Biodiversity of Marine Heterobranchia (Gastropoda) around North Sulawesi Indonesia

Dissertation zur Erlangung des Doktorgrades (Dr. rer. nat.)

der Mathematisch Naturwissenschaftlichen Fakultät

der Rheinischen-Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Bonn, Mai 2020

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität Bonn.

Die Arbeit wurde am Zoologischen Forschungsmuseum Alexander Koenig in Bonn durchgeführt.

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Tag der Promotion: 13.07.2020

Erscheinungsjahr: 2020

“Life is like riding a bicycle. To keep your balance you must keep moving”

Albert Einstein

Acknowledgements

First of all I would like to thank my supervisor Prof. Dr. Heike Wägele for providing and supervising this work. I am very grateful for the good time in her working group and her always open door. She supported me in all ways related to my work and beyond it. I was always impressed of her leadership abilities and networking skills. Thank you so much for your time and your patience. You allowed me to grow and to increase my scientific networks during this PhD. Hopefully, I was able to adopt some by her features during my years as PhD student.

Further, I want to thank Prof. Dr. Thomas Bartolomaeus for being the second referee for this thesis as well as two further referees.

I thank the Federal Ministry of Education and Research (BMBF) and the German Academic Exchange Service (DAAD) for funding my PhD project.

I also need to thank Dr. Astrid Gärdes and Matthias Birkicht of the Leibniz Center for Tropical Marine Research (ZMT) Bremen. I really appreciate working at their laboratory and am thankful all help they gave me.

Many thanks to the members of the molecular laboratory section in the Zoological Research Museum Alexander Koenig (ZFMK), in particular to Claudia Etzbauer and Sandra Kukowka for the intensive help in the laboratory.

I sincerely thank Prof. Grevo Gerung, Prof. Farnis Boneka, Prof Markus Lasut of the Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado for

their support and help in the ABS paper work to follow the Nagoya Protocol during my field trips.

I would also thank Dr. Gregor Christa who offered advice and suggestion whenever asked. Thanks to AG-Mollusca members: Dr. André Koch, Fatemeh Maniei, Dario Karmeinski, Dorothea Schillo, Adelfia Papu, Corinna Sickinger, Nilz Arenz, Antonia Schönleber for charming discussion and for all the fun we have had in the last four years.

Many of the above and some who are not named here, but all went with me through that times, are still connected to me in friendship, even if sometimes separated by distance and time, and I thank all these people that they have been at my side. I am so grateful to all the friends and friendly people who gave me support.

I want to thank for my parents, my brothers and sisters (Yani, Ully, Meiny and Nathan) for your pray, support throughout writing this thesis, for believing in me and for being there for me.

I dedicate this thesis to my lovely husband, Heffry, and my sons, Derrelyo and Hiroyuki. Thanks for giving me patience, strength, motivating, and being my inspiration.

Declaration

Declaration I hereby declare that I am the sole author of this thesis and that no other sources or learning aids, other than the ones listed, have been used. Furthermore, I declare that I have acknowledged the work of others by providing detailed references of said work.

Hereby I also declare, that this thesis has not been prepared for another examination or assignment, neither wholly nor partially.

Erklärung

Hiermit versichere ich, dass ich diese Arbeit selbständig verfasst habe, keine anderen als die angegebenen Quellen/Hilfsmittel verwendet habe und alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen wurden, als solche kenntlich gemacht habe. Ich versichere außerdem, dass ich die beigefügte Dissertation nur in diesem und keinem anderen Promotionsverfahren eingereicht habe und, dass diesem Promotionsverfahren keine entgültig gescheiterten Promotionsverfahren vorausgegangen sind

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Summary

North Sulawesi province is one of the most potential areas in terms of natural marine resources. In North Sulawesi there are many interesting tourist places with high marine biodiversity and many objects are currently under development, including around Bunaken National Marine Park (BNP), Lembeh Strait, in the island of Sangihe, and Bangka Archipelago.

Coastal areas of North Sulawesi are considered as the most diverse habitats in the world with high species diversity in coral reefs, but also in adjacent sea grass beds and mangroves. The high diversity also includes marine heterobranch sea slugs, which are known by an extremely large number of species of up to probably 2000, with many undescribed ones.

My study in Sangihe Island is the first survey of marine heterobranch around this island and included in the collection 23 species, with Phyllidiidae showing the highest dominance (Chapter 3). The amount of species is far lower than in the studies around BNP, or the Bangka Archipelago, probably due to unfavorite weather conditions.

The Chromodorididae is a large and colourful family of nudibranch sea slugs distributed across the tropical and temperate world's oceans. These sea slugs are known by many divers because of their beauty and by many pharmacists because of their high diversity in natural compounds.

For studying the Chromodorididae, 375 specimens were collected around North Sulawesi in 2015, 2016 and 2017. Chapter 4 and chapter 5 focus on this family. The phylogenetic hypothesis based on two mitochondrial genes, CO1 and 16S, is the only subsequent study after the first study published in 2012 by Johnson & Gosliner. The major result of my analyses is the confirmation of the results obtained by Johnson & Gosliner 2012 at that time.

Chromodoris is a genus of colourful nudibranchs that feed on sponges and is found across the Indo-Pacific. Chapter 5 in this study focuses on four species of this genus (i.e. *C. annae, C. dianae, C. willani, C. lochi*), of which hundreds of specimens were

collected. The results about *Chromodoris* species clearly show wide spread cryptic speciation.

Biodiversity is related to water quality, which was also addressed in this thesis. The results with regard to water quality indicate that all sampling points are within the range of normal values and no specific pollution can be seen (Chapter 6).

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Chapter 1

General Introduction

North Sulawesi, Indonesia

As an archipelagic country, Indonesia consists of thousands of islands, interconnected by water straits and seas with many different types of habitats and an extremely complicated geological history (von Rintelen et al. 2017). Currently, there are more than 13,000 islands that have been registered with valid coordinates in the United Nations statistic in 2012 (BPS-Statistic Indonesia 2019) with a coastline of nearly 100,000 km in length (National Geograpic 2013). The islands stretch along the equator in the tropic zone and are mainly surrounded by deep water basins and deep sea trenches.

The Indonesian archipelago comprises two of the world's biodiversity hotspots i.e. Sundaland and Wallacea by Myers et al. (2000) (areas with a high degree of endemic species that are highly threatened by loss of habitats): Its insular character and complex geological history led to the evolution of a megadiverse fauna and flora (Lohman et al. 2011). Furthermore, the importance of biodiversity is more and more acknowledged, because biodiversity contributes directly to human well-being by providing biological products, and indirectly through environmental services (Indonesian Ministry of National Development Planning (BAPPENAS) 2016)). However, the importance of this kind of knowledge accounts not only in Indonesia but also for other biodiversity-rich tropical countries.

Geographically, North Sulawesi Province, as one of the Republic of Indonesia provinces, is located between 00° 15' 51" - 05° 34' 06" N and 123° 07' 00" - 127° 10'30" E, on the border to the Republic of the Philippines in the north and the Maluku Sea on the east, the province of Gorontalo in the west and the Gulf of Tomini in the south. This province is an archipelago province consisting of 287 islands with 59 inhabited and 228 uninhabited islands. 1,664 villages, consisting of 627 coastal villages and 1,037 non-coastal villages are spread over the inhabited islands.

North Sulawesi region covers an area of 15,376.99 km^2, with an area of exclusive economic zone (EEZ) of 190,000 km2. 161,540 km^2 are territorial waters with a coast line of about 2,400 km in length. This geographical situation provides a great chance and challenge for North Sulawesi province as it is very rich in biological and non-biological resources.

North Sulawesi province, lying in the middle of the so-called Coral Triangle, exhibits an extraordinarily marine biodiversity (Allen 2000; Turak & DeVantier 2003). The area is described as being "clearly of global significances as the key reservoir of tropical marine biodiversity" (Turak & DeVantier 2003; p. 6). Therefore, North Sulawesi can be considered as a mega-diverse area. Currently, the development of marine tourism has become one of the major interests of Indonesian government and local politicians. North Sulawesi with its beautiful reefs is very popular for diving and snorkeling tourists. However, higher levels of tourists' activities are usually accompanied by more threats to ecosystems, such as increased farming, aquaculture, and fisheries due to additional needs of temporary visitors and/or permanent residents (Kaligis et al. 2018; Eisenbarth et al. 2018; Undap et al. 2019). These activities can cause biodiversity loss which has a significant impact on the functioning of ecosystems, the potential for recovering of damaged habitats, and recruitment of species (Worm et al. 2006).

Heterobranch sea slugs

The phylum Mollusca is very diverse with great variety of functional body plans evolved, and consists of eight classes, i.e. Gastropoda, Bivalvia, Scaphopoda, Cephalopoda, Monoplacophora, Polyplacophora, Solenogastres and Caudofoveata (World Register of Marine Species). With approximately 130,000 described species and about 70,000 fossils, Mollusca represent the second largest animal phylum (Haszprunar et al. 2008). Among Mollusca, the Gastropoda is the largest and most diverse class with more than 100,000 described species (Aktipis et al. 2008), and exhibit the highest diversity in morphology (Dinapoli 2009). According to Bouchet & Rocroi (2005), Gastropoda is divided into six main clades. Of these, Heterobranchia is a diverse and important clade of marine, freshwater and terrestrial snails and slugs. They encompass the former "Opisthobranchia" and are nowadays still often divided into "lower

Heterobranchia", "Opisthobranchia" and Pulmonata, although these groups are partly not monophyletic (Wägele et al. 2014).

Diversity and health of coral reefs is reflected by a diversity of marine organisms, including marine Heterobranchia (Eisenbarth et al. 2018). This group, which comprises all former "opisthobranch" taxa, collectively known as the "sea slugs" or still as opisthobranchs, is a highly diverse group of gastropod molluscs (Wägele & Klussmann-Kolb 2005). In total, the diversity of marine heterobranchs, including marine slugs without shell and their shelled relatives, is estimated in between 5,000 and 6,000 described species (Wägele & Klussmann-Kolb 2005). Out of these, roughly 3,000 belong to the Nudibranchia (Wägele & Willan 2000), which are the sea slugs in a strict sense. However, many species are still undescribed and many new are detected on a regular base, thus the overall number of species is certainly much higher (Gosliner et al. 2008, 2018; Eisenbarth et al. 2018; Papu et al. 2020).

Marine heterobranchs are very interesting to tourists, especially snorkelers and divers. Many of these sea slugs have reduced or lost their shells and thus were able to develop stunning body shapes and coloration (Haber et al. 2010). They also had to developed alternative defense or antifouling systems, by taking up natural compounds from their food (e.g. sponges), or by de novo synthesis (Cimino & Ghiselin 1999; Gavagnin & Fontana 2000; Cheney et al. 2016; Böhringer et al. 2017; Fisch et al. 2017). This made them very attractive for scientists, especially pharmacologists, searching for new drug leads. This is also one of the reason, why my project was financed by the German Ministry of Education and Research.

Indonesian marine heterobranch

The study of marine heterobranch diversity in Indonesia is still very uncommon. Nevertheless, a few studies on Indonesian marine heterobranch already indicate an extremely high diversity: 138 species were mentioned in the Ambon expedition in 1990 by Yonow (2001, 2011, 2017); 205 species from Bali (Tonozuka 2003); 45 species from Sempu Strait (Andrimida & Hermawan 2019). Burghardt et al. (2006) recorded around 75 species for the first time from North Sulawesi (Bunaken National Park, BNP). Further studies in this region followed: 8 species were mentioned from Manado Bay (Purba et al.

2013); 172 species from Bunaken National Park (BNP) in the years from 2015 to 2017 (Kaligis et al. 2018; Eisenbarth et al. 2018); 27 species from Lembeh Strait (Ompi et al. 2019); 23 species from the island of Sangihe (Undap et al. 2019) and most recently 150 species from Bangka Archipelago (Papu et al. 2020). Furthermore, many new and undescribed species are mentioned in these various publications, and one new species, *Moridilla jobeli* Schillo & Wägele in Schillo et al. (2019), is now described from BNP (Schillo et al. 2019).

Aims of the present study

The aim of this comprehensive study are to explore undersampled or unsampled habitats, such as the rarely explored sub tidal zone in North Sulawesi Indonesia coast in order to increase the knowledge of species biodiversity and their geographic distribution, as a baseline for future monitoring projects. Although I was involved on sampling in several other areas, resulting in publications, my focus was Sangihe Island in North Sulawesi. Because of the immense number of species and also new species, the second focus of this study was the thorough investigation of the largest nudibranch family in the tropics, the family Chromodorididae, including all samples collected in the frame of the project. By analyzing this taxon, it was meant to obtain the first comprehensive phylogenetic analysis of this family, by using 16S rDNA and CO1 sequences, and to search for evidences of putative species complexes.

This thesis is divided into 4 chapters, following this introduction and the general introduction into methods. After the introduction the second chapter follows and this is the material and method section. Chapter 3 deals with investigation of the marine heterobranch diversity around the island of Sangihe, North Sulawesi, Indonesia and compares the results of this first study from this area with former studies in Bunaken National Park (BNP) and other areas in and around Indonesia. This study is published in the journal Diversity in 2019 (Undap et al. 2019).

Chapter 4 and chapter 5 focus on the family Chromodorididae. Chapter 4 provides a phylogenetic hypothesis based on two mitochondrial genes, CO1 and 16S, and analyzing them by means of Maximum likelihood methods. In this analysis, several cryptic speciation events were detected.

Chapter 5 focuses on 4 species of the genus *Chromodoris*, of which hundreds of specimens were collected. To address the issue of cryptic speciation more in detail, a species delimitation algorithm is used to clarify species boundaries in *Chromodoris*. It gives information about cryptic speciation, the connectivity between the populations and geographic distribution or isolation. It is shown that earlier identification often is unreliable and it is likely that these pattern are also confounding species identification in many other genera.

Chapter 6 addresses the quality of the water along the shoreline of North Sulawesi by analyzing several physical, chemical and biological parameters. Diversity and health of marine habitats relies on high quality of the water. However, increased human activities, including population growth of locals, as well as increased tourism with all subsequent following problems, affect water quality especially around North Sulawesi. I therefore wanted to analyse the mentioned parameter in order to provide a baseline of the status quo, but also detect already problematic areas.

Chapter 2

Materials and Methods

Collection of specimens

The specimens were photo-documented in the field on the original substrate before being collected individually by snorkeling or scuba diving. Subsequently the animals were photo documented in the laboratory and the whole animal or at least a small piece of the foot was preserved in 96% alcohol for barcoding. Most specimens were identified before preservation using identification books. Further preservation methods included fixation in formaldehyde/seawater for histological investigation. Many specimens were subsequently handed over to other project partners for the various analyses. By barcoding only a tiny piece of the specimens allowed the further investigation with regard to chemistry or other scientific questions not pursued further in my own project. However, my correct identification via barcodes guarantees a correct species assignment within all other experiments.

DNA extraction and amplification

DNA was extracted usually from a small piece of the foot, or the whole animal, in case these were very small, using the QIAgen® DNeasy Blood and Tissue-Kit (QIagen, Hilden, Germany), following the manufacturer's instructions. DNA was then stored in 96% ethanol at -20ºC. Fragments of CO1 and 16S were amplified for all collected specimens. Partial sequences of mitochondrial CO1 (about 680 bp) and ribosomal 16 S (about 450 bp) were amplified by polymerase chain reaction using the primers LCO1490-JJ (5'–CHACWAAYCATAAAGATATYGG-3') and HCO2198-JJ (5'-AWACTTCVGGRTGVCCAAARAATCA-3') (Astrin and Stüben 2008) for CO1 and 16 Sar-L (5′-CGCCTGTTTATCAAAAACAT-3') and 16Sbr-H (5′-CCGGTCTGAACTCAGATCACGT-3') (Palumbi et al. 2002) for 16 S. Amplification of CO1 was performed by an initial step (95 °C for 15 min) followed by 40 touch-down

cycles of denaturation (94º C for 35 s), annealing (55 °C for 90 s) and extension (72 °C for 90 s), with a final extension step 72 °C for 10 min. For 16 S rRNA, the PCR started with an initial step (95 °C for 15 min), denaturation (94 °C for 45 s), followed by 34 touch-down cycles, annealing (56 °C for 45 s), extension (72 °C for 90 s), and final extension step at 72 °C for 10 min. PCR products were sequenced by Macrogen Europe Laboratory (Amsterdam, Netherlands).

Sequence analysis and alignment

The software GENEIOUS Pro 7.1.9 (Biomatters Ltd., Auckland, New Zealand) was used to extract the consensus sequence between the primer regions, and to construct the final alignments. To check if the correct genes have been amplified, and to uncover contamination, BLAST searches (Altschul et al. 1990) were performed to compare the amplified sequence with all sequences stored in the GenBank database (www.ncbi.nlm.nih.gov/Genbank/index.html). Subsequently all available sequences (mitochondrial CO1 and 16S) from the specimens of interest were downloaded and added to the sequences obtained in this study. A critical step of sequence-based phylogenetic analyses is the alignment of the data. Given that positions with a common ancestry have to be compared for reliable phylogenetic conclusions, homologous positions have to be arranged in common columns in correct alignment. Sequences were edited and aligned first separately for the 2 genes by using MAFFT (Katoh et al. 2002) in Geneious 7.1.9. Subsequently, CO1 and 16S sequences were concatenated according to the project's task and analysed in a similar way. More details for the various methods are provided in the specific chapters.

Chapter 3

First Survey of Heterobranch Sea Slugs (Mollusca, Gastropoda) from the Island Sangihe, North Sulawesi, Indonesia

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This is the author's version of the article originally published in Diversity, 2019, 11, 170; DOI: 10.3390/d11090170

Keywords: Sangihe; North Sulawesi; Indonesia; Heterobranchia; sea slugs; biodiversity; monitoring; tourism

Abstract:

Indonesia is famous for its underwater biodiversity, which attracts many tourists, especially divers. This is also true for Sangihe Islands Regency, an area composed of several islands in the northern part of North Sulawesi. However, Sangihe Islands Regency is much less known than, e.g., Bunaken National Park (BNP, North Sulawesi). The main island, Sangihe, has recently experienced an increase in tourism and mining activities with potentially high impact on the environment. Recently, monitoring projects began around BNP using marine Heterobranchia as indicators for coral reef health. No information about this taxon exists from the remote islands in North Sulawesi. The present study represents the first monitoring study ever and focuses on marine Heterobranchia around Sangihe. In total, 250 specimens were collected, which could be assigned to Sacoglossa (3), Anthobranchia (19), and Cladobranchia (1). Despite the low number (23 versus 172 in BNP), at least eight species (35%) are not recorded from BNP, probably indicating differences in habitat, but also influence of a strong El Niño year in 2016. Here we also report for the first time a *Chromodoris annae* specimen mimicking *C. elisabethina*, and the discovery of a new *Phyllidia* species.

Introduction

Indonesia is an archipelagic country with a coastline of more than 100,000 km. Coral reefs, sea grasses, and mangrove forests cover approximately 50,875 km^2, although this number does not take into consideration the remote areas (Suharsono 2008; Wilkinson 2008). These tropical ecosystems with a high species and habitat diversity have a tremendous ecological and economic value to nature and humans. They contribute substantially to the community's income as well as to the national economy. However, many anthropogenic activities form a threat to these natural habitats. Suharsono (1998) found that the condition of more than 20% of Indonesian coral reefs were in poor condition and only 6.5% were considered healthy. More recent studies in Indonesia suggest the additional decline of healthy reefs influenced by natural disturbances (e.g. Utama & Hadi 2018) and up to 50% are severely damaged (e.g. Rudianto & Bintoro 2019).

North Sulawesi is known as a mega-diverse area, and therefore very popular for diving and snorkeling tourists. Thus, the pressure on the reefs has increased dramatically in the last few years. Based on Badan Pusat Statistik Provinsi Sulawesi Utara (2018), the number of foreign visitors visiting North Sulawesi Province, via the International Airport of Sam Ratulangi, Manado, approached nearly 11,000 visitors alone in February 2018. This is an increase of 27% compared to January 2018. Comparing foreign visitors in February 2018 with February 2017, the number augmented by more than 100% (Badan Pusat Statistik Provinsi Sulawesi Utara 2018). Thus, the pressure on the reefs has increased dramatically in the last years. A few local studies conducted in Bunaken National Park (BNP), North Sulawesi, over 10 years clearly indicate a declining state of coral coverage and coral reef fish, and this is related to an increased number of local and foreign visitors, in addition to an increased number of permanent residents (Setiawan et al. 2013). Another study identified diving and snorkeling activities as a major source of the decline in living coral coverage by comparing different sites around Bunaken Island (Towoliu 2014). Undisturbed areas had a live coral coverage of nearly 55% in 3 m depths, while areas with snorkelers and divers showed coverage of only 17% at this depth.

Sangihe Islands in North Sulawesi Province is less known to tourists. It is one of the most northern groups of islands in Indonesia, with Sangihe as the largest island

covering an area of approximately 500 km^2. The area geographically connects North Sulawesi with Mindanao (Philippine Islands) and forms the eastern boundary of the Celebes Sea. However, biogeographically it is still part of the Wallacea, marked by the Wallace line, which runs between Sangihe Islands and the Philippines. Sangihe has come into focus recently by advertising adventurous diving tourism, including visits to the active underwater volcano Mahengetang in a depth of less than 10 m (Indonesia Tourism). Being promoted recently as one of the tourist destinations in the Sangihe Islands Regency, the area is liable to experience a huge pressure on its environment in the near future by many more visitors, both national and international, and a higher demand for hotels, resorts, and diving centers. Higher levels of tourist activities are usually accompanied by threats to ecosystems, such as increased farming, aquaculture, and fisheries due to additional needs of temporary visitors and/or permanent residents. Additionally, Sangihe has come into the focus of mining companies. Since 2007, East Asia Minerals Corporation and local partners were granted exploration permits from the local government within an area of 42,000 ha in the south of Sangihe. The first gold and silver production phase within this Sangihe Gold Project was scheduled for the end of 2018, but did not start yet in 2019 (East Asia Minerals Corporation). In terms of minimizing the negative impacts on the environment in the future and helping to build up a sustainable use of the natural resources on and around Sangihe, investigation of the biodiversity in this still rather undisturbed region is paramount. In contrast to BNP, which is already highly affected by diving and snorkeling tourism, monitoring activities in Sangihe Islands Regency with only 12 resorts (Badan Pusat Statistik Kabupaten Kepulauan Sangihe 2018) could provide a good opportunity to study the impact of new infrastructure for tourists and their activities in the marine habitats, as well as other economically important activities on the environment.

Diversity and health of coral reefs is reflected by a diversity of marine organisms, including marine Heterobranchia. These sea slugs use a highly diverse food spectrum, with a high affinity to their specific diet. This spectrum covers nearly all sessile organisms (algae, poriferans, cnidarians, ascidians, bryozoans, tunicates). Thus, this group was already used for monitoring coral reef diversity in North Sulawesi (Burghardt et al. 2006; Kaligis et al. 2018; Eisenbarth et al. 2018; Ompi et al. 2019). Because marine Heterobranchs are also very attractive to tourists, additional data are and will be available

through citizen science due to documentation in websites or personal information and provision of images on personal bases. This was shown lately by Nimbs et al. (2016) and Nimbs & Smith (2018) where long-term documentation of scientists and recreational divers led to the identification of new tropical species introduced in Port Stephens, on the central New South Wales coast of Australia, and Tasman Sea. In order to monitor potential damage to the environment around Sangihe, irrespective of its original cause, we have started with a first survey in 2016, focusing on marine Heterobranchia. Here we present the first results from this collecting period and compare our results with former studies in Bunaken National Park (Kaligis et al. 2018 and Eisenbarth et al. 2018) and other areas in and around Indonesia.

Materials and Methods

Sampling was carried out during daytime from 3 to 7 August 2016 at seven sites around the island Sangihe (Fig. 3.1, Table 3.1). Seven scientists and students (three with less and four with good collecting experience from former studies, including the BNP studies) collected in a depth range from the eulittoral to maximum of 28 m. On average, the bottom time for each collecting activity was 60 minutes. In total, underwater searching period correlated to approximately 50 working hours around the island. Additionally, about 10 working hours in total were spent collecting while snorkeling. Specimens were photo-documented in the field on the original substrate before being collected individually. Most specimens were identified before preservation using identification books and original literature (Debelius & Kuiter 2007; Gosliner et al. 2008, 2015; Rudman 1982, 1984, 1986, 1987; Yonow 1994, 1996, 2001, 2008, 2011, 2012), as well as websites (e.g. The Sea Slug Forum (www.seaslugforum.net)). Regarding species validity, the World Register of Marine Species (http://www.marinespecies.org) was used. The sea slugs were usually preserved in 96% alcohol for further study (including barcoding). All animals were recorded with metadata that are available in the database Diversity Collection (Part of Diversity Workbench) using the data brokerage service of the German Federation for Biological Data (GFBio) Diepenbroek et al. (2014). Geographic names were not available for all collection sites. We then used the name of the village close to the respective study area. This is the case for the villages of

Palahanaeng and Talengen. Available data on the distribution of respective sea slugs are downloaded from the Global Biodiversity Information Facility (GBIF). For visualization in maps, the geographic information system ArcGIS, release 10.0, was used. The material is registered in the Sam Ratulangi University (UNSRAT, Manado) reference collection under the number SRU2016/01.

Traditional barcoding genes (partial CO1 and partial 16 S) were analyzed for most specimens to verify identification. DNA-Isolation has been carried out by means of QIAgen® DNeasy Blood and Tissue-Kit (QIagen, Hilden, Germany), following the manufacturer's instructions. Partial sequences of mitochondrial CO1 (about 680 bp) and ribosomal 16 S (about 450 bp) were amplified by polymerase chain reaction using the primers LCO1490-JJ (5'–CHACWAAYCATAAAGATATYGG-3') and HCO2198-JJ (5'-AWACTTCVGGRTGVCCAAARAATCA-3') (Astrin and Stüben 2008) for CO1 and 16 Sar-L (5′-CGCCTGTTTATCAAAAACAT-3') and 16Sbr-H (5′-CCGGTCTGAACTCAGATCACGT-3') (Palumbi et al. 2002) for 16 S. Amplification of CO1 was performed by an initial step (95 °C for 15 min) followed by 40 touch-down cycles of denaturation (94^{o} C for 35 s), annealing (55 °C for 90 s) and extension (72 °C for 90 s), with a final extension step 72 °C for 10 min. For 16 S rRNA, the PCR started with an initial step (95 °C for 15 min), denaturation (94 °C for 45 s), followed by 34 touch-down cycles, annealing (56 °C for 45 s), extension (72 °C for 90 s), and final extension step at 72 °C for 10 min. PCR products were sequenced by Macrogen Europe Laboratory (Amsterdam, Netherlands). The software GENEIOUS Pro 7.1.9 (Biomatters Ltd., Auckland, New Zealand) was used to extract the consensus sequence between the primer regions, to construct the final alignments, including sequences from the National Center for Biotechnology Information (NCBI, Bethesda, Maryland, USA), in order to analyze species assignment.

Table 3.1 Details on collection sites (Fig. 3.1). When sites do not have a geographic name, we used the name of the village nearby. Abbreviations of localities are used in Table 3.2.

Name	Abbreviation	Area and Geographic Data	Date of Collection
Ship Wreck	ShW	3°36’28.00’’ N 125°29‘38.00“ E	04.08.2016
Tahuna Bay South	TBS	3°35’59.40’’ N 125°29‘23.40“ E	04.08.2016
Mendaku	Men	3°22’01.94’’ N 125°34‘26.67“ E	03.08.2016
Palahanaeng (village)	Pal	3°35’18.92’’ N 125°34‘26.67“ E	07.08.2016
Talengen (village)	Tal	3°34’49.92’’ N 125°34‘34.93“ E	05.08.2016
Manalu	Man	3°32’08.87’’ N 125°37‘25.46“ E	06.08.2016
Sapaeng	Sap	3°34’55.81’’ N 125°34‘49.04“ E	06.08.2016

Figure 3.1 Details on North Sulawesi with collection sites in Sangihe (upper insert, and see also Table 3.1) and the collection area around Bunaken Island (Kaligis et al. 2018; Eisenbarth et al., 2018) (lower insert) for comparison.

Results

250 specimens were collected comprising 23 species (Table 3.2, Figs. 3.2, 3.3). These can be assigned to the *Sacoglossa* (3) and within the *Nudibranchia* to *Anthobranchia* (19) and *Cladobranchia* (1) (Figs. 3.2, 3.3). Out of the 250 specimens, identification was verified by barcoding for 236 specimens (partial CO1 and 16 S genes, see NCBI accession numbers in Table 3.3). Distribution of the species based on data in GBIF, including the new results from the island Sangihe, are depicted in Figs 3.4–3.6 with a restriction to the Indian and Western Pacific Ocean.

Figure 3.2 Sacoglossa and Anthobranchia:
(A) *Elysia pusilla*, Elpu16Sa-3;
(B) *Thuridilla gracilis*, Thgr 16Sa-2;
(C) *Plakobranchus* cf. *papua*, Ploc16Sa-2;
(D) *Notodoris serenae*, Aese16Sa-2;
(E) *Chromodoris dianae*, Chdi16Sa-2;
(F) *Chromodoris annae*, Chan16Sa-2;
(G) *Chromodoris annae* mimicking *C. elisabethina*, Chel16Sa-1;
(H) *Chromodoris strigata*, Chst16Sa-1;
(I) *Glossodoris* cf. *cincta*, Glci16Sa-1;
(J) *Goniobranchus geometricus*, Goge16Sa-2;
(K) *Goniobranchus reticulatus*, Gore16Sa-1.

Table 3.2 Species records around Sangihe with details about specimens and locality, as well as first authorities. Species recorded in Eisenbarth et al. [14] around BNP are indicated in the last column.

Taxon	Species Name	Localities							Depths (m)	Number of Specimens	Size (mm)	Eisenbarth et al. [14]
		TBS	ShW	Man	Pal	Men	Sap	Tal				
Sacoglossa	*Elysia pusilla* (Bergh, 1871)	1	-	-	-	2	-	-	2	3	2–6	x
	Thuridilla gracilis (Risbec, 1928)	-	-	-	3	-	2	1	4–10	6	20–30	x
	Plakobranchus cf. *papua* (Meyers-Muñoz & van der Velde, 2016)	1	-	-	-	1	-	-	5–15	2	25,30	-
Anthobranchia	*Notodoris serenae* (Gosliner & Behrens, 1997)	-	-	2	-	-	-	-	24–27	2	60,90	x
	Chromodoris dianae (Gosliner & Behrens, 1998)	-	-	1	-	6	-	-	15–27	7	5–45	x
	Chromodoris annae (Bergh, 1877)	-	-	1	1	2	5	4	5–23	13	8–41	x
	Chromodoris strigata (Rudman, 1982)	-	-	-	-	1	-	-	15	1	10	x
	Glossodoris cf. *cincta* (Bergh, 1888)	-	-	-	1	-	-	1	8, 13	2	21, 48	x
	Goniobranchus geometricus (Risbec, 1928)	1	-	-	1	-	2	-	6–19	4	10–15	x
	Goniobranchus reticulatus (Quoy & Gaimard, 1832)	2	-	-	-	-	-	-	6, 9	2	25, 55	x
	Hypselodoris tryoni (Garret, 1873)	-	-	-	-	-	3	-	10, 16	3	25–60	x
	Phyllidia ocellata (Cuvier, 1804)	2	-	2	1	-	2	-	4–18	7	16–35	x
	Phyllidia picta (Pruvot-Fol, 1957)	6	-	3	2	2	6	2	1–15	21	13–30	-

	Phyllidia spec. (Phsp3_16Sa-1)	-	-	-	-	-	1	-	1	1	25	-
	Phyllidia madangensis (Brunckhorst, 1993)	-	-	-	-	-	-	1	8	1	28	-
	Phyllidia coelestis (Bergh, 1905)	3	-	1	1	-	1	3	3–12	9	7–32	x
	Phyllidia varicose (Lamarck, 1801)	19	-	2	10	2	15	10	3–15	58	7–87	x
	Phyllidiella lizae (Brunckhorst, 1993)	3	-	3	1	3	-	2	5–23	12	6–68	-
	Phyllidiella pustulosa (Cuvier, 1804)	19	4	11	15	2	15	11	1–23	77	12–47	x
	Phyllidiella nigra (van Hasselt, 1824)	-	-	-	-	-	1	-	8	1	29	x
	Phyllidiopsis krempfi (Pruvot-Fol, 1957)	1	-	2	6	-	6	-	6–28	15	14–50	-
	Phyllidiopsis shireenae (Brunckhorst, 1990)	-	-	1	-	-	1	-	8, 15	2	77, 81	-
Cladobranchia	Aeolidioidea (Flsp16Sa-1)	-	-	-	-	1	-	-	2	1	1	-

Table 3.3 Species used in this study, identification number, and GenBank accession numbers as also mentioned in Diversity Workbench.

Family	Species Name	ID	GenBank Accession Numbers	
			16 S	CO1
Chromodorididae (Bergh, 1891)	*Chromodoris dianae*	Chdi16Sa-1	MN104702	MN320502
	(Gosliner & Behrens, 1998)	Chdi16Sa-2	MN104703	MN320503
		Chdi16Sa-3	MN104704	MN320504
		Chdi16Sa-4	MN104705	MN320505
		Chdi16Sa-5	MN104706	MN320506
		Chdi16Sa-6	MN104707	MN320507
		Chdi16Sa-7	MN104708	MN320508
	Chromodoris annae	Chan16Sa-1	MN104690	MN124751
	(Bergh, 1877)	Chan16Sa-2	MN104691	MN124752
		Chan16Sa-3	MN104692	MN124753
		Chan16Sa-4	MN104693	MN124754
		Chan16Sa-5	MN104694	MN124755
		Chan16Sa-6	MN104695	MN124756
		Chan16Sa-7	MN104696	MN124757
		Chan16Sa-8	MN104698	MN124758
		Chan16Sa-9	MN104699	MN124759
		Chan16Sa-10	MN104700	MN124760
		Chan16Sa-11	MN104701	MN124761
		Chan16Sa-12	MN104702	MN124762
		Chel16Sa-1	MN104709	MN124763
	Chromodoris strigata (Rudman, 1982)	Chst16Sa-1	MN104710	MN365022

	Glossodoris cf. *cincta* (Bergh, 1888)	Glci16Sa-1	MN104711	MN339440
		Glci16Sa-2	MN104712	MN339441
	Goniobranchus geometricus (Risbec, 1928)	Goge16S-1	MN104715	MN339442
		Goge16S-2	MN104716	MN339443
		Goge16S-3	MN104717	MN339444
		Goge16S-4	-	MN339445
	Goniobranchus reticulatus (Quoy & Gaimard, 1832)	Gore16Sa-1	MN104719	MN339446
		Gore16Sa-2	MN104720	MN339447
	Hypselodoris tryoni (Garret, 1873)	Goca16S-1	MN104713	MN339448
		Goca16S-2	MN104714	MN339450
		Goku16Sa1	MN104718	MN339449
Phyllidiidae (Rafinesque, 1814)	*Phyllidia picta* (Pruvot-Fol, 1957)	Phpic16Sa-1	MN217674	MN248545
		Phpic16Sa-5	MN217680	MN248543
		Phpic16Sa-6	MN217675	MN248546
		Phpic16Sa-8	MN217671	MN248540
		Phpic16Sa-9	MN217669	MN248539
		Phpic16Sa-10	MN217672	MN248542
		Phpic16Sa-11	MN217679	MN248549
		Phpic16Sa-12	MN217678	MN248547
		Phpic16Sa-13	MN217676	MN248548
		Phpic16Sa-14	MN217681	MN248544
		Phsp616Sa-3	MN217677	MN248550
		Phspec116Sa-2	MN217670	MN248541
	Phyllidia spec.	Phsp316Sa-1	MN217673	MN265389

Phyllidia ocellata (Cuvier, 1804)	Phoc16S-1	MN173896	MN173896
	Phoc16S-2	MN173895	MN173895
	Phoc16S-4	MN173894	MN173894
	Phoc16S-5	MN173893	MN173893
	Phoc16S-6	-	MN173892
	Phoc16S-7	MN173891	MN173891
Phyllidia coelestis (Bergh, 1905)	Phco16Sa-1	MN172238	MN234119
	Phco16Sa-2	MN172237	MN234113
	Phco16Sa-3	MN172236	MN234115
	Phco16Sa-4	MN172235	MN234118
	Phco16Sa-5	MN172234	MN234112
	Phco16Sa-7	MN172233	MN234116
	Phco16Sa-9	MN172232	MN234114
	Phco16Sa-10	MN172231	MN234112
Phyllidia varicosa (Lamarck, 1801)	Phva16Sa-2	MN243776	-
	Phva16Sa-3	MN243779	-
	Phva16Sa-4	MN243778	MN248554
	Phva16Sa-5	MN243774	-
	Phva16Sa-7	MN243747	-
	Phva16Sa-8	MN243735	-
	Phva16Sa-9	MN243783	MN248572
	Phva16Sa-10	MN243750	-
	Phva16Sa-11	MN243761	-
	Phva16Sa-12	MN243781	-

Phva16Sa-13	MN243760	MN248571
Phva16Sa-15	MN243782	MN248555
Phva16Sa-16	MN243775	-
Phva16Sa-17	MN243759	-
Phva16Sa-18	MN243780	-
Phva16Sa-20	MN243758	MN248556
Phva16Sa-21	MN243734	MN248563
Phva16Sa-22	MN243777	-
Phva16Sa-23	MN243773	-
Phva16Sa-24	MN243757	MN248568
Phva16Sa-25	MN243746	-
Phva16Sa-26	MN243733	-
Phva16Sa-27	MN243771	MN248573
Phva16Sa-28	MN243748	-
Phva16Sa-29	MN243745	-
Phva16Sa-30	MN243740	-
Phva16Sa-31	MN243770	MN248567
Phva16Sa-32	MN243768	-
Phva16Sa-33	MN243767	MN248574
Phva16Sa-34	MN243756	-
Phva16Sa-36	MN243772	-
Phva16Sa-37	MN243755	MN248569
Phva16Sa-38	MN243763	-
Phva16Sa-39	MN243744	-

	Phva16Sa-40	MN243754	MN248557
	Phva16Sa-41	MN243739	-
	Phva16Sa-42	MN243749	MN248562
	Phva16Sa-43	MN243764	MN248565
	Phva16Sa-44	MN243766	MN248561
	Phva16Sa-45	MN243741	MN248564
	Phva16Sa-46	MN243738	-
	Phva16Sa-47	MN243737	MN248558
	Phva16Sa-48	MN243753	-
	Phva16Sa-49	MN243743	-
	Phva16Sa-50	MN243742	MN248559
	Phva16Sa-52	MN243765	MN248560
	Phva16Sa-53	MN243762	MN248570
	Phva16Sa-54	MN243752	-
	Phva16Sa-55	MN243751	-
	Phva16Sa-56	MN243769	MN248566
	Phva16Sa-57	MN243736	-
	Phva16Sa-58	MN243732	-
Phyllidiella lizae (Brunckhorst, 1993)	Phli16Sa-1	MN243971	MN248575
	Phli16Sa-2	MN243973	MN248577
	Phli16Sa-5	MN243972	MN248576
	Phli16Sa-6	MN243974	MN248578
Phyllidiella pustulosa (Cuvier, 1804)	Phpu16Sa-1	MN243977	MN248624
	Phpu16Sa-2	MN244015	MN248636

Phpu16Sa-3	MN243991	MN248601
Phpu16Sa-4	MN243992	MN248606
Phpu16Sa-5	MN243996	MN248608
Phpu16Sa-6	MN244006	MN248602
Phpu16Sa-7	MN244007	MN248594
Phpu16Sa-8	MN243999	-
Phpu16Sa-9	MN243980	MN248627
Phpu16Sa-13	MN243969	MN248581
Phpu16Sa-14	-	MN248580
Phpu16Sa-15	MN243960	MN248585
Phpu16Sa-18	MN243983	MN248632
Phpu16Sa-20	-	MN248590
Phpu16Sa-23	MN243962	MN248586
Phpu16Sa-24	MN243978	MN248625
Phpu16Sa-25	MN244008	MN248595
Phpu16Sa-26	MN243979	MN248626
Phpu16Sa-27	MN244009	MN248596
Phpu16Sa-28	MN243970	MN248591
Phpu16Sa-29	MN243955	MN248639
Phpu16Sa-30	MN244000	MN248620
Phpu16Sa-31	MN243963	MN248587
Phpu16Sa-33	MN243985	MN248614
Phpu16Sa-34	MN244017	MN248637
Phpu16Sa-35	MN243957	MN248640

Phpu16Sa-36	MN244011	MN248597
Phpu16Sa-38	MN243997	MN248609
Phpu16Sa-39	MN244010	MN248598
Phpu16Sa-40	MN243981	MN248628
Phpu16Sa-46	MN244001	MN248620
Phpu16Sa-48	MN243975	MN248590
Phpu16Sa-50	MN243958	MN248641
Phpu16Sa-52	MN244002	MN248621
Phpu16Sa-53	MN243968	MN248584
Phpu16Sa-55	MN244081	-
Phpu16Sa-56	MN243994	MN248605
Phpu16Sa-60	MN244014	MN248634
Phpu16Sa-61	MN244006	MN248613
Phpu16Sa-62	MN243995	MN248607
Phpu16Sa-68	-	MN248610
Phpu16Sa-69	MN244016	MN248635
Phpu16Sa-70	MN244018	MN248638
Phpu16Sa-71	MN243986	MN248616
Phpu16Sa-73	MN243998	-
Phpu16Sa-74	MN243956	MN248642
Phpu16Sa-75	MN243987	MN248617
Phpu16Sa-76	MN243989	MN248615
Phpu16Sa-77	MN243993	MN248604
Phpu16Sa-79	MN243988	MN248619

	Phpu16Sa-80	MN244019	MN248600
	Phpu16Sa-84	MN244012	MN248599
	Phpu16Sa-85	MN243990	MN248618
	Phpu16Sa-86	MN244003	MN248611
	Phpu16Sa-87	MN243982	MN248629
	Phpu16Sa-90	-	MN248630
	Phpu16Sa-91	MN243984	MN248631
	Phpu16Sa-92	MN243967	MN248592
	Phpu16Sa-94	-	MN248603
	Phpu16Sa-95	MN244004	MN248612
	Phli16Sa-4	MN243976	MN248623
	Phli16Sa-7	MN244013	MN248633
Phyllidiella nigra (van Hasselt, 1824)	Phpu16Sa-64	-	-
Phyllidiopsis krempfi (Pruvot-Fol, 1993)	Phfi16Sa-1	MN244067	MN248643
	Phfi16Sa-2	MN244068	MN248644
	Phpu16Sa-19	-	MN248652
	Phpu16Sa-47	MN244076	MN248654
	Phpu16Sa-54	MN244077	MN248653
	Phpu16Sa-57	MN244071	MN248651
	Phpu16Sa-58	MN244074	MN248650
	Phpu16Sa-65	MN244072	MN248649
	Phpu16Sa-66	MN244073	MN248647
	Phpu16Sa-67	MN244069	MN248645
	Phpu16Sa-72	MN244070	MN248646

	Phpu16Sa-82	-	MN248658
	Phpu16Sa-83	MN244080	MN248657
	Phpu16Sa-88	MN244075	MN248646
	Phpu16Sa-93	MN244078	MN248655
Phyllidiopsis shireenae (Brunckhorst, 1990)	Phsh16Sa-2	MN244082	MN248659

Figure 3.3 Anthobranchia and Cladobranchia/Aeolidioidea:
(A) *Hypselodoris tryoni*, Goku16Sa-1;
(B) *Phyllidia ocellata*, Phoc16Sa-3;
(C) *Phyllidia picta*, Phpic_16Sa-13;
(D) *Phyllidia* spec., Phsp3_16Sa-1;
(E) *Phyllidia madangensis*, Phma16Sa-1;
(F). *Phyllidia coelestis*, Phco16Sa-1;
(G) *Phyllidia varicosa*, Phva16Sa-6;
(H) *Phyllidiella lizae*, Phli16Sa-4;
(I) *Phyllidiella pustulosa* complex, Phpu16Sa-29;
(J) *Phyllidiella pustulosa* complex, Phpu16Sa-91;
(K) *Phyllidiella pustulosa* complex, Phpu16Sa-95;
(L) *Phyllidiella nigra*, Phpu16Sa-64;
(M) *Phyllidiopsis krempfi* Phpu16Sa-58;
(N) *Phyllidiopsis shireenae*, Phsh16Sa-2;
(O) Aeolidioidea Flsp16Sa-1.

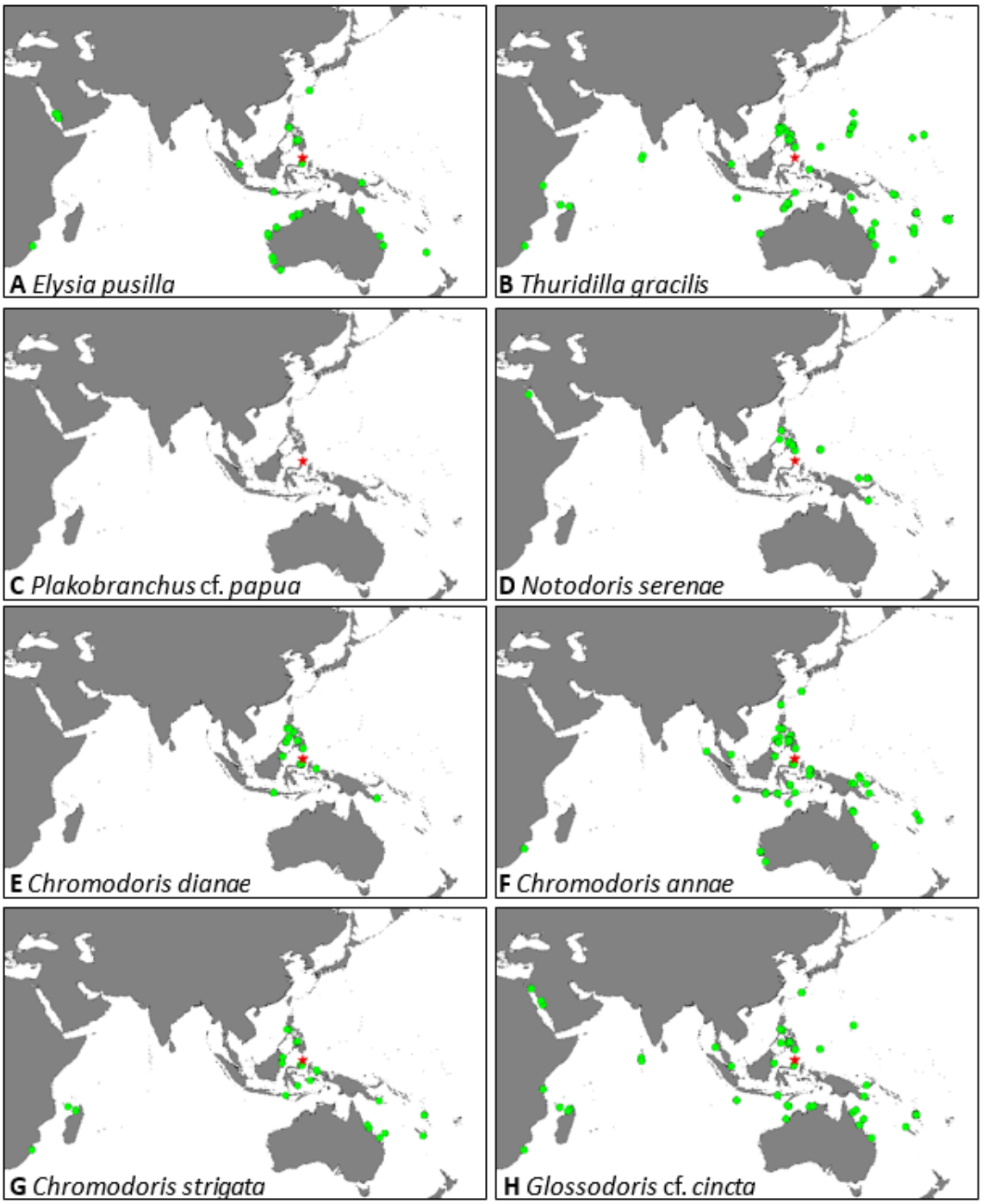

Figure 3.4 Distribution data of respective species in the Indo-Pacific Ocean. Data from this study (Sangihe) and downloaded from GBIF.
(A) *Elysia pusilla* (https://doi.org/10.15468/dl.mhkexk);
(B) *Thuridilla gracilis* (https://doi.org/10.15468/dl.a8jpj7);
(C) *Plakobranchus* cf. *papua* (no data in GBIF available yet)*;*
(D) *Notodoris serenae* (https://doi.org/10.15468/dl.3vlodn);
(E) *Chromodoris dianae* (https://doi.org/10.15468/dl.u1dqtv);
(F) *Chromodoris annae* (https://doi.org/10.15468/dl.2jdtpp)*;*
(G) *Chromodoris strigata* (https://doi.org/10.15468/dl.8sm78l)*;*
(H) *Glossodoris* cf. *cincta* (https://doi.org/10.15468/dl.lycuc9).

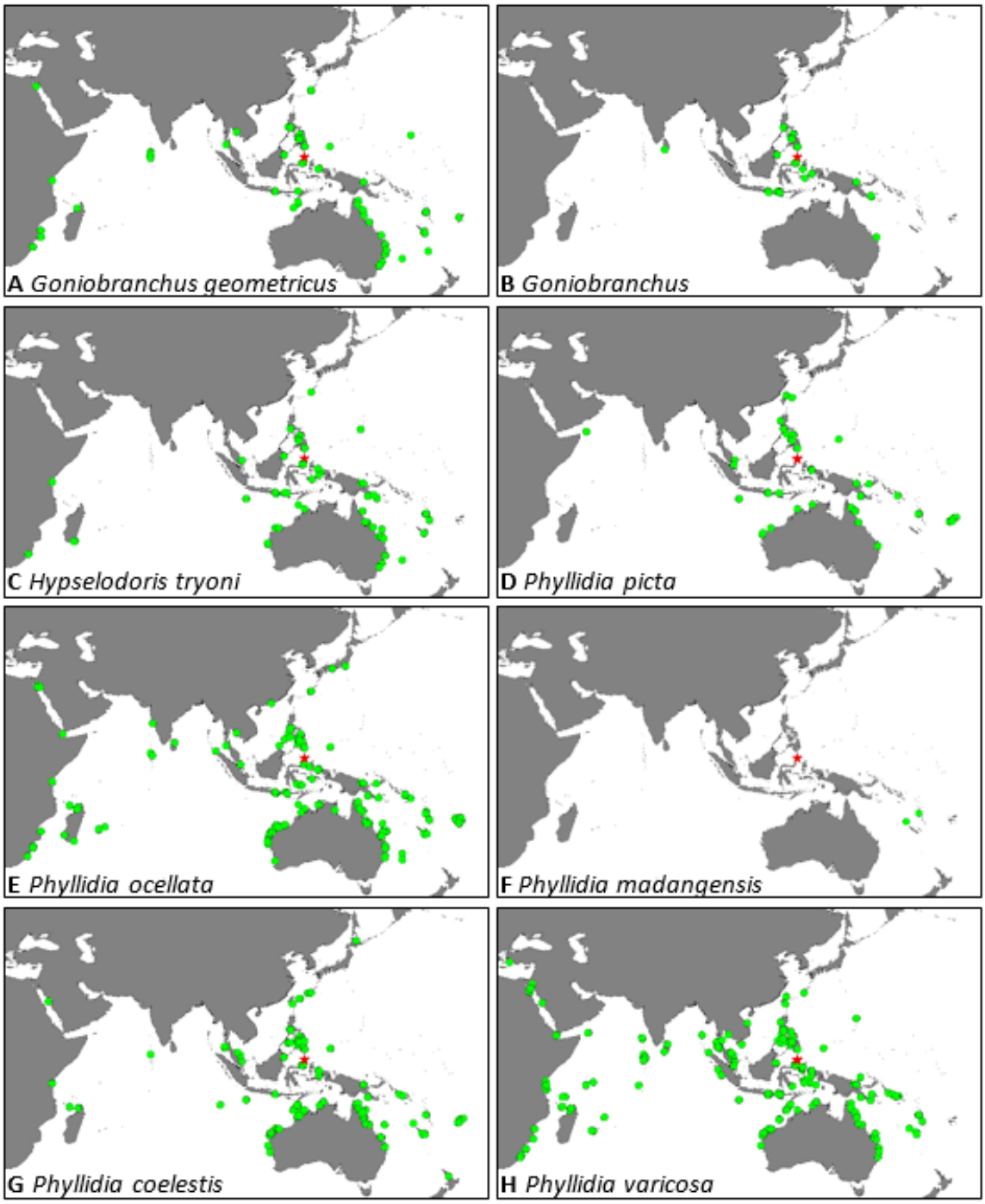

Figure 3.5 Distribution data of respective species in the Indo-Pacific Ocean. Data from this study (Sangihe) and downloaded from GBIF.
(A) *Goniobranchus geometricus* (https://doi.org/10.15468/dl.flgtfy)*;*
*(*B) *Goniobranchus reticulatus* (https://doi.org/10.15468/dl.2xa8go)*;*
(C) *Hypselodoris tryoni* (https://doi.org/10.15468/dl.3fmoxu);
(D) *Phyllidia ocellata* (https://doi.org/10.15468/dl.xplg0z);
(E) *Phyllidia picta* (https://doi.org/10.15468/dl.uzsfrc);
(F) *Phyllidia madangensis* (https://doi.org/10.15468/dl.hhqftx);
(G) Phyllidia coelestis (https://doi.org/10.15468/dl.4lqgzh);
(H) Phyllidia varicosa (https://doi.org/10.15468/dl.nhnbso).

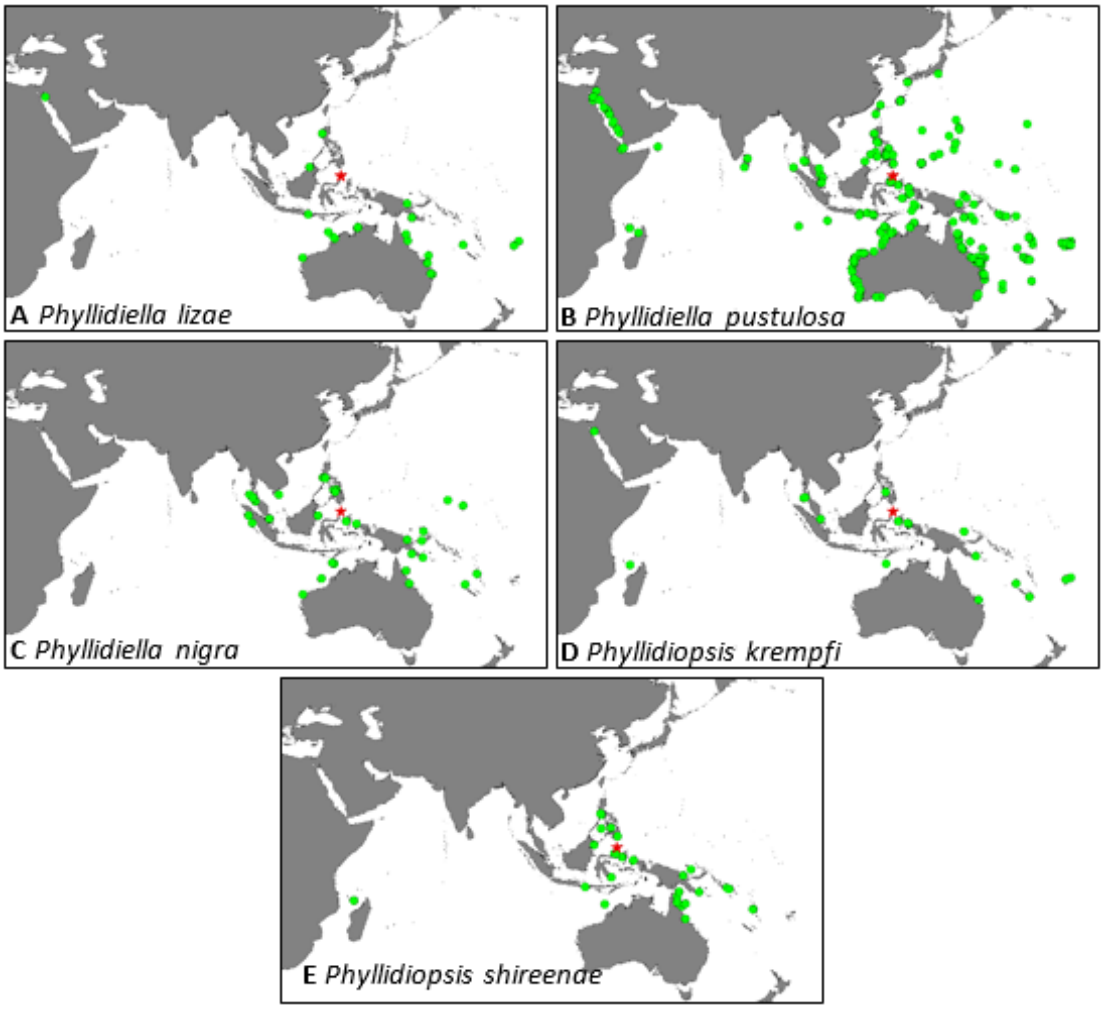

Figure 3.6 Distribution data of respective species in the Indo-Pacific Ocean. Data from this study (Sangihe) and downloaded from GBIF.
(A) *Phyllidiella lizae* (https://doi.org/10.15468/dl.y94qqg);
(B) *Phyllidiella pustulosa* (https://doi.org/10.15468/dl.108oy5);
(C) *Phyllidiella nigra* (https://doi.org/10.15468/dl.rgidut);
(D) *Phyllidiopsis krempfi* (https://doi.org/10.15468/dl.unlgk3);
(E) *Phyllidiopsis shireenae* (https://doi.org/10.15468/dl.mimhpp).

Systematics

HETEROBRANCHIA

SACOGLOSSA

PLAKOBRANCHOIDEA

Family: Plakobranchidae Gray, 1840

***Elysia*, Risso, 1818**

***Elysia pusilla*, Bergh, 1871 (Figures 3.2A, 3.4A, Table 3.2)**

Description

Three specimens of *Elysia pusilla* with lengths of 2–6 mm were collected from Mendaku and Tahuna Bay South (Fig. 3.2A). All three specimens had the typical green coloration with whitish rhinophores.

Remarks

One specimen from Mendaku was crawling out of a patch of the chlorophyte *Caulerpa racemosa*; another one at the same locality was sitting on a *Halimeda* species with small thalli. The third specimen from Tahuna Bay South was associated with *Halimeda* cf. *macroloba*. The species is widely distributed in the Indo-Pacific Ocean (Fig. 3.4A).

***Thuridilla*, Bergh, 1872**

***Thuridilla gracilis*, Risbec, 1928 (Figures 3.2B, 3.4B, Table 3.2)**

Description

Six specimens with lengths of 20–30 mm were collected in front of Palahanaeng village (3 specimens), Talengen village (1 specimen), and in Sapaeng (2 specimens). All specimens show the typical black background and whitish to light green colored fine longitudinal lines. The anterior part of the foot, the tips of the rhinophores, and the tip of the tail show the typical orange color, but the orange rim of the parapodia is only very narrow. No distinct blue spots, which are described from some specimens of the form *bayeri*, are visible.

Remarks

One specimen from Sapaeng was observed on an algae looking very similar to *Dictyota*, the other from the same locality and one animal from Palahanaeng village were sitting close to the same algal species. The specimen from Talengen village was crawling on the base of small *Halimeda* thalli. The remaining two specimens were crawling on unspecified sediment. The species is widely distributed in the Indian and Western Pacific Ocean (Fig. 3.4B).

***Plakobranchus*, van Hasselt, 1824**

***Plakobranchus* cf. *papua*, Meyers-Muñoz and van der Velde, 2016 (Figures 3.2C, 3.4C, Table 3.2)**

Description

Two specimens of *Plakobranchus* cf. *papua* with lengths of 25 and 30 mm were collected in Tahuna Bay South and Mendaku at depths of 5 and 15 m. Our animals show yellowish to white spots of various sizes arranged in a distinct pattern on a darker olive to green background. Our animals differ from the animals described and depicted by Meyers-Muñoz et al. (2016) in so far as that they exhibit more spots and thus appeared lighter in color than the animals described from West Papua, Indonesia. However, our animals match with regard to the rhinophores, which are nearly completely violet in color.

Remarks

Recently, Yonow & Jensen (2018) reviewed and discussed the complicated situation within the genus *Plakobranchus* with at least 14 species described from the Pacific Ocean. Many species have never been found again; descriptions were poor, rendering assignment of new material very difficult. The authors depict two specimens, one from Ambon, one from Malaysia, assigned tentatively to *P.* cf. *papua*. They look very similar to our specimens, especially in the number of spots and the arrangement of these. Eisenbarth et al. (2018) assigned their specimens from Bunaken Island to *P. ocellatus*. In contrast to our specimens which were collected in depths of 5 and 15 m, the animals collected in BNP lived in the eulittoral. Both animals from our collection were crawling

on sediment surrounded by various species of algae. No further distribution records are listed in GBIF (Fig. 3.4C).

NUDIBRANCHIA

DORIDINA

Family: Aegiridae P. Fischer, 1883

***Notodoris* Bergh, 1875**

***Notodoris serenae* Gosliner and Behrens, 1997 (Figures 3.2D, 3.4D, Table 3.2)**

Description

Two specimens of *Notodoris serenae* with lengths of 60 and 90 mm were collected in Manalu at depths of 24 and 27 m. They show the same typical coloration as depicted in Kaligis et al. (2018).

Remarks

Only *Notodoris serenae* from the family Aegiridae, which usually feeds on hexactinellid sponges, was collected during the present survey. Both animals were crawling on sediment. The species is mainly known from the Coral Triangle (Fig. 3.4D).

Family: Chromodorididae Bergh, 1891

***Chromodoris* Alder and Hancock, 1855**

***Chromodoris dianae* Gosliner and Behrens, 1998 (Figures 3.2E, 3.4E, Table 3.2)**

Description

Seven specimens of *Chromodoris dianae* with lengths of 5–45 mm were collected in Manalu (1 specimen) and Mendaku (6 specimens) at depths of 15–27 m. The body is elongate and the color of this species is white with a tinge of blue and a pattern of distinct interrupted black lines and spots. The rhinophores are yellowish to orange, whereas the gills are white with yellow tips.

Remarks

Gosliner and Behrens (1998) mentioned in their first description the similarity in color with *Chromodoris quadricolor* (Rüppell & Leuckart, 1830), another pale blue chromodorid. However, *C. quadricolor* has an orange marginal band. Our *Chromodoris dianae* specimens are very similar to those depicted in Yonow (2001) and Kaligis et al. (2018). Our specimens were also mainly collected from sponges. The mantle glands of *C. dianae*, which can be seen clearly in the live animal, are well separated from each other and are highly ramified with digitate branches. Species records are mainly confined to the Coral Triangle (Fig. 3.4E).

***Chromodoris annae* Bergh 1877 (Figures 3.2F, G, 3.4F, Table 3.2)**

Description

Thirteen specimens of *Chromodoris annae* with lengths of 8–41 mm were collected in Manalu, Palahanaeng village, Mendaku, Sapaeng, and Talengen village at depths of 5-23 m (Table 3.2). Our specimens show the typical blue color with darker miniature spots. They are lacking a mid-dorsal longitudinal line and any small black dots within the blue areas. The rhinophores exhibit the typical yellow color. However, one specimen shows differences in coloration by exhibiting a lighter blue and an interrupted black line in the middle.

Remarks

Some *Chromodoris* species are difficult to distinguish by color only (Kaligis et al. 2018). *Chromodoris elisabethina* Bergh, 1877 looks similar to *Chromodoris annae*, but *C. annae* usually does not have a median black line and the blue areas of the mantle are not uniform blue as is the case of *C. elisabethina* (Rudman 1982). However, we collected one animal in front of Talengen village (Fig. 3.2G) which is quite similar to *C. elisabethina*: the specimen shows the usual elongate bluish body with the mantle margin encircled by a black, a white, and finally a yellow band. However, the animal mimicking *C. elisabethina* had additionally a medially lying black line, which was interrupted several times. Barcoding and comparison with our unpublished sequences, and the few available

from NCBI, clearly indicate its correct assignment to *C. annae*, and therefore provides here the first example of mimicry involving *C. annae* and *C. elisabethina*. Mimicry forms between members of the Phyllidiidae and Chromodorididae are depicted in several identification books (Gosliner et al. 2008, 2015), and described in Cheney et al. (2016) and Padula et al. (2016). However, mimicry between closely related *Chromodoris* species was described in a broader context for the first time only recently (Layton et al. 2018). This is the first example of *Chromodoris annae* mimicking *C. elisabethina*. The species is widely distributed in the Indo-Pacific Ocean, including subtropical areas (Fig. 3.4F).

***Chromodoris strigata* Rudman, 1982 (Figures 3.2H, 3.4G, Table 3.2)**

Description

Only one specimen of *Chromodoris strigata* with a length of 10 mm was collected in Mendaku at a depth of 15 m. The mantle of this specimen shows a white background with bluish tinges. The gills and rhinophores are the same yellow to orange as the mantle border. The yellow band along the mantle rim is interrupted.

Remarks

Although having similarities to many bluish to white *Chromodoris* species, *C. strigata* is easily recognised in this color group by the fading blue on white background as well as the areas of light yellow to white in the yellow mantle rim. This renders the species paler than other species (W. B. Rudman 1982). Its distribution is recorded from the Indo-Pacific Ocean (Fig. 3.4G).

***Glossodoris* Ehrenberg, 1831**

***Glossodoris* cf. *cincta* (Bergh, 1888) (Figures 3.2I, 3.4H, Table 3.2)**

Description

Two specimens of *Glossodoris* cf. *cincta* with lengths of 21 and 48 mm were collected in Manalu and Mendaku at depths of 8 and 13 m. The animals show an elongate

to oval shape with mottled reddish brown and white on the notum. The gills and rhinophores are brown.

Remarks

Nudibranchs of the genus *Glossodoris* are moderately large and easily spotted. They are widely distributed in tropical and temperate reef environments around the world (Rudman 1986; Johnson and Gosliner 2012). Most recently several new species with similar color patterns to *G. cincta* were described (Matsuda and Gosliner 2018; Yonow 2018). *Doriprismatica kyanomarginata* Yonow 2018 differs from our specimen by having a diffuse inner yellow ribbon at the mantle margin, which is characteristic for this new species. Our animal is very close in coloration to *Glossodoris acosti* Matsuda and Gosliner 2018. Especially the coloration of the mantle margin with a light blue outermost ring, followed by a dark green and then a lighter yellow-green ring is very similar in both species. However, the rings are wider in *G. acosti* and furthermore, the gills are mentioned to be larger, forming an arch opening to the posterior and with two distinct spirals. Our animal had all gill branches on one level and the arrangement was forming a complete circle. It thus resembles the animal depicted as *Glossodoris* cf. *cincta* in Matsuda and Gosliner (2018). Bergh (1888) in his original description of *G. cincta* mentioned dark brown rhinophores with white dots and the gills with six larger branches in the anterior part, followed by eight smaller ones on each side in the posterior part of the circle. Thus, our specimen also differs from the original description. We therefore only tentatively assign our animal to *Glossodoris cincta*. The specimen was collected from a brownish sponge. According to GBIF data, *Glossodoris cincta* shows a broad distribution from the Red Sea until Fiji Islands (Fig 3.4H). However, difficulties in correct identification probably blur the correct distribution area.

***Goniobranchus* Pease, 1866**

***Goniobranchus geometricus* (Risbec, 1928) (Figures 3.2J, 3.5A, Table 3.2)**

Description

Four specimens of *Goniobranchus geometricus* with lengths of 10–15 mm were collected in Tahuna Bay South (1 specimen), Palahanaeng village (1 specimen), and

Sapaeng (2 specimens) at depths of 6–19 m. Our specimens are rose colored with opaque white tubercles and a network of thick black lines in between the tubercles. The mantle rim is whitish. The translucent white gills and rhinophores have bright green to yellow tips.

Remarks

The color pattern of *Goniobranchus geometricus* from Sangihe is very similar to that depicted in various identification books, and is also shown by Yonow(2001), Kaligis et al. (2018), and Eisenbarth et al. (2018). The slug usually can be found under stones or coral rubble (Gosliner et al. 2008), where we also found our animals. The species is widely distributed in the Indo-Pacific Ocean (Fig. 3.5A).

***Goniobranchus reticulatus* (Quoy and Gaymard, 1832) (Figures 3.2K, 3.5B, Table 3.2)**

Description

Two specimens of *Goniobranchus reticulatus* with lengths of 25 and 55 mm were collected in Sapaeng at depths of 6 and 9 m. The specimens show an elongate body with a reticulated network of red lines over the surface mantle. The mantle rim exhibits a narrow white area. The rhinophores are white with red tips. The gills are reddish with the inner rachis opaque white.

Remarks

Our specimen is very similar to the animals depicted by Kaligis et al. (2018) and Eisenbarth et al. (2018), which were also identified as *G. reticulatus*. Barcoding and comparison with our unpublished sequences, and the few available from NCBI, indicate its correct assignment to *G. reticulatus*. However, Yonow (2001) discussed *Chromodoris inopinata* Bergh, 1905 as a very common form in the Indo-Pacific and probably often misidentified as *G. reticulatus*. *C. inopinata* shows very similar color patterns as *G. reticulatus*. No CO1 sequences assigned to *C. inopinata* are available at NCBI GenBank yet. The records in GBIF show a more limited distribution than is known from *G. geometricus*, with findings mainly from the Coral Triangle (Fig. 3.5B).

***Hypselodoris* Stimpson, 1855**

***Hypselodoris tryoni* (Garrett, 1873) (Figures 3.3A, 3.5C, Table 3.2)**

Description

Three specimens of *Hypselodoris tryoni* with lengths of 25–60 mm were collected in Sapaeng at depths of 10–16 m. The specimens show a cream to dirty brown mantle with bluish to dark violet spots. These spots are surrounded by a ring of white pigment and then a paler area. The rim of the mantle is purple. The gill and rhinophores are translucent white with the rachis of the gills brownish and the rachis of the rhinophores purple.

Remarks

Hypselodoris tryoni, Goniobranchus leopardus (Rudman 1987), and *Goniobranchus cavae* (Eliot 1904) are very similar in external appearance, exhibiting a dark cream background color with dark violet round patches surrounded by a light colored area. Additionally, *G. cavae* can be highly variable in color (Yonow 2012; Tibiriçá et al. 2017). However, in *G. cavae* the gills and rhinophores are white with usually purple tips, whereas in *H. tryoni*, the rachis of the gills and rhinophores shows a purple coloration throughout the full length and the tips of the rhinophores are not distinctively purple. The species has a wide distribution in the Indo-Pacific Ocean with many records also from the subtropics (Fig. 3.5C).

Family: Phyllidiidae Rafinesque, 1814

***Phyllidia,* Cuvier, 1797**

***Phyllidia ocellata* Cuvier, 1804 (Figures 3.3B, 3.5E, Table 3.2)**

Description

Seven specimens of *Phyllidia ocellata* with lengths of 18–35 mm were collected in Tahuna Bay South (2 specimens), Manalu (2 specimens), Palahanaeng village (1 specimen), and Sapaeng (2 specimens) at depths of 4–18 m. All our animals exhibit the typical yellow coloration with white tubercles, some of which are surrounded by black

circles, followed by a thin white line. All other white tubercles are sticking out of the orange background color.

Remarks

Phyllidia ocellata with the yellow to orange background and the tubercles surrounded by black rings is unique in its coloration and therefore cannot be confused with any other *Phyllidia* species. Gosliner et al. (2015) depicted color morphs that lack white tubercles, which were not found during the present study. The species is very common in the Indo-Pacific with a range into subtropics of Australia (Fig. 3.5E).

***Phyllidia picta* Pruvot-Fol, 1957 (Figures 3.3C, 3.5D, Table 3.2)**

Description

Twenty-one specimens of *Phyllidia picta* with lengths between 13 and 30 mm were collected in Tahuna Bay South (6 specimens), Manalu (3 specimens), Palahanaeng village (2 specimens), Mendaku (2 specimens), Sapaeng (6 specimens), and Talengen village (2 specimens) at depths of 1–15 m. All of our animals show an oval shape, with black reticulate pattern and single yellow tubercles on a blue background. The rhinophores are yellow and the foot sole has no black stripe.

Remarks

Brunckhorst (1993) considered *Phyllidia picta* to be a junior synonym of *P. coelestis*, but Yonow (1996) and Stoffels et al. (2016) confirmed its validity. The species is not recorded from Bunaken National Park (Kaligis et al. 2018; Eisenbarth et al. 2018) but was reported from Yonow (2011) and is also recorded in GBIF from few other places in Indonesia down to Australia (Fig. 3.5D).

Phyllidia spec. (Figure 3.3D, Table 3.2)

Description

Figure 3.3D exhibits an unidentified *Phyllidia* specimen with a length of 28 mm. It was found only once in Talengen village. This specimen has an elongate to oval shape with greenish to greyish background and black lines between tubercles arranged in ridges. Tubercles are single rather than compound. The rhinophores are yellow. The foot sole shows a black line as is typical for *Phyllidia elegans* Bergh, 1869, to which it is very similar.

Remarks

The specimen cannot be assigned to any described species. Genetic information indicates no relationship to *P. elegans*, but to *Phyllidia picta*; however, therefore its assignment to the genus *Phyllidia* is confirmed. The specimen of the undescribed *Phyllidia* species depicted by Eisenbarth et al. (2018) looks very different from ours.

Phyllidia madangensis Bruncknorst, 1993 (Figures 3.3E, 3.5F, Table 3.2)

Description

Phyllidia madangensis was collected in front of Talengen village with one specimen with a length of 28 mm. Our animal shows the typical features, the lack of the dark stripe on the foot sole and its overall blackish color. Few white tubercles capped in bright yellow are scattered over the notum. The rhinophores are dark yellow.

Remarks

Phyllidia madangensis is very similar to *P. carlsonhoffi* Bruncknorst, 1993; however, our animal has smaller tubercles and is more blackish than *P. carlsonhoffi*, as is depicted by e.g. Gosliner, et al. (2015). Rudman (http://www.seaslugforum.net) illustrated a specimen of *P. madangensis* with whitish tubercles, similar to our specimen, whereas the tubercles of some *P. carlsonhoffi* can be more bluish. *Phyllidia carlsonhoffi* also has tubercles more evenly distributed over the notum, whereas *P. madangensis* has

sparsely scattered tubercles. Brunckhorst (1993) described rhinophoral tubercles to occur in all *Phyllidia* species, but the presence of a small tubercle directly in front of each rhinophoral pocket appears to be unique to *P. madangensis.* Our specimen did not really show this tubercle. However, the overall appearance and the coloration allow the assignment to P. madangensis, which is a rather rare species (Fig. 3.5F).

Phyllidia coelestis Bergh, 1905 (Figures 3.3F, 3.5G, Table 3.2)

Description

Nine specimens of *Phyllidia coelestis* with lengths of 7–32 mm were collected in Tahuna Bay South (3 specimens), Manalu (1 specimen), Palahanaeng village (1 specimen), Sapaeng (1 specimen), and Talengen village (3 specimens). The specimens display the typical background blue color with three black lines. The line in the middle is interrupted by few single yellow tubercles, whereas the outer two lines run lateral to the smaller yellow tubercles. The rhinophores are yellow.

Remarks

Phyllidia coelestis is a smaller and widely distributed species (Figure 3.5G), which has neither a foot stripe nor a median tuberculate ridge. The species can be distinguished from other similar looking phyllidiids, such as *P. varicosa*, by the central black stripe on the notum, interrupted by large yellow tubercles. Additionally, it has a characteristic black Y-shaped pattern between and in front of the rhinophores. Brunckhorst (1989) and Yonow (2011) mentioned a dark form that has a central oval region where the ground color is black and only a marginal band around it depicts the bluish-white color.

Phyllidia varicosa, Lamarck, 1801 (Figures 3.3G, 3.5H, Table 3.2)

Description

Fifty-eight specimens with lengths of 7–87 mm were collected at all sampling sites, except ship wreck, at depths of 3–15 m. All specimens show a light blue background

with yellow tubercles in rows and blackish lines between these tubercle ridges. The rhinophores are yellowish.

Remarks

Phyllidia varicosa is a large species that can be distinguished by its black stripe at the foot sole, which is absent in most *Phyllidia* species. It has three to six longitudinal, tuberculate notal ridges (Brunckhorst 1993). Our animals are quite similar to this description, with an elongate to oval shape, the yellow rhinophores, and the black stripe along the foot sole. The species is very common in the Indo-Pacific Ocean and also occurs in the subtropics of Australia (Fig. 3.5H).

***Phyllidiella*, Bergh 1869**

***Phyllidiella lizae* Brunckhorst, 1993 (Figures 3.3H, 3.6A, Table 3.2)**

Description

Twelve specimens with lengths of 6–68 mm were assigned preliminarily to *Phyllidiella lizae*. They were collected in Tahuna Bay South (3 specimens), Manalu (3 specimens), Palahanaeng village (1 specimen), Mendaku (3 specimens), and Talengen village (2 specimens). All specimens show the pale pink background, pale pink tubercles and irregular, narrow black lines on the dorsum like a pale ‘x’. The rhinophores are black with pink at the base.

Remarks

Brunckhorst (1993) stated that *Phyllidiella lizae* is recognizable by its pale pink notum with simple, rounded, pale pink tubercles and narrow black lines crossing the dorsum. The rhinophores are black at the tip, pink in the central area, and white at the base. Other distinguishing characters are the pale, pinkish white oral tentacles and foot sole. Our animals match this description, except that the rhinophores are more pinkish at the base, instead of white. However, molecular data indicate cryptic speciation (A.P. unpublished data). Records in GBIF are confined to the coral triangle and the northern parts of Australia (Fig. 3.6A).

Phyllidiella pustulosa (Cuvier, 1804) (Figures 3.3I–K, 3.6B, Table 3.2)

Description

In this study, 77 specimens of *Phyllidiella pustulosa* with lengths of 12–68 mm were found at all sampling sites in Sangihe in depths of 1–23 m. Our animals have elongate bodies, and diverse color variations; from reddish to pink or even green tubercles surrounded by black lines.

Remarks

Stoffels et al. (2016) described *Phyllidiella pustulosa* with a high intraspecific variation and cryptic speciation, based on molecular analyses. Already Brunckhorst, (1993) stated that ontogenetic variation also might have contributed to the confusion in the literature. Burghardt et al. (2006) assigned one specimen to *Phyllidiella nigra*, which actually looks very similar to *P. pustulosa*. In our collection, *P. pustulosa* is the species with the highest number of color-morphs and our own unpublished molecular data confirm cryptic speciation. Thus, the broad distribution data in GBIF in the tropic and subtropic Indo-Pacific Ocean probably reflect the distribution of several cryptic species (Fig. 3.6B).

Phyllidiella nigra (van Hasselt, 1824) (Figures 3.3L, 3.6C, Table 3.2)

Description

One specimen of *Phyllidiella nigra* with a length of 29 mm was collected in Sapaeng. This specimen has an elongate body, and its overall color appears blackish with pinkish to brownish tubercles. The tubercles are evenly scattered and not arranged in rows, however they cluster together, as typical for *Phyllidiella pustulosa*. The rhinophores are black.

Remarks

Brunckhorst (1993) distinguished *Phyllidiella nigra* from conspecifics by its tall, rounded, dark pink to red tubercles, which are evenly distributed (not clustered) over the

dorsum (e.g. specimens from Ambon in Yonow (2011)). Stoffels et al. (2016) already depicted several specimens with tubercles clustering and surrounded by black patterns. In our study, *P. nigra* appears blackish with darker pinkish tubercles, but the overall appearance is quite similar to the *P. nigra* specimens depicted in Stoffels et al. (2016). Our genetic analyses group this specimen with published sequences also assigned to *P. nigra*; however, the quality of our sequence is poor and needs repetition. The species is mainly recorded from the Coral Triangle and Northern Australia (Fig. 3.6C).

Phyllidiopsis Bergh, 1876

Phyllidiopsis krempfi Pruvot-Fol, 1957 (Figures 3.3M, 3.6D, Table 3.2)

Description

Fifteen specimens of *Phyllidiopsis krempfi* with lengths of 14–50 mm were collected in just one locality, Palahanaeng village, at depths of 13–16 m. The oral tentacles are fused, as is typical for the genus. The animals are elongate to oval and have two black lines on the dorsum extending around the rhinophores, meeting in front of the rhinophores. Additionally, black lines run from these longitudinal lines perpendicularly toward the notum margin, similar to the patterns depicted by Stoffels et al. (2016). The color of our animals varies from reddish (Fig. 3.3L) to pale pink. The rhinophores are black.

Remarks

Phyllidiopsis krempfi is characterised by a predominantly pink coloration and wide shape (Brunckhorst 1993). *Phyllidiopsis gemmata* (Pruvot-Fol, 1957) is very similar to *P. krempfi*, but Tibiriçá et al. (2017) described characteristic differences. *Phyllidiopsis krempfi* has pink rhinophores with only the apical part in black, while *P. gemmata* has mainly black rhinophores with only the base pinkish (Brunckhorst 1993). Our animals therefore more resemble *P. gemmata*. However, the tubercles are simple in *P. gemmata*, while they are compound in *P. krempfi* (Brunckhorst 1993), as this is the case in our animals. *P. gemmata* is also mentioned to be more elongate than *P. krempfi*. This character is difficult to distinguish, when no other material is available for comparison. Molecular data confirm the assignment to *P. krempfi* and indicate a higher color variation

as previously described. Records in GBIF are rare (Figure 6D) but reach from the Red Sea to Fiji Islands.

Phyllidiopsis shireenae Brunckhorst, 1990 (Figures 3.3N, 3.6E, Table 3.2)

Description

Two specimens of *Phyllidiopsis shireenae* with a length of 77 and 81 mm were collected in Manalu and Sapaeng at depth of 8 and 15 m. The body is elongate to oval, with a typical longitudinal mid-dorsal ridge, which is covered with large whitish tubercles. The body color is white with opaque white spots and a typical black lining. The foot is also white. The rhinophores are salmon pink.

Remarks

The specimens are very similar to the one depicted by Stoffels et al. (2016) from the northern Moluccas and from Yonow (2011). Ours have the two black transversal lines connecting the longitudinal stripes in common with them. One of our specimens shows a black dot in the middle of the white ridge, similar to the animal depicted by Gosliner et al. (2015). Brunckhorst (1993) considered the mid-dorsal crest as the characteristic feature of *Phyllidiopsis shireenae*, which is lacking in most other *Phyllidiopsis* species. Another characteristic is the salmon pink rhinophores. *Phyllidiopsis pipeki* Brunckhorst, 1993, *Phyllidiopsis burni* Brunckhorst 1993, and *Phyllidiopsis fissuratus* Brunckhorst, 1993 differ from *P. shireenae* in having large compound tubercles, black or pale pink rhinophores, and pink to grey ventral coloration (white in *P. shireenae*) (David J. Brunckhorst 1993). The species is mainly distributed from the Coral Triangle to Northern Australia (Fig. 3.6E).

Aeolidioidea spec. (Figure 3.3O, Table 3.2)

Description

A tiny aeolidid species, probably a juvenile, with a length of 1 mm was collected in Mendaku at 1 m depth. The animal (Fig. 3.3O) is whitish with orange rhinophores and

with orange to opaque white cerata. The rhinophores showed irregular swellings or rings. Oral tentacles are short.

Remarks

There are many members of the Aeolidioidea with similar rhinophores, and similar cerata shape and arrangement, but overall habitus resembles probably most a *Flabellina* species. Proper identification will need barcoding methods, resulting in the complete loss of this specimen for further investigation.

Discussion

This is the first study describing the diversity of marine Heterobranchia around the island of Sangihe, Sangihe Islands Regency, North Sulawesi Province. Collecting at different locations (Tahuna Bay South, Ship Wreck, Mendaku, and Manalu, on the eastern coastline; in front of the villages of Palahanaeng and Talengen, and Sapaeng, on the western coastline) ensured the cover of differing habitats and degrees of exposure. Strong currents did not allow extensive sampling in many exposed areas, especially at the outer reef areas and drop offs. This first record is based on a high number of specimens (250), which can be assigned to 23 species (Table 2). The species number cannot compare with the higher numbers of other recent studies at North Sulawesi (Fig. 3.7) (Kaligis et al. 2018; Eisenbarth et al. 2018), which might be due to several factors. Collecting time was lower than in BNP, but the differences in habitats were more pronounced. We observed a high sedimentation rate in the water column, resulting in many organisms (sponges, corals, and algae) being covered by a thin layer of silt or mud. This is probably caused by unusually heavy rainfall in this particular season and/or the higher impact of many small river systems close to the collection areas.

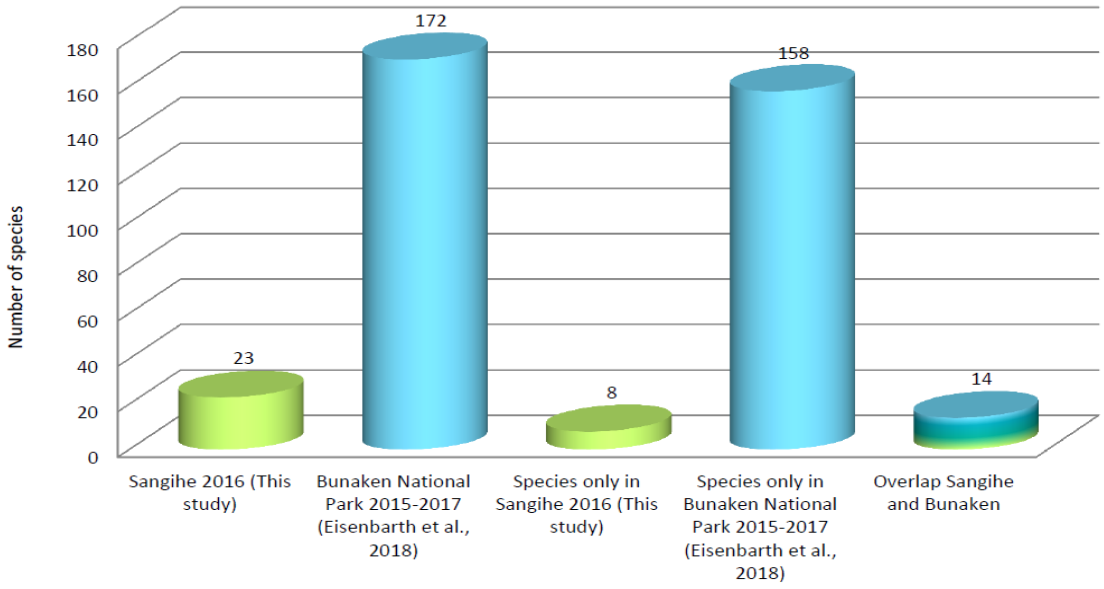

Figure 3.7 Comparison of species diversity in this study (Sangihe) with (Bunaken National Park) (Eisenbarth et al., 2018). Note that one-third of the species collected in Sangihe were not found during surveys in Bunaken National Park.

Figure 3.8 and Table 3.2 provide detailed information about numbers of species/specimens found at the various collection localities around Sangihe. Collection time and effort were similar for all localities. The highest number of sea slug species was found at Sapaeng (13 species, 60 specimens), followed by Tahuna Bay South (11 species, 58 specimens), Palahanaeng village (11 species, 42 specimens), Manalu (11 species, 29 specimens), Mendaku (10 species, 22 specimens), and Talengen village (9 species, 35 specimens). The lowest overall species number was recorded on the Ship Wreck (1 species, 4 specimens), a locality highly influenced by Tahuna harbor and the city of Tahuna. Members of the Anthobranchia (with 238 specimens assigned to 19 species) were present in all seven localities, followed by sacoglossans (11 specimens assigned to 3 species), present in five localities. The Cladobranchia was represented by only one specimen, an unidentified member of the Aeolidioidea.

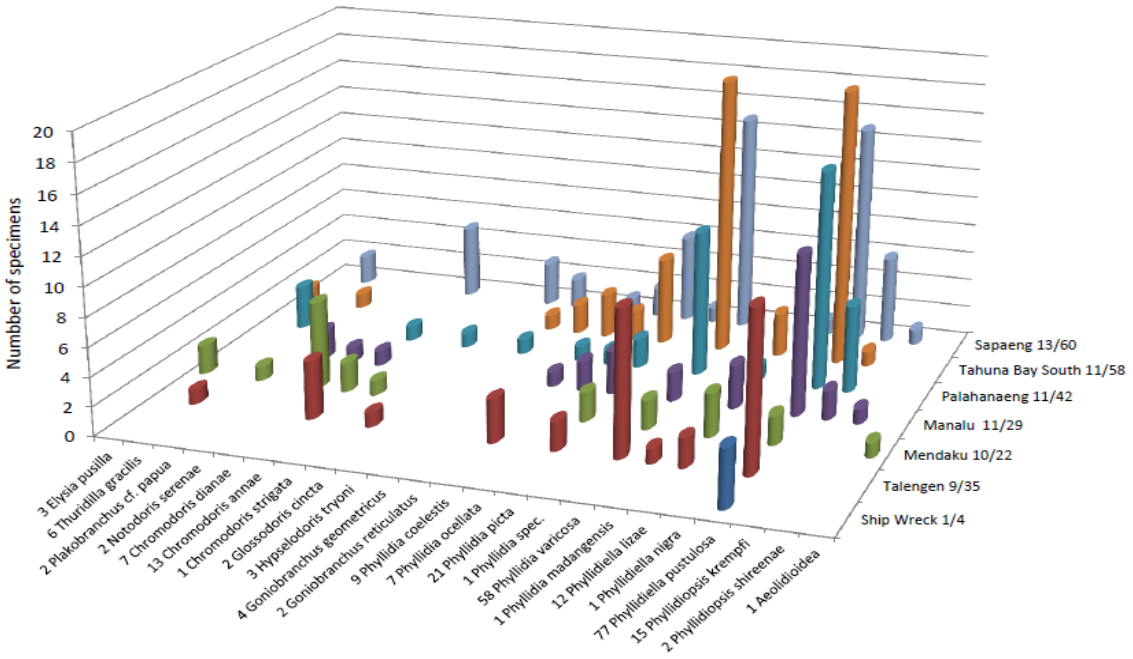

Figure 3.8 Comparison of marine Heterobranch species collected around the island Sangihe. The numbers in front of the species names indicate the number of collected specimens. The numbers after the locality names indicate the number of species collected, followed by the number of specimens.

Recent studies have shown that several species represent cryptic species complexes, while species treated earlier as different taxa are simply color-variants of the same species (Carmona et al. 2011; Ornelas-Gatdula et al. 2011; Pola et al. 2012; Pola et al. 2014; Padula et al. 2016; Matsuda & Gosliner 2017; Tibiriçá et al. 2017; Layton et al. 2018). With regard to our listed taxa, cryptic speciation has been recorded for the genus *Plakobranchus* (Yonow & Jensen 2018). We did not barcode our specimens, but the color patterns allow the tentative assignment to *P. papua*. We can confirm that *Phyllidiella pustulosa* is a species complex with similarly colored species or subspecies (Stoffels et al. 2016). Therefore, our animals are tentatively assigned to this species, although they group within different clades (unpublished data; see also Stoffels et al. (2016)). Color variation and mimicry appear quite common in Chromodorididae (e.g. Cheney et al. 2016; Padula et al. 2016; Layton et al. 2018; Johnson and Gosliner 2012; Epstein et al. 2019). Thus, identification only by color might lead to errors, and therefore we barcoded these taxa to verify identification by including sequences from our specimens into a preliminary phylogenetic analysis of this family (unpublished data). We could therefore

identify the first mimicry forms within the species *Chromodoris annae* exhibiting the color of *C. elisabethina*.

Phyllidiidae show the highest dominance (three genera represented by 11 species, with 205 specimens) in our study. Of the five valid phyllidiid genera, *Reticulidia* and *Ceratophyllidia* were not present in our study. These genera are also not recorded from BNP, but *Reticulidia halgerda* Brunckhorst and Burn in Brunckhorst was recorded from Ambon (Yonow 2011). The second most commonly recorded group is the family Chromodorididae. Seventeen chromodoridid genera are recorded by WoRMS. In our study three genera are represented by nine species with 35 specimens; therefore, this family is not well represented in our collection. Only one further anthobranch family besides Chromodorididae was found, the hexactinellid sponge-feeding Aegiridae with *Notodoris serenae*. Thus, in total 19 anthobranch species are now recorded from Sangihe, in contrast to the 69 anthobranch species mentioned by Eisenbarth et al. (2018) from BNP.

Interestingly, the number of cladobranchs with only one very tiny unidentified aeolidid species was extremely low, compared to other study areas close by, e.g. Ambon, Bali, Vietnam, Papua New Guinea, Taiwan, and Hong Kong (Table 3.4). According to these studies, usually one-quarter to one-third of collected nudibranchs comprise members of Cladobranchia (Table 3.4). A similar proportion of Anthobranchia to Cladobranchia as seen around Sangihe (20:1) was only recorded from Mauritius (Yonow & Hayward 1991). Eisenbarth et al. (2018), covering the Bunaken National Park, mentioned 28 species of Aeolidioidea and in total 47 cladobranch species, compared to 69 anthobranch species (Table 3.4). The low cladobranch number around Sangihe might be explained by the more sheltered sampling localities with a dominance of algae and sponges, and no hydro-dynamically exposed areas so typical of outer reefs and necessary for their hydrozoan prey. The number of sacoglossan species (3) with 11 collected specimens is also rather low. However, our overall numbers are in line with other studies from Indonesia, which show the general dominance of Nudibranchia and particularly the Anthobranchia, versus all other marine heterobranch groups (Table 3.4). This is also consistent with the overall diversity in these different groups (Wägele 2004; Goodheart et al. 2016). Comparing results from the collecting sites, a few species clearly dominate the various habitats: *Phyllidiella pustulosa* species complex (77 recorded specimens) was collected from all localities. The species has a high number of records (Fig. 3.6B) which

also indicates a very common distribution with high specimens' numbers; however, it has to be emphasized here that the map depicts actually a species complex with several cryptic species looking all very similar to *P. pustulosa*. The second most common species around Sangihe was *Phyllidia varicosa* (58), which is also very common in the Indo-Pacific (Fig. 3.5H). *Phyllidia picta* (21) was also collected from all sites around Sangihe except Ship Wreck. *Phyllidiopsis krempfi* was found only at four sampling sites. With 15 specimens, it was quite common around Sangihe, but this species probably is not so commonly distributed in the Indo-Pacific (Fig. 3.6C). It is also not recorded from BNP. *Chromodoris annae*, *Phyllidia coelestis*, and *Phyllidiella lizae* (13, 9, and 8 specimens respectively) were also found at only four sampling sites. *Phyllidia madangensis* seems to be very rare and our specimen probably represents the only record from Indonesia at the moment (Fig. 3.5F).

In comparison to the study by Eisenbarth et al. (2018) covering the Bunaken National Park (BNP) and including several collection periods between 2015 and 2017, the number of species is much lower (23 versus 172 species) (Fig. 3.7). When including a former collection period in 2003 (Burghardt et al. 2006), the total species number increases to 215 in BNP. Interestingly, we collected seven species that are not yet recorded from BNP (Fig. 3.7), despite the extensive studies around this area. Three of them were very common around Sangihe: *Phyllidia picta* (21 specimens), *Phyllidiella lizae* (12 specimens), and *Phyllidiopsis krempfi* (15 specimens). The other four were less common: *Phyllidia madangensis* (1 specimen), *Phyllidiella nigra* (1 specimen), *Phyllidiopsis shireenae* (2 specimens), and *Plakobranchus* cf. *papua* (2 specimens). An undescribed *Phyllidia* species was also collected, which is not recorded from BNP or any other locality. Since nothing can be said about the affiliation of the small aeolidid, the number might even be nine. Overlap of species when comparing these two areas in North Sulawesi was therefore less than 70%, despite the rather short distance of approximately 200 km.

By comparing our preliminary results on the largest island of the Sangihe Islands Regency, not only with the studies from North Sulawesi, but also with other studies from Indonesia and nearby countries, the overlap of species lies mainly in the most common phyllidiid species, including the *Phyllidiella pustulosa* complex and *Phyllidia varicosa*, as well as the chromodorid *Chromodoris annae*. Sangihe is still heavily under-sampled

and more collecting events are necessary to better understand the marine Heterobranch fauna from this highly remote area. However, differences outlined here between species composition clearly show the distinctiveness of this region from other areas close by. With this first sampling period, we have created the first baseline for future biodiversity studies and monitoring projects, especially with regard to human activities.

Table 3.4. Marine Heterobranch species records of several studies from the Indo-Pacific split into main taxa

	Acteonoidea	Cephalaspidea + Runcinacea	Anaspidea	Sacoglossa	Umbraculida	Pleurobranchomorpha	Anthobranchia	Cladobranchia	Total Species Number	References
Sangihe 2016	0	0	0	3	0	0	19	1	23	This Study
BNP 2015–2017	0	24	4	26	0	2	69	47	172	Eisenbarth et al. 2018
Ambon	0	11	6	12	0	4	90	15	138	Yonow 2001, 2011, 2017; pers. comm. Nathalia Yonow
Bali and Indonesia	3	12	7	11	0	9	128	35	205	Tonozuka 2003
Vietnam	0	11	7	6	1	6	95	25	151	Martynov and Korshunova 2012
Papua New Guinea	0	71	9	61	0	8	257	132	538	Gosliner, 1993
Taiwan	0	2	0	4	0	1	53	10	70	Huang et al. 2015]
Hong Kong	0	0	0	0	0	0	40	14	54	Orr 1981
Chagos Archipelago	0	2	1	2	0	0	30	6	41	Yonow et al 2002
Maldives	0	4	2	2	0	2	21	4	35	Yonow 1994
Marshall Islands	5	13	5	10	0	1	53	14	101	Johnson and Boucher 1983
Lizard Island	4	28	6	21	0	4	66	29	158	Wägele et al. 2006
Mauritius	0	5	5	0	0	2	22	1	35	Yonow and Hayward 1991
Western Australia	7	22	12	21	2	6	115	31	215	Wells and Bryce 1993
Fiji Islands	10	30	6	26	1	6	127	45	251	Brodie and Brodie 1990
New Caledonia	16	82	10	17	1	4	98	30	258	Bouchet et al. 2002
Heron Island	0	20	5	31	0	7	151	47	261	Marshall Willan 1999
Red Sea	7	41	17	16	0	8	140	65	294	Yonow, 2008
Great Barrier Reef	0	64	12	42	0	9	210	77	414	Marshall and Willan 1999

Lakshadweep Islands	1	6	5	9	0	4	27	8	60	Apte 2009
New Caledonia	4	19	12	25	0	11	237	65	373	Hervé 2010
New South Wales	0	35	17	27	2	12	209	80	378	Nimbs *et al.*, 2016
Tropical East Pacific	0	89	13	30	0	11	131	125	399	Bertsch 2010

Authors' Contributions

All authors except N.U. and A.P. were involved in collecting the animals. N.U., A.P., and H.W. analyzed the material; N.U. and H.W. wrote the manuscript and designed the figures; all other authors contributed with comments and corrections to the manuscript. All authors read and approved the final manuscript.

Funding

The study was funded by the Federal Ministry of Education and Research (BMBF) in the frame of the "Biodiversity and Health - from biodiversity to biomedical innovation" program (Indobio, grants 16GW0117K and 16GW0118) to H. Wägele, G.M. König, and T. F. Schäberle. Nani Undap and Adelfia Papu have grants provided by the German Academic Exchange System (DAAD).

Acknowledgments

The authors thank the Republic of Indonesia for making this research possible. We are very grateful to staff members of the Politeknik Nusa Utara, Sangihe, Indonesia who supported and helped in the fieldwork. All material was collected in accordance to the Nagoya Protocol, with all necessary permits and ABS papers provided by the director of the Politeknik Nusa Utara, Tahuna, Sangihe Islands Regency, North Sulawesi, Indonesia. No material collected during this study is listed in IUCN or CITES. Special thanks go to Katja Fisch (Bonn) helping in preparing the specimens after collection and Claudia Etzbauer (Bonn) for her support in the molecular laboratory. We thank Morris Flecks (Bonn) for assistance in using the ArcGIS program and Birgit Klasen for her support in data management in the program Diversity Workbench. We also want to thank two anonymous reviewers, as well as Nathalie Yonow (Swansea), who provided many valuable comments, and improved the English.

Conflicts of Interest

The authors declare that they have no conflict of interests.

Availability of data and materials

The material was made available by the late Fontje Kaligis. Some material is used for further studies within the project funded by the Federal Ministry of Education and Research, Germany. Metadata of each individual is documented in the database Diversity Collection (Part of Diversity Workbench) using the data brokerage service of the German Federation for Biological Data (GFBio) (Diepenbroek et al. 2014). Data are publicly available at www.gfbio.org for browsing and the archived data can be downloaded at https://doi.org/10.20363/heterobranchia-sangihe-prj-1.1. Photographs are available from Heike Wägele with copyright from Zoological Research Museum Alexander Koenig, Bonn. Material not used for further studies will be stored in the Sam Ratulangi University collection under the number SRU2016/01. Sequences are uploaded to NCBI.

Chapter 4

Biodiversity of Chromodorididae (Anthobranchia, Nudibranchia) around North Sulawesi

Introduction

Marine Heterobranchia are represented in North Sulawesi in great numbers, as has been shown recently by Kaligis et al. (2018), Eisenbarth et al. (2018), Ompi et al. (2019), Undap et al. (2019), and Papu et al. (2020). During these studies the diversity of certain Nudibranchia groups became very obvious. Especially the Phyllidiidae and Chromodorididae belong to the most common taxa not only in North Sulawesi, but also in the tropical and subtropical Indo-Pacific (Gosliner 2018).

Edmunds (1981) stated that the Chromodorididae are among the most gorgeously colored of all animals and they occur in greatest diversity in tropical reefs. They are not only of interest for divers, but also for many scientist, because they are famous for their secondary metabolites, which they mainly obtain from sponges, their major food item, and which they use as a defense system (Cimino & Ghiselin, 1999; Gavagnin & Fontana, 2000; Cheney et al. 2016; Böhringer et al. 2017; Fisch et al. 2017).

At least over 300 species are described in the family Chromodorididae, and it is thought that there are many more species yet to be discovered (Turner & Wilson, 2007). Color patterns have traditionally been used for species identification in Chromodorididae, which in general have a rather smooth mantle surface, only occasionally with small low tubercles (Edmunds, 1981; Turner & Wilson 2008; Johnson & Gosliner 2012; Layton et al. 2018). Recent work however has shown that color patterns in chromodorids can be identical, and conversely different color patterns can exist within a species (e.g. Almada et al. 2016; Padula et al. 2016). These findings suggest that the color patterns may be more unreliable than expected to identify the species in chromodorids (Layton et al. 2018).

Former taxonomic revisions of the family Chromodorididae based on morphological data (Rudman 1984; Gosliner & Johnson 1999). Their taxonomic work was recently revised by Johnson & Gosliner (2012) using molecular data (CO1 and 16S). Several paraphyletic clades were split and new subclades identified or named. Gosliner & Johnson (1999) also revised the phylogeny of the worldwide genus *Hypselodoris*, including some of the species that are more abundant in the northeastern Atlantic and Mediterranean Sea. These revisions were followed by a number of morphological (Alejandrino & Valdés 2006) and molecular studies (Alejandrino & Valdés 2006; Wollscheid-Lengeling et al. 2001; Turner & Wilson 2008; Johnson 2011), including the description of several new species (Dacosta et al. 2010; Ortigosa & Valdés 2012; Epstein et al. 2018).

Especially the genus *Chromodoris* Alder & Hancock 1855, was redefined by removing monophyletic groups into other new genera (Rudman 1982) (e.g. *Felimare* and *Felimida*). the genus is now estimated to contain approximately 200 species with up to 22% of known species still not described (Gosliner & Draheim 1996). Furthermore, *Chromodoris* is the most speciose genus in the nudibranch family Chromodorididae (Johnson & Gosliner 2012), and has a cosmopolitan distribution with the greatest diversity occurring at low latitudes (Wilson & Lee 2005).

Color patterns were also used to describe and identify *Chromodoris* species (Rudman 1983, 1985, 1987), with no expectation that these groupings reflected phylogenetic relationships (Gosliner & Behrens 1998; Wilson 2002).

At the moment 16 genera are recognized within the family according to the World Register of Marine Species (WoRMS), with *Chromodoris* still the largest group (Johnson & Gosliner 2012) (Fig. 4.1). Many of the genera are represented in North Sulawesi with several species and one aim of this study is the analysis of the Chromodorididae diversity in this hot spot of diversity.

Many chromodorid species have overlapping distributional ranges and similar colour patterns, which can confound species identifications (Rudman 1982). Therefore an extensive barcoding study was performed to investigate chromodoridid diversity in Bunaken National Park, Sangihe Island and Bangka Archipelago.

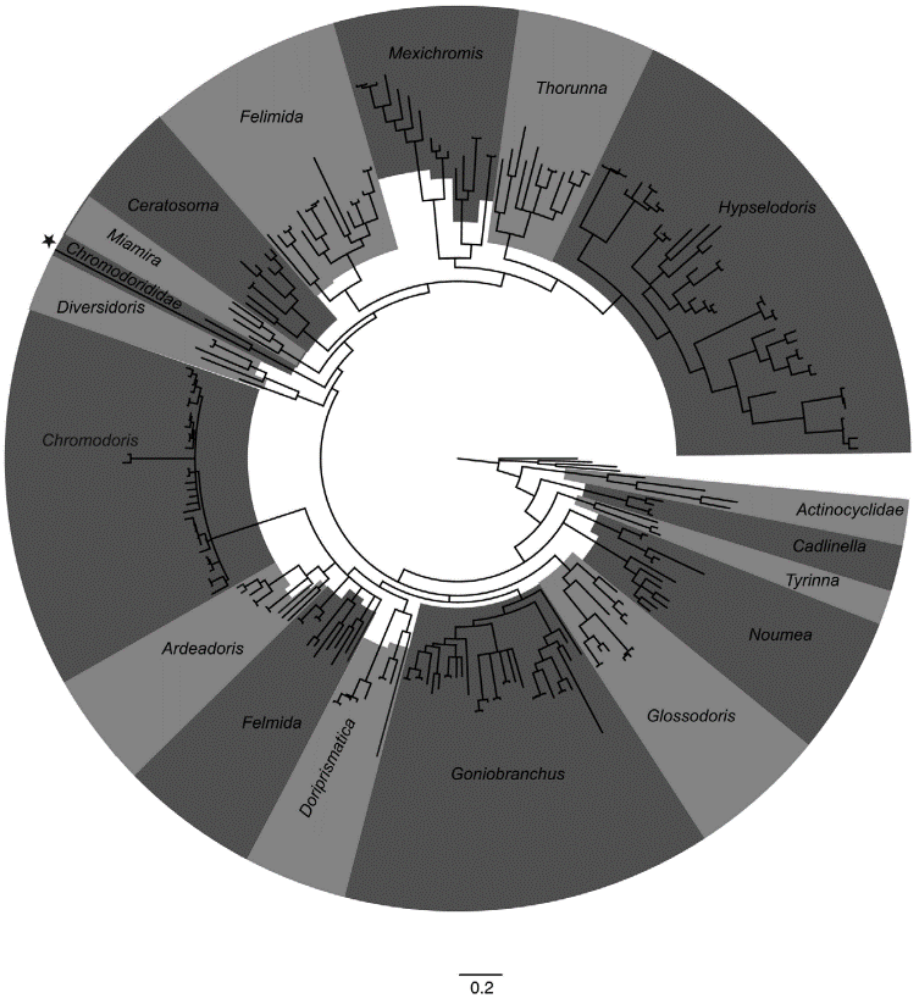

Figure 4.1 16S and CO1 of the Chromodorididae from Johnson & Gosliner (2012).

Materials and Methods

Collection of specimens

For this study, the specimens were collected around North Sulawesi (Bunaken National Park (BNP), the island Sangihe, and around Bangka Island (Fig. 4.2) in the years 2015, 2016 and 2017. Specimens were photo-documented in the field on the original substrate before being collected individually by snorkeling or scuba diving. Subsequently the animals were photo documented in the laboratory and the whole animal or at least a small piece of the foot was preserved in 96% alcohol for barcoding.

Figure 4.2 Details on North Sulawesi with collection (see also Table 4.1).

DNA extraction and amplification

DNA was extracted using the QIAgen® DNeasy Blood and Tissue-Kit (QIagen, Hilden, Germany), following the manufacturer's instructions and stored in 96% ethanol at -20°C. The samples were taken from preserved foot tissue. Fragments of CO1 and 16S were amplified for all collected specimens. Information about the processing of the extracted DNA is provided in chapter 2. PCR products were sequenced by Macrogen Europe Laboratory (Amsterdam, Netherlands).

Sequence analysis and alignment

The software GENEIOUS Pro 7.1.9 (Biomatters Ltd., Auckland, New Zealand) was used to extract the consensus sequence between the primer regions, and to construct the final alignments. To check if the correct genes have been amplified, and to uncover contamination, BLAST searches (Altschul et al. 1990) were performed to compare the amplified sequence with all sequences stored in the GenBank database (www.ncbi.nlm.nih.gov/Genbank/index.html). Subsequently all available sequences (mitochondrial CO1 and 16S) of chromodorids were downloaded and added to the sequences obtained in this study. A critical step of sequence-based phylogenetic analyses is the alignment of the data. Given that positions with a common ancestry have to be compared for reliable phylogenetic conclusions, homologous positions have to be arranged in common columns in correct alignment. Sequences were edited and aligned first separately for the 2 genes by using MAFFT (Katoh et al. 2002) in Geneious 7.1.9. The two complete alignments for CO1 and 16S including all available chromodorid sequences from NCBI (Alignment 1 with 644 sequences, 180 taxa, 600 bp, Alignment 2 with 718 sequences, 221 taxa, 542bp, respectively), were analysed separately. Subsequently a third alignment was created by concatenating my own sequences and combining them with a selection of sequences from NCBI (Alignment 3 with 462 sequences, 38 taxa and a length of 1216 bp). This Alignment 3, which comprises all chromodoridid genera, is used for the subsequent tree analysis. *Halgerda batangas* was selected as an outgroup based on the most recent molecular phylogeny of Chromodorididae (Padula et al. 2016; Layton et al. 2018) and these sequences were obtained from GenBank.

Tree reconstruction

The dataset was analysed in IQ-TREE web server for subsequent phylogenetic analysis, with following settings per default: substitution model set Auto; 1000 ultrafast bootstrap; SH-aLRT branch test (Trifinopoulos et al. 2016). The tree file was imported into Dendroscope v 3.5.9 to interpret results and subsequently in Figtree v 1.4.3 to collapse nodes with less than 60% support. The tree was further processed in Inkscape v 0.92.3 for graphical lay out.

Results

In this study, 375 specimens of Chromodorididae were collected in North Sulawesi waters. According to the tree reconstructed by using the concatenated Alignment 3 (16S and CO1) (Fig. 4.4), the specimens can be assigned to 26 species (Table 4.1). This is nearly 10 % of the known 300 species worldwide (Johnson & Gosliner 2012). They cover 9 out of the 16 recognized genera: *Ceratosoma* (2), *Chromodoris* (5), *Doriprismatica* (2), *Glossodoris* (2), *Goniobranchus* (4), *Hypselodoris* (6), *Miamira* (1), *Verconia* (2), and *Thorunna* (2). Similar results with regard to species assignment were obtained in the preliminary analyses of the separated genes and the full data sets (Alignment 1 and 2). Table 4.1 provides an overview of the species and the number of specimens collected in the three different areas. Figure 4.3 provides graphic information about the amount of specimens per species in comparison to the locality. Sangihe showed the lowest species number (7) and Bunaken the highest (19). Whereas the chromodorids collected around Sangihe are also found in Bunaken and Bangka, eight species were only collected around Bunaken (i.e. *Ceratosoma* spec., *Chromodoris willani, Doriprismatica stellate, Glossodoris hikuerensis, Miamira sinuata, Thorunna australis, Thorunna furtiva, Verconia* spec.) and seven species were only collected around Bangka (i.e. *Ceratosoma tenue, Doriprismatica atromarginata, Goniobranchus coi, Hypselodoris cerisae, H. lacuna, Hypselodoris maridadilus* and *Verconia simplex*).

According to the phylogeny with the reduced data set (Fig. 4.4), most genera are monophyletic, with the exception of *Goniobranchus*. The Mediterranean species *Felimida luteorosea* is clustering with the four represented *Goniobranchus* species.

Most species are represented as monophyletic clades, with two exceptions: *Chromodoris dianae* clusters as a paraphyletic group with *C. willani* nesting in between (Fig. 4.3). *Hypselodoris maculosa* is paraphyletic, with *H. tryoni* clustering in between. In the following, the genera are presented in more detail.

Table 4.1 Number of Chromodorid were collected in BNP, Sangihe, Bangka & Lembeh 2015-2017

Species Name	Number of Specimens	Bunaken 2015-2017	Sangihe 2016	Bangka 2017	Lembeh 2018	Size (mm)
Chromodoris annae Bergh, 1877	116	84	13	19	4	4-50
Chromodoris spec.	1	1				20
Chromodoris strigata Rudman, 1982	5	3	1	1		10-25
Chromodoris dianae Gosliner & Behrens, 1998	80	73	7			5-50
Chromodoris willani Rudman, 1982	40	40				18-70
Chromodoris lochi Rudman, 1982	43	38		5	2	15-50
Goniobranchus geometricus (Risbec, 1928)	11	6	4	1	3	6-40
Goniobranchus coi (Risbec, 1956)	2			2		30,40
Goniobranchus reticulatus (Quoy & Gaimard, 1832)	3	1	2		2	25-75
Goniobranchus fidelis (Kelaart, 1858)	2	1		1	1	13,16
Doriprismatica stellata (Rudman, 1986)	16	16				2.3-65
Doriprismatica atromarginata (Cuvier, 1804)	3			3		17-85
Glossodoris cf. *cincta* (Bergh, 1888)	11	8	2		1	20-60
Hypselodoris tryoni (Garret, 1873)	7	2	3	1	1	25-60
Hypselodoris maculosa (Pease, 1871)	6	3		3		4-23
Hypselodoris apolegma (Yonow, 2001)	2	1		1		33,70
Hypselodoris lacuna Gosliner & Johnson, 2018	2			2		6,8
Hypselodoris cerisae Gosliner and Johnson, 2018	1			1		16
Thorunna australis (Risbec, 1928)	1	1				17
Thorunna furtiva Bergh, 1878	1	1				10
Miamira sinuata (van Hasselt, 1824)	1	1				12
Ceratosoma spec.	4	4				4-12
Ceratosoma tenue Abraham, 1876	1			1		10
Verconia simplex (Pease, 1871)	1			1		6
Verconia spec.	1	1				4
Total	375	285	32	42	16	

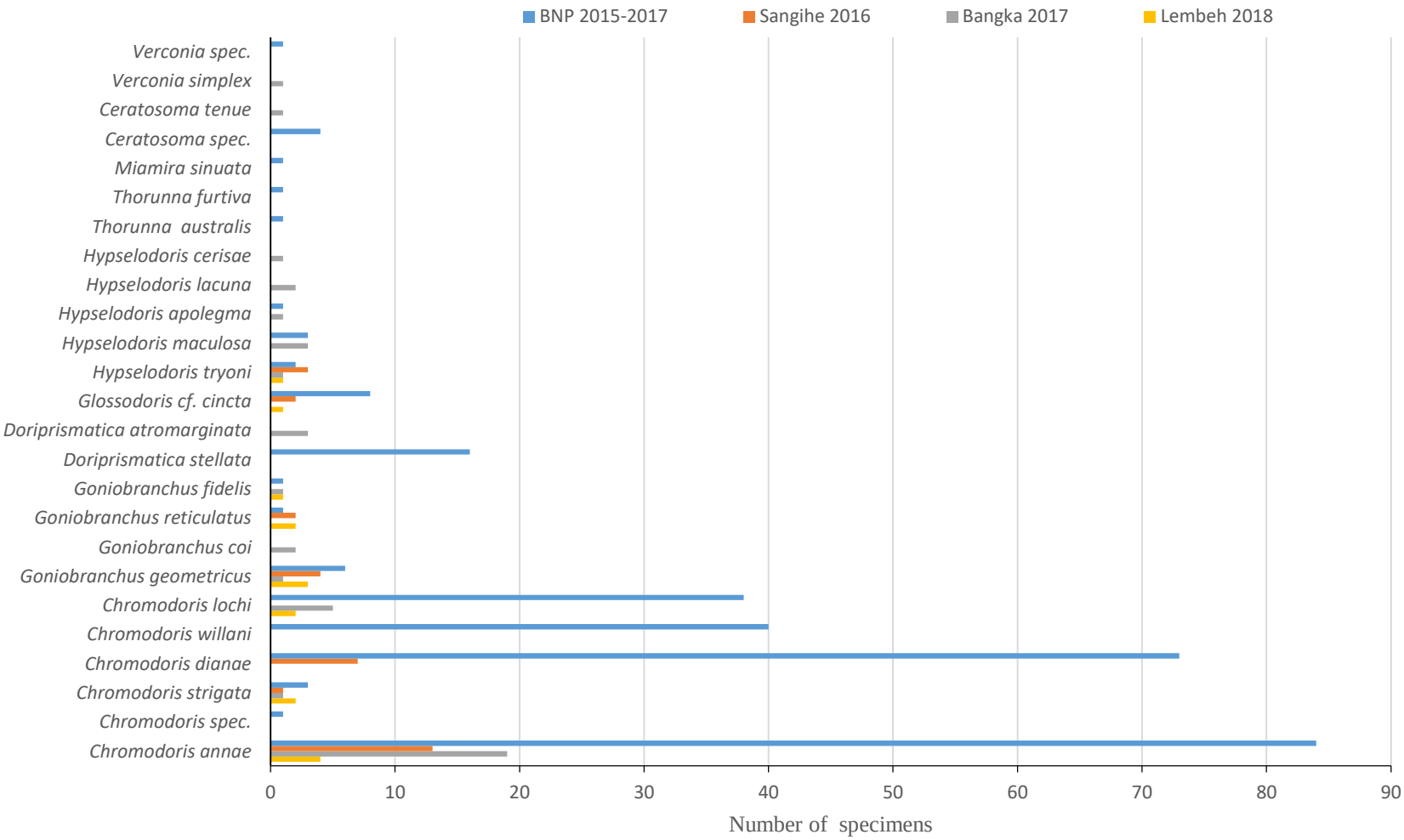

Figure 4.3 Comparison of Chromodorid species collected around Bunaken (BNP), Sangihe, Bangka and Lembeh

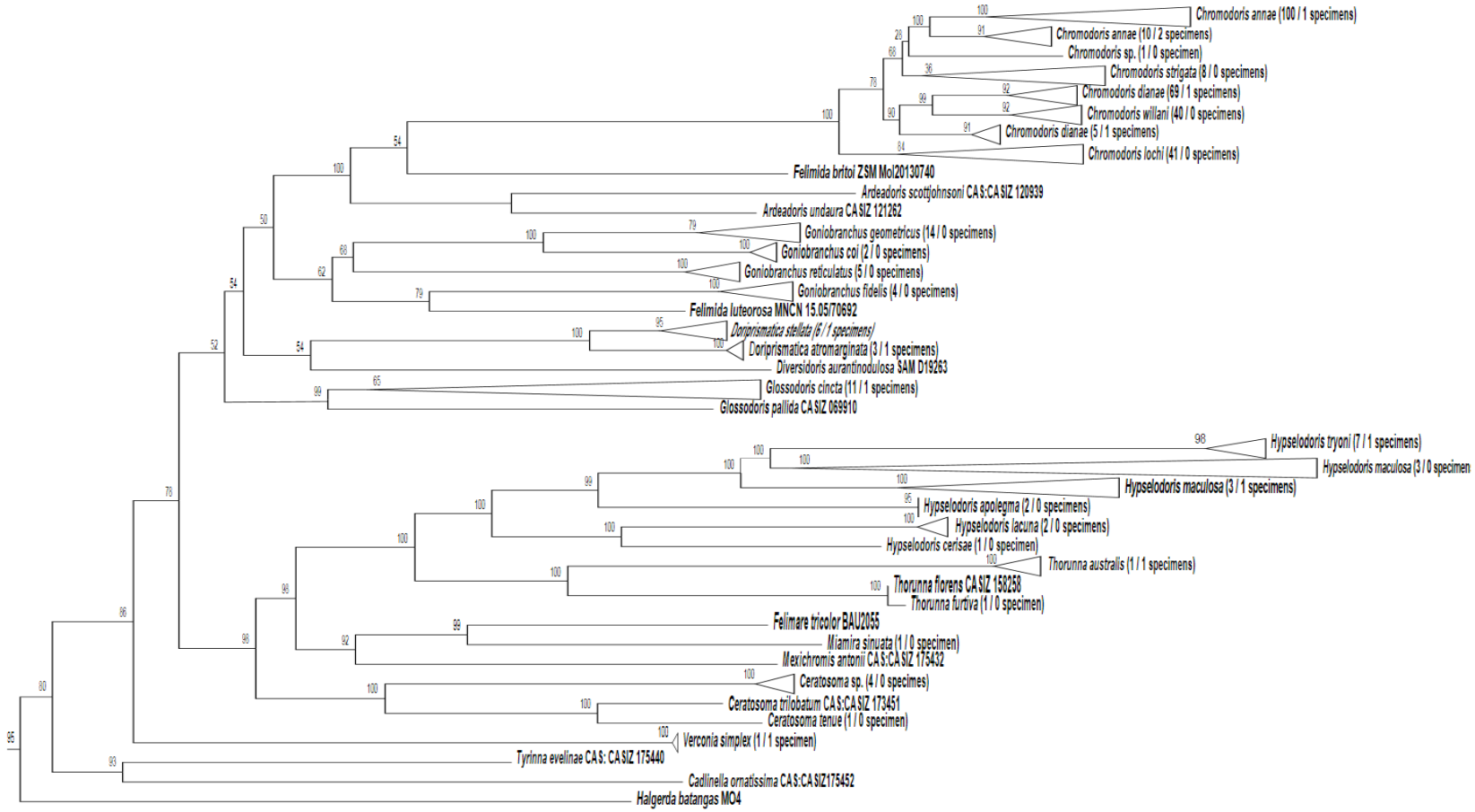

Figure 4.4 Concatenated 16S and CO1 genes. The first number in brackets indicate the number of specimens from this study, and the second, the number of specimens retrieved from NCBI

Chromodoris

The genus *Chromodoris* contains eight clades in this study, namely two clades of *Chromodoris annae*, *Chromodoris* sp., *Chromodoris strigata*, two clades of *Chromodoris dianae*, *Chromodoris willani*, and *Chromodoris lochi* (Fig. 4.5).

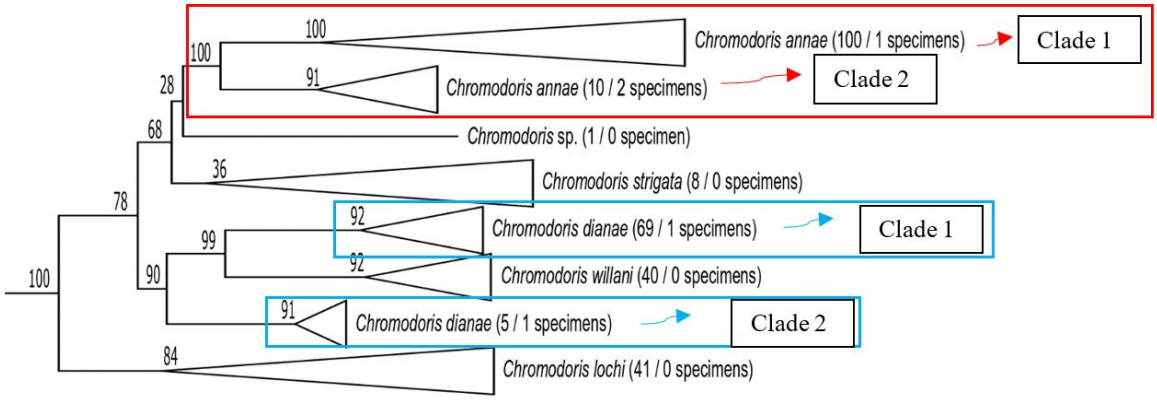

Figure 4.5. Clade *Chomodoris*.

Chomodoris annae

According to our results, there are 2 clades of *Chromodoris annae*. Clade 1 comprises the majority of collected specimens (100), clade 2 only 10 specimens. However, species delimitation tests need to be performed, to confirm the distinctiveness of the second clade. When analyzing my data together with available CO1 and/or 16S data from NCBI, three sequences retrieved from Genbank cluster with clade 1, and five sequences with clade 2, when applying the two genes in separate analyses. Chapter 5 will provide more details about *Chromodoris annae* in North Sulawesi and further localities.

Within clade 1 there is one specimen from Sangihe that was preliminarily identified as *Chromodoris elisabethina* (Fig. 4.6 B), but could be assigned to *Chromodoris annae* after barcoding. This *Chromodoris annae* specimen is mimicking *Chromodoris elisabethina* (Undap et al. 2019). A second specimen that shows aberrant color patterns and was first identified as a new *Chromodoris* species, could also be identified by barcoding as a *C. annae*.

It mimics a color morph of *Chromodoris lochi*. This is the first time that this phenomenon is described from Indonesian *Chromodoris* species.

Figure 4.6 *Chomodoris annae*: (A) typically color form of *Chomodoris annae*, Chan17Ba-13; (B) Specimen Chel16Sa-1 from Sangihe Island, misidentified first as *C. elisabethina*; (C) Chsp30_16Bu-1 identified as an unknown *Chromodoris* species. This color morph is typical for *C. lochi*.

Chromodoris spec.

One specimen from Bunaken National Park (BNP) was collected in 2016 that was preliminarily identified as a *Goniobranchus* sp. It groups as sister taxon to the *Chromodoris annae* clades (Fig. 4.7). This is unusual, because it does not have the typical blue, yellow and white coloration of the genus *Chromodoris*. Blasting the sequences against NCBI data base resulted in a low similarity to *Chromodoris quadricolor* with 96% (16S) and *Chromodoris colemani* 92% (CO1). In my separate tree analyses based on CO1 and 16S, they cluster also in both studies as sister taxon to *C. annae*. Further investigations are needed here to clarify this undescribed *Chromodoris* species, or, in case of contamination, its correct assignment, or whether it is another case of mimicry.

Figure 4.7 Gosp40_16Bu-1 identified preliminarily as a member of the genus *Goniobranchus*.

Chromodoris strigata

Eight specimens of *Chromodoris strigata* were collected in BNP (3 specimens), Sangihe (1 specimen), and Bangka Island (1 specimen). Additionally, 3 specimens were investigated from Lembeh Strait (see also Ompi et al. 2019). The species can easily be misidentified as *C. michaeli*. However, *C. michaeli* lacks the patches which look like shadow, a color pattern typical for *C. strigata*. Figure 4.8 show two specimens, one of them originally misidentified as *C. michaeli*.

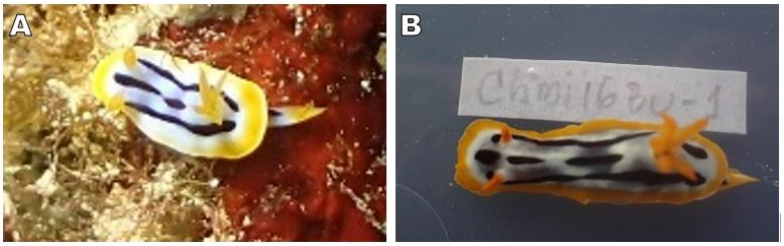

Figure 4.8 (A) *Chromodoris strigata*; (B) Chmi16Bu-1 misidentified preliminarily as *C. michaeli.*

Chromodoris dianae

According to our phylogenetic analysis *Chromodoris dianae* divides into two clades, which are not sister taxa, but are separated by the clade *Chromodoris willani*. The five sequences retrieved from NCBI cluster within the Clade 2 according to the separate gene analysis. None of my animals cluster within this clade, thus it is only composed of sequences

retrieved from GenBank. *Chromodoris dianae* has a distinct color pattern that differs from all other *Chromodoris* species. Therefore, the result is astonishing.

In Clade 1, which comprises only my sequences, three of the 69 specimens are preliminarily identified as *C. annae*. All three were collected around BNP. Genetic information indicates no relationship to *C. annae*, but to *C. dianae*; unfortunately, there are no pictures to confirm assignment of these specimens also by color pattern. Therefore we cannot say, whether this is again a form of mimicry of *C. dianae* specimens with *C. annae*.

One specimen was labeled wrong. It was collected from BNP, and depicted with a label of a *C. annae* (Chan15Bu-28, Fig. 4.9 B); however, according to barcoding and the result from the tree the specimen is confirmed as *C. dianae* (see Fig. 4.9). Also the available picture of this particular specimen shows a color pattern typical for *C. dianae* and thus confirms that labeling in the lab was wrong. For this particular animal, correct metadata are therefore not available.

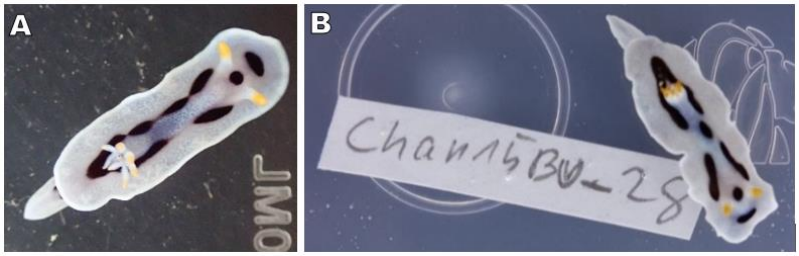

Figure 4.9 (A) *Chromodoris dianae*, Chdi17Bu-1; (B) wrong label assigned to one *C. dianae* specimen.

Chromodoris willani

Out of 40 specimens, only 30 could be successfully barcoded. They cluster in one clade. The typical color patterns, especially the sparkling white dots along the gills and rhinophores allow already the correct assignment to *Chromodoris willani* (Fig. 4.10). The reason, why 10 specimens could not be barcoded is not clear.

Figure 4.10. *Chromodoris willani*, Chwi15Bu-8.

Chromodoris lochi

Forty-three specimens were collected from BNP (38) and Bangka Island (5). Two different color morphs were represented. One showed the typical rose colored rhinophores and gills (Fig. 4.11 A). Another morph exhibited more yellow rhinophores and gills (Fig. 4.11 B, C & D). In the preliminary identification, the latter were considered as an undescribed species; however, molecular data clearly cluster these specimens (26 in number) with the typical color morph (15 specimens).

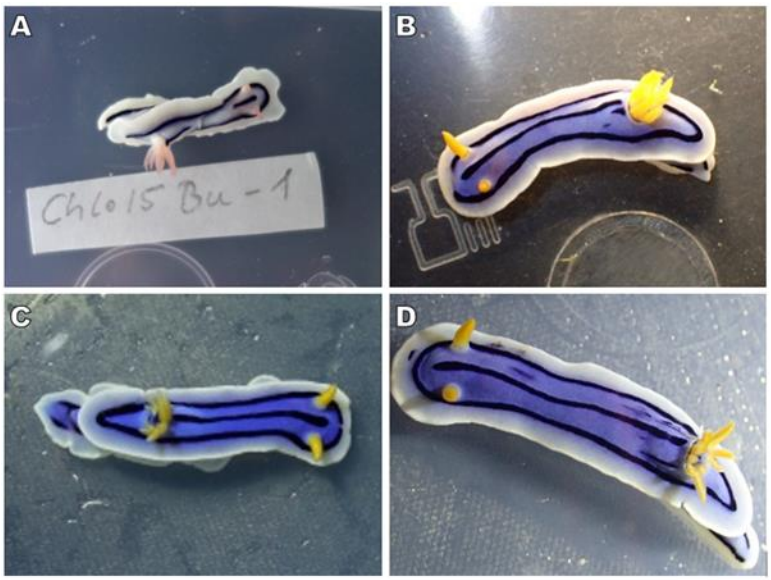

Figure 4.11 *Chromodoris lochi*: (A) Chlo15BU-1; (B) Chlo17Ba-5; (C) Chsp30_15BU-22 preliminarily identified as a new species; (D) Chsp30_16Bu-2 preliminarily identified as a new species.

Goniobranchus

The genus *Goniobranchus* comprises 4 clades (Fig. 4.12) in our study, namely *Goniobranchus geometricus* (Risbec 1928), *Goniobranchus coi* (Risbec 1956), *Goniobranchus reticulatus* (Quoy & Gaimard 1832), and *Goniobranchus fidelis* (Kelaart 1858) (Fig 4.13). The distinct color patterns of these species already allowed correct assignment, and barcoding confirmed the preliminary results.

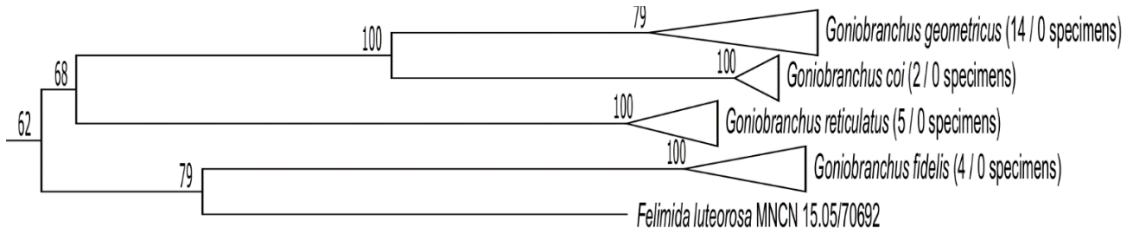

Figure 4.12 Clade *Goniobranchus*.

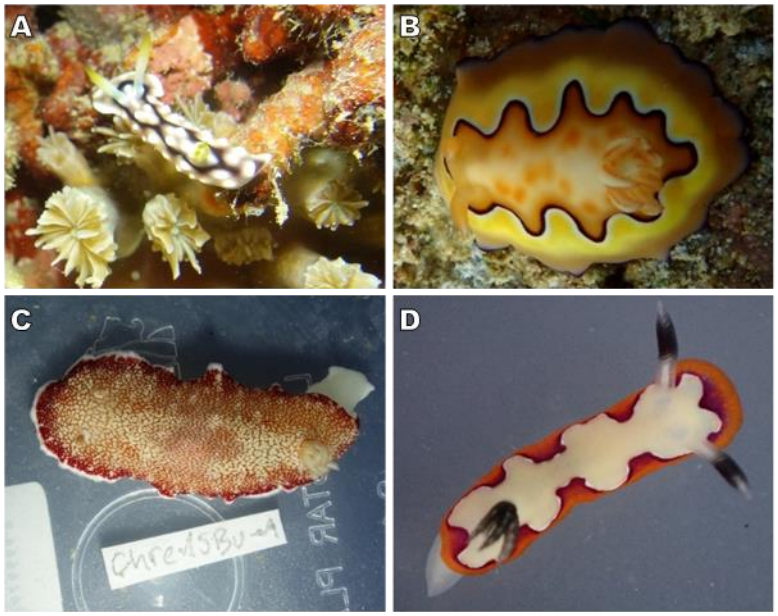

Figure 4.13 (A) *Goniobranchus geometricus* (Risbec 1928); (B) *Goniobranchus coi* (Risbec 1956); (C) *Goniobranchus reticulatus* (Quoy & Gaimard 1832); *Goniobranchus fidelis* (Kelaart 1858).

Doriprismatica

Nine specimens of *Doriprismatica* were collected from Bunaken, comprising two species, namely *Doriprismatica stellata* (Rudman 1986), and *Doriprismatica atromarginata* (Cuvier 1804) (Fig. 4.15). The phylogenetic tree confirms the presence of two species; they are separated in two distinct clades (Fig. 4.14). One specimen was preliminarily identified as *D. sibogae*. The species is very similar to *D. atromarginata*, but the rhinophores and gills have a cream colored rhachis, which is black in *D. atromarginata*. Our specimen showed actually the typical coloration of *D. sibogae*. Morphological characters that differentiate these two species is the much smaller radula of *D. sibogae* (W. B. Rudman 1986). More analyses are actually needed to clearly distinguish these two species.

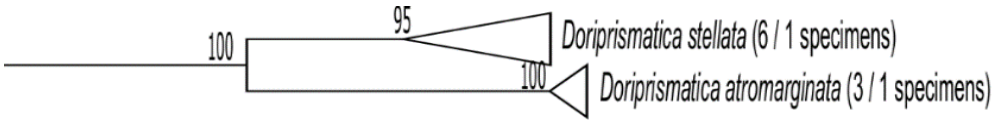

Figure 4.14 Clade *Doriprismatica*.

Figure 4.15 *Doriprismatica*: (A) *Doriprismatica stellata*, Dost16Bu-8; (B) Dosi17Ba-1 specimen preliminarily identified as *Doriprismatica sibogae* based on the cream colored gill rhachis. Molecular barcoding allows assignment to *D. atromarginata*; (C) *Doriprismatica atromarginata*, Doat17Ba-1 with typical dark coloration of gills.

Glossodoris

Eleven specimens could be assigned to *Glossodoris* cf. *cincta.* They were collected from Bunaken (9), Sangihe (2) and Lembeh (1) (Fig. 4.17) and form the sister group to

Glossodoris pallida (from GenBank) (Fig. 4.16). Unfortunately, no sequences from *Glossodoris cincta* are available from GenBank, and there are several other species looking similar to *G. cincta*, e.g. the most recently described *G. acosti* (Matsuda & Gosliner, 2018). The differences are mainly in the color of the outer rim of the mantle. Future analyses are needed when the sequences of the new species are available to confirm valid assignment of our specimens.

Figure 4.16 Clade *Glossodoris*.

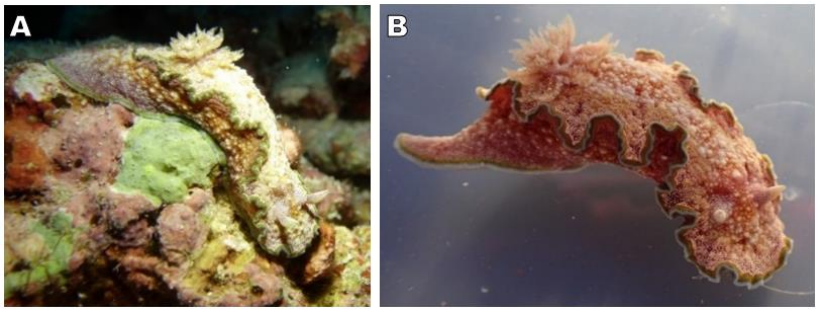

Figure 4.17 *Glossodoris cf. cincta* (Bergh, 1888), Glci16Bu2.

Hypselodoris

Eighteen specimens of *Hypselodoris* were collected from Bunaken, Bangka Archipelago, Sangihe Island and Lembeh Strait, which were assigned to following five species, namely *Hypselodoris tryoni*, *H. maculosa*, *H. apolegma*, *H. lacuna*, and *H. cerisae* (Fig. 4.19). However, the phylogenetic tree reveals that *Hypselodoris maculosa* can be divided in two distinct clades, separated by the species *H. tryoni*. Three from NCBI retrieved sequences of CO1/or 16S cluster with clade one (Fig. 4.18). *Hypselodoris tryoni* was misidentified after collecting several times and assigned e.g. to *Goniobranchus kuniei*, or *G. cavae*. These two *Goniobranchus* species show a similar pattern of dark dots with a white

rim in overall brownish back ground coloration. However, both *Goniobranchus* species have a much more distinct dark violet stained mantle rim, as has *H. tryoni*.

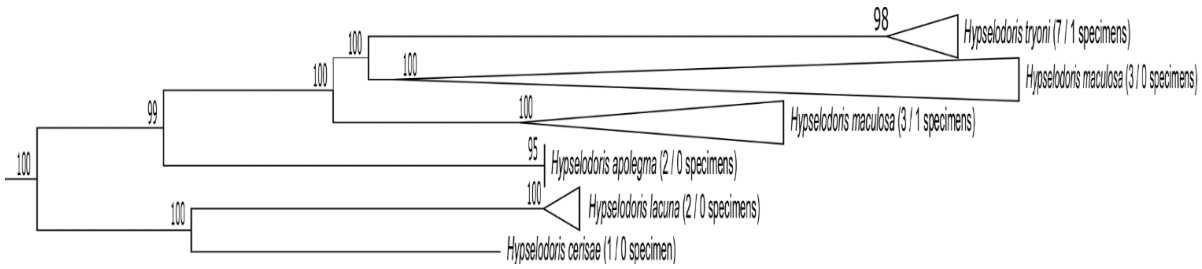

Figure 4.18 Clade *Hypselodoris*.

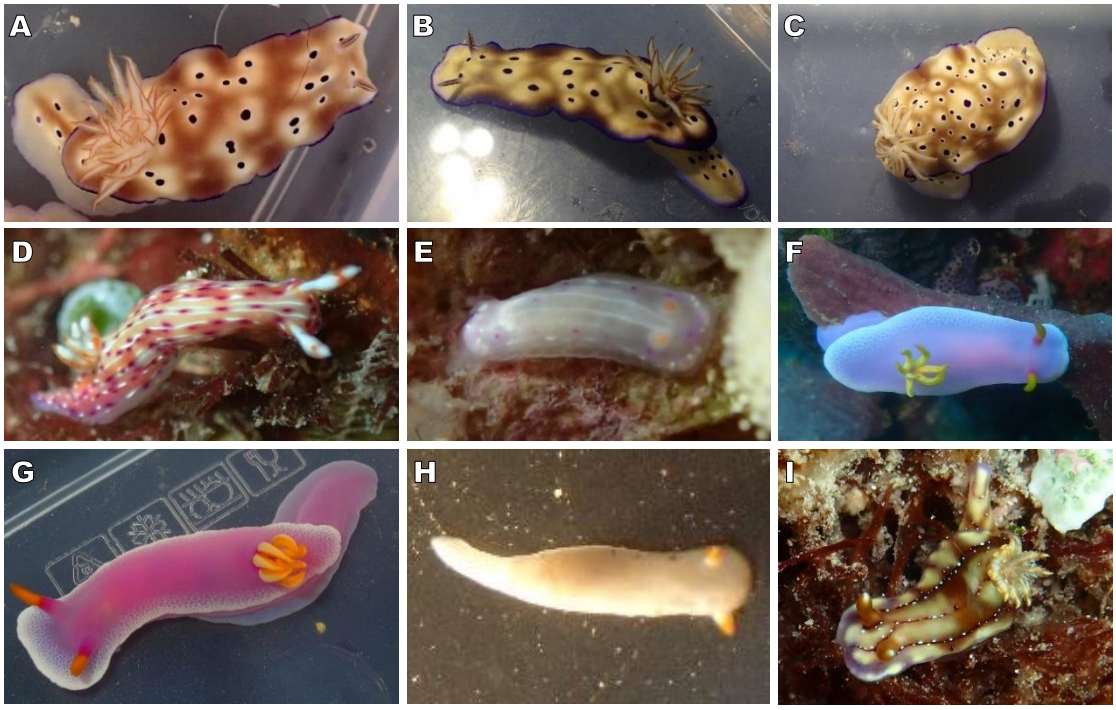

Figure 4.19 *Hypselodoris*: (A) *Hypselodoris tryoni*; (B) Goku16Sa-1 was preliminarily identified as *Goniobranchus kuniei*, but barcoding verified its correct assignment as *H. tryoni*; (C) Goca16Sa-1 identified as *H. tryoni*; (D) *Hypselodoris maculosa*, Hyma17Ba-1; (E) Hysp2_16Bu-1 identified as *H. maculosa*; (F) *Hypselodoris apolegma*, Hyap17Ba-1; (G) Hybu16Bu-1 identified as *H. apolegma;* (H) Hysp19_17Ba-1 identified as *Hypselodoris lacuna*; (I) Hysp1_17Ba-1 could not be assigned first to a certain species, but was subsequently identified as *Hypselodoris cerisae*.

Thorunna

There are two specimens of *Thorunna* which were collected from Bunaken, comprising two species, namely *Thorunna australis* and *Thorunna furtiva* (Fig. 4.21). In the phylogenetic tree they are separate in two clades (Fig. 4.20) and *T. furtiva* forms the sister clade to *T. florens*, a sequence retrieved from NCBI. There is nearly no genetic difference between *T. florens* and our *T. furtiva*. But *T. florens* is characterized by a violet ground color with orange rhinophores and gills. Our specimen of *T. furtiva* (Fig. 4.21 B) shows the typical white color with a yellowish mantle rim.

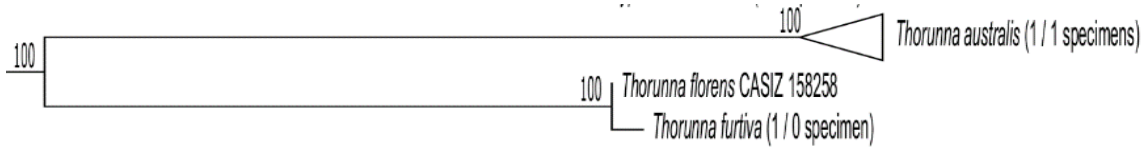

Figure 4.20 Clade *Thorunna*.

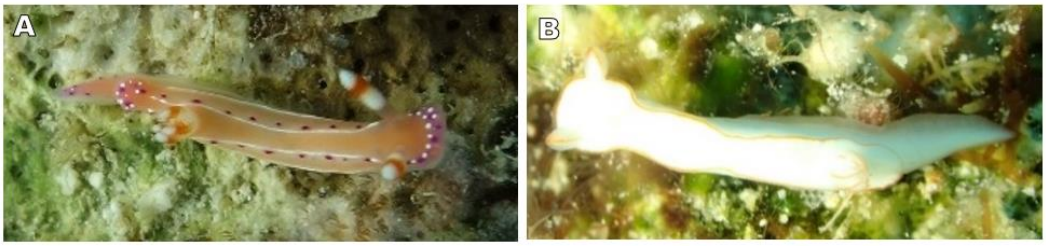

Figure 4.21 (A) *Thorunna australis*, Thau15Bu-1; (B) *Thorunna furtiva*, Thfu16Bu-1.

Miamira

In this study only one specimen of *Miamira sinuata was* collected from Bunaken (Fig. 4.23). In our analysis, which however only included the CO1 sequence of this specimen, it clusters with *Felimare tricolor* from the Mediterranean Sea (Fig. 4.22).

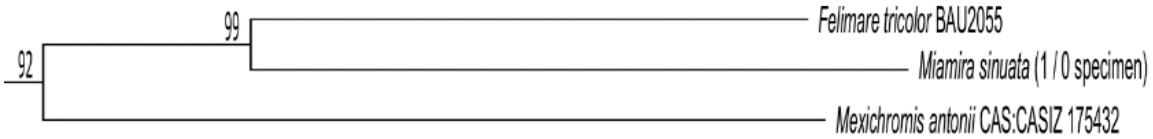

Figure 4.22 Clade *Miamira*.

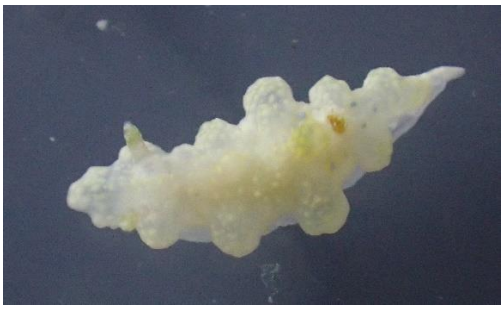

Figure 4.23 *Miamira sinuata,* Misi16Bu-1.

Ceratosoma

Five specimens of *Ceratosoma* were collected from Bunaken (4) and Bangka (1), comprising two species. *Ceratosoma* sp. (4 specimens), could not be assigned to any described *Ceratosoma* species yet. One specimen of *Ceratosoma tenue* (Figs. 4.24 & 4.25) was collected, which was preliminarily identified as a member of *Miamira*. It seems to be a juvenile, because the coloration differs from the color of adult specimens (Gosliner et al. 2018).

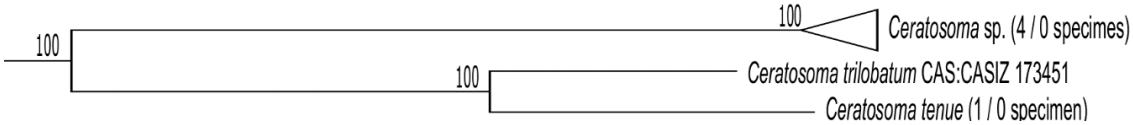

Figure 4.24 Clade *Ceratosoma*.

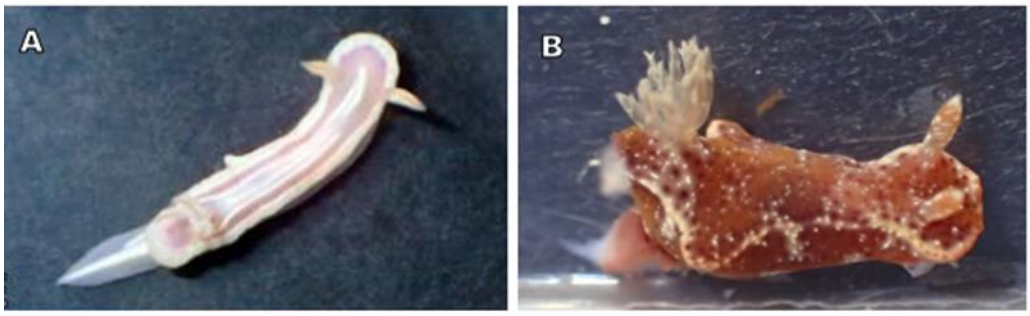

Figure 4.25 *Ceratosoma*: (A) *Ceratosoma* sp., Cesp1_17Bu-1; 1 (B) *Ceratosoma tenue*. (Misp17Ba-1).

Verconia

In this study only one specimen of *Verconia simplex* was collected from Bangka (Fig. 4.27). In our tree *Verconia* is sister taxon to all chromodorid genera (Fig. 4.26), with only *Tyrinna* and *Cadlinella* being more basal. The latter genera were not represented in my collection; however, I used one sequence each retrieved from NCBI.

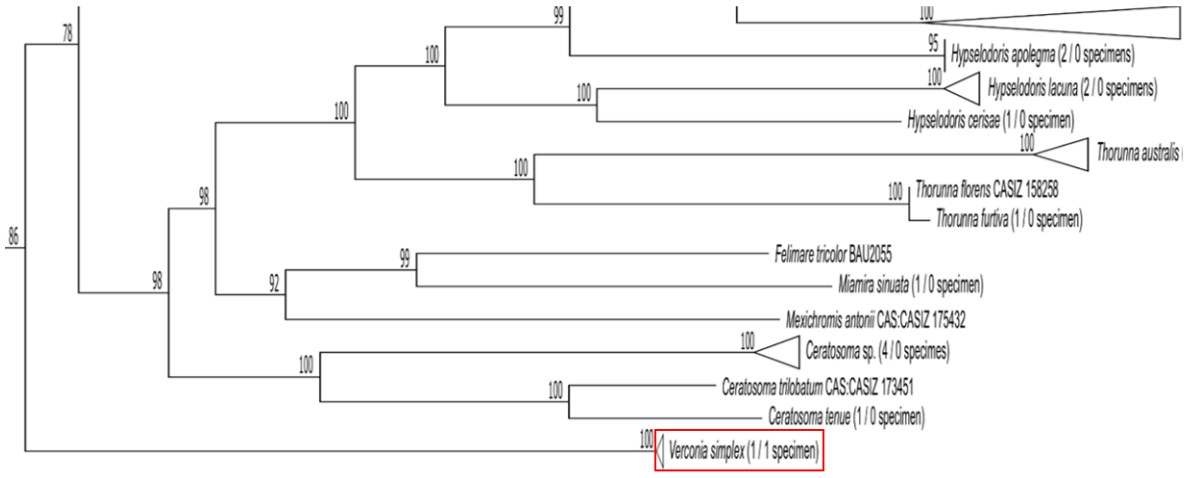

Figure 4.26 Clade *Verconia*.

Figure 4.27 *Verconia simplex*, Nosi17Ba-1.

Discussion

Cryptic species are a common phenomenon throughout the metazoan taxa, and can be found in all sorts of habitats and biogeographic zones (Bickford et al. 2007; Pfenninger & Schwenk 2007; Trontelj & Fiser 2009). Uncovering these cryptic species is fundamental for the understanding of evolutionary processes, historical biogeography, ecology, and also for conservation approaches. Cryptic speciation has an impact on the distribution ranges, which are smaller than initially assumed and thus, species are on a higher risk of local extinction (Bickford et al. 2007; Trontelj & Fiser 2009; Neusser et al. 2011). The lack of morphological characters to distinguish cryptic species should not lead to considerable parts of biological diversity remaining unaddressed (Jörger & Schrödl 2011).

Chromodorididae usually have very distinct color forms and can therefore quite well be identified. However, there are sometimes unforeseen problems, including cryptic speciation, but also ontogenetic color variability, where barcoding can help to clarify identification. First of all, the results show that within *Chromodoris annae*, cryptic speciation is occurring. This phenomenon is also known from other nudibranchs, some are listed here according to the date of description: E.g. *Doto*, Dotoidae (Morrow et al. 1992); *Hypselodoris*, Chromodorididae (Gosliner & Johnson, 1999); *Phestilla*, Trinchesiidae (Faucci et al. 2007); *Bosellia*, *Elysia*, *Thuridilla*, Sacoglossa (Carmona et al. 2011); *Notobryon*, Scyllaeidae (Pola et al. 2012); *Okenia*, Goniodorididae (Pola et al. 2014); *Glaucus*, Glaucidae (Churchill et al.

2014); *Spurilla*, Aeolidiidae (Carmona et al. 2014); Phyllidiidae (Brunckhorst, 1993; Stoffels et al. 2016); *Dendronotus*, Dendronotidae (Korshunova et al. 2017); *Glossodoris*, Chromodorididae (Matsuda & Gosliner 2017, 2018); *Flabellina*, Flabellinidae (Furfaro et al. 2018); *Piseinotecus*, Piseinotecidae (Furfaro et al. 2018); *Hypselodoris*, Chromodorididae (Epstein et al. 2019).

Furthermore, mimicry has now also been shown for the first time for a chromodorid from Indonesia. One specimen of *Chromodoris annae* mimics *C. elisabethina* (Undap et al. 2019). To verify this situation, the specimen was barcoded twice. Additionally, the color patterns of the life and preserved specimen were compared to exclude the possibility of incorrect handling of specimens and labels. Mimicry was only recently documented by Layton et al. (2018) for several other species of *Chromodoris*, and this phenomenon seems to be wide spread at least in Australia. Therefore, it is very likely, that this is also occurring in tropical regions, like Indonesia. Color variation and mimicry in Chromodorididae was also documented for *Glossodoris* (Valdés & Adams 2005), *Chromodoris* (Pasternak et al. 2011), *Hypselodoris* (Dacosta et al. 2010; Haber et al. 2010; Epstein et al. 2018), and *Felimida* (Padula et al. 2016). Furthermore, color variation also is known in Aeolidiidae (Carmona et al. 2013; Caballer & Buske 2016), e.g., *Phyllodesmium*, Mirrhinidae (Cheney et al. 2014), Phyllidiidae (Stoffels et al. 2016), and other marine Heterobranchia, like Aglajidae (Ornelas-Gatdula et al. 2011).

Quite surprising is the paraphyly of the very distinct *Chromodoris dianae*. No other species has a similar color pattern. Nevertheless, *Chromodoris willani* is sister group to *C. dianae* Clade 1, and *C. dianae* Clade 2 is sister group to the former two. We have only investigated mitochondrial genes, and it is shown by several other studies that closely related species living in sympatric populations can show introgression and hybridization (Wägele et al. 2010). To clarify this particular situation, analyses of the nuclear genes are warranted.

Problems in misidentification, or false assignment of metadata to certain species is a problem that might also contribute to queries, as we have outlined above. We also encountered this problem e.g. with one specimen of *C. dianae*, that was certainly labeled wrong and therefore correct metadata are not available for this particular animal.

Although the Chromodorididae are well presented in North Sulawesi, some species are dominating. These are the species *C. annae* (115 specimens), *C. dianae* (80 specimens), *C. lochi* (44 specimens) and *C. willani* (40 specimens). All of them are quite large in size (up to 80 mm) and also rather conspicuous, when sitting on their major food source, which is very often a brownish sponge. According to GBIF data, these *Chromodoris* species are widely distributed in the Indo-Pacific Ocean with many records also from the subtropics.

Few species have only been found once (i.e. *Ceratosoma tenue, Glossodoris hikuerensis, Hypselodoris cerisae, H. maridadilus, Miamira sinuata, Thorunna australis, T. furtiva, Verconia simplex, Verconia* spec.). Usually members of these species are rather tiny, and much more difficult to collect *in situ* under water. Nevertheless, animal less than 10 mm were still recognized and collected (e.g. *Verconia simplex* and *Verconia spec.*). The inability to recollect some species is certainly due to their cryptic appearance and might also be explained by different perceptions of the various collectors.

Due to the higher collection efforts in BNP (three collecting periods, versus two in BA), the specimen numbers are in general much higher in this area. However, there are also differences in species composition between these two areas (Papu et al. 2020). With regard to the Chromodorididae, six species were only collected from Bangka Island (i.e., *Goniobranchus coi, Hypselodoris lacuna, Hypselodoris cerisae, Doriprismatica atromarginata, Verconia simplex*). This is more than 25% of the total chromodoridid species number now recorded from BA. Papu et al. (2020) has already shown that Bangka Archipelago differs to a great extent from BNP, especially with the substrate composition. They have shown that species overlap is only about 30%.

The main task was to study the biodiversity of Chromodorididae in North Sulawesi. For this, two genes were analysed for barcoding, and tree reconstruction clarified assignment of specimens to genera and species. It is not the goal to analyse the phylogenetic relationship of the genera. However, the only comprehensive phylogeny of Chromodorididae, published by Johnson and Gosliner in 2012, also used 16S and CO1 for analyzing 244 chromodorid specimens. Their analysis resulted in the paraphyly of many well accepted genera, including *Chromodoris*, *Hypselodoris* and *Glossodoris*. Thus, they re-organized the systematics

according to their results, by transferring species into available genera. Now, Chromodorididae comprise 16 genera. We analysed these 16 genera, by combining data from our specimens, related to 9 genera, with data from NCBI, including especially sequences from those genera, which were not present in our collection. Here, I would like to discuss briefly the results of our concatenated tree with the concatenated tree published by Johnson & Gosliner (2012) (Fig. 4.1). Our tree included 375specimens (compared to the 244 specimens in (Johnson & Gosliner 2012).

Similar to their tree, we also retrieved two major clades within Chromodorididae, however with some differences. The first clade comprises the genera *Chromodoris*, *Ardeadoris*, *Goniobranchus*, *Doriprismatica* and *Diversidoris*. It also comprises *Felimida* with two species. One species, *F. britoi*, is sister to the genus *Chromodoris*, the other species, *F. luteorosea*, is sister taxon to *Goniobranchus fidelis*, thus rendering the genus *Goniobranchus* paraphyletic. Interesting, Johnson and Gosliner (2012) also did not retrieve a monophyly of the genus *Felimida*, but a monophyletic genus *Goniobranchus*. The single sequence of a *Diversidoris* species also clusters within the first clade, as sister taxon of *Doriprismatica*. This is in contrast to the results of Johnson and Gosliner, where the genus actually forms the most basal form of the second large clade within the Chromodorididae.

In our tree, *Glossodoris* is sister group to this first clade, whereas the position of the genus is not resolved in Johnson & Gosliner (2012).

The genera *Hypselodoris*, *Thorunna*, *Felimare*, *Mexichromis*, and *Ceratosoma* group into the second large clade. This again is similar to the results of Johnson & Gosliner (2012). Unfortunately, the authors erroneously labeled *Felimare* as *Felimida* in their figure. Differences can be found in the position of *Mexichromis*, which is sister taxon of the *Miamira*/*Mexichromis* clade in our analyses, whereas in the published analysis, *Mexichromis* forms the sister taxon of the *Thorunna*/*Hypselodoris* clade.

We can conclude with our new data that the systematics re-arranged by Johnson & Gosliner (2012) is rather stable and all genera valid. However, we also have to emphasize here, that we used the same mitochondrial genes. It cannot be ruled out, that by including nuclear genes, the phylogenetic relationships might change.

Chapter 5

Chromodoris of North Sulawesi

Introduction

Chromodoris, Alder and Hancock, 1855, is the most speciose genus in the nudibranch family Chromodorididae, and has a nearly cosmopolitan distribution with the greatest diversity occurring at low latitudes living from the intertidal to the deep sea (Johnson & Gosliner 2012; Wilson & Lee 2005). Their shell-less bodies show manifold forms and especially a broad array of colors and color patterns (Wollscheid-Lengeling et al. 2001; Layton et al. 2018; Tibiriçá et al. 2019).

In recent years, the first molecular studies focusing on chromodorid species were published. Turner and Wilson (2007) recovered evidence of paraphyly or polyphyly in different, widespread chromodorid genera, a view that was later confirmed and examined further, with the addition of more species (Johnson & Gosliner 2012). These latter authors resurrected old available names for new clades identified in their phylogenetic hypotheses.

Johnson and Gosliner (2012) proposed a new classification for the family, splitting the family now in 16 genera, including *Ardeadoris, Cadlinella, Ceratosoma, Chromodoris, Diversidoris, Doriprismatica, Felimida, Felimare, Glossodoris, Goniobranchus, Hypselodoris, Mexichromis, Miamira, Thorunna, Tyrinna,* and *Verconia*. They transferred many species of the genus *Chromodoris* into several of these different chromodorid genera. Thus, the number of species within the genus *Chromodoris* is now reduced to 22 species. *Chromodoris*, was previously thought to be circum-global distributed across temperate and tropical latitudes, but now it represents a radiation endemic to the Indo-West Pacific (Padula et al. 2016).

Recently, Layton et al. (2018) conducted a comprehensive molecular phylogenetic review of the genus *Chromodoris* that revealed several cryptic speciation events, increasing

the number of *Chromodoris* clades with now up to 39 putative species. Tibiriçá et al. (2019) confirmed cryptic speciation in the genus, however confirming only about 33 clades. In this chapter, I concentrate on the genus *Chromodoris*, since this genus was represented by many specimens in our collections, and because I could already show in chapter 3, that I also had cryptic species within the *Chromodoris* species represented in this phylogenetic analysis.

Materials and methods

Specimen collection and preservation

A total of 264 specimens of *Chromodoris* covering 5 species (i.e. *C. annae, C. dianae, C. willani, C. lochi, C. strigata*) were collected around North Sulawesi Bunaken National Park (BNP), the island Sangihe, Lembeh Strait and around Bangka Island, partly by myself and partly by colleagues participating in the project. Part of the results with regard to collection events at the various localities is already published (see Kaligis et al. 2018; Eisenbarth et al. 2018; Ompi et al. 2019; Undap et al. 2019; Papu et al. 2020). Specimens were collected directly from substrate in the field by scuba diving or by snorkeling and preliminary identified by various identification books (Gosliner et al. 2008; 2015, 2018) and additionally by the Sea Slug Forum (2019) (www.seaslugforum.net). The World Register of Marine Species (WORMS 2019) was used to check validity of species. All specimens were individually photographed, numbered and preserved in 96% alcohol for future barcoding. Available data on the distribution of respective sea slugs are downloaded from the Global Biodiversity Information Facility (GBIF). All material was collected with necessary permissions according to the Nagoya Protocol. The material is registered at Sam Ratulangi University collection and material is on loan.

DNA extraction and amplification

DNA was extracted as is outlined in detail in chapter 2 (general methods).

Sequence analysis and alignment

With regard to alignment methodologies, I would like to refer to chapter 2. For this particular analysis, a total of 259 *Chromodoris* specimens of the species *C. annae*, *C. dianae*, *C. lochi* and *C. willani* were used excluding 5 specimens of *C. strigata*. For this species, the number of sequences was not enough for a thorough analysis. Subsequently all available sequences of the mitochondrial CO1 of the 4 targeted *Chromodoris* specimens were downloaded from NCBI (in total 105 sequences) and added to the sequences obtained in this study, to construct the final alignments separately for each species, using other species as outgroups (see Appendix Table S1).

Phylogenetic reconstruction and species delimitation

Phylogenetic reconstruction was carried out using maximum likelihood methods. Analyses were performed with IQ-TREE web server, with following default settings: substitution model set Auto; 1000 ultrafast bootstrap; SH-aLRT branch test (Trifinopoulos et al. 2016). The tree file was imported into Dendroscope v 3.5.9 to interpret results and subsequently in Figtree v 1.4.3 to collapse nodes when necessary. The trees were further processed in Inkscape v 0.92.3 for graphical lay out.

Species delimitation tests were performed using the algorithms developed by (Puillandre et al. 2012): Automated Barcode Gap Definition (ABGD). This program was used to partition datasets into unique genetic clusters. Following default settings and parameters were applied: Pmin= 0.001, Pmax= 0.10, 10 steps, X=1.5, Nb bins of 20, and the evolutionary model of Kimura (K8o) TS/TV. ABGD algorithms were run with various outgroups, according to the various ingroups. This method was designed to detect the barcode gap in the distribution of pairwise distances calculated in a CO1 alignment (Puillandre et al. 2012), however can also be used for other genes. TCS haplotype networks (Clement et al. 2002) were generated in PopART (Leigh & Bryant 2015) using CO1 data and 5000 iterations.

Results

A total of 259 *Chromodoris* specimens covering the 4 species *Chromodoris annae*, *Chromodoris dianae*, *Chromodoris lochi* and *Chromodoris willani* and mainly collected in North Sulawesi waters were included in the analyses. Single sequences of the 5 specimens of *C. strigata* were only included as outgroups, when appropriate. In the following, the 4 *Chromodoris* species are presented in more detail.

Chromodoris annae

110 own sequences (CO1) of *C. annae*, and 20 sequences retrieved from GenBank, were analysed, with 3 specimens of *C. dianae* as outgroup, but also including one specimen of *C. strigata* and one specimen of *C. lochi* (ChloBuDS40). The alignment was 505 base pairs (bp) in length. A phylogenetic analyses using maximum-likelihood methods resulted in a tree that showed bootstrap supports ranging from 64-100 (Fig. 5.1). *Chromodoris annae* is not monophyletic, because the two sequences of *C. strigata* (ChstBuDS8) and *C. lochi* (see also in Fig. 5.1) appeared as sister group to a few *C. annae* sequences. However, bootstrap value of this relationship is very low, and the results are not confirmed in the network analysis.

The ABGD analysis based on this CO1 dataset using the default gap width (X=1.5) splitted all *C. annae* sequences in three clades (Figs. 5.1 & 5.2). When the gap width was adjusted to X=1, the initial partitions retrieved in total six groups with outgroup. Again, *C. strigata* (ChstBuDS8), *C. dianae* and *C. lochi* are own clades, showing their distinctness from *C. annae.*

In the following I concentrate on the 3 clades retrieved in the more conservative ABGD analysis. Intraspecific distances for CO1 of *C. annae* clade 1 (in total 130 specimens) ranged from 0% to 4.7%, thus intraspecific distances of clade 1 were less than 5% (Table 5.1). Intraspecific distances within clade 2 (2 specimens) was 0.8%, within clade 3 (8 specimens) was 3.4%.

Interspecific distances between the 3 clades (minimum and maximum values) are represented in Table 5.1. Interspecific distances of clade 1 and clade 2 ranged from 5.7% to 9.7%. Thus the barcode gap between the clades 1 and 2 is not very distinct in comparison to the intraspecific distances of 4.7%, but nevertheless it is present. Minimum interspecific distances of clade 1 and 2 to clade 3 were 7.8% and 9.7% respectively and thus representing a large barcode gap (Table 5.1). Therefore, the results indicate a cryptic speciation of *C. annae*, with 3 species involved.

Table 5.1 The distances of *C. annae*. Intraspecific distances within the different clades is shaded in green. Minimum and maximum values of interspecific differences between the clades are also provided.

	Clade 1 min-max distance	Clade 2 min-max distance	Clade 3 min-max distance	*C. strigata* min-max distance
Clade 1	0-4.7%	5.7-9.7%	9.7-12.1%	9.2-11.6%
Clade 2	5.7-9.7%	0-9.7%	7.8-11.2%	9.4-10.3%
Clade 3	9.7-12.1%	7.8-11.2%	0-3%	8.2-9.7%
C. strigata	9.2-11.6%	9.4-10.3%	8.2-9.7%	0
C. dianae (as outgroup)	8.7-11.8%	7.7-8.5%	7.2-10.4%	9.5-10%

These results are also reflected in the haplotype network analysis (Fig. 5.2). This graph reveals more the relationship of the various haplotypes in combination with the distribution of the *C. annae* clades. Only few results differ here from the ABGD test, or give a different perspective: *C. strigata* and *C. lochi* are not necessarily sister taxa and these 2 species are clearly separate from each other and not closely related with *C. annae*.

Chromodoris annae sequences were available from other areas of the Indo-Pacific. By including them in my analyses, I was able to assign them to the various clades. None of them appeared as a distinct or separate clade from the specimens collected around North Sulawesi. Clade 1 is now recorded from many areas in the Coral Triangle, including Philippines (4), Papua New Guinea (3), but also North Australia (Lizard Island at the Eastern

Coast, and Hibernia Reef at the Western Coast) and Marshall Islands (Fig. 5.3). Clade 2 with 2 specimens is only represented in Bunaken Island, North Sulawesi. Clade 3 comprises only 8 specimens, out of which 7 haplotypes are from North Sulawesi, but interesting one haplotype (retracted from NCBI) is from in Rottnest Island, South Western Australia (Fig. 5.3). *C. annae* was considered to be widely distributed in the Indo-Pacific Ocean. Looking at GBIF data, the distribution can be widened to the North and East and West (Fig. 5.3 blue area). Records in GBIF indicate a distribution even in South Africa and one specimen was recorded from Hawaii (Fig. 5.3). However, it is not known to which of the 3 clades of *C. annae* these records can be assigned.

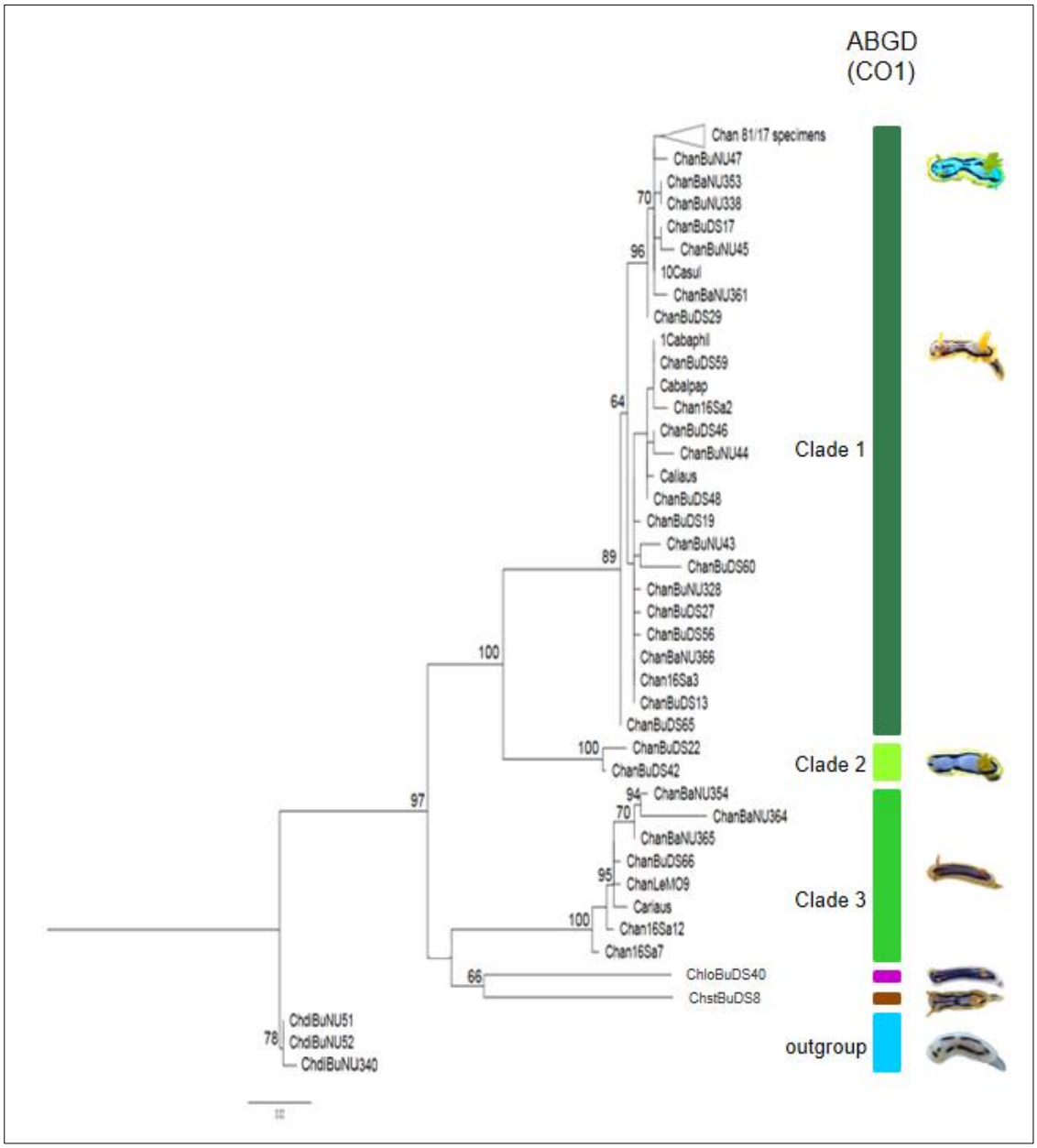

Figure 5.1 Maximum-likelihood phylogeny of the CO1 dataset for *Chromodoris annae*, with results of the species delimitation tests plotted on the right side. The triangle represents a collapsed clade with nearly now intraspecific variability. ABGD test reveals 3 *C. annae* clades (all colored in green), one *C. lochi* in purple, and one *C. strigata* in brown. The outgroup *C. dianae* is colored in blue.

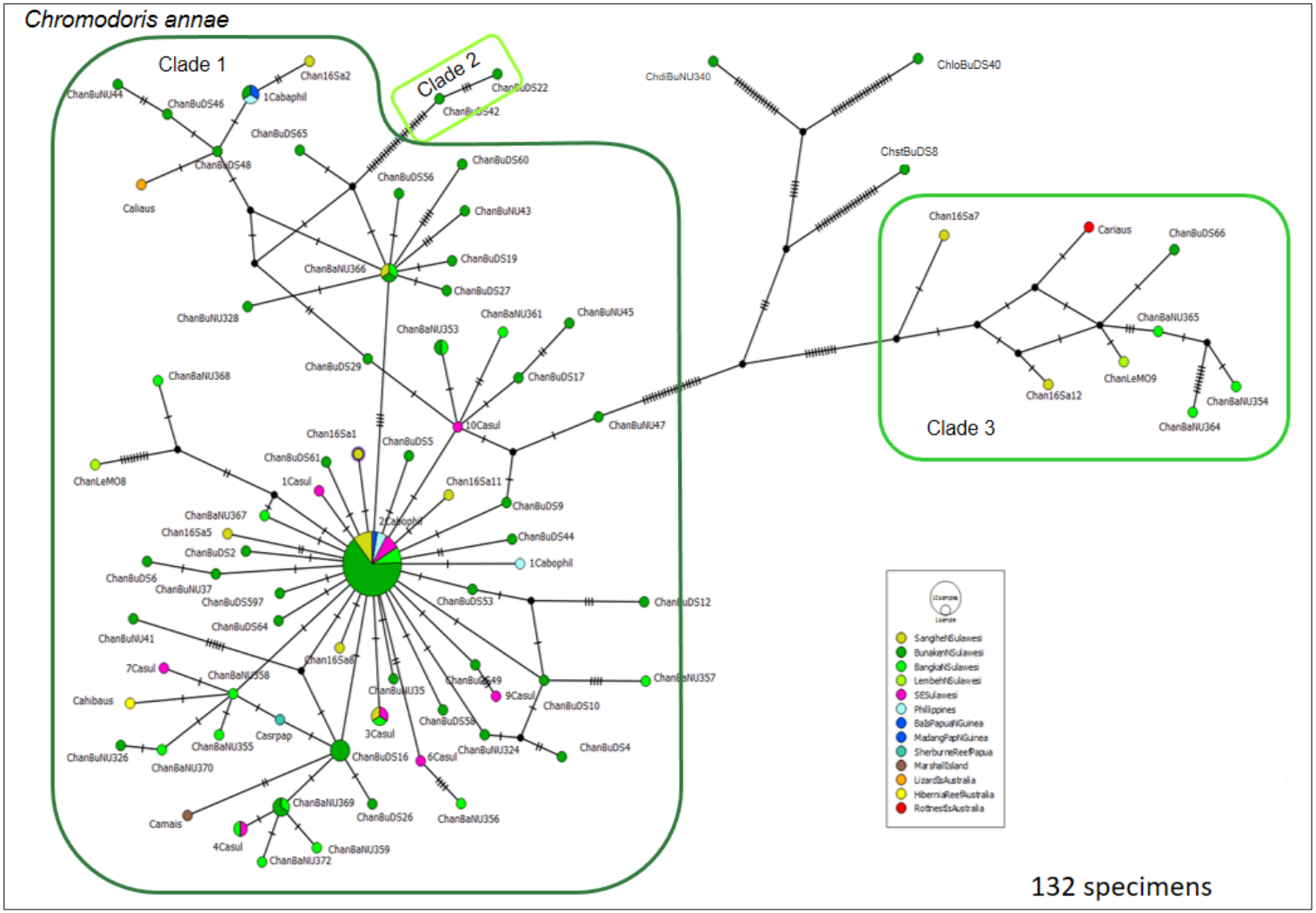

Figure 4.2 Haplotype network of *Chromodoris annae* showing the 3 different clades and the corresponding distribution. Included are one sequence of *C. lochi* and one of *C. strigata*. One *Chromodoris dianae* was used as outgroup.

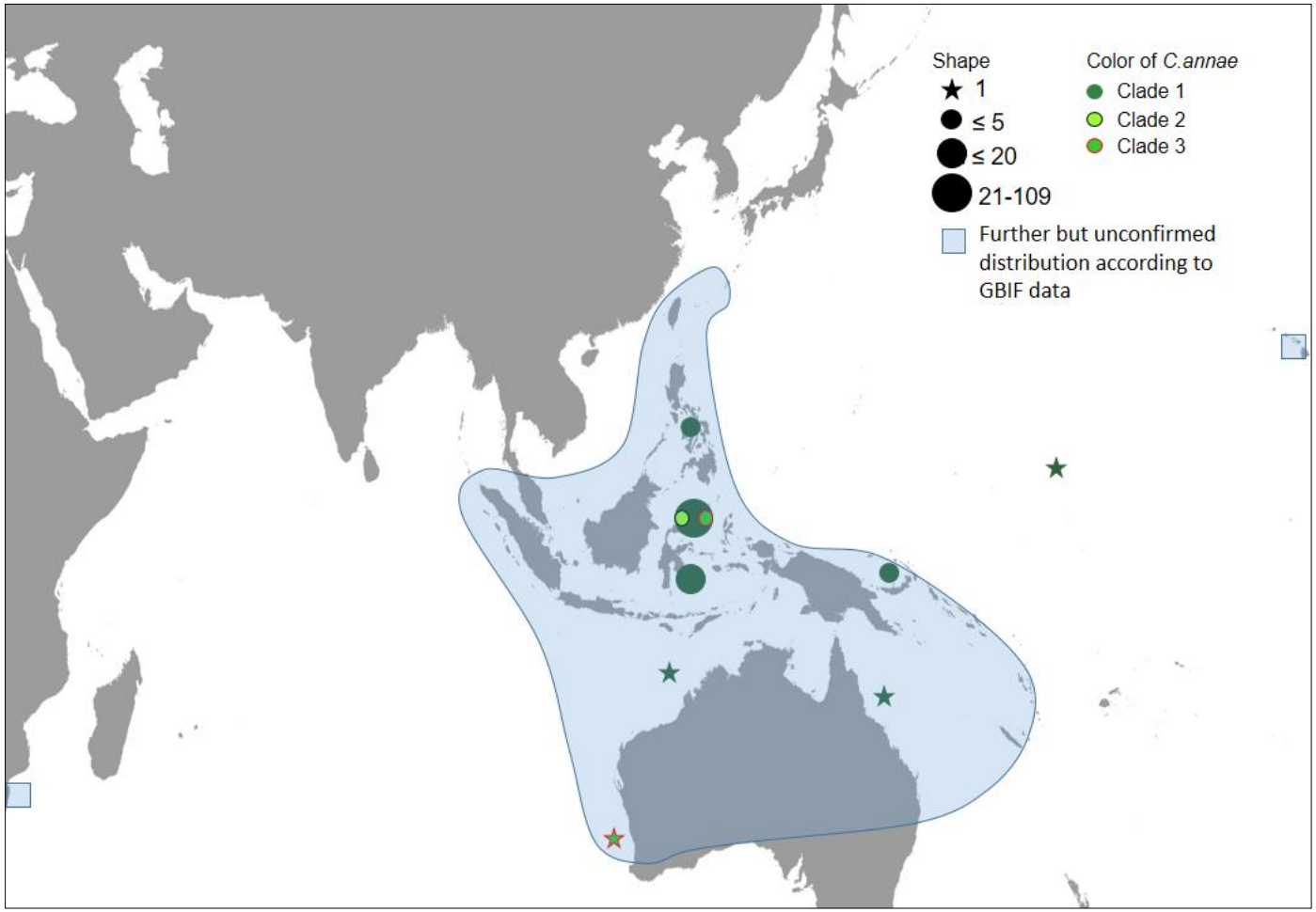

Figure 5.3 Distribution data of the 3 *Chromodoris annae* clades in the Indo-Pacific Ocean. Please note that clade 2 is only recorded from North Sulawesi. The shape of the symbols indicate the numbers of sequences included. The color indicates the clade. Additionally data from GBIF (latest update 2019) are included by delimitating the range recorded in this data facility. This range is shaded in blue. However, these record and range do not distinguish between the 3 clades.

Chromodoris dianae

The CO1 alignment for this analysis comprises 81 specimens of *C. dianae* with 73 specimens from North Sulawesi. 3 specimens from Southeast Sulawesi, 1 specimen from China, and 4 specimens from Philippines were retrieved from GenBank. One specimen of *C. lochi* was chosen as outgroup, and additionally one specimen of *C. willani* was included in the analysis, based on the results shown in chapter 4. The alignment had a length of the 507 bp. Maximum-likelihood analysis resulted in a tree shown in Fig. 5.4. Bootstrap support ranged from 66-99 with most specimens from Bunaken and Sangihe included in one clade (clade 1) with a bootstrap support of 99. The single sequence of *C. willani* already renders *C. dianae* paraphyletic, thus confirming the results from chapter 4.

The ABGD analysis based on the CO1 dataset using the default gap width (X=1.5) retrieved all *C. dianae* splitting in 4 clades, when including out groups, and 2 clades excluding outgroups (Fig. 5.4). Clade 1 comprises only specimens from North Sulawesi, whereas clade 2 includes all specimens retrieved from GenBank, as well as 5 new sequences from North Sulawesi. Comparing these results with a haplotype network analysis that only comprises those sequences that were clearly identified as *C. dianae* (clade 1 and clade 2 in Fig. 5.4), these results confirm the distinction into 2 clades (Fig. 5.5).

Intraspecific distances within clade 1 and clade 2 were less than 2% (Table 5.2). Interspecific distance between clade 1 and clade 2 were 7.6%, thus exceeding the barcode gap of 3% by far. Interestingly, all specimens retrieved from GenBank (China, Philippines and South Sulawesi) are assigned to clade 2, including also 5 sequences from Bunaken Island. The distribution of this clade at the moment is confined to China (1 specimen), Philippines (4), Southeast Sulawesi (3) and North Sulawesi (5) (Fig. 5.6). Interesting is the fact that Bunaken sequences share the same haplotypes as sequences obtained from Philippine specimens or South Sulawesi specimens. Sequences of the haplotype in clade 2 are quite similar with often only 1 difference in the base composition. Clade 1 with the highest number of represented specimens is now only known from Bunaken and Sangihe Island and thus

seems to be restricted to North Sulawesi. Sequences within this clade are also very similar, but differ from clade 2 by 25 different bases. When looking at GBIF data, the species records of the original *C. dianae* species are mainly confined to the Coral Triangle (Fig. 5.6, blue range). It is not clear, to which clade of the 2 clades within *C. dianae* these records can be assigned.

Table 5.2 The distances of *C. dianae*. Intraspecific distances within the different clades is shaded in green. Minimum and maximum values of interspecific differences between the clades are also provided.

	Clade 1 min-max distance	Clade 2 min-max distance
Clade 1	0-1.9%	5.3-7.6%
Clade 2	5.3-7.6%	0-1.1%
Outgroup1	6.5-7.8%	5.0-5.7%
Outgroup2	7.4-8.5%	6.5-7.0%

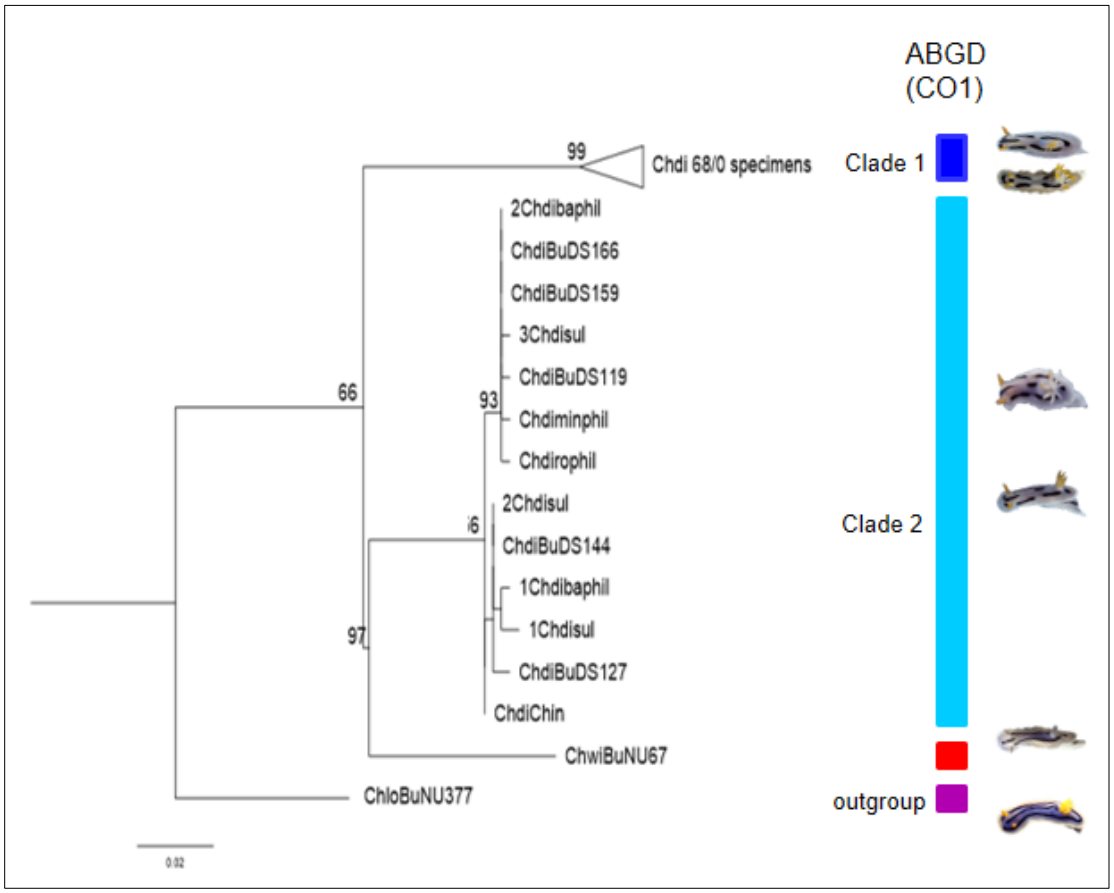

Figure 5.4 Maximum-likelihood phylogeny of the CO1 dataset for *Chromodoris dianae*. Triangle represent collapsed clades. For species delimitation analysis (ABGD), blue boxes splits within a clade, red as additional specimen of *C. willani* and purple boxes as outgroups.

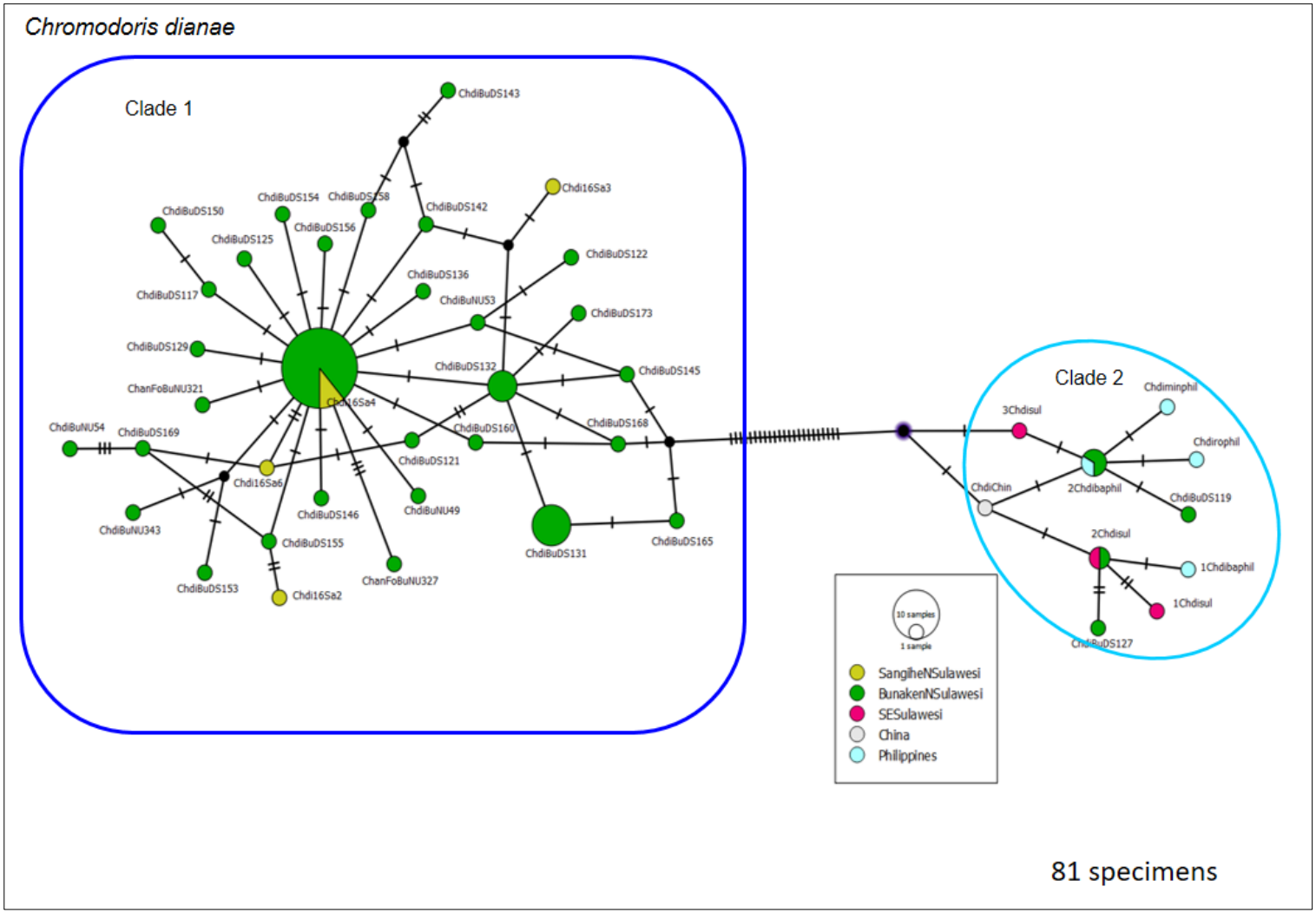

Figure 5.5 CO1 haplotype network of *Chromodoris dianae* showing the 2 different clades and the corresponding distribution.

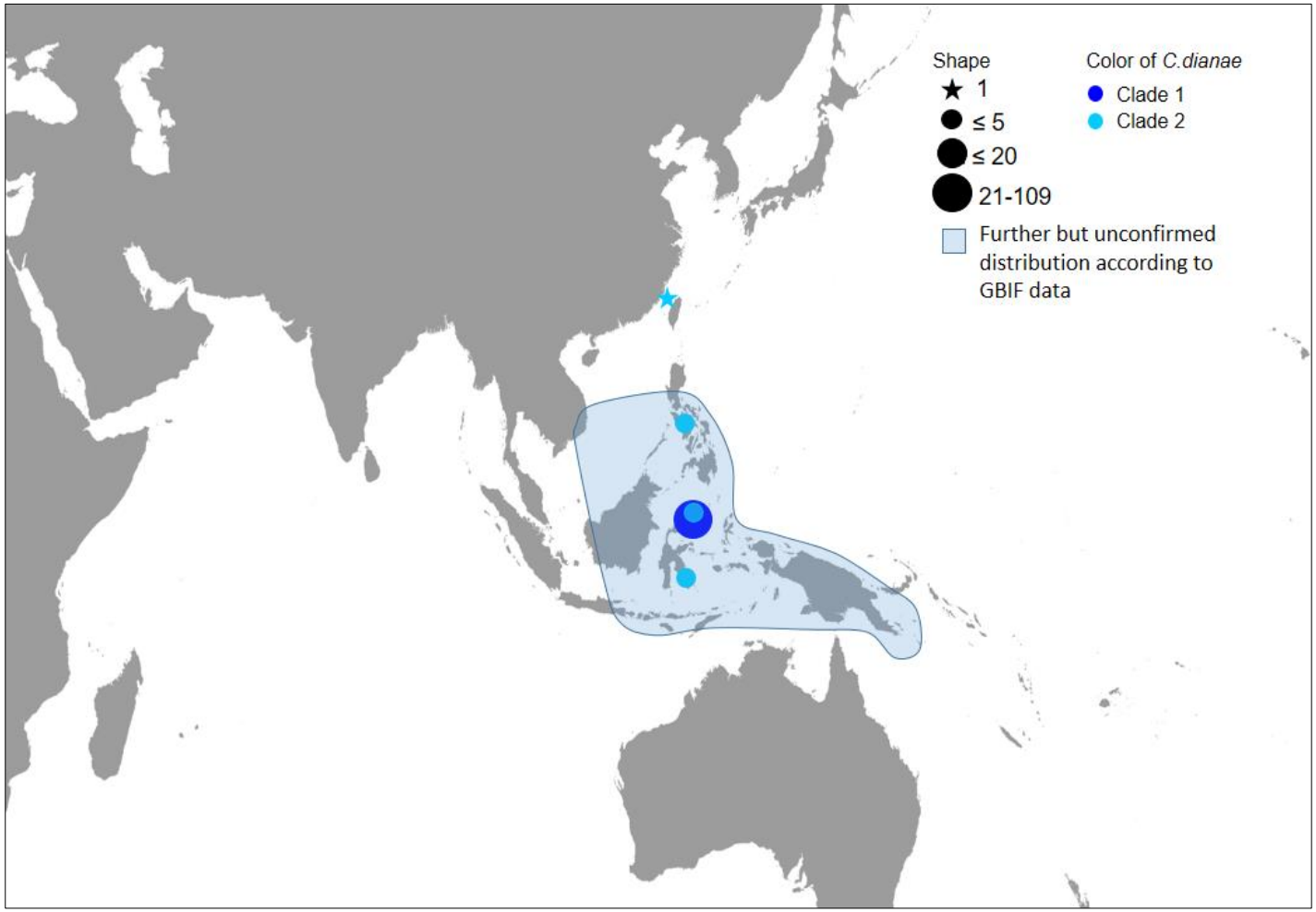

Figure 5.6 Distribution data of the 2 *Chromodoris dianae* clades in the Indo-Pacific Ocean. Please note that clade 1 is only recorded from North Sulawesi. The shape of the symbols indicate the numbers of sequences included. The color indicates the clade. Additionally data from GBIF (latest update 2019) are included by delimitating the range recorded in this data facility. This range is shaded in blue. However, these record and range do not distinguish between the 2 clades.

Chromodoris willani

A total 51 specimens of *C. willani* were included in the analysis, consisting of 30 specimens collected around North Sulawesi and 21 specimens retrieved from GenBank. Because of the results shown in chapter 3, *C. willani* is analysed together with the 81 sequences of *C. dianae*. The CO1 dataset resulted in an alignment with 502 bp. Fig. 5.7 shows the phylogeny (maximum-likelihood) with bootstrap support ranging between 75 and 99. The tree shows a similar result as was shown for *C. dianae* (s. above) including only one *C. willani* sequence. Again, *C. dianae* is paraphyletic, however *C. willani* is now sister group to clade 1 of *C. dianae* and not clade 2.

The ABGD analysis for *C. willani* was run with the same default settings and gap with as in the analyses of *C. annae* and *C. dianae,* i.e. X=1.5. All *C. willani* sequences, including own sequences, as well as those from GenBank, form one monophyletic clade with an intraspecific variety of 2.8% (Fig. 5.7, Table 5.3). Distances to the other 2 clades of *C. dianae* are 6.2 and 5.1 %, thus being far beyond the usual 3 % barcode gap between species. The network in Fig. 5.8 shows the connection of the haplotypes between *C. willani* and the 2 *C. dianae* clades: the distances are very long and no clear relationship between *C. willani* and the 2 *C. dianae* clades can be seen. The haplotypes of *C. willani* do not show a certain geographic distinctiveness with a distribution confined to certain localities. The species (which shows no cryptic species) is distributed mainly in the Coral Triangle with its most northern range in Japan and the most southern range in northern Australia. However, GBIF data extend its range into the East, including New Caledonia and Fiji Islands.

Table 5.3 The distances of *C. willani*. Intraspecific distances within the different clades is shaded in green. Minimum and maximum values of interspecific differences between the clades are also provided.

	C. willani Clade 1 min-max distance
C. willani Clade 1	0-2.8%
C. dianae Clade 1	6.2-9.8%
C. dianae Clade 2	5.1-7.4%

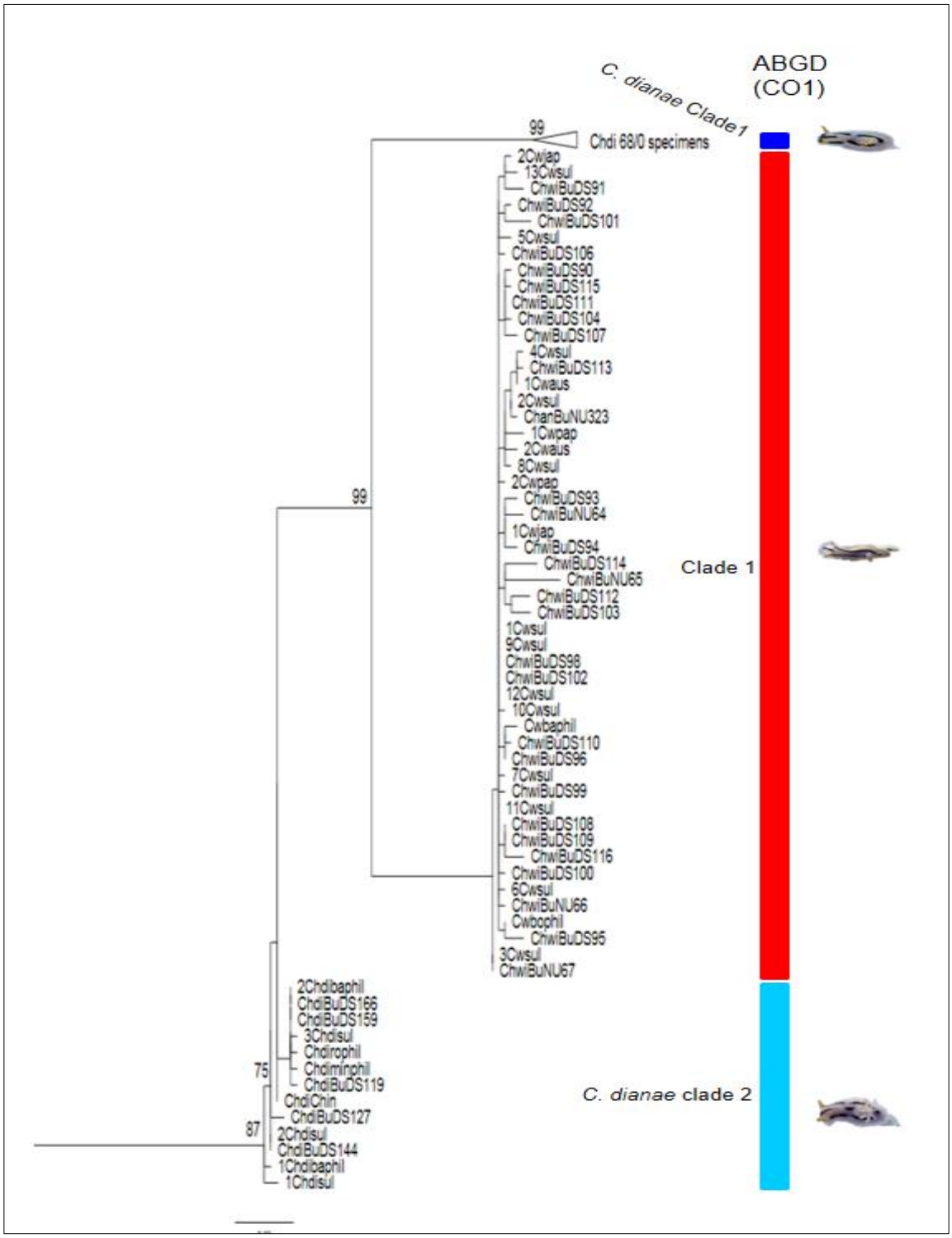

Figure 5.7 Maximum-likelihood phylogeny of the CO1 dataset for *Chromodoris willani*. For species delimitation analysis (ABGD), light blue boxes as outgroup.

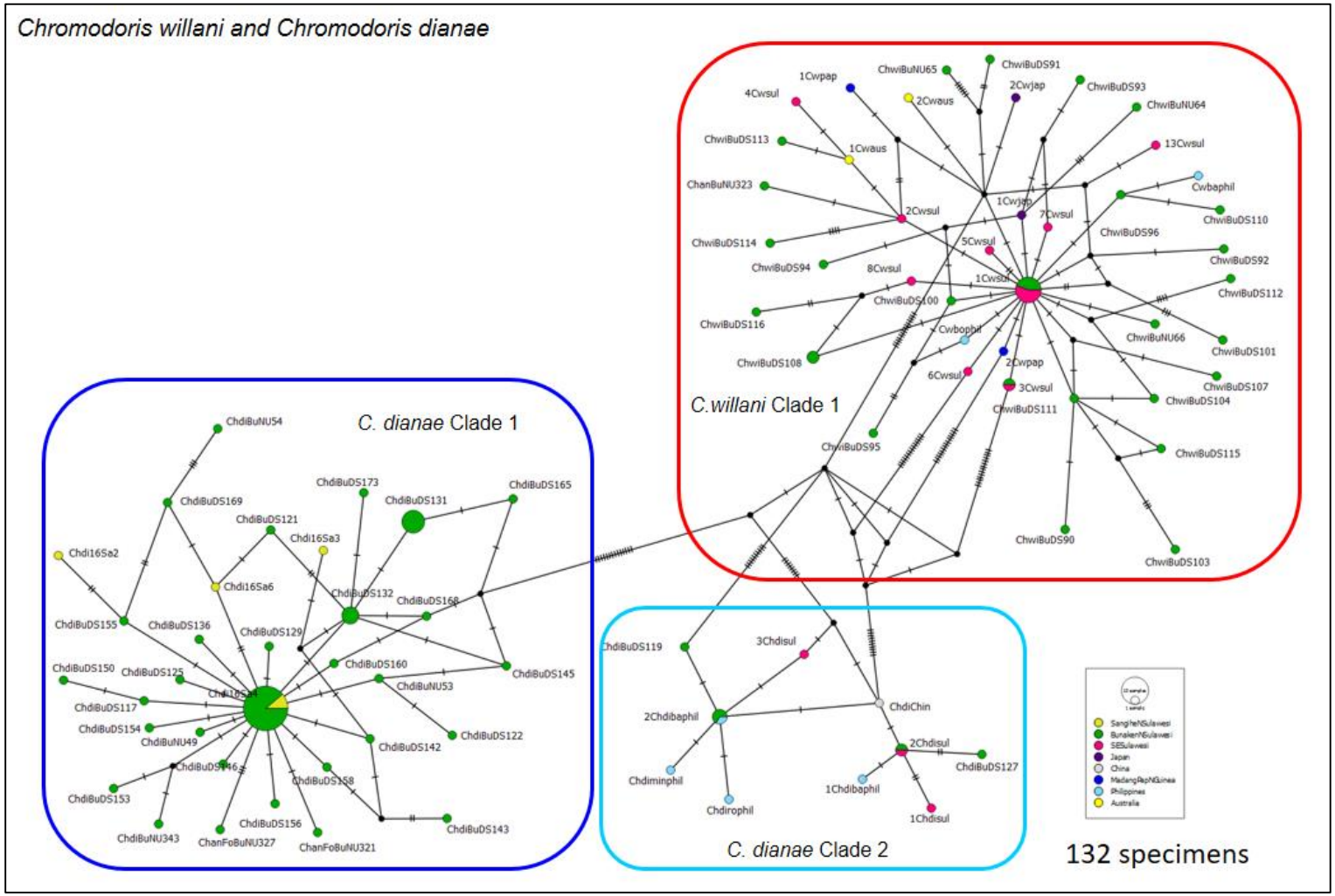

Figure 5.8 Haplotype network of *Chromodoris willani* the corresponding distribution. Included are sequences of *C. dianae* clade 1 and *C. dianae* clade 2.

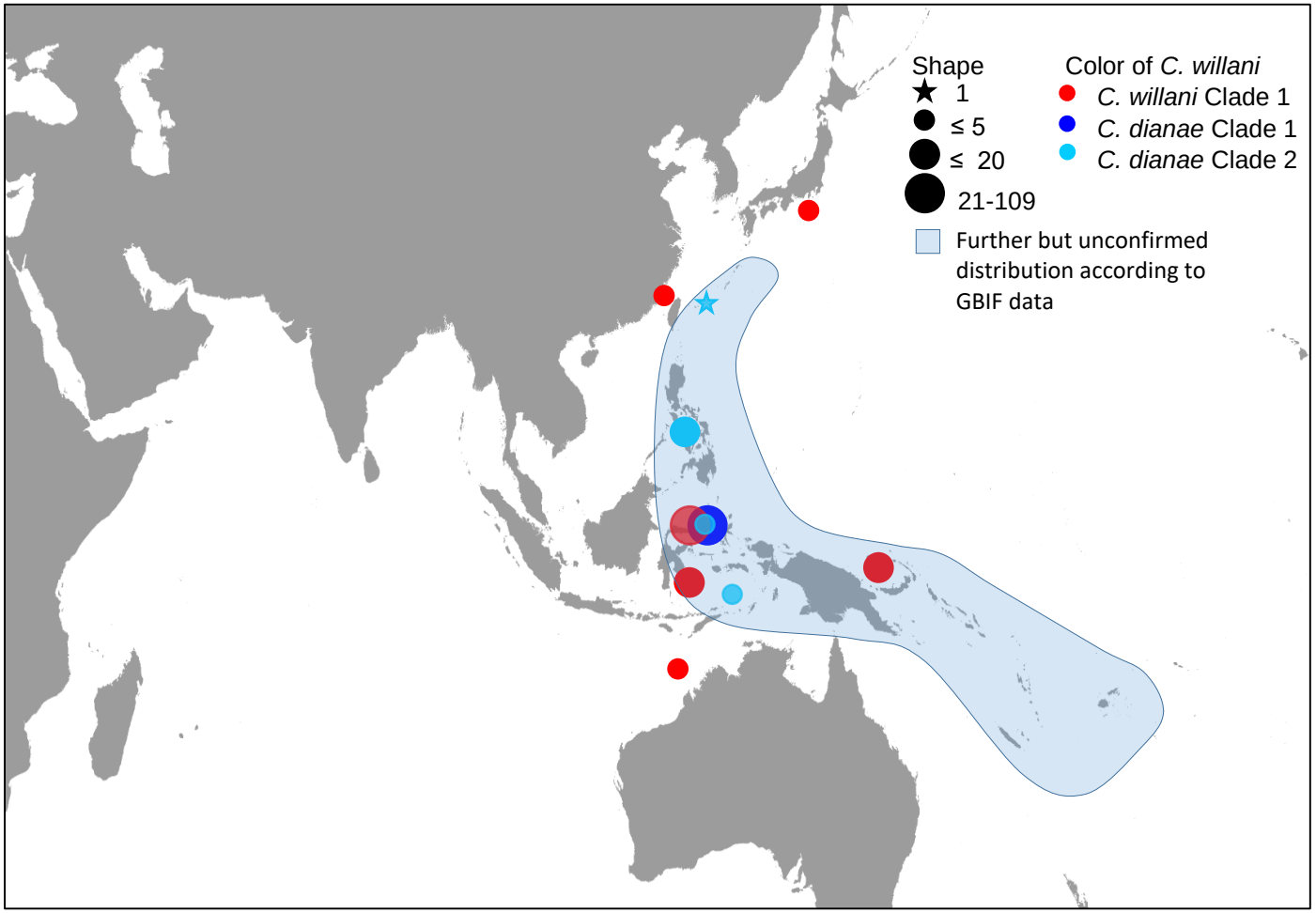

Figure 5.9 Distribution data of the *Chromodoris willani* clades in the Indo-Pacific Ocean. The shape of the symbols indicate the numbers of sequences included. The color indicates the clade. Additionally data from GBIF (latest update 2019) are included by delimitating the range recorded in this data facility. This range is shaded in blue. However, these record and range do not distinguish the clades.

Chromodoris lochi

38 specimens of *C. lochi* were collected in North Sulawesi and 56 specimens retrieved from GenBank. *C. dianae* was used as outgroup. The CO1 alignment was 390 bp in length. Maximum-likelihood analyses resulted in a tree (Fig. 5.10) with bootstrap support ranging from 69-99.

C. lochi was split into 3 clades when analyzing the sequences with ABGD test (Fig. 5.10). Our own sequences cluster with several sequences from GenBank, however clade 2 and clade 3 are only composed of specimens retrieved from GenBank.

Intraspecific distances of sequences within *C. lochi* clade 1 reached 6.2 %, within clade 2 and clade 3 distances reached 2.6 and 5% respectively (Table 5.4). Minimum interspecific distances were highest between clade 1 and clade 2 (9.4%), but were similar high between clade 2 and 3 (9.1 %). These distances are similar, or even higher than to the minimum distances of *C. lochi* to the outgroup (7.2 – 8.5 %).

Table 5.4 The distances of *C. lochi*. Intraspecific distances within the different clades is shaded in green. Minimum and maximum values of interspecific differences between the clades are also provided.

	Clade 1 min-max distance	Clade 2 min-max distance	Clade 3 min-max distance
Clade 1	0-6.2%	9.4-15.3%	6.9-14.4%
Clade 2	9.4-15.3%	0-2.6%	9.1-12.1%
Clade 3	6.9-14.4%	9.1-12.1%	0-5%
Outgroup	8.2-12.9%	8.5-10.3%	7.2-9.8%

The haplotype analysis confirmed the results of the ABGD test (Figs. 5.11). Clade 1 is a mixture of haplotypes with specimens from North Sulawesi sharing same haplotypes from Papua New Guinea, or Australia. The clade comprises in total sequences from North Sulawesi (38), Southeast Sulawesi (16), Papua (1), Papua New Guinea (5), Philippines (3), New Caledonia (1), and Fiji (1). Even haplotypes coming from far West, like Madagascar (1), and Mozambique (3) were very similar to haplotypes from North or South Sulawesi. Interesting is a group of haplotypes within this clade 1, that only come from Bunaken and

show several mutations unique only to these 13 sequences. They are closest related to one of the Mozambique haplotypes and to the single haplotype of Madagascar. Only specimens from Vanuatu (2) and French Polynesia (8) are grouped within clade 2, and 12 specimens from French Polynesia group within clade 3. Thus this analysis shows further 2 cryptic species, which are distributed only in the more eastern part of the Pacific. The original *C. lochi* has wide distribution in the Indo-Pacific Ocean with many records also from the subtropics, Southern Africa and Hawaii (Fig. 5.12). However, it has to be emphasized again, that the records in GBIF do not reflect here the real distribution of the 3 different clades. The results also show that the color morph of *C. lochi*, with yellow rhinophores and gills, is not restricted to certain haplotypes, but can be found throughout the network.

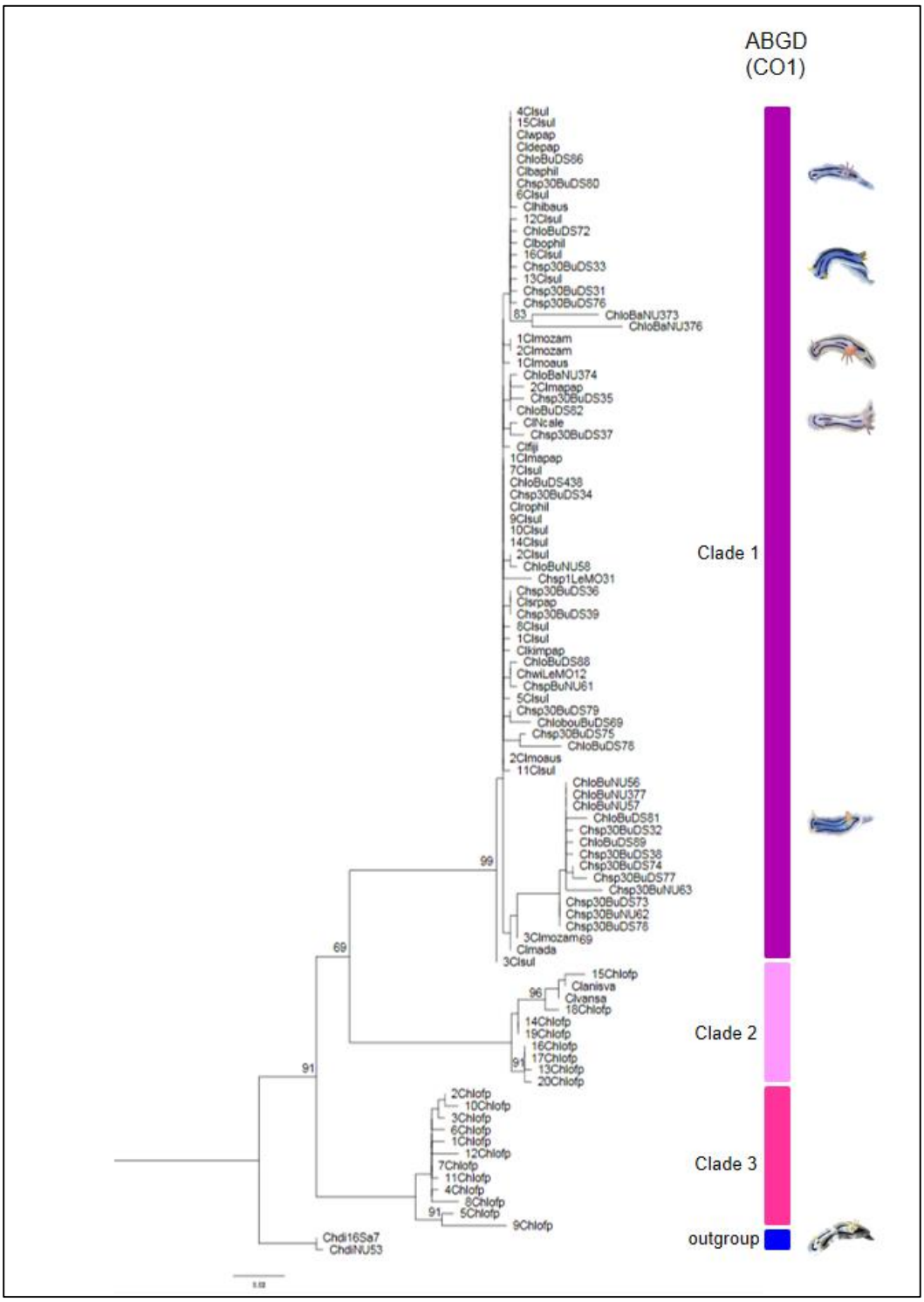

Figure 5.10 Maximum-likelihood phylogeny of the CO1 dataset for *Chromodoris lochi*. For species delimitation analysis (ABGD), purple boxes splits within three clades, blue boxes as outgroup.

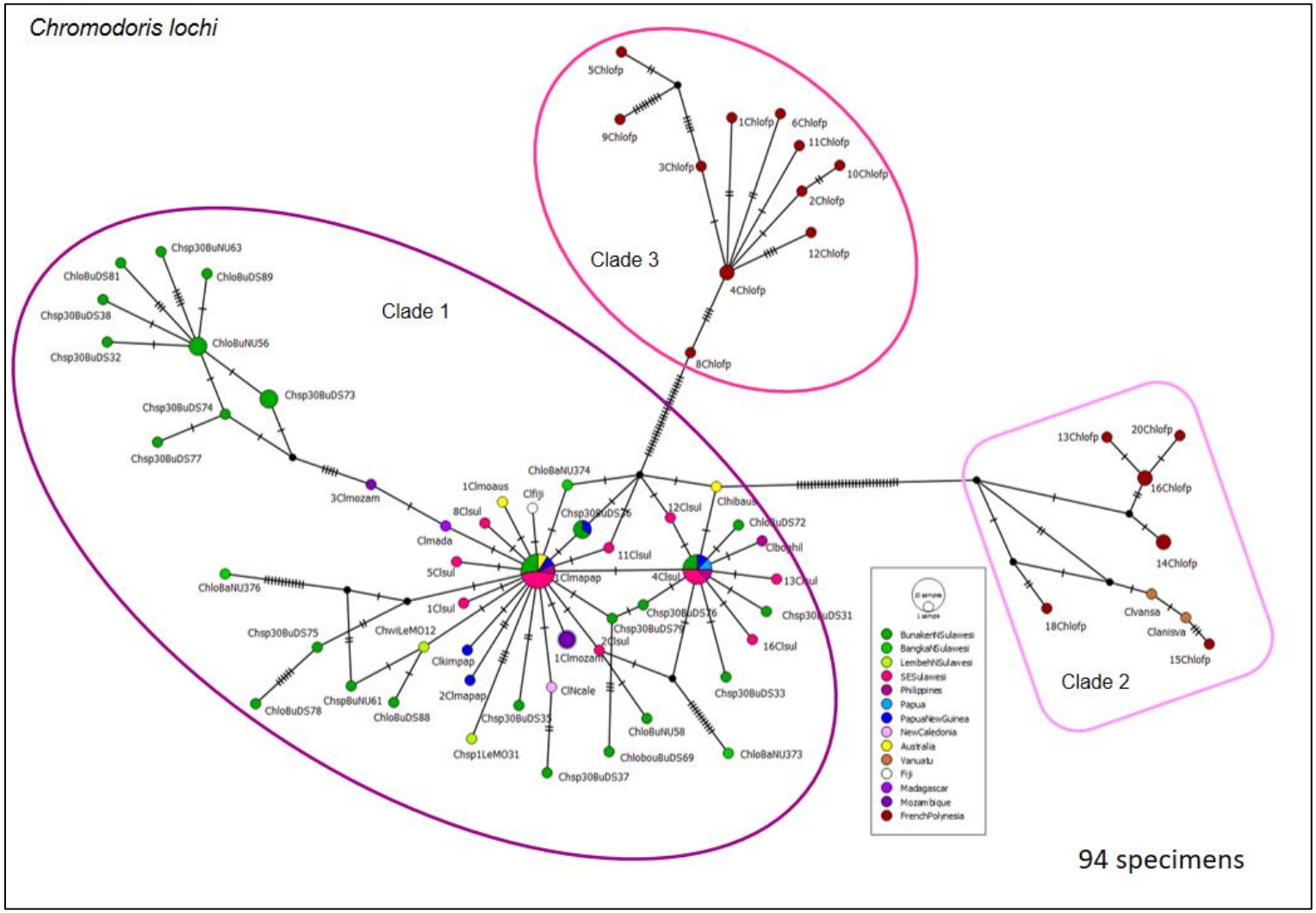

Figure 5.11 Haplotype network of *Chromodoris lochi* showing the 3 different clades and the corresponding distribution.

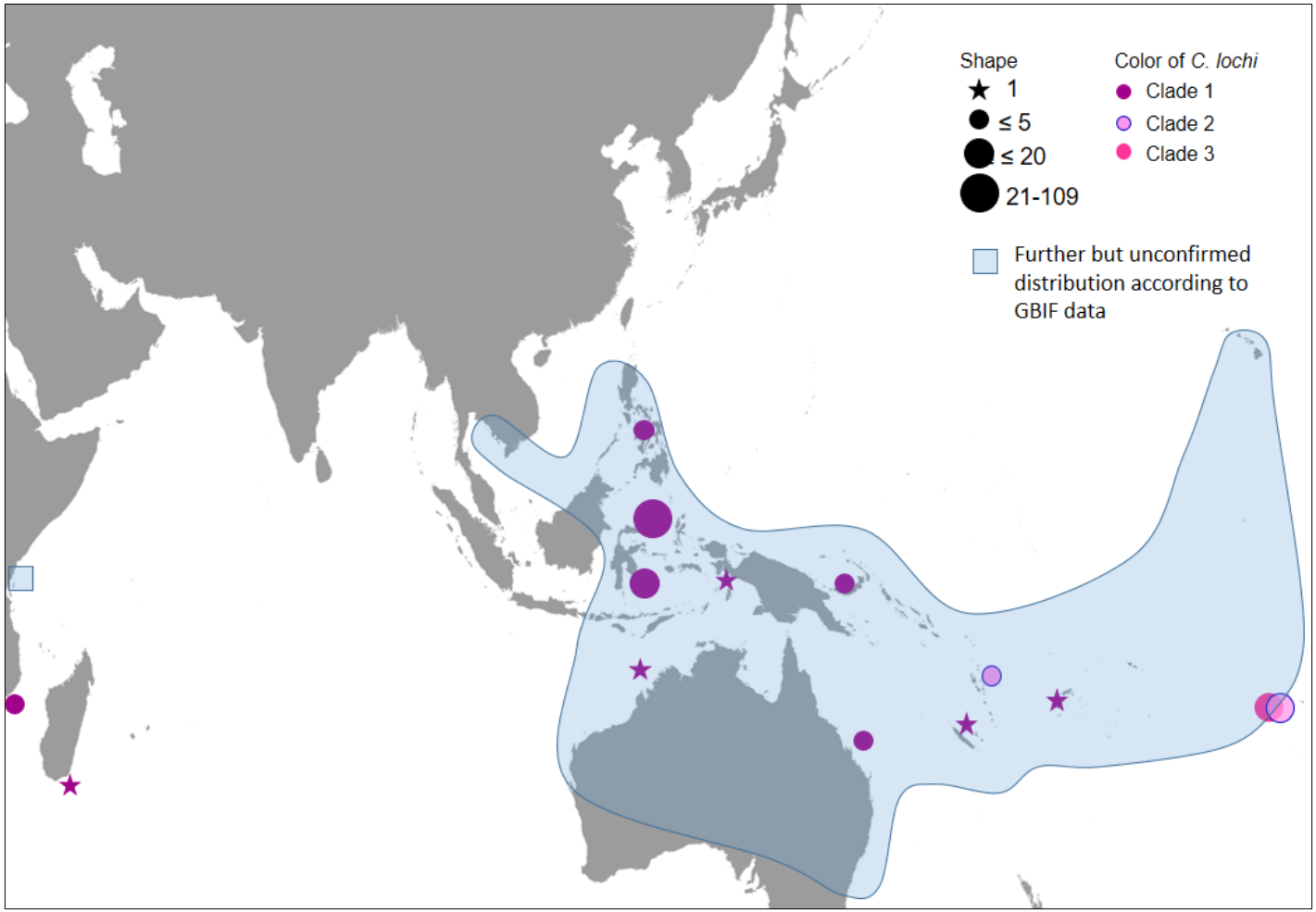

Figure 5.12 Distribution data of the *Chromodoris lochi* clades in the Indo-Pacific Ocean. The shape of the symbols indicate the numbers of sequences included. The color indicates the clade. Additionally data from GBIF (latest update 2019) are included by delimitating the range recorded in this data facility. This range is shaded in blue. However, these record and range do not distinguish the clades.

Discussion

Four *Chromodoris* species, which were very common around North Sulawesi, were investigated in detail. All analyses, using species delimitation tests and also haplotype networking indicate a high degree of cryptic species in at least 3 species. No cryptic speciation was only shown for *C. willani*.

In the molecular phylogenetic analysis, the majority of our *C. annae* specimens from North Sulawesi (96) clustered together with several *C. annae* specimens retrieved from GenBank. Two specimens from Bunaken Island, considered as an own species (clade 2), can be connected to clade 1, however, they differ enough with at least 25 mutations from the next haplotype that is present in North Sulawesi. Another 7 specimens of *C. annae* from North Sulawesi cluster together with one specimen of *C. annae* from Australia (clade 3) and are also clearly separate from the major clade 1. When looking at the color and color pattern of all these animals, they can clearly be assigned to the original description of *C. annae*. Similar situations are now shown for several other marine Heterobranchia. However, a recent analysis from Tibiriçá et al. (2019) did not reveal several distinct clades of *C. annae*. But this is certainly because of the very limited number of specimens that were included in their study. Although the animals still look the same, other characters, e.g., behavior, or physiological features, may differ within the 3 clades. It can also be the nutrition that differs. The animals were usually found on sponges and they also feed on sponges (Rudman 1991; Cimino and Ghiselin 1999; Rudman & Bergquist 2007: Gosliner et al. 2008, 2015; Geng-Ming Lin et al. 2017; Epstein et al. 2018; Layton et al. 2018; Ompi et al. 2019; Undap et al. 2019).

A similar situation seems to be existing in *Chromodoris lochi*. In this case, the two cryptic species are confined to localities far away from the Coral Triangle, in which the typical *C. lochi* with its 2 color morphs (clade 1) is occurring. Clade 2 and clade 3 are only present in French Polynesia, and clade 2 additionally in Vanuatu. Clade 2 thus shows a distribution with a potential overlap with the southern east most distribution of clade 1. Clade 3 is only known from 10 specimens and all sequences were retrieved from GenBank. Tibiriçá et al. (2019) also revealed 3 different clades, based on two mitochondrial genes. They also

considered the clade which I called clade 1 as the real *C. lochi*, whereas the other 2 clades are undescribed species.

Very interesting is the situation in *Chromodoris dianae*. This species is described by Gosliner et al. (2018) as present in the Western Pacific. My analyses show 2 distinct clades with animals that show the typical coloration of *C. dianae* in both clades (see also Fig. 5.4). However, *C. willani* is sister group to one of the clades (see also chapter 3). Network analyses, show that *C. willani* is closely related to both *C. dianae* clades, but is not nested within one clade, or shows exclusively mutations with only one *C. dianae* clade. It is therefore a distinct species. Tibiriçá et al. (2019) also shows 2 clades of *C. dianae*, one they call *C. dianae* and the other *C.* cf. *dianae*. However, the authors also retrieved these sequences from GenBank and from the collapsed tree, it is not possible which clade they consider the original and which one the cryptic species of *C. dianae*. In each clade they had mainly individuals from the Philippines Islands and one sequence each from Indonesia. All these sequences are clustering in our clade 2, together with own sequences from North Sulawesi. Our clade 1 comprises exclusively new sequences from North Sulawesi. Gosliner et al. (2018) also depicts a new species as *Chromodoris* sp. 7, which looks very similar to *C. dianae*, but has yellow dots along the mantle rim. Our new species (clade 1) from North Sulawesi does not show this coloration, but exactly the same as clade 2, without these yellow dots.

Many studies based on molecular work are now performed to study cryptic speciation. There are several studies comprising other marine Heterobranchia, which show unprecedented cryptic speciation. E.g. Lindsay and Valdés (2016) could show that the aeolid *Hermissenda crassicornis* consists actually of 3 species, Carmona et al. (2011) show that cryptic species are prevalent in the Sacoglossa (i.e. *Bosellia mimetica* consists of 2 species, *Elysia timida* consists of 4, and *Thuridilla picta* consists of 2 species), and Furfaro et al. (2018) show that the Flabellinidae, *Flabellina gracilis* consists of 2 species. Stoffels et al. (2016) identified several cryptic species in the *Phyllidiella pustulosa* complex, which is also confirmed by Papu et al. (2020).

In the analyses presented here, many new sequences of the 4 targeted species are now available. With the data from GenBank, we can now conclude that *C. lochi* has the widest distribution in the Indo-Pacific Ocean with localities reaching from the East Coast of Africa up to New Caledonia. The new and undescribed cryptic species with a similar coloration are much more restricted in distribution and occurs only in French Polynesia and Vanuatu.

C. willani is also widely distributed, but is not present (up to now) in the India Ocean. Both clades of *C. dianae* are restricted to the coral triangle. Within *C. annae*, clade 1 and clade 3 are wider distributed in the Coral Triangle down to Australia, however clade 2, which is only represented at the moment with 2 haplotypes, is confined to Bunaken Island.

I also included data records from GBIF for the 4 species. Usually, these data are used to understand species distribution, identify endemic species, or even help to understand evolution of species. By including the data, I could show, that using the data, the distribution of the species is probably incorrect for at least 3 species. *C. annae* records are also provided from east Coast of Africa and Hawaii-USA. However, no sequences are available for these records and therefore it is unclear, to which of my clades they belong too. Similarly, *C. dianae* is recorded in GBIF from Vietnam and Papua New Guinea. According to my data, these represent probably the real *C. dianae*, since one of my two identified clades is widely distributed in the Coral Triangle, whereas the other is only known at the moment from North Sulawesi. I could show that *C. willani* does not exhibit cryptic speciation. Its distribution now ranges from Okinawa-Japan, Vanuatu, Fiji, and Solomon Island. The situation in *C. lochi* is more complex again. GBIF records are also from Kenya, Solomon Island and Hawaii Islands. Probably the records from Kenya can be assigned to the widely distributed clade 1, whereas those from Solomon Island and Hawaii are more difficult to assign to a certain clade. For this sequences are necessary to establish the distribution of clades 2 and 3.

The color patterns to identify *Chromodoris* are usually stable, although there are rare case where a species does exhibit variation with some color variation with different color morphs (Rudman 1991). However, Layton et al. (2018) suggested that the color patterns are flexible in *Chromodoris* (as well as other chromodorids). In my study, I could confirm her

results at least for one *Chromodoris* species. *C. lochi* shows two distinct color morphs, one with the typical rose colored rhinophores and gills, the other with yellow rhinophores and gills. They were therefore in the beginning identified as 2 different species and the identifier sp30 was used. However, the phylogenetic analyses and also the haplotype network clearly indicate that these 2 color morphs are one and the same species. Interestingly, both morphs co-exist and it is not known, what triggers the different coloration. The presence of different color morphs is not comparable to the species mimicry that I could also show for at least *C. annae* (Undap et al. 2019), and which seems to be quite common in *Chromodoris* species (Layton et al. 2018). These authors describe species mimicry for *Chromodoris striatella*, which mimics *C.* aff. *striatella* WA A and *C.* aff. *striatella* WA B from Western Australia. However, the study mainly includes species from Australia, but not Indonesia, especially North Sulawesi. With this chapter, I could clearly show wide spread cryptic speciation of *Chromodoris* species with a probably much more limited distribution as was considered before. Some of the cryptic species co-occur (e.g. *C. annae*, *C. dianae*), whereas in *C. lochi*, the cryptic species seem to be confined to certain localities. It clearly shows that molecular analyses are essential for studying biodiversity in this region and that haplotype network analyses can give a deep insight in speciation processes.

Chapter 6

Water measurements around North Sulawesi for water quality assessment

Introductions

Coastal areas are rich in natural resources and their diverse and very productive ecosystems have an important role as habitats for many organisms. These resources, which provide a tremendous economic value to humans, comprise e.g., fish, and other marine life forms as food; reefs, seagrass beds, and mangroves have protective values for the shorelines, and non-biological natural resources can become important e.g., in mining. Natural coastal resources are important also in tourist development.

Coastal areas are complex systems, where the interaction between land and sea, with a variety of human activities in the surrounding areas, has an influence on the physical components, chemistry and biology of these waters. Various activities carried out along the coastal area usually greatly affect the carrying capacity of the coastal waters. Furthermore, with the paradigm of some people who regard the sea as a landfill, very often industrial and domestic wastes, as well as other wastes, are discharged in coastal waters. The high human activities and abuse of the water can potentially result in an environmental degradation with excessive transport of organic and inorganic substances into coastal ecosystems. Quality of sea water (physically, chemically and biologically) is deteriorating, which has a bad impact on the condition of the coastal ecosystems and adjacent oceanic areas. It affects the lives of many animals and plants, thus also the community structure, and with this also influences diversity, uniformity, abundance, dominance, biomass, and so on (Odum 1971; Warwick & Clarke 1993). The decline in water quality will degrade efficiency, effectiveness, productivity, and the carrying capacity of water resources, which in turn lowers the wealth

of natural resources. According to Gholizadeh et al. (2016), any anthropogenic activities result in vulnerable changes in the ecosystem with harm to fish and other aquatic organisms.

During its development over the last decades, the coastal area of North Sulawesi province experienced a wide range of interests and purpose, such as port activities, marine tourism, residential problems, maricultures and fisheries. These activities will certainly have affected the quality of the coastal region.

Nowadays, the coastal areas of North Sulawesi region are densely inhabited, with the residents increasingly involved in marine activities; maritime transport, ports, fisheries and tourism. One of the most pressing developments with regard to these economic activities are currently carried out by the North Sulawesi government: the development of coastal zone fishery (e.g. tuna fishery) and involved the subsequent development of large processing factories in Bitung, as well as the strong development of tourism in various regions in North Sulawesi. To monitor human activities and the resulting damages, it is essential to investigate the state of the quality of the water with regard to pollution and eutrophication. This would provide data about changes in quality of the water and thus provide facts and information for politicians or locals to counteract damaging activities.

This study aims to address the quality of the water along the shoreline of North Sulawesi by analyzing several physical, chemical and biological parameters. These data can be compared with the threshold of the sea water quality standards (Minister of Environment (KLH) 2004), which provide information about healthy habitats and which are relevant in designation of coastlines as a marine tourism area. These analyses were meant to provide a baseline and reference for the provincial governments through relevant agencies, to finally create programs and policies in coastal areas and islands around North Sulawesi. They can also serve as a reference in particular programs to control any activities, which lead to pollution or damages of the coastal environments.

Materials and Methods

In order to assess the condition of the coastal environment of North Sulawesi Province, observations were made in the waters around Bunaken Island (8 localities), Tasik Ria Beach (2 loc.), Moinit Beach (2 loc.), Bentenan Beach (1 loc.), Bangka Island (1 loc.) and Tumbak Beach (1 loc.) (Figure 6.1). Table 6.1 and 6.2 provide more detailed information about the localities, which are now called SP (sampling point). Following indicators were chosen: temperature, salinity, pH, phosphate, nitrate, particle organic matter (POM) and the coliform bacterium *Escherichia coli.*

Sampling was carried out in October 2016 and October 2017. However, not all analyses were performed in both years and sampling methods were adjusted in 2017 for better standardisation. Whereas data for temperature, salinity and pH are available now for both years, measurements of phosphate and *E. coli* were performed from samples taken in 2016, for nitrate and POM in 2017. Sampling points in 2016 covered only stations around Bunaken Island. In 2017, Bunaken Island was visited again; however, SPs differed from the localities covered in 2016, except for Muka Kampung. From this locality, measurements from both years exist, however taken with two different devices. In parallel to the documentation of the physical parameters, the water samples, were taken. Additional metadata, like geographic locality and characterization of the habitat were documented.

In October 2016, temperature, salinity and pH of the water was measured using a Multiparameter Water Quality Checker (Horiba U-50). The seawater samples for measuring phosphate and *E. coli* were taken at two different tide points from the surface down to a depth of approximately 30 cm, to assess the different water conditions. During high tide, when water was coming in, the influence from the open water was measured, and during low tide the influence from the shoreline. For the investigation of phosphate 650 ml water was filled in plastic bottles. Theses samples were then sent to the Center for Industry and Commerce (Baristand) in Manado. Baristand analysis phosphate by following the methodology of the Indonesian National Standard (SNI). At each SP, three samples were taken for repetition. For the methods of *E. coli* investigation, see below.

In October-November 2017, temperature, salinity and pH were measured using a Portable Water Quality Meter provided by the Leibniz Center for Tropical Marine Research (ZMT) Bremen. The samples for measuring phosphate, nitrate and POM were taken again at two different tide points at a depth of approximately 30 cm, using three flasks of 3 litre volume each. At each SP, three samples were taken again for repetition. While taking the water samples, metadata, like geographic locality, characterization of the habitat were documented and physical parameter (temperature and salinity) recorded.

Analyses of phosphate and nitrate collected in October 2017, were performed at the ZMT, Bremen. Methodology of water sampling techniques were following ZMT standard procedures. In the field, 125 ml of seawater taken from the 3 l flasks, was filtered using 47 mm diameters, 0.2 µm porosity glass microfiber filter (GF/F) in a Millipore® all-glass filtration apparatus. After filtration, I stored the samples in the plastic bottles (Low-Density Polyethylene (LDPE)) of 125ml volume and added 2-3 drops $HgCl_2$ (3.5g $HgCl_2$ diluted with 100ml distilled water) for preservation. To investigate phosphate and nitrate, the reagent standard solutions which are available in ZMT were used. The measurements were done using a Shimadzu UV-1700 spectrophotometer. For phosphate, 275µl samples and 55µl reagent standard solution of phosphate mix were added with a pipette and then waited for 30 minutes before measurement, which were read at a wavelength 880nm. For nitrate, 180µl sample and 150µl reagent standard solution of nitrate mix were put together, and heated to 40°C for 60 minutes. After cooling down to room temperature for 10 min, the measurements were taken using a wavelength of 540nm.

To investigate Particulate Organic Matter (POM) (only collected in 2017) the high temperature combustion (HTC) technique was performed (Wangersky 1978; Dafner & Wangersky 2002): As a preparation before going into the field, the filters were heated at 400^{o}C to ensure no carbon to be left on the filter and weighed before using in the field. Weight of the filters was determined using a precision balance (ME 36S, Sartorius, Göttingen, Germany). In the field, 2 L of seawater was filtered through these filters, using a 47 mm diameters, 0.7 µm porosity glass microfiber filter (GF/F) in a Millipore® all-glass filtration apparatus. After filtration, filters were allowed to dry at room temperature and were then

stored in plastic petri dishes at room temperature (22^{o}C). They were weighed again in the laboratory of ZMT Bremen. The concentration of POM per litre is determined as the difference in filter weight divided by the filtered seawater volume.

Measurement of *E. coli* were only taken in 2016. Sterilized glass bottles of 250 ml volume were provided from the Environmental Health and Engineering Centers for Disease Control (BTKL) Manado. They were filled with sea water that was collected in plastic containers from approximately of 30cm depth. The glass bottles were kept on dry ice to ensure no further growth of organisms in the water and subsequently sent to the BTKL in Manado. The company analysed my samples following the methodology of the Indonesian National Standard (SNI).

Table 6.1 Collection site were conducted in 2016 with abbreviations in high tide (HT) and low tide (LT)

Sampling points	Abbreviation	Geographic location (longitude/latitude)	Description of the location	Date of sampling	Time
Air Slobar	As1	1º37,021'/124 º45,846'	±200m from the land	16/10/16 21/10/16 21/10/16 24/10/16	08:43-09:00 (LT) 08:56-09:06 (LT) 13:52-13:58 (HT) 08:35-08:53 (HT)
Ron's Point	Rs2	1º37,089'/124 º44,945'	±50 m from the land	16/10/16 21/10/16 21/10/16 24/10/16	09:13-09:19 (LT) 09:19-09:30 (LT) 14:06-14:14 (HT) 08:59-09:19 (HT)
Tengah	Th3	1º37,089'/124 º44,945'	±200m from the land	16/10/16 21/10/16 21/10/16 24/10/16	09:41-09:49 (LT) 09:55-10:07 (LT) 14:39-14:48 (HT) 09:36-09:48 (HT)
Tanjung Parigi	Tp4	1º37,673'/124 º45,889'	±100m from the land, many sediment	16/10/16 21/10/16 21/10/16 24/10/16	10:00-10:12 (LT) 10:20-10:33 (LT) 15:00-15:08 (HT) 09:55-10:12 (HT)
Sachiko	Sa5	1º37,673'/124 º46,398'	±200m from the land	16/10/16 21/10/16 21/10/16 24/10/16	10:14-10:20 (LT) 10:45-10:53 (LT) 15:12 -15:21(HT) 10:17-10:35 (HT)
Pangalisang	Pg6	1º36,804'/124 º47,016'	±200m from the land	16/10/16 21/10/16 21/10/16 24/10/16	10:30-10:41 (LT) 11:10-11:23 (LT) 15:41 -15:52 (HT) 10:45-10:57 (HT)
Muka Kampung	Mk7	1º35,605'/124 º46,804'	±150m from the village, milky water	16/10/16 21/10/16 21/10/16 24/10/16	10:54-11:01 (LT) 11:35-11:48 (LT) 16:11-16:22 (HT) 10:01-11:20 (HT)
Likuan Tiga	Li8	1º36,312'/124 º46,025'	±500m from the land, so many organic material	16/10/16 21/10/16 21/10/16 24/10/16	11:13-11:21 (LT) 12:01-12:13 (LT) 16:37-16:50 (HT) 11:32-11:46 (HT)

Table 6.2. Collection site were conducted in 2017 with abbreviations in high tide (HT) and low tide (LT)

Sampling points	Abbreviation	Geographic location (longitude/latitude)	Description of the location	Date of sampling	Time
Bangka Island (Coral Eye)	Bi9	1º45,067'/125 º7,983'	In front of Coral Eye	22/09/2017	08:30-08:35 (HT) 14:00-14:10 (.LT)
Mikes Point	Mp10	1º37,247'/124 º44,079'	±50 m from the land	12/10/2017	09:22-09:30 (HT) 16:28-16:35 (.LT)
Alung Banua	Ab11	1º37,146'/124 º44,921'	±50 m from the land	12/10/2017	- (HT) 09:42-09:50 (.LT)
Muka Kampung	Mk7	1º35,605'/124 º46,804'	±150 m from the village	12/10/2017	08:45-08:50 (HT) 15:45-15:55 (.LT)
Moinit 1	Mo12	1º11,097'/124º29,447'	±50 m from the land	15/11/2017	16:05-16:15 (HT) 13:15-13:25 (.LT)
Moinit 2	Mo13	1º11,372'/124 º30,479'	±50 m from the land	15/11/2017	16:34-16:43 (HT) 12:30-12:38 (.LT)
Bentenan	Be14	1º00,372'/124 º53,671'	±50 m from the land	24/11/2017	10:06-10:14 (HT) 14:03-14:10 (.LT)
Tumbak	Tu15	0º58,300'/124 º53,083'	±50 m from the land	24/11/2017	08:36-08:43 (HT) 15:32-15:39 (.LT)
Tasik Ria 1	Tr16	1º24,650'/124 º42,429'	±50 m from the land	28/11/2017	10:44-10:50 (HT) 15:05-15:14 (.LT)
Tasik Ria 2	Tr17	1º24,916'/124 º42,337'	±50 m from the land	28/11/2017	11:23-11:30 (HT) 15:50-16:01 (.LT)

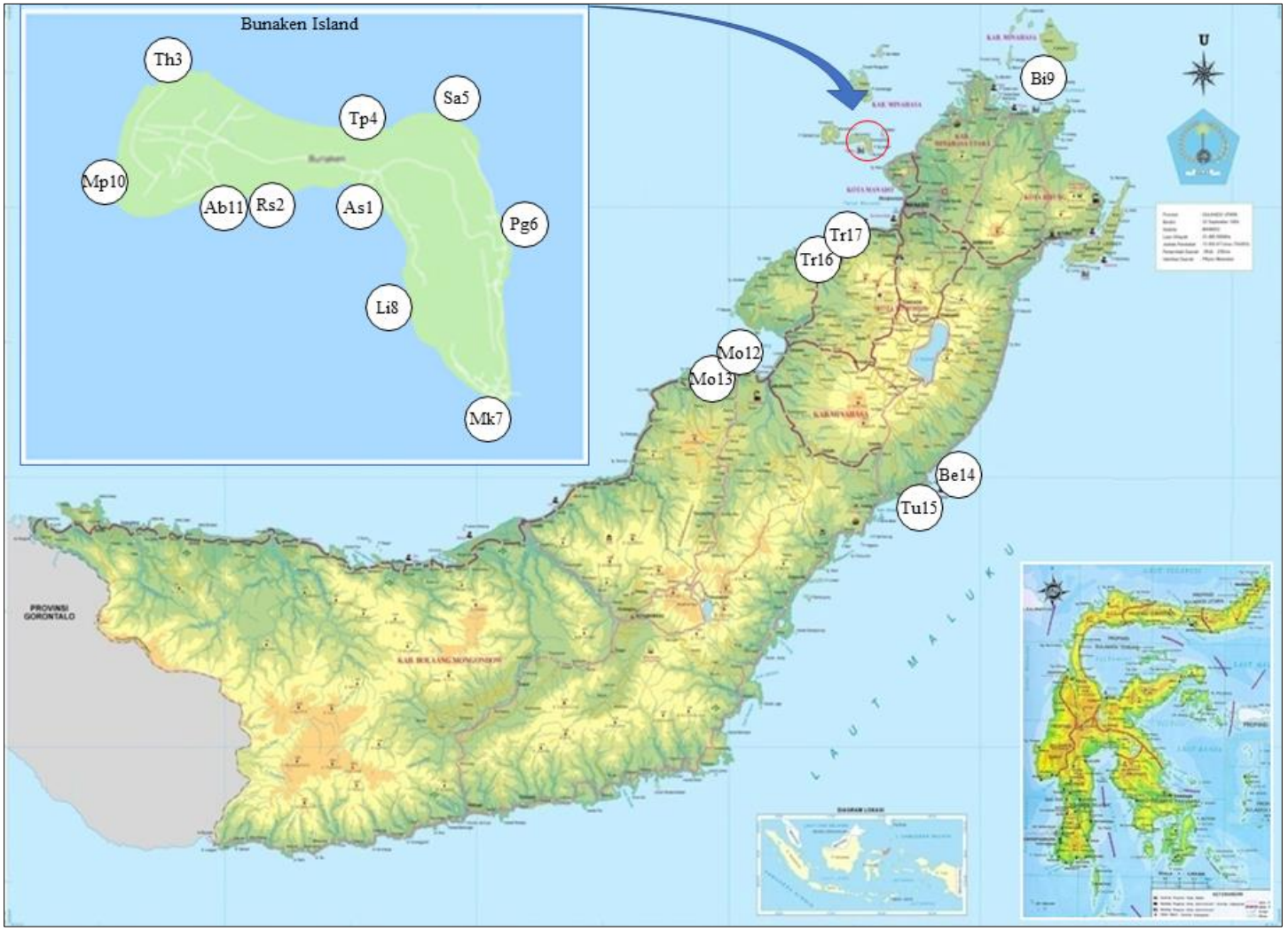

Figure 6.1 Sampling point around North Sulawesi (As1, Air Slobar; Rs2, Ron's Point; Th3, Tengah; Tp4, Tanjung Parigi; Sa5, Sachiko; Pg6, Pangalisang; Mk7, Muka Kampung; Li8, Likuan Tiga; Bi9, Bangka Island (Coral Eye); Mp10, Mikes Point; Ab11, Alung Banua; Mo12, Moinit 1; Mo13, Moinit 2; Be14, Bentenan; Tu15, Tumbak; Tr16, Tasik Ria 1; Tr17, Tasik Ria 2).

Results

Temperature

The water temperature measured in the coastal area of North Sulawesi ranged between 28.10 to 32.6°C (Table 6.3, Fig. 6.2). The highest temperature was measured during low tides (up to nearly 33°C), whereas during high tide, temperature was usually about 0.5 to 1°C lower.

Interesting is the low temperature during low tide in Bangka Island (Coral Eye). The low temperature in the waters of Bangka Island was probably due to weather conditions. When measurements were taken, the sky was cloudy, decreasing the effect of the sun heating the surface of the sea water.

Table 6.3 Temperature measurements around North Sulawesi in October 2016 (above line) and 2017 (below line). Highest va**l**ues are in bold and lowest in italics. (HT = high tide, LT = low tide).

Locality	Temperature (°C)	
	HT	LT
Air Slobar	31.53	31.48
Ron's Point	31.36	31.45
Tengah	31.14	31.01
Tanjung Parigi	31.22	31.31
Sachiko	31.23	31.08
Pangalisang	31.11	31.00
Muka Kampung	31.55	31.70
Likuan Tiga	31.46	31.73
Bangka Island	30.70	*28.10*
Mikes Point	30.80	30.60
Alung Banua	30.60	-
Muka Kampung	30.70	31.10
Moinit 1	30.30	31.10
Moinit 2	30.10	31.00
Bentenan	31.30	32.00
Tumbak	30.40	30.80
Tasik Ria 1	30.80	**32.60**
Tasik Ria 2	30.80	**32.60**

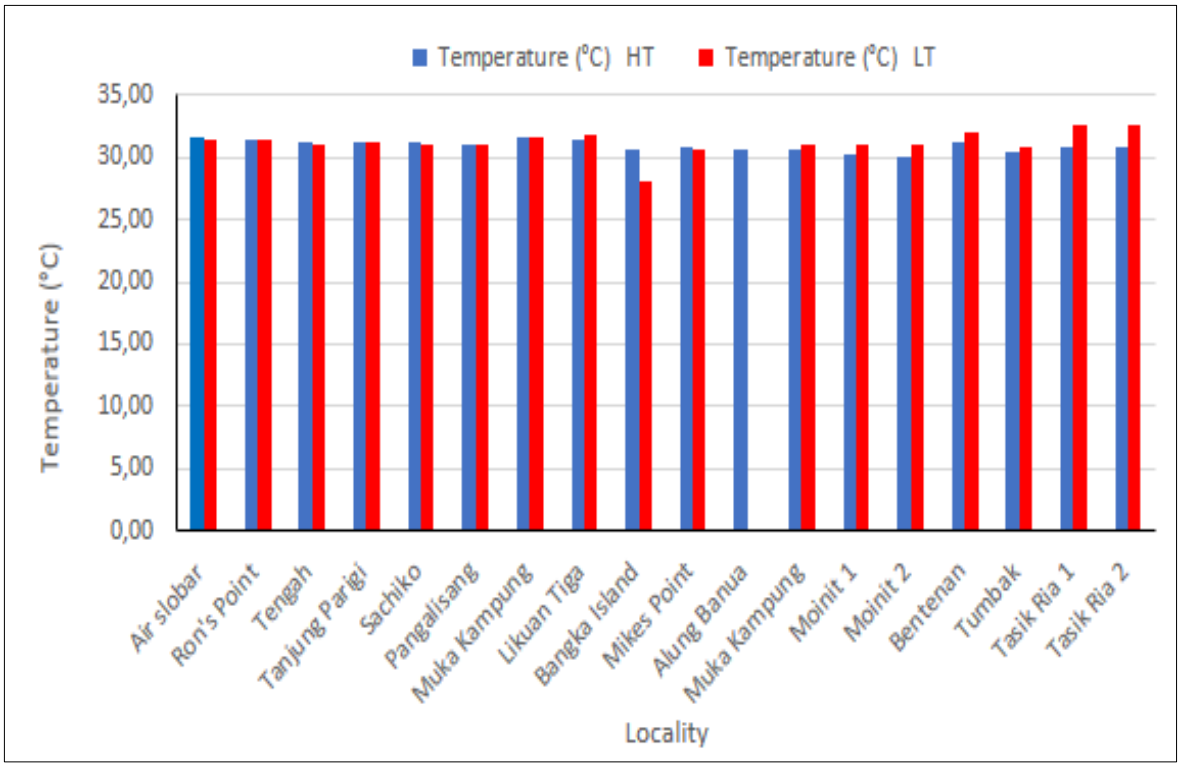

Figure 6.2 Comparison of temperature at each location during high tide and low tide.

Salinity

The salinity of the sea water along the sampling points ranged from 32.78-34.44 ‰ (Table 6.4, Fig. 6.3). The highest salinity values, higher than 34 ‰, were measured during low tide in Moinit 2. The lowest values were also measured during low tides and not as expected during high tides. However, the salinity was lower in most cases during high tides.

Table 6.4 Salinity measurements around North Sulawesi October 2016 (above line) and 2017 (below line). Highest values are in bold and lowest in italics.

Locality	Salinity (‰)	
	HT	LT
Air Slobar	*32.78*	33.00
Ron's Point	33.10	33.00
Tengah	33.13	32.95
Tanjung Parigi	33.27	33.03
Sachiko	33.12	33.10
Pangalisang	33.18	33.42
Muka Kampung	33.03	32.83
Likuan Tiga	33.20	33.13
Bangka Island	33.00	33.70
Mikes Point	33.00	33.10
Alung Banua	33.00	-
Muka Kampung	32.90	33.10
Moinit 1	33.87	33.30
Moinit 2	33.80	**34.44**
Bentenan	33.00	32.80
Tumbak	32.90	33.00
Tasik Ria 1	32.90	32.80
Tasik Ria 2	33.00	32.90

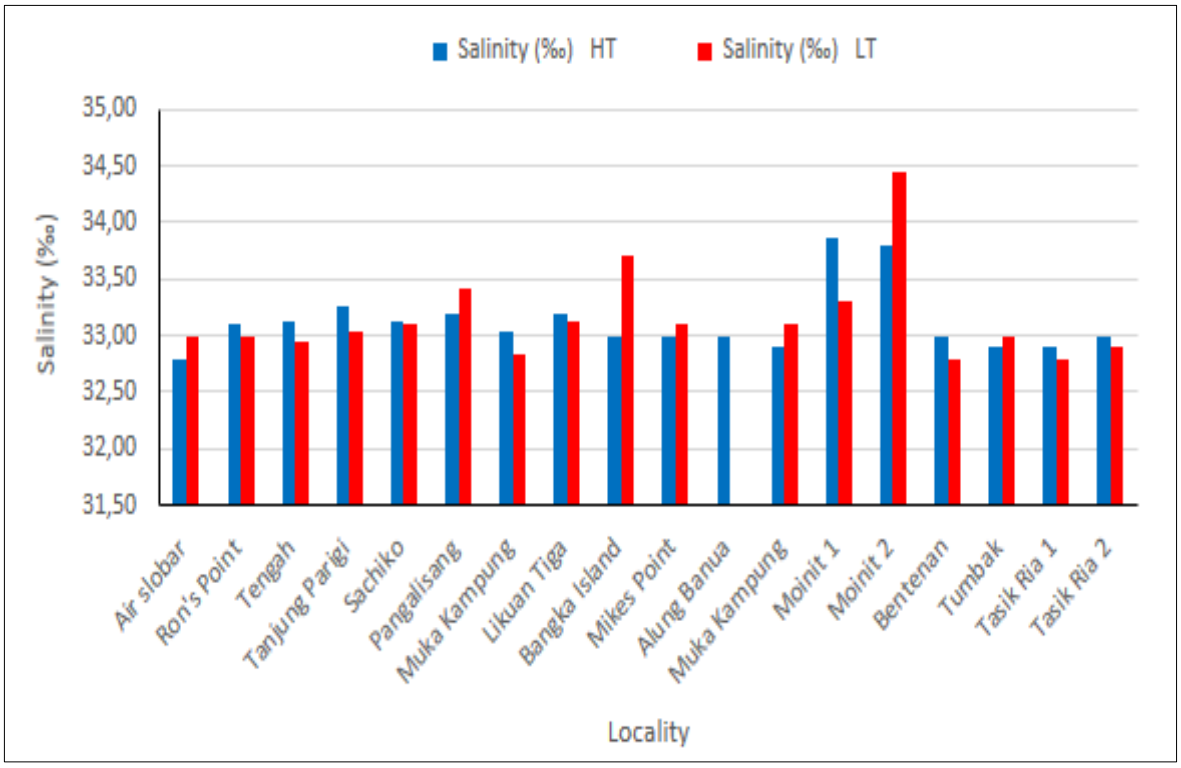

Figure 6.3 Comparison of salinity at each location during high tide and low tide

pH

Measurements were taken with a Horiba device (on loan from Faculty of Fisheries and Marine Sciences, Sam Ratulangi University, Manado) in 2016 and with a Portable Water Quality Meter (on loan from ZMT, Bremen) in 2017.

The pH values at the various sampling points ranged from 8.11 to 9.97 (Table 6.5, Fig. 6.4). Furthermore, the highest pH value was measured during low tides. These values were mainly taken around Bunaken Island. However, the pH was lower in most cases during high tides.

Table 6.5 pH was measured around North Sulawesi October 2016 (above line) and 2017 (below line). Highest values are in bold and lowest in italics.

Locality	**pH**	
	HT	LT
Air Slobar	8.87	9.36
Ron's Point	8.41	9.54
Tengah	8.87	9.18
Tanjung Parigi	8.93	**9.97**
Sachiko	9.36	9.67
Pangalisang	8.62	8.53
Muka Kampung	8.54	9.56
Likuan Tiga	9.14	9.71
Bangka Island	8.15	8.17
Mikes Point	8.18	8.15
Alung Banua	8.17	-
Muka Kampung	8.15	8.17
Moinit 1	8.30	8.30
Moinit 2	8.20	8.30
Bentenan	8.28	8.37
Tumbak	*8.11*	8.13
Tasik Ria 1	8.15	8.20
Tasik Ria 2	8.14	8.18

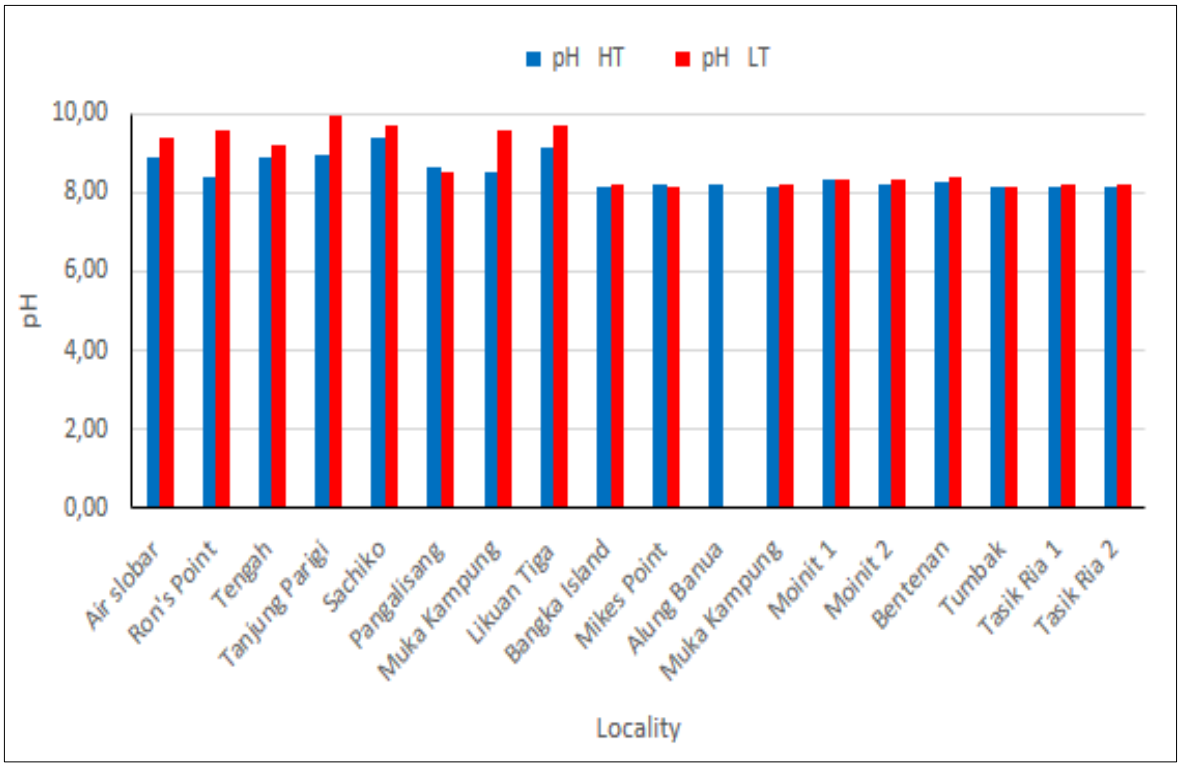

Figure 6.4 Comparison of pH values at each location during high tide and low tide measured in 2016 or 2017.

Phosphate

In order to assess the nutrient values along the coasts, phosphate was measured. Analyses of phosphate were performed in two different laboratories. The results ranged between 0.001 mg/l and 0.025 mg/l (Table 6.6, Fig. 6.5). The highest phosphate content was measured at Ron's Point during low tide and the lowest content in Muka Kampung and Likuan Tiga during high tide. Differences between low and high tides were much more pronounded in the year 2016, than in the year 2017. E.g. Muka Kampung, which was measured in both years, showed a really big difference in 2016, but nearly now difference in 2017.

Table 6.6 Measurement of phosphate around North Sulawesi October 2016 and 2017. Highest values are in bold and lowest in italics.

Locality	Phosphate (mg/l)	
	HT	LT
Air Slobar	0.008	0.010
Ron's Point	0.007	**0.025**
Tengah	0.002	0.012
Tanjung Parigi	0.004	0.014
Sachiko	0.005	0.012
Pangalisang	0.002	0.007
Muka Kampung	*0.001*	0.015
Likuan Tiga	*0.001*	0.020
Bangka Island	0.017	0.017
Mikes Point	0.011	0.012
Alung Banua	0.011	-
Muka Kampung	0.011	0.012
Moinit 1	0.012	0.012
Moinit 2	0.011	0.013
Bentenan	0.013	0.014
Tumbak	0.016	0.017
Tasik Ria 1	0.011	0.007
Tasik Ria 2	0.007	0.007

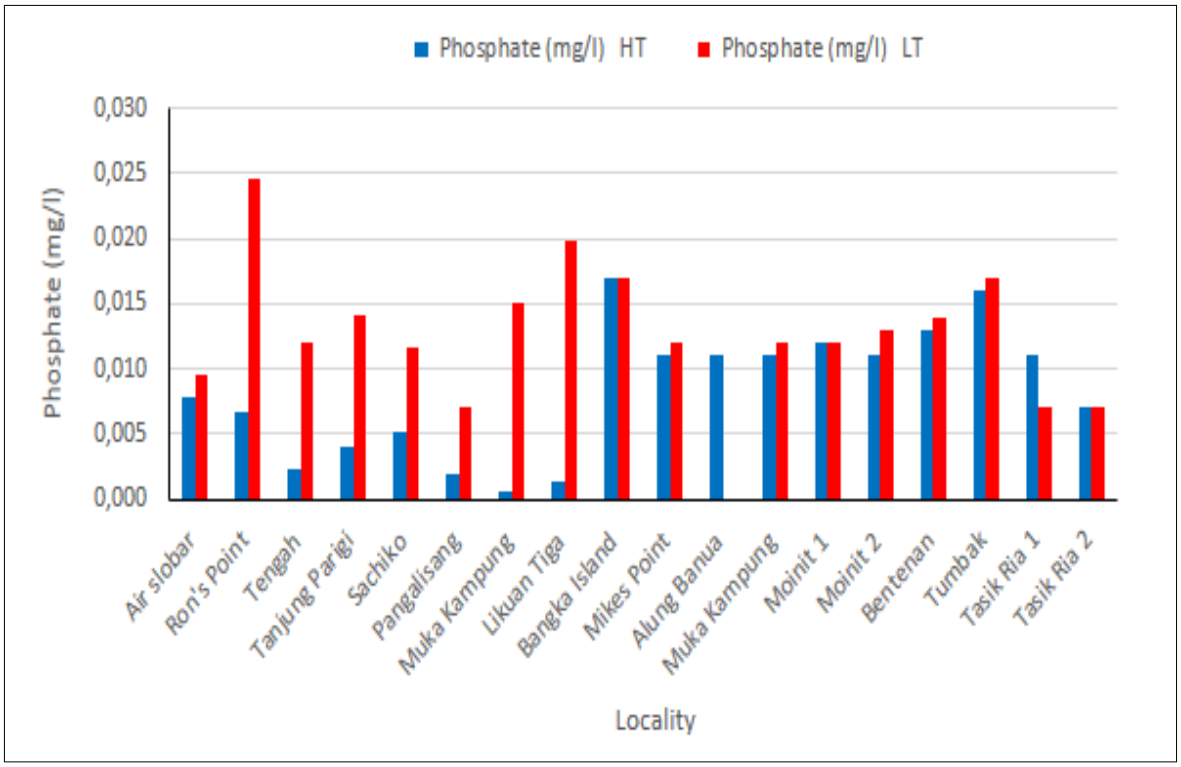

Figure 6.5 Comparison of phosphate at each location during high tide and low tide measured in 2016 or 2017.

Nitrate

As well as phosphate, nitrates are also chemical compounds that function as nutrients. To assess the nutrient situation, I investigated nitrate content in the samples taken in October 2017. The nitrate values along the coastline ranged between 0.0000 mg/l and 0.0017 mg/l (Table 6.7, Fig. 6.6). The highest nitrate was measured during low tides. The nitrate was lower in most cases during high tides.

Table 6.7 Measurement of nitrate around North Sulawesi in October 2017. Highest values are in bold and lowest in italics.

Locality	Nitrate (mg/l)	
	HT	LT
Bangka Island	0.0008	**0.0017**
Mikes Point	0.0001	0.0008
Alung Banua	0.0001	-
Muka Kampung	0.0001	0.0003
Moinit 1	0.0004	0.0002
Moinit 2	0.0002	0.0007
Bentenan	0.0003	0.0002
Tumbak	0.0010	0.0013
Tasik Ria 1	*0.0000*	*0.0000*
Tasik Ria 2	*0.0000*	*0.0000*

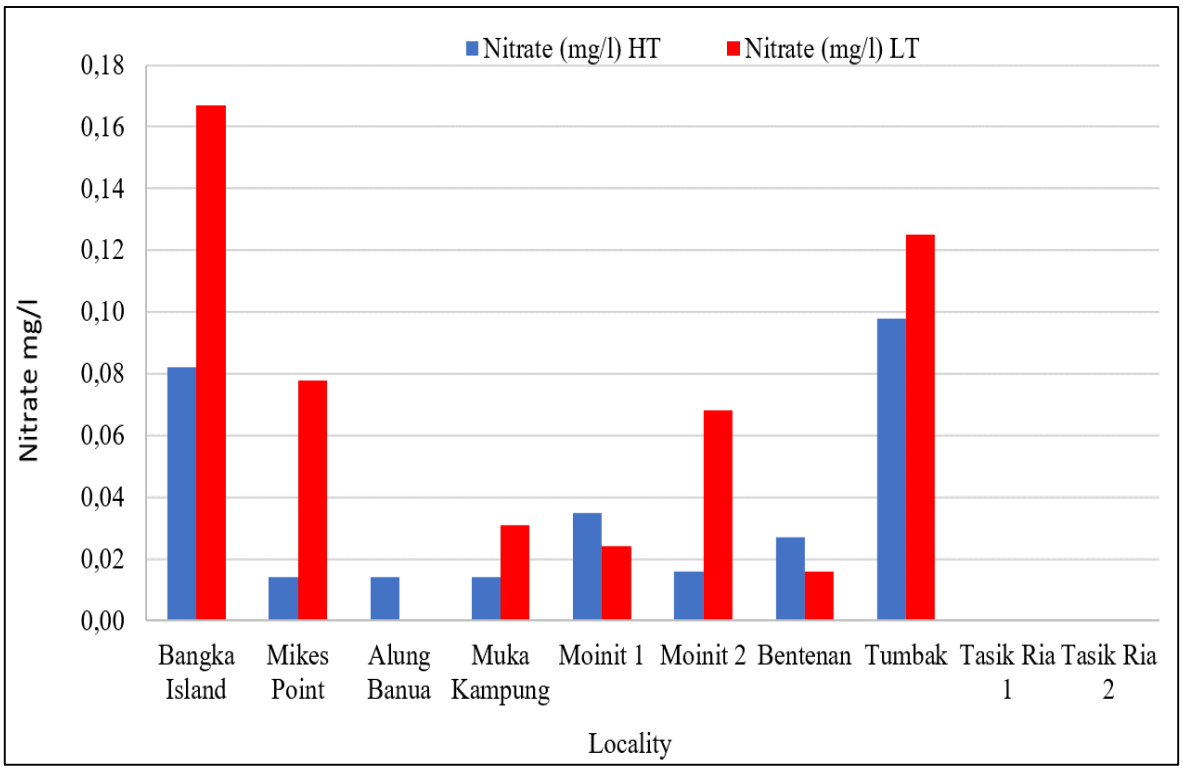

Figure 6.6 Comparison of nitrate at each location during high tide and low tide measured in 2017

Particulate Organic Matter

Particulate Organic Matter (POM) comprises suspended particles and includes phytoplankton and bacteria. The main elements are detritus that includes various substances and microorganisms that are usually associated with dead organic matter. The POM value ranged between 4.78 mg/l to 15.79 mg/l at the various localities (Table 6.8, Fig. 6.7). The highest and lowest POM values were measured during the high tide in Tasik Ria 1 and Bangka Island, respectively.

Table 6.8 Measurement of POM around North Sulawesi in October 2017. Highest values are in bold and lowest in italics

Locality	Particulate Organic Matter (POM) (mg/L)	
	HT	LT
Bangka Island	*4.78*	5.74
Mikes Point	5.83	5.87
Alung Banua	6.41	-
Muka Kampung	5.21	5.72
Moinit 1	7.00	7.00
Moinit 2	8.32	6.36
Bentenan	10.41	8.04
Tumbak	7.16	7.32
Tasik Ria 1	**15.79**	9.62
Tasik Ria 2	9.07	8.35

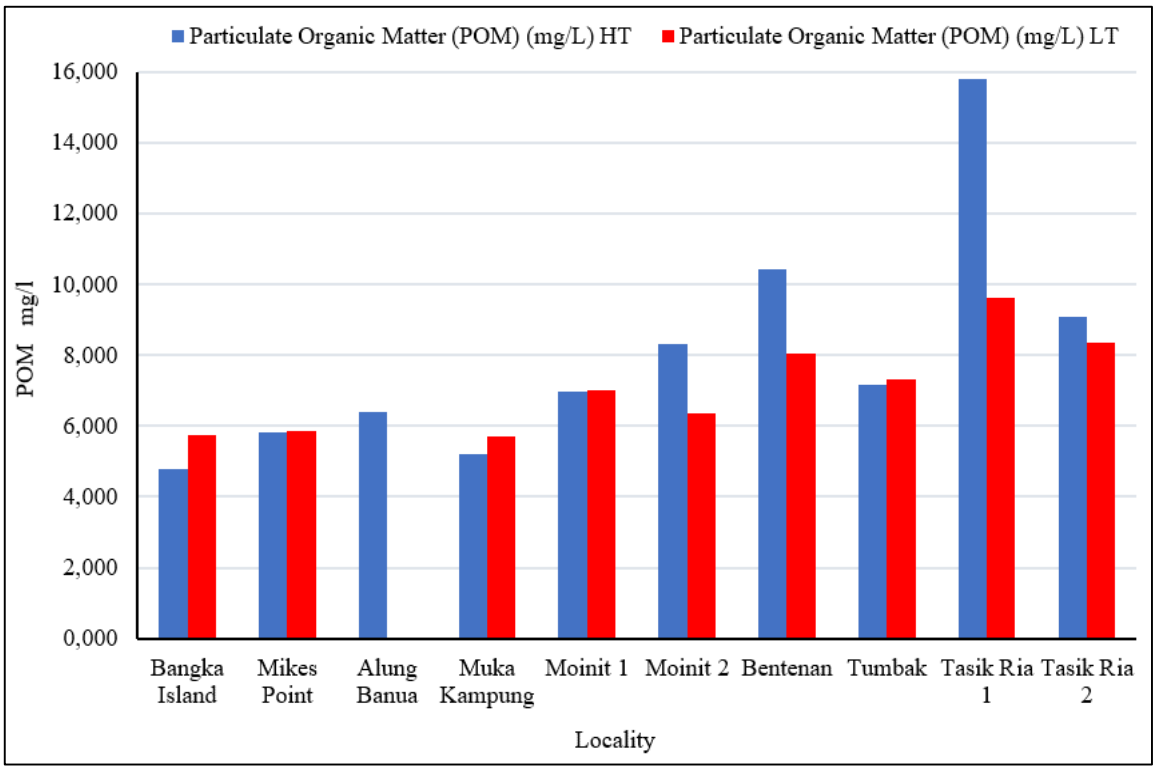

Figure 6.7 Comparison of POM at each location during high tide and low tide measured in 2017

Escherichia *coli*

E. coli indicates a pollution by human waste water and is a good indication of pollution. It was measured around Bunaken Island in October 2016. The results were negative in all sampling points (Table 6.9).

Table 6.9 Measurement of *E. coli* around Bunaken Island North Sulawesi in October 2016.

Locality	*E. coli*	
	HT	LT
Air slobar 1	Negative	Negative
Air slobar 2	Negative	Negative
Ron's Point	Negative	Negative
Tengah	Negative	Negative
Tanjung Parigi	Negative	Negative
Sachiko 1	Negative	Negative
Sachiko 2	Negative	Negative
Pangalisang	Negative	Negative
Muka Kampung	Negative	Negative
Likuan Tiga	Negative	Negative

Discussion

Temperature

Water temperature is an important parameter for the physical and biochemical processes occurring within water as well as in air-water interactions, because temperature regulates physical, chemical, and biological processes in water. Water temperature also influences the solubility, and thus availability of various chemical constituents in water. Most importantly, this parameter affects dissolved oxygen concentrations in water, as oxygen solubility decreases with increasing water temperature. Yulius et al. (2018) mentioned that the temperature in Indonesian waters generally ranges between 27-32°C, and this is confirmed in many different studies from various areas of Indonesia (e.g. Yusron 2008, Patty 2013, Guntur et al. 2017, Mudeng et al. 2015). In my study, the measurements show a similar variability, ranging around 28-32.6ºC, with the highest value in Tasik Ria (Table 6.3, Fig. 6.2). Similar or lower temperatures were observed around North Sulawesi by other studies.

Manado Bay ranged from 29-31ºC (Ijong 2011), or 28-29.7ºC (Kalangi et al. 2013). Both studies show lower temperatures, as my results, which raised up to nearly 33° C in Tasik Ria during low tide. This locality is close to Manado Bay. Other studies covering further localities, e.g. Bangka Strait, Sumbawa Island and West Nusa Tenggara, ranged from 29.20-31.57ºC (Saraswati et al. 2017); waters of Southern Bangka Strait ranged from 28.5-30.8 (Gaol et al. 2017); their results are similar to my results. Some studies in Indonesia indicate lower values, e.g. in Gerupuk Bay, West Nusa Tenggara, where temperature ranged between 26-29ºC (Putra et al. 2014), but these values differed from another study from the same area which showed a somewhat higher range from 27-30ºC (Erlania et al. 2014). Knauss (1997) and Effendi (2003) emphasized that the temperature of a water body is influenced by the position of the sun, geographical location, seasons and atmospheric conditions. Other factors that also affect the water temperature is bathymetry (Xie et al. 2002) and even the mountains on the mainland (Kitoh 2001). The water bodies around North Sulawesi are strongly influenced by Pacific Ocean conditions, where temperature changes are also often caused by El Niño events (NOAA 1994). Furthermore, some areas in North Sulawesi are upwelling zones, where cooler temperatures are typical. In general, temperature conditions around North Sulawesi's coastlines are still within the typical range known from tropical waters.

Salinity

Salinity has an important role in supporting the life of aquatic biota. The average salt content of seawater in the open ocean is around 35‰. Two properties, that are highly determined by the amount of salt in the sea (salinity), are electrical conductivity (conductivity) and osmotic pressure. It is mainly the ecosystems of the eulittoral, that show variances in salinity due to rain or evaporation (Hutabarat & Evans 1985). Estuary waters or the area around river systems transporting fresh water can have a complex salinity structure, with a wide range of salinity from brackish to marine, but because of the lower density, freshwater can also form surface layers of low salinity, which has an effect on plankton or intertidal reef flats. In these layers with homogeneous salinity, temperature is also usually

homogeneous. Tides can have a strong effect on these layer formations and are therefore crucial for the eulittoral and sublittoral habitats.

Hadikusumah & Sugiarto (2001) described salinity ranges in water bodies of North Sulawesi of 33.7-33.8‰, whereas Yusron (2008), described the salinity ranging from 31-32‰, and Patty (2013) described the salinity ranging from 28-33‰. These salinity values were lower than in my study. Hickey et al. (1998), Fong and Geyer (2001), Talley (2002), Garrison (2004), Kalangi (2008), and Kalangi et al. (2012) mentioned that horizontal layer formation with differing salinity is caused by patterns of water circulation, evaporation, rainfall and rivers. Thus differences in the salinity value of sea water from the same locality can be caused by the occurrence of disturbances (mixing) due to sea waves or the mass movement of water caused by wind (Banjarnahor 2000). Dahuri et al. (1996) mentioned that the salinity value in Indonesia waters range between 32-34‰, which seems very high, whereas Nonji (2005) mentioned that in general, the salinity value of the Indonesia waters range between 28-33‰. It is also mentioned in literature that high salinity in the surface layer is generally found in waters far from the coastline (Kalangi et al. 2013). In contrast to these statements, the highest salinity values in my study with 34.5‰ were measured at Moinit 2 near the coastline and close to hot springs during low tide, probably caused by high evaporation, as well as further increase of salts by the hot springs. The lowest salinity values (32.8‰) were still much higher than the lowest values mentioned for Indonesian water bodies and were measured in Air Slobar. Romimohtarto & Thayib (1982) suggested that for coastal areas salinity ranged from 32 to 34‰, which exactly shows the range of my measurements between 32.78 and 34.44‰. The measurements taken for this study were not performed during raining periods, and were not taken really close to any river systems, thus the values do not have a large range, with only slightly lower salinity during low tides in few cases, where a small influence of close by river systems might be seen (Air Slobar, Tasik Ria). Areas with larger intertidal reef systems, like Muka Kampung, showed a slightly increased salinity.

pH

The pH value is an important parameter in monitoring the stability of water. Changes in the pH value affects the life of the biota, because each biota has certain limitations on varying pH values (Simanjuntak 2012). It determines phytoplankton composition, which affects the level of primary productivity in the waters (Megawati et al. 2014).

The pH values measured in the frame of my project ranged from 8.11-9.97. Available pH values measured around Sangihe (7.8-7.9) (Mudeng et al. 2015), around Bali Strait (8.41-9.49) (Megawati et al. 2014) or east part of the coastal area of Surabaya (6.8-7,8) (Guntur et al. 2017), are always lower than my values, which were partly extremely high. Paramitha (2014) discussed that an increase of pH values from the estuary to the sea can be caused by the input of waste from land (river) to the aquatic environment. According to Salm (1984), the pH values in normal waters ranges from 8.0-8.3. In general, sea water is relatively more alkaline (alkaline) around 8.0, whereas Minister of Environment Decree (KLH, 2004) mention a range between 7-8.5 for Indonesia; however, these can deviate from the specific data provided for some localities, as is mentioned above. If the pH values are low (high acidity), the dissolved oxygen content will decrease, consequently oxygen consumption will decrease (Mudeng et al. 2015). US-EPA (1973) suggested that pH values ranging from 6.5-8.5 are still good for fish and according to Edward & Tarigan (2003) pH values between 6-9 are also still good for coral reefs. Many values measured in my project are lying within this range. However, in 2016, when measuring around Bunaken Island, very high values always above 9, and up to 9.97, were measured especially during low tide, whereas during high tide, values were lower. I have to emphasize here that in 2016, I used a device (Horiba), that was borrowed from Faculty of Fishery and Marine Sciences. It was said to be maintained, but measurements taken by another device, the Portable Water Quality Meter from ZMT Bremen, in the next year, indicate that the values taken with the Horiba device are far too high. The Portable Water Quality Meter is maintained by a technician in the ZMT, who also introduced me thoroughly in the measuring techniques. It can be assumed that the sensors, that actually have to be replaced on a regular schedule, were not properly calibrated. The values from 2017 always indicate a small increase of pH values during low tide.

Phosphate

Phosphate is an important nutrient and one of the indicators for determining the fertility of a water body (Makatita et al. 2014). Classification in terms of phosphate levels according to EPA (2002) is as follows: <0.048 mg/l, which is classified as low; between 0.048-0.096 mg/l classified as moderate, and >0.096 mg/l as high. Especially human activities on land can cause high values of phosphate, due to discharge and degradation of organic waste such as detergents, fertilizers or organic material (Saraswati et al. 2017). The values of phosphate in this study ranged between 0.001-0.025 mg/l. Patty (2014) mentioned average phosphate levels in North Sulawesi waters ranging from 0.0007-0.0246 mg/l. This shows that these water bodies are quite fertile (Patty 2014). Usually, higher nutrient levels can be observed at the sea floor, caused from the decomposition of flora and fauna (Edward & Tarigan 2003; Muchtar & Simanjuntak 2008). Whereas low values of phosphate in the surface layer can be caused by intensive phytoplankton growth (Patty 2015). Ilahude and Liasaputra (1980) suggested that the values of phosphate in the surface layers of the world's most fertile waters are close to 0.019 mg/l. Furthermore, Ketchum (1969) mentioned the values of phosphate of 0.087 mg/l as the upper limit in uncontaminated water. My measurements of the surface water indicate that this water is fertile, but not contaminated. Elevated levels during low tide indicate influence from the back land (especially here at Ron's Point). Very low levels especially during high tide indicate the exchange of the water masses. However, low values during low tides, as was seen in Tasik Ria, can also indicate a higher bacterial or phytoplankton growth during low tide in this specific area.

Nitrate

Nitrate is a chemical compound that serves as a nutrient and is very important in supporting the integrity of the aquatic ecosystem. Normal nitrate values in the sea waters generally ranges between 0.001-0.007 mg/l (Brotowijoyo et al. 1995) and the threshold values determined by US-EPA (1973) for nitrate is 0.07 mg/l. Hutagalung & Rozak (1997) stated that higher nitrate values are typical for water bodies close to the coast. In my study, the values of nitrate ranged between 0.0000 and 0.0017 mg/l (Table 6.7, Fig. 6.6). Therefore,

all sampled localities were within the normal range. Nevertheless, nitrate values differed between sampling points with the highest value on the island of Bangka. My specific sampling point on Bangka Island, Coral Eye, is close to a former mining area, with open land and earth erosion. This might lead to a higher nitrate content. On the other hand, Patty (2014) showed that the value of nitrate in Gangga Island ranged between 0,019-0,026 mg/l, therefore even higher than on Bangka Island. Gangga Island lies close to Bangka Island and also within the Strait and is thus exposed to similar water masses. In contrast, Siladen Island, which lies very close to Bunaken Island and which is much more exposed to the open ocean show less than 0,005 mg/l. This is conforming with my observations around Bunaken Island. Similar to phosphate, the amount of nitrate is influenced by bacterial and phytoplankton growth. Both organismal groups consume nitrate, and therefore, similar to low phosphate levels, low nitrate levels can also indicate plankton growth. To address this question, particulate organic matter (POM) was measured.

Particulate Organic Matter

By definition, organic material comprises dissolved organic material, suspended (particulate) organic material, and colloids (Yulius et al. 2018). Decomposition of organic matter is influenced by several factors such as residual composition, temperature, pH, and the availability of nutrients and oxygen (Arif 1999). According to Ministry of Environment Decree (KLH 1994), normal values of POM for coral reefs are 20 mg/l.

In my study, the values of particulate organic matter (POM) ranged between 4.78-15.79 mg/l. The highest values of POM were measured in Tasik Ria. A sea grass meadow and mangrove are close by, which might increase organic material (Arif 1999). The sampling point is also close to the estuary of a river, thus a higher import of phosphate and nitrate from land is very likely. However, interesting are the low values of phosphate and nitrate in Tasik Ria, actually the lowest measured (see Table 6.6 and Table 6.7). This indicates a rapid uptake of the nutrients and a bacterial and/or phytoplankton growth. Bangka Island exhibited the lowest POM values. This locality was also characterized by higher nitrate and phosphate levels.

Escherichia coli

E. coli was used as an indicator of human and animal faeces contamination. *E. coli* is a pathogen in the human intestinal tract that can cause diseases, like diarrhea. Therefore, the presence of *E. coli* beyond a certain threshold indicates that the sanitation conditions are insufficient (Sperling 2007). According to Minister of Environment Decree Republic of Indonesia number 51 of 2004 on water quality standard for marine life, the usual presence of *E. coli* with 200 MPN per 100ml is normal (Most Probable Number (MPN)/100 ml). Despite the presence of several resorts and small villages close to the sample points, no bacterial contamination was found.

Chapter 7

General Discussion

The surveys in North Sulawesi, Indonesia i.e. in the Bunaken National Park (Burghardt et al. 2006; Kaligis et al. 2018; Eisenbarth et al. 2018), Lembeh Strait (Ompi et al. 2019), in the island of Sangihe (Undap et al. 2019), and around Bangka Archipelago (Papu et al. 2020) revealed a previously unknown diversity of marine heterobranchs in these regions. My study in Sangihe Island is the first survey of marine heterobranch around this island and included in the collection 23 species, with Phyllidiidae show the highest dominance. The amount of species is far lower than in the studies around BNP, or the Bangka Archipelago. I was not involved in collection, but learned from the participating colleagues, that weather conditions were unfavorite. It was actually the year of the strong El Niño, with heavy rainfalls and a lot of land erosion, transporting lots of sediment into the habitats. It also did not allow the diving in more favorite habitats, and strong currents further hindered sampling. The overall number of species is certainly much higher and more collecting events are necessary to address diversity in this remote area. In my study here I could show one of the very few new records of a rather rare *Plakobranchus* species, which was only found recently as a cryptic form in the genus *Plakobranchus* (Yonow and Jensen 2018). I did not barcode the specimens but the color patterns allow the tentative assignment to *P. papua*. I was also able to record the first species mimicry in Indonesia, with *Chromodoris annae*, which is mimicking *C. elisabethina*. Mimicry in *Chromodoris* species is not well studied yet and only started in more detail with the extensive paper of (Layton et al. 2018), who investigated several mimicry rings in *Chromodoris* species. Another highlight of my study is the discovery of a new *Phyllidia* species, despite the few species records at all.

For studying the Chromodorididae, 375 specimens were collected around North Sulawesi in 2015, 2016 and 2017. Chapter 4 and chapter 5 focus on this family Chromodorididae. The phylogenetic hypothesis based on two mitochondrial genes, CO1 and

16S, is the most comprehensive one including 462 sequences and the only subsequent study after the first study published in 2012 by Johnson & Gosliner. The major result of my analyses is the confirmation of the results obtained by Johnson & Gosliner 2012 at that time. However it already revealed many cryptic species within clades of morphologically very similar specimens. This was more studied in detail for a few *Chromodoris* species, where a high number of sequences were available. Chapter 5 focuses on these species of the genus *Chromodoris* (i.e. *C. annae, C. dianae, C. willani, C. lochi*) of which hundreds of specimens were collected. Except of *C. willani,* all other species can be divided into 2 or more clades. Interesting in this context is the identification of clades that are only distributed in a small area, and thus rather endemic, like clade 1 of *Chromodoris dianae*. The close relationship with *C. willani* is also interesting, because haplotype networks indicate a kind of reticulate relationship of *C. willani* with both *C. dianae* clades. However, it has to be emphasized that for clarifying the relationships, nuclear markers should also be included in studies like this.

My water quality analyses showed normal results in all sampling points. Despite the assumed increased pollution due to higher populations, tourism and other human activities, the values for all abiotic factors were within the range of the Standards as outlined by Minister of Environment (2004). However, sampling of the water for the various analyses were performed with partly inadequate equipment, and statements about the quality of a habitat need much more and denser sampling schedule. It can only be a first glimpse on the area. However, it also showed the importance of adequate equipment for performing these studies. Maintenance and proper care of the devices is a critical issue, that needs more attendance in the future, if investigations like this should be performed in the future at Sam Ratulangi University.

Chapter 8

General Conclusion

In conclusion, the following major findings can be summarized:

The biodiversity of sea slugs around Sangihe is certainly under-sampled. However, first results indicate differences in comparison to the BNP; species composition marks a higher trend towards Anthobranchia. The results also indicate that molecular barcoding is very important to verify preliminary identification based on colour.

With the new data that the systematics re-arranged by Johnson & Gosliner (2012) is rather stable and all genera valid. However, to emphasize here, that the same mitochondrial genes were used. It cannot be ruled out, that by including nuclear genes, the phylogenetic relationships might change.

The results about *Chromodoris* species clearly show wide spread cryptic speciation of *Chromodoris* species with a probably much more limited distribution as was considered before. Some of the cryptic species co-occur (e.g. *C. annae*, *C. dianae*), whereas in *C. lochi*, the cryptic species seem to be confined to certain localities. It clearly shows that molecular analyses are essential for studying biodiversity in this region and that haplotype network analyses can give a deep insight in speciation processes.

The results with regard to water quality, indicate that all sampling points are within the range of normal values and no specific pollution can be seen. The higher amount of POM together with a higher temperature especially in Tasik Ria, seems to be the most problematic issue, indicating a eutrophication in contrast to all other places. However, these results are very preliminary and definitely need further investigation for final statements.

Outlook

Indonesia as a country that consists of thousands of islands, and the study of marine diversity in this country, including the one of marine Heterobranchia, is still very uncommon. In the future, investigating state of the art diversity of these sea slugs in the other areas around Indonesia that have not been yet explored is needed, with following monitoring programs, in order to minimize the negative impacts on the environment in the future and to help to build up a sustainable use of the natural resources on and around Indonesia. Furthermore, future studies should focus on producing formal descriptions for the undescribed species in this study and continue to explore understudied habitats that likely contain novel diversity.

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Appendix

1. Participation in other studies during my thesis

This project funded from the BMBF in order to find new drug leads was a joint project between 3 working groups, involving many students and different studies. Because we were working in a network, I also contributed to several other studies, where my results were used and are now already published (4 publication), or will be published. In the following, I explain my input into these studies:

1. Eisenbarth JH, **Undap N**, Papu A, Schillo D, Dialao J, Reumschüssel, S, Kaligis F, Bara R, Schäberle TF, König GM, Yonow N, Wägele H. 2018. Marine Heterobranchia (Gastropoda, Mollusca) in Bunaken National Park, North Sulawesi, Indonesia - a follow-up diversity study. Diversity 10, 127

   In this study, I helped in identification the material brought back from the first collecting trip in 2015. I already provided the first barcoding results mainly of the dorids and helped in the figures.

2. Ompi M, **Undap N**, Papu A, Wägele H. 2019.Monitoring marine Heterobranchia in Lembeh Strait, North Sulawesi (Indonesia), in a changing environment. AACL Bioflux 12, 664-677

   In this study, I sequenced all material brought back from Lembeh Strait, and performed the identification of many species and helped in writing the manuscript.

3. Schillo D, Wipfler B, **Undap N**, Papu A, Böhringer N, Eisenbarth J-H, Kaligis F, Bara R, Schäberle T, König GM, Wägele H. 2019. Description of a new *Moridilla* species from North Sulawesi, Indonesia (Nudibranchia: Cladobranchia, Aeolidioidea) – based on MicroCT, histological and molecular analyses. Zootaxa 4652, 265-295.

   For this study, I helped in collecting the animals, in identification and barcoding the specimens, thus providing also sequences.

4. Papu A, **Undap N**, Martinez NA, Segre, Mr. Datang IG, Kuada RR, Perin M, Yonow N, Wägele H. 2020. First study on marine Heterobranchia (Gastropod, Mollusca) in Bangka Archipelago, North Sulawesi, Indonesia. Diversity, 11.

   For this study, a large collection, that I sampled in 2017, was the base. I provided the sequences of all Chromodorididae, but also for many other species. I provided the identification of many of other species used in this study.

5. Schillo D, et al. (in preparation): On three new *Noumeaella* species from North Sulawesi

   In this study, I provided material from Bangka Island, that I collected and helped in sequencing. I also provided all metadata and pictures, that will be used for the publication

6. Hertzer Cora (in preparation): On defence systems in Chromodorididae

   In this study, which actually forms the main doctoral thesis of Cora Hertzer (Institute of Pharmaceutical Biology, Univ. Bonn), I provided material, as well as the proper identification, phylogenetic analyses of the Chromodorididae, information about specimens which are closer related and which are not. I re-sequenced material for this project and also provided DNA from targeted species. I was standing in close contact during two years, to provide the necessary information for the specific investigations of Cora.

7. Bara R. et al. (in preparation): On comparison of the antimicrobial properties from marine Heterobranchia (Gastropoda, Mollusca) collected from various regions of North Sulawesi, Indonesia.

   For this study, I helped in collecting the animals, in identification and barcoding the specimens, thus providing also sequences.

2. Table S.1 List of specimens used for the Chromodorididae analyses, with voucher ID, sampling localities, GenBank, accession numbers, abbreviation, and Area Geographic Data

Original name of species in NCBI	Voucher ID	Locality	CO1	Abbreviation	Latitude	Longitude
Chromodoris annae	CASIZ120926	Kwajalein Atoll, Marshall Island	MG883100	Camais	-	-
Chromodoris annae	CASIZ176672	Bohol, Philippines	MG883101	1Cabophil	9º00’31.10”N	123º00’41.30”E
Chromodoris annae	CASIZ181479	Bohol, Philippines	MG883102	2Cabophil	9º33.5”N	123º48.6”E
Chromodoris annae	CASIZ191402	Madang, Papua New Guinea	MG883103	Camapap	-	-
Chromodoris annae	UF322440	Baluan Island, Papua New Guinea	MG883105	Cabalpap	2º32’18.26”S	147º18’05.61”E
Chromodoris annae	UF323418	Sherburne Reef, Papua New Guinea	MG883106	Casrpap	2º53’38.61”S	147º20’33.72”E
Chromodoris annae	UQ1	Lizard Island, QLD, Austalia	MG883107	Caliaus	-	
Chromodoris annae	WAMS67513	Sulawesi, Indonesia	MG883108	1Casul	-	
Chromodoris annae	WAMS67514	Sulawesi, Indonesia	MG883109	2Casul	5º29’4”S	123º49’.7”E
Chromodoris annae	WAMS67515	Sulawesi, Indonesia	MG883110	3Casul		
Chromodoris annae	WAMS67516	Sulawesi, Indonesia	MG883111	4Casul	2º28’59.26”S	123º44’49.88”E
Chromodoris annae	WAMS67519	Sulawesi, Indonesia	MG883114	5Casul	5º28’24”S	123º44’.25”E
Chromodoris annae	WAMS67520	Sulawesi, Indonesia	MG883115	6Casul	5º28’24”S	123º44’.25”E
Chromodoris annae	WAMS67522	Sulawesi, Indonesia	MG883116	7Casul	5º28’24”S	123º44’.25”E
Chromodoris annae	WAMS67523	Sulawesi, Indonesia	MG883117	8Casul	5º28’24”S	123º44’.25”E
Chromodoris annae	WAMS67524	Sulawesi, Indonesia	MG883118	9Casul	5º28’24”S	123º44’.25”E
Chromodoris annae	WAMS67535	Sulawesi, Indonesia	MG883119	10Casul	-	-
Chromodoris annae	WAMS75456	Hibernia Reef, Australia	MG883120	Cahibaus	-	-
Chromodoris annae	CASIZ158677	Caban Island, Batangas, Philippines	JQ727829	2Cabaphil		
Chromodoris annae	CASIZ121261	Rottnest Island, Western Australia	JQ727830	Cariaus		
Chromodoris dianae	CASIZ158686	Batangas, Philippines	JQ727836	1Chdibaphil	-	-
Chromodoris cf. *dianae*	CASIZ177241	Batangas, Philippines	MG883143	2Chdibaphil	13º41’27.96”N	120º50’29.039”E
Chromodoris cf. *dianae*	CASIZ182289	Romblon, Philippines	MG883144	Chdirophil	12º36’57.564”N	122º15’8.315”E
Chromodoris cf. *dianae*	CASIZ200677	Occidental Mindoro, Philippines	MG883145	Chdiminphil	13º46’51.996”N	120º6’8.423”E
Chromodoris cf. dianae	WAMS67531	Sulawesi, Indonesia	MG883146	1ChdiSul	5º28’24”S	123º44’.25”E
Chromodoris cf. *dianae*	WAMS67532	Sulawesi, Indonesia	MG883147	2Chdisul	5º28’24”S	123º44’.25”E
Chromodoris cf. *dianae*	WAMS67536	Sulawesi, Indonesia	MG883148	3Chdisul	5º28’24”S	123º44’.25”E
Chromodoris cf. *dianae*	WAMS67592	Taiwan, China	MG883149	ChdiChin	21º59’44”59N	120º42’08.89”E
Chromodoris willani	CASIZ 159385	Mooloolaba, Queensland, Australia	JQ727861	1Cwaus		
Chromodoris willani	-	Mooloolaba, Queensland, Australia	JQ727862	2Cwaus		
Chromodoris willani	CASIZ176673	Bohol, Philippines	MG883370	Cwbophil	9°00'29.40"N	123°00'56.00"E

Chromodoris willani	CASIZ191052	Madang, Papua New Guinea	MG883371	1Cwpap	5°10'46.5"S	145°49'47.1"E
Chromodoris willani	CASIZ191103	Madang, Papua New Guinea	MG883372	2Cwpap		
Chromodoris willani	CASIZ202316	Batangas, Philippines	MG883373	Cwbaphil		
Chromodoris willani	UF352011A	Okinawa, Japan	MG883374	1Cwjap	26°43'49.63"N	127°49'25.93"E
Chromodoris willani	UF352011B	Okinawa, Japan	MG883375	2Cwjap	26°43'49.63"N	127°49'25.93"E
Chromodoris willani	WAMS67599	Sulawesi, Indonesia	MG883376	1Cwsul	5°28'13.54"S	123°45'24.85"E
Chromodoris willani	WAMS67600	Sulawesi, Indonesia	MG883377	2Cwsul		
Chromodoris willani	WAMS67601	Sulawesi, Indonesia	MG883378	3Cwsul		
Chromodoris willani	WAMS67602	Sulawesi, Indonesia	MG883379	4Cwsul		
Chromodoris willani	WAMS67603	Sulawesi, Indonesia	MG883380	5Cwsul		
Chromodoris willani	WAMS67604	Sulawesi, Indonesia	MG883381	6Cwsul		
Chromodoris willani	WAMS67605	Sulawesi, Indonesia	MG883382	7Cwsul		
Chromodoris willani	WAMS67606	Sulawesi, Indonesia	MG883383	8Cwsul		
Chromodoris willani	WAMS67607	Sulawesi, Indonesia	MG883384	9Cwsul	5°28'13.54"S	123°45'24.85"E
Chromodoris willani	WAMS67608	Sulawesi, Indonesia	MG883385	10Cwsul	5°28'13.54"S	123°45'24.85"E
Chromodoris willani	WAMS67609	Sulawesi, Indonesia	MG883386	11Cwsul	5°28'13.54"S	123°45'24.85"E
Chromodoris willani	WAMS67610	Sulawesi, Indonesia	MG883387	12Cwsul	5°28'13.54"S	123°45'24.85"E
Chromodoris willani	WAMS67611	Sulawesi, Indonesia	MG883388	13Cwsul		
Chromodoris lochi	CASIZ181566	Bohol, Philippines	MG883237	Clbophil	9º30.96”N	123º40.8”E
Chromodoris lochi	CASIZ182290	Romblon, Philippines	MG883238	Clrophil	12º36’57.564”N	122º15’8.315”E
Chromodoris lochi	CASIZ185077	West Papua, Indonesia	MG883239	Clwpap		
Chromodoris lochi	CASIZ191120	Madang, Papua New Guinea	MG883240	1Clmapap		
Chromodoris lochi	CASIZ191264	Madang, Papua New Guinea	MG883241	2Clmapap		
Chromodoris lochi	UF295733	Viti Levu, Fiji	MG883242	Clfiji	17º39’57.28”S	179º03’44.36”E
Chromodoris lochi	UF322447	Sherburne Reef, Papua New Guinea	MG883243	Clsrpap	2º53’38.61”S	146º20’33.72”E
Chromodoris lochi	UF323419	Kimbe Bay, Papua New Guinea	MG883244	Clkimpap	5º17’52.70”S	150º07’43.35”E
Chromodoris lochi	WAMS67551	Sulawesi, Indonesia	MG883247	1Clsul		
Chromodoris lochi	WAMS67552	Sulawesi, Indonesia	MG883248	2Clsul		
Chromodoris lochi	WAMS67553	Sulawesi Indonesia	MG883249	3Clsul		
Chromodoris lochi	WAMS67554	Sulawesi, Indonesia	MG883250	4Clsul		
Chromodoris lochi	WAMS67555	Sulawesi, Indonesia	MG883251	5Clsul		
Chromodoris lochi	WAMS67556	Sulawesi, Indonesia	MG883252	6Clsul	5º28’13.54”S	123º45’24.85”E
Chromodoris lochi	WAMS67557	Sulawesi, Indonesia	MG883253	7Clsul		
Chromodoris lochi	WAMS67558	Sulawesi, Indonesia	MG883254	8Clsul		
Chromodoris lochi	WAMS67564	Sulawesi, Indonesia	MG883255	9Clsul		
Chromodoris lochi	WAMS67565	Sulawesi, Indonesia	MG883256	10Clsul		
Chromodoris lochi	WAMS67566	Sulawesi, Indonesia	MG883257	11Clsul		

Chromodoris lochi	WAMS67567	Sulawesi, Indonesia	MG883258	12Clsul	5º28’13.54”S	123º45’24.85”E
Chromodoris lochi	WAMS67568	Sulawesi, Indonesia	MG883259	13Clsul	5º28’31”S	123º45’46”E
Chromodoris lochi	WAMS67570	Sulawesi, Indonesia	MG883260	14Clsul		
Chromodoris lochi	WAMS67571	Sulawesi, Indonesia	MG883261	15Clsul		
Chromodoris lochi	WAMS70793	Sulawesi, Indonesia	MG883262	16Clsul		
Chromodoris lochi	WAMS75448	Hibernia Reef, WA Australia	MG883263	Clhibaus	11º57’57.28”S	123º22’42.881”E
Chromodoris lochi	WAMS103143	Mooloolaba, QLD, Australia	MG883245	1Clmoaus	26º38.263’S	153º12.019’E
Chromodoris lochi	WAMS103144	Mooloolaba, QLD, Australia	MG883246	2Clmoaus	26º38.263’S	153º12.019’E
Chromodoris lochi	CASIZ173594	Madagascar, Iles de Radama	JQ727831	Clmada		
Chromodoris lochi	MB28-004633	Mozambique, Vamisi Island	MK994117	1Clmozam		
Chromodoris lochi	MB28-004929	Mozambique, Vamisi Island	MK994118	2Clmozam		
Chromodoris lochi	MHN-YT1551	Mozambique, Vamisi Island	MK994119	3Clmozam		
Chromodoris cf. *lochi* FP	SBMNH89007	French Polynesia, Tahaa	MG883150	1Chlofp	16º32’58.73”S	151º28’06.74”W
Chromodoris cf. *lochi* FP	SBMNH89038	French Polynesia, Bora-Bora	MG883151	2Chlolfp	16º30’10.57”S	151º45’20.75”W
Chromodoris cf. *lochi* FP	UF400236	French Polynesia, Moorea	MG883152	3Chlofp	17º29’10.50”S	149º45’40.93”W
Chromodoris cf. *lochi* FP	WAMS67559	French Polynesia	MG883153	4Chlofp	17º21’27.76”S	149º02’15.43”W
Chromodoris cf. *lochi* FP	WAMS67560	French Polynesia	MG883154	5Chlofp	17º21’27.76”S	149º02’15.43”W
Chromodoris cf. *lochi* FP	WAMS67561	French Polynesia	MG883155	6Chlofp	17º21’27.76”S	149º02’15.43”W
Chromodoris cf. *lochi* FP	WAMS67562	French Polynesia	MG883156	7Chlofp	17º21’27.76”S	149º02’15.43”W
Chromodoris cf. *lochi* FP	WAMS67563	French Polynesia	MG883157	8Chlofp	17º21’27.76”S	149º02’15.43”W
Chromodoris cf. *lochi* FP	MBIO41716	French Polynesia, Moorea	MG905089	9Chlofp	17º29’12.84”S	149º49’5.664”S
Chromodoris cf. *lochi* FP	MBIO41717	French Polynesia, Moorea	MG905090	10Chlofp	17º29’12.84”S	149º49’5.664”S
Chromodoris cf. *lochi* FP	MBIO41718	French Polynesia, Moorea	MG905091	11Chlofp	17º29’12.84”S	149º49’5.664”S
Chromodoris cf. *lochi* FP	MBIO41719	French Polynesia, Moorea	MG905092	12Chlofp	17º29’12.84”S	149º49’5.664”S
Chromodoris cf. *lochi* FPV	UF368685	Vanuatu, Sanma	MG883158	Clvansa	15º35’27.25”S	167º15’01.79”E
Chromodoris cf. *lochi* FPV	WAMS67572	French Polynesia, Moorea	MG883159	13Chlofp	17º29’27.13”S	149º49’33.27”W
Chromodoris cf. *lochi* FPV	WAMS67573	French Polynesia, Moorea	MG883160	14Chlofp	17º29’27.13”S	149º49’33.27”W
Chromodoris cf. *lochi* FP	MBIO41721	French Polynesia, Moorea	MG905093	15Chlofp	17º29’12.84”S	149º49’5.664”S
Chromodoris cf. *lochi* FP	MBIO41722	French Polynesia, Moorea	MG905094	16Chlofp	17º29’12.84”S	149º49’5.664”S
Chromodoris cf. *lochi* FP	MBIO41723	French Polynesia, Moorea	MG905095	17Chlofp	17º29’12.84”S	149º49’5.664”S
Chromodoris cf. *lochi* FP	MBIO41724	French Polynesia, Moorea	MG905096	18Chlofp	17º29’12.84”S	149º49’5.664”S
Chromodoris cf. *lochi* FP	MBIO41725	French Polynesia, Moorea	MG905097	19Chlofp	17º29’12.84”S	149º49’5.664”S
Chromodoris cf. *lochi* FP	MBIO41726	French Polynesia, Moorea	MG905098	20Chlofp	17º29’12.84”S	149º49’5.664”S
Chromodoris lochi	CASIZ158684	Eagle Pt. Batangas, Philippines	JQ727848	Clbaphil		
Chromodoris lochi	Paris Museum	Lifou, New Caledonia	JQ727849	ClNcale		
Chromodoris lochi	CASIZ167968	D’Entrecasteaux Islands, Papua New Guinea	JQ727850	Cldepap		
Chromodoris lochi	CASIZ167973	Aniwa Island, Vanuatu	JQ727851	Clanisva		