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

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Abstract: Cladobranchia is a diverse clade of nudibranch gastropods, with ~1,000 coastal (benthic) and off shore (pelagic) marine species. Their diversity may be tied to dietary specialization and the evolution of remarkable adaptations that are rare among animals, such as the sequestration of cnidarian nematocysts. Due to the deep divergences within Cladobranchia, and other higher-order taxa within Mollusca, phylogenetic analyses based on morphology or a few genes have been unable to resolve relationships within these clades with high support. The introduction of high-throughput sequencing technologies, however, has provided the opportunity to collect large genomic data sets, which have proven useful in resolving relationships within molluscs, as in other metazoan groups. As sequencing costs decrease, it has become easier to collect ever larger data sets in an attempt to resolve relationships within more recently diverged groups, such as Cladobranchia. The phyly and relationships of the three main groups within Cladobranchia (Arminida, Dendronotida, and Aeolidida) have been difficult to determine, primarily due to the rapid evolution of mtDNA and rDNA genes analyzed in traditional molecular phylogenetic studies. In this review, I discuss the systematics of Cladobranchia in regards to morphological data, molecular analyses with one or a few genes, and address the impact of genomic data. I then contend that the supported phylogenetic hypotheses provided by high-throughput sequencing data will provide a better framework for studying taxonomy and character evolution within Cladobranchia, as these data provide strong support for Cladobranchia itself, as well as the nested subclades within it. Although it is important to note that a broader taxon sampling is still needed for genomic data, it is clear that utilizing these data has provided the most-well-supported phylogenetic hypotheses of Cladobranchia to date. In doing so, these genomic data have challenged well-established relationships, including the monophyly of Facelinidae and inclusion of Tritoniidae in Dendronotida, and resolved the position of taxa that have been traditionally quite difficult to place, including *Melibe* Rang, 1829 and the families Proctonotidae and Dironidae. Finally, these genomic data provide a much stronger phylogenetic framework for studying character evolution within Cladobranchia, including the evolution of nematocyst sequestration, one of the quintessential characters in this clade.

Key words: high-throughput sequencing, nudibranchs, Mollusca

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 (Putz *et al.* 2010), the sequestration of symbiotic zooxanthellae (Wägele and Johnsen 2001), and independent evolution of rhythmic motor behavior (Lillvis and Katz 2013). 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 (Lee *et al.* 2012, Newcomb *et al.* 2012, Gunaratne *et al.* 2014, Xu *et al.* 2014). 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-molluscan, groups that include 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 (Wägele and Willan 2000, Wollscheid-Lengeling *et al.* 2001, Pola and Gosliner 2010, Goodheart *et al.* 2015a, Mahguib and Valdés 2015), 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., Helmkampf *et al.* 2008, Ruiz-Trillo *et al.* 2008, Hejnol *et al.* 2009, Struck *et al.* 2011, Kocot *et al.* 2011, 2013, Smith *et al.* 2011, Nesnidal *et al.* 2013, Feng *et al.* 2014, Telford *et al.* 2014, Zapata *et al.* 2015), 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., Misof *et al.* 2014), classes (e.g., Zhang and Lu 2007, O'Hara *et al.* 2014, Zapata *et al.* 2014) and others (e.g. Teasdale *et al.* 2016), including work on Cladobranchia specifically (Goodheart *et al.* 2015b). 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 (Table 1). The classification scheme that first divided Nudibranchia into two major groups (Holohepatica and Cladohepatica) originated with Bergh (Bergh 1890, 1902, 1906) based on the branching pattern of the digestive gland. However, Odhner (1934) 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 (1970) 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 (1970) 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 (1970) 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 (Minichev 1970, Willan and Morton 1984, Wägele and Willan 2000).

Following this separation of Anthobranchia from the rest of Nudibranchia, Willan and Morton (1984) introduced the name Cladobranchia for the group that contained Dendronotacea, Arminacea and Aeolidacea. Notably, Willan and Morton (1984) actually appear to give Odhner credit for establishing Cladobranchia, but a personal communication with Willan (Valdés and Bouchet 2005) specifies that this attribution to Odhner was an error. The authors do not provide a discussion regarding the renaming of this clade, but Willan and Morton (1984) 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.

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 Wägele and Willan 2000; Figure 1; Table 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 (Valdés and Bouchet 2005), along with some taxa the authors consider unassigned to any of these three groups, including Charcotiidae, Dironidae, Dotidae, Embletoniidae, Goniaeolididae, Heroidae and Madrellidae.

Table 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

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 samples sizes have found phylogenetic support for the monophyly of Cladobranchia (Thollesson 1999, Wollscheid-Lengeling *et al.* 2001, Shields 2009, Pola and Gosliner 2010, Goodheart *et al.* 2015a, 2015b, Mahguib and Valdés 2015), 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 (1999) – six; Wollscheid-Lengeling (2001) – fourteen), and Thollesson (1999) 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 (Pola and Gosliner 2010, Mahguib and Valdés 2015) and Arminida is well supported in a third (Goodheart *et al.* 2015a). Conversely, there is no support for Dendronotida as a monophyletic group in any molecular phylogeny thus far.

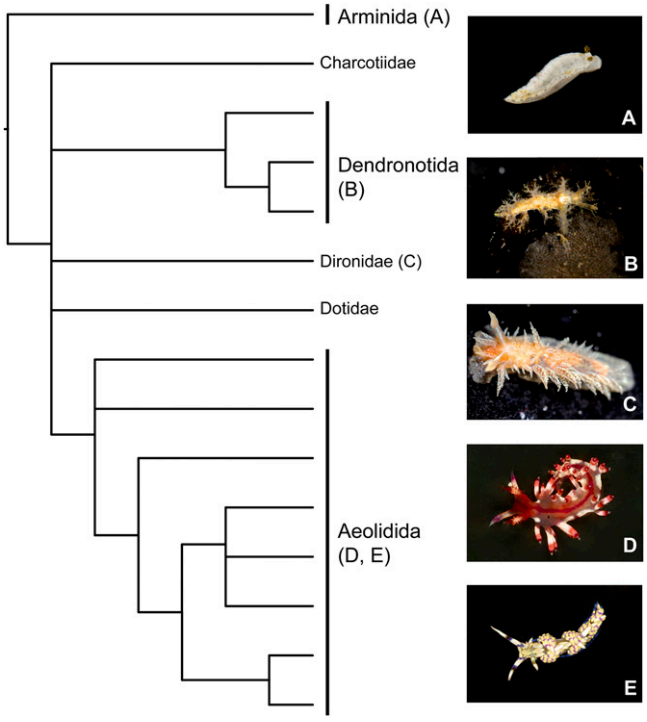

Figure 1. Phylogeny of Cladobranchia based on the most recent morphological analysis of Nudibranchia by Wägele and Willan (2000), and photographs of A) *Dermatobranchus albus* (Arminida), B) *Dendronotus venustus* (Dendronotida) (Goodheart *et al.* 2015b), C) *Dirona picta* (Dironidae) (Goodheart *et al.* 2015b), and D) *Flabellina rubrolineata* and E) *Godiva* sp. (Aeolidida).

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 (Pola and Gosliner 2010, Goodheart *et al.* 2015a, Mahguib and Valdés 2015), 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 (Mahguib and Valdés 2015). 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 (Carmona *et al.* 2013a), Arminidae (Kolb and Wägele 1998, Gosliner and Fahey 2011), Bornellidae (Pola *et al.* 2009), Dotidae (Shipman and Gosliner 2015), Glaucidae (Valdés and Campillo 2004), Scyllaeidae (Pola *et al.* 2012) and Tritoniidae (Bertsch *et al.* 2009, Hulett *et al.* 2015), and at the genus level, these subclades include *Anteaeolidiella* Miller, 2001 (Carmona *et al.* 2014a), *Babakina* Roller, 1973 (Gosliner *et al.* 2007, Carmona *et al.* 2011), *Berghia* Trinchese, 1877 (Carmona *et al.* 2014c), *Burnaia* Miller, 2001 (Carmona *et al.* 2015), *Dendronotus*

Alder and Hancock, 1845 (Stout *et al.* 2010, 2011, Ekimova *et al.* 2015), *Janolus* Bergh, 1884 (Chichvarkhin *et al.* 2016), *Limenandra* Haefelfinger & Stamm, 1958 (Carmona *et al.* 2013b), *Melibe* (Gosliner and Smith 2003), *Phyllodesmium* Ehrenberg, 1831 (Moore and Gosliner 2011, 2014) and *Spurilla* Bergh, 1864 (Carmona *et al.* 2014b). 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 (Goodheart *et al.* 2015a; Figure 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 (Goodheart *et al.* 2015b). The position and monophyly of Arminida was not assessed in these analyses, so inclusion of taxa within this clade will be critical in future analyses. Importantly, the genus *Melibe* (which has several deletions in the COI gene, making it difficult to place; Pola and Gosliner 2010) 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 (Goodheart *et al.* 2015b).

Regarding relationships among major subclades within Cladobranchia, two separate topologies were supported in the analyses using genomic data (Goodheart *et al.* 2015b). 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

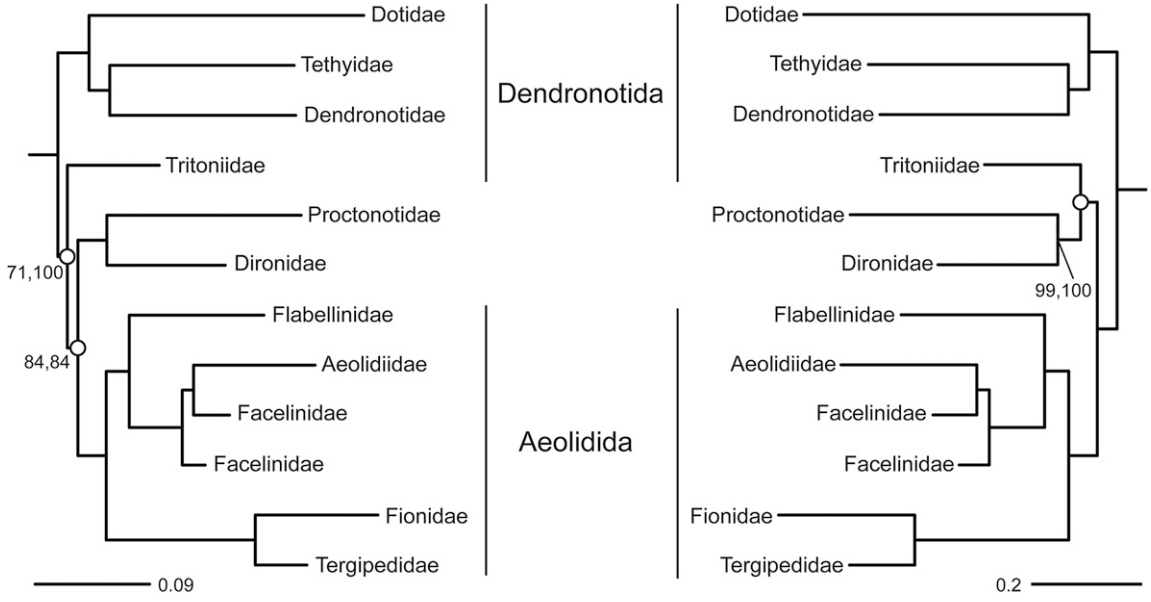

Figure 2. The two primary topologies for Cladobranchia based on analyses using 839 orthologous groups by Goodheart *et al.* (2015a). Bootstrap values for each node are 100% unless otherwise noted. The tree on the left was supported by two analyses – *degen* (703,665 nt positions, 46.4% complete, degeneration coding performed), first bootstrap value and *nt12_partitioned* (1,702,782 nt positions, 23.1% 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, 23.1% complete, partitioned by codon position), second bootstrap value. Open circles indicate the nodes at which the two topologies differ.

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 (Goodheart *et al.* 2015b), which are weakly indicated by one previous study (Carmona *et al.* 2013a). 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 phylogenetic 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 (Goodheart *et al.* 2015b). 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 (Wägele and Willan 2000), 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 (Goodheart *et al.* 2015b), 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 (Goodheart *et al.* 2015a, Mahguib and Valdés 2015), 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. 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.* (2015a) 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 (Wollscheid-Lengeling *et al.* 2001, Pola and Gosliner 2010, Mahguib and Valdés 2015), and thus genomic data may not lead to further changes within this taxon. 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 (Putz *et al.* 2010) and the independent evolution of rhythmic motor behavior (Lillvis and Katz 2013), 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 (Goodheart and Bely 2016, Greenwood 2009). The vast majority of cladobranchs feed on species of Cnidaria, primarily anthozoans (e.g., anemones, octocorals) and hydrozoans (hydroids) (McDonald and Nybakken 1991, 1997, 1999, Putz *et al.* 2010), but only species within the clade Aeolidida possess this ability, with one exception: members of the genus *Hancockia* Gosse, 1877 (Dendronotida) also sequester nematocysts (Putz *et al.* 2010). 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 (Edmunds 1966, Wägele 2004, Putz *et al.* 2010; Figure 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 (Goodheart and Bely 2016). In addition, diversification

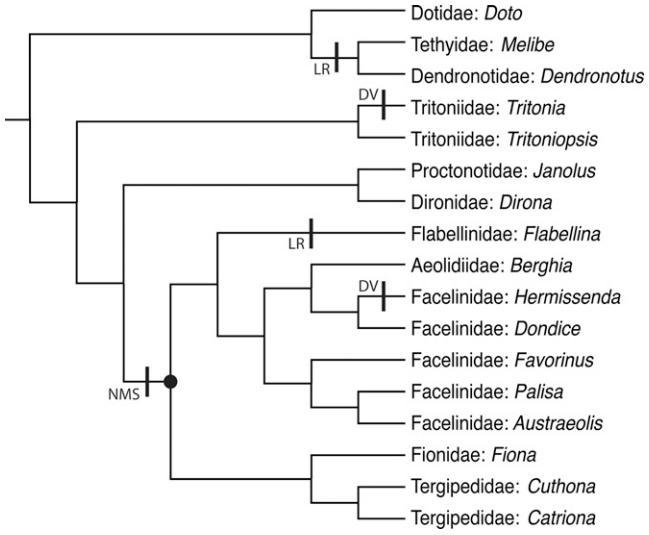

Figure 3. Inferred origins of multiple characters based on the current phylogenetic hypothesis for Cladobranchia. The closed black circle indicates the base of Aeolidida. Abbreviations: NMS indicates an origin of nematocyst sequestration, DV indicates an origin of dorsal-ventral swimming flexions, and LR indicates an origin of left-right swimming flexions.

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 (Rabosky and Goldberg 2015). 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.

A second character of interest is the independent evolution of rhythmic motor behavior within this group (Lillvis and Katz 2013). According to Lillvis and Katz (2013), the use of dorsal-ventral flexions for swimming is present in 17 taxa within Nudipleura, two of which (*Tritonia* Cuvier, 1798 and *Hermissenda* Bergh, 1879) are species within Cladobranchia. It has been estimated that this ability evolved between two to five (and potentially more) times within Nudipleura (Newcomb *et al.* 2012), 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,* and *Flabellina* Gray, 1833) (Newcomb *et al.* 2012, Katz 2016). Thus far, the genomic framework suggests that both dorsal-ventral and left-right flexions evolved at least twice within Cladobranchia (Figure 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 inclusion of type taxa within these analyses 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.

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