

Grateloupia ramosa Wang & Luan sp. nov. (Halymeniaceae, Rhodophyta), a new species from China based on morphological evidence and comparative *rbcL* sequences*

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Abstract *Grateloupia ramosa* Wang & Luan sp. nov. (Halymeniaceae, Rhodophyta) is newly described from Hainan Province, southern China. The organism has the following morphological features: (1) purplish red, cartilaginous and lubricous thalli 5–10 cm in height; (2) compressed percurrent axes bearing abundant branches with opposite arrangement; (3) claw-like apices on top, constricted to 2–4 cm at the base; (4) cortex consisting of 3–6 layers of elliptical or anomalous cells and a medulla covered by compact medullary filaments; (5) reproductive structures distributed throughout the thallus, especially centralized at the bottom of the end portion of the branches; and (6) 4-celled Carpogonial branches and 3-celled auxiliary-cell branches, both of the *Grateloupia*-type. The morphological differences were supported by molecular phylogenetics based on ribulose-1, 5-bisphosphate carboxylase/oxygenase (*rbcL*) gene sequence analysis. There was only a 1 bp divergence between specimens collected from Wenchang and Lingshui of Hainan province. The new species was embedded in the large *Grateloupia* clade of the Halymeniaceae. The pairwise distances between *G. ramosa* and other species within *Grateloupia* ranged from 26 to 105 bp, within pairwise distances of 13–111 bp between species of the large genus *Grateloupia* in Halymeniaceae. Thus, we propose this new species as *G. ramosa* Wang & Luan sp. nov.

Keyword: *Grateloupia*; *Grateloupia ramosa*; Halymeniaceae; morphology; *rbcL* sequence; Rhodophyta

1 INTRODUCTION

The genus *Grateloupia* was established based on generitype *G. flicina* (Lamouroux) C. Agardh from the Mediterranean Sea (Agardh, 1822). This genus is highly diverse, consisting of more than 90 species distributed worldwide in cold-temperate to tropical waters (Wang et al., 2000, 2001; Kawaguchi et al., 2001; Gavio and Fredericq, 2002; Faye et al., 2004; De Clerck et al., 2005a, b; Wilkes et al., 2005; Lin et al., 2008; Zhao et al., 2012; Guiry and Guiry, 2013). To date, 36 *Grateloupia* species have been reported in China (Xia, 2004; Zhang et al., 2012; Zhao et al., 2012; Yu et al., 2013; Liu et al., 2014; Wang et al., 2015). Among these, *G. orientalis* S.-M. Lin & H.-Y. Liang is a new record from Hainan, China (Zhang et al., 2012), and *G. dalianensis*, *G. yinggehaiensis*, *G. tenuis*, *G. boaoensis* and *G. huanghaiensis* are

recently reported species (Zhang et al., 2012; Yu et al., 2013; Liu et al., 2014; Wang et al., 2015). Classification of members of *Grateloupia* is extremely difficult because differences between the external morphology and internal structure have prevented development of uniform morphological classification criteria. Gargiulo et al. (2013) recently considered the formation of carpogonial and auxiliary cell ampullae and post fertilization events to be important characteristics for future generic reassessment of *Grateloupia*. Additionally, they found that the genus was strongly supported by *rbcL* phylogenies. Overall, they divided *Grateloupia* into eight subgroups,

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G. lanceolata-group, *G. americana*-group, *G. belangeri*-group, *G. phuquocensis*-group, *G. stipitata*-group, *G. filicina*-group, *G. doryphora*-group and *G. montagnei*-group.

G. catenata was first described in 1920 (Yendo, 1920), after which Howe (1924) described *G. filicina* (Lamouroux) C. Agardh var. *lomentaria* Howe with no reference to Yendo's (1920) publication. Okamura (1936) considered *G. filicina* var. *lomentaria* to be a mature stage of *G. filicina* var. *porracea* and proposed combination with *G. filicina* var. *porracea* f. *lomentaria* (Howe) Okamura. At the same time, Okamura (1936) reduced *G. catenata* to synonymy with *G. filicina* var. *porracea* f. *lomentaria*. Wang et al., (2000) then reinstated *G. catenata* based on morphological observations and *rbcL* analysis. *G. tenuis* was similar to *G. catenata* based on external morphology; however, Yu et al. (2013) confirmed a new species of *G. tenuis* through morphological observations and molecular phylogenetic studies.

The specimens investigated in the present study, which had an external morphology similar to *G. catenata* and *G. tenuis*, were collected from Lingshui (110°1'E, 18°30'N) and Wenchang (110°47'E, 19°32'N) of Hainan Province, southern China. The external and internal morphologies were distinguished from other species (*G. tenuis*, *G. catenata*, *G. filicina*, *G. ramosissima*, *G. orientalis*) for the first time based on observations. We also conducted *rbcL* sequence analysis to support the results of morphological observations. Herein, we propose that this alga be classified in the *Grateloupia* genus with the name *Grateloupia ramosa* Wang & Luan sp. nov.

2 MATERIAL AND METHOD

2.1 Morphological analysis

Specimens used in the present study were hand-

collected from Wenchang, Hainan Province, China. Voucher specimens have been deposited in the the Herbarium of the College of Life Sciences, Liaoning Normal University, Dalian, China (LNU) (1) (9 January 2009, leg. R. X. Luan; LNU20092016–LNU20092022), and (2) Lingshui, Hainan Province, China (5 February 2009, leg. R. X. Luan; LNU20092083–LNU20092088). We regarded *G. ramosa* (LNU20092086) as the holotype.

Morphological observations were made based on specimens preserved in 10% formalin/seawater using an anatomical lens or microscopic observation of several algae that contained the reproductive organs of algal specimens (LNU20092019–LNU20092020; LNU20092087–LNU20092089). To accomplish this, samples were selected, frozen, cut into small pieces of uniform size of about 1 cm, and placed in a petri dish. Photographs of the holotype specimen were taken with a Canon EOS 650D (Canon, Japan). Additionally, sections were made by cryostat microtome, stained with 0.5% (w/v) cotton blue and photographed using an Olympus BH₂ digital camera (Olympus Beijing Co. Ltd., China) mounted on a Nikon microscope (Nikon Corporation, Japan). The branch features, lateral branch formation, and cystocarp distribution were scanned using an anatomical lens.

2.2 Molecular analysis

DNA of LNU20092016, LNU20092017, LNU20092018, LNU20092083, LNU20092084, and LNU20092085 was isolated using a DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). Sequences of 41 additional related red algal species were selected from GenBank and included in the alignments (Table 1). DNA extraction and sequencing were performed as previously described (Wang et al., 2000). PCR (Applied Biosystems, USA) was conducted in a 50 µL reaction mixture that consisted

Table 1 Species used in *rbcL* gene analysis, collection location, GenBank accession number and references

Species	Collection location and site	Reference	GenBank accession number
* <i>Grateloupia ramosa</i> Wang & Luan	Wenchang, Hainan Province, China (LNU20092016)	This study	JF810596
* <i>G. ramosa</i> Wang & Luan	Lingshui, Hainan Province, China (LNU20092083)	This study	JF810598
* <i>G. ramosa</i> Wang & Luan	Lingshui, Hainan Province, China (LNU20092084)	This study	JF810597
* <i>G. ramosa</i> Wang & Luan	Wenchang, Hainan Province, China (LNU20092017)	This study	JX974606
* <i>G. ramosa</i> Wang & Luan	Wenchang, Hainan Province, China (LNU20092018)	This study	JX974607
* <i>G. ramosa</i> Wang & Luan	Lingshui, Hainan Province, China (LNU20092085)	This study	JX974608

* Deposited in the Herbarium of the College of Life Sciences, Liaoning Normal University, Dalian, China (LNU).

To be continued

Table 1 Continued

Species	Collection location and site	Reference	GenBank accession number
<i>G. tenuis</i> Wang et Luan	Lingshui, Hainan Province, China (LNU20092088)	Yu et al., 2013	KC918542
<i>G. yangjiangensis</i> Li et Ding	Yangjiang, Guangdong Province, China	Wang et al., 2013	HQ324236
<i>G. yinggehaiensis</i> H. W. Wang et R. X. Luan	Yinggehai, Hainan Province, China	Wang et al., 2012	HQ332513
<i>G. filicina</i> (Lamouroux) C. Agardh	Livorno, Italy	Wang et al., 2000	AB055472
<i>G. orientalis</i> S.-M. Lin et H.-Y. Liang	Linyuan, southwestern Taiwan, China	Lin et al., 2008	EU292744
<i>G. catenata</i> Yendo	Shimiao, Dalian, Liaoning Province, China	Wang et al., 2000	AB038617
<i>G. ramossissima</i> Okamura	Ho Ping Island, Keelung, North Taiwan, China	Gavio et al., 2002	AF488810
<i>G. carnosa</i> Yamada et Segawa	Oryuzako, Miyazaki Prefecture, Japan	Wang et al., 2000	AB038608
<i>G. montagnei</i> R. J. Wilkes, L. M. McIvor & Guiry	County Kerry, Ireland	Wilkes et al., 2004	AY435171
<i>G. minima</i> P. L. Crouan & H. M. Crouan	Brest, Atlantic France	De-Clerck et al., 2005a	AJ868468
<i>G. asiatica</i> Kawaguchi et Wang	Qingdao, Shandong Province, China	Kawaguchi et al., 2001	AB055488
<i>G. livida</i> (Harvey) Yamada	Izu-misaki, Miyake Island, Tokyo, Japan	Wang et al., 2000	AB038610
<i>G. acuminata</i> Holmes	Katase, Fujisawa, Kanagawa Prefecture, Japan	Wang et al., 2000	AB055480
<i>G. americana</i> Kawaguchi et Wang	Pigeon Point, San Matio County, California, USA	De-Clerck et al., 2005a	AY772037
<i>G. patens</i> (Okamura) Kawaguchi et Wang	Oohara, Chiba Prefecture, Japan	Wang et al., 2001	AB061392
<i>G. divaricata</i> Okamura	Oshoro, Hokkaido, Japan	Wang et al., 2000	AB038609
<i>G. lanceolata</i> (Okamura) Kawaguchi	Shikanoshima, Fukuoka Prefecture, Japan	Kawaguchi et al., 2001	AB055477
<i>G. elliptica</i> Homles	Goshikinohama, Usa, Tosa, Kochi Prefecture, Japan	Wang et al., 2000	AB055476
<i>G. chiangii</i> Kawaguchi et Wang	Izu-misaki, Miyake Island, Tokyo, Japan	Wang et al., 2001	AB061387
<i>G. cornea</i> (Okamura) E. Y. Dawson	Oohara, Chiba Prefecture, Japan	Wang et al., 2001	AB061382
<i>G. kurogii</i> Kawaguchi	Saikai-bashi, Nagasaki Prefecture, Japan	Wang et al., 2000	AB038606
<i>G. angusta</i> (Okamura) Kawaguchi et wang	Miyanoura, Hirado Island, Nagasaki Prefecture, Japan	Wang et al., 2001	AB061380
<i>G. imbricata</i> Holmes	Tsuyazaki, Fukuoka Prefecture, Japan	Wang et al., 2000	AB038607
<i>G. californica</i> Kylin	Monterey, California, USA	Miller et al., 2009	FJ013038
<i>G. versicolor</i> J. Agardh	Barra de Navidad, Mexico	Fredericq et al., 2000	AF212197
<i>G. lanceola</i> J. Agardh	Iberian Peninsula, Spain	Figueroa et al., 2007	AM422894
<i>G. capensis</i> De Clerck	Kommetjie, Western Cape Province, South Africa	De-Clerck et al., 2005a	AJ868465
<i>G. dichotoma</i> J. Agardh	Lugo, Galicia, Japan	De-Clerck et al., 2005a	AY772031
<i>G. longifolia</i> Kylin	Yzerfonteyn, Western Cape Province, South Africa	De-Clerck et al., 2005a	AY772023
<i>G. taiwanensis</i> Lin et Liang	Northeastern and southern Taiwan, China	Lin et al., 2008	EU292742
<i>G. filiformis</i> Kutzing	Marataizes Espiritu Santu, Brazil	Gavio & Fredericq, 2002	AF488822
<i>G. phuquocensis</i> Tanaka & Pham-Hoang	Kaalawai, Oahu, Hawaii, USA	De-Clerck et al., 2005a	AY772022
<i>G. sparsa</i> (Okamura) Chiang	Oohara, Chiba Prefecture, Japan	Wang et al., 2000	AB055473
<i>G. turuturu</i> Yamada	Muroran, Hokkaido, Japan	Wang et al., 2000	AB038611
<i>G. doryphora</i> (Montagne) M. A. Howe	Playa de San Francisco, Lima, Peru	Gavio & Fredericq, 2002	AF488817
<i>G. stipitata</i> J. Agardh	Lee Bay Stewart Island, New Zealand	Gavio & Fredericq, 2002	AF488816
<i>G. belangeri</i> (Bory) Setchell et Gardner	Platboom, Western Cape Province, South Africa	De-Clerck et al., 2005a	AY772027
<i>Yonagunia tenuifolia</i> Kawaguchi & Masuda	Yonaguni Island, Okinawa Prefecture, Japan	Kawaguchi et al., 2004	AB116250
<i>Carpopeltis phyllophora</i> (J. D. Hooker & Harvey) F. Schmitz	Port Arthur, Tasmania, Australia	Kawaguchi et al., 2004	AB116364
<i>Halymenia floresia</i> (Clemente) C. A. Agardh	Illes Formigues, Palamos, Girona, Spain	De-Clerck et al., 2005a	AY772019
<i>Polyopes constrictus</i> (Turner) J. Agardh	Point Lonsdale, Victoria, South Australia	Kawaguchi et al., 2001	AB055468



Fig.1 Holotype specimen of *G. ramosa* (female gametophyte collected from Lingshui, LNU20092086)

of 12.5 μ L of 2 \times Taq PCR Master Mix, 31.5 μ L of deionized water, 4 μ L of template DNA, and 1 μ L of each primer. PCR was carried out by subjecting the samples to 93°C for 1 min, followed by 35 cycles of amplification (denaturation at 94°C for 30 s, annealing at 53°C for 30 s and extension at 72°C for 45 s) and then final extension at 72°C for 5 min.

The *rbcL* sequences for phylogenetic analyses were aligned using Clustal X version 1.83 (Thompson et al., 1997). Phylogenetic trees were constructed using the Bayesian analysis (BA) Maximum Likelihood (ML), Maximum Parsimony (MP) and Neighbor-Joining (NJ) methods with PAUP version 4.0 (Swofford, 2003).

For MP, all sites were considered unordered and equally weighted. The heuristic search option was used, with 100 random sequence additions and tree-bisection and reconnection branch exchanging. For this analysis, missing data were treated as gaps. The ML and NJ analyses were estimated using the hierarchical likelihood ratio test. To obtain the optimal settings and ensure data reliability, we used Modeltest version 3.7 (Posada and Buckley, 2004) evolutionary analyses in MEGA5 (Tamura et al., 2011). All complex models of molecular evolution were evaluated using the methods outlined by Litaker et al. (1999) and Moncalvo et al. (2000). Node supports were estimated using the bootstrap proportion values

Table 2 Nucleotide substitution model parameter estimates of *rbcL* gene for Modeltest 3.7 analyses

Model selected	Base frequency	Rate matrix
GTR+I+G		R(a) [A-C]=0.722 5
-lnL=9 015.906 2		R(b) [A-G]=4.936 1
K=10	FreqA=0.316 9	R(c) [A-T]=0.557 0
	FreqC=0.142 2	
AIC=18 051.812 5	FreqG=0.194 0	R(d) [C-G]=0.822 2
	FreqT=0.346 8	
(I)=0.598 4		R(e) [C-T]=10.888 3
(G)=1.445 8		R(f) [G-T]=1.000 0

for calculation (Felsenstein, 1985) using ML (1 000 bootstrap resamplings), NJ (1 000 bootstrap resamplings) and MP (1 000 bootstrap resamplings) methods.

For Bayesian inference (BI), the dataset was partitioned according to codon positions and the prior probability distribution for the site-specific rates in the phylogenetic model was set as “variable”. Models of nucleotide substitution were estimated from Modeltest 3.7 (Posada and Buckley, 2004). Bayesian inference (BI) was performed with MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) using the GTR+I+G (Table 2). The BI results yielded 10 000 trees, 25% of which were burnin. The remaining trees were used to calculate the consensus tree.

3 RESULT

3.1 Morphological observations

Thalli were 5–10 cm in height, purplish red in color, cartilaginous and lubricous in texture. Blades had numerous branches and an opposite arrangement. Percurrent axes were compressed (Fig.1). Terminal branches consisted of up to five multiple claw-like branches that gradually tapered to the upper part (Fig.2a). The lateral branchlets were constricted to 2–4 mm at the base, frequently with proliferations from the margins and the surfaces (Fig.2b). The cortex consisted of 3–6 layers of elliptic or anomalous cells and a medulla composed of dense filaments. Gametophytes dioecious, reproductive structures distributed throughout the thallus and centralized at the bottom of the terminal branches (Fig.2c). Spermatangia were developed from cortical cells of male gametophytes. Mature spermatangia were ellipsoidal, measuring 4–6 mm in length and 3–4 mm in diameter. Carposporangia were produced from most cells of gonimoblasts and deeply immersed in the medulla. Mature cystocarps were spherical, 230–350 μ m in diameter. Tetrasporangia were divided

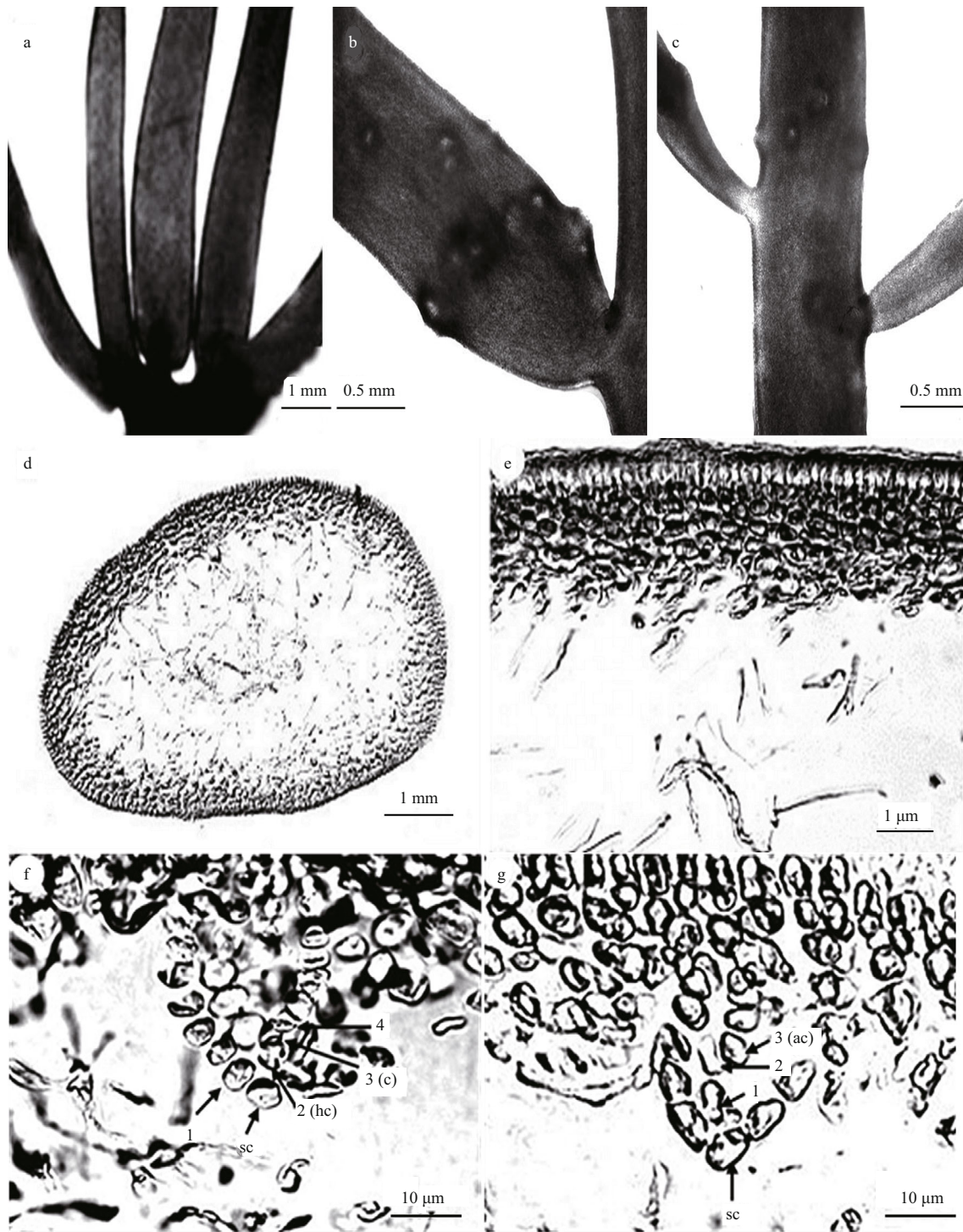
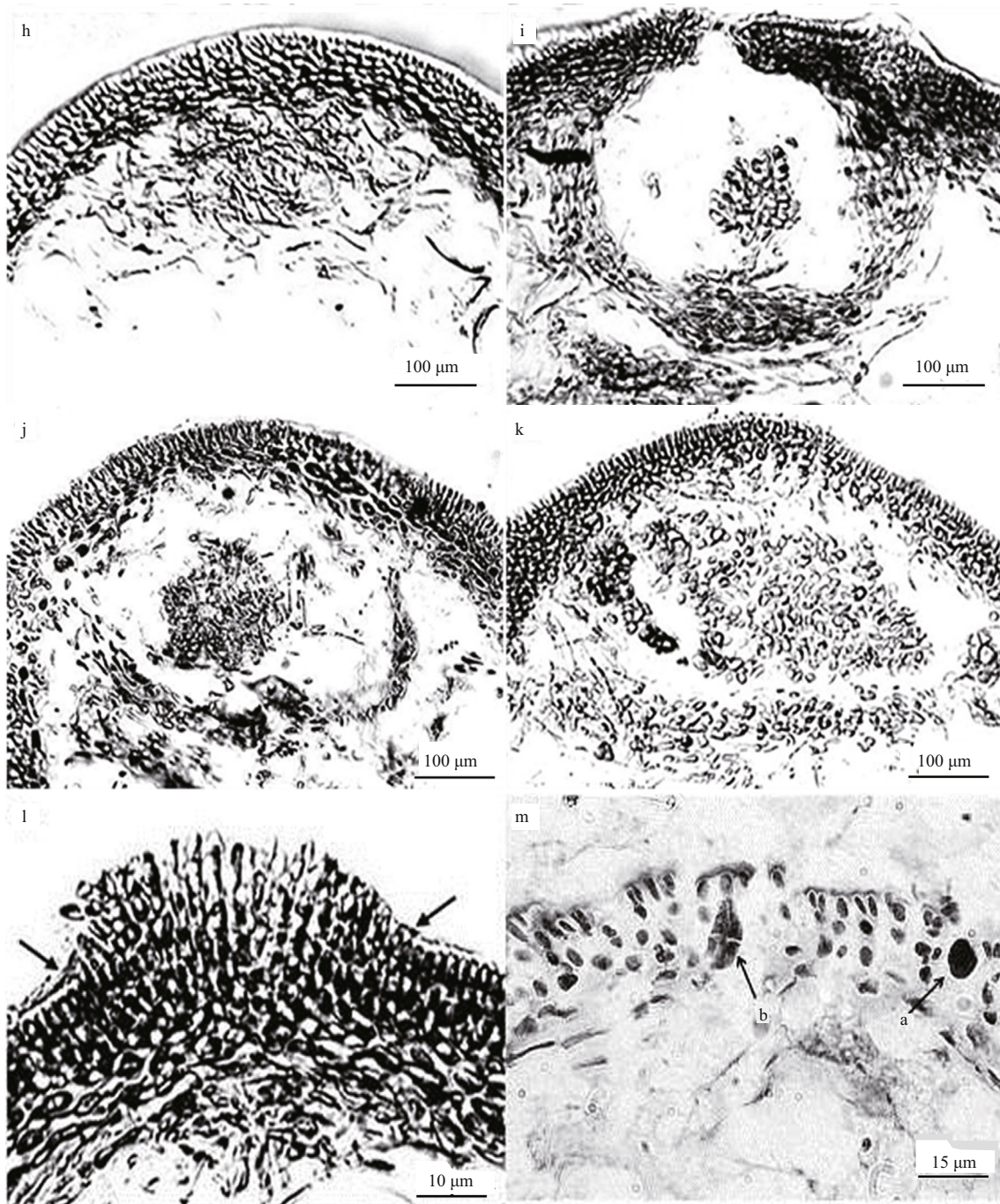


Fig.2 Branching characteristics of *G. ramosa*, distribution of cystocarps, and transverse sections and reproductive structure of *G. ramosa*

a. upper portion of proliferations on a cystocarpic plant, showing multiple branches; b. a branchlet showing the constriction at the base and proliferations from the margins and the surfaces; c. part of an erect axis, showing the arrangement of branches and the proliferations; d. a complete transverse section, showing the cortex and the medulla (LNU20092019, LNU20092020 and LNU20092088); e. part of a transverse section, showing the cortical cells and medullar filaments; f. transverse section of carpoogonial branch ampulla. Arrows and numbers show cells of the carpoogonial (sc: supporting cell, hc: hypogynous c: carpoogonium); g. transverse section of auxiliary cell ampulla. Arrows and numbers show the auxiliary-cell (ac); h. beginning period of cystocarp formation; i. developing stage; j. more advanced stage of cystocarp development; k. mature cystocarp immersed in cortex; l. structure of spermatangia (LNU20092022 and LNU20092087); m. tetrasporangia (arrowhead- a: young terasproocyte, b: cruciately divided terasporangium, LNU20092021 and LNU20092089).

To be continued

Fig.2 Continued



crucially, 35–45 μm long and 20–25 μm in diameter, and embedded in the outer cortex.

ETYMOLOGY: “*ramosa*” in reference to the numerous branches of thalli.

HOLOTYPE: Fig.1 A female specimen (LNU20092086).

TYPE LOCALITY: Lingshui, Hainan Province (108.7°E, 18.5°N), southern China, 5 February 2009 by R. X. Luan.

DISTRIBUTION: At present, known only in Hainan Province, Southern China (108.7°E, 18.5°N).

Transverse sections of the thallus displayed a cortex of 3–6 layers of elliptical or anomalous cells, and a medulla consisting of compact medullary filaments 2–7 μm in diameter that ran in various directions. The medulla cells approached the inner cortical layer, which consisted of rounded or irregularly shaped cells (Fig.2d). Spermatangia

developed from the outer cortex cells of the male gametophytes (Fig.2e). Mature spermatangia were ellipsoidal, measuring 4–6 μm in length and 3–4 μm in diameter. Both the carpogonial ampullae and auxiliary cell ampullae were initiated in the inner cortex. Specimens had a four-celled carpogonial branch borne on a supporting cell consisting of a hypogynous cell and a carpogonium (Fig.2f). The hypogynous cell (hc) bore a three-celled lateral branch. An oval cell (4) bore a five-celled lateral branch, with another cell (1) bearing a six-celled lateral branch. The four-celled carpogonial branch and the lateral branches constituted an ampulla. Auxiliary-cell ampullae were *Grateloupia*-type. Auxiliary cells were cup-shaped and remarkably ellipsoidal or ovate in shape. The auxiliary cell was surrounded by the cup-shaped auxiliary-cell branch and contained a three-celled branch. The gonimoblast initium was generally cut off from the fertilized body and gave rise to gonimoblast filaments. Carposporangia, which were the most developed gonimoblasts cells, were deeply immersed in the medulla. Cystocarps were inconspicuous and surrounded by dense clusters of cells. Cystocarp development is shown (Fig.2h–j). Mature cystocarps were spherical, with a diameter ranging from 400–600 μm (Fig.2j). Tetrasporangia were cruciately divided, 35–45 μm long, 5–15 μm in diameter, and embedded in the outer cortex (Fig.2k).

3.2 *rbcL* analyses

We compared the *rbcL* sequences of the six *G. ramosa* samples to the 37 selected species within *Grateloupia* and four species in other genera within Halymeniaceae (Figs.3, 4). Partial sequences of 1 251 base pairs (bp) corresponding to positions 101 to 1 351 of the full *rbcL* gene (1 467 bp) were used for the alignment.

Through *rbcL* gene sequence comparison, sequences from three samples, including the holotype of *G. ramosa* from the type locality of Lingshui, were identical, while the other three samples from Wenchang differed by only one nucleotide substitution. The pairwise divergence of these samples ranged from 0.0% to 0.08% (0–1 bp); thus, they can be treated as the same species. There were 62 or 63 bp changes (5.17%–5.26%) between *G. ramosa* and the generitype *G. filicina*. Additionally, there were 27–28 bp changes (2.19%–2.28%) between *G. ramosa* and *G. yinggehaiensis* H. W. Wang & R. X. Luan, which was the closest relative to *G. ramosa* in all

three phylogenetic trees. Finally, 44–45 bp changes (3.62%–3.71%) were observed between *G. ramosa* and *G. catenata*, which was most similar to *G. ramosa* in appearance. The pairwise distances between *G. ramosa* and *G. ramosissima* Okamura ranged from 56–57 bp changes (4.65%–4.74%), while there were 39–40 bp changes (3.12%–3.20%) between *G. ramosa* and *G. tenuis* Wang & Luan and between *G. ramosa* and *G. orientalis* S.-M. Lin & H.-Y. There were 48–49 bp changes (3.96%–4.04%) in Liang, and these two species were within the same subclade as our species. When compared with the outgroup species of *Yonagunia tenuifolia* Kawaguchi & Masuda, *Carpopeltis phyllophora* (J. D. Hooker and Harvey) F. Schmitz, *Halymenia floresia* (Clemente) C. Agardh and *Polyopes constrictus* (Turner) J. Agardh, the pairwise distances ranged from 106–132 bp changes (9.01%–11.49%).

The NJ, MP, ML and Bayesian phylogenetic trees are presented in Figs.3, 4, respectively. There were two main clades in all phylogenetic trees, *Grateloupia* clade and outgroups within the Halymeniaceae clade. In addition, there are eight subclades and several undefined branches of the selected species within the large *Grateloupia* sensu lato clade. The *G. ramosa* specimens formed an individual subclade in the *G. filicina*-clade within the large *Grateloupia* sensu lato clade. Finally, sister species to our species in the *G. filicina*-clade consisted of *G. yangjiangensis*, *G. orientalis*, *G. filiformis*, *G. yinggehaiensis*, and *G. tenuis*, with *G. yinggehaiensis* being most closely related to our species.

4 DISCUSSION

Characteristics of the auxiliary-cell ampullae and external morphology are used for the generic classification of Halymeniaceae. Chiang (1970) found that the developmental processes of auxiliary-cell ampullae and carpogonial branch ampullae were relatively stable, and were an important boundary for different genera within the Halymeniaceae. Gargiulo et al. (2013) reported almost all of the types of carpogonial and auxiliary cell ampullae within Halymeniaceae, while Lin et al. (2008) reported two different types of *G. taiwanensis* and *G. orientalis*. The auxiliary-cell ampullae types in Halymeniaceae include *Aeodes*, *Cryptonemia*, *Halymenia*, *Grateloupia* and *Thamnoclonium*. The *Grateloupia*-type structure is simplest, containing a single primary ampullary filament that produces two or three 7–13-celled unbranched secondary ampullary

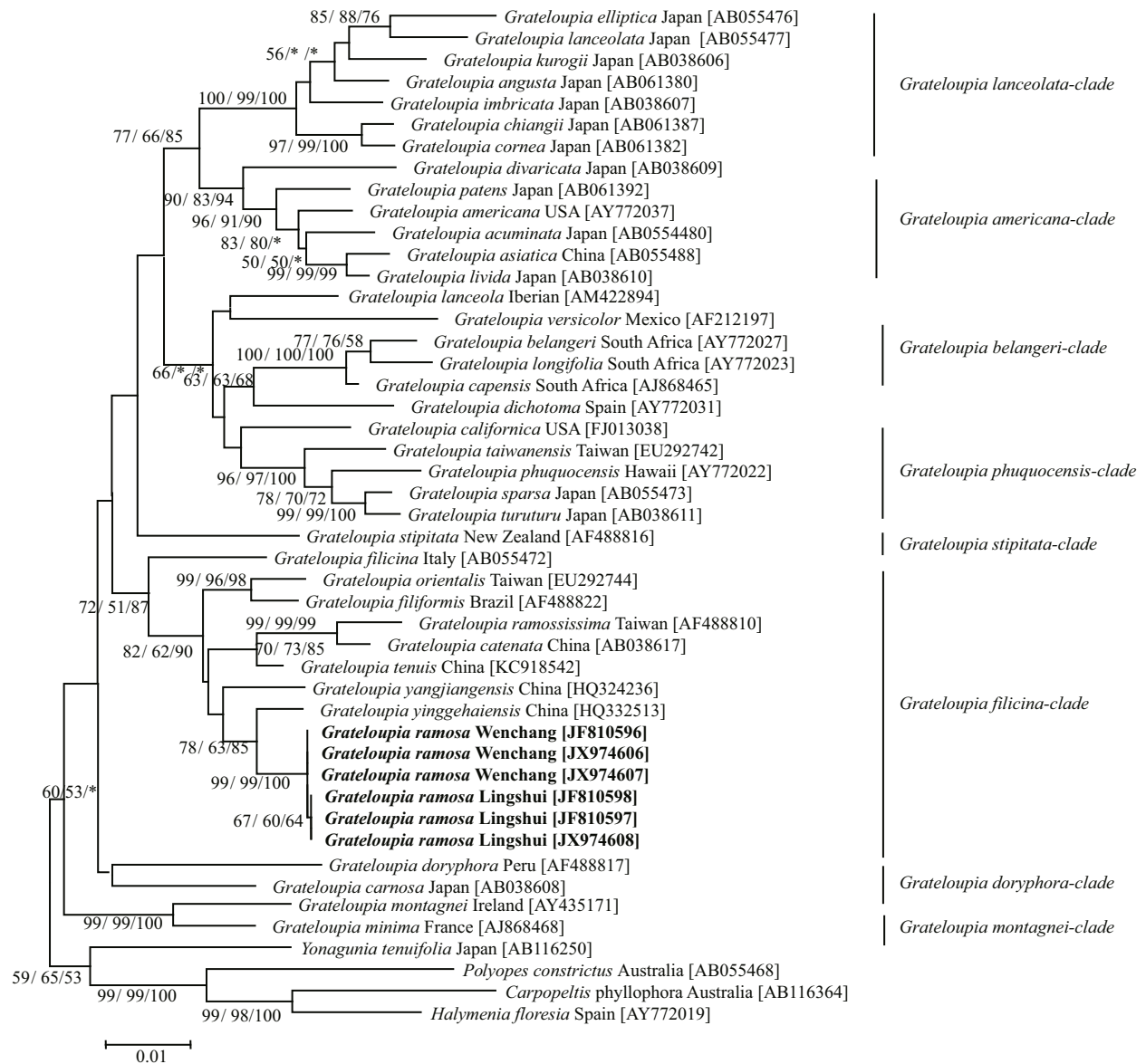


Fig.3 Maximum likelihood (ML) tree based on partial *rbcL* gene sequences data showing the phylogenetic relationship of *Grateloupia ramosa* with the other species within *Grateloupia* and species in other related genera in Halymeniaceae

Numerals at internal nodes are bootstrap values (1 000 replicates) inferred from ML (front), MP (middle) and NJ (behind). Only values above 50% bootstrap support are shown. “*” indicates the numbers of corresponding bootstrap values that lower than 50%. Boldface shows new species studied in this research.

filaments. Chiang (1970) reported that six genera in Halymeniaceae (*Grateloupia* C. Agardh, *Prionitis* J. Agardh, *Pachymeniopsis* Yamada ex Kawabata, *Dermocorynus* P. L. Crouan & H. M. Crouan, *Yonagunia* Kawaguchi & Masuda and *Phyllymenia* J. Agardh) had *Grateloupia*-type ampullae (Chiang, 1970; Kawaguchi et al., 2004; Mateo-Cid et al., 2005; Gargiulo et al., 2013). Wang et al. (2001) transferred *Pachymeniopsis* and *Prionitis* to *Grateloupia* (Kawaguchi, 1997; Wang et al., 2001), and *Phyllymenia* has also been merged to *Grateloupia* (De Clerck et al., 2005b). The genus *Sinotubimorpha*

also had simple *Grateloupia*-type ampullae, and was therefore transferred to *Grateloupia* after ampullary morphological observation and molecular analysis (Sheng et al., 2012). However, Gargiulo et al. (2013) recently resurrected the four groups based on the architecture of carpogonial and auxiliary cell ampullae and the early postfertilization events. Corresponding to Chiang (1970), Gargiulo et al. (2013) and other authoritative algal taxonomists investigating *Grateloupia* species (Wang et al., 2000; Faye et al., 2004; Lin et al., 2008; Gargiulo et al., 2013), the architecture of carpogonial and auxiliary cell ampullae

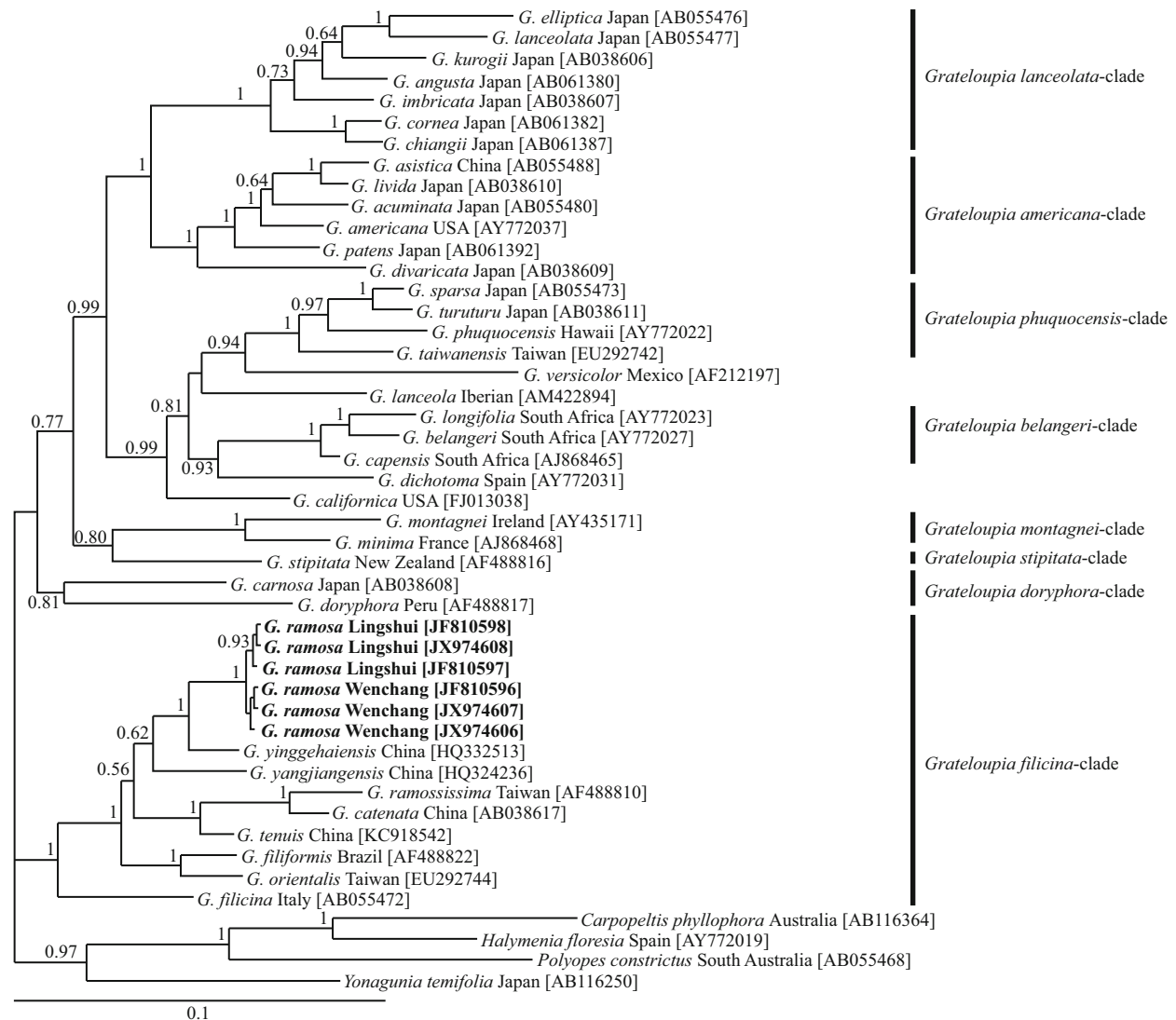


Fig.4 Bayesian tree based on partial *rbcL* gene sequences data showing the phylogenetic relationship of *Grateloupia ramosa* with the other species within *Grateloupia* and species in other related genera in Halymeniaceae

Bayesian posterior probabilities above 0.50 are indicated next to branches. Boldface type shows new species investigated in this research.

of our species (Fig.2f, g) shows that it is a member of the family Halymeniaceae that belongs to the genus *Grateloupia* based on its four-celled carpogonial branch and 3-celled auxiliary branch.

The proposed *G. ramosa* species is characterized by its linear thalli, median height of 5–10 cm, percurrent compressed axes, abundant branches that are opposite in arrangement, and constriction at the base. The terminal branches consisted of multiple claw-like branches gradually tapering to the apices. Reproductive structures are distributed throughout the thallus, especially centralize the bottom of the terminal branches. Table 3 shows a comparison of morphological characteristics of *G. ramosa* and the nearby species, *G. catenata*, *G. filicina*, *G. ramosissima*, *G. orientalis* and *G. tenuis*. The results demonstrate that the features

of this new species differed from other species, including the generitype *G. filicina*. *G. yinggehaiensis* is the most closely related to our species and has the same type of ampullae. However, these organisms are not similar morphologically. Specifically, *G. yinggehaiensis* is foliated and composed of several completely compressed branches bearing irregular pinnate branchlets, whereas *G. ramosa* is linear, with percurrent axes bearing vimineous claw-like branchlets. Prominent morphological diversity was also observed between *G. ramosa* and *G. catenata*. These species are somewhat similar based on their outer appearance and numerous proliferations, but have differing branching patterns, habits, and locations of reproductive structures and the medulla. The erect axes of *G. catenata* are up to 35 cm high. The erect

Table 3 Morphological comparison of *Grateloupia ramosa* and related species

Morphological characteristics	<i>G. ramosa</i>	<i>G. tenuis</i>	<i>G. catenata</i>	<i>G. filicina</i>	<i>G. ramosissima</i>	<i>G. orientalis</i>	<i>G. yinggehaiensis</i>
Branching pattern	Abundant branches; claw-like proliferated branches, 2–4 cm constricted at base. 5–10 cm high, 2–5 mm wide	Fewer branches; two or three dichotomous branches; 4–7 cm high, 0.2–0.6 mm wide	Irregular pinnate branchlets; 7–35 cm high, 2–3 mm wide	Di or trichotomously branched, numerous, pinnate arrangement, 7–75 cm high	Bifurcate or alternate branches, segments are linear, 13–22 cm high, 2–3 mm wide	Thalli are cylindrical, irregular or pinnate branches, 16cm high	Composed of several completely compressed branches bearing irregular pinnate branchlets; 5–10 cm high
Axes	Erect axes to 5–10 cm, compressed	Erect axes	Erect axes, terete to compressed branches	Percurrent axes are compressed terete to flattened blades	Erect axes, terete to compressed	Terete to slightly compressed	Erect axes, compressed to flattened upward
Texture	Cartilaginous and lubricous	Slippery and cartilaginous	Mucilaginous	Lubricous	Cartilaginous	Cartilaginous	Cartilaginous and slippery
Cortex (C)	C: 3–6 layers	C: 5–6 layers	C: 6–14 layers	C: 5–8 layers	C: 8–9 layers	C: 6–9 layers	C: 4–6 layers
Medullary structure	Unhollow	Hollow	Hollow	Unhollow	Unhollow	Hollow	Unhollow
Proliferations	Frequent proliferations throughout the thallus, numerous	Not numerous	Numerous	Not numerous	Numerous	Not numerous	Not numerous
Location of reproductive structures	Scattered over the entire thallus, centralized at the bottom of the terminal branches	Main axes and lower portions of branches	Proliferations and upper portions of thalli	Whole thalli	Scattered over the entire thallus	Scattered over the entire thallus except for the basal parts	Scattered over the entire thallus except for the basal parts
Native regions	Hainan Province, China	Hainan Province, China	China, Japan, Korea	Mediterranean, Italy	Korea, Vietnam, China, Japan, Philippines	Taiwan, China	Hainan Province, China
Reference	This paper	Yu et al., 2013	Wang et al., 2000; Lee et al., 2009	Kawaguchi et al., 2001	Lin et al., 2008	Lin et al., 2008	Zhao et al., 2012

axes are terete below, becoming compressed up to 2 mm wide above (Wang et al., 2000), whereas our species has shorter compressed erect axes 5–10 cm high. The proliferations arising from *G. catenata*'s erect axes have one or two constrictions (Wang et al., 2000), but our species only has claw-like proliferations and constrict at the base. *G. catenata* has a hollow medulla, but the medulla of *G. ramosa* has compact medullary filaments. These data indicated that the studied species was not identical to *G. catenata*.

The molecular phylogenetic data also support the declaration of a new *Grateloupia* species. These data show 27–28 bp (2.19%–2.28%) divergence between *G. ramosa* and *G. yinggehaiensis*, which was closest to *G. ramosa* in the phylogenetic tree, even though these species are not at all similar morphologically (Table 3). Moreover, the pairwise distance between species of *Grateloupia* was 13–111 bp (1.0%–8.8%) (Kawaguchi et al., 2002), indicating that the proposed species belonged within the *Grateloupia* and was not the same species as *G. yinggehaiensis*. There was also 44–45 bp (3.62%–3.71%) divergence between

G. ramosa and *G. catenata*, which was most similar to the proposed species in external morphology. *G. ramosa* and *G. tenuis* showed a 39–40 bp difference, but were similar in external morphology. These sequence divergences were well within the interspecific values observed within *Grateloupia* (García-Jiménez et al., 2008; Lee et al., 2009). The sequence divergence between our species and outgroups in other genera within Halymeniaceae [106–132 bp (9.01%–11.49%)] clearly indicate that *G. ramosa* belongs to the genus *Grateloupia*, family Halymeniaceae. The phylogenetic tree also clearly revealed eight subclades of the selected species within the large *Grateloupia* sensu lato clade, *G. lanceolata*-clade, *G. americana*-clade, *G. belangeri*-clade, *G. phuquocensis*-clade, *G. stipitata*-clade, *G. filicina*-clade, *G. doryphora*-clade and *G. montagnei*-clade. The *G. ramosa* specimens formed an individual subclade in the *G. filicina*-clade. The three samples collected from Wenchang and three from Lingshui, which are both in Hainan Province, China, only had 1 bp divergences and could therefore be considered one

species. These organisms also formed a single subclade within the *G. filicina*-clade with the sister related species *G. yangjiangensis*, *G. orientalis*, *G. filiformis*, *G. tenuis* and *G. yinggehaiensis*, among which *G. yinggehaiensis* was most closely related to our species.

The conclusions obtained in the paper support the point of view suggested by Gargiulo et al. (2013); thus, based on morphological and *rbcL* sequence data, it is proposed that these specimens belong to the genus *Grateloupia* and should be named *G. ramosa* Wang & Luan sp. nov.

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