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Research Article

RESEARCH ARTICLE

The specific and exclusive microbiome of the deep-sea bone-eating snail, *Rubyspira osteovora*

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One sentence summary: *Rubyspira osteovora* is an unusual snail found only at whalefalls in the deep-sea, with a gut microbiome dominated by bacteria not present in the surrounding environment.
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ABSTRACT

Rubyspira osteovora is an unusual deep-sea snail from Monterey Canyon, California. This group has only been found on decomposing whales and is thought to use bone as a novel source of nutrition. This study characterized the gut microbiome of *R. osteovora*, compared to the surrounding environment, as well as to other deep-sea snails with more typical diets. Analysis of 16S rRNA gene sequences revealed that *R. osteovora* digestive tissues host a much lower bacterial diversity (average Shannon index of 1.9; $n = 12$), compared to environmental samples (average Shannon index of 4.4; $n = 2$) and are dominated by two bacterial genera: *Mycoplasma* and *Psychromonas* (comprising up to 56% and 42% average total recovered sequences, respectively). These two bacteria, along with *Psychrilyobacter* sp. (~16% average recovered sequences), accounted for between 43% and 92% of the total recovered sequences in individual snail digestive systems, with other OTUs present at much lower proportions. The relative abundance of these three groups remained similar over 6 years of sampling (collection date was not shown to be a significant predictor of community structure), suggesting a long-term association. Furthermore, these bacterial genera were either not present (*Mycoplasma* and *Psychromonas*) or at very low abundance (<0.04% for *Psychrilyobacter*), in environmental samples and other deep-sea gastropods, supporting the uniqueness of the *R. osteovora* gut microbiome.

Keywords: deep sea; symbiosis; gastropod; whalefall; *Mycoplasma*; *Psychromonas*; *Rubyspira*

INTRODUCTION

Novel ecosystems often support unique biological communities, and their study helps to uncover Earth's true biodiversity. The deep sea is an unparalleled ecosystem, which remains largely unexplored and inaccessible, with combined physical conditions—high pressure, lack of light, and extreme low temperatures—unlike any environment on the planet. The deep sea was once thought to be devoid of life, yet discoveries have revealed thriving biological communities and unusual forms of life, from microbes to invertebrates, and thus fueled a surge of interest in deep-sea exploration (Lonsdale 1977). In recent years, the increased exploration of the deep sea, particularly along continental margins, has uncovered numerous ecosystems, including methane cold seeps and whale falls, which are host to animals with unusual life histories and nutritional strategies (Baco and Smith 2003; Dubilier, Bergin and Lott 2008; Levin *et al.* 2012).

When whales die, they sink to the seafloor and provide large amounts of energy in the form of organic material to an otherwise carbon-limited environment (excluding vents and seeps; Smith and Baco 2003). Thriving animal communities colonize these so-called whale falls, including mobile opportunistic scavengers, such as hagfish and crustaceans, and endemic polychaetes, for example, that colonize the organically enriched

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bones and sediments (Smith and Baco 2003; Goffredi *et al.* 2004a). The organisms in these communities often include specialist animals that have adapted exclusively to life at whale falls—20 species have been found solely on whale carcasses (Lundsten *et al.* 2010).

The Monterey Submarine Canyon, off of California, is home to a deep-sea whale fall community at 2893 m depth, discovered in 2002 (Goffredi *et al.* 2004a). At the time of discovery, this whale fall was in the enrichment-opportunistic stage, as described by Smith and Baco 2003, and was colonized by annelids, echinoderms and gastropods (Goffredi *et al.* 2004a; Lundsten *et al.* 2010). Early surveys of this whale fall revealed entirely novel organisms, like the bone-eating polychaete genus *Osedax* and its intracellular bacterial symbionts within the Oceanospirillales (Goffredi *et al.* 2005). Similarly, *Rubyspira osteovora* is a new genus of snail that was discovered in 2004 at this whale fall (Johnson *et al.* 2010). *Rubyspira osteovora* became abundant 3 years later (Braby *et al.*, 2007) and was observed in dense populations, both on the carcass and in the surrounding sediment (Lundsten *et al.* 2010). These gastropods are of particular interest since their main source of nutrition is bone, as revealed by stable isotope analysis, specialized radular mouthparts, and visual inspection of bone fragments in their digestive system (Johnson *et al.* 2010). Very few organisms rely exclusively on bone as their main source of nutrition, and other invertebrates that consume similarly indigestible substances, such as wood, often rely on microbial symbionts to supplement their dietary needs (Waterbury *et al.* 1983; Brune and Ohkuma 2010).

The microbial communities that reside both on and within animals, known as the microbiome, are often essential for digestion and nutrient supplementation, no matter the specific nature of the host nutritional strategy (Blaxter 1962; Douglas 1998; Brune and Ohkuma 2010). As we learn more about the diversity of life in understudied environments, we also uncover the diversity of microbial life associated with these organisms. Just as unique ecosystems on the planet can support novel biodiversity, the organisms within these ecosystems also support specific microbial communities. Animal microbiomes are often exceptional ecosystems—the human gut hosts its own unique assemblages of microbes and is unlike any on Earth (Costello *et al.* 2009). Viewing animals as host–microbe ecosystems nested within larger communities, rather than viewing animals in isolation, reveals the profound reciprocal influence between microbes and their hosts (McFall-Ngai *et al.* 2013).

Very little research has been conducted on the microbial communities associated with digestive tissues in deep-sea snails (Goffredi *et al.* 2004b), and surveying the microbiome of *R. osteovora* not only increases knowledge about biological diversity in the deep sea, but may also provide insight into their unusual nutritional strategy. This study aimed to characterize the microbial community associated with *R. osteovora* in comparison to microbes present in the surrounding environment and associated with other deep-sea gastropods that do not feed on bone. *Rubyspira osteovora* specimens were collected over the course of 6 years, and the microbial communities associated with digestive tissues were examined via sequencing of 16S rRNA genes, and both fluorescence microscopy and transmission electron microscopy (TEM). Although the role of the dominant microbes associated with *R. osteovora* is still undetermined, their distinctness from microbial communities from the environment and other deep-sea gastropods suggests a specific and exclusive microbiome within the bone-eating *R. osteovora*.

MATERIALS AND METHODS

Sample collection

Specimens ($n = 8$) were collected in 2004–2011 from a 2893 m depth whale fall in Monterey Submarine Canyon (36°36.8′N, 122°26.0′W; Table 1). *Rubyspira osteovora* samples were collected using the robotic submersibles ROV *Tiburon* and ROV *Doc Ricketts* (owned and operated by the Monterey Bay Aquarium Research Institute) via suction sample or sediment push core. Whale bone was collected via manipulator, and sediment was collected via push core. The scavenger *Bathymargarites symplector* and the predatory snail *Phymorhynchus* sp. were collected in April 2015 from the Gulf of California at 3651 m depth (23.358N; -108.542), using the ROV *Doc Ricketts*. Specimens for molecular analysis were preserved in 70% ethanol and stored at 4°C. Specimens for fluorescence *in situ* hybridization (FISH) microscopy and TEM were initially preserved in either 4% sucrose-buffered paraformaldehyde or 3% glutaraldehyde buffered with 0.1 M phosphate and 0.3 M sucrose, respectively, and kept at 4°C. Specimens were initially examined using a Leica S8APO stereomicroscope and photographed with a Nikon Coolpix P6000 digital camera (Figs 1C and 2A–D).

Molecular analyses

Snail tissues (digestive gland, intestine, stomach, fecal pellets) were dissected in 70% ethanol after removal of the shell (Table 1, Fig. 2A). Total genomic DNA was extracted from tissues using the Qiagen DNeasy kit (Qiagen, Valencia, CA) and from bone and sediment samples using the MoBio Powersoil DNA kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions. Total genomic DNA concentration was determined using the Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA), and ranged from 0.7 to 250 μg/mL. In some cases, successful PCR required up to 300× DNA dilution, most likely due to the presence of mucus within the snail tissue. PCR amplification was performed based on the specifications of the Earth Microbiome Project (EMP; Caporaso *et al.* 2011), except a first PCR was performed, for 25–30 cycles with primers lacking adapter, barcode, pad or linker (515f: GTGCCAGCMGCCG-CGGT AA; 806r: GGACTACHVGGGTWTCTAAT). For the second PCR step, 5 μl of the amplicon product from PCR#1 was used as template in a 5 cycle, 25 μl reconditioning reaction with the same EMP-recommended conditions and the full EMP primers (515f_barcode: AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA; 806r_barcode: CAAGCAGAAGACGGCATACGAGAT-X-AGTCAGTCAGCCG-GACTACHVGGGTWTCTAAT), where X indicates a unique 12-bp barcode. Adding the barcode indices at the second step minimizes PCR bias that would result from employing long primers over many cycles (Berry *et al.* 2011). Furthermore, the use of the 'reconditioning' PCR for barcoding, as well as the pooling of duplicate amplifications ahead of barcoding, was an attempt to minimize PCR errors and bias, respectively (Acinas *et al.* 2005; Kennedy *et al.* 2014). After all PCR reactions were completed, duplicate barcoded products were pooled and quantified. Samples were mixed together in equimolar amounts and purified in bulk through a Qiagen PCR Purification kit. At all PCR steps, amplification success and purity were checked by gel electrophoresis. Paired-end sequences (2×250 basepair) were generated from barcoded amplicon products at Laragen, Inc. on an Illumina MiSeq platform. At Laragen, the raw data were passed through a barcode filter which demultiplexed the library into individual samples and removed any

Table 1. Snail tissue and environmental samples analyzed via 16S rRNA sequencing.

Organism	Tissue	Sample ID[a,b]	Sample date	No. 16S rRNA sequences
Rubyspira osteovora	Digestive gland	Ruby204A-DG	Oct '10	35 153
		Ruby204B-DG	Oct '10	8615[c]
		Ruby204C-DG	Oct '10	34 835
		Ruby204D-DG	Oct '10	31 355
	Stomach	Ruby769A-St	Nov '04	31 217
		Ruby769B-St	Nov '04	42 488
		Ruby1162A-St	Dec '07	22 536
		Ruby204A-St	Oct '10	31 778
		Ruby204B-St	Oct '10	36 699
	Intestine	Ruby769A-Int	Nov '04	37 395
		Ruby769B-Int	Nov '04	44 141
		Ruby1162A-Int	Dec '07	21 615
		Ruby204D-Int	Oct '10	29 097
	Fecal pellets	Ruby204C-Fec	Oct '10	39 297
Other snails				
Bathymargarites sp.	Digestive gland	Bathym2-DG	Apr '15	35 878
	Intestine	Bathym1-Int	Apr '15	27 907
Phymorhynchus sp.		Phym751-Int	Apr '15	27 137
Environmental	Sediment	Sed204_PC44	Oct '10	28 108
	Whale bone	Bone_234_PC41	Jun '11	28 707

[a]Dives 769 and 1162 via remotely operated vehicle *Tiburon*, Dive 204 via remotely operated vehicle *Doc Ricketts*, both owned and operated by the Monterey Bay Aquarium Research Institute.
[b]A-D = separate individuals.
[c]Removed from data analysis.

sequences which had >1 basepair mismatch on the 12-basepair barcode sequence (Golay barcodes were chosen with Levenshtein distance ≥ 3; Caporaso *et al.* 2012). The resulting data were passed through Illumina MiSeq Recorder software, which assigned quality scores to each basepair call on every sequence. At the same time, adapter, barcode and primer sequences were removed. The raw sequence data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.5h1q1.

Sequence processing was performed in QIIME 1.8.0 (Quantitative Insights Into Microbial Ecology; Caporaso *et al.* 2010; Case *et al.* 2015). Raw sequences were aligned and quality control for unidentified base pairs and chimeras was performed (according to the specifics noted at http://sites.oxy.edu/sgoffredi/Goffredi_Lab/LabScripts). Sequences were clustered at 97% similarity and a representative sequence from each cluster was assigned a taxonomic identification using the Silva115 database. By barcode amplicon sequencing, 8615 to 57473 sequences were recovered from each sample (Table 1). After initial quality control and trimming, one sample with low sequence yield (*Rubyspira* 204B digestive gland with 8615 sequences) was removed from the data set. To avoid artifacts of sequencing depth, sequence data were then normalized to the next lowest sequence yield, which was the *Rubyspira* 1162A intestine, which resulted in 21 615 recovered sequences (Table 1). Normalized data were then used to calculate diversity indices and create principal components analysis (PCA) plots and bar charts of total bacterial OTU abundance. The normalized data for all samples is available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.5h1q1.

Mollicute-specific 16S rRNA PCR amplification was used to rapidly screen for the presence of Mollicutes, particularly *Mycoplasma*, in *R. osteovora* in digestive gland. A Mollicute FISH probe used by Duperron *et al.* (2013) was converted into a forward PCR primer (MolliB_F; 5′-CAGCAGTAGGGAATATT-3′) and combined with 1492R (5′-GGTTACCTTGTTACGACTT-3′) to amplify a specific 1142-bp Mollicute 16S rRNA gene product (with an anneal temperature of 59.6°C, initially determined via a temperature gradient amplification; 94 for 5 min, 94°C for 1 min, 59.6°C for 1 min, 72°C for 1 min, 35 cycles). The amplification product was sequenced directly using Sanger sequencing, via Laragen Inc., and was submitted to GenBank under accession number KY064173 (Fig. 6).

Statistical analyses

Diversity indices were calculated using normalized sequence data to compare the diversity across all samples (Table 2). Shannon–Wiener diversity ($H' = \sum_{i=1}^{s} p_i ln p_i$) and Simpson's diversity indices ($D = 1/\sum_{i=1}^{s} p_i^2$; where S is the total number of species in the community (richness), p_i is the proportion of S made up of the ith species) were calculated using Primer-E (Clarke and Warwick 2001). RStudio was used to make a PCA plot and perform analysis of variance (ANOVA) calculations using a script available at http://sites.oxy.edu/ sgoffredi/Goffredi_Lab/LabScripts. Alignments to close relatives were performed in Sequencher v4.10.1 and phylogenetic trees were created using MEGA v5.1. Close environmental and cultured relatives were chosen using top hits based on BLAST (www.ncbi.nlm.nih.gov).

A hierarchical approach was used to evaluate factors contributing to variance in microbial community dissimilarity among samples using both PCA and permutational multivariate analysis of variance using distance matrices (PERMANOVA). The contribution of sample type (*R. osteovora*, other snail species or environmental) and year (i.e. collection date) was evaluated for all samples. Analysis was restricted to only *R. osteovora* samples to evaluate differences among tissue types within *R. osteovora*

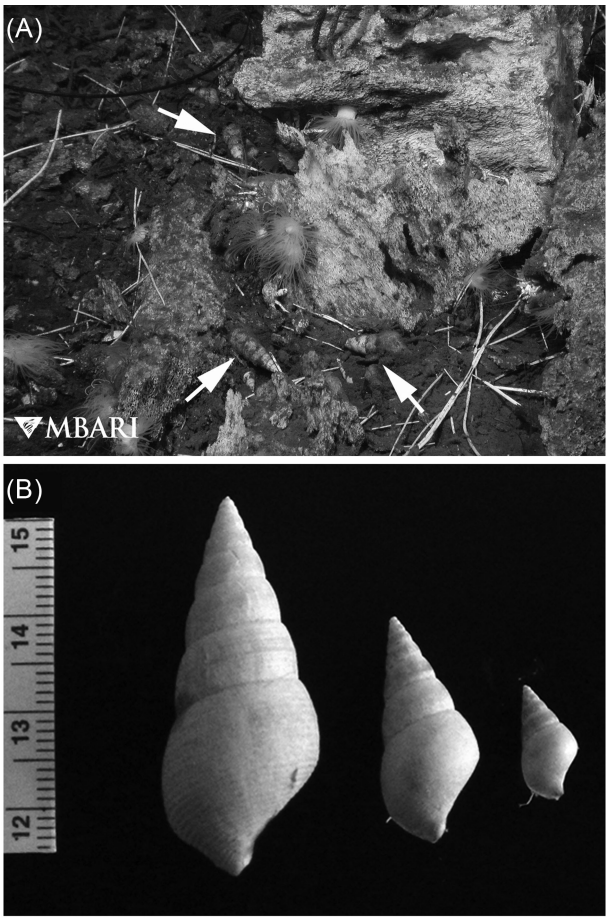

Figure 1. *Rubyspira osteovora*. **(A)** *Rubyspira osteovora* snails (arrows) on pieces of bone and sediment at a 2893 m whale fall at Monterey Submarine Canyon. **(B)** *Rubyspira* specimens, ruler (cm) shown for scale.

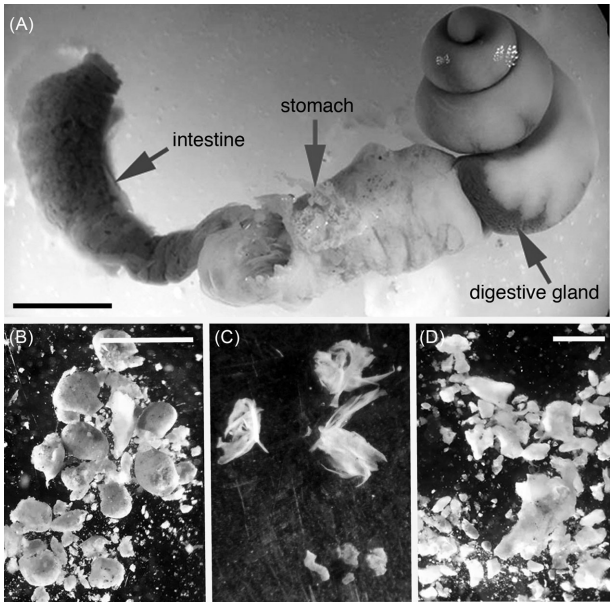

Figure 2. Anatomy of *R. osteovora* digestive tract and contents. **(A)** Dissected digestive tract of *Rubyspira* with intestine stomach and digestive gland (dark tissue; reproductive tissue is light colored). Scale bar, 5 mm. **(B)** Fecal pellets removed from the intestine. Scale bar, 2 mm. **(C)** Size comparison of bone fragments (top) and fecal pellets (bottom). **(D)** Bone fragments removed from the stomach. Scale bar, 2 mm.

(digestive gland, stomach and intestine) and year. The single fecal sample in *R. osteovora* was excluded from this analysis due to lack of replication. Bray–Curtis dissimilarity matrices were calculated using the 'vegan' R package (Oksanen *et al.* 2013), and PERMANOVA was conducted using the command 'adonis'.

To evaluate predictors of the proportions of the two most dominant microbial groups within the *Rubyspira osteovora* samples, *Mycoplasma* and *Psychromonas*, we conducted model selection analysis. A hierarchical approach was again used to first compare among sample types and then within *Rubyspira* alone. Four candidate models were considered for analysis (Table 3). For all samples, the models included sample type (*Rubyspira*, other snail species or environment), year (i.e. collection date), sample type + year, and a null model. For the *Rubyspira*-specific analysis, the models included tissue (digestive gland, stomach and intestine), year, tissue + year, and a null model. For each candidate model, we performed a linear model and calculated the second-order bias corrected Akaike's Information Criterion (AIC_C), a measure of the relative quality of a model based on the goodness of fit observed and the parsimony of that model, using the R package, 'AICcmodavg' (Mazerolle 2011). AIC has been shown to perform well as a model selection tool for complex ecological datasets (Aho, Derryberry and Peterson 2014). Akaike weights (ω_i) were calculated to assess the weight of evidence in favor of the hypotheses represented by the model (Burnham and Anderson 2002, 2004). Models in each set were ranked according to the difference in AICc (Δ_i) and ω_i. Models with $\Delta_i < 2$ were selected as best-fit models out of the candidate set. If only a single model received a $\Delta_i < 2$, this is considered comparable to a significant difference among alternate models. Higher ω_i values indicate better support for the model (as a percentage; Table 3). Variables included in the best-fit model(s) are considered to be the best predictors of the response variable. The analyses were repeated for both *Mycoplasma* and *Psychromonas*.

FISH microscopy

Paraformaldehyde-preserved *R. osteovora* specimens were rinsed with 1× PBS, transferred to 70% ethanol, and stored at –20°C. Digestive gland, stomach and intestine were dissected and embedded in Steedman's wax (1 part cetyl alcohol: 9 parts polyethylene glycol (400) distearate, mixed at 60°C; Steedman 1957). An ethanol:wax gradient of 3:1, 2:1 and 1:1, and eventually full strength wax, was used to embed the samples (1 h each treatment). Embedded samples were sectioned at 5–10 μm thickness using a Leica RM2125 microtome and placed on Superfrost Plus slides. Sections were dewaxed in 100% ethanol rinses. Hybridization buffers and wash buffers were prepared according to Pernthaler and Pernthaler (2005), using 30% formamide in the hybridization buffer and 450 mM NaCl in the wash solution, and fluorescent probes were at a final concentration of 5 μg mL^{-1}. A universal bacterial probe mix (Eub338˙I-III, Amann *et al.* 1990, Daims *et al.* 1999), a probe that targeted Mollicutes (5′- AATATTCCCTACTGCTG-3′; adapted from Duperron *et al.* 2013), and a genus-specific *Psychromonas* probe (NOR2-1453, 5′-GGTCATCGCCATCCCC-3′; Eilers *et al.* 2000) were used to observe the localization of *Mycoplasma* and *Psychromonas* in *R. osteovora* tissues, respectively. Probes were hybridized at 46°C for 4 h, followed by a 15 min wash at 48°C. Tissues were stained with 4′6′-diamidino-2-phenylindole (DAPI, 5 mg/mL) for 1 min, rinsed and mounted in Citifluor. Tissues were examined by

Table 2. Average bacterial diversity indices, based on 16S rRNA barcoding, for samples examined in the this study.

Organism	Tissue	Shannon index (x ±SE)[a]	Simpson index (x ±SE)[1]
Rubyspira osteovora	Digestive gland	1.29 ± 0.19	0.49 ± 0.07
	Intestine	1.77 ± 0.25	0.58 ± 0.08
	Stomach	2.12 ± 0.24	0.72 ± 0.04
	Fecal pellets	2.70	0.87
Bathymargarites symplector	Digestive gland	2.58	0.88
	Intestine	2.78	0.90
Phymorhynchus sp.	Intestine	1.32	0.50
Environment	Bone	4.57	0.98
	Sediment	4.16	0.98

[a]Sample sizes of 1 have no SE available. Data correspond to those shown in Fig. 3.

epifluorescence microscopy using a Nikon E80i epifluorescence microscope with a Nikon DS-Qi1Mc high-sensitivity monochrome digital camera.

Electron microscopy

Approximately 1 mm^3 digestive gland tissue samples for examination by TEM were fixed in 3% glutaraldehyde buffered with 0.1 M phosphate and 0.3 M sucrose (pH 7.8). Samples were washed in 0.1 M sodium cacodylate with 24% sucrose and post-fixed with 1% OsO_4 in 0.1 M sodium cacodylate for 1 h. Samples were stained in 3% uranyl acetate in 0.1 M sodium acetate buffer for 1 h, dehydrated in ethanol and embedded in Spurr's resin. Thick sections (0.4 μm) were stained with methylene blue and thin sections (70 nm) were stained with lead citrate. Sections were examined and photographed using a Nikon E80i epifluorescence microscope and Zeiss EM 109 TEM.

RESULTS

Rubyspira osteovora collections and anatomy

Rubyspira osteovora specimens were collected between 2004 and 2011 from a whale fall in Monterey Submarine Canyon (Table 1, Fig. 1). Snails ranged from 1 to 4 cm in total shell length. The stomach and distal intestine were easily identifiable, as their surfaces were visibly different from that of the digestive gland (Fig. 2). Samples of digestive gland were typically taken from the very tip of the coil to avoid adjacent reproductive tissue. Intestine samples, which usually contained dark fecal pellets, were taken from the distal end of the rectum closest to the gill. The stomach contained bone fragments (≤ 5 mm; Fig. 2D), which were noticeably different from fecal pellets in the intestine, as was observed by Johnson *et al.* 2010 (Fig. 2B–D).

A unique bacterial community associated with *Rubyspira osteovora*

Barcode sequencing of 16S rRNA genes revealed that *R. osteovora* digestive tissues contained 45–195 bacterial OTUs and 5–12 bacterial families (OTUs <0.5% and families <1% grouped as 'other'; Fig. 3). The digestive gland was the least diverse (average Shannon index of 1.29, $n = 3$), followed by the intestine (average Shannon index of 1.77, $n = 4$) and stomach (average Shannon index of 2.12, $n = 5$; Table 2). Fecal pellets most closely resembled intestine tissue based on PCA and contained 23% *Psychromonas*, which was present in high levels in the intestine (Figs 3 and 4). *Rubyspira osteovora* digestive gland, stomach and intestine bacterial communities were dominated by two genera: *Mycoplasma* and *Psychromonas*. A single *Mycoplasma* OTU was most dominant in *R. osteovora* tissues, comprising up to 75% total recovered sequences in the digestive gland, and averages of 56%, 40% and 11% in the digestive gland, stomach and intestine, respectively. A single *Psychromonas* OTU comprised up to 67% total recovered sequences in the intestine, with averages of 11%, 21% and 42% in the digestive gland, stomach and intestine, respectively. These two OTUs accounted for between 42% and 80% of the total recovered *R. osteovora* sequences, with other minor OTUs usually present at very low proportions (<0.5%). A single *Psychrilyobacter* OTU formed the next most dominant genus in *R. osteovora* tissues, up to 35% with averages of 16%, 3% and 6% in the digestive gland, stomach and intestine, respectively. Over the course of 6 years (2004–2011), similar microbial communities, with the same dominant three genera were recovered from *R. osteovora* (stomach and intestine Figs 4 and 5). Community dissimilarity analyses showed that tissue type was a significant predictor of community dissimilarity (PERMANOVA $R^2 = 0.42$, $P = 0.005$), while year (i.e. collection date) was non-significant (PERMANOVA $R^2 = 0.05$, $P = 0.13$), indicating the stability of the microbiome over time.

Conversely, microbial communities in sediment surrounding the whale fall and on a whale bone surface were found to be significantly more diverse, with Shannon indices of 4.2–4.6, compared to an average Shannon index of 1.3–2.1 for all *R. osteovora* tissues (Table 2; ANOVA $P = 0.0026$). Note that the diversity values for the environmental samples are further underestimated because a considerable number of OTUs (~60% of total recovered sequences) were eliminated during normalization of the entire dataset. For the bone and sediment samples from the Monterey

Table 3. Comparison of linear models for proportion of dominant microbial groups in microbial samples using AIC_C analysis.

Analysis	Species	Model[a]	K[b]	AIC_C	Δ_i	ω_i
All Samples	*Mycoplasma*	Type	4	167.2	0.00*	0.75
		NULL	2	169.4	2.22	0.25
		Year	6	177.5	10.24	0.00
		Type + year	7	179.2	11.94	0.00
Rubyspira Samples	*Mycoplasma*	Tissue	4	106.6	0.00*	0.94
		NULL	2	112.5	5.86	0.05
		Year	4	117.6	11.00	0.00
		Tissue + year	6	121.1	14.45	0.00
All Samples	*Psychromonas*	Type	4	156.5	0.00*	0.80
		NULL	2	159.7	3.14	0.17
		Year	6	163.3	6.79	0.03
		Type + year	7	166.9	10.40	0.00
Rubyspira Samples	*Psychromonas*	Tissue	4	106.3	0.00*	0.59
		NULL	2	107.1	0.82*	0.39
		Year	4	113.0	6.66	0.02
		Tissue + year	6	121.1	14.76	0.00

[a]The candidate models include the variables: sample type (*Rubyspira*, other snail species or environment), year (i.e. collection date) and tissue type (digestive gland, stomach or intestine). The results include the parameter count, K; the AIC_C score; the change in AIC_C scores relative to the lowest AIC_C score, Δ_i; the weight of each model (or % of variance that is explained by the parameter?), ω_i. (*) indicates values that are indicative of the model significance.
Parameter count includes intercept and variance.

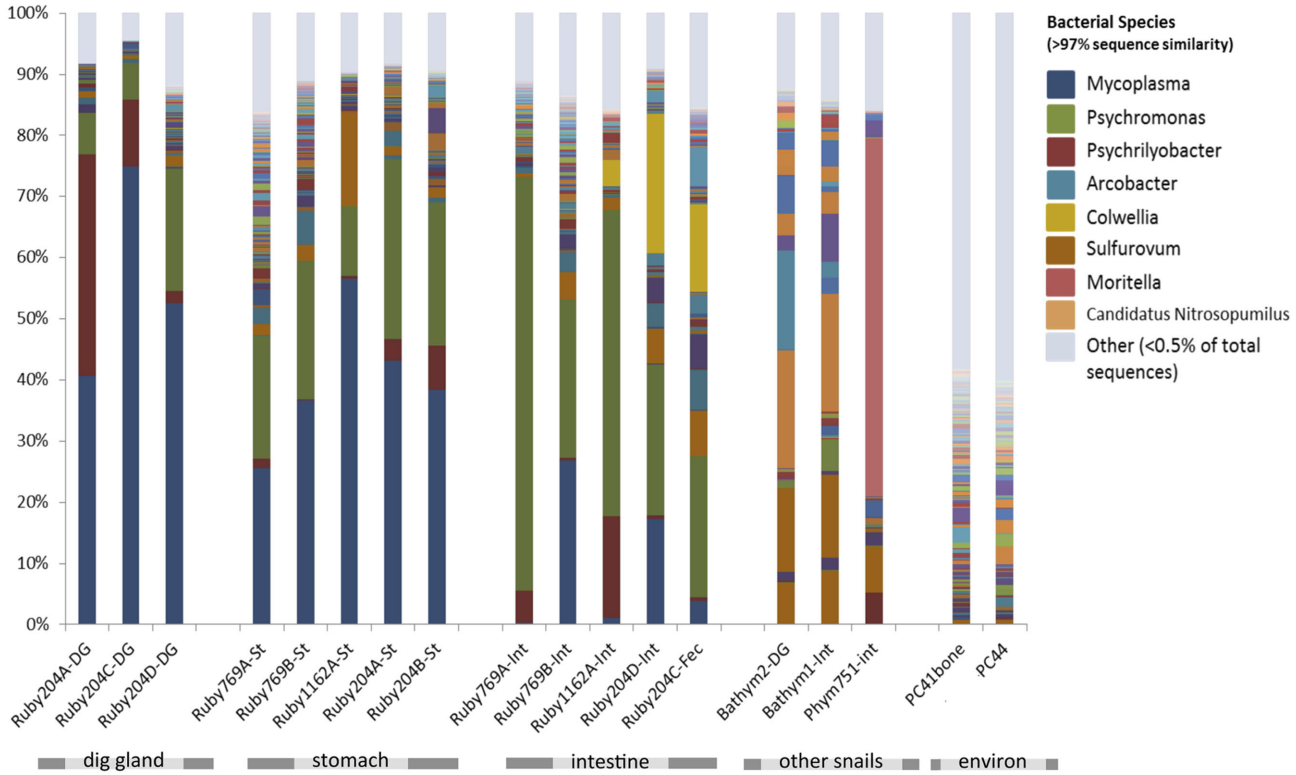

Figure 3. Relative abundance of bacterial community structure to the OTU level (97% 16S rRNA sequence similarity), from *R. osteovora* digestive gland, stomach and intestine, *B. symplector*, *Phymorhynchus* sp. and environmental samples (sediment and whale bone). Each color on the graph represents a distinct genus-level OTU or lowest level available. Dominant OTUs are indicated. Genera that constituted <0.5% of individual samples were grouped as 'Other' and are shown in gray.

Canyon whale fall, no single bacterial OTU (based on 97% similarity) accounted for more than 21% of recovered sequences. *Mycoplasma* and *Psychromonas* were not recovered from these environmental samples, while *Psychrilyobacter* comprised <0.04% of the bacterial community of bone and sediment samples.

The bacterial communities of other deep-sea non-bone-eating snails (*Phymorhynchus* sp. and *Bathymargarites symplector*) were also surveyed and found to be distinct from *R. osteovora* (Figs 3 and 4). The bacterial community in the *Phymorhynchus* intestine tissue was least diverse, with a Shannon index of 1.32, while the community diversity in the digestive gland and intestine of *B. symplector* was more diverse than *R. osteovora*, with Shannon indices of 2.58 and 2.78 for the digestive gland and intestine, respectively (Table 2, Fig. 3). *Mycoplasma, Psychromonas* and *Psychrilyobacter* were generally absent from *Phymorhynchus* and *Bathymargarites* tissues, with the exception of *Psychrilyobacter* in the intestine of *Phymorhynchus* (~5% of recovered sequences).

Rubyspira osteovora samples showed lower community dissimilarity than either of the other snail species samples, the environmental samples or the between sample type comparisons. PCA showed clear separation of *R. osteovora* tissue microbial communities from environmental microbial communities and those from other snail species (Fig. 4; the first two principal components accounted for 46% and 28% of the variation in microbial communities across samples). Sample type (*Rubyspira*, other snail species or environment) was a significant predictor of community dissimilarity (PERMANOVA: $R^2 = 0.45$, $P = 0.001$). In particular, mean proportions of *Mycoplasma* and *Psychromonas* were significantly different between *R. osteovora* tissues and the environment (ANOVA $P = 0.0002$ and 0.004, respectively; Fig. 5).

Dominant microbes associated with *Rubyspira osteovora*

Mycoplasma and *Psychromonas* were the most dominant bacterial genera associated with *Rubyspira* digestive tissues. Mean abundances of *Mycoplasma* and *Psychromonas* were significantly different between the three *R. osteovora* tissues types (ANOVA $P = 0.005$ and 0.032, respectively; Fig. 5). *Mycoplasma* proportions were highest in the digestive gland (average 56%) and lowest in the intestine (average 11%; Fig. 5A). Conversely, *Psychromonas* proportions were highest in the intestine (average 42%) and lowest in the digestive gland (average 11%; Fig. 5B). The stomach contained intermediate amounts of both microbes. Both digestive gland and stomach tissues showed higher within-tissue similarity in bacterial community composition, compared to either the intestine or the between tissue comparisons (Fig. 4). The best fit of the candidate model set for 'All Samples' included sample type only for proportions of both *Mycoplasma* ($\omega_i = 0.75$) and *Psychromonas* ($\omega_i = 0.80$) (e.g. tissue type essentially explains 75% and 80% of the bacterial community variability; Table 3). Each of the other models had Δ_i values greater than 2, suggesting that sample type is the best predictor of *Mycoplasma* and *Psychromonas* levels in samples, while year/collection date is not. For the *Rubyspira*-specific analyses, tissue type was identified in the top candidate models for both *Mycoplasma* ($\omega_i = 0.94$) and *Psychromonas* ($\omega_i = 0.59$) however, for *Psychromonas*, AIC_C analysis could not distinguish between the tissue type and null model ($\Delta_i < 2$ for both models; Table 3). Thus, while tissue type is a good predictor of the proportion of *Mycoplasma* in *Rubyspira* samples alone, there is not enough evidence to determine the best predictors of the proportion of *Psychromonas* in *Rubyspira* samples.

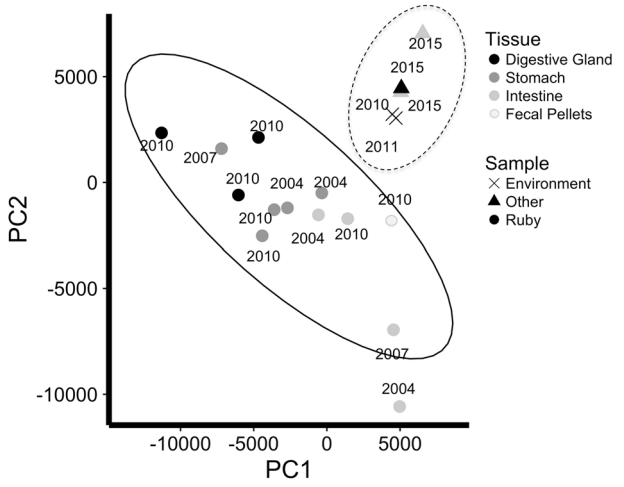

Figure 4. PCA results of individual samples at the level of OTUs (97% sequence similarity) showing microbial communities of *R. osteovora* tissues (circles), other snail species (triangles; *B. symplector*, *Phymorhynchus* sp.) and environmental samples (X, whale bone and sediment). The 95% confidence ellipse is shown for the *Rubyspira* samples (solid line). There were too few samples for 95% confidence ellipses for other species or environmental samples; however, an ellipse was drawn by hand (dashed line) around all non-*Rubyspira* samples, for clarity. *Rubyspira osteovora* samples clustered together and separately from other snail species and the environmental samples. Principal components 1 and 2 accounted for 46% and 28% of variation within microbial communities across samples, respectively.

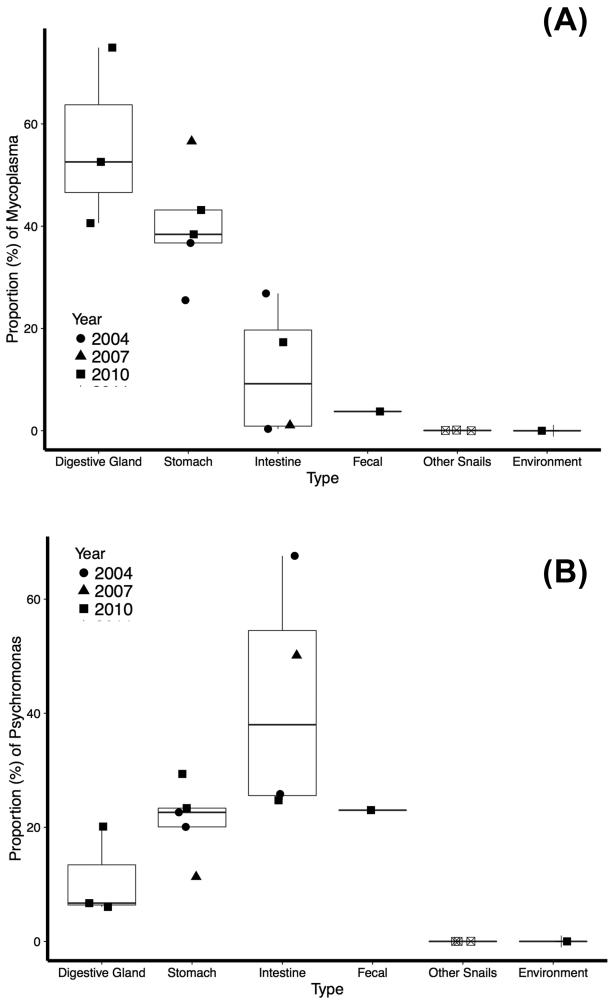

Figure 5. ANOVA of mean proportions of (A) *Mycoplasma* and (B) *Psychromonas* between tissue types and environmental samples. *Rubyspira osteovora* digestive tract tissues had significantly higher proportions of *Mycoplasma* and *Psychromonas* than the environment and other snails. ANOVA, $P = 0.0002$ (*Mycoplasma*), $P = 0.004$ (*Psychromonas*).

Five *Mycoplasma* phylotypes (which were 98%–99.6% similar to each other) were recovered in the barcode dataset, but one was dominant and represented >98% of the sequence reads in the 12 *Rubyspira* samples (all others represented 0.2%–1.2% of recovered *Mycoplasma* sequences). A phylogenetic tree of the dominant *Mycoplasma* OTU recovered from *R. osteovora*, via both barcode sequencing and the Mollicute-specific 16S rRNA amplification from the digestive gland, revealed relationships between this phylotype and other *Mycoplasma*-related bacteria. This *Mycoplasma* sequence was only 91%–94% similar to cultured and uncultured representatives, and clustered most closely to sequences from cage-cultured abalone intestine (GU070691 and HQ393429, both at 93% sequence similarity), distinct from human pathogens (Fig. 6). Although commonly considered to be a vertebrate pathogen, the closest relatives were observed from marine invertebrates. *Mycoplasma* has been only rarely reported for non-vertebrates; thus, the ribotype recovered from *R. osteovora* represents one of the only known instances of *Mycoplasma* in deep-sea molluscs and within deep-sea animals in general (Duperron *et al.* 2013).

Nine *Psychromonas* phylotypes were recovered in the barcode dataset (which were 98% similar to each other), but one was dominant and represented >96% of the sequence reads in all 12 *Rubyspira* samples (the others were 0.2%–1.3% of recovered *Psychromonas* sequences). The dominant *Psychromonas* sequence was >99% similar to cultured and uncultured representatives. *Psychromonas* recovered from *R. osteovora* clustered most closely with deep-sea environmental sequences from biocorroded marine metals (Dang *et al.* 2011), Arctic sediments (Prasad *et al.* 2014) and sequences recovered from leeches at a 600 m whale fall in Monterey Canyon (Goffredi, Morella and Pulcrano 2012) (Fig. 7A).

An additional genus *Psychrilyobacter* was common among *Rubyspira* digestive tissues. Four *Psychrilyobacter* phylotypes were recovered in the barcode dataset (which were 98.8% similar to each other), but one was dominant and represented 94.0% of the sequence reads in all 12 *R. osteovora* samples (the others were 0.9%–4.0% of recovered *Psychrilyobacter* sequences). The dominant *Psychrilyobacter* sequence was 100% similar to both uncultured and cultured *Psychrilyobacter atlanticus* strains (Fig. 7B; Zhao *et al.* 2004). Phylogenetic analysis grouped *Psychrilyobacter* from *R. osteovora* with deep-sea environmental samples such as those from hydrothermal vents (Reed, Lutz and Vetriani 2006; Hügler, Gärtner and Imhoff 2010) and cold seep sediments (Reveillaud *et al.* 2016; Fig. 7B), as well as marine invertebrates including a tunicate (Dishaw *et al.* 2014), oyster (Fernandez-Piquer *et al.* 2012) and *Paralvinella* worm, a hydrothermal vent polychaete (Alain *et al.* 2002).

FISH microscopy was used to confirm the presence of microbes within the digestive tissues of *R. osteovora* (Fig. 8). Light and fluorescence microscopy revealed distinct morphology for each tissue; intestinal epithelia contained ciliated columnar cells, stomach lining was highly convoluted and contained simple cuboidal cells with clear basal nuclei, and the digestive gland contained columnar cells arranged in tubules (Fig. 8). Positive FISH signals were obtained with the general eubacterial probe Eub338 for all three tissues, which indicates the presence of bac-

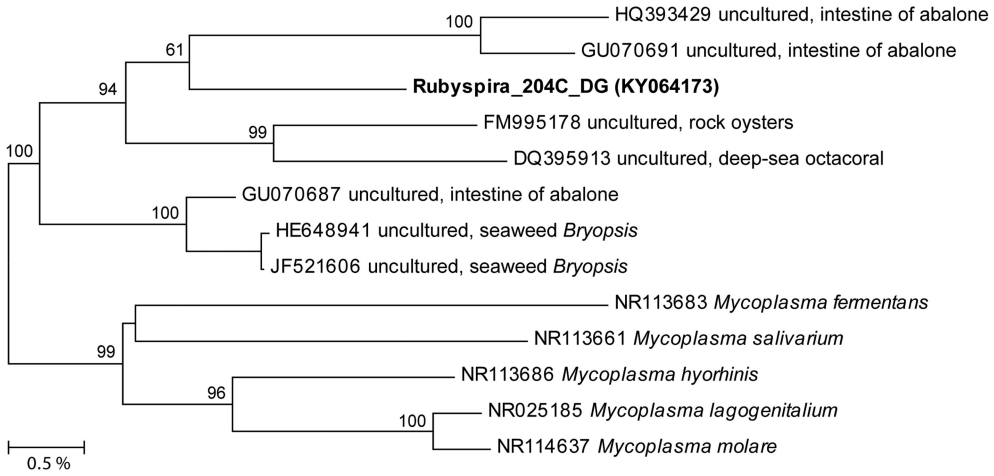

Figure 6. Phylogenetic tree of the dominant *Mycoplasma*-related OTU isolated from *R. osteovora* digestive gland (in bold). Five *Mycoplasma* phylotypes (which were 98%–99.6% similar to each other) were recovered in the barcode dataset, but one was dominant (shown in the tree) and represented >98% of the sequence reads in all 12 *Rubyspira* samples (all others represented 0.2%–1.2% of recovered *Mycoplasma* sequences). Close relatives were assigned using BLAST and include human pathogens and environmental samples. *Mycoplasma* from *Rubyspira* clustered most closely (61% bootstrap confidence) with *Mycoplasma* isolated from abalone intestine. The tree is based on neighbor-joining analysis was conducted with Olsen distance correction, and the resulting tree topology was evaluated by bootstrap analysis (numbers at nodes indicate bootstrap support from 1000 resampled data sets). The scale bar represents 0.05 substitutions per site.

teria in these areas. Bacteria were observed attached to the cilia of the stomach and intestine, as well as in the lumen. In the digestive gland, bacteria were localized within the lumen of the ducts. Qualitatively, greater numbers of bacteria were observed within the intestine and stomach, and fewer within the digestive gland. In the stomach and intestine, in particular, many of these bacteria were confirmed to be *Psychromonas* spp. via hybridization with a genus-specific probe (Fig. 8). Unfortunately, no positive signals were observed with the Mollicute-specific probe, even in the digestive gland. We suspect this is due to low numbers of bacteria in this tissue in particular, and perhaps also due to the enzymatic nature of the digestive gland and the compromised nature of the tissues, even with immediate preservation. Although intracellular bacteria-like objects in the digestive gland, detected by TEM, could be intracellular secretory vesicles, they are consistent with *Mycoplasma*, irregular in shape, size and without a cell wall (Rottem, Kornspan and Kosower 2012; Fig. 9).

DISCUSSION

It is now widely accepted that multicellular organisms form intricate cooperative associations with microbes. Bacteria live in the digestive systems, in particular, of nearly all animals that have been studied, and these bacteria play critical roles in the exchange of nutrients and digestion of food, and thus contribute to success and survival of the host. Discovering novel and diverse animals in unusual ecosystems has also uncovered the unique and specific microbial communities associated with these organisms. *Rubyspira osteovora* is a deep-sea gastropod that was discovered at a whalefall in 2004 (Johnson *et al.* 2010). 16S rRNA gene sequencing revealed that *R. osteovora* is colonized by a stable and specific microbiome, significantly less diverse than the surrounding microbial communities.

The unique and specific bacterial community of *Rubyspira osteovora*

Rubyspira osteovora has a specific microbial community dominated by three microbial genera: *Mycoplasma*, *Psychromonas* and *Psychrilyobacter*. The predominance of these three bacterial OTUs at 43%–92% of total recovered sequences is notable and indicates a specific relationship between *R. osteovora* and these microbes, although their role is still unknown. In contrast, the human gut microbiome, and other mammalian microbiomes, contain around 1000 bacterial species, and the 'core microbiome' is characterized at the phylum level, rather than the genus level because of high levels of diversity (Ley *et al.* 2008; Turnbaugh *et al.* 2009; Qin *et al.* 2010). Within invertebrates, the diversity level of the gut microbiome has been shown to be one to two orders of magnitude lower than that of mammals (Dillon and Dillon 2004; Dunn and Stabb 2005). For example, *Drosophila melanogaster* has five dominant species in its microbiome that are present at every host life stage, with only 17–71 total OTUs (Wong, Ng and Douglas 2011).

Rubyspira osteovora is thought to forage exclusively on bone, based on stable isotope analyses, radular morphology and the occurrence of bone fragments within the stomach (Johnson *et al.* 2010). It is still unclear whether the microbes associated with *R. osteovora* are acquired vertically or from the environment during foraging. If the microbes within the gut of *R. osteovora* were only transiently associated with the animals during feeding, we would expect to see a highly diverse microbiome that mirrored the composition of the environmental samples. Instead, the low bacterial diversity in *R. osteovora* tissues compared to nearby sediment and bone samples supports the argument for a specific microbial community, not present in the environment. *Psychrilyobacter* has been found in other deep-sea sediments and from sediments adjacent to whale falls (Goffredi *et al.* 2005; Reed, Lutz and Vetriani 2006; Reveillaud *et al.* 2016), and closely related *Psychromonas* has also been found in Monterey Canyon (Goffredi, Morella and Pulcrano 2012). Although *Psychrilyobacter* was found in the environmental communities in very low abundances (<0.04%), it is still possible that *R. osteovora* acquires them from the environment. A similar acquisition of a specific symbiont in very low abundance is seen in the *Euprymna* squid- *Vibrio fischeri* symbiosis (McFall-Ngai 1994). *Mycoplasma*, however, is an obligate pathogen, so it is likely transmitted from host to host. To study this, the microbiome of *Rubyspira* should be surveyed at

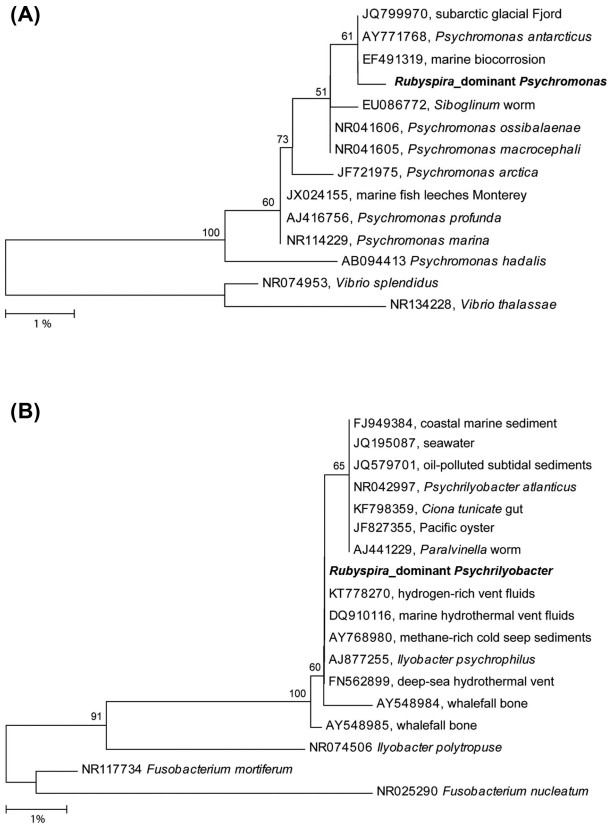

Figure 7. Phylogenetic trees of OTUs with high-sequence similarity to *Psychromonas* (**A**) and *Psychrilyobacter* (**B**) isolated from *R. osteovora* digestive tract tissue (in bold), based on 250-bp fragments from barcoded Illumina sequencing. Nine *Psychromonas* phylotypes were recovered in the barcode dataset (which were 98% similar to each other), but one was dominant (shown in the tree) and represented >96% of the sequence reads in all 12 *Rubyspira* samples (all others were 0.2%–1.3% of recovered *Psychromonas* sequences). Four *Psychrilyobacter* phylotypes were recovered in the barcode dataset (which were 98.8% similar to each other), but one was dominant (shown in the tree) and represented 94.0% of the sequence reads in all 12 *Rubyspira* samples (all others were 0.9%–4.0% of recovered *Psychrilyobacter* sequences). Trees based on neighbor-joining analysis were conducted with Olsen distance correction, and the resulting tree topology was evaluated by bootstrap analysis (numbers at nodes indicate bootstrap support from 1000 resampled data sets). The scale bars represent 0.1 substitutions per site.

different developmental stages—in the eggs, during larval development, as juveniles, and as adults. If these microbes are vertically transmitted, we would expect to see them throughout all stages of development, particularly in the eggs.

Additionally, the bacterial community composition of *R. osteovora* differed from other deep-sea, non-bone feeding gastropod microbiomes, despite diversity levels that were significantly different from those found in the environment. *Phymorhynchus* sp. is predatory and feeds on other gastropods, while *Bathymargarites symplector* is a scavenger on the remains of invertebrates, diatoms and undefined organic matter (Warén and Bouchet 1989). If a common microbiome existed among all deep-sea gastropods, we would expect to see similar microbial communities, despite differing nutritional strategies. However, these gastropods did not contain any of the abundant microbes observed in *R. osteovora*, except for *Phymorhynchus*, which contained 5% *Psychrilyobacter*. The absence of two of the dominant *R. osteovora* microbes from other gastropods supports the idea that *R. osteovora* hosts a specific microbial community in its digestive system.

Dominant microbes associated with *Rubyspira osteovora*

Mycoplasma (phylum Tenericutes; class Mollicutes) was the most dominant genus in the digestive gland of *R. osteovora*. Mycoplasmas are parasitic and have typically been found as obligate vertebrate pathogens, most notably the cause of several human diseases (*Mycoplasma genitalium* and *M. pneumoniae*; Razin, Yogev and Naot 1998). Normally, mycoplasmas have strict host and tissue specificity within vertebrates; however, recent research has observed *Mycoplasma* in high abundance in algae (Hollants *et al.* 2011, 2013) and invertebrates, including shallow-water molluscs, such as Sacoglossans (90% of total recovered sequences), aquacultured abalone and oysters (>80% of total sequences; Huang *et al.* 2010; King *et al.* 2012; Davis *et al.* 2013), deep-sea wood-eating chitons (50% of sequences in gut, 70% in gill) (Duperron *et al.* 2013) and the deep-sea polychaete *Riftia* (>80% of individuals; Scharhauser 2013). The abundance and role of *Mycoplasma* in these organisms is unclear, although authors have speculated that the microbes may help animals digest nutrient-poor foods (Fraune and Zimmer 2008; Duperron *et al.* 2013). In *R. osteovora*, *Mycoplasma* was dominant in nearly all digestive tract samples, but was most prevalent in the digestive gland, where intracellular enzymatic digestion occurs (Walker 1970). It is possible that nutrients or molecules involved in digestion facilitate *Mycoplasma* growth in this area. The *Mycoplasma* sequence isolated from *R. osteovora* is likely a novel species, as it was only 91%–94% similar to any close relatives. Whether *Mycoplasma* is a pathogen, beneficial symbiont or neutral associate is still unknown. However, evidence of *Mycoplasma* in snails collected over the course of 6 years indicates a relatively long-term relationship.

Psychromonas (class Gammaproteobacteria) was the most dominant genus in the intestine of *R. osteovora*, and was > 96% identical to sequences recovered from sediment adjacent to a sperm whale carcass (Miyazaki *et al.* 2008). While it seems to be a ubiquitous psychrophilic environmental microbe, there have been several reported instances of *Psychromonas* associating with marine invertebrates. For example, deep-sea leeches, from two different whale fall habitats in Monterey Canyon, were dominated by closely related *Psychromonas* (98% 16S rRNA sequence similarity; >90% of recovered sequences; Goffredi, Morella and Pulcrano 2012). Additionally, *Psychromonas* ribotypes have been recovered from the digestive tract of a wood-feeding gastropod from ~500 m depth in the Vanuatu archipelago (Zbinden *et al.* 2010). The close relatives of this isolate are anaerobic heterotrophs that are able to hydrolyze polymers such as starch, and the authors speculate that *Psychromonas*, along with other microbes in the digestive tract, have a role in wood digestion (Zbinden *et al.* 2010). The clear association of this bacterial genus with the digestive tissues of *R. osteovora* (Fig. 8) suggests a non-transient association.

Psychrilyobacter (Phylum Fusobacteria) was the third most abundant bacterial genus within *Rubyspira osteovora* tissue. *Psychrilyobacter* are psychrophilic, obligately anaerobic bacteria originally recovered from marine sediment at 215 m depth in 2009 (Zhao, Manno and Hawari 2009). Like *Psychromonas*, closely related *Psychrilyobacter* sequences were found in both environmental samples and in the microbiomes of invertebrates with no identified roles. The *Psychrilyobacter* sequence recovered from *R. osteovora* was 100% identical to uncultured bacteria from hydrothermal vent and cold seep sediments and over 99% similar

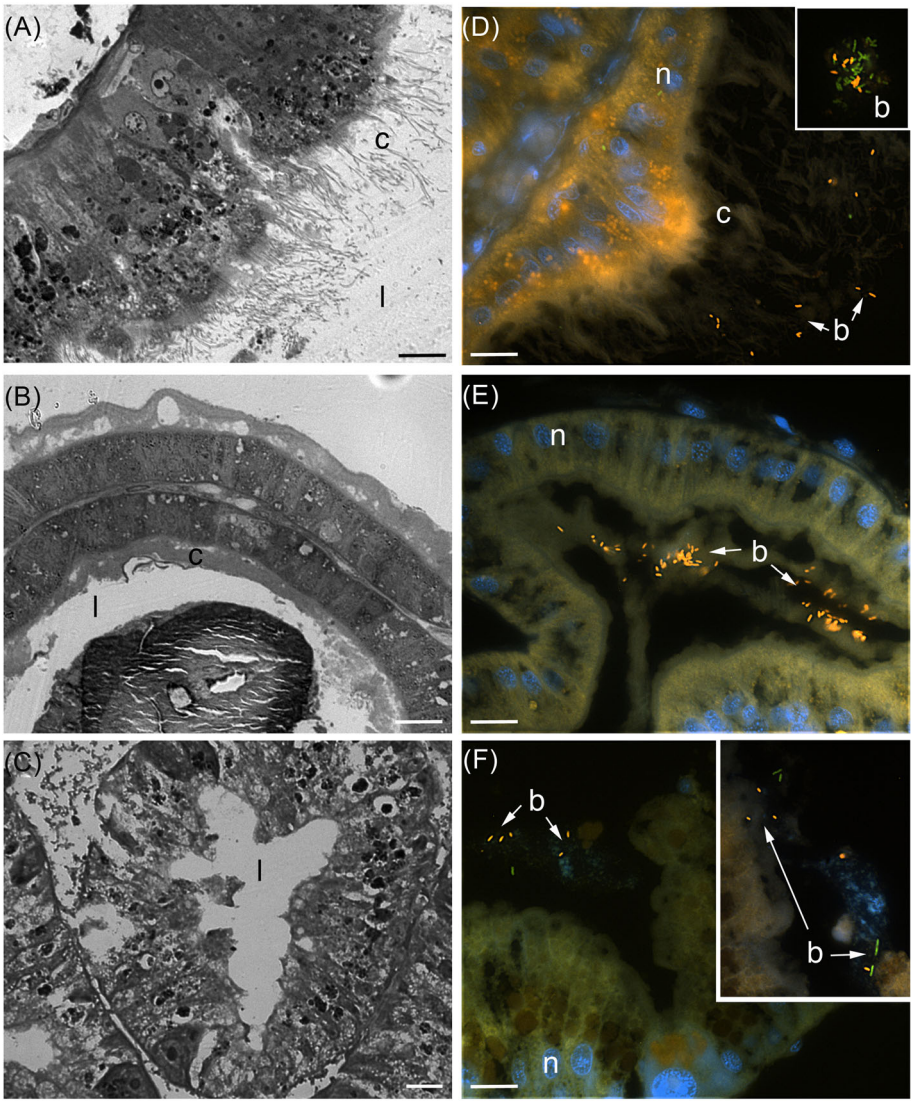

Figure 8. Light microscopy **(A-C)** and fluorescence microscopy **(D-F)** of *R. osteovora* digestive tract tissues. Methylene blue-stained thick sections (0.4 μm) were used for *R. osteovora* tissue morphology, and revealed intestinal epithelia columnar cells topped with very long cilia **(A),** stomach epithelia contained simple cuboidal cells, topped with short cilia **(B)** and a digestive gland with ducts of columnar cells **(C).** DAPI-stained nuclei of *R. osteovora* cells (blue), a general bacteria-probe Eub338-Alexa (green) and a *Psychromonas*-probe, NOR2-1453_Cy3 (orange) revealed that bacteria were attached to the cilia (c) of the intestinal epithelia **(D).** Large clusters of *Psychromonas* and other bacteria were also found in the lumen (l) of the intestine **(D-inset)**. In the stomach, bacteria were found in the lumen **(E).** Fewer bacteria were observed in the digestive gland, and localized within the duct lumen, close to the epithelia. **(F, inset)**. n, nucleus. l, lumen. c, cilia. b, bacteria. All scale bars are 10 μm.

to uncultured bacteria from Monterey whale fall sediments (Goffredi *et al.* 2005; Reed, Lutz and Vetriani 2006). *Psychrilyobacter* has limited known associations with metazoans. Three close relatives (>99% sequence similarity) were recovered, in low abundance, from *Paralvinella* polychaetes, Pacific oysters and *Ciona* tunicates (Alain *et al.* 2002; Fernandez-Piquer *et al.* 2012; Dishaw *et al.* 2014). Within the Pacific oysters, *Psychrilyobacter* dominated at 44% of the bacterial community, although its role was not determined (Fernandez-Piquer *et al.* 2012).

The stable bacterial community of *Rubyspira osteovora*

The discovery and elucidation of the role of symbiotic microorganisms to animal life has emerged as an important area of research, and, as such, there are increasing numbers of described animal–bacterial symbioses in the literature. One must be careful, however, to distinguish a true symbiosis involving two or more organisms from a transient interaction between an animal and the innumerable surrounding microbiota, with which they interact for part or all of their lives. 16S rRNA barcode sequencing of *R. osteovora* digestive tract tissues, including the digestive gland, intestine and stomach, revealed that three genera continuously dominated the gut microbiome over the course of 6 years, from 2004 to 2010. The presence of these microbes could be indicative of a persistent negative infection. However, this is unlikely because the proportions of the microbes do not change over time. The microbiome stability between tissue types implies that the specific conditions within these tissues are favorable for growth of these specific microbial species.

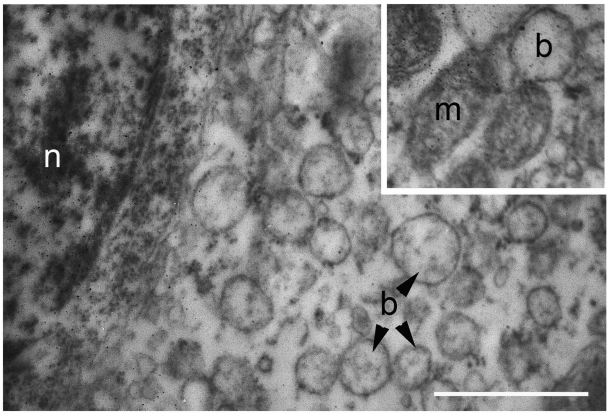

Figure 9. TEM of *R. osteovora* digestive gland, showing bacteria-like objects (b) that are small, intracellular, irregular in shape, without a cell wall, consistent with *Mycoplasma*. n, nucleus. m, mitochondria. Scale bar is 300 nm.

CONCLUSIONS

Overall, this study uncovered a very low-diversity gut microbiome of a gastropod with an unusual nutritional strategy. The discovery of *Rubyspira osteovora*, at two whale falls deep in Monterey Canyon, has revealed more about the exceptional biodiversity in the deep-sea, including the specific assemblage of microbes that associate with animals in these unique ecosystems. Importantly, the microbiome of *R. osteovora* is dominated by microbes not observed in either the environment or within other non-bone-feeding gastropods. This specificity, as well as the temporal stability of the microbiome over 6 years, indicates a microbiome that is exclusive to *R. osteovora*. Very few studies have examined the microbial communities associated with digestive tissues of deep-sea animals. While this study was able to characterize the microbiome of *R. osteovora*, uniquely dominated by *Mycoplasma*, *Psychromonas* and *Psychrilyobacter*, the role of these microbes within the nutrition and life history of *R. osteovora* remains largely speculative. The *Rubyspira* microbiome, in particular *Mycoplasma* and *Psychromonas*, could contribute to digestive functions, but specific digestive supplementation by these microbes has not yet been shown in any study. Future surveys of other deep-sea gastropods in different habitats and within whale falls may reveal new information about the specificity of the microbiome, nutrient supplementation and possibly new microbial host services. By considering the microbial community nested within marine animals, we will surely discover more about the reciprocal influence these organisms have on each other and further increase our understanding of biodiversity in deep-sea ecosystems.

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Conflict of interest. None declared.

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