frontiers IN EXTREME MICROBIOLOGY

# Widespread occurrence of two carbon fixation pathways in tubeworm endosymbionts: lessons from hydrothermal vent associated tubeworms from the Mediterranean Sea

Vera Thiel, Michael Hügler, Martina Blümel, Heike I. Baumann, Andrea Gärtner, Rolf Schmaljohann, Harald Strauss, Dieter Garbe-Schönberg, Sven Petersen, Dominique A. Cowart, Charles R. Fisher and Johannes F. Imhoff


| | |
|---|---|
| Journal Name: | Frontiers in Microbiology |
| ISSN: | 1664-302X |
| Article type: | Original Research Article |
| Received on: | 04 Oct 2012 |
| Accepted on: | 26 Nov 2012 |
| Provisional PDF published on: | 26 Nov 2012 |
| Frontiers website link: | www.frontiersin.org |
| Citation: | Thiel V, Hügler M, Blümel M, Baumann HI, Gärtner A, Schmaljohann R, Strauss H, Garbe-schönberg D, Petersen S, Cowart DA, Fisher CR and Imhoff JF(2012) Widespread occurrence of two carbon fixation pathways in tubeworm endosymbionts: lessons from hydrothermal vent associated tubeworms from the Mediterranean Sea. *Front. Microbio.* 3:423. doi:10.3389/fmicb.2012.00423 |
| Article URL: | http://www.frontiersin.org/Journal/Abstract.aspx?s=420&name=extreme%20microbiology&ART_DOI=10.3389/fmicb.2012.00423 |
| | (If clicking on the link doesn't work, try copying and pasting it into your browser.) |

# Widespread occurrence of two carbon fixation pathways in tubeworm endosymbionts: lessons from hydrothermal vent associated tubeworms from the Mediterranean Sea

Vera Thiel [1,5#*], Michael Hügler [2#], Martina Blümel [1,6], Heike I. Baumann [1], Andrea Gärtner [1], Rolf Schmaljohann [1], Harald Strauss [3], Dieter Garbe-Schönberg[4], Sven Petersen [1], Dominique A. Cowart[7], Charles R. Fisher[7], and Johannes F. Imhoff [1]

[1] GEOMAR, Helmholtz Centre for Ocean Research Kiel, Düsternbrooker Weg 20, 24105 Kiel, Germany

[2] Water Technology Center Karlsruhe (TZW), Karlsruher Str. 84, 76139 Karlsruhe, Germany

[3] Institut für Geologie und Paläontologie, Westfälische Wilhelms-Universität Münster (WWU), Corrensstr. 24, 48149 Münster, Germany

[4] Christian Albrechts Universität Kiel (CAU), Institut für Geowissenschaften, 24098 Kiel, Germany

[5] present address: Department of Biochemistry and Molecular Biology, The Pennsylvania State University (PSU), 231 South Frear Lab, University Park, PA 16802, USA

[6] present address: Christian Albrechts Universität Kiel (CAU), Institut für Pflanzenzüchtung, Am Botanischen Garten 1-9, 24118 Kiel, Germany

[7] Department of Biology, The Pennsylvania State University (PSU), 208 Erwin W. Mueller Laboratory, University Park, PA 16801, USA

* Correspondence: Dr. Vera Thiel, The Pennsylvania State University, Biochemistry and Molecular Biology, 231 South Frear, University Park, 16802, Pennsylvania, USA, vut1@psu.edu

[#] These authors contributed equally to this work.

Running title: carbon fixation in tubeworm endosymbionts

## ABSTRACT

Vestimentiferan tubeworms (siboglinid polychaetes) of the genus *Lamellibrachia* are common members of cold-seep faunal communities and have also been found at sedimented hydrothermal vent sites in the Pacific. As they lack a digestive system, they are nourished by chemoautotrophic bacterial endosymbionts growing in a specialized tissue called the trophosome. Here we present the results of investigations of tubeworms and endosymbionts from a shallow hydrothermal vent field in the Western Mediterranean Sea. The tubeworms, which are the first reported vent-associated tubeworms outside the Pacific, are identified as *Lamellibrachia anaximandri* using mitochondrial ribosomal and cytochrome oxidase I (COI) gene sequences. They harbor a single gammaproteobacterial endosymbiont. Carbon isotopic data, as well as the analysis of genes involved in carbon and sulfur metabolism indicate a sulfide-oxidizing chemoautotrophic endosymbiont. The detection of a hydrogenase gene fragment suggests the potential for hydrogen oxidation as alternative energy source. Surprisingly, the endosymbiont harbors genes for two different carbon fixation pathways, the Calvin-Benson-Bassham (CBB) cycle as well as the reductive tricarboxylic acid (rTCA) cycle, as has been reported for the endosymbiont of the vent tubeworm *Riftia pachyptila*. In addition to RubisCO genes we detected ATP citrate lyase (ACL – the key enzyme of the rTCA cycle) type II gene sequences using newly designed primer sets. Comparative investigations with additional tubeworm species (*Lamellibrachia luymesi*, *Lamellibrachia* sp. 1, *Lamellibrachia* sp. 2, *Escarpia laminata, Seepiophila jonesi*) from multiple cold seep sites in the Gulf of Mexico revealed the presence of *acl* genes in these species as well. Thus, our study suggests that the presence of two different carbon fixation pathways, the CBB cycle and the rTCA cycle, is not restricted to the *Riftia* endosymbiont, but rather might be common in vestimentiferan tubeworm endosymbionts, regardless of the habitat.


## INTRODUCTION

Vestimentiferan tubeworms are often dominant members of chemosynthetic communities present at reduced environments such as hydrothermal vents and cold seeps (Vrijenhoek, 2010). So far, hydrothermal vent-associated tubeworms have not been found outside the Pacific. In contrast, seep-associated tubeworms have been found in the Gulf of Mexico (GoM), the Mediterranean Sea and the margins of the Atlantic Ocean (Cordes et al., 2009; Vrijenhoek, 2010).

The Mediterranean Sea is the world's largest enclosed sea, and represents a hot spot of biodiversity with a considerable number of endemic species (Myers et al., 2000). Its only connection to the Atlantic Ocean is the narrow and shallow Strait of Gibraltar, which is the sole route for exchange of propagules between these two water bodies. The only vestimentiferan tubeworms documented to date in the Mediterranean Sea belong to the genus *Lamellibrachia* and specimens from several Mediterranean mud volcanoes were recently described as the new species *Lamellibrachia anaximandri* (Southward et al., 2011). The genus *Lamellibrachia* has a worldwide distribution, and occurs in several types of chemosynthetic environments from the shallow to the deep sea (e.g. Kojima et al., 2002). Within the Mediterranean Sea, *Lamellibrachia* spp. have been discovered in the vicinity of mud volcanoes in the Alboran Sea at 572 m depth (Hilário et al., 2011), from several mud volcanoes in the Eastern Mediterranean Sea at a depth of about 3,000 m (Olu-Le Roy et al., 2004; Bayon et al., 2009; Duperron et al., 2009; Southward et al., 2011) and also from two sunken ship wrecks in the Eastern and Western Mediterranean (Hughes and Crawford, 2008; Gambi et al., 2011) (Figure 1).

Hydrocarbon seep communities in the Gulf of Mexico (GoM) were among the first seep communities to be discovered, are extensively studied, and have a high diversity of tubeworm species (Kennicutt et al., 1985; Miglietta et al., 2010). The Louisiana Slope in the northern GoM area extends from the continental shelf to the salt deformation edge of the Sigsbee Escarpment, and ranges from about 300 to 3,000 m in depth. This area is home to at least six known morphospecies of vestimentiferan tubeworms (Miglietta et al., 2010), including the most commonly studied seep tubeworms, *Lamellibrachi luymesi* (van der Land and Nørrevang, 1975) and *Seepiophila jonesi* (Gardiner et al., 2001).

In contrast to the well-known hydrothermal vent tubeworm *Riftia pachyptila*, which inhabits hard substrate in hot sulfidic environments, members of the genus *Lamellibrachia* live in sedimented areas and are most common in cold seep environments. Seep habitats are generally much less dynamic than vent habitats and may be stable for centuries (Fisher et al., 1997). Compared to vent environments, emanating seep fluids are cooler, often enriched in methane and concentrations of dissolved sulfide may be quite low (Southward et al., 2011). *Lamellibrachia* tubeworms can obtain sulfide from the underlying sediments using the buried, permeable posterior region of the tube termed the "root" (Julian et al., 1999; Freytag et al., 2001). Since *Lamellibrachia*, like other siboglinid polychaetes, lack a digestive tract, they are dependent on

91 their endosymbionts for nutrition. Sulfide is transported via hemoglobin molecules in the blood
92 to the trophosome, a large organ that harbors dense populations of gammaproteobacterial
93 endosymbionts (reviewed by Childress and Fisher, 1992). These endosymbionts oxidize the
94 sulfide to obtain energy and reducing power for autotrophic carbon fixation. A portion of the
95 synthesized organic matter serves in turn as energy source for the host tubeworm (Bright et al.,
96 2000; Stewart and Cavanaugh 2006). *Lamellibrachia* spp. are not only found at cold seeps, but
97 also at sediment covered hydrothermal sites, e.g. *Lamellibrachia barhami* along the Juan de Fuca
98 Ridge (Juniper et al., 1992), *Lamellibrachia columna* near hydrothermal vents in the Lau Basin
99 in the southwest Pacific (Southward, 1991) and *Lamellibrachia satsuma* at hydrothermal sites off
100 southern Japan (Miake et al., 2006). Even though the so-called "vent" and "seep" tubeworm
101 genera are clearly specialized for their preferred *in situ* conditions, they have been found at the
102 same site, sometimes occurring only meters apart e.g., the seep tubeworm *L. barhami* and the
103 vent species *Ridgeia piscesae* at Middle Valley in 2,400 m depth in the northeast Pacific Ocean
104 (McMullin et al., 2003).
105 Vestimentiferan endosymbionts form a monophyletic cluster within the gammaproteobacteria.
106 They have been shown to cover very large geographic ranges, with nearly identical 16S rRNA in
107 hosts separated by thousands of kilometers. Within the endosymbiont cluster four different
108 groups (one "vent"-group, three "seep"-groups) are distinguishable. So-called 'vent'
109 endosymbionts appear to be specific for vent vestimentiferan hosts (e.g. *Riftia*, *Tevnia*, *Ridgeia*,
110 and *Oasisia*), while three different 16S rRNA gene clusters (groups 1-3), possibly representing
111 different strains, were found only in 'seep' vestimentiferans (Nelson and Fisher, 2000; McMullin
112 et al., 2003). Site depth has been postulated to be a factor in defining which of the three
113 endosymbiont strains is found in a particular 'seep' host, with "group 3" occurring only in
114 shallow water host specimens (McMullin et al., 2003).
115 The best-studied tubeworm endosymbiont is *Candidatus* Endoriftia persephone, the
116 gammaproteobacterial endosymbiont of the vent-associated tubeworm *Riftia pachyptila*. Its
117 metabolic capacities have been subject of detailed metagenomic, proteomic and enzymatic
118 studies (Felbeck 1981; Felbeck et al., 1981; Markert et al., 2007, 2011; Robidart et al., 2008;
119 Gardebrecht et al., 2012). *Candidatus* Endoriftia persephone is a sulfide-oxidizing
120 chemoautotroph. Sulfide is oxidized to sulfate via sulfite and adenosine phosphosulfate (APS).
121 The enzymes involved in the so-called APS pathway are dissimilatory sulfite reductase (DsrAB,

working in reverse as sulfide oxidase), APS reductase (AprAB) and ATP sulfurylase (Markert et al., 2007, 2011). Quite surprising is the presence of two alternative carbon fixation pathways in the *Riftia* endosymbiont, the Calvin-Benson-Bassham (CBB) cycle as well as the reductive tricarboxylic acid (rTCA) cycle (Felbeck, 1981; Markert et al., 2007). Both pathways show unique features. The CBB cycle seems more energy-efficient due to modified enzyme equipment (Markert et al., 2011; Gardebrecht et al., 2012; Kleiner et al., 2012) while the rTCA cycle harbors a novel type of ATP citrate lyase (Hügler and Sievert, 2011). In contrast to the *Riftia* endosymbiont there are no genomic, proteomic or metabolomic studies of the endosymbiont(s) of *Lamellibrachia* spp..

This study reports the recovery of vestimentiferan tubeworms from the Palinuro volcanic complex, a submarine volcano in the Tyrrhenian Sea (Western Mediterranean Sea), north of Sicily (Figure 1). The Palinuro complex is part of the active Aeolian Island Arc and consists of several volcanic edifices aligned over a strike length of 55 km (Petersen et al., 2008; Passaro et al., 2010). The volcanic complex is up to 25 km wide at its base and its shallowest portion rises from 3,000 m to a water depth of less than 100 meters. Iron and manganese-bearing precipitates were first documented at Palinuro by Kidd and Ármannson (1979) providing the first evidence for hydrothermal activity in the area. Hydrothermal sulfides were described by Minniti and Bonavia (1984) and Puchelt and Laschek (1987) within sediment sampled from the most westerly summit of Palinuro. The discovery of living vestimentiferan tubeworm colonies on top of the main volcanic edifices in this western summit in 2006 as well as temperatures of up to 60°C in sediment cores recovered from the seafloor indicated that active hydrothermal venting was taking place at the time although black smoker style venting has not been observed (Petersen et al., 2008; Monecke et al., 2009). Two colonies of these tubeworms were sampled in spring 2011.

We describe the results from detailed analyses of the Palinuro tubeworms and their endosymbionts, which are the first reported vent-associated tubeworms outside the Pacific Ocean. For comparison, several seep tubeworm species from the Gulf of Mexico were also analyzed (Figure 2), providing deeper insights into the geographic dispersal, phylogeny, and metabolic potential of tubeworms and their endosymbionts.

## MATERIALS AND METHODS

### Sampling site, sample collection and processing of Palinuro tubeworms

Vestimentiferan tubeworm specimens were retrieved from two different colonies termed colony #1 and #2 on the western summit of the Palinuro volcanic complex (Mediterranean Sea, 39°32.44´N, 14°42.38'E, depth: 630 m) during the Pos412 cruise of R/V *Poseidon* in spring 2011. Sampling was conducted using a Mohawk-type remotely operated vehicle (ROV) supplied by Oceaneering Inc. (Aberdeen, UK) fitted with a robotic arm. Locations of the tubeworm collections are given in Table 1.

The ROV was also equipped with a fluid sampling system (Kiel in situ pumping system, KIPS, Garbe-Schönberg et al., 2006) capable of acquiring four 550 mL water samples per dive with *in situ* filtration. Parallel to the sampling nozzle was a temperature probe attached to a data logger. Fluids and temperatures around the colonies were sampled using KIPS and the temperature probe by maneuvering the ROV´s robotic arm into the fluid in close proximity to the living tubeworms. Live tubeworms were sampled using the ROV´s robotic arm. Immediately after tubeworm sampling, the dive was terminated and the ROV was recovered. Upon recovery, the tubeworms obtained from colony #1 were put into sterile petri dishes using sterile tweezers followed by dissection with a sterile scalpel. The animal was then separated from the tube; subsamples were recovered from vestimentum (host tissue free of symbionts) and trophosome (endosymbiont) tissue, and then were stored at -20°C until further molecular analysis. Samples from colony #2 were immediately stored at -20°C until further processing in the home laboratory. As the tubeworms from colony #2 exhibited several morphotypes, these were stored in separate vials.

### Gulf of Mexico tubeworm sample collection and preparation

Gulf of Mexico vestimentiferan tubeworms were sampled from hydrocarbon seep sites during several research cruises between 1997 and 2011 (Table 2, Figure 2). *Lamellibrachia luymesi* and *Seepiophila jonesi* were collected using the Johnson Sea Link submersible from two sites on the Upper Louisiana Slope from about 540 m depth. *Lamellibrachia* sp. 1 and *Lamellibrachia* sp. 2 as well as *Escarpia laminata* were collected from three sites on the Lower Louisiana Slope ranging in depth from 1,975 m to 2,604 m. While on board the research vessel, tubeworms were

dissected and vestimentum (host tissue free of symbionts) and trophosome (endosymbiont) tissue was preserved at -80ºC or in 95% ethanol solution. All tissue samples were transported to the Pennsylvania State University where whole genomic DNA was obtained using a modified version of the high salt extraction protocol and ethanol precipitation as in Liao et al., 2007**.** Isolated DNA is currently stored at -80ºC at the Pennsylvania State University.

**DNA extraction, PCR amplification, cloning and sequencing**

Genomic DNA was extracted from the trophosome and vestimentum tissues of the vestimentiferan tubeworms. The tubeworms were dissected and washed three times in 0.2 µm filtered seawater prior to DNA extraction. DNA of Mediterranean tubeworm samples was isolated using the MoBio Power Biofilm Kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the protocol provided. DNA of Gulf of Mexico tubeworm samples was extracted following the protocol of Liao et al. (2007). Cytochrome c oxidase I (COI) genes, mitochondrial and bacterial ribosomal (16S rRNA) genes, as well as *cbbM* and ACL type II genes were analyzed from all individuals. Further functional genes and eukaryotic ribosomal (18S rRNA) genes were analyzed from one individual of colony #1 from the Palinuro volcanic complex.

For all gene amplifications of Mediterranean samples PCR reactions were conducted using Ready-To-Go PCR Beads (GE Healthcare, Munich, Germany) in a total volume of 25 µL. PCR from GoM samples were conducted using 1U BioBasic TaqPolymerase (BioBasic Inc., Markham Ontario, Canada) and 1x Thermopol Buffer (NEB Inc., USA) in a total volume of 50 µL. If not stated otherwise 10 pmol of each primer and 100 ng template DNA was used. For all amplifications, initial denaturation was 2 min at 94°C, final annealing was 1 min at annealing temperature and final elongation 5 min at 72°C. For the cycles denaturation was 40 sec at 94°C, annealing duration 40 sec at the respective annealing temperature and elongation was 1 min at 72°C. If not stated otherwise, 35 PCR cycles were applied. Fragments of the tubeworms´ 18S rRNA and mitochondrial 16S rRNA as well as COI genes were amplified using the (i) primer pairs 5´-start (5‘-GGT TGA TCC TGC CAG-3‘) and 1753rev (5’-GCA GGT TCA CCT ACG G-3’) targeting the 18S rRNA gene (30 cycles, 50°C annealing temperature), (ii) primer pair 16Sar/16Sbr (Palumbi et al., 2002) targeting the mitochondrial 16S rRNA gene (30 cycles, 50°C annealing temperature) and (iii) primers LCO 1490 (5’-GGT CAA CAA ATC ATA AAG ATA TTG G-3’) and HCO 2198 (5’-TAA ACT TCA GGG TGA CCA AAA AAT CA-3’) (40 cycles,

47°C annealing temperature (Folmer et al., 1994)) using DNA extracted from the symbiont-free vestimentum tissue. Gene fragments of the endosymbiont were amplified using DNA extracted from trophosome tissue as the template. Bacterial 16S rRNA gene fragments were amplified in a 30-cycle PCR at an annealing temperature of 50°C with the general bacterial primer set 27F and 1390R (Palinuro samples; 5'-GAC GGG CRG TGT GTA CAA-3') or 1492R (GoM samples) (Lane, 1991). Amplification for fragments of *dsrA* and *aprA* genes was performed using the primer sets r*dsrA*240F/r*dsrA*403R and *aps*1F/*aps*4R, respectively (Lavik et al., 2009; Meyer and Kuever, 2007b). Fragments of *soxB* were amplified using the primers *soxB*432F/*soxB*1446B (10 cycles with 55°C annealing temperature and 25 cycles with 47°C annealing temperature (Petri et al., 2001). For the amplification of fragments of the genes coding for the large subunit of RubisCO form I and II, the primer sets *cbbL*F/*cbbL*R and *cbbM*F/*cbbM*R were used (both include two initial cycles of 2 min annealing at 37°C and 3 min elongation at 72°C, as well as additional 35 cycles of 53°C and 58°C annealing temperature for *cbbL* and *cbbM* respectively (Campbell and Cary, 2004)). A fragment of the large subunit of the putative type II ATP citrate lyase gene was amplified using the newly designed primer *acl2*F1 (5‘-CGT CGC CAA GGA AGA GTG GTT C-3‘) and *acl2*R1 (5‘-GGC GAT GGC CTC AAA GCC GTT-3‘) in a 30 cycle PCR with annealing temperatures of 45-56°C (gradient). Fragments of the hydrogen uptake hydrogenase gene *hupL* were amplified with the primer set HUPLX1/HUPLW2 (Csaki et al., 2001). A fragment of the norCB gene for nitric oxide reductase subunits C and B was amplified using the primer set *nor*C21mF and *nor*B6R (Tank, 2005) in a 35 cycle PCR using annealing temperatures of 60°C-50°C (10 touchdown cycles 60°C/-1°C, 25 cycles of 50°C).

Additional primer pairs used in this study include: F2/R5 and 892F/1204R for the two subunits of ATP citrate lyase (Hügler et al., 2005; Campbell et al., 2003); MxaF1003, MxaR1555, MxaR1561 for methanol dehydrogenase gene *mxaF* (Neufeld et al., 2007; Kalyuzhnaya et al., 2008); *mmoXA/mmoXB* for genes encoding the conserved alpha-subunit of the hydroxylase component of the cytoplasmatic soluble methane monooxygenase (sMMO) (Auman et al., 2000); and A189F/MB661R for the particulate methane monooxygenase (pMMO) genes present in methanotrophs (Costello and Lidstrom, 1999).

All PCR products were purified via gel-extraction using QIAquick gel extraction kit (QIAgen, Hilden, Germany) for Mediterranean samples, and BioBasic EZ-10 spin columns (BioBasic Inc., Markham, Ontario, Canada) for GoM samples respectively, and either directly sequenced by

Sanger sequencing (18S rRNA gene fragments, COI and functional gene fragments) or cloned into pCR4-TOPO vectors with the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer before sequencing (16S rRNA gene fragments). Sequencing was conducted using amplification primers and additional internal primers in the case of 16S rRNA genes (342F, 534R (Muyzer et al. 1996); 790F (Thiel et al. 2007)). Amplification and sequencing of clones was conducted using vector specific primers M13 forward and M13 reverse (PCR) and T3 and T7 (sequencing), respectively. Sanger sequencing was performed using the BigDye Terminator v1.1 sequencing kit in a 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA) as specified by the manufacturer. Sequencing was conducted by the Institut für Klinische Molekularbiologie (IKMB), Universitäts-Klinikum Schleswig-Holstein (UK-SH), Kiel, Germany and the sequencing core facility at The Pennsylvania State University, University Park, PA, USA.

**Phylogenetic analysis**

All sequences were edited with ChromasPro c.c1.33 and compared to the NCBI database using BLAST (Altschul et al., 1997). Functional gene sequences nucleotides were also compared with the non-redundant protein sequence database using the blastx algorithm. The endosymbiont 16S rRNA gene sequences were aligned with the ARB software (www.arb-home.de) using the ARB FastAligner utility (Ludwig et al., 2004). The sequence alignment was manually refined based on known secondary structures. Sequences of functional genes as well as mitochondrial rRNA genes were aligned using Clustal X (Thompson et al., 1997) and manually adjusted using BioEdit (Hall, 1999). Maximum-Likelihood based trees and 100 bootstrap replicates were constructed using PhyML (Guindon and Gascuel, 2003). In order to verify the tree topology, further phylogenetic analyses using Neighbor-Joining and Maximum-Parsimony algorithms were conducted using MEGA5 (Tamura et al., 2011).

**Microscopy**

The morphology of endosymbiotic bacteria in trophosome tissue of the tubeworms was examined using light microscopy and scanning electron microscopy (SEM). Samples for light microscopy were prepared by removing small pieces of tissue from different parts of the

trophosome and subsequent squeezing preparation in a drop of particle-free seawater and examined under 100fold magnification using a Zeiss Axiophot Epifluorescence Microscope.

Samples for SEM were prepared by disrupting small trophosome samples in 0.2 µm-filtered seawater, and then concentrated by filtration on to 0.2 µm polycarbonate membrane filters followed by dehydration through ascending concentrations of ethanol. Subsequently, the samples were critical-point-dried using a Balzers CPD 030 and $CO_2$ as a transition medium. The filters were sputter-coated with gold-palladium using a Balzers SCD 004 and observed with a Zeiss DSM 940 electron microscope.

**Fluid chemistry**

After recovery of the ROV Mohawk all KIPS fluid samples were immediately transferred to the on-board ship lab and sub-sampled for subsequent analyses. Both pH and Eh of the fluids were determined immediately after sub-sampling using electrochemical techniques after calibration with certified standards. Dissolved oxygen was determined using standard Winkler titration protocols modified for small volumes. The concentration of dissolved sulfide was determined in 1 mL aliquots using a zinc acetate gelatin solution, which precipitates the dissolved sulfide as colloidal zinc sulfide. Subsequently, the color agent, N,N-dimethyl-1,4-phenylenediamine-dihydrochloride, and a catalyst, iron chloride solution, were added to form methylene blue (Cline, 1969). After 1 h, the solutions were measured photometrically at a wavelength of 660 nm using a Genesys Spectra 10 spectrophotometer. The detection limit was 1 µmol/L. Potential oxidation of dissolved hydrogen sulfide during sampling and sample recovery cannot be ruled out, but is likely minimal. Nonetheless, hydrogen sulfide concentration data given in this paper should be considered minimum values. Aliquots for the analysis of nutrients were stored in polypropylene bottles, sealed and stored in the dark at 4°C until analysis. Aliquots for cation and trace element analysis were pressure-filtrated through 0.2 µm Nucleopore polycarbonate (PC) membrane filters using Sartorius PC filtration units and high purity nitrogen. Samples were acidified with subboiled nitric acid to pH < 2 and stored in perfluoralkoxy (PFA) bottles until analysis. Multielement analysis for major ion composition (Cl, B, Si, Na, K, Ca, Mg, Fe, Mn) of the water samples was performed with a SPECTRO Ciros SOP ICP-OES spectrometer after 10fold dilution and using Y for internal standardization. Trace elements (As, Li, W) were determined by ICP-MS (Agilent 7500cs at University of Kiel) after 12.5fold dilution using

both In and Re for internal standardization. Certified reference materials NIST1643e, NASS-5, and IAPSO were used for validation and accuracy checks.

**Carbon isotope signature**

The organic carbon isotopic composition of tubeworm tissue ($\delta^{13}C_{ORG}$) was determined via continuous flow EA-IRMS using an elemental analyzer interfaced to a ThermoFinnigan Delta Plus isotope ratio mass spectrometer. Briefly, about 40-60 µg of freeze-dried worm tissue was weighed in tin capsules, combusted to $CO_2$, and chromatographically purified carbon dioxide was transferred to the mass spectrometer in a He gas stream. Results are reported in the standard delta notation as per mil difference to the Vienna Pee Dee Belemnite. Sample measurements were done in duplicate, and analytical performance was monitored with international reference materials (USGS 24; USGS 40) and lab standards (anthracite; brown coal) and the reproducibility was generally better than ±0.15 ‰.

The carbon isotopic composition of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) from vent fluids was measured using a ThermoFinnigan GasBench coupled to a ThermoFinnigan Delta Plus XL. Briefly, 0.5 – 1.0 mL of hydrothermal fluid was injected into an exetainer that contained phosphoric acid, liberating DIC as carbon dioxide. Prior to sample injection, the exetainer was flushed with helium. $CO_2$ was flushed from the exetainer with a stream of helium and injected into the mass spectrometer. Results are reported in the standard delta notation as per mil difference to the Vienna Pee Dee Belemnite. Sample measurements were done in duplicate, and analytical performance was monitored with a sodium carbonate lab standard.

**Nucleotide sequence accession numbers**

The sequence data have been submitted to EMBL/GenBank/DDBJ databases under accession numbers HE9744464-85, HE978212-46 and HE983327-53.

## RESULTS

### Biogeochemical characterization of the tubeworm habitat at Palinuro

For the present study, two colonies of vestimentiferan tubeworms as well as their biogeochemical environment were sampled by means of a remotely operated vehicle (ROV). The tubeworms occurred within a sediment-filled depression at the western summit of the Palinuro volcanic complex in water depths of around 630 m and formed small bushes, up to 1 $m^2$ in diameter, mainly on sedimented surfaces but some patches also occurred in areas where volcanic rocks were present at the seafloor. Frequently, shimmering water was observed rising above the tubeworm colonies suggesting active fluid emanation. The first colony (colony #1) appeared to be comprised mainly of equally sized animals. The second colony (colony #2), however, consisted of animals of different sizes, indicating different ages or variation in exposure to hydrothermal fluid at different places within the colony.

ROV assisted measurements of water temperature across and within the tubeworm colonies revealed a maximum recorded temperature of 19.4°C, about 5°C above ambient seawater temperatures of 14°C (Table 1). Diffusely venting warm hydrothermal fluids reached a maximum temperature of 58.4°C within small depressions in close proximity to the tubeworms. Chloride concentrations in these fluids were significantly higher than in ambient seawater indicating influx of hydrothermal brine eventually leading to the formation of small stratified brine pools in these depressions. Chemical analyses of vent and pore fluids sampled from sediment cores collected in the same area revealed that local hydrothermal fluids were anoxic, $H_2S$-rich, acidic and displayed an elevated salinity. Concentrations of dissolved alkali and alkali earth elements (potassium, calcium, lithium, cesium), silica, arsenic and tungsten ("Fluid endmember" in Table 3) were significantly higher when compared to normal bottom seawater. Fluid samples confirmed that this hydrothermal fluid was highly diluted with ambient seawater as it passed through the two tubeworm colonies leading to partly oxygenated waters (139 and 227 µmol/L dissolved $O_2$, Table 3) and reduced levels of sulfide. Still, dissolved sulfide concentrations of 32 and 72 µmol/L were measured in water samples from among the tubes in the tubeworm colonies. In contrast, a maximum concentration of dissolved sulfide of 5,172 µmol/L was measured for the "hottest" hydrothermal fluids (58°C) sampled at Palinuro.

### Characterization of the tubeworms

*Characterization of Palinuro tubeworms*

A wide range of sizes of vestimentiferan tubeworms were collected from the Palinuro volcanic complex (Figure 3). The tubes of the collected animals ranged up to 15 cm in length with a maximum exterior diameter of 3 mm at the anterior end, decreasing slightly to the posterior end. The anterior region was banded reddish brown and white, whereas the posterior region was a more uniform brownish color. The tube walls were thick and rigid in the anterior region, becoming thinner and more flexible in the posterior regions. The vestimentiferan tubeworm hosts were identified by molecular analyses of the 18S rRNA gene as well as the mitochondrial genes for ribosomal 16S rRNA and the cytochrome c oxidase I (COI). All three genes were amplified from DNA extracted from tubeworm vestimentum, which is free of endosymbionts. Based on COI and mitochondrial 16S rRNA nucleotide analyses, all individuals analyzed from tubeworm colony #1 and the four individuals exhibiting different morphologies from tubeworm colony #2 obtained from the Palinuro volcanic complex belonged to the same species. The maximum difference between the COI gene fragments sequences was 2 bases (total investigated length 650 bp) and 1 base for the mitochondrial 16S rRNA gene (total investigated length 529 bp). In accordance with 18S rRNA gene, mitochondrial 16S rRNA gene and COI sequence the tubeworm could be identified as the newly described species *Lamellibrachia anaximandri* (Southward et al., 2011).

*Characterization of Gulf of Mexico tubeworms*

Gulf of Mexico tubeworm samples were identified using mitochondrial 16S rRNA genes amplified from DNA extracted from the endosymbiont free vestimentum tissue. Phylogenetic results confirmed the initial morphological characterizations of *Lamellibrachia luymesi*/*Lamellibrachia* sp.1 (van der Land and Nørrevang, 1975), *Lamellibrachia* sp. 2, *Escarpia laminata* (Jones, 1985) and *Seepiophila jonesi* (Gardiner et al., 2001) (Table 2, Figure 4).

**Characterization of the tubeworm endosymbionts**

*Endosymbionts of L. anaximandri from Palinuro*

Microscopic studies on the Palinuro *L. anaximandri* revealed high numbers of coccoid bacterial cells in broken trophosome tissue. These endosymbiotic bacterial cells varied considerably in

size (2 – 10 µm diameter) and shape (spherical to irregularly coccoid). The color in the light microscope ranged from light to dark brown. Different modes of cell division were observed: equal division, unequal division and budding (Figure 5). The cell surface of many endosymbionts showed a characteristic pattern of small invaginations (0.2 – 0.5 µm diameter), while others had a completely smooth surface. Frequently it was observed that cells in the process of budding or unequal cell division had a structured surface in the larger (older) part of the cell, while the bud was smooth (Figure 5).

The bacterial endosymbionts of the tubeworms were identified by constructing 16S rRNA gene clone libraries from DNA extracted from the trophosome tissue of 11 tubeworm individuals. For each specimen at least 20 clones were sequenced and analyzed. The bacterial 16S rRNA gene sequences of each specimen had > 99% sequence identity, thus representing a single OTU. The consensus sequences (OTUs) from the different individuals were identical (100% sequence identity over a total length of 1387 bp), indicating that only one bacterial endosymbiont phylotype was present in the Palinuro tubeworms. BLAST analysis revealed the gammaproteobacterial sulfide-oxidizing "phylotype 2" bacterial endosymbiont of *L. anaximandri* from the Eastern Mediterranean mud volcanoes as the closest relative (FM165438, 99,7% sequence identity, 5 nucleotides differences over a total length of 1387 bp, (Duperron et al., 2009)). Other closely related sequences originate from other *Lamellibrachia* spp. and seep vestimentiferan endosymbionts from outside the Mediterranean (Figure 6). The endosymbionts of hydrothermal vent tubeworms *Riftia pachyptila* and *Tevnia jerichonana* were more distantly related and clustered on a separate branch within the 16S rRNA gene tree (Figure 6).

*Phylogeny of seep vestimentiferan endosymbionts from the Gulf of Mexico*

Bacterial endosymbionts from Gulf of Mexico tubeworm specimen were identified by amplification and direct sequencing of 16S rRNA genes from DNA extracted from the trophosome tissue.

The GoM vestimentiferan tubeworm's endosymbionts were affiliated with the three monophyletic groups of seep vestimentiferan tubeworm endosymbiont sequences described by McMullin et al, (2003). Three specimens from GoM site DC673 (*Lamellibrachia* sp. 1, *Lamellibrachia* sp. 2, and *E. laminata* (DC673_1211, DC673_1209, DC673_1170) shared the identical (100% 16S rRNA gene sequence) "group 2" endosymbiont, very closely related to the

sequences from *Lamellibrachia* sp. 1 and sp. 2 endosymbionts at site GC852 (GC852_L4, GC852_L1, GC852_L5) and *E. laminata* endosymbiont sequence from WR269 (WR269_E10). However, the endosymbiont sequence derived from an *E. laminata* specimen at site AC601 (AC601_E6) differed and clustered with "group 1" sequences. In two other tubeworm specimens (*E. laminata* and *Lamellibrachia* sp. 2) from site AC601 (AC601_L1, AC601_L20) we detected two different endosymbionts, one clustering with "group 1" (AC601_L1-PT1, AC601_L20-PT1) and the other with "group 2" (AC601_L1-PT2, AC601_L20-PT2). Endosymbiont sequences derived from *S. jonesi* and *L. luymesi* tubeworms from the shallower sites GC234 (GC234_4587, GC234_L7), and GC184 (GC184_L9) clustered with "group 3" sequences (Figure 6).

*Genes involved in endosymbiont energy metabolism*

In order to determine the potential energy-generating pathways for chemoautotrophic growth of the endosymbiont from the Palinuro *L. anaximandri*, we tried to amplify fragments of genes coding for key enzymes involved in the oxidation of sulfur compounds, hydrogen and methane.

The genetic potential for sulfur oxidation of the endosymbiont was analyzed by amplifying gene fragments coding for dissimilatory sulfite reductase (*dsrAB*), APS reductase (*aprA*) – both enzymes of the APS pathway – and sulfate thiohydrolase (*soxB*), an essential component of the Sox multienzyme complex (Friedrich et al., 2001). Fragments of all three genes (*dsrAB*, *aprA*, *soxB*) were recovered supporting a sulfide-oxidizing chemotrophic energy metabolism of the endosymbiont. Sequence similarities as well as phylogenetic analysis showed them to be very similar to the endosymbionts of other *L. anaximandri* (*apr* within symbiont cluster) and the vestimentiferans *Riftia pachyptila* and *Tevnia jerichonana* from hydrothermal vents on the East Pacific Rise (*dsrAB*, *soxB*) (Figure 7). The 397 bp *aprA* sequence showed highest similarity (97% nucleotide similarity, 100% amino acid similarity) to *L. anaximandri* endosymbiont "phylotype 1" described from seep specimens at the Amon mud volcano in the Eastern Mediterranean (Duperron et al., 2009). Phylogenetic analysis places the *Lamellibrachia aprA* sequences in a cluster of oxidizing lineage II APS reductase gene sequences of endosymbiotic and free-living beta- and gammaproteobacteria including endosymbionts of *Riftia* and *Tevnia* (Meyer and Kuever, 2007a; Markert et al., 2011; Gardebrecht et al., 2012). The *dsrAB* gene fragment (987 bp) was most closely related to dissimilatory sulfite reductase genes from the

457 *Riftia*/*Tevnia* endosymbiont (NZ_AFZB01000023, EGW53672 and EGV52261, 80% nucleotide
458 and 84% amino acid sequence similarity, Figure 7).
459 Likewise, the 986 bp *soxB* fragment from *L. anaximandri* from Palinuro showed highest
460 similarities to *soxB* from *Candidatus* Endoriftia persephone (EF618617, EGV50931 and
461 EGW54296, 84% nucleotide similarity, 90% amino acid similarity).
462 A fragment of the *hupL* gene, encoding the large subunit of a [NiFe] uptake hydrogenase was
463 amplified using the primer set W1 and Wxy. BLAST search as well as phylogenetic analysis
464 demonstrated highest similarity with reference sequences from the *Riftia*/*Tevnia* endosymbiont
465 (EGV51840, EGW53439, 82 % nucleotide identity, 93 % amino acid identity). This enzyme has
466 been shown to be involved in the oxidation of molecular hydrogen for energy generation
467 (Petersen et al., 2011; Kleiner et al., 2012).
468 Key genes for enzymes of methane oxidation (mxaF, mmoX, pmoA) were not successfully
469 amplified with the different primer sets (MxaF1003, MxaR1555, MxaR1561, mmoXA, mmoXB,
470 A189F, MB661R (Costello and Lidstrom, 1999; Auman et al., 2000; Neufeld et al., 2007;
471 Kalyuzhnaya et al., 2008) used in this study.
472
473 *Genes involved in Nitrate reduction*
474 A nitric oxide reductase (*norCB*) gene sequence was successfully amplified and sequenced from
475 the Palinuro *L. anaximandri* endosymbiont indicating the potential to reduce nitrate. The closest
476 relative was again the endosymbiont of *Riftia*/*Tevnia* (EMBL entry ZP_08818090) with 95%
477 amino acid sequence similarity. In the metagenomes of the *Riftia* and *Tevnia* endosymbionts, all
478 genes needed for a complete respiration of nitrate to dinitrogen gas have been detected, and it has
479 been suggested that the endosymbionts of these species could possibly use nitrate as alternative
480 electron acceptor (Gardebrecht et al., 2012).
481
482 *Genes involved in carbon fixation*
483 To investigate the autotrophic potential of the endosymbionts, we tried to amplify key genes of
484 two carbon fixation pathways, the Calvin-Benson-Bassham (CBB) cycle and the reductive
485 tricarboxylic acid (rTCA) cycle.
486 The $CO_2$ fixing enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) is the key
487 enzyme of the CBB cycle. In Proteobacteria, two different types are known, form I, encoded by

488 the *cbbL* gene, and form II encoded by *cbbM*. In accordance with other studies on
489 *Lamellibrachia* spp. endosymbionts (Elsaied and Naganuma, 2001; Elsaied et al., 2002), we
490 failed to amplify *cbbL* from the Palinuro tubeworm endosymbiont. In contrast, a fragment of the
491 *cbbM* gene was detected supporting the usage of the CBB cycle for carbon fixation by the
492 endosymbiont as was also previously demonstrated for other *Lamellibrachia* spp. endosymbionts
493 (Elsaied and Naganuma, 2001; Elsaied et al., 2002; Vrijenhoek et al., 2007). The *cbbM* sequence
494 displayed high similarity (96% nucleotide similarity, 100% amino acid similarity) to the bacterial
495 endosymbiont of *L. anaximandri* from the Eastern Mediterranean (FM165442 and CAQ63473,
496 Duperron et al., 2009), but was quite different from *cbbM* sequences of *Riftia/Tevnia*
497 endosymbionts (AF047688, 78% nucleotide similarity, 75% amino acid similarity).
498 Based on genomic, proteomic, enzymatic as well as isotopic data, the *Riftia pachyptila*
499 endosymbiont uses the rTCA cycle in addition to the CBB cycle for autotrophic carbon fixation
500 (Markert et al., 2007, 2011; Gardebrecht et al., 2012). Yet a novel type of ATP citrate lyase (type
501 II ACL) might be active in this case (Hügler and Sievert, 2011).
502 Newly designed primers were used to amplify a putative type II ACL from the endosymbionts of
503 the Palinuro tubeworms. Unexpectedly, amplified fragments of this type II ACL gene indicate
504 additional use of the rTCA cycle for carbon fixation in the Palinuro *L. anaximandri*
505 endosymbiont as well. BLAST results with sequence similarities of 78% amino acid identities, as
506 well as the phylogenetic analysis indicate the gene to be most closely related to the *Riftia* and
507 *Tevnia* endosymbiont (NCBI entry ZP_08829917 and ZP_08817421). In contrast genes coding
508 for a conventional ACL could not be detected using previously published primers for either
509 subunits (*aclA* or *aclB*, Campbell et al., 2003; Hügler et al., 2005).
510
511 *Carbon fixation genes in seep vestimentiferan endosymbionts from the Gulf of Mexico*
512 The discovery of ACL genes in the *L. anaximandri* endosymbiont from the Palinuro volcanic
513 complex raised the question about the further distribution of these genes in tubeworm
514 endosymbionts, especially seep species. Thus we analyzed endosymbionts from 13 tubeworms
515 from six different sites in the Gulf of Mexico. We discovered the type II ACL genes in all
516 tubeworm endosymbionts investigated, regardless of their host species identity or site in the
517 GoM (Table 2). Sequence analysis revealed the type II ACL gene to be highly conserved
518 between the different GoM tubeworm endosymbionts. Three different phylotypes were found to

519 be present in the 13 tubeworm samples in the GoM, and all three differed from the sequences
520 found in the Palinuro *L. anaximandri* endosymbionts (Figure 8A). All GoM endosymbionts of
521 host specimens from the sites DC673, WR269, and GC852 (DC673_1211, DC673_1209,
522 DC673_1170, WR269_E10, GC852_L4, GC852_L1, GC852_L5) shared identical (100% AA
523 similarity) type II ACL gene sequences (cluster 2) regardless of host species identity.
524 Endosymbionts of GoM host specimens from the shallower Green Canyon sites GC234 and
525 GC184 (GC234_4587, GC184_L9, GC234_L7) also bear one single type II ACL gene sequence
526 (cluster 3) (100% AA similarity), which differed from the deep water site sequence in 14 AA.
527 The third sequence type (cluster 1) was retrieved from endosymbionts of Alaminos Canyon site
528 AC601 (AC601_E6, AC601_L1, AC601_L20). Within AC601_L1 and AC601_L20 a second
529 ACL sequence type identical to the sequences of cluster 2 was also retrieved. In phylogenetic
530 analysis, the GoM tubeworm endosymbiont ACL type II sequences formed a cluster together
531 with the Mediterranean *L. anaximandri* endosymbiont sequences, and were clearly separated
532 from the *Riftia*/*Tevnia* sequences (Figure 8A).
533 In addition to the *acl* genes, we also amplified the *cbbM* gene of the GoM tubeworm
534 endosymbionts. As expected, all endosymbionts harbored a *cbbM* gene in addition to the *acl*
535 gene. Similar to the 16S rRNA and *acl* genes, the *cbbM* genes from the GoM endosymbionts
536 formed three different clusters (Figure 8B). Cluster 1 comprises the sequences of Alaminos
537 Canyon site AC601 specimens, AC601_E6, AC601_L1, and AC601_L20. The *cbbM* sequences
538 of DeSoto Canyon, Walker Ridge and Green Canyon specimens DC673_1211, DC673_1209,
539 WR269_E10, GC852_L4, GC852_L1, GC852_L5 form a second cluster (cluster 2), while the
540 cbbM sequence of sample DC673_1170 falls in between these two clusters. Cluster 3 (Green
541 Canyon samples GC234_4587, GC184_L9, GC234_L7) is clearly separated from the others. The
542 *cbbM* sequences from the Mediterranean tubeworm endosymbionts form a separate cluster. Quite
543 interestingly, the *cbbM* sequences of the *Riftia/Tevnia* endosymbionts are only distantly related
544 (Figure 8B).
545
546 **Isotopic signature**
547 Bulk organic carbon isotopes analyses of gill tissue from two Palinuro tubeworms resulted in
548 $\delta^{13}C$ values of -22.5 and -23.4‰, which are in accordance to previous measurements of
549 Mediterranean *Lamellibrachia* spp. (Olu-Le Roy et al., 2004; Carlier et al., 2010) but more

positive than most *Lamellibrachia* spp. from non-Mediterranean hydrocarbon seeps (Becker et al., 2011). The carbon isotopic composition of dissolved inorganic carbon in emanating diffuse fluids sampled at the tubeworm colonies display $\delta^{13}C$ values of -0.39 and -0.68‰. The $\delta^{13}C_{DIC}$ values of additional samples of shimmering water in the area range from -1.62 to +1.76‰.

## DISCUSSION

### Phylogeny and biogeography of the Mediterranean tubeworms

The discovery of living vestimentiferan tubeworm colonies associated with active hydrothermal venting during a seafloor survey of the Palinuro volcanic complex (Mediterranean Sea) in July 2006 (Petersen et al., 2008; Monecke et al., 2009) came as a surprise, as until then, vent-associated tubeworms were only known from the Pacific Ocean. Living individuals of the tubeworms were sampled during a research cruise in 2011 and this communication is the first description of the worms and their endosymbionts. Phylogenetic analyses of 18S rRNA, COI and mitochondrial 16S rRNA genes showed that the tubeworms from Palinuro are *Lamellibrachia anaximandri* recently described from mud volcanoes of the Eastern Mediterranean (Southward et al., 2011). The highest *in vivo* temperatures measured among the tubes in tubeworm aggregations at the Palinuro hydrothermal vent field were 15.6 – 19.4°C, elevated by as much as 5.4°C from the surrounding Mediterranean Seawater (14°C) and the previously published tubeworm-bearing locations in the Eastern Mediterranean (13 – 14°C), extending the previously described temperature range of the species (Southward et al., 2011). *L. anaximandri* has also been detected in a mud volcano field in the Western Mediterranean (Hilário et al., 2011), as well as on two ship wrecks in the Eastern Mediterranean (110 km southeast of Crete, Hughes and Crawford, 2008) and the Southern Tyrrhenian Sea (Gambi et al., 2011) (Figure 1). Even though this species has not been detected in the Northeastern Atlantic it has been hypothesized to occur at the West African and Lusitanian margins as well (Hilário et al., 2011).

The high diversity of habitats for the Mediterranean *Lamellibrachia* species is in accordance with *Escarpia* spp. and other *Lamellibrachia* spp.. Originally regarded as seep species, they were subsequently found in several non-seep habitats, i.e. at sediment covered hydrothermal sites in the Pacific (Juniper et al., 1992; Fujikura et al. 2006; Miake et al., 2006; Miura and Kojima, 2006), as well as ship wrecks (Dando et al., 1992) and whale falls (Feldman et al., 1998).

581 Considering the high diversity of so called seep-tubeworm habitats a high site-flexibility of these
582 organisms becomes apparent and supports the importance of different non-seep habitats in their
583 geographic distribution and the stepping stone hypothesis (Kimura and Weiss, 1964; Smith and
584 Kukert, 1989; Black et al., 1994; Olu et al., 2010). Larval survival of at least three weeks and
585 about five weeks has been demonstrated for the vestimentiferans *Riftia pachyptila* and
586 *Lamellibrachia luymesi* respectively, suggesting potential dispersal distances on the order of 100
587 km for seep and vent vestimentiferans (Young et al., 1996; Marsh et al., 2001; Tyler and Young,
588 2003). A variety of reducing habitats, functioning as dispersal stepping-stones separated by days
589 or weeks and connected by currents or shared water masses could facilitate the large species
590 ranges described for many vestimentiferans, including the seep species *L. barhami*, which has
591 been found in seep and low activity vent sites spanning at least 4,000 – 6,000 km of geographical
592 distance (McMullin et al., 2003).
593
594 **Phylogeny of endosymbionts**
595 The trophosome of the *L. anaximandri* specimens from Palinuro analyzed in this study harbored
596 a single gammaproteobacterial phylotype regardless of collection site or morphotype. The
597 endosymbiont was closely related (99.7%) to the phylotype 2 found in *L. anaximandri* from the
598 Amon mud volcano east of the Nile deep-sea fan (Duperron et al., 2009). The dominating
599 endosymbiont (phylotype 1) of the seep specimen from the Amon mud volcano was not found in
600 the tubeworms at the Palinuro hydrothermal vents.
601 This study is the first to characterize vestimentiferan tubeworm endosymbionts of shallow
602 hydrothermal vents in the Mediterranean Sea. The gammaproteobacterial endosymbiont clusters
603 with endosymbionts of other seep-associated tubeworms and are clearly distinct from the
604 endosymbionts of vent-tubeworms like *Riftia* and *Tevnia* (Fisher et al., 1997; Nelson and Fisher,
605 2000; McMullin et al., 2003; Vrijenhoek et al., 2007). The phylogenetic affiliation of the
606 Palinuro *L. anaximandri* endosymbiont with "group 3" endosymbiont 16S rRNA gene
607 sequences, as well as the affiliation of the dominating phylotype of *L. anaximandri* specimen
608 obtained from 1,157 m depth at Amon mud volcano with "group 1", suggest separation by depth,
609 as suggested for other seep-vestimentiferan endosymbionts (McMullin et al., 2003). However,
610 both Mediterranean phylotype sequences show considerable differences to the "group 1" and
611 "group 3" cluster sequences in signature nucleotide positions (Suppl. Table S1) and

612 Mediterranean and GoM tubeworms do not share identical endosymbiont phylotypes. Further, in
613 the Amon mud volcano specimen, phylotypes of "group 1" and "group 3" are present, yet in
614 assumed different abundances (deduced from the numbers of sequences in the clone libraries
615 (Duperron et al., 2009)). Endosymbionts of different groups are also present in individuals of
616 *Lamellibrachia* sp. 2 (AC601_L20) and *E. laminata* (AC601_L1) tubeworms from the GoM site
617 AC601 (this study). Thus separation by depth alone cannot explain these observations and
618 further studies are needed in order to reveal the question how endosymbionts are selected by
619 their vestimentiferan hosts.
620
621 **Metabolic characteristics of the endosymbiont**
622 Based on the functional gene analyses of this study, the endosymbiont of *Lamellibrachia*
623 *anaximandri* from Palinuro is a sulfide-oxidizing chemoautotroph. $\delta^{13}C$ values measured in this
624 study are in consistence with previous studies of *L. anaximandri* from Eastern Mediterranean
625 mud volcano fields (Olu-Le Roy et al., 2004; Carlier et al., 2010), and together with delta $\delta^{15}N$
626 and $\delta^{34}S$ from previous studies support a chemoautotrophic endosymbiont based nutrition for the
627 host tubeworm (Carlier et al., 2010). Due to the presence of *dsrAB* and *aprA* genes, sulfide
628 oxidation most likely is carried out via the APS pathway with sulfite and adenosine
629 phosphosulfate as intermediates. As in the *Riftia pachyptila* endosymbiont, *soxB* is also present
630 in the endosymbiont of *L. anaximandri* from Palinuro. In thiosulfate-utilizing bacteria, SoxB
631 functions as sulfate thiohydrolase. However, since tubeworm endosymbiont carbon fixation is
632 not stimulated by thiosulfate, its function in the tubeworm endosymbionts remains uncertain
633 (Fisher et al., 1989; Markert et al., 2011).
634 Although high methane fluxes were noted in the habitat of *L. anaximandri* in mud volcano
635 habitats (Olu-Le Roy et al., 2004), genes of methane oxidation were not successfully amplified
636 with the primer sets used in this study. In contrast, the potential to use hydrogen as an energy
637 source was suggested by the detection of the key gene for hydrogen oxidation, *hupL* in the
638 endosymbiont from the Palinuro *L. anaximandri*. The *hupL* gene was most similar to the
639 respective genes of the *Riftia* and *Tevnia* endosymbionts, where it is even expressed *in situ*
640 (Markert et al., 2011; Petersen et al., 2011). Hydrogen concentrations have not been measured in
641 the hydrothermal fluids from Palinuro volcano complex. However, hydrogen is present in the
642 fluids at many hydrothermal vents, and elevated hydrogen contents are present at vent systems

643 associated with ultramafic (mantle) rocks, or e.g. following a volcanic eruption (Wetzel and
644 Shock, 2000; Allen and Seyfried, 2003; Kumagai et al., 2008, Petersen et al., 2011). Such $H_2$-
645 rich fluids found at vent systems associated with ultramafic rocks have recently been shown to
646 be used as an energy source by the endosymbionts of a mussel, *Bathymodiolus puteoserpentis*
647 (Petersen et al., 2011). The endosymbionts in *Bathymodiolus* spp. mussels are located on the
648 external edge of the cells of gill filaments that are themselves only 2 cells thick. As a result,
649 passive diffusion of the energy source (hydrogen, sulfide, and/or methane in different species) is
650 sufficient to fuel the chemoautotrophic life style of these animals (Childress and Fisher, 1992). In
651 contrast, the endosymbionts in tubeworms are deep in an interior tissue and must rely on the host
652 blood to supply the electron donor for chemoautotrophy. Sulfide is transported in millimolar
653 concentrations to the trophosome, bound to hemoglobin molecules in vestimentiferan
654 tubeworms. Transport molecules for hydrogen or methane have not been found in these animals
655 and thus both hydrogen and methane are unlikely to contribute significantly to the metabolism of
656 the intact symbiosis in most environments (Childress and Fisher, 1992). However, the
657 gammaproteobacterial endosymbiont might use hydrogen as potential energy source in its free-
658 living stage. Thus, the potential for use of hydrogen by tubeworm endosymbionts deserves
659 additional study.
660 Detection of *cbbM* sequences coding for a form II RubisCO in all vestimentiferan tubeworms
661 reviewed in this study indicates that the potential to fix $CO_2$ via the CBB cycle is widespread in
662 vestimentiferan tubeworms (Elsaied and Naganuma, 2001; Elsaied et al., 2002; Naganuma et al.,
663 2005; Vrijenhoek et al., 2007; Duperron et al., 2009) and the detection of all genes of this cycle
664 in the metagenome of the *Riftia* endosymbiont suggest this pathway is fully functional in
665 vestimentiferans (see Markert et al., 2011 for further details). Enzyme activity measurements of
666 RubisCO and phosphoribulokinase add further evidence to the usage of the CBB cycle in *Riftia*
667 and *Lamellibrachia* endosymbionts (Felbeck, 1981; Felbeck et al., 1981). Up to now, there is no
668 evidence for the presence of RubisCO form I (*cbbL*) in either the *Riftia/Tevnia* or any
669 *Lamellibrachia* endosymbiont (this study; Elsaied and Naganuma, 2001; Elsaied et al., 2002;
670 Naganuma et al., 2005; Duperron et al., 2009).
671 In addition, *acl* genes, coding for ATP citrate lyase, the key enzyme of the rTCA cycle were
672 recovered from the Palinuro *L. anaximandri* endosymbiont using newly designed primers,
673 suggesting the presence of the rTCA cycle as alternate carbon fixation pathway. The operation of

674 the rTCA cycle in addition to the CBB cycle was first shown for the *Riftia* and *Tevnia*
675 endosymbiont using a combination of metagenomic, proteomic and enzymatic approaches
676 (Markert et al., 2007; Robidart et al., 2008; Gardebrecht et al., 2012). In the case of the
677 *Riftia/Tevnia* endosymbiont, citrate cleavage is accomplished by an unusual type of ATP citrate
678 lyase, tentatively named ACL type II (Hügler and Sievert, 2011). The recovered *acl* sequence
679 from the Mediterranean Palinuro *L. anaximandri* endosymbiont showed high similarities to the
680 sequence of the *Riftia*/*Tevnia* endosymbiont (Figure 8A). Subsequent analyses of seep
681 vestimentiferan (*Escarpia, Seepiophila* and *Lamellibrachia*) endosymbionts from different sites
682 at the Gulf of Mexico showed the presence of type II ACL genes there as well. This implicates a
683 wider distribution of these genes than previously thought. The presence of two different carbon
684 fixation pathways – the CBB cycle and the rTCA cycle – in a single bacterium seems not
685 restricted to the *Riftia*/*Tevnia* endosymbiont, but rather seems to be a common feature of
686 vestimentiferan tubeworm endosymbionts, regardless of genus or habitat.
687 Despite the still rather scarce dataset of type II ACL gene sequences, these sequences appear to
688 be monophyletic in tubeworm endosymbionts (Figure 8A). Similarly, ribosomal genes as well as
689 *apr*A genes support a monophyletic origin for the tubeworm endosymbionts (Figures 6, 7). In
690 contrast, a monophyletic origin of the *cbbM* gene of tubeworm endosymbionts is not clearly
691 supported by the phylogenetic analyses performed here (Figure 8B). This could mean that the
692 rTCA cycle is the evolutionary older $CO_2$ fixation pathway in the endosymbionts, and the *cbbM*
693 gene is acquired afterwards, e.g. via lateral gene transfer. This evolutionary aspect clearly
694 requires further studies.
695 The presence of two different carbon fixation pathways increases the metabolic versatility of the
696 tubeworm endosymbionts. In case of the *Riftia/Tevnia* endosymbiont proteomic data suggest the
697 usage of both pathways simultaneously (Markert et al., 2007, 2011; Gardebrecht et al., 2012).
698 This is also supported by the isotopic signature of the *Riftia* tubeworms (Markert et al., 2007).
699 The carbon isotopic fractionation associated with the rTCA cycle is generally smaller than the
700 one observed for the CBB cycle (House et al., 2003). Considering the carbon isotopic
701 composition of ca. -23 ‰ measured for plume tissue of two *L. anaximandri* tubeworms from
702 Palinuro low temperature diffuse vent sites and a respective carbon isotopic composition of
703 dissolved inorganic carbon ($\delta^{13}C_{DIC}$ between -0.7 and -0.4 ‰), the isotopic difference would be
704 consistent with the operation of the CBB cycle for autotrophic carbon fixation. Neither a greatly

attenuated isotopic fractionation characteristic for the rTCA cycle nor an isotopic signature reflecting a substantial contribution from methane-derived carbon is discernible at the site studied here. Yet, one has to keep in mind, that the isotopic signature provides only indirect evidence and neither the actual fractionation by the enzymes involved in the rTCA cycle present in tubeworm endosymbionts, nor fractionation during uptake and transport of DIC to the endosymbionts are known. Thus future studies are needed in order to determine the conditions for the usage, as well as the regulation of the two different carbon fixation pathways in vestimentiferan tubeworm endosymbionts (Hügler and Sievert, 2011).

## CONCLUSIONS

In this study we characterize vestimentiferan tubeworms and their endosymbionts from the Mediterranean Sea and the Gulf of Mexico. The tubeworms retrieved from a shallow water hydrothermal vent field in the Western Mediterranean – Palinuro volcanic complex – represent the first vestimentiferan tubeworms found associated with hydrothermal venting outside the Pacific Ocean. Our molecular studies of marker genes (18S rRNA, mitochondrial 16S rRNA and COI) identify the tubeworms as the recently described species *L. anaximandri*, the only vestimentiferan species described from the Mediterranean Sea to date.

Based on 16S rRNA gene surveys we conclude that the Palinuro *L. anaximandri* harbor a single gammaproteobacterial endosymbiont, closely related to endosymbionts of other *Lamellibrachia* spp. (Figure 6). Carbon isotopic data and the analysis of functional genes suggest a sulfide-oxidizing chemoautotrophic lifestyle. Energy can be generated by oxidizing reduced sulfur compounds via the APS pathway involving dissimilatory sulfite reductase and APS reductase. Due to the presence of a *hupL* gene one can speculate that the endosymbiont has the potential to use hydrogen as a supplemental energy source. Nitrate could potentially serve as alternative electron acceptor for the endosymbiont, as we detected a nitric oxide reductase gene sequence (*norCB*) and it was shown, that the metagenome of the *Riftia*/*Tevnia* endosymbiont includes all genes needed for the complete reduction of nitrate to dinitrogen gas (Gardebrecht et al., 2012).

Surprisingly, we were able to detect the key genes of two alternative carbon fixation pathways, namely *cbbM*, encoding RubisCO form II, the key enzyme of the CBB cycle, and a gene coding for ATP citrate lyase type II, the key enzyme of the rTCA cycle. Newly designed primers were used to amplify a gene sequence of the type II ACL. The presence of the rTCA cycle in addition

to the CBB cycle for carbon fixation was previously shown for the endosymbiont of the vent associated tubeworms *Riftia pachyptila* and *Tevnia jerichonana* (Markert et al., 2007; Gardebrecht et al., 2012). However, before this study, only the CBB cycle was documented as a carbon fixation pathway for *Lamellibrachia* spp. (Felbeck, 1981; Felbeck et al., 1981; Elsaied and Naganuma, 2001; Elsaied et al., 2002; Vrijenhoek et al., 2007). We also demonstrate the presence of the key genes of both carbon cycles in the endosymbionts from *Lamellibrachia luymesi, Lamellibrachia* sp. 1*, Lamellibrachia* sp. 2, *Escarpia laminata*, and *Seepiophila jonesi* from the Gulf of Mexico. These results suggest that the occurrence of two carbon fixation pathways in one bacterium may be a common feature of vestimentiferan tubeworm endosymbionts, which in turn indicates that this feature is more widely distributed than previously considered. It has already been shown, that carbon fixation through the rTCA cycle is important at deep-sea hydrothermal vent sites (cf. Hügler and Sievert 2011 and references therein). Our study indicates that the rTCA cycle could play an important role at seep sites as well.

**ACKNOWLEDGEMENTS**

The authors would like to thank the captain and crew of R/V *Poseidon* for their support during cruise Pos412. We also gratefully acknowledge the support of ROV pilots Jamie Norman and Paul Hastings during sampling as well as Peter Buchanan (Oceaneering Inc., Aberdeen, UK) for good cooperation during ROV Mohawk negotiations and adaptation for research purposes. We further thank Katrin Kleinschmidt (GEOMAR, Kiel, Germany) for onboard support. We also thank the captains and crews of the RV *Seward Johnson* and NOAA ship *Ronald Brown* as well as the pilots and support personnel for the Johnson Sea Link submersible and ROV Jason II for assistance with the collection of Gulf of Mexico Tubeworms. For Sanger sequencing, we would like to thank the teams from the IKMB at the UKSH, Kiel and from the sequencing facility at the Pennsylvania State University, PA, USA. Artur Fugmann and Ben Hindersmann (WWU Muenster, Germany) are thanked for their assistance during stable isotope measurements, and Ulrike Westernströer and Karen Bremer (CAU Kiel, Germany) for help with elemental analysis. Further, we thank Chunya Huang (Pennsylvania State University, PA, USA) for assistance during COI gene analysis and Costantino Vetriani (Rutgers University, NJ, USA) for fruitful discussion. JFI, SP, DGS, and HS acknowledge support through the German Science Foundation

767 (IM12/18). CRF acknowledges the support of the US NSF, the US Bureau of Ocean Energy
768 Management and US NOAA Office of Ocean Exploration for many years of support for the
769 study of Gulf of Mexico seep communities.
770

## FIGURE LEGENDS

Figure 1: Map with locations of vestimentiferan tubeworms of the genus *Lamellibrachia* described from previous studies (black circles) and this study (red circle) within the Mediterranean Sea.

Figure 2: Map of sampling locations for tubeworm specimens in the Gulf of Mexico included in this study. Scale is in degrees longitude; one degree = 111.12 km

Figure 3: *Lamellibrachia* sp. tubeworms recovered from the Palinuro volcanic complex (Mediterranean Sea). (A) in their natural habitat (photo obtained at Palinuro during cruise Pos340), (B) directly after ROW Mohawk recovery (Pos412) onboard, (C) individual from colony #1 dissected from its tube (not used for further analysis), (D) stere-micrograph of plume region, (E) stereo-micrograph of trophosome region.

Figure 4: Phylogenetic relationship of vestimentiferan tubeworms based on mitochondrial 16S rRNA gene sequences. The Maximum Likelihood tree was calculated using the GTR model. Numbers at the nodes indicate the proportion of occurrences in 100 bootstrap replicates. The scale represents 0.01 substitutions per nucleotide site.

Figure 5: Microscopic images of Palinuro *Lamellibrachia* sp. specimen endosymbionts. (A and B) Light micrographs showing trophosome content with large spherical prokaryotic cells (dark) of different size and shape. Various stages of equal and unequal cell division as well as budding can be recognized. (C, D, E, F) Scanning electron micrographs showing endosymbionts with characteristically structured cell surface. Probable budding stages (C and D) and unequal cell division (E and F).

Figure 6: Phylogenetic relationship of tubeworms endosymbionts based on 16S rRNA gene sequences. The Maximum Likelihood tree was calculated using the GTR model. Numbers at the nodes indicate the proportion of occurrences in 100 bootstrap replicates. The scale represents 0.01 substitutions per nucleotide site.

Figure 7: Phylogenetic tree based on dsrA (A) and aprA (B) protein sequences. The Maximum Likelihood tree was calculated using the JTT model. Numbers at the nodes indicate the proportion of occurrences in 100 bootstrap replicates.. Sequences obtained in the present

study are depicted bold and colored. The scale represents 0.1 substitutions per amino acid position.

Figure 8: Phylogenetic tree based on aclA (A) and cbbM (B) protein sequences. The Maximum Likelihood tree was calculated using the JTT model. Numbers at the nodes indicate the proportion of occurrences in 100 bootstrap replicates.. Sequences obtained in the present study are depicted bold and colored. The scale represents 0.1 substitutions per amino acid position.

Table 1: Position of tubeworm colonies sampled during RV *Poseidon* cruise Pos412 at Palinuro volcanic complex, Tyrrhenian Sea and temperatures measured within colonies.

Table 2: Sample identity, geographic origin and gene sequences accession numbers of tubeworm specimens and their endosymbionts included in this study.

Table 3: Composition of seawater-hydrothermal fluid mixtures inside tubeworm colonies #1 and #2.

Table S1: Signature nucleotide of tubeworm endosymbiont 16S rRNA gene sequences from vent- and seep vestimentiferan tubeworms. "Vent" vestimentiferan group includes endosymbiont rRNA gene sequences of *Riftia pachyptila*, *Tevnia jerichonana* and *Osasisia alvinae*. "Seep" vestimentiferan tubeworm endosymbiont cluster "seep-(1-3)" refer to grouping according to McMullin et al. (2003). PT-1 and PT-2 refer to *Lamellibrachia anaximandri* endosymbionts, phylotype 1 and phylotype 2 according to Dupperon et al. (2009). Pos412 refers to the *Lamellibrachia anaximandri* endosymbiont rRNA gene sequence phylotype obtained from the Palinuro volcanic complex during cruise Pos412 of RV *Poseidon* in this study. The position numbers refer to *E. coli* gene sequence positions.

## Reference List

Allen, D. E. and Seyfried, W. E. (2003). Compositional controls on vent fluids from ultramafic-hosted hydrothermal systems at mid-ocean ridges: An experimental study at 400°C, 500 bars. *Geochimica et Cosmochimica Acta*. 67, 1531 - 1542.

828 Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.
829 J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search
830 programs. *Nucleic Acids Research*. 25, 3389 - 3402

831 Auman, A. J., Stolyar, S., Costello, A. M., and Lidstrom, M. E. (2000). Molecular
832 characterization of methanotrophic isolates from freshwater lake sediment. *Applied and*
833 *Environmental Microbiology*. 66, 5259 - 5266.

834 Bayon, G., Loncke, L., Dupré, S., Caprais, J. C., Ducassou, E., Duperron, S., Etoubleau, J.,
835 Foucher, J. P., Fouquet, Y., and Gontharet, S. (2009). Multi-disciplinary investigation of
836 fluid seepage on an unstable margin: The case of the Central Nile deep sea fan. *Marine*
837 *Geology*. 261, 92 - 104.

838 Becker, E. L., Macko, S. A., Lee, R. W., and Fisher, C. R. (2011). Stable isotopes provide new
839 insights into vestimentiferan physiological ecology at Gulf of Mexico cold seeps.
840 *Naturwissenschaften*. 98, 169 - 174.

841 Black, M. B., Lutz, R. A., and Vrijenhoek, R. C. (1994). Gene flow among vestimentiferan tube
842 worm (*Riftia pachyptila*) populations from hydrothermal vents of the eastern Pacific.
843 *Marine Biology*. 120, 33 - 39.

844 Bright, M., Keckeis, H., and Fisher, C. R. (2000). An autoradiographic examination of carbon
845 fixation, transfer and utilization in the *Riftia pachyptila* symbiosis. *Marine Biology*. 136,
846 621 - 632.

847 Campbell, B. J. and Cary, S. C. (2004). Abundance of reverse tricarboxylic acid cycle genes in
848 free-living microorganisms at deep-sea hydrothermal vents. *Applied and Environmental*
849 *Microbiology*. 70, 6282 - 6289.

850 Campbell, B. J., Stein, J. L., and Cary, S. C. (2003). Evidence of chemolithoautotrophy in the
851 bacterial community associated with *Alvinella pompejana*, a hydrothermal vent
852 polychaete. *Applied and Environmental Microbiology*. 69, 5070 - 5078.

853 Carlier, A., Ritt, B., Rodrigues, C. F., Sarrazin, J., Olu, K., Grall, J., and Clavier, J. (2010).
854 Heterogeneous energetic pathways and carbon sources on deep eastern Mediterranean
855 cold seep communities. *Marine Biology*. 157, 2545 - 2565.

856 Childress, J. J. and Fisher, C. R. (1992). The biology of hydrothermal vent animals: physiology,
857 biochemistry, and autotrophic symbioses. *Oceanography and Marine Biology an Annual*
858 *Review*. 30, 337 - 441.

859 Cline, J. D. (1969). Spectrophotometric determination of hydrogen sulfide in natural waters.
860 *Limnology and Oceanography*. 454 - 458.

861 Cordes, E. E., Bergquist, D. C., and Fisher, C. R. (2009). Macro-ecology of Gulf of Mexico cold
862 seeps. *Annual Review of Marine Science*. 1, 143 - 168.

863 Costello, A. M. and Lidstrom, M. E. (1999). Molecular characterization of functional and
864 phylogenetic genes from natural populations of methanotrophs in lake sediments. *Applied*
865 *and Environmental Microbiology*. 65, 5066 - 5074.

866 Csaki, R., Hanczar, T., Bodrossy, L., Murrell, J. C., and Kovacs, K. L. (2001). Molecular
867 characterization of structural genes coding for a membrane bound hydrogenase in
868 *Methylococcus capsulatus* (Bath). *FEMS Microbiology Letters*. 205, 203 - 207.

869 Dando, P. R., Southward, A. F., Southward, E. C., Dixon, D. R., and Crawford, A. (1992).
870 Shipwrecked tube worms. *Nature*. 356, 667 - 667.

871 Duperron, S., De Beer, D., Zbinden, M., Boetius, A., Schipani, V., Kahil, N., and Gaill, F.
872 (2009). Molecular characterization of bacteria associated with the trophosome and the
873 tube of *Lamellibrachia sp.*, a siboglinid annelid from cold seeps in the Eastern
874 Mediterranean. *FEMS Microbiology Ecology*. 69, 395 - 409.

875 Elsaied, H., Kimura, H., and Naganuma, T. (2002). Molecular characterization and
876 endosymbiotic localization of the gene encoding D-ribulose 1, 5-bisphosphate
877 carboxylase/oxygenase (RuBisCO) form II in the deep-sea vestimentiferan trophosome.
878 *Microbiology*. 148, 1947 - 1957.

879 Elsaied, H. and Naganuma, T. (2001). Phylogenetic diversity of ribulose-1, 5-bisphosphate
880 carboxylase/oxygenase large-subunit genes from deep-sea microorganisms. *Applied and*
881 *Environmental Microbiology*. 67, 1751 - 1765.

882 Felbeck, H. (1981). Chemoautotrophic potential of the hydrothermal vent tube worm, *Riftia*
883 *pachyptila* Jones (Vestimentifera). *Science*. 213, 336 - 338.

884 Felbeck, H., Childress, J. J., and Somero, G. N. (1981). Calvin-Benson cycle and sulphide
885 oxidation enzymes in animals from sulphide-rich habitats. *Nature* 293, 291 - 293.

886 Feldman, R. A., Shank, T. M., Black, M. B., Baco, A. R., Smith, C. R., and Vrijenhoek, R. C.
887 (1998). Vestimentiferan on a whale fall. *The Biological Bulletin*. 194, 116 - 119.

888 Fisher, C. R., Childress, J. J., and Minnich, E. (1989). Autotrophic carbon fixation by the
889 chemoautotrophic symbionts of Riftia pachyptila. *The Biological Bulletin*. 177, 372 -
890 385.

891 Fisher, C. R., Urcuyo, I. A., Simpkins, M. A., and Nix, E. (1997). Life in the slow lane: growth
892 and longevity of cold–seep vestimentiferans. *Marine Ecology*. 18, 83 - 94.

893 Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. (1994). DNA primers for
894 amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan
895 invertebrates. *Molecular Marine Biology and Biotechnology*. 3:294–299.

896 Freytag, J. K., Girguis, P. R., Bergquist, D. C., Andras, J. P., Childress, J. J., and Fisher, C. R.
897 (2001). A paradox resolved: sulfide acquisition by roots of seep tubeworms sustains net
898 chemoautotrophy. *Proceedings of the National Academy of Sciences*. 98, 13408-134013.

Friedrich, C. G., Rother, D., Bardischewsky, F., Quentmeier, A., and Fischer, J. (2001). Oxidation of reduced inorganic sulfur compounds by bacteria: emergence of a common mechanism? *Applied and Environmental Microbiology*. 67, 2873 - 2882.

Fujikura, K., Fujiwara, Y., and Kawato, M. (2006). A new species of *Osedax* (Annelida: Siboglinidae) associated with whale carcasses off Kyushu, Japan. *Zoological Science* 23, 733-740.

Gambi, M. C., Schulze, A., and Amato, E. (2011). Record of *Lamellibrachia sp.*(Annelida: Siboglinidae: Vestimentifera) from a deep shipwreck in the western Mediterranean Sea (Italy). *Marine Biodiversity Records*. 4, e24.

Garbe-Schönberg, D., Koschinsky, A., Ratmeyer, V., Jähmlich, H., and Westernströer, U. (2006) KIPS - A new multiport valve-based all–Teflon fluid sampling system for ROVs. *Geophysical Research Abstracts* 8, 07032.

Gardebrecht, A., Markert, S., Sievert, S. M., Felbeck, H., Thürmer, A., Albrecht, D., Wollherr, A., Kabisch, J., Le Bris, N., Lehmann, R., Daniel, R., Liesegang, H., Hecker, M., and Schweder, T. (2012). Physiological homogeneity among the endosymbionts of *Riftia pachyptila* and *Tevnia jerichonana* revealed by proteogenomics. *The ISME Journal*. 6, 766-76.

Gardiner, S. L., McMullin, E., and Fisher, C. R. (2001). *Seepiophila jonesi*, a new genus and species of vestimentiferan tube worm (Annelida: Pogonophora) from hydrocarbon seep communities in the Gulf of Mexico. *Proceedings of the Biological Society of Washington*. 114, 694 - 707.

Guindon, S. and Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol*. 52, 696 - 704.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic acids symposium series*. 41, 95 - 98.

Hilário, A., Comas, M. C., Azevedo, L., Pinheiro, L., Ivanov, M. K., and Cunha, M. R. (2011). First record of a Vestimentifera (Polychaeta: Siboglinidae) from chemosynthetic habitats in the western Mediterranean Sea–Biogeographical implications and future exploration. *Deep Sea Research Part I: Oceanographic Research Papers*. 58, 200 - 207.

House, C. H., Schopf, J. W., and Stetter, K. O. (2003). Carbon isotopic fractionation by Archaeans and other thermophilic prokaryotes. *Organic Geochemistry*. 34, 345 - 356.

Hughes, D. J. and Crawford, M. (2008). A new record of the vestimentiferan *Lamellibrachia* sp. (Polychaeta: Siboglinidae) from a deep shipwreck in the eastern Mediterranean. *Marine Biodiversity Records*. 1, e21.

Hügler, M. and Sievert, S. M. (2011). Beyond the calvin cycle: autotrophic carbon fixation in the ocean. *Annual Review of Marine Science*. 3, 261-289.

Hügler, M., Wirsen, C. O., Fuchs, G., Taylor, C. D., and Sievert, S. M. (2005). Evidence for autotrophic $CO_2$ fixation via the reductive tricarboxylic acid cycle by members of the Epsilon- subdivision of proteobacteria. *Journal of Bacteriology*. 187, 3020 - 3027.

Jones, M. L. (1985). On the Vestimentifera, new phylum: six new species, and other taxa, from hydrothermal vents and elsewhere. *Bulletin of the Biological Society of Washington*. 117 - 158.

Julian, D., Gaill, F., Wood, E., Arp, A. J., and Fisher, C. R. (1999). Roots as a site of hydrogen sulfide uptake in the hydrocarbon seep vestimentiferan *Lamellibrachia sp*. *Journal of Experimental Biology*. 202, 2245 - 2257.

Juniper, S. K., Tunnicliffe, V., and Southward, E. C. (1992). Hydrothermal vents in turbidite sediments on a Northeast Pacific spreading centre: organisms and substratum at an ocean drilling site. *Canadian Journal of Zoology*. 70, 1792 - 1809.

Kalyuzhnaya, M. G., Hristova, K. R., Lidstrom, M. E., and Chistoserdova, L. (2008). Characterization of a novel methanol dehydrogenase in representatives of *Burkholderiales*: implications for environmental detection of methylotrophy and evidence for convergent evolution. *Journal of bacteriology*. 190, 3817 - 3823.

Kennicutt, M. C., Brooks, J. M., Bidigare, R. R., Fay, R. R., Wade, T. L., and McDonald, T. J. (1985). Vent-type taxa in a hydrocarbon seep region on the Louisiana slope. *Nature*. 317, 351 - 353.

Kidd, R. B. and Ármannson, H. (1979). Manganese and iron micronodules from a volcanic seamount in the Tyrrhenian Sea. *Journal of the Geological Society*. 136, 71 - 76.

Kimura, M. and Weiss, G. H. (1964). The stepping stone model of population structure and the decrease of genetic correlation with distance. *Genetics*. 49, 561 - 576.

Kleiner, M., Wentrup, C., Lott, C., Teeling, H., Wetzel, S., Young, J., Chang, Y. J., Shah, M., VerBerkmoes, N. C., Zarzycki, J., Fuchs, G., Markert, S., Hempel, K., Voigt, B., Becher, D., Liebeke, M., Lalk, M., Albrecht, D., Hecker, M., Schweder, T., and Dubilier, N. (2012). Metaproteomics of a gutless marine worm and its symbiotic microbial community reveal unusual pathways for carbon and energy use. *Proceedings of the National Academy of Sciences*. 109, E1173 - E1182

Kojima, S., Ohta, S., Yamamoto, T., Miura, T., Fujiwara, Y., Fujikura, K., and Hashimoto, J. (2002). Molecular taxonomy of vestimentiferans of the western Pacific and their phylogenetic relationship to species of the eastern Pacific. *Marine Biology*. 141, 57 - 64.

Kumagai, H., Nakamura, K., Toki, T., Morishita, T., Okino, K., Ishibashi, J. I., Tsunogai, U., Kawagucci, S., Gamo, T., Shibuya, T., Sawaguchi, T., Neo, N., Joshima, M., Sato, T., and Takai, K. (2008). Geological background of the Kairei and Edmond hydrothermal fields along the Central Indian Ridge: Implications of their vent fluids´ distinct chemistry. *Geofluids*. 8, 239 - 251.

Lane, D. (1991) "16S/23S rRNA sequencing.", in *Nucleic Acid Techniques in Bacterial Systematics*. ed. E. Stackebrandt, and M. Goodfellow (John Wiley & Sons, Chichester, UK), 115–175.

Lavik, G., Stührmann, T., Brüchert, V., Van der Plas, A., Mohrholz, V., Lam, P., Mußmann, M., Fuchs, B. M., Amann, R., Lass, U., and Kuypers, M. M. (2009). Detoxification of sulphidic African shelf waters by blooming chemolithotrophs. *Nature*. 457, 581 - 584.

Liao, X., Wang, D., Yu, X., Li, W., Cheng, L., Wang, J., and Tong, J. (2007). Characterization of novel microsatellite loci in rare minnow (*Gobiocypris rarus*) and amplification in closely related species in Gobioninae. *Conservation Genetics*. 8, 1003 - 1007.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, G., König, A., Liss, T., Lüssmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A. and Schleifer, K.H. (2004). ARB: a software environment for sequence data. *Nuleic Acids Research*. 32, 1363 - 1371.

Markert, S., Arndt, C., Felbeck, H., Becher, D., Sievert, S. M., Hügler, M., Albrecht, D., Robidart, J., Bench, S., Feldman, R. A., Hecker, M., and Schweder, T. (2007). Physiological proteomics of the uncultured endosymbiont of *Riftia pachyptila*. *Science*. 315, 247 - 250.

Markert, S., Gardebrecht, A., Felbeck, H., Sievert, S. M., Klose, J., Becher, D., Albrecht, D., Thürmer, A., Daniel, R., Kleiner, M., Hecker, M., and Schweder, T. (2011). Status quo in physiological proteomics of the uncultured *Riftia pachyptila* endosymbiont. *Proteomics*. 11,3106-3117.

Marsh, A. G., Mullineaux, L. S., Young, C. M., and Manahan, D. T. (2001). Larval dispersal potential of the tubeworm *Riftia pachyptila* at deep-sea hydrothermal vents. *Nature*. 411, 77 - 80.

McMullin, E. R., Hourdez, S., Schaeffer, S. W., and Fisher, C. R. (2003). Phylogeny and biogeography of deep sea vestimentiferan tubeworms and their bacterial symbionts. *Symbiosis*. 34, 1 - 41.

Meyer, B. and Kuever, J. (2007a). Molecular analysis of the distribution and phylogeny of dissimilatory adenosine-5-phosphosulfate reductase-encoding genes (aprBA) among sulfur-oxidizing prokaryotes. *Microbiology*. 153, 3478 - 3498.

Meyer, B. and Kuever, J. (2007b). Phylogeny of the alpha and beta subunits of the dissimilatory adenosine-5-phosphosulfate (APS) reductase from sulfate-reducing prokaryotes–origin and evolution of the dissimilatory sulfate-reduction pathway. *Microbiology*. 153, 2026 - 2044.

1008 Miake, H., Tsukahara, J., Hashimoto, J., Uematsu, K., and Maruyama, T. (2006). Rearing and
1009 observation methods of vestimentiferan tubeworm and its early development at
1010 atmospheric pressure. *Cahiers de biologie marine*. 47, 471 - 475.

1011 Miglietta, P. M., Hourdez, S., Cowart, D. A., Schaeffer, S. W., and Fisher, C. (2010). Species
1012 boundaries of Gulf of Mexico vestimentiferans (Polychaeta, Siboglinidae) inferred from
1013 mitochondrial genes. *Deep Sea Research Part II: Topical Studies in Oceanography*. 57,
1014 1916 - 1925.

1015 Minniti, M. and Bonavia, F. F. (1984). Copper-ore grade hydrothermal mineralization discovered
1016 in a seamount in the Tyrrhenian Sea (Mediterranean): Is the mineralization related to
1017 porphyry-coppers or to base metal lodes? *Marine Geology*. 59, 271 - 282.

1018 Miura, T. and Kojima, S. (2006). Two new species of vestimentiferan tubeworm (Polychaeta:
1019 Siboglinidae aka Pogonophora) from the Brothers Caldera, Kermadec Arc, South Pacific
1020 Ocean. *Species Diversity*. 11, 209 - 224.

1021 Monecke, T., Petersen, S., Lackschewitz, K., Hügler, M., and Hannington, M.D. (2009). Shallow
1022 submarine hydrothermal systems in the Aeolian volcanic arc, Italy. *Eos, Transactions,*
1023 *American Geophysical Union*. 90, 110 - 111.

1024 Myers, N., Mittermeier, R. A., Mittermeier, C. G., Da Fonseca, G. A. B., and Kent, J. (2000).
1025 Biodiversity hotspots for conservation priorities. *Nature*. 403, 853 - 858.

1026 Muyzer, G., Hottentrager, S., Teske, A., Wawer, C., (1996). "Denaturing gradient gel
1027 electrophoresis of PCR-amplified 16S rDNA. A new molecular approach to analyze the
1028 genetic diversity of mixed microbial communities." In: *Molecular Microbial Ecology*
1029 *Manual*, ed. A. D. L. Akkermans, J.D. van Elsas, F.J. Ž . de Bruijn, (Dordrecht, The
1030 Netherlands: Kluwer Academic Publishing), 3.4.4.1–3.4.4.22.Naganuma, T., Elsaied, H.
1031 E., Hoshii, D., and Kimura, H. (2005). Bacterial endosymbioses of gutless tube-dwelling
1032 worms in nonhydrothermal vent habitats. *Marine Biotechnology*. 7, 416 - 428.

1033 Nelson, K. and Fisher, C. R. (2000). Absence of cospeciation in deep-sea vestimentiferan tube
1034 worms and their bacterial endosymbionts. *Symbiosis*. 28, 1 - 15.

1035 Neufeld, J. D., Schäfer, H., Cox, M. J., Boden, R., McDonald, I. R., and Murrell, J. C. (2007).
1036 Stable-isotope probing implicates *Methylophaga* spp. and novel Gammaproteobacteria in
1037 marine methanol and methylamine metabolism. *The ISME Journal*. 1, 480 - 491.

1038 Olu, K., Cordes, E. E., Fisher, C. R., Brooks, J. M., Sibuet, M., and Desbruyères, D. (2010).
1039 Biogeography and potential exchanges among the Atlantic Equatorial Belt cold-seep
1040 faunas. *Plos One*. 5, e11967.

1041 Olu-Le Roy, K., Sibuet, M., Fiala-Médioni, A., Gofas, S., Salas, C., Mariotti, A., Foucher, J. P.,
1042 and Woodside, J. (2004). Cold seep communities in the deep eastern Mediterranean Sea:
1043 composition, symbiosis and spatial distribution on mud volcanoes. *Deep Sea Research*
1044 *Part I: Oceanographic Research Papers*. 51, 1915 - 1936.

Palumbi, S. R., Martin, A., Romano, S., McMillan, W. O., Stice, L., and Grabowski, G. (2002) *The simple fool's guide to PCR*. Department of Zoology and Kewalo Marine Laboratory, University of Hawaii.

Passaro, S., Milano, G., D'Isanto, C., Ruggieri, S., Tonielli, R., Bruno, P. P., Sprovieri, M., and Marsella, E. (2010). DTM-based morphometry of the Palinuro seamount (Eastern Tyrrhenian Sea): Geomorphological and volcanological implications. *Geomorphology*. 115, 129 - 140.

Petersen, J. M., Zielinski, F. U., Pape, T., Seifert, R., Moraru, C., Amann, R., Hourdez, S., Girguis, P. R., Wankel, S. D., Barbe, V., Pelletier, E., Fink, D., Borowski, C., Bach, W. and Dubilier, N. (2011). Hydrogen is an energy source for hydrothermal vent symbioses. *Nature*, 476, 176–180.

Petersen, S., Monecke, T., Augustin, N., De Benedetti, A. A., Esposito, A., Gärtner, A., Gardeler, A., Gemmell, J. B., Gibson, H., He, G., Hügler, M., Kayser, A., Kleeberg, R., Küver, J., Kummer, N., Lackschewitz, K., Lappe, F., Perrin, K.M., Peters, M., Sharpe, R., Simpson, K., Smith, D., and Wand, B. (2008). Drilling submarine hydrothermal systems in the Tyrrhenian Sea, Italy. *InterRidge News*. 17, 21 - 23.

Petri, R., Podgorsek, L., and Imhoff, J. F. (2001). Phylogeny and distribution of the soxB gene among thiosulfate–oxidizing bacteria. *FEMS Microbiology Letters*. 197, 171 - 178.

Puchelt, H. and Laschek, D. (1987). Massive sulphide ores in the Tyrrhenian Sea from Sonne cruise 41. *Terra Cognita*. 7, 188

Robidart, J. C., Bench, S. R., Feldman, R. A., Novoradovsky, A., Podell, S. B., Gaasterland, T., Allen, E. E., and Felbeck, H. (2008). Metabolic versatility of the *Riftia pachyptila* endosymbiont revealed through metagenomics. *Environmental Microbiology*. 10, 727 - 737.

Smith, C. R. and Kukert, H. (1989). Vent fauna on whale remains. *Nature*. 341, 27 - 28.

Southward, E. C. (1991). Three new species of Pogonophora, including two vestimentiferans, from hydrothermal sites in the Lau Back-arc Basin (Southwest Pacific Ocean). *Journal of Natural History*. 25, 859 - 881.

Southward, E. C., Andersen, A. C., and Hourdez, S. (2011). *Lamellibrachia anaximandri* n. sp., a new vestimentiferan tubeworm (Annelida) from the Mediterranean, with notes on frenulate tubeworms from the same habitat. *Zoosystema*. 33, 245 - 279.

Stewart, F. J. and Cavanaugh, C. M. (2006). "Symbiosis of thioautotrophic bacteria with *Riftia pachyptila*." in *Molecular basis of symbiosis*., ed. J. Overmann (Springer, Berlin, Germany), 197 - 225.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary

distance, and maximum parsimony methods. *Molecular biology and evolution*. 28, 2731 - 2739.

Tank, M. (2005) Vorkommen von Genen des dissimilatorischen Stickstoff-Stoffwechsels im Atlantischen Ozean. CAU Kiel.

Thiel, V., Neulinger, S. C., Staufenberger, T., Schmaljohann, R., and Imhoff, J. F. (2007). Spatial distribution of sponge-associated bacteria in theMediterranean sponge *Tethya aurantium*. *FEMS Microbiology Ecology*. 59, 47–63.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*. 25, 4876 - 4882.

Tyler, P. A. and Young, C. M. (2003). Dispersal at hydrothermal vents: a summary of recent progress. *Hydrobiologia*. 503, 9 - 19.

van der Land, J. and Nørrevang, A. (1975). The systematic position of Lamellibrachia (Annelida, Vestimentifera). *Zeitschrift für Zoologische Systematik und Evolutionsforschung*. 1, 86 - 101.

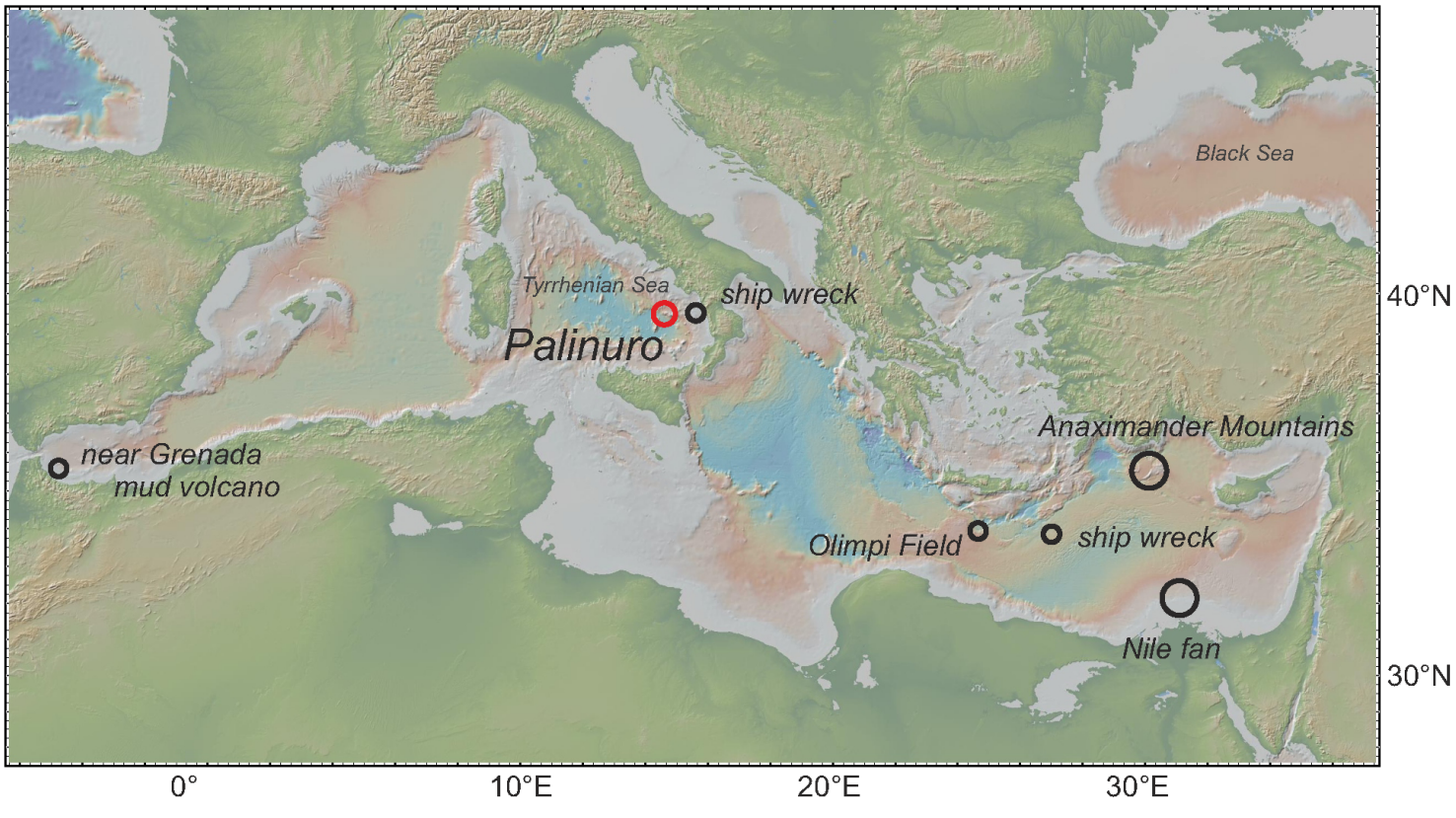
Vrijenhoek, R. C. (2010). Genetic diversity and connectivity of deep–sea hydrothermal vent metapopulations. *Molecular Ecology*. 19, 4391 - 4411.

Vrijenhoek, R. C., Duhaime, M., and Jones, W. J. (2007). Subtype variation among bacterial endosymbionts of tubeworms (Annelida:Siboglinidae ) from the Gulf of California. *The Biological Bulletin*. 212, 180 - 184.

Wetzel, L. R. and Shock, E. L. (2000). Distinguishing ultramafic-from basalt-hosted submarine hydrothermal systems by comparing calculated vent fluid compositions. *Journal of Geophysical Research*. 105, 8319 - 8340.

Young, C. M., Vázquez, E., Metaxas, A., and Tyler, P. A. (1996). Embryology of vestimentiferan tube worms from deep-sea methane/sulphide seeps. *Nature*. 381, 514 - 516.

Figure 1.TIF




*Thiel et al Figure 1*

Figure 2.TIF

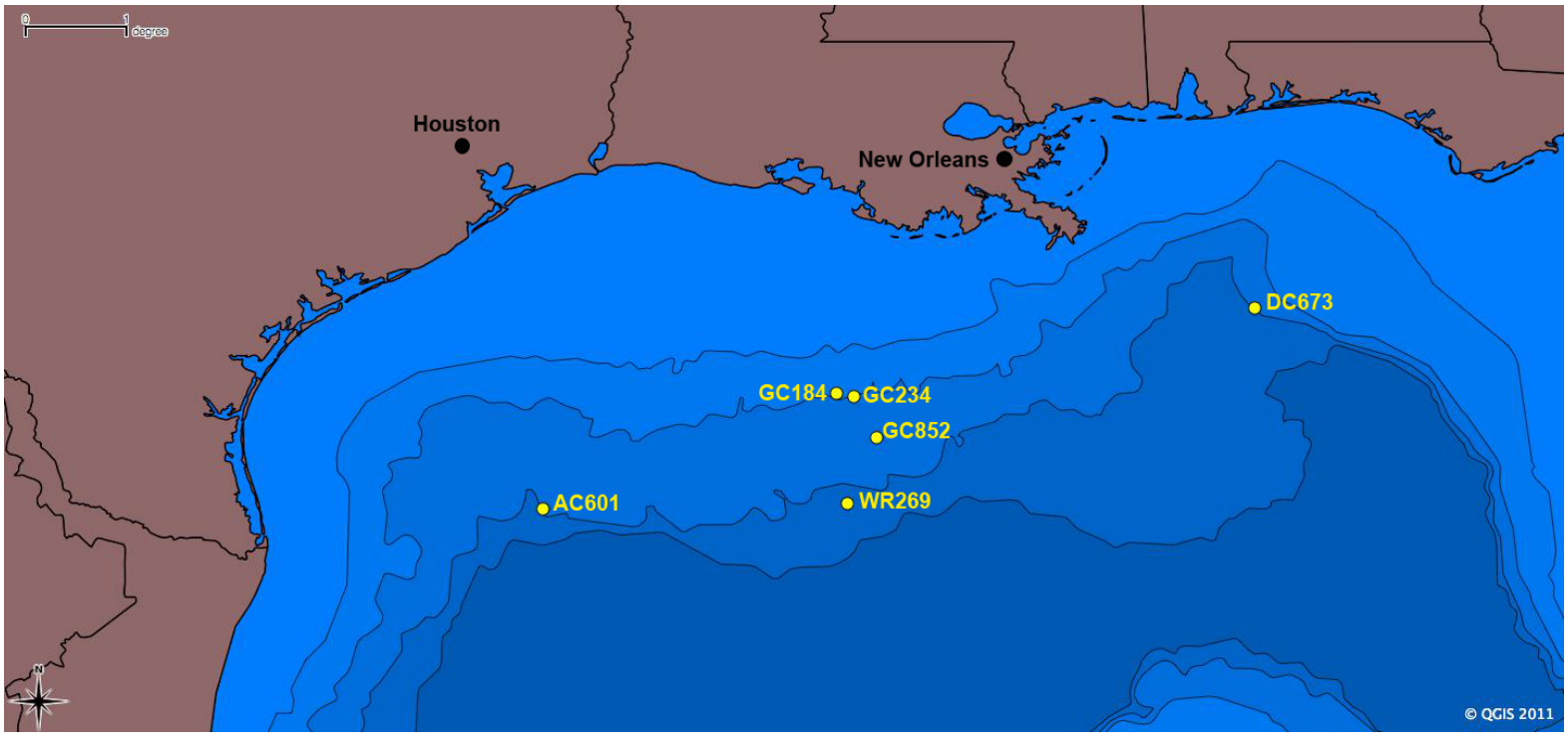

0
1
degree
Houston
New Orleans
DC673
GC184
GC234
GC852
AC601
WR269
N
© QGIS 2011

Figure 3.TIF

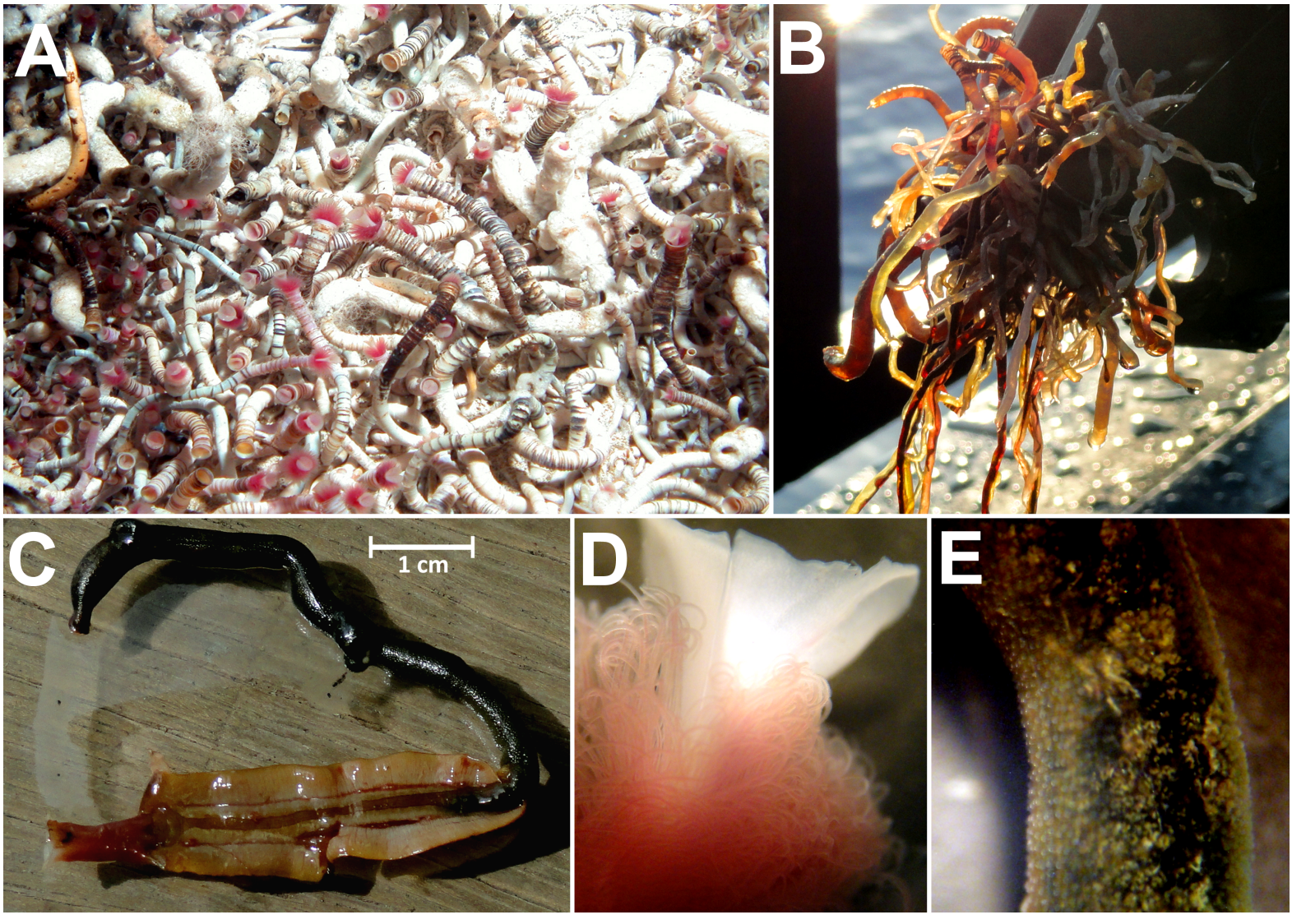


Figure 4.EPS

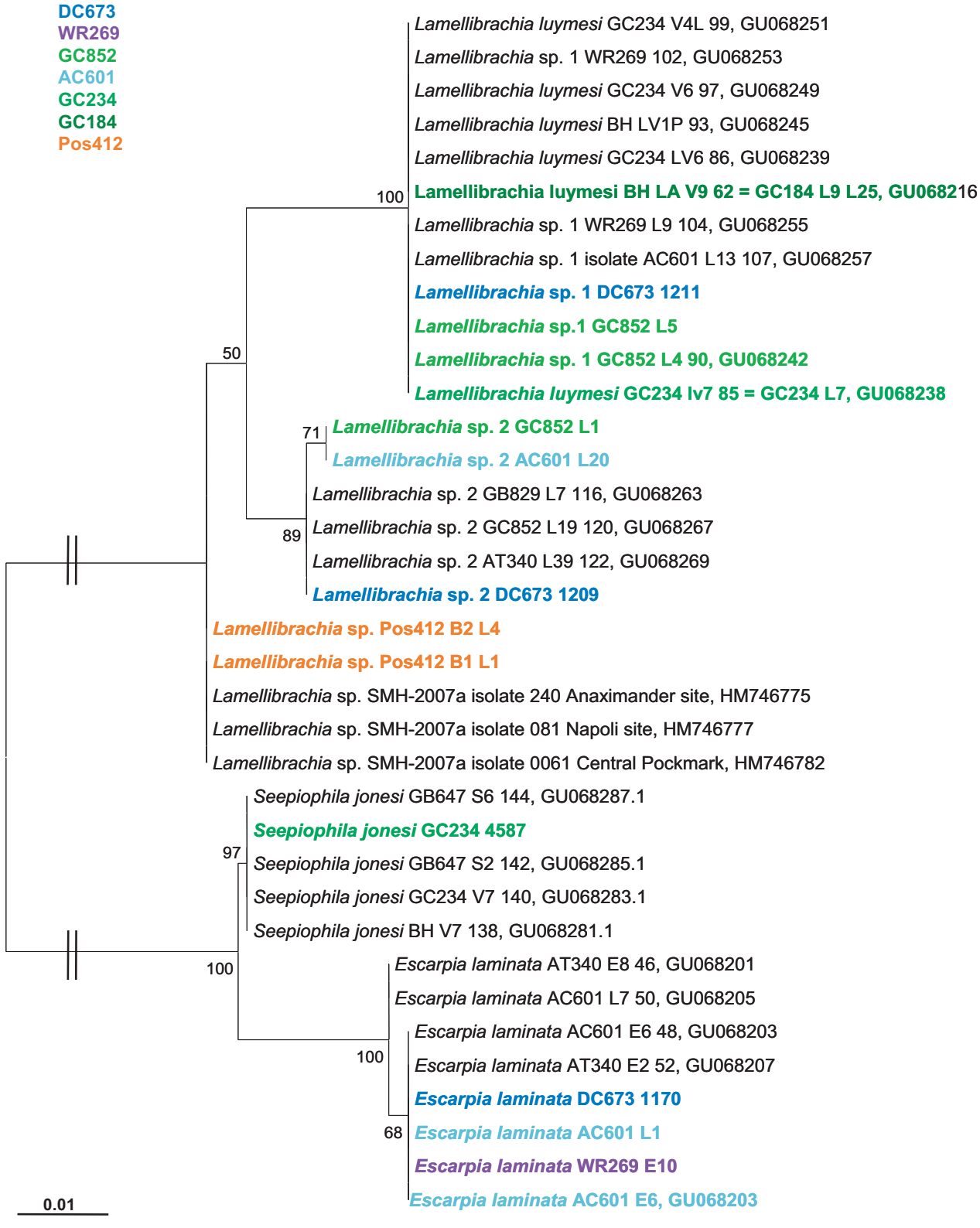

DC673
WR269
GC852
AC601
GC234
GC184
Pos412
Lamellibrachia luymesi GC234 V4L 99, GU068251
Lamellibrachia sp. 1 WR269 102, GU068253
Lamellibrachia luymesi GC234 V6 97, GU068249
Lamellibrachia luymesi BH LV1P 93, GU068245
Lamellibrachia luymesi GC234 LV6 86, GU068239
100
Lamellibrachia luymesi BH LA V9 62 = GC184 L9 L25, GU068216
Lamellibrachia sp. 1 WR269 L9 104, GU068255
Lamellibrachia sp. 1 isolate AC601 L13 107, GU068257
Lamellibrachia sp. 1 DC673 1211
Lamellibrachia sp.1 GC852 L5
50
Lamellibrachia sp. 1 GC852 L4 90, GU068242
Lamellibrachia luymesi GC234 lv7 85 = GC234 L7, GU068238
71
Lamellibrachia sp. 2 GC852 L1
Lamellibrachia sp. 2 AC601 L20
Lamellibrachia sp. 2 GB829 L7 116, GU068263
89
Lamellibrachia sp. 2 GC852 L19 120, GU068267
Lamellibrachia sp. 2 AT340 L39 122, GU068269
Lamellibrachia sp. 2 DC673 1209
Lamellibrachia sp. Pos412 B2 L4
Lamellibrachia sp. Pos412 B1 L1
Lamellibrachia sp. SMH-2007a isolate 240 Anaximander site, HM746775
Lamellibrachia sp. SMH-2007a isolate 081 Napoli site, HM746777
Lamellibrachia sp. SMH-2007a isolate 0061 Central Pockmark, HM746782
Seepiophila jonesi GB647 S6 144, GU068287.1
Seepiophila jonesi GC234 4587
97
Seepiophila jonesi GB647 S2 142, GU068285.1
Seepiophila jonesi GC234 V7 140, GU068283.1
Seepiophila jonesi BH V7 138, GU068281.1
100
Escarpia laminata AT340 E8 46, GU068201
Escarpia laminata AC601 L7 50, GU068205
Escarpia laminata AC601 E6 48, GU068203
100
Escarpia laminata AT340 E2 52, GU068207
Escarpia laminata DC673 1170
68
Escarpia laminata AC601 L1
Escarpia laminata WR269 E10
Escarpia laminata AC601 E6, GU068203
0.01

Figure 5.TIF

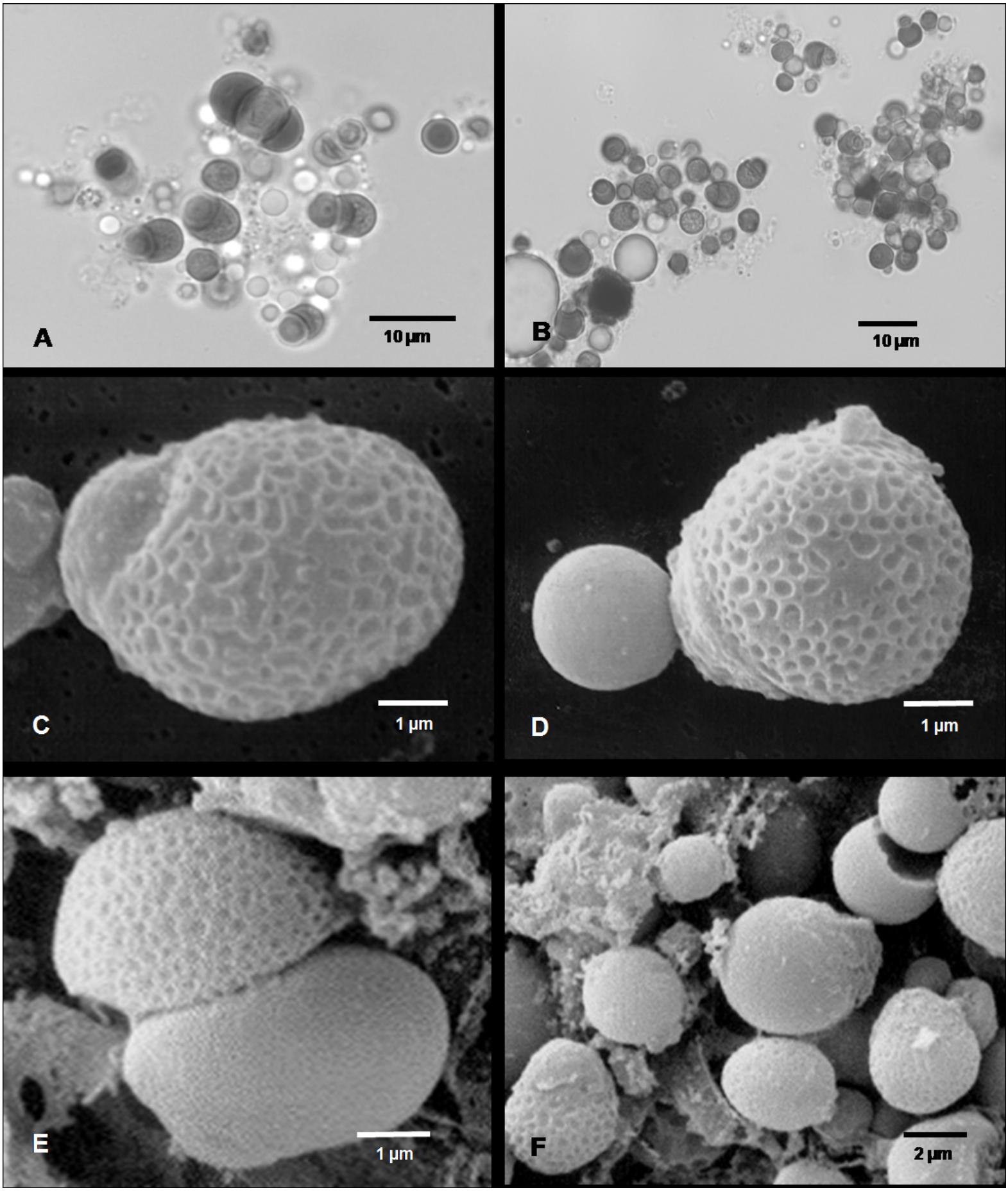


Figure 6.EPS

.

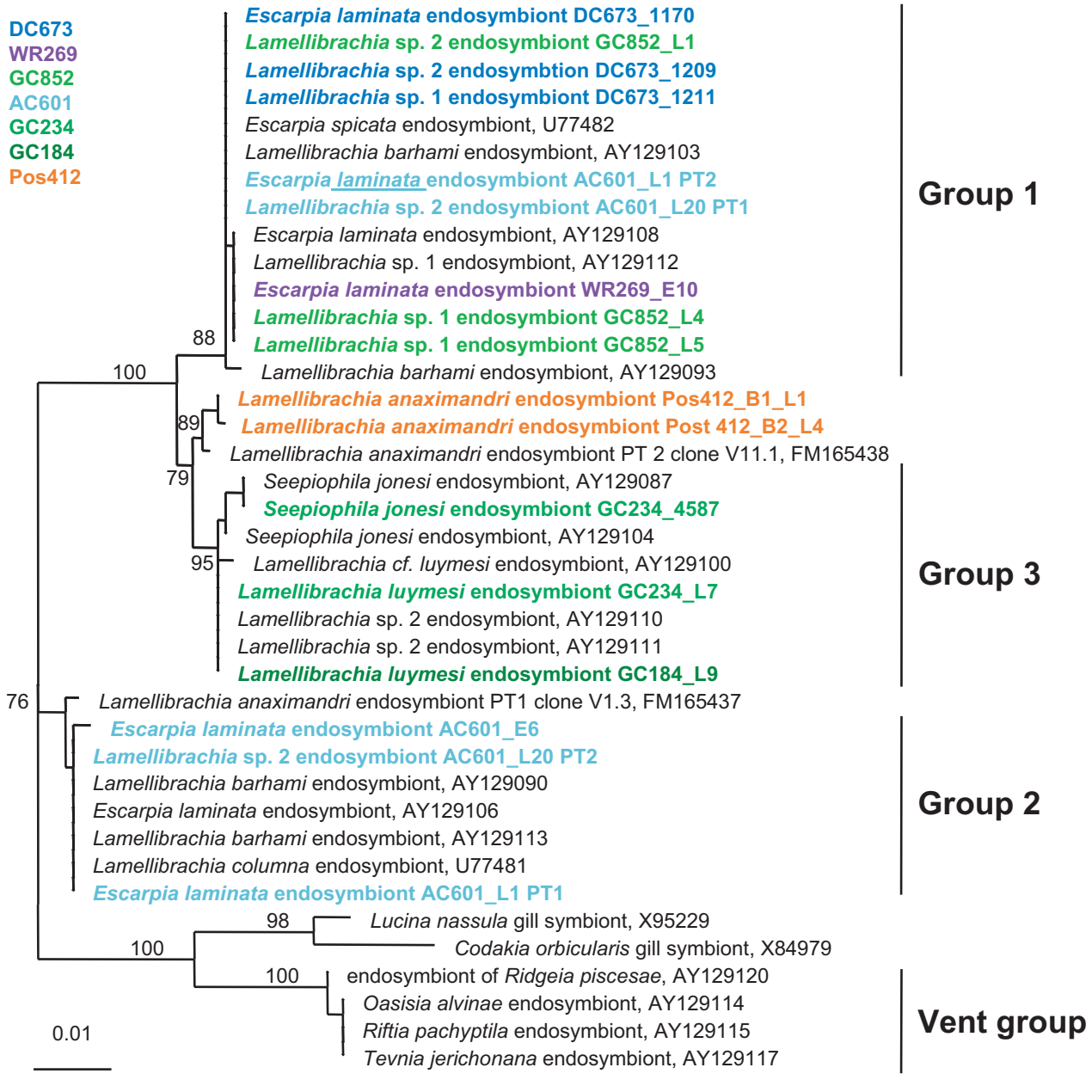

DC673
WR269
GC852
AC601
GC234
GC184
Pos412
Escarpia laminata endosymbiont DC673_1170
Lamellibrachia sp. 2 endosymbiont GC852_L1
Lamellibrachia sp. 2 endosymbtion DC673_1209
Lamellibrachia sp. 1 endosymbiont DC673_1211
Escarpia spicata endosymbiont, U77482
Lamellibrachia barhami endosymbiont, AY129103
Escarpia laminata endosymbiont AC601_L1 PT2
Lamellibrachia sp. 2 endosymbiont AC601_L20 PT1
Escarpia laminata endosymbiont, AY129108
Lamellibrachia sp. 1 endosymbiont, AY129112
Escarpia laminata endosymbiont WR269_E10
Lamellibrachia sp. 1 endosymbiont GC852_L4
Lamellibrachia sp. 1 endosymbiont GC852_L5
Lamellibrachia barhami endosymbiont, AY129093
Lamellibrachia anaximandri endosymbiont Pos412_B1_L1
Lamellibrachia anaximandri endosymbiont Post 412_B2_L4
Lamellibrachia anaximandri endosymbiont PT 2 clone V11.1, FM165438
Seepiophila jonesi endosymbiont, AY129087
Seepiophila jonesi endosymbiont GC234_4587
Seepiophila jonesi endosymbiont, AY129104
Lamellibrachia cf. luymesi endosymbiont, AY129100
Lamellibrachia luymesi endosymbiont GC234_L7
Lamellibrachia sp. 2 endosymbiont, AY129110
Lamellibrachia sp. 2 endosymbiont, AY129111
Lamellibrachia luymesi endosymbiont GC184_L9
Lamellibrachia anaximandri endosymbiont PT1 clone V1.3, FM165437
Escarpia laminata endosymbiont AC601_E6
Lamellibrachia sp. 2 endosymbiont AC601_L20 PT2
Lamellibrachia barhami endosymbiont, AY129090
Escarpia laminata endosymbiont, AY129106
Lamellibrachia barhami endosymbiont, AY129113
Lamellibrachia columna endosymbiont, U77481
Escarpia laminata endosymbiont AC601_L1 PT1
Lucina nassula gill symbiont, X95229
Codakia orbicularis gill symbiont, X84979
endosymbiont of Ridgeia piscesae, AY129120
Oasisia alvinae endosymbiont, AY129114
Riftia pachyptila endosymbiont, AY129115
Tevnia jerichonana endosymbiont, AY129117
88
100
89
79
95
76
98
100
100
0.01
Group 1
Group 3
Group 2
Vent group

Figure 7.EPS

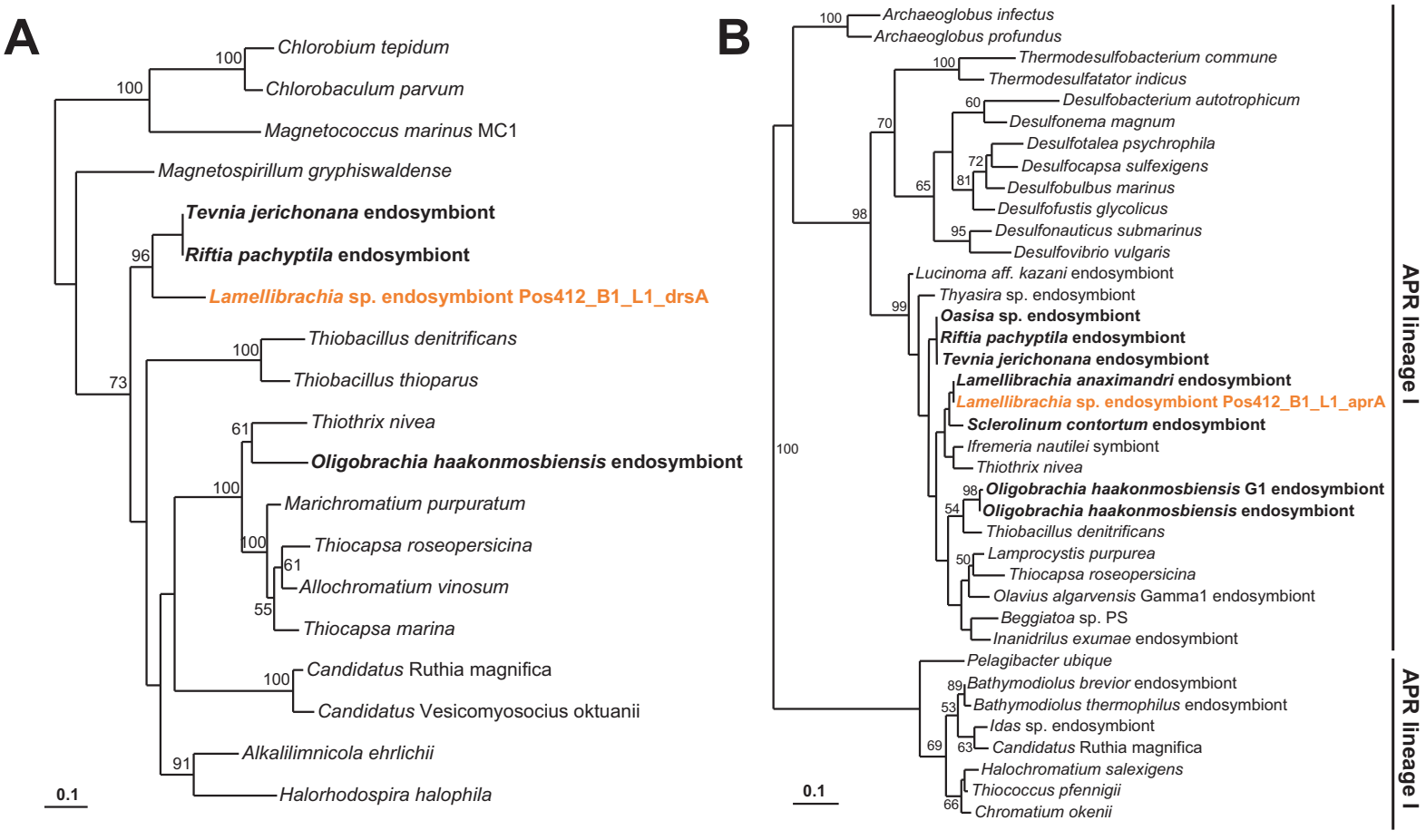
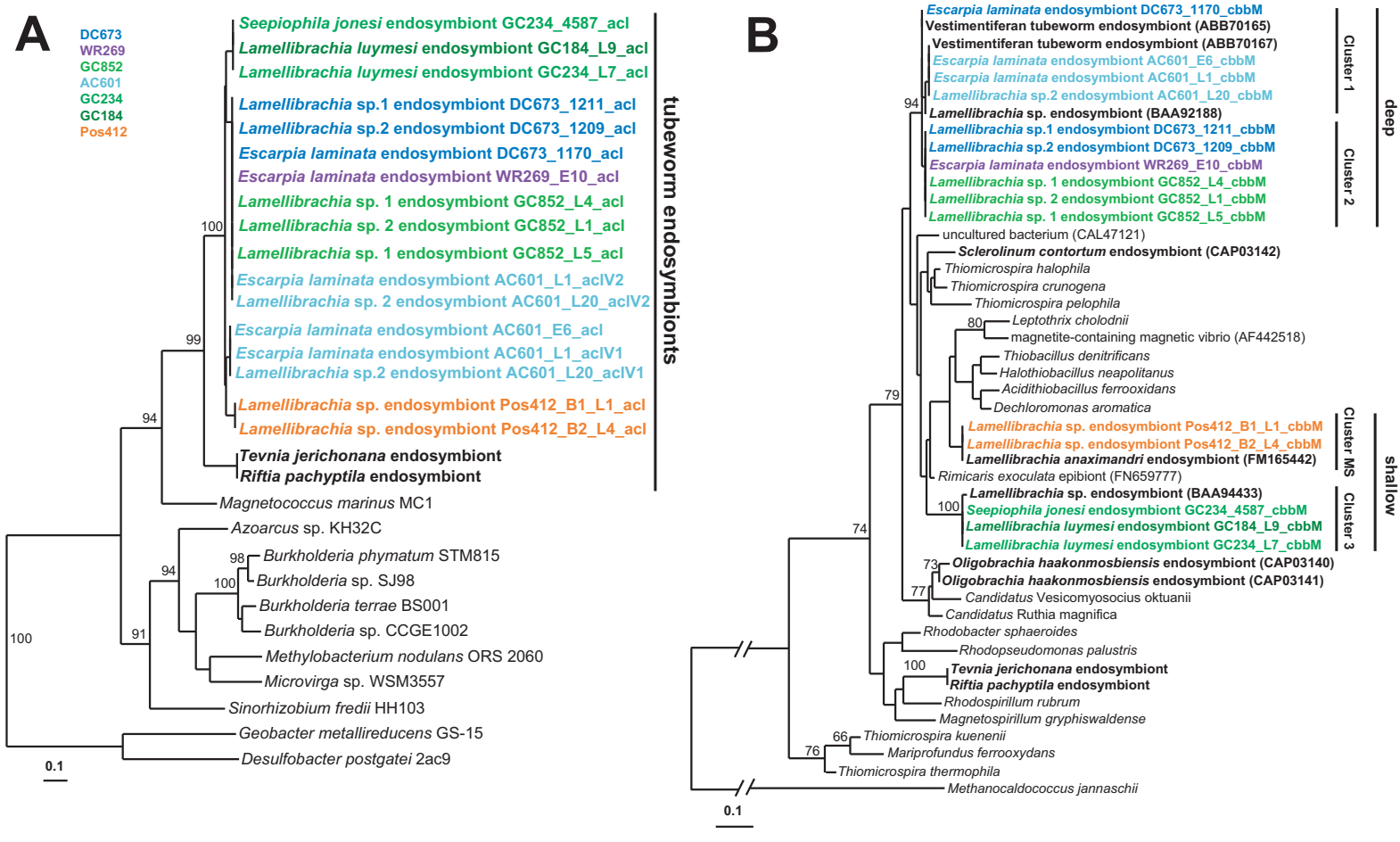

A
Chlorobium tepidum
Chlorobaculum parvum
Magnetococcus marinus MC1
Magnetospirillum gryphiswaldense
Tevnia jerichonana endosymbiont
Riftia pachyptila endosymbiont
Lamellibrachia sp. endosymbiont Pos412_B1_L1_drsA
Thiobacillus denitrificans
Thiobacillus thioparus
Thiothrix nivea
Oligobrachia haakonmosbiensis endosymbiont
Marichromatium purpuratum
Thiocapsa roseopersicina
Allochromatium vinosum
Thiocapsa marina
Candidatus Ruthia magnifica
Candidatus Vesicomyosocius oktuanii
Alkalilimnicola ehrlichii
Halorhodospira halophila
0.1
B
Archaeoglobus infectus
Archaeoglobus profundus
Thermodesulfobacterium commune
Thermodesulfatator indicus
Desulfobacterium autotrophicum
Desulfonema magnum
Desulfotalea psychrophila
Desulfocapsa sulfexigens
Desulfobulbus marinus
Desulfofustis glycolicus
Desulfonauticus submarinus
Desulfovibrio vulgaris
Lucinoma aff. kazani endosymbiont
Thyasira sp. endosymbiont
Oasisa sp. endosymbiont
Riftia pachyptila endosymbiont
Tevnia jerichonana endosymbiont
Lamellibrachia anaximandri endosymbiont
Lamellibrachia sp. endosymbiont Pos412_B1_L1_aprA
Sclerolinum contortum endosymbiont
Ifremeria nautilei symbiont
Thiothrix nivea
Oligobrachia haakonmosbiensis G1 endosymbiont
Oligobrachia haakonmosbiensis endosymbiont
Thiobacillus denitrificans
Lamprocystis purpurea
Thiocapsa roseopersicina
Olavius algarvensis Gamma1 endosymbiont
Beggiatoa sp. PS
Inanidrilus exumae endosymbiont
Pelagibacter ubique
Bathymodiolus brevior endosymbiont
Bathymodiolus thermophilus endosymbiont
Idas sp. endosymbiont
Candidatus Ruthia magnifica
Halochromatium salexigens
Thiococcus pfennigii
Chromatium okenii
0.1
APR lineage I
APR lineage I

Figure 8.EPS


A
DC673
WR269
GC852
AC601
GC234
GC184
Pos412
Seepiophila jonesi endosymbiont GC234_4587_acl
Lamellibrachia luymesi endosymbiont GC184_L9_acl
Lamellibrachia luymesi endosymbiont GC234_L7_acl
Lamellibrachia sp.1 endosymbiont DC673_1211_acl
Lamellibrachia sp.2 endosymbiont DC673_1209_acl
Escarpia laminata endosymbiont DC673_1170_acl
Escarpia laminata endosymbiont WR269_E10_acl
Lamellibrachia sp. 1 endosymbiont GC852_L4_acl
Lamellibrachia sp. 2 endosymbiont GC852_L1_acl
Lamellibrachia sp. 1 endosymbiont GC852_L5_acl
Escarpia laminata endosymbiont AC601_L1_aclV2
Lamellibrachia sp. 2 endosymbiont AC601_L20_aclV2
Escarpia laminata endosymbiont AC601_E6_acl
Escarpia laminata endosymbiont AC601_L1_aclV1
Lamellibrachia sp.2 endosymbiont AC601_L20_aclV1
Lamellibrachia sp. endosymbiont Pos412_B1_L1_acl
Lamellibrachia sp. endosymbiont Pos412_B2_L4_acl
Tevnia jerichonana endosymbiont
Riftia pachyptila endosymbiont
tubeworm endosymbionts
Magnetococcus marinus MC1
Azoarcus sp. KH32C
Burkholderia phymatum STM815
Burkholderia sp. SJ98
Burkholderia terrae BS001
Burkholderia sp. CCGE1002
Methylobacterium nodulans ORS 2060
Microvirga sp. WSM3557
Sinorhizobium fredii HH103
Geobacter metallireducens GS-15
Desulfobacter postgatei 2ac9
100
99
94
94
100
98
100
91
100
0.1
B
Escarpia laminata endosymbiont DC673_1170_cbbM
Vestimentiferan tubeworm endosymbiont (ABB70165)
Vestimentiferan tubeworm endosymbiont (ABB70167)
Escarpia laminata endosymbiont AC601_E6_cbbM
Escarpia laminata endosymbiont AC601_L1_cbbM
Lamellibrachia sp.2 endosymbiont AC601_L20_cbbM
Lamellibrachia sp. endosymbiont (BAA92188)
Lamellibrachia sp.1 endosymbiont DC673_1211_cbbM
Lamellibrachia sp.2 endosymbiont DC673_1209_cbbM
Escarpia laminata endosymbiont WR269_E10_cbbM
Lamellibrachia sp. 1 endosymbiont GC852_L4_cbbM
Lamellibrachia sp. 2 endosymbiont GC852_L1_cbbM
Lamellibrachia sp. 1 endosymbiont GC852_L5_cbbM
Cluster 1
Cluster 2
deep
uncultured bacterium (CAL47121)
Sclerolinum contortum endosymbiont (CAP03142)
Thiomicrospira halophila
Thiomicrospira crunogena
Thiomicrospira pelophila
Leptothrix cholodnii
magnetite-containing magnetic vibrio (AF442518)
Thiobacillus denitrificans
Halothiobacillus neapolitanus
Acidithiobacillus ferrooxidans
Dechloromonas aromatica
Lamellibrachia sp. endosymbiont Pos412_B1_L1_cbbM
Lamellibrachia sp. endosymbiont Pos412_B2_L4_cbbM
Lamellibrachia anaximandri endosymbiont (FM165442)
Cluster MS
Rimicaris exoculata epibiont (FN659777)
Lamellibrachia sp. endosymbiont (BAA94433)
Seepiophila jonesi endosymbiont GC234_4587_cbbM
Lamellibrachia luymesi endosymbiont GC184_L9_cbbM
Lamellibrachia luymesi endosymbiont GC234_L7_cbbM
Cluster 3
shallow
Oligobrachia haakonmosbiensis endosymbiont (CAP03140)
Oligobrachia haakonmosbiensis endosymbiont (CAP03141)
Candidatus Vesicomyosocius oktuanii
Candidatus Ruthia magnifica
Rhodobacter sphaeroides
Rhodopseudomonas palustris
Tevnia jerichonana endosymbiont
Riftia pachyptila endosymbiont
Rhodospirillum rubrum
Magnetospirillum gryphiswaldense
Thiomicrospira kuenenii
Mariprofundus ferrooxydans
Thiomicrospira thermophila
Methanocaldococcus jannaschii
94
80
79
100
74
73
77
100
66
76
0.1