Aligning evidence: concerns regarding multiple sequence alignments in estimating the phylogeny of the Nudibranchia suborder Doridina

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Molecular estimates of phylogenetic relationships rely heavily on multiple sequence alignment construction. There has been little consensus, however, on how to properly address issues pertaining to the alignment of variable regions. Here, we construct alignments from four commonly sequenced molecular markers (16S, 18S, 28S and cytochrome c oxidase subunit I) for the Nudibranchia using three different methodologies: (i) strict mathematical algorithm; (ii) exclusion of variable or divergent regions and (iii) manually curated, and examine how different alignment construction methods can affect phylogenetic signal and phylogenetic estimates for the suborder Doridina. Phylogenetic informativeness (PI) profiles suggest that the molecular markers tested lack the power to resolve relationships at the base of the Doridina, while being more robust at family-level classifications. This supports the lack of consistent resolution between the 19 families within the Doridina across all three alignments. Most of the 19 families were recovered as monophyletic, and instances of non-monophyletic families were consistently recovered between analyses. We conclude that the alignment of variable regions has some effect on phylogenetic estimates.
of the Doridina, but these effects can vary depending on the size and scope of the phylogenetic query and PI of molecular markers.

1. Introduction

The debate regarding multiple sequence alignment (MSA) construction [1–8] and molecular marker selection [9] for use in phylogenetic estimates has been well established in the literature. There is little consensus, however, on the most accurate and replicable approach when considering MSA assemblies [10]. The crux of the matter is how best to align and represent homology in highly variable regions of sequence data. Most approaches employ a combination of a mathematical algorithm and manual curation of variable regions. Unfortunately, in most instances manual curation is loosely defined—which makes subsequent studies difficult to replicate—and even though mathematical algorithms allow for studies to be easily replicated, they do not take into account evolutionary history and consider homology exclusively as similarity [3]. In addition, they often fail to accurately align variable regions [11].

The estimation of evolutionary relationships relies heavily on the ability to determine homology between any given set of characters. The wrongful designation of characters as homologous can confound interpretations of evolutionary relationships by creating homoplasies, which results in the loss of phylogenetic signal [12]. Thus, the determination of homology in either morphological character matrices or MSAs (computational or manually curated) has a direct impact on the accuracy and ability to replicate phylogenetic estimates. This has led to differing positions concerning variable regions and their importance in increasing [13,14] or decreasing [15–17] phylogenetic signal. These highly variable loop regions become increasingly more difficult to align as sequences become more divergent. This confounds the ability to determine accuracy in MSAs and may result in contradictory estimates of deeper evolutionary relationships. It has been proposed that the large sections created by highly variable loop regions in MSAs should be excluded because of the uncertainty in determining reliable estimates of positional homology [18]. Conversely, it has also been suggested that effects from MSAs should be explored in determining phylogenetic estimates due to their likelihood of containing phylogenetic signal [19,20].

Highly divergent sequences and a general lack of diverse molecular markers have made MSAs and resulting phylogenetic estimates for the Nudibranchia problematic [21–26]. For these reasons, we examine how MSA construction of common nDNA and mtDNA markers affect phylogenetic estimates of the diverse Nudibranchia clade Doridina. Dorid nudibranchs have been a robust model for investigating biochemical diversity [27–33], morphological evolution [34–42], colour evolution [43–49], population structure [50–52] and development [53–57]. Even though there have been previous phylogenetic estimates that included dorid nudibranchs (e.g. [35,36]), they did not include representatives of all 19 families within the Doridina, and thus, a comprehensive phylogenetic context for the evolution of these traits is deficient.

The Doridina suborder currently consists of 19 families and more than 2000 described species. The classification has been divided into five superfamilies (Bathydoridoidea, Doridoidea, Onchidoridoidea, Phyllidioidea and Polyceroidea) defined by morphological variation in gill and feeding structures [21,36,58,59]. Species that possess the ability to retract their gills into fully formed gill pockets, Eudoridoidea (=Cryptobranchia), are divided into two clades based on the presence or absence of radula, Labiostomata (=Doridoidea) and Porostomata (=Phyllidioidea), respectively [36]. Dorid nudibranchs that lack a fully formed gill pocket represent the Anthobranchia (=Phanerobranchia), which is subdivided into the Suctoria (=Onchidoridoidea) and the Non-Suctoria (=Polyceroidea) based on the presence or absence of a buccal pump. Even though morphological [36,59] and molecular [22,23,60,61] analyses have shown the Doridina to be monophyletic, no study has focused on a complete sampling of all currently recognized families. Thus, relationships between families are poorly understood due to conflicting phylogenies and inadequate sampling.

In this study, we use three common MSA methodologies: (i) strict mathematical algorithm; (ii) exclusion of variable or divergent regions and (iii) manually curated. We then discuss issues pertaining to the lack of resolution in phylogenetic estimates and limitations in resolving the base of the Doridina. We also address the possible effects alignment construction may have on phylogenetic informativeness (PI), and how phylogenetic signal may shift given alignment methodology. Lastly, we re-evaluate taxonomic classifications that were consistent between all three analyses.
2. Material and methods

2.1. Taxon sampling

We sampled (121 taxa) representatives from the five superfamilies and all 19 families that currently comprise the Doridina, and mined GenBank for specimens that had at least two sequences. To limit the amount of missing data, we obtained extractions or tissue samples from published specimens to sequence molecular markers that were previously unattained. *Pleurobranchaea meckeli* and *Berthella martensi* were chosen as outgroups because the Pleurobranchomorpha has been suggested to be sister to the Nudibranchia [23]. Additional members from the Aeolidina and Dendronotina were also used in testing the monophyly of the Doridina. Specimens, voucher numbers, collection sites and GenBank accession numbers are listed in table 1. Voucher specimens are located in the collections at California Academy of Sciences (CASIZ) and the Museum of National Scientific Center of Marine Biology, Vladivostok (MIMB).

2.2. Extraction, PCR protocols and sequencing

Genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue extraction kit (Valencia, CA). Amplification and sequencing for targeted gene fragments 16S, 18S, 28S and cytochrome c oxidase subunit I (COI) followed protocols used by Hallas & Gosliner [62]. Amplified fragments were sequenced at the Center for Comparative Genomics located at the California Academy of Sciences and National Scientific Center of Marine Biology.

2.3. Multiple sequence alignments

We assembled and edited sequences in Geneious Pro v. 9.1.7 [63] and BioEdit [64]. We aligned rDNA fragments (16S, 18S and 28S) using three different methodologies to examine conflicts regarding estimated phylogenies. For our first method, we used a computer algorithm E-INS-i in MAFFT (MA) [65]. The E-INS-i algorithm is designed to handle sequence data with several conserved regions embedded in long unalignable gapped regions. Our second method excluded all variable regions from the initial MAFFT alignment of rDNA using Gblocks (GA) [11]. We determined blocks using a less stringent selection by allowing for smaller final blocks, gap positions within the final blocks and less strict flank positions. For our third method, we used the initial MAFFT alignment from method one, but manually curated variable regions (CA). This was done by hand to correct for possible inappropriate alignment of sequence regions. For each alignment method, we concatenated all four targeted molecular markers into single MSAs that resulted in three separate concatenated datasets.

2.4. Phylogenetic informativeness

We estimated PI for each molecular marker per alignment [9] through the PhyDesign web interface [66], and estimated each PI profile using the site-rates model HyPhy [67]. First, we generated ultrametric trees by converting our concatenated Bayesian inference (BI) phylogenetic estimates in Mesquite [68]. Owing to the lack of a fossil record for the Doridina, our ultrametric trees are not in known time units. However, it has been shown that PI profiles can be used effectively even if divergence time estimates are absent [69]. Then, we analysed alignments in relation to each of their resulting estimated phylogeny to gain a greater understanding of how PI can change based on the alignment approach.

2.5. Phylogenetic analyses

We determined evolutionary models using PartitionFinder v.1.1.1 [70], and partitioned our concatenated datasets by rDNA fragment as well as codon position for COI. We also analysed our nDNA and mtDNA fragments separately to investigate possible conflicting evolutionary histories. We did this by aligning both nDNA and mtDNA datasets using the algorithm E-INS-i in MAFFT, and used the same partitioning scheme as in our concatenated datasets.

We analysed all our datasets using BI and maximum-likelihood (ML). BI searchers were run using MrBayes v. 3.2.1 [71], and convergence was checked in TRACER v.1.5 [72]. The datasets were run for $5 \times 10^7$ generations with Markov chains sampled every 1000 generations. The standard 25% burn-in was calculated and remaining tree estimates were used to create a 50% majority rule consensus tree.
Table 1. Specimens successfully sequenced and used for molecular analyses. Voucher numbers, GenBank accession numbers and collection localities.

| specimen                  | voucher | GenBank accession number | locality                                      |
|---------------------------|---------|--------------------------|----------------------------------------------|
|                            |         | 16S         | 18S         | 28S         | COI         |                            |
| **PLEUROBRANCHOIDEA**      |         |             |             |             |             |                            |
| Pleurobranchaea meckeli    |         | FJ917439    | FJ917449    | FJ917481    | FJ917499    | Mediterranean Sea, Spain  |
| Pleurobranchidae           |         |             |             |             |             |                            |
| *Berthella martensi*       | MZUCR6982 | HM162592 | MF958319    | MF958363    | HM162683    | Las Secas, Islas sin nombre, Panama |
| **AEOLIDINA**              |         |             |             |             |             |                            |
| Flabellinidae              |         |             |             |             |             |                            |
| *Flabellina pedata*        | AF249247 | AF124788    | —           | —           | AF249817    | North Sea, Helgoland      |
| **DENDRONOTINA**           |         |             |             |             |             |                            |
| Arminidae                  |         |             |             |             |             |                            |
| *Armina loveni*            |         | AF249243    | AF249196    | —           | AF249781    | North Sea, Kattegat       |
| **DORIDINA**               |         |             |             |             |             |                            |
| Bathydoirididea            |         |             |             |             |             |                            |
| Bathydoirididae            |         |             |             |             |             |                            |
| *Prodoris clavigera*       | CASIZ167553 | JX274067 | MF95832D    | MF958364    | JX274106    | Elephant Island, Antarctica |
| **Doridoidae**             |         |             |             |             |             |                            |
| Actinocyclus verrucomus    | CASIZ189448 | MF958311 | MF958392    | MF958397    | MF958438    | Kauai, Hawaii              |
| Hallina indecora           | CASIZ179600 | MF958302 | MF958340    | MF958386    | —           | South Lof Island, Marshall Islands |
| Hallina translucens         | CASIZ173447 | EU982814 | MF958341    | MF958387    | EU982760    | Iles Radama, Madagascar   |
| Hallina juju                | CASIZ175559 | EU982813 | —           | —           | EU982799    | Maui, Hawaii               |
| Cadlinidae                 |         |             |             |             |             |                            |
| *Aldisa sanguinea*         | CASIZ182031 | MF958309 | MF95830    | MF958394    | MF958435    | Marin Co., California      |
| *Aldisa sp.*               | CASIZ175733 | EU982818 | MF958301    | MF958395    | MF958436    | Tiger Reef, Malaysia       |
| Cadlina cf. luteomarginata | CASIZ188599A | KJ653679 | KP340317    | KP340350    | KM219678    | Parksville, Vancouver Island, British Columbia |
| Cadlina laevis             | CASIZ175444 | —         | MF958399    | MF958406    | —           | Scotland                   |
| Cadlina luna                | CASIZ175437 | EU982768 | —           | —           | EU982718    | Guanacaste, Costa Rica     |
| Cadlina modesta             | CASIZ1823286 | MF958310 | —           | —           | M958437     | Vista del Mar, San Luis Obispo Co., California |
| Cadlina pelucida            | CASIZ175448 | EU982774 | —           | MF958396    | EU982724    | Ilha de Pessegueiro, Baixo Alentejo Prov., Portugal |
| Cadlina sparsa              | CASIZ182932 | EU982776 | —           | —           | EU982726    | La Jolla, San Diego Co., California |

(Continued.)
Table 1. (Continued.)

| specimen | voucher | GenBank accession number | locality |
|----------|---------|--------------------------|----------|
| **Chromodorididae** | | | |
| Cadlinella omatissima (Risbec, 1928) | CASIZ 177420 | MF958284 MF958325 MF958371 MF958415 | Maricaban Island, Luzon, Philippines |
| Cadlinella omatissima (Risbec, 1928) | CASIZ 175452 | EU982779 — — EU982728 | Mooloolaba, Queensland, Australia |
| Chromodoris alternata (Burn, 1957) | SAM D19281 | AY458800 EF534031 — EF535120 | Port Phillip Bay, Victoria, Australia |
| Chromodoris ambiguus Rudman, 1987 | SAM D19260 | AY458801 EF534038 — EF535119 | Port Phillip Bay, Victoria, Australia |
| Chromodoris quadricolor (Rüppell & Leuckart, 1830) | | AF249241 AJ224773 — AF249802 | Red Sea, Egypt |
| Doriprismatica atromarginata (Cuvier, 1804) | | — AF249211 — AF249789 | Great Barrier Reef, Australia |
| ‘Felimare’ elegans (Cantraine, 1835) | | AF249238 AJ224779 — AF249787 | NE Atlantic, Spain |
| ‘Felimare’ midatlantica (Golinski, 1990) | CASIZ 175443 | JQ727789 — — JQ727898 | Islotes do Martinhal, Algarve, Portugal |
| ‘Felimare’ picta verdensis Ortea, Valds & Garca-Gmez, 1996 | CASIZ 179384 | HM162594 MF958346 MF958389 HM162685 | Gulf of Guinea, Ilha do Principe, São Tomé & Principe |
| ‘Felimare’ villafranca (Riaoa, 1818) | | AF249237 AJ224780 — — | NE Atlantic, Spain |
| ‘Felimida’ emundsi Cervera, Garcia-Gmez & Ortea, 1989 | CASIZ 179385 | HM162595 MF958347 MF958390 HM162686 | Gulf of Guinea, Ilha do Principe, São Tomé & Principe |
| ‘Felimida’ krohni (Verany, 1846) | | AF249239 AJ224774 AY427445 AF249805 | NE Atlantic, Spain |
| Goniobranchus geometricus (Risbec, 1928) | CASIZ 175549 | JQ727717 — — JQ727842 | Nosy Valiha, Iles Radama, Madagascar |
| Hypselodoris imperialis (Pease, 1860) | CASIZ 142952 | EU982807 — — EU982754 | Mala Wharf, Maui, Hawaii |
| Hypselodoris infulata (Rüppell & Leuckart, 1830) | | FJ917427 FJ917442 FJ917467 FJ917485 | NSW, Australia |
| Mirama magnaefico Eliot, 1904 | CASIZ 169951 | EU982781 — — EU982731 | Old Woman Island, Queensland, Australia |
| Thorunna danieleae (Kay and Young, 1969) | CASIZ 170055 | EU982809 — — EU982756 | Malaeaa Marina, Maui, Hawaii |
| Tynina evelinae (Marcus, 1958) | CASIZ 175440 | EU982811 — MF958391 EU982757 | Playa Ventana, Guanacaste, Costa Rica |
| Tynina nobilis Bergh, 1898 | ZSM M20050508 | EF34054 EF34035 — EF35127 | |
| **Dorididae** | | | |
| Aphelodoris sp. 1 | CASIZ 176920 | MF958293 MF958322 MF958379 MF958424 | Oudekraal, Cape Prov., South Africa |
| Aphelodoris sp. 1 | | — — GQ292033 GQ292034 | Auckland, New Zealand |
| Aphelodoris luctuosa (Cheeseman, 1882) | | — — GQ292044 | Auckland, New Zealand |
| Doris montereyensis (Cooper, 1862) | CASIZ 174493 | MF982894 MF958333 — MF958425 | Battery Point, Crescent City, Del Norte Co., California |
| Doris odhneri MacFarland, 1966 | CASIZ 188014 | MF982895 MF958334 MF958380 — | Duxbury Reef, Marin Co., California |
| Doris sp. 8 | CASIZ 192348 | MF98306 MF958345 — — | Red Sea, Saudi Arabia |

(Continued)
| specimen | voucher | GenBank accession number | GenBank accession number | GenBank accession number | GenBank accession number | locality |
|----------|---------|--------------------------|--------------------------|--------------------------|--------------------------|----------|
| Discodorididae | | | | | | |
| Asteronotus cespitosus (van Hasselt, 1824) | CASIZ 191096 | MF958288 | MF958328 | MF958375 | MF958419 | Kranket Island, Papua New Guinea |
| Atagema cf osseosa (Kelaart, 1859) | CASIZ 185142 | MF958296 | MF958335 | | MF958426 | Maui, Hawaii |
| Discodoris coerulescens Bergh, 1888 | CASIZ 182850 | MF958290 | MF958330 | MF958377 | MF958421 | Maricaban Island, Luzon, Philippines |
| Halgerda dalanghita Tahey & Gosliner, 1999 | CASIZ 181264 | MF958289 | MF958329 | MF958376 | MF958420 | Maricaban Island, Luzon, Philippines |
| Discodorididae | | | | | | |
| Peltodoris nobilis (MacFarland, 1905) | CASIZ 182223 | EU982816 | | | | Pillar Point, San Mateo Co., California |
| Platyodoris sanguinea Bergh, 1905 | CASIZ 177762 | MF958285 | MF958326 | MF958372 | MF958416 | Maricaban Island, Batangas Prov., Philippines |
| Rostanga calumus Rudman & Avern, 1989 | CASIZ 190788 | MF958286 | MF958327 | MF958373 | MF958417 | Madang Prov., Papua New Guinea |
| Sclerodoris tuberculata Eliot, 1904 | CASIZ 179590 | MF958287 | | MF958374 | MF958418 | Kwajalein Atoll, Marshall Islands |
| ONCHIDORIDOIDEA | | | | | | |
| Akiodorididae | | | | | | |
| Armodoris anudeorum Valdés, Moran & Woods, 2011 | LACM 3118 | KP340290 | GQ326879 | KP340355 | KP340387 | McMurdo Sound, Ross Sea, Antarctica |
| Calycidorididae | | | | | | |
| Calycidoris guentheri Abraham, 1876 | CASIZ 190966A | KP340301 | KP340338 | KP340371 | KP340397 | Chukchi Sea, Alaska |
| Diaphorodoris lurulacauda Milien, 1985 | CASIZ 184341 | KP340307 | KP340344 | KP340377 | KP340403 | Duxbury Reef, Marin Co., California |
| Diaphorodoris luteoancta (Sars, 1870) | LACM 8.7A | KP340308 | | KP340378 | KP340404 | Bahia de Algeciras, Cadiz Prov., Spain |
| Diaphorodoris cf mitsuii | CASIZ 185986 | KP340310 | KP340345 | KP340379 | KP340406 | Sepok Point, Philippines |
| Diaphorodoris papillata Portmann & Sandmeier, 1960 | LACM 8.6A | KP340311 | | | KP340407 | Bahia de Algeciras, Cadiz Prov., Spain Peninsula |
| Corambididae | | | | | | |
| Corambes obscura (Verrill, 1870) | CASIZ 183942 | KP340303 | KP340340 | KP340373 | KP340399 | | New Castle Portsmouth Bay, New Hampshire |
| Corambes pacifica MacFarland & O'Donoghue, 1929 | LACM 2007-2.6C | KP340305 | KP340342 | KP340375 | KP340401 | | Long Beach Marina, Los Angeles Co., California |
| Corambes steinbergae (Lance, 1962) | CASIZ 190508 | KP340306 | | | KP340402 | | Pillar Point, San Mateo Co., California |
| Goniodorididae | | | | | | |
| Anula gibbsae (Risso, 1818) | CASIZ 182028 | KP340291 | KP340322 | KP340356 | KP340388 | Cumberland Co., Maine |
| Anula gibbsae (Risso, 1818) | CASIZ 181271 | MF958291 | | | MF958422 | Duxbury Reef, Marin Co., California |
| Goniodoris nodosa (Montagu, 1808) | LACM 18625A | AF249226 | AJ224783 | | AF249788 | NE Atlantic, Spain |
| Okenia kendi Gosliner, 2004 | CASIZ 191431 | MF958303 | MF958342 | | MF958432 | | Tab Island, Papua New Guinea |

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| specimen | voucher | GenBank accession number | 16S | 18S | 28S | COI | locality |
|----------|---------|--------------------------|-----|-----|-----|-----|----------|
| Onchidorididae | Acanthodoris atrogrisea | Onchidoris atrogrisea | CASIZ 186000 | KJ653646 | KP340323 | KP340357 | KM219646 | Puget Sound, Kitsap Co., Washington |
| Acanthodoris hussoni | MacFarland, 1905 | Acanthodoris hussoni | CASIZ 179480 | KJ653652 | KP340324 | KP340359 | KM219650 | Asilomar, Monterey Co., California |
| Acanthodoris nanaimoensis | O'Donoghue, 1921 | Acanthodoris nanaimoensis | CASIZ 181569A | KJ653656 | KP340325 | KP340360 | KM219657 | Pillar Point, San Mateo Co., California |
| Acanthodoris pilosa | A. S. Milne-Edwards & J. J. Chevalier, 1889 | Acanthodoris pilosa | CASIZ 183941A | KJ653659 | KP340326 | KP340361 | — | — |
| Acanthodoris plana | Fahey & Valdés, 2005 | Acanthodoris plana | CASIZ 176116 | KJ653669 | KP340327 | KP340362 | KM219671 | Table Bay, Western Cape Prov., South Africa |
| Acanthodoris rhodoceras | Cockerell & Eliot, 1910 | Acanthodoris rhodoceras | CASIZ 181572 | KJ653671 | KP340328 | KP340363 | KM219673 | Pillar Point, San Mateo Co., California |
| Knoutsodonta brasilensis | Alvim, Padula & Pimenta, 2011 | Knoutsodonta brasilensis | — | — | — | — | — | — |
| Knoutsodonta depressa | Alder & Hancock, 1842 | Knoutsodonta depressa | CASIZ 186769A | KJ653676 | KP340315 | KP340347 | KM219680 | Asilomar, Monterey Co., California |
| Knoutsodonta jannae | Milten, 1987 | Knoutsodonta jannae | CASIZ 175578 | KJ653677 | KP340326 | KP340359 | KM219676 | Passamaquody Bay Eastport, Washington Co., Maine |
| Onchidoris bilamellata | Linnaeus, 1767 | Onchidoris bilamellata | CASIZ 18593 | KJ653680 | KP340317 | KP340349 | KM219678 | Puget Sound, Kitsap Co., Washington |
| Onchidoris macropompa | Korshunova, Sanamyan & Sanamyan, 2009 | Onchidoris macropompa | MIMB 34210 | MF958292 | MF958331 | MF958378 | MF958423 | Avacha Bay, Kamchatka |
| Onchidoris proxima | Alder & Hancock, 1854 | Onchidoris proxima | CASIZ 183921A | KJ653672 | KP340328 | KP340362 | KM219679 | Passamaquody Bay Eastport, Washington Co., Maine |
| Onchimira cavifera | Korshunova, Sanamyan & Sanamyan, 2009 | Onchimira cavifera | MIMB 34209 | MF958298 | MF958334 | MF958379 | MF958424 | Avacha Bay, Kamchatka |
| Onchimira cavifera | Korshunova, Sanamyan & Sanamyan, 2009 | Onchimira cavifera | MIMB 34209 | MF958298 | MF958334 | MF958379 | MF958424 | Avacha Bay, Kamchatka |

**Phyllidiidae**

| specimen | voucher | GenBank accession number | 16S | 18S | 28S | COI | locality |
|----------|---------|--------------------------|-----|-----|-----|-----|----------|
| Dendrodorididae | Dendrodoris arborescens | Dendrodoris arborescens | CMNH-ZM08965 | — | AB917459 | — | AB917459 | Oka, Tateyama |
| Dendrodoris atomaculata | Alder & Hancock, 1864 | Dendrodoris atomaculata | CASIZ 18123 | M958293 | MF958348 | MF958392 | MF958434 | Janao Bay, Luzon, Philippines |
| Dendrodoris denisoni | Angas, 1862 | Dendrodoris denisoni | CASIZ 17702 | M958308 | MF958349 | MF958393 | — | — |
| Dendrodoris fumata | Rüppell & Leuckart, 1830 | Dendrodoris fumata | CASIZ 192304 | M958358 | MF958405 | MF958444 | Red Sea, Saudi Arabia |
| Dendrodoris guttata | Othertobe, 1917 | Dendrodoris guttata | CMNH-ZM 08967 | — | AF249122 | FJ91470 | AF249799 | Great Barrier Reef, Australia |
| Dendrodoris nigra | Stimpson, 1855 | Dendrodoris nigra | CASIZ 182821 | M958318 | MF958357 | MF958404 | MF958443 | Maricaban Island, Luzon, Philippines |
| Dendrodoris nigra | Stimpson, 1855 | Dendrodoris nigra | AF249242 | MF958318 | MF958357 | MF958404 | MF958443 | Great Barrier Reef, Australia |

(Continued)
Table 1. (Continued.)

| Specimen | Voucher | GenBank Accession Number | 16S | 18S | 28S | COI | Locality |
|----------|---------|--------------------------|-----|-----|-----|-----|----------|
| Doriopsilla albopunctata (J. G. Cooper, 1863) | CPIC 00909 | KR002428 | —   | —   | —   | KR002480 | Long Beach, California |
| Doriopsilla bertschi Hoover, Lindsay, Goddard & Valdés, 2015 | CPIC 01058 | KR002462 | —   | —   | —   | KR002517 | Bahía de los Ángeles, Baja California, Mexico |
| Doriopsilla davibehrensi Hoover, Lindsay, Goddard & Valdés, 2015 | LACM 3419 | KR002476 | —   | —   | —   | —   | Bahía de los Ángeles, Baja California, Mexico |
| Doriopsilla fulva (MacFarland, 1905) | CPIC 00933 | KR002444 | —   | —   | —   | KR002498 | Malibu, California |
| Doriopsilla gemela Gosliner, Schaefer & Millen, 1999 | CPIC 00938 | KR002453 | —   | —   | —   | KR002506 | Malibu, California |
| Doriopsilla janaina Marcus &. Marcus, 1967 | CASIZ 173618 | MF958312 | MF958333 | MF958398 | —   | Galápagos Islands, Ecuador |
| Doriopsilla miniata (Alder & Hancock, 1864) | CMNH ZM008770 | —   | AB917464 | —   | AB917457 | Yoshio, Katsuura |
| Doriopsilla spaldingi Valdés & Behrens, 1998 | CPIC 00908 | KR002427 | —   | —   | —   | KR002479 | San Pedro, Los Angeles Co., California |
| Mandalidae | Mandalia mirocornata Valdés & Gosliner, 1999 | CASIZ 176266 | MF958278 | MF958321 | MF958365 | MF958411 | Oudekraal, Cape Prov., South Africa |
| Phyllidiidae | Ceratophyllidia sp. | CASIZ 181247 | MF958281 | MF958313 | MF958368 | MF958413 | Beatrice, Philippines |
| Phyllidia coelestis Bergh, 1905 | CASIZ 190982 | MF958279 | —   | MF958366 | MF958412 | —   | Kranket Island, Madang Prov., Papua New Guinea |
| Phyllidiella nigra (van Hasselt, 1824) | CASIZ 186196A | MF958280 | MF958322 | MF958367 | —   | Maricaban Strait, Batangas Prov., Luzon, Philippines |
| Phyllidiella pustulosa (Cuvier, 1804) | AF243232 | AF249208 | —   | —   | —   | —   | Great Barrier Reef, Australia |
| Phyllidopsis annae Brunkhorst, 1993 | CASIZ 186138 | MF958283 | MF958324 | MF958370 | —   | —   | Philippines |
| Reticulidia halgerda Brunkhorst & Burnin Brunkhorst, 1990 | CASIZ 186491 | MF958282 | —   | MF958369 | MF958414 | —   | Maricaban Island, Luzon, Philippines |
| Polyceridae | Aegires salopunctatus MacFarland, 1905 | CASIZ 182213 | MF958313 | MF958354 | MF958399 | MF958439 | Marin Co., California |
| Aegires citrinus Pruvot-Fol, 1930 | CASIZ 144027 | MF958314 | MF958355 | MF958400 | MF958440 | —   | Mooloolaba, Queensland, Australia |
| Aegires flores Fahey & Gosliner, 2004 | CASIZ 191244 | MF958316 | —   | MF958402 | MF958442 | —   | Papua New Guinea |
| Aegires serenae (Gosliner and Behrens, 1997) | CASIZ 192185 | MF958315 | —   | MF958401 | MF958441 | —   | Papua New Guinea |
| Aegires villosus Faran, 1905 | CASIZ 177563 | MF958317 | MF958356 | MF958403 | —   | —   | — |
| Gymnodorididae | Gymnodoris sp. | CASIZ 176781 | —   | MF958361 | —   | —   | Pulau Penang, Malaysia |
| Gymnothorax sp. | CAZIS 119381 | 18S 740 | —   | MF958343 | —   | —   | Kapalua Bay, Maui, Hawaii |
| Hexabranchidae | Hexabranthus sanguineus (Ruppell & Leuckart, 1828) | CASIZ 142942 | MF958304 | MF958343 | —   | —   | Papua New Guinea |
| Hexabranthus sanguineus (Ruppell & Leuckart, 1828) | CASIZ 142942 | MF958305 | MF958344 | MF958388 | MF958433 | —   | — |

(Continued)
| specimen | voucher  | GenBank accession number | GenBank accession number | GenBank accession number | GenBank accession number | locality |
|----------|----------|--------------------------|--------------------------|--------------------------|--------------------------|----------|
| Vayssiera sp. | CASIZ 190731 | — | MF958362 | MF958408 | — | Sunshine Coast, Kings Beach, Australia |
| Polyceridae | | | | | | |
| Kaloplocamus sp. 1 | CASIZ 194412 | MF958299 | MF958337 | MF958383 | MF983429 | South Madagascar, Madagascar |
| Limacia sp. 1 | CASIZ 176312 | HM162602 | KP340320 | KP340353 | HM162692 | False Bay, Western Cape Prov., South Africa |
| Limacia sp. 2 | CASIZ 176276 | HM162603 | — | — | HM162693 | Oudekraal, Cape Prov., South Africa |
| Nembrotha cristata Bergh, 1877 | CASIZ 191428 | MF958301 | MF958339 | MF958385 | MF958431 | Madang Prov., Papua New Guinea |
| Plocamopherus pecoso Valls and Gosliner, 2006 | CASIZ 191587 | MF958300 | MF958338 | MF958384 | MF958430 | Madang Prov., Papua New Guinea |
| Polycera quadrilineata (Müller, 1776) | CASIZ 173900 | AF249229 | AJ224777 | — | — | North Sea, Kattegat |
| Roboastra ricei Pola, Cervera & Gosliner, 2008 | CASIZ 170648 | HM162601 | — | — | — | Florida, 5 mi offshore of Loran Tower |
| Tambja marbellensis Schick & Cervera, 1998 | CASIZ 180379 | HM162600 | KP340321 | KP340354 | HM162690 | Setubal District, Oitao, Portugal |
| Triophacatalinae (Cooper, 1863) | CASIZ 181556 | HM162601 | — | — | — | Duxbury Reef, Marin Co., California |
of calculated posterior probabilities. Posterior probabilities (pp) that exceeded 0.95 were considered strongly supported, and values 0.94 and below were interpreted as having low support. For our ML analyses, we calculated non-parametric bootstrapping (bs) values and the ML tree simultaneously in RAxML v. 7.2.6 [73]. We used the same partitioning scheme as in our BI search, but used the evolution model GTR+I− and executed fast bootstrapping runs for $5 \times 10^4$ iterations. Bootstrap values 70 or higher were considered strongly supported, while all other values were evaluated as weakly supported [74].

3. Results

3.1. Molecular data

Sequences obtained for phylogenetic analyses and PI profiles are labelled in table 1, and all alignments have been deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S20396). As expected, our concatenated alignments (MA, GA and CA) varied in length as well as number of parsimony informative characters (table 2). Surprisingly, there were more parsimony informative characters in the MA than the CA. The GA had far fewer parsimony informative characters than the other two alignments generated. Evolutionary model GTR+I+I− was selected by PartitionFinder v. 1.1.1 for each partition based on the Akaike information criterion. Interestingly, specific species of Dendrodoris (D. nigra, D. arborescens, D. guttata and D. funata) had mtDNA regions that were highly divergent.

3.2. Phylogenetic informativeness

The net PI profiles depict the amount of signal through time of each molecular marker in relation to the respective estimated phylogeny (figure 1a–c). Regardless of the alignment, net PI profiles show most phylogenetic signal resides towards the tips of the trees. The amount of overall phylogenetic signal was lowest in our GA estimate, which suggests that information was lost when variable regions were excluded. Lastly, our MA appeared to have slightly more PI than our CA.

3.3. Phylogeny

BI posterior probability estimates generally resulted in higher overall support of relationships than ML non-parametric bootstrap values. Branch lengths for basal nodes were extremely short (figure 2a–c), especially in regions of the tree that resulted in low resolution for relationships between superfamilies (figure 3a–c). There were, however, some general similarities between topologies of both phylogenetic searches and among alignments.

The Doridina suborder was recovered as monophyletic with high support in all analyses estimates (CA: pp = 1.00, bs = 91; GA: pp = 1.00, bs = 86; MA: pp = 1.00, bs = 86; figures 4–6). GA was the only analysis that recovered the Aegiridae sister to all other members of the Doridina, but there was no support. Both MA and CA recovered Prodoris sister to the rest of the Doridina.

Onchidoridoidea and Phyllidioidae were the only two superfamilies recovered as monophyletic in any of the three analyses (figure 3a–c). Onchidoridoidea was recovered as monophyletic only in the CA (pp = 0.99, bs = 46). Phyllidioidae was monophyletic with little to no support in CA (pp = 0.94; bs = 47) and MA (pp = 0.75, bs = 34) analyses. Most families, however, were recovered as monophyletic with high pp and bs support across all three alignments analysed. The Phyllidiidae and Calycidorididae were the only two families that were not supported in all three analyses. In addition, Chromodorididae, Dendrodididae, Dorididae and Polyceridae were consistently recovered as not monophyletic.
Figure 1. Phylogenetic informativeness profiles estimated by PhyDesign. (a) MAFFT alignment. (b) Gblocks alignment. (c) Curated alignment.

The Dorispsilla clade was recovered more closely related (GA: pp = 1.00, bs = 50; MA: pp = 1.00, bs = 65) to a monophyletic Phyllidiidae than to the Dendrodorid clade. This strongly suggests the Dendrodorid clade is not monophyletic. Interestingly, Cadlinidae was recovered as sister to the Phyllidioidea in MA (pp = 0.67, bs = 13) and CA (pp = 0.64), but in our GA estimate Cadlinidae was sister (pp = 1.00, bs = 17) also to a clade formed by Phyllidiidae and Dorispsilla. Aphelodoris was recovered nested within Discodorididae and sister to Atagema cf. osseosa (CA: pp = 0.63, bs = 52; GA: pp = 0.98, bs = 73; MA: pp = 0.95, bs = 67). Furthermore, Vayssiera sp. and Gymnodoris sp. were recovered nested in the Polyceridae and sister to Polycentra quadridlineata (CA: pp = 1.00, bs = 64; GA: pp = 0.98, bs = 77; MA: pp = 0.53, bs = 86).

The Polyceroida, excluding Aegiridae and Hexabrandidae, was consistently recovered closely related to the Chromodorididae (CA: pp = 0.69; GA: pp = 0.99, bs = 36; MA: pp = 0.97, bs = 53). In addition, all three analyses recovered similar topologies that suggest Cadlinella ornitismus to be sister to Hexabrandus sanguineus. MA is the only phylogenetic estimate that recovered significant support for this relationship (pp = 0.96, bs = 82). Onchimira cavifera was recovered nested in the Onchidoridae and sister to Polycentra quadridlineata (CA: pp = 1.00, bs = 86; GA: pp = 1.00, bs = 87; MA: pp = 1.00, bs = 91).

There were only two instances where long branches were evident in the Doridina; Vayssiera sp. and a clade formed by Dendrodorid arborescens, D. fumata, D. guttata and D. nigra. Analysis of nDNA (electronic supplementary material, figure S1) and mtDNA data (electronic supplementary material, figure S2) suggests that the long branches may be an artefact of mtDNA data. The nDNA tree depicts no significantly long branches within the Doridina and recovers a monophyletic Dendrodorid clade, which contradicts our mtDNA tree that recovered a polyphyletic Dendrodorid clade.

4. Discussion

4.1. Resolving the Doridina

Disparity between phylogenetic estimates can confound interpretations and conclusions regarding processes and patterns of lineage diversification. Conflicting estimates are usually consequences of opposing methodologies, which have centred on taxonomic sampling [75–77], molecular markers [12,78], phylogenetic estimates [79–81] and alignment construction [2,3,14,79]. We examined molecular markers, which have been used in varying arrangements, that are most commonly used in nudibranch phylogenetics [21,23,24,49,60–62,82–92].

The large spikes observed in rDNA fragments for our MA and CA are probably a result of highly variable regions or ambiguous sequence calls, which ML is poor at estimating [66], thus overestimating PI towards the tips of their respective tree. Furthermore, PI profiles suggest that information was
lost when these regions were excluded from our analyses, and resulted in surprisingly high loss of parsimony informative characters (25%–55%) for rDNA markers. The removal of these variable regions has been shown to negatively affect phylogenetic estimates [9] and may explain why some relationships were not consistently recovered. This supports the position that highly variable loop regions can be vital in resolving some phylogenetic relationships [8]. Unfortunately, we were unable to increase the resolution of the dorid tree by any of our three MSA construction methods. All three PI profiles were fairly consistent in depicting similar curves, and as we have been able to show, these markers are more appropriate for phylogenetic estimates at family- or higher-level classifications.

Another issue we encountered was the potential noise that was incorporated into our estimates by additional taxon sampling. Even though PI profiles suggest these markers were informative at family level, our increased taxonomic sampling may have hindered our ability to recover consistency across analyses. For example, we only recovered a monophyletic Onchidoridoidea in our CA phylogenetic

**Figure 2.** Phylograms of Doridina phylogenetic estimates from all three different alignment constructions. Topologies represent Bayesian estimates. (a) MAFFT alignment. (b) Gblocks alignment. (c) Curated alignment.
Figure 3. Cladogram of Doridina phylogenetic estimates. Topology represents Bayesian estimate. Branches are coloured based on superfamily designations, which are pictured adjacent to the phylogenies. Only four of the five families are depicted due to the inability to obtain an image of Bathydoridoidea. Circles represent posterior probabilities (top) and non-parametric bootstrap support values (bottom). Closed circles indicate high Bayesian and ML support (pp ≥ 0.95; bs ≥ 75). Red circles indicate moderate support values (pp: 0.95–0.90; bs: 75–70). Open circles indicate no support (pp < 0.90; bs < 70). Relationships that were not recovered by ML analysis are represented by dashes. (a) MAFFT alignment. (b) Gblocks alignment. (c) Curated alignment.
Figure 4. Phylogenetic estimate from MAFFT Alignment. Topology represents Bayesian estimate, with posterior probabilities (pp) and non-parametric bootstrap (bs) support values depicted above and below each branch, respectively. Relationships that were not recovered by ML analysis are represented by dashes. Branches are coloured based on family designations and represented on the exterior of the phylogeny.
Figure 5. Phylogenetic estimate from Gblocks alignment. Topology represents Bayesian estimate, with posterior probabilities (pp) and non-parametric bootstrap (bs) support values depicted above and below each branch, respectively. Relationships that were not recovered by ML analysis are represented by dashes. Branches are coloured based on family designations and represented on the exterior of the phylogeny.
Figure 6. Phylogenetic estimate from Curated alignment. Topology represents Bayesian estimate, with posterior probabilities (pp) and non-parametric bootstrap (bs) support values depicted above and below each branch, respectively. Relationships that were not recovered by ML analysis are represented by dashes. Branches are coloured based on family designations and represented on the exterior of the phylogeny.
estimate. By contrast, Hallas & Gosliner [62] recovered a mostly resolved monophyletic Onchidoridoidea with significant pp and bs support. Their taxonomic sampling, however, was much more focused and included histone 3 as an additional molecular marker. These contradictory estimates illustrate issues that can result from inappropriate taxonomic sampling [93] and noise incorporated into analyses with inclusion of highly divergent taxa.

In a few instances, however, our expansive taxonomic sampling has illuminated the relationships of some problematic groups, specifically Aphelodoris, Cadlinidae, Cadlinella, Hexabranchidae and Polyceridae, but in relation to the Onchidoridoidea our estimates contradicted previously highly supported hypotheses. Even though we were able to include Onchimira cavifera, it is relevant to state that we were unable to procure other morphologically unique species that may have affected our ability to resolve some family relationships in the Doridina (e.g. Colga, Goslineria, Hoplodoris, Kalinga, Murphydoris, Otinodoris). These findings illustrate that each phylogenetic query has its own set of challenges and optimal sampling strategy [94], and that the focus for each investigation should be carefully calculated.

4.2. Doridina relationships

We were not able to confidently investigate patterns of biogeographical, morphological or chemical evolution due to the lack of resolution at the base of our phylogenetic estimates. The present study, however, offers some consistent new insights into Doridina relationships, in part due to our increased taxonomic sampling.

This work reinforces the conclusion from previous studies that traditional phanerobranch and cryptobranch groupings are not monophyletic [36,59,62]. Even though there was only moderate support in our GA, Cadlinidae does appear to be closely related to at least some members of the Porostomata, despite the ambiguous position of some porostomes such as Dendrodoris and Mandelia. In addition, we consistently recovered Gymnodorididae and Okadaiidae nested within the Polyceridae, which together are closely related to the Chromodorididae and Hexabranchidae. Unexpectedly, Cadlinella was recovered sister to the Hexabranchidae in our phylogenetic estimates. Cadlinella was originally included into the Chromodorididae based on morphological similarities [95] and further supported by molecular studies [84,93]. Our broader tax sampling, however, consistently recovered Cadlinella sister to Hexabranchus and that both of these taxa, together with the Polyceridae, are closely related to the Chromodorididae. This also is supported by the sperm ultrastructure of Cadlinella, which has been shown to be divergent from members of the Chromodorididae [96].

In addition, the yellowish northeastern Pacific species of Doriopsilla were thought to represent a species complex of closely related taxa, but this assumption was not tested by including any species from outside the complex, other than the outgroup taxon D. spauldingi [97]. In our analysis, we included D. miniata from Japan and D. janaina, another eastern Pacific species that has divergent colouration. In all three of our analyses, the ‘species complex’ suggested by Hoover et al. [97] includes members of two separate lineages, rather than a single radiation. This suggests that these species with yellowish colouration and white spots evolved similar colouration convergently rather than by means of radiation from a single common ancestor.

Lastly, the evolution of the gill pocket is further confounded by the recovery of Onchimira cavifera nested within the Onchidorididae. Onchimira cavifera was described as having both cryptobranch and phanerobranch characteristics, and hypothesized as a missing link in the current understanding of gill reduction [37,62,98,99]. Onchimira possess all the characteristics of a phanerobranch: buccal pump, rectangular rachidian tooth and hooked shaped first lateral tooth, but also possesses a fully formed gill pocket and retractable gill, which is typical of cryptobranch dorids. Surprisingly, Onchimira is not closely related to the only other two members of the Onchidorididae that possess similar gill structure to the Cryptobranchia, Calycidoris and Diaphorodoris [62], but instead nested within the Onchidorididae. Based on our estimates, it is unclear how or under what conditions the gill pocket might have evolved or was lost throughout the Doridina because of the lack of resolution at the base of the tree.

4.3. Molecular evolution

It is unclear why there are such large inconsistencies between mtDNA and nDNA phylogenies regarding Dendrodoris. To confirm if there was sequencing error, we examined additional specimens of D. fumata and D. nigra to compare to those on GenBank [61,100]. Surprisingly, all sequences collected were identical. Our inclusion of D. atromaculata and D. denisoni, however, suggested that there are possible highly divergent regions among mtDNA sequences. We were unable to compare other species of
Dendrodoris from Hirose et al. [100] because they only analysed COI. A complete sampling of Dendrodoris is needed to fully comprehend the discrepancies between mtDNA and nDNA sequences. Furthermore, there appears to be no molecular distinction between Aegires citrinus and A. serenae. Both species are clearly defined by morphological characteristics [101], but both the mtDNA and the nDNA suggest they are in fact the same species. Much like in the Dendrodorididae, it is unclear what molecular mechanisms might have influenced our observations. Further investigations are needed, but are beyond the scope of our data.

5. Conclusion

We decided to take an approach that used three common methods used in MSA construction for Nudibranchia phylogenetics. As expected, our findings suggested that MSA methodology affected phylogenetic estimates of the Doridina, especially regarding how we decided to align highly variable rDNA regions. We were able to show that the most commonly sequenced molecular markers for the Nudibranchia lacked the robustness to resolve the base of the dorid tree, and manipulation of highly variable regions affected our ability to recover consistent phylogenetic estimates. This effect, however, is most probably dependent upon the size and scope of the phylogenetic query and amount of missing data. These markers are better suited for higher-level classifications as suggested by our PI profiles. Even though the base of the Doridina was unresolved, family-level classifications were mostly supported across our three analyses, and families that were recovered as non-monophyletic were consistent between alignments. Our analyses suggest that the exclusion of variable regions may have weakened our ability to resolve the base of the Doridina, but previous studies that used much larger datasets have benefited from removing these regions (e.g. [102,103]).

Even though the focus of the present study was to understand MSA construction, our estimates of the Doridina also give a frame of reference for allowing more intensive queries into specific family evolutions. For example, the evolution of caryophyllidia in the Discodorididae, molecular evolution in Aegiridae and Dendroridoridae, or the relationships pertaining to the Polyceridae, Okakaiidae, Gymnodorididae, Hexabranchidae and Chromodorididae, which are some of the most morphologically unique and chemically distinct families.

Nudibranch studies unquestionably suffer from a lack of abundant and diverse molecular markers. Studies have argued that increasing molecular markers could resolve problematic relationships [94,104], but an increase in molecular markers does not resolve issues regarding homology and variable region alignments. Automated filtering protocols allow for MSAs to be easily replicated and eliminate the uncertainty of manual curation of alignments; however, these methods are not without error. In addition, there has been little consensus on the soundest method of increasing signal to resolve phylogenies [6]. Genomic tools, which only have recently been used to investigate nudibranch [105] and larger opisthobranch phylogenetics [106,107], have potential of resolving dorid relationships. However, genomic applications also suffer from alignment and homology issues [108,109]. Phylogenetic resolution of the Doridina can greatly benefit from a genomic approach, but it is important to emphasize the critical role MSAs and homology have on phylogenetic studies. Owing to the varying size and scope of molecular and taxonomic sampling, we strongly recommend the exploration of multiple MSA construction methods that can aid in the selection of an approach that best suits the data.

Data accessibility. Supporting files are accessible in the electronic supplementary material. Sequence alignments have been deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S20396). Finally, DNA sequences (listed in table 1) have been uploaded to GenBank accession ranges: 16S(MF958278-MF958318); 18S(MF958319-MF958362); 28S(MF958363-MF958409); COI(MF958411-MF958446).

Authors’ contributions. J.M.H. and T.M.G conceived the study. J.M.H and A.C. carried out molecular sequencing. J.M.H carried out analyses and wrote the manuscript. All the authors edited the manuscript.

Competing interests. We declare that we have no competing interests.

Funding. Portions of this research were supported by Research Program Far East, grant no. 15-I-6-014o to A.C., the US National Science Foundation (DEB, grant no. 12576304) grant to T.M.G., Richard Mooi, Luis Rocha and Gary Williams, and a generous donation from Lucy Jones.

Acknowledgements. This project would not have been possible without the help and support of the following people and institutions: Rebecca Johnson, Vanessa Knutson, Kelly Laughlin and Marta Pola for supplying unpublished sequences and DNA extractions for specimens; California Academy of Sciences (CAS) for help in acquiring specimens; all past and present graduate students from Nudi Central at CAS; everyone at the Center for Comparative Genomics at CAS, especially Anna Sellas, for their help with troubleshooting sequencing; Ryan Hulett and Nick Sinatra for editing and providing valuable suggestions; and, lastly, two anonymous reviewers for constructive comments. This work was
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