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Selection of suitable fragment from *rbc*L gene for DNA barcode analysis of family Halymeniaceae, Rhodophyta

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Abstract
Among the members of Halymeniaceae family, *Grateloupia sensu lato* occupies the largest composition in species. Classification based on morphological traits is difficult due to the highly variable terete to blade-like thalli among the members of this genus that usually leads to misidentification. Molecular systematics has been applied to classify *Grateloupia sensu lato* so that the taxonomists acquire a better understanding of the species diversity in general. The plastid gene encoding the large subunit of ribulose-1,5-bisphosphate-carboxylase-oxygenase (*rbc*L) was the focus of numerous marine algal studies concerning phylogeny and molecular evolution. However, using the full length of *rbc*L showed disadvantages such as cost and time consuming due to two times of sequencing and two times of PCR. In the present study, the shorter sequence, fragment 773 bp at 5' end and fragment 579 bp at 3' end of *rbc*L were applied and compared for the phylogenetic analysis of Halymeniaceae members. The results indicated there are no differences of topological phylogenetic trees, species resolution within genus and genus resolution within the family between fragment 773 bp at 5' and the full length of *rbc*L. Therefore, we conclude that fragment 773 bp at 5' should be used as DNA barcodes for the Halymeniaceae to reduce the cost and time during phylogenetic analysis. Two taxa *Grateloupia* newly collected in Vietnam were grouped to the known *Phyllymenia*, a new genus in Vietnam.

Keywords: DNA barcodes, fragments, Halymeniaceae, *Phyllymenia*, *rbc*L.

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INTRODUCTION

Halymeniaceae was considered as the highest species diversity family in Rhodophyta with 343 species which belong to 37 genera. *Grateloupia senso lato* shows the largest number in species (97 species) [1]. However, members of *Grateloupia* and closely related genera show highly diverse morphological traits, and it is one of the genera that present a difficult species classification. Therefore, it leads to misidentification among species within the genus and different genera [2]. Based on reproductive anatomy and postfertilization development of cystocarp, Gargiulo et al. [3] indicated that genus *Grateloupia* should be segregated into multiple genera including *Dermocorynus* P. L. Crouan et H. M. Crouan, *Pachymeniopsis* Y. Yamada *ex* S. Kawabata, *Phyllymenia* J. Agardh and *Prionitis* J. Agardh, all of which have been subsumed in *Grateloupia* by previous authors. In the recent studies of taxonomy based on detailed morphological observations, *Grateloupia senso lato* was segregated into eight genus including *Neorubra* M. S. Calderon, G. H. Boo et S. M. Boo; *Phyllymenia* [4]; *Prionitis*; *Pachymeniopsis*; *Grateloupia* C. Agardh; *Mariramirezia* M. S. Calderon, G. H. Boo, A. Mansilla et S. M. Boo [5]; *Yonagunia* (Okamua) Kawaguchi et Masuda and *Dermocarpus*.

Molecular systematics has been applied to classify marine plants so that the taxonomists acquire a better understanding of the species diversity in general. The plastid gene encoding PSII thylakoid protein D1 (*psb*A) was the focus of numerous brown algal studies concerning phylogeny and molecular evolution [6, 7] whereas, elongation factor Tu gene (*tuf*A) and the large subunit of ribulose-1,5-bisphosphate-carboxylase-oxygenase (*rbc*L) were used as DNA barcodes for green and red algae, respectively [8, 9]. In contrast, the nuclear internal transcribed spacer (ITS) region including the 5.8S sequence was applied to the molecular systematic of seagrass [10], mangroves [11] and phytoplankton [12]. Nowadays, molecular systematics and detailed morphological observations are two main tools for taxonomic studies.

Recently, molecular systematics was applied to study the taxonomy of various marine macrophytes in Vietnam such as seagrass [10, 13], mangroves [14]. Based on phylogenetic analysis of *rbc*L gene, Nguyen et al. [13] indicated that the red *Grateloupia taiwanensis* S. M. Lin et H. Y. Liang, the common species in Taiwan and USA was also found at Da Nang, Vietnam. The rare brown alga *Dictyota hauckiana* Nizamuddin was also recorded in Vietnam for the first time based on the concatenated *psb*A and *rbc*L genes [15]. Le et al., [16] published the new description of *Gracilaria phuquocensis* N. H. Le, N., Muangmai et G.C. Zuccarello with validation of *rbc*L gene. Therefore, DNA barcoding is an indispensable tool in term of classification of marine algae. DNA barcoding is an approach to identify and recognize species by using short orthologous DNA sequences, known as "DNA barcodes". The criteria for the development of reliable barcode data are that candidate loci should be suitable for a wide range of taxa, show a high variation between species, but should be conserved within species, so that the intra-specific variation will be insignificant [17]. It is well-known that the full length of *rbc*L was normally used for the phylogenetic analysis of the Halymeniaceae family, Rhodophyta. However, the disadvantages of the full length of *rbc*L (1,257 bp) approach were: (i) using three (Wang et al. [9]) or two (Lin et al., [18]) primer pairs for PCR of *rbc*L, (ii) costly and time consuming due to sequencing cost and two/three times of PCR and (iii) forming long concatenated sequences that increase and prolong steps in the bioinformatic analysis. This led to the hypothesis that phylogenetic analysis based on a short sequence (< 1,000 bp) of *rbc*L would resolve the taxonomy among members of Halymeniaceae, Rhodophyta instead of using the full length of *rbc*L (1,257 bp).

MATERIALS AND METHODS

Sample collection

The algal samples were collected at Da Nang City (16^{o}08'N; 108^{o}07') and Nha Trang City (12^{o}15'N; 109^{o}15'), Vietnam (fig. 1) in February 2019. Snorkelling was used to collect

the samples in the shallow water (3–5 m). Algal materials were washed with seawater in the field to remove the epiphytes and debris that were commonly attached to the algae. Each specimen was placed in a single plastic bag and kept on ice. Materials were transferred to the laboratory within one or two days. In the laboratory, materials were re-washed with de-ionized water to remove seawater. One specimen was divided into three parts, one part was pressed as a herbarium voucher specimen (G04-06DN; G40-42NT) deposited in the Museum of Oceanography, Nha Trang City, Vietnam, another part was fixed in formalin 7% for morphological observation later, and the small blades of herbarium voucher specimen were used for DNA extraction. Information of the samples is presented in Appendix 1.

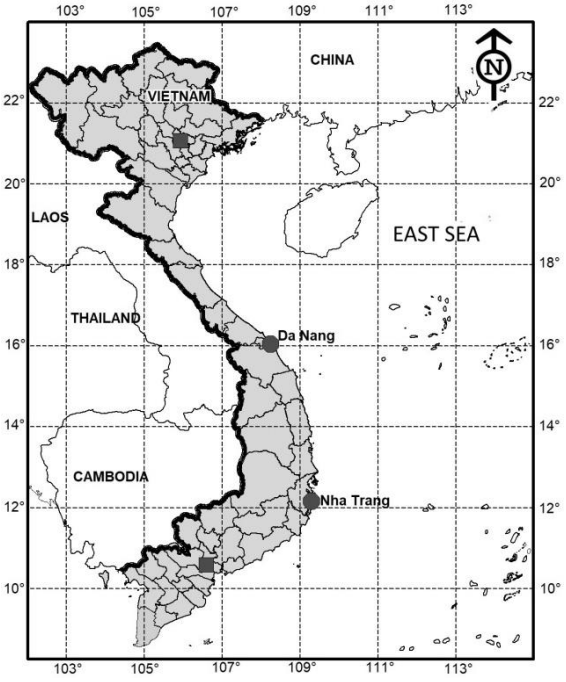

Fig. 1. The map of Vietnam and sampling sites (the black solid rounds). The map was processed by MapInfor Pro^{TM}, version 12.5.5 (Pitney Bowes Software Inc., NY, USA)

DNA extraction, polymerase chain reaction (PCR) and sequencing

The dried materials were rehydrated in sterile water for one hour. The materials were homogenized by a mortar and pestle in liquid nitrogen, and 100 mg of the finely powdered algal material was used for DNA extraction. The DNA extraction was carried out using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. DNA quality was checked on agarose gels stained with Midori Green Advance (Nippon Genetics Europe GmbH, Düren) and the concentration was measured by a spectrophotometer U-2900 (Hitachi, Tokyo, Japan). The primer pairs of F7 (5'-AACTCTGTAGAACGNACAAG-3') [19] and R898 (5'-GACGAGAATAAGTTGARTTAC C-3') [20], and the primer pairs of F762 (5'-GTATGAAAGAGCTGAATTTG-3') [20] and R1381 (5'-ATCTTTCCATAGATCTAAAGC-3') [21] were used to amplify the fragment of 773 bp at 5' end (Fragment 773 bp-F773) and 597 bp at 3' end (Fragment 597 bp-F597), respectively. Full length of *rbc*L (F1257) is a combination of F773 and F597. The PCR compositions and PCR conditions were followed in our previous study [13]. Two fragments were achieved from two independent PCR. All PCR reactions were repeated two to four times independently with the same individual to keep errors (possibly created by the *Taq* polymerase) in the final consensus sequence to a minimum. PCR products were cleaned using a GenEluteTM PCR Clean-Up kit (Sigma Aldrich, St. Louis, MI, USA) following the manufacturer's instruction. Direct sequencing of PCR product was done by 1ST BASE (Selangor, Malaysia) from both directions. The consensus sequence was achieved by Clone Manager 9 (Sci-Ed, Cary, NC, USA). For comparison, known *rbc*L sequences of members of Halymeniaceae were added to the dataset (Appendix 1).

Bioinformatics analysis

Six F773 sequences and six F597 sequences from three different taxa (from this study) and 62 *rbc*L sequences of Halymeniaceae were retrieved from the GenBank (Appendix 1). Three datasets (F773, F579 and F1257) were independently analyzed. For each dataset, 68 sequences were aligned by CLUSTAL W using MEGA X [21], and the alignment was further modified by eye. Gaps were considered as missing data. Identical

sequences within each species were excluded from the alignment. jModelTest [22] and the corrected AIC were 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+G model) performed in MrBayes v.3.2.2 [23]. In the BI, the two parallel runs with four chains each (three heated and one cold) were performed for 1 million generations, sampling a tree every 100 generations. Only trees sampled after convergence were used to make inferences about the phylogeny and to compute a 50% majority-rule consensus tree. In the analyses, trees were tested by the bootstrapping method with 1,000 replications. The consensus tree based on two different trees (achieved from the two methods) was constructed by Dendro Scope software, version 3.2.10 [24]. Comparisons of species boundaries among species within a genus, among genera within the family between two phylogenetic trees (F773 vs F1257; F579 vs F1257) were also performed by the tanglegram option in the Dendro Scope software.

RESULTS AND DISCUSSION

Phylogenetic analysis based on F1257, F773 and F579 fragments

Results of the phylogenetic analyses (Maximum Likelihood and Bayesian Inference) based on F1257 showed that all sequences were distributed into 18 main clades. *Grateloupia sensu lato* was segregated into nine clades consisting of *Phyllymenia* (I), *Neorubra* (II), *Pachymeniopsis* (III), *Prionitis* (IV), *Grateloupia stipitata* group (V), *Democorynus* (VI), *Grateloupia carnosa* (VII), *Grateloupia* (VIII) and *Mariramirezia* (IX). The unknown *Grateloupia* sp1 (G04DN-G06DN) specimen is sister species to *Phyllymenia huangiae* S. M. Lin et H. Y. Liang and *Grateloupia* sp2 (G40NT-G41NT) is sister species to *Phyllymenia proteus* Kützing. Specimen of *Grateloupia ramosissima* Okamura collected at Nha Trang was grouped to known *G. ramosissima*. The bootstrap values and posterior probability are very high (> 80% and 1.0, respectively) (fig. 2). So far, *Phyllymenia* was based on a single species, *Phyllymenia hieroglyphica* J. Agardh (1848), described from South Africa [25]. Several studies later placed *Phyllymenia* in different names such as *Iridaea* Bory., *Cryptymenia*, Schmitz, *Pachymenia*, *Grateloupia* [2]. Lin et al. [18, 26] suggested that *Grateloupia taiwanensis* and *G. huangiae* could be treated as *Phyllymenia* members due to similarities of cystocarp development. Based on morphological observation of vegetative and reproductive structures as well as phylogenetic analysis of the large subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase (*rbc*L) sequence, Calderon et al. [4] suggested that *Grateloupia taiwanensis*, *G. huangiae*, *G. phuquocensis* Tanaka et Pham-Hoang, *G. sparsa* (Okamura) Chiang, *G. turuturu* Yamada, *G. subpectinata* Holmes, *G. proteus* and *G. capensis* O. De Clerck are members of *Phyllymenia*.

Results of the phylogenetic analyses (Maximum Likelihood and Bayesian Inference) based on F773 indicated that there is no difference of topology from the F1257. Briefly, *Grateloupia sensu lato* was also segregated into nine clades consisting of *Phyllymenia* (I), *Neorubra* (II), *Pachymeniopsis* (III), *Prionitis* (IV), *Grateloupia stipitata* group (V), *Democorynus* (VI), *Grateloupia carnosa* (VII), *Grateloupia* (VIII) and *Mariramirezia* (IX). *Grateloupia ramosissima* collected at Nha Trang was grouped to known *Grateloupia ramosissima*, whereas *Grateloupia* sp1 and *Grateloupia* sp2 are sister species of *Phyllymenia huangiae* and *P. proteus*, respectively. The bootstrap values and posterior probability at the node *Grateloupia* sp1/ *Phyllymenia huangiae* are very high (100% and 1.0, respectively) whereas bootstrap values and posterior probability at the node *Grateloupia* sp2/ *P. proteus* are lower (< 50% and 0.75, respectively) (fig. 3).

For the result of the phylogenetic analyses based on F579, *Grateloupia sensu lato* was segregated into eight clades instead of nine clades. *Grateloupia stipitata* group and *Democorynus* formed a distinct clade, whereas the six remaining clades formed six distinct genera. *Grateloupia* sp1 and *Phyllymenia*

huangiae are sister species. However, *Grateloupia* sp2 is sister species with *Phyllymenia belangeri* instead of sister species of *P. proteus* like phylogenetic trees based on F1257 and F773. *Grateloupia ramosissima* collected at Nha Trang was also grouped to known *Grateloupia ramosissima* (fig. 4).

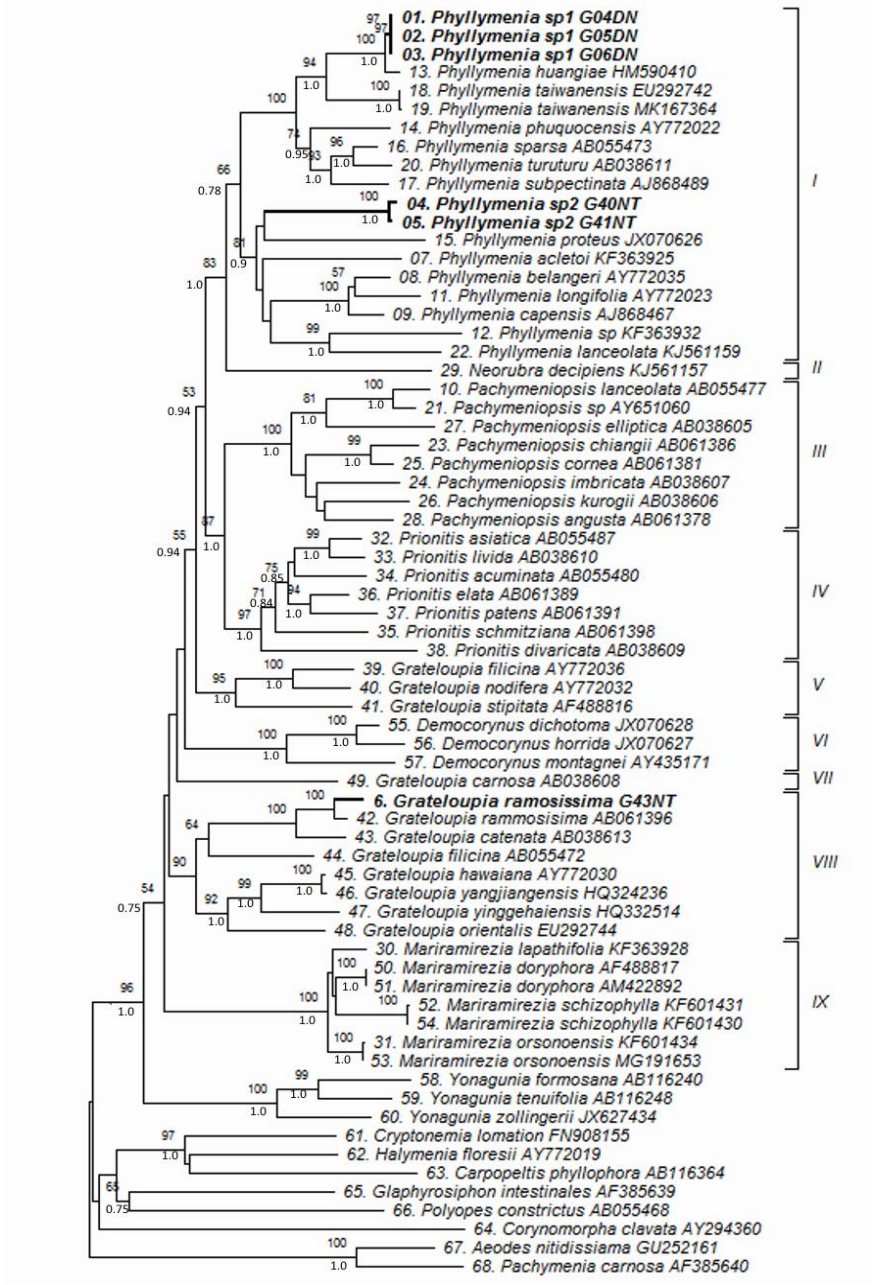

Fig. 2. Phylogeny of members of Halymeniaceae inferred from Bayesian Inference, Maximum Likelihood. The dataset is based on 1257 bp of *rbc*L. The posterior probability and bootstrap values of each method are shown in each node. **Bold**, samples collected at Vietnam. The consensus tree was constructed by Dendro Scope software. See Appendix 1 for the number in front of each taxon

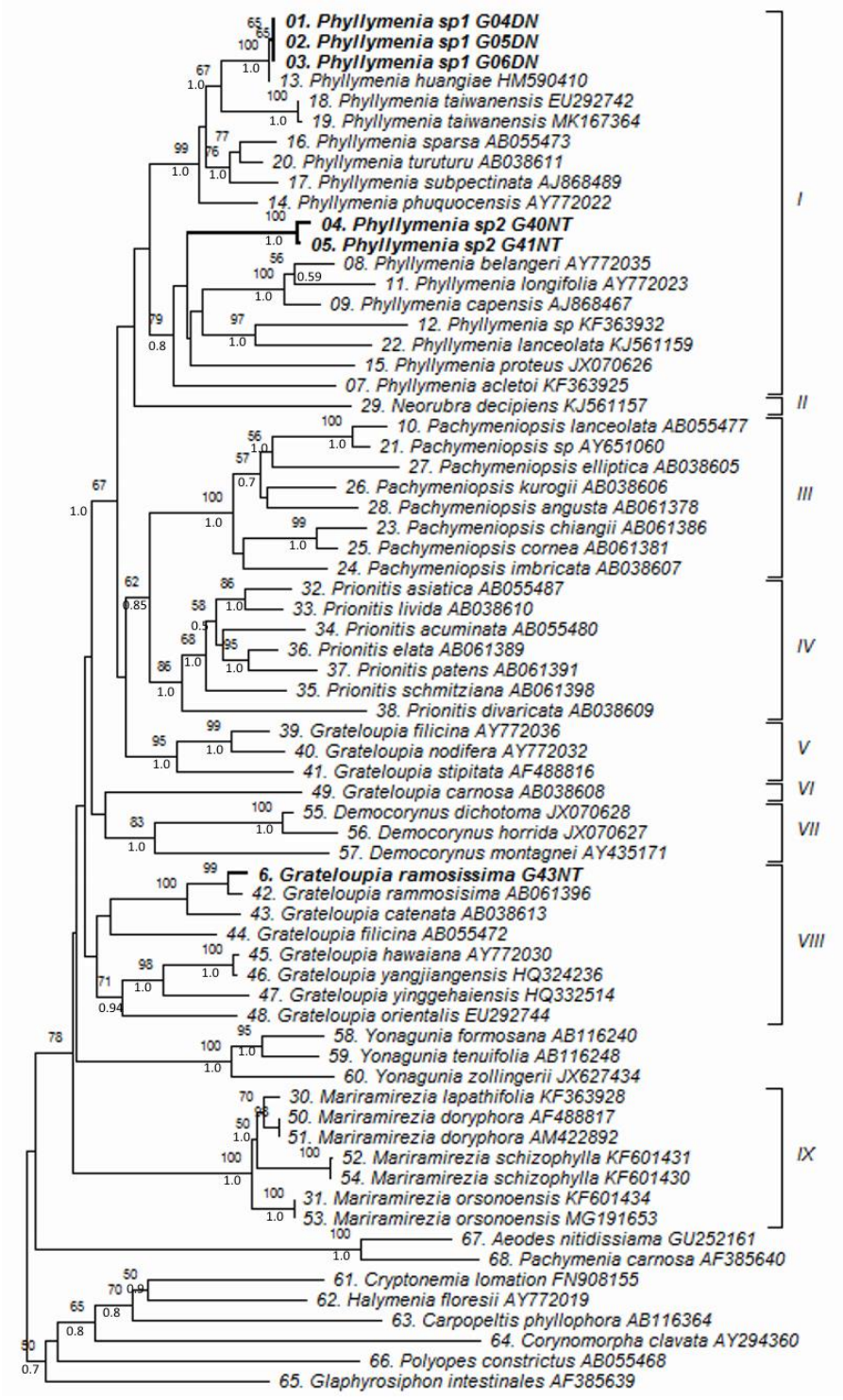

Fig. 3. Phylogeny of members of Halymeniaceae inferred from Bayesian Inference, Maximum Likelihood. The dataset is based on 773 bp of *rbc*L. The posterior probability and bootstrap values of each method are shown in each node. **Bold**, samples collected at Vietnam. The consensus tree was constructed by Dendro Scope software. See Appendix 1 for the number in front of each taxon

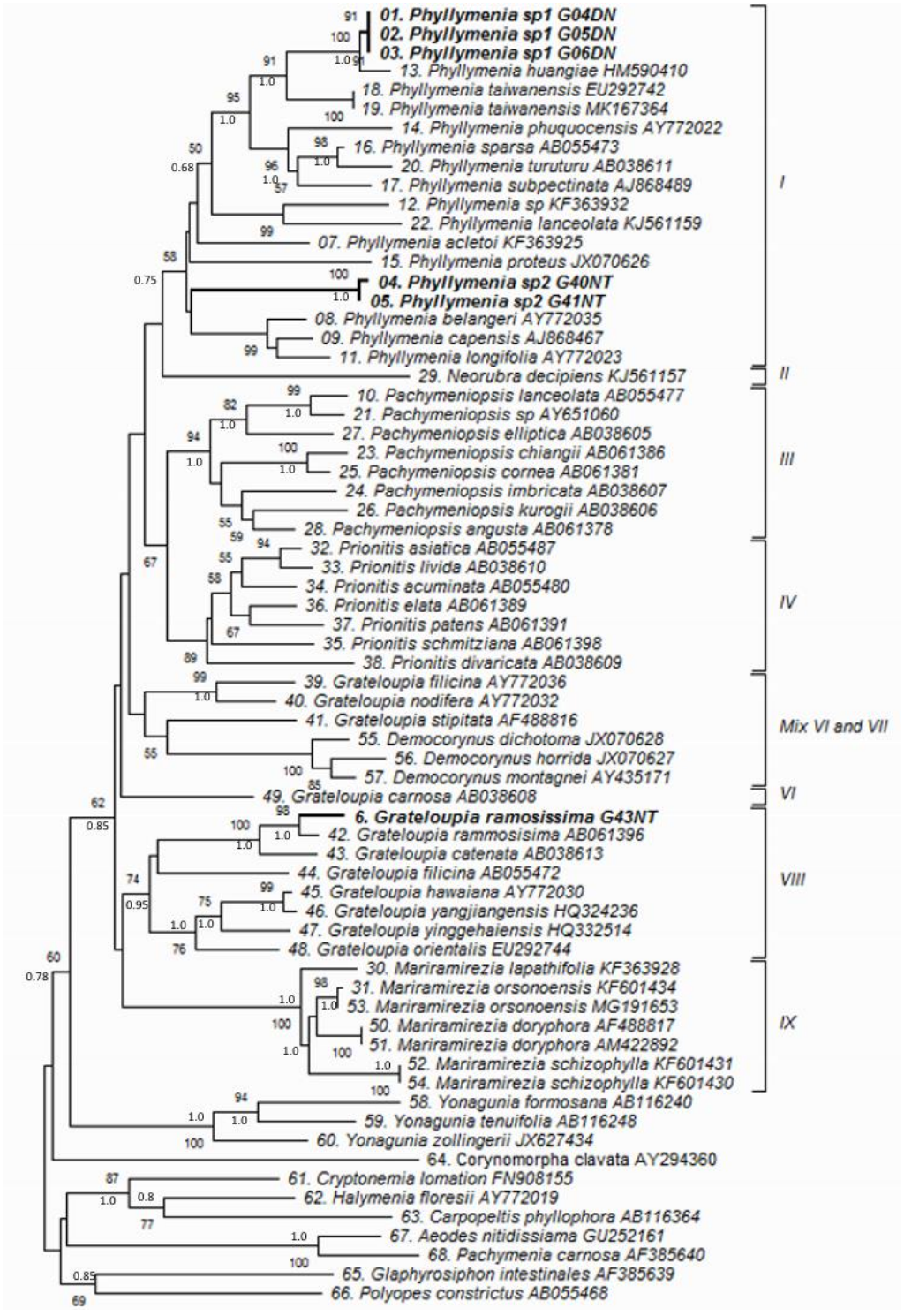

Fig. 4. Phylogeny of members of Halymeniaceae inferred from Bayesian Inference, Maximum Likelihood. The dataset is based on 579 bp of *rbc*L. The posterior probability and bootstrap values of each method are shown in each node. **Bold**, samples collected at Vietnam. The consensus tree was constructed by Dendro Scope software. See Appendix 1 for the number in front of each taxon

Comparison of species resolution between F1257 and F773, between F1257 and F579

The results of tanglegram phylogenetic tree indicated that there is no difference of topology of phylogenetic trees based on F1257 and F773. All species in this family are resolved in both F1257 (Panel A) and F773 (Panel B). Notably, the boundaries among genus based on F773 is very clear, it is similar to the phylogenetic tree based on F1257. Wang et al. [9] used three primer pairs to apply the full length of *rbc*L sequence. In the same way, the later studies used two different primer pairs to amplify the full length of *rbc*L sequence [13, 28]. That leads to cost and time consuming due to two times of sequencing and two times of PCR.

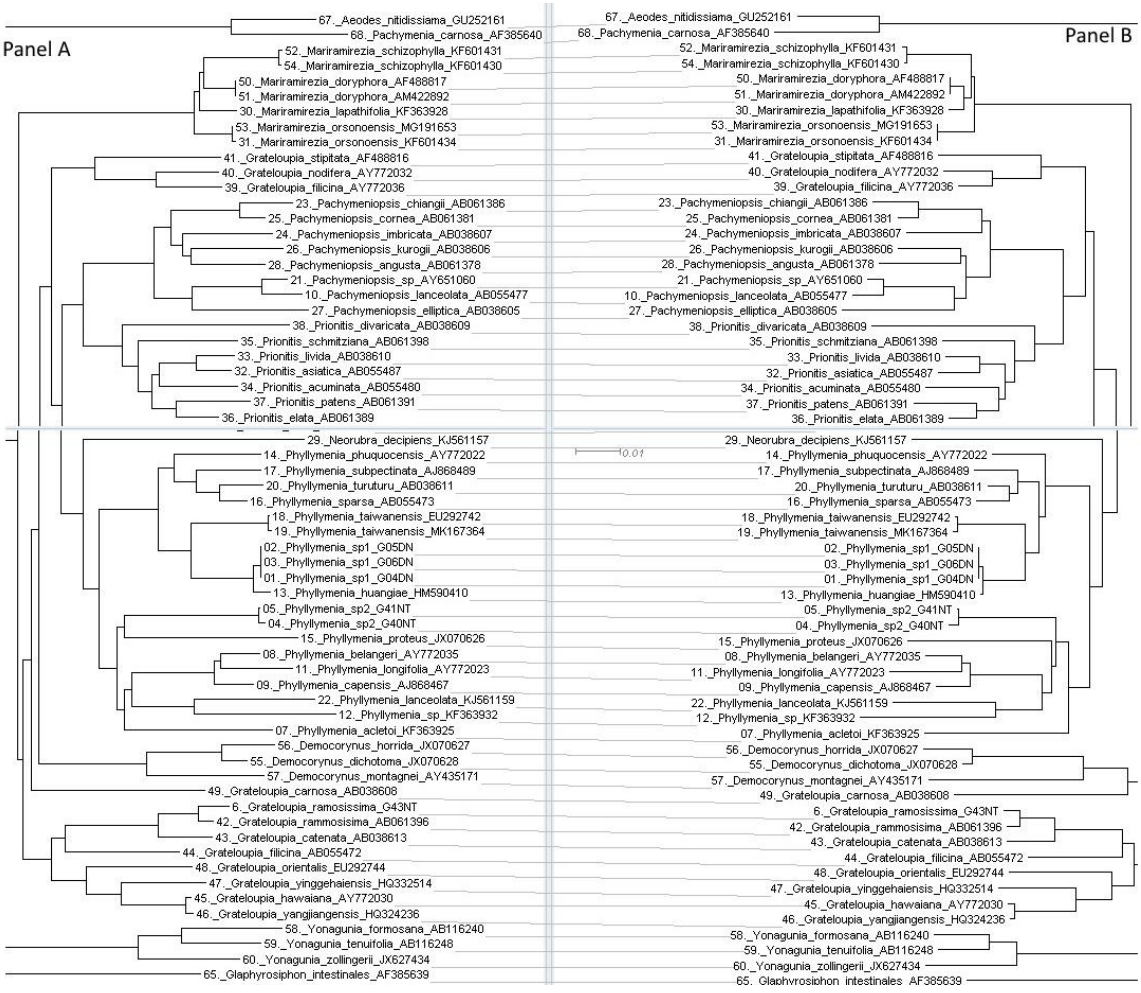

Fig. 5. Tanglegram of phylogenetic trees based on different fragments of *rbc*L. Panel A is phylogenetic tree based on 1,257 bp (including gaps). Panel B is phylogenetic tree based on 773 bp (including gaps) of *rbc*L. See Appendix 1 for the number in front of each taxon. See figures 3 and figures 4 for bootstrap values and posterior probability. The tanglegram phylogenetic tree was constructed by tanglegram method in Dendro Scope software

Comparison of phylogenetic trees based on F1257 and F579 indicated that there are two disadvantages of phylogenetic tree based on F579. The disadvantages are: (i) *Grateloupia stipitata* was grouped to members of genus *Democorynus* (Panel B, fig. 6) instead of being grouped to distinct *Grateloupia stipitata* group (Panel A, fig. 6); (ii) the bootstrap values and

posterior probability were much lower than F1257 although the species of two markers F1257 and F579 are the same. The main information, advantages and disadvantages among F1257, F773 and F579 were presented in table 1.

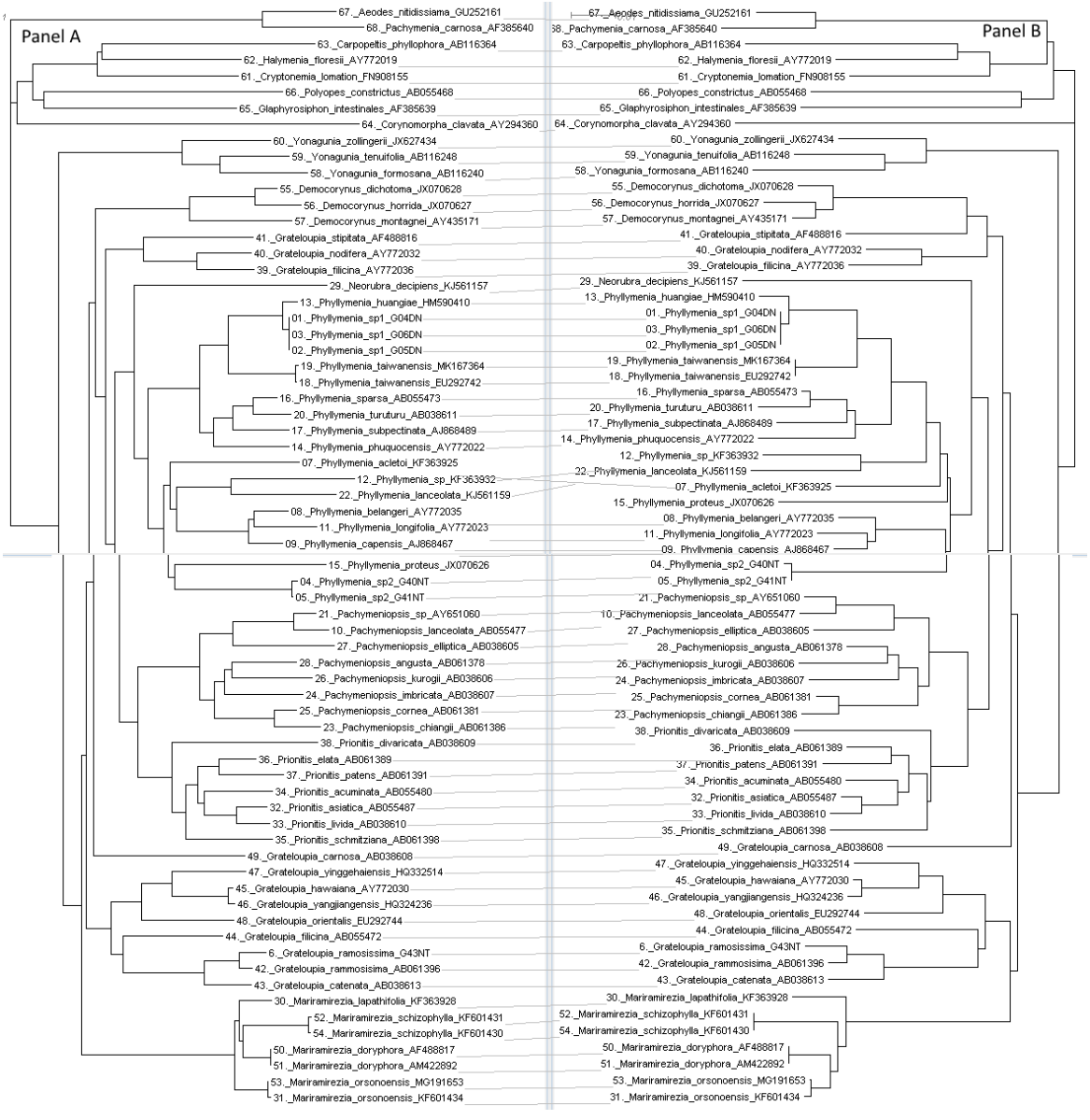

Fig. 6. Tanglegram of phylogenetic trees based on different fragments of *rbc*L. Panel A is phylogenetic tree based on 1,257 bp (including gaps). Panel B is phylogenetic tree based on 579 bp (including gaps) of *rbc*L. See Appendix 1 for the number in front of each taxon. See fig. 3 and 5 for bootstrap values and posterior probability The tanglegram phylogenetic tree was constructed by tanglegram method in Dendro Scope software

For other macrophytes like seagrass and mangroves, Nguyen et al. [10, 11] found that rDNA (ITS1-5.8S-ITS2) with the length of 699 bp had more advantages in species resolution than longer length of the concatenated *rbc*L and *mat*K (1,470 bp). Therefore, ITS could be

applied as a DNA barcode for seagrass and mangroves instead of the *rbc*L/*mat*K system previously proposed. Among three markers, F773 seems to be the best selection because it overcomes the disadvantages of both F579 and F1250. The cost and time consuming and species resolution are similar to F579, but the boundary of the genus is clearer than F579. Compared to F1257, the results of phylogenetic analysis are the same between F773 and F1257, but the cost to carry out the experiments of F1257 is two times higher than F773 due to two times of PCR and sequencing to achieve the length of 1.257 bp. The primers used for F773 can be applied to order family Gigartinales (personal information). Using a single primer pair from this study may fix the criteria of DNA barcoding [17]. The development of reliable barcode data is that candidate loci should be suitable for a wide range of taxa, show a high variation between species, but should be conserved within species, so that the intra-specific variation will be insignificant.

Table 1. Main information, advantages and disadvantages among three fragments: F1257, F773 and F579. **Bold:** Important information

	Markers		
Name	F773	F579	F1257
Length (bp)	773	597	1,257
Conservation site (%)	62.1	61.1	61.9
Variable sites (%)	37.9	38.9	38.1
Parsimony informative characters (%)	31.7	32.8	32.1
Singleton sites (%)	6.2	6.0	6.0
Genus resolution (%)	100	77	100
Species resolution (%)	100	100	100
Bootstrap values (%)	58–67	< 62	53–96
Posterior probability	> 0.5	< 0.5	> 0.5
Advantages	-Full species resolution -Full genus resolution -Low cost and time consuming -High bootstrap values and posterior probability	-Full species resolution -Low cost and time consuming	-Full species resolution -Full genus resolution -High bootstrap values and posterior probability
Disadvantages		-Not full genus resolution	-High cost and time consuming

CONCLUSION

The results and discussion presented above prove that i) F773 should be used as DNA barcodes for Halymeniaceae instead of the full length of *rbc*L to reduce cost and time consuming. ii) Fragment F571 should not be used as DNA barcodes for Halymeniaceae, and iii) Specimens of putative *Grateloupia* sp1 collected at Da Nang and *Grateloupia* sp2 collected at Nha Trang should be treated as *Phyllymenia* sp1 and *Phyllymenia* sp2, respectively. Our next studies will focus on description of these new records based on the development of cystocarps of *Phyllymenia* spp. found from this study.

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Appendix 1. List of the species included in the molecular analysis done in this study

No.	Taxa	Locations	Voucher specimens/GB number
1	*Phyllymenia sp1.*	Da Nang - Vietnam	G04DN
2	*Phyllymenia sp1.*	Da Nang - Vietnam	G05DN
3	*Phyllymenia sp1.*	Da Nang - Vietnam	G06DN
4	*Phyllymenia sp2.*	Nha Trang - Vietnam	G40NT
5	*Phyllymenia sp2.*	Nha Trang - Vietnam	G41NT
6	*Grateloupia ramosissima*	Nha Trang - Vietnam	G43NT
7	*Phyllymenia acletoi*	Peru	KF363925
8	*Phyllymenia belangeri*	South Africa	AY772035
9	*Phyllymenia capensis*	South Africa	AJ868467
10	*Pachymeniopsis lanceolata*	Japan	AB055477
11	*Phyllymenia longifolia*	South Africa	AY772023
12	*Phyllymenia sp*	Chile	KF363932
13	*Phyllymenia huangiae*	Taiwan	HM590410
14	*Phyllymenia phuquocensis*	Hawai'i	AY772022

15	*Phyllymenia proteus*	Italia	JX070626
16	*Phyllymenia sparsa*	Japan	AB055473
17	*Phyllymenia subpectinata*	Australia	AJ868489
18	*Phyllymenia taiwanensis*	Taiwan	EU292742
19	*Phyllymenia taiwanensis*	Da Nang - Vietnam	MK167364
20	*Phyllymenia turuturu*	Japan	AB038611
21	*Pachymeniopsis sp*	Italy	AY651060
22	*Phyllymenia lanceolata*	Chile	KJ561159
23	*Pachymeniopsis chiangii*	China	AB061386
24	*Pachymeniopsis imbricata*	Japan	AB038607
25	*Pachymeniopsis cornea*	China	AB061381
26	*Pachymeniopsis kurogii*	Japan	AB038606
27	*Pachymeniopsis elliptica*	Japan	AB038605
28	*Pachymeniopsis angusta*	Japan	AB061378
29	*Neorubra decipiens*	Peru	KJ561157
30	*Mariramirezia lapathifolia*	Chile	KF363928
31	*Mariramirezia orsonoensis*	Chile	KF601434
32	*Prionitis asiatica*	Japan	AB055487
33	*Prionitis livida*	Japan	AB038610
34	*Prionitis acuminata*	Japan	SAP 088107
35	*Prionitis schmitziana*	China	AB061398
36	*Prionitis elata*	China	AB061389
37	*Prionitis patens*	China	AB061391
38	*Prionitis divaricata*	Japan	AB038609
39	*Grateloupia filicina*	South Africa	AY772036
40	*Grateloupia nodifera*	South Africa	AY772032
41	*Grateloupia stipitata*	Peru	AF488816
42	*Grateloupia ramosissima*	China	AB061396
43	*Grateloupia catenata*	Japan	AB038613
44	*Grateloupia filicina*	China	AB055472
45	*Grateloupia hawaiana*	Hawaii	AY772030
46	*Grateloupia yangjiangensis*	China	HQ324236
47	*Grateloupia yinggehaiensis*	China	HQ332514
48	*Grateloupia orientalis*	Taiwan	EU292744
49	*Grateloupia carnosa*	Japan	AB038608
50	*Mariramirezia doryphora*	Peru	AF488817
51	*Mariramirezia doryphora*	Iberian	AM422892
52	*Mariramirezia schizophylla*	Peru	KF601431
53	*Mariramirezia orsonoensis*	Chile	MG191653
54	*Mariramirezia schizophylla*	Peru	KF601430
55	*Democorynus dichotoma*	Italy	JX070628
56	*Democorynus horrida*	Italy	JX070627
57	*Democorynus montagnei*	Ireland	AY435171
58	*Yonagunia formosana*	Vietnam	AB116240
59	*Yonagunia tenuifolia*	Japan	AB116248
60	*Yonagunia zollingerii*	Indonesia	JX627434
61	*Cryptonemia lomation*	France	FN908155
62	*Halymenia floresii*	Spain	AY772019
63	*Carpopeltis phyllophora*	Australia	AB116364
64	*Corynomorpha clavata*	Mexico	AY294360
65	*Glaphyrosiphon intestinales*	South Africa	AF385639
66	*Polyopes constrictus*	Japan	AB055468
67	*Aeodes nitidissiama*	New Zealand	GU252161
68	*Pachymenia carnosa*	South Africa	AF385640