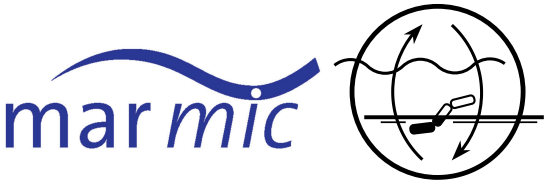

Phylogeny and diversity of symbionts from whale fall invertebrates

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Dissertation zur Erlangung des Grades eines Doktors der Naturwissenschaften

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Auf dem Deckblatt, *Osedax mucofloris* und Symbionten. Links, Wurm in situ Foto (von A. Glover). Mitte, Schema zeigt die Verteilung der Symbionten. Rechts, Photo der Symbionten mit FISH. / Sur la page de couverture: *Osedax mucofloris* et symbiontes. Gauche, photo in situ du vers (par A. Glover). Milieu, schema montrant la distribution des symbiontes. Droite, photo des symbiontes réalisée avec FISH.

‘Well may we affirm that every part of the world is habitable! Whether lakes of brine, or those of subterranean ones hidden beneath volcanic mountains—warm mineral spring—the wide expanse and depths of the ocean—the upper regions of the atmosphere, and even the surface of perpetual snow—all support organic beings.’

Charles Darwin - The Voyage of the Beagle

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Abstract

When whales die and sink to the seafloor, their decaying carcasses form oases at the bottom of the ocean that provide an energy source for a highly diverse and abundant fauna thriving at these unusual and ephemeral habitats. Among these species some are associated with chemoautotrophic symbionts, and were initially known from hydrothermal vents, cold seeps and wood falls, such as vestimentiferan tubeworms, bathymodiolin mussels, and vesicomiyd clams. In addition to these chemoautotrophic symbioses, a new symbiosis type was discovered with heterotrophic bacteria: *Osedax* worms are whale fall specialists that infiltrate whale bones with their root tissues. These roots are filled with endosymbiotic bacteria hypothesized to provide their hosts with nutrition by extracting organic compounds from the whale bones (i.e. heterotrophic bacteria). Although whale falls are a suitable habitat for different symbioses (chemoautotrophic and heterotrophic), symbiosis at whale falls remains mostly unexplored.

This thesis is made of three thematic parts. In the first part a review on the ecology and evolution of siboglinids worms is presented. Four siboglinids groups are known; Vestimentifera, Monolifera (Sclerolinum), Frenulata and *Osedax*. All siboglinids lack a mouth, gut and anus and rely on symbiotic bacteria for their nutrition. Siboliginid symbionts include different lineages of sulfur-oxidising and methane-oxidising Gammaproteobacteria, and heterotrophic Oceanospirillales bacteria. Siboglinids occur in a various range of reduced habitats: from organic rich sediment, to whale falls, vents and seeps. The diversity of their symbionts and the variety of habitats where they occur, have strongly influenced their ecology and their evolution. This review proposes several scenarios addressing how and when siboglinid ancestors, probably heterotrophic polychaetes, became obligate endosymbiotic species.

The second part focuses on the diversity of the symbionts associated with *Osedax mucofloris*, at shallow whale falls in the North Atlantic. Before this study, endosymbionts have been characterised in only five *Osedax* species from the Pacific Ocean. A high intraspecific symbiont diversity was found in each host species, which was associated with several bacterial groups within the Oceanospirillales bacteria. In *O. mucofloris* a higher diversity of Oceanospirillales bacteria was identified with eight monophyletic clusters and with considerable microheterogeneity within clusters. The symbiont clusters were not uniformly distributed, but one cluster dominated the population and each individual. In addition,

when several clusters co-occurred in one individual they were not mixed but were spatially separated. Multivariate statistical analyses showed that each *O. mucofloris* individual has a significant effect on symbionts diversity and distribution. Thus, each *O. mucofloris* individual has its own specific endosymbiont community. Our results suggest a horizontal transmission of the symbionts. Several scenarios explaining the observed symbionts distribution are considered including a flexible selection by the host, variability of the available symbionts in the environment, and competition between the symbionts.

The third part of this thesis focuses on the symbionts of another polycheate worm, a Ctenodrilidae, *Raricirrus beryli*. Bacteria associated with *R. beryli* gut and worm surface were highly diverse, belonging to several phyla, including Gammaproteobacteria, Epsilonproteobacteria, Firmicutes and Bacteroidetes. Furthermore, among the epibacteria, bacteria forming a monophyletic cluster with thiotrophic symbionts of bathymodiolin mussels were found. This is the first report of a polychaete host for these bacteria. It shows their ubiquity and allows new speculation on the dispersal capacities and host range of these bacteria.

Finally, this study gives new insights on symbioses at whale falls. The comparison of the epibiome of *O. mucofloris* and *R. beryli* extend the knowledge on the bacterial diversity found at whale falls. Futhermore, the finding of bacteria previously only associated with bivalves is highly surprising and raises many questions regarding bathymodiolin symbioses such as the diversity and ubiquity of their symbiotic free-living forms. Moreover, the description of *O. mucofloris* endosymbionts shows a pattern of diversity and spacial distribution previously unknown in marine invertebrates. This suggests that much more remains to be understood in invertebrate and bacterial symbioses.

Résumé

Après leur mort, l'ultime chute des baleines sur les fonds océaniques, crée des oasis éphémères dans ces environnements considérés comme désertiques. Ces carcasses attirent une faune diversifiée et abondante incluant des espèces caractéristiques d'autres habitats à base chimiosynthétiques. Parmi ces espèces on compte de nombreux invertébrés habritant des bactéries symbiotiques chimioautotrophes, comme les vers géants (annélides, siboglinidés) vestimentifères, les moules bathymodiolinées ou les bivalves vesicomyidés. En plus de ces symbioses chimioautotrophes, un nouveau type de symbiose entre des bactéries hétérotrophes et le siboglinidé *Osedax* a été découverte sur les chutes de baleines. Les *Osedax*, littéralement les mangeurs d'os, sont des annélides dont la partie postérieure prend la forme d'un tissu ramifié infiltrant les os. Les symbiontes sont présents dans ce tissu ramifié et aident vraisemblablement leur hôte dans la dégradation de molécules complexes présentes dans les os (tels que lipides et collagènes). La découverte récente des chutes de carcasses de baleines et la diversité des sources d'énergie possibles pour des bactéries symbiotiques suggèrent que de nombreuses symbioses restent à découvrir dans cet écosystème.

Cette thèse comprend trois parties. La première partie est une synthèse sur l'écologie et l'évolution des Siboglinidae. Cette famille d'annélides comprend les Vestimentifera, les Monolifera (*Sclerolinum*), les Frenulata et *Osedax*. Tous sont obligatoirement associés avec des bactéries symbiotiques qui contribuent à leur nutrition car ils n'ont ni bouche, ni système digestif, ni anus. Ils occupent divers habitats tels que les sédiments riches en composés organiques, les sources hydrothermales, les suintements froids, les bois coulés et les chutes de baleines. Leurs symbiontes sont diversifiés tant au niveau phylogénétique, avec plusieurs lignées de bactéries, qu'au niveau métabolique avec des sulfo-oxydants, des méthanotrophes et des hétérotrophes. Habitat et caractéristiques des symbiontes conditionnent probablement l'évolution et l'écologie des différents groupes de Siboglinidae. Ce qui soulève la question de la manière dont leur ancêtre commun, un polychète probablement hétérotrophe et dépourvu de symbiontes, est devenu un organisme obligatoirement associé à des bactéries.

La seconde partie présente les symbiontes d'*Osedax mucofloris*, une espèce présente sur des squelettes de baleines dans le nord de l'Atlantique. Les symbiontes ont été identifiés chez seulement cinq espèces d'*Osedax* du Pacifique. Ces symbiontes sont très diversifiés au sein d'une famille de bactéries, les Oceanospirillales. Chez chaque espèce d'*Osedax*

et même chez un individu unique, plusieurs symbiontes distincts peuvent coexister. Les symbiontes d'*O. mucofloris* sont encore plus diversifiés que ceux des autres *Osedax*, avec la présence de huit clades distincts de symbiontes et présentant également une diversité au sein de chaque clade. Un de ces clades domine la communauté bactérienne associée à *O. mucofloris*, et un clade seulement domine au sein de chaque individu. Lorsqu'ils coexistent, les clades de symbiontes sont spatialement restreints dans des zones différentes du tissu ramifié. Des analyses statistiques multivariées montrent que la population des symbiontes est significativement structurée au niveau de chaque individu, qui a donc sa propre population de symbiontes. De plus, l'ensemble des résultats de cette étude suggère que l'acquisition des symbiontes est environnementale et continue tout au long de la vie d'un individu. Pour expliquer la diversité et la distribution des symbiontes d'*Osedax*, plusieurs scénarios sont proposés, incluant la sélection des symbiontes par *Osedax*, la variabilité des symbiontes disponibles dans l'environnement et la compétition entre les symbiontes pour coloniser le tissu ramifié.

La troisième partie décrit les bactéries associées à un autre polychète de la famille Ctenodrilidae, *Raricirrus beryli*. Ces bactéries, présentes dans le système digestif et sur la surface de l'annelide, appartiennent à différentes divisions, Gammaproteobacteria, Epsilonproteobacteria, Firmicutes et Bacteroidetes. Parmi les bactéries présentes sur la surface du ver, certaines forment un groupe monophylétique avec des symbiontes de moules de la sous-famille Bathymodiolinae. Cette étude est la première à montrer que ces bactéries peuvent s'associer avec un polychète, donnant un nouvel éclairage sur leur versatilité et la diversité de leurs hôtes possibles.

Cette thèse contribue à une meilleure compréhension des symbioses dans les habitats consitués par les carcasses de baleines sur les fonds marins. La comparaison entre les épibiontes d'*O. mucofloris* et de *R. beryli* et les bactéries naturellement présentes permet de mieux caractériser la diversité et les dynamiques des bactéries de cet écosystème. De plus, la découverte de bactéries, auparavant seulement associées à des bivalves sur un nouvel hôte, soulève de nombreuses questions sur la capacité de ces bactéries à coloniser de nouveaux hôtes et de nouveaux habitats. Enfin, la symbiose entre *Osedax* et les bactéries Oceanospirillales est la première à montrer un tel degré de diversité et de structure chez les invertébrés marins suggérant sa pertinence en tant que modèle d'intractions complexes, et soulignant l'intérêt de poursuivre l'exploration des symbioses dans les habitats créés par l'ultime chute des baleines.

Zusammenfassung

Wenn Wale sterben und auf den Meeresboden absinken, bilden ihre sterblichen Überreste Oasen auf dem Grund des Meeres, welche Energie für eine vielfältige und reiche Fauna liefern. Einige Spezies dieser Fauna, z. B. Röhrenwürmer (Vestimentifera) oder Muscheln (Bathymodiolinae und Vesicomyidae), haben chemoautotrophe Symbionten und sind ursprünglich bekannt von hydrothermalen Quellen, Methanaustritten und Überresten von abgesunkenem Holz. Zusätzlich zu der chemoautotrophen Symbiose ist eine neue Art von Symbiose mit heterotrophen Bakterien entdeckt worden: *Osedax* Würmer sind Walknochenspezialisten, die den Walknochen mit ihrem Wurzelgewebe anbohren, das mit endosymbiontischen Bakterien gefüllt ist. Es wird angenommen, dass diese den Wirt mit Nährstoffen versorgen indem sie dem Knochenmaterial die organische Substanz entnehmen (d.h. heterotrophe Bakterien). Obwohl Walknochen ein geeignetes Habitat für verschiedene Symbiosen darstellen, sind diese weitgehend unerforscht.

Diese Arbeit besteht aus drei Teilen: Im ersten Teil wird die Ökologie und Evolution von siboglinіden Würmern abgehandelt. Vier Gruppen von Siboglinidae sind bekannt: Vestimentifera, Monolifera (*Sclerolinum*), Frenulata und *Osedax*. Alle Siboglinіden besitzen weder Mundöffnung, noch Verdauungstrackt oder After und sind daher auf symbiotische Ernährung durch Bakterien angewiesen. Symbionten von Siboglinіden umfassen verschiedene Linien von Schwefel- und Methan-oxidierenden Gammaproteobakterien, und heterotrophen Oceanospirillales. Siboglinidae kommen im Grenzbereich zwischen der oxischen und anoxischen Zone vor, z.B. in Sedimenten mit hohem Gehalt an organischem Material, in Walknochen, an hydrothermalen Quellen und Kohlenwasserstoffaustritten. Die Diversität ihrer Symbionten und die Vielfalt an Habitaten haben ihre Ökologie und Evolution stark beeinflusst. In dieser Synthese werden verschiedene Szenarien vorgeschlagen, wie Siboglinіden und ihre Vorfahren, die wahrscheinlich heterotrophe Polychaeten waren, obligat endosymbiontisch wurden.

Der zweite Teil befasst sich mit der Diversität der Symbionten, die mit *Osedax mucofloris* verwandt sind und in geringer Tiefe auf Walskeletten im Nordatlantik gefunden wurden. Bisher konnten fünf Spezies von Endosymbionten in *Osedax* aus dem Pazifik beschrieben worden. Eine hohe intraspezifische Diversität wurde in jedem dieser Wirtsorganismen festgestellt, welche mit verschiedenen Gruppen innerhalb der Oceanospirillales verwandt sind. In *O. mucofloris* wurde eine höhere Diversität von Oceanosprillales

mit acht monophyletischen Gruppen und beträchtlicher Mikroheterogenität nachgewiesen. Diese Gruppen waren nicht einheitlich verteilt, sondern eine Gruppe dominierte jeweils die Population in den einzelnen untersuchten Würmern. Wenn verschiedene Gruppen zusammen in einem Individuum vorkamen, waren diese jeweils räumlich voneinander getrennt. Mit Hilfe von multivarianter Statistik konnte gezeigt werden, dass jedes Individuum von *O. mucofloris* seine eigene Endosymbionten-Gemeinschaft besitzt. Unsere Resultate deuten auf eine horizontale Transmission der Symbionten hin. Verschiedene Szenarien werden vorgeschlagen, um die beobachteten Symbiontenverteilungen zu erklären: Eine flexible Auswahl durch den Wirt, Variabilität der vorhandenen Symbionten oder ein Wettbewerb zwischen den Symbionten.

Der dritte Teil dieser Arbeit konzentriert sich auf die Symbionten des Polychaeten *Raricirrus beryli*, der zur Familie der Ctenodrilidae gehört. Bakterien, die im Verdauungstrakt und auf der Oberfläche von *R. beryli* vorkommen, gehören zu verschiedenen Stämmen, einschließlich Gammaproterobacterien, Epsilonproteobacterien, Firmicutes und Bacteroidetes. Überdies wurden unter den Epibakterien Vertreter gefunden, welche eine monophyletische Gruppe mit thiotrophen Symbionten von Bathymodiolinae bilden. Vertreter dieser Gruppe wurden zum ersten Mal in einem Polychaeten nachgewiesen. Dieser Fund deutet auf eine weite Verbreitung hin, was neue Spekulationen über deren Verbreitungsart und die Vielfalt der Wirtsorganismen erlaubt.

Diese Studie ermöglicht eine neue Sichtweise über symbiotisches Leben auf Walskeletten. Ein Vergleich der Epibionten von *O. mucofloris* und *R. beryli* erweitert die Erkenntnisse über die Bakterienvielfalt auf Walskeletten. Zudem ist der Fund von Bakterien, die sonst nur von Muscheln (Bivalvia) bekannt sind, überraschend und es stellen sich neue Fragen bezüglich der Vielfalt und Verbreitung von Symbionten von Bathymodiolinae. Auch die Beschreibung von *O. mucofloris*-Symbionten zeigt ein bisher unbekanntes Verteilungsmuster in marinen Invertebraten. Dies zeigt auf, dass noch vieles über die Symbiose von Invertebraten mit Bakterien erforscht werden muss.

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Part I

Introduction

This introduction aims to describe in detail whale fall ecosystems, starting with their discovery. A brief overview of the ecological succession happening at a whale fall from the whale carcass sinking to the seafloor, to the whale being eaten is given. Focus is given on the description of the whale fall chemosynthetic habitat since it constitutes the longest and most complex stage of the ecological succession in terms of biogeochemistry, food web and species diversity.

Contributing to this species diversity, symbiosis between invertebrates and bacteria occurs at whale falls. These symbioses will be described in their generality, followed by a more detailed biology of the two whale fall polychaetes that are at the core of this thesis. Moreover, since some of the whale fall symbioses are also found in other chemosynthetic habitats, a comparison is made between whale falls and these other habitats. Finally, an estimation on whale fall frequency (past and actual) is given, to help understand how these animals are finding whale falls randomly located on the seafloor.

Chapter 1

History of whale fall studies: from discovery to sinking whale carcasses

1.1 First evidence of whale fall communities

1.1.1 Even before the first whale fall was found, scientists speculated on the impact of whale carcasses

Before the first whale fall discovery, scientists had speculated on the impact of whales sinking to the bottom of the ocean. It was proposed that these large dead animals falling to the deeps may "constitute the ultimate food for abyssal fauna" [61], and that a dead whale could attract scavengers for a long time [15]. Stockton & DeLaca (1982) [105] speculated that such a large food fall could lead to a specialised dense faunal community at the deep sea floor, and that these communities could last for several years. For a more detailed review on these speculations previous to the actual discovery of a whale fall see Smith & Baco (2003) [99].

In addition, some studies used more mathematical approaches to estimate the consequence of carcasses falling at the sea floor. Compared to the general background of particulate organic carbon (POC) reaching the deep sea floor, a whale is a huge particle [51, 97]. Smith & Baco (2003) [99] proposed that for the ∼50 m^2 sediment area that a whale carcasse covers, the dead whale is equivalent to about 2000 years of background POC rain at abyssal depth. For a review on the impact of large organic pulse on the deep sea fauna see Smith (1994) and Smith (2007) [96, 97].

1.1.2 Lucky findings

Trawling and bringing up whale pieces and bones represented the first evidence that a specific community was associated with whale carcasses. Indeed, many animals were attached to the trawled whale pieces including several species new to science and belonging to various taxa (table 1.1) such as a new limpet genus *Osteopelta*, bathymodiolin mussels including *Adipicola pelagica, Idas pelagica* and *Adipicola simpsoni*, and the new sipunculid *Phascolosoma saprophagicum* (Table 1.1) [23, 39, 67, 107, 119] (see [99] for a more detailed review). All species were supposed to feed on whale bones, whale blubber or bacteria on or near the bones [39, 67, 99].

The first whale fall was discovered by chance in 1987 off the coast of southern California, during a dive of the submersible Alvin [101], revealing a highly diverse faunal community, which confirmed the scientists speculations. Shortly after the first whale fall discovery another skeleton was discovered in the West Pacific close to Japan [37]. In both studies, the skeletons were colonised by a specific fauna and bacterial mats similar to chemosynthetic community at the deep sea [24, 75, 101]. The faunal abundance and species richness were very high. Illustrating the species richness, different animals were collected such as vesicomyid clams *Vesicomya gigas, Calyptogena cf. pacifica*, bathymodiolin mussels *Idasola washingtonia*, and a not yet described cocculinid limpet, the snail *Mitrella permodesta* and the lucinid *Lucinoma annulata* [101] (Table 1.1).

1.2 Sinking whale carcasses to study whale fall communities

To avoid the necessity of having to search for hours to find a whale fall, scientists decided to deliberately sink dead whales to the sea floor to further study community establishment, persistence and ecological succession. Stranded dead whales of different sizes, ages and species were sunk at different locations and depths (Table 1.2, Fig. 1.1) [8, 12, 20, 38, 45, 99].

Table 1.1: Species first recorded at large whale falls. From [97]

HigherTaxon	Species	Known only at whale falls[a]	Estimated pop. Size[b]	Location[c]	Reference
Mollusca					
Archaegastropoda	*Pyropelta wakefieldi*	×	>100	California	McLean 1992
	Cocculina craigsmithi		300–1100	California	McLean 1992
	Paracocculina cervae			New Zealand	Marshall 1994
	Osteopelta praeceps	×	>200	New Zealand	Marshall 1994
	Osteopelta ceticola			Iceland	Warén 1989
	Osteopelta mirabilis	×		New Zealand	Marshall 1987
	Protolira thorvaldsoni			Iceland	Warén 1996
Gastropoda	*Bruciella laevigata*	×		New Zealand	Marshall 1994
	Bruciella pruinosa	×		New Zealand	Marshall 1994
	Xylodiscula osteophila	×		New Zealand	Marshall 1994
	Hyalogyrina n.sp.			California	J. H. McLean and A. Warén, pers. comm.
Bivalvia					
Bathymodiolinae	*Adipicola pelagica*	×		South Atlantic	Dell 1987
	Myrina (Adipicola) Pacifica	×		Japan, Hawai'i	Dell 1987
	Adipicola (Idas) arcuatilis			New Zealand	Dell 1995
	Adipicola osseocola			New Zealand	Dell 1995
	Idas pelagica	×		North Atlantic	Warén 1993
	Idas ghisottii			North Atlantic	Warén 1993
Vesicomyid	New species?	×		California	Baco et al. 1999
Thyasiridae	*Axinodon* sp. nov.	×		California	P. Scott, pers. comm.
Aplacophora	New genus	×		California	Scheltema in prep.
Arthropoda					
Anomura	*Paralomis manningi*	×		California	Williams et al. 2000
Annelida					
Polychaeta					
Polynoidae	*Harmathoe craigsmithi*	×		California	Pettibone 1993
	Peinaleopolynoe santacatalina	×		California	Pettibone 1993
Chrysopetalidae	*Vigtorniella flokati*	×	1000–100,000	California	Smith et al. 2002, Dahlgren et al. 2004
Ampharetidae	New genus	×	>10	California	B. Hilbig, pers. comm.
	Asabellides sp. nov.	×	>10	California	B. Hilbig, pers. comm.
	Anobothrus sp. nov.	×		California	B. Hilbig, pers. comm.
Siboglinidae	*Osedax frankpressi*	×	>1,000	California	Rouse et al. 2004
	Osedax rubiplumus	×	>1,000	California	Rouse et al. 2004
	Osedax, 3 sp. nov.	×	>1,000	California	Pers. obs.
	Osedax mucofloris	×	>1,000	Sweden	Glover et al. 2005
Dorvilleidae[d]	*Palpiphitime* sp.nov.	×	>10,000	California	B. Hilbig pers. comm.
	Dorvilleid sp. nov.	×		California	B. Hilbig pers. comm.
Sipuncula	*Phascolosoma saprophagicum*	×	>20–>200	New Zealand	Gibbs 1987

NOTE: Modified from Smith and Baco (2003).

[a]The 28 species marked as "known only at whale falls" have been reported from no other habitat.

[b]Where available, estimated population sizes on whale falls are given.

[c]Note that more than half of these species have been collected from southern California whale falls, suggesting that whale-fall habitats in other regions may be grossly undersampled.

[d]In addition to *Palpiphitime* sp. nov., an estimated 38 unidentified species of dorvilleids, with population sizes ranging from 10s to 1000s of individuals per whale fall, have been collected from whale falls in the Santa Catalina Basin, San Diego Trough, San Clemente Basin, and Santa Cruz Basin (Baco and Smith 2003; C. Smith and Altamira, unpublished data). Many of these species appear to be new to science.

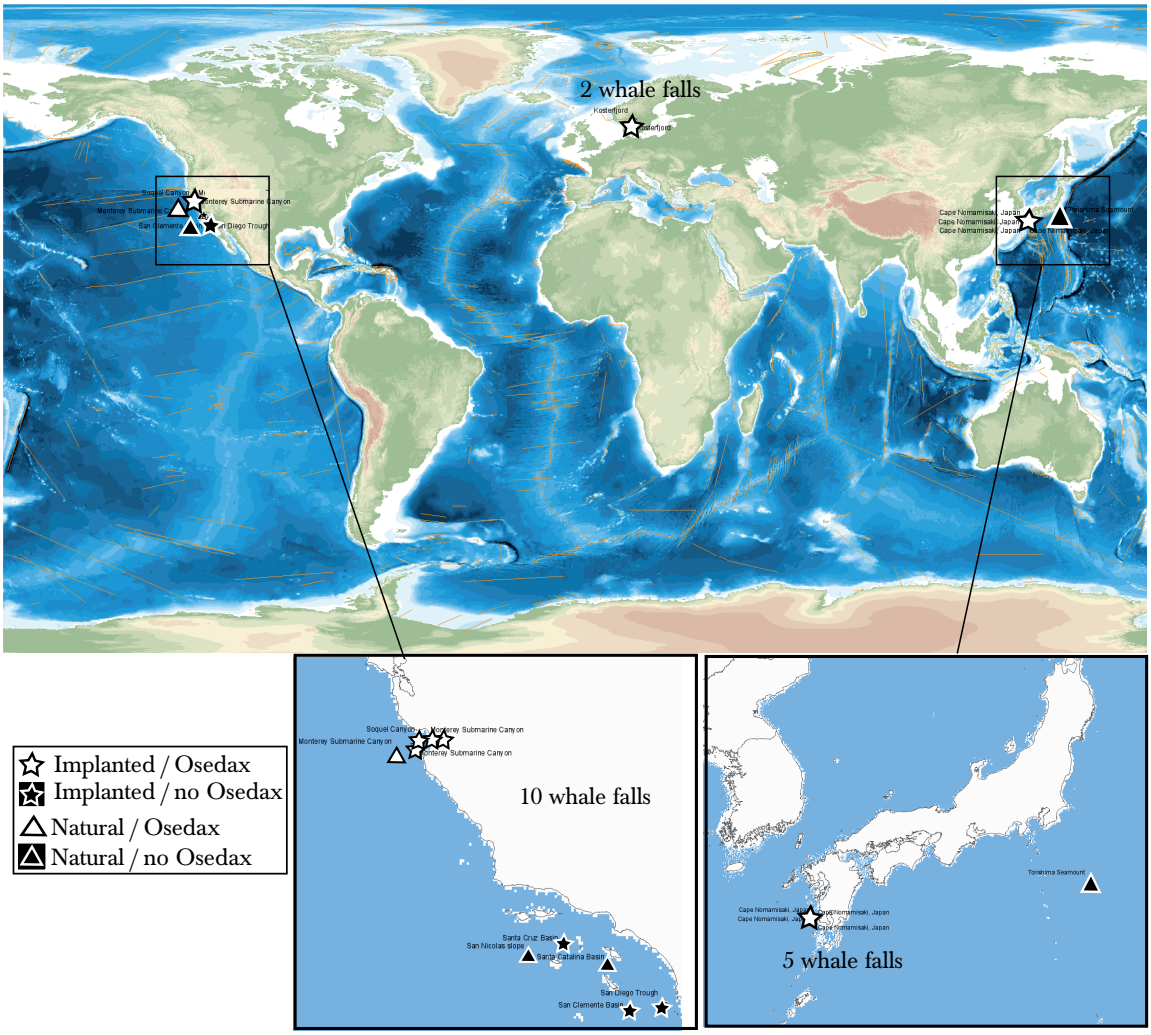


Figure 1.1: Location of known whale falls. This map was realised in collaboration with Renzo Kottmann with Megx.net: integrated database resource for marine ecological genomics [60], and will later be made public with links to the sequences information (such as metagenomic, 16S rRNA) at each site when available. Locations and whale fall descriptions can be found in the following references [12, 20, 37, 38, 45, 75, 86, 99, 101, 118]. More information on each whale is available in table 1.2.

Table 1.2: Known whale falls. Location and depth of each whale fall are given, as well as size, weight and species of the dead whale. It is also indicated if *Osedax* was found at a whale fall [12, 20, 37, 38, 45, 75, 86, 99, 101, 118].

Location	Type	Whale species	Estimated wet weight kg 10^3	Size in m	*Osedax*	Depth m	Latitude °	Longitude °
Santa Catalina Basin	natural	Blue or Fin	~60	20	not found	1240	33,2	-118,5
San Nicolas slope	natural	Balaenopterid?	~40	nd	not found	960	33,33	-119,98
San Clemente Basin	implanted	Gray	10	nd	not found	1960	32,43	-118,15
San Diego Trough	implanted	Gray	5	nd	not found	1220	32,58	-117,5
Santa Cruz Basin	implanted	Gray	35	nd	not found	1675	33,5	-119,36
Soquel Canyon	implanted	nd	nd	nd	present	633	36,802	-121,994
Monterey Submarine Canyon	implanted	Gray	nd	8	present	385	36.790	-121,887
Monterey Submarine Canyon	implanted	Blue	nd	17	present	1018	36,772	-122,083
Monterey Submarine Canyon	implanted	Gray	nd	10	present	1820	36,708	-122,105
Monterey Submarine Canyon	natural	Gray	~20	10	present	2893	36,613	-122,434
Torishima Seamount	natural	Bryde	nd	10 -11	not found	4037	32.9242	141.8287
Cape Nomamisaki, Japan	implanted	Sperm	23	12,2	present	219	31.3977	129.979
Cape Nomamisaki, Japan	implanted	Sperm	39	16	present	228	31.345	129.986
Cape Nomamisaki, Japan	implanted	Sperm	21,9	12,95	present	229	31.345	129.988
Cape Nomamisaki, Japan	implanted	Sperm	22,3	13,05	present	245	31.314	129.992
Cape Nomamisaki, Japan	implanted	Sperm	24,5	13,50	present	254	31.309	129.990
Kosterfjord	implanted	Minke	nd	5,3	present	125	58.885	11.067
Kosterfjord	implanted	Pilot	nd	4,5	present	30	58.886	11.104
Antartic	implanted bones	nd	nd	nd	present	~500	nd	nd

nd, information not available

Chapter 2

Whale fall succession: four successional stages

The study of several whale falls led scientists to characterise the ecological successions happening at whale falls which are proposed to go through four overlapping stages [97, 99, 102] (Fig. 2.1):

1. mobile scavenger stage
2. enrichment opportunist stage
3. sulphophilic stage
4. reef stage

Mobility and trophic shifts

Smith & Baco (2003) [99] suggested that the succession of the species on the whale should be seen more as a continuum with overlapping species; nevertheless, some rapid shifts in mobility pattern and trophic structure were observed: the mobile scavenger stage is dominated by active swimmers, the enrichment opportunistic stage by moderately mobile epibenthos, and the sulphophilic stage by sessile macrofauna and microbial mats (Fig. 2.1) [8, 9, 99]. The corresponding trophic shifts are from scavenger, to carnivore-scavenger-omnivore, and finally to macrofauna harbouring chemoautotrophic symbionts and chemoautotrophic microbes [8, 99, 100].

In the following sections, the predominant characteristics of each stage are listed.

2.1 Mobile scavenger stage

2.1.1 Description & duration of stage

The first whale fall stage, named the mobile scavenger stage, consist in the whale tissue being eaten up. It occurs at all experimental whale falls [12, 20, 45, 99]. Dead whales sink rapidly to the bottom and thus arrive almost intact on the sea floor attracting [97, 99] big and small mobile scavengers with the huge amount of available whale tissue (Fig. 2.1

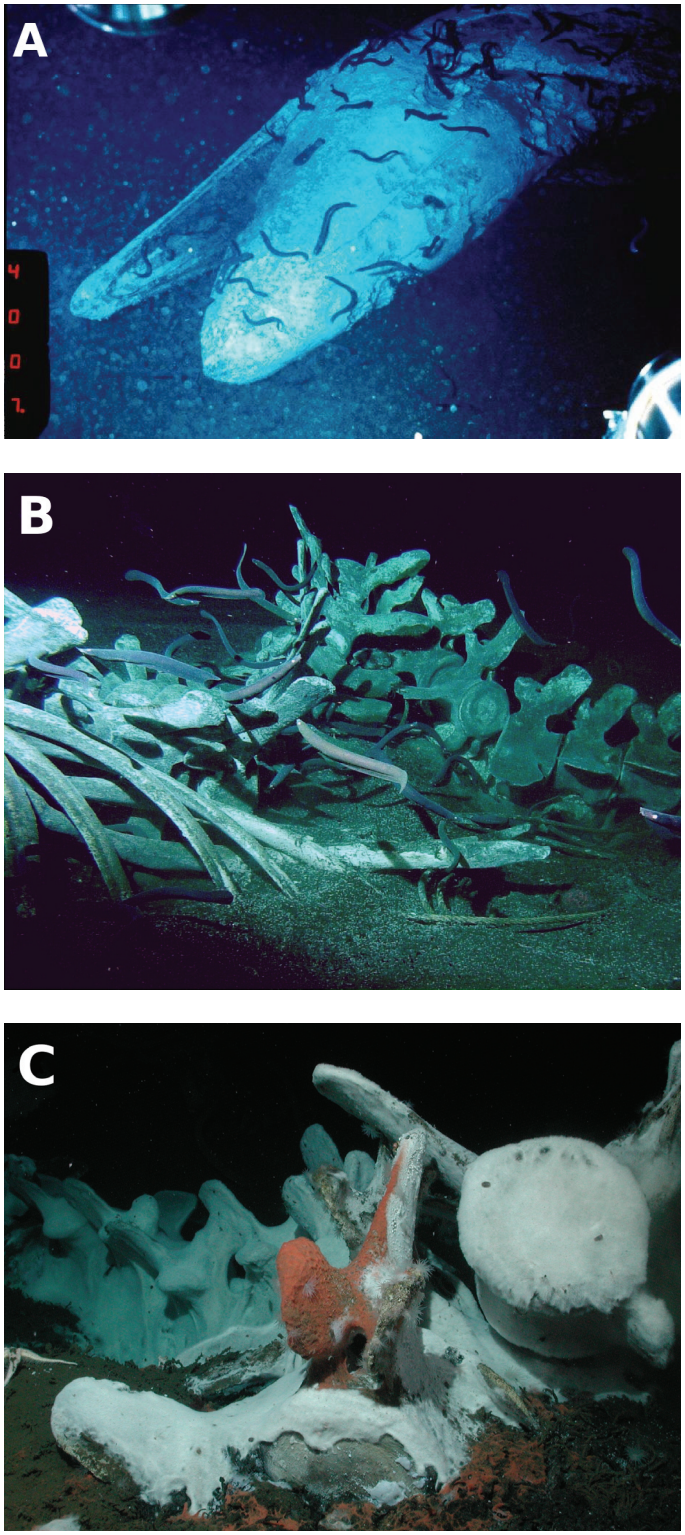


Figure 2.1: Photographs of whale falls at the sea floor on the California slope illustrating three successional stages. Pictures courtesy of Graig Smith, University of Hawaii. Pictures A and B also described in [97]
A. The Mobile scavenger stage: a 35 t gray whale carcass after 1.5 months on the sea floor at 1,675 m in the Santa Cruz Basin. Dozens of hagfishes (*Eptatretus deani*), each ∼30-cm long, are feeding on the white carcass. Large bite marks from sleeper sharks *(Somniosus pacificus)* are also visible.
B. The enrichment opportunist stage: the Santa Cruz carcass after 18 months on the sea floor. The whale soft tissue has been almost completely removed by scavengers, exposing vertebrae and ribs. The sediments around the skeleton are colonised by a dense assemblage of gastropods, juvenile bivalves, cumacean crustaceans, and dorvilleid polychaetes (visible as white dots).
C. The sulphophilic stage: the Santa Cruz whale after 4.5 years. The bones are covered by bacterial mats (white and orange).

A). Scavengers can be highly abundant and remove soft tissue at very high rates. Smaller whales are eaten faster than bigger ones: from few weeks to more than a year, respectively [12, 20, 99].

2.1.2 Scavenger diversity

Bigger whales seem to attract bigger scavengers (sharks and hagfishes) with higher feeding capacities than smaller whales [12, 20, 99]. As the soft tissue of the whale is consumed, the size of the scavenger also decreases from sharks and hagfishes (Fig.2.1 A) to lysianassid amphipods or lithotid crabs [99]. A stable isotope study showed that the food web is very simple during this stage, relying entirely on organic material from the whale [8].

Species from this stage are generalised scavengers, since the frequency of whale falls does not allow them to specialise on whale tissue only [99]. Species differ among different whale falls [20, 45, 100]. Potentially overlapping species are not known yet because they await future taxonomical identification, or simply because they have not yet been sampled [12]. Cryptic species might also change species richness estimations [120]. Many species are restricted to a certain depth range, which is why at very shallow (30 m) or very deep (>2000 m) sites some scavengers such as sharks and hagfishes, are often absent and soft tissue removal is taking longer [20, 45].

The removal of the soft tissue facilitates the arrival of other species, which are characteristic for the next succession stages, by spreading soft tissue on the sediment and allowing access to the bone [99].

2.2 Enrichment opportunist stage

2.2.1 Description

The second stage is the enrichment opportunist stage. During this stage opportunistic species are colonising the bones and the sediment enriched in whale organic material (Fig. 2.1.B). The colonisation begins first in the sediment near the carcass (1 - 3 m) where a high density of macrofauna can be found. The bones colonisation is secondary as they are being gradually exposed [99, 100]. This stage is characterised by a low diversity and a very abundant fauna, certain taxa can reach up to 20 000 - 45 000 individuals $\cdot\, m^{-2}$ [100]. Stable isotope studies showed that during this stage, faunal and microbial nutrition entirely relies on organic material from the whale [8, 44]. For example, *Osedax* (Polycheata), so-called bone-eating worms) occur on whale bones in high abundance, forming red carpets

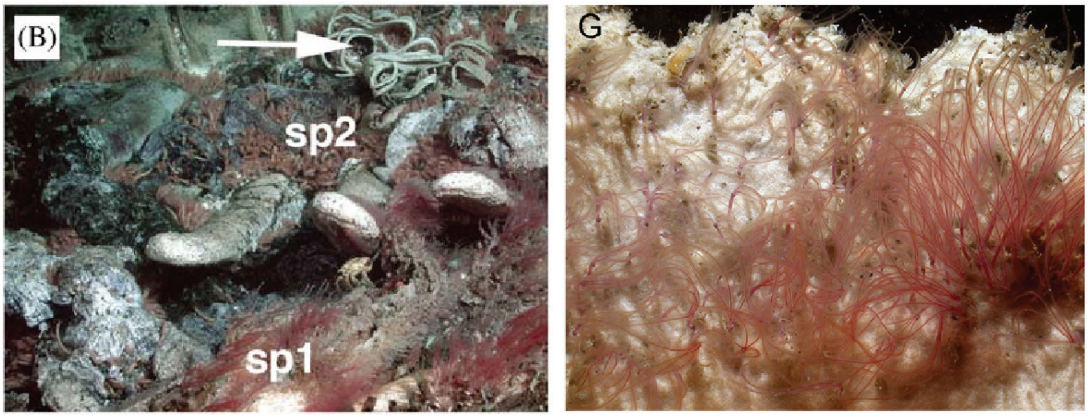


Figure 2.2: *Osedax* forming red carpets at Californian whale falls. **B.** Rib bones, with abundant *Osedax sp.* sp1 and sp2 along with whale tracheal rings (arrow). From [45]. **G.** *Osedax roseus* on lateral process of vertebra from a 1018 depth whale fall. From [12]

(Fig. 2.2) [12, 20, 45, 84]. Another polychaete species, *Vigtorniella flokati*, is also found in high abundance in the sediment and on the bones [19], as well as dorvilleid *Ophryotrocha sp.*, a cumacean, gastropods and juveniles bivalves [6, 8, 12, 19, 20, 40, 45, 69, 100].

2.2.2 Duration of stage

The colonisation occurs rapidly in weeks to months [12, 45]. Then, the duration of this stage depends on the size of the whale, ranging from months to several years [99, 100].

At a whale fall located at 385 m depth, Braby et al., 2007 [12] observed that no significant enrichment opportunist stage occurred. The lack of an enrichment opportunist stage at this particular whale fall is likely because most of the soft tissue was carried away by scavengers and the site was subjected to more disturbance than deeper sites, such as macrofaunal activity, sedimentation and currents.

2.2.3 Comparison to other organic falls

The assemblage of species found during the enrichment opportunistic stage of whale falls may be similar to other organic falls such as sewage outfalls, fish farms, fish falls and wood falls [97, 99]. Cocculinoform limpets, such as *Paracococculina cervae*, and bathymodiolin mussels are the two main taxa which are distributed throughout several other organic falls, including wood falls [32, 65, 99]. Lorion et al., (2009) showed that *Idas* and *Adipicola* mussels can colonise diverse organic substrates, including wood and bones [65]. Dorvilleid

polychaetes *Ophryotrocha* are found at sewage outfalls and the polynoid polychaetes *Peinaleopolynoe* in organic rich sediments [99]. Wiklund et al., (2009) described a novel species, chrysopetalid annelid *Vigtorniella ardabilia*, from fish farms in the Northeast Atlantic, which seems to graze on bacterial mats [120].

Many species found at organic fall sites may be generalists. However, it appears that some species, such as *Vigtorniella flokati*, remain whale fall specialists as they have been found only at whale falls [19, 84, 97, 99] (table 1.1).

2.3 Sulphophilic stage

The third successional stage is named the sulphophilic stage because of its similarity to other sulphide-rich habitats such as hydrothermal vents and cold seeps. It is the most complex stage of the ecological succession at whale fall in several aspects. Only a brief description is provided here as a more detailed description is given in Chapter 3.

2.3.1 Biogeochemistry

This stage comprises the anaerobic degradation of bone lipids by sulphate-reducing bacteria, the anaerobic degradation of organic material from the whale by sulphate-reducing bacteria and the activity of methanogenic archaea in the sediment. During sulphate reduction, hydrogen sulphide is produced in the sediment and bones. The activity of methanogenic archaea leads to the production of methane. Free-living sulphur-oxidising bacteria and sulphur-oxidising symbionts utilise the produced hydrogen sulphide as an energy source (Fig. 3.1) [24, 75, 97, 99, 110]. These processes are described in detail in section 3.1.

2.3.2 Faunal diversity

At whale falls older than five years, the sulphophilic stage is characterised by an abundant fauna and high species richness (Fig. 2.3) explained by a complex trophic structure with the presence of many different niches. This abundance of species is similar or higher than on other deep sea hard substrates, such as hydrothermal vents and cold-water coral reefs [6, 99, 100]. Niches include the bone matrix, the bone surface, the sediment and the sediment-water interface and the remaining soft tissue from the whale [6, 8, 24, 99]. The complex food web of the sulphophilic stage is described in section 3.2.

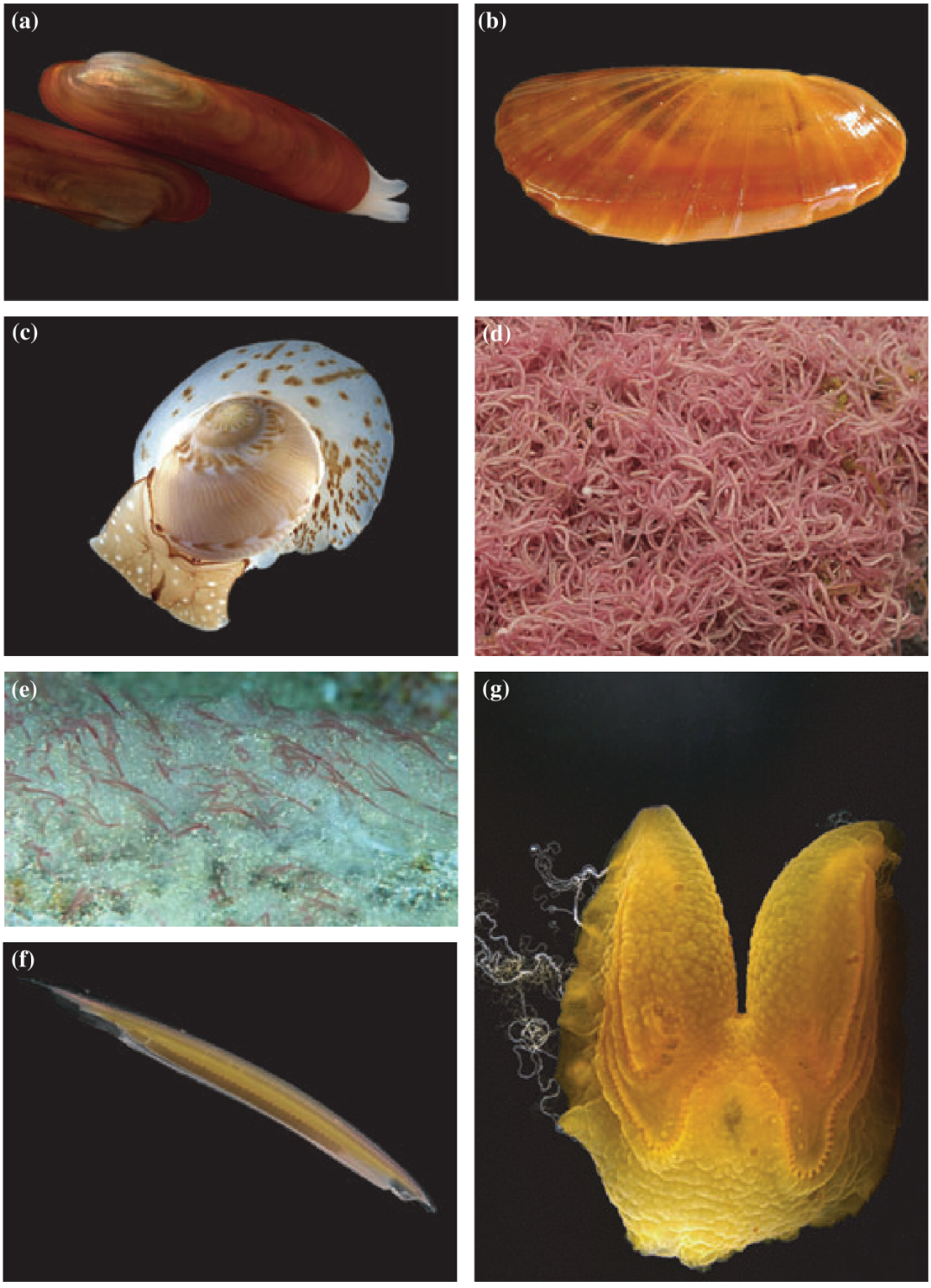


Figure 2.3: Benthic fauna at sperm whale falls. (a) Mussel *Adipicola crypta*, (b) protobranch *Solemya pervernicosa*, (c) gastropod *Tanea magnifluctuata*, (d) an unidentified protodrilid polychaete, (e) siboglinid *Osedax japonicus*,(f) lancelet *Asymmetron inferum* and (g) ctenophore *Lyrocteis imperatoris*. From [38]

2.3.3 Duration of stage

At a large whale the sulphophilic stage can last for more than 50 years [91, 99, 101]. There seems to be a minimum skeleton size and age for chemoautotrophic production to appear, probably due to different levels of bone calcification in juvenile and adult whales [8, 45, 99]. For example, the whale fall discovered in the Santa Catalina basins is thought to have been on the sea floor for more than 50 years [99, 101]. At sperm whales implanted off the Japanese coast, however, the sulphophilic stage seemed to last only for a few years [38].

On smaller cetaceans falls, the sulphophilic stage may never occur because small cetaceans probably lack enough organic material to sustain durably a sulphophilic fauna. Baco-Taylor (2002) reported that although species which can have chemoautotrophic symbionts were present on small cetacean carcasses, these species relied on organic carbon from the whale and not on chemoautotrophy [8].

In addition, it was proposed that *Osedax* accelerates the degradation of the skeleton and the use of the lipid rich bone, thus limiting or suppressing the sulphophilic stage compared to whale falls where *Osedax* are absent [12, 38]. Depth, temperature, and disturbance level of the community (such as high deposition of sediment and turbidity flow) may also influence the duration of the sulphophilic stage [12, 38].

2.4 Reef stage

When it was proposed that a whale fall would go through several successional stages by Smith et al., (1998) there was no direct evidence of a reef stage [102]. This stage was supposed to be dominated by suspension feeders using the enhanced flow conditions on the skeleton [99, 102]. Some suspension feeders already occur during the first stages on the skeleton and in the background fauna, and they are likely to persist on the skeleton after the organic matter is consumed [8, 99, 100]. Shallow sperm whale falls in the Northeast Pacific showed the first evidence of a reef stage: the ecological epifaunal succession was rapid and after two years evidence of a reef stage were observed, with the appearance of numerous suspension feeders [38]. It is not known yet how long this stage lasts since it has been only described once [38,99]. On small whales, rapid decomposition and mineralisation of the bone might shorten this stage [99]

Chapter 3

The sulphophilic stage: a chemosynthetic habitat

As previously described, at most whale falls, after a certain time a chemosynthetic habitat develops [24, 75, 99–101, 110]. First, microbial processes occurring in the sediment, in the bones, and in invertebrates will be presented. A second part describes the food web sustained by these microbial processes. Third, faunal abundance and diversity among whale falls are described and compared.

3.1 Biogeochemistry and microbes

3.1.1 Sulphide and methane production

In this section the following processes will be described in turn, hydrogen sulphide and methane production at whale falls (Fig. 3.1) [24,43,46,101,110]. Sulphate-reducing bacteria use sulphate from seawater and organic carbon from the whale to produce hydrogen sulphide and carbon dioxide in the bones as well as the sediments [24,43,110]. Methanogenic archaea are present only in the sediment, consuming organic carbon from the whale, and producing methane [46,110]. Since microbes and processes occurring in the bones and in the sediment are not identical, their description will be separated.

In the bones

Sulphate-reducing bacteria use sulphate (SO_4^{2-}) as an electron acceptor and hydrogen (H_2) [equation 3.1] or small organic molecules [equation 3.2] as electron donors. Some can oxidise fatty acids and other organic molecules all the way to carbon dioxide CO_2, thus remineralising organic compounds [equation 3.2](Fig. 3.1).

$$9H_2 + 2SO_4^{2-} + 2H^+ \rightarrow 2H_2S + 8H_2O \tag{3.1}$$

$$CH_3COO^- + SO_4^{2-} + 3H^+ \rightarrow 2CO_2 + H_2S + 2H_2O \tag{3.2}$$

Both reactions are exergonic and support the growth of sulphate reducers.

Whale bones contain up to 60% lipids (wet weight), thus the main electron acceptor in whale bones are fatty acids [equation 3.2] [24, 91]. The anaerobic decomposition of

these lipids by sulphate-reducing bacteria can produce high concentrations of hydrogen sulphide that diffuses out of the bones (Fig. 3.1A) [equation 3.2] [24, 110]. The bacteria are found mostly at the bone-water interface and are less abundant in the central portion of the bone [24]. Sulphate reduction rates are highest at the bone-water interface (in the first centimetre) [110]. Lipid concentrations inside the bone show an inverted pattern with higher lipid concentrations in the central part and lower lipid concentrations in the outer parts [24].

The degradation of lipids from the surface towards the inner core creates holes and passages in the bone matrix, which allows sulphate to diffuse deeper in the bone, and facilitates deeper settlement of sulphate-reducing bacteria inside the bone [4, 91, 93, 110]. Similarly, the presence of *Osedax* worms drilling into the bones down to some centimetres deep [84] facilitates the diffusion of sulphate into the bone matrix and enhances sulphate reduction [110].

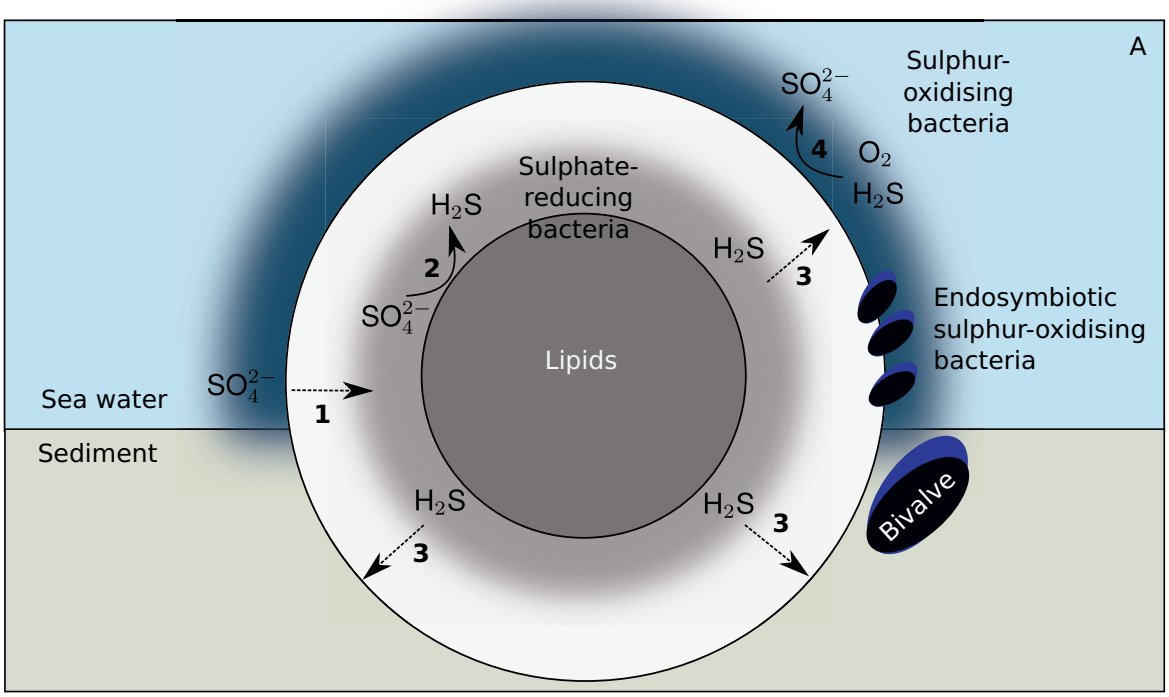
A slow degradation of the lipids explains the persistence of a sulphophilic stage for decades at some whale falls [99, 110].

In the sediment

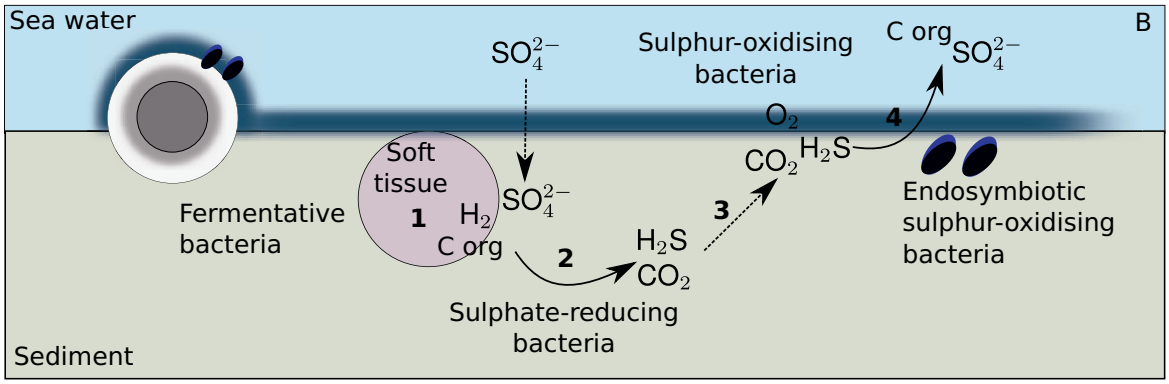
During the mobile scavenger stage, soft tissue is dispersed around the whale carcasses and buried into the sediment [99]. This organic carbon is then available for sulphate-reducing bacteria [43, 44, 110]. In the sediment associated with the whale fall, fermentative bacteria, sulphate-reducing bacteria and methanogenic archaea are more abundant than in the background sediment [43, 44]. High methane and hydrogen sulphide concentrations, and the highest microbial activity, can be measured in sediments close to the skeleton (0.5 to 3 m) [43, 46, 102, 110].

Fermentation Fermentative bacteria are usually present in the early stage of organic carbon breakdown, degrading macro-molecules. Fermentation facilitates the activity of other microbes such as sulphate reducers and methanogens by providing them with smaller organics molecules (fatty acids, alcohols) and hydrogen (H_2) (Fig. 3.1). In the associated sediments at two whale falls, high levels of total organic carbon were measured as well as high proteolytic activity, used as a proxy for macro-molecule breakdown. In addition, Bacteroidetes and Firmicutes bacteria were abundant. Many representatives from these two phyla are able to break down complex organic matter. Furthermore, elevated concentrations of hydrogen were measured in the sediment, which also indicates enhanced

In the bones :



In the sediment :



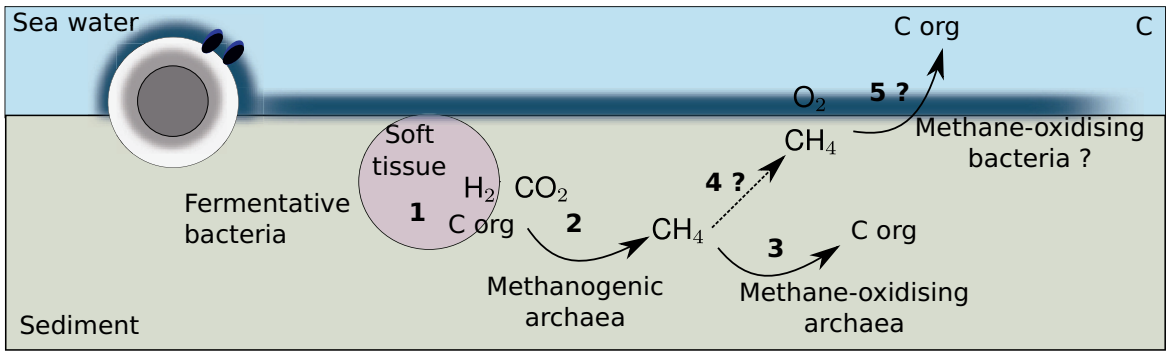


Figure 3.1: A. Schematic of cross section of a whale vertebra resting at the sea floor during the sulphophilic stage of succession (modified from [99]). The predominant decomposition processes occurring in the bones are illustrated, which include: (1) Diffusion of sulphate from sea water into the bone; (2) Sulphate reduction by anaerobic bacteria decomposing lipids in the lipid-rich bone core; (3) Diffusion of hydrogen sulphide outward from the bone core, (4) Sulphide oxidation, and organic matter synthesis, by sulphur-oxidising bacteria living on the bone surface and within the tissues (i.e. endosymbiotically) of vesicomyid clams, bathymodiolin mussels and other invertebrates [24, 95, 110].
B-C. Processes occurring at a whale fall during the sulphophilic stage in the sediment. Scheme by C. Verna based on data from the following references [24, 43, 46, 75, 95, 110].
B. (1) Fermentative bacteria breakdown complex molecules from whale soft tissue, producing hydrogen and small organic molecules. (2) Sulphate reduction by anaerobic bacteria; (3) Diffusion of hydrogen sulphide to the sediment-water interface (4) Sulphide oxidation, and organic matter synthesis, by sulphur-oxidising bacteria living in mats on the sediment surface and within the tissues (i.e. endosymbiotically) of invertebrates.
C. (1) Fermentative bacteria break down complex molecules from whale soft tissue, producing hydrogen and small organic molecules. (2) Methanogenesis by archaea; (3) Anaerobic methane oxidation coupled to sulphate reduction by archaea such as the ANME-3 group probably occurs. (4) Diffusion of methane to the sediment-water interface with (5) Aerobic methane oxidation by bacteria at the sediment-water interface might occur but was not directly measured.

fermentation activity [43].

Sulphate reduction As described above for the bones, sulphate reduction also occurs in the sediment were both hydrogen and small organic molecules are used as electron acceptors [equations 3.1, 3.2]. At several whale falls, high hydrogen sulphide concentrations underneath the bones were detected [24,38,75,99,102,110] as well as high sulphate reduction rates in the sediment [110]. In addition, fatty acids characteristic for sulphate-reducing bacteria were also detected [75].

Goffredi et al., (2010) analysed the bacterial community associated with two whale falls [43]. They discovered that Deltaproteobacteria from the families Desulfobacteraceae and Desulfobulbaceae, both known to perform sulphate reduction, were abundant [43]. Some bacteria capable of sulphur disproportionation, a process converting sulphur in intermediate oxidation states to sulphate and sulphide, were also indicated by bacterial 16S rRNA clustered with the family Desufobulbaceae [43]. Cultured representatives from Desulfobulbaceae are known to perform sulphur disproportionation.

Methanogenesis Methanogenesis is a strictly anaerobic process for the reduction of carbon dioxide (CO_2) to methane (CH_4). This process can occur with hydrogen as electron

donor [equations 3.3 & 3.4] or small organic molecules [equation 3.5]:

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O \tag{3.3}$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \tag{3.4}$$

In the absence of H_2:

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \tag{3.5}$$

High methane concentrations and methanogenesis rates were detected in sediment associated with a deep sea whale fall (Fig. 3.1) [110]. Goffredi et al., (2008) [46] investigated the archaeal diversity associated with a whale fall. They found that methanogens capable of using both C1 compounds (*Methanococcoides*) or hydrogen (*Methanogenium*) were abundant at a deep whale fall in Monterey Canyon. C1-utilising methanogens seemed to be established early, followed later by H_2-utilising ones.

The production of methane might support the establishment of methanotrophic (methane-oxidising) microbes (Fig. 3.1) [46, 75]. At a deep whale fall in the Northeast Pacific, fatty acids characteristic for methane-oxidising bacteria were found [75]. At another whale fall in the West Pacific methanotrophic archaea from the ANME-3 group were detected [44, 46].

Usually sulphate reduction and methanogenesis do not co-occur because they compete for small organic compounds and hydrogen. However, both were detected in the sediment below and surrounding whale falls with methanogenesis rates as high as 20 to 30% of sulphate reduction rates [43, 46, 110]. At whale falls, the excess of organic substrates as well as the spatial structure of the communities may allow both processes to co-occur [43, 46, 110].

3.1.2 Sulphide oxidation by free-living and symbiotic bacteria

The hydrogen sulphide (H_2S) produced in the bones and the sediments can sustain the growth of sulphur-oxidising bacteria which use H_2S as electron donor [equation 3.6] and CO_2 as carbon source (Fig. 3.1 B):

$$H_2S + 2O_2 \rightarrow SO_4^{2-} + 2H^+ \tag{3.6}$$

Sulphur-oxidising bacteria occur at whale falls as free-living bacteria or as symbiotic bacteria in marine invertebrates [8, 12, 24, 38, 75, 99, 101, 110]. Bacterial mats of sulphur-

oxidising bacteria can be observed directly on the bone surface and close to the bones on the sediment surface (Fig. 2.1 C), where both high fluxes of H_2S and oxygenated sea water are present [8, 12, 24, 45, 75, 101, 110]. The dominant microbes in the mats belong probably to *Beggiatoa* usually growing at oxic-anoxic interfaces [24]. Treude et al., (2009) [110] estimated the bacterial mat coverage to be ∼12% on the sediment within 0.5 m of the skeleton, and ∼50% on the bones [110]. Bacterial mats can appear as early as a few months after a whale is sunk [12, 20, 45] and can expand quickly (weeks) [45].

Diverse invertebrates known to have sulphur-oxidising symbionts have been reported from several whale falls and will be further described in section 4, dedicated to symbiosis at whale falls. However, although methane is available no species relying on methane-oxidising symbionts have been found at whale falls [8, 9, 24, 99]. The presence of high hydrogen concentrations at whale falls suggests that hydrogen could also be an energy source for symbioses, but, to our knowledge, this has not yet been investigated.

3.2 Complex food web

In contrary to the first two stages, where most of the nutrition is derived from the whale soft tissue [8, 99], during the sulphophilic stage, the food web becomes more complex with most of the nutrition being derived from the sulphide-oxidising microbes [8, 99]. This food web is composed of several trophic chains, each with different levels of complexity. Five different feeding strategies have been identified [9, 99]:

1. direct feeding on organic material (bones and soft tissue)
2. grazing on free-living bacteria (heterotroph or chemoautotroph)
3. chemoautotrophy using sulphide release (free-living and symbiotic bacteria)
4. predation
5. suspension feeding

Baco-Taylor (2002) used stable isotope (^{13}C and ^{15}N) on five whale falls to look in more detail at the food web composition during the sulphophilic stage [8]. The study of Baco-Taylor found three main trophic chains, the most complex trophic chain relied on sulphur-oxidising symbionts, and supported four trophic levels:

- producers with sulphur-oxidising endosymbionts

- primary consumers
- secondary consumers
- scavengers

The second trophic chain is dependent on the bacterial mats [8]:

- the bacterial mats (sulphur-oxidising and/or heterotrophic bacteria),
- primary consumers grazing on the mats

The third trophic chain based on the organic whale material was only represented by few species [8]. However, *Osedax* was absent from the studied sites. At sites where *Osedax*, which rely on organic carbon from the bones, are abundant, the trophic chain based on organic whale material would be proportionally more important in the food web [12, 44, 45, 84].

This complex food web based on various food sources allows a high diversity of species to co-occur at very high abundance. The following section describes this diversity.

3.3 High faunal abundance and high biodiversity

3.3.1 Abundance

During the sulphophilic stage invertebrate macrofauna can be highly abundant [75,99]. At several whale falls, it was estimated that on a single skeleton more than 30 000 individuals could be present, counting all taxa [8, 9, 38, 99, 100]. The most abundant taxa were mostly mytilid mussels with sulphur-oxidising symbionts, cocculinid limpets, dorvilleid and *Osedax* polychaetes (Figs. 2.2 & 4.3) [8, 12, 38, 45, 75, 99, 100].

3.3.2 High species richness

Species richness at whale falls seems to increase with time, thus, the sulphophilic stage is the most species-rich stage [12, 38, 99]. Species richness at one skeleton during the sulphophilic stage was as high as 190 species [99]. Global richness was estimated (for all four succession stages) to be 407 animal species [97]. The most species-rich taxa at several whale falls were the polychaetes [9, 99]. For example, new *Osedax* species are discovered almost at each new whale fall studied around the world (Table 1.2) [12, 36, 40, 42, 84, 117].

3.3.3 Few shared species among whale falls sulphophilic communities

In addition to the high biodiversity, not many species are shared between whale falls from different regions, possibly due to the lack of species-level identification and molecular characterisation, [12,38]. Northeast and Northwest Pacific whale falls share only one species (*Adipicola pacifica*) [38, 99]. In the Northeast Pacific, shallow and deep whale falls shared no species [38, 75]. However, sulphophilic communities were similar at the family level [12, 38, 99].

Numerous factors such as depth, disturbances created by high sedimentation rates and turbidity flow and local fauna diversity, could influence the number of species found at each whale fall explaining such a small overlap [12, 38, 45].

3.3.4 Whale fall specialists?

Different species can colonise whale falls: background fauna, whale fall specialists, broad chemosynthetic specialists, and refugees from other chemosynthetic habitats.

During the sulphophilic stage, few or no background fauna is found at whale falls [9, 12, 38, 99]. However, Naganuma et al. (1996) mentioned the presence of echinoderms which were more abundant at the whale fall than in the background fauna, and proposed that the whale fall could serve as an oasis for these species [75].

Few species found at other chemosynthetic habitats were reported at whale falls such as several clams species [7, 97, 98] which is described in Chapter 5 comparing the species found in the different habitats.

Most of the whale fall fauna appears to be specifically adapted to whale falls. Smith 2007 recorded 36 macrofaunal species that have been first found at a whale fall [97]. Of these species, 28 have not been reported in other habitats (table 1.1). In addition, five species may be dependent on whale falls as they are abundant at this habitat and scarce in others. These numbers are underestimated since many other whale fall communities have been described since then [9, 12, 38, 45, 52, 117].

Chapter 4

Whale falls and symbiosis

In this chapter, the notion of symbiosis will be briefly defined, followed by an overview of the different symbiotic species found at whale falls. Finally, the biology of the polychaetes *Osedax mucofloris* and *Raricirrus beryli*, which symbiont community are the focus of this thesis, will be presented.

4.1 Symbiosis diversity at whale falls

4.1.1 Symbiosis definition

The term symbiosis (from the Greek: $\sigma\upsilon\nu$ syn "with"; and $\beta\iota\omega\sigma\iota\varsigma$ biosis "living") was first used in 1879 by the German mycologist Heinrich Anton de Bary, who defined it as "the living together of unlike organisms" [21]. This definition includes different interaction types between partners, from positive interactions (e.g. mutualism) and neutral interactions (e.g. commensalism) to negative interactions (e.g. parasitism) [18]. In its stricter sense, symbiosis refers to mutualistic interactions only [18]. A broader definition may reflect better the reality, because one association can shift from beneficial to not beneficial as environmental conditions change. Therefore, this thesis defines symbiosis in its broader sense, as a close association between organisms including mutualism, commensalism and parasitism.

For a symbiotic association to persist over time, the symbionts need to be transmitted from one host generation to the next. Two main transmission modes are possible:

- Horizontal transmission: the symbionts are newly acquired at each generation from a free-living form in the environment or from co-occurring adult hosts. In this case the life cycle can be divided in two phases, symbiotic and aposymbiotic [13, 18]. For example, in the siboglinid *Riftia pachyptila* the transmission has been shown to be horizontal [77]. The larvae settle at a vent and, during a short time window, acquire their symbionts through their skin [77].

- Vertical transmission: the symbionts are directly transmitted from parent to offspring usually via the eggs or the larvae. In most case the whole cycle is symbiotic [13, 18].

An example of vertical transmission are the vesicomyid clams, where the symbionts are transmitted in the eggs [78, 104].

4.1.2 Chemosynthetic and heterotrophic symbioses co-occur at whales falls

In the marine environment, from shallow to deep sea (Fig. 4.1), many invertebrates are in a mutualistic symbiosis with microbes [14, 29]. Chemoautotrophic symbioses were first discovered at hydrothermal vents where very high fauna abundance were discovered to be mostly driven by symbiosis [29]. When first discovered whale falls were also suspected to harbor various symbioses [101]. As seen in Fig. 4.1, host species are highly diverse and often occur in more than one habitat including hydrothermal vents, cold seeps, whale falls, continental margins, and shallow water sediments [29].

The microbial partners of the symbiosis are also highly diverse, as for example the diversity of gammaproteobacterial symbionts (Fig. 4.2) [14, 18, 29]. In addition to the Gammaproteobacteria, Epsilonproteobacteria, Alphaproteobacteria, Deltaproteobacteria and Spirochetes are also found in these symbioses [14, 18, 29, 30]. The metabolic capacities of symbiotic microbes are diverse, ranging from sulphur oxidisers, sulphate reducers, and methane oxidisers to hydrogen oxidisers [29, 79].

At whale falls, chemoautotrophic symbioses are diverse and found in a wide variety of animals: Bathymodiolin mussels *Idas washingtonia, Adipicola crypta, Adipicola pacifica*, vesicomyid *Vesicomya gigas, Calyptogena kilmeri, Calyptogena elongata, Calyptogena sp.*, solemyid *Solemya pervernicosa* and thyasirid *Thyasira sp.*, lucinid *Lucinoma annulata* and even siboglinids tubeworms [12, 24, 33, 38, 75, 97, 99–101]. Transmission electron microscopy studies and enzyme assays confirmed the presence of sulphur-oxidising endosymbionts associated with *Idas washingtonia* and *Vesicomya gigas* present in high abundance at whale falls [24].

Bivalves relying on chemoautotrophic symbionts are mostly found during the sulphophilic stage, and are not abundant during the other successional stages [12, 38, 45, 99] (Fig. 4.3). Bathymodiolin mussels relying on sulphur-oxidising symbionts, typical of chemosynthetic habitats were estimated to be as abundant as $> 100\ 000$ individuals $\cdot\ \mathrm{m}^{-2}$ for *Adipicola pacifica* on some Japanese whale falls (Fig. 4.3) [38]. Smith & Baco (2003) estimated *Idas washingtonia* to be $> 10\ 000$ - $20\ 000$ individuals $\cdot\ \mathrm{m}^{-2}$ on three whale falls off the southern Californian coast [8, 99, 100].

Moreover, a novel type of symbiosis between *Osedax*, the so-called bone-eating worm, and heterotrophic bacteria was discovered at whale falls (Figs. 2.3 & 2.2) [12, 42, 84, 117].

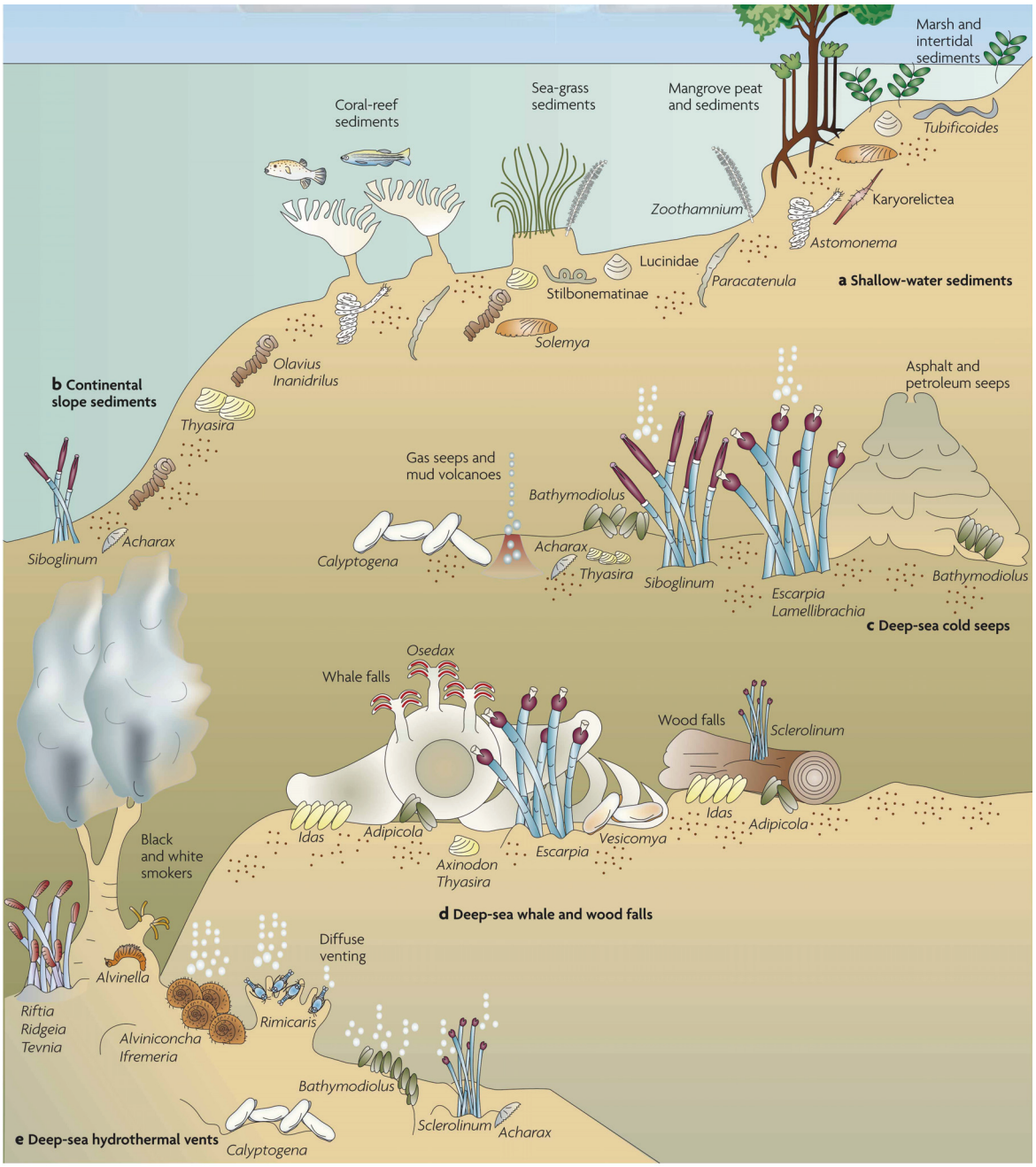


Figure 4.1: Chemosynthetic symbioses in different marine habitats (from [29]). Chemosynthetic symbioses occur in a wide range of marine habitats, including shallow-water sediments (a), continental slope sediments (b), cold seeps (c), whale and wood falls (d), and hydrothermal vents (e). Some host groups are found in only one habitat (such as *Osedax* on whale bones), whereas others occur in several different environments (such as thyasirid clams, which are found in shallow-water sea-grass sediments and in the deep sea at cold seeps, whale falls and hydrothermal vents). The animals are not drawn to scale; for example, *Idas* and *Adipicola* mussels are much smaller than *Bathymodiolus* mussels.

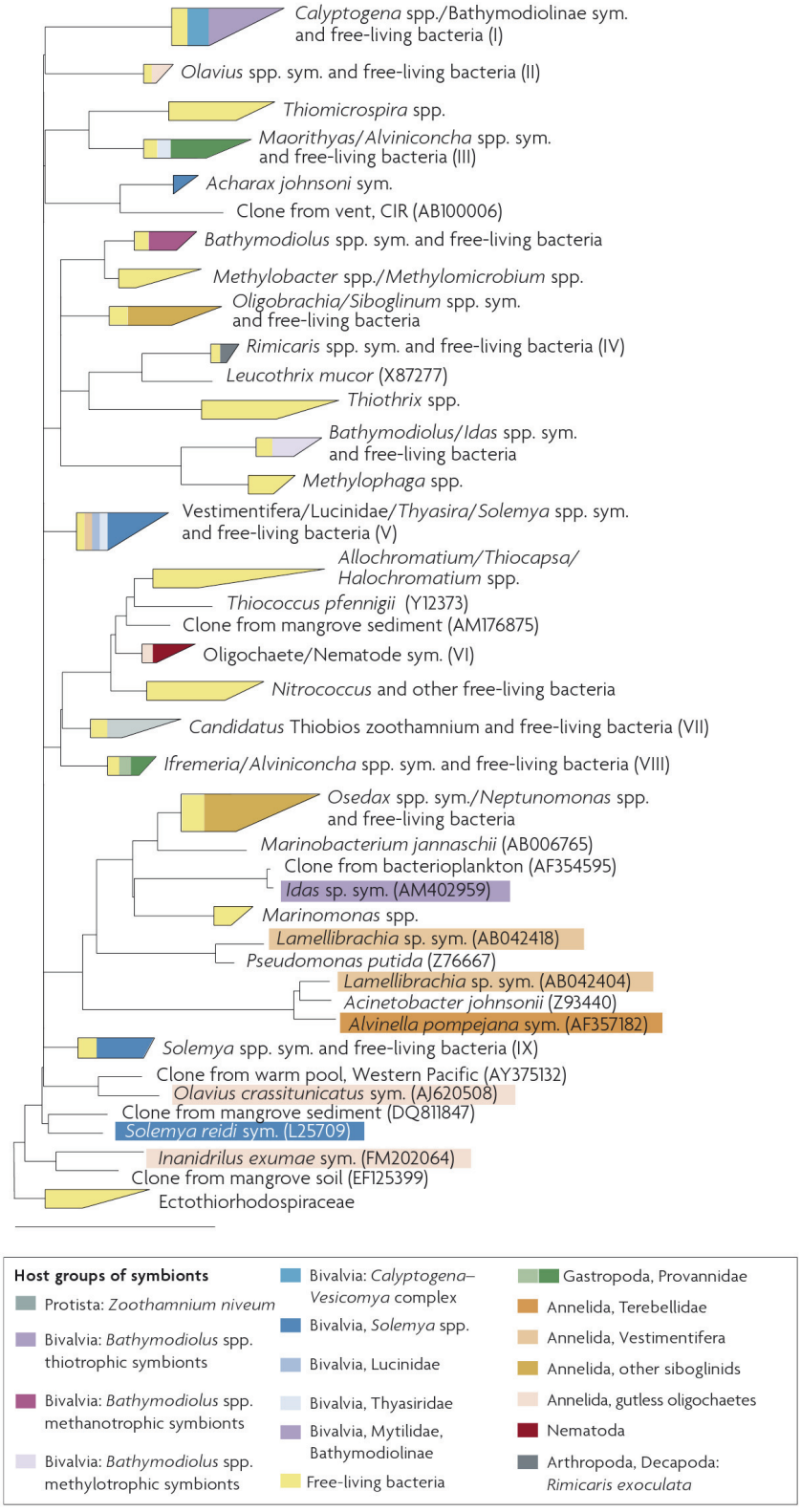


Figure 4.2: Phylogenetic diversity of gammaproteobacterial, chemosynthetic symbionts based on their 16S ribosomal rRNA gene sequences (from [29]). Symbionts from the same host group are shown in the same colour, and free-living bacteria are shown in yellow. Symbiont phylogeny is based on maximum likelihood analyses of 16S ribosomal RNA (rRNA) gene sequences.

Figure 4.3: Living specimens of the mytilid mussel *Adipicola pacifica* on a whale vertebra. From [38]

Osedax is associated with Oceanospirillales Gammaproteobacteria, which are supposed to help *Osedax* breaking down organic carbon of the bones (lipids and collagen) [42, 44].

Host diversity as well as phylogenetic and metabolic diversity of the symbionts are therefore high at whale falls, and similar to symbioses in other chemosynthetic habitats [29]

4.2 *Osedax*, the bone-eating siboglinid

Among siboglinids, this thesis focuses on *Osedax*. Therefore in this section *Osedax* is described in detail and other siboglinids only briefly.

4.2.1 Each female has a harem of dwarf males

Osedax larvae that settle on bones further develop into a female [84, 85]. Each female grows a root branching structure that infiltrates the bone matrix, and a trunk finishing with a crown of palps (or plume) and an oviduct that is free in the sea water (Fig. 4.4) [84]. The trunk and palps can be retracted quickly if the worm is disturbed [36, 40, 84]. As all siboglinids, *Osedax* lack mouth, anus and gut and rely on symbiotic bacteria for nutrition [81, 84]. The root contains the ovisacs with numerous developing oocytes and the bacteriocytes containing Oceanospirillales endosymbionts [36, 40, 44, 84, 86]. *Osedax* females become quickly mature and spawn eggs within a few months [40, 86]. Most of the

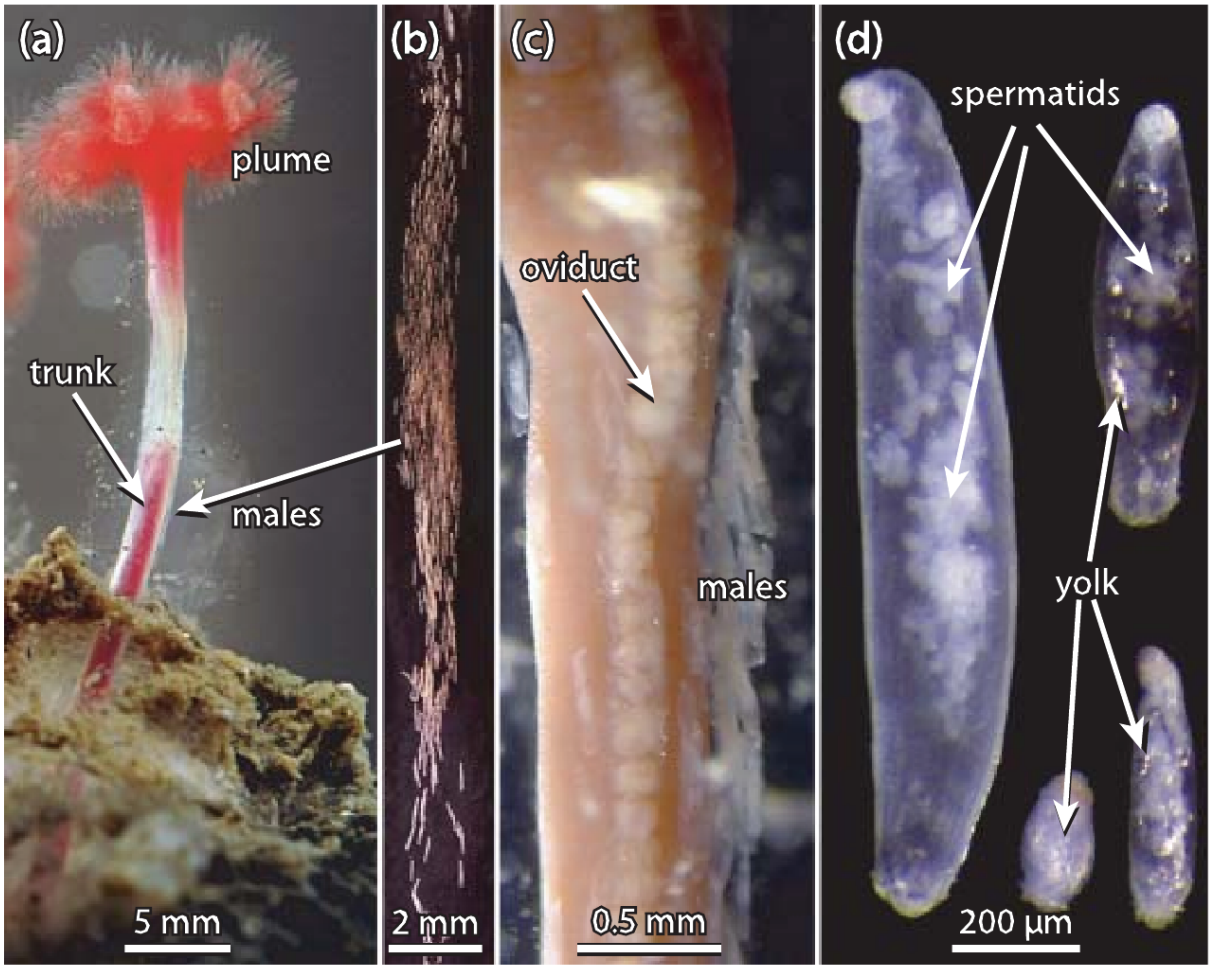


Figure 4.4: *Osedax rubiplumus* males and females. From [116] (a) Adult female on bone photographed in an aquarium immediately after recovery from ROV Tiburon. The female's plume has contracted from the normal condition in situ and has retracted slightly into the transparent tube surrounding the trunk. A harem of microscopic males lies next to her trunk in the lumen of the tube (arrow). (b) A harem of males attached to the transparent tube after removal of the female. (c) The anterior trunk of a female, showing a harem of males lying adjacent to her oviduct. (d) Four males from a single harem, illustrating the extent of size variation among males. The smallest male is still full of yolk, but the mid sized specimens have optically refringent yolk granules and spermatids. The largest male has no obvious yolk granules.

female volume is devoted to egg production [116].

Once the bone surface is covered by females, subsequently arriving larvae settle on a female and develop into dwarf males in the female's tubes (Fig. 4.4) [85]. Thus, *Osedax* exhibit an environmental sex determinism, depending on which substrate the larvae settle on, with larvae settling on bones becoming female and larvae settling on female becoming male [84–86].

Males do not harbor any symbionts [[116], this study Manuscript II], and do not seem to have any feeding activity. The males most probably rely on their yolk reserve, produce sperm and die as described by Vrijenhoek et al., (2008a) [116]. They observed that small males are packed with yolk droplets, intermediate size males have less yolk and contain mature sperm, the largest male have no more yolk and are packed with sperm. Male size varies between *Osedax* species and individuals, for example $200 - 1000\,\mu m$ for *Osedax rubiplumus*, probably representing the different development stages [116].

Males are continuously recruited during the life of the female, and with time each female accumulates a harem of dwarf males which grow bigger as the female gets older [85]. Up to 600 males were found in the tube of a single female (Fig. 4.4) [52, 84, 85, 116]. Fecundation is probably internal in *Osedax* females because spawned eggs are fertilised. However, it is not known how this process is happening [85].

The selection for fertility and limited surface available for settlement favoured rapidly growing females that could exploit ephemeral limited habitat, and simultaneously small, non-feeding and mobile male that breed very early.

4.2.2 *Osedax* species diversity and succession at whale falls

Osedax was first discovered at a deep sea whale fall in Monterey Bay in 2004 (Figs. 2.2 1.1, table 1.2) [84]. Six years later, fifteen *Osedax* species are known along the Californian coast alone [12, 52, 84, 86, 117] showing remarkable diversity in size, colours, and shape (Fig. 4.5) [117]. *Osedax* female size varies between species from about 1 cm to several centimetres for the bigger species (Fig. 4.5) [36, 40, 52, 84, 86, 117]. Within the whale fall habitat, *Osedax* occur on a variety of substrates including on whale blubber, in the sediment and on mammal bones deployed close to a whale skeleton (Fig. 4.5 g) [12, 36, 38, 52, 117]. The phylogeny of all known species show that *Osedax* clusters in five lineages sharing common morphological features but not grouping according to depth (Fig. 4.6) [117].

Depth, temperature and other environmental conditions probably play a role in *Osedax* distribution [12]. Some *Osedax* species might be restricted to certain depths. Thus, *O.*

mucofloris, O. japonicus, *Osedax* sp. 'yellow-patch' and *Osedax* sp. 'orange-collar' are found at rather shallow depths between 30 m - 600 m depth, and other species are found deeper and in a broader depth range 1820 - 2893 m for *O. rubiplumus* and 633 - 1820 m *O. roseus* [36, 40, 52, 86, 117]. *O. mucofloris* is the shallowest known species found at only 30 m [40] (Fig. 1.1).

In addition, *Osedax* species seem to follow a temporal succession pattern: *O. rubiplumus* might be an early coloniser followed by *O. frankpressi*. *Osedax* sp. 'spiral' is hypothesised to grow on degraded bone fragments buried in the sediment and might be a late coloniser (Fig. 4.5 g) [12, 52].

4.2.3 Other siboglinids: hosts, phylogeny, habitats

This section briefly introduces the Siboglinidae family, since ecology and evolution of the siboglinids are the topic of a review in Manuscript I.

Siboglinidae are composed of four major taxa, Vestimentifera, Frenulata, Monolifera (*Sclerolinum*) and *Osedax* [50, 81, 84]. Siboglinid classification and taxonomy has been controversial since their discovery [81]. Meanwhile they are confirmed by several morphological and molecular studies to be an annelid family (see Manuscript I) [81, 106, 123].

All siboglinid species share some common features. They lack a gut, mouth and anus; and are associated with endosymbionts which provide their nutrition [14, 45, 64, 84]. The symbionts are housed in a specialised organ called the trophosome [14]. In the trophosome the symbionts are hosted intracellularly in specialised cells called bacteriocytes [14, 44, 64, 77,81]. The phylogeny and roles of siboglinid symbionts are further described in Manuscript I. The morphology and cellular origin of the trophosome differs between siboglinids. In vestimentiferans, the trophosome makes a large proportion of the volume of the animal trunk, it is packed with symbionts [14]. It originates from the mesodermal tissue [77]. In contrast, monoliferans and frenulates trophosomes are smaller with patchy symbiont populations [62, 64]. In frenulates and monoliferans the trophosome probably originates from endodermal gut tissue [29]. Finally, in *Osedax* the trophosome is a completely different structure, it is the root branching tissue that invades the bone containing the worm ovisacs and the bacteriocytes (see 4.2) [29, 44, 84]. *Osedax* endosymbiont numbers vary greatly between species [42].

The four siboglinids groups are found in different habitats. *Osedax* and vestimentiferan are mostly found in patchy ephemerals habitat, cold seeps, hydrothermal vents and whale falls [12, 36, 40, 45, 50, 52, 81, 84, 92]. In contrast, Sclerolinum and frenulates are more

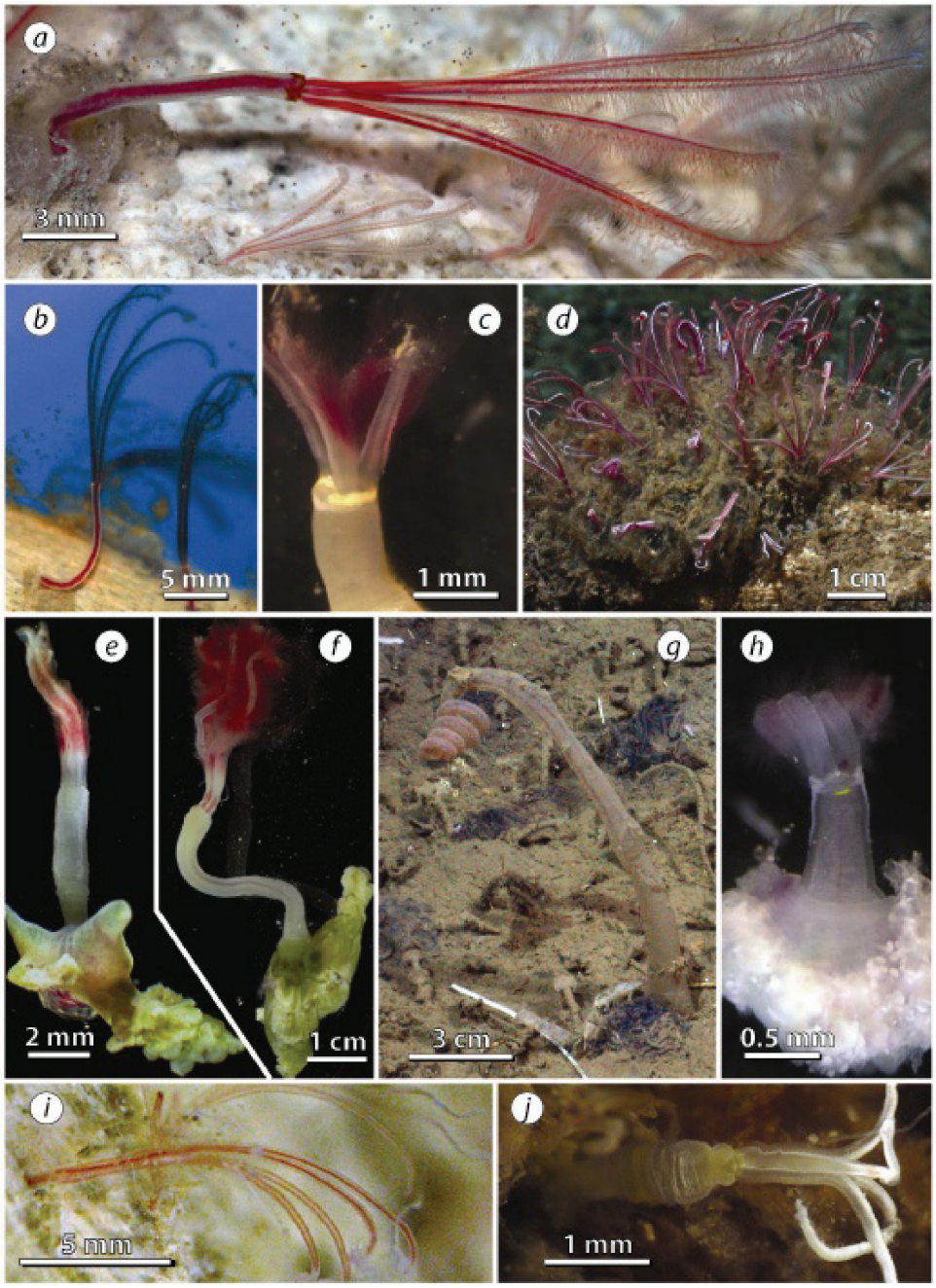


Figure 4.5: Morphological diversity among *Osedax* lineages from Monterey Bay. From [117]. (a) *Osedax* sp. orange collar from a whale at 633 m; (b) *Osedax* sp. yellow-collar from a whale at 385 m; (c) *Osedax* sp. white-collar from a whale at 1018 m; (d) *O. frankpressi* from a whale at 2893 m; (e) *O. roseus* from a whale at 1018 m; (f) *O. rubiplumus* from a whale at 2893 m; (g) *Osedax* sp. spiral from a whale at 2893 m; (h) *Osedax* sp. yellow-patch from a whale at 1018 m; (i) *Osedax* sp. nude-palp C from a whale at 1018 m; and (j) *Osedax* sp. nude-palp D from a whale at 1820 m. Approximate scale bars are provided in each panel.

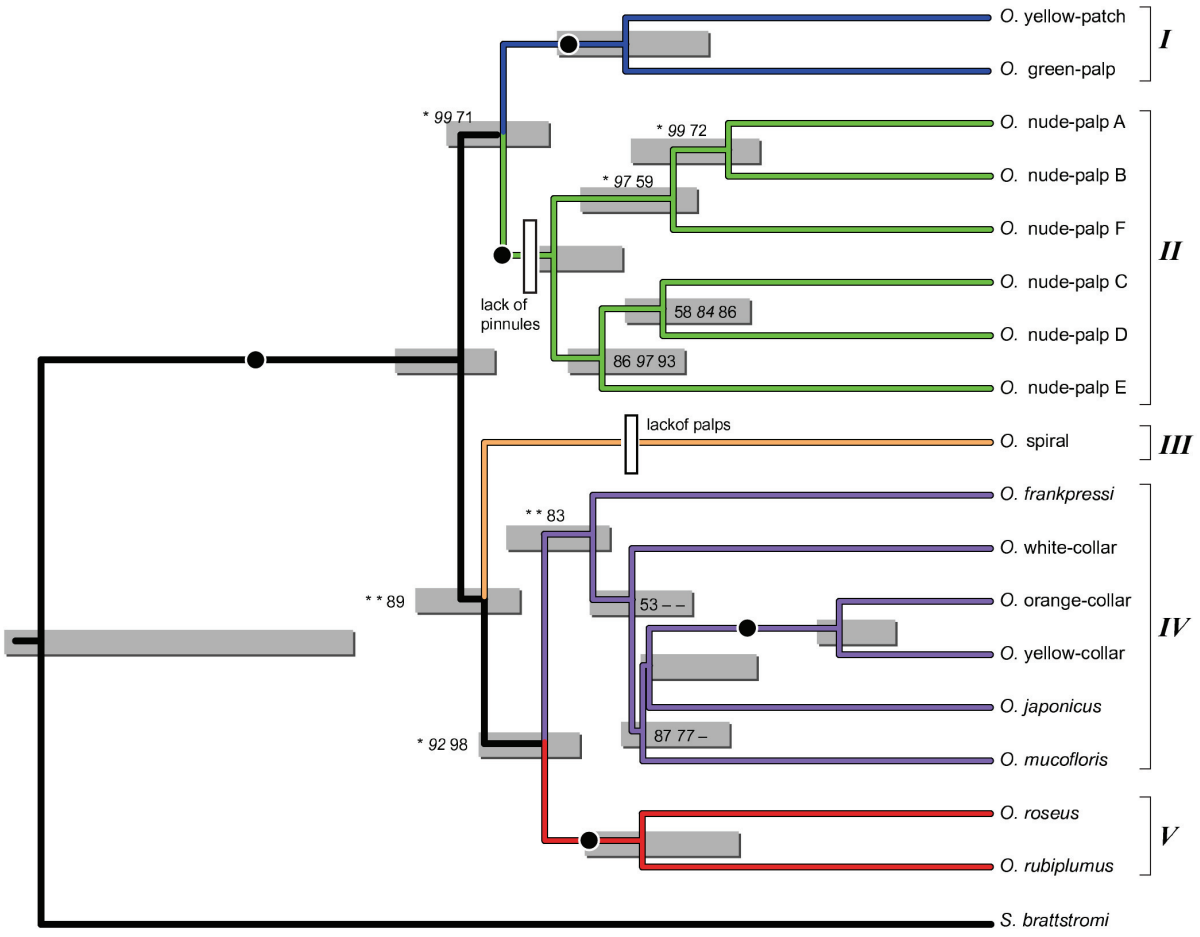


Figure 4.6: Phylogenetic relationships among *Osedax* species based on concatenated sequences from two protein-coding genes (COI and H3) and three ribosomal RNA genes (16S, 18S, and 28S). From [117]. Roman numerals at the right-hand margin delineate five *Osedax* species-groups. Three methods were used to denote the support for internal nodes: bayesian posterior probabilities (BPP), maximum parsimony (MP) jackknife, and RAxML bootstrap values. If all three methods produced values $\geq 95\%$, the node is marked with a large black dot. Where support values differ, the BPP, RAxML (italics) and MP values are shown in order, and asterisks (*) equal 100%. Nodes that were not recovered with RAxML or MP analyses are indicated by a dash. Support values ≤ 50 are not shown. Based on most parsimonious reconstructions, the white rectangles mark the loss of palps in *Osedax* sp. spiral and the loss of pinnules for the nude-palp species group.

Figure 4.7: *Raricirrus beryli* (Picture courtesy of Adrian Glover and Helena Wiklund).

ubiquitous, they occur at diverse reduced environments [50, 81, 92] (Fig. 4.1). For more details on the habitat of siboglinids refer to Manuscript I.

4.3 *Raricirrus* (Polychaeta: Ctenodrilidae): a reduced habitat specialist

Raricirrus beryli (Fig. 4.7) is a polychaete from the family Ctenodrilidae, a sister family to the Cirratulidae [80, 123]. This section presents what is known on *R. beryli* habitat, life style and dispersal capacities.

4.3.1 *Raricirrus* diversity and habitats

The described species of *Raricirrus* are specialists of organic rich environments: *Raricirrus maculata* habitats are described as fine sediment with organic enrichment [80] and a submarine waste discharge with hydrocarbon pollution (D. Montagne Personal Communication in [73]). *Raricirrus variabilis* is reported from a deep (4 000 m) wood fall in the Tongue of the Ocean, Virgin Islands [22].

4.3.2 *Raricirrus beryli* habitats

R. beryli habitats are sulphide rich habitats: oil fields, sulphide rich sediments and whalebones [73, 80], which are probably widespread in the North Sea (Fig. 4.8). *R. beryli* was first described in the 1980s, it was found in hydrocarbon polluted sediments under oil

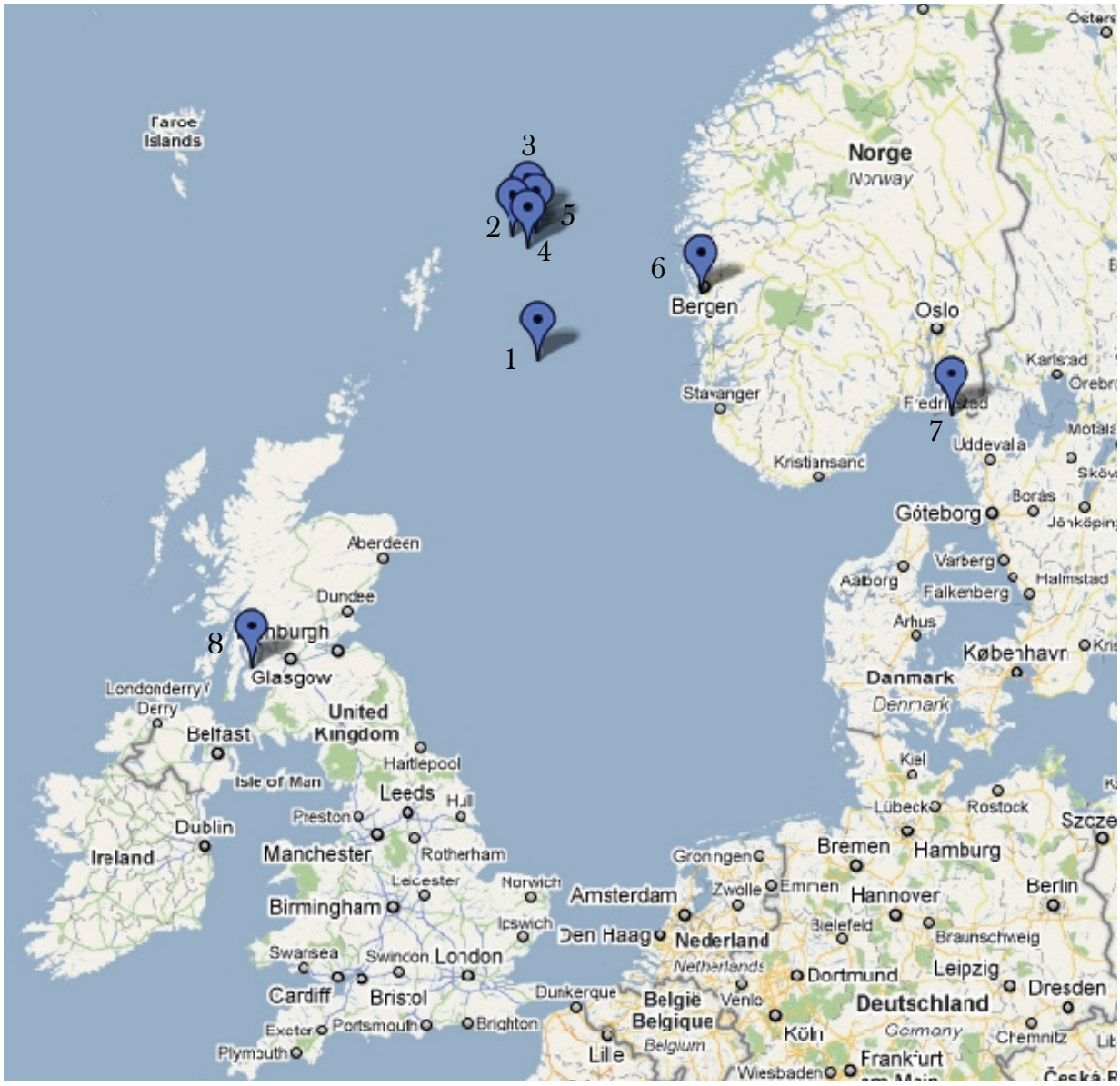


Figure 4.8: North Sea map with sampling sites. Locations of the whale falls studied in this thesis, and of *R. beryli* habitats: 1 - 5 = oil field (1 = Beryl, 2 = Cormorant, 3 = Thistle, 4 =Brent and 5 = Statfjord), 6 = off Bergen site with brown black mud, 7 = minke and pilot whale falls in the Kosterfjord, 8 = Garroch Head, sludge disposal with persistent oil. Map realised with Google Maps.

drilling stations in the North Sea (Fig. 4.8) [80]. In this thesis we report that *R. beryli* also occur at a shallow minke whale fall in the North Atlantic in freshly sampled whale bones, in whale bones kept in aquaria at Tjärnö laboratories and the bottom of the aquaria (Fig. 4.8) [20].

R. beryli was collected mainly on oil fields in the North Sea (Fig. 4.8), mainly east and south from Beryl platform (59°32.59'N, 01°32.26'E) at 100 - 115 m depth, from Brent Oil field about 140 m, Statfjord Oil field 145 m and Thistle Oilfield 160 m. In addition, the worms were found on brown black mud with high hydrogen sulphide off Bergen, in Norway at 58 m (60°19.02'N, 05°15.73'E) [80]. They were also found at Garroch Head, Clyde Sea off Scotland at 80 m, at a sludge disposal site with persistent oil [DAFS unpublished data in [73, 80]]. At the oil field stations, *R. beryli* were mainly present in the upper two centimetres of the sediment and could be found up to 4-6 cm deep [73]. Analysis of the gut content showed mainly sand particles. *R. beryli* is classified as a sub-surface, motile feeder [73].

R. beryli seems very tolerant of hydrocarbon pollution, since at one site, the abundance of *R. beryli* was positively correlated with the total oil in the sediment and with naphthalen or anthracene/phenanthrene, which are considered toxic components of aromatic hydrocarbons. A study by Vovelle et al (1994) showed that different compounds (mainly iron, and also sulphur, calcium, phosphorus and aluminium) were accumulated in the heart body of *R. beryli* (a gland like structure found in some polycheates) [114]. They propose that this is part of a detoxifying process because iron and sulphur accumulation in the heart body is higher in sites closer to hydrocarbon pollution [114].

Thus, *R. beryli* is considered by Moore 1991 an opportunistic species that occupies a discrete niche characterised by the presence of hydrocarbons [73]. However, its presence at whale falls give new insight in its habitat and is challenging the hydrocarbon niche hypothesis.

4.3.3 *R. beryli* life style and dispersal

R. beryli has sexual and asexual reproductions as reported by Petersen & George (1991). Asexual reproduction is not uncommon in annelids [88]. In the population analysed, four different adult forms were described by Petersen & George [80] and were found in different proportion in a population of the Beryl station [73]:

1. 'Normal' adult forms without eggs, or starting to have eggs, individuals showing no regeneration, 77% of the population

2. Epitoke, which is the sexually mature adult, spawning type, very few were found
3. Adult asexually regenerated, having regenerated the anterior or posterior segments, or both, 11% of the population
4. Dispersal form, smaller version of the epitoke, without gametes, and supposed to have good swimming capacities, 11% of the population. Most specimens classified as dispersal form have regenerated from one or few original fragments.

Thus, *R. beryli* by colonising a site with just few individuals is thought to rapidly increase population size by asexual reproduction [80]. This is suggested by the high population size found at different oil spill sites, where up to 20 000 individuals $\cdot$ m^{-2} where reported [73]. At different oil field stations, it is the dominant or one of the 10 dominants species [73]. The estimation of the population size at the studied whale fall is difficult because the worms are hidden in the bones, and not visible with the camera in situ. In one of the bone pieces, 5 individuals were extracted from a few cubic centimetres of bones suggesting that they are quite abundant. It is not known whether the larvae enter the plankton or not [73,80], larvae could maintain a population in the nearby area such within one whale fall or among close whale falls [80]. Dispersal between distant suitable habitats could be assured by the swimming adults only, or by both swimming adults and larvae [80].

Chapter 5

Whale falls compared to vents, seeps and wood falls

In the previous chapter, symbioses at whale falls were described, mentioning that symbiotic species occurred in other chemosynthetic habitats. It suggests that the sulphophilic stage is sharing many characteristics with other chemosynthetic habitats. Thus, in this chapter, the whale fall sulphophilic stage is compared to other chemosynthetic habitats such as hydrothermal vents, cold seeps, and wood falls for biogeochemistry, species overlap and biodiversity. Species could therefore use whale falls for dispersal among chemosynthetic habitats: dispersal stepping stone hypothesis. Furthermore, we present the evolutionary stepping stone hypothesis [101], which bases itself on these similarities to propose that

through time, shallow water species evolved out of their shallow water habitats to become deep sea specialist of chemosynthetic habitats by colonising shallow chemosynthetic habitats first and then jumping step by step to deep sea chemosynthetic habitats.

5.1 Comparison of biogeochemistry

In terms of biogeochemistry, sulphate reduction rates and sulphide concentration at whale falls are similar to some cold-seep environments, suggesting that whale falls provide a similar sulphophilic habitat [110]. However, whale falls represent ephemeral hard substrate habitats which are more similar in terms of habitat and persistence to some fast evolving hydrothermal vents [110]. Therefore, shared species between whale falls and hydrothermal vents or cold seeps can be expected.

5.2 Species overlap between chemosynthetic habitats

Smith (2003) proposed that a subset of vent and seeps species are likely to be found on whale falls and may use whale falls as dispersal stepping stones, i.e. using whale falls for geographical dispersal [99].

Smith (2007) reported that eleven whale fall species also occurred at hydrothermal vents, twenty species at cold seeps, and seven species at wood falls [97]. Other studies report as well species overlap among chemosynthetic habitats [7, 65, 97–99]. For example, Baco et al., (1999) studied the relationship between vesicomyid clams at whale falls, cold seeps and hydrothermal vents [7]. The authors show that one species was shared with vents (*Vesicomya gigas*) and one species shared with seeps (*Calyptogena kilmeri*). One potentially new species, clustering with the *'gigas/kilmeri'* species group was only found at a whale fall. To explain the overlap of fauna between those chemosynthetic habitats they proposed two hypotheses: i) clams have a broad tolerance range and can colonise several chemosynthetic habitats; ii) Whale fall habitat conditions overlap with seeps and vents conditions, enabling some vent and seep species to colonise whale falls as well [7].

This raised the question whether whale falls function as a sink habitat for the shared species, not enabling them to further disperse and colonise new sites; or whether whale falls constitute an important habitat for the shared species, enabling them to disperse and further colonise new sites [7, 101, 112]. The presence of a large (e.g. reproductively viable) population of vesicomyid clams at whale falls from the Northeast Pacific suggests that for at least some vesicomyid species, whale falls are important habitats contributing to the

dispersal of those species [7, 9, 99].

5.3 Comparison of species richness

Although whale fall have only recently been discovered, they have a high global species richness with 407 species reported in 2003. In comparison, cold seeps have an estimated species richness of $\sim$ 230 species, and hydrothermal vents 469 species [94, 97, 100, 112]. Since 2003, several whale fall communities have been discovered that share almost no species with previously described communities and that is why species diversity is likely to be underestimated [12, 20, 38, 52, 117].

5.4 Stepping stone hypothesis?

5.4.1 Evolutionary stepping stone hypothesis

Since chemosynthetic habitats share genera and even species, one of the questions is where did those organisms evolve first and in which of those habitats did they specialise first. After their discovery, whale fall communities were proposed to serve as evolutionary stepping stones for shallow water species to colonise deep sea seep and vent habitats [101], although this was controversial [112]. This hypothesis implies that:

1. Some shallow water species colonised shallow water whale falls and/or wood falls from adjacent habitat.
2. These species became adapted to these chemosynthetic habitats by gaining chemosynthetic symbionts.
3. Then, they could colonise deeper wood and whale falls.
4. Finally, some were able to colonise vents and seeps, and are potentially nowadays only found at these habitats.

In order to know whether this hypothesis is true for some taxa one needs to consider the ecology, phylogeny and fossil records for these taxa.

Since vesicomyid clams, bathymodiolin mussels and siboglinid worms occur in shallow and deep water, on wood and/or whale falls, as well as at cold seeps and hydrothermal vents, they represent possible species to test for the stepping stone hypothesis [7, 8, 101, 113]. For vesicomyids, molecular and fossil data do not support the hypothesis, because

vesicomyids were first present in seep fossils [4, 7, 8, 57, 58, 113]. For siboglinids, fossil evidence exists but scientists do not agree wether the found fossils are vestimentiferan tube fossils or fossils from other tube making invertebrates [63, 106, 113, 123].

Thus, after describing the actual knowledge on fossils chemosynthetic communities, bathymodiolin mussels will be used as an example of a family that could have used wood and whale falls as evolutionary stepping stones.

5.4.2 Fossil data

In the following, different findings important for the evaluation of how a species could have become a whale fall specialist and/or followed the evolutionary stepping stone hypothesis are summarised. A geological scale with events relevant for the stepping stone hypothesis are presented in Fig. 5.1.

- Seep and vent habitats are older than wood apparition on earth with trees, which is again older than whale apparition in ocean [55, 58, 59, 63]. This means that vents, seeps and wood falls could have been colonised by some species before there were whales, and even whale falls.

- Modern whales (basilosaurids) evolved about 40 My ago. A major radiation event occurred about 30 My ago in the family, and bigger whales appeared only about 11 My ago [59, 63, 103]. Some scientists thought that no chemosynthetic communities could have been supported before the evolution of bigger whales 11 My ago, because a minimum whale size was necessary [103]. However, the oldest known fossils of chemosynthetic whale fall communities are from 35 My ago, including a 3 m long fossil whale fall [3,4,41,57,82,93,103]. Therefore, lipid content in the bones rather then the skeleton size might have been a key factor for the establishment of chemosynthetic communities [82].

  To summarise, whale fall specialists could not have evolved before modern whale evolution 40 My ago, which is in theory a sufficient time for speciation to occur. Thus bathymodiolin mussels or *Osedax* might have evolved and specialised on whale falls in the last 40 My [32, 117] (Fig. 5.1). *Osedax* are soft body animals that do not leave fossils. Nevertheless, scientists have proposed to look in the future for boring in fossil whale bones and to compare them to *Osedax* boring at modern whales [117] (C. Little and N. Higgs, personal communication). Fossil data for bathymodiolin mussels are described below and indicated on Fig. 5.1. If fossil evidence and/or molecular

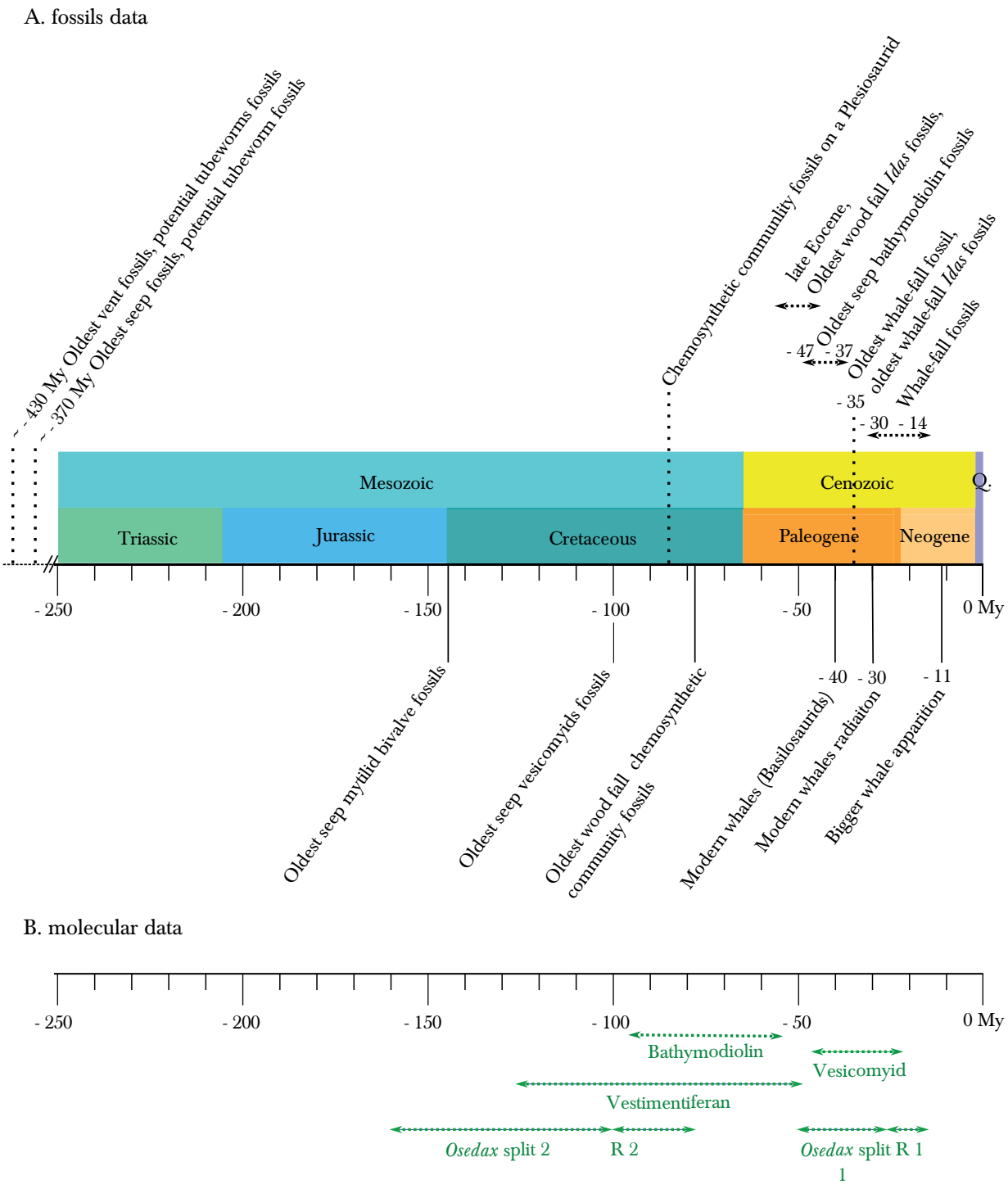


Figure 5.1: Geological scale with fossil and molecular data mapped for the evolutionary stepping stone hypothesis. Scheme by C. Verna based on data from the following references [3–5, 32, 41, 54, 55, 57–59, 63, 82, 103, 117]. A. fossils data. Q = quaternary. B. Estimated time of evolution for the different taxa based on molecular data. For *Osedax* two scenarios (1 & 2) from Vrijenhoek et al., (2009) [117] are shown with *Osedax* split from vestimentiferan and *Osedax* radiation (R). The two scenarios are based on different evolution rates for the analysed genes [117].

data show evidence that these species are older than whale evolution in oceans they would have evolved and specialised in another habitat than whale falls.

- Before the whales, other big animals populated the ocean, which could have also supported chemosynthetic communities. A plesiosaurid skeleton was found in association with provannid snail fossils. As provannids are also known at hydrothermal vents, this fossil suggested the presence of a chemosynthetic community on the plesiosaurid skeleton [54]. This suggests that before whales evolved, chemosynthetic communities could have been associated with other huge animal skeletons [54]. Thus, some species could have specialised on chemosynthetic plesiosaurid falls and survived on other habitats; and then colonised whale falls. However, there is currently not enough information on how similar plesiosaurid fall communities were similar to whale fall communities [54] (Fig. 5.1).

- Finally, a recent study showed that depth might be the most important factor behind whale fall specialist distribution based on an analysis of the abundance of molluscs at the family level on fossils and modern chemosynthetic communities [28]. Thus, depth could be the key factor determining the distribution of each species and not the habitat.

5.4.3 Bathymodiolin example

As mentioned before, bathymodiolin mussels are a good system to test for the evolutionary stepping stone hypothesis, because molecular and fossil data are available. This section discusses whether these mussels could have evolved from shallow water mussels and then colonised deep sea chemosynthetic habitats later.

Molecular data

A first study based on two mitochondrial genes showed that most primitive mussels were wood or whale falls specialists, and most derived mussels seeps or vents specialists [8]. Further studies on more taxa, including several organic fall specialists (such as *Idas*) and more genes confirm this trend [26, 31, 32, 53, 65, 90]. In addition, some primitive mussels species occur at both wood and whale falls, which suggests that at least some species can jump from one habitat to the next, strengthening the hypothesis [65] (Fig. 5.1).

Fossil data

The earliest fossils of bathymodiolin mussels are from the late Eocene mapped on Fig. 5.1 At seeps, fossils of *Bathymodiolus willapaensis* date from the late Eocene (in the range 47 My to 37 My ago) [55, 59], at wood falls first *Idas* fossils date from the late Eocene [4, 57, 58] and at whale falls the first *Idas* fossils date from 35 My ago, which is also the earliest record of a fossil whale fall community [4, 41, 57, 93, 103]. Based on these fossils it is difficult to answer the question if bathymodiolin mussels first colonised whale and wood falls and then seep and vent habitats because they are all from the same period. More data is needed to draw conclusions. However, it seems unlikely that bathymodiolin mussels evolved first on whales from shallow water mussels because present fossils data indicates that bathymodiolin mussels are found at wood falls and seeps before whale falls (Fig. 5.1) [2, 56, 57, 103]. Thus, a more likely explanation would be that they evolved at another chemosynthetic habitat such as seeps or wood falls and later colonised whale falls [2, 56–58] (Fig. 5.1).

Chapter 6

Biogeography of whale fall communities

6.1 Context

Whale falls are ephemeral islands at the bottom of the ocean. This raises the question of how whale fall species can find a whale carcass to colonise. Smith (2003) proposed that the reproductive and dispersal capacities of the whale fall fauna could be similar to vent and seeps fauna which also inhabit a patchy ephemeral habitat [99]. In oceans, different barriers exist that limit the contact between populations such as: frequency of the habitat, depth at which a species can be found (including pressure and temperature restrictions), physical barriers such as ridges, and currents [113]. The capacity to colonise a new whale fall depends on the whale frequency and dispersal capacities of each species [113]. In the following, after a presentation of past and modern whale fall frequency, an example of dispersal capacities for a whale fall species, *Osedax* is described.

6.2 Abundance of whale falls

6.2.1 Whale fall frequency

Whale fall frequency depends on the number of living whales (based on estimations), the mortality rates of each species, and the number of dead whales falling to the sea floor [97,101]. For the grey whale *Eschrichtius robustus*, it is estimated that 9 km separates two whale falls in the sulphophilic stage [99, 101]. Global calculation for nine large whale species estimates that 12 to 30 km separates two whale falls in the enrichment opportunist or the sulphophilic stages [99]. These estimates are assuming a uniform distribution over the whole ocean surface. Most probably, whale falls are not randomly distributed but found more often along migration routes and feeding grounds near ocean margins [16,101]. Along these axes, whale falls are therefore probably more frequent than in the middle of oceanic basins [16, 101].

6.2.2 Influence of whaling

Between 1920 and 1980 intensive whaling occurred on large whales with about 2 million great whales harvested in the ocean [97]. Jelmert and Oppen-Bernsten (1996) calculate that prior to whaling, 39 000 whales sank per year, making whale falls six times more abundant than nowadays [51, 99]. Whaling may have impacted deep sea diversity by reducing the number of dead whales sinking to the bottom, and ultimately leading to the extinction of whale fall specialists. A reduction in whale fall frequency may also have affected the population of species from diverse chemosynthetic habitats (vents and seeps) that also occur at whale falls [16, 17, 51, 97, 99, 112]. Intensified whaling varied between whale species and oceanic basins which leads to differences in the estimated impact on whale fall communities [97]. Smith (2007) estimates that habitat reduction and associated species extinctions might be greatest in the North Atlantic (with low whale populations for the last 150 years), substantial and accelerating in the Southern Ocean and least intense in the Northeast Pacific [97].

In addition, because of a change in hunting practices whaling probably had two other impacts: first, until 1910, it increased the number of whale skeletons on the bottom because the skeletons of hunted whales was left to sink. Second, intensive whaling highly decreased the flux of carcasses because whale populations were strongly reduced [16, 97]. Because the sulphophilic stage lasts longer (up to 50 years) a lag could exist between the reduction of whale populations and reduction of whale falls in the sulphophilic stage. Thus whale

fall communities are probably suffering only now from significant habitat loss, and from potential species extinction [97].

6.3 Dispersal capacities of whale fall fauna

For many marine animals without high mobile capacities, dispersal occurs through a planktonic larval stage. This enables gene flow across thousands of kilometres for some species (e.g. bathymodiolin mussels, clams, limpets, vestimentiferan and other polychaete) [113]. *Osedax* species are a good example of a whale falls species and their dispersal capacities will be described in the following part.

***Osedax* example** *Osedax* have been found in most oceanic basins and are distributed world wide (Fig. 1.1) [36, 40, 84, 117].

Osedax are sedentary and only their larvae can disperse and colonise a new whale fall [84]. Their capacity to colonise a new whale fall will depend on the number of eggs produced by a female and the larval lifespan. *Osedax* species are able to colonise available bone within 1 to 3 months [12, 40, 86] and within 3 months some mature female spawn fertilised eggs [85, 86, 116]. *Osedax* larvae are lecithotrophic (they do not feed but rely only on their yolk reserves) they can swim after 2 days, and settle after 10 to 16 days [85]. Bigger eggs with more reserves can potentially sustain larvae for a longer time, letting them disperse on a larger scale [85, 113].

Osedax sp. 'orange collar' spawns buoyant eggs (at atmospheric pressure) [116]. It seems that deeper *Osedax* species have bigger oocytes (*O. rubiplumus* (151×121 μm) and *O. frankpressi* (146×117 μm) [84]), than shallower species (*O. mucofloris* (85×90 μm) [40], *O. japonicus* (100 μm) [36], *Osedax* sp. 'orange collar' (96×63 μm), *Osedax* sp.'yellow collar' (92×72 μm) and *Osedax* 'nude palps' (84×81 μm) [85]). Size of eggs is not strictly correlated to depth as species found at the same whale fall have different egg sizes [85]. Deeper dwelling species live in colder water (3-4℃) than shallower species (5-7 ℃). This may also be a factor in larval development speed and lifespan [20, 38, 85], because in colder temperatures development is slower [85]. If larval lifespan correlates with egg size because they depend on yolk reserves, then *O. rubiplumus* with an egg volume 5 times bigger than species with small eggs, would have greater dispersal potential [85]. This is confirmed since *O. rubiplumus* was described in the East Pacific, off California, and is now also reported in the West Pacific, off Japan [85].

This suggests that at least some *Osedax* species have high dispersal capacities and are

able to colonise whale falls as far away as Northeast and Northwest Pacific. It is possible that species with smaller dispersal capacities occur within a more limited area. In fact, *O. mucofloris* and *O. japonicus* have not been reported away from their first description site which could reflect limited dispersal capacities or limited sampling.

Both males and females are recruited from a common larval pool, and are a product of random sexual mating [116]. The effective numbers of females that contribute to a given population has been calculated for several species and are large, ranging from $0,9 \cdot 10^6$ to $2,2 \cdot 10^6$ [40, 84, 86, 116]. This suggests that within an area such as the Monterey Bay Canyon the populations of several whale falls are connected by high gene flow [116].

Chapter 7

Aims

7.1 Siboglinid symbiosis, a bigger picture

In the context of this thesis, I participated in a review on ecology and evolution of siboglinid tubeworms. Siboglinid tubeworms are associated with symbiotic bacteria, and this symbiotic association shaped their evolution and strongly influenced their ecology. Four siboglinids taxa are known, Vestimentifera, Frenulata, Monolifera (*Sclerolinum*) and *Osedax*. Most of the attention went to Vestimentifera, with their star member, *Riftia patchyptila*, the giant tubeworm discovered at hydrothermal vents. Frenulata and Monolifera, smaller worms from the sediment were almost unnoticed and their symbionts have only been characterised for the 16S rRNA gene in the last five years. *Osedax*, the bone-eating worm, was the most surprising organism recently discovered at whale falls. The recent focus on several species of siboglinids has greatly extended our knowledge of this annelid family in the last years, making an integration in a review necessary. The aim of this review (Manuscript I) is to gain a better insight into the ecology and evolution of siboglinids by comparing symbiosis and habitat use among siboglinids.

7.2 Whale falls symbiosis

As described, at whale falls diverse invertebrates are involved in a symbiosis with several metabolic capacities, heterotrophic or chemoautotrophic. In addition, their symbiotic bacteria belong to diverse lineages, mostly within the Gammaproteobacteria. The high availability of various energy sources for microbes suggests that more symbioses remain to be discovered at whale falls. The deployment of two whale carcasses at shallow depths in the North Atlantic, close to the Swedish coast, allowed us a regular access for sampling whale fall fauna. This thesis focuses on the characterisation of the symbiotic communities of two whale falls polychaetes, *Osedax mucofloris* and *Raricirrus beryli*. *Osedax mucofloris* is found at both deployed whale falls, and *Raricirrus beryli* was found so far at only one (its presence is not yet investigated at the other). Thus, both worms co-occur at one of deployed whale falls and can survive in aquaria where whale bones were kept, making them good candidates for this investigation.

7.2.1 *O. mucofloris* symbiosis

Although many *Osedax* species are known, the symbionts of only five species have been characterised: *O. frankpressi, O. rubiplumus, O. roseus* and *Osedax* sp. 'yellow collar' from the East Pacific, and *O. japonicus* from the West Pacific [36, 42, 44]. These studies showed that all 5 species have endosymbionts that belong to the Oceanospirillales in the Gammaproteobacteria. In contrast to the well studied vestimentiferan symbiosis, where most endosymbionts belong to a limited number of bacterial lineages [25, 70], symbionts described for *Osedax* are highly variable within the Oceanospirillales [42]. However, this diversity has only been described using comparative 16S rRNA sequence analysis and the location and distribution of the symbionts in the root tissue was not confirmed with fluorescence in situ hybridisation (FISH) or equivalent techniques [42].

The discovery of *O. mucofloris*, at the deployed whales, allowed us to describe its symbiont community and compare it to the symbionts of the previously studied *Osedax* species from the Pacific. Furthermore, the objective was to investigate the diversity of the symbionts in more detail and assess their distribution in the host tissues. In addition to the classical comparative 16S rRNA sequence analysis we therefore designed probes for several of the symbiont lineages and did extensive fluorescence in situ analysis. Out of 28 individuals in total, twenty worms were used for clone libraries and 12 worms for FISH including serial sectioning of the whole root of three worms (Manuscript II).

7.2.2 A specific microbial fauna associated with *R. beryli*?

Microbial symbioses have evolved independently in diverse annelid families [14], and are common in Polychaeta and Oligochaeta from chemosynthetic habitats, such as the Siboglinidae, the Clitellata *Olavius* and *Inanidrilus*, and the Alvinellidae *Alvinella* [14]. Since *R. beryli* is found in various chemosynthetic habitats, and because preliminary investigations showed its surface was covered by microorganisms, it appeared to be a good candidate to look for the presence of symbionts. In this thesis, the diversity and phylogeny of the bacteria associated with several *R. beryli* individuals is therefore studied (Manuscript III).

7.2.3 Comparison of *O. mucofloris* and *R. beryli* epibiotic bacteria

Beside the resident endosymbionts, some *Osedax* species have been shown to be associated with epibacteria [36, 42]. This holds true for *O. mucofloris*, whose various body parts are associated with numerous bacteria. Since both *O. mucofloris* and *R. beryli* are associated with epibacteria, The question of how similar those epibiotic communities are will therefore be discussed. Furthermore, recent studies of free-living bacterial diversity at whale falls enables us to compare the epibionts diversity to the diversity of free-living bacteria (Chapter 8).

Part II

List of Publications

Contribution to the manuscripts presented in this thesis

1. **Ana Hilário, María Capa, Thomas G. Dahlgren, Kenneth M. Halanych, Crispin T.S. Little, Daniel J. Thornhill, Caroline Verna and Adrian G. Glover**. New perspectives on the ecology and evolution of siboglinid tubeworms.
   Review submitted to *PLoS ONE*.
   C.V., D.J.T and K.M.H : concept on siboglinids symbioses C.V.: graphics (Figure 6)

2. **Caroline Verna, Alban Ramette, Helena Wiklund, Thomas G. Dahlgren, Adrian G. Glover, Francoise Gaill and Nicole Dubilier**. (2010). High symbiont diversity in the bone-eating worm *Osedax mucofloris* from shallow whale falls in the North Atlantic.
   Environmental Microbiology 12(8): 2355-2370.
   C.V.: developed the concept, did the 16S rRNA sequencing and phylogeny, performed the FISH, conceived and wrote the manuscript C.V.,H.W.,T.G.G., A.G.G : provided samples, and did the COI sequencing A.R.: helped with the statistical analysis A.G.G: did the SEM microscopy F.G. edited the manuscript N.D. developped the concept with C.V, conceived and edited the manuscript

3. **Caroline Verna, Jillian M. Petersen, Thomas G. Dahlgren, Dennis Fink and Nicole Dubilier**. Extended host range of symbiotic bacteria previously found in bathymodiolin mussels.
   Manuscript in preparation.
   C.V.: developed the concept, did the 16S and COI sequencing, performed the FISH, conceived and wrote the manuscript. T.G.G: provided samples D.F: helped with the confocal microscopy J.M.P.-N.D.: developed the concept with C.V. edited the manuscript

4. **Caroline Verna, John Taylor, Alban Ramette and Nicole Dubilier**. Lucinid symbiont diversity: determining the influence of host selection, Geography, habitat, and depth
   Manuscript in preparation. This Manuscript is included in the Appendix
   C.V.: develop the concept, did the 16S sequencing, conceived and wrote the manuscript. A.R. will be involved in statistical analysis J.T.provided the samples J.T.-N.D: develop the concept with C.V.

Part III

Manuscripts

Manuscript I

New perspectives on the ecology and evolution of siboglinid tubeworms

Ana Hilário, María Capa, Thomas G. Dahlgren, Kenneth M. Halanych, Crispin T.S. Little, Daniel J. Thornhill, Caroline Verna and Adrian G. Glover

Review submitted to **PLoS ONE**

New perspectives on the ecology and evolution of siboglinid tubeworms

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Abstract

Siboglinids are tube-dweling annelids that are important members of deep-sea chemosynthetic communities, which include hydrothermal vents, cold seeps, whale falls and reduced sediments. As adults, they lack a functional digestive system and rely on microbial endosymbionts for their energetic needs. Recent years have seen a revolution in our understanding of these fascinating worms. Molecular systematic methods now place these animals, formerly known as the phyla Pogonophora and Vestimentifera, within the polychaete clade Siboglinidae. Furthermore, an entirely new radiation of siboglinids, *Osedax*, has just recently been discovered living on whale bones. The unique and intricate evolutionary association of siboglinids with both geology, in the formation of spreading centres and seeps, and biology with the evolution of large whales, offers opportunities for studies of vicariant evolution and the calibration of molecular clocks. Moreover, new advances in our knowledge of siboglinid anatomy coupled with molecular characterization of microbial symbiont communities are revolutionizing our knowledge of host-symbiont relationships in the Metazoa. Despite these advances, considerable debate persists concerning the evolutionary history of siboglinids. Here we review the morphological, molecular, ecological and fossil data in order to address when and how siboglinids evolved. We discuss the role of ecological conditions in the evolution of siboglinids and present possible scenarios of the evolutionary origin of the symbiotic relationships between siboglinids and their endosymbiotic bacteria.

Keywords: Pogonophora; Vestimentifera; Frenulata; Monolifera; Siboglinidae; *Riftia*; *Osedax*; hydrothermal vent; hydrocarbon seep; whale fall; deep-sea

INTRODUCTION

Deep-sea worms in the polychaete family Siboglinidae are not yet thought to be of any commercial or medical value to humans. Nevertheless, in 50 years of research, 27 publications have appeared in the top-cited science journals *Nature* and *Science* that deal exclusively with species in this group and these papers have been cited a total of 1621 times [1-27] (Figure 1). The highest-cited paper (for which metrics exist) on any siboglinid [13] has received 389 citations, 147 more than the next highest-cited paper in that same issue of Science, on the role of insulin in determining diabetes [28]. It is perhaps no surprise that of these 27 publications in *Nature* or *Science*, 13 of them focus exclusively on a single species of siboglinid worm, *Riftia pachyptila* Jones, 1980 (Figure 2a). This giant worm, discovered on hydrothermal vents at the Galapagos Rift in 1977, became the poster-child of deep-sea discovery; the 'lost world' of unknown animal lineages that the scientists on the Challenger deep-sea expedition 100 years previously had so wanted, but failed, to find. Arguably, this single species of worm launched the careers of a generation of deep-sea biologists.

Taxonomy and systematics have played a crucial, but unsung, role in the elevation of these discoveries to the international media. Early deep-sea biologists, the 'Challenger generation', were desperate to discover living fossils in the deep – trilobites crawling through abyssal muds, the lost world of the Mesozoic in the dark depths of the ocean. It was, perhaps, something of a disappointment to discover that although life was abundant and diverse in the deep sea, the majority of species were in the same families, and often congeneric with shallow-water forms. Hence the discovery of a new group of deep-sea creatures [29] and the creation of a new phylum, Pogonophora [30] grabbed media headlines in the 1950's [31], as did the discovery of a new family of Pogonophora – the Riftiidae – on hydrothermal vents in the 1970's [13]. Under much controversy [32], *Riftia pachyptila* was elevated to phylum 'status' [33] under the name Vestimentifera; its days there were numbered by new methods in cladistic analyses and the imminent arrival of molecular phylogenetics.

A series of papers through the last twenty years has supported the placement of tubeworms as a single family (Siboglinidae) within the annelid radiation, as originally postulated by Uschakov in 1933 [25,34-39], bringing the tale of Pogonophora and Vestimentifera full circle. However, the story of Siboglinidae has, in the last 5 years, received a new twist: the discovery of an entirely new species-rich clade of highly derived siboglinids, known as *Osedax*, that appear to live exclusively on mammal (typically whale) bones [40-42].

Currently most researchers recognize four main lineages within Siboglinidae: Frenulata, Vestimentifera, *Sclerolinum* and *Osedax* (Figure 3). *Sclerolinum* was originally regarded as a frenulate and later placed in its own taxon, Monilifera, equal in rank to Frenulata and Vestimentifera [43]. Recent molecular and morphological studies however, show that *Sclerolinum* is the sister clade to vestimentiferans [40,44]. Among these lineages, frenulates are by far the most diverse with 141 nominal species. By contrast, vestimentiferans have 18 species, *Sclerolinum* 6, and *Osedax* 5 (at the time of writing several new species for all groups were in the process of being described and thus the numbers are major underestimates) (Figure 4). Although biological generalizations are often problematic, each siboglinid clade is, in general, found in a certain type of habitat. Frenulates are typically found in muddy (often deep) environments, vestimentiferans in hydrothermal vent and hydrocarbon seep areas, *Sclerolinum* at sites with decaying organic matter (e.g., wood and rope) and *Osedax* at whale-falls.

With the exception of *Osedax,* the external anatomical characters are relatively constant among all siboglinids. These worms have a chitinous close-fitting tube of their own secretion that provides both protection and support (reviewed in [45]). The body can be divided into four main regions: an anterior region, a diaphragm, a trunk region and a segmented opisthosoma. In Vestimentifera, the anterior region is called the obturaculum, it functions as an operculum that closes the tube when the animal withdraws, and supports the large branchial plume. In frenulates and *Sclerolinum* the equivalent region includes a cephalic lobe and dorsal tentacles, two in *Sclerolinum* and from 1 to over 200 in frenulates. The second body region is responsible for the names Vestimentifera and Frenulata. In vestimentiferans it is called the vestimental region and is characterized by two dorsolateral folds with a ciliated field on the ventral side [46]. In frenulates and *Sclerolinum*, this region is called the forepart [47] and is characterized by the presence of a cuticular structure called the frenulum and the presence of a ventral ciliated band, respectively. Adjacent to the vestimentum/forepart is the elongated trunk region in which the gonads and the trophosome, the organ that holds the symbiotic bacteria, are enclosed. In all three groups the opisthosoma is divided by septa into coelomate segments, with regularly arranged chaeta. Most of the features shared with annelids are concentrated in the opisthosoma, including muscular septa, segmentally arranged chitinous chaetae, ganglia and blood vessels (reviewed in [45]).

In contrast to other siboglinids, the bone-eating *Osedax* species show a marked sexual dimorphism with dwarf paedomorphic males resembling other siboglinid larvae [40,48,49]. The females have a transparent mucous tube that encloses the trunk. The posterior portion of the trunk reaches into the bone and forms a complex system of “roots” that contain an ovisac covered

with tissue containing endosymbiotic bacteria. Although the microscopic males are provided with chaetae on the posterior portion of the body, the females have no opisthosome, which makes the morphological affinity with annelids more difficult to recognize.

Whilst there are many unanswered questions regarding the ecology and evolution of these strange deep-sea worms, three important facts are now accepted:

1) all adult siboglinids lack a gut, mouth, anus and conventional feeding ability,

2) all siboglinids studied thus far possess bacterial symbionts and

3) siboglinids form a well-supported monophyletic clade.

Given the conspicuous absence of a digestive system, many functional studies of siboglinids have concentrated on the question of nutrition. Early hypotheses centred on the possibility of dissolved organic matter (DOM) uptake across the body wall [50]. The twin papers of Cavanaugh et al. [13] and Felbeck [14] revolutionized this viewpoint by showing that larger siboglinids utilized symbiosis with chemoautotrophic bacteria. Although all siboglinids are assumed to house endosymbiotic bacteria for nutrition, symbionts have only been confirmed in a small minority of the 170 described siboglinid species. Furthermore, the discovery of unexpectedly different metabolic types of symbionts, with putatively heterotrophic metabolism opposed to chemoautotrophy, in the *Osedax* clade [51] and potential symbiont diversity in other gutless worms [52] has illustrated that much knowledge of the diversity and function of these relationships awaits discovery. Most of the work on endosymbiont evolution has focused on vestimentiferans [13,26,53] and considerable microbiological work has already been undertaken on *Osedax* [51,54,55]. In contrast, endosymbionts of frenulates and *Sclerolinum* have only recently been explored [56-59].

The evolutionary history of siboglinids has no doubt been a complex interaction of host and microbe evolutionary trajectories. Based on molecular genetic and morphological evidence [40,60], we may infer that over evolutionary time conventional heterotrophic polychaetes made the evolutionary leap to specialize as obligate endosymbiotic siboglinid species at chemosynthetic ecosystems. The aim of this paper is to address when and how this happened revising the available morphological, molecular, environmental and fossil data.

WHEN DID SIBOGLINIDS EVOLVE?

Clues from phylogenetic studies

The complex taxonomic story of the siboglinids has been recently well reviewed [40,61-63] and is, as Rouse [40] stated "one of the more fascinating tales in animal systematics." In the days prior to robust cladistic analysis or molecular evidence, a long scientific debate was held as to the possible origins of these enigmatic worms. Some of the early work was suggestive of a deuterostome origin (e.g., [30,64]) whilst others supported an annelid relationship (e.g., [34,65-67]. Initially, the debate centred on whether the position of the brain and nerve cord was dorsal, which is the classical deuterostome arrangement. The problem was the lack of a reference point (a gut) for determination of the dorsal or ventral position. The discovery of the opisthosome region at the posterior end of the worm, with its clear annelid-like segmentation and serially-arranged chaetae [67,68] should have been sufficient evidence to place the Pogonophora phylum, as it was then known, within the annelid radiation. However, supporters of the phylum designation maintained their stance for several more decades (e.g., [43,69]).

The incredible discoveries of the late 1970s of giant worms at hydrothermal vents pushed tubeworms, Pogonophora and the new group of Vestimentifera back onto journal covers and the popular press (Figure 1 and references therein). They also re-ignited the debate as to the origins of the Pogonophora, and in particular the relationships between the Pogonophora, Vestimentifera and annelids. For a time, the vestimentiferans were elevated to phylum status [33], although later studies found close links in the larval development of both Pogonophora and Vestimentifera [32]. To some, these discussions might have appeared as obscure taxonomic arguments of little relevance to modern day issues in biology. But they are relevant to our first major question – when did siboglinids evolve? Are the siboglinids an ancient lineage that branched from the rest of the Metazoa not long after the evolution of the major animal groups? Or are they a more recently-evolved branch of the tree of life, derived from more conventional filter-feeding polychaetes with which they share several morphological similarities?

Modern systematics has provided some answers to this difficult question. The first robust cladistic analysis of morphological characters in polychaete families [38] showed strong support for the placement of the pogonophorans and vestimentiferans as a clade within the polychaete group Sabellida. At a similar time, several early molecular studies also showed support for a polychaete-origin for siboglinids [37,70-72]. A taxonomic revision was undertaken [40] and together with more recent molecular studies [39,44,73-75] the name Siboglinidae is now firmly established as representative of the worms formally known as Vestimentifera and Pogonophora.

Whilst Siboglinidae as a clade of annelid worms is now well accepted, this improvement in the taxonomic situation has done little to help answer our primary question – when did siboglinids

evolve? Annelida is an ancient branch of the Metazoa that has probable lower-Cambrian origins at least [76]. However, these early, putative stem-group annelids resemble the errant polychaetes Phyllodocida, characterised by their clear segmentation and well-developed parapodia and chaetae. Although support for placement within current classifications is weak [77], current evidence suggests that Siboglinidae are likely affiliated with the Oweniidae within a clade of 'sabellimorph' species that include the Serpulidae and Sabellidae [39,73]. These polychaetes all share a similar sessile, tube-dwelling lifestyle and exhibit less pronounced segmentation and reduced chaetal structures. In general the fossil record of these animals is poor, with the main exception being the calcareous tube-forming Serpulidae, which have a slightly better fossil record dating back to the Late Triassic [78]. However, the presence of sabellimorph, tube-dwelling polychaetes in the Late Triassic (and perhaps earlier) does rather little to help narrow the window of geological history during which Siboglinidae may have evolved.

Molecular genetics can help. In theory, genetic differences between closely related taxa allow the establishment of a divergence time based on a known rate of accumulation of neutral genetic differences (the molecular clock). Intriguingly, the few studies of molecular clocks in annelids come from studies of Siboglinidae. The first attempt to age the Siboglinidae based on genetic data suggested a relatively recent Mesozoic or Cenozoic origin [70]. Molecular clocks for Siboglinidae can, in some instances, be calibrated as hydrothermal vent species are intrinsically linked with geology as mid-ocean ridges form and separate. A calibration of the molecular clock for siboglinid and ampharetid polychaetes, made using the genetic divergence between closely related species living on two different mid-ocean ridge systems, also suggested a recent origin of approximately 60 mya [79]. Apart from one other older estimate (126 mya [80,81]), work in this area has since stalled and more recent studies have focused mainly on direct evidence from fossils.

Clues from the fossil record

Establishing an unambiguous fossil record for the Siboglinidae is difficult because the characters that define the family and the contained taxa are based on soft tissues, and these soft tissues are not preserved in the geological record. However, the vestimentiferans, *Sclerolinum* and frenulates produce chemically stable tubes formed of a complex of proteins with inter-woven beta chitin crystallites (e.g., [45,82]). The tubes of most frenulates and *Sclerolinum* are small (usually only a few mm or less in diameter) and thin-walled (e.g., [83]), resulting in their rare detection within the fossil record. By contrast, many vestimentiferan tubes are large (up to 40 mm in diameter) and

robust, often having thick tube walls. Furthermore, vestimentiferans mostly live in environments where rapid mineralization occurs, including carbonates at seeps and sulphides at vents. Thus, vestimentiferan tubes might be expected to have better preservation potential than those of frenulates and moniliferans. Indeed, modern *Ridgeia piscesae* tubes at vents on the Juan de Fuca Ridge can be rapidly overgrown by initial barite and amorphous silica mineralization, which are later replaced by Fe, Zn and Cu sulphides during incorporation into growing sulphide chimneys [84]. A similar pattern of rapid mineralization of vestimentiferan tubes at seeps is found on the Congo deep-sea fan where some posterior 'root' tubes of *Escarpia southwardae* are partially to completely replaced by the carbonate mineral aragonite [85,86]. This replacement occurs from the outside of the tube wall inwards and leaves fine-scale relict textures of the original organic tube wall (Figure 5E). Similar carbonate replaced vestimentiferan tubes are known from seeps in the Gulf of Mexico and Eastern Mediterranean. The oldest fossil attributed to siboglinids is *Hyolithellus micans* from the Middle Cambrian (~500 Ma), based on tube morphology and the probable presence of chitin in the organic component of the tube wall [87,88]. However, subsequent authors have not followed this interpretation and attribute phosphatic walled *Hyolithellus* tubes to an unknown extinct order of animals (e.g., [89]). Slightly younger tubular fossils from Palaeozoic (542-251 Ma) hydrothermal vent and cold seep deposits have been formally and informally described as vestimentiferan tubes. Those from the vent deposits (e.g. the Silurian [~440 Ma] *Yamankasia rifeia* and Devonian [~393 Ma] *Tevidestus serriformis*) are large (up to 39 mm in diameter) external moulds formed by thin layers of pyrite, often preserving fine details of the external tube wall, including faint longitudinal striations, concentric growth lines and flanges [90]. Those tubular fossils from the seep deposits (e.g. the Devonian [~395 Ma] Hollard Mound and Carboniferous [~302 Ma] Ganigobis Limestone) are formed of carbonate and have distinctive concentrically laminated tube walls, often showing 'delamination' structures (Figure 5F) [85,91]. These taphonomic (i.e. preservational) features, which are identical to those seen in modern carbonate, replaced vestimentiferan tubes (Figure 5E).

Assigning these Palaeozoic vent and seep tubes specifically to the vestimentiferans raises a phylogenetic problem, because they are considerably older than the divergence estimates of the vestimentiferans from the frenulates based on mitochondrial cytochrome c oxidase subunit 1 (mtCO1), 18S rRNA and 28S rRNA gene studies [35,70,79]. These studies suggest that the origin of the vestimentiferans was less than 100 million years ago (i.e., Early Cretaceous), leaving a gap of about 300 million years between this date and the Silurian vent fossils. One explanation is that the Palaeozoic vent and seep tube fossils could represent earlier stem-group siboglinid lineages that are not ancestral to the extant vestimentiferans [81], another explanation is that the fossil

tubes are not vestimentiferans (or even siboglinids) and could be fossils of other, possibly extinct, tube forming worms [70,92]. It may also be the case that gene substitution rates are variable and hence the molecular dates are inaccurate; further work to calibrate the molecular clock in siboglinids is clearly needed.

A few fossil tubes from the Mesozoic (251-65 Ma) and Cenozoic (65-0 Ma) have also been formally described as siboglinid tubes. *Adekumbiella durhami* [93] is a small tube from late Eocene (~37 Ma) bearing some resemblance to frenulate tubes. The Neogene (23-3 Ma) *Palaeoriftia antillarum* is a large calcareous smooth tube with few features [94]. Tunnicliffe [95] questioned the interpretation of this fossil as a vestimentiferan due to incompleteness of the specimens. Tubular fossils from the early Jurassic (~185 Ma) Figueroa hydrothermal vent deposit have been assigned to the vestimentiferans [96]. These latter tubes share many morphological similarities with tubes from the younger Upper Cretaceous (91 Ma) Cypriot hydrothermal vent deposits [97], being external moulds of pyrite preserving an ornament of irregularly spaced flanges, concentric growth lines and longitudinal wavy striations with periodic bifurcations and plications where they cross the growth lines (Figure 5A,B) [96]. Identical longitudinal ridges can be seen in the tubes of modern vestimentiferan tubes, particularly at the anterior ends, in both vent and seep species (e.g., [96], fig. 8.8-10). Little et al. [96] took this to be a useful character to separate vestimentiferan from frenulate and moniliferan tubes, as neither of the latter groups are known to have this feature. Indeed, many frenulate tubes have distinctive regular constrictions along their length, giving them a 'bamboo cane'-like morphology (e.g., [83,96], fig. 8.11). Tubular fossils are also common in Mesozoic and Cenozoic cold seep deposits ([85], table 1, and references therein), some of which are undoubtedly of serpulid origin. However, most (e.g. Figure 4D) are morphologically similar to the modern carbonate replaced vestimentiferan tubes studied by Haas et al. [86] and some of the Palaeozoic seep fossil tubes in having concentrically laminated tube walls, often with 'delamination' structures (Figure 4F). Unfortunately this preservation style means that fine scale external ornament is not seen in these fossil cold seep tubes.

Although the majority of the fossil tubes from Mesozoic and Cenozoic seeps and vents are younger than the 100 Ma maximum molecular estimate for the origin of the vestimentiferans, it is difficult to be certain that these fossils belong are of vestimentiferan origin. The concentrically laminated tube walls with 'delamination' structures of the fossil cold seep tubes are a taphonomic feature, not a definitive morphological character, and thus, theoretically, could be a result of the calcification of any multi-layered organic-rich (and probably chitinous) tube (including those of

frenulates and *Sclerolinum*) [92]. Nonetheless, this preservational pathway has so far only been proven in the seep vestimentiferans (cf. [92]). The external ornament of longitudinal wavy ridges of the Mesozoic vent fossil tubes is identical to that seen on all modern vestimentiferan tubes, and not frenulates and *Sclerolinum*, so at present these seem to be among the best candidates for proving a vestimentiferan fossil record, which may thus go back 185 million years. As can be seen above, the fossil record of the frenulates and *Sclerolinum* is considerably poorer and very few fossils may be even tentatively assigned to these siboglinid clades.

Although entirely soft bodied, most species of *Osedax* bore into whale bone [25,41] and these borings have the potential to be recognized in the fossil record as a proxy for *Osedax* [98]. Indeed, recently borings in Oligocene (~30 Ma) whale bones from Washington, USA have been interpreted as *Osedax* borings [99] If correct this would constitute the oldest fossil record of this clade and the age is roughly the same as the first major radiation of whales, which strengthens the idea of an evolutionary link between *Osedax* and its main modern substrate [42].

HOW DID SIBOGLINIDS EVOLVE?

Adaptation 1: habitat and endosymbiosis

Insights into how siboglinids evolved can initially be derived from examining where these organisms live and commonalities in the physical and chemical parameters of those habitats. The hydrothermal vent habitat is often characterised as an 'extreme environment', where organisms must live on the side of mineralized hydrothermal chimneys in which hydrogen sulphide enriched fluids emanate at temperatures of up to 400°C. However, not all vents are like this, in particular many are characterised by more diffuse flow regimes and lower temperatures. In some cases, fluid flow may be through sediments and the organisms that are normally found on hard substrates must cope with this sedimentation. At cold seeps, siboglinids are almost always living within a sedimented environment, although hard substrates do form through carbonate precipitation. Frenulates are also found in sedimented environments, in the anoxic muds beneath organically-enriched regions, althought sulphide levels are generally lower than at vents and seeps. Finally, *Osedax* are found living on whale bones which may or may not be sitting on the sediment.

An important commonality in all these habitats is a reduction-oxidation (REDOX) boundary. Living at the REDOX boundary, vent, seep and frenulate siboglinids fuel their bacterial symbionts with oxygen, sulphide and carbon dioxide via some unique adaptations to their

circulatory system [45]. Bacterial symbionts then fix CO_2 into organic molecules using sulphide as the energy source [100,101]. At the strange whale-bone habitat of *Osedax*, rather less is known about the chemical milieu; the bacterial endosymbiosis and the nutitional pathways are not yet full understood. Nevertheless, a REDOX boundary and high levels of sulphide are also present at whale bones [102].

Siboglinids living in these different environments have evolved adaptations to exploit these differences in food and sulphide (or in some cases methane) availability. Whereas vestimentiferans living on hydrothermal vent chimneys absorb sulphide through a branchial plume that extends up to 2 m into the water column [103], vestimentiferans living in cold seeps obtain sulphide from the sediment, across the wall of the buried tube [104] (Figure 6). Frenulates, notwithstanding some exceptions, are found mainly in organic-rich, reduced sediments. Because frenulates can transport dissolved organic matter across their tube and body wall [105], sulphide is presumably transported across the thin tube that is buried in the sediment, but data supporting this are scarce. In the case of a few frenulates, for example *Siboglinum poseidoni*, methanogenesis is reported [106]. Sulphide levels or uptake location have not yet been investigated for *Sclerolinum* species, and for *Osedax*, the current evidence suggests that the endosymbionts are consuming collagen or lipids directly from bones rich in these energy sources [54]

A crucial adaptation in the evolution of siboglinids would appear to be a unique circulatory system that allows these chemicals to be delivered to the symbionts. Sulphide and oxygen are transported from the site of uptake (e.g the branchial plumes or body walls) via haemoglobin molecules that are freely dissolved in their blood or in the coelomic fluid surrounding the blood vessels [107-109]. These haemoglobin molecules exhibit some unique propertes. Three and two types of haemoglobin have been identified in vestimentiferans [109] and *Sclerolinum* [110], respectively. One is a hexagonal bilayer haemoglobin (HBL-Hb) that is capable of binding oxygen and sulphide simultaneously and reversibly [100,109], enabling the animals to transport and store both substances in large quantities while minimizing autoxidation and toxic effects [19]. A second type of haemoglobin detected in Siboglinidae is a ring-Hb that has been found in Vestimentifera, *Sclerolinum,* and Frenulata. Although sulphide binding has not been demonstrated for the ring-Hb, it has an extremely high affinity for oxygen [107,110,111] that enables the worm to take up and transport large amounts of oxygen while maintaining low internal dissolved O_2.

Equally important to adaptations within the circulatory system are the bacterial endosymbionts that are thought to provide the majority of energy to the hosts. Considering the diversity of both siboglinid worms and the habitats that they occupy, the existence of considerable bacterial endosymbiont diversity is perhaps unsurprising. Siboglinids engage in an obligate and persistent association with a numerically dominant phylotype of Gammaproteobacteria, referred to here as the "primary endosymbiont" ([53,58,59,112,113], but see [54,114,115]). Major siboglinid groups (i.e., frenulates, vestimentiferans/*Sclerolinum*, and *Osedax*) associate with a different monophyletic bacterial clade, reflecting host-symbiont specificity at higher taxonomic levels [57-59,116,117]. In vestimentiferans and *Sclerolinum* specifically, primary endosymbionts are two closely-related clades of chemoautotrophic bacteria within the Leucothrix-Methylococcaceae cluster. Information on symbiont diversity is more limited for frenulates. The three frenulate species examined to date harbour primary endosymbionts within a monophyletic clade of thiotrophic Leucothrix-Methylococcaceae Gammaproteobacteria [56-59]. Despite their apparent metabolic similarity to the vestimentiferan/*Sclerolinum* symbionts, the frenulate symbionts are phylogenetically distinct from symbionts of other siboglinids [57-59]. Notably, one species of frenulate, *Siboglinum poseidoni*, putatively harbours a methanotrophic endosymbiont [106,118] of unknown phylogenetic affinity. Finally, primary endosymbionts of *Osedax* belong to the Oceanospirillales cluster [51,54,55], a diverse bacterial group that is known for heterotrophic aerobic degradation of complex organic compounds. The role of the endosymbionts within *Osedax* is not clear, but they are hypothesized to provide nutrition to their hosts via the degradation of bone collagen [54].

In addition to the primary endosymbiont, bacterial consortia (referred to here as the "microflora") have been found in some siboglinids. These additional bacterial types consist of multiple bacterial phyla, including Alpha, Gamma, and Epsilonproteobacteria as well as members of the Bacteroidetes (e.g., [51,54,55,113-115]). The microflora typically occur at lower relative abundance compared to the primary endosymbiont and may not even be located within the host trophosome [54,55,57,113]. The nutritional contributions of these bacteria to their siboglinid hosts remain unknown and offer fertile ground for future research.

Despite the obligate nature of the siboglinid mutualism, available evidence supports horizontal transmission as the primary mode for establishment of the bacterial symbioses [119,120], but see [121]. This evidence includes: (1) a lack of symbionts in worms' gonadal tissues or larvae [13,55,122-124], (2) the presence of the motility-related flagellin gene in the vestimentiferan endosymbiont genome [117,125], (3) the detection of highly similar bacterial phylotypes (based

on 16S rRNA sequences analysis) in host and in the external environment [112,126-129], (4) the presence of heterotrophic metabolic pathways in the vestimentiferan endosymbiont that are not expressed *in hospite* [117], (5) direct confirmation of horizontal transmission in *Rifta pachyptila* [26], and (6) the absence of reciprocal phylogenies (i.e., co-evolution) between host and symbiont [112,130,131]. Thus, following a non-symbiotic larval stage, siboglinids must establish a new symbiosis each generation in order to survive. Despite the risk of failing to acquire an appropriate symbiont, horizontal transmission presumably enables the host to acquire a bacterial phylotype adapted to the local environmental conditions (e.g., sulphide concentration [60] or bone degradation stage [132]).

Following acquisition from the environment, bacterial symbionts migrate to the trophosome in some vestimentiferans [26,47]. Although it has previously been hypothesized that symbionts were acquired from the environment during the trochophore larval stage [32,133], recent work indicates that vestimentiferans are actually colonized by bacteria after larval settlement and development of a juvenile worm [26]. Remarkably, Nussbaumer et al. [26] showed that symbionts are able to enter the host through the epidermis during a symbiont-specific selective infection process and subsequently migrate into a mesoderm tissue that will develop into the trophosome. Once the trophosome is well established in juveniles, the infection ceases at the same time as apoptosis of skin and other non-trophosome tissues. The timing (larval or post settlement) and mechanism of symbiont acquisition from the environment are not known for other siboglinid groups. However, in *Osedax*, it has been proposed that infection would not be limited in time but continuous throughout the worm life, with symbionts infecting new root tissue as it grows into whale bones [55].

The obligate symbiosis in siboglinid tubeworms at deep-sea vents, seeps and whale-falls is a remarkable biological adaptation. However, many questions remain unanswered. In particular, the actual selective processes that occur during infection by the symbionts, and the final result of a dominant primary endosymbiont, are unknown. Unfortunately, symbiosis has only been investigated in a handful of siboglinid species. The question of nutrition in siboglinids has consumed research in this area, but results have been difficult to come by. For the first few decades, a handful of clever experimental studies suggested the paradigm of DOM uptake across the body wall. The following few decades have assumed that endosymbioses plays the primary role. Perhaps both of these paradigms are incorrect. Either way, the presence of luxuriant fields of giant tubeworms on the sulphide chimneys of the East Pacific Rise, without mouth or gut and reliant only on the chemistry of the moment to survive remains a compelling biological surprise.

Adaptation 2: reproduction and dispersal

The majority of deep-sea polychaetes live in the vast tracts of sedimented mud that dominate the abyssal seafloor. Habitat availability and stability are not, in general, a problem for organisms that can live on approximately 60% of the planet's surface. In contrast, many siboglinid habitats, including hydrothermal vents, cold seeps and whale-falls are extremely small and isolated habitats, often separated by 100s to 1000s of km. The evolutionary innovation of symbiosis that allowed siboglinids to invade and radiate on sulphide-rich 'island' habitats in the deep-sea must also have been coupled with equally innovative life-history strategies to ensure that the reproductive propagule can locate and colonize the "needle" in the oceanic "haystack".

While difficult logistics have so far precluded intensive time-series studies of the reproductive activity of any siboglinid species, much has been learned about the reproductive ecology through "snap-shot" analyses of, for example, gametogenic condition, population structure and population genetics [134-136]. Similarly, studies of early development based on spawning wild-caught individuals have provided insights into dispersal of all siboglinid clades [23,24,124,136,137]. Despite these increases in available data, very little is known about reproduction and dispersal of siboglinids in an evolutionary context.

Life-history theory predicts traits that maximize fitness of an organism in the particular environment where it lives. Therefore, differences between siboglinid habitats are expected to have a role in the evolution of life-history traits, including fecundity, breeding strategy and developmental mode. At present, we do not have estimates of lifetime fecundity for any siboglinid. However, instant fecundity data suggest that the Vestimentifera and *Osedax* have generally higher fecundity than Frenulata ([124]; Hilário pers. observ.). Although this could be related to body size (since small animals are expected to produce a small number of large eggs [138]), it is most likely related to the energy available in the environment and the insular and/or ephemeral nature of hydrothermal vents, cold seeps and whale falls. Siboglinids living in vents, seeps and whale falls have access to sufficient energy to invest in high fecundity, which in turn allows them to exploit these isolated and generally ephemeral habitats.

Fertilization is assumed to be internal for all siboglinid clades (no information is available for *Sclerolinum*). To further facilitate fertilization, Vestimentifera females store sperm in a spermatheca until eggs are mature (Figure 7a, [135]). *Osedax* have evolved a specialized strategy to ensure reproductive success; females host dwarf males in their tubes assuring sperm

availability (Figure 7b, [25,124]). Therefore, vestimentiferans and *Osedax* both utilize strategies in environments where periodic cues for gametogenesis and spawning synchrony are limited [139] and mate acquisition is not guaranteed.

Following fertilization and embryogenesis, planktonic larvae develop. Larval dispersal duration and distances are intuitively most likely related to habitat isolation. In vestimentiferans, small, yolky and slightly buoyant eggs develop into non-feeding trochophore larvae that are thought to disperse in the plankton for up to several weeks [23,24]. For instance, larvae of the vent species *Riftia pachyptila* are estimated to disperse more than 100 km over a 5-week period [24]. Whilst the vent and seep habitats of vestimentiferans are restricted geographically to areas such as mid-oceanic ridges and continental margins, the whale-fall habitats of *Osedax* may occur anywhere throughout the world's oceans where whales are present. As a result, *Osedax* are hypothesized to have shorter dispersal times and distances than vestimentiferans [124]. Although no estimates exist for larval dispersal distances and duration of Frenulata, it is known that some species incubate eggs in their tubes until settlement stage (Figure 7c) whereas others have planktonic larvae, although the latter have never been reared [48]. Brooding is favoured by natural selection on continuous habitats, such as anoxic sediments that are almost continuous along continental margins, as the great expanses of suitable substratum make colonization of new habitats unnecessary. Insufficient sampling of frenulates, however, does not allow robust comparisons between habitat isolation and developmental mode.

A detailed phylogenetic analysis of Siboglinidae is needed to provide a framework for understanding the evolution of life-history traits in the group. However, it does appear that the various reproductive strategies found in siboglinids are related to environmental conditions. Notwithstanding possible exceptions, the overall rank order of fecundity and dispersal distance of siboglinids is: Vestimentifera > Osedax > Frenulata corresponding to the degree of transience and isolation of the habitats occupied by these groups. The placement of *Sclerolinum* in this rank remains unknown, as no reproductive data is currently available.

DISCUSSION

The two questions posed by this review are when and how these worms evolved. How were these metazoans able to make the transition to an extreme habitat, apparently high in toxic sulphide and competing mats of free-living bacteria? When did this happen in Earths history? Was it driven by

the geological formation of spreading centres and hydrocarbon seeps? Or was there a long gap between the availability of the habitat and the biological adaptations necessary to colonise it?

These questions are not easy to answer, particularly so when it has taken over eighty years of detailed research even to determine what a siboglinid worm actually is. When confronted with a biological 'oddity', such as giant red tubeworms on a deep-sea volcanic vent, taxonomy is the first tool to be brought out. At several moments in the scientific history of siboglinid research, it has been a key taxonomic paper – often published in a high-impact journal – that has spurred research in the field. It is rare that deep-sea worm genera such as *Riftia* or *Osedax* are described in the pages of *Nature* or *Science*. However, in these cases, research into these animals was stalled until the names were published. It was the formal taxonomic publication, the creation of a compelling name and common language that allowed researchers to finally start linking together work on the biology of these unusual animals.

Following the name, the next part of the puzzle is determining an organism's closest relatives. Again, for siboglinids, this has challenged taxonomists, anatomists and evolutionary biologists. Only molecular genetics has provided convincing and consistent character sets, although with hindsight, the morphologial clues were always there. Molecular and morphological phylogeny studies now place frenulates in a basal position with vestimentiferans and *Sclerolinum* nested within this larger clade. Among vestimentiferans, vent species are nested within the clade of seep-dwelling species, which has led several authors to suggest that siboglinid evolution originated in soft substrates and progressed through to the species that live on sulphide-rich hydrothermal vents [35,44,60,140]. This seemingly ordered trend has been complicated by the discovery of the *Osedax* clade, specialist on whale bones and using heterotrophic rather than chemoautotrophic symbionts.

The evidence so far suggests that the last common siboglinid ancestor was likely either symbiotic or pre-adapted to symbioses with gamma proteobacteria. Given that there are, so far, only four major lineages of siboglinids known, and that symbionts within a major host lineage seem to be related, there are a limited number of alternative scenarios for the evolutionary origins of this symbiosis. The scenarios include: (1) an aposymbiotic ancestor, with endosymbiosis being established more than once independently in major siboglinid lineages, (2) a symbiotic ancestor that gave raise to major lineages that experienced switches in primary endosymbiotic phylotype, or (3) an ancestor that housed a consortia of bacteria and as major lineages emerged so did specialization in primary phylotype among lineages.

Available data support limited concordance between host and symbiont phylogenies. For example, although monophyletic clades of symbionts for vestimentiferans, *Sclerolinum*, frenulates, and *Osedax* are resolved, the deeper relationships between clades are not well resolved (Figure 3). Furthermore, the sister group relationship between *Osedax* and vestimentiferan hosts is tentatively supported in the phylogenetic analysis by Rouse et al. [25] but less in Glover et al. [41]. However, if one assumes that it is a greater number of evolutionary steps to transition from a chemoautotroph symbiont to a heterotroph symbiont than it is between two different types of chemoautotroph symbiont, parsimony arguments support a siboglinid ancestor with two possible chemoautotroph symbionts and the secondary loss of chemoautotrophy in *Osedax* (Figure 8).

If, as speculated, the evolution of host lineages may be driven by an evolutionary trend in the redox potential of the environments that host worms inhabit, this hypothesis would also explain why, from an evolutionary physiology point of view, the host would switch or specialize its symbiont community. As the host moved into new environments, different lineages of Gammaproteobacteria would allow more successful exploitation of the redox conditions within that environment. For example, consider that sulphide is available at whale-falls [102], whalebones often become sedimented, and that some species of *Osedax* have been found to specialize on bones buried in sediment [132]. An ancestor of *Osedax* may have contained a typical thiotrophic endosymbiont form that utilized sulphide rich sediment around whalebones. However, the energy reserves in the collagen of whalebones were a large untapped energy source offering a great selective advantage to, and rapid evolution of organisms that could utilize it. Thus, the thiotrophic *Osedax*-ancestor made the evolutionary transition to heterotrophy. One piece of evidence in support of this hypothesis is that vestimentiferans, with thiotrophic symbionts, have been recorded occasionally in sediments containing whalebones, although never ecologically dominant [141]. It may have been that this type of occasional habitat colonization, with overlapping sulphide conditions, was the necessary evolutionary step in the origin of *Osedax*.

Independently of how siboglinids evolved, their evolutionary age is one of the most intriguing subjects of chemosynthetic ecosystems biology. For now we are unable to confidently delineate a timeframe during which Siboglinidae split from its polychaete relatives or the age of the most recent common ancestor between clades. The fossil record suggests a Mesozoic or even Palaeozoic origin which largely disagree with molecular divergence phylogenies that point towards a much younger origin [70,92,96]. This raises several questions about the interpretation of both the molecular and fossil data. However, to investigate the origins and ages of siboglinids

in relation to their habitat the fossil record may provide valuable clues and validate hypotheses of divergence times such that *Osedax* origin coincided with that of its main modern substrate – the large oceanic cetaceans (e.g. [42]).

CONCLUSION & FUTURE DIRECTIONS

The circular story of Siboglinidae systematics is, as Pleijel et al. [63] have put, "one of humbleness… a reminder that we are all likely to make mistakes". None of the four major lineages of siboglinids have proved easy to sample, identify, classify or study. For almost 80 years, from their discovery in 1914 to the first molecular phylogenies in the 1990s, there was disagreement over what the frenulate pogonophore worms actually were. The more recently discovered vestimentiferan tubeworms also proved difficult to understand, despite their greater size. Even the most recently discovered group, *Osedax*, took over 10 years to be identified and described, from the first observations of small gelatinous tube worms attached to whale bones recovered from the Oregon subduction zone in 1994 (Dr. Eve Southward, pers. comm.) to the description and classification of the genus in 2004 [25].

Given the known diversity of siboglinds, one obvious issue in the study of siboglinid history is the lack of sampling among frenulate taxa. The fossil record is very poor, and in molecular phylogeny studies only five of the 140 described species have been used. Sampling constraints associated with the small size on the individuals, a shortage of taxonomic expertise, and the fact that for a long time specimens were routinely fixed in formaldehyde, which is incompatible with most molecular biology techniques, have all contributed to the current situation of frenulates being the least studied group of siboglinids. The lack of sampling among frenulate taxa has, in the last few years, stimulated new collections and research. Additional morphological and genetic information on frenulates is in the process of being disclosed [57,142,143].

In spite of the spectacular discoveries and extraordinary advances made in recent years the placement of siboglinids among the annelid tree is still poorly resolved and many other questions concerning the evolution and ecology of siboglinids remain unanswered. New challenges are presented to scientists on a daily basis, but because many siboglinids live in environments that are not easily accessible, understanding the larger picture of siboglinid evolution in relation to their habitat will require a concerted sampling effort from researchers from multiple disciplines and additional deep-sea exploration. Only a small fraction of the global ridge system (~65 000 km) and of the vast continental margin regions have been explored. We believe that the exploration of

new chemosynthetic environments, on planet earth and perhaps beyond, will include the discovery of new species capable of ecological and physiological attributes that cannot yet be imagined.

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FIGURE CAPTIONS

Figure 1. Cumulative citation count for papers published in the general science journals *Nature* or *Science* over the years 1958 to 2007 that deal exclusively with species in the annelid clade Siboglinidae (papers covering general vent/seep biology or symbiosis in general are not

included). Significant discoveries are highlighted by arrows and major increases in total citations. These include discoveries in feeding [10], the discovery of bacterial symbiosis [13], sulfide binding [18], tubeworms at shipwrecks [20], respiration [22], embryology [23], larval dispersal [24] and the new clade of siboglinids (*Osedax*) that consume whale bones [25].

Figure 2. Examples of siboglinid species and their habitat requirements. (a) *Riftia pachyptila* giant tubeworms growing on a hydrothermal vent in the north-east Pacific (Image courtesy of Richard Lutz), (b) *Lamellibrachia luymesi* at a cold seep in the Gulf of Mexico (Image courtesy of DT, KH, Kevin Fielman and Scott Santos) and (c) *Osedax mucofloris* living on a whale-bone found off the coast of Sweden.

Figure 3. Phylogenetic relationships amongst Siboglinidae, modified from [41]. A Bayesian analysis of 18S ribosomal RNA sequences reveals four major clades of siboglinids, from top, *Osedax* which are specialist on whale carcasses, the vestimentiferans, which are specialist on vents and seeps, *Sclerolinum* (here presented only by a single sequenced specimen), specialist on on organic-rich remains and the frenulates which specialise on organic-rich sediments. Images courtesy of Tomas Lundälv (whale-fall), Richard Lutz (vent site) and NOCS/JC10 (frenulate in sediment).

Figure 4. Cumulative number of species descriptions of since the discovery of the first siboglind [29]. With the exception of *Sclerolinum*, the curve does not asymptote showing that that new species have been (up to this day) continuously disclosed.

Figure 5. Tube fossils from ancient seep and vent deposits possibly attributable to vestimentiferans and modern vestimentiferan tubes for comparison. (a) Cluster of pyrite replaced tubes in matrix of pyrite, Kambia vent deposit, Cyprus, Early Cretaceous (91 Ma). (b) Pyrite replaced tube in pyrite matrix, Figueroa vent deposit, California, USA, Early Jurassic (~184 Ma), note fine concentric growth lines and wavy, periodically bifurcating longitudinal ridges. (c) Tube of holotype (NHM1996:1048) of vestimentiferan *Arcovestia ivanovi*, note external ornament of fine concentric growth lines and wavy, periodically bifurcating longitudinal ridges. (d) Carbonate tubes in matrix of carbonate minerals, Canyon River seep deposit, Washington, USA, Oligocene (~30 Ma), specimen courtesy of James Goedert. (e) Carbonate replaced tube of vestimentiferan (probably *Escarpia southwardae*) in transverse section from modern seep in the Kouilou pockmark field on the Congo deep-sea fan, 3100m water depth . The original organic tube has been ‘delaminated’ by the growth of aragonite crystals within it. (f) Carbonate tube in transverse section, Ganigobis seep deposits, Namibia, Late Carboniferous (~302 Ma), showing very similar

textures to the tube in E. Scale bars: A = 10mm, B = 1mm, C = 2mm, D = 10mm, E = 100µm, F = 100µm.

Figure 6. Sources of sulphide and respiratory pathways at contrasting habitats in siboglinid tubeworms. At hydrothermal vents, sulphide is produced through the inorganic reaction of sulphate with geothermal energy. By contrast, sulphide has a microbial origin at cold seeps, organic-rich sediments, and whale-falls. At cold seeps, the source of sulphide is the anaerobic oxidation of methane coupled to sulfate reduction. At organic-rich sediments, sulphide is produced during the anaerobic degradation of a range of organic compounds. At whale-falls, although sulphide is produced, *Osedax* worms are thought to rely only on heterotrophic digestion of bone by the endosymbionts. The trophosome (light grey) houses endosymbiotic bacteria (orange ovals). White open circles represent methane and hydrocarbon seepage. Full arrow = reaction, dashed arrow = diffusion, and dotted arrow = acquisition or excretion by the host/symbiont.

Figure 7. Life-history traits are found in various siboglinid groups. (a) Histological section through the spermatheca of *Riftia pachyptila* (Vestimentifera) (Gc = Gonocoel, PO = Primary oocyte, S = Clusters of spermatozoa, St = Spermatheca, scale bar: 200 µm) (from [135]), (b) two live males on the trunk of a female of an undescribed species of *Osedax* recovered in Antarctic waters (scale bar: 100 µm), (c) brooding larva inside the tube of *Siboglinum* sp. (Frenulata) (scale bar: 500 µm).

Figure 8. An evolutionary scenario for the origin of the four major siboglinid clades and their respective symbiont and habitat specialisation. Note that the sister-group relationship between *Osedax* and the vestimentiferan-*Sclerolinum* clade is currently only weakly supported. In this scenario, the putative siboglinid ancestor possessed chemoautotrophic symbionts that have been secondarily lost in *Osedax* and replaced by a heterotrophic symbiont. Images courtesy of DT, KH, Kevin Fielman and Scott Santos (vestimentiferan), Irmgard Eichinger (*Sclerolinum*).

Figure1
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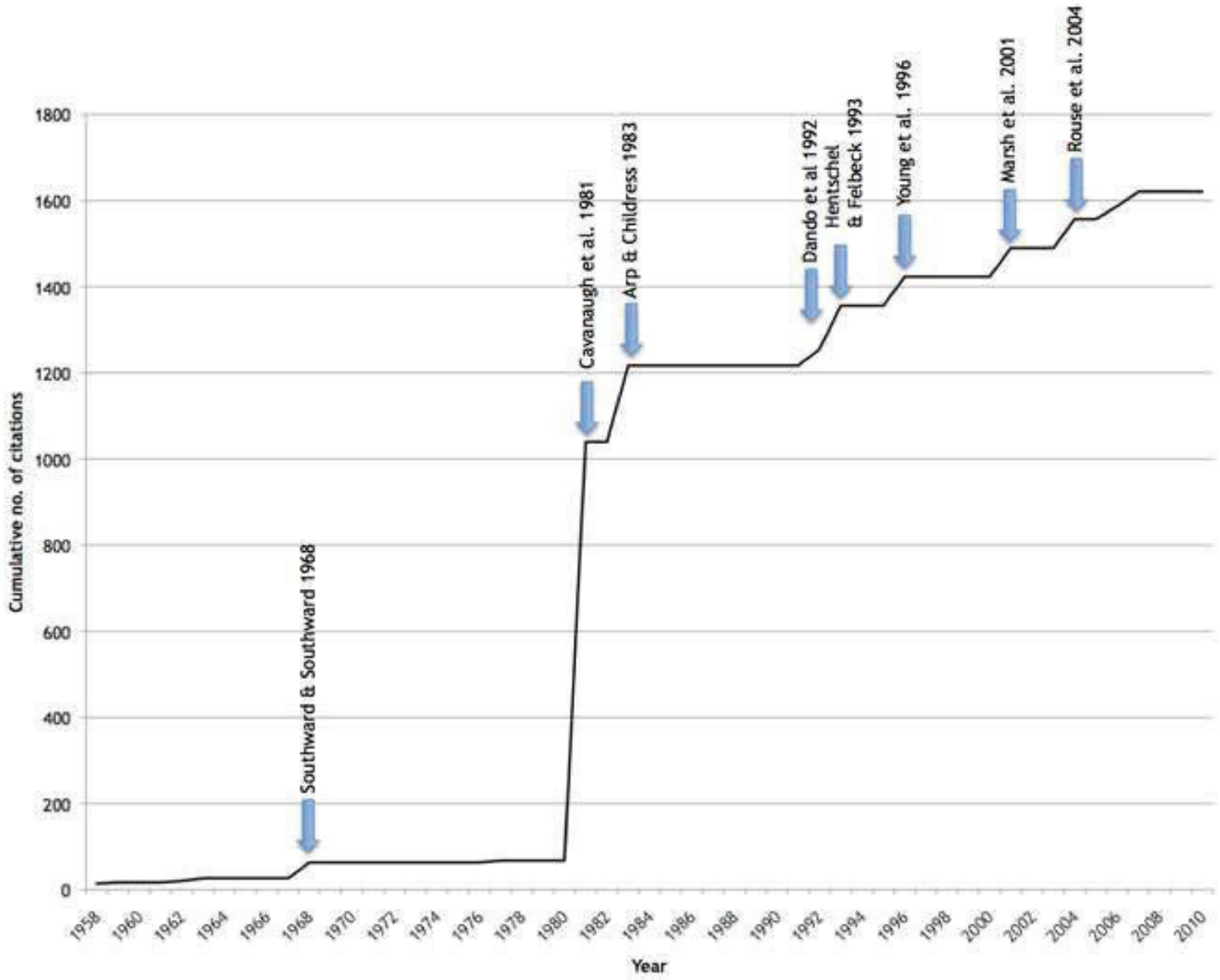

Figure2
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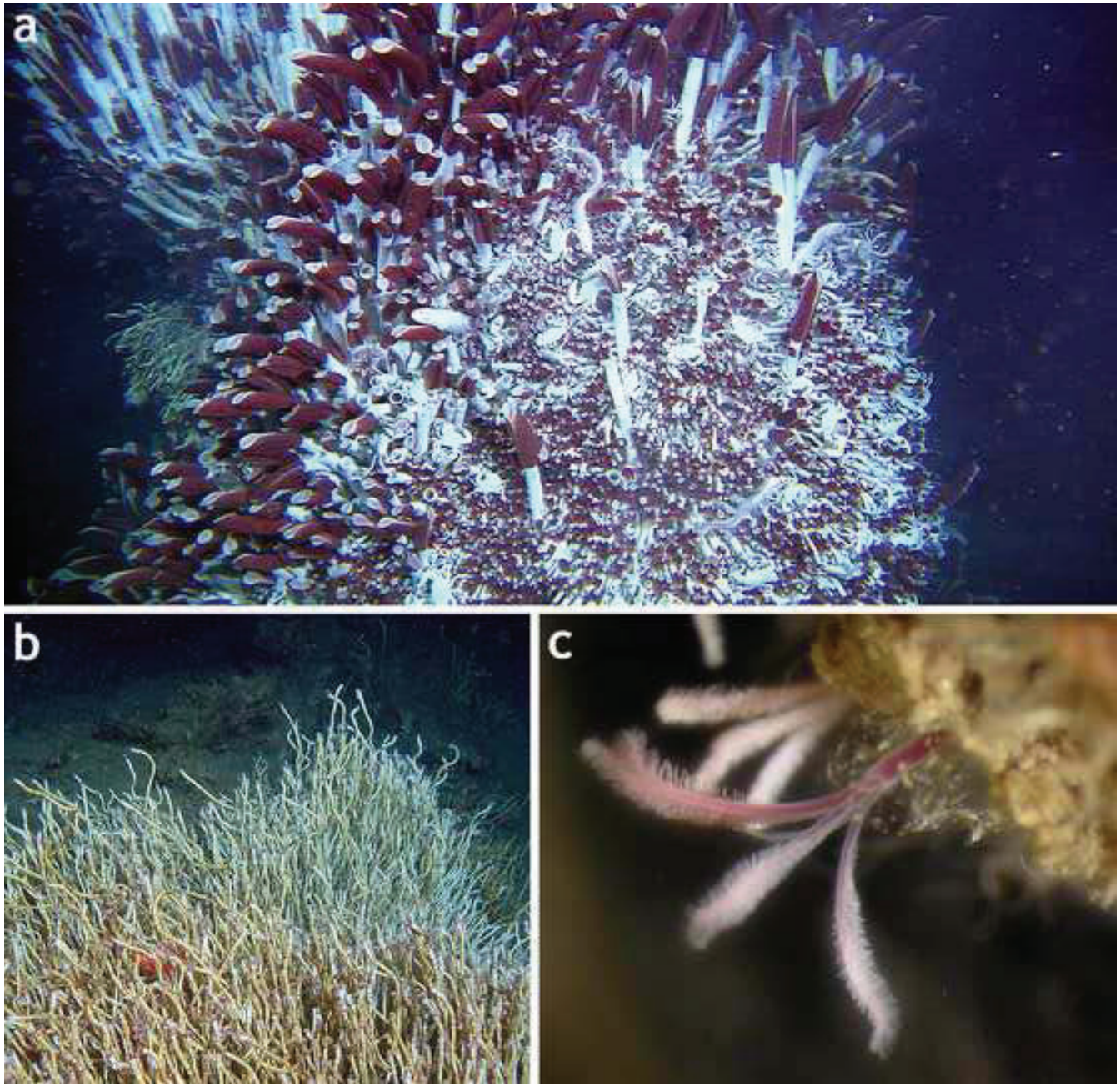

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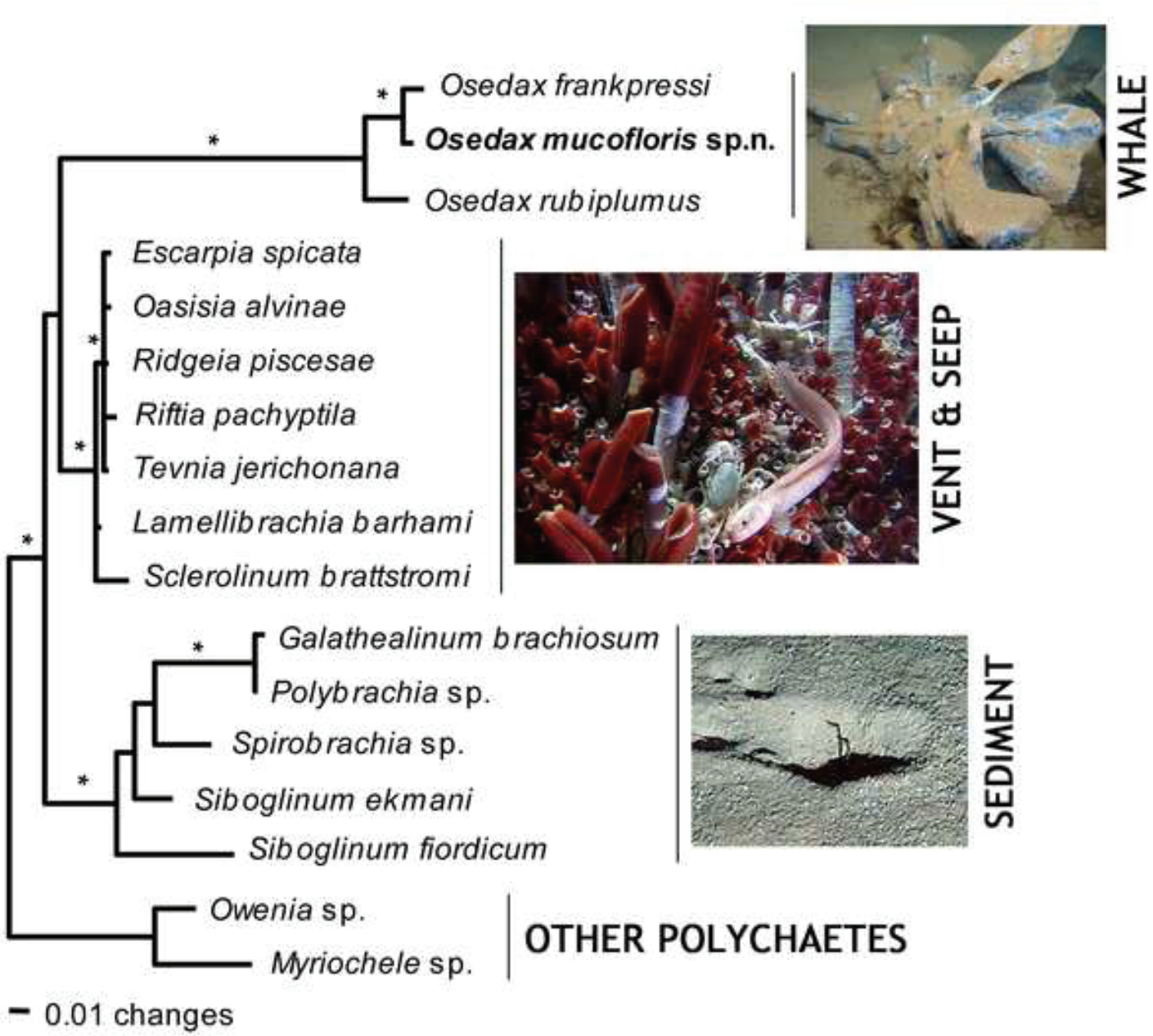

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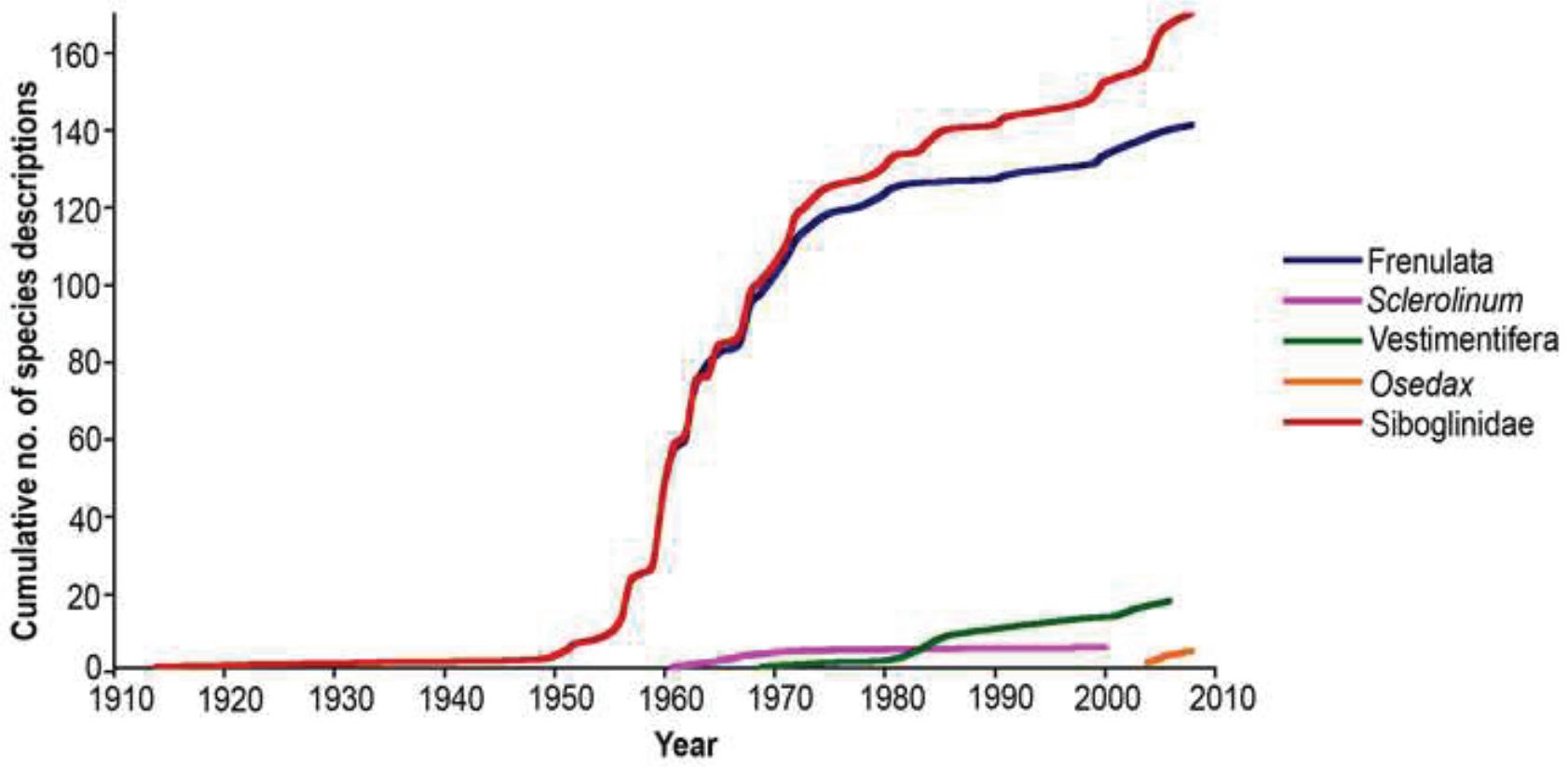

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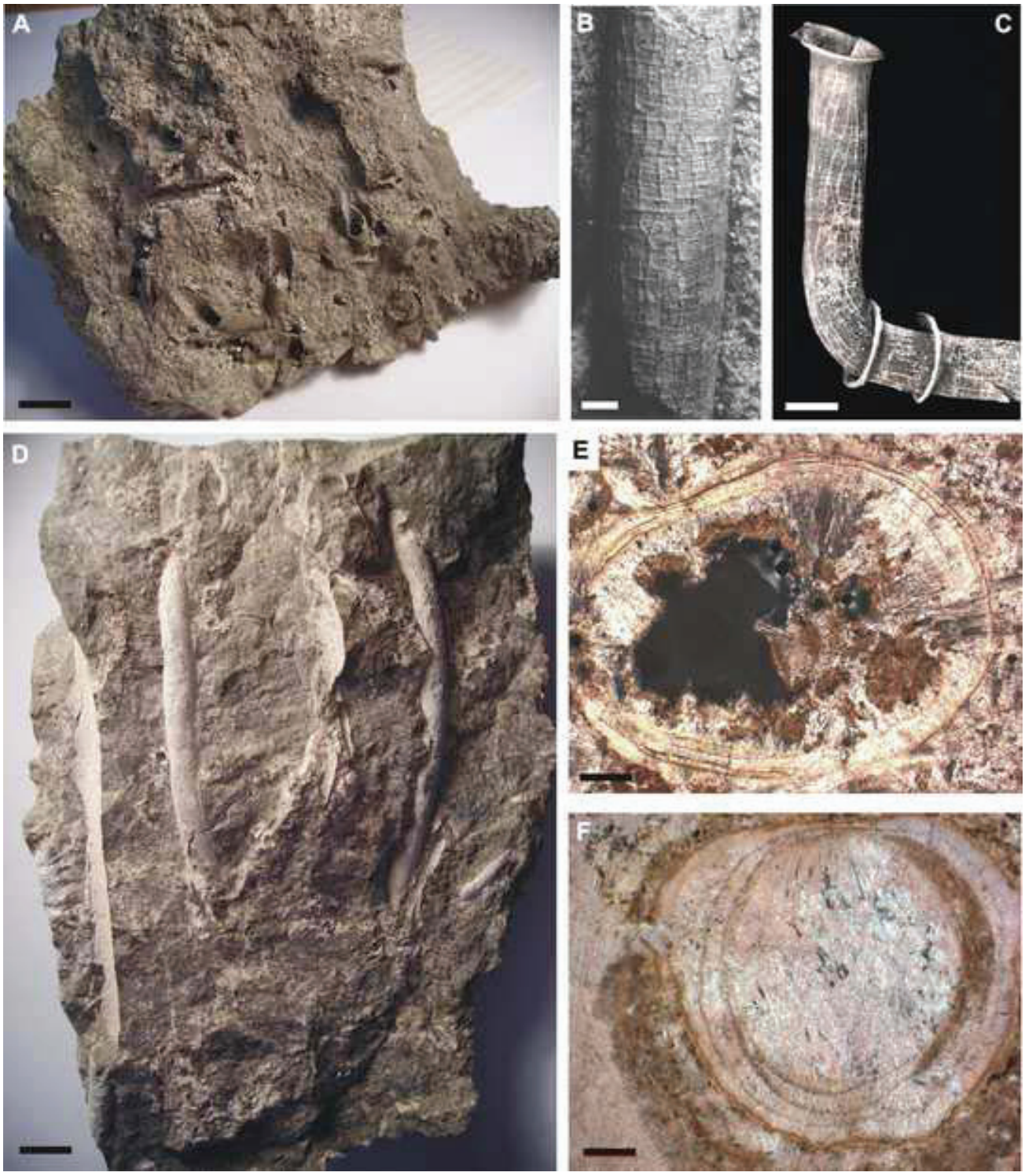

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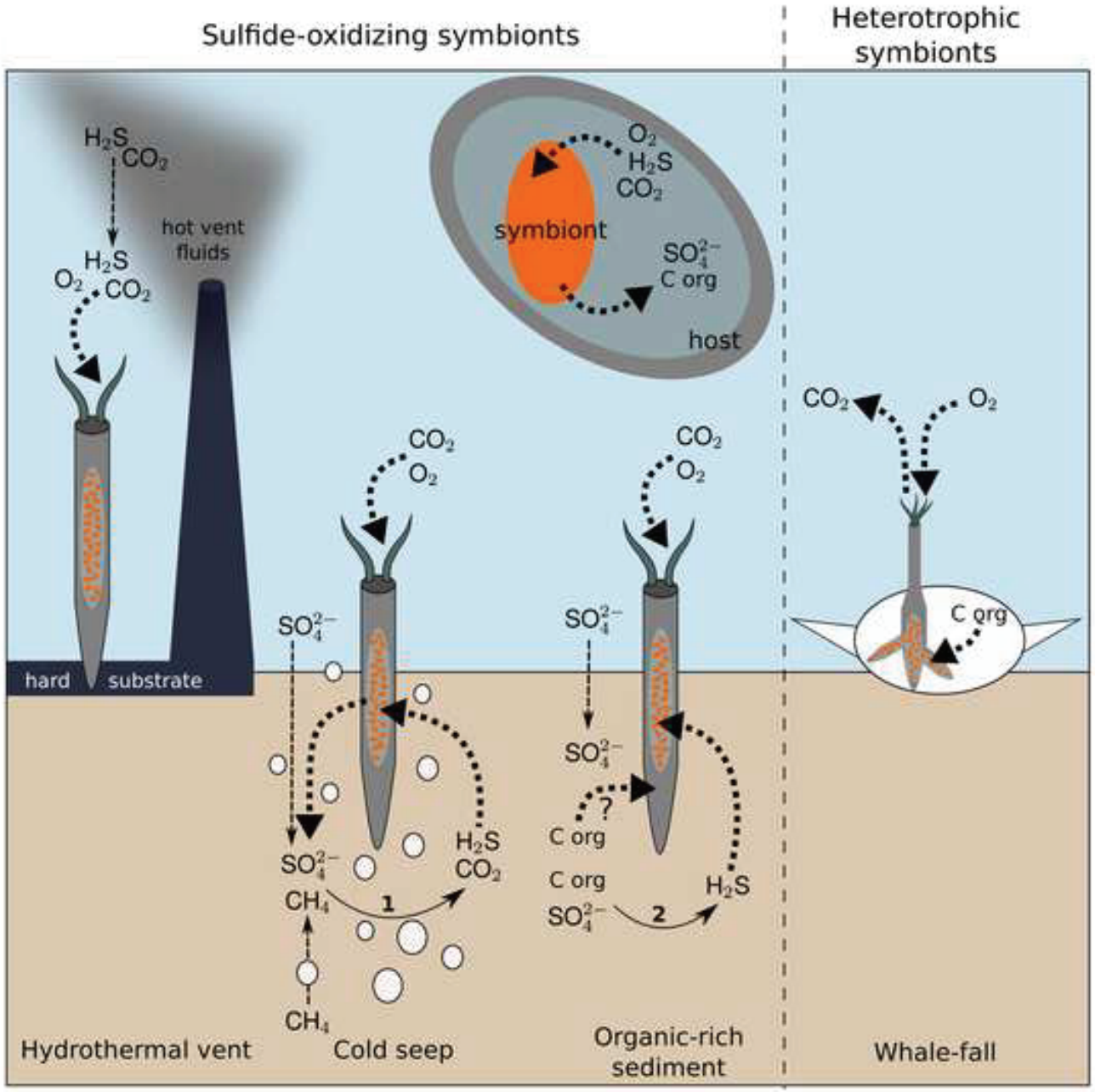

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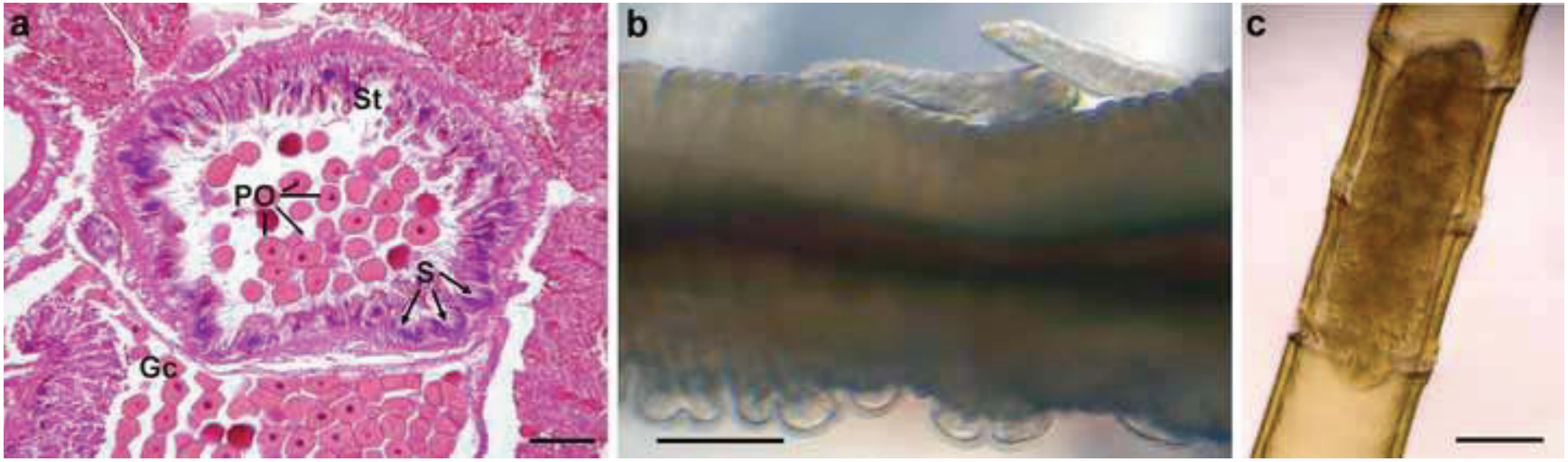

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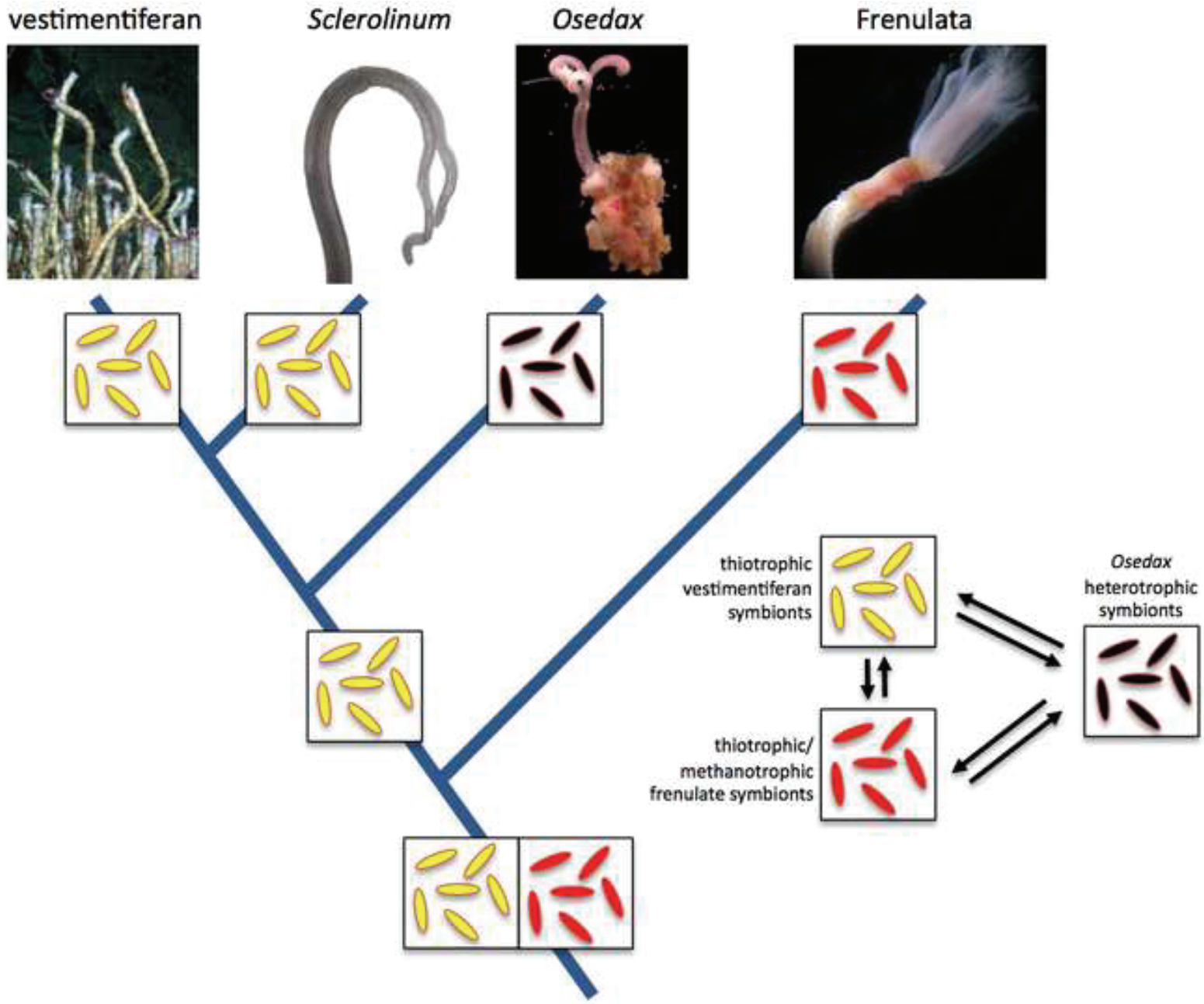

Manuscript II

High symbiont diversity in the bone-eating worm *Osedax mucofloris* from shallow whale-falls in the North Atlantic

Caroline Verna, Alban Ramette, Helena Wiklund, Thomas G. Dahlgren, Adrian G. Glover, Francoise Gaill, and Nicole Dubilier

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High symbiont diversity in the bone-eating worm *Osedax mucofloris* from shallow whale-falls in the North Atlantic

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Summary

Osedax **worms are whale-fall specialists that infiltrate whale bones with their root tissues. These are filled with endosymbiotic bacteria hypothesized to provide their hosts with nutrition by extracting organic compounds from the whale bones. We investigated the diversity and distribution of symbiotic bacteria in *Osedax mucofloris* from shallow-water whale-falls in the North Atlantic using comparative 16S rRNA sequence analysis and fluorescence *in situ* hybridization (FISH). We observed a higher diversity of endosymbionts than previously described from other *Osedax* species. Endosymbiont sequences fell into eight phylogenetically distinct clusters (with 91.4–98.9% similarity between clusters), and considerable microdiversity within clusters (99.5–99.7% similarity) was observed. Statistical tests revealed a highly significant effect of the host individual on endosymbiont diversity and distribution, with 68% of the variability between clusters and 40% of the variability within clusters explained by this effect. FISH analyses showed that most host individuals were dominated by endosymbionts from a single cluster, with endosymbionts from less abundant clusters generally confined to peripheral root tissues. The observed diversity and distribution patterns indicate that the endosymbionts are transmitted horizontally from the environment with repeated infection events occurring as the host root tissues grow into the whale bones.**

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Introduction

When whales die and sink to the seafloor, their decaying carcasses form oases at the bottom of the ocean that provide an energy source for species that are often highly specific to these unusual and ephemeral habitats (Smith *et al*., 1989; Baco and Smith, 2003). One of these whale-fall specialists is *Osedax*, the so-called 'bone-eating worm', that has a root-like structure at its posterior end with which it infiltrates the whale bones on which it grows (Rouse *et al*., 2004). These roots are filled with symbiotic bacteria that are hypothesized to degrade organic compounds in the whale bones to provide their host with nutrition (Goffredi *et al*., 2005; 2007). Phylogenetically, *Osedax* falls within the polychaete family Siboglinidae, a group of highly derived annelid worms that also includes the hydrothermal vent tubeworm *Riftia pachyptila*, and are characterized by the lack of a mouth and gut, and the presence of endosymbiotic bacteria (Ivanov, 1963; Cavanaugh *et al*., 1981; Jones, 1981; Pleijel *et al*., 2009).

One method to study whale-falls is to implant the remains of recently deceased stranded specimens, removing the problem of spending many hours looking for natural whale-falls on the seafloor (Smith and Baco, 2003; Dahlgren *et al*., 2006; Braby *et al*., 2007; Fujiwara *et al*., 2007). This approach has enabled scientists to discover numerous new *Osedax* species in the West and East Pacific, in the North Atlantic, and in the Antarctic with approximately 17 species currently described or under description (Rouse *et al*., 2004; Glover *et al*., 2005; Fujikura *et al*., 2006; Braby *et al*., 2007; Goffredi *et al*., 2007; Jones *et al*., 2008; Vrijenhoek *et al*., 2009). *Osedax mucofloris* was first discovered at a whale-fall close to the Swedish coast and is the only known *Osedax* species from the Atlantic (Glover *et al*., 2005; Dahlgren *et al*., 2006). It is also the only known *Osedax* species from very shallow waters (30–125 m), while all other species have been found at water depths below 224 m (Vrijenhoek *et al*., 2009).

The bacterial symbionts of *Osedax* have only been identified in five species, *O. japonicus* from the West Pacific off the coast of Japan (Miyazaki *et al*., 2008), and *O. frankpressi, O. rubiplumus, O. roseus* and *Osedax* sp. 'yellow collar' from Monterey Canyon off the coast of California in the East Pacific (Goffredi *et al*., 2005; 2007). These studies showed that all five *Osedax* species harbour endosymbionts that belong to the Oceanospirillales in the Gammaproteobacteria. While only a single endosymbiont 16S rRNA phylotype was found in the first host species studied, *O. frankpressi and O. rubiplumus* (Goffredi *et al*., 2005), subsequent studies revealed a higher diversity with two to three co-occurring endosymbiont lineages in *Osedax* sp. 'yellow collar' *O. roseus* and *O. frankpressi* (Goffredi *et al*., 2007). Intraspecific endosymbiont diversity was also observed with several phylotypes (unique 16S rRNA sequences) described from the same host species, or even the same individual (Goffredi *et al*., 2007). This endosymbiont diversity has, however, so far not been examined in detail with morphological methods such as fluorescence *in situ* hybridization (FISH), so that nothing is known about the distribution of these diverse endosymbiont phylotypes within a single individual.

In this study we describe the bacteria associated with *O. mucofloris* from shallow-water whale-falls off the coast of Sweden in the North Atlantic. Using comparative 16S rRNA sequence analysis and FISH we examined the diversity and phylogeny of the symbionts both within single *O. mucofloris* individuals as well as within the population. The results of these analyses together with multivariate statistical analyses were used to develop plausible explanations for the observed diversity and distribution patterns of symbionts in *O. mucofloris*.

Results

O. mucofloris *endosymbionts belong to eight phylogenetically distinct clusters*

Analyses of the 16S rRNA gene in 20 *O. mucofloris* individuals revealed that endosymbiont sequences belonging to the Oceanospirillales dominated the clone libraries of most individuals (Table S1) (sequences from other bacterial groups are described below). The endosymbiont sequences fell into eight phylogenetic groups called clusters A–H, with 99.5–99.7% sequence similarity within each cluster and 91.4–98.9% sequence similarity between clusters (Fig. 1). In most host individuals, 16S rRNA sequences from only a single cluster were found in the clone libraries (predominantly from Cluster A), but five individuals had sequences from two clusters, and one worm from three clusters (Fig. 1, Table S1).

The *O. mucofloris* endosymbiont clusters A–H did not form a monophyletic group, but were instead interspersed with 16S rRNA endosymbiont sequences from other *Osedax* species (Fig. 1). No geographic clustering of endosymbiont sequences was observed, with endosymbionts of *Osedax* species from the West Pacific (Japan) and East Pacific (California) more closely related to endosymbionts of *O. mucofloris* from the Atlantic Ocean than to those of other host species from their geographic region (Fig. 1).

O. mucofloris *endosymbiont microdiversity*

Within each *O. mucofloris* endosymbiont cluster, a pronounced microdiversity of 16S rRNA sequences was observed: 61 of the 76 fully sequenced endosymbiont clones were unique 16S rRNA phylotypes, that is differed by at least one nucleotide from all other endosymbiont sequences (Figs 1 and 2).

We examined if this microdiversity reflected the real diversity of endosymbiont 16S rRNA sequences in *O. mucofloris* or was instead caused by PCR and sequencing error. Substitution rates within endosymbiont clusters ranged from 8.2×10^{-4} to 2.7×10^{-3}. These values are 0.5 to 3 orders of magnitude higher than the error rates of the Taq polymerases we used for PCR amplifications (see *Experimental procedures*). Furthermore, most nucleotide differences occurred in variable regions of the 16S rRNA gene (Neefs *et al*., 1993; Pruesse *et al*., 2007), instead of randomly throughout the gene as one would expect from Taq and sequencing error. For example, the 25 unique phylotypes in Cluster A differed at 35 positions of which 31 were in variable regions. We therefore assume in the following that the diversity in *O. mucofloris* endosymbiont sequences is real and not an artifact of PCR or sequencing error.

Most host individuals had several phylotypes from the same cluster, with as many as nine unique endosymbiont phylotypes from a single cluster found in the 16S rRNA clone library of Individual Omu 3 (Fig. 2A). Most phylotypes were specific to the host individual, but some were shared by several individuals (Fig. 2).

Analyses of endosymbiont distribution with FISH

Oligonucleotide probes were designed for FISH analyses of the distribution of endosymbiont clusters within the *O. mucofloris* population as well as within single individuals. The designed probes enabled us to distinguish between endosymbionts from clusters A, B and/or C, D and/or E, F, and G and/or H (Table 1). FISH analyses of 12 *O. mucofloris* individuals showed that bacterial endosymbionts were present in the root tissues of all worms. In 10 of these, the endosymbionts could be clearly identified as belonging to clusters A, BC, DE or GH based on hybridization signals using the specific probes for these endo-

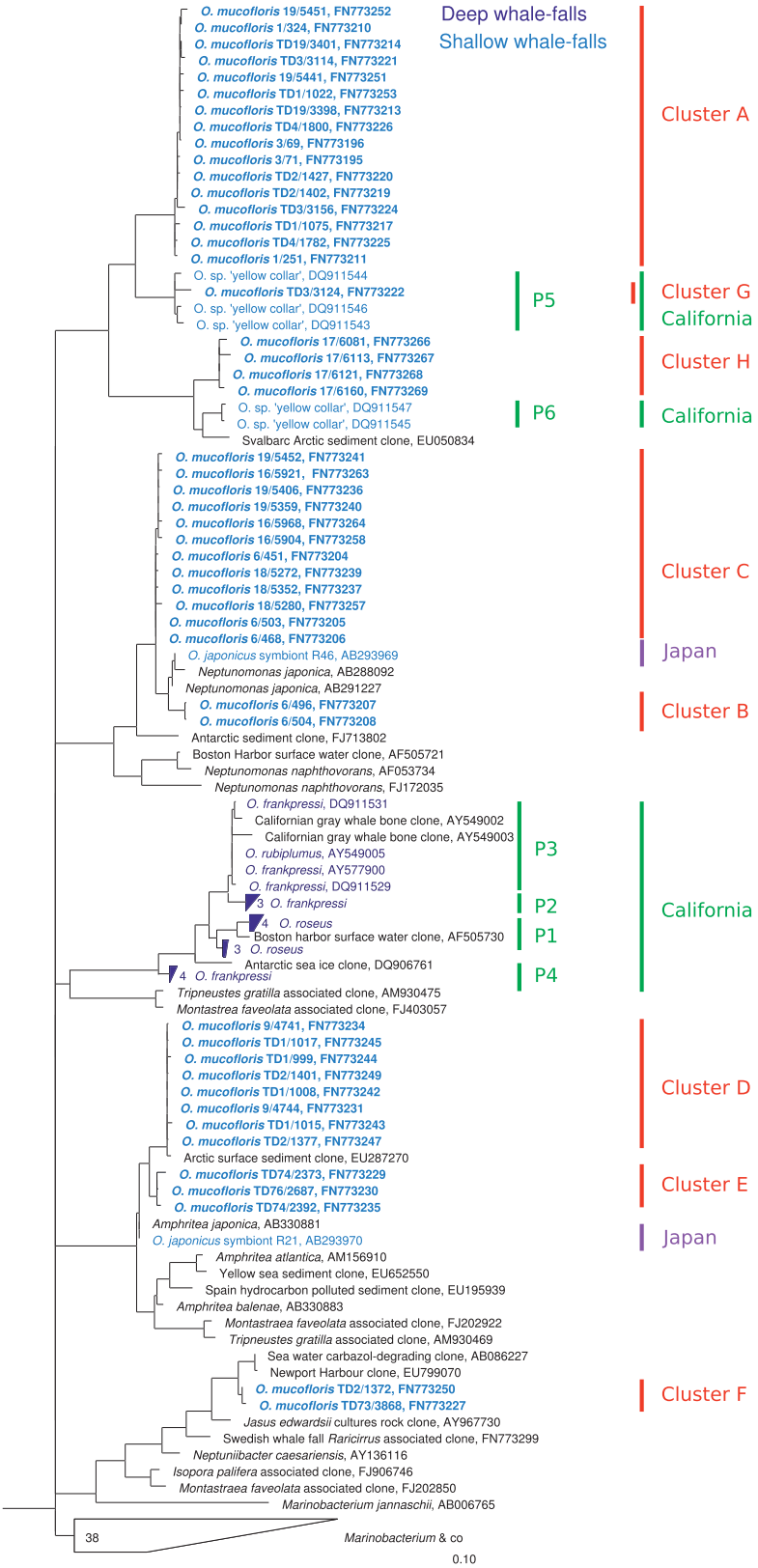


Fig. 1. 16S rRNA phylogeny of *O. mucofloris* Oceanospirillales endosymbionts. Consensus tree based on neighbour joining, maximum likelihood and maximum parsimony reconstructions. Sequences from this study are in bold. For clusters A and C, only some of the sequences used in the analyses are shown because of space limitations. Endosymbiont sequences from hosts collected below 1000 m are shown in dark blue and above 500 m in light blue. The colour bars on the right show the geographic location of the host collection site [red: North Atlantic, purple: West Pacific, green: East Pacific. For the Californian host species, the cluster names used by Goffredi *et al.*, 2007 (P1–P6) are shown in green]. The numbers following each *O. mucofloris* endosymbiont sequence show the individual number/clone number, and accession number. Scale bar = 0.10 estimated substitutions per site.

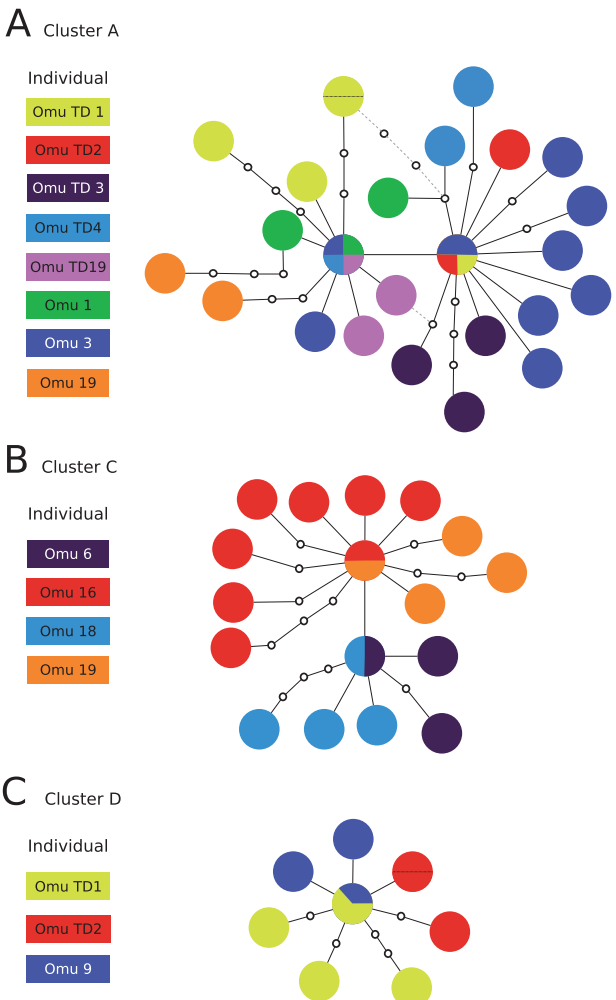


Fig. 2. Parsimony network of 16S rRNA sequences from endosymbiont clusters A, C and D in *O. mucofloris* individuals. Each unique 16S rRNA phylotypes is represented by a circle, lines connecting circles represent 1 nucleotide difference between phylotypes, and open circles on the lines show unsampled theoretical phylotypes. Colours show the host individual in which a given endosymbiont phylotype was found. If a phylotype was found more than once, the relative proportion of each colour within a circle shows how many times the phylotype occurred in each individual.

symbiont clusters (Table 2 and Table S1). Symbionts from Cluster F were not found with FISH, but symbionts from this cluster were very rare in the clone libraries and only found in two host individuals (Fig. 1, Table S1) (no tissues for FISH analyses were available from these two worms). In two individuals, the bacteria in the root tissues hybridized with the general *Osedax* endosymbiont probe (Gam140all in Table 1) but not with any of the probes for clusters A–H (Individuals Omu 2 and 4 in Table 2). This indicates that these worms had novel endosymbiont phylotypes not found in the 16S rRNA clone libraries of all other *O. mucofloris* worms. Unfortunately, no DNA from these individuals was available for examining the 16S rRNA genes of their bacterial endosymbionts.

The FISH analyses of the 10 *O. mucofloris* individuals for which endosymbiont clusters could be identified showed a similar distribution of endosymbionts as in the clone libraries (Table 2 and Table S1). Endosymbionts from Cluster A dominated the population, and in most individuals only endosymbionts from this cluster were found. Endosymbionts from clusters BC and DE were the second most dominant, and these co-occurred with Cluster A endosymbionts in two worms.

To better understand the distribution of endosymbionts within single host individuals, we analysed the nearly complete root tissues of three worms with FISH on serially cut sections (a small piece of root tissue from two worms was used for DNA analyses). The bacteriocytes of all three worms were dominated by endosymbionts from a single cluster (Figs 3 and 4). In the two individuals with endosymbionts from a second cluster, these secondary endosymbionts were only found occasionally in some bacteriocytes where they occurred in very low abundance (1–5 cells) (Figs 3D and 4D). In some peripheral root tissues, however, these secondary endosymbionts dominated the bacteriocytes and no other endosymbionts co-occurred with them (Figs 3 and 4).

Endosymbionts were also observed, although only very rarely, between the bacteriocyte layer and the epithelial cells, indicating that they occur outside of the bacteriocytes (Figs 3J and 4D). Symbionts were more regularly observed on the outside of the host in the mucus layer covering the root surface (Figs 3E and J and 4D). Within this mucus layer, symbionts from the cluster dominating the inside of the worm were the most abundant. However, the overall abundance of symbionts in the mucus layer was low in comparison to that of other bacteria.

Statistical analyses of endosymbiont distribution

We used distance-based redundancy analyses (db-RDA) to determine which factors could have influenced endosymbiont 16S rRNA diversity in the six *Osedax* host species for which enough data were available (see *Experimental procedures*). These analyses revealed a highly significant effect of host species and the water depth at which the hosts were collected (Table 3A). The influence of water depth on endosymbiont variance was supported by our 16S rRNA sequence analyses, showing that endosymbionts from *Osedax* species found in deep waters (> 1000 m) formed a monophyletic group and were phylogenetically distinct from endosymbionts of shallow-water hosts (< 500 m) (Fig. 1).

We also examined factors that could have affected endosymbiont diversity within the *O. mucofloris* population. No significant correlation was found between host

Table 1. FISH probes used in this study.

	Probe name	*O. mucofloris* target	Other bacterial targets	Probe sequence (5′–3′)	Position[a]	FA%[b]	Reference
O. mucofloris probes	Gam584A	Symbiont Cluster A		GTTGACTGACTTGACCAC	584	20–30	This study
	Gam579A	Symbiont Cluster A		ACTGACTTGACCACCTACG	579	20–30	This study
	Gam446A	Symbiont Cluster A		AAACGACACCCTTTCCTC	446	20–30	This study
	Gam823BC	Symbiont clusters B and C	*O. japonicus* symbiont R46, *Neptunomonas* bacteria, and few uncultured Oceanospirillales	GTTCCCCAACGGCTAGTT	823	20–30	This study
	Gam224DE	Symbiont clusters D and E	*O. japonicus* symbiont R21 and *Amphritea* bacteria and few uncultured Oceanospirillales	CCGACGCAGACUCAUCUA	224	20–30	This study
	Gam140F	Symbiont Cluster F	Uncultured Oceanospirillales in Cluster F	TCTGGCTTATCCCCCGCT	140	20–30	This study
	Sym435II	Symbiont clusters A, G and H	Some symbionts of *Osedax* sp. ‘yellow collar’ and few uncultured Oceanospirillales, 4500 hits outside Oceanospirillales mainly Gammaproteobacteria, 2000 Enterobacteriales, 1700 Alteromonadales	CTTTCCTCCTCGCTGAA	435	20–30	Modified from Goffredi *et al.* (2005)
	Sym435 (= Sym435I + Sym435III)	Symbiont clusters D and E	Symbionts of *O. frankpressi*, *O. rubiplumus*, *O. roseus*, *O. japonicus* symbiont R21, *Amphritea* bacteria	CTTTCCTCACWGCTGAA	435	20–30	Goffredi *et al.* (2007)
	Sym435I		Symbionts of *O. rubiplumus*, *O. roseus* and some *O. frankpressi*	CTTTCCTCACAGCTGAA	435	20–30	Goffredi *et al.* (2005)
	Sym435III	Symbiont clusters D and E	Symbionts of some *O. frankpressi*, *O. japonicus* symbiont R21, *Amphritea* bacteria and 400 hits in *Vibrio* bacteria	CTTTCCTCACTGCTGAA	435	20–30	Goffredi *et al.* (2007)
	Gam140all	Symbiont clusters A–H but not F	Symbionts of *O. frankpressi*, *O. rubiplumus*, some *O. roseus*, *Osedax* sp. ‘yellow collar’, *Neptunomonas* and *Amphritea* bacteria, and 3700 hits outside Oceanospirillales including 2000 Betaproteobacteria, 500 Alteromonadales	TCTGGGCTATCCCCCACT	140	20–30	This study
	Alf575	Trunk Alphaproteobacteria 1		CCAGCCCGCCTACGAACT	575	20–30	This study
	Alf189	Trunk Alphaproteobacteria 1		CTTTCACCCCCAAAATCC	189	20–30	This study
General group probes	Gam42a		Gammaproteobacteria	GCCTTCCCACATCGTTT	1027[c]	20–35	Manz *et al.* (1992)
	CF319a		Most Flavobacteria, some Bacteroidetes, some Sphingobacteria	TGGTCCGTGTCTCAGTAC	319	30	Manz *et al.* (1996)
	EPSY549		Most Epsilonproteobacteria, not *Arcobacter* cluster	CAGTGATTCCGAGTAACG	549	20–55	Lin *et al.* (2006)
	ARC1430		*Arcobacter* Epsilonproteobacteria	TTAGCATCCCCGCTTCGA	1430	30	Snaidr *et al.* (1997)
	EUBI-III		Most Bacteria	GCWGCCWCCCGTAGGWGT	338	20–40	Daims *et al.* (1999)
	Non338		Background control	ACTCCTACGGGAGGCAGC	338	20–35	Wallner *et al.* (1993)
Helpers and competitors	Helper-gam584		Helper for probe Gam584A	AAGCCCAGGGCTTTCACA		20–30	This study
	Comp-gam579G	Symbiont Cluster G	Competitor to probe Gam579A	ACTGACTCAGCCACCTACG		20–30	This study
	Comp-gam579DE	Symbiont clusters D and E	Competitor to probe Gam579A	ACTTAACAAACCGCCTACG		20–30	This study
	Comp-gam579BC	Symbiont clusters B and C	Competitor to probe Gam579A	ACTTACCAAGCCACCTACG		20–30	This study
	Helper-gam446A		Helper for probe Gam446A	TCACAGATGCCGTGTATT		20–30	This study
	Helper1-gam224		Helper for probe Gam224DE	ATAGCGAAAGGCCCGAAG		20–30	This study
	Helper2-gam224		Helper for probe Gam224DE	CCTCACCAACAAGCTAAT		20–30	This study

a. Position in the 16S rRNA of *E. coli.*
b. Formamide concentration in the FISH hybridization buffer in % (v/v).
c. Position in the 23S rRNA of *E. coli.*

Table 2. Distribution of symbiont clusters in *O. mucofloris* individuals using 16S rRNA clone analysis (left part of table) and FISH (right part of table).

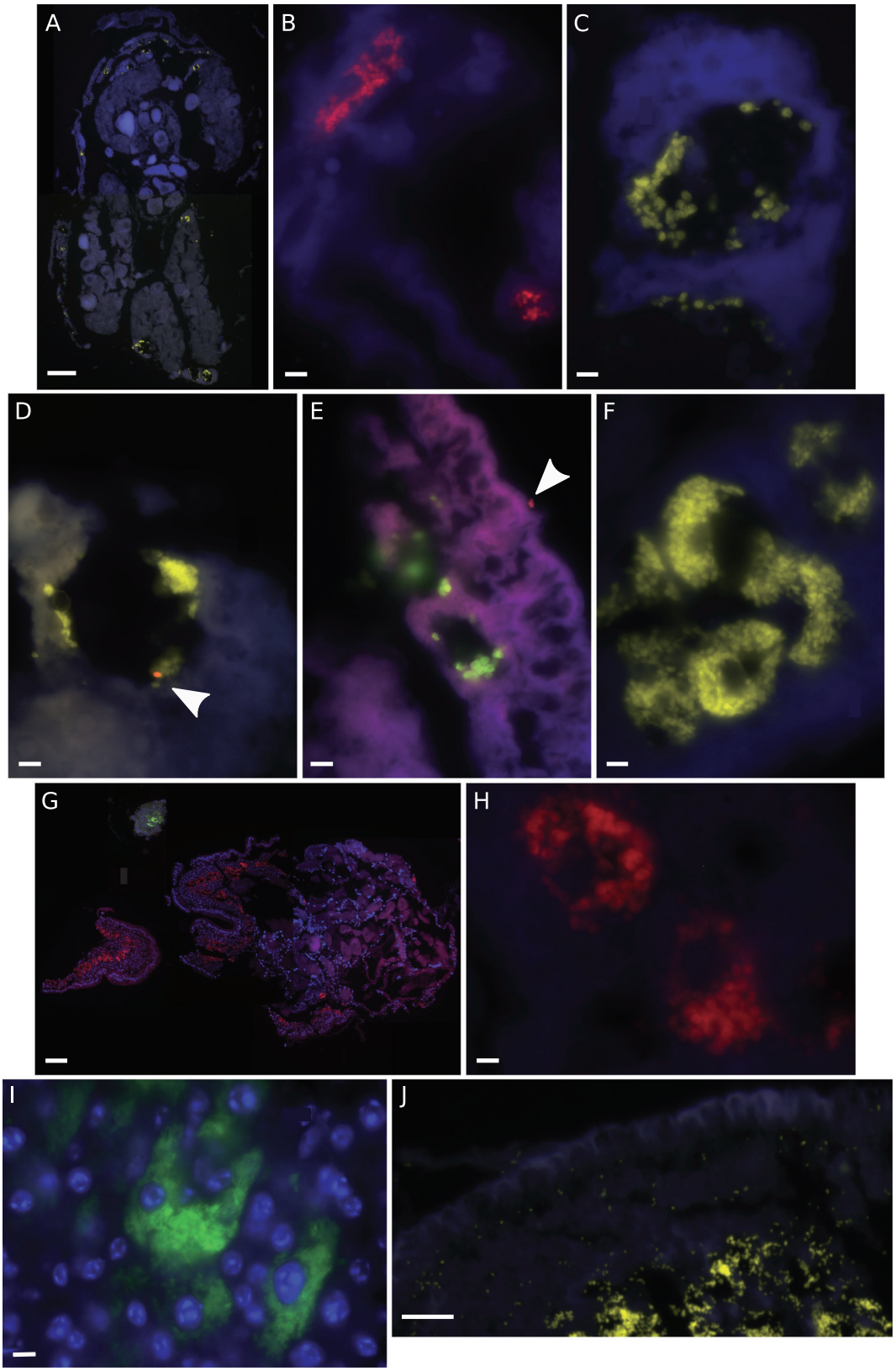
		Clone numbers found for each cluster								Symbiont clusters detected by FISH					
Whale and year	*O. mucofloris* individual	A	B	C	D	E	F	G	H	A	BC	DE	F	GH	Unknown
Minke 2004	Omu TD1	20			33										
	Omu TD2	29			3		1								
	Omu TD3	10						1							
	Omu TD4	12													
	Omu TD19	34													
Minke 2006	Omu 1	3													
	Omu 2														+++
	Omu 3	57													
	Omu 4														+++
	Omu 5									+++					
	Omu 6		28	9											
Minke 2007	Omu 7									+++					
	Omu 8									+++					
	Omu 9				40										
	Omu 10									+++					
	Omu 11									+++					
Minke 2008	Omu 15									+++					
	Omu 16			49						+[a]	+++	+			
	Omu 17								45					+++	
	Omu 18			35							+++				
	Omu 19	2		31						+++	+	+[a]			
Sperm 2006	Omu TD73						1								
	Omu TD74					3									
	Omu TD76			1		1									

a. Individual in which one symbiotic cell from the cluster was found.
+++, dominant symbiont cluster in the root tissue; +, symbiont cluster present in the root tissue.

and endosymbiont genetic distances based on the cytochrome c oxidase subunit I (COI) gene and 16S rRNA gene respectively ($P > 0.05$, Mantel test). Network analyses confirmed the lack of congruence between host COI haplotype and endosymbiont 16S rRNA phylotype (Fig. S1). In contrast, a very high proportion of endosymbiont diversity (68%) could be explained by host individual, that is each *O. mucofloris* individual generally had a specific endosymbiont population (Table 3B). At the minke whale-fall (one of the three whale-falls from which *O. mucofloris* were collected for this study, see *Experimental procedures*), *O. mucofloris* individuals were collected four times throughout 2004–2008 (Table 4), and there was a significant effect of sampling group on *O. mucofloris* endosymbiont diversity, although this only explained 31% of the variability (Table 3B).

Within *O. mucofloris* endosymbiont Cluster A, we observed a similar trend as in endosymbiont clusters A–H: there was no correlation between host COI haplotype and endosymbiont phylotype ($P > 0.05$, Mantel test), while a high proportion of endosymbiont diversity within Cluster A could be explained by host individual (40%) and to a lesser degree by sampling group (20%) (Table 3C). Network analyses of endosymbiont clusters A, C and D

Fig. 3. Endosymbiont distribution in *O. mucofloris* root tissues based on fluorescence *in situ* hybridization (FISH) analyses. FISH with probes specific to endosymbionts from Cluster A in yellow, Cluster BC in red and Cluster DE in green except where noted elsewhere.
A–E. Individual Omu 19 was dominated by endosymbionts from Cluster A [shown in (A) and (C) with probe Gam548A], while endosymbionts from Cluster BC were only found in high abundance in a peripheral part of the root tissues [shown in (C) with probe Gam823BC]. (D) A single endosymbiont from Cluster BC (shown with probe Gam823BC; arrowhead) was present in a bacteriocyte dominated by endosymbionts from Cluster A (EUBI-III probe). (E) A symbiont from Cluster DE on the root surface (arrowhead, shown with probe Gam224DE in red), with endosymbionts from Cluster A inside the root bacteriocytes (probe EUBI-III).
F. In Individual Omu 8, only endosymbionts from Cluster A were found (shown with probe Gam584A).
G–I. Individual Omu 16 was dominated by endosymbionts from Cluster BC [shown with probe Gam823BC in (G) and (H)], while endosymbionts from Cluster DE were only abundant in one of the root tips [shown in (G) and (I) with probe Gam224DE, host nuclei are stained blue with DAPI].
J. Individual Omu 16 with endosymbionts from Cluster BC in high abundance in the bacteriocytes, and in low abundance in the root tissues between the bacteriocytes and the worm's surface (shown in yellow with probe Gam823BC). Scale bars: (A) and (G) = 100 μm (J) = 50 μm (B–I) = 5 μm except (H) = 2.5 μm.


A
B
C
D
E
F
G
H
I
J

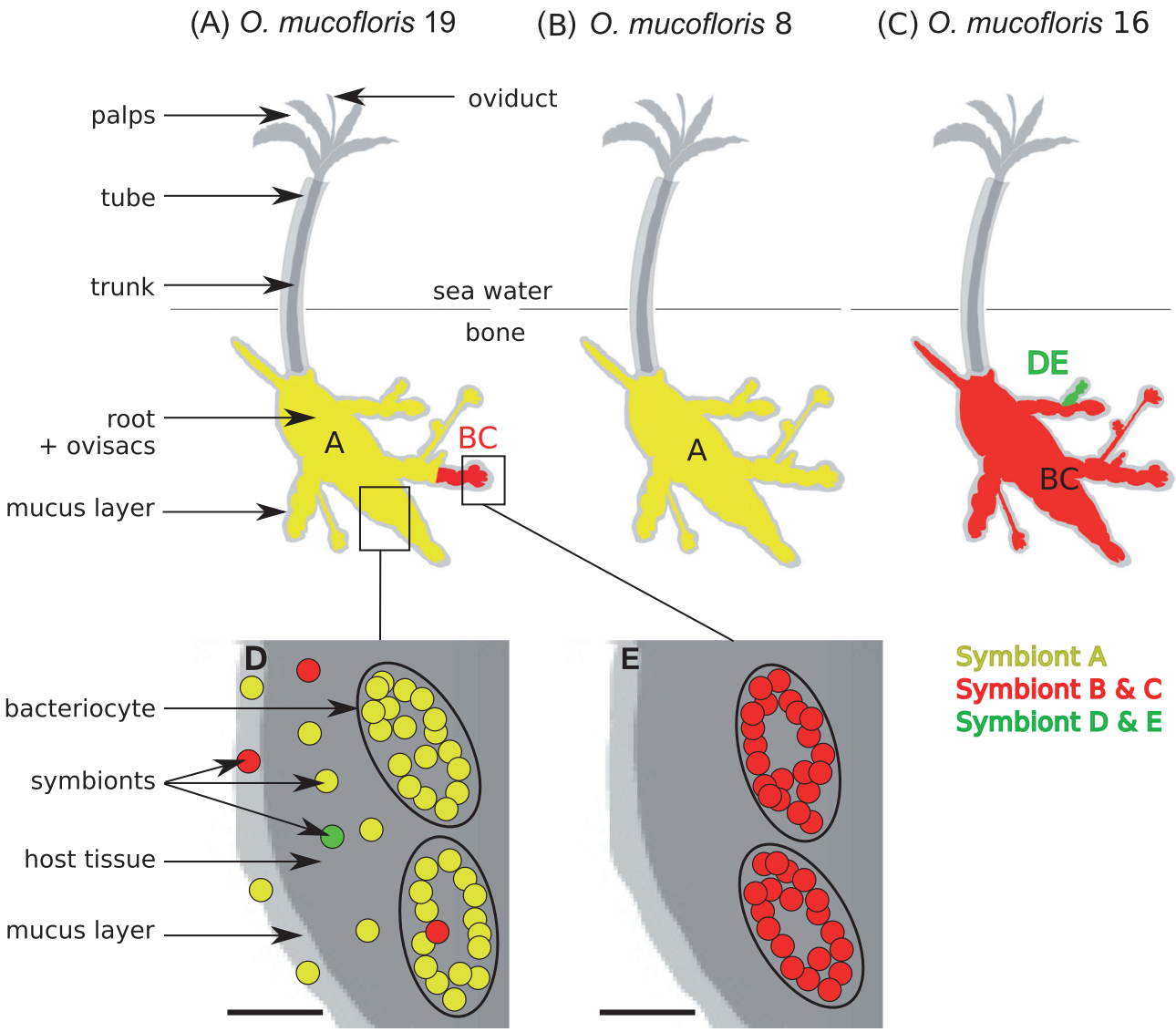


Fig. 4. Schematic diagram of endosymbiont distribution in *O. mucofloris* individuals Omu 19 (A), Omu 8 (B) and Omu 16 (C) based on FISH analyses. Colour scheme shows endosymbionts from Cluster A in yellow, from Cluster BC in red, and from Cluster DE in green. All three worms were dominated by endosymbionts from a single cluster (Cluster A in Omu 19 and 8, and Cluster BC in Omu 16). In two worms, endosymbionts from a second cluster were found, but only in the root tips (Cluster BC in Omu 19 and Cluster DE in Omu 16).
D. The bacteriocytes in most parts of the root tissues of Omu 19 were dominated by endosymbionts from Cluster A, with endosymbionts from Cluster BC found in low abundance in only some bacteriocytes.
E. In one root tip, all bacteriocytes contained endosymbionts from clusters BC. Scale bars: (D) and (E) = 5 μm.

confirmed the strong effect of host individual on endosymbiont diversity (Fig. 2). For example, of the 17 unique 16S rRNA phylotypes in Cluster C, 15 of these were host specific and only two were shared between two host individuals (Fig. 2B).

Other bacteria associated with O. mucofloris

In addition to the endosymbionts in the root tissues, other bacteria were also associated with *O. mucofloris*. 16S rRNA sequences belonging to the Bacteroidetes and Alphaproteobacteria were found in the clone libraries of 17 and 8 host individuals respectively (Table S1). In contrast to the Oceanospirillales endosymbionts in the root tissues, we observed much less heterogeneity in the Bacteriodetes and Alphaproteobacteria sequences (Fig. S2A). The diversity of Epsilonproteobacteria associated with *O. mucofloris* was higher, and included sequences related to *Arcobacter* and *Sulfurospirillum arcachonense* (Table S1, Fig. S2B). Bacteria closely related to the alphaproteobacterial, epsilonproteobacterial and Bacteroidetes sequences included (i) bacteria asso-

Table 3. Statistical analysis of 16S rRNA sequence variability in *Osedax* symbionts using distance-based redundancy analysis with nested designs.

	d.f.	*F*-ratio	Explained variation (%)
(A) *Osedax* symbionts (6 species)			
Depth (< 500 m or > 1000 m)	1	23.17***	18.51
Host species	5	6.97***	26.22
(B) *O. mucofloris* symbionts (clusters A–H)			
Sampling group	4	10.01***	30.90
O. mucofloris individual	15	10.10***	67.60
(C) *O. mucofloris* Cluster A symbionts[a]			
Sampling group	2	3.60**	20.44
O. mucofloris individual	7	2.19*	40.00

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.
a. Of the 8 symbiont clusters found in *O. mucofloris*, only Cluster A contained enough sequences for statistical analyses.
d.f., degrees of freedom.

ciated with other *Osedax* species (Goffredi *et al*., 2005; 2007); (ii) free-living bacteria found at whale-falls (Goffredi *et al*., 2005; Tringe *et al*., 2005; Goffredi and Orphan, 2010); (iii) free-living bacteria from the Tjärnö aquarium in Sweden where the whale bones were kept (Grünke *et al*., 2010); and (iv) symbionts of other marine invertebrates (Fig. S2B).

The FISH with the general probe for Bacteria (Table 1), DAPI staining and scanning electron microscopy revealed abundant bacteria on the root and trunk surfaces as well as in and on the mucus tube (Fig. S3). Bacteroidetes, detected with the general CF319a probe, were dominant on the root surface, and abundant on the trunk surface as well as on and within the mucus tube (Fig. S3). FISH with probes specific to the alphaproteobacterial sequence Alpha 1 (Fig. S2A, Table 1) showed that this sequence originated from bacteria colonizing the trunk surface of *O. mucofloris* (data not shown). A probe targeting epsilonproteobacterial *Arcobacter* species (Arc1430) only rarely revealed bacteria on the worm trunk and root surface

Table 4. Summary of sampling sites and dates of the *O. mucofloris* individuals investigated in this study.

O. mucofloris individual	Site	Sampling date	FISH	16S rRNA clone library	COI
Omu TD1	Minke	August 2004		X	7
Omu TD2	Minke	August 2004		X	4
Omu TD3	Minke	August 2004		X	1
Omu TD4	Minke	August 2004		X	13
Omu TD8	Minke	August 2004		X	14
Omu TD18	Minke	August 2004		X	11
Omu TD19	Minke	August 2004		X	12
Omu 1	Minke	October 2006		X	18
Omu 2	Minke	October 2006	X		5
Omu 3	Minke	October 2006		X	4
Omu 4	Minke	October 2006	X		18
Omu 5	Minke	October 2006	X		17
Omu 6	Minke	October 2006		X	20
Omu 7	Minke	August 2007	X		13
Omu 8	Minke	August 2007	X		7
Omu 9	Minke	August 2007		X	1
Omu 10	Minke	August 2007	X		7
Omu 11	Minke	August 2007	X		19
Omu 15	Minke	May 2008	X		22
Omu 16	Minke	May 2008	X	X	23
Omu 17	Minke	May 2008	X	X	4
Omu 18	Minke	May 2008	X	X	1
Omu 19	Minke	May 2008	X	X	1
Omu TD42	Pilot	July 2005		X	10
Omu TD73	Sperm	February 2006		X	1
Omu TD74	Sperm	February 2006		X	2
Omu TD75	Sperm	February 2006		X	3
Omu TD76	Sperm	February 2006		X	4

Each number in the last column (COI: cytochrome c oxidase subunit I) corresponds to a unique COI haplotype (e.g. COI haplotype 7 was found in Omu TD1, Omu 8 and Omu 10).

(data not shown), while no signal was observed with a general probe targeting many Epsilonproteobacteria including the *Sulfurospirillum* but not the *Arcobacter* (EPSY549; Table 1).

Discussion

General diversity of bacteria associated with O. mucofloris

In addition to the Oceanospirillales endosymbionts in *O. mucofloris* root tissues, a diverse microbial community was associated with these hosts, particularly with their tubes and the mucus layer covering their root tissues. In contrast to the intracellular endosymbionts, these other bacteria were always epibiotic, i.e. on the worm's surface and never observed within its body or cells. The same morphological differentiation between Oceanospirillales endosymbionts and epibiotic bacteria was also observed in other *Osedax* host species (Fujikura *et al*., 2006; Goffredi *et al*., 2007).

It appears as if the dominant members of the *O. mucofloris* epibiotic community are more than just casual associates of these hosts. They were regularly found in numerous *O. mucofloris* individuals collected from different sites and at different sampling times throughout 2004–2008 (Table S1), indicating their pervasiveness within the host population and persistence throughout time. Bacteria closely related to the epsilonproteobacterial and Bacteriodetes 1 epibionts of *O. mucofloris* were also found in the 16S rRNA clone libraries of other *Osedax* species from Monterey Canyon off the coast of California (Goffredi *et al*., 2005; 2007). The recurrent presence of epibiotic bacteria on *Osedax* species from both shallow and deep waters of the Pacific and Atlantic suggests that these may be regular members of the bacterial community associated with these hosts. The role of these epibionts is not currently known. Many of the *Osedax* associated Epsilonproteobacteria and Bacteriodetes fall in clades that include bacteria found on whale bones or sediments surrounding the whale bones (Fig. S2A and B), indicating a general affinity of these bacteria for these organic-rich, reducing environments (Goffredi and Orphan, 2010).

Endosymbionts from deep-water Osedax *hosts are phylogenetically distinct from those of shallow-water hosts*

In our phylogenetic analyses of endosymbiont diversity in the 6 *Osedax* host species for which 16S rRNA sequence data are available, there was no congruence between endosymbiont phylogeny and host geography (Fig. 1). In contrast, there was a clear phylogenetic grouping of endosymbionts from hosts found at water depths below 1000 m (Fig. 1), and statistical analyses confirmed the significance of water depth on endosymbiont variability (Table 3). The hosts from the deep-water clade, *O. frankpressi*, *O. rubiplumus* and *O. roseus* are not phylogenetically more closely related to each other than to the other *Osedax* species examined in this study (Vrijenhoek *et al*., 2009). It is therefore unlikely that host phylogeny affected the observed clustering of endosymbionts from deep-water *Osedax* species. An alternative explanation, based on the assumption of horizontal endosymbiont transmission (see below) is that the distribution of the free-living stages of *Osedax* endosymbionts is affected by depth. The influence of water depth on microbial population structure is well described (DeLong *et al*., 2006; Konstantinidis *et al*., 2009), and it is possible that deep-water hosts take up their endosymbionts from an environmental population that is phylogenetically distinct from the shallow-water population. Vestimentiferan tubeworms are known to take up their endosymbionts from the environment (Nussbaumer *et al*., 2006), and in cold seep vestimentiferans there is also evidence that water depth affects endosymbiont phylogeny (McMullin *et al*., 2003).

Endosymbiont diversity in O. mucofloris *compared with other* Osedax *host species*

The diversity of Oceanospirillales endosymbionts in *O. mucofloris* is higher than previously reported from other *Osedax* host species. Most *Osedax* species harbour endosymbionts from two phylogenetically distinct lineages with the highest diversity described in *O. frankpressi* with three endosymbiont lineages (Goffredi *et al*., 2007). The presence in *O. mucofloris* of 8 distinct endosymbiont lineages (clusters A–H in Fig. 1) is thus unprecedented among the known *Osedax* associations. Given the strong effect of the host individual on endosymbiont diversity (see below), the higher number of host individuals examined in this study in comparison to previous studies could explain the higher diversity found in *O. mucofloris*. Alternatively, it is possible that shallow-water *Osedax* species have a higher diversity of endosymbionts than those from deeper waters. Of the six *Osedax* species for which endosymbiont sequences are available, *O. mucofloris* is the only species collected from the euphotic zone (125 m in this study), while all other species were found at depths below 225 m (Goffredi *et al*., 2007; Miyazaki *et al*., 2008). In other siboglinid hosts, water depth might also affect endosymbiont diversity. McMullin and colleagues (2003) predicted that shallow-water vestimentiferan tubeworms have a higher diversity of endosymbionts than their deep-water relatives, and in the frenulate tubeworms, endosymbiont 16S rRNA sequence diversity was considerably higher in

hosts collected at shallower water depths (*Oligobrachia mashikoi* from 25 m, and *Siboglinum fiordicum* from 30–250 m water depth) than in a species from deeper waters (*Oligobrachia haakonmosbiensis* from 1250 m) (Kubota *et al*., 2007; Lösekann *et al*., 2008; Thornhill *et al*., 2008). However, only three host individuals were examined in the Lösekann and colleagues' (2008) study, and the analysis of more specimens might have revealed a higher diversity.

Endosymbiont uptake and distribution in O. mucofloris

Several results from this study support the assumption that endosymbionts are transmitted horizontally, that is taken up from the environment by *O. mucofloris*, as assumed previously for other *Osedax* species (Goffredi *et al*., 2007; Rouse *et al*., 2009) and proven in other siboglinid worms (Nussbaumer *et al*., 2006). The high diversity of endosymbionts in *O. mucofloris* is consistent with horizontal transmission as diversity is low in most vertically transmitted symbioses (Bright and Bulgheresi, 2010). In our FISH analyses of 12 worms, including serial sectioning through three of these, we never observed bacteria in the eggs or in the sperm of the single male we found. Finally, there was no congruence between the genetic distances of endosymbionts and hosts (Fig. S1), a common feature of horizontally transmitted symbioses (McMullin *et al*., 2003; Won *et al*., 2008; Bright and Bulgheresi, 2010).

Assuming the environmental transmission of endosymbionts in *O. mucofloris*, our results provide support for the following scenario. Most host individuals take up endosymbionts from only a single cluster, most commonly from Cluster A (Table 2). The high intraclustal microdiversity of endosymbionts within each host individual (Fig. 2) indicates either the uptake of a large pool of genetically heterogeneous endosymbionts during a single infection event, or repeated infection events during the individual's lifetime. Support for the latter comes from our FISH analyses showing the presence of symbionts on the worm's surface as well as in the epithelial tissues between the worm's surface and the bacteriocytes (Fig. 3J and H). (For the latter, ultrastructural evidence is needed to conclusively prove that the endosymbionts occur outside of the bacteriocytes and not inside unusually elongated bacteriocytes that extend into the epithelial tissues.) The distribution of endosymbionts from two different clusters within *O. mucofloris* individuals provides additional support for repeated infection events. The dominant endosymbionts from the primary cluster were found throughout most of the root tissues while the less abundant endosymbionts from the secondary cluster only occurred in high numbers in some peripheral root tissues (Figs 3 and 4). The most parsimonious explanation for this distribution is that the primary endosymbionts colonize the worm at an early developmental stage when the root tissues are still small, while the secondary endosymbionts enter the peripheral root tissues later as these grow into the bones. If all endosymbionts were taken up during a single event, one would expect a more even distribution of the primary and secondary endosymbiont throughout the root tissues. Goffredi and colleagues (2007) also found indirect evidence for repeated infection events during the lifetime of an *Osedax* host individual: juvenile *O. frankpressi* individuals had only a single endosymbiont phylotype, while adults harboured several endosymbiont phylotypes. The continuous uptake of endosymbionts throughout the lifetime of a host individual, has to our knowledge not been previously described in animals with intracellular bacterial endosymbionts, but is well known from corals that harbour multiple clades of symbiotic algae (Little *et al*., 2004; Stat *et al*., 2006). In an intriguing parallel to the *Osedax* symbiosis, continuous infection events are also known from the bacterial symbioses of hortwort thalli and leguminous plants, hosts that also have roots which continuously grow throughout their lifetime (Bright and Bulgheresi, 2010).

To each its own: endosymbiont diversity is determined at the level of the host individual

Which factors can best explain endosymbiont diversity in *O. mucofloris*? Our statistical analyses showed that two variables significantly affected endosymbiont diversity: (i) sampling group and (ii) host individual (Table 3B and C).

i. The variable sampling group was defined as the four collections of *O. mucofloris* individuals from the minke whale bones in 2004, 2006, 2007 and 2008 (Table 4). This variable explained 31% of the endosymbiont diversity in the eight endosymbiont clusters A–H, and 20% within Cluster A (Table 3). Several explanations for this effect are possible, including (i) the observed effect is an artefact caused by the low number of individuals available for each sampling group (1–5 per group; Table 2); (ii) the free-living population from which the endosymbionts were taken up varied over time, either randomly or because of environmental changes in the chemical and biological milieu at the whale-fall; and (iii) choice of endosymbionts by host individuals varied over time either stochastically or because of specific selection processes driven by factors such as changes in the host's environment. In coral symbioses, it is well known that changes in environmental conditions such as temperature or light can affect the composition of the zooxanthellae symbiont community (Rowan, 2004; Stat *et al*., 2006; LaJeunesse *et al*., 2010).

ii. The strongest factor influencing endosymbiont diversity and distribution in the *O. mucofloris* population appears to be the host individuals themselves. This effect explained 68% of the variability in the endosymbiont clusters A–H, and 40% within Cluster A (Table 3B and C). Our network analyses confirmed this effect on intracluster variability and showed that most endosymbiont phylotypes were specific to a given host individual and very few were shared between individuals (Fig. 2). In the siboglinid tubeworms *O. mashikoi* and *S. fiordicum* that also have heterogeneous endosymbiont communities, each host individual is dominated by only a single 16S rRNA endosymbiont phylotype (Kubota *et al*., 2007; Thornhill *et al*., 2008). This suggests that in these associations there is also a strong effect of the host individual on endosymbiont distribution within the host population.

How can we explain the observed effect of the host individual on endosymbiont diversity? As discussed above for the variable sampling group, several scenarios are possible. In the first scenario, each host individual takes up the dominant endosymbiont at a given time or a given location. Uptake of endosymbionts from the surrounding waters would be unlikely in this scenario, because mixing processes would prevent the establishment of spatially or temporally separated bacterial populations. In contrast, free-living stages of the endosymbiont could easily be structured if they occur on or in the bone, where clonal growth could occur without physical disruption. In the second scenario, host individuals are exposed to a genetically heterogeneous pool of endosymbionts from which they take up a limited number of endosymbiont phylotypes. Because of the large size of the free-living endosymbiont population in comparison to the host population, any given host individual ends up with a specific assemblage of endosymbiont phylotypes that differs from that of its neighbour. In a third scenario, one could imagine that endosymbionts from different clusters compete with each other during host colonization, leading to their mutual exclusion in most bacteriocytes (Fig. 3), while endosymbionts from the same cluster are genetically similar enough to allow their co-occurrence within a host individual. These scenarios are not mutually exclusive and could all be involved to varying degrees in determining the observed diversity at the level of the host individual.

Conclusions and outlook

In this study, we described a number of factors that could influence endosymbiont diversity and distribution in *O. mucofloris*, including host specificity, endosymbiont competition and the genetic variability of the free-living endosymbiont population. Remarkably, little is currently known about these factors in *Osedax* and other siboglinid worms. Future studies that could provide a better understanding of these factors include in-depth analyses of the free-living endosymbiont population over time and space, high-throughput analyses of the genetic diversity of endosymbionts in high numbers of host individuals from different developmental stages, and detailed analyses of endosymbiont uptake in the worms with FISH and electron microscopy. The relative easiness with which these shallow-water hosts and their environment can be sampled and the ability to maintain *O. mucofloris* for extended periods in aquaria make them an ideal model for examining how symbiont diversity is established and maintained in siboglinid worms.

Experimental procedures

Sample collection and fixation

A total of 28 *O. mucofloris* individuals were examined in this study of which all 28 were used for COI analyses, 20 for 16S rRNA gene analyses, and 12 for FISH analyses (Table 4).

The *O. mucofloris* individuals were collected from three whale-falls:

i. The first whale-fall was the carcass of a minke whale, *Balaenoptera acutorostrata* Lacépède, 1804, deployed in the Kosterfjord, Sweden (58°53.1′N; 11°06.4′E) at 125 m depth in October 2003 (Dahlgren *et al*., 2006). Whale vertebrate bones were collected in 2004, 2006, 2007 and 2008 (Table 4) with a Phantom XL and Speere Sub-Fighter Remotely Operated Vehicle and transferred to aquaria at the Tjärnö laboratory (Sweden) with flow-through seawater at 8.0°C for hours to months (Dahlgren *et al*., 2006).
ii. The second whale-fall was the carcass of a pilot whale, *Globicephala melas* Traill 1809, also deployed in the Kosterfjord (58°53′09″N; 11°06′14″E) at 30 m depth in January 2005 (Dahlgren *et al*., 2006). Whale vertebrate bones were collected and transferred to aquaria as described above in July 2005.
iii. The location of the third whale-fall is unknown. A sperm whale bone was found by fishermen in coastal waters off Tjärnö in February 2006. No live *Osedax* were found but dead worms were picked from the bones and later identified as *O. mucofloris* using cytochrome c oxidase subunit I (COI) gene analyses (Table 4).

Samples for DNA analyses were fixed and stored in 96% ethanol or frozen and stored at –80°C. Samples for FISH were fixed at 4°C for 1–20 h in 1–4% formaldehyde in 1× phosphate buffered saline (PBS), washed three times in 1× PBS and stored in 0.5× PBS/50% ethanol at 4°C.

DNA extraction, PCR amplification, cloning and sequencing

DNA was extracted with the DNAeasy Tissue kit (Qiagen, Hilden, Germany). The COI gene was amplified with primers

OsCO1f and OsCO1r (Glover *et al*., 2005) using the following PCR cycling conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, followed by a final elongation step at 72°C for 10 min. PCR products (about 500 bp) were sequenced directly (both strands) as described below.

Bacterial 16S rRNA genes were amplified with primers GM3F and GM4R (Lane, 1991; modified in Muyzer *et al*., 1995) using the following PCR cycling conditions: initial denaturation at 94°C for 5 min, followed by 20–25 cycles at 94°C for 1 min, 43°C for 1:30 min, and 72°C for 2 min, followed by a final elongation step at 72°C for 10 min. Two types of Taq were used, recombinant Taq DNA polymerase (5 Prime, Gaithersburg, MD, USA), and for samples that did not amplify easily, the high fidelity DNA polymerase Takara ex Taq polymerase (Takara Bio, Shiga, Japan). The error rates for these DNA polymerases are 2.1×10^{-4}–1×10^{-6} for recombinant Taq polymerase (Tindall and Kunkel, 1988; Hengen, 1995; Li *et al*., 2006) and 8.7×10^{-6} for Takara ex Taq (Takara Bio). At least five parallel PCR reactions from each host individual were pooled to minimize the effects of PCR bias. PCR products were purified with the QiaQuick PCR Purification Kit (Qiagen, Hilden, Germany), loaded on a 1% agarose gel, and bands of the correct size (about 1500 bp) excised and purified using the Qiaquick Gel Purification protocol (Qiagen). For cloning, PCR products were ligated with the PCR4 TOPO vector (Invitrogen, Carlsbad, CA, USA) and transformed into *E. coli* TOP10 cells (Invitrogen) according to the manufacturer's recommendations. Clones were checked for the correct insert size by PCR with vector primers M13F and M13R (Invitrogen). Partial sequencing of the 16S rRNA gene was performed with primer 907R (Lane *et al*., 1985) and representative clone sequences chosen for full sequencing. For these clones, plasmid preparations were grown overnight and purified with the Qiaprep Spin miniprep kit (Qiagen). The plasmid inserts were fully sequenced in both directions using the following primers M13F and M13R (Invitrogen), 1114F (Lane, 1991), with GM5F (Lane, 1991; modified in Muyzer *et al*., 1993) and GM4R (Lane, 1991; modified in Muyzer *et al*., 1995) or with GM1F (Lane, 1991) and GM12R (Buchholz-Cleven *et al*., 1997). Sequencing was done with the Bigdye v3.1 cycle sequencing kit (Applied Biosystems) and the sequencer 3130xL genetic analyser (Applied Biosystems). Full sequences were assembled with DNA Baser Sequence Assembler v2.x (2009) (HeracleSoftware, http://www.DnaBaser.com/index.html). Sequences were checked manually after alignment in ARB (Ludwig *et al*., 2004) using the Silva database (Pruesse *et al*., 2007).

Phylogenetic analyses

Of the 448 partially sequenced (about 500–900 bp) 16S rRNA endosymbiont clones, 76 representative endosymbiont clones were fully sequenced, and only these were used for phylogenetic and statistical analyses (see below). Phylogenetic trees were calculated with the ARB software package (Ludwig *et al*., 2004) using neighbour-joining, maximum likelihood (phyML) and maximum parsimony analysis with filters that exclude highly variable regions and gap regions. For tree reconstructions, only 16S rRNA sequences > 1200 bp were used. Tree topologies derived from the different approaches were compared and a consensus tree generated. Branching orders that were not supported by all methods are shown as multifurcations.

All sequence comparisons are given as percentage sequence identity (% identical nucleotides). Similarity within and between clusters of sequences were calculated using MEGA (Tamura *et al*., 2007) and were based on pairwise p-distances (number of substitutions standardized to sequence length).

Network analyses

The network analyses (Figs 2 and S1) were performed with the TCS software (Clement *et al*., 2000) using nearly full-length 16S rRNA sequences for the endosymbionts and COI sequences for the host.

FISH and probe design

Oligonucleotide probes were designed with ARB for the endosymbiont clusters A–H found in the 16S rRNA clone libraries (Table 1). Sequences from some clusters were too closely related to allow the design of probes specific to a single cluster and for these, probes targeting the sequences in two or more clusters were designed (e.g. clusters B and C in Table 1). Probes were fluorescently labelled with cy3 or cy5 (Biomers, Ulm, Germany) and their specificity tested with clone-FISH as described by Schramm and colleagues (2002).

FISH-fixed *O. mucofloris* individuals were dehydrated in an ethanol series and embedded in paraffin. Samples were sectioned serially (3–8 μm thick sections) and mounted on SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany) or polysine slides (Menzel-Gläser). Sections were baked to slides by incubating these for 2 h at 58–60°C. The paraffin was removed from sections in 3–4 Roti-Histol (Carl Roth, Karlsruhe, Germany) washes of 10 min each, and the sections rehydrated in an ethanol series. Sections were encircled with a liquid-repellent pen (Super Pap Pen, Kisker Biotechnology, Steinfurt, Germany) and hybridized in a buffer (0.9 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDS, with the appropriate formamide concentration) containing probes at an end concentration of 5 ng ml^{-1}. Sections were hybridized for 2–28 h at 46°C, washed for 20 min at 48°C in buffer (0.1 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDA, 5 mM EDTA), and rinsed in distilled water. For DNA staining, sections were covered in a 1% DAPI solution containing 1% SDS for 10 min and rinsed with distilled water.

Of the 12 *O. mucofloris* individuals investigated with FISH, 9 were examined by hybridizing and analysing 5–15 randomly distributed sections per individual. Three individuals were examined in more detail by serial sectioning through the entire root tissue. A total of 60–300 slides per individual (depending on its size) with *ca*. 5 sections per slide of 4–8 μm thickness were prepared. Every 10th slide (corresponding to a distance between examined sections of 200–400 μm) was hybridized and analysed with FISH.

Statistical analyses

Two statistical tests were used to examine the factors influencing endosymbiont diversity, the Mantel test (Legendre and

Legendre, 1998) and distance-based redundancy analysis (db-RDA; Legendre and Anderson, 1999). For both analyses, only nearly full-length 16S rRNA endosymbiont sequences were used (> 1200 bp). Genetic distance matrices were calculated with MEGA (Tamura *et al*., 2007) based on pairwise p-distances (number of substitutions standardized to sequence length).

The Mantel test was used to examine if there was a significant correlation between the genetic distances within the *O. mucofloris* population (based on COI) and their endosymbiotic bacteria (based on 16S rRNA).

Distance-based RDA (db-RDA) was used to examine the effect of the following factors on 16S rRNA endosymbiont diversity: (i) for all *Osedax* species: water depth of the whale-fall and host species; and (ii) for *O. mucofloris*: sampling group and host individual. A nested design was used for (i) and (ii) for the following reasons. In (i), each host species was collected from only a single water depth so that the variable 'host species' was nested within the variable 'water depth'. The water depths of the whale-falls were divided into two categories: shallow < 500 m or deep > 1000 m. In (ii), the variables 'host individual' and 'sampling group' are hierarchically structured: a given *O. mucofloris* individual belonged to only one of the four sampling groups from the minke whale-fall (Table 4). In nested versus unnested designs, significance levels but not R^2 values (explained variation) can differ. The effect of geography was not tested with dbRDA because all *Osedax* species for which endosymbiont sequences are available occur at only three sites with very large distances between them (off the coasts of California, Japan, and Sweden). However, a plot of 16S rRNA genetic distances versus geographic distances showed no correlation (data not shown), and phylogenetic analyses confirmed the lack of congruence between endosymbiont diversity and host geography (Fig. 1). The effect of whale type could not be tested with db-RDA because endosymbiont sequences were only retrieved from two whale types, with only six endosymbiont sequences found in one of the two whale types (Table S1).

For db-RDA, all explanatory, qualitative variables were treated as sets of dummy variables (Ramette, 2007), and significances of full and partial (i.e. when controlling for the effects of other factors in the models) db-RDA models were assessed by multivariate analyses of variance based on 1000 permutations of the data response tables. All calculations were implemented within R (R Foundation for Statistical Computing, http://www.R-project.org) with the package *vegan*.

Nucleotide accession numbers

The sequences from this study are available through GenBank under the accession numbers FN773194–FN773299 for the symbiont 16S rRNA gene, and FN773300–FN773315 for the host COI gene.

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Supporting information

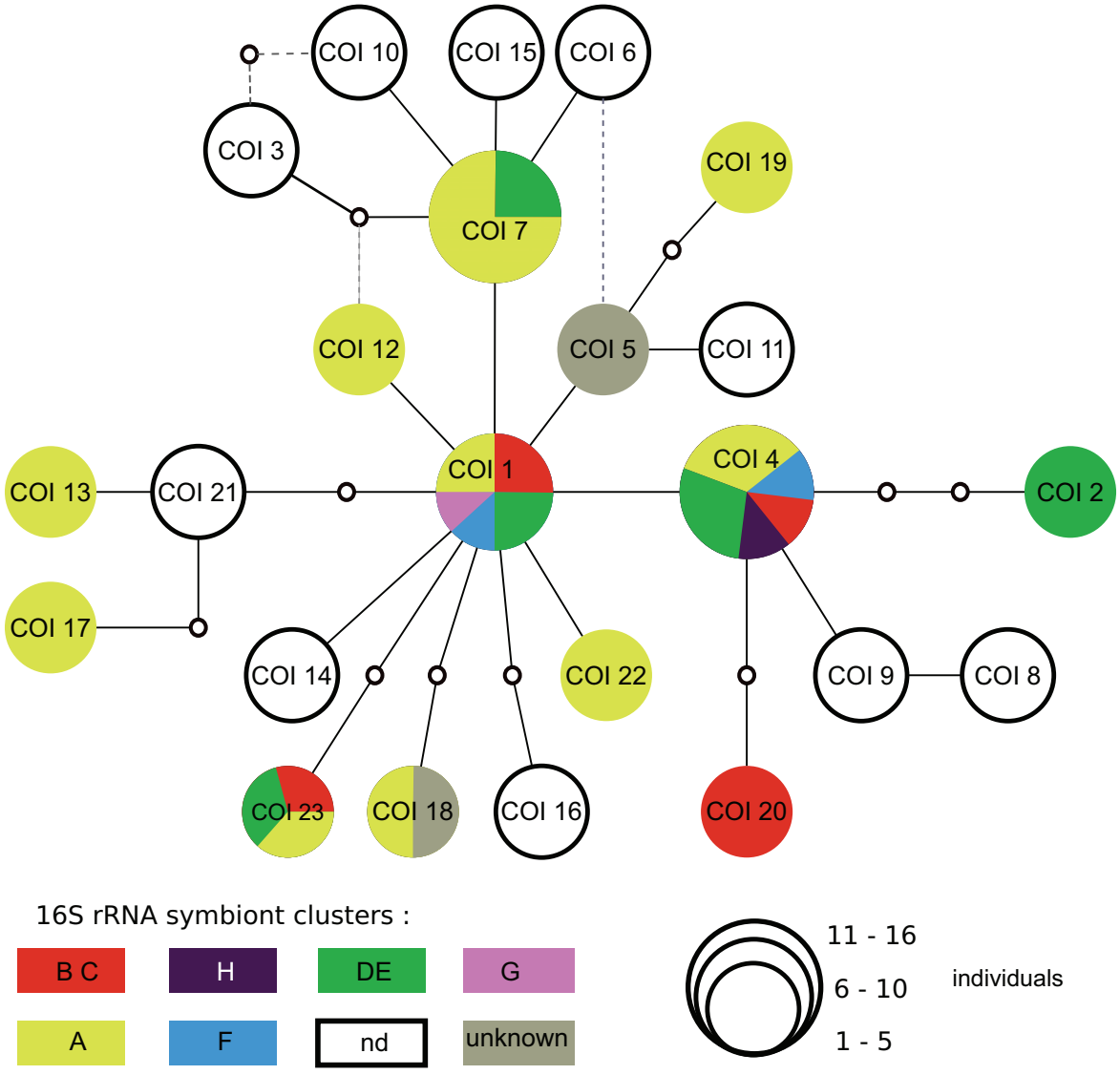
Additional Supporting Information may be found in the online version of this article:

Fig. S1. Parsimony network of *O. mucofloris* COI haplotypes (69 individuals from 3 whale-falls, with 28 individuals from this study and 41 individuals from previous studies including Glover *et al*., 2005). COI haplotype numbers correspond to those shown in Table 4. Each circle shows a unique COI haplotype; circle size shows the number of individuals that share the haplotype. Lines connecting circles represent 1 nucleotide difference between haplotypes, open circles on lines represent unsampled theoretical haplotypes, dashed lines show alternative connections between haplotypes. Colours represent endosymbiont clusters A–H; the proportion of a colour within a circle shows the number of host individuals that had the endosymbiont cluster. Unknown: endosymbionts that hybridized with the general *Osedax* endosymbiont probe but not with probes specific to clusters A–H. nd: Endosymbiont identity not determined.
Fig. S2. Phylogeny of bacteria from the (A) Alphaproteobacteria and Bacteroidetes, and (B) Epsilonproteobacteria associated with *O. mucofloris*. Only 16S rRNA sequences > 1200 bp were used in maximum likelihood (phyML) analyses with 100 bootstraps (values > 70% to the left of a given node). Shorter sequences were added afterwards without changing the tree topology using the ARB parsimony add function. Sequences from this study in bold. Scale bars = 0.10 estimated substitutions per site.
Fig. S3. *O. mucofloris* epibiotic bacteria.
A. Fluorescence *in situ* hybridization. Epifluorescence micrograph of cross section through the root tissues of Individual Omu 16 showing abundant Bacteriodetes (arrow) in the mucus layer covering the worm (shown in green with probe CF319a) and endosymbionts (arrowhead) (shown in yellow with probe EUBI-III) in the epithelial cells (e). Host nuclei stained blue with DAPI.
B. Scanning electron micrograph of epibiotic bacteria on the trunk surface of *O. mucofloris*. Such a dense covering of epibiotic bacteria was not observed on other worm species prepared in the same way. Specimens were critical point dryed, coated in gold and imaged using a Phillips XL30 SEM. Scale bars: (A) = 20 μm and (B) = 10 μm.
Table S1. Clone library 16S rRNA sequences. Oceanospirillales symbiont sequences were grouped in a cluster if they had at least 99.5% sequence identity. For Epsilonproteobacteria, Alphaproteobacteria and Bacteroidetes sequences, only those found in several host individuals are shown, all other sequences are grouped under 'others'. Number of nearly full-length sequences shown in parentheses (both strands were sequenced).

Table S1. Clone library 16S rRNA sequences. Oceanospirillales symbiont sequences were grouped in a cluster if they had at least 99.5% sequence identity. For Epsilon-, Alphaproteobacteria, and Bacteroidetes sequences, only those found in several host individuals are shown, all other sequences are grouped under "others". Number of nearly full-length sequences shown in parentheses (both strands were sequenced).

		Number of clones													
		Gammaproteobacteria Oceanospirillales								Epsilonproteobacteria		Alphaproteo-bacteria	Bacteroi-detes		
Whale and year	*O. mucofloris* individual	Cluster A	Cluster B	Cluster C	Cluster D	Cluster E	Cluster F	Cluster G	Cluster H	*Arcobacter 1*	*Sulfuro-spirillum*	Alpha 1	Bacteroi-detes 1&2	Others	Total count
Minke 2004	Omu TD1	**20** (5)	0	0	**33** (6)	0	0	0	0	**2** (1)	0	0	0	4 (2)	59
	Omu TD2	**29** (2)	0	0	**3** (3)	0	**1** (1)	0	0	**11** (5)	**10** (1)	0	**3** (1)	6	63
	Omu TD3	**10** (3)	0	0	0	0	0	**1** (1)	0	**12**	**4**	0	**1**	29	57
	Omu TD4	**12** (3)	0	0	0	0	0	0	0	**8** (3)	**1**	**2** (2)	**3** (2)	11 (1)	37
	Omu TD8	0	0	0	0	0	0	0	0	**40**	**4**	**3**	0	15	62
	Omu TD18	0	0	0	0	0	0	0	0	**29**	0	**8**	0	1	38
	Omu TD19	**34** (3)	0	0	0	0	0	0	0	**20**	**2**	**2**	**3**	3	64
Minke 2006	Omu 1	**3** (3)	0	0	0	0	0	0	0	**6**	**2**	**1**	**39**	88	139
	Omu 3	**57** (11)	0	0	0	0	0	0	0	**4**	0	**18** (3)	**3** (2)	16 (1)	98
	Omu 6	0	**28** (2)	**9** (3)	0	0	0	0	0	0	**1**	0	**37**	10	85
Minke 2007	Omu 9	0	0	0	**40** (4)	0	0	0	0	**3**	**1**	0	**1**	4	49
Minke 2008	Omu 16	0	0	**49** (8)	0	0	0	0	0	0	0	0	**6**	0	55
	Omu 17	0	0	0	0	0	0	0	**45** (4)	0	0	0	**12**	12	69
	Omu 18	0	0	**35** (4)	0	0	0	0	0	0	0	0	**3**	2	40
	Omu 19	**2** (2)	0	**31** (4)	0	0	0	0	0	0	0	0	**1**	0	34
Pilot 2005	Omu TD42	0	0	0	0	0	0	0	0	**3**	0	0	**3**	4	10
Sperm 2006	Omu TD73	0	0	0	0	0	**1** (1)	0	0	**1**	**7**	0	**24**	33	66
	Omu TD74	0	0	0	0	**3** (3)	0	0	0	**1**	**2**	0	**24**	42	72
	Omu TD75	0	0	0	0	0	0	0	0	0	**2**	**1**	**23**	32 (1)	58
	Omu TD76	0	0	**1**	0	**1** (1)	0	0	0	**2**	**3**	**1**	**12**	89	109
	Total	167 (31)	28 (2)	125 (19)	76 (13)	4 (4)	2 (2)	1 (1)	45 (4)	142 (9)	39 (1)	36 (5)	198 (5)	401 (5)	1264

Figure S1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COI 16
COI 20
16S rRNA symbiont clusters :
B C
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A
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nd
unknown
11 - 16
6 - 10
1 - 5
individuals

Figure S2A

O. mucofloris associated clones
Osedax associated clones
Marine invertebrates associated clones
Whale-fall and whale bone associated clones

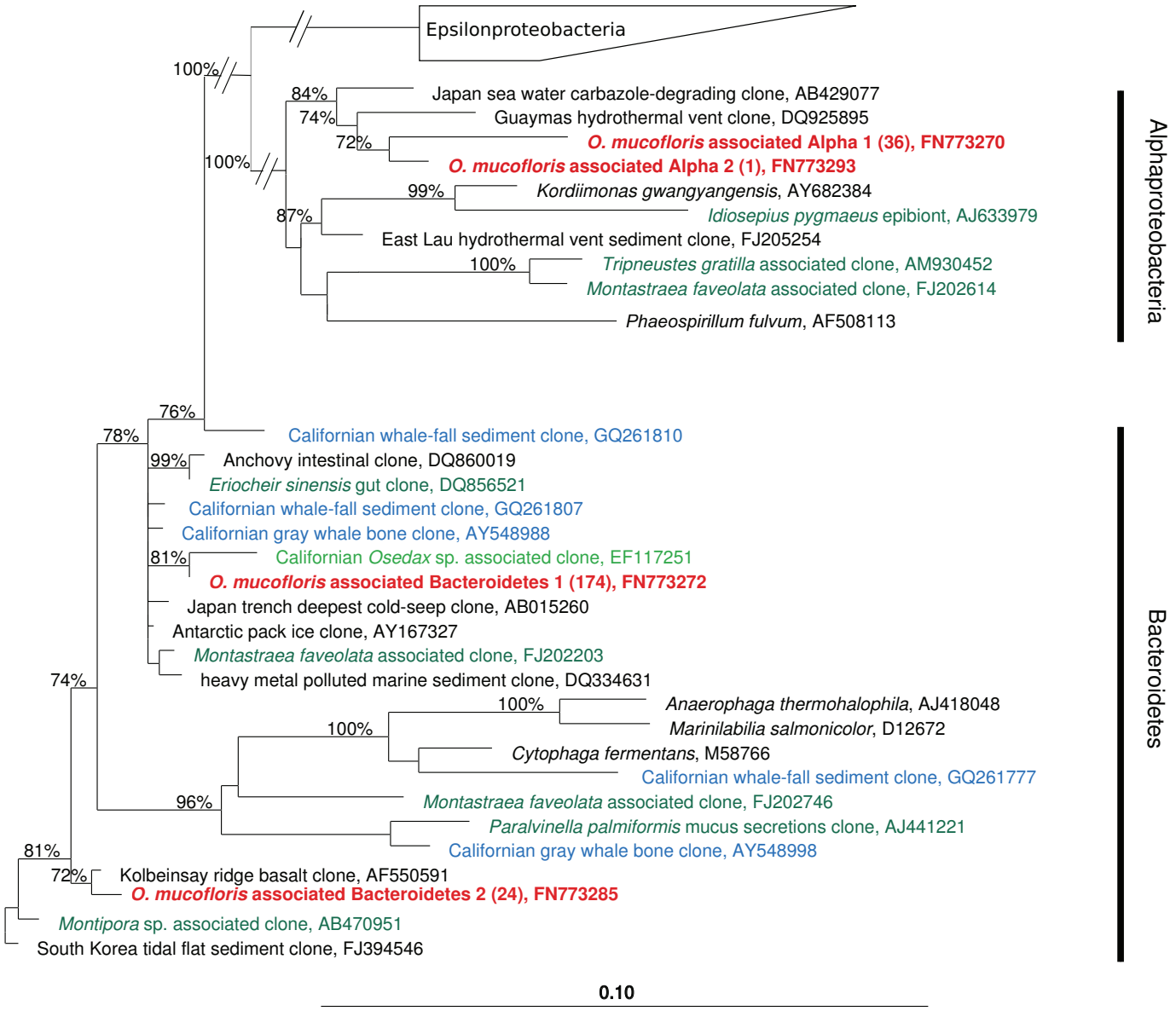


Figure S2B

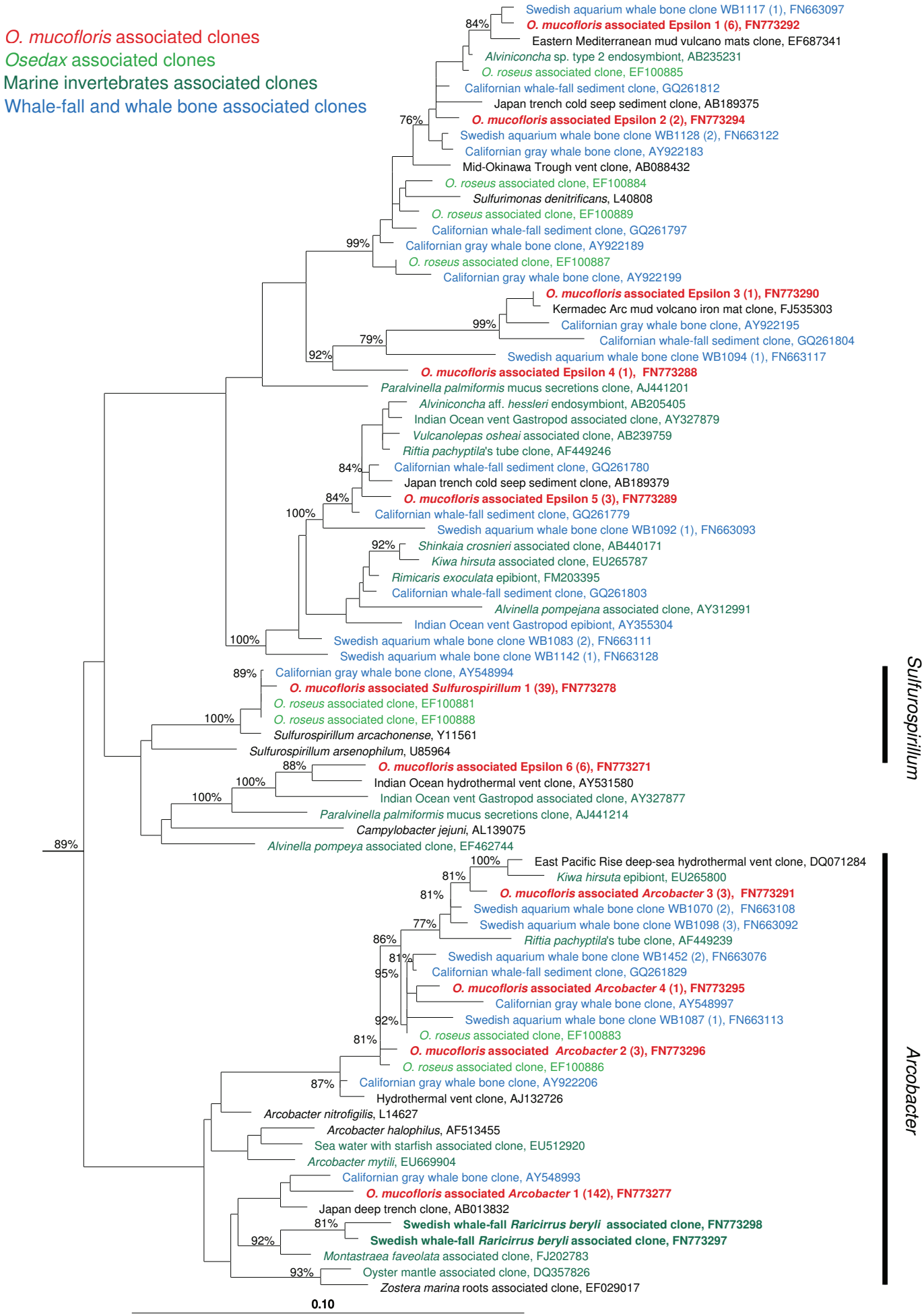
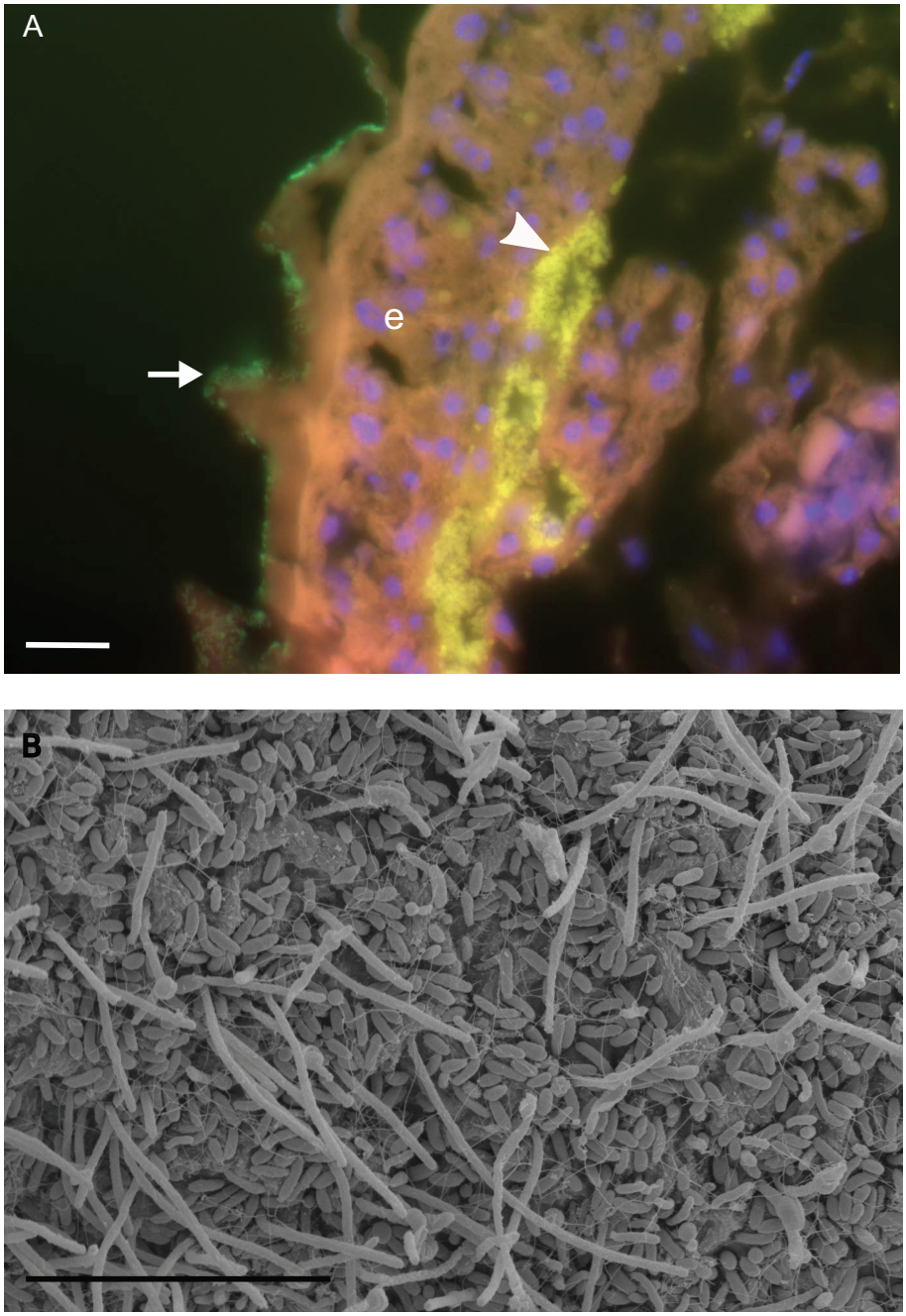

O. mucofloris associated clones
Osedax associated clones
Marine invertebrates associated clones
Whale-fall and whale bone associated clones
Swedish aquarium whale bone clone WB1117 (1), FN663097
O. mucofloris associated Epsilon 1 (6), FN773292
Eastern Mediterranean mud vulcano mats clone, EF687341
Alviniconcha sp. type 2 endosymbiont, AB235231
O. roseus associated clone, EF100885
Californian whale-fall sediment clone, GQ261812
Japan trench cold seep sediment clone, AB189375
O. mucofloris associated Epsilon 2 (2), FN773294
Swedish aquarium whale bone clone WB1128 (2), FN663122
Californian gray whale bone clone, AY922183
Mid-Okinawa Trough vent clone, AB088432
O. roseus associated clone, EF100884
Sulfurimonas denitrificans, L40808
O. roseus associated clone, EF100889
Californian whale-fall sediment clone, GQ261797
Californian gray whale bone clone, AY922189
O. roseus associated clone, EF100887
Californian gray whale bone clone, AY922199
O. mucofloris associated Epsilon 3 (1), FN773290
Kermadec Arc mud volcano iron mat clone, FJ535303
Californian gray whale bone clone, AY922195
Californian whale-fall sediment clone, GQ261804
Swedish aquarium whale bone clone WB1094 (1), FN663117
O. mucofloris associated Epsilon 4 (1), FN773288
Paralvinella palmiformis mucus secretions clone, AJ441201
Alviniconcha aff. hessleri endosymbiont, AB205405
Indian Ocean vent Gastropod associated clone, AY327879
Vulcanolepas osheai associated clone, AB239759
Riftia pachyptila's tube clone, AF449246
Californian whale-fall sediment clone, GQ261780
Japan trench cold seep sediment clone, AB189379
O. mucofloris associated Epsilon 5 (3), FN773289
Californian whale-fall sediment clone, GQ261779
Swedish aquarium whale bone clone WB1092 (1), FN663093
Shinkaia crosnieri associated clone, AB440171
Kiwa hirsuta associated clone, EU265787
Rimicaris exoculata epibiont, FM203395
Californian whale-fall sediment clone, GQ261803
Alvinella pompejana associated clone, AY312991
Indian Ocean vent Gastropod epibiont, AY355304
Swedish aquarium whale bone clone WB1083 (2), FN663111
Swedish aquarium whale bone clone WB1142 (1), FN663128
Californian gray whale bone clone, AY548994
O. mucofloris associated Sulfurospirillum 1 (39), FN773278
O. roseus associated clone, EF100881
O. roseus associated clone, EF100888
Sulfurospirillum arcachonense, Y11561
Sulfurospirillum arsenophilum, U85964
O. mucofloris associated Epsilon 6 (6), FN773271
Indian Ocean hydrothermal vent clone, AY531580
Indian Ocean vent Gastropod associated clone, AY327877
Paralvinella palmiformis mucus secretions clone, AJ441214
Campylobacter jejuni, AL139075
Alvinella pompeya associated clone, EF462744
East Pacific Rise deep-sea hydrothermal vent clone, DQ071284
Kiwa hirsuta epibiont, EU265800
O. mucofloris associated Arcobacter 3 (3), FN773291
Swedish aquarium whale bone clone WB1070 (2), FN663108
Swedish aquarium whale bone clone WB1098 (3), FN663092
Riftia pachyptila's tube clone, AF449239
Swedish aquarium whale bone clone WB1452 (2), FN663076
Californian whale-fall sediment clone, GQ261829
O. mucofloris associated Arcobacter 4 (1), FN773295
Californian gray whale bone clone, AY548997
Swedish aquarium whale bone clone WB1087 (1), FN663113
O. roseus associated clone, EF100883
O. mucofloris associated Arcobacter 2 (3), FN773296
O. roseus associated clone, EF100886
Californian gray whale bone clone, AY922206
Hydrothermal vent clone, AJ132726
Arcobacter nitrofigilis, L14627
Arcobacter halophilus, AF513455
Sea water with starfish associated clone, EU512920
Arcobacter mytili, EU669904
Californian gray whale bone clone, AY548993
O. mucofloris associated Arcobacter 1 (142), FN773277
Japan deep trench clone, AB013832
Swedish whale-fall Raricirrus beryli associated clone, FN773298
Swedish whale-fall Raricirrus beryli associated clone, FN773297
Montastraea faveolata associated clone, FJ202783
Oyster mantle associated clone, DQ357826
Zostera marina roots associated clone, EF029017
0.10
Sulfurospirillum
Arcobacter

Figure S3



Manuscript III

Extended host range of symbiotic bacteria mostly associated with bathymodiolin mussels

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Manuscript in Preparation

Extended host range of symbiotic bacteria mostly associated with bathymodiolin mussels

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1 Abstract

At whale falls numerous species with symbionts are known, such as bathymodiolin mussels with chemoautotrophic symbionts and *Osedax* bone-eating worms with heterotrophic symbionts. Diverse polychaetes worms have evolved to become symbiotic. Therefore, the high polychaete diversity at whale falls suggests that many polychaetes symbiosis are yet to be discovered.

In this study we assess the diversity of bacteria associated with *Raricirrus beryli*, a whale fall polychaete, using 16S rRNA gene analysis and catalyzed reporter deposition fluorescence in situ hybridisation. We show that the bacteria associated with *R. beryli* are highly diverse, and are located in its gut and on its surface.

Furthermore, we discovered the presence on the worm of bacteria forming a monophyletic cluster with bathymodiolin thiotrophic symbionts. These bacteria, although they are only occasionally found on the worm surface, are consistently associated with all individuals investigated during this study. This is the first report of a polychaete host for these bacteria, showing that they are quite ubiquitous.

2 Introduction

Several invertebrates with chemosynthetic symbionts occur at whale falls such as vestimentiferan tubeworms, vesicomyid clams and bathymodiolin mussels [2, 12, 36]. So far species with thiotrophic but no methanotrophic symbionts have been found at whale falls [2,12,35] although both hydrogen sulphide and methane are produced and could sustain chemosynthetic symbiosis [37]. Bathymodiolin mussels such as *Idas washingtonia, Adipicola crypta* and *Adipicola pacifica* can even be found in very high abundance on whale bones [2,14,34].

In addition to invertebrates with chemoautotrophic bacteria, *Osedax*, a siboglinid worm which can be highly abundant at whale falls, is associated with heterotrophic bacteria probably involved in the worms nutrition by degrading complex organic carbon [17, 18, 33]. The presence of a high diversity of polychaetes at whale-falls, and the presence of diverse symbiosis types (heterotrophic and chemoautotrophic) suggests that whale falls are a suitable habitat to discover new symbiotic association [14, 18, 29, 34, 35].

Raricirrus beryli Petersen & George (Ctenodrilidae, Polychaeta) is a worm originally described from sediments with high hydrocarbon pollution found under oil drilling platforms in the North Sea [27, 31]. We have now discovered *R. beryli* at an implanted whale fall in the North Atlantic close to the Swedish coast [5]. Whale falls could therefore be one of the natural habitats of *R. beryli*. The finding of this worm in high numbers at two organic rich habitat made it a good candidate to look for symbionts, since numerous symbiotic invertebrates are found at chemosynthetic habitats [3, 9].

Bacterial presence on the worms surface was confirmed and suggested the presence of bacteria closely related to symbionts from other invertebrates. This led us to further investigate the diversity of the bacteria associated with *R. beryli* using 16S rRNA gene analysis and catalyzed reporter deposition fluorescence in situ hybridisation (CARD FISH). Since the sampled worms did not have a uniform morphology, we will also examine if they belong to one species by analysing the cytochrome c oxidase subunit I (COI) gene.

3 Materials and Methods

3.1 Site description and specimen collection

A total of 9 *R. beryli* were examined during this study (Table. 1).

The collection site was a 5.3 m long carcass of a female minke whale, *Balaenoptera acutorostrata* Lacépède 1804, sunk in the Kosterfjord, Sweden (58°53.1'N; 11°06.4'E) at 125 m depth in October 2003 [5]. Using a Phantom XL Remotely Operated Vehicles (ROVs) we recovered whale bones in 2004, 2006, 2007 and 2008. The recovered bones were kept in seawater and transferred to aquaria in the laboratory with flow-through seawater at 8.0°C for hours to months. *R. beryli* individuals were mostly found inside the whale bones where they were first recorded in 2006. Occasionally some *R. beryli* were seen at the bottom of the aquaria where the whale bones were kept, that had probably fallen from the bones (H. Wiklund, Pers. Comm.). Extracting live *R. beryli* from the bones was extremelly difficult because the worm were so mobile (C. Verna and H. Wiklund Observation). Therefore, most individuals used in this study were sampled from the aquarium floor (see Table 1).

R. beryli was identified based on its morphology (Gordon Paterson, Pers. Comm.) and COI analyses see bellow.

For each sample preparation and fixation are listed in Table 1. One sample collected in 2007 was fixed in 96% ethanol for DNA analysis only. All other *R. beryli* individuals were fixed for DNA extraction and CARD FISH as follows. Samples were first washed in sterile sea water. The fixation was done in phosphate buffered saline (PBS) with 4% formaldehyde 30 min - 24 h at 4°C . The samples were then washed three times in 1X PBS and stored in 1X PBS / 50% ethanol.

3.2 Gene analysis

For 8 individuals, DNA was extracted with the DNAeasy Tissue kit (Qiagen, Hilden, Germany) from a whole worm or from a fragment of a worm (Table 1).

For all 8 individuals, the cytochrome c oxidase subunit I (COI) gene was amplified with primers forward LCO1490 and reverse LCO2190 [13] using the following PCR cycling condition: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 42°C for 1 min, and 72°C for 1 min, followed by a final elongation step at 72°C for 10 min. 3-4 reactions were pooled to minimise PCR bias. All sequences obtained during this study were sequenced using the Bigdye v3.1 cycle sequencing kit (Applied Biosystems) along with sequencer 3130xL genetic analyzer (Applied Biosystems). The PCR product was sequenced

Table 1: Summary of sampling site and collection dates of the *R. beryli* individuals investigated in this study. Bone refers to individuals that were found inside freshly sampled bones from the minke whale falls. Aquarium refers to individuals found at the bottom of the aquarium where whale bones where kept. Further processing of each individual for DNA extraction or FISH analysis is indicated.

R. beryli individual	Year	Site	DNA extraction	Paraffin
Rber 031	2007	Aquarium	X	
Rber 1	2008	Bone		X
Rber 2	2008	Bone	X*	
Rber 3	2008	Bone	X*	
Rber 5	2009	Aquarium	X*	
Rber 6	2009	Aquarium	X*	
Rber 7	2009	Aquarium	X*	
Rber 8	2009	Aquarium	X*	
Rber 10	2009	Aquarium	X*	

* Partial individuals were used, a piece of each worm being kept for further analyses

directly (both strands) and assembled with DNA Baser Sequence Assembler v2.x (2009) (HeracleSoftware, http://www.DnaBaser.com/index.html). Sequences obtained were of about 500 bp. For 2 individuals (Rber 5 and Rber 8) sequencing did not work and therefore, we cloned the PCR product as described below, and then sequenced two clones of each worm.

Three *R. beryli* individuals (Rber 031, Rber 2 and Rber 5) were used for 16S rRNA sequences analysis using primers GM3F and GM4R specific for the domain Bacteria ([21] modified in [28]) using the following PCR cycling conditions: initial denaturation at 94°C for 5 min, followed by 20 - 25 cycles at 94°C for 1 min, 43°C for 1 min 30 s, and 72°C for 2 min, followed by a final elongation step at 72°C for 10 min. The Takara ex Taq polymerase (Takara Bio Inc., Shiga, Japan) was used. At least five amplified PCR products from each host individual were pooled, purified with the QiaQuick PCR Purification Kit (Qiagen), and run on 1% agarose gels. The gel was stained with ethydium-bromide and a band of the 16S rRNA gene size was extracted. The gel piece was then purified using the Qiaquick Gel Purification protocol (Qiagen). For cloning, PCR products were ligated into the PCR4 TOPO vector (Invitrogen, Carlsbad, CA) or into the vector pGMTeasy

(Promega, Wisconsin, USA) and transformed into E. coli TOP10 cells (Invitrogen) according to the manufacturer's recommendations. Clones were checked for the correct insert size by PCR with vector primers M13F and M13R (Invitrogen). Sequencing was first performed with primer 907R only [22]. For individual Rber 2 and Rber 5 sequencing was done by an external company (GATC Biotech AG, Kontanz, Germany). Sequences of 500 to 900 pb lenght were aligned in ARB [24] using the Silva database [32]. For some clusters of sequences, representative clones were chosen for plasmid preparation using Qiaprep Spin miniprep kit (Qiagen). The plasmid inserts were fully sequenced (both strands) using the following primers M13F (Invitrogen), M13R (Invitrogen), 1114F [21]. The full sequences were assembled with DNA Baser Sequence Assembler v2.x (2009) (HeracleSoftware, http://www.DnaBaser.com/index.html) obtaining a size of about 1460 bp which were added in ARB.

For 6 *R. beryli* individuals (Table 3), bacterial 16S rRNA genes were amplified for about 1300 bp with a forward primer specific for thiotrophic bathymodiolin symbionts from the North Atlantic, as shown on Fig. 1, designed initially as a FISH probe BMARt193 (reverse complement sequence from BMARt193 used) [10] and reverse primer GM4R specific for the domain Bacteria using the following PCR conditions: Initial denaturation at 94°C for 5 min, followed by 45 cycles at 94°C for 1 min, 50°C for 1:30 min, and 72°C for 2 min, followed by a final elongation step at 72°C for 10 min. Takara ex Taq polymerase (Takara Bio Inc.) was used for the amplification. The obtained PCR product were then cloned as previously described. Plasmid were extracted using Qiaprep Spin miniprep kit (Qiagen) and plasmid insert sequenced with primer 907R [22]. For representative clones, the plasmid inserts were fully sequenced in (both strands) using the primers M13F (Invitrogen), M13R (Invitrogen) and the primer Thio_4_800_F 5'_ACTAGCCGTTGGGAGGATTT_3' (from Dennis Fink) specific for thiotrophic bathymodiolin symbionts. The full sequences were assembled with DNA Baser Sequence Assembler v2.x (2009) (HeracleSoftware) obtaining a size of about 1300 bp which were added in ARB.

3.3 Phylogenetic analysis

Sequence data were analyzed and phylogenetic trees were calculated with the ARB software package [24] using the Silva database [32]. Sequence alignments were manually checked. Phylogenetic trees of rRNA gene sequences were calculated by maximum likelihood analysis and 100 bootstrap analyses, with filters that exclude gaps and badly aligned regions. For tree reconstruction, only long 16S rRNA sequences (>1400 bp) were used. Branching

orders that were not supported are shown as multifurcations in phylogenetic tree (Fig. 1). Shorter sequences (<1400 bp) were added with ARB quick add function without changing the tree topology.

All sequence comparisons are given as percentage sequence identity (% identical nucleotides) calculated in ARB.

3.4 CARD_FISH

The fixed individuals were dehydrated in an ethanol series and embedded in paraffin (melting temperature 58-60°C). Samples were sectioned serially (sections 3-8 μm) and mounted on SuperFrost plus slides (Menzel-Gläser, Braunschweig, Germany). Before dewaxing, the slides were incubated two hours at paraffin melting temperature. Paraffin was removed in three to four Roti-Histol (Carl Roth, Karlsruhe, Germany) baths (each 10 min) and then the sections were rehydrated in an ethanol series. To prevent mixing or loss of different solutions during hybridisation, each section was encircled with a liquid-repellent slide marker pen (Super Pap pen, Kisker Biotechnology, Steinfurt, Germany)

CARD FISH was done on individual Rber 1 with hybridisation of horseradish peroxidase (HRP) Labeled probes (Biomers, Ulm, Germany) as described in [1, 30]. The following probes EUBI-III targeting most bacteria [6], Gam42a targeting most Gammaproteobacteria [26], CF319a targeting most Flavobacteria, some Bacteroidetes, some Sphingobacteria [25] and BMARt193 specific for the thiotrophic symbionts of several bathymodiolin mussels [10] were used. Probe Non338 [39] was used as a control for background autofluorescence. Sections were additionally stained with 4',6-diamidino-2-phenylindole (DAPI) 1μg / ml for 10 - 20 minutes at 37°C.

4 Results

4.1 *R. beryli* diversity based on the cytochrome c oxidase subunit I gene analysis

We sequenced the COI gene of 8 *R. beryli* individuals. The obtained sequences showed a low diversity, only two haplotypes that differed at two positions were found. The first haplotype was found in 6 host individuals and the second haplotype in 2 individuals. Blast results showed that the closest relatives in the public database were Canalipalpata polychaetes with 75% similarity to *R. beryli* COI sequences. Raricirrus is part of the Ctenodrilidae family which belong to the Canalipalpata polychaetes. However, no Ctenodrilidae COI sequences are available in the public databases.

4.2 *R. beryli* associated bacteria are highly diverse

Analyses of the 16S rRNA clone libraries from 3 *R. beryli* individuals revealed that associated bacteria sequences are highly diverse (Table 2). The bacteria belonged mostly to the Gammaproteobacteria, Bacteroidetes and Firmicutes (Table 2). No dominant population could be identified based on the abundance in the clone library. Most bacterial groups were only found in clone libraries from one or two individuals. Only two bacterial groups were found in all three individuals. One belonged to Firmicutes closely related to bacteria of the genus *Fillifactor*. The second belonged to a monophyletic cluster of thiotrophic symbionts from bathymodiolin mussels and one thyasirid bivalve. We will refer to this second cluster as the "bathymodiolin thiotrophic Cluster" (Table 2, Fig. 1).

CARD-FISH using probe EUBI-III and DAPI staining showed that bacteria were attached to the surface of the worms and in its gut (Fig. 2). Our clone libraries therefore represent gut-associated and surface associated bacteria. CARD-FISH with probe Gam42a showed the dominance of Gammaproteobacteria on the surface of Rber 1 individual (Fig. 2). Finally, CARD-FISH with probe CF319a showed no signal for Bacteroidetes on the worm surface, but few in the gut (data not shown).

Table 2: Clone library 16S rRNA sequences obtained with general primer for Bacteria from 3 *R. beryli* individuals. Only 16S rRNA gene sequences from bacterial group with 4 clones and more, or in several individuals are shown (sequences at lower abundances are grouped under 'others'). Numbers of nearly full-length sequences shown in parentheses (both strands were sequenced)

		Sampling site and year	Aquarium 2006	Bone 2008	Aquarium 2009		
		R. beryli individual	Rber 031	Rber 2	Rber 5	Total	
Phyla	*R. berily* bacterial groups	Similarity [a] %					Closely related bacteria (similarity %) [a]
Gammaproteo-bacteria	Bathymodiolin thiotrophic cluster	> 99.5	**18** (3)	**1**	**24** (3)	43 (6)	Bathymodiolin thiotrophic symbionts (94 - 99)
	Oceanospirillales III	> 99.5	**13** (2)	0	0	13 (2)	Uncultured marine bacteria (93 - 95)
	Colwellia 1	> 99.5	0	**7**	0	7	*Colwellia psychrerythraea (95)*
	Colwellia 2	> 99.5	0	**4** (1)	0	4 (1)	*Colwellia psychrerythraea (95)*
	Oceanospirillales I	91 – 98	0	**5** (1)	0	5 (1)	*Osedax* endosymbionts (92 - 97)
	JTB23	> 99.5	**16** (1)	0	0	16 (1)	Marine invertebrate associated & chemosynthetic environments bacteria (89 - 96)
	Acidithiobacillales	> 99.5	0	**14** (1)	**30** (2)	44 (3)	Chemosynthetic environments bacteria (90)
Bacteroidetes	Marinilabiaceae	98 – 99	**2**	0	**5**	7	*Cytophaga fermentans (89)*
	Flexibacteraceae	> 99.5	**5**	0	0	5	Chemosynthetic environments bacteria (92 – 96)
Firmicutes	Clostridia, *Fillifactor*	92 – 99.5	**2** (2)	**1**	**16** (2)	19 (4)	Oral cavity and gut bacteria (80 - 83)
	Mollicute	> 99.0	**11** (2)	0	**4** (2)	15 (4)	Invertebrates gut bacteria (84 - 89)
	Others		19	57 (7)	11	87 (7)	
	Total count		86 (10)	89 (10)	90 (9)	265 (29)	

Number of clones

[a] Pourcentage of nucleotide substitutions between two sequences

Table 3: Clone library 16S rRNA sequences from 6 *R. beryli* individuals amplified with primer BMARt193 (specificity shown in Fig. 1) and GM4R (general primer for Bacteria). Only 16S rRNA gene sequences corresponding to the target sequences are shown (the rest of the sequences are grouped under 'others'). Numbers of nearly full-length sequences shown in parentheses (both strands were sequenced)

		Number of clones		
Sampling site and year	*R. beryli* individual	Bathymodiolin thiotrophic Cluster	Others	Total count
Bone 2008	Rber 2	**6 (3)**	1	7
Bone 2008	Rber 3	**6**	2	8
Bone 2008	Rber 6	**4 (3)**	0	4
Aquarium 2009	Rber 7	**1**	14	15
Aquarium 2009	Rber 8	**8 (5)**	0	8
Aquarium 2009	Rber 10	**1**	14	15

4.3 *R. beryli* associated bacteria belong to a cluster of bathymodiolin thiotrophic symbionts

CARD-FISH on Individual Rber 1 using probe BMARt193 specific for bathymodiolin thiotrophic symbionts from the North Mid-Atlantic Ridge confirmed the presence of the targeted bacteria (Fig. 2). These bacteria were found on the surface of the worm. They were rare, not present on every analyzed section and showed a patchy distribution with a maximum of 1 - 5 cells grouped together (Fig. 2 D).

To further investigate the presence of the bathymodiolin thiotrophic Cluster, we made clone libraries from 6 *R. beryli* individuals using the specific probe BMARt193 (See Material and Methods). Sequences belonging to the bathymodiolin thiotrophic Cluster were found in all 6 individuals (Table 3, Fig. 1).

Comparison of all *R. beryli* sequences in the bathymodiolin thiotrophic Cluster, obtained from both clone libraries with primers general for Bacteria and specific for some bathymodiolin symbionts, showed that out of the 1300 to 1500 positions, there were only 0-3 nucleotide substitutions in each sequences. The majority of nucleotide substitutions were unique and not found in other sequences or individuals, and thus probably due to sequencing or PCR errors. However, all sequences from Individual Rber 5 were consistently different from sequences from other *R. beryli* individuals at two variable positions.

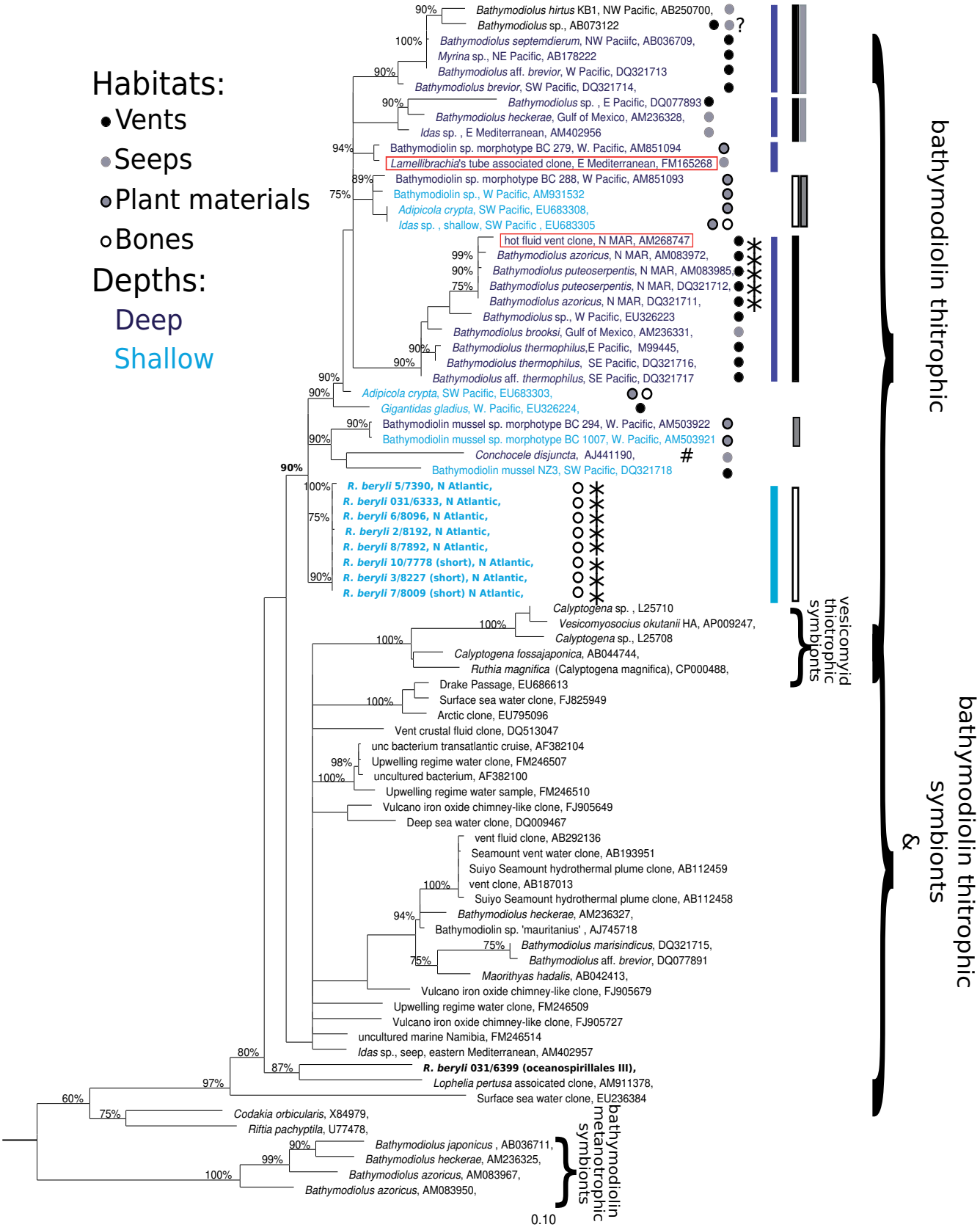
Habitats:
Vents
Seeps
Plant materials
Bones
Depths:
Deep
Shallow
Bathymodiolus hirtus KB1, NW Pacific, AB250700,
Bathymodiolus sp., AB073122
Bathymodiolus septemdierum, NW Paciifc, AB036709,
Myrina sp., NE Pacific, AB178222
Bathymodiolus aff. brevior, W Pacific, DQ321713
Bathymodiolus brevior, SW Pacific, DQ321714,
Bathymodiolus sp. , E Pacific, DQ077893
Bathymodiolus heckerae, Gulf of Mexico, AM236328,
Idas sp. , E Mediterranean, AM402956
Bathymodiolin sp. morphotype BC 279, W. Pacific, AM851094
Lamellibrachia's tube associated clone, E Mediterranean, FM165268
Bathymodiolin sp. morphotype BC 288, W Pacific, AM851093
Bathymodiolin sp., W Pacific, AM931532
Adipicola crypta, SW Pacific, EU683308,
Idas sp. , shallow, SW Pacific , EU683305
hot fluid vent clone, N MAR, AM268747
Bathymodiolus azoricus, N MAR, AM083972,
Bathymodiolus puteoserpentis, N MAR, AM083985,
Bathymodiolus puteoserpentis, N MAR, DQ321712,
Bathymodiolus azoricus, N MAR, DQ321711,
Bathymodiolus sp., W Pacific, EU326223
Bathymodiolus brooksi, Gulf of Mexico, AM236331,
Bathymodiolus thermophilus,E Pacific, M99445,
Bathymodiolus thermophilus, SE Pacific, DQ321716,
Bathymodiolus aff. thermophilus, SE Pacific, DQ321717
Adipicola crypta, SW Pacific, EU683303,
Gigantidas gladius, W. Pacific, EU326224,
Bathymodiolin mussel sp. morphotype BC 294, W. Pacific, AM503922
Bathymodiolin mussel sp. morphotype BC 1007, W. Pacific, AM503921
Conchocele disjuncta, AJ441190,
Bathymodiolin mussel NZ3, SW Pacific, DQ321718
R. beryli 5/7390, N Atlantic,
R. beryli 031/6333, N Atlantic,
R. beryli 6/8096, N Atlantic,
R. beryli 2/8192, N Atlantic,
R. beryli 8/7892, N Atlantic,
R. beryli 10/7778 (short), N Atlantic,
R. beryli 3/8227 (short), N Atlantic,
R. beryli 7/8009 (short) N Atlantic,
Calyptogena sp. , L25710
Vesicomyosocius okutanii HA, AP009247,
Calyptogena sp., L25708
Calyptogena fossajaponica, AB044744,
Ruthia magnifica (Calyptogena magnifica), CP000488,
Drake Passage, EU686613
Surface sea water clone, FJ825949
Arctic clone, EU795096
Vent crustal fluid clone, DQ513047
unc bacterium transatlantic cruise, AF382104
Upwelling regime water clone, FM246507
uncultured bacterium, AF382100
Upwelling regime water sample, FM246510
Vulcano iron oxide chimney-like clone, FJ905649
Deep sea water clone, DQ009467
vent fluid clone, AB292136
Seamount vent water clone, AB193951
Suiyo Seamount hydrothermal plume clone, AB112459
vent clone, AB187013
Suiyo Seamount hydrothermal plume clone, AB112458
Bathymodiolus heckerae, AM236327,
Bathymodiolin sp. 'mauritanius' , AJ745718
Bathymodiolus marisindicus, DQ321715,
Bathymodiolus aff. brevior, DQ077891
Maorithyas hadalis, AB042413,
Vulcano iron oxide chimney-like clone, FJ905679
Upwelling regime water clone, FM246509
Vulcano iron oxide chimney-like clone, FJ905727
uncultured marine Namibia, FM246514
Idas sp., seep, eastern Mediterranean, AM402957
R. beryli 031/6399 (oceanospirillales III),
Lophelia pertusa assoicated clone, AM911378,
Surface sea water clone, EU236384
Codakia orbicularis, X84979,
Riftia pachyptila, U77478,
Bathymodiolus japonicus , AB036711,
Bathymodiolus heckerae, AM236325,
Bathymodiolus azoricus, AM083967,
Bathymodiolus azoricus, AM083950,
0.10
bathymodiolin thitrophic
vesicomyid thiotrophic symbionts
bathymodiolin thitrophic symbionts &
bathymodiolin metanotrophic symbionts

Figure 1: 16S rRNA phylogeny of bathymodiolin thiotrophic symbionts. Tree based on maximum likelihood (phyML) analyses with 100 bootstraps (branches with support values < 40% were collapsed in multifurcations; values > 75% to the left of a given node). In the bathymodiolin thiotrophic cluster, sequences from hosts collected below 800 m are shown in dark blue and above 800 m in light blue for those sequences where information was available. In addition, their habitat is indicated by circles at the right of each sequences: closed black circle = hydrothermal vents, closed grey circle = cold seeps, open white cicles = bones, open grey circle = plant remain including wood and sugar canes. Finally, their geographic location is indicated in the sequence name, N = North, S = South, E = East, W = West, MAR = Mid Atlantic Ridge. Two red boxes indicates sequences obtained from environmental clones in the habitats of the mussels. The bars on the right indicate groups of sequences that were mostly found at one depth or in one habitat type. A star * shows sequences targeted by the BMARt193 probe and primer used in this study. A # indicates a thyasirid bivalve symbiont. Sequences from this study are in bold, one sequence is shown for each *R. beryli* individual. Short refers to single strand sequences of 700 bp. The numbers following each *R. beryli* symbiont sequence show the individual number / clone number. Scale bar = 0.10 estimated substitutions per site.

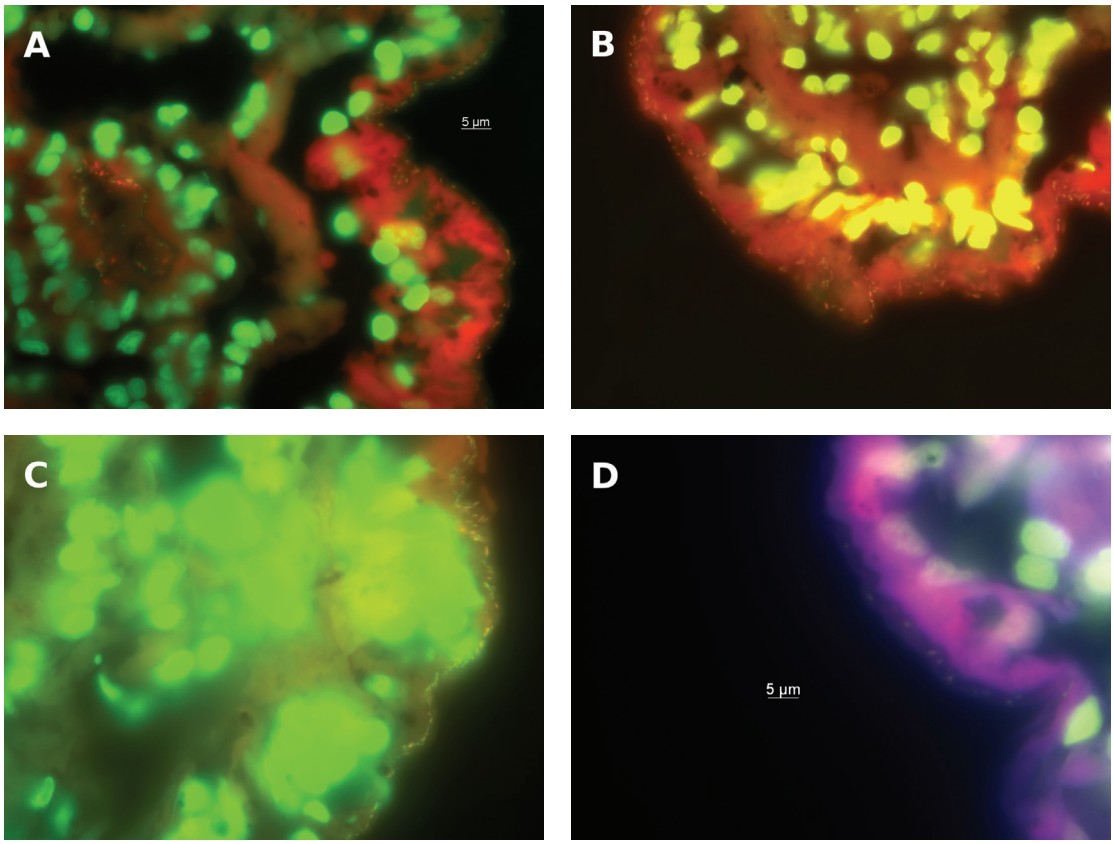

Figure 2: Fluorescence in situ hybridisation. A - D Epifluorescence micrographs of cross sections through *R. beryli*. **A.** Bacteria are visible on *R. beryli* surface and in its gut (EUBI-III probe shown in red and DAPI in green) **B.** Bacteria on *R. beryli* surface (EUBI-III probe shown in red and DAPI in yellow). **C.** Dominance of Gammaproteobacteria on the *R. beryli* surface (Gam42a probe shown in orange and DAPI in green). **D.** Few bacteria from the bathymodiolin thiotrophic Cluster are present on *R. beryli* surface (BathyMARt193 probe shown in pink and DAPI in green). Scale bar in A valid for all pictures = 5 μm

5 Discussion

5.1 *R. beryli* associated bacteria diversity

The high diversity, based on 16S rRNA sequences analysis, of the bacteria associated with *R. beryli* probably comes from both gut and surface bacteria:

In part the bacteria found in the clone libraries represent the diversity of ingested bacteria. *R. beryli* found on the oil spill habitat were deposit feeders, ingesting sand particles and digesting the epibacteria [27, 31]. At the whale fall, *R. beryli* can probably ingest small particles such as degraded bone pieces with the bacteria covering them. For example Firmicutes and Bacteroidetes bacteria were found in abundance in the sediment at a whale fall and could be found also in the whale bones [16]

In general, the bacteria on *R. beryli* surface could be commensalist, with the bacteria inhabiting the surface of the worm without any harmful effects: i) Heterotrophic bacteria could degrade organic molecules produced by the worm ii) Chemoautotrophic bacteria could use the worm to bridge the oxic-anoxic interface [4]. Indeed, *R. beryli* worms are particularly mobile and active [C. Verna, H. Wiklund pers. comm.] and can migrate from inside the bone matrix (sulphide rich) to the bone surface (oxygenated water). Such a migration along a sulphide - oxygen gradient is known for shrimps, nematodes, and oligochaetes with chemoautotrophic symbionts; thus, they are giving their symbionts access to both hydrogen sulphide and oxygen [4, 15]. Similarly, chemoautotrophic bacteria on *R. beryli* should be able to access both hydrogen sulphide and oxygen while the worm is crawling between bone and the bone-water interface. Such a behaviour could explain the presence of the bacteria closely related to bathymodiolin thiotrophic symbionts [4, 9]. The diversity of the surface bacteria and the role of the association is difficult to assess since no bacteria are dominant and found in association with all hosts. Any benefit for *R. beryli* of the presence of the surface bacteria is unclear.

5.2 Bacteria previously only found in bathymodiolin mussels are associated with *R. beryli*

5.2.1 A surprising finding

It is highly surprising to find bacteria belonging to the bathymodiolin thiotrophic Cluster on the worms surface, because that cluster is monophyletic and mostly contains bacteria associated with bathymodiolin mussels and one thyasirid bivalve. Only two sequences from

environmental clones are in the cluster, and they are from the mussels habitat, thus we can not exclude a contamination from the mussels themselves (Fig. 1). In addition, at the whale-fall where *R. beryli* was sampled, no bathymodiolin mussels have been found so far, and we can therefore exclude a contamination from co-occurring bathymodiolin mussels.

At whale-falls, bathymodiolin mussels are exclusively associated with thiotrophic symbionts and not with methanotrophic bacteria [2, 8, 23, 35]. Since in bathymodiolin mussels, indirect evidence indicates a horizontal transmission of their thiotrophic symbionts [11, 20, 23, 40, 41], free-living form of the symbionts should exist. However, several studies of the microbial diversity associated with bone and sediment at whale-falls did not detect them [16, 17, 19, 38]. The bathymodiolin thiotrophic cluster bacteria detected on *R. beryli* are probably enriched on the worm compared to the rest of the whale fall environment.

5.2.2 Implications for the biology and dispersal capacities of these bacteria

Bacterial dispersal is independent of bathymodiolin mussels Since bacteria from the bathymodiolin thiotrophic Cluster were detected in absence of bathymodiolin mussels their dispersal is independent from the bathymodiolin hosts. One hypothesis is that *R. beryli* participate in the dispersal of these bacteria. Indeed, the worms are known to have adults with different morphologies, including a swimming dispersal form [27, 31]. The adult dispersal form could allow the worm to colonise new favorable environments with the transport of its epibiotic bacteria. It is not known whether *R. beryli* larvae enter the plankton or not [27, 31]. However, the dispersal of the bacteria from the bathymodiolin thiotrophic Cluster on *R. beryli* seem unlikely since this species is not known at vents, seeps and wood falls where the bathymodiolin mussels occur. Thus, the more likely hypothesis is that bacteria of the bathymodiolin thiotrophic Cluster have high dispersal capacities and are able to colonise new environments by themselves.

Confirmation of a diverse habitat range: ubiquitous bacteria The ubiquity of bacteria from the bathymodiolin thiotrophic Cluster is extended by this study, to polychetes hosts, as these bacteria were previouly only known to be symbionts of bivalves from different habitats including hydrothermal vents, cold seeps and various organic substrates [11, 23, 41].

Factors potentially structuring the distribution of these bacteria: habitat, geography, depth, and host specificity Several factors were proposed to affect the

distribution of bacteria from the bathymodiolin thiotrophic Cluster.

- With this study we confirm that the host species is unlikely to affect the distribution of the symbionts [40, 41].
- Geography has been proposed to influence the distribution of the symbionts [7]. However, *R. beryli* sequences from the bathymodiolin thiotrophic Cluster are not most closely related to bathymodiolin symbionts from the North Atlantic such as those from the Mid Atlantic Ridge but from the West Pacific ones (Fig. 1), indicating that geography does not play a role in the distribution of these bacteria or that they are isolated off the Swedish shallow coast from the rest of the Northern Atlantic.
- Within the bathymodiolin thiotrophic Cluster, most branches are poorly supported. Depth and/or habitat where the bacteria are found could influence the bacterial distribution. Some bacterial groups in the phylogeny contain bacteria mostly found at deep sites, and some groups contain bacteria mostly found at shallow sites (Fig. 1). Similarly, some bacterial groups contain bacteria found mostly at seeps and vents or mostly at organic falls, respectively. Groups containing 2 two 10 sequences can be seen on Fig. 1. For example, symbionts from *Bathymodiolus puteoserpentis, Bathymodiolus azoricus, Bathymodiolus thermopilus, Bathymodiolus brooksi* and an environmental clone are forming a monophyletic cluster and all these are from deep sea vents except *Bathymodiolius brooksi* from a deep sea seep. Similarly, bathymodiolin sp. morphotype BC 288 (AM851093), bathymodiolin sp. (AM931532), *Adipicola crypta* (EU683305) and *Idas* sp. (EU683305) are also forming a monophyletic group, they all occur at organic falls (wood and bones).

As was already proposed, the 16S rRNA gene might not be variable enough in the bathymodiolin thiotrophic Cluster to resolve the phylogeny, and looking at more variable markers such as rRNA internal transcribed spacer (ITS) may improve our understanding of the distribution of the bacteria within that cluster [7, 11, 40].

6 Outlook & conclusion

The role of the diverse bacteria associated with *R. beryli* is not clear. We will check more individuals with CARD-FISH, and use more specific probes to find which bacteria are dominant in the host gut and on its surface. Assessing the metabolic capacities of the dominant bacteria would also be of interest to elucidate the nature of the association.

Although surprising, the presence of the bathymodiolin thiotrophic Cluster at whale falls could be expected since bathymodiolin mussels were found at whale falls (even if not at the studied site), and since bathymodiolin symbionts are most likely horizontaly transmitted [20, 40, 41]. In further studies, we aim to look for the bacteria belonging to the bathymodiolin thiotrophic Cluster on whale bones where *R. beryli* was sampled. We are also planing to use ITS rRNA to get a better insight into the diversity of these bacteria associated with *R. beryli*, and the bones.

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Part IV

Discussion and conclusion

In the following, I aim at bringing together the results and conclusions from the different parts composing this thesis. This discussion focuses on three topics. First, we will compare the microbial diversity among two whale-falls micro-habitats ˜free-living˝and ˜host-associated˝. Then we will discuss how recent research on siboglinid symbiosis, including this study, gives a new perspective on siboglinid evolution. Finally, we will speculate on the role of symbiont diversity in *Osedax* symbiosis as well as the potential function of *Osedax* endosymbionts.

Chapter 8

Microbial diversity at whale falls: comparison between free-living bacteria and epibacteria associated with two polychaetes species

Before comparing the microbial diversity of epibacteria from *R. beryli* and *O. mucofloris*, and whale-fall free-living bacteria we summarise the distribution of several bacterial groups with each micro-habitats. Then, we propose scenarios to explain the observed distributons: including bacterial ubiquity and a specific host-bacteria association.

8.1 Results: which bacteria in which micro-habitat

8.1.1 Summary of bacterial diversity associated with each species

As shown in Manuscripts II and III, the two polychaetes studied here, *O. mucofloris* and *R. beryli*, are covered by epibacteria. Table 8.1 summarizes which bacterial groups where associated with *O. mucofloris* and *R. beryli* and compares these epibacteria to the diversity of free-living bacteria found at whale fall environments including whale bones [44, 111], whale fall associated sediments [43] and whale bones kept in aquaria [47]. Only bacterial groups that were dominant in the clone libraries from at least one of the two polychaete species are considered. To complement the table, a summary of microscopy results from the previous chapter follows.

On *O. mucofloris*, epibacteria were abundant in and on a mucus layer covering trunk and root surfaces, and in its tube. Scanning electron microscopy showed that the trunk

is covered by various bacterial morphotypes (filaments and cocci) (Manuscript II Figure S3). Clone libraries revealed a high diversity of bacteria besides the known *Osedax* Oceanospirillales endosymbionts (Table 8.1). A subsequent FISH analysis revealed that Gammaproteobacteria were detected mostly on the trunk and in the tube. Several *Osedax* endosymbiont clusters were also occasional member of the epibacteria in the root mucus layer and in the tube. *Arcobacter* were only rarely detected in the mucus layer from both trunk and root. A probe targeting Epsilonproteobacteria, which excludes *Arcobacter* but includes the *Sulfurospirillum* group gave no signal. The *O. mucofloris* Alpha 1 bacteria were detected on the trunk with a specific probe, where they were mostly associated with tissue invaginations. A probe targeting most of Alphaproteobacteria, but excluding *O. mucofloris* Alpha 1, gave no signal. Bacteroidetes bacteria were dominant in the mucus layer covering the root tissue (Manuscript II Figure S3), present on the trunk surface and in the tube.

Table 8.1: Comparison of the bacterial groups found free-living at whale falls, and associated with *O. mucofloris* and *R. beryli*.

		Whale fall free-living bacteria	*O. mucofloris* epibionts		*R. beryli* epibionts		closest relatives
Phyla	Bacterial groups [a]	Clone library	Clone library	FISH	Clone library	FISH	
Gammaproteobacteria	General	+	+	+	+	+	
	Bathymodiolin thiotrophic Cluster	-	-	/	+	+	A
	R. beryli Oceanospirillales III	-	-	/	+	/	E
	R. beryli Colwellia 1	-	-	/	+	/	E
	R. beryli Colwellia 2	-	-	/	+	/	E
	Osedax endosymbiont Cluster [b]	+ *	+	+	+	/	A & E
	R. beryli JTB23	-	-	/	+	/	A & E
	R. beryli Acidithiobacillales	-	-	/	+	/	E
Epsilonproteobacteria	General	+	+	-	+	/	
	O. mucofloris Arcobacter 1	+	+	+	+	/	A & E
	O. mucofloris Sulfurospirillum	+	+	-	-	/	A & E
Alphaproteobacteria	General	+	+	-	+	/	
	O. mucofloris Alpha 1	-	+	+	-	/	E
Bacteroidetes	General	+	+	+	+	+	
	O. mucofloris Bacteroidetes 1	+	+	/	+	/	A & E
	O. mucofloris Bacteroidetes 2	+	+	/	-	/	A & E
	R. beryli Marinilabiaceae	+	-	/	+	/	E
	R. beryli Flexibacteraceae	+	-	/	+	/	E
Firmicutes	General	+	+	/	+	+	
	R. beryli Clostridia, *Fillifactor*	-	-	/	+	/	A
	R. beryli Mollicute	-	-	/	+	/	A

+ The bacterial group was detected. - The bacterial group was not detected. / No FISH with a probe targting the bacterial group was done. A animal associated clones. E free-living environmental clones

* Oceanospirillales closely related to *Osedax* endosymbionts were isolated from the sediment at several japanese whale-falls

[a] Those bacterial groups correspond to group described in precedent chapters

[b] Since those bacteria where found with FISH on *O. mucofloris* root tissue in the mucus layer, they are here considered as epibacteria

Based on DAPI staining and FISH, epibacteria on *R. beryli* seemed less abundant than on *O. mucofloris*. In addition, bacteria were also found in *R. beryli* guts (Manuscript III Figure 2). Both epibacteria and gut bacteria were included since we could not separate them based on the clone library results. FISH revealed that Gammaproteobacteria were dominant on the surface of the worm, and bacteria from the bathymodiolin thiotrophic Cluster were occasionally found in small patches (1 - 5 cells) (Manuscript III Figure 2). Bacteroidetes were detected in the gut.

8.1.2 Comparison of host-associated and free-living bacterial groups

Gammaproteobacteria, Epsilonproteobacteria, Alphaproteobacteria, Bacteroidetes, and Firmicutes were all detected in the environment and as symbionts associated with both *O. mucofloris* and *R. beryli* (Table 8.1). Here, in more details:

- At a lower taxonomic level, only three bacterial groups were found associated with both *O. mucofloris* and *R. beryli*: bacteria from the *Osedax* endosymbiont cluster, *O. mucofloris Arcobacter* 1 associated bacteria, and *O. mucofloris* Bacteroidetes 1 associated bacteria. All other 13 bacterial groups were only found within one host (Table 8.1)

- Except for the Osedax endosymbiont Cluster, none of the gammaproteobacterial groups associated with the worms was detected in the environment. *O. mucofloris* associated Alpha 1 and *R. beryli* associated Firmicutes were also not detected as free-living in the whale fall habitat. However, all Bacteroidetes and Epsilonproteobacteria were detected in a free-living stage.

8.2 Conclusions: Hypotheses explaining the observed epibacterial distribution

Based on the occurence of these bacterial groups, and where known their abundance in the three habitats (*O. mucofloris, R. beryli* and free-living), different strategies can be defined for the bacteria and the host, explaining some of the observed patterns.

8.2.1 Ubiquitous bacteria

Bacteria found on both worms and free-living could be ubiquitous bacteria, living in different niches of the whale fall habitat. Further FISH studies comparing the abundance in

each habitat (free-living, *O. mucofloris* associated and *R. beryli* associated) could show if the bacteria are ubiquitous, or display preferences for a certain habitat.

For example, *O. mucofloris* associated *Arcobacter* were rarely found with FISH on *O. mucofloris*, and only two clones were found in one *R. beryli* individual. Members from this group were closely related to environmental clones from chemosynthetic environment. It is thus possible that the Arcobacter found on *O. mucofloris* and *R. beryli* are casual and occasional associates from the surrounding chemosynthetic environment rather than true epibionts.

8.2.2 Bacterial association: mutualism or commensalism?

Most bacterial groups were detected only in association with one polychaete species. This shows an enrichment of the bacteria on one worm species compared to the other species or the environment, where they could not be detected. These bacterial groups may show a preference for one of the three habitats and likewise each worm may show a preference for certain bacterial groups. Several interaction effects between each host and its epibionts can be envisaged. Effects range from positive, neutral to negative effects for each partner. Several advantages can be considered for the epibionts: for example a new space available in the limited bone space and access to nutritive elements. For the hosts, advantages could could be protection against pathogens, nutritional benefits, or recycling of waste products. Moreover, interactions among the epibiotic bacteria probably also affect the bacterial diversity, with mutualistic effects (including exchange of nutrients), neutral effects, or negative effects (competition for space and/or nutrition).

Without more knowledge of each bacterial group, we cannot speculate in more depth on the interactions among the epibionts. However, three scenarios can be considered for the worm and epibiont interactions. None of these scenarios are mutually exclusive. The first scenario describes several commensalist interactions where the effect is neutral for the host and positive for the epibionts. In the second and third scenarios, positive effects for the host are considered, indicating potential mutualistic symbioses: in the second scenario between the epibionts and their host and in the third scenario between the gut microbiome and the host.

First scenario: habitat preferences of the epibionts The epibiont's preference for a specific host suggests that the worms are structuring the bone bacterial community, each worm creating one or more micro-niches favorable to certain bacteria only. Each worm

species may structures the bacterial communities in different ways:

The bone matrix is an anoxic and sulphidic habitat compared to the bone-water interface which is oxic. Both *O. mucofloris* and *R. beryli* occur in both oxic and anoxic habitats. *O. mucofloris* is fixed in the bone by its branching root tissue, and reaches the oxic water with its palps and trunk. In comparison, *R. beryli* is a highly mobile species (C. Verna and H. Wiklund personal observation) which can crawl through holes in the bone matrix, and reach the oxic interface. *R. beryli* is thought to be a deposit feeder [73, 80].

Thus, the following micro-niches could be created by *O. mucofloris* and *R. beryli*: i) bacteria inside the tube of *O. mucofloris* could be protected from current and sedimentation; ii) both host species might provide more favourable biochemical conditions (O_2, pH, H_2S) compared to the environment; iii) a surface for the bacteria to attach to; iv) protection from grazing by other invertebrates; v) the worms could produce organic compound on which certain bacteria could feed, such as *O. mucofloris* mucus.

For example, *O. mucofloris* Alpha 1 bacteria could be aerobic or microaerophilic degrading organic compounds from the mucus layer covering *O. mucofloris*. These bacteria were found in the mucus layer on trunk surface invaginations which probably correspond to canals connected to excreting gland like structures [36]. Based on 16S rRNA gene analysis, the *O. mucofloris* Alpha 1 were most closely related to carbazole degrading bacteria; indicating that these could degrade complex organic molecules probably present in the worm mucus.

Second scenario: host selection of the epibionts or vice versa The specific epibionts found on each species suggests selection by the host for its epibionts, or by the epibionts for its host. The role of the bacteria, and the way a selection would occur between partners needs more investigations. *Osedax* epibionts could be an example of host defense: in situ and in the aquaria, *Osedax* worms and bacterial mats never co-occur on the bones [45] (H. Wiklund personal observation). This indicates that they compete for the limited space on each bone. *Osedax* epibionts could be deterring the growth of bacterial mats e.g. by antibiotic production for example thus protecting their host.

Bacteroidetes group 1 were recurrently found with *O. mucofloris*. They were also found in association with several *Osedax* species from Californian whale falls [42, 44]. This indicates that *Osedax* might select this particular epibiont, or that this epibiont specifically colonise *Osedax*.

Third scenario, a resident gut microbiome *R. beryli* Firmicutes associated bacteria might be part of a resident gut microflora. We proposed that some of the bacterial group found with *R. beryli* are ingested during feeding. However, both Firmicutes groups associated with *R. beryli* were not detected in the environment, and are therefore probably not ingested by the worm. In addition, based on 16S rRNA gene analysis, they were related to gut microflora from other animals. Thus, they could be *R. beryli* gut symbionts.

Chapter 9

On siboglinid symbiosis and evolution

The characterisation of the endosymbionts from frenulates and *Osedax* gives a new perspective on the evolution of symbiosis in siboglinids. First, comparing endosymbiont and host diversity suggests an evolutionary trend of increasing diversity of endosymbiont and decreasing diversity of host. Furthermore, symbiosis probaly strongly influence the evolution of siboglinids and their endosymbionts. Low or high endosymbionts diversity suggest different recognition and colonisation pathway for these host.

9.1 Symbiosis: host and symbiont diversity

Although all siboglinid species studied up to date live in a obligate symbiosis, in many of them the symbiont has not yet been characterised (Manuscript I). Siboglinid phylogeny is not completely resolved: Vestimentiferan species are the most derived, and monoliferans are the sister group of vestimentiferans [50, 81]. Rouse et al., (2004) placed *Osedax* as the sister group of the vestimentiferan and monoliferan taxa [84]. However, in Glover et al., (2005) this placement is poorly supported [40]. It is therefore uncertain whether *Osedax* or the frenulates are more basal in the siboglinids. Thus, it is difficult to assess how host and symbiont have evolved in within the siboglinids. Nethertheless, by comparing the data available for some species, some general trends seem to emerge suggesting that although siboglinids and their endosymbionts do not co-speciate [25,42,70,76,108], evolution of hosts and endosymbionts is not independent of each other.

For example, vestimentiferans species show a low endosymbiont diversity compared to

Osedax and frenulate species. In vestimentiferans, 16S rRNA analyses show that only one endosymbiontic phylotype is present in a given population and in each individual, but intraspecific variations exist in different habitats (i.e. hydrothermal vents, seeps, and whale falls) and according to depth. In addition, two species found at the same site can even share a common endosymbiont [25, 33, 70, 76]. Likewise endosymbiont intraspecific variations are low for vestimentiferan ($\sim 0.5\%$) based on 16S rRNA [25, 70, 76]. Furthermore, a metagenomic analysis of *Riftia pachyptila* endosymbionts suggested that a single symbiont strain composed the trophosome population from two individuals, based on the 16S-ITS-23S rRNA operon, GC content and low nucleotide polymorphisms in the metagenome [83]. In vestimentiferans, the analysis of more variable markers than the 16S and ITS rRNA genes might detect more intraspecific endosymbiont variations [64, 115]. In frenulates, within one population, the presence of different symbiont phylotypes has been detected in *Siboglinum fiordicum* and *Oligobrachia mashikoi* [62, 109] and is probable in *Oligobrachia haakonmosbiensis* [64], although not enough specimens were analysed to conclude this with certainty in the latter. In addition, in *S. fiordicum* more than one 16S rRNA phylotype seems to be present in each individual [109], showing a pattern similar to *O. mucofloris* and other *Osedax* symbiont diversity [42]. Indeed in comparison to vestimentiferan species, the three frenulates *O. mashikoi*, *S. fiordicum* and *O. haakonmosbiensis* have higher endosymbiont intraspecific variations, respectively 2.4% and 2-3% for *O. mashikoi* and *O. haakonmosbiensis* [62, 64, 108]. Thus, in frenulates, intraspecific endosymbiont variations are comparable to *Osedax* ones, which are on average 4% for *Osedax* sp. 'yellow collar', 1.2% for *O. roseus*, 2% for *O. frankpressi* [42], and for *O. mucofloris* 1% to 9% between two symbiont clusters (Manuscript II).

Furthermore, low or high symbiont diversity may be coupled with host diversity. Halanych (2005) reported that diversity within the vestimentiferans is limited [50], and rRNA data suggests that vestimentiferan genes evolved more slowly than those of frenulates [48, 49]. This could also be the case for *Osedax*, i.e. that these have higher evolutionary rates than vestimentiferans. Vrijenhoek et al., (2009) showed that species diversity is high in *Osedax* [117]. Furthermore, COI sequence divergences between Osedax species are higher than those of vestimentiferans (from 8 to 24 % between *Osedax* species, and 13 to 20 % between vestimentiferan genera) [11, 117]. These differences in evolutionary rates are most likely linked to the life spans of these two annelid groups. It has been proposed for mammals that longer lived species (like elephants) have slower gene evolution rates than shorter lived species (like mice) [74]. Likewise, vestimentiferans with low gene evolution

rates are very long lived in comparison to Osedax [81]. However, unclear is how symbiont diversity could have influenced host evolutionary rates or vice versa. This might be an artifact due to two independent patterns evolving in the same direction.

In conclusion, although *Osedax* and frenulates species have different endosymbionts (heterotrophs versus chemotrophs), each host seems to have a high intraspecific diversity of endosymbionts at the species level, population level and even in some cases at the host individual level [42, 62, 109]. The endosymbionts of more *Osedax* and frenulate species need to be studied to confirm if this pattern is representative for the entire *Osedax* genus and Frenulata. Thus, symbiont specificity in the siboglinid could have evolved from a high diversity in primitive siboglinids toward a more specific symbiosis is the more derived siboglinids.

9.2 Symbiosis in siboglinid ancestors?

How did symbioses evolve in siboglinids? At this point in time, much remains to be resolved. It seems likely that the last common siboglinid ancestor was either symbiotic or pre-adapted to symbioses with Gammaproteobacteria. Possible scenarios include: (1) an aposymbiotic ancestor, with the independent evolution of endosymbiosis in the major siboglinid groups. (2) At first, an association with varied bacteria, this association becoming specific later on with sulfur-oxidising or Oceanospirillales bacteria. (3) At first, an association with sulfur oxidisers and then a switch to heterotrophy or vice versa. Since sulphide is available at whale falls [110] and can sustain chemosynthetic symbiosis, *Osedax* heterotrophy in a chemosynthetic environment remains a mystery as to when and where it evolved.

The contrast between the high intra-population symbiont diversity in frenulates and *Osedax* species and the low intra-population symbiont diversity in vestimentiferans would fit better with the second and third hypotheses. As mentioned in Manuscripts I and II, each *Osedax* and frenulate species seem to be able to interact with several symbiont types. The capacity to interact with various symbionts could thus be an ancestral character still retained in *Osedax* and frenulates, while vestimentiferans would be more specialized and restricted in the number of symbionts they can interact with.

Symbionts are horizontally transmitted in vestimentiferans [77] and a horizontal transmission is also highly probable in *Osedax* [85]. The low symbiotic diversity in vestimentiferans was hypothesised to come from a low symbiotic diversity in the environment or from a highly selective colonisation process [29]. In *Riftia pachyptila*, less than 20 bacteria infect

the juvenile host through its skin [77]. Furthermore the colonisation process is reminiscent of colonisation patterns in pathogenic infection, indicating a strong selection for a highly specific mode of transmission [29].

Such a colonisation process differs from the one we propose for *O. mucofloris*. In *O. mucofloris*, the high symbiont diversity and the segregation of the symbiont types in different areas of the tissue suggest that the colonisation process is continuous through the worm's life. The recognition mechanisms in *O. mucofloris* are therefore probably different than those of *R. pachyptila*. Therefore, in terms of evolution, not only is there a symbiont type switch between *Osedax* and vestimentiferans from heterotrophy to chemoautotrophy, but the recognition patterns between partners are likely different, which suggests a strong adaptation of vestimentiferans to a low symbiont diversity and of *Osedax* to a high symbiont diversity.

To better resolve this, the diversity of free-living bacteria in different siboglinids habitats [1], as well as the symbiont characterisation of more *Osedax*, frenulates and *Sclerolinum* species would be needed [50, 108].

Chapter 10

Osedax **symbiosis**

In the following, we compare *Osedax* symbiont diversity to other symbioses before discussing the potential role or effect of such a diversity. Then, we discuss the potential function of the host and the endosymbionts in *Osedax* symbiosis.

10.1 Symbiont diversity is high

10.1.1 Comparison to other symbioses

To date, the high diversity of *O. mucofloris* endosymbionts is rarely found in other marine symbioses [29]. Molecular methods are revealing that many host species are associated with a higher diversity of symbiotic bacteria than was previously thought [29]. Several examples of species with multiple symbionts exist both in terms of diversity of

the bacterial lineages involved and metabolic capacities of those symbionts [29]. However, the diversity observed within *Osedax* is different because its symbionts belong to a single lineage in the Gammaproteobacteria and are not known to have complementary metabolic capacities [42, 71, 72]. Bathymodiolin mussels are for example mainly associated with a sulphur-oxidising symbiont or a methane-oxidising symbiont or both. Duperron et al., (2008) showed that an *Idas* mussel was associated with four additional symbionts, 3 Gammaproteobacteria including a methylotroph and an additional sulfur oxidiser, a symbiont of unknown function, and a Bacteroidetes [30]. In two oligochaete worms multiple symbionts co-occur including Gammaproteobacteria, Deltaproteobacteria and a spirochete, and these symbionts have different metabolic capacities including sulphur reduction and sulphur oxidation [87,122]. Recently, Petersen et al., (2010), reported that the vent shrimp *Rimicaris exoculata* was associated with a gammaproteobacterial symbiont in addition to the already known epsilonproteobacterial symbiont [79]. This symbiosis also show complementary metabolic capacities with the use of different carbon fixation cycles.

In wood-boring bivalves (Teredinidae), so-called shipworms, symbiont diversity is similarly high as in *Osedax*. Symbionts of the shipworms provide the host with cellulase and nitrogenase to help digest the wood and supplement the host diet [27]. Luyten et al., (2006) [66] showed that shipworms are associated with a high diversity of heterotrophic bacteria that fall into one gammaproteobacterial lineage. Within that bacterial lineage, different symbiont clusters are found in a single host population and in a single host individual, with each individual dominantly associated with one cluster. Two clusters dominate in most of the individuals and seem to exclude one another, i.e. in one individual when cluster one is dominant cluster two is in low abundance or absent, and vice versa. Thus, *Osedax* and shipworms share a similar pattern of both symbiont diversity and the dominance of one symbiont in one individual [29, 42].

10.1.2 On the role of symbiont diversity: a flexible adaptation of the host or the presence of cheaters?

It has been proposed that symbiont diversity in a host would give it more flexibility in a variable environment and more efficiency in its nutrition by accessing more than one food source [29,121]. The co-occurrence of sulphur-oxidising and methane-oxidising symbionts in bathymodiolin mussels is proposed to improve the fitness of the symbiosis [29,32]. The two symbionts use different energy sources (methane or hydrogen sulphide). They therefore do not compete, and might possibly cooperate [29]. The host could survive change in

the availability of these energy sources at vents or seeps, and even adapt by varying the abundance of each respective symbiont [29, 32, 34]. Such an adaptation is unlikely in *Osedax* because its different symbionts are from a single lineage. Nevertheless some *Osedax* symbiont clusters share only 91% similarity based on 16S rRNA and, as described below, isolated Oceanospirillales bacteria in that cluster differ slightly in their metabolism. Some differences could therefore exist in the role of each *Osedax* symbiont cluster, with different symbionts being better adapted to different conditions the host might encounter.

Another hypothesis is that some of the *O. mucofloris* symbionts cheat. *Osedax* branching root systems could share another analogy with plants. Plant roots are associated with mycorrhizal fungi symbionts in nodule structures, which can fix nitrogen, thereby enhancing the plant's access to nitrogen. In return, the plant allocates resources to its symbiont. However, not all symbiotic mycorrhiza are beneficial at the same level: they range from highly beneficial to cheaters that benefit from the host without providing something in return [10]. In reaction, the plant can reward the nodules of beneficial symbionts more by providing them with more resources [10]. However, this selective rewarding is not possible anymore if the symbiont population is mixed [10]. Bever et al., (2009) observed that plants maintain a spacial separation of the different symbionts in order to discriminate against cheaters [10]. If some *Osedax* symbionts are less efficient than others, the spacial distribution of the symbionts in the root of each individual could be a way for the host to control the presence of cheaters.

Alternatively, Friesen & Matthias (2010) proposed another model in plant - mycorrhizal fungi where mixed infection could promote the diversification of mutualistic symbionts [35]. They use an adaptive model of the symbiotic interaction which shows that complete cheaters (non-fixing symbionts) do not evolve. However, in a mixed population, competition leads to diversification with the co-existing symbionts developing new strategies, where less mutualistic strains exploit the benefits generated by better mutualistics. Following this second model high diversity in *Osedax* symbiosis could be the result of such a competition among its symbionts driving their diversification. These two models (spacial separation or symbiont competition) give new insights on how *Osedax* symbiosis could maintain such a high diversity of symbionts.

10.2 Who does what? Endosymbionts and host roles in the *O. mucofloris* symbiosis a mutualism?

10.2.1 Symbiont's role

Based on several lines of evidence, including the lack of a gut, mouth and anus, previous studies proposed that *Osedax* relies for its nutrition on Oceanospirillales endosymbionts for the degradation of the whale bones to organic compounds (lipids and collagen) [42, 44, 84]. Lipid composition analysis of both symbiotic (root) and symbiont free (trunk and palps) host tissues, whale bones and whale flesh, supported a trophic interaction between the host and the symbionts [44]. Furthermore, energy transfer was proposed to occur through direct digestion of the symbionts [44] which was observed in both *O. rubiplumus* and *O. frankpressi* (S. Katz and M. Bright, unpublished data cited in [42]).

Based on ^{13}C and ^{15}N stable isotopes analyses, *Osedax* was shown to rely on whale bone collagen instead of whale bone lipids [44]. Collagen is a protein, one of the main components of bone, that can be degraded by collagenase in gelatin and then further degraded by gelatinase. Collagenase and gelatinase activity can be tested together [42]. For *O. roseus, O. frankpressi* and *Osedax* sp. 'yellow collar', collagenolytic (collagenase & gelatinase) activity was detected only in the symbiotic root tissue, indicating that the endosymbionts could be responsible for the degradation of whale bone collagen [42]. However, direct evidence that *Osedax* endosymbionts are involved in bone collagen degradation is lacking:

- Both the host and its symbionts are heterotrophs and could be able to degrade bone collagen (or bone lipids). The detection of collagenolytic activity in the symbiotic (root) and not in the symbiont free (trunk and palp) host tissue could be an indication of the specialisation of *Osedax* root to degrade the bones, and not come directly from the symbionts.

- The symbionts are not in direct contact with the bone matrix, but separated from it by epithelial host cells (Manuscript II Figures 3 & 4). Collagen is not soluble, and known collagenase are usually extracellular [42]. Thus, if the symbionts produce collagenase, it would need to be transported through the epithelial cells to the bone worm interface. Goffredi et al., (2007) proposed that the proteins involved may not be true collagenases but instead proteases with the capability of cleaving amino acids from the collagen molecule [42]. The characterisation of the type of collagenase would help determine its symbiotic or host origin.

- Oceanospirillales bacteria were successfully isolated from whale fall sediments [71, 72]. Two of these Oceanospirillales bacteria, *Amphritea japonica* and *Neptunomonas naphthovorans*, lacked a collagenase/gelatinase activity which is therefore not shared by all Oceanospirillales bacteria [72]. In addition, *A. japonica* is closely related to the *O. japonicus* symbiont R21 (100% 16S rRNA similarity) [72] and *O. mucofloris* symbiont clusters D and E (∼99% 16S rRNA similarity). This 16S rRNA similarity to *A. japonica* indicates that those particular symbionts of *O. japonicus* and *O. mucofloris* may not have a collagenase/gelatinase activity.

With such a diversity of endosymbionts associated with *O. mucofloris*, it is difficult to assess what the nature of their interaction with the host is.

On the one hand, if the symbionts help the host degrade the bones, the diversity of the bacteria could correspond to different states of bone degradation: it has been shown that *Osedax* species colonise the whale bones in a succession from early colonisers to late colonisers [12]. Furthermore, some *Osedax* species were not found on the bones but on whale blubber (containing mainly lipids) or in the sediment surrounding the whale skeleton [12, 36, 38, 52]. The characterisation of symbionts from early and late colonisers, as well as species living on a different substrate than bones could therefore help answer the question of the symbionts role.

On the other hand, we hypothesise that the symbiont role could not be related to bone degradation at all. For example, the symbionts could supply molecules that the host can not synthesize de novo, such as certain amino acids, or fatty acids. Vaccenic acids can for instance not be produced de novo by the worms and were abundant in the host, probably with a transfer from the symbiotic root tissue to the asymbiotic trunk and palps [44]. Such symbioses are known in sap-feeding insects: the sap on which the symbionts are feeding lack essential nutritional compounds which are synthesised by the bacterial symbionts of the aphids. For example, sharpshooters feeding on xylem are in metabolic interdependence with mostly two symbionts *Sulcia muelleri* and *Baumannia cicadellinicola*, which provide their host with essential amino-acids and vitamins [68]. Symbionts of cockroaches, feeding on plant sap, recycle nitrogen compounds (urea and ammonia) and can produce all essential amino acids and some vitamins [89].

10.2.2 Host role

As Oceanospirillales bacteria are aerobic heterotrophs and the bone is an anaerobic environment [110], the worm probably provides the symbionts with oxygen diffusing through

its highly vascularised palps in contact with the oxic water. Depending on the nature of the nutritional interaction between host and symbionts, the host role will differ. On the one hand, if the symbionts are involved in collagen or other complex molecule degradation, we can speculate on two scenarios: i) The host provides the symbiont with complex organic compounds that it can not degrade itself and then digests the symbionts for nutrition. ii) The symbionts produce proteins to degrade the complex compounds. These enzymes are then transported through the host cells to the bone matrix. Carbon from the bone is digested extracellularly and the host then assimilates smaller organic molecules (such as gelatin which is soluble). In return, the host feeds the symbionts and there is no explanation as to why the host digests the symbionts.

On the other hand, if the host itself can degrade complex molecules from the bone, it provides the symbionts with organic molecules, and the symbionts synthesise organic compounds missing in the host diet (which could include essential amino acids, fatty acids or vitamins).

Chapter 11

Summary and conclusion

In this study, different aspects of symbioses at whale falls were considered. With the characterisation of *R. beryli* and *O. mucofloris* epibionts, new insights into associations between bacteria and polychaetes were gained. The comparison of the two polychaetes epibionts to the free-living bacteria from whale falls shows that these worms provide unique ecosystems for their epibionts. However, the exact role of these associations remain unclear: we suspect a commensalism or a mutualism, but more data is needed on the abundance of each bacteria on each worm species and in the environment, as well as of their metabolic capacities. The epibiont diversity suggests that these symbioses are more complex than previously thought and possibly varying over time.

The finding on *R beryli* of bacteria from a monophyletic cluster dominated by bathymodiolin mussels symbionts is intriguing. Besides the report of a new host for these bacteria, it raises several questions regarding their ubiquity, host specificity, and dispersal

capacities. Here again, a better knowledge of their abundance at whale falls would be useful to answer some of these questions.

Remarkable is the diversity of symbionts in *O. mucofloris*. In contrast to other marine symbioses, the diversity of *O. mucofloris* symbionts does not seems to allow the utilisation of several energy sources, but seem redundant in term of metabolic capacities since all symbionts belong to the Oceanospirillales. Symbiont distribution in the population and in each worm showed a unique pattern, unknown in other invertebrate - bacteria symbioses to our knowledge. Moreover, it suggested that new mechanisms of symbionts recognition, symbiont transmission, and control of the symbiont population exist in *Osedax* compared to other siboglinids.

With so many challenges, whale falls are ideal habitats for exploring symbioses. The deployment of, and access to several whale falls, as well as the possibility of keeping whale fall fauna in aquarium will help in the understanding of the symbioses. In the future, characterisation of the free-living symbionts, their diversity, the metabolism of the symbionts will give new insights on symbiosis and may well reveal more diversity.

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Part V

Appendix

Manuscript IV

Lucinid symbiont diversity: determining the influence of host selection, geography, habitat, and depth

Caroline Verna, John Taylor, Alban Ramette and Nicole Dubilier

Manuscript in Preparation

This project is a collaboration with John Taylor from The Natural History Museum in London. My part is to work on the characterisation of the symbionts from several lucinid species. The host phylogeny will soon be published by John Taylor and collaborators. Alban Ramette will help with the phylogeography and statistical analysis of the data once the lab work is complete.

Lucinid symbiont diversity: determining the influence of host selection, geography, habitat, and depth

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Abstract

Lucinidae are a diverse bivalve family occuring wordlwide in various habitats. All species are known to be associated with sulphur-oxidising endosymbionts, that are horizontaly transmitted. However, symbionts have only been characterised in very few species, mostly from shallow sea-grass bed and mostly from the Caribbean. Based on these species, symbiont diversity seemed low among lucinid species, with more than one species sharing a unique symbiont, based on the 16S rRNA gene. In this study we aim to characterise the dominant symbiont from lucinid species from diverse habitats and locations worldwide, as well as from several lineages within the Lucinidae, using comparative 16S rRNA sequences analysis. Preliminary results indicates a higher diversity of symbionts than previously found with symbiont belonging to more than one bacterial group in the Gammaproteobacteria. Several factors seem to influence the symbiont diversity. Although host and symbiont phylogenies are not congruent, each sub family within the Lucinidae show preference for a single gammaproteobacterial group. In addition, depth and habitat but not geography seem to influence host-symbiont associations at the genus/species level.

Context

The project focuses on the symbionts of lucinids, a group of bivalves found in a variety of reducing environments: mangrove, coral reef and sea-grass bed sediments, deep sea sediments, cold seeps and recently hydrothermal vents [8, 9, 19]. Lucinid bivalves are one of the most species rich bivalve families [8,17–20]. Their phylogeny is not well characterised yet [19], however John Taylor and collaborators are currently working on a new phylogeny based on several genes to better understand the evolutionary history of the group.

All lucinid bivalves investigated up to date have sulphur-oxidising symbionts but the symbionts have been characterised in only very few species [1, 4, 6, 7, 11]. Lucinid symbionts are horizontally transmitted, as shown in shallow water species from the Caribbean [3, 10, 12, 13]. Horizontal transmission is thought to lead to a higher diversity of symbionts than in vertically transmitted symbionts [2, 5, 16]. Nevertheless, in lucinids, symbiont diversity seems low with a unique symbiont in each host species [4,6,7,11]. In addition, several species have been found to share a single symbiont strain based on 16S rRNA gene analysis [6,7], and symbionts from one species have even been shown to colonise the juveniles of other species [12]. In contrast, Ball et al., (2009) [1] detected several symbiont morphotypes in the gills of *Anodontian ovum*, and the symbiont sequences did not cluster with the previously known lucinid symbionts. This suggests that symbiont diversity in this species is higher than in all previously characterised host species.

In this context, we have decided to characterise the primary symbionts of lucinid species, from sampling locations around the world, in various habitats, and widespread in several sub-families of Lucinidae. We thus hope to assess the symbiont diversity in this understudied symbiosis, and assess the influence of different factors on the lucinid symbiont diversity: host selection, habitat, depth and geography.

Materials and methods

Most of the available materials were provided by John Taylor and collaborators. The samples were mostly gill tissue both fixed and stored in ethanol. The objective being to characterise the dominant symbiont in each specimen, we used direct sequencing of the 16S rRNA gene. Then, if ambiguities in the obtained sequences suggested that more than one symbiont was present and dominant, symbiont diversity was further assessed with cloning and sequencing.

Sixty different individuals from 40 different species were available. For most species, a single individual from a single location was available, but for about 7 species, several locations, or up to three specimens from one location were available. For now the symbionts of 17 specimens have been characterised including four with clone libraries to better assess the symbiont diversity.

Preliminary results

Preliminary results showed that indeed lucinid diversity has been underestimated, and showed several interesting patterns (Fig. 1). The obtained sequence clusters grouped in four monophyletic groups. The first group contained the symbionts from previously studied lucinid species such as *Codakia orbicularis, Codakia costata, Lucinoma annulata*. This group contained three distinct clusters named 1 - 3. The second group, cluster 4, was a monophyletic cluster containing the symbionts from *Anondontia* lucinids. The third group, cluster 5, was a monophyletic cluster containing the symbionts from one lucinid species *Phacoides pectinatus* (previously named *Lucina pectinata*). In the last group, cluster 6, sequences formed a monophyletic cluster including symbionts from vent and seep lucinid, *Maorithyas*, and *Alviniconcha*, species (Fig. 1).

Preliminary conclusions

Based on the preliminary analysis of the 16S rRNA genes, several conclusions can be drawn.

First, to a certain degree, host and symbiont phylogenies are congruent. The host phylogeny showed that *Anodontia* spp. are distinct from other lucinids (Taylor and col.). In addition, the position of *Phacoides pectinatus* in the host phylogeny is unclear, as it is forming a long branch whose position is not well supported (Taylor and col.). Therefore, three monophyletic symbiont groups (clusters 1-3, cluster 4 and cluster 5) in the symbiont phylogeny (Fig. 1) correspond to three monophyletic groups in the host phylogeny.

Within each cluster, congruence between host and symbiont phylogeny is not supported since distinct species share a single symbiont, for example in cluster 3, *Codakia paytenorum, Divalinga bardwelli, Codakia orbicularis, Ctena eburnea*, and *Lucina pensilvanica* all share one identical symbiont sequence (Fig. 1).

In addition, geography does not appear to play a role in the distribution of the symbionts. Indeed the 5 species aboved-mentioned, sharing a single symbiont in cluster 3, were sampled in distinct locations, namely Japan, West Australia, Guadeloupe, Cape Verde and Florida. (Fig. 1)

Depth and habitat could influence the symbiont diversity in lucinids, since two lucinids from deep sea seeps and vents have completely distinct symbionts in cluster 6, which contain symbionts of several seep and vent species. However, *Lucinoma* aff. *kasani*, also from a seep, is in cluster 2. (Fig. 1)

Hopefully, the characterisation of the symbionts from more species, including other deep sea species, will help to better understand the symbiont diversity in the lucinids and the factor influencing their distribution. In addition, we plan to make rigorous statistical analysis of the symbiont phylogeny and factors influencing their distribution (host, geography, depth and habitat).

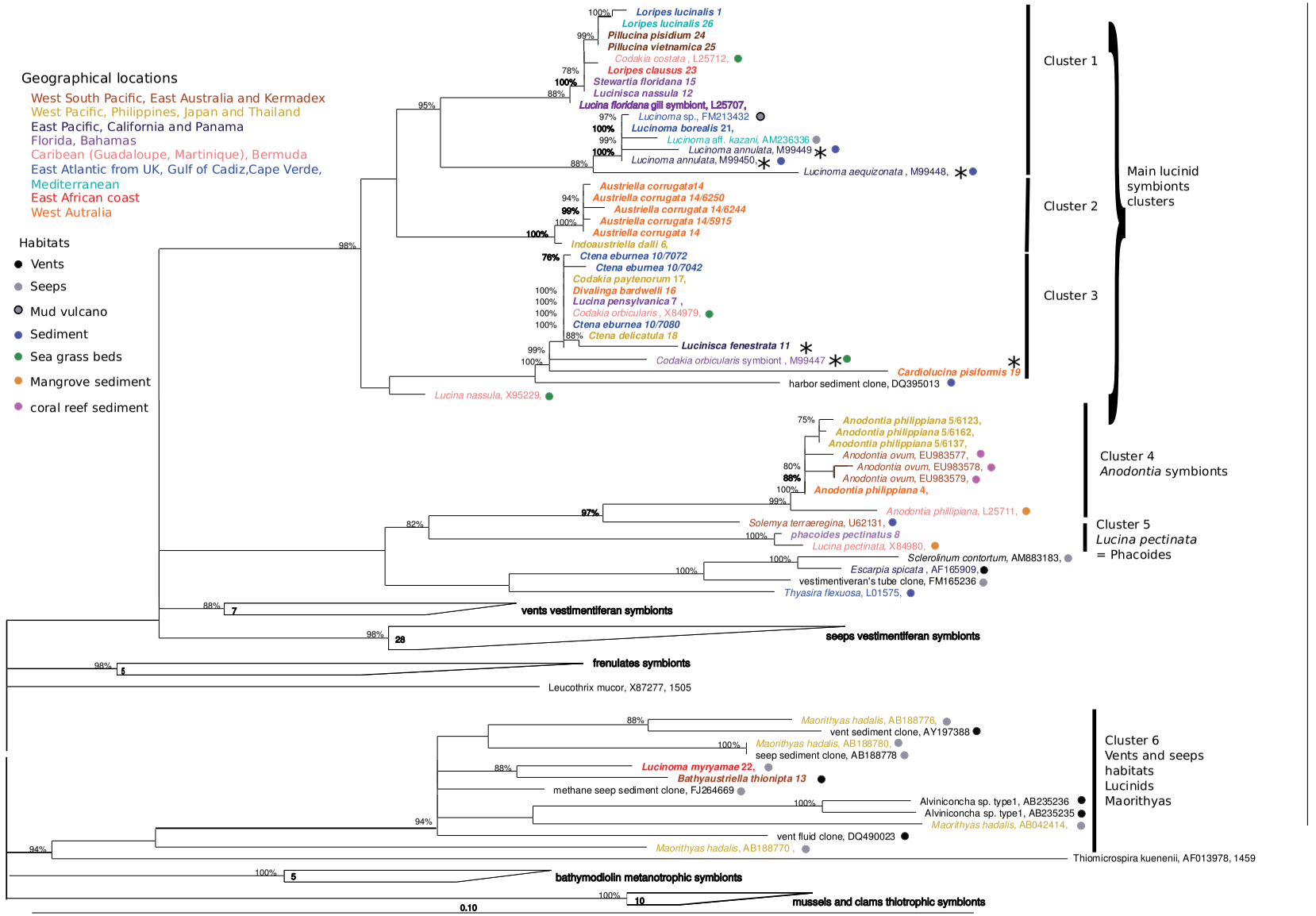
Geographical locations
West South Pacific, East Australia and Kermadex
West Pacific, Philippines, Japan and Thailand
East Pacific, California and Panama
Florida, Bahamas
Caribean (Guadaloupe, Martinique), Bermuda
East Atlantic from UK, Gulf of Cadiz,Cape Verde,
Mediterranean
East African coast
West Autralia
Habitats
Vents
Seeps
Mud vulcano
Sediment
Sea grass beds
Mangrove sediment
coral reef sediment
Loripes lucinalis 1
Loripes lucinalis 26
Pillucina pisidium 24
Pillucina vietnamica 25
Codakia costata , L25712,
Loripes clausus 23
Stewartia floridana 15
Lucinisca nassula 12
Lucina floridana gill symbiont, L25707,
Lucinoma sp., FM213432
Lucinoma borealis 21,
Lucinoma aff. kazani, AM236336
Lucinoma annulata, M99449
Lucinoma annulata, M99450,
Lucinoma aequizonata , M99448,
Austriella corrugata14
Austriella corrugata 14/6250
Austriella corrugata 14/6244
Austriella corrugata 14/5915
Austriella corrugata 14
Indoaustriella dalli 6,
Ctena eburnea 10/7072
Ctena eburnea 10/7042
Codakia paytenorum 17,
Divalinga bardwelli 16
Lucina pensylvanica 7 ,
Codakia orbicularis , X84979,
Ctena eburnea 10/7080
Ctena delicatula 18
Lucinisca fenestrata 11
Codakia orbicularis symbiont , M99447
Cardiolucina pisiformis 19
harbor sediment clone, DQ395013
Lucina nassula, X95229,
Cluster 1
Cluster 2
Cluster 3
Main lucinid symbionts clusters
Anodontia philippiana 5/6123,
Anodontia philippiana 5/6162,
Anodontia philippiana 5/6137,
Anodontia ovum, EU983577,
Anodontia ovum, EU983578,
Anodontia ovum, EU983579,
Anodontia philippiana 4,
Anodontia phillipiana, L25711,
Cluster 4
Anodontia symbionts
Solemya terraeregina, U62131,
phacoides pectinatus 8
Lucina pectinata, X84980,
Cluster 5
Lucina pectinata
= Phacoides
Sclerolinum contortum, AM883183,
Escarpia spicata , AF165909,
vestimentiveran's tube clone, FM165236
Thyasira flexuosa, L01575,
7
vents vestimentiferan symbionts
28
seeps vestimentiferan symbionts
5
frenulates symbionts
Leucothrix mucor, X87277, 1505
Maorithyas hadalis, AB188776,
vent sediment clone, AY197388
Maorithyas hadalis, AB188780,
seep sediment clone, AB188778
Lucinoma myryamae 22,
Bathyaustriella thionipta 13
methane seep sediment clone, FJ264669
Alviniconcha sp. type1, AB235236
Alviniconcha sp. type1, AB235235
Maorithyas hadalis, AB042414,
vent fluid clone, DQ490023
Maorithyas hadalis, AB188770 ,
Thiomicrospira kuenenii, AF013978, 1459
Cluster 6
Vents and seeps
habitats
Lucinids
Maorithyas
5
bathymodiolin metanotrophic symbionts
10
mussels and clams thiotrophic symbionts
0.10

Figure 1: 16S rRNA phylogeny of lucinid symbionts. Tree based on maximum likelihood (phyML) analyses with 100 bootstraps (branches with support values $< 40\%$ were collapsed in multifurcations; values $> 75\%$ to the left of a given node). Calculated with ARB [14], using the Silva (100) alignment [15]. When information was available, habitats are indicated by circles at the right of each sequences: closed black circle = hydrothermal vents, closed grey circle = cold seeps, open grey circle = Mud Vulcano, Green circle = see grass bed sediment, blue circle = sediment, orange circle = Mangove sediment. The color of the sequences indicate their geographic location (see graphic). The bars on the right indicate the 6 clusters where lucinid symbionts are founds. Sequences from this study are in bold. For sequences obtained with direct sequencing, the number following each symbiont sequence is an individual number. For sequences obtained with clone libraries, both individual / clone numbers are given. Accession numbers for public database sequences are given. A star * mark short sequences (< 1000 bp) or sequences with numerous ambiguities which position is not clear in the tree. Scale bar = 0.10 estimated substitutions per site

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