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The first molecular phylogeny of cladobranchian opisthobranchs (Mollusca, Gastropoda, Nudibranchia)

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ABSTRACT

The first molecular phylogeny is presented for the highly diverse, opisthobranch molluscan Cladobranchia. This study, the most comprehensive for Cladobranchia to date, used new sequences of two mitochondrial and one nuclear genes for 95 specimens from 22 families and 38 genera with a species of Pleurobrancoidea as outgroup. Although our results do not resolve all the relationships within the Cladobranchia, there are significant findings that have implications for the systematics of the Cladobranchia. Cladobranchia represents a monophyletic group within the Nudibranchia with the exception of a clade containing species of *Melibe*. These species share a deletion of four codons in the COI gene that may account for their strong divergence from the remainder of the Cladobranchia. Bornellidae is the sister group to the rest of Cladobranchia, but this relationship is weakly supported. A series of well-supported clades within Cladobranchia show little structure as to their relationships to each other in the current analysis. The relationships of *Tethys* and Hancockidae to other Cladobranchia remain unresolved. *Pseudobornella orientalis* is here transferred to *Dendronotus* as *D. orientalis*. With this systematic change Bornellidae and Dendronotidae are now monophyletic. *Lomanotus* appears as the sister group to a monophyletic Aeolidida, but this relationship is not strongly supported. Scyllaeidae is monophyletic in this study with *Scyllaea* being sister taxon to *Notobryon*. The Proctonotidae are monophyletic and are clearly nested in the Cladobranchia. Dotoidae is monophyletic when *Pinufius* is included in this clade. *Doriodomorpha* is sister taxon to the Arminidae. Within Arminidae, *Dermatobranchus* and *Armina*, as they are presently constructed, are not monophyletic. There is an interesting potential sister group relationship between *Dirona albolineata* and *Lemina millecra* that requires additional exploration with expanded taxon sampling. In this study, *Marianina rosea* is nested within Tritoniidae, thus we consider Aranucidae, and its synonym Marianinidae, as a junior synonym of Tritoniidae to preserve the monophyly of Tritoniidae.

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1. Introduction

Opisthobranch mollusks are among the most ecologically and morphologically diverse of all gastropod clades. They are also important in terms of biomedical research as they provide models for neurophysiological studies, produce secondary metabolites that have potential pharmaceutical value and are indicators of ecosystem health. Our understanding of the phylogenetic relationships of opisthobranchs has grown in recent years with clarification of monophyletic taxa and relationships between clades (Wägele et al., 2003; Grande et al., 2004; Vonnemann et al., 2005; Wägele and Klussmann-Kolb, 2005; Klussmann-Kolb and Dinapoli, 2006;

Martynov and Schrödl, 2008; Klussmann-Kolb et al., 2008; Malaquias et al., 2009).

The clade containing the largest proportion of opisthobranch species is the Nudibranchia. The traditional classification of Nudibranchia includes four suborders, the Doridina, Aeolidina, Dendronotina, and Arminina (Odhner, 1939). More recently, Wägele and Willan (2000) recognized two major clades of Nudibranchia: the Anthobranchia (Bathydoridodea and Doridoidea) and the Cladobranchia (Dendronotoidea, Aeolidoidea, and the paraphyletic taxon Arminoidea). The Anthobranchia is characterized by having the digestive gland that forms a single compact, ovoid mass while in the Cladobranchia, the digestive gland is variably ramified. The phylogeny of the nudibranchs is not well understood. A morphological phylogeny (Wägele and Willan, 2000) demonstrated that Dendronotina and Arminina were not monophyletic, but few exemplars of these clades were included in the analysis. Wollscheid and Wägele (1999) constructed a molecular phylogeny of the Nudibranchia that also indicated that Dendronotina was paraphyletic, but it only

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included two exemplars of Dendronotina and no representatives of the Arminina.

Recently the phylogenetics of the Doridina has been clarified (Valdés and Gosliner, 1999; Valdés, 2002; Fahey and Gosliner, 2004). All these studies suggest that Doridina is monophyletic, but that of the two traditional higher dorid taxa, Phanerobranchia and Cryptobranchia, the Phanerobranchia is paraphyletic while Cryptobranchia appears to be monophyletic. Pola et al. (2007) also confirmed these results.

Wägele and Willan (2000) and Wollscheid and Wägele (1999) demonstrated that the Doridina (Anthobranchia) is the sister group to the Cladobranchia, but there is little resolution of the relationships between taxa traditionally placed within the Arminina and Dendronotina. Few other phylogenetic studies of Arminina and Dendronotina have been undertaken and are limited to studies of specific clades: *Melibe* (Gosliner and Smith, 2003), Arminidae (Kolb and Wägele, 1998; Gosliner and Fahey, in press), Tritoniidae (Bertsch et al., 2009), Bornellidae (Pola et al., 2009). While adding much to our understanding of relationships within these taxa, none of these studies provide much insight into the resolution of the relationships of taxa within the Cladobranchia. The morphological phylogenetic work of Fahey and Gosliner (in press) also supports previous studies that Arminida is not monophyletic.

Based on the preliminary work undertaken by Wägele and Willan (2000), which demonstrated that Dendronotida and Arminida were not monophyletic, Bouchet and Rocroi (2005) provided a revised classification of the these groups, which we adopt in this study (Table 1). Within the traditional Arminina, Arminidae and Doridomorphidae are included in the Euarminida, while Charcotiidae, Dironiidae, Goniaeolididae, Heroidae, Madrellidae, Pinufiidae, and Proctonotidae are left as unassigned taxa. Within the traditional Dendronotina, Tritoniidae, Aranucidae, Bornellidae, Dendronotidae, Hancockiidae, Lomanotidae, Phylliroidae, Scyllaeidae, and Tethyidae are included in the Dendronotida. Dotoidae and Embletoniidae are not assigned to any higher taxon.

In order to further determine phylogenetic relationships of cladobranch taxa, we undertook a study to compare the molecular phylogeny of Cladobranchia, including a broad range of 94 ingroup taxa to further explore their phylogenetic relationships. By examining DNA sequences in a broad spectrum of taxa for two mitochondrial and one nuclear gene, we tested the monophyly of the three traditional cladobranch taxa, Aeolidida, Arminida, and Dendronotida and determined the phylogenetic relationships among all of the constituent taxa included within these higher taxa.

2. Materials and methods

2.1. Taxon sampling

Sampling included 95 individuals representing 59 described species, 38 genera, 22 families, and 33 un-described species. The classification of all the species used in this study is listed in Table 1 and arranged based on Bouchet and Rocroi's 2005 classification of the Gastropods. The numbers following sp. in the names of un-described species, refer to the identification system used by Gosliner et al. (2008). Un-described species labeled as sp. followed by a letter and a locality refer to new species not included in Gosliner et al. (2008), but which voucher photos are available at the Invertebrate Zoology and Geology research website of the California Academy of Sciences (<http://research.calacademy.org/redirect?url=http://researcharchive.calacademy.org/research/izg/nudibranchs/>). The letters following sp. for other un-described species not included in Gosliner et al. (2008) have not been designated by these letters outside the context of this work. Most of the tissues used in this study came from specimens collected on recent field trips, as well as specimens collected from different people around the world and

specifically sent to us for this study. All these specimens were preserved in 95% EtOH. In addition to the specimens collected specifically for molecular study, we were also able to use museum material that was, either preserved in 70–75% EtOH or where the original fixation method is unknown. We used all specimens for all genera available for molecular studies representing the super families (SPF) Dendronotida, Euarminida and those included in the Unassigned SPF Bouchet and Rocroi (2005). We also included a few species of the SPFs Aeolidida, Porostomata, Cryptobranchia, and Polyceridae for comparative purposes and to provide a more explicit test of the monophyly of Cladobranchia.

Berthella martensi (Pilsbry, 1896), was included as the outgroup as members of the Pleurobranchioidea have been shown to be the sister taxon to Nudibranchia (Dinapoli and Klussmann-Kolb, 2010). All of the species and sequences used in this study (including specimens retrieved from GenBank) are listed in Table 1. Voucher specimens are held either at the California Academy of Sciences, CASIZ (San Francisco, USA), Natural History Museum of Los Angeles County, LACM (Los Angeles, USA), Museo de Zoología de la Universidad de Costa Rica, MZUCR (San Jose, Costa Rica) or Museo de Zoología da Universidade São Paulo, MZSP (São Paulo, Brazil).

2.2. DNA extraction, amplification, and sequencing

Genomic DNA was extracted from small pieces of foot tissue for most samples using Quiagen DNeasy Tissue Kits. In those cases where animals were tiny, small pieces from the dorsal processes were used. Amplification of DNA was conducted on BioRads MyCycler™ Thermocycler (software version 1.065, Bio-Rad Laboratories). Partial sequences of the mitochondrial genes cytochrome c oxidase subunit I and 16S rRNA and the nuclear gene Histone 3 were amplified using pairs LCO1490 and HCO2198 (Folmer et al., 1994), 16Sar-L and 16Sbr-H (Palumbi et al., 1991) and H3a F and H3a R (Colgan et al., 1998), respectively. PCR amplifications were carried out in a 25 µl reaction volume including 1 µl of 10× PCR buffer, 0.2 µl dNTPs (10 mM stock), 1.5 µl MgCl (25 mM stock), 0.025 µl Taq (1.25 U/µl)-Apex, 0.2 µl of each primer (25 µM stock), and 1 µl of genomic DNA. Standard PCRs for COI consisted of: an initial denaturing step at 94 °C for 3 min; 40 cycles of denaturing at 94 °C for 30 s, annealing at 48–50 °C for 30 s; and final extending at 72 °C for 5 min. The partial 16S amplifications followed the following parameters: an initial denaturing step at 94 °C for 3 min; 39 cycles of denaturing at 94 °C for 30 s, annealing at 50–52 °C for 30 s; and extension at 72 °C for 2 min and 25 °C for 2 min. Finally, the PCR conditions for the H3 amplification consisted of an initial denaturing step at 94 °C for 3 min; 35 amplification cycles (94 °C for 35 s, 50 °C for 1 min, and 72 °C for 1 min and 15 s), and a final step at 72 °C for 2 min. Double-stranded amplified product was electrophoresed in a 0.5% TBE agarose gel stained with ethidium bromide. Amplified products were purified with ExoSAP-IT (USB Scientific). Cycle-sequencing reactions were performed using ABI Prism Big Dye Terminator (Applied Biosystems) (total volume 10 µl) and analyzed using the automated sequencer ABI 3130 Genetic Analyzer (Applied Biosystems) in the Center for Comparative Genomics at the California Academy of Sciences (San Francisco, USA). All new DNA sequences have been deposited in GenBank (Table 1).

2.3. Sequence alignment and analysis

H3 and COI sequences were edited and aligned using Geneious Pro 4.5.4 (Drummond et al., 2009) and checked by eye. Protein-coding sequence were translated into amino acids using MacClade 4.08 (Maddison and Maddison, 2005) for confirmation of alignment. 16S sequences were aligned using MUSCLE (Edgar, 2004),

Table 1
Specimens used for molecular analyses, collection sites, dates, vouchers and GenBank Accession Nos.

Family	Species	Locality	Collection dates	Voucher	GenBank Accession Nos.		
					H3	COI	16S
Pleurobranchidae (Gray, 1827) Dorididae (Rafinesque, 1815)	<i>Berthella martensi</i>	Panama: Las Secas, Islas sin nombre	20Mar06	MZUCR6982	HM162498	HM162683	HM162592
	<i>Peltodoris nobilis</i>	California: San Mateo County, Pillar Point	28May05	CASIZ 182223	HM162499	HM162684	HM162593
Chromodorididae (Bergh, 1891)	<i>Hypselodoris picta</i>	Gulf of Guinea: Sao Tome & Principe, Ilha do Principe	19Jan09	CASIZ 179384	HM162500	HM162685	HM162594
	<i>Glossodoris edmundsi</i>	Gulf of Guinea: Sao Tome & Principe, Ilha do Principe	19Jan09	CASIZ 179385	HM162501	HM162686	HM162595
Mandeliidae (Valdés and Gosliner, 1999)	<i>Mandelia microcornata</i>	South Africa: Cape Province, Atlantic Coast, Oudekraal, Coral Gardens, Hottentot's Huisie	05Jan08	CASIZ 176263	HM162502	–	HM162596
Polyceridae (Alder & Hancock, 1845)	<i>Polycera capensis</i>	South Africa: Western Cape Province, Atlantic Coast, Hout Bay	12Jan08	CASIZ 176907	HM162503	HM162687	HM162597
	<i>Roboastra ricei</i>	Florida, 5 mi off shore of Loran Tower	04Mar06	CASIZ 173900	HM162504	HM162688	HM162598
	<i>Tambja marbellensis</i>	Portugal: Atlantic Coast, Setubal District, Outao	20Apr09	CASIZ 180379	HM162505	HM162689	HM162599
	<i>Triopha catalinae</i>	California: San Francisco, San Francisco Yacht Harbor	15Jul04	CASIZ 170648	HM162506	HM162690	HM162600
	<i>Triopha maculata</i>	California: Marin County, Duxbury Reef	22Jul09	CASIZ 181556	HM162507	HM162691	HM162601
	<i>Limacia</i> sp.1	South Africa: Western Cape Province, False Bay	06Jan08	CASIZ 176312	HM162508	HM162692	HM162602
	<i>Limacia</i> sp.2	South Africa: Cape Province, Atlantic coast, Oudekraal	05Jan08	CASIZ 176276	HM162509	HM162693	HM162603
	<i>Piseinotecus</i> sp.	Philippines: Luzon: Batangas, Maricaban, Caban Is.,Layag Layag	22Apr08	CASIZ 177740	HM162510	HM162694	HM162604
Glaucidae (Gray, 1827)	<i>Favorinus elenalexiarum</i>	Costa Rica: Guanacaste, Isla Plata	17Apr07	CASIZ 178875	HM162588	HM162755	HM162679
	<i>Sakuraeolis enosimensis</i>	California: San Francisco Bay, Marin, Richardson Bay	13Dec07	CASIZ 178876	HM162591	HM162758	HM162682
	<i>Babakina indopacifica</i>	Philippines: Batangas, Luzon, Calumpan, Mainit Point	20Mar08	CASIZ 177458	HM162587	HM162754	HM162678
	<i>Godiva quadricolor</i>	South Africa: Western Cape Province, Knysna Lagoon	09Jan08	CASIZ 176385	HM162589	HM162756	HM162680
Doridomorphidae (Marcus & Marcus, 1860) (1908) Arminidae (Iredale & O'Donoghue, 1923) (1841)	<i>Phyllodesmium horridum</i>	South Africa: Western Cape Province, False Bay, Miller's Point	03Jan08	CASIZ 176127	HM162590	HM162757	HM162681
	<i>Doridomorpha gardineri</i>	Malaysia: Pulau Tioman Pulau Renggis	02Oct07	CASIZ 178233	HM162511	HM162695	HM162605
	<i>Armina semperi</i>	Philippines: Batangas, Anilao, Luzon, Mainit Point	21Mar08	CASIZ 177534	HM162512	HM162696	HM162606
	<i>Armina</i> sp.3	Philippines: Batangas, Anilao, Luzon, Bethlehem	18Apr08	CASIZ 177661	HM162513	–	HM162607
	<i>Armina</i> sp.9	Philippines: Batangas, Anilao, Luzon, Mainit Bubbles	22Apr08	CASIZ 177753	HM162514	–	HM162608
	<i>Dermatobranchus pustulosus</i>	Philippines: Batangas, Anilao, Luzon Island, Ligpo	18Apr08	CASIZ 177637	HM162516	–	HM162610
	<i>Dermatobranchus</i> sp.7	Malaysia: Pulau Chimbe off Tioman	05Oct07	CASIZ 178239	HM162517	–	HM162611
	<i>Dermatobranchus</i> sp.12	Philippines: Batangas, Anilao, Luzon, Mainit Point	20Mar08	CASIZ 177481	HM162518	–	HM162612
	<i>Dermatobranchus</i> sp.16	Malaysia: Pulau Labus Tioman	02Oct07	CASIZ 178238	HM162519	–	HM162613
	<i>Dermatobranchus</i> sp.17	Philippines: Luzon, Batangas, Twin Rocks	22May09	CASIZ 179491	HM162520	–	HM162614

(continued on next page)

Table 1 (continued)

Family	Species	Locality	Collection dates	Voucher	GenBank Accession Nos.		
					H3	COI	16S
Tethydidae (Rafinesque, 1815)	<i>Dermatobranchus</i> sp.17	Philippines: Batangas, Anilao, Luzon Island, Ligpo	18Mar08	CASIZ 177643	HM162521	–	HM162615
	<i>Dermatobranchus</i> sp.21	Philippines: Batangas, Anilao, Maricaban Is, Bethlehem	18Mar08	CASIZ 177375	HM162522	HM162698	HM162616
	<i>Dermatobranchus</i> sp.A	South Africa: Hottentot's Huisie, Coral gardens, Ouderkaal, Atlantic coast, Cape Province	05Jan08	CASIZ 176273	HM162515	HM162697	HM162609
	<i>Tethys fimbria</i>	–	–	–	EF133468	AY345035	AY345035
	<i>Melibe digitata</i>	Philippines: Batangas, Anilao, Luzon Is, Twin Rocks	20Mar08	CASIZ 177478	HM162523	HM162699	HM162617
	<i>Melibe viridis</i>	Philippines: Batangas, Anilao, Luzon Is, Mainit Point	21Mar08	CASIZ 177524	HM162524	HM162700	HM162618
Tethydidae (Rafinesque, 1815)	<i>Melibe engeli</i>	Philippines: Batangas, Anilao, Luzon Is, Mainit Bubbles	17Apr08	CASIZ 177625	HM162525	–	HM162619
	<i>Melibe rosea</i>	South Africa: Tsitsikamma	10Jan08	CASIZ 176392	HM162526	HM162701	HM162620
Hancockiidae (MacFarland, 1923)	<i>Hancockia californica</i>	Costa Rica: Guanacaste	15Apr04	CASIZ 175722	HM162527	HM162702	HM162621
Bornelliidae (Bergh, 1874)	<i>Hancockia cf. uncinata</i>	Italy: Calae cicale	14Jul07	CASIZ 175721	HM162528	–	HM162622
	<i>Bornella stellifer</i>	Hawaii: Lanai	Feb03	CASIZ 167989	HM162529	HM162703	HM162623
	<i>Bornella johnsonorum</i>	Marshalls Is: Kwajalein Atoll	14Apr07	CASIZ 175406	HM162530	HM162704	HM162624
	<i>Bornella hermanni</i>	Malaysia: Tokong Kamundi	29Sep07	CASIZ 175743	HM162531	HM162705	HM162625
	<i>Bornella valdae</i>	South Africa: Durban, Kwazulu-Natal	Apr08	CASIZ 176832	HM162532	HM162706	HM162626
	<i>Bornella calcarata</i>	Brazil: Bahía, Marau, Barra Grande de Camamu	12Nov06	MZSP 84448	HM162533	HM162707	HM162627
Dendronotidae (Allman, 1845)	<i>Pseudobornella orientalis</i>	China: Daisong Bay	01Apr07	CASIZ 174989	HM162534	–	HM162628
	<i>Dendronotus regius</i>	Philippines: Batangas, Anilao, Tingloy Is. Kirby's Rock	17May09	CASIZ 179492	HM162535	HM162708	HM162629
	<i>Dendronotus venustus</i>	California: Santa Monica, Redondo Canyon	Dec07	LACM 174850	HM162536	HM162709	HM162630
	<i>Dendronotus iris</i>	Washington: Gig Harbor	29Jul06	CASIZ 174471	HM162537	–	HM162631
	<i>Dendronotus lacteus</i>	Scotland: Garvellachs Islands	04Nov07	LACM 174877	HM162538	HM162710	–
	<i>Dendronotus subramosus</i>	Washington: Hudson's Pt.	12Mar07	LACM 174854	HM162539	–	HM162632
Scyllaeidae (Alder & Hancock, 1855)	<i>Scyllaea pelagica</i>	Texas: Galveston	28Jun07	CASIZ 175651	HM162540	HM162711	HM162633
	<i>Notobryon wardi</i>	Philippines: Batangas, Anilao, Luzon, Mainit Bubbles	16Apr08	CASIZ 177591	HM162544	–	HM162637
	<i>Notobryon wardi</i>	Philippines: Batangas, Anilao, Luzon, Mainit Bubbles	21Mar08	CASIZ 177537	HM162545	HM162714	HM162638
	<i>Notobryon wardi</i>	Marshall Islands: Kwajalein Atoll, South Loi Island	18Nov09	CASIZ 180378	HM162546	–	HM162639
	<i>Notobryon</i> sp.B	Philippines: Batangas, Anilao, Luzon, Mainit Bubbles	22Apr08	CASIZ 177759	HM162541	–	HM162634
	<i>Notobryon</i> sp.C	Mexico: Jalisco, Bahia de Banderas, Mismaloya	03Apr09	CASIZ 180376	HM162542	HM162712	HM162635
	<i>Notobryon</i> sp.D	South Africa: Dale Brooks, Western False Bay, Cape Pr.	07Jan08	CASIZ 176363	HM162543	HM162713	HM162636

Lomanotidae (Bergh, 1890)	<i>Lomanotus</i> sp.E	Mexico: Jalisco, Puerto Vallarta, Los Arcos		LACM 174962	HM162547	HM162715	HM162640
Tritoniidae (Lamarck, 1809)	<i>Tritonia nilsodhneri</i>	South Africa: Cape Province, False Bay, Gordon's Bay	04Jan08	CASIZ 176219	HM162548	HM162716	HM162641
	<i>Tritonia pickensi</i>	Costa Rica: Islas Catalinas	17Jan01	CASIZ 175718	HM162549	HM162717	HM162642
	<i>Tritonia antarctica</i>	Atlantic Ocean: Bouvetoya	30Jun04	CASIZ 171177	HM162550	HM162718	HM162643
	<i>Tritonia festiva</i>	Oregon: Coos County	28Jul06	CASIZ 174491	HM162551	HM162719	-
	<i>Tritonia</i> sp.F	Philippines: Batangas, Luzon Is. Tingloy, Bethlehem	May09	CASIZ 179495	HM162552	HM162720	HM162644
	<i>Marionia blainvillea</i>	Portugal: Azores	20Aug07	CASIZ 176812	HM162553	HM162721	HM162645
	<i>Marionia arborescens</i>	Philippines: Batangas, Maricaban Is., Red Palm	22Mar08	CASIZ 177578	HM162554	HM162722	HM162646
	<i>Marionia levis</i>	Madagascar: kalakajoro	21Oct05	CASIZ 173454	HM162555	HM162723	HM162647
	<i>Marionia elongoviridis</i>	Philippines: Panglao	03Jul04	CASIZ 173308	HM162556	HM162724	-
	<i>Marionia distincta</i>	Philippines: Panglao	04Jul04	CASIZ 173317	HM162557	HM162725	HM162648
	<i>Marionia</i> sp.	Mexico: Jalisco	03Jun03	CASIZ 166891	HM162558	HM162726	HM162649
	<i>Marionia</i> sp.5	Philippines: Batangas, Maricaban Is., Aphol's Point	21Mar08	CASIZ 177513	HM162559	HM162727	HM162650
	<i>Marionia</i> sp.10	Philippines: Panglao	04Jul04	CASIZ 173349	HM162560	HM162728	HM162651
	<i>Marionia</i> sp.14	Philippines: Batangas, Anilao, Luzon Is., Bethlehem	18Apr08	CASIZ 177659	HM162561	HM162729	HM162652
	<i>Tritonia</i> sp.G	South Africa: Cape Province, False Bay, Gordon's Bay	04Jan08	CASIZ 176233	HM162562	HM162730	HM162653
<i>Tritonia</i> sp.3	Philippines: Batangas, Anilao, Luzon Is., Twin Rocks	21Mar08	CASIZ 177523	HM162563	HM162731	HM162654	
<i>Tritonia</i> sp.4	Philippines: Batangas, Anilao, Luzon Isl., Sepok Point	19Apr08	CASIZ 177668	HM162564	HM162732	HM162655	
Aranucidae (Odnher, 1936)	<i>Marianina rosea</i>	Malaysia: P. Tioman	02Oct07	CASIZ 175746	HM162565	HM162733	HM162656
Dotidae (Gray, 1853)	<i>Doto coronata</i>	South Africa: Mushroom Rock: Oudekraal, Cape Pr.	05Jan08	CASIZ 176278	HM162566	HM162734	HM162657
	<i>Doto koenneckeri</i>	Portugal: Azores Islands, Sao Miguel, Ilheu do Vila Franca do Campos	08Sep08	CASIZ 178247	HM162567	HM162735	HM162658
Dotidae (Gray, 1853)	<i>Doto ussi</i>	Philippines: Batangas, Anilao, Luzon Is, Mainit Point.	19Mar08	CASIZ 177438	HM162568	HM162736	HM162659
	<i>Doto</i> sp.2	Philippines: Batangas, Anilao, Luzon Is, Mainit Point.	21Mar08	CASIZ 177543	HM162569	HM162737	HM162660
	<i>Doto</i> sp.7	Philippines: Batangas, Anilao, Luzon Is, Mainit Point.	21Mar08	CASIZ 177542	HM162570	HM162738	HM162661
	<i>Doto</i> sp.15	Philippines: Batangas, Anilao, Luzon Is, Mainit Point.	21Mar08	CASIZ 177545	HM162571	HM162739	HM162662
	<i>Doto</i> sp.H	Mexico: Jalisco, Puerto Vallarta, Los Arcos		LACM 174964	HM162572	HM162740	HM162663
	<i>Doto</i> sp.I	South Africa: Millers Point: W. False Bay, Cape Pr.	03Jan08	CASIZ 176123	HM162573	HM162741	HM162664
	<i>Doto</i> sp.J	Italy: Sardegna	14Jul07	CASIZ 175711	HM162574	HM162742	HM162665
<i>Doto</i> sp.K	Philippines: Batangas, Anilao, Luzon Is, Mainit Point.	20Mar08	CASIZ 177460	HM162575	HM162743	HM162666	

(continued on next page)

Table 1 (continued)

Family	Species	Locality	Collection dates	Voucher	GenBank Accession Nos.		
					H3	COI	16S
Pinufiidae (Marcus & Marcus, 1960)	<i>Pinuffius rebus</i>	Philippines: Batangas, Anilao, Aphol's Rock	23Apr08	CASIZ 177763	HM162576	HM162744	HM162667
Dironidae (Eliot, 1910)	<i>Dirona albolineata</i>	Washington: Pierce County, Gig Harbor	30Jul06	CASIZ 174466	HM162577	-	HM162668
Charcotiidae (Odhner, 1926)	<i>Leminda millecra</i>	South Africa: Western Cape Province: False Bay, Smitswinkel Bay, wreck of HMSAS Good Hope	06Jan08	CASIZ 176348	HM162578	HM162745	HM162669
Proctonotidae (Gray, 1853)	<i>Bonisa nakaza</i>	South Africa: Percy's Hole: Gordon's Bay, Eastern False Bay, Cape Province	04Jan08	CASIZ 176146	HM162579	HM162746	HM162670
	<i>Janolus barbarensis</i>	California: Santa Barbara Harbor	24Jun05	CASIZ 176833	HM162580	HM162747	HM162671
	<i>Janolus capensis</i>	South Africa: Millers Point, Western False Bay, Cape Pr.	03Jan08	CASIZ 176129	HM162581	HM162748	HM162672
	<i>Janolus longidentatus</i>	South Africa: A-Frame: Western False Bay, Cape Prov.	06Jan08	CASIZ 176320	HM162582	HM162749	HM162673
	<i>Janolus mirabilis</i>	Philippines: Batangas, Anilao, Tingloy Is., Layaalayag	19May09	CASIZ 179494	HM162583	HM162750	HM162674
	<i>Janolus</i> sp.1	Philippines: Batangas, Anilao, Maricaban Is. Red Palm	22Mar08	CASIZ 177573	HM162584	HM162751	HM162675
	<i>Janolus</i> sp.2	Philippines: Batangas, Anilao, Luzon, Maimit Bubbles	21Mar08	CASIZ 177533	HM162585	HM162752	HM162676
	<i>Janolus</i> sp.7	Philippines: Batangas, Anilao, Maricaban Is. Bethlehem	22Mar08	CASIZ 177562	HM162586	HM162753	HM162677

using the default settings. Alignments were further optimized by eye using Geneious (Biomatters) and MacClade. To test for the possible saturation types, we plotted the number of transition (Ti) and transversion (Tv) against the uncorrected pairwise distances. Saturation plots were also examined separately for the first, second, and third positions of proteins-coding genes. For the 16S rRNA locus, two types of tests were run in which we either took into account or did not consider the most variable regions. Results were not different from each other so these ambiguously aligned regions were finally included in the analysis.

2.4. Model selection and phylogenetic analyses

Two different phylogenetic methods, maximum likelihood (ML) and Bayesian inference (BI) were used to infer evolutionary relationships. The analyses were performed using the combined molecular datasets but each gene was also analyzed independently. Incongruence length differences (ILD) tests (Farris et al., 1994) were conducted using the partition homogeneity test in PAUP* 4.0b10 (Swofford, 2002) to determine the congruence between the different sets of data (Cunningham, 1997). Test settings consisted of 10 random stepwise additions (100 replicates) with TBR branch swapping. Analyses were performed using parsimony (with the heuristic search option) as the optimality criterion. Evolutionary models for each dataset and partition (combined datasets) were selected using MrModelTest 2.3 (Nylander et al., 2004) under the Akaike information criterion (Akaike, 1974). All full datasets and truncated alternatives, shortened so that no missing taxa and characters were present, consistently yielded the same evolutionary models (GTR+I+G), so the full set (including missing characters for some taxa) was used in the analysis. Support for nodes in the ML analysis was assessed with non-parametric bootstrapping (BP) using both RAxML v7.0.4 (Stamatakis et al., 2008) and Garli v.0.951 (Zwickl, 2006) with 1000 pseudoreplicates, random starting trees, and parameters estimated from each dataset under the model selected for the original dataset. RAxML automatically determined the number of necessary bootstrapping runs. Support values from both programs were very similar. Fig. 1 shows values obtained with RAxML. The combined dataset was also analyzed in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) after partitioning the data. Models for each partition were chosen in MrModelTest as explained above. Analyses were initiated with random starting trees and run for 5×10^7 generations and four chains. Markov chains were sampled at intervals of 1000 generations. The program TRACER v1.3 (Drummond and Rambaut, 2007) was used to determine when the log likelihood (ln L) of sampled trees reached a stationary distribution. Generations sampled before the chain reached stationary were discarded as burn-in, and the remaining trees were used to create 50% majority-rule consensus tree and to estimate Bayesian posterior probabilities. Bayesian posterior probabilities of 0.95 or higher are considered strongly supported while maximum likelihood values of 75 or higher are considered strongly supported. Values below these are considered weakly supported.

3. Results

As indicated in Table 1, amplifications were not successful for some gene fragments in isolated taxa. After alignment, 1470 bp were used: 328 for H3, 658 for COI, and 484 for 16S (including gaps and variable regions). We obtained 94 new sequences for H3, 76 for COI, and 91 for 16S, and we used 3 sequences (H3, COI, and 16S) from GenBank (only for the species *Tethys fimbria*) (Table 1). The saturation plots of uncorrected differences against corrected sequences divergences divided by codon indicated no saturation for any gene (not shown).

The congruency of the H3, COI, and 16S trees ($p = 0.99$) allowed these markers to be combined into a single analysis (Cunningham, 1997). The combined tree provides better resolution of overall topology than H3, COI, or 16S separately. Fig. 1 shows the phylogenetic hypothesis based on the combined dataset represented by BI. The topology of the tree obtained by ML (not shown) is exactly the same as the one represented by Bayesian Inference except that the basal relationships obtained by BI for the clade containing specimens of Proctonotidae, Aeolidina plus *Lomanotus*, *Dirona*, *Leminda*, and Tritoniidae is not recovered under ML analyses.

Fig. 1 strongly supports the monophyly of Doridacea as the sister group to most of the Cladobranchia (except *Melibe*). Within Doridacea, the Cryptobranchia is paraphyletic while our limited sample of Phanerobranchia (only Polyceridae were sampled) is monophyletic, but weakly supported (Pp = 0.61, ML = 56). Bornelliidae is the sister group to the rest of Cladobranchia, but this relationship is weakly supported (Pp = 0.63, not recovered in ML). A series of well-supported clades within Cladobranchia show little structure as to their relationships to each other in the current analysis. The relationships of *Tethys* and Hancockidae to other Cladobranchia remain unresolved. Dendronotidae is strongly supported as a clade (Pp = 1.0, ML = 99) when *Pseudobornella* is included in Dendronotidae rather than Bornelliidae. *Pseudobornella orientalis* is here transferred to *Dendronotus* as *D. orientalis* (Baba, 1932), **comb. nov.** With this systematic change Bornelliidae and Dendronotidae are now both monophyletic. *Lomanotus* appears as the sister group to a monophyletic Aeolidida, but this relationship is not strongly supported (Pp = 0.66, ML = 46). Aeolidida is monophyletic and strongly supported (Pp = 1.0, ML = 94). Within this clade, the Piseinotididae (represented by the species *Piseinotecus* sp.) is basal and sister to the rest of the Aeolidida. Scyllaeidae is monophyletic and strongly supported (Pp = 0.99, ML = 93) in this study, with *Scyllaea* being sister taxon to *Notobryon*. The Proctonotidae are monophyletic and are clearly nested in the Cladobranchia (Pp = 1.0, ML = 93). Dotoidae is monophyletic when *Pinufius* is included in this clade (Pp = 0.97, ML = 56) but is only weakly supported in the maximum likelihood tree. *Doriodomorpha* is strongly supported as sister taxon to the Arminidae (Pp = 0.98, not recovered in ML) in the Bayesian tree but not recovered in the maximum likelihood tree. Within Arminidae, *Dermatobranchus* and *Armina*, as they are presently constructed, are not monophyletic. A sister group relationship between *Dirona* plus *Leminda* and the Tritoniidae is weakly supported (Pp = 0.54, not recovered in ML). There is a sister group relationship between *Dirona albolineata* and *Lemina millecra* that is strongly supported (Pp = 0.96, ML = 44). Tritoniidae is monophyletic but very weakly supported (Pp = 0.56, not recovered in ML). This clade is divided in two: one clade including species of the genus *Tritonia* plus *Marianina rosea* (Pp = 1, ML = 88) and another clade including species of the genus *Marionia* (Pp = 1, ML = 88). In the tree the later clade appears to include species previously identified as *Tritonia*, these un-described species are clearly *Marionia* species and they will be described in a forthcoming paper. In this study, *Marianina rosea* is nested within Tritoniidae, thus we consider Aranucidae, and its synonym Marianinidae, as a junior synonym of Tritoniidae to preserve the monophyly of Tritoniidae.

4. Discussion

The primary aim of this study was provide the first estimate of relationships within the diverse clade Cladobranchia focusing on the Arminoidea and Tritonioidea as well as members of most of the unassigned taxa and some species of the Aeolidioidea. We used the widely used COI and 16S markers that have been used successfully for elucidating relationships among species and genera for

different phyla. We also added data on the nuclear histone H3 gene. This extremely conservative H3 protein has previously been used to clarify arthropod molecular evolution (Colgan et al., 1998) and later in polychaete annelid (Brown et al., 1999), gastropod (Colgan et al., 2000, 2003; Dinapoli et al., 2007), polyplacophoran (Okusu et al., 2003), cephalopod (Lindgren et al., 2004), and hexapod (Kjer et al., 2006) phylogenies.

Within Gastropods, very few species representing Cladobranchia have been used in phylogenetic analyses. Wollscheid and Wägele (1999) in their paper on the molecular phylogeny of the Nudibranchia, using 18S rDNA data only, included *Tritonia plebeia*, *Melibe leonina* and three species of Aeolidida as representatives of Cladobranchia. They obtained a highly supported clade in which *Tritonia plebeia* was basal and sister to a clade including *Melibe leonina* and the remaining Cladobranchia species included in the analysis. Wägele and Willan's phylogenetic analysis on Nudibranchia, based on morphological data, included 16 Cladobranchia species (Wägele and Willan, 2000). In these analyses high bootstraps values were recovered for Cladobranchia (96) and Arminidae (99). Resolution within Cladobranchia was particularly low. Dendronotida always turned out to be monophyletic and the recent unassigned groups (Dotidae, Charcotiidae, Dironidae) never clustered on any branch. Wollscheid-Lengeling et al. (2001) confirmed these morphological results using three molecular markers (18S, 16S, and COI). Dinapoli et al. (2007) only included *Tethys fimbria* and *Armina neapolitana* in order to resolve the phylogenetic reconstruction of heterobranch Gastropoda based on histone H3 data.

Our paper is the first to focus on the phylogenetic relationships of the Cladobranchia and to use broad sampling across the group including a large number of representative taxa. Although our results do not resolve all the relationships within the Cladobranchia, there are significant findings that have implication to the systematics of the Cladobranchia. The following relationships are suggestive of systematic revision within the Cladobranchia.

4.1. Doridacea

Doridacea is the sister group to the Cladobranchia and represents a monophyletic group. Within the Doridacea, the Cryptobranchia is paraphyletic, as *Peltochelis nobilis* is the sister group to the Phanerobranchia. Phanerobranchia is monophyletic in the present analysis, but it should be noted that all of the phanerobranch exemplars included in this study are representatives of the Triophinae, Nembrothinae and the Polycerinae, all subgroups within the Polyceridae. Other phanerobranch taxa, such as Goniodorididae, Onchidorididae, Gymnodorididae and Aegiridae, were not included in this study as phylogeny of the Cladobranchia was the primary focus of this work rather than an overall phylogeny of the Nudibranchia. A number of dorid taxa were included in the present analysis largely to test the monophyly of Cladobranchia by having more potential outgroup taxa included. Increased resolution of dorid phylogeny will require inclusion of other phanerobranch and cryptobranch taxa.

4.2. Cladobranchia

Cladobranchia represents a monophyletic group within the Nudibranchia, with the exception that species of *Melibe* cluster outside of the traditional Nudibranchia or could be considered as the sister group to the remainder of the Nudibranchia. These taxa also are contained on a very long branch owing to the fact that all of these species share a deletion of 12 nucleotides on the COI gene. Their placement on the combined tree is likely an artifact of this deletion. Further resolution of the phylogenetic placement of *Melibe* will require study of additional genes to determine these relationships. The sister group to the Bornelliidae includes all

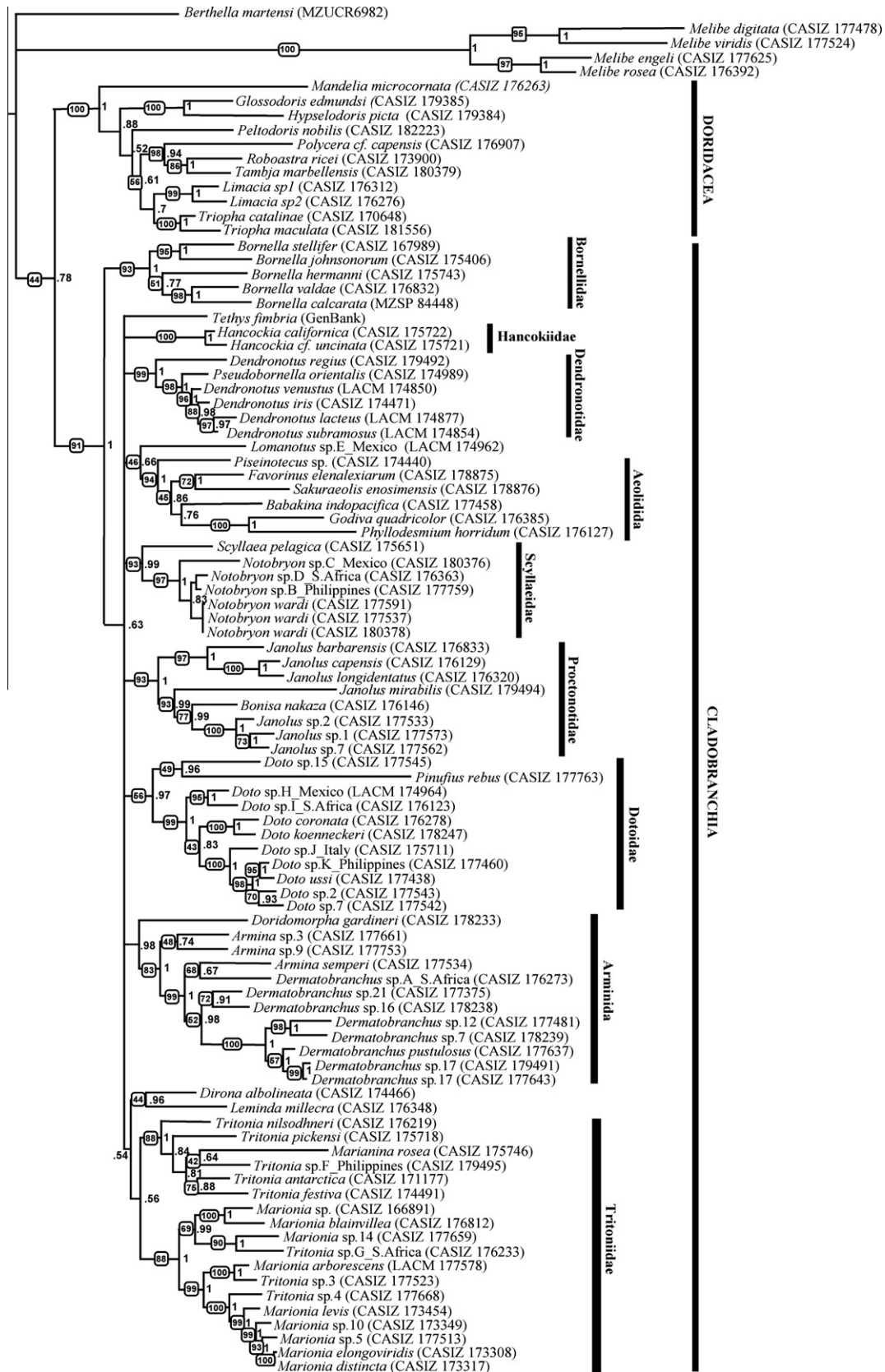


Fig. 1. Phylogenetic hypothesis based on combined molecular data (H3+COI+16S) represented by Bayesian inference. Numbers after branches represent posterior probabilities from Bayesian inference. Numbers on branches, they indicate bootstrap values for ML.

remaining Cladobranchia and there is little basal structure within this large cladobranch clade. This study reconfirms the initial

findings of Wollscheid and Wägele (1999) and Wägele and Willan (2000) using a much broader sampling of taxa. Cladobranchia

remains as the sister group to the Anthobranchia (Eucteniidae of Bouchet and Rocroi (2005)).

4.3. Bornellidae

The five species of *Bornella* included in this analysis form a clade. Interestingly, the only Atlantic species, *B. calcarata* is sister to a tropical western Indian Ocean taxon. Greater taxon sampling of *Bornella* is required to further resolve relationships within this clade. The fact that *Pseudobornella* is not a member of Bornellidae, but rather Dendronotidae, as suggested by Pola et al. (2009), is confirmed by molecular data as well as morphological study.

4.4. Tethyiidae

Traditionally, *Melibe* and *Tethys* are included together in the Tethyiidae. In this study, *Melibe* is located quite basally and has a long branch length. This position is likely to do the presence of 12 nucleotides that have been deleted from the *Melibe* genome. While the relationships between *Tethys* and *Melibe* remain unresolved, *Melibe* clearly represents a clade, with three Indo-Pacific species and one temperate South African species. Employing other genes that lack major deletions or insertions is a promising avenue to resolve the issue of the phylogenetic relationships of *Melibe* to other nudibranchs, especially other cladobranchs. The relationship of *Tethys* to other cladobranchs is presently unresolved.

4.5. Hancockiidae

The two species of *Hancockia*, including one taxon from Costa Rica and another from Italy, form a clade. The relationships of Hancockiidae to other Cladobranchia remain unresolved.

4.6. Dendronotidae

Traditionally, *Pseudobornella* has been included in the Bornellidae. Based on morphological data a recent study by Pola et al. (2009) has shown that *Pseudobornella* is more closely related to Dendronotidae than to Bornellidae. Inclusion of *Pseudobornella* in Bornellidae renders Bornellidae paraphyletic. That view is also supported by the molecular data presented here. In this study, *Pseudobornella* is clearly nested within *Dendronotus* species. *Pseudobornella orientalis* is here transferred to *Dendronotus*. With this systematic change Bornellidae and Dendronotidae are now monophyletic.

4.7. Aeolidida

Aeolidida is monophyletic in this study. All of the aeolid taxa are nested within this clade and are the sister group of *Lomanotus* sp. The relationship between *Lomanotus* and Aeolidida needs to be investigated further and may be an artifact of the fact that the only aeolids represented are members of Facelinidae, Piseinotecidae and Babakinidae, which are more highly derived aeolids. Members of the Notaeolidiidae and Flabellinidae have been shown to occupy a more basal position within the Aeolidida (Wägele and Willan, 2000) but no representatives of these taxa were included in this study. Additionally, no species of Tergipedidae or Eubranchidae are included in the present analysis. A more comprehensive study of aeolid phylogeny is certainly required, but is not the primary focus of this study.

4.8. Scyllaeidae

In the present analysis both Scyllaeidae and *Notobryon* are monophyletic and *Scyllaea* is the sister group to *Notobryon*. Inclusion of

Crosslandia species in future analyses would be worthwhile in more fully resolving relationships within this family.

4.9. Proctonotidae

Members of this family have traditionally been included in the Arminida and more recently, Gosliner and Fahey (in press) suggested that Proctonotidae might form a clade that is sister to the Doridacea, based on a morphological phylogeny. In the present analysis, the Proctonotidae are monophyletic and are clearly nested in the Cladobranchia rather than forming a sister taxon to the Doridacea. Their relationships to other cladobranch taxa remain unresolved.

4.10. Dotoidae

Our analysis of Dotoidae includes 10 species of *Doto*. The majority of taxa form a distinct clade with the exception of *Doto* sp. 15, which is sister taxon to *Pinufius rebus*. *Pinufius rebus*, which is situated on a relatively long branch, has been traditionally considered as a species of Arminida, but also bears some resemblance to aeolid nudibranchs. Some species of tergipedid aeolids of the genus *Phestilla* also feed on corals of the genus *Porites* and may be more closely related to *Pinufius*. No tergipedid aeolids were included in the present analysis. Further study of these taxa will help resolve this apparent relationship.

4.11. Dironidae/Charcotiidae

There is an interesting potential sister group relationship between *Dirona albolineata* MacFarland in Cockerell & Eliot, 1905 and *Leminda millecra* (Griffiths, 1985). These two taxa are morphologically very divergent, with *Dirona* having numerous elongate papillae on the notum, while *Leminda* has undulating margins of the notum devoid of papillae. Both of these taxa have been included in the traditional Arminida and were included as unassigned families in Bouchet and Rocroi, where Lemindidae was considered a synonym of Charcotiidae. There are no published sequences for *Charcotia* or *Pseudotritonia* to compare with *Leminda*. It would be desirable to include sequences of these taxa together with the other species of *Dirona* to test further this relationship. *Dirona* plus *Leminda* are sister to the Tritoniidae, in the present analysis, but this relationship is poorly supported. This relationship requires further investigation, especially in light of the weak support for the monophyly of the Tritoniidae.

4.12. Arminida

The Arminidae plus Doridomorphidae forms a distinct clade within the Cladobranchia, and this clade is strongly supported. The other taxa traditionally included in Arminida are not the closest relatives of the Arminidae and Doridomorphidae. This confirms the initial studies of Wägele and Willan (2000) and Gosliner and Fahey (in press), both based on morphological data. Bouchet and Rocroi (2005) included Arminidae and Doridomorphidae in the clade Euarminida, but this taxon is unnecessary, as Arminida can be applied to this clade and has been more widely used. It is unclear why these authors included Arminidae and Doridomorphidae in the same clade and no basis is included in their extensive footnotes. Despite this lack of basis, the relationship is clearly supported by this study. Interestingly, Arminidae and Doridomorphidae are the only Arminida taxa that feed on octocoral anthozoans. This possible relationship between *Doridomorpha* and Arminidae certainly requires additional study.

4.13. Arminidae

Within Arminidae, it is apparent that *Dermatobranchus* and *Armina*, as they are presently constructed, are not monophyletic. In the present analysis only three species of *Armina* are included and all of them are endemic to the Indo-Pacific tropics. *Armina* species are known from most polar, temperate and tropical regions of the world. Including additional *Armina* taxa representing a greater diversity of taxa might provide additional resolution to the tree. It is likely that *D. albineus* is not part of a monophyletic *Dermatobranchus*, despite the fact that it shares morphological synapomorphies with other species of *Dermatobranchus* (Gosliner and Fahey, in press). Clearly, some revision of *Armina* and *Dermatobranchus* is necessary once more taxa from a diversity of localities have been studied. While taxa can be added or eliminated to *Dermatobranchus* to make this taxon a monophyletic, *Armina* may represent a grade rather than a clade. Further investigation with greater taxon sampling may resolve some of the remaining systematic issues within Arminidae.

This study further substantiates the monophyly of Cladobranchia and establishes its sister group relationship with Doridacea when a broad spectrum of cladobranch taxa are studied. While the monophyly of the Cladobranchia is strongly supported, relationships of its component clades show little resolution. The monophyly of most major groups of cladobranchs that have been traditionally treated as “family-level taxa” is largely supported, while the higher taxa of Dendronotida and Arminida are shown to be clearly paraphyletic or unresolved in their relationships. The Aeolidida is monophyletic in this study but diverse taxon sampling of Notaeolidiidae, Flabellinidae, Eubranchidae and anomalous taxa such *Glaucus*, *Fiona* and *Cumanotus* need to be sampled. The taxa Bornellidae, Hancockiidae, Scyllaeidae, Dendronotidae, Dotoidae Arminidae and Proctonotidae are well supported as clades. Unresolved relationships of *Melibe*, *Tethys*, Hancockiidae *Lomanotus*, *Pinufius* require additional study with additional genes and broader taxon sampling. The monophyly of Tritoniidae also requires additional testing with expansion of genetic markers and inclusion of additional taxa.

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