ABSTRACT

Title of Dissertation: PHYLOGENOMICS, SYSTEMATICS, AND EVOLUTION WITHIN THE NUDIBRANCH GROUP CLADOBRANCHIA (MOLLUSCA: GASTROPODA)

Jessica A. Goodheart, Doctor of Philosophy, 2017

Dissertation directed by: Dr. Michael P. Cummings, Professor
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To truly understand evolution, we must document patterns of variation in traits – ranging from anatomical features of individuals to geographic ranges of species – to gain insights into the mechanisms that lead to changes in diversity through time. This type of work requires a robust historical context of evolutionary relationships in order to make comparisons across taxa and inferences about past events. My dissertation provides a thorough phylogenetic analysis of the marine gastropod group Cladobranchia (Mollusca) to better understand the evolution of defensive capabilities within the clade. In the absence of a protective shell, lineages within Cladobranchia

have evolved a diverse array of alternative defense mechanisms, including the use of stinging organelles (nematocysts) acquired from their cnidarian prey. It has been hypothesized that incorporation of nematocysts as a defensive strategy may have been an evolutionarily important event that led to large-scale diversification within this group. As such, understanding the steps involved in the evolution of this ability is necessary for evaluating this hypothesis. A major objective for my dissertation has been to use transcriptome (RNA-Seq) data from 37 species in Cladobranchia in order to generate a well-supported phylogenetic hypothesis of Cladobranchia. This research has produced the most highly supported phylogenetic tree of Cladobranchia thus far and contributes to confidence in the efficacy of genomic data to resolve relationships among gastropod lineages. As I have been able to expand this phylogenetic hypothesis with additional taxon sampling, including molecular data from a further 60 species, I have been able to provide context for understanding the evolutionary steps that led to the ability to sequester nematocysts. This phylogeny was then combined with morphological data from 50 nematocyst sequestering species within Cladobranchia to allow for a more detailed reconstruction of the evolution of nematocyst sequestration and prey preference within this clade. Overall, this work builds knowledge of the relationships among major lineages within Cladobranchia, and has substantially increased understanding of the evolution of morphological and ecological characters in this group.

PHYLOGENOMICS, SYSTEMATICS, AND EVOLUTION WITHIN THE NUDIBRANCH GROUP CLADOBRANCHIA (MOLLUSCA: GASTROPODA)

by

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2017

Advisory Committee:

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Preface

This dissertation is based, in part, on manuscripts completed with the assistance of co-authors. The student, Jessica Goodheart, was the primary contributor to all aspects of these publications, justifying their inclusion in this dissertation. Following are the references for those publications, listed by chapter:

Chapter 2

Jessica A. Goodheart, Adam L. Bazinet, Allen G. Collins & Michael P. Cummings. 2015. Phylogeny of Cladobranchia (Gastropoda: Nudibranchia): a total evidence analysis using DNA sequence datafrom public databases. *Digital Repository at the University of Maryland,* DOI: 103016/M-20K9X.

Chapter 3

Jessica A. Goodheart, Adam L. Bazinet, Allen G. Collins, Michael P. Cummings. 2015. Relationships within Cladobranchia (Gastropoda: Nudibranchia) based on RNA-Seq data: An initial investigation. *Royal Society Open Science* 2: 150196.

Chapter 4

Jessica A. **Goodheart**, Ryan A. Ellingson, Xochitl G. Vital, Hilton C. Galvão Filho, Jennifer B. McCarthy, Sabrina M. Medrano, Vishal J. Bhave, Kimberly García-Méndez, Lina M. Jiménez, Gina López, Craig A. Hoover, Jaymes D. Awbrey, Jessika M. De Jesus, William Gowacki, Patrick J. Krug & Ángel Valdés. 2016. Identification

guide to the heterobranch sea slugs (Mollusca: Gastropoda) from Bocas del Toro, Panama. *Marine Biodiversity Records* 9: 56

Chapter 5

Jessica A. Goodheart & Alexandra E. Bely. 2017. Sequestration of nematocysts by divergent cnidarian predators: mechanism, function, and evolution. *Invertebrate Biology* 136: 75-91.

Chapter 6

Jessica A. **Goodheart**. 2017. Insights into the systematics, phylogeny and evolution of Cladobranchia (Gastropoda: Heterobranchia). *American Malacological Bulletin* 35: 73-81.

Chapter 7

Jessica A. **Goodheart,** Adam L. Bazinet, Ángel Valdés, Allen G. Collins, & Michael P. Cummings. 2017. Feeding follows phylogeny: prey choice and relationships in Cladobranchia. *BMC Evolutionary Biology* 17: 221.

Chapter 8

Jessica A. **Goodheart,** Sabrina Bleidißel, Dorothee Schillo, Daniel Ayres, Allen G. Collins, Michael P. Cummings, Ellen E. Strong, & Heike Wägele. In preparation. Comparative morphology and evolution of the cnidosac in Cladobranchia (Gastropoda: Heterobranchia: Nudibranchia).

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This letter is written to signify that the dissertation committee, committee chair, and the graduate director have all approved the use of previously published co-authored work in the final dissertation of Jessica Goodheart, Biological Sciences Graduate Program, 112853686. In accordance with the Graduate School's policy the dissertation committee has determined that they made substantial contributions to the included work.

The citations for the published work is/are:

Chapter 2
Jessica A. Goodheart, Adam L. Bazinet, Allen G. Collins & Michael P. Cummings. 2015. Phylogeny of Cladobranchia (Gastropoda: Nudibranchia): a total evidence analysis using DNA sequence datafrom public databases. *Digital Repository at the University of Maryland,* DOI: 103016/M-20K9X.

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2017. Feeding follows phylogeny: prey choice and relationships in Cladobranchia. *BMC Evolutionary Biology* 17: 221.

Per Graduate School policy the dissertation forward will identify the scope and nature of the student's contributions to the jointly authored work included in the dissertation and a copy of this letter will be submitted with the dissertation.

Sincerely,

Dr. Michael Cummings, Dissertation Committee Chair,
Professor, Department

Dr. Michelle Brooks,
Associate Director, Biological Sciences Graduate Program

Jessica Goodheart,
Graduate Student, Biological Sciences

Dedication

To my parents, Theresa and Steve Goodheart. Thanks for putting up with my shenanigans for all these years.

Acknowledgements

I am very grateful to the many people that have made this dissertation work possible. I am truly blown away by the generosity and support that I have received during my PhD.

First and foremost, I would like to thank my co-advisors, Dr. Michael Cummings and Dr. Allen Collins. I have been privileged to work with and learn from both of these phenomenal scientists, and the experiences I have had in the Laboratory of Molecular Evolution and the National Museum of Natural History have been invaluable. Both Michael and Allen are tireless and effective mentors that pushed me throughout my PhD to become a more effective writer and a more thoughtful scientist.

I would also like to recognize the contributions of two other colleagues that have contributed to my scholarly development, Dr. Alexa Bely and Dr. Ellen Strong. Both have been valuable collaborators, and each provided me with copious amounts of advice and assistance. Thank you for putting up with my frequent visits to your offices!

I am also grateful to the rest of my committee members, Drs. Marjorie Reaka, Carlos Machado, and Mihai Pop for their assistance throughout this process. My past and current lab mates, Dr. Adam Bazinet, Dr. Cheryl Ames and Leann Biancani, must also be thanked for their assistance, and for all of the stimulating discussions that made my time here that much more fruitful. I would also like to thank all of the faculty, curators, staff and students from BISI-BEES, Department of Biology, and CBCB at the University of Maryland and the Department of Invertebrate Zoology, the

Laboratories of Analytical Biology, and the National Systematics Laboratory at the National Museum of Natural History for all of the support and camraderie.
I would like to offer a special thanks to all of my BEESsties for making this a truly enjoyable four years.

Finally, I am enormously grateful to my family and friends for all of the support throughout the years and during my time at UMD: My parents and grandparents, Theresa, Steve, and Joyce Goodheart, and Bill and Carol LaPlante, my brother Austin, all of my aunts and uncles, my Grand Oaks family, and Grace DiRenzo, Daniel Escobar-Camacho, Natalia Umaña, Gabriel Arellano, and Denise Mammen. You all are very special to me.

The acknowledgements for the individuals and organizations that supported the research in this dissertation can be found at the end of each chapter.

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Chapter 1: Introduction

Motivation and scope of this work

Research in evolutionary biology is critically important to our understanding of biodiversity. This type of work provides specific insights into the mechanisms of evolution that lead to changes in diversity, and provides a broader context for interpreting why particular traits and characters evolve. However, research in evolutionary biology requires a solid grasp of the relationships among organisms, otherwise known as systematics, to identify the relative timing of evolutionary changes or provide hypotheses for the reasons such changes might have occurred. This is true for studies conducted across the diversity of life, from single cell to multicellular organisms. This dissertation enhances our understanding of systematics and evolution within invertebrate groups in Metazoa, and specifically focuses on the marine gastropod group Cladobranchia (Mollusca), using modern phylogenomics and bioinformatics tools. Overall, this work encompasses research into the use of transcriptomes for building phylogenies in marine gastropods, builds knowledge of the relationships among major lineages within Cladobranchia, and follows the evolution of morphological and ecological characters in this group. In particular, this dissertation looks at the evolution of nematocyst sequestration and prey preference within Cladobranchia, and elucidates the changes involved in the evolution of each using comparative phylogenetics tools.

Phylogenomics

Introduction to phylogenetics and phylogenomics

Estimation of the evolutionary relationships between groups of organisms is a task completed indirectly, by comparing characters among primarily extant organisms. These relationships are most commonly represented graphically as a phylogenetic tree (i.e., dendrogram, cladogram). As representations of the evolutionary history of organisms, phylogenetic trees are now integral to the study of systematics and taxonomy. This is primarily the result of the work of Willi Hennig (often called the founder of phylogenetic systematics), who first suggested that groups of organisms, or taxa, should be recognized and formally named only in cases where they are evolutionarily real entities (i.e., monophyletic groups) [1]. But the utility of phylogenetic trees in regards to the evolution of organisms is not limited to systematics and taxonomy. Phylogenies also enable scientists to infer geographic distribution and life history changes along lineages, the historical age of individual groups, the progression of adaptation to local ecological conditions, the existence of radiations, and the influence of climate changes, migrations or the evolution of other species on the evolution of a group. As such, phylogenetic trees are now ingrained in the study of the evolutionary biology.

The field of phylogenetics has been changing at a rapid pace. It began with the use of morphological and ecological characters to build phylogenetic trees, an obvious extension to the use of these characters in taxonomic work. These types of data were then replaced (to some extent) by the use of allozyme data (which consists

of allele frequencies obtained via gel electrophoresis of proteins), followed closely by the use of DNA sequences, to construct phylogenies [2]. Advances in two areas led to these improved methodologies. First, the technology necessary for collecting allozyme data and sequencing DNA from organisms became available. Second, mathematical models of sequence evolution were improved greatly and better statistical testing was used to evaluate evolutionary hypotheses [3]. This was necessary to extricate as much information as possible from both types of data in order to facilitate a more robust phylogenetic hypothesis.

One limitation of DNA sequence data in particular has often been the cost associated with sequencing small fragments of genes. More recently however, our ability to sequence ever-greater amounts of sequence data has increased exponentially with the advent of genomic sequencing technologies [4]. The subsequent decrease in cost has meant that the cost of sequencing per base is far less [5], and as such has provided more researchers with more data for analyzing phylogenetic relationships. This use of high-throughput sequencing data (often called genomic data) for phylogenetic inference is referred to as phylogenomics. Phylogenomics as a whole is not only beneficial due to the low cost per base pair sequenced, however. Phylogenetics as a field relies on polymorphic sites among lineages to infer relationships, and the ability to sequence millions of sites per lineage allows for more thorough and more highly supported phylogenetic inferences [6]. Although phylogenomics often provides much more confidence in our inferences, challenges in orthology determination and the lower cost-effectiveness for non-model organisms have presented some difficulties in its widespread use.

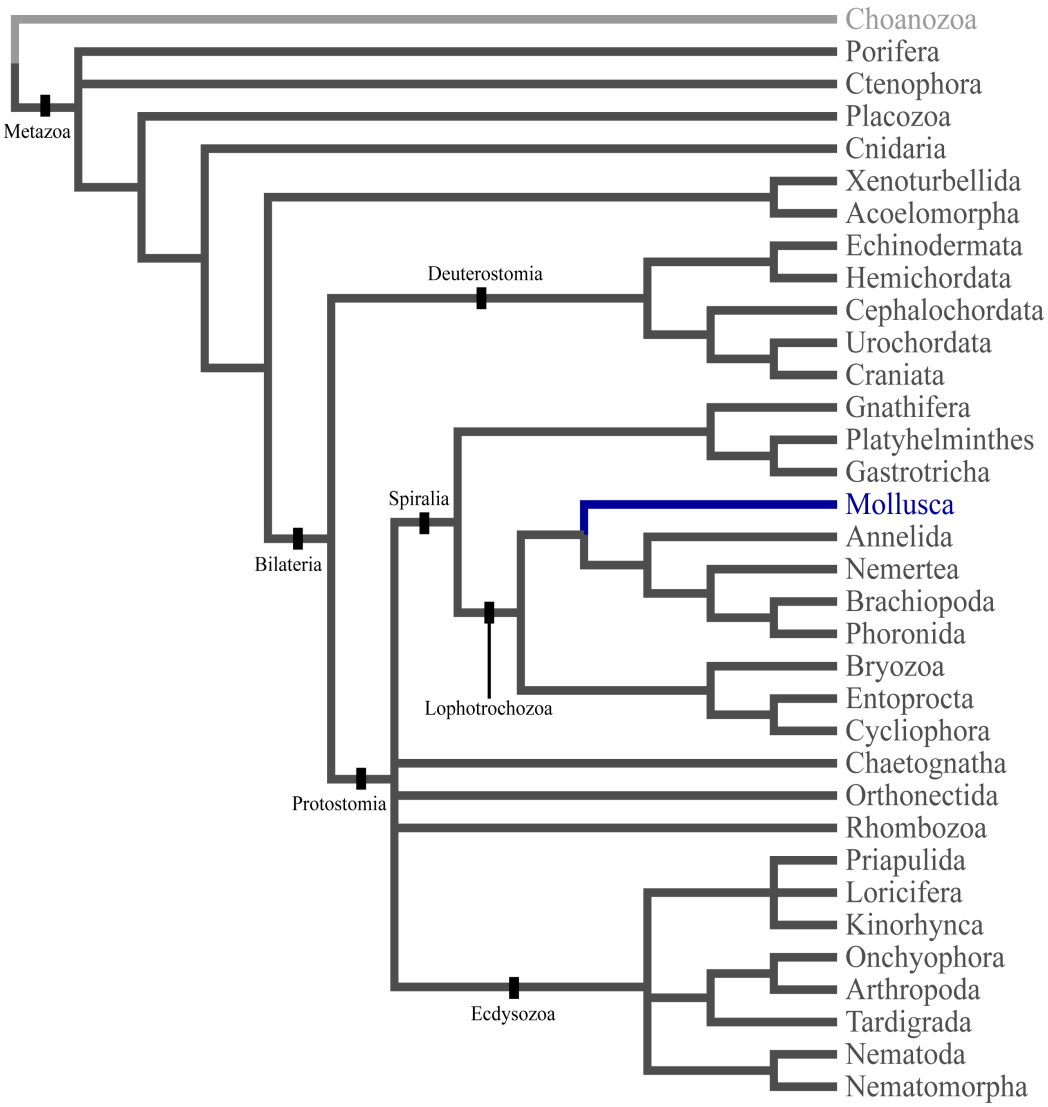

Figure 1.1. A phylogenetic tree adapted from [7–9] depicting the relationships among the phyla in Metazoa. The main clades are labeled with black marks, and the position of Mollusca is highlighted in blue within Lophotrochozoa.

Phylogenomics and Metazoa

Animals are multicellular, eukaryotic organisms of the clade Metazoa (1.1). The systematics of metazoans in particular has dramatically shifted with the introduction of high-throughput sequencing technologies, and most of the attention has focused on relationships at the phylum level or above (e.g., [10–20]). This is likely due to the ability of these data to resolve the deeper relationships of many groups that have

historically been in flux. However, it is important to note that controversies still exist, and some relationships may never be resolved with confidence [9]. With the cost of sequencing continuing to decrease, more researchers now have the ability to collect large data sets in an attempt to resolve relationships within more recently diverged groups, such as subphyla (e.g., [21]), classes (e.g., [22–24]) and others [25]. As more phylogenies are completed using these big genomic data sets, as with any other type of data, inevitably the systematics and taxonomy must be updated to reflect shifts in support for previous or new hypotheses.

Introduction to Cladobranchia systematics and evolution

Cladobranchia is a clade composed of exclusively marine gastropod mollusks within Heterobranchia (Figures 1.1 and 1.2). These taxa are nudibranchs, which are characterized by the lack of a shell as adults, unlike most other gastropods [26]. Nudibranch defensive strategies include the uptake or synthesis of biochemically active compounds [27,28], the presence of warning (aposematic) coloration [29] or cryptic coloration to deter or hide from predators, respectively, and the use of stinging organelles (nematocysts) acquired from cnidarian prey [30]. Nudibranch gastropods have been identified as solid indicators of climate change [31], and due to their conspicuous nature (often as a result of aposematic coloration) would be ideal for monitoring by both biologists and citizen scientists.

The roughly 1000 species within Cladobranchia can be found on coastal reefs and some pelagic habitats worldwide [32], and most families are broadly distibuted. A number of interesting characters among nudibranchs evolved within this clade, such as the development of multiple defensive strategies, including nematocyst

sequestration [33], the sequestration of symbiotic zooxanthellae [34], and independent evolution of rhythmic motor behavior (e.g., swimming) [35]. Many species within this clade are also commonly utilized in various types of neurological research [36–39]. These taxa are exclusively carnivorous, and tend to feed on a variety of prey items, including cnidarians (most common), bryozoans, crustaceans, and the eggs of other gastropods [40], and the majority of cladobranchs are often specialists on individual prey species [41].

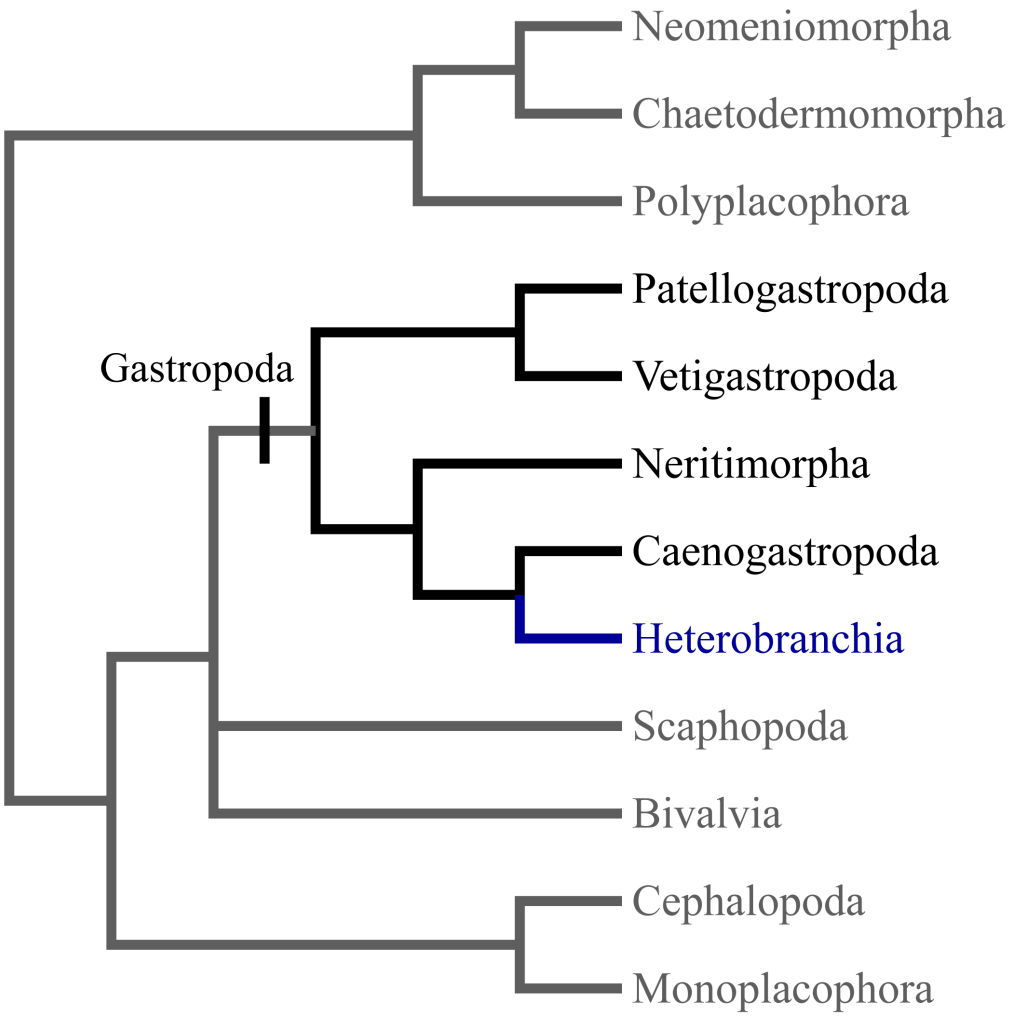

Figure 1.2. Phylogeny of Mollusca (based on [10,11,42]), highlighting the position of Heterobranchia within Gastropoda, which is where Cladobranchia falls.

Cladobranchia has been characterized by four synapomorphies: (i) branched digestive glands [43], (ii) the loss of the primary gills (ctenidia), (iii) the loss of the bursa copulatrix, and (iv) the loss of the blood gland, though none of these characters are unique to Cladobranchia [26]. Nevertheless, multiple studies using various morphological and molecular data sets have found phylogenetic support for the clade [26,43–50]. It should be noted, however, that Pola and Gosliner [43] resolved Cladobranchia only to the exclusion of the genus *Melibe*.

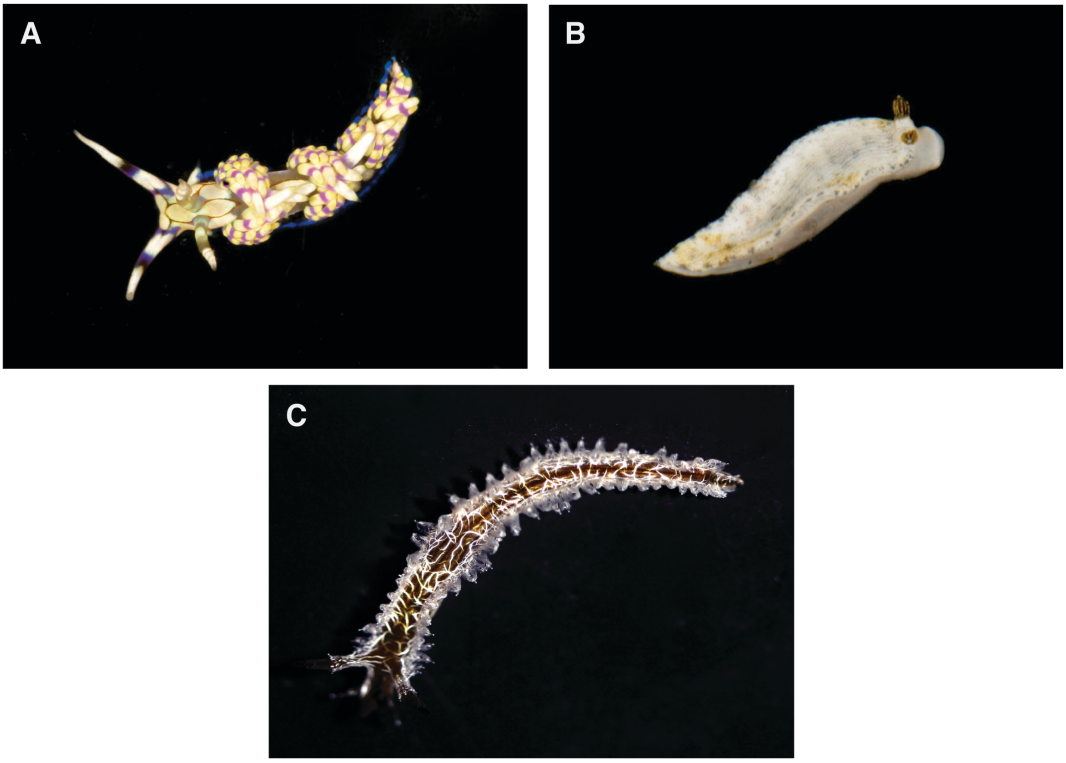

Figure 1.3. Exemplar taxa representing each of the three major morphological clades. A) *Godiva* sp. representing Aeolidida, B) *Dermatobranchus* sp. representing Arminida, and C) *Lomanotus vermiformis* representing Dendronotida.

Although support for Cladobranchia as a monophyletic clade is high, the resolution of relationships among the major lineages within it has been more problematic. There are three long-recognized major clades within this group: Aeolidida [51], Arminida [43], and Dendronotida [52] (Figure 1.3). Aeolidida is

recognized by the presence of a cnidosac (a structure that houses sequestered stinging organelles from their prey) and the shift from an oral veil to oral tentacles. Dendronotida is characterized by the presence of rhinophoral sheaths, possession of a cuticle lining the stomach, and the presence of tentacular extensions of the oral veil. Arminida has been more challenging to characterize morphologically, and has been hypothesized to be paraphyletic if certain groups (e.g. the genera *Janolus* and *Dirona*) are included [26]. As such, there are currently no synapomorphies described for this group. Though some attempt to determine the relationships among these three groups has been made using morphological data, those relationships had low support [26].

Hypotheses of monophyly and relationships of the lineages within Cladobranchia have long needed testing with molecular data. Although the monophyly of Aeolidida is well supported across multiple molecular and morphological analyses, support for Dendronotida and Arminida as monophyletic groups has often been lacking [26,43–46,49,53]. Due to the uncertainty surrounding the monophyly of Arminida, and in some cases Dendronotida, multiple families within Cladobranchia have essentially been considered "unassigned" taxa in recent classifications, primarily stemming from questions of inclusiveness [43,53]. In addition to a lack of support for the monophyly of these lineages, relationships among these groups have remained unresolved.

The issues described here regarding the relationships of higher-level groups within Cladobranchia have led to a lack of understanding of the evolutionary pathways and trends that have developed historically within this group. One example is that of prey preference evolution. In the marine realm, where barriers to genetic

exchange are less obvious than in terrestrial or freshwater systems [54], non-allopatric divergence and speciation may play a fundamental role in the generation of biodiversity (e.g., [55,56]). In this context, shifts between major prey types could constitute important factors explaining the biodiversity of marine taxa, particularly in groups with highly specialized diets, like Cladobranchia. Previous work on prey preference evolution within gastropods (and Heterobranchia specifically) has suggested two hypotheses related to prey preference evolution and diversification. The first suggests that transitions to new prey types correlated with morphological evolution specific to that prey type can lead to an increase in diversity within those groups [57–59], and the second focuses more on the increase in speciation associated with more specific shifting between host species [60]. Some authors have also suggested that the preference for cnidarian prey within the nudibranch group Cladobranchia may have led to the evolution of the ability to sequester cnidarian nematocysts [57], which is displayed by numerous members of Cladobranchia. A more stable and well-supported phylogenetic tree for Cladobranchia would allow for better assessments of these evolutionary hypotheses.

Dietary specialization has also been considered a contributing factor in the species richness of Nudibranchia as a whole [58], and especially cladobranchs [33,57], in conjunction with the evolution of nematocyst sequestration. As previously mentioned, the sequestration of cnidarian nematocysts has long been considered a defense mechanism, and the only gastropods with this ability are within the group Cladobranchia. To sequester the nematocyst, the cnidocyte (the cell) is separated from its nematocyst (the organelle) during ingestion of cnidarian tissues. Nematocysts

are then passed through the digestive gland and incorporated into epithelial cells lining the cnidosac [30,61], a distal extension of the digestive gland within dorsal body outgrowths termed cerata [62]. However, there are a number of seemingly morphologically intermediate species of uncertain or unstable affinity. For example, species of the family Embletoniidae have been placed within both Aeolidida and Dendronotida by different authors [63] and possess cnidosac-like structures. Consequently, it remains unclear if this defensive strategy has a single unique origin. An integrated synthesis of morphological analyses and a robust phylogenetic tree would allow for the evaluation of the origin and evolution of this ability, as can be seen in this dissertation.

Overview of this dissertation

This dissertation addresses the uncertainty in the relationships among major lineages within Cladobranchia in order to more effectively describe the evolution of both prey preference and nematocyst sequestration. In particular, this work marks the first attempt to use genomic data in resolving the relationships of taxa within Nudibranchia. These new phylogenetic hypotheses are then used to assess the evolution of prey preference in Cladobranchia and the evolution of the morphological adaptations associated with the ability to sequester nematocysts (stinging organelles) from prey. This dissertation also includes a review of the historical systematics and evolution research in Cladobranchia and and a record of the cladobranch (and other heterobranch) gastropods in the coastal waters of Bocas del Toro, Panama.

In Chapter 2, I assess the value of public sequencing data that was available in GenBank [64] in 2014 for resolving the deeper lineages within Cladobranchia. It was

made clear through this study that the genes currently being sequenced for phylogenetic inference within Cladobranchia would not provide the information necessary for a well-supported hypothesis [45]. I conceived of the study that is the basis for this chapter and led the design, performed sequence downloads, and with the help of my co-authors performed data processing and analysis, and drafted the manuscript.

Chapter 3 provides the first assessment of the utility of genomic (specifically RNA-Seq) data for resolving lineages within Cladobranchia [50]. This work has provided the first well-supported molecular-based hypothesis of the relationships between the deeper lineages in this group. I conceived of this study, collected samples and field data, and carried out the molecular lab work; I, along with my co-author Adam Bazinet, performed the bioinformatics analyses; all co-authors participated in study design and data analysis and helped draft the manuscript.

In Chapter 4, I provide an identification guide for the marine Heterobranchia gastropods found along the coast of Bocas del Toro, Panama. This work serves as a record of the fieldwork that some colleagues and I completed during a sea slug taxonomy course at the Smithsonian Tropical Research Station. Overall, we found a total of 82 species belonging to five groups of heterobranchs, an increase from the previously known 19 species. I was the lead author on this work, and I was responsible for the organizing, and a large portion of the writing, of this manuscript. All co-authors, including myself, participated in the collection and identification of the animals described in this work.

Chapter 5 is a review of the sequestration of cnidarian nematocysts performed by multiple phyla within Metazoa, including Ctenophora, Acoelomorpha, Platyhelminthes, and Mollusca. In it, my co-author (Alexandra Bely) and I describe the phylogenetic distribution, mechanisms, and possible functions of nematocyst sequestration in each of these phyla. We also highlight several traits that are common to Ctenophora, Acoelomorpha, Platyhelminthes and Mollusca and suggest hypotheses for how these traits could have played a role in the evolution of nematocyst sequestration. I performed the majority of the literature review for this work and my co-author and I both provided substantial contributions to the intellectual content and writing of the manuscript.

In Chapter 6, I describe the historical context for the systematics and evolution research within Cladobranchia, and provide insights for how genomic data has already, and might in the future, impact this type of work. Genomic data have challenged well-established relationships within Cladobranchia and resolved the position of taxa that have been traditionally quite difficult to place, including *Melibe* and the families Proctonotidae and Dironidae. These phylogenetic hypotheses also provide a much stronger framework for studying character evolution within Cladobranchia, including the evolution of nematocyst sequestration, one of the quintessential characters in this clade. As the sole author of this work, I am responsible for all of the intellectual content found therein.

Chapter 7 provides a return to phylogenetic and evolution analyses within Cladobranchia. In this study, next generation sequence (RNA-Seq) data is used to produce a more expansive phylogeny of Cladobranchia, which feed on a diverse array

of prey taxa but mostly specialize on cnidarians. This phylogeny, along with ancestral state reconstruction analysis, is then used to better understand the evolution of prey preference within this clade. These analyses answer several fundamental questions regarding the evolutionary relationships within Cladobranchia and make clear that there is strong phylogenetic correlation in regards to prey preference within this group. I, along with my co-author Ángel Valdés, conceived of this study; I collected samples and field data, and carried out the molecular lab work; I performed the bioinformatics analyses with my co-author Adam Bazinet; all co-authors participated in study design and data analysis and helped draft the manuscript.

Finally, Chapter 8 looks more closely at the comparative morphology and evolution of the cnidosac, the structure necessary for the sequestration of cnidarian nematocysts in Cladobranchia. In it, the general structure of most cnidosacs is described, and a structure is presented for the use of more precise and consistent terminology when describing the cnidosac. The evolution of these characters is then mapped, and we hypothesize that the cnidosac may be an extension of the terminal sac present in the sister group to Aeolidida. I, along with my co-author Heike Wägele, conceived of this study; my co-authors Sabrina Bleidißel and Dorothee Schillo and I collected samples and data; phylogenetic analyses were completed by myself and my co-author Daniel Ayres; all co-authors participated in study design and data interpretation and helped draft the manuscript.

Chapter 2: Phylogeny of Cladobranchia (Gastropoda: Nudibranchia): a total evidence analysis using DNA sequence data from public databases

Introduction

Cladobranchia is a diverse and charismatic clade of exclusively marine slugs. These organisms live in globally distributed habitats from the intertidal to the deep ocean, and are characterized by having branched digestive glands [43]. Though not as speciose as some other gastropod clades, cladobranchs have developed remarkable biological features that are rare among animals, many of which are related to defensive strategies. As this is a clade within Nudibranchia, which is characterized by the loss of the shell in adult animals [26], selection likely favored the evolution of defense mechanisms to compensate for the loss of a protective shell. The development of many different chemical and physical defense mechanisms has been hypothesized to have led to the large-scale diversification of Nudibranchia, and within it, Cladobranchia [33]. In order to understand this diversity, as well as the evolution of the ecological roles of taxa within Cladobranchia, an accurate phylogenetic framework is needed. However, given the depth of the evolutionary divergences and the diversity within this clade, reconstruction of the phylogenetic relationships among taxa in this group has proven difficult.

Both Cladobranchia (~1000 species) and its sister taxon, Anthobranchia (~2000 species) [65], have been supported as monophyletic by molecular data [26,43,44], but thus far there has been little resolution among the higher-level groups

within these two clades. Within Cladobranchia, there are three traditional taxa characterized on the basis of morphology: Dendronotida, Euarminida and Aeolidida [44]. Though a number of studies on the evolutionary history of Cladobranchia have been undertaken, the majority have been limited to specific clades, often at the family or genus level (e.g., Scyllaeidae [66], Aeolidiidae [67], Tritoniidae [68], and *Babakina* [69]). Due to this focus on more recent divergences within Cladobranchia, there is little data that either support or reject the traditional classification of the three major taxa, making it difficult to understand the deeper evolutionary history within these groups.

To date, there has been only one large-scale phylogeny attempted for Cladobranchia [43], which was based on the three most commonly used genes in nudibranch systematics: mitochondrial 16S rRNA and Cytochrome Oxidase I, and nuclear Histone 3. In this phylogeny, the majority of relationships between higher-level taxa remained unresolved, both between and within the three traditional taxonomic divisions of Cladobranchia. Consequently, the evolution of traits within Cladobranchia remains poorly understood.

A robust phylogeny of Cladobranchia is necessary to provide a framework for our understanding of adaptations within this clade. Here we present the "current state of knowledge": a phylogeny for Cladobranchia as inferred from all publicly available DNA sequence data.

Materials and Methods

Taxon and Data Selection

The Cladobranchia sequence data used in our analyses (Dendronotina [=Dendronotida], Arminina [=Euarminida], and Aeolidina [=Aeolidida]; taxa in brackets reflect equivalent taxonomic designations in the literature) were downloaded from GenBank [10] in February 2014. These data comprise 297 species and five genes, including the mitochondrial genes coding for cytochrome oxidase I (COI) and 16S rRNA, and nuclear genes coding for Histone 3 (H3), 18S rRNA and 28S rRNA (Appendix Table A2). The two outgroups for this analysis, *Discodoris atromaculata* and *Cadlina laevis*, were selected to maximize the number of genes for each outgroup as well as provide some taxonomic breadth from within Anthobranchia, the sister taxon to Cladobranchia. *D. atromaculata* was the only species in GenBank from Anthobranchia for which sequences were available for all five genes, and *C. laevis* was the only remaining species for which four of the genes were available.

Multiple sequence alignment and data matrix construction

Alignments were generated for each gene using the auto function in MAFFT 7.130 [70] (Appendix Table A1). In each gene alignment, multiple sequences from the same taxon (identified by GenBank taxon ID) were reduced to a single consensus sequence, using nucleotide ambiguity codes [71] as necessary. The GenBank taxon ID number is the most accurate identifier of species in GenBank because it reflects taxonomic rearrangements (e.g., a genus change), and as such was used to identify taxa.

Consensus sequences were generated by providing the nucleotide coding sequence alignment as input to the consensus_iupac BioPerl subroutine [72].

There are a few principal motivations for using consensus sequences. The first is a desire to incorporate all information about the variability of specific nucleotide states for positions in each gene, both within species and within individuals. A second motivation is to mitigate the effects of mistaken taxon identification within GenBank and prevent errors resulting from the incorrect choice of a single representative sequence. A major challenge of working with previously published sequences is the lack of access to morphology and other means of confirming the identification of samples; the use of consensus sequences can mitigate the effects of possible taxonomic misidentification. Finally, by utilizing more available sequence data, the consensus procedure yields somewhat longer final sequences for each taxon.

The individual gene alignments were concatenated into a single matrix, and sites containing data for fewer than four taxa were removed. This matrix (ALL_TAXA) contained 297 species. Three additional data matrices were generated using subsets of this data: one that contained only taxa for which two or more genes were present (MIN_TWO_GENES; 271 species), a second that contained only taxa for which three or more genes were present (MIN_THREE_GENES; 196 species), and a third that includes all species for which either COI, H3 or 16S rRNA genes are present, thereby eliminating taxa for which only 18S or 28S were present (THREE_GENES; 290 species). An additional matrix was generated (MIN_149_TAXA; 297 species) to minimize missing data. For this matrix, the five genes were concatenated and sites containing data for fewer than 149 taxa (~50%)

were removed. All five alignments, plus each separate gene consensus alignment (for a total of ten) are available as supplementary files.

Phylogenetic analyses

To complete the phylogenetic analyses we used GARLI 2.0 (Genetic Algorithm for Rapid Likelihood Inference; [73]) through the GARLI web service hosted at molecularevolution.org [74]. We used a general time reversible nucleotide model [75] with a proportion of invariant sites and an among site rate heterogeneity model with a discrete gamma distribution (GTR+I+G) together with GARLI default settings, including stepwise-addition starting trees. Three analyses were run for all matrices except MIN_149_TAXA: one without data partitioning; another with data partitioned into four possible subsets by type of gene: 1) COI mitochondrial, 2) H3 nuclear, 3) 16S mitochondrial rRNA, and 4) 18S and 28S nuclear rRNA, for a total of at most three partitions; and a third, unpartitioned, with all sequences from the genus *Melibe* removed (due to an extremely long *Melibe* branch in our analyses). For MIN_149_TAXA, only a full, unpartitioned analysis was run. Two analyses were also run for each gene, one including and one excluding *Melibe*. For all analyses, non-parametric bootstrap values were determined using 2000 bootstrap replicates with five search replicates per bootstrap replicate. Post-processing of the phylogenetic inference results was done by the GARLI web service at molecularevolution.org using DendroPy [76] and the R system for statistical computing [77], which includes the construction of a bootstrap consensus tree for each analysis. The estimation of the number of replicates required to recover the "best" topology follows Regier et al. [78].

Results

Data matrix properties

The matrix of five genes containing 297 species (ALL_TAXA) contained 6,475 nucleotide positions and was 26.9% complete, while the MIN_TWO_GENES (271 taxa) and MIN_THREE_GENES (196 taxa) data matrices each contained 6,484 nucleotide positions and were 28.0% and 29.7% complete, respectively. The THREE_GENES data matrix (290 taxa) contained 2,920 sites and was 41.0% complete. Finally, the MIN_149_TAXA data matrix (297 taxa) contained 1,419 sites and was 78.0% complete (Table 2.1). The full data matrix represented at least 65 genera (62.5%) and 20 families (66.7%) of all known families and genera within Cladobranchia.

Table 2.1. Size and completeness of aligned data matrices from GenBank sequences.

	Five Genes				Three Genes
Matrix name	ALL_TAXA	MIN_2_GENES	MIN_3_GENES	MIN_149_TAXA	THREE_GENES
Number of taxa	297	271	196	297	290
Number of nucleotide positions	6,475	6,484	6,484	1419	2,920
Number of nucleotides (non-gap characters) in alignment	1,923,075	1,757,164	1,270,864	328, 771	846,800
Matrix completeness (number nt ÷ number *possible* nt)	26.9%	28.0%	29.7%	78.0%	41.0%
Percentage of ambiguous nucleotides (non-gap, non-A/C/G/T chars)	0.10%	0.11%	0.13%	0.51%	0.21%

Percentage of all possible internal nodes with bootstrap ≥ 80 (non-partitioned)	36.4%	40.4%	41.3%	32.4%	38.4%
Percentage of all possible internal nodes with bootstrap ≥ 80 (partitioned)	36.4%	37.4%	39.8%	Not applicable	23.9%

Phylogenetic analyses

We performed two phylogenetic analyses for four of our five data matrices (ALL_TAXA, MIN_TWO_GENES, MIN_THREE_GENES, THREE_GENES). For the MIN_149_TAXA matrix, only an unpartitioned analysis was run (Appendix20). The MIN_TWO_GENES tree represented the best combination of comprehensive taxon sampling and proportion of well-supported nodes (those with a bootstrap value ≥ 80), in which Cladobranchia had high bootstrap support. The ALL_TAXA tree contained a smaller percentage of resolved nodes, and the MIN_THREE_GENES tree contained 75 fewer taxa and only a slightly higher percentage of resolved internal nodes (+0.9%) (Table 2.1). Therefore, we consider the bootstrap consensus tree from the MIN_TWO_GENES analysis to be the most reliable current inference of relationships within Cladobranchia based on molecular data, and present it in Figure 2.1. Phylogenetic trees based on the other data sets are presented in Appendix A, and all trees showed a lack of resolution among most branches. The genes that seem to have contributed the most information are 16S and H3, due to a larger amount of resolution in the topology of these gene trees, followed by 18S and 28S, which had low taxon representation but a considerable amount of resolution, and COI, in which the topology was simply poorly resolved.

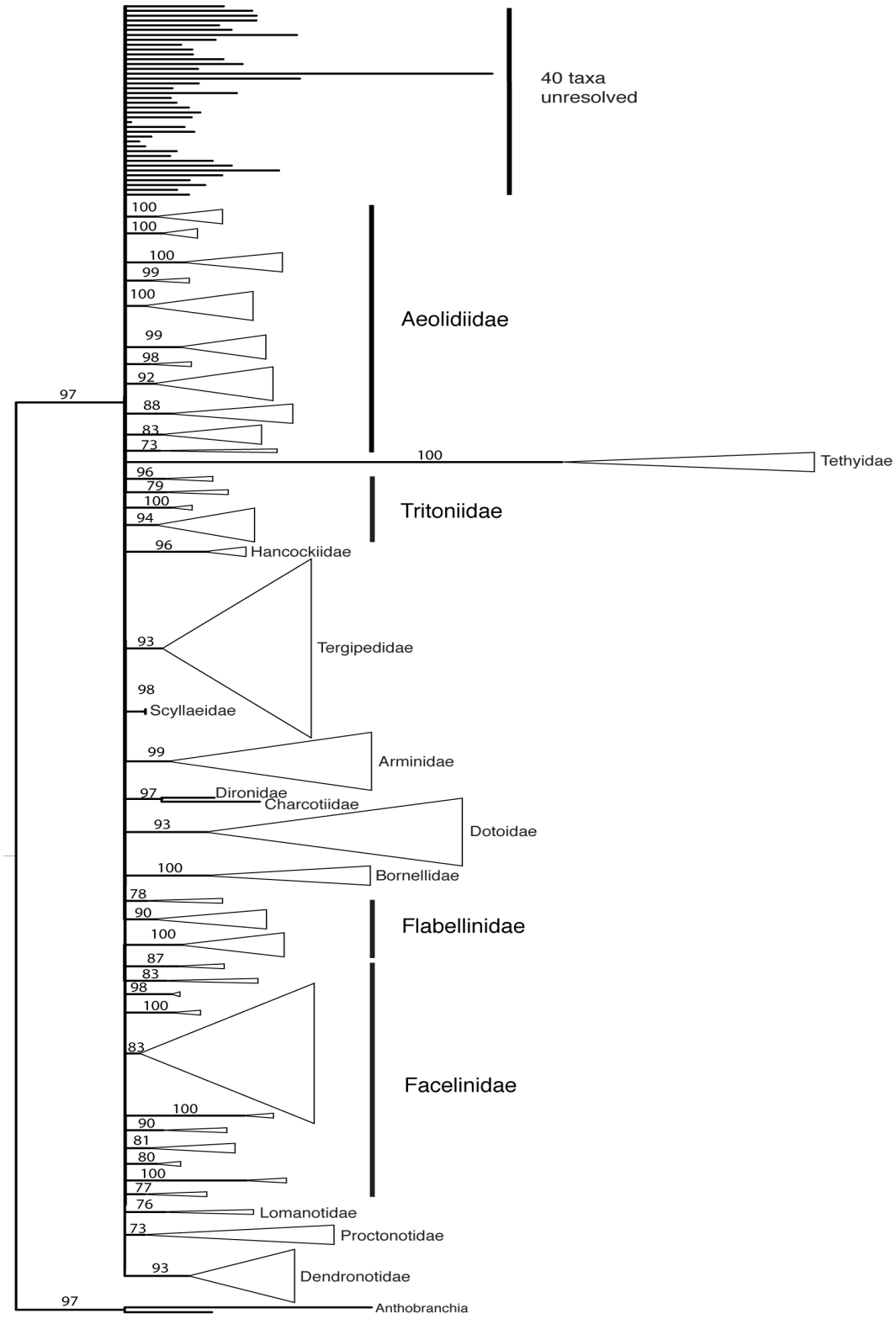

Figure 2.1. The 70% majority-rule bootstrap consensus tree of Cladobranchia using sequence data from five genes (COI, H3, 16S, 18S, 28S) in the MIN_TWO_GENES data matrix. Bootstrap values are provided above each branch.

The tree generated using the MIN_TWO_GENES matrix (Figure 2.1) supported the monophyly of Cladobranchia (bootstrap value = 97), including *Melibe* (Tethyidae). Relationships within Cladobranchia, however, were still largely unresolved; our tree included a massive polytomy at the base of Cladobranchia consisting of 41 small clades and 40 individual taxa. Many of these taxa and groups form non-monophyletic assemblages of species at all levels, including genus, family, superfamily and infraorder according to current taxonomic divisions. Five families were well supported (bootstrap value ≥ 80) as monophyletic: Bornellidae (bootstrap value = 100), Hancockiidae (bootstrap value = 96), Tergipedidae (bootstrap value = 93), Dotidae (bootstrap value = 93) and Dendronotidae (bootstrap value = 93). One family had relatively low support (80 > bootstrap value ≥ 70): Lomanotidae (bootstrap value = 76). Two families in the analysis were represented by only one taxon (Dironidae and Charcotiidae), and these were well supported as sister taxa in our analysis (bootstrap value = 97). All other taxa included in this analysis were from families that were not supported as monophyletic, with as yet unresolved evolutionary histories and taxonomic disarray.

Discussion

In this section, we review our findings on the bootstrap support for both shallow and deep divergences in Cladobranchia. We then discuss the importance of our results and how they affect the current understanding of the group relationships themselves.

Support levels

In our trees, the bootstrap support values are highly varied. A major point of interest is the high support for the monophyly of Cladobranchia itself, including *Melibe*, a genus excluded from Cladobranchia in Pola & Gosliner [43]. However, the backbone of the tree within the group is rife with polytomies and low bootstrap values. Some genera and a few families are well supported in our analysis, but the majority of support and resolution comes at a very shallow phylogenetic level. Additionally, specific placement of roughly 15% of the taxa had had bootstrap values of less than 50%, thus forming a comb along the backbone of our majority-rule consensus tree. There are several explanations for this lack of resolution and low support in our analyses. One possibility is that our relatively small amount of data and sparse data matrices (with at most 78.0% completeness) may have prevented our likelihood analyses from performing well [79]. The problems may concern the specific number or type of genes that were sampled for each taxon and included in the analyses. For example, although species of the same genus were included in the analysis, there may be only one gene for one or more of those taxa. If these genes are different, there can be no comparison of similar characters to place them together on the phylogeny. There are a number of cases in our analyses where this could potentially be an issue, including multiple species of *Eubranchus* (*E. exiguous*, 76182; *Eubranchus* sp., 252571; *E. rustyus,* 763125; and *E. sanjuanensis,* 763126) and one species of *Protaeolidiella* (*P. atra,* 1154746).

Prior research suggests that this missing data may not be as much of a problem as previously suspected. In Cho et al. [80], a data matrix with 45%

intentionally missing data yielded no signs of the contradictory groupings that missing data would supposedly produce. This result is consistent with those of three other studies from across a broad taxonomic range, including frogs [81], angiosperms [82], and an entire phylum of eukaryotes [83]. Other literature has also indicated that missing data is not always a substantial problem [84]. From Wiens and Morrill [84]: "Overall, our results confirm previous simulation and empirical studies showing that taxa with extensive missing data can be accurately placed in phylogenetic analyses and that adding characters with missing data can be beneficial (at least under some conditions)." In support of this, the tree obtained from the analysis of our MIN_149_TAXA matrix (78% complete) is actually less resolved than any of the other analyses (Table 2.1, Appendix Figure A20). This indicates that missing data are not the major issue, at least in this case, but rather that the available data are insufficient for the problem.

An alternative to the sparse data matrix hypothesis for the lack of resolution in our trees is possible contamination or specimen misidentification. Based on the location of certain taxa in the tree, either some identifications may be incorrect in GenBank, or these taxa may have been routinely placed in the wrong genus or family, including: *Caloria indica* (376200), *Piseinotecus* sp. (797203), *Pinufius rebus* (797256), *Flabellina baetica* (934968), *Calma glaucoides* (1154735), *Flabellina cacaotica* (1287503), *Piseinotecus gabinierei* (1287625), and *Fiona pinnata* (1287638). These taxa can be found in unexpected locations on both the ALL_TAXA and MIN_TWO_GENES phylogenies, often some distance from others within the same genus or family. The exact reasons for these instances of taxonomic discord are

unknown, but may be due to misidentifications, contamination, or taxonomic misplacement. One point to note, however, is that not a single taxon on this list is associated with a higher proportion of ambiguous characters. As such, their placements are likely not artifacts of the consensus procedure.

A third possible explanation for a lack of resolution is that conflicting gene tree topologies may be confounding the true species tree in our analyses. This is a common problem in phylogenetic studies [85]. Our individual gene trees (COI, H3, 16S, 18S and 28S) all resolve different topologies (Appendix Figure A8-12), which would be consistent with this hypothesis. However, most of our trees are poorly resolved (both those based on single gene and those based on multiple genes) and include a large number of polytomies. This, in turn, indicates not that the data are necessarily inconsistent, but that they are insufficient to resolve relationships within Cladobranchia.

Another plausible reason for the low bootstrap support and lack of resolution is that certain taxa are particularly troublesome and can negatively affect bootstrap support values [86]. The genus *Melibe* is a possible example, with long branches for COI and 16S and absent sequences for 18S and 28S. Taxa with vastly elevated rates of evolution, such as *Melibe*, tend to move around, eroding support in bootstrap analyses. To address these concerns, analyses were run excluding *Melibe* from all data matrices where it was formerly included. These tended to have slightly higher support values for most nodes as compared to when *Melibe* was included. However, the exclusion of *Melibe* did not affect the overall resolution for each tree (Appendix

Figure A13-19), and thus inclusion of this taxon is not likely a strong contributing factor to poor resolution.

Alternatively, gene sequences for a few taxa in our analyses contained slightly more ambiguous characters in their consensus sequence compared to other taxa, including those from *Glaucus marginatus* (1154738, 16S and COI), *Doto coronata* (154624, COI), *Aeolidia papillosa* (195873, 16S), *Favorinus elenalexarium* (797222, H3), *Dondice banyulensis* (869980, 16S), *Spurilla neapolitana* (929453, 16S and COI) and *Phyllodesmium macphersonae* (869973, 16S and COI). This could also have an effect on both the resolution and support for phylogenetic trees estimated by likelihood [87]. However, none of these taxa appear to be in an unexpected place on the MIN_TWO_GENES tree. This is most likely because the percentage of ambiguous characters in all of our matrices is extremely small: between 0% and 0.51% (Table 2.1). This indicates that for the majority of our taxa that have multiple sequences, there are few differences between those sequences. Indeed, one thing we note above is that the taxa identified as potential problems are not the same taxa with a larger percentage of ambiguity in their consensus sequence. On a final note, the taxon with the highest percentage of ambiguous characters in the full matrix, *Spurilla neapolitana* (9.7%), was still appropriately placed within a clade with other members of the genus *Spurilla* in all analyses, affirming that our consensus procedure had little to no deleterious effects on our results.

As has been suggested in Regier et al. [78], a final possibility is that insufficient search effort on each bootstrap pseudo-replicate may have played a role in the low bootstrap values found in our tree. However, this is unlikely in our case, as

our analysis included a total of five GARLI searches on each pseudo-replicate, in contrast to the single search replicate used in the analyses in Regier et al. [78]. As suggested in Debry & Olmstead [88], this type of resampling results in more precise estimates of bootstrap values, likely because insufficient search effort during bootstrapping has been shown to artificially lower bootstrap values.

In summary, we conclude that the most likely reason for our lack of resolution is simply that the data do not have sufficient phylogenetic signal to successfully reconstruct deep phylogenetic relationships. These might be obtained, however, using high-throughput sequencing for greater genomic data sampling; i.e., "phylogenomics." Rather than yielding only a few genes, these sequencing assays often provide hundreds of genes and have been successfully used to resolve relationships within many groups [89–93]. Thus, a phylogenomics study may be more successful at mapping the evolutionary history of Cladobranchia.

Current understanding of the phylogeny of Cladobranchia

Our results indicate that there is presently a severe lack of data useful for addressing deep evolutionary divergences within Cladobranchia. A result novel to this study, however, is the monophyly of Cladobranchia, which was recovered as paraphyletic (if the genus *Melibe* was included) in the only previous molecular phylogeny of this group [43]. Our analyses resolve Cladobranchia (including *Melibe*) as monophyletic, with high bootstrap support (bootstrap value = 97, MIN_TWO_GENES). However, much like the study of Pola & Gosliner [43], we find little resolution of relationships within Cladobranchia.

Six families within Cladobranchia were resolved as monophyletic, including Bornellidae, Hancockiidae, Tergipedidae, Dotidae, Dendronotidae and Lomanotidae. Three of these families (Bornellidae, Hancockiidae and Dendronotidae) are consistent with the results from a previous study [43], while the monophyly of Tergipedidae, Dotidae and Lomanotidae are novel results. However, given the relatively low support of the monophyly of Lomanotidae, greater taxon and or gene sampling within this family is probably necessary to better establish its position and status. The lower bootstrap support for this clade could be the result of including only two species from Lomanotidae in the analysis. The remaining families within Cladobranchia that were included in the analysis appear as non-monophyletic species assemblages. The monophyly of some of these families, such as Aeolidiidae [67,94], was previously determined by morphological and molecular evidence, while the monophyly of other families, such as Arminidae [95] or Scyllaeidae [66], was determined using only morphological characters. Still other families in Cladobranchia have been weakly supported by morphological data, including Tritoniidae [68]. The lack of support for these clades in this analysis could be due to low taxon sampling in some cases. In other cases, the molecular data may have simply revealed paraphyly or polyphyly within groups previously well supported by morphology.

It is abundantly clear that the evolutionary history of Cladobranchia remains to be understood. Our results provided some additional support for some relationships in this group, but the majority of the relationships in our trees remain unresolved (Table 2.1). In order to better understand evolution within this diverse group, as with any group of organisms, a well-resolved and well-supported phylogenetic tree is

necessary. The recent advances in phylogenomic approaches may hold the key to our understanding of taxonomic relationships within Cladobranchia.

Acknowledgements

I would like to thank my co-authors, Adam Bazinet, Allen Collins and Michael Cummings, as well as the researchers who generated and contributed sequence data to GenBank. I am also grateful to two anonymous reviewers for comments on an earlier version of this paper.

Chapter 3: Relationships within Cladobranchia (Gastropoda: Nudibranchia) based on RNA-Seq data: An initial investigation.

Introduction

Cladobranchia is a diverse (~1000 species) but understudied and poorly understood group of sea slug mollusks. It is also a clade within Nudibranchia, which is a group of marine gastropods defined by the loss of the adult shell [33]. In the absence of a protective shell, these slug lineages have evolved a diverse array of alternative defense mechanisms. The development of chemical and physical defense mechanisms has allowed for the occupation of new ecological niches [96] and has been hypothesized as having been a primary driver in the diversification of Nudibranchia [33]. Defensive strategies found in Cladobranchia include the uptake or synthesis of biochemically active compounds [28,97], the presence of warning (aposematic) coloration [29] or cryptic coloration to deter or hide from predators, respectively, and the use of stinging organelles (nematocysts) acquired from cnidarian prey [30,33]. The theft and synthesis of biochemically active compounds has provided a pool of materials that have potential uses in the synthesis of new pharmaceuticals (e.g. Zalypsis, currently in Phase II clinical trials for the treatment of various cancers; made from a chemical isolated from *Jorunna funebris*) [98–100]. Strong phylogenetic hypotheses of Cladobranchia will be useful in understanding the evolution of these

chemical defenses and the evolution of other character traits, such as the ability of many cladobranchs species to sequester nematocysts [30] and the evolution of swimming behaviors [37].

To date, there have been only two large-scale phylogenies published specifically on Cladobranchia [43,45], the first of which used the three genes most commonly used in nudibranch phylogenetics (mitochondrial 16S rRNA and Cytochrome Oxidase I, and nuclear Histone H3) [43]. The second study used all data publicly available through GenBank [101] to understand the evolutionary relationships of members of this group, thus adding two additional genes and increasing taxon sampling by ~200 taxa [45]. In both of these phylogenetic inferences of Cladobranchia, the majority of relationships remained unclear, both between and within the three traditional taxonomic divisions of Cladobranchia (Arminida, ~100 species; Dendronotida, ~250 species; and Aeolidida, ~600 species). The name of the taxon Arminida was changed by Bouchet & Rocroi [53], who introduced the taxon Euarminida for the two families Arminidae and Doridomorphidae. Though the analysis by Pola & Gosliner [43] supported the inclusion of only those families within a clade, they disagreed with the name change and retained Arminida in an altered sense, which we follow.

In addition to the phylogenies specifically aimed at understanding relationships within Cladobranchia, phylogenetic inferences have been attempted to address the evolutionary history of Nudibranchia or the larger clade Euthyneura [26,44,46,47,49,102,103]. The results regarding the major groupings within Cladobranchia were inconsistent in all of these studies, with all three major divisions

considered both paraphyletic and monophyletic in different publications. The most recent and comprehensive work by Wollscheid-Lengling et al. [44], based on the 18S, 16S and COI genes, suggested that Aeolidida is monophyletic and both Dendronotida and Arminida are paraphyletic.

Though the more basal evolutionary history of Cladobranchia has been problematic for phylogenetic inference, a number of studies (both morphological and molecular) have provided evidence to support relationships at both the family and generic levels. Phylogenies of individual families have been published on Aeolidiidae [67], Arminidae [95,104], Bornellidae [105], Glaucidae [106], Scyllaeidae [66] and Tritoniidae [68], and at the genus level publications have focused on *Antaeolidiella* [107], *Babakina* [69,108], *Berghia* [109], *Burnaia* [110], *Dendronotus* [111–113], *Limenandra* [114], *Melibe* [115], *Phyllodesmium* [116,117] and *Spurilla* [118]. All of these studies were focused on particular aspects of the Cladobranchia tree, and although they have supported some relationships, it is clear that the overall relationships of higher-level groups in Cladobranchia are not well understood. In addition, individually these studies cover very little of the overall diversity of Cladobranchia.

Most importantly for the purposes of this paper, the phylogenies estimated in all previous studies on taxa within Cladobranchia have lacked the support needed for confidence in suprageneric taxonomic relationships, with the exception of a small subset of familial-level phylogenies, mentioned above. This lack of resolution and low overall bootstrap support demonstrates that the relatively small, multi-gene strategies that have been previously used for phylogenetic reconstruction are

insufficient for comprehensive analyses and deep phylogenetic inferences regarding the relationships of this particular group. To address this, our paper presents a preliminary exploration into the use of RNA-Seq data to generate well-supported hypotheses regarding the evolutionary relationships within Cladobranchia. This study includes the publication of 16 new cladobranchian transcriptomes.

Methods

Organismal sampling

Two specimens of each representative species (a total of 16) were collected in tide pools or via snorkeling or SCUBA (under AAUS certification) using a variety of methods (direct collection, substrate collection and non-destructive collecting under

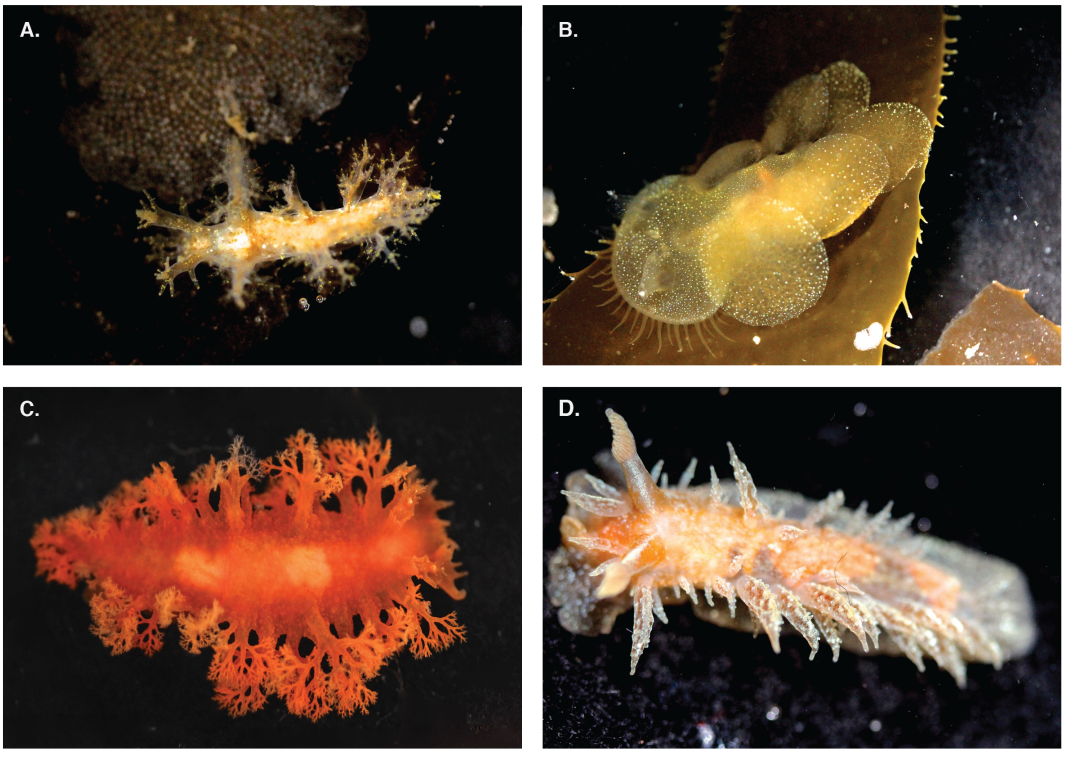

Figure 3.1. Select photographs of dendronotid and unassigned taxa used in this project, including: A) *Dendronotus venustus* (SRR1950948), B) *Melibe leonina*, C. *Tritoniopsis frydis* (SRR1950954), and D. *Dirona picta* (USNM1276030).

rocks) with one individual used for RNA-Seq and one individual preserved as a voucher and deposited in the Smithsonian National Museum of Natural History (NMNH). Some photographs specimen are shown in Figure 3.1 and Figure 3.2. We generated raw transcriptome data by RNA-Seq for 16 Cladobranchia species, and downloaded data for one additional Cladobranchia species from the NCBI Sequence Read Archive (SRA). Three outgroup transcriptomes were also obtained from the SRA: two representatives of Anthobranchia (the sister taxon of Cladobranchia), and one of Pleurobranchoidea (the sister taxon to Nudibranchia). Specimen and sequence data are listed in Table 3.1.

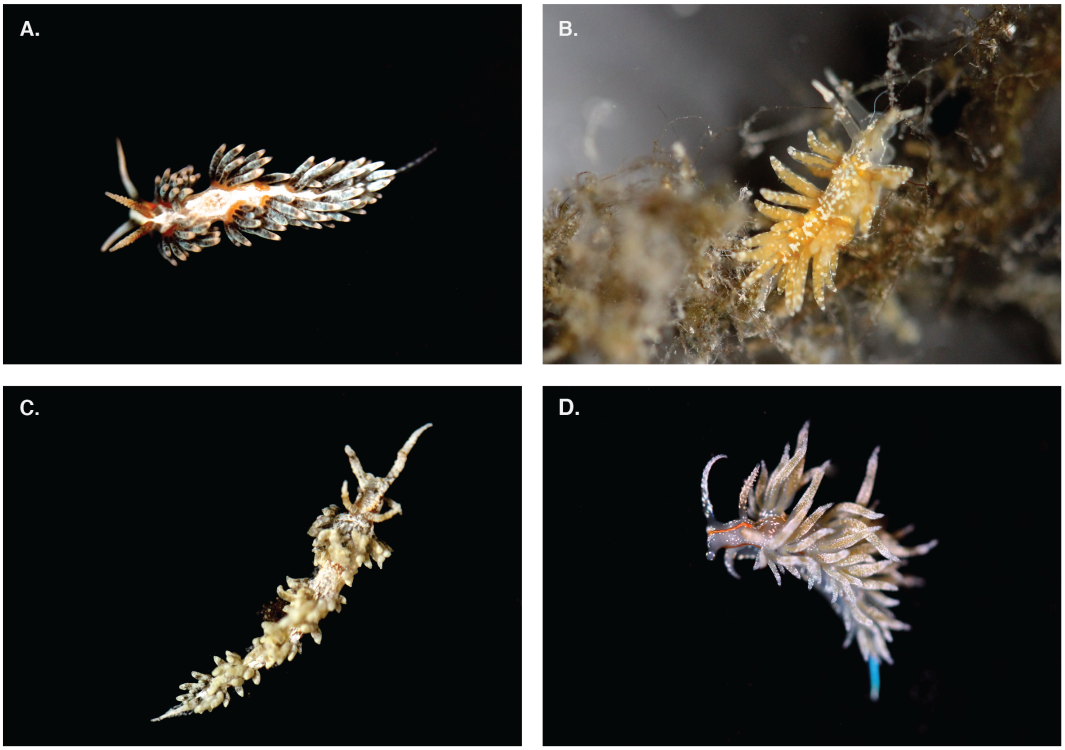

Figure 3.2. Select photographs of aeolid taxa used in this project, including: A) *Berghia stephanieae* (SRR1950951), B) *Favorinus auritulus* (USNM1276034), C. *Palisa papillata* (SRR1950952), and D. *Dondice occidentalis*.

A visual examination was used for confirmation of identity using field guides published by Valdes et al. [119] (for the Caribbean) and Behrens & Hermosillo [120]

(for the Indo-Pacific), as well as expert opinions when the placement of species was uncertain. One of the two specimens was placed in RNAlater solution (Qiagen, Hilden, Germany) for RNA preservation and frozen within one week of collection in -80°C to prevent RNA degradation. Some specimens in RNAlater were refrigerated or placed in a -20°C within 24 hours, others were kept at room temperature for up to one week. A second specimen of each species was preserved as a voucher for morphological analysis, first in Bouin's Fixative and subsequently transferred to 70% ethanol for long-term storage. Voucher specimens were depositied in the Smithsonian National Museum of Natural History (SI-NMNH) and are available for study under the catalog numbers provided in Table 3.1.

Table 3.1. List of specimens examined in this study, including species name, locality, and morphological voucher information. Sequence Read Archive accession numbers are also provided for each transcriptome.

Species	Locality	Morphological voucher	SRA accession no.
Bathydoris clavigera		NCBI SRA	SRR1505104
Doris kerguelenensis		NCBI SRA	SRR1505108
Fiona pinnata		NCBI SRA	SRR1505109
Pleurobranchaea californica		NCBI SRA	SRR1505130
Austraeolis stearnsi	Point Loma, San Diego, CA, USA	USNM1276025	SRR1950943
Berghia stephanieae	Key Largo, FL, USA	-	SRR1950951
Catriona columbiana	Redondo Beach, CA, USA	-	SRR1950949
Cuthona albocrusta	Mission Bay, San Diego, CA, USA	USNM1276026	SRR1950944
Dendronotus venustus	Morro Bay, CA, USA	USNM1276033	SRR1950948
Dirona picta	Redondo Beach, CA, USA	USNM1276030	SRR1950946
Dondice occidentalis	Riviera Beach, FL, USA	USNM1276036	SRR1950953
Doto lancei	Mission Bay, San Diego, CA, USA	USNM1276027	SRR1950945
Favorinus auritulus	Key Largo, FL, USA	USNM1276034	SRR1950950
Flabellina iodinea	Point Loma, San Diego, CA, USA	USNM1276023	SRR1950940
Hermissenda crassicornis	Sunset Cliffs, San Diego, CA, USA	USNM1276022	SRR1950939
Janolus barbarensis	Point Loma, San Diego, CA, USA	USNM1276029	SRR1950942
Melibe leonina	Morro Bay, CA, USA	USNM1276031	SRR1950947
Palisa papillata	Key Largo, FL, USA	-	SRR1950952
Tritonia festiva	Point Loma, San Diego, CA, USA	USNM1276024	SRR1950941
Tritoniopsis frydis	Pompano Beach, FL, USA	USNM1276038	SRR1950954

RNA extraction and sequencing

A 20–100 mg tissue sample was taken from the anterior of each animal and manually homogenized using a motorized pestle. After 1–2 minutes of homogenizing, the tissue was flash frozen in liquid nitrogen for subsequent homogenizing, until tissue mixture was fully uniform. 500 µL of TriZOL Reagent (Life Technologies, Carlsbad, CA, USA) was then added and the mixture was homogenized again. This procedure was repeated until the solution was fully homogenized. Once this process was complete, an additional 500 µL of TriZOL Reagent was added to the solution and the mixture was left at room temperature for five minutes.

100 µL of BromoChloroPropane was then added to the solution, which was subsequently mixed thoroughly. The mixture was left at room temperature for five minutes, then centrifuged at 16,000g for twenty minutes at 8°C. Following this step, the top aqueous phase was removed and placed in another tube where 500 µL of 100% isopropanol was added. This tube was stored overnight at -20°C for RNA precipitation.

After overnight precipitation, the samples were centrifuged at 17,200g for ten minutes at 4°C. The supernatant was then removed and the pellet washed with freshly prepared 75% ethanol. The sample was then centrifuged at 7,500g for five minutes at 4°C. The supernatant was removed and the pellet air-dried for one to two minutes (or until it looked slightly gelatinous and translucent). The total RNA was then re-suspended in 10–30 µL of Ambion Storage Solution (Life Technologies, Carlsbad, CA, USA), and 1 µL of RNase inhibitor (Life Technologies, Carlsbad, CA, USA) was added to prevent degradation.

Total RNA samples were submitted to the DNA Sequencing Facility at University of Maryland Institute for Bioscience and Biotechnology Research, where quality assessment, library preparation and sequencing were completed. RNA quality assessment was done with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), and total RNA samples with a concentration higher than 50 ng/μL were used for library construction. Library preparation used the Illumina TruSeq RNA Library Preparation Kit v2 (Illumina, San Diego, CA, USA) and 200 base pair inserts; and 100 base pair, paired-end reads were sequenced with an Illumina HiSeq1000 (Illumina, San Diego, CA, USA).

Quality control and assembly of reads

Reads that failed to pass the Illumina "Chastity" quality filter were excluded from our analyses. Subsequent quality assessment and control were performed using autoadapt (version 0.2; [44]) with default settings, which in turn used FastQC (version 00; [45]) and cutadapt (version 1.3; [46]) to remove overrepresented sequences and to trim and remove low-quality reads. Reads passing quality control were assembled using Trinity (version r20140717; [47]) with default settings, which required assembled contigs to be at least 200 base pairs long.

Orthology assignment

Translated transcript fragments were organized into orthologous groups corresponding to a custom gastropod-specific core-ortholog set (3,854 protein models) using HaMStR (version 13.2.2; [48]), which in turn used FASTA (version 36.3.6d; [49]), GeneWise (version 2.2.0; [50]) and HMMER (version 3.0; [51]). In

the first step of the HaMStR procedure, substrings of assembled transcripts (translated nucleotide sequences) that matched one of the gastropod protein models were provisionally assigned to that orthologous group. To reduce the number of highly divergent, potentially paralogous sequences returned by this search, we set the E-value cutoff defining an HMM hit to 1e-05 (the HaMStR default is 1.0), and retained only the top-scoring quartile of hits. In the second HaMStR step, the provisional hits from the HMM search were compared to our reference taxon, *Aplysia californica*, and retained only if they survived a reciprocal best BLAST [52] hit test with the reference taxon using an E-value cutoff of 1e-05 (the HaMStR default was 10.0). In our implementation, we substituted FASTA [49] for BLAST because FASTA programs readily accepted our custom substitution matrix (GASTRO50).

Table 3.2. HaMStR statistics for the subset of orthologous groups passing our paralogy filter, given for each taxon.

Species	Sequences matching orthologous groups	Unique orthologous groups represented	Mean length (in amino acids)
Bathydoris clavigera	782	621	215
Doris kerguelenensis	622	530	190
Fiona pinnata	723	623	232
Pleurobranchaea californica	918	698	217
Austraeolis stearnsi	607	578	238
Berghia stephanieae	629	587	228
Catriona columbiana	540	513	224
Cuthona albocrusta	630	576	239
Dendronotus venustus	763	674	263
Dirona picta	589	558	227
Dondice occidentalis	621	578	227
Doto lancei	470	446	205
Favorinus auritulus	673	626	255
Flabellina iodinea	660	613	264
Hermissenda crassicornis	619	593	226
Janolus barbarensis	356	351	174
Melibe leonina	627	591	229
Palisa papillata	615	581	226
Tritonia festiva	469	453	168
Tritoniopsis frydis	407	388	210

The gastropod core-ortholog set was generated by first downloading all available gastropod clusters with 50% similarity or higher from UniProt [121]

(39,403 clusters). Excluding clusters that contained only one sequence left 6,160 clusters. We calculated the sequence similarity of each cluster and as a heuristic, decided to remove clusters whose percent identity was less than 70%, which left 6,015 clusters. We then assessed the number of times each taxon was represented within those clusters. *Aplysia californica* was identified as the most abundant taxon (3,854 associated clusters with 70% similarity or higher), and was therefore selected as the reference taxon for the custom HaMStR database. We constructed the gastropod HaMStR database by following the steps given in the HaMStR README file, which included generating profile hidden Markov models for each cluster using HMMER. Our gastropod HaMStR database contained 3,854 orthologous groups. All protein sequences for *Aplysia californica* (Uniprot/NCBI taxon ID 6500) were downloaded from UniProt and used to generate the BLAST database for HaMStR.

Construction of the custom substitution matrix (GASTRO50) followed the procedure outlined in Lemaitre et al. [122], and used the 50%-similarity gastropod clusters downloaded from UniProt. In this protocol, a block is defined as a conserved, gap-free region of the alignment. Our blocks output file contained 34,109 blocks and a total of 2,442,130 amino acid positions. (This was after we removed one large block from the blocks output file that contained 1,388 sequences, which prevented the scripts from executing properly.)

Construction of data matrix and paralogy filtering

Protein sequences in each orthologous group were aligned using MAFFT (version 787; [70]). We used the --auto and --addfragments options of MAFFT to add transcript fragments to the *Aplysia californica* reference sequence, which was

considered the existing alignment. We converted the protein alignments to corresponding nucleotide alignments using a custom Perl script. A maximum likelihood tree was inferred for each orthologous group using GARLI (version 2; [73]), and was given as input to PhyloTreePruner (version 1.0; [20]). Following the workflow of Bazinet et al. [89], orthologous groups that showed evidence of out-paralogs for any taxa were discarded; for those with in-paralogs, multiple sequences were combined into a single consensus sequence for each taxon. This process left 839 orthologous groups eligible for inclusion in data matrices. Individual orthologous group alignments were then concatenated (*nt123_unfiltered* matrix). Positions not represented by sequence data in at least four taxa were then removed (*nt123* matrix), which resulted in more compact data matrices. To address potential issues in regards to missing data, three additional matrices were generated by increasing the required representation for a position to remain within the data matrices: *nt123_min80percentcomplete* (position must have been represented in at least 16 taxa), *nt123_min90percentcomplete* (positions must have been represented in at least 18 taxa) and *nt123_100percent complete* (positions must have been represented in all taxa).

The *nt123* nucleotide matrix was then subjected to degen1 encoding (version 1.4; [123]), which we refer to as our *degen* matrix. "Degen" uses degeneration coding to eliminate all synonymous differences among species from the data set, resulting in phylogeny inference based only on non-synonymous nucleotide change. This procedure was shown in a previous study [124] to generally improve recovery of deep nodes.

Phylogenetic analyses

To conduct the phylogenetic analyses we used GARLI (Genetic Algorithm for Rapid Likelihood Inference version 2; [73]) through the GARLI web service hosted at molecularevolution.org [74]. We used a general time reversible nucleotide model [75] with a proportion of invariant sites and among site rate heterogeneity modeled with a discrete gamma distribution (GTR+I+G) together with GARLI default settings, including stepwise-addition starting trees. We first analyzed the *nt123_unfiltered* data matrix, partitioned by codon position (*nt123_partitioned*), followed by the unpartitioned *nt123* and *degen* data matrices (*nt123* and *degen* analyses, respectively). We then analyzed the partitioned *nt123_unfiltered* data matrix including only the first and second codon positions (*nt12_partitioned*) and the three more complete matrices *nt123_min80percentcomplete* (*nt123_min80*), *nt123_min90percentcomplete* (*nt123_min90*) and *nt123_100percentcomplete* (*nt123_100percent*). For each analysis, we ran 10 best tree searches and 1,000 bootstrap replicates. Post-processing of the phylogenetic inference results was performed by the GARLI web service at molecularevolution.org using DendroPy [76] and the R system for statistical computing [77], which included the construction of bootstrap consensus trees.

Results

Read quality statistics

The raw number of 101 base pair reads for each newly sequenced transcriptome ranged from 44,805,574 to 65,504,176 (mean: ~55 million reads; Appendix Table B1). Read processing (filtering and trimming) removed 1.40% to 94% of reads per

sample (mean: 3.96%); thus, the number of reads provided as input to assembly ranged from 43,047,096 to 63,155,516 (mean: ~52 million).

Assembly and data matrix properties

The number of transcript fragments per sample ranged from 56,091 to 242,632 (mean: 108,957; Appendix Table B2). N50 ranged from 408 to 921 bases (mean: 714). HaMStR results for the 839 orthologous groups used in our analyses are presented in Table 3.2 (HaMStR results for the complete set of orthologous groups are presented in Appendix Table B3). The number of sequences from each assembly that matched the HaMStR database ranged from 662 to 1,765 (mean: 1,172). However, the number of matches to unique orthologous groups ranged from 599 to 1,126 (mean: 916). The mean length of matching sequences was 249 amino acids. When concatenated and filtered, the final data matrices contained 9,354-1,702,782 nucleotide positions from 839 orthologous groups and were 23-100% complete (Appendix Table B4).

Phylogenetic results

Our analyses supported Cladobranchia as a monophyletic group with a bootstrap (BS) value of 100% (Figure 3.3). This result remains consistent across topologies derived from all seven analyses.

Aeolidida is also monophyletic (BS = 100%) across all topologies, containing *Flabellina* (Flabellinidae), *Berghia* (Aeolidiidae), *Hermissenda* (Facelinidae), *Dondice* (Facelinidae), *Favorinus* (Facelinidae), *Palisa* (Facelinidae), *Austraeolis* (Facelinidae), *Fiona* (Fionidae), *Cuthona* (Tergipedidae) and *Catriona*

(Tergipedidae). Facelinidae is paraphyletic due to the inclusion of *Berghia* in a clade with the members of this family. This clade is sister to Flabellinidae (BS = 100%). In the sister group, Tergipedidae is monophyletic (BS = 100%) and as the sister taxon to Fionidae (BS = 100%).

Dendronotida is paraphyletic in all topologies. The *degen* and *nt12_partitioned* analyses (Figure 3.3A) supported three clades within Dendronotida, and the *nt123 and nt123_partitioned* analyses (Figure 3.3B) supported two clades. The clade found in both topologies (BS = 100%) contained *Doto* (Dotidae), *Melibe* (Tethyidae) and *Dendronotus* (Dendronotidae) and placed Tethyidae and Dendronotidae as sister group (BS = 100%). The topology returned by the *nt123* analyses included a clade sister to Aeolidida (BS = 100% in *nt123* analyses) that contained *Tritonia* (Tritoniidae), *Tritoniopsis* (Tritoniidae), *Janolus* (Proctonotidae) and *Dirona* (Dironidae), whereas the *degen* and *nt12_partitioned* analyses both supported a clade containing Proctonotidae and Dironidae (BS = 100%) as sister to Aeolidida (BS = 84%, *degen*; 86%, *nt12_partitioned*), and the Tritoniidae clade (BS = 100%) as sister to the Aeolidida + Proctonotidae + Dironidae assemblage (BS = 77%, 100%).

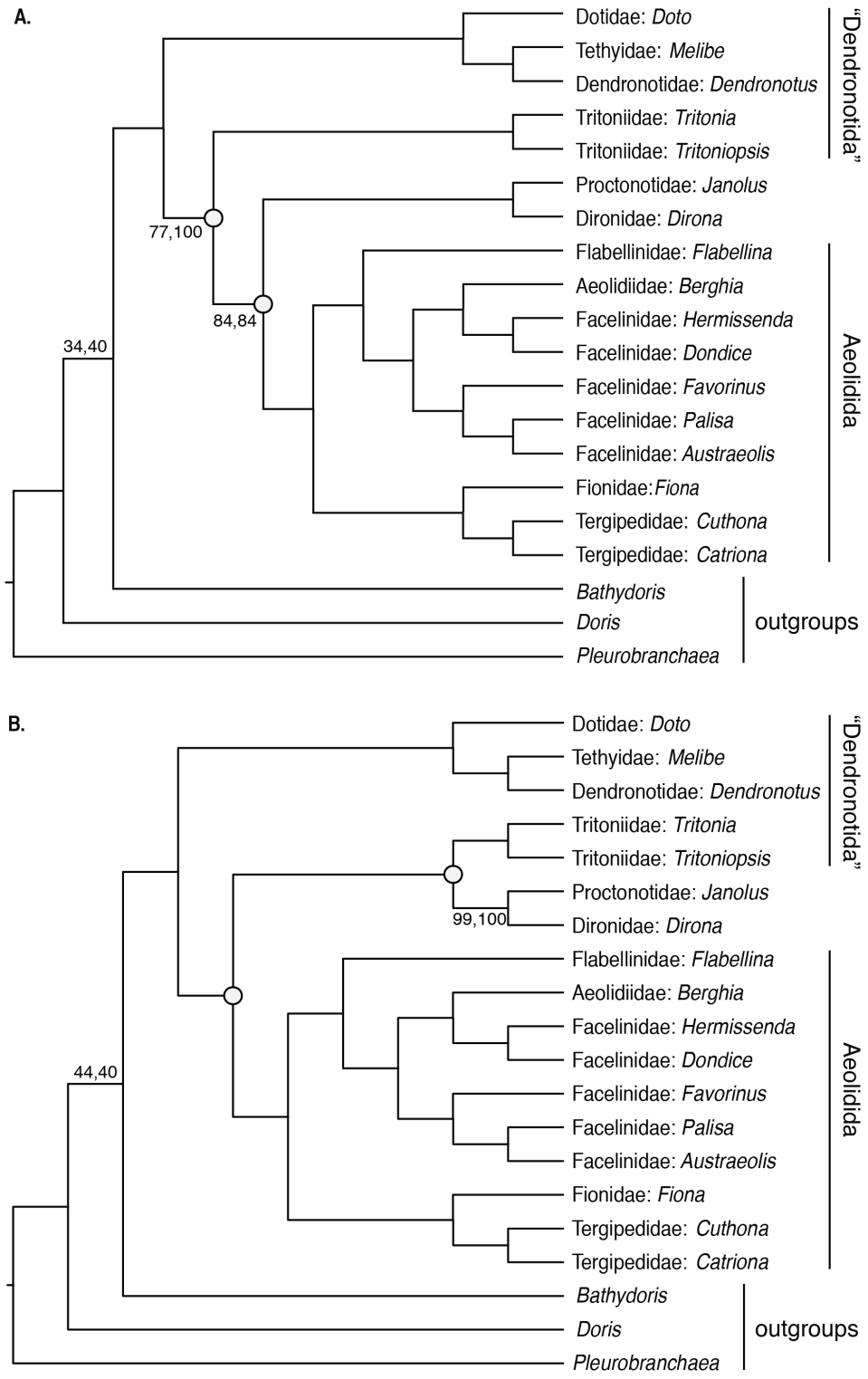

Figure 3.3. A) The maximum likelihood tree from the *degen* (first bootstrap value) and *nt12_partitioned* (second bootstrap value) analyses; B) The maximum likelihood tree from the *nt123* (first bootstrap value) and *nt123_partitioned* (second bootstrap value) analyses. All unlabeled nodes have 100% bootstrap support in both

Discussion

In this section, we first review the characteristics of our data across our bioinformatics pipeline and the construction of our data matrix. We then address the efficacy of transcriptome data for inferring phylogenetic relationships within Cladobranchia, and examine potential methodological concerns. Finally, we discuss the novel results produced by our analyses and compare them to previous studies.

Bioinformatics pipeline and data matrix construction

The number of reads from newly sequenced transcriptomes was higher than from transcriptomes downloaded from the SRA (Appendix Table B1). This is possibly due to a greater depth of sequencing in our pipeline compared to the original paper that published those transcriptomes [22]. Alternatively, the data retrieved from the SRA had already been filtered, with many reads removed prior to download. Though the average number of transcript fragments from the Trinity assembly was lower in newly generated data sets, the average length of the fragments was higher (Appendix Table B2). Importantly, the number of sequences that matched to unique orthologous groups, and the average length of these sequences was sufficient for all taxa (Appendix Table B3), indicating that sequencing depth may not be a limiting factor. After we removed sites that were represented in fewer than four taxa, overall matrix completeness was slightly higher than in the previous five-gene, 296-taxon analysis [45]. Most importantly, the number of loci used in this analysis was orders of magnitude higher than in the two previous phylogenetic studies on the evolutionary history of Cladobranchia [43,45].

Use of phylotranscriptomics to understand the evolution of Cladobranchia

Phylogenetic inference of Cladobranchia has been a difficult part of the larger problem of understanding the evolutionary history of Nudibranchia [43–45,67]. However, the high bootstrap values among the ingroup taxa in our analyses suggest that RNA-Seq will be useful in generating a well-supported hypothesis of the phylogenetic relationships among genera in Cladobranchia, thereby providing a basis for establishing a classification at infraorder-, superfamily- and family-levels that reflects evolutionary history. Our tree topologies are almost fully resolved by our data, and the nodes that are recovered consistently across all trees all have 100% bootstrap support. Thus, a phylotranscriptomic approach for understanding the phylogeny of Cladobranchia seems to be extremely effective in providing evidence to support relationships that were previously uncertain.

Bootstrap support levels

Though our methodology seems to be have produced good results, the possibility exists that some of our relatively sparse data matrices (with at most 46.8% completeness) may have misled our likelihood analyses [79]. To address this, we ran analyses with more complete matrices (87.9-100%). In these results, the bootstrap support values are quite high across the phylogenies, even with a matrix length of 48,426 nucleotides (93.6% complete), although the values decrease considerably in the 100% complete matrix analysis. The overall topology of the phylogenies from these analyses was also consistent with the other analyses, with Dendronotida supported as paraphyletic and Aeolidida and Cladobranchia supported as monophyletic. In addition, some research suggests that missing data may not be as

much of a problem as some have suspected. In Cho et al. [80], a data matrix with 45% intentionally missing data yielded no signs of the contradictory groupings that missing data might produce. This result is consistent with those of three other studies from across a broad taxonomic range, including frogs [81], angiosperms [82], an entire phylum of eukaryotes [83] and strains of the HIV virus [84].

In addition to concerns regarding missing data, multiple studies have been published that suggest that bootstrap values in phylogenomic analyses may be inflated, primarily due to incongruent gene topologies [125,126]. However, others have suggested that these issues are not as problematic as they may seem. In particular, Simmons & Norton [127] specifically state that bootstrap methods do not seem to have an elevated false-positive rate, and Betancur et al [128] found that the incongruence in the data of Salichos & Rokas [125] is likely the result of sampling error, and thus not likely to be responsible for inflated bootstrap values. In the case of our data and analyses, it is important to note that the divergences within Nudibranchia are much more recent than those addressed by Salichos & Rokas [125] and the number of genes in our analyses surpasses those in Dell'Ampio et al [126].

The phylogeny of Cladobranchia

These analyses have resolved several questions regarding the evolutionary relationships within Cladobranchia. First and foremost, the monophyly of Cladobranchia is reinforced with 100% bootstrap support. Though monophyly was indicated in previous morphological [26] and molecular [44,45] analyses, there have also been studies suggesting paraphyly [43] when the genus *Melibe* was included.

Of the three traditional taxonomic divisions within this group, members of Dendronotida (*Melibe, Dendronotus, Tritonia* and *Tritoniopsis*) and Aeolidida (*Flabellina, Berghia, Hermissenda, Dondice, Favorinus, Palisa, Austraeolis, Fiona, Cuthona* and *Catriona*) are included in our analyses, as well as three taxa (*Doto, Dirona* and *Janolus*) that were recently classified as unassigned to any of the three groups [43,44,49]. Both *Janolus* and *Dirona* were originally considered to be within Arminida, and *Doto* was once placed under Dendronotida before newer molecular analyses rejected those classifications [26,44,46]. In our analyses, Dendronotida is not supported as monophyletic, which is consistent with previous morphological [26] and molecular [43–45,49] phylogenetic hypotheses. Our results indicate a serious need for complete taxonomic revision of the taxa within this group. The monophyly of Aeolidida has also been uncertain. Molecular analyses of Nudibranchia have supported Aeolidida as both paraphyletic and monophyletic, depending on the genes used for the analysis [44]. Other molecular studies on the evolution of Cladobranchia have suggested that Aeolidida is monophyletic [43,49], with very low support, although another did not support Aeolidida as monophyletic [45]. Our analyses strongly support the hypothesis in Pola & Gosliner [43], with Aeolidida being monophyletic with extremely high bootstrap support.

Given our taxon sampling, the earliest diverging lineage within Cladobranchia is a clade containing Dotidae (*Doto*), Tethyidae (*Melibe*) and Dendronotidae (*Dendronotus*), which is a result novel to this study. An exciting result is the inclusion of *Melibe* well within Cladobranchia. This particular genus of filter feeders, which captures crustaceans using a dome-like oral hood fringed by sensory tentacles [129],

was excluded from Cladobranchia in a previous study [43]. In that study, the authors attributed this result to a deletion in a section of the COI fragment used in the phylogenetic analyses. Further examination included *Melibe* within Cladobranchia [45], but the *Melibe* clade was at the end of a long branch, and might therefore have caused issues in phylogenetic inference [130].

Elsewhere among the dendronotid taxa, the family Tritoniidae (*Tritonia* and *Tritoniopsis*) is supported as monophyletic, as is a group composed of Proctonotidae (*Janolus*) and Dironidae (*Dirona*). In previous molecular analyses, Tritoniidae has been revealed as both paraphyletic [45] and monophyletic [43], though the monophyly results was poorly supported, with a posterior probability below 0.6. In our analyses, the exact positions of these two clades are uncertain. In one of our analyses (*nt123_unfiltered*), these two clades are sister taxa (Figure 3B), whereas other data sets (*nt123* and *degen*) suggest that the clade consisting of Proctonidae and Dironidae is the sister taxon of Aeolidida. Neither of these cases were supported in any previous analyses, but the tree presented in Figure 3.3B clearly provides stronger support for the two clades as sister taxa, and these analyses included both synonymous and non-synonymous substitutions.

Within Aeolidida, the families Tergipedidae (*Cuthona* and *Catriona*) and Fionidae (*Fiona*) are sister taxa, supporting a previous hypothesis [67]. The study of Carmona et al. [43] weakly supported these taxa as an early diverging lineage within Aeolidida. Here, our phylogenomic data strongly favor this clade as the sister group of the remaining aeolid taxa in our analysis. An especially interesting result within Aeolidida is the paraphyly of Facelinidae (*Hermissenda, Dondice, Favorinus, Palisa*

and *Austraeolis*), which forms a clade with Aeolidiidae (*Berghia*) that is sister to Flabellinidae (*Flabellina*). The paraphyly of Facelinidae with respect to Aeolidiidae is consistent with the results of Carmona et al. [67] and Mahguib & Valdés [49]. Our data strongly support the hypothesis that Aeolidiidae is derived from within Facelinidae and that the closest relative to Aeolidiidae, among the facelinid taxa we have been able to include, is a clade consisting of *Hermissenda* and *Dondice*, two genera that were not included in the Carmona et al. study [18]. Our lone representative of Flabellinidae was revealed as the sister group of Facelinidae plus Aeolidiidae. Similarly, Carmona et al. [18] found flabellinids in a clade sister to a clade consisting of Facelinidae, Babakinidae and Aeolidiidae, but their study revealed Flabellinidae to be polyphyletic, being interspersed with taxa from the family Piseinotecidae, which has not yet been sampled for phylogenomic data. Hence, broader taxon sampling will be necessary to better understand the specificity of these evolutionary relationships within Aeolidida.

Though this study supports some previous hypotheses and provides new support for others, more work remains to be done. It is critical for future studies to increase taxon sampling. These analyses represent only 10 of the 32 families and 17 of the over 100 genera from Cladobranchia that are accepted in the World Register of Marine Species [131]. It will be especially important to include taxa from Arminida, which would allow for a more complete evaluation of the placement of families with uncertain affinities. Additionally, a representative from Doridoxidae would allow for evaluation of the placement of *Doridoxa* within Cladobranchia [49], for which existing molecular data yield inconsistent results [103]. Overall, an increase in the

number of genera and families represented in these analyses will allow for a more complete hypothesis regarding the evolutionary history of Cladobranchia, which will in turn allow for assessments of character evolution through detailed comparative analysis.

Acknowledgements

I would first like to thank my co-authors, Adam Bazinet, Allen Collins and Michael Cummings. We are grateful to Constance Gramlich at San Diego State University (SDSU), Craig Hoover at California State Polytechnic University, Pomona (CPP), Jermaine Mahguib at Iowa State University, Ariane Dimitris and José Victor Lopez at Nova Southeastern University for assistance with specimen collection. We would also like to thank Nathan Robinette at SDSU and Emma Ransome and Vanessa Gonzalez at the Smithsonian National Museum of Natural History (NMNH) for help with RNA extractions. Finally, we are grateful to Ángel Valdés at CPP and Forest Rohwer at SDSU for the use of their laboratory facilities for specimen processing. Portions of the laboratory work were also conducted in, and with the support of, the L.A.B. facilities of the National Museum of Natural History. This work was supported by the Conchologists of America, the Society of Systematic Biologists, a University of Maryland Graduate School Dean's Fellowship, a Smithsonian Institution Small Grant award, funding from University of Maryland, and NSF Partnerships for International Research and Education program Award 1243541.

Chapter 4: Identification guide to the heterobranch sea slugs (Mollusca: Gastropoda) from Bocas del Toro, Panama

Introduction

The Bocas del Toro Archipelago is located on the Caribbean coast of Panama, near the Costa Rican border. The major islands of the archipelago include Isla Colón, Bastimentos, Solarte, Cristóbal, Popa and Cayo Aqua. The archipelago has a predominantly wet climate, receiving an average precipitation of 2870 mm per year [132] and a maximum of 7000 mm [133]. The primary marine ecosystems in the archipelago consist of mangroves (dominated by red mangroves), seagrass beds and coral reef patches [134–136].

The Bocas del Toro Research Station, a well-known marine station of the Smithsonian Tropical Research Institute (STRI), is located on Isla Colón. Numerous researchers at this station, both past and present, have utilized the waters surrounding the archipelago for various studies. However, this research has often been hampered by a lack of accurate and updated identification/field guides. This is particularly problematic for researchers studying heterobranch sea slugs, for which the taxonomy and systematics have changed dramatically in recent years. The only available field guide for Caribbean heterobranch sea slugs [119] is outdated and in need of revision.

Although the Caribbean Sea is inhabited by hundreds of heterobranch sea slug species [119], only 19 species have been formally identified and documented in the

Bocas del Toro Archipelago [136], representing only a fraction of the total diversity of sea slugs in the Caribbean.

In this paper we present an updated record of the diversity of heterobranch sea slugs in the Bocas del Toro Archipelago, resulting from several research trips to the area and a field course organized by STRI in July and August of 2015. We report new records for Bocas del Toro and provide updated information for previous records from the overall area, increasing the total number of observed heterobranch sea slug species in the region to 82.

Materials and Methods

The STRI course on the taxonomy of sea slugs took place from July 24th to August 5th, 2015 in the Bocas del Toro Archipelago, Panama. Collecting effort for this expedition was documented and completed by a total of 16 observers with various levels of experience in searching for sea slugs (the minimum and maximum number of observers at any given time was 7 and 15, respectively). Although the amount of substrata collected was not measured, search time and the number of observers in each location were recorded. Therefore, "collecting effort" refers to the total searching time through direct observations for all observers. The results below represent an estimation of the species found using both direct and indirect methods.

Records from two previous field expeditions in Bocas del Toro are reported here as well, the first occurring in December 2004 and the second in July 2006. Collecting effort during these trips was not quantified, thus is not documented in this paper.

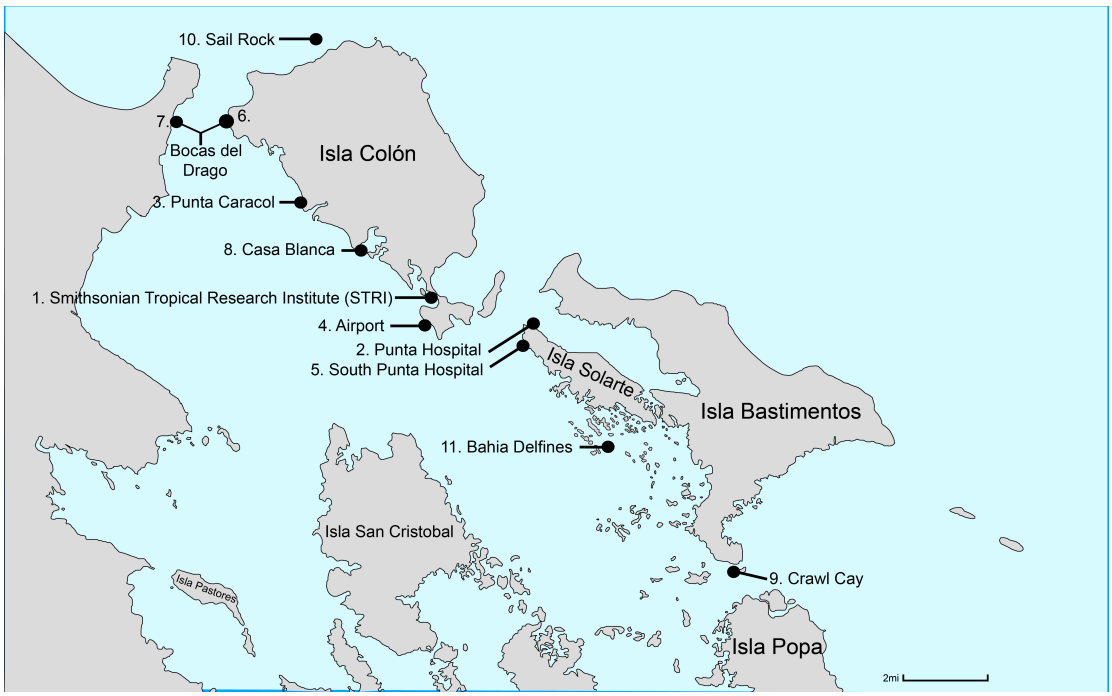

Figure 4.1. Map of localities sampled in Bocas del Toro, Panama.

Eleven sites in Bocas de Toro were explored, exclusively during the daytime (Figure 4.1). Specimens were documented via: (1) direct observation in the field (mainly snorkeling, but also SCUBA diving), or (2) substrate collection (primarily various species of algae and hydroids). After the substrate was collected and searched, materials were separated into trays with fresh seawater and left to rest overnight to allow for further examination and collection of sea slugs the following day.

Most specimens were identified in the field using the field guide by Valdés *et al.* [119] or in the laboratory using primary literature from the Caribbean region. A few problematic specimens were identified based on unpublished sequence data. Some specimens were collected and preserved for further study.

Results

After approximately 307.5 person-hours of field searching, a total of 82 species belonging to five clades of heterobranch sea slugs were found, some of which have not yet been described (Table 4.1). The clade Nudibranchia had the highest number of species (n=40, ~49% of total) and was present in all eleven localities, followed by Sacoglossa (n=28, ~34% of total), which was present in all localities but one. In contrast, Pleurobranchomorpha had the lowest number of species (n=2, <3% of total) and was found in only three localities (Table 4.1).

Table 4.1. Number and proportion of species found per clade in Bocas del Toro, Panama.

Order	Number of species	Percentage of total	Localities
Cephalaspidea	6	7.3	1, 8–11
Anaspidea	6	7.3	1, 6, 10
Sacoglossa	28	34	1–10
Pleurobranchomorpha	2	2.4	2, 4, 9
Nudibranchia	40	48.8	1–11

The highest number of species (n=22) was found at STRI (locality 1), followed by Crawl Cay (locality 9) and Sail Rock (locality 10), and the lowest overall species number (n=2) was recorded in the Panamanian mainland side of Bocas del Drago (locality 7) (Table 4.2). All sites had species belonging to the clades Nudibranchia and Sacoglossa, except for Little Cay in Bahía Delfines (locality 11), in which sacoglossans were not found. The average search time and number of nudibranch species found per locality were almost 28 h and n=5, respectively. The locality with the highest collecting effort was STRI (locality 1) and the lowest was the Panamanian mainland side of Bocas del Drago (locality 7) (Table 4.2), which might explain the highest and lowest number of species found. It is also important to note that locality 7 was the only collecting site located off the Panamanian mainland,

which contains numerous rivers and is strongly influenced by terrestrial runoff and turbidity in the water. These factors likely reduced the overall abundance of heterobranch sea slugs and impeded attempts to find them. In Sail Rock (locality 10) all the species were found by indirect methods.

Table 4.2. Search time and number of species found in each of the 11 sites explored in Bocas del Toro, Panama.

Site	Search time (h)	Nudibr-anchia	Anaspidea	Pleurobran-chomorpha	Cephalaspidea	Sacoglossa	Total
1	75.25	10	3	0	1	8	22
2	34.75	3	0	1	0	5	9
3	36	3	0	0	0	1	4
4	25.5	7	0	2	0	2	11
5	18.42	1	0	0	0	4	5
6	21.42	6	1	0	0	2	9
7	2.67	1	0	0	0	1	2
8	22.5	3	0	0	1	2	6
9	44.5	13	0	1	1	3	18
10	4.5	9	2	0	2	3	16
11	22	4	0	0	2	0	6

In the systematics section below, summarized descriptions and illustrations are provided for described species as well as for those species previously recognized as distinct in other studies. For most species the habitat information (substrate or food source on which specimens were found) is provided. In cases in which the food source is important for field collection or identification, but the animals were not found in association with specific substrates, this information is provided with references. Several sacoglossan species were kept in captivity and the egg masses obtained and examined; brief descriptions of the egg masses are also included. For some species egg mass information is provided with references meaning that these data were not obtained in the course of this study.

SYSTEMATICS

Clade NUDIPLEURA Wägele & Willan, 2000

Order PLEUROBRANCHOMORPHA Pelseneer, 1906

Suborder PLEUROBRANCHOIDEA Gray, 1827

Family PLEUROBRANCHIDAE Gray, 1827

Genus *Pleurobranchus* Cuvier, 1804

Pleurobranchus areolatus Mörch, 1863

(Figure 4.2A)

SYNONYMS

Pleurobranchus crossei Vayssière, 1896; *Pleurobranchus atlanticus* Abbott, 1949; *Pleurobranchus reesi* White, 1952; *Susania gardineri* White, 1952; *Pleurobranchus evelinae* Thompson, 1977; *Pleurobranchus emys* Ev. Marcus, 1984.

DESCRIPTION

Body oval. Rhinophores rolled and fused at the base, with horizontal striations from base to tip. Dorsum with numerous small, polygonal and flat tubercles. Shell internal. Background color ranges from light brown to deep violet, with varying degrees of opaque white pigment on the tubercles. In some cases the opaque white pigment is arranged in a symmetrical pattern across the body. Up to 150 mm long.

DISTRIBUTION

Mexico, Costa Rica, Venezuela, Brazil, Jamaica, Puerto Rico, St. Thomas, Aruba, St. Maarten/St Martin, Bahamas, Bermuda [119,137] and Panama [136].

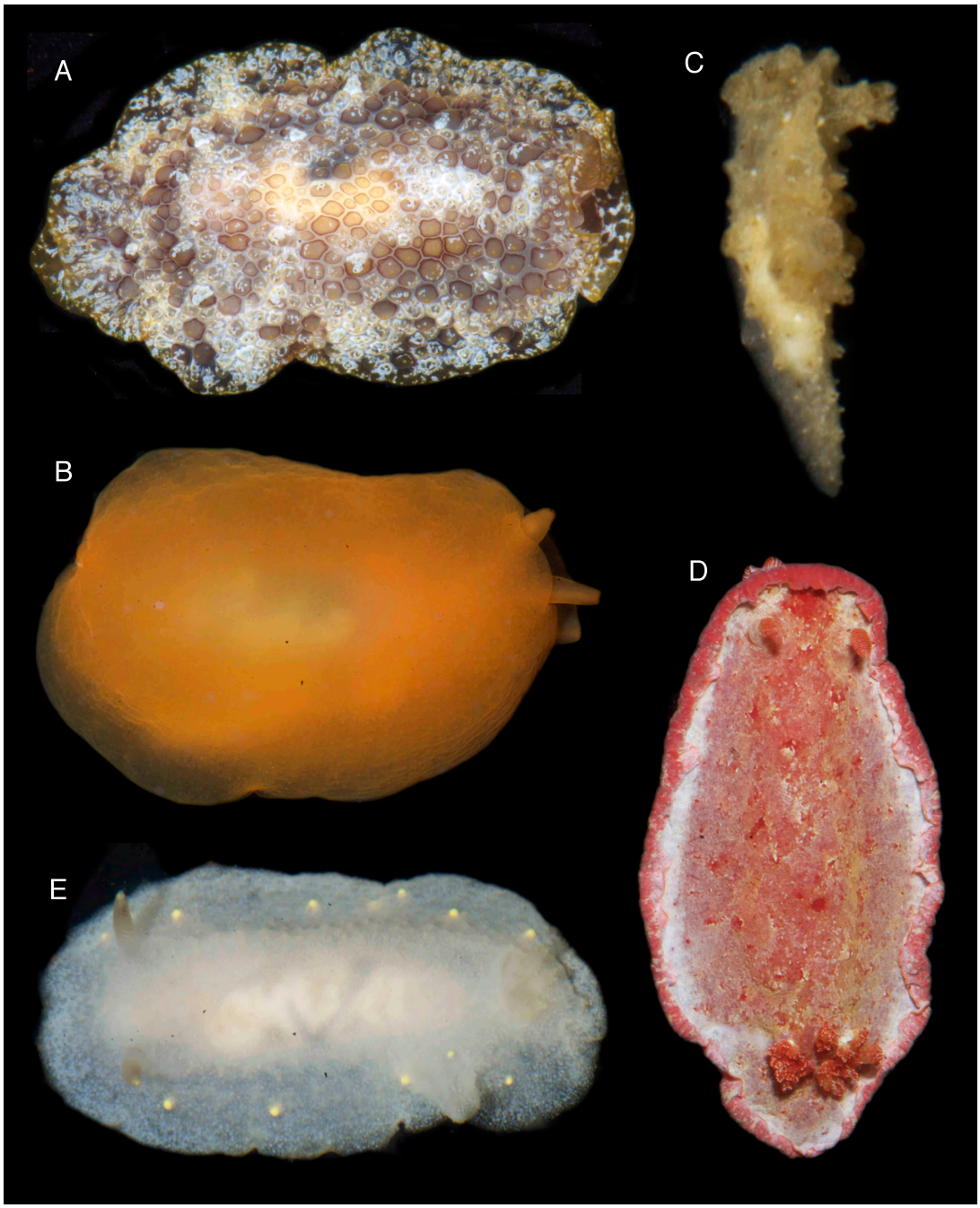

Figure 4.2. Nudipleura: Pleurobranchidae, Hexabranchidae, Aegiridae and Cadlinidae. A) *Pleurobranchus areolatus* Mörch, 1863; B) *Berthellina quadridens* (Mörch, 1863); C) *Aegires ortizi* Templado, Luque & Ortea, 1987; D) *Hexabranchus morsomus* Ev. Marcus & Er. Marcus, 1962; E) *Cadlina rumia* Er. Marcus, 1955.

NOTES

This species is found under rocks and coral rubble and probably feeds on ascidians [119,138]. Although there were believed to be six species of *Pleurobranchus* in the Caribbean, the other five (*Pleurobranchus atlanticus* Abbott, 1949, *Pleurobranchus evelinae* Thompson, 1977, *Pleurobranchus crossei* Vayssière, 1896, *Susania gardineri* White, 1952, *Pleurobranchus reesi* White, 1952 and *Pleurobranchus emys* Ev. Marcus, 1984) were recently synonymized with *P. areolatus,* based on molecular and morphological evidence [137].

Genus *Berthellina* Gardiner, 1936

Berthellina quadridens (Mörch, 1863)

(Figure 4.2B)

DESCRIPTION

Body oval, inflated. Dorsum smooth covering the internal shell, which is located over the anterior portion of the viscera. Anterior end of the body with a large oral veil, rhinophores rolled emerging between the veil and the dorsum. Color yellow to orange, semi-translucent. Up to 25 mm long.

DISTRIBUTION

Mexico, Belize, Colombia, Costa Rica, Panama, Venezuela, Aruba, Curaçao, Haiti, Jamaica, Puerto Rico, Virgin Islands, St. Maarten/St. Martin, St. Lucia, Guadeloupe, Martinique, Barbados, St. Vincent and the Grenadines, Grenada, Trinidad and Tobago, Brazil [119,139].

NOTES

Possibly feeds on sponges [138] and likely on the corals *Orbicella faveolata* (Ellis & Solander, 1786) and *Orbicella annularis* (Ellis & Solander, 1786) (see [140]) as well as on anemones [141].

Order NUDIBRANCHIA Odhner, 1984

Infraorder ANTHOBRANCHIA Wägele & Willan, 2000

Family AEGIRIDAE P. Fischer, 1883

Genus *Aegires* Lovén, 1844

Aegires ortizi Templado, Luque & Ortea, 1987

(Figure 4.2C)

DESCRIPTION

Body elongate. Tubercles large, varying from conical to mushroom-shaped, with flat tops in some individuals. Gill leaves forming a semicircle on the posterior portion of the dorsum. Background color usually mottled white, sometimes with noticeable brown spots. Up to 8 mm long.

DISTRIBUTION

Cayman Islands, Bahamas, Venezuela, Cuba [119,142] and Panama (present study).

NOTES

A single specimen was found on cyanobacteria over coral rubble and sand patches. In the Bahamas this species has been found on algae of the genera *Cladophora* Kützing, 1843 and *Sargassum* Agardh, 1820 (see [143]).

Family HEXABRANCHIDAE Bergh, 1891

Genus *Hexabranchus* Ehrenberg, 1828

Hexabranchus morsomus Ev. Marcus & Er. Marcus, 1962

(Figure 4.2D)

DESCRIPTION

Body oval to elongate. Dorsum with small conical tubercles. Rhinophores club shaped. Gill large, composed of several multi-pinnated leaves. Background color reddish with mottled white and yellow patches on the dorsum. Mantle margin usually curled up over small portion of dorsum covering white areas. Up to 400 mm long.

DISTRIBUTION

Honduras, Costa Rica, Venezuela, Aruba, Puerto Rico, Virgin Islands, St. Maarten/St. Martin, St. Lucia, Martinique, Antigua, Grenada, St. Vincent and the Grenadines, Trinidad and Tobago [119] and Panama [136].

NOTES

Found under rocks or coral rubble, primarily on living reefs [119]. Defensive behavior consists of the unrolling of the mantle margins to expose bright white areas followed by swimming by contracting the body and mantle margin [136]. Species of the genus *Hexabranchus* prey on a variety of sponges [40].

Family CADLINIDAE Bergh, 1891

Genus *Cadlina* Bergh, 1879

Cadlina rumia Er. Marcus, 1955

(Figure 4.2E)

DESCRIPTION

Body oval, flat, covered with numerous small tubercles. Background color usually translucent white with a few yellow spots (mantle glands). Rhinophores and gill often yellowish brown. Up to 15 mm long.

DISTRIBUTION

Amphiatlantic. Western Atlantic: Florida, Belize, Panama, Venezuela, Bahamas, Dominican Republic, Jamaica, Puerto Rico, Curaçao, St. Maarten/St. Martin, St. Lucia, St. Vincent & the Grenadines, Grenada, Brazil [119,142] and Panama [136].

NOTES

This is the only species of *Cadlina* in the tropical western Atlantic [119,144,145]. The genus *Cadlina* was recently transferred from the Chromodorididae to the Cadlinidae [146]. In our study *C. rumia* was found under rocks and on various sponges. This species feeds on several types of sponges from different orders (including spiculate and non-spiculate species), exhibiting a not specialized diet preference among the spongivorous dorid nudibranchs [147].

Family CHROMODORIDIDAE Bergh, 1891

Genus *Tyrinna* Bergh, 1898

Tyrinna evelinae (Er. Marcus, 1958)

(Figure 4.3A)

SYNONYMS

Cadlina burnayi Ortea, 1988.

DESCRIPTION

Body oval to elongate. Background color usually translucent white with a number of orange spots. Mantle margin edged by an opaque white line and white mantle glands with orange tips. Rhinophores and branchial leaves translucent white with opaque white tips. Up to 30 mm long.

DISTRIBUTION

Eastern Atlantic, Eastern Pacific, and Western Atlantic: Costa Rica, Venezuela, Jamaica, Puerto Rico, Dominican Republic, Brazil [119,142] and Panama (present study).

NOTES

Found under rocks and on various sponges in this study. Belmonte et al. [147] found that *Tyrinna evelinae* in Brazil feeds primarily on dysideid sponges, but also upon an unidentified chalinid species of the order Haplosclerida. This species has planktotrophic development. Caribbean populations are morphologically indistinguishable from Eastern Pacific and Eastern Atlantic populations [119].

Genus *Felimida* Ev. Marcus, 1971

Felimida clenchi (Russell, 1935)

(Figure 4.3B)

DESCRIPTION

Body oval. Dorsum smooth. Background color pale blue with a dense pattern of red covering the dorsum, but leaving small circular uncovered areas. The red becomes yellow near the rhinophores and gill. Mantle margin with a submarginal white band edged with a red line. Rhinophores and gill white with purple rachises. Up to 30 mm long.

DISTRIBUTION

Florida, Costa Rica, Panama, Colombia, Venezuela, Bermuda, Cayman Islands, Jamaica, Curaçao, St. Lucia, St. Vincent and the Grenadines [119,142].

NOTES

Found under rocks or on sponges in this study. Originally a member of the genus *Chromodoris* Alder & Hancock, 1855, this species was recently transferred to *Felimida* by Johnson & Gosliner [146]. This species is part of a complex that comprises *Felimida binza* (Ev. Marcus & Er. Marcus, 1963), *Felimida britoi* (Ortea & Pérez, 1983) and *Felimida neona* (Er. Marcus, 1955). All these species share a similar reticular pattern of yellow and red pigment and morphology [139,148,149].

Felimare fregona (Ortea & Caballer *in* Ortea *et al*., 2013)

(Figure 4.3C)

DESCRIPTION

Body elongate, narrow, with the posterior portion of foot extending beyond the mantle margin. Background color white with irregular shades of pale blue and gray. Dorsum with three longitudinal yellow lines. Mantle margin edged by an opaque white line with a narrow submarginal band of yellow and a series of black circular spots. Rhinophores white with a purple longitudinal line up from the base. Up to 40 mm long.

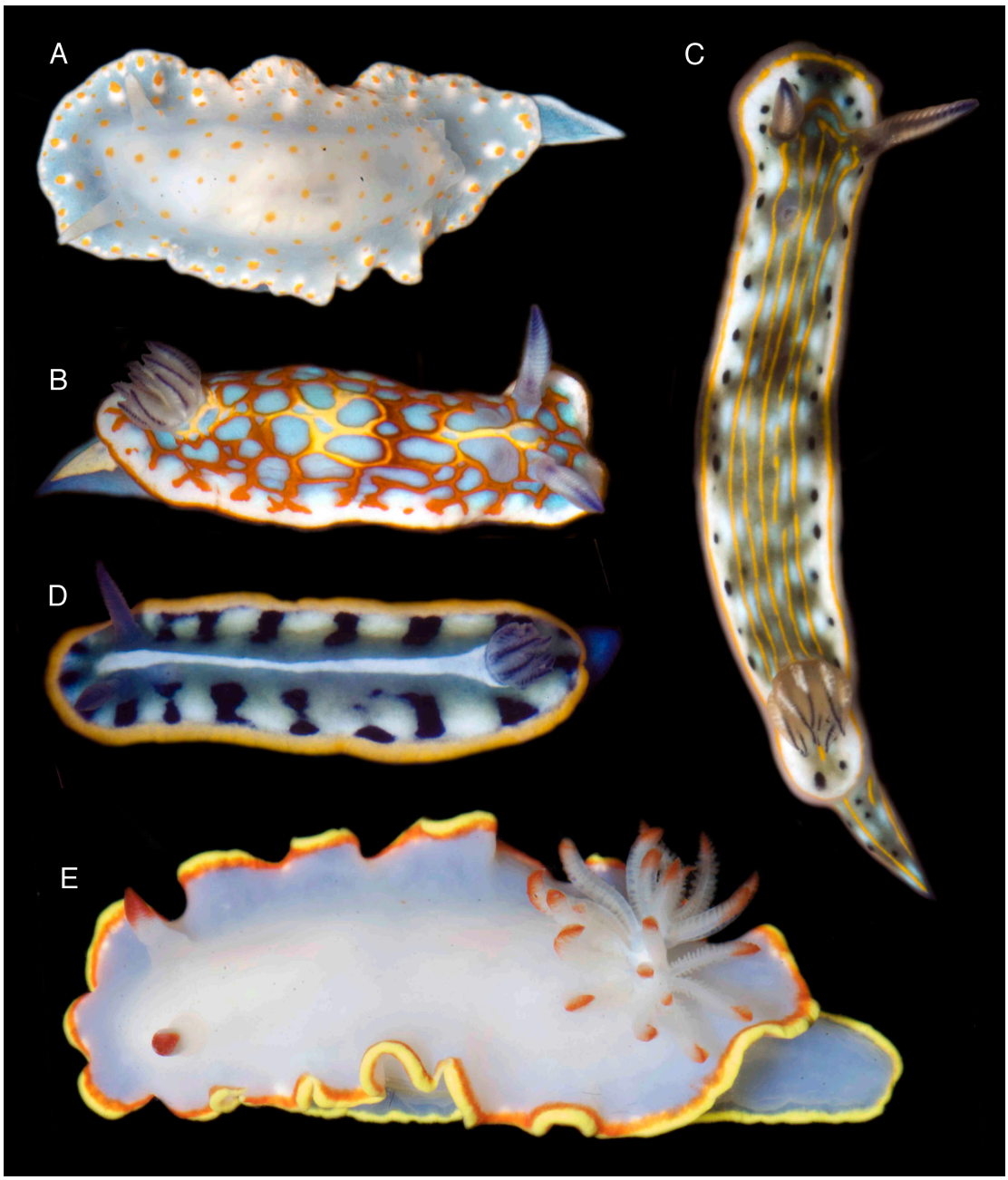

Figure 4.3. Nudipleura: Chromodorididae. A) *Tyrinna evelinae* (Er. Marcus, 1958); B) *Felimida clenchi* (Russell, 1935); C) *Felimare fregona* (Ortea & Caballer *in* Ortea *et al*., 2013); D) *Felimare kempfi* (Ev. Marcus, 1971); E) *Doriprismatica sedna* (Ev. Marcus & Er. Marcus, 1967).

DISTRIBUTION

Venezuela, Puerto Rico, Virgin Islands, Curaçao [119], Guadeloupe [150], and Panama (present study).

NOTES

Feeds on a blue sponge [119]. Recently described by Ortea & Caballer *in* Ortea *et al*. [150] from Guadeloupe. Appears to be the same morphotype illustrated by Valdés *et al*. [119] as *Hypselodoris* sp. 3.

Genus *Felimare* Ev. Marcus & Er. Marcus, 1967

Felimare kempfi (Ev. Marcus, 1971)

(Figure 4.3D)

DESCRIPTION

Body elongate, narrow, with the posterior portion of foot extending slightly beyond the mantle margin. Background color bright blue with a thick yellow line around the mantle margin. A central white line and a series of large black and white spots extend down the dorsum. Rhinophores and gills blue, branchial leaves with black rachises. Up to 20 mm long.

DISTRIBUTION

Florida, Mexico, Costa Rica, Venezuela, Brazil, Puerto Rico [119,142] and Panama [136].

NOTES

This species has previously been placed in the genera *Chromodoris* Alder & Hancock, 1855 (see [136]) and *Mexichromis* Bertsch, 1977. It was recently transferred to *Felimare* by Johnson & Gosliner [146].

Genus *Doriprismatica* d'Orbigny, 1839

Doriprismatica sedna (Ev. Marcus & Er. Marcus, 1967)

(Figure 4.3E)

SYNONYMS

Chromodoris fayae Lance, 1968.

DESCRIPTION

Body oval. Mantle margin ruffled. Background color white with two colored bands (inner red and outer yellow) bordering the foot and mantle. Upper half of the rhinophoral clubs and tips of the branchial leaves of the gill red. Up to 65 mm long.

DISTRIBUTION

Eastern Pacific: from the Gulf of California to the Galapagos Islands [151] and Western Atlantic: Florida, Belize, Bahamas [119] and Panama (present study).

NOTES

Found on mangrove roots covered with sponges in this study. The diet of *Doriprismatica sedna* was studied by Padilla-Verdín et al. [152] on the Pacific coast of Mexico. By examining the stomach content and feces, they found that this species feeds exclusively on spiculated sponges and exhibits a variable diet, which includes 16 different species. Originally described from the Eastern Pacific, records from the Caribbean are considered the result of a recent introduction, presumably human-induced. This species has previously been placed in the genus *Glossodoris* Ehrenberg, 1831 (see [119]), but was recently transferred to *Doriprismatica* by Johnson & Gosliner [146].

Family DISCODORIDIDAE Bergh, 1891

Genus *Discodoris* Bergh, 1877

Discodoris branneri MacFarland, 1909

(Figure 4.4A–B)

SYNONYMS

Discodoris evelinae Er. Marcus, 1955; *Discodoris hedgpethi* Ev. Marcus & Er. Marcus, 1960.

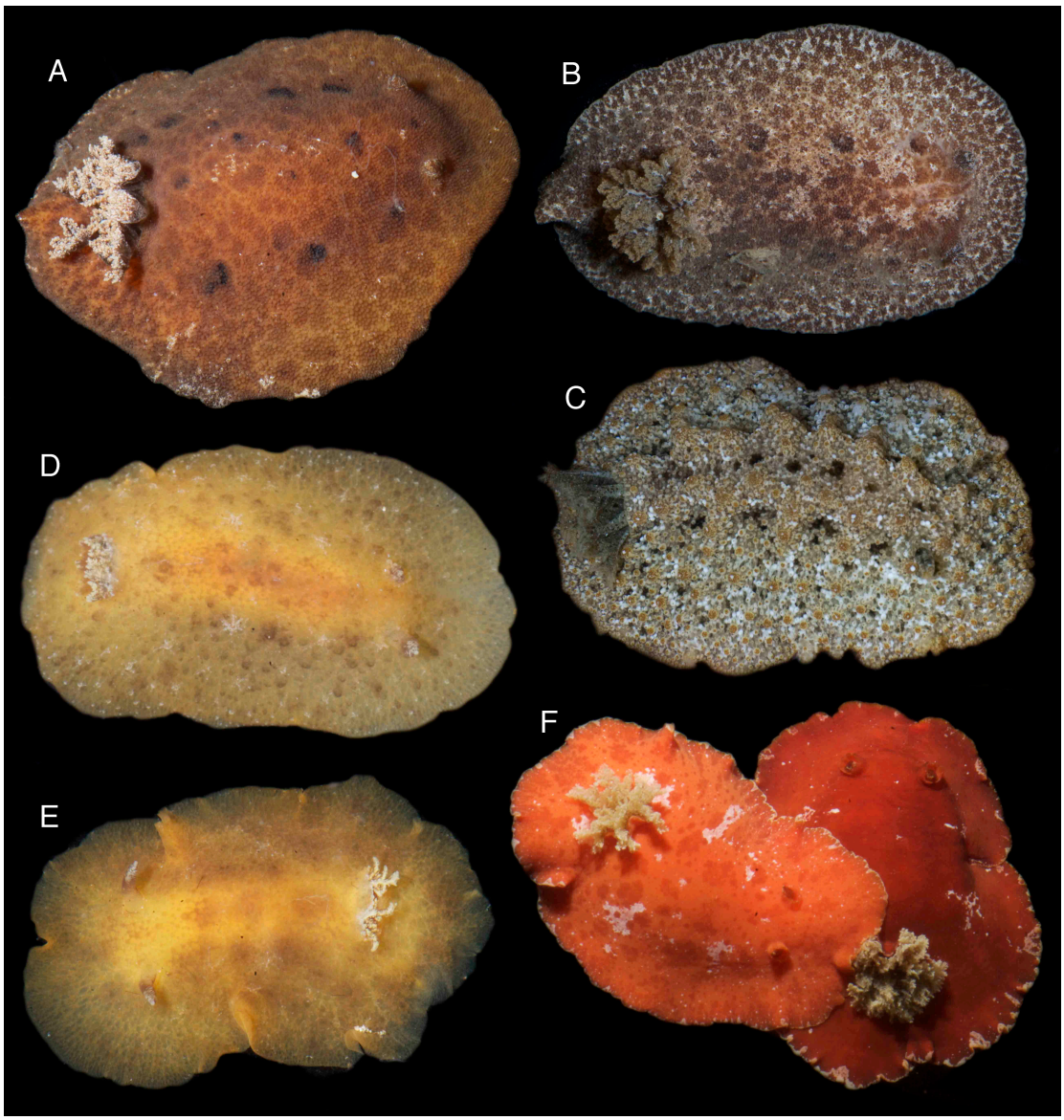

Figure 4.4. Nudipleura: Discodorididae. A–B) *Discodoris branneri* MacFarland, 1909; C) *Sclerodoris prea* (Ev. Marcus & Er. Marcus, 1967); D) *Geitodoris* cf. *planata* (Alder & Hancock, 1846); E) *Geitodoris immunda* Bergh, 1894; F) *Platydoris angustipes* (Mörch, 1863).

DESCRIPTION

Body oval, moderately rigid. Dorsum covered by numerous conical tubercles. Background color variable, from cream to purplish brown, sometimes with black or white patches and spots. Rhinophores and gill usually the same color as the dorsum with white tips. Up to 110 mm long.

DISTRIBUTION

Florida, Texas, Costa Rica, Honduras, Panama, Colombia, Venezuela, Bahamas, Cayman Islands, Puerto Rico, Jamaica, Barbados, Martinique, St. Lucia, Guadeloupe, St. Vincent and the Grenadines, Brazil [119].

NOTES

Found under rocks in this study. Members of this family feed on sponges. When disturbed, this species autotomizes parts of the mantle [119]. This species previously identified as *Discodoris evelinae* Er. Marcus 1955, but is now accepted as *Discodoris branneri* (see [153]).

Genus *Sclerodoris* Eliot, 1904

Sclerodoris prea (Ev. Marcus & Er. Marcus, 1967)

(Figure 4.4C)

DESCRIPTION

Body oval, mantle rigid. Dorsum covered with numerous caryophyllidia. Larger tubercles arranged in two rows along the visceral hump, with a longitudinal depression in the center. Rhinophores elongate, gill composed of multipinnate branchial leaves. Background color cream-brown with numerous dark brown spots. Black patches present along the center of the visceral hump. Rhinophores cream with dark spots and gill gray with opaque white spots. Up to 40 mm long.

DISTRIBUTION

Florida, Venezuela, Bahamas, Jamaica and Barbados [119,142] and Panama (present study).

NOTES

Found under rocks in this study. This species probably feeds on sponges.

Geitodoris cf. *planata* (Alder & Hancock, 1846)

(Figure 4.4D)

SYNONYMS

Doris testudinaria Risso, 1826; *Doris complanata* Verrill, 1880.

DESCRIPTION

Body oval, mantle rigid. Dorsum covered by rounded, stalked tubercles. Background color grayish-brown with some dark brown irregular patches. The color fades and becomes more translucent towards the mantle margin. Larger tubercles surrounded with opaque white pigment. Rhinophores and gill usually the same color as the dorsum with white tips. Up to 65 mm long.

DISTRIBUTION

Mediterranean Sea, North Atlantic Ocean, North Sea [154]; Western Atlantic: New Jersey, St. Lucia [119] and Panama (present study).

NOTES

Found in coral rubble in a predominately sea grass habitat in this study. Feeds on sponges [40]. Originally described from Europe, Caribbean populations are morphologically similar but almost certainly distinct. Alvim & Pimenta [153] regarded Caribbean animals as *Geitodoris pusae* (Er. Marcus, 1955), but no molecular studies have been conducted to compare animals from both sides of the Atlantic Ocean. Further research is necessary to clarify the status of this species.

Genus *Geitodoris* Bergh, 1891

Geitodoris immunda Bergh, 1894

(Figure 4.4E)

DESCRIPTION

Body oval, mantle moderately rigid. Dorsum with a complex network of low ridges covering the entire surface, with some conical tubercles at the intersections. Branchial sheaths with characteristic wavy edges. Background color grayish-brown with numerous opaque white dots and some darker brown areas. Rhinophores and gill brown with white tips. Up to 43 mm long.

DISTRIBUTION

Gulf of Mexico, Costa Rica, Venezuela, Brazil [119,155] and Panama (present study).

NOTES

Found under coral rubble in a reef habitat in this study. This species as well as the preceding one are similar to *Geitodoris pusae* (Er. Marcus, 1955), redescribed by Alvim & Pimenta [153]. Further review is necessary to clarify the taxonomic status of these taxa.

Genus *Platydoris* Bergh, 1877

Platydoris angustipes (Mörch, 1863)

(Figure 4.4F)

SYNONYMS

Platydoris alaleta Bergh, 1877; *Platydoris rubra* White, 1952.

DESCRIPTION

Body oval, mantle rigid. Dorsum flattened, covered with caryophyllidia. Background color ranges from reddish-brown to red or orange with scattered white specks often clustered in 3–4 dense groups. Mantle margin often darker or lighter than the rest of the mantle with proportionally more white patches. Rhinophores dark brown with cylindrical apex. Gill translucent straw-colored often with numerous opaque white spots. Up to 150 mm long.

DISTRIBUTION

Central American mainland, from Florida to Panama, also Greater Antilles, Cayman Islands, Lesser Antilles, Turks and Caicos, and Brazil [119,139,142].

NOTES

Found under rocks in this study. This species possibly has lecithotrophic development. Additional information and descriptions provided by Alvim & Pimenta [153].

Genus *Diaulula* Bergh, 1878

Diaulula phoca (Ev. Marcus & Er. Marcus, 1967)

(Figure 4.5A)

DESCRIPTION

Body oval, mantle rigid. Dorsum covered with small caryophyllidia. Body, rhinophores, and gill dark purplish brown with numerous small opaque white dots. Up to 50 mm long.

DISTRIBUTION

Florida, Honduras, Costa Rica, Brazil [119,145] and Panama (present study).

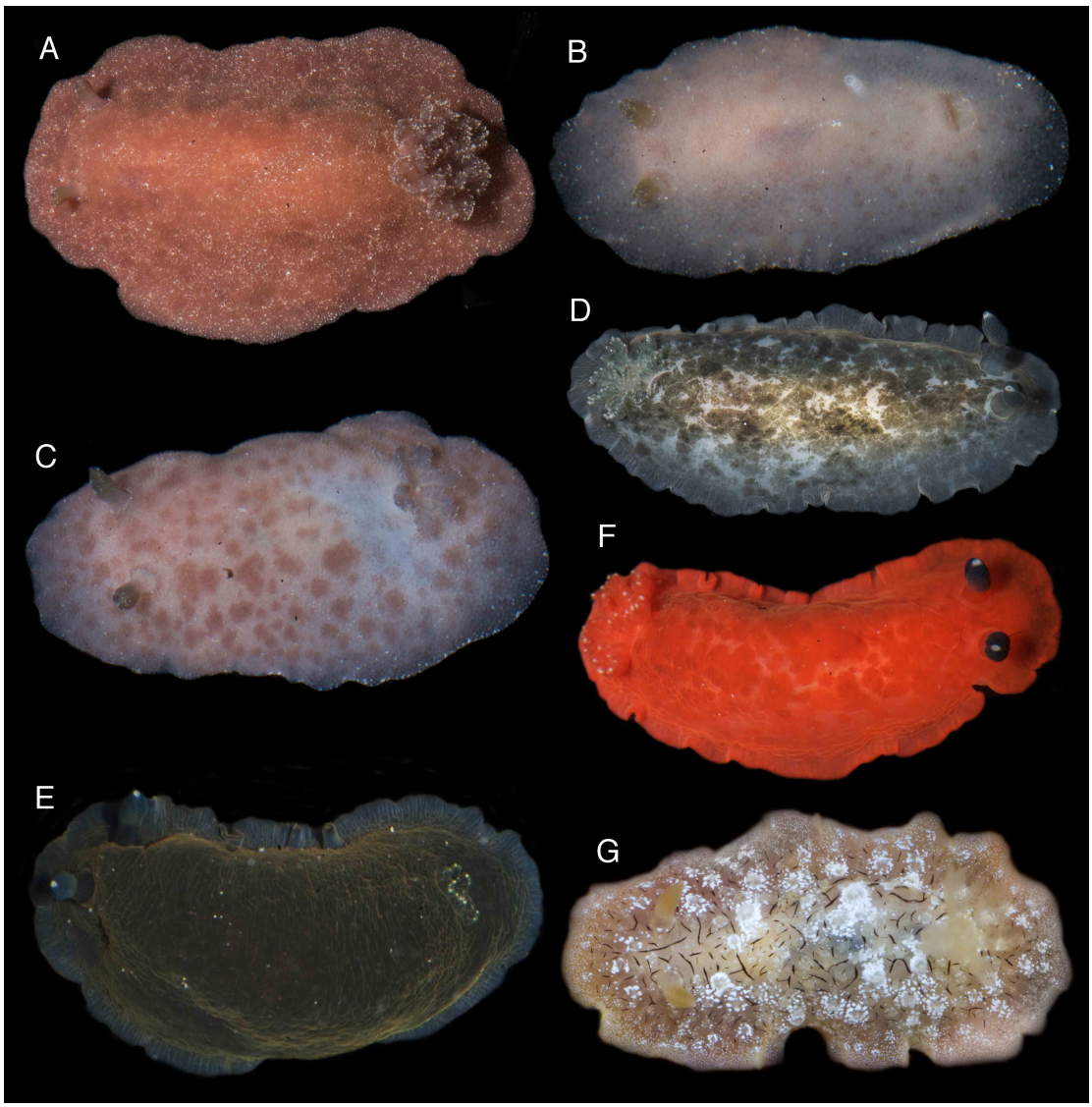

Figure 4.5. Nudipleura: Discodorididae and Dendrodorididae. A) *Diaulula phoca* (Ev. Marcus & Er. Marcus, 1967); B–C) *Jorunna* cf. *spazzola* (Er. Marcus, 1955); D–F) *Dendrodoris krebsii* (Mörch, 1863) G) *Doriopsilla nigrolineata* Meyer, 1977.

NOTES

Feeds on sponges (Ev. Marcus & Er. Marcus, 1967). Originally named *Discodoris phoca* Ev. Marcus & Er. Marcus, 1967 it is considered a member of *Diaulula* because of the presence of caryophyllidia.

Genus *Jorunna* Bergh, 1876

Jorunna cf. *spazzola* (Er. Marcus, 1955)

(Figure 4.5B–C)

SYNONYMS

Discodoris mortenseni Ev. Marcus & Er. Marcus, 1963.

DESCRIPTION

Body oval, mantle rigid. Dorsum flattened, covered with small caryophyllidia. Branchial leaves very short. Background color translucent gray with a few darker gray or brown patches over the dorsum. Mantle margin surrounded by small opaque white glands. Rhinophores and gill the same color as the rest of the body. Up to 18 mm long.

DISTRIBUTION

Florida, Honduras, Costa Rica, Venezuela, Cuba, Curaçao, Barbados, Virgin Islands, Turks and Caicos, Brazil [119,139,142] and Panama (present study).

NOTES

Found under rocks in this study. Known to feed on sponges of the order Haplosclerida [147] on which it is well camouflaged. This species is able to quickly change colors as a response to unknown environmental cues [119]. Camacho-García *et al*. [139] suggested Caribbean animals identified as *Jorunna spazzola* could

constitute a distinct species, because they display external differences with the original description from southern Brazil.

Family DENDRODORIDIDAE O'Donoghue, 1924

Genus *Dendrodoris* Ehrenberg, 1831

Dendrodoris krebsii (Mörch, 1863)

(Figure 4.5D–F)

DESCRIPTION

Body oval to elongate, dorsum soft, lacking tubercles. Background color extremely variable, white, black, orange, red or light green, with or without spots of red, black, gray or white. Rhinophores and gill usually the same color as the rest of the body with white tips. Up to 150 mm long.

DISTRIBUTION

North and south American mainland from Georgia to Brazil, Bahamas, Cuba, Cayman Islands, Jamaica, Dominican Republic, Virgin Islands, St. Martin, Antigua, Guadeloupe, Martinique, St. Lucia, St. Vincent and the Grenadines, Barbados, Aruba, Curaçao, Bonaire, Grenada [119,139].

NOTES

Found under coral rubble or rocks in this study. This is one of the most common species of Nudibranchia in the Caribbean. Members of this family are suctorial

sponge feeders. Belmonte et al. [147] recorded active feeding of *Dendrodoris krebsii* upon a haplosclerid sponge.

Genus *Doriopsilla* Bergh, 1880

Doriopsilla nigrolineata Meyer, 1977

(Figure 4.5G)

SYNONYMS

Doriopsilla areolata nigrolineata Meyer, 1977.

DESCRIPTION

Body oval to elongate. Dorsum rigid, covered with rounded tubercles. Background color translucent white to orange, with a series of irregular black lines over the entire dorsum. Bases of tubercles densely spotted with white, rhinophores and gill yellow. Up to 30 mm long.

DISTRIBUTION

Panama, Honduras [119].

NOTES

Found in 3–6 m of water. Previously considered a subspecies of *Doriopsilla areolata* Bergh, 1880 by Valdés & Ortea [156], but Valdés & Hamann [157] confirmed that it is a distinct species.

Infraorder CLADOBRANCHIA Willan & Morton, 1984

Family TRITONIIDAE Lamarck, 1801

Genus *Tritonia* Cuvier, 1798

Tritonia hamnerorum Gosliner & Ghiselin, 1987

(Figure 4.6A)

DESCRIPTION

Body elongate and narrow. Rhinophoral sheaths elevated with an irregular edge. Cerata short and branched. Edge of the oral veil with relatively long appendages, rhinophores long, branched. Background color translucent gray with a series of irregular, longitudinal, thin white lines that run along the length of the dorsum. Up to 15 mm long.

DISTRIBUTION

Florida, Mexico, Belize, Bahamas, Cayman Islands [119] and Panama (present study).

Figure 4.6. Nudipleura: Tritoniidae, Lomanotidae and Dotidae. A) *Tritonia hamnerorum* Gosliner & Ghiselin, 1987 on substrate with egg mass; B) *Tritonia bayeri* Ev. Marcus & Er. Marcus, 1967. C) *Lomanotus vermiformis* Eliot, 1908, on substrate with egg mass; D) *Doto escatllari* Ortea, Moro & Espinosa, 1998, on substrate; E) *Doto chica* Ev. Marcus & Er. Marcus, 1960, on substrate; F) *Doto* cf. *wildei* Er. Marcus & Ev. Marcus, 1970.

NOTES

Found on gorgonian sea fans in dense aggregations in this study. This species reportedly feeds on the octocorals *Gorgonia ventalina* Linnaeus, 1758 and *Gorgonia flabellum* Linnaeus, 1758. It sequesters chemicals from the sea fans and stores them for its own defense [158].

Tritonia bayeri Ev. Marcus & Er. Marcus, 1967

(Figure 4.6B)

SYNONYMS

Tritonia bayeri misa Ev. Marcus & Er. Marcus, 1967.

DESCRIPTION

Body elongate and narrow. Rhinophoral sheaths elevated with an irregular edge. Cerata relatively short and branched. Edge of the oral veil with relatively long appendages, rhinophores long, branched. Background color translucent gray with a distinctive reticulate network of opaque white across the dorsum. Up to 11 mm long.

DISTRIBUTION

Georgia, Florida, Belize, Honduras, Cayman Islands, Virgin Islands, Guadaloupe, Barbados [119] and Panama (present study).

NOTES

Found on gorgonians and coral rubble in this study. Inhabits reefs down to 77 m depth. This species feeds on the octocorals *Briareum asbestinum* (Pallas, 1766), *Leptogorgia virgulata* (Lamarck, 1815) and *Pseudopterogorgia* sp. [159].

Family LOMANOTIDAE Bergh, 1890

Genus *Lomanotus* Vérany, 1844

Lomanotus vermiformis Eliot, 1908

(Figure 4.6C)

SYNONYMS

Lomanotus stauberi Clark & Goetzfried, 1976.

DESCRIPTION

Body very elongate and narrow. Rhinophoral sheaths with papillae and elevated to cover three quarters of the rhinophores. Cerata very short and pointed. Background color brown with dark brown spots and opaque yellow lines. Opaque white reticulations also present across the body. Up to 40 mm long.

DISTRIBUTION

Circumtropical. Western Atlantic: Florida, Bahamas [119] and Panama [136].

NOTES

This species feeds on hydroids of the genus *Macrorhynchia* Kirchenpauer, 1872 [159]. In this study was found feeding on an unidentified species of hydroid (illustrated), on which it is extremely cryptic. This species can swim with lateral flexions of the body when disturbed [119].

Family DOTIDAE Gray, 1853

Genus *Doto* Oken, 1815

Doto escatllari Ortea, Moro & Espinosa, 1998

(Figure 4.6D)

DESCRIPTION

Body short and narrow. Rhinophores smooth. Rhinophoral sheaths with small frontal extensions. Cerata large with rounded tubercles; apical tubercles much larger than the rest. Background color translucent gray with a series of dark brown spots on the dorsum. Cerata with dark brown branches of the digestive gland and bluish tubercles, rhinophores with opaque white dots. Up to 5 mm long.

DISTRIBUTION

Costa Rica, Barbados [119] and Panama (present study).

NOTES

Found on hydroids in this study.

Doto chica Ev. Marcus & Er. Marcus, 1960

(Figure 4.6E)

SYNONYMS

Doto fragilis umia Ev. Marcus & Er. Marcus, 1969.

DESCRIPTION

Body narrow and elongate. Rhinophores smooth, rhinophoral sheaths with small posterior extensions. Cerata large, with rounded tubercles; apical tubercles much larger than the rest. Background color translucent gray with a dense series of dark brown spots and a less dense set of opaque white spots on the dorsum. Cerata with orange extensions of the digestive gland. Up to 5 mm long.

DISTRIBUTION

Florida, Mexico, Costa Rica, Venezuela, Puerto Rico, Curaçao, Cuba, Brazil [119,145,160] and Panama (present study).

NOTES

Found on hydroids in this study. Known to feed on hydroids of the genus *Eudendrium* Ehrenberg, 1834 [161].

Doto cf. *wildei* Er. Marcus & Ev. Marcus, 1970

(Figure 4.6F)

SYNONYMS

Doto caramella wildei Er. Marcus & Ev. Marcus, 1970.

DESCRIPTION

Body narrow and elongate. Rhinophores smooth with tight rhinophoral sheaths. Cerata with rounded tubercles; apical tubercles much larger than the rest. Cerata spaced out along the dorsum. Background color translucent gray with a series of opaque white spots on the dorsum. Cerata with cream or white extensions of the digestive gland. Up to 4 mm long.

DISTRIBUTION

Curaçao [119] and Panama (present study).

NOTES

Found on hydroids. The identification of this specimen is uncertain; it looks most similar to *Doto wildei* but lacks pseudogills on the cerata. The systematics of *Doto* in the Caribbean region is in need of major revision and until the taxonomy is clarified many species identifications remain tentative.

Family FLABELLINIDAE Bergh, 1889

Genus *Flabellina* Gray, 1833

Flabellina engeli Ev. Marcus & Er. Marcus, 1968

(Figure 4.7A)

DESCRIPTION

Body elongate, narrowing posteriorly. Rhinophores lamellate, club-shaped, oral tentacles long. Cerata arranged into clusters in two rows along the dorsum. Background color translucent gray with thick white or yellow patches running between the cerata clusters, on the margin of the dorsum. A submarginal row of opaque white spots present along the sides of the body. Three white or yellow patches on the head. Oral tentacles translucent, white at the tips; rhinophores with white bands. Cerata translucent with a brown or orange band about a third of the way down from the tip. Up to 25 mm long.

DISTRIBUTION

Florida, Costa Rica, Colombia, Venezuela, Barbados, Cuba, Puerto Rico, Curaçao, St. Lucia, Martinique, Granada, Brazil [119,142,145] and Panama (present study).

NOTES

One specimen found on a living blade of sea grass in 1 m of water.

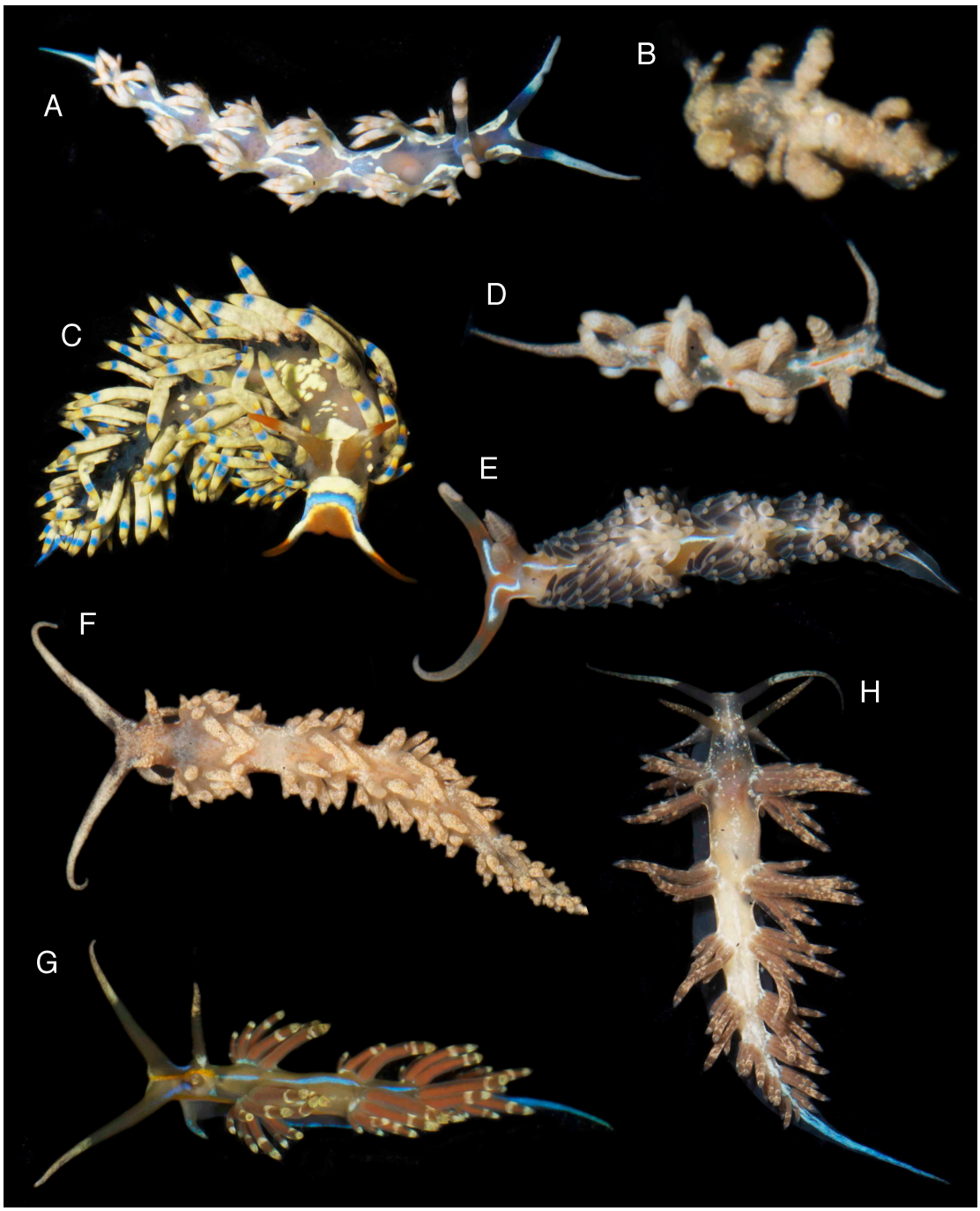

Figure 4.7. Nudipleura: Flabellinidae, Eubranchidae, Tergipedidae, and Facelinidae. A) *Flabellina engeli* Ev. Marcus & Er. Marcus, 1968; B) *Eubranchus conicla* (Er. Marcus, 1958); C) *Cuthona* cf. *caerulea* (Montagu, 1804); D) *Nanuca sebastiani* Er. Marcus, 1957; E) *Phidiana lynceus* Bergh, 1867; F) *Palisa papillata* Edmunds, 1964; G) *Dondice occidentalis* (Engel, 1925); H) *Dondice parguerensis* Brandon & Cutress, 1985.

Family EUBRANCHIDAE

Genus *Eubranchus* Forbes, 1838

Eubranchus conicla (Er. Marcus, 1958)

(Figure 4.7B)

SYNONYMS

Eubranchus convenientis Ortea & Caballer, 2002.

DESCRIPTION

Body elongate. Rhinophores smooth, oral tentacles short. Cerata tuberculate, few in number, arranged in two simple rows. Background color translucent gray or brown with numerous white dots. Rhinophores and oral tentacles sometimes ringed with brown. Cerata white, sometimes with brown or green spots. Up to 4 mm long.

DISTRIBUTION

Florida, Honduras, Costa Rica, Venezuela, Jamaica, Barbados, Tobago, Brazil [119,142] and Panama (present study).

NOTES

Found on *Sargassum* in less than 3 m of water.

Family TERGIPEDIDAE Bergh, 1889

Genus *Cuthona* Alder & Hancock, 1855

Cuthona cf. *caerulea* (Montagu, 1804)

(Figure 4.7C)

SYNONYMS

Eolidia bassi Vérany, 1846; *Eolis glotensis* Alder & Hancock, 1846; *Eolis deaurata* Dalyell, 1853; *Eolis molios* Herdman, 1881.

DESCRIPTION

Body elongate. Rhinophores smooth, oral tentacles relatively short. Cerata numerous on both sides of the dorsum with a small empty space down the middle of the dorsum. Background color translucent gray with a bright blue patch, and sometimes a yellow patch, on the head. Oral tentacles yellow at the base, orange at the tips. Rhinophores with a translucent base, a central white or yellow area and orange-brown tips. Cerata translucent white, gray or yellow, with a blue band followed by a bright yellow band near the apex. Up to 25 mm long.

DISTRIBUTION

Possibly amphiatlantic. Western Atlantic: Florida to Brazil [119] including Panama (present study).

NOTES

The animals here illustrated are tentatively identified as the European species *Cuthona caerulea*, but the coloration of the head, with a conspicuous blue patch, is

different; they probably constitute an undescribed species. Found on hydroids in this study. This species has been recorded feeding upon hydroids of several different genera [159].

Family FACELINIDAE Bergh, 1889

Genus *Nanuca* Er. Marcus, 1957

Nanuca sebastiani Er. Marcus, 1957

(Figure 4.7D)

DESCRIPTION

Body elongate. Rhinophores annulate; oral tentacles long. Cerata arranged in two rows of clusters (with 3–5 cerata each) on the dorsum. Background color translucent green with numerous opaque white spots and a series of areas with blue and/or white with orange spots forming a cross-like pattern. Cerata with longitudinal opaque white lines with a white, narrow tip. Up to 12 mm long.

DISTRIBUTION

Mexico, Costa Rica, Venezuela, Curaçao, Bonaire, Martinique, Cuba, Cayman Islands, Virgin Islands, Barbados, Bermuda, Brazil [119,142] and Panama (present study).

NOTES

This species was found in *Sargassum* algae with sertularid hydroids in this study.

Genus *Phidiana* Gray, 1850

Phidiana lynceus Bergh, 1867

(Figure 4.7E)

SYNONYMS

Phidiana selencae Bergh, 1879; *Phidiana brevicauda* Engel, 1925.

DESCRIPTION

Body elongate and narrow. Rhinophores annulate, oral tentacles long. Cerata elongate densely covering the dorsum, except for the dorsal mid-line. Background color translucent gray with a dorsal white line that splits on the head and continues into the oral tentacles. The line can be narrow, broad or absent. Cerata with white apices. Orange pigment on the oral tentacles and rhinophores. Up to 45 mm long.

DISTRIBUTION

Florida, Mexico, Costa Rica, Panama, Colombia, Venezuela, Curaçao, Aruba, Bonaire, Jamaica, Bahamas, Virgin Islands, Guadeloupe, Martinique, St. Maarten/St. Martin, St. Lucia, Barbados, St. Vincent and the Grenadines, Brazil. Ghana, Canary Islands [119,145].

NOTES

Found under rocks in this study. Known to feed on hydroids [162]. Shows intraspecific variation in rhinophores and head morphology [119].

Genus *Palisa* Edmunds, 1964

Palisa papillata Edmunds, 1964

(Figure 4.7F)

DESCRIPTION

Body elongate. Rhinophores tuberculate; oral tentacles long. Cerata arranged in clusters forming a single row along each side of the dorsum. Background color translucent gray with numerous opaque white spots on both the dorsum and cerata. Cerata with a pale blue digestive gland and characteristic black or dark brown spots at the base. Up to 15 mm long.

DISTRIBUTION

Florida, Jamaica [119] and Panama (present study).

NOTES

Found among algae in this study, probably feeding on epiphytic hydroids.

Genus *Dondice* Er. Marcus, 1958

Dondice occidentalis Engel, 1925

(Figure 4.7G)

DESCRIPTION

Body elongate, tapering toward the posterior end. Rhinophores annulate, long; oral tentacles longer than the rhinophores. Cerata arranged in clusters along two rows on the dorsum. Background color translucent gray with a yellow or orange median line of variable width, running from the head to the anterior end, between the rhinophores. A white or blue broken line down the dorsal mid-line from behind the rhinophores to the posterior end of the body is sometimes present. Opaque white spots sometimes present on the dorsum. Oral tentacles translucent or light blue at the base, becoming white towards the tips. Cerata translucent gray, often with large blue or white bands covering the upper two-thirds of each ceras. Up to 50 mm long.

DISTRIBUTION

Florida, Mexico, Belize, Costa Rica, Colombia, Venezuela, Curaçao, Bonaire, Venezuela, Bermudas, Bahamas, Cayman Islands, Jamaica, Turks and Caicos, Grenada, St. Maarten/St. Martin, Martinique, Trinidad, Brazil [119,142] and Panama (present study).

NOTES

Found on hydroids in this study. This species feeds on hydroids of the genus *Eudendrium* and *Amathia* Lamouroux, 1812 (see [159]). It easily sheds the cerata when disturbed. According to Gonzalez et al. [163], *Dondice occidentalis* and

Dondice parguerensis probably represent an example of incipient sympatric speciation. Molecular analyses support partially the differentiation of these species, but are inconclusive. Further research is needed in order to resolve this species complex.

Dondice parguerensis Brandon & Cutress, 1985

(Figure 4.7H)

DESCRIPTION

Body elongate, tapering toward the end. Rhinophores annulate, oral tentacles long. Cerata abundant, arranged in clusters along two rows on the dorsum. Background color translucent brown with a white median line from the head that extends posteriorly. Oral tentacles and rhinophores both translucent brown at the base and white on the distal half. Cerata translucent brown with white tips. Up to 48 mm long.

DISTRIBUTION

Puerto Rico, Panama, Venezuela, Guadeloupe [119,136,150,164].

NOTES

This species is found exclusively on the tentacles of the upside-down jellyfish *Cassiopea* Péron & Lesueur, 1810 in shallow mangrove areas. The divergence of this species from the close relative *Dondice occidentalis* was recently investigated by Gonzalez et al. [163](see above). Previously reported from Panama as *D. occidentalis* [136].

Family AEOLIDIIDAE Gray, 1827

Genus *Berghia* Trinchese, 1877

Berghia rissodominguezi Muniain & Ortea, 1999

(Figure 4.8A)

DESCRIPTION

Body narrow and elongate. Oral tentacles longer than the rhinophores. Cerata moderately elongate, cylindrical, with round apices and constant diameter throughout most of their length. Rhinophores densely papillate on the posterior side. Background color translucent white with oblique orange lines on the borders of the insertion of the cerata. Cerata translucent with reddish brown diverticula and white to yellow apices. Rhinophores bright orange with yellow or cream pigmentation on the apical portion. Up to 52 mm long.

DISTRIBUTION

Florida, Venezuela, Curaçao, Jamaica, St. Lucia, Guadeloupe, Brazil, Argentina [109,119,142,150] and Panama (present study).

Figure 4.8. Nudipleura: Aeolidiidae. A) *Berghia rissodominguezi* Muniain & Ortea, 1999; B) *Berghia creutzbergi* Er. Marcus & Ev. Marcus, 1970; C) *Anteaeolidiella lurana* (Ev. Marcus & Er. Marcus, 1967).

NOTES

Found under rocks in intertidal areas in this study. Feeds on anemones. It can autotomize the cerata when being handled. Carmona *et al*. [109] clarified the misidentifications that had been published for the western Atlantic.

Berghia creutzbergi Er. Marcus & Ev. Marcus, 1970

(Figure 4.8B)

SYNONYMS

Milleria ritmica Ortea, Caballer & Espinosa, 2003.

DESCRIPTION

Body elongate. Rhinophores tuberculate. Cerata arranged in two rows of clusters along the dorsum. Background color translucent gray or brown with numerous opaque white spots covering the majority of the dorsum and cerata. Cerata with longitudinal opaque white lines and white, narrow tips. Up to 30 mm long.

DISTRIBUTION

Tropical western Atlantic, Florida, Costa Rica, Venezuela, Cuba, Barbados, Bahamas, Cayman Islands, Curaçao, Brazil [109,119] and Panama (present study).

NOTES

The single specimen in this study was found under a rock in a seagrass bed. The cerata of this species rock from side to side distinctively while the animal is in motion [119]. The genus *Berghia* was recently confirmed as the correct placement for this species [109].

Genus *Anteaeolidiella* M.C. Miller, 2001

Anteaeolidiella lurana (Ev. Marcus & Er. Marcus, 1967)

(Figure 4.8C)

DESCRIPTION

Body elongate. Rhinophores smooth, about the same length as the oral tentacles. Cerata covering most of the dorsum except for the dorsal mid-line. Background color

translucent gray with orange pigmentation on the head, behind the rhinophores, and along the edges of the dorsum. Rhinophores and oral tentacles with cream or yellow tips. Cerata translucent with orange digestive diverticula and white cnidosacs. Up to 10 mm long.

DISTRIBUTION

Amphiatlantic. Western Atlantic: Caribbean Sea, Brazil, Bermuda [107,142] and Panama (present study).

NOTES

Carmona *et al.* [67] recently confirmed the validity of the genus *Anteaeolidiella*. Additional information on this species can be found in Carmona et al. [107].

Clade EUOPISTHOBRANCHIA Jörger, Stöger, Kano, Fukuda, Knebelsberger & Schrödl, 2010

Order CEPHALASPIDEA P. Fischer, 1883

Family HAMINOEIDAE Pilsbry, 1895

Genus *Haminoea* Turton & Kingston, 1830

Haminoea elegans (Gray, 1825)

(Figure 4.9A)

SYNONYMS

Bulla guildinguii Swainson, 1840; *Bulla diaphana* Gould, 1852; *Haminoea taylorae* Petuch, 1987.

DESCRIPTION

Shell external, thin, translucent. Body wide and elongate, with large parapodia covering the anterior part of the shell. Shell with numerous and conspicuous spiral grooves crossed by growth lines. Cephalic shield deeply notched with reduced tentacles. Background color translucent yellowish gray with numerous black and opaque white. Up to 35 mm long.

DISTRIBUTION

Florida to Brazil, Greater and Lesser Antilles, Bermuda and Bahamas [119,139].

NOTES

Found on dense bacterial mats in shallow water, about 1 m depth.

Haminoea succinea (Conrad, 1846)

(Figure 4.9B)

SYNONYMS

Haminoea solidor Vanatta, 1901.

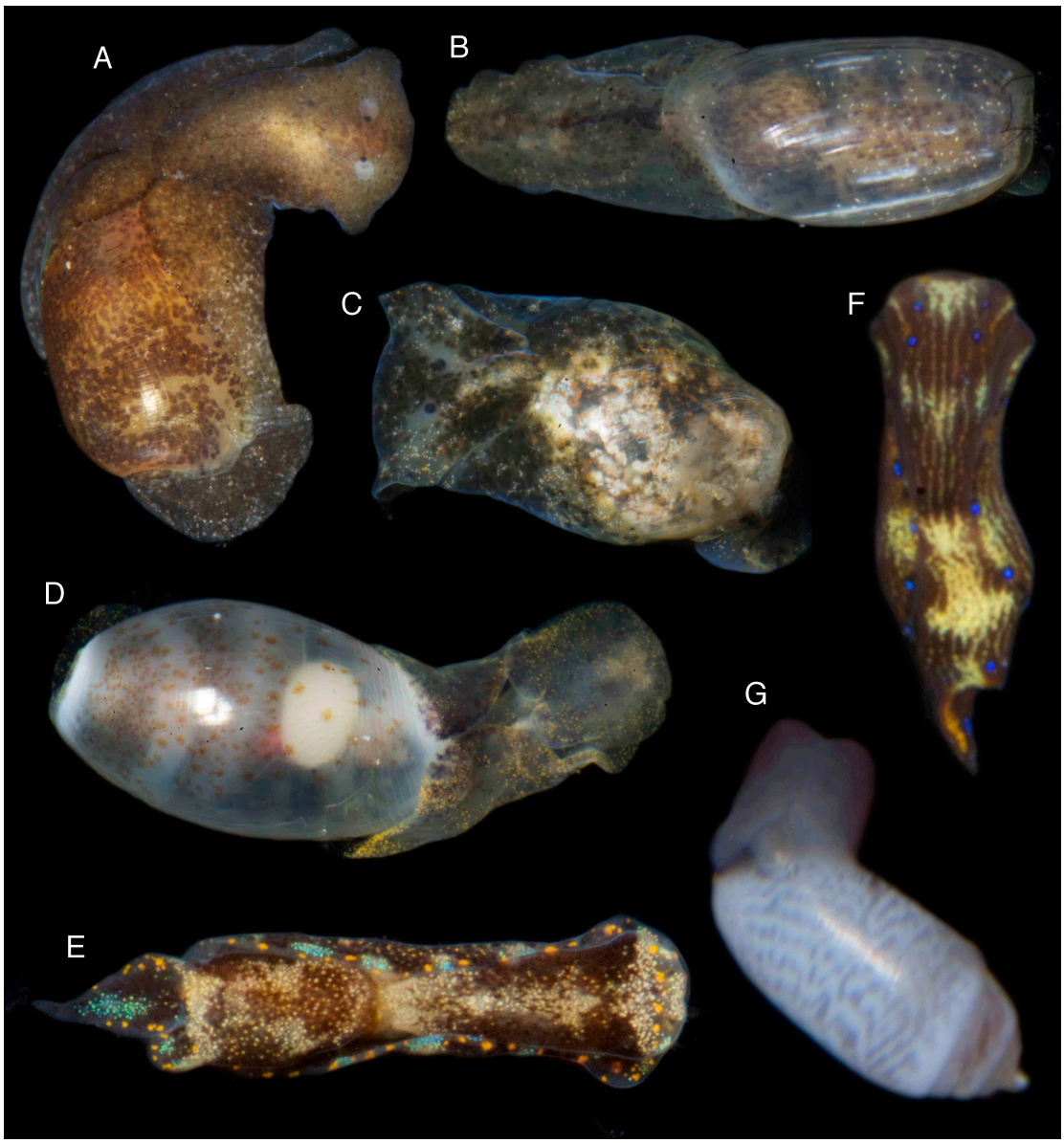

Figure 4.9. Euopisthobranchia: Haminoeidae, Aglajidae and Acteocinidae. A) *Haminoea elegans* (Gray, 1825); B) *Haminoea succinea* (Conrad, 1846); C) *Haminoea antillarum* (d'Orbigny, 1841); D) *Atys caribaeus* (d'Orbigny, 1841); E) *Chelidonura berolina* Er. Marcus & Ev. Marcus, 1970; F) *Navanax gemmatus* (Mörch, 1863); G) *Acteocina candei* (d'Orbigny, 1842).

DESCRIPTION

Shell external, rounded, translucent white. Body elongate, with a short and broad cephalic shield having two lateral, very short and wide extensions. Parapodia very short, not covering any portion of the shell. Posterior end of the foot broad and

rounded. Background color translucent gray with numerous black spots and some opaque white dots. Up to 20 mm long.

DISTRIBUTION

Florida. Louisiana, Texas, Mexico, Colombia, Venezuela, Bermuda, Puerto Rico, St. Maarten/St. Martin, St. Barthelemy [119] and Panama (present study).

NOTES

Found on soft bottoms in protected areas near mangrove roots in this study.

Haminoea antillarum (d'Orbigny, 1841)

(Figure 4.9C)

SYNONYMS

Bulla cerina Menke, 1853; *Haminea guadaloupensis* G.B. Sowerby II, 1868.

DESCRIPTION

Shell external, rounded, lacking any marking other than light growth lines. Shell translucent, showing through the viscera with orange and dark brown spotting. Cephalic shield with two extensions visible. Head with two conspicuous eye spots on the dorsal side. Foot broad on the posterior end. Small parapodial flaps cover the anterior portion of the shell. Up to 40 mm long.

DISTRIBUTION

From Florida to Brazil, including Cuba, Cayman Islands, Jamaica, Puerto Rico, Virgin Islands, Guadeloupe, Bermuda [119,142,145].

NOTES

Typically found on red algae in highly turbulent areas. As with other Haminoeidae, this species is herbivorous [165]. It is a common species in the intertidal zone.

Genus *Atys* Leach, 1816

Atys caribaeus (d'Orbigny, 1841)

(Figure 4.9D)

SYNONYMY

Bulla speciosa A. Adams, 1850.

DESCRIPTION

Shell external, elongate, translucent, with conspicuous spiral grooves near the anterior and posterior ends. Body very elongate, with a deeply notched cephalic shield (posteriorly). Parapodia short, covering a small portion of the anterior end of the shell. Background color translucent white with irregular opaque white spots and some black dots, sometimes with a dense covering of brown dots. Shell sometimes with brown patches. Up to 20 mm long.

DISTRIBUTION

North Carolina, Florida to Brazil, Greater and Lesser Antilles [119].

NOTES

Found on soft bottoms near mangrove roots in this study.

Family AGLAJIDAE Pilsbry, 1895

Genus *Chelidonura* A. Adams, 1850

Chelidonura berolina Er. Marcus & Ev. Marcus, 1970

(Figure 4.9E)

DESCRIPTION

Shell reduced, internal. Body elongated with a cephalic shield slightly longer than the visceral hump. Posterior end of the body with two lobes, the left one being much longer. Background color black with a submarginal yellow band on the parapodial edge, posterior end of the cephalic shield and anterior and posterior ends of the body. Anterior edge of the body translucent. Dorsum covered by white and yellow patches in some specimens, not present in others. Up to 12 mm long.

DISTRIBUTION

From Mexico to Colombia, Cayman Islands, Cuba, Jamaica, Martinique, Puerto Rico, Bermuda, Bahamas [119,166,167].

NOTES

Common on shallow sandy areas as it buries itself in the sand, can be found crawling among seagrass at daytime [119,167]. A taxonomic revision of *Chelidonura* in the Caribbean was recently published [166].

Genus *Navanax* Pilsbry, 1895

Navanax gemmatus (Mörch, 1863)

(Figure 4.9F)

SYNONYMS

Aglaja hummelincki Er. Marcus & Ev. Marcus, 1970.

DESCRIPTION

Shell reduced, internal. Body elongated with well-formed cephalic shield and parapodia. Posterior end of the body with two lobes, the left one with a thin elongate projection. Background color from opaque yellow to dark brown. Dorsum with white and brown longitudinal lines and some whitish areas. Edge of the parapodia with a row of bright blue spots. Up to 50 mm long.

DISTRIBUTION

From Florida to Brazil, Lesser Antilles, Cuba, Jamaica, Bahamas, Bermuda [119,139,142,168].

NOTES

Feeds on other sea slugs and inhabits rocky areas [119]. Ornelas-Gatdula et al. [168] studied the *Navanax aenigmaticus* (Bergh, 1893) species complex using morphological and molecular data and proposed that the valid species name for the Western Atlantic species was *Navanax gemmatus.*

Family ACTEOCINIDAE Dall, 1913

Genus *Acteocina* Gray, 1847

Acteocina candei (d'Orbigny, 1842)

(Figure 4.9G)

DESCRIPTION

Shell external, solid, oval to elongate. Spire long, conical, with 2–3 channeled whorls. Umbilicus absent. Columellar margin thickened, slightly oblique, with a small, simple fold. Head with two large posterior lobes, parapodia absent. Background color translucent white. Shell translucent white with the viscera visible as an irregular pattern of white pigment on a slightly reddish background. Up to 5.3 mm long.

DISTRIBUTION

North Carolina, Texas, Florida to Brazil, Argentina, Bermuda, Bahamas, Cuba, Jamaica, Puerto Rico, Virgin Islands, Guadeloupe, Martinique, Guyana [119,142] and Panama (present study).

NOTES

Found on soft bottoms near mangrove roots in this study.

Order ANASPIDEA Fischer, 1883

Suborder APLYSIOIDEA Lamarck, 1809

Family APLYSIIDAE Lamarck, 1809

Genus *Aplysia* Linnaeus, 1767

Aplysia dactylomela Rang, 1828

(Figure 4.10A)

SYNONYMS

Aplysia protea Rang, 1828; *Aplysia schrammi* Deshayes, 1857; *Aplysia aequorea* Heilprin, 1888; *Aplysia megaptera* Verrill, 1900.

DESCRIPTION

Shell reduced, internal. Body elongated with two tough and leathery parapodia that cover the mantle cavity. Rhinophores rolled, oral tentacles reduced. Background color usually greenish brown with large dark or black rings, which are characteristic of this species. Dark or black reticulate lines also present. Up to 200 mm long.

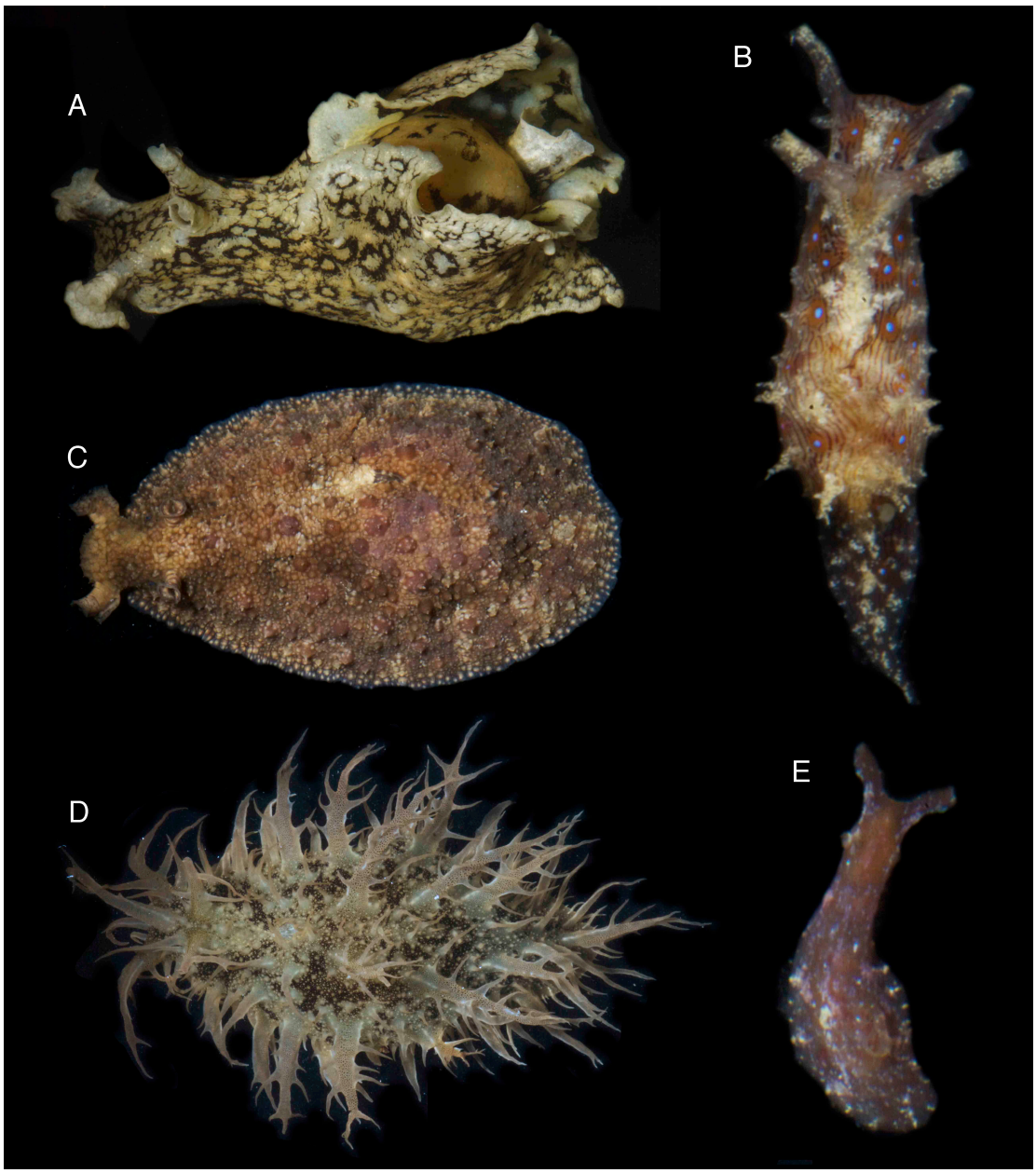

Figure 4.10. Euopisthobranchia: Aplysiidae. A) *Aplysia dactylomela* Rang, 1828; B) *Stylocheilus striatus* (Quoy & Gaimard, 1832); C) *Dolabrifera dolabrifera* (Rang, 1828); D) *Bursatella leachii* Blainville, 1817; E) *Phyllaplysia engeli* Er. Marcus, 1955.

DISTRIBUTION

Amphiatlantic. Western Atlantic: from Florida to Brazil, Greater and Lesser Antilles [119,142,169].

NOTES

This species produces purple ink when disturbed and animals mate in chains [119]. Atlantic populations were recently classified as *Aplysia dactylomela* and separated from the Indo-Pacific *Aplysia argus* Rüppell & Leuckart, 1830 [169]. *Aplysia dactylomela* has been introduced into the Mediterranean sea [170]. Found on algae and an algae covered reef in this study.

Genus *Stylocheilus* Gould, 1852

Stylocheilus striatus (Quoy & Gaimard, 1832)

(Figure 4.10B)

SYNONYMS

Notarchus polyomma Mörch, 1863; *Stylocheilus lineolatus* Gould, 1852.

DESCRIPTION

External shell present in juveniles but lost in adults. Body elongated with numerous branched papillae. Background color translucent with shades of cream, brown, and grey. Body with longitudinal or interrupted dark lines and scattered spots. Adults often have bright pink or blue ocelli that are not found in juveniles. Up to 45 mm long.

DISTRIBUTION

Circumtropical. Western Atlantic: from Florida to Brazil, Greater Antilles and Lesser Antilles, Bermuda, Bahamas [119,139,142].

NOTES

Organism feeds on algae and is common in shallow waters. This species has been often assigned to *Stylocheilus longicaudus* (Quoy & Gaimard, 1825), which is a pelagic species associated with floating algae [119].

Genus *Dolabrifera* Gray, 1847

Dolabrifera dolabrifera (Rang, 1828)

(Figure 4.10C)

SYNONYMS

Aplysia ascifera Rang, 1828; *Aplysia oahouensis* Souleyet, 1852; *Dolabrifera cuvieri* H. & A. Adams, 1854; *Dolabrifera maillardi* Deshayes, 1863; *Dolabrifera nicaraguana* Pilsbry, 1896; *Dolabrifera olivacea* Pease, 1860; *Dolabrifera sowerbyi* G. B. Sowerby II, 1868; *Dolabrifera swiftii* Pilsbry, 1896; *Dolabrifera virens* Verrill, 1901.

DESCRIPTION

Body flattened and tapered anteriorly, with the posterior end usually broader and more rounded. Parapodia fused except for a very small region in the posterior mid-line. Dorsum covered with low tubercles. Background color varies from mottled green to brown (light or dark) to even pink. Up to 90 mm long.

DISTRIBUTION

Circumtropical. Western Atlantic: North and south American mainland, from Florida to Brazil, Greater and Lesser Antilles, Bermuda, Bahamas [119,139].

NOTES

Feeds on patches of algae on rocks. This species can be very abundant in intertidal rocky areas and crawls with a leach-like movement [119].

Genus *Bursatella* Blainville, 1817

Bursatella leachii Blainville, 1817

(Figure 4.10D)

SYNONYMS

Notarchus laciniatus Rüppell & Leuckart, 1830; *Aplysia bursatella* Rang, 1834; *Aclesia glauca* Cheeseman, 1878; *Notarchus intrapictus* Cockerell, 1893; *Aclesia africana* Engel, 1926; *Aclesia rosea* Engel, 1926; *Bursatella lacinulata* Gould, 1852; *Bursatella leachii lacinulata* Gould, 1852.

DESCRIPTION

Body rounded, wider towards the posterior end. Head with two rhinophores on the dorsal side and two oral tentacles one on either side of the mouth. Dorsum covered with many papillae along, which gives the animal a fuzzy appearance. Body color

dark green to dark brown with some lighter colored spots. The gill is on the dorsal side covered by two parapodial flaps. Up to 120 mm long, but typically 75–100 mm.

DISTRIBUTION

Circumtropical. Western Atlantic: from North Carolina to Brazil, Virgin Islands, Jamaica, Aruba, Curaçao, Bermuda, Trinidad [119,142].

NOTES

Found in tide pools, lagoons and estuaries. This species lives in sea grass beds, feeds on algae and lays long, thin, ribbon-like egg masses. It is currently considered to be circumtropical species, but made up of several subspecies. The subspecies found in the Caribbean is *Bursatella leachii pleii* Rang, 1828 [119].

Genus *Phyllaplysia* P. Fischer, 1872

Phyllaplysia engeli Er. Marcus, 1955

(Figure 4.10E)

DESCRIPTION

Body flattened and oval, some specimens with low papillae. Parapodia fused. Background color translucent with varying patches and spots of pink, brown, white and some green. Some specimens have white longitudinal lines. Up to 15 mm long.

DISTRIBUTION

Florida to Brazil, Curaçao, Bahamas, Puerto Rico, Jamaica, St. Maarten/St. Martin, Barbados [119,142].

NOTES

Found on sea grasses of the genera *Thalassia* Banks ex König, 1805 and *Halodule* Endlicher, 1841 on which they are extremely cryptic [119].

Clade PANPULMONATA Jörger, Stöger, Kano, Fukuda, Knebelsberger & Schrödl, 2010

Order SACOGLOSSA Ihering, 1876

Family VOLVATELLIDAE Pilsbry, 1895

Genus *Ascobulla* Ev. Marcus, 1972

Ascobulla ulla (Er. Marcus & Ev. Marcus, 1970)

(Figure 4.11A)

DESCRIPTION

External shell slightly calcified with a cylindrical shape and flat apex. Eyes present and positioned in the upper region of the head, covered by the cephalic shield during locomotion and digging, making it difficult to observe in living animals. Cephalic shield has two lobes divided by a deep groove. Translucent shell, mantle and visceral mass define the body color, varying between brown and orange. Head shield has white coloration and opaque white dots on its surface. Up to 6 mm long.

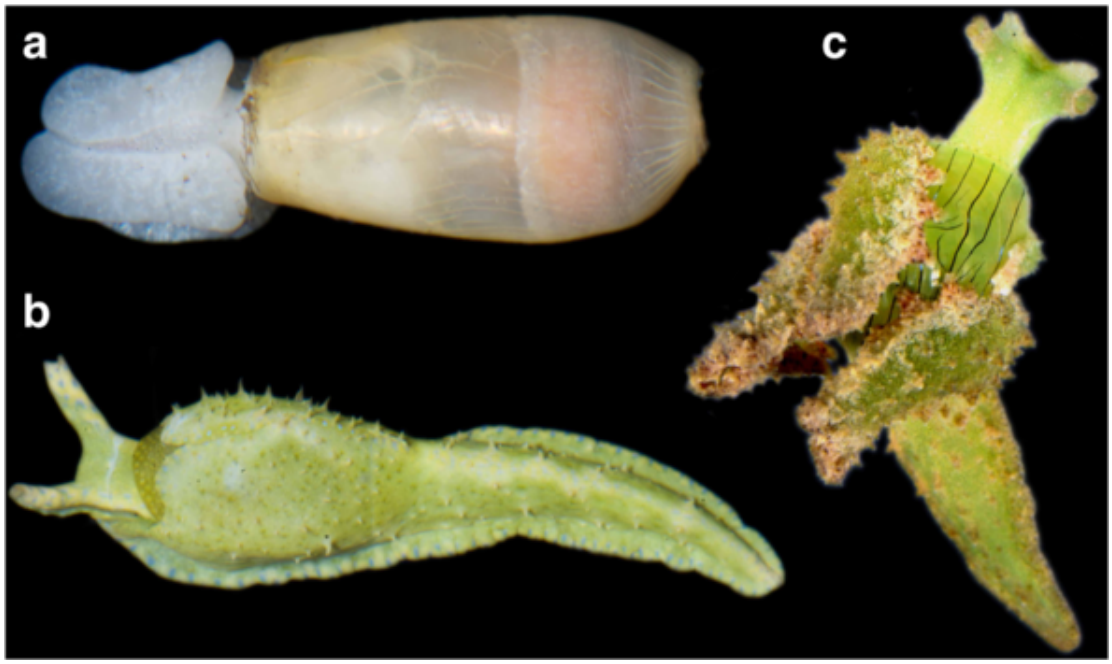

Figure 4.11. Panpulmonata: Volvatellidae and Oxynoidae. A) *Ascobulla ulla* (Er. Marcus & Ev. Marcus, 1970); B) *Oxynoe antillarum* Mörch, 1863; C) *Lobiger souverbii* Fischer, 1857.

DISTRIBUTION

Florida, Mexico, Belize, Costa Rica, Venezuela, Bermuda, Bahamas, Turks and Caicos, Cayman Islands, Virgin Islands, Brazil [119,139] and Panama (present study).

NOTES

Found on different species of *Caulerpa* Lamouroux, 1809, most commonly on rhizoids of *Caulerpa racemosa* Agardh, 1873 or crawling on sand next to algae. May exude a milky substance when disturbed. Fragile shell easily cracked or broken when handled.

Family OXYNOIDAE Stoliczka, 1868 (1847)

Genus *Oxynoe* Rafinesque, 1814

Oxynoe antillarum Mörch, 1863

(Figure 4.11B)

DESCRIPTION

Body elongate with long posterior end of foot resembling a tail. Shell globose and translucent, fragile with a wide opening and partially or fully covered by parapodia in adults. Smaller juvenile parapodia expose much of the shell. Rolled rhinophores prominent. Groove runs horizontally from base of rhinophores through each lateral region of the head. Body color light green with white papillae on parapodial margins and running down midline of tail. White and blue patches on rhinophores, sides of head, and parapodial margins. Juvenile body more elongated and smooth, ground color solid yellow to green with white and blue patches along, or at base of, rhinophores; some blue spots on mantle may be visible through shell. Up to 20 mm long.

DISTRIBUTION

Florida, Mexico, Belize, Honduras, Costa Rica, Panama, Venezuela, Bahamas, Curaçao, Bermuda, Cayman Islands, Jamaica, Dominican Republic, Puerto Rico, Virgin Islands, Martinique, St. Lucia, Barbados, St. Vincent and the Grenadines, Grenada, Trinidad and Tobago, Brazil [119,139].

NOTES

Very common throughout the tropical western Atlantic coast on many species of *Caulerpa,* but primarily on *Caulerpa racemosa*. Specimens sometimes found under rocks or crawling next to algae. Exudes white secretion and may autotomize tail when disturbed. High intra-specific variation and a lack of diagnostic differences in external morphology make it difficult to distinguish from *Oxynoe azuropunctata* Jensen, 1980.

Genus *Lobiger* Krohn, 1847

Lobiger souverbiei P. Fischer, 1857

(Figure 4.11C)

SYNONYMS

Lobiger pilsbryi Schwengel 1941.

DESCRIPTION

Shell extremely fragile with apex directed posteriorly to left side. Some small papillae scattered on rhinophores and lateral sides of the head. Each parapodium expands narrowly in two leaf-like projections upwards from shell, with white papillae on parapodial margins. Rolled rhinophores shorter than head. Foot extends posteriorly to form thick tail and anteriorly to form two lobes. Body coloration light green with yellowish-brown papillae on tail and parapodia. Light green mantle with black longitudinal lines and a few scattered blue dots visible through translucent shell. Up to 30 mm long.

DISTRIBUTION

Florida, Mexico, Honduras, Costa Rica, Venezuela, Curaçao, Cayman Islands, Jamaica, Puerto Rico, Guadeloupe, Barbados, St. Vincent and the Grenadines, Grenada, Brazil [119,139] and Panama (present study).

NOTES

Feeds on *Caulerpa* spp., most commonly found on *Caulerpa racemosa* in high-flow areas. May exude milky secretion and sometimes autotomize parapodial extensions when disturbed.

Family LIMAPONTIIDAE Gray, 1847

Genus *Ercolania* Trinchese, 1872

Ercolania coerulea Trinchese, 1892

(Figure 4.12A)

SYNONYMS

Stiliger cricetus Er. Marcus & Ev. Marcus 1970.

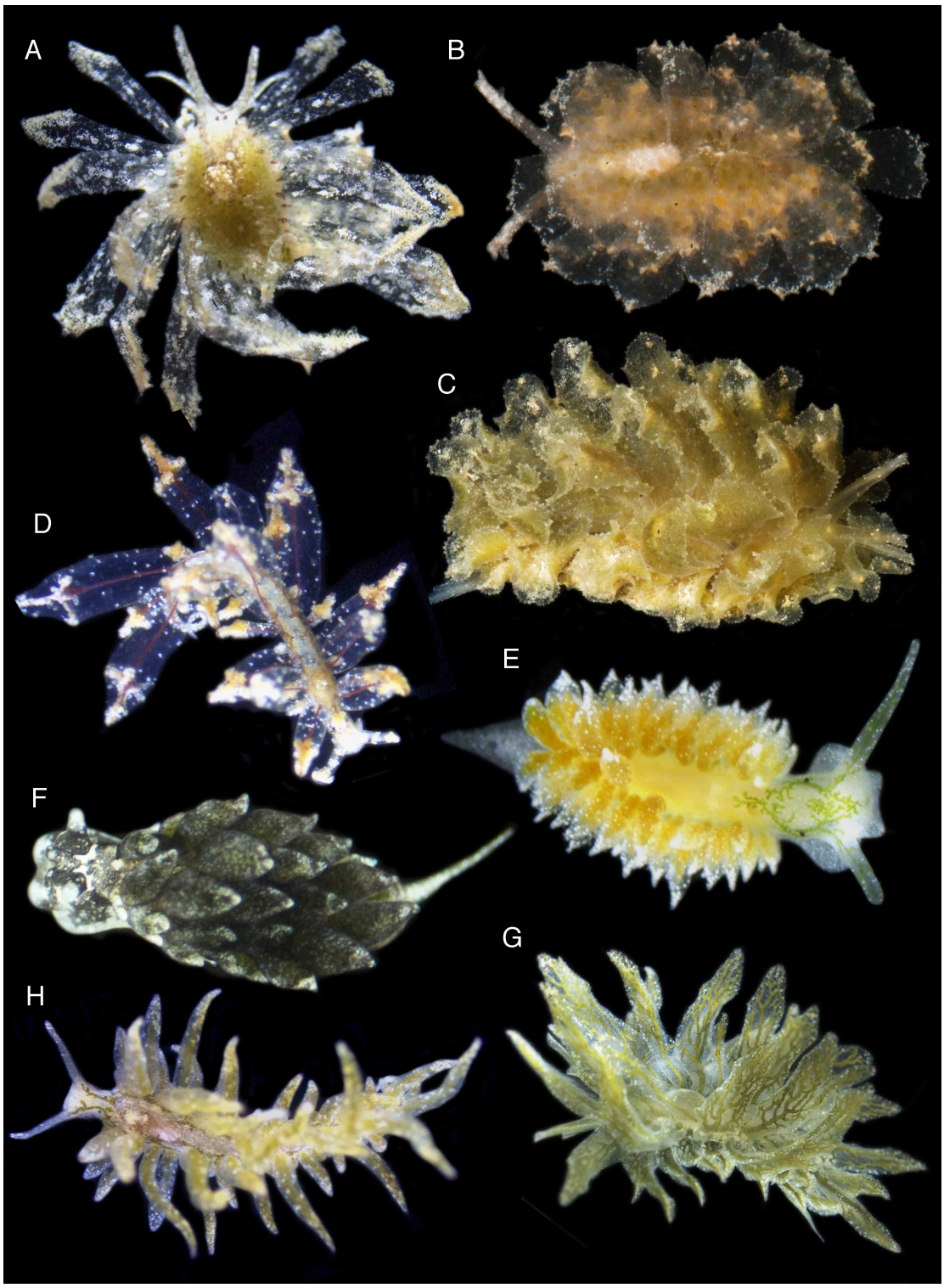

Figure 4.12. Panpulmonata: Caliphyllidae, Costasiellidae, Hermaeidae and "Limapontiidae." A) *Cyerce antillensis* Engel, 1927; B) *Cyerce* cf. *antillensis* Engel, 1927; C) *Polybranchia viridis* (Deshayes, 1857); D) *Hermaea cruciata* Gould, 1870; E) *Ercolania coerulea* Trinchese, 1892; F) *Costasiella nonatoi* Ev. Marcus & Er. Marcus, 1960; G) *Caliphylla mediterranea* Costa, 1867; H) *Placida kingstoni* Thompson, 1977.

DESCRIPTION

Cerata short and fusiform arranged in rows on both sides of body. Digestive diverticula irregular inside each ceras. Rhinophores simple, smooth, longer than head. Eyes in dorsolateral position. Pericardial hump short, not covered by cerata. Foot forms thick short tail and large rounded anterior expansion. Opaque translucent body with posterior region colored by yellow digestive gland. Small white dots scattered across entire body, highly concentrated on cerata and rhinophore tips. Some individuals have blue spots clustered at tips of cerata and rhinophores and on top of head. Two longitudinal light green branches of digestive diverticula run anteriorly, branching in cephalic region and inside rhinophores. Body length typically 8–10 mm, up to 16 mm.

DISTRIBUTION

Florida, Venezuela, Curaçao, Jamaica, Virgin Islands, St. Kitts, St. Lucia, Brazil [119] and Panama (present study).

NOTES

Found in clumps of the green bubble algae *Dictyosphaeria cavernosa* Børgesen, 1932 and *Valonia* Agardh, 1823. Originally described from Mediterranean Sea, only species of the genus *Ercolania* recorded from the Atlantic and Indo-Pacific [171]. Recent phylogenetic analysis confirmed placement of *E. coerulea* within a larger clade that includes most other species in the genus [172].

Family COSTASIELLIDAE K. B. Clarke, 1984

Genus *Costasiella* Pruvot-Fol, 1951

Costasiella nonatoi Ev. Marcus & Er. Marcus, 1960

(Figure 4.12B)

DESCRIPTION

Grooved rhinophores shorter than head. Foot elongated posteriorly in sharp tail longer than half the body length and anteriorly wide and bilobate. Eyes mid-dorsally positioned behind rhinophores. Largest fusiform cerata arranged in dorsal region, while small ones in one row next to foot corner. Overall external color almost entirely black, except for tail, periocular area, border of foot, and tips of rhinophores and cerata, which are transparent or opaque white. Bright whitish or yellowish dots dispersed through foot, rhinophores, cerata, and tail. Up to 4 mm long.

DISTRIBUTION

North Carolina, Florida, Costa Rica, Venezuela, Cayman Islands, Puerto Rico, Bahamas, Bermuda, Brazil [119,139,142] and Panama (present study).

NOTES

Feeds and reproduces on *Avrainvillea* Descaisne, 1842 spp. and crawls among its filaments. Phylogenetic analysis places *C. nonatoi* outside monophyletic group formed by other species of the genus [172]. Characteristics such as grooved

rhinophores, eyes positioned behind rhinophores, and absence of photosynthetic ability also distinguish it from other species of *Costasiella* [173,174].

Genus *Placida* Gray, 1847

Placida kingstoni Thompson, 1977

(Figure 4.12C)

DESCRIPTION

Opaque translucent body scattered with small white dots, brown dorsal region. Two longitudinal yellow-green digestive system branches run along dorsal region, branching anteriorly next to rhinophores. Elongated fusiform cerata, each containing one unbranched ramification of the digestive diverticula extending almost to tip. Rhinophores enrolled at base, smooth, longer than head. Eyes in a dorsolateral position closer to pericardial hump than to rhinophores. Prominent pericardial hump visible on dorsal region, longer than head. Anal papillae positioned mid-anteriorly on pericardial hump. Foot forms a short tail and small square anterior expansion. Up to 15 mm long.

DISTRIBUTION

Florida, Costa Rica, Jamaica, Martinique, Bermuda [119] and Panama (present study).

NOTES

Found on green algae in the genus *Bryopsis* Lamouroux, 1809.

Family HERMAEIDAE Adams & Adams, 1854

Genus *Hermaea* Lovén, 1844

Hermaea cruciata Gould, 1870

(Figure 4.12D)

SYNONYMS

Hermaea coirala Er. Marcus 1955.

DESCRIPTION

Rhinophores bifurcated and slightly longer than the head. Foot forms sharp tail posteriorly and projects anteriorly into small foot corner extensions. Cerata fusiform with a conical tip, variable in size. Largest cerata reaching more than half the body length. A duct of the digestive system run inside each ceras and branches highly only in the apex under the yellow gland. Translucent body with scattered small white dots. Dark red tubules of digestive diverticula, yellow glands at tips of cerata, whitish gonads, and other internal organs visible through translucent body wall. Up to 5 mm long.

DISTRIBUTION

Massachusetts, New York, Florida, Costa Rica, Trinidad and Tobago, Brazil [119] and Panama (present study).

NOTES

Species of *Hermaea* often feed on filamentous red algae [175], as opposed to the green algae that serve as host for most sacoglossans.

Family CALIPHYLLIDAE Tiberi, 1881

Genus *Caliphylla* A. Costa, 1867

Caliphylla mediterranea A. Costa, 1867

(Figure 4.12E)

DESCRIPTION

Each side of the body has four rows of leaf-shaped cerata. Dorsal midline lacking cerata, starting at the pericardium. Digestive diverticula branch within cerata, bifurcating at margin of each ceras. Oral veil is present. Anus at apex of papilla on right side, at eye level anterior to pericardium. Bifid rhinophores long and grooved. Eyes positioned on median side behind rhinophores. Male genital pore at base of rhinophores, female aperture anus and male pore. Digestive diverticula, varying from dark green to brown, visible through translucent elongated body. Numerous black and white dots scattered throughout body. Up to 35 mm long.

DISTRIBUTION

Amphiatlantic; Western Atlantic: Florida, Curaçao, Virgin Islands, Trinidad and Tobago, Brazil [119] and Panama (present study).

NOTES

Associated with filamentous green algae *Bryopsis plumosa* Agardh, 1823 growing in sheltered areas of rocks, reef corals or mangrove roots. Readily shed cerata and extrude adhesive substance when disturbed. Monotypic genus with type specimen from Mediterranean Sea, but other morphotypes from West Atlantic coast may reveal one or more additional species [119], and at least one cryptic species exists in the Pacific [172].

Genus *Cyerce* Bergh, 1870

Cyerce antillensis Engel, 1927

(Figure 4.12F)

SYNONYMS

Cyerce habanensis Ortea & Templado 1989.

DESCRIPTION

Body broad and oval-shaped. Eye spots behind base of rhinophores. Body translucent with light green to yellow-white viscera showing through. Pericardium opaque white.

Cerata wide and inflated, almost transparent with scattered white spots that concentrate at the tips and irregular edges. Up to 60 mm long.

DISTRIBUTION

Florida, Mexico, Belize, Honduras, Costa Rica, Curaçao, Bermuda, Cayman, Islands, Cuba, Bahamas, Jamaica, Puerto Rico, Virgin Islands, Barbados, Tobago [119,139] and Panama (present study).

NOTES

Feeds on green algae in the genus *Penicillus* Lamarck, 1813; older reports of other hosts (*Udotea* Lamouroux, 1812, *Halimeda* Lamouroux, 1812) likely reflect unrecognized cryptic species that eat other host genera [176,177]. May autotomize cerata when disturbed.

Cyerce cf. *antillensis* Engel, 1927

(Figure 4.12G)

DESCRIPTION

Body broad and oval-shaped. Eye spots behind base of rhinophores. Body translucent with light green to yellow-white viscera showing through. Pericardium opaque white. Cerata wide and short, almost transparent with orange spots and white at the tips and irregular edges. Up to 30 mm long.

DISTRIBUTION

Panama (present study).

NOTES

Feeds on *Halimeda* green algae. Similar to *Cyerce antillensis* but is genetically distinct (unpublished data), has a white pericardium and broader cerata; it may constitute an undescribed species. Autotomizes the cerata when disturbed.

Genus *Polybranchia* Pease, 1860

Polybranchia viridis (Deshayes, 1857)

(Figure 4.12H)

DESCRIPTION

Body oval-shaped. Rhinophores bifid for half of their length or more, cerata and rhinophores covered with small papillae. Body almost transparent with internal viscera giving the animal a light green to pale gold tint. Flattened cerata translucent with opaque white spots and characteristic fold in middle and numerous white glands on edges. Up to 80 mm long.

DISTRIBUTION

Florida, Costa Rica, Curaçao, Bonaire, Jamaica, Virgin Islands, Guadeloupe, Barbados [119] and Panama (present study).

NOTES

Feeds on green algae in the genus *Caulerpa*. Found under rocks during the day and active at night.

Family PLAKOBRANCHIDAE Gray, 1840

Genus *Elysia* Risso, 1818

Elysia crispata Mörch, 1863

(Figure 4.13A–B)

SYNONYMS

Elysia schrammi Ørsted & Mörch, 1863; *Tridachia whiteae* Er. Marcus, 1957; *Elysia clarki* Pierce, Curtis, Massey, Bass, Karl & Finney, 2006.

DESCRIPTION

Most conspicuous and one of the largest sacoglossans in the Caribbean. Parapodia highly undulated, resembling lettuce (hence the common name lettuce sea slug). Highly variable in body color, ranging from light to dark green with small or large white spots, to dark green or purple with white spotting (described as *Elysia clarki*), to entirely blue. Parapodial margins also highly variable in color – often white but can also be lined with yellow, red, and/or blue. Foot may be uniformly pale cream, or green with small to large white spots. Dorsal surface between parapodia generally pale green, often with pale cream to white spots. Up to 50 mm long.

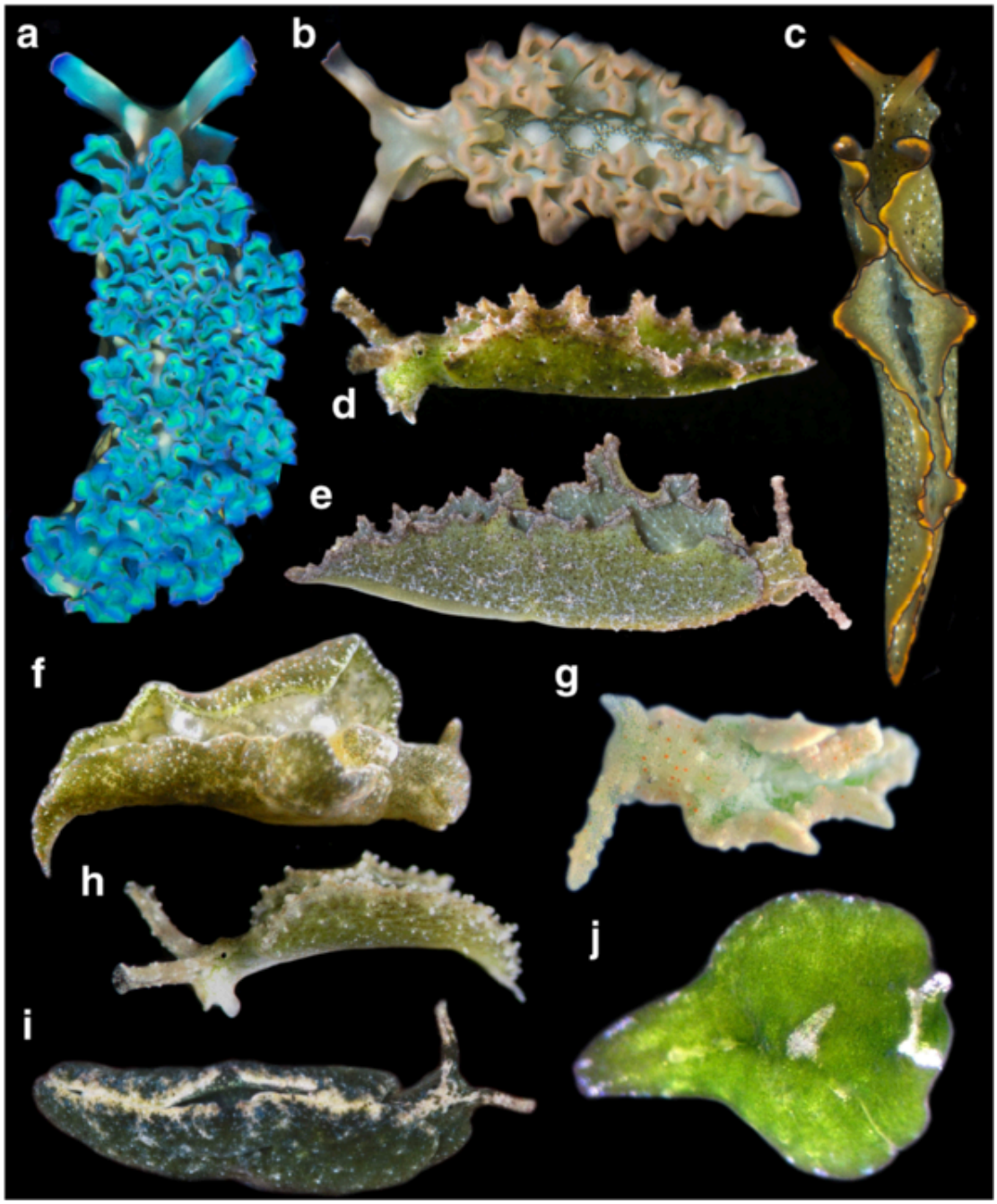

Figure 4.13 Panpulmonata: Plakobranchidae. A–B) *Elysia crispata* Mörch, 1863; C) *Elysia ornata* (Swainson, 1840); D) *Elysia papillosa* Verrill, 1901; E) *Elysia subornata* Verrill, 1901; F) *Elysia canguzua* Er. Marcus, 1955; G) *Elysia cornigera* Nuttall, 1989; H) *Elysia zuleicae* Ortea & Espinosa, 2002; I) *Elysia velutinus* Pruvot-Fol, 1947; J) *Elysia marcusi* (Ev. Marcus, 1972).

DISTRIBUTION

Florida, Dry Tortugas, Mexico, Belize, Honduras, Costa Rica, Colombia, Venezuela, Aruba, Curaçao, Bonaire, Venezuela, Bermuda, Cayman Islands, Bahamas, Jamaica,

Haiti, Puerto Rico, Virgin Islands, St. Maarten/St. Martin, Antigua, St. Lucia, Martinique, Guadeloupe, Turks and Caicos, St. Vincent and the Grenadines, Barbados, Trinidad and Tobago [119,139] and Panama [136].

NOTES

Very common throughout the Caribbean and easily spotted on rocks, coral rubble, or sediment, often crawling or sometimes stationary with parapodia opened giving the appearance of basking in the sun. Uncertainty surrounds feeding ecology, but thought to be highly polyphageous for a sacoglossan. The most recent work using field and lab methods confirmed feeding on one or more species in the genera *Bryopsis*, *Penicillus*, *Halimeda*, *Acetabularia* Lamouroux, 1812, and *Derbesia* Solier, 1846 [178–181]. Originally described as *Elysia* (*Tridachia*) *crispata*, now formally recognized as a member of the genus *Elysia*.

Elysia ornata (Swainson, 1840)

(Figure 4.13C)

SYNONYMS

Pterogasteron marginatum Pease 1871.

DESCRIPTION

Parapodia highly arched to form a prominent raised “chimney” halfway along the body, then unite at posterior end of body to a pointed tail. Rhinophores short and taper to a blunt point at rolled tips. Olive green with small black and white spots on

dorsal surface and outer parapodia. Sharp black band runs along entire parapodial margin, with more diffuse orange submarginal band on inner and outer parapodia. Rhinophores match orange coloration of parapodia but may lack black edges. Up to 50 mm long.

DISTRIBUTION

Florida, Belize, Honduras, Costa Rica, Colombia, Venezuela, Bahamas, Curaçao, Bermuda, Jamaica, Puerto Rico, Virgin Islands, Martinique, Turks & Caicos, Barbados, St. Vincent and the Grenadines, Grenada, Trinidad and Tobago, Brazil [119,139] and Panama (present study).

NOTES

Commonly found feeding inside clumps of *Bryopsis plumosa* and can be surprisingly cryptic despite relatively large size and bright coloration. This species was thought to have a cosmopolitan distribution, but recent molecular work suggests that the Caribbean population is genetically distinct from several undescribed species in the Indo-Pacific [182].

Elysia papillosa Verrill, 1901

(Figure 4.13D)

SYNONYMS

*Elysia anneduponta*e Ortea, Espinosa & Caballer, 2005.

DESCRIPTION

Outer parapodial surface covered with rows of white papillae. Parapodial margin tan to dark brown, bears many light tan to brown papillae, with scalloped edge forming several siphonal openings. One large pair of sperm-storage vesicles visible on dorsal surface of large adults, usually near the 6th dorsal vessel. Highly variable external body coloration, generally light green but can range from white/tan to olive green. Sides of head lighter green to white. One or two large white papillae between the eyes on most specimens. Inner parapodial surface and dorsum lightly to heavily speckled with brown or black spots, and with scattered white, rounded papillae. Pericardium round, with brown streaks and spots and low white papillae. Up to 30 mm long.

DISTRIBUTION

Mexico, Panama, Cuba, Jamaica, Florida, Bahamas, U.S. Virgin Islands, Antigua, Curaçao [183].

NOTES

When disturbed, specimens readily swim by undulating their parapodia. Specializes on green algae in the genus *Penicillus*. One of the most abundant sacoglossans in the Caribbean – sometimes visible in the field and often found on collections of *Penicillus* spp. Often confused with *E. zuleicae*, which may be distinguished by its longer rhinophores and extended tail. Also readily confused with *E. patina*, which is externally very similar but can be distinguished by its host alga *Halimeda opuntia*

Lamouroux, 1816 and its egg masses – *E. papillosa* produces relatively more numerous, larger eggs (planktotrophic development) with white extra-zygotic yolk, while *E. patina* has larger, fewer eggs (lecithotrophic development) with flat, orange ribbons of extra-zygotic yolk [183].

Elysia subornata Verrill, 1901

(Figure 4.13E)

DESCRIPTION

Coloration ranges from yellow to olive to dark green. Sides of parapodia dusted with white to varying degrees, with white pigment often arranged in star-shaped clusters around base of white papillae. Tiny black or brown dots scattered all over head and body. Some specimens have few papillae, others are densely covered in elongated white papillae. Rhinophores short relative to body length, with tan to lavender to dark brown coloration and white tips. Distinctive fine black line along the edge of parapodia, with tan to dark brown margin, sometimes with white speckling. Inner parapodia green with white speckling. Mostly symmetrical, simple vessels extending from center of dorsal surface. Up to 50 mm long.

DISTRIBUTION

Florida, Mexico, Belize, Costa Rica, Bermuda, Bahamas, Aruba, Cayman Islands, Jamaica, Puerto Rico, Virgin Islands, Martinique, Grenada, Trinidad and Tobago, Brazil [119,139,184,185] and Panama (present study).

NOTES

Egg masses have continuous string of bright orange extra-zygotic yolk, larvae metamorphose inside egg capsules [183]. Slugs are usually found in association with *Caulerpa* and are known to feed on at least eight different species in the genus. Adults do not swim when disturbed.

Elysia canguzua Er. Marcus, 1955

(Figure 4.13F)

SYNONYMS

Elysia eugeniae Ortea & Espinosa 2002.

DESCRIPTION

Rhinophores short, blunt-tipped, same color and texture as head but with white patch at tip. Three siphonal openings in parapodial folds at head, middle, and posterior end. Dark to olive green on head and outer parapodia, mostly smooth with low sparse papillae. Body densely covered with distinctive red/orange spots, and smaller iridescent blue specks. Uneven rows of white spots on head and across sides of parapodia. Up to 12 mm long.

DISTRIBUTION

Costa Rica, Brazil [119,139] and Panama (present study).

NOTES

Found feeding on *Bryopsis* sp. growing on loose sediment 1–2 m depth. Preferred hosts reported to be both *Bryopsis plumosa* and *Codium* Stackhouse, 1797 [176]. Parapodia typically held open when resting, adults do not swim when disturbed.

Elysia cornigera Nuttall, 1989

(Figure 4.13G)

DESCRIPTION

Rhinophores long and curled, white to light green with red dots and many white papillae. White to grey on parapodia and head with numerous warty papillae. Red granules dotting head and rhinophores, smaller red dots scattered on parapodia. Densely enervated green digestive diverticula inside of parapodia. Up to 8 mm long.

DISTRIBUTION

Florida, Cuba, Cayman Islands, Bahamas [119] and Panama (present study).

NOTES

One small specimen found on coral rubble near *Caulerpa racemosa*, but only confirmed host is *Acetabularia crenulata* Lamouroux, 1816. Formerly synonymized with *Elysia timida* Risso, 1818, subsequently resurrected based on genetic,

morphological, and developmental characteristics, and differences in photosynthetic ability [182,186]. Upper surface of egg mass has flat ribbon of white to translucent ribbon of extra-zygotic yolk with granular appearance [183].

Elysia zuleicae Ortea & Espinosa, 2002

(Figure 4.13H)

DESCRIPTION

External morphology quite variable. Rhinophores long and rolled. Narrow tail often extends a few millimeters beyond posterior end of parapodia, but some specimens have no tail. Parapodia are thin, sometimes with slight undulation but no siphonal openings. Body coloration typically olive to dark green. Head light to dark green, sometimes with rust-colored patches. Rhinophores colored white to brown-purple with scattered white papillae and white patches of pigment concentrated at tips. Outer surface of parapodia are green with scattered white specks and low white papillae. White papillae run along parapodial margin, sometimes forming crown-like clusters that appear to rise and fall along the margin. Some specimens, particularly juveniles, have a thin black line along parapodial margins surrounded by thicker white submarginal bands.

DISTRIBUTION

Cuba, Costa Rica, Jamaica, Venezuela [119,142] and Panama (present study).

NOTES

Specializes on the green alga *Udotea flabellum* Howe, 1904. Juveniles hold parapodia flat against algal blade are distinctly darker green than adults. Adults swim readily by undulating parapodia when disturbed. Egg masses have a thin, white ribbon of extra-zygotic yolk.

Elysia velutinus Pruvot-Fol, 1947

(Figure 4.13I)

DESCRIPTION

Parapodia form one small siphonal opening about halfway down the body. Body coloration varies from light to dark green, with spots or large patches of white or tan pigment. Head has large Y-shaped white to tan patch of pigment, starting anterior of pericardium and running up to the base of each rhinophore. Rhinophores are green at the base but distally become white or tan, sometimes with small papillae. Panamanian specimens tend to have less white pigmentation/fewer papillae on external surface of parapodia compared with those found in the Bahamas. Up to 15 mm long.

DISTRIBUTION

Florida, Mexico, Honduras, Costa Rica, Panama, Colombia, Venezuela, Bermuda, Bahamas, Curaçao, Cayman Islands, Jamaica, Puerto Rico, Virgin Islands, St. Maarten/St. Martin, St. Lucia, Barbados, St. Vincent and the Grenadines, Grenada, Brazil [119,142,167].

NOTES

Typically associated with *Halimeda* spp., most commonly the upright branching species *H. incrassata* J.V. Lamouroux, 1816 and *H. monile* J.V. Lamouroux, 1816. Parapodia held together when resting, slugs do not swim when disturbed. Egg masses have continuous ribbon of bright yellow extra-zygotic yolk. This species was previously known as *Elysia tuca* Ev. Marcus & Er. Marcus, 1967 but Krug et al. [183] found that *Elysia velutinus* is a senior synonym.

Elysia marcusi Ev. Marcus, 1972

(Figure 4.13J)

DESCRIPTION

Small bodied. Parapodia fused to body with fusion line visible running dorsally down the body. Uniformly light to dark green, sometimes with white patches. Rhinophores solid white, simple, flat (not rolled), and fully retractable into head. Up to 5 mm long.

DISTRIBUTION

Florida, Costa Rica, Bahamas, Jamaica [119] and Panama (present study).

NOTES

Found on mixed collection of *Caulerpa racemosa* and *Halimeda* sp., but preferred host is *Halimeda opuntia* [183]. Resting slugs flatten into perfectly round circles, superficially resembling *Bosellia mimetica* Trinchese, 1891. Crawling slugs elongate into form more typical of *Elysia* spp.

Discussion

Few studies of heterobranch sea slugs have reported collecting effort. In the Eastern Pacific, Nybakken [187] searched for sea slugs for 120 hours and found 31 species in a California intertidal assemblage. Hermosillo [188] searched for 750 hours and found 140 species in Bahía de Banderas, Pacific coast of Mexico, while Bertsch [189] in Bahía de Los Ángeles, Pacific coast of Mexico found 81 species in 229.3 hours of searching. For the Caribbean, Thompson [190] reported a total of 61 species for a searching time of approximately 298 hours, mostly in Jamaica. A recent study conducted in a Mexican Caribbean coral reef reported 32 species observed in a total of 74.4 hours of search [191], however in this case indirect methods were also included. The preceding studies also found that the highest number of species belonged to the clade Nudibranchia, which is consistent with the greater overall diversity in this group [32].

Based on the information provided in these prior studies, the collecting effort of our study (307.5 hours) represents one of the highest recorded for sea slugs not only in the Caribbean but also in tropical regions generally. Despite this large collecting effort, relatively few species were found compared to the total known diversity in the Caribbean. Only 82 out of the 308 species reported by Valdés *et al.* [119] or the 329 species reported by García & Bertsch [192] were found; this

represents about 25% of known Caribbean species diversity. All the species reported here were included in Valdés *et al*. [119] except for those that could not be identified at the species level. From the 19 species recorded by Collin *et al*. [136] five were not observed during the newly conducted field work in Panama: *Atys macandrewii* E. A. Smith, 1872, *Elysia flava* Verrill, 1901, *Aphelodoris antillensis* Bergh, 1879, *Paradoris adamsae* Padula & Valdés, 2012 [as *Paradoris mulciber* (Ev. Marcus, 1971)] (see [184]) and *Doto* cf. *caramella* Er. Marcus, 1957.

The total diversity of sea slugs documented in this study, as well as the total diversity in the Caribbean region is much lower than in the Indo-Pacific region, which is the center of tropical diversity. For example, Gosliner et al. [32] reported 815 sea slug species just in the region of Anilao, located in the Philippine Islands. The total diversity in other Indo-Pacific regions increases dramatically from peripheral areas such as Tanzania (258 spp.), Guam (474 spp.) or French Polynesia (504 spp.) to the Coral Triangle where according to Gosliner et al. [32] diversity reaches unprecedented levels (Philippines 1006 spp., Papua New Guinea 646 spp.). Unfortunately, there are no sea slug diversity studies in the Indo-Pacific region documenting collecting effort and therefore comparisons with the present study are not possible.

For heterobranch sea slugs the experience of the observers in finding species while conducting surveys/inventories is critical, as these animals are difficult to find. Many sea slugs are very small and well-camouflaged, making them nearly invisible to the untrained eye. Even experienced observers often have difficulties finding species in the Caribbean because the abundances of sea slugs in this region are typically

lower than in other tropical regions of the world [119]. Our results are consistent with this observation, as the total number of specimens found was relatively low and many species were only represented by one specimen.

Most of the sacoglossan species were found by indirect methods. Individuals of these species are very small and remarkably cryptic on their host algae. In spite of this, a few species were found by direct observations (e.g. *Elysia crispata* and *Polybranchia viridis*), primarily due to their more conspicuous size and tendency to periodically leave their algal food sources. *Elysia crispata* is particularly common in the area and throughout the Caribbean [119,136]. In contrast, most species belonging to other clades (Table 4.2) were found by direct methods due to their (mostly) larger size and more observer experience finding these groups, especially nudibranchs.

This paper represents a substantial increase in the knowledge of heterobranch sea slug diversity in Bocas del Toro, Panama as compared to the single previous publication from Collin et al. [136]. This increase in known diversity strongly suggests that the distribution of species within the Caribbean is still poorly known (at least in regards to some localities), and thus species ranges may need to be modified as more surveys are conducted.

Acknowledgements

I would first like to thank my co-authors on this publication. The course "Taxonomy and Biology of Sea slugs" was funded by awards from the US National Science Foundation DEB-1355190 to PJK and DEB-1355177, and organized by Rachel Collin of the Smithsonian Tropical Research Institute in Bocas del Toro. The two NSF grants also supported the travel of several students. Other funding agencies

made travel possible for other student participants: Conchologists of America and the University of Maryland Biological Sciences Department Genie Clark Research Award, CSULA Culminating project fund, Smithsonian Tropical Research Institute fellowships, Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP 2014/06979–1, International Affairs Office of the Universidad de Antioquia, the British Ecological Society, travel grant 5585-6629, and Bombay Natural History Society. Several staff members of the Smithsonian Tropical Research Institute in Bocas del Toro were extremely helpful supporting the fieldwork and course activities, these are: Plinio Gondola, Urania Gonzalez, Tanyusha Grenald, Arcadio Castillo, Deyvis Gonzalez and Nerea Nieto.

Chapter 5: Sequestration of nematocysts by divergent cnidarian predators: mechanism, function, and evolution.

Introduction

Adaptations to avoid predation are extremely common and diverse among animals. Such adaptations can take a variety of forms, including behavioral, physical, or chemical defenses, and can be generated through diverse mechanisms [193–196]. Although defenses are often produced endogenously, being entirely encoded by the genome of the organism [197], in some cases animals acquire defenses from exogenous sources. For example, some animals associate with other species that are themselves well-defended, thus garnering protection from their associate's defenses (e.g., [198]). Strikingly, certain animals have evolved the ability to actually sequester the defenses of other species, integrating them into their own tissues [158,199].

A number of animals can sequester predator-deterring chemicals or structures from other species, such as their symbionts or prey. The ability to sequester defensive chemicals is widespread among animals, being well documented in Arthropoda [200–202], Mollusca [158,193] and Chordata [199,203,204], among others. Although much less common, some animals have evolved the ability to sequester not just chemicals but entire structures, such as whole organelles or cells, from other organisms [205,206]. In most cases investigated, the structures are sequestered from prey, and are inferred to be a source of energy, nutrients and/or carbon for the sequestering

animal. An example of this is kleptoplasty (or chloroplast "theft") by some marine gastropods that feed on algae. These gastropods possess the ability to sequester functional chloroplasts from their algal food sources, which are then used as a source of energy [207]. The sequestration of exogenous structures explicitly for defense purposes, however, is rare and much less well documented; the clearest example of this is the sequestration of nematocysts, the stinging organelles of cnidarians.

Several divergent animal lineages have evolved the ability to sequester nematocysts from their cnidarian prey and incorporate these organelles into their own bodies. Nematocyst sequestration has been documented in Ctenophora, Acoelomorpha, Platyhelminthes and Mollusca (Figure 5.1, [30,208]), indicating multiple origins of this ability. For most species, information on nematocyst sequestration remains limited to a basic description of the location and appearance of nematocysts within the sequestering animal's body. Nematocyst sequestration has been studied further in only a few species, yet such studies provide important insights about the mechanism by which nematocysts are sequestered and the ecological consequences of sequestration.

Here, we review nematocyst sequestration in the four animal groups in which it is known: Ctenophora, Acoelomorpha, Platyhelminthes and Mollusca. For each group, we review the distribution of nematocyst sequestration across the phylum and, where this is known, the mechanism and potential ecological function of sequestration. We also provide context about the structure and function of nematocysts in Cnidaria. Based on the information available, we highlight similarities

among sequestering species, propose a general model for the evolution of sequestration, and highlight important avenues for future research.

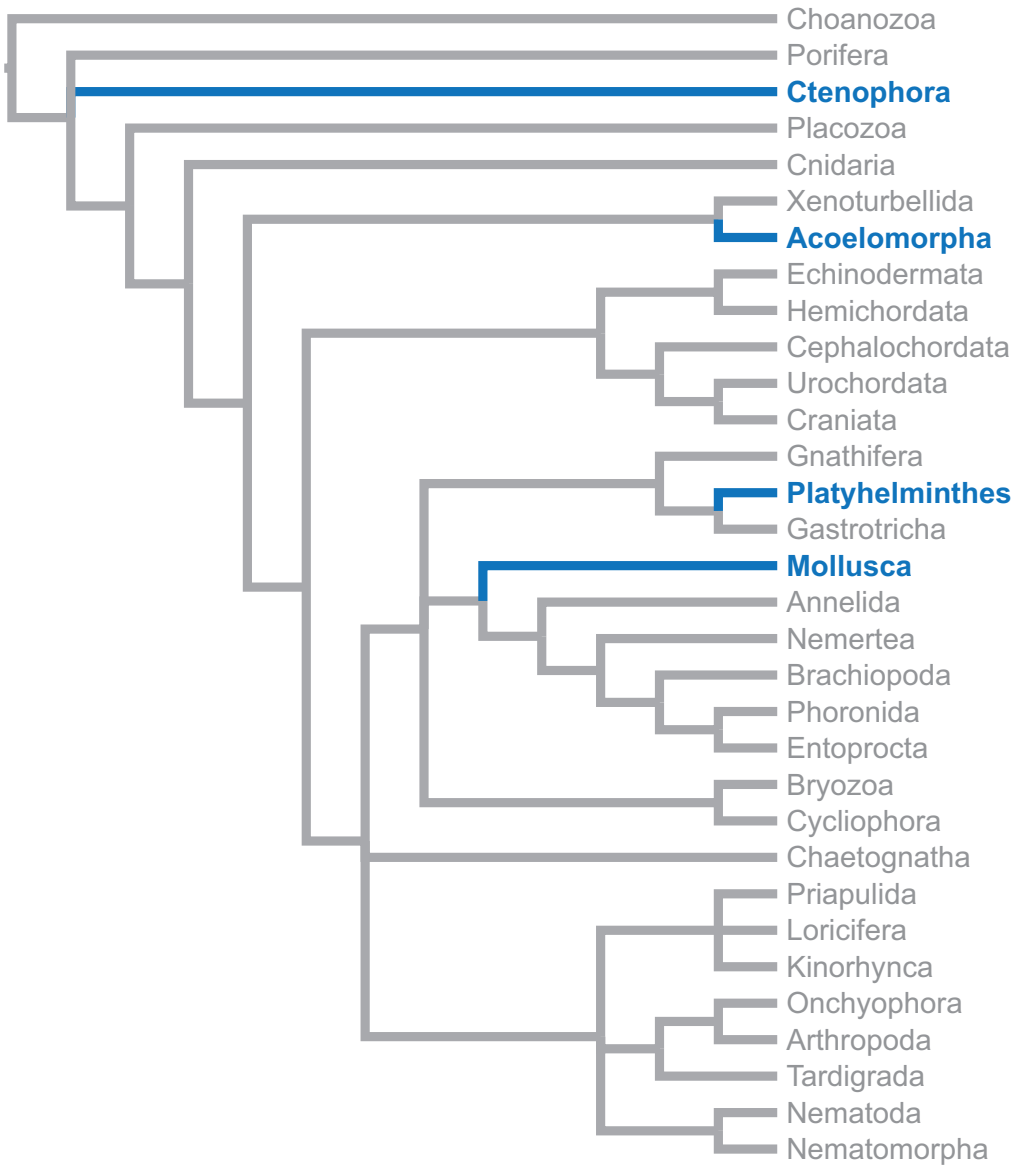

Figure 5.1. Phylogeny of Metazoa indicating lineages that are known to sequester nematocysts. Phyla in which sequestering species are known are shown in blue. Relationships are based on Edgecombe et al. (2011) and Dunn et al. (2014).

What are nematocysts?

Animals within Cnidaria, a large, diverse clade of over 13,000 species [209,210], sting predators and capture food with complex intracellular organelles called cnidae

[211]. These structures are found within cells called cnidocytes, which are most commonly found in the epithelial lining of tentacles but may also occur in other regions of the body (Figure 5.2A). Cnidae are of several forms, the most common being the nematocyst, which is likely the ancestral form given its widespread distribution across the phylum [210]. Nematocysts are small venom-filled capsules containing an eversible tubule (Figure 5.2B), often with spines or barbs, that can be discharged into the tissues of other organisms with very high accelerations, up to 5 million *g* [212,213].

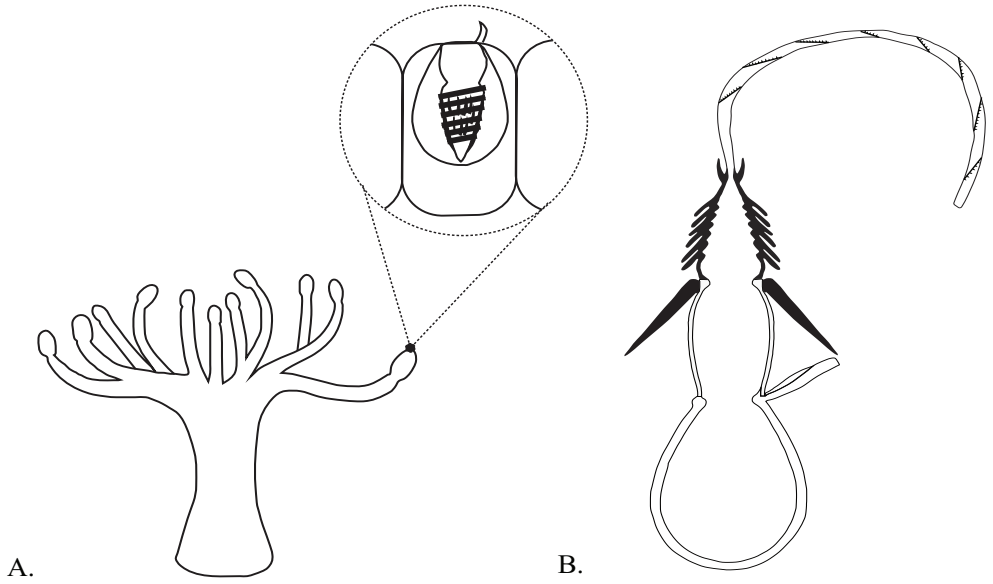

Figure 5.2. Location and morphology of cnidarian nematocysts. A) A generalized cnidarian polyp showing the location of nematocysts in the tentacle epithelium. Within the enlarged region is a nematocyst located inside an epithelial cell. B) An everted nematocyst (specifically a stenotele nematocyst) after it has fired.

The discharge of these structures is triggered by the stimulation of the cnidocil (a modified cilium on the outside of the cnidocyte) by chemical and/or mechanical mechanisms [214–216]. Based largely on the shape of the tubule and its shaft, as well as the presence and shape of their armaments, several subtypes of nematocysts are recognized, including isorhizas (in which the tubule is of largely uniform thickness

across most of its length and does not have a well-defined shaft), mastigophores (in which the tubule extends well beyond a well-defined shaft), and stenoteles (in which the tubule shaft possesses three large spines), among others [214,217].

The efficacy of nematocysts for defense in cnidarians

Although the primary function of nematocysts in cnidarians is thought to be prey capture, a fundamental question is whether they are also effective as defensive structures [196,217–225]. The few studies attempting to address this question have provided some evidence supporting the hypothesis that nematocysts can indeed have a defensive function. Stachowicz & Lindquist [196] and Bullard & Hay [222] showed that several species of predatory fish were deterred by nematocysts, with the fish showing a preference for consuming tissues in which nematocysts were either absent or had previously been discharged. As discussed by Mariscal [217], it is difficult to experimentally separate the effect of nematocysts from that of other potential defenses such as chemicals that may be present within cnidarian tissues. However, in both of these fish studies, experiments that were designed to address this issue (by testing palatability with and without nematocysts and each chemical compound) identified nematocysts as a major contributor to predation deterrence, with a greater relative effect than alternative chemical defenses. Additionally, in the nudibranch mollusc *Spurilla neapolitana*, Conklin & Mariscal [220] noted that nematocysts from cnidarian prey can potentially cause death of the nudibranch if the nematocyst concentration is sufficiently high. Although further experimental studies of this effect are needed, this observation suggests that nematocysts can have a potent effect against this cnidarian predator. Some cnidarian predators also have developed what

appear to be counter defenses to cnidarian nematocysts (e.g., [223,226]), implying that there is a need for such protection. Together, such studies provide evidence strongly suggesting that nematocysts can provide a defensive function to the cnidarians that synthesize these structures.

Nematocyst sequestration in four metazoan lineages

Although cnidarian nematocysts can serve as defensive structures, a diverse array of cnidarians are preyed on by species from a range of animal phyla [227,228]. Predators of cnidarians include Chordata (specifically fish, reptiles and birds), Chaetognatha, Arthropoda, Ctenophora, Acoelomorpha, Platyhelminthes and Mollusca. Focusing on the last four phyla (these being the focus of this paper, Figure 5.3), these four groups alone are known to feed on diverse cnidarians representing four of the five major cnidarian clades: ctenophores are known to feed on anthozoans, scyphozoans, and hydrozoans such as narcomedusans; platyhelminths and acoelomorphs are known to feed on scyphozoans and hydrozoans such as *Hydra*; and mollusks are known to feed on anthozoans such as soft corals, hard corals, anemones

Table 5.1. Taxonomic distribution of nematocyst sequestration within Metazoa.

Phylum	Order	# of Species	Inferred # of Origins
CTENOPHORA	Cydippida	3	1
ACOELOMORPHA	Acoela	1	1
PLATYHELMINTHES	Catenulida	1	
	Macrostomorpha	12	
	Proseriata	5	6-13
	Prolecithophora	4	
	Polycladida	9	
	Rhabdocoela	1	
MOLLUSCA	Nudibranchia	~600	1-2

and sea pens, hydrozoans such as hydroids and siphonophores, scyphozoans, and staurozoans (the stalked jellyfish) [227–229].

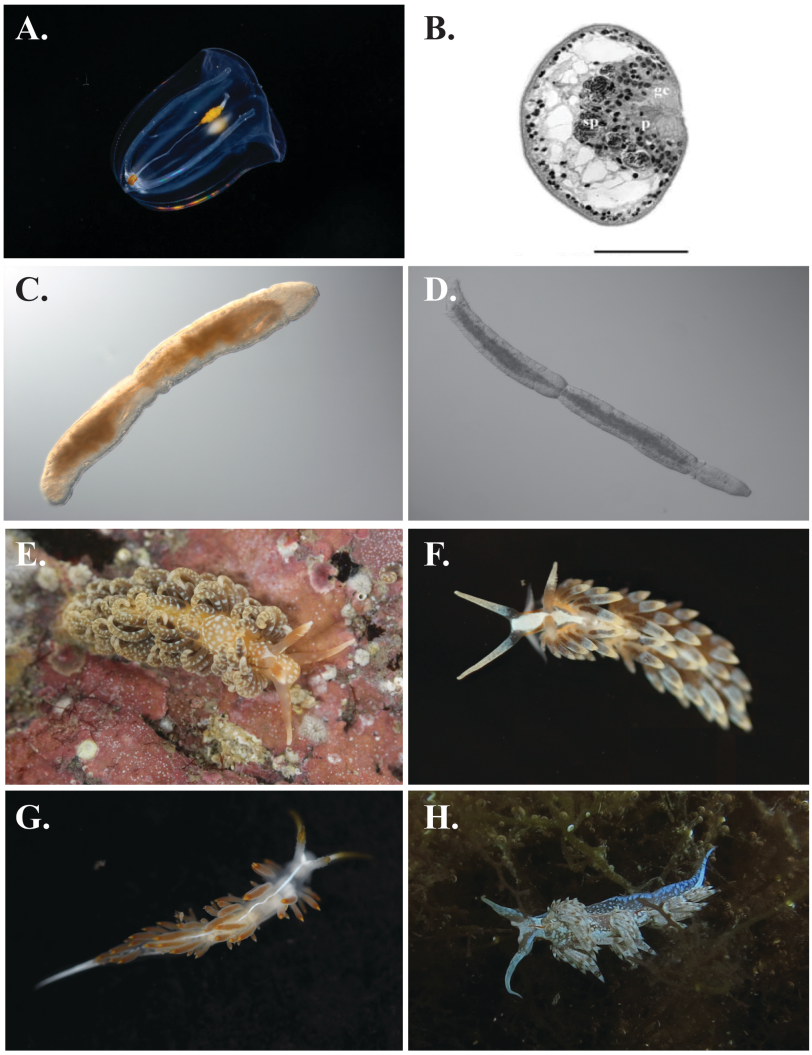

Figure 5.3. Photographs of species from the four metazoan phyla known or thought to sequester nematocysts. A) Ctenophora: *Haeckelia rubra.* B) Acoelomorpha: *Childia dubium* (in cross section; scale bar = 250 mm; gc, glandular complex; p, penis; sp, sperm), reproduced from Tekle (2006) with permission from the author. C-D) Platyhelminthes: *Microstomum* spp. E-H) Mollusca: *Spurilla braziliana* E), *Berghia stephanieae* F), *Flabellina trilineata* G) and *Dondice occidentalis* H). Photo credits: Steve Haddock (*H. rubra*), Yonas Tekle (*C. dubium*), Julian Smith III (*Microstomum* spp.) and Jessica Goodheart (*S. braziliana*, *B. stephanieae*, *F. trilineata* and *D. occidentalis*).

In addition to simply feeding on cnidarians, some Ctenophora, Acoelomorpha, Platyhelminthes and Mollusca encapsulate cnidarian nematocysts and sequester these structures within their own body tissues [30] (Table 5.1, Figure 5.4). These

sequestered nematocysts, often called “kleptocnidae”, are thought to provide a defensive function to the predator, thus representing exogenously produced defenses. Below, we review nematocyst sequestration in animals, focusing on the four phyla in which this phenomenon is known or thought to occur. For each phylum, we provide an overview of the group and its natural, endogenous defenses, review the phylogenetic distribution of sequestration, and, where data are available, review what is known about the mechanism of sequestration, the location of sequestered nematocysts in the predator’s tissues, and evidence for sequestered nematocysts providing a defensive function.

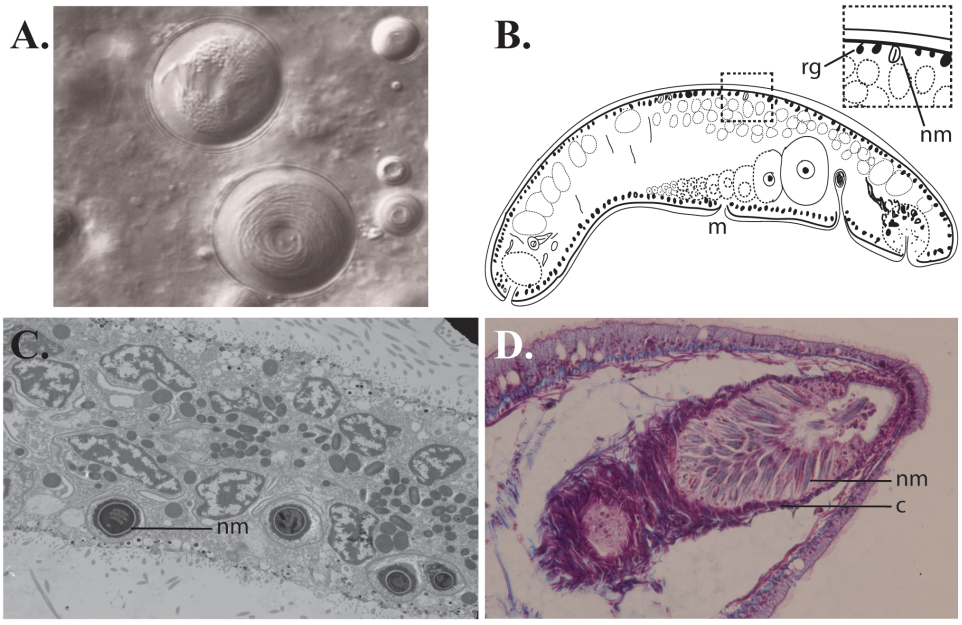

Figure 5.4. Nematocysts within the tissues of sequestering species. A) Ctenophora – *H. rubra* tentacle. B) Acoela – *C. dubium.* Illustration is adapted from Westblad (1942), anterior is to the left. C) Platyhelminthes – *Theama* sp. epithelium. D) Mollusca – *Aeolidia papillosa* cerata tip. Abbreviations are as follows: c, cnidosac; m, mouth; nm, nematocyst; rg, rhabdoid gland. Photo credits: Claudia Mills (*H. rubra*), Julian Smith III (*Theama sp.*) and Jessica Goodheart (*Aeolidia papillosa*).

Ctenophora

Ctenophores, also known as comb jellies, are mostly planktonic marine predators with gelatinous, transparent and relatively fragile bodies and, in some species, tentacles that are used for prey capture [232]. To protect themselves, ctenophores primarily employ defensive behaviors, such as intimidation or escape behaviors [233]. In addition, the nutritional quality of some ctenophores is known to be low [222], which may make them undesirable prey. Nematocyst sequestration has been suggested in only one genus in the phylum, *Haeckelia*. Nematocysts have been found in the tissues of three of the four species in this genus, *H. rubra* [234], *H. bimaculata* [235] and *H. beehleri* (S. H. D. Haddock, pers. comm.), while nematocyst storage in the fourth (*H. filigera*) has not been described.

Although the presence of nematocysts within *Haeckelia* has never been significantly questioned, the source of these nematocysts (specifically within *H. rubra*) was for a while debated in the literature. Some authors regarded the nematocysts as endogenous to the ctenophore, and cited this as a useful phylogenetic character supporting Coelenterata, a clade containing Cnidaria and Ctenophora [236–241]. Other authors, however, viewed nematocysts as exogenous structures, likely originating in cnidarians [242–244].

The exogenous origin of nematocysts in the ctenophore is now well established for this genus. Carré & Carré [245,246] were the first to provide solid evidence for this, describing in *H. rubra* the sequestration of nematocysts from the primary prey of this species, the hydrozoan narcomedusa *Aegina citrea.* The nematocysts were described from the ctenophore's tentacles, and the distribution of

nematocysts within tentacles was interpreted as indicating that nematocysts are integrated there by way of the tentacular canals, which are connected to the stomach where the prey would be located [246]. Furthermore, it has been shown that the eggs of *H. rubra* are surrounded by nematocysts, and that these structures are ingested by the larvae during development [247].

Within the tentacles, the nematocyst capsules are transported through the tissue of the ctenophore within endocytotic vesicles [235]. However, the nematocysts that reach the tentacles are no longer within a cnidocyte and are without a cnidocil according to Carré & Carré [245,247]; the lack of a cnidocil raises the question of how nematocyst discharge is triggered in ctenophores [216]. Sequestered nematocysts appear to be associated with a sensory cell of the ctenophore that might serve this purpose of controlling nematocyst firing [245]. Nematocysts incorporated in the tissues of *H. rubra* appear to be primarily a subset of the nematocysts present in the prey. Specifically, *H. rubra*'s prey, *A. citrea*, and other similar narcomedusans possess microisorhizas (small isorhizas with a mean diameter of ~4 μm) and macroisorhizas (large isorhizas with a mean diameter of ~8 μm) yet most sequestered nematocysts are microisorhizas [235]; this finding suggests that particular subtypes of nematocysts are selectively sequestered by *H. rubra*. As very few macroisorhizas appear to be sequestered, it is assumed that most of the larger nematocysts present in *A. citrea* are not sequestered but are instead digested or passed through the gut undigested, although there is no direct evidence for this.

Beyond the information described above, there is virtually no information regarding the process of sequestration in *Haekelia,* and no studies have attempted to

determine the actual function of the nematocysts in these ctenophores [248]. However, the finding that nematocysts are present solely in the tentacles of these ctenophores is consistent with the hypothesis that sequestered nematocysts are used for prey capture rather than defense.

Acoelomorpha

Acoelomorpha is a clade of soft-bodied, primarily marine worms comprised of two subgroups, Acoela and Nemertodermatida [8,249]. The most well-characterized defensive structures of acoelomorphs are sagittocysts, needle-shaped secretory structures that can be ejected from the epidermis [250], though rhabdoids (secretory inclusions which may release protective coatings) and mucous-producing frontal glands may also provide a defensive function within acoelomorphs [251]. A single species of Acoelomorpha, the acoel *Childia dubium* (Mecynostomidae) from the Mediterranean Sea, appears to possess the ability to sequester nematocysts from its cnidarian prey.

Westblad [231] originally identified structures within *C. dubium* as "cnidocytes," although he provided no details regarding the structures other than their location just below the epidermis. Additional information was provided by Karling [208], who identified them as nematocysts and indicated that the number of nematocysts sequestered within *C. dubium* is small (though he gave no actual numbers). Karling also identified undischarged nematocysts in the syncitial gut, within both the central and peripheral parenchyma; these nematocysts were not enclosed in any special cells or cysts, in contrast to where these organelles occur in cnidarians. Interestingly, no nematocysts have been described from the epidermal

epithelium [208,231], unlike nematocysts sequestered within ctenophores and platyhelminths.

Platyhelminthes

Platyhelminthes is a large phylum (roughly 20,000 species) of soft-bodied worms, often referred to as flatworms, that include both free-living and parasitic species [252]. Free-living platyhelminths are typically small aquatic worms and are known from nearly every body of water on the planet [253,254], while parasitic worms live on or within the tissues of a wide range of hosts. Platyhelminths have a relatively simple body organization, lacking a coelom, hemal system, and cuticle (among other traits). Among free-living platyhelminths, primary defensive strategies are based on exocytic organelles including both paracnids (refractive glands that sometimes also have an eversible tubule) and rhabdites (rod-like structures in the epidermis that release mucous or potentially repellant substances) [255–257].

Nematocyst sequestration has been described in multiple species from a variety of groups within Platyhelminthes, suggesting multiple origins of this feature within the phylum. The first mention of nematocysts in Platyhelminthes appears to have been by Lang [258], who described the presence of nematocysts and bundles of needle structures in the dorsal epidermis of *Anonymus virilis* (Polycladida). Several additional studies have identified nematocysts in other groups of Platyhelminthes [259–267] and a comprehensive overview was published by Karling [208]. In total, 33 species from 13 families are known to sequester nematocysts, and we estimate, based on the distribution of this trait, that nematocyst sequestration likely evolved

between 6 and 13 times within Platyhelminthes (Table 5.2, Figure 5.5). In all but the monotypic or very small genera, nematocyst sequestration is indicated in only some, but not all, of the species in the genus, suggesting that most if not all origins of this ability are relatively recent. Interestingly, one sequestering species, *Wahlia macrostylifera* (Rhabdocoela), is a commensal parasite within a holothurian host [265], representing the only known instance of nematocyst sequestration in a commensal organism.

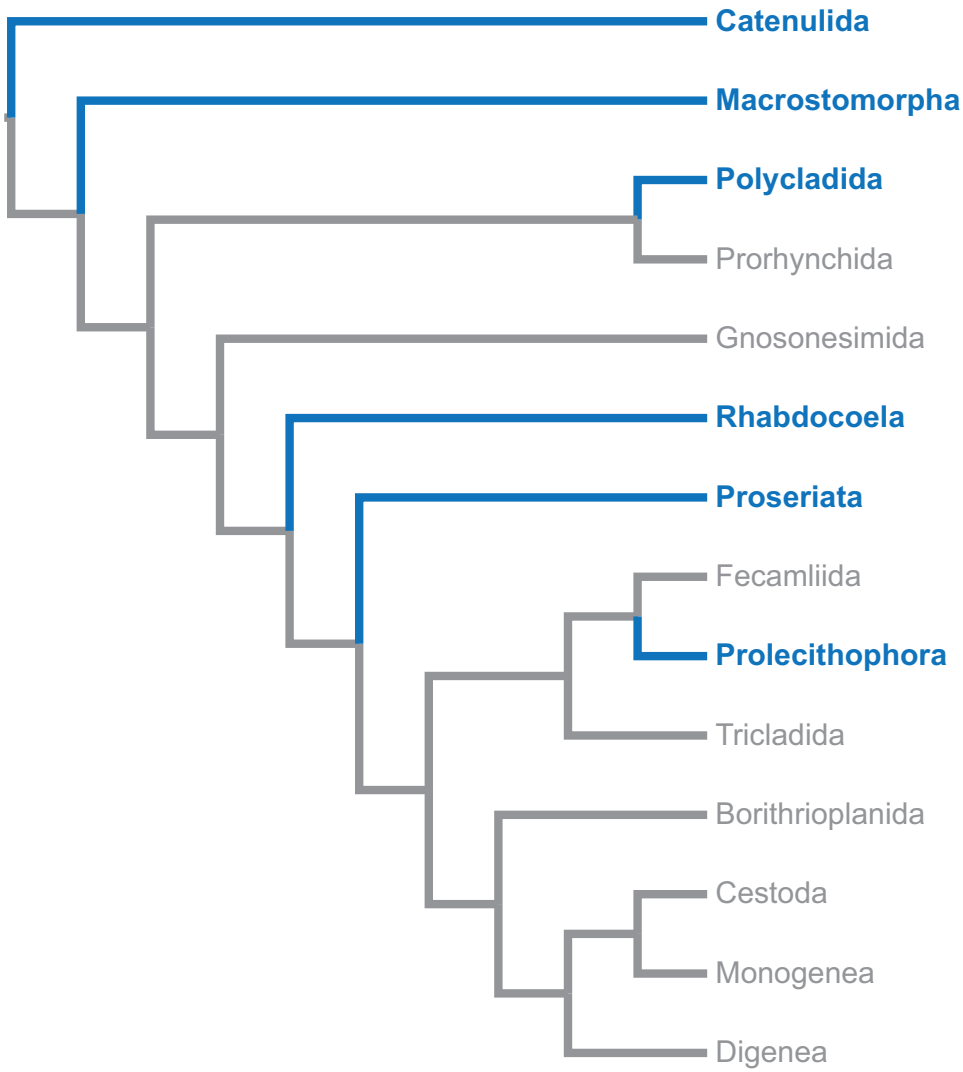

Figure 5.5. Phylogeny of Platyhelminthes indicating lineages that are known to sequester nematocysts. Taxa in which sequestering species are known are shown in blue. Relationships are based on Laumer et al. [254].

Table 5.2. Taxonomic distribution of nematocyst sequestration within Platyhelminthes. Asterisks denote genera in which all of the species are known to sequester nematocysts.

Order	Family	Genus	Species	Reference
Catenulida	Stenostomidae	*Stenostomum*	*sieboldi*	Martin (1914)
Macrostomorpha	Microstomidae	*Microstomum*	*lineare*	Martin (1914)
			papillosum	Martin (1908)
			rubromaculatum	Martin (1914)
			mundum	Karling (1966)
			mortenseni	Karling (1966)
			gabriellae	Karling (1966)
			jenseni	Karling (1966)
			ulum	Marcus & Marcus (1951)
			breviceps	Marcus & Marcus (1951)
			spiriferum	Karling (1966)
			hamatum	Karling (1966)
			caudatum	Kepner & Barker (1924)
Prolecithophora	Ulianiniidae	*Ulianinia*	*mollissima*	Martin (1914)
			westbladi	Karling (1966)
	Pseudostomidae	*Pseudostomum*	*klostermanni*	von Graff et al. (1908)
	Cylindrostomidae	*Cylindrostoma*	*monotrochum*	Martin (1914)
Proseriata	Archimonicelidae	*Archimonocelis*	*mediterranea*	Karling (1966)
			bathycola	Karling (1966)
			koinocystis	Karling (1966)
			semicircularis	Karling (1966)
			coronata	Karling (1966)
Polycladida	Stylochoplanidae	*Stylochoplana*	*tarda*	Martin (1914)
			inquilina	Poulter (1975)
	Anonymidae	*Anonymus**	*kaikourensis*	Holleman (1998)
			multivirilis	Holleman (1998)
			virilis	Karling (1966)
	Chromoplanidae	*Chromoplana*	*bella*	Karling (1966)
	Prosthiostomidae	*Amakusaplana*	*acroporae*	Rawlinson et al. (2011)
	Amyellidae	*Amyella**	*lineata*	Bock (1922)
	Theamatidae	*Theama*	sp.	J. P. S. Smith III, pers. comm.
Rhabdocoela	Umagillidae	*Wahlia*	*macrostylifera*	Snyder (1980)

The acquisition and storage of nematocysts shows some similarities across sequestering platyhelminths. First, nematocysts from the consumed cnidarian prey are taken up by the gastrodermal phagocytes, and some of these nematocysts remain both unfired and undigested. Nematocyst-bearing gastrodermal cells, which become known as "cyst cells" or "cnidophages", then move away from the gastrodermis, passing through the parenchyma. Finally, the undischarged nematocysts make their way into the epidermis and come to reside amongst the epidermal cells. Usually nematocysts are located above the basement membrane, but in some species, such as those in *Microstomum* and *Archimonocelis*, nematocysts can also be found sub-epidermally (beneath the basement membrane) and/or in the digestive epithelium [208].

In many cases, including in *Microstomum, Archimonocelis*, and *Anonymus,* the nematocysts in or near the epidermis are grouped into clusters (often with 2-3 nematocysts per cluster). In both *Archimonocelis* and at least two species of *Microstomum* (*M. hanatum* and *Microstomum* cf. *lineare*), the membrane around the bundle of nematocysts is surrounded by musculature [208], which could potentially be used to actively and rapidly expel the organelles. In the case of *Microstomum* cf. *lineare*, the musculature involves a single-celled muscle sheath basally and laterally and is associated with parenchymal muscle cells [271]. In each animal, there are typically multiple such muscular bundles of nematocysts, which Karling [208] refers to as cnidosacs.

Although nematocyst sequestration is well documented in several platyhelminths, nothing is known about how nematocyst-sequestering flatworms

protect themselves against the firing of nematocysts within the gut or about the ultimate function of sequestered nematocysts. Karling [208] suggested that mucus secretions may provide some protection from nematocysts and Bock [262] suspected that sequestered nematocysts help to protect the worms, but these hypotheses have not been tested. Some further discussion of nematocyst sequestration is available in Kepner [272] and Kepner & Barker [273], who also provide evidence, primarily anectodal, suggesting that sequestered nematocysts are used for both defense and prey capture in *Microstomum*. The behavior of sequestering species of *Microstomum* also suggests that nematocysts are in some way useful, as these species appear to only feed on *Hydra* when the supply of undischarged nematocysts in their own tissues is low [273]. Given that nematocyst sequestration appears to have evolved many times within this phylum (Figure 5.5), Platyhelminthes represent a particularly useful group to further investigate the evolution of this ability.

Mollusca

Mollusca is a highly diverse and species-rich group, with approximately 100,000 extant species described and including organisms with a wide diversity of body forms. This group comprises seven classes: Aplacophora, Polyplacophora, Monoplacophora, Cephalopoda, Scaphopoda, Bivalvia and Gastropoda [10,11]. Of these, Gastropoda (snails and slugs) is the most diverse group, making up approximately 80% of the species richness within the phylum [253].

Although gastropods are known for their coiled shell, which is retained in most species, multiple gastropod lineages have lost the shell [96]. One of these lineages is Nudibranchia, an order of shell-less gastropods known for their bright

coloration and charismatic patterns. Species within this clade use several types of defensive strategies, including the synthesis or uptake of biochemically active compounds in tissues [27,28], warning coloration to deter predators [29], cryptic coloration to avoid detection, and the use of nematocysts acquired from their cnidarian prey [30]. These defensive strategies are widespread throughout Nudibranchia, with one exception: nematocyst sequestration in this group is found only within some species of Cladobranchia, a group of nudibranchs known for their characteristically branched digestive glands [43]. More specifically, the sequestration of cnidarian nematocysts occurs primarily in one group of cladobranchs, Aeolidida, a group that appears to be monophyletic [50]. Additional species within the cladobranch genus *Hancockia* are also known to sequester nematocysts, but the position of this genus in the cladobranch tree is still uncertain. Thus, nematocyst sequestration appears to have evolved at least once, and possibly twice, within Mollusca.

Nematocyst sequestration in aeolid nudibranchs has been relatively well studied and is far better characterized than in the three phyla previously discussed here. A detailed review of nematocyst sequestration in aeolids has recently been published by Greenwood [30]. Below, we present a relatively brief overview of this phenomenon, referring the reader to this earlier review for more thorough coverage of the topic.

Aeolids feed on a variety of cnidarians, including corals, anemones and hydroids, and must protect themselves from nematocysts of their prey. Two forms of protection have been proposed to be present in aeolids. The first is a physical

protection: a chitinous cuticle covers the epithelium of the buccal cavity and the esophagus [226,274]. The second is a chemical protection: certain chemicals present in nudibranch mucus appear to prevent nematocysts from firing [223].

Once cnidarian tissue containing nematocysts is ingested by a nudibranch, some nematocysts (both discharged and undischarged) are excreted as waste. Other nematocysts are retained and passed through the branched digestive tract to diverticula of digestive glands located within dorsal body outgrowths, named cerata. These dorsal appendages, including the nematocysts within them, can be autotomized (released) if the animal perceives danger [275].

Once inside the digestive gland of a ceras, nematocysts are moved to a muscular sack, the cnidosac, located at the tip of each ceras, and are stored there until release or digestion [30,220]. During this process, nematocyst packaging and transport appear to occur in different ways in different species. In most aeolids, individual nematocysts are passed through the digestive gland and are encapsulated in cells called cnidophages as they are moved into the cnidosac [30,276]. In others, such as *Cratena peregrina*, nematocysts have been found within large vacuoles inside the digestive cells of the lining of the lumen of the digestive gland [277]. In *Hancockia*, nematocysts appear to be encapsulated in cnidophages within the lumen of the digestive gland and transported to the cnidosacs within the cnidophages [278]. In the aeolid *Spurilla neapolitana*, the release of nematocysts is triggered by non-motile, sensory cilia on the external surface of the ceras. Once the signal is received, nematocysts are extruded through an opening at the tip of the ceras called the

cnidopore. When the nematocysts reach the external environment, they are then fired from their capsule [220].

In some nudibranch species, immature nematocysts are sequestered from cnidarian prey and these nematocysts continue to mature within the nudibranch itself. In *S. neapolitana*, only immature nematocysts are sequestered, and it has been proposed that this strategy is less dangerous to the nudibranch because nematocysts being moved to the cerata would be less likely to fire, and thus less likely to cause damage, within the animal [279]. Recently, the mechanism of nematocyst maturation was investigated in *Berghia stephanieae* by Obermann et al. [280], who determined that an accumulation of protons, causing a decrease in pH, is involved in initiating maturation of sequestered nematocysts.

Although it is clear that aeolid nudibranchs retain and store nematocysts from their cnidarian prey, there is much less evidence for the use of sequestered nematocysts for defense. It is known that aeolids will release stored nematocysts when they are threatened, and that these nematocysts can sting and damage predators [274,281]. Another study found that several potential predators of nudibranchs, including several fish and a shrimp, fed more quickly on aeolids that had had their cerata removed than on those that were intact, suggesting that the cerata and their nematocysts may have some defensive value [282]. However, it has been difficult to distinguish between the defensive effects and relative contributions of nematocysts fired from the cerata from that of other defenses, such as chemical defenses, that are present in some species [283]. For this reason, the defensive function of sequestered nematocysts is still viewed as tentative by some authors [284,285], and it has even

been suggested that cnidosacs may simply be excretory organs rather than defensive ones [286]. It does appear, though, that the particular combination of nematocyst types within a species, referred to as the cnidome, can differ between the nudibranch and its prey. Specifically, the nudibranch cnidome often comprises only a subset of the nematocyst types present in a particular prey item [220,274,277,287], and the nudibranch cnidome can even change depending on the particular predator that is threatening the individual [288]. This suggests that there may be selectivity in nematocyst sequestration and that the selection of particular nematocysts could be a type of inducible defense. These findings lend further support to the hypothesis that nematocysts within nudibranchs have a defensive role.

In spite of the potential defensive benefits of sequestration ability, the evolutionary loss of nematocyst sequestration is suggested within at least one group of aeolids. Species in the genus *Phyllodesmium* have switched to feeding on octocorals, a group of corals known to have less potent nematocysts than other cnidarians. Species of *Phyllodesmium* do not appear to sequester nematocysts from their food as their cnidosacs are consistently devoid of nematocysts, suggesting they are no longer functional [67,289]. Thus, although nematocyst sequestration may be an important defense mechanism across most of Aeolidida, it is not present in all species in this group and appears to have been lost at least once.

The evolution of nematocyst sequestration in Metazoa

Similarities in nematocyst sequestration across disparate groups

Although nematocyst sequestration has evolved independently in the four groups discussed above, several similarities in the process are apparent across these different

groups, suggesting some convergent evolution. Specifically, there are similarities regarding the mode of transport of nematocysts, selectivity of particular nematocyst types in the sequestration process, and the storage of groups of nematocysts in larger muscular sacks.

First, in sequestering ctenophores, platyhelminths, and molluscs, nematocysts are transported from the gut to a storage destination, and sometimes are transported in similar ways. Specifically, in each of these groups, available data suggest that in some cases nematocysts are transported within cells often called cnidophages (in endocytotic vesicles) from tissues near the gut to their storage location, namely the tentacles (*Haeckelia*), epidermis (Platyhelminthes) and cerata (Nudibranchia) [220,235]. In nudibranchs, these cnidophages continue to house the nematocysts until they are ejected from the muscular storage sack (the cnidosacs) or digested [223].

Second, sequestering species from two groups appear to preferentially sequester certain nematocyst types over others from their prey. Nudibranchs in the genera *Spurilla, Aeolidia* and *Aeolidiella* appear to selectively retain the mastigophores from their anemone prey [220,274], and species in other nudibranch genera, such as *Catriona*, *Cuthona*, *Eubranchus*, *Godiva* and *Tergipes*, appear to preferentially sequester long isorhizas from their hydroid prey [274]. It is important to note, however, that these preferences might be dependent on the particular prey item. Research on the cnidome of the nudibranch *Flabellina verrucosa* indicates a preferential uptake of a particular type of mastigophore (microbasic mastigophores) when feeding on *Obelia*, but this nudibranch does not appear to selectively sequester particular types of nematocysts when feeding on *Tubularia* [287]. In ctenophores, *H.*

rubra appears to consistently sequester the smaller microisorhizas over macroisorhizas from their cnidarian prey [235], though no data exist on the other species within *Haeckelia*. The selectivity of sequestration of particular nematocyst types is strongly suggestive of sequestration having a defensive function, especially considering that in at least some species (e.g., *Flabellina verrucosa*), the cnidome of the sequestering species is sensitive to the type of predator [288]. Currently, it is not known how the cnidome of acoels and platyhelminths compares to that of their prey; such information would be particularly interesting to obtain so that it can be compared to that from the other groups.

Third, in both nudibranchs and some platyhelminths, sequestered nematocysts are stored in muscular sacks, referred to as cnidosacs [30,208,220,271]. Although these structures appear somewhat similar morphologically, in platyhelminths the precise function of cnidosacs, including the associated musculature, remains uncertain and should be studied further so that it can be better compared to that in nudibranchs.

Common features of sequestering taxa

Many groups are known to prey upon cnidarians, including other cnidarians, ctenophores, acoelomorphs, platyhelminths, mollusks, arthropods, chaetognaths, fish, reptiles, and birds [228]. Among these, only species of the four phyla discussed above (Ctenophora, Acoelomorpha, Platyhelminthes, and Mollusca) have evolved the ability to sequester nematocysts from their cnidarian prey. Below, we highlight several similarities among these four groups that we hypothesize may have contributed to the evolution of their ability to sequester nematocysts.

One obvious similarity between sequestering groups is that they are all soft-bodied, without any hard exteriors. Although this feature is common within Metazoa, the fact that sequestering organisms are soft-bodied implies that they have little in the way of external physical protection from predators. Soft-bodied species without a hard exterior often evolve alternative modes of protection, such as physical weapons (e.g., rhabdites, sagittocysts), chemical defenses, aposematism and crypsis [27–30,250,255]. This is particularly apparent in the Mollusca, where loss of the shell is quite tightly associated with gain of alternative defenses [193]. This general pattern suggests the hypothesis that nematocyst sequestration is favored in soft-bodied taxa via selection for defense from predators.

A second character shared by many nematocyst-sequestering species is a branched (i.e., diverticulated) digestive system. Such a feature is present in *Haeckelia* in Ctenophora [290], in Polycladida within Platyhelminthes [291], and in Cladobranchia within Mollusca [26]. In *Haeckelia*, the gut includes a system of multiply branched gastrovascular canals that connect to the tentacular canals, which extend into the tentacles; in polyclads, the gut consists of multiple branched tubes each extending toward the surface of the body [292]; and in Cladobranchia, the gut includes branches which extend dorsally from the main body into the cerata. The sequestering acoel *C. dubium* has a central mass of digestive tissue rather than a true gut cavity [293], but this digestive mass is typically in close proximity to the epidermis [251]. Thus, in the digestive systems of each of these groups, there is the possibility for contents of the gut to be passed relatively easily into tissues close to the body surface where nematocysts are held, such as the tentacles, epidermis, or

dorsal cerata. Although a branched gut occurs in many nematocyst-sequestering taxa, it is important to note that not all sequestering species possess this gut feature; for example many Platyhelminthes, including some species that sequester nematocysts, do not have a branched digestive system [251]. However, collectively, these observations lead to the hypothesis that a branched gut may facilitate nematocyst sequestration by providing a relatively easy way for gut contents, including nematocysts, to be distributed to other body regions.

A third feature that is shared across many sequestering taxa is the ability to regenerate. Regeneration of structures and/or tissues has been documented in all four phyla known to sequester nematocysts [294,295], and, more specifically, the particular structures in which sequestered nematocysts are stored are known to be able to regenerate in at least some species of these phyla. Many adult tentaculate ctenophores can regenerate tentacles and other tissues or structures that have been damaged or lost [296]; some acoels and many platyhelminths can regenerate every part of the body and also continually regrow the epidermis from stem cells [295,297,298]; and nudibranchs can regenerate cerata that have been autotomized [275,299]. The ability to regenerate tissues, and specifically tissues in which nematocysts are stored, could be of considerable advantage to nematocyst-sequestering organisms. This is because most sequestering species do not appear to be capable of controlling the firing of nematocysts, and thus predators will likely be affected by nematocysts only after attempting to eat tissue of sequestering species. The ability to replace these lost body parts would therefore be advantageous. Thus, another hypothesis to consider is that, because structures and tissues that sequester

nematocysts are vulnerable to predation, selection may favor both nematocyst sequestration and regeneration of these body regions.

Although many animal groups possess one or more of the features we highlight as being associated with sequestration (namely, a soft body, a diverticulated digestive system, and the ability to regenerate), to our knowledge no groups other than the four phyla discussed here both consume cnidarians and possess all three of these features. The only exceptions are cnidarians that prey on other cnidarians, yet these already possess nematocysts (or other types of cnidae) and thus would not be expected to derive a benefit from sequestering nematocysts from their prey. It is worth noting, however, that studies aimed at assessing whether all nematocysts within a cnidarian are made endogenously (by the species possessing them) are rare, and thus that detection of nematocyst sequestration in cnidarians could easily be overlooked.

Proposed steps in the evolution of sequestration

The evolution of nematocyst sequestration is expected to be a process involving multiple evolutionary steps, with intermediate steps each presenting some advantages. We propose a possible model for this process, hypothesizing plausible intermediate steps in the evolution of nematocyst sequestration (Figure 5.6). A necessary initial step is a species transitioning to feeding on cnidarian prey. As soon as a species transitions to feeding on cnidarians, the potential arises for protection by nematocysts; this is because whether nematocysts are actively sequestered or not, a predator of the cnidarian predator may still be harmed by ingesting tissue in which active nematocysts are present. Subsequently, likely steps would be the evolution of

chemical or physical mechanism(s) to protect the species from the nematocysts that are ingested during feeding and/or the ability to package ingested nematocysts. Evolved forms of chemical or physical protection could involve, for example, mechanisms to prevent nematocysts from firing or a cuticle barrier to protect the lining of the gut from nematocyst firing. Packaging of nematocysts into vesicles by cells in contact with gut contents could involve newly evolved cellular processes or could result from modifications of previously existing digestive processes, and, regardless of the mechanism, could provide strong protection from the nematocysts.

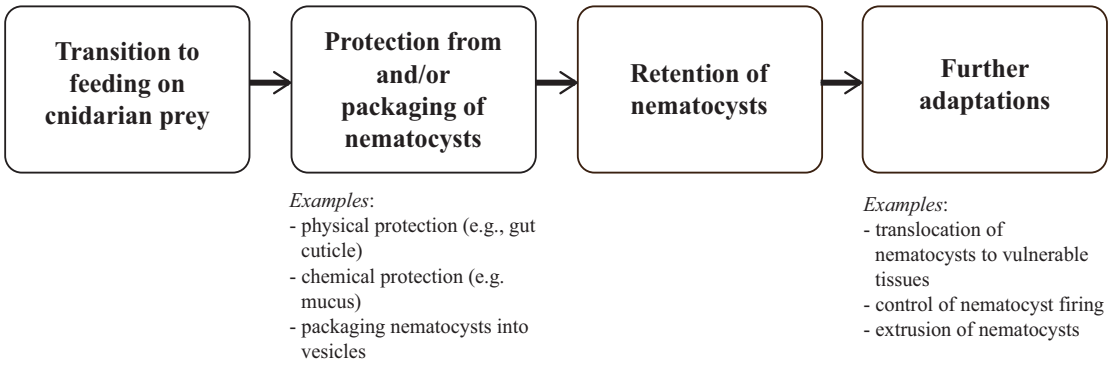

Figure 5.6. Hypothesized evolutionary steps leading to the ability to sequester nematocysts. The third step, retention of nematocysts, is viewed as the step that establishes nematocyst sequestration in a species. Sequestering species in all four phyla reviewed here have features of the first three steps; species of Aeolidida (Mollusca) and some sequestering species within Platyhelminthes additionally have features of the fourth step.

The retention of intact (undigested) nematocysts could then evolve, which would establish nematocyst sequestration in the species. Once retention of nematocysts has evolved, further adaptations associated with nematocyst sequestration could then be acquired, such as mechanisms to transport vesicles or cells containing nematocysts to body regions vulnerable to predator attacks, mechanisms to control nematocyst firing, mechanisms to actively excrete sequestered nematocysts even from undamaged tissues, and the evolution of specific structures for nematocyst storage. Evaluating

this model will require a combination of comparative analyses to evaluate the order of possible steps, as well as functional studies to evaluate the possible selective advantage of each step.

Conclusions and future directions

The ability to sequester nematocysts from cnidarians appears to be rare, and certainly is much less common than chemical sequestration, which is widespread across the Metazoa. However, nematocyst sequestration has clearly evolved multiple times and in divergent groups of animals, likely as a form of defense. Nematocysts have been described from four metazoan phyla (Ctenophora, Acoelomorpha, Platyhelminthes and Mollusca) and sequestration appears to have evolved 9–17 times. The literature on nematocyst sequestration is relatively small, but some similarities in the mechanism of sequestration among divergent groups are apparent, such as the transport of nematocysts within cnidophages from the gut to more distal locations of the animal. We have presented hypotheses regarding traits that may be associated with the evolution of this ability as well as proposed a model of possible steps leading to the evolution of nematocyst sequestration. We hope that by reviewing existing literature on nematocyst sequestration and by proposing these hypotheses and this model, this review will stimulate further research into the evolution of nematocyst sequestration.

In addition to testing the broad hypotheses and the general model we propose, future studies should focus on filling a number of important knowledge gaps regarding the evolutionary patterns and specific processes of nematocyst sequestration. In particular, important questions remain regarding the phylogenetic

distribution of predation on cnidarians, the phylogenetic distribution and mechanism for preferential sequestration of certain nematocyst types, the mechanisms of controlling nematocyst firing, and the possible function of sequestered nematocysts.

First, what is the phylogenetic distribution of predation on cnidarians within the four phyla in which nematocyst sequestration has evolved? Knowledge of feeding habits and preferences within these clades will allow evaluation of how prevalent sequestration is among groups feeding on cnidarians, and can elucidate the relative timing between a transition to feeding on cnidarians and the evolution of nematocyst sequestration.

Second, is there preferential sequestration of particular types of nematocysts? If so, in which species and by what mechanisms does this occur? Selectivity in sequestration could be an adaptive mechanism for effective defense in nature. Testing whether the nematocysts that are selectively sequestered are more effective against particular types of predators would provide further insight into the evolutionary function of sequestration.

Third, what are the molecular and cellular mechanisms involved in controlling nematocyst firing? Specifically, what mechanisms are involved in preventing the firing of nematocysts following ingestion and, in certain cases, controlling the later firing of sequestered nematocysts? Answers to these questions are needed to assess the diversity of sequestration and defense mechanisms used by sequestering animals. Even in non-sequestering species, mechanisms to protect the predator from ingested cnidae are known. For example, planktonic larvae of smooth fan lobsters encase jellyfish cnidae inside an additional membrane within their digestive tract, providing

protection from the cnidae [300]. Among sequestering taxa, some species encapsulate nematocysts within special cells (cnidophages), providing a possible mechanism for controlling nematocyst discharge. Some sequestering species are also known to strip the nematocyst capsules from the cnidocyte, in which cases the nematocysts become devoid of the cnidocil, the structure that typically affects nematocyst firing in cnidarians [301]. In such organisms, if nematocysts are used for defense, how do they control the discharge of nematocysts? Such questions will need to be investigated in other sequestering groups in order to understand the similarities and differences between the mechanisms for controlling nematocysts.

Finally, what is the ultimate function of sequestered nematocysts, and how does sequestration affect the ecology of sequestering species? Answering these questions is critical for evaluating the evolutionary advantages of nematocyst sequestration, yet such questions have received scant attention. Only a few studies have focused on these questions in Nudibranchia (e.g., [274,281]), and these studies have suggested a defensive function is possible, yet no further effort has been made to address the possible selective advantage of sequestration. Nematocysts are clearly powerful weapons for the cnidarians that produce them, and the use of nematocysts by non-cnidarian species suggests they can provide a selective value to such species, even in the absence of sequestration. For example, *Dardanus* hermit crabs which have *Calliactus* anemones living commensally on their shells have been shown to be protected from octopus predation [198] and the cephalopod *Tremoctopus violaceus* has been found to use *Physalia* tentacles as offensive weapons for prey capture [302]. These examples do not involve actual sequestration yet they demonstrate the potential

of nematocysts for both defensive and offensive functions in organisms that did not actually produce these structures. Elucidating the proximate and ultimate mechanisms leading to sequestration of nematocysts will thus broaden understanding of the many uses of these potent biotic weapons.

Acknowledgements

I am grateful to my fantastic co-author Alexa Bely. We thank Michael Cummings and Allen Collins for helpful comments on an early version of the manuscript and are particularly grateful to two anonymous reviewers who thoroughly and constructively critiqued this manuscript, helping us to improve it considerably.

Chapter 6: Insights into the systematics, phylogeny and evolution of Cladobranchia (Gastropoda: Heterobranchia.

Introduction

Cladobranchia is a diverse clade of gastropods within the order Nudibranchia, with ~1,000 coastal and off shore marine species. Cladobranchs have evolved remarkable biological features that are rare among animals, such as the development of multiple defensive strategies, including nematocyst sequestration [33], the sequestration of symbiotic zooxanthellae [34], and independent evolution of rhythmic motor behavior [35]. Many species within this clade are also commonly utilized in various types of neurological research, and a better understanding of the relationships among these taxa can better inform neurobiological studies [36–39]. In studying Cladobranchia, much can also be learned about hypothesized relationships between adaptations such as diet specialization and nematocyst or zooxanthellae sequestration, and rates of diversification in lineages with and without those traits. This allows for comparative analyses that can test whether diversification rate shifts that occur in Cladobranchia are present in other, non-mollusk, groups of nematocyst or zooxanthellae sequestering organisms as well.

It is clear that a reliable phylogeny for this group has the potential to provide important insight when addressing broader questions in other fields of science. However, well-supported phylogenetic hypotheses have been difficult to come by,

due simply to a lack of useful genetic data for this group. Although previous molecular hypotheses have mostly recovered a monophyletic Cladobranchia [26,43–45,49], very few relationships among taxa within this group have been resolved with strong support. Recently, however, the systematics of metazoans such as Cladobranchia has dramatically changed with the introduction of high-throughput sequencing technologies. Most of the attention has focused on relationships at the phylum level or above (e.g., [10–20]), likely due to the ability of newly available phylogenomic approaches to resolve the deeper relationships of many groups, of which our knowledge has historically been in flux, and the greater general interest of these relationships. With the cost of sequencing continuing to decrease, it is now easier to collect large data sets in an attempt to resolve relationships within more recently diverged groups, such as subphyla (e.g., [21]), classes (e.g., [22–24]) and others (e.g., [25]), including work on Cladobranchia specifically [50]. As more phylogenies are completed using these large genomic data sets, as with any other type of data, inevitably the systematics and taxonomy will need to be updated to reflect shifts in support for previous or new hypotheses.

Here, I review the systematics, phylogeny, and evolution of Cladobranchia, placing particular emphasis on the insights that have been provided from phylogenomic analyses. This provides the context necessary for us to better understand the origin of Cladobranchia and the original definition of this clade, which can give insight into results obtained using genomic data. In addition to questions of systematics, I address the potential for this phylogeny to address broader biological

questions in regards to character evolution of defensive strategies and motor function in Cladobranchia.

Morphological classification of Nudibranchia and Cladobranchia

The classification and taxonomy of groups within Nudibranchia has historically been complex (see Table 6.1 for a nomenclature guide). The classification scheme that first divided Nudibranchia into two major groups (Holohepatica and Cladohepatica) originated with Bergh [303–305] based on the branching pattern of the digestive gland. However, Odhner [51] suggested that this character was too variable to be of taxonomic use and instead focused on four major groups, where he reaffirmed the validity of the older groups (Doridacea, Aeolidacea and Dendronotacea), and described a new one (Arminacea). Thirty-six years later, Minichev [306] removed Anthobranchia (dorids) from Nudibranchia and considered the two clades to be separate orders, which then left the three suborders Dendronotacea, Arminacea and Aeolidacea within Nudibranchia. Minichev [306] separated Anthobranchia from the rest of Nudibranchia based on multiple morphological characters, including the presence of the mantle organs on the dorsal side of the body, the asymmetry of the internal organs, the structure of the circulatory system, and the presence of a triaulic reproductive system, defined by three reproductive openings (as opposed to diaulic, which has two). Beyond just these distinctions, however, Minichev [306] decided that these groups were not sister taxa, and suggested that Anthobranchia was actually more closely related to Cephalaspidea (headshield slugs and bubble snails), whereas the three groups within Nudibranchia were likely most closely related to Pleurobranchidae (side-gilled sea slugs). Although few authors studying the

morphology of Nudibranchia agreed with the hypothesis that Anthobranchia and Nudibranchia were separate orders, they appear to have concurred that Anthobranchia is a separate entity from the other three groups within Nudibranchia [26,306,307].

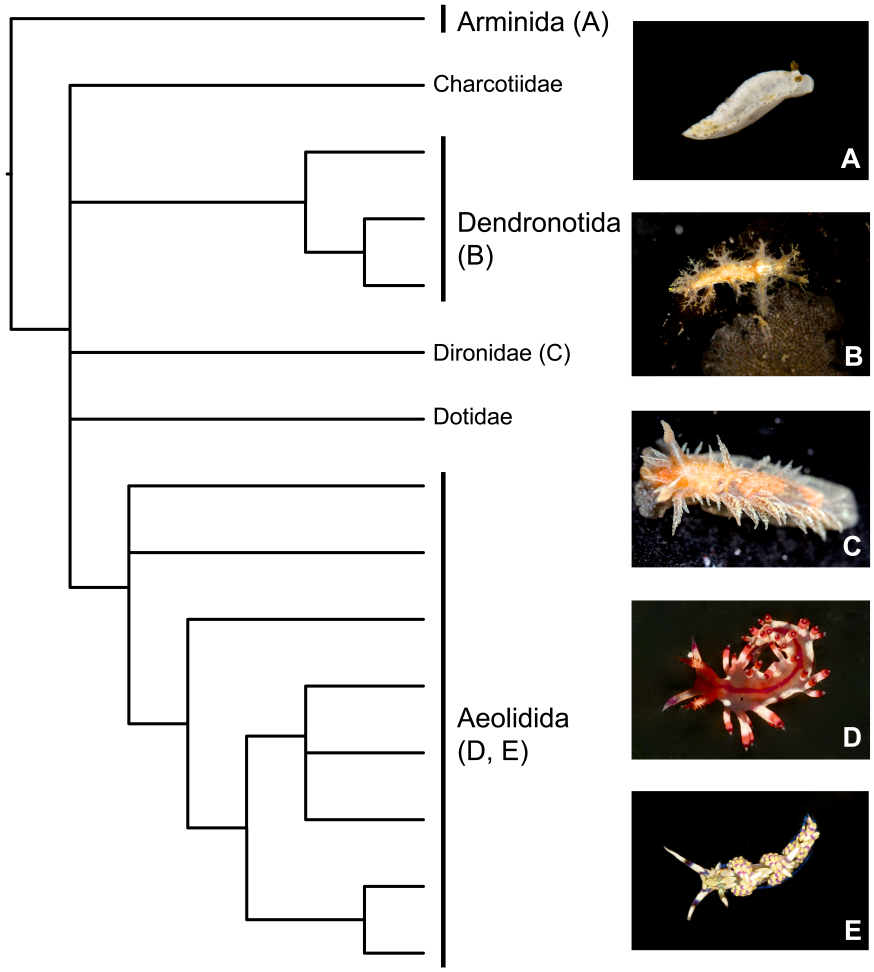

Figure 6.1. Phylogeny of Cladobranchia based on the most recent morphological analysis of Nudibranchia by Wägele and Willan (2000), and photographs of A) *Dermatobranchus albus* (Arminida), B) *Dendronotus venustus* (Dendronotida) [50]**, C) *Dirona picta* (Dironidae)** [50]**, and D) *Flabellina rubrolineata* and E) *Godiva* sp. (Aeolidida).**

Following this separation of Anthobranchia from the rest of Nudibranchia, Willan & Morton [307] introduced the name Cladobranchia for the group that contained Dendronotacea, Arminacea and Aeolidacea. Notably, Willan & Morton [307] actually appear to give Odhner credit for establishing Cladobranchia, but a

personal communication with Willan [308] specifies that this attribution to Odhner was an error. The authors do not provide a discussion regarding the renaming of this clade, but Willan & Morton [307] do contribute the first formal definition for Cladobranchia, stating that the anus is on the right side of the body and the gills are formed by various kinds of "outgrowth" from the body.

Table 6.1. Current classification of Nudibranchia, along with previously used classifications and names for each group.

Current clade	Bergh (1890), (1902), (1906)	Odhner (1934)	Minichev (1970)	Willan and Morton (1984) Wägele and Willan (2000)	Valdés and Bouchet (2005)
Anthobranchia	Holohepatica	Doridacea	–	Anthobranchia	Euctenidiacea
Cladobranchia	Cladohepatica	–	–	Cladobranchia	Cladobranchia
Arminida		Arminacea	Arminacea	Arminoidea	Euarminida
Dendronotida		Dendronotacea	Dendronotacea	Dendronotoidea	Dendronotida
Aeolidida		Aeolidacea	Aeolidacea	Aeolidoidea	Aeolidida

The most recent morphological analysis of nudibranchs maintains the two primary divisions of Nudibranchia (Anthobranchia and Cladobranchia), and three main groups within Cladobranchia (Aeolididoidea, Dendronotoidea and Arminoidea)(see [26]; Figure 6.1; Table 6.1). The authors describe Cladobranchia as characterized by branched digestive glands and the loss of the primary gills (ctenidia), though they specifically state that neither of these characters is actually unique to Cladobranchia. This remains the most descriptive definition of Cladobranchia thus far. The most recent classification for Cladobranchia based on previous morphological work includes Arminida [=Euarminida] (Arminidae and Doridimorphidae), Dendronotida (Tritoniidae, Aranucidae, Bornellidae, Dendronotidae, Hancockiidae, Lomanotidae, Phylliroidae, Scyllaeidae and Tethyidae) and Aeolidida (Flabellinidae, Notaeolidiidae, Fionidae, Calmidae, Eubranchidae,

Pseudovermidae, Tergipedidae, Aeolidiidae, Facelinidae, Glaucidae and Piseinotecidae) as monophyletic groups [308], along with some taxa the authors consider unassigned to any of these three groups, including Charcotiidae, Dironidae, Dotidae, Embletoniidae, Goniaeolididae, Heroidae and Madrellidae.

Molecular phylogenies of Cladobranchia

Single- and few-gene based methods

Multiple studies using various molecular data sets (one to five genes, both mitochondrial and nuclear genes) and sample sizes have found phylogenetic support for the monophyly of Cladobranchia [43–45,47–50], but there are few data that either support or reject the traditional classification of the three major taxa, Aeolidida, Dendronotida and Arminida, making it difficult to understand the deeper evolutionary history among and within these groups. The earliest of these phylogenies included only a small number of species within Cladobranchia (Thollesson [47] – six; Wollscheid-Lengeling [44] – fourteen), and Thollesson [47] was unable to resolve the monophyly of any of the three groups. In two more recent molecular analyses, however, the monophyly of Aeolidida and Arminida are well supported [43,49] and Arminida is well supported in a third [45]. Conversely, there is no support for Dendronotida as a monophyletic group in any molecular phylogeny thus far.

In regards to the position of particular subclades within Cladobranchia, there is little information in the literature. In most cases the position of Aeolidida is unresolved [43,45,49], and in light of the fact that support for the monophyly of Arminida and Dendronotida have consistently been low and nonexistent, respectively,

the placement of each of these two groups within Cladobranchia is even more uncertain.

In many cases, family and genus-level relationships, and the assignment of taxa to Cladobranchia, have been resolved even though deeper relationships within the Cladobranchia phylogeny have remained elusive. The enigmatic family Doridoxidae is a prime example, as it was recently supported as a member of Cladobranchia, though it is still unassigned to any particular clade [49]. This is likely because a number of studies published on the evolutionary history of taxa within Cladobranchia have focused on phylogenetic relationships within specific subclades. At the family level, these subclades include Aeolidiidae [67], Arminidae [95,104], Bornellidae [105], Dotidae [309], Glaucidae [106], Scyllaeidae [66] and Tritoniidae [68,310], and at the genus level, these subclades include *Antaeolidiella* [107], *Babakina* [69,108], *Berghia* [109], *Burnaia* [110]*, Dendronotus* [111–113], *Janolus* [311], *Limenandra* [114], *Melibe* [115], *Phyllodesmium* [116,117] and *Spurilla* [118]. These studies vary slightly in gene selection, with most favoring the most commonly used genes in nudibranch phylogenetics, which is primarily the genes that code for Cytochrome c oxidase subunit I (COI), Histone 3 (H3) and 16S ribosomal RNA (16S). Although levels of support also vary, the majority of these studies found that the subclades assessed (originally defined using morphology) are monophyletic.

High-throughput sequencing based methods

A phylogenomic study was recently published for Cladobranchia (17 ingroup taxa and 839 orthologous groups), which was the first to provide a well-supported and well-resolved phylogenetic hypothesis for this clade [50] (Figure 6.2). In this study,

Cladobranchia was strongly supported as monophyletic, and strongly supported hypotheses were provided in answer to some questions regarding the deeper relationships Cladobranchia. The analyses presented in this phylogenomic study supported the hypothesis that Aeolidida is monophyletic, but yielded a paraphyletic Dendronotida [50]. The position and monophyly of Arminida was not assessed in these analyses, so inclusion of taxa within this clade will be critical to include in future analyses. Importantly, the genus *Melibe* (which has several deletions in the COI gene, making it difficult to place; [43]) was resolved well within Cladobranchia, closely related to Dotidae and Dendronotidae, with a branch length and placement that fit better with morphological expectations than in previous analyses [50].

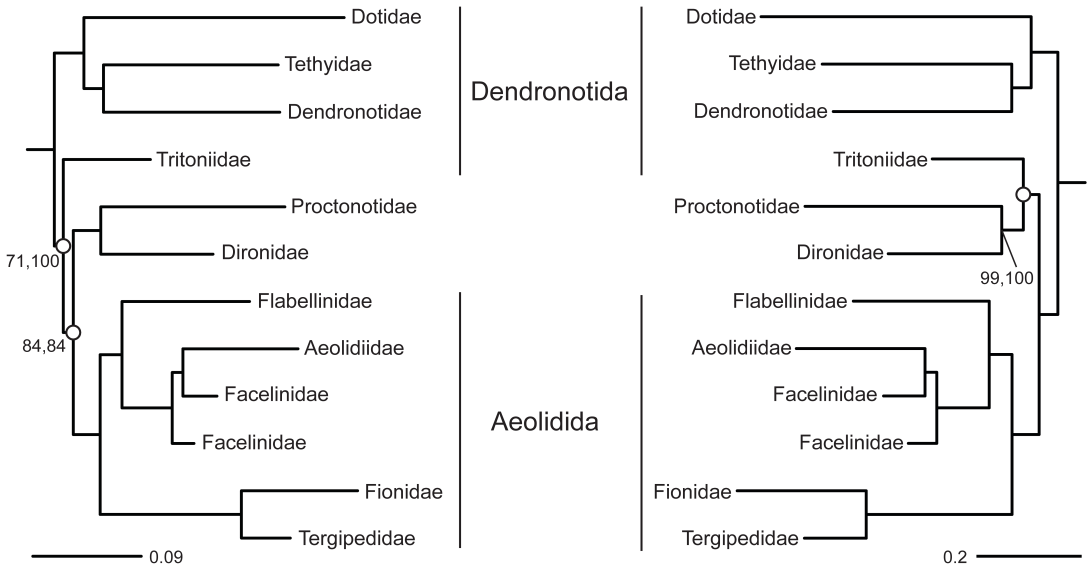

Figure 6.2. The two primary topologies for Cladobranchia based on analyses using 839 orthologous groups by Gooheart et al. [50]. Bootstrap values for each node are 100% unless otherwise noted. The tree on the left was supported by two analyses – *degen* (703,665 nt positions, 46.4% complete, degeneration coding performed), first bootstrap value and *nt12_partitioned* (1,702,782 nt positions, 23% complete, only first and second positions considered, partitioned by codon position), second bootstrap value, as was the tree on the right – *nt123* (703,665 nt positions, 46.4% complete, positions not present in at least 4 taxa removed), first bootstrap value and *nt123_partitioned* (1,702,782 nt positions, 23% complete, partitioned by codon position), second bootstrap value. Open circles indicate the nodes at which the two topologies differ.

Regarding relationships among major subclades within Cladobranchia, two separate topologies were supported in the analyses using genomic data [50]. In the first, Aeolidida was supported as sister to a clade containing Proctonotidae and Dironidae. This clade (Aeolidida + Proctonotidae + Dironidae), in turn, was supported as sister to Tritoniidae. In the second, Aeolidida is sister to a clade containing Proctonotidae, Dironidae and Tritoniidae. The second topology was more strongly supported. The final subclade in both topologies is sister to all other taxa within Cladobranchia, and includes other taxa previously assigned to Dendronotida (Dotidae, Tethyidae and Dendronotidae).

In addition to providing a strongly supported hypothesis for higher-level relationships of Cladobranchia, genomic data were also useful in resolving and confirming some of the family- and genus-level relationships. Included is strong support for the close relationship between the families Fionidae and Tergipedidae, and a decided lack of support for the monophyly of the family Facelinidae, among others [50], which are weakly indicated by one previous study [67]. Due to the effective use of genomic data to provide strongly supported phylogenetic hypotheses, we can now better address necessary systematic changes and assess character evolution through detailed comparative analyses within Cladobranchia.

Implications of recent molecular phylogenetics studies

Systematics and Taxonomy of Cladobranchia

The new phylogenetic hypotheses supported by molecular data provide the opportunity to address potential systematic changes in Cladobranchia. Although it is clear from these analyses that Aeolidida is strongly supported as a monophyletic

group and should likely be retained pending formal tests for monophyly, our understanding of relationships and group designations within Cladobranchia will likely shift through the coming years.

First, Dendronotida is strongly indicated to be non-monophyletic in phylogenomic analyses [50]. There is strong and consistent bootstrap support for two separate clades of species assigned to Dendronotida: one that contains taxa such as Dotidae, Dendronotidae and Tethyidae, and another that appears to only include Tritoniidae. This topology renders Dendronotida paraphyletic, though formal tests for monophyly remain to be done. Assuming this result is supported by monophyly tests, it is unclear whether either of these clades should retain the name Dendronotida. Taxa that fall under this group have consistently been characterized by rhinophoral sheaths [26], but this appears to potentially be a plesiomorphic (or convergent) character. Since this is the case, either a new definition for Dendronotida would be necessary, or a new name for each of the two clades would need to be designated. In either case, morphological synapomorphies for the new groupings would need to be identified.

Second, some unassigned taxa such as Proctonotidae, Dotidae and Dironidae have been placed within highly supported monophyletic clades with other cladobranch taxa. Two of these taxa form one clade, the Proctonotidae + Dironidae assemblage, which is a novel result of the phylogenomics study [50], but the position of this clade is still somewhat uncertain, as it may or may not be closely related to Tritoniidae. Additionally, previous molecular phylogenies found strong support for Dironidae as sister to Charcotiidae [45,49], and it seems likely that Charcotiidae will also turn out to be a member of this clade. The uncertainty regarding the position of

this Proctonotidae + Dironidae clade renders a decision as to whether or not it should be named unclear, but future genomic work may provide a more stable hypothesis for its position and make this decision simpler. Regardless, morphological analyses need to be completed in order to determine what synapomorphies define this clade. The third family previously considered unassigned is Dotidae, which in all phylogenomic analyses in Goodheart et al. [50] is strongly supported as sister to Dendronotidae + Tethyidae, taxa assigned to Dendronotida.

Finally, Arminida (Arminidae and Doridimorphidae) is missing from current phylogenomic analyses, and will need to be included in future analyses to better address deep relationships within Cladobranchia. Arminida is supported as monophyletic in multiple molecular analyses [43,44,49], and thus genomic data may not lead to further changes within these taxa. However, the position of Arminida is still not clear, and phylogenomic analyses will likely provide a stronger hypothesis for the phylogenetic placement of this clade within Cladobranchia.

Character evolution within Cladobranchia

As stated above, cladobranchs have evolved novel biological adaptations, including nematocyst sequestration [33] and the independent evolution of rhythmic motor behavior [35], that have yet to be studied in the context of a well-resolved phylogeny. The well-supported phylogenetic topologies provided by genomic data will likely allow for the study of these characters in a new light.

The first of these two, nematocyst sequestration, is the ability that some cladobranchs possess to sequester the stinging organelles (nematocysts) from their

cnidarian prey [30,61]. The vast majority of cladobranchs feed on species of Cnidaria, primarily anthozoans (e.g., anemones, octocorals) and hydrozoans (hydroids) [33,159,162,312], but only those within the clade Aeolidida possess this feature, with one exception: members of the genus *Hancockia* (Dendronotida) also sequester nematocysts [33]. Due to the diversity within Aeolidida (~600 species) as compared to Arminida (~150 species) and Dendronotida (~250 species), it has been hypothesized that nematocyst sequestration may have led to a burst of diversification at the base of the Aeolidida tree [33,57,274] (Figure 6.3). The more resolved phylogenetic framework provided by genomic data will likely allow for a better hypothesis regarding the number of origins of this process (via the placement of *Hancockia*) and ancestral state reconstruction of the characters associated with nematocyst sequestration, which can provide evidence for the steps involved in the evolution of this ability in nudibranchs [61]. In addition, diversification analyses can be run to test the hypothesis that nematocyst sequestration has led to an increase in diversification within this group. One caveat with diversification analyses, however, is the low power provided when evolutionary replication for a trait is limited [313]. As such, it will be critical to test for an increase in diversification not just in nudibranchs that sequester nematocysts, but in other clades that have evolved this ability such as ctenophores, flatworms, and acoels as well.

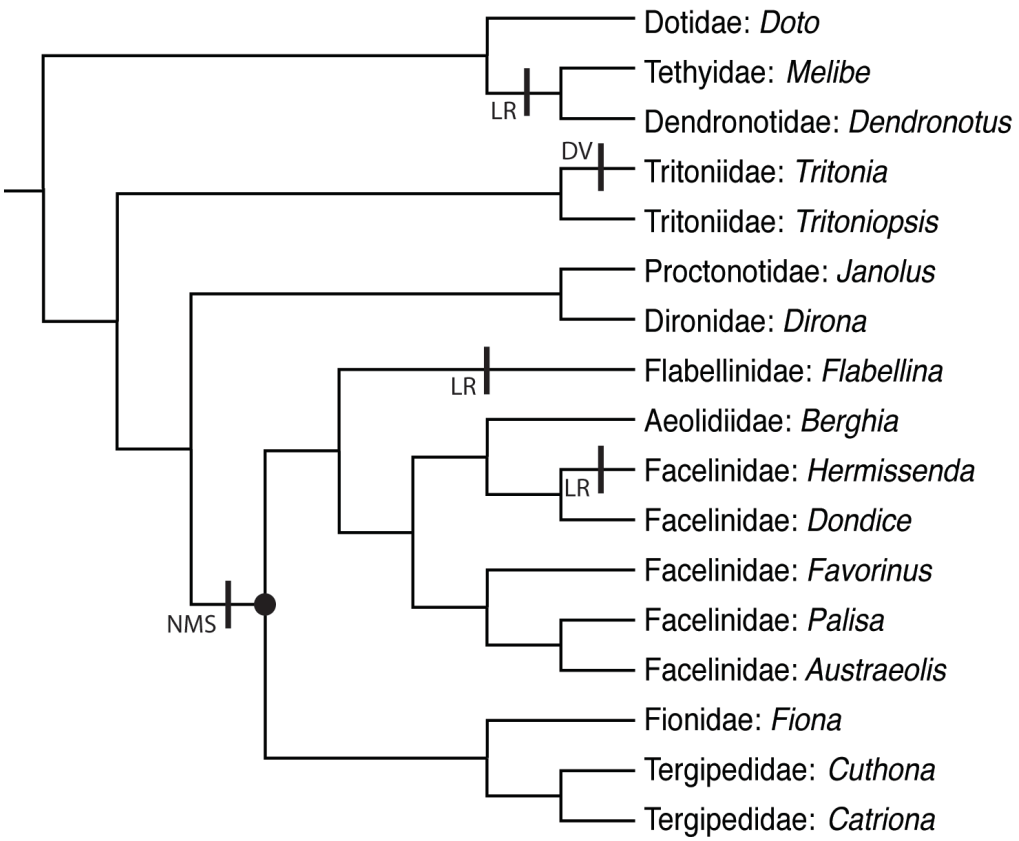

Figure 6.3. Inferred origins of multiple characters based on the current phylogenetic hypothesis for Cladobranchia. The closed black circle indicates the base of Aeolidida. Abbreviations: NMS indicates an origin of nematocyst sequestration, DV indicates an origin of dorsal-ventral swimming flexions, and LR indicates an origin of left-right swimming flexions.

A second character of interest is the independent evolution of rhythmic motor behavior within this group [35]. According to Lillvis and Katz [35], the use of dorsal-ventral flexions for swimming is present in 17 taxa within Nudipleura, one of which (*Tritonia* Cuvier, 1798) is a group within Cladobranchia. It has been estimated that this ability evolved between two to five (and potentially more) times within Nudipleura [37], but the lack of a strong phylogenetic hypothesis for this group has made evolutionary analyses problematic. More interesting still, the use of rhythmic left-right flexions for swimming is also present within Cladobranchia (found within at least *Melibe*, *Dendronotus, Hermissenda* Bergh, 1879, and *Flabellina* Gray, 1833)

[37,314]. Thus far, the genomic framework suggests that left-right flexions evolved at least three times within Cladobranchia (Figure 6.3). This robust phylogeny also suggests closely related taxa to these other groups that may also be good for testing hypotheses related to swimming behavior.

Conclusions

Morphological data and analyses have provided well-reasoned hypotheses for Cladobranchia and its subgroups, such as the presence of three major subclades, that we have been able to test using molecular data. The use of these molecular data for resolving the phylogeny of nudibranchs has great potential, and has already proved useful in improving our understanding of relationships within Cladobranchia. At least one of the larger clades in Cladobranchia supported by morphological analyses (Aeolidida) is also supported in genomic analyses (and some smaller molecular studies), and the same studies have demonstrated that Dendronotida is likely a non-monophyletic group. In addition, strongly supported phylogenetic hypotheses provide a better framework for studying character evolution within Cladobranchia, including characters like nematocyst sequestration and rhythmic motor behavior.

Although these data have proved particularly useful in many ways, there are some considerations for the future. First, a broader taxon sampling will provide a more thorough look at the evolution of cladobranchs. The inclusion of members of Arminida is especially critical, as genomic data have not been used to assess the monophyly or position of this group. Second, the inclusion of morphological analyses and taxonomic revisions will provide a stronger classification of Cladobranchia that will be incredibly important for future work. It would be better served, however, to

undertake these analyses after a broader sampling of taxa is present in the phylogeny. This will provide stronger classification hypotheses that will likely hold up better over time, and the presence of type taxa within an analysis can be useful when dealing with questions of nomenclatural precedence.

The systematics of most metazoan clades has shifted with the introduction of high-throughput sequencing technologies, and taxa within Mollusca are no exception. It is clear that genomic data have been beneficial when it comes to resolving phylogenetic trees of Cladobranchia with strong support, as is true in other mollusc and metazoan groups. However, a combination of genomic (and genetic) data, strong morphological analyses and careful taxonomy will be critical for the future of systematics and classification of Cladobranchia and other metazoan taxa.

Acknowledgements

I would like to thank the American Malacological Society for funding to travel to the 49th Western Society of Malacologists and 82nd American Malacological Society Annual Meetings held in Ensenada, Mexico, and I am grateful to Kevin Kocot for the invitation to participate in the symposium. I would also like to express my gratitude to Michael Cummings, Allen Collins, Alexandra Bely, Ángel Valdés, and an anonymous reviewer for their suggestions and helpful comments, which improved the quality of this paper. This work was funded through a Peter Buck Fellowship from the Smithsonian Institution.

Chapter 7: Prey preference follows phylogeny: evolutionary dietary patterns within the marine gastropod group Cladobranchia (Gastropoda: Heterobranchia: Nudibranchia)

Introduction

Predator-prey interactions are among the most fundamental processes in ecology and constitute the fabric of community structure and ecosystem function [315,316]. However, the role of those interactions in evolution, and their impacts on biodiversity, is less well understood in marine systems [317,318]. The most widely accepted hypothesis to explain the origin of biological diversity traces its origins to Mayr [319,320], who proposed that the ranges of organisms are fragmented by the formation of physical barriers, resulting in isolation and divergence in allopatry. However, in the marine realm, where barriers to genetic exchange are less obvious than in terrestrial or freshwater systems [54], non-allopatric divergence and speciation may play a fundamental role in the generation of biodiversity (e.g., [55,56]). In this context, shifts between major prey types (e.g., different cnidarian classes) could constitute important factors explaining the biodiversity of marine taxa, particularly in groups with highly specialized diets. The greatest obstacle to testing these ideas is the lack of well-supported phylogenies for groups of specialized consumers.

In this study, we generate RNA-Seq data to test the role of prey preference shifting in the evolution of Cladobranchia (Mollusca: Gastropoda: Hererobranchia:

Nudibranchia), a group of marine invertebrates with at least 1000 species [32]. Cladobranch sea slugs occupy various marine environments, from coastal reefs, where diversity is highest, to the deep sea, as well as highly specialized pelagic and neustonic niches [119,321–323]. Species of Cladobranchia are exclusively carnivorous, and exhibit diverse dietary specializations, preying on a variety of animal taxa, including bryozoans and crustaceans, eggs of fishes and molluscs, and cnidarians (Figure 7.1) [57,58,312]. However, the vast majority of cladobranchs prey on species of the two most diverse clades within Cnidaria, Anthozoa (e.g., anemones, stony corals, and octocorals) and Hydrozoa (hydroids, siphonophores, and hydromedusae) [33,159,162,312]. This preference for cnidarian prey is hypothesized to have facilitated the evolution of the ability to sequester cnidarian nematocysts in Cladobranchia [61], which is believed to have evolved only once within this group [57].

Based on recent classifications, two of the three main groups in Cladobranchia (Aeolidida and Dendronotida [26]; the third being Arminida, as defined in [43]) contain taxa that prey on animals distributed across the list given above. These classifications suggest that shifts in prey type preference are relatively common throughout Cladobranchia over evolutionary timescales. There also exist many well-documented cases where cladobranch species are tightly associated with specific prey types or species (e.g., [158,324–328]). In many of these cases the prey species might even be considered a host as defined by Coyne & Orr [329], due to similarities that many sea slugs share with herbivorous insects, including their small size relative to

their hosts and the use of hosts for both food and shelter [317].

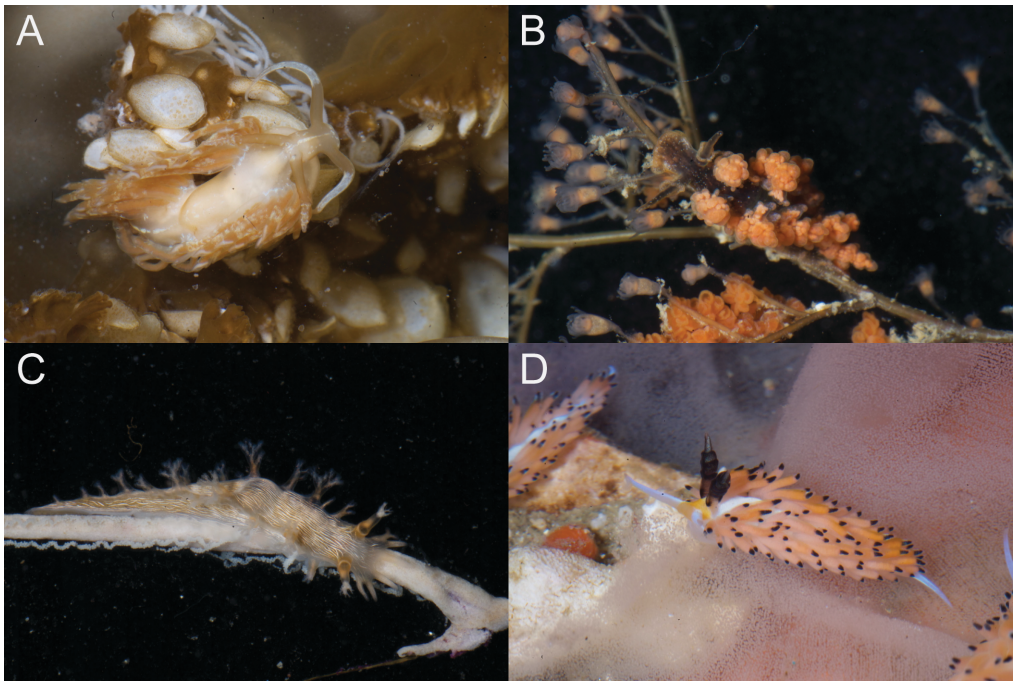

Figure 7.1. Select photographs of cladobranch taxa on their food source, including: A) *Dondice parguerensis* on the scyphozoan jellyfish *Cassiopea* sp., B) *Doto chica* on the hydroid *Eudendrium* sp.; C) *Tritonia hamnerorum* on the octocoral *Gorgonia ventalina*; and D) *Favorinus tsuruganus* on an opisthobranch egg mass (Photo credits: Ángel Valdés).

Two main hypotheses have emerged regarding the roles that dietary specialization and prey shifts have played in the evolution of heterobranch sea slugs (formerly called opisthobranchs). The first of these hypotheses suggests that increased speciation occurs due to species-specific prey switching in groups where specialization is prevalent [60]. This leads to clades consisting of many taxa that specialize on individual prey species. In many metazoan groups studied, mainly involving terrestrial symbiotic and parasitic systems [330–332], host shifting (shifting between prey species) has been implicated as a driver of diversification, with colonization of new hosts often leading to bursts of cladogenesis. Speciation of taxa by host or prey shifting may also be important in a handful of specialized marine consumers such as some bivalves [333], amphipods [334], barnacles [335], gobies

[336], and gastropods [324]. We do not assess this hypothesis here, as it requires broad taxon sampling across Cladobranchia and solid evidence of dietary specialization, both of which are lacking in many cases.

The second hypothesis relating to dietary specialization is that major radiations within heterobranch sea slugs may be related to the evolution of particular morphological structures necessary for feeding on different types of prey [57–59], such as the distinct radular morphology present in members of Aeolidida. This hypothesis suggests that shifting to new prey items leads to an increase in niche availability, similar to the effect of habitat preference shifts in some groups [337]. This hypothesis is broader than the first, in that it refers to switches between prey types at higher levels of organization. The consequences of this type of switching relate to species that, following a switch, are able to prey on multiple taxa within a general prey type, rather than explicitly focusing on those that specialize on certain prey species. This pattern has been found in only a few taxa [333,338,339]. Diversification in this context relates more to the expansion of possible prey types rather than specialization.

Given the variety of prey type preferences exhibited by its members, Cladobranchia constitutes an excellent system to explore the relationship between prey shifting and cladogenesis. Until now, it has not been possible to test how prey choice has evolved through the history of this group, because existing cladobranch phylogenies are notoriously poorly resolved. Support for Cladobranchia as a monophyletic group is high [43,44,50], but the relationships among major lineages within Cladobranchia have long been problematic [26,43–46,49,308]. However, a

recent phylogenomic study provided evidence that these lineages could be resolved with RNA-Seq data [50]. In addition to the growing availability of RNA-Seq data for this group, the prey preferences of the majority of species within this group have been published (e.g., [33,40,57,159,162,312]).

To address the role of dietary specialization and host shifts in the evolution of this group and resolve outstanding systematics issues, we reconstruct the phylogeny of Cladobranchia using RNA-Seq data. In this study, we increase the taxon sampling compared to previous phylogenomic work on Cladobranchia [50] by incorporating additional diversity from both previously sampled clades (Aeolidida and Dendronotida) and the previously unsampled Arminida (as defined in [43], though we only include members of Arminidae). In addition, we seek to address patterns of prey type switching among and within the major lineages of cladobranchs by assessing prey type preference for each taxon included in the phylogenetic analyses, and using these data to reconstruct the most likely ancestral prey type preference for each node in the tree. These analyses provide the means to examine the prevalence of prey type switching within Cladobranchia in order to provide a framework for studying how dietary preferences may have affected evolution within this group.

Materials and Methods

Organismal sampling

One or two specimens of each of 16 representative species were collected in tide pools or via snorkeling or SCUBA (self-contained underwater breathing apparatus; under AAUS certification) using a variety of methods (direct collection, substrate collection, and non-destructive collecting under rocks). A visual examination was

used for confirmation of identity using field guides for the Caribbean [119] and the Indo-Pacific [323]. Barcode sequences and expert opinions were used when the identity of specimens was still uncertain. Images of select specimens are in Figure 7.2 and Figure 7.3. One of the two specimens was placed in RNAlater solution (Qiagen, Hilden, Germany) for RNA preservation and frozen at -80°C within one week of collection to prevent RNA degradation. Some specimens in RNAlater were instead

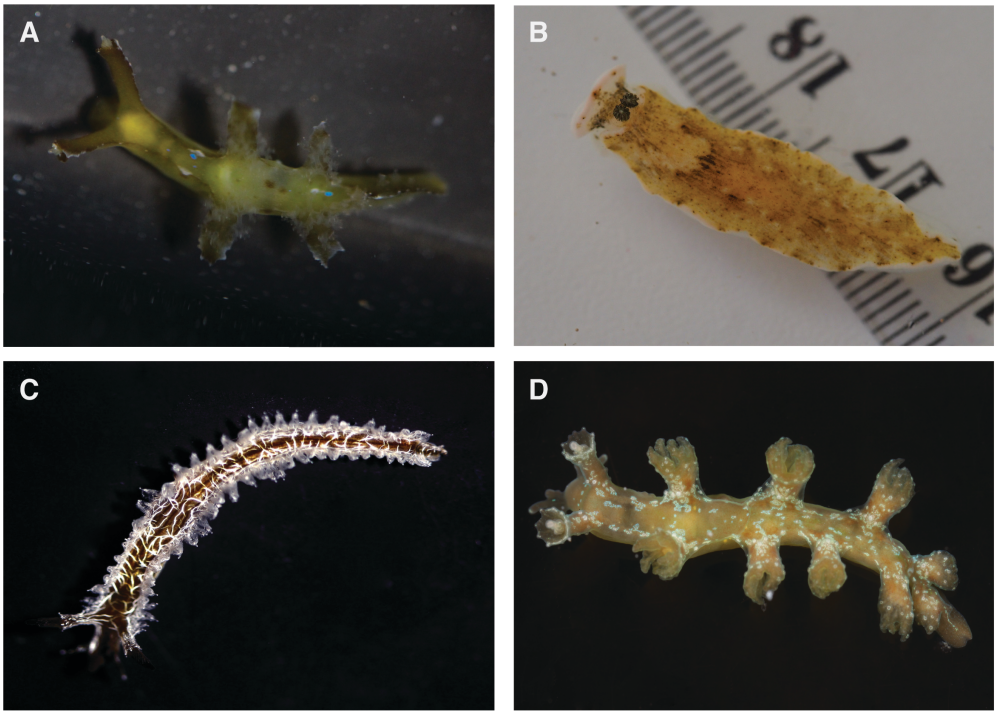

Figure 7.2. Select photographs of dendronotid and unassigned taxa used in this project, including: A) *Scyllaea fulva* (SRR3726701), B) *Dermatobranchus* sp. (SRR3726698; Photo credit: Karen Cheney), C) *Lomanotus vermiformis* (SRR3726706) and D) *Hancockia uncinata* (Photo credit: David Fenwick III).

stored at -20°C within 24 h and remained there for up to a month. A second specimen of each species, when available, was fixed as a voucher for morphological analysis, first in 10% formalin and subsequently preserved in 70% ethanol for long-term storage. Voucher specimens were deposited in the Smithsonian National Museum of Natural History (NMNH) and are available for study under the catalog numbers

provided in Appendix Table C5.

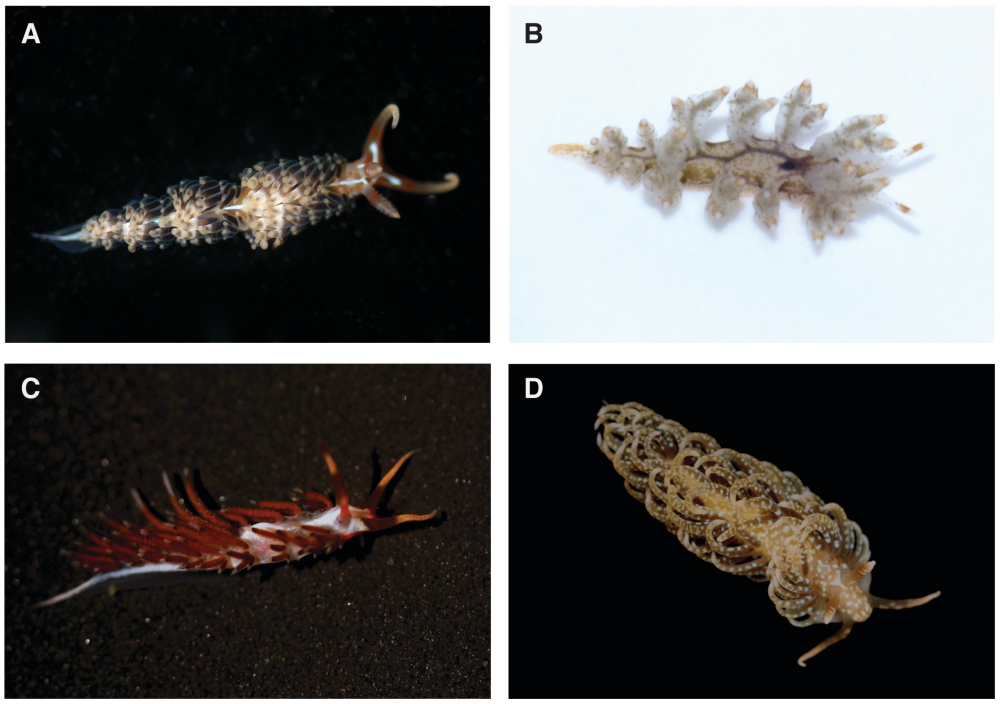

Figure 7.3. Select photographs of aeolid taxa used in this project, including: A) *Phidiana lynceus*, B) *Eubranchus rustyus* (SRR3726692), C) *Learchis evelinae* (SRR3726693), and D) *Spurilla braziliana*.

For *Bulbaeolidia alba*, *Hancockia uncinata*, *Unidentia angelvaldesi*, *Bornella anguilla*, *Dermatobranchus* sp., *Phestilla* sp., and *Eubranchus rustyus* we were unable to obtain morphological vouchers. Cella et al. [340] proposed to use the genus name *Tenellia* for species previously assigned to *Catriona*, *Cuthona* and *Phestilla*, but recognized that the assignment of species to *Tenellia* is problematic due to the absence of morphological synapomorphies. Thus, we chose to temporarily maintain species in the genera *Catriona*, *Cuthona* and *Phestilla* until the additional studies suggested by Cella et al. [340] are carried out. We generated RNA-Seq data for 16 Cladobranchia species, downloaded data for 19 additional Cladobranchia species from the NCBI Sequence Read Archive (SRA) and obtained two RNA-Seq datasets

from colleagues at Georgia State University. Three outgroup RNA-Seq datasets were also obtained from the SRA: two representatives of Anthobranchia (the sister taxon of Cladobranchia; [26,49]), and one of Pleurobranchoidea (the sister taxon to Nudibranchia [22]). Specimen data, SRA numbers and barcode GenBank numbers are listed in Appendix Table C5 and Appendix Table C6.

RNA extraction and sequencing

A 20–100 mg tissue sample was taken from the anterior of each animal and homogenized using a motorized pestle. In some cases, the specimen was so small the entire animal was used. After homogenizing for 1–2 min the tissue was flash-frozen in liquid nitrogen for subsequent homogenizing until tissue mixture was fully uniform. 500 μL of TriZOL Reagent (Life Technologies, Carlsbad, CA, USA) was then added and the mixture was homogenized again. This procedure was repeated until the solution was deemed fully homogenized. Once this process was complete, an additional 500 μL of TriZOL Reagent was added to the solution and the mixture was left at room temperature for five min.

Following the five min incubation, 100 μL of 1-Bromo-3-chloropropane was added to the solution, which was subsequently mixed thoroughly. The mixture was then left at room temperature for five min, and then centrifuged at 16,000 g for 20 min at 8°C. The top aqueous phase was then removed and placed in another tube where 500 μL of 100% isopropanol was added, and stored overnight at -20°C for RNA precipitation.

After precipitation, the samples were centrifuged at 17,200 g for 10 min at 4 C. The supernatant was then removed and the pellet washed with freshly prepared

75% ethanol. The sample was then centrifuged at 7,500 g for 5 min at 4°C. The supernatant was removed and the pellet air-dried for 1 to 2 min (or until it looked slightly gelatinous and translucent). The total RNA was then re-suspended in 10–30 μL of Ambion Storage Solution (Life Technologies, Carlsbad, CA, USA), and 1 μL of SUPERase•In (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was added to prevent degradation.

Total RNA samples were submitted to the DNA Sequencing Facility at University of Maryland Institute for Bioscience and Biotechnology Research, where quality assessment, library preparation, and sequencing were performed. RNA quality assessment was done with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), and samples with a concentration higher than 20 ng/μL were used for library construction. Library preparation used the Illumina TruSeq RNA Library Preparation Kit v2 (Illumina, San Diego, CA, USA) and 200 bp inserts; 100 bp, paired-end reads were sequenced with an Illumina HiSeq1000 (Illumina, San Diego, CA, USA).

Quality control and assembly of reads

Reads that failed to pass the Illumina "Chastity" quality filter were excluded from our analyses. Reads passing the quality filter were assembled using Trinity (version 2; [341]) with default settings, which required assembled transcript fragments to be at least 200 bp in length.

Orthology assignment

Translated transcript fragments were organized into orthologous groups corresponding to a custom gastropod-specific core-ortholog set of 3,854 protein models [50] using HaMStR (version 13.2.2; [342]), which in turn used FASTA (version 36.3.6d; [343]), GeneWise (version 2.2.0; [344]), and HMMER (version 3b2; [345]). In the first step of the HaMStR procedure, substrings of assembled transcript fragments (translated nucleotide sequences) that matched one of the gastropod protein models were provisionally assigned to that orthologous group. To reduce the number of highly divergent, potentially paralogous sequences returned by this search, we set the E-value cutoff defining an HMM hit to 1e-05 (the HaMStR default is 1.0), and retained only the top-scoring quartile of hits. In the second HaMStR step, the provisional hits from the HMM search were compared to the reference taxon, *Aplysia californica*, and retained only if they survived a reciprocal best BLAST hit test with the reference taxon using an E-value cutoff of 1e-05 (the HaMStR default was 10.0). In our implementation, we substituted FASTA [343] for BLAST [346] because FASTA programs readily accepted our custom amino acid substitution matrix (GASTRO50; [50]).

Construction of data matrix and paralogy filtering

Protein sequences in each orthologous group were aligned using MAFFT (version 7.187; [70]). We used the `--auto` and `--addfragments` options of MAFFT to align transcript fragments to the *Aplysia californica* reference sequence, which was considered the existing alignment. We converted the protein alignments to corresponding nucleotide alignments using a custom Perl script. A maximum

likelihood tree was inferred using GARLI (Genetic Algorithm for Rapid Likelihood Inference version 2; [73]) for each orthologous group where at least 75% of the taxa were present (716 orthologous groups), and was given as input to PhyloTreePruner (version 1.0; [347]). Orthologous groups that showed evidence of out-paralogs for any taxa (352 orthologous groups out of 716) were pruned according to the default PhyloTreePruner protocol, which removes all additional sequences outside of a maximally inclusive sub-tree. For orthologous groups containing in-paralogs, multiple sequences were combined into a single consensus sequence for each taxon, and orthologous groups for which fewer than 75% of taxa remained were discarded. This process left 406 orthologous groups eligible for inclusion in data matrices. Individual orthologous group alignments were concatenated (*nt123* matrix) (Table 7.1). Codons not represented by sequence data in at least four taxa were then removed (*nt123sitesremoved* matrix).

Table 7.1. Data matrix statistics for each of the two data matrices.

Data matrix	# of nucleotide positions	% complete	% of ambiguous sites
nt123	966,888	33	0.08
nt123sitesremoved	605,934	50.7	03

Phylogenetic analyses

Four separate phylogenetic analyses were completed in this study: (i) an analysis with the *nt123* data matrix partitioned by codon position (*nt123partitioned*) by assigning different model parameters and rates to the three types of codon positions, (ii) an analysis with the *nt123sitesremoved* data matrix partitioned by codon position (*nt123sitesremoved_partitioned*), (iii) an analysis of the *nt123* matrix partitioned by codon position, but excluding the third position (*nt12partitioned*), and (iv) an analysis of the unpartitioned *nt123sitesremoved* data matrix

(*nt123sitesremoved_unpartitioned*). To conduct all four phylogenetic analyses we used GARLI (version 2; [73]) through the GARLI web service hosted at molecularevolution.org [74]. We used the default settings in GARLI, including a general time reversible substitution model (GTR; [75]) with a rate heterogeneity model with a proportion of invariant sites estimated (+I; [348]) and the remainder with a gamma distribution (+G; [349]), along with stepwise-addition starting trees. Post-processing of the phylogenetic inference results was performed by the GARLI web service at molecularevolution.org using DendroPy [76] and the R system for statistical computing [350]. For all analyses, 1000 bootstrap replicates were generated and a best tree search was performed with 10 search replicates.

Ancestral state reconstruction

We conducted a literature search to collect prey preference data for all nudibranch taxa in our phylogeny and coded each species as Anthozoa: Octocorallia, Anthozoa: Hexacorallia, Hydrozoa, Scyphozoa, Bryozoa, Crustacea, Gastropoda eggs, or generalist, for a total of eight states (Appendix Table C4). In the cases where more than one type of prey is fed upon by an individual species, we provide that information and run additional analyses to test the effect of these alternatives on the final results. The final analysis incorporates the prey type for each species that that species has been observed to feed on more than 50% of the time. Data was compiled primarily from review papers on feeding and defense in nudibranchs [33,57,159,162,312], field guides [119,351], one additional paper [352], and web sources where necessary [353–356]. Though limited, the taxon selection in this study represents a large portion of the morphological and ecological diversity of

Cladobranchia, including the diversity of prey type preferences. Using these character states, we compared the fit of three discrete trait models using the AICcmodavg 2.0-4 package [357] in R 3.3.1 [350]. We assessed fit for models where: (i) all transition rates were equal (ER); (ii) forward and reverse transitions were equal between states (i.e. symmetrical, SYM); and (iii) all transition rates were different (ARD) using the corrected Akaike information criterion (AICc). The ER model (AICc = 100.61) was a better fit to the data than either the SYM model (AICc = 118.53) or the ARD model (AICc = 165.16). The final ancestral state reconstruction analysis was completed using the ace function, in the APE package [358], under the ER model using default parameters and a joint reconstruction approach. The ace function uses a Markov model employing a maximum likelihood approach. In this analysis, the reconstructed ancestral states that are returned are the marginal ancestral states, which are given as the proportion of the total likelihood calculated for each state for each node.

Results

Assembly and data matrix properties

The raw number of reads for each RNA-Seq dataset ranged from 25,756,442 to 133,156,930 ($\overline{x} \approx$ 49M reads; Appendix Table C1). Once assembled, the number of transcript fragments per sample ranged from 71,967 to 295,127 ($\overline{x}$ = 146,403; Appendix Table C2). N50 ranged from 395 to 1,058 bp ($\overline{x}$ = 716 bp). HaMStR results are presented in Appendix Table C3. The transcript fragments from each assembly that matched the HaMStR database ranged from 615 to 2,013 ($\overline{x}$ = 1,282). However, the number of matches to unique orthologous groups ranged from 512 to 1,198 ($\overline{x}$ =

935). The mean length of transcript fragment matches to the HaMStR database was 282 amino acids.

Phylogenetic results

Results from all analyses supported Cladobranchia as a monophyletic group with a bootstrap (BS) value of 100% (Figure 7.4). Arminidae (Arminida) is also supported as monophyletic (BS = 100%), and is sister (BS = 100%) to Tritoniidae (BS = 100%).

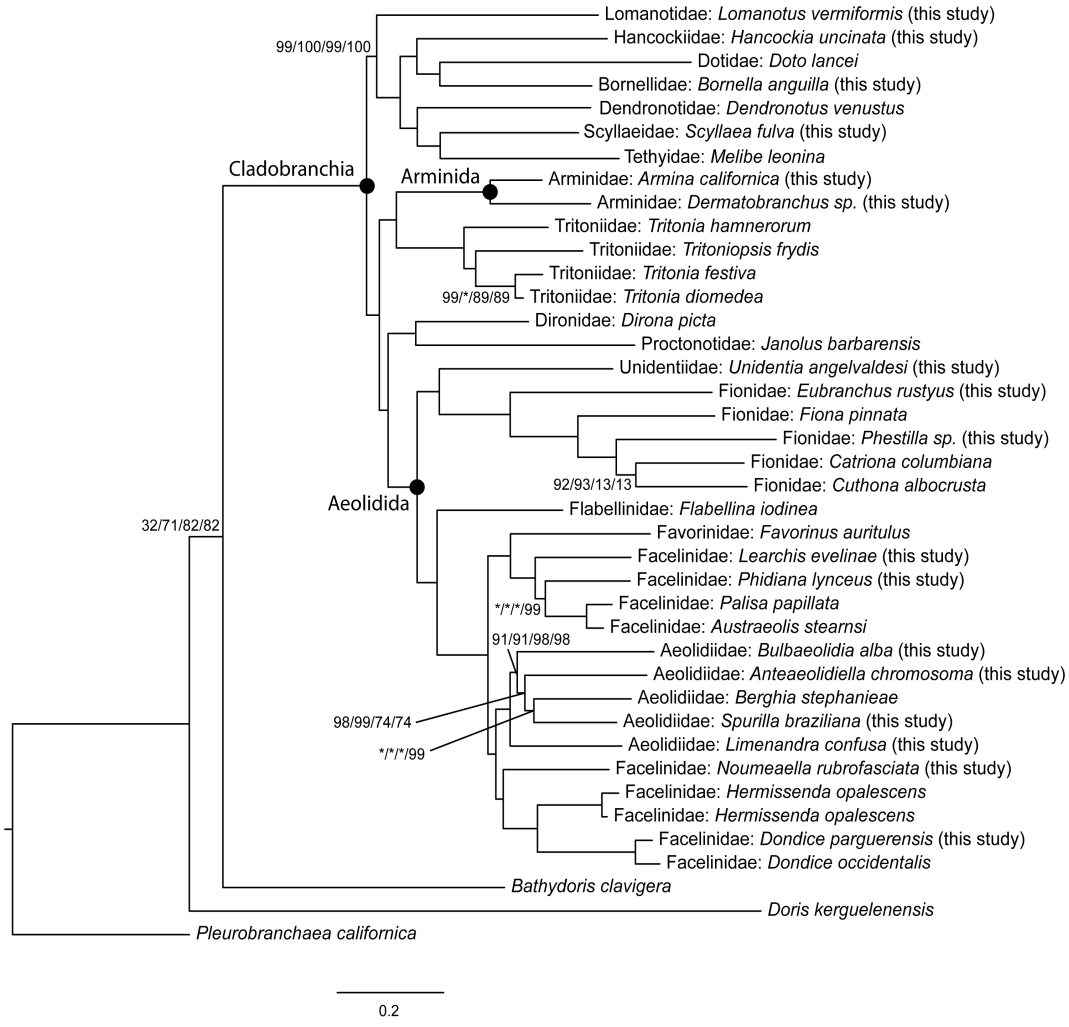

Figure 7.4. The maximum likelihood topology from the *nt123_partitioned* analysis, with bootstrap support values from each analysis labeled on some nodes (*nt123_partitioned / nt123sitesremoved_partitioned / nt123sitesremoved_ unpartitioned / nt12partitioned*). All unlabeled nodes have 100% bootstrap support in all analyses.

Dendronotida is non-monophyletic across all topologies, with dendronotid taxa comprising two major clades. The first of these clades is sister to all other cladobranchs (BS = 100%) and contains *Doto* (Dotidae), *Bornella* (Bornellidae), *Hancockia* (Hancockiidae), *Scyllaea* (Scyllaeidae), *Melibe* (Tethyidae), *Dendronotus* (Dendronotidae), and *Lomanotus* (Lomanotidae).

Aeolidida is supported as monophyletic (BS = 100%) across all topologies, containing *Flabellina* (Flabellinidae), *Berghia*, *Spurilla*, *Bulbaeolidia*, *Anteaeolidiella,* and *Limenandra* (Aeolidiidae)*, Hermissenda*, *Dondice*, *Noumeaella*, *Favorinus*, *Palisa*, *Austraeolis*, *Learchis,* and *Phidiana* (Facelinidae)*, Fiona, Cuthona*, *Catriona, Phestilla*, and *Eubranchus* (Fionidae), and *Unidentia* (Unidentiidae). All families within Aeolidida where multiple taxa from the same family are included are supported as monophyletic, with the exception of Facelinidae, which is paraphyletic and forms two separate clades.

Two taxa previously unassigned to any of the three major clades, *Dirona* (Dironidae) and *Janolus* (Proctonotidae), are supported as sister taxa (BS = 100%) and form a clade that is sister to Aeolidida (BS = 100%).

Ancestral state reconstruction analysis

The ancestral state reconstruction results support the hypothesis that the most recent common ancestor (MRCA) of Cladobranchia preyed upon species of Hydrozoa (Figure 7.3). A cladobranch that preyed upon Hydrozoa also appears to be the MRCA for Aeolidida and the clade composed of most of the taxa assigned to Dendronotida, as well as the rest of the MRCAs along the backbone of the tree. However, a taxon that preyed upon Octocorallia species is the likely MRCA for the Arminidae +

Tritoniidae clade (88.78% of the scaled likelihood), a taxon that preyed upon bryozoans or hydrozoans is the most likely MRCA for the *Dirona* + *Janolus* clade (74.44% of the scaled likelihood; Figure 7.3, Table 7.2), and the MRCA for Aeolidiidae most likely fed upon species within Hexacorallia (98.03% of the scaled likelihood). Additional ancestral state reconstruction analyses were completed to evaluate the effects of alternative prey types for certain taxa on the overall reconstruction of ancestral states (Appendix Tables C7—C11). With the exception of the ancestral node of the *Dirona* + *Janolus* clade, which changes to >97% of the scaled likelihood supporting a Hydrozoa feeding ancestor in three of the alternative analyses, the results are robust to these changes. The scaled likelihoods across all other nodes within each of the alternative analyses remain within 5% of the value in the original analysis.

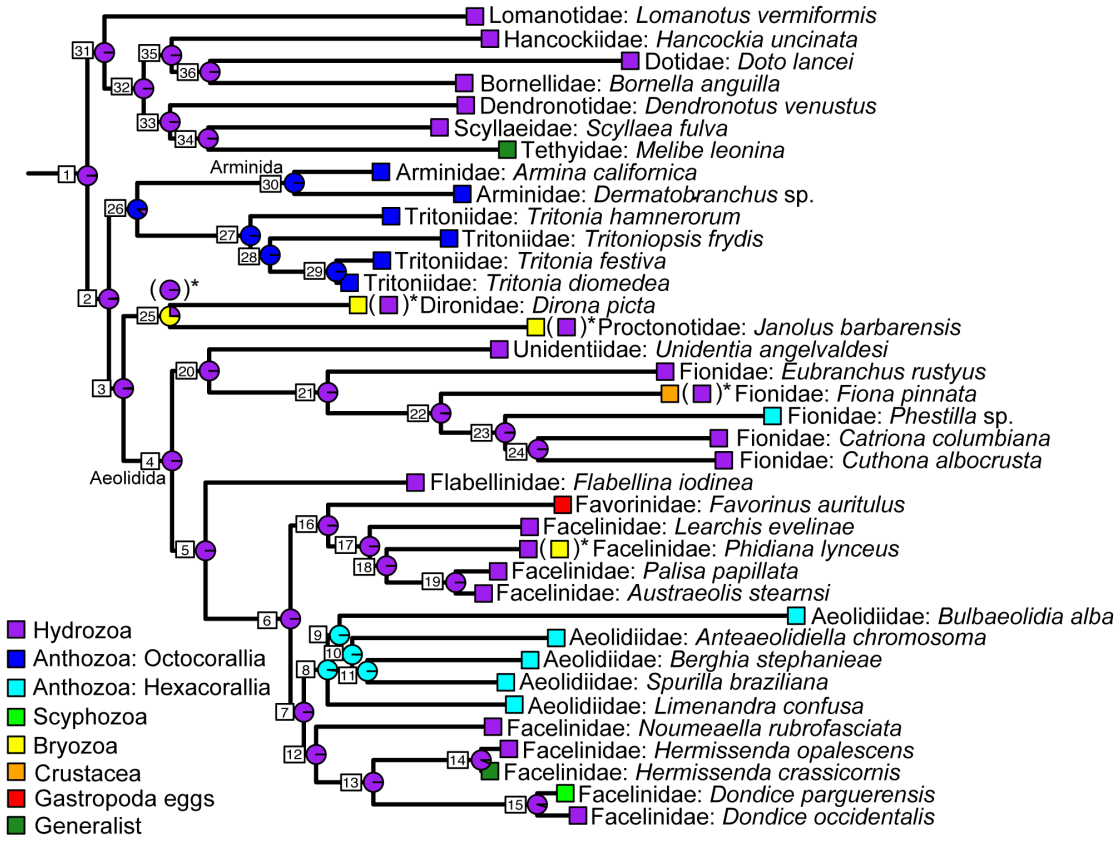

Figure 7.5. Ancestral state reconstruction results for the evolution of diet preference in Cladobranchia. Pie charts on the nodes are scaled marginal likelihoods calculated using the ace function in APE. Alternative states and results are indicated in parentheses with an asterisk at the tips of the tree and nodes, and only alternative node states with greater than or equal to 5% difference from the original reconstruction are shown. Nodes are also labeled with numbers consistent with Table 7.2.

Table 7.2. Ancestral state reconstruction results for the evolution of diet preference in Cladobranchia. This table provides the percentage (%) of the total likelihood assigned to each state for each node. The node numbers correspond to those provided in Figure 7.3. Bold values are those on nodes different by greater than or equal to 5% in at least one alternative analysis. Abbreviations: Alt1, analysis using all alternative states; Alt2, analysis using the alternative state for *Dirona picta*; Alt3, analysis using the alternative state for *Janolus barbarensis*.

Node	Octocorallia	Hexacorallia	Hydrozoa	Bryozoa	Scyphozoa	Crustacea	Gastropoda eggs	Generalist
1	0.8141	0.0100	98.8615	0.2744	0.0100	0.0100	0.0100	0.0100
2	1.6933	0.0096	97.6957	0.5631	0.0096	0.0096	0.0096	0.0096
3	1.1047	0.0113	97.8230	1.0160	0.0113	0.0113	0.0113	0.0113
4	0.0069	0.0003	99.9855	0.0064	0.0002	0.0003	0.0002	0.0002
5	0.0010	0.0007	99.9957	0.0010	0.0004	0.0004	0.0004	0.0004
6	0.0002	0.0163	99.9808	0.0002	0.0003	0.0002	0.0018	0.0004
7	0.0007	0.1722	99.8198	0.0007	0.0017	0.0007	0.0014	0.0027
8	0.0080	98.0323	1.9194	0.0080	0.0081	0.0080	0.0080	0.0081

9	0.0013	99.7978	0.1946	0.0013	0.0013	0.0013	0.0013	0.0013
10	0.0001	99.9881	0.0111	0.0001	0.0001	0.0001	0.0001	0.0001
11	0.0002	99.9958	0.0028	0.0002	0.0002	0.0002	0.0002	0.0002
12	0.0004	0.0418	99.9485	0.0004	0.0026	0.0004	0.0006	0.0051
13	0.0071	0.0202	99.5668	0.0071	0.1256	0.0071	0.0071	0.2590
14	0.0095	0.0103	93.6216	0.0095	0.0159	0.0095	0.0095	6.3140
15	0.0225	0.0236	95.2642	0.0225	4.5788	0.0225	0.0225	0.0434
16	0.0051	0.0128	99.8505	0.0051	0.0051	0.0051	0.1112	0.0052
17	0.0002	0.0003	99.9974	0.0002	0.0002	0.0002	0.0015	0.0002
18	0.0001	0.0001	99.9991	0.0001	0.0001	0.0001	0.0003	0.0001
19	0.0000	0.0000	99.9997	0.0000	0.0000	0.0000	0.0000	0.0000
20	0.0023	0.0014	99.9885	0.0022	0.0011	0.0022	0.0011	0.0011
21	0.0086	0.0269	99.8573	0.0086	0.0084	0.0735	0.0084	0.0084
22	0.0452	0.3201	98.4388	0.0452	0.0452	1.0152	0.0452	0.0452
23	0.0284	0.5362	98.9631	0.0284	0.0284	0.3586	0.0284	0.0284
24	0.0066	0.0935	99.8105	0.0066	0.0066	0.0631	0.0066	0.0066
25	**0.4701**	**0.2032**	**24.0771**	**74.4369**	**0.2032**	**0.2032**	**0.2032**	**0.2032**
25^{alt1}	**0.0049**	**0.0021**	**99.9846**	**0.0021**	**0.0021**	**0.0000**	**0.0021**	**0.0021**
25^{alt2}	**0.0664**	**0.0287**	**99.4038**	**0.3865**	**0.0287**	**0.0287**	**0.0287**	**0.0287**
25^{alt3}	**0.1369**	**0.0585**	**97.8795**	**1.6908**	**0.0585**	**0.0587**	**0.0585**	**0.0585**
26	88.7837	0.0555	10.8222	0.1165	0.0555	0.0555	0.0555	0.0555
27	99.9377	0.0016	0.0525	0.0019	0.0016	0.0016	0.0016	0.0016
28	99.9924	0.0003	0.0056	0.0004	0.0003	0.0003	0.0003	0.0003
29	99.9998	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
30	99.7727	0.0076	0.1805	0.0086	0.0076	0.0076	0.0076	0.0076
31	0.1248	0.0022	99.8216	0.0425	0.0022	0.0022	0.0022	0.0023
32	0.0009	0.0001	99.9982	0.0003	0.0001	0.0001	0.0001	0.0003
33	0.0020	0.0016	99.9689	0.0017	0.0016	0.0016	0.0016	0.0210
34	0.0316	0.0314	99.2906	0.0314	0.0314	0.0314	0.0314	0.5209
35	0.0005	0.0004	99.9972	0.0004	0.0004	0.0004	0.0004	0.0004
36	0.0032	0.0032	99.9776	0.0032	0.0032	0.0032	0.0032	0.0032

Discussion

In this study we significantly increased the breadth of RNA-Seq sampling in Cladobranchia in order to generate a robust phylogenetic hypothesis, and provide a framework for the evolution of prey type preference within this group.

Prey preference evolution in Cladobranchia

Well-supported clades recovered within Cladobranchia appear to be strongly associated with prey groups. Most of the larger clades recovered in the phylogenetic tree prey almost exclusively on particular types of organisms (Figure 7.3), such as Aeolidiidae on Hexacorallia, Arminida + Tritoniidae on Octocorallia, and multiple clades that prey on Hydrozoa. This result is in opposition to previous studies [26,46], which indicated that groupings within Cladobranchia contained taxa that fed on a broad range of prey types. The results here support the idea that prey preference within Cladobranchia may be a taxonomically useful trait for placing taxa into some groups. Past taxonomic work on Cladobranchia has focused on different anatomical features to diagnose groups, such as the presence of rhinophoral sheaths (Dendronotida) or oral veils (Arminida) [26]. In the future, incorporating feeding adaptations to particular prey taxa may accelerate taxonomic progress in the group.

Our results indicate that prey preference shifts from one major taxon to another are relatively rare in Cladobranchia. The ancestral state reconstruction unambiguously supports an ancestor for Cladobranchia that preyed upon Hydrozoa (Figure 7.3). This analysis also suggests at least five transitions from hydrozoan to other prey taxa, such as Hexacorallia (Anthozoa), Octocorallia (Anthozoa), and Scyphozoa. Interestingly, the clade containing *Dirona* and *Janolus* has expanded to feeding on Bryozoa in addition to Hydrozoa, rather than shifting to Bryozoa exclusively. This expansion is mirrored in *Phidiana*, which is also able to feed on members of both taxa. Overall, expansions to feeding on multiple types of prey have occurred at least six times in Cladobranchia, leading to multiple generalist taxa

(*Melibe* and *Hermissenda*) and those that can feed on both Hydrozoa and either Bryozoa or Crustacea. These are cases in which diversification might be related more to an increase in options rather than specialization.

The mechanism by which the evolution of prey preference is constrained is unknown, but it could result from relative difficulty in evolving specific traits for protection against nematocysts (or other defenses) from various cnidarian prey groups. Although it is possible for some species (e.g., *Phidiana hiltoni*) to prey upon different cnidarian species [359], there are few examples of cladobranchs preying on multiple, taxonomically distant cnidarians (only *H. crassicornis* in this study). Cladobranchs require a series of adaptations to prevent cnidarian nematocysts from firing or minimizing the damage in the case of firing [30], including mucous secretions. These secretions appear to be specific to the prey species in one studied case [360], and may be why switching between types of prey is much more challenging and occurs much less frequently than previously thought. Species of Cladobranchia that do not prey on cnidarians, such as *Favorinus*, can easily switch between egg masses of distantly related gastropods, including aplysiids, sacoglossans, and other nudibranchs [40], lending support to this hypothesis.

Previous work has suggested that dietary specialization on particular prey types was crucial in the evolution of Euthyneura [57,58,361], and has been proposed as a "driving force" in heterobranch sea slug evolution [59,362]. Dietary specialization has also been considered a contributing factor in the species richness of Nudibranchia as a whole [58], and especially cladobranchs [33,57] in conjunction with the evolution of nematocyst sequestration. This hypothesis is entirely plausible

when looking at the numbers of species in prey groups and how those correlate with species diversity in the cladobranch predators. Although Bryozoa has nearly 6,000 species [253], there are fewer than 50 species within Cladobranchia that prey on members of this group [40]. Conversely, more than 700 species of cladobranchs prey on Hydrozoa, a clade of cnidarians with ~3,500 species [253]. This drastic difference is primarily due to Aeolidida, which contains a large proportion of the taxa that prey on Hydrozoa, and which appears to have diversified primarily while preying on hydrozoans. Our results do not support the hypothesis that prey type shifts lead to morphological adaptations that increase diversity, as the distinct radular morphology found in Aeolidida is not associated with a prey type shift according to our ancestral state reconstruction. However, Aeolidida is also one of two lineages where nematocyst sequestration is known to have evolved within Cladobranchia. Given that hydrozoans are known to have the highest diversity of nematocyst types [363], the ability to sequester nematocysts may have had an impact on diversification.

The hypothesis that the diversity of larger clades within Cladobranchia is related to the frequency of major prey type shifts is not supported by these results. Instead, we suspect that if shifts in diversification associated with diet in Cladobranchia are going to be found, these may occur within groups that prefer a major prey type (e.g., at the family or genus level), where more species-specific prey shifting is likely to occur [57,324]. The literature indicates that in groups where taxa are specialized on particular prey species, shifting to a new prey (or host) species often leads to speciation and diversification [364–366]. This pattern is found in many metazoan taxa, including flies [367], amphipods [334,368,369], alpheid shrimp

[370,371], barnacles [335], whelks [372], gobies [336], and sacoglossan gastropods [373,374], and has been extensively investigated in phytophagous insects (reviewed in [329]). Within Nudibranchia, a large subset of taxa exhibit specialization on a single species, with many others preferring only two or three prey species [41]. We suspect that in the case of Cladobranchia, this specialization and prey shifting at the species level may be the primary impact that prey preference has on the diversification rate across lineages, rather than shifts to new prey types, as is true in many herbivorous insect lineages (reviewed in [329]). Tests of this hypothesis require a broader sampling of members of Cladobranchia for both the phylogenetic inference and species-specific prey preference data.

Systematics of Cladobranchia and prey preference within individual clades

Based on the phylogenetic hypothesis presented here, the monophyly of Cladobranchia is reinforced with full bootstrap support across all analyses. Though monophyly was indicated in previous morphological [26] and molecular [44–47,49,50] analyses, there has also been a study suggesting paraphyly [43], though the authors of that paper contended that this might be due to a deletion of a string of nucleotides within one lineage (*Melibe*) that was biasing the results.

Arminida

The most significant systematics results from this study involve Arminida, a group not included in the one previous phylogenomic study of Cladobranchia [50]. Arminida, when first described, comprised the genera *Janolus* and *Dirona*, among

other taxa, including Arminidae [51]. The inclusion of *Janolus* and *Dirona* within Arminida renders this group paraphyletic in both morphological and molecular analyses [26,95], and they, along with others (Charcotiidae and Pinufiidae), have since been removed from Arminida and are considered unassigned members of Cladobranchia [26]. The analyses presented here support this exclusion of *Janolus* and *Dirona* from Arminida, consistent with recent studies [26,44,310]. Both of these genera primarily prefer bryozoan prey, but also feed on members of Hydrozoa.

There is strong support for Arminidae (one of two families within Arminida) as the sister group to Tritoniidae, which is a novel result. This result is in agreement with only one previous phylogenetic hypothesis, which was generated using 18S rDNA data [44]. In all other previous studies, taxa from Arminida had been either unplaced within the Cladobranchia phylogeny [47,49,310] or supported as sister to various other combinations of taxa from Dendronotida and Aeolidida [44]. The position of Tritoniidae in the previous phylogenomics study of Cladobranchia was uncertain [50]. It appears that prey preference is particularly relevant for the evolution of Tritoniidae + Arminidae as species within this group prey exclusively on Octocorallia. Species within Octocorallia are known for their noxious chemical defenses in addition to the nematocysts present in their tissues [375,376], and these defenses could help explain why a switch to octocorals has occurred rarely within Cladobranchia.

A caveat does exist, however, in regard to the classification of these clades. Both of the taxa from Arminida included within the present analyses (*Armina* and *Dermatobranchus*) are members of the family Arminidae; thus, the monophyly of

Arminida (containing Arminidae and Doridomorphidae) as a whole has not yet been rigorously tested. That said, the un-sampled family within Arminida, Doridomorphidae, is monotypic and its sole species lives on the blue coral *Heliopora coerulea*, an octocoral with a massive calcium carbonate skeleton [323,377]. This is congruent with the dietary evolution results offered here.

"Dendronotida"

With regard to Dendronotida, the analyses presented here strongly contradict monophyly. The majority of Dendronotida form a single clade (Lomanotidae, Hancockiidae, Dotidae, Bornellidae, Scyllaeidae, Tethyidae, and Dendronotidae) that is sister to all other species within Cladobranchia. This clade and the relationships within it are fully supported (BS = 100%) by all analyses presented here. Lomanotidae as sister to the rest of the species within this clade is a result novel to this study, with most previous morphological and molecular analyses [43,49] supporting alternative topologies, though support was mostly low for these hypotheses.

The rest of the group contains two clades, the first of which is one where Bornellidae is sister to Dotidae and Hancockiidae is sister to the Dotidae + Bornellidae assemblage. This result is also novel as compared to most previous studies [49,66,105,113,309,310]. In morphological analyses in particular, both *Hancockia* and *Doto* have been considered "problematic" genera [26], and as such have mostly been unplaced (*Hancockia*) [26] or unassigned (*Doto*) [308] within the Cladobranchia phylogeny. The analyses presented here, however, very strongly

support the position of these genera in the tree, and therefore provide a much stronger hypothesis for their relationships. The second clade within this grouping contains sister groups Scyllaeidae and Tethyidae, as well as Dendronotidae, which is sister to the Scyllaeidae + Tethyidae assemblage. These relationships are consistent with most previous studies [43,49,50,66], but similar to the Dotidae + Bornellidae + Hancockiidae clade, in other cases Scyllaeidae has been previously supported as sister to Dendronotidae, with Tethyidae (usually *Melibe* specifically) as an early branching lineage [111,309,310].

This clade containing the members of "Dendronotida" appears to be almost exclusively composed of taxa that prey on hydrozoans, with the exception of *Melibe*, which prefers crustaceans that it catches with a remarkable oral hood [129]. Species within Tritoniidae (originally assigned to Dendronotida) form a separate monophyletic group in all analyses as sister to Arminida, as discussed above. In addition, this topology supports the hypothesis that nematocyst sequestration evolved at least twice, because the genus *Hancockia* (the only non-aeolid genus to sequester nematocysts; [278]) does not form a clade with Aeolidida.

Aeolidida

Aeolidida is fully supported as monophyletic, consistent with previous studies [26,44,46,50].

The first of two clades within Aeolidida is made up of taxa from Facelinidae, Aeolidiidae and Flabellinidae. The family Facelinidae forms two separate clades within this group, while Aeolidiidae is monophyletic. The relationships within this

clade are consistent with most previous molecular studies [49,50,67,69,109,110,310]. Based on these results, Facelinidae should likely be split into two separate families, with one clade retaining the name Facelinidae and the other assigned a more appropriate identifier. However, until a member of the genus *Facelina* (the type genus for this family) is included in the analyses (ideally the type taxon *Facelina auriculata*), it is impossible to say which clade should receive the Facelinidae designation. These results also include support for Aeolidiidae as a monophyletic group, at the base of which is one of two shifts to Hexacorallia prey within Aeolidida. The other shift occurs within the family Fionidae.

The second clade within Aeolidida is fully supported across all analyses (BS = 100%), and contains taxa from two families (Fionidae and Unidentiidae) [340]. The relationships between these taxa are also fully supported across all analyses, with the exception of the relationships within Fionidae (though the family itself is monophyletic with full support). Sister to the Fionidae is Unidentiidae. This position for Unidentiidae is a novel result. Only one study previously addressed the phylogenetic position of this family, using morphological data, and in that case Unidentiidae was found to be more closely related to members of Flabellinidae, Piseinotecidae, and Babakinidae [352]. This Fionidae + Unidentiidae clade in particular has multiple shifts to different prey types, including shifts to Crustacea (*Fiona*) and Hexacorallia (*Phestilla*).

Conclusion

RNA-Seq data have recovered a well-supported phylogeny for Cladobranchia. The results of this study include a robust hypothesis of relationships between the major

cladobranch clades, and indicate that some taxonomically diverse groups, such Dendronotida and Facelinidae, are not monophyletic. The ancestral state reconstruction indicates a strong phylogenetic correlation with prey preference within this group, indicating that host shifts are much more rare than previously thought. The mechanism causing evolution of prey preference to be constrained remains unknown, but it could result from difficulties in evolving specific traits for protection against nematocysts from various cnidarian prey groups and chemical compounds from Octocorallia. Future research of Cladobranchia would benefit from combined analyses of prey specialization and prey switching, nematocyst sequestration evolution, and diversification using broader sample coverage. The present study provides a framework for understanding major evolutionary trends in Cladobranchia and indicates that prey type specialization within this group has phylogenetic inertia.

Acknowledgements

We are grateful to Craig Hoover (California State Polytechnic University, Pomona), Hans Bertsch (Universidad Autónoma de Baja California), Karen Cheney (University of Queensland), David Fenwick III, and Ariane Dimitris for providing specimens and/or collecting assistance, and Paul Katz, Jonathan Boykin, Aastha Vashist and Amirah Hurst (Georgia State University) for providing two of the RNA-Seq datasets for analysis. We would also like to thank Vanessa Gonzalez at the Smithsonian National Museum of Natural History for help with RNA extractions. Finally, we are grateful to the Laboratories of Analytical Biology of the National Museum of Natural History for use of the laboratory facilities, and the staff of the Smithsonian Tropical Research Institute in Panama and the Richard B. Gump South Pacific Research

Station in French Polynesia for use of their facilities and their help in acquiring the proper permits. Lastly, we want to thank four anonymous reviewers for their detailed and constructive criticism on previous versions of this manuscript. This work was supported by a Smithsonian Peter Buck Pre-doctoral Fellowship, the Conchologists of America, the Society of Systematic Biologists, a University of Maryland Graduate School Dean's Fellowship and Summer Research Fellowship, a Smithsonian Institution Small Grant award, funding from University of Maryland, and NSF Partnerships for International Research and Education program Award 1243541.

Chapter 8: Comparative morphology and evolution of the cnidosac in Cladobranchia (Gastropoda: Heterobranchia: Nudibranchia).

Introduction

Shell-less gastropods are known to use internally generated (endogenous) or externally obtained (exogenous) biochemically active compounds [28,97] and nematocysts [30,33], as well as crypsis and aposematism in a defensive capacity [29]. Lineages that are known to possess such defenses include the heterobranch groups Sacoglossa (both chemical defenses and crypsis [378]), Anaspidea (chemical and behavioral defenses, e.g., inking [379]), and Nudipleura (aposematism, crypsis and chemical defenses [29,158,380]), among others [96,381,382]. Within Nudipleura, a group of nudibranchs called Cladobranchia possess such alternative defenses [26,33], which have been hypothesized to have contributed to the large-scale diversification of Cladobranchia [33]. In particular, some taxa within Cladobranchia possess the ability to sequester nematocysts from their cnidarian prey. Termed kleptocnides once sequestered, these small venom-filled capsules contain an eversible tubule, often with spines or barbs, that can be discharged into the tissues of other organisms [212,213] and are used by members of Cnidaria to sting predators and capture food [211].

The sequestration of cnidarian nematocysts occurs primarily in one group of cladobranchs, Aeolidida, which appears to be monophyletic [383]. Additional species within the non-Aeolidida cladobranch family Hancockiidae and the currently unplaced Embletoniidae are also known to sequester nematocysts, but the relationships of these two families to nematocyst sequestering taxa in Aeolidida has been uncertain [43,45,67]. Based on a recent phylogenomics study, there is now confidence in the position of *Hancockia* within a non-aeolid group of cladobranchs [383]. These results support the hypothesis that nematocyst sequestration has originated twice within Cladobranchia [61,384]. However, the position of *Embletonia* is still unclear.

The process of nematocyst sequestration has been described previously [30,61], so we will discuss it only briefly here. The nematocyst is a particular type of cnidae, which are complex intracellular organelles enclosed within cnidocytes. To sequester the nematocyst, the cnidocyte (the cell) is separated from its nematocyst (the organelle) during ingestion of cnidarian tissues. Nematocysts are then passed through the digestive gland and incorporated into epithelial cell lining the cnidosac [30,61] (Figure 8.1), a distal extension of the digestive gland within dorsal body outgrowths termed cerata [62]. Nematocysts are not functional when taken up by the cnidophages but mature via a proton transport [280]. The cnidosac is often surrounded by musculature, which allows the nematocysts to be extruded through an opening in the tip of each ceras as necessary [30,61]. Though the cnidosac is critical for nematocyst storage, it has been described in relatively few species of Aeolidida. Similar “cnidosac-like” structures have been found in *Hancockia* and *Embletonia*, but

whether they are homologous to true cnidosacs remains untested [63,220,274,277,278,385].

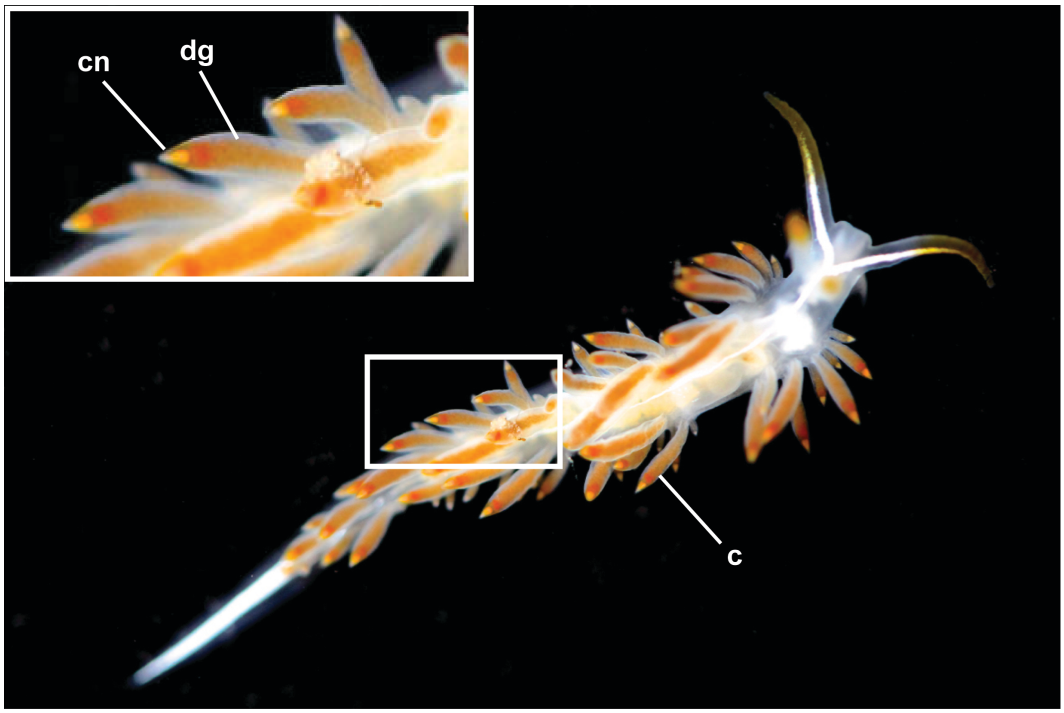

Figure 8.1. Dorsal view of the living animal of the aeolid nudibranch *Flabellina trilineata* (USNM 1408860). Inset: detail of the cerata. Abbreviations: c, ceras; cn, cnidosac; dg, digestive gland.

In regards to the comparative morphology and evolution of the cnidosac and cnidosac-like structures within Cladobranchia, many questions remain. The incorporation of an immature organelle that can be subsequently matured and used by the animal sequestering it allows for the assumption that the structures associated with this ability may be specific to particular types of that organelle. Since there is diversity in nematocyst morphology, we hypothesize that the cnidosac may have evolved specialized structures to cope with the variation in nematocyst type. In this paper, we describe cnidosac morphology across the main groups of Cladobranchia in which it occurs and discuss possible functions for the variation in structural characters in a phylogenetic context. We then use molecular data (including RNA-Seq and PCR-

based sequencing data) to infer a phylogeny for Cladobranchia and combine this with morphological data to provide a better understanding of the evolution of nematocyst sequestration within this group.

Materials and Methods

Morphological data collection

Individuals were relaxed in 10% magnesium chloride when possible, followed by fixation in 10% Bouin's solution or ~4-6% saltwater formalin. For plastic sectioning, whole animals were dehydrated in ethanol and embedded in Hydroxypropyl methacrylate [386]. Serial sections (2.5 μm) were stained with Toluidine blue for 15-20 seconds (for the majority of specimens), which stains neutral mucopolysaccharides, nucleic acids and proteins shades of blue, and acidic mucopolysaccharides red to violet. For paraffin sectioning, individual cerata were dehydrated in ethanol and embedded in paraffin. Serial sections of 6 μm were made and stained with a modified Masson's trichrome stain [387].

Taxon sampling

Molecular data was collected for a total of 92 individual cladobranchs and four outgroups (Appendix Table D1). The majority of these taxa are from Aeolidida, with some taxa from the other major clades in Cladobranchia to assess nematocyst sequestration across this group. This molecular data includes RNA-Seq data for 40 taxa taken from the NCBI Sequence Read Archive [101], along with additional PCR-

based Sanger sequencing data from GenBank [64] (10 taxa) and 42 newly sequenced individuals.

Molecular data collection – PCR-based

Specimens were fixed in 96% ethyl alcohol and stored partly at room temperature or in a refrigerator around 7°C. DNA isolation was carried out by means of *DNeasy Blood and Tissue-Kit* (QIAgen®), DNeasy Plant Mini Kit (QIAgen®), or E.Z.N.A. Invertebrate DNA Kit (Peqlab), following the included instructions. Under sterile conditions slices of the foot or preferably dorsal parts in a size of approximately 5mm^2 were taken and ground with a stamp. Proteinase K was added to assist with lysis. To ensure efficient lysis, the samples were placed in a 56°C shaking bath and lysed over night. The contents of the reaction tube were then transferred to a silica-membrane mini spin-column with collection tube and centrifuged. Two washing steps were performed to eliminate the remaining contaminant and enzyme inhibitors. The purified DNA was then eluted in two successive steps using 50 µL of low-salt buffer each. The extracted DNA was then stored at -20°C.

For some PCR reactions the QIAGEN® Multiplex PCR Kit was used according to manufacturer instructions. Each PCR reaction used 2.3 µL RNAse-free H_2O, 2.0 µL 5x Q-Solution, 10.0 µL 1x QIAGEN® Multiplex PCR Master Mix, and 1.6 µL of each primer at a concentration of 10 pmol/µL. The primers used for each gene fragment can be found in Appendix Table D2. The thermocycler parameters for the PCR reactions for each gene are presented in Appendix Table D3. In some cases as indicated in Appendix Table D3, a touchdown PCR protocol was used to ensure enrichment of the correct product and minimize non-specific binding. . The

QIAquick™ PCR Purification Kit, ExoSAP-IT™, or E.Z.N.A. Cycle-Pure Kit (Omega) were then used for PCR product purification.

Bi-directional sequencing was completed by IIT Biotech/Bioservice, Bielefeld or Eurofins MWG Operon.

Extraction of sequences from transcriptome data

To extract the sequences for COI, 16S, and 18S from each of the transcriptomes, the datasets were first used to create BLAST databases using makeblastdb from the BLAST [346] command line applications. Sequences from the most closely related organisms in GenBank [64] were then aligned to the transcriptome databases using tblastn (COI) or blastn (16S and 18S). The top hit with the lowest e-value and the longest sequences were then selected from the hits. These were then manually trimmed to match the most common sequence lengths for each gene.

Alignments and construction of sequence matrix

Sequences from each gene (COI, 16S, and 18S) were aligned using MAFFT version 7.187 [70] using the `--auto` option. The COI, 16S, and 18S individual gene alignments were then concatenated and merged with the *nt123* matrix from Goodheart et al. [383]. Sites not represented by sequence data in at least four taxa were then removed from the matrix

Phylogenetic analyses

Phylogenetic analysis included the following partitioning scheme: 1) protein coding genes were partitioned by codon position, and 2) the two mitochondrial rDNA genes (16S and 18S) were partitioned by gene. To conduct the phylogenetic analysis we

used MrBayes (v3.2.6; [388]), incorporating BEAGLE [389] to make use of highly-parallel processors to speed up the core calculations for the phylogenetic analysis. We used a general time reversible substitution model (GTR; [75]) with a rate heterogeneity model with a proportion of invariant sites estimated (+I; [348]) and the remainder with a gamma distribution (+G; [349]), along with stepwise-addition starting trees. The analysis was run for 6 million generations and sampled every 1000 generations. The first 25% of trees were discarded as burn-in. The consensus tree was built using the default parameters in MrBayes.

Ancestral state reconstruction

Ancestral states were reconstructed for two characters: (i) the presence or absence of a sac (a bag-like structure larger than an individual cell) at the distal edge of the digestive gland, and (ii) the presence or absence of kleptocnides in the distal tissues (Table 8.2). Using these character states, we compared the fit of three discrete trait models using the AICc from the AICcmodavg 2.0-4 package [357] in R 3.3.1 [350]. We assessed fit for two models, where: (i) all transition rates were equal (ER); (ii) forward and reverse transitions were different between states (symmetrical, ARD). The ARD model (kleptocnides AICc = 64.50144; sac AICc = 59.07113) was a slightly better fit to the data than the ER model (kleptocnides AICc = 65.01457; sac AICc = 59.12095) for each character. The final ancestral state reconstruction analysis was completed using the ace function, in the APE [358] package, under the ARD model using default parameters. The ace function uses a Markov model employing a maximum likelihood approach. In this analysis, the reconstructed ancestral states that

are returned are the marginal ancestral states, which are given as the proportion of the total likelihood calculated for each state at each node.

Table 8.2. Morphological data on the distal sac and presence of kleptocnides for all species evaluated in this study.

Family	Species	Distal sac in cerata	Kleptocnides	Reference
Lomanotidae	*Lomanotus vermiformis*	Absent	Absent	Wagele & Willan 2000
Dotidae	*Doto lancei*	Absent	Absent	Wagele & Willan 2000
Bornellidae	*Bornella anguilla*	Absent	Absent	Wagele & Willan 2000
Dendronotidae	*Dendronotus venustus*	Absent	Absent	Wagele & Willan 2000
Scyllaeidae	*Scyllaea fulva*	Absent	Absent	Bergh, 1875
Tethyidae	*Melibe leonina*	Absent	Absent	Agersberg 1923
Arminidae	*Armina californica*	Absent	Absent	Wagele & Willan 2000
	Dermatobranchus sp.	Absent	Absent	This study
Tritoniidae	*Tritonia hamnerorum*	Absent	Absent	Wagele & Willan 2000
	Tritoniopsis frydis	Absent	Absent	Wagele & Willan 2000
	Tritonia festiva	Absent	Absent	Wagele & Willan 2000
	Tritonia diomedea	Absent	Absent	Wagele & Willan 2000
Charcotiidae	*Charcotia granulosa*	Present	Absent	Wägele et al., 1995
	Pseudotritonia gracilidens	Present	Absent	Wägele, 1991
	Pseudotritonia telarma	Present	Absent	Wagele & Willan 2000
Dironidae	*Dirona albolineata*	Absent	Absent	Cockerell & Eliot, 1905; MacFarland, 1912
	Dirona picta	Absent	Absent	This study
Proctonotidae	*Janolus barbarensis*	Absent	Absent	This study
	Janolus capensis	Present	Absent	This study
	Janolus cristatus	Present	Absent	This study
Hancockiidae	*Hancockia uncinata*	Present	Present	Martin et al. 2009
Embletoniidae	*Embletonia gracilis*	Present	Present	This study
	Embletonia pulchra	Present	Present	Martin et al. 2010
Aeolidiidae	*Aeolidia papillosa*	Present	Present	This study
	Bulbaeolidia alba	Present	Absent	This study
	Anteaeolidiella chromosoma	Present	Present	This study
	Berghia stephanieae	Present	Present	This study
	Cerberilla amboinensis	Present	Present	This study
	Limenandra confusa	Present	Present	This study
	Spurilla neapolitana	Present	Present	This study
Facelinidae 1	*Austraeolis stearnsi*	Present	Present	This study
	Caloria elegans	Present	Present	This study
	Cratena peregrina	Present	Present	This study

	Facelina rubrovittata	Present	Present	This study
	Favorinus auritulus	Present	Absent	This study
	Glaucus atlanticus	Present	Present	This study
	Learchis evelinae	Present	Present	This study
	Palisa papillata	Present	Present	This study
	Phidiana lottini	Present	Present	This study
	Phidiana lynceus	Present	Present	This study
	Pruvotfolia pselliotes	Present	Present	This study
	Pteraeolidia ianthina	Present	Present	This study
Facelinidae 2	*Dondice occidentalis*	Present	Present	This study
	Hermissenda crassicornis	Present	Present	This study
	Hermissenda opalescens	Present	Present	This study
	Noumeaella rubrofasciata	Present	Present	This study
	Phyllodesmium cf. magnum	Present	Absent	This study
	Phyllodesmium colemani	Present	Absent	This study
	Phyllodesmium jakobsenae	Present	Present	This study
	Phyllodesmium koehleri	Present	Absent	This study
	Phyllodesmium macphersonae	Present	Absent	This study
Flabellinidae 1	*Calmella cavolinii*	Present	Present	This study
	Flabellina affinis	Present	Present	This study
	Flabellina babai	Present	Present	This study
	Flabellina falklandica	Present	Absent	This study
	Flabellina gracilis	Present	Present	This study
	Flabellina ischitana	Present	Present	This study
	Flabellina salmonacea	Present	Present	This study
	Piseinotecus gabinierei	Present	Present	This study
Flabellinidae 2	*Flabellina iodinea*	Present	Present	This study
Flabellinidae 3	*Flabellina pedata*	Present	Present	This study
Fionidae	*Cuthona albocrusta*	Present	Present	This study
	Cuthona caerulea	Present	Present	This study
	Cuthona kanga	Present	Present	This study
	Fiona pinnata	Absent	Absent	This study
	Phestilla sp.	Present	Present	This study
	Tergipes antarcticus	Absent	Absent	This study
	Tergipes tergipes	Absent	Absent	This study
Notaeolidiidae	*Notaeolidia depressa*	Present	Present	This study

Results

Terminology

The terminology used to describe the structure of the cnidosac has varied over time [30,274,276,390–393]. In this paper, we clarify the terminology and define how we use each term in order prevent ambiguity and to encourage consistency in descriptions of the cnidosac in the future. A summary of the terminology as described here and how it relates to terms used in previous publications is provided in Table 8.1 [30,220,274,276,282,390–393]. As the table suggests, the term cnidosac (to describe the structures that house kleptocnides in the tips of the cerata) has been in use for over 100 years, but terminology to describe the many parts of the cnidosac has been insufficient. Our use of the term kleptocnides is particularly deliberate. No study exists that clearly identifies the types of nematocysts that may be incorporated into the cnidosac, and in fact it is impossible to say whether similar types of cnidocysts (spirocysts or ptychocysts) may also be sequestered. This is largely due to the fact that researchers have previously identified kleptoncnides based on the definition that nematocysts have a tubule wrapped around a shaft, which can also be used to describe spirocysts [394]. As such, use of the term "nematocyst" may be more precise, but possibly incorrect. However, both additional cnidocyst types are present only in Hexacorallia (Anthozoa), and so would not be found in any nudibranch that feeds on other types of cnidarian prey.

Table 8.1. Equivalency table for terminology related to the cnidosac.

Author Herein	Cnidosac	Kleptocnides	Entrance/ Channel	Exit/ Cnidopore	Cnidophage	Digestive gland
Hancock & Embleton 1845	ovate vesicle/sac	elliptical bodies with slender, hair-like filaments	ciliated channel	external orifice	little transparent ellipsoidal membranous bags	liver caecum
Wright 1863	ovoid vesicle/sac	cnidae, thread cells	-	-	-	biliary sac
Herdman & Clubb 1889	cnidophorous sac	cnida	connecting tube, ciliated canal	terminal opening	cnidocyst	hepatic caecum
Herdman 1890	cnidophorous sac	cnida, thread cells	slender tube with thin walls & few muscle fibers	clearly defined aperature at the apex	cnidocyst	hepatic diverticulum
Grosvenor 1903	cnidophorous sac/cnidosac/ cnidophore	nematocysts	ciliated canal	terminal opening	cnidocyst/ cnidoblast	alimentary canal/ diverticula of the gastric gland
Edmunds 1966	cnidosac	nematocysts	-	cnidopore	cells	digestive gland
Conklin & Mariscal, 1977	cnidosac	nematocysts	-	cnidopore	cnidocyst	-
Ohkawa & Yamasu, 1993	cnidosac	nematocysts	-	-	cnidophage cell	digestive diverticulum
Greenwood 2009	cnidosac	nematocysts/ kleptocnidae	-	-	cnidophage cell	digestive diverticula

General structure of cnidosacs

The cnidosac is a sac-like structure formed by a thin epithelium and is located in the apex of each ceras; one per ceras in the case of aeolids, and multiple per ceras in species of *Hancockia* (Figure 8.2). The cnidosac is found within members of Aeolidida, *Hancockia* and *Embletonia*, and is a prolongation of the digestive gland that contains nematocysts (termed kleptocnides once sequestered) from the nudibranchs' cnidarian prey. However, not all species that possess cnidosacs

sequester nematocysts, which are missing in most members of the genera *Favorinus* and *Phyllodesmium* (Figure 8.3). Differences in the various structures within the cnidosac are quite obvious in relation to the uptake, storage and release of the kleptocnides.

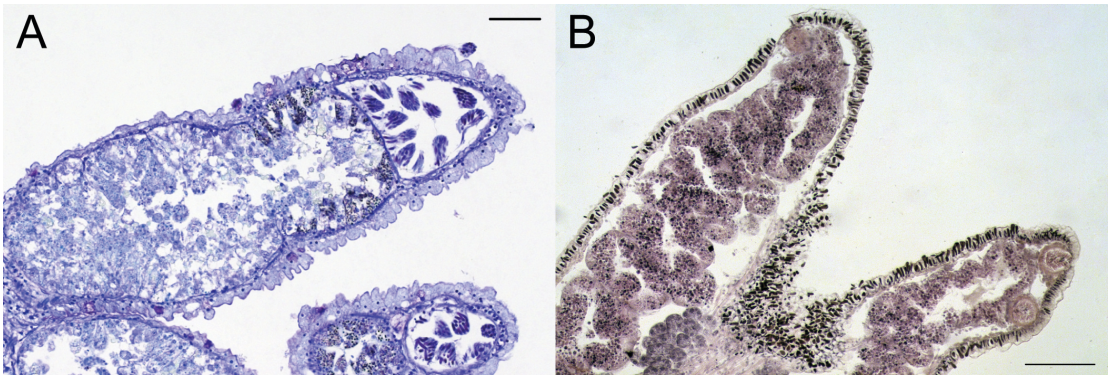

Figure 8.2. Longitudinal sections of cerata showing variation in number of cnidosacs: A) a single cnidosac in one ceras from *Caloria elegans* (scale bar = 50 µm), and B) multiple cnidosacs in the dendronotid *Hancockia californica* (scale bar = 100 µm).

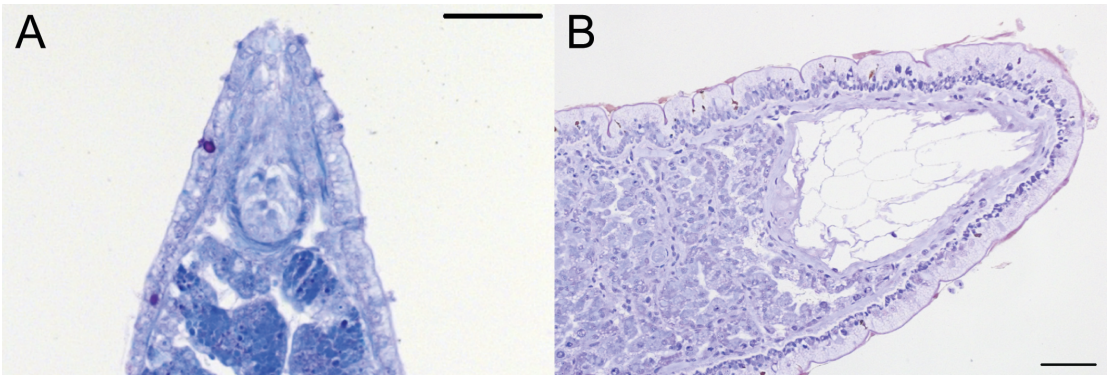

Figure 8.3. Longitudinal sections of cerata tips with cnidosacs lacking kleptocnides in A) *Favorinus auritulus* (USNM1276034), and B) *Phyllodesmium colemani*. Scale bars = 50 µm.

The cnidosac consists of an epithelium that may be surrounded by muscle fibers, which can be present in multiple layers (Figure 8.4). The epithelium of the cnidosac consists of cells, termed cnidophages, that may be differentiated according to their position within the cnidosac, from proximal to distal. Close to the entrance of the cnidosac these cells usually still show distinct nuclei, but their appearance

becomes more atypical in the distal part of the cnidosac (Figure 8.5B), likely owing to the incorporation of kleptocnides. Although we did not observe a distinct epithelial lining within the cnidosac in all species investigated, this may be due in part to artifacts in the preservation process. Kleptocnides are usually located within vacuoles inside the cnidophages, and the number of kleptocnides may vary within a vacuole.

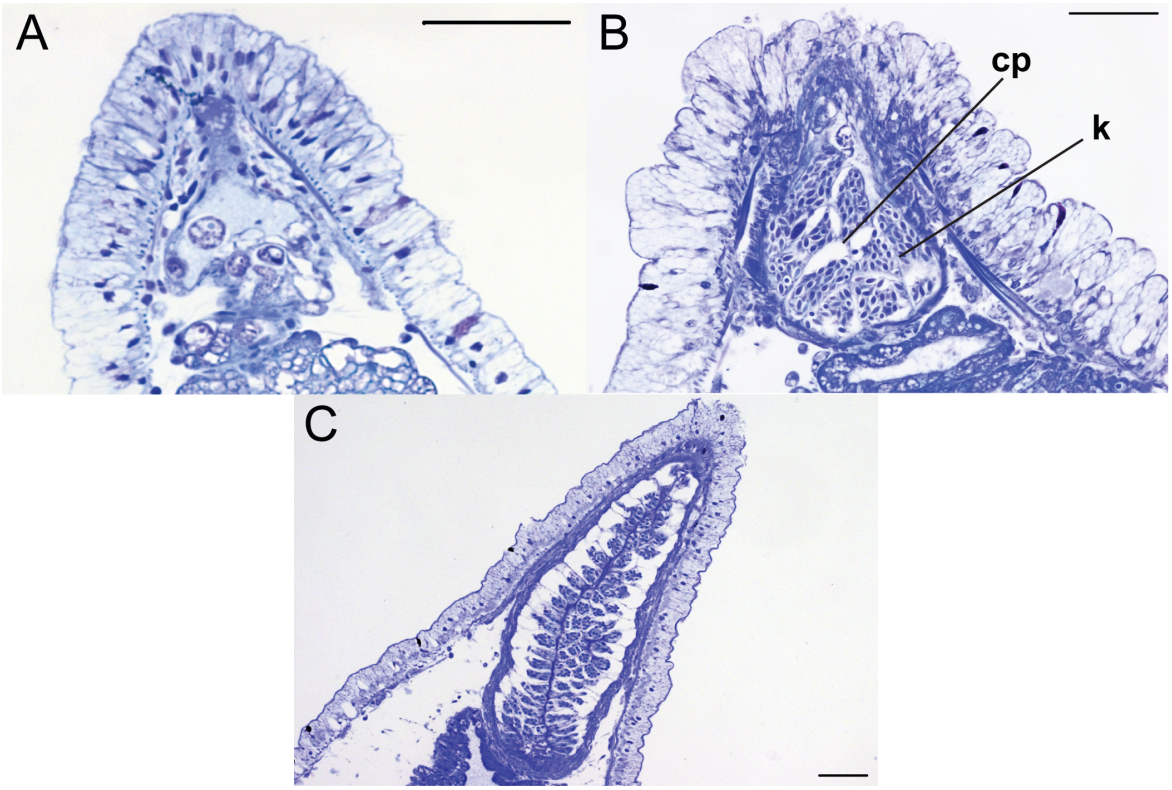

Figure 8.4. Longitudinal sections of cerata tips showing variation in cnidosac musculature: A) no muscle in *Bulbaeolidia alba*, B) a single muscle layer in *Flabellina gracilis*, and C) a multi-layered musculature in *Flabellinia affinis*. Abbreviations: cp, cnidophage; k, kleptocnides. Scale bars = 50 µm.

Table 8.3. Morphological data on the cnidosac and cnidosac-like structures of nematocyst sequestering species evaluated in this study.

Species	Musculature	Proliferation zone	Entrance	Exit	Constriction near the tip
Hancockia uncinata	multi-layered	No	Entrance	Exit	Absent
Hancockia schoeferti	thick	No	Entrance	Exit	Absent
Hancockia californica	multi-layered	No	Entrance	Exit	Absent
Embletonia gracilis	Absent	No	Unobserved	Unobserved	Absent
Embletonia pulchra	Absent	No	Unobserved	Unobserved	Absent
Aeolidia papillosa	multi-layered	Yes	Channel	Pore	Absent
Bulbaeolidia alba	Absent	No	Unobserved	Unobserved	Absent
Anteaeolidiella chromosoma	multi-layered	Yes	Inferred	Pore	Absent
Berghia stephanieae	multi-layered	Yes	Inferred	Unobserved	Absent
Cerberilla amboinensis	multi-layered	Yes	Channel	Pore	Present
Limenandra confusa	multi-layered	Yes	Inferred	Exit	Present
Spurilla neapolitana	multi-layered	Yes	Entrance	Unobserved	Absent
Austraeolis stearnsi	single layer	No	Unobserved	Pore	Present
Caloria elegans	two layers	Yes	Unobserved	Unobserved	Absent
Cratena peregrina	multi-layered	Yes	Channel	Exit	Absent
Facelina rubrovittata	multi-layered	No	Unobserved	Exit	Present
Favorinus auritulus	multi-layered	Yes	Unobserved	Exit	Absent
Glaucus atlanticus	multi-layered	Yes	Unobserved	Unobserved	Inconclusive
Learchis evelinae	multi-layered	Yes	Entrance	Exit	Absent
Palisa papillata	multi-layered	Inconclusive	Unobserved	Exit	Inconclusive
Phidiana lottini	multi-layered	No	Unobserved	Exit	Inconclusive
Phidiana lynceus	multi-layered	Yes	Unobserved	Exit	Present
Pruvotfolia pselliotes	multi-layered	Yes	Unobserved	Exit	Absent
Pteraeolidia ianthina	multi-layered	Yes	Channel	Exit	Absent
Dondice occidentalis	single layer	Yes	Entrance	Unobserved	Absent
Hermissenda crassicornis	multi-layered	Inconclusive	Entrance	Exit	Absent
Noumeaella sp. 2	multi-layered	Yes	Entrance	Exit	Absent
Phyllodesmium cf. magnum	single layer	No	Unobserved	Unobserved	Absent
Phyllodesmium colemani	single layer	No	Unobserved	Unobserved	Absent
Phyllodesmium jakobsenae	single layer	Yes	Entrance	Unobserved	Absent
Phyllodesmium koehleri	single layer	No	Unobserved	Unobserved	Absent
Phyllodesmium macphersonae	single layer	No	Entrance	Unobserved	Absent
Calmella cavolinii	single layer	Yes	Entrance	Exit	Absent
Flabellina affinis	multi-layered	Yes	Entrance	Unobserved	Absent

Flabellina babai	multi-layered	No	Unobserved	Unobserved	Absent
Flabellina falklandica	multi-layered	Inconclusive	Unobserved	Unobserved	Absent
Flabellina gracilis	single layer	Inconclusive	Unobserved	Unobserved	Absent
Flabellina ischitana	multi-layered	Yes	Entrance	Exit	Absent
Flabellina salmonacea	multi-layered	Yes	Channel	Unobserved	Absent
Piseinotecus gabinierei	multi-layered	No	Entrance	Unobserved	Absent
Flabellina iodinea	multi-layered	Yes	Entrance	Exit	Absent
Flabellina pedata	multi-layered	Yes	Entrance	Unobserved	Absent
Cuthona albocrusta	single layer	Yes	Entrance	Unobserved	Absent
Cuthona caerulea	single layer	Inconclusive	Entrance	Exit	Absent
Cuthona kanga	multi-layered	Yes	Unobserved	Exit	Absent
Fiona pinnata	-	-	-	-	-
Phestilla sp.	multi-layered	Inconclusive	Entrance	Exit	Absent
Tergipes antarcticus	-	-	-	-	-
Tergipes tergipes	-	-	-	-	-
Notaeolidia depressa	single layer	No	Entrance	Exit	Absent

The transition from the digestive gland to the cnidosac may vary in length and diameter between taxa. We refer to this as a channel in the presence of ciliated cells along the length of this transition, or at least cuboidal cells without vacuoles producing enzymes, as is typical for digestive gland cells (Figure 8.6A). If there are no discernable ciliated epithelial cells, we consider this transition to simply be an entrance (Figure 8.6B). This entrance is extremely small and may not be captured in sections, and must be inferred based on changes in orientation of cells in the vicinity of the transition between the digestive gland and the cnidosac, indicated in Table 8.3. Adjacent to the entrance at the base of the cnidosac is a proliferation zone, where small cells from the proximal cnidosac epithelium seem to form and grow larger as they move toward the distal end, likely to accommodate nematocysts (Figure 8.5A). In some cases the contents of the cnidosac may obscure the proliferation zone in sections, making it unclear whether the zone is not present or is simply unobservable (e.g., *Hancockia* spp.; Figure 8.2B); in other cases the cnidosac is mostly, or

completely, empty (e.g., *A. stearnsi*, *F. rubrovittata*, and *Phyllodesmium* spp.; Figure 8.5D). A very simple exit from the cnidosac was found in many of the, covered in some cases with a thin epithelial lining. We refer to a distinct exit from the cnidosac, in the sense that the epithelium of the cnidosac connects to that of the epidermis, as a cnidopore (Figure 8.5C). If this structure is not distinguishable, however (e.g., the epidermis shows only small cuboidal cells at the tip, instead of the elongate vacuolated cells), we refer to this simply as an exit (Figure 8.5D).

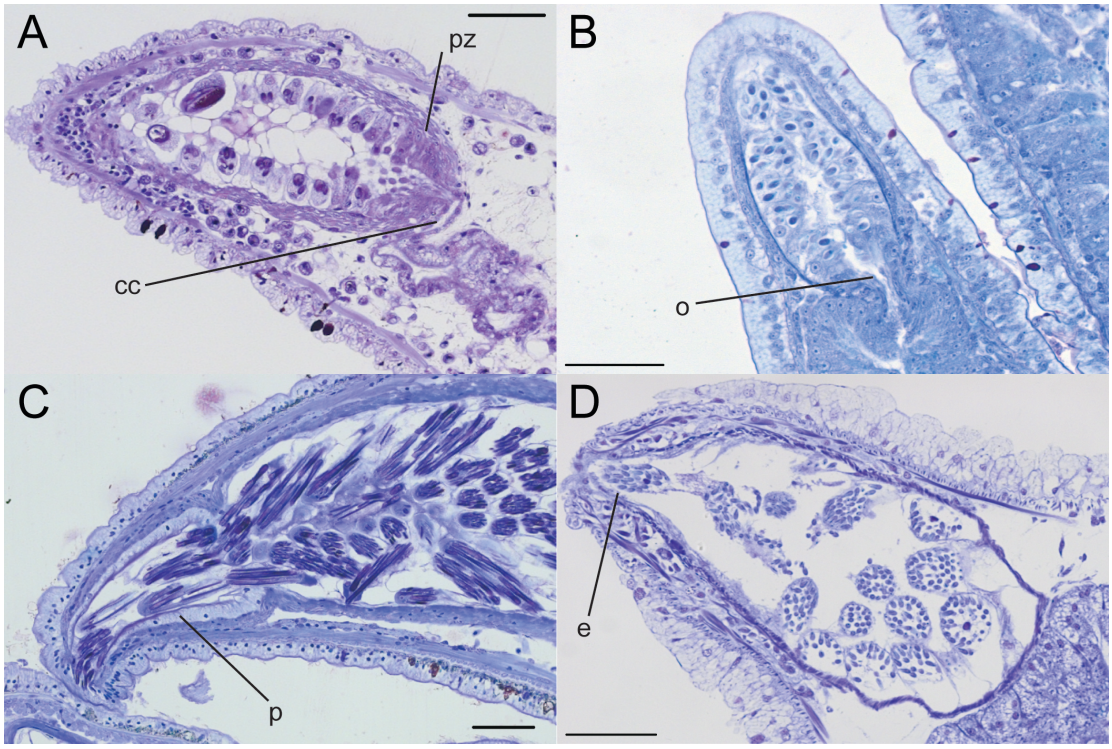

Figure 8.5. Longitudinal sections of cerata tips showing cnidosac entrances and exits: A) a ciliated channel in *Pteraeolidia ianthina* (scale bar = 50 µm), B) an entrance from *Dondice occidentalis* (USNM1276036; scale bar = 50 µm), C) a cnidopore in *Cerberilla amboinensis* (scale bar = 100 µm), and D) an exit in *Cratena peregrina* (scale bar = 50 µm)..

However, a few taxa that we evaluated do not fit comfortably within the general scheme outlined above, including *Hanockia* spp., *Embletonia* spp., *Bulbaeolidia alba*,

Favorinus auritulus, *Phyllodesmium* spp., and the two included Fionidae, *Fiona pinnata* and *Tergipes* spp.

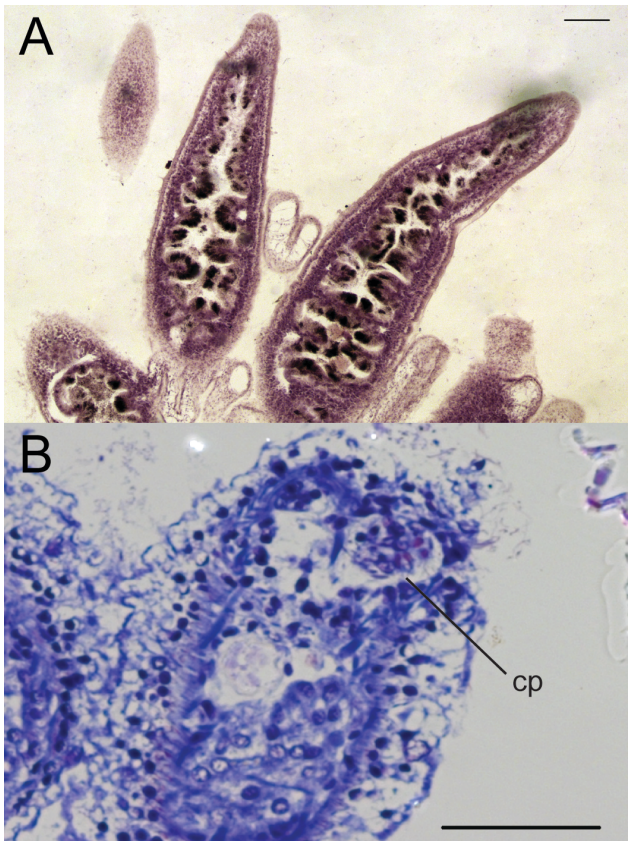

Figure 8.6. Longitudinal sections of cerata from: A) *Fiona pinnata*, which lacks cnidosacs (scale bar = 100 µm), and B) *Embletonia gracilis*, showing a cnidophage (scale bar = 50 µm).

In the Fionidae (*F. pinnata*, *Tergipes tergipes*, and *T. antarcticus*), a cnidosac is not present (Figure 8.6A). In contrast, in *B. alba*, *F. auritulus*, and the majority of *Phyllodesmium* species (except *P. jakobsenae*), a cnidosac was found to be present but no kleptocnides were observed (Figure 8.3). The structure of the cnidosac in several of these taxa was further observed to present several unique differences compared to those species that harbor kleptocnides. For example, in *Phyllodesmium*, the cnidosac closely resembles those of other aeolids, but typically has only a single

layer of musculature, no obvious proliferation zone, and in most cases no exit was observed. *Favorinus auritulus* on the other hand has a multi-layered musculature, an obvious exit, and appears to possess a proliferation zone similar to species that have kleptocnides. Instead of a muscular cnidosac *Bulbaeolidia alba* possesses a membrane-bound sac at the tip of the ceras which lacks an exit or cnidopore. This sac attaches to the digestive gland and contains only zooxanthellae. In *Embletonia gracilis*, the cnidosac as defined here is lacking; nematocysts are housed at the tip of the cerata and seem to be contained within a single cnidophage (Figure 8.6B).

Phylogenetic results

The Bayesian phylogeny inferred in this study has varied support among its branches (Figure 8.7) across all parts of the tree. Support for Cladobranchia is high (PP = 1), but support values for other major clades, including Aeolidida (PP = 0.89) is lower.

Ancestral state reconstruction

Ancestral state reconstruction supports the hypothesis that the sequestration of nematocysts has originated twice within Cladobranchia, once at the base of Aeolidida and once in *Hancockia* (Figure 8.8). The sac at the distal edge of the digestive gland also seems to have originated at least twice, once in *Hancockia*, and with a second origin possibly at the node uniting Aeolidida with Charcotiidae + Dironidae + Proctonotidae. Loss of nematocyst sequestration has occurred three times (in *Phyllodesmium*, *Fiona*, and *Favorinus*) and the distal sac has been lost at least twice (in Fionidae and *Janolus*).

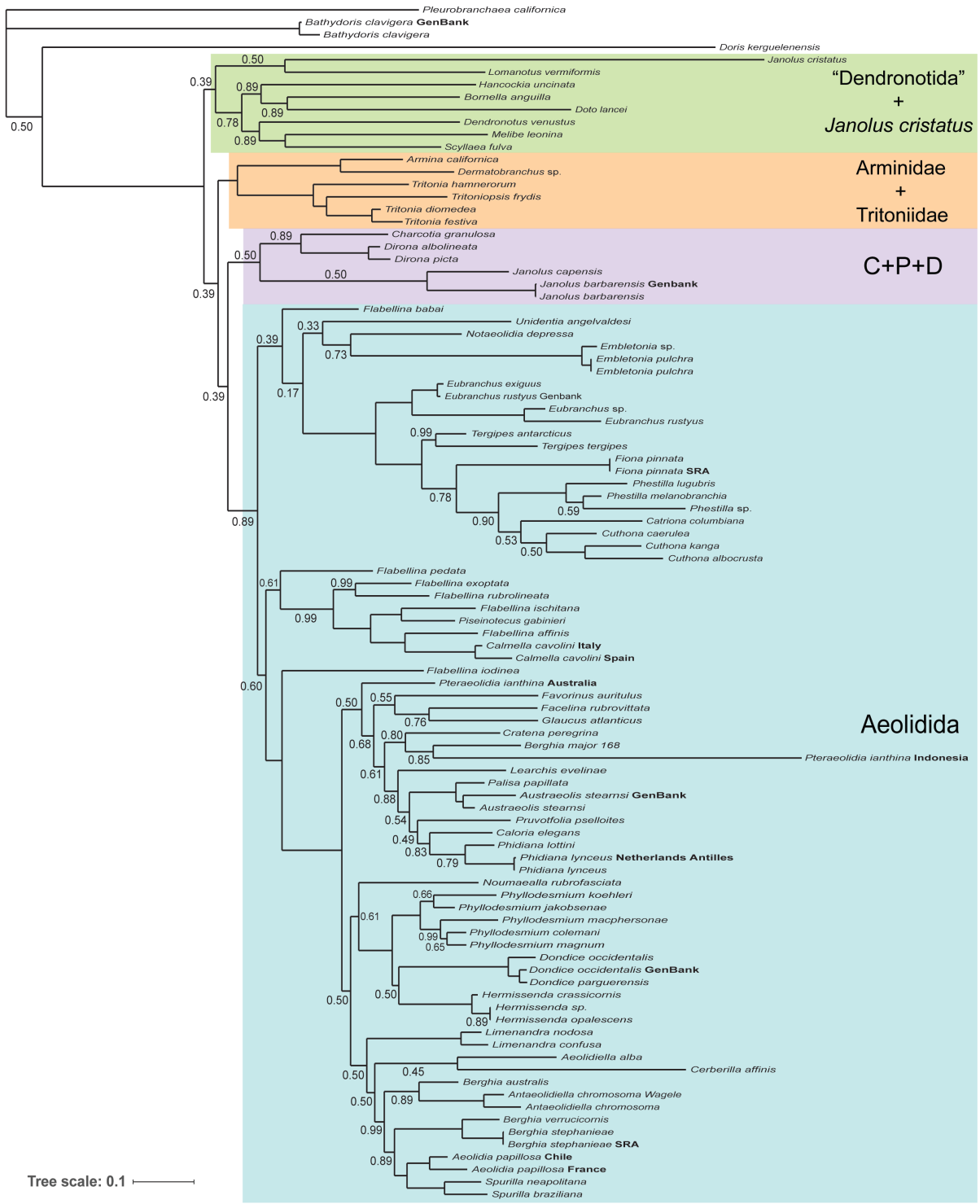

Figure 8.7. Bayesian phylogeny of Cladobranchia using the taxa and genes presented in Appendix Table D1. Posterior probabilities are represented on the branches; nodes with a posterior probability of 1 are empty of notes. Abbreviations: C+P+D, Charcotiidae + Dironidae + Proctonotidae.

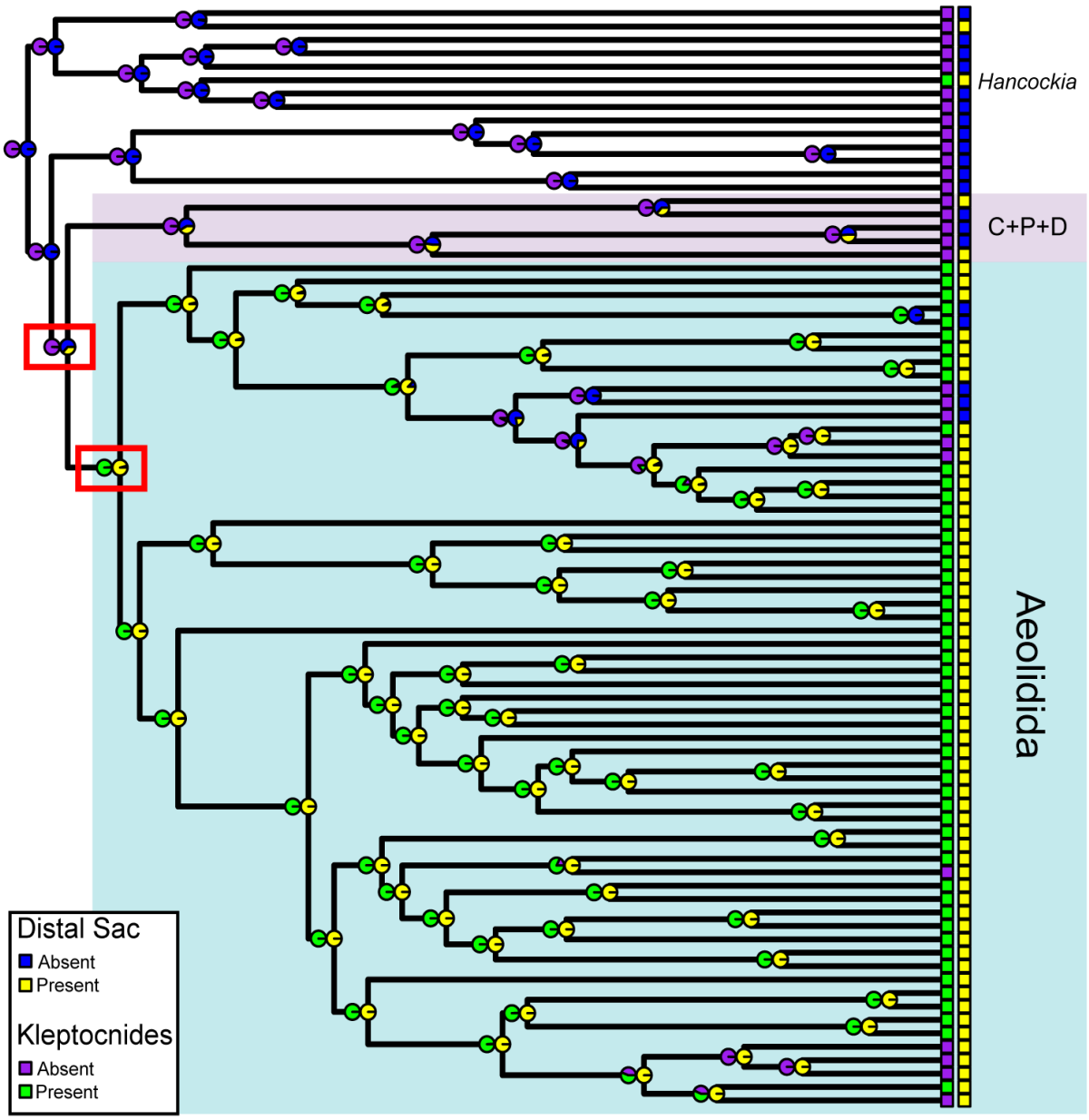

Figure 8.8. Ancestral state reconstruction analysis for the presence of kleptocnides and a distal sac off of the digestive gland. Pie charts on the nodes are scaled marginal likelihoods calculated using the ace function in APE. The red boxes indicate: the node at the base of Aeolidida; and the base of the clade of Aeolidida and Charcotiidae + Dironidae + Proctonotidae (C+P+D).

Discussion

The cnidosac in Cladobranchia

Prior to this study, numerous assumptions have been made about the uniformity of cnidosac character states among cladobranchs that sequester nematocysts [30,61,274]. Edmunds [274] in particular provides drawings that are remarkably consistent across various species from Fionidae, Favorinidae, Facelinidae and Aeolidiidae, though cnidosac descriptions were not the primary purpose of that publication. All of the species illustrated in that work possess a clear entrance connecting the digestive gland with the cnidosac (though in some cases a slight elongation of the entrance is depicted, similar to a channel) and a pore at the tip of the ceras connecting the cnidosac to the exterior. Additionally, all cnidosacs possess nematocysts, and the way the musculature is presented is also very consistent. This has been the most detailed work on aeolid cnidosacs thus far, but it does not truly capture the variation that can be found in this structure across the roughly 600 species of aeolids [33]. In addition, work on the cnidosac-like structures in *Hancockia* spp. and *Embletonia* spp. began only recently [63,278].

In this study, we find that the length, size and structure of the entrance to the cnidosac can vary more than expected based on previous work, as can the structure of the exit, or cnidopore, the musculature surrounding the cnidosac, and the position and orientation of the kleptocnides [30,220,274,385,390,392,395]. The ways in which the nematocysts are taken up by cnidophages also appears to vary across taxa. The transition from the digestive gland to the cnidosac can vary from a wide, open, and simple entrance, such as that found in *Dondice occidentalis* (Figure 8.6B), to a long,

narrow ciliated channel (e.g., *Pteraeolidia ianthina*; Figure 8.6A). Previous work presents only a simple and large entrance to the cnidosac [220,274,392], likely due to the selection of taxa that possess this condition simply by chance. Our work suggests that this is the most common transition between the digestive gland and the cnidosac. However, Hancock and Embleton [390] mention the presence of a ciliated channel in *Aeolidia* (= *Eolis*) *papillosa* and Herdman and Clubb [392] note the presence of a "long, curved connecting duct" in what is now *Facelina bostoniensis* (= *Facelina drummondi)*. A few taxa possess a ciliated channel, including the species from *Aeolidia papillosa*, *Cerberilla amboinensis*, *Cratena peregrina*, *Pteraeolidia ianthina*, and *Flabellina ischitana*. These taxa are not closely related, and therefore the channel is not homologous among the taxa that possess it, suggesting a functional explanation for its presence. We initially suspected that the presence of this elongated structure was related to the size of the kleptocnides, as *A. papillosa*, *C. amboinensis*, and *P. ianthina* all sequester larger kleptocnides. However, this is not supported by *C. peregrina* and *F. ischitana*, as these species sequester smaller nematocysts. Consequently, the possible functional significance of the ciliated channel is unclear.

Similarly, there is little pattern amongst the taxa that possess a proliferation zone versus those that did not. The one exception is the absence of a proliferation zone in taxa from the genus *Phyllodesmium*, which do not sequester nematocysts. The only species within *Phyllodesmium* in which we identified a proliferation zone is *P. jakobsenae*, which is the only species of *Phyllodesmium* known to possess kleptocnides. It is still unclear why other taxa seem to have a proliferation zone while some do not, but we suspect that in some cases artifacts in the preservation of some

samples have led to the destruction of this region, which leads to membrane fragments and free-floating kleptocnides within the cnidosacs of some species (e.g., *Cratena peregrina*; Figure 8.5D). At least one study mentioned the presence of this region [385], but it was not discussed in detail. It is clear that this region is where nematocysts are taken up by cnidophages before migrating towards the distal end of the cnidosac. What is less clear is precisely where the proliferation zone begins. In some species, this appears to be within the cnidosac (e.g., *Pteraeolidia ianthina*; Figure 8.5A), but in others this zone seems to begin within the digestive gland (e.g., *Dondice occidentalis*; Figure 8.5B). In the majority of taxa that sequester nematocysts, we found only very simple exits from the cnidosac, which in some cases is covered with a thin epithelial lining. This lining contains cells similar to that of the epidermis of the cerata, which is composed of elongated columnar cells with many specialized vacuoles. This simple exit is the most common case, both in our study and seemingly in others [274,392]. Additionally, in select taxa within Aeolidiidae, including *Aeolidia papillosa*, *Anteaeolidiella chromosoma*, and *Cerberilla amboinensis*, a cnidopore is present (Figure 8.5C). This pore is complex with an epithelial lining that connects to that of the epidermis, and has never been described in detail before. Previously, the term cnidopore was used to refer to all exits from the cnidosac [220,274]. We hypothesize that the cnidopore may be a special adaptation for releasing the exceptionally long and narrow kleptocnides sequestered from anemones.

Although the musculature surrounding the cnidosac also varies across Aeolidida, the significance of this variation is unclear. Muscle is lacking entirely in

only a few species, including *Embletonia gracilis, Embletonia pulchra* and *Bulbaeolidia alba*. When present, musculature thickness varies across species, ranging from one to multiple layers. This variation in the thickness of the musculature is illustrated in one previous study [274], though not as precisely as we describe here. There is no obvious functional explanation for the variation in muscle thickness or number of muscle layersacross taxa [275,299].

The differentiation of cnidophages from a functional and active cell into a "container or larder" of nematocysts at the tip of the cnidosac may be necessary for the use of these structures, as can be explained by the fact that nematocysts are not functional when taken up by the cnidophages but mature via proton transport while inside [280]. Previous workers have attempted to address the origin of the membrane of the cnidophage [30,282], and recently have concluded that it is a phagosome, a specialized vesicle formed by the cell membrane [30]. Within the cnidophages, the number of kleptocnides may vary both within and among taxa. This appears to be associated with the size of the nematocysts; there tend to be fewer large kleptocnides within a given cnidophage compared to those with smaller kleptocnides. An example can be found in *Pteraeolidia ianthina* (Figure 8.5A), which sequesters nematocysts of multiple size classes.

The cnidosac morphological characters assessed in this study appear to be quite variable within families, but most cnidosacs generally vary on a theme that is conserved across Aeolidida. However, there are still others that have lost some particular strucutes or even the entirety of the cnidosac altogether. For example, species from the genus *Phyllodesmium* (except for *P. jakobsenae*) possess muscle

bound cnidosacs that appear to be devoid of kleptocnides. There are also no obvious entrances from the digestive diverticulum or exits to the external environment, and we hypothesize that the cnidosacs lose this connection to the digestive gland when no nematocysts are sequestered. These species sequester chemicals rather than structures [289], and thus do not necessarily require a structured entrance. In this way, the cnidosacs in *Phyllodesmium* are similar to the Mantle dermal formations in Charcotiidae, which never open directly but release contents when squeezed [396,397]. In species from the genus *Favorinus*, the overall structure of the cnidosac (including the entrance from the digestive gland and muscles around the cnidosac) remains the same, but no kleptocnides are present due to the proclivity of these species for feeding on the eggs of other gastropods [312].

Even more extreme deviations within Aeolidida are found in *Bulbaeolidia alba*, *Embletonia* spp., and species within the genus *Fiona*. *Bulbaeolidia alba,* which has a sac at the distal end of the digestive gland that contains nothing other than occasional cases of zooxanthellae (*Symbiodinium*). In addition, this sac contains no obvious entrance or exit, and appears to be surrounded by a few thin muscle filaments. We hypothesize that the lack of kleptocnides within this sac may be due to the very small size of *B. alba*, which might therefore possess a lower defense requirement, or potentially the size or utility of the nematocysts found within the anemones on which this species feeds [312]. Species of Fionidae within the genus *Fiona* (this study) do not possess cnidosacs, ostensibly because species in this genus prefer non-cnidarian prey items [312].

Sequestered nematocysts have also been found in other families within Cladobranchia, namely Embletoniidae and Hancockiidae. In this study, we find that there are nematocysts housed at the tip of the cerata that seem to be contained within a single cnidophage in *Embletonia gracilis*. This is consistent with previous work on *Embletonia pulchra* and *E. gracilis* [=*E. gracile*], which also found that nematocysts were housed only within cnidophages, without the "organized muscular bags" (i.e., cnidosacs) normally found within aeolids [63]. We also see structures in *Hancockia californica* that are very similar to cnidosacs overall, with kleptocnides housed in cnidophage-like structures within a muscular sac (or multiple muscular sacs) at the tip of each ceras. These structures have also been found in the species *Hancockia uncinata* and *H. schoeferti* [398], and in some cases cnidosacs were found in both the cerata and the rhinophoral sheaths. A discussion regarding questions of homology between the structures found in Embletoniidae and Hancockiidae and those in Aeolidida is provided in the next section.

Phylogeny of Cladobranchia and evolution of the cnidosac

Not unexpectedly, given that much of the molecular data included here is derived from previously published sources, the topology inferred in our phylogenetic analysis (Figure 8.7) is consistent with that found in both recent phylogenomic studies [45], with the exception of one apparently rogue taxon (*Janolus cristatus*). We suspect that this was primarily due to incomplete data, as this taxon is missing 16S, which we have from other taxa within this genus. This work also includes taxa not previously analyzed in the phylogenomic studies, namely taxa from the genera *Phyllodesmium*, *Caloria*, *Pruvotfolia*, *Pteraeolidia*, *Cratena*, *Facelina*, *Glaucus*, *Calmella*,

Piseinotecus, *Tergipes*, *Notaeolidia*, *Embletonia*, and *Charcotia*. The majority of these genera fall within the clades we would expect based on previous studies: *Phyllodesmium* is closely related to *Dondice* within the facelinid clade that is sister to Aeolidiidae [49,110]; *Caloria* is supported within the second facelinid clade and is closely related to species of *Pruvotfolia* [69]; *Facelina*, *Glaucus* and *Cratena* are closely related within the second facelinid clade [110]; *Calmella* is closely related to *Flabellina* and *Piseinotecus* [108]; and *Tergipes* falls within what is now Fionidae [340]. However, the placement of *Pteraeolidia* as closely related to *Cratena* within the second facelinid clade and the position of *Charcotia* within the sister group to Aeolidida are novel to this study (though this position of *Charcotia* was supported by morphology [26].

The position of *Notaeolidia* and *Embletonia* still remains unclear, as has long been the case [63]. In this phylogeny, support for these two genera as sister taxa is poor, and appears to be contributing to the low posterior probabilities at the base of the Aeolidida tree. This may be due to long-branch attraction among the *Notaeolidia, Embletonia,* and *Unidentia* lineages. As such, it is impossible to say with confidence that these are among the earliest branching lineages within Aeolidida, and this uncertainty has implications for our understanding of the evolution of the cnidosac. However, morphological characters suggest a basal position of Notaeolidia within Aeolidida [26,399].

The sequestration of cnidarian nematocysts has originated twice within Cladobranchia based on the phylogeny presented here (Figure 8.8). However, as stated, low support for long branches at the base of Aeolidida containing members of

Notaeolidiidae and Embletoniidae appears to be affecting the support values at the origin of Aeolidida. This uncertainty leaves further questions in regards to the position of the apparently one evolutionary origin at the base of Aeolidida, and does not discount the possibility of additional evolutionary origins of this ability. This result also indicates that species within Aeolidida that do not sequester nematocysts have lost this ability, which seems to have occurred at least three times.

The presence of a sac at the distal end of the digestive gland may have originated prior to that of the sequestration of nematocysts (Figure 8.8), although this result relies on the assumption that the terminal sacs found in Charcotiidae and Proctonotidae [396,397,400,401] are homologous to the sacs found in the cerata of species within Aeolidida. The terminal sacs found in Charcotiidae and Proctonotidae are considered to function as excretory structures, and some have hypothesized that the cnidosac in aeolids is a special modification of this sac which has been adapted for defense [397]. Although the homology in this case remains uncertain, our ancestral state reconstruction provides some support the origin of this character at the ancestral node connecting Aeolidida with the clade containing these two families, in support of this modification hypothesis. One concern may be that low support for the branch leading to this ancestor (PP = 0.39) calls this conclusion into question. However, strong support for this branch when using genomic data lends further strength to this hypothesis [383]. More morphological and molecular data from additional species in the Charcotiidae + Proctonotidae + Dironidae group is necessary to further test this hypothesis. The cnidosac-like structure in Hancockiidae [63,278] appears to have evolved independently from the sac structure in these groups. We

suspect that low support values at the base of Aeolidida (PP = 0.89) and the branch clustering Aeolidida with Charcotiidae + Proctonotidae + Dironidae (PP = 0.39) are due to difficulties placing Embletoniidae and Notaeolidiidae, which form long branches with Unidentiidae at the base of the tree. Our inability to place Embletoniidae is particularly problematic, as this presents challenges in reconstructing the evolution of the cnidosac. There are similarities between this structure [63] and the cnidophage in taxa with cnidosacs, but the structure in Embletoniidae that houses nematocysts appears to be distinct from the cnidosac. A stronger placement of Embletoniidae in the cladobranch phylogeny is necessary before further inferences can be made.

Finally, more than one taxon in Aeolidida seems to have independently lost the cnidosac, including members of *Fiona* and *Tergipes*. This appears to be due to a switch to preying mostly on the non-cnidarian Crustacea in *Fiona* [312], but the precise reason remains unclear for species of *Tergipes*, as these taxa are expected to prey upon hydroids [312].

Conclusions

Here, we describe the morphology of the cnidosac and cnidosac-like structures across all groups in Cladobranchia in which it has been identified, and we discuss possible functions for the variation in structural characters. Overall, we find that cnidosac morphological characters are variable across Cladobranchia, and we provide hypotheses in many cases that might explain the evolutionary patterns found. We find that the sequestration of nematocysts has originated at least twice within Cladobranchia and the sac at the distal end of the digestive gland may have originated

prior to that of the sequestration of nematocysts, but the lack of strong support for our phylogenetic inferences hinders our ability to draw robust conclusions regarding these origins. However, support for the origin of a distal sac prior to that of nematocyst sequestration suggests that the terminal sacs found in Charcotiidae and Proctonotidae are homologous to the cnidosacs found in Aeolidida. Taken together, this research provides a more thorough understanding of the evolution of morphological characters among nematocyst sequestering taxa in Cladobranchia.

Acknowledgements

I would first like to thank my co-authors: Sabrina Bleidißel, Dorothee Schillo, Daniel Ayres, Allen G. Collins, Michael P. Cummings, Ellen E. Strong, and Heike Wägele. We are grateful to Michael Schrödl (Zoologische Staatssammlung München), Craig Hoover (California State Polytechnic University, Pomona), Hans Bertsch (Universidad Autónoma de Baja California), Jeffery Goddard (University of California Santa Barbara Marine Science Institute) and Ariane Dimitris for providing specimens and/or collecting assistance. We would also like to thank Freya Goetz at the Smithsonian National Museum of Natural History for her assistance with histological techniques and Elizabeth Kools for arranging access to the slide collections at the California Academy of Sciences. We are grateful to four high school students that assisted with the histology in this manuscript: Emma Rebour, Maiss Mohamed, Jolanthe Stürmer, and Birthe Priesmann. Lastly, we would like to thank the staff of the Smithsonian Tropical Research Institute in Panama and the Richard B. Gump South Pacific Research Station in French Polynesia for use of their facilities and their help in acquiring the proper permits.

Appendix A: Supplementary Material for Chapter 2

Table A1. MAFFT Alignment parameters used for each gene.

Gene	Alignment parameter	Method
16S	FFT-NS-2	Progressive
H3	FFT-NS-i	Iterative Refinement
COI	FFT-NS-2	Progressive
18S	FFT-NS-i	Iterative Refinement
28S	L-INS-i	Iterative Refinement

Table A2. List of taxa and GenBank sequences used in this study.

Taxon ID	Taxon Name	COI GenBank IDs	H3 GenBank IDs	16S GenBank IDs	18S GenBank IDs	28S GenBank IDs
195873	*Aeolidia papillosa*	JQ997042 JQ997039 JX087536 JQ699565 GQ292049 JQ997038 JQ997041 JX087535 JX087534 JQ997040 AY345028	JQ996934 JX087596 JQ996935 JX087597 JX087598 JQ996936 JQ996937 JQ699385	JX087464 JQ996836 JQ996834 JQ996835 JX087462 JQ996833 JQ699475 JX087463	GU227371	JQ699293
1290779	*Aeolidia* sp. A	JX087532 JX087533 JQ997037 JX087531	JX087593 JQ996933 JX087594 JX087595	JX087459 JQ996832 JX087460 JX087461		
1290780	*Aeolidia* sp. B	JQ997035 JQ997036	JQ996931 JQ996932	JQ996831 JQ996830		
1154711	*Aeolidiella alba*		JQ699386			JQ699294
934974	*Aeolidiella alderi*	HQ616766 HQ616765	HQ616794 HQ616795 JQ996910	HQ616729 JQ996811 HQ616728		
1287571	*Aeolidiella sanguinea*	JX087538 JX087537	JX087599 JX087600	JX087465 JX087466		
1287507	*Aeolidiella stephanieae*	JQ997044	JQ996940	JQ996839		
1288050	Aeolidiidae gen. sp. 'alba'	JQ997016 JQ997017 JQ997013 JQ997015 JQ997012 JQ997014	JQ996909 JQ996908 JQ996907 JQ996905 JQ996906 JQ996904	JQ996806 JQ996805 JQ996810 JQ996808 JQ996809		
1288051	Aeolidiidae gen. sp. 'japonica'	JQ997033	JQ996929	JQ996828		
1290799	Aeolidiidae gen. sp. A	JQ997011	JQ996902 JQ996903	JQ996803 JQ996804		
1290800	Aeolidiidae gen. sp. B	JQ997022 JQ997023	JQ996917 JQ996916	JQ996818 JQ996817		
1394325	Aeolidiidae sp.	KC706903				
1287505	*Aeolidiopsis ransoni*	JQ997043	JQ996938 JQ996939	JQ996837 JQ996838		
1287503	*Anteaeolidiella cacaotica*	JX087528 JQ997030	JQ996926 JX087590	JX087455 JQ996825 JX087457		
1330516	*Anteaeolidiella lurana*	JQ997027 JQ997031	JQ996922 JQ996923 JQ996927	JQ996822 JQ996821 JQ996826		

1287504	*Anteaeolidiella oliviae*	JQ997034	JQ996930	JQ996829		
1291184	*Anteaeolidiella saldanhensis*	JQ997032	JQ996928	JQ996827		
1290782	*Anteaeolidiella* sp. A	JQ997029	JQ996924	JQ996823		
		JQ997028	JQ996925	JQ996824		
1290783	*Anteaeolidiella* sp. B	JQ997020	JQ996914	JQ996815		
1330517	*Anteaeolidiella takanosimensis*	JX087529	JX087592	JX087458		
		JX087530	JX087591	JX087456		
763115	*Armina californica*	GQ292055			GQ326884	
71480	*Armina lovenii*	AF249781		AF249243	AF249196	
1400840	*Armina maculata*	KF369111				
431601	*Armina neapolitana*		EF133469			
797211	*Armina semperi*	HM162696	HM162512	HM162606		
797168	*Armina* sp. 3		HM162513	HM162607		
797169	*Armina* sp. 9		HM162514	HM162608		
930957	*Armina* sp.	HQ010504	HQ010473	HQ010539		
869979	*Austraeolis ornata*	GQ403774		GQ403752		
1154718	*Austraeolis stearnsi*	JQ699571	JQ699395	JQ699483		JQ699303
934965	*Babakina anadoni*	HQ616767	HQ616796	HQ616710		
		HQ616746	HQ616776	HQ616730		
		HQ616747	HQ616806	HQ616711		
		HQ616748	HQ616775	HQ616742		
			HQ616807	HQ616743		
			HQ616805	HQ616744		
			HQ616777	HQ616709		
934966	*Babakina festiva*		HQ616802	HQ616736		
			HQ616801	HQ616735		
			HQ616803			
797244	*Babakina indopacifica*	HM162754	HM162587	HM162678		
929452	*Baeolidia australis*				GU227367	
1287509	*Baeolidia japonica*	JQ997058	JQ996954	JQ996856		
		JQ997059	JQ996956	JQ996855		
		JQ997057	JQ996957	JQ996854		
			JQ996955	JQ996853		
1287510	*Baeolidia moebii*	JX087550	JX087619	JQ996857		
		HQ616771	JQ996958	JQ996858		
		JQ997060	JX087618	HQ616733		
		JQ997061	JQ996959	JX087481		
		JX087551	HQ616800	HQ616734		
		HQ616770	HQ616799	JX087482		
934972	*Baeolidia nodosa*	JQ997080	JX087629	HQ616731	GU339155	
		JX087560	JQ996992	JQ996886		
		HQ616768	JX087630	JX087493		
		JX087559	HQ616797	JX087527		
		JQ997081	JQ996991	JX087494		
1290784	*Baeolidia* sp. A	JQ997056	JQ996953	JQ996851		
		JQ997051	JQ996948	JQ996850		
		JQ997054	JQ996952	JQ996852		
		JQ997055	JQ996951	JQ996847		
1290785	*Baeolidia* sp. B	JQ997046	JQ996943	JQ996842		

1290786	*Baeolidia* sp. C	JQ997045	JQ996941	JQ996840		
1287537	*Berghia cf. salaamica*	JQ997048	JQ996945	JQ996843		
		JQ997047	JQ996944	JQ996844		
1287511	*Berghia coerulescens*	JQ997049	JQ996946	JQ996845		
			JX087604	JX087470		
1287631	*Berghia columbina*	JX087543	JX087608	JX087473		
		JX087545	JX087609	JX087472		
		JX087544	JX087605	JX087474		
		JX087542	JX087606	JX087471		
			JX087607			
1287632	*Berghia rissodominguezi*	JX087552	JX087621	JX087484		
			JX087620	JX087483		
1287506	*Berghia salaamica*	JQ997062	JQ996962	JQ996860		
			JQ996960	JQ996859		
				JQ996862		
1290787	*Berghia* sp. A	JX087549	JX087617	JX087480		
929456	*Berghia verrucicornis*	HQ616750	JX087623	HQ616713	GU227364	
		JX087553	HQ616779	JX087487		
		JX087554	JX087624	JX087486		
		HQ616749	JX087610	JX087485		
			JX087622	HQ616712		
			HQ616778	JX087488		
797246	*Bonisa nakaza*	HM162746	HM162579	HM162670		
1170240	*Bornella anguilla*		JN869424			
			JN869425			
797212	*Bornella calcarata*	HM162707	HM162533	HM162627		
			JN869427			
797213	*Bornella hermanni*	JN869448	JN869421	JN869403		
		JN869446	JN869422	JN869404		
		HM162705	JN869423	JN869402		
		JN869447	HM162531	HM162625		
			JN869420			
797214	*Bornella johnsonorum*	HM162704	JN869419	HM162624		
		JN869445	HM162530	JN869401		
1170241	*Bornella sarape*		JN869428			
219659	*Bornella stellifer*	HM162703	JN869418	JN869400	AY165756	
			JN869417	HM162623	AY165755	
			HM162529			
797215	*Bornella valdae*	JN869449	HM162532	HM162626		
		HM162706	JN869426	JN869405		
71296	*Cadlina laevis*	AY345034		AY345034		
1154735	*Calma glaucoides*	JQ699567	JQ699388	JQ699477		JQ699296
929458	*Calmella cavolini*	HQ616772		HQ616737	GU227361	
929450	*Caloria elegans*	HQ616751	HQ616780	HQ616714	GU227363	
				HQ616738		
376200	*Caloria indica*	DQ417325	JQ699389	DQ417273		JQ699297
1287538	*Caloria* sp. 4	JQ997064	JQ996966	JQ996865		
		JQ997063	JQ996965	JQ996864		
1291185	*Catriona* sp. A	JQ997021	JQ996915	JQ996816		

1290775	*Catriona* sp. B	JQ997024	JQ996919 JQ996918	JQ996819		
929460	*Cerberilla affinis*		JQ996964	JQ996863	GU227366	
1287512	*Cerberilla annulata*		JQ996967	JQ996866		
1287633	*Cerberilla bernadettae*	JX087555	JX087625	JX087489		
1287543	*Cerberilla cf. affinis*	JQ997065	JQ996968	JQ996867		
1287539	*Cerberilla* sp. 3		JQ996976	JQ996873		
1290788	*Cerberilla* sp. A	JQ997069 JQ997070	JQ996978 JQ996975 JQ996977	JQ996874		
1290789	*Cerberilla* sp. B	JQ997068	JQ996973	JQ996872		
1290790	*Cerberilla* sp. C		JQ996974			
696318	*Cf. Tergipes antarcticus*			GU227007		
763139	*Charcotia granulosa*	GQ292060			GQ326885	
154642	*Cratena peregrina*	AF249786 HQ616752	HQ616781	HQ616715	GU339156	
763116	*Cratena pilata*	GQ292053 KC785096				
1170246	*Crosslandia daedali*		JN869444			
1154713	*Cuthona abronia*	JQ699568	JQ699390	JQ699478		JQ699298
154644	*Cuthona caerulea*	AF249807			AF249199	
763117	*Cuthona cocoachroma*	GQ292071			GQ326893	
763118	*Cuthona concinna*	GQ292072			GQ326898	
1154714	*Cuthona divae*	JQ699569	JQ699391	JQ699479		JQ699299
1154715	*Cuthona fulgens*		JQ699392	JQ699480		JQ699300
1154716	*Cuthona lagunae*		JQ699393	JQ699481		JQ699301
219662	*Cuthona nana*				AY165760	AY427448
252567	*Cuthona ocellata*	AY345043		AY345043		
219663	*Cuthona sibogae*				AY165761	
763533	*Cuthona* sp. 1	GQ292068			GQ326899	
763534	*Cuthona* sp. 2	GQ292078			GQ326908 GQ326907	
763535	*Cuthona* sp. 3	GQ292066			GQ326902 GQ326903	
1287545	*Cuthona* sp. 35	JQ997026	JQ996921	JQ996820		
763536	*Cuthona* sp. 4	GQ292069			GQ326901	
763537	*Cuthona* sp. 5	GQ292067			GQ326900	
763538	*Cuthona* sp. 6	GQ292070			GQ326896 GQ326895 GQ326897 GQ326894	
763539	*Cuthona* sp. 7	GQ292074			GQ326892	
763540	*Cuthona* sp. 8	GQ292073			GQ326891	
763541	*Cuthona* sp. 9	GQ292076 GQ292075 GQ292077			GQ326906	
1290791	*Cuthona* sp. A	JQ997019	JQ996913	JQ996814		
763120	*Dendronotus albopunctatus*	GQ292064			GQ326861	

904359	*Dendronotus albus*		HQ267088	GU339186 GU339185		
154605	*Dendronotus dalli*	AF249800		AF249252	AY165757	AY427450
71302	*Dendronotus frondosus*	JN869450 GQ292063 AY345041	HQ267089 JN869429	JN869406 AF249251 GU339187 AY345041	GQ326860 AF249206	
219661	*Dendronotus iris*	GQ292062	HM162537 HQ267090 JN869431	HM162631 GU339188 GU339189 GU339190	AY165758	
797216	*Dendronotus lacteus*	HM162710	HM162538			
1170242	*Dendronotus orientalis*		JN869432			
1054385	*Dendronotus patricki*	HQ225828		HQ225829	HQ225830	
797217	*Dendronotus regius*	JN869451 HM162708	JN869430 HM162535	HM162629 JN869407		
904360	*Dendronotus rufus*		HQ267091	GU339191		
763135	*Dendronotus* sp. 1	GQ292061				
797218	*Dendronotus subramosus*		HM162539 HQ267092	HM162632 GU339197 GU339194 GU339192 GU339195 GU339196 GU339193		
797219	*Dendronotus venustus*	HM162709	HQ267093 HM162536	GU339199 GU339198 GU339200 HM162630		
797220	*Dermatobranchus pustulosus*		HM162516	HM162610		
154607	*Dermatobranchus semistriatus*			AF249244	AF249195	
797170	*Dermatobranchus* sp. 12		HM162518	HM162612		
797171	*Dermatobranchus* sp. 16		HM162519	HM162613		
797172	*Dermatobranchus* sp. 17		HM162520 HM162521	HM162614 HM162615		
797173	*Dermatobranchus* sp. 21	HM162698	HM162522	HM162616		
797174	*Dermatobranchus* sp. 7		HM162517	HM162611		
797175	*Dermatobranchus* sp. A	HM162697	HM162515	HM162609		
934976	*Dicata odhneri*	HQ616773		HQ616739		
763122	*Dirona albolineata*	GQ292058	HM162577	HM162668	GQ326888	
329893	*Dirona picta*	DQ026831				
120394	*Discodoris atromaculata*	AF120637	DQ280013	DQ280054	AF120521	AF120577

1154717	*Dondice occidentalis*	JQ699570	KC526527 KC526536 KC526528 KC526530 KC526533 KC526526 KC526524 KC526529 JQ699394 KC526532 KC526534 KC526531 KC526525 KC526523	KC526513 KC526510 KC526509 KC526517 KC526518 KC526514 KC526512 KC526508 JQ699482 KC526507 KC526506 KC526515 KC526511 KC526519 KC526516		JQ699302
1353478	*Dondice parguerensis*		KC526535	KC526521 KC526522 KC526520		
763123	*Doto antarctica*	GQ292025			GQ326882	
763124	*Doto columbiana*	GQ292026			GQ326881	
154624	*Doto coronata*	HM162734 AF249794	HM162566	HM162657	AF249203	
154610	*Doto eireana*			AF249248	AF249204	
154645	*Doto floridicola*	AF249820			AY165759	
154611	*Doto koenneckeri*	HM162735 AF249797	HM162567	HM162658 AF249249	AF249205	
154612	*Doto pinnatifida*	AF249793		AF249250	AF249202	
797176	*Doto* sp. 15	HM162739	HM162571	HM162662		
797177	*Doto* sp. 2	HM162737	HM162569	HM162660		
797178	*Doto* sp. 7	HM162738	HM162570	HM162661		
797179	*Doto* sp. H	HM162740	HM162572	HM162663		
797180	*Doto* sp. I	HM162741	HM162573	HM162664		
797181	*Doto* sp. J	HM162742	HM162574	HM162665		
797182	*Doto* sp. K	HM162743	HM162575	HM162666		
797221	*Doto ussi*	HM162736	HM162568	HM162659		
76182	*Eubranchus exiguus*	AF249792		AF249246		
763125	*Eubranchus rustyus*	GQ292065			GQ326905	
763126	*Eubranchus sanjuanensis*	GQ292079			GQ326909	
252571	*Eubranchus sp. A*	AY345046		AY345046		
76181	*Eubranchus sp. B*	AF249791				
1287513	*Facelina annulicornis*	JQ997076	JQ996986 JQ996987	JQ996881		
219665	*Facelina bostoniensis*	AY345031		AY345031	AY165763 GU339157	
154649	*Facelina punctata*	AF249816				
1291186	*Facelina* sp. A	JQ997052	JQ996949	JQ996848		

1290792	*Facelina* sp. B	JQ997066 JQ997067	JQ996972 JQ996971 JQ996970 JQ996969	JQ996868 JQ996871 JQ996870 JQ996869 JQ996882		
1290793	*Facelina* sp. C	JQ997072 JQ997093 JQ997073 JQ997092	JQ997004 JQ996981 JQ996982 JQ997005	JQ996898 JQ996877 JQ996876 JQ996897		
1290794	*Facelina* sp. D	JQ997074	JQ996983	JQ996878		
1287568	*Facelinidae* sp. 2	JQ997071 JQ997075	JQ996985 JQ996984	JQ996880 JQ996879		
1290781	*Facelinidae* sp. A	JQ997025	JQ996920			
934967	*Favorinus brachialis*	HQ616761 AY345042	HQ616790	HQ616724 HQ616741 AY345042		
797222	*Favorinus elenalexiarum*	HM162755	JQ699396 HM162588	HM162679 JQ699484		JQ699304
929454	*Favorinus* sp.				GU227369	
1287638	*Fiona pinnata*	JX087558	JX087628	JX087492		
154626	*Flabellina affinis*	HQ616753 AF249783 AY345055	HQ616782	HQ616716 AY345055	AY165767	
763127	*Flabellina amabilis*	GQ292022			GQ326912	
219672	*Flabellina babai*	HQ616754	HQ616783	HQ616717	AY165768	AY427449
934968	*Flabellina baetica*	HQ616755	HQ616784	HQ616718		
929451	*Flabellina bilas*				GU227368	
1287634	*Flabellina confusa*	JX087556 JX087557	JX087627 JX087626	JX087490 JX087491		
1154719	*Flabellina exoptata*	JQ699572	JQ699397	JQ699485		JQ699305
1154720	*Flabellina fusca*	JQ699573	JQ699398	JQ699486		JQ699306
154627	*Flabellina ischitana*	HQ616757 HQ616756 AF249814	HQ616785 HQ616808 HQ616786	HQ616719 HQ616720 HQ616745		
76183	*Flabellina pedata*	HQ616758 AF249817	HQ616787	AF249247 HQ616721		
219673	*Flabellina sp.*				AY165769	
763128	*Flabellina trilineata*	GQ292024	JQ699399	JQ699487	GQ326911	JQ699307
763129	*Flabellina trophina*	GQ292023			GQ326910	
154613	*Flabellina verrucosa*	AF249790		AF249245	AF249198	

1154737	*Glaucus atlanticus*	JQ699595	JQ699403	JQ699510	JQ699312
		JQ699590	JQ699400	JQ699514	JQ699314
		JQ699598	JQ699415	JQ699511	JQ699327
		JQ699576	JQ699414	JQ699508	JQ699337
		JQ699594	JQ699427	JQ699492	JQ699336
		JQ699596	JQ699406	JQ699500	JQ699316
		JQ699588	JQ699416	JQ699495	JQ699317
		JQ699586	JQ699401	JQ699496	JQ699326
		JQ699581	JQ699422	JQ699499	JQ699324
		JQ699583	JQ699409	JQ699490	JQ699329
		JQ699575	JQ699426	JQ699512	JQ699318
		JQ699578	JQ699425	JQ699489	JQ699330
		JQ699574	JQ699412	JQ699513	JQ699321
		JQ699585	JQ699407	JQ699497	JQ699335
		JQ699600	JQ699421	JQ699506	JQ699322
		JQ699602	JQ699405	JQ699507	JQ699308
		JQ699601	JQ699428	JQ699517	JQ699333
		JQ699589	JQ699413	JQ699491	JQ699310
		JQ699580	JQ699410	JQ699494	JQ699332
		JQ699587	JQ699417	JQ699501	JQ699315
		JQ699597	JQ699402	JQ699505	JQ699325
		JQ699599	JQ699419	JQ699488	JQ699323
		JQ699603	JQ699423	JQ699503	JQ699334
		JQ699579	JQ699408	JQ699498	JQ699313
		JQ699592	JQ699429	JQ699502	JQ699331
		JQ699584	JQ699404	JQ699509	JQ699311
		JQ699593	JQ699411	JQ699516	JQ699309
		JQ699591	JQ699418	JQ699504	JQ699319
		JQ699577	JQ699420	JQ699493	JQ699320
		JQ699582	JQ699424	JQ699515	JQ699328

1154738	*Glaucus marginatus*	JQ699607	JQ699459	JQ699552		JQ699339
		JQ699618	JQ699442	JQ699519		JQ699347
		JQ699613	JQ699446	JQ699523		JQ699354
		JQ699616	JQ699451	JQ699518		JQ699342
		JQ699623	JQ699438	JQ699551		JQ699344
		JQ699625	JQ699450	JQ699550		JQ699349
		JQ699605	JQ699435	JQ699529		JQ699370
		JQ699608	JQ699445	JQ699533		JQ699368
		JQ699609	JQ699441	JQ699541		JQ699357
		JQ699604	JQ699436	JQ699549		JQ699338
		JQ699622	JQ699454	JQ699544		JQ699343
		JQ699612	JQ699457	JQ699545		JQ699364
		JQ699610	JQ699432	JQ699543		JQ699346
		JQ699611	JQ699461	JQ699548		JQ699358
		JQ699606	JQ699448	JQ699539		JQ699371
		JQ699627	JQ699433	JQ699521		JQ699345
		JQ699624	JQ699439	JQ699540		JQ699361
		JQ699620	JQ699465	JQ699546		JQ699348
		JQ699619	JQ699463	JQ699528		JQ699362
		JQ699626	JQ699434	JQ699542		JQ699372
		JQ699614	JQ699460	JQ699537		JQ699351
		JQ699615	JQ699447	JQ699530		JQ699366
		JQ699628	JQ699453	JQ699522		JQ699353
		JQ699629	JQ699458	JQ699532		JQ699359
		JQ699617	JQ699464	JQ699553		JQ699341
		JQ699621	JQ699430	JQ699535		JQ699340
			JQ699455	JQ699526		JQ699373
			JQ699440	JQ699547		JQ699355
			JQ699449	JQ699524		JQ699350
			JQ699437	JQ699525		JQ699369
			JQ699462	JQ699520		JQ699365
			JQ699452	JQ699527		JQ699363
			JQ699443	JQ699538		JQ699360
			JQ699431	JQ699531		JQ699352
			JQ699456	JQ699534		JQ699356
			JQ699444	JQ699536		JQ699367
869980	*Godiva banyulensis*	GQ403773	HQ616804	GQ403751	AY165764	
		AF249782		HQ616740		
797223	*Godiva quadricolor*	HM162756	HM162589	HM162680		
797263	*Hancockia californica*	HM162702	HM162527	JN869408		
		JN869452	JN869433	HM162621		
797208	*Hancockia cf. uncinata*		HM162528	HM162622		
252574	*Hancockia uncinata*	AY345047		AY345047		
205593	*Hermissenda crassicornis*	JQ699630	JQ699466	JQ699554		JQ699374
		GQ292054				
1154740	*Hermosita hakunamatata*	JQ699631	JQ699467	JQ699555		JQ699375
797226	*Janolus barbarensis*	HM162747	HM162580	HM162671		
797227	*Janolus capensis*	HM162748	HM162581	HM162672		
154651	*Janolus cristatus*	AF249813			AF249194	
763130	*Janolus fuscus*	GQ292048			GQ326887	
797228	*Janolus longidentatus*	HM162749	HM162582	HM162673		
797229	*Janolus mirabilis*	HM162750	HM162583	HM162674		
797183	*Janolus* sp. 1	HM162751	HM162584	HM162675		

797184	*Janolus* sp. 2	HM162752	HM162585	HM162676		
797185	*Janolus* sp. 7	HM162753	HM162586	HM162677		
1154742	*Learchis poica*	JQ699632	JQ699468	JQ699556		JQ699376
797250	*Leminda millecra*	HM162745	HM162578	HM162669		
1287514	*Limenandra fusiformis*	JQ997077	JQ996988	JQ996883		
		JQ997078	JQ996989	JQ996884		
1290776	*Limenandra* sp. A	JQ997082	HQ616798	JQ996887		
		HQ616769	JQ996993	HQ616732		
1291187	*Limenandra* sp. B	JX087540	JQ996947	JQ996846		
		JX087539	JX087601	JX087469		
		JX087541	JX087602	JX087468		
		JQ997050	JX087603	JX087467		
1290795	*Limenandra* sp. C	JQ997079	JQ996990	JQ996841		
			JQ996942	JQ996885		
797197	*Lomanotus* sp. E	HM162715	HM162547	HM162640		
1170243	*Lomanotus* sp.	JN869453	JN869434	JN869409		
1170244	*Lomanotus vermiformis*		JN869435			
797254	*Marianina rosea*	HM162733	HM162565	HM162656		
797230	*Marionia arborescens*	HM162722	HM162554	HM162646		
154647	*Marionia blainvillea*	AF249812	HM162553	HM162645		
		HM162721				
797231	*Marionia distincta*	HM162725	HM162557	HM162648		
797232	*Marionia elongoviridis*	HM162724	HM162556			
797233	*Marionia levis*	HM162723	HM162555	HM162647		
797188	*Marionia* sp. 10	HM162728	HM162560	HM162651		
797189	*Marionia* sp. 14	HM162729	HM162561	HM162652		
797190	*Marionia* sp. 5	HM162727	HM162559	HM162650		
904361	*Marionia* sp. A			GU339201		
857010	*Marionia* sp. B	HM162726	HM162558	HM162649		
1370030	*Melibe arianeae*	KC992314	KC992315	KC992313		
797234	*Melibe digitata*	HM162699	HM162523	JX306061		
		JX306069	JX306076	HM162617		
797235	*Melibe engeli*		HM162525	JX306062		
			JX306077	HM162619		
76178	*Melibe leonina*	GQ292059		GU339202		
797236	*Melibe rosea*	JX306070	JX306081	HM162620		
		JX306071	JX306082	JX306063		
		JX306073	JX306079	JX306064		
		JX306072	JX306080	JX306066		
		JX306074	JX306078	JX306065		
		HM162701	HM162526			
1239314	*Melibe sp.*		JX306084			
499938	*Melibe viridis*	HM162700	JX306083	HM162618		
		JX306075	HM162524	JX306068		
1287517	*Moridilla brockii*	JQ997083	JQ996994	JQ996888		
1154744	*Nanuca sebastiani*	JQ699633	JQ699469	JQ699557		JQ699377
219676	*Notaeolidia depressa*	GQ292057			AY165770	
					GQ326886	
1171428	*Notobryon panamica*		JN869440			

797199	*Notobryon* sp. B		HM162541	HM162634
797200	*Notobryon* sp. C	HM162712	HM162542	HM162635
797201	*Notobryon* sp. D	HM162713	HM162543	HM162636
1171429	*Notobryon thompsoni*	JN869455	JN869438	JN869412
		JN869456	JN869439	JN869413
797237	*Notobryon wardi*	JN869454	HM162546	HM162637
		HM162714	JN869436	HM162639
			HM162545	JN869411
			JN869437	JN869410
			HM162544	HM162638
1287519	*Noumeaella isa*	JQ997084	JQ996995	JQ996889
1290774	*Noumeaella rehderi*		JQ996961	JQ996861
1287556	*Noumeaella* sp. 3	JQ997087	JQ996999	JQ996893
		JQ997088	JQ996998	JQ996892
1287557	*Noumeaella* sp. 4	JQ997085	JQ997003	JQ996894
		JQ997090	JQ997000	JQ996890
		JQ997091	JQ997001	JQ996891
		JQ997086	JQ996997	JQ996896
		JQ997089	JQ996996	JQ996895
			JQ997002	
1290796	*Noumeaella* sp. A	JQ997053	JQ996950	JQ996849
1290797	*Noumeaella* sp. B	JX087548	JX087616	JX087479
376196	*Phestilla lugubris*	DQ417299		DQ417253
		DQ417300		DQ417252
		DQ417298		DQ417254
376192	*Phestilla melanobrachia*	DQ417281		DQ417228
		DQ417277		DQ417233
		DQ417274		DQ417231
		DQ417280		DQ417236
		DQ417282		DQ417235
		DQ417278		DQ417230
		DQ417279		DQ417232
		DQ417275		DQ417229
		DQ417276		DQ417234
376197	*Phestilla minor*	DQ417311		DQ417263
		DQ417301		DQ417257
		DQ417307		DQ417258
		DQ417308		DQ417262
		DQ417310		DQ417264
		DQ417305		DQ417260
		DQ417303		DQ417256
		DQ417304		DQ417261
		DQ417313		DQ417259
		DQ417309		DQ417255
		DQ417302		
		DQ417312		
		DQ417306		

376195	*Phestilla sibogae*	DQ417297 DQ417292 DQ417293 DQ417296 DQ417288 DQ417287 DQ417294 DQ417291 DQ417290 DQ417295 DQ417289		DQ417245 DQ417249 DQ417242 DQ417251 DQ417248 DQ417241 DQ417246 DQ417247 DQ417244 DQ417250 DQ417243		
376198	*Phestilla* sp. 1	DQ417320 DQ417324 DQ417322 DQ417314 DQ417316 DQ417315 DQ417319 DQ417323 DQ417318 DQ417317 DQ417321		DQ417272 DQ417270 DQ417269 DQ417266 DQ417268 DQ417267 DQ417271 DQ417265		
376194	*Phestilla* sp. 2	DQ417286 DQ417285 DQ417284 DQ417283		DQ417238 DQ417237 DQ417239 DQ417240		
1154721	*Phidiana hiltoni*		JQ699470	JQ699558		JQ699378
219669	*Phidiana lynceus*	JX087562 JX087563	JX087633 JX087634	JX087497 JX087498	AY165765	
1287508	*Phidiana militaris*		JQ996979	JQ996875		
219671	*Phyllodesmium briareum*	HQ010492 HQ010480 GQ403775 HQ010491	HQ010442 HQ010459 HQ010460	HQ010510 HQ010528 GQ403753 HQ010527	GU339158	
869976	*Phyllodesmium cf. magnum*	GQ403785 GQ403784		GQ403762 GQ403763		
869965	*Phyllodesmium colemani*	HQ010499 GQ403777 HQ010498 GQ403776	HQ010466 HQ010467	GQ403755 GQ403754 HQ010534	GU339159	
869966	*Phyllodesmium crypticum*	HQ010507 HQ010502	HQ010477 HQ010470 HQ010471	HQ010537 GQ403770 HQ010536 HQ010543	GU339160	
797238	*Phyllodesmium horridum*	HM162757	HQ010445 HM162590	HQ010513 HM162681 HQ010514		
869967	*Phyllodesmium hyalinum*	GQ403778		GQ403756		

869968	*Phyllodesmium jakobsenae*	HQ010488 HQ010489 GQ403779 GQ403781	HQ010455 HQ010456	GQ403757 GQ403759 HQ010524	GU339162	
869969	*Phyllodesmium kabiranum*		HQ010454 HQ010444	HQ010512 HQ010523 GQ403766 GQ403767		
930959	*Phyllodesmium karenae*	HQ010483 HQ010508	HQ010478 HQ010448	HQ010544 HQ010517		
869970	*Phyllodesmium koehleri*	HQ010494 GQ403782	HQ010462	GQ403760 HQ010530		
869971	*Phyllodesmium lembehensis*	GQ403780		GQ403758 GQ403771		
674072	*Phyllodesmium lizardensis*	HQ010505 HQ010496	HQ010464 HQ010474	GQ403772 HQ010540 HQ010532		
869972	*Phyllodesmium longicirrum*	JQ699634 GQ403783	JQ699471	GQ403761 JQ699559	GU339161	JQ699379
869973	*Phyllodesmium macphersonae*	HQ010487 HQ010479 HQ010482	HQ010446 HQ010453 HQ010441	GQ403769 HQ010522 HQ010509 HQ010515 GQ403768		
869964	*Phyllodesmium magnum*	HQ010500 HQ010481	HQ010443 HQ010468	HQ010511		
930960	*Phyllodesmium opalescens*	HQ010484 HQ010485	HQ010450 HQ010449 HQ010451	HQ010519 HQ010518 HQ010520		
930961	*Phyllodesmium parangatum*	HQ010501 HQ010506 JQ699635	JQ699472 HQ010475 HQ010469 HQ010476	JQ699560 HQ010542 HQ010535 HQ010541		JQ699380
930962	*Phyllodesmium pinnatum*		HQ010458	HQ010526		
869974	*Phyllodesmium poindimiei*	GQ403786 HQ010495 HQ010486	HQ010452 HQ010463	HQ010531 GQ403764 HQ010521		
869975	*Phyllodesmium rudmani*	GQ403787 HQ010493	HQ010461	HQ010529 GQ403765		
930958	*Phyllodesmium* sp. 2		HQ010447	HQ010516		
930963	*Phyllodesmium tuberculatum*	HQ010490 HQ010497	HQ010465 HQ010457	HQ010525 HQ010533		
797256	*Pinufius rebus*	HM162744	HM162576	HM162667		
1287625	*Piseinotecus gabinierei*	JX087561	JX087632 JX087631	JX087496 JX087495		
934969	*Piseinotecus gaditanus*	HQ616759	HQ616788	HQ616722		
797203	*Piseinotecus* sp.	HM162694	HM162510	HM162604		
1154746	*Protaeolidiella atra*			JQ699561		JQ699381
1287515	*Protaeolidiella juliae*	JQ997094	JQ997007	JQ996899		

934978	*Pruvotfolia longicirrha*	HQ616760	HQ616789	HQ616723		
934979	*Pruvotfolia pselliotes*	HQ616762	HQ616791	HQ616725		
1290777	*Pruvotfolia* sp. A		JQ997008			
1290778	*Pruvotfolia* sp. B		JQ996980			
797258	*Pseudobornella orientalis*		HM162534	HM162628		
929462	*Pteraeolidia ianthina*		JQ699473 JQ997006	JQ699562	GU227370	JQ699382
797260	*Sakuraeolis enosimensis*	HQ010503 HM162758	HQ010472 HM162591	HQ010538 HM162682		
797262	*Scyllaea pelagica*	HM162711 JN869458 JN869459 JN869457	HM162540 JN869441 JN869442 JN869443	JN869415 JN869414 HM162633 JN869416		
1449875	*Spurilla braziliana*	JX087575 JX087568 JQ997095 JQ997097 JX087567 JX087578 JQ997096	JQ997009 JX087639 JX087638 JX114844 JX087644 JX087647	JX087503 JQ996900 JX087508 JX087511		
1154712	*Spurilla chromosoma*	JQ699566 JQ997018	JQ996912 JQ699387 JQ996911	JQ996812 JQ699476 JQ996813		JQ699295
1287630	*Spurilla creutzbergi*	JX087547 JX087546	JX087614 JX087613 JX087615 JX087612	JX087475 JX087477 JX087478 JX087476		
910326	*Spurilla major*				GU227365	

929453	*Spurilla neapolitana*	JX087574 JX087583 JX087586 JX087566 JX087576 JX087570 JQ699636 JX087569 HQ616764 JX087572 JX087571 JX087581 JX087577 HQ616763 JX087564 JX087585 JX087584 JX087582 JX087587 JX087573	JX087655 JX087656 JX087646 JX087643 JX087645 JX087640 JX087661 JX087651 JX087642 HQ616792 JX087652 JX087658 JX087635 JX087650 JX114845 JX087654 JX087660 JX087637 JX087641 JX087659 JX087657 JQ699474 JX087662 JX087653 HQ616793	JX087509 HQ616726 JX087502 HQ616727 JX087517 JX087521 JX087523 JX087520 JX087504 JX087519 JX087518 JX087506 JX087499 JX087516 JX087510 JX087524 JX114843 JX087507 JX087522 JX087514 JX087515 JQ699563 JX087500 JX087505 JX114842	GU227362	JQ699383
1154722	*Spurilla sargassicola*	JX087589 JX087588 JQ997098	JX087663 JX087664 JQ997010 JX087665	JQ996901 JX087525 JQ699564 JX087526		JQ699384
1290798	*Spurilla* sp. A	JX087565 JX087580 JX087579	JX087649 JX087636 JX087648	JX087501 JX087513 JX087512		
530585	*Tergipes antarcticus*	EU727251 EU727252 EU727253 GU227106 EU727250		KF713480		
157144	*Tergipes tergipes*	AY345032		AY345032	AF249197	
252556	*Tethys fimbria*		EF133468			
797240	*Tritonia antarctica*	HM162718	HM162550	HM162643		
763132	*Tritonia challengeriana*	GQ292052			GQ326904	
70853	*Tritonia diomedea*	GQ292050		GU339203	GQ326890	
763133	*Tritonia festiva*	GQ292051 HM162719	HM162551		GQ326889	
157146	*Tritonia nilsodhneri*	HM162716	HM162548	HM162641	AF249200	
797241	*Tritonia pickensi*	HM162717	HM162549	HM162642		
797192	*Tritonia* sp. 3	HM162731	HM162563	HM162654		
797193	*Tritonia* sp. 4	HM162732	HM162564	HM162655		
797194	*Tritonia* sp. F	HM162720	HM162552	HM162644		

797195	*Tritonia* sp. G	HM162730	HM162562	HM162653	
157148	*Tritoniella belli*	GQ292056		GU227002	AF249201
		GU227111			GQ326883

Appendix B: Supplementary Material for Chapter 3

Table B1. Table of sequence read information for each sample before and after autoadapt filtering.

Sample	Species	Number of reads	Read 1 Length (R1)	Read 2 Length (R2)	R1 after autoadapt	R2 after autoadapt	# of reads after autoadapt	Reads removed
SRR1505104	*Bathydoris clavigera*	25,756,442	100	100	12,813,017	12,813,017	25,626,034	0.51%
SRR1505108	*Doris kerguelenensis*	29,570,328	90	90	14,631,290	14,631,290	29,262,580	1.04%
SRR1505109	*Fiona pinnata*	32,901,186	100	100	16,264,740	16,264,740	32,529,480	13%
SRR1505130	*Pleurobranchaea californica*	37,446,108	130	130	18,642,322	18,642,322	37,284,644	0.43%
SRR1950943	*Austraeolis stearnsi*	44,805,574	101	101	21,523,548	21,523,548	43,047,096	3.92%
SRR1950951	*Berghia stephanieae*	49,132,190	101	101	24,114,455	24,114,455	48,228,910	1.84%
SRR1950949	*Catriona columbiana*	56,575,094	101	101	26,266,693	26,266,693	52,533,386	74%
SRR1950944	*Cuthona albocrusta*	64,049,004	101	101	31,577,758	31,577,758	63,155,516	1.40%
SRR1950948	*Dendronotus venustus*	56,770,684	101	101	27,923,122	27,923,122	55,846,244	1.63%
SRR1950946	*Dirona picta*	53,926,908	101	101	26,346,637	26,346,637	52,693,274	2.29%
SRR1950953	*Dondice occidentalis*	64,970,546	101	101	30,641,247	30,641,247	61,282,494	5.68%
SRR1950945	*Doto lancei*	51,615,104	101	101	25,081,463	25,081,463	50,162,926	2.81%
SRR1950950	*Favorinus auritulus*	53,995,048	101	101	26,582,960	26,582,960	53,165,920	1.54%
SRR1950940	*Flabellina iodinea*	65,504,176	101	101	29,757,962	29,757,962	59,515,924	94%
SRR1950939	*Hermissenda crassicornis*	54,646,466	101	101	25,563,781	25,563,781	51,127,562	6.44%
SRR1950942	*Janolus barbarensis*	48,949,876	101	101	23,311,257	23,311,257	46,622,514	4.75%
SRR1950947	*Melibe leonina*	52,242,406	101	101	25,285,526	25,285,526	50,571,052	3.20%
SRR1950952	*Palisa papillata*	47,519,642	101	101	22,685,063	22,685,063	45,370,126	4.52%
SRR1950941	*Tritonia festiva*	54,723,130	101	101	26,341,769	26,341,769	52,683,538	3.73%
SRR1950954	*Tritoniopsis frydis*	54,963,702	101	101	26,589,409	26,589,409	53,178,818	3.25%

Table B2. Trinity-assembled transcriptome details, including number of transcript fragments and total number of bases assembled, as well as the N50 and L50 for each transcriptome.

Sample	Species	number of transcript fragments	total bases	N50	L50
SRR1505104	*Bathydoris clavigera*	139042	63990068	521	35319
SRR1505108	*Doris kerguelenensis*	126589	50303325	408	33722
SRR1505109	*Fiona pinnata*	99009	58162124	766	20300
SRR1505130	*Pleurobranchaea californica*	242632	115142632	548	58832
SRR1950943	*Austraeolis stearnsi*	94712	56736760	800	18037
SRR1950951	*Berghia stephanieae*	156675	76998938	571	34802
SRR1950949	*Catriona columbiana*	86556	46777284	689	17293
SRR1950944	*Cuthona albocrusta*	117918	63368478	677	23970
SRR1950948	*Dendronotus venustus*	126739	73686296	788	23103
SRR1950946	*Dirona picta*	71876	43786699	853	13242
SRR1950953	*Dondice occidentalis*	108858	57951959	667	22149
SRR1950945	*Doto lancei*	82816	48555738	784	16510
SRR1950950	*Favorinus auritulus*	116780	66416388	754	22449
SRR1950940	*Flabellina iodinea*	108593	67954563	893	19097
SRR1950939	*Hermissenda crassicornis*	92712	57790886	871	17077
SRR1950942	*Janolus barbarensis*	56091	27833962	592	12294
SRR1950947	*Melibe leonina*	77391	49868130	921	13784
SRR1950952	*Palisa papillata*	104095	58566669	727	20746
SRR1950941	*Tritonia festiva*	75944	41890333	707	16064
SRR1950954	*Tritoniopsis frydis*	94124	53070144	748	19061

Table B3. HaMStR statistics for each transcriptome.

Sample	Species	Sequences matching orthologous groups	Unique orthologous groups represented	Mean length (in amino acids)
SRR1505104	*Bathydoris clavigera*	1537	997	239
SRR1505108	*Doris kerguelenensis*	1236	899	213
SRR1505109	*Fiona pinnata*	1405	997	277
SRR1505130	*Pleurobranchaea californica*	1765	1126	237
SRR1950943	*Austraeolis stearnsi*	1168	948	264
SRR1950951	*Berghia stephanieae*	1209	978	243
SRR1950949	*Catriona columbiana*	997	847	254
SRR1950944	*Cuthona albocrusta*	1191	939	268
SRR1950948	*Dendronotus venustus*	1409	1075	282

SRR1950946	*Dirona picta*	1076	901	243
SRR1950953	*Dondice occidentalis*	1163	950	237
SRR1950945	*Doto lancei*	865	736	235
SRR1950950	*Favorinus auritulus*	1307	1034	272
SRR1950940	*Flabellina iodinea*	1221	992	301
SRR1950939	*Hermissenda crassicornis*	1218	966	253
SRR1950942	*Janolus barbarensis*	662	599	190
SRR1950947	*Melibe leonina*	1102	953	273
SRR1950952	*Palisa papillata*	1191	948	250
SRR1950941	*Tritonia festiva*	913	775	184
SRR1950954	*Tritoniopsis frydis*	799	651	232

Table B4. Data matrix statistics for each of our six data matrices.

Data Matrix	Number of nucleotide positions	Percent complete	Percentage of ambiguous sites
nt123_unfiltered	1,702,782	23	0.04
nt123	703,665	46.4	0.08
degen	703,665	46.4	17
nt123_min80percentcomplete	100,953	87.9	02
nt123_min90percentcomplete	48,426	93.6	06
nt123_100percentcomplete	9,354	100	08

Appendix C: Supplementary Material for Chapter 7

Table C1. Table of sequence read information for each sample.

Sample	Species	Number of reads	Read 1 Length (R1)	Read 2 Length (R2)
SRR3726702	*Aeolidiella alba*	40,239,806	101	101
SRR3726695	*Anteaeolidiella chromosoma*	44,057,108	101	101
SRR4124996	*Armina californica*	49,137,024	125	125
SRR1950943	*Austraeolis stearnsi*	44,805,574	101	101
SRR1505104	*Bathydoris clavigera*	25,756,442	100	100
SRR1950951	*Berghia stephanieae*	49,132,190	101	101
SRR3726697	*Bornella anguilla*	30,903,660	101	101
SRR1950949	*Catriona columbiana*	56,575,094	101	101
SRR1950944	*Cuthona albocrusta*	64,049,004	101	101
SRR1950948	*Dendronotus venustus*	56,770,684	101	101
SRR3726698	*Dermatobranchus sp.*	38,595,038	101	101
SRR1950946	*Dirona picta*	53,926,908	101	101
SRR1950953	*Dondice occidentalis*	64,970,546	101	101
SRR3726707	*Dondice parguerensis*	41,188,580	101	101
SRR1505108	*Doris kerguelenensis*	29,570,328	90	90
SRR1950945	*Doto lancei*	51,615,104	101	101
SRR3726692	*Eubranchus rustyus*	44,900,716	101	101
SRR1950950	*Favorinus auritulus*	53,995,048	101	101
SRR1505109	*Fiona pinnata*	32,901,186	100	100
SRR1950940	*Flabellina iodinea*	65,504,176	101	101
SRR3726694	*Hancockia uncinata*	44,066,522	101	101
SRR1719366	*Hermissenda crassicornis*	54,646,466	101	101
SRR1950939	*Hermissenda opalescens*	109,387,186	100	100
SRR1950942	*Janolus barbarensis*	48,949,876	101	101
SRR3726693	*Learchis evelinae*	39,094,232	101	101
SRR3726703	*Limenandra confusa*	39,556,892	101	101
SRR3726706	*Lomanotus vermiformis*	47,217,216	101	101
SRR1950947	*Melibe leonina*	52,242,406	101	101
SRR3726700	*Noumeaella rubrofasciata*	37,270,102	101	101
SRR1950952	*Palisa papillata*	47,519,642	101	101
SRR3726699	*Phestilla sp.*	42,380,642	101	101
SRR3726705	*Phidiana lynceus*	39,230,598	101	101
SRR1505130	*Pleurobranchaea californica*	37,446,108	130	130

SRR3726701	*Scyllaea fulva*	43,374,630	101	101
SRR3726704	*Spurilla braziliana*	41,223,098	101	101
SRR1721590	*Tritonia diomedea*	133,156,930	100	100
SRR1950941	*Tritonia festiva*	54,723,130	101	101
SRR4190242	*Tritonia hamnerorum*	51,064,334	125	125
SRR1950954	*Tritoniopsis frydis*	54,963,702	101	101
SRR3726696	*Unidentia angelvaldesi*	37,237,790	101	101

Table C2. Trinity-assembly details, including number of transcript fragments and total number of bases assembled, as well as N50 and L50 statistics for each transcriptome.

Sample	Species	Number of transcript fragments	Total bases	N50	L50
SRR3726702	*Aeolidiella alba*	135,658	68,829,944	587	30,234
SRR3726695	*Anteaeolidiella chromosoma*	105,807	54,712,282	609	23,104
SRR4124996	*Armina californica*	164,304	112,937,294	1,058	25,105
SRR1950943	*Austraeolis stearnsi*	117,007	72,595,298	828	22,000
SRR1505104	*Bathydoris clavigera*	178,144	77,513,189	468	47,797
SRR1950951	*Berghia stephanieae*	193,197	98,802,980	595	42,296
SRR3726697	*Bornella anguilla*	93,537	51,612,871	696	19,285
SRR1950949	*Catriona columbiana*	131,762	70,968,477	659	25,940
SRR1950944	*Cuthona albocrusta*	166,300	89,001,674	652	33,678
SRR1950948	*Dendronotus venustus*	159,696	95,693,636	811	28,459
SRR3726698	*Dermatobranchus sp.*	107,195	46,826,108	460	27,642
SRR1950946	*Dirona picta*	91,926	56,135,288	835	16,979
SRR1950953	*Dondice occidentalis*	138,158	74,488,947	658	29,076
SRR3726707	*Dondice parguerensis*	129,030	69,460,123	655	24,857
SRR1505108	*Doris kerguelenensis*	141,651	55,197,023	395	40,075
SRR1950945	*Doto lancei*	113,713	69,028,271	820	21,676
SRR3726692	*Eubranchus rustyus*	113,670	69,002,480	821	20,256
SRR1950950	*Favorinus auritulus*	160,725	89,596,195	695	32,274
SRR1505109	*Fiona pinnata*	124,230	68,609,332	680	26,095
SRR1950940	*Flabellina iodinea*	137,325	86,800,292	870	24,392
SRR3726694	*Hancockia uncinata*	131,924	77,477,062	762	24,038
SRR1719366	*Hermissenda crassicornis*	120,902	78,490,056	932	20,668
SRR1950939	*Hermissenda opalescens*	244,299	136,233,417	712	42,866
SRR1950942	*Janolus barbarensis*	71,967	36,318,001	581	16,144
SRR3726693	*Learchis evelinae*	136,824	69,829,187	580	29,383
SRR3726703	*Limenandra confusa*	229,944	130,831,914	749	46,534

SRR3726706	*Lomanotus vermiformis*	101,986	53,386,921	604	21,134
SRR1950947	*Melibe leonina*	99,326	66,000,458	965	16,878
SRR3726700	*Noumeaella rubrofasciata*	121,254	71,634,931	771	23,062
SRR1950952	*Palisa papillata*	141,805	80,435,497	722	27,851
SRR3726699	*Phestilla sp.*	195,785	102,593,153	642	39,279
SRR3726705	*Phidiana lynceus*	128,619	79,261,824	827	22,894
SRR1505130	*Pleurobranchaea californica*	295,127	135,826,770	508	75,183
SRR3726701	*Scyllaea fulva*	151,905	88,809,531	767	26,965
SRR3726704	*Spurilla braziliana*	159,038	86,406,689	659	30,498
SRR1721590	*Tritonia diomedea*	243,272	147,329,750	809	41,106
SRR1950941	*Tritonia festiva*	100,613	57,165,768	730	20,555
SRR4190242	*Tritonia hamnerorum*	227,362	153,676,892	990	35,869
SRR1950954	*Tritoniopsis frydis*	126,224	70,763,758	721	25,955
SRR3726696	*Unidentia angelvaldesi*	124,907	73,100,445	757	22,728

Table C3. HaMStR statistics for each RNA-Seq dataset.

Sample	Species	Sequences matching orthologous groups	Unique orthologous groups represented	Mean length (in amino acids)
SRR3726702	*Aeolidiella alba*	1086	820	227
SRR3726695	*Anteaeolidiella chromosoma*	991	795	227
SRR4124996	*Armina californica*	1609	1025	442
SRR1950943	*Austraeolis stearnsi*	1226	953	268
SRR1505104	*Bathydoris clavigera*	1641	1017	226
SRR1950951	*Berghia stephanieae*	1249	959	261
SRR3726697	*Bornella anguilla*	911	770	228
SRR1950949	*Catriona columbiana*	1122	876	269
SRR1950944	*Cuthona albocrusta*	1306	956	266
SRR1950948	*Dendronotus venustus*	1617	1085	294
SRR3726698	*Dermatobranchus sp.*	615	512	193
SRR1950946	*Dirona picta*	1207	927	244
SRR1950953	*Dondice occidentalis*	1258	969	250
SRR3726707	*Dondice parguerensis*	1344	1021	296
SRR1505108	*Doris kerguelenensis*	1312	899	207
SRR1950945	*Doto lancei*	974	760	234
SRR3726692	*Eubranchus rustyus*	1222	973	291
SRR1950950	*Favorinus auritulus*	1400	1041	280
SRR1505109	*Fiona pinnata*	1446	997	282

SRR1950940	*Flabellina iodinea*	1358	1019	320
SRR3726694	*Hancockia uncinata*	1351	1031	351
SRR1719366	*Hermissenda crassicornis*	1828	1077	399
SRR1950939	*Hermissenda opalescens*	1468	1004	264
SRR1950942	*Janolus barbarensis*	722	623	187
SRR3726693	*Learchis evelinae*	1280	964	248
SRR3726703	*Limenandra confusa*	1400	980	253
SRR3726706	*Lomanotus vermiformis*	1015	862	261
SRR1950947	*Melibe leonina*	1228	968	289
SRR3726700	*Noumeaella rubrofasciata*	1265	971	265
SRR1950952	*Palisa papillata*	1275	960	254
SRR3726699	*Phestilla sp.*	1453	1071	258
SRR3726705	*Phidiana lynceus*	1376	1016	280
SRR1505130	*Pleurobranchaea californica*	1938	1138	240
SRR3726701	*Scyllaea fulva*	1377	1032	346
SRR3726704	*Spurilla braziliana*	1284	983	303
SRR1721590	*Tritonia diomedea*	1883	1089	474
SRR1950941	*Tritonia festiva*	1031	797	191
SRR4190242	*Tritonia hamnerorum*	2013	1198	499
SRR1950954	*Tritoniopsis frydis*	901	685	232
SRR3726696	*Unidentia angelvaldesi*	1337	1027	328

Table C4. Prey preference data used for the ancestral state reconstruction.

Species	Prey preference	Reference
Aeolidiella alba	Anthozoa: Hexacorallia	McDonald & Nybakken 1997, Rudman 2002
Anteaeolidiella chromosoma	Anthozoa: Hexacorallia	McDonald & Nybakken 1997 [as *Aeolidiella chromosoma*], Rudman 2002 [as *Spurilla chromosoma*]
Armina californica	Anthozoa: Octocorallia	McDonald & Nybakken 1997
Austraeolis stearnsi	Hydrozoa	Wägele 2004
Berghia stephanieae	Anthozoa: Hexacorallia	Wägele 2004, Valdés *et al.* 2006
Bornella anguilla	Hydrozoa	McDonald & Nybakken 1997, Wägele 2004, Putz *et al*. 2010
Catriona columbiana	Hydrozoa	McDonald & Nybakken 1997, Wägele 2004
Cuthona albocrusta	Hydrozoa	McDonald & Nybakken 1997, Wägele 2004
Dendronotus venustus	Hydrozoa	McDonald & Nybakken 1997, Wägele 2004 [as *D. frondosus*]
Dermatobranchus sp.	Anthozoa: Octocorallia	Wägele 2004, Putz *et al.* 2010
Dirona picta	Bryozoa & Hydrozoa	McDonald & Nybakken 1997

Dondice occidentalis	Hydrozoa	McDonald & Nybakken 1997
Dondice parguerensis	Scyphozoa	McDonald & Nybakken 1997
Doto lancei	Hydrozoa	McDonald & Nybakken 1997, Wägele 2004
Eubranchus rustyus	Hydrozoa	McDonald & Nybakken 1997, Wägele 2004
Favorinus auritulus	Gastropoda eggs	McDonald & Nybakken 1997
Fiona pinnata	Crustacea & Hydrozoa	McDonald & Nybakken 1997
Flabellina iodinea	Hydrozoa	McDonald & Nybakken 1997, Wägele 2004, Putz *et al*. 2010
Hancockia uncinata	Hydrozoa	Picton & Morrow 1994, McDonald & Nybakken 1997, Wägele 2004
Hermissenda crassicornis	Hydrozoa	McDonald & Nybakken 1997
Hermissenda opalescens	Hydrozoa	Putz *et al.* 2010
Janolus barbarensis	Bryozoa & Hydrozoa	McDonald & Nybakken 1997, Wägele 2004
Learchis evelinae	Hydrozoa	Valdés *et al.* 2006
Limenandra confusa	Anthozoa: Hexacorallia	McDonald & Nybakken 1997 [as *Baeolidia nodosa*], Rudman 2002 [as *L. nodosa*]
Lomanotus vermiformis	Hydrozoa	McDonald & Nybakken 1997, Rudman 1999b, Wägele 2004
Melibe leonina	Crustacea	McDonald & Nybakken 1997, Putz *et al*. 2010
Noumeaella rubrofasciata	Hydrozoa	Wägele 2004
Palisa papillata	Hydrozoa	Wägele 2004, Valdés *et al.* 2006
Phestilla sp.	Anthozoa: Hexacorallia	Putz *et al.* 2010 [multiple *Phestilla* spp.]
Phidiana lynceus	Hydrozoa & Bryozoa	McDonald & Nybakken 1997
Scyllaea fulva	Hydrozoa	McDonald & Nybakken 1997 [as *S. pelagica*]
Spurilla braziliana	Anthozoa: Hexacorallia	McDonald & Nybakken 1997, Rudman 1999a [as *Spurilla neapolitana*]
Tritonia diomedea	Anthozoa: Octocorallia	McDonald & Nybakken 1997
Tritonia festiva	Anthozoa: Octocorallia	McDonald & Nybakken 1997
Tritonia hamnerorum	Anthozoa: Octocorallia	McDonald & Nybakken 1997
Tritoniopsis frydis	Anthozoa: Octocorallia	McDonald & Nybakken 1997, Rudman 2001
Unidentia angelvaldesi	Hydrozoa	Millen & Hermosillo 2012

Table C5. List of specimens examined in this study, including species name, locality, and morphological tissue voucher information.

Species	Locality	Morphological voucher
Pleurobranchaea californica	-	SRA
Bathydoris clavigera	-	SRA
Doris kerguelenensis	-	SRA
Bornella anguilla	Mujimba (Old Woman Island), Mooloolaba, SE QLD, Australia	-
Dendronotus venustus	-	SRA
Doto lancei	-	SRA
Hancockia uncinata	Newlyn Marina, Newlyn, Cornwall, England, UK	-
Lomanotus vermiformis	Crawl Cay, Bocas del Toro, Panama	-
Melibe leonina	-	SRA
Scyllaea fulva	Gump Station, Cook's Bay, Moorea, French Polynesia	USNM1408879
Tritonia diomedea	-	SRA
Tritonia festiva	-	SRA
Tritonia hamnerorum	-	SRA
Tritoniopsis frydis	-	SRA
Armina californica	-	SRA
Dermatobranchus sp.	Mujimba (Old Woman Island), Mooloolaba, SE QLD, Australia	-
Aeolidiella alba	Back Reef near Maharepa, Moorea, French Polynesia	-
Antaeolidiella chromosoma	Punta Belcher, Bah'a Magdalena, Baja California, Mexico	-
Austraeolis stearnsi	-	SRA
Berghia stephanieae	-	USNM1408872
Catriona columbiana	-	SRA
Cuthona albocrusta	-	SRA
Dondice occidentalis	-	SRA
Dondice parguerensis	Smithsonian Tropical Research Institute, Bocas del Toro, Panama	-
Eubranchus rustyus	Neptune's/County Line, Malibu, CA, USA	-
Favorinus auritulus	-	SRA
Fiona pinnata	-	SRA
Flabellina iodinea	-	SRA
Hermissenda crassicornis	-	SRA
Hermissenda opalescens	-	SRA
Learchis evelinae	Blue Heron Bridge, Riviera Beach, FL, USA	USNM1408873
Limenandra confusa	Temae Beach, Moorea, French Polynesia	USNM1408882
Noumeaella rubrofasciata	Paradise Cove, Malibu, CA, USA	-
Palisa papillata	-	USNM1408874

Phestilla sp.	Lorenso House Reef, Pulau Bunaken, Sulawesi, Indonesia	-
Phidiana lynceus	Canal del Drago, Isla Colón, Panama	-
Spurilla braziliana	Isla Urabá, Panama	-
Unidentia angelvaldesi	Gold Coast Seaway, Queensland, Australia	-
Dirona picta	-	SRA
Janolus barbarensis	-	SRA

Table C6. List of specimens examined in this study, including species name, molecular tissue voucher and barcode information. Sequence Read Archive accession numbers are also provided for each RNA-Seq dataset.

Species	SRA accession no.	Catalog Number	COI Sequence
Pleurobranchaea californica	SRR1505130	-	-
Bathydoris clavigera	SRR1505104	-	-
Doris kerguelenensis	SRR1505108	-	-
Bornella anguilla	SRR3726697	USNM1408886	KX889723
Dendronotus venustus	SRR1950948	USNM1408861	KX889726
Doto lancei	SRR1950945	USNM1408854	KX889731
Hancockia uncinata	SRR3726694	USNM1408884	KX889735
Lomanotus vermiformis	SRR3726706	-	KX889740
Melibe leonina	SRR1950947	USNM1408859	KX889741
Scyllaea fulva	SRR3726701	USNM1408878	KX889746
Tritonia diomedea	SRR1721590	-	-
Tritonia festiva	SRR1950941	USNM1408850	KX889748
Tritonia hamnerorum	SRR4190242	-	-
Tritoniopsis frydis	SRR1950954	USNM1408870	KX889749
Armina californica	SRR4124996	-	-
Dermatobranchus sp.	SRR3726698	USNM1409026	KX889727
Aeolidiella alba	SRR3726702	-	KX889719
Antaeolidiella chromosoma	SRR3726695	USNM1409024	KX889720
Austraeolis stearnsi	SRR1950943	USNM1408852	KX889721
Berghia stephanieae	SRR1950951	USNM1408862	KX889722
Catriona columbiana	SRR1950949	-	KX889724
Cuthona albocrusta	SRR1950944	-	KX889725
Dondice occidentalis	SRR1950953	USNM1408868	KX889729
Dondice parguerensis	SRR3726707	-	KX889730
Eubranchus rustyus	SRR3726692	-	KX889732
Favorinus auritulus	SRR1950950	-	KX889733
Fiona pinnata	SRR1505109	-	-

Flabellina iodinea	SRR1950940	USNM1408849	KX889734
Hermissenda crassicornis	SRR1719366	-	-
Hermissenda opalescens	SRR1950939	-	KX889736
Learchis evelinae	SRR3726693	-	KX889738
Limenandra confusa	SRR3726703	USNM1408880	KX889739
Noumeaella rubrofasciata	SRR3726700	-	KX889742
Palisa papillata	SRR1950952	USNM1408863	KX889743
Phestilla sp.	SRR3726699	-	KX889744
Phidiana lynceus	SRR3726705	-	KX889745
Spurilla braziliana	SRR3726704	-	KX889747
Unidentia angelvaldesi	SRR3726696	USNM1409025	KX889750
Dirona picta	SRR1950946	USNM1408856	KX889728
Janolus barbarensis	SRR1950942	USNM1408851	KX889737

Table C7. Ancestral state reconstruction results for the evolution of diet preference in Cladobranchia with the alternative prey type states.

Node	Octocorallia	Hexacorallia	Hydrozoa	Bryozoa	Scyphozoa	Crustacea	Gastropoda eggs
1	0.1633%	0.0028%	99.8228%	0.0028%	0.0028%	0.0000%	0.0028%
2	0.3386%	0.0019%	99.6521%	0.0019%	0.0019%	0.0000%	0.0019%
3	0.0119%	0.0001%	99.9875%	0.0001%	0.0001%	0.0000%	0.0001%
4	0.0001%	0.0000%	99.9997%	0.0000%	0.0000%	0.0000%	0.0000%
5	0.0004%	0.0006%	99.9976%	0.0004%	0.0004%	0.0000%	0.0004%
6	0.0002%	0.0162%	99.9809%	0.0003%	0.0002%	0.0000%	0.0019%
7	0.0007%	0.1684%	99.8245%	0.0008%	0.0016%	0.0000%	0.0014%
8	0.0077%	98.1028%	1.8587%	0.0077%	0.0077%	0.0000%	0.0077%
9	0.0012%	99.8148%	0.1794%	0.0012%	0.0012%	0.0000%	0.0012%
10	0.0001%	99.9896%	0.0099%	0.0001%	0.0001%	0.0000%	0.0001%
11	0.0002%	99.9965%	0.0024%	0.0002%	0.0002%	0.0000%	0.0002%
12	0.0004%	0.0394%	99.9519%	0.0004%	0.0025%	0.0000%	0.0006%
13	0.0066%	0.0188%	99.5929%	0.0066%	0.1208%	0.0000%	0.0067%
14	0.0092%	0.0099%	93.7787%	0.0092%	0.0154%	0.0000%	0.0092%
15	0.0216%	0.0226%	95.4166%	0.0216%	4.4541%	0.0000%	0.0216%
16	0.0052%	0.0131%	99.8378%	0.0142%	0.0053%	0.0000%	0.1190%
17	0.0007%	0.0012%	99.9675%	0.0213%	0.0007%	0.0000%	0.0078%
18	0.0041%	0.0045%	99.8140%	0.1600%	0.0041%	0.0000%	0.0092%
19	0.0001%	0.0001%	99.9992%	0.0005%	0.0001%	0.0000%	0.0001%
20	0.0008%	0.0008%	99.9950%	0.0008%	0.0008%	0.0000%	0.0008%
21	0.0031%	0.0038%	99.9808%	0.0031%	0.0031%	0.0000%	0.0031%
22	0.0019%	0.0138%	99.9768%	0.0019%	0.0019%	0.0000%	0.0019%

23	0.0084%	0.1651%	99.7927%	0.0084%	0.0084%	0.0000%	0.0084%
24	0.0022%	0.0282%	99.9606%	0.0022%	0.0022%	0.0000%	0.0022%
25	0.0049%	0.0021%	99.9846%	0.0021%	0.0021%	0.0000%	0.0021%
26	89.0223%	0.0530%	10.7124%	0.0530%	0.0530%	0.0000%	0.0530%
27	99.9416%	0.0015%	0.0510%	0.0015%	0.0015%	0.0000%	0.0015%
28	99.9932%	0.0003%	0.0053%	0.0003%	0.0003%	0.0000%	0.0003%
29	99.9999%	0.0000%	0.0000%	0.0000%	0.0000%	0.0000%	0.0000%
30	99.7891%	0.0072%	0.1751%	0.0072%	0.0072%	0.0000%	0.0072%
31	0.0242%	0.0006%	99.9727%	0.0006%	0.0006%	0.0000%	0.0006%
32	0.0002%	0.0000%	99.9995%	0.0000%	0.0000%	0.0000%	0.0000%
33	0.0015%	0.0015%	99.9727%	0.0015%	0.0015%	0.0000%	0.0015%
34	0.0299%	0.0299%	99.3350%	0.0299%	0.0299%	0.0000%	0.0299%
35	0.0004%	0.0003%	99.9980%	0.0003%	0.0003%	0.0000%	0.0003%
36	0.0029%	0.0029%	99.9827%	0.0029%	0.0029%	0.0000%	0.0029%

Table C8. Ancestral state reconstruction results for the evolution of diet preference in Cladobranchia with the alternative prey type state for *Dirona picta*.

Node	Octocorallia	Hexacorallia	Hydrozoa	Bryozoa	Scyphozoa	Crustacea	Gastropoda eggs
1	0.1822%	0.0029%	99.7989%	0.0042%	0.0029%	0.0029%	0.0029%
2	0.3782%	0.0021%	99.6067%	0.0047%	0.0021%	0.0021%	0.0021%
3	0.0529%	0.0005%	99.9392%	0.0053%	0.0005%	0.0005%	0.0005%
4	0.0004%	0.0001%	99.9993%	0.0001%	0.0000%	0.0001%	0.0000%
5	0.0004%	0.0006%	99.9973%	0.0004%	0.0004%	0.0004%	0.0004%
6	0.0002%	0.0154%	99.9819%	0.0002%	0.0002%	0.0002%	0.0017%
7	0.0007%	0.1664%	99.8261%	0.0007%	0.0016%	0.0007%	0.0013%
8	0.0076%	98.0858%	1.8683%	0.0076%	0.0077%	0.0076%	0.0077%
9	0.0012%	99.8084%	0.1845%	0.0012%	0.0012%	0.0012%	0.0012%
10	0.0001%	99.9891%	0.0102%	0.0001%	0.0001%	0.0001%	0.0001%
11	0.0002%	99.9962%	0.0025%	0.0002%	0.0002%	0.0002%	0.0002%
12	0.0004%	0.0393%	99.9517%	0.0004%	0.0025%	0.0004%	0.0005%
13	0.0066%	0.0188%	99.5877%	0.0066%	0.1199%	0.0066%	0.0066%
14	0.0091%	0.0098%	93.7744%	0.0091%	0.0152%	0.0091%	0.0091%
15	0.0215%	0.0225%	95.3841%	0.0215%	4.4662%	0.0215%	0.0215%
16	0.0048%	0.0121%	99.8548%	0.0048%	0.0049%	0.0048%	0.1088%
17	0.0001%	0.0002%	99.9976%	0.0001%	0.0001%	0.0001%	0.0015%
18	0.0001%	0.0001%	99.9991%	0.0001%	0.0001%	0.0001%	0.0002%
19	0.0000%	0.0000%	99.9997%	0.0000%	0.0000%	0.0000%	0.0000%
20	0.0010%	0.0012%	99.9919%	0.0010%	0.0010%	0.0020%	0.0010%
21	0.0078%	0.0253%	99.8662%	0.0078%	0.0078%	0.0697%	0.0078%
22	0.0429%	0.3107%	98.4859%	0.0429%	0.0429%	0.9889%	0.0429%
23	0.0269%	0.5216%	98.9954%	0.0269%	0.0269%	0.3484%	0.0269%

24	0.0061%	0.0889%	99.8206%	0.0061%	0.0061%	0.0599%	0.0061%
25	0.0664%	0.0287%	99.4038%	0.3865%	0.0287%	0.0287%	0.0287%
26	88.9000%	0.0529%	10.7821%	0.0532%	0.0529%	0.0529%	0.0529%
27	99.9402%	0.0015%	0.0510%	0.0015%	0.0015%	0.0015%	0.0015%
28	99.9929%	0.0003%	0.0053%	0.0003%	0.0003%	0.0003%	0.0003%
29	99.9998%	0.0000%	0.0000%	0.0000%	0.0000%	0.0000%	0.0000%
30	99.7813%	0.0072%	0.1756%	0.0072%	0.0072%	0.0072%	0.0072%
31	0.0274%	0.0007%	99.9684%	0.0009%	0.0007%	0.0007%	0.0007%
32	0.0002%	0.0000%	99.9994%	0.0000%	0.0000%	0.0000%	0.0000%
33	0.0016%	0.0015%	99.9711%	0.0015%	0.0015%	0.0015%	0.0015%
34	0.0299%	0.0299%	99.3121%	0.0299%	0.0299%	0.0299%	0.0299%
35	0.0004%	0.0003%	99.9976%	0.0003%	0.0003%	0.0003%	0.0003%
36	0.0030%	0.0030%	99.9793%	0.0030%	0.0030%	0.0030%	0.0030%

Table C9. Ancestral state reconstruction results for the evolution of diet preference in Cladobranchia with the alternative prey type state for *Fiona pinnata*.

Node	Octocorallia	Hexacorallia	Hydrozoa	Bryozoa	Scyphozoa	Crustacea	Gastropoda eggs
1	0.8329%	0.0105%	98.8425%	0.2826%	0.0105%	0.0000%	0.0105%
2	1.7300%	0.0100%	97.6508%	0.5790%	0.0100%	0.0000%	0.0100%
3	1.1286%	0.0118%	97.7802%	1.0439%	0.0118%	0.0000%	0.0118%
4	0.0071%	0.0003%	99.9854%	0.0065%	0.0002%	0.0000%	0.0002%
5	0.0011%	0.0007%	99.9959%	0.0010%	0.0004%	0.0000%	0.0005%
6	0.0002%	0.0171%	99.9800%	0.0002%	0.0003%	0.0000%	0.0019%
7	0.0008%	0.1776%	99.8147%	0.0008%	0.0018%	0.0000%	0.0015%
8	0.0084%	98.0133%	1.9449%	0.0084%	0.0084%	0.0000%	0.0084%
9	0.0013%	99.7973%	0.1961%	0.0013%	0.0013%	0.0000%	0.0013%
10	0.0001%	99.9880%	0.0113%	0.0001%	0.0001%	0.0000%	0.0001%
11	0.0003%	99.9959%	0.0028%	0.0003%	0.0003%	0.0000%	0.0003%
12	0.0004%	0.0436%	99.9467%	0.0004%	0.0028%	0.0000%	0.0006%
13	0.0075%	0.0212%	99.5579%	0.0075%	0.1306%	0.0000%	0.0075%
14	0.0100%	0.0107%	93.5194%	0.0100%	0.0167%	0.0000%	0.0100%
15	0.0234%	0.0246%	95.2153%	0.0234%	4.6447%	0.0000%	0.0234%
16	0.0053%	0.0134%	99.8494%	0.0053%	0.0054%	0.0000%	0.1157%
17	0.0002%	0.0003%	99.9974%	0.0002%	0.0002%	0.0000%	0.0016%
18	0.0001%	0.0001%	99.9991%	0.0001%	0.0001%	0.0000%	0.0003%
19	0.0000%	0.0000%	99.9997%	0.0000%	0.0000%	0.0000%	0.0000%
20	0.0022%	0.0010%	99.9918%	0.0021%	0.0010%	0.0000%	0.0010%
21	0.0036%	0.0044%	99.9779%	0.0036%	0.0035%	0.0000%	0.0035%
22	0.0021%	0.0151%	99.9741%	0.0021%	0.0021%	0.0000%	0.0021%
23	0.0092%	0.1732%	99.7806%	0.0092%	0.0092%	0.0000%	0.0092%
24	0.0025%	0.0307%	99.9566%	0.0025%	0.0025%	0.0000%	0.0025%

25	0.4776%	0.2068%	23.9164%	74.7787%	0.2068%	0.0000%	0.2068%
26	88.7381%	0.0574%	10.9115%	0.1207%	0.0574%	0.0000%	0.0574%
27	99.9371%	0.0017%	0.0543%	0.0020%	0.0017%	0.0000%	0.0017%
28	99.9924%	0.0003%	0.0059%	0.0004%	0.0003%	0.0000%	0.0003%
29	99.9998%	0.0000%	0.0000%	0.0000%	0.0000%	0.0000%	0.0000%
30	99.7734%	0.0080%	0.1857%	0.0090%	0.0080%	0.0000%	0.0080%
31	0.1275%	0.0023%	99.8195%	0.0437%	0.0023%	0.0000%	0.0023%
32	0.0009%	0.0001%	99.9982%	0.0003%	0.0001%	0.0000%	0.0001%
33	0.0020%	0.0017%	99.9694%	0.0018%	0.0017%	0.0000%	0.0017%
34	0.0327%	0.0325%	99.3006%	0.0326%	0.0325%	0.0000%	0.0325%
35	0.0005%	0.0004%	99.9975%	0.0004%	0.0004%	0.0000%	0.0004%
36	0.0033%	0.0033%	99.9802%	0.0033%	0.0033%	0.0000%	0.0033%

Table C10. Ancestral state reconstruction results for the evolution of diet preference in Cladobranchia with the alternative prey type state for *Janolus barbarensis*.

Node	Octocorallia	Hexacorallia	Hydrozoa	Bryozoa	Scyphozoa	Crustacea	Gastropoda eggs
1	0.2102%	0.0032%	99.7650%	0.0089%	0.0032%	0.0033%	0.0032%
2	0.4370%	0.0024%	99.5366%	0.0143%	0.0024%	0.0026%	0.0024%
3	0.1101%	0.0011%	99.8613%	0.0228%	0.0011%	0.0014%	0.0011%
4	0.0025%	0.0003%	99.9927%	0.0006%	0.0002%	0.0033%	0.0002%
5	0.0102%	0.0143%	99.7161%	0.0090%	0.0087%	0.2236%	0.0093%
6	0.0002%	0.0230%	99.9715%	0.0002%	0.0003%	0.0016%	0.0026%
7	0.0007%	0.1725%	99.8188%	0.0007%	0.0016%	0.0013%	0.0017%
8	0.0075%	98.1092%	1.8458%	0.0075%	0.0075%	0.0075%	0.0075%
9	0.0011%	99.8130%	0.1802%	0.0011%	0.0011%	0.0011%	0.0011%
10	0.0001%	99.9895%	0.0099%	0.0001%	0.0001%	0.0001%	0.0001%
11	0.0002%	99.9963%	0.0024%	0.0002%	0.0002%	0.0002%	0.0002%
12	0.0004%	0.0403%	99.9505%	0.0004%	0.0025%	0.0005%	0.0006%
13	0.0064%	0.0189%	99.5953%	0.0064%	0.1177%	0.0064%	0.0065%
14	0.0089%	0.0096%	93.8401%	0.0089%	0.0149%	0.0089%	0.0089%
15	0.0210%	0.0220%	95.4355%	0.0210%	4.4178%	0.0210%	0.0210%
16	0.0048%	0.0158%	99.8501%	0.0048%	0.0049%	0.0054%	0.1093%
17	0.0001%	0.0003%	99.9976%	0.0001%	0.0001%	0.0001%	0.0015%
18	0.0001%	0.0001%	99.9991%	0.0001%	0.0001%	0.0001%	0.0002%
19	0.0000%	0.0000%	99.9997%	0.0000%	0.0000%	0.0000%	0.0000%
20	0.0012%	0.0009%	99.9931%	0.0009%	0.0008%	0.0014%	0.0008%
21	0.0030%	0.0038%	99.9781%	0.0030%	0.0030%	0.0030%	0.0030%
22	0.0018%	0.0134%	99.9757%	0.0018%	0.0018%	0.0018%	0.0018%
23	0.0082%	0.1599%	99.7912%	0.0082%	0.0082%	0.0082%	0.0082%
24	0.0022%	0.0273%	99.9596%	0.0022%	0.0022%	0.0022%	0.0022%
25	0.1369%	0.0585%	97.8795%	1.6908%	0.0585%	0.0587%	0.0585%

26	89.0230%	0.0519%	10.6645%	0.0531%	0.0519%	0.0519%	0.0519%
27	99.9416%	0.0014%	0.0499%	0.0014%	0.0014%	0.0014%	0.0014%
28	99.9931%	0.0003%	0.0052%	0.0003%	0.0003%	0.0003%	0.0003%
29	99.9998%	0.0000%	0.0000%	0.0000%	0.0000%	0.0000%	0.0000%
30	99.7864%	0.0070%	0.1719%	0.0070%	0.0070%	0.0070%	0.0070%
31	0.0312%	0.0007%	99.9636%	0.0016%	0.0007%	0.0007%	0.0007%
32	0.0002%	0.0000%	99.9994%	0.0000%	0.0000%	0.0000%	0.0000%
33	0.0015%	0.0014%	99.9718%	0.0014%	0.0014%	0.0014%	0.0014%
34	0.0293%	0.0292%	99.3212%	0.0292%	0.0292%	0.0292%	0.0292%
35	0.0003%	0.0003%	99.9977%	0.0003%	0.0003%	0.0003%	0.0003%
36	0.0029%	0.0029%	99.9800%	0.0029%	0.0029%	0.0029%	0.0029%

Table C11. Ancestral state reconstruction results for the evolution of diet preference in Cladobranchia with the alternative prey type state for *Phidiana lynceus*.

Node	Octocorallia	Hexacorallia	Hydrozoa	Bryozoa	Scyphozoa	Crustacea	Gastropoda eggs
1	0.9378%	0.0132%	98.6836%	0.3116%	0.0131%	0.0144%	0.0131%
2	1.9286%	0.0126%	97.3749%	0.6310%	0.0125%	0.0153%	0.0125%
3	1.2712%	0.0150%	97.5190%	1.1306%	0.0148%	0.0198%	0.0148%
4	0.0330%	0.0018%	99.9143%	0.0296%	0.0012%	0.0175%	0.0013%
5	0.0332%	0.0221%	99.5946%	0.0311%	0.0130%	0.2791%	0.0138%
6	0.0006%	0.0370%	99.9531%	0.0010%	0.0007%	0.0026%	0.0041%
7	0.0012%	0.2235%	99.7625%	0.0013%	0.0026%	0.0020%	0.0027%
8	0.0105%	97.7162%	2.2206%	0.0105%	0.0105%	0.0105%	0.0105%
9	0.0019%	99.7292%	0.2592%	0.0019%	0.0019%	0.0019%	0.0019%
10	0.0002%	99.9814%	0.0173%	0.0002%	0.0002%	0.0002%	0.0002%
11	0.0004%	99.9930%	0.0047%	0.0004%	0.0004%	0.0004%	0.0004%
12	0.0007%	0.0628%	99.9217%	0.0008%	0.0041%	0.0009%	0.0011%
13	0.0103%	0.0311%	99.4326%	0.0104%	0.1614%	0.0104%	0.0105%
14	0.0123%	0.0134%	92.7352%	0.0123%	0.0203%	0.0123%	0.0123%
15	0.0291%	0.0308%	94.5634%	0.0291%	5.2340%	0.0291%	0.0291%
16	0.0073%	0.0252%	99.7872%	0.0195%	0.0073%	0.0082%	0.1379%
17	0.0012%	0.0025%	99.9531%	0.0291%	0.0012%	0.0013%	0.0105%
18	0.0056%	0.0065%	99.7724%	0.1863%	0.0056%	0.0056%	0.0124%
19	0.0001%	0.0001%	99.9987%	0.0007%	0.0001%	0.0001%	0.0001%
20	0.0078%	0.0018%	99.9732%	0.0072%	0.0017%	0.0048%	0.0017%
21	0.0056%	0.0064%	99.9618%	0.0055%	0.0051%	0.0054%	0.0051%
22	0.0031%	0.0195%	99.9619%	0.0031%	0.0031%	0.0031%	0.0031%
23	0.0118%	0.1938%	99.7355%	0.0118%	0.0118%	0.0118%	0.0118%
24	0.0036%	0.0385%	99.9397%	0.0036%	0.0036%	0.0036%	0.0036%
25	0.5983%	0.2567%	26.7653%	71.3516%	0.2567%	0.2580%	0.2567%
26	87.3007%	0.0710%	12.1959%	0.1480%	0.0710%	0.0713%	0.0710%

27	99.9176%	0.0023%	0.0680%	0.0027%	0.0023%	0.0023%	0.0023%
28	99.9887%	0.0005%	0.0082%	0.0006%	0.0005%	0.0005%	0.0005%
29	99.9998%	0.0000%	0.0000%	0.0000%	0.0000%	0.0000%	0.0000%
30	99.7013%	0.0111%	0.2305%	0.0125%	0.0111%	0.0111%	0.0111%
31	0.1625%	0.0033%	99.7662%	0.0546%	0.0033%	0.0035%	0.0033%
32	0.0014%	0.0001%	99.9971%	0.0005%	0.0001%	0.0001%	0.0001%
33	0.0030%	0.0024%	99.9575%	0.0026%	0.0024%	0.0024%	0.0024%
34	0.0413%	0.0408%	99.1644%	0.0410%	0.0408%	0.0408%	0.0408%
35	0.0008%	0.0006%	99.9955%	0.0007%	0.0006%	0.0006%	0.0006%
36	0.0049%	0.0048%	99.9661%	0.0049%	0.0048%	0.0048%	0.0048%

Appendix D: Supplementary Material for Chapter 8

Table D1. Specimen information for molecular data analysis in this study.

Species	Locality	SRA accession no.	Catalog number	COI	16S	18S
Pleurobranchaea californica	NCBI Sequence Read Archive	SRR1505130	-	Yes	Yes	Yes
Bathydoris clavigera	NCBI Sequence Read Archive	SRR1505104	-	Yes	Yes	Yes
Doris kerguelenensis	NCBI Sequence Read Archive	SRR1505108	-	-	Yes	Yes
Bornella anguilla	NCBI Sequence Read Archive	SRR3726697	USNM1408886	KX889723	To submit	To submit
Dendronotus venustus	NCBI Sequence Read Archive	SRR1950948	USNM1408861	KX889726	To submit	To submit
Doto lancei	NCBI Sequence Read Archive	SRR1950945	USNM1408854	KX889731	To submit	To submit
Hancockia uncinata	NCBI Sequence Read Archive	SRR3726694	USNM1408884	KX889735	To submit	To submit
Lomanotus vermiformis	NCBI Sequence Read Archive	SRR3726706	-	KX889740	To submit	To submit
Melibe leonina	NCBI Sequence Read Archive	SRR1950947	USNM1408859	KX889741	To submit	To submit
Scyllaea fulva	NCBI Sequence Read Archive	SRR3726701	USNM1408878	KX889746	To submit	To submit
Tritonia diomedea	NCBI Sequence Read Archive	SRR1721590	-	-	To submit	To submit
Tritonia festiva	NCBI Sequence Read Archive	SRR1950941	USNM1408850	KX889748	To submit	To submit
Tritonia hamnerorum	NCBI Sequence Read Archive	SRR4190242	-	-	To submit	To submit
Tritoniopsis frydis	NCBI Sequence Read Archive	SRR1950954	USNM1408870	KX889749	To submit	To submit
Armina californica	NCBI Sequence Read Archive	SRR4124996	-	-	To submit	To submit
Dermatobranchus sp.	NCBI Sequence Read Archive	SRR3726698	USNM1409026	KX889727	To submit	To submit
Aeolidiella alba	NCBI Sequence Read Archive	SRR3726702	-	KX889719	To submit	To submit
Antaeolidiella chromosoma	NCBI Sequence Read Archive	SRR3726695	USNM1409024	KX889720	To submit	To submit
Austraeolis stearnsi	NCBI Sequence Read Archive	SRR1950943	USNM1408852	KX889721	To submit	To submit
Berghia stephanieae	NCBI Sequence Read Archive	SRR1950951	USNM1408862	KX889722	To submit	To submit
Catriona columbiana	NCBI Sequence Read Archive	SRR1950949	-	KX889724	To submit	To submit
Cuthona albocrusta	NCBI Sequence Read Archive	SRR1950944	-	KX889725	To submit	To submit
Dondice	NCBI Sequence	SRR1950953	USNM1408868	KX889729	To submit	To submit

occidentalis	Read Archive					
Dondice parguerensis	NCBI Sequence Read Archive	SRR3726707	-	KX889730	To submit	To submit
Eubranchus rustyus	NCBI Sequence Read Archive	SRR3726692	-	KX889732	To submit	To submit
Favorinus auritulus	NCBI Sequence Read Archive	SRR1950950	-	KX889733	To submit	To submit
Fiona pinnata	NCBI Sequence Read Archive	SRR1505109	-	-	To submit	To submit
Flabellina iodinea	NCBI Sequence Read Archive	SRR1950940	USNM1408849	KX889734	To submit	To submit
Hermissenda crassicornis	NCBI Sequence Read Archive	SRR1719366	-	-	To submit	To submit
Hermissenda opalescens	NCBI Sequence Read Archive	SRR1950939	-	KX889736	To submit	To submit
Learchis evelinae	NCBI Sequence Read Archive	SRR3726693	-	KX889738	To submit	To submit
Limenandra confusa	NCBI Sequence Read Archive	SRR3726703	USNM1408880	KX889739	To submit	To submit
Noumeaella rubrofasciata	NCBI Sequence Read Archive	SRR3726700	-	KX889742	To submit	To submit
Palisa papillata	NCBI Sequence Read Archive	SRR1950952	USNM1408863	KX889743	To submit	To submit
Phestilla sp.	NCBI Sequence Read Archive	SRR3726699	-	KX889744	To submit	To submit
Phidiana lynceus	NCBI Sequence Read Archive	SRR3726705	-	KX889745	To submit	To submit
Spurilla braziliana	NCBI Sequence Read Archive	SRR3726704	-	KX889747	To submit	To submit
Unidentia angelvaldesi	NCBI Sequence Read Archive	SRR3726696	USNM1409025	KX889750	To submit	To submit
Dirona picta	NCBI Sequence Read Archive	SRR1950946	USNM1408856	KX889728	To submit	To submit
Janolus barbarensis	NCBI Sequence Read Archive	SRR1950942	USNM1408851	KX889737	To submit	To submit
Phyllodesmium koehleri	Calangaman Island, Cabilao, Phillipines	-	-	GQ403782	GQ403760	To submit
Berghia stephanieae	Aquarium shop	-	-	To submit	To submit	To submit
Aeolidia papillosa	Chile	-	-	To submit	To submit	GU227371
Aeolidia papillosa	Roscoff, France	-	-	To submit	To submit	To submit
Antaeolidiella chromosoma	GenBank	-	CASIZ173060	JQ997018	JQ996812	-
Austraeolis stearnsi	GenBank	-	-	JQ699571	JQ699483	-
Baeolidia australis	Albany, Australia	-	-	-	yes	GU227367
Berghia major	Dingo Beach, Australia	-	-	To submit	GU550051	GU227365
Baeolidia nodosa	Elba Island, Italy	-	-	To submit	To submit	GU339155
Bathydoris clavigera	Wedell Sea, Antarctica	-	-	To submit	To submit	To submit
Berghia verrucicornis	San Andrea, Elba Island, Italy	-	-	To submit	To submit	GU227364
Cerberilla affinis	Orpheus Island, Australia	-	-	To submit	To submit	GU227366
Cuthona caerulea	Elba Island, Italy	-	-	To submit	To submit	AF249199
Calmella cavolini	Giglio, Italy	-	-	To submit	To submit	GU227361

Caloria elegans	Giglio, Italy	-	-	To submit	To submit	To submit
Cuthona kanga	Dingo Beach, Australia	-	-	To submit	To submit	To submit
Cratena peregrina	Rosas, Spain	-	-	AF249786	To submit	GU339156
Calmella cavolini	Blanes, Spain	-	-	To submit	To submit	To submit
Charcotia granulosa	Wedell Sea, Antarctica	-	-	To submit	-	To submit
Dirona albolineata	Vancouver Island, British Columbia, Canada	-	-	To submit	To submit	To submit
Dondice occidentalis		-	-			
Eubranchus exiguus	NCBI GenBank	-	-	AF249792	AF249246	AJ224787
Embletonia pulchra	Rovinj, Croatia	-	-	To submit	To submit	To submit
Eubranchus rustyus	NCBI GenBank	-	-	GQ292065	-	GQ326905
Eubranchus sp.	NCBI GenBank	-	-	AF249791	-	AJ224786
Embletonia pulchra	Rovinj, Croatia	-	-	To submit	To submit	To submit
Embletonia sp.	Banyuls-sur-Mer, France	-	-	To submit	To submit	To submit
Flabellina affinis	Costa Brava, Spain	-	-	AF249783	To submit	AY165767
Flabellina babai	Italy, Mediterranean Sea	-	-	To submit	To submit	AY165768
Flabellina exoptata	Orpheus Island, Australia	-	-	To submit	To submit	To submit
Flabellina falklandica	Huniay, Chile	-	-	To submit	To submit	To submit
Flabellina ischitana	Elba Island, Italy	-	-	To submit	To submit	To submit
Flabellina rubrolineata	Lizard Island, Australia	-	-	To submit	To submit	To submit
Facelina rubrovittata	Blanes, Spain	-	-	To submit	To submit	To submit
Fiona pinnata	Struckbroke Island, Queensland, Australia	-	-	To submit	To submit	-
Flabellina pedata	Ferrol, Spain	-	-	To submit	To submit	-
Glaucus atlanticus	Fuertoventura, Spain	-	-	To submit	To submit	To submit
Hermissenda sp.	-	-	-	To submit	To submit	
Janolus capensis	GenBank	-	-	HM162748	HM162672	-
Janolus cristatus	Osterschelde, Netherlands	-	-	To submit	-	To submit
Janolus barbarensis	GenBank	-	-	HM162747	HM162671	-
Notaeolidia depressa	Wedell Sea, Antarctica	-	-	To submit	To submit	AY165770
Phyllodesmium colemani	Moalboal, Cebu, Phillipines	-	-	GQ403776	GQ403754	GU339159
Phyllodesmium crypticum	Cockle Bay, Australia	-	-	-	GQ403770	GU339160
Phyllodesmium jakobsenae	Bunaken Island, Sulawesi, Indonesia	-	-	GQ403779	GQ403757	GU339162
Phestilla lugubris	Sambangan Island Karimunjawa,	-	-	To submit	To submit	To submit

	Indonesia					
Phidiana lynceus	Netherlands Antilles, Netherlands	-	-	To submit	To submit	AY165765
Phyllodesmium macphersonae	Lizard Island, Australia	-	-	-	GQ403768	To submit
Phyllodesmium magnum	Dahab, Egypt	-	-	GQ403785	GQ403763	To submit
Phestilla melanobranchia	Sulawesi, Indonesia	-	-	To submit	To submit	To submit
Piseinotecus gabinieri	GenBank	-	MCNCN/ ADN52000	JX087561	JX087495	-
Pruvotfolia pselloites	GenBank	-	MNCN15.05/ 53705	HQ616762	HQ616725	-
Pteraeolidia ianthina	Botany Bay, Australia	-	-	To submit	To submit	GU227370
Pteraeolidia ianthina	Sulawesi, Indonesia	-	-	To submit	To submit	To submit
Spurilla neapolitana	Elba Island, Italy	-	-	To submit	To submit	To submit
Tergipes antarcticus	Wedell Sea, Antarctica	-	-	To submit	To submit	To submit
Tergipes tergipes	GenBank	-	-	AY345032	AY345032	AF249197

Table D2. Primers used for fragments of CO1, 16S and 18S.

Primer	Direction	Sequence 5' à 3'	Reference
CO1 Partial Fragment (length 680 bp)			
LCO (1490)	Forward	GGT CAA CAA ATC ATA AAG ATA TTG G	Folmer et al. 1994
HCO (2198)	Reverse	TAA ACT TCA GGG TGA CCA AAA AAT CA	Folmer et al. 1994
C1-N-2329	Forward	ACT GTA AAT ATA TGA TGA GCT CA	Simon et al. (1994)
C1-J-1718	Reverse	GGA GGA TTT GGA AAT TGA TTA GTT CC	Simon et al. (1994)
16S Partial Fragment (length 650)			
LR-J-12887 (16S1)	Forward	GGA GCT CCG GTT TGA ACT CAG ATC	Simon et al. 1994
LR-N-13398 (16S2)	Reverse	CGG CCG CCT GTT TAT CAA AAA CAT	Simon et al. 1994
18S Complete (length 1800)			
18A1	Forward	CTG GTT GAT CCT GCC AGT CAT ATG C	Spears et al. 1994
400F	Forward	ACG GGT AAC GGG GAA TCA GGG	Spears et al. 1994
700F	Forward	GTC TGG TGC CAG CAG CCG CG	Spears et al. 1994
1155F	Forward	CTG AAA CTT AAA GGA ATT GAC GG	Spears et al. 1994
700R	Reverse	CGC GGC TGC TGG CAC CAG AC	Spears et al. 1994
1155R	Reverse	CCG TCA ATT CCT TTA AGT TTC AG	Spears et al. 1994
1800R	Reverse	GAT CCT TCC GCA GGT TCA CCT ACG	Spears et al. 1994

Table D3. Polymerase chain reaction (PCR) cycling protocols for each of the three genes.

	COI-1	**COI-2**	**18S-1**	**18S-2**	**16S**
Initial Step (95°C)	4 min	15 min	15 min	15 min	15 min
Denaturation (94°C)	45 sec	35 sec	25 sec	45 sec	45 sec
Annealing	45 sec	90 sec	25 sec	45 sec	45 sec
Elongation (72°C)	2 min	90 sec	35 sec	90 sec	90 sec
Final Elongation (72°C)	6 min	10 min	10 min	10 min	10 min
Number of cycles	30	30	30	25	25
Touchdown phase cycles	N/A	15	N/A	9	9
Annealing temperature	52°C	55°C (-1°C)	54°C	56°C (-1°C)	56°C (-1°C)

Appendix E: Links for online supplementary materials

Chapter 2. All sequence alignments and tree files for this chapter are available at http://hdl.handle.net/1903/16863.

Chapter 3. Transcriptomes sequenced for this chapter can be accessed at the Sequence Read Archive (SRA) at NCBI: SRA accession numbers SRR1950939-SRR1950954. The GASTRO50 substitution matrix used in this chapter, as well as all sequence alignments and tree files for this chapter, are available at http://rsos.royalsocietypublishing.org/content/2/9/150196.figures-only.

Chapter 7. RNA-Seq sequence data for this chapter can be accessed at the NCBI Sequence Read Archive (SRA) with the following accessions: SRA accession numbers SRR1505104, SRR1505108, SRR1505109, SRR1505130, SRR1719366, SRR1721590, SRR1950939–SRR1950954, SRR3726692–SRR3726707, SRR4124996 and SRR4190242. Aligned data matrices and tree files for this chapter can be accessed at the Dryad Digital Repository (DOI: 10.5061/dryad.7kh2n).

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