Microbial insights from Antarctic and Mediterranean shallow-water bone-eating worms

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Received: 18 August 2019 / Revised: 8 July 2020 / Accepted: 7 August 2020 / Published online: 20 August 2020
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Abstract

Bone-eating worms of the genus *Osedax* (Annelida, Siboglinidae) form unique holobionts (functional entity comprising host and associated microbiota), highly adapted to inhabit bone tissue of marine vertebrates. These gutless worms have developed nutritional symbioses housing intracellular, horizontally acquired, heterotrophic bacteria hypothesised to harness nutrients from organic compounds, sequestered within the bone. Despite previous efforts, critical mechanisms mediating activity and acquisition of diverse bacterial assemblages remain unclear. Using 16S rRNA amplicon sequencing, we performed detailed taxonomic and predicted functional analyses shedding light on the microbial communities of two shallow-water *Osedax* species (*Osedax deceptionensis* and *Osedax* ‘mediterranea’) from contrasting habitats (Antarctic and Mediterranean Sea), in two tissue types (roots and palps). Comparative assessments between host species revealed distinct microbial assemblages whilst, within host species and body tissue, relative symbiont frequencies retained high variability. We reported relatively high abundances of microbes previously classified as primary endosymbionts, Ribotype 1 (order Oceanospirillales), and diverse likely secondary epibionts warranting further exploration as recurrent *Osedax* associates. Surprisingly, *O.* ‘mediterranea’ exhibited relatively low abundance of Oceanospirillales, but increased abundance of other potentially hydrocarbon degrading bacteria from the family Alteromonadaceae. We hypothesise the presence of functionally similar, non-Oceanospirillales primary endosymbionts within *O.* ‘mediterranea’. Functional metagenomic profiling (using 16S rRNA sequences) predicted broad metabolic capabilities, encompassing relatively large abundances of genes associated with amino acid metabolism. Comparative analyses between host body tissue communities highlighted several genes potentially providing critical functions to the *Osedax* host or that confer adaptations for intracellular life, housed within bone embedded host root tissues.

Keywords Endosymbiont · Symbiosis · Siboglinidae · Holobiont · Oceanospirillales · Whale fall

Introduction

Unique, specialist adaptations are critical for the survival of diverse organisms within nutrient limited, competitive marine zones. The death and fall of large marine mammals can bring temporary and successional oases within such environments, as macrofaunal communities compete to exploit these large influxes of organic matter (Smith and Baco 2003). One such specialist, *Osedax* (Annelida, Siboglinidae) (Goffredi et al. 2004; Rouse et al. 2004) have successfully colonised these unusual habitats, feeding and living within vertebrate bones from shallow and deep waters of the Pacific, Atlantic and Antarctic Oceans, and even the Mediterranean Sea (see Fujiwara et al. 2019). Commonly known as the ‘bone-eating’ worms, free-swimming *Osedax* larvae settle upon vertebrate carcasses and develop a distinct network of vascularised ‘roots’ which proliferate into the

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00300-020-02731-1>) contains supplementary material, which is available to authorised users.

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bone (Goffredi et al. 2005, 2007; Miyamoto et al. 2013). Without a mouth or gut, *Osedax* rely upon a community of endosymbionts to supply nutrition through the degradation of high energy compounds (Goffredi et al. 2005, 2007; Rouse et al. 2009; Taboada et al. 2015), including collagen and possibly lipids, made available by enzymes secreted from within the roots (Tresguerres et al. 2013; Miyamoto et al. 2017). However, the mechanisms by which endosymbionts acquire such nutrients remain to be fully understood (Goffredi et al. 2014). We now know of 26 *Osedax* species (Rouse et al. 2004, 2008, 2015; Glover et al. 2005, 2013; Fujikura et al. 2006; Braby et al. 2007; Fujiwara et al. 2007; Goffredi et al. 2007; Vrijenhoek et al. 2008, 2009; Amon et al. 2014; Taboada et al. 2015; Sumida et al. 2016), of which 15 were formally described within the last few years (Rouse et al. 2018; Fujiwara et al. 2019), and a few, as yet remain undescribed, demonstrating the increased knowledge of *Osedax* prevalence and diversity.

Since their discovery in Monterey Bay, California (Rouse et al. 2004), the unique relationship of *Osedax* and its symbionts have received much attention. Female *Osedax* house primary endosymbionts (intracellular symbionts) from the order Oceanospirillales (class Gammaproteobacteria) within specialised bacteriocytes of their interior root tissues, located close to the ovisac (Goffredi et al. 2005). Conversely, with the exception of *Osedax priapus* (Rouse et al. 2015), paedomorphic dwarf males are developmentally arrested and live as harems within the female's tube, with no endosymbiotic bacteria ever documented within them (Rouse et al. 2004, 2008). Pre-settling *Osedax* larvae and gametes are aposymbiotic, strongly indicating horizontal acquisition (Goffredi et al. 2007; Rouse et al. 2009; Verna et al. 2010). Indeed, consistent with horizontally acquired communities (Bright and Bulgheresi 2010), *Osedax* exhibit diverse endosymbiont assemblages (Goffredi et al. 2005, 2007; Fujikura et al. 2006), particularly within shallow-water hosts (30 m and 125 m; Verna et al. 2010). To date a total of 25 endosymbiont ribospecies have been reported (Goffredi et al. 2004; Rouse et al. 2004; Glover et al. 2005; Fujikura et al. 2006; Braby et al. 2007; Vrijenhoek et al. 2009; Salathé and Vrijenhoek 2012; Taboada et al. 2015), with up to eight distinct endosymbiont clusters (91.4–98.9% similarity between clusters) occurring within a single species (*Osedax mucofloris*; Verna et al. 2010).

The extent of host *Osedax* microbial diversity is yet to be fully understood. One popular hypothesis suggests diverse assemblages result from repeated microbial uptake throughout *Osedax* root development (Verna et al. 2010; Salathé and Vrijenhoek 2012) and their opportunistic, yet selective, mechanisms of acquisition (Salathé and Vrijenhoek 2012; Goffredi et al. 2014). Nonetheless, we are unlikely to fully comprehend such mechanisms without broader assessments encompassing the total *Osedax* holobiont community (Bosch

and McFall-Ngai 2011; Bordenstein and Theis 2015) of which considerably less is known. In addition to primary endosymbionts, several secondary (extracellular) bacterial symbionts belonging to Alphaproteobacteria, Deltaproteobacteria, Epsilonbacteraeota, and Bacteroidetes have also repeatedly been observed from shallow- and deep-water species across a large geographical range (Fujikura et al. 2006; Goffredi et al. 2007; Verna et al. 2010). Despite this consistent association, there are currently no known potential roles of secondary symbionts. Indeed, nine of the above-mentioned studies were conducted ten or more years ago, since which time genomic analysis tools have significantly improved, allowing more detailed assessments. The diverse and complex nature of *Osedax* microbial relationships have become increasingly apparent. However, key questions regarding *Osedax* primary and secondary symbiont evolution, functional capacity and mode of acquisition remain unknown and warrant extensive exploration (Verna et al. 2010; Salathé and Vrijenhoek 2012; Goffredi et al. 2014).

Here, we conduct the first holistic assessment of the *Osedax* holobiont using 16S rRNA amplicon sequencing, to shed light on total microbial community structure and function for two geographically separated shallow-water *Osedax* species, namely, *Osedax deceptionensis* from Deception Island, Antarctica and an undescribed species located in the Mediterranean, referred to as *Osedax* 'mediterranea' (Taboada et al. 2015). Unlike previous descriptions, our analyses focus not only on the primary endosymbionts Oceanospirillales but encompass broad scale descriptions of the total holobiont community. We provide detailed microbial community descriptions and predictive functional profiles using PICRUSt2, making comparative analyses between two host species for root samples, and between root and palp body tissues for *O. deceptionensis*. To the best of our knowledge, *Osedax* palp tissues are not considered to house primary endosymbionts, and thus provide comparative context for our root tissue analyses. Here we elucidate primary and secondary symbiont structures, detailing microbial diversity at the species and individual level. We aim to provide an initial assessment that may facilitate further investigation into the nature of a microbial partnership, which has likely contributed significantly to the success for members of this genus.

Methods

Sample collection, DNA extraction and amplification

Individual *O. deceptionensis* and *O. 'mediterranea'* were collected and processed for 16S rRNA amplicon sequencing using DNA extractions from the study by Taboada et al. (2015). Briefly, *O. deceptionensis* individuals were obtained

from the caudal fin of a de-fleshed common minke whale (*Balaenoptera acutorostrata*), placed at ca. 10 m depth on the seabed of Whalers Bay at Port Foster, Deception Island (South Shetland Islands, Antarctica). All bones were collected using SCUBA in January 2013 after approximately a year of deployment, and there after maintained in filtered sea water (0.22 µm) at ambient temperature (0–5 °C) during a maximum of 5 days whilst bones were observed to investigate the occurrence of *Osedax*; sea water temperature at the collection site was 0–1 °C. Individuals of *O. 'mediterranea'* were found in 2014 in association with a minke whale bone experimentally deployed at 53 m at the head of the Blanes submarine canyon (NW Mediterranean; 41°40' 15.5" N 2°53' 23.28" E) after 6 months of deployment, and were kept alive for 5 days in a container with filtered sea water at ambient temperature (20 °C); sea water temperature at the collection site was 17 °C.

For DNA extraction, individuals of *O. deceptionensis* and *O. 'mediterranea'* were preserved in 96% ethanol and stored at – 20 °C until further processing. Total DNA was extracted using the DNeasy Blood & Tissue Kit (Qia-gen) following the manufacturer's instructions. A total of 7 different individuals of *O. deceptionensis* (comprising 4 palps and 3 roots) and 2 two different individuals of *O. 'mediterranea'* (all roots) were processed (Table 1). From each individual either palps or roots were sampled, but not both tissue parts, to ensure independent random sampling. About 272 bp amplicon of the 16S rRNA gene was generated using general bacterial primers 515F-Y (Parada et al. 2016) and 806R (Apprill et al. 2015). We used the PCR BIO HiFi Polymerase (PCR Biosystems Ltd, UK) under the following conditions: 95 °C for 3 min, followed by 25 cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 30 s, after

which a final elongation step at 72 °C for 5 min was performed. After amplification, PCR products were checked in 1% agarose gel to determine the success of amplification and the relative intensity of bands. PCR products were purified with Agencourt AMPure XP Beads (Beckman Coulter Inc., USA), and libraries prepared with Nextera XT DNA Library Preparation Kit (Illumina Inc., USA). An equimolar pool of DNA was generated by normalising all samples at 4 nM for the sequencing. Next generation, paired-end sequencing was performed at the NHM (<https://www.nhm.ac.uk/>) on an Illumina MiSeq device.

Analysis of prokaryote 16S rRNA gene amplicon sequences

Raw 16S rRNA amplicon reads were imported for processing into Mothur version 1.41.3 (Schloss et al. 2009). As a quality filter, we removed any read with ambiguous bases, homopolymers longer than 15 nucleotides, or that did not pass the assembled quality score test (average quality score 25). Sequences were pre-clustered with UNOISE3 (Edgar 2016) implemented in Mothur, allowing 1 difference for every 100 bp. This step infers amplicon sequence variants (ASVs), without imposing any arbitrary threshold (Callahan et al. 2017). Remaining singletons were removed at this point. Chimeric sequences were identified with UCHIME (Edgar et al. 2011), referenced to the SILVA database, and discarded. Taxonomies were assigned by classifying sequences with the NR SILVA database v.132 as a reference. Any residual sequences identified as chloroplasts, mitochondria, or eukaryote were excluded from further downstream analysis (1.14% of total derived sequences). Sequences were

Table 1 Total reads and unique ASVs based on filtered library sizes for individual microbial communities of *O. deceptionensis* and *O. 'mediterranea'*

Sample	Abbreviation	Reads	ASVs	Chao1	Shannon	Simpson
<i>O. deceptionensis</i> -8Roots	DR8	43,978	503	817	2.6	0.82
<i>O. deceptionensis</i> -13Roots	DR13	51,082	819	1174	2.6	0.69
<i>O. deceptionensis</i> -21Roots	DR21	46,645	691	1006	4.4	0.97
Total <i>O. deceptionensis</i> roots		141,705	1144			
<i>O. 'mediterranea'</i> -1Roots	MR1	42,902	353	454	2.9	0.89
<i>O. 'mediterranea'</i> -2Roots	MR2	48,850	314	452	3.0	0.84
Total <i>O. 'mediterranea'</i> roots		91,752	432			
<i>O. deceptionensis</i> -3Palps	DP3	54,546	519	710	2.4	0.78
<i>O. deceptionensis</i> -6Palps	DP6	26,947	398	468	3.1	0.87
<i>O. deceptionensis</i> -9Palps	DP9	30,174	267	338	1.7	0.69
<i>O. deceptionensis</i> -11Palps	DP11	46,900	187	263	1.6	0.55
<i>O. deceptionensis</i> -16Palps	DP16	30,038	261	335	1.4	0.57
Total <i>O. deceptionensis</i> palps		188,605	923			
Grand total		422,062	1551			

Species richness and diversity estimates based on rarefied library sizes for individual microbial communities of *O. deceptionensis* and *O. 'mediterranea'*

imported as ASVs into R v.3.5.2 for downstream analysis described below.

We assessed the microbial community structure within our samples using the R package ‘Vegan’ and based on datasets rarefied to the smallest sampling effort. Datasets were filtered, removing any ASV present only in one sample. We visualised variation in community structure amongst samples using both a heat map of the rarefied counts of the 100 most abundant ASVs and a hierarchical clustering dendrogram based on Euclidian distances and average linkage with the R package pheatmap (Kolde 2015). For measures of Alpha diversity, we calculated Chao1, Shannon, and Simpson indices. To evaluate alpha diversity measures between species and body tissues we performed analysis of variance on two separate linear models. Beta diversity was estimated on rarefied counts based on Bray–Curtis dissimilarity and unweighted UniFrac distance separately. Non-metric multidimensional scaling (NMDS) was used to obtain the ordination of community similarity. Samples were compared by performing two separate permutational multivariate analyses of variance (using the *adonis* function of the R package *vegan*), comparing roots and palps of *O. deceptionensis*, and roots of *O. ‘mediterranea’* and *O. deceptionensis*. For analysis of differential abundance, we used the software *edgeR* (Robinson et al. 2010; McCarthy et al. 2012). Samples were compared by performing two separate exact tests comparing roots and palps of *O. deceptionensis*, and roots of *O. ‘mediterranea’* and *O. deceptionensis* as before.

Sequences classified as order Oceanospirillales were assessed for phylogenetic analysis. These sequences were imported into Geneious Prime 2019.1 (<https://www.geneious.com>) and aligned using MAFFT multiple alignment against 168 previously aligned *Osedax* endosymbiont sequences (Taboada et al. 2015) and some sequences recently published by Taboada et al. (2020). Bayesian inference (BI) analyses were run in MrBayes v.3.2.2 (Ronquist et al. 2012) in the NHMUK cluster, using the most appropriate evolutionary model (GTR + I + G) and consisted of two chains run for 10 million generations, sampled every 1000 generations, and a 25% burn-in. Results were visualised using iTOL 5.5 (Ciccarelli et al. 2006; Letunic and Bork 2019).

Gene content predictions from the 16S rRNA SSU gene

Metagenomic functional profiles of *Osedax* microbial communities were estimated using PICRUSt2 based on the filtered ASVs (Douglas et al. preprint). PICRUSt2 predicts metagenomic functional abundances based on 16S rRNA data. Briefly, in PICRUSt2 ASV sequences are first aligned (HMMER) and placed within a 16S rRNA reference tree (EPA-NG: Barbera et al. 2019; gappa: Czech and Stamatakis

2019), before implementing the R package, Caster (Louca and Doebeli 2018) to predict gene family abundances (i.e. copy number) for each ASV. Metagenomes are then inferred by dividing the read depth per ASV by predicted 16S rRNA copy numbers and multiplied by predicted gene family copy number for each ASV. Lastly, MetaCyc (Caspi et al. 2014) pathway-level abundances were inferred based on Enzyme Commission number (EC) abundances with MinPath (Ye and Doak 2009). We implemented the script, ‘picrust2_pipeline.py’ following online documentation, <https://github.com/picrust/picrust2>. ASVs above the nearest-sequenced taxon index (NSTI) cut-off of 2.0 were subsequently removed from further downstream analysis. Predictive functional abundance tables were classified by MetaCyc metabolic pathways (Caspi et al. 2014), EC enzymes, and Kyoto encyclopaedia of genes and genomes (KEGG). Relative abundances down to the sub pathway level were inferred using BURRITO (McNally et al. 2018) from predicted KEGG ortholog (KO) abundances and KEGG BRITE hierarchy (Kanehisa and Goto 2000). Comparative assessments between palp (*O. deceptionensis*) and root (*O. ‘mediterranea’* and *O. deceptionensis*) tissues were performed on datasets first transformed by relative abundance, before using Welch’s two-sided T-test in the software STAMP (Parks et al. 2014). Differentially abundant pathways, enzymes and KEGG orthologs were visualised with extended error bar plots in STAMP.

Results

Initially we identified 6,381 bacterial ASVs from 465,118 sequences across all samples (ASV sequences, abundances, and taxonomy available online at <https://doi.org/10.6084/m9.figshare.12611735>). After filtering, we retained a total of 1,551 ASVs from 422,062 sequences across all samples (Table 1). Of these, 1,144 ASVs were identified within *O. deceptionensis* roots, 923 within *O. deceptionensis* palps, and 432 within *O. ‘mediterranea’* roots (Table 1). Except for individuals, DR21 and MR1, rarefaction curves based on total sequence counts displayed asymptotic behaviour, indicating sequencing depth was sufficient for representative read counts (Online Resource 1). Microbial communities within our samples were distinct between *Osedax* species and body tissue as displayed by the dendrogram (Fig. 1). In fact, we identified only 3 core ASVs that were present in all individuals (avg: 1.8% of total relative abundance (*ra.*); Online Resource 2a). A total of 14 core ASVs were present in all root samples from both species (avg: 15% of total root *ra.*) and 16 core ASVs were present in all root and palp samples from *O. deceptionensis* (avg: 19% of total *O. deceptionensis ra.*).

Fig. 1 Heatmap of the 100 most abundant ASVs based on rarefied counts. Horizontal orange and blue bars indicate samples belonging to species *O. 'mediterranea'* and *O. deceptionensis*, respectively. Horizontal light grey and dark grey bars indicate samples from root

and palp tissues, respectively. Scale bar indicates log₂ counts per million (cpm). Hierarchical clustering dendrogram is based on Euclidian distances and average linkage. DR, *O. deceptionensis* roots; MR, *O. 'mediterranea'* roots; DP, *O. deceptionensis* palps

Alpha diversity indices were calculated over rarefied datasets (i.e. 26,947 sequences). The greatest diversity was found amongst *O. deceptionensis* roots (Shannon H=4.4); however, there was no statistically significant difference amongst roots of both species (ANOVA; $p > 0.05$; Online Resource 3). Conversely species diversity was lowest within *O. deceptionensis* palps (Table 1), but again there was no statistically significant difference between palp and root diversity.

Our assessment of beta diversity based on Bray Curtis dissimilarity and Unifrac distance measures detected distinct microbial community compositions between *O.*

deceptionensis palps and roots only (Fig. 2; adonis: $df = 1, 6$; $p = 0.039$; and Online Resource 4; Unifrac $p = 0.044$). No significant differences were found between the roots of both species (adonis, $df = 1, 3$; $p = 0.1$), probably due to the low number of samples available (2 samples from *O. 'mediterranea'*, and 3 from *O. deceptionensis*).

Total microbial community composition was dominated by Proteobacteria (80% average relative abundance (*ara.*); Fig. 3a), specifically classes Gammaproteobacteria (54% *ara.*), followed by Alphaproteobacteria (13.5% *ara.*), Deltaproteobacteria (10% *ara.*), and Betaproteobacteria (2% *ara.*) (Fig. 3b). Other prominent phyla within the total

Fig. 2 NMDS of beta diversity between *O. deceptionensis* roots, *O. deceptionensis* palps and *O. 'mediterranea'* roots. Bray–Curtis measures of dissimilarity estimated based on rarefied library sizes of the smallest sampling size. *Osedax deceptionensis* palps (DP) and roots (DR) samples exhibited significantly different microbial communities (adonis: $p=0.04$)

microbial community included Epsilonbacteraeota (8.5% *ara.*), Bacteroidetes (6.4% *ara.*), Actinobacteria (1.2% *ara.*), Kiritimatiellaeota (0.7% *ara.*), and Firmicutes (0.6% *ara.*) (Fig. 3a). Within *Osedax* individuals Gammaproteobacteria

was consistently the most abundant. Only in the two most diverse individuals (*O. deceptionensis*-DR21 and *O. mediterranea*-MR1), where sequencing depth was the lowest, abundance of Proteobacteria dropped below 80% *ara.* (with Gammaproteobacteria comprising, 21% and 22% *ara.*, respectively; Fig. 3a), whilst relative abundance of Epsilonbacteraeota was much greater than within other individuals (21% and 22% *ara.*, respectively; Fig. 3a).

Comparing total root and palp samples separately, we observed much lower average abundance of classes, such as Deltaproteobacteria and Bacteroidia within palp samples (4.5% and 0.9% *ara.*) compared to root samples (*O. deceptionensis*, 16.2% and 12.3% *ara.*; *O. 'mediterranea'*, 12.6% and 8.4% *ara.*; Fig. 3b). Conversely, abundance of Alphaproteobacteria was lower in *O. deceptionensis* root samples (2.9% *ara.*) than *O. deceptionensis* palp (17.3% *ara.*) and *O. 'mediterranea'* root samples (22.3% *ara.*).

We identified 10 dominant ASVs present at relative abundances of 5% or greater in at least one of *O. deceptionensis* roots, *O. deceptionensis* palps or *O. 'mediterranea'* roots (Fig. 4; Online Resource 2b). Six of these belonged to three orders of class Gammaproteobacteria, four within the order Oceanospirillales, (ASV1903 and ASV5088 *Profundimonas*; ASV960 *Oleispira*; ASV1445 Kangiellaceae uncultured), and the other two within the orders, Alteromonadales (ASV944 *Alkalimarinus* but see below) and Thiomicrospirales (ASV5513 cluster SUP05 of family Thioglobaceae).

Fig. 3 Individual *O. deceptionensis* and *O. 'mediterranea'* taxonomic microbial composition shown by relative abundance. **a** Phylum composition for phyla of at least 0.5% total relative abundance. **b** Class composition for members of Proteobacteria and two classes with the highest relative abundance outside Proteobacteria. **c** Order composition

for orders of at least 4% total relative abundance within one of *O. deceptionensis* roots, *O. deceptionensis* palps or *O. 'mediterranea'* roots. Letters in brackets indicate phylum (upper case letter) and class (lower case letter)

Fig. 4 Relative abundance of ASVs above 1% abundance within at least one of *O. deceptionensis* roots, *O. deceptionensis* palps or *O. 'mediterranea'* roots. Bubble sizes are proportional to the relative abundance of an ASV within an individual and are coloured according to their order, as assigned using NR SILVA reference database. ASVs displayed on the y-axis are denoted first by their associated

family and genus, followed by their unique ASV number. ASV names displayed in blue are the 10 most abundant ASVs (present in abundances of at least 5% within one of *O. deceptionensis* roots, *O. deceptionensis* palps or *O. 'mediterranea'* roots). DR, *O. deceptionensis* roots; MR, *O. 'mediterranea'* Roots; DP, *O. deceptionensis* palps

The remaining ASVs belonged to the order Campylobacteriales (ASV6298 *Arcobacter*; ASV140 *Sulfurospirillum*) and unclassified order(s) of Alphaproteobacteria (ASV4230 and ASV4953). Dominant ASVs were largely specific to *Osedax* species and body region. Additionally, except for ASV1903, none of these 10 dominant ASVs were present in abundances greater than 1% within more than one of the samples types (i.e. *O. deceptionensis* roots, *O. deceptionensis* palps or *O. 'mediterranea'* roots), and only three (ASV944, ASV960, and ASV1445) were also present within the identified core root microbiota. However, ASV1903 was abundant within *O. deceptionensis* roots and palps.

Osedax deceptionensis root microbial communities were largely dominated by only two ASVs, belonging to the order Oceanospirillales: ASV1903 comprising 29% *ara.* and ASV960, comprising 9.1% *ara.* (Fig. 4). The relative frequencies of these two ASVs were not consistent between individuals (Fig. 4). Remaining *O. deceptionensis* root taxa composition comprised many less abundant ASVs including ASV3078 (3.3% *ara.*) and ASV568 (3.0% *ara.*) within

Bacteroidetes, and ASV1442 (3.1% *ara.*) within class Deltaproteobacteria. These generally less dominant ASVs were observed in much greater relative abundances within the most diverse individual, *O. deceptionensis*-DR21 (Fig. 4). Conversely, *O. deceptionensis* palp microbial communities consisted of several dominant ASVs, in which ASV5513 (assigned to the family Thioglobaceae) was most abundant, with 19.8% *ara.* (Fig. 4). ASV5513 was largely unique to palp samples, only found outside the palps in very low numbers of *O. deceptionensis*-DR21 (Fig. 4). Also abundant within *O. deceptionensis* palps were ASV1445 (16.9% *ara.*) and ASV1903 (12.6% *ara.*) belonging to the order Oceanospirillales, ASV4230 (13.3% *ara.*) an unclassified Alphaproteobacterium, and ASV6298 (5% *ara.*) belonging to Campylobacteriales (Fig. 4). Within *O. 'mediterranea'* root microbial communities, order Oceanospirillales was present only at 7.1% *ara.*, of which ASV5088 represented 6.8% *ara.* (Figs. 3c, 4). Instead, the most dominant ASV was ASV944 (Gammaproteobacteria), which comprised 21.3% *ara.* We observed high similarity of ASV944 to *Alkalimarinus*

sediminis (99.6% identity), a recently described species within family Alteromonadaceae that clusters separately but shares similarity with *Marinobacter* (92.8% identity; Zhao et al. 2015). Microbial communities of *O. 'mediterranea'* roots also included high average relative abundances of ASV4953 (9.6% *ara.*), an unclassified Alphaproteobacterium, and ASV140 (8.9% *ara.v*), belonging to order Campylobacteriales (Fig. 4).

Differential abundance analysis of counts in edgeR, highlighted 19 differentially abundant ASVs between host species (*O. deceptionensis* roots vs. *O. 'mediterranea'* roots) and 30 between body tissue (palp vs. roots of *O. deceptionensis*) ($p < 0.01$; Online Resource 2c). We observed the highest log fold change values between host species. These ASVs included, significantly greater abundance of ASV1903 and ASV960 (order Oceanospirillales) within *O. deceptionensis* roots, whilst abundance of ASV4953 and ASV518 (class Alphaproteobacteria) were significantly greater within *O. 'mediterranea'* roots (Online Resource 2c).

Metagenomic predictive functional profiling

All 1,551 ASVs were initially included in the PICRUSt2 analysis. After removal of 802 ASVs above the NSTI cut-off 2.0, we had a dataset of 749 ASVs. A total of, 4011 KEGG orthologs (KO), 1,327 enzymes, and 285 metabolic pathways were predicted from these ASVs. There were 226 KO uncharacterized proteins. A comparison of KEGG orthologs predicted from our samples revealed highly comparable functions to those identified within dominant *Osedax* symbionts, Rs1 and Rs2, as well as the closest described free-living relative *Neptumonas japonica* (Goffredi et al. 2014; Online Resource 5a). Within our samples, the super pathway amino acid metabolism comprised 25% of total abundance for metabolism classified pathways (Table 2). Specifically, amino acid metabolism was comprised most abundantly of the sub pathway, Phenylalanine, tyrosine and tryptophan biosynthesis (5.4% of total metabolism; Table 2), but also included metabolism of sulphur containing (Cysteine and methionine), and collagen-derived amino acids (Glycine, Proline, Hydroxyproline) (Online Resource 5b). Metagenomic predictions revealed high metabolic flexibility in general, with the potential to metabolise monosaccharides, disaccharides, amino sugars, nucleotide sugars, fatty acids, and organic acids. We identified a total of 252 ABC transporters, including those for oligopeptides (*opp*ABCDF), dipeptides (*dpp*ABCDF), nickel (*nik*ABCDE), vitamin B12 (*btu*CDF), zinc (*znu*ABC), iron (*afu*ABC), and multiple amino acids (*aot*Q, *arg*T, *art*IJMPQ, *gln*HPQ, *his*JMPQ, *liv*GFHMK, *met*INQ, *pro*VWX, *tau*ABC, *tcy*BCKLM, *thi*BPQ, *aap*JMPQ) (Online Resource 5c).

Comparative analysis between root and palp tissue samples highlighted differential abundance of 11 metabolic

pathways, 54 enzymes and, 217 KEGG orthologs (Welch's *t* test, $p < 0.05$; Online Resource 6). We identified several functions significantly more abundant within root samples that may shed light on the adaptive traits of root tissue symbionts. These included, urea cycling (urease; EC:3.5.1.5 & *nik*ABCD), vitamin biosynthesis (biotin biosynthesis II; PWY-5005), sulphur detoxification (superpathway of sulphur oxidation; PWY-5304), and intracellular host infection of free-living symbionts (*clf*AB, *fnb*AB, *sdr*CDE) (Online Resource 5d & e).

Phylogenetic assessment of ASV's belonging to order Oceanospirillales

In total we identified 100 ASVs belonging to 8 families within the order Oceanospirillales. Of these, 82 were identified within *O. deceptionensis* roots, 16 within *O. 'mediterranea'* roots, and 64 within *O. deceptionensis* palps (Fig. 5). We identified three core Oceanospirillales ASVs shared across all palp and root samples of *O. deceptionensis* (ASV960, ASV1445, ASV2484), but none shared between all root samples of *O. deceptionensis* and *O. 'mediterranea'* (Online Resource 2a).

Oceanospirillales abundance within *O. deceptionensis* and *O. 'mediterranea'* roots was dominated by ASV1903 and ASV5088, respectively. Both clustered within the monophyletic lineage, Ribotype 1 (distinguished following Salathé and Vrijenhoek 2012) and most closely associated with Nitrospiraceae *Profundimonas* (formerly Oceanospirillaceae; Fig. 5). Both host species also harboured many low abundant ASVs closely associated to either Nitrospiraceae *Profundimonas* or Nitrospiraceae unclassified. The second most abundant within *O. deceptionensis* roots, ASV960 most closely associated with Saccharospirillaceae *Oleispira*, but did not cluster significantly within any described *Osedax* primary endosymbiont ribotypes. Conversely, *O. 'mediterranea'* roots comprised no other dominant Oceanospirillales ASVs. Within *O. deceptionensis* palps, Oceanospirillales was dominated by ASV1445, belonging to the family Kangiellaceae and did not cluster with any known *Osedax* endosymbiont Ribotype.

Discussion

Microbial community structure

The shallow-water annelids *O. deceptionensis* and *O. 'mediterranea'* house diverse primary and secondary microbial assemblages dominated by Proteobacteria (Fig. 3a). Within this bacterial phylum, class Gammaproteobacteria was predominant, distantly followed by Alphaproteobacteria, Deltaproteobacteria, and other non-Proteobacteria groups such as Epsilonbacteraeota and Bacteroidetes (Fig. 3). Such high

Table 2 Metagenomic predictive functional abundances as a percentage of Metabolism

Super pathway	Sub pathway	As percentage of category (metabolism)
Amino acid metabolism		25.2
	Alanine, aspartate and glutamate metabolism	2.5
	Amino acid-related enzymes	4.3
	Arginine biosynthesis	2.6
	Cysteine and methionine metabolism	2.6
	Glycine, serine and threonine metabolism	2.8
	Histidine metabolism	2.9
	Lysine biosynthesis	2.0
	Phenylalanine, tyrosine and tryptophan biosynthesis	5.4
Carbohydrate metabolism		8.4
	Amino sugar and nucleotide sugar metabolism	3.6
	Glyoxylate and dicarboxylate metabolism	2.7
	Pyruvate metabolism	2.1
Energy metabolism		10.1
	Carbon fixation pathways in prokaryotes	2.5
	Oxidative phosphorylation	7.6
Enzyme families		6.7
	Peptidases	6.7
Glycan biosynthesis and metabolism		9.1
	Lipopolysaccharide biosynthesis	2.8
	Lipopolysaccharide biosynthesis proteins	2.9
	Peptidoglycan biosynthesis and degradation proteins	3.4
Lipid metabolism		2.9
	Glycerophospholipid metabolism	2.9
Metabolism of cofactors and vitamins		20.0
	Biotin metabolism	1.9
	Folate biosynthesis	4.3
	Nicotinate and nicotinamide metabolism	3.4
	Pantothenate and CoA biosynthesis	4.1
	Porphyrin and chlorophyll metabolism	3.7
	Thiamine metabolism	2.6
Metabolism of terpenoids and polyketides		3.4
	Terpenoid backbone biosynthesis	3.4
Nucleotide metabolism		14.3
	Purine metabolism	8.1
	Pyrimidine metabolism	6.2

Functional abundances predicted using PICRUSt2 and classified by the KEGG BRITE hierarchy using BURRITO (McNally et al. 2018)

prevalence of Gammaproteobacteria is unsurprising considering its global dominance within other *Osedax* and closely related Vestimentiferan tube worms (Thornhill et al. 2008). Conversely, our assessments of species level (i.e. ASVs) diversity revealed highly variable assemblages, both within and between host species (Fig. 4). Individual *O. deceptionensis* and *O. 'mediterranea'* were characterised by a limited number of dominant ASVs in distinct frequencies, and a large pool of diverse, low abundant ASVs, comprising only a minimal core community (Fig. 4; Online Resource 2a). The

taxonomic composition and diversity we observed here is largely consistent with previous *Osedax* microbial descriptions such as, *O. roseus* and *Osedax* sp. 'yellow collar' (Goffredi et al. 2007; *Osedax lehmani* in Rouse et al. 2018). Conversely, other Siboglinidae including *Riftia* and *Tevnia*, housing chemotrophic bacteria, display much less diversity, harbouring only a single dominant symbiont phylotype, *Candidatus* Endoriftia persephone, despite also acquiring their symbionts horizontally within highly disparate

Fig. 5 Phylogenetic tree based on Bayesian inference analysis of 100 sequences classified as order Oceanospirillales aligned against 168 previously aligned *Osedax* endosymbiont sequences (Taboada et al. 2015, 2020). Branches and text are coloured according to the presence of each sequence within respective samples, *Blue*: *O. deceptionensis* palp and root samples, *Purple*: *O. deceptionensis* palp samples only, *Orange*: *O. 'mediterranea'* root samples, *Black*: Sequences

used in Taboada et al. (2015, 2020). Stars indicate the most abundant Oceanospirillales sequences for each sample type, *O. deceptionensis* roots (blue), *O. deceptionensis* palps (purple), and *O. 'mediterranea'* roots (orange). Tree based on evolutionary model (GTR + I + G) and two chains run for 10 million generations, sampled every 1000 generations, and a 25% burn-in

environmental extremes (Edwards and Nelson 1991; Di Meo et al. 2000; Gardebrecht et al. 2012; Forget et al. 2015).

Individual *Osedax* microbial community compositions were diverse, yet distinct between host species and body tissue (Figs. 3, 4). Within *O. deceptionensis* root communities, consistent with all other described *Osedax* microbiome assemblages (Goffredi et al. 2005; 2007; Verna et al. 2010; Salathé and Vrijenhoek 2012), were predominantly dominated by primary endosymbionts Oceanospirillales, with the exception of the most diverse individual DR21 (Fig. 3c), which may be a reflection of the poorer sampling depth for this individual. The most abundant symbiont, ASV1903 (*Profundimonas*) lies within family Nitrospiraceae, known to comprise other primary *Osedax* endosymbionts (Goffredi et al. 2007). Additionally, ASV1903 most closely associated with Ribotype 1, a monophyletic lineage that globally dominates *Osedax* endosymbionts (Fig. 5; Salathé and Vrijenhoek 2012) and was the most abundant ASV within *O. deceptionensis* (Fig. 4), indicating it may be a primary endosymbiont involved in nutrient acquisition. The second most abundant symbiont, ASV960 (*Oleispira*) belongs to the known *Osedax* endosymbiont family Oceanospirillales, and thus is also a likely functionally significant endosymbiont, though its ribotype placement could not be supported in our phylogenetic tree (Fig. 5). Indeed, we do note the limitations from using relatively short length 16S rRNA markers and the small sample size of this study, when interpreting such results. All root samples of *O. deceptionensis* harboured both ASV1903 and ASV960; however, their relative abundances of each were distinct (Fig. 4). Despite close spatial and temporal proximity of the *O. deceptionensis* individuals (they were collected from the same bone and at the same time of the year), relative abundance of ASV960 was much greater (*on par* with ASV1903) within DR8 than either DR13 or DR21, providing insight into the plasticity of *Osedax* endosymbiont assemblages.

Little is known regarding the processes by which *Osedax* horizontally acquire their primary endosymbionts; however, their characteristically diverse assemblages may provide some insight. Unlike other Siboglinidae, *Osedax* likely acquire their endosymbionts repeatedly during root tissue proliferation, enabling uptake of variable endosymbionts through multiple mechanisms (Goffredi et al. 2007; Verna et al. 2010; Salathé and Vrijenhoek 2012). For instance, the temporally dynamic microbial assemblages within associated whale fall sediments (Goffredi et al. 2008a, b) may facilitate acquisition of distinct lineages through time (Verna et al. 2010; Salathé and Vrijenhoek 2012; Goffredi et al. 2014). That said, here we report distinct frequencies of potential endosymbionts, within individuals of *O. deceptionensis* and *O. 'mediterranea'* despite collection from a single time point. Alternatively, diverse communities may arise from small-scale variability within the bone-associated

microbiota. Verna et al. (2010) hypothesised that a lack of disturbance enables free-living endosymbiont populations within the bone to form structured, clonal growth. Subsequently, because of the large available free-living symbiont populations, any given *Osedax* individual may acquire a distinct endosymbiont assemblage to that of its neighbour (Verna et al. 2010). Indeed, in their assessment of 20 *Osedax mucifloris* individuals, Verna et al. (2010) found host individual explained the majority (68%) of observed endosymbiont diversity for this shallow-water *Osedax* species. Assessment of *O. deceptionensis* and *O. 'mediterranea'* supports the hypothesis that, at least for shallow-water species, acquisition is not random but selectivity encompasses broad ribospecies diversity through opportunistic acquisition of the best adapted, free-living bacteria accessible within an individual's immediate environment (Salathé and Vrijenhoek 2012; Goffredi et al. 2014).

Palp tissues within *O. deceptionensis*, were less diverse and dominated by unique ASVs, only present in very low numbers within other root tissues. Specifically, these included ASV5513 (Thioglobaceae), most closely related with sulphur oxidising, thioautotrophic endosymbionts of the cold seep mussel *Idas* sp. (identity 98.8%) which inhabits sunken wood and whale bones (Duperron et al. 2008). This was followed by Oceanospirillales, ASV1445 (Kangiellaceae), and ASV4230 (unclassified Alphaproteobacteria), most closely related with *Pseudovibrio* sp. (identity 99.2%) (Fig. 3). It is unsurprising to detect such distinct root and palp microbial assemblages given their disparate environment and function (Huusgaard et al. 2012). Embedded within vertebrate bone, root tissues are highly active, exposed to the build-up of toxic sulphide and strong oxygen depletion, whilst palps facilitate ventilation over large diffusive surfaces in the water column (Huusgaard et al. 2012). *Osedax* palp microbial communities have not previously been explored, and thus the potential functional significance of their ectosymbionts is currently unknown.

Microbial communities were distinct between *O. 'mediterranea'* and *O. deceptionensis* root tissues (Figs. 3c, 4). Within *O. deceptionensis* roots, ASVs belonging to the order Oceanospirillales comprised 40% relative abundance, but only 7% (6.8% ASV5088) within *O. 'mediterranea'*, none of them being common to both species. Such low abundance of Oceanospirillales in *O. 'mediterranea'* is surprising given its critical role proposed for *Osedax* nutrient acquisition (Goffredi et al. 2007). If hosts progressively acquire endosymbionts throughout root growth (Verna et al. 2010; Salathé and Vrijenhoek 2012), low Oceanospirillales abundance might be a result of limited root proliferation. Alternatively, in a depth associated comparison of species sampled 1000 m or deeper with those sampled shallower than 500 m, Verna et al. (2010) demonstrated distinct depth-related *Osedax* microbial communities. Conversely, here depth disparity

was not so distinct, with *O. deceptionensis* and *O. 'mediterranea'* sampled at 10 m and 53 m, respectively. Subsequently instead we suggest that the species' distinct geographical regions likely exhibit a greater influence on their microbial community composition. Indeed, within warm Mediterranean waters, increased endosymbiont metabolic activity may reduce host minimal endosymbiont requirements for the host to sustain sufficient nutrition (Sarmiento et al. 2010). However, primary endosymbiont acquisition and activity remains largely unclear (Goffredi et al. 2014). Furthermore, whilst abundance of Oceanospirillales was relatively low, abundance of family Alteromonadaceae (ASV944 *Alkalimarinus* and ASV5580 *Alteromonas*) was relatively high and observed only in limited numbers apart from *O. 'mediterranea'* (Fig. 4). Consequently, we suggest that *O. 'mediterranea'* may house additional primary endosymbionts, other than Oceanospirillales, capable of hydrocarbon degradation, and which may compensate for relatively low Oceanospirillales abundance. As in Oceanospirillales, family Alteromonadaceae, are obligate heterotrophs associated with nutrient rich environments, including marine snow (López-Pérez and Rodríguez-Valera 2014). Additionally, they possess large genomes containing several degradative genes and numerous members of the family, including several species of *Marinobacter* (96.8% identity to ASV944) and *Alteromonas*, have been described as hydrocarbon degrading (Jin et al. 2011; López-Pérez and Rodríguez-Valera 2014). Considering such attributes, ASV944 and ASV5580 may feasibly possess the required functionality for *Osedax* nutrient acquisition, indicating a potential role for non-Oceanospirillales symbionts. Even so, the placing of ASV944 warrants investigation, considering that its closest known relative, *Alkalimarinus sediminis* shares equally high identity scores with members of both Oceanospirillaceae and Alteromonadaceae (Zhao et al. 2015). Nevertheless, to further explore the functional significance of Alteromonadaceae, we must first determine whether they reside as intracellular endosymbionts. To the best of our knowledge, no free-living bacteria closely related with ASV944 and ASV5580 have previously been detected within associated whale fall sediments (Goffredi and Orphan 2010), or indeed within the microbial mats from which these *O. 'mediterranea'* individuals were sampled (Taboada et al. 2020). Thus, we suggest family Alteromonadaceae might not simply be a casual associate and suggest further exploration as primary endosymbionts within the shallow-water host *O. 'mediterranea'*.

Osedax also house a rich community of secondary bacterial symbionts of which current knowledge is severely lacking. Within both root and palp tissues of *O. deceptionensis* and *O. 'mediterranea'* roots, we detected relatively high abundances of likely secondary symbionts belonging to Alphaproteobacteria, Deltaproteobacteria, Epsilonbacteraeota and Bacteroidetes (root tissues only Fig. 3). Whilst

members of these, including families and genera detected here (*Desulfobacteraceae*, *Desulfobulbaceae*, *Sulfurospirillum*, *Arcobacter*, *Sulfurimonas*; Fig. 4), are strongly associated to surrounding whale fall sediments (Tringe et al. 2005; Goffredi and Orphan 2010; Taboada et al. 2020), they are notably recurrent within numerous spatially and temporally separated species of *Osedax* (Goffredi et al. 2005, 2007; Verna et al. 2010). We suspect these bacteria to be epibionts, since several closely related species have been detected across root and trunk surface tissues (Fujikura et al. 2006; Goffredi et al. 2007; Verna et al. 2010). Unusually, Fusobacteria, another recurrent secondary phylum (Goffredi et al. 2005, 2007; Verna et al. 2010), were only detected here within *O. deceptionensis* roots (Fig. 4). Currently there are no known functional roles of *Osedax* secondary symbionts. However, considering their consistent association within *Osedax* microbiota, we suggest investigating whether they are functionally significant as either beneficial, commensal, parasitic or competitive symbionts. Since *Osedax* are hypothesised to house dominant endosymbionts within core root structures and less abundant assemblages within their peripheral roots (Verna et al. 2010), visualising the spatial distribution of such bacteria may provide an initial proxy for their relative functional significance.

Microbial community functional profile

Metagenomic analysis (predicted from the 16S rRNA genes) of *O. deceptionensis* and *O. 'mediterranea'* microbial communities using PICRUSt2, revealed predicted functional profiles largely consistent with *Osedax*'s most dominant symbionts, Rs1 and Rs2 (Goffredi et al. 2014; Online Resource 5a). Similar to Rs1 and Rs2, our analysis indicated broad metabolic flexibility, encompassing metabolism of monosaccharides, disaccharides, amino sugars, nucleotide sugars, fatty acids and organic acids (Online Resource 5b). Additionally, PICRUSt2 analysis predicted large abundances of KEGG orthologs associated with the metabolism of amino acids. Specifically, amino acid metabolism accounted for 25% of the total abundance for metabolism associated pathways (Table 2). These included collagen-derived amino acids (glycine, proline, hydroxyproline), despite observing only limited abundances of collagenase. However, collagenase has been reported from root tissue samples (Goffredi et al. 2007), and indeed Miyamoto et al. (2017) identified proteases (*mmp* and *ctsB* genes) capable of collagen degradation, accessing peptides and other nutrients within root epidermal cells. Thus, our data support the hypothesis that *Osedax* endosymbionts metabolise collagen-derived amino acids or peptides, which are first liberated by the host (Goffredi et al. 2014; Miyamoto et al. 2017). Additionally, oligopeptide ABC transporters (*oppABCDF*), suggested to facilitate uptake of potentially collagen-derived

peptides (Goffredi et al. 2014), were particularly abundant in our samples. The full cassette of dipeptide transporters (*dppABCDF*) was also present but much less abundant. Expression of SLC genes encoding oligopeptide and amino acid transport have also been reported within *Osedax japonicus* host root epidermis tissue (Miyamoto et al. 2017). Thus, this supports the hypothesis that host derived, small-peptides and amino acids from the collagenous bone matrix may indeed play a critical role in nutritional acquisition within the *Osedax* holobiont.

Comparisons of functional abundances between root and palp tissue microbial communities, highlighted several potential adaptations, specific to root tissue symbionts. Within root tissue samples we observed significantly greater prevalence of the urea cycle pathway (PWY-4984; Online Resource 5d). Considering, the urea cycle is a product of amino acid catabolism, and that root tissues house *Osedax*'s primary endosymbionts, this may provide further indication for amino acid metabolism as a dominant nutritional resource. Additionally, urease (EC:3.5.1.5, subunits *ureABC* and *ureDEFG*), a catalyst for the production of ammonia from urea (Varner 1960; Andrews et al. 1984), and transport proteins *nikABCDE* and *nixA*, for Nickle, of which urease is reliant on, were significantly more abundant within root tissue samples (Dixon et al. 1976; Blakeley and Zerner 1984; Online Resource 5e). The activity of urease is significant since the assimilation of ammonia (to glutamate and glutamine) facilitates nitrogen recycling, increasing amino acid turnover. PICRUST2 analysis revealed the potential for de novo microbial synthesis of 6 essential (lysine, valine, leucine, isoleucine, phenylalanine, tryptophan) and three non-essential amino acids within our samples. We hypothesise that *Osedax* root microbial populations exhibit nitrogen cycling, enabling increased synthesis of amino acids that may be transferred and utilised by their host. Urea recycling has already been suggested in other marine invertebrates hosting microbial endosymbionts, such as in the tropical sponge *Xestospongia testudinaria* (Su et al. 2013). This mechanism was proposed as a potential source of nitrogen for the host to overcome living in habitats with limited nitrogen availability (Su et al. 2013). Additionally, the production of amino acids through urea recycling has been demonstrated within gut bacterial symbionts of the insect *Melolontha hippocastani* (Forest Cockchafer; Alonso-Pernas et al. 2017). Similarly, symbiont vitamin biosynthesis may also be critical for the host. For instance, the pathway for Biotin biosynthesis (PWY-5005) was significantly more prevalent within root tissues samples (Online Resource 5d). Biotin is an essential cofactor within cellular metabolism, but which animals cannot produce (Patton et al. 1996; Schneider and Lindqvist 2001) and leads us to speculate whether host *Osedax* acquire such essential vitamins from their symbionts.

Highly active root tissues are exposed to the build-up of toxic sulphide and strong oxygen depletion (Husgaard et al. 2012), yet there has been no described mode of detoxification. Within root tissue samples, our analysis predicted significantly greater prevalence of the super pathway for sulphur oxidation (PWY-5304) and five sulphur metabolism classified orthologs, including the sulphite reductase flavoprotein (*cysJ*) for sulphur assimilation and cystathionine gamma-synthase *metB*, capable of essential amino acid synthesis for methionine (Ostrowski et al. 1989; Ravanel et al. 1998; Online Resource 5d & e). Consistent with our results, Waits et al. (2016) demonstrated the likely biochemical capacity of *O. mucofloris* to produce sulphur-binding haemoglobins (Hbs). They also showed that *O. mucofloris* possess Hb linkers with greater similarity to vestimentiferan siboglinids than sulphide-tolerant polychaetes. Indeed the use of sulphur containing amino acids for detoxification of sulphur has been a suggested mechanism within *Riftia pachyptila*, a closely related vestimentiferan siboglinid (Pruski et al. 1998). Interestingly, genes involved with metabolism of sulphur derived amino acids, cysteine and methionine, were particularly abundant within our samples and never reported, to our knowledge, in previous studies on *Osedax* microbes. Thus, investing in microbial mechanisms of sulphur detoxification is likely to be critical for the *Osedax* holobiont.

We also identified several genes that might be involved in facilitating the infection of free-living microbial symbionts intracellularly within the host (Online Resource 5e). Endosymbiont adaptations for intracellular life likely share analogy with those of intracellular pathogens. Comparative analysis revealed several *Staphylococcus aureus* associated genes that were significantly more abundant within root tissue samples. These included, serine-aspartic acid repeat proteins *sdrCDE*, clumping factors *clfAB*, and fibronectin-binding proteins *fnbAB*, enabling adhesion of *S. aureus* to host squamous epithelial and elastin cells, respectively (Corrigan et al. 2009; Roche et al. 2004; Online Resource 5e). Additionally, membrane damaging leukotoxins and haemolysins (*hlg*, *hld* and *hlyII*) associated with pathogenic host infection were also significantly more abundant in root tissue samples (Barrio et al. 2006; Vandenesch et al. 2012; Online Resource 5e). Similarly, within endosymbionts Rs1 and Rs2, Goffredi et al. (2014) also observed haemolysin homologs, encoding large surface exoproteins. Successful mechanisms for host infection are likely to be key for *Osedax* symbionts, considering their continual horizontal acquisition throughout host root growth (Goffredi et al. 2007; Verna et al. 2010; Salathe and Vrijenhoek 2012).

Conclusion

Broadly, the microbial communities of *O. deceptionensis* and *O. 'mediterranea'* share some key *Osedax* microbiome patterns described previously. These include, a dominance of Gammaproteobacteria, high relative abundance of Oceanospirillales within *O. deceptionensis* root tissues (ASV1903 which closely associated with Ribotype 1, and ASV960), and comparable functional predictions to those observed in Ribotype 1 and Ribotype 2 (Goffredi et al. 2014). Conversely, within *O. 'mediterranea'*, abundance of Oceanospirillales was surprisingly low. Thus, we hypothesise that some *Osedax* species may in fact house other, non-Oceanospirillales functionally significant primary endosymbionts. Within *O. 'mediterranea'*, we suggest members belonging to the family Alteromonadaceae as potential candidates.

Deeper assessment, at ASV level revealed a high degree of disparity between individuals, both between and within species, despite individuals of the same species having been sampled from close proximity on the bone matrix. Subsequently, our analyses provide insight to the extent of complexity that comprises the *Osedax* holobiont and additionally, emphasises the significant gap in the current understanding regarding mode of *Osedax* symbiont acquisition. Indeed, we document relatively high and consistent abundance of several microbes (including orders Campylobacteriales, Desulfobacteriales, and Bacteroidales) that we suspect to be more than simply transient, and hence merit further investigation. To date, *Osedax* microbiome research has largely focussed on primary endosymbionts belonging to order Oceanospirillales. However, the observable holobiont and its functioning is a result of the total nested ecosystem interactions of all microbial members (whether commensal, parasitic, or mutualistic), and thus we stress the importance of such holistic approach in future *Osedax* holobiont research.

Acknowledgements We are indebted with Dr Ana Riesgo, who provided all the resources needed to conduct all the laboratory analysis. We also would like to acknowledge the project ACTIQUIM-II (CTM2010-17415/ANT) and the environmental association S'Agulla Educació Mediambiental, which contributed with the samples used in this study. Last, but not least, we thank all the members of the Riesgo Lab, Martín Taboada and Otilia Moreno for all the help they provided during the sample processing and writing of the manuscript, and Lorna Page for whose enthusiastic discussions helped inform many of the thoughts presented here.

Funding This work was supported by the SponGES project (Grant Agreement No. 679848)—European Union Framework Programme for Research and Innovation, H2020. OHH received funding from the European Erasmus + Traineeship programme (2018-1-BE02-KA103-046670). CDV received funding from the EU Horizon 2020 MSCAs DeepSym (grant agreement: 796011). ST received funding from the Juan de la Cierva-Incorporación programme (IJCI-2017-33116), Spanish Government.

Data availability The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, BioProject Accession Number PRJNA644051, sample accession numbers SAMN15446574 to SAMN15446583, raw 16S rRNA sequences, absolute abundance, relative abundance and taxonomical assignments online at <https://doi.org/10.6084/m9.figshare.12611735>.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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