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Introgressive hybridization in two Indo-West Pacific *Rhizophora* mangrove species, *R. mucronata* and *R. stylosa*

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ABSTRACT

Natural hybridization is common in plants, including mangroves. Three *Rhizophora* mangrove species are recognized in the Indo-West Pacific region, namely *R. apiculata*, *R. mucronata*, and *R. stylosa*. So far, *R. apiculata* has been known to form sterile hybrid offspring with *R. mucronata* (= *R. x annamalayana*) and with *R. stylosa* (= *R. x lamarckii*). A third hybrid between morphologically similar *R. mucronata* and *R. stylosa* was only recently shown to exist, via DNA sequencing. However, it has been suspected that this newly discovered hybrid may be fertile, with potential to interbreed to give rise to advanced-generation hybrids in locations where both parental species occur. In this study, inter-simple sequence repeat (ISSR) markers were used to survey several stands where *R. mucronata* and *R. stylosa* co-occur. By screening through 26 ISSR primers, a set of species-specific diagnostic bands for each species were first identified from standard samples before being used to genotype the test samples. A chloroplast DNA locus was also sequenced in the test samples to determine the direction of hybridization. Results showed that hybridization occurred between *R. mucronata* and *R. stylosa* in all investigated locations with differing levels of introgression, and that it could happen in any direction.

1. Introduction

Natural hybridization is common in plants and has been known to play an important role in the evolution of species through introducing novel genetic variations and promoting speciation (reviewed by Mallet, 2007; Wissemann, 2007; Soltis and Soltis, 2009). Nonetheless, repeated hybridization and introgression can also facilitate gene flow between species, reversing the process of speciation.

Mangrove species are no exception to natural hybridization. Putative mangrove hybrids have been reported within the major genera of *Rhizophora*, *Sonneratia*, *Lumnitzera*, and *Bruguiera* (Tomlinson, 1986; Duke and Ge, 2011). Most of these hybrids were identified based on intermediate morphology of two or more co-occurring putative parental species (Chan, 1996; Duke, 2010; Kathiresan, 1995, 1999). As morphological identification is often unreliable when it comes to establishing the identities of hybrids, a majority of recent studies have turned to molecular methods for confirmation (Parani et al., 1997; Zhou et al., 2005; Qiu et al., 2008; Guo et al., 2011; Tsai et al., 2012). So far, most of these hybrids have been found to be limited to the F_1 generation, and few actually find inter-specific introgression in mangrove species (Cerón-Souza et al., 2010; Sun and Lo, 2011; Ng et al., 2013).

The Indo-West Pacific *Rhizophora* comprises of three species, namely *R. apiculata*, *R. mucronata*, and *R. stylosa*. Unlike *R. apiculata* and *R. mucronata* that co-occur in most mangrove areas in South-east Asia, *R. stylosa* often prefers hard sandy soil or rocks and is best adapted to exposed marine locations (Mohd Nasir and Safiah Yusmah, 2007; Ng and Chan, 2012b; Duke, 2006). Based on our survey of Indo-West Pacific *Rhizophora* sites on the Malay Peninsula, few, if any, *R. apiculata* and *R. mucronata* occur at sites where *R. stylosa* grows in abundance (Ng and Chan, 2012b). When these species co-occur on the same site, they sometimes interbreed and form natural hybrids. So far, it is known that sterile hybrids can form via crossing of *R. apiculata* with *R. mucronata* (= *R. x annamalayana*) and *R. apiculata* with *R. stylosa* (= *R. x lamarckii*) (Lo, 2010; Ng and Chan, 2012a; Tyagi, 2002). Our recent study showed that a third hybrid, a cross between the morphologically similar *R. mucronata* and *R. stylosa*, exists and may be fertile (Ng et al., 2013). In the study using DNA sequences of six nuclear DNA loci, several individuals from populations where *R. mucronata* and *R. stylosa* co-occur showed patterns of parental haplotype segregation, a sign of advanced-generation hybridization (i.e. hybrids of the F_2 generation or more, and backcrosses) (Ng et al., 2013).

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Realizing the importance of mangrove forests, several programs to reforest mangrove areas have been carried out worldwide. The correct identification of the native and introduced species is always a priority in such efforts. In *Rhizophora*, *R. mucronata*, *R. stylosa*, and their hybrids with *R. apiculata* are sometimes misidentified for one another due to similarity in their morphology (Duke, 2006; Kathiresan, 1995, Ng, personal observation). Our previous finding that *R. mucronata* and *R. stylosa* do cross in nature to produce fertile hybrid offspring further poses a question on the possible disfavor that species misidentification during reforestation does to the extant natural population. More specifically, translocation of a foreign species into the ecosystem could potentially lead to the production of hybrids that will negatively affect locally adapted populations by reducing or replacing native species (reviewed by Vila et al., 2000; Seehausen et al., 2008). An understanding of the capability of hybridization between the two closely related species is therefore crucial not only to understand the evolutionary aspects of speciation in mangrove species, but also in assessing the potential impacts of human intervention on the continued survival of natural populations.

While our earlier study was able to show the existence of hybrids between *R. mucronata* and *R. stylosa* (Ng et al., 2013), the few loci used were not sufficient to further infer the stages of hybridization in those individuals. Inter-simple sequence repeat (ISSR) markers are in the form of DNA fragments amplified by single-primer PCR reactions. The technique uses microsatellites as primers to amplify DNA segments located between two identical microsatellite repeat regions. Despite claims of lack of reproducibility, being time- and cost-effective and highly variable – a combination of the advantages of AFLP, microsatellite, and RAPD markers (reviewed by Reddy et al., 2002 and Agarwal et al., 2008) – ISSR markers continue to be an attractive choice for studies that require a high number of loci and especially on species that lack genetic information. Wolfe et al. (1998) were among the first who demonstrated the utility of ISSR markers in assessing hybridization in natural populations. This method was recently used to study hybridization in *Bruguiera* (Sun and Lo, 2011) and *Rhizophora* (Lo, 2010) mangroves.

Here we report the use of ISSR markers to study the hybridization between *R. mucronata* and *R. stylosa* in an attempt to evaluate their ability to hybridize in natural populations. Chloroplast DNA sequences were also obtained from the samples to determine the direction of hybridization. Findings in this study will provide insights into the ability of the sister species to form hybrids, thereby contributing to the further understanding and subsequent management of these unique floras.

2. Materials and methods

2.1. Sampling and DNA extraction

First, 16 *R. mucronata* and 16 *R. stylosa* samples from various locations were randomly chosen to act as species standards (hereinafter "standard samples"). Four individuals were randomly chosen from each of four locations where only one of the two species occurs. This minimizes the possibility of the standard samples being hybrids. Then, a set of sampling criteria was used to identify suitable samples to be tested in this study: (1) Only individuals identified as *R. mucronata* or *R. stylosa* (morphotypes) from leaf and floral characteristics were included. *Rhizophora apiculata* and its hybrids with *R. mucronata* and *R. stylosa* can easily be identified and excluded (see Ng et al., 2013 for details on morphological identification). (2) Samples were collected from locations where both morphotypes co-occur, and sampling at each location covered both morphotypes as well as their intermediates. A total of 85 samples (hereinafter "test samples") from five locations in Peninsular Malaysia and Indonesia were included in this study. The details on the standard and test samples are shown in Table 1 and Fig. 1.

Table 1
Standard and test samples used in this study. Standard samples were chosen from locations where either *R. mucronata* or *R. stylosa* occurs to act as species standards to establish species-specific diagnostic markers, while test samples were samples obtained from locations where *R. mucronata* and *R. stylosa* co-occur.

Sampling site	Location	Sample size (No. of individuals)	
		R. mucronata	*R. stylosa*
Standard samples			
PHU	Phuket, Thailand	4	–
KRA	Krabi, Thailand	4	–
SS	Samut Songkhram, Thailand	4	–
SP	Samut Prakan, Thailand	4	–
PBM	Pulau Besar, Melaka, Malaysia	–	4
PMJ	Pulau Mawar, Johor, Malaysia	–	4
FNR	Funaura Bay, Iriomote, Japan	–	4
URC	Urauchi Estuary, Iriomote, Japan	–	4
Total		16	16
Test samples			
KRT	Kurong Tengar, Perlis, Malaysia	10	
BLS	Bagan Lalang, Selangor, Malaysia	18	
PBS	Pulau Burung, Negeri Sembilan, Malaysia	11	
JK	Jakarta, Indonesia	18	
MEN	Menjangan, Bali, Indonesia	28	
Total		85	

Leaf samples were collected and dried in silica gel. Genomic DNA was extracted from approximately 20 mg of dried leaf material using the DNeasy Plant Mini Kit (QIAGEN) following manufacturer's instructions.

2.2. Chloroplast DNA sequencing

One chloroplast DNA (cpDNA) region, the *atpB-rbcL* intergenic spacer, was amplified from the genomic DNA of the *Rhizophora* samples. Only cpDNA sequences for populations JK and MEN were obtained in this study. The cpDNA sequences for the other populations (KRT, BLS, and PBS) and the standard samples were obtained in our previous and ongoing studies, i.e. Ng et al. (2013) and Ng et al. (unpublished). The primers used for PCR were *F*: 5′-GAAATGGAAGTTAGCACTCG-3′ and *R*: 5′-AAGATTCAGCAGCTACCGCA-3′ (Inomata et al., 2009). 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 45 °C 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).

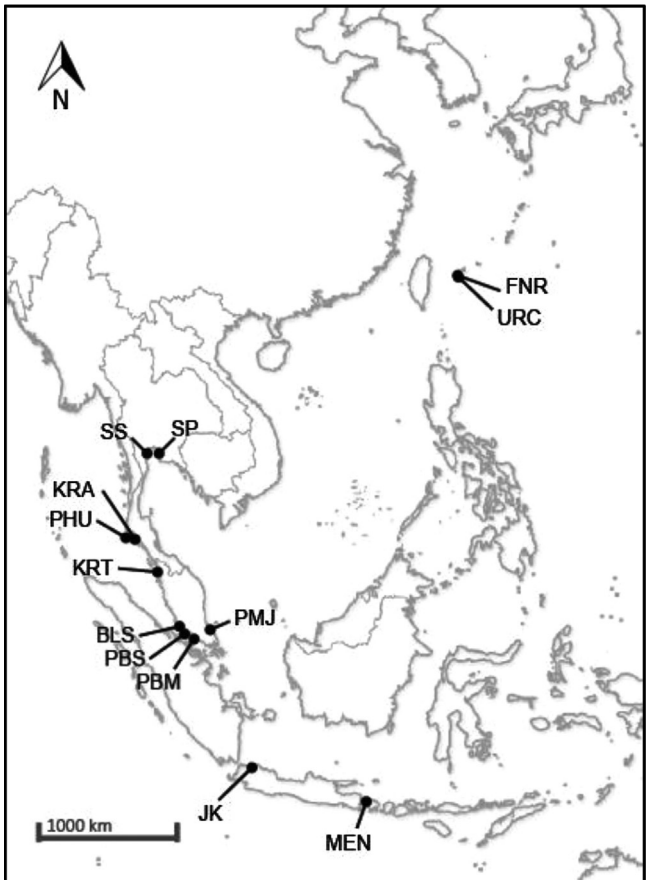

Fig. 1. Map showing sampling sites for this study.

2.3. ISSR genotyping

Twenty six ISSR primers (Supplementary Table S1) from Lo (2010) and Wolfe et al. (1998) were used to genotype the standard samples to obtain a set of species-specific diagnostic markers. Single-primer PCR reactions were performed in 10 μl mixtures, each containing ~20 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.), 0.4 μM primer, and 0.5 U of Ex-Taq DNA polymerase (TaKaRa Bio Inc.). Either one of the following PCR reaction profiles were used: (1) Usual PCR–Comprised of an initial denaturation of 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 45 s at T_a, and 1.5 min at 72 °C, and finally an extension step at 72 °C for 7 min; (2) Touchdown PCR–Comprised of an initial denaturation of 5 min at 95 °C, followed by 13 cycles of 30 s at 95 °C, 30 s at 58–46 °C (−1 °C/cycle), and 1.5 min at 72 °C, 25 cycles of 30 s at 95 °C, 30 s at 45 °C, and 1.5 min at 72 °C, and finally an extension step at 72 °C for 7 min. PCR amplifications were replicated and negative controls were included in each run to verify the consistency of the genotyping. Finally, 3 μl each of the PCR amplicons were analyzed by electrophoresis on 2.0% (weight/volume) agarose gel, stained with ethidium bromide (EtBr) and viewed under UV illumination. Only clear, non-ambiguous, and reproducible bands between 200 and 3000 bp that were specific to either *R. mucronata* or *R. stylosa* standard samples were chosen. ISSR primers that produced reliable diagnostic bands between the two species were subsequently used to genotype the test samples. Only diagnostic bands were scored as either present (+) or absent (−) for each individual to generate a binary ISSR data matrix for each population to be used in subsequent analyses. Failed amplifications were treated as missing data. Individuals (of both standard and test samples) that contained five or more missing data points were excluded from subsequent analyses.

2.4. Data analyses

For the cpDNA locus, nucleotide sequences were assembled and edited using the software ATGC ver. 6.0 (GENETYX CORPORATION). Sequence alignments were performed using Clustal W (Thompson et al., 1994) implemented in the software MEGA 5.0 (Tamura et al., 2011) and corrected manually. 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 examine the genetic composition of the individuals based on their ISSR genotypes, the Bayesian clustering software, STRUCTURE ver. 2.3.4 (Pritchard et al., 2000; Falush et al., 2003), was used to assign the individuals to a given number of (K) populations based on the ISSR data. The program was run with a burn-in of 100,000 iterations followed by 100,000 MCMC iterations. Ten independent runs were performed under the admixture model for K = 1–7 clusters. The web-based software Structure Harvester ver. 0.6.93 (Earl and vonHoldt, 2012) was then 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.

Another Bayesian analysis software, NewHybrids (Anderson and Thompson, 2002) ver. 1.1 beta3 (which includes support for dominant markers), was used to analyze the same ISSR data matrix. The program estimates the posterior probability of each sample's membership in six genotype classes, assuming two generations of hybridization. The six genotype classes were: pure parent *R. mucronata* (P_M), pure parent *R. stylosa* (P_S), 1st generation hybrid progeny (F_1), 2nd generation hybrid progeny (F_2), backcross with pure *R. mucronata* parent (B_M), and backcross with pure *R. stylosa* parent (B_S). The *z* and *s* options were chosen during data input to establish that the *R. mucronata* and *R. stylosa* standard samples are of pure origin, but at the same time preventing these individuals from influencing the estimation. The program was left to run with 100,000 sweeps after burn-in, for 3 replicates.

DNA sequences of the different cpDNA haplotypes have been deposited in GenBank with the accession numbers KM261785-KM261787. The total cpDNA alignment and the ISSR binary data matrices used in this study have been deposited as Supplementary Material.

3. Results

3.1. Analysis of cpDNA locus

The lengths of the amplified cpDNA *atpB-rbcL* intergenic spacer region ranged from 812 to 828 bp, resulting in four haplotypes (CP1, CP2, CP3, and CP4). Fig. 2 shows the relationship among all the observed haplotypes. Generally, the different cpDNA haplotypes reflected the two *Rhizophora* standard samples used in this study identified through morphology and confirmed via molecular (i.e. ISSR) data. The CP1 (812 bp) haplotype was shared between *R. mucronata* and *R. stylosa*, but found mostly in *R. mucronata*. The CP2 (813 bp) haplotype was specific to *R. mucronata*, while the CP3 (827 bp) haplotype was specific to *R. stylosa*. The CP4 (828 bp) haplotype was observed only in the MEN test population (mainly *R. stylosa*). Supplementary Table S2 lists the cpDNA *atpB-rbcL* haplotypes corresponding to the samples analyzed in this study.

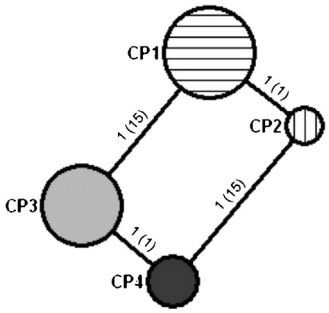

Fig. 2. Haplotype network showing the relationship among the four cpDNA *atpB-rbcL* intergenic spacer haplotypes (CP1–CP4) observed in all samples. Circle size is proportional to the frequency of the haplotype. The number of indel events is labeled at the lines. Number in parenthesis represents the number of base pairs involved in the indel event. Line-shaded circles = haplotypes found mostly in *R. mucronata*. Grey-colored circles = haplotypes found in *R. stylosa*.

3.2. Analysis of ISSR data

Results of the ISSR markers were reproducible. Out of the 26 ISSR primers used to genotype the standard samples, only 14 produced diagnostic bands that distinguished *R. mucronata* from *R. stylosa*. Finally, a total of 24 reliable diagnostic bands were identified. These diagnostic bands were relatively evenly distributed between *R. mucronata* and *R. stylosa* (10 bands for *R. mucronata* and 14 bands for *R. stylosa*), reducing bias towards any species in the following analyses. The 14 ISSR primers and the resulting banding results among standard samples are listed in Table 2. After removing individuals with five or more missing data points, a total of 12 *R. mucronata* standard samples, 15 *R. stylosa* standard samples, and 77 test samples were included in the subsequent analyses.

In the STRUCTURE analysis, the plot of Ln $P(D)$ plateaued after $K = 2$, while the Evanno's ΔK peaked also at $K = 2$ (Fig. 3). In the barplot, $K = 2$ perfectly grouped *R. mucronata* and *R. stylosa* standard samples into two clusters. In every other test population, several individuals displayed different levels of genotypic admixture, a clear sign of introgressive hybridization (Fig. 3).

NewHybrids generated similar results in all three replicates. It assigned all *R. mucronata* and *R. stylosa* standard samples as pure parents P_M and P_S, respectively, with high support (i.e. posterior probability $\geq$0.9). Among the 77 test samples, 20 were assigned as pure *R. mucronata* (P_M), 39 as pure *R. stylosa* (P_S), four as backcrosses to *R. mucronata* (B_M), and two as backcrosses to *R. stylosa* (B_S) with high support. The other 12 test samples could not be assigned to a particular genotype class with posterior probability <0.9. Among these, two were assigned as P_S, one as F_2, one as B_M, and three as B_S with substantial support, i.e. posterior probability $\geq$0.8 but <0.9 (Fig. 4 and Supplementary Table S3). The samples that could not be assigned confidently to a particular genotype class may represent introgressants of more advanced generations (i.e. more than two generations of hybridization).

4. Discussion

4.1. ISSR genotyping

The number of diagnostic bands identified between *R. mucronata* and *R. stylosa* in this study was lower than between *R. apiculata* and *R. mucronata* or between *R. apiculata* and *R. stylosa* (half the number of bands, or lower; data not shown). This could be because *R. mucronata* and *R. stylosa* are more closely related to each other than to *R. apiculata*. In this study, we used the same primers as reported by Lo (2010), who scored a high number of diagnostic bands among several *Rhizophora* species. Between *R. mucronata* and *R. stylosa* for example, the total number of diagnostic bands reported in that study were 25 for *R. mucronata* and 30 for *R. stylosa*, more than double that of what we found in this study (10 for *R. mucronata* and 14 for *R. stylosa*). Unfortunately, the experimental methods were not sufficiently detailed in that study for us to make further comparisons. Various factors could have affected the results of band-scoring across different studies, including the geographical coverage of sampling (samples from different geographic regions can yield different levels of polymorphism), different PCR conditions (e.g. types and concentrations of reagents, PCR reaction temperatures, etc.) and different band-scoring criteria (e.g. how bright a band should appear before being scored as "present"). Also, many studies use relatively low annealing temperatures in PCR reactions, e.g. 43–49 °C (Wolfe et al., 1998; Pannacciulli et al., 2009; Lo, 2010; Sun and Lo, 2011), presumably to increase the number of bands amplified, but at the same time subjecting the amplification results to possible fluctuations. Therefore, while results may be reproducible using the same equipment and protocol, we recommend caution when comparing band-scoring results across studies.

4.2. Introgressive hybridization of R. mucronata and R. stylosa

Results of this study provided evidence that *R. mucronata* and *R. stylosa* frequently hybridize at sites where they co-occur. We previously used DNA sequences of six nuclear loci to show that *R. mucronata* and *R. stylosa* do hybridize in nature, and found seven *R. mucronata-R. stylosa* hybrids in populations BLS and PBS (Ng et al., 2013). In the current study, aside from two samples (PBSsty02 and PBSsty03) that were removed due to insufficient data, the other five (BLSmuc01, PBSmuc02, PBSmuc03, PBSsty01, and PBSsty04) were assigned as hybrids, concurring with the earlier finding. This further demonstrated the utility of ISSR markers in assessing hybridization between the two closely related species. Other molecular markers may also be useful, but are often technically cumbersome and/or costly for large-scale applications (e.g. DNA sequencing, AFLP, RFLP, etc.).

In this study, morphological characters were initially used to distinguish *R. mucronata*/*R. stylosa* from *R. apiculata* and its hybrids during collection of the test samples in the field. However, distinguishing *R. mucronata*, *R. stylosa*, and their hybrids was difficult, since morphological keys use continuous traits to tell apart the sister species (e.g. bigger versus smaller leaves, shorter versus longer flower styles, etc.), which often suffer from morphological plasticity (Ng and Chan, 2012a; Ragavan et al., 2011; Duke et al., 2002). We thus relied on molecular data generated in this study for species and hybrid identification, without morphological data for comparison. In the original study describing the Bayesian method behind NewHybrids, Anderson and Thompson (2002) showed that 20 "nearly diagnostic loci" was sufficient to produce good inferences to identify hybrids in mixed populations. We therefore believe that with the 20 or more diagnostic marker bands used in this study, NewHybrids could give a reliable estimation to identify and characterize the hybrids of *R. mucronata* and *R. stylosa*. In addition, both the outcomes of the STRUCTURE and NewHybrids analyses showed highly concurrent results—although unable to give estimations of the level of hybridization, STRUCTURE estimated admixed genotypes in all individuals assigned by NewHybrids as possible hybrid individuals.

Rhizophora apiculata, *R. mucronata*, and *R. stylosa* have similar flowering periods (Duke, 2006). Their flowers are thought to be pollinated by wind or general pollinators like bees (Tomlinson, 1986), facilitating hybridization when the different species co-occur. However, hybrids involving *R. apiculata* are rare (Ng and Chan, 2012a) and have been shown to be limited to the F_1 generation (Ng and Chan, 2012a; Tyagi, 2002). The presence of simple F_1 hybrids as well as introgressants in the test samples as assigned

Table 2
ISSR primers that resulted in diagnostic bands used in this study.

Primer	Primer sequence (5′–3′)	T_a (°C)	Diagnostic bands[a]	Source
UBC 809	$(GA)_8T$	TD	3 (1/2)	Lo (2010)
UBC 811	$(GA)_8C$	TD	1 (0/1)	
UBC 817	$(CA)_8A$	49	1 (1/0)	
UBC 818	$(CA)_8G$	49	1 (1/0)	
UBC 834	$(AG)_8CTT$	49	1 (0/1)	
UBC 836	$(AG)_8CTA$	55	3 (1/2)	
UBC 842	$(GA)_8CTG$	49	1 (0/1)	
UBC 844	$(CT)_8AGC$	49	2 (1/1)	
UBC 846	$(CA)_8AGT$	49	1 (1/0)	
UBC 847	$(CA)_8AGC$	TD	4 (1/3)	
UBC 889	$(AC)_7AGT$	55	1 (1/0)	
UBC 890	$(GT)_7ACG$	49	2 (1/1)	
814.1	$(CT)_8TG$	TD	1 (0/1)	Wolfe et al. (1998)
17902	$(GT)_6AY$	49	2 (1/1)	
Total			24 (10/14)	

T_a = Annealing temperature. TD = Touchdown PCR protocol.

[a] Number of diagnostic bands between *R. mucronata* and *R. stylosa*. If a band is present in one species and absent in the other, the band is counted. If a band is polymorphic in one species and fixed in another, the band is not counted. If a band is polymorphic in one species and absent in another, the band is counted. Numbers in parentheses = (Number of bands specific for *R. mucronata*/Number of bands specific for *R. stylosa*).

by NewHybrids suggested a lack of pre- and post-zygotic reproductive barrier between *R. mucronata* and *R. stylosa*. There was also no tendency for the assigned hybrids to specifically inherit the cpDNA haplotype (usually maternally inherited in plants) of any parental species, suggesting that hybridization could happen in a bidirectional manner. Thus, habitat preference of the sister species, as mentioned earlier, could have been one of the major barriers that have promoted speciation between them. There is currently no information on the evolutionary potential of these hybrids, but new species have been known to arise from hybridization in other plant species (reviewed by Soltis and Soltis, 2009; Wissemann, 2007; Mallet, 2007).

The only other large-scale study on Indo-West Pacific *Rhizophora* hybrids was by Lo (2010), who also used ISSR markers to study putative hybrids collected from several locations, four of which had co-occurring *R. mucronata* and *R. stylosa*. In those locations, previously described putative Indo-West Pacific *Rhizophora* hybrids (i.e. *R. x lamarckii* and *R. x annamalayana*) and their putative parents (i.e. *R. apiculata*, *R. mucronata*, and *R. stylosa*) were morphologically identified in the field and collected. Consequently, any individual

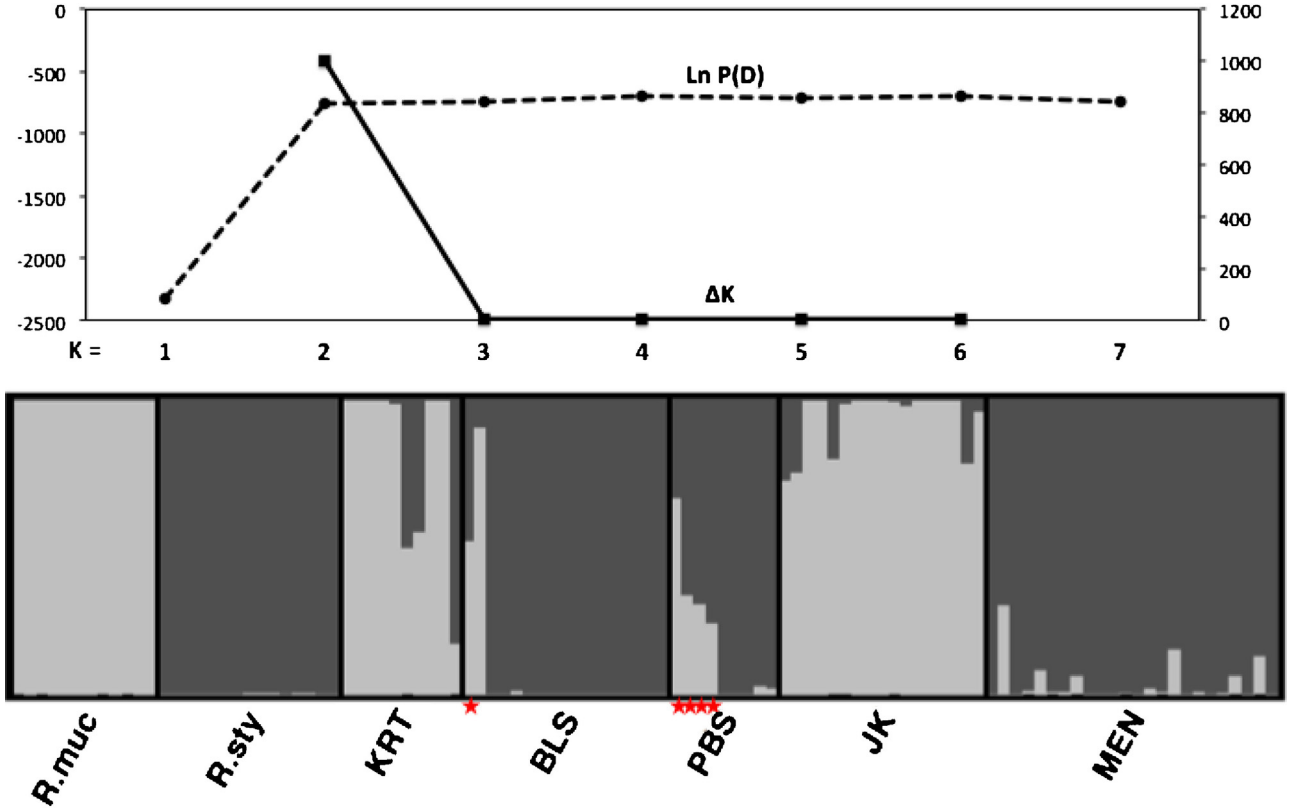

Fig. 3. Outcome of structure analysis. Barplot of the optimum $K = 2$ is based on the Ln $P(D)$ and Evanno's ΔK data plots. Red-colored stars indicate individuals that were also identified by Ng et al. (2013) as *R. mucronata-R. stylosa* hybrids. R.muc: *R. mucronata* standard samples; R.sty: *R. stylosa* standard samples; KRT, BLS, PBS, JK, MEN: test populations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

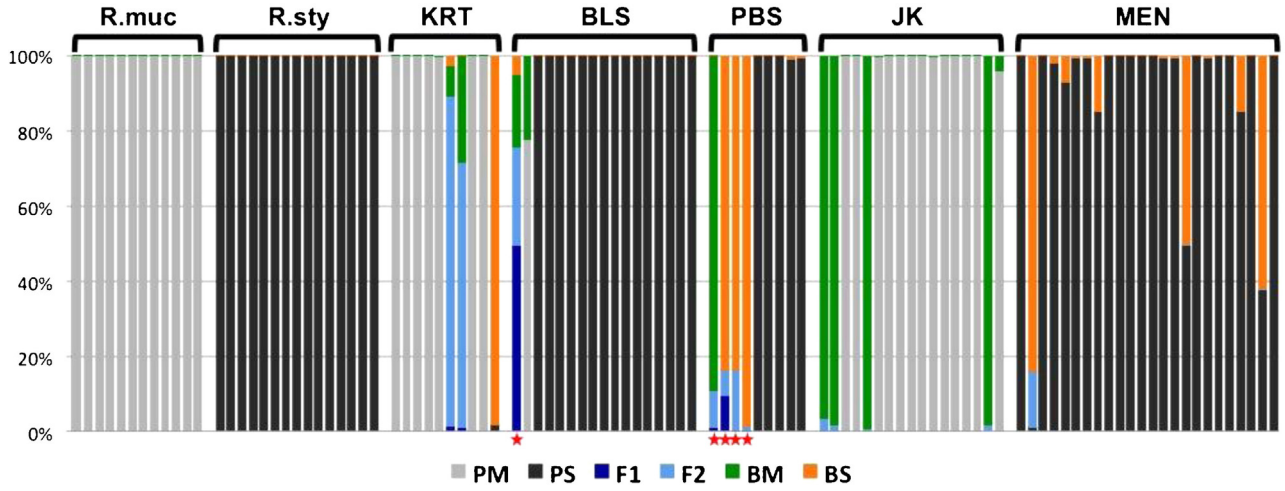

Fig. 4. Outcome of NewHybrids analysis. Each bar represents an individual sample, while the different colors represent posterior probability of membership in each genotype class—P_M, P_S, F_1, F_2, B_M, and B_S. Red-colored stars indicate individuals that were also identified by Ng et al. (2013) as *R. mucronata*-*R. stylosa* hybrids. R.muc: *R. mucronata* standard samples; R.sty: *R. stylosa* standard samples; KRT, BLS, PBS, JK, MEN: test populations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

that displayed ambiguous traits, particularly between *R. mucronata* and *R. stylosa*, would have been excluded from the study. This reduces the possibility of stumbling upon *R. mucronata*-*R. stylosa* hybrids, which explains the lack of detection of such a hybrid in that study. Looking closer (Fig. 2 of Lo, 2010), we actually observed one individual that showed genetic admixture between *R. mucronata* and *R. stylosa* (i.e. a sign of being a hybrid between the two species) but the observation was not discussed in that study. This highlights the importance of this study in clarifying the hybridization between *R. mucronata* and *R. stylosa*, giving new perspectives for further research on mangrove species hybridization and its ecological consequences.

4.3. Implications for conservation

As shown until now, Indo-West Pacific *Rhizophora* species do hybridize in nature, i.e. between *R. apiculata* and *R. stylosa* (= *R. x lamarckii*; sterile), between *R. apiculata* and *R. mucronata* (*R. x annamalayana*; sterile), and between *R. mucronata* and *R. stylosa* (fertile). During a field trip to a small mangrove island featured in this study (Pulau Burung; PBS), we found through morphological identification that *Rhizophora* hybrids (showed by Ng et al., 2013 to be *R. x lamarckii*) were the most abundant (69.4%), followed by *R. stylosa* (16.7%) > *R. mucronata* (8.3%) > *R. apiculata* (5.6%) (Ng and Chan, 2012b). Unfortunately, we also found educational signboards on the island misidentifying this rare *Rhizophora* hybrid population as *R. mucronata*, and hundreds of *R. mucronata* seedlings were recently planted, presumably in an attempt to reforest the bare areas of the island. Given that *R. mucronata* starts flowering at 3–4 years old (Hung Tuck Chan, personal communication), the planting on Pulau Burung may change the dynamics of species interaction on the island and facilitate hybridization with the extant *R. stylosa* and/or *R. apiculata*. Although the fitness of natural hybrids between *R. mucronata* and *R. stylosa* is not known, one could speculate that hybrid offspring may outperform and displace the parental species, i.e. through hybrid vigor. If that is the case, an increase in hybridization between *R. mucronata* and *R. stylosa* may negatively impact the already rare *R. stylosa*. The effect on the existing population of *R. x lamarckii* remains unclear.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquabot.2014.07.006.

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