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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).

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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-

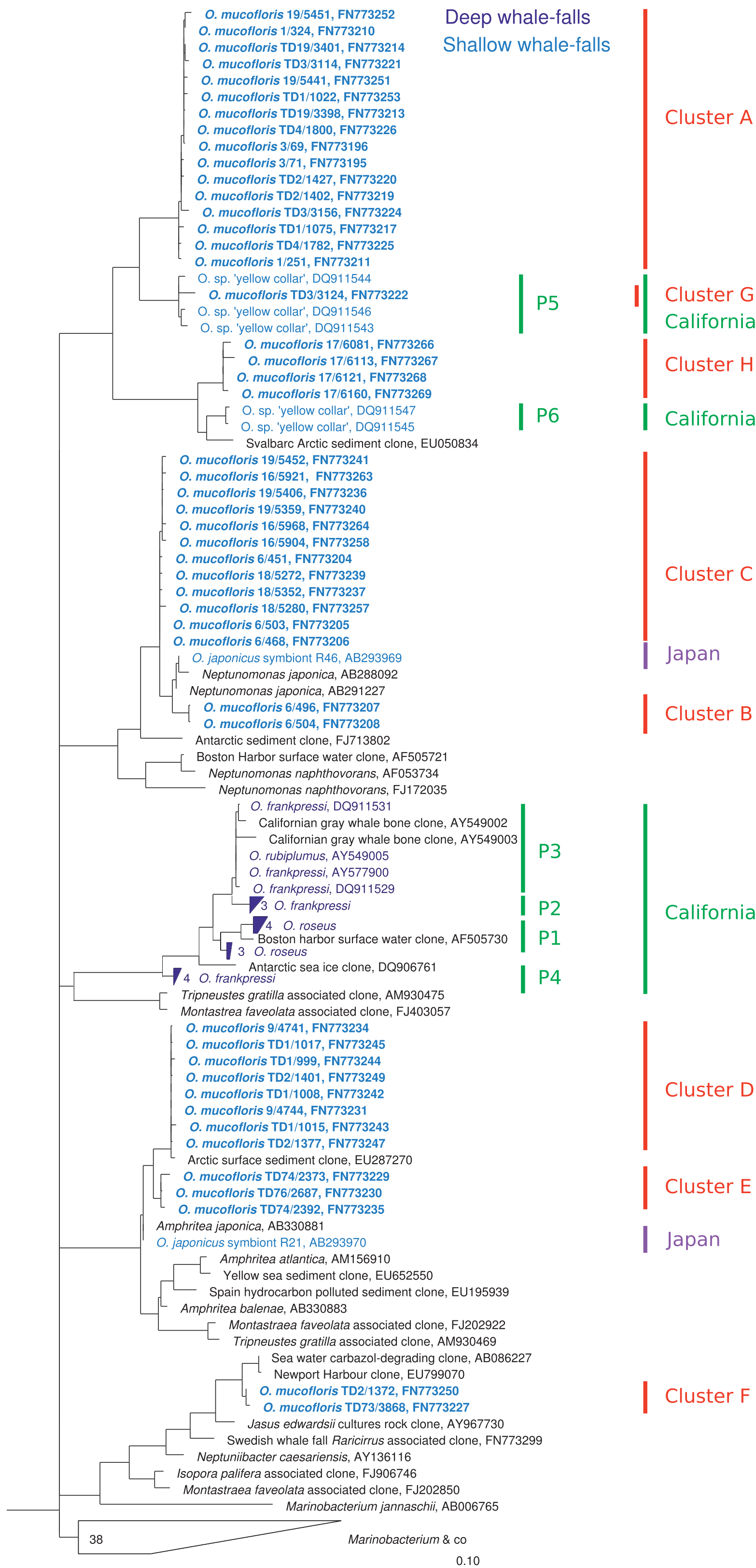

Fig. 1. 16S rRNA phylogeny of *O. mucofloris* Oceanospirillales endosymbionts. Consensus tree based on neighbour joining, maximum likelihood and maximum parsimony reconstructions. Sequences from this study are in bold. For clusters A and C, only some of the sequences used in the analyses are shown because 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.

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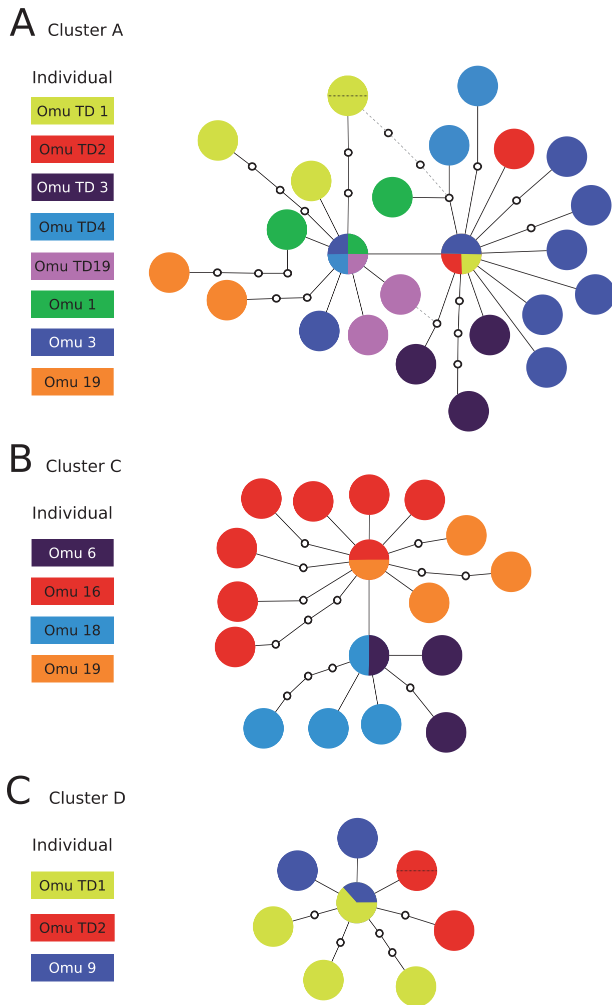

Fig. 2. Parsimony network of 16S rRNA sequences from endosymbiont clusters A, C and D in *O. mucofloris* individuals. Each unique 16S rRNA phylotypes is represented by a circle, lines connecting circles represent 1 nucleotide difference between phylotypes, and open circles on the lines show unsampled 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.*

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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).

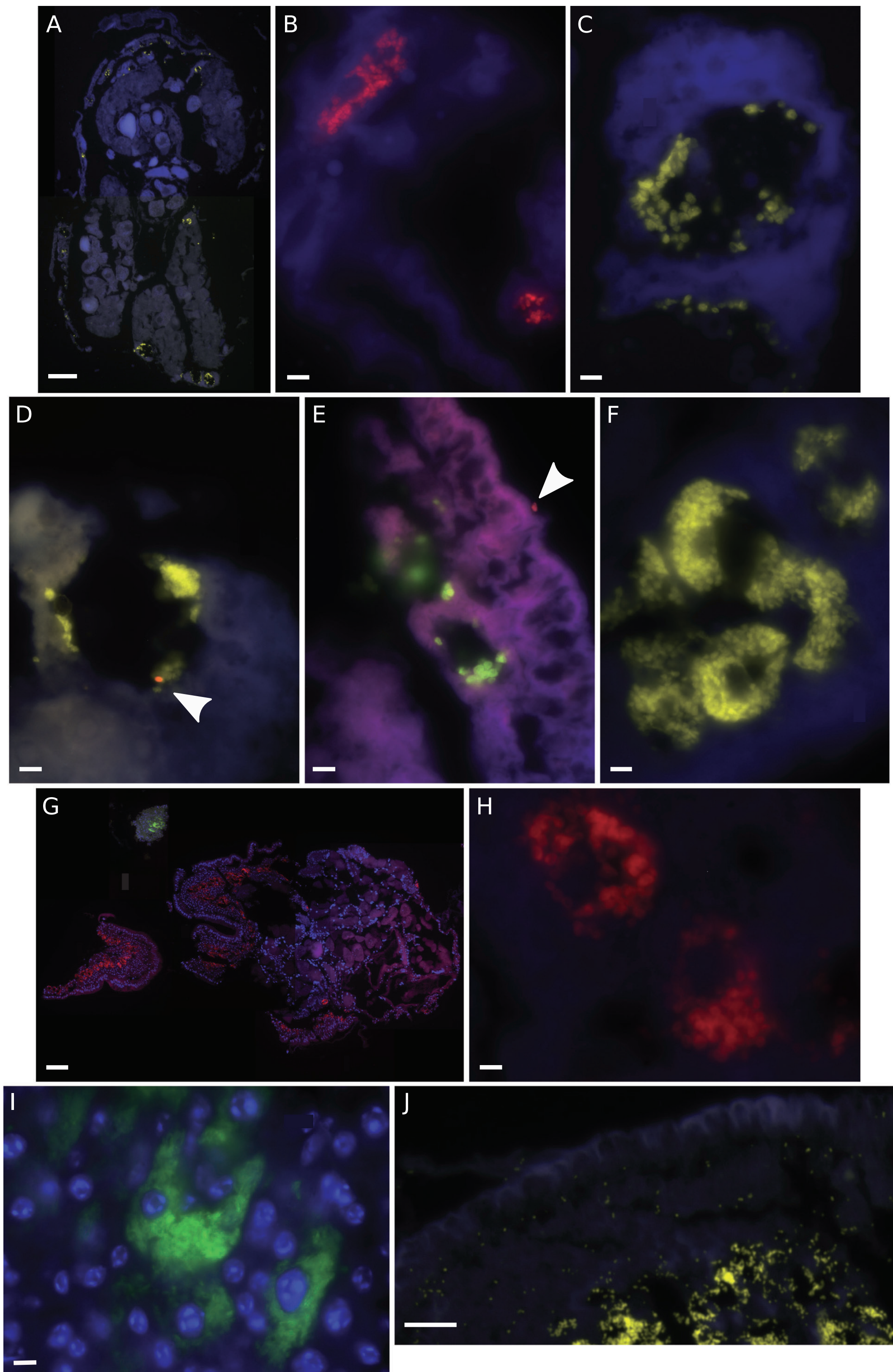
		Clone numbers found for each cluster								Symbiont clusters detected by FISH					
Whale and year	*O. mucofloris* individual	A	B	C	D	E	F	G	H	A	BC	DE	F	GH	Unknown
Minke 2004	Omu TD1	20			33										
	Omu TD2	29			3		1								
	Omu TD3	10						1							
	Omu TD4	12													
	Omu TD19	34													
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
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G
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J

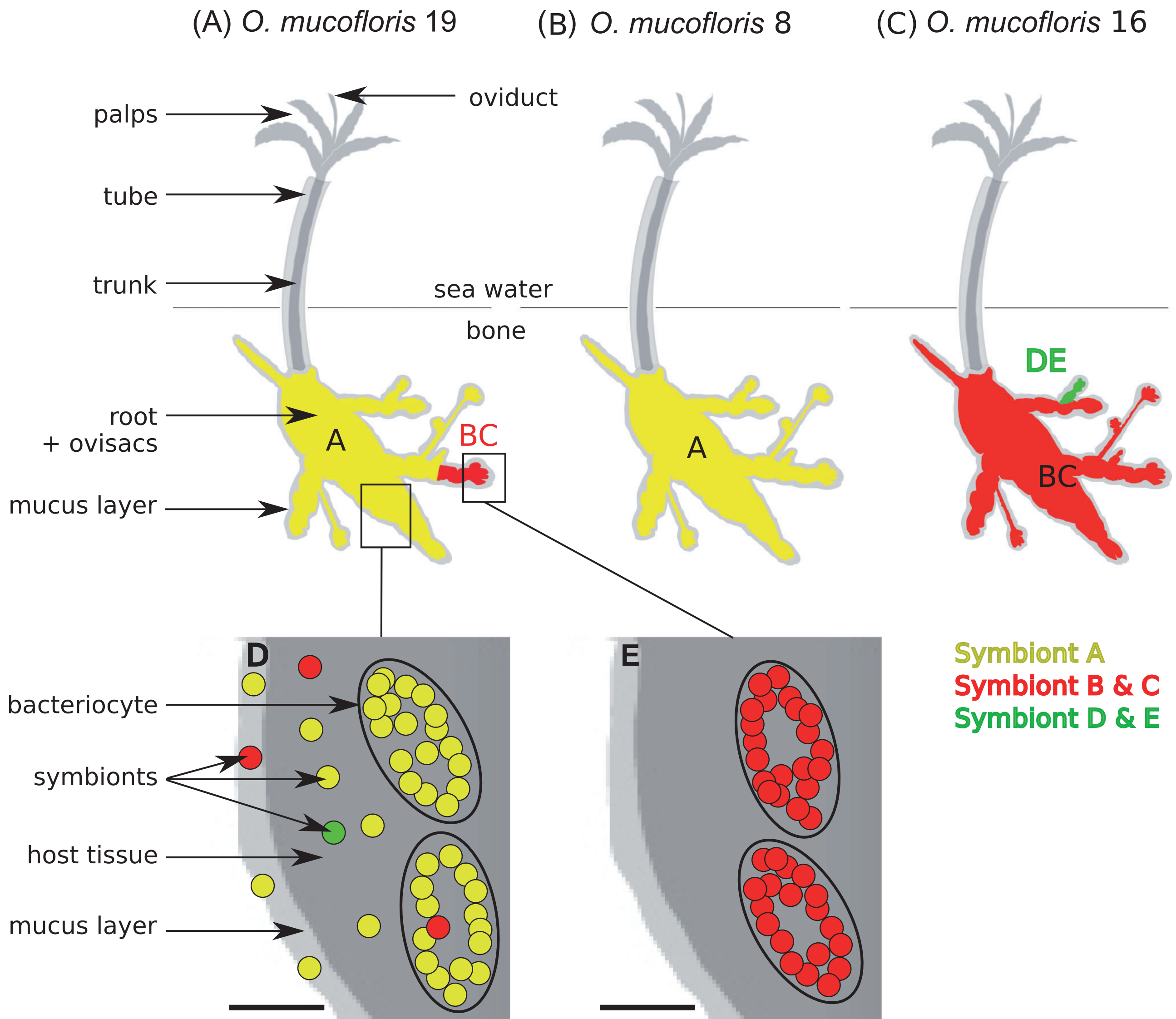

Fig. 4. Schematic diagram of endosymbiont distribution in *O. mucofloris* individuals Omu 19 (A), Omu 8 (B) and Omu 16 (C) based on FISH analyses. Colour scheme shows endosymbionts from Cluster A in yellow, from Cluster BC in red, and from Cluster DE in green. All three worms were dominated by endosymbionts 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).

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