Research Article

Grateloupia tenuis Wang et Luan sp. nov. (Halymeniaceae, Rhodophyta): A New Species from South China Sea Based on Morphological Observation and rbcL Gene Sequences Analysis

Ling Yu,1 Hongwei Wang,1 and Rixiao Luan2

1 College of Life Sciences, Liaoning Normal University, Dalian 116029, China
2 Dalian Natural History Museum, Dalian 116023, China

Correspondence should be addressed to Hongwei Wang; kitamiwang@163.com

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1. Introduction

Red algae Grateloupia C. Agardh is a species-rich genus in the family Halymeniaceae, which not only exhibits highly diverse external morphology, but also is one of the genera in which species discrimination is more difficult. This genus is widely distributed in tropical and temperate coastal areas and includes more than 80 species worldwide and a total of 35 species in China [1–12]. The genus Grateloupia was originally built on the foundation of three species, namely, G. ornata, G. hybrida, and G. filicina, with G. filicina identified as the genus generitype [13].

Species morphology is significantly varied in Grateloupia, determining difficulties in species identification and controversy in species classification between algae taxonomists. Yendo [14] described Grateloupia with lomentaceous features as “catenate” and proposed G. catenata as a new species; however, Howe [15] regarded Grateloupia with lomentaceous characteristics as G. filicina (Lamouroux) C. Agardh var. lomentaria Howe. Okamura [16] opined that G. filicina var. lomentaria was a mature stage of G. filicina var. porracea, and integrated them into G. filicina var. porracea f. lomentaria (Howe) Okamura, with G. catenata being a synonym. Wang et al. [1] inspected type material of G. catenata Yendo and proposed to reinstate the Yendo name. G. ramosissima Okamura was discovered by Lin et al. [9], and its morphology resembles the algae researched in the present study. G. orientalis was described based on studies of cystocarp development and rbcL gene sequence analysis [9]. Zhang et al. [12] confirmed a new record of G. orientalis in the Hainan province of China through morphological observations, development of reproductive structures and molecular phylogenetic studies.

In our current research, the above species (G. filicina, G. catenata, G. ramosissima and G. orientalis) showed close relationships with G. tenuis, however, due to the particularly large differences in external and internal morphologies between G.
tenuis and other known algae, this species has not been mentioned in previous reports. Based on these differences and analysis of ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcL) gene sequences, we recommend that this alga is a new species of Grateloupia and should be defined as Grateloupia tenuis Wang et Luan sp. nov.

2. Materials and Methods

2.1. Morphological Analysis. Specimens were collected from the coast of Lingshui, Hainan Province, South China Sea (5 February 2009, leg. R. X. Luan; LNU20092087, LNU20092088, LNU20092089, and LNU20092090). Voucher herbarium specimens are reserved in the Herbarium of the College of Life Sciences, Liaoning Normal University, Dalian, China (LNU). We took G. tenuis (LNU20092088) as the holotype.

Morphological observations were made on algal specimens preserved in 10% seawater or pressed on herbarium sheets, and molecular analysis was conducted on samples desiccated in silica gel. Photographs of the holotype specimen were taken with a Canon EOS 650D (Canon, Japan). Hand sections were made by cryostat microtome, stained with 0.5% (w/v) cotton blue and discolored with 45% acetic acid. Photomicrographs were taken on an Olympus BH2 digital camera (Olympus Beijing Co. Ltd., China) mounted on a Nikon microscope (Nikon Corporation, Japan).

2.2. DNA Extraction and Phylogenetic Tree Construction. DNA samples of LNU20092087, LNU20092088, LNU20092089, and LNU20092090 were extracted using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, Beijing). The procedures for PCR amplification and sequencing were executed as described previously [1]. The rbcL gene sequences of 32 extra relevant Halymeniaceae species and two additional related family (Gelidiaceae and Gracilariaeae) species were selected from GenBank for analysis and were involved in the alignment (Table 1). Gelidiella ligulata Dawson and Gracilaria tenuistipitata Chang et Xia were treated as outgroups.

The rbcL sequences were aligned and compiled with Clustal X version 1.83 [21] for phylogenetic analysis. Phylogenetic tree construction and nucleotide differences analyses were conducted using MEGA 5.0. Maximum parsimony (MP), neighbor joining (NJ), and maximum likelihood (ML) were adopted to construct the phylogenetic tree. The MP analysis used heuristic searches for evaluating tree likelihoods, which was carried out with 1000 replicates, employed random addition sequence of taxa and used tree-bisection-reconnection (TBR) branch swapping [22]. The NJ analysis used the ratio test for estimation to seek optimal settings and ensure data dependability and was performed with Modeltest version 3.06 [23]. For ML analysis, a variety of cumulatively complex models of molecular evolution were assessed, as summarized by Litaker et al. [24] and Moncalvo et al. [21]. Bootstrap support values were calculated using 1000 samplings of the dataset [25] to estimate statistical reliability for MP, NJ, and ML methods.

3. Results

3.1. Taxonomic Descriptions. Thallus simplex, linear, purplish red in color, cartilaginous and slippery in texture, 4–7 cm in height, main axes about 1 mm in width, branches about 0.2–0.6 mm in width, with fewer branches than other thalli (Figure 1). Main axes of thalli and branches relatively slight, erect axes cylindrical or subcylindrical and bearing alternation or second branches, twice or thrice dichotomously branched (Figures 2(a) and 2(b)). Cortex 5-6 layers, cells elliptic or polygonal and arranged densely; medulla solid with relatively intensive filaments when young, but hollow in the center in old branches. Gametophytes dioecious with reproductive structures dispersed in main axes of thalli and lower portion of branches, and cystocarps of main axes significantly fewer than branches (Figures 2(a)–2(c)). Carposporangium developed from gonimoblasts cells, profoundly immersed inside medulla and revolved by branched ampullar filaments. Mature cystocarps 90–110 μm in diameter. Spermatangium shaped from outermost cortex cells of male gametophytes. Mature tetrasporangium cruciately partitioned, 10–16 μm long, 6–8 μm in diameter, and inserted in outer cortical cells.

Consider the following.

Etymology; “tenuis” refers to the morphology of the thallus.

Holotype; appointed here is a female specimen (LNU20092088 Figure 1).

Type locality; Lingshui, Hainan Province, China, 5 Feb. 2009, collected by R. X. Luan.

Habitat and seasonality; plant collections were seasonal from January to March, and were attached on rocky reefs or stony marsh of coastal intertidal zone.

Distribution; currently only known in Lingshui, Hainan Province, China (E109.9°, N18.4°).

3.2. Vegetative and Reproductive Structures. Cross-section of the thallus showed a densely arranged cortex and solid
Table 1: List of species for rbcl sequences analysis, collection data, and accession numbers in GenBank.

| Species                  | Collection data (location, site, and reference)                           | GenBank accession numbers |
|--------------------------|----------------------------------------------------------------------------|----------------------------|
| Grateloupia tenuis Wang et Luan | Lingshui, Hainan Province, China (LNU20092087)                          | KC918541                   |
| Grateloupia tenuis Wang et Luan | Lingshui, Hainan Province, China (LNU20092088)                          | KC918542                   |
| Grateloupia tenuis Wang et Luan | Lingshui, Hainan Province, China (LNU20092089)                          | KC918543                   |
| Grateloupia tenuis Wang et Luan | Lingshui, Hainan Province, China (LNU20092090)                          | KC918544                   |
| G. catenata Yendo         | Shimiao, Dalian, Liaoning Province, China (Wang et al., 2000) [1]         | AB038617                   |
| G. ramosissima Okamura   | Province, China (Gavio and Fredericq, 2002) [4]                          | AF488810                   |
| G. filicina (Lamouroux) C. Agardh | Livorno, Italy (Wang et al., 2000) [1]                                    | AB055472                   |
| G. orientalis Lin et Liang | Linyuan, southwestern Taiwan Province, China (Lin et al., 2008) [9]       | EU292744                   |
| G. filiformis Kutzing    | Marataizes, Espiritu Santo, Brazil (Gavio and Fredericq, 2002) [4]        | AF488822                   |
| G. carnosus Yamada et Segawa | Oryuzako, Miyazaki Prefecture, Japan (Wang et al., 2000) [1]              | AB038608                   |
| G. stipitata J. Agardh   | Lee Bay, Stewart Island, New Zealand (Gavio and Fredericq, 2002) [4]      | AF488816                   |
| G. acuminata Holmes      | Katase, Fujisawa, Kanagawa Prefecture, Japan (Kawaguchi et al, 2001) [3] | AB055480                   |
| G. americana Kawaguchi et Wang | California, USA (De Clerck et al, 2005) [7]                             | AY772037                   |
| G. asiatica Kawaguchi et Wang | Qingdao, Shandong Province, China (Kawaguchi et al., 2001) [3]          | AB055488                   |
| G. livida (Harvey) Yamada | Izu-misaki, Miyake Island, Tokyo, Japan (Wang et al., 2000) [1]           | AB038610                   |
| G. patens (Okamura) Kawaguchi et Wang | Oohara, Chiba Prefecture, Japan (Wang et al., 2001) [2]                  | AB061392                   |
| G. divaricata Okamura    | Oshoro, Hokkaido, Japan (Wang et al., 2000) [1]                          | AB038609                   |
| G. schmitziana (Okamura) Kawaguchi et Wang | Shichirigahama, Kamakura, Kanagawa, Japan (Wang et al., 2000) [1]      | AB061398                   |
| G. lanceolata (Okamura) Kawaguchi | Shikanoshima, Fukuoka, Japan (Kawaguchi et al., 2001) [3]               | AB055477                   |
| G. elliptica Holmes      | Goshikinohama, Usa, Tosa, Kochi Prefecture, Japan (Kawaguchi et al., 2001) [3] | AB055476                   |
| G. kurogii Kawaguchi     | Saikai-bashi, Nagasaki Prefecture, Japan (Wang et al., 2001) [2]         | AB038606                   |
| G. phuquocensis Tanaka et Pham-Hoang | Kaalawai, Oahu, Hawaii (De Clerck et al., 2005) [6] | AY772022                   |
| G. sparsa (Okamura) Chiang | Oohara, Chiba Prefecture, Japan (Wang et al., 2000) [1]                  | AB055473                   |
| G. imbricata Holmes      | Tsuyazaki, Fukuoka Prefecture, Japan (Wang et al., 2000) [1]              | AB038607                   |
| G. longifolia Kylin      | Yzerfonteyn, Western Cape Province, South Africa (De Clerck et al., 2005) [6] | AY772023                   |
Table 1: Continued.

| Species                        | Collection data (location, site, and reference)                                                                 | GenBank accession numbers |
|-------------------------------|------------------------------------------------------------------------------------------------------------------|---------------------------|
| G. belangeri (Bory de Saint-Vincent) Setchell et Gardner | Platboom, Western Cape Province (De Clerck et al., 2005) [6]                                                  | AY772027                  |
| G. angusta (Okamura) Kawaguchi et Wang | Miyanoura, Hirado Island, Nagasaki Prefecture, Japan (Wang et al., 2001) [2]                                      | AB061380                  |
| G. capensis De Clerck          | South Africa (De Clerck et al., 2005) [6]                                                                       | AJ868465                  |
| G. somalensis Hauck            | Plage de Monseigneur, Fort Dauphin, Madagascar (De Clerck et al., 2005) [7]                                     | AY772021                  |
| G. taiwanensis Lin et Liang    | Northeastern and southern Taiwan (Lin et al., 2008) [9]                                                         | EU292742                  |
| G. subpectinata Holmes         | Irago-misaki, Atumi, Aichi, Japan (Faye et al., 2004) [5]                                                        | ABI14213                  |
| G. turuturu Yamada            | Muroran, Hokkaido, Japan, South Africa (Wang et al., 2000) [1]                                                  | AB038611                  |
| Halymenia durvillei Bory       | Beruwela, Sri Lanka (De Clerck et al. 2005) [6]                                                                   | AB0772020                 |
| Halymenia floresia (Clemente) C. Agardh | Pulau Rebak Besar, Langkawi, Kedah, Malaysia (Wang et al., 2000) [1]                                              | AB038603                  |
| Polyopes constrictus (Turner) J. Agardh | Point Lonsdale, Victoria, Australia (Kawaguchi et al., 2001) [3]                                                 | AB055468                  |
| Polyopes lancifolius (Harvey) Kawaguchi et Wang | Inoshiri, Usa, Tosa, Kochi, Japan (Kawaguchi et al., 2002) [17]                                                  | AB084543                  |
| Gelidiella ligulata Dawson     | Miyake Island, Tokyo, Japan (Shimada et al., 1998) [18]                                                          | AB017678                  |
| Gracilaria tenuistipitata Chang et Xia | Viet Nam (Gurgel et al., 2008)                                                                                 | EU380718                  |

Figure 2: Branching characteristics and distribution of reproductive structures. (a) Second branches and distribution of cystocarp. (b) Alternation branches and distribution of cystocarp. (c) Cystocarp only dispersed in lower portion of branches.

The thallus had Grateloupiatype auxiliary cell ampullae. Auxiliary cells (ac) and carpogonial branches were produced in independent ampulla initiated from inner cortical cells. Two-celled carpogonial branches existed in each carpogonial branch ampulla and contained a terminal carpogonium and hypogynous cell (Figure 3(d)). The auxiliary cell ampullae were narrowly bottle-shaped and comprised of two or three secondary filaments. The mature auxiliary cell (ac) was elliptical in shape and obviously larger than other ampullary cells, and was located at the basal part of the ampulla (Figure 3(e)). Successive stages of the cystocarp development are displayed in Figures 3(f)–3(i). Terminal cells of the gonimoblast filaments gradually matured (Figure 3(f)). Maturing carposporangia developed from gonimoblasts cells, and were surrounded by branched ampullar filaments (Figure 3(g)).
As cystocarps proceeded with development, they became increasingly larger and more deeply embedded in the medulla (Figure 3(h)). Mature cystocarps were spherical or ellipsoidal and 90–110 μm in diameter (Figure 3(i)). The mature cystocarp released carpospores (Figure 3(j)). Spermatangia of Gracilaria tenuis were shaped from the outermost cortex cells (Figure 3(k)). Mature tetrasporangia formed from the male gametophytes were shaped from the outermost cortex cells (Figure 3(l)).

3.3. Molecular Analysis. In the phylogenetic tree, we compared four G. tenuis rbcL gene sequences with a total of 34 extra rbcL sequences from taxa comprising 28 species of Grateloupia, with four species from other genera (Halymenia C. Agardh and Polyopes J. Agardh) in Halymeniaceae and two species from Gelidiaceae and Gracilariaeae treated as outgroups, which were selected from GenBank for analyses (Table 1). The rbcL sequences of the four Lingshui samples were uniform. The rbcL sequence alignment consisted of 1322 base pairs (bp), but since many rbcL sequences were incomplete at the 5' and 3' ends, the first 67 bp and last 15 bp were excluded from analyses.

The maximum likelihood phylogenetic tree (Figure 4) was obtained through the NJ, MP, and ML analysis. The species aggregate of the ML phylogenetic tree was divided into three main clades with high bootstrap support values and included a large Grateloupia clade; a Halymenia/Polyopes clade, which were all within Halymeniaceae; and a Gelidiella/Gracilaria clade, which were in Gelidiaceae and Gracilariaeae, respectively. The four G. tenuis samples and the 28 kinds of Grateloupia genera formed together into one large clade. The Grateloupia clade was divided into a small and large clade, and G. tenuis formed a monophyletic group within the small clade.

The rbcL analyses of pairwise base differences between G. tenuis and generitype G. filicina were 68 bp changes (5.72%). Sequences among G. tenuis and G. catenata and G. ramosissima differed by 39 bp (3.22%) and 48 bp (3.98%). There were 45 bp (3.73%) and 50 bp (4.17%) differences between G. tenuis and G. orientalis and G. filiformis, respectively. Sequences between G. tenuis and other samples in Grateloupia ranged from 81–108 bp changes (6.88%–9.34%). Divergence between G. tenuis specimens and other genera of Halyeniaceae ranged from 114–133 bp differences (9.86%–11.66%). When compared to outgroups, the G. tenuis specimens exhibited 190 bp (17.17%) and 196 bp (17.75%) differences from Gracilaria tenuistipitata (Gracilariaeae) and Gelidiella ligulata (Gelidiaceae), respectively.

4. Discussion

The family Halymeniaceae exhibits high species richness, especially within Grateloupia, which makes taxonomic species identification difficult. Nevertheless, the formation of auxiliary cell ampullae is a remarkable characteristic for distinguishing genera within Halymeniaceae [2, 3, 6, 26].

The newly described G. tenuis species is morphologically similar to some species, especially G. catenata. Table 2 shows a comparison in morphological features among G. tenuis and the closely related species G. catenata, G. ramosissima, G. orientalis, G. filicina. It was easy to differentiate G. tenuis from G. catenata by its significantly smaller size (4–7 cm high compared to 35 cm high, resp.) and by its dispersed reproductive structures in the main axes of thalli and lower portions of branchlets compared to the scattered reproductive structures.
over the whole thallus in *G. catenata*. It was possible to separate *G. tenuis* from generitype *G. filicina* by its slippery and cartilaginous texture compared with the mucilaginous and hard texture of *G. filicina*, and by its dichotomous branches compared with pinnate branchlets. Distinction with *G. ramosissima* and *G. orientalis* showed that they had abundant branches. *G. tenuis* also had representative *Gratelouphia*-type auxiliary cell ampullae, which demonstrated that it was a new species of the family Halymeniaceae and pertained to the genus *Gratelouphia*.

The *rbCL* sequence data also strongly supported *G. tenuis* as a new species. In the ML phylogenetic tree (Figure 4), *G. tenuis* specimens were embedded in the large clade of *Gratelouphia* and clustered into a single monophyletic group. The small clade of *Gratelouphia* included *G. catenata*, *G. ramosissima*, *G. filiformis*, and *G. orientalis* from China,
and genotypic *G. filicina* from Italy. The *G. catenata*/*G. ramosissima* subclade was the most closely related sister taxon to *G. tenuis* and the most similar in appearance. In addition, *G. tenuis* produced a high bootstrap support value with the *G. catenata*/*G. ramosissima* subclade. The genotypic species, *G. filicina*, belonged to the sister position of *G. tenuis*, which strongly supported that *G. tenuis* phylogenetically approached *G. filicina*. Moreover, *G. orientalis*, and *G. filiformis* formed a sister clade with *G. tenuis*. All *Grateloupia* species were phylogenetically different to the two clades, especially *Gelidiella ligulata* and *Gracilaria tenuistipitata* serving as outgroups.

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**Figure 4:** Maximum likelihood tree showing phylogenetic relationships of *G. tenuis* and relevant genera within *Grateloupia*, inferred from partial *rbcL* gene sequences (1240 bp). *Gelidiella ligulata* and *Gracilaria tenuistipitata* were treated as outgroups. Numbers at internal nodes are bootstrap proportion values (1000 replicates) for ML, NJ, and MP, which only show above 50% bootstrap support. Branch lengths are proportional to the amount of sequence change. Boldface displays new species depicted in this research.
5. Conclusions

From morphological observation and rbcl gene sequence analysis, we concluded that the studied specimen was a new species of genus *Grateloupi*a, defined as *Grateloupi*a *tenuis* Wang et Luan sp. nov. Currently, due to algae species diversity, traditional taxonomic methods are not the most effective way to identify species. With the rapid development of molecular biology and gene sequencing technology; however, taxonomists are increasingly using combined morphological observation with DNA barcoding for algae classification. In recent years, human impact on marine ecosystems has increased the urgent need to conserve aquatic resources, and accurate species identification is a basic prerequisite in helping protect marine algae.

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