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Molecular identification of natural mangrove hybrids of *Rhizophora* in Peninsular Malaysia

Wei Lun Ng · Hung Tuck Chan · Alfred E. Szmidt

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Abstract Natural hybridization is common in plants. Very often, the identity of a putative hybrid is inferred based on the observation of morphological features intermediate between two possible parental species occurring in a specific location. However, due to plasticity of morphological features and the co-occurrence of more than two possible parental species, molecular markers would be most useful to establish the origin of a putative hybrid. In mangroves, three *Rhizophora* species (*Rhizophora apiculata*, *Rhizophora mucronata*, and *Rhizophora stylosa*) and two putative hybrids (*Rhizophora* × *lamarckii* and *Rhizophora* × *annamalayana*) are distributed in the Indo-West Pacific region. Leaf samples of *Rhizophora* were obtained from two locations in Peninsular Malaysia, namely, Bagan Lalang and Pulau Burung, where all three species grow in sympatry. We analyzed sequences of one chloroplast and six nuclear DNA regions. Our results confirmed earlier claims that the morphologically identified putative hybrids growing in Pulau Burung are *R.* × *lamarckii*, a cross between *R. apiculata* and *R. stylosa*. Our data also pointed to the possible discovery of a new *Rhizophora* hybrid—a cross between *R. mucronata* and *R. stylosa*—the identification of which would have been difficult based on morphological features alone. The directions and the stages of hybridization are also discussed.

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W. L. Ng · A. E. Szmidt (✉)
Graduate School of Systems Life Sciences, Kyushu University, Fukuoka, Japan
e-mail: aszmiscb@kyushu-u.org

H. T. Chan
International Society for Mangrove Ecosystems (ISME), Okinawa, Japan

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Introduction

Natural hybridization is common in flowering plants. Mallet (2005) reported that at least 25 % of the plant species in the UK are involved in interspecific hybridization or introgression. Hybridization plays an important role in the evolution of the earth's biodiversity, introducing new genetic variation within species and driving the emergence of new species (Arnold et al. 1999; Mallet 2007; Rieseberg 1997; Soltis and Soltis 2009; Wissemann 2007). Well-known examples of plant hybrid speciation can be found in sunflower *Helianthus* (Rieseberg et al. 1991) and pines *Pinus* (Ma et al. 2006; Ren et al. 2012)

Mangrove species are no exception in terms of hybridization. There are approximately 70 known mangrove taxa distributed worldwide, including naturally occurring putative hybrids (Duke et al. 1998; Spalding et al. 2010). Putative hybrids have been reported within the major genera of *Rhizophora*, *Sonneratia*, and *Lumnitzera* (Tomlinson 1986) and more recently in *Bruguiera* (Duke and Ge 2011). Three mangrove species of *Rhizophora*, including *Rhizophora apiculata*, *Rhizophora mucronata*, and *Rhizophora stylosa*, and two putative hybrids, *Rhizophora* × *lamarckii* (= *R. apiculata* × *R. stylosa*) and *R.* × *annamalayana* (= *R. apiculata* × *R. mucronata*), occur in the Indo-West Pacific (IWP) region. The morphologically recognized putative hybrids are said to occur throughout the region (Duke et al. 2002 and references therein) and have so far been thought to be sterile and/or limited to the F_1 stage (Chan 1996; Lo 2010; Ng and Chan 2012a; Tyagi 2002). Despite the huge overlap in the geographical distribution of *R. apiculata*, *R. mucronata*, and *R. stylosa* (Duke 2006), their putative hybrids have been reported only in specific locations around the world. For

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instance, although *R. apiculata* and *R. mucronata* are widespread and co-occur in many mangrove areas of Peninsular Malaysia, their putative hybrid (= *R.* × *annamalayana*) has only been observed in the Merbok Mangroves, Kedah (Ong 2003). Similarly, putative hybrids showing intermediate morphology between *R. apiculata* and *R. stylosa* (= *R.* × *lamarckii*) were only observed in Pulau Burung (Chan 1996; Ng and Chan 2012a) out of the several locations where both species co-occur (Nasir and Yusmah 2007; Ng and Chan 2012b). To our knowledge, there are currently no reports on possible hybrids between *R. mucronata* and *R. stylosa*.

Conventionally, the identification of mangrove hybrids has been based on observation of intermediate morphology between, and co-occurrence of, putative parental species (Chan 1996; Duke 2010; Kathiresan 1995; Kathiresan 1999). However, morphological features are often under the influence of environmental conditions and thus can be unreliable and misleading (Filppula et al. 1992; Hegarty and Hiscock 2004; Mallet 2005). Moreover, due to plasticity in the morphology of the putative hybrids (Cerón-Souza et al. 2010; Ng and Chan 2012a) and variation within the putative parental species (Duke et al. 2002; Ragavan et al. 2011), there often is a lack of consensus on the correct description of putative mangrove hybrids. As an example, putative *Rhizophora* hybrids discovered in India were initially identified as *R.* × *lamarckii*, based on intermediate morphology between *R. apiculata* × *R. stylosa* (Kathiresan 1995 and references therein). Kathiresan (1995, 1999) later named the hybrid as *R.* × *annamalayana*, a cross between *R. apiculata* and *R. mucronata*, since *R. stylosa* does not occur at the site. Subsequent genetic analysis has confirmed that the hybrids were indeed crosses between *R. apiculata* and *R. mucronata* (Parani et al. 1997). The correct identification of hybrids is crucial to answer taxonomic, ecological, and evolutionary questions which will further contribute towards the conservation and management of this rapidly declining group of flora.

Recognizing the inadequacy of morphological approach alone in studying mangrove natural hybridization, many recent studies used various molecular methods to identify and characterize such hybrids. The majority of studies used hypervariable markers such as AFLP (Zhou et al. 2005), RAPD, RFLP (Parani et al. 1997), and ISSR (Lo 2010; Sun and Lo 2011). However, the use of such markers is continuously marred by problems such as homoplasy and poor reproducibility (Agarwal et al. 2008; Caballero et al. 2008; Hardig et al. 2000; Mondini et al. 2009). On the other hand, single- or low-copy nuclear genes have so far shown promising results in confirming interspecific hybridization of various taxa (Ishiyama et al. 2008; Kamiya et al. 2011; Kusumi et al. 2012; Pacheco et al. 2002) including mangroves (Guo et al. 2011; Qiu et al. 2008). Theoretically, in diploid organisms, an F_1 hybrid would inherit one allele from each parent at every nuclear DNA (nDNA) locus. They can thus be detected with the analysis of a single nDNA locus. According to Pacheco et al. (2002), the detection of F_2 and backcrossed hybrids at high probability ($P \geq 95$ %), however, would require at least three and five nDNA loci, respectively. This is because the probability that an F_2 hybrid is homozygous for alleles from either parent at n loci is 0.25^n and $0.25^3 < 0.05$, while the probability that a backcrossed hybrid is homozygous for alleles from either parent at n loci is 0.5^n and $0.5^5 < 0.05$. While nDNA is biparentally inherited and thus can be used to determine the occurrence of hybridization, chloroplast DNA (cpDNA), being maternally inherited in most plant species (Mogensen 1996), is useful to determine the direction of hybridization.

This study presents molecular evidence of natural hybridization among IWP *Rhizophora* species. In Peninsular Malaysia, *R. apiculata*, *R. mucronata*, and *R. stylosa* are known to co-occur only in Pulau Burung (an islet in Port Dickson, Negeri Sembilan) and in Bagan Lalang (Selangor), making them unique sites for studies on IWP *Rhizophora* hybridization. *R.* × *lamarckii* was reported to be growing in Pulau Burung alongside its putative parents, *R. apiculata* and *R. stylosa* (Chan 1996). The morphological features of these putative hybrid individuals make them easily recognizable, with characteristics that are intermediate to their putative parents.

During a recent survey, several mature trees that appeared to be *R. mucronata* were found growing in Pulau Burung (Ng and Chan 2012a). *R. mucronata* and *R. stylosa* have similar morphological features with few differentiating characters (Duke 2006). Had putative hybrids of *R. apiculata* × *R. mucronata* and *R. apiculata* × *R. stylosa* always displayed intermediate characteristics, the products from different crosses would not be readily discernible through morphological identification alone. In addition, the morphology of the putative hybrid individuals in Pulau Burung varied (Ng and Chan 2012a), throwing into question the real identities of these individuals. However, no morphologically recognizable putative hybrids were found in Bagan Lalang (Ng and Chan 2012a, b), which is ca. 30 km from Pulau Burung. Comparing data from these two sites may provide more insight on the hybridization of *Rhizophora* species.

We analyzed nucleotide variation at one cpDNA and six nDNA loci to investigate natural hybridization in IWP *Rhizophora* using samples from Bagan Lalang and Pulau Burung. We aimed to address the following questions: (1) Do genetic data agree with the morphological classification of the different IWP *Rhizophora* species? (2) Do the putative hybrid samples from Pulau Burung exhibit hybrid genotypes (combination of parental haplotypes) at the nDNA loci analyzed? If yes, what are the possible parental species? (3) Could possible "cryptic" hybrids between the morphologically close *R. mucronata* and *R. stylosa* be detected in

the populations using DNA sequence markers? (4) What are the directions and extent of hybridization in the hybrid individuals?

Materials and methods

Plant materials

Sampling was performed at two locations in Peninsular Malaysia, i.e., Bagan Lalang, Selangor (BLS; 2°35′N, 101° 41′E) and Pulau Burung, Negeri Sembilan (PBS; 2°32′N, 101°47′E). All taxa were identified based on leaf and floral characteristics as listed in Table 1. For the "pure" species, identification relied heavily on leaf shape, the size and arrangement of black/brown dots at the under surface of the leaf, and the structure of the inflorescence, while for the putative hybrid individuals in Pulau Burung, identification was straightforward, simply those bearing *R. mucronata*/*R. stylosa*-like leaves (often with venation patterns on the leaves) with *R. apiculata*-like inflorescence. In total, we collected leaf samples of 4 *R. apiculata*, 3 *R. mucronata*, and 15 *R. stylosa* from Bagan Lalang and 2 *R. apiculata*, 2 *R. mucronata*, 9 *R. stylosa*, and 13 *Rhizophora* putative hybrid individuals from Pulau Burung. Leaf samples were collected and stored with silica gel before further analyses.

Sampling for this study was limited by two main factors: (1) There are only two known sites in Peninsular Malaysia where all three IWP *Rhizophora* species co-occur, both of which were sampled for this study; (2) *R. stylosa* is known to occur on very specific sites in Peninsular Malaysia with hard sandy soil or rocks (Nasir and Yusmah 2007) and usually few, if any, *R. apiculata* and *R. mucronata* occur in those sites (Ng and Chan 2012b). Therefore at both sites, we were only able to collect a few available *R. apiculata* and *R. mucronata* samples as compared to *R. stylosa* or to the putative *Rhizophora* hybrid(s) in Pulau Burung.

DNA extraction, PCR amplification, and sequencing

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

One cpDNA region: *atpB-rbcL* intergenic spacer and six nDNA regions: *PAL1*, *DLDH*, *SBE2*, *mang-1*, *FMRrm11*, and *RPB2* were PCR-amplified in all sampled individuals. The primers used for PCR and the corresponding annealing temperatures (T_a) are listed in Table 2. Primers for loci *atpB-rbcL*, *PAL1*, *DLDH*, *SBE2*, and *mang-1* were previously described by Inomata et al. (2009), while the primers for the *FMRrm11* locus were described by Cerón-Souza et al. (2010). Primers for the *RPB2* locus were designed in this study based on sequences obtained from initial amplification using primers described by Denton et al. (1998). 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 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 v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed on an ABI 3730 DNA Analyzer (Applied Biosystems).

To obtain a wider representation of IWP *Rhizophora* genetic variation, DNA sequences of three samples of *R.*

Table 1 Diagnostic morphological features used to identify *R. apiculata*, *R. mucronata*, *R. stylosa*, and the putative hybrid(s) in Pulau Burung

Feature	Taxon			
	R. apiculata	*R. mucronata*	*R. stylosa*	Putative hybrid(s)
Leaf[a]	Sublanceolate, wider towards the middle of the blade; apex pointed with mucronate tip; fine and dense black/brown dots under the surface	Broadly oblong, wider towards the middle of the blade; apex pointed with mucronate tip; fine and dense black/brown dots under the surface	Broadly elliptic, wider towards the apex; apex blunt with prominent mucronate tip; dark black/brown dots under the surface, less dense; leaf size often smaller than that of *R. mucronata*	Broadly oblong, with shape and size generally resembling that of *R. mucronata*; often with variations in leaf size, color tone, and venation pattern
Inflorescence	Flower buds always as single pairs, greenish/yellowish, globular, and borne on a stout peduncle; style short and stubby	Flower buds pendulous, 4–8, cream-colored, and borne on a long peduncle; style intermediate in length	Flower buds pendulous, 4–8, cream-colored, and borne on a long peduncle; style elongated	Flower buds resemble those of *R. apiculata*, 2–4, and borne on slightly elongated peduncle; style intermediate in length

[a] See Ng and Chan (2012a) for photos and Ng and Chan (2012b) for measurements, comparing leaf morphologies of *R. apiculata*, *R. mucronata*, *R. stylosa*, and the putative hybrid(s) in Pulau Burung

Table 2 PCR primers used in this study

Locus	Primer sequence (5′–3′)	T_a (°C)	Source
cpDNA region			
atpB-rbcL spacer	F: GAAATGGAAGTTAGCACTCG	45	Inomata et al. (2009)
	R: AAGATTCAGCAGCTACCGCA		
nDNA region			
PAL1	F: GAGCGCCAATTGGGTTGCTTT	55	Inomata et al. (2009)
	R: TGAGCAAACATGAGCTTTCCTAT		
DLDH	F: TGGATGGTCATATAGCTCT	50	Inomata et al. (2009)
	R: GAACAAGCTCCCCTGCATTAG		
SBE2	F: CAAAGTTTGTGAGTCTTATC	50	Inomata et al. (2009)
	R: GTCCTGACATTAAAACAGCC		
mang-1	F: CTGCTCTGAGAACCGTCTCTTCTTC	50	Inomata et al. (2009)
	R: GCCTTGGCCGCCGGCATCGGCT		
FMRrm11	F: TTTCTATTTATGATCCCATCATCTC	55	Cerón-Souza et al. (2010)
	R: GCGTTTAACTGCCACAATTC		
RPB2	F: CATTGCAATTTTATCGTCCT	50	Designed in this study based on Denton et al. (1998)
	R: GACCAAGCTTTCCATCATAG		

F forward, *R* reverse, T_a annealing temperature

mucronata per location from Krabi (KRA), Phuket (PHU), Samut Prakan (SP), and Samut Songkram (SS) (all in Thailand) and four samples of *R. stylosa* per location from Funaura Bay (FNR) and Urauchi Estuary (URC) (both in Iriomote, Japan) were included in the analysis. A map of the sampling sites and list of all samples included in this study are shown in Fig. 1 and Table 3, respectively. Sequences of these additional samples at loci *atpB-rbcL*, *PAL1*, *DLDH*, *SBE2*, and *mang-1* were determined in a separate study (unpublished), while the sequences of loci *FMRrm11* and *RPB2* of the same samples were determined in this study.

For all nDNA loci, individual haplotypes were inferred through computational means (described in the next section). Two putative *Rhizophora* hybrid samples from Pulau Burung (PBSxxx01 and 03) were randomly chosen to undergo cloning of all six nDNA loci to verify the results of the haplotype inference: Purified PCR-amplified 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.

Data analyses

Nucleotide sequences were assembled and edited using the software ATGC version 6.0 (GENETYX CORPORATION). Cloning was not performed for sequences containing indels or more than one polymorphic site. The portion of the sequences containing the largest number of polymorphic sites, before an indel, was used for subsequent analyses. To investigate the relationship among sequences, haplotypes at each locus were first inferred using PHASE 2.1 (Stephens et al. 2001; Stephens and Donnelly 2003) implemented in DNAsp version 5.10 (Librado and Rozas 2009). Sequences have been deposited in GenBank with the accession numbers KC996868-KC997175, and in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.dn584. Sequences of each locus were then aligned using ClustalW (Thompson et al. 1994), before the construction of neighbor-joining trees, using MEGA 5.0 (Tamura et al. 2011). To illustrate the relationship among cpDNA haplotypes, a haplotype network was constructed using the median-joining model (Bandelt et al. 1999) implemented in NETWORK version 4.6.1.0 (fluxus-engineering.com).

Results

Analysis of nDNA loci

Sequence lengths of loci *PAL1*, *DLDH*, and *FMRrm11* were identical across all the *Rhizophora* samples. On the other hand, sequence lengths of loci *mang-1*, *SBE2*, and *RPB2* differed between samples of *R. apiculata* and *R. mucronata* or *R. stylosa* (samples identified as *R. mucronata* and *R. stylosa* had similar sequence lengths at these loci). Direct sequencing of the amplified nDNA loci from the morphologically identified putative hybrid samples from Pulau Burung revealed indels at these loci. The final aligned lengths of the nDNA loci were 940 bp for *PAL1*, 1221 bp for *DLDH*, 947 bp for *SBE2*, 438 bp for *mang-1*, 609 bp for *FMRrm11*, and 126 bp for *RPB2*. Species-specific sites were observed at all the loci (Fig. 2). The nDNA loci analyzed exhibited limited variation among the samples. After the

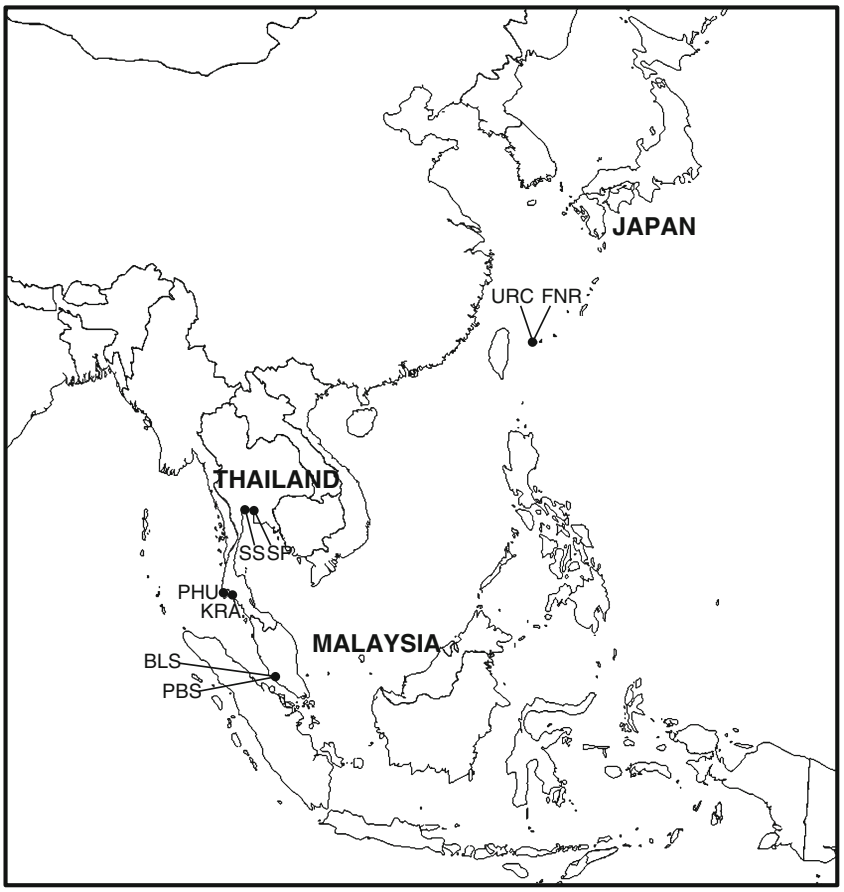

Fig. 1 Map showing the sampling sites of this study

inference of haplotypes from genotype data, a total of five, five, four, five, four, and three haplotypes were found at loci *PAL1*, *DLDH*, *SBE2*, *mang-1*, *FMRrm11*, and *RPB2*, respectively. These haplotypes formed distinct clusters on each nDNA phylogeny, mostly concordant with the morphological classification of the *Rhizophora* species (Supplementary Fig. S1).

When two diploid individuals cross to form a hybrid, one allele at a certain locus of the hybrid offspring would have originated from each parent. As seen in Fig. 2, all morphologically identified putative hybrid samples from Pulau Burung (PBSxxx01-13) were consistently heterozygous for the species-specific sites at which *R. apiculata* and *R. stylosa* differ. To investigate the relationship among sequences, inferred haplotypes were used to construct neighbor-joining trees (Supplementary Fig. S1). On each of the nDNA phylogenies, the putative hybrids had one of their haplotypes clustered with the *R. apiculata* haplotypes and the other with the *R. stylosa* haplotypes. These findings provided evidence

Table 3 Samples analyzed in this study

Location	Taxon[a], number of samples, and sample ID			
	R. apiculata	*R. mucronata*	*R. stylosa*	*R.* hybrid(s)
Bagan Lalang, Selangor (BLS), Malaysia	4 (BLSapi01-04)	3 (BLSmuc01-03)	15 (BLSsty01-15)	–
Pulau Burung, Negeri Sembilan (PBS), Malaysia	2 (PBSapi01-02)	2 (PBSmuc02-03)	9 (PBSsty01-09)	13 (PBSxxx01-13)
Phuket (PHU), Thailand	–	3	–	–
Krabi (KRA), Thailand	–	3	–	–
Samut Prakan (SP), Thailand	–	3	–	–
Samut Songkhram (SS), Thailand	–	3	–	–
Funaura bay (FNR), Iriomote, Japan	–	–	4	–
Urauchi estuary (URC), Iriomote, Japan	–	–	4	–

[a] As identified through morphology, i.e., leaf and floral characteristics

		nDNA locus																																			cpDNA locus
		PAL1 (940 bp)											*DLDH* (1221 bp)																	*mang-1* (438 bp)								*atpB-rbcL*
	Position	5	22	58	118	124	163	307	571	665	667	757	1	23	62	64	102	117	314	468	575	639	710	711	767	782	800	1154	1163	211	231	252	296	348	366	376	409	
Taxa	Sample no.																																					
R. apiculata	N = 6	G	C	T	T	C	A	T	G	C	A	G	G	T	C	G	G	A	T	G	C	C	C	A	T	C	C	G	G	C	A	G	A	T	A	T	C	cp1; cp2
R. mucronata	N = 14	A	T	T	A	C	T	C	T	G	A	A	T	G	G	T	A	G	T	A	T	T	T	G	C	G	T	C	A	T	C	C	G	C	G	C	T	cp3; cp4
R. stylosa	N = 28	A	T	A	A	T	T	C	T	G	G	A	T	G	G	T	A	G	C	A	T	C	T	G	C	G	C	C	A	T	C	C	G	C	G	T	T	cp3; cp5
R. apiculata × *R. stylosa*	N = 13	R	Y	W	W	Y	W	Y	K	S	R	R	K	K	S	K	R	R	Y	R	Y	C	Y	R	Y	S	C	S	R	Y	M	S	R	Y	R	T	Y	cp5
R. mucronata × *R. stylosa*	BLSmuc01	A	T	W	A	Y	T	C	T	G	R	A	T	G	G	T	A	G	Y	A	T	Y	T	G	C	G	Y	C	A	T	C	C	G	C	G	Y	T	cp3
	PBSmuc02	A	T	W	A	Y	T	C	T	G	R	A	T	G	G	T	A	G	Y	A	T	Y	T	G	C	G	Y	C	A	T	C	C	G	C	G	C	T	cp3
	PBSmuc03	A	T	W	A	Y	T	C	T	G	R	A	T	G	G	T	A	G	Y	A	T	Y	T	G	C	G	Y	C	A	T	C	C	G	C	G	Y	T	cp3
	PBSsty01	A	T	W	A	Y	T	C	T	G	R	A	T	G	G	T	A	G	Y	A	T	Y	T	G	C	G	Y	C	A	T	C	C	G	C	G	T	T	cp3
	PBSsty02	A	T	W	A	Y	T	C	T	G	R	A	T	G	G	T	A	G	Y	A	T	Y	T	G	C	G	Y	C	A	T	C	C	G	C	G	T	T	cp3
	PBSsty03	A	T	A	A	T	T	C	T	G	G	A	T	G	G	T	A	G	C	A	T	C	T	G	C	G	C	C	A	T	C	C	G	C	G	Y	T	cp5
	PBSsty04	A	T	A	A	T	T	C	T	G	G	A	T	G	G	T	A	G	T	A	T	T	T	G	C	G	T	C	A	T	C	C	G	C	G	T	T	cp3

		nDNA locus																													
		SBE2 (947 bp)																			*FMRrm11* (609 bp)										*RPB2* (126 bp)	
	Position	28	51	53	92	93	162	267	299	301	310	341	396	575	677	706	786	787	838	917	98	152	173	178	285	345	347	533	536	606	7	43
Taxa	Sample no.																															
R. apiculata	N = 6	C	T	G	T	A	T	T	G	T	T	T	G	C	A	A	T	T	T	A	A	A	C	G	A	G	T	T	T	G	C	T
R. mucronata	N = 14	T	C	G	G	G	C	G	A	A	C	C	A	T	G	G	T	C	T	T	G	G	C	C	G	A	G	C	C	A	G	C
R. stylosa	N = 28	T	C	T	T	G	C	G	A	A	C	C	A	T	G	G	G	C	G	T	G	G	A	C	G	A	G	C	T	A	C	C
R. apiculata × *R. stylosa*	N = 13	Y	Y	K	T	R	Y	K	R	W	Y	Y	R	Y	R	R	K	Y	K	W	R	R	M	S	R	R	K	Y	T	R	C	Y
R. mucronata × *R. stylosa*	BLSmuc01	T	C	K	K	G	C	G	A	A	C	C	A	T	G	G	K	C	K	T	G	G	M	C	G	A	G	C	Y	A	S	C
	PBSmuc02	T	C	K	K	G	C	G	A	A	C	C	A	T	G	G	K	C	K	T	G	G	M	C	G	A	G	C	Y	A	C	C
	PBSmuc03	T	C	K	K	G	C	G	A	A	C	C	A	T	G	G	K	C	K	T	G	G	M	C	G	A	G	C	Y	A	S	C
	PBSsty01	T	C	K	K	G	C	G	A	A	C	C	A	T	G	G	K	C	K	T	G	G	A	C	G	A	G	C	T	A	C	C
	PBSsty02	T	C	K	K	G	C	G	A	A	C	C	A	T	G	G	K	C	K	T	G	G	A	C	G	A	G	C	T	A	C	C
	PBSsty03	T	C	T	T	G	C	G	A	A	C	C	A	T	G	G	G	C	G	T	G	G	A	C	G	A	G	C	T	A	C	C
	PBSsty04	T	C	K	K	G	C	G	A	A	C	C	A	T	G	G	K	C	K	T	G	G	A	C	G	A	G	C	T	A	C	C

Fig. 2 Species-specific nucleotide positions at nDNA loci *PAL1*, *DLDH*, *mang-1*, *SBE2*, *FMRrm11*, and *RPB2* for the *Rhizophora* samples investigated in this study. *R* = A or G, *Y* = C or T, *S* = G or C, *W* = A or T, *K* = G or T, *M* = A or C. Nucleotide positions at which *R. mucronata* and *R. stylosa* differ are *shaded*. The cpDNA haplotypes found in each taxon are also shown

that the individuals are crosses between *R. apiculata* and *R. stylosa*.

Interestingly, samples BLSmuc01, PBSmuc02, PBSmuc03, PBSsty01, PBSsty02, PBSsty03, and PBSsty04 had sequences that were heterozygous at all or some species-specific sites at which *R. mucronata* and *R. stylosa* differ (Fig. 2). Their haplotypes also did not cluster consistently according to morphological classification on the nDNA phylogenies. These samples, at every locus (BLSmuc01 and PBSmuc03) or at certain loci (PBSsty01, 02, 03, 04, and PBSmuc02), had one haplotype clustered within the clade consisting mainly of *R. mucronata* haplotypes and the other haplotype in the clade consisting mainly of *R. stylosa* haplotypes (Supplementary Figure S1). These showed patterns of additional hybrid genotypes—crosses between *R. mucronata* and *R. stylosa*.

Analysis of cpDNA locus

The lengths of the amplified cpDNA *atpB-rbcL* intergenic spacer region ranged from 809 to 827 bp, resulting in five haplotypes (cp1, cp2, cp3, cp4, and cp5) among all the *Rhizophora* samples (Supplementary Fig. S2). Figure 3 shows the relationship among all the observed haplotypes. Generally, the different cpDNA haplotypes reflected the different taxa

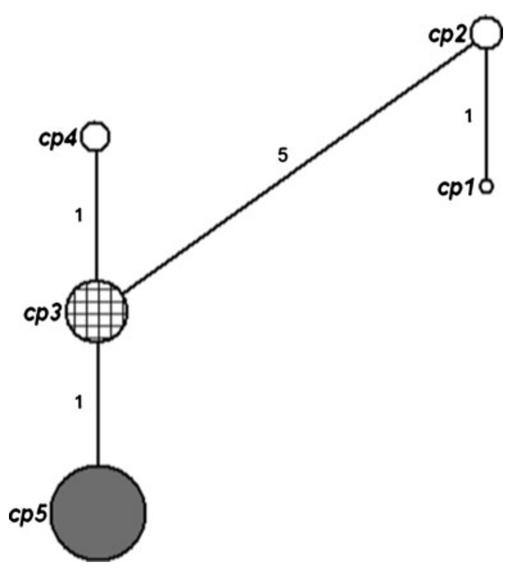

Fig. 3 Haplotype network showing the relationship among the five cpDNA *atpB-rbcL* intergenic spacer haplotypes (cp1–cp5) observed in the *Rhizophora* samples. Circle size is proportional to frequency of the haplotype. The number of mutation steps is labeled at the lines. Indels are included in the calculation of mutation steps, and a continuous indel is considered as a single mutation step. *Line-shaded circle* = haplotype found in *R. mucronata* × *R. stylosa* hybrids, *gray-colored circle* = haplotype found in *R. apiculata* × *R. stylosa* hybrids

identified in this study (Supplementary Table S1). The cp1 (809 bp) and cp2 (810 bp) haplotypes were specific to the *R. apiculata* samples. All *R. mucronata* samples from Bagan Lalang and Pulau Burung had the cp3 (812 bp) haplotype, while the cp4 (813 bp) haplotype was only found among the *R. mucronata* samples from Thailand. Most samples identified as *R. stylosa* and all the putative hybrids from Pulau Burung had the cp5 (827 bp) haplotype. Exceptions were samples PBSsty01, PBSsty02, PBSsty04, and URCsty01, which had the cp3 haplotype. Of these exceptions, PBSsty01, PBSsty02, and PBSsty04 were suggested by nDNA data to be crosses between *R. mucronata* and *R. stylosa*.

Discussion

Genetic variability and computational inference of haplotypes

PHASE 2.1 functions by estimating possible haplotypes from known haplotypes (e.g., of homozygous sequences) in a pool of samples (Stephens and Donnelly 2003). Harrigan et al. (2008) had previously compared haplotype inference using PHASE 2.1 with cloning of a highly variable nDNA locus of three closely related bird species and found that the computational inference was highly accurate. The *Rhizophora* samples analyzed in this study exhibited limited genetic variability, suggested by the low number of haplotypes at each locus across all taxa. This could be due to the small sample sizes, especially of *R. apiculata* and *R. mucronata*. However, similarly low levels of genetic variation have been reported in several other studies on mangrove species that used DNA sequence markers (Huang et al. 2008; Inomata et al. 2009; Minobe et al. 2010). Additionally, a previous study on *R. apiculata* and *R. mucronata* (Inomata et al. 2009) and an ongoing study on all three IWP *Rhizophora* species across the Malay Peninsula (unpublished) have also provided strong indication that low intraspecific genetic variability may be the general feature of IWP *Rhizophora* around the Malay Peninsula region. The low genetic variability in the *Rhizophora* samples provided fewer possibilities for PHASE 2.1 to "guess" from, thereby contributing towards a lower error rate in the estimation, producing accurate inferences for the purpose of this study. For verification, we cloned and sequenced all six nDNA loci of two putative *R. apiculata* × *R. stylosa* hybrid samples (PBSxxx01 and PBSxxx03). The haplotype sequences obtained through cloning were found to be identical to those predicted by PHASE 2.1.

Species delimitation

Mangrove species of the pantropical genus *Rhizophora* can be divided into the IWP and Atlantic-East Pacific (AEP) geographical zones (Tomlinson 1986). Using microsatellite and DNA sequence markers, Cerón-Souza et al. (2010) found overall discordance between genetic and morphological data in the classification of AEP *Rhizophora*, namely, *Rhizophora mangle*, *Rhizophora racemosa*, and the putative hybrid *Rhizophora* × *harrisonii*. Their findings suggested that *R. mangle* and *R. racemosa* sampled along the same side of the Central American Isthmus (CAI) were more closely related to each other than to conspecifics sampled along the other side of the CAI, raising doubt on the current taxonomical classification of AEP *Rhizophora* (Cerón-Souza et al. 2010).

In this study, we investigated *Rhizophora* samples from two locations in Peninsular Malaysia where all three IWP *Rhizophora* species coexist. Conspecific populations of IWP mangrove species have been shown to be genetically differentiated across the Malay Peninsula (Ge and Sun 2001; Inomata et al. 2009; Liao et al. 2007; Minobe et al. 2010; Zhang et al. 2008). Nonetheless, our genetic data showed strong concordance with the morphological classification of the IWP *Rhizophora* species, even after the inclusion of additional samples from both sides of the peninsula, although *R. mucronata* and *R. stylosa* were clearly more genetically similar (fewer between-species nucleotide differences) compared to either species with *R. apiculata*. When analyzed using a single nDNA locus identical to the one used by Cerón-Souza et al. (2010), genetic data still supported three clades corresponding to *R. apiculata*, *R. mucronata*, and *R. stylosa*. This consistency in morphological and genetic differentiation among the different taxa suggests that IWP *Rhizophora* species are better established and differentiated compared to AEP *Rhizophora*.

Hybrid identification and patterns of hybridization

Using a similar approach as several recent studies on hybrids (Guo et al. 2011; Kamiya et al. 2011; Kusumi et al. 2012; Pacheco et al. 2002), this study demonstrated the utility of nDNA sequences for the verification and identification of IWP *Rhizophora* hybrid individuals. The identity of the morphologically intermediate individuals in Pulau Burung (Chan 1996; Ng and Chan 2012a) was not confirmed through genetic analysis until now. All morphologically identified putative hybrids sampled from Pulau Burung were heterozygous at nDNA nucleotide sites specific to *R. apiculata* and *R. stylosa*. Inferred haplotypes of the putative hybrids also clustered with haplotypes of *R. apiculata* and *R. stylosa*, confirming that they are hybrids between the two species, recognized as *R.* × *lamarckii*. Hybrid genotypes observed at all nDNA loci investigated in this study suggested that all the putative hybrid samples from Pulau Burung were simple F_1s of *R. apiculata* × *R. stylosa* crosses. Moreover, no morphologically intermediate individuals were found bearing mature fruits/propagules,

and their flowers often had abnormal development (Ng and Chan 2012a). We therefore agree with claims from earlier observations and molecular studies that *R. apiculata* × *R. stylosa* hybrids are usually sterile (Duke 2010; Lo 2010; Tomlinson 1986). Interestingly, genetic data in this study did not show any possible *R. apiculata* × *R. mucronata* crosses among the morphologically identified putative hybrid samples, although mature trees identified as *R. mucronata* were found to also grow in Pulau Burung (Ng and Chan 2012a). This could be due to the difference in the abundance among parental species in the islet, where *R. stylosa* > *R. mucronata* (Ng and Chan 2012b). Relative abundance has been known to affect hybridization patterns in other plant species (Lepais et al. 2009). Other pre- or post-zygotic barriers in *R. apiculata* × *R. mucronata* crosses that render them less fit compared to *R. apiculata* × *R. stylosa* crosses in certain habitats could also cause such hybridization bias. *R. apiculata* × *R. mucronata* crosses do, however, exist in several other habitats (Kathiresan 1995, 1999; Lo 2010; Ong 2003).

The observation of several individuals morphologically identified as *R. mucronata* or *R. stylosa* with hybrid genotypes in this study provided convincing evidence of hybridization between the two sibling species. Although a few morphological key features are commonly used to discriminate *R. mucronata* and *R. stylosa*, differences are often subtle and classification becomes inconclusive when individuals display ambiguous characteristics. The presence of intraspecific morphological variation in these continuous traits complicates the situation even more. Duke (2006) suggested that the only distinguishing feature between *R. mucronata* and *R. stylosa* is the flower style length. Even then, such feature was recently found to be quite variable in IWP *Rhizophora* (Ragavan et al. 2011). Subsequently, morphological identification of any putative hybrids between *R. mucronata* and *R. stylosa* would be almost impossible, explaining the lack of reporting on possible hybridization between the species. Previous studies using molecular markers have confirmed hybridization events of *R. apiculata* × *R. mucronata* and *R. apiculata* × *R. stylosa* (Lo 2010; Parani et al. 1997). It is thus highly possible that *R. mucronata* and *R. stylosa*, being more closely related while having overlapping distributions and flowering periods (Duke 2006; Ng and Chan 2012a), also cross in nature.

To further strengthen our hypothesis, we included additional samples from other locations, i.e., samples of *R. mucronata* from Thailand and *R. stylosa* from Japan. So far, there have not been reports of the occurrence of *R. stylosa* in Thailand or *R. mucronata* in Japan (FAO 2007; Spalding et al. 2010) where the additional samples were obtained. The inclusion of such samples in our analysis not only helped avoid the underestimation of genetic variation in both species but also tested if genetic data agreed with morphological identification. These additional pure species samples were homozygous at most nDNA loci, and all their haplotypes clustered on the nDNA phylogenies in concordance with the morphological classification of the species. The sequences were also very conservative across distant populations, suggesting that the hybrid-like genotypes found in several *R. mucronata* and *R. stylosa* sampled from Bagan Lalang and Pulau Burung did not result from intraspecific mating.

To our knowledge, this study represents the first account of molecular evidence of the existence of natural hybrids between *R. mucronata* and *R. stylosa*. Unlike in other *Rhizophora* hybrids where pollen viability was found to be low (Tyagi and Singh 1998; Tyagi 2002), findings in this study suggested that the possible hybrids from *R. mucronata* × *R. stylosa* crosses are capable of further interbreeding and/or backcrossing. While an F_1 hybrid would be expected to show combined genotypes made up of haplotypes from both parental species at all the nDNA loci, an advanced-stage hybrid would show segregation of parental haplotypes across the different (presumably unlinked) loci (Rentsch and Leebens-Mack 2012), although recombinant alleles may also result from frequent backcrossing (Ishiyama et al. 2003, 2008). Then, of the seven possible hybrids between *R. mucronata* and *R. stylosa*, two would be F_1s while the other five would be advanced-stage hybrids.

This study is also the first to document advanced-stage hybrids in IWP *Rhizophora*. Introgressive hybridization has been found in *Bruguiera* (Sun and Lo 2011) and AEP *Rhizophora* (Cerón-Souza et al. 2010), although it was not explicitly discussed in the latter study due to difficulty in the classification of species. Given the low number of *R. mucronata* × *R. stylosa* hybrids detected in both populations, hybridization between the two seems to be rare. However, due to the small sample sizes in terms of the number of individuals, number of populations, and the number of loci surveyed in this study, such hypothesis could not be accurately tested. Notwithstanding, the fact that this hybrid may look like either parent calls for alternative methods to be used in line with currently established morphological keys to aid in field management and conservation of *Rhizophora* populations.

Finally, the uniparental inheritance of cpDNA is useful to determine the direction of hybridization in the putative hybrids. In this study, we assumed that the chloroplast is maternally inherited like in most plant species (Mogensen 1996). Maternal inheritance of cpDNA has been proven in some mangrove species, i.e., *Sonneratia alba* and *Bruguiera gymnorrhiza* (Zhou et al. 2008). As it was not possible to tell the direction of cpDNA inheritance in advanced-stage hybrids, only F_1-stage hybrids were taken into account. The 13 F_1-stage *R. apiculata* × *R. stylosa* and 2 F_1-stage *R. mucronata* × *R. stylosa* hybrid samples appeared to be products of unidirectional hybridization. *R. stylosa* was the maternal parent for *R. apiculata* × *R. stylosa* crosses, while *R. mucronata* was the maternal parent of *R. mucronata* × *R.*

stylosa crosses. The direction of hybridization for *R. apiculata* × *R. stylosa* hybrids found in this study contrasted with the general conclusion of an earlier study that suggested bidirectional hybridization in IWP *Rhizophora* (Lo 2010). However, in most cases, *R. stylosa* was the maternal parent (Lo 2010), similar to the findings in this study. The ratio of the number of individuals of putative parental species present on the site is thought to have an influence on the direction of hybridization in various plant species (Burgess et al. 2005; Edwards-Burke et al. 1997; Zhou et al. 2008).

During our field survey in Pulau Burung, we observed that the putative *Rhizophora* hybrids usually had better growth compared to their putative parents (Ng and Chan 2012a). This is consistent with observations by Tyagi (2002), who reported that putative hybrids between *R. mangle* and *R. stylosa* normally grew faster and produced better timber. However, despite the robustness observed in these individuals, their establishment and subsequent growth seemed to be limited by yet unknown environmental factors, having been observed only in restricted locations among all areas where two or more *Rhizophora* species co-occur. Between both locations featured in this study, the abundance of putative hybrids differed strongly; through morphological observation, putative *Rhizophora* hybrids (shown in this study to be *R.* × *lamarckii*) were reported to flourish in Pulau Burung with a relative abundance of 69.4 % compared to other *Rhizophora* species (Ng and Chan 2012b). No such individuals were identified in Bagan Lalang. Also, the relative frequencies of putative hybrids of *R. mucronata* × *R. stylosa* in both locations detected in this study (one per 18 *R. mucronata* and *R. stylosa* in Bagan Lalang, and six per 11 *R. mucronata* and *R. stylosa* in Pulau Burung) seem to suggest that hybridization events are more pronounced in Pulau Burung. This is intriguing as both locations have the same *Rhizophora* species composition. Given the tendency to only occur in certain areas, mangrove populations with hybrids should be given due attention and management for the conservation of these unique habitats. More studies should also be conducted in order to understand and explore the evolutionary potentials of these mangrove hybrids.

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