dominance was derived that mirrored the congruency pattern of these phylogenetic trees. Taken together, these data suggest that cospeciation has occurred during the evolution of the squid-vibrio symbioses and that initial recognition processes are key specificity determinants in these associations. These results provide the first experimentally derived support of cospeciation between animal hosts and their bacterial symbionts.

MATERIALS AND METHODS

Generation of molecular phylogenies. Squid specimens and their bacterial symbionts were collected alive at nine different geographic locations (Table 1). In addition, formalin- or ethanol-preserved specimens of Euprymna scolopes, Sepiola atlantica, Sepiola aurantica, Sepiola ligulata, Sepiola rondoletti, Rossia macrosoma, and Heteroteuthis dispar were obtained from various museum and private collections. Bacterial symbionts were identified as either Vibrio fischeri, Vibrio logei, or Photobacterium leiognathi as previously described (7, 25). Only light organ isolates of sepiolid species that were identified as V. fischeri were compared in this study (7).

Squid DNA was extracted from fresh tissue (1 to 10 g) by homogenization in 100 mM Tris-HCl (pH 8), containing 1.4 M NaCl, 20 mM EDTA, 2% (wt/vol) hexadecyltrimethylammonium bromide, 0.2% (vol/vol) β-mercaptoethanol, 0.1% (wt/vol) polyvinylpyrrolidone, and 0.1% (wt/vol) sodium dodecyl sulfate (SDS). The homogenate was incubated at 80°C for 60 min, an equal volume of a chloroform-isooctyl alcohol mixture (24:1) was then added, and the suspension was centrifuged at 10,000 × g for 10 min. A two-thirds volume of cold isopro-
Individual colonies were homogenized in 200 μl of TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 8.0]) containing 2.6 g of CsCl, and centrifuged at 270,000 × g for 8 h at 20°C. The isolated genomic DNA fraction was then precipitated with 95% ethanol, washed with 70% ethanol, dried, and resuspended in a buffer composed of 20 mM NaCl, 1 mM Tris-HCl, 1 mM EDTA (pH 7.5), and 0.2% (vol/vol) SDS, to which was added 250 μl of 7 M ammonium acetate (12). The homogenate was centrifuged, and the DNA was precipitated, washed, and resuspended in TE buffer as described above.

DNA templates for sequence analyses were obtained by PCR amplification. Each 100-μl PCR mixture contained 1 μl of template DNA solution, 0.01 μM (each) PCR primer, and 200 μM total deoxynucleoside triphosphates. DNA was isolated from strains of symbiotic bacteria (Table 1) that were cultured from the luminescent organs of freshly collected squid hosts as previously described (17, 32). Briefly, newly hatched Euprymna scolopes squid were placed in vials containing 5 ml of seawater without symbiotic bacteria. The progress of light organ colonization was monitored by measuring the luminescence of each squid with a sensitive photometer (model 3000; Biospherical Instruments, San Diego, Calif.). Because bacterial cells from light organ homogenates have essentially a 100% plating efficiency (27), the actual extent of colonization could be calculated from the number of CFU arising from aliquots of light organ homogenates that were plated on seawater nutrient agar medium (3). Different clutches of E. scolopes eggs were used in at least three replicate competition experiments to ensure that the results were not affected by any interclutch variation in colonization characteristics. Forty-eight hours after inoculation, the relative degree of colonization by the two competing bacterial strains from different host species was quantified by plating homogenates of light organs of E. scolopes juveniles exposed to mixtures of the strains (17). As previously described (23), the relative abundance of each strain in the light organ was determined by the visually distinct and genetically stable differences in the luminescence intensities of colonies of these different strains.

<table>
<thead>
<tr>
<th>Species</th>
<th>Collection site</th>
<th>Representative luminescent organ symbiont strains</th>
<th>Luminescence on seawater nutrient agar medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euprymna morsyi</td>
<td>Seto Sea, Japan</td>
<td>EM17&lt;sup&gt;a&lt;/sup&gt; and EM24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Bright</td>
</tr>
<tr>
<td>Euprymna scolopes</td>
<td>Kane‘ohe Bay, Hawai‘i</td>
<td>ES114&lt;sup&gt;a&lt;/sup&gt; and ES12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Nonvisible</td>
</tr>
<tr>
<td>Euprymna stenodactyla</td>
<td>Solomon Islands</td>
<td>NA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Dim</td>
</tr>
<tr>
<td>Euprymna tannarctica</td>
<td>Crib Point, Australia</td>
<td>ET101&lt;sup&gt;c&lt;/sup&gt; and ET104&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Dim</td>
</tr>
<tr>
<td>Heteroteuthis dispar</td>
<td>Atlantic Ocean</td>
<td>NA</td>
<td>Bright</td>
</tr>
<tr>
<td>Loloius noctiluca</td>
<td>Crib Point, Australia</td>
<td>LN101&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Bright</td>
</tr>
<tr>
<td>Sepiotta oweniana</td>
<td>Banyuls-sur-mer, France</td>
<td>No organ</td>
<td>Moderately bright</td>
</tr>
<tr>
<td>Sepiola affinis</td>
<td>Banyuls-sur-mer, France</td>
<td>SA1&lt;sup&gt;c&lt;/sup&gt; and SA18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Moderately bright</td>
</tr>
<tr>
<td>Sepiola atlantica</td>
<td>Vigo, Spain</td>
<td>NA</td>
<td>Moderately bright</td>
</tr>
<tr>
<td>Sepiola aurantica</td>
<td>Adriatic Sea</td>
<td>SI&lt;sup&gt;c&lt;/sup&gt; and SI&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Moderately bright</td>
</tr>
<tr>
<td>Sepiola ligulata</td>
<td>Banyuls-sur-mer, France</td>
<td>NA</td>
<td>Moderately bright</td>
</tr>
<tr>
<td>Sepiola robusta</td>
<td>Banyuls-sur-mer, France</td>
<td>NA</td>
<td>Moderately bright</td>
</tr>
<tr>
<td>Sepiola rondeleti</td>
<td>Adriatic Sea</td>
<td>NA</td>
<td>No organ</td>
</tr>
<tr>
<td>Rossia macrosoma</td>
<td>Atlantic Ocean</td>
<td>NA</td>
<td>No organ</td>
</tr>
<tr>
<td>Rossia pacifica</td>
<td>San Diego, Calif.</td>
<td>NA</td>
<td>No organ</td>
</tr>
</tbody>
</table>

* V. fischeri.
* NA, symbiont isolates not available.
* P. leognathi.

RESULTS

Molecular phylogenies. The ITS and COI sequences of squid specimens were compared for within-species variation and between-species divergence. No differences were found in the >1,400-bp ITS locus between separate clones derived from an individual animal, and less than 0.1% sequence variation occurred between any two or three individuals of the same squid species at the ITS locus. Sequence divergence rates ranged from 2% (between species) to 12% (between species in the same genus and outgroups). Similarly, there was no variation observed between individuals of the same species at the COI locus, and the sequence divergence rates ranged from 3.5% to 22%.

The branching patterns of phylogenetic trees derived from sequences of each of these two loci, by either the parsimony or maximum-likelihood method of analysis, were similar. However, while in all cases Euprymna species clustered together, as did species of Sepiola (Fig. 1), the ITS and COI data sets revealed some minor differences in the species relationships within these clades. Specifically, the relative position of Euprymna morsyi varied slightly between the trees. In addition,
whereas the ITS data resolved the relationship between the species *Sepiola affinis*, *Sepiola intermedia*, and *Sepiola robusta*, the COI data did not. In both trees, species of the Pacific genera, *Euprymna*, which bear light organs, and *Rossia*, which do not, clustered more closely together than *Euprymna* and *Sepiola*, which both bear light organs. Similarly, species of the Mediterranean genera, *Sepiola*, which bear light organs, and *Sepietta*, which do not, clustered more closely together. The ITS locus of *Heteroteuthis dispar*, a mesopelagic squid from the Atlantic, could not be completely recovered for sequencing. However, the analyses of COI sequences of this species supported it as an outgroup to all species examined other than *Lolilolus noctiluca*, a loliginid squid with a bacterial light organ, that formed the family-level outgroup for both the ITS and COI loci.

The 700-bp gapA sequences obtained from either of the two bacterial isolates that were obtained from the same squid species (Table 1) were identical. Bacterial symbionts isolated from squid species in either the *Euprymna* clade (strains EM17, ES114, and ET101) or the *Sepiola* clade (strains SA1, SI6, and SR5) showed a 94 to 98% gapA sequence identity within each clade, but exhibited only an 81 to 88% sequence identity between the two clades (Table 2).

Phylogenetic analysis of the gapA locus of the symbiotic bacteria revealed a branching pattern that aligned exactly with that of the host ITS tree (Fig. 1A), whereas the symbiont tree aligned similarly, but not exactly, with the host COI tree (Fig. 1B).

**Competitive dominance.** When presented individually, all bacterial strains isolated from *Euprymna* species were capable of initiating and maintaining a typical level of symbiotic colonization of juvenile *E. scolopes*; i.e., at 24 and 48 h postinoculation, there were between $3 \times 10^5$ and $10^6$ CFU per light organ (data not shown and reference 28). In contrast, SA1, the *Sepiola* light organ symbiont strain, while able to colonize *E. scolopes* juveniles, was less effective, reaching a population level of no more than $3 \times 10^5$ CFU per light organ. Strain LN101, the light organ symbiont of *L. noctiluca*, belongs to the distinct luminous species *P. leiognathi* (Table 1) and has no ability to colonize the *E. scolopes* light organ.

In all of the competition experiments using symbionts from sepiolid squids, it was observed that 12 h following inoculation, the two strains were present in the light organ at approximately a 1:1 ratio, indicating that these strains were equally competent in initiating a symbiotic colonization (data not shown). However, after 48 h, the native strain, ES114, had achieved a greater than 20-fold advantage over any of the nonnative strains tested (Table 3). When strains from the other two *Euprymna* species were presented to juveniles of *E. scolopes*, the strain from *E. morsei* (EM17), exhibited a fourfold competitive dominance over the *Euprymna tasmanica* strain (ET101); however, the *E. tasmanica* strain outcompeted the symbiont from *S. affinis* (SA1). Thus, the hierarchy among symbiont strains isolated from sepiolid species mirrors the relatedness between the squid-symbiont pairs as derived from the molecular phylogenetic analyses (Fig. 1).

**DISCUSSION**

In this paper, we present (i) the derived molecular phylogenies of an array of sepiolid squids and their symbiotic bacterial isolates. Each tree was inferred by comparison of the unambiguously aligned positions of either the ITS, the COI, or the gapA sequence. Identical branching patterns were obtained for each locus when analyzed by either the parsimony (30) or maximum-likelihood (24) method. The values given at each node are the percentages of 100 bootstrap resamplings and subsequent heuristic searches that support the relationships between the clades.

![Phylogenetic trees of squid host species and their symbiotic bacterial isolates. Each tree was inferred by comparison of the unambiguously aligned positions of either the ITS, the COI, or the gapA sequence. Identical branching patterns were obtained for each locus when analyzed by either the parsimony (30) or maximum-likelihood (24) method. The values given at each node are the percentages of 100 bootstrap resamplings and subsequent heuristic searches that support the relationships between the clades.](image)

**TABLE 2.** Percent gapA sequence identity between different strains of bacterial symbionts

<table>
<thead>
<tr>
<th>Symbiont strain</th>
<th>% Identity to symbiont strain (squid host)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SR5 (S. robusta)</td>
</tr>
<tr>
<td>SR5</td>
<td>100</td>
</tr>
<tr>
<td>SI6</td>
<td>100</td>
</tr>
<tr>
<td>SA1</td>
<td>100</td>
</tr>
<tr>
<td>ES114</td>
<td>100</td>
</tr>
<tr>
<td>EM17</td>
<td>100</td>
</tr>
<tr>
<td>ET101</td>
<td>100</td>
</tr>
<tr>
<td>LN101</td>
<td>100</td>
</tr>
</tbody>
</table>
TABLE 3. Infection of juvenile E. scolopes with mixed inocula of squid light organ symbionts

<table>
<thead>
<tr>
<th>Strains used in competition</th>
<th>No. of animals tested</th>
<th>Mean proportion found in light organs at 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES114 and EM17</td>
<td>37</td>
<td>98:2</td>
</tr>
<tr>
<td>ES114 and ET101</td>
<td>35</td>
<td>95:5</td>
</tr>
<tr>
<td>EM17 and ET101</td>
<td>17</td>
<td>75:25</td>
</tr>
<tr>
<td>ET101 and SA1</td>
<td>30</td>
<td>90:10</td>
</tr>
<tr>
<td>ES114 and LN101</td>
<td>21</td>
<td>100:0</td>
</tr>
</tbody>
</table>

a 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.

b Mean averages of individual ratios of bacterial strains were arc sine transformed (31) to determine the significance of each treatment. All mean values were significantly different to within P ≤ 0.05.

demonstration that a hierarchy exists in the ability of bacterial symbionts from different species of squid to colonize the Hawaiian squid, E. scolopes.

The systematic relationships among the Sepiolidae have not been adequately revealed by morphological data alone (2), and the fossil record of this group is limited. Thus, molecular data present a particularly valuable approach to resolving the relationships within this family. The phylogenies derived from ITS and COI sequences separated the Sepiola species and the Euprymna species into monophyletic genera (Fig. 1). In addition, the arrangement of squid species on these trees suggests either that luminescent organs arose independently in the evolution of these two genera or that they were lost within members of the genera Sepiella and Rossia.

Although the ITS and COI data resolved the Sepiola species and Euprymna species into two independent groupings, some variability existed between the branching patterns at the species level. Specifically, in the phylogeny derived by using the COI data, E. morsei was more closely related to E. tasmanica and E. sternodactyla than it was to E. scolopes, whereas the ITS data suggested that E. morsei is the sister taxon to E. scolopes (Fig. 1). In addition, the COI tree did not resolve the relationship of the Sepiola species; however, although the COI locus was less useful in certain aspects of these analyses, consideration of the ITS and the COI loci together provided a stronger basis of support for possible coordinate processes occurring in the evolution of these symbioses. The value of data derived from nuclear (ITS), relative to mitochondrial (COI), gene sequences in revealing phylogenies is a controversial issue (14, 20, 21). Thus, analyses based on these two separate gene trees still leave the species tree unresolved. Even with additional molecular phylogenetic data and more knowledge of the life histories of the animals, this issue may not be resolved. However, other results in this paper may be viewed as providing additional support for the ITS tree, i.e., the gapA phylogeny for the bacterial symbionts (Fig. 1B) and the competitive hierarchy from the colonization experiments (Table 3).

The phylogenies derived from the analyses of the squid ITS and the symbiont gapA loci support congruent evolution of host-symbiont pairs. Similar studies with other symbiotic relationships using molecular and/or morphological data have provided evidence that many associations (e.g., chemosymbiotic symbioses, ant-fungal mutualisms, and aphid-Buchnera relationships) are phylogenetically congruent (1, 4, 5, 13). While molecular phylogenetic analysis has been a powerful method to approach these questions, under some circumstances, it has not been able to resolve patterns of parallel cladogenesis (11). Such problems with phylogenetic analyses would benefit from independent measures of congruent evolution.

The ability to manipulate experimentally the sepiolid-bacterial associations offers a series of methods that provide a powerful complementation to phylogenetic analyses. In the present study, we tested whether parallel cladogenesis was reflected in the dynamics of the initiation of this symbiosis. The hierarchy that was observed among the symbionts of the seven squid species tested directly reflected the relative ITS-gapA phylogenies derived for the host animals and their specific symbionts and provided additional support for the evolution of strain specificity and partner fidelity in squid light organ associations. The hierarchical patterns that we observed in the colonization experiments may also provide insight into the processes that occur during divergence of coevolving species. The data showed two patterns: (i) Euprymna symbionts were capable of infecting E. scolopes juveniles fully, but demonstrated a competitive dominance hierarchy; and (ii) Sepiola symbionts were less competitive than the Euprymna strains. These data suggest either that the Euprymna and the Sepiola associations evolved independently or that their divergence included a two-step process, i.e., a change in symbiotic characters that influenced competitiveness, followed by a loss of traits that allowed full colonization.

Because these symbioses can be experimentally initiated, study of the sepiolid associations promises to advance our understanding of host-symbiont evolution. Future studies will pursue two directions: (i) to continue to use the variety of sepiolid species and their symbionts to study parallel evolutionary relationships and (ii) to study the mechanisms that drive the molecular basis for specificity and speciation. For example, examination of the several sympatric species of Sepiola in the Mediterranean (18) will reveal whether each of these squid species is co-evolving with a specific lineage of symbionts, or whether instead they all share a common pool of symbionts. In addition, the discovery that symbionts from divergent Euprymna species express different degrees of colonization dominance provides a model for identifying the biochemistry underlying the evolution of specificity and recognition in a host-bacterial interaction. Finally, as has been the case in the plant root nodule symbioses, molecular genetic manipulation of bacterial light organ symbionts has begun to reveal the genes involved in symbiotic competency (26, 32) and should lead ultimately to an understanding of the biochemical mechanisms underlying species specificity in this cooperative partnership.

ACKNOWLEDGMENTS

We thank P. Baumann, N. Davies, G. Roderick, and members of the M.M.N. and E.G.R. laboratories for their comments and suggestions. Squid specimens were caught or donated by S. von Boletzky, A. Guerra, F. G. Hochberg, M. Norman, T. Okutani, R. Villanueva, and R. Young. The ITS external primers were obtained from L. Goff. Automated sequencing was performed with the assistance of G. Bernardi. PAUP analysis was performed under the auspices of D. Eernisse.

This research was supported by National Science Foundation grants OCE-9321645 (Marine Biotechnology Fellowship) to M.K.N. and IBN-96-01155 (to M.M.N. and E.G.R.), as well as by Office of Naval Research awards N00014-93-1-1357 and N00014-93-1-0846 to M.M.N. and E.G.R., respectively, and NIH R01 RR10926 (to E.G.R. and M.M.N.).

REFERENCES
