DNA Barcoding of Thiosulfate-Citrate-Bile Salts-Sucrose Agar-Selective Bacterial Species in the Mucus of Acropora millepora from Guimaras, Philippines

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Abstract – The purpose of this study was to identify Thiosulfate-citrate-bile salts-sucrose (TCBS) agar-selective bacterial species present in the mucus of *Acropora millepora* collected in Taklong-Island Marine Reserve, Nueva Valencia, Guimaras. Bacteria sample cultured in TCBS agar was sent to the Philippine Genome Center for the extraction and sequencing of the 16s rRNA gene. DNA Barcoding was done with the use of BioEdit software and BLAST. The bacteria sample cultured and subcultured in TCBS agar plates displayed green colonies, each having a diameter of 0.3 cm. The bacterial 16s rRNAgene was successfully extracted using Quick-DNA Fungal/Bacterial Miniprep Kit, amplified by PCR, and sequenced by Capillary Electrophoresis. The GenBank sequence database result displayed a 98 percent match with *Pseudomonas aeruginosa* 16S ribosomal RNA gene, partial sequence. This study will help assess the health of the *Acropora millepora* by further studying the implications of *Pseudomonas aeruginosa* found in its mucus.

Introduction. – Coral reefs support a high diversity of marine life, serving as home to 25 percent of allmarine lives on the planet, yet only taking up less than 0.25 percent of the entire marine environment. Seventy-five percent of the worlds coral reefs are threatened [13] and most are degrading [1]. Southeast Asias coral reefs are the most threatened in the world, and the Philippines, part of the Coral Triangle, has over 95 percent of reefs at risk. This makes the countrys coral reefs part of the most endangered coral reefs in the region [4]. In recent decades, there has been a dramatic increase on the part of threatened corals. Out of 845 reef building coral species, 32.8 percent of the 704 species assigned with conservation status are at an elevated risk of extinction [5].

The decline of coral cover results to the decrease in marine biodiversity. Unfortunately, even marine reserves are not enough to guarantee the survival of the organisms that greatly rely on corals [11]. A decline greater than 10 percent in coralcover will reduce the abundance of up to 62 percent fish species within three years [22]. Furthermore, a devastating decline reduces over 75 percent of reef fish species, of which 50 percent will halve their original numbers [11], putting rare species at a high risk of extinction [10].

Damage in coral reef systems are linked with coral bleaching where skeletal growth, reproductive activity, capacity to shed sediments, and resistance to competing species and diseases of the corals are reduced [8]. Coral reef bleaching has been observed on coral reefs such as the Great Barrier Reef [17]. Small scale bleaching events have been attributed with particular stressors such as increase in sea water temperatures [7] [3], whereas large scale bleaching cannot be explained by temperature alone [6]. Bleaching has been attributed to changes in ultraviolet radiation (UVR) and sometimes diseases such as Aspergillosis and Dark Spots [3] [14]. Some coral diseases and coral bleaching are caused by certain microorganisms, and findings show that pathogenic bacteria can cause coral bleaching to some corals [2] [12] [19] [20]. For example, bacteria under the genus of Vibrio have been observed to be pathogenic to coral *Oculina patagonica* [21]. These bacteria lives on the mucus of the host coral.

The aim of this research is to identify what TCBS agar-selective bacteria live in the mucus of *Acropora millepora*.

Methods. – *Overview.* The Methods is composed of three main parts, which is the culturing, extraction and sequencing, and dna barcoding.

Thiosulfate-Citrate-Bile Salts-Sucrose or TCBS Agar was prepared to be used for the bacterial culture. This

agar is a form of selective agar that selectively feeds only vibrio species.

For the preparation of the agar plate, the agar powder and water were poured in a media bottle, the agar powder and water having a ratio of 89.09 grams: 1 mL, respectively. After which, the mixture was set to boil and then poured into petri dishes for the bacterial culture.

The mucus samples were collected using sterilized syringes from two colonies of the hard coral, *Acropora millepora*, which was approximately a meter below the surface, from Taklong Island National Marine Reserve in Guimaras. The coral mucus were placed inside 1.5 mL microcentrifuge tubes and then transported to the laboratory in aseptic condition within two hours after collection.

Upon arrival at the laboratory, the mucus samples in the microcentrifuge tubes were then transferred to two centrifuge tubes. The centrifuge tubes were placed in a centrifuge, making sure that the two test tubes face each other to make sure that the centrifuge is balanced in the inside while it is rotating. The centrifuge was set at 2675 rpm for 3 minutes, causing the mucus to suspend on top of the liquid.

The samples then underwent a ten-fold serial dilution using 9mL of distilled water and 1mL of the mucus acquired through centrifugation.

To culture the bacteria, a triangular, glass hockey was used. Initially, the hockey has to be sterilized with the use of heat. After which, 0.1mL of the diluted coral mucus was placed on the agar plate, and then spread throughout the plate using the sterilized triangular, glass hockey. The agar plate was then closed and sealed, and then incubated at room temperature for three days.

After three days, the cultured bacteria were isolated using the Four Quadrant Streaking Method. A sterilized inoculating loop was used to streak the bacteria onto three new agar plates, after which they were then incubated for another three days at room temperature. This method was done once again after three days in order to ensure the purity of the samples. Morphological assessment was then conducted visually with the use of a vernier caliper, a compound microscope and an ultraviolet lamp.

After the assessment, an agar plate, containing the cultured bacteria with isolated colonies was packed tightly then secured to be delivered to the Philippine Genome Center in the National Institute of Molecular Biology and Biotechnology in the University of the Philippines Diliman for the 16S ribosomal RNA Extraction to Sequencing.

When the results arrived, the FASTA-formatted sequence was checked using the BioEdit software and was then inputted in the NCBI-GenBank. Basic Local Alignment Search Tool (BLAST) was used to find matching sequences in the NCBI databank. The highest matching sequence identifies the species of the bacteria.

Results. – This study aimed to culture and isolate TCBS agar-selective bacteria present in the mucus of *Acropora millepora* and extract and sequence their 16S rRNA for barcoding. Samples of *Acropora millepora*'s mucus was collected, prepared and plated on a TCBS agar plate for three days of incubation. Bacterial colonies were streaked on separate agar plates for subculturing after the third day. Bacterial morphology was assessed visually and with the use of a vernier caliper, compound microscope, and ultraviolet lamp. The petri dish containing the subcultured bacteria with isolated colonies was sent to the Philippine Genome Center for the bacterial 16S rRNA gene extraction to sequencing. The sequenced 16S rRNA was then analyzed using the BioEdit Version 7.1.9 and uploaded in NCBI-GenBank to identify the organism and gene using BLAST.

Culture TCBS agar-selective Bacteria. After three (3) days of incubation, all three petri dishes, A, B and C, plated with the coral mucus showed signs of bacterial colony growth. Only petri dish C showed isolated colonies numbering up to 12 distinct isolated colonies while the other two petridishes (A and B) had coalesced colonies. The colonies were green in color and around 0.3 cm in diameter. There was no bioluminescence observed after exposing the petri dishes to ultraviolet lamp. Under the microscope, there were no distinct differences observed; however, bacterial morphology was not thoroughly assessed. The subcultured petri dishes C1, C2, and C3 streaked from the isolated colonies of petri dish C also showed bacterial growth after three days of incubation. Subcultured petri dishes SC1, SC2, and SC3 streaked from petri dishes C1, C2, C3 respectively also showed bacterial growth after three days of incubation. SC2 and SC3 had isolated colonies.

DNA Extraction and Sequencing of 16s rRNA. The extraction to sequencing was done by experts of Philippine Genome Center. The sequencing result includes four files, two of which are of AB1 file and the other two of SEQ file. BioEdit shows a query length of 955 bases.

DNA Barcoding. The FASTA formatted sequence is a nucleic acid with query length of 955. The GenBank sequence database displayed *Pseudomonas aeruginosa* 16S ribosomal RNA gene, partial sequence. The description section shows a max and total score of 1555 and a 98 percent similarity, in terms of residues at the same positions in an alignment, with the FASTA formatted sequence emailed by the PGC. In the alignments section, the partial sequence of *Pseudomonas aeruginosa* displays 1555 bits(842) in score, 869/886(98 percent) in identities, and 4/886(0 percent) in gaps. Other alignments also shows *Pseudomonas aeruginosa* partial sequences with 98 percent identities.

Discussion. After the DNA Barcoding, the cultured bacteria was identified to be *Pseudomonas aeruginosa.* This indicates that the green colonies that grew in the TCBS agar are colonies of *Pseudomonas aeruginosa* and that no *Vibrio* bacteria is present in the mucus of *Acropora millepora* collected in Taklong Island National Marine Reserve. TCBS Agar is usually used to culture Vibrio species but inhibited only *Pseudomonas aeruginosa* which

could also grow in the TCBS agar [18] but with poor to no growth and green in colony color [16]. This result shows the presence of *Pseudomonas aeruginosa* in the mucus of *Acropora millepora* in TINMR.

Other studies conducted regarding coral-associated bacteria such as the study of Littman et. al (2009) [15] also received *Pseudomonas*-affiliated sequences from Magnetic and Orpheus Island clone libraries when they were studying the diversity of coral-associated bacteria in acroporid corals in the Great Barrier Reef. In a study conducted by Guimaraes et. al (1993) [9] on marine waters, they found out that as coliform, fecal streptococci, presumptive pathogenic yeast and heterotrophic bacteria count increase, there is also a notably increase in the occurrence of *Pseudomonas aeruginosa*. This could possibly explain the presence of *Pseudomonas aeruginosa* in the marine water; thus it is not impossible but that they could have possibly been present on the coral mucus too.

A correlation between the health of the water and the health of the coral cannot be made since our study focused on just identifying the bacterial species thriving on the coral mucus using a TCBS agar and not necessarily its abundance.

Conclusion. – Culturing of bacteria from the coral mucus in TCBS agar, extraction to sequencing of the bacterial 16S rRNA, and DNA Barcoding the result gives a 98 percent match with the bacteria *Pseudomonas aeurigonosa*.

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