

Gracilaria phuquocensis sp. nov., a new flattened *Gracilaria* species (Gracilariales, Rhodophyta), previously recognized as *G. mammillaris*, from the southern coast of Vietnam

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SUMMARY

Flattened *Gracilaria* species are widely distributed along the coasts of the South China Sea with more than 20 species recorded. Within the South China Sea, *Gracilaria mammillaris* has only been reported from Vietnam, but this species is likely restricted to the western Atlantic. This study aimed to reevaluate the taxonomic status of Vietnamese specimens of '*G. mammillaris*' using combined morphological and molecular data. Our data clearly indicated that Vietnamese specimens were morphologically and genetically distinct from authentic *G. mammillaris* from the western Atlantic, and also other described flat *Gracilaria* species. We, therefore, propose that specimens from Vietnam originally identified as *G. mammillaris* be designated as a new species, *Gracilaria phuquocensis* sp. nov. Morphologically, *G. phuquocensis* can be distinguished from other flat *Gracilaria* species by its small thallus size, narrower blades, many medullary layers, abundant basal nutritive filaments within mature cystocarps, and tetrasporangial nemathecium. Our *rbcl* sequence analyses showed that the new species was sister to *Gracilaria rhodymenioides* from Thailand, and these two species formed a clade with cylindrical *Gracilaria* species. Our study contributes to clarification of the taxonomic status of misidentified specimens attributed to the flattened *Gracilaria* species in the South China Sea.

Key words: agarophyte, nemathecium, Pacific, phylogeny, seaweed.

INTRODUCTION

The marine red algal genus *Gracilaria* Greville has a wide distribution in tropical and temperate regions of the world, and currently contains 178 taxonomically accepted species (Guiry & Guiry 2019). Due to its economic importance, the taxonomy and systematics of *Gracilaria* have been extensively studied (Núñez-Resendiz *et al.* 2015; Lyra *et al.* 2015a; Ng *et al.* 2017; Gurgel *et al.* 2018). Recently, new species of *Gracilaria* have been discovered on the basis of combined morphological and molecular data (Muangmai *et al.* 2014; Lyra *et al.*, 2015b; Ardito *et al.* 2017; Muangmai *et al.* 2017;

Dreckmann *et al.* 2018; Hardesty & Freshwater 2018; Soares *et al.* 2018).

Along the coastal areas of the South China Sea, more than 40 species of *Gracilaria* have been reported to date, about 20 of which are flattened species (Phang *et al.* 2016; Muangmai *et al.* 2017). In Vietnam, approximately 18 species of *Gracilaria* have currently been recorded, including seven with flattened thalli: *Gracilaria cuneifolia* (Okamura) I.K. Lee & Kurogi, *Gracilaria mammillaris* (Montagne) M. Howe, *Gracilaria punctata* (Okamura) Yamada, *Gracilaria spinulosa* (Okamura) Chang & B.M. Xia, *Gracilaria textorii* (Suringar) De Toni, *Gracilaria vieillardii* P.C. Silva and *Gracilaria yamamotoi* Zhang & B.M. Xia (Le & Nguyen 2006, Nguyen *et al.* 2013).

Among flat species, *G. mammillaris* has only been reported in Southeast Asian waters from Vietnam (Phang *et al.* 2016). *Gracilaria mammillaris* was originally described from Martinique in the eastern Caribbean Sea, and the distribution of this species seems to be restricted to the western Atlantic from the Caribbean south to Brazil (Gurgel *et al.* 2004; Hardesty & Freshwater 2018). Considering this, it is feasible that the Vietnamese record of '*G. mammillaris*' is based on misidentified specimens. Previous studies have also indicated that flattened *Gracilaria* specimens from the tropical Western Atlantic were misidentified as *G. mammillaris* and based on morphological and molecular evidences new species were proposed (Gurgel *et al.* 2004; Hardesty & Freshwater 2018). Studies on the taxonomy of *Gracilaria* in Vietnam have relied mostly on morphology (Ohno *et al.* 1999; Le & Nguyen 2006) and this can lead to incorrect identification. The objective of this study is to reassess the taxonomic identity of Vietnamese specimens identified as '*G. mammillaris*' using morphological and *rbcl* gene sequence analyses.

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MATERIAL AND METHODS

Sampling

Specimens initially identified as *G. mammillaris* were collected from Phu Quoc Island, Kien Giang province, southern Vietnam (105°42'58.86"E, 19°6'10.75"N) (Fig. S1 in the Supporting Information). Algal samples were either preserved as dry materials on herbarium sheets or fixed in 4% formalin/seawater. Type and voucher specimens were deposited in the Kasetsart University Museum of Fisheries (KUMF), Faculty of Fisheries, Kasetsart University, Bangkok, Thailand.

Morphological investigation

Transverse sections were made by hand using a razor blade, stained with 1% aniline blue, acidified with 1 N HCl, and mounted in 40% Karo syrup on microscope slide. Digital images were photographed using Olympus DP20 (Olympus, Tokyo, Japan) microscope digital camera and edited using Photoshop Elements 6 (Adobe, San Jose, CA, USA).

Molecular analysis

Apical portion of each voucher specimens was placed in silica gel until DNA extraction. Additional samples of *G. yamamotoi* from Quynh Tien, Nghe An province, northern Vietnam, and flat *Gracilaria* from Hainan Island, China were also genetically analyzed in this study. Total DNA was extracted using a 5% Chelex resin solution following Zuccarello *et al.* (1999). DNA amplification was carried out using the polymerase chain reaction (PCR) for the chloroplast *rbcL* gene. PCR and sequencing of *rbcL* gene were performed with two combinations of primers: *rbcL*-for and R753, plus F577 and *rbcL*-rev (Freshwater & Rueness 1994; Nam *et al.* 2000). PCR procedures followed Zuccarello *et al.* (2002), products were confirmed by gel electrophoresis in 1% agarose gels. The PCR products were purified using ExoSAP-IT (USB, Ohio, USA) according to the manufacturer's instructions and subsequently sent for commercial Sanger sequencing (Macrogen Inc., Seoul, Korea).

The five newly determined sequences were edited and assembled using Geneious 11.0 software package (Biomatters, <http://www.geneious.com>). Data matrix were also prepared using Geneious 11.0 software and further modified manually. Data set of *rbcL* included an additional 69 published sequences of the Gracilariaceae retrieved from GenBank (Table S1 in the Supporting Information). The genus *Gracilariopsis* was selected as an outgroup following Gurgel *et al.* (2004). All new sequences generated in this study were submitted to GenBank (accession number MK935560–MK935564).

Phylogenetic trees were reconstructed using maximum likelihood (ML) and Bayesian Inference (BI). An appropriate model of sequence evolution was selected using Kakusan 4 (Tanabe 2011) for each analysis. The *rbcL* dataset was partitioned by codon position, and separate DNA substitution models were chosen for each position. For ML analysis, phylogenetic trees were reconstructed using raxmlGUI v1.5b3 (Silvestro & Michalak 2012) under the option 'ML + thorough bootstrap' with 10 runs of random additional sequence

generating 10 000 nonparametric bootstrap replicates. BI analysis was implemented through MrBayes v3.2 (Ronquist *et al.* 2012). BI tree was reconstructed with two runs and four Markov Chain Monte Carlo (MCMC) chains with 10 million generations, sampling every 100 generations. Two independent runs were checked for stabilization of overall likelihood via Tracer v1.5 (Rambaut & Drummond 2009). The first 25% of trees were discarded as burn-in, and the remaining trees were used to calculate the Bayesian posterior probability (PP) for each clade. ML and BI trees were visualized and edited using FigTree v1.4.2 (Rambaut 2012).

RESULTS

A dataset of 1112 bp of *rbcL* from various *Gracilaria* specimens was used for phylogenetic analysis (Fig. 1). The phylogenetic tree clearly indicated that our samples from Vietnam were placed in the major clade of *Gracilaria* and were evolutionarily distant from the type of *G. mammillaris* from Martinique in the eastern Caribbean Sea. Two *rbcL* sequences of Vietnamese samples were distinct from other *Gracilaria* sequences by at least 1.4% (16 bp) and differed from the type of *G. mammillaris* by about 5%. Both ML and BI analyses yielded an identical topology, and therefore only the ML tree (–ln L score of –10097.0070) was presented (Fig. 1). The Vietnamese species was sister to *Gracilaria rhodymenioides* A.J.K. Millar from Thailand, and these two species formed a clade with two terete species, *Gracilaria salicornia* (C. Agardh) E.Y. Dawson and *Gracilaria arcuata* Zanardini. In contrast, *G. yamamotoi* from Vietnam and two unknown flat *Gracilaria* from Hainan Island, China were phylogenetically clustered with other flat species from Western Pacific species (Fig. 1).

Our phylogenetic analysis clearly showed that '*G. mammillaris*' from Vietnam did not group with the type specimen of *G. mammillaris* and was distinct from all other *Gracilaria* species. We proposed that it be described as a new species:

Gracilaria phuquocensis N.H. Le,
N. Muangmai & G.C. Zuccarello sp. nov.
(Figs 2–3)

Diagnosis

Plants were perennial, solitary, and flattened, 3–7 cm long, 3–5 mm wide and 250 µm thick. Plants were dichotomously branched. Blades were composed of one to two pigmented cortical cells and five to eight unpigmented medullary cells. Tetrasporangia mostly occurred in nemathecium. Cystocarps were subglobose with variable levels of rostrate tips and constricted bases. Pericarps consisted of 17–25 cell layers. Nutritive filaments were abundant at the basal part of cystocarps.

Type locality

Kien Giang, Phu Quoc Island, Vietnam (105°42'58.86"E, 19°6'10.75"N).

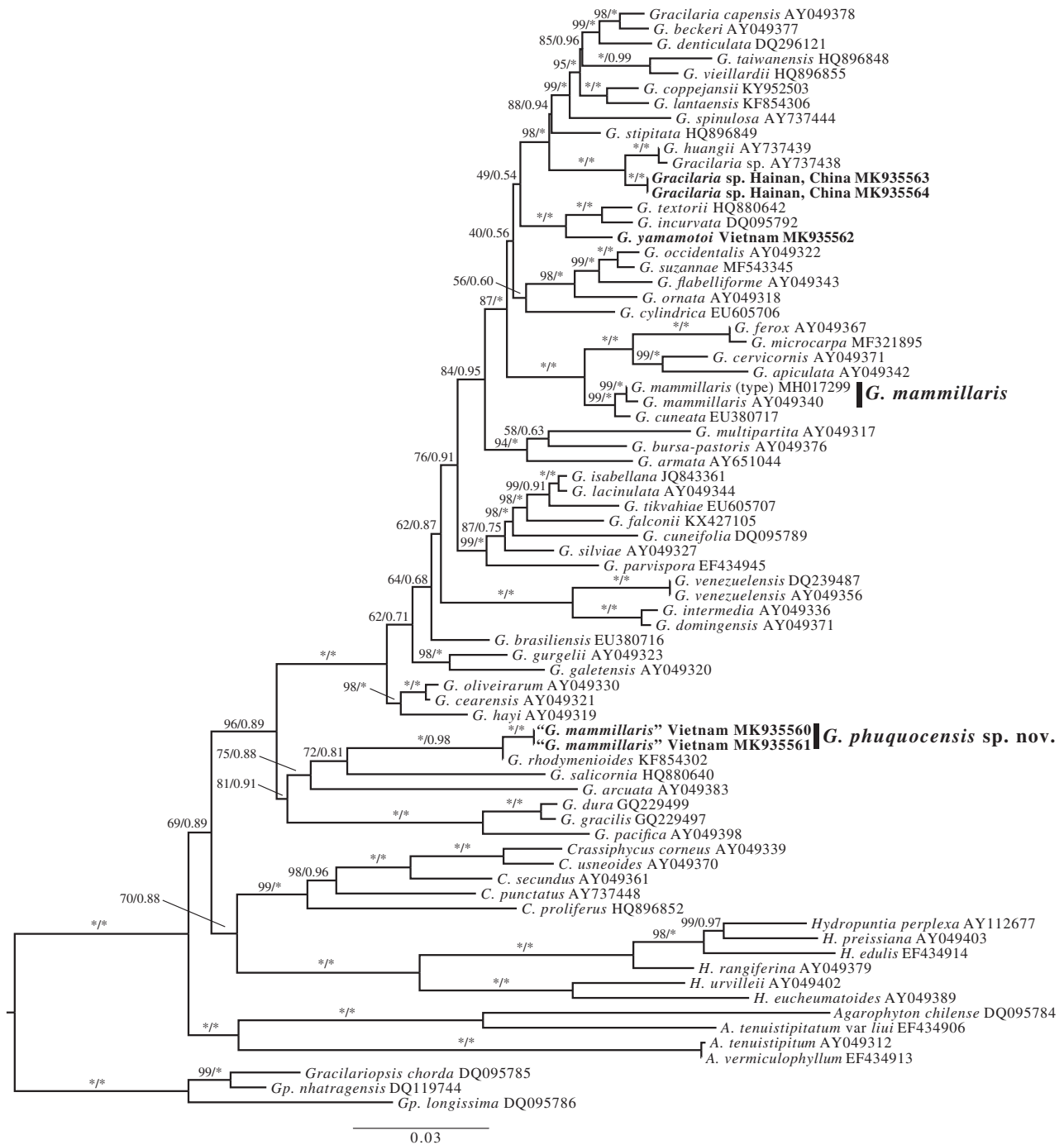


Fig. 1. Maximum-likelihood tree of partial *rbcL* sequences. Support values at each node are bootstrap values from ML (left) and Bayesian posterior probability (right). Asterisk (*) indicates full support (100%, 1.0) in both analyses. Taxa in bold correspond to new sequences in this study.

Type

KUMF-NM1811001 (tetrasporophyte, Fig. 2a) deposited at KUMS, 5 October 2010, coll. Hau Nhu Le. Genbank accession number for *rbcL*: MK935560.

Isotypes

Hau 200122 (female gametophyte, Fig. 2b) (Genbank accession number for *rbcL*: MK935561), Hau 200105

(tetrasporophyte, Fig. 2c) and KUMF-NM1811002 (vegetative, Fig. 2d), deposited at KUMS, 5 October 2010, coll. Hau Nhu Le.

Etymology

The species epithet referred to the locality of Phu Quoc Island, where this species was found.

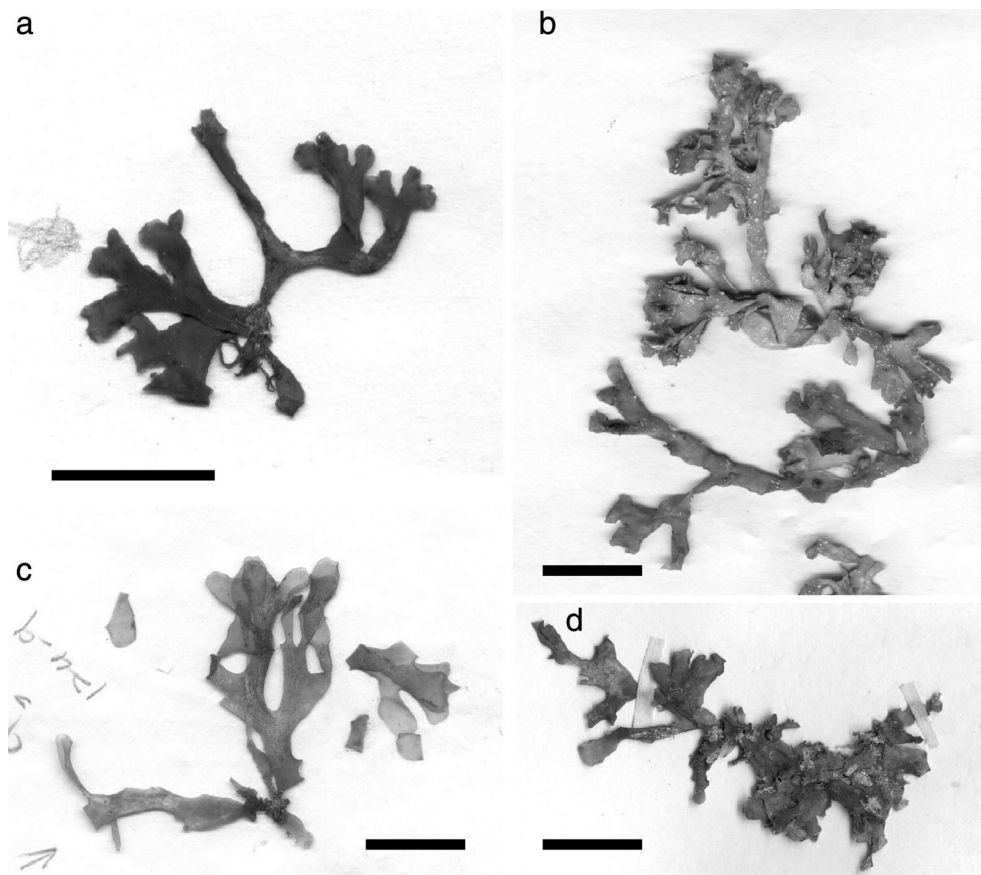


Fig. 2. Habit of *Gracilaria phuquocensis* sp. nov. (a) Holotype, tetrasporophyte (KUMF-NM1811001), scale bar = 3 cm. (b) Isotype, female gametophyte (Hau 200122), scale bar = 1 cm. (c) Isotype, tetrasporophyte (Hau 200 105), scale bar = 2 cm. (d) Isotype, sterile plant (KUMF-NM1811002), scale bar = 1 cm.

Description

Plants grew in the upper sublittoral zone in the rocky, wave-exposed localities. Plants were flat with smooth margin, 3–7 cm long and 3–5 mm broad (Fig. 2a–d). Plants were reddish brown or pale red and mostly dichotomously branched (Fig. 2a,b). Blades were linear-lanceolate and broad, and occasionally flabellate (Fig. 1a,c). In cross section, blades were composed of one to two cell layers of oval or round cortical cells, 4–8 μm in diameter. Medullary layers consisted of five to eight cells, 80–120 μm long by 25–50 μm wide (Fig. 3a). Transition between cortex and medulla was abrupt (Fig. 3a).

Tetrasporophytes mostly bore many tetraspores in nemathecia (tetrasporic sori), which were raised areas of the surface of thalli (Fig. 2b,c). Nemathecia occurred only on one side of the blades. Mature tetrasporangia were 25–40 μm long by 15–30 μm wide (Fig. 3c). Female plants possessed small cystocarps, 1.2–1.5 mm in diameter, scattered on both surfaces of the blade. Cystocarps were subglobose with or without rostrate apices (Fig. 3d,e). Non-rostrate cystocarps were slightly constricted (Fig. 3d) at their bases, whereas rostrate cystocarps were clearly constricted (Fig. 3e). Pericarps were thick, composed of 17–25 cell layers of spherical and radially elongated cells. Gonimoblasts were mostly composed of

relatively large cells, 60–80 μm in diameter. Numerous nutritive filamentous cells occurred mostly in basal parts of the carposporophyte (Fig. 3f).

DISCUSSION

Our phylogenetic and morphological analyses clearly indicate that flattened *Gracilaria* specimens identified as *G. mammillaris* in Vietnam are distinct from true *G. mammillaris* from Martinique in the eastern Caribbean Sea. The morphological features that distinguish Vietnamese *G. phuquocensis* from *G. mammillaris* are thallus size and blade width (both smaller and narrower in *G. phuquocensis*), medullary cell layer (more layers in *G. phuquocensis*), and the occurrence of tetrasporangial nemathecia in *G. phuquocensis*, while they are embedded in the cortex in *G. mammillaris*. Also, the genetic distance between the two species is large, and they are in different clades within the *rbcl* phylogeny. Moreover, the Vietnamese *G. phuquocensis* differs morphologically and genetically from any other described flattened species of *Gracilaria* in Southeast Asian waters (Table 1).

A unique feature observed in *G. phuquocensis* is the arrangement of tetrasporangia in well-developed nemathecia,

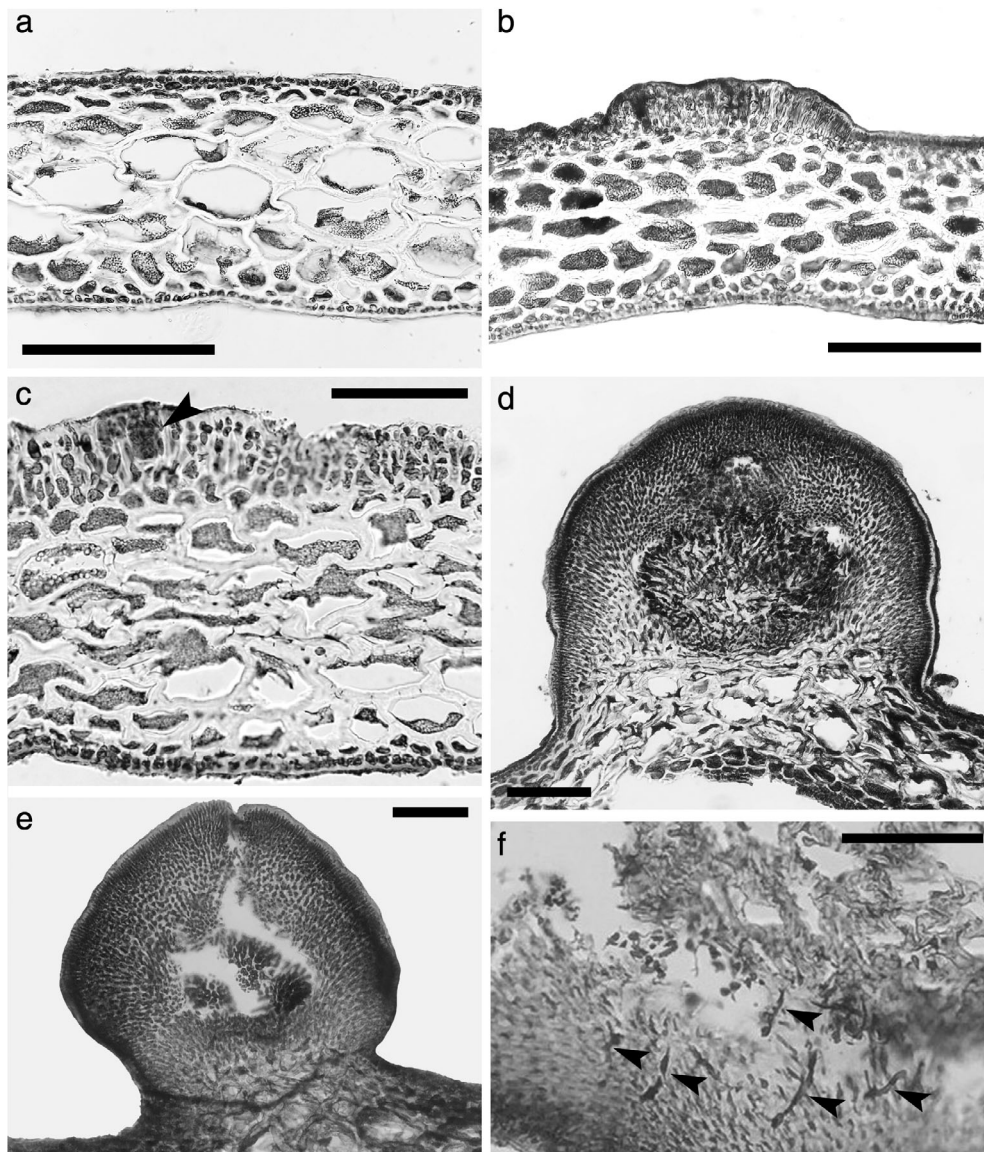


Fig. 3. Morphological characteristics of *Gracilaria phuquocensis* sp. nov. (a) Section of sterile plant, showing medullary cells and cell size transition, scale bar = 350 μm . (b) Tetrasporangial nemathecium in the surface of thallus, scale bar = 250 μm . (c) Close-up of tetrasporangia (arrowhead) in well-developed nemathecium, scale bar = 100 μm . (d) Cross-section of non-rostrate mature cystocarp, with a slight constriction at base, showing large gonimoblast cells and thickened pericarp cell layers, scale bar = 200 μm . (e) Section of rostrate mature cystocarp, with a conspicuous constriction at base, scale bar = 200 μm . (f) Mature cystocarp with numerous basal nutritive filaments (arrowheads) connecting to pericarp cells, scale bar = 200 μm .

and this characteristic has not been reported for any flattened *Gracilaria* species in Southeast Asian waters. On the other hand, a tetrasporangial nemathecium is relatively common for some flat *Gracilaria* species in the northeastern Pacific, for example, *G. skottsbergii* W.R.Taylor and *G. rubrimembra* E.Y. Dawson (Dawson 1949).

Gracilaria phuquocensis has only been found at Phu Quoc Island, southern Vietnam. *Gracilaria phuquocensis* could also be mistaken two other flattened species, *G. cuneifolia* and *G. yamamotoi*, from Vietnam. Although these three species are similar in external appearance, they can be distinguished based on the thickness of the medulla, cystocarp structure

and tetrasporangia arrangement (Table 1). *Gracilaria phuquocensis* consists of more medullary layers (five to eight cells) than those two species (two to three cells in *G. cuneifolia* and four to five cells in *G. yamamotoi*). Cystocarps of *G. phuquocensis* mostly have its nutritive filamentous cells in the basal parts of carposporophytes, while other two species have upper and lateral nutritive cells (Table 1). *Gracilaria phuquocensis* also has more pericarp layers (17–25 cells) than found in *G. cuneifolia* (12–15 cells). Furthermore *G. phuquocensis* produces tetrasporangial nemathecium on only one side of the blade, whereas the other two species bear tetrasporangia immersed in the cortex on both sides of the

Table 1. Comparisons of morphological features of *Gracilaria phuquocensis* and other related flat *Gracilaria* species

Characters	<i>G. phuquocensis</i> ^a	<i>G. mammillaris</i> ^{b,c}	<i>G. cuneifolia</i> ^{d,e,f,g}	<i>G. yamamotoi</i> ^{d,f,h}	<i>G. rhodymenioides</i> ^{i,j,k}
Thallus height (cm)	3–7	up to 40	4–12	5–6	2–5
Blade width (mm)	3–5	up to 150	3–10	3–5	2–7
Branching pattern	dichotomous	pseudodichotomous/ irregular	dichotomous/ irregular	dichotomous/ trichotomous	dichotomous/ trichotomous
Medullary cell layers	5–8	3–4	2–4	4–5	3–4
Nutritive filaments within cystocarp	abundant in basal parts	unknown	scattered in all parts	absent/ abundant in lateral and basal part	abundant in lateral and basal parts
Pericarp layers	17–25	unknown	12–15	14–18	20–25
Spermatangia	unknown	textorii-type	textorii-type	textorii-type	textorii-type
Tetrasporangia	borne in nemethesia	embedded in cortex	embedded in cortex	embedded in cortex	embedded in cortex
Distribution	Vietnam	Western Atlantic	Western Pacific	Western Pacific	Indian Ocean and Western Pacific

^a This study.^b Gurgel *et al.* 2004.^c Gurgel *et al.* 2008.^d Zhang & Xia 1994.^e Kim *et al.* 2006.^f Le & Nguyen 2006.^g Muangmai *et al.* 2012.^h Terada & Ueno 2004.ⁱ Millar 1997.^j Lewmanomont & Chirapart 2004.^k Muangmai *et al.* 2014.

blade. Phylogenetic analyses of *rbcl* sequences also revealed that these three species are not closely related to each other. *Gracilaria phuquocensis* forms a clade with *G. rhodymenioides* and two other cylindrical species. *Gracilaria cuneifolia* grouped with many flattened *Gracilaria* species from the western Atlantic, and *G. yamamotoi* grouped with two Asian flattened species, *G. incurvata* Okamura and *G. textorii* (Suringar) Hariot.

Apart from similarities to other Vietnamese species, *G. phuquocensis* resembles *G. rhodymenioides* from Thailand. Even though both species are very similar in size, shape and branching pattern (Table 1), these two species are distinguishable based on cellular characteristics and cystocarp and tetrasporangia configuration. Blades of *G. phuquocensis* are thicker, consisting of more medullary cells, than *G. rhodymenioides* (three to four cells). While *G. phuquocensis* produces numerous basal nutritive filaments within cystocarps, in *G. rhodymenioides* nutritive filaments are both lateral and basal. *Gracilaria phuquocensis* possesses tetrasporangia in nemathecial sori, while *G. rhodymenioides* produces tetrasporangia embedded in an unmodified cortex.

Our molecular data also demonstrated that two flat *Gracilaria* specimens collected from Hainan Island, southern China were genetically distinct from other described *Gracilaria* species, and sister to two flattened species from Taiwan, an undescribed *Gracilaria* species and *Gracilaria huangii* S.-M. Lin & De Clerck. It is obvious that further morphological analyses are required to confirm the taxonomic status of flattened Chinese specimens. Our study reveals that morphological similarity cannot alone be used to determine regional species diversity, such as that of the flattened *Gracilaria* species in the South China Sea, and a combination molecular and morphological examination is essential for these diversity studies.

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SUPPORTING INFORMATION

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

Fig. S1. Map showing the sampling site.

Table S1. List of taxa used in this study and their GenBank accession numbers.