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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).