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Endosymbionts of *Siboglinum fiordicum* and the Phylogeny of Bacterial Endosymbionts in Siboglinidae (Annelida)

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Abstract. Siboglinid worms are a group of gutless marine annelids that are nutritionally dependent upon endosymbiotic bacteria. Four major groups of siboglinids are known—vestimentiferans, moniliferans, *Osedax* spp. and frenulates. Although endosymbionts of vestimentiferans and *Osedax* spp. have been previously characterized, little is currently known about endosymbiotic bacteria associated with frenulate and moniliferan siboglinids. This is particularly surprising given that frenulates are the most diverse and widely distributed group of siboglinids. Here, we molecularly characterize endosymbiotic bacteria associated with the frenulate siboglinid *Siboglinum fiordicum* by using 16S rDNA ribotyping in concert with laser-capture microdissection (LCM). Phylogenetic analysis indicates that at least three major clades of endosymbiotic γ-proteobacteria associate with siboglinid annelids, with each clade corresponding to a major siboglinid group. *S. fiordicum* endosymbionts are a group of γ–proteobacteria that are divergent from bacteria associated with vestimentiferan or *Osedax* hosts. Interestingly, symbionts of *S. fiordicum*, from Norway, are most closely related to symbionts of the frenulate *Oligobrachia mashikoi* from Japan, suggesting that symbionts of frenulates may share common evolutionary history or metabolic features.

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Introduction

Siboglinids, previously known as the phyla Pogonophora and Vestimentifera, are now recognized as a monophyletic clade within annelid worms (McHugh, 1997; Black *et al.,* 1997; Halanych *et al.,* 2001; Rouse *et al.,* 2004; Struck *et al.,* 2007). As adults, they lack a mouth, gut, and anus and instead have evolved a storage organ for microbial endosymbionts (Southward *et al.,* 2005). This organ, called a trophosome, is perfused with a blood-vascular system to facilitate transport of nutrients to and from endosymbionts (Southward *et al.,* 2005). As currently recognized, siboglinids comprise four major lineages and encompass more than 150 recognized species (Rouse *et al.,* 2004; Glover *et al.,* 2005; Halanych, 2005). Vestimentifera, the best-known lineage, includes large (up to 2 m in length) tubeworms from hydrothermal vents (*e.g., Riftia pachyptila*) and hydrocarbon cold-seeps (*e.g., Lamellibrachia* and *Escarpia*). Other groups include moniliferans, *Sclerolinum* spp., which live on decaying wood and other organic matter (Halanych *et al.,* 2001), and the recently discovered whale-bone-eating worms, *Osedax* spp. (Rouse *et al.,* 2004; Glover *et al.,* 2005). Last are Frenulata (a.k.a. perviates), composing about 75% of all recognized siboglinids. They are found throughout the world's oceans, along continental margins, continental slopes, fjords, trenches, at least one mud volcano, and the periphery of vents and seeps (*e.g.,* Ivanov, 1963; Webb, 1963; Southward, 1979, 1988, 1991; Southward *et al.,* 1981; Schmaljohann and Flügel, 1987). In

comparison to vestimentiferans, frenulates are typically diminutive, about the thickness of a coarse human hair, and more than 10–20 cm long (Southward *et al.,* 2005). Surprisingly, the symbiotic biology of frenulates, which are found in many more marine habitats than vestimentiferans, has been largely ignored.

Most siboglinids are thought to host a single species of chemosynthetic endosymbiont, which they obligately depend upon for organic nutrition (Distel *et al*., 1988; but see Chao *et al.,* 2007; Vrijenhoek *et al.,* 2007). Despite this obligate association, there is no evidence for vertical transmission of symbionts from parents to offspring in Siboglinidae. For those hosts examined to date (mainly vestimentiferans), no bacteria have been found in the gonadal tissues, sperm, eggs, or early-stage larvae (Cavanaugh *et al.,* 1981; Cary *et al.,* 1989, 1993), and phylogenies of the host and the symbiont are not congruent (Feldman *et al.,* 1997; Di Meo *et al.,* 2000; Nelson and Fisher, 2000). If symbionts were transmitted from parent to offspring, a tightly coevolved phylogeny would be predicted (*i.e.,* Clark *et al.,* 2000; Degnan *et al.,* 2004), as has been observed in species of the vesicomyid clam *Calyptogena* (Peek *et al.,* 1998). Furthermore, symbionts of the vestimentiferan *R. pachyptila* possess an apparently functional flagellin gene; this may indicate a motile, and therefore nonsymbiotic, stage of this endosymbiont's life cycle (Millikan *et al.,* 1999). Although no molecular studies have identified free-living siboglinid symbionts thus far (Southward *et al.,* 2005), bacteria of similar ultrastructure and size have been identified from both the trophosome of *Siboglinum poseidoni* and the external environment (Schmaljohann and Flügel, 1987). Symbionts are apparently newly acquired from the environment each generation.

Siboglinid endosymbionts are typically thought to fall into one of three general metabolic categories: (1) thiotrophic symbionts that oxidize sulfide or other reduced inorganic compounds and fix CO_2 by the Calvin-Benson cycle, (2) methanotrophic symbionts that use methane as both the source of energy for metabolism and the carbon for assimilation, or (3) heterotrophic symbionts found in the bone-eating worms, *Osedax* spp. (Fisher, 1990; Stewart *et al.,* 2005; Goffredi *et al.,* 2005, 2007). However, *R. pachyptila* symbionts also have enzymes for both the energy-generating tricarboxylic acid (TCA) cycle and an alternative carbon fixation pathway *via* the reverse TCA cycle, indicating that the metabolic mode of these symbionts is more complicated than previously believed (Markert *et al.,* 2007). In theory, the *R. pachyptila* symbiont community could switch between RuBisCO-autotrophy, heterotrophic oxidation of carbon storage compounds by the TCA cycle, and autotrophic growth by the reverse TCA cycle (Markert *et al.,* 2007). Regardless of the metabolic mode, the host is primarily dependent upon endosymbionts for its nutritional and energetic requirements (Southward *et al.,* 1986), although some host species may also absorb organic materials from the surrounding environment (Southward and Southward, 1970). The host provides a stable environment and access to necessary substrates (*e.g.,* sulfide, methane, oxygen, carbon dioxide) for bacterial metabolism, while the bacteria provide energy *via* the release of small nutritive molecules (Felbeck and Jarchow, 1998) or possibly by digestion of symbionts (Bright *et al.,* 2000; Goffredi *et al.,* 2005).

Studies to date have focused on endosymbionts associated with the larger vestimentiferans (reviewed in McMullin *et al.,* 2003) and *Osedax* spp. (Goffredi *et al.,* 2005, 2007), with few molecular studies addressing frenulate endosymbiont diversity *(i.e., Oligobrachia mashikoi* [Kimura *et al.,* 2003a; Kubota *et al.,* 2007] and *Oligobrachia* "sp. JT-1" [Naganuma *et al.,* 2005]). Molecular analyses based on 16S rDNA place the majority of known vestimentiferan and *Osedax* spp. endosymbionts within the γ–proteobacteria (*e.g.,* McMullin *et al.,* 2003; Stewart *et al.,* 2005; Goffredi *et al.,* 2007). Other bacterial groups have been detected *in hospite* with vestimentiferans as well, such as α, β, and ε –proteobacteria in *Lamellibrachia* spp. (Naganuma *et al.,* 1997a, b; Elsaied and Naganuma, 2001; Elsaied *et al.,* 2002; Kimura *et al.,* 2003b) and α–proteobacteria and *Cytophaga-Flavobacterium-Bacteroides* in *Ridgeia piscesae* (Chao *et al.,* 2007). However, the stability and persistence of these non-γ-proteobacteria associations is not clear at this time (Naganuma *et al.,* 2005; Stewart *et al.,* 2005; Chao *et al.,* 2007). Species of *Osedax* symbiotically associate with *Oceanospirillales*-like γ-proteobacteria endosymbionts (Goffredi *et al.,* 2005, 2007). Currently, there are no studies of endosymbiont diversity in moniliferans.

Because the symbiosis of frenulates is poorly understood, we molecularly characterized bacteria associated with the frenulate siboglinid *Siboglinum fiordicum* Webb 1963. To this end, we used standard molecular and phylogenetic approaches with laser-capture microdissection (LCM). *S. fiordicum* endosymbionts are a distinct group of γ–proteobacteria not associated with the endosymbionts of vestimentiferan or *Osedax* hosts.

Materials and Methods

Data collection

In September 1998, *Siboglinum fiordicum* was collected with a Smith-MacIntyre benthic grab sampler near Bergen, Norway. Table 1 lists the localities of *S. fiordicum* used in this study. For six worms, samples were divided into two subsamples and processed as follows. For the first subsample, worms were carefully removed from their tubes and rinsed in seawater. Genomic DNA was extracted from trophosome tissue, using a modified CTAB protocol (Doyle and Dickson, 1987). The ~1458-bp fragment of the bacterial 16S rRNA gene was amplified using bacterial 16S rRNA primers GM3F 5'-AGAGTTTGATCMTGGC-3' and

Table 1

Collection information and GenBank accession numbers of Siboglinum fiordicum *samples used in this study*

Individual number	Depth (m)	Lat/Long	Location in Norway	Clone number	GenBank accession number
12	33	60°33.274′N/5°01.264′E	Ypsesund Bay	1	EU086760
				2	EU086761
				3	EU086762
16	33			1	EU086763
				2	EU086764
				3	EU086765
31	36	60°16.166′N/5°05.529′E	Skoge Inlet	1	EU086766
				2	EU086767
32	36			1	EU086768
				2	EU086769
				3	EU086770
41	243	60°16.512′N/5°08.648′E	Raunefjorden	1	EU086771
				2	EU086772
49	243			1	EU086773
				2	EU086774

GM4R 5'-TACCTTGTTACGACTT-3' as described in Muyzer *et al.* (1995). All PCR cycling conditions were as follows: initial denaturation, 94 °C, 3 min; 30 cycles of denaturation, 94 °C, 45 s; annealing, 50 °C, 1 min; extension, 72 °C, 1.5 min; final extension, 72 °C, 5 min. PCR products were verified by agarose-gel electrophoresis, purified using spin columns (Promega, Inc.), and cloned using the pGEM-Teasy cloning kit (Promega, Inc.). The primers used for amplification and sequencing are described in Muyzer *et al*. (1995). Dye-labeled automated sequencing was carried out on either a Licor 4000 using the Excel Seqitherm II kit (Epicentre Inc.) or on a Beckman CEQ 8000 using Genome Lab Quick Start mix (Beckman Coulter). About three clones from each individual were completely sequenced in both directions.

To unambiguously demonstrate that obtained sequences were from host trophosome, we localized endosymbionts with laser-capture microdissection (LCM) (Espina *et al.,* 2006) on the second subsample from each of the six worms. This procedure permits target cell populations from histological sections to be isolated and "captured" uncontaminated by surrounding cells. Tissues were fixed in 75% ethanol prior to being dehydrated and embedded in Paraplast. Tissues were sectioned (~7 μm) and affixed to individual membrane-mounted metal frame slides (Molecular Machines and Industries, Inc. [MMI, Inc.], Manchester, NH) for LCM. Prior to LCM and capture of targeted cells, sections were deparaffinized, rehydrated, and stained using the DNA/RNA-Friendly Basic Staining kit (MMI, Inc). Imaging, LCM, and cell capture were performed on an MMI-CellCut LCM workstation. Mid-trophosome sections were used to confirm that symbiont identification was derived from host trophosome rather than from contaminating bacteria from the surrounding environment or on the exterior of the worm. DNA extraction and 16S rRNA gene amplification was conducted as described above for the first subsample from each worm. Because of the small size of the LCM sections, a whole-genomic amplification step was included (using a Genomiphi kit from GE Healthcare) prior to PCR.

Phylogenetic analysis

Taxa used in the phylogenetic analysis and their GenBank accession numbers are listed in Tables 1 and 2. Employed taxa represent a range of γ-subdivision organisms, but due to the endosymbiotic nature of the bacteria housed in *S. fiordicum*, this analysis focused on symbiotic organisms. Furthermore, because initial BLAST (Altschul *et al.,* 1997) results returned γ-subdivision sequences, the taxonomic breadth of the analysis was restricted to this subdivision. Taxa choice was, in part, based on the previous analysis of Teske *et al.* (1999) and on the most similar (*i.e.,* BLASTN) sequences available from GenBank (www.ncbi.org). 16S rRNA gene sequences of *Thiobacillus* spp. bacteria from the border of the β- and γ- proteobacteria subdivisions were used as outgroups (Teske *et al.,* 1999).

The Clustal X program, ver. 2.0 (Thompson *et al.,* 1997), was used to align the obtained sequences to available data (Table 2). The resultant data set was manually corrected on the basis of ribosomal secondary structure available from the Ribosomal Database Project II (accessed July 2007: http://rdp.cme.msu.edu/). Ambiguously aligned regions were excluded. Topologies were produced under Bayesian inference using MrBayes (Huelsenbeck and Ronquist, 2001) implementing the GTR+I+G model of substitution, suggested by MrModeltest 2.2 (Nylander, 2004). Two sets of four chains (3 hot, 1 cold) were run for 2×10^6 gener-

Table 2

Bacterial 16S sequences used in the Bayesian inference phylogeny

Bacteria	GenBank accession number
Acidithiobacillus ferrooxidans strain Nfe-3	X75268
Acinetobacter johnsonii	X89775
Bathymodiolus japonicus gill symbiont	AB036711
Bathymodiolus platifrons gill symbiont	AB036710
Bathymodiolus thermophilus gill symbiont	M99445
Beggiatoa alba strain B15LD	L40994
Beggiatoa sp. Bay of Concepción	AF035956
Calyptogena magnifica symbiont	M99446
Calyptogena sp. symbiont	L25708
Escarpia laminata endosymbionts	AY129102, AY129106–AY129109
Escarpia spicata endosymbiont	U77482
Escherichia coli	J01859
Halothiobacillus hydrothermalis strain r3	M90662
Lamellibrachia barhami endosymbionts	AY129090, AY129093, AY129103, AY129113
Lamellibrachia columna endosymbiont	U77481
Lamellibrachia cf. *luymesi* endosymbiont	AY129100
Lamellibrachia sp. 1 endosymbiont	AY129112
Lamellibrachia sp. 2 endosymbionts	AY129110, AY129111
Lucina floridana symbiont	L25707
Lucinoma aequizonata symbiont	M99448
Methylosarcina	AY007296
Ridgeia piscesae endosymbionts	AY129119, U77480
Riftia pachyptila endosymbionts	AY129115, U77478
Oasisia alvinae endosymbiont	AY129114
Oligobrachia mashikoi endosymbionts	AB057751, AB271120–AB271125
Oligobrachia sp. JT1 "endosymbiont"	AB070215
Osedax frankpressi endosymbionts	AY549004, DQ919529–DQ919536
Osedax rubiplumis endosymbiont	AY549005
Osedax sp. MB3 endosymbionts	DQ919537–DQ919542
Osedax sp. MB4 endosymbionts	DQ919543–DQ919547
Pseudomonas tolaasii	AF320990
Seepiophila jonesi endosymbionts	AY129089, AY129101, AY129104, AY129105
Siboglinum fiordicum	EU086760–EU086774
Solemya occidentalis symbiont	U41049
Solemya reidi symbiont	L25709
Solemya velum symbiont	M90415
Tevnia jerichonana endosymbiont	AY129117
Thiobacillus denitrificans	AJ243144
Thiomicrospira crunogena	NC_007520
Uncultured gamma proteobacterium	AY225630
Vestimentiferan "Shinkai" endosymbiont	AF165907
Vesicomya chordata symbiont	L25713

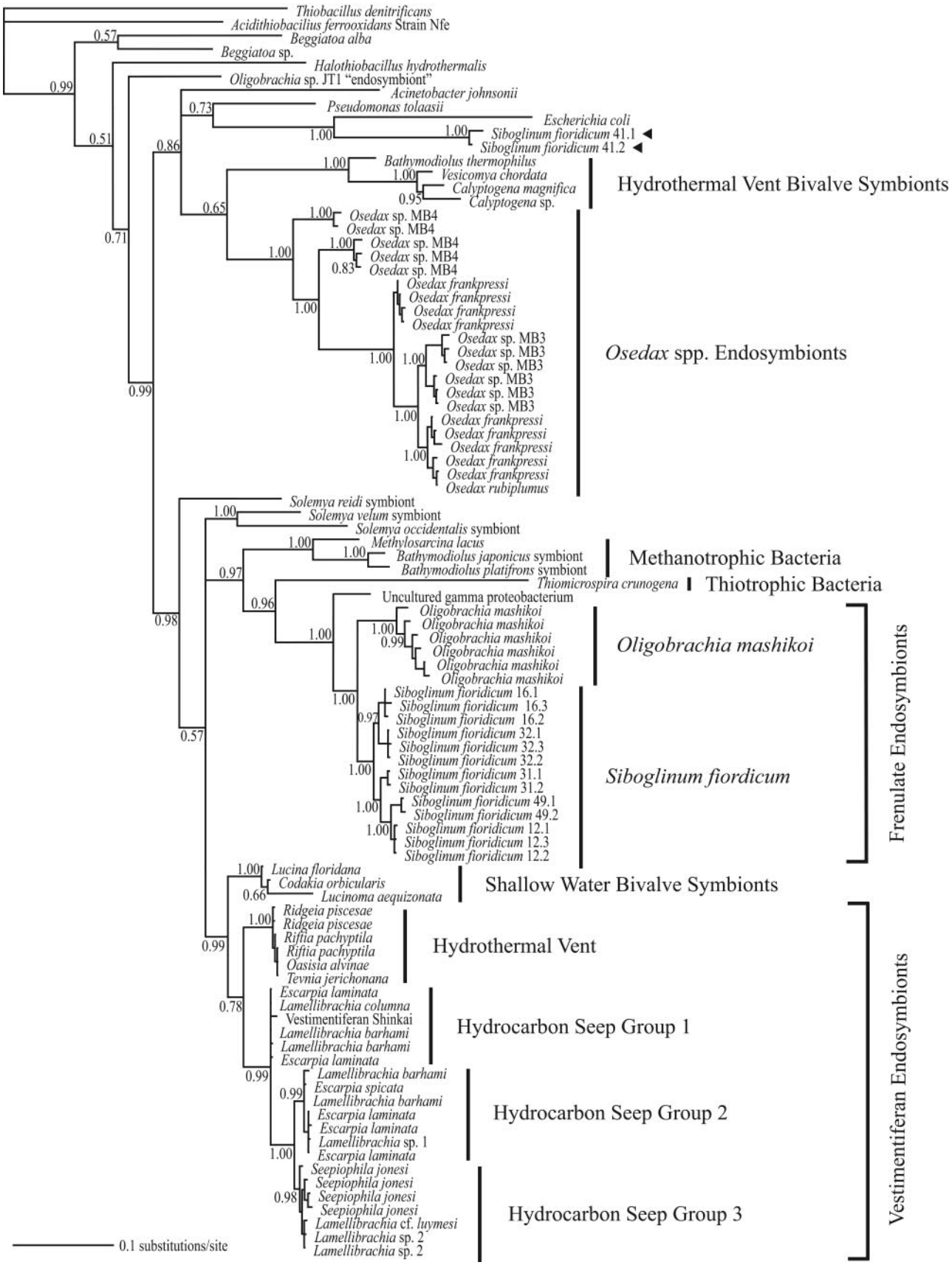
ations and sampled every 100 generations. The first 100,000 generations were discarded as burn-in, and a 50% majority-rule consensus tree was calculated from the resulting 38,000 trees (19,000 trees from each run). Nodal supports (posterior probabilities) were recorded for each recovered clade to assess the reliability of recovered nodes.

Results

Phylogenetic analysis

The data set consisted of 1458 nucleotide positions, of which 1376 could be unambiguously aligned. Of these, 618 positions (44.9%) were variable and 443 (32.2%) were

Figure 1. Bayesian inference phylogeny of known γ-proteobacteria siboglinid endosymbionts based on 1458-bp 16S rRNA gene sequences. Posterior probability values indicated next to the relevant node. Important taxa are labeled for siboglinid endosymbionts and other γ-proteobacteria of interest. Unusual *Siboglinum fiordicum* "endosymbionts" not recovered by laser-capture microdissection are designated by a black triangle. Numbers next to the *Siboglinum* branches indicate individual and clone numbers (separated by a decimal).


Thiobacillus denitrificans
Acidithiobacilius ferrooxidans Strain Nfe
Beggiatoa alba
Beggiatoa sp.
Halothiobacillus hydrothermalis
Oligobrachia sp. JT1 "endosymbiont"
Acinetobacter johnsonii
Pseudomonas tolaasii
Escherichia coli
Siboglinum fioridicum 41.1
Siboglinum fioridicum 41.2
Bathymodiolus thermophilus
Vesicomya chordata
Calyptogena magnifica
Calyptogena sp.
Hydrothermal Vent Bivalve Symbionts
Osedax sp. MB4
Osedax frankpressi
Osedax sp. MB3
Osedax rubiplumus
Osedax spp. Endosymbionts
Solemya reidi symbiont
Solemya velum symbiont
Solemya occidentalis symbiont
Methylosarcina lacus
Bathymodiolus japonicus symbiont
Bathymodiolus platifrons symbiont
Methanotrophic Bacteria
Thiomicrospira crunogena
Thiotrophic Bacteria
Uncultured gamma proteobacterium
Oligobrachia mashikoi
Siboglinum fioridicum 16.1
Siboglinum fioridicum 16.3
Siboglinum fioridicum 16.2
Siboglinum fioridicum 32.1
Siboglinum fioridicum 32.3
Siboglinum fioridicum 32.2
Siboglinum fioridicum 31.1
Siboglinum fioridicum 31.2
Siboglinum fioridicum 49.1
Siboglinum fioridicum 49.2
Siboglinum fioridicum 12.1
Siboglinum fioridicum 12.3
Siboglinum fioridicum 12.2
Siboglinum fiordicum
Frenulate Endosymbionts
Lucina floridana
Codakia orbicularis
Lucinoma aequizonata
Shallow Water Bivalve Symbionts
Ridgeia piscesae
Riftia pachyptila
Oasisia alvinae
Tevnia jerichonana
Hydrothermal Vent
Escarpia laminata
Lamellibrachia columna
Vestimentiferan Shinkai
Lamellibrachia barhami
Hydrocarbon Seep Group 1
Escarpia spicata
Lamellibrachia sp. 1
Hydrocarbon Seep Group 2
Seepiophila jonesi
Lamellibrachia cf. luymesi
Lamellibrachia sp. 2
Hydrocarbon Seep Group 3
Vestimentiferan Endosymbionts
0.1 substitutions/site

parsimony informative. The nucleotide composition of recovered sequences was 26.0% adenines, 22.0% cytosines, 31.5% guanines, and 20.5% thymines and does not appear to have exhibited a strong bias. The aligned data set is available from TREEBASE (http://www.Treebase.org study accession number S2007; matrix accession number M3746).

The topology produced by Bayesian inference is shown in Figure 1. Most *Siboglinum fiordicum* 16S endosymbiont sequences clustered as a well-supported (posterior probability = 1.0), monophyletic clade. This clade was most closely related to the endosymbionts of the frenulate *Oligobrachia mashikoi* and more distantly related to an uncultured γ-proteobacterium, thiotrophic *Thiomicrospira* spp. bacteria (represented here by *T. crunogena*), methanotrophic *Bathymodiolus* spp. endosymbionts, and methanotrophic *Methylosarcinalacus* sp. bacteria. *Siboglinum fiordicum* endosymbiont 16S sequences did not cluster with known vestimentiferan or *Osedax* spp. endosymbionts. Only sequences derived from individual 41 from Raunefjorden fell outside of this *S. fiordicum* endosymbiont lineage. These particular sequences were also not detected in extractions taken by LCM (see below). Instead, bacteria from individual 41 clustered with *E. coli* and other nonsymbiotic γ-proteobacteria. Therefore, we interpret the individual 41 sequences cautiously, as they possibly represent contaminant bacteria from the surrounding environment or the external surface of the host, and not host-associated endosymbionts. Alternatively, these bacteria could represent an additional lineage of mutualistic endosymbionts. Further investigation is warranted to determine the nature of the bacterial lineage associated with individual 41.

Clones from within an individual worm were highly similar to one another, differing by 2 to 11 bp (uncorrected genetic distance $p = 0.0014$ to 0.0075), which is consistent with a single bacterial lineage per host (Coenye and Vandamme, 2003). No additional evidence of mixed endosymbiont communities was detected in any of the hosts examined.

Laser-capture microdissection

Figure 2 illustrates a representative example of trophosome and other internal host tissues extracted *via* LCM without contamination from the external environment or host epidermis. Excised cells were PCR amplified, sequenced, and compared to sequences obtained from the six extractions from the rinsed host organism. Sequences obtained from LCM-extracted cells were 100% identical to sequences from the corresponding whole-organism preparation (except individual 41, see above), indicating that recovered 16S sequences originated from trophosome tissue.

Discussion

Our results, combined with those from previous studies of siboglinid endosymbionts, indicate that three major clades of γ-proteobacteria are associated with siboglinid annelids. Athough these bacterial clades are not closely related to one another, each clade corresponds to a major group of siboglinids: frenulates, vestimentiferans, and *Osedax* spp. This correspondence suggests host-symbiont specificity at higher taxonomic levels in Siboglinidae. Furthermore, differences in endosymbiont population may play a role in driving host evolution, similar to the hypothesis Schulze and Halanych (2003) based on habitat and sulfide tolerance. Given that very little is known about frenulate endosymbionts, the close relationship of *Siboglinum fiordicum* symbionts from Norway to symbionts of another frenulate, *Oligobrachia mashikoi* from Japan, is interesting. Perhaps bacterial symbionts of frenulates share a common evolutionary history, metabolic features, or both.

Within the frenulate endosymbiont clade, two well-supported subgroups were identified: one group associated with *S. fiordicum* from Norwegian fjords and a second group associated with *O. mashikoi* from the coastal waters of Japan. Whether these subgroups are separated on the basis of specificity at the level of host species, geographic isolation, or both of these factors is not clear at this time. On the basis of electron microscopy studies, E. C. Southward suggested that each species of frenulate may harbor unique endosymbionts (Southward, 1982). In comparison, vestimentiferan endosymbionts show little specificity at the level of the host species. For example, site-specific symbiont phylotypes were found in multiple host species, including *Riftia pachyptila*, *R. piscesae*, *Oasisia alvinae*, and *Tevnia jerichonana* (Feldman *et al.,* 1997; Di Meo *et al.,* 2000; Nelson and Fisher, 2000) and *Lamellibrachia* spp. and *Escarpia* spp. (Nelson and Fisher, 2000; Vrijenhoek *et al.* 2007). Instead, habitat, geography, and host specificity to vent *versus* seep taxa (or a combination of these) appear to govern symbiotic associations in vestimentiferans. Although vestimentiferan symbionts form one large clade, this clade is divided into several significant subgroups, or phylotypes. The deepest division in the vestimentiferan endosymbiont clade occurs between symbionts found in vents and those found in seep hosts or environments (Feldman *et al.,* 1997; Nelson and Fisher, 2000; Di Meo *et al.,* 2000; Vriejenhoek *et al.,* 2007). This pattern holds true even from geographically proximate vent and seep species, such as at the Middle Valley segment of Juan de Fuca Ridge in the northeast Pacific, where *R. piscesae* (a vent species) and *L. barhami* (a seep species) occur in proximity but harbor different symbionts (Nelson and Fisher, 2000). Among seep vestimentiferan endosymbionts, three well-supported subgroups have been identified (Nelson and Fisher, 2000; McMullin *et al.,* 2003). All three groups are found in symbioses

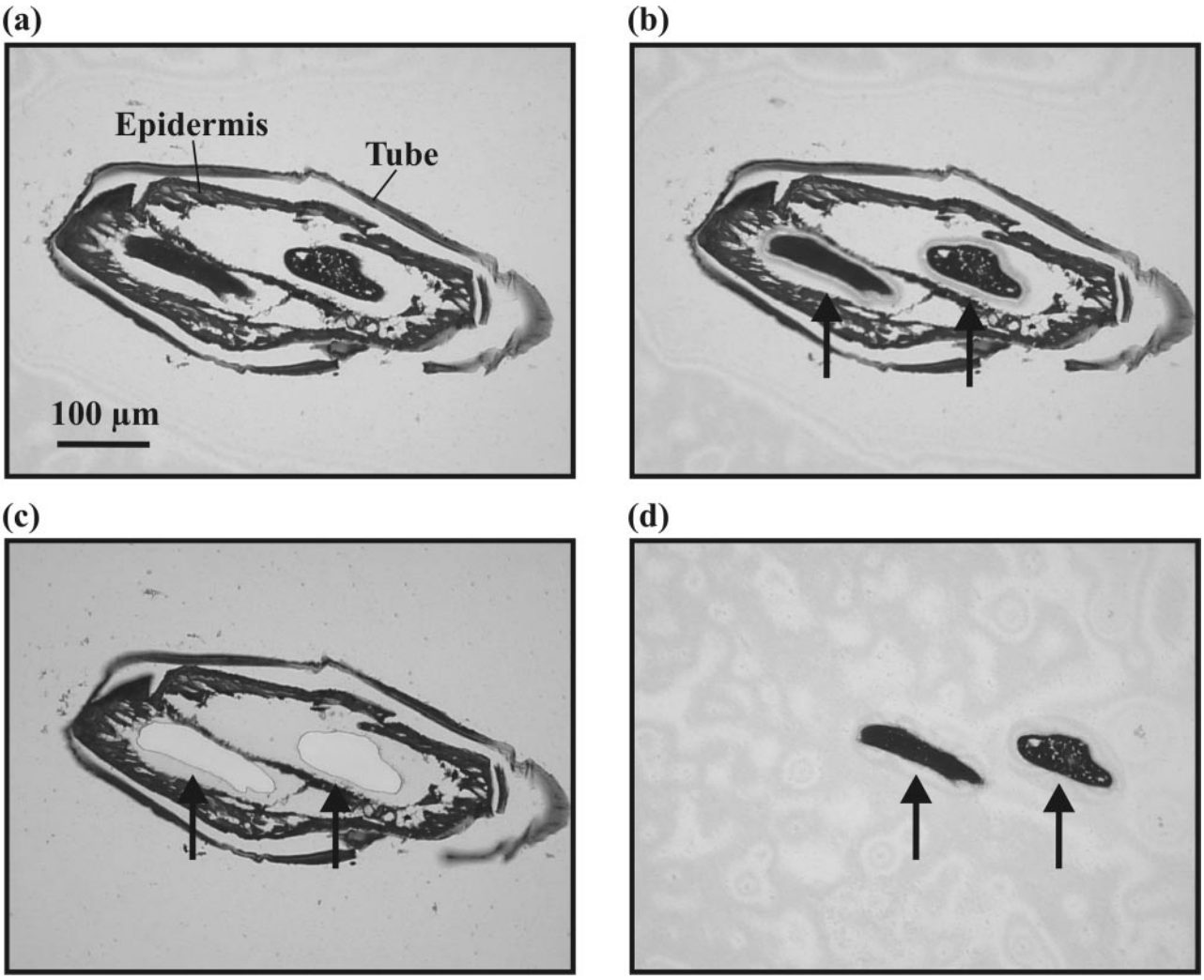


Figure 2. Laser microdissection and capture of endosymbiotic bacteria from *Siboglinum fiordicum*. Note that preservation in 70% ethanol resulted in dehydration of tissues, potentially altering the worm's internal morphology. Original magnification 20×. (a) Cross section of *S. fiordicum* including bacteriocytes within the host trophosome. Epidermis and external protein/chitin tube labeled for reference. (b) Region after laser excision of worm tissue including bacteriocytes containing numerous bacteria (note grey excision lines surrounding targeted cells; arrow). (c) Region after excision and capture of targeted bacteriocytes (arrow). (d) Excised and captured bacteriocytes prior to nucleotide extraction.

with *Lamellibrachia* and *Escarpia* spp. and are distinguished by their biogeographic or bathymetric distribution (reviewed in McMullin *et al.,* 2003). Although it seems likely that habitat and geography similarly govern symbiotic associations of frenulate endosymbionts, including those of *S. fiordicum*, it is entirely possible that different patterns, such as specificity to different host species, have evolved in this group. Additional information from other frenulate endosymbionts will be required to test such hypotheses.

Within a broad bacterial tree spanning five subdivisions, Naganuma *et al.* (2005) placed a new endosymbiont from an undescribed species of frenulate siboglinid, *Oligobrachia* sp. JT1, as closely related to vestimentiferan endosymbionts. In contrast, our endosymbiont topology agrees in most respects with those reported in previous studies (*e.g.,* McMullin *et al.,* 2003; Goffredi *et al.,* 2007), and we find the *Oligobrachia* sp. JT1 endosymbiont to be more basal and unassociated with vestimentiferan endosymbionts. The discrepancy in these findings may result from the breadth of bacterial types included or the rooting with *E. coli* (within γ-proteobacteria) in the phylogeny proposed by Naganuma *et al*. Additionally, the *Oligobrachia* sp. JT1 endosymbiont was not localized to host trophosome, and therefore it is unclear whether this bacterium represents a novel endosymbiont lineage or a bacterium from the external environment.

Phylogenetic proximity of *S. fiordicum*'s endosymbiont to known thiotrophs, including *Thiomicrospira crunogena* (Scott *et al.,* 2006), suggests thiotrophy as a likely metabolic

mode for this bacteria. Previous studies similarly indicated thiotrophy in *S. fiordicum*'s endosymbiont. For instance, Southward *et al.* (1986) detected enzymatic activity consistent with thiotrophy, including RuBisCO, adenylylsulphate reductase, and sulphate adenylyltransferase, in the trophosome region of *S. fiordicum*. Fixation of CO_2 was also enhanced in the presence of sulfide and thiosulfate but not methane (Southward *et al.,* 1986). Similarly, Dando *et al.* (1986) detected sulfur-oxidizing enzyme activity in S. *fiordicum,* suggesting probable thiotrophy. However, Kimura *et al.* (2003a) interpreted the closely related endosymbiont of *O. mashikoi* as a possible methanotroph on the basis of phylogenetic affinity (also seen in our proposed phylogeny) to known methanotrophs, *Methylosarcina* sp. and symbionts of *Bathymodiolus japonicus* and *B. platifrons* (Fujiwara *et al.,* 2000). Despite this, further evidence for methanotrophy, including methane monooxygenase genes (*pmoA* and *mmoX*) and intracytoplasmic-stacked membranes (Cavanaugh *et al.,* 1992; Lidstrom, 1992), was not detected in this endosymbiont (Kimura *et al.,* 2003a; Deguchi *et al.,* 2007). Additionally, a RuBisCO gene was detected in *O. mashikoi*'s trophosome, consonant with a thiotrophic endosymbiont (Kimura *et al.,* 2003a).

Consistent with the suggestion that each species harbors a single type of bacterial symbiont (Southward, 1982), multiple endosymbiont lineages were not detected within an individual *S. fiordicum* sample. However, our sampling regime was directed only at determining the numerically dominant symbiont in these hosts. Admittedly, with only a few clones sequenced per host, it is possible that other bacterial lineages went undetected in this survey. Other siboglinids appear to harbor multiple bacterial species in the trophosome of a single host. In vestimentiferans, both substrate oxidation and TEM observations suggest the presence of mixed symbiont communities (Fisher and Childress, 1984; Southward, 1988; de Burgh *et al.,* 1989). Multiple metabolic pathways detected in the proteomics study by Markert *et al.* (2007) could also be interpreted as the presence of multiple symbionts. Most definitively, molecular studies indicate that *E. spicata*, *L. barhami*, the undescribed species *Lamellibrachia* sp. L1, and *R. piscesae* all host multiple endosymbionts (Elsaied *et al.,* 2002; Kimura *et al.,* 2003b; Chao *et al.,* 2007; Vrijenhoek *et al.,* 2007).

Herein we characterized the dominant bacterial endosymbiont associated with the frenulate siboglinid *S. fiordicum* as a group of γ–proteobacteria. Bayesian analysis indicated that these bacteria are closely related to endosymbionts of *O. mashikoi*, the only previously characterized frenulate endosymbiont. Both phylogenetic affinity and results from previous studies also suggest thiotrophy as the metabolic mode in these endosymbiotic bacteria. Using laser-capture microdissection (LCM), we were able to definitively localize putative symbionts to host trophosome. To our knowledge, this is the first application of LCM to marine symbiosis research. The successful targeting of nucleic acids from specific tissue regions by LCM may be further applicable in future investigations of marine symbioses, such as tissue-specific studies of symbiotic gene expression or microhabitat niche partitioning among different symbionts within a host. Much remains to be discovered about the biology of endosymbionts in the globally distributed and diverse Frenulata and Monilifera siboglinids. Additional studies of their genetic and metabolic diversity will greatly improve our understanding of the ecology and evolution of these important marine symbioses.

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