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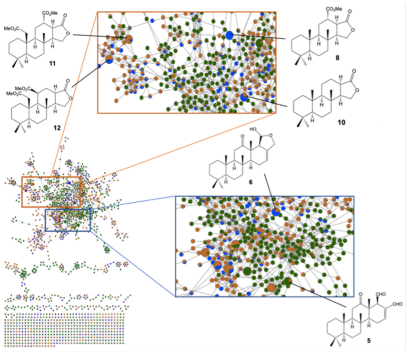
Marine Biodiversity Insights from Indonesia: Cryptic Species and Chemical Profiles in the Nudibranch Genus *Chromodoris*

Muhammad Rizki Ramadhan [1,*] and Sofyatuddin Karina [1]

[1] Department of Marine Science, Faculty of Marine and Fisheries, Syiah Kuala University, Banda Aceh 23111, Indonesia
* Correspondence: muhammadrizkiramadhan1@outlook.com



Abstract: During our ongoing biodiversity investigation on Weh Island, we encountered the marine Gastropod mollusks genus *Chromodoris*. Phylogenetic analysis revealed the presence of cryptic species of *Chromodoris lochi* on 82 specimens. We also collected their prey-specific sponge, *Spongian* sp., to evaluate their chemical contents. As a result, the chemical profile showed well-known scalarane diterpenoids on either nudibranch or the sponge by spectroscopy analysis. Further analysis with molecular networks revealed that the chemical profile of the genus *Chromodoris* had inadvertently been mixed with other clades to introduce chemical variations. Detailed analysis by descriptive statistical approach indicated that the biochemical parameters have a higher concentration in the northern than in the southern area of Weh Island.

Keywords: Biodiversity, *Chromodoris*, Molecular Network, Weh Island.

1. Introduction

Chromodoris is the largest genus of colorful sea slugs primarily found in tropical and subtropical Indo-Pacific regions. This genus has been shown to be genetically distinct [1,2]. Members of *Chromodoris* are known to inhabit coral reefs and prey on specific sponges. During this interaction, nudibranchs can ingest sponge-derived compounds and store them in mantle dermal formations (MDFs), where they are converted for chemical defense purposes [3]. This behavior indicates the presence of a detoxification mechanism that facilitates the transport and sequestration of toxic metabolites into the MDFs. Recent genome mining has uncovered the role of sponge-associated microbes in producing secondary metabolites [4]. These microbes, particularly uncultured marine microorganisms residing on sponge surfaces, are likely ingested along with sponge tissue, enabling nudibranchs to acquire microbial-derived compounds.

Several studies have reported such sponge-microbe associations. For example, *Lamellodysidea herbacea* and *Dysidea granulosa* host the cyanobacterium *Synechococcus elongatus*, which biosynthesizes polybrominated diphenyl ethers [5]. Similarly, *Theonella swinhoei* harbors polyketide-producing *Pseudomonas* species [6], while the actinomycete *Micromonospora* sp. found in *Acanthostrongylophora ingens* produces manzamine A [7]. Chemical investigations of nudibranchs have identified prey-specific compounds. *C. luteorosea* diterpenoids from *Dendrilla* sp., *Dysidea* sp., *Chelonaplysilla* sp., and *Aplysilla polyrhaphis* [8]; *C. elisabethina* and *C. magnifica* prey on *Heteronema* sp. containing puupehenone, and *C. lochi* feeds on *Luffariella variabilis* containing manoalide [9]. In our recent expedition around Weh Island, *Chromodoris* specimens were found to contain scalarane-type diterpenoids, known for antibacterial, antifungal, antifouling, anticancer, and pheromonal activities [10–12]. However, the ecological and chemical relationship between nudibranchs and their sponge prey, particularly involving microbial mediation, remains underexplored. This study aims to elucidate the biological and chemical diversity of *Chromodoris* and associated uncultured sponge microbes through molecular networking.

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2. Results

The identification of 70 specimens of *Chromodoris lochi* indicated the presence of cryptic species confirmed by molecular phylogenetics (Figure 1). On the other hand, all *C. lochi* prey sponges were identified as *Spongian* sp.. On the other hand, the characterization of the chemical profile on the extracts was carried out by ^{1}H NMR and MS/MS analysis. As a result, we identified well-known diterpenoid and sesterterpenoid classes confirmed by those reported data. In addition, the molecular network and environmental correlation are described above.

Figure 1. Molecular phylogenetic of Chromodoris nudibranchs

2.1. Identification of the chemical profile of Chromodoris lochi

All lipophilic extracts were identified as well-known compounds as scalarane diterpenoids by comparison of ^{1}H NMR and HRESIMS data with previously reported (Gonzalez, 2010). The presence of chemical diversity in the extracts suggests that common chemical reactions such as oxidation, cyclization, Michael addition, and Diel-Alder reaction may be involved during enzymatic reactions on the nudibranch (Figure 2).

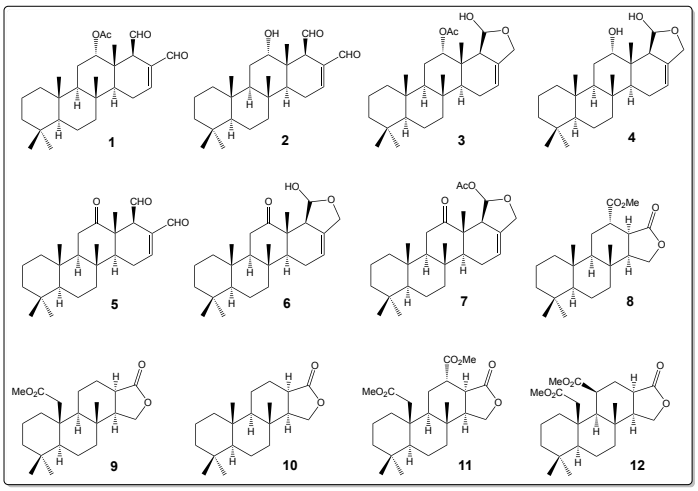
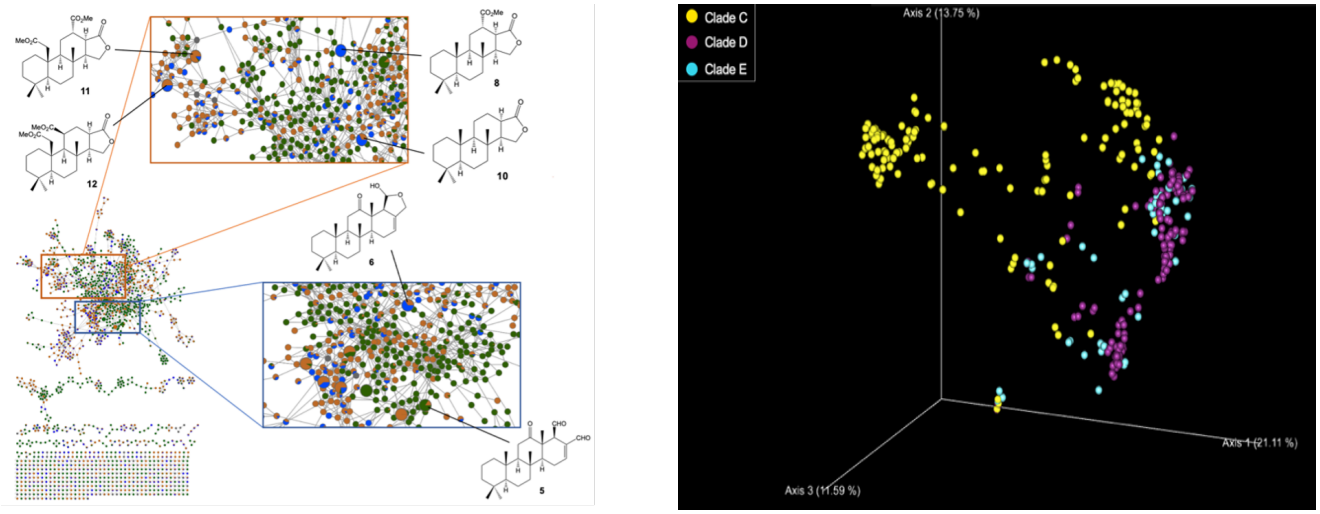


Figure 2. Scalarane diterpenoids from *Chromodoris* nudibranchs

2.2. Metabolites Examination by MS/MS and molecular network

The lipophilic extracts of *C. lochi* together with the references compound (1-12), were subjected to MS/MS to obtain their chemical profile, which was grouped by clade. As a result, the trace of *C. lochi* clade C showed diverse chemotypes with other clades, D and F. Moreover, we organized their chemical profile into a few chemical variations. To obtain a molecular network, the MS/MS raw data of *C. lochi* was analyzed by MZmine2, resulting in 1852 and 1624 features, respectively (Figure 3a). To understand further, we subjected MS/MS results to PCA analysis. By modification of the quantitative feature table (.CSV format), the data was uploaded to Metaboanalyst (www.metaboanalyst.ca) to give PCA data results (Figure 3b). The result showed that the clade C was separated clearly toward D and E but not from D and E. We suspect that the cryptic species in clades D and E may produce similar chemical contents.



(**a**) (**b**)

Figure 3. (**a**) Feature-based molecular networking; (**b**) Principal Component Analysis result.

3. Discussion

The current study presents compelling evidence for cryptic speciation within the nudibranch *Chromodoris lochi*, as supported by molecular phylogenetic analyses of 82 specimens. This discovery adds to the growing body of literature demonstrating that traditional morphological approaches may underestimate true species diversity, particularly in soft-bodied marine invertebrates such as nudibranchs. Cryptic species, while genetically distinct, often exhibit negligible morphological divergence, making molecular tools essential for accurate taxonomic resolution [12-13].

Molecular phylogenetic data, as depicted in Figure 1, revealed the existence of multiple well-supported clades within what was traditionally recognized as a single species. The presence of distinct clades, such as clades C, D, and E, highlights the evolutionary divergence that has occurred despite apparent morphological stasis. This pattern is consistent with allopatric or sympatric speciation mechanisms in marine environments, where ecological or chemical selection pressures may drive speciation even in the absence of physical barriers. Ecologically, all *C. lochi* specimens were found to prey upon the same sponge taxon (*Spongian* sp.), suggesting a strong trophic linkage across clades [13]. This conserved feeding preference raises interesting questions about the role of diet in nudibranch diversification. While one might expect dietary divergence to accompany speciation events, our data suggest that even genetically divergent clades may maintain similar ecological niches. This could be due to the specialized nature of sponge feeding in *Chromodoris*, where selectivity for bioactive, chemically defended prey provides both nutrition and a source of secondary metabolites for chemical defense [10].

The chemical analysis further reveals significant insight into intra- and interspecific metabolic diversity. Using ^{1}H NMR and high-resolution MS/MS analyses, we identified a suite of scalarane diterpenoids and other terpenoid structures, confirming previous reports on sponge-derived metabolites in nudibranch tissues. These metabolites are not synthesized de novo by the nudibranchs but are sequestered from their sponge prey. This process of dietary sequestration and subsequent chemical modification is a hallmark of the genus and supports the role of chemical ecology in nudibranch evolution [14].

Notably, the MS/MS analysis and molecular networking, aided by software tools such as MZmine2 and MetaboAnalyst, uncovered over 1,800 distinct molecular features. Principal Component Analysis (PCA) of these data revealed that

clade C forms a chemically distinct group from clades D and E, although clades D and E remain chemically similar. These findings suggest that while some cryptic species may diverge in both genotype and chemical phenotype (e.g., clade C), others may retain conserved metabolomic profiles despite genetic differentiation [14]. This pattern may indicate shared biosynthetic pathways or similar prey processing mechanisms. The implications of this chemical divergence are multifaceted. First, the presence of distinct chemotypes among cryptic species supports the idea that chemical profiles can serve as auxiliary taxonomic markers, complementing molecular and morphological data. Second, it raises the possibility that chemical divergence may precede or coincide with speciation events, potentially acting as a selective force in ecological adaptation or mate recognition. Finally, the retention of similar metabolite profiles among certain clades may reflect evolutionary constraints or shared ecological pressures that maintain chemical functionality across species boundaries. In sum, this study integrates phylogenetic, ecological, and metabolomic data to elucidate the hidden diversity within *C. lochi*. The presence of cryptic species, their conserved ecological interactions, and their partially overlapping but distinct chemical signatures underscore the complexity of nudibranch biodiversity. These findings highlight the need for integrative approaches in marine taxonomy and open new avenues for research into the evolutionary drivers of chemical diversity in marine organisms.

4. Materials and Methods

4.1. General

NMR spectra analysis was performed on a Bruker 600 MHz NMR spectrometer in deuterated chloroform (Cambridge Isotope Laboratory) at ambient temperature with internal standard as Tetramethylsilane (TMS). The NMR data processing was performed using the TopSpin 4.0 Bruker NMR software package. HRESIMS were performed by an Amazon Ion Trap system (Bruker Daltonics, Bremen, Germany) connected to an Agilent 1260 Infinity LC system (Agilent, Santa Clara, CA, USA) incorporated with a reversed-phase C18 analytical HPLC column (5 µm, 250 mm × 4.6 mm, Phenomenex, Torrance, CA, USA). The data were then interpreted by Compass Data Analysis 4.2 Bruker software.

4.2. Biomaterial

The cryptic species of *Chromodoris lochi* and its prey sponges were collected around Weh Island in 2018. *Chromodoris lochi* and sponge species have been identified and deposited by our collaborator at Naturalis Biodiversity Center, Leiden, the Netherlands

4.3. Isolation and Identification of secondary metabolite

Fresh specimens were extracted immediately with acetone and partitioned between dichloromethane-water to give a lipophilic extract. Furthermore, a stepwise gradient of n-hexane-dichloromethane was applied to silica gel to obtain a nonpolar portion. The portion was subjected to MS/MS to obtain the feature of the molecular network on *Chromodoris* nudibranchs.

4.4. The feature-based molecular networking approach

The detection, grouping, and alignment of the MS/MS spectra were generated by MZmine2 software to reconstruct the molecular network. After processing, the quantitative feature table in CSV format and MS/MS spectra for each feature in MGF formats were obtained. The MS/MS data were available online as MSV000087358 (https://massive.ucsd.edu). The precursor ion mass and product tolerance were set to 0.05 Da. In addition, Molecular networks were set using a minimum of 10 matched peaks together with 0.6 for the cosine score. Furthermore, the data were uploaded in GNPS (https://gnps.ucsd.edu), and the results were visualized by Cytoscape version 3.7.2.

5. Conclusions

This study demonstrates the presence of cryptic species within the nominal taxon *Chromodoris lochi*, highlighting the limitations of traditional morphological taxonomy in accurately capturing the true diversity of marine nudibranchs. Molecular phylogenetic analyses revealed distinct genetic clades, each potentially representing separate species, despite shared external morphology. Interestingly, these genetically divergent clades maintain a conserved ecological niche, specifically in their dietary preference for *Spongian* sp. sponges. This trophic uniformity suggests that ecological stasis may persist despite genetic divergence, or that dietary specialization in these nudibranchs exerts a strong stabilizing selection pressure. The chemical profiling of these clades revealed significant intra-specific variation, with some clades exhibiting distinct

chemotypes while others retained overlapping metabolomic profiles. These findings underscore the role of chemical ecology not only in defense mechanisms but also as a supplementary tool in taxonomic and evolutionary studies. The application of MS/MS-based molecular networking and PCA provided an effective means of visualizing chemodiversity and distinguishing between cryptic species.

Altogether, this integrative approach, combining phylogenetics, metabolomics, and ecological data, offers a more comprehensive framework for understanding biodiversity in marine systems. It emphasizes the need for multi-dimensional species concepts and sets the stage for further exploration of the evolutionary and ecological drivers behind cryptic speciation and chemical diversity in nudibranchs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.scientifia.com/article/doi/.

Author Contributions: M.R.R. and S.K. initiated the conceptual development of the study. M.R.R. undertook the experimental work, including methods design, data analysis, curation, and writing of the initial draft. Validation was collaboratively handled by M.R.R., S.K., and S.K. Each author has reviewed and consented to the manuscript's publication.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

NMR	Nuclear Magnetic Resonance
MS/MS	Mass Spectrometry
PCA	Principal Component Analysis

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