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TECHNICAL NOTE

A simple and inexpensive molecular assay for species identification of Indo-West Pacific *Rhizophora* mangroves for conservation and management

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Abstract Mangrove species of the genus *Rhizophora* are predominant and highly sought-after for the reforestation of mangrove areas in Southeast Asia. Of the three *Rhizophora* species occurring in the Indo-West Pacific (IWP) region, *R. mucronata* and *R. stylosa* have similar morphological features, making species identification difficult. Their respective hybrids with *R. apiculata* are also difficult to tell apart morphologically. Based on DNA sequence alignment of the partial nuclear starch-branching enzyme II (*SBE2*) gene obtained in a recent study, we designed species-specific primers for a multiplex PCR assay for rapid species identification, allowing us to differentiate among samples of *R. apiculata*, *R. mucronata*, *R. stylosa*, and their respective hybrids. The method described here makes use of simple molecular techniques; we envision that this simple and inexpensive method will improve the efficiency of efforts to conserve and manage IWP *Rhizophora* populations.

Keywords Indo-West Pacific *Rhizophora* · Mangrove · Molecular identification · Multiplex PCR

In mangroves, *Rhizophora* represents one of the most common and important mangrove genera across the tropical region (Duke 2006), and is popular for reforestation of mangrove areas (Polidoro et al. 2010). In the Indo-West Pacific (IWP), three species of *Rhizophora*, namely *R. apiculata*, *R. mucronata*, and *R. stylosa*, can be found, together with two putative hybrids, *R.* × *lamarckii* (=*R. apiculata* × *R. stylosa*) and *R.* × *annamalayana* (=*R. apiculata* × *R. mucronata*). Owing to similarities in morphology between *R. mucronata* and *R. stylosa* (Duke 2006), the two species are difficult to tell apart at times. The two described putative hybrids also look similar (Kathiresan 1995, and references therein). Moreover, a third hybrid between *R. mucronata* and *R. stylosa* was only recently identified through DNA sequences (Ng et al. 2013).

Many recent studies have relied on the power of molecular methods for species identification. Probably the most popular method nowadays is through species-specific DNA "barcodes" that involves the sequencing of a fragment of organellar DNA. However, there are two major issues with the application of this method on IWP *Rhizophora*: (1) Uniparental inheritance of chloroplast in plants means that the identification of species through cpDNA sequences works only when the tested samples are of "pure" species. Given that IWP *Rhizophora* hybrids are rare, they too, warrant attention for identification and subsequent conservation. (2) While DNA sequencing is accurate, its cost limits the number of samples to be screened. Furthermore, a cpDNA "barcode" has not been identified for all *Rhizophora*. *Rhizophora mucronata* and *R. stylosa* for example, have been found to share similar haplotypes at several cpDNA loci commonly used for phylogenetic studies, *i.e. trnG-trnS*, *trnH-rpl2* (Lo 2010), and *atpB-rbcL* (Ng et al. 2013). Therefore, a nuclear DNA (nDNA) marker would be most useful to discriminate the different IWP *Rhizophora* species and their hybrids.

In this study, we describe a simple, rapid, and inexpensive multiplex PCR assay for the identification of

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Table 1 PCR primers used in this study

Forward primer (5′–3′)	Species-specific reverse primers (5′–3′)	Target species	Amplicon size (bp)
TGGATGGTCATATAGCTCT	SBE2.RevRa: GAAAGCTGAAACACAAGA**A**A**C**	*R. apiculata*	707
	SBE2.RevRm: TACAGGGGAAGAGGACAT**C**C	*R. mucronata*	498
	SBE2.RevRs: CACAACAAGTCATCTGAAAG**C**	*R. stylosa*	1,245

For the reverse primers, bolded letters represent species-specific SNP matches; underlined letters represent introduced mismatches

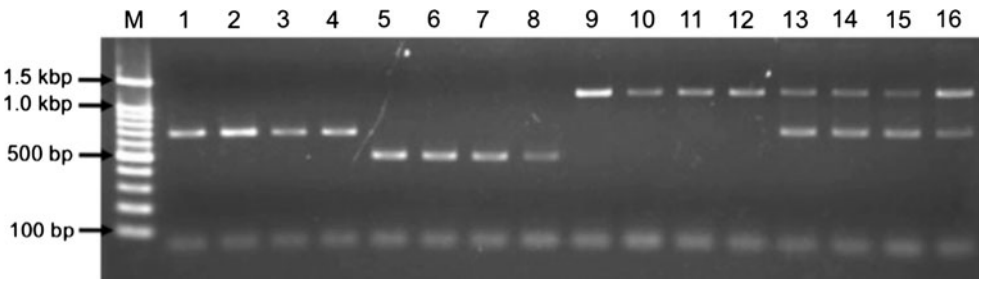

Fig. 1 Amplification results of several *Rhizophora* samples using the multiplex assay. M = 100 bp DNA ladder (GeneDireX); *lanes 1–4* = *R. apiculata*; *lanes 5–8* = *R. mucronata*; *lanes 9–12* = *R. stylosa*; *lanes 13–16* = *R.* × *lamarckii*

R. apiculata, *R. mucronata*, *R. stylosa*, and their respective hybrids through amplification of species-specific nDNA fragments. Based on alignment of the starch-branching enzyme II (*SBE2*) partial nuclear gene sequences of IWP *Rhizophora* obtained in a recent study (Ng et al. 2013), species-specific single nucleotide polymorphisms (SNPs) were identified. Reverse primers specific to each of the species were then designed: the 3′ end of a reverse primer was designed to perfectly match the targeted species-specific SNP. An additional mismatch was introduced at the third nucleotide position from the 3′ end to increase the power of selective amplification during PCR, following Suharyanto and Shiraishi (2011). The primers and their approximate amplicon sizes are listed in Table 1.

Multiplex PCR, using the designed reverse primers together with a forward primer, was conducted on known *Rhizophora* samples collected from distant populations. This included 24 *R. apiculata*, 24 *R. mucronata*, 24 *R. stylosa*, and 13 *R.* × *lamarckii* samples (Supplementary Table S1) that were not included in the initial DNA alignment for primer design. 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 (total of four primers), and 1.0U of *Ex-Taq* DNA polymerase (TaKaRa Bio Inc.). The PCR reaction comprised of an initial denaturation of 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 57 °C and 2 min at 72 °C, and finally an extension step at 72 °C for 7 min. PCR amplicons were analyzed by electrophoresis on 2.0 % (weight/volume) agarose gel, stained with ethidium bromide (EtBr), and viewed under UV illumination.

The samples yielded the intended banding patterns on the agarose gel. Results were consistent in all populations tested, indicating the universal applicability of the species-specific primers. As shown in Fig. 1, the species-specific bands were clear and can easily be sized on a 2.0 % w/v agarose gel (~700 bp for *R. apiculata*, ~500 bp for *R. mucronata*, and ~1200 bp for *R. stylosa*). For verification, resultant bands were excised from one sample of each taxon and sequenced. The DNA sequences corresponded exactly to the intended fragment of the *SBE2* locus.

Theoretically, diploid F_1 hybrids carry one allele from each parent at each locus. As hybridization progresses, inter-mating of hybrids or backcrossing will result in the segregation of alleles at each locus and introgression. Therefore the marker chosen for this assay can only be used to reliably discriminate "pure" species and F_1 hybrids, but not advanced-stage hybrids (F_2 or later, and backcrosses). *Rhizophora* × *lamarckii* and *R.* × *annamalayana* have so far been thought to be sterile, limiting them to the F_1 stage (Chan 1996; Lo 2010; Parani et al. 1997). The assay described in this study can thus be employed with confidence to identify these hybrids.

While conservation and reforestation efforts are highly encouraged, the correct identification of the native species and the introduced species is crucial. The assay described in this study can potentially be employed for small-scale species identification as well as for large-scale screening of *Rhizophora* populations to understand their species composition, thereby effectively identifying areas of conservation priority.

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