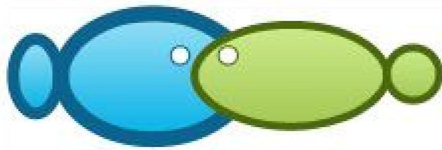

Molecular identification and antibacterial screening of nudibranch-symbiont bacteria from North Sulawesi waters

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Abstract. This study aims to find bacterial isolates of nudibranchs from North Sulawesi waters, to identify microscopic and macroscopic characteristics of the isolates, to screen the antibacterial activity of nudibranch-symbiont bacteria, to identify the isolate potential based upon 16S rRNA gene, and to detect the presence of nonribosomal peptide synthase (NRPS) and polyketide synthase (PKS). Isolation of the bacteria used four-way streak-plate method, whereas determination of microscopic and macroscopic characteristics was carried out through observations on the colony morphology, such as shape, color, and height. Gram staining was also done to know the cell type of the isolate. Antibacterial activity assay was done on *Escherichia coli* and *Staphylococcus aureus* using Kirby and Bauer disc diffusion method, whereas molecular identification of potential isolate used DNA barcoding of 16S rRNA gene, and detection of PKS-1 and NRPS encoding genes utilized primer pair of K1F and M6R. From 4 species of nudibranchs, *Phyllidia picta, Phyllidia varicosa, Phyllidiella pustulosa,* and *Phyllidiella nigra,* there were 121 single colonies isolated. The colony screening found 6 isolates with strong antibacterial activity against *S. aureus* and *E. coli.* DNA barcoding of the selected isolates based on 16S rRNA gene succeeded to identify the nudibranch symbiont bacteria as *Bacillus aryabhattai*, *Bacillus licheniformis*, *Bacillus tequilensis*, and *Alcaligenes faecalis*. Detection of PKS and NRPS occurrence indicated that one isolate had PKS gene, one had NRPS gene, two isolates had both PKS and NRPS genes, and one did not have any gene.

Key Words: NRPS, PKS, *Bacillus* spp., *Alcaligenes faecalis*.

Introduction. Marine invertebrates-associated microorganisms were considered to be important sources of marine bioactive products (Leal et al 2014). Nudibranchs (Gastropod, Heterobranchia, Opistobranchia) are one of the unique and interesting marine resources of Indonesia to be studied. Unlike other group of Gastropods, nudibranchs do not have a protective shell. The function of the shell is replaced by a chemical protection system in the form of secondary metabolites which are active in the body. These active compounds are used to protect themselves from predators and disturbances in their environment (Avila et al 1991; Wägele & Klussmann-Kolb 2005). Nudibranchs are rich in secondary metabolites (Dean & Prinsep 2017) as functional active compounds with various bioactivities, such as antimicrobial against *Pseudomonas* sp. (Ramya et al 2014), *Klebsiella*, *Escherichia coli*, *Enterobacter* (Pringgenies et al 2015), cytotoxic (Dewi et al 2016), antitumor and anticancer (Carbone et al 2010), ichthyotoxic and antifeedant (Garson 2010; Mudianta et al 2014; Bogdanov et al 2017), and antioxidant (Nurjanah 2010; Hafiluddin 2011). There is strong belief that bioactive compounds in nudibranchs are produced by the associated microorganisms, such as bacteria or fungus. Previous studies have showed the potential of nudibranch-symbiont microbes in producing antibacterial compounds (Riyanti et al 2009; Böhringer et al 2017; Kristiana et al 2019; Kristiana et al 2020; Sarjito et al 2020).

In general, bioactive biosynthesis involves complex gene encoding clusters of polyketide synthase (PKS) and nonribosomal peptide synthase (NRPS) that occur in bacteria. Polyketide and ribosomal peptide compound groups or the hybrid of both are bioactive groups held by several bacteria, such as *Alcaligenes faecalis* (Kapley et al 2016), *Baccilus* sp. (Caulier et al 2019), cyanobacterium *Nodularia spumigena* (Moffitt & Neilan 2004), and sponge-symbiont bacterium and its microbiom (Kurnia et al 2017).

Nudibranch explorations from North Sulawesi waters have been conducted by a number of researchers (Purba et al 2013; Fisch et al 2017; Pungus et al 2017; Kaligis et al 2018; Marpaung et al 2019; Ompi et al 2019), but studies on antibacterial potential of nudibranch symbiont bacteria from North Sulawesi waters are still very limited (Böhringer et al 2017; Arie et al 2020; Dajoh et al 2020; Doringin et al 2020; Ukar et al 2020), and detection of PK-NRPS gene in nudibranch symbiont bacteria is only reported by Böhringer et al (2017).

This study aims to find bacterial isolates from nudibranchs in North Sulawesi waters, to identify microscopic and macroscopic characteristics of the isolate, to screen the antibacterial activity of nudibranch-symbiont bacteria, to identify potential isolates based on 16SrRNA gene, and to detect the presence of NRPS and PKS genes of the potential isolates.

Material and Method

Sample collection and handling. This study was carried out from October 2018 to October 2020. Nudibranchs were collected from marine habitat by SCUBA diving (Figure 1) in Malalayang waters, Manado, Bangka Island, North Minahasa, and Bulo coast, Minahasa. Sample collections in Malalayang waters were done in October 2018, in Bangka Island, Likupang, in February 2019, in Bulo waters, Tateli, in September 2019. The samples were put in the ziplock plastic bags and brought to the laboratory in live condition. They were snapped and labelled for species identification. Morphological identification of samples, isolation and characterization processes of isolates, antibacterial activity screening, extraction and partitioning of bioactive compounds were carried out at the Laboratory of Molecular Biology and Marine Pharmacetics, Faculty of Fisheries and Marine Sciences, Sam Ratulangi University Manado, while molecular identification and detection of PKS and NRPS encoding genes were carried out at the Laboratory of PT Genetika Science Indonesia, Tangerang and First BASE Malaysia.

Morphological identification of nudibranch. This work was carried out by observing the color, shape, and body size, the presence of tubercle, 'rhinophore', gill, feet, body line pattern following Gosliner et al (2015), Brunckhorst (1993), then confirmed with WORMS (http://www.marinespecies.org/) and Sea Slug Forum (http://www.seaslugforum.net/).

Isolation and purification of nudibranch symbiont bacteria. Nudibranch samples were cleaned in running water to remove the dirt, and sterilized in 70% alcohol (Böhringer et al 2017). They were then rinsed in sterile seawater, chopped, planted on Nutrient Agar medium (HiMedia®) dissolved in 50% seawater, and incubated at room temperature for 3 days (Kjer et al 2010). Bacterial isolate purification was carried out using four-way streak plate technique, a dilution technique that involves spreading a loopful of culture over the surface of an agar plate (Cappuccino & Welsh 2017).

Antibacterial activity screening. Potential antibacterial was obtained through screening using Gram positive bacteria *Staphylococcus aureus* and Gram negative bacteria *Escherichia coli.* As much as 1 loopful of bacterial isolate was dropped on Nutrient Agar medium (HiMedia®) supplemented with test bacteria, incubated at room temperature for 3x24 hours to observe the antibacterial activity. The isolates that potentially have antibacterial activity are indicated with clear zone formation around the colony. The potential isolates were then screened using Kirby-Bauer disc diffusion method (Ortez 2005). Positive control used antibiotic Chloramphenycol (Pharos®) at 1 mg mL^{-1}.

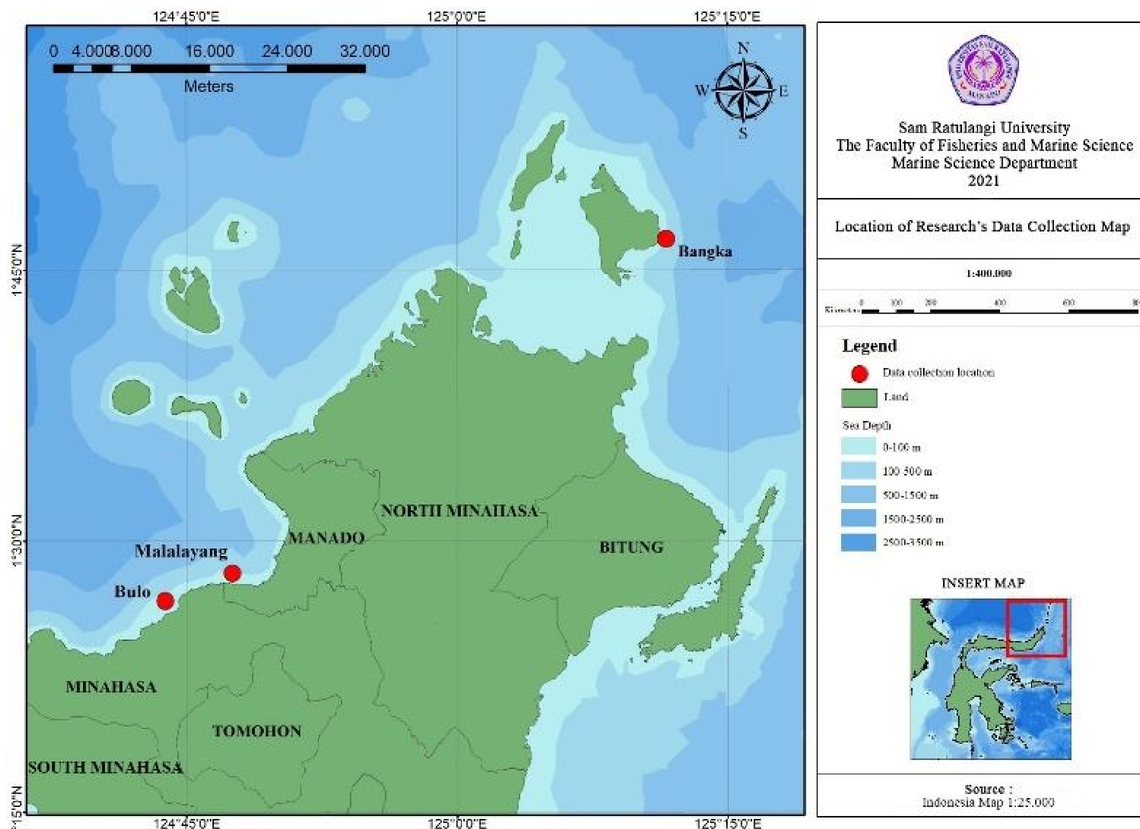

Figure 1. Sampling sites.

DNA barcoding-based symbiont bacterial identification. Bacterial DNA isolation and extraction were accomplished in Genetika Science Lab, Jakarta, following Zymo Research company protocols using Quick-DNA Fungal/Bacterial Miniprep kit. The quantity and purity of DNA were measured through spectrophotometric test using Nanodrop 2000 spectrophotometer at the wavelength of 230 nm, 260 nm, and 280 nm. Amplification of 16S rRNA gene used Enzim Taq Polymerase My Taq HS Red Mix (Bioline) with primer pair of 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-GGTTAC CTTGTTACGACTT-3) at 1400-1500 pb target fragment (Weisburg et al 1991). The PCR product was purified using Zymoclean^{TM} Gel DNA Recovery Kit (Zymo Research) and sent to First BASE Malaysia for nucleotide sequencing using Sanger method. The sequence data were compared with the Genbank data at the NCBI (National Centre for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool) program at https://blast.ncbi.nlm.nih.gov/Blast.cgi. The phylogenetic tree was reconstructed using Neighbour Joining with MEGA 7 software program.

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Detection of PKS and NRPS genes. Detection of PKS and NRPS genes used primer of Ayuso-Sacido & Genilloud (2005). PKS I gene was amplified with Kapa Taq EXtra PCR Kit (KK3009, Kapa Biosystem), using primer K1F (5′TSAAGTCSAACATCGGBCA3′) and M6R (5′CGCAGGTTSCSGTACCAGTA3′) pair with fragment target of 1200-1400 pb. Total volume used for the amplification was 10 µL, consisting of 0.1 µL of Taq polymerase, 2 µL Buffer, 1 µL dNTP Mix, 0.3 µL of each primer, 1 µL $MgCl_2$, 1 µL DNA, 4.3 µL ddH_2O. The PCR condition was set for 40 cycles at pre-denaturation temperature of 95°C for 3 min, denaturation of 95°C for 30 sec, annealing of 55°C for 30 sec, extension of 72°C for 45 sec, and final extension of 72°C for 3 min. The PCR product target of 1200 pb was separated in 0.8% agarose gel.

NRPS gene was amplified with MyTaq HS Red Mix (Bioline) using primer A3F (5′GCSTACSYSATSTACACSTCSGG3′) and A7R (5′SASGTCVCCSGTSCGGTAS3′) pair with fragment target of 700-800 pb. Total volume of the amplification was 10 µL, consisting of 5 µL Master Mix, 0.5 µL of each primer, 1 µL DNA template, and 3 µL µl ddH_2O. The PCR condition was set for 35 cycles at pre-denaturation of 95°C for 2 min, denaturation of 94°C for 15 sec, annealing of 55°C for 30 sec, extension of 72°C for 45 sec, and final extension of 72°C for 3 min. The PCR product target of 700 bp was separated in 0.8% of agarose gel.

Results and Discussion. Nudibranchs were morphologically identified as *Phyllidia picta*, *Phyllidia varicosa*, *Phyllidiella pustulosa*, and *Phyllidiella nigra* (Table 1 and Figure 2).

Screening nudibranch-symbiont bacterial pure isolates found 6 potential isolates having specific antibacterial compounds against *E. coli* and *S. aureus*, RL-2, RL-6, RL-8, RL-10, RL-11, and RL-14, respectively (Table 2).

Table 1

Morphological characteristics of nudibranch

Species	*Morphology characteristics*
Phyllidia picta Pruvot-Fol, 1957	Oval in shape, has a black pattern on the back interrupted by large yellow tubercles surrounded by pale blue patches. The blue areas converge towards the mantle edge and the tubercles become smaller, the smallest without the yellow cap. Rhinophores are yellow.
Phyllidia varicosa Lamarck, 1801	The body is 4-5 cm long, hard with tubercles without external gills on the back. Distinguished by 3-6 ridges of bumps along the body. The bumps are blue-grey where they join the body and tip yellow. The short rhinophores are orange. At the bottom there is a dark line or lines of spots/dark lines along the soles of the feet. It secretes harmful substances from glands in the white part of its body.
Phyllidiella pustulosa (Cuvier, 1804)	The body is 4-5 cm long, hard with tubercles that are clustered. The bumps may be pink, red, gray, green or blue. There is a colored margin around the edges of the body. The short rhinophores are black. There is no line along the bottom of the foot. The black background forms more of a grid pattern.
Phyllidiella nigra (van Hasselt, 1842)	The body is 4-5 cm long, hard with tubercles that are evenly distributed throughout the body (not in clusters or ridges along the body). The tubercles are tall and round, pink to red near the tip, but black where they join the body. The short rhinophores are black. When disturbed, it secretes a milky substance. The broad legs on the underside are bluish in color and have no midline.

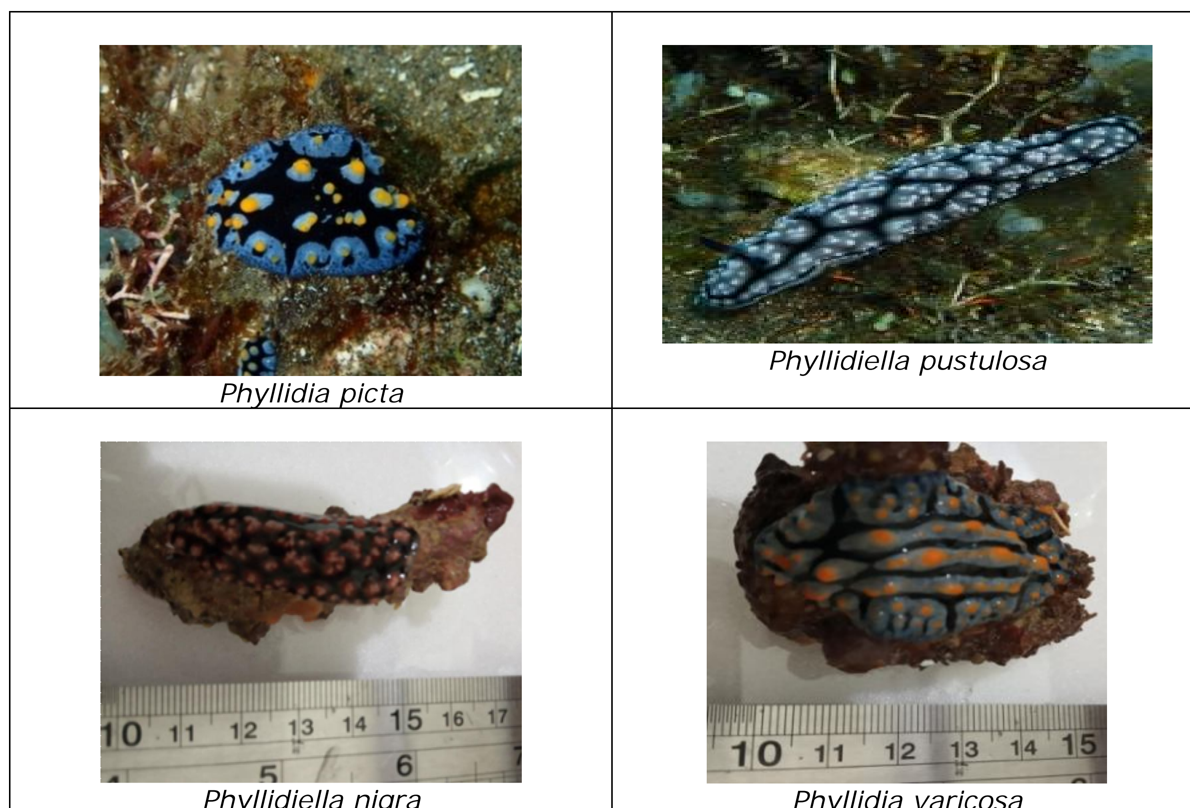

Figure 2. Nudibranch species collected (sample photographs).

Table 2

Antibacterial activity of nudibranch-symbiont isolate and host

Isolate code	*Diameter of inhibition zone*		*Sample code*
	E. coli	*S. aureus*	
RL-2	+	++	19-Ba-Pva-3
RL-6	+	++	18-Mal-Ppi-1
RL-8	+	++	19-Ba-Pva-3
RL-10	-	++	19-Ba-Pni-1
RL-11	+	++	18-Mal-Ppu-2
RL-14	++	++	18-Mal-Ppi-1
Control +	Chloramphenycol		

Notes: - no activity; + diameter of inhibition zone (7-8.5 mm); ++ diameter of inhibition zone (8.6-10 mm); Control: antibiotic Chloramphenicol 1 mg mL^{-1}. Ba-Pva – *P. varicosa* from Bangka waters; Ba-Pni – *P. nigra* from Bangka waters; Mal-Ppi – *P. picta* from Malalayang waters; *P. pustulosa* from Malalayang waters.

Table 2 demonstrates that all isolates can inhibit the growth of *S. aureus* better than *E. coli*, and RL-14 can inhibit both test bacteria, but RL-10 cannot inhibit *E. coli.* Based on the work spectrum, antibiotics comprise two major groups, i.e. broad spectrum antibiotics and narrow spectrum one (Black 2012). Optimization of potential nudibranch symbiont bacterial isolates is still needed to develop as antibiotic source.

***Morphological characteristics of nudibranch-symbiont isolate*.** Morphological observations on the bacterial isolate colony showed variations in colony form, but it had relatively same margin, elevation, and color. All bacterial isolates had basil form, whereas Gram staining indicated that four isolates, RL-2, RL-6, RL-8, RL-10, and RL-11, were Gram positive and one isolate, RL-14, was Gram negative (Table 3).

Table 3

Morphological characteristics of antibacterial-producing isolate colony and cell

Isolate code	*Colony morphology*				*Cell morphology*	
	Form	*Margin*	*Elevation*	*Color*	*Shape*	*Gram*
RL-2	Circular	Undulate	Raised	Milky white	Basil	Positive
RL-6	Irregular	Undulate	Raised	Milky white	Basil	Positive
RL-8	Circular	Undulate	Raised	Milky white	Basil	Positive
RL-10	Rhizoid	Lobate	Flat	Milky white	Basil	Positive
RL-11	Circular	Undulate	Raised	Milky white	Basil	Positive
RL-14	Irregular	Undulate	Raised	Milky white	Basil	Negative

Morphological observations on the colony need to be done because the morphological characteristics of the colony and the pure isolate could become one of the basis for proper identification, continued with chemical test and molecular work. According to Cappuccino & Sherman (2014), Gram positive bacteria can sustain the main dye in Gram staining, gentian violet, so that purple colour appears since the cell wall of the bacteria is mostly composed of peptidoglycan that is able to bind the dye and not damaged when washed with alcohol. Gram negative bacteria have the cell wall mostly composed of lipid layer, so that it cannot maintain the dye at the Gram staining, particularly when washed with alcohol, and this bacteria group gives red colour (the second colour, safranin) at the end of Gram staining.

Molecular identification of nudibranch-symbiont bacteria. ***DNA amplification of 16SrRNA gene***. Six nudibranch-symbiont bacterial isolates that had antibacterial activity were amplified using primer 27F and 1492R with 1400-1500 bp target fragments (Figure 3).

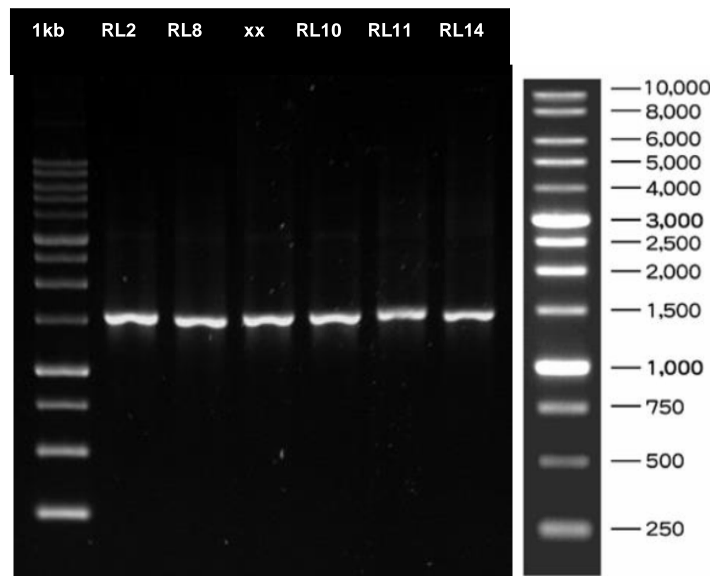

Figure 3. Electrogram of partial amplification of 16S rRNA gene on 1% agarose gel.

Partial sequence based on 16S rRNA gene succeeded to obtain 1416 bp of RL-2, 1391 bp of RL-8, 1400 bp of RL-10, 1410 bp of RL-11, and 1394 bp of RL-14, respectively. BLAST analysis with GenBank database showed the identity of RL-2, RL-8, RL-10, and RL-11 isolates had 100% homologous genes with *Bacillus* as follows: RL2 - *B. aryabhattai*, R-8 - *B. licheniformis*, RL-10 - *B. tequilensis*, and RL-11 - *B. licheniformis,* whereas RL-14 had 99% similarity to *Alcaligenes faecalis* (Table 4).

Table 4
Homology of 16S rRNA gene partial sequence of nudibranch symbiont bacteria based on GenBank database

Isolate code	*Homologous species to the GenBank*	*Accession*	*Identity (%)*	*E-value*
RL-2	*B. aryabhattai* strain 3595	MT538470	100	0
RL-8	*B. licheniformis* strain CY-012	KR349358	100	0
RL-10	*B. tequilensis* strain JLS12	MT501810	100	0
RL-11	*B. licheniformis* strain QX13	MT367732	100	0
RL-14	*A. faecalis* subsp. *phenolicus* strain CPE1	MK928387	99.9	0

In BLAST analysis, a species of bacterium with 16S rRNA encoding gene is identical if it has the identity above 97.5%, and at the genus level, the identity is above 95% (Stackebrandt & Goebel 1994), so that the isolates obtained in the present study had very close kinship to *B. aryabhattai*, *B. licheniformis*, *B. tequilensis*, *B. licheniformis, Alcaligenes faecalis* respectively, as shown in the phylogentic tree (Figure 4).

The present study found *B. aryabhattai* (RL-2) and *B. licheniformis* (RL-8) isolated from *P. varicosa*, *B. tequilensis* (RL-10) from *P. nigra,* and *B. licheniformis* (RL11) from *P. pustulosa*, whereas *A. faecalis* (RL-14) was obtained from *P. picta*. RL-6 was unidentified. Some of these are also reported by Böhringer et al (2017) from different nudibranch species. In North Sulawesi, they succeeded to isolate *Bacillus subtilis* from *Chromodoris dianae* and *B. aryabhattai* isolated from egg mass of *Chromodoris annae*. Kristiana et al (2020) also found bacterium *Virgibacillus salarius* isolated from nudibranch *P. varicosa* from Bali waters and *B. kochii* with other bacteria, *V. marismortui, V. dokdonensis, Vibrio algynoliticus,* and *Pseudoalteromonas piscicida*, in nudibranchs from Saparua waters (Kristiana et al 2019; Sarjito et al 2020). Kristiana et al (2020) found isolate strain close to *Pseudoalteromonas rubra* in nudibranch *Phyllidia coelestis* as well. *Bacillus* sp. shows various biological activity, such as antimicrobial (Sumi et al 2015; Caulier et al 2019), anticancer, antialga (Mondol et al 2013), enzyme producer (Hatmanti 2000; Ibrahim et al 2013; Purwani 2018). *B. licheniformis* produces wide spectral antibacterial activity against Gram positive or Gram negative bacteria (Kuta et al 2009; Kannahi & Eshwari 2016). *B. aryabhattai* TBRC8450 isolated from shrimp pond has antimicrobial activity against pathogenic strains *Vibrio harveyi* and *V. parahaemolyticus* (Tepaamorndech et al 2019), whereas *B. subtilis* isolated from gastropod has antibiofilm properties (Viju et al 2020), and *B. licheniformis* isolated from marine sediment produces bacteriostatic lipopeptide (Chen et al 2017).

Detection of NRPS and PKS genes. PCR amplification of NRPS and PKS I encoding genes of 5 potential symbiont bacterial isolate as antibacterial revealed that the target tape of 700-800 bp for NRPS could be detected in RL-8 and RL-11 (*B. licheniformis*) and RL-10 *B. tequilensis* with A3F/A7R primer, while the target band of 1200-1400 bp to indicate the presence of PKS I gene could not be clearly detected using K1F/M6R primer since other non target bands were amplified as well (Figure 5) that could result from non specific primer. According to Ayuso-Sacido & Genilloud (2005), appropriate primer selection highly determines the PCR product because it will also determine the success of gene detection. K1F/M6R is more specific for keto synthase (KS) sequence and methyl-malonyl-CoA transferase (Ayuso-Sacido & Genilloud 2005), but the use of other primers may need to be considered as well, such as KSDPQQF and KSHGTGR primer pair as used by Kurnia et al (2017).

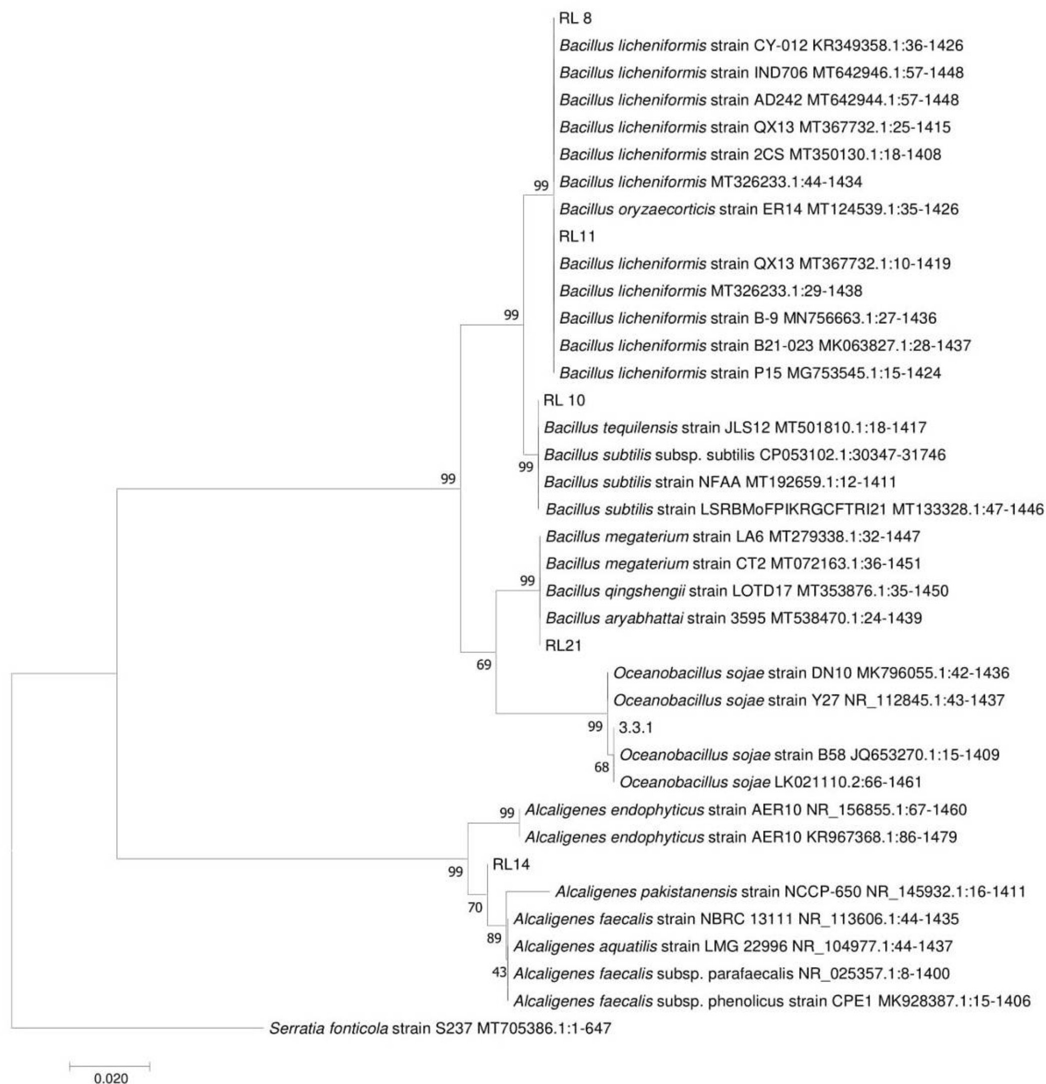

Figure 4. Phylogenetic tree of nudibranch symbiont bacteria based on 16S rRNA sequence analysis, *Serratia fonticola* strain S237 as outgroup.

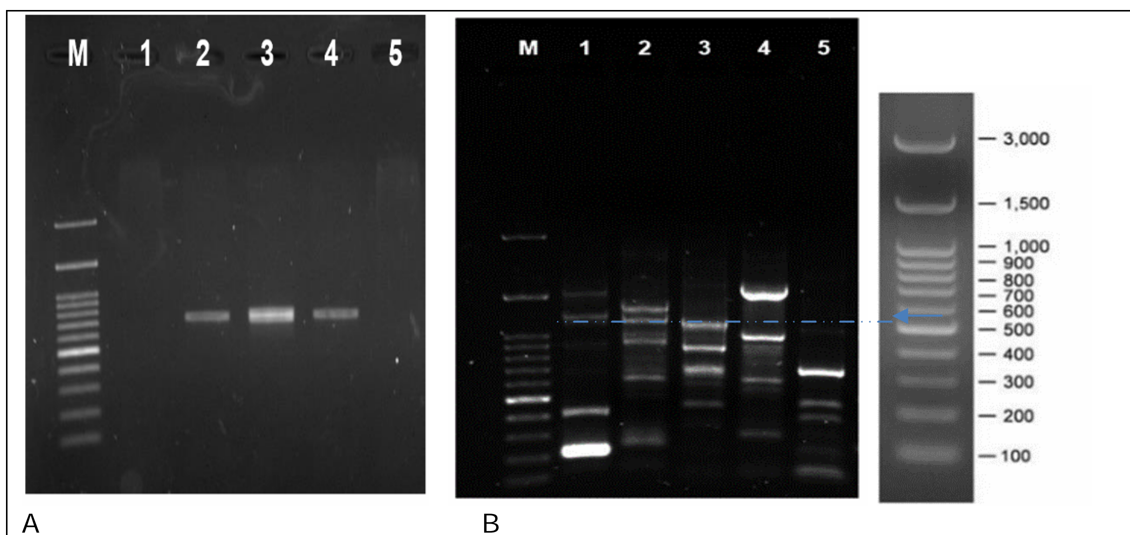

Figure 5. Electrogram of NRPS (A) and PKS (B) gene amplification. M = 1kb, 1 = RL2, 2 = RL8, 3 = RL10, 4 = RL11, 5 = RL14.

The presence of NRPS gene band indicates the presence of nonribosomal peptide compound catalyzed by NRPS enzyme produced by *Baccilus* bacteria. Previous finding informed that NRPS gene is held by *Baccilus* (Caulier et al 2019; Sudarmono et al 2019). The present study found that RL-2 isolate only contained PKS encoding gene, RL-11 isolate only had NRPS gene, whereas RL-14 isolate was not detected to have either PKS or NRPS encoding genes. Nevertheless, not all bioactive compounds are produced through PK and NRP syntheses (Basharat et al 2018), so that no PKS and NRPS genes do not mean that the bacteria do not have antibacterial activity. Therefore, RL14 isolate identified as *A. faecalis* is not formed through PKS or NRPS biosynthetic path. Kapley et al (2016) found 6 gene clusters related with antimicrobial compound biosynthesis in *Alcaligenes* sp. genome, HPC 1271, i.e. ectoine, butyrolactone, phosphonate, terpene, 1-typed PKS, and NPRS, whereas Basharat et al (2018) found 3 gene clusters, i.e. butyrolactone, ectoine, and NPRS in *A. faecalis* subsp. *phenolicus* genome, MB207. The isolates of RL-8 and RL-10 that hold both NRPS and PKS encoding genes enable to have NRPS-PKS hybrid gene. The hybrid gene of NRPS-PKS could yield new bioactive compound in hybrid structure (Ansari et al 2004; Fisch 2013).

Conclusions. From nudibranchs, *Phyllidia picta*, *Phyllidia varicosa*, *Phyllidiella nigra*, and *Phyllidiella pustulosa*, a total of 121 single colonies were obtained, 6 isolates showed strong inhibition ability on *Escherichia coli* and *Staphylococcus aureus*. The bacteria potential to produce compounds showed variations in morphological characteristics, such as shape, margin, elevation, and color, whereas cell morphology indicated that 5 isolates were Gram positive bacteria and one was Gram negative bacterium. Based on DNA barcoding, 4 isolates were identified as *Bacillus aryabhattai*, *Bacillus licheniformis*, *Bacillus tequilensis*, and *Alcaligenes faecalis.* PKS and NRPS gene detection showed that *Bacillus aryabhattai* had only PKS gene, *Bacillus licheniformis* RL-11 had only NRPS gene, *Bacillus licheniformis* RL-8 and *Bacillus tequilensis* had both PKS and NRPS, and *Alcaligenes faecalis* did not have either PKS or NRPS gene.

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