Polyphyly across oceans: a molecular phylogeny of the Chromodorididae (Mollusca, Nudibranchia)

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The Chromodorididae is a large and colourful family of nudibranch sea slugs distributed across the world's oceans. Most diversity is centred in the Indo-Pacific, but several genera are present in multiple ocean basins, or across regions separated by biogeographical barriers. The monophyly of these widespread genera had not been tested previously. We used 18S rDNA, 16S rDNA and COI sequence data to generate a molecular phylogeny for this group. We recovered evidence of paraphyly or polyphyly in all of the widespread genera examined (*Hypselodoris*, *Mexichromis*, *Chromodoris* and *Glossodoris*). East Atlantic *Hypselodoris* and west Atlantic + east Pacific *Mexichromis* species were more closely related to each other than they were to their Indo-Pacific congeners. The addition of Southern Ocean species of *Digidentis* demonstrated an interesting alternative to this relationship, becoming the sister group for the east Atlantic *Hypselodoris* on the basis of 16S and 18S data, but not COI data. Sister group relationships were recovered for most monotypic or enigmatic genera. *Ardeadoris* is linked to *Glossodoris*, as is *Diversidoris*; *Pectenodoris* is sister to the Indo-Pacific *Mexichromis* clade, and *Verconia* is the sister to *Noumea haliclona*. Controversy surrounding the placement of the three most basal genera was only partially resolved. Using *Actinocyclus* to root the mitochondrial trees, *Cadlinella* was the unsupported sister to the Chromodorididae (excluding *Cadlina*), and *Tyrinna* occupied a relatively basal position, although this also did not receive significant statistical support. Adding nuclear 18S data gave support for *Cadlina* as the sister group to the rest of the Chromodorididae s.s. Otherwise, like previous molecular studies, mitochondrial genes supported an alternative position for *Cadlina* (with other dorid genera).

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Introduction

The Chromodorididae Bergh, 1891 is one of the largest and most spectacularly coloured groups of opisthobranch molluscs. Representatives are found at all latitudes, with the highest diversity occurring in tropical regions. The family is known to comprise over 300 described species, and it is thought that there are many more species yet to be discovered, and many more awaiting description (Gosliner & Draheim 1996). In recent years, scientific attention has been drawn to chromodorid defence mechanisms, in particular, the toxic chemicals they contain (Cimino & Ghiselin 1999; Gavagnin & Fontana 2000) and the role which they play in the development of new drugs (Avila 1995). This is connected to chromodorids' bright colouration and the evolution of warning colouration and mimicry systems (Rudman 1991; Giménez-Casalduero *et al*. 1999). Some authors (Wägele *et al*. 2003, 2006) considered that the synapomorphy most often invoked for uniting the Chromodorididae — the presence of mantle dermal formations — was not valid given that it occurred widely in the Opisthobranchia. Therefore, the monophyly of the family is not certain, and a robust phylogeny of the group would underpin the investigation and testing of many future evolutionary questions.

The classification of the Chromodorididae has a confused history (Rudman 1984). The first revisions on the group were based primarily on the examination of radular morphology and external colouration (Bertsch 1977, 1978b,a,c; Edmunds 1981), but there remained a poor knowledge of the comparative

anatomy of many species within the family. Most species were originally attributed to *Chromodoris*, and the phylogenetic validity of further division was argued to be artificial until quite recently (Edmunds 1971). Rudman (1984) carried out a comprehensive revision of the family, utilizing previously untapped reproductive characters, together with external colouration and radular morphology. In that revision, Rudman erected several new genera, and proposed that the Chromodorididae could be split into three general subgroups: the *Cadlina* subgroup, which he considered to contain the three genera exhibiting the most plesiomorphic characters (*Cadlina*, *Cadlinella* and *Tyrinna*); the *Chromodoris* subgroup, containing six genera (*Chromodoris*, *Ardeadoris*, *Glossodoris*, *Noumea*, *Pectenodoris* and *Verconia*); and the most derived *Hypselodoris* subgroup, containing seven genera (*Hypselodoris*, *Ceratosoma*, *Digidentis*, *Durvilledoris*, *Mexichromis*, *Risbecia* and *Thorunna*). This work remains the most comprehensive revision of the family to date, although it focused on representatives from the Indo-west Pacific. Gosliner & Johnson (1999) later produced the first cladistic hypothesis for the family. The resulting phylogeny was broadly consistent with Rudman (1984) study, but the authors did not report any support values for the recovered clades. One major difference between the two hypotheses was that Gosliner and Johnson's phylogeny depicted *Ceratosoma* in a clade with *Chromodoris* and *Glossodoris*, as opposed to grouping with the hypselodorid group species as shown in Rudman (1984) hypothesis.

One area of contention in the hypothesized phylogeny of the Chromodorididae is the positioning of *Cadlina*, *Cadlinella* and *Tyrinna*. These three genera are all currently considered to be basal genera associated with the Chromodorididae. However, they have also been previously placed in a separate family Cadlinidae (Bergh 1891) or subfamily, Cadlininae (Odhner 1968). Despite much morphological investigation (Rudman 1984; Muniain *et al*. 1996; Schrödl 2000; Valdés & Campillo 2000; Schrödl & Millen 2001; Valdés & Afonso 2003), as well as recent work examining the sperm ultrastructure of representatives from *Cadlina*, *Cadlinella* and *Tyrinna* (Wilson & Healy 2002, 2006), the relationship among these three genera remains unresolved, and requires attention.

The different genera of the Chromodorididae vary in their distribution patterns, but most diversity is centred in the Indo-Pacific (Table 1). Six of the 17 genera are widespread and occur in multiple ocean basins (*Cadlina*, *Chromodoris*, *Glossodoris*, *Hypselodoris*, *Mexichromis* and *Tyrinna*). Nine are essentially restricted to the Indo-Pacific although may have some species extend into southern waters (*Ardeadoris*, *Cadlinella*, *Ceratosoma*, *Diversidoris*, *Durvilledoris*, *Noumea*, *Pectenodoris*, *Risbecia* and *Thorunna*), and two are restricted or show greatest abundance in areas influenced by the Southern Ocean (*Digidentis*, *Verconia*). Although the Southern Ocean is subject to influences from different geographical regions, our study only involves representatives from the Australian region. In recent years, detailed phylogenies have been produced for a few genera in the Chromodorididae: *Ceratosoma* (Gosliner 1996; Valdés & Gosliner 1999), *Hypselodoris* (Gosliner & Johnson 1999; Johnson & Valdés 2001; Alejandrino & Valdés 2006) and *Chromodoris* (Wilson & Lee 2005). The latter study suggested that *Chromodoris* was paraphyletic, although broad geographical sampling was lacking. In general, the comparison of congeners from different ocean basins and/or regions is yet to be addressed with appropriate outgroups, and the monophyly of broadly distributed genera remains largely untested.

Table 1 Global distribution of Chromodorididae genera. Those considered widespread are in bold font.

	Indo-West Pacific (IW)	East Pacific (EP)	West Atlantic (WA)	East Atlantic (EA)	Southern Ocean (SO)	Arctic Ocean (AO)
Ardeadoris	X					
Cadlina		X	X	X	X	X
Cadlinella	X					
Ceratosoma	X				X	
Chromodoris	X	X	X	X	X	
Digidentis					X	
Diversidoris	X					
Durvilledoris	X					
Glossodoris	X	X	X	X	X	
Hypselodoris	X	X	X	X	X	
Mexichromis	X	X	X	X	X	
Noumea	X				X	
Pectenodoris	X					
Risbecia	X					
Thorunna	X					
Tyrinna		X	X	X		
Verconia	X				X	

The aim of this study was to reconstruct chromodorid phylogeny using mitochondrial ribosomal 16S and mitochondrial protein-coding cytochrome oxidase I (COI) genes, as well as the nuclear ribosomal 18S gene. Mitochondrial 16S and COI genes have proven suitable for resolving lower taxonomic levels in the Opisthobranchia (Medina & Walsh 2000; Thollesson 2000; Wollscheid-Lengeling *et al*. 2001; Valdés 2003; Wägele *et al*. 2003; Grande *et al*. 2004a,b; Wilson & Lee 2005), and it was anticipated that 18S would provide information related to the deeper nodes (Wollscheid & Wägele 1999). We wanted to determine the phylogenetic relationships between chromodorid genera and to test the monophyly of the more widely distributed genera of the Chromodorididae.

Materials and methods

Taxa

In this study, a total of 68 species were investigated, with 60 of these being chromodorid species (Fig. 1). The additional

Fig. 1 A–F. Chromodorid specimens used in this study (all photos N. Wilson, except B, by G. Rouse). —A. *Ardeadoris egretta*, SAMD19257, Sulawesi, Indonesia. —B. *Durvilledoris similaris*, Queensland, Australia. —C. *Diversidoris aurantionodulosa* on prey sponge, SAMD19263, Queensland, Australia. —D. *Mexichromis kempfi* showing trailing behaviour, SIOM11641, Florida, USA. —E. *Glossodoris sedna* on prey sponge, SIOM11636, Florida, USA. —F. *Risbecia tryoni* showing trailing behaviour, Sulawesi, Indonesia.

eight species (from Actinocyclidae, Dorididae and Discodorididae) were used as outgroups. *Actinocyclus* has been hypothesized to be the sister group to chromodorids (Gosliner & Johnson 1994), and further members of the Dorididae and Discodorididae were included as these groups are hypothesized to be the sister of Actinocyclidae + Chromodorididae (Valdés 2002). Table 2 provides details of the samples used, and the additional sequences drawn from GenBank. An effort was made to use the type species of genera wherever possible, given that they are name-bearing. Although it appears likely that *Glossodoris edmundsi* is a junior synonym of *G. ghanensis* (Rudman 2003), we have retained the original use until an explicit synonymy is published.

DNA extraction, amplification and sequencing

Total cellular DNA was extracted from tissue preserved in 95% alcohol following a modified CTAB DNA extraction method (Sokolov 2000) or using Chelex (see Wilson & Lee 2005). All regions were directly amplified by polymerase chain reaction (PCR) with primers listed in Table 3.

The 18S rDNA fragments were amplified using a Biometra® T1 thermal cycler with ~60 ng template and a reaction mix of 50 µL of 1.25 U *Taq* polymerase (Qiagen), 1 mM each dNTP, 0.1 µM each primer, 10 µL of Q-Solution (Qiagen) and 5 µL of 10× PCR buffer (Qiagen). The thermal cycling conditions were: 95 °C for 5 min, followed by 38 cycles of 30 s at 94 °C, 30 s at 53.5 °C, 2.5 min at 72 °C and a final extension for 10 min at 72 °C. PCR products were purified using the *micro*CLEAN system (Microzone) according to the manufacturer's instructions.

The 16S rDNA fragments were amplified using either a Biometra® TGradient or MJResearch PTC-200 thermal cycler. Each PCR was performed with ~60 ng template in a 25-µL total volume reaction mix containing 1 U of *Taq* polymerase (Promega), 1 mM each dNTP, 0.01 µM each primer, 2 µL of 10× PCR buffer (Promega) and 2 µL of $MgCl_2$ (Promega). The thermal cycling conditions were 94 °C for 4 min, followed by 36 cycles of 1 min at 94 °C, 1 min at 41.5 °C, 1.5 min at 72 °C and a final extension for 10 min at 72 °C.

COI fragments were amplified using either a MJResearch PTC-225 or a DNA Engine Tetrad® 2 Peltier thermal cycler. Each PCR was performed with ~60 ng template in a 25-µL total volume reaction mix containing 1 U of *Taq* polymerase (Promega), 1 mM each dNTP, 0.01 µM each primer, 2 µL of 10× PCR buffer (Promega) and 2 µL of $MgCl_2$ (Promega). The thermal cycling conditions were 94 °C for 4 min, followed by 38 cycles of 1 min at 94 °C, 1 min at 49.9 °C, 1.5 min at 72 °C and a final extension for 10 min at 72 °C. Purification and sequencing was carried out by Macrogen (www.macrogen.com) using a magnetic bead protocol.

Sequencing for 16S and 18S was performed using the chain termination method with a fluorescent-labelled terminator cycle sequencing kit (BigDye™ Terminator Cycle Sequencing Ready Reaction Kit, PE Applied Biosystems). Products were run on an automated capillary sequencer (ABI PRISM® 3100, ABI 3730XL or ABI 3700) following the manufacturer's

Table 2 Species used in this study, with collection sites, voucher numbers and GenBank accession numbers. Taxonomy for outgroups follows Valdés (2002). The type species of each chromodorid genus has been indicated in bold. Abbreviations for museum vouchers are AM, Australian Museum, Sydney; SAM, South Australian Museum, Adelaide; SIO, Scripps Institution of Oceanography Benthic Invertebrate Collection, California; WAM, Western Australian Museum, Perth. Other abbreviations from Table 1.

Species	Locality	Area	Voucher	18S rDNA	16S rDNA	COI
Actinocyclidae Pruvot-Fol, 1934						
Actinocyclus verrucosus Ehrenberg, 1831	Mooloolaba, Queensland, Australia	IWP	SAM D19274	—	AY458799	EF535108
Hallaxa indecora (Bergh 1905)	Mooloolaba, Queensland, Australia	IWP	SAM D19275	—	EF534071	—
Dorididae Rafinesque, 1815						
Doris pseudoargus1 Rapp, 1827	North Sea, Helgoland	EA	—	AF249217	—	—
Doris pseudoargus2 Rapp, 1827	Asturias, Spain	EA	—	—	AF430347	—
Doris pseudoargus3 Rapp, 1827	Kingsbarns, Scotland	EA	—	—	—	AY345030
Doris kerguelenensis (Bergh 1884)	Weddell Sea, Antarctica	SO	—	AJ224771	AF249233	AF249780
Discodorididae Bergh, 1891						
Discodoris concinna (Alder & Hancock, 1864)	Great Barrier Reef, QLD, Australia	IWP	—	AF249213	AF249228	AF249801
Jorunna tormentosa (Cuvier, 1804)	Kristineberg, Bohuslän, Sweden	EA	—	—	AJ225191	AJ223267
Peltodoris atromaculata1 Bergh, 1880	Mediterranean, Turkey	M	—	—	—	AF249784
Peltodoris atromaculata2 Bergh, 1880	Ibiza, Spain	M	—	—	AF430360	—
Platydoris argo (Linnaeus, 1767)	Ceuta, Spain	M	—	—	—	AY345037
Chromodorididae Bergh, 1891						
Ardeadoris egretta Rudman, 1984	Sulawesi, Indonesia	IWP	SAM D19257	EF534022	EF534068	EF535140
Cadlina flavomaculata MacFarland, 1905	Palos Verdes, California, USA	EP	AM C203860	—	EF534041	EF535109
Cadlina laevis1 Linnaeus, 1767	St. Andrews, Scotland	EA	—	EF534039	EF534040	—
Cadlina laevis2 Linnaeus, 1767	Kinkell Braes, Scotland	EA	—	—	—	AY345034
Cadlina luarna (Marcus & Marcus, 1967)	Costa Rica	EP	—	—	AF430348	—
Cadlina cf. *luteomarginata* MacFarland, 1966	North Atlantic, USA	WA	—	AJ224772	AF249231	AF249803
Cadlinella ornatissima1 (Risbec, 1928)	Heron Island, Queensland, Australia	IWP	AM C203859	—	AY458802	—
Cadlinella ornatissima2 (Risbec, 1928)	Heron Island, Queensland, Australia	IWP	SIO-BIC M11631	EF534030	—	—
Ceratosoma amoena (Cheeseman, 1886)	Eden, New South Wales, Australia	IWP	SAM D19258	EF534021	—	—
Ceratosoma trilobatum (J. E. Gray, 1827)	Amity, Queensland, Australia	IWP	SAM D19259	EF534025	EF534070	EF535142
Chromodoris alternata (Burn, 1957)	Port Phillip Bay, Victoria, Australia	SO	SAM D19281	EF534031	AY458800	EF535120
Chromodoris ambiguus (Rudman, 1987)	Port Phillip Bay, Victoria, Australia	SO	SAM D19260	EF534038	AY458801	EF535119
Chromodoris aspersa (Gould, 1852)	Mooloolaba, Queensland, Australia	IWP	SAM D19282	EF534026	AY458813	—
Chromodoris collingwoodi Rudman, 1987	North Stradbroke Island, Australia	IWP	SAM D19283	—	AY731181	—
Chromodoris daphne (Angas, 1864)	Moreton Bay, Queensland, Australia	IWP	SAM D19284	—	AY458803	—
Chromodoris epicuria (Basedow & Hedley, 1905)	Triabunna, Tasmania, Australia	SO	SAM D19285	—	AY458804	EF535114
Chromodoris geometrica1 Risbec, 1928	Mooloolaba, Queensland, Australia	IWP	SAM D19286	—	AY458805	—
Chromodoris geometrica2 Risbec, 1928	Heron Island, Queensland, Australia	IWP	SIO-BIC M11632	EF534029	—	—
Chromodoris krohni (Verany, 1846)	NE Atlantic, Spain	EA	—	AJ224774	AF249239	AF249805
Chromodoris kuiteri1 Rudman, 1982	Mooloolaba, Queensland, Australia	IWP	SAM D19287	—	—	AF249804
Chromodoris kuiteri2 Rudman, 1982	Great Barrier Reef, Australia	IWP	—	AF249214	AF249240	—
Chromodoris kuniei Pruvot-Fol, 1930	Heron Island, Queensland, Australia	IWP	SAM D19261	EF534033	AY458807	EF535112
Chromodoris leopardus Rudman, 1987	Mooloolaba, Queensland, Australia	IWP	SAM D19288	—	AY458808	EF535116
Chromodoris lochi (Rudman 1982)	Mooloolaba, Queensland, Australia	IWP	SAM D19289	EF534027	AY458810	—
Chromodoris luteorosea (von Rapp, 1827)	Cadiz, Andalusia, Spain	EA	—	—	AJ225183	AJ223259
Chromodoris magnifica (Quoy & Gaimard, 1832)	Whitsundays, Queensland, Australia	IWP	SAM D19290	EF534028	EF534042	EF535110
Chromodoris purpurea (Risso in Guérin, 1831)	Cadiz, Andalusia, Spain	EA	—	—	AJ225184	AJ223260
Chromodoris quadricolor (Rüppell & Leuckart, 1828)	Red Sea, Egypt	IWP	—	AJ224773	AF249241	AF249802
Chromodoris roboi Gosliner & Beherens, 1998	Heron Island, Queensland, Australia	IWP	SAM D19291	—	AY458814	—
Chromodoris splendida (Angas, 1864)	Mooloolaba, Queensland, Australia	IWP	SAM D19292	—	AY458815	EF535115
Chromodoris striatella Bergh, 1876	Mooloolaba, Queensland, Australia	IWP	SAM D19293	—	AY458809	EF535111
Chromodoris strigata Rudman, 1982	Heron Island, Queensland, Australia	IWP	SAM D19294	—	AY458816	—
Chromodoris tasmaniensis Bergh, 1905	Triabunna, Tasmania, Australia	SO	SAM D19295	EF534032	AY458817	EF535113
Chromodoris tinctoria (Rüppell & Leuckart 1828)	—	IWP	—	AF188676	—	—
Digidentis cf. *arbutus* (Burn, 1961)	Point Puer, Tasmania, Australia	SO	—	EF534015	EF534043	EF535143
Digidentis perplexa (Burn, l957)	Bicheno, Tasmania, Australia	SO	SIO-BIC M11633	—	EF534044	EF535144
Diversidoris aurantinodulosa Rudman, 1987	Mooloolaba, Queensland, Australia	IWP	SAM D19263	EF534011	EF534069	EF535141
Durvilledoris pusilla (Bergh, 1874)	Tab Island, Papua New Guinea	IWP	—	—	AJ225193	AJ223269
Durvilledoris similaris Rudman, 1987	Lizard Island, Queensland, Australia	IWP	—	—	EF534055	EF535128
Glossodoris atromarginata (Cuvier, 1804)	Great Barrier Reef, Australia	IWP	—	AF249211	—	AF249789

Table 2 *Continued.*

Species	Locality	Area	Voucher	18S rDNA	16S rDNA	COI
Glossodoris cincta (Bergh, 1888)	Heron Island, Queensland, Australia	IWP	—	EF534034	EF534064	EF535136
Glossodoris edmundsi Cervera *et al.* 1989	Ilheu Cabra, São Tomé	EA	—	—	EF534061	EF535133
Glossodoris hikuerensis (Pruvot-Fol, 1954)	Heron Island, Queensland, Australia	IWP	SIO-BIC M11634	EF534024	EF534065	EF535137
Glossodoris pallida (Ruppell & Leuckart, 1828)	Heron Island, Queensland, Australia	IWP	SIO-BIC M11635	EF534023	EF534066	EF535138
Glossodoris sedna (Marcus & Marcus, 1967)	Florida Keys, Florida, USA	WA	SIO-BIC M11636	—	EF534062	EF535134
Glossodoris sibogae (Bergh, 1905)	French Polynesia	IWP	SIO-BIC M11637	—	EF534063	EF535135
Hypselodoris bennetti (Angas, 1864)	Wilsons Prom., Victoria, Australia	IWP	—	EF534019	EF534059	EF535131
Hypselodoris bilineata (Pruvot-Fol, 1953)	Madeira, Portugal	EA	—	—	EF534052	EF535125
Hypselodoris obscura1 Stimpson, 1855	Amity, Queensland, Australia	IWP	AM C379393	EF534012	EF534058	—
Hypselodoris obscura2 Stimpson, 1855	Amity, Queensland, Australia	IWP	SIO-BIC M11638	—	—	EF535130
Hypselodoris orsinii (Verany, 1846)	Cadiz, Andalusia, Spain	EA	—	—	AJ225189	AJ223265
Hypselodoris picta (Schultz, 1836)	NE Atlantic, Spain	EA	—	AJ224779	AF249238	AF249787
Hypselodoris villafranca (Risso, 1818)	NE Atlantic, Spain	EA	—	AJ224780	AF249237	AJ223266
Hypselodoris zephyra1 Gosliner & Johnson, 1999	Mooloolaba, Queensland, Australia	IWP	—	EF534013	EF534057	EF535129
Hypselodoris zephyra2 Gosliner & Johnson, 1999	Cook Is., New South Wales, Australia	IWP	SIO-BIC M11639	—	EF534056	—
Mexichromis festiva (Angas, 1864)	Coffs Harbour, NSW, Australia	IWP	SIO-BIC M11640	—	EF534051	EF535124
Mexichromis kempfi (Ev. Marcus, 1970)	Florida Keys, Florida, USA	WA	SIO-BIC M11641	—	EF534047	EF535121
Mexichromis macropus Rudman, 1983	Dampier, Western Australia	IWP	WAM S12634	EF534016	EF534050	EF535123
Mexichromis mariei (Crosse, 1872)	Amity, Queensland, Australia	IWP	SAM D19268	—	EF534049	—
Mexichromis porterae (Cockerell, 1902)	Palos Verdes, California, USA	EP	SIO-BIC M11642	EF534014	EF534067	EF535139
Noumea haliclona1 (Burn, 1957)	Port Phillip Bay, Victoria, Australia	SO	SAM D19269	EF534037	EF534045	—
Noumea haliclona2 (Burn, 1957)	Port Phillip Bay, Victoria, Australia	SO	SIO-BIC M11644	—	—	EF535117
Pectenodoris trilineata (Adams & Reeve, 1850)	Heron Island, Queensland, Australia	IWP	—	EF534017	EF534048	EF535122
Risbecia tryoni1 (Garrett, 1873)	Heron Island, Queensland, Australia	IWP	SIO-BIC M11643	—	EF534060	—
Risbecia tryoni2 (Garrett, 1873)	Sulawesi, Indonesia	IWP	—	EF534018	—	EF535132
Thorunna furtiva Bergh, 1878	Sulawesi, Indonesia	IWP	—	EF534020	EF534053	EF535126
Tyrinna nobilis Bergh, 1898	Región de Los Lagos, Chile	EP	ZSM M20050508	EF534035	EF534054	EF535127
Verconia verconis (Basedow & Hedley, 1905)	Port Phillip Bay, Victoria, Australia	SO	SAM D19270	EF534036	EF534046	EF535118

Table 3 Primers used in this study.

Primer name	Primer sequence	Reference
18SF 5–457	5′-ATCTGGTTGATCCTGCCAGT-3′	This study
18SR 5–457	5′-CTTGGATGT GGTAGCCGTTT-3′	This study
18SF 438–1044	5′-AAACGGCTACCACATCC AAG-3′	This study
18SR 438–1044	5′-CGCCTCTGACTTTCGTTCTT-3′	This study
18SF 989–1667	5′- CTGCGAAAGCATTTGTCAAG-3′	This study
18SR 989–1667	5′-TAGCACGAAGGGGATTCAAC-3′	This study
18A1	5′-CCT AYCTGGTTGATCCTGCCAGT-3′	Wollscheid & Wägele (1999)
1800	5′-TAATGATCCTTCCGCAGGTT-3′	Wollscheid & Wägele (1999)
16Sar-L	5′-CGCCTGTTTATCAAAAACAT-3′	Palumbi *et al.* (1991)
16Sbr-H	5′-CCGGTCTGAACTCAGATCACGT-3′	Palumbi *et al.* (1991)
LCO-1490	5′-GGTCAACAAATCATAAAGATATTGG-3′	Folmer *et al.* (1994)
HCO-2198	5′-TAAACTTCAGGGTGACCAAAAAATCA –3′	Folmer *et al.* (1994)

protocol. All three genes were sequenced in both directions using the PCR primers.

Sequence alignment

Reconciliation of forward and reverse reads was carried out in BioEdit (Hall 1999) or AlignIR Version 1.2 (LI-COR). Sequences were initially aligned using ClustalX (Thompson *et al*. 1997), and then imported into BioEdit or Se-Al (Rambaut 1996) where manual adjustments were made by eye. The alignments and resulting trees (including those discussed but not figured here) are deposited in TreeBASE (www.treebase.org/). Sequences are deposited in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) (see Table 2 for accession details).

Statistical tests and phylogenetic reconstruction

Base composition and alignment parameters were investigated using paup* (Swofford 2002). The degree of substitution

saturation was examined, using the test of Xia *et al*. (2003), as implemented in the package DAMBE (Xia & Xie 2001). hLRT's in MODELTEST Version 3.07 (Posada & Crandall 1998) were used to calculate the proportion of invariable sites for this test. The incongruence length distance (ILD) test (Farris *et al*. 1994) was carried out in PAUP* where it is known as the 'partition homogeneity' test. We chose a *P* level of 0.01 (Cunningham 1997) to assess if the data were significantly incongruent. We implemented this test using maximum parsimony heuristic searches with 1000 replicates, using 100 random sequence additions in each, and retaining no more than 1000 trees greater than 100 steps in each replicate. We also investigated substitutional rate variation among taxa, using the relative rate test in K2WULI (Jermiin 1997), to help identify taxa that may be affected by long-branch attraction. Transition and transversion frequencies were calculated using MEGA Version 3.1 (Kumar *et al*. 2004) and plotted against uncorrected sequence distances to examine saturation.

Phylogenetic reconstruction was carried out with maximum parsimony and Bayesian inference approaches, with the most appropriate model of sequence evolution determined using hLRT's in MRMODELTEST Version 3.07 (Posada & Crandall 1998; Posada 2005) (see Table 4). Maximum parsimony analyses were carried out in PAUP* (Swofford 2002) using a heuristic search (stepwise addition = random, branch swapping option = TBR) with 100 random sequence additions. For 18S and 16S data, gaps were treated as a fifth state character, which improves topological accuracy (Ogden & Rosenberg 2007). Clade support was calculated using bootstrapping (Felsenstein 1985) with replacement (1000 replicates), heuristic search as above, but with 10 random sequence addition replicates. Bayesian inference was conducted with the software MRBAYES v3.1.2 (Huelsenbeck & Ronquist 2001) setting the data as unlinked partitions when data were combined, using a Metropolis Chain Monte Carlo search with two sets of six chains (one cold, five heated). The model was selected as outlined above and parameters were estimated in MRBAYES from default priors. One million generations were produced from each set, sampling every 1000 generations. The first 200 trees (= 200 000 generations) were removed as burn-in, and TRACER Version 1.3 (Rambaut & Drummond 2005) was used to ensure that the trees removed actually represented prestationarity burn-in. To minimize missing data in the three-gene data set, ingroup taxa were required to have 18S rDNA coverage. Only clades with significant support values (defined here as > 70 bootstrap; > 0.90 posterior probabilities) are discussed in the Results section. Relationships recovered in the analyses but not supported by significant bootstrapping or posterior probability values are discussed as though they are collapsed.

Table 4 Comparison of data sets used in this study.

Data set	Length of alignment (bp)	No. of excluded positions	No. of parsimony-informative positions	Substitution saturation	Base composition	Estimated model of sequence evolution	Estimated model of sequence evolution
						MODELTEST	MRMODELTEST
18S rDNA	1667	—	66	$I_{ss} < I_{ss.c}$ (0.694 < 0.712) $P = 0.4634$	$P = 1.0$	TrN + I + Γ $P_{invar} = 0.6970$ $\gamma = 0.8731$	GTR + I + Γ $P_{invar} = 0.7002$ $\gamma = 0.9000$
16S rDNA	387	44	162	$I_{ss} < I_{ss.c}$ (0.391 < 0.684) $P = 0.0000$	$P = 1.0$	TVM + I + Γ $P_{invar} = 0.2986$ $\gamma = 0.5830$	GTR + I + Γ $P_{invar} = 0.2621$ $\gamma = 0.4281$
COI	590	196	50	Pos. 1 $I_{ss} < I_{ss.c}$ (0.198 < 0.666) $P = 0.0000$ Pos. 2 $I_{ss} < I_{ss.c}$ (0.021 < 0.666) $P = 0.0000$ Pos. 3 $I_{ss} > I_{ss.c}$ (0.949 > 0.666) $P = 0.0000$	$P = 1.0$	Position 1 & 2 only GTR + I + Γ $P_{invar} = 0.8044$ $\gamma = 0.8217$	Position 1 & 2 only GTR + I + Γ $P_{invar} = 0.8014$ $\gamma = 0.7135$
16S + COI	977	240	405	NA	NA	NA	GTR + I + Γ $P_{invar} = 0.4457$ $\gamma = 0.4343$
18S + 16S + COI	2642	240	262	NA	NA	NA	GTR + I + Γ $P_{invar} = 0.6890$ $\gamma = 0.4137$

Results

Data set comparisons and parameters

Total analysed alignment length for 18S was 1667 positions, 16S was 343 and COI was 590 positions. The 16S alignment was improved by utilizing secondary structure models (unpubl. data), but ultimately 44 positions could not be aligned unambiguously and were excluded. The base composition was homogeneous in all three data sets (Table 4). Significant saturation was identified in the third codon position for COI using the conservative assumption of an asymmetrical tree (see also Fig. 2C), and we excluded this data from all analyses. When only first and second codon positions were plotted (Fig. 2D), there were more transitions than transversions for all taxa, and both increased in a linear fashion. Saturation was not evident for the 18S and 16S data sets. These scatter plots (Fig. 2A,B) showed for the most similar sequences (indicated by low uncorrected genetic distance values) there are more transitions than transversions. However, for the most divergent sequences (inroup/outgroup comparisons) this pattern began to break down in the more conservative 18S data set, and was mostly reversed in the 16S data set. When the COI data were translated into proteins, much phylogenetic information was lost. Only 19 amino acid changes were parsimony-informative, and this was not enough to resolve relationships between taxa. ILD tests showed no significant incongruence between genes, and the data were combined for subsequent analyses.

The relative rate test (Sarich & Wilson 1973) revealed that evolutionary rates are significantly different in the three genes ($P = 0.0000$). The 16S rDNA and COI genes for all taxa were tested against *Actinocyclus verrucosus* (Actinocyclidae) with the outgroup *Discodoris concinna* (Table 5). *Actinocyclus verrucosus* was chosen as it has been hypothesized to be sister to the chromodorids (Gosliner & Johnson 1994). 18S rDNA sequence was not available for *A. verrucosus*. All three genes were also tested against *D. kerguelenensis* (Dorididae) (with *D. concinna* as outgroup) as the Dorididae has been hypothesized to be the sister of Actinocyclidae + Chromodorididae (Valdés 2002).

For ingroup taxa, the highest rate recorded for 18S data was between *C. geometrica* and *D. kerguelenensis* (Z score = 2.508969). When this irregular result was removed, *Chromodoris* as a group had Z scores of between 0 and 0.824461, which were similar to those recorded for other groups of taxa (Wägele *et al.* 2003) and, instead, *Cadlinella ornatissima* had the highest recorded evolutionary rate (Z score = 1.336661).

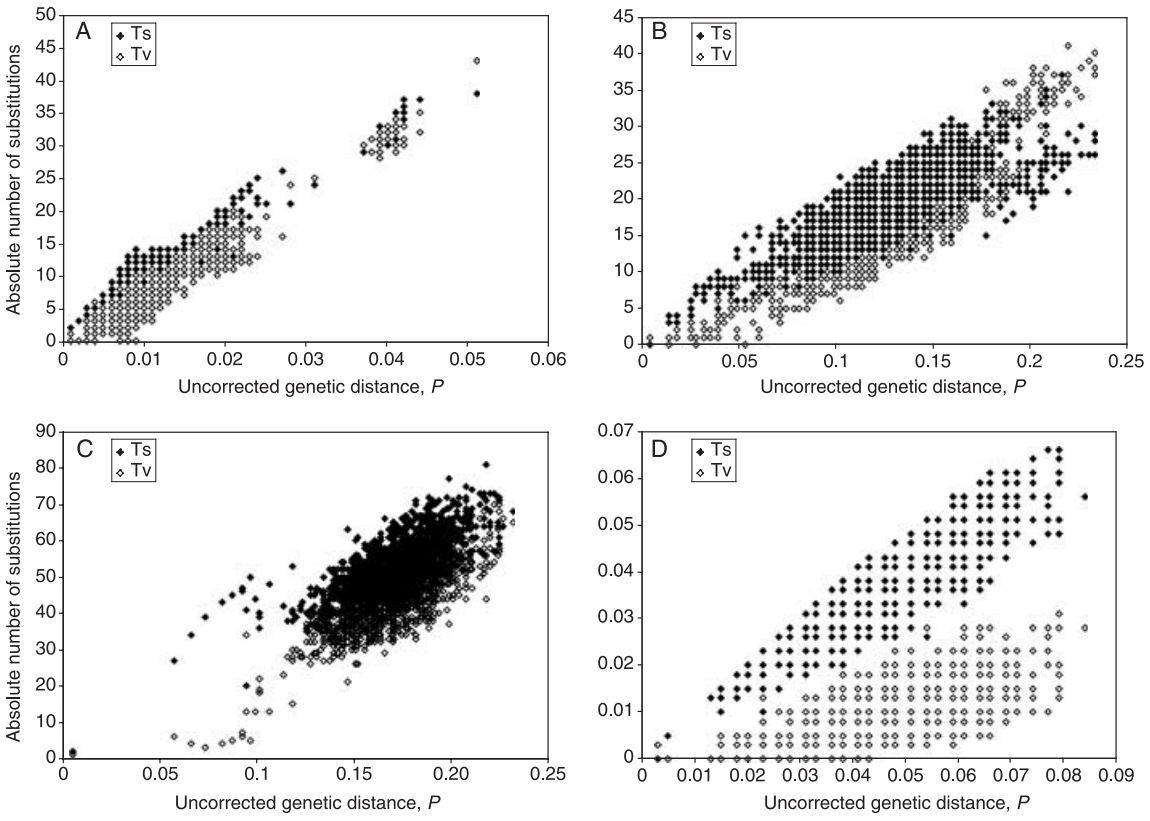


Fig. 2 Plot of observed number of transitions (Ts) and transversions (Tv) versus uncorrected genetic distance, P (A) 16S rDNA, (B) 18S rDNA, (C) COI and (D) COI with third positions excluded.

Table 5 Results of relative rate test (*Z* score values) comparing species and major taxonomic groups in the Chromodorididae. Species with deviating values are shown in bold.

	***Z* score values (range)**				
	18S rDNA	**16S rDNA**		**COI**	
Reference species	*Doris kerguelenensis*	*Actinocyclus verrucosus*	*Doris kerguelenensis*	*Actinocyclus verrucosus*	*Doris kerguelenensis*
Taxon					
Chromodorididae					
Ardeadoris	0.805818	1.107134	1.829912	2.114806	0.243346
Cadlina	0.001819–0.005899	0.104099–1.428799	0.16697–1.646835	0.356824–0.578408	1.352388–1.538095
Cadlinella	1.336661	1.273784	0.554309	—	—
Ceratosoma	0.197114–0.1018758	1.023072	2.925609	2.538318	0.63963
Chromodoris	0–2.508969	0.013763–4.652045	1.554759–6.140406	0.817379–2.668503	0.015349–1.322999
C. alternata		**4.652045**	**6.140406**		
Chromodoris minus *C. alternata*		0.013763–1.315393	1.554759–3.275934		
C. geometrica	**2.508969**				
Chromodoris minus *C. geometrica*	0–0.824461				
Digidentis	0.204076	0.993073–1.954857	2.704062–3.199977	0.623892–0.79245	1.072526–1.234329
Diversidoris	0.378491	0.877366	2.66487	2.32236	0.646915
Durvilledoris	—	1.227389–1.685439	3.071975–3.613425	1.584578–1.887809	0.027713–0.186389
Glossodoris	0.18005–0.731874	0.24759–1.009755	1.245533–2.64772	1.049397–2.873693	0.281978–1.241527
Hypselodoris	0.180013–0.377001	0.264835–2.699602	2.106427–4.364473	1.053832–1.896693	0.201018–0.816639
Mexichromis	0.188146–0.722544	0.614999–1.824998	2.322438–3.899788	0.505413–2.684109	0.238845–1.550238
Noumea	0.155736	2.559325	4.250495	2.216985	0.646915
Pectenodoris	0.54315	0.485468	2.319721	1.266823	0.55512
Risbecia	0.194505	2.200698	4.006379	1.206812	0.563161
Thorunna	0.601556	0.996361	2.802137	1.022718	0.782335
Tyrinna	0.536907	0.498999	2.208819	1.322856	0.402536
Verconia	1.010623	1.862944	3.717203	1.764017	0.017139

For ingroup taxa, the highest rates recorded for 16S rDNA was between *C. alternata* and *D. kerguelenensis* (tested against *A. verrucosus*: *Z* score = 4.652045, tested against *D. kerguelenensis*: *Z* score = 6.140406). If *Chromodoris alternata* was subsequently removed, *Chromodoris* had *Z* scores that were similar to those recorded for other groups of taxa (Wägele *et al*. 2003) and, instead, *Hypselodoris* had the highest recorded evolutionary rate (tested against *A. verrucosus*: *Z* score = 2.699602, tested against *D. kerguelenensis*: *Z* score = 4.364473). The high evolutionary rates recorded for *C. alternata* and *C. geometrica* indicated that these species positions in phylogenetic analyses may be influenced by long-branch attraction.

Single gene analyses

18S rDNA. This data set (analyses not shown) contained very few parsimony-informative sites (66 of 1667, Table 4) and, as such, showed very little structure or supportable clades in maximum parsimony analyses. Notable ingroup relationships that were supported by bootstrapping were *Verconia* + *Noumea* (100), and *Hypselodoris picta* + *villafranca* (100).

Bayesian inference methods recovered a monophyletic Chromodorididae *sensu* Rudman (1984) (1.00). Relationships were supported for *Verconia* + *Noumea* (1.00), *Hypselodoris picta* + *villafranca* (1.00), *Cadlina laevis* + *luteomarginata* (0.98) and *Chromodoris ambiguus* + *alternata* (1.00).

16S rDNA. The maximum parsimony strict consensus tree showed *Cadlina* forming a monophyletic group (100) together with members of the Discodorididae. Bootstrapping did not support basal nodes, and other values are shown on Fig. 3. Bootstrapping did support the monophyly of Indo-Pacific *Mexichromis* (100), *Durvilledoris* (100), Indo-Pacific *Hypselodoris* + *Risbecia* (92), *Digidentis* (98) and east Atlantic *Hypselodoris* (97). The planar spawning *Chromodoris* were supported as monophyletic (98), as was *Noumea* + *Verconia* (100), *Chromodoris collingwoodi* + *kuniei* (87), *Chromodoris tasmaniensis* + *epicuria* (98), *Chromodoris leopardus* + *geometrica* + *roboi* (92) and *Glossodoris edmundsi* + *sedna* (73).

Posterior probabilities on the Bayesian inference tree (Fig. 3) supported *Doris pseudoargus* as sister to all the rest, and a monophyletic *Cadlina* (1.00) formed a clade with several outgroup species (1.00). If unsupported relations are collapsed, this clade, together with *Doris kerguelenensis* and *Cadlinella*, formed a highly supported polytomy (0.97) with the rest of the Chromodorididae (defined as Chromodorididae *sensu stricto*) (0.91). Although the consensus tree in Fig. 3 showed

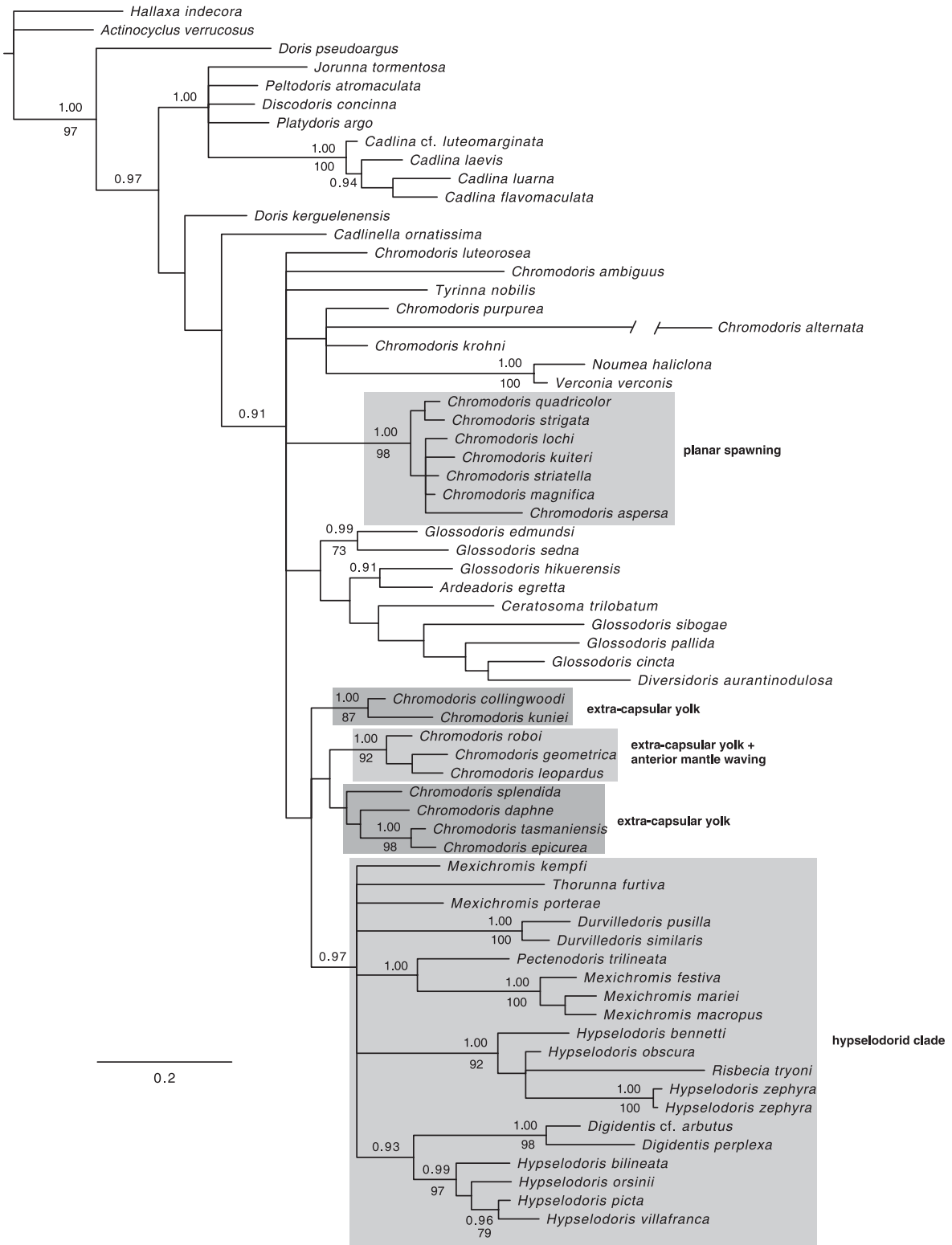


Fig. 3 Bayesian majority-rule consensus phylogram of 16S rDNA data set. Numbers above are posterior probabilities from Bayesian analysis; numbers below represent bootstrap values (1000 replicates) for nodes obtained by parsimony analysis. Only values equal or greater to 0.90 and 70 are shown. Branch length for *Chromodoris alternata* has been shortened for ease of publication.

Cadlinella as the sister to Chromodorididae s.s., this relationship was not supported by posterior probabilities. The relationships within Chromodorididae s.s. were mostly unresolved, but supported groups included *Glossodoris edmundsi* + *sedna* (0.99), *Glossodoris hikuerensis* + *Ardeadoris egretta* (0.91), *Noumea* + *Verconia* (1.00), planar spawning *Chromodoris* (1.00), *Chromodoris collingwoodi* + *kuniei* (1.00), *Chromodoris leopardus* + *geometrica* + *roboi* (1.00), *Chromodoris tasmaniensis* + *epicuria* (1.00), and a large clade containing the hypselodorid subgroup of Rudman (1984) (0.97). Within this latter group, *Pectenodoris* was the sister group (1.00) to the Indo-Pacific *Mexichromis* (1.00), *Digidentis* (1.00) was the sister group (0.93) to the east Atlantic *Hypselodoris* (0.99), and other monophyletic groups were *Durvilledoris* (1.00) and the Indo-Pacific *Hypselodoris* + *Risbecia* (1.00).

COI. Without third positions, the maximum parsimony strict consensus tree showed almost no resolution. Rooting with *Actinocyclus* placed the remaining taxa in a major polytomy (analyses not shown). The Bayesian analysis of COI also showed very little resolution that was supported by posterior probabilities. Rooting with *Actinocyclus* formed a basal polytomy with that taxon, *Discodoris* and *Peltodoris*. The rest of the outgroup formed a polytomy together with *Cadlina* (1.00) and the clade containing the rest of the Chromodorididae. None of these basal nodes were supported. Groups within Chromodorididae s.s. that did show considerable posterior probability support were *Noumea* + *Verconia* (1.00), *Chromodoris ambiguus* + *alternata* (1.00), *Pectenodoris* + Indo-Pacific *Mexichromis* (0.97), *Durvilledoris* (0.92) and *Digidentis* (1.00).

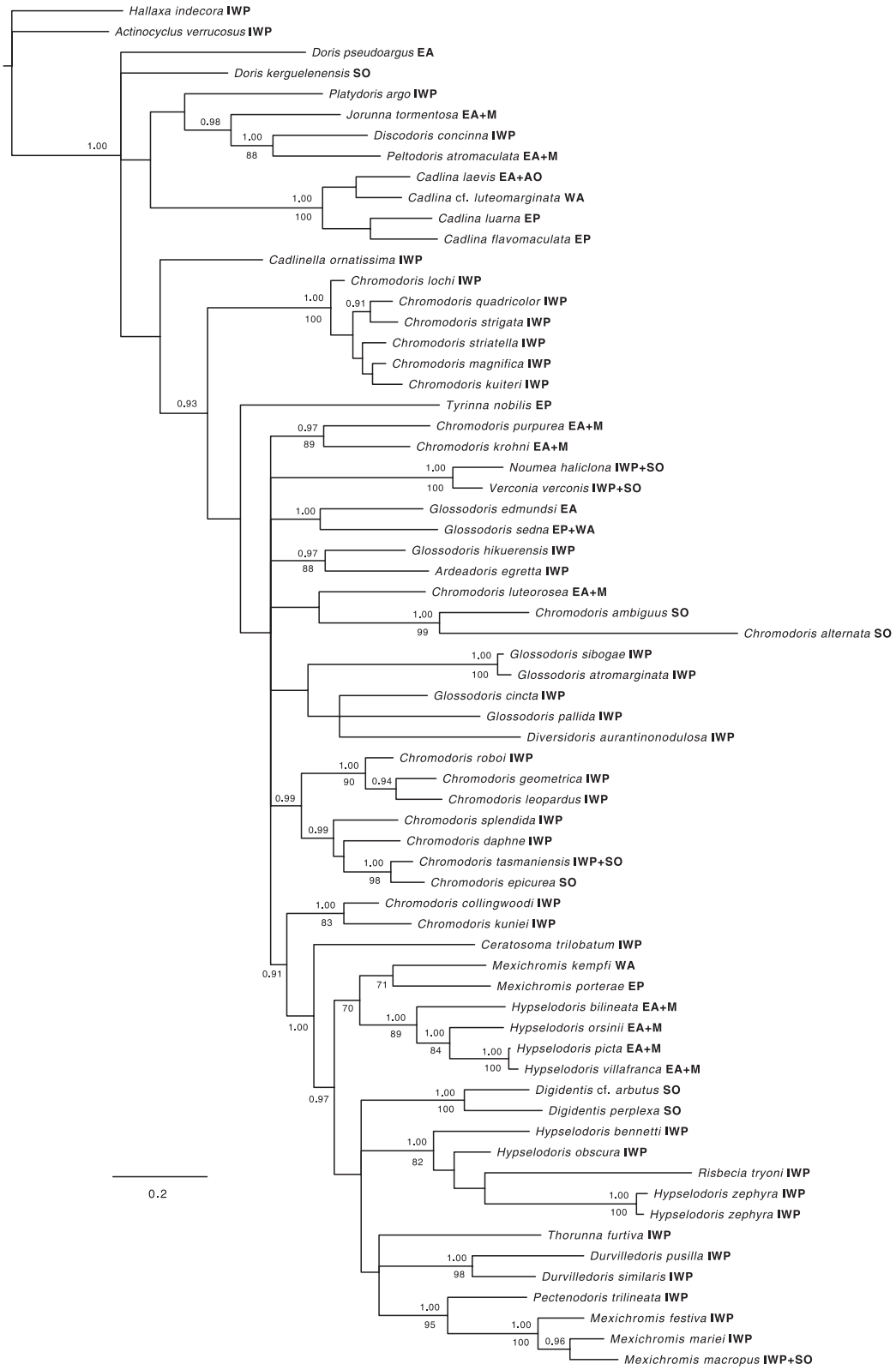
Combined analyses

16S rDNA and COI. The combined data set of 16S + COI showed the most resolution overall, and all of the well-supported components of the single gene analyses were congruent with its topology. Rooting the maximum parsimony analysis with *Actinocyclus* placed *Cadlina* + *Doris* + *Platydoris* outgroups as sister to the remaining Chromodorididae, and *Cadlinella* as basal to these two groups. *Jorunna* + *Discodoris* were sister to all the rest, although none of the deeper nodes were supported. Bootstrapping values were imposed on the Bayesian tree (Fig. 4) and supported a monophyletic *Cadlina* (100), *Chromodoris ambiguus* + *alternata* (99), *Noumea* + *Verconia* (100), *Glossodoris atromarginata* + *sibogae* (100), *Chromodoris purpurea* + *krohni* (89), *Chromodoris leopardus* + *geometrica* + *roboi* (90), *Chromodoris tasmaniensis* + *epicurea* (98), *Glossodoris hikuerensis* + *Ardeadoris egretta* (88), *Chromodoris collingwoodi* + *kuniei* (83), planar spawning *Chromodoris* (100), *Digidentis* (100), *Pectenodoris* + Indo-Pacific *Mexichromis* (95), Indo-Pacific *Mexichromis* (100), *Mexichromis kempfi* + *porterae* (71), east Atlantic *Hypselodoris* (89), *Durvilledoris* (98) and Indo-Pacific *Hypselodoris* + *Risbecia* (82).

The Bayesian majority-rule consensus tree (Fig. 4) showed a polytomy containing *Doris* species, a clade of *Cadlina* + *Platydoris* + *Jorunna* + *Discodoris* + *Peltodoris*, and a clade of the remaining members of the Chromodorididae. *Cadlina* was supported as monophyletic (1.00), as was Chromodorididae *sensu stricto* (excluding *Cadlina*) (0.93). *Cadlinella ornatissima* was the sister taxon to the rest of the Chromodorididae s.s., although this position was unsupported. Resolution between major lineages in the Chromodorididae s.s. was poor, and it was only reasonable to describe those lineages or groupings that did gain some statistical support. All of the species pair associations seen in the previous analyses were present again. An additional relationship recovered here was the placement of *Diversidoris* with *Glossodoris cincta* + *pallida*, although this only gained support (0.95) when the data were not partitioned for analyses. *Glossodoris edmundsi* + *sedna* formed a sister pair (1.00), as did *Glossodoris sibogae* + *atromarginata* (1.00). *Chromodoris purpurea* + *krohni* formed a sister pair (0.97), as did *Chromodoris ambiguus* + *alternata* (1.00), *Noumea haliclona* + *Verconia verconis* (1.00) and the planar spawning *Chromodoris* (1.00). The clade containing *Chromodoris geometrica* + *leopardus* + *roboi* (1.00) was shown as sister (0.99) to *Chromodoris tasmanienesis* + *epicurea* + *splendida* + *daphne* (0.99). *Chromodoris collingwoodi* + *kuniei* formed a sister pair (1.00) and so did *Chromodoris epicurea* + *tasmaniensis* (1.00). In the most derived part of the tree, *Ceratosoma* was sister (1.00) to the two clades containing *Mexichromis*, *Hypselodoris*, *Thorunna*, *Digidentis*, *Pectenodoris*, *Durvilledoris* and *Risbecia*. One clade contained the east Atlantic *Hypselodoris* (1.00) sister to east Pacific and west Atlantic *Mexichromis*. The other clade contained only Indo-Pacific and Southern Ocean representatives: *Durvilledoris* (1.00), *Thorunna*, *Digidentis* (1.00), *Pectenodoris* + Indo-Pacific *Mexichromis* (1.00), Indo-Pacific *Hypselodoris* + *Risbecia* (1.00).

18s, 16s and COI. Maximum parsimony analysis and Bayesian inference (Fig. 5) resulted in almost identical topologies and were discussed together. These analyses showed *Cadlina* as monophyletic (1.00/100) and supported as the sister group (1.00/–) to a monophyletic Chromodorididae (1.00/–), which included *Tyrinna* and *Cadlinella*. The Chromodorididae (excluding *Cadlina*) formed a large polytomy, with little resolution aside from the planar spawning *Chromodoris* (1.00/100),

Fig. 4 Bayesian majority-rule consensus phylogram of the combined 16S rDNA and COI data set. Numbers above are posterior probabilities from Bayesian analysis; numbers below represent bootstrap values (1000 replicates) for nodes obtained by parsimony analysis. Only values equal or greater to 0.90 and 70 are shown. Species distribution data has been added using the abbreviations from Table 1, with the addition of M, Mediterranean. For species marked with multiple biogeographical regions, check Table 2 for locality data.


Hallaxa indecora IWP
Actinocyclus verrucosus IWP
Doris pseudoargus EA
Doris kerguelenensis SO
Platydoris argo IWP
Jorunna tormentosa EA+M
Discodoris concinna IWP
Peltodoris atromaculata EA+M
Cadlina laevis EA+AO
Cadlina cf. luteomarginata WA
Cadlina luarna EP
Cadlina flavomaculata EP
Cadlinella ornatissima IWP
Chromodoris lochi IWP
Chromodoris quadricolor IWP
Chromodoris strigata IWP
Chromodoris striatella IWP
Chromodoris magnifica IWP
Chromodoris kuiteri IWP
Tyrinna nobilis EP
Chromodoris purpurea EA+M
Chromodoris krohni EA+M
Noumea haliclona IWP+SO
Verconia verconis IWP+SO
Glossodoris edmundsi EA
Glossodoris sedna EP+WA
Glossodoris hikuerensis IWP
Ardeadoris egretta IWP
Chromodoris luteorosea EA+M
Chromodoris ambiguus SO
Chromodoris alternata SO
Glossodoris sibogae IWP
Glossodoris atromarginata IWP
Glossodoris cincta IWP
Glossodoris pallida IWP
Diversidoris aurantinonodulosa IWP
Chromodoris roboi IWP
Chromodoris geometrica IWP
Chromodoris leopardus IWP
Chromodoris splendida IWP
Chromodoris daphne IWP
Chromodoris tasmaniensis IWP+SO
Chromodoris epicurea SO
Chromodoris collingwoodi IWP
Chromodoris kuniei IWP
Ceratosoma trilobatum IWP
Mexichromis kempfi WA
Mexichromis porterae EP
Hypselodoris bilineata EA+M
Hypselodoris orsinii EA+M
Hypselodoris picta EA+M
Hypselodoris villafranca EA+M
Digidentis cf. arbutus SO
Digidentis perplexa SO
Hypselodoris bennetti IWP
Hypselodoris obscura IWP
Risbecia tryoni IWP
Hypselodoris zephyra IWP
Hypselodoris zephyra IWP
Thorunna furtiva IWP
Durvilledoris pusilla IWP
Durvilledoris similaris IWP
Pectenodoris trilineata IWP
Mexichromis festiva IWP
Mexichromis mariei IWP
Mexichromis macropus IWP+SO
0.2

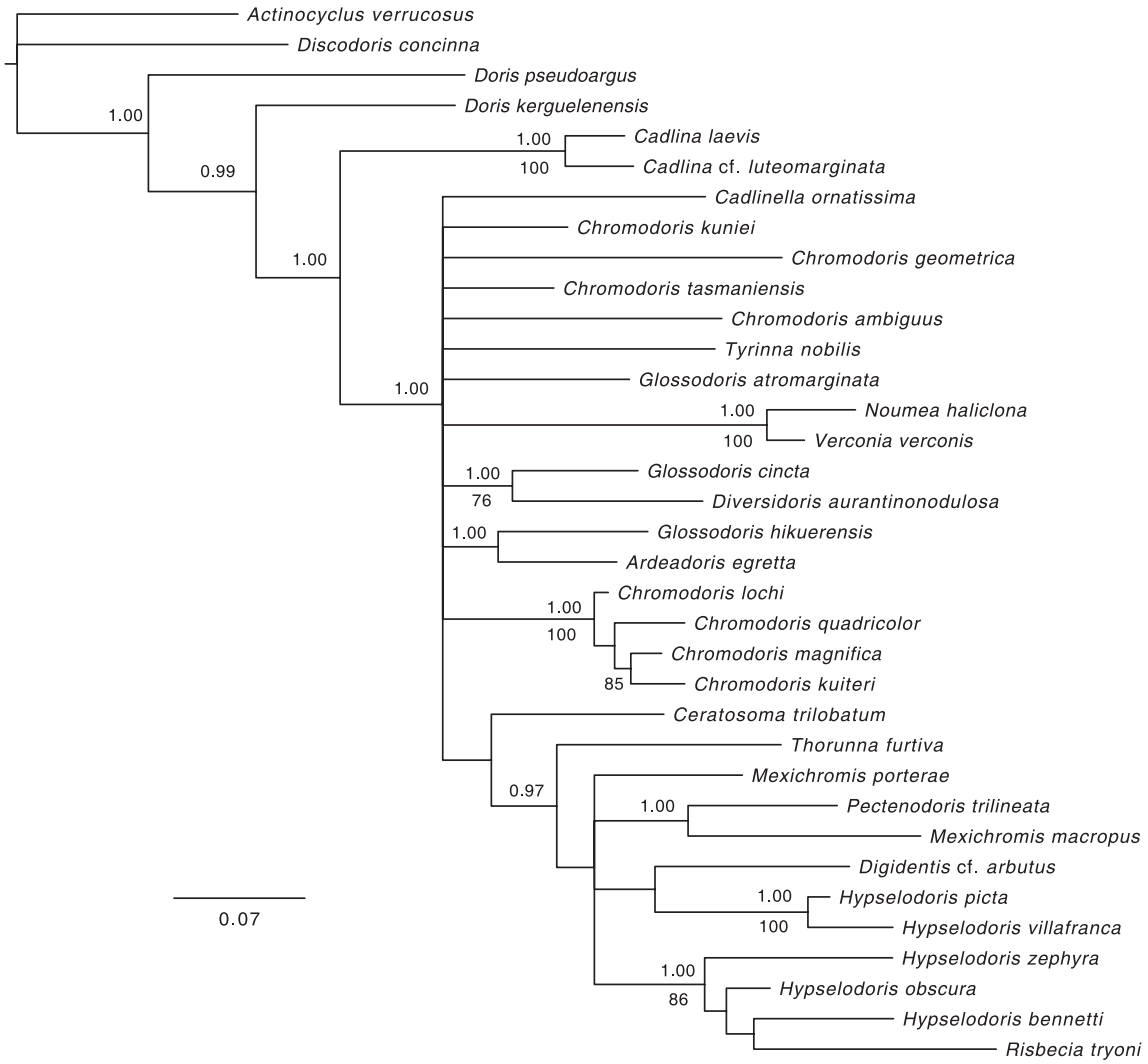


Fig. 5 Bayesian majority-rule consensus phylogram of the combined 18S rDNA, 16S rDNA and COI data set. Numbers above are posterior probabilities from Bayesian analysis; numbers below represent bootstrap values (1000 replicates) for nodes obtained by parsimony analysis. Only values equal or greater to 0.90 and 70 are shown.

and the hypselodorid clade (0.97) containing *Thorunna* + *Mexichromis* + *Pectenodoris* + *Digidentis* + *Hypselodoris* + *Risbecia*. The sister group relationships that were supported in addition to these two clades were *Glossodoris cincta* + *Diversidoris aurantionodulosa* (1.00/76), *Glossodoris hikuerensis* + *Ardeadoris egretta* (1.00/–) and *Noumea* + *Verconia* (1.00/100). Relationships supported within the hypselodorid group were *Pectenodoris* + Indo-Pacific *Mexichromis* (1.00), *Hypselodoris picta* + *villafranca* (1.00/100) and Indo-Pacific *Hypselodoris* + *Risbecia* (1.00/86).

Relative contributions of different markers. There was no significant conflict of the topology recovered by single gene vs. combined data sets. Not surprisingly, 18S rDNA contained the least amount of information, and could only differentiate outgroup and ingroup taxa. Even though the aligned fragment was quite short, 16S rDNA performed surprisingly well against the combined data sets. Once the third position was removed from the COI data because of substitution saturation, much of the information was stripped from the data set. Consequently, the overall topology of the consensus COI trees was less resolved than other analyses, but not incongruent, and clade support was usually lacking. The combined 16S + COI data set provided the most comprehensive sampling, but the three-gene data set showed increased resolution in the basal nodes.

The position of *Cadlina* and *Digidentis* were obviously affected by the inclusion of different data. *Cadlina* was not

recovered as the sister group to the rest of the Chromodorididae in any mitochondrial data analyses. This relationship was recovered after the addition of 18S nuclear data. Similarly, *Digidentis* was recovered as the sister group to east Atlantic *Hypselodoris* in the single gene analyses using 16S. Adding COI collapsed this relationship, but it re-appeared in Bayesian analyses after the addition of 18S.

Discussion

Phylogeny of the widespread chromodorid genera

The primary aim of the present study was to test the interoceanic monophyly of some of the more widely distributed genera of the Chromodorididae. *Hypselodoris*, *Mexichromis*, *Chromodoris*, *Glossodoris*, *Cadlina* and *Tyrinna* are all known to occur in multiple ocean basins and/or regions (Table 1). The latter two genera were not sampled enough, or their position was not resolved enough, to discuss fully here. The discussion for the remaining widespread genera will be based on the results of the data set of 16S + COI data (Fig. 4), which was chosen as the focal data set as it is the combination that best maximizes data and taxa. The other analyses are referred to where relevant.

Hypselodoris

This genus appears polyphyletic and/or paraphyletic in all analyses presented here. The Indo-Pacific *Hypselodoris* species never form a monophyletic clade with the remaining species of east Atlantic *Hypselodoris*. Instead, each geographical group of *Hypselodoris* species remains as separate subclades within parts of a larger hypselodorid polytomy (Fig. 5), or as sister groups to other hypselodorid genera (Figs 3 and 4). The biogeographically intriguing relationship of *Digidentis* as the sister group to east Atlantic *Hypselodoris* species in Fig. 3 cannot be easily explained by vicariant events and warrants further investigation.

Two existing morphological phylogenies for *Hypselodoris* (Gosliner & Johnson 1999; Alejandrino & Valdés 2006) showed an Indo-Pacific clade as the sister to an Atlantic + east Pacific clade, but did not include additional chromodorid outgroups that are necessary for rigorously evaluating monophyly. Despite the congruence of clade topology with ocean in our study, the morphology-based phylogeny of Alejandrino & Valdés (2006) revealed a complex biogeographical pattern of east Atlantic, west Atlantic and east Pacific *Hypselodoris* species. It will be very interesting to test these hypotheses further with molecular data, since they highlight potential dispersal events in contrast to prevalent vicariant patterns. Gosliner & Johnson (1999) outlined two uniquely derived characters defining an Atlantic + east Pacific *Hypselodoris* clade, relating to the muscularization and width of the vaginal duct. A wide and muscular vaginal duct is also present in *Digidentis* (Rudman 1984), which adds further support to this potential relationship identified by molecular data. The Indo-Pacific *Hypselodoris* clade exhibited the synapomorphy of a minute receptaculum seminis (Gosliner & Johnson 1999). The type species of the genus (and thus the name bearer) is *Hypselodoris obscura*, in the Indo-Pacific clade. Therefore, it is the Atlantic and east Pacific *Hypselodoris* species that require further clarification of morphological synapomorphies, and subsequent reclassification.

Additionally, the Indo-Pacific *Hypselodoris* species are also identified here as paraphyletic, with *Risbecia tryoni* nested within. Other studies using morphology have identified *Risbecia* as monophyletic, forming the sister group to *Hypselodoris* (Rudman 1984; Gosliner & Johnson 1999), and this is the first indication that *Risbecia* may nest inside the Indo-Pacific species of *Hypselodoris*.

Mexichromis

The phylogeny of *Mexichromis* has not been previously studied in detail, either from a morphological or molecular perspective. There are currently around 11 valid species in the genus, five species of which were used in this study. The results of this study indicate that *Mexichromis* is also polyphyletic. The three Indo-Pacific species form a highly supported clade (Figs 3 and 4), and show a sister group relationship with *Pectenodoris* (Figs 3–5). The east Pacific and west Atlantic *Mexichromis* species appear more closely related to other east Pacific and Atlantic *Hypselodoris* species. *Mexichromis porterae* (east Pacific) and *M. kempfi* (west Atlantic) showed a variety of positions in analyses; a polytomy in the hypselodorid clade (Figs 3 and 5) or as sister group to the east Atlantic *Hypselodoris* (Fig. 4). Gosliner & Johnson (1999) recovered *Mexichromis* as sister to *Hypselodoris* + *Risbecia* in their morphological analysis. This relationship is supported for east Pacific and Atlantic taxa in this study, but not for the Indo-Pacific taxa. Here, the name-bearing type is *Mexichromis antonii* from the east Pacific, and it is the Indo-Pacific *Mexichromis* clade that likely requires a new name.

Mexichromis kempfi was originally described as a species of *Chromodoris* (Marcus 1970), but Rudman (1984) suggested it might be more accurately placed in *Mexichromis* due to the multicuspid jaw rodlets and small and narrow radular ribbon. This change was later made (Ortea *et al.* 1996), and the molecular results here confirm *M. kempfi* as a member of the hypselodorid clade, although further work on the classification of '*Mexichromis*' as a whole is clearly required.

Chromodoris

Species of *Chromodoris* included in this study never formed a monophyletic group and instead formed a series of subclades whose relative positions were not well resolved. These subclades typically corresponded with geographical locality, although several Indo-Pacific subclades were identified that did not cluster together. Wilson & Lee (2005) identified two major clades of *Chromodoris* based on their 16S data — the

'planar spawner' clade and the 'erect spawner' clade — in reference to the shape of the egg masses laid by the respective members of each clade. The planar spawner clade was also found to be monophyletic in all analyses in this study (see Fig. 3), and likely represents the genus *Chromodoris* in the strictest sense. Because this planar group contains the name-bearing type of *Chromodoris*, reclassification of the erect spawning '*Chromodoris*' cannot be carried out until its phylogenetic relationships are more resolved.

The erect spawning *Chromodoris* species did not form a clade in any of the analyses in the present study, unlike in the study of Wilson & Lee (2005). However, the subclades found in that study are recovered again here. The Indo-Pacific subclade (*roboi* + *geometrica* + *leopardus*) and the Indo-Pacific/Southern Ocean clade (*tasmaniensis* + *epicurea* + *splendida* + *daphne*) were recovered as sister groups and (0.99, Fig. 4), although support was absent where 18S data was used, likely due to the anomalous substitution rate in *Chromodoris geometrica*. Both of these groups share the reproductive trait of including extra-capsular yolk in their egg masses (Fig. 3), but only the Indo-Pacific *roboi* + *geometrica* + *leopardus* clade maintain the additional behavioural characteristic of anterior mantle lifting (Fig. 3). There is another pair of Indo-Pacific species (*C. collingwoodi* + *kuniei*) that also utilizes extra-capsular yolk, but their relationship to the former clade is not resolved. The relationship of a Southern Ocean sister pair (*C. tasmaniensis* + *epicurea*) with Indo-Pacific species (*daphne* + *splendida*) concurs with Briggs (2003) supposition that Southern Oceans areas in Australia and New Zealand house relict species of tropical ancestry. It is also interesting to note that the three east Atlantic *Chromodoris* species do not form a single clade, although *C. purpurea* and *C. krohni* form a well-supported sister pair. Previous 16S data had suggested *C. ambiguus* and *C. alternata* showed an affinity to *Cadlina* (maximum parsimony and maximum likelihood) (Wilson & Lee 2005). This result was not maintained in any analyses here, and the expanded taxon sampling supports these two *Chromodoris* species with others in the Chromodorididae s.s. clade.

Rudman & Bergquist (2007) noted that the diversity of sponges fed on by *Chromodoris* as a whole was broader than that demonstrated for other genera. However, some species groups within *Chromodoris* did show specific habits, for example, the planar spawning *Chromodoris* fed on sponges containing the chemical Latrunculin A. However, no fine-scale correlation can be made for the subclades within the erect spawning group found in this study, and the feeding data Rudman & Bergquist (2007) presented.

Glossodoris

As in the case of *Mexichromis*, no *a priori* hypotheses of phylogeny exist for *Glossodoris*, although Rudman (1987) identified four subgroups based predominantly on radular morphology and body shape. The (i) *atromarginata* subgroup is characterized by small teeth, no central rachidian tooth, a high profile body, thick body wall and reduced mantle overlap; (ii) the *pallida* subgroup by larger teeth, a central rachidian tooth, inner lateral tooth cusp broad and triangular with many fine denticles, and an intermediate body profile and mantle overlap; (iii) the *sedna* subgroup by larger teeth, a central rachidian tooth, inner lateral tooth cusp pointed with fine denticles, sometimes a broader radular ribbon and a low body profile with mantle overlap; and (iv) the *cincta/hikuerensis* subgroup which essentially consists of species that do not fit easily into the other subgroups. *Glossodoris* is the third largest genus of the Chromodorididae, with more than 30 species known (www.seaslugforum.net).

Five Indo-Pacific *Glossodoris* species were used in the present analysis, together with an east Atlantic species, and one found in both the east Pacific and the west Atlantic. The results of the present study indicate that *Glossodoris* is not monophyletic, and may contain *Diversidoris* and *Ardeadoris*. Four main supported subclades or sister pairs are recovered in most analyses (see Fig. 4). These relationships are never conflicted elsewhere, although support varies. *Diversidoris* is sister to *Glossodoris cincta* + *pallida*; *Glossodoris edmundsi* + *sedna* form a sister pair, as do *Glossodoris atromarginata* + *sibogae*; and finally *Ardeadoris* forms a sister pair to *Glossodoris hikuerensis* (Fig. 4). The single gene 16S analysis (Fig. 3) placed all *Glossodoris* (+ *Ceratosoma*, *Ardeadoris*, *Diversidoris*) into a single, albeit unsupported clade. Subsequent addition of COI and 18S data collapsed this grouping, and it is still not known how these subclades relate to each other. This information will determine whether the genus *Glossodoris* is paraphyletic or polyphyletic and guide any necessary ammendments to the current classification. The four groups (not colour groups) identified by Rudman (1987) are all represented here, and a good correlation exists between those and the subclades identified here, with the exception of *Glossodoris cincta* and *G. hikuerensis*. Rudman noted that these two species did not fit easily into a simple evolutionary scenario, as each have unusual radular morphology, not shared with each other or any other *Glossodoris* (*G. cincta* — high pointed inner lateral teeth; *G. hikuerensis* — lacks any denticles).

Phylogeny of genera restricted to the Indo-Pacific or Southern Ocean

Despite the previous morphological analyses of the Chromodorididae (Rudman 1984; Gosliner & Johnson 1999), the relationships between most genera remain unsupported, unknown or in conflict. In Rudman (1984) hypothesis, the monotypic *Ardeadoris* was considered to be present in a clade with *Glossodoris* and *Verconia*. In Gosliner & Johnson (1999) phylogeny, *Ardeadoris* was hypothesized to be basal to the clade containing *Ceratosoma*, *Chromodoris* and *Glossodoris*.

Results from the present study show *A. egretta* grouping with *G. hikuerensis* (Figs 3–5), although the pairs' relationship to other *Glossodoris* species remains essentially unresolved. Despite *Glossodoris* and *Ardeadoris* both having a distinctive wide folding mantle (see Fig. 1) and other morphological features in common (Rudman 1984, 1985, 1990), *A. egretta* was considered sufficiently distinct to warrant the creation of a separate genus (Rudman 1984). As the position of *Glossodoris* species relative to each other remains unsupported or unresolved, and more work will be needed to show if *Ardeadoris* consistently shows a sister group relationship to some or all *Glossodoris* species and should retain its generic validity, or if it nests inside the genus and should be synonymized with *Glossodoris*.

Our analyses show some support for *Diversidoris* grouping in a clade with the Indo-Pacific *Glossodoris cincta* and sometimes *G. pallida* (Figs 3–5). There were no *a priori* phylogenetic hypotheses regarding the monotypic *Diversidoris aurantionodulosa* Rudman's (1987). It was described after Rudman (1984) review and was not included in the cladistic analysis by Gosliner & Johnson (1999). *Diversidoris aurantionodulosa* has a wide geographical distribution throughout the Indo-Pacific (Rudman 1987; Rudman & Darvell 1990). Rudman (1987) tentatively proposed that *D. aurantionodulosa* was related to the *Thorunna/Digidentis* group of Chromodorididae. He did note that the 'folding or undulating mantle' of *Diversidoris* is shared with *Glossodoris* (Fig. 1) and *Thorunna*, but saw no other anatomical features that suggested a close relationship with these genera. Like *Ardeadoris*, more work is needed on the phylogeny of *Glossodoris* before the status of the taxon *Diversidoris* can be determined.

There is very strong support in all analyses for *Verconia verconis* and *Noumea haliclona* grouping together in a clade (Figs 3–5). Rudman (1984) suggested *Verconia* showed a similar reproductive morphology to *Glossodoris*, which is reflected in his phylogenetic hypothesis, but in text, only specifies that it belongs to the *Chromodoris* subgroup (which includes *Noumea*). Subsequent revision of *Noumea* highlighted the probability that *Verconia* was derived from *Noumea* (Rudman 1987). Gosliner & Johnson (1999) showed *Verconia* as sister to the clade containing *Hypselodoris*, *Risbecia*, *Mexichromis*, *Digidentis*, *Thorunna*, *Durvilledoris*, *Noumea* and *Pectenodoris*, which is not congruent with our results. Interestingly, *Verconia verconis* and *Noumea haliclona* share the same prey sponges (Rudman & Bergquist 2007), and both exhibit pink and yellow colour forms matching the sponge colour (Rudman 1987). Genetic control of colouration is likely since direct uptake of sponge pigment has been disproved (Avern 1986). Both species are known to occur in south and south-east Australia (www.seaslugforum.net). In our study, only a single species of *Noumea* was included, so much greater sampling will be required to determine whether *Verconia* remains as the sister group to *Noumea*, or more likely given the diversity of *Noumea*, nests inside and requires synonymy.

Pectenodoris Rudman (1984) was erected for a single Indo-Pacific species. Rudman indicated that *Pectenodoris* was the sister genus of *Chromodoris* (Rudman 1984), although he noted foregut and reproductive similarities with *Noumea*. A second species was later described (Johnson & Gosliner 1998), and the cladistic analysis by Gosliner & Johnson (1999) placed the genus as sister to *Noumea*. *Pectenodoris* is known to prey on the sponge *Dysidea* (Rudman & Bergquist 2007), along with members of the hypselodorid subgroup of Rudman. In our study, *Pectenodoris trilineata* consistently formed a well-supported clade with the Indo-Pacific species of *Mexichromis*, in nearly all analyses in this study (Figs 3–5). This places it firmly with derived chromodorid species, and supports no close relationship to *Chromodoris* or *Noumea*.

The position of *Ceratosoma* was the major difference in chromodorid phylogenies published to date. It has been placed with a *Glossodoris* + *Chromodoris* clade (Gosliner & Johnson 1999), or as the sister to the rest of the hypselodorid subgroup (Rudman 1984). Interestingly, we recover evidence for both of these conflicting positions, depending on the genes or analyses conducted. 16S only data puts *C. trilobatum* in the middle of an unsupported *Glossodoris* clade (including *Ardeadoris* and *Diversidoris*) with Bayesian inference, and as the unsupported sister to *Tyrinna nobilis* in a large polytomy in the strict consensus tree using Maximum Parsimony. 16S + COI Baysian analyses places *C. trilobatum* as the highly supported sister to the derived hypselodorid clade (1.00) but maximum parsimony places it as the unsupported sister to *Tyrinna nobilis* in an unsupported *Glossodoris* clade. Finally, the three-gene Baysian analysis places *C. trilobatum* as the unsupported sister to the derived hypselodorid clade and parsimony leaves it in a large chromodorid polytomy. The variety of positions assigned to this taxon gives little confidence about its true affinities. *Ceratosoma trilobatum* has been demonstrated as quite derived in a morphological phylogeny (Valdés & Gosliner 1999), and this may have affected its position in the analyses.

Durvilledoris and *Thorunna* were recovered in the hypselodorid clade along with *Digidentis*, *Pectenodoris* + *Mexichromis* and *Hypselodoris* + *Risbecia*. No well-supported sister group relationships were determined for either of these two genera. Thollesson's (1999, 2000) analyses showed *Durvilledoris* with a basal position in the Chromodorididae (and sometimes outside the family), which was likely caused by long-branch attraction. It appears that the addition of more derived Indo-Pacific taxa allowed a more accurate placement of *Durvilledoris* and subsequently allowed us to understand more about its position in the family. *Thorunna* typically assumed an unsupported basal position in the hypselodorid clade.

The position of *Digidentis*, a genus restricted to the colder Southern Ocean waters in Australia, was often unresolved, but also showed a potential relationship with east Atlantic *Hypselodoris*. Both the 16S (parsimony and Bayesian) and the three-gene analyses (Bayesian, support in unpartitioned analyses) resolved *Digidentis* as the sister group to the east Atlantic *Hypselodoris* species (0.93 and 0.98, respectively). This somewhat surprising relationship was noted in the context of *Hypselodoris* polyphyly. A biogeographical connection between the east Atlantic and the Australian Southern Ocean is unexpected, and yet the molecular results that suggest this are supported by morphology (shared wide vaginal duct and large exogenous sperm sac/receptaculum seminis).

Digidentis is a small genus, comprising of only three valid species, all sharing unique radular morphology and a distribution restricted to southern Australia. However, all three have very distinct colouration patterns (*kulonba*, white with thin yellow mantle edge; *arbutus*, bright pink with diffuse beige markings; *perplexa*, white with orange spots and purple mantle band) and differing mantle gland morphology (*kulonba* digitate; *arbutus* and *perplexa* rounded) (Burn 1966; Rudman 1984, 1985, 1987). When first re-examining *Hypselodoris kulonba* Burn (1966), Rudman (1985) noted that the body shape, mantle glands and radular morphology were suggestive of *Hypselodoris*, but also that the large exogenous sperm sac was not typical of the genus, and the radular seemed unique. He tentatively referred it as '*Hypselodoris*' *kulonba*. Later, Rudman (1987) redescribed *Glossodoris perplexa* (which had also previously been in *Chromodoris*), placed it in *Digidentis* alongside *D. arbutus*, and predicted '*H*' *kulonba* to also be congeneric. Resolving the position of this genus will likely require the addition of more hypselodorid taxa (and presumably more data), and the conclusions of either Figs 3 and 5, with the surprising EA + SO relationship, or alternatively, Fig. 4 with the more predictable EA + IWP/SO be upheld.

The position of the basal genera

One of the key questions in this study was to determine the positioning of the presumed basal genera *Cadlina*, *Cadlinella* and *Tyrinna*, and their relationships to each other. By rooting the combined data set with *Actinocyclus*, *Cadlina* consistently appears as a monophyletic group with high support values outside a strict chromodorid clade (Figs 3–5). It remains either as a part of a mostly unresolved group of dorid and discodorid outgroup taxa (Figs 3 and 4), or as the sister to the rest of the Chromodorididae (Fig. 5). Support for the sister group relationship arose solely from the inclusion of nuclear data (18S rDNA). Previous molecular analyses using mitochondrial sequence data have indicated *Cadlina* cannot be included in a monophyletic Chromodorididae (Thollesson 1999, 2000; Wollscheid-Lengeling *et al*. 2001; Wägele *et al*. 2003; Grande *et al*. 2004b) although in many cases, sampling of chromodorid taxa was very limited. Previous 18S data have given some evidence for *Cadlina* appearing as a basal chromodorid (Wollscheid & Wägele 1999; Wollscheid-Lengeling *et al*. 2001; Wägele *et al*. 2003), but these results were never supported by bootstrapping assessments.

On a morphological basis, the position of *Cadlina* in relation to other chromodorid taxa is still disputed. Bergh (1891) initially created a separate Cadlinidae and Chromodorididae. Subsequently, some authors have accepted this division (regardless of the rank assigned) (Eliot 1910; Odhner 1968; Lance in Keen 1971; Ros 1975; Bertsch 1977; Vaught 1989), although some consider it unnecessary (Thiele 1931; Marcus & Marcus 1967; Edmunds 1981; Boss 1982; Rudman 1984; Gosliner & Johnson 1999; Schrödl 2000; Valdés & Campillo 2000; Schrödl & Millen 2001; Domínguez *et al*. 2006). Rudman (1984) correctly rejected the concept of a separate Cadlinidae on the grounds that *Cadlina*, *Tyrinna* and *Cadlinella* were not closely related to each other, and it is likely that this secondary inclusion of *Cadlinella* and *Tyrinna* into Cadlinidae may have prevented its wider usage, and aided synonymization with Chromodorididae. In Gosliner & Johnson (1999) morphological phylogeny, *Cadlina* was found to be the sister to a clade containing *Tyrinna* and *Cadlinella*, when rooting their tree with *Actinocyclus*. In the present study, these three genera never grouped together in any of the analyses conducted; yet, support for *Cadlina* as the sister group to Chromodorididae *sensu stricto* was present when 18S data were included.

Cadlina possesses several plesiomorphic features including spiculose body, rachidian teeth and a serial seminal receptacle (Valdés & Campillo 2000), and species usually occur in cold temperate or arctic waters unlike the majority of other chromodorids. To date, no morphological (Gosliner & Johnson 1999) or histological (Wägele *et al*. 2006) synapomorphies are known that could at present provide reasons for resurrection of the family name Cadlinidae Bergh (1891). However, its inclusion in Chromodorididae remains provisional because only a single nuclear gene supports this, while data from several mitochondrial genes conflict with this and actively support an alternative position with the Dorididae and Discodorididae.

Cadlinella ornatissima appeared as the unsupported sister group to the rest of the Chromodorididae (excluding *Cadlina*) on the basis of 16S, but when 18S data were added, the branches collapsed, rendering it as simply another part of the large chromodorid polytomy. In Rudman's (1984) review of the Chromodorididae, he was suggested that *Cadlinella* was the most basal genus within the family. This conclusion was based on morphological evidence that linked several plesiomorphic characteristics with *Cadlinella*, *Cadlina* and *Tyrinna* and also that *Cadlinella* could not be closely morphologically linked with any other genus of the Chromodorididae. This argument has been supported by Gosliner & Johnson (1994)

who considered the Actinocyclidae to be the sister group of the Chromodorididae. However, Schrödl & Millen (2001) argued that this arrangement did not support a basal position for *Cadlinella* within the Chromodorididae. Additional work examining the sperm ultrastructure of *Cadlinella ornatissima* (Wilson & Healy 2002) demonstrated a number of morphological features that separate this species from other members of the Chromodorididae and from other nudibranchs. The present study indicates that *Cadlinella* is not closely related to *Cadlina* or *Tyrinna*, although its position in the Chromodorididae is far from being understood.

Tyrinna nobilis is supported as part of the Chromodorididae (excluding *Cadlina*) in all analyses. In the 16S + COI tree (Fig. 4), only *Cadlinella* and the planar spawning *Chromodoris* are more basal than *Tyrinna nobilis*, although these positions are not statistically supported. *Tyrinna* possesses many plesiomorphic characteristics and there is general agreement that it is basal in the Chromodorididae (Rudman 1984; Muniain *et al*. 1996; Gosliner & Johnson 1999; Schrödl & Millen 2001). Despite this, identifying the precise relationship of *Tyrinna* to the rest of the Chromodorididae has proved problematic (Schrödl & Millen 2001). Recent work on secondary metabolites has revealed that *Tyrinna* probably feeds on dysideid sponges (Fontana *et al*. 1998), like many of the derived genera of the Chromodorididae (*Ceratosoma*, *Mexichromis*, *Hypselodoris*, *Risbecia*, *Thorunna*, *Digidentis*, *Pectenodoris* and *Durvilledoris*) (Rudman & Bergquist 2007). The MP combined 16S + COI strict consensus tree does show *Tyrinna* as sister to *Ceratosoma*, but this relationship does not maintain support through bootstrapping. It is possible that *Tyrinna's* position closer to the base of the Chromodorididae may be influenced by long-branch attraction, and its real affinities lie with the *Hypselodoris* subgroup. Using feeding preference data, Rudman & Bergquist (2007; Fig. 3) have updated Rudman (1984) non-cladistic phylogenetic hypothesis to reflect this change.

Biogeography and paraphyly in the Chromodorididae

Vicariant events involving the closure of the Tethys Sea and the succeeding rise of dispersal barriers such as the Isthmus of Panama and the east Pacific Barrier have affected global biogeographical patterns in a reasonably predictable way, typically resulting in an east Pacific + Atlantic clade that is sister to an Indo-west Pacific clade. This generalized pattern has been identified, based on morphology, in all the widespread nudibranch genera that have been examined (e.g. *Hypselodoris* Gosliner & Johnson 1999; *Rostanga* Garovoy *et al*. 2001; *Platydoris* Dorgan *et al*. 2002; *Phyllidiopsis* Valdés 2001, 2002), although the east Pacific + Atlantic clade was usually nested inside an Indo-Pacific grade. The exceptions to this overall pattern has been when deep water or cold water (sub-Arctic or South African) species have been basal to the rest (Garovoy *et al*. 2001; Dorgan *et al*. 2002), and this is not altogether unexpected given that plesiomorphic Indo-west Pacific species can be driven to the periphery of habitats (Briggs 2003).

The predicted biogeographical pattern is apparent in parts of our phylogenetic analyses (e.g. *Cadlina*), with some notable exceptions. East Atlantic *Hypselodoris* are not sister to their Indo-Pacific congeners, and west Atlantic and east Pacific *Mexichromis* are also not sister to their Indo-Pacific congeners. This renders these taxa non-monophyletic, and indicates widespread changes are necessary for the current classification of the Chromodorididae. The conclusions drawn from this study are too preliminary to recommend these types of changes, but serve to highlight necessary subsequent work.

The same pattern of among-ocean non-monophyly was recently discovered in Atlantic corals, where genera conventionally assigned to different families were more closely related to each other than they were to their respective Pacific 'congeners' (Fukami *et al*. 2004). These parallel results indicate that the pattern may hold for additional widespread and speciose invertebrate groups, and will likely change our ideas about endemicity in the Atlantic. This comparison study also serves to highlight the disparity of taxon ranks across invertebrate taxa. Presumably, the same vicariant events separated these ancestral corals and nudibranchs, and yet it has resulted in taxonomic confusion at the family level for corals, and the generic level for nudibranchs.

The remaining two genera with species in multiple oceans, *Chromodoris* and *Glossodoris*, do not show the same pattern, although their positions in the phylogeny were not resolved enough for rigorous discussion. The Atlantic species of *Chromodoris* and *Glossodoris* did not form a clade with the Atlantic *Hypselodoris* and *Mexichromis* species, and must therefore represent a lineage/s that had diverged prior to the closure of communication between the Indo-west Pacific and the Atlantic in the early Miocene (16–24 Myr ago). Clearly, better sampling is needed to solve the phylogeny of *Chromodoris* and *Glossodoris*, and subsequently understand the pattern of evolution in the Chromodorididae.

Although vicariance has undoubtably caused divergences recognizable between larger clades examined in this study, radiations within these clades have occurred along coastlines, or smaller biogeographical regions. This highlights increasing evidence that sister species may occur sympatrically, and in the absence of any evidence for significant distributional changes over time points toward speciation occurring in the absence of any obvious geographical barriers (e.g. *Tigriopus* copepods, Burton 1998; *Tegula* snails, Hellberg 1998; calyptraeid limpets, Collin 2003). Whether these speciation events occurred sympatrically through ecological specialization or other means, or by periods of transient allopatry, remains unknown. In either case, molecular phylogenies are challenging the prevailing view that marine speciation and biogeographical

patterns are characterized by vicariance or broadscale dispersal (Mayr 1954, 1970).

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References

Alejandrino, A. & Valdés, A. (2006). Phylogeny and biogeography of the Atlantic and eastern Pacific *Hypselodoris* Stimpson, 1855 (Nudibranchia, Chromodorididae) with the description of a new species from the Caribbean Sea. *Journal of Molluscan Studies*, *72*, 189–198.

Avern, G. J. (1986). *The biology of the nudibranch Noumea haliclona (Burn, 1957) (Family Chromodorididae)*. M.Sc. Sydney: University of Sydney.

Avila, C. (1995). Natural products of opisthobranch molluscs: a biological review. *Oceanography and Marine Biology an Annual Review*, *33*, 487–559.

Bergh, L. S. R. (1891). Die cryptobranchiaten Dorididen. *Zoologische Jahrbücher*, *6*, 103–144.

Bertsch, H. (1977). The Chromodoridinae nudibranchs from the Pacific coast of America — Part I. Investigative methods and supra-specific taxonomy. *Veliger*, *20*, 107–118.

Bertsch, H. (1978a). The Chromodoridinae nudibranchs from the Pacific coast of America — Part II. The genus *Chromodoris*. *Veliger*, *20*, 307–327.

Bertsch, H. (1978b). The Chromodoridinae nudibranchs from the Pacific coast of America — Part III. The genera *Chromolaichma* and *Mexichromis*. *Veliger*, *21*, 70–86.

Bertsch, H. (1978c). The Chromodoridinae nudibranchs from the Pacific coast of America — Part IV. The genus *Hypselodoris*. *Veliger*, *21*, 236–250.

Boss, K. J. (1982). Mollusca. In S. P. Parker (Ed.) *Synopsis and Classification of Living Organisms* (pp. 945–1166). New York: McGraw-Hill.

Briggs, J. C. (2003). Marine centres of origin as evolutionary engines. *Journal of Biogeography*, *30*, 1–18.

Burn, R. (1966). On three new Chromodoridinae from Australia (Opisthobranchia: Nudibranchia). *Veliger*, *8*, 191–197.

Burton, R. S. (1998). Intraspecific phylogeography across the Point Conception biogeographic boundary. *Evolution*, *52*, 734–745.

Cimino, G. & Ghiselin, M. (1999). Chemical defense and evolutionary trends in biosynthetic capacity among dorid nudibranchs. *Chemoecology*, *9*, 187–207.

Collin, R. (2003). Phylogenetic relationships among calyptraeid gastropods and their implications for the biogeography of marine speciation. *Systematic Biology*, *52*, 618–640.

Cunningham, C. W. (1997). Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution*, *14*, 733–740.

Domínguez, M., García, F. J. & Troncoso, J. S. (2006). Some aspects of the family Chromodorididae (Opisthobranchia: Nudibranchia) from Brazil, with description of a new species. *Scientia Marina*, *70*, 621–634.

Dorgan, K. M., Valdés, A. & Gosliner, T. M. (2002). Phylogenetic systematics of the genus *Platydoris* (Mollusca, Nudibranhcia, Doridoidea) with descriptions of six new species. *Zoologica Scripta*, *31*, 271–319.

Edmunds, M. (1971). Opisthobranchiate Mollusca from Tanzania. *Zoological Journal of the Linnean Society*, *50*, 339–396.

Edmunds, M. (1981). Opisthobranchiate Mollusca from Ghana: Chromodorididae. *Zoological Journal of the Linnean Society*, *72*, 175–201.

Eliot, C. N. E. (1910). *A Monograph of the British Nudibranch Mollusca*. London: Ray Society.

Farris, J. S., Källersjö, M., Kluge, A. G. & Bult, C. (1994). Testing significance of incongruence. *Cladistics*, *10*, 315–319.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, *39*, 783–791.

Folmer, O., Black, M., Hoen, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*, 294–299.

Fontana, A., Muniain, C. & Cimino, G. (1998). First chemical study of patagonian nudibranchs: a new seco-11, 12-spongiane, tyrinnal, from the defensive organs of *Tyrinna nobilis*. *Journal of Natural Products*, *61*, 1027–1029.

Fukami, H., Budd, A. F., Paulay, G., Solé-Cava, A. M., Chen, C. A., Iwao, K. & Knowlton, N. (2004). Conventional taxonomy obscures deep divergence between Pacific and Atlantic corals. *Nature*, *427*, 832–835.

Garovoy, J. B., Valdés, A. & Gosliner, T. M. (2001). Phylogeny of the genus *Rostanga* (Nudibranchia), with descriptions of three new species from South Africa. *Journal of Molluscan Studies*, *67*, 131–144.

Gavagnin, M. G. & Fontana, A. (2000). Diterpenes from marine opisthobranch molluscs. *Current Organic Chemistry*, *4*, 1201–1248.

Giménez-Casalduero, F., Thacker, R. W. & Paul, V. J. (1999). Association of colour and feeding deterrence by tropical reef fishes. *Chemoecology*, *9*, 33–39.

Gosliner, T. M. (1996). Phylogeny of *Ceratosoma* (Nudibranchia: Chromodorididae), with descriptions of two new species. *Proceedings of the California Academy of Sciences*, *49*, 115–126.

Gosliner, T. M. & Draheim, R. (1996). Indo-Pacific opisthobranch gastropod biogeography: how do we know what we don't know? *American Malacological Bulletin*, *12*, 37–43.

Gosliner, T. M. & Johnson, S. (1994). Review of the genus *Hallaxa* (Nudibranchia: Actinocyclidae) with descriptions of nine new species. *Veliger*, *37*, 155–191.

Gosliner, T. M. & Johnson, R. F. (1999). Phylogeny of *Hypselodoris* (Nudibranchia: Chromodorididae) with a review of the monophyletic

clade of Indo-Pacific species, including descriptions of twelve new species. *Zoological Journal of the Linnean Society*, *125*, 1–114.

Grande, C., Templado, J., Cervera, J. L. & Zardoya, R. (2004a). Molecular phylogeny of Euthyneura (Mollusca: Gastropoda). *Molecular Biology and Evolution*, *21*, 303–313.

Grande, C., Templado, J., Cervera, J. L. & Zardoya, R. (2004b). Phylogenetic relationships among Opisthobranchia (Mollusca: Gastropoda) based on mitochondrial *cox1*, *trnV*, and *rrnL* genes. *Molecular Phylogenetics and Evolution*, *33*, 378–388.

Hall, T. A. (1999). Bioedit: a user-friendly biological sequence editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98.

Hellberg, M. E. (1998). Sympatric sea shells along the sea's shore: the geography of speciation in the marine gastropod *Tegula*. *Evolution*, *52*, 1311–1324.

Huelsenbeck, J. P. & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, *17*, 754–755.

Jermiin, L. S. (1997). *K2WuLi: a Program to Conduct Relative-Rate Tests from DNA Sequences*: No longer available from http://jcsmt.anu.edu.au/dmm/humgen/lars/k2wulisub.htm.

Johnson, R. F. & Gosliner, T. M. (1998). The genus *Pectenodoris* (Nudibranchia: Chromodorididae) from the Indo-Pacific, with the description of a new species. *Proceedings of the California Academy of Sciences*, *50*, 295–306.

Johnson, R. F. & Valdés, A. (2001). The *Hypselodoris infucata*, *H. obscura* and *H. saintvincentius* species complex (Mollusca, Nudibranchia, Chromodorididae), with remarks on the genus *Brachychlanis* Ehrenberg, 1831. *Journal of Natural History*, *35*, 1371–1398.

Keen, A. M. (1971). *Sea Shells of Tropical West America*. California: Stanford University Press.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, *5*, 150–163.

Marcus, E. (1970). Opisthobranchs from northern Brazil. *Bulletin of Marine Science*, *24*, 922–951.

Marcus, E. & Marcus, E. (1967). American opisthobranch mollusks. *Studies in Tropical Oceanography*, *University of Miami*, *6*, 1–256.

Mayr, E. (1954). Geographic speciation in tropical echinoids. *Evolution*, *8*, 1–18.

Mayr, E. (1970). *Populations*, *Species*, *and Evolution*. Cambridge, MA: Harvard University Press.

Medina, M. & Walsh, P. J. (2000). Molecular systematics of the order Anapsidea based on mitochondrial DNA sequences (12S, 16S, and COI). *Molecular Phylogenetics and Evolution*, *15*, 41–58.

Muniain, C., Valdés, A. & Ortea, J. (1996). Redescription of *Tyrinna nobilis* Bergh, 1898 (Opisthobranchia: Chromodorididae) from Patagonia, Argentina. *Journal of Molluscan Studies*, *3*, 265–273.

Odhner. N. H. J. (1968). Systematique. In P. Grassé (Ed.) *Sous-Classe Des Opisthobranches*, *Traité de Zoologie* (pp. 834–888). Masson, Paris.

Ogden, T. H. & Rosenberg, M. S. (2007). How should gaps be treated in parsimony? A comparison of approaches using simulation. *Molecular Phylogenetics and Evolution*, *42*, 817–826.

Ortea, J., Valdés, A. & Garcia-Gomez, J. C. (1996). Revision de las especies atlantica de la familia Chromodorididae (Mollusca: Nudibranchia) del grupo cromatico azul. *Avicennia supplement*, *1*, 1–160.

Palumbi, S., Martin, A., Romano, S., McMillan, W. O., Stice, L. & Grabowski, G. (1991). *The simple fool's guide to PCR*, *Version 2.0*. Hawaii: Department of Zoology and Kewalo Marine Laboratory, University of Hawaii.

Posada, D. (2005). *Modeltest*, Version 3.7 documentation. Available via http://darwin.uvigo.es/software/modeltest.html.

Posada, D. & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics*, *14*, 817–818.

Rambaut, A. (1996). *Se-Al: Sequence Alignment Editor*. Available via http://evolve.zoo.ox.ac.uk.

Rambaut, A. & Drummond, A (2005). *Tracer: MCMC Trace Analaysis Tool. 1.3*. University of Oxford, UK.

Ros, J. (1975). Opistobranquios (Gastropoda: Euthyneura) del litoral ibérico. *Investigación Pesquera*, *39*, 269–372.

Rudman, W. B. (1984). The Chromodorididae (Opisthobranchia: Mollusca) of the Indo-West Pacific: a review of the genera. *Zoological Journal of the Linnean Society*, *81*, 115–273.

Rudman, W. B. (1985). The Chromodorididae (Opisthobranchia: Mollusca) of the Indo-West Pacific: *Chromodoris aureomarginata*, *C. verrieri and C. fidelis* colour groups. *Zoological Journal of the Linnean Society*, *83*, 241–299.

Rudman, W. B. (1987). The Chromodorididae (Opisthobranchia: Mollusca) of the Indo-West Pacific: *Chromodoris epicura*, *C. aureopurpurea*, *C. annulata*, *C. coi*, *and Risbecia tryoni* colour groups. *Zoological Journal of the Linnean Society*, *90*, 305–407.

Rudman, W. B. (1990). The Chromodorididae (Opisthobranchia: Mollusca) of the Indo-West Pacific: further species of *Glossodoris*, *Thorunna and* the *Chromodoris aureomarginata* colour group. *Zoological Journal of the Linnean Society*, *100*, 263–326.

Rudman, W. B. (1991). Purpose in pattern: the evolution of colour in chromodorid nudibranchs. *Journal of Molluscan Studies* (Suppl.). *T.E. Thompson Memorial Issue*, *57*, 5–21.

Rudman, W. B. (2003). (May 22). *Glossodoris edmundsi and G. ghanensis*. Available via http://www.seaslugforum.net/find.cfm?id=10008.

Rudman, W. B. & Bergquist, P. R. (2007). A review of feeding specificity in the sponge-feeding Chromodorididae (Mollusca: Nudibranchia). *Molluscan Research*, *27*, 60–88.

Rudman, W. B. & Darvell, B. W. (1990). Opisthobranch molluscs of Hong Kong: Part 1. Goniodorididae, Onchidorididae, Triophidae, Gymnodorididae, Chromodorididae (Nudibranchia). *Asian Marine Biology*, 7, 31–79.

Sarich, V. M. & Wilson, A. C. (1967). Immunological time scale for hominid evolution. *Science*, *158*, 1200–1203.

Schrödl, M. (2000). Revision of the nudibranch genus *Cadlina* (Gastropoda: Opisthobranchia) from the southern Ocean. *Journal of the Marine Biology Association of the U.K.*, *80*, 299–309.

Schrödl, M. & Millen, S. V. (2001). Revision of the nudibranch gastropod genus *Tyrinna* Bergh, 1898 (Doridoidea: Chromodorididae). *Journal of Natural History*, *35*, 1143–1171.

Sokolov, E. P. (2000). An improved method for DNA isolation from mucopolysaccharide-rich molluscan tissues. *Journal of Molluscan Studies*, *66*, 573–575.

Swofford, D. L. (2002). *PAUP*, Version 4b10*. Sunderland, Massachusetts: Sinauer Associates Inc.

Thiele, J. (1931). *Handbuch der Systematischen Weichtierkunde*. Jena1(2): Fischer.

Thollesson, M. (1999). Phylogenetic analysis of dorid nudibranchs (Gastropoda: Doridacea) using the mitochondrial 16s rRNA gene. *Journal of Molluscan Studies*, *65*, 335–353.

Thollesson, M. (2000). Increasing fidelity in parsimony analysis of

dorid nudibranchs by differential weighting, or a tale of two genes. *Molecular Phylogenetics and Evolution*, *16*, 161–172.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgens, D. G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, *25*, 4876–4882.

Valdés, A. (2001). Depth-related adaptations, speciation processes and evolution of colour in the genus *Phyllidiopsis* (Mollusca: Nudibranchia). *Marine Biology*, *139*, 485–496.

Valdés, A. (2002). A phylogenetic analysis and systematic revision of the cryptobranch dorids (Mollusca, Nudibranchia, Anthobranchia). *Zoological Journal of the Linnean Society*, *136*, 535–636.

Valdés, A. (2003). Preliminary molecular phylogeny of the radula-less dorids (Gastropoda: Opisthobranchia), based on 16S MTDNA sequence data. *Journal of Molluscan Studies*, *69*, 75–80.

Valdés, A. & Afonso, C. M. L. (2003). Synonymy of *Cadlina burnayi* Ortea, 1988, from the Cape Verde Islands (Opisthobranchia: Chromodorididae) with *Tyrinna evelinae* (Marcus, 1958). *Veliger*, *46*, 176–185.

Valdés, A. & Campillo, O. J. (2000). Redescription and reassessment of *Cadlina luarna* (Ev. Marcus and Er. Marcus, 1967) comb. nov. (Mollusca, Opisthobranchia, Doridina). *Proceedings of the California Academy of Sciences*, *52*, 77–85.

Valdés, A. & Gosliner, T. M. (1999). Reassessment of the systematic status of *Miamira* Bergh, 1875 and *Orodoris* Bergh, 1875 (Nudibranchia: Chromodorididae) in light of phylogenetic analysis. *Journal of Molluscan Studies*, *65*, 33–45.

Vaught, K. C. (1989). *A Classification of the Living Mollusca*. Florida: American Malacologists, Inc.

Wägele, H., Ballesteros, M. & Avila, C. (2006). Defensive glandular structures in opisthobranch molluscs — from histology to ecology. *Oceanography and Marine Biology: An Annual Review*, *44*, 197–276.

Wägele, H., Vonnemann, V. & Wägele, W. (2003). Toward a phylogeny of the Opisthobranchia. In C. Lydeard & D. R. Lindberg (Eds) *Molecular Systematics and Phylogeography of Mollusks* (pp. 185–228). Washington and London: Smithsonian Books.

Wilson, N. G. & Healy, J. M. (2002). Is *Cadlinella ornatissima* a chromodorid? Sperm ultrastructure in an enigmatic nudibranch (Opisthobranchia, Mollusca). *Invertebrate Reproduction and Development*, *42*, 179–188.

Wilson, N. G. & Healy, J. M. (2006). Basal chromodoridid sperm ultrastructure (Mollusca, Gastropoda, Nudibranchia). *Zoomorphology*, *125*, 99–107.

Wilson, N. G. & Lee, M. S. Y. (2005). Molecular phylogeny of *Chromodoris* (Mollusca, Nudibranchia) and the identification of a planar spawning clade. *Molecular Phylogenetics and Evolution*, *36*, 722–727.

Wollscheid, E. & Wägele, H. (1999). Initial results on the molecular phylogeny of the Nudibranchia (Gastropoda, Opisthobranchia) based on 18S rDNA data. *Molecular Phylogenetics and Evolution*, *13*, 215–226.

Wollscheid-Lengeling, E., Boore, J., Brown, W. & Wägele, H. (2001). The phylogeny of Nudibranchia (Opisthobranchia, Gastropoda, Mollusca) reconstructed by three molecular markers. *Organisms Diversity and Evolution*, *1*, 241–256.

Xia, X. & Xie, Z. (2001). DAMBE: Data analysis in molecular biology and evolution. *Journal of Heredity*, *92*, 371–373.

Xia, X., Xie, Z., Salemi, M., Chen, L. & Wang, Y. (2003). An index of substitution saturation and its application. *Molecular Phylogenetics and Evolution*, *26*, 1–7.