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Morphological observation and *rbc*L gene sequences studies of two new species, *Grateloupia dalianensis* H.W.Wang et D.Zhao, sp. nov. and *G. yinggehaiensis* H.W.Wang et R.X.Luan, sp. nov. (Halymeniaceae, Rhodophyta) from China

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Abstract

A few species in the genus *Grateloupia* (Halymeniaceae, Rhodophyta) have been investigated in detail with respect to morphological observations and molecular analyses. In this study, the authors document the vegetative and reproductive structures of two new species of *Grateloupia, G. dalianensis* H.W.Wang et D.Zhao, sp.nov. and *G. yinggehaiensis* H.W.Wang et R.X.Luan, sp.nov. They both have the morphological character that carpogonial ampullae and auxiliary cell ampullae are the simple *Grateloupia*-type. The two species can be distinguished from other species of the genus by their distinctive morphological features respectively. Based on ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) gene sequences, the phylogenetic tree obtained in the study indicated that they are both embedded within the *Grateloupia* clade. *G. dalianensis* clusters a subclade with *G. asiatica*, and *G. yinggehaiensis* forms a single monophyletic subclade with *G. hawaiiana*.

Key words: Halymeniaceae, *Grateloupia, Grateloupia dalianensis, Grateloupia yinggehaiensis*, morphology, *rbc*L gene

1 Introduction

The marine red algal genus *Grateloupia* (Halymeniales, Rhodophyta), containing more than 80 species, is the most species-rich genus and distributed widely throughout the world, whether in temperate or 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; see also Guiry and Guiry, 2007; Lin et al., 2008). The generitype, *G. filicina* (Lamouroux) C. Agardh, was first reported by C. Agardh in 1822 from the Mediterranean Sea when the genus *Grateloupia* was established. Thirty-two species were distributed in China (Xia, 2004). *Grateloupia* has been considered problematic in environmental changes and different stages of physical impact because the species is diverse in gross morphology, ranging from small to large, soft to hard, irregularly to frequently branched and with or without pinnate branchlets. Morphological classification criteria have not been in unity. Sometimes two individuals of the same species have been identified as different types, or two different types have been identified artificially as one species. Because of this, *Grateloupia* is regarded as one of the most difficult subjects classified by algal taxonomists in the world. In recent years, some entities have clarified the taxonomic status by combining molecular and morphological analysis (Wang et al., 2000; Kawaguchi et al., 2001; Gavio, 2002; Gavio and Fredericq, 2002; Mateo-Cid et al., 2005) and obtained positive results very quickly, but because of the diversity, many taxa in the genus still require a critical reassessment.

During the course of investigation to assess the diversity of benthic marine macroalgae along the coast of China, we encountered two entities. Through careful observation, we found that not only the external morphology but also the internal structure of the two entities are different from other algae respectively, and we have not found any reports about them up until

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now. Simple auxiliary cell and carpogonial ampullary filaments are typical of the *Grateloupia*-group in the Halymeniaceae (Chiang, 1970). We preliminary judged them to be two new species. Furthermore, the *rbc*L sequence analysis also strongly supports this conclusion. Herein, we define the alga as two new species with the name of *G. dalianensis* H.W.Wang et D.Zhao, sp.nov. and *G. yinggehaiensis* H.W.Wang et R.X.Luan, sp.nov.

2 Materials and methods

2.1 *Morphological observations*

Specimens were collected along the coast of China. Voucher herbarium specimens are deposited in the Herbarium of the College of Life Sciences, Liaoning Normal University, Dalian, China (LNU).

G. dalianensis: (1) Fujiazhuang, Dalian, Liaoning Province, China (20 June 2009, leg. H.W.Wang and R.X.Luan; LNU20096046). (2) Xinghai Park, Dalian, Liaoning Province, China (20 June 2008, leg. H.W.Wang and R.X.Luan; LNU20086047). (3) Zhangzi Island, Dalian, Liaoning Province, China (10 July 1996, leg. H.W.Wang and R.X.Luan; LNU19960787). (4) Guanglu Island, Dalian, Liaoning Province, China (10 July 1983, leg. H.W.Wang and R.X.Luan; LNU19830710).

G. yinggehaiensis: (1) Yinggehai, Hainan Province, China (24 February 2009, leg. R.X. Luan and X.K.Zhu; LNU20092045). (2) Lingshui, Hainan Province, China (28 February 2009, leg. R.X.Luan and X.K.Zhu; LNU20092050).

Morphological observations were made on specimens preserved in 10% formalin/sea water, or rehydrated herbarium specimens. Sections were made by hand, using a razor blade and pith stick, stained with 0.5% (w/v) cotton blue in a lacticacid/phenol/glycerol/water (1:1:1:1) solution, and mounted in 50% glycerol/seawater on microscope slides. Drawings were made using an Olympus BH_2 digital camera mounted on a Nikon DLC300-L (Japan) microscope.

2.2 *Molecular analysis*

Total DNA was extracted from four samples of *G. dalianensis* and two samples of *G. yinggehaiensis*. The procedures for DNA extraction, PCR amplification and sequencing were those as described previously (Wang et al., 2000). Sequence analyses were performed manually because no insertion/deletion mutations were detected. *rbc*L genes sequence from an additional 41 species of Halymeniaceae were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/), and were used for sequence alignments (Table 1). *Halymenia floresia* and *Polyopes constrictus* were used as outgroups for the sequence analysis.

Phylogenetic trees were constructed by Neighbour Joining (NJ) methods and maximum parsimony (MP) methods, using a Macintosh G4 personal computer. The parsimony analysis was performed with PAUP 4.0b10 (Swofford, 2002). All sites were treated as unordered and equally weighted. In order to seek the best settings for the NJ analysis, all kinds of increasingly complex models of molecular evolution were regarded as outlined by Litaker et al. (1999) and Moncalvo et al. (2000).

3 Results

3.1 *Molecular analyses*

In the phylogenetic tree, four *G. dalianensis rbc*L sequences and two *G. yinggehaiensis rbc*L sequences were compared with additional 41 species of Halymeniaceae including two outgroups (Table 1). Because sequences were incomplete at the 5′ and 3′ ends, 1 173 base pairs (bp) corresponding to positions 181 to 1 354 of the full *rbc*L gene (1 462 bp) were used for the alignment. Sequences of four samples, respectively from four different localities of China, of *G. dalianensis* were found to be identical, and sequences of the samples of *G. yinggehaiensis* from Yinggehai and Lingshui in Hainan Province were found to be identical.

The phylogenetic trees obtained from NJ and Maximum Parsimony (MP) analysis are presented in Fig.1 and Fig.2. In both NJ and MP analysis, three main clades, which are supported with high bootstrap values, were recognized within the Halymeniaceae, which is a *Halymenia* clade, a *Polyopes* clade and a large *Grateloupia* clade.

Topologies of the NJ and MP trees are almost congruent. In the phylogenetic tree obtained in the study (Figs 1 and 2) the existence of a large *Grateloupia* clade is evident. Both in the NJ and MP analysis, within the large *Grateloupia* clade, *G. dalianensis* from China is a sister taxon to *G. asiatica* from China with some supports and with 66 base pairs (bp) difference (5.02%). The pair-wise distance difference between *G. dalianensis* and the species clustered in the same subclade ranged from 5.55% to 10.79% (73 to 142 bp). Sequences among *G. dalianensis* and *Halymenia*

Table 1. List of species used in *rbc*L analysis, their collection data and GenBank accession number

Species	Collection data (Location and site and reference)	Accession no.
Halymenia floresia (Clemente) C. Agardh	Pulau Rebak Besar, Langkawi, Kedah, Malaysia (Wang et al., 2000)	AB038603
Polyopes constrictus (Turner) J. Agardh	Point Lonsdale,Victoria, Australia (Wang et al., 2000)	AB055468
G. belangeri South Afrien	Platboom,Western Cape Province (De Clerck et al., 2005a)	AY772027
G. orientalis S.-M.Lin et H.-Y.Liang	S.Taiwan (Lin et al., 2008)	EU292744
G. catenata Yendo	Shimiao, Dalian, Liaoning Province, China (Wang et al., 2000)	AB038617
G. ramossissima Okamura	Ho Ping Island, Keelung, North Taiwan (Gavio et al., 2002)	AF488810
G. hawaiiana Dawson	Maui, Hawai (De Clerck et al., 2005a)	AY772030
G. filicina (Lamouroux) C. Agardh	Livorno, Italy (Wang et al., 2000)	AB055472
G. carnosa Yamada et Segawa	Oryuzako, Miyazaki Prefecture, Japan (Wang et al., 2000)	AB038608
G. doryphora (Montagne) M. A. Howe	Playa de San Francisco, Lima, Peru (Gavio et al., 2002)	AF488817
G. acuminata Holmes	Katase, Fujisawa, Kanagawa Prefecture, Japan (Wang et al., 2000)	AB055480
G. americana Kawaguchi et Wang	Pigeon Point, San Matio County, California, USA (De Clerck et al., 2005a)	AY772037
G. asiatica Kawaguchi et Wang	Qingdao, Shandong Province, China (Kawaguchi et al., 2001)	AB055488
G. livida (Harvey) Yamada	Izu-misaki, Miyake Island, Tokyo, Japan (Wang et al., 2000)	AB038610
G. patens (Okamura) Kawaguchi et Wang	Oohara, Chiba Prefecture, Japan (Wang et al., 2001)	AB061392
G. divaricata Okamura	Oshoro, Hokkaido, Japan (Wang et al., 2000)	AB038609
G. schmitziana (Okamura) Kawaguchi et Wang	Shikanoshima, Prefecture, Japan (Wang et al., 2000)	AB061398
G. lanceolata (Okamura) Kawaguchi	Shikanoshima, Fukuoka Prefecture, Japan (Kawaguchi et al., 2001)	AB055477
G. elliptica Homles	Goshikinohama, Usa, Tosa, Kochi Prefecture, Japan (Wang et al., 2000)	AB055476
G. chiangii Kawaguchi et Wang	Izu-misaki, Miyake Island, Tokyo, Japan (Wang et al., 2001)	AB061387
G. cornea Okamura	Izu-misaki, Miyake Island, Tokyo, Japan (Wang et al., 2001)	AB061382
G. angusta (Okamura) Kawaguchi et Wang	Miyanoura, Hirado Island, Nagasaki Prefecture, Japan (Wang et al., 2001)	AB061380
G. kurogii Kawaguchi	Saikai-bashi, Nagasaki Prefecture, Japan (Wang et al., 2001)	AB038606
G. imbricata Holmes	Tsuyazaki, Fukuoka Prefecture, Japan (Wang et al., 2000)	AB038607
G. longifolia Kylin	Yzerfonteyn, Western Cape Province, South Africa (De Clerck et al., 2005a)	AY772023
G. capensis De Clerck	South Africa (De Clerck et al., 2005a)	AJ868465
G. dichotoma J. Agardh	Lugo, Galicia, Spain (De Clerck et al., 2005a)	AY772031
G. sparsa (Okamura) Chiang	Oohara, Chiba Prefecture, Japan (Wang et al., 2000)	AB055473
G. turuturu Yamada	Muroran, Hokkaido, Japan, South Africa (Wang et al., 2000)	AB038611
G. subpectinata Holmes	Japan (Faye et al., 2004)	AB114213
G. taiwanensis Lin et Liang	Northeastern and southern Taiwan (Lin et al., 2008)	EU292742
G. lanceola (J. Agardh) J. Agardh	Iberian (Figueroa et al., 2007)	AM422894
G. somalensis Hauck	Plage de Monseigneur, Fort Dauphin, Madagascar (De Clerck et al., 2005a)	AY772021
G. versicolor J. Agardh	Barra de Navidad, Mexico1)	AF212197
G. phuquocensis Tanaka et Hoang Hoang	Kaalawai, Oahu, Hawaiian Islands (De Clerck et al., 2005a)	AY772022
G. filiformis Kutzing	Marataizes, Espiritu Santu, Brazil (Gavio and Fredericq, 2002)	AF488822
G. heurtana Mateo-Cid, Mendoza-González & Gavio	Oaxaca, Pacific Mexico LUZ ELENA (Mateo-Cid et al., 2005)	AY178760
G. stipitata J. Agardh	Lee Bay, Stewart Island, New Zealand (Gavio et al., 2002)	AF488816
G. californica Kylin	California, USA (Miller et al., 2009)	FJ013038
G. minima P. Crouan et H. M. Crouan	Brest, Atlantic France (De Clerck et al., 2005a)	AJ868468
G. montagnei P. Crouan et H. M. Crouan	Ireland2)	AY435171
G. yinggehaiensisi H.W.Wang et R.X.Luan	Yinggehai, Hainan Province, China (LNU20092045)	HQ332513
G. yinggehaiensisi H.W.Wang et R.X.Luan	Lingshui, Hainan Province, China (LNU20092050)	HQ332514
Grateloupia dalianensis H.W.Wang et D.Zhao	Fujiazhuang, Dalian, Liaoning Province, China (LNU20096046)	HQ385503

To be continued

Continued from Table 1

Species	Collection data (Location and site and reference)	Accession no.
Grateloupia dalianensis H.W.Wang et D.Zhao	Xinghai Park, Dalian, Liaoning Province, China (LNU20086047)	HQ385504
Grateloupia dalianensis H.W.Wang et D.Zhao	Zhangzi Island, Dalian, Liaoning Province, China (LNU19960787)	HQ385505
Grateloupia dalianensis H.W.Wang et D.Zhao	Guanglu Island, Dalian, Liaoning Province, China (LNU19830710)	HQ385506

Notes: 1) Fredericq S, Moe D L, Ramirez M E. Trematocarpus antarcticus comb.nov. (Sarcodiaceae, Rhodophyta) from the Antarctic Peninsula: Characterization of the alga known as *Kallymenia antarctica* Hariot. 2) Wilkes R J, McIvor L M, Guiry M D. Speciation in *Grateloupia* (Cryptonemiales, Rhodophyta) with particular reference to some cryptic and introduced species.

floresia, Polyopes constrictus, differ by 145–151 bp (11.03%–11.48%). Two samples of *G. yinggehaigensis* from Hainan and *G. hawaiiana* from Hawaii form a single monophyletic subclade. The position of *G. filicina* from Italy comes to the sister position of *G. yinggehaigensis/G. hawaiiana* subclade. It is evident that *G. yinggehaigensis*, which is phylogenetically approaching *G. filicina*, occupies the basal position of the large *Grateloupia* clade. The pairwise distances in *rbc*L sequences between *G. yinggehaiensis* from China and *G. hawaiiana* from USA are 45 bp changes (3.6%), and the pairwise distances in *rbc*L sequences between *G. yinggehaiensis* from China and *G. filicina* from Italy are 73 bp changes (5.8%); those between *G. yinggehaiensis* and other species in *Grateloupia* range from 53–139 bp changes. Sequences among *G. yinggehaigensis* and *H. floresia, P. constrictus*, differ by 117–125 bp (9.73%–10.74%).

3.2 *Taxonomic descriptions*

Grateloupia dalianensis H.W.Wang et D.Zhao, sp.nov. (Figs 3 and 4).

Thalli raggruppati, erectae in colore atroruberae puniceus, cartilaginous lubricus in texture. 15–30 cm altae, dichotome velsubdichotome ramosae quinquiens ad octiens, medulla laxe, non alveus; cortex 2–6 cellulas; Gametophyta dioecia, structuras reproductivas super partes superas ad medias laminae efferentia; spermatangia 3–4 µm longa et 1–4 µm diametro; ampullae ramorum carpogonialium filis secundariis tribus vel quatuor simplicibus de cellula auxiliari; cupulatae, cellula auxiliari ovoidea; cellula auxiliaries ceters leviter grandior; matura cystocarpia sphericalia 200–290 µm diametro; matura tetrasporophytes gero cruciately vel decussately tribuo tetrasporangia postulo 34–56 µm longa et 12–24 µm lata, in cortice externo inclusa.

Thalli cluster in upright blades, bright red and purple in color, cartilaginous and gelatinous in texture, 15–30 cm high, and dichotomously or subdichotomously branched two to three times. The medulla is lax but not hollow and the cortex is 2–6 cells thick. Gametophytes are dioecious, producing reproductive structures demarcate the upper or middle portion of the blades. Spermatangia are 3–4 µm long and 1–3 µm in diameter. Carpogonial branches are auxiliary with 1–4 secondary filaments and a two-celled Carpogonial branch; auxiliary cell with one to two simple secondary filaments and an auxiliary cell, cup-shaped, with a large, spheroidal auxiliary cell. The mature auxiliary cell is a little larger than other ampullary cells. Oval mature cystocarps are 200–290 µm in diameter. Mature tetrasporophytes bear cruciately or decussately divided tetrasporangia and measure 34–56 µm long by 12–24 µm wide, embedded in the outer cortex.

Holotype: Tetrasporaphite specimen (LNU20096046), collected by H.W. Wang on 20 June 2009.

Type locality: Dalian in Liaoning Province, southern China (38.5°N, 120°E).

Etymology: Distributed in Liaoning and Shandong Province, China.

Habit and vegetative morphology: *G. dalianensis* grows on rocks or pebbles at middle intertidal zones or low-tide terraces. Algae tufts survive alone. The texture is cartilaginous, slimy and soft, and the color is purple (occasionally somewhat greenish). The main branch is flat, 3–6 cm wide, 20–30 cm high and plumed. There is a 2–3 mm handle in the basal part of thalli, and a discoid holdfast at the bottom. Occasionally there is a prominence at the surface of the blades. The width of the blades is 1–2 cm (Fig. 3). There are some black protuberances, which barrayed at the surface of the blades (called sporangium) (Fig. 4b). The blades are composed of filamentous medulla and cellular cortexes (Fig. 4c). The medulla is comp-

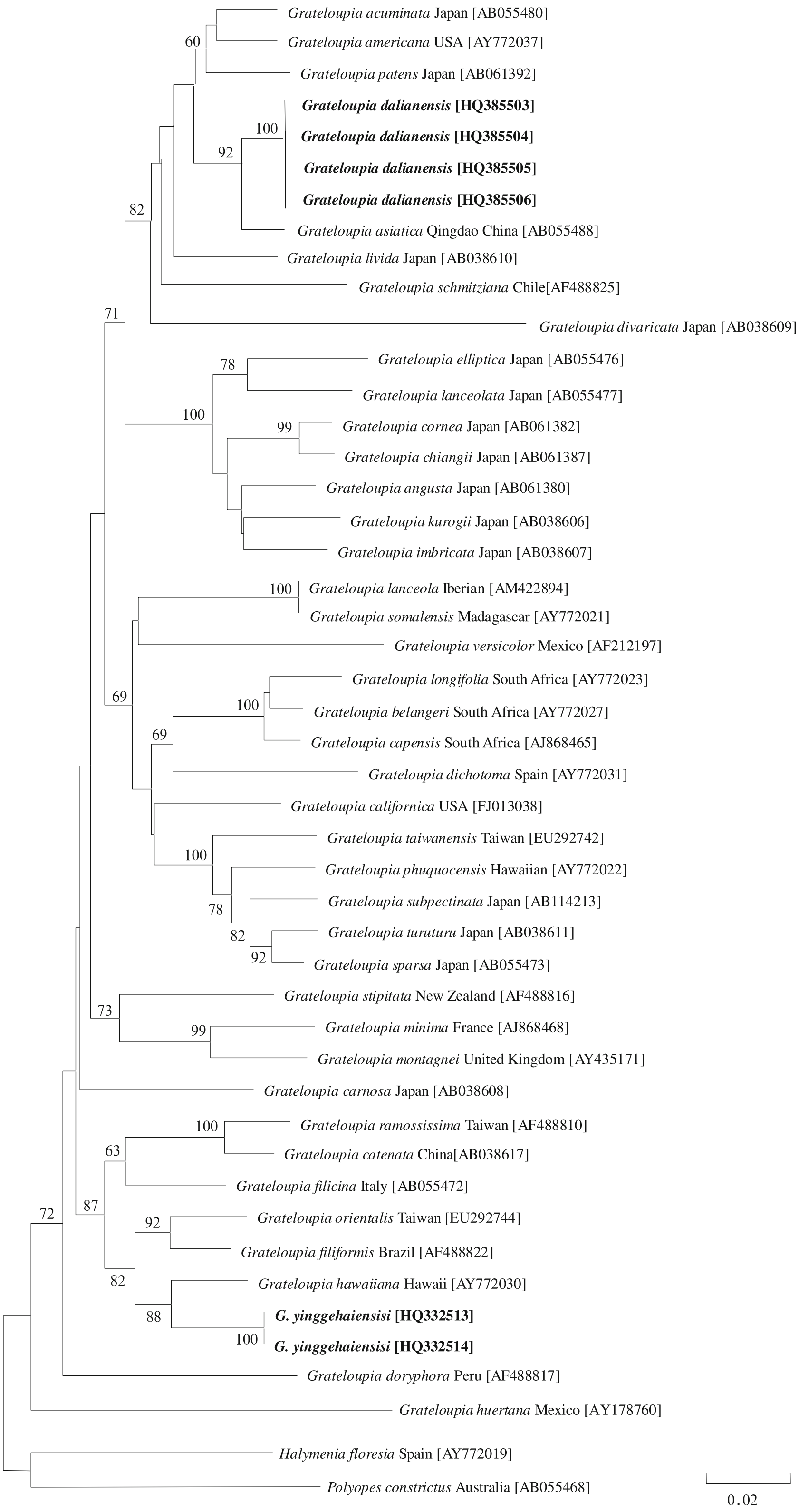

Fig.1. NJ tree based on partial *rbc*L gene sequences (**173** bp). *Halymenia floresia* and *Polyopes constrictus* were used as outgroups. Numerals at internal nodes (percentages) are bootstrap values (100 replicates). Only values above 60% bootstrap support are shown. Scale bar is 0.01 substitutions per site. Boldface shows new species studied in this study.

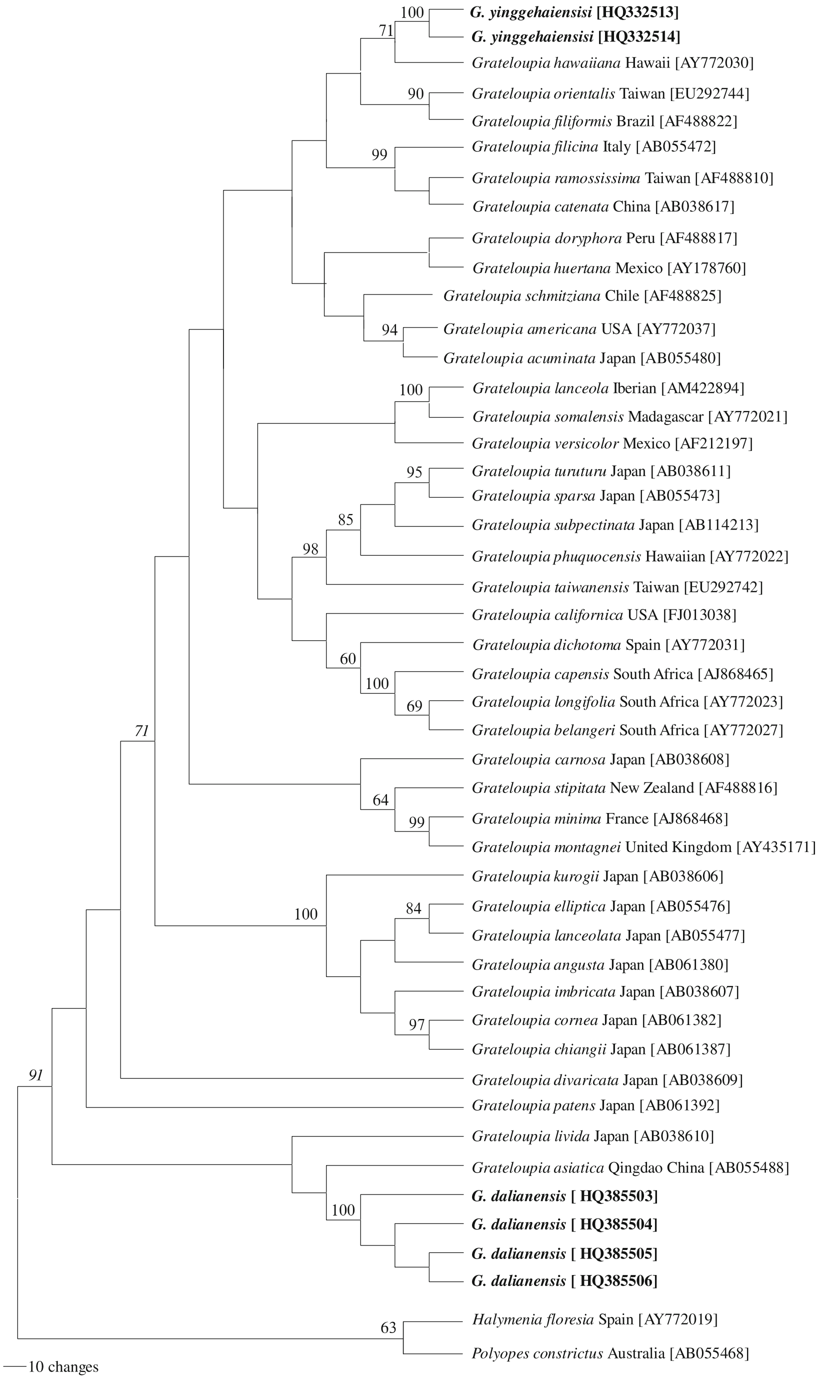

Fig.2. Most parsimonious tree of partial *rbc*L gene sequences (1 173 bp). *Halymenia floresia* and *Polyopes constrictus* were used as outgroups. All sites were treated as unordered and equally weighted; only values above 60% bootstrap support are shown. Boldface shows new species studied in this study.

osed of rather dense filaments, which are 10–40 μm in length and 2–5 μm in width. The Cortex consists of 2–6 layers, anticlinally arrayed. The outer cortex is composed of four layers of ellipsoidal to rounded cells, and the inner cortex is composed of two or three irregularly round or oval cells. Outer cortex cells metamorphically develop into carpogonium (Fig. 4e), whose upside is trichogyne, and the bottom is ovum.

Reproductive morphology: The Gametophytes are dioecious and reproductive structures range from the middle to upper portions of its blades. Carpogonial branches are auxiliary with 1–4 secondary filame-

Fig.3. Holotype specimen of *Grateloupia dalianensis* collected from Fujiazhuang of Dalian (Tetrasporaphite, LNU20096046).

nts and a two-celled Carpogonial branch; two or four simple secondary filaments compose the auxiliary cell ampulla, which are narrowly flask-shaped (Fig. 4f). Carposporangia is formed by abundant gonimoblast cells. Cystocarps are deeply immersed in the medulla, with a distinct pore above and a loose network of involucral filaments. Mature cystocarps are spherical or hemispherical, 200–290 mm in diameter (Figs 4g-k). Tetrasporangia are formed from the cortical cells in the second or forth layer from the surface and are diffused over the middle to the upper portions of the blades. Mature tetrasporangia are cruciately or decussately divided, narrowly ellipsoidal in shape, 34–56 μm height and 12–24 μm width, and are embedded in the outer cortex (Fig. 4l). There are many spermatangiums at the surface of the thalli with white speckles (Fig. 4a). Spermatangia (arrows) were produced from the outermost cortical cells (Fig. 4d). Spermatangia are 3–4 μm long and 1–3 μm in diameter.

Grateloupia yinggehaiensis H.W.Wang et R.X.Luan, sp.nov. (Figs 5 and 6).

Thalli foliosus, colore puniceus ad atrum rutilus, cartilaginous quod lubricus in texture, haptero parvo disciformi oriens, usque ad 10 cm altus; molior axes es decens cogo ut palpo sursum paro per multiplex incomposite pinnate branchlets. Proliferationibus paucis e marginibus er paginis. medulla laxe vel profundus, non alveus; cortex 4–6 cellulas crassus. Gametophyta dioecia (raromonoecia), structuras reproductivas super thallum omnem praeter partem basalem efferentia; ampullae ramorum carpogonialium filis secundariis duobus vel tribus et ramo carpogoniali bicellulari; ampullae cellulae auxiliaries filis secundariis tribus vel quatuor simplicibus de cellua auxiliary cupulatae, cellula auxiliary ovoidea vel ellipsoidali grandi; cellula auxiliaries ceters leviter grandior; matura cystocarpia sphericalia 230–350 μm diametro; matura tetrasporophytes gero cruciately vel decussately tribuo tetrasporangia postulo 50–65 μm longa et 20–23 μm lata, in cortice externo inclusa.

Thalli foliose are purple to dark red in color, cartilaginous and slippery in texture and arise from a small discoid holdfast up to 10 cm high. The erect axes are compressed to flatten upward, set with numerous irregular pinnate branchlets with proliferations from margins and surfaces. The medulla is loose or dense but not hollow and the cortex is 4–6 cells thick. Gametophytes are dioecious and reproductive structures are scattered over the thallus except for the basal portion. Carpogonial branches are auxiliary with two or three secondary filaments and a two-celled carpogonial branch. The auxiliary cell has three or four simple secondary filaments and an auxiliary cell, cup-shaped, with a large, ovoid or ellipsoidal auxiliary cell; mature auxiliary cells are a little larger than other ampullary cells; mature cystocarps are spherical, 230–350 μm in diameter. Mature tetrasporophytes bear cruciately or decussately divided tetrasporangia and measure 50–65 μm long by 20–23 μm wide, embedded in the outer cortex.

Holotype: A female gametphyte (LNU20092045 Fig.5a), collected by R.X. Luan on 24 February 2009.

Typelocality: Yinggehai in Hainan Province, sourthen China (18.5°N, 108.7°E).

Etymology: Distributed in Hainan Province, China.

Vegetative morphology: Thalli are foliose and attached to solid bedrock by a discoid holdfast in the tide pools in the mid-to lower intertidal zone. Short stipe is 1–5 mm long and bears 2–6 flattened blades 5–10 cm high and 1–3 cm wide. Blades are purple to dark red when exposed in the blazing sun, cartilaginous and slippery in texture. Blades are 0.3–0.6 cm broad near the base, gradually broadening upward, and then narrowing toward blunt to blade-like apices and sickle-like apices. The blades are 500–700 μm thick and the

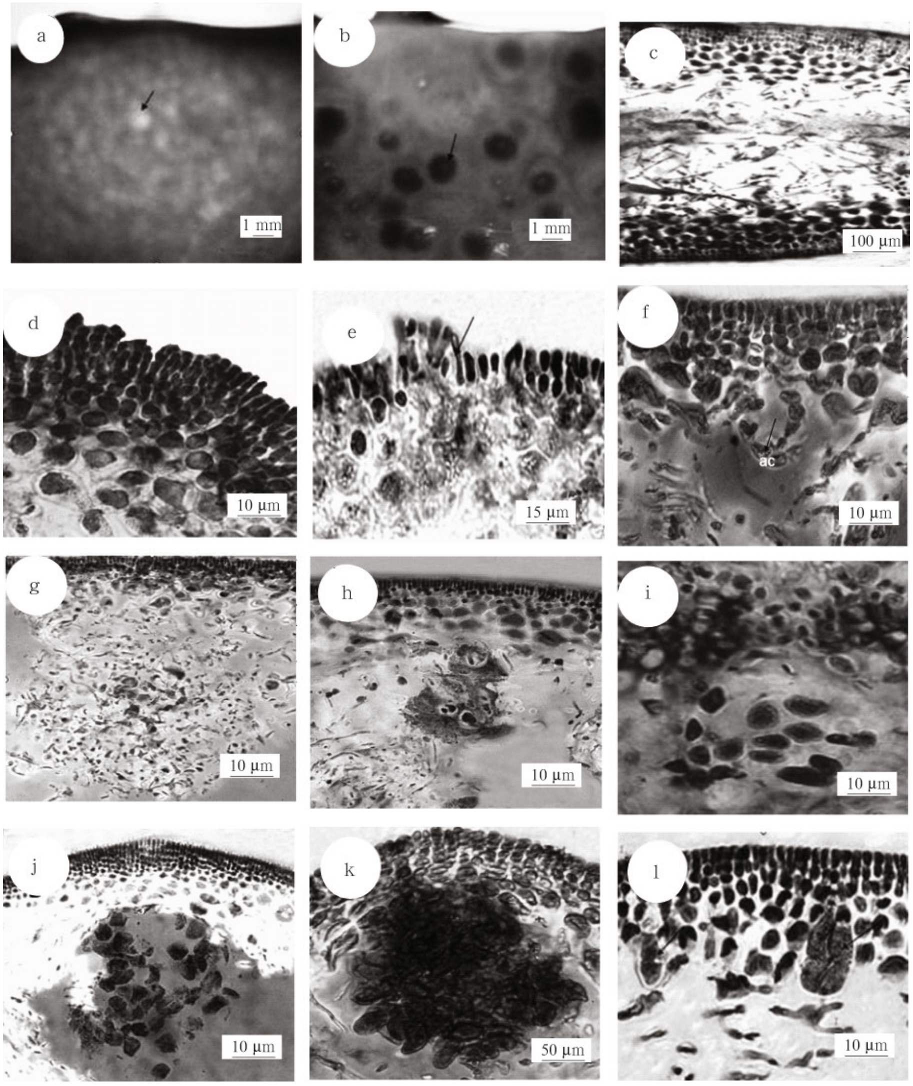

Fig.4. a. Spermatangia of *Grateloupia dalianensis*. b. Carposporangium of *Grateloupia dalianensis*. c. Internal structure of a blade, showing cellular cortex and medullar filament. d. Spermatangia (arrows) produced from the outermost cortical cells. e. Nutritive cell of exodermis metamorphos is development carpogonium. f. Cross-section shows mature auxiliary ampulla. g-k. Development of the cystocarp. l. Mature tetrasporangia.

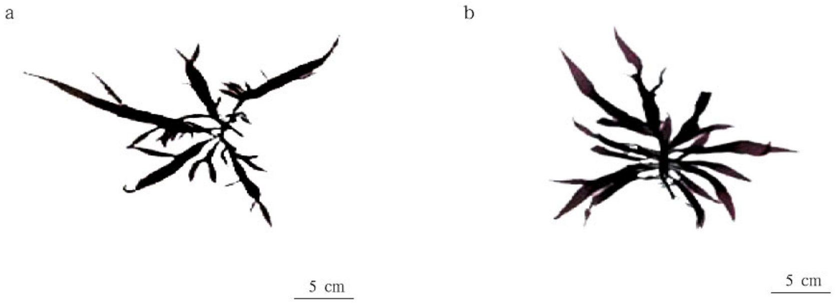

Fig.5. External morphology of *Grateloupia yinggehaiensis*. a. Holotype specimen of the *G. yinggehaiensis* collected from Yinggehai (A female gametophyte, 24 February 2009, leg. R.X. Luan LNU 20092045). b. Herbarium specimen of the *G. yinggehaiensis* collected from Lingshui (Tetrasporangia, 28 February 2009, leg. R.X. Luan LUN 20092050.

margins are occasionally swollen up to 850 mm thick. The margin of the blades often generate more or less irregular proliferations scattered over the thallus except for the basal portion on both young and older attached or drift specimens (Fig. 6a).

The thallus is not hollow. Internally it is composed of compacted cortex and a loosely or densely constructed filamentous medulla. The cortex is 60–80 μm in diameter and formed by 4–6 cell layers (Fig. 6b). The outer portion is composed of 2–3 layers of narrowly ellipsoidal cell, arranged in anticlinal rows and the inner portion consists of 2–3 layers of irregular or rounded cells. These cells are laterally connected by secondary pit-connections. The medulla is slender, very dense, and 3–10 mm in diameter and runs in various directions (Fig. 6b). Another medulla automatically merges into the inner cortex that consists of rounded or irregularly shaped cells.

Reproductive morphology: Reproductive structures are scattered over the thallus branches and proliferated but not found in their basal parts and the distal ends. Gametophytes are dioecious. Carpogonial branches and auxiliary cells are formed in separate ampullae which are produced from an inner cortex and then produce two to three unbranched filaments that grow straight towards the surface. Compared with the auxiliary-cell ampullae, carpogonial-branch ampullae are smaller and are branched to the third order. The carpogonia branches are two-celled in each carpogonial branch ampulla (Fig. 6c). Gonimoblasts are formed from the auxiliary cell toward the thallus surface after contact with a connecting filament (Fig. 6e). Different steps of the cystocarp development are shown (Figs 6d-h).

Mature cystocarps are spherical (Fig. 6h), 230–350 μm in diameter and may be compassed by more or less filaments that are mainly derived from ampullary cells and their derivatives. The cystocarps are deeply immersed in the medulla, and have a distinct pore above. Carpospores are released by a central ostiole in the overlying pericarp (Fig. 6h).

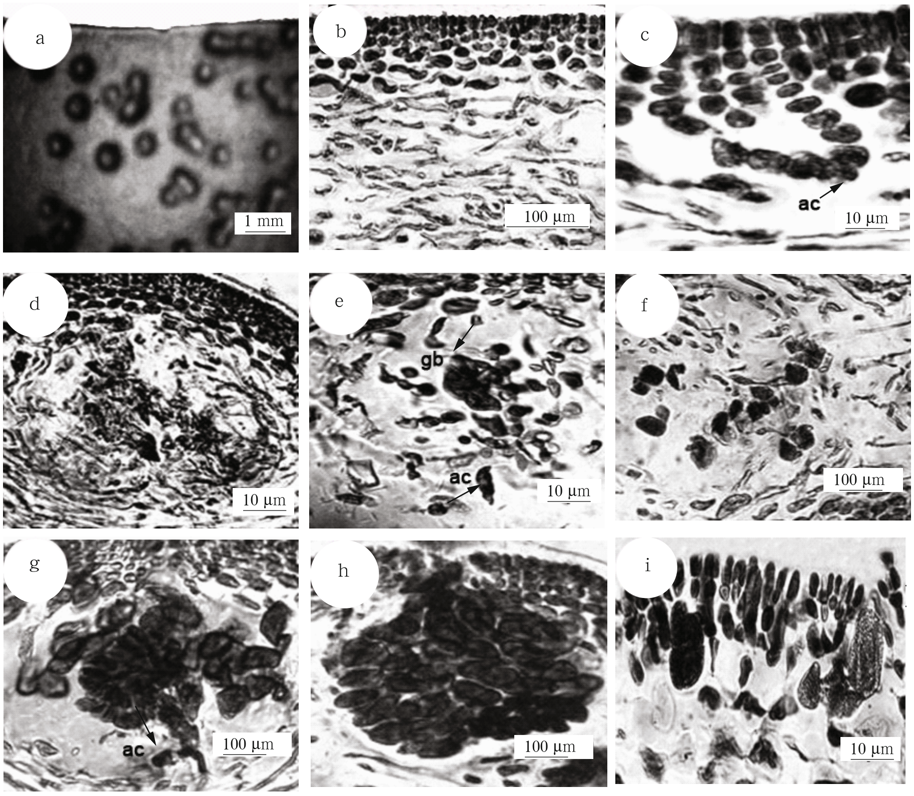

Fig.6. Vegetative anatomy of the *Grateloupia yinggehaiensis.* a. Part of proliferations at thalli. b. Detail of the cortex and medullar filaments. c. Mature auxiliary ampulla. d. Beginning period in the formation of cystocarp. e. Young gonimoblast produced from the auxiliary cell. f. Developing cystocarp. g. More advanced stage of gonimoblast development. h. Mature cystocarp is immersed in cortex. i. Tetrasporangia embedded in the cortex. ac represents auxiliary cell and gb gonimoblast.

Mature tetrasporophytes are cruciately or decussately divided, measure 50–65 μm long by 20–23 μm wide (Fig. 6i), are restricted to the surface of the blades and embedded in the inner cortex. Each tetrasporangia is a transformed surface cell which is cut from the second or third cortical cell from the surface (Fig. 6i) and then continues to grow the thallus outward gradually.

4 Discussion

The structure of the auxiliary cell ampulla is a good feature for separating genera within the Halymeniaceae (Chiang, 1970; Kawaguchi et al., 2001; Wang et al., 2001; De Clerck et al., 2005a). The *Grateloupia*-type is characterized by a single primary ampullary filament, simple ampullae, and a few secondary filaments. These characteristics are accepted by most algal taxonomists who study the *Grateloupia* species in the world (Kawaguchi et al., 2001; Wang et al., 2001). The species which we studied *G. dalianensis* and *G. yinggehaiensis* both have a primary ampullary filament and two to three secondary filaments forming flask-shaped ampullae (Fig.6c). They are similarity to the *Grateloupia*-type.

The new alga *G. dalianensis* is unique among species of *Grateloupia* in its (a) cartilaginous and slimy in texture; (b) cortex cells are skimp, containing 2–6 layers; (c) the surface of proliferation cells are scarce; (d) the reproductive structures are located in the middle to the upper portion of the blades. The great difference in size, the recent description of *G. asiatica* from Qingdao, China, includes blades 14–24 cm high and 1–5 mm wide, and *G. dalianensis* from Dalian, China, includes blades 20–30 cm high and 3–6 mm wide. Besides there are some other morphological differences, the thalli of both entities is fundamentally mucilaginous, but *G. asiatica* is much softer than *G. dalianensis.* Furthermore, the medulla of *G. asiatica* is generally densely constructed compared with *G. dalianensis* in which the medulla is tarnal laxly constructed with sparse filaments. The scattered reproductive structures of *G. dalianensis* also contrasts with *G. asiatica*, which has a tendency for reproductive structures to be restricted to the midst of the upper portion of the blades. Apart from *G. asiatica* from Qingdao (Kawaguchi et al., 2001), three species of *Grateloupia* are distinguished from *G. dalianensis* by reproductive structures and blade shape (Table 2). Among these, the generitype *G. filicina* (Lamouroux) C.Agardh, described from Italy, has blades 9–12 cm high and 2–5 mm in width. *G. auminata* Holmes from Japan, is 20–30 cm in height and up to 1–2 cm in width. *G. livida* (Harvey) Yamada from Japan is 10–25 cm high and 0.5–2.5 cm wide. Reproductive structures of *G. filicina* are beset with numerous proliferations that are irregularly pinnately arranged along the margins. But the other two are over nearly the whole blade.

Our new species *G. yinggehaiensisis* distinguished from other members of the genus because it is completely compressed, has a pinnate to foliose habit, is cartilaginous, slippery and has blade-like apices or sickle-like apices in many *Grateloupia.* *G. yinggehaiensis* is slightly similar to *G. turuturu*, because both of them are flaky. They can be easily separated by size. *G. yinggehaiensis* is very small (up to 10 cm in height by up to 3 cm in width); *G. turuturu* is relatively big (up to 40 cm in height by up to 10 cm in width), and they are different in texture (Table 3). Our phylogenetic tree reveals that the relationship among *G. yinggehaiensis, G. hawaiian, G. orientalis* and *G. filicina* is close. In morphology, they can be separated very easily by their size and thallus habit (Table 3). These facts indicate that *G. yangjiangensis* is a new species.

Our *rbc*L sequence analysis supports our conclusion that *G. dalianensis* and *G. yinggehaiensis* are two new species. Within the *Grateloupia* clade, *G. dalianensis* grouped with high bootstrap support with *G. asiatica* from Qingdao of China. Sequences between *G. dalianensis* and *G. huertana* have 154 bp changes (11.71%), which was newly reported by Mateo-Cid et al. (2005). But the pairwise distances between *G. dalianensis* and other species of *Grateloupia* except for *G. huertana* ranged from 66–142 bp (5.02%–10.79%) in the Halymeniaceae. The pairwise distances among *G. dalianensis* and *H. floresia, P. constrictus* ranged from 145 to 151 bp (11.03%–11.48%) in the Halymeniaceae. *G. yinggehaigensis* from China and *G. hawaiiana* from Hawaii form a single monophyletic subclade. The pairwise distances between them is 3.6% (45 bp changes) whereas pairwise distances between *G. yinggehaigensis* from China and *G. filicina* from Italy is 5.8% (73 bp changes), which correspond to those of *Grateloupia* species.

Ultimately from morphological and molecular analysis, we conclude that the specimen of our research belongs to two new species of *Grateloupia*.

Table 2. Comparison of morphological features between *Grateloupia dalianensis* from Dalian and other related species

Morphological feature	*G. dalianensiss*	*G. asiatica*	*G. filicina*	*G. acuminata*	*G. livida*
Habit	discoid holdfast and erect axes, 15–30 cm high	discoid holdfast and erect axes, 7–75 cm high	discoid holdfast and erect axes, 9–12 cm high	discoid holdfast and erect axes, 7–75 cm high	discoid holdfast and erect axes, 7–75 cm high
Texture	mucilaginous	mucilaginous	mucilaginous	mucilaginous	leathery
Thickness of cortex	78–155 μm	90–120 μm	62–87 μm	50–70 μm	55–75 μm
Cortex	2–6 layers	3–6 layers	4–6 layers	4–6 layers	7–10 layers
Medulla	solid	solid	solid	solid	solid
Tetrasporangia	(34–56)× (12–24) μm	(35–45)× (15–20) μm	(30–45)× (15–18) μm	(25–40)× (15–20) μm	(30–40)× (10–18) μm
Distribution	Dalian, China	Qingdao, China	Trieste in the Adriatic Sea	Japan	Japan
Reference	present paper	Kawaguchi et al. (2001)	Kawaguchi et al. (2001)	Yoshida (1998)	Yoshida (1998)

Table 3. Comparison of morphological features between *Grateloupia yinggehaiensis* from Dalian and other related species

Morphological feature	*G. yinggehaiensis*	*G. hawaiian*	*G. orientalis*	*G. turuturu*	*G. filicina*
Thallus habit	bushy, composed of several completely compressed branches bearing irregular pinnate branchlets; cartilaginous and slippery; 5–10 cm high	clump of subdichtomously divided, flattened blades with irregular pinnate branchlets; fleshy and cartilaginous; 9–12 cm high	bushy, composed of terete to slightly compressed branches bearing irregular pinnate branchlets; gelatinous and cartilaginous; 10–16 cm high	simple linear to lanceolate blades with entire margins or bearing few soft and gelatinous; 30–40 cm high	composed of several erect to flattened blades with bladelets; irregular pinnate branchlets; gelatinous and hard; 9–12 cm high
Location of reproduction structures	scattered over the thallus except for the basal portion	scattered over the thallus except for the basal portion	scattered over the thallus except the basal portion	scattered over the thallus	proliferations and upper portion of axes
Thickness of cortex	four to six cells thick	seven to eleven cells thick	six to eight cells thick	five to six cells thick	five to eight cells thick
Tetrasporangia (length by diameter)	50–65 μm by 20–23 μm	45–50 μm by 15–18 μm	35–38 μm by 12–14 μm	20–30 μm by 10–15 μm	20–25 μm by 40–45 μm
Geographical distribution	Yinggehai, China	Hawaii, America	Linyuan, Taiwan	Japan, Mediterranean Sea	Mediterranean Sea
Reference	present study	Dawson (1958) and Abbott (1999)	Lin et al. (2008)	Yoshida (1998), Gavio et al. (2002), John et al. (2004), and Xia (2004)	Kawaguchi et al. (2001)

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References

Abbott I A. 1999. Marine red algae of the Hawaiian Island. Honolulu, Hawaii: Bishop Museum Press, 140-142, fig. 33C-E

Agardh C A. 1822–1823. Species Algarum Rite Cognitae. v 1. part 2. Lund, Sweden: Lundae. Lundae. [I–VIII] 169–398 (1822)

Chiang Y M. 1970. Morphological studies of red algae of the family Cryptonemiaceae. University of California Publications in Botany, 58: vi-95, 10 pls

Dawson E Y. 1958. Notes on Pacific coast marine algae, VII. Bull. Southern California Acad. Science, 57: 65–80

De Clerck O, Gavio B, Fredericq S, et al. 2005a. Systematics of *Grateloupia filicina* (Halymeniaceae, Rhodophyta), based on *rbc*L sequence analyses and morphological evidence, including the reinstatement of *G. minima* and the description of *G. capensis* sp. nov. Phycologia, 41: 391–410

De Clerck O, Gavio B, Fredericq S, et al. 2005b. Systematic reassessment of the red algal genus *Phyllymenia* (Halymeniaceae, Rhodophyta). European Journal of Phycology, 40: 169–178

Faye E T, Wang Hongwei, Kawaguchi S, et al. 2004. Reinstatement of *Grateloupia subpectinata* (Rhodophyta, Halymeniaceae) based on morphology and *rbc*L sequences. Phycologia, 52: 59–68

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 39: 783–791

Figueroa F L, Korbee N, De Clerck O, et al. 2007. Characterization of *Grateloupia lanceola* (Halymeniales, Rhodophyta), an obscure foliose *Grateloupia* from the Iberian Peninsula, based on morphology, comparative sequence analysis and mycosporine-like amino acid composition. European Journal of Phycology, 42: 231–242

Gavio B. 2002. Systematics of the Rhodymeniales–Cryptonemiales complex (Rhodophyta), with emphasis on taxa from the western Atlantic [dissertation]. Lafayette, LA, USA: University of Louisiana at Lafayette, 276

Gavio B, Fredericq S. 2002. *Grateloupia turuturu* (Halymeniaceae, Rhodophyta) is the correct name of the non-native species in the Atlantic known as *Grateloupia doryphora*. European Journal of Phycology, 37: 349–360

Guiry M D, Guiry G M. 2007. Algae Base Version 4.2. Worldwide electronic publication, National University of Ireland, Galway. http://www.algaebase.org/2007-05-09/2009-06-05

John D M, Prud' homme van Reine W F, Lawson G W, et al. 2004. A taxonomic and geographical catalogue of the seaweeds of the western coast of Africa and adjacent islands. Nova Hedwigia, 127: 1–339

Kawaguchi S, Wang Hongwei, Horiguchi T, et al. 2001. A comparative study of the red alga *Grateloupia filicina* (Halymeniaceae) from the northwestern Pacific and Mediterranean with the description of *Gratloupia asiatica*, sp. nov. Phycologia, 37: 433–442

Kawaguchi S, Wang Hongwei, Horiguchi T, et al. 2002. Rejection of Sinkoraena and transfer of some species of Carpopeltis and Sinkoraena to Polyopes (Rhodophyta, Halymeniaceae). Phycologia, 41: 619–635

Lin Xiumei, Liang Hongyiang, Max H Hommersand. 2008. Two types of auxiliary cell ampullae in *Grateloupia* (Halymeniaceae, Rhodophyta), including *G.taiwanensis* sp.nov. and *G. orientalis* sp. nov. from Taiwan based on *rbc*L gene sequence analysis and cystocarp development. Phycologia, 44: 196–214

Litaker R W, Tester P A, Colorni A, et al. 1999. The phylogenetic relationship of Pfiesteria piscicida, cryptoperidiniopsoid sp. Amyloodinium ocellatum and a Pfiesterialike dinoflagellate to other dinoflagellates and apicomplexans. Phycologia, 35: 1379–1389

Mateo-Cid L E, Mendoza-González A C, Gavio B, et al. 2005. *Grateloupia huertana* sp. nov. (Halymeniaceae, Rhodophyta), a peculiar new prostrate species from tropical Pacific Mexico. Phycologia, 44: 4–16

Moncalvo J M, Lutzoni F M, Rehner L S, et al. 2000. Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. Systems Biology, 49: 278–305

Swofford D L. 2002. Paup. Phylogenetic Analysis Using Parsimony, Version 4. Sunderland, Massachusette: Sinauer Associates

Wang Hongwei, Kawaguchi S, Horiguchi T, et al. 2000. Reinstatement of *Grateloupia catenata* (Rhodophyta, Halymeniaceae) on the basis of morphology and *rbc*L sequences. Phycologia, 39: 228–237

Wang Hongwei, Kawaguchi S, Horiguchi T, et al. 2001. A morphological and molecular assessment of the genus *Prionitis* J. Agardh (Halymeniaceae, Rhodophyta). Phycologia, 49: 251–261

Wilkes R J, McIvor L M, Guiry M D. 2005. Using *rbc*L sequence data to reassess the taxonomic position of some *Grateloupia* and *Dermocorynus* species (Halymeniaceae, Rhodophyta) from the north-eastern Atlantic. European Journal of Phycology, 40: 53–60

Xia Bangmei. 2004. Flora Algarum Marinarum Sinicarum (in Chinese). Beijing: Science Press, 1: 135–138

Yoshida T. 1998. Marine Algae of Japan (in Japanese). Tokyo: Uchida Rokakuho Publishing, 1222