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A PHYLOGENETIC RE-APPRAISAL OF THE FAMILY LIAGORACEAE SENSU LATO (NEMALIALES, RHODOPHYTA) BASED ON SEQUENCE ANALYSES OF TWO PLASTID GENES AND POSTFERTILIZATION DEVELOPMENT[1]

Showe Mei Lin[2]

Institute of Marine Biology, National Taiwan Ocean University, Keelung 20224, Taiwan

Conxi Rodríguez-Prieto

Department of Environmental Sciences, Faculty of Sciences, University of Girona, Campus de Montilivi, Girona 17071, Spain

John M. Huisman

School of Veterinary and Life Sciences, Murdoch University, Murdoch, Western Australia 6150, Australia
Western Australian Herbarium, Science Division, Department of Parks and Wildlife, Bentley Delivery Centre, Locked Bag 104, Bentley, Western Australia 6983, Australia

Michael D. Guiry

The AlgaeBase Foundation, c/o Ryan Institute, National University of Ireland, University Road, Galway, Ireland

Claude Payri

Institut de Recherche pour le Développement, Nouméa 98848, New Caledonia

Wendy A. Nelson

National Institute of Water and Atmospheric Research (NIWA), Private Bag 14-901, Kilbirnie, Wellington 6241, New Zealand
School of Biological Sciences, University of Auckland, Private Bag 92-019, Auckland, New Zealand

and Shao Lun Liu

Department of Life Science, Tunghai University, Taichung 40704, Taiwan

The marine red algal family Liagoraceae sensu lato is shown to be polyphyletic based on analyses of a combined *rbc*L and *psa*A data set and the pattern of carposporophyte development. Fifteen of eighteen genera analyzed formed a monophyletic lineage that included the genus *Liagora*. *Nemalion* did not cluster with Liagoraceae sensu stricto, and Nemaliaceae is reinstated, characterized morphologically by the formation of the primary gonimolobes by longitudinal divisions of the gonimoblast initial. *Yamadaella* and *Liagoropsis*, previously placed in the Dermonemataceae, are shown to be independent lineages and are recognized as two new families Yamadaellaceae and Liagoropsidaceae. Yamadaellaceae is characterized by two gonimoblast initials cut off bilaterally from the fertilized carpogonium and diffusely spreading gonimoblast filaments. Liagoropsidaceae is characterized by at least three gonimoblast initials cut off by longitudinal septa from the fertilized carpogonium. In contrast, Liagoraceae sensu stricto is characterized by a single gonimoblast initial cut off transversely or diagonally from the fertilized carpogonium. Reproductive features, such as diffuse gonimoblasts and unfused carpogonial branches following postfertilization, appear to have evolved on more than one occasion in the Nemaliales and are therefore not taxonomically diagnostic at the family level, although they may be useful in recognizing genera.

Key index words: **carposporophyte development; Liagoraceae sensu stricto; Liagoropsidaceae fam. nov.; molecular phylogeny; Nemaliaceae; *psa*A; *rbc*L; Yamadaellaceae fam. nov**

***Abbreviations*: bp, base pairs; BP, Bootstrap proportion values; ML, maximum likelihood; MP, maximum parsimony; PP, posterior probabilities**

The Liagoraceae Kützing (1843) sensu lato is the largest family of the marine red algal order Nemaliales F. Schmitz (1892 in Engler, as "Reihe Nemalionales," Kylin 1956, as "Nemalionales") and contains some 25 genera, including several that have been previously placed in the families Nemaliaceae (Farlow) De Toni and Levi (1886), Helminthocladiaceae

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[2]Author for correspondence: e-mail linsm@ntou.edu.tw.
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J. Agardh (1851), and Dermonemataceae (Schmitz & Hauptfleisch) I.A. Abbott (Abbott 1976, 1985, Kraft 1988, 1989, Tseng 2005, Huisman 2006, Lin et al. 2011a,b). The life history of members of the Liagoraceae has been shown to have an alternation of microscopic, filamentous tetrasporophytes with macroscopic gametophytes. The filamentous phase produces either monosporangia or tetrasporangia or both, the tetraspores generally germinating to form monoecious or dioecious gametophytes (Guiry 1990). The taxonomic features used for separating the previously established families Nemaliaceae, Helminthocladiaceae (=Liagoraceae sensu stricto), and Dermonemataceae were based largely on differences in postfertilization stages, such as the orientation of the division plane of gonimoblast initials (= cells directly arising from the zygote), the morphology of gonimoblast filaments, and the absence or presence and sites of origin of involucral filaments associated with the carposporophyte (Abbott 1976, Huisman 2006 for a review). Doty and Abbott (1964) revised the taxonomic concepts of the Nemaliaceae and the Helminthocladiaceae, and subsequently, Abbott (1976) proposed elevating the helminthocladiaceous tribe Dermonemeae Schmitz and Hauptfleisch (1896) to family level as the Dermonemataceae, to include the genera *Dermonema* Harvey ex Heydrich, *Yamadaella* (Decaisne) I.A. Abbott, and the new genus *Dotyophycus* I.A. Abbott. She considered that the manner in which the gonimoblast initials were cut off from the fertilized carpogonium and the subsequent fusion of cells of the carpogonial branch plus zygote at the base of gonimoblasts to be different from the families Nemaliaceae and Helminthocladiaceae (= Liagoraceae sensu stricto). In his compendium of algal family names, Silva (1980:85) noted that the Liagoraceae of Kützing (1843: 321, 328 as "Liagoreae") was the earliest correct name for the Helminthocladiaceae, but proposed conservation of the latter, as the name Liagoraceae had "seldom been used." This proposal was subsequently rejected by the Nomenclature Committee of the International Code of Botanical Nomenclature. Silva (1993:707) later remarked that "Since *Liagora* is the better known of the two generic names, it is not surprising that [conservation of] Helminthocladiaceae failed to receive the necessary two-thirds majority."

The taxonomic importance of female reproductive features for the characterization of genera in the Liagoraceae has been controversial (Kraft 1988, 1989). Abbott (1985) proposed to merge the families Nemaliaceae and Dermonemataceae with the Liagoraceae at an international phycological conference presentation in Copenhagen, but did not herself adopt this proposal in her subsequent Hawaiian marine red algal flora (Abbott 1999:58).

In recent years, Lin et al. (2011a,b) reexamined numerous species of *Liagora* J.V. Lamouroux (the type genus of the Liagoraceae), using *rbc*L sequence data to support their morphological observations. Species in *Liagora* have diverse carposporophyte morphologies, including diffuse or compact gonimoblasts and cells of carpogonial branches fusing or remaining discrete during carposporophyte development. The results showed that the generitype, *Liagora viscida* (Forsskål) C. Agardh, which has a diffuse gonimoblast and fused carpogonial branch cells, was closely related to a subset of *Liagora* species that also displayed those features. Conversely, other species with diffuse gonimoblasts and unfused carpogonial branch cells were found to be only distantly related to liagoroid species with either diffuse or compact gonimoblasts but with fused carpogonial branch cells, with the result that several segregate genera were described (Lin et al. 2013, 2014). However, the phylogenetic relationships among genera in the Liagoraceae sensu stricto (bearing compact gonimoblasts, e.g., *Helminthocladia* J. Agardh) and the Dermonemataceae (with diffuse gonimoblast filaments, e.g., *Yamadaella*) remained obscure and largely unexplored.

A further reproductive feature used to distinguish genera of the Liagoraceae sensu lato is the presence and nature of involucral filaments that densely or laxly surround developing carposporophytes. In the present study, we examined genera that lack such structures altogether (viz *Nemalion*, *Yamadaella*, *Liagoropsis,* and *Dermonema*) and focused on postfertilization events, providing additional information on *Helminthocladia*, the type genus of the previously recognized family Helminthocladiaceae. The significance of the differences in carposporophyte development among genera in the *Liagoraceae* sensu lato is further assessed, as well as their phylogenetic relationships as inferred from *rbc*L and *psa*A sequence analyses.

MATERIALS AND METHODS

Specimens were collected from intertidal reefs and tidal pools or subtidally by snorkeling or SCUBA diving. Samples used in morphological studies were preserved in 5% formalin in seawater or pressed on herbarium sheets. Voucher specimens were deposited in the Seaweed Laboratory at the National Taiwan Ocean University, Taiwan, herbaria of the University of Girona, Spain (HGI), and of the National University of Ireland, Galway (GALW), and the Museum of New Zealand Te Papa Tongarewa (WELT). Herbarium abbreviations follow Thiers (2014; continuously updated). The species names used herein were either based on the marine floral studies made by the various authors or followed the studies of Lin et al. (2011a,b, 2013, 2014). The collection information and the herbarium numbers are seen in Table S1 in the Supporting Information. Hand sections were stained with 1% aniline blue acidified with 1% HCl and mounted in 25%–30% Karo® syrup (CPC International, Inc., Englewood Cliffs, NJ, USA) or were treated with full or reduced concentration of Wittmann's aceto-iron-hematoxylin-chloral hydrate (Wittmann 1965) and mounted in 50% Hoyer's mounting medium or in 40% Karo® light corn syrup (Lin et al. 2014). Habit pictures were taken with a Nikon D300 (Nikon Corp., Tokyo, Japan). Photomicrographs were

taken on an Olympus BX51 microscope with a Q-imaging digital camera (Burnaby, BC, Canada) or made with an AxioCam MRc attached to an Axioskop 2 plus microscope (Zeiss, Oberchohen, Germany).

DNA materials were extracted either from newly collected specimens dried in silica gel or from the DNA collections of the Liagoraceae deposited at the Seaweed Laboratory, NTOU, Taiwan, as used in Lin et al. (2011a,b, 2013, 2014). DNA samples were prepared using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the instructions of the manufacturer. DNA amplification procedures and the *rbc*L primers (F7+R753, F7+R1150, F64+R753, F64+R1150, F492+R1150, F645+*Rbc*S start, F993+*Rbc*S start) were as described by Freshwater and Rueness (1994), Lin et al. (2001), and Gavio and Fredericq (2002). The *psa*A gene was amplified and sequenced using the primers (*psa*A130F, *psa*A180F & *psa*A-3) as described by Yoon et al. (2002) and combined with the primers designated by Yang and Boo (2004) (*psa*A1110R & *psa*A971F) as well as two newly designed primers *psa*A1492F (5′-ACA GCA CCT AAT GCC TTA A-3′) and *psa*A1155F (5′-AGG AAA TAA AGT GGC AAT GA-3′) specifically for the genera *Nemalion*, *Helminthocladia,* and *Yamadaella.* New sequence data and those available from GenBank (Lin et al. 2011a,b, 2013, 2014 for some Liagoraceae, Wang et al. 2005 for some Galaxauraceae, Müller et al. 2002 and Yoon et al. 2002 for *Thorea violacea* Bory de Saint-Vincent, and Yang and Boo [unpublished data] for *Acrochaetium savianum* [Meneghini] Nägeli and *Batrachospermum gelatinosum* [Linnaeus] De Candolle) were compiled and aligned with Sequencher (Gene Codes Corp., Ann Arbor, MI, USA) and exported for phylogenetic analysis.

18 *rbc*L and 50 *psa*A sequences were newly generated (see Table S1). Phylogenetic analyses were performed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian analysis. MP and nonparametric bootstrapping followed Lin et al. (2011a). We used PAUP* v4.0 (Swofford 2003) for MP analyses and MEGA 5.03 (Tamura et al. 2011) for ML analyses. Bootstrap proportion values (BP) involved 1,000 and 500 replicates for MP and ML analyses, respectively. The substitution model for ML was the general-time-reversible with gamma-distributed rate heterogeneity (GTR+I+Γ) as suggested by running "Find best DNA model" implemented in MEGA 5.03. A Bayesian analysis (BA) was performed in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using a GTR+I+Γ model, which allowed for rate variation among different codon positions. The analysis consisted of four chains (one hot and three cold), which were run for 2×10^6 generations with sampling every 100 generations. Burn-in values (Bayesian posterior probabilities, PP) were set at 25,000 generations, in which the split frequency of standard deviation is greater than 0.01. A 50% consensus tree (majority rule as implemented by PAUP* v4.0) was computed from the 7,500+1 trees saved after the burn-in point. The Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999) implemented in the PAUP program was used to determine whether the different tree topologies derived from the two molecular phylogenetic data sets (*rbc*L and *psa*A) were statistically congruent.

RESULTS

Molecular analyses. DNA sequences were newly generated for 18 genera in the Liagoraceae sensu lato (*Liagora*, *Izziella* Doty, *Stenopeltis* Itono & Yoshizaki, *Neoizziella* Showe M. Lin, S. Y. Yang, & Huisman, *Macrocarpus* Showe M. Lin, S. Y. Yang, & Huisman, *Titanophycus* Huisman, G.W. Saunders, & A.R. Sherwood, *Akalaphycus* Huisman, I.A. Abbott, & A.R. Sherwood, *Helminthora*, *Trichogloeopsis* I.A. Abbott & Doty, *Hommersandiophycus* Showe M. Lin & Huisman, *Dotyophycus*, *Ganonema* K. C. Fan & Y. C. Wang, *Helminthocladia*, *Cumagloia* Setchell & N.L. Gardner, *Dermonema*, *Yamadaella*, *Nemalion,* and *Liagoropsis* Yamada), four species of *Scinaia* in the Scinaiaceae, and four genera (*Galaxaura*, *Dichotomaria*, *Tricleocarpa,* and *Actinotrichia*) in the Galaxauraceae, including 50 taxa for *psa*A and 18 taxa for *rbc*L (see Table S1). The genera *Acrochaetium*, *Thorea,* and *Batrachospermum* were selected as outgroups, and their sequences were obtained from GenBank based on the studies of Yang and Boo (unpublished data), Müller et al. (2002), and Yoon et al. (2002), as well as 36 *rbc*L sequences of the Liagoraceae previously published by Lin et al. (2011a,b, 2014). The analyzed data matrix included 1,383 base pairs (bp) for *rbc*L with 517 parsimony-informative sites and 1,932 bp for *psa*A with 796 parsimony-informative sites. No insertion or deletion mutations were found in the two data sets of *rbc*L and *psa*A.

The topology of the MP, ML, and BA trees was largely congruent, and only the ML tree was shown for the *rbc*L and *psa*A combined data set (Fig. 1). Both ML topologies of the *rbc*L phylogeny and *psa*A phylogeny alone were similar to the *rbc*L+*psa*A combined data but with weaker statistical supports. Genera from the Liagoraceae sensu lato were clustered into four assemblages (I-IV, Fig. 1), but their phylogenetic relationships were not resolved. Clade I contained the majority of the genera analyzed, including the generitypes of *Liagora*, *Helminthocladia,* and *Dermonema*, the type genera for the families Liagoraceae, Helminthocladiaceae, and Dermonemataceae, respectively. The genera *Yamadaella* (Clade II), *Nemalion* (Clade III), and *Liagoropsis* (Clade IV) formed separate lineages and did not cluster with *Liagora* (Clade I). Although *Yamadaella* and *Nemalion* were clustered together with the family Scinaiaceae and *A. savianum*, their relationships were not supported (less than 50% BP or 0.5 PP). The *rbc*L sequence divergence values among the genera analyzed from the Liagoraceae sensu lato ranged from 8.9% (*Neoizziella* vs. *Macrocarpus*) to 19.5% (*Nemalion* vs. *Yamadaella*), whereas the *psa*A gene sequence divergence values among the same genera ranged from 13.3% (*Neoizziella* vs. *Macrocarpus*) to 21.6% (*Nemalion* vs. *Liagora*).

Morphological observations. Nemalion elminthoides (Velley) Batters (1902: 59) (Fig. 2)

Basionym: *Fucus elminthoides* Velley (in Withering 1792: 255)

Type locality: "off the Beal, at the extremity of Portland," Dorset, England (Velley in Withering 1792). Lectotype: in the Liverpool Museum herbarium (LIV) (selected by Womersley 1994: 81). Nomenclatural note 1: Batters (1902: 59) mentioned seeing authentic material: "*e spec. auth. in Herb. Kew.*" (now at BM), which could be considered

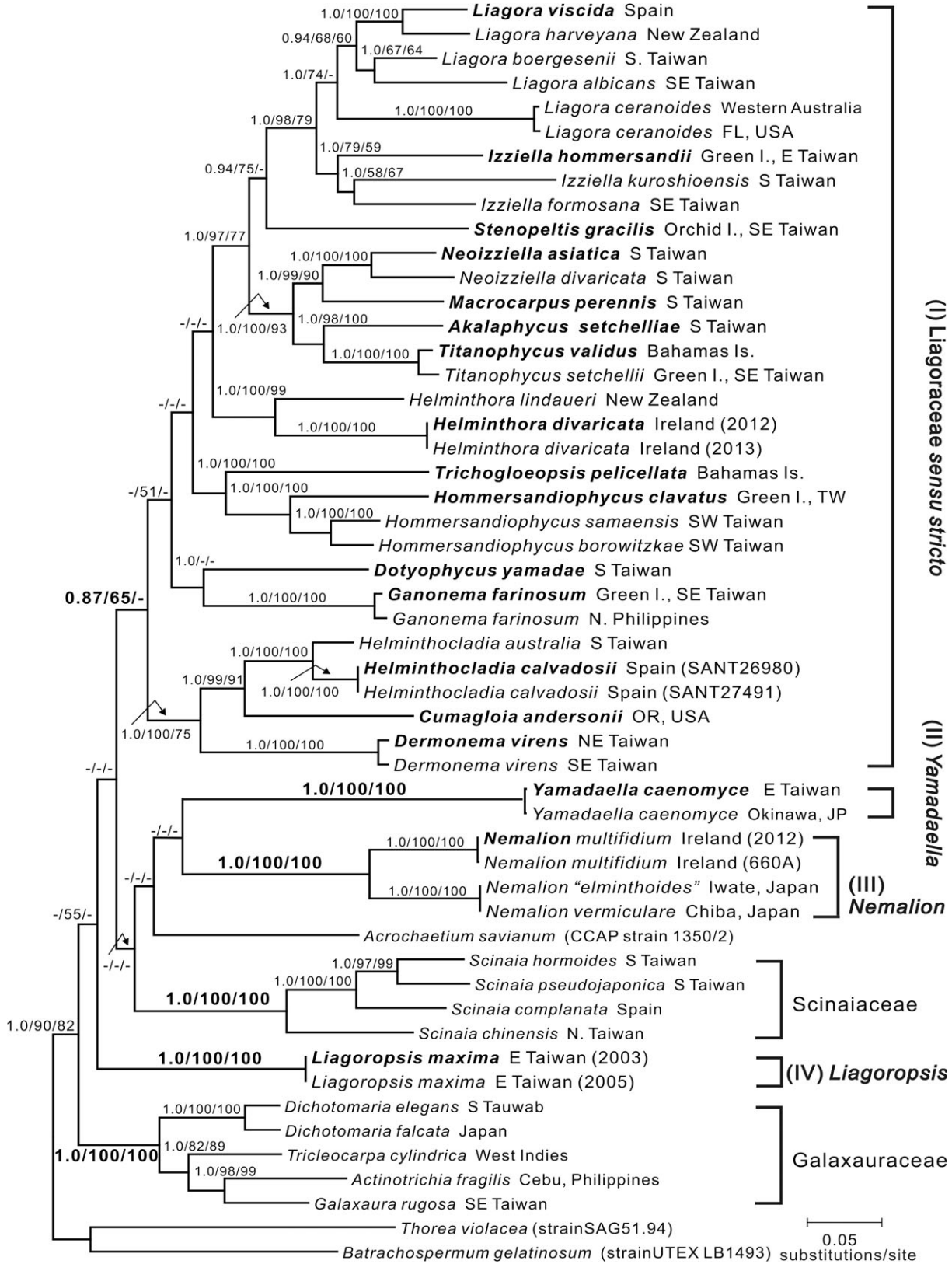

FIG. 1. *Psa*A+*rbc*L phylogeny: Global ML tree (In L = −48817.0031) of Liagoraceae, using selection of genera from related families Scinaiaceae and Galaxauraceae within Nemaliales. Numbers above branches are Bayesian posterior probabilities (PP), ML, and MP bootstrap values (BP), respectively. Dash refers to BP values less than 50% or PP values below 0.5. Note: the names of families, genera, and the species in bold type were traditionally classified in the Liagoraceae sensu lato.

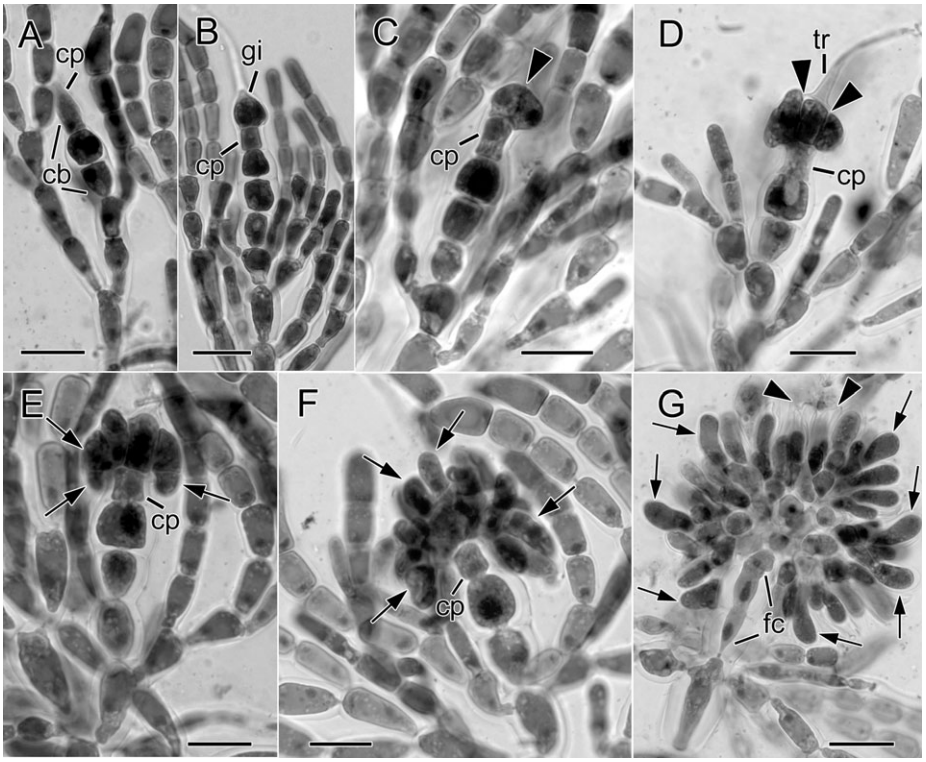

Fig. 2. *Nemalion elminthoides*. Carposporophyte development (A, C–G: HGI-A 9170; B: HGI-A 9169); scale bars: A–G = 20 μm. (A) Three-celled carpogonial branch (cb) with terminal carpogonium (cp). (B) Early postfertilization showing gonimoblast initial (gi) cut off transversely from fertilized carpogonium (cp). (C) Later stage of early postfertilization showing remnants of fertilized carpogonium (cp) and longitudinal division (arrowhead) of gonimoblast initial. (D) Early carposporophyte showing primary gonimoblast cells produced by longitudinal cell division (arrowheads) and remnants of trichogyne (tr). Note pit connection between carpogonium (cp) and hypogynous cell beginning to break down. (E) Later stage showing formation of gonimoblast filaments (arrows) bilaterally. Note carpogonium (cp) fusing with hypogynous cell. (F) Young carposporophyte showing fused inner gonimoblast cells and production of gonimoblast filaments (arrows). (G) Fully developed carposporophyte bearing terminal carposporangia (arrows) and some remnant walls of discharged carposporangia (arrowheads). Note carpogonial branch cells fused (fc).

a prior lectotypification to that of Womersley (see Guiry and Guiry 2014), but this requires further investigation.

Nomenclatural note 2: This species has long passed under the epithet "*helminthoides*," even though Velley (in Withering 1792: 255) did not use "h," and Batters (1902: 59) retained this spelling when he made the transfer to *Nemalion*. The epithet is derived from the Greek ελμινθος or ἕλμινθος (meaning, relating to worms). Generally, when new names were formed, the "*spiritus asper*" ("rough breathing" or what is called an "[h]aitch" in English; see Stearn 2004:255) would have been utilized to make the prefix "helmint-" rather than "elminth-" as in, for example, the genus *Helminthochorton* Zanardini or *Batrachospermum helminthosum* Bory de Saint-Vincent. However, Art. 60.3 of the ICN (McNeill et al. 2012: 128) is quite firm on the matter: "The liberty of correcting a name is to be used with reserve, especially if the change affects the first syllable and, *above all, the first letter of the name* [our emphasis]"; this original spelling has long been employed by Paul Silva in his *Index Nominum Algarum*.

Nomenclatural note 3: The generitype of *Nemalion*, *Nemalion lubricum* Duby (1830), for many years considered to be a heterotypic synonym of *N. elminthoides*, has been restored as a separate species by Le Gall and Saunders (2010) based on molecular evidence.

Examined specimens: Atlantic France, Saint Michel de Plouguerneau, attached on intertidal rocks, 15 July 2010, leg. C. Rodríguez-Prieto (HGI-A 9169 & HGI-A 9170, only *rbc*L successfully sequenced).

Habit and carposporophyte development: Thalli simple or branched, with terete axes, arising from a discoid holdfast on intertidal rocks. Carpogonial branches straight, 3- to 4-celled, borne on shoulder of supporting cell (Fig. 2A). After fertilization, gonimoblast initial cut off transversely from fertilized carpogonium; several sterile filaments cut off from supporting cell or nearby cortical cells (Fig. 2B); gonimoblast initial enlarges and divides longitudinally (Fig. 2C, arrowhead) producing on one side first gonimolobe initial and then divides longitudinally again on another side (Fig. 2D, arrowheads);

two gonimolobe initials divide obliquely or transversely producing gonimoblast filaments and cells of carpogonial branch begin to fuse (Fig. 2E); gonimolobes cut off radially new filaments by both transverse and oblique cell divisions (Fig. 2F); when mature, terminal gonimoblast cells differentiate into carposporangia; pit connections between carpogonial branch cells break down to form fusion cell (Fig. 2G).

Dermonema virens (J. Agardh) Pedroche and Avila Ortíz (1996: 77) (Fig. 3)

Basionym: *Nemalion virens* J. Agardh (1847:8).

Type locality: San Agustín, Oaxaca, Mexico.

Nomenclatural note: The generitype (monotype) of *Dermonema*, *Dermonema dichotomum* Harvey ex Heydrich (1894:289), was described by Heydrich on the basis of material collected at Kelung on the north coast of Formosa (now Keelung, Taiwan). However, Heydrich (1894:289) opted to adopt an unpublished name of Harvey's in an exsiccata, "Algae from Ceylon, Friendly Islands and Australia. Collected during the years 1852, 1853, 1854 and 1855." This name is illegitimate as Heydrich placed it in synonymy under *Gymnophlaea gracilis* Martens, thus rendering it superfluous. This is discussed in great detail by Silva in Silva et al. (1996, pp. 915–6). Nevertheless, the type of the genus is *D. dichotomum* Harvey ex Heydrich, regardless of its legitimacy and is typified by material collected by Ferguson from Ceylon and distributed by Harvey. The question of the conspecificity of Heydrich's material from Taiwan (upon which the concept of the genus is essentially based), Harvey's material from Sri Lanka, and Agardh's material of *N. virens* from México remains

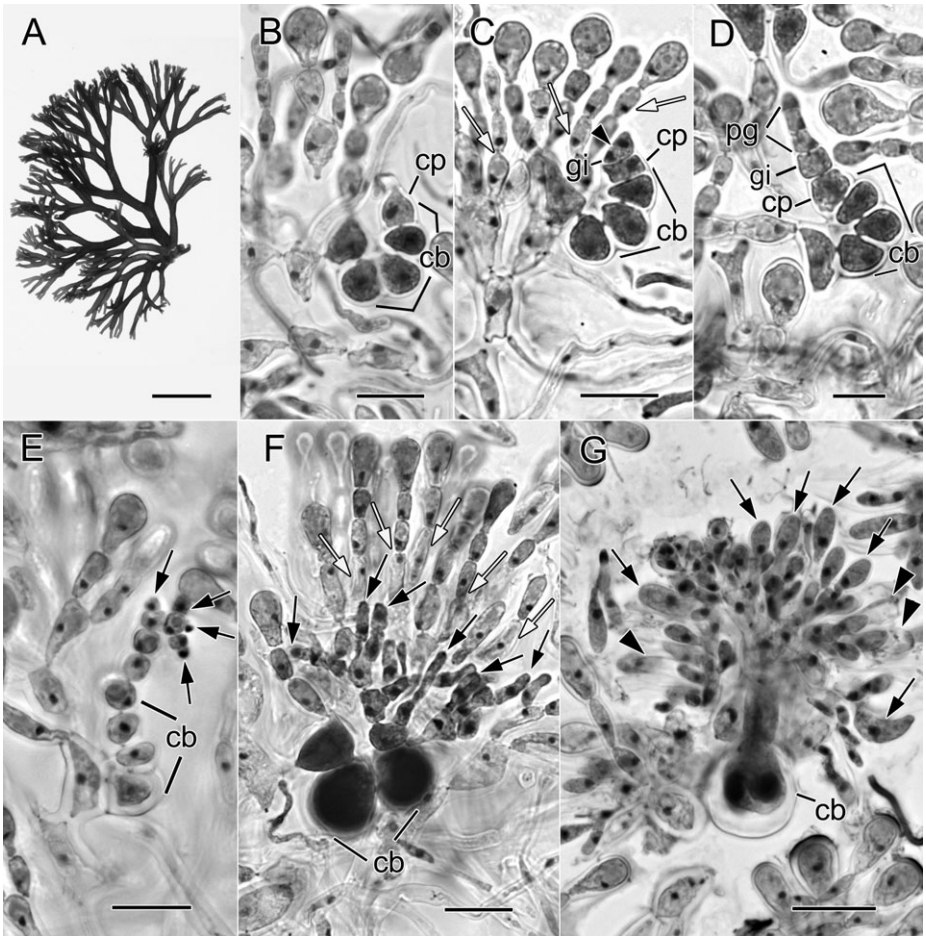

FIG. 3. *Dermonema virens*. Carposporophyte development (A, E: NTOU28032006Dv; B–D, G: NTOU09032008; F: NTOU432006); scale bars: A = 1 cm; B–C, E–G = 25 μm; D = 10 μm. (A) Thallus habit. (B) Prefertilization four-celled carpogonial branch (cb) with carpogonium (cp). (C) Early postfertilization showing carpogonial branch (cb) and presumably fertilized carpogonium (cp) cutting off a gonimoblast initial (gi) that divide obliquely (arrowhead) to produce a young gonimoblast cell. Note cortical cells (white arrows) adjacent to carpogonial branch slightly elongated. (D) Early postfertilization stage showing carpogonial branch (cb), fertilized carpogonium (cp), gonimoblast initial (gi), and young gonimoblast cells (pg) growing acropetally. (E) Later early postfertilization stage showing lateral and outward spread of gonimoblast filaments (arrows) and unmodified carpogonial branch (cb). (F) Young carposporophyte showing unilaterally developing gonimoblast filaments (black arrows) and elongating cortical filaments (white arrows). Note enlarged and darkly stained cells of carpogonial branch (cb). (G) Mature carposporophyte bearing terminal carposporangia (arrows), remaining cell walls (arrowheads) of released carposporangia and flask-shaped, fused carpogonial branch (cb).

unresolved at this time. The identity of *G. gracilis* also remains to be resolved.

Examined specimens: Taiwan: Chao Jing, Keelung City, attached on intertidal rocks, March 28, 2006 (NTOU28032006Dv, sequenced for both *rbc*L and *psa*A), leg. S. M. Lin; Lungkeng, Kenting National Park, Pingtung Co., attached on intertidal rocks, March 9, 2008 (NTOU09032008, sequenced for both *rbc*L and *psa*A), leg. S. M. Lin; San Xin Tai, Taitung Co., attached on intertidal rocks, March 4, 2006 (NTOU432006), leg. S. M. Lin.

Habit and carposporophyte development: Thalli with terete, dichotomous axes arising from discoid holdfast (Fig. 3A) attached to rocks or coral reefs in intertidal zone. Carpogonial branches curved, three- or four-celled, medial on assimilatory filaments (Fig. 3B). After presumed fertilization, gonimoblast initial cut off transversely from fertilized carpogonium; inner cortical cells near supporting cell elongated slightly (Fig. 3C); gonimoblast initial divided transversely several times resulting in a uniseriate series of four to six cells (Fig. 3D); these cut off gonimoblast filaments laterally (Fig. 3E) and then branched radially and carpogonial branch cells enlarged (Fig. 3F); when carposporophyte matures, terminal cells of gonimoblast filaments differentiated into carposporangia; pit connections between carpogonial branch cells broadened, forming elongated fusion cell (Fig. 3G).

Helminthocladia australis Harvey (1863:39)

Type locality: Fremantle, Western Australia. Lectotype: TCD 0011731.

Examined specimens: Taiwan: Lintou Park, Penghu Co. in Taiwan Strait, growing in subtidal zone at 1–2 m water depths, April 9, 2010 (NTOU09042010, only *rbc*L successfully sequenced), leg. S. M. Lin; Chuan Fan Shi, Pingtung Co., growing in subtidal zone at 2–3 m water depths, January 15, 2006 (NTOU15012006Ha, both *rbc*L and *psa*A sequenced), leg. S. L. Liu.

Habit and carposporophyte development: Specimens examined agreed with the description and illustrations of Huisman and Womersley (2006:48–54) except for the morphology of the carpogonial branch in mature carposporophytes. Carpogonial branches four-celled and borne on supporting cells in proximal portions of assimilatory filaments; after presumed fertilization, carpogonium enlarged slightly (Fig. 4A) and two lobes of sterile initials cut off from two cortical cells near middle portion of carpogonial branch (Fig. 4, A and B, arrows); gonimoblast initial cut off transversely from carpogonium, and gonimoblast initial divided obliquely giving rise to gonimolobe initial, which grew to form compact gonimolobe; trichogyne disintegrated (Fig. 4C); meanwhile, two sterile lobes produced earlier on adjacent cortical cells enlarged (Fig. 4C) and became multinucleate (Fig. 4D); similar situation seen in species currently recognized as generitype of *Helminthocladia*, *H. calvadosii* (J.V. Lamouroux ex Duby) Setchell (Fig. 4F, arrow); early in gonimoblast development, only few branched involucral filaments produced from cortical cells in vicinity of carpogonial branch (illustrated also by Womersley 1994, fig. 23C); as gonimoblast matured, pit connections between carpogonial branch cells broadened but fully developed fusion cell not formed (Fig. 4E).

Yamadaella caenomyce (Decaisne) I.A. Abbott (1970: 117; Fig. 5).

Basionym: *Liagora caenomyce* Decaisne (1842:119).

Type locality: Manila, Luzon, the Philippines (fide Silva et al. 1996:130). Lectotype: H. Cuming 2222; L (fide Abbott 1970:117, Huisman 2006:86).

Nomenclature note: Although we were not able to study the type specimen, Abbott (1970: 117–118) did examine some specimens of *Yamadaella caenomyce* collected from Formosa (now Taiwan) and from the Ryukyu Archipelago by the Japanese phycologists Yamada and Tanaka.

Examined specimens: Taiwan: Taitung Co.: Three Fairy, attached on intertidal rocks, March 20, 2010 (NTOU20032010), leg. S. M. Lin; Yalulan, attached on intertidal rocks, June 15, 2007 (NTOU15062007), leg. S. M. Lin; Shanyuan, attached on intertidal rocks, June 9, 2005 (NTOU09062005, only *rbc*L successfully sequenced), leg. S. M. Lin; Jeefui, attached on intertidal rocks, March 1, 2009 (NTOU01032009, sequenced for both *rbc*L and *psa*A), leg. S. M. Lin.

Habit and carposporophyte development: Thalli 2–3 (-4) cm tall, strongly calcified, branched subdichotomously to 4–6 orders, and occur in decumbent clumps or mats in patches (Fig. 5A) or on tops of coral reefs intertidally; only monoecious gametophytes found in field. Carpogonial branches three-celled, straight, lateral from basal or proximal portion of assimilatory filaments, carpogonia with relatively short, terminal trichogynes (Fig. 5B); post-fertilization stages rarely seen, restricted to lightly calcified tips of branches; after presumed fertilization, zygote cut off a gonimoblast initial laterally (Fig. 5C) followed by another lateral gonimoblast initial (Fig. 5D) from opposite side; at early stage of gonimoblast development, gonimoblast initials elongated and branched adaxially producing gonimoblast filament initials (Fig. 5E); as carposporophytes matured, gonimoblast filaments branched radially and loosely (Fig. 5F); pit connections between cells of carpogonial branch broadened to form slender fusion cell (Fig. 5G); at later stage of carposporophyte development, gonimoblast filaments further elongated and branched once or twice (Fig. 5G); up to this stage, assimilatory filaments remained unmodified and no involucral filaments cut off from cortical cells in vicinity of carposporophyte (Fig. 5G); when mature, terminal cells of gonimoblast filaments differentiated into carposporangia and two gonimoblast initial cells remained distinct (Fig. 5H, arrows); carposporangia undivided in Taiwan specimens, decussately or cruciately divided in

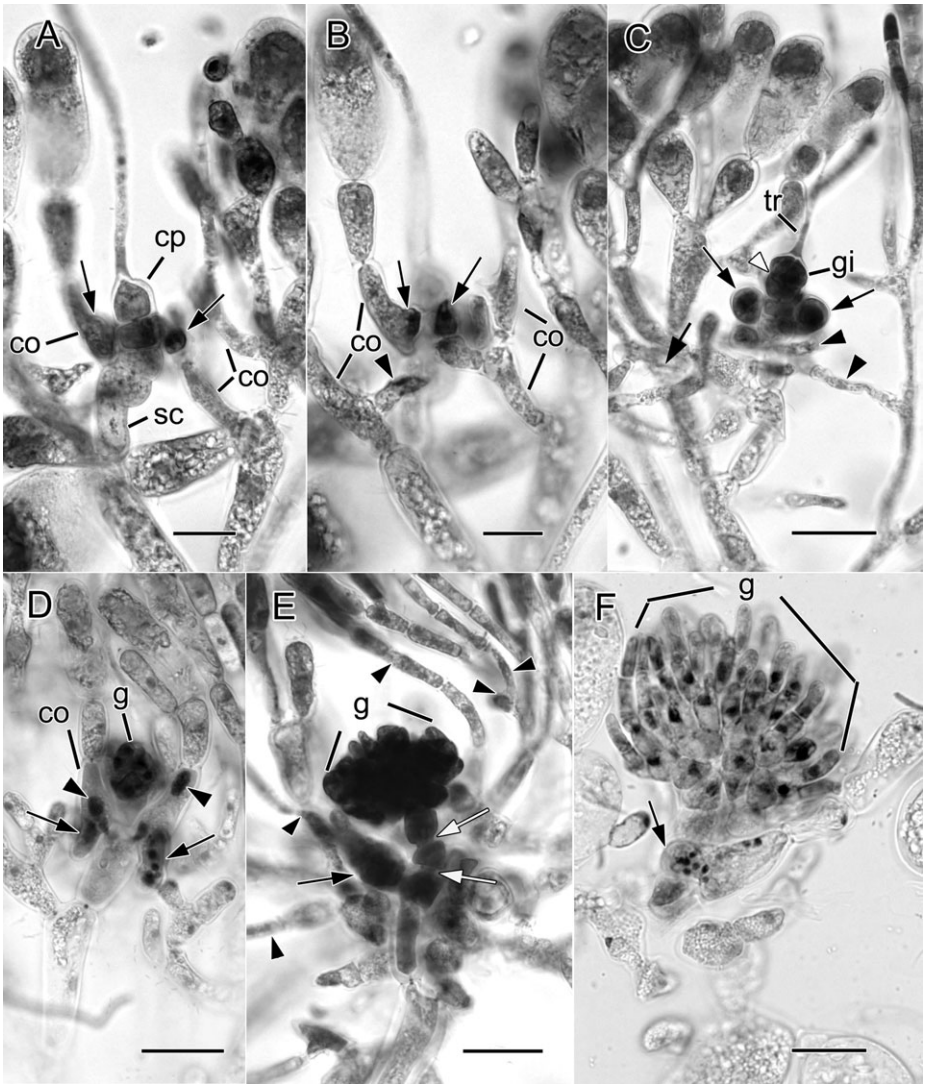

FIG. 4. *Helminthocladia australis* (A–E: NTOU09042010) & *Helminthocladia calvadosii* (F; HGI-A 14538). Carposporophyte development; scale bars: A–B = 10 μm; C–F = 20 μm. (A) Early postfertilization stage showing straight four-celled carpogonial branch borne on supporting cell (sc), and undivided carpogonium (cp). Note initials of involucral filaments (arrows) produced from cortical cells (co) adjacent to carpogonial branch. (B) Different focal plane of A showing two involucral filament initials (arrows) flanking carpogonial branch and one involucral initial (arrowhead) arising from lower cortical cell. (C) Early postfertilization showing trichogyne (tr) and gonimoblast initial (gi), enlarged and darkly stained initials of involucral filaments (arrows) flanking carpogonial branch, and fine involucral filaments (arrowheads) produced from lower cortical cells. (D) Later stage of early postfertilization showing enlarged nuclei (arrowheads) in cortical cells bearing multinucleate involucral cells (arrows) flanking carpogonial branch. (E) Young carposporophyte showing compact gonimoblasts (g), unfused cells of carpogonial branch with broadened pit connections (white arrows), enlarged, multinucleate involucral cell (arrow) flanking carpogonial branch, and elongated inner cortical cells (arrowheads). (F) Carposporophyte prior to differentiation of carposporangia and showing compact gonimoblasts (g) and enlarged, multinucleate involucral cell (arrow) flanking carpogonial branch.

specimens from Kenya (Abbott 1970, fig. 8; Abbott 1999, p. 62).

Liagoropsis maxima Yamada 1944: 19 (Fig. 6)

Type locality: Babukutu, Taitung Co., Taiwan. Holotype: SAP026365.

Examined specimens: Taiwan: Taitung Co., Shenglan (= southern part of Babukutu), growing in subtidal zone at 1–2 m depths, March 11, 2008 (NTOU11032008), leg. S. M. Lin; Shanyuan, growing in subtidal zone at 1–2 m depths, August 3, 2003 (NTOU03082003, sequenced for both *rbc*L and *psa*A), leg. S. M. Lin, June 9, 2005 (NTOU09062005, sequenced for both *rbc*L and *psa*A), leg. S. Y. Yang.

Habit and carposporophyte development: the topotype specimens agreed with the description and illustrations of Yamada (1944) and Doty and Abbott (1964). Thalli slightly calcified (Fig. 6A), 15–50 cm tall, with one to several, cylindrical to flattened axes arising from short, terete stalk with discoid holdfast attached on coral reefs at 1–2 m depth or in tidal pools. Carpogonial branches 7- to 8-celled, single or

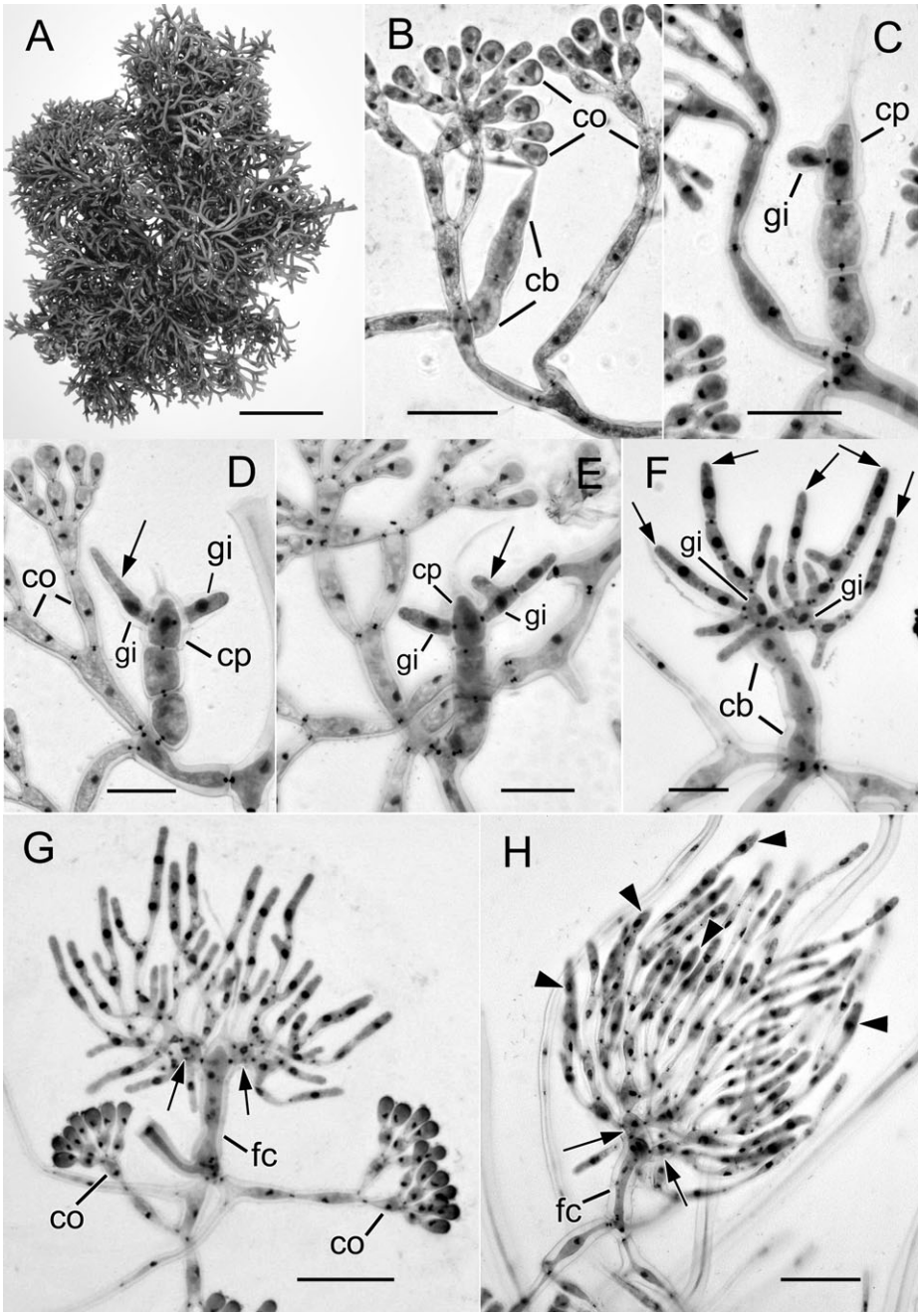

Fig. 5. *Yamadaella caenomyce*. Carposporophyte development (A: NTOU20032010, B, C: NTOU15062007, D, E: NTOU09062005, F, H: NTOU01032009); scale bars: A = 2 cm; B–F = 20 μm; G, H = 50 μm. (A) Thallus habit, wet preserved. (B) Cortical filaments (co) bearing a carpogonial branch (cb). (C) Early postfertilization stage showing fertilized carpogonium (cp) cutting off a first gonimoblast initial (gi) laterally. (D) Early postfertilization stage showing fertilized carpogonium (cp) cutting off two gonimoblast initials (gi) bilaterally, and unmodified cortical cells (co). Note elongation of one of two gonimoblast initials (arrow). (E) Later stage of early postfertilization development showing fertilized carpogonium (cp) and elongating gonimoblast initials (gi), one laterally branched (arrow). (F) Young carposporophyte showing branched gonimoblast filaments (arrows) and fusing carpogonial branch (cb). Note gonimoblast initials (gi) remain distinct. (G) Immature carposporophyte showing loosely developed gonimoblasts, fused carpogonial branch (fc), and unmodified cortical cells (co). Note gonimoblast initials (arrows) remain distinct and no involucral filaments produced. (H) Nearly mature carposporophyte showing diffuse gonimoblast filaments, terminal carposporangia (arrowheads), and fused carpogonial branch (fc). Note gonimoblast initials (arrows) remain distinct.

in clusters on basal portion of assimilatory filaments (Fig. 6B); after presumed fertilization, zygote divided several times longitudinally (Fig. 6C, arrowhead) to produce gonimoblast initials, at least three gonimoblast initials cut off directly from zygote; these gonimoblast initials elongated upwardly and then divided several times transversely to produce young gonimoblast filaments (Fig. 6D); gonimoblast

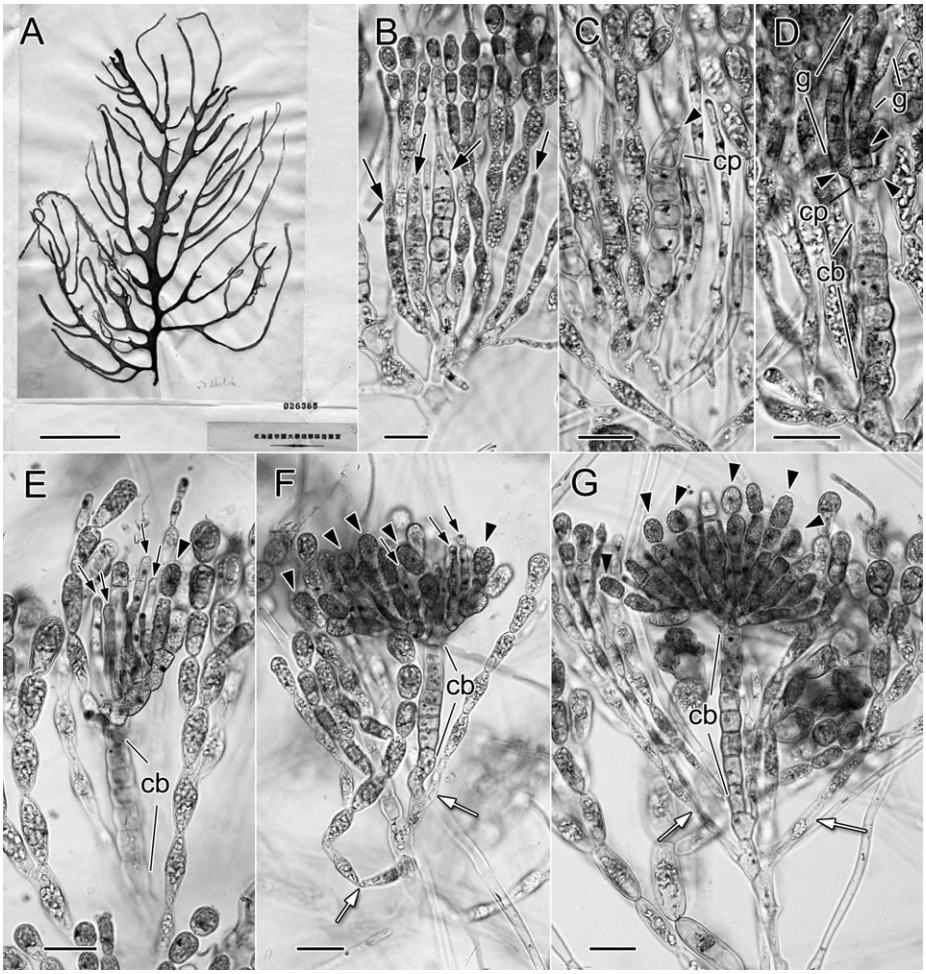

FIG. 6. *Liagoropsis maxima*. Habit and carposporophyte development (B–G: NTOU09062005); scale bars: A = 5 cm; B–G = 25 μm. (A) Holotype (SAP026365, Babukutsu, Taitung Co., Taiwan). (B) Cluster of straight carpogonial branches (arrows). (C) Early postfertilization stage showing fertilized carpogonium (cp) dividing longitudinally (arrowhead). (D) Later stage of C, showing two primary gonimoblast filaments (g) cut off from fertilized carpogonium (cp) and unfused cells of carpogonial branch (cb). Note fertilized carpogonium cutting off at least three gonimoblast initials (arrowheads). (E) Young carposporophyte showing elongated gonimoblast filaments (arrows), differentiating carposporangium (arrowhead) and unfused carpogonial branch (cb). (F) Immature carposporophyte showing gonimoblast filaments (black arrows), differentiating carposporangia (arrowheads) and unfused carpogonial branch (cb). Note slight elongation (white arrow) of inner assimilatory cells. (G) Mature carposporophyte showing most terminal cells of gonimoblast filaments differentiated into carposporangia (arrowheads) and unfused cells of carpogonial branch (cb). Note basal cells of assimilatory filaments adjacent to carpogonial branch have elongated (white arrows).

filaments at first branched unilaterally (Fig. 6E) and then radially (Fig. 6F); no involucral filaments produced during carposporophyte development; carposporophytes fastigiate when fully developed (Fig. 6G); carposporangia differentiated successively from terminal cells of gonimoblast filaments (Fig. 6, E–G); no remaining cell walls from discharged carposporangia found; basal cells of assimilatory filaments adjacent to carpogonial branches elongated at carposporophyte maturity; pit connections not breaking down between cells of carpogonial branch, so no fusion cell formed at any stage (Fig. 6G).

Note: Doty and Abbott (1964) placed *L. maxima* in synonymy with *Liagoropsis schrammii* (P.L. Crouan & H.M. Crouan) Doty & I.A. Abbott (basionym: *Helminthocladia schrammii* P.L. Crouan & H.M. Crouan; type locality, Guadeloupe, Lesser Antilles, Caribbean), recognizing only a single widespread species in the genus. Given the considerable geographic separation and the lack of any sequence data for Caribbean populations, we prefer to retain Yamada's name until further collections become available.

DISCUSSION

Nemaliales, type order of subclass Nemaliophycidae T.A. Christensen, has been regarded as a natural assemblage characterized by carposporophytes that are produced directly from the carpogonium after fertilization without a generative auxiliary cell. Most families previously included in the order by Fritsch (1945) and Kylin (1956; as "Nemalionales")

have been raised to a higher taxonomic rank (see Huisman 2006: 2, Le Gall and Saunders 2010 for reviews), except for the Galaxauraceae and the Liagoraceae (Chaetangiaceae and Helminthocladiaceae, respectively, in Fritsch and Kylin). Chaetangiaceae was elevated to ordinal status by Desikachary (1964), but this has largely been ignored. Monophyly of the order has been demonstrated by Huisman et al. (2004a,b) based on large-subunit ribosomal DNA sequences, and Huisman et al. (2004a) also added a third family, the Scinaiaceae. However, relationships among genera in the family Liagoraceae sensu lato have remained mostly unexplored (Le Gall and Saunders 2010, Lin et al. 2011a,b).

In the present study, phylogenetic analyses of combined *rbc*L and *psa*A sequences have shown that the Liagoraceae sensu lato is polyphyletic as the genera examined were grouped in four independent clades (see Fig. 1). Clade I contained the generitype of *Liagora* and the majority of genera with more-or-less well-developed involucral filaments and in which gonimoblast initials are cut off transversely or obliquely (i.e., *Akalaphycus*, *Dermonema*, *Izziella*, *Titanophycus*, *Helminthocladia*, *Trichogloeopsis*). The other three clades (II-IV) were monogeneric: *Yamadaella* (Clade II), *Nemalion* (Clade III), and *Liagoropsis* (Clade IV). Genera in Clade I (the Liagoraceae sensu stricto as defined herein) have the widest geographic distribution, occurring from temperate northern Atlantic and southern Pacific Oceans through warm-water regions of the Indo-Pacific and Caribbean Sea (Abbott and Hollenberg 1976, Huisman 2006, Lin et al. 2011a, 2013, 2014). By contrast, *Yamadaella* (Clade II) and *Liagoropsis* (Clade IV) are restricted to warm-water regions of the Indo-Pacific and western Atlantic Oceans (Yamada 1944, Doty and Abbott 1964, Abbott 1970), whereas *Nemalion* (Clade III) is found only in temperate regions but is widespread in both hemispheres (Womersley 1994, p. 81, Guiry and Guiry 2014, see Le Gall and Saunders 2010).

Our molecular analyses do not support the proposed subfamilial/familial relationships among genera placed in the Liagoraceae by Doty and Abbott (1964: 451) (table 2), in which *Liagoropsis*, *Nemalion*, *Trichogloea* Kützing, and *Trichogloeopsis* were placed in Nemaliaceae (as "Nemalionaceae") based on their gelatinous thalli as well as their simply constructed, compact gonimoblasts. Based on our analyses, the phylogenetic relationships among the families recognized herein are incompletely resolved. However, the resurrected family Nemaliaceae includes the single genus *Nemalion* and is not in the clade of Liagoraceae sensu stricto. Doty and Abbott (1964) included *Dermonema* and *Cumagloia* in the subfamily Dermonemae, which they did not formally place in a family although they suggested that "it would seem perhaps to be more closely related to the Helminthocladiae than to the Nemalioneae." The genera *Dermonema*, *Dotyophycus,* and *Yamadaella*, which possess diffuse gonimoblasts, were later placed in the newly proposed family Dermonemataceae by Abbott (1976), but these genera did not prove to be closely related based on our *psa*A+*rbc*L sequence analyses. Thus, strictly morphological characters such as thallus and gonimoblast morphology are clearly inadequate for grouping genera into families. However, the orientation of cell divisions of the carpogonia after presumed fertilization is clearly phylogenetically significant. For the genera clustered in Liagoraceae sensu stricto (Clade I), the first cell division of the zygote is always transverse (i.e., *Liagora* spp., see Lin et al. 2011a, figs 27, 30; *H. australis*, Fig. 4C in this study) or slightly oblique (i.e., *D. virens*, Fig. 3 C and D, this study), and only a single gonimoblast initial is produced. Although the gonimoblast initial is also cut off transversely in *Nemalion*, the primary gonimolobe cell is produced longitudinally, rather than transversely or obliquely as is seen in the Clade I members. By contrast, two gonimoblast initials are cut off laterally from the zygote in *Yamadaella* (Clade II, Fig. 5 D–F), and at least three are produced by longitudinal division of the zygote in *Liagoropsis* (Clade IV, Fig. 6 C and D).

Although not the focus of the present study, our analyses (Fig. 1) placed *A. savianum* of the order Acrochaetiales as sister to the Nemaliaceae and clearly embedded within the Nemaliales. In their investigation of the Acrochaetiales, Harper and Saunders (2002: 469) highlighted the close relationship between that order and the Nemaliales, suggesting that a single order could be argued, but preferring to adopt a multiorder classification based on the considerable morphological differences between the taxa. We prefer not to speculate given our very limited sample size, but this result should be investigated further.

Molecular phylogenetic analyses based on combined *rbc*L and *psa*A sequences of the currently circumscribed Liagoraceae demonstrate that the family is polyphyletic (Fig. 1). *Yamadaella* (Clade II), *Nemalion* (Clade III), and *Liagoropsis* (Clade IV) are shown to be three independent natural assemblages. Although the genera *Dermonema*, *Helminthocladia,* and *Cumagloia* formed a strong clade sister to the other genera of Liagoraceae sensu stricto, the postfertilization stages examined in this study did not show significant differences from the majority of Liagoraceae sensu stricto, in which only a single gonimoblast initial is cut off transversely from the fertilized carpogonium and their involucral filaments are produced from cortical cells adjacent to carposporophytes. Accordingly, we propose to emend the family Liagoraceae; to reinstate the family Nemaliaceae for *Nemalion*; and to establish the two new families Yamadaellaceae Showe M. Lin, Rodríguez-Prieto & Huisman based on *Yamadaella* and Liagoropsidaceae Showe M. Lin, Rodríguez-Prieto & Huisman based on *Liagoropsis*.

TAXONOMIC CONCLUSIONS

Key to the families of the Nemaliales.

1a Carposporophyte surrounded by a consolidated, ostiolate, pericarp 2
1b Carposporophyte naked or surrounded by unconsolidated sterile or involucral filaments 3
2a Thalli calcified; gonimoblast filaments spreading over inner walls of pericarp; life history an isomorphic,
dimorphic (gametophytes and sporophyte both macroscopic but unlike morphologically) or heteromorphic alternation of generations . . . . . Galaxauraceae
2b Thalli noncalcified; gonimoblast filaments clustered at base of cystocarp and not spreading over the inner walls of pericarp; life history heteromorphic with diminutive, filamentous, or loosely crustose tetrasporophytes . . . . . Scinaiaceae
3a Single gonimoblast initial cut off transversely from zygote 4
3b Two or more gonimoblast initials cut off from zygote 5
4a. Primary gonimoblast cells dividing longitudinally from gonimoblast initial . . . . . Nemaliaceae
4b Primary gonimoblast cells dividing transversely or obliquely from gonimoblast initial . . . . . Liagoraceae
5a Two gonimoblast initials cut off laterally from zygote; gonimoblast filaments diffuse and slender . . . . . Yamadaellaceae fam. nov.
5b At least three gonimoblast initials cut off longitudinally
from zygote; gonimoblast filaments compactly arranged . . . . . Liagoropsidaceae
fam. nov.

Liagoraceae Kützing emend. Showe M. Lin, Rodríguez-Prieto & Huisman

Thalli variable in habit and calcification, ranging from noncalcified to lightly or heavily calcified, composed of terete to compressed axes and laterals. Carpogonial branches either straight or curved to varying degrees, generally four- or five-celled, but occasionally up to 6- to 9-celled, borne laterally or terminally/subterminally from cells of assimilatory filaments. Gonimoblast initial single, cut off transversely or obliquely from zygote. Gonimoblasts diffuse or compact. Carposporophytes mostly flanked by involucral filaments derived from proximal portions of assimilatory cells or by elongated or modified cortical cells, gonimorhizoids only seen in *Trichogloeopsis*. Carposporangia produced mostly in distal ends of gonimoblast filaments. In some instances, most cells of gonimoblast filaments differentiate into carposporangia, for example, *Trichogloeopsis* and *Gloiocallis*. Spermatangia mostly cut off from distal ends of cortical cells or sometimes produced from subcortical cells. Life history heteromorphic, with conspicuous gametophytes (monoecious or dioecious) alternating with minute, loosely and irregularly filamentous tetrasporophytes that produce monosporangia and/or tetrasporangia with cruciately or decussately arranged tetraspores (Guiry 1990).

Type genus: *Liagora* J.V. Lamouroux (1812: 185).

Included genera: *Akalaphycus*, *Cumagloia*, *Dermonema*, *Dotyophycus*, *Ganonema*, *Gloiocallis* Showe M. Lin, Huisman & D.L. Ballantine, *Helminthocladia*, *Helminthora*, *Hommersandiophycus*, *Izziella*, *Liagora*, *Macrocarpus*, *Neoizziella*, *Patenocarpus* Yoshizaki, *Stenopeltis*, *Titanophycus*, *Trichogloea*, *Trichogloeopsis*, *Yoshizakia*. Although the genera *Cylindraxis* Kraft, *Gloiotrichus* Huisman & Kraft, *Sinocladia* C.K. Tseng & W. Li have not been sequenced, their fertilized carpogonia all divide transversely to produce gonimoblast initials, and these genera should be included in the emended Liagoraceae.

Nemaliaceae (Farlow) De Toni and Levi (1886: 212) (as "Nemalieae")

Description: Thalli gelatinous and flaccid, uncalcified, composed of cylindrical, simple or dichotomous axes arising from discoid holdfasts. Carpogonial branches straight and three-celled borne on upper parts of assimilatory filaments, sometimes in place of assimilatory filaments. Gonimoblast initial single, cut off transversely from fertilized carpogonium and producing gonimolobe initials longitudinally first then obliquely and radially. Carposporophytes of compactly arranged gonimoblast filaments; few sterile or involucral filaments produced from assimilatory filaments surrounding carposporophytes. Cells of mature carposporophytes mostly differentiate into carposporangia; pit connections between carpogonial branch cells break down to form fusion cells. Life history heteromorphic with large, dioecious gametophytes and diminutive filamentous tetrasporophytes.

Type and only known genus: *Nemalion* Duby (1830: 959)

Note: Silva (1980: 85) concluded that the name Nemaliaceae was superfluous, since the family to which it was applied "implicitly included" *Liagora*, the type of the preexisting, valid family name Liagoraceae Kützing. Under provisions of the International Code of Botanical Nomenclature in force at the time (Stafleu et al. 1978), the name Nemaliaceae was thus illegitimate and could not be used without conservation. However, a subsequent revision of the Code removed this requirement (see Nicolson and Norris 1983: 290) and the name Nemaliaceae is available for our concept of the family (i.e., excluding *Liagora*).

Yamadaellaceae Showe M. Lin, Rodríguez-Prieto & Huisman **fam. nov**.

Description: Thalli highly calcified, forming decumbent clumps of subdichotomous axes attached by multiple holdfasts. Carpogonial branches three-celled, borne laterally on lower portions of assimilatory filaments. Cell sizes of carpogonial branches relatively large compared to members of Liagoraceae. Two gonimoblast initials produced laterally from carpogonia after fertilization, then

elongating and branching outwardly, producing young gonimoblast filaments. Involucral filaments lacking. Pit connections between cells of carpogonial branches breaking down to form a slender fusion cell at early postfertilization stages. Fully developed carposporophytes composed of lax, laterally spreading, coarse gonimoblast filaments bearing terminal undivided or cruciately divided carposporangia. Spermatangial parental cells cut off directly from terminal cells of assimilatory filaments; each spermatangial parental cell producing 2–3 spermatangia. Life history probably heteromorphic; isomorphic tetrasporophytes unknown.

Type and currently only known genus: *Yamadaella* I.A. Abbott (1970: 117).

Liagoropsidaceae Showe M. Lin, Rodríguez-Prieto & Huisman **fam. nov.**

Description: Thalli strongly lubricous, with internal reticula of light calcification; axes erect, with or without a short stalk. Carpogonial branches straight, 6- to 7 (-8)-celled, borne on basal parts of assimilatory filaments, sometimes in place of ordinary assimilatory filaments. At least three gonimoblast initials cut off longitudinally from zygote, these initially producing young gonimoblast filaments unilaterally, the subsequent filaments then dividing radially. Involucral filaments absent, assimilatory filaments in vicinity of carposporophytes elongating only slightly. Mature gonimoblast filaments fastigiate. Carposporangia differentiated successively from terminal cells of gonimoblast filaments. Fusion cell not formed. Life history probably heteromorphic with large, dioecious gametophytes; tetrasporophytes unknown.

Type genus and currently only known genus: *Liagoropsis* Yamada (1944:19).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Table S1. List of species sequenced for rbcL and LSU analysis in this study and their accession numbers in GenBank.