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ORIGINAL PAPER

Recent expansion led to the lack of genetic structure of *Sargassum aquifolium* populations in Southeast Asia

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Abstract Phylogeographical study of the brown macroalga, *Sargassum aquifolium* using nuclear internal transcribed spacer 2, plastidal RuBisCo spacer, and mitochondrial cytochrome oxidase subunit-III revealed the populations in Southeast Asia to be homogeneous. On the other hand, genetic differences were detected between populations from Southeast Asia and western Pacific Islands/Guam, suggesting the presence of genetic break between these regions. This further suggests that populations of *S. aquifolium* may have survived east of Sunda Shelf during the Last Glacial Maximum and recent recolonization led to homogeneity of the populations in the Sunda Shelf region. Recolonization could be facilitated by year-round reproduction of the populations and dispersal of germlings on floating thalli by coastal currents. Restricted current flow across Maluku Sea and directional equatorial current flows could have isolated the Pacific Island and Guam populations from those of Southeast Asia. Our results support the presence of multiple refugia as the source of different lineages of *S. aquifolium* populations with a lack of secondary contact in the post-glacial dispersal between Southeast Asia and western Pacific as the mechanisms behind the phylogeographical patterns observed.

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Introduction

Southeast Asia is well known for having a complex geological history with fluctuating sea level during the glacial periods. The low sea level, up to 120 m below the present level in Pleistocene, resulted in formation of land bridges due to exposure of land masses in relatively shallow waters (Voris 2000). The largest land mass exposed, i.e., the Sunda Shelf, which included Malay Peninsula, Sumatra, Kalimantan, Java, Madura, Bali, and other smaller islands, created barriers for marine organisms (McManus 1985). Enclosed by Sunda Shelf and the surrounding land bridges were deeper basins, e.g., the South China Sea to the north, Sulu Sea in the northeast, and Celebes Sea and Flores Sea to the east. These basins served as refugia where isolation and differentiation of populations occurred (Barber 2000; Barber et al. 2006; Timm and Kochzius 2008). Post-glacial oceanographic conditions also contributed in shaping population structure of marine organisms. Monsoon currents promoted genetic

connectivity of derived lineages (Wyrtki 1961). The strong downward Indonesian Throughflow (20 million m^3/s), connecting Celebes Sea and Flores Sea through Makassar Strait (Fig. 1, Gordon and Fine 1996; Gordon 2005), has been identified as one of the most important directional current flows that contributed to high genetic connectivity among marine organisms in this region (Barber et al. 2006). On the other hand, this and other localized directional currents also serve as barriers blocking gene flows between populations (Barber et al. 2006). This complex geological history and the present oceanographic system are the key causes that contributed to Southeast Asia as one of the biodiversity hotspots and centers of marine endemism in the world (Hughes et al. 2002).

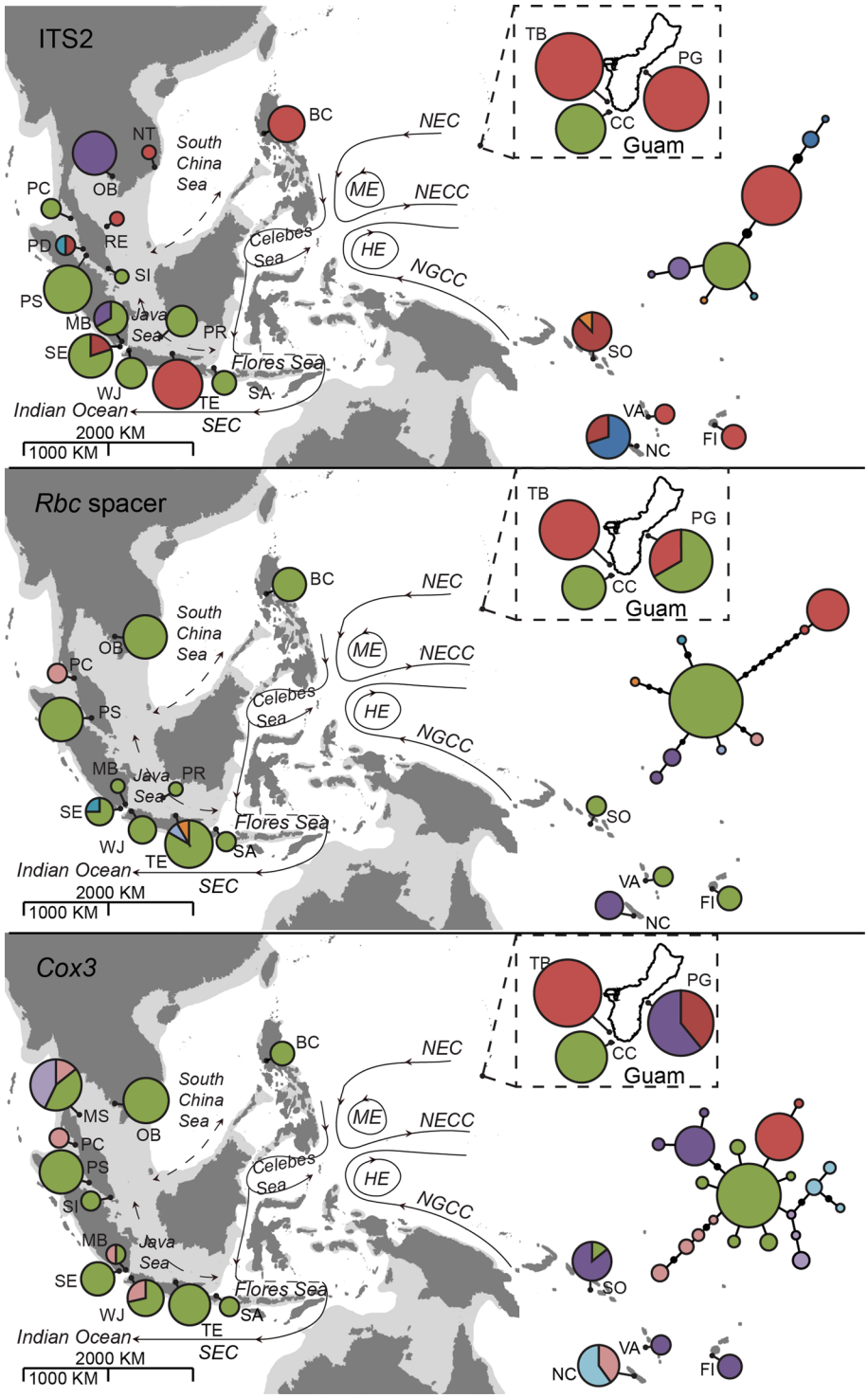

Fig. 1 Haplotype distribution and haplotype network of *Sargassum aquifolium* for ITS2, *Rbc* spacer, and *Cox3*. Abbreviations of sample sites are given in Table 1. Pie chart size is proportional to sample size. Dominant currents are shown in *solid lines* (after Gordon and Fine 1996) and seasonally reversing currents in *dashed lines* (after Wyrtki 1961). *Light gray* area shows the coastal outline during Pleistocene maximum low sea level of −120 m (after Voris 2000). *SEC* South Equatorial Current; *NEC* North Equatorial Current; *NECC* North Equatorial Counter Current; *NGCC* New Guinea Coastal Current; *ME* Mindanao Eddy; *HE* Halmahera Eddy

Phylogeographical studies in Southeast Asia have revealed the populations of marine organisms to be highly structured. Genetic break between Sulawesi and Sunda Shelf was found in populations of false clown anemone fish (Timm and Kochzius 2008; Timm et al. 2012) and giant clam (Kochzius and Nuryanto 2008), with further genetic diversity contributed by subsequent recolonization. For stomatopods, genetic break was revealed between Papua New Guinea and Northern Indonesia where gene flow was restricted by limited current flow in Maluku Sea (Barber et al. 2006). These studies, however, mainly focused on the Coral Triangle region, and extensive sampling in Sunda Shelf region itself was only available for four species of seahorses (Lourie et al. 2005) wherein their population structures and genetic diversity were found to be related to the individual ecologies of these species. More study in the Sunda Shelf region itself is necessary to provide better understanding of its phylogeography and to lend weight to the suggestion of it as an independent unit for conversation management in Southeast Asia (Carpenter et al. 2011).

Most of the phylogeographical studies in Southeast Asia were on animals. Their lineage diversification and population connectivity among sites were highly dependent on the duration of their larval stages and direction of current flows (Crandall et al. 2008). Macroalgae, on the other hand, exhibited different mode of dispersal. Their immobile germlings with shorter dispersal distances (<km) than those of animal larvae may lead to higher differentiation between their populations. They could therefore provide a different perspective on understanding the forces shaping population structure of different marine organisms in the region. Extensive studies on macroalgae using multilocus molecular markers (Coyer et al. 2003; Provan et al. 2005; Olsen et al. 2010; Andreakis et al. 2007, 2009) have provided comprehensive information on the evolutionary history of marine organisms in North Atlantic. Recent phylogeographical studies using macroalgae (e.g., Cheang et al. 2010a) also revealed the importance of land bridges and oceanic current in contributing to the genetic diversification as well as mixing of genetic populations in northwestern Pacific.

The genus Sa*rgassum* (Phaeophyceae) is widely distributed in Southeast Asia. Members of this genus have a unique mode of dispersal. The dispersal distance of their germlings is very short, <1 m (Kendrick and Walker 1995), but they can also disperse for long distance in the form of drifting fronds with germlings attached (Ohno 1984). Southeast Asian region has a complex current system with a combination of monsoonal and directional current flows that may contribute to the dispersal, hence the mixing of populations of *Sargassum* spp.

Sargassum aquifolium (Turner) C. Agardh is a common brown macroalga growing on lower intertidal areas in many tropical reefs. It has been recorded in most Southeast Asian countries (Phillips 1995; Stiger et al. 2000; Ang et al. 2008). In this study, the nuclear internal transcribed spacer 2 (ITS2), plastidal RuBisCo spacer (*Rbc* spacer), and mitochondrial cytochrome oxidase subunit-III (*Cox3*) were used as molecular markers to investigate its phylogeographical pattern in Southeast Asia.

Materials and methods

Sample collection and DNA extraction

Samples were collected from 15 sampling sites within Southeast Asia by snorkeling and/or sampling during low tide (Fig. 1; Table 1). Young leaf tips were preserved in silica gel, and voucher plants were dried and stored as reference collections in the Simon F. S. Li Marine Science Laboratory Herbarium, Chinese University of Hong Kong. DNA was extracted following the methods described by Cheang et al. (2010a). Briefly, samples were crushed in liquid nitrogen, and genomic DNA was extracted by modified cetyltrimethylammonium bromide (CTAB) method. It was further purified by GENECLEAN II kit (Obiogene Inc.), following the manufacturer's instructions.

Polymerase chain reaction and sequencing

The ITS2 and *Cox3* were amplified with the following PCR profile: initial 2 min at 94 °C, 4 cycles of 30 s at 94 °C, 1 min at 47 °C and 1 min at 68 °C, 29 cycles of 25 s at 94 °C, 25 s at 55 °C, and 35 s at 72 °C. *Rbc* spacer was amplified with the following profile: initial 1 min at 94 °C, 40 cycles of 40 s at 94 °C, 30 s at 42 °C, and 45 s at 72 °C with final extension of 7 min at 72 °C. PCR was performed in a C1000™ thermal cycler (Bio-Rad Laboratory, Inc.). Primers for ITS2 and *Rbc* spacer were described in Mattio et al. (2008). Primers for *Cox3* were newly designed (CoxF: GGGCCAGCATACTGTTGCGGT, CoxR: ACCAAGCG GCTGCTTCAAATCCA). Each 25 μL PCR contained 1.5 μL of DNA template, 2.5 μL of 10× buffer, 0.2 mM of each of dNTP, 0.2 μM of each primer, and 0.625 U of *TaKaRa Ex Taq* DNA polymerase (5 U/μL). PCR products were sequenced in both directions with the same primers by Macrogen (Macrogen Inc., Seoul, Korea). Sequences were aligned and edited together with the sequences of *S. aquifolium* available from GenBank by naked eyes using MEGA ver. 5 (Tamura et al. 2011).

Phylogeny and haplotype network construction

Samples of *S. aquifolium* were initially screened to confirm the identity before conducting subsequent analysis.

Table 1 Sampling sites, diversity indices, and neutrality tests of *Sargassum aquifolium* using ITS2, *Rbc* spacer, and *Cox3* (from top to bottom for each site). Sequences from Genbank are included as indicated

Locality	N*	H ± SD*	π ± SD*	*D**	*Fs**
1. Balibago, Calatagan, Philippines (BC) (14°27′49.08″N, 120°44′49.48″E)	7	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	6	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	3	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
2. Surat Thani Mud Sum Island, Thailand (MS) (9°16′11.16″N, 99°51′13.33″E)	0				
	0				
	14	0.868 ± 0.059	0.0891 ± 0.0630	2.15	−2.78
3. Otres Beach, Sihanoukville, Cambodia (OB) (11°7′14.90″N, 103°0′0.68″E)	10	0.200 ± 0.154	0.0333 ± 0.0507	−1.11	−0.34
	10	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	11	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
4. Singapore (SI) (0°40′31.48″N, 104°5′31.52″E)	1	1.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	0				
	2	1.000 ± 0.500	0.0556 ± 0.0786	0.00	0.00
5. Pulau Serimbun, Melaka, Malaysia (PS) (2°42′46.30″N, 101°25′33.71″E)	12	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	10	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	10	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
6. Pantai Cenang, Langkawi, Malaysia (PC) (6°51′7.12″N, 99°36′20.10″E)	2	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	2	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	2	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
7. Sanur, Bali, Indonesia (SA) (8°8′28.16″S, 115°22′39.15″E)	3	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	2	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	2	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
8. Merak Balantung, Indonesia (MB) (5°47′18.44″S, 105°21′40.77″E)	6	0.533 ± 0.172	0.0889 ± 0.0977	0.85	0.63
	1	1.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	2	1.000 ± 0.500	0.1667 ± 0.1925	0.00	1.10
9. Teluk Awur, Indonesia (TE) (6°32′30.58″S, 110°42′2.00″E)	13	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	12	0.318 ± 0.164	0.2601 ± 0.0562	**−1.75**	−0.10
	9	0.389 ± 0.164	0.0216 ± 0.0251	0.156	0.48
10. Sebuku, Indonesia (SE) (5°53′57.33″S, 105°29′59.40″E)	10	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	4	0.500 ± 0.265	0.0910 ± 0.0901	−0.71	1.10
	6	0.600 ± 0.215	0.0370 ± 0.0376	−1.13	−0.86
11. Merak, West Java, Indonesia (WJ) (5°59′44.19″S, 105°57′35.60″E)	5	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	4	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	7	0.476 ± 0.171	0.0794 ± 0.0627	0.75	2.51
12. Palau Pramuka, Indonesia (PR) (5°6′54.56″S, 109°18′13.91″E)	5	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	1	1.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	0				
13. Cocos Island, Guam (CC) (13°12′6.55″N, 144°35′16.76″E)	13	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	10	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	14	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
14. Pago Bay, Guam (PG) (13°25′3.50″N, 144°48′4.91″E)	22	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	21	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	23	0.498 ± 0.053	0.0830 ± 0.0580	2.14	4.41
15. Toguan Bay, Guam (TB) (13°29′4.37″N, 144°41′23.73″E)	24	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	19	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	24	0.083 ± 0.075	0.0046 ± 0.0100	−1.16	−1.03
Genbank					
16. Palau Redang, Terengganu, Malaysia (RE) ITS2: AB043118 (5°46′26.93″N, 102°58′52.91″E)	1	1.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	0				
	0				

Table 1 continued

Locality	N*	H ± SD*	π ± SD*	*D**	*Fs**
17. Port Dickson, Malaysia (PD) ITS2: AB043116, EU833443 (3°26′19.26″N, 101°4′40.30″E)	2	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	0				
	0				
18. Fiji (FI) ITS2: EU833431-3 *Rbc*: EU833464-6 *Cox3*: EU833406, EU833409, EU833411 (17°32′26.65″S, 177°24′54.73″E)	3	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	3	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	3	0.667 ± 0.314	0.0370 ± 0.4619	0.00	0.20
19. Cau Da, Nhatrang, Vietnam (NT) ITS2: AB043115 (12°3′0.90″N, 109°15′41.48″E)	1	1.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	0				
	0				
20. Solomon Islands (SO) ITS2: EU833441-2, EU833444-9 *Rbc*: EU833462-3 *Cox3*: EU833392-8, EU833400 (9°23′39.19″S, 159°55′59.80″E)	8	0.250 ± 0.180	0.0412 ± 0.0591	−1.05	−0.18
	2	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	8	0.464 ± 0.200	0.0655 ± 0.0532	−1.03	0.51
21. Vanuatu (VA) ITS2: EU833455-6 *Rbc*: EU833476-7 *Cox3*: EU833412-3 (17°46′23.13″S, 168°18′5.30″E)	2	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	2	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
	2	0.000 ± 0.000	0.0000 ± 0.0000	0.00	N.A.
22. New Caledonia (NC) ITS2: EU882252-3, FJ170431-7,HQ416053 *Rbc*: EU882262-3, FJ170380-3 *Cox3*: EU882241-3, FJ170405-10 (21°4′49.65″S, 165°36′52.75″E)	10	0.600 ± 0.131	0.1889 ± 0.1509	0.25	0.72
	6	0.533 ± 0.172	0.0485 ± 0.0533	0.85	0.63
	9	0.806 ± 0.089	0.1574 ± 0.1037	0.45	1.16
Whole data set	160	0.230 ± 0.043	0.0576 ± 0.0631	**−1.41**	**−3.46**
	115	0.182 ± 0.049	0.0370 ± 0.0381	**−2.05**	**−4.50**
	151	0.782 ± 0.025	0.1023 ± 0.0654	−1.16	**−10.67**

* Number of individuals (*N*), haplotype (*h*), and nucleotide (π) diversities, Tajima's D, Fu's Fs is given per site. Significant values ($p < 0.05$) are marked in bold. N.A. = not applicable as Fs cannot be calculated from single haplotype. Sequences from Malaysia, Fiji, Vietnam, Solomon Islands, Vanuatu, and New Caledonia are pooled for each site and accession number shown

Representative sequences from each haplotype of combined ITS2, *Rbc* spacer, and *Cox3* sequences were initially screened to ensure they formed a monophyletic lineage with no misidentification. The sequences were aligned with those of other members of subgenus *Sargassum* spp. (Mattio et al. 2008, 2009) using Bayesian inference (BI) and maximum likelihood (ML) methods by MRBAYES 3.2.1 (Ronquist et al. 2012) and PhyML 3.0 (Guindon et al. 2010), respectively. Best-fit substitution model was determined by corrected Akaike information criterion (AICc) implemented in jMODELTEST 2.1.1 (Darriba et al. 2012). *Turbinaria ornata* (Turner) J. Ag., as suggested by Stiger et al. (2003) and Phillips et al. (2005), was used as outgroup. Any sequence that was not monophyletic was discarded to ensure consistency in subsequent analysis.

Phylogenetic relationship within *S. aquifolium* was investigated with ML using PhyML 3.0 and BI using MRBAYES 3.2.1. Best-fit substitution models were determined by jMODELTEST 2.1.1. *Sargassum ilicifolium* (Turner) C. Ag. was used as outgroup as it is closely related, being within the same subgenus *Sargassum* (Mattio et al. 2009). Significance of the branching was supported by 1,000 bootstrap in ML and posterior probability in BI analysis. For BI, two independent Markov Chain Monte Carlo (MCMC) searches were conducted until the divergence became small and stationary. Trees were sampled every 100 cycles in 1,500,000 generations, and first 25 % of trees were discarded as burn-in. Haplotype networks for the three markers were generated by TCS 1.21 (Clement et al. 2000).

Population structure analysis

Haplotype (*h*) and nucleotide (π) diversity (Nei 1987; Nei and Li 1989) were calculated in ARLEQUIN 3.5.1.2 (Excoffier and Lischer 2010). Tajima's (1989) and Fu's (1997) were calculated to test for the selective neutrality of the markers. Recent population bottleneck and population expansion can be indicated by negative Tajima's D and Fu's Fs. Pairwise Φ_{ST} was calculated to estimate the level of gene flow between populations. Hierarchical analysis of molecular variance (AMOVA) with 10,000 permutations was conducted based on Φ_{ST} to find out the best spatial groupings of populations. A Mantel test (Rousset 1997) with 10,000 permutations was used to

test for the isolation by distance model by comparing the pairwise Φ_{ST} and the matrix of geographical distances among sampling sites. Geographical distances were measured using the shortest distance between two sites by Google Earth (http://www.google.com/earth/index.html).

Demographic history

Demographic history of *S. aquifolium* was only conducted for *Cox3* as no molecular clock is available for ITS2 and *Rbc* spacer. Mutation rate of *Cox3* was calibrated to be 1.035×10^{-9}–1.555×10^{-9} substitutions per site per year (Chan et al. 2013). The generation time of *S. aquifolium* was assumed to be 1 year as most *Sargassum* species have an annual cycle of regeneration (Ang 2006). Mismatch distribution was used to detect recent population expansion as categorized by unimodel distribution (Rogers and Harpending 1992) generated in ARLEQUIN 3.5.1.2. Time of expansion (in generation), t, can be calculated with $\tau = 2\ \mu t$, where τ is the crest of distribution calculated by ARLEQUIN and μ is the mutation rate of the marker per generation. A Bayesian skyline plot was used to estimate the demographic history of *S. aquifolium*. It was generated with BEAST 1.5.3 (Drummond et al. 2005) using MCMC sampling procedures with 10^8 steps in every 1,000 steps. Runs were repeated until effective sample size >200 in all parameters was reached, as recommended in the user manual (Drummond and Rambaut 2007). All runs were pooled in LogCombiner 1.7.3 with first 10 % of generations discarded as burn-in. Bayesian skyline plots were generated and visualized in Tracer 1.5.

Results

Genetic diversity

A total of 160, 115, and 151 sequences were obtained from 15 sampling sites and Genbank for ITS2 (297 bp), *Rbc* spacer (559 bp), and *Cox3* (404 bp), respectively (Table 1; Fig. 1). *Cox3* showed the highest variability ($H = 0.782 \pm 0.025$, $\boldsymbol{\pi} = 0.1023 \pm 0.0654$). ITS2 and *Rbc* spacer showed similar variability ($H = 0.230 \pm 0.043$, $\boldsymbol{\pi} = 0.0576 \pm 0.0631$ and $H = 0.182 \pm 0.049$, $\boldsymbol{\pi} = 0.0370 \pm 0.0381$, respectively). A total of 8, 9, and 21 haplotypes were revealed for ITS2 (Genbank accession numbers for new sequences produced: KF826917–KF826920), *Rbc* spacer (KF826921–KF826927), and *Cox3* (KF826928–KF826941), respectively, with 6, 11, and 18 polymorphic sites. No intragenomic variation was observed for all the markers as all the chromatographs showed clear and consistent peaks in both sequencing directions.

Interspecific and intraspecific phylogenetic relationship

HKY + G model was determined by jMODELTEST to be the best-fit substitution model to analyze the taxonomic status of *S. aquifolium*. Phylogenetic trees inferred from BI and ML with other members of the subgenus *Sargassum* showed all samples to be monophyletic (Fig. 2). All sequences were therefore used for subsequent analysis. HKY, GTR + G, and GTR + G models were determined by jMODELTEST to be the best-fit substitution model for ITS2, *Rbc* spacer, and *Cox3*, respectively. ML and BI analyses gave same tree topology and were supported by 1,000 bootstrap value and posterior probability (Figs. S1-S3, Electronic Supplementary Material).

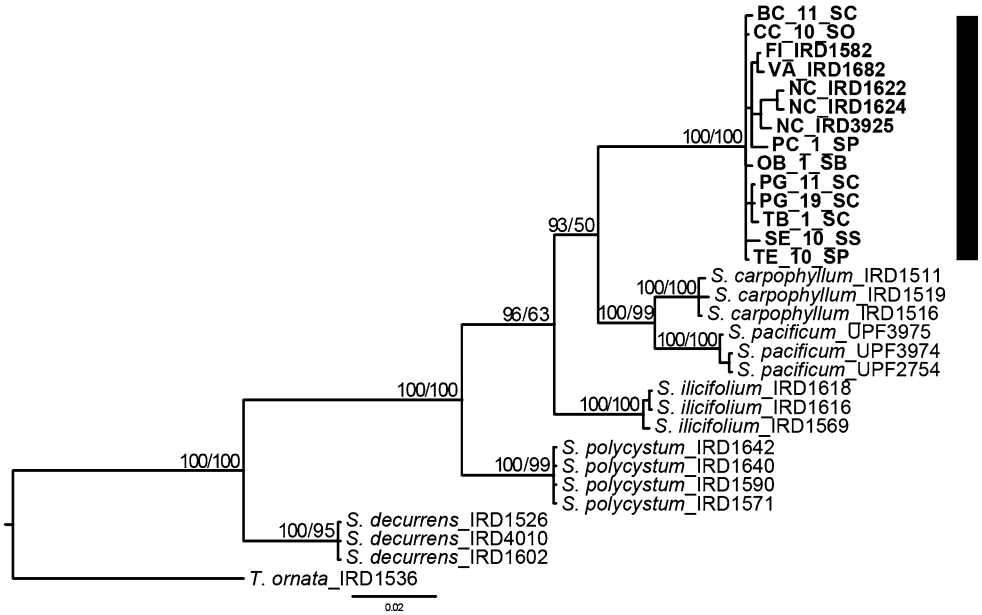

Fig. 2 Phylogenetic tree of representative sequences from each haplotype of combined ITS2, *Rbc* spacer, and *Cox3* sequences from *Sargassum aquifolium* with other *Sargassum* spp. in subgenus *Sargassum*. Voucher numbers of sequences from Genbank are presented after species name. Representative sequences from each haplotype of *S. aquifolium* are labeled in *black bar*. *Turbinaria ornata* is used as outgroup. Posterior probabilities of Bayesian inference and bootstrap value of maximum likelihood are shown

Table 2 Pairwise Φ_{ST} among populations of *Sargassum aquifolium* based on *Cox3*

Site	BC	CC	FI	MB	MS	NC	OB	PC	PG	PS	SA	SE	SO	TB	TE	SI	VA	WJ
BC																		
CC	**1.000**																	
FI	0.900	**0.959**																
MB	0.571	0.763	0.466															
MS	**0.502**	**0.438**	**0.628**	0.166														
NC	**0.542**	**0.659**	**0.423**	0.204	**0.451**													
OB	**1.000**	0.000	**0.949**	0.711	**0.402**	**0.620**												
PC	1.000	**1.000**	0.868	0.250	**0.631**	**0.453**	**1.000**											
PG	**0.544**	**0.472**	0.175	0.282	**0.492**	**0.481**	**0.445**	**0.616**										
PS	**1.000**	0.000	**0.945**	0.688	**0.389**	**0.605**	0.000	**1.000**	**0.436**									
SA	1.000	0.000	0.813	0.000	0.148	0.360	0.000	1.000	0.261	0.000								
SE	**0.663**	0.153	**0.750**	0.214	**0.219**	**0.481**	0.108	**0.830**	**0.376**	0.091	0.000							
SO	**0.701**	**0.762**	0.000	0.323	**0.546**	**0.402**	**0.729**	**0.677**	0.099	**0.716**	0.501	**0.561**						
TB	**0.963**	**0.949**	**0.960**	**0.892**	**0.723**	**0.798**	**0.945**	**0.980**	**0.583**	**0.943**	**0.925**	**0.847**	**0.873**					
TE	**0.759**	0.195	**0.823**	0.466	**0.355**	**0.561**	0.156	**0.895**	**0.415**	0.141	0.000	0.068	**0.635**	**0.865**				
SI	0.769	0.763	0.724	0.000	0.077	0.383	0.711	0.857	0.336	0.688	0.000	0.082	0.519	**0.909**	0.259			
VA	1.000	**1.000**	0.676	0.571	**0.686**	**0.490**	**1.000**	1.000	0.428	**1.000**	1.000	**0.838**	**0.433**	**0.980**	**0.895**	0.857		
WJ	0.480	0.303	**0.546**	0.000	**0.212**	**0.383**	0.254	0.537	**0.306**	0.235	0.000	0.034	**0.399**	**0.770**	0.184	0.039	0.651	

Abbreviations for sites are given in Table 1. Φ_{ST} with $p < 0.05$ are in bold

Population structure

Tajima's D gave significant negative value for ITS2 and *Rbc* spacer, but gave nonsignificant negative value for *Cox3* (Table 1). Fu's Fs gave significant negative value for all markers. Result of the two neutrality tests showed there has been population expansion in *S. aquifolium*. Most of the populations showed nonsignificant pairwise Φ_{ST} values in ITS2 and *Rbc* spacer with shallow population structure (Tables S1–S2, Electronic Supplementary Material). Significant population differentiation was found in New Caledonia (NC) and Otres Beach, Cambodia (OB), and between these and other populations for ITS2 (pairwise Φ_{ST}: 0.516–0.752 and 0.564–0.944, respectively, $p < 0.05$). For *Rbc* spacer, significant population differentiation was found in NC and Pantai Cenang, Malaysia (PC), and between these and other populations (pairwise Φ_{ST}: 0.747–0.952 and 0.752–1.000, respectively, $p < 0.05$). In contrast, most pairwise Φ_{ST} showed significant values for *Cox3* (Table 2). High pairwise Φ_{ST} values with other populations were found in Balibago, Calatagan, Philippines (BC, 0.502–1.000), Cocos Island (CC, 0.438–1.000), Fiji (FI, 0.423–0.960), Otres Beach (OB, 0.402–1.000), Pantai Cenang (PC, 0.453–1.000), Toguan Bay, Guam (TB, 0.583–0.980), and Vanuatu (VA, 0.433–1.000). Hierarchical AMOVA result is summarized in Table 3. For ITS2, highest interpopulation variation (%var = 51.08, $\Phi_{CT} = 0.51$) was found in the groupings: (1) Gulf of Thailand and South China Sea; (2) western Malaysian Peninsula and Java Sea; (3) Vanuatu, Solomon Islands and Fiji; (4) Guam; (5) NC. For *Rbc* spacer, highest interpopulation variation (%var = 58.46, $\Phi_{CT} = 0.58$) was found with the groupings: (1) Southeast Asia; (2) Vanuatu, Solomon Islands and Fiji; (3) Guam; (4) NC. For *Cox3*, shallower population structure was revealed with the same groupings as *Rbc* spacer (%var = 31.68, $\Phi_{CT} = 0.32$). Mantel test for ITS2, *Rbc* spacer, and *Cox3* showed low regression coefficient ($r = 0.1 \times 10^{-5}$, $p > 0.05$; $r = 2.6 \times 10^{-5}$, $p > 0.05$; $r = 0.5 \times 10^{-5}$, $p > 0.05$) that did not fit into the isolation by distance model.

Demographic history

Demographic analysis was conducted in *Cox3* only as no molecular clock is available for ITS2 and *Rbc* spacer. Mismatch distribution of *Cox3* fits into the sudden expansion model (Fig. 3, sum of squared deviation 0.0059, $p > 0.05$). Estimated expansion time was 2.04–3.06 Mya, late Pliocene to early Pleistocene ($\tau = 2.559$). Bayesian skyline plot showed gradual increase in population size since 0.25 Mya, late Pleistocene (Fig. 3).

Discussion

Genetic variability

Overall, genetic variability found in *S. aquifolium* from Southeast Asia is comparable to that for other species of

Table 3 Hierarchical analysis of molecular variance (AMOVA) based on ITS2, *Rbc* spacer, and *Cox3* for *Sargassum aquifolium*

Number of groups	Groupings	% var*	Φ_{CT}
ITS2			
3	(BC, NT, OB, PC, PD, PS, RE, SA, SE, TE, SI, MB, WJ, PR)(SO, NC, VA, FI)(TB, PG, CC)	10.41	0.10
4	(NT, OB, PC, PD, PS, RE, SA, SE, TE, SI, MB, WJ, PR)(SO, NC, VA, FI)(TB, PG, CC)(BC)	5.56	0.05
4	(WJ, SI, TE, MB, SE, PC, PD, SA, PR, PS)(NT, OB, RE, BC)(VA, SO, FI, NC)(CC, TB, PG)	24.20	0.24
4	(WJ, SI, TE, MB, SE, PC, PD, SA, PR, PS, RE, BC, OB, NT)(FI, VA, SO)(TB, CC, PG)(NC)	38.39	0.38
5	(WJ, SI, TE, MB, SE, PC, PD, SA, PR, PS)(NT, OB, RE, BC)(VA, SO, FI)(CC, TB, PG)(NC)	**51.08**	0.51
Rbc spacer			
3	(WJ, TE, SE, SA, PS, PR, PC, OB, MB, BC)(VA, SO, NC, FI)(TB, PG, CC)	18.54	0.19
4	(BC, OB)(PC, PS, MB, TE, SE, SA, WJ)(CC, PG, TB)(VA, SO, FI, NC)	8.86	0.09
5	(WJ, TE, SE, SA, PS, PR, PC, OB, MB, BC)(VA, SO, FI)(TB, PG, CC)(NC)	**58.46**	0.58
Cox3			
3	(BC, MB, MS, OB, PC, PS, SA, SE, TE, SI, WJ)(CC, TB, PG)(SO, VA, FI, NC)	24.44	0.49
4	(BC, MB, MS, OB, PC, PS, SA, SE, TE, SI, WJ)(CC, TB, PG)(SO, VA, FI)(NC)	**31.68**	0.32

*Largest percentage of variance in bold. *p* values are all <0.005

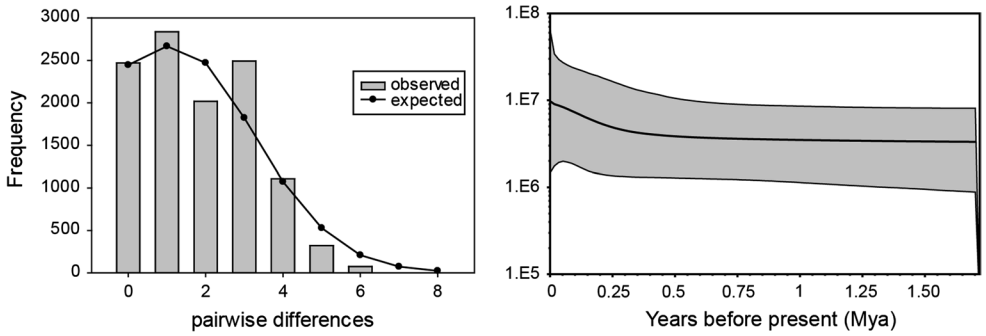

Fig. 3 Mismatch distribution (*left*) and Bayesian skyline plot (*right*) of *Sargassum aquifolium* from *Cox3* sequences. Bar chart and line graph in mismatch distribution indicate the observed and expected frequency, respectively. Bayesian skyline plots are shown in effective population size with function of time (year before present in ka). 95 % confidence interval is shaded in *gray*

brown macroalgae. For example, 21 haplotypes of *Cox3* were revealed in the present study, 17 in *Ishige okamurae* (Lee et al. 2012), and 9 in *Undaria pinnatifida* (Uwai et al. 2006) in northwestern Pacific. In contrast, genetic diversity among *Sargassum* spp. varies across regions and among species. For ITS2, eight haplotypes were revealed in the present study, two in *S. hemiphyllum* (Cheang et al. 2010a) in northwestern Pacific and 1 in *S. muticum* (Cheang et al. 2010b) from northwestern Pacific to Europe and North America. For *Rbc* spacer, nine haplotypes were revealed in the present study, three in *S. hemiphyllum* (Cheang et al. 2010a), one in *S. muticum* (Cheang et al. 2010b), and nine haplotypes in *S. horneri* (Hu et al. 2011). It was suggested that *S. muticum* evolved too recently to accumulate mutations causing the low genetic variability across a wide region (Bae et al. 2013). The genetic variability may thus reflect the difference in the evolutionary history experienced by these species across regions and/or the dispersal ability of the species that led to the present genetic pattern observed.

Among the three molecular markers used, *Cox3* was found to have the highest variability in this study and was widely used in phylogeography of macroalgae in northwestern Pacific (Uwai et al. 2006; Hu et al. 2011; Lee et al. 2012). ITS2 and *Rbc* spacer have relatively lower variability than *Cox3*, but consistent genetic patterns could still be observed based on groupings of hierarchical AMOVA. They can serve as alternative markers besides mitochondrial genome for *S. aquifolium*, but markers with higher resolution power may be required for different species of *Sargassum* with different genetic variability.

Population structure

In general, lack of population structure was revealed within Southeast Asia, especially along the Sunda Shelf region, including the Gulf of Thailand. All populations within this region form a single group in hierarchical AMOVA based on *Rbc* spacer and *Cox3* (Table 3). For ITS2, hierarchical

AMOVA revealed one more group in Southeast Asia (i.e., South China Sea/Gulf of Thailand and West Peninsular Malaysia/West Java) which is mainly contributed by the differentiation of OB population from the other populations, as shown in pairwise differences (Table S1, Electronic Supplementary Material). However, shared haplotypes were observed between the two regions, suggesting that gene flow is not limited. More sampling sites would be needed to reveal any genetic break that may be present between South China Sea/Gulf of Thailand and Java Sea, as indicated in false clown anemonefish (*Amphiprion ocellaris*) and giant clam (*Tridacna crocea*) (Kochzius and Nuryanto 2008; Timm and Kochzius 2008).

The lack of population structure of *S. aquifolium* in Southeast Asia is unexpected given that complex geological history in the region is well established, involving fluctuating sea levels during Pleistocene that potentially restricted gene flow. Generalized concordant breaks found along the edge of Sunda Shelf on the Indian Ocean side and also along northern Java in the Java Sea support the presence of genetic barrier (i.e., the Sunda Shelf) (Carpenter et al. 2011). The incongruence in genetic pattern for *S. aquifolium* may be related to its rapid and successful dispersal after the Last Glacial Maximum so that no divergence of lineages could be revealed. *Sargassum* is capable of dispersing for long distance in the form of drifting fronds with germlings (Komatsu et al. 2007). Unlike other *Sargassum* spp. that exhibit only a single-peak reproductive season once a year (Ang 2006), *S. aquifolium* is capable of active reproduction throughout the whole year so that continuous production of germlings is possible and new recruits could be found every month (Yeong and Wong 2012). This reproductive strategy is in sharp contrast to animals with limited larval mobility (Carpenter et al. 2011) and allowed rapid re-establishment of gene flow with present-day monsoonal currents after the Glacial Maxima (Wyrtki 1961). While it is also possible that the variability of the markers used in this study may be too low to reveal any divergence of lineages, this is unlikely given that the same markers were used to reveal a high variability in the genetic lineage of *S. ilicifolium*, a closely related species (Chan et al. submitted).

Hierarchical AMOVA showed differentiation in the spatial grouping of Guam (TB, PG, and CC), Pacific Islands (SO, VA, and FI), and NC, indicating that there may be genetic breaks elsewhere but not within the sampling area covered in this study. Samplings were carried out around southern Philippines, Sulawesi, and Java in Indonesia in the Coral Triangle region, but no populations of *S. aquifolium* were found. Further efforts need to be carried out to fill up this sampling gap between Southeast Asia and the Pacific Islands in order to confirm the presence of this genetic break. Nonetheless, private haplotypes were found in NC in all markers indicating isolation and limited gene flow in this site. Branches of south equatorial counter current (SECC) connect Fiji, Vanuatu, and Solomon Islands together and combine to form the New Guinea Coastal Current (NGCC) (Fig. 4, Benzie and Williams 1997; McGregor et al. 2008). NC is influenced by the downward branch of SECC so that upward gene flow may not be likely. In addition, NGCC flows along and east of Papua New Guinea to form the North Equatorial Counter Current (NECC). While this current circulation may allow genetic exchange between Pacific Islands, it may limit the gene flows to the west of

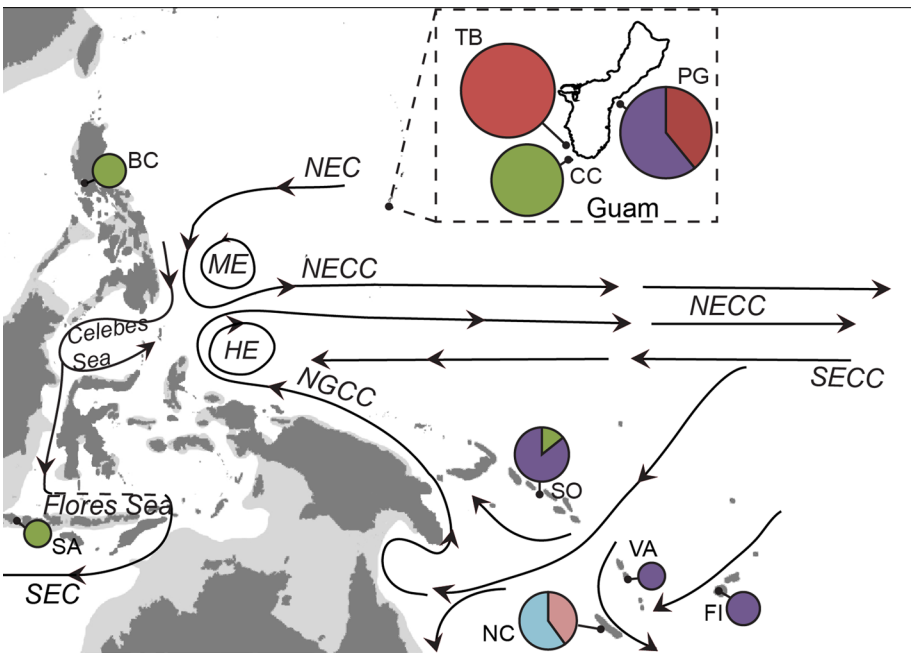

Fig. 4 Detailed current flow among Pacific Islands with haplotype distribution of *Cox3*. Detailed current flows are modified from Benzie and Williams (1997) and McGregor et al. (2008). *SEC* South Equatorial Current; *SECC* South Equatorial Counter Current; *NEC* North Equatorial Current; *NECC* North Equatorial Counter Current; *NGCC* New Guinea Coastal Current; *ME* Mindanao Eddy; *HE* Halmahera Eddy

Coral Triangle. Similarly, Guam populations were isolated from the west of Coral Triangle where private haplotypes were revealed. Such restricted current flow has also been suggested to limit genetic exchange in animal populations in the Coral Triangle region. In the study of three stomatopods, deep genetic divergence was revealed between Papua New Guinea and Indonesia and restricted current flow in the Maluku Sea in between was suggested to be a barrier that limited larval dispersal (Barber et al. 2006).

Demographic history

Estimated expansion time of *S. aquifolium* from mismatch distribution was 2.04–3.06 Mya, late Pliocene to early Pleistocene. During that period, sea level was fluctuating within 50–120 m below present level (Miller 2009). The scenario of *S. aquifolium* expansion and recolonization into the Sunda Shelf region resembles model III of contemporary phylogeographical pattern suggested by Maggs et al. (2008). The population structure of *S. aquifolium* observed today may be the result of populations deriving from two or more refugia between Southeast Asia and Pacific Islands/Guam. No secondary contact was achieved because of isolation by limited current exchange. It is hypothesized that *S. aquifolium* survived in east of Sunda Shelf during the Last Glacial Maximum and expanded to Guam and Pacific Islands. Recent recolonization to Sunda Shelf occurred, as indicated by gradual increase in population size in Bayesian skyline plot since 0.25 Mya, late Pleistocene (Fig. 3). Lack of population structure within the Sunda Shelf region may be the result of most recent recolonization. Previous population expansion cannot be revealed in Bayesian skyline plot (2.04–3.06 Mya from mismatch distribution analysis) as it is erased by the most recent signal in the Last Glacial Maximum (Grant et al. 2012). Therefore, the flat shape of Bayesian skyline plot before the Last Glacial Maximum cannot truly reflect the demographic history of this species.

Conclusion

Populations of *S aquifolium* were found to be genetically homogeneous within the Sunda Shelf region, but different from those from Guam and the western Pacific Islands. It is suggested that a genetic break likely exists in the middle of Coral Triangle where limited current flow through the Maluku Sea would have restricted gene flow between these regions. A scenario is proposed in which populations of *S. aquifolium* are suggested to persist east of Sunda Shelf and western Pacific during the Last Glacial Maximum and more recently recolonized in the Sunda Shelf, resulting in homogeneity of its population in the Sunda Shelf region due to recent expansion. Rapid expansion may be facilitated by year-round reproduction of this species, where germlings carried on floating thalli could be dispersed by complex oceanographic currents within this region.

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