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Research Article

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Phylogeography of the marine benthic alga *Gracilaria salicornia* (Gracilariales, Rhodophyta) in Southeast Asia

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Abstract: Recent and future coastal habitat degradation and climate change can result in the loss of genetic diversity, especially in Southeast Asia. Despite its economic importance for the agar industry, the phylogeography of the intertidal red alga *Gracilaria salicornia* from Vietnam remains unexplored. Here we used 399 mitochondrial COI sequences, including 55 newly generated from Vietnam, to decipher the phylogeographic pattern of *G. salicornia*. The COI phylogeny and haplotype network revealed the presence of three haplogroups in *G. salicornia*. Haplogroup I, comprising 18 haplotypes, predominantly inhabited the west of the South China Sea, while haplogroup II, consisting of eight haplotypes from Japan and the Philippines, likely reflecting the influence of the Kuroshio Current. A single haplotype from the Philippines was placed in haplogroup III. *G. salicornia* exhibited high genetic diversity, with neutrality test, mismatch distribution, and Bayesian skyline plot suggesting a sudden demographic or range expansion during the mid-Pleistocene. The divergence of haplogroups I and II, occupying the eastern and western sides of the South China Sea, likely resulted from sea currents and glacial sea-level changes. Our study on historical phylogeography offers valuable insights that will encourage further research into the population history of tropical red algae in Southeast Asia, a region for its remarkable marine biodiversity.

Keywords: agarophyte; COI-5P; population genetics; Pleistocene expansion; Vietnam

1 Introduction

Southeast Asia is one of the most important centers of marine biodiversity and species distribution (Ludt and Rocha 2015). Complex geography and oceanography have contributed to the various patterns of species diversity and distributions (Gallagher et al. 2024). Historical phylogeography assumes a central role in assessing current distribution patterns by analyzing the genetic relationships between different populations (Grant 2015). Surface seawater currents have influenced genetic connectivity between distant population, while geographical barriers, sea-level fluctuations, and other factors have resulted in genetic fragmentation of population (Ludt and Rocha 2015).

Previous studies on Southeast Asian seaweeds have highlighted various patterns of population structure and phylogeography, as mentioned below. Their distribution patterns have been influenced by various driving factors; sea-level rise (Kantachumpoo et al. 2014), geographical barrier (Bulan et al. 2022; Muangmai et al. 2023; Wichachucherd et al. 2014), and regional sea circulation or ocean currents (Bulan et al. 2022; Fontana et al. 2024; Hu et al. 2017; Muangmai et al. 2023). Historical phylogeographic studies revealed post-glacial dispersals or mid-Pliocene divergence (Chan et al. 2013, 2014; Fontana et al. 2024; Hu et al. 2017).

The intertidal red alga *Gracilaria salicornia* (C.Agardh) E.Y.Dawson (Gracilariales) is a good example for the historical phylogeography because it's common in Southeast Asia and mitochondrial COI-5P data were accumulated in previous studies (Ferrer et al. 2019; Muangmai et al. 2023; Ng et al. 2015; Yang et al. 2013). Lim et al. (2001), using random amplified polymorphic DNA method, reported some genetic difference between the articulated and the non-articulated morphotypes within *G. salicornia*. However, the genetic difference between the two morphotypes has not been supported by subsequent molecular studies (Ferrer et al. 2019; Ng et al. 2015; Yang et al. 2013). Genetic diversity has been documented for populations from Malaysia, the Philippines, and Thailand (Ferrer et al. 2019; Muangmai et al. 2023; Ng et al. 2015; Yang et al. 2013). Muangmai et al. (2023)

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showed contrasting patterns of genetic diversity and population discontinuity in *G. salicornia* by geographical barrier between the Andaman Sea and the Gulf of Thailand. The aim of the present study was to analyze COI-5P sequence from seven populations of *G. salicornia* in Vietnam and to infer the phylogeography of Southeast Asian populations. This is the first historical phylogeographic study of *G. salicornia* utilizing both mismatch distribution and Bayesian skyline plot analyses.

2 Materials and methods

2.1 Sampling

Gracilaria salicornia specimens were collected at seven locations, ranged from Nui Thanh (15.5113 N, 108.6724 E), Quang Nam in the north to Ha Tien (10.3886 N, 104.4452 E), Kien Giang in the south (Figure 1; Supplementary Table S1). Depending on their frequency, 5–16 specimens were collected about 50 cm apart. Individuals were mostly green in shaded locations, however, plants in the exposed intertidal flat were yellow (Figure 1). Both articulated and non-articulated morphotypes were collected, but the articulated morphotype was most frequent. Branch tips from each individual were cleaned of epiphytes and kept with silica gel for DNA extraction. Vouchers were deposited in the Marine Ecology Laboratory of the Institute of Tropical Biology, Ho Chi Minh City, Vietnam.

2.2 DNA extraction and COI-5P sequencing

DNA extraction, PCR amplification, and sequencing procedures followed Boo et al. (2019). Genomic DNA was extracted from ~5 mg cleaned fragments ground in liquid nitrogen using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The primers GazF2 and GazR2 (Saunders 2005) were used for amplifying and sequencing COI-5P. The polymerase chain reaction (PCR) was performed using the Gotaq Green Master Mix (Promega, Madison, WI, USA) with 5 µl of DNA template. The PCR thermocycling protocol consisted of an initialization step of 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s (denaturation), 45 °C for 30 s (annealing), and 72 °C for 1 min (extension), and a final extension of 72 °C for 10 min. PCR products were purified using the LaboPass Gel & PCR purification Kit (Cosmo Genetech, Seoul, Korea) and sequenced commercially by the 1st Base Company (Selangor, Malaysia). All sequences were aligned using the MUSCLE algorithm in MEGA7 (Kumar et al. 2016) with default parameters, followed by manual adjustments and trimming to match the length of the shortest sequence (557 bp). A total of 399 sequences including 55 newly generated in this study were used in further analyses (Supplementary Table S1).

2.3 Genetic diversity and phylogeographic analyses

The genetic diversity indices were calculated using DnaSP v.6 (Rozas et al. 2017) at population, country, and ecoregion levels: the number of haplotypes (h), the number of polymorphic sites (S), haplotype diversity (Hd), and nucleotide diversity (π). Haplotype network was constructed with PopART v.1.7 (Leigh and Bryant 2015) using the median-joining network (MJN) (Bandelt et al. 1999). Phylogeny of COI-5P haplotypes was reconstructed using the Maximum Likelihood (ML) optimality criterion on the W-IQ-tree webserver (Trifinopoulos et al. 2016). The best-fitting substitution model was determined with the model test option (auto), followed by the ML tree search, and 1,000 ultrafast bootstrap replicates. The Bayesian inference analysis was performed with MrBayes v3.2.1 (Ronquist et al. 2012) using the Metropolis-coupled Markov Chain Monte Carlo (MC3) with the best-fitting substitution model. Four million generations of two independent runs were performed with four chains and sampling trees every one hundred generations. Twenty-five percent of saved trees were removed as burn-in, and the remaining trees were used to infer Bayesian posterior probabilities (BPP). *Gracilaria caerensis* (A.B.Joly et Pinheiro) A.B.Joly et Pinheiro and *Gracilaria parva* Freshwater, B. Williamson, P.W. Gabrielson et Margarita Brandt were selected as outgroups based on their previously determined evolutionary relationship to *G. salicornia* (Lyra et al. 2021; Wang et al. 2023). Non-hierarchical and hierarchical analyses of molecular variance (AMOVA) were performed using Arlequin v.3.5 (Excoffier and Lischer 2010) with Φ-statistics to quantify the proportion of total genetic variance, with significance of fixation indices tested using 10,000 permutations. Hierarchical AMOVA analysis was conducted to identify genetic variations among the haplogroups revealed by the haplotype network, following the criteria of Ferrer et al. (2019).

Mismatch distribution analysis (MDA) was conducted to tested the null hypotheses of spatial expansion using Arlequin v.3.5. For the expansion model, goodness-of-fit was tested with the sum of squared deviations (SSD) and Harpending's raggedness index (H_{Rag}) using 1,000 parametric bootstrap replicates (Schneider and Excoffier 1999). The neutrality tests (Tajima's D and Fu's F_{S}) were also performed to infer potential

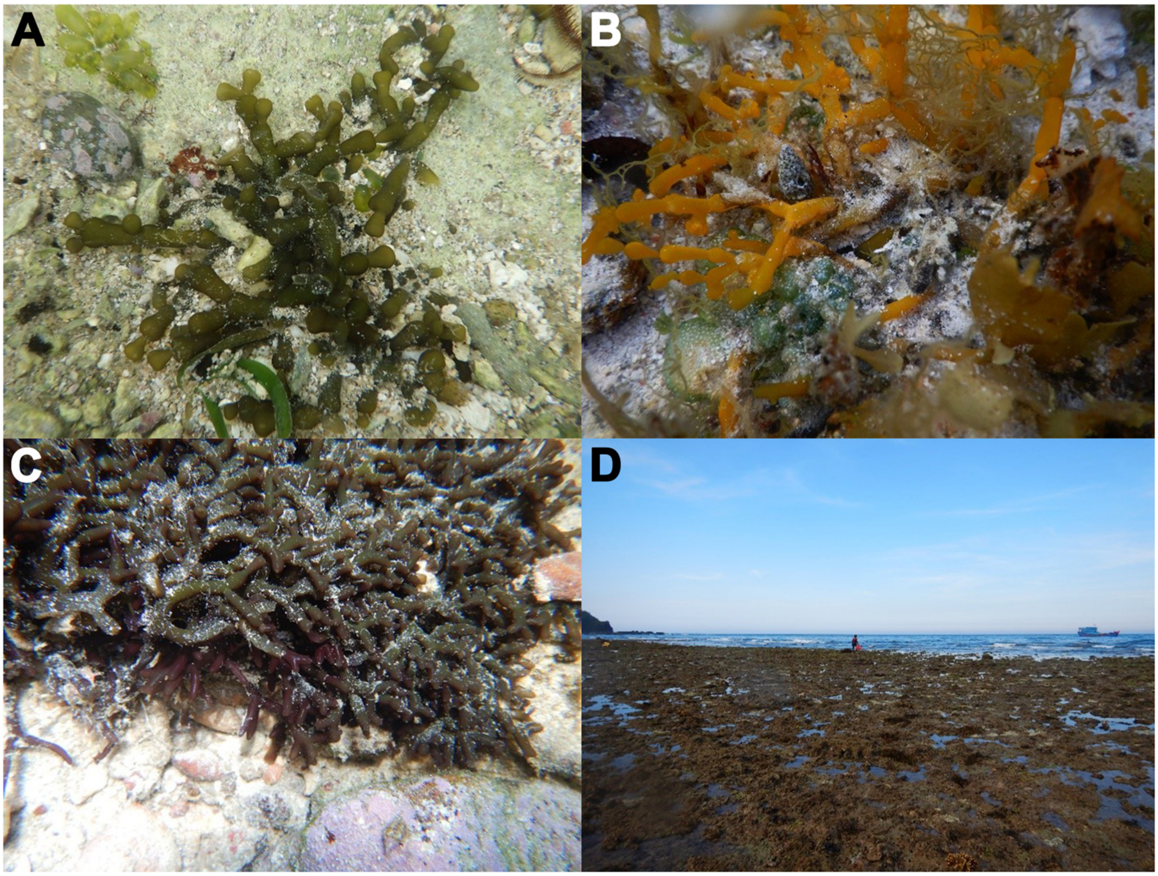

Figure 1: Habitat of *Gracilaria salicornia.* (A) Green plants with articulated axes (Ninh Hoa, Vietnam; 7 December 2023). (B) Yellow plants with articulated axes (Ly Son, Vietnam; 22 June 2022). (C) Dark green clump with compressed to cylindrical axes (Ha Tien, Vietnam; 1 April 2022). (D) Intertidal flat where *G. salicornia* commonly occurs (Nui Thanh, Vietnam; 16 June 2022).

population growth and expansion (Fu 1997; Tajima 1989); Tajima's D is conservative and Fu's F_S is powerful.

BEAST v.2.7.3 (Bouckaert et al. 2019) was used to infer the demographic histories by constructing Bayesian Skyline Plots (BSPs) of effective population size through time (Heled and Drummond 2009). The substitution rate for COI-5P was set at 7.6×10^{-9} substitutions/site/year, a value calculated in the red algae (Bringloe and Saunders 2019). The Markov chain Monte Carlo (MCMC) was run for 1×10^7 generations with trees sampled every 1,000 generations and the first 10 % of the samples were discarded as burn-in. The result was visualized by Tracer v.1.7 (Rambaut et al. 2018). Three replicate runs using different random seeds were conducted to confirm convergence.

3 Results

We generated 629–857 bp of COI-5P from 55 samples of *G. salicornia* from Vietnam (Supplementary Table S1). A total of 399 sequences, including published sequences from GenBank, were used for genetic diversity and phylogeographic analyses. A total of 27 haplotypes were identified, including five haplotypes found in Vietnam, three of which were private. Pairwise divergence ranged from 0.18–1.8 % between populations from different countries.

Gracilaria salicornia had high haplotype diversity (Hd = 0.765 ± 0.018) and low nucleotide diversity (π = 0.00456 ± 0.00023) (Table 1). Vietnamese populations (Hd = 0.352 ± 0.079, π = 0.00074 ± 0.00019) and the Chinese populations (Hd = 0.331 ± 0.143, π = 0.00082 ± 0.00039) had similar haplotype and nucleotide diversities. Genetic diversity values were twice as high in the Philippines and Thailand populations (Hd = 0.615 ± 0.031, π = 0.00553 ± 0.00034, and Hd = 0.670 ± 0.024, π = 0.00172 ± 0.00012, respectively).

The ML tree of *G. salicornia* haplotypes was monophyletic and well resolved (100 % ML bootstrap value, 1.0 for BPP) (Supplementary Figure S1). *G. salicornia* was divided into three haplogroups (I–III), following the criteria of Ferrer et al. (2019); haplogroup I comprised 18 haplotypes from

Table 1: Genetic characteristics of populations of *Gracilaria salicornia* from 10 countries based on mitochondrial COI-5P sequences.

Population	N	h	S	Hd ± SD	π ± SD	Tajima's D	Fu's F_S
China	17	4	3	0.331 ± 0.143	0.00082 ± 0.00039	−1.37718	−1.936**
Hawaii (USA)	8	1	0	0.000 ± 0.000	0.00000 ± 0.00000	NA	NA
India	1	1	0	NA	NA	NA	NA
Indonesia	3	2	1	0.667 ± 0.314	0.00120 ± 0.00056	NA	NA
Japan	5	2	1	0.600 ± 0.175	0.00108 ± 0.00031	1.22474	0.626
Malaysia	31	3	3	0.374 ± 0.092	0.00127 ± 0.00033	−0.12813	0.818
Philippines	95	10	19	0.615 ± 0.031	0.00553 ± 0.00034	−0.48965	0.940
Singapore	6	2	2	0.333 ± 0.215	0.00120 ± 0.00077	−1.13197	0.952
Thailand	178	6	6	0.670 ± 0.024	0.00172 ± 0.00012	−0.16247	−0.030
Vietnam	55	5	5	0.352 ± 0.079	0.00074 ± 0.00019	−1.47717*	−2.460*
Haplogroup I (H1–H18)	306	18	19	0.636 ± 0.028	0.00175 ± 0.00132	−1.72366*	−11.841**
Haplogroup II (H20–H27)	92	8	12	0.590 ± 0.030	0.00489 ± 0.00290	0.41963	1.708
Haplogroup III (H19)	1	1	0	NA	NA	NA	NA
Total	399	27	36	0.765 ± 0.018	0.00456 ± 0.00023	−1.46097*	−8.913*

N, number of analyzed samples; *h*, number of haplotypes; *S*, number of variable sites; Hd, haplotype diversity; π, nucleotide diversity; SD, standard deviation; NA, not applicable; *$p < 0.05$, **$p < 0.01$.

China, Hawaii, Indonesia, Malaysia, the Philippines, Singapore, Thailand, and Vietnam with statistical support. Haplogroup II comprised eight haplotypes from Japan and the Philippines, but it was not statistically supported. A single haplotype (H19) from the Philippines was placed in haplogroup III.

Of 27 haplotypes found in *G. salicornia*, two (H1, H2, and H4) were shared with other countries and 15 were private, each found in a single population (Figure 2). The haplotype network consisted of the same three haplogroups as the ML tree. Haplotype H1 ($n = 175$) was the most dominant and widely distributed haplotype in China, Indonesia, Malaysia, the Philippines, Singapore, Thailand, and Vietnam, and we designated it as the ancestral haplotype. Haplotype 2 was shared between Hawaii and the Philippines, and haplotype 4 occurred in China, the Philippines, and Vietnam.

Non-hierarchical AMOVA showed that 55.92 % ($p < 0.001$) of the genetic variation was found among countries (Table 2). The hierarchical AMOVA showed that 65.6 % ($p < 0.01$) of variation occurred between three haplogroups, followed by within-haplogroup variation (25.9 %, $p < 0.001$).

Under the sudden expansion model of the mismatch distribution analysis, the population expansion of *G. salicornia* was estimated to start about 251,976 years ago (95 % CI: 105,522–787,488) with low and non-significant sum of squared deviation (SSD = 0.02090) and Harpending's raggedness ($H_{\mathrm{Rag}} = 0.03970$) values (Table 3). The expansion time of haplogroup I was estimated to be 78,742 years ago (95 % CI: 68,900–96,890). Both the Chinese and Vietnamese populations were estimated to have expanded about 236,000 years ago.

The pairwise mismatch distribution was bimodal for the overall *G. salicornia* populations (Figure 3A). Haplogroup I was unimodal (Figure 3B), being closely aligned with the expected pattern under the sudden expansion model. Both Tajima's D and Fu's Fs values were statistically significant for haplogroup I ($D = -1.72366$, $p < 0.05$; $F\text{s} = -11.841$, $p < 0.01$) (Table 1), indicating a recent demographic or range expansion. In the remaining two haplogroups (II and III), the Tajima's D and Fu's Fs values were not statistically significant; therefore, a mismatch distribution analysis was not performed.

The generalized skyline plot showed that the divergence time from its recent common ancestor was about 0.5 Ma, and indicated population growth up to about 0.2 Ma, followed by stable population sizes until the present (Figure 3C). Haplogroup I showed a steady population growth over about 0.175 Ma (Figure 3D). Both haplogroups II and III were not able to analyze, as mentioned in mismatch analysis.

4 Discussion

Our sampling of 55 specimens across seven localities represented a diverse range of habitats for *G. salicornia* in Vietnam, which comprised five haplotypes, three unique (H6–H8) and two (H1, H4) shared with neighboring countries. Both shared and unique haplotypes are equally important in phylogeographic analysis; shared haplotypes indicate genetic connectivity between Vietnam and other countries, while unique haplotypes indicate mutation accumulation specific to Vietnam. The genetic diversity of

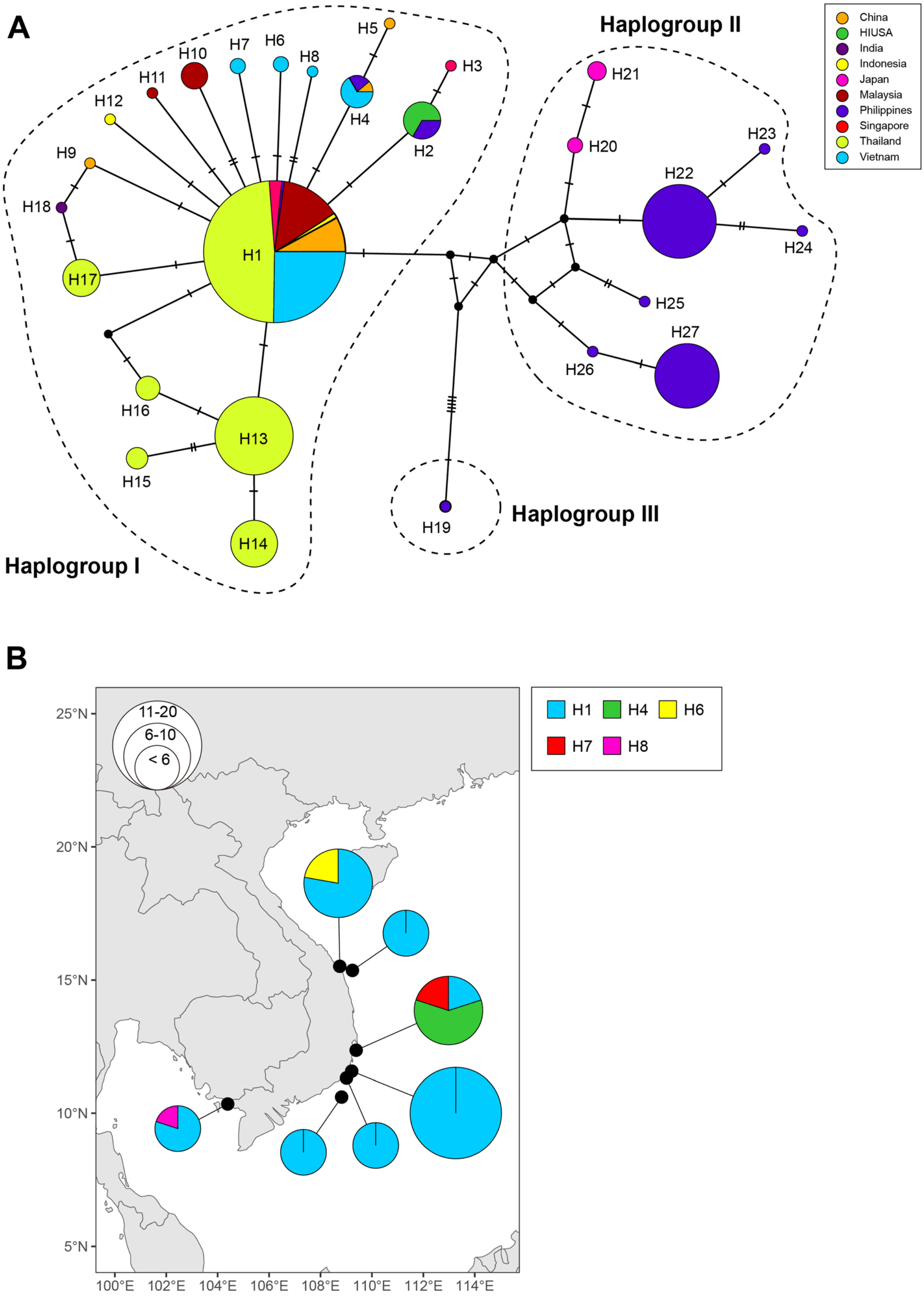

Figure 2: Haplotype network and geographic distribution of COI-5P haplotypes in *Gracilaria salicornia*. (A) Median-joining network. Each circle denotes a single haplotype with size proportional to frequency. Small cross lines represent single mutation steps and black circles represent extinct or unsampled haplotypes. Haplotypes are colored by country, as shown in the key. (B) A map showing haplotype distribution in Vietnam. Pie charts denote the proportion of haplotypes present in each site. Haplotypes are colored as shown in the key.

Table 2: Analyses of molecular variance (AMOVA) of mitochondrial COI-5P sequences.

Hierarchical	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*F*-statistics
Non-hierarchical	Among countries	9	247.211	0.84368	55.92	0.55921***
	Within countries	389	258.699	0.66504	44.08	
Hierarchical (haplogroup)	Among haplogroups	2	233.508	1.49774	65.59	0.65585**
	Among populations within haplogroups	9	43.908	0.19549	8.56	0.24874***
	Within haplogroups	387	228.495	0.59043	25.85	0.74146***

$^{**}p < 0.01$, $^{***}p < 0.001$.

Table 3: Summary of mismatch distribution parameters and expansion time of *Gracilaria salicornia* under sudden expansion model.

Group (generation time)	Parameter (τ)	Expansion time (t, yr)	SSD	H_{Rag}
Species	3.2 (1.34009–10.00079)	251,976 (105,522–787,488)	0.02090	0.03970
Haplogroup I	1.0 (0.87500–1.23047)	78,742 (68,900–96,890)	0.00023	0.05485
China	3.0 (0.40430–3.50000)	236,227 (31,835–275,599)	0.00169	0.22908
Vietnam	3.0 (0.42188–3.50000)	236,227 (33,219–275,599)	0.00610	0.18724

Vietnamese populations is lower, than that of the Philippines and Thailand populations, due to the high frequency (44/55 specimens examined) of the ancestral H1. Our results indicate a recent expansion of the Vietnamese population that is supported by both Tajima's *D* and Fu's *F*s. This demographic or spatial expansion was estimated to start in the middle Pleistocene (about 236,000 years ago). The Vietnamese population is an important piece in the phylogeographic puzzle of *G. salicornia* from Southeast Asia.

The presence of three haplogroups (I–III) within *G. salicornia* is supported by our phylogeny, haplotype network, and distribution of haplotypes, as was also reported in a previous study (Ferrer et al. 2019; Muangmai et al. 2023). Haplogroup I is distributed mostly in Indonesia, Malaysia, Thailand, Vietnam, and China, being on the west side of the marginal sea in the South China Sea. Haplogroup II occurs in the Philippines and Japan, being on the east side of the marginal sea, and likely reflecting the influence of the Kuroshio current. Despite comprising a relatively high number of mutation steps in the haplotype network, haplogroup II was broadly defined due to its restricted geographic distribution in the Philippines and Japan (Okinawa). Further study is required to decipher the genetic structuring of haplogroup II, which was not statistically supported in the present study, despite detailed collections in many locations in the Philippines (Ferrer et al. 2019; Yang et al. 2013). Haplogroup III includes a single specimen from the Philippines (Ferrer et al. 2019). Despite extensive collections across multiple localities in China, Malaysia, Thailand, the Philippines (Ferrer et al. 2019; Muangmai et al. 2023; Ng et al. 2015; Wang et al. 2023; Yang et al. 2013), and Vietnam, in the present study, no additional specimens of haplogroup III were found. We infer that the intervals of low sea level in the South China Sea, which became an inland sea during the Pleistocene (Wang et al. 1995), may have introduced west-east distribution pattern of *G. salicornia*. Demographic or range expansion related to sea-level fluctuations in the Quaternary are not uncommonly reported in tropical marine animals and the brown algal genus *Sargassum* (Chan et al. 2013, 2014; Grant 2015; Ludt and Rocha 2015; Wang et al. 2016; Zhang et al. 2014).

The recent chronogram of *Gracilaria* showed that the most recent common ancestor (MRCA) of *G. salicornia* began to diverge in a period of time between the Eocene and the Quaternary (approximated from Figure 3 in Lyra et al. 2021). This phylogeny was constructed using protein-coding genes of plastid and mitochondrial genomes of specimens from Hawaii and Singapore (Lyra et al. 2021). Haplotype network in the previous study (Ferrer et al. 2019) as well as the present study revealed that a single mutation event occurred between H2 (KF824534 from Hawaii, and KT357381, KY7774623-5 from the Philippines) and H3 (MZ336093 from Singapore) (Supplementary Tables S1 and S2), both being included in the haplogroup I (Figure 2). Evolutionary estimation of the MRCA of *G. salicornia* will highlight its phylogeography, however, it is beyond the focus of the present study because of the lack of organellar genomes from representatives of haplogroups II and III.

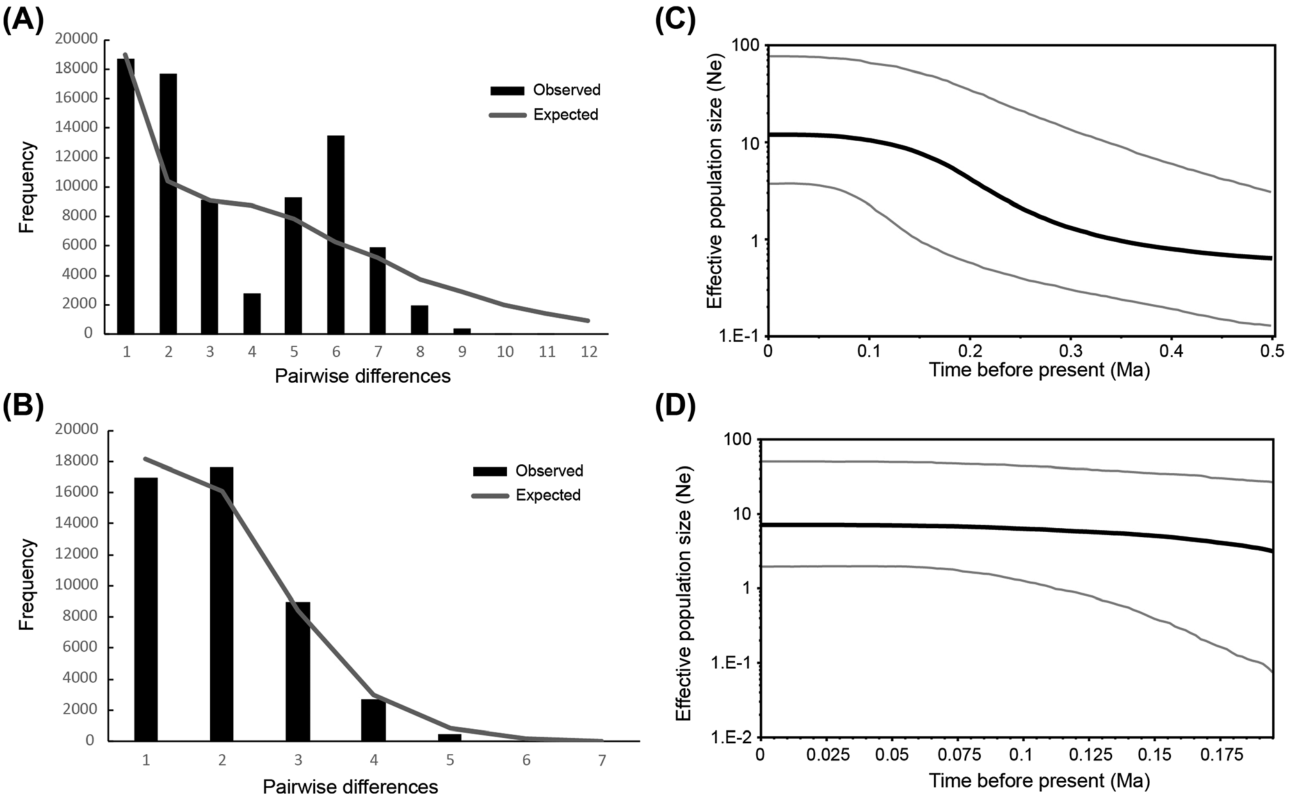

Figure 3: Mismatch distribution for *Gracilaria salicornia* (A) and *G. salicornia* haplogroup I (B); and Bayesian skyline plots for *G. salicornia* (C) and *G. salicornia* haplogroup I (D). For mismatch distributions, the black bar chart represents observed distributions, whereas the grey line represents simulated data under a spatial expansion model. For Bayesian skyline plots, *x*-axis indicates time since the present in years and *y*-axis indicates estimated effective population size. Bold line is the median estimate, and thinner lines indicate the 95 % highest posterior density (HPD) interval.

There are two secondary contact areas in northern Philippines, where two geographically different haplogroups (I and II) of *G. salicornia* are geographically reunited; one area is Batanes Islands, Bashi Channel, where H1 and H2 populations (Haplogroup I) may contact H25 population (Haplogroup II), and the other is Balaoan, La Union, where H4 population (Haplogroup I) likely contact H27 population (Haplogroup II). This result indicates the range expansion of Haplogroup I from west to east, suggesting the potential of reestablishment of gene flow between two groups (Bertl et al. 2018).

Our haplotype network revealed that most haplotypes especially in haplogroup I were linked by a single mutation to the ancestral haplotype. This might be a result of habitat preference or sea surface circulation by seasonal monsoons (Fumo and Sherwood 2023; Grant 2015; Hewitt 2000; Ludt and Rocha 2015; Ng et al. 2017). Muangmai et al. (2023) reported that genetic divergence within Thailand populations of *G. salicornia* was likely due to the geographic barrier of the Thai-Malay Peninsula and complex ocean currents.

Gracilaria salicornia was supposedly introduced from the Philippines to Hawaii (Abbott 1999; Smith et al. 2004). Ferrer et al. (2019) found an identical haplotype between the Philippines (four specimens from Batanes, Batangas, and Pangasinan) and Hawaii (eight specimens from Hawaii, Molokai, Oahu of the Hawaiian Islands, Sherwood et al. 2010), and supported the likelihood of anthropogenic transport from the Philippines to Hawaii. However, *Acanthophora spicifera* (M.Vahl) Børgesen (Rhodomelaceae) is a well-known case of a red algal introduction from Guam to Hawaii (Sherwood and Guiry 2023). To confirm whether the Hawaiian specimens originated in the Philippines or Guam, COI-5P analysis of Guam specimens of *G. salicornia* is required.

The occurrence of *G. salicornia* in India was demonstrated based on published COI-5P data (MT939890). However, although its occurrence in South Africa was reported in a previous study based on RuBisCO spacer and morphology (Iyer et al. 2005), the sequence in GenBank (AY241149) fell outside the monophyletic *G. salicornia* clade, as also shown by Muangmai et al. (2023). Our sequencing of three specimens from Lala Neck, Durban, South Africa failed due to contamination by marine bacteria. The occurrence of *G. salicornia* in South Africa to Tanzania and Red Sea (e.g., Einav et al. 2021; Iyer et al. 2005; Silva et al. 1996) requires molecular confirmation. Sequence data is lacking from Australia and Pacific Islands, although the morphology of the species was well documented (Meneses and Abbott 1987; Withell et al. 1994).

In conclusion, our data from Vietnamese *G. salicornia* is a significant addition to the phylogeography of the species in Southeast Asia, a tropical biodiversity hotspot (Boag et al. 2021) and brings together previous studies of other regions for an important contribution to our understanding of the genetic diversity and biogeography of global populations from outside Southeast Asia. This is the first study to examine the genetic variability and structure of *G. salicornia* in Southeast Asia, incorporating publicly available sequences from GenBank. Furthermore, to our knowledge, this represents the second report on the historical phylogeography of red algae in Southeast Asia using both mismatch distribution and Bayesian skyline plot analyses following Fontana et al. (2024) who studied *Dichotomaria elegans* (Nemaliales) from Taiwan, at the northeastern boundary of Southeast Asia. While our findings highlight genetic variability and haplogroup patterns across Southeast Asia waters, we advocate for additional sampling in surrounding regions to fill haplotype gaps and to further investigate population growth and range expansion scenarios in *G. salicornia*, an agar-yielding species.

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Data availability: DNA sequences were submitted to the public database (GenBank).

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