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Molecular phylogeny of foliose Halymenia and Austroepiphloea (Halymeniaceae, Rhodophyta) from the Indo-Pacific including H. taiwanensis sp. nov.

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Abstract:	Many species of Halymenia from the Indo-Pacific have been described in the past decade, but their phylogenetic relationships are not well discussed. In this study, we inferred these relationships for the available species of Halymenia with an emphasis on the foliose species from the western Pacific Ocean and Western Australia based on rbc L sequence analyses. Our analyses show that most foliose Halymenia from the Indo-Pacific are clustered in a natural assemblage that also includes a new species (Halymenia taiwanensis sp. nov.) found in northern Taiwan as well as the monospecific genus Austroepiphloea (single species A. bullosa) from Western Australia. The architecture of carpogonial branch (composed of a 2-celled carpogonial branch and two orders of ampullar filaments and a basal, nutritive cellular cluster) and auxiliary cell ampullae in Halymenia taiwanensis is similar to that found in the generitype H. floresii . We therefore propose the new combination Halymenia bullosa comb. nov. that is closely related to H. taiwanensis both genetically and in sharing a similar thallus morphology. However, H. taiwanensis can be separated from H. bullosa by possessing thinner blades and bearing surface bladelets, and in lacking a long cartilaginous stipe. Based on the rbc L phylogeny, most foliose Halymenia are seemingly more range-restricted than previously thought, except for a few species that are shown to have a wide distribution in the Western Pacific and Indian Oceans. In addition, H. dilatata , a species widely recorded in the Western Pacific Ocean, may include cryptic species and requires further investigation.

Molecular phylogeny of foliose *Halymenia* and *Austroepiphloea* (Halymeniaceae, Rhodophyta) from the Indo-Pacific including *H. taiwanensis* sp. nov.

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Running title: **Systematics of *Halymenia* and *Austroepiphloea***

ABSTRACT

Many species of *Halymenia* from the Indo-Pacific have been described in the past decade, but their phylogenetic relationships are not well discussed. In this study, we inferred these relationships for the available species of *Halymenia* with an emphasis on the foliose species from the western Pacific Ocean and Western Australia based on *rbc*L sequence analyses. Our analyses show that most foliose *Halymenia* from the Indo-Pacific are clustered in a natural assemblage that also includes a new species (*Halymenia taiwanensis* sp. nov.) found in northern Taiwan as well as the monospecific genus *Austroepiphloea* (single species *A. bullosa*) from Western Australia. The architecture of carpogonial branch (composed of a 2-celled carpogonial branch and two orders of ampullar filaments and a basal, nutritive cellular cluster) and auxiliary cell ampullae in *Halymenia taiwanensis* is similar to that found in the generitype *H. floresii*. We therefore propose the new combination *Halymenia bullosa* comb. nov. that is closely related to *H. taiwanensis* both genetically and in sharing a similar thallus morphology. However, *H.*

taiwanensis can be separated from *H. bullosa* by possessing thinner blades and bearing surface bladelets, and in lacking a long cartilaginous stipe. Based on the *rbc*L phylogeny, most foliose *Halymenia* are seemingly more range-restricted than previously thought, except for a few species that are shown to have a wide distribution in the Western Pacific and Indian Oceans. In addition, *H. dilatata*, a species widely recorded in the Western Pacific Ocean, may include cryptic species and requires further investigation.

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INTRODUCTION

In recent years, the taxonomy of the Halymeniaceae has been the subject of investigation and revision, with several small genera established based on species previously placed in *Halymenia* C.Agardh [e.g. *Amalthea* D'Archino & W.A.Nelson in D’Archino *et al*. (2014); *Neofolia* Showe M.Lin, Rodríguez-Prieto, De Clerck & Huisman in Rodríguez-Prieto *et al.* (2018); *Nesoia* H.W.Lee & M.S.Kim in Lee & Kim (2019)]. *Halymenia* is widely distributed in both temperate and tropical regions and is the largest genus in the family, containing more than 70 species (see a summary in Guiry & Guiry 2021). Recent studies based on *rbc*L sequence analyses (Hernández-Kantún *et al*.; 2012; Rodríguez-Prieto *et al.* 2018, 2020) have indicated that *Halymenia* is polyphyletic, and that two monotypic genera, *Austroepiphloea* Molinari, Sánchez Ocharan & Guiry and *Gelinaria* Sonder (both based on Western Australian species), are nested within a larger clade of *Halymenia* species. The name *Austroepiphloea* is a recently proposed replacement for *Epiphloea* J.Agardh (1890: 18), an illegitimate later homonym as the name *Epiphloea* was first used for a fungus by Trevisan (1880: 73) (see Molinari-Novoa *et al.*, 2021). *Austroepiphloea bullosa* was originally described by Harvey (1863: plate CCLXXVII) as *Schizymenia? bullosa* Harvey, based on a large and bullate, membranous thallus with a conspicuous and cartilaginous, rigid stipe. Harvey had collected the species during his trip to Australia, but was later presented with a ‘fragment’ collected by George Clifton that led Harvey to speculate that “the lamina, when full grown … may perhaps be two or three feet across!” The species was transferred to *Epiphloea* by De Toni (1905), under which name it has been generally recorded (e.g., Huisman 2019). The second Western Australian taxon, *Gelinaria ulvoidea* Sonder, possesses a branched and compressed thallus with a cuneate stipe (Womersley & Lewis 1994). The species was once transferred to *Halymenia* as *H. ulvoidea* (Sonder) Kützing, however Womersley & Lewis (1994) maintained *Gelinaria* and included three heterotypic synonyms (*Nemastoma? gelinarioides* Harvey, *Halymenia speciosa* Zanardini & *Gelinaria harveyana* J.Agardh, the latter a renaming of Harvey’s *N. gelinarioides*) under *G. ulvoidea*. Womersley & Lewis (1994) characterized *Gelinaria* as having: a bi- to tripinnate thallus with broad axes, tapering to narrow ramuli, a broad cortex, moderate to densely filamentous medulla, and auxiliary cell ampullae with much branched filaments scarcely converging above.

In this study, we continue our systematic studies of the Halymeniaceae and focus on the *Halymenia/Austroepiphloea* complex based on our newly collected and herbarium specimens from the Indo-Pacific. In particular, we examine the vegetative and reproductive structures of *A. bullosa* and a new species of *Halymenia* (= *H. taiwanensis* sp. nov. herein), whose thallus morphology resembles that of *Austroepiphloea bullosa*. The phylogenetic relationships among the available species of *Halymenia* and related genera are inferred and discussed based on *rbc*L and LSU sequence analyses.

MATERIALS AND METHODS

Specimens of *Halymenia* used in this study were collected subtidally by SCUBA from 4 to 15 m depths. For morphological studies, samples were preserved in 3-5% Formalin in seawater or preserved as herbarium sheets. A fragment of each specimen was preserved in silica gel for subsequent DNA extraction. Hand sections were stained with 1% aniline blue acidified with 1% HCl or treated with Wittmann’s aceto-iron-hematoxylin-chloral hydrate (Wittmann 1965) and mounted in 50% Hoyer's mounting medium modified by Rodríguez-Prieto & Hommersand (2009). *In situ* images were taken with a Sony RX100iii camera (Sony, Minato, Japan) and an Olympus underwater camera (Tough, Tokyo, Japan), while laboratory habit photographs were taken with a Nikon Z5 (Nikon, Tokyo, Japan), a Canon EOS 350D (Canon, Tokyo, Japan), and an Epson scanner (Epson, Tokyo, Japan). Photomicrographs were made with a Nikon DS-L4 camera attached to a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) and an AxioCam MRc attached to an Axioskop 2 plus microscope (Carl Zeiss, Oberkochen, Germany). Voucher specimens were deposited in the seaweed laboratory at the Institute of Marine Biology, National Taiwan Ocean University ("NTOU"), the Herbarium of University of Girona, Spain (HGI), and the Western Australian Herbarium (PERTH). Herbarium abbreviations follow Thiers (2021).

DNA samples were prepared using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. DNA amplification and sequencing procedures of the LSU rDNA and *rbc*L genes are as described in Freshwater *et al.* (1999), Saunders and Moore (2013) and Lin *et al.* (2008, 2020). Newly generated sequences for *Halymenia* and related genera from Australia and Taiwan were integrated with a selection of sequences available from GenBank (see Supplementary table S1) and aligned with the computer software Sequencher (Gene Codes Corp., Ann Arbor, MI, USA). The taxon sampling aimed to present the species diversity of *Halymenia* as comprehensively as possible (see Table S1). A maximum likelihood (ML) species tree was generated using the computer software MEGA v. 10.1.7 (Kumar *et al*. 2018) under a Tamura 3-parameter model, as suggested by running "Find best DNA model" implemented in MEGA. The robustness of the resulting phylogeny was tested using 1000 bootstrap replicates of a ML heuristic implemented in MEGA. For the *rbc*L dataset, a Bayesian analysis (BA) was estimated using MrBayes 3.2. (Ronquist *et al.* 2012), applying the default GTR+GAMMA model applied to every partition. Two runs consisting of 4 chains each were run for 10^6 generations with sampling every 100 generations. the average standard deviation of split frequencies decreased below 0.01 within 25,000 generations, indicating that the two runs had reached convergence. Inferences about the phylogeny were based on those trees sampled after generation 25,000. A 50% consensus tree (majority rule as implemented by PAUP* (v4.0, Swofford, 2003) was computed from the 9750+1 trees saved after the burn-in point.

RESULTS

Molecular analyses

A total of 63 *rbc*L sequences, including the newly generated new species (*H. taiwanensis*) and the two monospecific genera (*Gelinaria* and *Austroepiphloea*) from Western Australia, from representative taxa belonging to *Halymenia* and related genera in the family Halymeniaceae were selected for analysis, with the genus *Tsengia* K.C.Fan & Y.P.Fan, serving as the outgroup (Table S1). The alignment included 1257 sites, omitting 106 and 103 base pairs from the 5' and 3' end of the *rbc*L (1467 bp) gene due to missing information. The maximum likelihood (ML) and Bayesian trees (BA) (Fig. 1) are overall congruent, differing in the relative placement of clades that received only low support. The inter-generic relationships of the Halymeniaceae are weakly to strongly supported by bootstrapping values (BP) and Bayesian posterior probabilities (PP). The majority of species currently placed in *Halymenia* including the generitype, *H. floresii*, are clustered together to form a large clade with moderate support (BP = 75%; PP = 88%), whereas "*H.*" *abyssicola* is closely related to the genus *Amalthea* and *"H." floridana* is positioned in the major clade of *Cryptonemia*. On the other hand, the Australian endemics *Austroepiphloea bullosa* and *Gelinaria ulvoidea* are positioned in the major clade of *Halymenia*. Moreover, *H. taiwanensis* from northern Taiwan is closely related to *A. bullosa* with 2.78% of pairwise distance. The interspecific genetic divergence of *Halymenia* ranges from 1.72 - 6.44 %, whereas no intraspecific genetic difference is detected among the populations of *H. taiwanensis*. The circumscribed species "*Gelinaria ulvoidea*" from Australia contains at least three different species with 1.88 – 3.84 % pairwise distances (see Fig. 1). The *rbc*L+LSU dataset for phylogenetic analyses consisted of 3666 characters (LSU = 2409 bp; *rbc*L = 1257 bp) and 43 taxa. The new species, *H. taiwanensis*, Australian endemics *Austroepiphloea bullosa* and *Gelinaria ulvoidea* are all positioned in the major clade of *Halymenia* including the generitype with a strong support (BP = 97%, see Fig. S1).

The molecular phylogenetic analyses support the merge of the monospecific *Austroepiphloea bullosa* from Western Australia into *Halymenia* (name priority). Accordingly, we propose the new combination *Halymenia bullosa* to accommodate the species. The vegetative and reproductive structure of *H. bullosa* and *H. taiwanensis* are documented in detail in the next section.

Morphological observations

Halymenia bullosa* (Harvey) Huisman, C.Rodríguez-Prieto & Showe M.Lin *comb. nov.

Figs 2-10

DESCRIPTION: Thalli are composed of single large, circular, reniform to irregularly shaped, unbranched fleshy blade with smooth and thicker margins, to 30 cm (at least) long, arising from a prominent cartilaginous stipe (Figs 2-4). Old blades are bullate (Figs 2-4), often red-purple *in situ* and fading to yellow in color when mature. Thallus blade growth is multiaxial (Fig. 5) led by many obliquely dividing apical cells. Young blades are 170-200 µm thick and become 350-600 µm thick when mature. Cortex is composed of 5-6 cell layers, including the inner 3-4 of irregularly stellate cells (Fig. 5, arrowheads), subtending a layer of spherical cells that each bear 1-3 elongate cells in a palisade, 12-17 µm long. Surface cortical cells are obovoid and protruding at the thallus surface. Medulla is consisting of irregularly arranged filaments, 3-6 µm in diameter, with occasional anticlinal thicker filaments, 10-12 µm in diameter, that traverse the medulla and link the cortices. Darkly staining stellate cells bear long arms are present in the medulla (Fig. 5, arrow). Gametophytes and tetrasporophytes are isomorphic. Tetrasporangia initials (Fig. 6, arrowheads) are originally cut off terminally from subcortical cells (Fig. 6, arrows), then enlarge and develop into cruciately or decussately divided mature tetrasporangia (Fig. 7), 12-20 µm wide x 25-34 µm long. At this stage, the outermost cortical cells in the vicinity of the tetrasporangia elongate and form a layer of paraphyses surrounding the tetrasporangia. Carpogonial branch ampullae and early diploidization stages were not found. A fully developed auxiliary cell ampulla is composed of two or three orders of branched ampullar filaments (Fig. 8). After presumed diploidization, the ampullar filaments in the auxiliary cell ampulla become highly branched, composed of rounded to ovoid cells (Fig. 9, black arrows), and the diploidized auxiliary cell enlarges and cuts off a gonimoblast initial distally (Fig. 9). Meanwhile, secondary medullary filaments are produced from the innermost cortical cells. Fully developed carposporophytes are surrounded by a mixture of elongated ampullar filaments and secondary medullary filaments (Fig. 10, black and white arrows). A mature gonimoblast is composed of at least 2 gonimolobes and most gonimoblast cells differentiated into carposporangia, 10-18 µm diameter (Fig. 10). Cystocarps are spherical to ovoid, 100-200 µm in diameter, immersed in the medulla, sometimes causing a slight swelling at the thallus surface.

BASIONYM: *Schizymenia bullosa* Harvey, *Phycologia Australica* Vol. 5: xlvii, pl. CCLXXVII (1863).

HOMOTYPIC SYNONYMS: *Platymenia bullosa* (Harvey) Kuntze (1891: 910); *Epiphloea bullosa* (Harvey) De Toni (1905: 1578); *Austroepiphloea bullosa* (Harvey) Molinari, Sánchez Ocharan & Guiry (2021: 2).

TYPE LOCALITY: Fremantle, Australia, May, 1858, collected by George Clifton.

TYPE: Lectotype: TCD0011815 (designated here); isolectotype: BM000640393. Note: In the protologue Harvey mentioned his own collections from 1854 and the later collections of George Clifton. The lectotype is selected here in accordance with an annotation on TCD0011815 by Bryan Womersley, added during a visit to Trinity College in 1952 [as ‘Type (H.B.S.W.)’].

SPECIMENS EXAMINED: Western Australia: (1) West End, Rottnest Island, -15 m, 24 February 1990, coll. J.M. Huisman (PERTH 06549683, female), (2) The Basin, Rottnest Island, -4 m, 3 January 2018, coll. J.M. Huisman (tetrasporic), (3) Outer Rocks, Jurien Bay, 24 October 2000, coll. J.M. Huisman (JB665, tetrasporic), (4) Easter Group, Houtman Abrolhos, -4 m, 18 January 1983, coll. B.G. Hatcher (PERTH 07138288, sterile).

DISTRIBUTION, HABITAT AND SEASONALITY: South-west coast of Australia, from Esperance north to the Houtman Abrolhos Islands. Thalli have been collected from October to

January in shallow waters (4-15 m deep). Records from other areas are likely misidentifications and should be re-examined.

Halymenia taiwanensis Showe M.Lin, C. Rodríguez-Prieto & Huisman *sp. nov.*

Figs 11-35

DESCRIPTION: Thalli are erect, rose, bright to brick red in colour, consisting of 1-3 deeply divided or undivided, fleshy blades with dark spots (Figs 11-13). Blades shape and sizes are variable, 10-18 (-25) cm wide and 12-28 (-32) cm long (Fig. 11). Blades are 119-460 µm thick, arising from discoid holdfasts with or without short stipes (Figs 11-12). Margins of blades are entire or dentate, and surface and marginal proliferations and bladelets are frequently present on old blades (Figs 12-13). Fresh blades are soft in texture and become tough after dried. Surface of old blades are rough and bullate (Fig. 13). Thallus structure is multiaxial (Fig. 14) and the growth is led by many obliquely dividing apical cells. The cortex is composed of 4-6 cell layers with the cell size decreasing progressively towards to the thallus surface (Fig. 15). Outer cortical cells (Fig. 15, white arrows) are compactly arranged, rounded to polygonal in surface view, obovoid in transverse view, 3.6-8.1 µm in diameter and 7.1-16.9 µm long. Secondary pit connections are absent between outer cortical cells (Fig. 15). The outer subcortical cells, which do not form any secondary pit connections, are ovoid or spherical (Fig. 15, black arrowheads), whereas the inner subcortical cells are secondarily pit-connected to one another (Fig. 15, white arrowheads). The innermost subcortical cells are stellate, with a cell body up to 8-23 µm in diameter and short arms (Fig. 15), and form a network parallel to thallus surface. The medulla is either hollow or loosely filled with a network of irregularly arranged filaments, 1.5-2.5 µm wide x 25-91 µm long (Fig. 14, black arrows), some anticlinally arranged filaments, 2.5-6.0 µm wide x 9-35 µm, that join the cortices of both sides of the blade (Fig. 14, white arrows), and a few stellate cells, 9-19 µm wide by 22-27 µm long, with lightly to darkly stained long arms (Fig. 16, arrow). Gametophytes and tetrasporophytes are isomorphic. Tetrasporangia are initially produced from subcortical cells and scattered over the thallus surface (Fig. 17). The developing tetrasporocytes enlarge and initially divide transversely, and the sterile surface cells elongate slightly (Fig. 18). Mature tetrasporangia (Fig. 19, black arrowheads) are attached basally to subcortical cells (Fig. 19, white arrowheads) and are cruciately divided, 9-16 µm wide by 14-27 µm long. The species is monoecious as spermatangia and cystocarps can be found in the same fertile blade. Spermatangial parent cells are initially produced from surface cells and formed in sori scattered over the fertile blade (Fig. 20). Each spermatangial parent cell divides obliquely or anticlinally to produce one or two spermatangia (Fig. 21). In a fully developed spermatangial sorus, more spermatangia can be produced from the subcortical cells (= the parental cells) at the distal ends (Fig. 22). The female reproductive system is non-procarpic, with carpogonial branches (Figs 23-25) and auxiliary cells (Figs 26-28) born in separate ampullae. The development of the carpogonial branch and auxiliary cell ampulla is similar to that is seen in the generitype, *H. floresii*. The carpogonial branch ampullae are rare in an early stage when the trichogyne is not well developed (Fig. 23). In the development of the carpogonial branch ampulla, a carpogonial branch initial is produced from the supporting cell, which is differentiated from the basal cell of the first-order ampullar filament (Fig. 23, af1i). Following this, the carpogonial branch initial undergoes an oblique cell division: the upper cell differentiating into a carpogonium bearing a trichogyne at the distal end and the lower one acting as a hypogynous cell (Fig. 24). Prior to presumed fertilization, a nutritive cellular cluster is secondarily produced from the innermost cortical cells at the basal part of the carpogonial branch ampulla (Figs 24-25). The fully developed carpogonial branch ampulla is composed of a 2-celled carpogonial branch with a distally long trichogyne and two orders of branched ampullar filaments (Fig. 25). Auxiliary cell ampullae are abundant in the inner cortex of young parts of fertile blades and are composed of three orders of ampullar filaments (Figs 26-27). Very early stages of the auxiliary cell ampullae were not found. During development, the second-order ampullar filament is cut off from the first cell of the first-order ampullar filament, whereas the third-order ampullar filament is produced from the first cell of the second-order ampullar filament (Fig. 26). When fully developed, the basal cell of the third-order ampullar filament differentiates into an auxiliary cell

(Fig. 27) and all ampullar filaments branch two or three times to form short laterals developing towards the thallus surface (Figs 27-28). Direct and very early diploidization stages were not found. At early cystocarp development, a few gonimoblast cells are produced from the gonimoblast initial borne on the fusion cell, which is formed from a fusion of the diploidized auxiliary cell and neighboring ampullar cells (Fig. 29). At this stage, most cells of the ampullar filaments remain rounded or ovoid, while some elongate and branch laterally one or two times, and are pit-connected to nearby formed secondary medullary filaments (Fig. 29, white arrows). In later stages, cells of the ampullar filaments elongate and the ampullar filaments may branch 4-5 times (Fig. 30), and loosely surround the developing gonimoblast (Fig. 31). As gonimoblast development continues, the fusion cell fuses with more basal ampullar cells and most ampullar cells elongate slightly but do not branch further, whereas the basal secondary medullary filaments are weakly developed (Figs 32). Noticeably, the ampullar cells in the vicinity of the fusion cell or fused with it become darkly stained and enlarge slightly (Fig. 33, black arrow at the bottom). The gonimoblast initial remains distinct through maturation of cystocarps and can produce at least two gonimolobes (Figs 34, 35). The pericarp is composed of elongated and extended ampullar filaments (Figs 30-35) with their distal ends forming secondary pit connections to the inner cortical cells (Fig. 34, white arrow). Most cells of gonimolobes differentiate into spherical to ovoid carposporangia, 8.0-9.5 μm wide by 10.5-15.0 μm long. Cystocarps with ostioles are scattered over the fertile blade surface, immersed in the thallus or slightly protruding at maturity. Mature carposporophytes are 100-150 μm in diameter.

HOLOTYPE: NTOU-001649, female gametophyte, deposited in the seaweed laboratory of National Taiwan Ocean University, collected from Chaojng, a rocky bottom off the east of Keelung city, Taiwan, R.O.C. (25.142690N, 121.804170E) on 27 May 2010 by L.-C. Liu, at 6-7 m depth.

ISOTYPES: NTOU-001650~ NTOU-00162, gametophytes & tetrasporophytes, deposited in the seaweed laboratory of National Taiwan Ocean University. GenBank accession number for the isotype (NTOU-001650): *rbc*L, to be added.

ADDITIONAL SPECIMENS EXAMINED: Taiwan: (1) Keelung city, Chaojing, 5-10 m, 08 July 1994, coll. S.-M. Lin (sterile), 02 May 2008, coll. X.-R. Hsieh [NTOU-001632 (= HGI-A 20874) male and female], 06 August 2009, coll. X.-R. Hsieh [NTOU-001639 (= HGI-A 20997) male and female, NTOU-001640~001642 sterile & tetrasporic], 27 May 2010, coll. L.-C. Liu (NTOU-001649~001662 tetrasporic & female), 28 May 2013, coll. L.-C. Liu (NTOU-001663~001666 sterile & tetrasporic); (2) New Taipei City, Long Dong, 3-10 m deep, 30 June 2009, coll. S.-M. Lin [NTOU-001643~001647 sterile & tetrasporic, HGI-A 20999 tetrasporic).

ETYMOLOGY: “*taiwanensis*” refers to the location, Taiwan, in which the new species was found.

DISTRIBUTION, HABITAT AND SEASONALITY: Known only from northern Taiwan (New Taipei City & Keelung City). Plants were found in May through August, growing on shallow rocky bottoms (5-10 m deep).

DISCUSSION

The species diversity of *Halymenia* in the Indo-Pacific regions is rapidly increasing with accumulated marine floral surveys in the past decades (e.g. Abbott 1999; Xia 2004) and many new species of *Halymenia* have been described based on a combination of morphological comparisons and *rbc*L sequence analyses (e.g. Hernández-Kantún *et al*. 2012; Tan *et al*. 2015; Huisman & De Clerck 2018). Based on *rbc*L and *rbc*L+LSU analyses (see Fig. 1 & Fig. S1), the majority of *Halymenia* species, including the generitype (*H. floresii* having a branched thallus) and the new species described from Taiwan (= *H. taiwanensis* having a foliose thallus), clustered together to form a large, natural assemblage together with the generitype species of two monotypic genera, *Austroepiphloea bullosa* (treated as *Halymenia bullosa* comb. nov. herein) and *Gelinaria ulvoidea*, originally described from Western Australia. Remarkably, the four available sequences attributed to *G. ulvoidea* (two newly generated as part of this study) form a clade comprising three species level groups. The specimen from Cottesloe (PERTH 08921792) appears to be morphologically closest to the holotype and is regarded as authentic, but further investigation is required, including an assessment of the taxa currently regarded as synonyms. Whatever the outcome, it is clear that *Gelinaria* should not be retained, and the species should be known as *Halymenia ulvoidea* (Sonder) Kützing. With the exception of a few bladed species (i.e. *H. porphyriformis*, *H. villosa*), for which *rbc*L sequences are not available, most foliose species of *Halymenia* (*H. bullosa, H. taiwanensis, H. dilatata, H. maculata, H. malaysiana, H. stipitata*) from the Indo-Pacific are shown to be closely related (see Fig. 1). The molecular phylogenetic analyses do not support the record of *H. bullosa* from Lord Howe Island, off the coast of New South Wales, Australia, as reported by Withall & Saunders (2006, as *Epiphloea bullosa*) as it is distantly related to *H. bullosa* from Western Australia.

Halymenia taiwanensis has similar carpogonial branch ampullae and development of auxiliary cell ampullae pre- and post-diploidization to the generitype *H. floresii*, as shown in Rodríguez-Prieto *et al*. (2018). Namely, the carpogonial branch ampulla is composed of a 2-celled carpogonial branch with a distally long trichogyne and two orders of branched ampullar filaments and a nutritive cellular cluster is secondarily produced from the innermost cortical cells at the basal part of the carpogonial branch ampulla [see Figs 12-15 in Rodríguez-Prieto *et al*. (2018), Figs 23-25 (this study)]. The auxiliary cell ampullae consist of three orders of branched filaments and the ampullar filaments elongate and branch to form a weak pericarp (see Figs 27-28, 33-25, this study). As shown in the *rbc*L phylogenetic tree, *H. bullosa* is a sister clade to *H. taiwanensis*. Both species possess fairly large blades (up to 30 cm or more) and share a similar blade morphology. However, *H. taiwanensis* differs from *H. bullosa* in having surface proliferations and thinner thallus blades (less than 500 µm thick in the former *vs*. up to 600 µm in the latter).

Halymenia taiwanensis was previously misidentified as *Halymenia dilatata* Zanardini in Huang (1999), a species originally described from the Red Sea, and the two species share some morphological similarities. Zanardini's original protologue (1851, p. 35) included a short description of *H. dilatata* as having subflabellately broadened (= dilated) blades with rounded and wavy, toothed margins. However, no information on thallus dimensions or illustrations of *H. dilatata* were included. A few years later, a shorter description and illustration (depicting a membranous blade bearing toothed margins arising from a basal stipe) of *H. dilatata* were provided in Zanardini (1858, p. 72, tab. III, fig. 1). De Smedt *et al.* (2001, fig. 7A) illustrated the lectotype of *H. dilatata*, possessing a broadened foliose and stipitate blade, deposited at the Museo Civico di Storia Naturale, Venezia. *Halymenia dilatata* has been reported to be widely distributed in the Asian Pacific (Kawaguchi & Lewmanomont 1999; Xia & Wang 1999; De Smedt *et al.* 2001; Abbott *et al.* 2002). However, the two available *rbc*L sequences attributed to *H. dilatata* from Japan (Wang *et al.* 2000) and the Philippines (Tan *et al.* 2015) are genetically different (see Fig. 1 in this study), and neither match the sequences of *H. taiwanensis*. In the absence of sequences from topotype specimens of *H. dilatata* for comparison, we cannot unequivocally remove it from contention. Nevertheless, it is not possible to molecularly define authentic *H. dilatata* without sequencing a topotype specimen, or unequivocally ascertain whether or not it occurs in the Asian Pacific. We anticipate that further studies of *Halymenia* from the Indo-Pacific Oceans, combining molecular analyses with detailed morphological comparisons, will reveal additional undescribed and possibly cryptic species.

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Fig. 1. Global Maximum likelihood phylogeny of the genus *Halymenia* based on the *rbc*L dataset. Numbers at nodes indicate bootstrap support (first value) and Bayesian posterior probabilities (second value). “-” refers the supporting values is less than 50%. The species names in bold refer to the new sequences generated in this study.

Figs 2-10. *Halymenia bullosa* (Harvey) Huisman, C.Rodríguez-Prieto & Showe M.Lin. *comb. nov.* Thallus morphology and vegetative and reproductive structure. Stained with aniline blue (Figs 5-10).

Fig. 2. Lectotype (designated here), a non-reproductive plant from Fremantle, Western Australia. Note the smooth darker margin (black arrows) in places where the plant has not been chewed or broken (TCD0011815). Scale bar = 5 cm.

Fig. 3. Plant photographed *in situ* at 10 m depth at Roe Reef, Rottnest Island, Western Australia. Note the bullate shape (black arrows) of the blades (PERTH 08187932). Scale bar = 5 cm.

Fig. 4. Monoecious specimen from West End, Rottnest Island fixed with a prominent cartilaginous stipe (black arrow) (PERTH 06549683). Scale bar = 5 cm.

Fig. 5. Transverse section through a thallus blade showing the multiaxial structure, a cortex of several layers of cells, a subcortex of multinucleate stellate cells of short arms (arrowheads), and a lax filamentous medulla. Note the presence of some stellate darkly staining cells with long arms (arrow) in the medulla (PERTH 06549683). Scale bar = 50 µm.

Fig. 6. Transverse section through a tetrasporangial sorus showing tetrasporangial initials (arrowheads) cut off terminally from subcortical cells (arrows) (Huisman JB665). Scale bar = 25 µm.

Fig. 7. Cross section through another tetrasporangial sorus showing developing tetrasporangia (arrowheads) borne on subcortical cells (black arrows). Note that the outermost cortical cells (white arrows) in the vicinity of the tetrasporangia become very slender and act as paraphyses for protecting the tetrasporangia (Huisman JB665). Scale bar = 25 µm.

Fig. 8. Detail of an auxiliary cell ampulla soon after fertilization showing enlarged diploidized auxiliary cell (aux) beginning to divide and branched ampullar filaments (black arrows) composed of rounded-ovoid cells (PERTH 06549683). Note the basal cells of the ampullar filaments also slightly enlarged (arrowheads) and the innermost cortical cells slightly to strongly elongated (white arrows). Scale bar = 25 µm.

Fig. 9. Detail of an auxiliary cell ampulla after diploidization showing the diploidized auxiliary cell (aux) which has transversely cut off a gonimoblast initial (gi). Note the cells (black arrows) of ampullar filaments are profusely branching and the secondary medullary filaments (white arrows) are produced from innermost cortical cells (PERTH 06549683). Scale bar = 25 µm.

Fig. 10. Cross section through a mature cystocarp showing well-developed carposporophyte bearing two gonimolobes (gl1 and gl2) and the broadened pit connection (pc) between the gonimoblast initial (gi) and fusion cell (fc). Note that cells of the ampullar filaments largely elongate (black arrows), forming a conspicuous filamentous pericarp, and are distally

pit-connecting to surrounding cortical cells (arrowheads) and more secondary medullary filaments (white arrows) are produced below the fusion cell (PERTH 06549683). Scale bar = 50 µm.

Figs 11-19. *Halymenia taiwanensis* Showe M.Lin, C.Rodríguez-Prieto & Huisman *sp. nov.* Thallus morphology, vegetative structure and tetrasporophyte. Stained with haematoxylin (Figs 14, 15); stained with aniline blue (Figs 16-19).

Fig. 11. Holotype (NTOU-001649), a female plant, showing a large foliose thallus attached to the substrate by a small discoid holdfast (white arrow). Note the typical plant has smooth (black arrows) and dentate margins (arrowheads). Scale bar = 5 cm.

Fig. 12. An isotype (NTOU-001661), a tetrasporangial plant, showing a large foliose thallus with many marginal dentation (black arrowheads) and lobes (black arrows) and surface bladelets (white arrowheads), and the small discoid holdfast (white arrow). Scale bar = 5 cm.

Fig. 13. Plant photographed *in situ* at ca. 5 m depth showing a large, brick red blade with bullate spots (arrowheads) and surface proliferations (arrows). (HdCJ14v2012-1). Scale bar = 5 cm.

Fig. 14. Transverse section through a thallus blade showing the subcortical stellate cells with short arms (arrowheads) and the medullary filaments that can be either irregularly orientated (black arrows), or anticlinally arranged (white arrows), and in this case joining the subcortical cells from both sides of the cortex (HGI-A 20997). Scale bar = 50 µm.

Fig. 15. Transverse section of another thallus blade showing the obovoid, outer cortical cells (white arrows), outer (black arrowheads) and innermost (white arrowheads) subcortical cells, and medullary filaments (black arrows). Note that the outer cortical cells do not form any secondarily pit connections, whereas the innermost cortical cells are secondarily pit-connected one another and become stellate, with short arms (HGI-A 20874). Scale bar = 20 µm.

Fig. 16. Another transverse section of the blade showing a darkly staining medullary cell (arrow), which has bigger size and bears longer arms compared to the stellate subcortical cells (arrowheads) (HGI-A 20874). Scale bar = 20 µm.

Fig. 17. Transverse section through a tetrasporangial sorus showing the tetrasporangial initials (black arrows) cut off from the subcortical cells (white arrowheads). Note that the sterile surface cells in the vicinity of the tetrasporangial initials are relatively slender (white arrows) (HGI-A 20999). Scale bar = 20 µm.

Fig. 18. Another transverse section of the tetrasporangial sorus showing the tetrasporocytes (black arrows) borne on the subcortical cells (white arrowheads) as well as an enlarged and dividing tetrasporocyte (black arrowhead). Note that the sterile surface cells (white arrows) are elongating (HGI-A 20999). Scale bar = 20 µm.

Fig. 19. Another transverse section through the tetrasporangial sorus showing the developing tetrasporangia (black arrowheads) borne on the subcortical cells (white arrowheads) sub-terminally. Note that the further elongated sterile cortical cells (white arrows) act as paraphyses for protecting the tetrasporangia (HGI-A 20999). Scale bar = 20 µm.

Figs 20-29. *Halymenia taiwanensis* Showe M.Lin, C.Rodríguez-Prieto & Huisman *sp. nov.* Development of spermatangia, carpogonial branch and auxiliary cell ampullae and early diploidization stages. Stained with haematoxylin (Figs 20-24, 26-29); stained with aniline blue (Fig. 25).

Fig. 20. Transverse section through a spermatangial sorus showing spermatangial parent cells (arrows) differentiated from the cortical cells (HGI-A 20874). Scale bar = 10 µm.

Fig. 21. Detail of the spermatangia (arrowheads) cut off from the spermatangial parent cells (arrows) singly or in a pair (HGI-A 20874). Scale bar = 10 µm.

Fig. 22. Transverse section through a well-developed spermatangial sorus showing many spermatangia (arrowheads) cut off from the differentiated cortical cells (HGI-A 20874). Scale

bar = 10 µm.

Fig. 23. Cross section through a fertile blade showing an early stage of young carpogonial branch ampulla which is composed of a carpogonial branch initial (cbi) borne on a supporting cell flanked by two branched ampullar filaments (af1 & af2). Note that the supporting cell (sc) borne on the initial (af1i) of the (af1) is differentiating from the first cell of the af2 (HGI-A 20874). Scale bar = 10 µm.

Fig. 24. A later stage of Fig. 23 showing a 2-celled carpogonial branch composed of a carpogonium (cp) bearing a trichogyne (tr) at the distal end and a hypogynous cell (hy), the two branched ampullar filaments (af1 & af2) and the supporting cell (sc). Note that a basal cellular cluster (arrows) is secondarily produced from the innermost cortical cells (HGI-A 20874). Scale bar = 10 µm.

Fig. 25. Detail of a fully developed carpogonial branch ampulla showing the elongated trichogyne (tr) borne on the carpogonium (cp), the hypogynous cell (hy) and the two further branched ampullar filaments (af1 & af2). Note that the cells of the basal cellular cluster (arrows) become much larger at this stage (HGI-A 20874). Scale bar = 20 µm.

Fig. 26. Detail of a developing auxiliary cell ampulla, composed of three orders of ampullar filaments (af1, af2 & af3). Note that the auxiliary cell (aux) is differentiated from the basal cell of the af3, which is cut off the first cell (afi) of the af2 (HGI-A 20874). Scale bar = 20 µm.

Fig. 27. A developed auxiliary cell ampulla showing further branched ampullar filaments (af1, af2, af3). Note that the af2 is borne on the first cell (af1i) of the af1 and the auxiliary cell (aux) is borne on the first cell (af2i) of af2 (HGI-A 20874). Scale bar = 20 µm.

Fig. 28. A fully developed auxiliary cell ampulla showing enlarged auxiliary cell (aux) and the highly branched ampullar filaments (arrows) before diploidization (HGI-A 20874). Scale bar = 20 µm.

Fig. 29. Detail of an early stage of post-diploidization showing few gonimoblast cells (g) borne on the gonimoblast initial (gi), the multinucleate fusion cell (fc) and the neighbouring

ampullar cells (black arrowheads). Note that the elongated, branched ampullar filaments (black arrows) are pit-connected to secondary medullary filaments (white arrows) (HGI-A 20997). Scale bar = 20 µm.

Figs 30-35. *Halymenia taiwanensis* Showe M.Lin, C.Rodríguez-Prieto & Huisman *sp. nov.* Cystocarp development. Stained with haematoxylin (Figs 30, 32, 35); stained with aniline blue (Figs 31, 33, 34).

Fig. 30. Cross section through a young cystocarp showing developing gonimoblast (g) borne on the gonimoblast initial (gi) flanked by darkly staining, branched ampullar filaments (black arrows). Note that the pit connection between the newly formed fusion cell (fc) and the gonimoblast initial (gi) remains distinct and the basal secondary medullary filaments (white arrow) are only weakly developed (HGI-A 20874). Scale bar = 20 µm.

Fig. 31. A later stage of Fig. 30, showing developing gonimoblast (g) and the broadened pit connection (pc) between the enlarged gonimoblast initial (gi) and fusion cell (fc). Note that the cells (black arrows) of the ampullar filaments are elongating further and some more secondary medullary filaments (white arrows) are produced (HGI-A 20997). Scale bar = 20 µm.

Fig. 32. Cross section through a young cystocarp, showing the ostiole (o), a compact gonimoblast (g) and a secondary connecting filament (cf’) produced from the basal part of the enlarged fusion cell (fc). Note that the fusion cell (fc) is flanked by the elongated ampullar filaments (black arrows) and many more secondary medullary filaments (white arrows) are derived from the floor of the cystocarp (HGI-A 20997). Scale bar = 50 µm.

Fig. 33. Cross section through an immature cystocarp showing a cone-shaped gonimoblast (g) borne on the gonimoblast initial (gi) and the elongated fusion cell (fc). Note that the gonimoblast is flanked by the loosely arranged ampullar (black arrows) and secondary medullary (white arrows) filaments (HGI-A 20997). Scale bar = 50 µm.

Fig. 34. Cross section through a mature cystocarp showing the well-developed gonimoblast with two gonimolobes (gl1 and gl2) and the gonimoblast initial (gi) borne on the branched fusion cell (fc). Note that the gonimoblast is blanketed by a slightly protruding pericarp (p) and surrounded by the loosely arranged ampullar (black arrows) and secondary medullary (white arrows) filaments (HGI-A 20997). Scale bar = 50 µm.

Fig. 35. Cross section through another mature cystocarp showing the oval shaped gonimoblast with two gonimolobes (gl1 and gl2), the remaining gonimoblast initial (gi) and the branched fusion cell (fc) are loosely surrounded by the darkly stained ampullar (black arrows) and secondary medullary (white arrows) filaments. Note that most cells of the gonimoblast are differentiated into carposporangia (HGI-A 20997). Scale bar = 50 µm.

Fig. S1. Global Maximum likelihood phylogeny of the genus *Halymenia* based on the *rbc*L+LSU dataset. Numbers at nodes indicate bootstrap support and Bayesian posterior. The species names in bold refer to the new sequences generated in this study.

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Figure 1

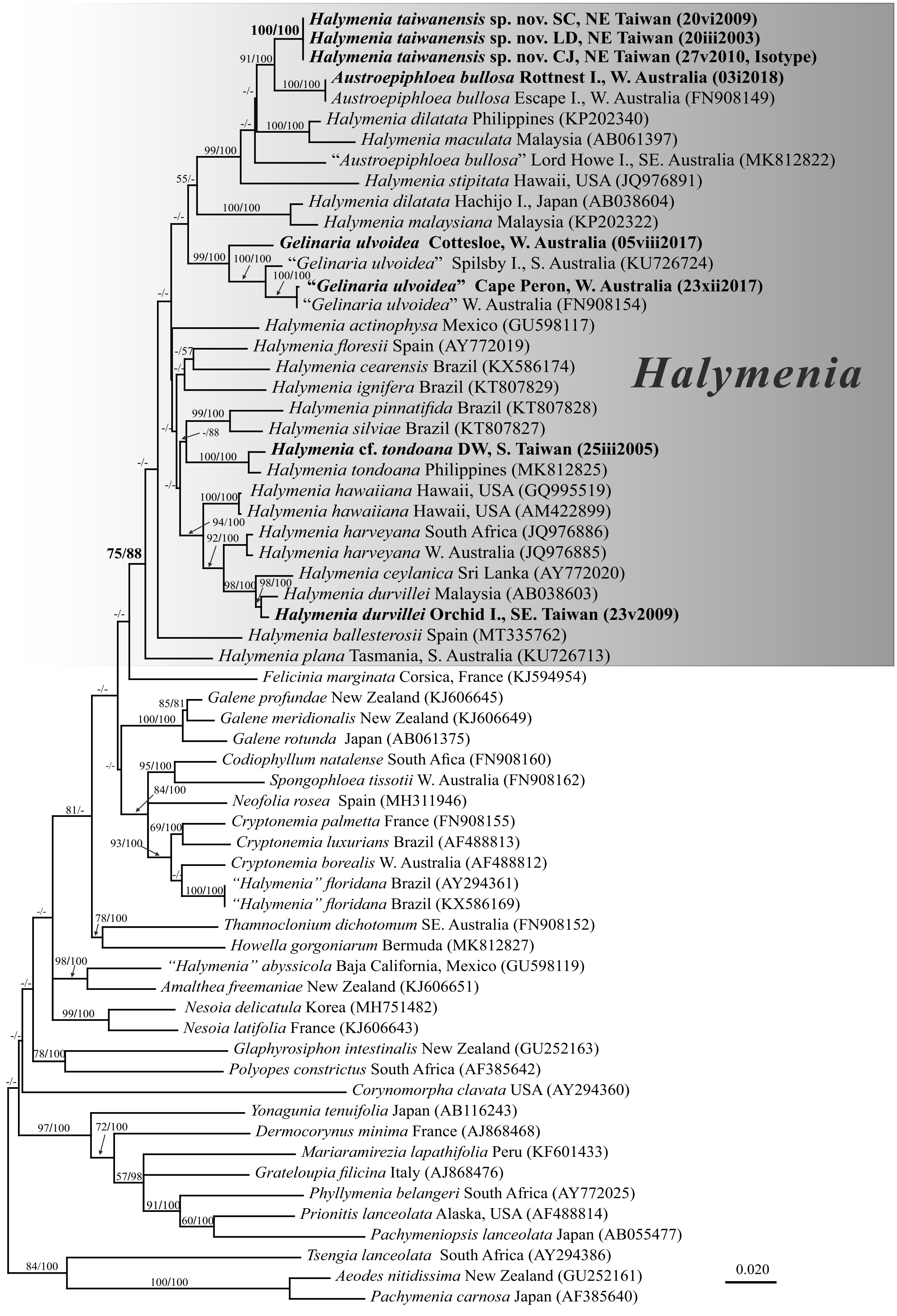

100/100
Halymenia taiwanensis sp. nov. SC, NE Taiwan (20vi2009)
Halymenia taiwanensis sp. nov. LD, NE Taiwan (20iii2003)
91/100
Halymenia taiwanensis sp. nov. CJ, NE Taiwan (27v2010, Isotype)
Austroepiphloea bullosa Rottnest I., W. Australia (03i2018)
-/-
100/100
Austroepiphloea bullosa Escape I., W. Australia (FN908149)
-/-
100/100
Halymenia dilatata Philippines (KP202340)
Halymenia maculata Malaysia (AB061397)
99/100
"Austroepiphloea bullosa" Lord Howe I., SE. Australia (MK812822)
55/-
Halymenia stipitata Hawaii, USA (JQ976891)
100/100
Halymenia dilatata Hachijo I., Japan (AB038604)
-/-
Halymenia malaysiana Malaysia (KP202322)
Gelinaria ulvoidea Cottesloe, W. Australia (05viii2017)
99/100
100/100
"Gelinaria ulvoidea" Spilsby I., S. Australia (KU726724)
100/100
"Gelinaria ulvoidea" Cape Peron, W. Australia (23xii2017)
-/-
"Gelinaria ulvoidea" W. Australia (FN908154)
Halymenia actinophysa Mexico (GU598117)
-/57
Halymenia floresii Spain (AY772019)
-/-
Halymenia cearensis Brazil (KX586174)
Halymenia
Halymenia ignifera Brazil (KT807829)
99/100
Halymenia pinnatifida Brazil (KT807828)
-/-
-/88
Halymenia silviae Brazil (KT807827)
Halymenia cf. tondoana DW, S. Taiwan (25iii2005)
100/100
-/-
Halymenia tondoana Philippines (MK812825)
-/-
100/100
Halymenia hawaiiana Hawaii, USA (GQ995519)
Halymenia hawaiiana Hawaii, USA (AM422899)
94/100
Halymenia harveyana South Africa (JQ976886)
92/100
75/88
Halymenia harveyana W. Australia (JQ976885)
Halymenia ceylanica Sri Lanka (AY772020)
98/100
98/100
Halymenia durvillei Malaysia (AB038603)
Halymenia durvillei Orchid I., SE. Taiwan (23v2009)
-/-
Halymenia ballesterosii Spain (MT335762)
Halymenia plana Tasmania, S. Australia (KU726713)
Felicinia marginata Corsica, France (KJ594954)
-/-
85/81
Galene profundae New Zealand (KJ606645)
100/100
Galene meridionalis New Zealand (KJ606649)
Galene rotunda Japan (AB061375)
-/-
95/100
Codiophyllum natalense South Afica (FN908160)
Spongophloea tissotii W. Australia (FN908162)
84/100
Neofolia rosea Spain (MH311946)
81/-
69/100
Cryptonemia palmetta France (FN908155)
93/100
Cryptonemia luxurians Brazil (AF488813)
Cryptonemia borealis W. Australia (AF488812)
-/-
100/100
"Halymenia" floridana Brazil (AY294361)
"Halymenia" floridana Brazil (KX586169)
-/-
78/100
Thamnoclonium dichotomum SE. Australia (FN908152)
Howella gorgoniarum Bermuda (MK812827)
98/100
"Halymenia" abyssicola Baja California, Mexico (GU598119)
-/-
Amalthea freemaniae New Zealand (KJ606651)
99/100
Nesoia delicatula Korea (MH751482)
Nesoia latifolia France (KJ606643)
-/-
78/100
Glaphyrosiphon intestinalis New Zealand (GU252163)
Polyopes constrictus South Africa (AF385642)
-/-
Corynomorpha clavata USA (AY294360)
Yonagunia tenuifolia Japan (AB116243)
97/100
72/100
Dermocorynus minima France (AJ868468)
Mariaramirezia lapathifolia Peru (KF601433)
57/98
Grateloupia filicina Italy (AJ868476)
Phyllymenia belangeri South Africa (AY772025)
91/100
Prionitis lanceolata Alaska, USA (AF488814)
60/100
Pachymeniopsis lanceolata Japan (AB055477)
Tsengia lanceolata South Africa (AY294386)
84/100
0.020
Aeodes nitidissima New Zealand (GU252161)
100/100
Pachymenia carnosa Japan (AF385640)

Figure 2-10

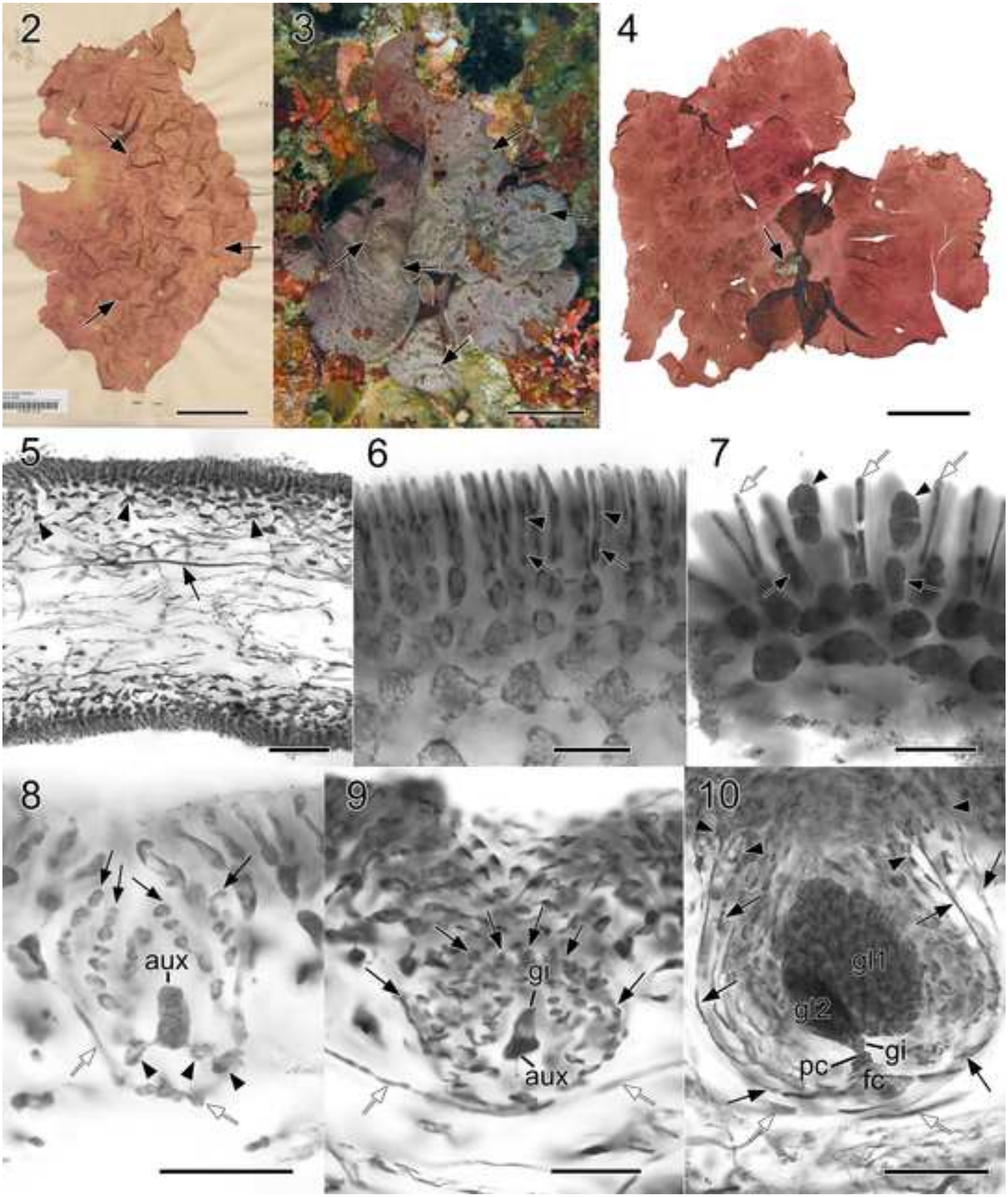
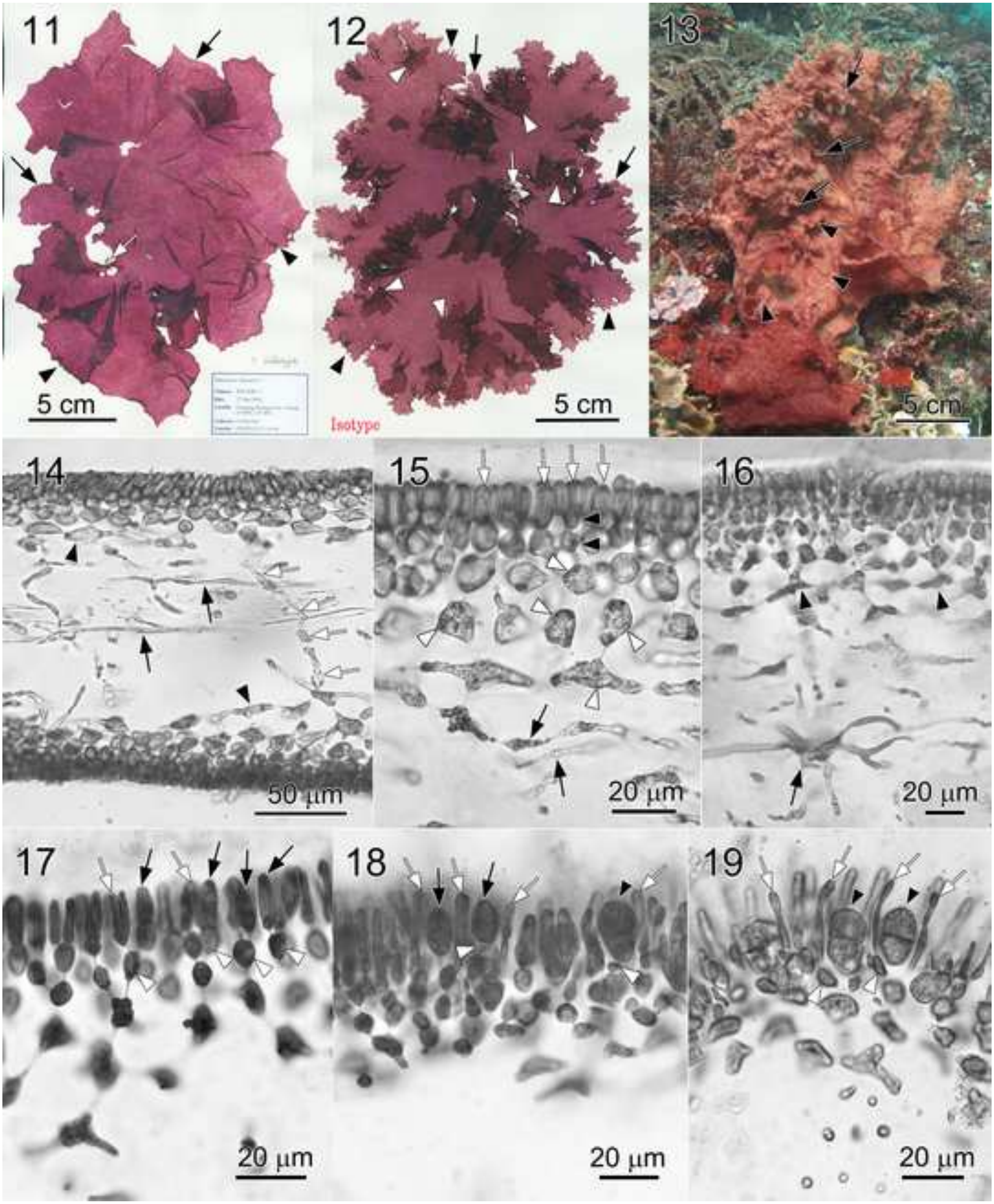


Figure 11-19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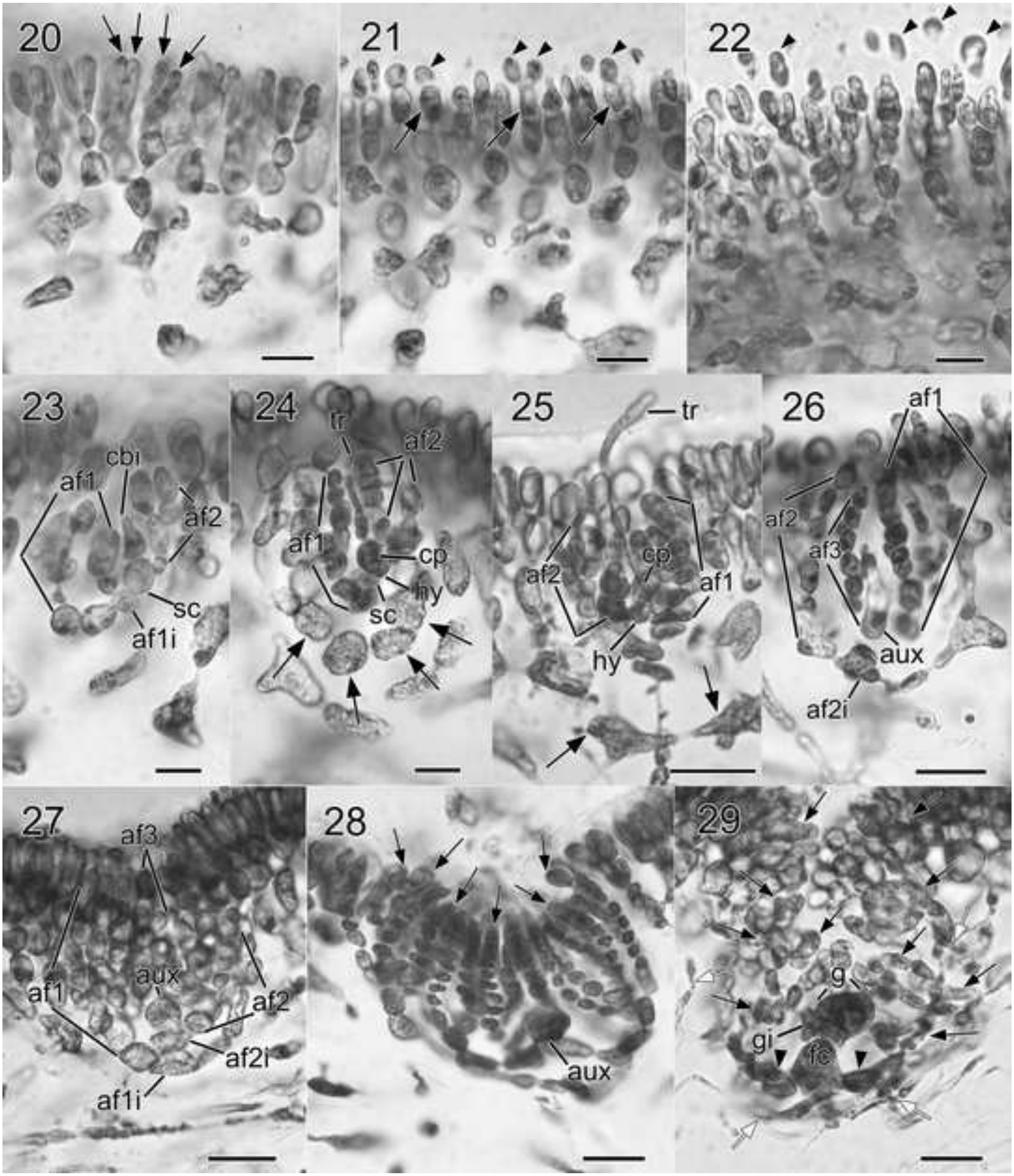
5 cm
12
5 cm
Isotype
13
5 cm
14
50 µm
15
20 µm
16
20 µm
17
20 µm
18
20 µm
19
20 µm

Figure 20-29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21
22
23
cbi
af1
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af1i
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tr
af2
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hy
sc
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af2
cp
af1
hy
26
af1
af2
af3
aux
af2i
27
af3
aux
af1
af2
af2i
af1i
28
aux
29
g
gi
fc

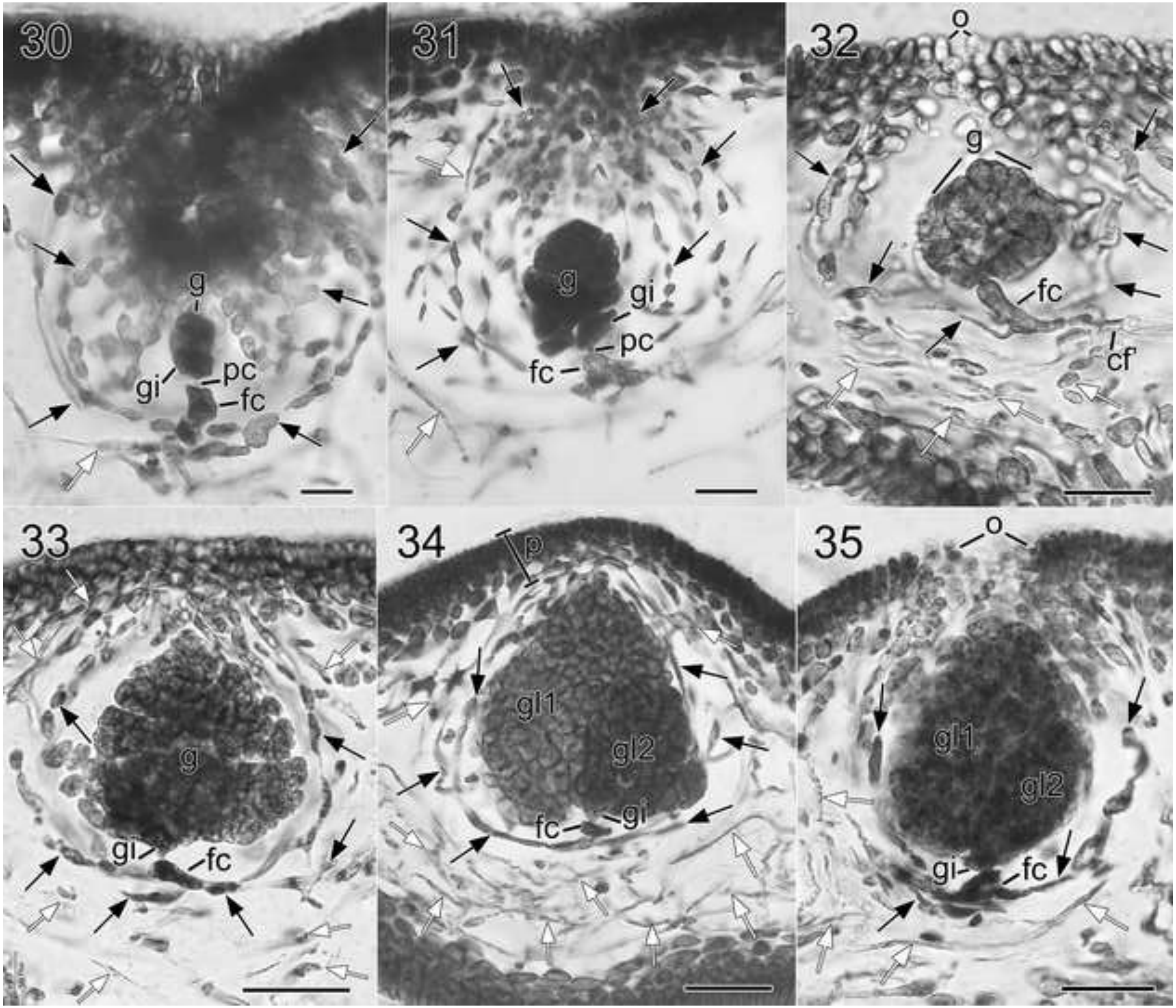
30
g
gi
pc
fc
31
g
gi
pc
fc
32
o
g
fc
cf
33
g
gi
fc
34
p
gl1
gl2
fc
gi
35
o
gl1
gl2
gi
fc

Table S1

Fig. S1