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RESEARCH ARTICLE

Closely related and sympatric but not all the same: genetic variation of Indo-West Pacific *Rhizophora* mangroves across the Malay Peninsula

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Abstract Members of the mangrove genus *Rhizophora* represent the most commonly occurring and highly valued species in the Indo-West Pacific region. However, to date, few studies have been directed towards the understanding of their genetic variation. The levels and patterns of genetic variation at chloroplast and nuclear gene regions were studied in *R. apiculata*, *R. mucronata*, and *R. stylosa* sampled from Southeast Asia and Japan. All three species were characterized by low intraspecific genetic variation and a deficiency of heterozygotes in populations within the region, consistent with findings in studies on other mangrove species. *Rhizophora mucronata* and *R. stylosa* were also found to be more closely related than any of them with *R. apiculata*. During the Last Glacial Maximum, sea levels dropped to 120 m below the current levels, exposing part of the Sunda Shelf that became a barrier that limited gene flow between marine species living in the Pacific and Indian Oceans. Today, the Malay Peninsula is thought to still serve as a barrier to gene flow between populations occurring on its coasts. The pattern of genetic differentiation of *R. apiculata* supports the hypothesis of the land barrier effect of the Malay Peninsula, but such patterns were not found in *R. mucronata* and *R. stylosa*. Our findings suggest that *R. apiculata*, *R. mucronata*, and *R. stylosa* have different demographic histories despite being closely related and having sympatric distributions today. Furthermore, all three species appear to have high levels of inbreeding due to limited pollen and propagule dispersal, and that both these factors contributed to population differentiation.

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Introduction

Mangroves are typically tropical or occasionally subtropical plants (Duke et al. 1998; Giri et al. 2011), and can be broadly categorized into the Atlantic East Pacific (AEP) and the Indo-West Pacific (IWP) zones (Duke et al. 1998; Tomlinson 1986). Mangrove forests have been shown to be important coastal resources (reviewed by Kathiresan 2012) and an integral part in supporting various marine ecosystems (Lee and Kwok 2002; Wolanski et al. 1997) and mitigating natural disasters (Osti et al. 2009). Unfortunately, due to overexploitation and rapid development of coastal regions in many parts of the world, these forests are facing rapid decline. In the regions of South and Southeast Asia, the main causes of deforestation of the mangroves are agriculture, aquaculture, and urban development (Giri et al. 2008). Other contributing factors include those that impoverish the quality of the mangrove forests (Polidoro et al. 2010), such as pollution (Valiela et al. 2001), specific harvesting of highly valued mangrove species like *Rhizophora* (Polidoro et al. 2010), and natural hazards (e.g. 2004 Indian Ocean tsunami disaster in Aceh, Indonesia; I.Z. Siregar, personal observation in 2007) that further deteriorate the forests. Duke et al. (2007) suggested that with the current rate of deterioration and clearing of mangrove areas, the mangrove ecosystem will be gone in 100 years.

With the rapid loss of mangrove coverage area, it is important to understand the genetic variation of the various mangrove species in order to identify areas of conservation priority. Many earlier studies have reported low genetic variation and high differentiation between mangrove populations of both sides of the Malay Peninsula (MP) in Southeast Asia, including *Ceriops tagal* (Ge and Sun 2001; Liao et al. 2007), *Bruguiera gymnorrhiza* (Minobe et al. 2010), *Excoecaria agallocha* (Zhang et al. 2008), and *Rhizophora apiculata* (Inomata et al. 2009). For mangrove species, the ability to disperse propagules via sea currents also limits their dispersal across a land barrier. One commonly regarded land barrier in the Southeast Asian region is the Sundaland that formed most recently during the Last Glacial Maximum (LGM) period approximately 20,000 years ago when sea levels dropped to about 120 m below the current level and exposed part of the Sunda Shelf (Voris 2000). The Sundaland was thought to be a major land barrier to the dispersal of many marine species between the Indian Ocean and the Pacific Ocean, causing high genetic differentiation between species present on the coasts of both oceans (Crandall et al. 2008; Fitzpatrick et al. 2011). Today, the MP that was part of the Sundaland is thought to still serve as a land barrier for gene flow between populations occurring on its coasts.

In Southeast Asia, *Rhizophora* mangroves predominate and are of economic importance, especially for the local communities living near the mangroves (Mohd Nasir and Safiah Yusmah 2007). Despite having different total ranges, all three species of IWP *Rhizophora* (namely *R. apiculata*, *R. mucronata*, and *R. stylosa*) co-occur in Southeast Asia (Duke 2006). In a preliminary study on *R. apiculata* and *R. mucronata* using samples from three populations, Inomata et al. (2009) found low genetic diversity, and excess of homozygotes as compared to Hardy–Weinberg expectations. They attributed those results to inbreeding and/or the Wahlund effect. They also found significant genetic differentiation between populations of *R. apiculata* on the west and east coasts of the MP but such pattern was not strongly supported in *R. mucronata*, possibly due to low genetic variation. In the current study, we included additional samples of *R. apiculata* and *R. mucronata*, as well as samples of a close relative, *R. stylosa*, to better test the effects of the reproductive system and the MP as a land barrier, on the IWP *Rhizophora*. Understanding the levels and patterns of genetic variation in these mangrove species is an important step towards understanding the distribution of mangrove genetic diversity, which in turn assists in the better management of mangrove forests.

Molecular methods used for genetic variation studies in mangrove species so far mainly include hypervariable markers such as microsatellites (Arnaud-Haond et al. 2006; Jian et al. 2010; Maguire et al. 2000; Salas-Leiva et al. 2009), AFLP (Maguire et al. 2002), and RAPD (Lakshmi et al. 2000). Under these methods, markers are usually selected for showing high variation and are usually species-specific. This poses questions on the feasibility and consistency of comparing genetic variation data among species genotyped using different markers. On the other hand, studying mangrove genetic variation at coding gene regions is useful not only in estimating genetic variation and inferring population structure (Huang et al. 2008; Inomata et al. 2009; Minobe et al. 2010), but also for the estimation of demographic parameters (Urashi et al. 2013; Zhou et al. 2007) and the detection of natural selection (Zhou et al. 2011). Moreover, the same gene regions can be amplified across different species and compared to yield other useful inferences, such as for hybrid identification (Guo et al. 2011; Ng et al. 2013; Zhou et al. 2005).

In this study, we analyzed genetic variation in chloroplast and nuclear gene regions of *R. apiculata*, *R. mucronata*, and *R. stylosa* sampled from Southeast Asia and Japan, with a focus on populations around the Malay Peninsula. We then discuss the important effects of the

Table 1 List of sampling sites and samples analyzed in this study

No.	Sampling site	Location	Relative location (to the Malay Peninsula)	Sample size (No. of individuals)		
				R. apiculata	*R. mucronata*	*R. stylosa*
1.	PHU	Phuket, Thailand	West	25	20	–
2.	KRA	Krabi, Thailand	West	22	4	–
3.	TR	Trang, Thailand	West	9[a]	14[a]	–
4.	SP	Samut Prakan, Thailand	East	29	15	–
5.	SS	Samut Songkhram, Thailand	East	32	16	–
6.	BK	Bangkok, Thailand	East	11[a]	12[a]	–
7.	ST	Surat Thani, Thailand	East	13[a]	13[a]	–
8.	KRT	Kurong Tengar, Perlis, Malaysia	West	5	3	7
9.	SGM	Sungai Merbok, Kedah, Malaysia	West	3	8	–
10.	MTG	Matang, Perak, Malaysia	West	10	2	–
11.	BLS	Bagan Lalang, Selangor, Malaysia	West	4[b]	3[b]	15[b]
12.	PBS	Pulau Burung, Negeri Sembilan, Malaysia	West	2[b]	2[b]	9[b]
13.	PBM	Pulau Besar, Melaka, Malaysia	West	–	–	11
14.	PMJ	Pulau Mawar, Johor, Malaysia	East	5	–	15
15.	LBS	Labuk Bay, Sandakan, Sabah, Malaysia	East	10	–	–
16.	JK	Jakarta, Indonesia	South	2	–	–
17.	MEN	Menjangan, Bali, Indonesia	South	14	–	–
18.	PND	Panacan, Davao City, Philippines	East	20	–	–
19.	PPF	Pearl Farm, Davao City, Philippines	East	4	–	–
20.	FNR	Funaura Bay, Iriomote, Okinawa, Japan	East	–	–	20
21.	URC	Urauchi Estuary, Iriomote, Okinawa, Japan	East	–	–	20
		Total		220	112	97

Also included are samples used in studies by [a] Inomata et al. (2009) and [b] Ng et al. (2013)

reproductive system and migration history on the levels and patterns of genetic variation of the species. In particular, we show that due to different migration routes of individual species, the MP could have played different roles in shaping patterns of genetic variation in all mangrove species, even closely related ones that occur sympatrically today. We also suggest that limited pollen and propagule dispersal contributed to population differentiation, and that inbreeding appears to be common in *Rhizophora* species.

Materials and methods

Plant material

Leaf samples of 187 *R. apiculata*, 73 *R. mucronata*, and 97 *R. stylosa* individuals were collected from Thailand, Malaysia, Indonesia, the Philippines, and Japan, in addition to the materials collected for the study by Inomata et al. (2009). As much as possible, the different *Rhizophora* species were sampled in the same locations. However, *R. stylosa* has been observed to occur at very specific sites, and usually few, if any, *R. apiculata* or *R. mucronata* occur at those sites. The details on the total samples and their sampling locations are shown in Table 1 and Fig. 1. Leaf samples were collected and stored in silica gel before further analyses.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from approximately 20 mg of silica gel-dried leaf material using the DNeasy Plant Mini Kit (QIAGEN)

One chloroplast DNA (cpDNA) region: *atpB–rbcL* intergenic spacer; and five partial nuclear gene (nDNA) regions: *DLDH* (dihydrolipoamide dehydrogenase), *LAS* (lipoic acid synthase), *mang-1* (mangrin), *PAL1* (phenylalanine ammonia lyase), and *SBE2* (starch branching enzyme), were amplified from the genomic DNA of *R. apiculata*, *R. mucronata*, and *R. stylosa*. The primers used for PCR and the corresponding annealing temperatures (T_a) are listed in Supporting Table S1. PCR amplifications were performed in 20 µl reaction mixtures, each containing 10–50 ng of genomic DNA, 1 × *Ex-Taq* buffer (2 mM of Mg^{2+}; TaKaRa Bio Inc.), dNTP mixture (0.2 mM of each dNTP; TaKaRa Bio Inc.), 0.2 µM of each primer, and 1.0 U of *Ex-Taq* DNA polymerase (TaKaRa Bio Inc.). The

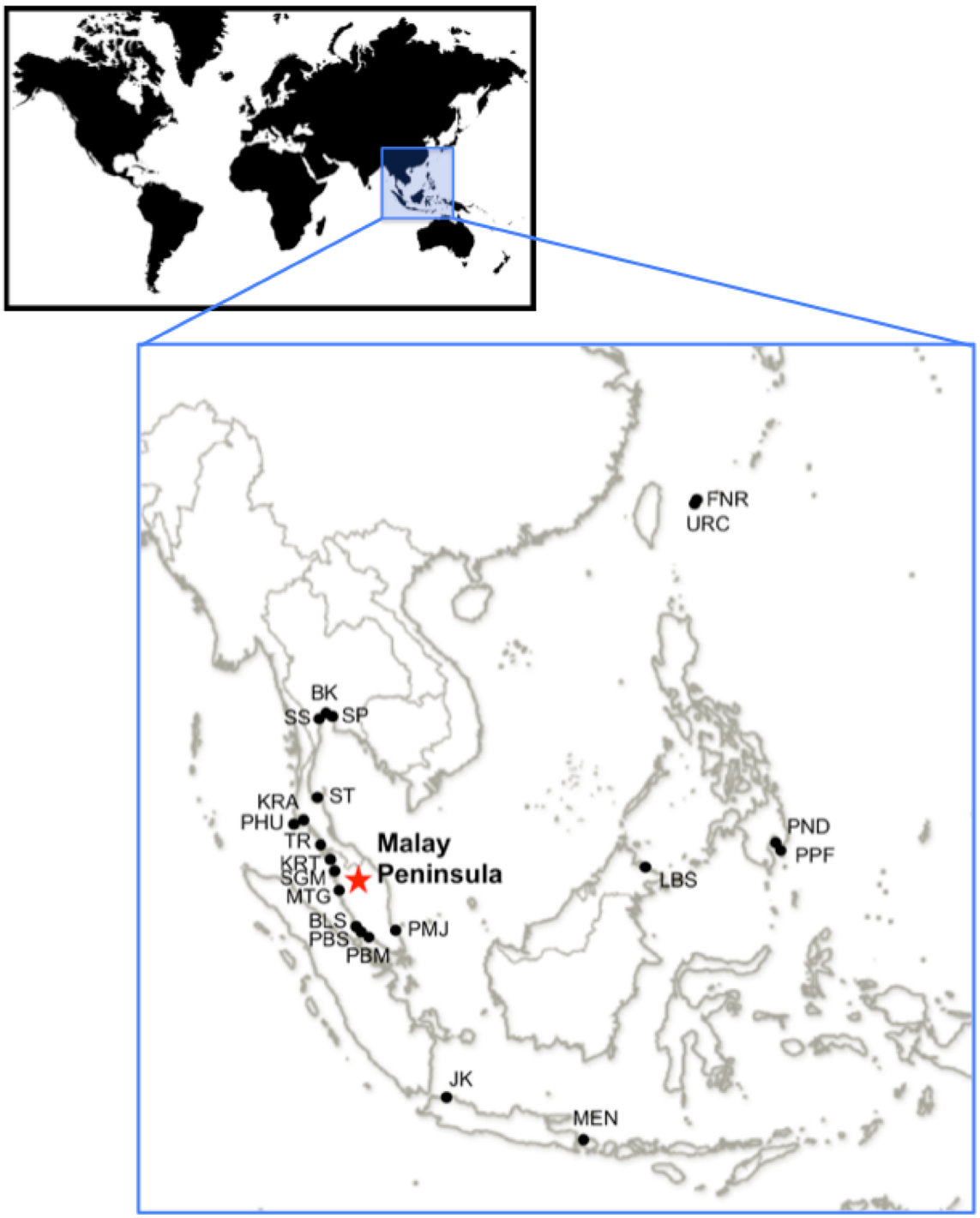

Fig. 1 Map showing sampling sites for this study

PCR reaction profile comprised of an initial denaturation of 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at T_a and 2 min at 72 °C, and finally an extension step at 72 °C for 7 min.

Purified PCR products were used for direct sequencing. Sequencing reactions were carried out using the BigDye® Terminator ver.3.1 Cycle Sequencing Kit (Applied Biosystems) and the products were analyzed on an ABI 3730 DNA Analyzer (Applied Biosystems). For sequences obtained through direct sequencing that had only one or no heterozygous sites, sequences of both haplotypes were directly inferred. When two or more heterozygous sites or

indels were detected by direct sequencing, the purified amplification products were cloned into the pGEM T-easy vector (Promega) and sequenced. To eliminate PCR artifacts, a haplotype was confirmed only when identical sequences from two or more clones were found. The different sequences obtained in this study have been deposited in GenBank with the accession numbers KM288624-KM288694, and the full sequence alignments were deposited as Supplementary Material.

Data analyses

Nucleotide sequences were assembled and edited using the software ATGC ver. 6.0 (GENETYX CORPORATION). For the purpose of having a more thorough study, sequences obtained in the studies by Inomata et al. (2009) and Ng et al. (2013) were included into our total data set. Sequence alignments were performed using Clustal W (Thompson et al. 1994) implemented in MEGA 5 (Tamura et al. 2011) and corrected manually. Standard population genetic statistics, including the number of segregating sites (S), number of insertion-deletion (indel) mutations, number of haplotypes (H), haplotype diversity (Hd) (Nei 1987), nucleotide diversity (π) (Nei 1987), and the Tajima's D (Tajima 1989) were determined using DnaSP ver. 5.10 (Librado and Rozas 2009). Nucleotide divergence between species at every nDNA locus was also estimated using DnaSP ver. 5.10. To illustrate the relationship among cpDNA haplotypes, a haplotype network was constructed using the median-joining model (Bandelt et al. 1999) implemented in NETWORK ver. 4.6.1.1 (fluxus-engineering.com).

To investigate the population structuring within each species of *Rhizophora*, the partitioning of genetic variation at three hierarchical levels—among populations, among individuals within populations, and within individuals, was estimated. Fixation indices (F_{ST}, F_{IS}, and F_{IT}) were estimated from the allele frequencies of the multilocus nDNA data (combination of the five nDNA loci) using the locus-by-locus analysis of molecular variance (AMOVA) implemented in Arlequin ver. 3.5 (Excoffier and Lischer 2010). Then, pairwise F_{ST} between pairs of populations were estimated. The significance of the results of AMOVA and fixation indices was tested by 10,000 permutations. Neighbor-Joining (NJ) trees were also constructed from the population pairwise F_{ST} matrix of each species using MEGA 5 to better visualize the relationship among populations as suggested by the population pairwise F_{ST} values.

To further infer the patterns of population structuring in each species, the Bayesian clustering software STRUCTURE ver. 2.3.4 (Falush et al. 2003; Pritchard et al. 2000) was used to assign individuals of the same species to a given number of (K) populations according to their multilocus nuclear genotypes. The program was run with 100,000 burn-ins followed by 100,000 MCMC iterations. Ten independent runs were performed under the admixture model for each number of K, according to the total number of populations sampled for each species (i.e. $K = 1$–18 for *R. apiculata*, $K = 1$–12 for *R. mucronata*, and $K = 1$–7 for *R. stylosa*). The web-based software Structure Harvester ver. 0.6.93 (Earl and vonHoldt 2012) was used to obtain the average log likelihood Ln P(D) and ΔK (Evanno et al. 2005) for each K from the results of STRUCTURE. Finally, the CLUMPP (Jakobsson and Rosenberg 2007) and DISTRUCT (Rosenberg 2004) programs were used to graphically visualize the clustering results.

Later, populations of each species were grouped based on different clustering scenarios, and the genetic differentiation among clusters (F_{CT}) was estimated using Arlequin ver. 3.5. We first arbitrarily grouped populations of each species located on the MP into the "west coast" and "east coast" clusters to estimate genetic differentiation across the MP. Then, populations were grouped into different clusters based on the STRUCTURE inference to test the significance of such population clustering.

Results

DNA variation and test of neutrality

DNA sequence data from a total of 220 *R. apiculata*, 112 *R. mucronata*, and 97 *R. stylosa* samples were included in this study. Of the one cpDNA and five nDNA loci used as markers, some could not be amplified in several individuals and were subsequently treated as missing data. The results of the alignment length, and nucleotide variation of sequences obtained at each locus for each species are summarized in Table 2.

The levels of nucleotide variation varied across the loci studied in *R. apiculata*, *R. mucronata*, and *R. stylosa*. For the cpDNA locus, only indel mutations and no segregating sites were found in each of the species. In total, six cpDNA haplotypes were found in all IWP *Rhizophora* samples. Three haplotypes (cp1–cp3) were specific to *R. apiculata*, two haplotypes (cp4–cp5) were found mainly in *R. mucronata* and several *R. stylosa* individuals, and one haplotype (cp6) was specific to *R. stylosa*. Supporting Fig. S1 shows the relationship among all the observed cpDNA haplotypes. The cpDNA haplotypes of *R. apiculata* were differentiated from those of the other two *Rhizophora* species by at least five mutations, while the majority of cpDNA haplotypes found in *R. mucronata* and *R. stylosa* were only one mutation step apart. For the nDNA loci, the number of nucleotide differences per silent site (π_s) of the

Table 2 Nucleotide variation in *R. apiculata*, *R. mucronata*, and *R. stylosa*

Locus	Species	Length/L_s (bp)	*n*	*S*	Indel	*H*	*Hd*	π_t	π_s	π_a
cpDNA										
atpB–rbcL	*R. apiculata*	811/685.50	208	0	2	3	0.137	0	0	0
	R. mucronata	813/688.50	104	0	1	2	0.348	0	0	0
	R. stylosa	828/688.50	97	0	2	3	0.284	0	0	0
nDNA										
DLDH	*R. apiculata*	1,221/385.00	422	6	0	8	0.156	0.00013	0.00029	0.00006
	R. mucronata	1,221/385.89	188	4	0	4	0.221	0.00046	0.00090	0.00026
	R. stylosa	1,221/386.32	190	3	0	3	0.029	0.00019	0.00047	0.00006
LAS	*R. apiculata*	1,022/565.79	418	8	2	8	0.700	0.00132	0.00238	0
	R. mucronata	983/533.17	202	3	0	2	0.225	0.00069	0.00127	0
	R. stylosa	983/533.17	188	3	0	2	0.120	0.00037	0.00068	0
mang-1	*R. apiculata*	883/527.46	428	3	2	5	0.673	0.00109	0.00176	0
	R. mucronata	880/556.00	220	2	0	3	0.211	0.00026	0.00041	0
	R. stylosa	880/556.00	192	3	0	4	0.548	0.00116	0.00183	0
PAL1	*R. apiculata*	864/210.67	434	6	0	6	0.317	0.00043	0.00050	0.00041
	R. mucronata	864/210.42	224	5	0	5	0.290	0.00063	0.00025	0.00075
	R. stylosa	864/210.65	194	3	0	3	0.109	0.00033	0.00103	0.00011
SBE2	*R. apiculata*	1,274/1077.83	424	3	2	6	0.352	0.00022	0.00026	0
	R. mucronata	1,265/1,077.50	202	4	0	2	0.029	0.00009	0.00011	0
	R. stylosa	1,265/1,077.50	192	4	0	2	0.118	0.00037	0.00044	0
Average across 5 nDNA loci	*R. apiculata*	1052.80/553.35	n/a	5.2	1.2	6.6	0.440	0.00064	0.00104	0.00009
	R. mucronata	1,044.40/552.60	n/a	3.6	0	3.2	0.195	0.00043	0.00059	0.00020
	R. stylosa	1,044.40/552.73	n/a	3.2	0	2.8	0.185	0.00048	0.00089	0.00003

Length/L_s (bp): Sequence alignment length including gaps/number of silent sites excluding alignment gaps

n no. of sequences. *S* no. of segregating sites excluding indels. *Indel* no. of indel mutations

A continuous alignment gap was considered as a single indel. *H* no. of haplotypes, indels included. *Hd* haplotype diversity. π_t no. of nucleotide differences per total site (nucleotide diversity) with the Jukes and Cantor (JC, 1969) correction, indels not included. π_s no. of nucleotide differences per silent site (synonymous or non-coding site) with the JC (1969) correction, indels not included. π_a no. of nucleotide differences per nonsynonymous site with the JC (1969) correction, indels not included. *n/a* not applicable

total samples for each locus, with the Jukes and Cantor (1969) correction, ranged from 0.00026 (*SBE2*) to 0.00238 (*LAS*) for *R. apiculata* (average $\pi_s = 0.00104$), from 0.00011 (*SBE2*) to 0.00127 (*LAS*) for *R. mucronata* (average $\pi_s = 0.00059$), and from 0.00044 (*SBE2*) to 0.00183 (*mang-1*) for *R. stylosa* (average $\pi_s = 0.00089$). There were few indel mutations in the sequences, ranging from 0 to two in both the cpDNA and nDNA sequences of *R. apiculata*; while in *R. mucronata* and *R. stylosa*, indel mutations were only found in the cpDNA sequences. Given that genetic variation was much higher at the nDNA loci compared to cpDNA locus and thus more informative, only the nDNA data was used for most subsequent analyses.

All Tajima's *D* values were not significant at the species level across all five nDNA loci, thus there was no evidence for deviation from neutrality at the loci analyzed in this study. Nucleotide divergence at the silent sites (K_{sil}) averaged at about 2.8 % for *R. apiculata–R. mucronata*, 3.0 % for *R. apiculata–R. stylosa*, and 0.4 % for *R. mucronata–R. stylosa*. The results of nucleotide divergence are summarized in Supporting Table S5.

Population structure

Significant genetic differentiation among populations (F_{ST}) was detected over the cpDNA and nDNA data of all species, indicating the presence of population structuring among the sampled populations. The inbreeding coefficient (F_{IS}) values for all species were positive and significant, indicating departure from the Hardy–Weinberg equilibrium with a deficiency of heterozygotes in the populations studied. AMOVA revealed that most of the genetic variation at the nDNA loci resided among populations in *R. apiculata* (56.0 %) and *R. stylosa* (72.7 %), but not in *R. mucronata* (42.1 %). Low genetic variation was found to reside within populations of all species (6.8–13.8 %). Results of the fixation indices and AMOVA are summarized in Supporting Table S2 and Supporting Table S3, respectively.

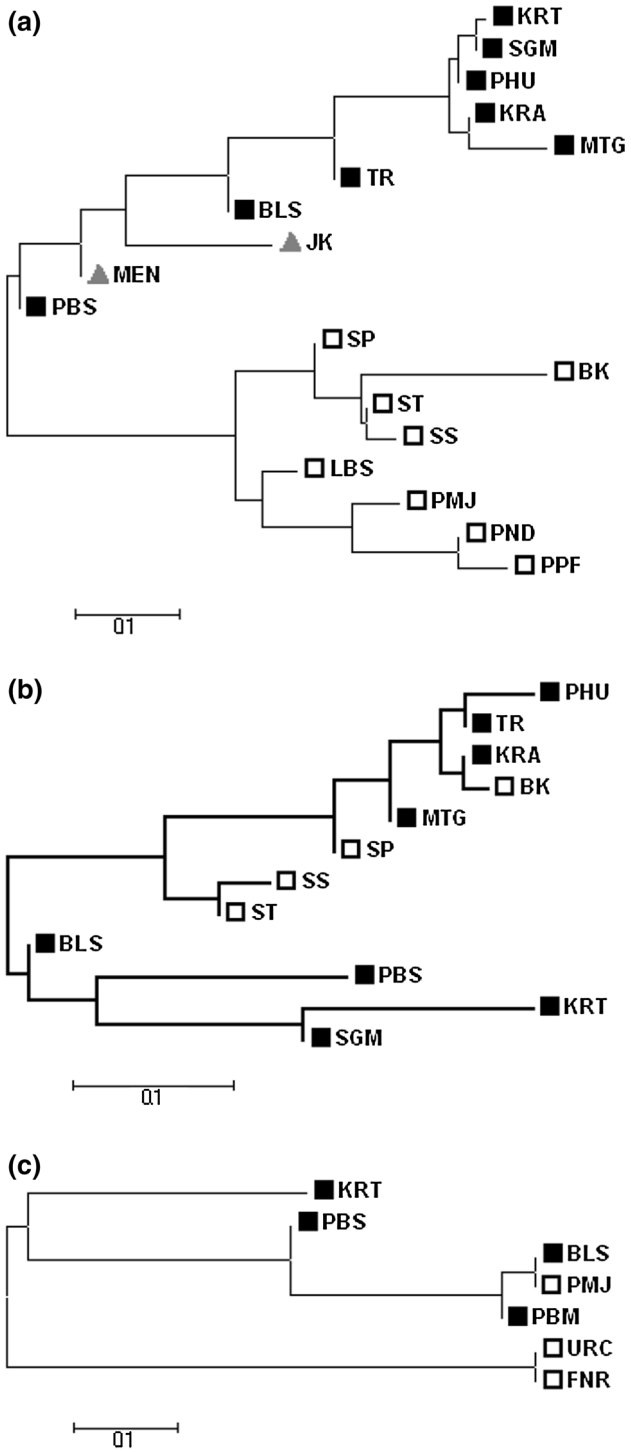

Fig. 2 Neighbor-joining trees constructed using population pairwise F_{ST} values for **a** *R. apiculata*, **b** *R. mucronata*, and **c** *R. stylosa*. *Filled square* population located to the west of the MP; *open square* population located to the east of MP; *grey triangle* population located to the south of the MP

Population pairwise F_{ST} matrices are summarized in Supporting Table S4. In *R. apiculata*, population pairs involving populations located to the east and to the west of the MP usually generated higher pairwise F_{ST} estimates than population pairs involving populations of the same side of the MP. Such pattern was not found in *R. mucronata* or *R. stylosa*. *Rhizophora stylosa* populations of the peninsula, however, were clearly differentiated from the populations of Japan (pairwise $F_{ST} = 0.684–1.000$). The resultant NJ trees gave a better picture for the interpretation of relationship among populations based on the estimated pairwise F_{ST} values. As observed from the NJ trees in Fig. 2, *R. apiculata*, *R. mucronata*, and *R. stylosa* populations grouped differently. *Rhizophora apiculata* populations grouped into two main clusters: one consisting of populations to the west and to the south of the MP, and the other consisting of populations to the east of the MP. *Rhizophora mucronata* populations grouped into two clusters without any particular order—populations to the east and to the west of the MP were intermixed within the clusters. *Rhizophora stylosa* grouped into two main clusters: one consisting of populations on the MP, and the other consisting of populations in Japan.

The STRUCTURE analysis revealed different patterns of population clustering among species. For *R. apiculata*, the plot of Ln $P(D)$ plateaued after $K = 2$ or 4, while the Evanno's ΔK peaked at $K = 2$. Using the smallest K (i.e. $K = 2$) to capture the major population structure, *R. apiculata* populations to the west of the MP clustered together with the populations located to the south of the MP, away from the other cluster containing populations to the east of the MP. For *R. mucronata*, the plot of Ln $P(D)$ plateaued after $K = 2$, while ΔK peaked at $K = 2$. Although this seems to suggest an optimal of two population clusters, no spatial clustering pattern was observed. Admixture was observed in most of the *R. mucronata* populations. For *R. stylosa*, the plot of Ln $P(D)$ plateaued after $K = 3$, while ΔK peaked at $K = 3$, confidently placing the number of clusters as $K = 3$. Using $K = 3$, admixture was observed in populations KRT and PBS, and *R. stylosa* populations on the MP clustered away from those in Japan. The plots and the resulting graphical representation of population clusters are shown in Fig. 3.

From the estimation of genetic differentiation across the MP, populations located on the west and east coasts of the MP were found to be significantly differentiated in *R. apiculata*, but not in *R. mucronata* and *R. stylosa*. When *R. apiculata* and *R. stylosa* populations were grouped based on the STRUCTURE output, significant genetic differentiation was observed among the clusters. Due to the lack of a clustering pattern in the STRUCTURE output, only one scenario was tested in *R. mucronata*. The estimates of genetic differentiation under each scenario are summarized in Table 3.

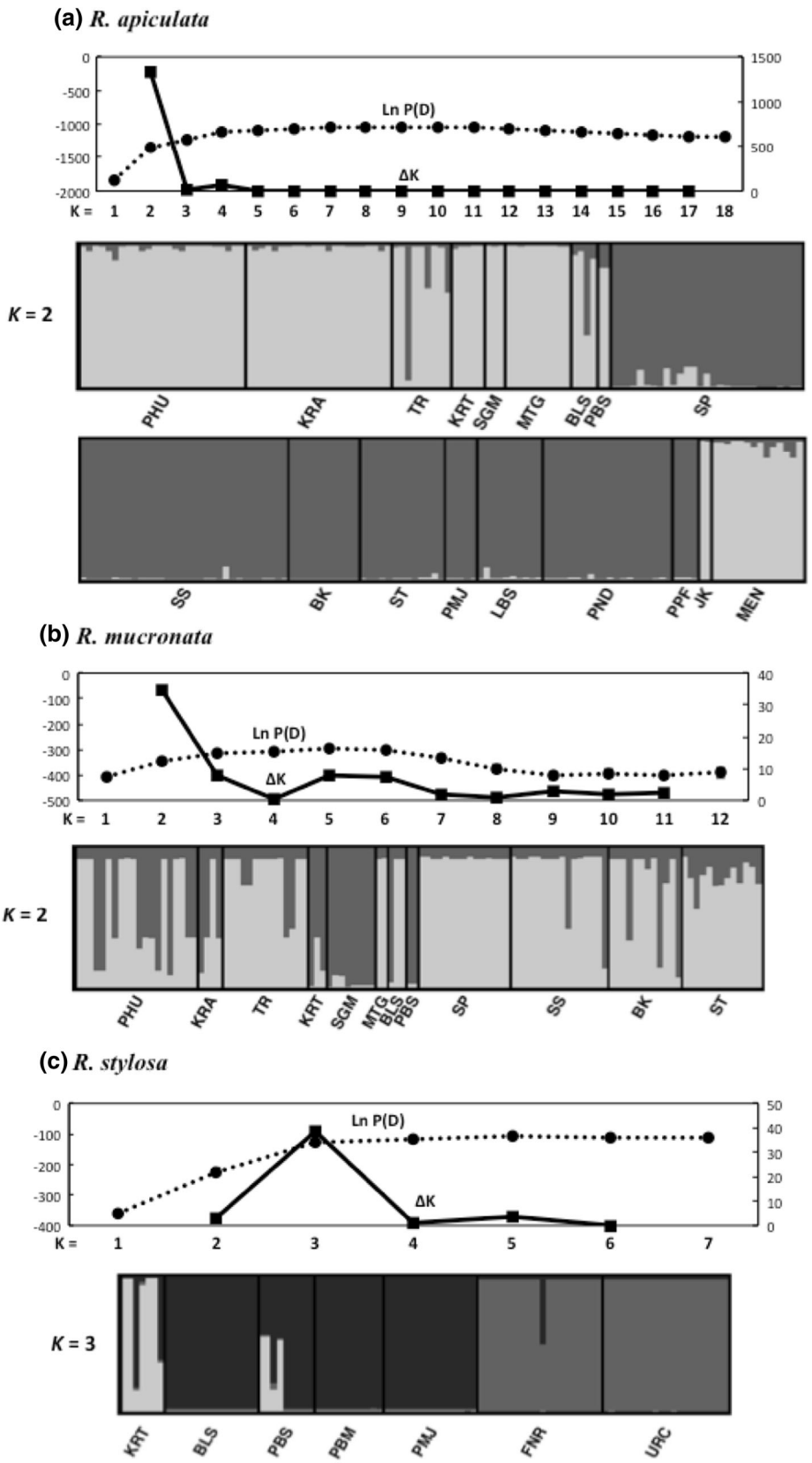

Fig. 3 Outcome of STRUCTURE analysis for **a** *R. apiculata*, **b** *R. mucronata*, and **c** *R. stylosa*. Barplots of the optimum K, based on the Ln $P(D)$ and Evanno's ΔK data plots, are shown: $K = 2$ for *R. apiculata* and *R. mucronata*, $K = 3$ for *R. stylosa*

Table 3 Genetic differentiation among clusters (F_{CT}) in each *Rhizophora* species

Species/Clustering scenario	Genetic differentiation, F_{CT}
R. apiculata	
1) West coast vs. East coast	0.6911**
2) West MP and South MP vs. East MP	0.5597**
R. mucronata	
1) West coast vs. East coast	0.0183
R. stylosa	
1) West coast vs. East coast	−0.5001
2) West coast (excluding KRT and PBS) vs. East coast	0.0000
3) MP populations vs. Japan populations	0.4650*
4) MP populations (excluding KRT and PBS) vs. Japan populations	1.0000*

West coast populations on the MP, west coast; *East coast* populations on the MP, east coast; *West MP* populations located to the west of the MP; *East MP* populations located to the east of the MP; *South MP* populations located to the south of the MP

* $p < 0.05$; ** $p << 0.001$

Discussion

Intraspecific genetic variation

In this study, we used one cpDNA region and five nDNA regions to assess the genetic variation in all three IWP *Rhizophora* species. Generally, our analysis revealed low levels of genetic variation in all three species. Among IWP mangrove species, an observation of low intraspecific genetic variation seems to be usual, e.g. in *Bruguiera* (Minobe et al. 2010), *Ceriops* (Ge and Sun 2001; Huang et al. 2008, 2012; Tan et al. 2005), *Nypa* (Jian et al. 2010), and *Sonneratia* (Zhou et al. 2010), unless when a species is sampled across its whole distribution range and studied with a considerable number of loci, e.g. in *Bruguiera* (Urashi et al. 2013).

At the cpDNA locus, only indel mutations were found within each IWP *Rhizophora* species, generating few haplotypes within each species. In most plant species, chloroplast DNA generally mutates at a low rate and so is expected to exhibit less variation within species. The amount of variation found at the cpDNA locus in this study was therefore consistent with the general expectation, although exceptions have been found in other studies (e.g. Liao et al. 2007). However, we think that a finding of high intraspecific variation at such cpDNA loci may be due to species misidentification, as many controversies still exist over the relationships within and among mangrove species (Duke 2006; Tomlinson 1986). At the nDNA loci, the average nucleotide diversity at silent sites (π_s) of all three IWP *Rhizophora* species were found to be even lower than in other tropical woody species such as the threatened *Shorea* species (Ishiyama et al. 2003, 2008; Iwanaga et al. 2012) and the endangered tropical pine *Pinus krempfii* (Wang et al. 2014). The observed low levels of genetic variation in local populations of mangrove species have been attributed to past evolutionary processes such as repeated bottlenecks and founder events that can greatly reduce genetic variation (Arnaud-Haond et al. 2006). In the case of Southeast Asia, sea level fluctuations like those on the Sunda Shelf (Voris 2000) may have caused repeated local extinctions and subsequent re-establishment of mangrove populations in the region by founder individuals from nearby refuge populations.

In this study, the levels of intraspecific nucleotide variation seem to correlate with the geographic range of sampling of the species (sampling range and π of *R. apiculata* > *R. stylosa* > *R. mucronata*). When comparing with the results of the study by Inomata et al. (2009) that used the same nDNA loci, nucleotide diversity increased with additional sampling of *R. apiculata* ($\pi_t = 0.00041 \rightarrow 0.00064$; $\pi_s = 0.00059 \rightarrow 0.00104$) and *R. mucronata* ($\pi_t$ =nbsp;0.00013 → 0.00043; $\pi_s = 0.00003 \rightarrow 0.00059$). This suggested that as the geographic range of sampling increases, so does the total level of genetic variation. Thus, when sampled throughout the distribution, the level of genetic variation of most mangrove species may be comparable to other tree species, as demonstrated in *Sonneratia alba* ($\pi_t = 0.00432$), *S. caseolaris* ($\pi_t = 0.01003$) (Zhou et al. 2007), and *Bruguiera gymnorrhiza* ($\pi_t = 0.00411$; $\pi_s = 0.00800$) (Urashi et al. 2013). Together with the outcome of the AMOVA analysis and high F_{ST} values, it is clear that much of the genetic variation in IWP *Rhizophora* species reside among populations, different from most other woody plant species that usually have high intra-population genetic variation and low inter-population genetic variation (Hamrick et al. 1992). The same has been shown in many mangrove species such as *Ceriops* (Huang et al. 2008), *Nypa* (Jian et al. 2010), and *Bruiguiera* (Urashi et al. 2013). In particular, in the AEP *Rhizophora* species Cerón-Souza et al. (2010) and Takayama et al. (2013) found strong population structure, which they attributed to the land barrier effect of the Central and South American Isthmuses.

The low intra-population and high inter-population genetic variation appear to be typical features of *Rhizophora* species. It may be attributed to two main reasons: (1) life history—i.e. high inbreeding and limited seed dispersal, and (2) demographic history. In this study, *R. apiculata*, *R. mucronata*, and *R. stylosa* were found to have positive F_{IS} (inbreeding coefficient) estimates in the nDNA data. This indicates a deficiency of heterozygotes within subpopulations of the species, which could be explained by inbreeding and/or the Wahlund effect. Deficiency of heterozygotes was also found in our previous study on *R. apiculata* and *R. mucronata* (Inomata et al. 2009). Furthermore, two studies

on AEP *Rhizophora* species based on microsatellite markers gave similar results. Nearly all populations studied by Cerón-Souza et al. (2010) and roughly one third of populations investigated by Takayama et al. (2013) showed heterozygote deficiency. However, in both studies null alleles were detected. Therefore, it is difficult to decide to what extent the presence of such alleles affected reported heterozygosity estimates. *Rhizophora* species are self-compatible (Kondo et al. 1987), have limited pollen dispersal (Kusmana, personal communication) and can be predominantly inbreeding (Lowenfeld and Klekowski 1992). Lowenfeld and Klekowski (1992) reported high levels of albinism in *R. mangle* native to AEP. Albino mutants are also common in *R. apiculata* and *R. mucronata* and *R. stylosa* (Szmidt, personal observation). In plants, albino mutants are usually associated with recessive homozygotes, which further indicates that reproductive system of *Rhizophora* species involves high levels of inbreeding. Moreover, although the viviparous propagules of *Rhizophora* are buoyant and could be dispersed by sea current, their dispersal is likely to be hindered by the structure of the mangrove stands. The floor of the *Rhizophora* stand is made up of a massive network of prop roots, potentially trapping and reducing the mobility of the propagules, limiting dispersal and further promoting inbreeding. This produces stands that consist of closely related individuals (family-structured subpopulations) or even monoculture mangrove stands over time (Klekowski 1998). Lo (2010) found that most propagules travelled less than 10 km before establishing themselves and growing into adult trees. Assuming that most extant *Rhizophora* populations were founded by very few drifting propagules that "escaped" from nearby refuge populations, then these populations would have very small effective population sizes, further producing inbred populations within their ranges while maintaining certain levels of differentiation with distant populations. However, Cerón-Souza et al. (2010) and Takayama et al. (2013) suggested that propagules of AEP *Rhizophora* species can occasionally migrate several thousands kilometers. This suggestion was based on the presence of identical cpDNA haplotypes in distant populations. In our opinion the presence of such haplotypes is better explained by ancestral polymorphism than by long-distance propagule migration. Clearly, taking into account the aforementioned conflicting results, more studies are required to elucidate the true extent of propagule migration in *Rhizophora* species.

Genetic relationship among R. apiculata, R. mucronata, *and* R. stylosa

Differences at the cpDNA *atpB–rbcL* intergenic spacer, frequently used to resolve relationships of plants at the higher taxonomic order, and the presumably neutral nDNA loci used in this study can be indicators of the genetic relatedness among the IWP *Rhizophora* species. Simple observations of the cpDNA haplotype network and nucleotide divergences at each nDNA locus suggested that *R. mucronata* and *R. stylosa* are indeed very closely related with each other than with *R. apiculata*. Even after the removal of populations with potential hybrids (discussed below) between *R. mucronata* and *R. stylosa*, the nucleotide divergence at nDNA loci was still very low between the two "pure" species ($K_{sil} = 0.4$ %; Supporting Table S6). Phylogenies built with multiple nDNA genes also showed close relationship between *R. mucronata* and *R. stylosa* (Ng et al. 2013). The cpDNA and nDNA data thus agree with the currently established relationship among the three IWP *Rhizophora* species based on morphological classification, i.e. [*R. apiculata* (*R. mucronata*, *R. stylosa*)] (Duke 2006).

Several *R. stylosa* individuals were found to share *R. mucronata*-majority cpDNA haplotypes in populations KRT, PBS, FNR, and URC. Similar instances were observed at the nDNA loci, whereby several individuals of *R. mucronata* had *R. stylosa*-majority haplotypes, and vice versa, in any or all of the nDNA loci (samples from populations KRT, SGM, BLS, and PBS). Interestingly, almost all of the *R. mucronata* or *R. stylosa* individuals that possessed nDNA haplotypes of each other occur in locations where both species are found, i.e. KRT, BLS, and PBS. An example can be clearly observed in Fig. 3c in which the *R. mucronata*-like component (light grey) in populations KRT and PBS formed a different cluster from the other *R. stylosa*-like components (grey and dark grey). Our earlier (Ng et al. 2013; Ng and Szmidt, in press) studies have shown that such individuals in locations KRT, BLS, and PBS could be hybrids between *R. mucronata* and *R. stylosa*, and so are products of recent/ongoing hybridization. This can be clearly observed from a joint STRUCTURE analysis of *R. mucronata* and *R. stylosa* samples (Supporting Fig. S2). Several *R. stylosa* individuals in populations FNR and URC, on the other hand, had *R. mucronata*-majority cpDNA haplotypes but not the nDNA haplotypes. Given that *R. stylosa* is the only *Rhizophora* species found on the islands of Japan (Duke 2006; FAO 2007; Spalding et al. 2010) it is unlikely that the individuals in the Japan populations had obtained the *R. mucronata*-majority haplotype through recent/ongoing hybridization. One possible explanation would be that the *R. mucronata* chloroplast was transmitted to *R. stylosa* during the speciation process. Such chloroplast-capture events via ancient hybridization are well documented in plants (Rieseberg and Soltis 1991).

The close relationship between *R. mucronata* and *R. stylosa* and their ability to form seemingly fertile hybrids (Ng et al. 2013; Ng and Szmidt, in press) raise obvious questions as to how these two species are maintained in the face of ongoing genetic exchange. Although both species

could be correctly assigned most of the time by examining morphologies of their leaves and inflorescences, grey areas still exist (Duke 2006; Ng et al. 2013; Tomlinson 1986). *Rhizophora mucronata* is common in most mangrove areas in Southeast Asia, while *R. stylosa* has patchy distributions, usually preferring sandy/rocky habitats (Mohd Nasir and Safiah Yusmah 2007; Ng and Chan 2012). At places where *R. stylosa* was observed, few, if any, *R. mucronata* individuals were found. In fact, out of the seven sites where *R. stylosa* was sampled for this study, only three were found to contain *R. mucronata*, all in small numbers. We therefore think that their adaptation to different habitats could be a possible factor for speciation and continued isolation.

Population structures and demographic histories of IWP Rhizophora across the Malay Peninsula

As mentioned earlier, several species of mangroves that also occur in the Southeast Asian region showed significant genetic differentiation between populations located to the east and the west coasts of the MP. This demonstrated the potential of the MP as a present-day land barrier that continues to limit gene flow between populations of a species occurring on both sides of the peninsula. Results in this study showed that the Malay Peninsula as a land barrier significantly affected *R. apiculata*, while having minimal effect on the cross-peninsula dispersal of *R. mucronata* and *R. stylosa*.

For *R. apiculata*, populations of the east and the west coasts of the MP were differentiated from each other, evident from the population pairwise F_{ST} values and clustering analyses (STRUCTURE and F_{CT}). Interestingly also, the Indonesian populations clustered with populations of west MP. This was despite the sea level rise after the LGM first connected the Indonesian island of Java to the rest of the South China Sea (i.e. east of the MP), and then to the west of the MP (Voris 2000). For *R. mucronata*, except those populations with possible *R. mucronata* × *R. stylosa* hybrids (KRT, SGM, BLS, and PBS), most population pairs showed low, if any significant, genetic differentiation (most have $F_{ST} < 0.2$, with a few exceptions). Even when STRUCTURE results of *R. mucronata* were viewed at $K = 3$–11, or when analysis was repeated with additional MCMC iterations (data not shown) no spatial clustering patterns were observed. Careful examination of the haplotypes showed that a majority of the populations had similar haplotypic compositions across all five nDNA loci, further eliminating the possibility of $K = 12$ (i.e. the possibility that every population is genetically unique). This goes to suggest that the possible number of clusters for *R. mucronata* is $K = 1$ (i.e. no population clustering) within our sampling range. For *R. stylosa*, results were similar to those of *R. mucronata* for populations of the MP, but genetically differentiated from the populations of Japan. Although one may argue that the only *R. stylosa* population sampled on the east coast of the MP is close to the tip of the MP and hence gene flow with populations on the west coast may have been frequent, *R. apiculata* samples from the same location remained genetically differentiated from the other populations on the west coast. *Rhizophora apiculata* and *R. stylosa* have similar propagule shapes and sizes (i.e. torpedo-shaped, ~30 cm long; cf. *R. mucronata*: torpedo-shaped, ~60 cm long), which are important determinants of how long and how far they can float on sea (Drexler 2001). Their propagules should theoretically have similar abilities to disperse across the peninsula. The "propagule size" argument hence does not explain the lack of genetic differentiation observed among *R. stylosa* populations of the MP. Besides, there is currently no known *R. stylosa* population north of the sampled location along the east coast of the MP (Mohd Nasir and Safiah Yusmah 2007; H.T. Chan, personal communication), so the sampled population was the best we had as far as the MP is concerned. One possibility for this observation is that the MP is located at the edge of the *R. stylosa* distribution (see Duke 2006 for distribution map of species), and mangrove populations located at the distribution margins have been shown to harbor lower genetic variation than populations located at the core of the species' distribution (Arnaud-Haond et al. 2006).

Recently, Yahya et al. (2014) and Wee et al. (2014) proposed that surface sea currents may have contributed to the population structuring observed in *R. apiculata* and *R. mucronata*, respectively, in Southeast Asia. However, given the similar flowering and fruiting period, and dispersal strategy shared by all three IWP *Rhizophora* species, the species should have been affected in a similar fashion, and so have similar population structuring patterns. This is clearly not the case, and so some other forces may have been at play. Based on fossil and morphological records, Duke et al. (2002) proposed model paths for the radiation and dispersal of ancestral *Rhizophora* taxa in the IWP. Simply, *R. apiculata* is thought to have first migrated into Southeast Asia from the north and travelled south from opposite sides of the MP, while *R. mucronata* and *R. stylosa* first established in the south and migrated northwards. Supporting Figure S3 is a simplified adaptation of the proposed model. It thus seems that the repeated sea level fluctuations in the region could have forced *R. apiculata* to retreat back north and form at least two isolated refuge populations on opposite sides of the MP, each individually accumulating mutations through time. *Rhizophora mucronata* and *R. stylosa* on the other hand, could have retreated back and formed one large, connecting, refuge population south of the MP. When the most recent LGM ended, all three species migrated back onto the MP, forming populations with the contrasting structuring

patterns observed in this study. At the same time, the high genetic differentiation between *R. stylosa* populations of Japan and the MP could be explained by isolation-by-distance. Results from our study thus fit the model, but we propose that populations from throughout the distribution of all three species be studied to further test this hypothesis. Other possible factors could also have contributed to the breakdown of the population structuring in *R. mucronata* and *R. stylosa* across the MP, such as hybridization (i.e. between the two species) and anthropogenic factors (e.g. human-mediated movement of propagules/seedlings).

Implications for conservation

Rhizophora apiculata, *R. mucronata*, and *R. stylosa* are currently listed as "Least Concern (LC)" with their population trends rated "Decreasing" on the IUCN Red List (www.iucnredlist.org, accessed 13 December 2013). Although *Rhizophora* mangroves are hardy, fast-growing (Polidoro et al. 2010), and produce viviparous propagules that are relatively more viable compared to propagules of other mangrove species (Duke et al. 1998), their management and conservation should not be overlooked. These species, usually dominating the low intertidal zones (Duke et al. 1998), have been shown to be effective in protecting the shorelines (Alongi 2008), thus influence the presence and distribution of other mangrove species (Duke et al. 1998). Given the findings in this study of low genetic variation in *Rhizophora* populations, protection of the currently established mangrove areas is essential in preserving these unique floras, as most species are not driven to extinction before genetic factors impact them (Spielman et al. 2004). Any future actions (e.g. reforestation or delineation of seed zones) should also take into account the genetic structure of the intended species to avoid homogenizing the mangrove gene pool.

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