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Isolation and identification of bacterial endosymbionts in the brooding brittle star Amphipholis squamata

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Isolation and identification of bacterial endosymbionts in the brooding brittle star *Amphipholis squamata*

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Abstract:

Symbiotic associations with subcuticular bacteria (SCB) have been identified and studied in numerous echinoderms, including the SCB of the brooding brittle star, *Amphipholis squamata*. These SCB, however, have not been studied using current next generation sequencing technologies. Previous studies on the SCB of *A. squamata* placed these bacteria in the genus *Vibrio* (γ-*Proteobacteria*), but subsequent studies suggested that the SCB are primarily composed of α-*Proteobacteria*. The present study examines the taxonomic composition of SCB associated with *A. squamata* from the Northwest Atlantic. DNA was extracted using a CTAB protocol and 16S rRNA sequences were amplified using gene-targeted PCR on an Illumina HiSeq at the UNH Genomics Center. Results show the presence of a single dominant bacterial type, within the family *Rhodobacteraceae*, which composes 70-80% of the *A. squamata* microbiome. The majority of sequences recovered from *A. squamata* were identified as members of the genus *Octadecabacter* (97% similarity). By comparison, adjacent seawater and sediment bacterial communities were significantly more diverse, hosting bacteria in the phyla *Proteobacteria, Bacteroidetes, Cyanobacteria, Verrucomicrobia,* and *Actinobacteria.* Thus, a distinct SCB community is clearly evident in *A. squamata*. Here, we hypothesize the potential functions of this symbiotic community, in addition to what may be driving the unique dominance of a member of the family *Rhodobacteraceae.* Although metatranscriptome studies are needed to characterize the functional attributes of the SCB community, we have identified a specific and potentially beneficial symbiont that may support metabolic requirements and nutrient uptake vital to the reproduction of *A. squamata*.

Introduction:

Symbiotic relationships are found throughout all marine ecosystems. In particular, oligotrophic environments such as coral reefs often require symbiotic relationships for adequate nutrition and maintenance of species fitness (e.g., Lesser, 2004). Classic examples of symbioses include the anemonefish, also known as the clownfish, and its host anemone, where the anemone provides a protective structure and toxins for the fish's mucus coating and in return the fish drives off intruders, cleans the anemone of parasites, and provides food scraps to the anemone (Collard, 2016), a second example is the endosymbiotic dinoflagellate (*Symbiodinium* sp.) that forms an obligate mutualism with most scleractinian corals, where each provides nutritional byproducts to the other (Lesser, 2004). Over the past decade, research has demonstrated that most eukaryotes also rely on complex and dynamic symbiotic associations with microbes; the commonly referred to microbiome. Microorganisms often provide critical resources such as protection against pathogenic bacteria or fungi, essential inorganic and organic nutrients, vitamins and amino acids (Fiore et al. 2010, 2015), which are necessary for success in the marine environment. Host-associated microbial symbioses are now widely studied in many habitats, including tropical and temperate marine environments. These studies coupled with novel molecular techniques have revealed the diverse microbial assemblages harbored by many marine invertebrates (de Ridder and Foret, 2001). This study focuses on one group in particular, the Phylum *Echinodermata*, which has garnered significant interest in the study of symbioses.

For some time, the integumentary system of echinoderms has been found to host symbionts of unclear identity and purpose (Féral, 1979), specifically under the outer cuticle of the epidermis (Holland and Nealson, 1978, Lawrence *et al.*, 2010). All echinoderms have a cuticle secreted by epidermal cells, ranging from one to three layers dependent on the species (Holland and Nealson, 1978). The function of the cuticle is to protect the organism and separate it from the surrounding environment, and in some species facilitate nutrient uptake and excretion. Subcuticular bacteria have been found in 50-65% of echinoderms with their presence linked to the metabolism and functions of the host, especially regarding the uptake of nutrients from the marine environment (Burnett and McKenzie, 1997, Kelly *et al.*, 1994, Walker and Lesser, 1989, Lesser and Walker, 1992, de Ridder and Foret, 2001). The phylum *Echinodermata* hosts five classes that all contain SCB, with highest prevalence in the classes *Ophiuroidea* (i.e. brittle stars) and *Echinoidea* (i.e. sea urchins and sand dollars) (Kelly *et al.*, 1994, de Ridder and Foret 2001). Bacteria may function in the early life stages as endosymbionts of larvae of a brooding ophiuroid brittle star, aiding in nutrient uptake from dissolved organic matter (DOM) as described by Fontaine and Chia (1968). Reproduction in *Amphipholis squamata* occurs in bursal sacs which function as both a site for gas exchange and for brooding embryos (Lesser and Blakemore, 1990). The epithelial cells of embryonic *A. squamata* tissues can incorporate amino acids in small amounts (Burnett and McKenzie, 1997, Fontaine and Chia, 1968), but it has been suggested that the amounts are not enough to sustain brooding larvae (Whitehill, 2005), whereas marine bacteria can take up DOM more efficiently (Lesser and Walker, 1992). Input from yolk stores is also likely low, supporting the suggestion that nutrients are acquired from the surrounding environment by bacterial endosymbionts (Walker and Lesser, 1989, Kelly *et al.*, 1994, Burnett and McKenzie, 1997, Whitehill, 2005). Additional studies are needed to define whether SCB are essential to normal embryonic growth and development within *A. squamata*.

Extensive research has described SCB presence and distribution patterns in *A. squamata* from coastal waters in the United States, Scotland, New Zealand, and the British Isles (Lesser and Blakemore, 1990, Lesser and Walker, 1992, Walker and Lesser, 1989, Kelly *et al.*, 1994, McKenzie and Kelly, 1994). The cosmopolitan distribution of *Amphipholis squamata* is unique compared to other benthic marine invertebrates (Sponer and Roy, 2002). For example, 16 genetically similar coastal populations were identified in New Zealand with an apparent North/South split consistent with the islands biogeographical zones (Sponer and Roy, 2002). Populations of *A. squamata* have also been studied in the United States along the coast of New England, where the SCB are morphologically indistinguishable from the SCB of the British Isles populations (McKenzie and Kelly, 1994). Lesser and Blakemore (1990) successfully cultured a single isolate (AS1) from *A. squamata* using Zobell's 2216E agar and described it as a species of *Vibrio* spp. based on multiple phenotypic characteristics while subsequent attempts to culture SCB from *A. squamata* failed (e.g., McKenzie and Kelly, 1994). The SCB of *A. squamata* from the coast of New Hampshire, USA were described as Gram-negative, facultative anaerobes with a hypothesized role in nutrient uptake (Lesser and Blakemore, 1990, Burnett and McKenzie, 1997). The SCB were described by Lesser and Blakemore (1990) using structural, biochemical, and immunological characteristics, but no molecular sequencing information was available at the time. Thus, additional genetic information is required to further clarify the taxonomic status of these isolates (Burnett and McKenzie, 1997). Another study examined Northeast Atlantic populations of brittle stars and found that 17 of the 19 species sampled contained SCB (Kelly *et al.*, 1994). Light and electron microscopy identified morphological consistency within the genera found in the SCB, which suggested the potential for coevolution between the SCB and their hosts (McKenzie and Kelly, 1994). The observed bacteria were long rods (2-6 um) with vacuoles and "holes" (McKenzie and Kelly, 1994), consistent with the hypothesis that these bacteria were also affiliated with *Vibrio* spp. Morphologically similar SCB consisting of long rods (3-4 um), as well as

shorter rods (2 um), often forming pairs of two, were identified in New Zealand populations (Kelly *et al.*, 1994).

The SCB has only been examined using molecular approaches in one other ophiuroid, *Ophiactis balli,* in which *α-Proteobacteria* were identified using 16S rRNA phylogenetic analyses (Burnett and McKenzie, 1997). The authors noted that the *α-Proteobacteria* found in *O. balli* were related to nitrogen-fixing symbionts of plants and plant pathogens (Burnett and McKenzie, 1997). Here, we examine the SCB of *A. squamata* (Figure 1), as well as the surrounding sediments and seawater, to identify the symbionts of this cosmopolitan brittle star from coastal New Hampshire using Illumina based 16S rRNA sequencing. Results are used to develop hypotheses regarding the functions of the SCB and their potential roles as symbionts. The present study also suggests the presence of a dominant symbiont taxon within the SCB, as was previously described (Lesser and Blakemore, 1990). However, the new identification based on the 16S rRNA gene places the bacterium within the family *Rhodobacteraceae* and genus *Octadecabacter* within the class *α-Proteobacteria*, not as members of the genus *Vibrio* (class γ-*Proteobacteria*).

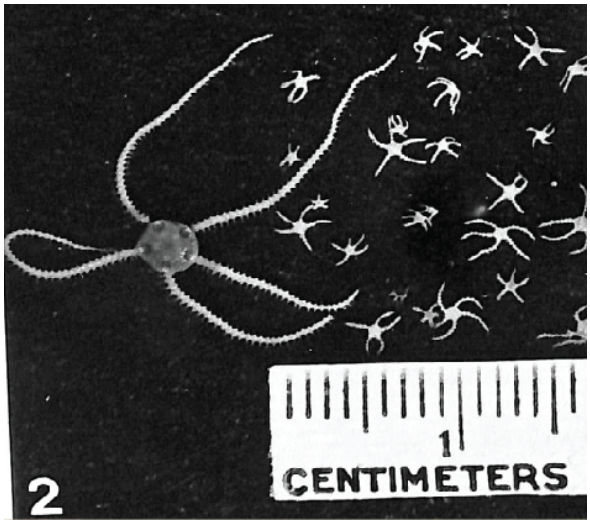


Figure 1: *A. squamata* with brooded offspring removed (Lesser and Blakemore, 1990)

Figure 2: *A. squamata* collection site, Rye, NH at low tide

Materials and Methods:

Sample collection and preservation

Samples of the brittle stars *Amphipholis squamata* (n=6), sediment (n=3) and seawater (n =3) were collected in September of 2014 north of the coastal New Hampshire seacoast science center in Rye, NH. *A. squamata* were collected with forceps under emergent rocks in tidal pools (Figure 2), rinsed, and stored in 25 mL DNA preservation buffer (0.25M Ethylenediaminetetraacetic acid [EDTA; pH 7.5], 20% dimethylsulfoxide [DMSO] in 50 mL Falcon tubes, and saturated sodium chloride [NaCl]; Seutin et al. 1991) at -20°C until later DNA isolation. Sediment and seawater were collected in 50 mL Falcon tubes and were kept on ice until returning to the laboratory at UNH where they were refrigerated until processing. Upon return to the laboratory, 25 ml of the seawater and pore water from the sediment samples were filtered onto GFF filters ready for DNA extraction and stored in DNA preservation buffer.

DNA isolation and processing

Genomic DNA was isolated from *A. squamata*, seawater and sediments using a 2X CTAB (1.21 g Tris, 8.18 g NaCl, 0.744 g EDTA, and 2 g CTAB per 100 mL of DI water) protocol (Fiore, 2013). In a laminar flow hood, the volume of CTAB required for all samples was mixed on a stir plate at level three and 2 µL of 2-mercaptoethanol were added for each mL of CTAB. The samples were homogenized with a sterile plastic pestle and put into 1.5 mL microcentrifuge tubes, followed by the addition of 600 uL CTAB, and 5 µL of Proteinase K (20 mg/mL, freshly prepared). Tubes were briefly mixed by vortex at level 7 and incubated on a heat block at 65°C for 3 hrs. After incubation, an equal volume (600 uL) of chloroform was added to each tube (carefully, in hood) and inverted to mix several times. With hinges facing outwards, the samples were then centrifuged at 12,000 rpm for 10 min. The top aqueous layer was removed, without touching the second layer, and put into a new 1.5 mL tube. One mL of ethyl alcohol (EtOH 100%) was added to each new tube and inverted to mix. The samples were then centrifuged at 12,000 rpm for ten minutes. Following centrifugation, the liquid was decanted without disturbing the pellet. DNA was washed with one mL of cold 70% EtOH, briefly vortexed to resuspend the pellet, and centrifuged at 12,000 rpm for 10 minutes. A second wash was performed following the same protocol. Supernatant was decanted without disturbing the pellet. DNA was dried on a SpeedVac (Thermo Scientific, Waltham, MA) at medium heat for 10 minutes to evaporate excess EtOH. The DNA pellet was eluted in 50 µL of sterile molecular-grade water and vortexed briefly to mix. Genomic DNA (gDNA) concentration and quality was evaluated with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA).

16S rRNA PCR amplification and Sequencing

Samples were checked for amplification with universal primers designed to amplify Bacteria and Archaea consisting of the forward primer 8F (AGA GTT TGA TCC TGG CTC AG) and reverse primer 1492R (CGG TTA CCT TGT TAC GAC TT). Tips, tubes, and nuclease-free water were UV-sterilized with the micropipettes prior to PCR set-up. Template DNA and PCR reagents were thawed on ice. PCR reactions included 20.25 µL nuclease-free H_2O, 2.5 µL 10x Titanium Taq Buffer, 0.5 µL dNTPs (0.2 mM) (Promega, Madison, WI), 1.0 µL of each primer (8F, 1492R, concentration 10µM/L), 0.25 µL of 50X Titanium Taq Polymerase (Clontech, Mountain View, CA), and 1.0 µL of template DNA. Reactions were performed with a Thermocycler (Eppendorf Mastercycler, Wesseling-Berzdorf, Germany) using the following program: initial denaturation for 5 min at 95°C, followed by 25 cycles of 95°C for 30 sec, 53°C for 30 sec, and 72°C for 45 sec, followed by a final elongation step at 72°C for 6 min and indefinite hold at 14°C until removal of samples. PCR products were then electrophoresed on a 1% agarose gel with ethidium bromide and *Bioline Hyperladder II*. Genomic DNA from a total of 12 samples of genomic DNA which generated a band at the appropriate product length (~1400 bp) were sent to the University of New Hampshire Genomics Center for Illumina sequencing using the prokaryote-specific primers, 515F (GTG CCA GCM GCC GCG GTA A) and 806R (GGA CTA CHV GGG TWT CTA AT), which amplify the V4 hypervariable region and broadly target both Bacteria and Archaea (Caporaso et al. 2010).

Sequence Processing and Statistical Analysis

Starting with demultiplexed .fastq files with barcodes removed, the forward and reverse reads were paired using the Quantitative Insights Into Microbial Ecology (QIIME; version 1.9.1) script 'multiple_join_paired_ends.py' with a min_overlap of 20 base pairs (bp) and max_diff of 25 bp, followed by 'multiple_split_libraries_fastq.py' with a phred quality threshold of 19 or better. The raw reads were submitted to the NCBI Sequence Read Archive.

An operational taxonomic unit (OTU) table was generated in QIIME by passing the script 'pick_open_reference_otus.py' against the 'gg_13_8_otus' greengenes database at 97% identity using default settings. Core alpha and beta diversity analyses were conducted using 'core_diversity_analyses.py' and the core microbiome community members were examined with 'compute_core_microbiome.py' at a 50% cut-off value. From the OTU table, total relative abundance counts were converted to ratios by dividing by the total number of reads to adjust for variability between samples. Bray-Curtis distance matrices were built to examine additional patterns of community structure and visualized using principal coordinate analyses (PCoA). Pearson correlation vectors were overlayed to demonstrate which taxa have strong positive or negative correlations with either PCO axis, indicative of sample type differences (brittle star, seawater, sediment). A permutational analysis of variance (PERMANOVA, 9999 permutations) determined whether spatial separation between sample types was statistically significant. All multidimensional statistical analyses were performed in PRIMER V7 with the PERMANOVA+ add-on (PRIMER-E Ltd., Devon, UK).

***Sequencing of the Original Bacterial Symbiont* (AS1)**

The bacterial isolate from *A. squamata* denoted as AS1 (Lesser and Blakemore, 1990) was obtained from -80 F stocks of log phase cultures in 2216E medium plus 50% glycerol. Three glycerol stock bacteria samples were diluted ten fold three times, spread plated on marine broth agar (2216E), and incubated at room temperature for 48 hours. Translucent colonies resembling the descriptions of the original isolate were observed and three individual colonies were streaked for isolation on marine agar plates and incubated at room temperature for 48 hour. Ten isolated translucent colonies were collected and inoculated into 4 mL of liquid marine broth and incubated at room temperature for 48 hours. Three mL of 48-hr culture were pelleted at 16,000 xg and the marine broth decanted. DNA from the cell pellet was isolated using the MOBIO PowerSoil DNA Isolation kit (MOBIO laboratories, Inc. Carlsbad, CA) following manufacturers instructions. Genomic DNA (gDNA) concentration and quality was evaluated with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) and 10 ul was sent to Genewiz (South Plainfield, NJ) for sequencing using the 16S rRNA gene primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT). Forward and reverse sequences were quality trimmed and assembled using Geneious R9 (Biomatters ltd.). Assembled sequences were blasted against the NCBI nucleotide collection (nr/nt) and optimized for highly similar sequences (megablast). A FASTA file was generated with the top 100 hits (> 95% identity) and a tree was constructed using Randomized Axelerated Maximum Likelihood (RAxML) with bipartitions and 99 bootstrap support. The resulting tree was visualized using FigTree v1.4.2.

Results:

Microbial community diversity was assessed for brittle stars (N=6), seawater (N=3) and sediment/pore water (N=3) communities using 16S rRNA gene amplicon sequencing. A total of 1,434,612 high-quality 16S rRNA gene Illumina tagged reads were recovered from all samples, with an average of 119,551 ± 47,141 SD reads per sample (Figure 3). The mean sequence length for all 12 samples was 253 bp ± 1.4 (mean +/- SD). The *Amphipholis squamata* SCB was represented by an average of 8.5 OTUs ± 1.9 SD and demonstrated a considerably lower Shannon diversity (H' = 1.3 ± 0.3) than either seawater (H' = 5.2 ± 4.1) or adjacent sediments (H' = 6.7 ± 2.0).

Alpha Diversity Statistics (Average ± SD)

Sample Type	n	Trimmed Seqs	Unique OTUs	Chao1	Observed	Shannon *H'*
Brittle Stars	**6**	77483 ± 8479	8.5 ± 1.9	127 ± 38	99 ± 25	1.3 ± 0.3
Seawater	**3**	173891 ± 28719	597 ± 19.3	11685 ± 1744	7827 ± 893	5.2 ± 4.1
Sediments	**3**	149347 ± 12149	587 ± 12.1	11975 ± 263	7528 ± 281	6.7 ± 2.0

Figure 3: Statistical data on sequencing results

Eighteen total OTUs were obtained from the six brittle star samples and communities were found to be 81% similar (SIMPER analysis; Figure 4). Overall, the majority of the sequences recovered from the *A. squamata* SCB were represented by a single dominant bacterial species identified as a member of the genus *Octadecabacter* (class α-*Proteobacteria,* family *Rhodobacteraceae*), which composed 78% ± 4.5 (mean ± SD) of the SCB microbiome (Figure 5). Additionally, bacterial isolates cultured and sequenced from the *A. squamata* SCB (sample AS1; Lesser and Blakemore, 1990) were all a > 98% match for the genus *Octadecabacter spp.* (Genbank accession # FJ889525.1) found in marine sediments of the Artic Ocean. A phylogenetic tree was constructed representing the top NCBI blastn hits related to the bacterial cultivar isolate suggesting that *Octadecabacter* is indeed the dominant endosymbiont found within the *A. squamata* SCB (Figure 6). Other bacterial taxa that represented > 1% of the SCB included sequences related to the phyla *Firmicutes* (*Bacillus* spp.) and *Proteobacteria* (*Marinobacter* spp; Figure 5). Additional sequences that represented < 1% of the SCB microbiome were affiliated with the phyla *Proteobacteria*, *Bacteriodetes*, *Firmicutes*, *Actinobacteria*, and *Acidobacteria* (Figure 5).

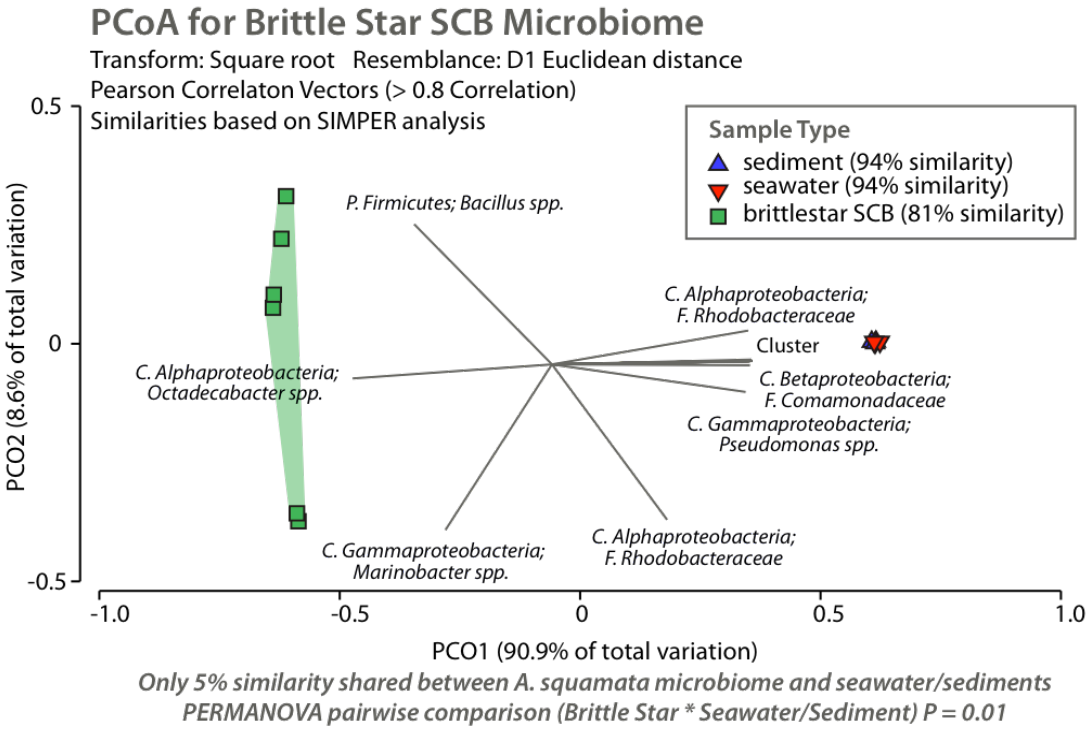


Figure 4: Principle coordinate analysis of the Subcuticular bacteria of *A. squamata* and environmental samples

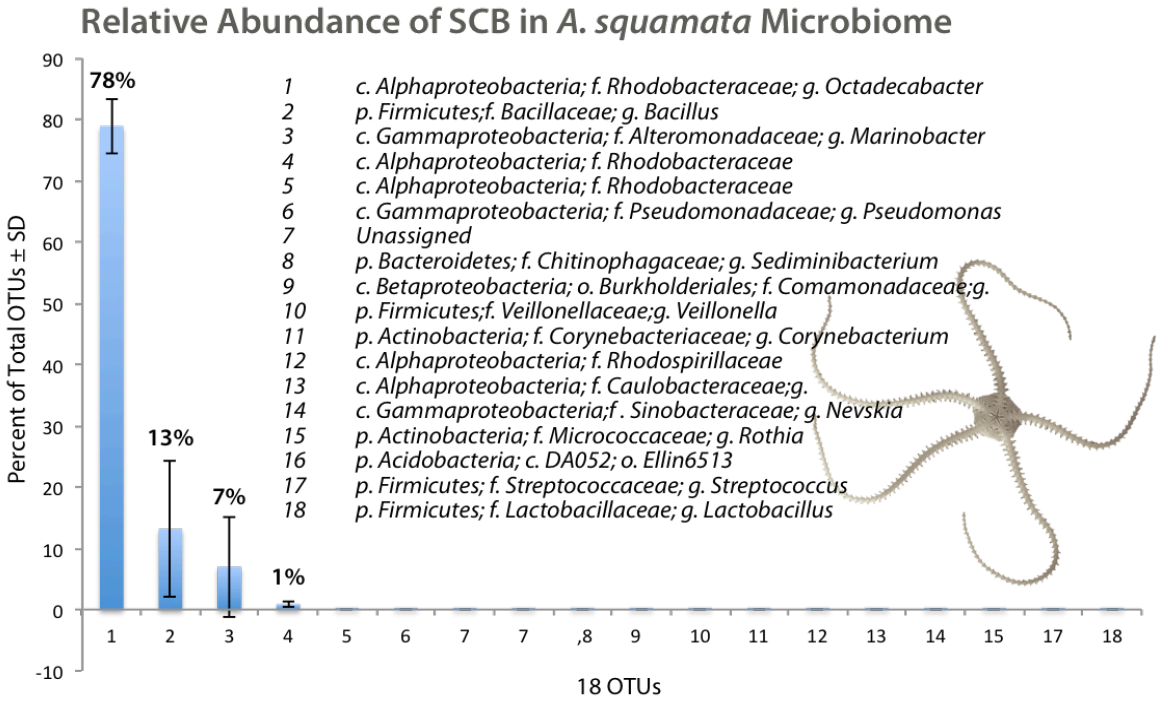


Figure 5: Top eighteen operational taxonomic units (OTUs) identified from the microbiome of *A. squamata*

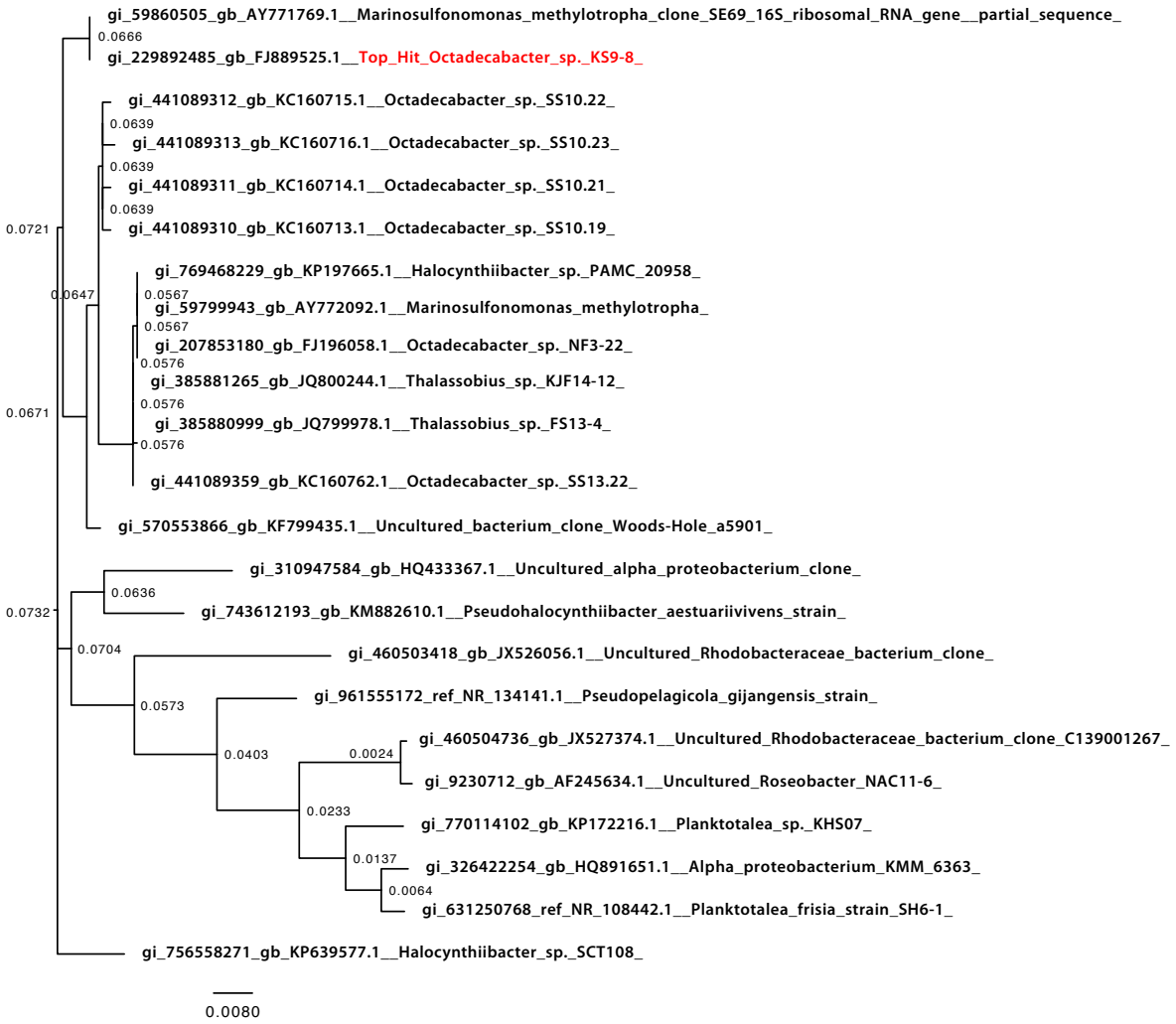
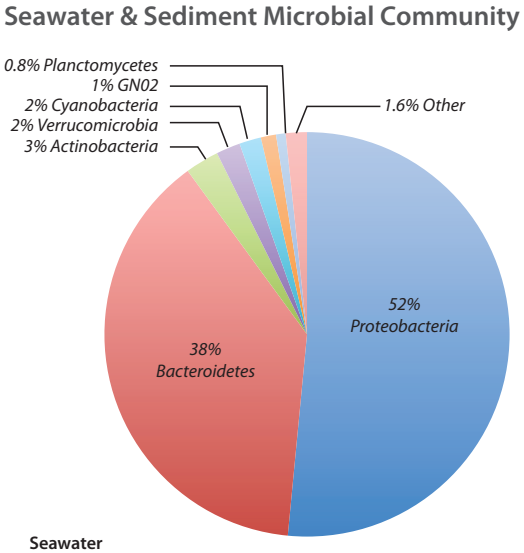


Figure 6: Phylogenetic tree of top NCBI Blast hits related *A. squamata* isolates

The adjacent seawater and sediment pore water bacterial communities were significantly more diverse, hosting a number of unique OTUs with an average of 597 OTUs ± 19.3 SD in the seawater and 587 OTUs ± 12.1 SD in the sediments (Figure 3). Bacteria affiliated with the phyla *Proteobacteria*, *Bacteriodetes*, *Cyanobacteria*, *Verrucomicrobia*, and *Actinobacteria* were recovered (Figure 7). However, sequences affiliated with *Octadecabacter* spp. represented less than 0.5% of all recovered sequences in both the seawater and sediment communities. The top 10 OTUs represented within the seawater and sediment communities were similar, but their order of prominence varied. Both the seawater and sediment communities were significantly different from the brittle star communities with only 5% similarity.



Seawater

Top 10 OTUs	%	Taxonomy
OTU140	*16.3*	*p. Bacteroidetes; f. Flavobacteriaceae; g. Polaribacter*
OTU580	*10.5*	*c. Gammaproteobacteria; f. Colwelliaceae*
OTU120	*6.5*	*p. Bacteroidetes; f. Flavobacteriaceae*
OTU113	*4.3*	*p. Bacteroidetes; f. Cryomorphaceae*
OTU408	*4.3*	*c. Alphaproteobacteria; f. Rhodobacteraceae*
OTU413	*4.2*	*c. Alphaproteobacteria; f. Rhodobacteraceae; g. Octadecabacter*
OTU172	*2.5*	*p. Bacteroidetes; f. Saprospiraceae*
OTU644	*2.5*	*c. Gammaproteobacteria; f. Oceanospirillaceae; g. Marinomonas*
OTU24	*2.1*	*p. Actinobacteria; c. Acidimicrobiia; f. OCS155;g.*
OTU690	*1.9*	*c. Gammaproteobacteria; f. Vibrionaceae*

Sediments

Top 10 OTUs	%	Taxonomy
OTU140	*16.7*	*p. Bacteroidetes; f. Flavobacteriaceae;g. Polaribacter*
OTU580	*10.5*	*c. Gammaproteobacteria; f. Colwelliaceae*
OTU120	*6.5*	*p. Bacteroidetes; f. Flavobacteriaceae*
OTU413	*4.3*	*c. Alphaproteobacteria; f. Rhodobacteraceae; g. Octadecabacter*
OTU113	*4.2*	*p. Bacteroidetes; f. Cryomorphaceae*
OTU408	*4.3*	*c. Alphaproteobacteria; f. Rhodobacteraceae*
OTU644	*2.6*	*c. Gammaproteobacteria; f. Oceanospirillaceae; g. Marinomonas*
OTU172	*2.4*	*p. Bacteroidetes; f. Saprospiraceae*
OTU590	*2.0*	*c. Gammaproteobacteria; o. Alteromonadales; f. OM60*
OTU24	*2.1*	*p. Actinobacteria; c. Acidimicrobiia; f. OCS155;g.*

Figure 7: Top ten OTUs identified from the surrounding sediments and seawater of *A. squamata*

Discussion:

The SCB of *Amphipholis squamata* is dominated by the α-*Proteobacteria* family *Rhodobacteraceae* by a member of the genus *Octadecabacter* spp., which composes 70-80% of the *Ampipholis squamata* microbiome. The similarity of this *in hospite* bacterium was confirmed by the molecular identification of the original isolate (i.e., AS1) described in Lesser and Blakemore (1990) as *Octadecabacter* spp. using 16S rRNA clone sequencing.

Adjacent seawater and sediment bacterial communities were significantly more diverse, hosting bacteria in the phyla *Proteobacteria, Bacteroidetes, Cyanobacteria, Verrucomicrobia,* and *Actinobacteria.* Thus, *A. squamata* SCB communities are distinct and significantly differ from the surrounding environment (Figure 4). Dominant SCB in *A. squamata* including *Octadecabacter* spp., *Marinobacter* spp., and *Bacillus* spp. are all found in low numbers in the seawater and sediment. Thus, it is possible that *A. squamata* sources their symbionts from the surrounding environment.

Members of the genus *Octadecabacter* are known to produce the antimicrobial compound octadecanoic acid (Gosink *et. al*, 1997, Rahman *et. al*, 2014), which may function in preventing other bacteria from becoming significant components of the *A. squamata* microbiome. The subcuticular space of *A. squamata* provides an environment where *Octadecabacter* spp. can grow and divide, supporting the hypothesis of a specific symbiotic relationship. The mechanism by which bacteria are transmitted from the parent to the brooded embryos is still largely unknown. Possibilities include vertical transmission from parent to larvae during the brooding period, or horizontal transmission whereas offspring acquire their symbionts from the environment after release (Walker and Lesser, 1989).

Another potential role *Octadecabacter* sp. may play as endosymionts is in increasing metabolic and nutritional efficiency, including the uptake of DOM and free amino acids for the brooding embryo as originally described in Walker and Lesser (1989). Known species of *Octadecabacter* include *Octadecabacter arcticus*, *Octadecabacter antarcticus* (Gosink *et. al*, 1997), and *Octadecabacter temperatus* (Billerbeck *et. al*, 2015). These bacteria are heterotrophic, rod-shaped, and produce white to beige (Billerbeck *et. al*, 2015), circular colonies (Gosink et. *al*, 1997), as was observed in our cultures from Lesser and Blakemore (1990). Both *O. arcticus* and *O. antarcticus* genomes contain genes important in nitrogen metabolism and the production of cyanate hydratases (Volmers *et. al* 2013). Genes responsible for cyanophycin degradation and synthesis are found in *O. arcticus*, and genes for the assimilation pathways of nitrite and nitrate are found in *O. antarcticus* (Volmers *et al.* 2013). Both *O. arcticus* and *O. antarcticus* are from the Arctic and Antarctic respectively, but *O. temperatus* was isolated from surface water of the southern North Sea (Atlantic ocean, Billerbeck *et. al* 2015) and Walker and Lesser (1989) isolated AS1 from the coast of New Hampshire.

In future studies, the genome of the most prominent *Octadecabacter* spp. found within the SCB of *A. squamata* should be sequenced and compared to other known species of *Octadecabacter* in order to identify a novel species or novel habitat of the known species. Identifying genes related to nitrogen or other nutrient assimilation and metabolism is essential for identifying the nature of the relationship between *Octadecabacter* spp. and *A. squamata*. Additional experiments to study any natural antimicrobial properties of the SCB would aid in further characterizing the functions of the symbiotic relationship.

Future research would also benefit from metagenomic and metatranscriptomic approaches to quantify the functional attributes and specific roles the SCB plays as symbionts. However, based on these and other studies we hypothesize that the relationship is likely related to nutrient assimilation and metabolism, particularly nitrogen, for brooding offspring. The host, *A. squamata*, likely also provides a protective environment for *Octadecabacter* spp. to thrive in numbers much higher than seawater and sediments

allow, while *Octadecabacter* spp. assimilates nutrients from the environment to nourish developing offspring. A protective environment for *Octadecabacter* spp. and nutrients for early life stages are both necessary for proliferation of the individual populations, so a mutualistic relationship may have developed over time to sustain both organisms. Further research is needed to more definitively describe mechanisms of SCB integration, selective propagation of *Octadecabacter* spp., and the exact functions of the relationship of *A. squamata* individuals and its symbiotic bacteria.

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