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Application of multigene phylogenetics and site-stripping to resolve intraordinal relationships in the Rhodymeniales (Rhodophyta)[1]

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ABSTRACT

Previous molecular assessments of the red algal order Rhodymeniales have confirmed its monophyly and distinguished the six currently recognized families (viz. Champiaceae, Faucheaceae, Fryeellaceae, Hymenocladiaceae, Lomentariaceae, and Rhodymeniaceae); however, relationships among most of these families have remained unresolved possibly as a result of substitution saturation at deeper phylogenetic nodes. The objective of the current study was to improve rhodymenialean systematics by increasing taxonomic representation and using a more robust multigene dataset of mitochondrial (COB, COI/COI-5P), nuclear (LSU, EF2) and plastid markers (*psbA*, *rbc*L). Additionally, we aimed to prevent phylogenetic inference problems associated with substitution saturation (particularly at the interfamilial nodes) by removing fast-evolving sites and analyzing a series of progressively more conservative alignments. The Rhodymeniales was resolved as two major lineages: (i) the Fryeellaceae as sister to the Faucheaceae and Lomentariaceae; and (ii) the Rhodymeniaceae allied to the Champiaceae and Hymenocladiaceae. Support at the interfamilial nodes was highest when 20% of variable sites were removed. Inclusion of *Binghamiopsis, Chamaebotrys* and *Minium*, which were absent in previous phylogenetic investigations, established their phylogenetic affinities while assessment of two genera consistently polyphyletic in phylogenetic analyses, *Erythrymenia* and *Lomentaria,* resulted in the proposition of the novel genera *Perbella* and *Fushitsunagia*. The taxonomic position of *Drouetia* was reinvestigated with re-examination of holotype material of *D. coalescens* to clarify tetrasporangial development in this genus. In addition, we added three novel Australian species to *Drouetia* as a result of ongoing DNA barcoding assessments—*D. aggregata* sp. nov., *D. scutellata* sp. nov., and *D. viridescens* sp. nov.

***Key Index words: Drouetia*; *Erythrymenia*; *Fushitsunagia*; *Lomentaria*; Multigene phylogenetics; *Perbella*; Rhodymeniales; SiteStripper.**

Abbreviations: small subunit ribosomal gene (SSU) PartitionFinder (PF)

INTRODUCTION

The Rhodymeniales is a well-studied, species-rich florideophycean order of extremely diverse frond morphologies united by a uniform procarpic female reproductive system (i.e. the auxiliary cell is produced prior to fertilization and in close proximity to the carpogonium within a single branch system) and outward carposporophyte development. Published phylogenetic analyses of florideophycean taxa regard the Rhodymeniales as monophyletic and closely related to the Halymeniales and Sebdeniales (e.g. Saunders & Hommersand 2004, Withall & Saunders 2006, Le Gall & Saunders 2007, Verbruggen et al. 2010). Historically, subordinal classification of the Rhodymeniales has been complicated by conflicting perspectives regarding the most useful anatomical features for taxonomic distinction. A brief summary of rhodymenialean systematics is provided below; the reader is referred to Saunders et al. (1999) and Le Gall et al. (2008) for a more comprehensive review.

When Schmitz (1889) established the Rhodymeniales, he included six families: Bonnemaisoniaceae, Ceramiaceae, Delesseriaceae, Rhodomelaceae, Rhodymeniaceae and Sphaerococcaceae. Soon after, all of those families were removed except for the Rhodymeniaceae (Oltmanns 1904, Sjöstedt 1926). Bliding (1928) subsequently added the Champiaceae, which was differentiated from the Rhodymeniaceae according to thallus construction (hollow thalli with longitudinal filaments bordering the cavity present vs. solid thalli or hollow thalli but lacking longitudinal filaments), tetrasporangial division patterns (tetrahedral vs. cruciate), number of cells in the carpogonial branch (four vs. three) and the degree to which cells differentiated into carposporangia (only the terminal cells vs. most of the gonimoblast). Kylin (1931) maintained Bliding's two families and further defined them by assigning subfamilies to each. Sparling (1957) rejected Kylin's subclassifications and emphasized the presence (Champiaceae) or absence (Rhodymeniaceae) of longitudinal filaments bordering the hollow parts of thalli for family-level distinction. Later, Lee (1978) used a combination of vegetative (thallus septation) and reproductive criteria (number of cells in the carpogonial branch and the position of the tetrasporangia) to define the Champiaceae, Rhodymeniaceae, and a third family, Lomentariaceae. Lee's three-family classification system was widely accepted; however, some rhodymenialean genera featured diagnostic characters of more than one family and, therefore, could not be classified

confidently (e.g., *Dictyothamnion, Ceratodictyon, Gelidiopsis, Hymenocladia, Hymenocladiopsis* and *Semnocarpa*, see Saunders et al. 1999).

The application of molecular techniques to systematic studies of the red algae has enabled a more objective means of assessing evolutionary relationships within the Rhodymeniales. Although rhodymenialean taxa were represented in some of the earliest red algal molecular phylogenetic investigations (e.g., Freshwater et al. 1994, Ragan et al. 1994, Saunders & Kraft 1994, Saunders & Kraft 1996), these studies only included enough taxa to establish the order as monophyletic and allied to the Halymeniales. Subordinal relationships remained largely unresolved until Saunders et al. (1999), using the nuclear small subunit ribosomal gene (SSU), embarked on the first extensive molecular systematic study specific to the Rhodymeniales. That study included 56% of the recognized genera (Saunders et al. 1999). Challenging Lee's three-family system, Saunders et al. (1999) resolved the Rhodymeniales as six monophyletic assemblages distributed in two major lineages – the first included only a reduced Rhodymeniaceae while the second consisted of the Champiaceae, Lomentariaceae, their newly established Faucheaceae, and two unassigned lineages. In light of their results, Saunders et al. (1999) emphasized reproductive features, including the number of cells in the carpogonial branch, tetrasporangial cleavage patterns and tetrasporangial position for family-level classification. Although that study was significant for providing a more natural system of classification for the Rhodymeniales, it was limited by its low representation of rhodymenialean taxa (both the absence of entire genera and many generitype species) and lack of resolution among interfamilial nodes (Saunders et al. 1999). Poor resolution was partially due to gene choice as SSU displayed highly variable rates in some families, which increased the potential for long-branch attraction artefacts (Saunders et al. 1999). In a subsequent study Le Gall et al. (2008) sought to circumvent potential long-branch attraction artefacts by increasing taxonomic representation (by ~13%) and basing phylogenetic inference on two nuclear genes: elongation factor 2 (EF2) and the large-subunit nuclear ribosomal DNA (LSU). Le Gall et al. (2008) again resolved six fully supported family-level lineages. Although their newly described Fryeellaceae was strongly allied to the sister families

Faucheaceae and Lomentariaceae, the relationships among the remaining families remained inconclusive (Le Gall et al. 2008).

The inability of the previous molecular phylogenetic analyses to generate meaningful interfamilial support was due at least in part to the general difficulty of obtaining strong phylogenetic signal for lineages that have diverged deeper in evolutionary time (Verbruggen et al. 2009, Pisani et al. 2012, Zeng et al. 2014). Although a common approach for improving phylogenetic inference is to use larger datasets (Holton & Pisani 2010), increasing the number of taxa and genes does not guarantee that signal at deeper epochs of interest will be enhanced and this approach may actually increase the potential for systematic errors (Jeffroy et al. 2006, Sperling et al. 2009, Pick et al. 2010). One of the major challenges for resolving deep evolutionary relationships is overcoming issues associated with substitution saturation at quickly evolving sites (Cocquyt et al. 2010). Typically deep phylogenetic nodes are recorded in the historical signal of the more slowly evolving positions in an alignment; however, the stochastic noise introduced by quickly evolving sites can overwhelm this signal, which can lead to incorrect or poorly supported deep phylogenetic nodes (Ho & Jermiin 2004, Kostka et al. 2008). Fast site removal (site-stripping) is one technique that has been applied to reduce issues associated with systematic errors and substitution saturation to facilitate resolution of deeper relationships (Ruiz-Trillo et al. 1999, Morgan-Richards et al. 2008, Cocquyt et al. 2010). By removing quickly evolving sites, the signal to noise ratio is increased so that the signal of the historically informative more slowly evolving sites is maximized (Brinkmann & Philippe 1999, Waddell et al. 1999, Delsuc et al. 2005, Rodríguez-Ezpeleta et al. 2007, Kostka et al. 2008). The command line program SiteStripper (Verbruggen 2012) was originally designed to better resolve deep nodes within the Ulvophyceae (Chlorophyta). SiteStripper systematically removes variable sites from an alignment, 5% each time, to create a series of progressively more conservative sub-alignments that can be analyzed by phylogenetic inference techniques (Cocquyt et al. 2010). One of those sub-alignments should have enough of the quickly evolving sites removed while retaining sufficient signal to elucidate deeper relationships (Cocquyt et al. 2010).

The goals of the present study were: 1) to reassess and improve rhodymenialean systematics by expanding taxonomic representation (in conjunction with the Red Algal Tree of Life project, http://dblab.rutgers.edu/redtol/home.php) and generating a more robust multigene dataset of mitochondrial (COB & COI/COI-5P), nuclear (EF2 & LSU) and plastid (*psbA* & *rbc*L) markers; 2) to minimize phylogenetic inference problems associated with substitution saturation and improve resolution at interfamilial nodes by assessing this dataset with a fast-site removal reconstruction technique (SiteStripper); 3) to revisit the classification system proposed by Le Gall et al. (2008) based on our generated topology; 4) to establish the phylogenetic affinities of some genera (*Binghamiopsis, Chamaebotrys,* and *Minium*) absent from previous large-scale phylogenetic studies; 5) to assess genera consistently polyphyletic in published molecular analyses (notably *Erythrymenia* and *Lomentaria*); 6) to reinvestigate the taxonomic position of the problematic genus *Drouetia*; and, 7) to characterize novel taxa included in our analyses.

MATERIALS AND METHODS

Phylogenetic Analyses. Specimens used in phylogenetic analyses (Table S1) were pressed on herbarium paper or dried in silica to serve as vouchers with subsamples preserved in silica for molecular analyses. Genomic DNA was extracted following Saunders & McDevit (2012) with amplification of the following markers following Saunders & Moore (2013): the mitochondrial cytochrome b gene (COB); the mitochondrial cytochrome *c* oxidase subunit 1 extended fragment or barcode region (COI and COI-5P, respectively); the nuclear elongation factor 2 gene (EF2); the nuclear large-subunit ribosomal RNA gene (LSU); the plastid photosystem II thylakoid membrane protein D1 (*psbA*); and the plastid ribulose-1, 5-biphosphate carboxylase large subunit gene (*rbc*L) (Table S1). Amplicons were sequenced by the Génome Québec Innovation Centre and raw data were edited in Sequencher™ 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned in Geneious R7 version 7.1.7 (http://www.geneious.com, Kearse et al. 2012) with additional sequences acquired from GenBank (Table S1 in the Supporting Information).

Six single-gene alignments were produced [COB (49% of ingroup taxa, 942 bp); COI/COI-5P (90% of ingroup taxa, 1232 bp); EF2 (72% of ingroup taxa, 1641 bp); LSU

(100% of ingroup taxa, 2588 of 2841bp included in analyses,); *psbA* (62% of ingroup taxa, 953 bp) and *rbc*L (89% of ingroup taxa, 1358 bp); (Table S1)] and analyzed independently using Bayesian inference and Maximum likelihood under the GTR+I+G model with partitioning by codon for protein-coding genes. Bayesian analyses were completed using MrBayes v.3.2.1 (Ronquist & Huelsenbeck 2003) in Geneious R7 version 7.1.7 with parameter settings (Tratio, Revmat, Statefreq, Pinvar, Shape, Switchrates) unlinked, rate differentiation across the partitions enabled and branch lengths unconstrained with the branch length priors set to exponential (default exponential prior setting: 10.0). Analyses were run in parallel for 1,000,000 generations with sampling performed every 100 generations. The burn-in was determined when both analyses converged into the stationary phase. Maximum likelihood analyses were performed using RAxML in Geneious R7 version 7.1.7 with a non-parametric bootstrap of 1,000 replicates.

All genes resolved congruent phylogenetic relationships and were combined to generate a multigene alignment (8,714 bp, 78% complete by site) for further phylogenetic analyses. Percent completeness for each taxon included in this study was recorded in Table S1. The multigene alignment was analyzed by Bayesian inference and Maximum likelihood under the GTR+I+G model with parameters settings as specified above with the exception of running Bayesian analyses for 3,500,000 generations with sampling performed every 3,500 generations. The impact of partitioning was evaluated by completing analyses first by partitioning data by gene and codon (noPF) and then according to the evolutionary models and partitioning schemes determined by implementing PartitionFinder (PF) (Lanfear et al. 2012) under the BIC model selection criteria with linked branch length estimation. To assess the impact of missing data, Bayesian and Maximum likelihood analyses were repeated after first removing taxa less than 50% (n= 8) complete by site and then removing taxa less than 70% (n= 25) complete by site.

Site-Stripping. To assess the impact of substitution saturation on phylogenetic inference, site-specific rates were calculated for the multigene alignment using the program HyPhy (Pond et al. 2005) under the JC69 model with a Bayes phylogram as a guide tree. SiteStripper (Verbruggen 2012) was used to order sites by rate and then remove quickly

evolving sites, 5% at a time, to generate a series of progressively more conservative sub-alignments that were analyzed using RAxML version 7.3.5 (Stamatakis 2012) with a command line script available through SiteStripper. Analyses were partitioned by gene and then codon (noPF) under a GTR+I+G model and with the partitioning schemes and models of evolution as determined by PartitionFinder (PF). Branch support was estimated by 1,000 non-parametric bootstrap replicates.

To determine if site-stripping biases downstream analyses towards the phylogeny used as a guide tree, we recalculated site rates using an alternative starting tree topology (Supplementary Fig. S1) that was generated using a neighbor-joining analysis under the HKY genetic distance model in Geneious R7. The resulting site rates were used by SiteStripper to generate a series of subalignments, which were analyzed by RAxML. To assess the neighbor-joining tree against the original starting tree (Bayesian Inference tree with partitioning by gene and codon), the Shimodaira-Hasegawa test ("-f h" option) was implemented using RAxML.

Barcode analyses

A total of 16 collections field identified to the genus *Drouetia* were collected from the subtidal in Coffs Harbour, New South Wales, Australia (Table 1) and dried in silica gel with voucher preservation, DNA extraction, and COI-5P amplification, sequencing, editing and aligning as outlined above. The DNA barcode alignment (664 bp) was analyzed in BOLD to determine intraspecific variation and nearest neighbour distances.

Morphological analyses. Vegetative and reproductive features were analyzed by rehydrating algal tissue in 5% formalin and seawater and preparing sections using a freezing microtome (Leica CM1850, Leica Microsystems, Wetzlar, Germany). Sections were stained with 1% analine blue solution in 6% 5N hydrochloric acid, rinsed with distilled water and permanently mounted in 50% corn syrup with 4% formalin. Photographs were taken using a Leica digital camera (DFC480) mounted to a Leica microscope (CTR5000) and plates were made using Adobe Photoshop Elements 11 (2012).

RESULTS

To resolve subordinal relationships among the Rhodymeniales, a six gene concatenated alignment (80 species representing 42 genera) was assessed using Bayesian analysis and Maximum likelihood (ML). Analyses were completed using the model GTR+I+G with partitioning by gene and codon. Then, analyses were performed again according to the model (GTR+I+G) and partitioning scheme (LSU) (COB1) (COB2, *psbA*1) (COB3, COI3) (COI1, *rbc*L1) (COI2, *psbA*2, *rbc*L2) (EF21) (EF22) (EF23) (*psbA*3) (*rbc*L3) as determined by PartitionFinder. The phylogram inferred by ML analysis with partitioning by gene and then codon is presented (Fig. 1) with bootstrap support values shown for subfamilial branches. Familial and interfamilial branch support are presented separately (Table 2). The topology in Fig. 1 was resolved for all analyses of the full alignment except Bayesian inference with partitioning by gene and then codon, which failed to resolve branch C (lacked support in all full alignment analyses) (Table 2). Posterior probabilities and bootstrap support values were essentially not changed whether the alignment was fully partitioned or used the scheme selected by PartitionFinder. For both Bayesian and ML analyses, tree scores were slightly better when data were partitioned by gene and codon rather than the partitioning schemes as defined by PartitionFinder (Table 2). Exclusion of incomplete taxa (both those < 50% and < 70% complete by site, Table S1) did not change the tree topology (Fig. 1) and had little impact on posterior probabilities and bootstrap support values (data not shown). Maximum likelihood analyses of the progressively more conservative sub-alignments (partitioned by gene and codon only) produced the same tree topology (Fig. 1) as analyses of the full dataset; however, interfamilial branch support increased at three key branches (Table 2, branches B, C & I) with optimal signal gained when 20% of the quickly evolving sites were eliminated (i.e. 80% of the sites were retained). The results of the Shimodaira-Hasegawa test indicated that the neighbor-joining guide tree topology (likelihood -144156.83, Fig. S1) was significantly different ($p<0.01$) than the Bayes guide tree topology (likelihood -143596.69). Despite the previous, use of the neighbor-joining topology as the guide tree for calculating sites rates did not impact downstream site-stripping analyses as support for interfamilial and familial branches was consistent with SiteStripping analyses that used a Bayes guide tree.

All analyses (i.e. full alignment, site-stripped alignments, those for which incomplete taxa were removed) solidly resolved the Rhodymeniales (branch A, Fig. 1, Table 2) as comprised of six monophyletic families distributed in two major lineages. The first lineage (branch B, Fig. 1) was strongly resolved in all analyses and included the Faucheaceae (branch G, Fig. 1), Fryeellaceae (branch D, Fig. 1) and Lomentariaceae (branch F, Fig. 1). The second lineage (branch C, Fig. 1) included the Champiaceae (branch J, Fig. 1), Hymenocladiaceae (branch K, Fig. 1) and Rhodymeniaceae (branch H, Fig. 1) and was variably supported in analyses of the full alignment; however, support at branch C increased (up to 75%, Table 2) as quickly evolving sites were removed. Within the first lineage the sister relationship of the Faucheaceae to the Lomentariaceae (interfamilial branch E; Fig. 1) was fully supported across all analyses (Table 2). Within the second lineage, the Champiaceae was moderately resolved (Table 2) as sister to the Hymenocladiaceae (interfamilial branch I, Fig. 1). Support at branch I (Fig. 1; Table 2) initially decreased (down to 71%, Table 2) when 5% of the quickly evolving sites were removed (i.e. 95% of the sites were retained) and continued to fluctuate up and down with the generation of each sub-alignment; however, support was highest (81%, Table 2) when 20% of the sites were eliminated.

Analyses resolved phylogenetic relationships for genera included in a molecular context for the first time. The monospecific genus *Binghamiopsis* was positioned within the Lomentariaceae and strongly associated with *Lomentaria hakodatensis* (Fig. 1). Within the Rhodymeniaceae, *Chamaebotrys* (represented by the generitype *C. boergesenii* and an unidentified species from Australia) was solidly allied to the sister genera *Halichrysis* and *Halopeltis* (Fig. 1), while collections assigned to the genus *Drouetia* were resolved as a fully supported monophyletic lineage distantly related to a lineage containing species of *Chrysymenia* and *Maripelta* (Fig. 1). The monospecific genus *Minium* was fully resolved within the Fryeellaceae as an unexpectedly close sister to *Fryeella gardneri* (Fig. 1).

Inclusion of select generitype species enabled assessment of monophyly for a few genera. Within the Hymenocladiaceae, species of *Erythrymenia* failed to form a monophyletic lineage with *Erythrymenia minuta* sister to *Hymenocladia* spp. rather than the type of the genus, *Erythrymenia obovata* (Fig. 1). The genus *Perbella* was established

(below) to accommodate *Erythrymenia minuta* and render *Erythrymenia* monophyletic. Within the Lomentariaceae, species of *Lomentaria* were resolved as four independent lineages: (i) the generitype *L. articulata* joined *L. clavellosa* and *L. orcadensis* to form a fully supported monophyletic lineage; (ii) *L. catenata* and *Ceratodictyon* spp. were allied with full support; (iii) *L. divaricata* resolved as sister to *Stirnia*; and (iv) *L. hakodatensis* was fully resolved as sister to *Binghamiopsis* (Fig. 1). The genus *Fushitsunagia* was established to accommodate *L. catenata* while the generic assignments of *L. divaricata* and *L. hakodatensis* could not formally be determined at this time (discussed below).

Analyses resolved some additional genera as polyphyletic (Fig 1). Rather than resolving with other species of *Botryocladia*, the species *B. leptopoda* was more closely allied with *Chrysymenia wrightii* (which failed to resolve with other included *Chrysymenia* spp.; Fig. 1). The species *R. delicatula* was resolved as relatively distinct from other species of *Rhodymenia* (including the generitype) and was most closely related to *Cordylecladia erecta* (Fig. 1).

Sixteen collections from Coffs Harbour, New South Wales, Australia were resolved as three genetic species groups and assigned to the genus *Drouetia* as novel taxa (see below). Maximum COI-5P intraspecific divergence for these species was 0.31% (Table 3). The nearest neighbour to *D. aggregata* was *D. scutellata* (7.19% divergent, Table 3) while the closest species to *D. scutellata* was *D. viridescens* (2.5% divergent, Table 3). The species *D. scutellata* and *D. viridescens* were included in multigene analyses where they were resolved in the Rhodymeniaceae and distantly related to an unidentified species of *Drouetia* from South Africa (Fig. 1).

Taxonomic Treatment

Drouetia aggregata Filloramo & G.W. Saunders, **sp. nov.** (Fig. 2)

Description. Blades irregular in outline, peltate, typically anastomosing where they overlap (Fig. 2a), stipe 12-15 mm long x 2 mm wide (Fig. 2b); blades ca. 950 µm in transverse section at mid-thallus with 9-13 medullary layers of large axially elongated, non-pigmented cells (250-400 µm long x 80-100 µm wide) with frequent secondary pit connections (Fig. 2c) and distinct aggregations of smaller, round cells (50 µm in diameter; Fig. 2d);

medullary cells typically reducing in size toward the ~2 inner cortical layers of smaller cells subtending a dorsal cortex of 2-3 layers of densely packed, columnar-shaped, darkly staining cells (~10 µm in diameter) and a single-layered ventral cortex of larger round cells (15-18 µm in diameter; Fig. 2c); dorsal and ventral cuticle, 10 µm and 7.5 µm thick, respectively (Fig. 2c). Reproductive structures not observed.

Holotype. GWS032793 collected on December 12, 2012 at Mutton Bird Island (South), Coffs Harbour, New South Wales, Australia (-30.30467, 153.14796), by G.W. Saunders and K. Dixon and deposited in the Connell Memorial Herbarium (UNB) of the University of New Brunswick, Fredericton, Canada. Images of holotype Figures 2, a-c.

Holotype COI-5P Barcode. KU707873.

Isotype. GWS032792 (deposited in UNB, Table 1).

Etymology. Named for the aggregates of small cells interspersed throughout the larger cells of the medulla – this feature being pronounced when compared to other species of *Drouetia*.

Geographical Distribution. Thus far only known from the type location.

Comments. *Drouetia aggregata* is distinguished from the other species of *Drouetia* in Australia owing to its long stipe, thick thallus in cross section, larger medullary cells, and the aggregation of small round cells interspersed among the other medullary cells.

Drouetia scutellata Filloramo & G.W. Saunders, **sp. nov.** (Fig. 3)

Description. Peltate blades depressed at center and borne on short, narrow stipes 6-10 mm long x 0.5 mm wide (Fig. 3a); blades typically remaining saucer-shaped upon maturity, at times anastomosing at the margins (Fig. 3b); blades ca. 250-375 µm thick in transverse section at mid-thallus with (3-) 4-5 medullary layers of large elongated cells (90-145 µm long x 72-87.5 µm wide) grading to 1-2 inner cortical layers of elongated cells subtending 2 dorsal cortical layers of darkly staining cells (12.5 µm in diameter) and 2 ventral layers of slightly larger rounded cells (17.5 µm in diameter; Fig. 3c8). Plants dioecious. Cystocarps clustered centrally on dorsal (upper) blade surface (Fig. 3b), rounded, ostiolate, 800-1000 µm in diameter, nutritive tissue present at base (Fig. 3d). Spermatangial sori formed on the dorsal surface with spermatangia cut off from elongated spermatangial mother cells (Fig. 3e). Tetrasporophytes not observed.

Holotype. Female gametophyte GWS032729 collected on December 11, 2012 at Mutton Bird Island (North), Coffs Harbour, New South Wales, Australia (-30.304706, 153.150959), by G.W. Saunders and K. Dixon and deposited in the Connell Memorial Herbarium (UNB) at the University of New Brunswick, Fredericton, Canada. Images of holotype Figure 3, b and d.

Holotype COI-5P Barcode. KU707880

Isotype. Male gametophyte GWS032752 (deposited in UNB; Table 1).

Etymology. Named for the plant's typically saucer-shaped blades.

Representative specimen examined. GWS032664 (deposited in UNB, see Table 1 for collection details).

Geographical Distribution. Thus far known only from the type location and nearby South Solitary I., Coffs Harbour, New South Wales, Australia.

Comments. *Drouetia scutellata* features a thin thallus in cross section with few medullary cell layers rendering it rather distinct when compared to other species of *Drouetia* from Australia. Differences in internal anatomy are especially useful for distinguishing between *D. scutellata* and *D. viridescens*, which also has a single peltate blade with a depressed center and short stipe. Additionally, while some reproductive plants of *D. scutellata* have been encountered as single blades, reproductive plants of *D. viridescens* have only been observed as anastomosed blades (below).

Drouetia viridescens Filloramo & G.W. Saunders, **sp. nov.** (Fig. 4)

Description. Juvenile blades peltate with shallow central depression (Fig. 4a), becoming stellate or lobed with age and typically anastomosing at margins and where blades overlap (Fig. 4b), borne on a short stipes 3-6 mm long x 1 mm wide. Transverse sections at mid-thallus 310-500 µm thick with 9-12 medullary layers of large, axially elongated cells (90-140 µm long x 50-80 (-120) µm wide) decreasing in size to 2 dorsal cortical layers of small, densely packed, darkly staining cells (10-15 µm in diameter) and 1-2 ventral cortical layers of round darkly staining cells (17.5-20 µm in diameter); outgrowths of the ventral cortical cells presumably involved in secondary attachments to substratum and other blades (Fig. 4, c and d); dorsal (~7.5 µm thick) and ventral cuticles (5 um thick) obvious (Fig. 4c).

Tetrasporangial sori confined to the dorsal surface (Fig. 4d), tetrasporangia cruciately divided (30-40 µm long x 15-20 µm wide; Fig. 4e), differentiating from existing cortical cells (Fig. 4, f-g); all outer cortical cells appear capable of differentiating into sporangia but do not convert simultaneously causing undifferentiated cortical cells to become elongated and compressed between developing tetrasporangia giving the appearance of paraphyses (Fig. 4, f-g). Cystocarps and spermatangia not observed.

Holotype. Tetrasporophyte GWS032612 collected on December 9, 2012 in Split Solitary Island (Northwest) Coffs Harbour, New South Wales, Australia (-30.24210, 153.17921), by G.W. Saunders and K. Dixon and deposited in the Connell Memorial Herbarium (UNB) at the University of New Brunswick, Fredericton, Canada. Images of holotype Figure 4, b, d-g .

Holotype COI-5P Barcode. KU707855

Isotype. GWS032624 (Table 1).

Etymology. The species epithet acknowledges the plant's green iridescent glow underwater observed at the time of collection.

Representative specimens examined. GWS032599, GWS032600, GWS032642, GWS032643, GWS032665, GWS032672, GWS032682, GWS032740, GWS032790 (deposited in UNB, see Table 1 for collection details).

Geographical Distribution. Thus far known only from the type locality and nearby sites in Coffs Harbour, New South Wales, Australia.

Comments. This species has the shortest stipe and the center of the blade typically "glows" green when encountered underwater. Although *D. viridescens* has a similar number of medullary cell layers as *D. aggregata*, the former is much thinner in cross section with smaller medullary cells. The upper range of thallus thickness for *D. scutellata* overlaps with the lower range for *D. viridescens*; however, the fewer number of medullary cell layers for *D. scutellata* distinguishes it from the latter.

Generitype observations of Drouetia coalescens (Farlow) G. De Toni (Fig. 5)

Holotype material of *D. coalescens* was provided on loan from the Farlow Herbarium (FH) (Fig. 5a). Blades were ca. 500 µm thick with 8-11 medullary cell layers of typically axially

elongated cells of varying sizes (Fig. 5b). The dorsal blade surface was ca. 3 cortical layers thick while the ventral surface was composed of up to 6 layers of small, round, tightly packed cells subtending a thick cuticle (Fig. 5b). Tetrasporangia were observed as deeply embedded in only the dorsal cortex (Fig. 5, b and c), cruciately divided (52-55 µm long x 15-18 µm wide; Fig. 5d) with the tetrasporangial initials arising from the cells of the inner cortex (Fig. 5e). As the tetrasporangia matured, they lengthened and swelled causing neighboring cortical cells to become compressed and elongated between the developing tetrasporangia, taking on the appearance of paraphyses (Figs. 5c-e).

Comments. The generitype species shares a similar gross morphology with the novel species *D. aggregata* and *D. viridescens* in that all three species feature decumbent, peltate blades, which commonly anastomose at the margins. Compared to *D. aggregata*, the generitype is thinner in cross section and lacks aggregations of small cells within the medulla. The generitype species features longer tetrasporangia compared to those of *D. viridescens* and has never been reported to "glow" green underwater as is diagnostic of *D. viridescens*. Although the habitat of the type specimen of *D. coalescens* is unknown, Taylor (1945) collected material from the low-intertidal that was observed by Saunders et al. (2006) who recognized it as morphologically consistent with *D. coalescens*. According to the previous, *D. coalescens* is distinct in its intertidal habitat relative to the three Australian *Drouetia* species, which have only been collected subtidally.

Fushitsunagia Filloramo & G.W. Saunders, **gen. nov.**

Diagnosis: Lomentariaceaen algae with hollow thalli divided by multi-rowed cellular septa. Spermatangial sori are borne on specialized fertile ramuli. The cells of the medulla become elongated and stretched appearing almost filamentous as they form a conspicuous stellate network surrounding the developing tetrasporangia, which, when mature, are tetrahedrally divided.

Etymology: In recognition of the Japanese name "*Fushitsunagi*" ("fushi"= joint, knot, and node; "tsunagi"= connection) that was assigned to *Lomentaria catenata* by Okamura (Lee 1978).

Type and only species: ***Fushitsunagia catenata*** (Harvey) Filloramo & G.W. Saunders, **comb. nov**.

Basionym: *Lomentaria catenata* Harvey 1857 (Algae. *In*: Account of the Botanical specimens. Gray, A., [Eds.] *Narrative of the expedition of an American squadron to the China Seas and Japan, performed in the years 1852, 1853 and 1854, under the command of Commodore M.C. Perry, United States Navy. Volume II – with illustrations.* Anon. [Eds.]. Senate of the Thirty-third Congress, Second Session, Executive Document. House of Representatives, Washington, USA, pp. 331-332).

Perbella Filloramo & G.W. Saunders, **gen. nov.**

Diagnosis: Hymenocladiaceaen algae with smaller cells intermixed among the larger medullary cells from which they likely develop. Tetrasporangia are decussately divided.

Etymology: Latin for "very beautiful", attributed to the breathtaking appearance of the type species when observed underwater or immediately after collection.

Type and only species: ***Perbella minuta*** (Kylin) Filloramo & G.W. Saunders, **comb. nov**.

Basionym: *Erythrymenia minuta* Kylin 1931 [Die Florideenordung Rhodymeniales. *Acta Universitatis Lundensis* 27(11):1-48].

DISCUSSION

Interfamilial relationships

The present study represents the most comprehensive molecular phylogenetic analysis of the Rhodymeniales based on the number of taxa included and markers used. Further, we have applied a technique to remove the most rapidly evolving sites from our multigene alignment to enhance resolution at the interfamilial nodes (Fig. 1). Results were largely congruent with previously published phylogenetic assessments of the order (Saunders et al. 1999, Le Gall et al. 2008) in that all of our analyses resolved the Rhodymeniales as comprised of six monophyletic families (Fig. 1); however, those previous studies consistently resolved only an alliance between the Faucheaceae and Lomentariaceae (Saunders et al. 1999, Le Gall et al. 2008) while the current study resolved most interfamilial relationships with solid support (Fig. 1, Table 2). The Faucheaceae and

Lomentariaceae were resolved as sister groups and allied to the Fryeellaceae while the Champiaceae and Hymenocladiaceae were resolved together and associated with the Rhodymeniaceae (Fig. 1). Site-Stripping had the greatest impact on the short internal branch (branch C, Fig. 1) leading to the Champiaceae, Hymenocladiaceae and Rhodymeniaceae. It is possible that this branch represents a lineage that radiated over a short period of evolutionary time. Generally, the limited signal associated with short branches is masked by more recent substitution events, which impacts accurate resolution of evolutionary relationships occurring over those short time intervals (Pisani et al. 2012). Removing the quickly evolving sites (i.e. those prone to substitution saturation) from our original alignment reduced the stochastic noise and uncovered the historical signal. We were able to provide meaningful resolution for previously unresolved relationships within the Rhodymeniales. The interfamilial relationships resolved herein were consistent with the classification system proposed by Le Gall et al. (2008) emphasizing ontogeny of the tetrasporangia.

Taxa included in molecular analyses for the first time

Molecular data solidly resolved the monospecific genus *Binghamiopsis* within the Lomentariaceae (Fig. 1), which was expected given that it has the diagnostic characteristics of this family (tetrahedrally divided tetrasporangia, three-celled carpogonial branches and characteristic lomentariaceous fusion cell in carposporophyte development, Lee et al. 1988, Saunders et al. 1999, Le Gall et al. 2008). The only species, *B. caespitosa*, was closely allied to *Lomentaria hakodatensis* (Fig. 1). While *Binghamiopsis* features thalli with a multilayered cortex and loosely interwoven medullary filaments in a central cavity (Lee *et. al.* 1988), *L. hakodatensis* is characterized by thalli with cortical and medullary layers surrounding a central cavity that is articulated at various intervals by multi-rowed cellular septa (Lee 1978). As *L. hakodatensis* was polyphyletic relative to *Lomentaria* sensu stricto, its proper generic-level assignment awaits future assessments of *Lomentaria* (see below); however, such substantial differences in thallus construction between *B. caespitosa* and *L. hakodatensis* warrant their recognition as belonging to distinct genera.

The generitype of *Chamaebotrys, C. boergesenii,* was resolved within the Rhodymeniaceae (Fig. 1) where it was placed by Huisman (1996) when he established this genus to accommodate some taxa originally assigned to another rhodymeniaceaen genus, *Coelarthrum.* Based on the species included here, his decision to recognize *Chamaebotrys* was fully supported (Fig. 1). Unfortunately, our analyses did not include the generitype of *Coelarthrum, C. cliftonii,* and therefore, the distinction of *Coelarthrum* and *Chamaebotrys* remains untested by molecular analyses.

At the time that Moe (1979) described the only crustose rhodymenialean taxon, *Minium parvum,* only two families (Champiaceae and Rhodymeniaceae) were recognized in the order. The criteria delimiting these families was greatly contested and poorly understood and Moe (1979) struggled to assign the monospecific genus *Minium* to either group especially after observing a variety of characteristics (tetrasporangial nemathecia, auxiliary cell branches with their associated sterile cells and the arrangement of the cells in the three-celled carpogonial branch) that did not ally *Minium* to either family. Moe (1979) determined that the genus was best placed in the Rhodymeniaceae on account of a solid thallus construction, cruciately divided tetrasporangia and the overall appearance and development of the carposporophyte. Our molecular results solidly resolved *Minium* within the Fryeellaceae as an unexpected close sister to *Fryeella gardneri* (Fig. 1). Analysis of tetrasporangial collections will confirm if one of the diagnostic features of the Fryeellaceae, the adventitious growth of the cortex in the formation of tetrasporangia and short, 3-4 celled paraphyses, is also characteristic of *Minium.* Those observations will be included in a future manuscript specific to rhodymenialean diversity in Canada.

Assessment of select polyphyletic genera

The present study was consistent with a previous molecular study that failed to resolve the type of the genus *Erythrymenia*, *E. obovata* from South Africa, and *E. minuta* from Australia as monophyletic (Saunders et al. 2006). To accommodate *E. minuta* we established the novel monospecific genus *Perbella*, which was solidly resolved as sister to *Hymenocladia* (Fig. 1). The latter two genera are distinguished primarily by tetrasporangial division patterns, which are tetrahedral in *Hymenocladia* and cruciate in *Perbella*,

534 respectively. Additionally, *Perbella* is characterized by obovate blades while
535 *Hymenocladia* features pinnate blades (e.g. *H. usnea*) or complanate, broad, palmate,
536 subdichotomously divided blades (e.g. *H. chondricola*, Womersley 1996). Nonetheless, we
537 uncovered inconsistencies and taxonomic confusion in the literature related to the
538 identification of *E. minuta* (= *P. minuta*) relative to species of *Hymenocladia*. Specimens of
539 *H. conspersa* collected by Harvey from Garden Island, Western Australia (Trinity College
540 Dublin Herbarium, TCD) included foliose, irregularly divided plants with abundant laminar
541 and marginal tooth-like proliferations [*Harvey 132*, TCD, annotated "type" by Womersley,
542 lectotypified by Lewis (1994)] and separately mounted smaller obovate plants (*Harvey*
543 *300-A*, TCD). Harvey (1862) considered the former to be mature *H. conspersa* while the
544 latter were juveniles. Additional juvenile specimens were collected from Port Fairy,
545 Victoria (e.g. *Harvey 300-D*, TCD). Lewis (1994) and Womersley (1996) concluded that
546 Harvey's "juveniles" were actually *E. minuta* (= *P. minuta*). Further, they speculated that
547 the juvenile *H. conspersa* specimens (that they attributed to *E. minuta*), reportedly from
548 Western Australia, were likely collected from Port Fairy, Victoria (Lewis 1994). Owing to
549 the previous, Lewis (1994) and Womersley (1996) interpreted *H. conspersa* as limited to
550 Western Australia with *E. minuta* (= *P. minuta*) restricted to Victoria.
551 As part of an ongoing barcode (COI-5P) survey of southern Australia (South
552 Australia, Victoria and Tasmania), three genetic species groups were resolved for
553 *Hymenocladia* including *H. chondricola*, *H. conspersa* and *H. usnea*. The first two were
554 closely related species groups (1.24% maximum interspecific variation in COI-5P) and are
555 in need of further molecular study. Our specimens identified as *H. chondricola* sensu stricto
556 (Fig. 6) were restricted to South Australia while collections attributed to *H. conspersa* were
557 more widely distributed from South Australia, Tasmania and Victoria and included mature
558 lanceolate morphs (Fig. 6b), intermediate morphs (Fig. 6c) and small, obovate juvenile
559 morphs (Fig. 6d) similar to those collected by Harvey and superficially reminiscent of *P.*
560 *minuta* specimens (Fig. 6e). As the type of *H. conspersa* is from Western Australia and our
561 isolates from southern Australia lacked the laminar papillae considered by Harvey (1862) to
562 be characteristic of this species (Fig. 6b), our assignment of *H. conspersa* remains tentative
563 pending inclusion of topotype material. If our collections do represent *H. conspersa*, we

provide genetic evidence that this species is indeed found in southern Australia and that small plants resembling *P. minuta* should be considered with caution as they are potentially juveniles of *H. conspersa*.

Previous and current phylogenetic assessments have consistently resolved the speciose genus *Lomentaria* as polyphyletic (e.g., Le Gall et al. 2008). Our molecular analyses resolved species assigned to *Lomentaria* as four independent lineages in the Lomentariaceae (Fig. 1). The type of *Lomentaria*, *L. articulata*, was joined by *L. clavellosa* and *L. orcadensis* (Fig. 1). As *L. articulata* features branches with multilayered septa and *L. clavellosa* and *L. orcadensis*, have multilayered plugs only at the branch base, results herein do not support previously recommended revisions of this genus based on thallus construction and septation (Irvine & Guiry 1983). Thus, *Lomentaria* sensu stricto remains a broadly defined genus characterized by various modes of thallus construction, tetrahedrally divided tetrasporangia in invaginated sori and conical cystocarps with prominent pores (Lee 1978).

The novel genus *Fushitsunagia* was established to accommodate *L. catenata*, which was resolved as sister to *Ceratodictyon* (Fig. 1). These genera are distinguished by tetrasporangial division patterns, which are tetrahedral in *F. catenata* (Lee 1978) and cruciate in *Ceratodictyon* (Price & Kraft 1991). The genus *Fushitsunagia* is unique from other lomentariaceaen taxa in that during tetrasporangial development, medullary cells become modified and form a conspicuous filamentous network that surrounds the developing tetrasporangia (Lee 1978).

The remaining *Lomentaria* species included in our multigene analyses, *L. divaricata* and *L. hakodatensis*, were resolved as sister to *Stirnia* and *Binghamiopsis*, respectively (Fig. 1). *Lomentaria divaricata* is distinguished from *Stirnia* according to thallus structure (hollow in the former and solid in the later) and the respective production of spermatangia and tetrasporangia on specialized fertile proliferations described only for the latter genus (Wynne 2001). *Lomentaria hakodatensis* is separated from *Binghamiopsis* based on overall thallus construction (see above). There are two available names to accommodate *L. divaricata* and *L. hakodatensis*: *Hooperia* J. Agardh (1896), which would apply directly to its generitype taxon *L. divaricata* and, *Chondrosiphon* Kützing (1843) for which the

generitype species, *L. firma*, was not available for inclusion in our molecular analyses. As *L. firma* shares numerous vegetative and reproductive characteristics with *L. hakodatensis* (flattened thalli, anastomosed intricate branches and well-developed cortication formed of a single layer of pigmented cells and 3-4 inner layers of subcortical cells, Curiel et al. 2006) it seems likely that these taxa will resolve together in the resurrected genus *Chondrosiphon*; however, it is possible that *L. firma* will resolve with *L. divaricata*, which would render *Hooperia* a synonym of *Chondrosiphon* necessitating a new genus for *L. hakodatensis.* Until *L. firma* is molecularly assessed, *L. divaricata* and *L. hakodatensis* should remain *incertae sedis* in the Lomentariaceae. While we have established *Lomentaria* sensu stricto as a monophyletic lineage and clarified some of its taxonomic conflicts, there remains significant molecular and morphological work until this genus and the many species attributed to it are properly assigned.

Recent molecular assessment of Australian species assigned to *Gloiosaccion* [*G. brownii* Harvey (including *G. brownii* var. *firmum* and *G. brownii* var. *coriaceum*) and *G. pumilum* J. Agardh], in relation to species of *Chrysymenia* [including the generitype species *C. ventricosa* (J.V. Lamouroux) J. Agardh] warranted transfer of the former genus to the latter (Schmidt et al. 2016). In that study, *"G." brownii* var. *firmum* (collected from South Australia) was supported as distinct from *G." brownii* var. *coriaceum* (collected from Western Australia) and the latter variety was raised to specific rank (Schmidt et al. 2016). Our routine barcoding studies in Australia have resolved collections (n= 31) field assigned to *"G". brownii* in five genetically distinct morphologically cryptic species groups (data not shown). Two of those groups (one being our concept of *"G." brownii*, which included specimens collected from near the type locality, Tasmania) were included in the current study (Fig. 1, Table S1). The *rbc*L data from our two *"G." brownii* genetic groups did not match the GenBank *rbc*L data representative of *"G." brownii* or *"G." coriacea* from the Schmidt et al. (2016) study and further study of *"G." brownii* from Australia is needed.

Consistent with Le Gall et al. (2008), our analyses resolved the genus *Chrysymenia* as polyphyletic with one of the species, *C. wrightii* most closely related to *Botryocladia leptopoda* (rendering this genus polyphylectic as well). Assessment of that conundrum was beyond the scope of this study. Future molecular assessments including the generitype taxa

for *Botryocladia*, *Chrysymenia* and *Cryptarachne* (previously recognized as a subgenus or a section of *Chrysymenia*, Guiry & Guiry 2015) are needed to clarify the species appropriately assigned to those genera.

Former morphological and molecular assessments of rhodymenialean taxa also identified the close alliance between *Cordylecladia erecta* and species of *Rhodymenia*, which are distinguished primarily according to habit (Brodie & Guiry 1988, Millar et al. 1996, Saunders et al. 1999). The addition of *R. delicatula* here goes further by resolving as sister to *C. erecta* and calling into question monophyly of *Rhodymenia* (Fig. 1). While *C. erecta* features fully terete axes, species of *Rhodymenia* are typically compressed or foliose; however, the recent description of the novel Australian species *R. compressa*, which features compressed and sparsely branched thalli (Filloramo & Saunders *in press*), challenges the utility of thallus habit for distinguishing *C. erecta* from *Rhodymenia* spp. and more study is needed.

Taxonomic reassessment of Drouetia

Prior to this study inclusion of the monotypic genus, *Drouetia* in molecular analyses has been uncertain due to frequent misidentifications of numerous taxa currently assigned to *Asteromenia* and *Halichrysis* as the reportedly cosmopolitan generitype of *Drouetia, D. coalescens* (Saunders et al. 2006). A recent study resolved material from South Africa that was vegetatively representative of *Drouetia* and identified as "*D. coalescens*" as an independent lineage in the Rhodymeniaceae (Saunders et al. 2006). In that study, Saunders et al. (2006) viewed a prepared mica slide of holotype tetrasporangial material for the generitype, *D. coalescens*, and reported what they interpreted as adventitious growth of the cortex to form nemathecia of paraphyseal filaments and tetrasporangia on the dorsal surface only. Those observations were inconsistent with reports of tetrasporangial development for the South African samples (terminal tetrasporangia on both surfaces, Norris 1991) which, as a result, were not regarded as representative of *D. coalescens* (Saunders et al. 2006). The present study resolved three novel species from Australia as morphologically assignable to the genus *Drouetia* (none were consistent with *D. coalescens*) and two of these were included in multigene phylogenetic analyses where they were resolved in the

Rhodymeniaceae sister to the previously discussed South African collection (Fig. 1). Our observation of tetrasporangial development for the novel species *D. viridescens* did not suggest adventitious cortical growth in the formation of the tetrasporangial nemathecia (i.e., true paraphyses were absent) as reported by Saunders et al. (2006) for the generitype, which prompted re-examination of holotype material of *D. coalescens* to clarify tetrasporangial development for this genus. That material was provided on loan from the Farlow Herbarium (FH) and freshly prepared sections were examined. We observed cruciately divided tetrasporangia deeply embedded in the dorsal cortex only (Fig. 5, b and c). Tetrasporangial initials developed from the cells of the inner cortex and during maturation, became elongated and swollen, which resulted in neighbouring cortical cells becoming compressed and elongated between the developing tetrasporangia, taking on the appearance of paraphyses (Fig. 5, c-e). This mode of development was more consistent with that observed in *D. viridescens* and other rhodymeniaceaen taxa, which are typically characterized by tetrasporangia that differentiate from pre-existing cortical cells and lack true adventitious filaments (paraphyses; Le Gall et al. 2008, Saunders & McDonald 2010). More study of tetrasporangial development for the South African collections is needed to determine if previous reports of tetrasporangial sori occurring on both sides of the blade (Norris 1991) are incorrect and confounded by taxonomic misidentifications or if that species has an atypical mode of development. Either way, the South African species represents a fifth species best assigned to *Drouetia*, a conclusion that ultimately awaits inclusion of *D. coalescens* in phylogenetic analyses to provide molecular confirmation, as well as our decision to assign the three Australian species *D. aggregata, D. scutellata* and *D. viridescens* to *Drouetia* as distinct from the generitype.

CONCLUSIONS

The intention of this study was to enhance understanding of evolutionary relationships in the Rhodymeniales. Fast-site removal successfully improved resolution at a key node and provided clarification of interfamilial relationships. By expanding taxonomic representation we have addressed the phylogenetic affinities of rhodymenialean genera absent from previous molecular assessments and have clarified two consistently polyphyletic genera.

Additionally, re-examination of holotype material for *Drouetia coalescens* clarified rhodymeniacean tetrasporangial development for the genus and provided evidence that the three novel Australian species are correctly assigned to this genus, and that this genus is correctly assigned to the Rhodymeniaceae.

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FIGURE LEGENDS

Figure 1. RAxML phylogeny for full multigene alignment partitioned by gene and then codon with subfamilial bootstrap support values appended. Branch letters correspond to the support values in Table 2. Taxa in bold represent generitype species. Asterisks denote fully supported branches. Species transferred to new genera have the previous genus name in brackets. Outgroup species of the Halymeniales and Sebdeniales were compressed to ordinal level to facilitate presentation.

Figure 2. *Drouetia aggregata* Filloramo & G.W. Saunders sp. nov.
a. Top view of vegetative holotype (GWS032793). Scale bar = 8.75 mm.
b. Side view of vegetative holotype with distinct stipes subtending anastomosed blades (GWS032793). Scale bar = 8 mm.
c. Transverse section at mid-thallus showing medulla and dorsiventral aspect (GWS032793). Scale bar = 200 µm.

d. Transverse section at mid-thallus showing ventral surface and aggregates of small cells interspersed among the larger cells of the medulla (GWS032792). Scale bar = 100 µm.

Figure 3. *Drouetia scutellata* Filloramo & G.W. Saunders sp. nov.
a. Habit of single peltate blades showing distinct stipes and central depressions, lighter patches indicate spermatangial sori (arrows) (Isotype, GWS032752). Scale bar = 1 cm.
b. Habit of cystocarpic holotype (GWS032729). Scale bar = 1 cm.
c. Transverse section at mid-thallus showing medulla and dorsiventral aspect (GWS032729). Scale bar = 100 µm.
d. Transverse section through mature cystocarp (GWS032729). Scale bar = 250 µm.
e. Transverse section of male plant showing spermatangial development on the dorsal surface (GWS032752). Scale bar = 10 µm.

Figure 4. *Drouetia viridescens* Filloramo & G.W. Saunders sp. nov.
a. Habit of single peltate blades attached to substratum by single stipe (not pictured) (GWS032740). Scale bar = 1 cm.
b. Habit of tetrasporic holotype showing anastomosed blades that form an irregular outline (GWS032612). Scale bar = 1 cm.
c. Transverse section at mid-thallus of vegetative plant (GWS032682). Scale bar = 93 µm.
d. Transverse section at mid-thallus of tetrasporic holotype (GWS032612). Scale bar = 108 µm.
e. Mature cruciately divided tetrasporangium (GWS032612). Scale bar = 33 µm.
f. Transverse section at mid-thallus of tetrasporic holotype showing cortical cell (arrow) compressed between developing and mature tetrasporangia creating the appearance of paraphyses. Tetrasporangial initials pit connected (arrowheads) to inner cortical cell (GWS032612). Scale bar = 25 µm.
g. Intercalary tetrasporangial initial pit connected (arrowheads) to two neighboring cortical cells (GWS032612). Scale bar = 20 µm.

Figure 5. Tetrasporangial holotype of *Drouetia coalescens* (Farlow) G. De Toni (Harvard University Herbaria & Libraries, No. 00777108).

a. Herbarium label and gross morphology of holotype. Scale bar = 21 mm.

b. Transverse section of tetrasporangial thallus showing dorsal tetrasporangial sorus. Scale bar = 91 µm.

c. Developing tetrasporangia deeply embedded in the dorsal cortex. Scale bar = 47 µm.

d. Cruciately divided tetrasporangia (arrows). Scale bar = 23 µm.

e. Pit connection (arrow) between two cortical cells modified into tetrasporangia surrounded by compressed cortical cells creating the appearance of paraphyses. Scale bar = 18 µm.

Figure 6. *Hymenocladia chondricola* (Sonder) J. Lewis, *Hymenocladia conspersa* (Harvey) J. Agardh and *Perbella minuta* (Kylin) Filloramo & G.W. Saunders comb. nov.

a. Gross morphology of a vegetative specimen of *H. chondricola* from South Australia (GWS029560). Scale bar = 25 mm.

b. Mature morphology of a vegetative specimen of *H. conspersa* from Victoria (GWS017300). Scale bar = 25 mm.

c. Intermediate morphology of vegetative specimen of *H. conspersa* from Tasmania (GWS015238). Scale bar = 19 mm.

d. Juvenile morphology of vegetative specimen of *H. conspersa* from Tasmania (GWS016539). Scale bar = 13 mm.

e. Gross morphology of a vegetative specimen of *Perbella minuta* from Tasmania (GWS015206). Scale bar = 25 mm.

Table 1. Collection details and COI-5P GenBank accession numbers for Australian *Drouetia* samples used in molecular and morphological analyses. All samples were collected from Coffs Harbour, New South Wales, Australia. All data was generated for the present study.

Table 2. Bayesian posterior probabilities, RAxML bootstrap values and likelihood scores for the full multigene alignment with partitioning by gene and then codon (noPF) and with partitioning as determined by PartitionFinder (PF). RAxML bootstrap values and likelihood scores for the site-stripper subalignments with partitioning by gene and then codon (noPF). Dashes (-) indicate branch support below 50% and n/a indicates a branch that was not resolved.

Table 3. Intraspecific COI-5P divergence and distance to nearest neighbour for Australian species of *Drouetia* included in this study.

SUPPLEMENTARY MATERIAL

Figure. S1. An alternative topology produced by neighbor-joining analysis with the HKY model to assess the impact of the starting tree on SiteStripper analyses.

Table S1. List of species and voucher numbers used in this study. GenBank sequences in bold were generated for this study. Generitype species are noted with an asterisk. ND indicates data not generated for this study. Percent (%) complete is based on the number of included sites out of the 8714 bp alignment. Collection details for all specimens are available on the BOLD website in the public dataset DS-RHDYPHY.

Table 1. Collection details and COI-5P GenBank accession numbers for Australian *Drouetia* samples used in molecular and morphological analyses. All samples were collected from Coffs Harbour, New South Wales, Australia. All data was generated for the present study

Species	Voucher Number	Habitat	Geography	COI-5P GenBank Accession
Drouetia aggregata Filloramo & G.W. Saunders	GWS032792	4 m on rock	Mutton Bird Island (South)	**KU707859**
	GWS032793	4 m on rock	Mutton Bird Island (South)	**KU707873**
Drouetia scutellata Filloramo & G.W. Saunders	GWS032664	6 m on invert	South Solitary Island (Southeast)	**KU707850**
	GWS032729	5 m on rock	Mutton Bird Island (North)	**KU707846**
	GWS032752	5 m on invert	Mutton Bird Island (North)	**KU707880**
Drouetia viridescens Filloramo & G.W. Saunders	GWS032599	5 m on rock	Korora Beach	**KU707869**
	GWS032600	5 m on rock	Korora Beach	**KU707868**
	GWS032612	8 m on rock	Split Solitary Island (Northwest)	**KU707855**
	GWS032624	8 m on rock	Split Solitary Island (Northwest)	**KU707881**
	GWS032642	10 m on invert	Split Solitary Island (East)	**KU707878**
	GWS032643	10 m on coral rubble	South Solitary Island (East)	**KU707874**
	GWS032665	6 m on invert	South Solitary Island (Southeast)	**KU707863**
	GWS032672	10 m on invert	Black Rock	**KU707870**

Table 1. Collection details and COI-5P GenBank accession numbers for Australian *Drouetia* samples used in molecular and morphological analyses. All samples were collected from Coffs Harbour, New South Wales, Australia. All data was generated for the present study

Species	Voucher Number	Habitat	Geography	COI-5P GenBank Accession
	GWS032682	6 m on invert	Black Rock	**KU707847**
	GWS032740	5 m on invert	Mutton Bird Island (North)	**KU707852**

Table 2. Bayesian posterior probabilities, RAxML bootstrap values and likelihood scores for the full multigene alignment with partitioning by gene and then codon (noPF) and with partitioning as determined by PartitionFinder (PF). RAxML bootstrap values and likelihood scores for the site-stripper subalignments with partitioning by gene and then codon (noPF). Dashes (-) indicate branch support below 50% and n/a indicates a branch that was not resolved.

	Bayes		RAxML		RAxML					
% of sites retained	**100%**				**95%**	**90%**	**85%**	**80%**	**75%**	**70%**
	noPF	**PF**	**noPF**	**PF**	**noPF**					
BRANCHES	**-143267**	**-143395**	**-143595**	**-143787**	**-112089**	**-86686**	**-65296**	**-47116**	**-32139**	**-20852**
A	1	1	100	100	100	100	100	100	100	100
B	1	1	95	95	93	95	97	99	98	79
C	n/a	-	-	50	n/a	51	68	75	65	62
D	1	1	100	100	100	100	100	100	100	100
E	1	1	100	100	100	100	100	100	100	100
F	1	1	100	100	100	100	100	100	100	100
G	1	1	100	100	100	100	100	100	100	98
H	1	1	100	100	100	100	100	100	100	100
I	1	1	79	80	71	78	71	81	71	80
J	1	1	100	100	100	100	100	100	100	100

Table 3. Intraspecific COI-5P divergence and distance to nearest neighbour for Australian species of Drouetia included in this study.

Species	Max intraspecific divergence (%)	Nearest species	Distance to nearest neighbour (%)
D. aggregata (n= 2)	0	D. scutellata	7.19
D. scutellata (n= 3)	0.15	D. viridescens	2.5
D. viridescens (n= 11)	0.31	D. scutellata	2.5

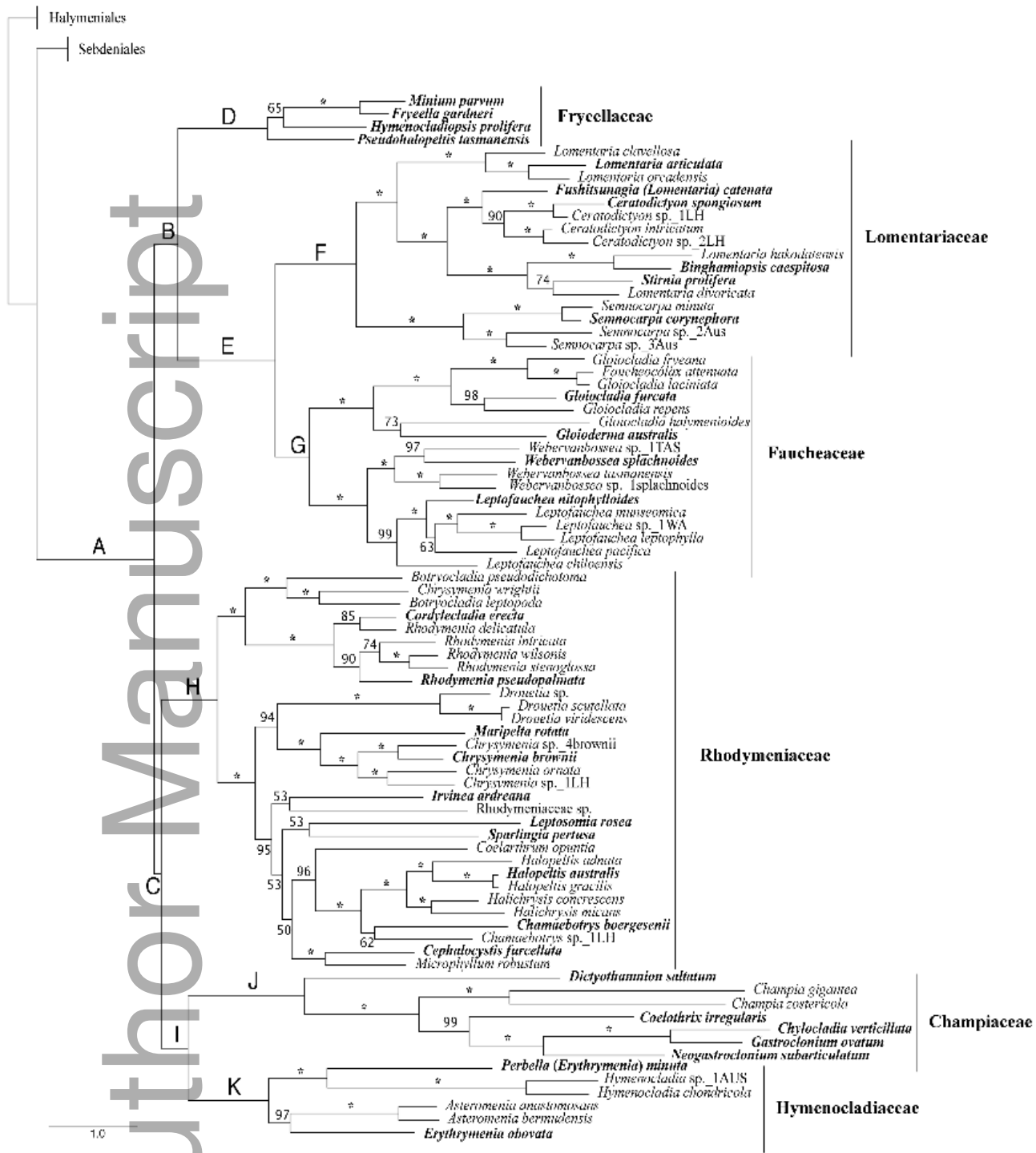

Figure 1.

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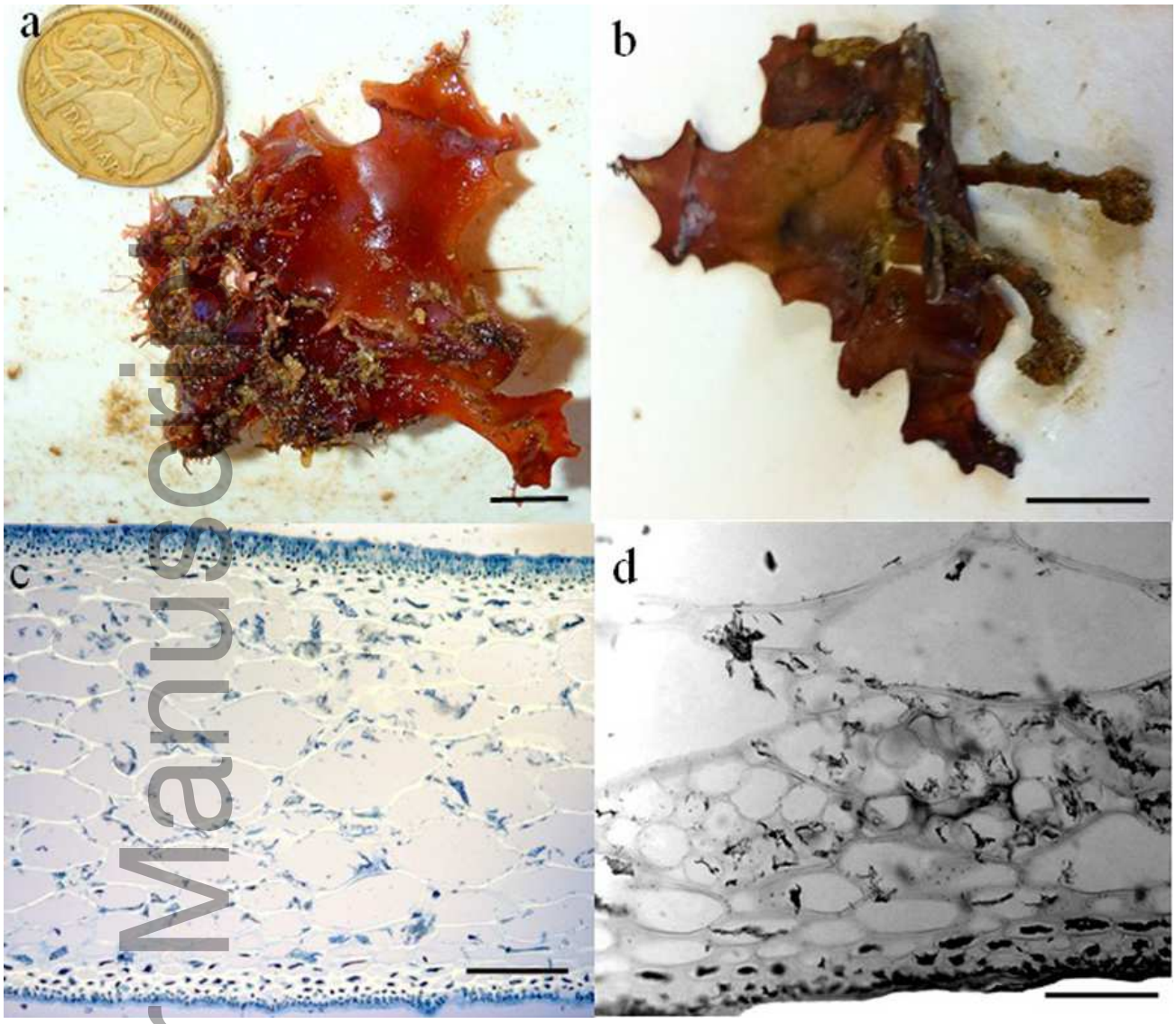

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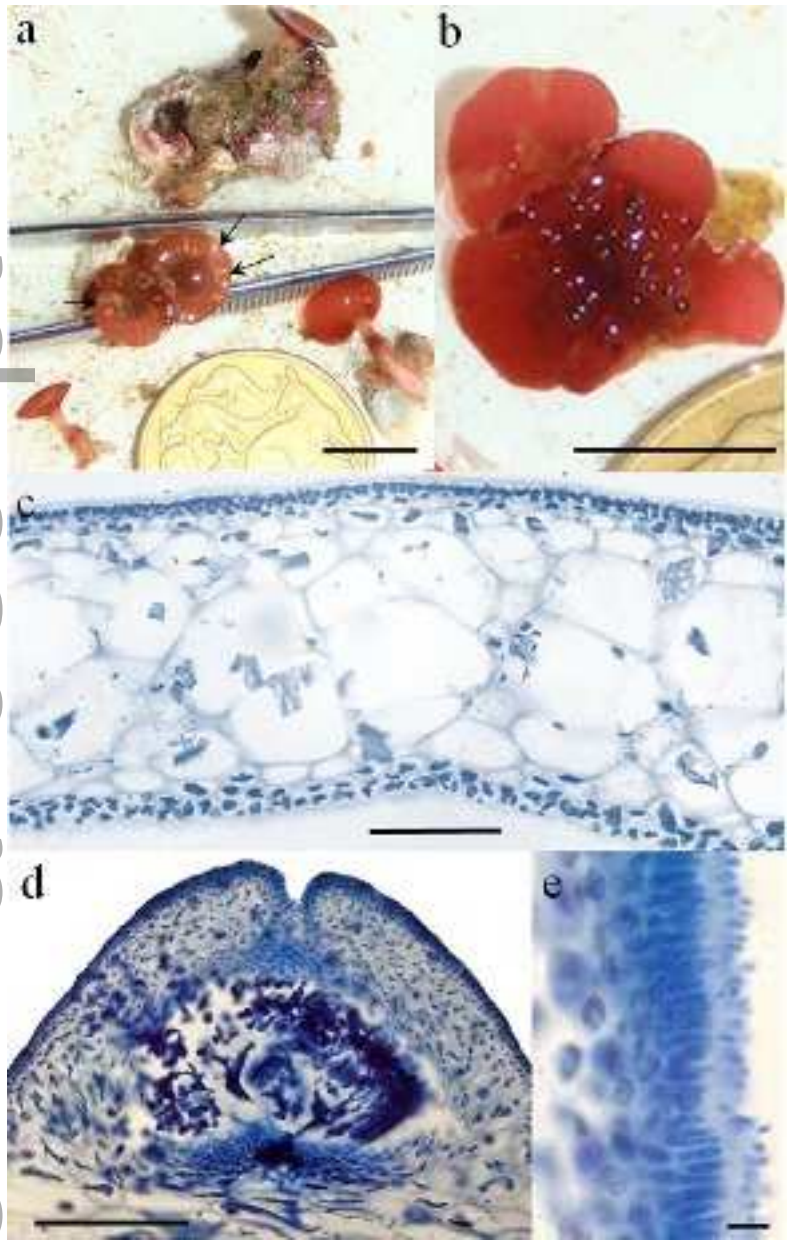

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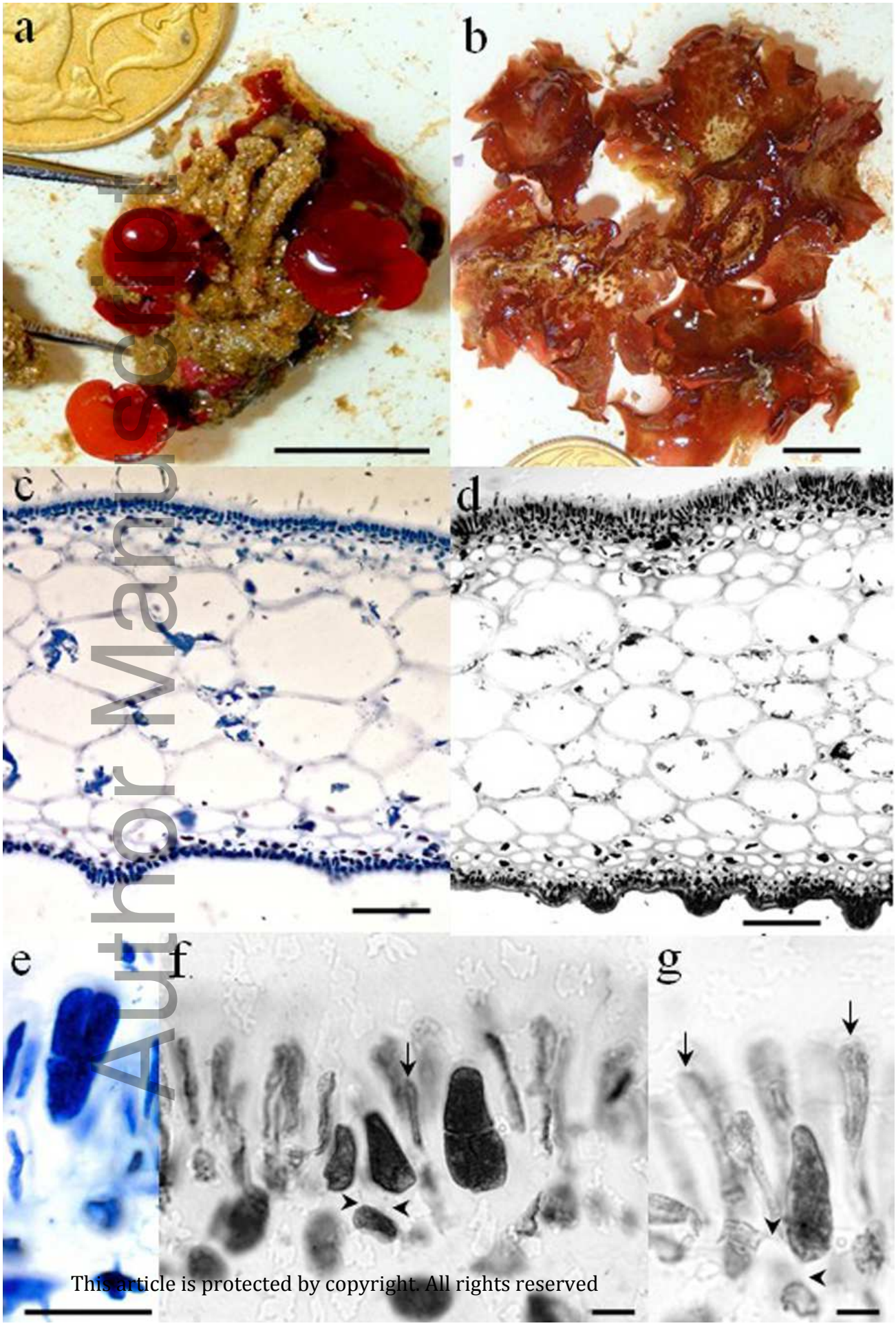

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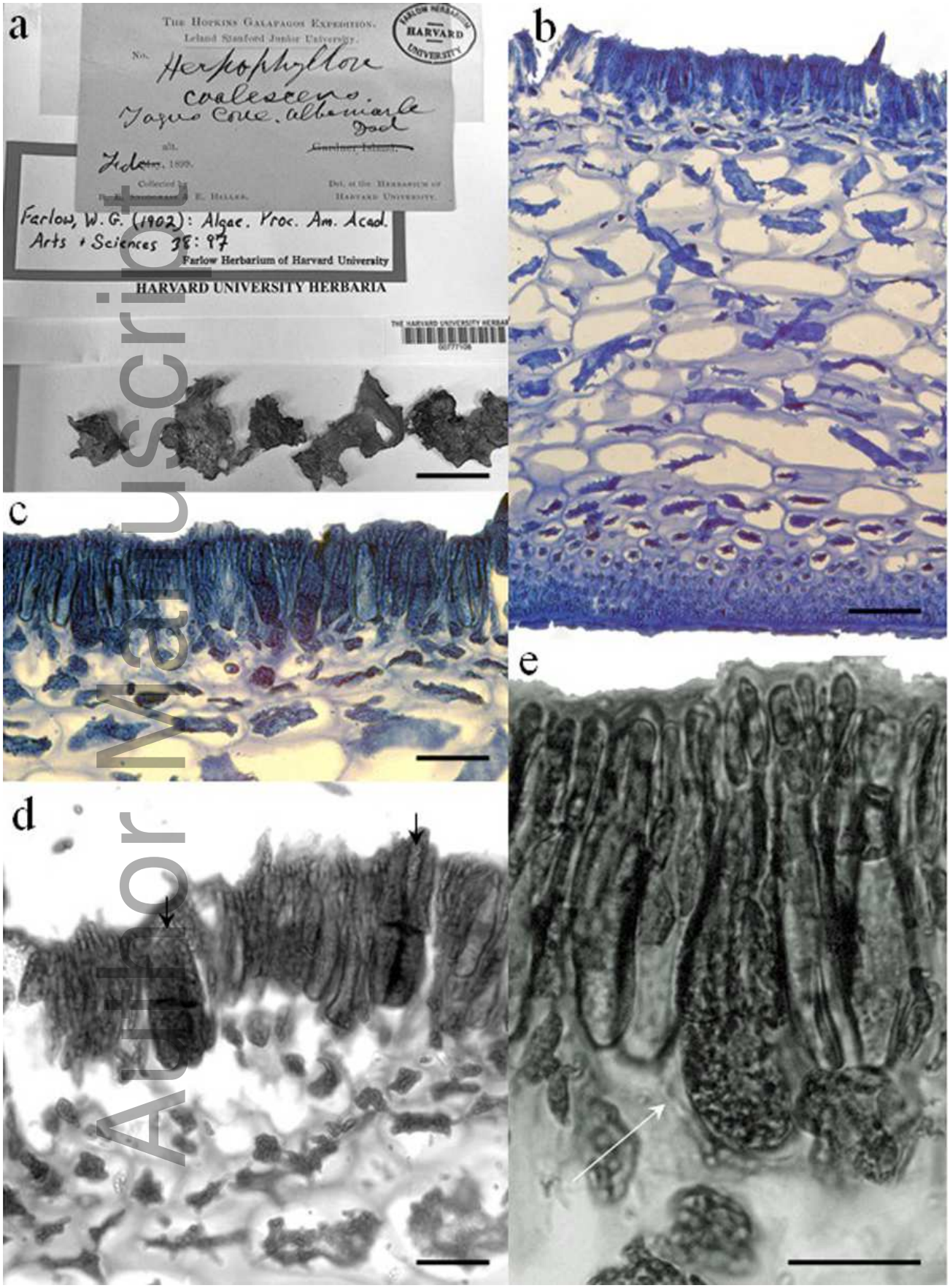

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Figures 23-27

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