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Short communication

Molecular phylogeny of *Chromodoris* (Mollusca, Nudibranchia) and the identification of a planar spawning clade

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1. Introduction

Chromodoris, Alder and Hancock, 1855, is the most speciose genus in the nudibranch family Chromodorididae, and has a cosmopolitan distribution with the greatest diversity occurring at low latitudes. It is estimated to contain approximately 200 species with up to 22% of known species still not officially described (Gosliner and Draheim, 1996); presumably many remain to be discovered. Some species have shown unique chemical defenses (Gavagnin and Fontana, 2000) and future screening would benefit from an evolutionary perspective. A phylogeny will also facilitate analysing colour evolution, as within *Chromodoris* there appear instances of both convergent (due to mimicry: Rudman, 1991) and homologous colour traits (Gosliner and Behrens, 1998). Currently, there are no phylogenetic hypotheses for this large and important genus. Many colour groups were described in *Chromodoris* to facilitate identification using external colour patterns (e.g., Rudman, 1983, 1985, 1987), with no expectation that these groupings reflected phylogenetic relationships. However, recent work has suggested one of these groups shares features in internal anatomy and reproduction, and may represent a real clade (Gosliner and Behrens, 1998; Wilson, 2002).

The monophyly of *Chromodoris* has also not yet been rigorously tested and cannot be assumed: in phylogenetic studies that contained 2–3 *Chromodoris* species, the genus appeared monophyletic in only some (Thollesson, 1999; Wollscheid-Lengeling et al., 2001) or none (Thollesson, 2000) of the optimal trees. Miller (1980) emphasised the difficulty in separating the typically bipolar genus *Cadlina* Bergh, 1879 from *Chromodoris*, and suggested synonymy.

We used mitochondrial DNA sequence data (partial 16S rDNA) to create a phylogeny for a group where convergent evolution of colour patterns could hinder phylogenetic reconstruction based on the most widely used diagnostic characters.

2. Materials and methods

2.1. Taxon sampling

Seventeen species of *Chromodoris* and two outgroups were collected and sequenced directly for partial 16S rDNA. Sequences of five other species of *Chromodoris* and three *Cadlina* species were taken from GenBank (see Table 1). These exemplars from *Chromodoris* were chosen so that many putative species-groups and divergent forms were represented. Specimens were identified by comparison to published literature and dissected when necessary. The sequenced specimens have been deposited in the Australian and South Australian Museum collections (Table 1).

Small portions of ethanol-preserved foot tissue were washed in STE buffer and transferred to 10% Chelex solution. After adding proteinase-K (10 mg/ml), the solution was incubated at 50–55 °C for a few hours, with occasional mixing, then raised to 90 °C, and the cooled extraction buffered with 10% TE. The 16S rDNA fragments were amplified using the primers 16Sar-L and 16Sbr-H (Palumbi et al., 1991). PCR amplifications were

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Table 1
Species used in this study, asterisk indicates sequence acquired from GenBank

Species	Locality	Accession number	Voucher number
Actinocyclus verrucosus	Mooloolaba, Queensland, Australia	AY458799	SAM D19274
Cadlina laevis	Marstrand, Bohuslän, Sweden	AJ225182*	—
Cadlina luarna	Costa Rica	AF430348*	—
Cadlina luteomarginata	North Atlantic, USA	AF249231*	—
Cadlinella ornatissima	Heron Island, Queensland, Australia	AY458802	AM C203859
Chromodoris alternata	Port Phillip Bay, Victoria, Australia	AY458800	SAM D19281
Chromodoris ambiguus	Port Phillip Bay, Victoria, Australia	AY458801	SAM D19260
Chromodoris aspersa	Mooloolaba, Queensland, Australia	AY458813	SAM D19282
Chromodoris collingwoodi	North Stradbroke Island, Australia	AY731181	SAM D19283
Chromodoris daphne	Moreton Bay, Queensland, Australia	AY458803	SAM D19284
Chromodoris epicurea	Triabunna, Tasmania, Australia	AY458804	SAM D19285
Chromodoris geometrica	Mooloolaba, Queensland, Australia	AY458805	SAM D19286
Chromodoris krohni	NE Atlantic, Spain	AF249239*	—
Chromodoris kuiteri1	Mooloolaba, Queensland, Australia	AY458806	SAM D19287
Chromodoris kuiteri2	Great Barrier Reef, Australia	AF249240*	—
Chromodoris kuniei	Heron Island, Queensland, Australia	AY458807	SAM D19261
Chromodoris leopardus	Mooloolaba, Queensland, Australia	AY458808	SAM D19288
Chromodoris lochi	Mooloolaba, Queensland, Australia	AY458810	SAM D19289
Chromodoris luteorosea	Cadiz, Andalusia, Spain	AJ225183*	—
Chromodoris magnifica	Whitsundays, Queensland, Australia	AY458811	SAM D19290
Chromodoris purpurea	Cadiz, Andalusia, Spain	AJ225184*	—
Chromodoris quadricolor	Red Sea, Egypt	AF249241*	—
Chromodoris roboi	Heron Island, Queensland, Australia	AY458814	SAM D19291
Chromodoris splendida	Mooloolaba, Queensland, Australia	AY458815	SAM D19292
Chromodoris striatella	Mooloolaba, Queensland, Australia	AY458809	SAM D19293
Chromodoris strigata	Heron Island, Queensland, Australia	AY458816	SAM D19294
Chromodoris tasmaniensis	Triabunna, Tasmania, Australia	AY458817	SAM D19295

carried out using whole genomic preparations. The thermal cycling conditions were an initial denaturation step at 94 °C for 3 min, followed by 39 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, extension for 5 min at 72 °C, and soaked at 25 °C for 2 min. The PCR product was gel purified and sequenced directly in both directions using ABI automated sequencing.

2.2. Alignment and analyses

The sequences were reconciled using Sequencher (Gene Codes, Ann Arbor, MI) and aligned in ClustalX (Thompson et al., 1997). Initially, a sensitivity analysis (Giribet et al., 2001) was performed, where several alignment parameter combinations were used (gap opening penalty 2–15, gap extension cost 1–6.66; and transition weight 0.5–1), and each resultant alignment analysed (using parsimony for computing speed). The alignments and trees were all very similar, suggesting that the influence of alignment variation was small. One of these alignments (using default parameters in Clustal, i.e., 15:6.66:0.5) was chosen for more detailed analysis and small manual adjustments were made by eye.

The alignment was imported into MacClade (Maddison and Maddison, 2000) and then PAUP* (Swofford, 2002) for phylogenetic analyses. Hypervariable regions (69 sites) that were considered unalignable were excluded from analysis, leaving 416 sites (178 variable, 124 parsimony-informative). The total alignment was 485 sites long, and has been deposited in TreeBASE (www.treebase.org/). Sequences have been deposited in the NCBI GenBank database (see Table 1).

All trees were rooted using *Actinocyclus verrucosus* and *Cadlinella ornatissima*, which are related to, but outside, a putative *Chromodoris*-*Cadlina* clade (Gosliner and Johnson, 1994; Rudman, 1984). Parsimony analyses employed heuristic searches with 500 random sequence additions, with gaps treated as missing data. Clade support was calculated using bootstrapping and decay indices. Parsimony-uninformative characters were excluded from MP bootstraps (10,000 replicates) following discussion in Felsenstein (2004). Branch support (Bremer, 1988) was calculated using Tree Rot v2b (Sorenson, 1999).

The most appropriate model of DNA substitution for these data was chosen using Modeltest v3.06 (Posada and Crandall, 1998). Both hierarchical likelihood-ratio tests (HLTs) and the Akaike Information Criterion converged on the TVM+I+G model (first named in Posada, 2004). Bayesian MCMC analyses using MrBayes v3 (Ronquist and Huelsenbeck, 2001) used the most similar available model (erring on the side of simplicity): HKY+I+G (Hasegawa et al., 1985). Four chains (one heated) were run simultaneously, burn-in was 50,000 generations (well after likelihood reached stationarity), and the remaining 1,000,000 trees sampled every 100 trees.

3. Results and discussion

Uncorrected pairwise distance (alignment–ambiguous sites excluded) ranged from 0.013 between the two specimens of *Chromodoris kuiteri* to 0.228 (between *Chromodoris alternata* and *Chromodoris roboi*). The MP analyses resulted in a single optimal tree (Fig. 1), with three major clades discussed below. The Bayesian analysis also returned a very similar tree (Fig. 2), but contained minor topological differences that affected nodes poorly supported in both analyses.

These results indicate that *Chromodoris* is paraphyletic. The most basal ingroup clade is reasonably supported (branch support 5, bootstrap 74, and Bayesian 76) and contains *Chromodoris ambiguus* and *C. alternata* as well as included cadlinid species, i.e., *Cadlina luarna*, *Cadlina laevis*, and *Cadlina luteomarginata*. The second clade (branch support 8, bootstrap 96, and Bayesian 100) contains the type species *Chromodoris magnifica* and all the included members of the *Chromodoris quadricolor* colour group, as well as *Chromodoris aspersa*. This clade is hereafter termed the 'planar spawner' clade, in reference to the flat egg mass laid by its members (Wilson, 2002). While the clade itself is strongly supported, 16SrDNA provides little resolution for its internal relationships. MP analyses suggest *C. aspersa* as the most basal taxon while Bayesian analyses indicate *Chromodoris lochi*. The third major clade (branch support 4, bootstrap 68, and Bayesian 100) contains the remaining *Chromodoris* species from southeastern Australia, the Atlantic/Mediterranean, and the Indo-Pacific, and is termed the 'erect spawner' clade after their upright egg masses. Many of these subclades are also diagnosed by unusual morphological and behavioural features, as discussed below, although the polarity of these traits needs to be rigorously assessed.

3.1. Cadlina and the monophyly of Chromodoris

The monophyly of *Chromodoris* (*sensu* Rudman) is not supported by this study. Constraining *Chromodoris* to be monophyletic results in an MP tree only six steps longer, but one that is significantly worse according to both Templeton's nonparametric test and the Kishino–Hasegawa (K–H) test ($p = 0.014$ for both) (see Felsenstein, 2004). The

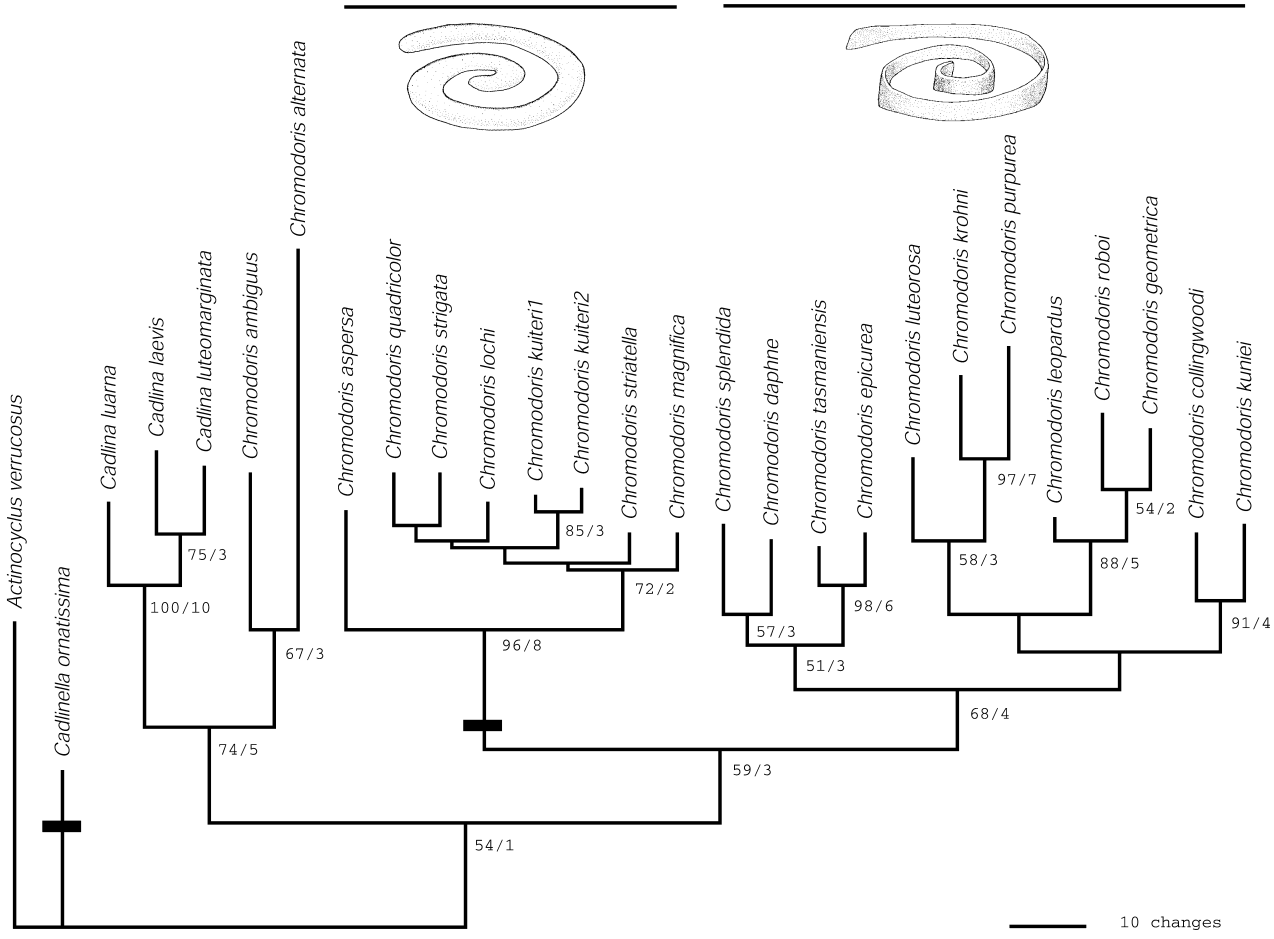

Fig. 1. Parsimony phylogram based on 16S rDNA for *Chromodoris* species. Closed box indicates acquisition of planar egg mass. The numbers to the right represent Bremer support; numbers to the left of each branch represent bootstrap percentages. Bootstrap values are only shown for clades present in the majority-rule consensus tree. Branch lengths reconstructed under ACCTRAN optimisation in PAUP*. CI: 0.52, RI: 0.61, and $L = 517$.

grouping of the southern temperate taxa *C. ambiguus* and *C. alternata* with *Cadlina* is inconsistent with the current generic classification. Despite the fact that the included *Cadlina* species are from the northern hemisphere, and the two *Chromodoris* species are from the Southern Ocean, the clade is reasonably supported. *C. ambiguus*, *C. alternata*, and most *Cadlina* species are restricted to very cold waters, and this physiological trait appears generally characteristic of this clade. The grouping of these temperate *Chromodoris* species with *Cadlina* is consistent with Rudman's (1987) observation that the exogenous sperm sac in *C. ambiguus* and *C. alternata* is the same as that seen in some *Cadlina* species. However, Miller's (1980) proposal that *Cadlina* be synonymised with *Chromodoris* is not the only taxonomic solution, since an alternative is to transfer *C. ambiguus* and *C. alternata* to another genus.

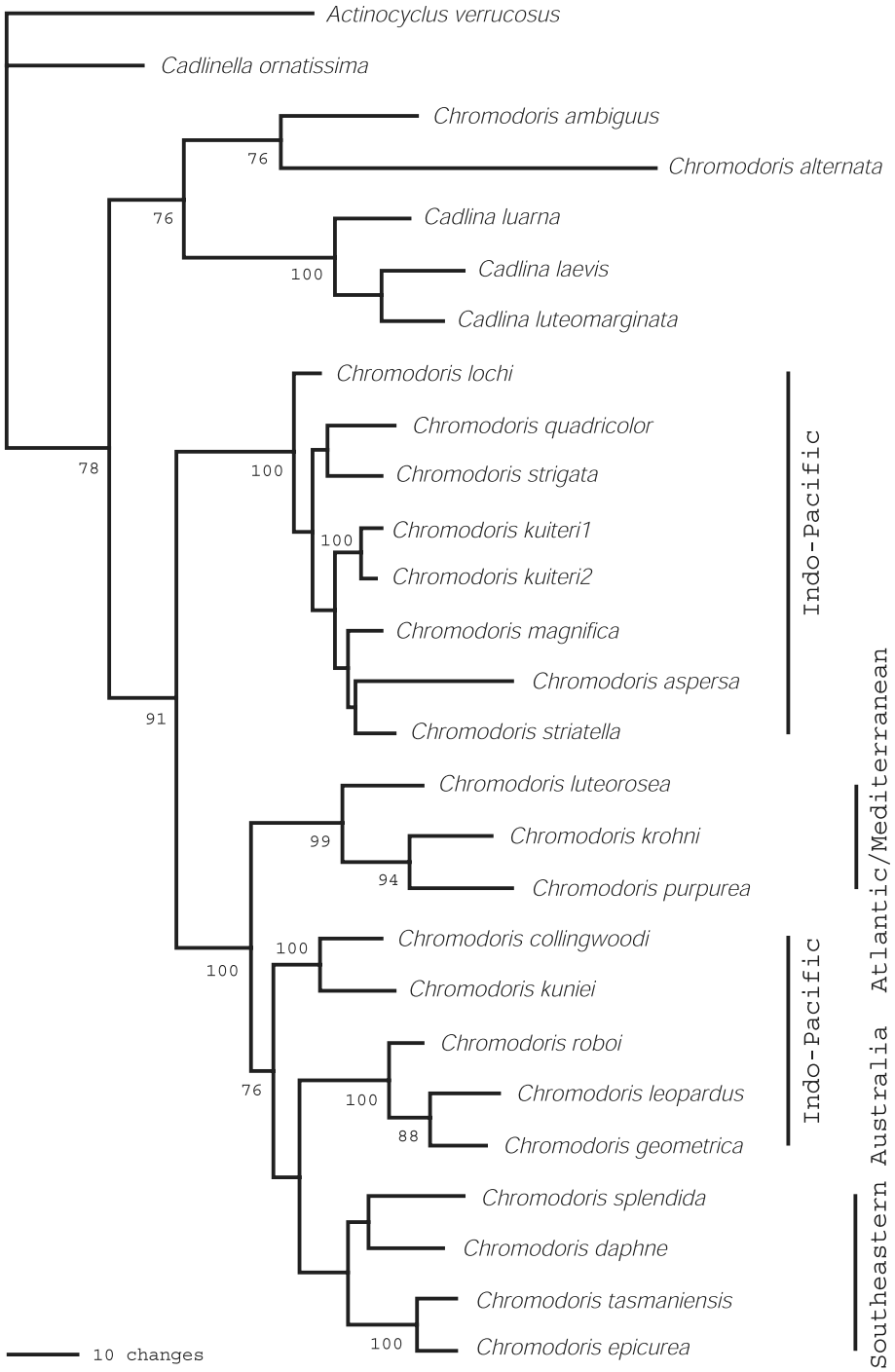

Fig. 2. Bayesian MCMC majority-rule consensus tree for *Chromodoris* species based on 16S rDNA and gap information, and the substitution model (HKY+I+G) most similar to the model selected using HLTs. Numbers represent the posterior probabilities.

3.2. Planar spawners

The planar spawning clade is strongly supported and species are predominately found in the tropical Indo-West Pacific. The best trees that lacked this clade were eight steps longer and these four trees were significantly worse than the MP tree according to both Templeton's nonparametric test and the K–H test ($p < 0.05$). The clade contains all included members of the *C. quadricolor* colour group, described by Rudman (1977, 1982). It also includes *C. aspersa* that has been linked with the *C. quadricolor* group previously based on the shared planar egg masses (Wilson, 2002). A report of *C. magnifica* laying an upright egg mass (Klussman-Kolb and Wägele, 2001) is erroneous, and further observation has shown that *C. magnifica* lays a planar egg mass like others in this clade (Wilson, pers. obs). Planar spawning is arguably a derived reproductive character diagnosing this clade, since *Actinocyclus*, *Cadlina*, and the other *Chromodoris* in this analysis (where known) lay erect egg masses, although *Cadlinella* lays planar egg masses (Boucher, 1983).

3.3. Erect spawners

This clade consists of the majority of species of *Chromodoris*, excluding the planar spawners and the close relatives of *Cadlina*. There were no previous existing hypotheses of phylogenetic relationships within this clade. In both analyses, the erect spawners are divided into four geographically coherent subclades: southeastern Australian endemics (*Chromodoris splendida*, *Chromodoris daphne*, *Chromodoris tasmaniensis*, and *Chromodoris epicurea*), Atlantic/Mediterranean taxa (*Chromodoris luteorosea*, *Chromodoris krohni*, and *Chromodoris purpurea*), and two clades containing Indo-Pacific forms (*Chromodoris collingwoodi* and *Chromodoris kuniei*) (*Chromodoris leopardus*, *C. roboi*, and *Chromodoris geometrica*). The latter two subclades, excluding *C. collingwoodi*, exhibit the unusual behavioural trait of rhythmically lifting their mantles. Relationships between these four clades are labile: the Australian clade is basal in the MP analyses, while the Atlantic/Mediterranean clade is basal in the Bayesian analysis. Indo-Pacific species occur in both the planar and upright spawning clades of *Chromodoris*. This contrasts directly with the phylogenetic structure of another chromodorid genus, *Hypselodoris*, where the two major clades correspond to the Indo-Pacific and the Atlantic/Eastern Pacific regions (Gosliner and Johnson, 1999).

3.4. Colouration, convergence, and common descent

The diversity and similarity of colour patterns in chromodorid nudibranchs have been attributed in part to warning colouration (Edmunds, 1991), mimicry (Rudman, 1991), or phylogenetic constraints (Gosliner and Behrens, 1998; Johnson and Gosliner, 1998). The current phylogeny provides some suggestions as to how these processes might be responsible for particular aspects of colouration. The production of pure black pigment exhibits strong phylogenetic conservativism: it occurs in all planar spawners with the exception of *C. aspersa*, and is absent from almost all erect spawners (present only in *C. geometrica*). Other aspects of colour patterns appear to correlate with presence of black pigment, with the planar species all showing longitudinal black stripes or swathes of black. *C. aspersa*, the only member of the planar clade that lacked black pigment, also lacked any longitudinal striped pattern. *C. geometrica*, the lone species from the erect spawning clade with black pigment, had a different (reticulated) pattern.

Background body colour also shows strong phylogenetic conservatism within the erect spawners. The Mediterranean clade all share pink bodies, while the southeastern Australian endemics have white bodies, regardless of pattern. A white background colour is also present in the *Cadlina* clade and may represent the plesiomorphic state.

Mullerian mimicry (the evolution of similar warning colouration in toxic species) has been invoked to explain nudibranch colour patterns common to geographical regions (Rudman, 1991). All of Rudman's examples used changes in spot size or distribution, mostly in *Chromodoris* in southeastern Australia. The phylogeny here reveals some evidence of mimicry (orange spots common to both *C. ambiguus* and *C. tasmaniensis*) but also that most homologous colour traits are restricted to particular geographical regions. Thus, similarities in both colour pattern and geographic distribution within *Chromodoris* might be explained by phylogeny, rather than by convergence. Indeed, when faced with similarly patterned taxa in the same locality, in the absence of a rigorous phylogeny the most reasonable null hypothesis would be that they are all descended from a common ancestor which exhibited that colour pattern and lived in that locality, rather than invoking dispersal of unrelated (and differently coloured) lineages into the locality and subsequent convergent evolution of colouration. Detailed data on geographic co-variation in colour patterns of putative models and mimics in a phylogenetic framework are required to provide solid proof of mimicry (Greene, 1997).

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