Microscopic and Genetic Characterization of Bacterial Symbionts With Bioluminescent Potential in Pyrosoma atlanticum

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Microscopic and Genetic Characterization of Bacterial Symbionts With Bioluminescent Potential in *Pyrosoma atlanticum*

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The pelagic tunicate pyrosome, *Pyrosoma atlanticum*, is known for its brilliant bioluminescence, but the mechanism causing this bioluminescence has not been fully characterized. This study identifies the bacterial bioluminescent symbionts of *P. atlanticum* collected in the northern Gulf of Mexico using several methods such as light and electron microscopy, as well as molecular genetics. The bacteria are localized within the pyrosome light organs. Greater than 50% of the bacterial taxa present in the tunicate samples were the bioluminescent symbiotic bacteria Vibrionaceae as determined by utilizing current molecular genetics methodologies. A total of 396K MiSeq16S rRNA reads provided total pyrosome microbiome profiles to determine bacterial symbiont taxonomy. After comparing with the Silva rRNA database, a *Photobacterium* sp. r33-like bacterium (which we refer to as “*Photobacterium* Pa-1”) matched at 99% sequence identity as the most abundant bacteria within *Pyrosoma atlanticum* samples. Specifically designed 16S rRNA V4 probes for fluorescence in situ hybridization (FISH) verified the *Photobacterium* Pa-1 location as internally concentrated along the periphery of each dual pyrosome luminous organ. While searching for bacterial *lux* genes in two tunicate samples, we also serendipitously generated a draft tunicate mitochondrial genome that can be used for *Pyrosoma atlanticum* identification. Scanning (SEM) and transmission (TEM) electron microscopy confirmed the presence of intracellular rod-like bacteria in the light organs. This intracellular localization of bacteria may represent bacteriocyte formation reminiscent of other invertebrates.

**Keywords:** symbiosis, bioluminescence, microscopy, high throughput sequencing, 16S rRNA, tunicate

**INTRODUCTION**

Bioluminescence is an important adaptive trait in ocean dwelling taxa that appears to be more prevalent than originally thought (Martini and Haddock, 2017). Over 700 animal genera are known to include luminous species, with more than 80% being marine organisms (Widder, 2010). Within this group, 90% of pelagic organisms that live between 200 and 1000 m are known to have bioluminescent capabilities. In addition, fishes, squid, and shrimp are able to modify aspects of their...
light production, such as the intensity, kinetics, wavelength, and angular distribution. The emission of light by organisms has evolved independently over 40 times in marine and terrestrial organisms (Haddock et al., 2010). This emphasizes the evolutionary importance of the bioluminescence mechanism. There are several critical ways bioluminescence can aid an organism’s survival, such as facilitating food location and capture, attracting mates, allowing for species recognition, and functioning as a defense mechanism (Widder, 2010).

Pyrosomes derive their name from the Greek words pyro (“fire”) and soma (“body”) from the “fiery” bioluminescence that is so visible at night (Sutherland et al., 2018). Pyrosomes were classified by Lamarck and Huxley under the subphylum Tunicata (previously known as Urochordata) due to the zooids, which compose these organisms, being encased by a tunic (Huxley, 1851; Lemaire and Piette, 2015). Pyrosomes are approximately 95% water and are extremely well adapted for rapid growth and efficient energy use. Transparency makes pyrosomes difficult to see at any depth, which is why they can be found throughout the pelagic realm. Aside from being transparent and of limited nutritional value, pyrosomes have few sensory or predator-avoidance adaptations. Most biological processes, such as feeding, respiration, and swimming occur simultaneously through contraction of the same muscles (Allredge and Madin, 1982).

Pyrosomes, in the class Thaliacea, remain one of the least studied planktonic grazers, despite their widespread distribution and ecological significance. Pyrosomes are characterized as highly successful planktonic grazers, and swarms of colonies can consume substantial amounts of phytoplankton (Alldredge and Bone, 1978; Haygood, 1993), but this has never been verified. It has also been hypothesized that symbionts in the dual light organs of each Pyrosoma atlanticum zooid are intracellular and thus will likely have undergone some metabolic integration with host cells (Mackie and Bone, 1978; Holland, 2016). Mackie and Bone based this statement on the fact that luminous bacteria glow continuously, and fishes that utilize luminous bacterial symbionts possess shutters to block the light. Pyrosoma appears able to directly control the light emissions from their putative bacterial symbionts by an as yet unidentified mechanism, but very likely involving the supply of an essential metabolite. However, since these symbionts have not been successfully isolated and cultivated, their role in pyrosome bioluminescence remains unconfirmed. The goal of the present study is to determine if the bioluminescence of Pyrosoma atlanticum, is from bacterial origin by identifying the location, anatomy, and classification of its symbiotic bacteria.

**MATERIALS AND METHODS**

**Sample Collection and Fixation**

Tunicate samples were collected on cruises conducted by the Deep Pelagic Nekton Dynamics of the Gulf of Mexico (DEEPEND) consortium (Sutton et al., 2020). In 2017, midwater trawling was conducted on DEEPEND Cruise DP05, during which fishes, crustaceans, cephalopods, large gelatinous zooplankton, and other pelagic taxa were collected from the Gulf of Mexico, including Pyrosoma atlanticum. On shipboard, specimens were frozen or stored in dimethyl sulfoxide (DMSO), then transferred and stored in the Microbiology and Genetics Laboratory at Nova Southeastern University. Twenty-nine additional samples were collected from the Gulf of Mexico on the July 2018 DEEPEND Cruise DP06 and stored in DMSO or RNALater. Samples from both cruises were collected from depths of 0 to 1,500 m at multiple collection sites. Of the samples stored in RNALater, two samples were utilized for genetic sequencing (Table 1). In 2019, 12 additional P. atlanticum samples were collected in the Gulf of Mexico during a NOAA Ocean Exploration and Research-funded cruise aboard the R/V Point Sur (Supplementary Figure 1). Water samples used in comparative analysis were collected from the DEEPEND cruise DP05 using Niskin bottles deployed on a CTD (Baker et al., 2019; Easson and Lopez, 2019; Freed et al., 2019). These samples were taken at the same time and location as the DMSO samples were collected. Water samples were not collected for both 2019 cruises for this study.

**Light and Fluorescence Microscopy (Histology)**

Samples were fixed for light microscopy in 2% glutaraldehyde with a sodium cacodylate-buffered seawater fixative, washed with a buffer rinse, placed in 70% EtOH overnight, processed through a graded series of ethanol, cleared, and infiltrated with molten Paraplast Plus®, and embedded in Paraplast Xtra®. Using a Leica RM 2125 microtome, 4 μm thick sections were cut, mounted

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[1] www.deependconsortium.org
TABLE 1 | Collection data for the 15 samples used in this study.

| Sample ID | Sample type | Counts | Cruise designation | Sample date | StartLat | StartLon | Trawl depth (m) | Protocol Used |
|-----------|-------------|--------|-------------------|-------------|----------|----------|----------------|----------------|
| DP0625UL18-MOC10-1208.40 | RNAlater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1209.40 | RNALater | 1 | - | 26-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1506 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1210.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1211.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1212.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1213.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1214.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1215.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1216.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1217.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1218.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1219.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1220.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1221.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1222.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1223.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1224.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |
| DP0625UL18-MOC10-1225.40 | RNALater | 1 | - | 25-Jul-18 | 27.79°32.76° N | 88.30°36.61° W | 0–1503 | 16S Sequencing and Genome sequencing |

Scanning and Transmission Electron Microscopy

Samples utilized for SEM were stored in a 2% glutaraldehyde with a sodium cacodylate-buffered seawater fixative. Pyrosomes were dissected in the fixative and divided into three sections per sample. They were rinsed three times in sodium cacodylate-buffered seawater, postfixed in 1% osmium tetroxide, rinsed in the buffer, dehydrated through a graded series of ethanol (20, 50, 70, 95, and 100%), and dried in hexamethyldisilazane (HMDS). Dried samples were outgassed overnight, coated with palladium in a sputter coater, and examined in a Philips XL-30 Field Emission SEM at the University of Miami Center for Microscopy (UMCAM) located in the Chemistry Department at the University of Miami Coral Gables Campus.

TEM samples were prepared similarly as for SEM, except that samples at the last dehydration step (100% EtOH) were embedded in Spurr resin and polymerized for 3 days at 60°C. Blocks were trimmed, sectioned, sections were floated onto grids, stained with either Uranyl Acetate and/or Lead Citrate and examined in a JEOL 1,400X TEM located at the University of Miami Miller School of Medicine TEM Core Lab. Semi-thin sections of TEM prepared samples were examined in an IX-70 fluorescent microscope to examine gross structures.

DNA Extraction and Polymerase Chain Reaction (PCR)

Total microbiome DNAs were extracted with the standard protocol for the Qigen PowerLyzer PowerSoil kit. Whole body slices of the pyrosome with at least two or three zooids included in the section were used for extraction. This study focused on amplifying the 16S gene in order to identify luminescent bacteria the pyrosome. Once DNA extractions were completed, polymerase chain reactions (PCR) were run using Invitrogen Platinum Hot Start PCR Master Mix (2×) and the universal primers 515F and 806R. Each sample was given a unique 806R primer in order to identify and separate the samples during the post run analysis. The 515F and 806R primers were used to amplify the 200 bp sequence of the V3 and V4 region of the 16S gene (Caporaso et al., 2011; Easson and Lopez, 2019). The V3 and V4 region are two of nine hypervariable regions (V1-V9) found within the 16S gene on microscope slides, and stained with Harris’s hematoxylin and eosin. Slides were examined using an Olympus BX43 light microscope at 4–60 × magnification. Fluorescence microscopy was performed on an Olympus IX70 Fluorescence Microscope with green (500–570 nm) and red (610 ~750 nm) filter cubes. Bacteriocytes were counted on light micrographs of the histology sections. We define bacteriocytes as membrane bound structures that surround more than one individual bacterium (Sen et al., 2018). A structure was considered a bacteriocyte if it was a dark cluster of multiple bacteria about 5 µm in size with a discernable membrane within the interior of the light organ. Additional estimation of the quantity of bacteria within the light organ was determined on TEM and SEM micrographs.
(Gray et al., 1984). The V4 region provides the lowest error rates for identification (Wang et al., 2007). The PCR products were cleaned using AMPure XP beads. This process was used to purify the 16S V3 and V4 amplicon away from free primers and primer dimer species (Chakravorty et al., 2007). The final DNA concentration was checked using a Qubit 2.0 fluorometer.

Illumina High-Throughput 16S Sequencing and Analysis

The 16S rRNA gene fragment was the target for bacterial identification via high throughput DNA sequencing (O’Connell et al., 2018; Easson and Lopez, 2019). Samples were prepared for sequencing following the 16S Illumina Amplicon Protocol per the Earth Microbiome Project (Kuczynski et al., 2011; Thompson et al., 2017). The final DNA concentrations from the PCR extractions were used to dilute each sample to a normalization of 4 pM. All DNA samples were library pooled and rechecked. DNA concentration was checked using a Qubit 2.0 fluorometer to make sure the concentration was at 4 pM. All DNA samples were library pooled and rechecked. Extractions were used to dilute each sample to a normalization of 4 pM. All DNA samples were library pooled and rechecked.

The 16S metagenomics DNA at 500 cycles using V2 chemistry. The final product was loaded into an Illumina MiSeq system for identification via high throughput DNA sequencing (O’Connell et al., 2017). The final DNA concentrations from the PCR extractions were used to dilute each sample to a normalization of 4 pM. All DNA samples were library pooled and rechecked.

The Quantitative Insights into Microbial Ecology v.2 (QIIME2) pipeline was used to demultiplex, quality filter, assign taxonomy, reconstruct phylogeny, and produce diversity analysis and visualizations from the FASTQ DNA sequence files (Caporaso et al., 2010). The quality filtering and trimming of the data was conducted in DADA2, which was used to create a feature table that was analyzed in RStudio. The QIIME2-generated sequences were assigned taxonomy through a learned k-mer based approach to metagenomics. FASTQ files were uploaded to CosmosID®, a bioinformatic pipeline used for microbial analysis that employs a phylogenetic and k-mer based approach to metagenomics. FASTQ files were uploaded to CosmosID®, a bioinformatic pipeline used for microbial analysis that employs a phylogenetic and k-mer based approach to metagenomics. FASTQ files were uploaded to CosmosID®, which provided relative abundance of the microbial community described in a heatmap comparison. Further data analysis used 16S rRNA multiple sequence alignments with MAFFT (Katoh and Standley, 2013), in order to generate a phylogeny to compare the extracted 16S sequence from the MiSeq run with known luminescent bacterial species. Pyrosome microbiome sequences have been deposited to the NCBI Sequence Read Archive (PRJNA636187).

Fluorescence in situ Hybridization (FISH)

*Pyrosoma atlanticum* samples were stored in paraformaldehyde and dehydrated through an ethanol series, cleared in xylene and infiltrated with paraffin. Serial sections were cut at 4 and 8 µm, mounted on slides, and deparaffinized with xylene and ethanol series (100–70%). Specific probes were then added to localize the bacteria within the light organs. Optimal probes sequences were designed using MAFFT alignment (Katoh and Standley, 2013) of the new tunicate 16S rRNA sequences obtained from our Illumina MiSeq run, with previously determined 16S sequences from various bacterial species from NCBI database (DQ889917, DQ889916, DQ889915, DQ889914, DQ889913).

The 16S rRNA sequences from samples previously mentioned were aligned using MAFFT. The sequence we had generated, TCTAGGTTGAGCCGTTGAAATGC, was chosen because it appeared to match closest to *Photobacterium* sequences and was also the most variable part of the V4 region alignment. This also indicated that it would be unique with less similarity to other sequences in the fixed tissues. The high specificity was required to specifically detect the *Photobacterium* in FISH prepared slides. The probes were then tested on the NCBI PROBE Database2 and Microbial Ribosomal Databases Probe Match3 (Negandhi et al., 2010).

Labeled probes for FISH were manufactured by IDT Inc. (Iowa, United States). The dye used for the *Photobacterium*-specific probe was Cy3, which is a standard orange-fluorescent label for nucleic acids and was attached at the 5′ end (Table 2).

The control probe EUB338 is a universal bacteria probe and was dyed with 6-FAM (fluorescein) (Negandhi et al., 2010). FAM is the most commonly used fluorescent dye attachment for oligonucleotides and this particular dye was attached at the 3′ end and appears green. The probes attach to one end or the other to allow for overlap. This is possible because the two probes’ nucleotide sequences are at different location on the ribosome (either the 5′ or 3′ end). When imaging the samples, only orange and green fluorescence should appear, and red fluorescence should be excluded due to double binding. This means both probes should bind to the targeted *Photobacterium* sp. and present the orange fluorescence while the rest of the bacteria appear green.

FISH protocols utilized here followed previously described methods (Sharpe et al., 2007; Negandhi et al., 2010). FISH hybridization buffer (35% formamide) contained 5 M NaCl, 40 µl 1 M Tris-HCl, 700 µl formamide, 900 µl H2O, and 2 µl 10% SDS, and applied as 45 µl mixed with 5 ng/µl of the desired probe, for a total of 50 µl per slide. Pyrosome tissues were then incubated inside a humidity chamber with a paper towel that was saturated.

### TABLE 2 | FISH probe sequences and dye used to identify *Photobacterium* in the samples.

| Probe     | Sequence with TAG | Base pairs | 5′ or 3′ attachment | Absorbance max | Emission max |
|-----------|-------------------|------------|---------------------|----------------|--------------|
| Photobact | /5Cy3/TCTAGGTTGAGCCGTTGAAATGC | 22         | 5′ End              | 550 nm         | 564 nm       |
| EUB338    | GCTGCCTCCCGTTGAGGAGT/3′-FAM/  | 18         | 3′ End              | 495 nm         | 520 nm       |

2. [www.ncbi.nlm.nih.gov/probe](http://www.ncbi.nlm.nih.gov/probe)
3. [http://rdp.cme.msu.edu/probematch/search.jsp](http://rdp.cme.msu.edu/probematch/search.jsp)
with the hybridization buffer for 2 h at 46°C. After hybridization, slides were put in a buffer wash (700 µl 5 M NaCl, 1 ml 1 M Tris-HCl, 500 µl 0.5 EDTA, 50 ml H2O, and 50 µl 10% SDS) for 20 min at 48°C, then rinsed with dH2O and air dried.

FISH was performed on three samples with two sections each. The control runs utilized probe EUB338. In addition to the control, a slide with no probes as well as slides with both EUB338 and Photobacterium probes were hybridized. This allowed for an autofluorescence assessment and aided in eliminating background noise. Slides were examined using an Olympus IX70 Fluorescence Microscope with green (500–570 nm) and red (610–750 nm) filter cubes.

RESULTS

Structure and Morphology of the Light Organ: Light and Fluorescence Microscopy

Two luminous light organs per Pyrosoma atlanticum zooid along with potential symbiotic microbes were identified with light and fluorescent microscopy. The bacteria within each light organ were well resolved and clearly identifiable on each side of the buccal siphon (Figure 1A). The left and right light organs (30–50 μm in diameter) were oval shaped structures, usually fully intact with each exhibiting a nodule capping the end (Figure 1A). Within the light organ, a clear, possibly open space in the center appeared surrounded by bacteria-filled cells delineating the boundaries of each light organ. At higher magnification, the bacteria clustered in the light organ were found to be bacteriocytes (Figure 1B). As many as 72 bacteriocytes were identified within a single light organ. This value was calculated by counting the number of dark structures within the light organ.

Sequencing of P. atlanticum Microbiomes and Partial Mitochondrial Genome

Microbiomes based on 16s rRNA amplicons were sequenced from three different Pyrosoma atlanticum individuals and compared to 10 seawater samples. The sampling design provided a background environmental microbial profile for comparison with the pyrosome microbiomes. Each pyrosome sample was matched to a previously sequenced seawater microbiome collected at two sites and depths of 1,500 m, corresponding to the pyrosome collections. The seawater samples were previously sequenced as a part of another DEEPEND consortium project (Easson and Lopez, 2019). A total of 396K MiSeq reads and 497 Amplicon Sequence Variant (ASV) were produced. In all three pyrosome samples, the most abundant 16S rRNA sequences matched Photobacterium sp. r33 in the pyrosome microbiomes (Table 3). In order to confirm the identity of the symbiont, the sequence derived from the MiSeq run was aligned with the sequence of Photobacterium sp. r33 from NCBI (Supplementary Figure 2). This was done through the NCBI BLAST program. This alignment exhibited a 99% match, with only a single nucleotide difference. However, until bacterial identity is confirmed with deeper genomic sequencing, the most abundant bacterium taxon will be referred to as Photobacterium Pa-1.

In the samples analyzed, matches to a Photobacterium sp. r33- like had a range of abundances. In the 10 seawater samples, Photobacterium sp. r33 appeared less than 0.12% at the highest abundance, while in the tissue samples, the same Photobacterium appeared in DMSO1 at 74.20%, RNALater6 at 70.88%, and in RNALater7 at 39.60%. These abundances in the pyrosomes were calculated through the CosmosID® pipeline and showed significant differences in beta diversity of bacterial ASV’s between the pyrosome and water samples after an ANOSIM test (R = 0.8704, p = 0.0001) (Figure 2), with the water samples having a considerably more diverse bacterial community (Supplementary Figure 3). The top two bacterial taxa in the three pyrosome samples were Photobacterium sp. r33 and Vibrio_us (unidentified species). There were over 1,100 ASV’s found in the water samples.

In an effort to detect and characterize lux genes of a bacterial photosymbiont producing bioluminescence, we conducted a whole-genome (metagenomic) Illumina sequencing run. This

4https://blast.ncbi.nlm.nih.gov/Blast.cgi
was based on the evidence that most of the *Pyrosoma atlanticum* 16S rRNA profiles appeared to be dominated by just a few bacterial taxa. Unfortunately, bacterial *lux* genes were not detected in the resulting assemblies, although a partial mitochondrial DNA contig of 14,302 base pairs (bp) was serendipitously generated with 26X coverage. The mtDNA sequences provided an opportunity to gain a genetic basis for the taxonomic identity of *Pyrosoma atlanticum*. The next closest sequence to *P. atlanticum* was another tunicate in class Thaliacea, pelagic order *Doliolida*, *Doliolum nationalis*. A preliminary phylogeny based on the mitochondrial cytochrome oxidase subunit 1 (COI) genes supports the affinity of *P. atlanticum* with *Doliolum nationalis* (Supplementary Figure 4).

**Fluorescence in situ Hybridization (FISH)**

Under fluorescence microscopy, pyrosome tissue exhibited autofluorescence, but the bacteria were still clearly discernable (Figure 1). When histology sections were compared with those prepared for fluorescence in situ hybridization (FISH) analyses, similar orientation and morphology of the two light organs were evident (Figure 3). Both methods revealed putative “bacteriocytes” containing bacteria concentrated at the outer edges of the organ with a clear space in the center. FISH controls included pyrosome sections that were incubated with no fluorescent probes. The control sections reflected native background autofluorescence and did not display the same intensity (brightness) as seen in sections hybridized with probes (Figure 3A). This comparison demonstrated that the probes annealed specifically to their respective DNA targets and produced a signal after stringent washing. The pair of light organs fluoresced in the absence of a probe, but not as brilliantly as when the EUB338 and *Photobacterium* probes were used (Figure 3B). The signal produced with both probes was very intense and, as anticipated, bacteria surrounding the light organ as well as the light organ emitted a signal above background. This was likely a result of the universal EUB338 bacterial probe being designed to bind to almost all bacteria within the sample (Figure 3C). When the *Photobacterium* probe was employed, only the light organ emitted a signal (Figure 3D). Other areas of the tunic did not emit a signal, indicating that the photobacteria were concentrated in the light organ. The morphology of bacteria differed in the tissue throughout the section, ranging from coccoid in the light organ to bacterium with flagella-like structures in the tunic. Due to the high light intensity, the shutter on the microscope was partially closed for the sections hybridized with probes while the sections with no probes were imaged with the shutter remaining fully open.

When the EUB338 and *Photobacterium* probes were combined, variability in the intensity of signal emission was evident. *Photobacterium Pa-1* fluorescence was brightest when both probes were combined. For example, under the green filter the bacteria are seen concentrated around the outer edges of the light organ with a clear space in the center similarly to previous observations (Figure 3E). Under the red filter, the same outer edges are packed with *Photobacterium Pa-1* fluorescing orange (Figure 3F). This filter and orange fluorescence strongly indicates the presence of *Photobacterium Pa-1* in the light organ.
Fine Structure and Bacterial Cluster Location in *P. atlanticum* (SEM)

A three-dimensional view of the pyrosome light organ and associated microbes was obtained using SEM imaging (Figure 4). This imaging shows the relationship between the tissue surrounding the light organ with the intracellular bacteria present. The light organ was approximately 20 µm in diameter and confirms diameter estimates made using light microscopy. The light organ is divided into two distinct areas confirming our light microscope observations. In the organ shown, the bacteria were clustered in an area of the light organ and include approximately 25 spherical 1–2 µm cells. In this SEM image it is not possible to discern if the microbial cells were intracellular but was better addressed in the TEM images.

Ultrastructure of the Microbial Population in *P. atlanticum* (TEM)

The light organ bacterial symbionts were intracellular and associated with mitochondria (Figure 5A). Their intracellular location was confirmed by observation of a cell membrane that encased both bacterial symbionts and mitochondria (Figure 5A). Cells containing these bacteria and mitochondria were also associated with abundant endoplasmic reticulum (Figures 5A,B). The mitochondria were sometimes closely associated with the bacteria. However, when comparing clusters of microbes and mitochondria, the bacteria could be easily distinguished from the mitochondria by the presence of prominent cristae in the mitochondria and the bacterial cells resembling bacteriocytes (Figure 5A). In some cases, the bacteria were clustered around...
FIGURE 3 | The light organs (green arrows) with no probe and the shutter wide open (A) vs. 4 µl of both EUB338 and Photobacterium probe with the shutter partially closed (B). The EUB338 probe (C) binds to many bacteria (green arrow) within the tunic (white arrow) and the light organ (yellow arrow). In contrast, the Photobacterium probe (D) only illuminated the light organ. EUB338 and Photobacterium probes in green (E) vs. red (F). The orange fluorescence in Photobacterium Pa-1 is found exclusively concentrated around the edges of the light organ (yellow arrow). Scale bar = 100 µm in (A–C), while the scale bar is 50 µm (D), and 20 µm in (E,F).

an “opening” that suggests excretion activity (Figure 5C). It appears that fluid filled vesicles may pinch off and move to the extracellular environment. The nature of these vesicles are unknown, or whether these excretory products are associated with bioluminescence. Within a single bacteriocyte, 5–7 bacteria were visualized (Figure 5) and therefore we estimated that
a minimum of 480 ~ 1,200 putative photosymbiotic bacteria could be found within a single *Pyrosoma atlanticum* light organ. These are approximate numbers since we cannot fully view all bacteriocytes and their contents in one micrograph.

**DISCUSSION**

**Structure of the Light Organ in *P. atlanticum***

Our imaging of the light organ of *Pyrosoma atlanticum* supported previous descriptions (Mackie and Bone, 1978; Nealson et al., 1981). Previous studies had micrographs of semi-thin sections showing “photic” organelles in the light organ (Mackie and Bone, 1978). This study is the first to combine detailed light and electron microscopic imaging of *P. atlanticum* with genetic analyses (light, TEM, SEM, FISH, and 16s rRNA sequencing) simultaneously. This combination demonstrated clearly that *Photobacterium* sp. r33-like bioluminescent symbionts are contained intracellularly within bacteriocyte resembling structures intracellularly in the light organ. The specialized adipocytes are heavily concentrated around the outer edges of the light organ and have intracellular vacuoles containing multiple bacteria, which have been found in several different marine holobionts, including tunicates (Kwan et al., 2012; Lopez, 2019). The intracellular location of the bacterial symbionts is highly unusual here, as most bioluminescent bacterial symbionts are extracellular (Mackie and Bone, 1978).

**Source of Bioluminescence in *P. atlanticum***

We have demonstrated a likely bacterial source for bioluminescence in *Pyrosoma atlanticum*, a taxon that is genetically closest to *Photobacterium* sp. r33. However, we cannot unequivocally identify the species without additional genomic data, since 100% identity to a single gene such as 16s rRNA does not confirm species identity (Fox et al., 1992; Janda and Abbott, 2007). The genus *Photobacterium* is known to show substantial ecophysiological diversity, which includes free-living, symbiotic, and parasitic lifestyles (Labella et al., 2017). The bioluminescent *Photobacterium* species, in particular *P. aquimaris*, *P. damselae*, *P. kishitanii*, *P. leiognathi*, and *P. phosphoreum*, exhibit free-living and symbiotic lifestyles. They can be found in dense populations associated with tissues in the light organs of their selective hosts (Labella et al., 2017). These tissues could be reflectors, shutter lens, or other tissues that are used to control, focus, and/or diffuse the bacterial light produced from the organism’s body (Urbanczyk et al., 2011). Some of the hosts of *P. kishitanii* and *P. leiognathi* are marine fishes, cephalopods, and octopods. However, *P. leiognathi* has established a highly specific
Photobacterium habitats encompassed by Photobacterium sp. hosts. The EUB338 probe fluoresced a greenish tint under the green filter cube (500–570 nm) and produced more signals than 2010). The EUB338 probe showed the probe-specific bacteria fluorescing orange. The orange fluorophores confirmed that Photobacterium Pa-1 was located in the light organ.

Light microscopy revealed microbial localization within the luminous organ, and the bacterial symbionts were identified by FISH. TEM clearly indicated intracellular bacteria concentrated in the organ. There were approximately 60 bacteriocytes found in a single light organ in light microscopy. Precise estimates of bacteriocyte numbers are likely affected by the plane in which tissues were sectioned, so there may be more Photobacterium sp. per cell than that observed using EM. In each micrograph, regardless of the type of microscopy used, the bacteria were concentrated on the interior border of the cells, while the bacteriocytes made up the periphery of light organ itself, surrounding a non-cellular space in the center. This begs the question as to why bacteria concentrate at the edges. To determine if the orientation of bacteria in the luminous organ plays a role, or stages, in the production of luminescence would pose an interesting question for future research.

**P. atlanticum** Bacterial Symbiont Morphology

Bacterial symbionts have been described in many marine invertebrates (McFall-Ngai et al., 2013; Lopez, 2019); however, only two papers have produced a description of the ultrastructure of photogenic organelles assumed to be bacteria in pyrosomes (Mackie and Bone, 1978; Nealon et al., 1981), and one paper describes bacterial luciferase activity similar to that of Photobacterium in Pyrosoma sp. (Leisman et al., 1980). There is precedence for bacterial symbionts to be contained intracellularly or within bacteriocytes, especially in tunicates such as *Lissoclinum patella* (Kwan et al., 2012). Bioluminescent bacterial symbionts, however, are often found to be extracellular.

Extracellular and free living bacterial symbionts are typically rod-shaped and more elongated (Nealon et al., 1981) than the bacteria present in pyrosomes. With the morphological similarities to gram-negative bacteria, thick cell walls and double membranes around each cell, our results provide strong support that the cells identified in the light organ are of microbial origin, aiding in the validation of the hypothesis that *Pyrosoma atlanticum* uses bacterial symbiosis as their bioluminescence mechanism (Dunlap and Schaechter, 2009). Gram staining could not be applied directly on the bacteria because neither...
they, nor the light organ, could be isolated. Nonetheless, the proposed intracellular luminescent bacteria in *Pyrosoma* differ morphologically and biochemically from almost all other bioluminescent bacteria, which are typically longer than oval or as subspherical rods and without granules (Mackie and Bone, 1978). These photobacteria in *Pyrosoma atlanticum* were exclusively coccoid in morphology and 1–2 μm in diameter, in agreement with previous bacterial ultrastructural descriptions of *Pyrosoma atlanticum* (Nealson et al., 1981).

The microscopy images in the current study produced a more detailed description of the bacteria found in *Pyrosoma atlanticum* than any previous work done on pyrosomes. Moreover, the current results can be added and contrasted to the continuing debate over whether *P. atlanticum* bioluminescence stems from bacterial or eukaryotic sources. For example, Tessler et al. (2020) recently described a potential luciferase, an Rluc-like protein in *Pyrosoma*. They provide compelling phylogenetic support that this enzyme is endogenous, decreasing the likelihood of contamination from orthologs. It is known that several non-luminous organisms such as *Strongylocentrotus*, *Ciona*, *Trichoplax*, or *Saccoglossus*, and likely other unidentified pelagic organisms have orthologs to Rluc luciferase which could also bind to the antibody (Tessler et al., 2020). However, the specific luciferase function of the proteins in these other organisms and pyrosomes was not confirmed, and there are many examples of "gene or protein sharing" throughout molecular evolution (Shimizu et al., 1998; Horwitz, 2000). This scenario indicates that a conserved luciferase protein sequence may be present, but still may have different functions that depend on tissue or taxonomic context. For example, although Tessler et al. (2020) did express the recombinant pyrosome Rluc homolog and show that it generated light with coelenterazine, the binding of their antibody did not unequivocally confirm that the target is the native luciferase. Lastly Tessler et al. (2020) did not unequivocally demonstrate that there is any coelenterazine in the native system in *P. atlanticum* that would be used by the endogenous luciferase. These represent major missing pieces that could support a primary host role in bioluminescence.

Moreover, Tessler et al.'s immunostained confocal micrographs indicated the presence of a strong signal to *Renilla* luciferase antibody in each zooid, in a circular region below the incumbent siphon containing nuclei. This strongly stained region was in the same regions of the luminous organ described by Mackie and Bone (1978). These nucleated cells may be from the eukaryotic pyrosome bacteriocytes in which the bacteria are localized. However, when the micrographs from Tessler et al. (2020) are compared with those from this study (Figures 3E,F), it is evident that different areas of the tissue and cells are examined. The incumbent siphons of the zooids are near the light organs, but the light organs are not shown in these figures. The immunostained confocal localization with some observations of nuclei (Tessler et al., 2020), does not preclude the presence of intracellular bacteria.

Continuing controversy exists in the literature concerning the relationship between bacteria and pyrosome luminescence. This may persist with the recent results and until unequivocal evidence appears [e.g., direct observation of bioluminescence, vertical transmission of photosymbionts (Sharp et al., 2007)]. For example, some suggest that pyrosome luminescence is due to an “intrinsic system” not related to bacterial symbionts, with the source of light being a type of tunic cell (Aoki et al., 1989). Our ultrastructural characterization of the putative bacterial cells for the most part coincided with published TEM work on *Pyrosoma atlanticum* (Mackie and Bone, 1978; Nealson et al., 1981; Aoki et al., 1989). The overall size and morphology of the microbial cells found in our study are similar to those in the literature in terms of ultrastructural characteristics and are found in the tunic cells packed with organelles and not innervated (Mackie and Bone, 1978).

### Distribution and Acquisition of Bacteria in Organisms Related to Bioluminescence

Previous work on *Pyrosoma atlanticum* had not determined whether the bacteria are intra- or extracellular, and only two studies have hypothesized an intracellular organization for pyrosome bacterial symbionts (Mackie and Bone, 1978; Nealson et al., 1981). Our genetic and imaging data is consistent with the hypothesis that intracellular bacteria are present in the pyrosome light organs and provide strong evidence that intracellular bacterial symbionts are potentially responsible for light production in *Pyrosoma atlanticum*. The visualizations of the light organs in light, fluorescence, and electron microscopy showed that bacterial symbionts belonging to the bioluminescent genera *Photobacterium* were found in same region where the bacteriocytes were observed, encased by a cell membrane with the mitochondria. Intracellular organization, in conjunction with host mediated bacteriocyte structure, indicates a highly interdependent and specialized biochemical relationship between the bacteria and host cells (Nealson et al., 1981). Intracellular symbionts represent the most highly adapted of bacterial symbionts (Nealson et al., 1981; Shigenobu et al., 2000) and our current microscopy data provides the first evidence of *Pyrosoma atlanticum* harboring these highly adapted intracellular symbionts.

There is much to be learned on how and when the hosts of *Photobacterium* initiate symbioses. The intracellular occurrence of these symbionts brings up the question of whether *Photobacterium* Pa-1 symbiont may be acquired via horizontal or vertical transmission. Horizontal transmission is the acquisition of symbionts from the environment, while vertical transmission is the inheritance of symbionts from previous generations (Bright and Bulgheresi, 2010). In deep-sea ceratioid fishes it is reported that the bioluminescent symbionts are acquired from the environment during the larval migration of the fish from surface waters to the bathypelagic water, albeit in low levels of abundance (Freed et al., 2019). These symbionts were found in low levels of abundance in both mesopelagic and bathypelagic zones, which suggest that the microbes are not obligately dependent on the hosts for growth. Anglerfishes appear to not acquire their photosymbionts from the environment.
Pyrosoma atlanticum sequences. Since which contained a large concentration of 40–74% of these like sequences (0.0–0.12%) compared to pyrosome samples, previously been viewed as “an alternative” electron transport (Rees et al., 1998; Bourgois et al., 2001). Bacterial luciferase has respiratory chain of mitochondria and bioluminescent bacteria with mitochondria inside pyrosome cells (Nealson et al., 1981). Photobacterium Pa-1 Mitochondrial Association

Microbial symbionts occur in almost every metazoan, although many partnerships have not been sufficiently studied (McFall-Ngai et al., 2013; Aprill, 2017). With most microbial mutualisms, the host relies nutritionally on the microbial symbiont, such as chemosynthetic bacterium or photosynthetic algae, and without these symbionts, the host growth suffers significantly. Bioluminescent symbiosis differs from other types of symbiotic associations, as lumen production from the microbe is the currency of exchange rather than metabolic nutrients for the host (Dunlap and Schaechter, 2009). Some bioluminescent symbioses also appear facultative as the host or bacterial symbionts can survive without each other ex situ and in laboratory settings (Dunlap and Schaechter, 2009). Many bacterial bioluminescent symbionts appear to be extracellular, suggesting a facultative association, whereas in obligate symbiosis the bacteria are found intracellularly. Luminous bacteria are characterized as Gram-negative, non-spore-forming, motile, have cell walls difficult to penetrate, and are generally chemoorganotrophic.

Photobacteria have previously been found to be associated with mitochondria inside pyrosome cells (Nealson et al., 1981). It has been noted that there are several similarities between the respiratory chain of mitochondria and bioluminescent bacteria (Rees et al., 1998; Bourgois et al., 2001). Bacterial luciferase has previously been viewed as “an alternative” electron transport pathway, however, it is more recently considered an “alternative” oxidase (Bourgois et al., 2001). This is why the entire photogenic system of bioluminescent bacteria scavenges not only reducing equivalents (luciferase), but also ATP and NADPH. The close association also ties into the fact that the organism needs to consume a certain amount of energy to produce the visible spectrum of the bioluminescent light (Rees et al., 1998). In most cases it would be the blue photon (~470 nm), which requires about 255 J mol⁻¹. The fact that bioluminescence requires a lot of energy and mitochondria produce ATP, might explain why the mitochondria and microbes are so closely associated and densely packed into the cells (Bourgois et al., 2001).

CONCLUSION

This study provides new insights into the bioluminescent mechanism of Pyrosoma atlanticum, a dominant colonial tunicate found in tropical to temperate pelagic waters worldwide. Our findings support the hypothesis that bioluminescence in these tunicates is bacterial-based, specifically associated with Photobacterium Pa-1. The Family Vibrionaceae is known to contain three genera of bioluminescent bacteria, including Photobacterium. Photobacterium Pa-1 are found intracellularly and within the light organs of Pyrosoma atlanticum. They were found dominating the microbiome in greater relative abundances of about 40–74% in these pyrosomes. Future studies should focus on bioluminescent mechanisms of the light organ itself: regulation of bioluminescence, comparing the microbiome of the whole tunicate to that of the light organ to show the selectivity of its environment, and finally the acquisition and retention of bacterial symbionts if they proven to be major source of bioluminescence (Guindon and Gascuel, 2003).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found at NCBI repositories https://www.ncbi.nlm.nih.gov/. This includes Sequence Read Archives under PRJNA636187 and PRJNA635880.

AUTHOR CONTRIBUTIONS

AB and JL were responsible for project and experimental design. AB conducted the above research. PB lead the electron microscopy work as well as imaging for all micrographs. TF guided and directed the bioluminescence work, and analysis. JL carried out 16S primer selection for FISH and phylogenetic analysis. TS, NP, and NS collected samples and metadata from the various research cruises for this project. JL, PB, TF, and TS edited manuscript drafts and helped prepare for press. All authors contributed to the article and approved the submitted version.
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REFERENCES
Alldredge, A., and Madin, L. (1982). Pelagic tunicates: unique herbivores in the marine plankton. *Bioscience* 32, 655–663. doi: 10.2307/1308815
Aoki, M., Hashimoto, K., and Watanabe, H. (1989). The intrinsic origin of bioluminescence in the ascidian. *Clavelina miniata*. *Biolog. Bull.* 176, 57–62. doi: 10.2307/1541889
Apprill, A. (2017). Marine animal microbiomes: toward understanding host–microbiome interactions in a changing ocean. *Front. Mar. Sci.* 4:222. doi: 10.3389/fmars.2017.00222
Baker, L. J., Freed, L. L., Easson, C., Fenolio, D., Sutton, T. T., et al. (2019). Diverse deep-sea anglerfishes share a genetically reduced luminous symbiont that is acquired from the environment. *eLife* 8:e47606.

Bourgois, J.-J., Sluse, F., Baguet, F., and Mallefet, J. (2001). Kinetics of light emission and oxygen consumption by bioluminescent bacteria. *J. Bioenerg. Biomembr.* 33, 353–363.

Bowlby, M. R., Widder, E. A., and Case, J. F. (1990). Patterns of Stimulated Bioluminescence in 2 Pyrosomes (Tunicata, Pyrosomatidae). *Biol. Bull.* 179, 340–350. doi: 10.2307/1542326
Bright, M., and Bulgheresi, S. (2010). A complex journey: transmission of microbial symbionts. *Nat. Rev. Microbiol.* 8, 218–230. doi: 10.1038/nrmicro2262
Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7:335.
Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci.* 108(Suppl. 1), 4536–4522. doi: 10.1073/pnas.1000083
Chakraverty, S., Hellb, D., Burday, M., Connell, N., and Alland, D. (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J. Microbiol. Methods*. 69, 330–339. doi: 10.1016/j.mimet.2007.02.005
Chiba, K., Hoshi, M., Isobe, M., and Hirose, E. (1998). Bioluminescence in the tunic of the colonial ascidian, *Clavelina miniata*: identification of luminous cells in vitro. *J Exp. Zool.* 281, 546–553. doi: 10.1002/(SICI)1097-010X(19980815)281:6<546::AID-JEZ2>3.0.CO;2-n
Décima, M., Stukel, M. R., López–López, L., and Landry, M. R. (2019). The unique ecological role of pyrosomes in the Eastern Tropical Pacific. *Limnol. Oceanogr.* 64, 728–743. doi: 10.1002/ino.11071
Dunlap, P., and Schaechter, M. (2009). Microbial bioluminescence. *Encyclop. Microbiol.* 2009, 45–61. doi: 10.1016/b978-012373944-5.00066-3
Dunlap, P. V., Ast, J. C., Kimura, S., Fukui, A., Yoshino, T., and Endo, H. (2007). Phylogegetic analysis of host–symbiont specificity and codivergence in bioluminescent symbioses. *Cladistics* 23, 507–532. doi: 10.1111/j.1096-0031.2007.00157.x

SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.606818/full#supplementary-material

Easson, C. G., and Lopez, J. V. (2019). Depth-Dependent environmental drivers of microbial plankton community structure in the Northern Gulf of Mexico. *Front. Microbiol.* 9:3175. doi: 10.3389/fmicb.2018.03175
Fox, G. E., Wisotzkey, J. D., and Jurthshuk, J. (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int. J. Syst. Evol. Microbiol.* 42, 166–170. doi: 10.1099/00277133-42-1-166
Freed, L. L., Easson, C., Baker, L. J., Fenolio, D., Sutton, T. T., Khan, Y., et al. (2019). Characterization of the microbiome and bioluminescent symbions across life stages of Ceratioid Anglerfishes of the Gulf of Mexico. *FEMS Microb. Ecol.* 95:fi146.
Galt, C., and Flood, P. (1998). Bioluminescence in the Appendicularia. *Biol. P ing. 1998, 215–229.
Galt, C., and Sykes, P. (1983). Sites of bioluminescence in the appendicularians Oikopleura dioica and O. labradoriensis (Urochordata: Larvacea). *Mar. Biol.* 77, 155–159. doi: 10.1007/bf00396313
Gray, M. W., Sankoff, D., and Cedergren, R. J. (1984). On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. *Nucleic Acids Res.* 12, 5837–5852. doi: 10.1093/nar/12.14.5837
Guindon, S., and Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* 52, 696–704. doi: 10.1080/10635150393253520
Haddock, S. H., Moline, M. A., and Case, J. F. (2010). Bioluminescence in the sea. *Ann. Rev. Mar. Sci.* 2, 443–493.
Hagood, M. G. (1993). Light organ symbioses in fishes. *Crit. Rev. Microbiol.* 19, 191–216. doi: 10.3109/10408419309113529
Hirose, E. (2009). Ascidian tunic cells: morphology and functional diversity of free cells outside the epidermis. *Invert. Biol.* 128, 83–96. doi: 10.1111/j.1744-7410.2008.00153.x
Holland, L. Z. (2016). Tunicates. *Curr. Biol.* 26, R146–R152. doi: 10.1016/j.cub.2015.12.024
Horwitz, J. (2000). The function of alpha-crystallin in vision. *Semin. Cell Dev. Biol.* 2000, 53–60. doi: 10.1006/scdb.1999.0351
Huxley, T. H. (1851). XXIV. Observations upon the anatomy and physiology of salpa and pyrosoma. *Philosop. Transac. R Soc. Lon.* 141, 567–593. doi: 10.1098/rstl.1851.0027
Janda, J. M., and Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J. Clin. Microbiol.* 45, 2761–2764. doi: 10.1128/jcm.01228-07
Kaeding, A. J., Ast, J. C., Pearce, M. M., Urbanczyk, H., Kimura, S., Endo, H., et al. (2007). Phylogegetic diversity and cosymbiosis in the bioluminescent symbioses of "Photobacterium mandapamensis". *Appl. Environ. Microbiol.* 73, 3173–3182. doi: 10.1128/aem.02212-06
Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software ver 7: improvements in performance and usability. *Molecular biology and evolution* 30, 772–780. doi: 10.1093/molbev/ms3010
Kuczynski, J., Stombaugh, J., Walters, W. A., González, A., Caporaso, J. G., and Knight, R. (2011). Using QIIME to analyze 16S rRNA gene sequences from microbial communities. *Curr. Protoc. Bioinform.* 36, 11–10.

Kwan, J. C., Donia, M. S., Han, A. W., Hirose, E., Haygood, M. G., and Schmidt, E. W. (2012). Genome streaming and chemical defense in a coral reef symbiosis. *Proc. Natl. Acad. Sci.* 109, 20655–20660. doi: 10.1073/pnas.1213820109

Labella, A. M., Arahal, D. R., Castro, D., Lemos, M. L., and Borrego, J. J. (2017). Revisiting the genus Photobacterium: taxonomy, ecology and pathogenesis. *Int. Microbiol.* 20, 1–10. doi: 10.1007/978-94-017-2901-7_1

Leisman, G., Cohn, D. H., and Nealon, K. H. (1980). Bacterial Origin of Luminescence in Marine Animals. *Science* 208, 1271–1273. doi: 10.1126/science.208.4449.1271

Lemaire, P., and Piette, J. (2015). Tunicates: exploring the sea shores and roaming the open ocean. A tribute to Thomas Huxley. *Open Biol.* 5:150053. doi: 10.1098/rsob.150053

Lopez, J. V. (2019). After the taxonomic identification phase: addressing the functions of symbiotic communities within marine invertebrates in Symbiotic Microbiomes of Coral Reefs, Sponges and Corals. Berlin: Springer. 105–144.

Mackie, G. O., and Bone, Q. (1978). “Luminescence and Associated Effector Activity in Pyrosoma (Tunicata Pyrosomida),” in *Proceedings of the Royal Society Series B-Biological Sciences*, Vol. 202:0081. doi: 10.1098/rspb.1978.0081

Martini, S., and Haddock, S. H. (2017). Quantification of bioluminescence from the surface to the deep sea demonstrates its predominance as an ecological trait. *Sci. Rep.* 7:45750.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., et al. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci.* 110, 3229–3236.

Nealson, K., Cohn, D. L., Leisman, G., and Tebo, B. (1981). Co—evolution of luminous bacteria and their eukaryotic hosts. *Ann. N Y Acad. Sci.* 361, 76–91. doi: 10.1111/j.1749-6632.1981.tb46512.x

Negandhi, K., Blackwelder, P. L., Ereskovsky, A. V., and Lopez, J. V. (2010). Florida reef sponges harbor coral disease-associated microbes. *Symbiosis* 51, 117–129. doi: 10.1007/s13199-010-0059-1

Nelson, J. S., Grande, T. C., and Wilson, M. V. (2016). *Fishes of the World*. John Wiley & Sons

Nyholm, S. V., and McFall-Ngai, M. (2004). The winnowing: establishing the squid–Vibrio symbiosis. *Nat. Rev. Microbiol.* 2, 632–642. doi: 10.1038/nrmicro957

O’Connell, L., Gao, S., McCorquodale, D., Fleisher, J., and Lopez, J. V. (2018). Fine grained compositional analysis of Port Everglades Inlet microbiome using high throughput DNA sequencing. *PeerJ* 6:e6671.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P., O’Hara, R., et al. (2013). *Community ecology package. R package version*, 2.0-2.

Rees, G. N., Baldwin, D. S., Watson, G. O., Perryman, S., and Nielsen, D. L. (2004). Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: application of multivariate statistics. *Antoni van Leeuwenhoek* 86, 339–347. doi: 10.1007/s10482-005-0498-5

Rees, J.-F., De Wergifosse, B., Noiset, O., Dubuissou, M., Janssens, B., and Thompson, E. M. (1998). The origins of marine bioluminescence: turning oxygen defense mechanisms into deep-sea communication tools. *J. Exp. Biol.* 201, 1211–1221.

Robison, B. H., Raskoff, K. A., and Sherlock, R. E. (2005). Ecological substrate in midwater: Dolioluma equus, a new mesopelagic tunicate. *J. Mar. Biol. Assoc. U K* 85, 655–663. doi: 10.1071/mj04075

Sen, A., Duperron, S., Hourdez, S., Piquet, B., Léger, N., Gebruk, A., et al. (2018). Cryptic frenulates are the dominant chemosymbiotrophic fauna at Arctic and high latitude Atlantic cold seeps. *PLoS One* 13:e0209273. doi: 10.1371/journal.pone.0209273

Sharp, K. H., Eam, B., Faulkner, D. J., and Haygood, M. G. (2007). Vertical transmission of diverse microbes in the tropical sponge *Corticium sp.* *Appl. Environ. Microbiol.* 73, 622–629. doi: 10.1128/aem.01493-06

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., and Ishikawa, H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. *APS. Nat. 407*, 81–86. doi: 10.1038/35024074

Shimizu, K., Cha, J., Stucky, G. D., and Morse, D. E. (1998). Silicate: α cathepsin L-like protein in sponge biosilica. *Proc. Natl. Acad. Sci.* 95, 6234–6238. doi: 10.1073/pnas.95.11.6234

Sutherland, K. R., Sorenson, H. L., Blondheim, O. N., Brodeur, R. D., and Galloway, A. W. (2018). Range expansion of tropical pyrosomes in the northeast Pacific Ocean. *Ecology* 99, 2397–2399. doi: 10.1002/ecy.2429

Sutton, T. T., Frank, T., Judkins, H., and Romero, I. C. (2020). “As gulf oil extraction goes deeper, who is at risk? community structure, distribution, and connectivity of the deep-pelagic fauna,” in *Scenarios and Responses to Future Deep Oil Spills*, (Berlin: Springer), 403–418. doi: 10.1007/978-3-030-12963-7_24

Tessler, M., Gaffney, J. P., Oliveira, A. G., Guaraccina, A., Dobi, K. C., Gujarati, N. A., et al. (2020). A putative chordate luciferase from a cosmopolitan tunicate indicates convergent bioluminescence evolution across phyla. *Sci. Rep.* 10, 1–11.

Thompson, L. R., Sanders, J. G., McDonald, D., Amir, A., Ladau, J., Loccy, K. J., et al. (2017). A communal catalogue reveals Earth’s multiscale microbial diversity. *Nature* 551:457.

Urbanczyk, H., Ast, J. C., and Dunlap, P. V. (2011). Phylogeny, genomics, and symbiosis of photobacterium. *FEMS Microbiol. Rev.* 35, 324–342. doi: 10.1111/j.1574-6976.2010.00250.x

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of RNA sequences into the new bacterial taxonomy. *Appl. Environ. Microb.* 73, 5261–5267. doi: 10.1128/aem.00602-07

Widder, E. A. (2010). Bioluminescence in the Ocean: Origins of Biological, Chemical, and Ecological Diversity. *Science* 328, 704–708. doi: 10.1126/science.1174269

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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