

# Molecular Identification and Phylogenetic Analysis of Bioluminescent Bacteria Isolated from Selected Marine Crustacean in Manila Bay, Philippines

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## Abstract

Bioluminescent bacteria (BLB) are the most widely distributed light-emitting microorganisms, most of which are found as symbionts of free-living marine organisms, including the crustacean species. In this study, 4 out of 7 marine crustaceans in the northeastern section of Manila Bay were found to contain bioluminescent bacteria. Marine crustacean species namely *Thenus orientalis* (Oriental Slipper Lobster), *Oratosquilla oratoria* (Mantis Shrimp), *Penaeus monodon* (Giant Tiger Prawn), *Litopenaeus vannamei* (Whiteleg shrimp), *Scylla serrata* (Mud Crab), *Portunus pelagicus* (Blue Swimming Crab), and *Charybdis feriata* (Crucifix crab), were swabbed after collection. Bioluminescent bacteria were isolated from the inner surface of the carapace, eyes, abdomen, and abdominal segments of the crustacean samples. All glowing colonies were isolated and upscaled. Only bioluminescent bacteria from *T. orientalis*, *O. oratoria*, and *P. monodon* completed the isolation process and were identified using 16S rRNA gene sequencing for phylogenetic analysis. Furthermore, results from molecular identification through Nucleotide BLAST identified that it was *Vibrio alginolyticus* and *Mucus bacterium*. At the same time, the presence of *Vibrio rumoiensis* was also identified which was proved to be understudied and needs exploration. This study was aimed to identify the present bioluminescent bacteria in selected marine crustaceans in Manila Bay, Philippines through microbiological isolation, molecular identification, and phylogenetic reconstruction.

**Keywords:** *Vibrio alginolyticus*, *Vibrio rumoiensis*, Marine Crustaceans, Manila Bay, 16S rRNA Gene Phylogeny

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**Citation:** Santos NC, Cabrera MEB, Flores JD, et al. Molecular Identification and Phylogenetic Analysis of Bioluminescent Bacteria Isolated from Selected Marine Crustacean in Manila Bay, Philippines. *J Pure Appl Microbiol.* 2023;17(4):2119-2130. doi: 10.22207/JPAM.17.4.08

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## INTRODUCTION

*Vibrionaceae* is the most abundant family of bacteria which includes several species of microorganisms that cause pathogenic intestinal tract infection following consumption of contaminated food and water by both humans and animals.<sup>1</sup> *Vibrionaceae* symbiosis is widespread whether it is mutualism, host-pathogen relationship, or commensalism. They are also a part of marine sediments and form a sand biofilm microflora, and are attached to debris, zooplankton, phytoplankton, and carrion.<sup>2</sup> *V. harveyii* is the most common causative agent of vibriosis affecting the fishes and crustaceans. Other *Vibrio* strains including *V. vulnificus*, *V. alginolyticus*, *V. vulnificus*, *V. mimicus*, and *V. parahaemolyticus* are present in most marine crustacean species.<sup>3</sup> In aquaculture, pathogenic strains of *Vibrio* sp. cause mortality of *Penaeus monodon* in hatcheries and coastal areas, which initiates a wide economic loss in agriculture and danger to the public health. *V. alginolyticus*, *V. harveyii*, *V. anguillarum*, and *V. parahaemolyticus* are the major causing species.<sup>4,5</sup> Numerous cases of economic loss and risk to public health were documented caused by Vibriosis outbreaks on both human and animal. Consumption of raw or undercooked seafood and water which are exposed to the natural environment are highly associated with diarrhea, and other intestinal infections.<sup>6</sup> Bioluminescent *Vibrio* sp. is one of the major etiological agents associated with the vibriosis in the pond-cultured *P. monodon* and its pathogenicity was obtained through the intramuscular injection to shrimp. The most dominant luminescent *Vibrio* species from *P. monodon* was *V. harveyii*, followed by *V. logei*, *Photobacterium* sp., and *V. orientalis*.<sup>7</sup>

Some of the *Vibrio* spp. in the family *Vibrionaceae* could produce a bluish-green cold light known as bioluminescence. These bioluminescent organisms produce light through a chemical reaction with the help of an enzyme and proteins in the presence of oxygen.<sup>8</sup> These bacteria, either wild-type or genetically engineered bacteria, produce and emit cold light for communication, capturing prey, attracting mates and for predatory activities.<sup>8-11</sup> Bioluminescent bacteria may be present in some terrestrial and aquatic organism such as annelids and worms, fungi, dinoflagellates,

cnidarians, crustaceans, tunicates, echinoderms, and mollusks, which is regulated by different biochemical reactions.<sup>10-14</sup> These bacteria act as gut symbionts in the digestive tracts of marine fishes, and some act as free-living in marine and brackish water ecosystems.<sup>15-17</sup> They modified their forms and functions to adapt to the challenging environment; for example, the bioluminescence of some species is used as their cloaks of invisibility for security and some were used for communication.<sup>16,14</sup>

Bioluminescent bacteria could be used in different applications, they could be used as the toxin detection in water due to their high sensitivity to pollution,<sup>18,19</sup> and they could also be used for analytical work in bioluminescent imaging and cancer cell therapies, and other biomedical applications for the detection of infection, and malignancies or cancers, because of their enzymes (Luciferase) and reactants which are induced to the bacteria to produce a light signal.<sup>15,20-23</sup> Recent findings from different studies done in the past showed that extracted secondary metabolites from *Vibrio* spp. contain antibiotic properties against gram-positive and gram-negative nosocomial bacteria.<sup>24</sup> This report can be explored to alleviate the current threats of antibiotic resistance and nosocomial infections in clinical settings in the Philippines.

Food and waterborne diseases are the most common outbreaks which remain the leading causes of morbidity and mortality to the public health of the Philippines. In unclean water, microorganisms, toxic substances, and heavy metals mostly cause it. For an instance, in the Philippine Island of Western Mindanao, the villagers have the common symptoms including memory loss, heart and respiratory complications which are signs caused by mercury poisoning of "Minamata Disease". This has been caused by the waste from abandoned mining area that was distributed among the landfill and water sources.<sup>25</sup> Luminescent *Vibrio* sp. has high sensitivity to different concentration of heavy metals which are normally found in the contaminated water and soil. These bacteria could be utilized as biosensor for examining the quality of water, detection of heavy metals and toxic substances present in soil and water.<sup>18,19</sup>

The main objective of the study was to isolate and identify the bioluminescent bacteria that can be found in selected marine crustaceans from Manila Bay, Philippines based on molecular data and phylogenetic relationships. The study may contribute to provide more data on the presence of pathogenic bioluminescent bacteria in marine aquatic organisms. Moreover, this study may contribute to greater knowledge of the pond-culture fishermen on early detection and prevention of these disease-causing pathogens to their fishes and crustaceans that may lead to economic loss.

## MATERIALS AND METHODS

### Sample collection of marine crustacean

The marine crustaceans were collected from Navotas Fishport Complex, Navotas City (14°38'51.797" N, 120°56'52.331" E) northeastern section of Manila Bay. It is the first fishing port that was placed under supervision of the Philippine Fisheries Development Authority and is dubbed as the fishing capital of the Philippines.

### Morphological identification of the marine crustaceans

Each freshly caught marine crustacean species was identified based on the standard protocol for morphological identification of the fish samples using taxonomic keys and morphometric analysis. The verification was based on the journal website of World Register of Introduced Marine Species (WRiMS) and Global Biodiversity Information Facility (GBIF) for the appropriate classification of each crustacean sample.<sup>26</sup>

### Isolation and purification of bioluminescent bacterial isolates

Seven crustacean samples: *O. oratoria* (Mantis shrimp), *T. orientalis* (Slipper lobster), *P. monodon* (Giant tiger prawn), *S. serrata* (Mud crab), *P. pelagicus* (Blue swimming crab), *C. ferriata* (Crucifix crab), and *P. vannamei* (Whiteleg shrimp) were dissected and specific parts -the inner surface of carapace, eyes, abdomen, abdominal segments were swabbed separately. The cotton swabs were streaked on standard formulation of bioluminescent agar components: NaCl, peptone, K<sub>2</sub>HPO<sub>4</sub>, MgSO<sub>4</sub>, glycerol, agar and

distilled water,<sup>19,24</sup> incubated at 28°C for 24 hours. Bioluminescent colonies were picked and purified by standard microbiological streaking.

### Bacterial genomic DNA extraction

A 1 mL of fresh upscaled bioluminescent bacterial cells with estimated cell density (12.0 x 10<sup>8</sup>) based on McFarland turbidity standards were collected. The instructions of the QIAprep® Spin Miniprep Kit were used to extract the genomic DNA (gDNA) of the collected bioluminescent bacterial cells. A 3µL isolated gDNA from the samples was run on a 1.2% agarose gel in 100V for 40mins with DNA marker 1kb plus ladder (Invitrogen).

### 16S rRNA gene amplification

Gene amplification by PCR, components include genomic DNA, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'GGTTACCTTGTACGACTT-3') universal 16S primers.<sup>27</sup> Cycling parameters on thermal cycler: 95°C 5 min; 30 cycles of 95°C 1 min, 60°C 45 secs, 70°C 1 min; 72°C 10 min; hold at 4°C. Post-PCR amplicon exhibited faint smear. Samples were purified prior to sequencing.

### Sequencing and data analysis

Sanger di-deoxy sequencing was carried out. Cycle sequencing involves the incorporation of fluorescently labelled chain terminator ddNTPs; components include: amplicons, primers, and ABI BigDye® Terminator v3.1 Cycle Sequencing Kit. The cycling parameters on Bio-Rad T100 Thermal Cycler: pre-hold at 4°C; 96°C in 1 minute; 25 cycles of 96°C in 10 seconds, 50°C in 5 seconds, 62°C in 4 minutes; hold at 4°C. Ethanol precipitation was added to remove unincorporated ddNTPs, excess primers and primer dimers. Capillary electrophoresis was done on the ABI 3730xl DNA Analyzer using a 50cm 96-capillary array, POP7TM Polymer, and 3730xl Data Collection Software v3.1base calling on the Sequencing Analysis Software v5.4.

Quality control was done by initially converting the raw AB1 files to FASTQ format. The resulting FASTQ files were then subjected to quality control by trimming the first and last 30 base calls (low quality) and additionally filtering for base calls with PHRED quality score of 15 (>97% accuracy)

or better. Sequences with <40% of the remaining bases passing the quality filter were filtered out from the analysis. After quality control of the sequences, multiple sequence alignment (MSA) was carried out. In the MSA, the sample sequences (total of 5), the unique set of top three blast hits (17 sequences), and the downloaded sequences from the genus *Photorhabdus* (3 sequences) were aligned using the L-INS-i algorithm implemented in the MAFFT tool. The resulting alignment was further trimmed to remove gapped regions using the tool TRIMAL, and the resulting alignments in FASTA format were converted to PHYLIP format using a custom script based on Biopython.

#### Molecular Identification using 16S rRNA gene

The sequences of a 16S rRNA gene region amplified from three bacterial samples were obtained. The amplicons were sent to the sequencing laboratory of the Philippine Genome Center (PGC) and had sequenced using 27F and 1492R (Forward and Reverse primer sequences). All sequences that passed the quality control procedure were then converted to FASTA format. All of the sequences that passed the quality control step were used as input to the Basic Local Alignment Search Tool (BLAST). The sequences were queried against the nucleotide database of NCBI GenBank using the BLASTN algorithm.

For each query, the top three database hits were recovered and included in the subsequent analysis and three 16S rRNA gene sequences from the genus *Photorhabdus* were also downloaded for the outgroup analysis of the phylogenetic tree.

#### Phylogenetic tree reconstruction of isolated bioluminescent bacteria

For the phylogenetic tree reconstruction, multiple sequence alignment was carried out as input to reconstruct a phylogenetic tree using the PhyML as a tool, under the K80+I nucleotide substitution model, with 1000 bootstrap replicates. The substitution model was chosen because it has the lowest Bayesian Information Criterion (BIC) for finding the best fit substitution model for the analysis. The resulting maximum likelihood tree was generated initially in Newick format (phym\_ tree.txt) and viewed using the FigTree as a tool. The tree was viewed in rectangular orientation.

## RESULTS

#### Morphologically-identified marine crustaceans

The four out of seven marine crustaceans with bioluminescent isolates collected from northeastern section of Manila Bay belonging to order Decapod were identified and verified based on journal website of World Register of Introduced

**Table 1.** Four marine crustaceans collected from Manila Bay that exhibited luminescence and its bacterial colonies; *O. oratoria* (Mantis shrimp), *T. orientalis* (Slipper lobster), *P. monodon* (Giant tiger prawn), *S. serrata* (Mud crab)

Species	<i>O. oratoria</i>	<i>T. orientalis</i>	<i>P. monodon</i>	<i>S. serrata</i>
Inner surface of carapace	+	+	+	+
Eyes	+	+	+	+
Abdomen	+	+	-	+
Abdominal segments	+	+	-	-

\*(+) Present; (-) Not present luminescent bacterial colonies

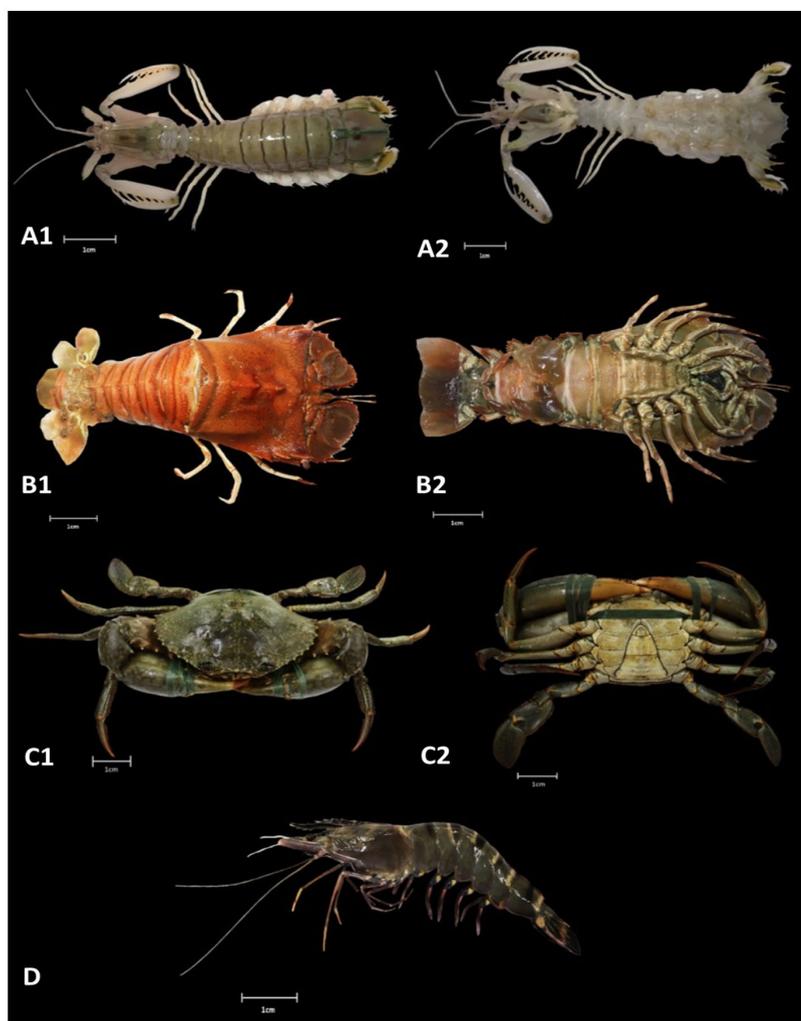
**Table 2.** Significant hits of each bioluminescent bacterial isolates from each species of crustacean and their homologies based on BLASTN with the given accession number

Crustacean Species	Parts of isolation	Identity (%)	Orthologues	Accession Number
<i>O. oratoria</i>	inner carapace	95.010%	<i>V. alginolyticus</i>	MH611367.1
<i>T. orientalis</i>	inner carapace	98.159%	<i>V. alginolyticus</i>	MK167375.1
<i>T. orientalis</i>	eyes	97.917%	<i>V. alginolyticus</i>	MH643643.1
<i>P. monodon</i>	inner carapace	98.082%	<i>Mucus bacterium</i>	AY654787.1
<i>P. monodon</i>	eyes	97.945%	<i>V. rumoiensis</i>	MH712966.1

Marine Species (WRiMS) and Global Biodiversity Information Facility (GBIF)<sup>26</sup> were *Oratosquilla oratoria* (*O. oratoria*)- Mantis Shrimp, *Thenus orientalis* (*T. orientalis*) - Oriental Slipper Lobster, *Scylla serrata* (*S. serrata*) -Mud Crab and *Penaeus monodon* (*P. monodon*)- Giant Tiger Prawn. Figure 1 presents the dorsal and ventral structures of the crustacean specimen, respectively. (A-B) *O. oratoria*- Mantis Shrimp, (C-D)(*T. orientalis*) - Oriental Slipper Lobster, (E-F)(*S. serrata*) -Mud Crab and (G)(*P. monodon*)- Giant Tiger Prawn.

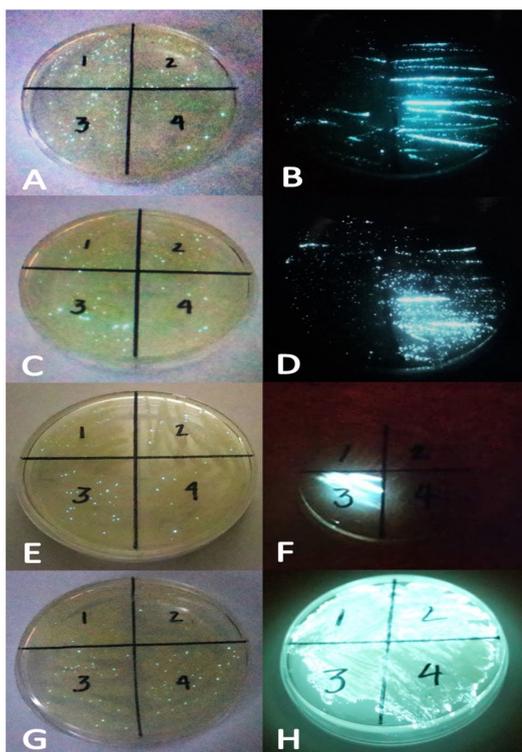
#### Presence of bioluminescent bacterial isolates

The seven marine crustaceans were swabbed from the inner carapace, eyes, abdomen, abdominal segments and were streaked separately in bioluminescent agar. However, four out of seven marine crustaceans *O. oratoria*, *T. orientalis*, *S. serrata*, and *P. monodon* contained bioluminescent bacteria which were successfully isolated. Table 1 shows the presence of BLB in the inner surface of carapace and the eyes, the abdomen of *O. oratoria*, *T. orientalis* and *S. serrata* and the abdominal



**Figure 1.** Four crustaceans species collected from Manila Bay: *O. oratoria* or Mantis Shrimp Lobster (A1. Dorsal and (A2. Ventral); *T. orientalis* or Slipper Lobster (B1. Dorsal and B2. Ventral); *S. serrata* or Mud Crab (C1. Dorsal and; C2. Ventral); *P. monodon* or Giant Tiger Prawn (D)

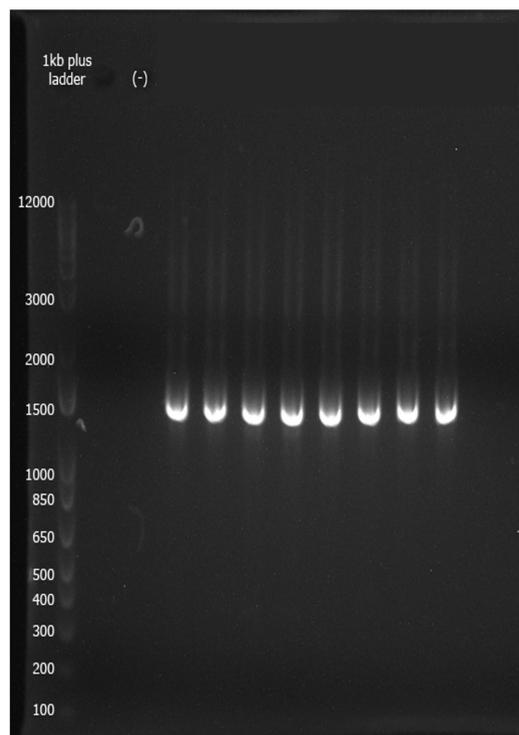
segments of *O. oratoria* and *T. orientalis*. On the other hand, Figure 2 shows the first and second isolation of bioluminescent bacteria from four (4) marine crustacean species collected.



**Figure 2.** The first and second isolation of bioluminescent bacteria from four of the crustacean species collected from Manila Bay: *O. oratoria* or Mantis Shrimp Lobster (A. First isolation and B. Second); *T. orientalis* or Slipper Lobster (C. First isolation and D. Second); *S. serrata* or Mud crab (E. First isolation and F. Second); *P. monodon* or Giant Tiger Prawn (G. First isolation and H. Second)

#### Molecular-based identification of bioluminescent bacterial isolates

The isolated bacteria from the parts of the four crustacean species were upscaled; however, the BLB from *S. serrata* showed no visible pellet, hence was not extracted. The genomic DNA (gDNA) isolation followed the protocols from QIAprep® Spin Miniprep Kit. A 3 µl of the resolved genomic DNA was analyzed by electrophoresis on a 1.2% agarose gel in 100V for 40mins with DNA marker 1kb plus ladder (Invitrogen). All samples showed partially degraded gDNA with thick smeared bands from 1,000bp to



**Figure 3.** Agarose Gel Electrophoresis of amplified 16S rRNA gene region with ~1,500bp size. Post-PCR product was loaded to 1.2% agarose run in 100V for 35mins DNA marker 1kb plus ladder (Invitrogen)

~12,000bp. Isolated gDNA were subjected to gene amplification by polymerase chain reaction (PCR). Components include genomic DNA, 27F and 1492R universal 16S primers, Taq buffer, DNA polymerase, and dNTP mix. Amplicons exhibited intense band smear for each sample—loaded to 1.2% agarose gel run in 100V for 35 minutes DNA marker 1kb plus ladder (Invitrogen). The AGE of amplified 16s rRNA gene showed with region ~1,500bp size (Figure 3). Bioluminescent bacterial strains with complete isolation were processed for the identification based on amplified 16S rRNA gene sequence with an average length of ~770bp size. Based on the analysis, BLAST hits of the five 16S rRNA gene sequences found from the genera of *Vibrio alginolyticus* and *Vibrio rumoiensis*.

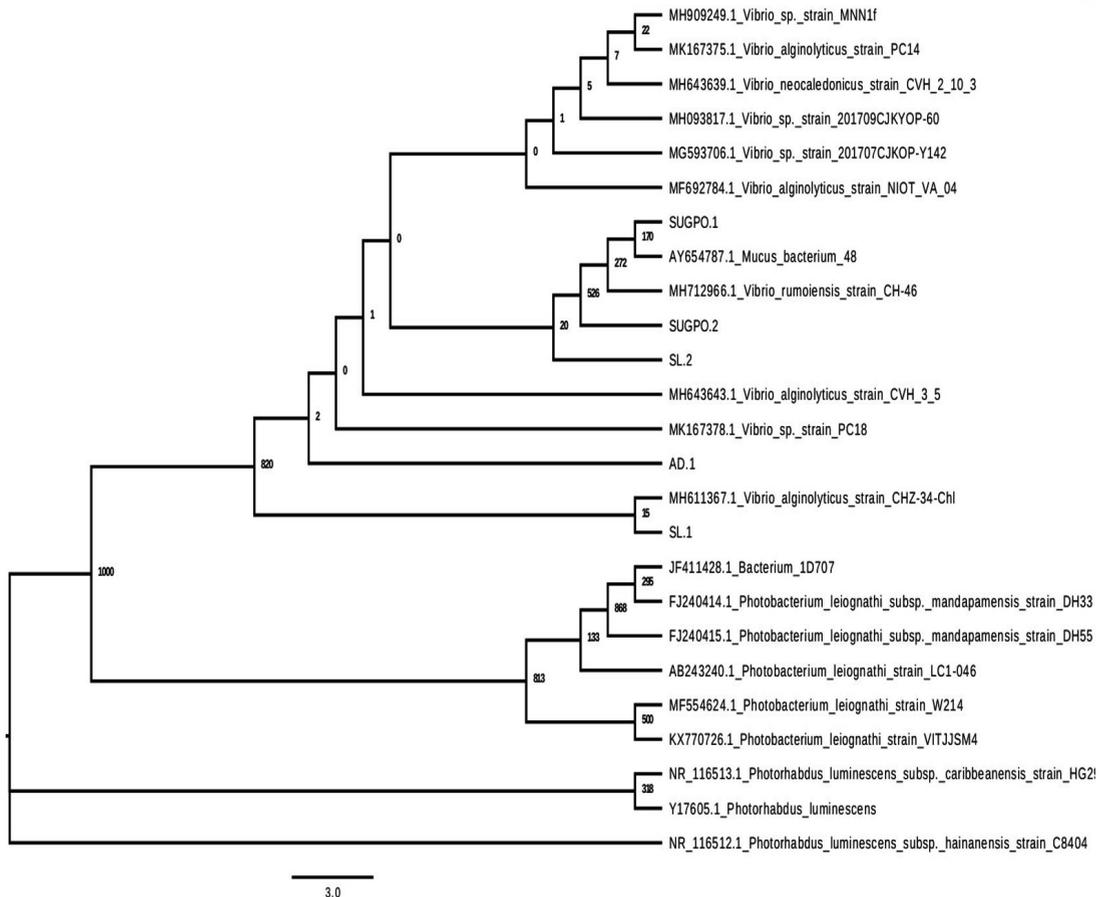
#### DISCUSSION

Bioluminescence is a phenomenon that is widely observed among the diverse groups of

organisms from bacteria to vertebrates.<sup>10,12</sup> One of the largest groups of bioluminescent bacteria is the genera of *Vibrio* which is composed of greater than 100 species clustered into 14 clades under the family of *Vibrionaceae* that are naturally residing in fresh and saline aquatic environments. Approximately 12 out of >100 known *Vibrio* spp. cause infections to humans, infections are normally acquired through exposure to seawater or consumption of undercooked or raw seafoods.<sup>28</sup> For several years, *vibrio* disease outbreaks create an impact on the economic, personal, and public health. The risk factor of consuming raw or undercooked seafood and shellfish exposed to natural environments are strongly associated with

diarrhea, and other intestinal infections.<sup>29,30</sup> In the Philippines, the most common *Vibrio* species are *V. cholerae*, known for its disease-causing outbreak due to consumption of water or undercooked or raw meat and seafood products, followed by *V. parahaemolyticus*, *V. alginolyticus*, *V. harveyii*, and *V. anguillarum* which are commonly found in marine organisms.<sup>4,6,31</sup>

Vibriosis is one of the major disease problems in aquaculture that resulted in high mortality and severe economic loss in the production of shrimps and other crustacean species. The most common causing species are *V. alginolyticus*, *V. harveyii*, *V. anguillarum*, and *V. parahaemolyticus*.<sup>4,31</sup> From the two-year (1983-



**Figure 4.** Maximum likelihood phylogenetic tree of the 16S rRNA gene of the isolated bioluminescent bacteria. The tree was inferred from 16S rRNA gene sequences using MEGA 10 with a 1000 bootstrap replicates. Sample ID were named on the following in the phylogenetic tree (SUGPO.1 – *Mucus bacterium* from *P. monodon* host, SUGPO.2 – *Vibrio rumoiensis* strain from *P. monodon* host, SL.2 – *Vibrio alginolyticus* strain from *T. orientalis* host, AD.1 – *Vibrio alginolyticus* strain from *O. oratoria* host, SL.1 – *Vibrio alginolyticus* strain from *T. orientalis* host).

1984) survey of etiologic survey of diarrheal disease conducted at Manila, Philippines, the prevalence of *Vibrio* pathogens in 2,908 patients with diarrhea admitted was determined: 3.8% *V. cholerae* biotype eltor; 2.8% non-O1 *V. cholerae*; 1.7% *V. parahaemolyticus*; 1.1% other *Vibrio* spp.<sup>6</sup> Aquaculture and fisheries in the Philippines had secured the contribution of food, employment, and foreign exchange. *P. monodon*, together with the *S. serrata* are the major aquaculture species in the Philippines. This is growing much faster than the capture fisheries. In the early 1990s, due to the over production of *P. monodon* using high stocking densities these had led to disease outbreak and mass mortalities, which is mainly caused by luminous vibriosis.<sup>32</sup>

In this study, the bioluminescent bacteria *V. alginolyticus*, *Mucus bacterium*, and *V. rumoiensis* were isolated from the four crustacean species, *O. oratoria*, *T. orientalis*, *S. serrata*, and *P. monodon*, respectively. They are mostly observed in the eyes and inner carapace (head region) of all host organisms. *Vibrionaceae* symbioses are widespread in most marine organisms, whether it is mutualism, host-pathogen relationship, or commensalism. Some *Vibrio* spp. is known for its blue or green-light bioluminescence. The presence of these microorganisms in crustaceans' eyes indicates a symbiotic interaction between the symbiont and its host, which is employed for communication, prey capture, and attracting mates.<sup>9,10,14,16</sup> In most cases, these pathogenic strains of *Vibrio* species had caused mass mortalities on the marine organisms that led to countless economic losses and risk to the public health.<sup>4,5</sup> These bioluminescent bacteria are not only harmful to both human and animal, in other cases, these could be useful for various applications, medically and environmentally. For biomedical application, bioluminescence in bacteria is achieved through the regulation of the oxidative enzymes called luciferase. These enzymes could induce to other bacteria to produce light signal for the bioluminescence imaging used to detect infections, malignancies and cancers.<sup>20-23</sup> Secondary metabolites of non-pathogenic *Vibrio* sp. also have the potential of combating deadly pathogens including *Staphylococcus aureus* and *Klebsiella pneumoniae*, which could greatly contribute to the medical field in the Philippines.<sup>24</sup>

Due to the high sensitivity of bioluminescent bacteria, it could be used in detection of heavy metals and toxic substances present in food and water. For instance, heavy metals from the abandoned mining area have been distributed to the landfill and water sources of the residence in the Philippine Island of Western Mindanao. Early detection of heavy metal present in water and food sources including mercury could possibly prevent the mass poisoning or complications in the community.<sup>18,19,25</sup>

Bioluminescent microorganisms could also serve as bioindicator species on the totality of marine ecosystems.<sup>33</sup> Pathogenic *Vibrio* spp. were isolated from the *Pernaviridis* (Green Mussels) in Bacoor bay, a large inlet of south-eastern Manila Bay, the isolates include *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. Manila bay marine ecosystem is highly contaminated due to the presence of pathogenic *Vibrio* species that may affect the development of juvenile crustacean species and other marine organisms which could severely affect the production of seafood leading to economic loss and danger to public health.<sup>34</sup>

Approximately 1,500bp size of the 16S rRNA gene was amplified from all the marine crustaceans' bioluminescent isolates; indicates a positive relevance of genomic DNA to bioluminescent bacteria with 1,500bp size. In this study, it was determined that the bacteria isolated from the four crustacean species belongs to the genera of *Vibrio*. The 16s rRNA gene sequences of the *V. alginolyticus* isolated from the eyes and inner carapace region of *O. oratoria* and *T. orientalis* showed significant hits: 95.010%, 98.159%, and 97.917%, respectively (Table 2). Molecular identification of *Vibrio* species, including *V. alginolyticus* has been studied and still need exploration for possible intervention in its pathogenesis especially in aquaculture settings. In the study of Xue *et al.*,<sup>35</sup> using a thorough pangenome analysis, the genomes of seven *V. alginolyticus* strains isolated from shrimp larviculture ponds in four provinces of Southeast China were thoroughly characterized. The toxicity of the seven isolates to shrimp postlarvae and their chitin utilization were also determined. These findings may aid in the development and prevention of vibriosis in penaeid shrimp by a thorough examination of the

phylogeny and genetic composition of various *V. alginolyticus* strains, along with their pathogenicity and chitinolytic capacity.

*V. alginolyticus* is a halophilic pathogenic bacterium which is formerly known as biotype 2 of *V. parahaemolyticus* that could be acquired by exposure to contaminated salt water and consumption of raw or undercooked seafood. Pathogenic *V. alginolyticus* can cause severe infections to different crustacean species, as well as in humans. In marine species, pathogenic *Vibrio* rate of virulence and mortality are mainly higher to the crustacean's larval stage due to their ability to produce exotoxins which invade the larval tissues causing necrosis that could lead to mass mortalities. This pathogenic *vibrio* species also devastated the *P. monodon* and shell clam (*Ruditapes decussatus*) hatcheries in the Philippine water that resulted in a mass mortality of juveniles.<sup>7,36</sup> Aside from *P. monodon* as crustacean host for *V. alginolyticus*, a study was carried out in *Penaeus vannamei* (White leg shrimp). High-throughput sequencing was used to study the apoptosis and autophagy involved in the white leg shrimp's response to bacterial infection by identifying miRNA-mRNA interactions in the haemocyte of *Penaeus vannamei* after *Vibrio alginolyticus* injection. As a result of the injection of *Vibrio alginolyticus*, the DNA damage in *Penaeus vannamei* hemocytes increased, and immunological reactions such autophagy and apoptosis significantly increased. The identification of mRNAs and miRNAs from *Penaeus vannamei* infected with *V. alginolyticus* also utilized high-throughput sequencing methods. After the *V. alginolyticus* challenge, a substantial number of miRNAs displayed changed expression, and the majority of them were connected to immunity.<sup>37</sup>

Extremely potent poison known as TTX or Tetrodotoxin are originally found in the ovary and liver of puffer fish are mainly the toxin produced by the marine bacteria *V. alginolyticus* and other *Vibrio spp.*<sup>38</sup> TTX is a potent neurotoxin which blocks voltage-gated sodium channels that causes heart failure, paralysis and consequently death. Due to its paralysis effect, this neurotoxin could be potentially used as an analgesic to treat severe various types of pain.<sup>39</sup> The *V. alginolyticus* isolated from crustaceans in this study can be

explored and further studies should be conducted on the isolation of this neurotoxin. Another study conducted regarding *V. alginolyticus*, they looked at the histological changes in *Macrobrachium rosenbergii* groups that had received experimental intramuscular injections of 50L of 107 CFU of *V. alginolyticus* between the second and third abdominal segment. The study also shown the importance of using immunohistochemistry and histology to diagnose vibriosis in giant freshwater prawns in order to develop a successful control and treatment plan.<sup>40</sup>

In Bangladesh, a study conducted to detect the presence of antimicrobial-resistant *Vibrio* species. The findings supported thorough studies on the prevalence and antimicrobial resistance profiles of *Vibrio* species, particularly isolates of *V. cholerae*, *V. parahaemolyticus*, and *V. alginolyticus* derived from shrimp and shrimp habitats. This shows that antimicrobial-resistant *Vibrio* species are present in shrimp and shrimp settings, necessitating a thorough surveillance program including numerous farms and samples from all across the country.<sup>41</sup> In non-crustacean marine organisms, an outbreak of vibriosis characterized by serious ulcers in the skin was reported in cultured seahorses. It emphasizes the role of *Vibrio harveyi* and *Vibrio alginolyticus* in the disease outbreak in the aquaculture of farmed seahorses and emphasizes the significance of creating early diagnostics and suitable prevention measures to lessen the harm caused by *Vibrio spp.* in a setup for seahorse farming.<sup>42</sup>

Another disease caused by *Vibrio spp.* are extraintestinal, ear, and wound infections when exposed to *V. alginolyticus*, clinical features may include diarrhea, otitis, mild cellulitis and seropurulent exudate.<sup>43</sup> Bacterial isolates from the inner carapace region of *P. monodon* showed a significant hit of 98.082% with 16s ribosomal rRNA gene sequence of *Mucus bacterium*. Isolates from the eyes of the *P. monodon* showed a significant hit of 97.945% (Table 2) with 16S ribosomal rRNA gene sequence of *V. rumoiensis*, a rod-shaped and non-flagellated microorganism with numerous blebs on its surface. Presence of these blebs on its surface could be associated with its ability to grow in a H<sub>2</sub>O<sub>2</sub>-containing environment. The *V. rumoiensis* isolated from the eyes of *P. monodon*

have a symbiotic relationship to thrive in a H<sub>2</sub>O<sub>2</sub>-containing environment. The pathogenicity and function of the *V. rumoiensis* are still unknown; however, this bacterial strain shown a higher catalase activity than other bacterial species such as *Escherichia coli*, *V. parahaemolyticus*, and *Micrococcus luteus*, which are well known for their high catalase activity.<sup>44</sup>

The phylogenetic tree reconstruction analysis of the bacterial isolates illustrates a clear separation of the genera under the Family *Vibrionaceae* in correlation of their hosts and habitats where they are usually observed (Figure 4). The class of Proteobacteria including the genera of *Vibrio*, *Photobacterium*, and *Photorhabdus* were genera of bacteria known to produce bioluminescence or green light.<sup>8-10,16</sup> The constructed phylogenetic tree shows seven major clades. Clade 1 includes *Vibrio sp.*, *V. alginolyticus*, and *V. neocaledonicus* strains. These bacterial strains are commonly found in different marine organisms such as fish, corals, sea urchins, and crustaceans.<sup>2</sup> The phylogenetic analysis of the three bacterial isolates *Mucus bacterium*, *V. rumoiensis*, and *V. alginolyticus* which are respectively isolated from *P. monodon* and *T. orientalis* host, are existed in the Clade 2 which showed a closer phylogenetic relatedness. This could highly be expected since *V. alginolyticus* has been considered as a pathogenic bacterium of some marine crustacean species, including *P. monodon*.<sup>7</sup> Based on the 16S rRNA gene sequences of the bacterial isolate, *Mucus bacterium*, acquired from the NCBI database of GenBank, this isolate was taken from the mucus DNA of *Oculinapatagonica*. Based on the mucus DNA analysis, the most abundant bacteria present in the mucus of *O. patagonica* was *V. splendidus* from winter and summer.<sup>45</sup> The bacterial isolates *V. alginolyticus* from the host of *O. oratoria* and *T. orientalis* has two distinct clades; Clade 3 and 4, respectively. The three 16S rRNA gene sequences from the genus *Photorhabdus* used as an outgroup for the analysis of phylogenetic tree.

## CONCLUSION

The present study through molecular identification and phylogenetic tree reconstruction

revealed that the isolated bioluminescent symbionts in the Philippine marine crustacean hosts were closely related to the Family *Vibrionaceae*—*V. alginolyticus*, *V. rumoiensis* and *Mucus bacterium*. This highlights the evidence of the presence of bioluminescent symbionts in the Philippine marine crustacean hosts. This report presents different bioluminescent bacterial species present in crustaceans and would be of great help in the formulation of techniques for conserving the marine species inhabiting the Manila Bay. The Philippine marine crustaceans were established as bioluminescent symbionts. This study will contribute to the very limited knowledge of the great diversity of microorganisms in the enormous marine organisms thriving in the Philippine marine ecosystem and provide greater knowledge for future researchers about various sources of bioluminescent species that are significant for medical and environmental applications.

## ACKNOWLEDGMENTS

The authors would like to express their sincere gratitude to the people that led to the completion of the study and their Alma Mater, Bulacan State University and to Angeles University Foundation Integrated School, where most of the laboratory work was conducted.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS' CONTRIBUTION

NCS, MEBC, and JDF conceptualized the study and conducted the experiment. NCS, MEBC, and JDF wrote original draft. NCAS, MEBC, JDF and CJNO revised original draft. NCS, MEBC, JDF and CJNO wrote, reviewed and edited the manuscript. RFJ, ORA, AJM and CJNO performed supervision. All authors read and approved the final manuscript for publication.

## FUNDING

None.

## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

**ETHICS STATEMENT**

This article does not contain any studies with human participants or animals performed by any of the authors.

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