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Morphological variation and haplotype diversity of *Halimeda macroloba* and *H. opuntia* (Chlorophyta: Halimedaceae) from Southern Vietnam

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ABSTRACT

Plasticity in morphology is a common phenomenon of aquatic plants. *Halimeda* (Chlorophyta: Halimedaceae) is usually supersaturated with calcium carbonate and is found in tropical and subtropical regions. Among members of *Halimeda*, both species including *Halimeda macroloba* and *H. opuntia*, often occur in different habitats. Haplotype diversity and network of *Halimeda* were reported in the different sea and oceanic systems. However, there are no reports of the genetic diversity of *Halimeda* in Vietnamese waters. This present study carried out sample collections along the coast of Southern Viet Nam, including the coast sites, offshore islands, and Spratly (Truong Sa) islands. External morphological and anatomical characteristics of two dominant species, *Halimeda macroloba* and *H. opuntia*, were caparisoned. The genetic marker *tuf*A was applied to find the haplotype diversity and network among Vietnamese and worldwide populations. The results showed that high morphology exists in both species. In contrast, the genetic variation in *H. macroloba* is very low, and *H. opuntia* tends to form a distinct group. We suggest that more samples of two species from other locations in Northern Vietnam be included.

Keywords: Genetic variation, *Halimeda*, morphology, Southern Vietnam, *tuf*A.

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INTRODUCTION

The genus *Halimeda* (Chlorophyta: Bryopsidales) is common in tropical and subtropical regions [1]. It was usually supersaturated with calcium carbonate [2]. For the geographic distribution, most *Halimeda* members are restricted to the Atlantic Ocean or the Indo-Pacific Ocean by the continents of Africa, and the Americas limit their dispersal [3]. Worldwide, 49 species of *Halimeda* were recorded [4]. However, *Halimeda* has a complex taxonomy, and the morphological characters overlap between species. Morphological plasticity leads to identification problems. Within 1 species, morphological characters showed variation based on ecological factors and their habitats. For example, the thallus morphology of *H. macroloba* Decaisne from Thai waters showed variation in different habitats such as subtidal, intertidal, or high light intensity areas [5]. The surface utricles within individual segments also showed variation in the case of *H. tuna* [6]. Therefore, genetic markers and morphological observation are used to identify the genus into genetically and morphologically distinct species [7].

In taxonomy studies, separated the genus *Halimeda* into five sections, including *Rhipsalis*, *Halimeda*, *Micronesicae*, *Opuntia*, and *Pseudo-Opuntia* [8]. In the Southeast Asian countries, the review of Phang et al., [9] showed that there are 20–30 species of *Halimeda* within the waters of Malaysia, Thailand, Vietnam, Singapore, Philippines, and Indonesia in the East Vietnam Sea. Based on morphological observation and molecular maker, there are 8 species along the coast of Thailand [10]. Arina et al., [1] reported 6 species of *Halimeda* after recording *H. taenicola* for Malaysian flora. 14 species of *Halimeda* were listed in the waters of the Philippines [11]. A checklist of the marine macroalgae of Vietnam showed 13 species of *Halimeda* [12]. However, most reported species were based on a single morphological observation, without molecular confirmation, while morphological variations appear to correlate with habitats in *Halimeda*. Molecular markers and morphology have been globally useful for algal species delimitation [13]. Recently, several new species and records of marine algae were presented for marine macroalgae of Vietnam, mainly red and brown algae, based on the combination of molecular markers and morphology [14–16]. Therefore, the actual members of *Halimeda* from Vietnam are still questionable. Some *Halimeda* members' cases reported a distribution break line, except for *H. macroloba* [5]. The authors also indicated the significant differences in morpho-anatomical variation between populations of *H. macroloba* from Thai waters. Worldwide, several haplotypes were recorded in different regions. However, data on haplotypes from Vietnamese water are still lacking.

This present study aims to evaluate the morphological variation and haplotype diversity of two dominant species of *Halimeda*: *H. macroloba* and *H. opuntia* (Linnaeus) J. V. Lamouroux, based on genetic marker *tuf*A.

MATERIALS AND METHODS

Sample collection

Specimens (40) of *Halimeda* were collected from six sites along the coast of Southern Vietnam (Figure 1). Snorkeling and SCUBA diving were used for sampling in April 2021. Water depths varied in different sites (from 4–12 m). For the morphological study, algal samples were preserved in 5% formalin seawater or pressed on herbarium sheets, whereas materials used in the molecular studies were preserved in DESS solution (dimethyl sulfoxide, disodium EDTA, and saturated NaCl). Voucher specimens are deposited in the Institute of Oceanography (ION), Nha Trang city, Vietnam (Appendix 1).

Morphological observation

Specimen identification was carried out by defining external morphological and anatomical characteristics. External traits, including the specimen height, segment size, were observed [17]. For anatomical identification, segments were decalcified using 20% hydrochloric acid [1]. The tissues were then stained with Lacto-phenol cotton blue to distinguish the utricle and medullary filaments. The stained surface was observed under an

Olympus CH30 microscope in both longitudinal and cross-sectional views, and photomicrographs were taken on a microscope with a Q-imaging digital camera (Burnaby, BC, Canada). Habit views were reproduced with an Epson scanner (Tokyo, Japan) at the Institute of Oceanography, Vietnam. The utricle was also observed after the decalcified segment was dissected and compared to existing keys from previous works. Morphological data were assessed and checked by Levene's test for homoscedasticity. Levene's test, one-way analysis of variance (ANOVA), Tukey test and whisker plots were carried out by Minitab software (State College, PA, USA).

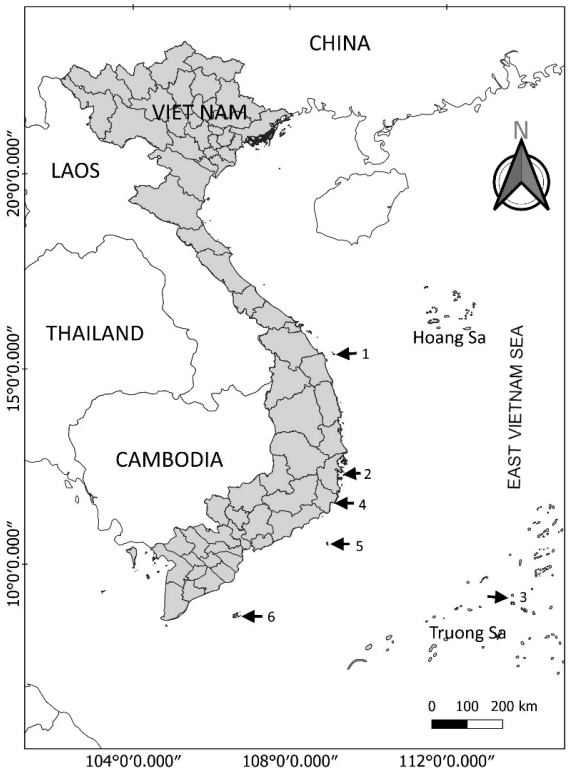

Figure 1. The map of Vietnam and sampling sites. 1: Ly Son island, 2: Nha Trang bay, 3: Spratly islands, 4: Ninh Thuan, 5: Phu Quy, 6: Con Dao. The map was processed by MapInfor Pro™, version 12.5.5 (Pitney Bowes Software Inc., NY, USA)

Molecular data acquisition

A bead mill homogenized samples fixed in DESS solution at 22 Hz in 2 min, and 100 mg of the fine powdered plant material was used for DNA extraction. The DNA extraction was performed using the Quick-DNA™ Miniprep Plus Kit (Zymo Research, CA, USA), following the manufacturer's instructions. The regions selected for PCR amplification were portions of plastid genes encoding the elongation factor Tu gene (*tuf*A). The total volume of 25 µL included 12.5 µL 2x OneTag® Master Mix (New England Biolabs, Ipswich, MA, USA), 10–30 ng template DNA, one pmol of each primer, and sterile water up to 25 µL. PCR was performed in an Applied Biosystems 2720 thermocycler (Applied Biosystems, Foster, CA, USA) with a heated lid under the following conditions: initial denaturation for 4 min at 94°C followed by 35 cycles of denaturation for 60 s at 94°C, primer annealing for 2 min at 55°C, and extension for 2 min at 72°C, and a final extension at 72°C for 7 min. In this present study, the primer pair *tuf*A F and *tuf*A R was suggested by Famà et al., [18] to obtain a length of about 900 bp. All PCR reactions were repeated two to four times independently to keep errors (possibly created by the *Taq* polymerase) in the final consensus sequence to a minimum. Sequencing was done by 1ST BASE (Selangor, Malaysia) from both directions. The consensus sequence was achieved by Clone Manager 9 (Sci-Ed, Cary, NC, USA).

In total, 55 *tuf*A sequences obtained during this study and retrieved from GenBank (www.ncbi.nlm.nih.gov) were used for the analysis (Appendix 1). These sequences were aligned by the MAFFT algorithm with the selection of the q-ins-i option [19]. jModelTest version 2.1.6 [20] and the corrected AIC (Akaike Information Criterion) was used to find the best model for the analysis. Phylogenetic analyses were performed using Maximum Likelihood (ML) in RAxML version 8.1 with the General Time Reversible (GTR) model and Bayesian Inference (BI) (Metropolis-coupled Markov chain Monte Carlo method, GTR model) performed in MrBayes version 3.2.2 [21]. Two parallel runs with four chains each (three heated and one cold) were performed for one million generations, sampling a tree every

1,000 generations. The 400,000 burn-in periods were identified graphically using Tracer version 1.7.1 [22] by tracking likelihoods at each generation to determine whether the likelihood values had reached a plateau and the average deviation of split frequencies fell below 0.01. The consensus tree based on two different trees (achieved from the two methods) was constructed by Dendro Scope version 3.2.10 [23].

Haplotype network analysis

In this present study, two different haplotype network analyses were applied for two species: *Halimeda macroloba* and *H. opuntia*. For the *H. macroloba* dataset, five *tuf*A sequences from various sites and published data from other regions including Australia, New Caledonia, Tazania and Japan were used for the analysis. For the *H. opuntia* dataset, three new sequences were achieved from three sites and 10 sequences from different regions, including Belgium, Brazil, French Polynesia, Japan, Philippines, and New Caledonia. Datasets were loaded to DnaSP version 6 [24] to find the distinct haplotype. The haplotypes containing a 714 bp *tuf*A sequence were defined based on transitions, transversions, indels, and mononucleotide repeats. Indels were considered a single event and recoded as proposed by Müller [25]. Haplotype frequency distribution and genotype networks were performed by PopART software (http://popart.otago.ac.nz) with the method Minimum Spanning Networks [26].

RESULTS AND DISCUSSION

Morphological observation

Morphological observation of five main traits, including thallus height, segment height, segment width, the diameter of utricle at the surface view, and utricle height from both species, revealed high variations. For *H. macroloba*, the average thallus height of samples collected from Spratly islands (20.8 cm) was much higher than four other locations (< 14 cm) (Figure 2A). Segment height and width showed low variation among five populations, from 0.83–1.25 cm, and 1.14–1.56, respectively (Figures 2B, 2C). The trait of the diameter of utricle at surface view showed high variation among populations. Samples collected from Con Dao island (60.5 µm) are the biggest, while the samples collected from Phu Quy island showed the smallest (27.5 µm). Those values from the three remaining populations are from 32.4–53.4 µm (Fig. 2D). In the same way, the utricle height also depicted the high variation among populations; the population from Con Dao island is highest (105 µm) while data from Spratly island is lowest (40 µm) (Figures 2E, 3). The result of ANOVA for the five above traits indicated that statistically significant differences were found among populations for three features, including thallus height, the diameter of the utricle at the surface view, and utricle height (*p*-values < 0.05). In contrast two traits, including segment height and width, did not show statistically significant differences.

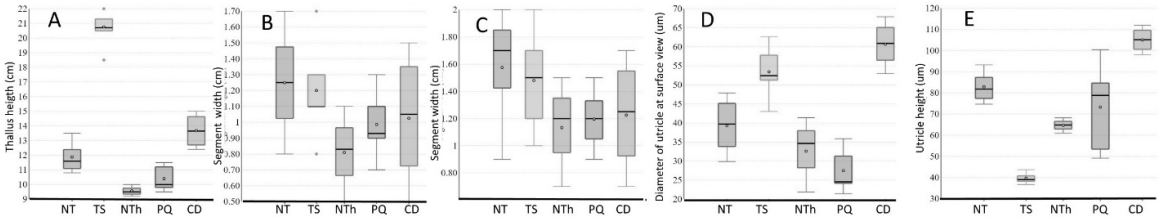

Figure 2. Morphological characters of *H. macroloba* collected from five locations. A = Thallus height, B = Segment height, C = Segment width, D = Diameter of utricle at surface view, E = Utricle height. NT = Nha Trang bay, TS = Spratly islands, NTh = Ninh Thuan, PQ = Phu Quy island, CD = Con Dao

For *H. opuntia*, the average thallus height at Ly Son island (9.9 cm) is a little bit higher than two populations at Ninh Thuan (7.58–8.04 cm) (Figure 4A). The average size of segment

(height and width) of samples collected from site 1, Ninh Thuan (0.47 × 0.68 cm) revealed to be bigger than Ly Son island (0.39 × 0.52 cm) and site 2, Ninh Thuan (0.33 × 0.49 cm) (Figure 4B, 4C). In the same way, the diameter of the utricle at the surface view of samples collected from site 1, Ninh Thuan (24.8 µm), is bigger than the two remaining populations (16–18 µm) (Figure 4D). However, there is a low variation of utricle height among three populations, from 23.4–26.2 µm (Fig. 4E). Two traits, including thallus height and diameter of utricle at the surface view showed statistically significant differences (ANOVA test, *p*-values < 0.05). In comparison, three traits, including segment height, segment wide, and utricle height, did not show significant differences (ANOVA test, *p*-values > 0.05).

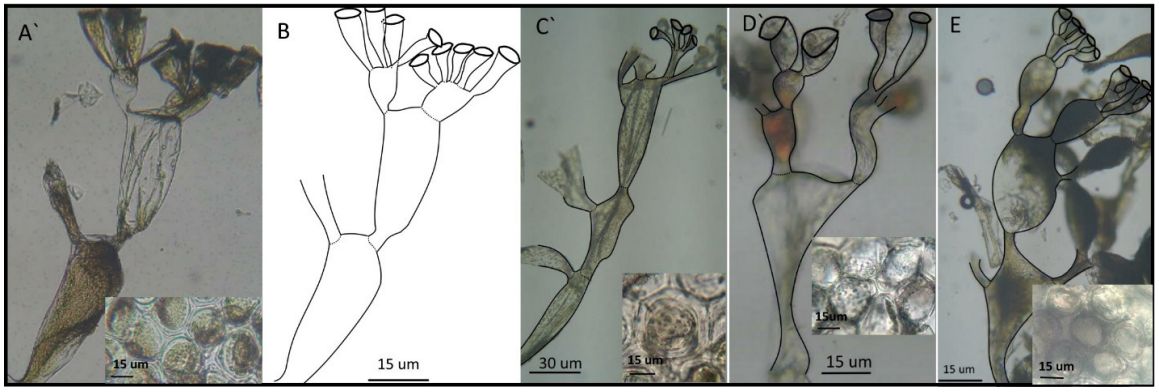

Figure 3. Morphology of utricle and surface cell shape (low right corner) of *H. macroloba* collected from different locations. A = Nha Trang bay, B = Redrawing from A, C = Spratly islands, D = Ninh Thuan, E = Phu Quy

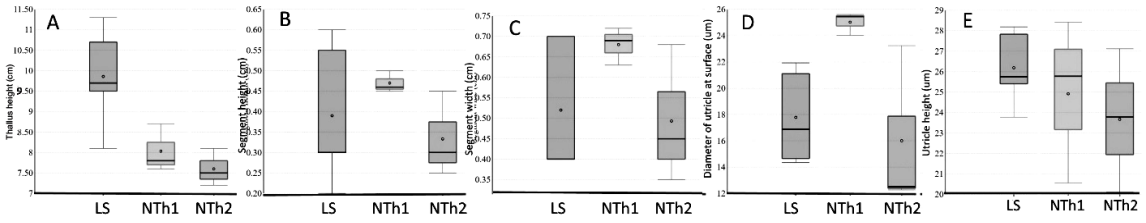

Figure 4. Morphological characters of *H. opuntia* collected from three locations. A = Thallus height, B = Segment height, C = Segment width, D = Diameter of utricle at surface view, E = Utricle height. LS = Ly Son island, NTh1 = Ninh Hai, Ninh Thuan, NTh2 = Ca Na, Ninh Thuan

This present study showed a morphological variation of *H. macroloba* in a different location in Southern Vietnam. Previously, Pongparadon et al., [5] reported that *H. macroloba* collected from the Andaman Sea (Indian Ocean) and Gulf of Thailand (Pacific Ocean) had high morpho-anatomical variability, especially the height and number of layers of the utricle. Southern Vietnam samples showed 4–5 layers, whereas the Thai materials had 4–7 layers. Marine algae respond to the environmental fluctuation by changing their morphologies that may be adaptive *i* the new changes [27]. Plasticity in the morphology of surface utricles is also found in *H. tuna* [6]. *H. macroloba* belongs to section *Rhipsalis*, exhibiting high morphological plasticity [28]. Arina et al., [1] also depicted two morphotypes of *H. bonerensis* from Malaysia based on the morphology of utricle and medullary filaments. Morphotype was also reported in our previous studies in marine plants such as *Halophila ovalis* [29] and *Halophila major* [30].

Phylogenetic tree of *Halimeda*

A final alignment of 666 bp was generated for the *tuf*A marker, of which 461 (69.2%) were conserved sites, 245 (36.8%) were variable sites, and 184 (27.6%) were parsimony-informative characters, and 61 (9.2%) were singletons. Results of the two algorithms applied (ML, BI) showed that all *tuf*A sequences of *Halimeda* were divided into five main clades that reflected five sections: Section *Rhipsalis* includes *H. macroloba*, *H. heteromorpha*, *H. kalaloana*, *H. borneensis*, *H. cylindrica*, *H. incrassata*, and *H. simulans*; Section *Opuntia* includes *H. opuntia*, *H. minima*, and *H. renschii*; Section *Pseudo-Opuntia* consists of two species *H. lacrimosa* and *H. gracilis*; Section *Micronesia* includes three species *H. cryptica*, *H. pygmaea*, and *H. Micronesia*; Section *Halimeda* consists of the remaining species. Materials *H. macroloba* and *H. opuntia* collected from Vietnamese waters grouped to the known *H. macroloba* and *H. opuntia.* (Figure 5). For *H. macroloba*, the evolutionary divergence as measured by estimated total fragment and per nucleotide differences are very low, 1–6 nucleotides and < 0.01, respectively. For *H. opuntia*, however, the evolutionary divergence as measured by estimated total fragment and per nucleotide differences are significantly higher, 1–14 nucleotides and 0.02–0.29, respectively (data not shown).

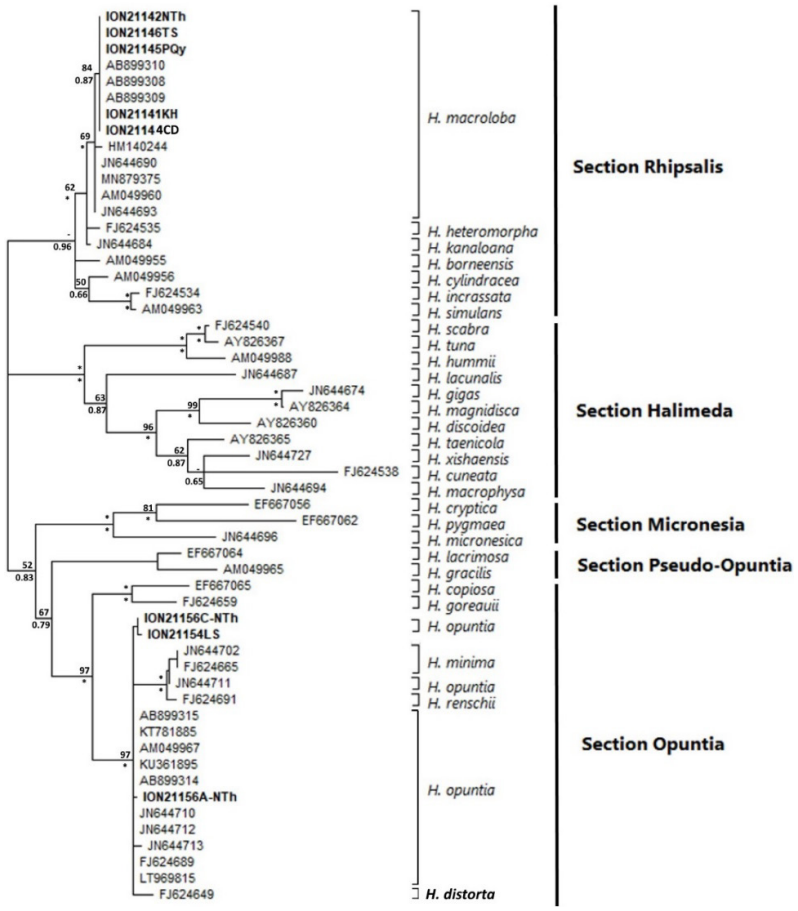

Figure 5. Phylogenetic tree of members of *Halimeda* inferred from Maximum likelihood and Bayesian inference. Data set based on 666 bp of *tuf*A. Bootstrap values and posterior probability of each method are shown at each node; *: full support (bootstrap value = 100, posterior probability = 1). See Appendix 1 for more information. The consensus tree was constructed by Dendro Scope software, version 3.2.10

Haplotype networks

For *Halimeda macroloba*, eight haplotypes (Hap) were found globally. Hap1 was shared by Southern Vietnam and Japan, while Hap3 was only found in Australia. In the same way, Hap4–6 and 8 were presented in Guam only, and Hap7 was only found in Japan. Notably, among haplotypes, frequencies of Hap2 were highest, as it occupied 46.4% of the total number. Frequencies of Hap2 were also higher than the remaining haplotypes with 32.1% (Fig. 6, panel A). The most parsimonious network did not reveal any distinct groups. Hap2 seems to be the presumed ancestral haplotype, and seven variants (Hap1, Hap3–8) were raised from Hap2. There were 1–3 mutations between Hap2 and other haplotypes (Figure 6, panel B).

For *H. opuntia*, eight haplotypes were found. There are three haplotypes in Southern Vietnam (Hap1-3). Among haplotypes, the frequencies of Hap6 were highest, as it occupied 64.3% of the total number. Hap4 and Hap5 occur in French Polynesia and Belgium, respectively. In contrast, Hap6 seems to be global distribution in New Caledonia, the Philippines, Japan, and French Polynesia (Figure 7, panel A). The haplotype network analysis revealed that Hap6 might be the presumed ancestral haplotype. Five variants form two groups, including Hap4 and Hap1–3, 5. There were 14 mutations between Hap6 and Hap4; it is much higher than Hap6 and Hap1–3, 5 (7–9 mutations) (Figure 7, panel B).

Our finding indicated minimal genetic variation in *H. macroloba* based on *tuf*A sequences. Only two haplotypes were found along the coast of Southern Vietnam and Spratly islands. The haplotype number is the same as Japan [13], lower than the haplotype number from Thai waters with three haplotypes [5]. In contrast, *H. opuntia* showed three haplotypes, which do not share any locations worldwide. *H. opuntia* samples collected from Southern Vietnam seem to form a smaller group based on the phylogenetic tree and the network. Recently, Rindi et al., [31] indicated that populations of *H. tuna* in the Adriatic/Ionian region shared a haplotype unique to this region and formed a group separated from all western Mediterranean regions. In this present study, *H. opuntia* was collected from two locations. Therefore, more samples from the Spratly islands, Northern Vietnam, and the Gulf of Thailand should be collected. Another plastid marker *rpl*2-*rpl*14 region, should be applied.

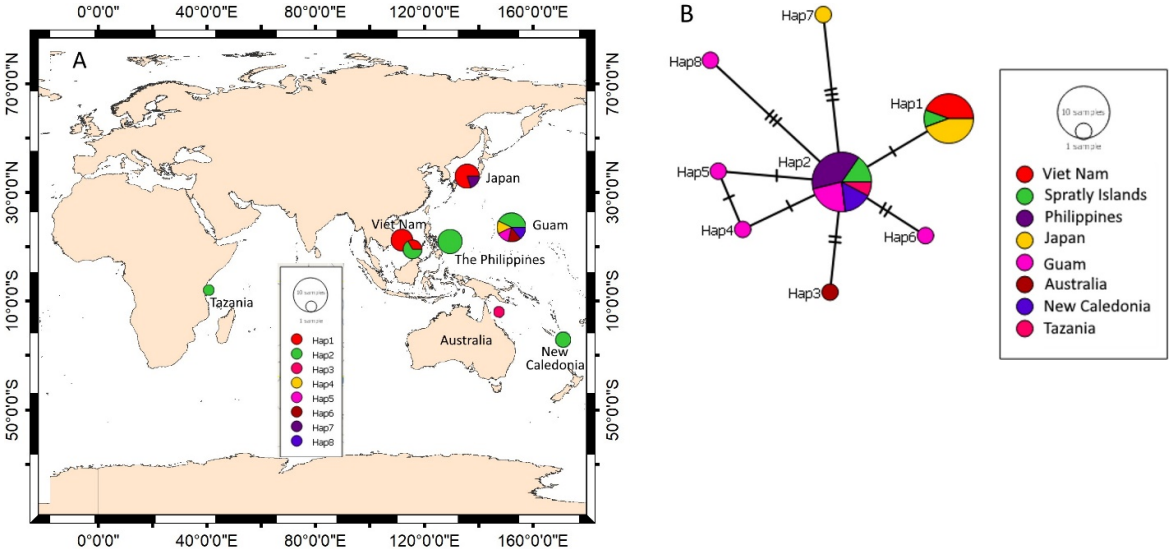

Figure 6. Distribution of haplotype frequency of *H. macroloba*. Eight haplotypes are defined by different colors (panel A); and its network, each short segment in the distance between two haplotypes is a single mutation (panel B)

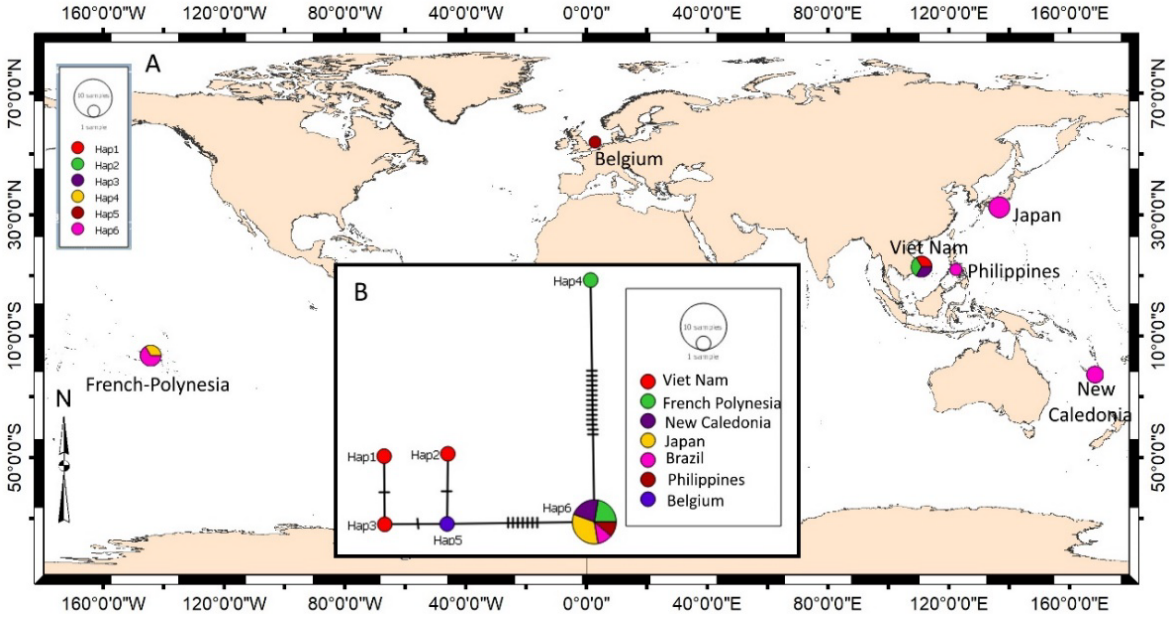

Figure 7. Distribution of haplotype frequency of *H. opuntia*. Six haplotypes are defined by different colors (panel A); and its network, each short segment in the distance between two haplotypes is a single mutation (panel B)

CONCLUSION

This report is the first on the combination of morphological observation and genetic marker of *Halimeda* in Vietnam. Both *H. macroloba* and *H. opuntia* showed high morphological variations among populations. The genetic variation in *H. macroloba* is very low, and *H. opuntia* from Vietnam tends to form a distinct smaller group.

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Appendix 1. GenBank accession/Herbarium voucher specimens numbers of the sequences used in the alignment. Bold in face: sample collected in Vietnam; -: as above

No.	Species	Voucher specimens/GenBank	Locations	Coordinates	
				Longitude (^{o}E)	Latitude (^{o}N)
1	*Halimeda borneensis*	AM049955	Polynesia		
2	*H. copiosa*	EF667065	Jamaica		
3	*H. cryptica*	EF667056	Jamaica		
4	*H. cuneata*	FJ624538	Australia		
5	*H. cylindracea*	AM049956	Yemen		
6	*H. discoidea*	AY826360	-		
7	*H. distorta*	FJ624649	Philippines		
8	*H. gigas*	JN644674	Maldives		
9	*H. goreauii*	FJ624659	Panama		
10	*H. gracilis*	AM049965	Polynesia		
11	*H. heteromorpha*	FJ624535	Philippines		
12	*H. hummii*	*AM049988*	Panama		
13	*H. incrassata*	FJ624534	British Virgin islands		
14	*H. kanaloana*	JN644684	Caledonia		
15	*H. lacrimosa*	EF667064	Bahamas		
16	*H. lacunalis*	JN644687	New Caledonia		
17	*H. macroloba*	AB899310	Japan		
18	-	AB899309	-		
19	-	AB899308	-		
20	-	AM049960	Tazania		
21	-	JN644690	New Caledonia		
22	-	JN644693	-		
23	-	HM140244	Australia		
24	-	MN879375	Truong Sa, Vietnam		
25	-	**ION21146TS**	**Truong Sa, Vietnam**	**114.22320**	**10.10550**
26	-	**ION21145PQy**	**Phu Quy, Vietnam**	**108.95426**	**10.55250**
27	-	**ION21142NTh**	**Ninh Thuan, Vietnam**	**109.17560**	**11.65868**
28	-	**ION21141NT**	**Nha Trang, Vietnam**	**109.25467**	**12.22152**
29	-	**ION21144CD**	**Con Dao, Vietnam**	**106.61980**	**08.68864**
30	*H. macrophysa*	JN644694	New Caledonia		
31	*H. magnidisca*	AY826364	Guam		
32	*H. minima*	FJ624665	Tanzania		
33	-	JN644702	Maldives		
34	*H. micronesica*	JN644696	Maldives		
35	*H. opuntia*	JN644710	French Polynesia		
36	-	JN644711	-		
37	-	JN644712	-		
38	-	AM049967	-		
39	-	FJ624689	Philippines		
40	-	LT969815	Belgium		
41	-	JN644713	New Caledonia		
42	-	KT781885	Brazil		
43	-	AB899314	Japan		
44	-	AB899315	-		
45	-	KU361895	-		
46	-	**ION21154LS**	**Ly Son, Vietnam**	**109.11692**	**15.39425**
47	-	**ION21156A**	**Ninh Thuan, Vietnam**	**109.17560**	**11.65868**

No.	Species	Voucher specimens/GenBank	Locations	Coordinates	
				Longitude (^{o}E)	Latitude (^{o}N)
48	**-**	**ION21156C**	**-**	**108.83871**	**11.32345**
49	*H. pygmaea*	EF667062	Fiji		
50	*H. renschii*	FJ624691	Tanzania		
51	*H. scabra*	FJ624540	Bahamas		
52	*H. simulans*	AM049963	Jamaica		
53	*H. taenicola*	AY826365	French Polynesia		
54	*H. tuna*	AY826367	Mexico		
55	*H. xishaensis*	JN644727	Maldives		