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FIRST RECORD OF *Gracilaria phuquocensis* (Gracilariales, Rhodophyta) IN THE EAST SEA OF VIETNAM

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ABSTRACT

The red algal genus *Gracilaria* is an important biomaterial for its use in seaweed vegetable and agar industry in Southeast Asia. However, due to the morphological plasticity, the taxonomy and distribution of flattened *Gracilaria* species remain understudied. *Gracilaria phuquocensis* N.H.Le, N.Muangmai & G.C.Zuccarello is one of the flattened species described for specimens in the Phu Quoc Islands, Vietnam. Our study aimed to provide morphological details and ecology, and to analyze mitochondrial COI-5P and plastid *rbc*L sequences of recent collections at My Hoa, Ninh Thuan province. The COI-5P, first generated in *G. phuquocensis* in the present study, was identical to the sequence from Malaysia. Plastid *rbc*L sequence divergence of specimens from Malaysia, Philippines, Thailand as well as Vietnam was 0–0.5%, a value similar to intraspecific divergence in *Gracilaria* species. This result indicates the broad distribution of *G. phuquocensis* across Southeast Asian waters. Our study provides an intriguing issue for further study on the origin and dispersal of *G. phuquocensis* in Southeast Asia.

Keywords: Ecology, Genetic variation, Red algae, Seaweed, Southeast Asia, Tropic.

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INTRODUCTION

The *Gracilaria* species is an essential economic genus that includes about 206 species distributed from tropical to temperate waters (Wang et al., 2023; Gury & Gury, 2024). Several species have been extensively studied in their general biology and chemical diversity (Fredericq & Hommersand, 1989; Torres et al., 2019). In Vietnam, approximately 30 species of *Gracilaria* have been recorded (Nguyen et al., 2013; Nguyen et al., 2023). Of these, some cylindrical species have been described in detail with specimens and localities where specimens were collected. However, four flattened species have been understudied: *Gracilaria cuneifolia* (Okamura) I.K.Lee & Kurogi, *Gracilaria textorii* (Suringar) De Toni, *Gracilaria yamamotoi* Zhang & B.M.Xia, and *Gracilaria phuquocensis* N.H.Le, N.Muangmai & G.C.Zuccarello (Ohno et al., 1999; Le & Nguyen, 2006; Le et al., 2019).

G. phuquocensis was described for specimens from the Phu Quoc Islands based on morphology and *rbc*L sequence (Le et al., 2019). It was previously misidentified as *Gracilaria mammillaris* (Montagne) M.Howe (Pham, 1985), which is restricted to the western Atlantic. *G. phuquocensis* was reported to be found only in Phu Quoc Island (Le et al., 2019), and later, it was reported to occur in the Philippines (Dumilag et al., 2024).

Repeated collections along the long coast of Vietnam have widened the current knowledge on the species diversity and distribution of various seaweeds (Nguyen, 2013; Kim et al., 2022; Nguyen et al., 2024). During collection trips in southern Vietnam, we collected a foliose species of *Gracilaria* on the lower intertidal rocky beds. Both plastid *rbc*L and mitochondrial COI-5P sequences demonstrated their identification as *G. phuquocensis*, which was restricted to Phu Quoc Island, Vietnam. The aim of the present study was to compare the morphology, DNA sequences of *G. phuquocensis* from Vietnam, the Philippines, and other species known as '*Gracilaria rhodymenioides'* from Thailand, and to discuss the species boundary and distribution of the species

MATERIALS AND METHODS

Specimen collection and morphology observation

Specimens were collected from Son Hai on June 14th 2022, and My Hoa, Ninh Thuan province, on December 7th 2023, during a regular field trip to study the diversity and seasonality of *Gracilaria* species in the South-central coast of Vietnam. All specimens were dried and preserved as vouchers, a part of the thallus cleaned and dried in silica gel for molecular analysis, and other parts were preserved for morphological study. The morphology of the collections was observed in detail by the microscope Olympus ZS51. Sections were made by hand and stained with 1% cotton blue in 50% glycerol/seawater. Voucher specimens are deposited in the Herbarium of the Institute of Tropical Biology (VNM).

DNA extraction, amplification and sequencing

Clean fragments were extracted for molecular analysis. DNA extraction, polymerase chain reaction amplification, and sequencing procedures followed Boo et al. (2022). Total genomic DNAs were extracted following the Promega kit (M1125, Promega, USA). Primers used for amplifying and sequencing were GAZF1 and GAZR1 for COI-5P (Saunders and Moore, 2013), and R753 and R1442 for *rbc*L (Freshwater & Rueness, 1994; Kim et al., 2010).

All amplified products were cleaned and sequenced commercially (Jenotek, Daejeon, Korea). Sequences were edited, assembled, and aligned using Bioedit 7.0 and Mafft 7.

Phylogenetic analysis

The phylogenetic relationship was inferred by using the Maximum Likelihood method and the Tamura-Nei model (Tamura & Nei, 1993). The tree with the highest log likelihood was shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and

then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The proportion of sites where at least 1 unambiguous base was present in at least 1 sequence for each descendant clade was shown next to each internal node in the tree. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1383 positions in the final dataset for rbcL and 696 positions in the final dataset for COI-5P. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

RESULTS

Gracilaria phuquocensis N.H.Le, N.Muangmai & G.C.Zuccarello

Description. Thallus (Fig. 1a) reddish-brown to pale red roses, a foliose, erect solitary or clustered, slightly irregular dichotomous with entire margins, 2.0–5.0 cm tall, 3.0 –5.0 mm wide and 0.2–0.3 mm thick. Blades arising from a short cylindrical stipe that is attached to the substrate by a rhizoidal base, irregular to dichotomous, three to five branched. In cross section, blades were composed of the cortex and medulla parts, cortices comprising up to two layers of pigmented globular cells, 4–6 µm (Fig. 1c). Medulla was composed of 2–4 unpigmented spherical or ovoid cells, cell size variable from 50–100 µm (Fig. 1d).

Tetrasporangia are widely distributed on the surface of blades, arising from out cortical cells, and are divided irregularly to cruciately. Female and male thalli not found (Fig. 1b).

A morphological comparison of *G. phuquocensis* from Vietnam and the Philippines and '*G. rhodymenioides*' is given in Table 1.

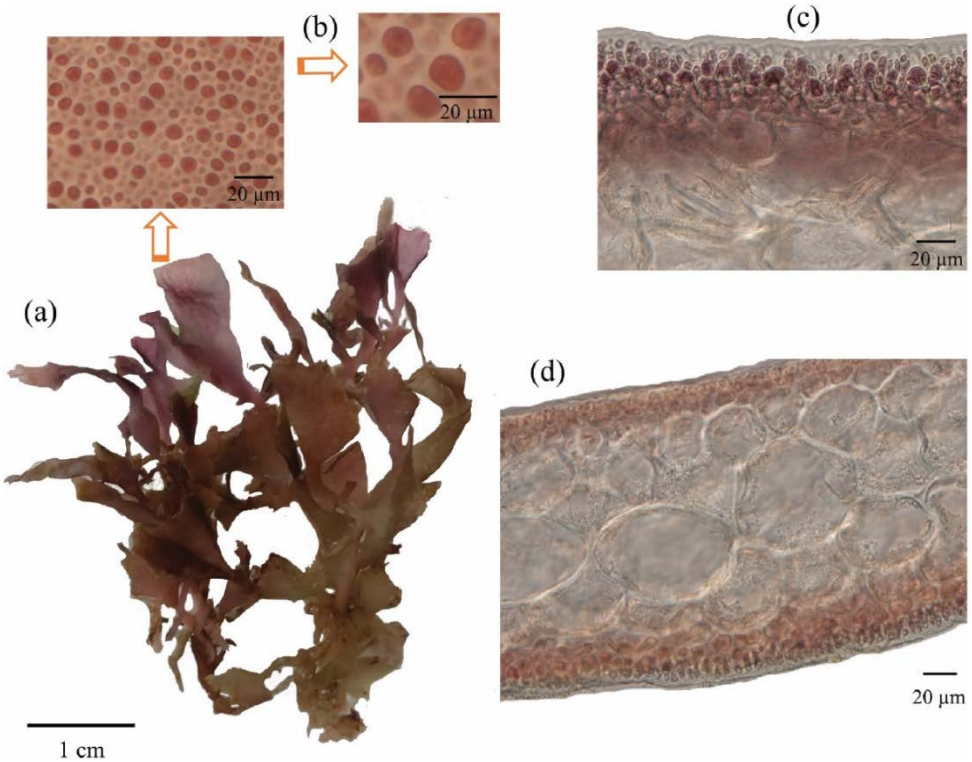

Figure 1. *Gracilaria phuquocensis* collected at My Hoa village on December 7th, 2023. (a) Habit of a specimen from My Hoa village; (b) Tetrasporangia in surface view; (c); cross section of blade showing tetrasporangia in the upper and medulla cells in the centre, (d) enlargement of tetrasporangia

Table 1. A comparison of *Gracilaria phuquocensis* from Southeast Asia with *Gracilaria rhodymeniodes* from Thailand

Species	*Gracilaria phuquocensis*	*Gracilaria phuquocensis*	*Gracilaria phuquocensis*	*Gracilaria rhodymeniodes*
Area studied	Phu Quoc Island, Vietnam, type locality	My Hoa Village, Vietnam	Bulusan, Philippines	Prachuap Khiri Khan, Thailand
Marker used for identification	*rbc*L	*rbc*L, COI-5p	*rbc*L	*rbc*L
Length of thalli	3–7 cm	2–5 cm	1.5–5 cm	2–5 cm
Width of blades	3–5 mm	3–5 mm	1–3 mm	3–7 mm
Branching pattern	dichotomous	irregular to dichotomous	dichotomous	irregular to di- or trichotomous
Medulla	5–8 layers of cells	4–6 layers of cells	2–4 layers of cells	3–4 layers of cells
Cystocarp	rostrate, constricted	-	-	globose, constricted at base, non-rostrate
Nutritive filament	abundant in basal cells	-	-	Many, basal
Pericarp	17–25 layers of cells	-	-	up to 18 layers of cells
Spermatangia	-	-	-	*textorii*-type
Tetrasporangia	borne in nemethecia	scattered on the surface	-	borne in nemathecia
References	Le et al., 2019	This study	Dumilag et al., 2024	Lewmanomont & Chirapart, 2004; Muangmai et al., 2014

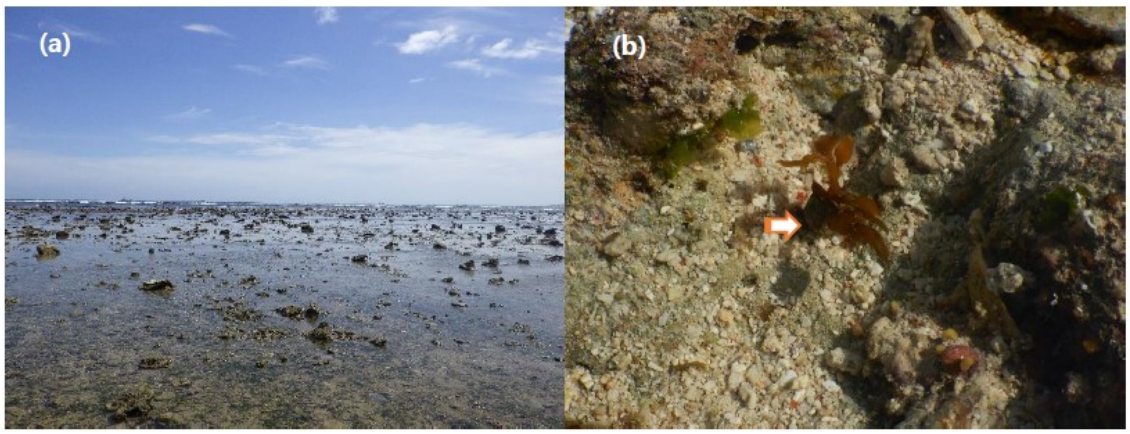

Figure 2. Field observation of *Gracilaria phuquocensis.* (a). tidal bed at My Hoa village (b). thallus attached on gravel dead coral bottom in summer 2024

Specimens observed. My Hoa village, Ninh Thuan; December 7th, 2023; specimen codes: VNM231294, VNM 231294-2, VNM 071215-1, and G071512-3. GenBank accession number, PX354556 for *rbc*L and PX354557 for COI-5P.

Ecology. *G. phuquocensis* was found in the intertidal zone (Fig. 2a) with moderate wave action. Species occurred sparsely with small clumps, and was normally attached on rock (Fig. 2b), gravel or dead coral. The exposed thallus was paler red than the one in a deeper, less-sunlit area. The morphology of *G. phuquocensis* revealed slight variation depending on location and season. Thalli were smaller and relatively thicker in summer than in winter.

Phylogenetic relationship

The optimal tree of the Maximum Likelihood (ML) based on partial *rbc*L gene sequences is shown in Figure 3 and the tree of the Maximum Likelihood (ML) based on partial COI-5P sequences is shown in Figure 4. *G. phuquocensis* was well resolved in *rbc*L phylogeny, including specimens from Vietnam, the Philippines, and Thailand. The *rbc*L sequence was identical between specimens in My Hoa village and Malaysian species. (JQ026035). However, the pairwise divergence in *rbc*L was 0.2 between specimens in My Hoa village and Phu Quoc Islands, and 0.5% between specimens in My Hoa Village and the Philippines (OR427952-4), and between My Hoa and Thailand (KF854302).

The mitochondrial COI-5P sequence of *G. phuquocensis*, newly generated from a specimen collected in My Hoa, was compared to those of nine other *Gracilaria* species available in GenBank (Fig. 4). A perfect match was found between My Hoa village specimens and *Gracilaria* sp. (JQ026068) from Malaysia.

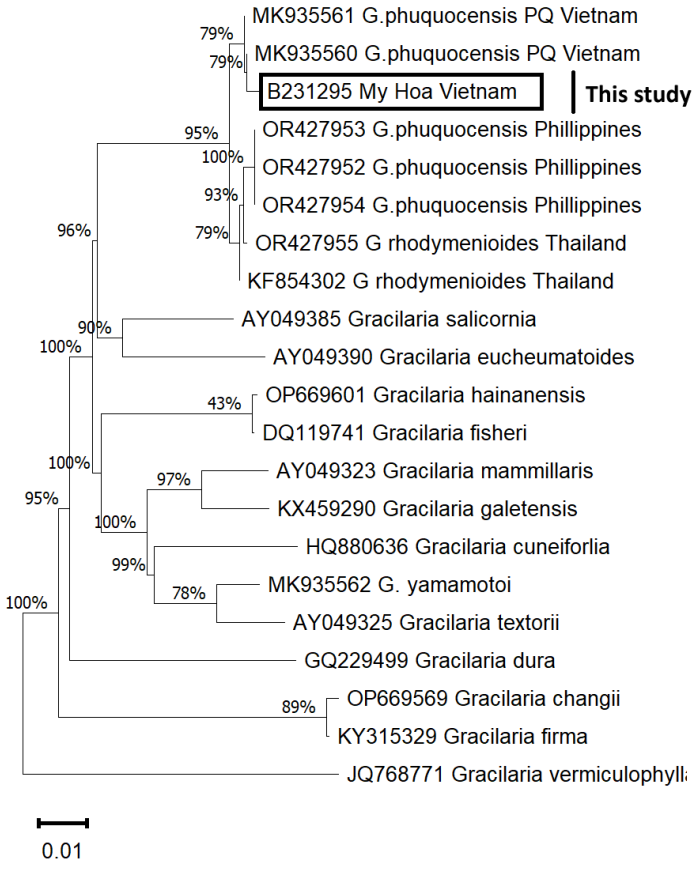

Figure 3. Maximum Likelihood (ML) of *Gracilaria* based on partial *rbc*L gene sequences showing the position of *Gracilaria phuquocensis* from My Hoa Village, Ninh Thuan, Vietnam. ML bootstrap values are indicated at the nodes

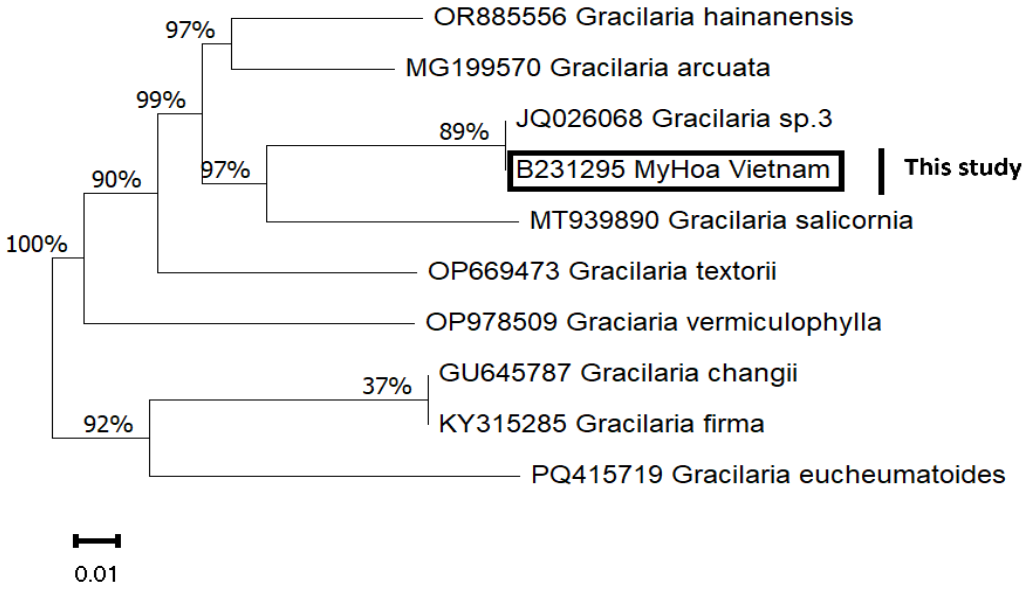

Figure 4. Maximum Likelihood (ML) of *Gracilaria* based on partial COI-5P gene sequences showing the position of *Gracilaria phuquocensis* from My Hoa Village, Ninh Thuan, Vietnam. ML bootstrap values are indicated at the nodes

DISCUSSION

G. phuquocensis was described for the first time for specimens from Phu Quoc Island by Le et al. (2019), which was likely misidentified as *G. mammillaris* in Southern Vietnam (Pham, 1985). Small and thick summer plants to winter ones in My Hoa village reveal slight variations depending on ecological conditions, highlighting the temperature sensitivity of *G. phuquoensis*. Le et al. (2019) reported that thalli grew in the upper sublittoral zone in the rocky, wave-exposed areas in October, while Pham (1985) documented their occurrence in March. We collected specimens from the East Sea of Vietnam in June and December. Specimens from My Hoa, Ninh Thuan province, were found in intertidal and shallow subtidal zones, frequently attached to rocks, dead corals, or other solid substrates. These plants thrive in areas with moderate water flow, wave action, likely due to their robust thalli. Given all these collections in Vietnam, *G. phuquocensis* occurs throughout the year.

Seaweed and other marine species are likely affected by water currents, especially upwelling. This phenomenon alters ecological conditions, including temperature and nutrient availability, which can significantly affect the growth and distribution of marine organisms (Lobban & Harrison, 1994). Summer upwelling, replacing warm, nutrient-poor surface waters with cool, nutrient-rich deep waters, commonly occurs in South Vietnam (Yao & Wang, 2021), and it provides favorable conditions for the growth of various seaweeds and contributes to the appearance of new records or new species and enhances biodiversity compared to other areas (e.g., Nguyen & Boo, 2020; Do et al., 2023).

Both COI-5P and *rbc*L gene sequences demonstrated the occurrence of *G. phuquocensis* in My Hoa, Ninh Thuan province. This is the first report of the species on the east coast of Vietnam, outside of Phu Quoc Island since its description in Phu Quoc Islands (Le et al., 2019). The morphology of the My Hoa collections aligns closely with that of the Phu Quoc Island specimens, characterized by typically flattened, slightly irregular dichotomous blades composed of distinct cortex and medulla layers (Pham, 1985; Le et al., 2019).

Phylogeny of *rbc*L revealed that *G. phuquocensis* is a widespread species from Vietnam to the Philippines and likely Thailand. This topology was supported by pairwise divergence.

The pairwise divergences in *rbc*L between Vietnam and the Philippines (OR427952-4) or Thailand (KF854302) was in a range of 0.5%, a very close value (0.43-0.51%) reported in a previous study (Dumilag et al, 2024). This result reveals a signal of geographical structure in *G. phuquocensis* from Southeast Asia, as similarly reported by Dumilag et al. (2024).

The COI-5P sequence from My Hoa was identical to that of *Gracilaria* sp3. (JQ026068) from Penang, Malaysia (Yang & Kim, 2015), suggesting they are highly likely the same species. The phylogeny of COI-5P from nine species available in GenBank clearly affirms the separation of *G. phuquocensis* from other species. Our study suggests that *G. phuquocensis* is likely widespread in tropical Southeast Asia, ranging in Malaysia (as *Gracilaria* sp3.), the Philippines, Thailand (as *G. rhodymenioides*), and Vietnam. According to Le et al. (2019), *G. phuquocensis* could also be mistaken with two other flattened *Gracilaria* species, *G. cuneifolia* and *G. yamamotoi*, also recorded from Vietnam. Our results show *G. phuquocensis* from My Hoa was genetically distinguished from these species through partial *rbc*L gene sequence analysis (Fig. 3).

Because of morphological variability in *Gracialaria* species, plastid *rbc*L and COI-5P markers greatly contribute to the taxonomy (Gurgel & Fredeicq 2004; Muangmai et al., 2014; Le et al., 2019). Based on *rbc*L phylogeny and pairwise divergence shown in the present study and previous reports in the Philippines and Vietnam (Le et al., 2019; Dumilag et al., 2024), the species boundary and range of *G. phuquocensis* are greatly expanded to include specimens from Malaysia, the Philippines, and Vietnam. Although merging '*G. rhodymenioides*' from Thailand into *G. phuquocensis* should be careful because of the difference in female structure (Table 1). Herein, we included it in *G. phuquoensis* based on *rbc*L phylogeny and pairwise divergence values, as discussed above. Additional sampling in Thailand and surrounding countries will provide further resolution between the two species.

The present study demonstrates the occurrence of *G. phuquocensis* outside Phu Quoc Island, the type locality since its description in 2019. Additional surveys may expand its range along the long coast of Vietnam. Both *rbc*L and COI-5P phylogenies provide an intriguing issue on the species boundary and range of *G. phuquocensis*. Further intensive collections and DNA data are needed to enhance the current knowledge of flattened species of *Gracilaria*, including *G. phuquocensis* in Southeast Asian waters. Our study highlights the important tool of molecular markers for the identification of *G. phuquocensis*, and proposes reexamination of many other species of *Gracilaria* recorded only in floristic studies in Vietnam. Describing the morphology and distribution of *Gracilaria* species along the entire coast of Vietnam is challenging and important for the conservation of economically valuable species in relation to anthropogenic causes of climate change.

CONCLUSION

G. phuquocensis has been recorded at My Hoa, Ninh Thuan province, in the East Sea of Vietnam. The first generated COI-5P sequence was identical with the sequence from Malaysia, and the *rbc*L phylogeny of *G. phuquocensis* from this study shares a branch with specimens from the Philippines and Thailand. Further investigation is needed to clarify the evolutionary history of *G. Phuquocensis.*

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