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Evidence of bacterial bioluminescence in a Philippine squid and octopus hosts

Maureen Anne A. Naguit, Kirk C. Plata, Rhea G. Abisado, Ronie J. Calugay

Department of Biology, School of Science and Engineering, Ateneo de Manila University, Loyola Heights, Quezon City, 1108 Philippines. Corresponding author: R. J. Calugay, rcalugay@ateneo.edu

Abstract. Bioluminescence is the light emission from living organisms. Despite the tremendous biodiversity existing in the vast and numerous aquatic habitats of the Philippines, bioluminescent bacteria are one of the most poorly studied organisms in the country. In this study, Philippine marine organisms were investigated for the presence of symbiotic bioluminescent bacteria. Bioluminescent bacteria were consistently isolated from marine squid and octopus samples. Based on morphology, the squid commonly known in the Philippines as *pusit lumot* was identified as *Photololigo* sp. of family Loliginidae, and the octopus commonly known in the Philippines as *pugita* was identified as *Cistopus* sp. belonging to family Octopodidae. Sequence analysis of the 16S rRNA gene of the bioluminescent bacterial isolates from the squid and octopus samples, designated as strains ADMU-01 and ADMU-02 respectively, showed that they are both closely related to both *Photobacterium mandapamensis* and *Photobacterium leiognathi* with 99% identity. Phylogenetic analysis showed that the isolates clustered with known representatives of marine bioluminescent bacterial symbionts under the Family Vibrionaceae under which *Photobacterium* spp. belong. To date, this is the first report on the molecular identification and phylogenetic analysis of Philippine bioluminescent bacterial isolates. Most remarkably, this is the first report on the evidence of bacterial luminescence in an octopus, an organism whose bioluminescent counterparts are typically known to emit light only through surface photophores.
Key Words: bioluminescent bacteria, *Photobacterium mandapamensis*, *Photobacterium leiognathi*, *Photololigo* sp., *Cistopus* sp.

Introduction. Bioluminescence is the emission of light from a living organism for attracting mates, capturing prey, and for counterillumination to evade predators. The biochemical reaction is catalyzed by the enzyme luciferase, and luciferin as the substrate in the presence of oxygen (Nealson 1977; Widder 2010). Bioluminescent organisms such as fireflies, annelids, fungi, dinoflagellates, mollusks, and fish emit their own light regulated by biochemical, neural, or endocrine control (Ruby & Morin 1979; Herring 1987; Brejc et al 1997; Widder 2010; Zarubin et al 2012). Bioluminescence, however in certain fish and squids, are typically provided by symbiotic bioluminescent bacteria which thrive in nutrient-rich skin, intestinal tracts, gills, and light organs which the hosts developed over time (Ruby & Morin 1979; Herring 1987; Meighen 1991; Widder 2010; Zarubin et al 2012). These bacteria however, can also exist as free swimming organisms in coastal and open ocean waters (Nealson & Hastings 1979; Widder 2010; Urbanczyk et al 2011). Bioluminescent bacteria rely on quorum sensing, a cell density-dependent process in which the bacteria reach a high cell-density population to accumulate a sufficient concentration of autoinducers to switch on the lux operon comprised by the luxCDABEG genes and express luciferase and other pertinent enzymes for light production (Nealson et al 1970; Nealson 1977; Miller & Bassler 2001; Waters & Bassler 2005; Xavier & Bassler 2005; Dunlap & Kita-Tsukamoto 2006).

Bioluminescence studies on octopus specimens have only been limited to the genera *Japetella* of family Bolitaenidae, commonly found in the oceans of Sri Lanka and Sumatra, and *Stauroteuthis* of Family Stauroteuthidae, found in the North and South Atlantic (Johnsen et al 1999; Sarfati 1999; Zylinski & Johnsen 2011). The bioluminescence in *Japetella* is found in its luminous oral ring used to lure their prey, and

evade predators by photophores or varying its pigmentation via expanding and contracting chromatophores on its arms and mantle (Robison & Young 1981; Zylinski & Johnsen 2011); while in *Stauroteuthis*, are found in their sucking muscles (Johnsen et al 1999; Sarfati 1999).

Bioluminescence has been extensively studied typically in squids and fish hosts, and in other aquatic organisms (Nealson & Hastings 1979; Ruby & Morin 1979; Herring 1987; Brejc et al 1997; Dunlap et al 2008; Widder 2010; Urbanczyk et al 2011; Zarubin et al 2012). Despite the tremendous variety of aquatic organisms from the vast and numerous bodies of water in the Philippines, the presence and diversity of bioluminescent bacteria within local organisms remain virtually uninvestigated. This study, therefore, embarked on the investigation of the presence of bioluminescent bacteria in a Philippine squid, *Photololigo* sp. and in another cephalopod, an octopus *Cistopus* sp. Bioluminescent bacteria were found in the squid samples, and surprisingly, also in the octopus samples. While reported bioluminescent octopods typically emit light through photogenic organs or surface photophores, this study highlights the isolation of a bioluminescent bacterium from an octopus thriving near the eastern coast of the island of Palawan, Philippines. The bacteria from the octopus and squid samples were purified and identified through 16S rRNA gene sequence analysis and found both to be closely related, with 99% identity, to both *Photobacterium leiognathi* and *Photobacterium mandapamensis*, which are reported as common symbionts of squid and fish hosts. To date, this is the first report on the evidence of the presence of bacterial luminescence in an octopus, an organism whose bioluminescent counterparts are typically known to emit light through surface photophores.

Material and Method

Collection of marine and freshwater samples. To determine the presence of symbiotic bioluminescent bacteria in different aquatic hosts, marine and freshwater organisms were purchased from the seafood Farmers Market, Cubao, Quezon City, Metro Manila, Philippines during the period of April to June 2012 early in the mornings at 4:00 am to 6:00 am to obtain freshly caught samples. The sample organisms purchased in triplicates include (as called also by their common names in the Philippines) squid, *Photololigo* sp. (pusit lumot), and octopus, *Cistopus* sp. (pugita). Only samples found with bioluminescent bacteria were brought to the service laboratory of The National Museum of the Philippines, Metro Manila and Center for Research and Development, Angeles University Foundation, Angeles City, Pampanga, Philippines for identification based on morphological features.

Isolation of bioluminescent bacteria. Swabs were taken inside the head and inside the vitreous sac of the eyes of the squid and octopus samples in triplicates and streaked on luminescence agar (Nealson 1978) (4 mL glycerol, 10 g peptone, 1 g $MgSO_4$, 15 g bacteriological agar, 4 g K_2HPO_4, 30 g NaCl, 1 L distilled water). The plates were incubated overnight at room temperature (~27°C). The colonies were observed for bioluminescence in the dark. Distinct luminescent colonies were picked and purified following standard bacterial isolation by repeated streaking on luminescence agar. Gram staining and microscopic examination were done to observe the general morphology of the cell.

Amplification of the 16S rRNA gene of bioluminescent bacterial isolates. Genomic DNA from the bioluminescent bacterial cells was extracted using the method of Wang et al (1994). The universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Abulencia et al 2006) were used to amplify the 16S rRNA gene of the isolates with Kapa Taq PCR kit (Kapa Biosystems, Inc., MA, USA) using the following amplification profile for 20 cycles: 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 60 seconds. Agarose gel electrophoresis of the genomic DNA samples and PCR products was performed in 0.7% and 1% agarose gel (Vivantis) respectively at 100 V for 30 minutes.

Molecular identification and phylogenetic analysis of bioluminescent bacterial isolates. The PCR products were sent to the sequencing service laboratory of AITBiotech, Singapore. The 16S rRNA gene of the isolates was sequenced using 27F and 1492R primers (Abulencia et al 2006). Chromas software was used for the chromatogram editing of the resulting sequences (Goodstadt & Ponting 2001). The resulting forward and reverse 16S rRNA gene sequences were assembled using CAP3 (Huang & Madan 1999). The Database Enabled Code for Ideal Probe Hybridization Employing R (DECIPHER) (Wright et al 2012) was used to determine the presence of chimera in the 16S rRNA gene sequences. The gene sequences were then compared to deposited sequences in the public databases using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul et al 1990).

The 16S rRNA gene sequences of known species of bioluminescent bacteria were downloaded from NCBI database (accession numbers: AB243250.1, AF003548.1, AF493804.1, AJ746357.1, AY455872.1, FJ240416.1, and X74685.1) and were aligned with the 16S rRNA gene sequences of the isolates of this study using ClustalW. The Molecular Evolutionary Genetics Analysis version 6 software (MEGA6) was used to construct the molecular phylogenetic tree of the bioluminescent bacterial isolates (Tamura et al 2013). The Neighbor-Joining method was used to determine the evolutionary history (Saitou & Nei 1987). The evolutionary distance was calculated using Maximum Composite Likelihood (Tamura et al 2004). Bootstrap test was conducted at 1000 replicates (Felsenstein 1985). Only branches with more than 50% bootstrap support were marked with bootstrap values. The tree was rooted with the terrestrial bioluminescent bacterium *Photorhabdus* sp. (AY278667.1).

Results

Isolation of bioluminescent bacteria. Among the aquatic organisms collected, the octopus samples were found to harbor bioluminescent bacteria. The octopus thrives near the eastern coasts of the island of Palawan, Philippines with the common name *pugita* (according to fishermen and local merchants interviewed). Authorized experts of The National Museum, Metro Manila and Center for Research and Development, Angeles University Foundation, Angeles City, Pampanga, Philippines identified the octopus based on morphological features as *Cistopus* sp. of the Family Octopodidae (Figure 1A to D). Bioluminescent bacteria from the octopus were isolated from inside the vitreous sac of the eyes but consistently more inside the head region (Figure 2).

Squid samples with the common name *pusit lumot* (also caught in the coastal waters of Palawan according to fishermen and local merchants interviewed) identified to be *Photololigo* sp. of Family Loliginidae (Figure 1E to H), also harbored bioluminescent bacteria from the head region. To further confirm the presence of bioluminescent bacteria in the said body part and hosts, 17 more samples each of octopus and squid samples were swabbed and yielded the same results. Two bioluminescent bacterial isolates were finally obtained, one from the squid and the other from the octopus sample. The bacterial isolates were observed to be Gram-negative rods.

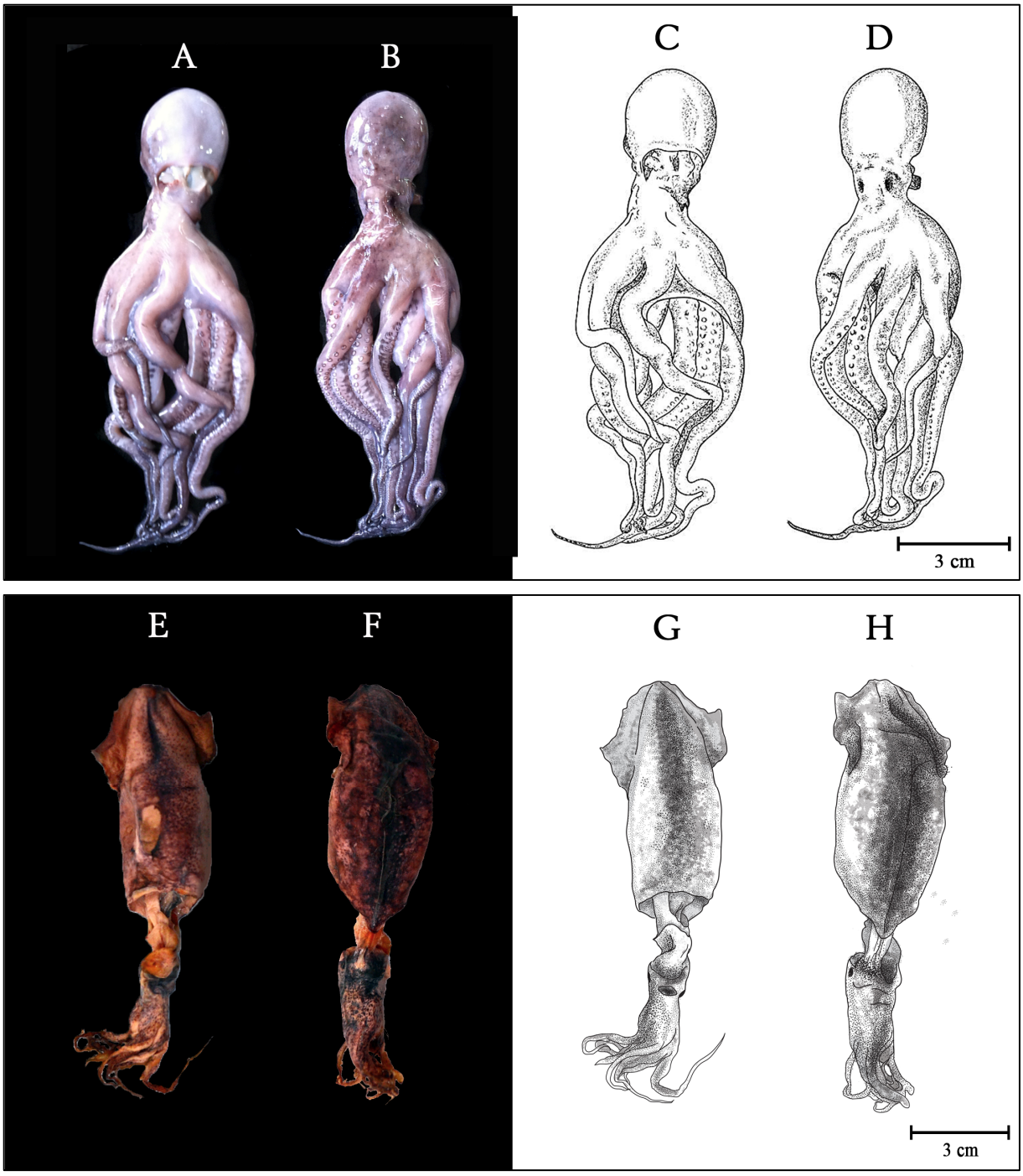

Figure 1. The ventral (A) and the dorsal (B) view of the octopus *Cistopus* sp., with their corresponding sketches (C and D respectively). The posterior (E) and the anterior (F) view of the squid *Photololigo* sp. with their corresponding sketches (G and H respectively).

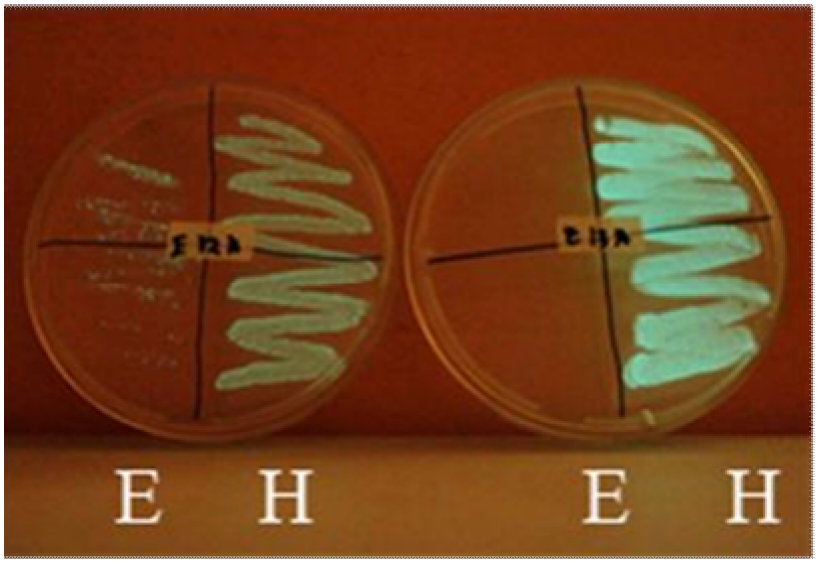

Figure 2. Swabs from inside the vitreous sac of the eyes (E) and inside the head (H) regions of the octopus samples were streaked on luminescent agar and incubated at room temperature (27°C) overnight (shown here in duplicate plates).

Molecular identification and phylogenetic analysis of bioluminescent bacterial isolates. Approximately 22,000 bp genomic DNA was extracted from the bioluminescent bacterial isolates from the octopus and squid samples. Approximately 1,400 bp size of the 16S rRNA gene was amplified from both the octopus and squid bioluminescent bacterial isolates and sequenced. BLAST analysis of the 16S rRNA gene sequences showed that both the octopus and squid isolates showed significant hits (99% identity) with 16S rRNA gene sequences of *Photobacterium* sp. deposited in GenBank. The bioluminescent bacteria isolated from the squid and octopus samples were designated in this study as strains ADMU-01 and ADMU-02, respectively. Figure 3 shows the phylogenetic tree of the bioluminescent bacterial isolates. Both the bacterial isolates from the squid and octopus hosts clustered with representative members of Family Vibrionaceae (*Photobacterium* and *Vibrio*) with a closer evolutionary relationship with species of *Photobacterium*. Among the most commonly known luminescent species of *Photobacterium*, both the 16S rRNA gene sequences of both the octopus and squid isolates showed high similarity with *P. mandapamensis* and *P. leiognathi*. The results also showed that *P. leiognathi* and *P. mandapamensis* are more closely related to the octopus isolate than to the squid isolate.

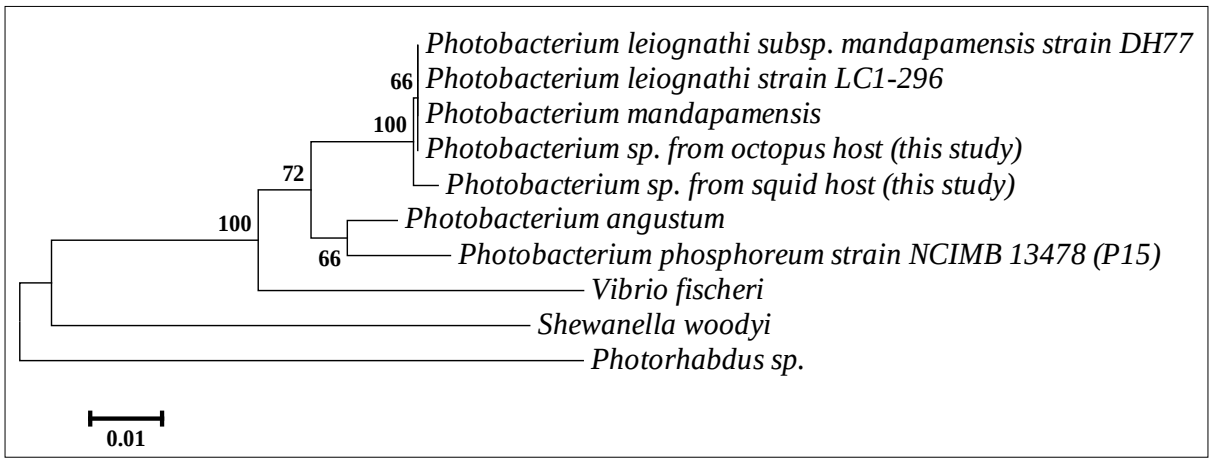

Figure 3. Phylogenetic tree (Neighbor-Joining method) of the bioluminescent bacterial isolates isolated from Philippine octopus and squid host samples based on ~ 1,400 bp16S rRNA gene sequence. The branch lengths were drawn in the units of the number of base substitutions per site. The tree was rooted using *Photorhabdus* sp. (AY278667.1). Bootstrap values, which are based on 1000 replicates, are shown for branches with more than 50% bootstrap support.

Discussion

Isolation of bioluminescent bacteria. As stated before, bioluminescence is the emission of light from living organisms. This biological phenomenon is observed across diverse groups of organisms from bacteria to vertebrates (Nealson & Hastings 1979; Ruby & Morin 1979; Herring 1987; Brejc et al 1997; Widder 2010; Zarubin et al 2012). Among the vertebrates, numerous marine fish and a few families of sharks are bioluminescent (Urbanczyk et al 2011; Claes et al 2014). Bioluminescence is generally formed in two ways: (1) by neural or endocrine control in which light is emitted through chemical reactions within surface cells of the organism or special light organs equipped with lenses and reflectors called photophores (Brejc et al 1997; Widder 2010; Claes et al 2014), and (2) by symbiosis with luminescent bacteria; some bioluminescent fish and squids also use photophores but most have been reported to emit light through symbiotic bioluminescent bacteria present in their skin, intestinal tracts, gills, and light organs (Nealson & Hastings 1979; Haygood & Distel 1993; Dunlap & Kita-Tsukamoto 2006; Widder 2010; Zarubin et al 2012).

There are three large families of γ-Proteobacteria with bioluminescent members: (1) Vibrionaceae, under which are the genera *Vibrio*, *Aliivibrio*, and *Photobacterium,* (2) Shewanellaceae, under which is the genus *Shewanella*, and (3) Enterobacteriaceae, under which include the bioluminescent members of genus *Photorhabdus* (Nealson & Hastings 1979; Dunlap & Kita-Tsukamoto 2006; Yoshizawa et al 2009). These bacteria share a common morphology of being Gram-negative and motile rods, and are facultatively aerobic. *Aliivibrio*, *Vibrio*, *Photobacterium*, and *Shewanella* are usually found in marine than in freshwater environments while *Photorhabdus* is terrestrial. Many marine fish and squid species form symbiotic relationship with three *Photobacterium* species: *P. kishitanii*, *P. leognathi*, and *P. mandapamensis* which usually thrive in the intestinal tracts, gills, skin, and light organs of their hosts (Ast & Dunlap 2005; Flodgaard et al 2005; Dunlap & Kita-Tsukamoto 2006; Wada et al 2006; Kaeding et al 2007; Dunlap et al 2008). Recently, *Photobacterium* symbionts were also found in corals, oysters, and crab haemolymphs (Thompson et al 2005; Chimetto 2010; Gomez-Gil et al 2011).

In this study, bioluminescent bacteria were isolated from a marine squid, and surprisingly from a marine octopus (Figure 1), designated as strains ADMU-01 and ADMU-02 respectively. The bioluminescent bacteria were observed in the eyes but more consistently isolated from the head region of both host organisms (Figure 2). This could be related to the disruptive illumination which some marine fishes employ to evade predators. Since the head has a greater surface area, the density of bioluminescence may be greater in the cephalic region in comparison to the eyes (McFall-Ngai & Morin 1991).

Molecular identification and phylogenetic analysis of bioluminescent bacterial isolates. It was determined in this study that the closest match of the 16S rRNA gene sequence for both strains ADMU-01 and ADMU-02 was *Photobacterium* sp. with 99% identity. This is consistent with previous reports that *Photobacterium* is one of the currently known genera of marine Gram negative γ-Proteobacteria that include luminescent species and considered as common symbionts of marine organisms such as fishes and squids (Reichelt et al 1977; Dunlap and Kita-Tsukamoto 2006; Wimpee et al 1991).

The species of strains ADMU-01 and ADMU-02 are not yet established using the approximately 1,400 bp 16S rRNA gene sequences obtained in this study. Based on the significant hits observed in BLAST analysis, there is high probability that both the squid and octopus isolates are closely related to *P. mandapamensis* or *P. leiognathi*. A 99% identity was shared by strains ADMU-01 and ADMU-02 with both species.

P. mandapamensis and *P. leiognathi* are actually regarded as synonymous to each other because of their niche and phenotypic similarity (Reichelt & Baumann 1975). However *luxF* gene, with function still unknown, is present only in *P. mandapamensis* and absent in *P. leiognathi* (Ast & Dunlap 2004; Kaeding et al 2007; Urbanczyk et al 2011).

In *Photobacterium* species, the *lux* operon genes are followed by *ribEBHA*, genes involved in riboflavin production and in generating luciferase substrate (FMNH2). Such arrangement is referred to as *Photobacterium lux-rib* operon (Lin et al 2001; Ast et al 2007). The genes *lumQ* and *lumP*, coding for the proteins needed in the lumazine operon, are found upstream of the *lux-rib* operon in *P. mandapamensis*. These proteins, when combined with luciferase, are responsible for the change in the spectrum of bioluminescence from blue-green light to blue, and in *P. leiognathi*, *lumP* is not found (Ast et al 2007; Urbanczyk et al 2011). Hence, it is recommended for future studies to analyze the full (1,500 bp) 16S rRNA gene sequences of these isolates and also use additional gene markers such as the *luxF* or *lumP* to completely establish the taxonomic identity of these local bioluminescent bacterial isolates at the species level.

The phylogenetic analysis of the squid and octopus bioluminescent bacterial isolates obtained in this study also demonstrates a clear separation of the two of the three genera under the Family Vibrionaceae related to the habitats where they are usually observed. The constructed phylogenetic tree (Figure 3) reveals three major clades: Clade 1 consists of two sister groups. The first sister group contains strains ADMU-01 and ADMU-02 clustered together with *Photobacterium leiognathi* and *P. mandapamensis*. These species are known to be symbionts of coastal fish and squids thriving in warm and shallow marine waters (Wada et al 2006; Kaeding et al 2007; Dunlap et al 2008). This is consistent with the fact that the squid and octopus hosts in this study were derived from the coastal waters near the island of Palawan. Non-bacterial bioluminescent octopods usually inhabit cold deep sea environments, and they use their ventrally located photophores for counterillumination making them invisible from upward-looking predators (Young 1981; Claes et al 2014). They are also capable of adjusting the light intensity depending on the variable pelagic conditions unlike bioluminescent bacteria which can only emit light in shallow-dwelling nocturnal hosts, such as those in the squid and octopus samples in this study, at nightime when their cell density reaches its peak since they operate via quorum sensing (Nealson & Hastings 1979; Widder 2010; Claes et al 2014).

P. leiognathi and *P. mandapamensis* however are more closely related to strain ADMU-01 isolated from the squid host than to strain ADMU-02 isolated from the octopus host (Figure 3). This may imply that both species may not have distinct host-preference among shallow-dwelling cephalopods such as the squid and the octopus obtained in this study. Among fish hosts, *P. mandapamensis* is predominant in *Acropoma japonicum* and *P. leiognathi* in *Leiognathus* spp. (Kaeding et al 2007). This host-specificity of *Photobacterium* spp., among the diverse Philippine cephalopod hosts, is another interesting aspect for future studies.

Both *P. mandapamensis* and *P. leiognathi* have been extensively reported to have symbiotic associations with marine animals such as fishes and squids, and remarkably, to date, *Photobacterium* spp. or any bioluminescent bacterium has never been reported in any octopod host (Herring & Morin 1978; Kaeding et al 2007; Widder 2010; Urbanczyk et al 2011; Zarubin et al 2012; Claes 2014).

Strains ADMU-01 and ADMU-02 did not cluster with the other sister group of clade 1 which contains *P. phosphoreum* and *P. angustum*. Consistently, *P. phosphoreum* and *P. angustum* do not thrive in the coastal waters but in the open ocean, in mesopelagic, colder marine waters unlike *P. mandapamensis* and *P. leiognathi* (Reichelt et al 1977; Budsberg et al 2003; Ast & Dunlap 2005). *P. phosphoreum* has not been identified or established for bioluminescent symbiosis, but some strains transit between seawater and host and may associate with marine animals as commensals (Reichelt & Baumann 1973; Ast & Dunlap 2005; Dunlap et al 2008). Also, in *P. angustum,* only certain strains are luminescent (Urbanczyk et al 2011).

Clade 2 includes *Vibrio fischeri*. Strains ADMU-01 and ADMU-02 did not cluster with this clade as expected because *Photobacterium* was resolved as a distinct lineage from *Vibrio* by 16S RNA gene analysis (Haygood & Distel 1993). Although both *Photobacterium* and *Vibrio* (Family Vibrionaceae) are commonly reported as bioluminescent bacterial symbionts for marine organisms (Dunlap & Kita-Tsukamoto 2006), the squid and octopus isolates obtained in this study showed closer phylogenetic

relationship with *Photobacterium*. This could be highly expected for the squid since *P. leiognathi* has been considered as an established bioluminescent bacterial symbiont of some loliginid squids (Herring & Morin 1978).

Clade 3 is formed by *Shewanella woodyi*. As an established member of Family Shewanellaceae, *Shewanella woodyi*, a marine bioluminescent bacterium isolated from squid ink, was also resolved as separated from Family Vibrionaceae based on 16S rRNA gene sequence (Haygood & Distel 1993; Urbanczyk et al. 2011). The terrestrial bioluminescent bacterium *Photorhabdus* sp. (Family Enterobacteriaceae) that includes species in symbiotic relationship with nematodes (Fischer-Le Saux et al 1999), was used as an appropriate outgroup for the phylogeny of the bioluminescent bacterial isolates in this study.

No symbiotic bioluminescent bacteria were observed in our preliminary study of the freshwater fish samples collected (data not shown). Almost all species of luminous bacteria emit light in culture media containing over 2% NaCl and these are the marine species. Freshwater bioluminescent bacteria such as *Vibrio albensis* or sometimes referred to as *Vibrio cholerae* biovar *albensis*, have low sodium ion requirement only at 50 mM (Nealson & Hastings 1979; Kasai 2006). Haddock et al (2010) enumerated reasons why bioluminescent bacteria are common in ocean waters: (a) comparatively stable environmental conditions; (b) in large portions of the habitats that receive no more than dim light or exists in conditions of darkness, in murky freshwater, most fish usually utilize their long whiskers to sense their environment; (c) occurrence of interactions among inhabitants; and (d) the ocean environment is much older and more evolved than the freshwater environments. This study, however, does not dismiss the presence of bioluminescent bacteria in other Philippine freshwater organisms.

Conclusions. The Philippines is tagged as the 'center of the center' of the world's hotspots for biodiversity. Its 7,700 islands yield numerous and diverse aquatic ecosystems which harbor highly diverse organisms and symbiotic microorganisms that remain undiscovered and unidentified. Hence, this study aimed to contribute in providing pioneering and foundational information on the diversity of aquatic bioluminescent bacteria in the Philippines. Taken all together, this study highlights the evidence of the presence of bacterial bioluminescence in a Philippine octopod host and squid as well. Molecular identification and phylogeny based on 16S rRNA gene sequence analysis reveal that the isolated bioluminescent bacterium is closely related to *P. mandapamensis* or *P. leiognathi* species. These bioluminescent species are established symbionts of coastal fish and squids, and notably, never been reported before to exist in any octopus, a marine organism known to use photophores for bioluminescence and chromatophores for camouflage. This study contributes to the very limited knowledge of the tremendous diversity of microorganisms existing in the vast and numerous aquatic organisms thriving in Philippine aquatic environments.

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Authors:
Maureen Anne A. Naguit, Department of Biology, School of Science and Engineering, Ateneo de Manila University, Loyola Heights, Quezon City, 1108 Philippines, e-mail: maunaguit@yahoo.com
Kirk C. Plata, Department of Biology, School of Science and Engineering, Ateneo de Manila University, Loyola Heights, Quezon City, 1108 Philippines, e-mail: kirkplata@yahoo.com
Rhea G. Abisado, Department of Biology, School of Science and Engineering, Ateneo de Manila University, Loyola Heights, Quezon City, 1108 Philippines, e-mail: rabisado@ateneo.edu
Ronie J. Calugay, Department of Biology, School of Science and Engineering, Ateneo de Manila University, Loyola Heights, Quezon City, 1108 Philippines, e-mail: rcalugay@ateneo.edu

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