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Levels and patterns of DNA variation in two sympatric mangrove species, *Rhizophora apiculata* and *R. mucronata* from Thailand

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In mangrove species the past geomorphic changes in coastal regions and reproductive systems are important factors of their distribution and genetic structure of populations. However, very little is known about the levels of genetic variation of *Rhizophora* species in Southeast Asia. In this study, we surveyed levels and patterns of genetic variation as well as population structure of two sympatric mangrove species, *Rhizophora apiculata* and *R. mucronata* in Thailand, using five nuclear genes and two cpDNA regions. In all investigated DNA regions, nucleotide variation within species was low, while nucleotide divergence between the two species was considerable. The nuclear genes evolved 10 times faster than the cpDNA regions. In both *R. apiculata* and *R. mucronata*, significant positive F_{IS} values were found, indicating deviation from Hardy-Weinberg proportions and a deficiency of heterozygotes. In both species, we found significant genetic differentiation between populations. However, the pattern of population differentiation (F_{ST}) of *R. apiculata* differed from that of *R. mucronata*. Our results suggest that the two investigated species have different demographic history, even though they are sympatric and have similar reproductive systems.

Key words: mangrove, nuclear and cpDNA genes, nucleotide variation, population differentiation, *Rhizophora*

INTRODUCTION

Mangrove is a general term for plant species distributed in tropical and subtropical coastal regions. Mangrove forests are important for preventing erosion and are habitat of a vast number of species, and thus constitute a unique ecosystem. They are also used for various economically important products such as e.g., charcoal (Lanly and Lindquist, 1985). In addition, some mangrove tree species have important morphological and physiological traits such as e.g., viviparous propagules, aerial roots and salt tolerance. These traits are believed to be adaptation to severe coastal environments. Revealing genetic structure of mangrove species provides useful information not only for the management of the mangrove forests but also for the understanding of evolutionary forces leading to the present biodiversity and adaptation.

In plants, reproductive system is one of the important factors affecting species distribution and genetic structure of populations. Dispersal of pollen and seeds, corresponding to gene flow, could determine the range of species distribution and the level of population differentiation. In Southeast Asia, two mangrove species of the *Rhizophoraceae*, *Rhizophora apiculata* and *R. mucronata* are predominant and sympatric. Distribution of *R. mucronata* reaches further east than that of *R. apiculata* (Tomlinson, 1986). Both species are restricted to regions with wet climate (Tomlinson, 1986). In the Indo-Malayan region *R. mucronata* grows with *R. apiculata* but becomes less conspicuous as one moves eastward (Tomlinson, 1986). *R. apiculata* is often dominant component of mangrove forest in the Malaysian region (Tomlinson, 1986). As far as we know, *R. apiculata* and *R. mucronata* are diploid species. At least, their close relatives, *R. mangle* and *R. stylosa*, are diploid species

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(Tyagi, 2002). *R. apiculata* and *R. mucronata* have similar reproductive systems and appear to be self-compatible (Tomlinson, 1986). Pollination of *Rhizophora* species is somewhat controversial. According to Tomlinson (1986) they are wind-pollinated. However, some other authors concluded that they may also be pollinated by insects (e.g., Coupland et al., 2006). The viviparous propagules of *R. mucronata* are much longer (up to 70 cm) than those of *R. apiculata* (20–30 cm) (Tomlinson, 1986). The propagules of both species spread by ocean currents (Tomlinson, 1986), but the efficiency of dispersal is poor (Aksornkoae et al., 1992).

In the Southeast Asia land connections and disconnections of the Malay Peninsula, Sumatra, Java and Borneo islands have repeatedly occurred by exposure and sinking of their continental shelf due to sea level changes in association with climatic changes (Voris, 2000). These past geomorphic changes in the coastal regions could have affected distribution of *R. apiculata* and *R. mucronata* and genetic structure of their populations, because their propagules spread by ocean currents.

Patterns of haplotype distribution of chloroplast DNA (cpDNA) regions in *Ceriops tagal* (Huang et al., 2008; Liao et al., 2007), a species of the *Rhizophoraceae* family, is consistent with the so-called land barrier hypothesis of the Malay Peninsula, in which the past and/or present land barrier of the Malay Peninsula is expected to prevent gene flow between mangrove species occurring along the coasts of the Pacific and Indian Oceans leading to population differentiation between western and eastern populations of the Malay Peninsula (Duke et al., 2002). The levels of polymorphism of cpDNA regions in *C. tagal* were unexpectedly high: however, it is not clear whether those cpDNA regions are also highly variable in other mangrove species. Although genetic markers such as AFLP (e.g., Giang et al., 2003), microsatellites (e.g., Maguire et al., 2000), cpDNA (e.g., Chiang et al., 2001) and mitochondrial DNA (e.g., Chiang et al., 2001) were developed for surveys of genetic structure, phylogeography and molecular phylogeny of some mangrove species, the amount and pattern of genetic variation of functional nuclear genes in mangrove species are largely unknown. Investigation of such regions is necessary for understanding of evolutionary forces shaping genetic structure and distribution of extant populations, finding genetic factors responsible for adaptation, and providing guidelines for conservation of mangrove forests.

In this study, we examined nucleotide variation of five nuclear genes and two cpDNA regions in *R. apiculata* and *R. mucronata*. Plant materials were collected in natural populations from three coast areas (Bangkok, Surat Thani and Trang) in Thailand. We addressed the following questions: (1) are DNA sequence data for chloroplast and nuclear DNA regions useful for population genetic studies on the two investigated species? (2) are populations genetically structured? (3) do sympatric species with similar reproductive systems have similar patterns of genetic variation?

MATERIALS AND METHODS

Sampling Leave samples of *R. apiculata* and *R. mucronata* were collected from two eastern coastal populations in the gulf of Thailand (Bangkok, BK: 13° 44'N, 100°34'E, Surat Thani, ST: 09°07'N, 099°21'E) and one western coastal population in the Andaman Sea site of Thailand (Trang, TR: 07°31'N, 099°37'E). In *R. apiculata*, 11, 13 and 7 individuals from population BK, ST and TR were used. In *R. mucronata*, 12, 13 and 14 individuals from population BK, ST and TR were used.

Genomic DNA extraction, PCR and sequencing Genomic DNA was isolated from leaves with a modified CTAB method (Murray and Thompson, 1980) and further purified with a GenElute Mammalian Genomic DNA Kit (Sigma). Five nuclear genes *PAL*1 (phenylalanine ammonia-lyase, EC 4.3.1.24), *SBE*2 (starch branching enzyme II, EC 2.4.1.18), *DLDH* (dihydrolipoamide dehydrogenase, EC 1.8.1.4), *mang*-1 (mangrin) and *LAS* (lipoic acid synthase), and two cpDNA regions of *atp*B-*rbc*L spacer and *trn*L-*trn*F spacer regions were amplified in *R. apiculata* and *R. mucronata*.

In *Bruguiera gymnorrhiza* the *DLDH* and *LAS* genes were identified as genes involved in response to salt stress (Banzai et al., 2002). The *mang*-1 gene of *B. sexangula* was a homolog of *AOC* (allene oxide cyclase, EC5.3.99.6) and was identified as a salt tolerance gene (Yamada et al., 2002). PCR primers for the investigated nuclear gene regions were designed based on comparisons with corresponding sequences from other plant species: *PAL* (*PAL*1 and *PAL*2 genes of *R. mangle*, *PAL* gene of *Populus kitakamiensis*), *SBE*2 (*Shorea* species by Ishiyama, personal communication), *DLDH* (*DLDH* genes of *B. gymnorrhiza*, *Lycopersicon esculentum*, *Pisum sativum* and *Arabidopsis thaliana*), *mang*-1 (*mang*-1 gene of *B. sexangula*, *AOC* genes of *Nicotiana tabacum* and *A. thaliana*), *LAS* (*LAS* genes of *B. gymnorhiza*, *P. sativum* and *A. thaliana*). The efficiency of all these primers was poor. We therefore redesigned them based on sequences obtained from *R. apiculata* and *R. mucronata*. The investigated cpDNA regions are identical to those used in the previous study on *C. tagal* (Liao et al., 2007). The primers specific to each DNA region are listed in Table 1.

PCR amplifications were performed in a reaction volume of 20 μl with an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 45°C, 50°C or 55°C for 30 sec and polymerization at 72°C for 2 min and a final extension at 72°C for 7 min. The purified PCR products were directly sequenced. The sequences of both strands were deter-

Table 1. PCR primers and annealing temperature

Locus	Primer name	Sequence (5'-3')	Annealing temperature
Nuclear genes			
*PAL*1	PAL1-For	GAGCGCCAATTGGGTTGCTTT	
	PAL-Rev	TGAGCAAACATGAGCTTTCCTAT	55°C
*SBE*2	SBE2-F2	CAAAGTTTGTGAGTCTTATC	
	SBE2-R2	GTCCTGACATTAAAACAGCC	45°C
DLDH	DLDH-F1.5	TGGATGGTCATATAGCTCT	
	DLDH-R1.2	GAACAAGCTCCCCTGCATTAG	50°C
or	DLDH213For	GGATCTGACGACAACGACGT	
	DLDH1597Rev	GATGTGAATGGGCTTGTCAT	55°C
mang-1	manFor2	CTGCTCTGAGAACCGTCTCTTCTTC	
	manRev	GCCTTGGCCGCCGGCATCGGCT	45°C
or	manFor2	CTGCTCTGAGAACCGTCTCTTCTTC	
	manR1.2	AAGGGTGAGGCTCAACCGGC	50°C
LAS	LAS-F1.2	TGGTCTGGCGGCGAGACAGG	
	LAS-RaR2	GATTGGATCTTGAATATACC	45°C
cpDNA regions			
*atp*B-*rbc*L	atpB-F1.2	GAAATGGAAGTTAGCACTCG	
	rbcL-R1.2	AAGATTCAGCAGCTACCGCA	45°C
or	atpB-Samuel	GAAGTAGTAGGATTGATTCTC	
	rbcL-F259R	TCTCCAACAACACGCTCGAT	45°C
*trn*L-*trn*F	trnL-c	CGAAATCGGTAGACGCTACG	
	trnF-f	ATTTGAACTGGTGACACGAG	45°C or 50°C

mined using ABI Prism 3100 automatic sequencer (Applied Biosystems) and a DNA sequencing kit (BigDye terminator v. 3.1/1.1 cycle sequencing kit, ABI) with the PCR primers and the internal primers for sequencing. Of the five nuclear genes and two cpDNA regions, PCR products from some regions could not be amplified for some individuals. The number of sequences obtained for each gene is summarized in Table 2.

In this study, we did not determine sequences of both haplotypes of an individual with two or more heterozygous sites. When the sequences obtained by direct sequencing had no or only one heterozygous site, we inferred sequences of both haplotypes of an individual. Although the sequences of both haplotypes for each individual with two or more heterozygous sites cannot be determined, we can determine the genotype of each heterozygous site for such individuals. Using haplotype and genotype information, we can still estimate some measures of population genetic parameters such as the number of segregating sites and heterozygosity without determination of sequences of individual haplotypes. Therefore, in the following analyses we used all sequence information including both haplotypic and genotypic data.

Data analyses DNA sequences were aligned using the CLUSTAL X program (Thompson et al., 1997) and further edited by hand. Molecular population genetic parameters were estimated, and Tajima's test (Tajima, 1989) and MK test (McDonald and Kreitman, 1991) were performed using the DNAsp program, version 4.20 (Rozas et al., 2003). The population structure was examined using the Arlequin software ver. 3.11 (Excoffier et al., 2005). *F*-statistics, F_{IS} and F_{ST}, for each nuclear gene were estimated by the locus-by-locus analysis of molecular variance (AMOVA) approach (e.g., Weir and Cockerham, 1984). In addition, F_{ST} for each cpDNA region was estimated by the locus-by-locus AMOVA approach. Overall values of *F*-statistics for the five nuclear genes and two cpDNA regions were obtained by summing variance components over the genes. In the AMOVA analyses, genotypic data, where gametic phase of genotypes is unknown, were used for nuclear genes and haplotypic data were used for the cpDNA regions. Sequences of individuals that failed to amplify were treated as missing data. A continuous alignment gap was counted as a single indel. For nuclear genes, the significance of F_{IS} values was tested by 10,000 permutations of haplotype sequences among individuals within populations and the significance of F_{ST} values was

Table 2-1. Nucleotide variation in *R. apiculata*

Nuclear genes	Alignment length/Number of silent sites (bp)	Population	n	S	Indel	π_t	π_s	π_a
*PAL*1	864/210.67							
		BK	22	1	0	0.43	0.00	0.56
		ST	24	1	0	0.34	0.00	0.44
		TR	14	0	0	0.00	0.00	0.00
		Total	60	1(0/1)	0	0.30	0.00	0.40
*SBE*2	1267/1078.83							
		BK	22	1	0	0.36	0.42	0.00
		ST	22	1	0	0.33	0.39	0.00
		TR	12	0	0	0.00	0.00	0.00
		Total	56	1(1/0)	0	0.38	0.44	0.00
DLDH	1221/385.00							
		BK	22	0	0	0.00	0.00	0.00
		ST	20	1	0	0.22	0.70	0.00
		TR	12	0	0	0.00	0.00	0.00
		Total	54	1(1/0)	0	0.09	0.28	0.00
mang-1	1083/676.72							
		BK	22	0	0	0.00	0.00	0.00
		ST	26	1	0	0.30	0.48	0.00
		TR	12	2	0	0.43	0.70	0.00
		Total	60	2(2/0)	0	0.63	1.01	0.00
LAS	1071/577.17							
		BK	22	0	0	0.00	0.00	0.00
		ST	14	0	0	0.00	0.00	0.00
		TR	10	0	1	0.00	0.00	0.00
		Total	46	2(2/0)	1	0.65	1.21	0.00
Total	5506/2928.39		—	7	1	2.05	2.94	0.40
Average	1101/585.68		55	1.4	0.2	0.41	0.59	0.08
cpDNA region								
*atp*B-*rbc*L	815/689.50							
		BK	10	0	0	0.00	0.00	0.00
		ST	9	0	1	0.00	0.00	0.00
		TR	2	0	1	0.00	0.00	0.00
		Total	21	0	2	0.00	0.00	0.00
*trn*L-*trn*F	743/743.00							
		BK	11	0	0	0.00	0.00	0.00
		ST	11	0	0	0.00	0.00	0.00
		TR	5	0	0	0.00	0.00	0.00
		Total	27	0	0	0.00	0.00	0.00
Total	1558/1432.50		—	0	2	0.00	0.00	0.00
Average	779/716.25		24	0	1	0.00	0.00	0.00

Population: BK: Bangkok, ST: Surat Thani, TR: Trang.
Alignment length/Number of silent sites: Sequence length includes alignment gaps. Number of silent sites does not include alignment gaps.
n: Number of sequences.
S: Number of segregating sites excluding indels. Number of silent/replacement differences are shown in parentheses.
Indel: Number of indels. Continuous alignment gap was counted as a single indel.
π_t: Number of nucleotide differences per total site (nucleotide diversity; Nei 1987) with the Jukes and Cantor correction (1969). Indels are not included. The value was multiplied by 10^3.
π_s: Number of nucleotide differences per silent site with the Jukes and Cantor correction (1969). Indels are not included. The value was multiplied by 10^3.
π_a: Number of nucleotide differences per nonsynonymous site with the Jukes and Cantor (1969) correction. Indels are not included. The value was multiplied by 10^3.
Total, π: Sum of π values across loci.
Average, π: Total π values divided by the number of loci.

Table 2-2. Nucleotide variation in *R. mucronata*

Nuclear gene	Alignment length/Number of silent sites (bp)	Population	n	S	Indel	π_t	π_s	π_a
*PAL*1	864/210.41							
		BK	24	1	0	0.26	0.00	0.35
		ST	26	2	0	1.03	0.00	1.36
		TR	28	2	0	0.17	0.00	0.22
		Total	78	3(0/3)	0	0.57	0.00	0.75
*SBE*2	1265/1077.50							
		BK	22	0	0	0.00	0.00	0.00
		ST	12	0	0	0.00	0.00	0.00
		TR	24	0	0	0.00	0.00	0.00
		Total	58	0	0	0.00	0.00	0.00
DLDH	1221/385.83							
		BK	16	0	0	0.00	0.00	0.00
		ST	2	0	0	0.00	0.00	0.00
		TR	24	0	0	0.00	0.00	0.00
		Total	42	0	0	0.00	0.00	0.00
mang-1	880/555.67							
		BK	24	1	0	0.09	0.15	0.00
		ST	22	0	0	0.00	0.00	0.00
		TR	28	1	0	0.16	0.25	0.00
		Total	74	1(1/0)	0	0.09	0.14	0.00
LAS	1036/544.50							
		BK	20	0	0	0.00	0.00	0.00
		ST	12	0	0	0.00	0.00	0.00
		TR	24	0	0	0.00	0.00	0.00
		Total	56	0	0	0.00	0.00	0.00
Total	5266/2773.91		—	4	0	0.66	0.14	0.75
Average	1053/554.78		62	0.8	0	0.13	0.03	0.15
cpDNA region								
*atp*B-*rbc*L	817/692.50							
		BK	9	0	0	0.00	0.00	0.00
		ST	10	0	0	0.00	0.00	0.00
		TR	12	0	1	0.00	0.00	0.00
		Total	31	0	1	0.00	0.00	0.00
*trn*L-*trn*F	743/742.00							
		BK	11	0	0	0.00	0.00	0.00
		ST	10	0	0	0.00	0.00	0.00
		TR	13	0	1	0.00	0.00	0.00
		Total	34	0	1	0.00	0.00	0.00
Total	1560/1434.50		—	0	2	0.00	0.00	0.00
Average	780/717.25		33	0	1	0.00	0.00	0.00

tested by 10,000 permutations of individual genotypes between populations. For cpDNA regions, the significance of F_{ST} values was tested by 10,000 permutations of haplotype sequences between populations. The multilocus HKA test was performed using the HKA program obtained from Jody Hey's web site (http://lifesci.rutgers.edu/~heylab/). DNA sequences obtained in this study were deposited in the DDBJ. Their accession numbers are AB446550–AB447246.

RESULTS

DNA polymorphism in *R. apiculata* and *R. mucronata* We determined sequences of partial regions of the five nuclear genes, *PAL*1, *SBE*2, *DLDH*, *mang*-1 and *LAS*, in *R. apiculata* and *R. mucronata*. We also determined sequences of two cpDNA regions, *atp*B-*rbc*L spacer and *trn*L-*trn*F spacer, in both species. The levels of DNA polymorphism in *R. apiculata* and *R. mucronata* were estimated and the results are summarized in Table 2.

Sequence alignment was first performed species-wise.

Sequence alignment length in three regions *PAL*1, *DLDH* and *trn*L-*trn*F spacer was identical between the two species. In the other four regions, length variations between the two species were found (Table 2). The level of nucleotide variation was surprisingly low in both species. For example, the number of nucleotide differences per silent site with the Jukes and Cantor (1969) correction (π_s) ranged from 0 to 0.00121 for the nuclear genes in *R. apiculata* (Table 2-1), 0 to 0.00014 in *R. mucronata* (Table 2-2). Among the five nuclear genes, no shared polymorphism was found between *R. apiculata* and *R. mucronata*. The cpDNA regions were monomorphic when indels were excluded in both *R. apiculata* and *R. mucronata* (Table 2-1 and 2-2). In the *atp*B-*rbc*L region a single indel was shared polymorphism between *R. apiculata* and *R. mucronata*. In both nuclear and cpDNA regions the number of indels was small, at most one in the two species. Extremely low polymorphism observed in the cpDNA regions of both *R. apiculata* and *R. mucronata* contrasts with the high polymorphism reported in *C. tagal* (Liao et al., 2007).

Divergence between *R. apiculata* and *R. mucronata* In contrast to low polymorphism, nucleotide divergence between *R. apiculata* and *R. mucronata* was considerable (Table 3). Nucleotide divergences were higher in nuclear genes than cpDNA regions. The number of nucleotide substitutions per silent site between the species (*Ks*, Nei and Gojobori, 1986) with the Jukes and Cantor (1969) correction ranged from 0.01821 to 0.04561 for the nuclear genes, and 0.00135 or 0.00445 for the cpDNA regions (Table 3), indicating that the nuclear genes evolve faster than the cpDNA regions. Roughly, the nuclear genes evolved 10 times faster than the cpDNA regions. The number of nucleotide substitutions per replacement site between the species (*Ka*, Nei and Gojobori, 1986) with the Jukes and Cantor (1969) correction ranged from 0.00205 to 0.00601 for the nuclear genes (Table 3). Thus, *Ka*/*Ks* value ranged from 0.045 for the *LAS* to 0.297 for the *SBE*2, indicating these nuclear sequences are not pseudogenes. In most nuclear genes included in this study the number of fixed nucleotide differences between the species was much larger than the number of polymorphic nucleotide differences within species (Table 2 and Table 3).

Neutrality tests The standard neutral model was tested by the Tajima's test. In *R. apiculata* Tajima's *D* values of the five nuclear genes, *PAL*1, *SBE*2, *DLDH*, *mang*-1 and *LAS*, were 0.269, 1.557, –0.675, 1.002 and 0.963, res-

Table 3. Nucleotide divergence between *R. apiculata* and *R. mucronata*

Nuclear genes	Length (bp)	*n*	Fix	Indel	*Ks*	*Ka*
*PAL*1	864	138	5(4/1)	0	19.25	4.78
*SBE*2	1267	114	20(19/1)	1	18.21	5.40
DLDH	1221	96	16(11/5)	0	31.65	6.01
mang-1	1083	134	11(10/1)	2	21.65	3.14
LAS	1071	102	24(23/1)	1	45.61	2.05
Total	5506	—	76	4	136.37	21.38
Average	1101	117	15.2	0.8	27.27	4.28
cpDNA region						
*atp*B-*rbc*L	830	52	3	2	4.45	0.00
*trn*L-*trn*F	743	61	1	0	1.35	0.00
Total	1573	—	4	2	—	—
Average	786	57	2	1	2.90	0.00

Length: Sequence length includes alignment gaps.
n: Number of sequences.
Fix: Number of fixed nucleotide differences between the species excluding indels. Number of silent/replacement differences are shown in parentheses.
Indel: Number of fixed indels between the species. A continuous alignment gap was counted as a single indel.
Ks: Number of nucleotide substitutions per silent site between the species with the Jukes and Cantor (1969) correction. Indels are not included. The value was multiplied by 10^3.
Ka: Number of nucleotide substitutions per replacement site between the species with the Jukes and Cantor (1969) correction. Indels are not included. The value was multiplied by 10^3.
Total, π: Sum of π values across loci.
Average, π: Total π values divided by the number of loci.

pectively. In *R. mucronata*, Tajima's *D* values were –0.372 and –0.752 in *PAL*1 and *mang*-1, respectively, and those of other three genes were not obtained simply because of no variation. All Tajima's *D* values in the two species were not significant. The number of polymorphic silent/replacement differences and that of fixed ones is shown in Table 2 and Table 3, respectively. In the MK test the result was significant for only one gene (*PAL*1, $P = 0.047$, Fisher's exact test), indicating an excess of replacement polymorphism. We also performed the multilocus HKA test for the five nuclear genes. The result was significant ($P = 0.043$), indicating the ratio of the levels of polymorphism to divergence is heterogeneous among the gene regions. Probably the heterogeneous ratio is due to low and uniform polymorphism among the gene regions.

Estimates of *F*-statistics At first, *F*-statistics, F_{IS} and F_{ST}, among the three populations (BK, ST and TR) were estimated by the AMOVA approach. In *R. mucronata*, three of the five nuclear genes (*SBE*2, *DLDH* and *LAS*) were monomorphic and thus they did not give any information on population structure. In both *R. apiculata* and *R. mucronata*, overall values of the five nuclear genes were significant for both F_{IS} and F_{ST} (Table 4), although the significance level of F_{IS} in *R. apiculata* was weak. Significant overall F_{IS} value indicates the deviation from Hardy-Weinberg proportions within subpopulations. Positive F_{IS} value indicate a deficiency of heterozygotes. Significant overall F_{ST} value indicates genetic differentiation among the three populations in both species.

Actually, the number of heterozygotes appeared to be small. For example, in the *PAL*1 of *R. apiculata*, five individuals were heterozygous of 30 individuals in total. We also tested the deviation from Hardy-Weinberg proportions, where the alternative hypothesis is heterozygote deficiency, using the GenePop software ver. 4.0 (Rousset, 2008). We found highly significant heterozygote deficiency in the BK population of *R. apiculata* ($P = 0.0078$) and significant heterozygote deficiency in the ST population of *R. mucronata* ($P = 0.0336$).

For further examinations of population structure, we estimated *F*-statistics for pairs of populations. In *R. apiculata*, overall values for the five nuclear genes were significant for all *F*-statistics except for F_{IS} values in population pairs: ST-BK and ST-TR (Table 5). Overall F_{ST} value for the five nuclear genes was lowest between ST and BK populations ($F_{ST} = 0.140$, $P = 0.018$, Table 5), and highest between BK and TR populations ($F_{ST} = 0.875$, $P << 0.001$, Table 5). This tendency in the nuclear genes was consistent with overall F_{ST} values of the two cpDNA regions. Overall F_{ST} value was lowest between ST and BK populations ($F_{ST} = 0.012$, Table 5), and highest

Table 4. *F*-statistics, F_{IS} and F_{ST}, among three populations (BK, ST and TR)

R. apiculata		
Nuclear genes	F_{IS}	F_{ST}
*PAL*1	0.352 (0.145)	0.021 (0.469)
*SBE*2	0.071 (0.581)	0.366 (0.001)
DLDH	–0.119 (1.000)	0.101 (0.105)
mang-1	0.439 (0.059)	0.742 (<<0.001)
LAS	0.000 (1.000)	1.000 (<<0.001)
Overall (5 loci)	0.221 (0.049)	0.728 (<<0.001)
cpDNA region		
*atp*B-*rbc*L	na	0.278 (0.090)
*trn*L-*trn*F	na	0.000
Overall (2 loci)	na	0.278 (0.090)
R. mucronata		
Nuclear genes	F_{IS}	F_{ST}
*PAL*1	0.456 (0.003)	0.188 (<0.001)
*SBE*2	0.000	0.000
DLDH	0.000	0.000
mang-1	–0.023 (1.000)	–0.008 (0.766)
LAS	0.000	0.000
Overall (5 loci)	0.381 (0.005)	0.163 (<0.001)
cpDNA region		
*atp*B-*rbc*L	na	0.154 (0.091)
*trn*L-*trn*F	na	0.143 (0.095)
Overall (2 loci)	na	0.148 (0.016)

Indels were included in this analysis. Continuous alignment gap was counted as a single indel. *P*-values are in parentheses. na: not applicable.

Table 5. Pairwise *F*-statistics, F_{IS} and F_{ST}

R. apiculata				
		BK	ST	TR
BK	Nuclear gene		0.167 (0.134)	0.592 (0.003)
	cpDNA region		na	na
ST		0.140 (0.018)		0.036 (0.515)
		0.012 (0.480)		na
TR		0.875 (<<0.001)	0.811 (<<0.001)	
		0.688 (0.167)	0.353 (0.326)	
R. mucronata				
		BK	ST	TR
BK	Nuclear gene		0.484 (0.003)	–0.046 (1.000)
	cpDNA region		na	na
ST		0.213 (0.002)		0.487 (0.002)
		0		na
TR		0.027 (0.389)	0.170 (0.010)	
		0.144 (0.074)	0.145 (0.051)	

F_{IS} and F_{ST} values are given above and below the diagonal, respectively, with their *P* values in parentheses. Upper and lower F_{ST} values indicate nuclear gene and cpDNA region, respectively. Overall mean (five nuclear or two cpDNA loci) was shown. na: not applicable.

between BK and TR populations (F_{ST} = 0.688, Table 5), although they were not significant. These results indicate high population differentiation in *R. apiculata*, suggesting strong differentiation between populations from the western and eastern coasts of Thailand.

In *R. mucronata*, overall values for the five nuclear genes were significant for all *F*-statistics except for pairs of BK-TR populations (Table 5). In contrast to *R. apiculata*, overall F_{ST} value of the five nuclear genes was highest between ST and BK populations (F_{ST} = 0.213, P = 0.002, Table 5), and lowest between BK and TR populations (F_{ST} = 0.027, Table 5). Overall F_{ST} value of two cpDNA regions in the pair of populations ST-TR (F_{ST} = 0.170, Table 5) was similar to that in pair BK-TR (F_{ST} = 0.144, Table 5). In *R. mucronata*, the pattern of population differentiation did not appear to be consistent between the nuclear genes and cpDNA regions.

DISCUSSION

Patterns of nucleotide variation We examined nucleotide variation of the cpDNA region that was identical to the one studied in *C. tagal* (Liao et al., 2007). In *C. tagal*, π values of cpDNA regions excluding alignment gaps were 0.00307 for the *atp*B-*rbc*L and 0.02515 for the *trn*L-*trn*F (Liao et al., 2007). In contrast to that study, π values of cpDNA regions were zero in both *R. apiculata* and *R. mucronata*, and even when indels were included, both species still showed low polymorphism (Table 2-1 and 2-2). The cpDNA regions, in particular the *trn*L-*trn*F region, investigated in this study are frequently used to reconstruct phylogenetic trees across a wide range of plant taxa, because they are conservative. Therefore, we can expect that the regions show low polymorphism. In this context, variation found in *C. tagal* seems to be unexpectedly high.

In some pairs of populations, we found some degree of population differentiations, but F_{ST} values of the two cpDNA regions were not significant. Only when the three populations were considered together, F_{ST} value was significant in *R. mucronata* (Table 4). Unlike the previous study on *C. tagal*, our results indicate that in both *R. apiculata* and *R. mucronata* the cpDNA regions are not very useful for surveys of population structure. On the other hand, although variation of the five nuclear genes was low in both *R. apiculata* and *R. mucronata*, specifically in *R. mucronata* three of the five genes were monomorphic, significant *F*-statistics values were found between populations in both species, indicating that these nuclear genes are more useful for such purpose than the cpDNA regions.

In contrast to most previous studies on nucleotide variation in plant populations the level of polymorphism in all nuclear gene regions used in our study was very low. The average π_s values (0.00059 and 0.00003 in *R. apiculata* and *R. mucronata*, respectively) were one or two orders lower than those in other plant species (e.g., *Cryptomeria japonica*: π_s = 0.0038 (Kado et al., 2003); *A. thaliana*: π_s = 0.0083 (Schmid et al., 2005); *Pinus tabuliformis*, *P. densata* and *P. yunnanensis*: π_s = 0.0087–0.0128 (Ma et al., 2006). Taking into account that five different nuclear loci totaling more than 5500 bp including more than 2900 bp of introns were investigated, the observed low levels of polymorphism appear to be a general feature of both *R. apiculata* and *R. mucronata*. Some previous studies on other mangrove tree species based on genetic markers also suggested that they harbor particularly little genetic variation (Arnaud-Haond et al., 2006).

Low variation found in this study could be caused by failures of PCR amplification. Both low quality of template DNA and mismatches of PCR primers at priming sites in distinct sequences could result in the missing data (failure of amplification). However, in this study, most individuals with the missing data failed to amplify in the multiple loci (only a single individual failed to amplify in a single locus out of 7 loci). In addition, sequences of the distinct haplotypes or heterozygotes found here showed very low number of segregating sites. These results are more likely to support the possibility of low quality of template DNAs rather than the presence of undetected very distinct haplotypes.

In contrast to low nucleotide polymorphism within both *R. apiculata* and *R. mucronata*, the nucleotide divergence between the two species was considerable (Table 3). *R. apiculata* and *R. mucronata* are not likely to be the most closely related species (Lakshmi et al., 2002). Our results (low polymorphism and high divergence) indicate that the investigated gene regions are not conservative or do not evolve at extremely low evolutionary rate.

Reproductive system *R. apiculata* and *R. mucronata* appear to be self-compatible (Tomlinson, 1986). Our results showed positive F_{IS} values indicating a deficiency of heterozygotes within subpopulations in both species. In addition, when we tested the deviation from Hardy-Weinberg proportions, where the alternative hypothesis is heterozygote deficiency, we found highly significant heterozygote deficiency in the BK population of *R. apiculata* (P = 0.0004) and the ST population of *R. mucronata* (P = 0.0006). The observed heterozygote deficiency across the loci could be explained by inbreeding, the Wahlund effect, or both. In plants, albino individuals are usually recessive homozygotes and their frequent occurrence may be associated with increased levels of inbreeding. High rates of albinism and selfing were reported for a closely related species *R. mangle* from Central America (Lowenfeld and Klekowski, 1992). Albino mutants were also observed in *R. apiculata* and *R. mucronata* (Szmidt, personal observation). In both *R. apiculata* and *R. mucronata* pollen release is very limited

(Kusmana, personal communication), which could cause increased levels of inbreeding. Therefore, our results suggest the effect of inbreeding within populations of both species included in our study.

Population structure In *R. apiculata*, the patterns of population differentiation of the nuclear genes were consistent with those of the cpDNA regions. That is, population differentiation was smallest in the population pair of ST-BK and largest in the population pair of BK-TR (Table 5). This is likely to be consistent with the land barrier hypothesis of the Malay Peninsula, in which the Malay Peninsula prevents gene flow between mangrove species occurring along the coasts of the Pacific and Indian Oceans leading to population differentiation between western and eastern populations of the Malay Peninsula (Duke et al., 2002). Results reported for the *Ceriops* species are also consistent with this hypothesis (Huang et al., 2008; Liao et al., 2007). In contrast, in *R. mucronata* the pattern of population differentiation of the nuclear genes was inconsistent with that of the cpDNA regions. In addition, the geographic pattern of population differentiation was different from that of *R. apiculata*, and the degree of differentiation was not strong. This pattern of population differentiation of the nuclear genes did not give much support for the land barrier hypothesis of the Malay Peninsula, because the F_{ST} value between BK and TR populations was lowest and not significant. Probably genetic variation detected in our study was not sufficient to estimate *F*-statistics of *R. mucronata*. Indeed, three of the five nuclear genes were monomorphic in *R. mucronata*. Unfortunately, in both species only three populations from Thailand were examined in this study. To test the land barrier hypothesis, it is necessary to survey additional nuclear gene regions and populations collected from western and eastern coasts of the Malay Peninsula.

The levels of seas surrounding Malay Peninsula and its neighboring islands have experienced frequent and substantial fluctuations in the past 20,000 years (Voris, 2000). As a result, Malay Peninsula was repeatedly separated and reconnected with the surrounding islands. These changes of the coastal lines may have caused frequent and repeated extinctions and re-colonization events of mangrove species. Frequent and repeated extinctions and re-colonization events are known to greatly reduce effective population size (Maruyama and Kimura, 1980). Unfortunately, probably because of low variation, we did not obtain significant Tajima's *D* values. However, a significant result of the multilocus HKA test, which indicates extremely low and uniform level of intra-specific variation relative to inter-specific variation, is consistent with such scenario.

In this study we revealed unexpectedly low level of polymorphism in contrast to considerable divergence between *R. apiculata* and *R. mucronata* and the usefulness of the developed nuclear gene markers for population surveys. In addition, our results suggest that the demographic history of the investigated species is not similar even though they are sympatric and have similar reproductive systems.

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