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First Record of Bioluminescence in a Sipunculan Worm

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During a search for bioluminescent marine annelids around the island of Carrie Bow Cay, Belize, we discovered bioluminescence in the sipunculan worm *Nephasoma pellucidum*. The identification of *N. pellucidum* was verified by DNA barcoding of ∼650 bp of the mitochondrial cytochrome c oxidase I gene, using sequences of the same species from previous studies as references. Our study constitutes the first record of a bioluminescent sipunculan species. Green luminescence was detected in four of the five individuals of *N. pellucidum* collected from coral rubble from a shallow reef flat and one deeper (14 m) site. To test for bioluminescent activity, all collected annelids (including sipunculans) were sorted into morphotypes and identified to the lowest taxonomic level possible. After several hours of dark adaptation, they were mechanically stimulated, and luminescent reactions were noted and recorded by photography and/or video whenever possible. Of a total of nine examined sipunculan species, *N. pellucidum* was the only one that showed bioluminescent activity. The underlying biochemical processes for the bioluminescence are so far unknown, but transcriptome data indicate the presence of proteins with similarities to *Renilla*-type luciferases. We did not find similarities to any known photoproteins. Our findings broaden the known diversity of bioluminescent annelid taxa and support the notion that bioluminescence arose multiple times in annelids. The bioluminescent properties of *N. pellucidum* add a new reason to pursue this species as a model species, as has been previously proposed.

Keywords: annelida, luciferin, luciferase, photoprotein, marine invertebrate

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INTRODUCTION

Bioluminescence, the biochemical generation of cold, visible light, is a very common phenomenon in marine organisms, especially in the deep sea (Haddock et al., 2010; Widder, 2010). Lau and Oakley (2021) estimate that bioluminescence has evolved 94 times across the tree of life. In animals, bioluminescence has been reported in at least 12 phyla and often appears to have multiple evolutionary origins within a given phylum (Haddock et al., 2010; Waldenmaier et al., 2012; Lau and Oakley, 2021). While the bioluminescent properties of some phyla, especially cnidarians and arthropods, have been studied extensively, comparatively little is known about bioluminescence in the phylum Annelida. Annelids include at least 13 family-level taxa with reported bioluminescent representatives, as well as several additional anecdotal reports (Verdes and Gruber, 2017). Annelid bioluminescence spans terrestrial taxa and diverse marine groups. There is no single habitat in which marine annelids are particularly prone to displaying bioluminescence nor a particular body

region that produces light. Some examples of bioluminescent annelids include the tube-dwelling chaetopterids (Nicol, 1952b,c,a, 1957; Mirza et al., 2020), epifaunal taxa such as polynoid scale worms (Nicol, 1954; Herrera, 1979; Plyuscheva and Martin, 2009; Moraes et al., 2021), and pelagic forms such as tomopterids (Dales, 1971; Gouveneaux et al., 2017). Additionally, there is a wide variety of wavelengths of emitted light, from yellow to blue (Verdes and Gruber, 2017). Ecological functions of bioluminescence include intraspecific communication, especially mate attraction (e.g., Galloway and Welch, 1911; Fischer and Fischer, 1995) and predation deterrence (e.g., Livermore et al., 2018) but in many cases the ecological roles of annelid bioluminescence are not well understood.

The classical biochemical pathway for the generation of light involves a small molecule, generically called luciferin, and an enzyme, known as luciferase, which catalyzes the oxidation of luciferin to oxyluciferin. This reaction produces an unstable complex (usually a cyclic peroxide) that breaks down to produce a compound called oxyluciferin and gives off a large amount of energy as light (Shimomura, 2006). Some luciferases form complexes with the substrate, known as photoproteins, which may also depend on cofactors such as Ca^{2+}, Fe^{2+}, H_2O_2, and ATP, among others (Daunert and Deo, 2006). In annelids, the best studied bioluminescent pathway is that of *Odontosyllis*. *Odontosyllis* is a genus of syllid polychetes inhabiting shallow coastal warm-water habitats. Benthic as adults, they periodically produce bioluminescent swimming stages that rise to the surface and release likewise bioluminescent gametes. The bioluminescent properties are retained post-spawning, suggesting that the ecological function of bioluminescence is not limited to reproduction (Fischer and Fischer, 1995). This green/blue bioluminescent system has been identified as a unique luciferin/luciferase system which requires no cofactor and shows little resemblance to those of other organisms (Brugler et al., 2018; Schultz et al., 2018; Kotlobay et al., 2019).

Here we present the first report of a bioluminescent sipunculan worm. Sipunculan worms, also known as peanut worms or star worms, represent an understudied group of unsegmented marine worms, characterized by a simple sac-like body and a retractable introvert with a crown of tentacles at its tip (Cutler, 1994; Schulze et al., 2019; Schulze and Kawauchi, 2021). Sipuncula were long regarded as a distinct phylum but recent phylogenetic and phylogenomic studies (Struck et al., 2007; Mwinyi et al., 2009; Dordel et al., 2010; Weigert et al., 2014), have placed them as a basal branch in the annelid radiation, despite their lack of segmentation (Carrillo-Baltodano et al., 2019). Sipunculans generally have a cryptic lifestyle and are rarely seen unless actively sought after. One habitat where sipunculans are particularly abundant and diverse is coral rubble, or eroded pieces of coral skeleton where sipunculans either borrow into the calcareous material or inhabit existing holes and crevices (Rice, 1975; Rice and MacIntyre, 1982; Gómez et al., 2013). Many of these species have long introverts, with recurved hooks that they likely use to scrape epiphytic organisms from surfaces (Rice, 1975, 1976; Cutler, 1994), but otherwise little is known about their biology.

The discovery of bioluminescence in a single sipunculan species from Carrie Bow Cay (CBC), Belize was the result of a search for novel bioluminescent annelid systems focusing on shallow-water tropical taxa. Although the region around CBC has been thoroughly sampled for sipunculans during previous expeditions (Rice and MacIntyre, 1982; Schulze and Rice, 2004), the bioluminescent species we observed, *Nephasoma pellucidum* (Keferstein, 1865), has not previously been reported from CBC. However, it has been reported from more southern Caribbean locations, such as Bocas del Toro, Panama (Schulze, 2005), Curaçao and Venezuela (Dean et al., 2007), as well as in the western Atlantic (Schulze and Rice, 2009), eastern Pacific (Dean and Cutler, 1998) and occasionally from the Indo-Pacific (Cutler, 1994; Kawauchi et al., 2012). Although it is not a particularly abundant species in any of the known Caribbean/Atlantic locations, *N. pellucidum* has been proposed as a model species for spiralian development (Schulze and Rice, 2009; Boyle and Rice, 2014), because it is relatively easy to keep in laboratory culture. Males and females from the Florida population readily spawn in the lab and larval development is well documented (Schulze and Rice, 2009), although attempts to complete the life cycle through metamorphosis have so far been unsuccessful. Here, we report on our methods of collection and eliciting the bioluminescent response.

MATERIALS AND METHODS

Collections and Morphological Identification

Field collections were conducted at the Smithsonian CBC Field Station in February of 2020. CBC is a small island located at 16.8000°N, 88.0833°W, about 24 kilometers off the coast of Belize, on the southern portion of the Meso-American Barrier Reef. The east side of the island is flanked by a reef flat, followed by the reef crest and a drop-off to deeper waters. The landward (west) side of the island is dominated by seagrass and patch reefs. Coral reef, seagrass, and intertidal coral rubble habitats were all sampled following a similar protocol. Pieces of coral rubble were collected in mesh dive bags or buckets either by SCUBA, snorkel, or wading into the water. The collected material was kept in free-flowing saltwater tables until processing. Pieces of coral rubble were carefully cracked using a hammer and chisel. Sipunculans were found either embedded in the calcium carbonate skeletons or nestled in grooves within the rubble. Specimens were extracted using forceps and blunt probes.

All specimens were then kept in small plastic dishes covered with seawater which was periodically replaced to prevent fouling. Individuals were sorted into morphotypes and identified to the lowest taxonomic level possible. Multiple individuals of the same identification were pooled. All dishes were placed underneath a dark box. Specimens were left in this configuration for the dark acclimation period prior to bioluminescence screening. The identification of the bioluminescent species was confirmed after return from CBC by a closer inspection of the morphology, dissections to observe the internal anatomy,

and through molecular taxonomy (DNA barcoding, see section "Molecular Taxonomy").

Molecular Taxonomy

We used DNA the barcoding sequence of the cytochrome *c* oxidase subunit I gene (COI) (Hebert et al., 2003) to confirm the morphology-based identification. DNA was extracted from small pieces of the introvert retractor muscles and body wall from the two injured specimens. DNA extraction was performed using the Qiagen DNEasy Blood and Tissue kit, following the instructions of the manufacturer. The COI barcoding sequence was amplified using the primer combination Poly-LCO (GAYTATWTTCAACAAATCATAAAGATATTGG) and Poly-HCO (TAMACTTCWGGGTGACCAAARAATCA) (Carr et al., 2011). The PCR mix consisted of 12.5 μl OneTaq Mastermix (New England Biolabs), 0.5 μl magnesium chloride (25 mM), 1 μl of each primer (10 μM), 9 μl water, and 1 μl of genomic DNA template. The cycling protocol was as follows: 94°C for 30 s, 35 cycles of 94°C for 30 s, 42°C for 30 s, and 68°C for 60 s; followed by a final extension at 68°C for 5 min. Successful amplification was confirmed through electrophoresis on 1% agarose gels. Amplification was only successful for one of the two specimens. The PCR product was enzymatically cleaned using the ExoSapIt Express reagent (ThermoFisher) and sequenced in both directions at Genewiz[1]. The forward and reverse fragments were assembled in CodonCode Aligner v9.0.2[2]. The single sequence with a length of 658 bp obtained from one specimen is available at GenBank under accession number MZ852770. A BLAST search for this sequence in GenBank resulted in multiple close hits, therefore we performed a phylogenetic analysis to determine the specimen's closest relatives. The obtained sequence was aligned with publicly available COI sequences from other members of the genera *Nephasoma*, including two identified as *N. pellucidum*, and *Golfingia*, the presumed sister taxon to *Nephasoma*, as well as one sequence for *Siphonosoma cumanense* as an outgroup. Alignment was performed using Muscle algorithm in Geneious Prime® 2021.2.2. The final alignment was 649 bp long. Phylogenetic analysis was conducted using Bayesian Inference in MrBayes on XSEDE through the CIPRES Science Gateway (Miller et al., 2010). Bayesian analysis was performed under a GTR+I+G model. Two runs with four chains each were run for 10,000,000 generations of MCMC, sampling every 1,000th tree. A 50% consensus tree was calculated after discarding trees from the first 2,500,000 generations.

Observation and Documentation of Bioluminescence

Specimens were acclimated to the dark for at least 5 h. The dark acclimation effectively eliminates any potential stress from an increase in non-directional light intensity caused by the extraction out of their concealed burrows (embedded in rock, or rubble). We retrofitted the field station's laboratory into a functional darkroom by covering all windows and light sources. We then spent 15–20 min acclimating our eyes to the darkness. At this point, specimens were removed from the dark box and screened for bioluminescence by mechanically irritating them with blunt probes or forceps for 5–10 s. Any sighting of visible light in response to this stimulation was recorded as a positive display of bioluminescence.

Additional individuals of positive bioluminescent species were then targeted in field collections and similarly screened. Bioluminescent activity was documented using a Sony α7 s digital camera attached to a Sony SEL50M28 lens, with an ISO setting of 32,000 and a wide aperture (f/2.8), producing a video of 24 frames per second. We used red light, which does not seem to interfere with light production, to position the specimens for video and photo documentation. Post-processing of video contrast and exposure were enhanced for visualization in the video editor Adobe Premiere Pro 2019. Individuals with confirmed bioluminescent displays were preserved in either 95% ethanol or RNAlater for genetic analysis, or 10% formaldehyde for taxonomic purposes.

Transcriptome Analysis

The transcriptome raw datasets already sequenced and available in a public database for nine Sipuncula species (Andrade et al., 2015; Lemer et al., 2015; **Table 1**), including one of *N. pellucidum* from Florida, were used for the prospection of genes associated with the bioluminescence in *N. pellucidum*. The read datasets were downloaded and filtered to remove low-quality reads and adaptors with SeqyClean v.1.10.09 (Zhbannikov et al., 2017) and used for *de novo* assembly in Trinity v.2.11.0 (Grabherr et al., 2011), with the default settings and *in silico* normalization. The transcripts with more than 200 bp were translated to amino acids by TransDecoder v.5.5.0 and were subjected to a similarity search against the non-redundant (nr) SWISS-PROT v.5 database using the BLASTp (Altschul et al., 1997) algorithm, with a cut-off *e*-value of ≤10–5. We also conducted a second annotation step using specific gene products related to luciferase/photoprotein and GFP (green fluorescent protein) present in public databases. For that, we promoted a search in the non-redundant NCBI database using the terms "Luciferase," "Photoprotein," "Aequorin," "Obelin," " Mnemiopsin," "GFP," and "Green Fluorescent Protein" (Access retrieve: October 5, 2021) and created a local database using makeblastdb function. The transcripts abundance was calculated by the align_and_estimate_abundance.pl script, which is present in the Trinity package. We estimated the completeness of the transcriptome against the metazoa database using BUSCO v.4.0.1 (Simão et al., 2015) software. The transcriptomes assembly and the photoprotein/luciferase sequences databases were deposited in the FigShare repository (10.6084/m9.figshare.16574831).

RESULTS

Collection and Identification

In addition to *N. pellucidum*, we collected eight other sipunculan species from rubble around CBC: *Aspidosiphon* sp., *Aspidosiphon* cf. *steenstrupii*; *Aspidosiphon* cf. *laevis*; *Aspidosiphon* cf. *elegans*; *Aspidosiphon cristatus*, *Phascolosoma nigrescens*; *Phascolosoma*

[1] genewiz.com

[2] www.codoncode.com

TABLE 1 | RNA-Seq assembly details of Sipuncula species used in this study.

Species	SRA access	Total of putative genes	Total of the coding sequence	Completeness (%)
Antillesoma antillarum	SRR1646260	46,777	5,151	14.1 (C:2.7%[S:2.7%, D:0.0%], F:11.4%, and M:85.9%)
Aspidosiphon parvulus	SRR1646391	136,231	8,566	13.3 (0.4%[S:0.4%, D:0.0%], F:12.9%, and M:86.7%)
Golfingia vulgaris	SRR1797875	128.071	90.629	99.2 (C:96.1%[S:70.6%, D:25.5%], F:3.1%, and M:0.8%)
Nephasoma pellucidum	SRR1646439	115,867	13,815	39.2 (C:8.6%[S:7.8%, D:0.8%], F:30.6%, and M:60.8%)
Phascolion cryptum	SRR1646440	85,590	3,005	13.00 (1.2%[S:0.8%, D:0.4%], F:11.8%, and M:87.0%)
Phascolopsis gouldii	SRR1654498	203,972	29,524	38.8 (C:10.6%[S:9.8%, D:0.8%], F:28.2%, and M:61.2%)
Phascolosoma perlucens	SRR1646442	47,741	2,582	8.2 C:0.4%[S:0.4%, D:0.0%], F:7.8%, and M:91.8%
Siphonosoma cumanense	SRR1646441	74,738	4,316	7.1 (C:0.0%[S:0.0%, D:0.0%], F:7.1%, and M:92.9%)
Sipunculus nudus	SRR619011	169.32	24,681	52.5 (C:13.3%[S:8.6%, D:4.7%], F:39.2%, and M:47.5%)

C, complete gene; S, single genes; D, duplicate genes; F, fragmented genes; M, missing genes.

perlucens, and *Antillesoma antillarum*. Members of the genus *Nephasoma* have an array of tentacles arranged around the mouth at the tip of the introvert which is generally shorter than or approximately equal in length to the trunk. In addition, they have two introvert retractor muscles and two nephridia. Although the introverts in our preserved samples were retracted, we had observed the extended introverts with the tentacles in the live specimens (Visible in **Supplementary Videos**). The dissection of the preserved material showed the two retractor muscles and paired nephridia, confirming the genus identification. The two preserved specimens had trunk lengths of 34.4 and 45 mm, respectively (note that the length depends on the contraction status, **Figures 1A,C**). The trunk and proximal introvert of *N. pellucidum* are pale in color and covered by raised, often dark papillae. The two retractor muscles insert in the ventral body wall approximately 50% down the trunk. The nephridia are dark and not attached to the body wall. Nephridiopores are located at the level of the anus. Cutler (1994) mentions the presence of two dark eyespots on the brain which we observed as well. Over the course of 1 week, five specimens of *N. pellucidum* were recovered from coral rubble in shallow water (<2 m) around CBC and one specimen from coral rubble at Raph's Wall (16.77961°N; 88.075117°W) from ∼14 m depth. Two of them were injured during retrieval from the rock, one of which was immediately fixed in 95% ethanol.

Observed Bioluminescence

Out of the nine sipunculan species identified, only *N. pellucidum* produced light upon stimulation (**Figure 1B,D**). We observed bioluminescence in four different specimens, one from Raph's wall and three from the shallow water rubble. The remaining fifth individual of *N. pellucidum* was not tested for bioluminescence due to injury upon extraction. Each specimen produced a pulse of visible greenish light upon perturbations with a blunt probe (**Supplementary Video**). The glow was localized on the body wall with no apparent patterning, although the intensity was not even throughout the body.

Molecular Taxonomy

The phylogenetic analysis placed *N. pellucidum* from Belize in a clade with *N. pellucidum* from New Caledonia and Florida, plus one *N. flagriferum* sequence (**Figure 2**). This clade is strongly supported by 100% posterior probability. Kimura-2-Parameter distances among the *N. pellucidum* sequences are: Belize-New Caledonia: 22.8%; Belize-Florida: 22.4%; and Florida-New Caledonia: 21.9%. Sequence variation is almost entirely in the third codon position, with only a single amino acid difference between the Florida sequence and the other two.

Transcriptomic Findings

A total of 115,867 gene products were obtained for *N. pellucidum*, which correspond to 39.22% of transcriptome completeness. After the amino acid translation filtering, we obtained a total of 13,815 coding sequences, in which 7,172 (∼52%) were annotated. Among them, we identified gene products associated with the bioluminescence in *Renilla*, echinoderms, starfish, and firefly, which are related to luciferase, coelenterazine, and *Renilla* luciferin/luciferase. We have also carried out the second round of annotation using a constrain search against luciferases, from metazoa to bacteria (identified as responsible for luminescent emission in some marine species, Delroisse et al., 2021), and Fluorescent Proteins FPs such as GFPs (green fluorescent proteins, Rodriguez et al., 2017), which may explain the greenish emission in this species. We observed gene product similarity to *coelenterazine 2-monooxygenase*, *Renilla-luciferin monooxygenase*, *Renilla luciferase*, *firefly luciferase/4-Coumarate CoA-ligase,* and *firefly luciferin sulfotransferase* present in bioluminescent (eg., *Photinus pyralis* and *Luciola cruciata*) and non-bioluminescent species (eg., *Strongylocentrotus purpuratus* and *Lytechinus variegatus*; **Supplementary Table 1**), however, no similarity was observed for other photoproteins and green fluorescent proteins. The evaluation of transcript abundance, in FPKM (Fragment per Kilobase per Million mapped bases), showed lower values for all the transcripts identified (**Supplementary Table 1**).

We also carried out the assembly and annotation of the RNA-Seq available from the other 8 species of Sipuncula for comparative purposes (details about the assembly results available in **Supplementary Table 1**). As described for *N. pellucidum*, no similarity was observed to photoproteins, green fluorescent proteins, or bacterial luciferase. However, we have found gene products related to luciferase and

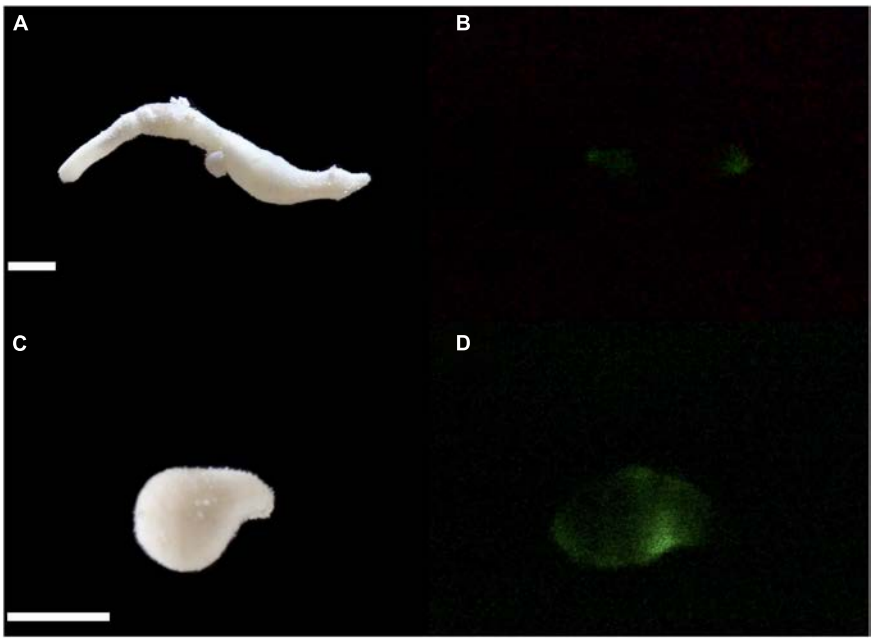

FIGURE 1 | Images of *N. pellucidum* with introvert everted **(A)**, retracted **(C)** and in the dark displaying bioluminescence by mechanical stimulation **(B,D)**. Scale bars = 0.5 cm. Photos: Oliveira G. Anderson. Images were processed using Adobe Premiere Pro 2019.

Renilla luciferin/luciferase in five of them (*Sipunculus nudus*, *A. antillarum*, *Aspidosiphon parvulus*, *Golfingia vulgaris*, and *Phacolopsis gouldii*). The other three species (*Phascolion cryptum*, *P. perlucens*, and *S. cumanense*) displayed a low number of coding sequences, which may explain why we did not recover any of these gene products.

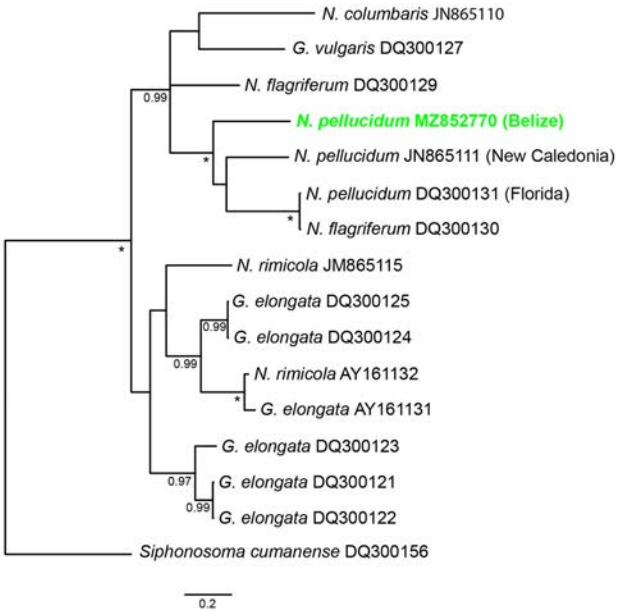

FIGURE 2 | 50% majority rule consensus tree resulting from Bayesian analysis of available COI sequences for *Nephasoma* and *Golfingia* species. Posterior probabilities over 90% are indicated at the notes; asterisks (*) indicate 100% posterior probability. All sequences are labeled with the species name and Genebank accession code.

DISCUSSION

Implications for the Diversity of Bioluminescent Systems in Annelids

The unexpected discovery of bioluminescence in Sipuncula highlights the current lack of knowledge about the distribution, evolution, ecological functions, and mechanisms of bioluminescence in annelids. Although we did not observe bioluminescence in any of the other sipunculan species we collected, sipunculans should be considered in future exploratory studies of marine invertebrate bioluminescence. *N. pellucidum* is a derived species within the Sipuncula, belonging to a larger clade (referred to as Clade III) with poorly resolved taxonomy (Kawauchi et al., 2012; Lemer et al., 2015; Schulze et al., 2007, 2019). None of the other species we collected in Belize fall into this clade; therefore, Clade III may be of particular interest. For many sipunculan species, the COI barcode sequences can be highly divergent within morphospecies, often leading to the detection of cryptic species (Schulze and Kawauchi, 2021). Based on the morphological and anatomical features of the specimens we collected, we are confident with the identification as *N. pellucidum*, despite the relatively high genetic distances to other specimens identified as such. The single sequence for *Nephasoma flagriferum* that falls into the same clade is suspiciously similar (a single nucleotide difference)

to the Florida specimen of *N. pellucidum* and a mistake or sample contamination cannot be excluded for this sample. The transcriptome data analyzed here originated from an *N. pellucidum* specimen from Florida (Lemer et al., 2015). It is unknown whether this specimen had the same bioluminescent properties as our samples from Belize. Future studies on *N. pellucidum* specimens from other locations, combined with transcriptomic analysis from confirmed luminescing specimens, will therefore be important.

Assuming that bioluminescence evolved multiple times independently in annelids, it is difficult to make parallels between the few mechanisms already discovered (Haddock et al., 2010; Verdes and Gruber, 2017). For example, the bioluminescent system of the tubeworm *Chaetopterus variopedatus* has been studied since the early 1960s (Shimomura, 2006). Shimomura and Johnson isolated and crystallized a photoprotein that emitted light in the presence of Fe^{2+}, peroxides and molecular oxygen (Shimomura and Johnson, 1968). Additionally, two other unknown activating factors also stimulated light emission *in vitro*. However, knowledge about the bioluminescence of the *C. variopedatus* is still fragmented and incomplete, due mainly to the numerous difficulties related to the isolation of the luminous components involved in this process (Mirza et al., 2020).

Regarding the Polynoidae family, Nicolas et al. (1982) isolated and partially purified a photoprotein that would be responsible for the light emission in the scale worm *Harmothoe lunulata*. According to some authors, the homogenate prepared from the luminous scales was capable of emitting light through the addition of various reagents, such as sodium dithionite (Herrera et al., 1974), ferrous ions (Lecuyer and Arrio, 1975), xanthine-xanthine oxidase system, and Fenton reaction (Nicolas et al., 1979). The isolated 66 kDa photoprotein was named polynoidin, but there is no additional information available on the chemical studies of bioluminescence in *Harmothoe*, nor any proposal for a chemical structure for its luciferin or methodologies described to isolate it (Nicolas et al., 1982; Shimomura, 2006; Moraes et al., 2021).

Recently, the isolation and structural characterization of a novel luciferin from the fireworm *Odontosyllis* (Syllidae) was published by Kotlobay et al. (2019). By using the polychete *Odontosyllis undecimdonta* as a source of luminogenic material, the authors showed that an unusual tricyclic sulfur-containing heterocycle serves as a substrate in the light emission by this syllid (Kotlobay et al., 2019). Also, Mitani et al. (2019) demonstrated that the luciferase gene of *O. undecimdonta* has a different evolutionarily origin, since this gene has no homology to any other known sequence from a marine bioluminescent system.

The evaluation of transcriptome analyses may suggest three hypotheses to explain the bioluminescence in *N. pellucidum*: (i) a possible association among the enzymes related to firefly and/or *Renilla* luciferase to the bioluminescence emission, (ii) the bioluminescence is caused by distinct sources, such as symbiotic bacteria, although we did not recover any gene product associated with the bacterial luciferase, or (iii) the presence of an unknown (and new) bioluminescent system.

Regarding hypothesis (i), our results obtained from the *N. pellucidum* transcriptome suggest the presence of gene products associated with firefly ligases and sulfotransferases. However, it appears unlikely that any firefly luminescent protein is involved in the light emission mechanism of *N. pellucidum*, since the biochemistry system (including an unknown luciferin) and the photophore morphologic structure are quite distinct between these species. Additionally, other genes related to bioluminescent products from the luminous sea pansy *Renilla reniformis,* such as a *Renilla*-type luciferase, were also found. Considering the green bioluminescence of the *N. pellucidum* species and the low expression of the *Renilla*-type luciferase candidates it is important to clearly state that while these *Renilla*-type luciferases will be interesting to investigate in the future, there is no indication of the potential involvement of these enzymes in the bioluminescence of the species.

The luciferase present in the soft coral *Renilla* is responsible for catalyzing the oxidation of the coelenterazine molecule, a luciferin found in at least eight different aquatic phyla, producing blue light (Jiang et al., 2016; Vacher et al., 2018). Whilst, a *Renilla*-type luciferase presents great tertiary structure similarity, as well as homology, with bacterial haloalkane dehalogenases (Loening et al., 2006; Delroisse et al., 2017). Recently, *Renilla*-type luciferases have been identified in the brittle star *Amphiura filiformis* (Echinodermata, Ophiuroidea) (Delroisse et al., 2017) as well as in the tunicate *Pyrosoma atlanticum* (Chordata, Thaliacea) (Tessler et al., 2020). The bioluminescence color attributed to *Renilla*-type luciferases present in *A. filiformis* and *P. atlanticum* is blue, which does not explain the green color produced by *N. pellucidum*.

In corals such as *Renilla*, the blue light generated by the oxidation of coelenterazine is modulated to a green color through a GFP (Green Fluorescent Protein), through a process of resonance energy transfer (Shimomura, 2006). However, no gene homologous to a GFP or any other fluorescent protein was found in the *N. pellucidum* transcriptome. Yet, some coelenterazine-dependent photoproteins present in a few species of ctenophores are capable of emitting light at wavelengths shifted to the color green, but again no homologous photoprotein genes were detected (Haddock and Case, 1999). Future biochemical experiments will be necessary to corroborate a potential role, or not, of coelenterazine in the bioluminescence of *N. pellucidum*. In addition, in the case of a new bioluminescent system, the development of *in vitro* light emission assays will be necessary to isolate and purify the luminogenic molecular components of this system.

Considering the great diversity of bioluminescence mechanisms in marine annelids, the light emission process in *N. pellucidum* might also involve a new luminescent system (hypothesis ii). A final possibility (hypothesis iii) to be explored is that the bioluminescence in sipunculans is produced by bioluminescent bacteria, i.e., extrinsic bioluminescence. In fact, strains of marine bacteria of the *Shewanella* genus, known to contain bioluminescent species, have been isolated from

sipunculans, such as *Phascolosoma japonicum* (Ivanova et al., 2003). For example, *Shewanella baltica* is a bioluminescent bacterium capable of emitting blue-greenish light (Burtseva et al., 2020). Evidently, all the hypotheses presented here require further investigation.

Conclusions and Future Research Directions

We have now reported on a novel bioluminescent system from a basal branch within the annelid radiation. This discovery has implications for further understanding the evolution, be it convergent or not, of this trait within Annelida. Future research directions include isolating and purifying the luminogenic substances and describing their properties, as well as exploring possible ecological functions. As for our transcriptome analysis, we now have targeted species, containing the luciferase-like gene products, for collection and bioluminescent screening. Additionally, as *N. pellucidum* can be maintained in laboratory culture, there is potential for studying the development of such trait or determining if the light production is diet derived.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MZ852770. The transcriptomes assembly and the photoprotein/luciferase sequences databases are available in FigShare (doi: 10.6084/m9.figshare.16574831).

AUTHOR CONTRIBUTIONS

AO and AS conceived the project. AO, AS, and MH conducted the field and laboratory work. DA performed the bioinformatics analysis. All authors contributed to the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.762706/full#supplementary-material

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