Solemya pervernicosa Lives in Sediment underneath Submerged Whale Carcasses: Its Biological Significance

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Abstract: *Solemya pervernicosa*, which had previously been regarded as a bathyal species restricted to northeastern Japan, occurred in the sediment underneath whale carcasses submerged experimentally on the shelf off Cape Noma-misaki, Satsuma Peninsula, southwestern Japan, at depths of 219–254 m. This occurrence represents a significant range extension. Transmission electron microscopic observations of the gill tissue revealed dense bacteria in the epithelial cells. A molecular phylogenetic analysis strongly indicated that these bacteria are thioautotrophs closely related to the thioautotrophic symbionts associated with *Solemya reidi* and *Acharax johnsoni*. Analysis of carbon and sulfur isotopes of soft tissues of *S. pervernicosa* proved that this bivalve depends nutritionally on thioautotrophic symbionts. However, it is not clear at present whether the symbiont is transmitted vertically, as with some other chemosynthetic bivalves, or environmentally.

Keywords: *Solemya pervernicosa*, whale-fall ecosystem, sulfur isotope, thioautotrophic symbiont

Introduction

For the purpose of corroboration of the geographic "stepping stone" hypothesis in regard to dispersal of sulfide-dependent/chemosynthesis-based biota (Smith *et al.*, 1989), the biological communities on and around experimentally submerged sperm whale bodies have been investigated for three years (Fujiwara *et al.*, 2007). The whale bodies were placed on the shelf slope off Cape Noma-misaki on the Satsuma Peninsula in Kyushu in 2002, and were examined in 2003, 2004 and 2007 with the ROV *Hyper-Dolphin* of the Japan Agency for Marine-Earth Science & Technology (JAMSTEC).

The unexpected discovery of huge mass aggregations of the mytilid *Adipicola pacifica* on these whale carcasses has already been reported (Okutani *et al.*, 2004). An examination of the sediments underneath the carcasses resulted in the additional discovery of the awning clam *Solemya* (*Petrasma*) *pervernicosa* Kuroda, 1948, which belongs to the family Solemyidae. Solemyids are known to depend nutritionally on chemoautotrophic symbionts in a wide variety of reducing environments, such as hydrothermal vents, seeps and organic-rich sediments. The finding of this solemyid in a whale-fall ecosystem provides additional information concerning the ecology

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of this group, and is worth reporting. Furthermore this paper discusses the biological significance of this occurrence in terms of biochemical environmental processes and the characters of the symbiotic bacteria, based on transmission electron microscopy and molecular analyses of the bivalve.

Abbreviations: HD – ROV *Hyper-Dolphin* Dive number; SL – shell length.

Material and methods

Material

Specimens of *Solemya* (*Petrasma*) *pervernicosa* were collected on and around sperm whale bodies. Twelve of fourteen whales (11.85–16.00 m in body length) that originally beached on the west coast of the Satsuma Peninsula, Kyushu in February 2002 were placed on the shelf slope off Cape Noma-misaki (Fig. 1), at depths of 219–254 m. They were explored repeatedly by the ROV *Hyper-Dolphin* in the periods July 26 to August 1, 2003 and July 26 to 29, 2004, about 17 and 29 months after submergence. Bivalves were also collected from surrounding sediments, which were scooped up for analyses of infauna and monitoring of the physico-chemical environment around the whale bodies. The specimens used for molecular analyses were initially deep-frozen, then fixed and preserved in 99% ethanol. Following biochemical analyses, they were transferred to 70% ethanol. The empty shells and dead collected specimens are preserved dry. All voucher specimens are to be deposited in the biological collection of JAMSTEC, Yokosuka City, Japan.

Material examined

Live-taken specimens — 1 ex. (34.9 mm SL), HD #192, 28 July 2003, 31°20.7´N, 129°59.3´E, 229 m (Fig. 2A); 1 ex. (26.7 mm SL), HD #226, 28 July 2004, 31°21.0´N, 129°59.2´E, 226 m; 1 ex. (19.7 mm SL), HD #332, 29 July 2004, 31°21.0´N, 129°59.2´E, 225 m.

Empty shells — 1 ex. (24.0 mm SL), HD #196, 30 July 2003, 31°21.0´N, 129°59.2´E, 228 m (Fig. 2B); 3 exs. (36.4, 36.5, 39.0 mm SL), HD #328, 27 July 2004, 31°20.7´N, 129°59.3´E, 220 m; 1 ex. (20.2 mm SL), HD #331, 28 July 2004, 21°21.0´N, 129°59.2´E, 226 m.
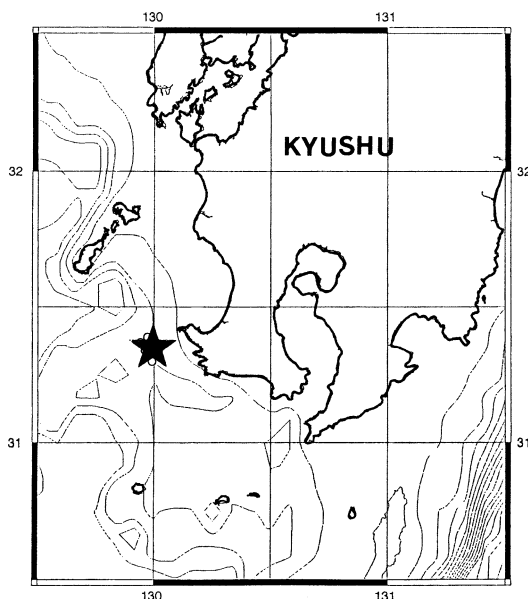

Fig. 1. Location of submerged whale carcasses, where the ROV *Hyper-Dolphin* surveyed (star).
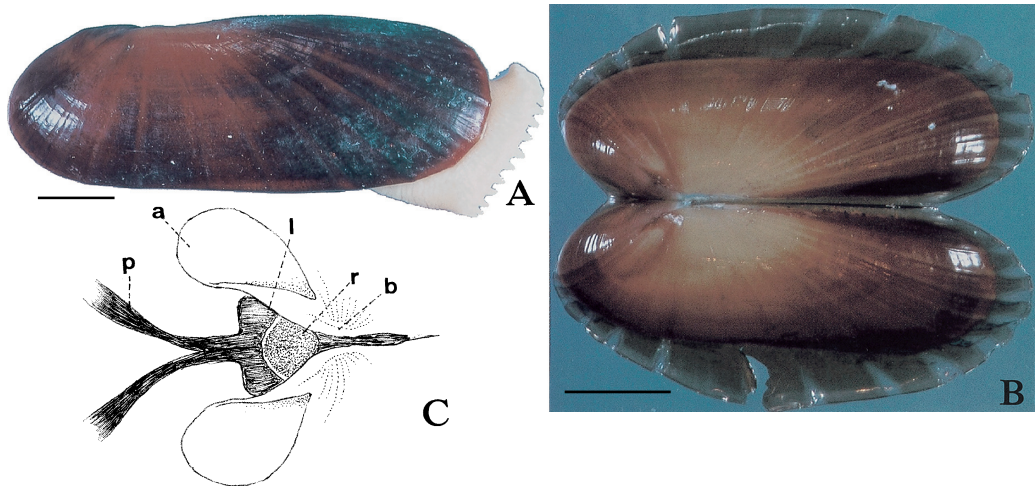
Fig. 2. *Solemya pervernicosa*. **A**. Specimen collected alive by HD #192 (SL 34.9 mm). Scale bar = 5 mm. **B**. Empty shell collected by HD #196 (SL 24.0 mm). Scale bar = 5 mm. **C**. Hinge from ventral view. Abbreviations: a, posterior adductor scar; b, buttress; l, ligament; p, periostracum; r, resilium.

Isotope analysis

The concentration and isotopic composition of sulfide-sulfur were measured following the methods described by Yamanaka *et al*. (2003a). Concentrations of ammonium in the pore water from the substrate sediment samples were measured on board ship using the colorimetric method described by Gamo & Gieskes (1992). Samples in which higher concentrations of ammonium were detected in the water had the ammonium extracted with 2M KCl solution in the shipboard laboratory. The extracted ammonium was recovered by a steam distillation technique using a gas-tight container (semi-micro Kjeldahl apparatus) and finally converted into the water-insoluble form tetra-phenyl-borate, as described by Sakata (2001). Nitrogen isotope ratios in the recovered borates were analyzed using a mass spectrometer in continuous-flow mode (NA2500 elemental analyzer as a gas preparation system and Delta-plus mass spectrometer with Conflo II, Thermo Electron, USA). The carbon, nitrogen and sulfur isotope compositions of the soft body parts of the clam were measured according the method described by Yamanaka *et al*. (2003b), except for carbon and nitrogen isotopes that were measured using the mass spectrometer in continuous-flow mode. Sulfur isotopic compositions were measured using composite sample of several individuals due to their low recovery from soft tissues. All the isotopic results were expressed by d notations (δ^{13}C, δ^{15}N and δ^{34}S), per mil (‰) variation relative to international stable isotope standards. Analytical errors associated with the overall procedure are less than +0.2‰.

Treatment for TEM observations

Small pieces of gill tissue were fixed with 2.5% glutaraldehyde in filtered seawater at 4°C for one week. Blocks were rinsed 10 times with 0.05M phosphate buffer (pH7.8) for 10 minutes at room temperature and post fixed in 1% OsO_4 in 0.05M phosphate buffer (pH7.8) at 4°C for 2 hours. Then, tissues were dehydrated and embedded in EPON 812 resin (TAAB, Aldermaston, UK). Ultra-thin sections of the specimens were stained with uranyl acetate and lead citrate, and were observed by a JEOL JEM-1210 transmission electron microscope at an acceleration voltage of 80 kV.

DNA preparation

DNA was extracted from the gill tissue of the host species. To eliminate surface contaminants,

each gill was thoroughly washed in autoclaved and filtered (0.22 μm) seawater. DNA extraction from tissue samples were conducted using a DNeasy kit (Qiagen Japan, Tokyo, Japan).

PCR amplification

The bacterial 16S rRNA gene was amplified by PCR using the Ex Taq PCR kit (TaKaRa, Kyoto). Two oligonucleotide primers (0.2 μM each) and <1 μg of DNA template were added to the reaction mixtures. Thermal cycling was as follows: denatured at 96°C for 20 seconds, annealed at 55°C for 45 seconds, and extended at 72°C for 2 minutes for a total of 35 cycles. The oligonucleotide primer sequences used for bacterial 16S rRNA gene amplification were Bac27F and 1492R (Lane, 1991). Molecular size of the PCR products was checked by 1.2% Agarose S (Nippon Gene, Toyama) gel electrophoresis.

Cloning of amplified 16S rRNA genes

The PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA). For bacterial genes, the PCR amplicons were cloned into the pCR-TOPO vectors using the TOPO TA cloning kit (Invitrogen, San Diego, USA). The DNA constructs were transferred into *Escherichia coli* TOP10 cells (Invitrogen, San Diego, USA). Blue/white screening was performed to select colonies of *E. coli* that had vectors with the inserted sequence.

Sequencing of amplified 16S rRNA genes

DNA sequencing of the amplified 16S rRNA genes was performed using the BigDye Terminator Cycling Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, USA). Nine universal 16S rRNA gene-specific primers were used in sequencing reactions (Kato *et al.*, 1997). Sequencing was performed using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, USA). The sequences reported here have been deposited in the DDBJ database under the accession number AB499617.

Sequence and phylogenetic analyses

Nearly complete sequences of the 16S rRNA gene were analyzed using the gapped-BLAST search algorithm (Altschul *et al.,* 1997; Benson *et al*., 2000) to estimate the degree of similarity to other 16S rRNA gene sequences. A single 1426 bp sequence was used to run the similarity analysis. The database used for similarity analysis was the non-redundant nucleotide sequence database from GenBank. Sequences were manually aligned and phylogenetic analysis was restricted to nucleotide positions that were unambiguously alignable in all sequences. The genetic distance between operational taxonomic units (OTUs) was calculated by Kimura's two-parameter method (Kimura, 1980). Neighbor-joining analysis was accomplished using the CLUSTAL X software package (Thompson *et al.,* 1997). Bootstrap analysis was used to provide confidence estimates for phylogenetic tree topologies.

Results

Gross morphology

The general morphology agrees with other members of *Solemya*. The periostracum is ornamented by dark bands radiating from the umbo to shell margin. The ligament is situated internally in front of beak, and is triangular in shape. The resilium is large. The buttress hemmed dorsal margin of posterior adductor scar (Fig. 2C). The sole is broad with a crenulated margin.

Isotope analysis

Carbon isotope composition (δ^{13}C) values obtained from the soft body parts of the *Solemya*

specimens ranged from –30.3 to –32.7‰ ($n = 5$). These lower $\delta^{13}C$ values indicated the existence of a carbon fixation pathway involving the Calvin-Benson cycle (Nelson & Fisher, 1995) and confirmed the previous results for *Solemya* symbionts ($\delta^{13}C = -31.0 \sim -35.4$‰: Yamanaka *et al.*, 2008).

Sulfur isotope composition ($\delta^{34}S$) values of the soft body parts were +2.5 and +3.7‰, while the $\delta^{34}S$ values of recovered sulfide-sulfur in the substrate sediments ranged from +1.8 to +8.9‰ ($n = 5$). These values are significantly lower than those for general seawater sulfate ($\delta^{34}S = +21$‰: Rees *et al.*, 1978). These low $\delta^{34}S$ values in the soft body parts indicate a sulfur source other than ambient seawater sulfate.

Nitrogen isotope composition ($\delta^{15}N$) values of the soft body parts ranged from +3.3 to +4.7‰ ($n = 5$), while the $\delta^{15}N$ values of recovered ammonium in the substrate sediments ranged from +5.5 to +7.5‰ ($n = 4$). In addition, the $\delta^{15}N$ values of suspended material obtained from near the habitat were +2.5 ± 1‰ ($n = 8$). Relative to common marine clams (> +5‰: *e.g.*; Kwak & Zedler, 1997; Page & Lastra, 2003), all the soft body parts examined in this study showed slightly low $\delta^{15}N$ values. The ammonium concentration in the water of the substrate sediment ranged from 0.1 to 2.9 mM. This is a significantly higher value relative to the common marine sediments overlying oxic seawater. Such an unusual concentration is related to the microbial decomposition of the whale remains.
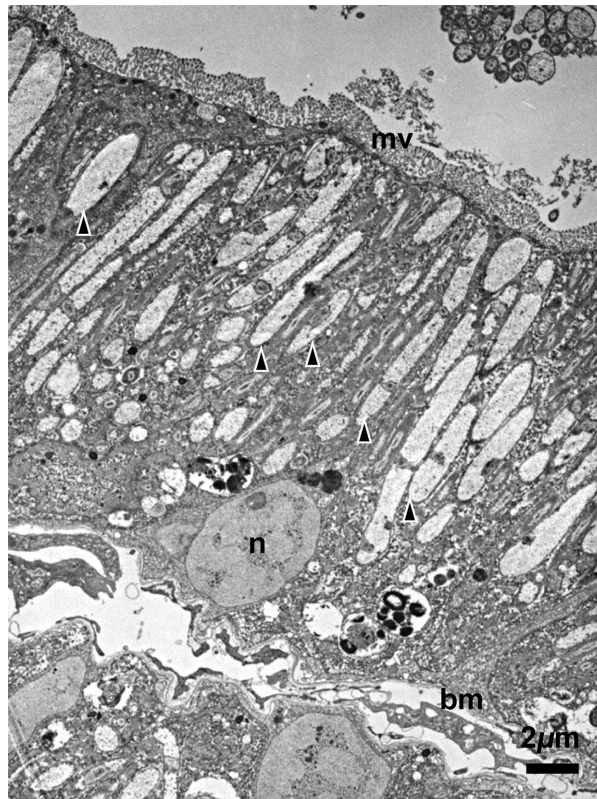

Fig. 3. *Solemya pervernicosa.* TEM photo of a transverse section of a gill filament showing intracellular gram-negative bacterial symbionts (arrowheads) without stacked internal membranes. Abbreviations: bm, basal membrane; mv, microvilli; n, nucleus.
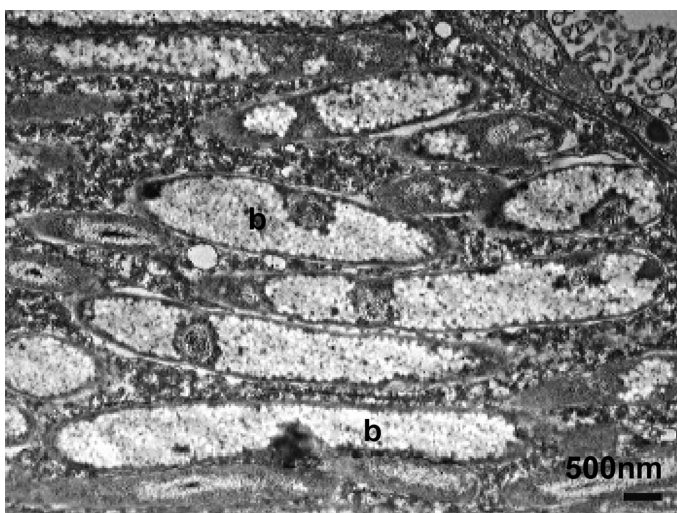
Fig. 4. *Solemya pervernicosa*. High magnification of TEM photo of intracellular bacterial symbionts within a bacteriocyte of epithelium of host gill. The symbionts were gram-negative and contained no stacked internal membranes. Abbreviation: b, bacteria.

TEM observations

Numerous bacteria were observed in the epithelial cells of the gill tissue of *S. pervernicosa* using the TEM (Figs. 3, 4). Mono-morphological bacteria were visible within the vacuoles of the host cells. A trilamellar cell envelope, typical of gram-negative bacteria, surrounded the bacteria (Fig. 4). Possible divisional stages of the bacteria were only occasionally observed (data not shown). The bacteria lacked internal membranes and in form were small cocci or rods that averaged 5.05 μm (SD = 1.49, n = 34) in the length of the major axis. The external surface of each bacteriocyte was fringed by well-developed microvilli.

16S rRNA gene sequences

Partial sequencing (~500 bp) of the bacterial 16S rRNA gene was performed on 85 appropriately sized inserts, which were amplified from three specimens of *S. pervernicosa* gills using Bac27F and 1492R as primers. All inserts were homogeneous. Nearly complete sequences of 16S rRNA gene from nine clones were also homogenous (100% identical between the three sequences), and the lengths of the inserts were 1426 bp.

Phylogenetic analysis of 16S rRNA gene sequences

Phylogenetic analysis performed by the neighbor-joining method placed the 16S rRNA gene sequence of the *S. pervernicosa* symbiont within the γ subdivision of the Proteobacteria, which encompasses the majority of thioautotrophic symbionts in marine invertebrate hosts (Fig. 5). The symbiont formed a monophyletic group supported by a bootstrap value of 100% with the symbionts of *Solemya reidi*, which are the sibling clade of the *Acharax johnsoni* symbionts, but bootstrap analysis did not support monophyly with the latter group.

Discussion

Since Kuroda (1948) originally described *S. pervernicosa* from off Cape Erimo, Hokkaido, at a depth of ca. 250 fathoms (holotype: 53 mm SL), there have been few reliable records of its
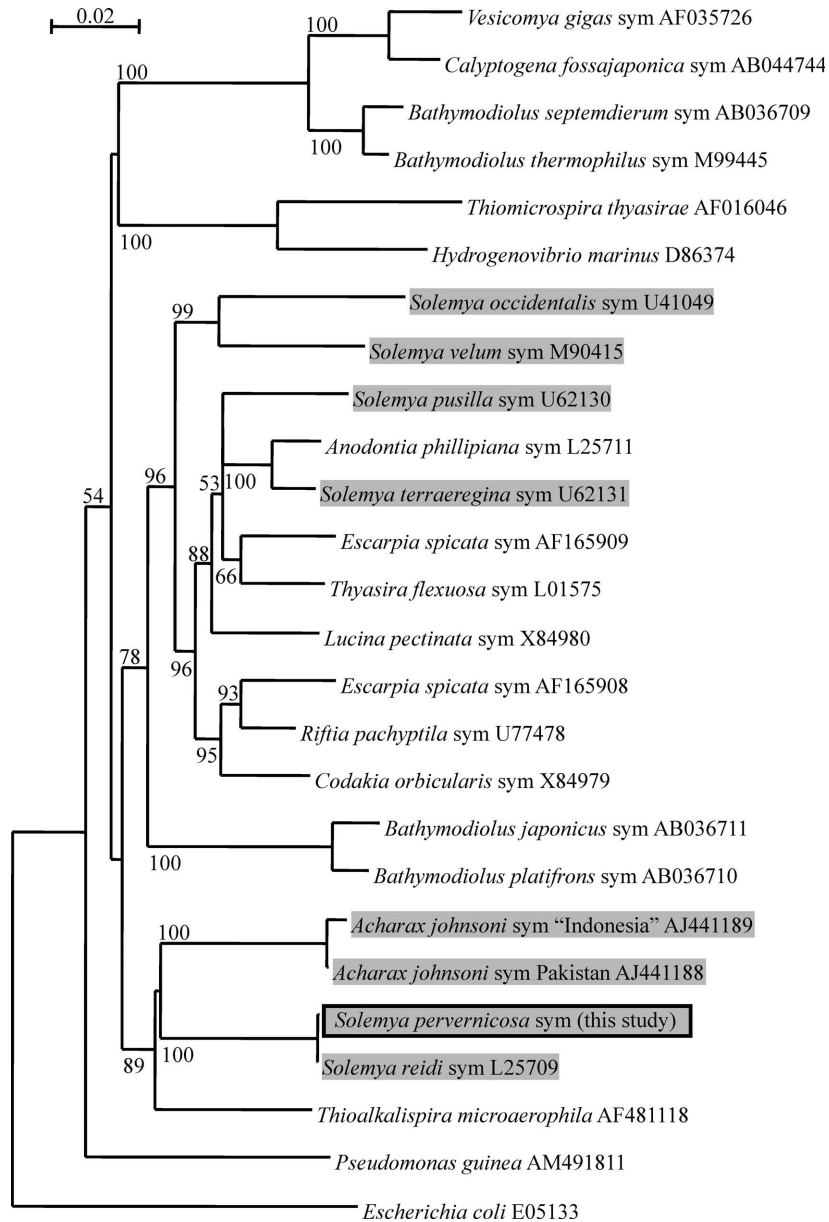
Fig. 5. Phylogenetic topology inferred by neighbor joining-analysis of 1130 homologous positions of 16S ribosomal RNA gene sequences of the symbionts of *Solemya pervernicosa* and representative free-living and symbiotic bacteria within the gamma subdivision of Proteobacteria. Scale bar represents 0.02 nucleotide substitutions per sequence position. The percentage of 1000 bootstrap resamplings is indicated. Bootstrap values for nodes supported in greater than 500 of 1000 trees are shown. Symbiont of the clam examined in this study is highlighted. All solemyid symbionts are shaded.

occurrence. Habe (1977) stated that the species is distributed in "the Sea of Japan", and from off Choshi, Chiba Prefecture northwards to Hokkaido on the Pacific coast, on sandy mud bottoms at depths of 100–500 m. Okutani *et al.* (1989) recorded it from Sagami Bay at a depth of 1510 m (35

mm SL) and from off Kuriya in Fukui Prefecture on the Sea of Japan, at an unknown depth (50 mm SL). It was also once recorded from off Wakayama Prefecture, though without detailed data (Habe (?), 1981: A Catalogue of Molluscs of Wakayama Prefecture). The present finding thus represents a significant range extension southwards to the west coast of Kyushu in the East China Sea. Furthermore, the association with whale carcasses observed in the present study represents a new habitat for any known species in the family(?).

It is noteworthy that both *Adipicola pacifica*, which was found to inhabit the present whale carcasses in huge masses (Okutani *et al*., 2004), and *S. pervernicosa* were also recorded from off the coast of Fukui Prefecture (Kuroda, 1933; Okutani, 1989). This may indicate the presence of whale remains in the area, although there is no evidence to support this.

Yamanaka *et al*. (2004) mapped the chemical environmental settings in the biota around whale bones on the sea floor (Fig. 6). A reducing environment with prevailing hydrogen sulfide and ammonium occurs only directly beneath the bones. Sulfide-sulfur and ammonium-nitrogen in the sediments show unique isotopic signatures such as $\delta^{34}S$ values ranging from +1.8 to +8.9‰, and $\delta^{15}N$ values from +5.5 to +7.5‰. Soft body parts of clams inhabiting this environment show common isotopic values ($\delta^{34}S$ = +2.5, +3.7‰, $\delta^{15}N$ = +3.3 ~ +4.7‰) with those reductive chemical species. The $\delta^{15}N$ values of soft body parts, however, were also similar to organic nitrogen levels in suspended material obtained from the area ($\delta^{15}N$ = +2.5 ± 1‰). In addition, the carbon isotope values ($\delta^{13}C$ = –30.3 ~ –32.7‰) of the soft parts of the clam are distinctly lower than those of common marine animals, and lie within the range documented for thioautotrophy-based animals (Mizota and Yamanaka, 2003). Since animals with thioautotrophic endosymbionts assimilate sulfide-sulfur, the soft-body part sulfur isotope signature is the same as that of the surrounding substrate sulfides (Mizota & Yamanaka, 2003). Both carbon and sulfur isotope values of the present soft body parts suggest that the clam harbors thioautotrophic symbionts that provide nutrition to their host.

The presence of endosymbiotic bacteria in the gill of *S. pervernicosa* indicates that this species belongs to a chemosynthesis-based community created around the whale remains.

This is the first record of chemosynthetic symbiosis to date for solemyid clams in whale-fall ecosystems. Morphological observations and phylogenetic results suggest strongly that the bacteria in this symbiosis are thioautotrophic, which is borne out by the stable isotopic analyses. The morphological features of the gill tissue of this clam are consistent with previous studies of other solemyid clams (reviewed by Fisher, 1990). Both the large size and the structure of the gill tissue clearly indicate it to be the primary organ involved in nutrition. TEM observations showed that the structure of the gill tissue is similar to that of other solemyid clams (Powell & Somero, 1985, Gustafson & Reid, 1988, Conway *et al*., 1992, Krueger *et al*., 1996a; Krueger *et al*., 1996b). Numerous bacteria were visible in the epithelial cells of the gill tissue (Figs. 4, 5). Divisional stages of bacteria were extremely rare, indicating a "milking"-type carbon transfer from the symbionts to the host, as reported in other solemyid clams (Fisher & Childress, 1986). The bacteria were gram-negative and had no stacked internal membranes. These features match those of other thioautotrophic symbionts found in marine invertebrates (Fisher, 1990). The size of the bacteria (5.05 μm mean length in major axis) is within the range of other solemyid clam symbionts (Fisher 1990).

The 16S rRNA gene sequence analysis of the symbionts of *S. pervernicosa* suggested that they are phylogenetically related to thioautotrophic symbionts from *S. reidi* and *Acharax johnsoni*. However, many other solemyid clam symbionts, such as those of *S. occidentalis*, *S. velum*, *S. pusilla* and *S. terraeregina*, are less closely related to the *S. pervernicosa* symbionts than to those from lucinid and thyasirid clams and vestimentiferan tubeworms. This polyphyly of symbionts implies that solemyid hosts and their symbionts have evolved independently. However, the thioautotrophic symbionts of *S. reidi* have been reported to be transmitted vertically through the
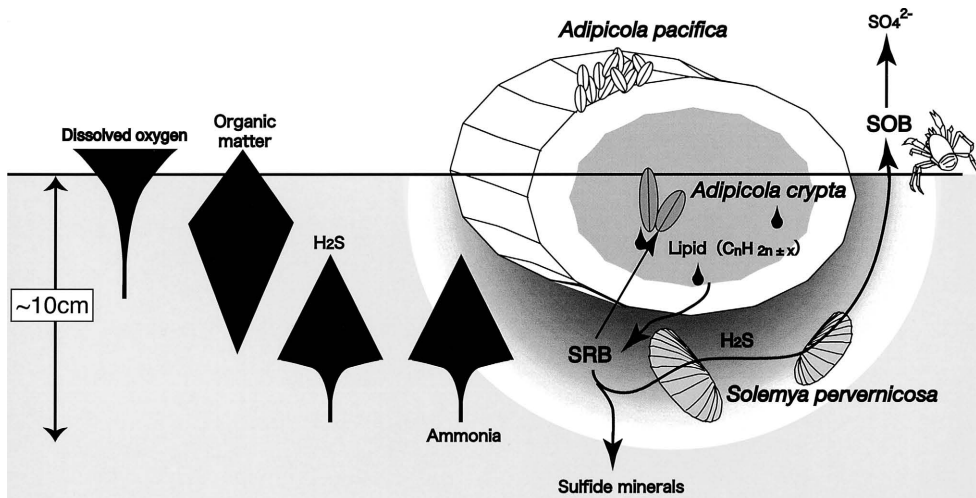
Fig. 6. Diagram of chemical environment setting around whale bone (Yamanaka *et al*., 2004). Abbreviations: SOB, Sulfur oxidizing bacteria; SRB, sulfur reducing bacteria.

ovary to progeny (Cary, 1994; Krueger *et al*., 1996b) (Fig. 6). Symbionts in vesicomyid clams are also transmitted vertically (Endow & Ohta, 1990; Cary & Giovannoni, 1993) and cospeciation between vesicomyid hosts and their bacterial symbionts due to their long-term, indivisible association has been reported (Peek *et al*., 1998). The symbiont transmission mechanism in solemyid clams is thus still not completely resolved.

Acknowledgements

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鯨骨の下にアブラキヌタレが棲む―その生物学的意義

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河戸　勝・山本智子・大越健嗣

要　約

　鹿児島県野間岬沖に沈下された鯨の遺骸上にはヒラノマクラの大群が棲むことを既報したが，今回は同じ遺骸下の海底沈積物のなかにアブラキヌタレが棲息することを報告する。

　アブラキヌタレはこれまで，襟裳岬沖，銚子沖，相模湾，和歌山県沖および福井県厨（くりや）沖から知られていたが，今回の発見は本種の分布域の拡大のみならず特異な棲息環境についての新情報となる。また，本種は鰓に硫黄細菌を共生させていて，本種の炭素と硫黄同位体組成の分析の結果から化学合成依存性であることが証明された。更にその中に棲む共生細菌の超微細構造観察を行い，分子系統を調べた結果，その共生細菌は既報のキヌタレガイ科二枚貝の共生細菌と形態的に類似し，また分子系統解析の結果も *Solemya reidi* とスエヒロキヌタレの共生細菌とよく一致した。しかしながら，現時点では共生細菌の獲得方法を示唆する知見は得られていない。