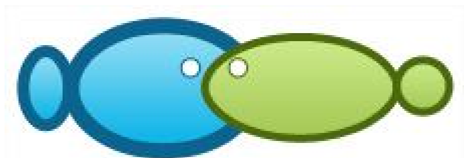

Culture-dependent and 16S metagenomics analyses of blue-bell tunicate *Clavelina coerulea* microbiome

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Abstract. The season diversity of the microbial associated with the blue bell tunicate, *Clavelina coerulea* was investigated in this study. Marine tunicate samples were collected from Pulau Bidong, Terengganu during the pre-monsoon (October) and post-monsoon seasons (May). Using the culture dependent approach, bacteria were isolated, morphologically characterized, and genetically identified by sequencing the 16S rDNA. Forty-six (46) bacteria isolates were obtained with significant variation observed in both seasons. However, majority of the bacterial isolated belonged to the class γ-Proteobacteria. Microbiota enumeration was also done using the culture-independent approaches and Next Generation Sequencing (NGS) technology. Result obtained revealed 764,742 valid reads belonging to 343 total operational taxonomic units (OTUs). Of all the 11 phyla associated with the tunicate, Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria and Acidobacteria were the most prevalent in both seasons. Also, 49 families were found in both season out of the 91 microbiota identified. The families left were exclusive to the post and pre-monsoon season (20 and 22 families respectively). Since salinity was the most varied water quality observed in the current study, it was thought to be one of the major drivers of the seasonal variation in bacterial diversity associated with the blue bell tunicate, *C. coerulea*.
Key Words: blue bell tunicate, microbiota culture, microbial analysis, NGS technology.

Introduction. The ocean is considered the most biologically diverse ecosystem on the planet as it covers more than 70% of the earth surface (Larsen et al 2005). Microbiota of the marine environment is an important component of the ecosystem and can thrive as free living organism or attached to abiotic surfaces or in/on biotic components of the seawater (Nurul et al 2019, 2020; Okomoda et al 2020). Studies on the microbiota composition of different biotic components of the ecosystem are important as this sort of interaction influences several biological processes (Arrieta & Finlay 2012; Buchon et al 2013; Arrieta et al 2014; Dishaw et al 2014). More so, since the ecological communities of the ocean are very dynamic, the aquatic microbial communities therefore change in response to seasonal variation and period (Magurran et al 2010; Gilbert et al 2012; Hatosy et al 2013). Although, annual surveys by Fuhrman et al (2006) had demonstrated that the structure of marine free-living bacterial communities can be predicted from ocean conditions, the same cannot be assumed for microbiota communities attached/associated with marine vertebrate or invertebrates (Bunse & Pinhassi 2017). Unfortunately, this area of research has not been given much attention.

Benthic organisms such as tunicate have been reported to have surfaces rich in nutrient which are uniquely suited for the colonization of different microbiota (Egan et al

2008; Goecke et al 2010). The survival of many tunicates is directly dependent on the symbiotic relationship with bacteria colonies on their surfaces as this helps in the primary and secondary metabolic functions needed by the invertebrate (Lambert 2005). Among the metabolic functions is the production of bioactive metabolites which mediates ecological stressors such as micro/macro-fouling organisms, competition for food and space, as well as predator/invader deterrence (Donia et al 2011). This characteristic has made tunicate a choice destination for the exploitation of potential bioactive compounds and natural product used in pharmaceuticals products (Paul et al 1990; Schmidt et al 2012; Ali & Tamilselvi 2016). These compounds are effective antimicrobial, antineoplastic, antitumor, antifouling, antioxidant, anti-inflammatory and anti-multidrug resistant agents (Ayuningrum et al 2019). They have also been reported to possess plant growth regulatory activity, deterrent activity, insect control, wound healing activity, hepatic productive activity and immune stimulating activity (Fattoruso et al 2012; Kim 2015). However, only a few of the tunicates species have been analyzed for their microbiological communities and content.

Ascidians are about the most diverse group of tunicates in the marine ecosystem with an estimated number of 3000 species (Lambert 2005; Kim et al 2012). These sea squirts are wildly distributed around the world and can be found tied to rocks and high-current fields (Shenkar & Swalla 2011). Despite the fact that ascidians are ecologically important components of many marine ecosystems, the microbiota composition still remains largely unexplored with exception of a few model species (Erwin et al 2014). One of such understudied and less known ascidians is *Clavelina coerulea* of the colonial ascidians belonging to the family Clavelinidae. It is one of the major dominant tunicates species of the Bidong Island, Terengganu, in Malaysia (Saari 2014). The Bidong Island is also home to a number of unique marine invertebrates such as sponges, sea cucumber, and other tunicates. This study was designed in an attempt to explore the seasonal diversity of bacteria associated with the tunicate *C. coerulea* in Malaysian waters (especially in Bidong Island) by employing both the culture-dependent and in-dependent approach. Using the culture-dependent method, all isolates from the tunicates samples in this study were identified by the sequencing of the 16S rRNA gene to examine the diversity of culturable bacteria isolates. The culture-independent method however, employed the 16S metagenomic analysis by Next Generation Sequencing (NGS). The combination of both approaches is believed to be a robust strategy in obtaining a more comprehensive view of the bacterial diversity of the tunicates sample, since the limitation of one approach might be mitigated by the use of the other (Ward et al 1995).

Material and Method

Tunicates sample collection. The sampling for this study was carried out in October 2012 and May 2013, respectively at the Bidong Island, Terengganu, Malaysia (N $05^{o}36.815'$ E $103^{o}03.418'$) before the start of the raining season i.e. pre-monsoon (October 2012) and shortly after the raining season, i.e. post-monsoon (May 2013). The northeast monsoon season usually commences in the early part of November and ends in March (Malaysian Meteorological Department 2012). Hence, the sampling for this study was dictated in accordance with the period when Scuba diving is permitted within the Bidong Island (i.e. after the rains stops and before it begins). The water quality readings of the seawater such as salinity (ppt), temperature (°C), dissolved oxygen (DO) (mg L^{-1}) and pH (units) were also determined on site using the YSI Handheld Multiparameter Instrument.

During the sampling for this study, colonies of *C. coerulea* were collected by SCUBA divers from a depth of 10-15 m in the Island. The specimens were placed into plastic bags to avoid prolonged exposure to atmospheric oxygen when brought to the surface. The plastic bags were then placed on ice and transported to the Anatomy and Physiology Laboratory of the Faculty of Fisheries and Food Sciences, Universiti Malaysia Terengganu for analysis. Samples that were not analyzed immediately were stored in -80°C till it was convenient to analyze them. The collected tunicates species were identified using Ascidiacea World Database (Shenkar et al 2012) and tunicate atlas (Monniot & Monniot 1997; Kott 2004).

Isolation of the bacteria associated with Clavelina coerulea using culture dependent approach. Approximately 5 g of the tunicate sample was rinsed with sterilized seawater to remove the loosely attached bacteria. Thereafter, sterilized seawater was added to the sample in a ratio of 9:1 and cut into small pieces using a sterilized scalpel before been homogenized using sterilized mortar. The homogenate was serially diluted up to 6-fold, then 100 μL of each dilution was spread onto the surface of Marine Agar (Difco, USA). All the plates were then incubated for 14 days at 28°C to ensure higher yield and increase the detection of pigmented bacteria. After the 14 days incubation, a representative of each colony morphotype was serially streaked on Marine Agar until pure cultures were obtained. All these procedures were carried out in a laminar air flow cabinet aseptically. Morphological characterization of each pure colony (i.e. bacterial cell morphology) was determined by the Gram-staining (Gram 1884).

PCR amplification of 16S rDNA and sequence analysis. The preparation of the DNA template for PCR amplification was according to the method described by Abdullah (2009). Briefly, single colony was picked from an overnight culture and re-suspended in 500 μL TE buffer (pH 7.8). Then, 200 μL of the bacteria suspension was transferred to a PCR tube and boiled at 100°C for 10 min. Thereafter, 4 μL of the boiled bacteria suspension were used as the DNA templates for the 16S rDNA amplification through the polymerase chain reaction (PCR). The PCR amplification of the 16S rRNA gene from the chromosomal DNA was achieved using the primer pair 8F, 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R, 5′-GTTTACCTTG TTACGACTT-3′ (Wellinghausen et al 2004). This was done in a 50 μL reaction containing DNA template, 1× Reaction Buffer, 2.5 mM $MgCl_2$, 200 μM of each dATP, dCTP, dGTP and dTTP (Vivantis Technologies, Malaysia), 0.5 μM of each primer and 2 U of *Taq* polymerase (Vivantis Technologies, Malaysia) using the PTC-0200G thermo cycler (Bio-Rad Laboratories, Inc., USA).

The PCR condition used was as follows: preheating at 95°C for 5 min; 26 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 2 min; followed by 72°C for 10 min. Fragment sizes of the PCR products were determined by electrophoresis using 1% agarose gel stained with 1× SYBR Safe DNA Gel Stain (Life Technologies, USA) in 1 × TAE buffer. The gel was visualized under the ultraviolet light and images taken using Alpha Imager 2200 (Alpha Innotech, USA). Direct sequencing of the PCR products were performed using an ABI 3730XL automated DNA sequencer (PE ABI, USA). Bidirectional sequencing was performed by FirstBase Sdn. Bhd. The raw and unaligned 16S rDNA full sequences were edited using sequence assembly software DNA Baser Sequence Assembler v3.x (2012), (Heracle BioSoft SRL Romania). The sequences were then compared with sequences from EZ-Taxon-e server (http://eztaxon-e.ezbiocloud.net/) (Kim et al 2012) and GenBank database to obtain the closest phylogenetic neighbours. All obtained sequences have been deposited in the GenBank with the following accession numbers obtained: MG896123 - MG896168.

Analysis of bacteria community by 16S metagenomic sequencing (culture independent approach). About 10 mL of the homogenate tunicate sample (following the procedure earlier described in the culture dependent method) was used to extract the bulk DNA in this study. The bulk DNA was extracted using GeneJET™ Genomic DNA Purification Kit (Fermentas, USA). In the first PCR, the 16S rRNA gene was amplified using universal bacterial primer set 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1389R (5′-ACG GGC GGT GTG TAC AAG-3′) (Hongoh 2003). The PCR reagent mixture and protocol (Ruh et al 2009) was done using the the following conditions: 2 min initial denaturation at 95°C followed by 24 cycles of denaturation (30 s at 95°C), annealing (1 min at 53°C), extension (2 min at 72°C) and a final extension at 72°C for 10 min. This study also included a negative control to the PCR amplification to confirm that the reagents were not contaminated during the course of this study.

Illumina library generation: amplicon PCR. A second PCR was used to amplify the V3 region of the 16S rRNA gene in accordance with the method previously described by Bartram et al (2011). This method was selected because of its taxonomic resolution (Huse et al 2008), conserved flanking regions (Muyzer & de Waal 1993), and length

(Gloor et al 2010) (≥ 170 to 190 nucleotides) which is compatible with paired-end 125-base read assembly. The amplification of the V3 [aatcatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctCCTACGGGAGGCAGCAG (V3_F = used for all samples); caagcagaagacggcatacgagat**TCAAGT**gtgactggagttcagacgtgtgctcttcccgatctATTACCGCGGCTGCTGG (V3_8R = used for post-monsoon); caagcagaagacggcatacgagat**TCGCTT**gtgactggagttcagacgtgtgctcttcccgatctATTACCGCGGCTGCTGG (V3_16R = used for pre-monsoon)] region of the 16S rRNA gene was therefore done using the 341F (CCTACGGGAGGCAGCAG) and 518R (ATTACCGCGGCTGCTGG) universal primers (Muyzer & de Waal 1993; Bartram et al 2011). Also, the V3 specific priming regions primers were complementary to the standard Illumina forward and reverse primers. Hence, the primers contained a 6-bp index (bold) allowing for multiplexing.

This second PCR amplification was done using 25 µL reaction mixtures containing 0.5 µL KAPA HiFi DNA Polymerase (1 U $µL^{-1}$), 5.0 µL of PCR buffer (1X), 0.75 µL of each primers (10 mM), 0.5 µL DNA templates (10-100 ng) and PCR-grade water up to 25 µL. The PCR conditions involved an initial denaturation step at 95°C for 5 min followed by 15 cycles of 98°C for 20 sec, 67°C for 15 sec, and 72°C for 1 min and ended with an extension step at 72°C for 7 min in a DNA Engine thermocycler (Bio-Rad, Mississauga, Ontario, Canada). Following separation of products from primers and primer dimers by electrophoresis on a 2% agarose gel, PCR products of the correct size were recovered using a Thermoscientific Gel Extraction kit (Thermo Scientific, Wilmington, DE). The purified PCR products with purity between 1.8 and 2.0 and weigh about 30-100 µg were then sequenced using Illumina MiSeq Desktop Sequencer (Illumina, Inc.) at the Science Vision Sdn. Bhd., Malaysia.

16S rRNA-based taxonomic analysis. The trimming and assembling of the generated multi-million reads was done using the Mothur software (Schloss et al 2009). Overlapping regions within Illumina paired-end reads were aligned to generate "contigs." In an even that a mismatch was sported, the paired-end sequences associated with such assembly were discarded. In the same vein, all sequences with ambiguous base calls were also not used. Sequences were then assigned taxonomic affiliations based on naïve Bayesian classification (RDP classifier) (Wang et al 2007).

After the trimming, screening, and alignment of the sequences, they were assigned to operational taxonomic units (OTUs) of the 16S rRNA gene fragments. The sequenced data was connected to the server and the fastq file was downloaded. A tab-delimited "oligos" file containing the primer and barcode information was created. Then, the Greengenes reference files from the Mothur website (http://www.mothur.org/wiki/Taxonomy_outline) were used to analyze the data. The OTUs of the microbiota were defined by a pairwise similarity cutoff of 97% using the Ribosomal Database Project pyrosequencing pipeline according to Cole et al (2009). All the sequence reads generated were deposited in the GeneBank with Accession numbers SAMN15516361, and SAMN15516362.

Results. The water quality parameter variations between the two seasons are presented in Table 1. Out of the parameters recoded, salinity was the most varied with the highest value observed in the post-monsoon season compared to the pre-monsoon season (34.74 vs 31.40 ppt respectively).

Table 1

Water parameters during post-monsoon and pre-monsoon seasons

Parameter	*Post-monsoon season*	*Pre-monsoon season*
Temperature (°C)	29.12±0.82	28.20±0.54
Dissolved oxygen (mg L^{-1})	8.02±0.43	8.74±0.52
pH	6.51±0.23	6.70±0.39
Salinity (ppt)	34.74±0.22	31.40±0.82

Bacterial colony morphology isolated from Clavelina coerulea using culture dependent approach. Table 2 shows the 46 bacteria isolates of the blue bell tunicate, *C. coerulea* (comprising of 22 and 24 isolate for post-monsoon season and pre-monsoon season respectively) recorded in this study. Thirty-nine (39) of these isolates were Gram-negative while the others (7 isolates) were Gram-positive. Based on the colony morphological traits most of the bacteria isolates had vibrio-like shaped cell (52.17%); others were short rod or coccobacilli, cocci, and bacilli shape.

Table 2

Colony and cell morphology of the microbiota isolated from *Clavelina coerulea*

Bacteria isolate	*Colony and cell morphology*			
	Gram's reaction	*Cell shape*	*Colony shape*	*Colony colour*
UMTBB101	Negative	Vibrio	Round	Light orange
UMTBB102	Negative	Bacilli	Round	Light brown
UMTBB103	Negative	Vibrio	Round	Light yellow
UMTBB104	Negative	Vibrio	Round	Yellowish orange
UMTBB105	Negative	Vibrio	Round	Creamy orange
UMTBB106	Negative	Vibrio	Irregular	Creamy orange
UMTBB107	Negative	Vibrio	Irregular	Milky white
UMTBB108	Negative	Vibrio	Irregular	Translucent yellow
UMTBB109	Negative	Vibrio	Round	Whitish orange
UMTBB110	Negative	Vibrio	Round	Creamy yellow
UMTBB111	Negative	Vibrio	Round	Cream
UMTBB112	Positive	Cocci	Irregular	Light yellow
UMTBB113	Negative	Bacilli	Irregular	Creamy yellow
UMTBB114	Negative	Vibrio	Irregular	Yellowish cream
UMTBB115	Negative	Vibrio	Round	Creamy yellow
UMTBB116	Negative	Bacilli	Irregular	Light orange
UMTBB117	Negative	Bacilli	Irregular	Translucent brown
UMTBB118	Negative	Vibrio	Irregular	Creamy yellow
UMTBB119	Negative	Vibrio	Irregular	Creamy orange
UMTBB120	Negative	Coccobacilli	Round	Orange red
UMTBB121	Negative	Coccobacilli	Round	Dark green
UMTBB122	Negative	Coccobacilli	Round	Dark purple
UMTBB201	Negative	Vibrio	Round	Brown
UMTBB202	Negative	Vibrio	Round	Cream
UMTBB203	Negative	Vibrio	Round	Cream
UMTBB204	Negative	Vibrio	Round	Yellowish cream
UMTBB205	Positive	Bacilli	Round	Creamy orange
UMTBB206	Positive	Cocci	Round	Yellow
UMTBB207	Negative	Bacilli	Round	Orange cream
UMTBB208	Negative	Vibrio	Round	Opaque orange
UMTBB209	Negative	Bacilli	Round	Translucent cream
UMTBB210	Positive	Cocci	Round	Creamy yellow
UMTBB211	Negative	Vibrio	Round	Whitish cream
UMTBB212	Negative	Vibrio	Round	light cream
UMTBB213	Negative	Vibrio	Round	Opaque cream
UMTBB214	Negative	Vibrio	Round	Cream
UMTBB215	Positive	Bacilli	Round	Cream
UMTBB216	Negative	Coccobacilli	Round	Translucent orange red
UMTBB217	Negative	Coccobacilli	Round	Dark green
UMTBB218	Negative	Coccobacilli	Round	Dark purple
UMTBB219	Negative	Coccobacilli	Round	Cream
UMTBB220	Negative	Coccobacilli	Round	Light orange
UMTBB221	Negative	Coccobacilli	Irregular	White
UMTBB222	Positive	Cocci	Round	Cream
UMTBB223	Negative	Vibrio	Round	Cream
UMTBB224	Positive	Cocci	Irregular	Yellow cream

Genetic confirmation and phylogenetic relationships of the microbiota associated with Clavelina coerulea isolated using the culture dependent approach. All the sequence of the isolated microbiota gotten in this study has been deposited in the GeneBank (Accession numbers MG896123 - MG896168). Table 3 showed the identity of the isolates based on the partial 16S rRNA gene sequences (1290 to 1574 nucleotide positions) available in the EZ-taxon database (Table 3a) and the GenBank database using the BLAST tool (Table 3b).

Table 3a

Phylogenetic affiliation of bacteria isolated from Blue bell tunicate, *Clavelina coerulea* based on EZ-Taxon database

Bacteria isolate	*Closest nucleotide sequences in EZ-Taxon database*	*Percent of similarity (%)*	*Phylogenetic affiliation*
	Post-monsoon season		
UMTBB101	*Vibrio campbellii* (XZ74692)	97.6	γ-Proteobacteria
UMTBB102	*Ferrimonas marina* (AB193751)	99.8	γ-Proteobacteria
UMTBB103	*Vibrio communis* (GU078672)	99.6	γ-Proteobacteria
UMTBB104	*Ferrimonas balearica* (CP002209)	99.9	γ-Proteobacteria
UMTBB105	*Photobacterium damselae* (X78105)	99.7	γ-Proteobacteria
UMTBB106	*Photobacterium damselae* (X78105)	99.7	γ-Proteobacteria
UMTBB107	*Vibrio communis* (GU078672)	99.8	γ-Proteobacteria
UMTBB108	*Vibrio tubiashii* (X74725)	99.6	γ-Proteobacteria
UMTBB109	*Vibrio rotiferianus* (AJ316187)	99.9	γ-Proteobacteria
UMTBB110	*Photobacterium damselae* (X78105)	99.7	γ-Proteobacteria
UMTBB111	*Vibrio harveyi* (X74706)	99.2	γ-Proteobacteria
UMTBB112	*Micrococcus yunnanensis* (FJ214355)	98.7	Actinobacteria
UMTBB113	*Paramoritella alkaliphila* (AB364966)	99.6	γ-Proteobacteria
UMTBB114	*Vibrio rotiferianus* (AJ316187)	99.9	γ-Proteobacteria
UMTBB115	*Vibrio owensii* (GU018180)	99.9	γ-Proteobacteria
UMTBB116	*Ruegeria conchae* (HQ171439)	98.7	α-Proteobacteria
UMTBB117	*Ferrimonas kyonanensis* (AB245514)	97	γ-Proteobacteria
UMTBB118	*Vibrio campbellii* (XZ74692)	100	γ-Proteobacteria
UMTBB119	*Vibrio ponticus* (AJ630103)	96.8	γ-Proteobacteria
UMTBB120	*Pseudoalteromonas rubra* (X82147)	99.7	γ-Proteobacteria
UMTBB121	*Pseudoalteromonas luteoviolacea* (X82144)	99.6	γ-Proteobacteria
UMTBB122	*Pseudoalteromonas luteoviolacea* (X82144)	99.9	γ-Proteobacteria
	Pre-monsoon season		
UMTBB201	*Photobacterium jeanii* (GU065209)	97.8	γ-Proteobacteria
UMTBB202	*Vibrio rotiferianus* (AJ316187)	99.1	γ-Proteobacteria
UMTBB203	*Vibrio campbellii* (X74692)	99.9	γ-Proteobacteria
UMTBB204	*Vibrio campbellii* (X74692)	99.9	γ-Proteobacteria
UMTBB205	*Bacillus aquimaris* (AF483625)	98.8	Firmicutes
UMTBB206	*Micrococcus yunnanensis* (FJ214355)	99.6	Actinobacteria
UMTBB207	*Ruegeria arenilitoris* (JQ807219)	99.3	α-Proteobacteria
UMTBB208	*Vibrio campbellii* (X74692)	100	γ-Proteobacteria
UMTBB209	*Bacillus aquimaris* (AF483625)	98.2	Firmicutes
UMTBB210	*Micrococcus yunnanensis* (FJ214355)	99.9	Actinobacteria
UMTBB211	*Vibrio ichthyoenteri* (AJ421445)	95	γ-Proteobacteria
UMTBB212	*Vibrio shilonii* (ABCH01000080)	99.7	γ-Proteobacteria
UMTBB213	*Photobacterium jeanii* (GU065209)	99.8	γ-Proteobacteria
UMTBB214	*Vibrio rotiferianus* (AJ316187)	99.2	γ-Proteobacteria
UMTBB215	*Bacillus vietnamensis* (AB099708)	98.6	Firmicutes
UMTBB216	*Pseudoalteromonas rubra* (X82147)	99.7	γ-Proteobacteria
UMTBB217	*Pseudoalteromonas luteoviolacea* (X82144)	99.6	γ-Proteobacteria
UMTBB218	*Pseudoalteromonas luteoviolacea* (X82144)	99.9	γ-Proteobacteria
UMTBB219	*Ruegeria arenilitoris* (JQ807219)	100	α-Proteobacteria
UMTBB220	*Ruegeria arenilitoris* (JQ807219)	99.1	α-Proteobacteria
UMTBB221	*Vibrio sinaloensis* strain F17C1 (JX999950.1)	99.9	γ-Proteobacteria
UMTBB222	*Brachybacterium paraconglomeratum* (AJ415377)	99.5	Actinobacteria
UMTBB223	*Vibrio brasiliensis* (AEVS01000097)	99.4	γ-Proteobacteria
UMTBB224	*Gordonia westfalica* (AJ312907)	100	Actinobacteria

Table 3b

Phylogenetic affiliation of bacteria isolated from Blue bell tunicate, *Clavelina coerulea* based on BLAST analysis

Bacteria isolate	*Closest relative in GenBank database*	*Percent of similarity (%)*	*Phylogenetic affiliation*
	Post-monsoon season		
UMTBB101	*Vibrio* sp. (JQ679094.1)	99	γ-Proteobacteria
UMTBB102	*Ferrimonas* sp. (AB193754.1)	97	γ-Proteobacteria
UMTBB103	*Bacterium* M7 (JN119259.1)	99	γ-Proteobacteria
UMTBB104	*Ferrimonas balearica* (AB193753.1)	99	γ-Proteobacteria
UMTBB105	*Photobacterium damselae* (KF529965.1)	99	γ-Proteobacteria
UMTBB106	*Photobacterium damselae* (KF529965.1)	99	γ-Proteobacteria
UMTBB107	*Bacterium* 7 (JN119259.1)	99	γ-Proteobacteria
UMTBB108	*Bacterium* 3H101 (JF411528.1)	99	γ-Proteobacteria
UMTBB109	*Vibrio haryeyi* (AM422800.1)	99	γ-Proteobacteria
UMTBB110	*Photobacterium damselae* (KF529965.1)	99	γ-Proteobacteria
UMTBB111	*Vibrio rotiferianus* (KC534401.1)	99	γ-Proteobacteria
UMTBB112	*Micrococcus* sp. (KF585026.1)	99	Actinobacteria
UMTBB113	*Paramoritella alkaliphila* (AB364967.1)	99	γ-Proteobacteria
UMTBB114	*Vibrio harveyi* (AM422800.1)	99	γ-Proteobacteria
UMTBB115	*Vibrio* sp. (GQ406679.1)	99	γ-Proteobacteria
UMTBB116	*Silicibacter* sp. (GQ391987.1)	100	α-Proteobacteria
UMTBB117	*Ferrimonas kyonanensis* (NR_041387.1)	99	γ-Proteobacteria
UMTBB118	*Vibrio campbellii* (HM771344.1)	98	γ-Proteobacteria
UMTBB119	*Vibrio ponticus* (KC884589.1)	100	γ-Proteobacteria
UMTBB120	*Pseudoalteromonas* sp. (AB571949.1)	97	γ-Proteobacteria
UMTBB121	*Bacterium* T25 (JN119279.1)	99	γ-Proteobacteria
UMTBB122	*Bacterium* T25 (JN119279.1)	99	γ-Proteobacteria
	Pre-monsoon season		
UMTBB201	*Bacterium* 3H107 (JF411534.1)	99	γ-Proteobacteria
UMTBB202	*Vibrio harveyi* (GU974342.1)	100	γ-Proteobacteria
UMTBB203	*Vibrio campbellii* (CP006605.1)	100	γ-Proteobacteria
UMTBB204	*Vibrio campbellii* (CP006605.1)	99	γ-Proteobacteria
UMTBB205	Bacterium BW3PhS7 (KC012879.1)	99	Firmicutes
UMTBB206	*Micrococcus luteus* (FJ816022.1)	99	Actinobacteria
UMTBB207	Bacterium 1H212 (JF411473.1)	99	α-Proteobacteria
UMTBB208	*Vibrio* sp. *Persian* (KC505639.2)	100	γ-Proteobacteria
UMTBB209	Bacterium BW3PhS7 (KC012879.1)	99	Firmicutes
UMTBB210	*Micrococcus luteus* (FJ816022.1)	99	Actinobacteria
UMTBB211	Uncultured bacterium clone (KF798666.1)	97	γ-Proteobacteria
UMTBB212	*Vibrio mediterranei* (HF541962.1)	100	γ-Proteobacteria
UMTBB213	Bacterium 3H107 (JF411534.1)	99	γ-Proteobacteria
UMTBB214	*Vibrio* sp. PaH2.06c (GQ406738.1)	100	γ-Proteobacteria
UMTBB215	Bacterium BW3PhS7 (KC012879.1)	99	Firmicutes
UMTBB216	*Pseudoalteromonas* sp. (AB571949.1)	99	γ-Proteobacteria
UMTBB217	Bacterium T25 (JN119279.1)	99	γ-Proteobacteria
UMTBB218	Bacterium T25 (JN119279.1)	99	γ-Proteobacteria
UMTBB219	*Ruegeria* sp. (KF277768.1)	100	α-Proteobacteria
UMTBB220	Bacterium 1H113 (JF411452.1)	99	α-Proteobacteria
UMTBB221	Bacterium *M14* (JN119263.1)	99	γ-Proteobacteria
UMTBB222	*Brachybacterium* sp. (EU912470.1)	99	Actinobacteria
UMTBB223	Bacterium M14 (JN119263.1)	99	γ-Proteobacteria
UMTBB224	*Gordonia westfalica* (NR_025468.1)	99	Actinobacteria

It was observed that the 22 isolate in the post-monsoon season were composed of 16 microbiota species, while 15 were identified in the pre-monsoon season (of 24 isolates) using the EZ-taxon database. The Genbank database however, revealed 18 and 16 species respectively in the post and pre-monsoon season. Similarity percentage for most of the isolate was about 97% and above except for UMT 211 which was observed to be only 95% similar to *Vibrio ichthyoenteri* (AJ421445) according to the using the EZ-taxon database. The microbiota identified during the post-monsoon season belongs to the phyla

γ-Proteobacteria, α-Proteobacteria, and Actinobacteria (Figure 1a). In addition to these phyla, species belonging to Firmicutes, were also identified in the post-monsoon season (Figure 1b).

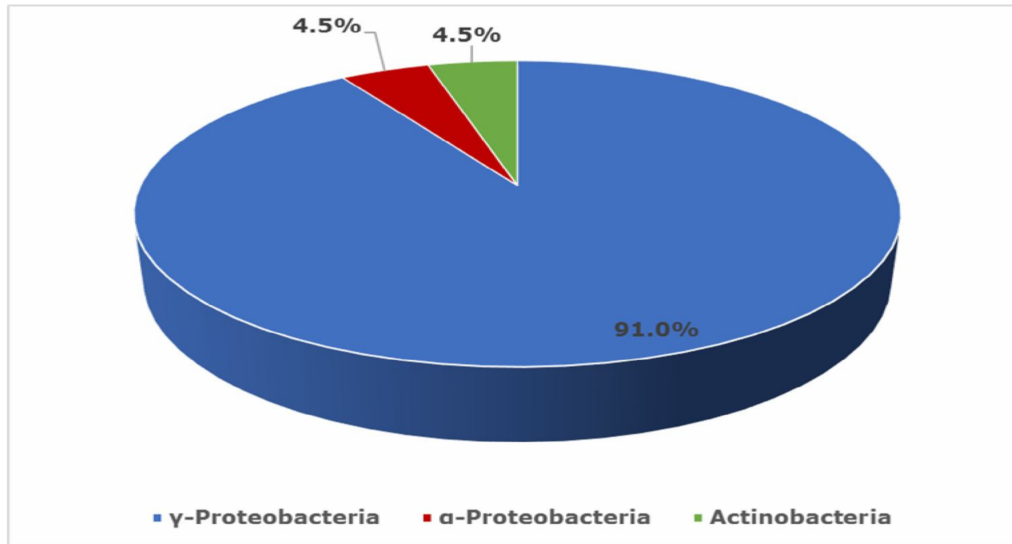

Figure 1a. Total abundance of bacteria flora in blue bell tunicate, *Clavelina coerulea* during post-monsoon season.

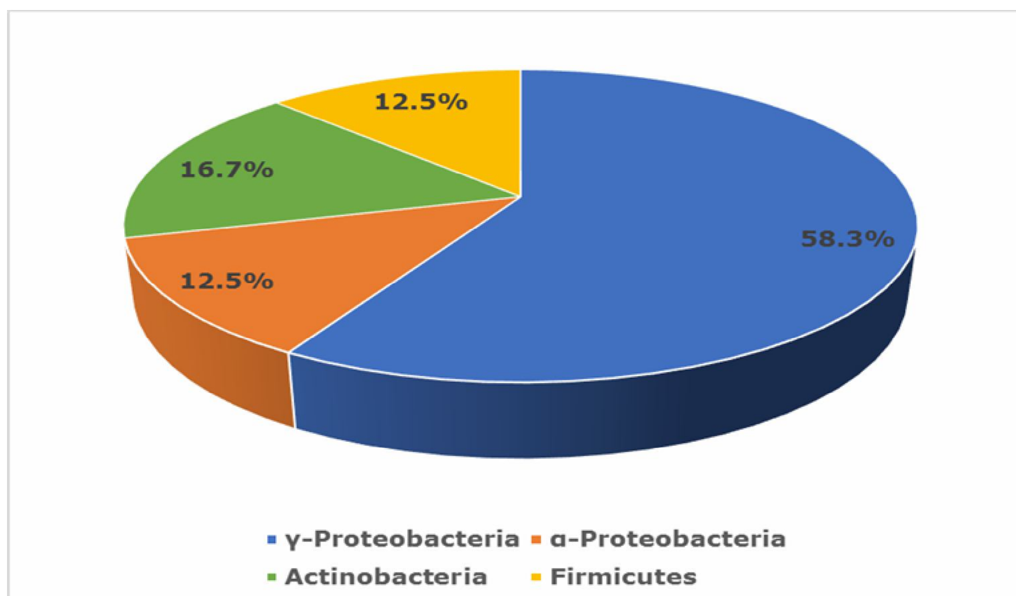

Figure 1b. Total abundance of bacteria flora in blue bell tunicate, *Clavelina coerulea* during pre-monsoon season.

The phylogenetic relationships of isolated bacteria from *C. coerulea* during the post and pre-monsoon seasons as inferred by the neighbour-joining analysis of the 16S rRNA gene sequences as presented in Figure 2 (2a and 2b respectively for post and pre-monsoon seasons). The result of the phylogenetic analysis indicated that bacterial were group into three and four phylotypes respectively for the post and pre-monsoon season according to the phyla previously mentioned. In summary, the microbiota isolate demonstrated that majority of them were gram-negative bacterial from the phyla and genus *Proteobacteria* and *Vibrio* sp. respectively.

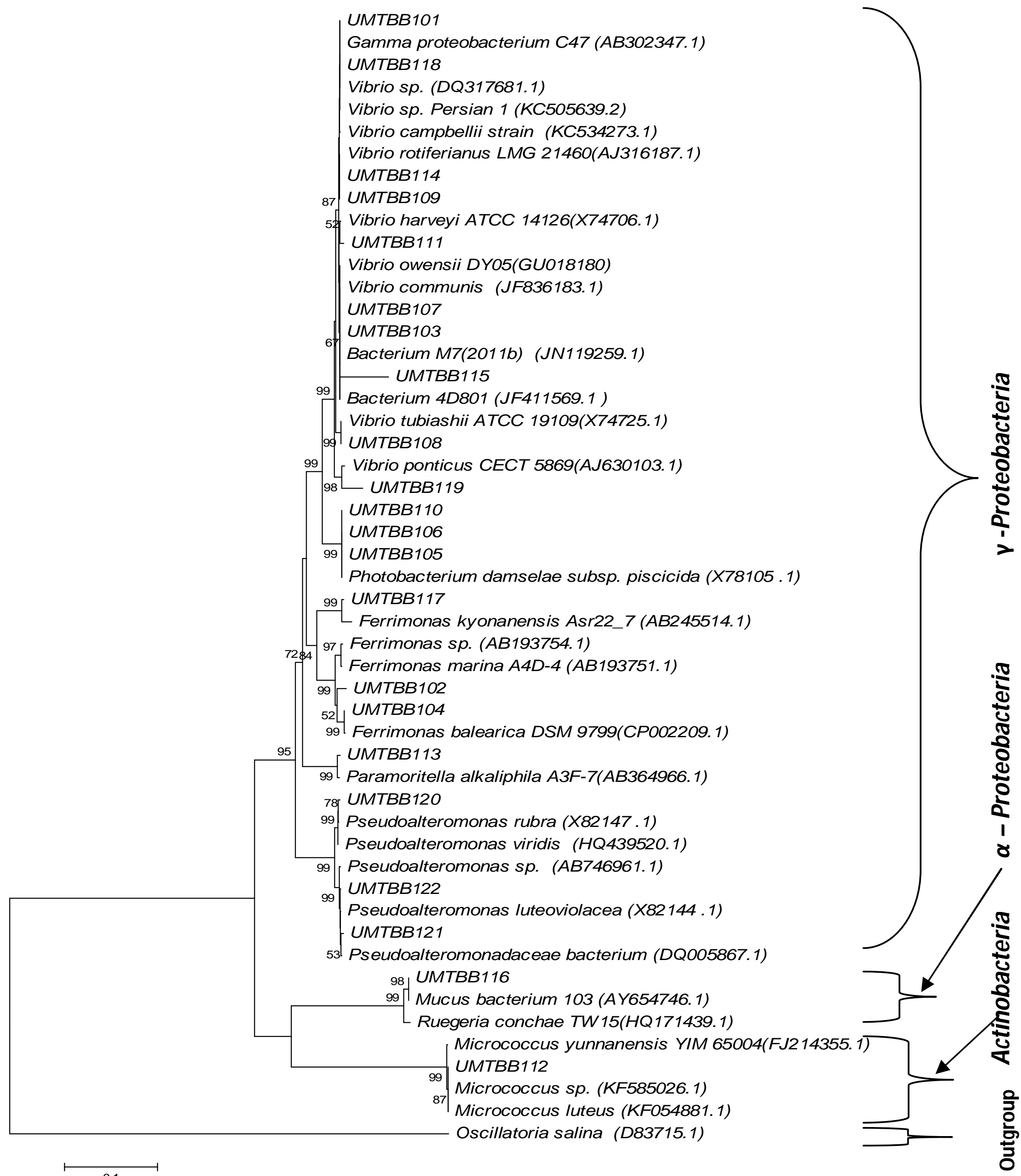

Figure 2a. Phylogenetic tree, inferred from neighbour-joining analysis based on 16S rRNA gene sequences obtained in this study and related sequences. Sequences of the bacteria isolates from *Clavelina coerulea* during post-monsoon are indicated by UMTBB1 Bootstrap values greater than 50% (based on 1000 resamplings) are shown at branch points. Bar, 0.1 substitutions per nucleotide position.

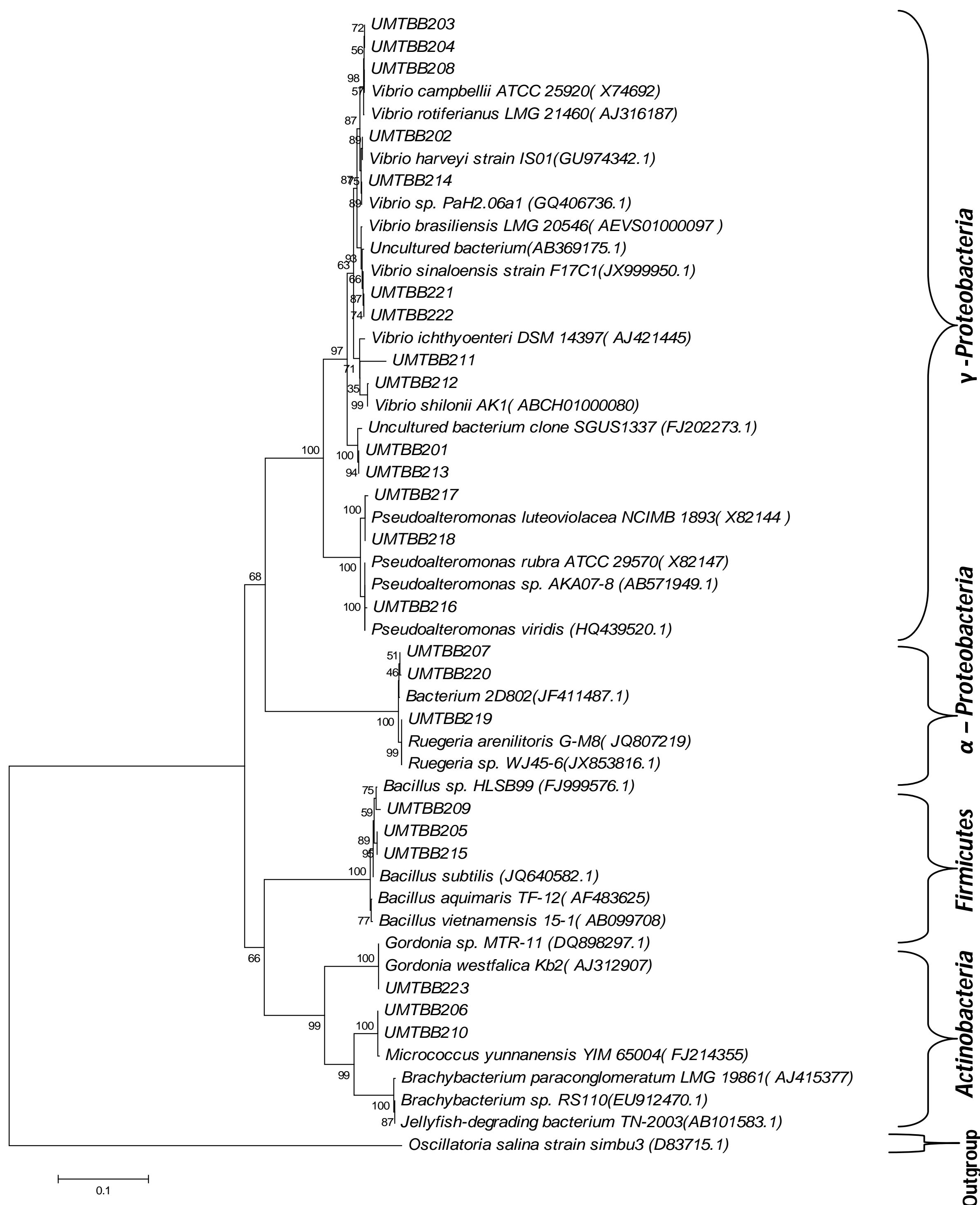

Figure 2b. Phylogenetic tree, inferred from neighbour-joining analysis based on 16S rRNA gene sequences obtained in this study and related sequences. Sequences of the bacteria isolates from *Clavelina coerulea* during pre-monsoon are indicated by UMTBB2 Bootstrap values greater than 50% (based on 1000 resamplings) are shown at branch points. Bar, 0.1 substitutions per nucleotide position.

Bacteria composition in the marine tunicate Clavelina coerulea using the New Generation Sequencing Method. Table 4 shows the total number of amplicon sequences reads obtained in this study to be 764,742 belonging to 343 total operational taxonomic units (OTUs). All sequence reads from this study were deposited in the GeneBank (Accession numbers SAMN15516361, and SAMN15516362). A total number of 10 classifiable phyla were identified in this study (Figure 3). Among these phyla, only

Proteobacteria (66.9 vs 63.9%), Bacteroidetes (12.9 vs 17.8%), Firmicutes (6.13 vs 5.57%), Actinobacteria (4.3 vs 6.67%), Fusobacteria (2.45 vs 2.22%), and Acidobacteria (4.9 vs 2.77%) were predominantly observed in both post and pre-monsoon season (respectively). Although Nitrospira, Planctomycetes, and TM7 were identified in the tunicates during the post-monsoon season, their occurrence was in trace amount (0.61%). Also Tenericutes was also found exclusively during the pre-monsoon season (0.56%). Less than 1% of OTUs was unclassifiable at the phylum level.

Table 4

Characteristics of 16S rDNA metagenomic libraries

Characteristics	*Sample designation*	
	Post-monsoon	*Pre-monsoon*
Amplicon sequences	281005	483737
OTUs	163	180
Numbers of single reads OTUs	30	19
(Percentage of total)	18.4%	10.6%

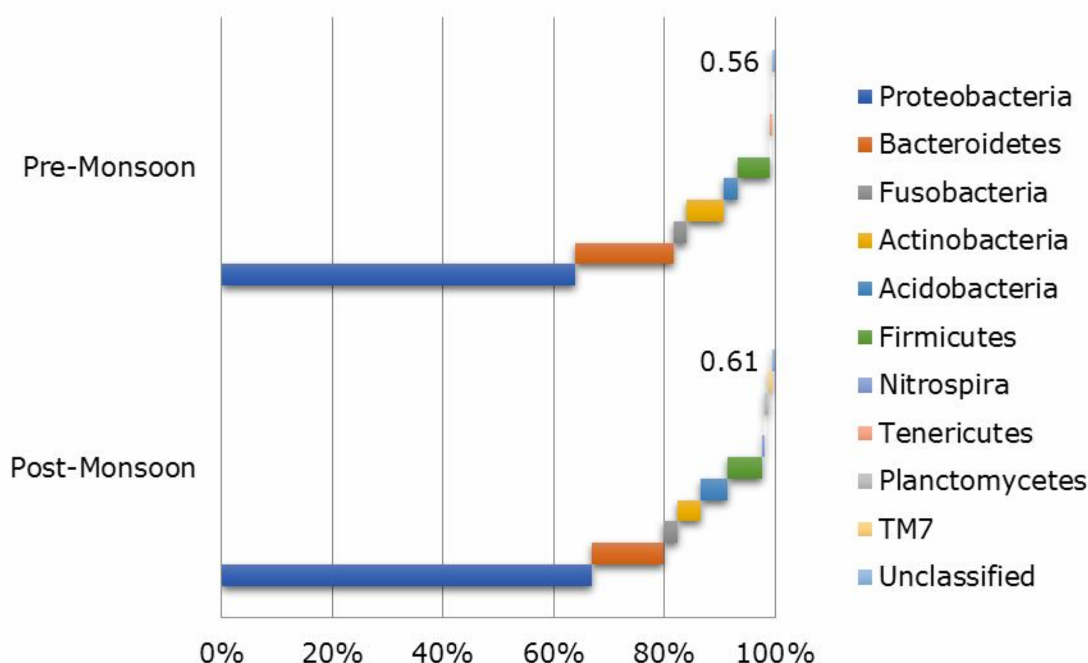

Figure 3. Relative abundance of the dominant bacterial phyla associated with the blue-bell tunicate, *Clavelina coerulea*.

Class, Order and Family distribution of microbiota associated with the marine tunicate Clavelina coerulea. In general, a total of 17 different classes of microbiota were associated with the marine tunicate reported in the current study. *α* – Proteobacteria, γ – Proteobacteria, Flavobacteria, β – Proteobacteria, δ – Proteobacteria Sphingobacteria, Actinobacteria, Bacteroidia, and ε - Proteobacteria were commonly found in both seasons of the study (Figure 4). However, associated microbiota during the post-monsoon season was more diverse (16 classes) compared to the pre-monsoon season (14 classes).

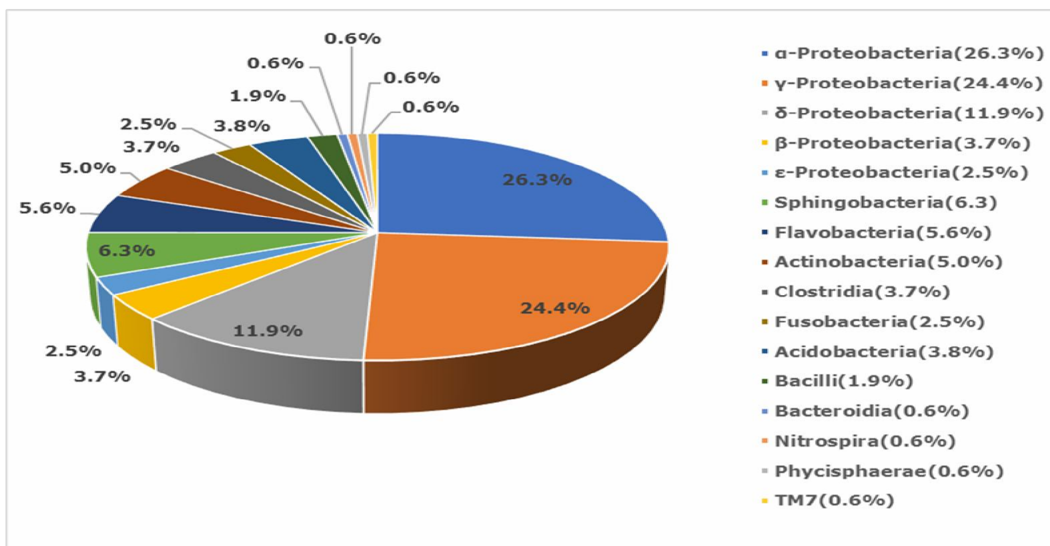

Figure 4a. Relative abundance at the class level of bacteria associated with the blue-bell tunicate, *Clavelina coerulea* during the post-monsoon season.

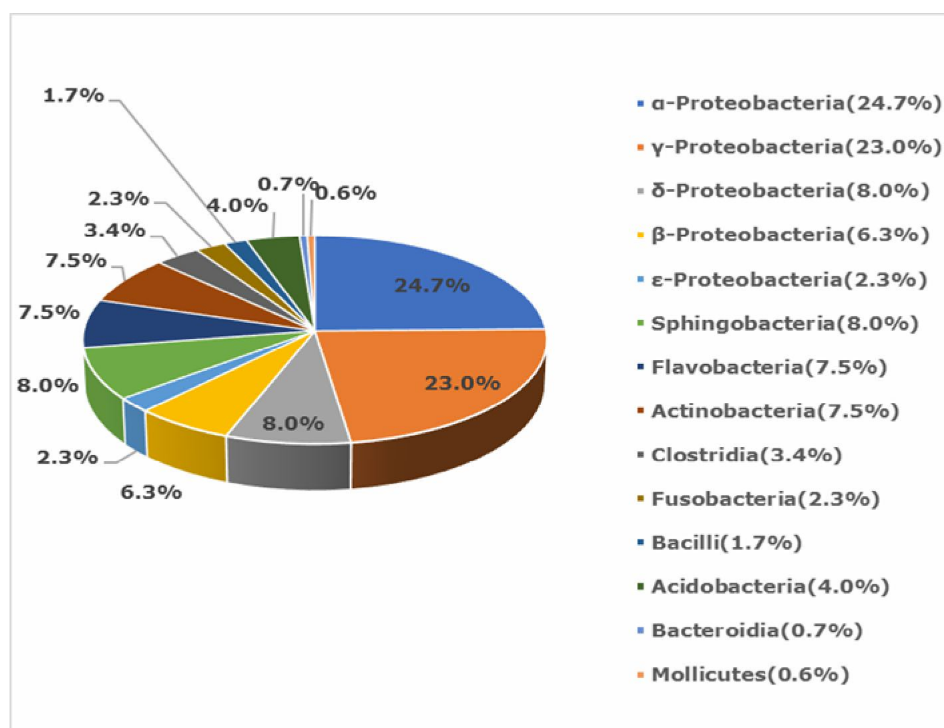

Figure 4b. Relative abundance at the class level of bacteria associated with the blue-bell tunicate, *Clavelina coerulea* during the pre-monsoon season.

To further understand the microbial diversity of the marine tunicates in this study, each class of the α –, β –, δ –, γ – Proteobacteria were characterized to the order level, while all the remaining orders were grouped as "other" as shown in Figure 5. The most prominent members of the α – Proteobacteria observed were the Rhizobiales and Rhodobacterales while Burkholderiales dominated the β – Proteobacteria. The δ – Proteobacteria were largely represented by the members of the Desulfobacterales and Myxococcales. The γ – Proteobacteria on the other hand was dominated by the Alteromonadales and the Vibrionales. The Sphingobacteriale and Flavobacteriales were the dominant orders classified as "Others" in the current study.

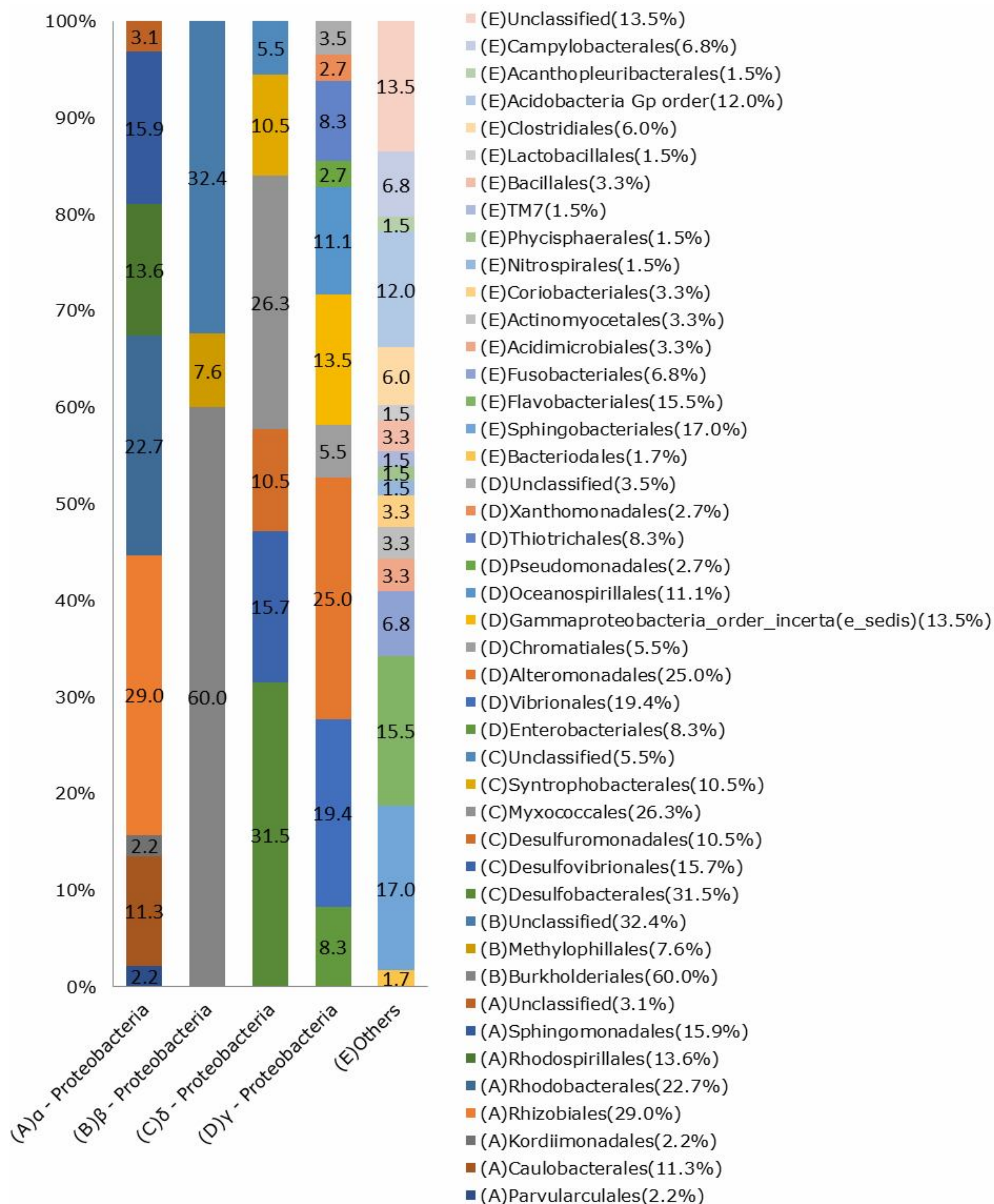

Figure 5a. Relative abundance at the order level of bacteria associated with blue-bell tunicate, *Clavelina coerulea* during the post-monsoon season.

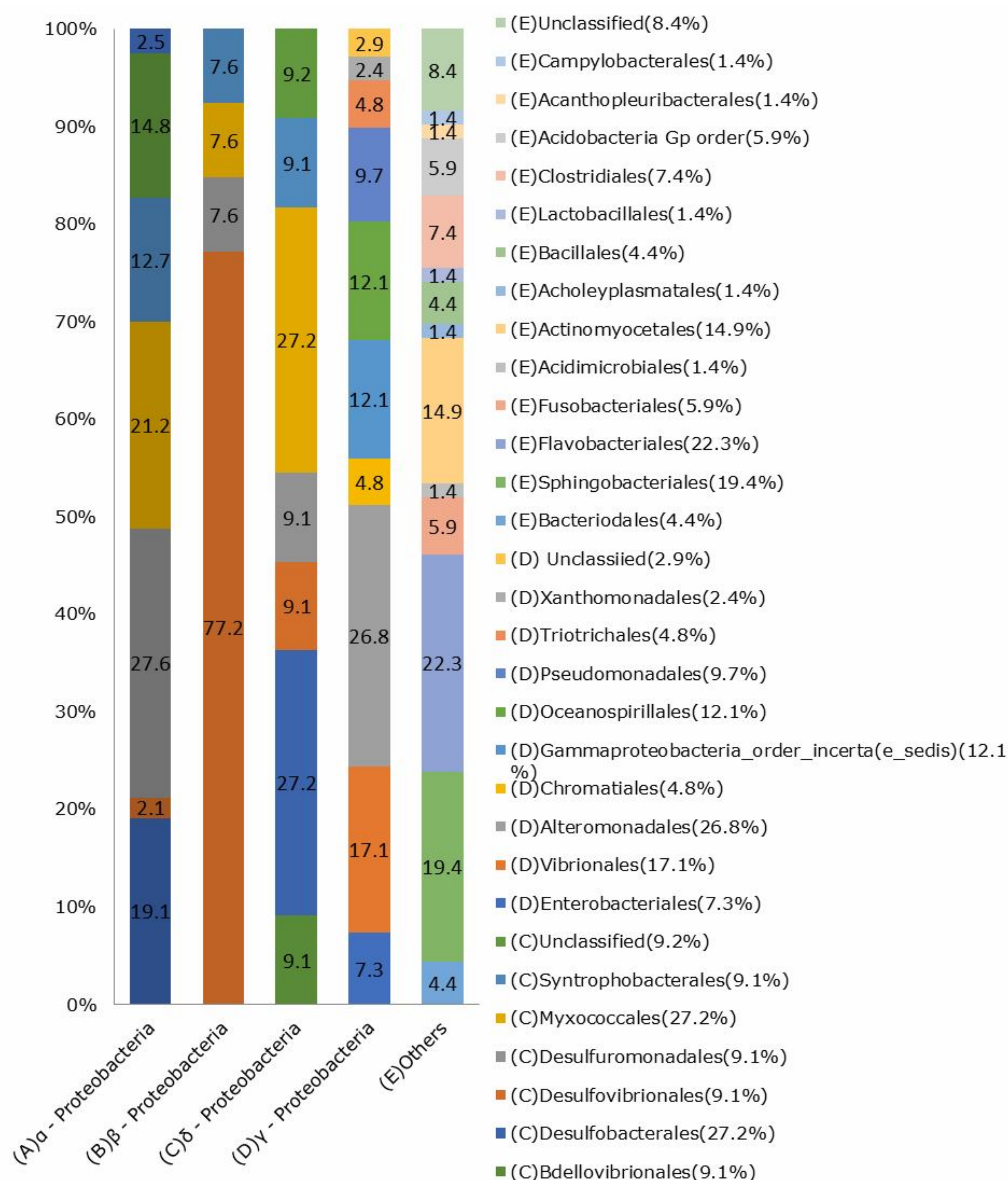

Figure 5b. Relative abundance at the order level of bacteria associated with blue-bell tunicate, *Clavelina coerulea* during the pre-monsoon season.

A total of 91 bacteria families were identified in this study (Figure 6). Forty-nine of these microbiota (54%) were present in both seasons, while remaining families were exclusive to the post and pre-monsoon season representing 22% and 24% of the total families respectively.

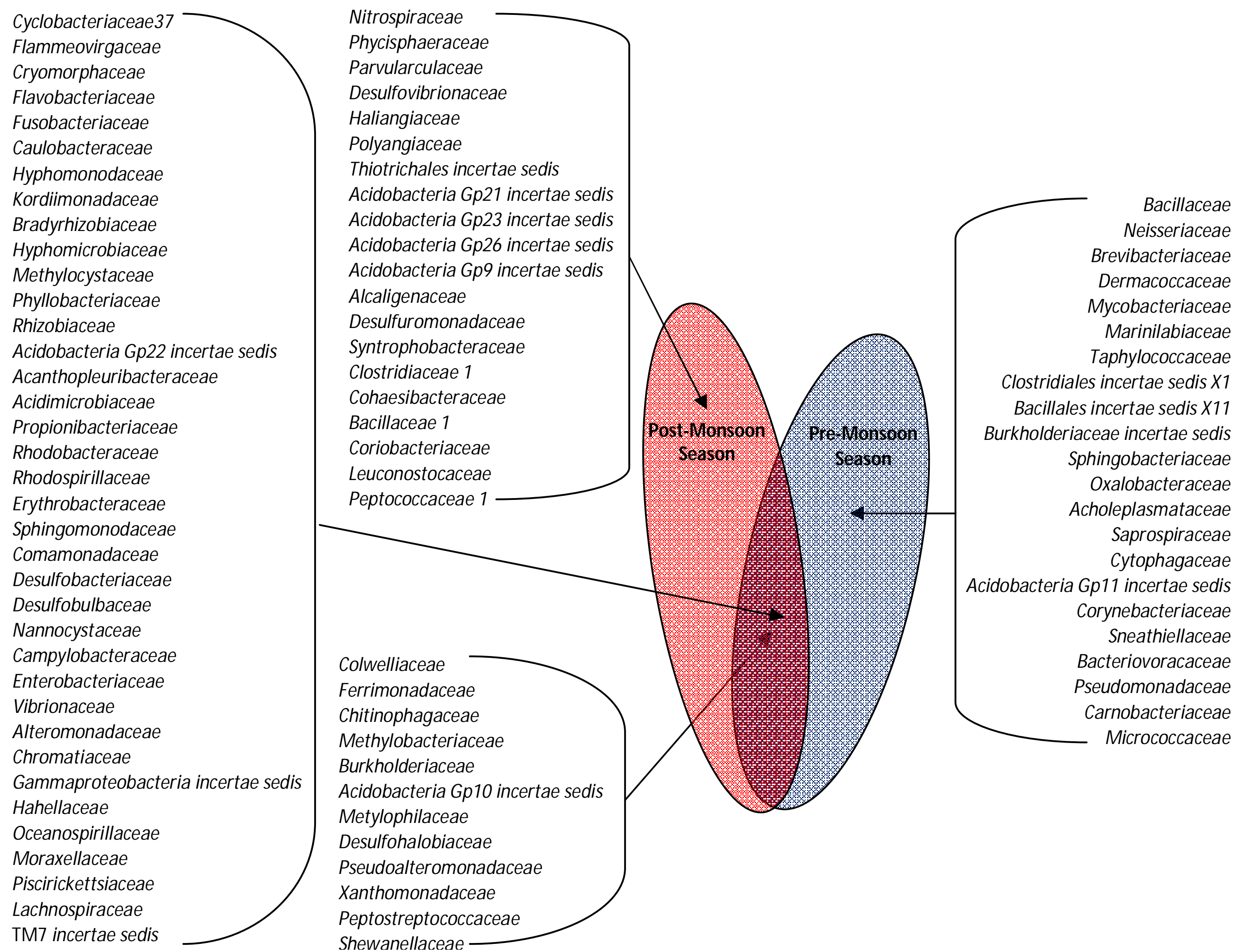

Figure 6. Venn diagram comparison of bacterial families unique and shared in the blue bell tunicate *Clavelina coerulea* in both seasons.

Discussion. Many earlier studies had adopted both culture-dependent and independent approaches to investigate the microbial communities established on the surfaces of seawater organisms such as tunicates, corals, sponges, and macroalgae (Rohwer et al 2002; Taylor et al 2004; Wegley et al 2007; Wilson et al 2010; Erwin et al 2011). This has enabled an in-depth view of the culturable, dominant, as well as less abundant taxa that may not be detected using only traditional clone sequencing and cultivation methods (Pedrós-Alió 2012). The estimation of marine bacterial diversity for different tunicate species is particularly important, as the abundance, distribution and structure is uneven and may be host-specific in nature (Erwin et al 2013, 2014; Evans et al 2017) or differs among geographical locations (Sunagawa et al 2015; Louca et al 2016), and seasons as demonstrated in the current study. According to Deepanjali et al (2005), water temperatures are relatively stable over different seasons in the tropics, hence, are of less importance as causes of microbiota variation. This is therefore suggestive that any seasonal variations in the abundance of bacteria in the tropics are probably dictated by factors other than temperature. Salinity was observed to be the most varied among the water quality parameters reported and may be partly responsible for the seasonal variation in microbiota isolated in our study. This assumption is different from observation earlier made for temperate seawater where changes in the bacteria dynamics has been proven to be governed by changes in day length, temperature, nutrients, as well as chlorophyll-a (Pinhassi et al 2006; Gilbert et al 2009; Andersson et al 2010).

As observed with the culture-dependent technique, most bacterial isolates showed sequence identities of about 97% and above when compared with the stored sequences available in the GenBank database and the EZ-taxon database. However, 95.0% sequence similarity observed in UMTBB211 may be suggestive that it belongs to a novel taxa following the threshold value of 97% similarity suggested for species delineation of prokaryotic species by Stackebrandt & Goebel (1994). The current study revealed that the majority of the isolates fell into the phylum Proteobacteria, however with significant variation during the post-monsoon (91%) and pre-monsoon (75%) season. This is also similar to the findings of da Silva Oliveira et al (2012) who reported that the bacterial symbionts associated with ascidians are mainly from Proteobacteria. Their study revealed that the α-Proteobacteria dominated the *Didemnum galacteum* (51%) while γ-Proteobacteria were much higher in *Cystodytes* sp. (90%) which was in line with finding of the current study during the post-monsoon season. The research by Thiel & Imhoff (2003) in sponges has also shown the dominance of Proteobacteria, especially the α-Proteobacteria and γ-Proteobacteria.

Most of the groups of bacteria affiliated with the γ-Proteobacteria were related to marine *Vibrio* species in both seasons. This is similar to the report of Santavy et al (1990) on marine sponges when they revealed that not less than 78% of the bacteria isolates of the group γ-Proteobacteria were from the genus *Vibrio.* Although several species of this genus are pathogens of fishes and shellfishes, they are also a typical component of micro fouling in most aquatic habitats (Israely et al 2001). Hence, they are of great importance for the remineralization of organic matter in the sea (Fukami et al 1985). Some *Vibrio* strains earlier found associated with *Hyatella* sp. are reported to produce peptide antibiotics (Oclarit et al 1994). Since tunicate bacterial communities have been demonstrated as a potential source of new natural products (Blasiak et al 2014), future research can therefore examine the possibility of obtaining these from the various *Vibrio* strains reported in the current study. Another genus of the family Vibrionaceae isolated in this study worth mentioning is the *Photobacterium*. Although largely known to be free-living organisms, they also are a symbiont in the light organs of marine animals (Farmer & Hickman-Brenner 2006). One of the isolated species in the current study namely *Photobacterium damselae*, is a pathogen of aquatic animals (Rivas et al 2013).

This study also observed that the genera *Paramoritella* and *Ferrimonas* were found only in the post-monsoon season. *Brachybacterium* sp. was also found exclusively associated with *C. coerulea* sampled during the pre-monsoon season suggesting an exclusively temporal association with the tunicate. Previous study by Orsod et al (2012) had earlier isolated the *Brachybacterium* sp. from Asian sea bass (*Lates calcarifer*) reared in an open sea aquaculture facility located in Gelang Patah, Johore, Malaysia. Much

earlier study Zhang et al (2006) had also recovered this bacterium from marine invertebrates such as sponges without giving attention to season of sampling. In addition to *Brachybacterium*, *Micrococcus* was the other Actinobacteria represented in the culturable community of *C. coerulea* isolated in the current study. These Actinobacteria and other members of this bacterial phylum have considered valuable producers of biologically active secondary metabolites, such as antibiotics, vitamins, enzymes and other therapeutic compounds (Mincer et al 2005; Jensen et al 2007; Sun et al 2010). They also function in the marine environment by breakdown the recalcitrant organic matter, which is an important role in the ocean biogeochemical cycles (Das et al 2006). Although we are unable to substantially explain the reason exclusive occurrence of these bacterial species in both pre and post monsoon season in this study, however, salinity which was the main variable water quality measure might be a culprit among other factors. This can be justified in future studies.

Pigmented bacteria isolates belonging to the *Pseudoalteromonas* genus were also found associated with *C. coerulea* during pre-monsoon (UMTBB120, UMTBB121 and UMTBB122) and the post-monsoon seasons (UMTBB216, UMTBB217 and UMTBB218). Two of the sequences were matched with red pigmented bacteria *Pseudoalteromonas rubra*, while the remaining four were matched with the violet pigmented bacteria *Pseudoalteromonas luteoviolacea*. Many *Pseudoalteromonas* species had also been isolated from various animals, such as mussels (Ivanova & Mikhailov 2001), pufferfish (Simidu et al 1990) and tunicates (Holmström et al 1998). In addition to pigmentation, they also display a broad range of effective antibiotic activities. This was demonstrated by Gauthier (1976) and Gauthier & Flatau (1976) who produced high molecular mass anti-bacterial compounds from *P. luteoviolacea* and *P. rubra* respectively (Baumann et al 1984).

Detailed investigations of microbial diversity through analysis of 16S metagenomic in this study were done in order to provide broader information of the bacteria diversity associated with tunicates. Similar approach has been used to reveal the highly diverse and host-specific communities of prokaryotic symbionts associated with other ascidians (Erwin et al 2013, 2014; Evans et al 2017). Hence, allowing the accurate detection and characterization of both dominant and rare members of the bacteria community (Ghanbari et al 2015). The most abundant phyla in both seasons in decreasing order were Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Fusobacteria, and Acidobacteria. Phylum-level composition of the ascidian microbiota retrieved from other ascidian species had been reported to comprise of mostly Proteobacteria, Bacteroidetes and Planctomycetes (Martinez-Garcia et al 2007; Tait et al 2007). In addition to these bacterial phyla, the sponge-associated bacterial community reported by Hentschel et al (2006) also included six others namely; Nitrospira, Cyanobacteria, Actinobacteria, Chloroflexi, Acidobacteria, Poribacteria. Some studies with fish have also demonstrated the abundant Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria phyla associated in different parts of the fish organs (Boutin et al 2013; Gerzova et al 2014; Larsen et al 2014; Nurul et al 2019, 2020; Okomoda et al 2020).

Based on class abundance, the α-Proteobacteria and γ-Proteobacteria were the most dominant in *C. coerulea* both in the post-monsoon (50.7%) and pre-monsoon season (47.7%). Hagström et al (2002), Cottrell & Kirchman (2000), had earlier postulated that α−Proteobacteria comprises one of the largest fractions of heterotrophic bacteria in the marine environment. It is however noteworthy that the post-monsoon season had more class diversity than the pre-monsoon season and noticeable changes in bacterial class composition and dominance (excluding the α-Proteobacteria and γ-Proteobacteria which were relatively same for both seasons). Observations in temperate regions suggest that the stratification of water column in summer is characterised by the domination of Cyanobacteria, Roseobacter, SAR86, and SAR11 (Schauer et al 2003; Alonso-Sáez et al 2007; Lindh et al 2015). However, in the winter and spring, the dominate bacterial are Flavobacteria, Roseobacter, and some Gammaproteobacteria due to the mixing of the water column (Teeling et al 2012; Buchan et al 2014; Taylor et al 2014).

To further reveal microbial diversity, each class of Alpha, Gamma, and Delta - Proteobacteria was characterized to the order level. When compared to the other class

designations, the γ-Proteobacteria had the highest number of orders, including Alteromonadales, Chromatiales, Enterobacteriales, Pseudomonadales, Oceanospirilales, Xanthomonadales. The γ-Proteobacteria order was however dominated by the Thiotrichales and the Vibrionales aside the unclassified orders. The β-Proteobacteria on the other hand was dominated by Burkholderiales, most of which were of the family Comamonadaceae. Previous study by Devine et al (2012) had also found that the β-Proteobacteria in sea slug samples was dominated by order Burkholderiales. In α-Proteobacteria, the order of Rhizobiales was the most dominant in both seasons, while the order Rhizobiales was the biggest group within the α-Proteobacteria. Similarly, in Deltaproteobacteria, Dellovibrionales and Desulfovibrionales were the most dominant orders during the post-monsoon and pre-monsoon season.

We observed significant variation not only in the order of microbiota of the tunicates but also in the family composition. Only, about 54% of the bacterial families were shared in both the pre and post-monsoon seasons. According to Grube et al (2009), shared bacteria communities are a fundamental reflection of the symbiotic interactions between the bacteria and the host usually exploited for metabolic benefits. Hence, the shared microbiota in the current study may constitute part of the mature or permanent microbiota associated with the tunicate according to the bacterial categories earlier stated by Sugita et al (1998) and de Paula Silva et al (2011). In the same light, the exclusive microbiota observed per season (i.e. either the post or pre-monsoon season) during this study is likely transitory in nature. The inability of hypothesized "transitory bacterial" to be established on the tunicates in both seasons may be linked to the self-cleaning phenomenon of the flowing seawater. This has been shown to affect the OTUs as well as the composition of microbiota (Rasmussen & Sorensen 2001; Horner-Devine et al 2004; Leflaive et al 2008; Sala et al 2006, 2008; Nurul et al 2019, 2020). Sullam et al (2012) and Zarkasi et al (2014) have also stated earlier that changes in environmental factors due to seasons and geographic location could severely influence the composition of free-living and symbiotic bacteria communities. This assumption was justified in this study as the composition of shared bacterial phylum; class and order were varied in both seasons.

Conclusions. The present study revealed that bacterial community associated with blue bell tunicate *Clavelina coerulea* varied seasonally. Through the culture-dependent method, a total of 46 bacterial isolates were found to be associated with the tunicate host. While 91 bacterial families were found to be associated with the tunicate using 16S metagenomics analysis. Most of the bacterial communities revealed by using both culture-dependent and 16S metagenomics methods were affiliated to the phylum Proteobacteria. Since salinity was the most varied water quality parameter observed in the current study, it was thought to be one of the major drivers of the seasonal variation in bacterial diversity associated with *C. coerulea*. In conclusion, our study has successfully provided insights into the diversity, structure, and composition of bacterial communities associated with *C. coerulea*. It is recommended that future study to be carried out using other "omics" approaches to better understand the interactions between the marine ascidians and their associated microbiome.

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