Depik
Jurnal Ilmu-Ilmu Perairan, Pesisir dan Perikanan
p-ISSN: 2089-7790, e-ISSN: 2502-6194 http://jurnal.unsyiah.ac.id/depik

RESEARCH ARTICLE

DOI: 10.13170/depik.9.1.15126

Biological and chemical diversity of the Indonesian marine nudibranchs based on MS/MS molecular networking approach

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Received: 04 December 2020
Accepted: 19 March 2020

Abstract. *The collection of 337 specimens of the Chromodoris species was conducted in Sabang island, Indonesia, from 2013 to 2019. The purpose of this study to investigate the biodiversity and their secondary metabolites related by molecular structures. There are 21 species together with the chemical diversity detected by MS/MS molecular networking approach. The results showed that the nudibranch species of C. willani and C. geometrica have the highest abundance species, while C. aureopurpurea has the lowest abundance species. The result of the chemical investigation showed that the class of diterpene derivatives was found from all specimens of Chromodoris genus in Sabang island, the western part of the Indonesian Archipelago.*
Keywords*: Nudibranch, biodiversity, chemical diversity, MS/MS molecular networking, Chromodoris genus*

Introduction

The family of Chromodorididae or known as Chromodoris nudibranchs have a worldwide distribution, which primarily found in tropical and subtropical sea waters in the Indo-Pacific region (Gosliner *et al.*, 2018). They are the carnivorous species like prey on sponges, corals, sea anemones, hydroids, barnacles, fish eggs, sea slugs, and other nudibranchs on the seabed (Kara *et al.*, 2018). Because they have unique eating habits, they are susceptible to all changes to their food sources (Johnson and Gosliner, 2012). This phenomenon explains that they are likely to respond very rapidly to environmental change such as pH change due to ocean acidification, and they have potential as a natural bioindicator. On the other hand, climate change dramatically affects the existence of marine organisms (Alheit and Bakun, 2010). In consequence, the chemical reaction arising from the ocean acidification can damage chemical equilibrium in seawater and change the marine environment condition (Albright *et al.*, 2018; Robert, 2012).

In the fact that the corals have long-lived, and they can respond the global warming by bleaching the zooxanthellae out of their tissues (Hughes *et al.*, 2017). However, nudibranchs have a short-lived animal which their lifespan is two months to one year for most species. They respond the global warming by their chemoreception so they would seem extremely more potent as sensitive bioindicators of climate change (Seroy and Grünbaum, 2018; Korshunova *et al.*, 2017; Jeffrey *et al.*, 2016).

Chemoreceptors or known as rhinophore is a pair of chemosensory rod-shaped or ear-like structures that are the most prominent part of the external head anatomy in nudibranchs as the scent or taste receptors. The rhinophores detect all of the scents and tastes from the chemicals dissolved in the seawater. It allows the nudibranchs to stay close to their food source such as sponges and to find mates. However, the rhinophore unable to detect the

scents and tastes. Also, the marine species are likely to generate stress that could impact metabolic activity due to the shift of seawater environment such as the decrease of the pH level due to ocean acidification (Lewis and Michèle, 2017; Valles-Regino *et al*., 2015; Scott *et al*., 2009).

Nudibranchs of the genus *Chromodoris* has been known to have the highest abundance of scalarane class diterpene that has high biological activity as anticancer (Wu *et al*., 2019). Even though no published paper related to the nudibranch research in Sabang island so far, but we expect that this genus abundant in Sabang island as an active volcanic island that is known has high biodiversity on the coral reefs in the western part of the Indonesian Archipelago. Here, we investigated the natural products of various species of this genus and also which can be used as natural bioindicators of global climate change caused by the ocean acidification effect.

Materials and Methods

General experimental procedures

Analysis of NMR spectra were performed on a Bruker 500 MHz NMR spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively; Bruker BioSpin, Billerica, MA, USA) in deuterated $CHCl_3$ (Cambridge Isotope Labs) at ambient temperature, and the residual solvent signals were detected at ^{1}H 7.26 ppm and ^{13}C 77.16 ppm to Tetramethylsilane (TMS). The Offline NMR data processing was performed using the MNova 8.1 NMR software package (Mestrelab Research, Santiago de Compostela, Spain). The data of ESI-Ion Trap MS by High-resolution method were obtained using an Amazon Ion Trap (Bruker Daltonics, Bremen, Germany) MS system coupled to an Agilent 1260 Infinity LC system (Agilent, Santa Clara, CA, USA) incorporated with a reversed-phase C_{18} analytical HPLC column (5 µm, 250 mm × 4.6 mm, Phenomenex, Torrance, CA, USA). Further, the data analyzed with Bruker Compass DataAnalysis 4.2 software.

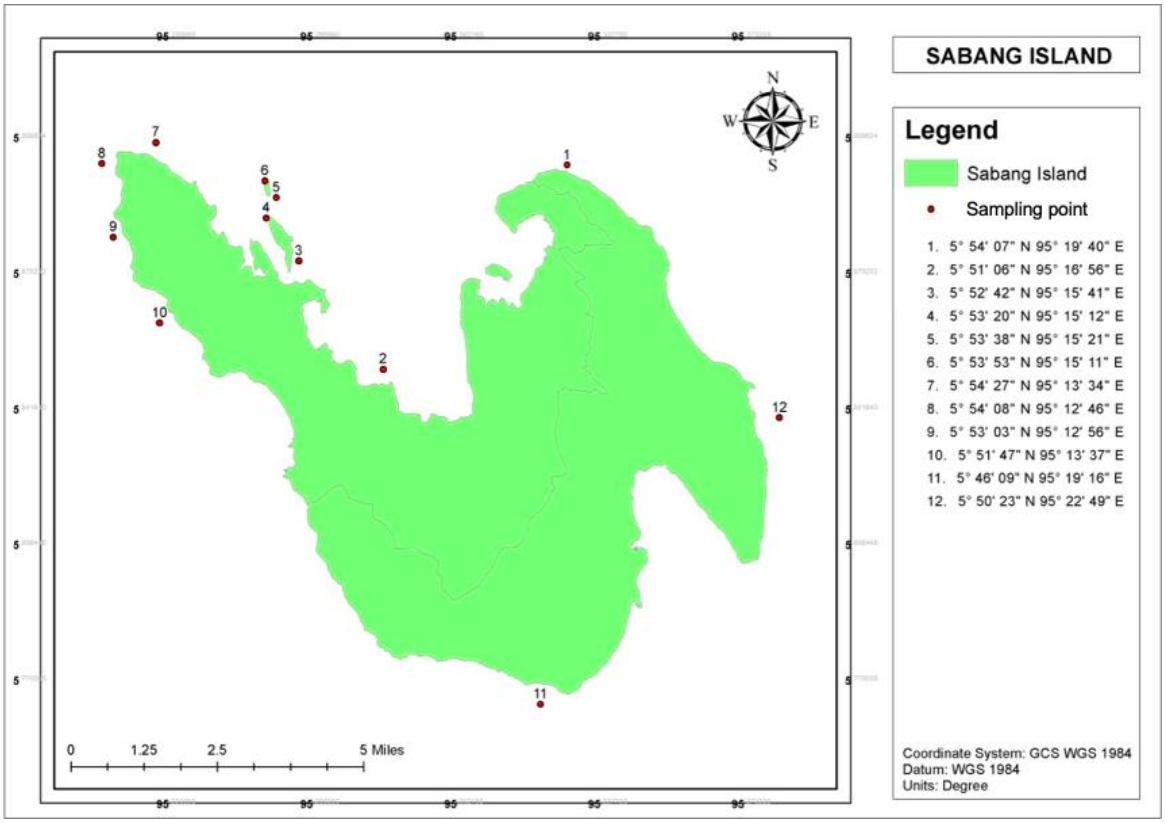
Collection and extraction of specimens

A total of 337 specimens of *Chromodoris* (Table 1) were collected by hand using SCUBA equipment at 10-45 meters from 2013-2019 in Sabang island, Indonesia (Figure 1). The number of specimens from each collection site were as follows: 32 (Sumur Tiga), 20 (Gapang), 32 (Rubiah Island), 25 (Arus Paleeh), 15 (Seulako), 40 (Batee Tokong), 23 (Batee Gla), 42 (The Canyon), 38 (Pante Peunateung), 27 (Bak Kopra), 16 (Batee Meuduroe), and 27 (Anoi Itam) (Figure 1).

The fresh specimens were kept frozen until extraction by acetone and partitioned using ethyl acetate and water to obtain ethyl acetate layer as organic material that contains secondary metabolites.

General methods of chemical analyses

Each ethyl acetate extract was subjected first for ^{1}H Nuclear Magnetic Resonance (NMR) analyses to examine whether a dominant marker of scalarane existed. Furthermore, the purification procedures using silica gel thin-layer chromatography (TLC) and open column chromatography to obtain major constituent. Then, the presence of a major scalarane constituent was confirmed qualitatively by a gradient High Performance Liquid Chromatography (HPLC) system equipped with a photodiode array detector using a silica gel column with linear gradient elution profile in 20 minutes from 100 % *n*-hexane to 100% ethyl acetate. ^{1}H and ^{13}C NMR spectra were taken on a Bruker 500 MHz by dissolving extracts or pure compounds in deuterated chloroform using tetramethylsilane as an internal standard. Infrared (IR) spectra were taken on a Jasco FTIR-300, MS spectra on a Hitachi M-2500 instrument, and ultraviolet-visible (UV-Vis) spectra on a Jasco Uvidec 610 equipments.



(1) Sumur Tiga; (2) Gapang; (3) Rubiah Island; (4) Arus Paleeh; (5) Seulako Island; (6) Batee Tokong; (7) Batee Gla; (8) The Canyon; (9) Pantee Peunateung; (10) Bak Kopra; (11) Batee Meuduroe; (12) Anoi Itam.

Figure 1. Site of Sampling in Sabang Island, Indonesia

Analysis extracts by LC-MS/MS

The lyophilized sample (10 mg) was crushed and sonicated in 1:1 methanol: dichloromethane for 15 minutes, and centrifuged for 10 minutes at 14000 rpm. The supernatant was transferred to 96-well plates, and rotary evaporation used to remove the solvent. The residue was resuspended in 200 µL of methanol, including 2 µM sulfamethazine as an internal standard, sonicated for 10 minutes, and centrifuged for 10 minutes at 2000 rpm. The supernatant was diluted twofold in methanol solution and add 2 µM sulfamethazine to give a total of four times (to obtain a final concentration of 0.0625X relative to the original resuspended extract). Next, 2 µL of the diluted extract was injected for LC-MS/MS analysis using an UltiMate 3000 UPLC system (Thermo Scientific, Waltham, MA) using a Kinetex reverse phase C_{18} column (1.7 um × 50 mm × 2.1 mm, Phenomenex, Torrance, CA, USA), coupled to a Maxis Impact HD Q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The column was equilibrated with 35% solvent B (LC-MS grade acetonitrile) for 2 min, followed by a linear gradient from 35% B to 100% B in 12 min, held at 100% B for 3 min, then adjusted back to 35% B over 2.0 min and maintained at 35% B for 2 min. The flow rate was maintained at 0.5 mL/min throughout the run. The positive ion mode on MS spectra was needed in the range of 50-2000 m/z. The data were collected by a dependent data acquisition method with an advanced stepping method, and the raw data files were converted to mzXML format using Bruker Compass DataAnalysis 4.2 software (Chong *et al.*, 2019; Myers *et al.*, 2017; Teta *et al.*, 2015).

Molecular networking approach

The molecular networks are visual displays of the chemical space present in tandem mass spectrometry (MS/MS) experiments. This visualization approach can detect sets of spectra from related molecules based on the molecular weight and the parent cluster of significant constituents from the extracted sample without purification steps. The nodes can be supplemented with metadata, including dereplication matches or information that is provided by the user, such as abundance, the origin of the product, biochemical activity, or hydrophobicity, which can be reflected in a node's size or color. The visualization of a node's size or color related to the significant constituents as the big nodes and minor components as the small nodes (Protsyuk *et al*., 2018; Wang *et al*., 2016).

This map of all related molecules is visualized as a global molecular network. The detection for featuring, grouping, and alignment was performed by MZmine2 format data. The CSV file (quantitative feature table) and MGF file (MS/MS spectra for each feature) generated in MZmine2 were uploaded and used for the feature-based molecular networking workflow in GNPS (http://gnps.ucsd.edu.). These files are also available as part of the MassIVE dataset described above. The precursor ion mass tolerance and production mass tolerance were both set to 0.05 Da (Nothias *et al*., 2018; Pluskal *et al*., 2010).

Chemoreception approach

The acidity effect on seawater for chemoreception of *Chromodoris* genus was tested in the aquarium without current. There is a mate specimen as a stimulus, five specimens as individually tested, and five specimens as negative control were placed in the different aquarium, which contains 20 cm of seawater level from the bottom of the aquarium. The stimulus was placed in a cage, and the individual tested was set 30 cm from the cage. The seawater was maintained by Neptune Systems Apex-EL Aquarium Controller. The treatment for the acidic condition, the pure CO_2 was added into pH = 7.50, and the control of seawater was treated at pH = 8.00 without CO_2 for 48 H. The effect of seawater acidity on nudibranch determined by the movement of the nudibranch in responding to a stimulus by environment condition. Movements that lead to the stimulus are considered to detect a chemical stimulus, while movements that move away from stimulus are considered not to detect a chemical stimulus (Lewis and Michèle, 2017; Smith, 2005).

Results

Identification of secondary metabolite extracts based on LC-MS/MS and molecular networking approach

The crude extracts from each nudibranch specimen analyzed by LC-MS/MS and preprocessed by MZmine2, resulting in 1425 features used for feature-based molecular networking using the Global Natural Product Social (GNPS) website platform and Metaboanalyst approach used for statistical analysis. To obtain a global figure of the secondary metabolite from nudibranchs, we use a feature-based molecular networking approach using the GNPS website (Figure 2-4). GNPS molecular networking approach used for the rapid analysis of large mass spectra datasets. In the output data, the nodes represent fragmentation spectra corresponding to individual molecular ions. The data of molecular ions which have similar mass fragmentation patterns in the MS/MS (measured as cosine similarity) are linked together (via edges) to form clusters that show the molecular families (Nothias *et al*., 2018).

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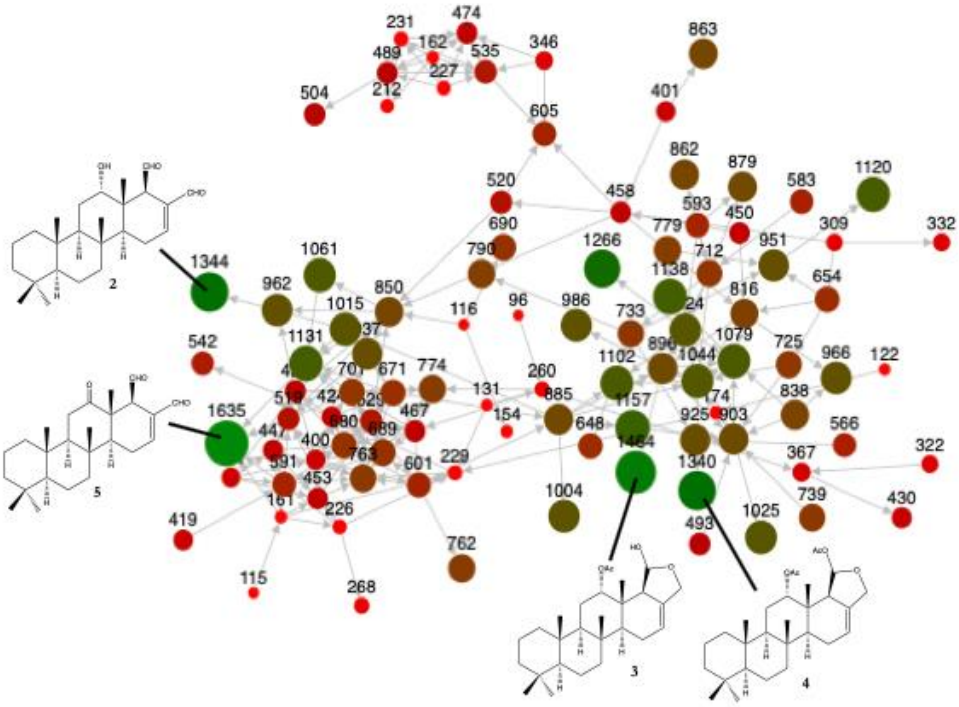

Figure 2. Parent cluster I

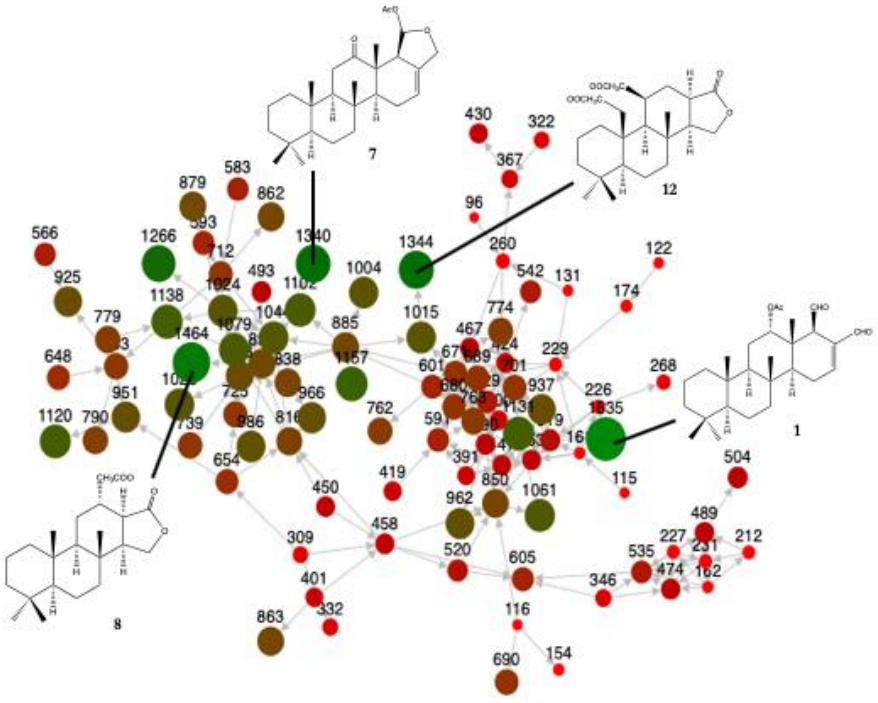

Figure 3. Parent cluster II

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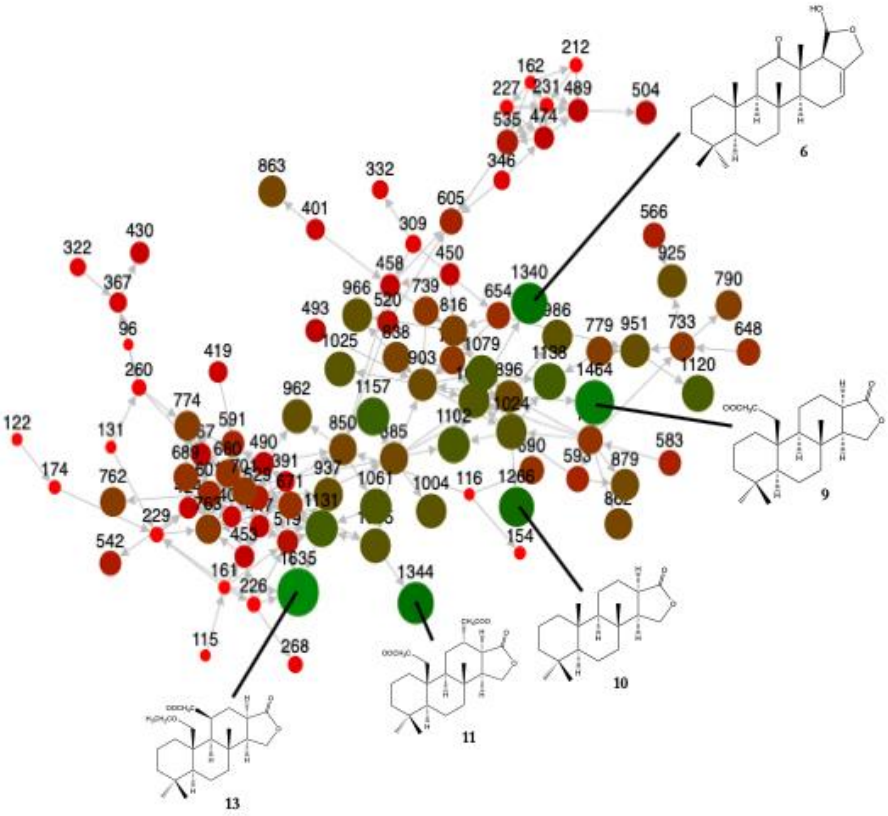

Figure 4. Parent cluster III

Based on the parent cluster I to III (Figure 2-4), the size of nodes indicate as a major and minor constituent of the extract. The highest similar molecular ions and mass fragmentation patterns as molecular families show the chemotypes from compounds 2, 3, 4, and 5 (Figure 5). On the other hand, the parent cluster II (Figure 3) shows chemotypes from compound 1, 7, 8, and 12 (Figure 5). And also, for parent cluster III (Figure 4) suggest containing chemotypes from compound 6, 9, 10, 11, and 13 (Figure 5).

Discussion

Structure identification of scalarane diterpene

The all compounds were identified as known compounds (Figure 5) followed by NMR spectra comparison with those previously published (Forster *et al.*, 2016; Katavic *et al.*, 2012; Gavagnin *et al.*, 2004; Fontana *et al.*, 2000; Hambley *et al.*, 1990; Kernan *et al.*, 1990; Cimino *et al.*, 1974).

Biological and chemical diversity of nudibranchs of the *Chromodoris* genus

The number of each species from a total of 337 specimens collected in Sabang island are follows: *C. willani* (31), *C. geometrica* (28), *C. kuniei* (22), *C. albopunctata* (21), *C. lochi* (20), *C. magnifica* (20), *C. albonares* (18), *C. annae* (16), *C. roboi* (15), *C. rufomaculata* (14), *C. collingwoodi* (13), *C. dianae* (13), *C. tinctoria* (13), *C. colemani* (12), *C. fidelis* (11), *C. vibrata* (11), *C. decora* (10), and *C. aureopurpurea* (7). Based on this data, we describe the chemotypes for each species related to the variation of their environment.

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Table 1. Number of specimens at each collection sites

Species/ Location	1	2	3	4	5	6	7	8	9	10	11	12	Total
C. albonares					4	10		2	1			1	18
C. albopunctata	3	9		2	2			3				2	21
C. annae	1			4		6		2		3			16
C. aspersa	1		4			8		1					14
C. aureopurpurea				3				1	3				7
C. burni	5		2						4	3			14
C. coi	5		1			1	3	2				2	14
C. colemani			4			3	2			2	1		12
C. collingwoodi				2	3		3	1				4	13
C. decora	1		4					2	3				10
C. dianae	2		2					1	6	2			13
C. fidelis		5					2		2		2		11
C. geometrica	7			4		8	4	5					28
C. kuniei			4					3	5	5	2	3	22
C. lochi			3	3				5			8	1	20
C. magnifica	2		3			1		7		4		3	20
C. roboi	1			2			4		3			5	15
C. rufomaculata	4		5			3			2				14
C. tinctoria				1					4	8			13
C. vibrata				2	4			3				2	11
C. willani		6		2	2		5	4	5		3	4	31
Total	32	20	32	25	15	40	23	42	38	27	16	27	337

(1) Sumur Tiga; (2) Gapang; (3) Rubiah Island; (4) Arus Paleeh; (5) Seulako Island; (6) Batee Tokong; (7) Batee Gla; (8) The Canyon; (9) Pantee Peunateung; (10) Bak Kopra; (11) Batee Meuduroe; (12) Anoi Itam.

Table 2. Number of collected specimens based on the chemotypes

Species/ chemotype	1	2	3	4	5	6	7	8	9	10	11	12	13
C. albopunctata	2	-	-	-	18	4	-	-	-	-	-	-	-
C. albonares	3	-	-	-	2	-	-	-	-	-	-	-	-
C. annae	-	-	-	-	3	-	-	-	-	-	-	-	-
C. aspersa	-	-	-	8	-	-	-	-	-	-	-	-	3
C. aureopurpurea	-	-	-	-	-	-	2	-	-	-	-	-	-
C. burni	-	-	-	1	-	-	-	-	-	-	-	3	-
C. coi	-	-	-	1	-	-	-	-	-	-	-	7	-
C. colemani	-	-	-	-	-	-	3	-	-	-	-	-	4
C. collingwoodi	-	8	-	-	2	-	-		-	-	2	-	-
C. decora	-	1	-	-	-	-	2	4	-	-	-	-	-
C. dianae	1		-	-	-	-	-	3	-	-	-	-	2
C. fidelis	1	4	-	-	4	-	-	-	-	-	-	6	-
C. geometrica	-	3	-	-	-	-	-	-	-	-	-	-	-
C. kuniei	-	-	12	-	-	-	-	3	-	-	-	-	-
C. lochi	-	-	-	-		-	-	-	2	8	-	-	-
C. magnifica	-	5	-	6	-	-	-	3	-	-	-	-	-
C. roboi	-	-	3	-	-	-	-	-	-	12	-	-	-
C. rufomaculata	2	-	-	-	-	-	-	6	-	-	5	-	-
C. tinctoria	-	-	-	-	-	-	-	-	7	4	-	-	-
C. vibrata	-	-	-	-	-	-	5	-	-	2	-	-	-
C. willani	-	-	5	-	-	-	-	-	-	-	-	-	-

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A total of 21 species were observed, *C. willani* and *C. geometrica* had the highest number of specimen collected as the most abundant species (31 and 28 specimens, respectively). At the same time, *C. aureopurpurea* was the least abundant species (7 specimens). Based on NMR spectra analysis, the secondary metabolite which contains scalarane diterpene class were separate based on the type of chemical structure were including: chemotype 1 (9 by 5 specimens), chemotype 2 (21 by 5 specimens), chemotype 3 (20 by 3 specimens), chemotype 4 (16 by 4 specimens), chemotype 5 (29 by 5 specimens), chemotype 6 (4 by 1 specimens), chemotype 7 (12 by 4 specimens), chemotype 8 (19 by 5 specimens), chemotype 9 (9 by 2 specimens), chemotype 10 (26 by 4 specimens), chemotype 11 (7 by 2 specimen), chemotype 12 (16 by 3 specimen), and chemotype 13 (9 by 3 specimen). Interestingly, the chemotypes of 8 to 13 also were found in the marine sponge (Hambley *et al.*, 1990; Kernan *et al.*, 1990). This fact indicates that all nudibranch eat sponges so that they will contain similar chemotypes from the sponges in their environment (Proksch, 1994).

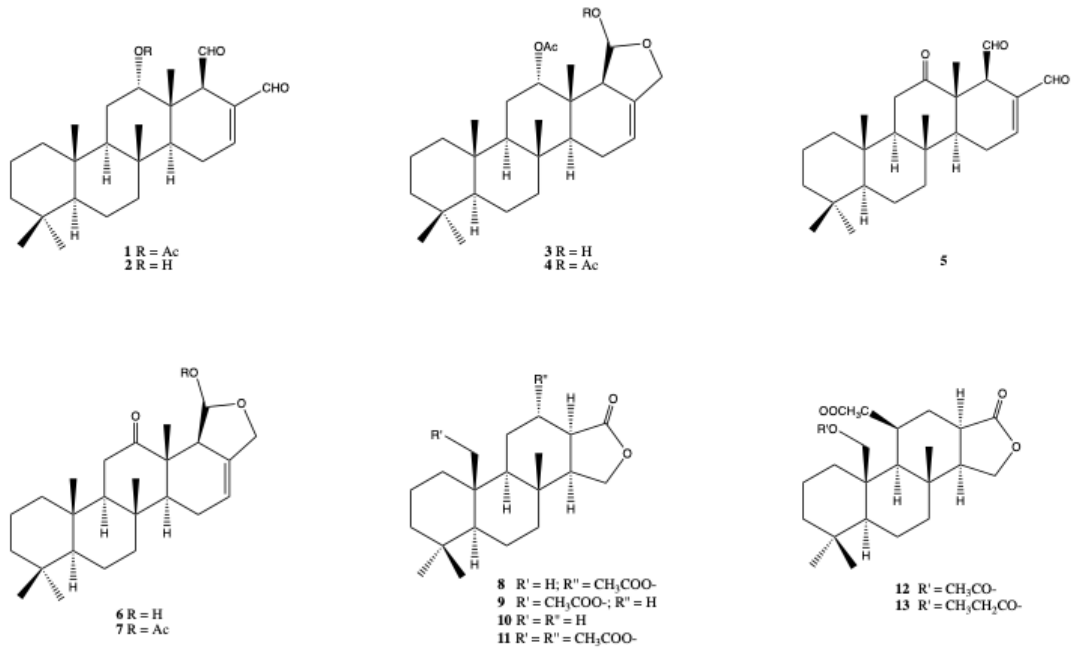

Figure 5. Chemotypes of scalarane from 21 *Chromodoris* nudibranch species in Sabang island

Chemoreception approach as a bioindicator

The results of the only chemoreception approach show that the nudibranch of the genus *Chromodoris* treated in normal seawater moves to the stimulus. However, nudibranchs treated in acidic seawater move away from the stimulus. This fact indicates that nudibranchs treated by acidic seawater unsuccessful at detecting a chemical stimulus. As has also been reported in previous research which shows that a decrease in pH has the potential to inhibit chemoreception so that they unable to find their food source (Kump *et al.*, 2009; Munday *et al.*, 2009).

Chemoreception plays an essential role in natural bioindicator. The nudibranchs in areas that have decreased pH values cannot find food sources due to reduced sensitivity of chemoreception will reduce the rate of metabolism so that primary metabolites will experience interference. Although they have secondary metabolites that are unique as a self-defense system to survive in the marine environment due to the influence of predators, temperature, pressure, and sunlight, they unable to use the secondary metabolites for survival (Jensen *et al.*, 2014).

When nudibranchs experience primary metabolite disorders, they will experience an immediate death. We conclude that nudibranchs can be used as natural bioindicators of ocean acidification as a result of global warming based on chemoreception approach. On the other hand, we suggest that the mass coral bleaching up to 60% in the coral reef system as a result of ocean acidification since early 2010 in Sabang Island plays an important role in decreasing the nudibranchs population (Rudi *et al*., 2012).

Further analysis, the chemoreception approach of nudibranchs closely related to the abundant of chemotypes containing compounds 5, 10, 2, 3 and 8, respectively. However, the less abundant chemotype comes from compounds 1 and 9, 11, and 6, respectively. The nudibranchs use the chemoreception as chemical cues. Because of their heavy reliance on the chemoreception, we suggest that nudibranchs are especially prone to interference from nature. The presence of acid caused by the presence of carbon dioxide from the atmosphere makes chemical equilibrium in seawater should be achieved, so that dissociates into a bicarbonate ion and a hydrogen ion resulting in increasing the ocean acidity. The effects of ocean acidification on chemoreception make the chemical compound as chemoreception was decomposed, so the nudibranchs are likely to be less successful at finding the foods and the mates (Lewis and Michèle, 2017; Valles-Regino *et al*., 2015; Scott *et al*., 2009; Kump *et al*., 2009; Karuso, 1987).

On the other hand, the other reasons for this diversity due to the reproductive stage of the nudibranch which causes genetic differences that result in hybridization between species, including biosynthetic pathways by environmental such as symbiotic algae, etc. (Ekimova *et al*., 2015; Ritson-Williams and Paul, 2007; Barsby *et al*., 2002).

Conclusion

We collected 337 specimens from 21 Chromodoris nudibranch species during the collection at 12 sampling sites of Sabang island in 2013 – 2019. The most abundant species were *C. willani* and *C. geometrica*, meanwhile, *C. aureopurpurea* was the least abundant species. The chemotypes of scalarane class diterpene are follows chemotype 1 (9 by 5 specimens), chemotype 2 (21 by 5 specimens), chemotype 3 (20 by 3 specimens), chemotype 4 (16 by 4 specimens), chemotype 5 (29 by 5 specimens), chemotype 6 (4 by 1 specimens), chemotype 7 (12 by 4 specimens), chemotype 8 (19 by 5 specimens), chemotype 9 (9 by 2 specimens), chemotype 10 (26 by 4 specimens), chemotype 11 (7 by 2 specimen), chemotype 12 (16 by 3 specimen), and chemotype 13 (9 by 3 specimen). The highest numbers of compounds were from chemotypes 5, 10, 2, 3, and 8, while the lowest numbers of compounds were from chemotypes 1, 9, 13, 11, and 6, respectively.

Acknowledgments

The authors thank to Osaka University, the University of the Ryukyus, and Lampung University for the collection of specimens in 2013-2019. We also thank to the Laboratory of Marine Chemistry and Fisheries Biotechnology, Syiah Kuala University for supporting the laboratory equipment during specimens collection, and to the Ministry of Marine Affairs and Fisheries of the Republic of Indonesia in Sabang for supporting the SCUBA equipment.

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How to cite this paper:

Kurnianda, V., D.A. Winahyu, R. Firdaus, E. Wahyudi, M. Musman. 2020. Biological and chemical diversity of the Indonesian marine nudibranchs based on MS/MS molecular networking approach. Depik Jurnal Ilmu-Ilmu Perairan, Pesisir dan Perikanan, 9(1): 83-94.