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RESEARCH ARTICLE

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

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Abstract

During a biodiversity survey on Weh Island, Indonesia, marine gastropods of the genus *Chromodoris* were investigated. Phylogenetic analysis of 82 specimens revealed cryptic diversity within *Chromodoris lochi*, indicating hidden genetic lineages. Their prey-specific sponge (*Spongian* sp.) was also collected to evaluate chemical composition. Spectroscopic analysis identified scalarane diterpenoids in both nudibranchs and sponges, suggesting dietary sequestration or shared biosynthetic origins. Molecular networking further showed that chemical profiles of *Chromodoris* were partially mixed among different clades, contributing to apparent metabolite variation. Descriptive statistical analysis demonstrated higher concentrations of biochemical compounds in specimens from northern compared to southern Weh Island. These findings emphasize the integration of phylogenetics and metabolomics to understand spatial and cryptic diversity in marine chemical ecology.

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

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1 Introduction

Chromodoris is one of the largest genera of brightly colored nudibranchs distributed throughout tropical and subtropical Indo-Pacific regions. Molecular phylogenetic studies have demonstrated that this genus comprises genetically distinct lineages despite morphological similarity among species [1,2]. Members of *Chromodoris* commonly inhabit coral reef ecosystems and display selective feeding behavior, preying on specific sponge taxa. During feeding, these nudibranchs sequester sponge-derived secondary metabolites and accumulate them in mantle dermal formations (MDFs), where the compounds are modified and utilized for chemical defense [3]. This process indicates the presence of detoxification and transport mechanisms that enable the safe storage of toxic metabolites.

Recent genome mining studies have revealed that sponge-associated microorganisms play a crucial role in the biosynthesis of secondary metabolites [4]. These uncultured marine microbes, often residing on sponge surfaces, may be co-ingested with sponge tissue, thereby contributing microbial-derived compounds to nudibranch chemical profiles. Several sponge–microbe symbioses have been reported. For example, *Lamellodysidea herbacea* and *Dysidea granulosa* harbor the cyanobacterium *Synechococcus elongatus*, which produces polybrominated diphenyl ethers [5]. Similarly, *Theonella swinhoei* contains polyketide-producing *Pseudomonas* species [6], while

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Acanthostrongylophora ingens hosts *Micromonospora* sp., known to biosynthesize manzamine A [7].

Chemical investigations of nudibranchs have consistently demonstrated prey-specific metabolite patterns. For instance, *C. luteorosea* accumulates diterpenoids from *Dendrilla* sp., *Dysidea* sp., *Chelonaplysilla* sp., and *Aplysilla polyrhaphis* [8]. Likewise, *C. elisabethina* and *C. magnifica* prey on *Heteronema* sp., which contains puupehenone, whereas *C. lochi* feeds on *Luffariella variabilis*, a known source of manoalide [9]. During our recent expedition around Weh Island, *Chromodoris* specimens were found to contain scalarane-type diterpenoids, compounds recognized for antibacterial, antifungal, antifouling, anticancer, and pheromonal activities [10–12].

Despite increasing evidence of sponge-derived metabolites in nudibranchs, the ecological and chemical relationships among nudibranchs, sponge prey, and associated microbes remain insufficiently understood. Therefore, this study aims to elucidate the biological diversity of *Chromodoris* and characterize the chemical profiles of both nudibranchs and associated uncultured sponge microbes using molecular networking approaches.

2 Methodology

2.1 General

NMR spectra were recorded on a Bruker 600 MHz spectrometer using deuterated chloroform (CDCl, Cambridge Isotope Laboratories) as solvent at ambient temperature. Tetramethylsilane (TMS) was used as the internal reference standard. Spectral acquisition and data processing were performed using TopSpin 4.0 (Bruker BioSpin).

High-resolution electrospray ionization mass spectrometry (HRESIMS) analyses were conducted on an Amazon Ion Trap mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled to an Agilent 1260 Infinity LC system (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was achieved using a reversed-phase C18 analytical HPLC column (Phenomenex, Torrance, CA, USA; 5 m, 250 × 4.6 mm). Mass spectral data were processed and interpreted using Compass DataAnalysis 4.2 (Bruker Daltonics).

2.2 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.

2.3 Isolation and Identification

Fresh specimens were immediately extracted with acetone at room temperature to prevent metabolite degradation. The crude extracts were concentrated under reduced pressure and subsequently partitioned between dichloromethane and water to afford a lipophilic organic fraction. The dichloromethane extract was subjected to silica gel column chromatography using a stepwise gradient of n-hexane–dichloromethane to obtain the non-polar fraction. The resulting fractions were analyzed by LC–MS/MS to generate fragmentation data for molecular networking analysis. MS/MS features were processed and organized to construct a molecular network, enabling visualization of chemical diversity and metabolite distribution among *Chromodoris* nudibranch specimens.

2.4 Feature-Based Molecular Networking

MS/MS data were processed using MZmine 2 for peak detection, deconvolution, alignment, and feature grouping to reconstruct the molecular network. Following data processing, a quantitative feature table was exported in CSV format, and MS/MS spectra for each feature were generated in MGF format. The raw MS/MS dataset has been deposited in the MassIVE repository under accession number MSV000087358 (https://massive.ucsd.edu). For molecular networking, precursor ion mass tolerance and fragment ion tolerance were set to 0.05 Da. Network construction parameters included a minimum of 10 matched fragment peaks and a cosine similarity score threshold of 0.6. The processed data were uploaded to the Global Natural Products Social Molecular Networking (GNPS) platform (https://gnps.ucsd.edu) for network generation. The resulting molecular networks were visualized and analyzed using Cytoscape version 3.7.2.

3 Results

3.1 Identification of Cryptic species

Molecular phylogenetic analysis of 70 *Chromodoris lochi* specimens revealed the presence of cryptic lineages, supporting hidden genetic diversity within this morphospecies (Figure 1). In contrast, all prey sponges associated with *C. lochi* were taxonomically identified as *Spongian* sp. Chemical characterization of the crude extracts was performed using 1H

NMR spectroscopy and MS/MS analysis. These analyses led to the identification of well-known diterpenoid and sesterterpenoid metabolites, consistent with previously reported compounds in related nudibranch–sponge systems. Furthermore, feature-based molecular networking enabled visualization of metabolite distribution patterns, while environmental correlation analysis provided insight into spatial variations in chemical composition.

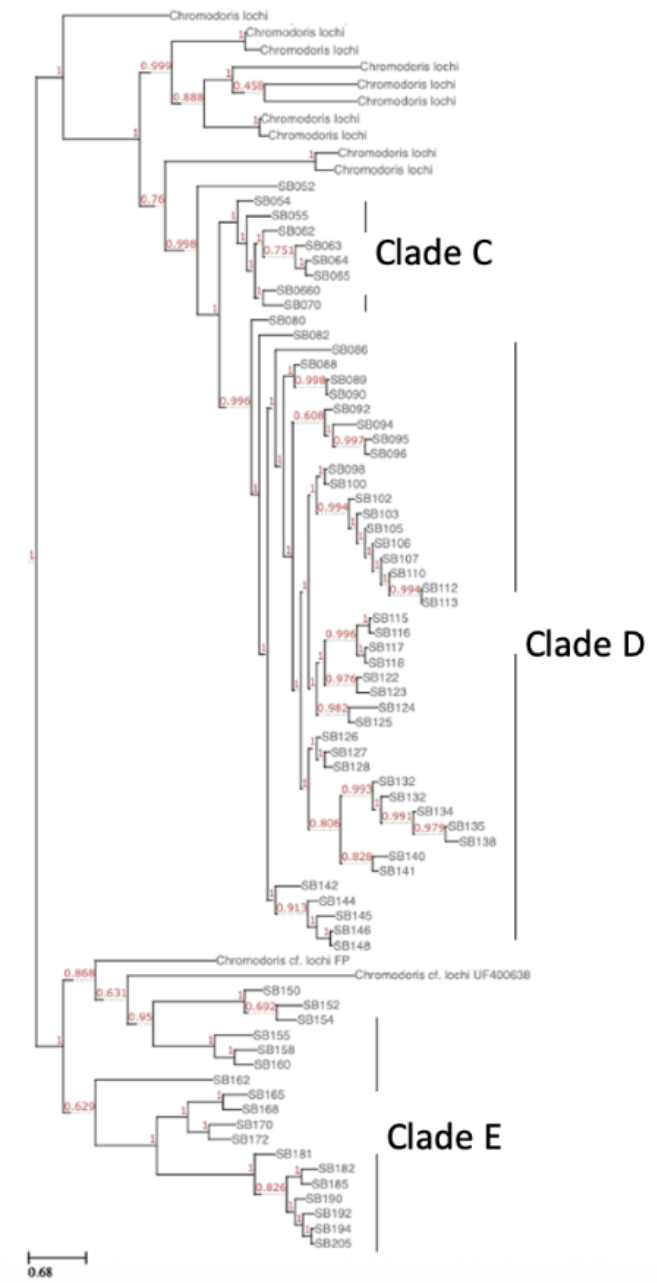

Figure 1. Molecular phylogenetic of *Chromodoris* nudibranchs

3.2 Chemical Profiling

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

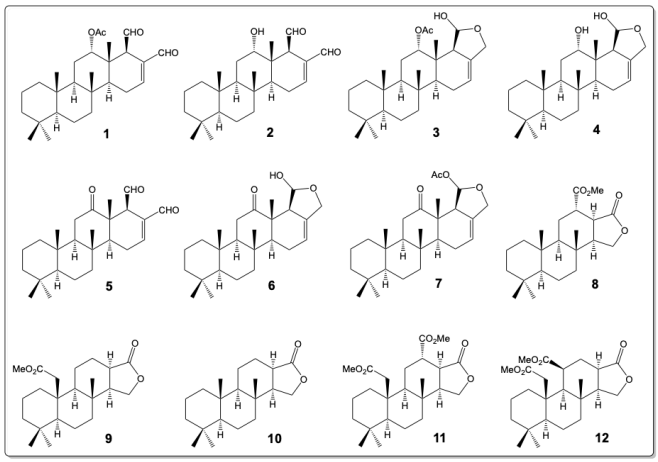

Figure 2. Scalarane diterpenoids from *Chromodoris* nudibranchs

3.3 Metabolomic Profiling by MS/MS and Molecular Networking

The lipophilic extracts of *C. lochi*, together with reference compounds (**1–12**), were subjected to MS/MS analysis to comprehensively characterize their secondary metabolite profiles. Features were subsequently organized according to phylogenetic clades to assess chemotaxonomic patterns. Notably, clade C exhibited a distinct and more chemically diverse metabolomic signature compared to clades D and F, indicating potential lineage-specific metabolic differentiation.

For molecular network construction, raw MS/MS datasets were processed using MZmine2, yielding 1,852 and 1,624 aligned features, respectively (Figure 3). The resulting quantitative feature table (CSV format) was curated and imported into MetaboAnalyst for multivariate statistical evaluation. Principal component analysis (PCA) revealed a clear clustering pattern, with clade C distinctly separated from clades D and E along the principal components (Figure 4). In contrast, clades D and E showed considerable overlap, suggesting high chemical similarity between these groups.

The observed clustering pattern supports the hypothesis that cryptic species within clades D and E may share closely related biosynthetic capacities, whereas clade C likely undergoes divergent metabolic regulation. These findings highlight the relevance of integrating molecular phylogenetics with metabolomics to resolve chemotype differentiation within morphologically similar taxa.

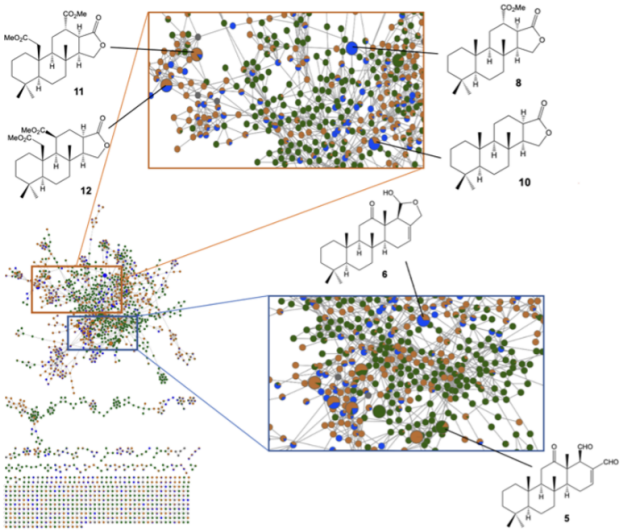

Figure 3. Feature-based molecular networking of *Chromodoris* nudibranchs.

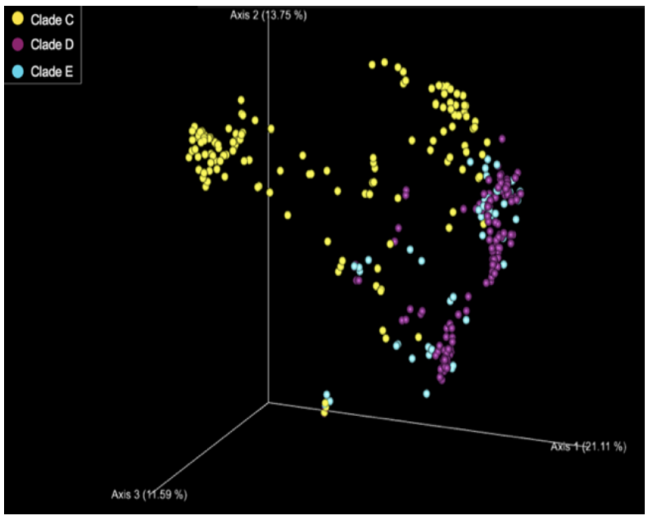

Figure 4. Principal Component Analysis result.

4 Discussion

The present study provides robust evidence for cryptic speciation within the nudibranch *Chromodoris lochi*, supported by molecular phylogenetic analyses of 82 specimens. These findings contribute to the growing recognition that morphology-based taxonomy may substantially underestimate species diversity, particularly among soft-bodied marine invertebrates such as nudibranchs. Cryptic species, genetically distinct yet morphologically indistinguishable, underscore the necessity of molecular approaches for accurate taxonomic resolution [12–13].

Phylogenetic reconstruction revealed multiple well-supported clades within what has historically been treated as a single species (Figure 1). The identification of distinct lineages (clades C, D, and E) indicates substantial evolutionary divergence despite apparent morphological stasis. Such patterns are consistent with both allopatric and sympatric speciation processes in marine systems, where ecological specialization or chemically mediated selection pressures may promote divergence even in the absence of obvious geographic barriers. Ecological observations demonstrated that all *C. lochi* specimens preyed upon the same sponge taxon (*Spongian* sp.), indicating a conserved trophic association across clades [13]. This dietary uniformity suggests that genetic divergence has occurred without detectable shifts in primary prey selection. Given the highly specialized sponge-feeding behavior characteristic of *Chromodoris*, selective retention of bioactive, chemically defended prey likely confers both nutritional and defensive advantages, reinforcing trophic conservatism across lineages [10].

Metabolomic profiling provided additional resolution into intra- and interclade chemical variation. Based on 1H NMR and high-resolution MS/MS analyses, they identified a suite of scalarane diterpenoids and related terpenoid metabolites, consistent with previous reports of sponge-derived compounds sequestered by nudibranch tissues. These metabolites are not biosynthesized de novo but are acquired through dietary sequestration and may undergo subsequent enzymatic modification. Such trophically mediated chemical acquisition is a defining feature of the genus and highlights the central role of chemical ecology in nudibranch diversification [13].

Comprehensive MS/MS analysis combined with molecular networking (MZmine2 and MetaboAnalyst workflows) detected over 1,800 aligned molecular features. Principal Component Analysis (PCA) revealed that clade C forms a chemically distinct cluster relative to clades D and E, whereas clades D and E exhibit substantial overlap in metabolomic

space. This pattern suggests that some cryptic lineages diverge at both genetic and chemical levels (e.g., clade C), while others retain conserved metabolomic profiles despite clear genetic differentiation. The chemical similarity between clades D and E may reflect shared biosynthetic modification pathways, comparable prey-derived metabolite processing, or similar ecological constraints.

The evolutionary implications of these findings are multifaceted. First, distinct chemotypes among cryptic lineages support the use of metabolomic signatures as auxiliary taxonomic markers that complement phylogenetic data. Second, chemical divergence may act as a driver or consequence of speciation, potentially influencing ecological adaptation, predator deterrence efficiency, or reproductive signaling. Conversely, the persistence of conserved metabolite profiles across genetically distinct clades may indicate stabilizing selection maintaining functional chemical defenses.

Collectively, this study integrates phylogenetic, ecological, and metabolomic evidence to reveal previously unrecognized diversity within *C. lochi*. The coexistence of genetic divergence, trophic conservatism, and partial chemical differentiation underscores the complexity of nudibranch biodiversity and highlights the value of integrative approaches in marine systematics. These findings open new perspectives on the evolutionary mechanisms shaping chemical diversity in marine organisms.

5 Conclusion

This study provides compelling evidence for the existence of cryptic species within the nominal taxon *Chromodoris lochi*, underscoring the limitations of morphology-based taxonomy in accurately resolving biodiversity among marine nudibranchs. Molecular phylogenetic analyses revealed multiple well-supported genetic clades that likely represent distinct evolutionary lineages, despite their shared external morphology.

Notably, these genetically divergent clades maintain a conserved ecological niche, particularly in their specialized feeding on *Spongian* sp. sponges. Such trophic conservatism suggests that ecological stasis may persist despite genetic divergence, potentially driven by strong stabilizing selection associated with dietary specialization and chemically mediated defense strategies.

Metabolomic profiling further revealed both shared and clade-specific chemical signatures. While certain clades displayed distinct chemotypes, others retained overlapping metabolomic profiles, indicating that chemical divergence does not uniformly accompany genetic differentiation. These findings highlight the complex interplay between phylogeny, ecology, and chemical acquisition in shaping nudibranch diversity. Moreover, the application of MS/MS-based molecular networking combined with multivariate statistical analyses proved highly effective in resolving chemodiversity patterns and supporting taxonomic inference.

Collectively, the integration of phylogenetic, ecological, and metabolomic datasets provides a multidimensional framework for understanding species boundaries in marine systems. This study reinforces the importance of integrative taxonomy and advances our understanding of the evolutionary processes underlying cryptic speciation and chemical diversification in nudibranchs.

Data Availability Statement

Data will be made available on request.

Author Contributions

M.R.R. and S.K. conceptualized and designed the study. M.R.R. conducted the experimental work, including methodology development, data analysis, data curation, and preparation of the original draft. Validation was carried out collaboratively by M.R.R. and S.K. All authors have reviewed and approved the final version of the manuscript and consent to its publication.

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Conflicts of Interest

The authors declare no conflicts of interest.

Ethical Approval and Consent to Participate

Not applicable.

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