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Absence of Cospeciation in Deep-Sea Vestimentiferan Tube Worms and Their Bacterial Endosymbionts

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Abstract

Molecular evolutionary relationships among tube worm hosts and their sulfur-oxidizing chemoautotrophic bacterial endosymbionts were deduced from sequences of the mitochondrial cytochrome oxidase I gene and the small subunit rDNA gene, respectively. Evolutionary relationships among host taxa reflect traditional morphological taxonomic species boundaries. Cold seep tube worms were more closely related to each other than to tube worms from hydrothermal vents. There is a single rDNA-type of hydrothermal vent symbiont that occurs in *Oasisia alvinae*, *Ridgeia piscesae*, *Riftia pachyptila*, and *Tevnia jerichonana*. This type is distinct from the lineage of symbionts found within seep tube worms. Within the diversity of symbionts found in tube worms from cold seeps, there were three groups, which reflect, with a single exception, the geographic locations. One group of symbionts was exclusive to *Lamellibrachia* sp. nov. 2 and an "escarpid-like" tube worm of the shallow Gulf of Mexico (500–700 m), while the second was found in *Lamellibrachia* sp. nov. 1 and *Escarpia laminata* from Alaminos Canyon in the Gulf of Mexico (2,020 m). The third group of cold-seep symbionts was found in *Escarpia laminata* from the Florida Escarpment (3,700 m) and *Lamellibrachia barhami* from Middle Valley in the Northeast Pacific (2,420 m). The lack of congruence between

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phylogenies of host tube worms and their symbionts supports the hypothesis that the endosymbionts of tube worms are acquired de novo each generation from environmental sources.

Keywords: Vestimentiferan tube worms, endosymbiosis, cospeciation, de novo acquisition, molecular phylogeny

1. Introduction

With the discovery of thriving communities of metazoans living in the deep sea in association with hydrothermal vents and cold seep environments, scientists have sought to understand this ecosystem that is based on chemosynthesis. Of the diverse organisms found at these sites, the vestimentiferan tube worms with their chemoautotrophic sulfur-oxidizing bacterial symbionts are one of the more unusual.

In many hydrothermal vent and cold seep communities, tube worms are ecosystem-structuring organisms, and as a result of symbiosis with chemoautotrophic bacteria, they are one of the de-facto primary producers. For the adult host tube worm, the symbiotic relationship is obligate. An adult tube worm has no mouth, gut or anus, and all available evidence indicates that tube worms obtain most, if not all of their nutritional needs from the symbionts (Childress and Fisher, 1992; Nelson and Fisher, 1995). The tube worms have evolved numerous anatomical, ultrastructural, physiological, and biochemical adaptations to their symbionts (Felbeck and Childress, 1988). A conspicuous feature of their anatomy is the trophosome, a large mass of tissue that houses the intracellular symbionts, which occur at densities of about 10^{11} bacteria per gram. These bacteria can account for 15% or more of the wet weight of the trophosome (Powell and Somero, 1986). Within the trophosome, specialized host cells, bacteriocytes, are packed with symbionts but are almost devoid of the usual eukaryotic cellular machinery (Bosch and Grasse, 1984a; b; Hand, 1987). Several investigators have noted a dramatic gradient of increasing size of bacteria across a transect from the host cells in the center of a lobule of trophosome to those near the surface. Dividing bacteria are relatively rare throughout the tissue (Bosch and Grasse, 1984a; b; Gardiner and Jones, 1993). Regardless of this size variation of the bacteria, only a single species of bacterium is present in an individual tube worm (Distel et al., 1988). An important adaptation of the tube worm to the symbiosis is the presence of hemoglobins that can bind and transport sulfide and oxygen simultaneously, thereby providing an efficient delivery system for both host and bacterial metabolic needs (Arp et al., 1987; Fisher et al., 1988). While open to interpretation, these observations argue that the tube worm and the bacteria

that it houses are well adapted to each other and to chemoautotrophic life.

Because of the obligate nature of the symbiosis and the degree of integration between the two partners, one might assume that along with other adaptations, a method of direct transmission of the symbionts to the next generation of tube worms would have also evolved. Direct parent-to-offspring transmission of bacterial symbionts through the eggs occurs in clams that inhabit hydrothermal vents and cold seeps (Cary and Giovannoni, 1993; Peek et al., 1998). On the other hand, a shallow water lucinid bivalve re-acquires it's sulfur-oxidizing gill symbiont from the environment each generation (Gros et al., 1996). A growing body of negative and indirect evidence supports the hypothesis that the tube worm symbionts are not vertically transmitted between generations. Ultrastructural studies of sperm and eggs have failed to locate structures resembling symbiotic bacteria (Cavanaugh et al., 1981; Cary et al., 1989), and neither polymerase chain reaction amplification of the DNA from eggs nor *in-situ* hybridization with symbiont-specific oligonucleotides have yielded evidence of symbionts associated with gonadal tissue or freshly released eggs or sperm (Cary et al., 1993). Two research groups have independently identified an early vestimentiferan juvenile stage with presumptive mouth, gut, and anus, and have suggested that the symbionts are acquired from the environment at this stage of development (Jones and Gardiner, 1988; 1989; Southward, 1988). Neither group saw intracellular endosymbionts in these early juvenile stages, but both groups observed unidentified bacteria present in the gut epithelium (Gardiner and Jones, 1993).

In the absence of direct evidence for the vertical transmission of tube worm symbionts between generations, one can turn to historical evidence of past associations between the two members of a symbiosis. One such method is to compare the evolutionary histories of the hosts and symbionts derived from molecular data. Concordant host and symbiont phylogenies are expected if vertical transmission and cospeciation occurs. Previous molecular evolutionary studies of tube worms and their endosymbionts have focussed primarily on the hydrothermal vent species, *Riftia pachyptila, Tevnia jerichonana, Oasisia alvinae,* and *Ridgeia piscesae* (Edwards and Nelson, 1991; Williams et al., 1993; Feldman et al., 1997; Laue and Nelson, 1997). RFLP analyses with three symbiont-specific gene probes concluded that the widely divergent hosts, *Riftia, Ridgeia,* and *Tevnia,* harbor the same species of symbiont (Laue and Nelson, 1997). Likewise, SSU rDNA sequences from the symbionts found in different species of hydrothermal vent tube worms were virtually identical, differing at one or a few nucleotide sites (Feldman et al., 1997). Included in the SSU rDNA study were three representatives of cold-seep symbionts and a symbiont from a tube worm collected off of a whale carcass; the three cold-seep symbionts were nearly identical in sequence and a variant of this symbiont was found in the vestimentiferan from the whale carcass (Feldman et al., 1997).

Cold seep vestimentiferans have a broad geographic distribution, and in the Gulf of Mexico are found at sites that vary in depth from approximately 500 m to over 2,000 m. This study examines the molecular evolutionary relationships of the bacterial symbionts of vent and seep vestimentiferans in conjunction with those of their hosts, and includes a broad representation among known seep tube worm populations. It was expected that the molecular phylogeny of symbionts from cold-seep tube worms would not be concordant with the phylogeny of their hosts, and, like the hydrothermal-vent symbionts, would reflect geographic occurrence.

2. Materials and Methods

Specimens

Vestimentiferan tube worms were collected from the following deep-sea hydrothermal vent and cold seep sites (Fig. 1). (1) Middle Valley, Northeast Pacific (NEP), 2420 m, *Lamellibrachia barhami*, n = 1; *Ridgeia piscesae*, n = 1. (2) Gorda Ridge, NEP, 2720 m, *Ridgeia piscesae*, n = 1. (3) 9°N, East Pacific Rise (EPR), 2500 m, *Oasisia alvinae*, n = 1; *Riftia pachyptila*, n = 2; *Tevnia jerichonana*, n = 2. (4) Alaminos Canyon, Gulf of Mexico (GoM), 2020 m, *Escarpia laminata*, n = 2; *Lamellibrachia* sp. nov. 1., n = 1. (5) Bush Hill, GoM, 545 m, "escarpid-like", n = 1; *Lamellibrachia* sp. nov. 2., n = 1; and Green Canyon 234, GoM, 540 m, "escarpid-like", n = 1; *Lamellibrachia* sp. nov. 2., n = 1 (Bush Hill and Green Canyon 234 are separated by approximately 10 km.). (6) Florida Escarpment, GoM, 3700 m, *Escarpia laminata*, n = 2. The tube worms were collected by manned submersibles DRSV ALVIN and the Johnson Sea Link II.

Nucleic acid manipulations

Total nucleic acids were extracted from the symbiont-containing trophosome tissue by previously described methods (Nelson and Selander, 1994). Most of the bacterial small subunit ribosomal gene (SSU rDNA) was amplified by PCR with primers (fD1 and rP1) that are highly conserved among eubacteria (Weisburg et al., 1991). The sequence of the 3' end of the SSU rDNA was obtained by amplification with primers (1406f and 242r) located in the 3' end of the SSU rDNA and the 5' end of the large subunit rDNA, respectively (Lane, 1991).

Nearly complete SSU rDNA sequences (exclusive of the 5' primer site) were obtained by *Taq* cycle-sequencing, and the resulting products were analyzed on an ABI 373 automated sequencer. Internal primers for *Taq* cycle sequencing were

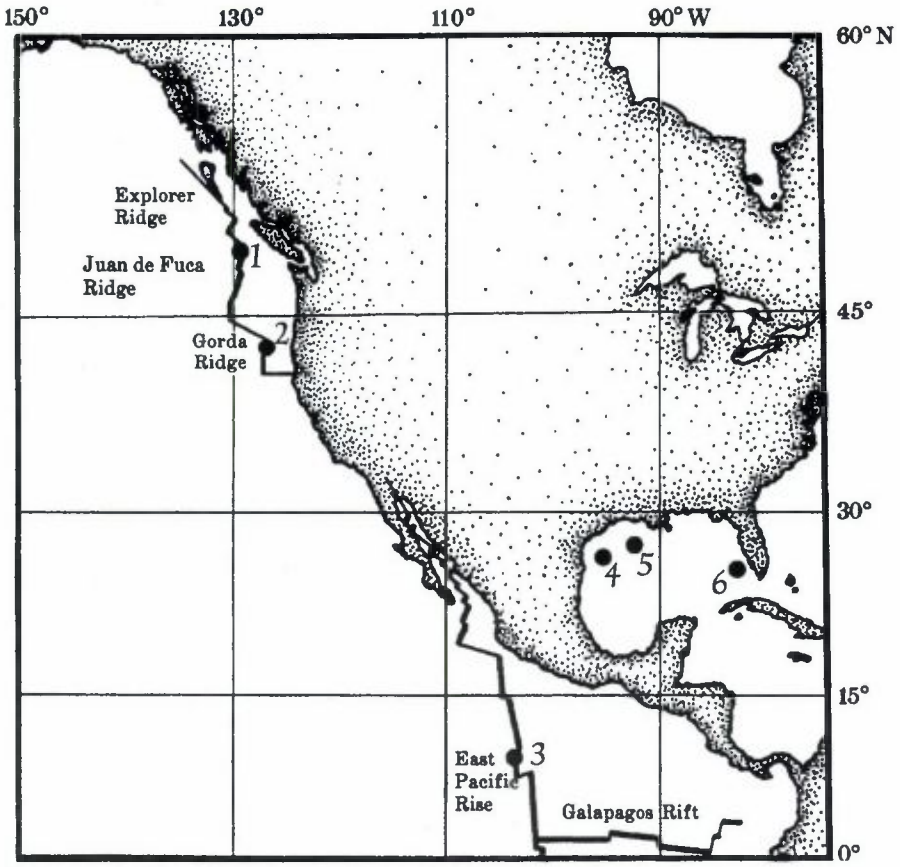

Figure 1. Geographic locations of the vestimentiferan tube worms. Localities 1, 2 and 3 are hydrothermal vent habitats and localities 4, 5, and 6 are cold seep habitats.

selected from a list of conserved eubacterial primers (Lane, 1991). For each symbiont, 1515–1516 base pairs (bp) of the SSU rDNA gene were completely sequenced with multiple overlapping sequences on both strands, with the exception of the approximately 30 bp near the 5' most primer, which was single-stranded. Individual sequencing runs were assembled and edited by the use of SEQMAN and SEQMANED (DNASTAR, Inc.). Final symbiont sequences were aligned and compared with the Eyeball Sequence Editor (Cabot and Beckenbach, 1989).

To establish host relationships for five of our tube worm taxa, we sequenced a portion of the mitochondrial cytochrome oxidase I gene (COI) and compared it with published data for other vestimentiferans (Black et al., 1997; Kojima et

al., 1997). For these studies, total nucleic acids were extracted from vestimentum tissue. The COI gene was amplified by PCR with primers designed from conserved sequences of other invertebrates {COIf: TC(CA)ACTAATCA(CT)AA(GA)GA(CT)ATTGG(ATGC)AC and COIr: CC(ATG)CTTAG(TA)CCTA(GA)(GA)AA(GA)TGTTG(ATCG)GG].

These primers amplified an approximately 1250-bp segment, and from this segment 350 bp were sequenced as described above. Sequences were obtained from the following individuals and localities (Fig. 1): (1) *Lamellibrachia barhami*, Middle Valley, site 1; (2) *Escarpia laminata*, Alaminos Canyon, site 4; (3) "escarpid-like", Bush Hill, site 5; (4) *Lamellibrachia* sp. nov. 2., Bush Hill, site 5; and (5) *Escarpia laminata*, Florida Escarpment, site 6.

Phylogenetic analyses

Molecular evolutionary relationships among sequences were examined by the neighbor-joining method of tree construction (Saitou and Nei, 1987), based on pairwise genetic distances estimated from the proportion of differences and corrected for multiple substitutions by the Jukes and Cantor formula (Jukes and Cantor, 1969). The significance of the branching order was evaluated by bootstrap analysis of 1000 computer-generated trees. All analyses were performed with MEGA (Kumar et al., 1993).

To determine the branching pattern of the deep phylogenetic relationships, we utilized four-cluster analysis (Rzhetsky et al., 1995). This method may be used to infer the relationships among groups of species, of which each group may contain many species.

3. Results and Discussion

Evolutionary relationships between seep and vent taxa

Anatomically and physiologically the vent and seep vestimentiferans are similar in gross detail (Childress and Fisher, 1992), and their habitats are similar in that both sulfide and oxygen are present. However, the hydrothermal vent environment is much more variable and ephemeral, and levels of sulfide and temperature are much higher than at a typical seep locality (Scott and Fisher, 1995). This is reflected in very different growth rates, longevity, and other ecological, anatomical, and physiological adaptations to their environments (Lutz et al., 1994; Fisher, 1996; Fisher et al., 1997). These fundamental differences in the animals and their habitats are reflected in the molecular phylogeny of the vestimentiferans, which indicates

that the vent species represent a separate radiation from the seep species (Fig. 2 and associated references).

Four-cluster analysis (Rzhetsky et al., 1995) of the order of branching of vent symbionts, seep symbionts and two groups of shallow water bivalve symbionts yields strong support for the distinction of the tube worm symbionts to the exclusion of other sulfur-oxidizing chemoautotrophic symbionts (Fig. 3). This analysis also supports a single origin for all tube worm symbionts. A previous study utilizing SSU rDNA sequences from three symbionts from cold seep tube worms did not show strong support for the single origin hypothesis of tube worm symbionts, and suggested a group that contained the symbionts from the seep vestimentiferans and shallow water marine bivalves, albeit with low bootstrap support (Feldman et al., 1997).

In agreement with Feldman et al. (1997), we find that relatively large genetic distances (average of 65.1 sites or 4.3% divergence, Fig. 3), separate the symbionts of the seep and vent vestimentiferans. This level of genetic divergence of SSU rDNA sequences reflects an old divergence of the bacteria, and has been suggested to be sufficient for classification of bacterial strains as different species (Stackebrandt and Goebel, 1994). If one assumes an average rate of divergence of 1% per 50 million years for bacterial SSU rDNA sequences (Ochman and Wilson, 1987; Moran, 1996), then the sulfur-oxidizing chemoautotrophic bacteria that are now endosymbionts of vestimentiferans from vents and seeps diverged 108-215 Mya.

Evolutionary relationships among vent taxa

The four species of hydrothermal vent vestimentiferans (*Oasisia alvinae, Ridgeia piscesae, Riftia pachyptila,* and *Tevnia jerichonona*) found in the eastern Pacific are readily distinguished from each other anatomically and with the use of molecular markers (Fig. 2; average divergence for the COI gene is 14%; Jones, 1985; Black et al., 1997). In contrast, the symbionts of all hydrothermal vent vestimentiferans examined to date are very closely related (Fig. 3; Edwards and Nelson, 1991; Feldman et al., 1997; Laue and Nelson, 1997). The average SSU rDNA divergence among symbionts of the four vent species is 2.1 sites (0.14%), and differences in the symbiont population within a species can exceed those between species (Fig. 3). Among the three EPR species, the average divergence is only 0.73 sites (0.05%), and thus, we conclude that *Oasisia alvinae, Riftia pachyptila,* and *Tevnia jerichonona* from 9°N on the EPR all harbor symbionts from the same species and likely the same population.

The symbiont from *Ridgeia piscesae* from the NEP spreading centers is slightly divergent from the vent symbionts of the EPR species (Fig. 3; 0.2%).

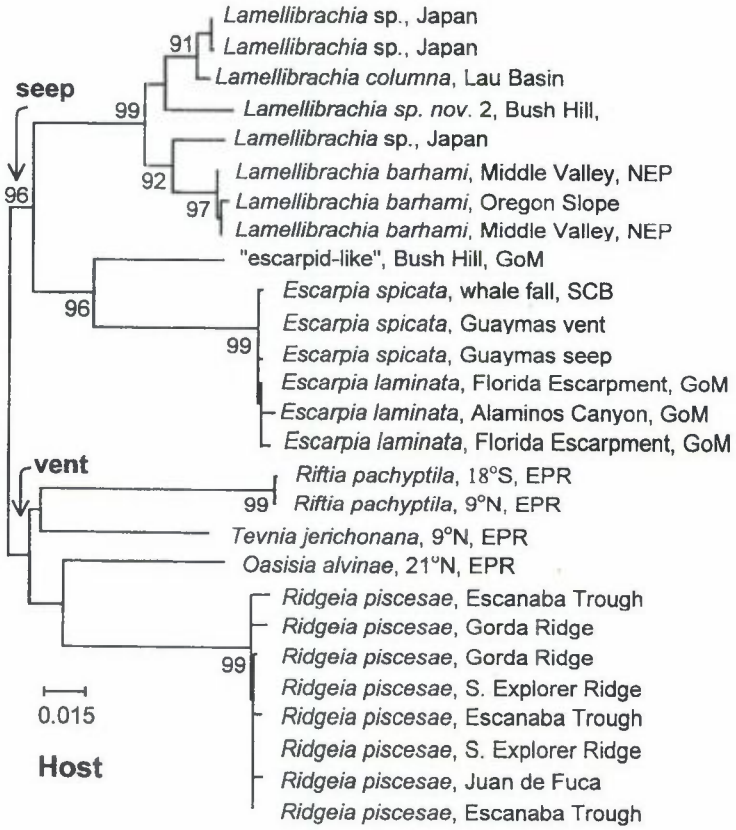

Figure 2. Neighbor-joining tree depicting the molecular evolutionary relationships among tube worms based on sequences of the mitochondrial cytochrome oxidase I gene (this study; Black et al., 1997; Kojima et al., 1997). Numbers at the nodes indicate the proportion of occurrences in 1000 bootstrap replications. Bootstrap percentages below 90% are not shown.

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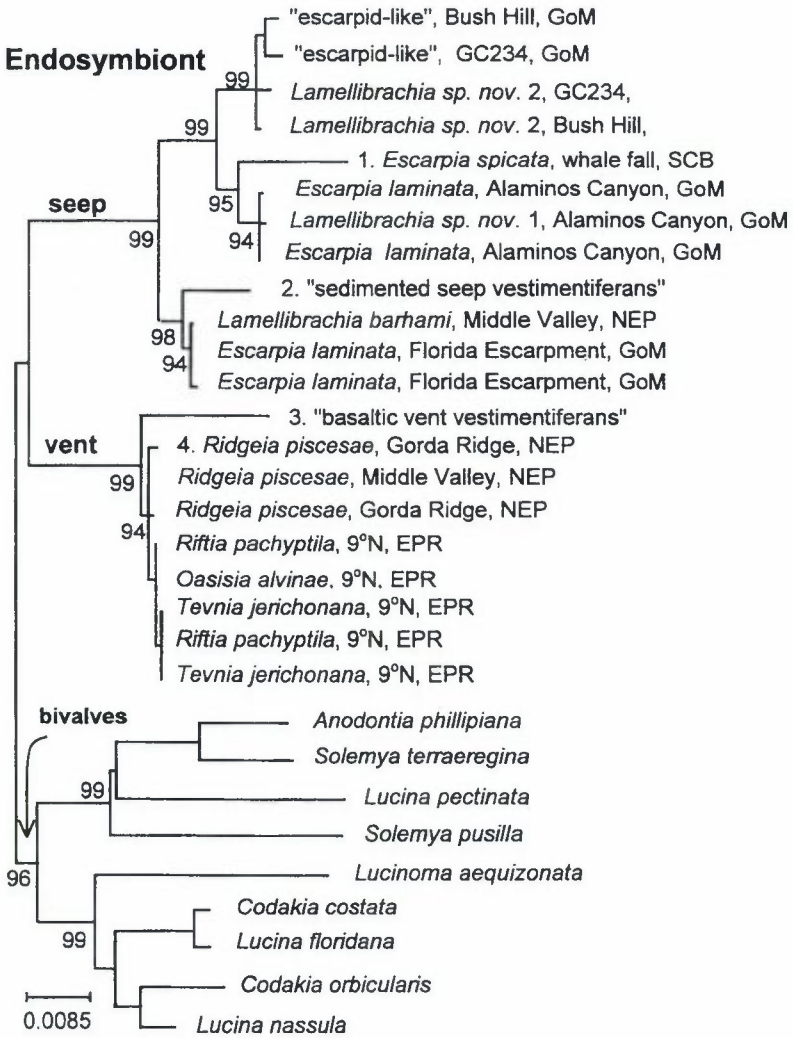
Figure 3. Neighbor-joining tree depicting the molecular evolutionary relationships among symbionts based on sequences of the SSU rDNA gene (this study; Feldman et al., 1997). 1. SSU rDNA sequence from the symbiont of *Escarpia spicata* from a rotting whale carcass (Feldman et al., 1997; Smith et al., 1989). 2. "Sedimented seep vestimentiferans" includes sequences of symbionts from Escarpia laminata from the Florida Escarpment, *Lamellibrachia columna* from the Lau Basin, and a reported sequence from *Lamellibrachia* sp. nov. 2 from Green Canyon in the GoM (This symbiont should be similar in sequence to one of our four symbionts from shallow GoM tube worms. We note that this species is variously referred to in Feldman et al. (1997) as either *Escarpia* or *Lamellibrachia*, and we do not interpret this sequence as indicative of shallow GoM symbionts). 3. "Basaltic



vent vestimentiferans" includes sequences of symbionts from the following tube worm species: *Oasisia alvinae* from 21°N on the EPR; *Riftia pachyptilia* from 18°S on the EPR, Galapagos Rift, 9°N on the EPR and Guaymas Basin; *Tevnia jerichonana* from 9°N on the EPR (Feldman et al., 1997). 4. SSU rDNA sequence from the symbiont of *Ridgeia piscesae* from Gorda Ridge in the NEP (Feldman et al., 1997). The sequences of Feldman et al. (1997) as reported in GenBank appear basal to our sequences and have long branches, presumably due, in part, to sequencing problems at the 3' ends of their SSU rDNA sequences and in regions of insufficient coverage on both strands. Numbers at the nodes indicate the proportion of occurrences in 1000 bootstrap replications. Bootstrap percentages below 90% are not shown.

This is likely a reflection of the split of the Pacific-Farallon Ridge during the mid-Tertiary, approximately 30 Mya, and the resultant discontinuity that occurs at the Mendocino Transform Fault and the San Andreas Fault. Mid-ocean ridges and faults are suggested to be the primary avenues of larval dispersal between vent sites (Kim et al., 1994; Tunnicliffe and Fowler, 1996), and after the split of the Pacific-Farallon Ridge this avenue is no longer available for dispersal of vent propagules. Restriction-fragment-length polymorphism data from three genes, including the SSU rDNA, also indicate that the symbiont of *Ridgea* has diverged from that of *Riftia* and *Tevnia* (Laue and Nelson, 1997). We conclude that the symbionts of *Ridgeia piscesae* differ at the population/strain level from the symbionts of the three EPR vestimentiferan species.

Evolutionary relationships among seep taxa

The seep vestimentiferans fall into two easily distinguishable groups. One group consists entirely of species in the genus *Lamellibrachia*. The other includes all of the described species in the genus *Escarpia* and an undescribed "escarpid-like" species, which is distantly related to the named species of *Escarpia* (Fig. 2; S. Gardiner pers. com.). *Lamellibrachia* and *Escarpia* (or the "escarpid-like" species) co-occur at many seep sites in the GoM as well as at several locations around Japan (Kojima et al., 1997).

In contrast to the evolutionary relationships among the hosts, three groups of symbionts from seep vestimentiferans are distinguished that do not correlate with host taxonomic classification (Fig. 3). The first group includes the symbionts from *Lamellibrachia* sp. nov. 2. and the "escarpid-like" tube worms from shallow seep sites in the GoM (Fig. 1, GC234 and Bush Hill, site 5). In fact, sequence differences between symbionts from two individuals of the same host species are the same or greater than the differences between the sequences of symbionts of the two different host species sampled from the same site (Fig. 3). Average divergence (5.8 sites, 0.38%) among the symbionts in four host individuals from the shallow seep sites in the GoM is approximately three times the divergence (2.1 sites, 0.14%) found among all vent symbionts analyzed.

A second group of cold seep symbionts is found in vestimentiferans collected from Alaminos Canyon, a seep site that occurs in the deeper waters of the GoM (Fig. 1, site 4; Brooks et al., 1990). Similar to the shallow sites, two species of tube worms, *Lamellibrachia* sp. nov. 1. and *Escarpia laminata*, occur at the site, and harbor symbionts from the same population. Only a single nucleotide difference was found among the three symbiont sequences analyzed from Alaminos Canyon tube worms. These sequences differed from those of the

shallow GoM species by an average of 19 nucleotide differences (1.3%).

Escarpia laminata was collected from 3,700 meters on the Florida Escarpment in the GoM (Fig. 1, site 6). The SSU rDNA sequences of the symbionts from the two individuals analyzed from this site were quite similar (99.9%) but differed by 1.8% from symbionts of the other GoM sites (Fig. 3). The large divergence (27.1 nucleotide differences) between the symbionts of *Escarpia laminata* from the Florida Escarpment and *Escarpia laminata* from Alaminos Canyon is not reflected in the evolutionary relationships of the hosts, which show few differences in the COI gene (Fig. 2, 2.5 sites). Clearly, the seep symbiont phylogeny is not congruent with that of the hosts, but rather with geography.

The relationships depicted among seep symbionts in this study contrast with the observations of Feldman et al. (1997). Their sample of three seep symbiont sequences led them to conclude that a single species of endosymbiont inhabits seep tube worms. Their symbionts cluster at the base of one of our three groups, which is composed of symbionts from *Escarpia laminata* from the Florida Escarpment and *Lamellibrachia barhami* from Middle Valley in the NEP (Fig. 3).

The lack of concordance between the evolutionary relationships of the seep lineages of hosts and symbionts argues strongly against vertical transmission of symbionts from one generation to the next. Although this lack of congruence stands in contrast to other deep-sea metazoan endosymbioses that have been studied to date (Cary and Giovannoni, 1993; Distel et al., 1994; Kreuger et al., 1996; Peek et al., 1998), it is not without precedence. An environmental transmission of the sulfur-oxidizing gill symbiont of the shallow water lucind bivalve has been proposed (Gros et al., 1996; Durand et al., 1996). However, a strategy that requires the de-novo acquisition of symbionts each generation would seem risky for a host that is absolutely dependent on the symbiont for it's nutritional needs as an adult, and for a host that occupies a habitat that is as patchy and ephemeral as the hydrothermal vents. Thus, there must be some mechanism of host-symbiont recognition that assures the uptake and establishment of a symbiont population within larval or early juvenile tube worms.

An unexpected finding was the close relationship between the *Escarpia laminata* symbionts from the Florida Escarpment and *Lamellibrachia barhami* from Middle Valley in the North East Pacific. Not only are the two locations separated by land masses, but Middle Valley is a hydrothermal vent site located on a mid-ocean ridge. However, Middle Valley is a sedimented site with some areas of very low flow (Southward et al., 1996). Thus, in some ways, areas of Middle Valley may be very similar to a cold seep environment. The same species, *L. barhami*, is found at the cold seep sites (400 km distant) on the continental margins off the coast of Oregon and California (Webb, 1969; Suess et al., 1985; Southward et al., 1996). At the Middle Valley site where these

collections were made, the hydrothermal vent species, *Ridgeia piscaesae*, appeared emaciated, apparently reflecting the relatively inactive status of this site (J.J. Childress, pers. com), and harbored the expected vent symbiont and not the seep symbiont hosted by *L. barhami*. This site is only one that we know of where two taxa of tube worms co-occur but do not host the same species of symbiont. (At Guyamas Basin, *Riftia pachyptila* and *Escarpia spicata* co-occur, and we predict that the symbionts from these two species would also be very different, but this has yet to be tested.) The fact that they house very divergent symbionts argues for host/symbiont co-evolution at least at the level of the divergence into the two very different habitats.

Thus, the emerging picture of sulfur-oxidizing chemoautotrophic bacterial endosymbionts and their tube worm hosts points to habitat specificity and the acquisition of the symbiont from the surrounding environment. The mechanism by which a symbiont population is established within an individual host remains to be elucidated.

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