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Review article

Phylogeny and Biogeography of Deep Sea Vestimentiferan Tubeworms and Their Bacterial Symbionts

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Abstract

The present study combines previously published morphological descriptions and molecular-based characterizations of vestimentiferans and their symbionts with new molecular data to summarize and extend the understanding of vestimentiferan host and symbiont phylogeny and biogeography. Host cytochrome oxidase I (COI) and symbiont 16S ribosomal gene (16S) DNA sequences were used to explore evolutionary relationships among the vestimentiferans and their symbionts. Lamellibrachids of the northern Gulf of Mexico (GOM) are identified as a single species, *Lamellibrachia* cf *luymesi*, and new data and analyses are presented for *Lamellibrachia barhami*, *Paraescarpia echinospica*, and *Arcovestia ivanovi*. In general, both vestimentiferan hosts and symbionts have very large species ranges that are interrupted by depth. No evidence for cospeciation was found between vestimentiferans and their symbionts, supporting an environmental acquisition of the symbionts. Symbiont acquisition depends on host type (vent or seep), depth of site, and possibly host species. A test of evolutionary rate showed that vent

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vestimentiferans had a significantly faster COI sequence evolution than lamellibrachids, and symbionts from vent and seep vestimentiferans from deep water sites had a significantly slower rate of evolution than those from mid-depth and shallow sites.

Keywords: Vestimentiferan, symbiont, phylogenetics, biogeography, COI, 16S

1. Introduction

Vestimentiferan tube worms were first described by Webb in 1969 when a deep sea trawl recovered large worms in thick tubes from deep waters off the coast of California. These large animals bore a resemblance to a previously described group, the Pogonophora. Though first described in 1969, vestimentiferans were found only twice before 1977 (Webb, 1969; van der Land and Norrevang, 1975) in trawl and dredge collections, their habitats remaining unknown. In the late 1970s extremely high concentrations of organisms, including the 'giant tube worm' *Riftia pachyptila*, were found clustered around the hydrothermal vents of the Galapagos Rift spreading center (Lonsdale, 1977; Corliss et al., 1979). *Riftia pachyptila* were similar to the vestimentiferans originally described by Webb (1969) and van der Land (1975), but were much larger than those previously seen. In the early and mid 1980s aggregations of vestimentiferan tube worms were found at a variety of 'cold seep' sites worldwide, including the Oregon subduction zone (Suess et al., 1985), on the Florida Escarpment (Paull et al., 1984), off the coast of San Diego (Jones, 1985) and at the hydrocarbon seeps in the northern Gulf of Mexico (Kennicutt et al., 1985).

The energy source for most deep sea life is organic matter generated by photosynthesis in the upper water column, very little of which reaches the bottom of the ocean. The high biomass at hydrothermal vent and cold seep sites are a direct result of the unique chemistry of these environments. The reduced chemicals present in vent and seep fluid, specifically sulfide and methane, are an energy source for chemoautotrophic bacteria, providing a rich local food source for deep sea organisms able to live in these extreme environments (Fisher, 1996). Though chemically similar, vents and seep environments differ in the geological setting that leads to the production of reduced chemicals. At hydrothermal vents seawater interacts with hot, newly formed rock several kilometers deep in the crust. As a result of interactions with hot basalts, the superheated water exiting the seafloor is devoid of oxygen and carries high concentrations of reduced metals and chemicals like hydrogen sulfide. Because of their tectonic nature, vent habitats have extreme

temporal and spatial fluctuations of temperature (2–60°C) and chemistry, and are ephemeral, normally lasting only years or decades (Tunnicliffe et al., 1998). The reduced chemicals present at cold seeps are a result of thermogenic or biogenic degradation of organic matter. Seeps can be associated with hydrocarbon reservoirs, subduction zone accretionary prisms, landslides, slumps, and other geologic features that create compressed sediments (MacDonald et al., 1990). Unlike vent environments, seep habitats are generally near ambient temperature, are highly sedimented, and may be stable for centuries to millennia (Fisher et al., 1997).

Though the relationships of species and genera within the vestimentiferans are fairly well understood, the higher order classification of the vestimentiferans has been a continued source of debate. Alternately considered a phylum, class, or order of their own (Jones, 1985; Mañé-Garzón and Montero, 1985; Webb, 1969; van der Land and Nørrevang, 1977), new molecular data (Williams et al., 1993; Kojima et al., 1993; Winnepenninckx et al., 1995; McHugh, 1997; Halanych et al., 2001) and a reevaluation of morphological data (Rouse, 2001) support the placement of vestimentiferans in the polychaete family Siboglinidae. Siboglinidae is therefore comprised of the vestimentiferans, the pogonopharans and *Sclerolinum brattstromi*, a species previously thought to be a basal pogonophoran but recently found to be more similar to the vestimentiferans (Rouse, 2001; Halanych et al., 2001). This classification of vestimentiferans places them as a clade within the Family Siboglinidae, within the Class Polychaeta, under Phylum Annelida (Black et al., 1997; Kojima et al., 1997; McHugh, 1997, 2000; Southward, 1999; Bartolomaeus, 1999; Halanych et al., 1998, 2001; Rouse and Fauchald, 1997; Rouse, 2001).

The vestimentiferans described to date fall into seven higher order taxa (previously families) and ten genera. The seven higher order taxa will be referred to here by a contraction of their now outdated family names (e.g. Lamellibrachiidae becomes lamellibrachid). Four species from three different taxa, the ridgeids, tevnids, and riftids, are known from hydrothermal vents on the east Pacific and northeast Pacific spreading centers (Jones, 1981, 1985), and two additional monospecific taxa, the arcovestids and alaysids, are known from hydrothermal vent sites in the Lau back arc basin (Southward, 1991; Southward and Galkin, 1997). Alaysid-like vestimentiferans have also been reported from diffuse flow sites near Japan (Kojima, 2002; Kojima et al., 2002). The two groupings of cold seep vestimentiferans, the escarpids and lamellibrachids (Jones, 1985), are generally more widely distributed than the hydrothermal vent taxa, and live in sedimented habitats of near-ambient temperatures with more diffuse sulfide seepage. Escarpids, containing four described species, have been found off the coast of Japan, off the North American Pacific coast, in Guaymas Basin, in the Gulf of Mexico, and off the

coast of Barbados. Members of the lamellibrachids, with five described species, are more widespread than the escarpids, and have been collected from both sides of the Pacific Ocean (near Japan, in Lau Basin, off the North American Pacific coast) and of the Atlantic (in the Gulf of Mexico, in the Mediterranean, and off the coasts of Barbados, Uruguay, Portugal, and West Africa). Seep vestimentiferan communities have been reported at depths ranging from 82 to 3,300 m (Hashimoto et al., 1993; Paull et al., 1984).

Vestimentiferan tube worms, like their pogonophoran relatives, lack a digestive tract, and rely directly on primary production by sulfide oxidizing eubacterial symbionts (reviewed in Nelson and Fisher, 1995). A large amount of the vestimentiferan body is occupied by the trophosome, a specialized tissue containing large numbers (10^{11} bacteria/gram) of intracellular sulfide-oxidizing bacteria (gamma proteobacteria) (Powell and Somero, 1986). Researchers report the presence of a single species of bacterium within an individual (Distel et al., 1988), although one report suggests the presence of a second bacterial type, an epsilon proteobacterium, in the trophosome of a cold seep vestimentiferan (Naganuma et al., 1997a, 1997b). Highly integrated symbioses such as these involve a well-coordinated interweaving of responses between a host and symbiont pair that evolves over many generations. Vertical transmission of symbionts in the egg, such as that seen in some vent and seep bivalves (Peek et al., 1997), provides offspring with the specific strain of bacteria that was in the parent, guaranteeing the presence of a successful symbiont in the next generation. On the other hand, hosts like the lucinid bivalve *Codakia orbicularis* (Gros et al., 1996) acquire symbionts from the environment with each generation, a process known as environmental or horizontal symbiont transmission.

The highly integrated and obligate nature of the symbiosis between vestimentiferans and their endosymbionts suggests that evolution would favor a mechanism by which offspring are guaranteed the presence of the symbiont so critical for vestimentiferan life. Despite the apparent benefit of a direct transmission of vestimentiferan endosymbionts, evidence does not support this mode of symbiont transmission between generations. No bacteria have been found in either vestimentiferan sperm or eggs (Cavanaugh et al., 1981), and molecular detection by bacterial-specific PCR and in situ hybridizations with gonadal tissue and freshly released sperm and eggs have both failed (Cary et al., 1993). Additionally, a functional form of the flagellin gene, which is involved with motility in free living bacteria, has been isolated from the endosymbiont of *Riftia pachyptila*. The presence of this gene is interpreted as evidence for a motile stage within the endosymbiont's lifecycle (Millikan et al., 1999). Vertical transmission of symbionts links host and symbiont DNA between generations, resulting in phylogenies with a degree of congruent evolution, where host speciation events are reflected in the phylogenetic gene

tree of the symbionts (Cary, 1993, 1994; Distel et al., 1994; Durand et al., 1996; Funk et al., 2000). No such congruence has been seen in the molecular phylogenetic trees of symbionts and their vestimentiferan hosts (Feldman et al., 1997; Di Meo et al., 2000; Nelson and Fisher, 2000). Indeed, three different vent vestimentiferan species share one symbiont type (Laue and Nelson, 1997; Nelson and Fisher, 2000) and multiple species of seep vestimentiferan share a second (Feldman et al., 1997; Nelson and Fisher, 2000), while different symbionts are seen in the same host species collected from different geographic regions (Nelson and Fisher, 2000).

The present study combines previously published morphological descriptions and molecular-based characterizations of vestimentiferans and their symbionts with new molecular data to summarize and extend the understanding of vestimentiferan host and symbiont phylogeny and biogeography.

2. Materials and Methods

The phylogenies of vestimentiferan hosts presented here were generated based on the mitochondrial cytochrome oxidase I gene (COI), whose gene product is involved in aerobic respiration, a critical metabolic pathway. Twelve new vestimentiferan COI sequences were generated in this study, and were aligned with those vestimentiferan COI sequences previously available in GenBank, using the pogonophoran *Galathealinum brachiosum* (GenBank #AF178679) COI sequence as an outgroup. The GenBank accession numbers and collection locations for the sequences used in this study are listed in Table 1. The observed variation in vestimentiferan COI sequences generated no amino acid changes in the vestimentiferan COI protein sequence. These silent DNA substitutions should be selectively neutral; changes at these sites are expected to accumulate between two reproductively isolated species at a rate dependent on the mutation rate, the generation time, and the population size of each species (Kimura, 1983).

Symbiont phylogeny was investigated using the sequence of the small subunit ribosomal RNA gene (16S), a gene whose product is an RNA molecule involved in the machinery of protein production. Though the shape of the 16S rRNA gene product is important to ribosome function, portions of the 16S sequence are not as constrained as the COI gene. The eight new seep symbiont 16S sequences generated for the current study were aligned with data presented in Feldman (1997) and Nelson and Fisher (2000), using the 16S sequence of the sulfide oxidizing endosymbiont of the bivalve *Thyasira flexuosa* (Genbank #L01575) as an outgroup. GenBank accession numbers for all symbiont sequences used in this study are shown in Table 1. Several sequences available in GenBank (e.g. U77479 in Feldman (1997)) and four sequences from DiMeo et al. (2001) were

Table 1. GenBank Accession numbers of sequences used in vestimentiferan host and symbiont phylogenetic analyses

Species	Location	Host	Reference	Symbiont	Reference
Escarpia laminata	Alaminos Canyon	AY129128-30	This study	AY129102,8,9	Nelson & Fisher 2000; this study
Escarpia laminata	Florida Escarpment	U74063, AY129131	Black 1997	AY129106,7	Nelson & Fisher 2000; this study
Escarpia spicata	Santa Catalina Island	U84262	Feldman 1998	U77482	Feldman 1997
Seepiophila jonesi	Louisiana Slope	AF317287-90	Gardiner 2001	AY129101,4,5	Nelson & Fisher 2000; this study
Second escarpid	Louisiana Slope	AY129134	This study	AY129089	This study
Parescarpia echinospica	Manus Basin	N/A	Southward 2002	N/A	
Parescarpia echinospica	Nankai Trough	D50594	Kojima 1997	N/A	
Parescarpia echinospica	Okinawa Trough	D50595	Kojima 1997	N/A	
New escarpid	Nankai Trough	D50593	Kojima 1997	N/A	
Lamellibrachia barhami	Middle Valley	U74055	Black 1997	AY129113	Nelson & Fisher 2000
Lamellibrachia barhami	Vancouver Island Margin	AY129147	This study	AY129103	This study
Lamellibrachia barhami	Oregon Margin	U74054	Black 1997	AY129090	This study
Lamellibrachia barhami	Monterey Canyon	AY129137	This study	AY129093	This study
Lamellibrachia cf *luymesi*	Louisiana Slope	AY129124, 32,36,39	This study	AY129100,10,11	Nelson & Fisher 2000; this study
Lamellibrachia satsuma	Nankai Trough	D38030	Kojima 1997	N/A	
Lamellibrachia satsuma	Kagoshima Bay	D38030	Kojima 1997	N/A	
Lamellibrachia satsuma	Nikko Seamount	U74078	Black 1997	AF165907	DiMeo 2001
Lamellibrachia columna	Nankai Trough	D38029	Kojima 1997	N/A	
Lamellibrachia columna	Nankai Trough	D50592	Kojima 1997	N/A	
Lamellibrachia columna	Lau Basin	U74061	Black 1997	U77481	Feldman 1997
Lamellibrachia sp.	Alaminos Canyon	N/A	Nelson & Fisher 2000	AY129112	Nelson & Fisher 2000

Table 1. Continued

Species	Location	Host	Reference	Symbiont	Reference
New lamellibrachid	Kuroshima Knoll	AB055210	Kojima 2001	N/A	
New lamellibrachid	Manus Basin	AB055209	Kojima 2001	N/A	
Riftia pachyptila	EPR 9°N	U74053	Black 1997	U77478, AY129115	Feldman 1997; Nelson & Fisher 2000
Tevnia jerichonana	EPR 9°N	U74075	Black 1997	AY129117	Nelson & Fisher 2000
Oasisia alvinae	EPR 21°N	U74069	Black 1997	AY129114	Nelson & Fisher 2000
Ridgeia piscesae	Juan de Fuca	U74073	Black 1997	U77480	Feldman 1997; Nelson & Fisher 2000
Ridgeia piscesae	Middle Valley	AF022234	Black 1997	AY129119	Nelson & Fisher 2000
Arcovestia ivanovi	Lau Basin	N/A	Kojima et al. 2002	N/A	

N/A indicates no sequence available.

Table 2. Vestimentiferan collections worldwide, including site depth, habitat type (V = vent, S = seep, SH = shipwreck, W = whalefall), and vestimentiferan species present (species abbreviations are the same as in Fig. 2). Column headings E, L, and V indicate the three main vestimentiferan clusters, escarpids, lamellibrachids, and vent vestimentiferans, respectively.

Collection location	Depth (m)	Type	Latitude	Longitude	E	L	V	Reference
E Atlantic								
Eastern Mediterranean	1700–2000	S	?	?		L		Olu-LeRoy 2002
Vigo Spain	1160	SH	42°08'N	9°30'W		L		Dando 1992
Congo-Angola Margin	?	S	?	?		L		Olu-LeRoy 2002

Table 2. Continued

Collection location	Depth (m)	Type	Latitude	Longitude	E	L	V	Reference
W Atlantic								
Louisiana Slope, Viosca Knoll	550	S	29°09'N	88°02'W	Sj	L cf l		This study
Louisiana Slope	540–640	S	27°47'N	91–92°W	Sj	L cf l		This study
Louisiana Slope, western site	600	S	27°26'N	93°35'W	Sj			This study
Alaminos Canyon	2200	S	26°21'N	94°30'W	El	L		Brooks 1990; Nelson 2000
Florida Escarpment	3300	S	26°02'N	84°54'W	El			Paull 1984; Black 1997
Near Barbados	1000–2000	S	?	?	Ecf l, E	L		Olu 1997; Sibuet & Olu 1998
Near Guyana	500	S	8°10'N	57°24'W		Ll		van der Land & Norrevang, 1975
Uruguay cont. shelf	300	S	35°11'S	52°10'W		Lv		Mane-Garzon & Montero 1985
E Pacific								
Juan de Fuca, Explorer Ridge	1870	V	49°46'N	139°16'W			Rp	Tunnicliffe 1991
Juan de Fuca, Middle Valley	2400	V	48°27'N	128°42'W		Lb	Rp	Tunnicliffe 1991; Black 1997
Juan de Fuca, Endeavor	2250	V	47°57'N	129°08'W			Rp	Tunnicliffe 1991
North Gorda Ridge	2700	V	42°45'N	126°43'W			Rp	Tunnicliffe 1991
Vancouver Island Margin	1300	S	48°42'N	126°55'W		Lb		This study
Oregon Margin	1800–2200	S	44°29'N	125°13'W		Lb		Suess 1985; Kennicutt 1989
Oregon Margin	1390	S	44°36'N	125°11'W		Lb		Paull 1984
Monterey Canyon	1000	S	36°46'N	122°05'W		Lb		Barry 1996; Vrijenhoek 1994; this study
Santa Catalina Island	1240	W	33°12'N	118°30'W	Es			Feldman 1998

Table 2. Continued

Collection location	Depth (m)	Type	Latitude	Longitude	E	L	V	Reference
California	1125	S	32°20'N	117°19'W		Lb		Webb 1969
Guaymas Basin	1653	S	27°35'N	111°28'W	Es			Black 1997
Guaymas Basin	2020	V	27°00'N	111°25'W	Es		R	Tunnicliffe 1998; Black 1997
EPR 21°N	2600	V	20°51'N	109°05'W			R, O	Tunnicliffe 1998
EPR 13°N	2600	V	12°38–54'N	103°54–58'W			T, R, O	Tunnicliffe 1998
EPR 9°N	2500	V	9°50'N	104°17'W			T, R, O	Tunnicliffe 1998
Costa Rican coast	1408	S	9°02'N	84°37'W		Lb		H. Sahling, per. com.
Galapagos Rise	2480	V	0°47–48'N	86°01–14'W			R	Tunnicliffe 1998
EPR 17°S	2700	V	17–21°S	113–114°W			T, R, O	Tunnicliffe 1998; Hurtado 2002
EPR 32°S	2300	V	31–32°S	111–112°W			T, R, O	Hurtado 2002; Hurdato et al. 2002
NW Pacific								
Sagami Knoll	1400	S	35°06'N	139°20'E		Lc		Kojima 2001, 2002a, 2002b
Nankai Trough, Kinsu-no-se	300	S	34°17'N	138°15'E	E	Lc, Ls		Kojima 2001, 2002a, 2002b
Nankai Trough, Omaezaki	1200	S	34°15'N	138°02'E	Pe	Lc		Kojima 2001, 2002a, 2002b
Nankai Trough, Yukie Ridge	2000	S	33°50'N	137°54'E		Lc	Asp	Kojima 2001, 2002a, 2002b
Nankai Trough, Muroto Point	3200	S	32°35'N	134°41'E		Lc		Saito, per. com. in Kojima 2001
Kagoshima Bay	82	V	31°40'N	130°48'E		Ls		Kojima 2001, 2002a, 2002b

Table 2. Continued

Collection location	Depth (m)	Type	Latitude	Longitude	E	L	V	Reference
Okinawa Trough, Iheya Knoll	1000	V	27°47'N	126°54'E		Lc	Asp	Yamamoto 1999; Kojima 2001
Okinawa Trough, Iheya Ridge	1400	V	27°33'N	126°59'E	Pe	Lc	Asp	Yamamoto 1999; Kojima 2001
Okinawa Trough, Minami-Ensei Knoll	680	V	18°24'N	127°38'E	"vestimentiferans"			Kojima 2001, 2002a, 2002b
Kikaijima Island	1500	S	27°27'N	130°19'E	Pe			Kojima 2001, 2002a, 2002b
Kuroshima Knoll	680	S	24°07'N	124°12'E		Lc, L		Matsumoto 1999; Kojima 2001
Nikko Seamount	433		23°05'N	142°20'E		Ls		Black 1997
Mariana Trough, TOTO Caldera	?	V	12°43'N	143°32'E	"vestimentiferans"			Mitsuzawa 2000
SW Pacific								
Papua New Guinea	1600	S	2°53'S	142°16'E	Pe	L	Ai	Kojima et al. 2002
Lihir Island	1650	V	3°19'S	152°36'E	Pe			Southward 2002
Manus Basin	1660–1900		3°42–44'S	151°4–5'E	Pe	L	Ai, Asp*	Hashimoto 1999; Kojima 2001, 2002b
Java Trench	1100–1500		6°25'S	104°50'E	Pe			Southward 2002; H. Sahling, per. com.
Fiji Basin	2000	V	17°S	173°55'E		Lc	Ai	Southward 2002
Lau Basin	1890	V	22°32'S	176°43'W		Lc	Ai, As	Southward 1991; Black 1997

*Also reports of a *Ridgeia*-like species (Hashimoto 1999).

excluded from the analysis because the host species was unclear, or the sequences differed markedly from all others analyzed. The DiMeo symbiont sequences may represent entirely new groups of vent and seep symbionts, but the absence of additional sequences to support the observations make these data worrisome.

To obtain the new COI and 16S rRNA sequences, genomic DNA was isolated from paired vestimentum (symbiont free) and trophosome (symbiont containing) tissue samples. The paired samples were frozen immediately after collection in liquid nitrogen, and were stored at –80°C. A small piece of either vestimentum or trophosome was digested overnight in a proteinase K digestion buffer, and DNA extracted by the standard phenol/chloroform technique (Ausubel et al., 1989). The density of bacterial cells in the trophosome ensured the isolation of predominantly symbiont genomic DNA, while the symbiont-free vestimentum generated pure host genomic DNA. Amplification of the COI and 16S was done directly from the isolated genomic DNA. A 1250 base pair fragment of the COI gene was amplified from vestimentum genomic DNA using primers based on COI regions conserved in invertebrates (Nelson and Fisher, 2000). 1550 base pairs of the 16S rRNA gene were amplified from the trophosome genomic DNA using universal bacterial primers published by Lane (1991). A single PCR band was generated with each primer set, COI or 16S.

The two outer COI primers and four additional inner COI primers were used to sequence the COI fragment for a total of three overlapping sequence fragments on each strand. The COI sequencing primers used are as follows:
fw2: GC(CT)GG(AG)ACAGGATGAAC(AT)GT,
fw3: TTCTTTGA(TC)CC(TC)GCAGGAGG,
rv2: GC(GA)AAAT(GA)GCTA(GA)ATCAATGCATGG,
rv3: AC(AT)GTTCATCCTGT(CT)CC(AG)GC.

The 1550 base pair16S PCR product was sequenced with the two original 16S PCR primers as well as six additional internal 16S primers based on conserved eubacterial regions, generating four overlapping fragments on each strand (Lane, 1991; Weisburg et al., 1991). Nucleotide sequences generated from these primers were unambiguous, indicating the presence of a single PCR product, COI or 16S, amplified from the genomic DNA. The COI and 16S sequences obtained by this method were similar to those previously published for vestimentiferans and their symbionts. Sequences were replicable, with the same animal consistently generated the same COI and 16S sequence.

Sequences for the two genes were generated using chain terminating fluorescently labeled nucleotides (Beckman DTCS Quick Start Kit), and cycle sequenced according to the manufacturer's protocol. Products of this cycle sequencing reaction were run on a Beckman CEQ2000 automated sequencer (Beckman, Palo Alto, CA). The sequence fragments for each gene were assembled in Seqman (Lasergene, Madison, WI) and were edited by eye,

generating a 1200 bp contig of the COI gene and a 1550 bp contig of the 16S rRNA gene. Newly generated COI or 16S sequences were aligned (Lasergene, Madison, WI) with those available from GenBank (Table 1) using Clustal (Thompson et al., 1994) and were then adjusted by eye. The COI sequences used in this analysis overlapped for 600 to 1090 base pairs, while the 16S sequences overlapped for 1300 base pairs. Phylogenetic analyses were performed with MEGA 2.1 (Kumar et al., 2001). Pairwise genetic distances among the COI or 16S sequences were calculated using the proportion of differences between samples, and the Kimura two parameter correction (Kimura, 1980) was applied to correct for multiple substitutions. Gene trees were generated using both the neighbor joining (NJ) (Saitou and Nei, 1987) and minimum evolution (ME) methods (Rzhetsky and Nei, 1993). A maximum likelihood (ML) COI tree was also generated using PHYLIP (version 3.6a2, Felsenstein, 2001). The significance of the branching order in the COI and 16S neighbor joining and minimum evolution trees was evaluated by the bootstrap analysis of 1000 computer-generated trees.

The substitution rates among both host and symbiont lineages were compared using two methods. First, Tajima's relative rate test (Tajima, 1993) was used within the MEGA package to compare substitution rates in pairs of sequences versus an outgroup (*Galathealinum brachiosum* or the endosymbiont of *Thyasira flexuosa*). A second program, RRTree (Robinson et al., 1998; Robinson-Rechavi and Huchon, 2000) was used to compare substitution rates between lineages, rather than individual sequences. RRTree calculates the probability of the substitution rates of two lineages being identical by comparing the mean substitution rate of each lineage to that of an outgroup. For RRTree a reduced data set of vestimentiferan COI sequences was produced using a 589 base pair fragment from one individual of each vent and seep species, excepting *Arcovestia ivanovi*, which did not overlap in this region.

A test of coevolution, TREEMAP 1.0 (Page, 1995), was performed on the 15 vestimentiferans and symbionts for which paired host and symbiont sequences were available. TREEMAP evaluates the congruence of the phylogenies for host and symbiont pairs. The number of predicted cospeciation events between the 15 pairs was inferred and compared to the distribution obtained from a set of 1,000 randomly permuted tree topologies.

3. Results and Discussion

Vestimentiferan hosts

Fig. 1 shows the neighbor joining tree of available COI sequences for vestimentiferan tube worms. Data presented here include published sequences

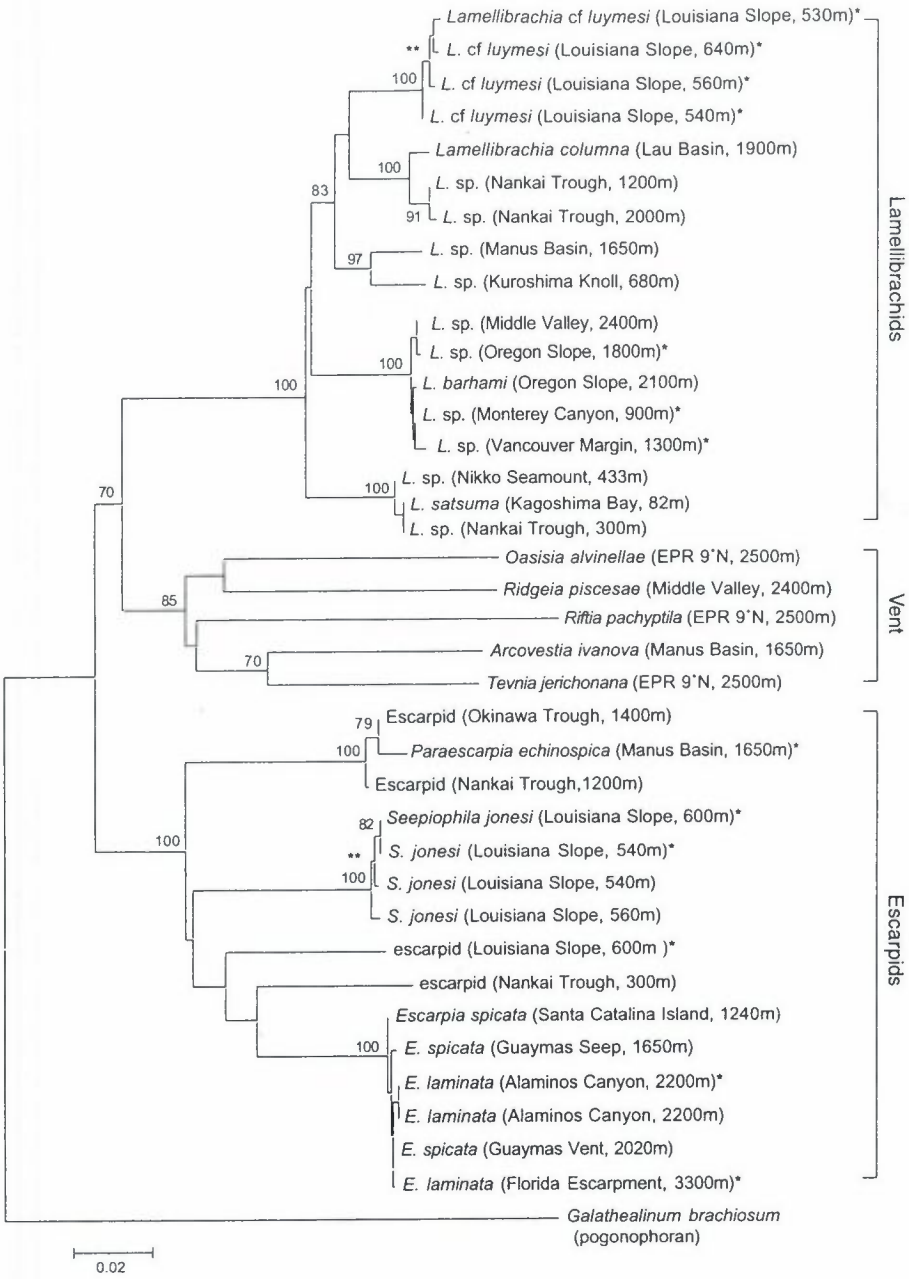

Figure 1. Neighbor joining tree showing molecular evolutionary relationships among vestimentiferan cytochrome oxidase I sequences. Collection locations and depths are given next to each sample. Numbers at the nodes indicate the proportion of occurrences in 1000 bootstrap replications. Bootstrap values below 70% are not shown. EPR = East Pacific Rise; * new data generated for this study; ** Gulf of Mexico collections span 480 km (*L.* cf. *luymesi*) and 580 km (*S. jonesi*).

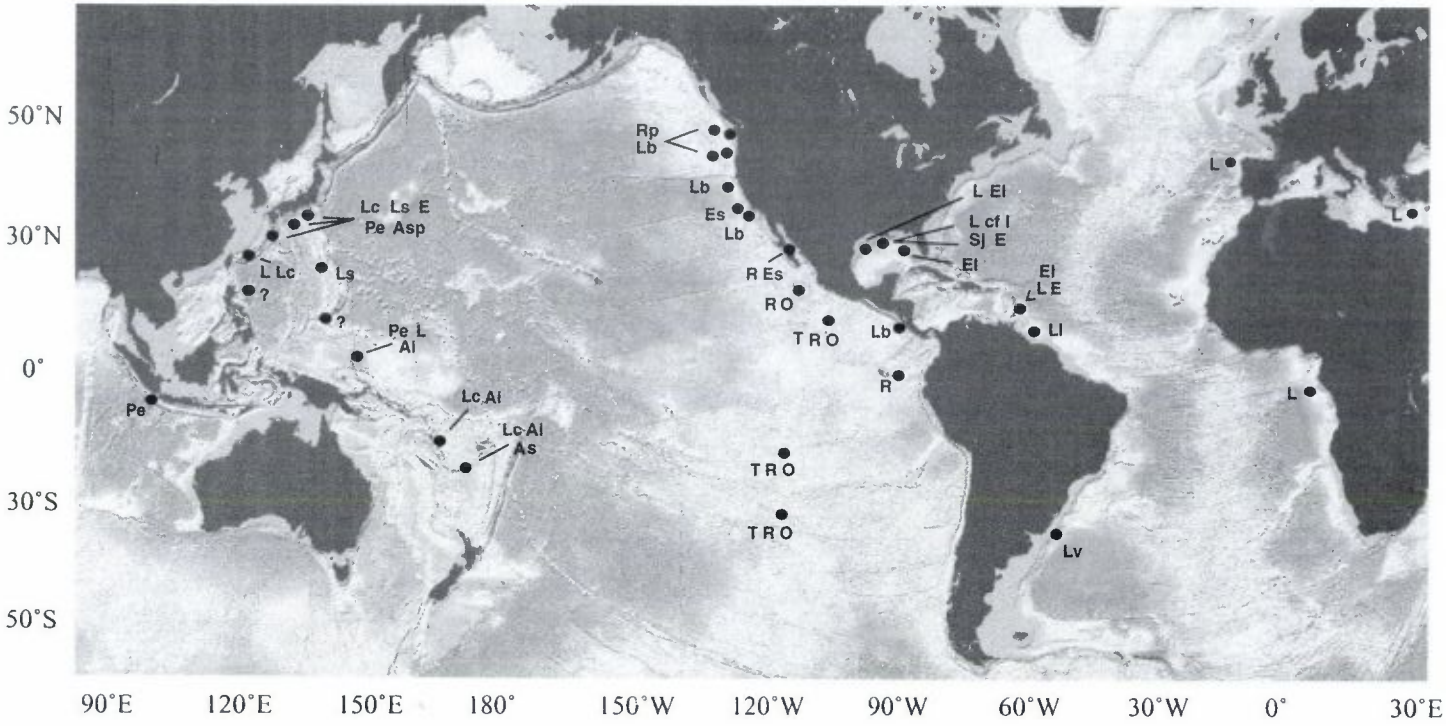

Figure 2. Worldwide distribution of vestimentiferans labeled by species when known. Abbreviations are as follows: Seep vestimentiferans: E = Escarpid; El = *Escarpia laminata*; Es = *Escarpia spicata*; Lb = *Lamellibrachia barhami*; Lc = *Lamellibrachia columna*; Ll = *Lamellibrachia luymesi*; L cf l = *Lamellibrachia* cf *luymesi*; Ls = *Lamellibrachia satsuma*; L = Lamellibrachid; Lv = *Lamellibrachia victori*; Pe = *Paraescarpia echinospica*; Sj = *Seepiophila jonesi*. Vent vestimentiferans: Ai = *Arcovestia ivanovi*; As = *Alaysia spiralis*; Asp = *Alaysia* sp.; R = *Riftia pachyptila*; Rp = *Ridgeia piscesae*; O = *Oasisia alvinae*; T = *Tevnia jerichonana*; ? = unidentified vestimentiferan.

from previous studies (Black et al., 1997; Miura et al., 1997; Feldman et al., 1998; Kojima et al., 1997, 2002), as well as new data (marked with an asterisk). Though the family names Escarpiidae and Lamellibrachiidae are no longer applicable under the new classification of vestimentiferans as a clade within the Family Siboglonidae (Halanych et al., 2001; Rouse, 2001), the former families maintain two distinct and highly supported (100% bootstrap values) groups in all (NJ, ME, and ML) COI phylogenetic trees. The ML tree differed from the NJ and ME trees only in its deep branches, placing the vent vestimentiferans basal to the lamellibrachids and escarpids. The five species of vent vestimentiferans compared here form a third group in the COI tree with lower bootstrap support (85%). Previously published data were combined with the molecular identifications presented here to generate a map of worldwide vestimentiferan species distribution (Fig. 2). Table 2 is a detailed table of the collection locations, depth, and habitat type of vestimentiferan collections.

A test of evolutionary rate constancy, the RRTree relative rate test, found a significant difference ($p=0.028$) in substitution rates between lamellibrachids (0.217 substitutions/site) and the vent species (0.252 substitutions/site). RRTree substitution rates are the number of substitution per site between a particular lineage and an outgroup. Tajima's relative rate test revealed a significant difference in evolutionary rate only between *Lamellibrachia satsuma* and *Riftia pachyptila* ($P=0.02$). Because *R. pachyptila* and *L. satsuma* have the fastest and slowest substitution rates they may affect RRTree comparisons between their respective groups. An RRTree test comparing evolutionary rates between vent vestimentiferans and lamellibrachids with *R. pachyptila* and *L. satsuma* removed still showed a significant difference. No significant difference was found between the escarpid group (0.243 substitutions/site) and either the vent species or lamellibrachids. However, the new escarpid species from 300 m Nankai showed a significantly higher substitution rate than both the new escarpid from the Louisiana Slope and the lamellibrachid group at ($p<0.05$). From these data lamellibrachids appear to have the slowest evolutionary rate of the three vestimentiferan groups, and vent species the fastest (Fig. 3). The escarpid substitution rate is somewhat less than is seen in the vent species, but substitution rates vary significantly within the group.

The escarpids

Members of the escarpids appear basal to both the lamellibrachids and the vent species in Fig. 1, though this branching pattern is not supported by strong bootstrap values (70%). Five distinct branches are seen within the escarpids. COI sequence data are available for at least one individual of each of the four described escarpid species. Sequences of two described escarpids, *Paraescarpia*

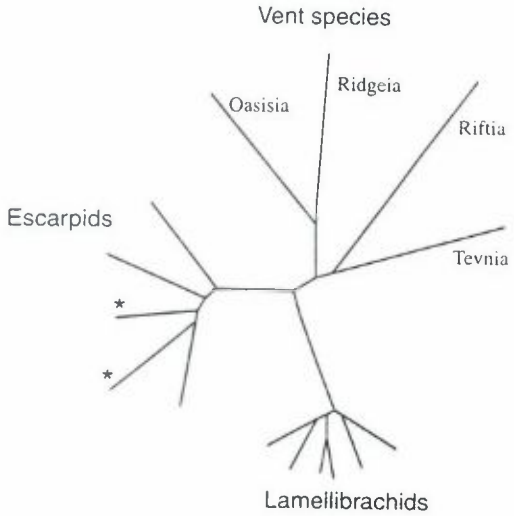

Figure 3. An unrooted neighbor joining tree of the vestimentiferan COI sequences used in the relative rates test. Branch length reflects number of nucleotide changes between species. Asterisks (*) indicate the two escarpid species with significantly different substitution rates.

echinospica (Southward et al., 2002) and *Seepiophila jonesi* (Gardiner et al., 2001), fall into separate and highly supported groups with bootstrap values of 100%. A third highly supported group (100% bootstrap) is composed of the two described species, *Escarpia laminata* and *Escarpia spicata* (Jones, 1985). The remaining COI sequences cluster with either *P. echinospica*, *S. jonesi*, and *E. laminata/E. spicata*, with the exception of a single escarpid from the Nankai Trough (300 m), and a second escarpid morphotype from the Louisiana Slope in the Gulf of Mexico. The available COI sequence data do not clearly define the deeper branching pattern within the escarpids, though some affinity appears to exist between *E. laminata/^* and the sequences from the two undescribed shallow water species.

Escarpia laminata and Escarpia spicata

The COI sequence of two described species, *Escarpia laminata* and *Escarpia spicata*, form a highly supported group (100% bootstrap value). *E. laminata* was described from the Florida Escarpment (3300 m) (Jones, 1985), and found subsequently in Alaminos Canyon (2200 m) (Brooks et al., 1990) and from the Barbados accretionary prism (Olu et al., 1997). *E. spicata* is found on the opposite side of the Panama isthmus (Jones, 1985), off the southern coast of

California (type specimen, Jones, 1985), in both seep and vent sites of the Guaymas Basin (Black et al., 1997), as well as at a whalefall off Santa Catalina Island, California (Feldman et al., 1998). The two species of escarpids look very similar but differ by some key morphological characters, including the obturacular process, the ventral ridge (present in *E. spicata* only), the anterior margin of the vestimentum, and the number of paired branchial lamellae (Jones, 1985). As previously reported, *E. laminata* and *E. spicata* have extremely similar COI sequences (Black et al., 1997; Feldman et al., 1997; Nelson and Fisher, 2000), and form a single cluster with 100% bootstrap values (Fig. 1). Three new sequences of *E. laminata* COI, two from Alaminos Canyon and one from the Florida Escarpment, were used here to further explore the diversity of this gene in the two species. Of the seven sequences used (each 1050 bp in length), the variation observed within each species (0.1% to 0.3%) was similar to that seen between the two species (0.2% to 0.4%). In comparison, the genetic distance between the next most similar COI sequences, *Lamellibrachia* cf *luymesi* and *Lamellibrachia columna*, is an order of magnitude greater (3.2% to 4.8%).

The connection between the Gulf of Mexico and the Pacific Ocean was broken by the formation of the Panama Isthmus roughly 3 million years ago, with deep water connections disappearing as many as 10 million years ago (Knowlton et al., 1993). Both *E. laminata* and *E. spicata* are currently found in very deep water (2000 to 3300 m) and were likely deep water species at the time of the Isthmus formation. In general, the estimated accumulation of mitochondrial mutations for many species is between 1% and 2% every 1 million years (Avise, 1991), and shallow water urchin species divided by the closing of the Panama Isthmus show COI divergence of 1.6% to 3.4% per million years (Bermingham and Lessios, 1993; McCartney et al., 2000). If *E. laminata* and *E. spicata* COI genes are accumulating mutations at 1% per million years, an expected difference of 3% in COI sequences is a conservative estimate of expected divergence. The observed difference between *E. laminata* and *E. spicata* COI sequences, however, is no more than 0.4%. Recent work suggests that the evolutionary rate of the COI gene within vent annelids may in fact be nearer to 0.2% per million years (Chevaldonné et al., 2001), generating an expected difference of 0.6% to 2.0% for *E. laminata* and *E. spicata* COI sequences, a divergence somewhat greater than the observed value. A test of evolutionary rate variation among the different vestimentiferan species did not show an unusually slow evolutionary rate for these two species compared to other vestimentiferans. The low divergence between *E. laminata* and *E. spicata* appears to be the result of a generally slow evolutionary rate in vestimentiferan COI genes rather than of a particularly slow rate in these two species.

Seepiophila jonesi

Seepiophila jonesi, a recently described species (Gardiner et al., 2001), is found at the hydrocarbon seep sites at relatively shallow depths (550–650 m) on the Louisiana Slope (MacDonald et al., 1989; Brooks et al., 1990). Previous molecular data (Gardiner et al., 2001) supported the existence of a single escarpid species within four Louisiana Slope sites which spanned 100 km. New sequences from animals collected 350 km to the east and 100 km to the west of the original sites were used in this study. COI sequences from all but one of the escarpids from the northern Louisiana Slope cluster in a single group, supported by 100% bootstrap values, with low genetic variation (0.03%) among the sequences. These data support the existence of a single dominant escarpid species inhabiting seep sites in the northern Gulf of Mexico as much as 580 km apart and 540 m to 650 m in depth.

Paraescarpia echinospica

COI sequence from an escarpid from a 1200 m deep seep site on the Nankai Trough and a second individual from 1400 m deep a vent site on the Okinawa Trough (Kojima et al., 1997) form a single highly supported cluster with the COI sequence from an individual identified as *Paraescarpia echinospica* from a 1650 m deep vent site off Lihir Island (Southward et al., 2002, sequence provided by Ken Halanych). The COI sequences among these three individuals show very little divergence (0.4%). This same morphotype of escarpid has also been found near Japan in the Ryuko Canyon (1100 m), a seep site on the Omaezaki Spur (1200 m), and in a seep area off Kikaijima Island (1400 m) (Kojima, 2002). This species has also been reported from a 1500 m site in the Java Trench (Southward et al., 2002), as well as from a second site within the Manus Basin and from near Papua New Guinea (Kojima et al., 2002). Given the high degree of similarity in sequence between the samples reported here and in Kojima et al. (2000), and assuming based on morphological analysis that the individual from the Java Trench is also *P. echinospica*, this appears to be a single species of escarpid from intermediate depths (1200–1650 m) that spans at least the 8000 km from the Nankai Trough to the Java Trench. Excluding the Java Trench individual, for which no sequence data are available, the sequence data from the remaining samples support the existence of a single species that spans 4500 km.

Other escarpid species

Kojima (Kojima et al., 1997, 2002) reported the COI sequence for a second morphotype of escarpid collected from a shallow site (300 m) in the Nankai Trough. The molecular phylogeny of these sequences shows a very distinct division between this second escarpid morphotype and the *P. echinospica*

individuals collected at deeper sites in the Nankai Trough (1200 m) and the Iheya Ridge (1400 m) (Kojima et al., 1997). Given the different morphology and the COI sequence divergence (8.5%) of this morphotype from *P. echinospica*, the individuals from the 300 m site in the Nankai Trough appear to be a new and undescribed species. This as yet undescribed shallow water escarpid has only been reported at one site, with a higher genetic diversity among those individuals than is seen in other vestimentiferans (Kojima et al., 2002).

A second morphologically and genetically distinct escarpid has been collected, though rarely, from the Louisiana Slope sites in the Gulf of Mexico. Only one sample of this morphotype was preserved for genetic analysis, while three others were fixed for morphologic description. The COI sequence from this individual is significantly different from both *Seepiophila jonesi* (7.8%) of the Louisiana Slope (600 m) sites, and *Escarpia laminata* (6.8%), which is found at the Florida Escarpment (3300 m) and Alaminos Canyon (2200 m). The COI neighbor joining tree places this new GOM escarpid well outside of the *S. jonesi* cluster and with the *E. laminata*/*E. spicata* group at very low bootstrap values (<70%). The genetic and morphological differences between this morphotype and previously reported escarpids support it as a new species. This rare escarpid may be a representative of an intermediate depth escarpid species, which occurs only rarely at the shallower Louisiana Slope sites.

Another new escarpid species was reported from collections near Barbados, but again no sequence data are available (Olu et al., 1997; Sibuet and Olu, 1998).

The lamellibrachids

Fig. 1 shows a possible evolutionary association between the lamellibrachids and the vent species, though this is supported with only intermediate bootstrap values (70%). A similar branching pattern was reported by Nelson (2000). Within the lamellibrachids five species have been named and described: *Lamellibrachia luymesi* (van der Land), *L. victori* (Mane-Garzon), *L. columna* (Southward), *L. barhami* (Webb), and *L. satsuma* (Miura), although no sequence data are available for either *L. luymesi* or *L. victori*. The lamellibrachid group in Fig. 1 contains six branches, three of which contain a sequence from one of the three described lamellibrachid species. A fourth highly supported (100% bootstrap value) cluster of sequences is comprised of sequences from individuals from the shallow water seep sites in the Gulf of Mexico, samples which bear a strong morphological resemblance to *L. luymesi*. Two additional sequences, the first from Manus Basin and the second from Kuroshima Knoll, did not cluster with any previously known species and were

different enough from each other to suggest they are separate species, though together they form a highly supported (97% bootstrap value) cluster within the lamellibrachids.

Lamellibrachia barhami

Lamellibrachia barhami was first described by Webb (1969) (emended by Jones, 1985) from the coast of California near San Diego. It has since been reported from a variety of sites along the west coast of the North America, including the Monterey Canyon (Barry et al., 1996), the Oregon Slope (Suess et al., 1985), and from a low flow vent site in Middle Valley (Williams et al., 1993). The COI sequence has been published (Black et al., 1997) for *L. barhami* from the Oregon Slope (2100 m) and from Middle Valley (2400 m). Additional COI sequences were generated in this study from lamellibrachids sampled from 1300 m depth on the Vancouver Island Margin, from 1800 m and 2200 m depths off the Oregon Slope, and from 1000 m depth in Monterey Canyon. Samples of lamellibrachids from all sites on the North American Pacific coast fall within a single cluster, which includes individuals identified as *Lamellibrachia barhami*, supported by 100% bootstrap values. Only two nucleotide substitutions are seen in 1050 bp of COI sequence among the samples, and both substitutions are seen in individuals from the northern Vancouver Margin site as well as the more southern Monterey Canyon site. Additionally, a newly obtained sample from 1400 m depth off the coast of Costa Rica is identical to the *L. barhami* COI sequences presented here. Overall, samples within the highly supported *Lamellibrachia barhami* cluster (100% bootstrap values) show a genetic distance of 0.3% to 0.5%, similar to the genetic distance seen within *Seepiophila jonesi*, *Lamellibrachia* cf *luymesi*, and *Lamellibrachia columna* (0.1% to 0.5%). This single species of lamellibrachid is found in multiple seep and low activity vent sites spanning 6000 km, from the Vancouver Island Margin to the coast of Costa Rica, at depths ranging from 900 m (Barry et al., 1996) to 2400 m (Tunnicliffe, 1991).

Lamellibrachia luymesi

Lamellibrachia luymesi was described in 1975 from a 500 m deep site near Guyana (van der Land and Norrevang, 1975). No type sample of *L. luymesi* is available for morphological comparison to other lamellibrachid species; however a lamellibrachid found on the Louisiana Slope of the Gulf of Mexico is morphologically very similar to the description of *L. luymesi*. The morphology of the single specimen of *L. luymesi* as described by van der Land and Norrevang (1975) falls within the morphological variation exhibited by the Louisiana Slope species. The Louisiana Slope species will therefore be referred to as *Lamellibrachia* cf *luymesi*. A redescription of *L. luymesi*, based on material

collected from sites on the Louisiana Slope, is in progress (Gardiner and Hourdez, submitted). This shallow water lamellibrachid co-occurs with *Seepiophila jonesi*, forming bush-like aggregations composed of both species (MacDonald et al., 1990). *Lamellibrachia* cf *luymesi* were sampled from sites spanning 480 km, and depths of 550 m to 650 m. COI sequences from all samples were very similar, with a genetic distance of 0.2% to 0.4% within the group, and are supported as a single phylogenetic group with 100% bootstrap values. Similar to *L. barhami* on the west coast of North America, *L. luymesi* appears to maintain a single species in a range exceeding 4000 km, between the Guyana shelf and the northern Gulf of Mexico.

A second species of *Lamellibrachia*, *L. victori*, has been reported from the coast of Uruguay (Mañé-Garzón and Montero, 1985). The description of *L. victori*, trawled from approximately 300 m depth, is also similar to that of *L. luymesi*. The two species mainly differ in the number of sheath lamellae: 6 pairs for *L. luymesi* and 7 for *L. victori* (Mañé-Garzón and Montero, 1985) in samples of one and possibly two individuals, respectively. The number of sheath lamellae in *L.* cf *luymesi* of the Louisiana Slope typically varies from 4–8 pairs (Gardiner, submitted). Sheath lamellae appear to be a variable trait; Southward (1991) documented 8–16 pairs of sheath lamellae for *L. columna*. The three species are also found at similar depths, which may be a strong factor in defining species range. Without additional samples of *L. victori* available for morphological studies and genetics, however, the identity of this species with regard to those in the Gulf of Mexico and Caribbean cannot be determined. If this animal is indeed the same species as the Louisiana Slope lamellibrachid, then *L.* cf *luymesi* has a species range of 8000 km, comparable to that seen in *Paraescarpia echinospica*.

Lamellibrachia satsuma

Lamellibrachia satsuma is one of two species of *Lamellibrachia* described from vent and seep sites around Japan. *L. satsuma*, described by Miura et al. (1997) from 82–110 m depth vent sites in Kagoshima Bay, is the shallowest species of vestimentiferan known (Hashimoto et al., 1993). Two different morphologies of vestimentiferan were found at a slightly deeper Nankai Trough site (Kinsu-no-se, 300 m). The COI sequence of the first of these morphotypes matched that of *L. satsuma* from Kagoshima bay, while the COI sequence of the second morphotype matched the sequence for *L. columna* (Black et al., 1997) from Lau Basin (Kojima et al., 1997). A third vestimentiferan, a lamellibrachid (Black et al., 1997) from a 433 m deep vent community on Nikko Seamount, also matches that from *L. satsuma* (Kojima et al., 2001). Kojima (2001) reports a very low genetic diversity among the COI sequences from *L. satsuma* of Kagoshima Bay, and argues that this low diversity may be the

result of a relatively recent colonization by a small founding population. The co-occurrence of *L. columna* and *L. satsuma* at the 300 m deep Kinsu-no-se site also suggests that perhaps this is a transition depth for the two species, with *L. satsuma* becoming dominant at shallower depths and *L. columna* dominant at deeper depths.

Lamellibrachia columna

Lamellibrachia columna was first described by Southward (1991) from the Lau Basin, and the COI sequence of a Lau Basin *L. columna* was published by Black (1997). Kojima (2001) has shown that samples of lamellibrachids from eight different sites near Japan, both vents and seeps, are very similar to the published sequence of *L. columna*, a grouping which is supported in the current study with high (100%) bootstrap values. Though COI sequences from these eight sites are very similar (0.1% to 0.5% genetic distance), a marked division appears between individuals from shallow and deep collections. No overlap of COI haplotypes was seen between 45 shallow (300–1400 m) and 22 deep water (2000–3270 m) individuals sampled within or near the Nankai Trough. Of 14 animals sampled from 680–1400 m in the Okinawa Trough and Kuroshima Knoll, two had unique COI haplotypes and twelve had a haplotype seen in shallow Nankai Trough lamellibrachids; no deepwater haplotypes were seen within these collections (Kojima et al., 2001). The division of COI haplotype by depth indicates that *L. columna* from these sites may in fact be genetically isolated from each other. The pairwise genetic distance between the three distinct clusters within the *L. columna* group have a genetic distance of 0.5% to 1.1% (Kojima et al., 2001), larger than the distance between *E. laminata* and *E. spicata*, but less than that seen within the vent species *Ridgeia piscesae*. If these three clusters do indeed represent a single species, albeit subdivided, then *Lamellibrachia columna* is found over a distance of 8000 km and in a depth range of 300 to 3270 m. Kojima's data (2001), however, raise the possibility that these are three separate sister species with very low levels of COI divergence.

Possible new species of Lamellibrachia

Kojima (2001) reports COI sequences for two additional vestimentiferans which, though they cluster within the lamellibrachids, are sufficiently different from all other known sequences to suggest that they are both from new species. The first new lamellibrachid sequence is from a sample collected from a 1650 m deep seep site in the Manus Basin, a region where *Arcovestia ivanovi* (Southward and Galkin, 1997), an escarpid-like, a ridgeid-like, and an alaysid-like species (Hashimoto et al., 1999; Southward et al., 2002) were

previously reported. A second unique sequence is from an individual sampled from a vent site (680 m) on the Kuroshima Knoll where *L. columna* is also found. While only one sequence is available from each of these new vestimentiferans (Kojima et al., 2001), the divergence between each sequence and other lamellibrachid sequences indicates the two individuals represent two new lamellibrachid species. Additional endemic species of *Lamellibrachia* may also exist in the Manus Basin and the seep area off the New Guinea Island (Kojima, 2002).

A vestimentiferan collected from a shipwreck 1160 m deep off the coast of Portugal (Dando et al., 1992) was identified by tube morphology and 28S sequence as a *Lamellibrachia* sp. Tissue from the sample was too degraded for morphological identification, and no COI sequence was obtained, limiting comparisons to other known lamellibrachid species. The 28S data, however, did differentiate this sample from samples from the Gulf of Mexico, from Canadian samples (most likely *L. barhami*) (Williams et al., 1993), and from the Japanese *L. satsuma* (Brown et al., 1999). Because this sample is from a very different geographic region from the only remaining described species, *L. columna* of the southeast Pacific, it is likely to be another new species of lamellibrachid.

Lamellibrachids have been collected from the Alaminos Canyon (2200 m) in the Gulf of Mexico (Brooks et al., 1990), but unfortunately no samples were available for molecular study. Given the division of species based on depth seen in sample sites near Japan, these deep water Gulf of Mexico lamellibrachids are likely a separate species from those found on the shallower Louisiana Slope (Brooks et al., 1990). Sibuet (1998) also reports a species of *Lamellibrachia* collected from below 1000 m off the coast of Barbados, again with no morphological species identification or samples for genetics. Additional reports of lamellibrachids collected from the Mediterranean (Olu-LeRoy et al., 2001b) and from the west coast of Africa (Olu-LeRoy et al., 2001a) are intriguing, but no species descriptions or genetic data have yet been published.

The vent vestimentiferans

Four vestimentiferan species, *Riftia pachyptila*, *Tevnia jerichonana*, *Oasisia alvinae*, and *Ridgeia piscesae* were described by Jones (1985) from venting sites in the east and northeast Pacific. Two additional vent species, *Arcovestia ivanovi* and *Alaysia spiralis*, were subsequently described from hydrothermal vent sites at the Lau back arc basin (Southward, 1991; Southward and Galkin, 1997). COI sequences from the four east Pacific vestimentiferans and newly published COI sequence from *Arcovestia ivanovi*

(Kojima et al., 2002) form a single moderately supported vent clade (85%) (Fig. 1). This vent clade in turn pairs with the lamellibrachids, though at a low bootstrap value (70%). Deep branching patterns among the three vestimentiferan clades are not clearly resolved by COI sequences, as previous studies with this gene have placed vent vestimentiferans either outside of an escarpid/lamellibrachid seep clade (Nelson and Fisher, 2000) or within an escarpid/vent clade to the exclusion of the lamellibrachids (Black et al., 1997; Feldman et al., 1997). A similar phylogenetic tree using 28S sequence also failed to resolve the issue, grouping three vent genera, *Tevnia, Ridgeia,* and *Riftia,* in a cluster with *Escarpia* at low bootstrap values (63%) (Williams et al., 1993). Within the vent clade, *Oasisia* and *Ridgeia* form a group with low bootstrap support and *Riftia, Tevnia,* and *Arcovestia* form a second group. *Oasisia/Ridgeia* and *Riftia/Tevnia* pairings have been reported by a number of researchers (Black et al., 1997; Feldman et al., 1997; Nelson and Fisher, 2000). The new *Arcovestia ivanovi* COI sequence is most similar to that from *Tevnia jerichonana,* and the two sequences form a moderately supported (70%) cluster.

Riftia pachyptila

Riftia pachyptila is a dominant organism at venting regions throughout the east Pacific and is found at Guaymas Basin and Rift, on the Galapagos Rise, and from 32°S to 21°N on the East Pacific Rise (Tunnicliffe et al., 1998). Like *R. piscesae, R. pachyptila* shows a degree of morphological placticity not reflected in molecular data (Black et al., 1994). Based on genetic analysis, *Riftia pachyptila* appears to maintain a single species over thousands of kilometers (Black et al., 1994; Tunnicliffe et al., 1998), though the *Riftia* at 32°S EPR appear be reproductively isolated from individuals from the northern sites (Hurtado, 2002). *R. pachyptila* often co-occurs on the EPR with two other vestimentiferan species, *Tevnia jerichonana* and *Oasisia alvinae.*

Tevnia jerichonana and Oasisia alvinae

Tevnia jerichonana and *Oasisia alvinae* co-occur with the larger *R. pachyptila* at most east Pacific hydrothermal sites. The three species overlap on the East Pacific Rise from 32°S to 21°N, though *T. jerichonana* is absent from the northernmost EPR site, and only *R. pachyptila* is present on the Galapagos Rise (Segonzac et al., 1997; Tunnicliffe et al., 1998; Hurtado, 2002). *R. pachyptila* and *T. jerichonana* dominate different successional stages of the short-lived EPR communities (Lutz et al., 1994; Shank et al., 1998; Mullineaux et al., 2000), with *T. jerichonana* the first to colonize newly opened vent sites, only to be subsequently replaced by *R. pachyptila. O. alvinae,* found only sporadically and hard to distinguish from *T. jerichonana* (Lutz et al., 1994; Shank et al., 1998; Mullineaux et al., 2000), may occupy a different ecological

niche from the two other EPR species (pers. obs. C.R.F.). As seen in *R. pachyptila*, *T. jerichonana* and *O. alvinae* from 32°S EPR have limited gene flow with the more northern EPR populations. *O. alvinae* at this site show a particularly high genetic divergence from the northern *Oasisia*, and may in fact be a second *Oasisia* species (Hurtado et al., 2002).

Ridgeia piscesae

Hydrothermal vent vestimentiferans found in the northeast Pacific were initially described as two different species, *Ridgeia phaeophiale* and *Ridgeia piscesae* (Jones, 1985). As many as six additional species were suspected based on differences in tube and body morphology among samples (Jones, 1985; Tunnicliffe, 1991). *Ridgeia* are found in areas of high and diffuse flow, with different morphotypes found in these different flow regimes (Tunnicliffe, 1991). For example, a long and thin *Ridgeia* morphotype is found on basaltic substrate in diffuse, low flow environments where temperatures are barely above ambient (2°C) and sulfide is often undetectable, while a short and thick morphotype is found on sulfide structures in higher flow environments (sulfide ~200 μM, temperature ~30°C) (Sarrazin et al., 1997; Urcuyo et al., 1998). Genetic studies, however, failed to find any evidence for reproductive isolation among the various *Ridgeia* morphotypes (Black, 1991; Southward et al., 1995; Black et al., 1998). *Ridgeia phaeophiale* and *R. piscesae* have subsequently been redescribed (Southward et al., 1995) as a single species, *R. piscesae*, with a number of highly variable morphological features. The distinct phenotypes of *R. piscesae* may be induced by local environmental conditions (Southward et al., 1995; Tunnicliffe et al., 1998). Ongoing studies are investigating physiological and gene expression differences that may explain how this highly polymorphic tubeworm species can inhabit such a broad range of microhabitats (Carney et al., 2002; Flores et al., 2002).

Arcovestia ivanovi and Alaysia spiralis

Arcovestids and alaysids were originally reported from sedimented hydrothermal vent sites at the Lau back arc basin (Southward, 1991; Southward and Galkin, 1997). More recent collection sites of these two vent species often overlap collections of escarpids and lamellibrachids, classically considered the 'seep' vestimentiferans. Arcovestids have been collected from three vent sites within Manus Basin (Hashimoto et al., 1999), a vent site in the North Fiji Basin (Southward et al., 2002) and from a vent site near Papua New Guinea (Kojima et al., 2002). Genetic analysis of the COI sequence of samples from two sites within the Manus Basin and a third near Papua New Guinea show no genetic differentiation (Kojima et al., 2002) suggesting that samples from these three sites comprise a single species, *Arcovestia ivanovi*. These

newly released COI data (Kojima et al., 2002), when added to the aligned sequences used here, place *Arcovestia ivanovi* firmly within the 'vent' vestimentiferans, with *Tevnia jerichonana* as its closest relative.

Though no sequence data have yet been published for *Alaysia spiralis*, reports and collections of this second western Pacific vent vestimentiferan have increased. A possible new *Alaysia* species was reported from Iheya Knoll as a dominant species. In 2000 a tentative new species of *Alaysia* was collected from a new vent field at 1350 m on the Daiyon Yonaguni Knoll (south Okinawa Trough) (Kojima, 2002). Kojima et al. (2002) report that molecular analysis is being done on an alaysid vestimentiferan sampled from a 2000 m deep seep site near Sagami Bay (the Yukie Ridge), and that preliminary data show that this alaysid species is closely related to *Arcovestia ivanovi* (Kojima et al., 2002). This close relationship would place *Alaysia* as well as *Arcovestia* within the existing vent vestimentiferan clade, supporting a common origin for all species of vent vestimentiferans.

Vestimentiferan symbionts

Previous work has shown that vestimentiferan endosymbiont phylogenies do not parallel the phylogenies of their host species. For example, *Escarpia laminata* and a novel species of lamellibrachid from Alaminos Canyon have indistinguishable symbionts, while *E. laminata* from the Florida Escarpment harbors a different symbiont (Nelson and Fisher, 2000). These data, as well as the apparent lack of symbionts in vestimentiferan eggs and early larva (Cavanaugh et al., 1981; Cary et al., 1989b, 1993), support a horizontal or environmental transmission of symbionts from generation to generation. A number of factors affect symbiont acquisition in species with horizontal transmission, the most fundamental of which is that such species can only acquire a bacterial symbiont if it is present in the environment of the host. Because vestimentiferan larvae must interact directly with potential symbionts, motile bacteria found in the immediate vicinity of the larvae are the prime candidates for initiating a symbiosis. The physical presence of a potential symbiont is not enough to form a symbiosis; the host and symbiont must communicate in a specific way to initiate the process, and the host must be able to provide the appropriate conditions for bacterial growth while screening out unwanted bacteria (Smith and Douglas, 1987; Hirsch and McFall-Ngai, 2000). Varying degrees of specificity are seen in different host/symbiont associations. Some host/symbiont interactions are extremely specific: the host will only acquire a specific bacterial strain even if it is at low densities in the environment (e.g. the squid *Euprymna scolopes* (Hirsch and McFall-Ngai, 2000)). Other interactions are less specific: hosts will acquire any one of a variety of symbionts, depending on which strains of bacteria are available in

the environment (e.g. Ectomycorrhizae (Smith and Douglas, 1987)). New data presented here further explore the interplay of geography, host species, and the physical characteristics of a site in defining which strain or species of endosymbiont is found in a particular vestimentiferan host.

The deepest divergence in the vestimentiferan endosymbiont 16S rRNA tree (Fig. 4) is between endosymbionts sampled from vent species and those from seep species. This division of vent and seep symbionts has been previously reported (Feldman et al., 1997; Nelson and Fisher, 2000; Di Meo et al., 2000), and is clearly illustrated by the two species *L. barhami* and *R. piscesae,* which occur in close proximity in the venting regions of Middle Valley but which contain very different symbionts (Nelson and Fisher, 2000). The 16S sequences of endosymbionts of the four east Pacific vent vestimentiferans form a single highly supported group, and have an average of 4.6% difference in 16S sequence from endosymbionts of seep vestimentiferans. Nelson and Fisher (2000) report a similar value, and argue that this level of difference between 16S sequences reflects an old divergence between these two symbiont groups (108 to 215 mya), perhaps indicating that seep and vent symbionts are different bacterial species. Identical 16S sequences were found in symbionts from three different host species at 9°N on the East Pacific Rise, and very slightly divergent symbionts were found in *Ridgeia piscesae* from the NEP spreading centers (Nelson and Fisher, 2000). This slight level of divergence is also supported by restriction fragment length polymorphism data (Laue and Nelson, 1997) and suggests that the symbionts of *Ridgeia piscesae* may be of a different population or strain from those found at 9°N on the EPR (Nelson and Fisher, 2000).

The 16S sequences of endosymbionts from seep vestimentiferans form three distinct clusters, each supported with 100% bootstrap values. This result differs from the findings of Feldman (1997), who found that the symbionts from four sedimented seep host samples comprise a single bacterial species. The data are in agreement with Nelson (2000), who also found that the seep symbiont clade was composed of three separate clusters. The most basal cluster (Group 1) of symbionts is composed of samples from *Lamellibrachia barhami* from the Oregon Slope and from Middle Valley, *E. laminata* from the Florida Escarpment, and *L. columna* from Fiji-Lau (Fig. 5). The 16S sequences of symbionts within Group 1 show very low divergence (0.1% or 1.3 nucleotides), and differ by 2% (25.6 nucleotide differences) from the two other seep symbionts groups.

The remaining two clusters of seep symbionts in turn form a highly (99%) supported subgroup. The first of these (Group 2) includes the symbionts of *Lamellibrachia* sp. and *E. laminata* collected from Alaminos Canyon, *L. barhami* from the Vancouver Island Margin and Monterey Canyon, and *E. spicata* from a whalefall near Santa Catalina California (Fig. 5). As reported

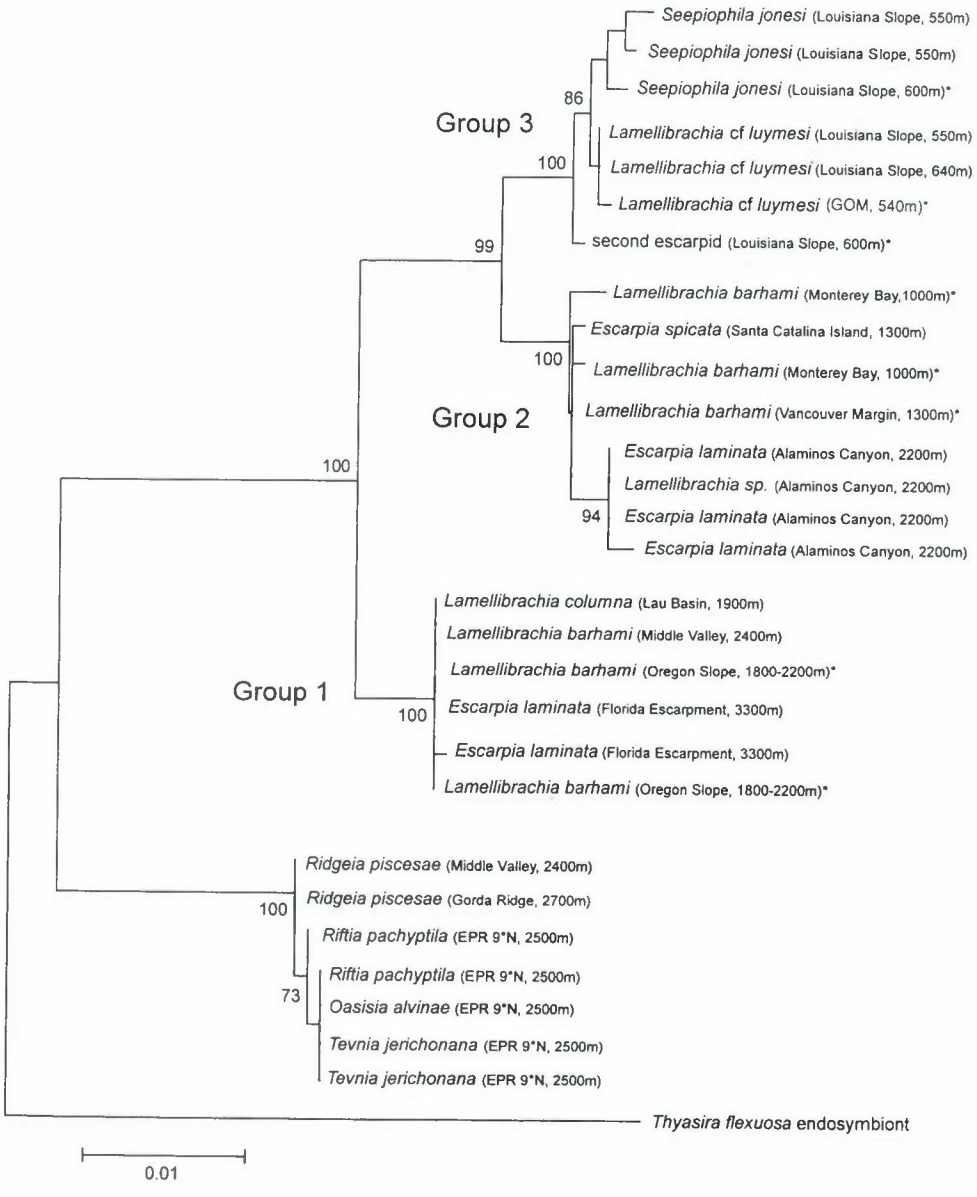

Figure 4. Neighbor joining tree showing molecular evolutionary relationships among vestimentiferan symbionts rDNA 16S sequences. Numbers at the nodes indicate the proportion of occurrences in 1000 bootstrap replications. Bootstrap values below 70% are not shown. Asterisks (*) indicate new data generated for this study.

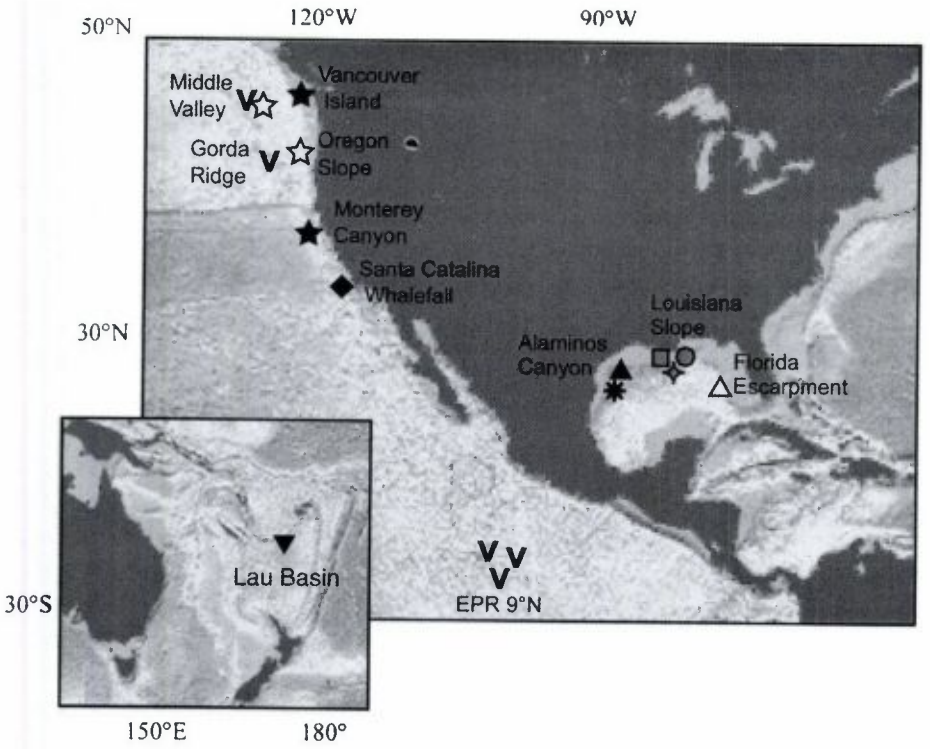

Figure 5. Distribution of vestimentiferan host species and symbiont strains in paired samples. Vestimentiferan host species are identified by symbol shape: *Seepiophila jonesi* (o), *Lamellibrachia* cf. *luymesi* (❑), *Escarpia spicata* (◇), *Escarpia laminata* (Δ), *Lamellibrachia columna* (∇), *Lamellibrachia barhami* (☆), an undescribed lamellibrachid (✲), and an undescribed escarpid (✧). Symbiont strain is identified by symbol color: black = Group 1 symbiont, white = Group 2 symbiont, grey = Group 3 symbiont. Vent vestimentiferan species all harbor vent symbionts, and are simply labeled 'V'.

in Nelson and Fisher (2000) symbionts of two species, *E. laminata* and a *Lamellibrachia* sp. from Alaminos Canyon, have only one nucleotide replacement among them. These symbionts form a single branch with high bootstrap values, and are 0.2% (2.6 nucleotides) different from the other symbionts within Group 2, data which suggest that the symbionts of Alaminos Canyon are a separate population of bacteria. Group 3 symbionts are from the three species of the Louisiana Slope (GOM), *Seepiophila jonesi*, *Lamellibrachia* cf *luymesi*, and a new escarpid species (Fig. 5). Symbionts from Group 2 and Group 3 have a 1% (or 12.8 bp) difference in their 16S sequences, while within each group individuals are between 0.2% and 0.3% different.

Previous authors (Feldman et al., 1997; Di Meo et al., 2000; Nelson and Fisher, 2000) have used the lack of congruence between host and symbiont phylogenies as evidence for environmental transmission of symbionts in the vestimentiferans. In the present study, a test for coevolution (TREEMAP) between the host COI tree and the symbiont 16S tree found five cospeciation events between the two phylogenetic trees. This result, however, is not significant when compared to a normal distribution of cospeciation events generated with 1000 random tree topologies. Vestimentiferans appear to acquire the locally available symbiont strain independent of host species, as illustrated by the collections from Alaminos Canyon, the Louisiana Slope, and EPR 9°N, which each have only one discernable symbiont but multiple host species (Fig. 5).

Vestimentiferans do not, however, take up bacterial symbionts in an indiscriminate fashion. Only four strains or species of bacteria have been found in vestimentiferans (with the possible exception of the Guaymas Basin samples not included in this analysis), yet countless other bacteria are available in the water surrounding larval vestimentiferans. In particular, sulfide oxidizing gamma proteobacterial symbionts from local vent and seep bivalve species may be available to the vestimentiferan larvae, yet these particular symbionts are never found in a vestimentiferan host. Vestimentiferans are clearly discriminating among an array of available bacteria, acquiring only bacteria with a narrow range as symbionts. An even finer level of host/symbiont specificity is apparent in the animals collected from Middle Valley in the northeast Pacific. At this site *Lamellibrachia barhami* and *Ridgeia pisceseae,* collected just meters apart, harbor different species of symbionts. Because the hosts are in such close proximity, both symbiont types are presumably available to both vestimentiferan species, yet the vent species *R. piscesae* contains the classic 'vent' symbiont while the *L. barhami* symbiont falls into the seep symbiont cluster. Discrimination therefore occurs between very similar 'vent' and 'seep' symbionts species, and the appropriate association between vestimentiferans and their symbionts results even when numerous other bacterial species are available.

The symbiont that a vestimentiferan acquires from its environment is therefore a product of three filtering events. First, the bacteria must be available in the host's environment; second, the bacteria must be one of a limited set of bacteria which can be vestimentiferan symbionts; and third, the potential bacterial symbiont must be of the same environmental type, vent or seep, as its host. Questions of symbiont distribution can therefore be answered with a data set such as the one presented here. Within the two groups of vestimentiferans, vent and seep, symbiont acquisition appears to be defined by the availability of the free living symbiont. Mapping the locations and site characteristics of vent and/or seep host collections may reveal boundaries,

geographic, physical, or chemical, that define symbiont distribution. Little information can be obtained from the vent symbionts because of their low 16S rRNA genetic variation. The three different strains of seep symbionts, however, are informative for such a comparison.

Symbiont distribution does not appear to be strongly affected by geography (Fig. 5). The 16S sequences of symbionts of *L. barhami* from the Vancouver Island Margin and from Middle Valley, only 135 km apart, are 1.8% different from each other, whereas sequences of symbionts from three different host species from Fiji-Lau (*L. columna*), Middle Valley (*L. barhami*), and the Florida Escarpment (*E. laminata*), separated by as much as 11,000 km, are only 0.2% different. Symbionts from Group 1 and Group 2 are found in both the Atlantic and the Pacific, and representatives of all three groups are found within the Gulf of Mexico, separated at most by 1000 km. These data suggest that symbionts of Group 1 and Group 2 are available globally, and that some factor other than distance between hosts controls the presence of each strain.

One possible environmental feature that could limit the range of a free living symbiont is depth. Group 1 symbionts were collected from generally deeper sample sites (1800 to 3300 m), while Group 2 is comprised of samples from intermediate depths (900m to 2200 m), and Group 3 from shallower sites (550 to 650 m) (Fig. 4). The collections depths of Group 1 and 2 show some overlap; these two strains may not have a clear depth boundary between them. Additionally, the deeper Vent and Group 1 symbiont collections, with mean substitution rates of 0.061 and 0.062 nucleotides/site respectively, show a significantly slower evolutionary rate ($p<0.001$) than the intermediate Group 2 (0.085 nucleotides/site) and shallow Group 3 (0.087 nucleotides/site) collections.

Though the discussion above relies on the assumption that, on a local level, symbiont strain is defined first by collection site, new data presented here (Fig. 4) also suggest fine scale division of symbiont strain between vestimentiferan hosts within a collection site. The GOM symbiont sequences within Group 3 appear to be divided by host species. The three *S. jonesi* symbiont sequences form a single cluster (71% bootstrap), while the three sequences from *L.* cf *luymesi* form a second cluster, with low bootstrap support (61%). The symbiont from the second GOM escarpid species falls basal to these two clusters in its own well supported branch (87% bootstrap). While Group 1 and Group 2 Alaminos Canyon sequences have very little 16S rRNA variation, and the remaining Group 2 symbionts are from a single host species, Group 3 symbionts may have enough genetic variation in their 16S sequences for fine scale divisions to be seen. Preliminary data from RFLP analysis of 16S variation within the Louisiana Slope tubeworms shows a statistically significant tendency for one symbiont strain to be found within *S. jonesi* and a second symbiont strain within *L.* cf *luymesi*.

The above observations lead to a number of predictive hypotheses that may be tested with known vestimentiferan collection sites, as well as with new collections in the future. First, we predict that deep water symbionts, both vent and seep, will show a lower diversity and slower evolutionary rate than intermediate and shallow water symbionts. Second, all samples of vent vestimentiferans will harbor symbionts of the 'vent' symbiont group. This prediction includes the alaysids and arcovestids of Lau Basin and the waters near Japan; a large species range in vent symbionts would not be unprecedented as similar ranges are seen in both Group 1 and Group 2 symbionts. Third, seep vestimentiferan symbiont type will depend on the depth of collection: all seep vestimentiferans species collected from >2000 m will harbor Group 1 seep symbionts, those from intermediate depths (1000–2000 m) will harbor Group 2 symbionts, and shallow water vestimentiferans (<1000 m) will harbor Group 3 symbionts. The predictions for shallow water symbionts are based at present on a limited amount of data, and while Group 3 symbionts may be found worldwide in shallow water seep sites, they may also be found only locally in the northern Gulf of Mexico. Samples of *L. luymesi* from the shallow water site near Guyana would be particularly useful in answering this question. Finally, different host species from the same sample site may show a tendency toward having one or another symbiont strain, though not every individual of a given host species will have the preferred symbiont.

A number of collection sites could be particularly useful in addressing the above questions. It would be interesting to see if symbionts from the two west Pacific vent vestimentiferans, *Alaysia spiralis* and *Arcovestia ivanovi*, are the same symbiont strain as the vent vestimentiferans of the eastern Pacific. Sites with co-occurring vent and seep species, such as in Guaymas Basin and in the waters near Japan, could be used to further test the specificity of these vestimentiferans for their respective symbiont types. Vestimentiferan communities near Japan include multiple vent and seep vestimentiferan species which span a wide range of depths and include seep, vent, and whalefall sites. These sites provide an exciting and extensive opportunity for studies concerning symbiont strain and evolutionary rate as a function of host species, depth, or other physical characteristics of a site.

4. Conclusion

While symbiont evolutionary rates appear to decrease with depths, vent vestimentiferans have the fastest COI evolutionary rate, while the slowest rate is in the shallow *L. satsuma*. Evolutionary rate is the accumulation of mutations in a gene per generation, and is therefore influenced by such factors as the mechanics of DNA replication and repair, and the generation time of the

species (Hartl and Clark, 1997). The generation time effect may explain why the relatively short-lived vent species have a faster COI sequence evolution than the very long-lived lamellibrachids (Bergquist et al., 2000), but does not explain why the escarpids have an evolutionary rate more similar to vent vestimentiferans when evidence suggests that escarpids live as long as, if not longer than, the lamellibrachids (Bergquist et al., 2002). In fact, the two seep vestimentiferan with the slowest evolutionary rate and lowest genetic diversity (*L. satsuma*), and the fastest evolutionary rate and highest genetic diversity (the escarpid), are from the same 300 m deep site of the Nankai Trough. Though the low diversity in *L. satsuma* of the Nankai Trough may be, as Kojima et al. (2002b) argue, the result of a recent colonization event by a small founding population and/or a stressful environment, the same low diversity is not seen in the escarpid. Most likely the differences in evolutionary rate and genetic diversity between these two species are the result of a combination of different physiologies, life history traits, and population histories. The slower COI evolution rate in lamellibrachids may explain why COI-based trees show *L. columna* from the Nankai Trough as a single species while detailed genetic studies reveal two genetically isolated communities. Lamellibrachid species with large geographical and/or depth ranges (*L. barhami* and *L. luymesi*) should therefore be subjected to more detailed genetic studies to reveal genetic isolation undetected by COI-based phylogenetics.

We have combined information from the current literature on vestimentiferan biogeography and genetics with new genetic data to present a more complete description of vestimentiferan host and symbiont occurrence worldwide. While vent vestimentiferans are notably absent from the Atlantic and the recently explored Indian Ocean vents, seep vestimentiferans are found in a variety of sulfidic environments in the Atlantic, the Pacific, and the Mediterranean, over a wide range of depths (82 m to 3300 m). Often multiple species are found at the same site, with seep and vent species at times occurring meters apart, and multiple vent or seep species inhabiting the same site or even aggregation. In general, vestimentiferans (eg. *L. barhami*, *L. columna* and *R. pachyptila*) have very large species ranges that are interrupted by changes in depth (eg. the four species of the Nankai Trough). Symbionts also have very large ranges, with nearly identical 16S sequence found in hosts separated by thousands of kilometers. Vent symbionts appear specific to vent vestimentiferan hosts, while three different symbiont strains inhabit seep vestimentiferans. Site depth appears to be a factor in defining which of these three strains is found in a particular seep host, and different hosts may show a preference for variants within a symbiont strain. Depth may also be directly influencing seep and vent symbiont diversity and evolutionary rate. A number of known sample sites, particularly in the western Pacific, offer an exciting opportunity for genetic studies of host and symbiont biogeography similar to

those presented here, which would be greatly advanced by similar analyses of samples from the eastern Atlantic. It will be interesting to see if the trends in host and symbiont phylogenetics, biogeography and evolutionary rate shown in this study are supported in future samples.

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